Immune modulation using probiotics and multiple sclerosis peptides

A thesis submitted for the degree of

Doctor of Philosophy

by

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ABSTRACT

Changes in physiological, immunological and gut microbiome can lead to a range of chronic conditions in humans. The 'hygiene hypothesis' identifies the increasing trend of immune-mediated disorders to possibly be a consequence of intestinal dysbiosis, that in turn results in a dysfunctional immune system leading to conditions such as, eczema, asthma, allergies and autoimmune diseases. Therefore, utilization of beneficial probiotic bacteria can increase their abundance within the gastrointestinal lumen, and subsequently modulate immune cells, such as, T helper (Th)-1, Th2, Th17, regulatory T (Treg) cells, B cells, macrophages, dendritic cells and monocytes. Modulation of immune cells are directly related to human health and pathogenesis of immune disorders. Chapter 1a describes the cross talk between probiotics and the gastrointestinal immune system, and their effects in relation to inflammatory bowel disease, multiple sclerosis (MS), allergies and atopic dermatitis.

MS is one of the debilitating autoimmune disease of the central nervous system which has been increasing during the past decades. MS severely affects patients' health, work and quality of life, and its treatment has changed over the last 20 years. In chapter 1b the immunopathology of MS and various available treatments have been investigated. As all the MS immunotherapeutic drugs target relapsing remitting MS (RRMS), in particular developing a treatment for progressive forms of MS is a medical challenge and medical specialists and clinicians are in constant battle with serious treatment challenges for MS. Although β-interferons 1a or 1b and glatiramer acetate are accounted as the most commonly used injectable disease modifying therapies in RRMS, however, one of the major challenge of these types of therapies has been the lack of devotion to treatment among MS patients, with approximately 50% of patients ceasing their therapy plan within the first year. This chapter revisits the basics of the immune-pathophysiology of MS to gain insights in the development of innovative improved drug treatments and presents current drug treatments and new and emerging immune modulating approaches for the immunotherapy of MS. This chapter provides groundwork for vaccine (or immune modulation) development research and the investigation of new potential vaccines (immune modulators) against MS which are used in chapters 5a and 5b.

Backtracking probiotics beneficial effects to the host that occur through their contribution to the development and maintenance of a healthy immune system, tracked the

steps to the use of some probiotics in the food industry as starter or secondary starter cultures to ferment dairy products. These probiotics include *Streptococcus thermophilus* (ST); in chapter 2, ST1275, ST285 and ST1342 bacteria were used to determine their modulatory effects on U937 human promonocytic cell line which exhibited differential cytokine induction, in particular, increased secretion of anti-inflammatory IL-4 and IL-10 cytokines were noted. ST also stimulated an increase in the production of CXCL8 and GM-CSF, as well as expression of cell surface markers, CD11c, CD86, C206, CD209, MHC-1. ST285 was determined the most potent probiotic, therefore was considered to investigate further in chapters 3, 4 and 6. The main objective of next study was to assess modulatory and anti-inflammatory properties of ST285 using human peripheral blood mononuclear cells (PBMC) from healthy donors. To fulfil this objective, modifications in the mRNA expression of genes related to innate and adaptive immunity were assessed and results showed strong immune modulatory effects of ST285 to human PBMC with an array of anti-inflammatory properties. ST285 reduced mRNA expression of IL-18, IFNγR1, CCR5, CXCL10, TLR-1, TLR-2, TLR-4, TLR-8, CD14, CD40, CD86, C3, GATA3, ITGAM, IRF7, NLP3, LYZ, TYK2 and IFNR1. ST285 upregulated IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-23, IFNγ, TNFα, CSF-2 to human PBMC; no changes to mRNA expression of IFNA1, IFNB1, IL-4, IL-5, IL-13, CCL2, CCL5, CCL8, CCR4, CCR8, CXCR3, TLR-3, TLR-5, TLR-6, TLR-9, CD4, CD80, FOXP3, STAT3, CD40LG, HLA-A, HLA-E and RORC were noted. These data demonstrated a predominant anti-inflammatory profile exhibited by ST285, hence, ST285 was validated for further investigation on human monocytes.

Some of the beneficial effects attributed to probiotics may be through modulation of the immune system; the effect of ST285 to human monocytes was assessed and a range of immune modulating effects of ST285 by human monocytes was demonstrated. This included significant downregulation in the mRNA expression of IL-1R, IL-18, IFNγR1, IFNαR1, CCL2, CCR5, TLR-1, TLR-2, TLR-4, TLR-5, TLR-6, TLR-8, CD14, CD86, CD4, ITGAM, LYZ, TYK2, IFNR1, IRAK-1, NOD2, MYD88, ITGAM, SLC11A1, and significant upregulation in the mRNA expression of IL-1α, IL-1β, IL-2, IL-6, IL-8, IL-23, IFNγ, TNFα, and CSF-2. ST285 is used in the dairy industry, survives during cold storage, well tolerates upon ingestion, and their consumption may have beneficial effects with potential implications in inflammatory and autoimmune disorders, such as, multiple sclerosis.

In order to determine a suitable autoimmune setting to investigate the effects of ST285 in an animal model, it was required to revisit MS treatments to determine the effects of recently developed agonist and antagonist MS vaccines. Encephalitogenic T cells are greatly implicated in the pathogenesis of MS, stimulation of these T calls is triggered by the formation of a tri-molecular complex among the human leukocyte antigen (HLA), an immunodominant myelin basic protein (MBP) epitope, and the T cell receptor (TCR). This next study (chapter 5) concentrated on the rational design and synthesis of non-peptide mimetic molecules, based on the immunodominant $MBP_{83–96}$ epitope that is recognized by the TCR in complex with HLA, with a focused attention on the inhibition of the tri-molecular complex formation which can consequently lead to the inhibition of proliferation of activated T cells. In view of the interactions between the TCR and the HLA-$MBP_{83–96}$ complex, a structure-based pharmacophore model was generated and the newly candidate molecules were obtained through the ZINC database, six molecules were synthesized and further evaluated *in vitro* as TCR antagonists. Analogues 15 and 16 were able to inhibit the stimulation of T cells by the immunodominant $MBP_{83–99}$ peptide from immunized mice to some extent, and to a lesser degree by analogues 17 and 18 and then by analogue 19, presenting the lead compounds 15 and 16 may be used for immunotherapy against MS. In addition in chapter 5b, the immune modulatory effects of MBP_{83-99} peptide conjugated or not conjugated to carrier mannan, in either linear or cyclic forms were determined. It was shown that MBP_{83-99} modulated the immune responses in SJL/J immunized mice which resulted in cytokine secretion by immunized spleen cells which was protective in an experimental autoimmune encephalitis model and protection against axonal spinal damage. Molecular modelling was used to gain insights into the binding mode of the peptide to MHC class II.

In the chapter 6, the effects of ST285 to agonist MBP_{83-99} peptide immunized mouse spleen cells was determined. Agonist peptide induced a Th1 profile, however in the presence of ST285 a significant increase in the expression of anti-inflammatory IL-4, IL-5, IL-10 cytokines, and decreased pro-inflammatory IL-1β and IFN-γ were noted. Regular consumption of probiotic bacteria such as ST285 in the form of capsules, fermented food or dairy products may therefore be beneficial in the management and treatment of autoimmune diseases such as multiple sclerosis. Consumption of probiotics contributes to a healthy microbiome of the GIT leading to many health benefits. They also contribute to the modulation of the immune system and are becoming popular for the treatment of a number of immune and inflammatory diseases.

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Doctor of Philosophy Student Declaration

Doctor of Philosophy Declaration
"I, **Narges Dargahi**, declare that the PhD thesis entitled "***Immune modulation using probiotics and multiple sclerosis peptides***" is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work".

Signature [redacted] Date 28.08.2019

Updated March 2018

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PART A:

DETAILS OF INCLUDED PAPERS: THESIS BY PUBLICATION

Please list details of each Paper included in the thesis submission. Copies of published Papers and submitted and/or final draft Paper manuscripts should also be included in the thesis submission

Item/ Chapter No.	Paper Title	Publication Status (e.g. published, accepted for publication, to be revised and resubmitted, currently under review, unsubmitted but proposed to be submitted)	Publication Title and Details (e.g. date published, impact factor etc.)
1	1a:Immunomodulatory effects of probiotics: Can they be used to treat allergies and autoimmune diseases? 1b:Multiple Sclerosis: immunopathology and treatment update	1a: Published 12 November 2018 1b: Published 7 July 2017	**1a: Maturitas-Impact Factor 2.942-Open Access** **1b: Brain Sciences- Impact Factor 2.876-Open Access**
2	Immunomodulatory effects of Streptococcus thermophilus on U937 monocyte cell cultures	Published 31 August 2018	**Journal of Functional Foods** **Impact Factor 3.197**
3	Streptococcus thermophilus alters the expression of genes associated with innate and adaptive immunity in human peripheral blood mononuclear cells	To be revised and resubmitted by 20 November 2019	**PLOS ONE - Impact factor: 2.776**
4	Immune modulatory effects of probiotic Streptococcus thermophilus in human monocytes	Currently under review	PLOS ONE - Impact factor: 2.776
5	5a:Immune modulation of linear and cyclic MBP83-99 peptide conjugated to mannan 5b:Design and Synthesis of Non-Peptide Mimetics Mapping the Immunodominant Myelin Basic Protein (MBP83–95) Epitope to Function as T-Cell Receptor Antagonists International Journal of Molecular Sciences	5a: Currently under review 5b: Published 26 April 2017	5a: International Journal of Molecular Sciences- Impact Factor: 4.183-Open Access 5b: International Journal of Molecular Sciences - Impact Factor: 4.183
6	***Streptococcus thermophilus*** **ST285 alters pro-inflammatory to anti-inflammatory cytokine secretion against myelin basic protein (MBP_{83-99}) peptide in mice**	Currently under review	Brain Sciences- Impact Factor 2.876-Open Access

Declaration by [candidate name]: **Signature:** **Date:**

Narges Dargahi [redacted] 30.10.2019

Citation & links to published versions of included articles

Ch. 1a.
Narges Dargahi, Joshua Johnson, Osaana Donkor, Todor Vasiljevic, Vasso Apostolopoulos, Immunomodulatory effects of probiotics: Can they be used to treat allergies and autoimmune diseases?, Maturitas. 2019: 119. https://doi.org/10.1016/j.maturitas.2018.11.002.

Ch. 1b.
Dargahi N, Katsara M, Tselios T, Androutsou M-E, De Courten M, Matsoukas J, Apostolopoulos V. Multiple Sclerosis: Immunopathology and Treatment Update. *Brain Sciences*. 2017; 7(7):78. https://doi.org/10.3390/brainsci7070078

Ch. 2.
Narges Dargahi, Joshua Johnson, Osaana Donkor, Todor Vasiljevic, Vasso Apostolopoulos, Immunomodulatory effects of Streptococcus thermophilus on U937 monocyte cell cultures, Journal of Functional Foods, 2018: 49. https://doi.org/10.1016/j.jff.2018.08.038.

Ch. 3.
Dargahi N, Johnson J, Apostolopoulos V (2020) Streptococcus thermophilus alters the expression of genes associated with innate and adaptive immunity in human peripheral blood mononuclear cells. PLOS ONE 15(2):e0228531. https://doi.org/10.1371/journal.pone.0228531

Ch. 5b.
Yannakakis, M.-P.; Simal, C.; Tzoupis, H.; Rodi, M.; Dargahi, N.; Prakash, M.; Mouzaki, A.; Platts, J.A.; Apostolopoulos, V.; Tselios, T.V. Design and Synthesis of Non-Peptide Mimetics Mapping the Immunodominant Myelin Basic Protein ($MBP_{83–96}$) Epitope to Function as T-Cell Receptor Antagonists. *Int. J. Mol. Sci.* **2017**, *18*, 1215. https://doi.org/10.3390/ijms18061215

Ch. 6.
Dargahi, N.; Matsoukas, J.; Apostolopoulos, V. *Streptococcus thermophilus* ST285 Alters Pro-Inflammatory to Anti-Inflammatory Cytokine Secretion against Multiple Sclerosis Peptide in Mice. *Brain Sci.* **2020**, *10*, 126. https://doi.org/10.3390/brainsci10020126

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I am very grateful to my beloved parents and my sisters and brothers for their infinite love, inspiration, tolerance, support and all the sacrifice they gave to me throughout and beyond my PhD. A very special word of gatitude to my beloved late Dad, to whom that I have missed every single minute of each day since the day I left home, and I will always miss you. Your

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'To my beloved dad who left my family and myself too early'

And

'To my lovely mum who is the symbol of love, affection, tolerance and persistant hard work'

Publications

Peer-reviewed journal articles

Dargahi N., *Joshua CJ., Donkor NO., Vasiljevic T., Apostolopoulos V., "*Immunomodulatory effects of probiotics: Can they be used to treat allergies and autoimmune diseases?" (2018) (Review) Maturitas. January 2019;119:25-38. https://doi: 10.1016/j.maturitas.2018.11.002. Epub 2018 Nov 12.

Dargahi N., *Apostolopoulos V.,* "Multiple sclerosis: Immunopathology and treatment update" (Invited review), (2017). Brain Sciences 2017, 7(7), 78; https://doi.org/10.3390/brainsci7070078.

Dargahi N., *Joshua CJ., Donkor NO., Vasiljevic T., Apostolopoulos V.,* "Immunomodulatory effects of *Streptococcus thermophilus* on U937 monocyte cell cultures*"* (2018) (Research paper). Journal of Functional Foods. Volume 49, October 2018, Pages 241-249. https://doi.org/10.1016/j.jff.2018.08.038

Yannakakis MP, Simal C, Tzoupis H, Rodi M, ***Dargahi N.*** *, Prakash M, Mouzaki A, Platts JA, Apostolopoulos V, Tselios TV.* "Design and Synthesis of Non-Peptide Mimetics Mapping the Immunodominant Myelin Basic Protein (MBP83–96) Epitope to Function as T-Cell Receptor Antagonists" (2017). (Research paper) (Co-author). International Journal of Molecular Sciences. 2017 Jun 8;18(6). pii: E1215. https://doi: 10.3390/ijms18061215.

Ahmadifar E., Sheikhzadeh N., Roshanaei K., ***Dargahi N.****, Faggio C.,* "Can dietary ginger (*Zingiber officinale*) alter biochemical and immunological parameters and gene expression related to growth, immunity and antioxidant system in zebrafish (*Danio rerio*)?" (Research paper) (April 2019) (Co-author). Aquaculture. Volume 507, 30 May 2019, Pages 341-348 https://doi.org/10.1016/j.aquaculture.2019.04.049

Faisal Md., ***Dargahi N.****, Vasiljevic T., Donkor NO.,* "Immunomodulatory properties of selectively processed prawn protein fractions assessed using human peripheral blood mononuclear cells (PBMCs)". (Research paper). AID - IJFS14331 International Journal of Food Science & Technology. Manuscript ID - IJFST-2019-27786.R1(Research paper) (August 2019) (Co-author). https://doi.org/10.1111/ijfs.14331

Dargahi M., Jamilian H., Heydari H., Davoodi H., Apostolopoulos V., ***Dargahi N.****, Polman R.* "A comparative study of anxiety sensitivity and positive psychological capital in Iranian patients with irritable bowel syndrome and normal participants". (Research paper) (Submitted June 2019) (Co-author). BMC Gastroenterology

Dargahi N., *Joshua CJ., Apostolopoulos V.,* "Immunomodulatory effects of *Streptococcus thermophilus* on gene expression of human peripheral blood mononuclear cells" (Research paper). PLOS ONE (Submitted July 2019).

Dargahi N., *Joshua CJ., Apostolopoulos V.,* "Immune modulatory effects of probiotic *Streptococcus thermophilus* in human monocytes" (Research paper). Food Research International (Submitted July 2019).

Dargahi N., *Matsoukas J., Apostolopoulos V., "Streptococcus thermophilus ST285 alters pro-inflammatory to anti-inflammatory cytokine secretion against myelin basic protein (MBP83–99) peptide in mice"* (Research paper) Journal of Brain Sciences (Submitted September 2019).

Dargahi N., *Matsoukas J., Tselios T., Androutsou M., Apostolopoulos V., "Immune modulation of linear and cyclic MBP83-99 peptide conjugated to mannan.*" (Research paper) Journal of Molecules (To be submitted October 2019).

Awards

2018 Best Paper Award from Brain Sciences for "Multiple sclerosis: Immunopathology and treatment update"

Conferences Presentations (Oral and Posters)

3MT presentation at Victoria University, (Melbourne Australia). Title: ***"****Probiotics, are they the answer to inflammation and autoimmune disease?"* (August 2019).

Conference presentation at Victorian Post graduate conference, Victoria University, (Melbourne Australia). Abstract title: ***"****Effect of Streptococcus thermophilus ST1342, 287, 127 probiotics on promonocytic U937 cells".* (2017).

Poster presentation at the 9th Australian Society for Medical Research (ASMR) Student Research Symposium, Victoria University, (Melbourne Australia). Abstract title: ***"****Effect of Streptococcus thermophilus probiotics on human promonocytic U937 cell line".* (1st June 2018).

3MT presentation at Victorian Post graduate conference, Victoria University, (Melbourne Australia). Abstract title: ***"****Probiotics in health and disease".* (201

Table of contents

Thesis structure

This thesis is structured as a general introduction and eight chapters; with one literature chapter containing 2 published literature reviews listed as Literature review 1 and Literature review 2. There are five data chapters and one conclusions chapter. Every individual data chapter is self-contained and is either published or under review for publication in peer-reviewed international journals. There is no overlap in the data between chapters, except for the probiotic bacteria species used in different research chapters.

General Introduction This chapter presents an introduction to and an overview of this thesis, current knowledge of probiotic bacteria and their utility in health enhancement.

Chapter One This Chapter presents two extensive literature reviews. The first literature review has been published in the journal of MATURITAS. It presents detailed insight into probiotics general effects on health, their immune-modulatory effects on immune cells, and their effects of probiotics in the treatment of allergies, inflammatory disorders and autoimmune diseases. The second review paper has been published in the journal of BRAIN SCIENCES and presents detailed insight into multiple sclerosis, the immunopathology of this disease, different treatments and immune modulation using vaccine candidates.

Chapter Two has been published in the JOURNAL OF FUNCTIONAL FOODS, and presents data on the effects of three strains of *Streptococcus thermophilus* on immunomodulation of U937 human monocyte cell line. This chapter provides a guideline for choosing the best strain of *Streptococcus thermophilus* among the three utilized in this study.

Chapter Three has been submitted for publication in the journal of PLOS ONE. The chapter presents research data related to effects of *Streptococcus thermophilus* on immunomodulation and the gene expression of human peripheral blood mononuclear cells (PBMC). This paper is the first to analyze 84 different genes related to human innate and adaptive immune responses simultaneously using *S. thermophilus* to stimulate/ modulate PBMC. Comments made by the jornal editor and reviewers have been recived and the manuscript is in the process of addressing those comments to be finalized for being published.

Chapter Four has been submitted for publication in the journal of PLOS BIOLOGY and assesses changes in immune modulation caused by probiotic *Streptococcus thermophilus* in human monocytes. This paper is also one of the first to analyze 84 different genes related to human innate and adaptive immune responses simultaneously using *S. thermophilus* ST285 to modulate monocyte cells.

Chapter Five, integrates the findings from two research papers; the first paper I to be submitted for publication in the journal of MOLECULES and reports data on the structural studies on Immune modulation of aginst linear and cyclic MBP_{83-99} peptide conjugated to mannan (vaccines), as well as extensive analysis of the cytokine profiles produced by spleen cells following vaccine injections into the mice. The manuscript 5a is the main section of this chapter that includes all the animal studies and immunological studies.

The second research paper has been published in the INTERNATIONAL JOURNAL OF MOLECULAR SCIENCES and presents data on the design and synthesis of non-peptide mimetics mapping the immunodominant Myelin Basic Protein (MBP_{83-99}) epitope to function as T-Cell receptor antagonists. This chapter also includes data on molecular structure of the vaccine and the effect of some of the non-peptide vaccine in mouse animal model. Chapter 5b is included to show how the peptides were modulated in a different way and to develop the full story of what had been conducted. The animal and related *in vitro* immunological experimentations in this chapter have been conducted, analysed and interpreted by Narges Dargahi and chemical structural studies have been conducted by collaborators in the University of Patras, Greece. Due to the university regulation for a minimum 60% contribution by candidate as requirement for including published paper in the thesis, this paper has been removed from this chapter and included in the appendix.

Chapter Six presents a research paper that has been submitted for publication in the journal of BRAIN SCIENCES. The paper presents data on the animal model of MS injected with myelin basic protein MBP_{83-99} and exposed to *Streptococcus thermophilus* ST285, and findings presents alterations of the pro-inflammatory to anti-inflammatory cytokine secretion against myelin basic protein MBP_{83-99} peptide in mice spleens cells.

Chapter Seven presents a general discussion for the entire thesis, and gives some insights into future research.

Chapter Eight presents references used for this thesis.

Appendix 1 to 7 contain the cover page and abstracts of published papers resulted from research studies in this thesis or collaborations with other Victoria University and non- Victoria University colleagues. Appendix 8 is the 2018 best paper award for the review paper published in the journal of Brain Sciences.

The writing style of the data chapters has followed the requirements of the journals to which they were submitted to, however, the format, section headings, numbering and referencing have been amended to be consistent across the thesis.

General Introduction

General Introduction

This section presents an overview to this thesis. In the modern age the incidence of immune-mediated disorders has been growing significantly (Bach, 2018; Versini et al., 2015). The hygiene hypothesis rationalises this incident by stating that reduced exposure to microorganisms and microbial products is associated with higher chance of developing atopic disorders such as, dermatitis, eczema, asthma (Bach, 2018; Dargahi, Johnson, Donkor, Vasiljevic, & Apostolopoulos, 2019). In addition, there are links to other inflammatory disorders including irritable bowel syndrome (IBS), Crohn's disease, ulcerative colitis and autoimmune disorders such as multiple sclerosis (MS) and type-1 diabetes (Bach, 2018). This happens in particular when the natural microbial ecosystem is imbalanced due to the absence or lack of adequate natural body microflora. The incidence of asthma in the young has augmented by 59% in Australia, 56% in Scotland between 1964 and 1990 and by 38% in the United States between 1980 and 2003 (Versini et al., 2015). Similarly, the prevalence of inflammatory bowel disease (IBD) has increased so much that ulcerative colitis (UC) has gone up from 8 in 100,000 to 14/100,000 individuals, and that of Crohn's disease (CD) has risen from 6 to 15 in 100,000 individuals (Cosnes, Gowerrousseau, Seksik, & Cortot, 2011). There is also an increasing trend for the incidence of type-1 diabetes and MS globally, extending from 2.9% to 5.4% per annum between 1989–2003 for type-1 diabetes (Bach, 2018) and from 2.1 million in 2008 to 2.3 million in 2013 for MS (Kurtzke, 2008).

The hygiene hypothesis, in parallel, is reinforced by the robust epidemiological data and evidence noted through individuals with different immune and inflammatory disorders diagnosed with low composition or imbalanced gastrointestinal (GIT) microflora. It has also become evident that there is a cross-talk between the GIT and its microbiota (Dargahi et al., 2019). A number of clinical trials have been conducted with oral administration of microbial products which results in neutralization of atopic disorders (Dargahi et al., 2019), for instance, lactic acid bacteria (LAB) have regularly been administered to modulate the immune system for the purpose of preventing and/or treating allergies, lactose intolerance, infections, irregular bowel movements (constipation or diarrhea) and autoimmune diseases (Dargahi et al., 2019). It has also been established that in the absence of enough stimuli from body's micro-biome, the person becomes more prone to pathogenic bacteria and other trigger factors that cause

immune-inflammatory responses and in turn become more susceptible to many immune-related diseases (Dargahi et al., 2019).

Chronic inflammation has been generally accepted as a common hallmark of neuro-degenerative diseases, including MS, Alzheimer's disease and Parkinson's disease (Dargahi et al., 2017). It has been established that there is cross-talk between the GIT, the central nervous system (CNS) and the immune system, known as the gut-brain-immune axis, thus, gut microbiota interact with the CNS (Dargahi et al., 2017; Russo et al., 2018; Sandhu et al., 2017). Any dysfunction in the gut-brain axis is associated with the pathogenesis of a number of diseases inside and outside the GIT (Dargahi et al., 2019; Sandhu et al., 2017).

Since the discovery of $CD4^+$ T cell subtypes in particular, T helper (Th) 17 cells and $FOXP3^+$ CD4 regulatory T cells (Treg) the original Th1/Th2 paradigm has changed. Characterization of Th1, Th2, Th17 and Treg cells has improved our understanding of the underlying pathogenesis of many autoimmune diseases, including MS (Dargahi et al., 2019). It is clear that some cytokines secreted by these cells (IL-17 and IL-23) may have a key role in inflammation and autoimmune diseases due to their role in differentiation and stabilization of Th1/Th2 and Th17 phenotype cells which are involved in those diseases, whereas Treg cells can have a suppressive effect on Th1 and Th17 cells through secreting transforming growth factor-β (TGF-β) and IL-10 (Dargahi et al., 2019). It has been revealed that probiotics influence differentiation and proliferation of Th cells, their expression of surface markers and cytokines associated with immune responses. The rise of chronic diseases was a mayhem for researchers and the emergence of data showing association of a balanced microflora (symbiosis in microbiome and the GIT) resulting in functional healthy immune system, and a dysbiosis microflora leading to dysfunctional immune system, which again was the wakeup call for the scientists to investigate the role of microflora in human health.

According to the most recent definition of probiotics "Probiotics are bacteria which, when eaten with food or in capsules, reach the intestine either intact or as fragments and exert actions beneficial to the host, such as exclusion of pathogens from access to the mucosa, production of short chain fatty acids, and modulation of the immune response". The only members of Streptococcus species having the GRAS (in USA) and QPS (in Europe) status is *S. thermophiles*. *S. thermophilus* was approved as 'Generally Recognized as Safe' (GRAS) status in the USA and the 'Qualified Presumption of Safety' (QPS) status in the European

Union due to its safe use in food production over the years, and was authorized for human consumption (Hols et al., 2005). In spite of all the evidence for the positive benefits of probiotics, current literature still lacks data related to the effects of probiotics to immune cells as well as in most autoimmune diseases inducing MS. We therefore, studied the effects of probiotic strains of *Streptococcus thermophilus* (ST) on U937 monocyte cell line, on human peripheral blood mononuclear cells (PBMC) and to human monocyte cells isolated from human PBMC. Although insufficient, there are several studies that provide data in favour of beneficial properties of some lactic acid bacteria in experimental autoimmune encephalomyelitis (EAE), the animal model of human MS, but, data related to immune modulatory or therapeutic effects of probiotic bacteria in MS disease is scarce. Here, probiotic ST bacteria were used and tested for their effects to an inflammatory profile of mice immunized with MS pathogenic peptides. In fact, mice were injected with pathogenic myelin basic protein ($MBP_{83–99}$) peptide conjugated to the carrier mannan 3 times, and spleens isolated and restimulated with $MBP_{83–99}$ peptide. This induced a Th1 inflammatory profile, however, in the addition of ST probiotic bacteria, the profile was diverted from Th1 dominant to Th2 anti-inflammatory. These data suggest that probiotic ST bacteria have immune modulating properties.

Chapter 1

Litereture review

Chapter 1

Literature reviews

Review paper manuscript 1.

1a- Immunomodulatory effects of probiotics: Can they be used to treat allergies and autoimmune diseases?

ABSTRACT

As the population ages, physiological, immunological and gut microbiome changes collectively result in an array of chronic conditions. According to the 'hygiene hypothesis' the increasing trend of immune-mediated disorders may be the result of intestinal dysbiosis, leading to immune disfunction such as, eczema, asthma, allergies and autoimmune diseases. Consequently, utilization of beneficial probiotic bacteria can increase their abundance within the gastrointestinal lumen, and in turn modulate immune cells, such as, T helper (Th)-1, Th2, Th17, regulatory T (Treg) cells and B cells, which have direct relevance to human health and pathogeneis of immune disorders. Here, we describe the cross talk between probiotics and the gastrointestinal immune system, and their effects in relation to inflammatory bowel disease, multiple sclerosis, allergies and atopic dermatitis.

Key words: Probiotics*;* Dysbiosis*;* Lactic acid bacteria*;* Gastrointestinal tract*;* Inflammatory bowel disease*;* Multiple sclerosis*;* Allergies *;* Atopic dermatitis

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DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION: PAPERS INCORPORATED IN THESIS

This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

1. PUBLICATION DETAILS (to be completed by the candidate)

Title of Paper/Journal/Book: Immunomodulatory effects of probiotics: Can they be used to treat allergies and autoimmune diseases?
Maturitas

Surname: Dargahi First name: Narges

Institute: Institute for Health and Sport Candidate's Contribution (%): 75

Status:

Accepted and in press: ☐ Date:

Published: ☑ Date: 12/11/2018

2. CANDIDATE DECLARATION

I declare that the publication above meets the requirements to be included in the thesis as outlined in the HDR Policy and related Procedures – policy.vu.edu.au.

[redacted] 23.08.2019

Signature **Date**

3. CO-AUTHOR(S) DECLARATION

In the case of the above publication, the following authors contributed to the work as follows:

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;

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3. There are no other authors of the publication according to these criteria;
4. Potential conflicts of interest have been disclosed to a) granting bodies, b) the editor or publisher of journals or other publications, and c) the head of the responsible academic unit; and
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Immunomodulatory effects of probiotics: Can they be used to treat allergies and autoimmune diseases?

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ABSTRACT

As a person ages, physiological, immunological and gut microbiome changes collectively result in an array of chronic conditions. According to the 'hygiene hypothesis' the increasing prevalence of immune-mediated disorders may be related to intestinal dysbiosis, leading to immune dysfunction and associated conditions such as eczema, asthma, allergies and autoimmune diseases. Beneficial probiotic bacteria can be utilized by increasing their abundance within the gastrointestinal lumen, which in turn will modulate immune cells, such as, T helper (Th)-1, Th2, Th17, regulatory T (Treg) cells and B cells, which have direct relevance to human health and the pathogenesis of immune disorders. Here, we describe the cross-talk between probiotics and the gastrointestinal immune system, and their effects in relation to inflammatory bowel disease, multiple sclerosis, allergies and atopic dermatitis.

1. Introduction

Regular ingestion of probiotic bacteria, has been used for tapping into the health benefits exerted by microbiota of the gastrointestinal tract (GIT) within a healthy subject. Use of probiotics in the form of fermented milk dates back to ancient times and in Middle Eastern traditions, with claims that Abraham's longevity was due to the consumption of fermented milk [1,2]. The physiological changes of the GIT and alterations in the gut microbiome associated with age, along with changes in life style and dietary behaviors, leads to changes to the immune system [3]. Crosstalk between gut microflora and the immune system enables a balanced gut homeostasis in healthy individuals, however, alterations in the gut ecosystem due to the aged gut or diseases, causes changes in GIT microflora homeostatic equilibrium, resulting in a number of chronic diseases [3]. In fact, there are decreased anaerobic and *Bifidobacteria* populations, and increased *Enterobacter* species in the intestine of ageing individuals [4]. Advances in the gut microbiota probiotics and its synbiotics may be beneficial to the ageing population [5]. Probiotics consumed by humans must be non-pathogenic and survive GI transit to render their health benefits [6]. Whether or not probiotic strains should be of human origin is a matter of debate; although, it is accepted that if a strain can survive and colonize the human large intestine, its origin is not important [6,7]. The survival of probiotics is crucial within the gastric acid environment, and as such, new genes are activated to encode a number of stress proteins for their survival. Once in the lower small intestine and colon, probiotics confer health benefits (Tables 1 and 2), although there is evidence that dead cells can also induce beneficial outcomes [7].

Probiotic bacteria, mainly belong to the lactic acid bacteria (LAB) family, which are commonly found in decomposing milk products and secrete lactic acid, fermentating carbohydrates. In fact, LAB have been used in fermentation and storage of certain foods (milk, vegetables, meat) for thousands of years [8]. LAB have also been shown to contribute to the healthy microflora of the human gut [7,9]. As such, the genera *Lactobacilli* (L.) (L. *rhamnosus*, *L. helveticus*), *Bifidobacterium (B.)*, *Streptococcus* (*S.*) and *Enterococcus* species have been used as probiotic strains, i.e. supplementing foods with live microorganisms, and subsequent improvements in a number of human health conditions [9,10]. The basis for these benefits include, the detoxification of xenobiotics [11], microbial toxins, host metabolites i.e. bile salts and food components [12], biosynthesis of vitamin K_1, folic acid, biotin, vitamin B12, an increase in the absorption of calcium, iron and magnesium, fermentation of lactose, modulation of intestinal gas production [13,14] and production of short-chain fatty acids (SCFAs; acetate, propionate, butyrate, lactate) [15–17]. SCFAs are used as a source of energy to favour the growth and differentiation of GI epithelial cells, in addition to, modulating the immune system by regulating proliferation and cytokines of T cells, T helper (Th)-17 cells and T regulatory (Treg) cells of

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1. Introduction

Regular ingestion of probiotic bacteria, has been used for tapping into the health benefits exerted by microbiota of the gastrointestinal tract (GIT) within a healthy subject. Use of probiotics in the form of fermented milk dates back to ancient times and in Middle Eastern traditions, with claims that Abraham's longevity was due to the consumption of fermented milk (Ahtesh, Stojanovska, & Apostolopoulos, 2018; Vasiljevic & Shah, 2008). The physiological changes of the GIT and alterations in the gut microbiome associated with age, along with changes in life style and dietary behaviors, leads to changes to the immune system (E. Biagi et al., 2010). Crosstalk between gut microflora and the immune system enables a balanced gut homeostasis in healthy individuals, however, in the aged gut, this homeostatic equilibrium is altered, resulting in a number of chronic diseases (E. Biagi et al., 2010). In fact, there are decreased anaerobic and Bifidobacteria populations, and increased *Enterobacter* species in the intestine of ageing individuals (Tiihonen, Ouwehand, & Rautonen, 2010). Advances in the gut microbiota probiotics and its synbiotics may be beneficial to the ageing population (Elena Biagi et al., 2017). The probiotic microorganisms consumed by humans must be non-pathogenic and survive GI transit to render their health benefits (Hardy, Harris, Lyon, Beal, & Foey, 2013). Whether or not the consumption of probiotic strains should be of human origin is a matter of debate; although, it is generally accepted that if a strain can survive and colonize the human large intestine, its human origin is not a matter of concern (Hardy et al., 2013; Ljungh & Wadstrom, 2006). The survival of probiotics is crucial within the gastric acid environment, and as such, new genes are activated to encode a number of stress proteins for their survival. Once in the lower small intestine and colon, probiotics confer health benefits (Table 1, 2), although there is evidence that dead cells can also induce beneficial outcomes (Ljungh & Wadstrom, 2006).

Probiotic bacteria, mainly belong to the lactic acid bacteria (LAB) family, which are commonly found in decomposing milk products and secrete lactic acid, a metabolic end product from the fermentation of carbohydrates. In fact, LAB have been used in fermentation and storage of certain foods (milk, vegetables, meat) for thousands of years (Reis, Paula, Casarotti, & Penna, 2012). LAB have also been shown to contribute to the healthy microflora of the human gut (Sytze de Roock et al., 2011; Ljungh & Wadstrom, 2006). As such, the genera Lactobacilli (*L.*) (*L. rhamnosus*, *L. helveticus*), *Bifidobacterium (B.), Streptococcus* (*S.*) and *Enterococcus* species have been used as probiotic strains, *i.e.* supplementing foods with live microorganisms, and subsequent improvements in a number of human health conditions (Butel,

2014; Sytze de Roock et al., 2011). The basis for these benefits include, the detoxification of xenobiotics (Maurice, Haiser, & Turnbaugh, 2013), microbial toxins, host metabolites *i.e.* bile salts and food components (Kiseleva & Novik, 2013), biosynthesis of vitamin K_1, folic acid, biotin, vitamin B12, an increase in the absorption of calcium, iron and magnesium, fermentation of lactose, modulation of intestinal gas production (Macpherson & Harris, 2004; Salminen, Von Wright, & Ouwehand, 2004) and production of short-chain fatty acids (SCFAs; acetate, propionate, butyrate, lactate) (Asarat, Apostolopoulos, Vasiljevic, & Donkor, 2015, 2016; Asarat, Vasiljevic, Apostolopoulos, & Donkor, 2015). SCFAs are used as a source of energy to favour the growth and differentiation of GI epithelial cells, in addition to, modulating the immune system by regulating proliferation and cytokines of T cells, T helper (Th)-17 cells and T regulatory (Treg) cells of peripheral blood (Asarat, Apostolopoulos, et al., 2015; Asarat et al., 2016; Asarat, Vasiljevic, et al., 2015; Kiseleva & Novik, 2013). A non-pathogenic (probiotic) strain of *Escherichia coli* (G3/10) has been shown to suppress pathogenic bacterial growth, and secrete potent antimicrobial peptides (bacteriocin, microsin S) which are harmful to gastroenteric pathogens (*i.e. Helicobacter pylori, Campylobacter jejuni*, *Clostridium difficile* and *rotavirus*) (Zschüttig et al., 2012) (Figure 1). LAB compete with enteric pathogenic bacteria for binding to mucin (primarily MUC2) sites on the surface of epithelial cells, which may be a mechanism for inhibiting pathogenic bacteria translocating from the gut to different organs including the liver; identified as one of the benefits of some probiotic strains (Giannelli et al., 2014). The impact of LAB on the immune system has also drawn much attention in the last decade leading to their use in several pre-clinical and clinical studies in allergic and autoimmune disorders (Goudarzvand, Rasouli Koohi, Khodaii, & Moghadam, 2016; Ljungh & Wadstrom, 2006). In 2001, the first randomized placebo controlled clinical study was published on the use of *L. rhamnosus*, to modulate immune responses in late stages of pregnancy or to newborns, with high risk factors of allergic responses, and showed a significant decrease in the prevalence of atopic eczema (Kalliomäki et al., 2001). In addition, using probiotic supplements alone, or in combination with vitamins and minerals have been recommended for the prevention and treatment of multiple sclerosis (MS) (Goudarzvand et al., 2016); in particular, *L. plantarum* and *Bifidobacterium* B94 (BB94) were shown to positively impact the spatial memory and learning of rats (Goudarzvand et al., 2016). Herein, we describe the effects of probiotics to immune cells and their use in allergic and autoimmune disorders.

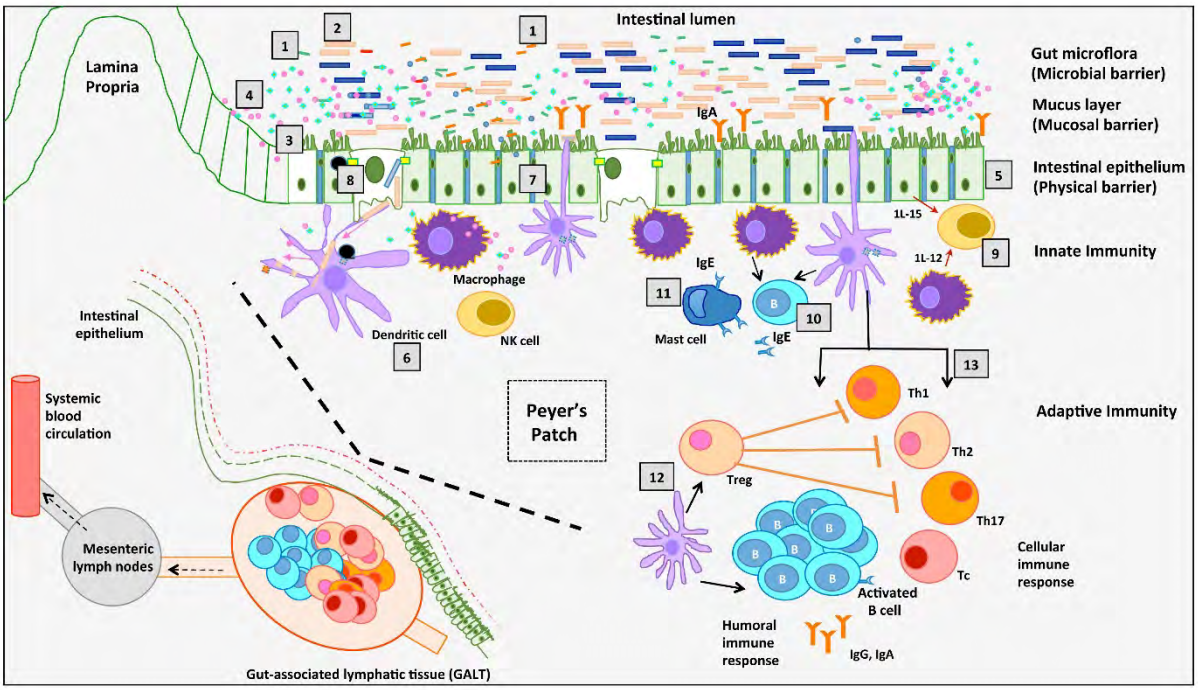

Figure 1. The immunological complexity of the gut associated lymphoid tissue (GALT) and its interaction and activation with probiotics and gut microbiota. Probiotic and commensal microflora (1) collaboratively perform barrier function and compete with microbial pathogens (2) for nutrients and adhesion to the epithelial surface (3). Probiotics stimulate mucin secretion by goblet cells which protects the mucosal barrier. Antimicrobial peptides (4) such as, bacteriocin and microsin S by probiotics have antagonistic effects against pathogens. Probiotics can also induce mucosal epithelial cells (5) to secrete defensins. Dendritic cells (DCs) (6) endocytose bacterial products either via extending into the enteric lumen throughout epithelial tight junctions (7), or via bacterial transit through microfold M cells (8), or via pinocytosis of probiotics/microflora by epithelial cells (5). The innate immune response (natural killer (NK) cells), are triggered by IL-12 secreted by macrophage/DCs and IL-15 by epithelial cells (9). Immune response to extracellular pathogens via B cells are activated by macrophage/DC and secrete IgE (10) which stimulate mast cells (11) contributing to innate immunity. Adaptive immune responses are triggered by DCs, macrophages, and epithelial cells which process and present probiotics that stimulate regulatory T (Treg) cells (12) and tolerogenic TGFβ, IL-10 cytokines leading to suppression of IgA secretion and effector T helper (Th)-1, Th2, Th17 and cytotoxic T (Tc) cell responses (13). If pathogens bypass the epithelial barrier, pathogenic peptides presented by DC/macrophages trigger a series of immune responses; Th1 and Th17 pro-inflammatory and Th2 anti-inflammatory responses which can lead to pathogenicity of the immune systems such as inflammation, allergic reactions, autoimmune disorders, and cancer. Such disorders can be modulated with probiotics supplementation. Thus, probiotics confer an array of benefits on epithelial barrier activities and the ensuing responses of the underlying mucosal immune and GALT systems.

2. Methodology

Searches were conducted using PubMed, Google Scholar and Medline using the following key terms: Probiotics OR lactic acid bacteria AND human health, probiotics OR lactic acid bacteria AND immune disorders OR allergies AND therapy, probiotics OR lactic acid bacteria AND immune cells, probiotics OR lactic acid bacteria AND immune cells differentiation, probiotics OR lactic acid bacteria AND inflammation, probiotics AND inflammatory diseases, probiotics AND Inflammatory bowel disease, probiotics OR lactic acid bacteria AND multiple sclerosis, probiotics AND atopic dermatitis. Studies from all years were included, specifically those published within the last 10 years. Additionally, terms such as therapeutic, health benefits, and specific immune cells (i.i. monocytes, T cells, natural killer cells, and dendritic cells) were searched in the context of probiotic effects and gastrointestinal microflora. Some review articles and their reference lists were also searched to identify related articles. Over 1,000 papers were retrieved and the most relevant were included in this article.

3. Cross talk between probiotics, intestinal epithelium and immune development

The beneficial effects of probiotics was first explained by the 'hygiene hypothesis', suggesting a lack of exposure to microbial stimuli early in childhood was the major factor behind allergic reactions (Clarke, O'Mahony, Dinan, & Cryan, 2014; Kelly, King, & Aminov, 2007). Probiotics and intestinal microflora interact and confer an array of positive effects on the epithelial layer to maintain gastrointestinal and systemic health, by interacting with the gut-associated lymphoid tissues (GALT) which mediate oral tolerance and mucosal immunity (Rhee, Sethupathi, Driks, Lanning, & Knight, 2004). Within the GALT are Peyer's patches, specialized areas of the intestinal immune system consisting of numerous lymphoid follicles surrounded by specialized epithelial cells of the mucosa-associated lymphoid tissues (M-cells), which are involved in the translocation of most antigens and bacteria, including probiotics, which transit via the intestinal lumen into the patches (Britti, Roselli, Finamore, Merendino, & Mengheri, 2006) (Figure 1, Table 1). Interestingly, specific bacteria in the intestinal microflora, such as combination of *Bacteroides fragilis* and *Bacillus subtilis,* can induce the development of GALT and pre-immune antibody repertoire (Rhee et al., 2004). Germ-free mice, that lack intestinal bacteria, display major immune deficiencies, such as, structural lymphoid deficiencies in the spleen and Peyer's patches and lymph nodes, with no germinal zones (Pollard & Sharon, 1970). Yet, after exposure to microbial antigenic stimuli such as,

Salmonella paratyphi, germinal zones are developed, and antibodies are present in the circulation (Pollard & Sharon, 1970). In addition, germ-free mice show abnormal T cells, intraepithelial lymphocytes, macrophages and dendritic cells (DCs). However, colonization with commensal flora early in life allows proper development of immune cells (D'Souza et al., 2010; Williams et al., 2006; Yamamoto et al., 2012). Thus, it is believed that establishing a balanced gut microflora and maintaining it by adequate exposure to commensal bacteria at birth and early in childhood provides challenges to the immune system by infections or other immune insults resulting in impacts on the microbiome population, colonization, and host health.

Both probiotics and commensal bacteria enforce the functions of the mucosal barrier of the GIT epithelia, induce mucus secretion, and stimulate secretion of IgA which neutralizes pathogens inside the lumen (Figure 1) (Macpherson & Harris, 2004). Correspondingly, cross talk between epithelial cells and residing epithelial immune cells are mediated and enforced by probiotics, and contribute to their effector functions (Hardy et al., 2013). Probiotics are also able to induce expression of adhesion molecules, similarly important for residing immune cells for their regulatory functions. Probiotics stimulate the innate immune system, antigen presenting cells (APC) and natural killer (NK) cells in both mice and humans (Mortha & Diefenbach, 2011). Adaptive immunity is stimulated by probiotics, such that IgG and IgA antibodies are produced in response to probiotic consumption, as demonstrated with *L. acidophilus, L. bulgaricus and B. bifidum* (Figure 1) (Delcenserie et al., 2008; Michałkiewicz et al., 2003). In addition, macrophages, $CD8^+$ T cells, Treg cells and cytokines (*i.e.* interferon (IFN)-gamma and interleukin (IL)-10) are stimulated by probiotics (Fink et al., 2007). If APCs are exposed to probiotics or commensal bacteria it leads to presentation of harmless peptides to T cells subsequently inducing Treg cells, to produce anti-inflammatory cytokines including, transforming growth factor beta (TGF-beta), IL-10 and retinoic acid. In addition, Treg cells suppress effector Th1, Th17 and cytotoxic T (Tc) cells and IgA secretion (Josefowicz et al., 2012). However, if APCs present invasive pathogenic peptides, it leads to the initiation of effector, pro-inflammatory Th1/Th17 responses. Thus, it is believed that probiotic bacteria can control the 'on/off switch' of immune responses in a strain-dependant manner, modulating the host immune system at the mucosal level. Lack of sufficient probiotic bacteria and the subsequent stimulatory impact they have on the immune system, leads to inadequate or inappropriate immune modulation. Insufficient probiotic bacteria alone, or inadequacy, together with stimulation of immune system by invasive pathogens (which bypass the mucosal barrier), are associated with a range of immunopathogenic disorders such as, allergies, asthma,

atopic disorders, inflammatory bowel diseases (IBD), cancer, type-2 diabetes and autoimmune disorders (Table 1) (Aumeunier et al., 2010; Hardy et al., 2013; Knight, Campbell, & Rhodes, 2008; Ott & Schreiber, 2006).

4. Probiotic organisms and immune cells

Probiotic bacteria have numerous beneficial immune and health effects. They not only enhance the bioavailability of nutrients and moderate health, they also aid in regulating the gastrointestinal ecosystem and stimulate immunomodulatory properties to a number of immune cells (Table 1, Figure 1, 2).

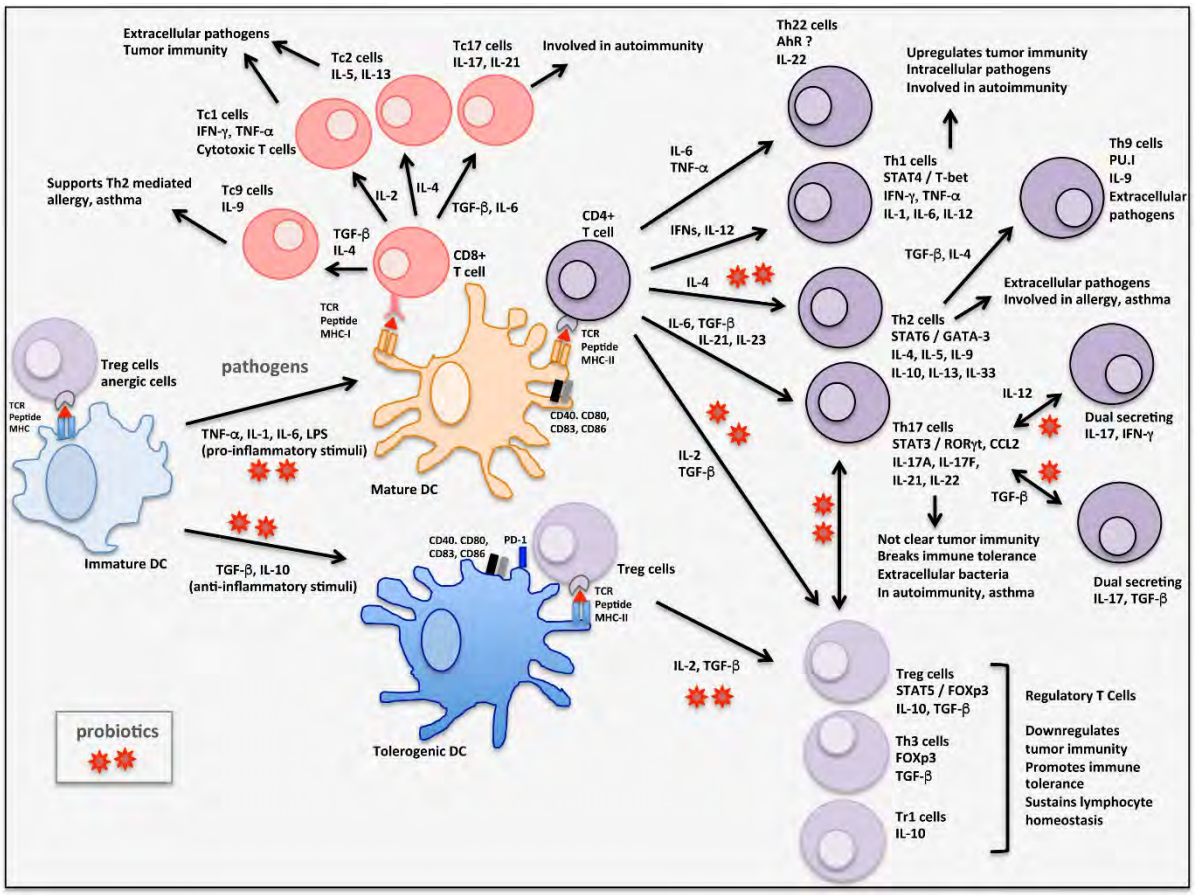

Figure 2. Immunomodulatory effects of probiotics stimulating T helper (Th)-1, Th2, Th17 cells or T regulatory (Treg) cells. Immature dendritic cells (DC) either mature following pro-inflammatory stimuli (IL-1, IL-6, lipopolysacharride (LPS), TNF-alpha or pathogens) or become tolerogenic DCs in the presence of anti-inflammatory stimuli TGF-beta and IL-10. The differentiation of immature DCs into mature or tolerogenic DCs is also regulated in the presence of probiotics. Mature DCs confer an array of stimulatory functions whereby they stimulate Th or Treg cells depending on the cytokine produced. Mature DCs interact with naïve CD4+ T cells and

depending on the resulting cytokine produced by the CD4+ T cells they differentiated into either, (i) inflammatory Th1 (secrete IL-1, IL-6, IL-12, IFN-gamma, TNF-alpha) or Th17 (secrete IL-17A, IL-17F, IL-21, IL-22) cells; (ii) anti-inflammatory Th2 (secrete IL-4, IL-5, IL-9, IL-10, IL-13, IL-33) or Th9 (secrete IL-9) cells; (iii) regulatory Treg (secrete IL-10, TGF-beta), Th3 (secrete TGF-beta) or Tr1 (secrete IL-10) cells or (iv) Th22 cells which secrete IL-22. In addition, Th17 cells can be further divided into dual secreting cells of either IL-17, IFN-gamma or IL-17, TGF-beta. Probiotics have been shown to regulate Treg cells and/or Th2 cells depending on the strain and disease. Tolerogenic DCs interact with T cells and can directly stimulate Treg cells in the presence of IL-2 and TGF-beta (or probiotics). Furthermore, mature DCs stimulate CD8+ T cells into Tc1, Tc2, Tc9 or Tc17 cells however, it is not clear whether probiotics are involved in their differentiation / stimulation.

4.1. Dendritic cells

DCs play a key role in directing immune responses to self and non-self antigens. Upon endocytosis of antigens, DCs mature functionally and phenotypically. As mature cells, they prime T cells towards Th1 or Th2 phenotypes, however, in their immature state (tolerogenic DCs) they cause the deletion of T cells or stimulation of Treg cells (Figure 2, Table 2) (R. M. Steinman, Hawiger, & Nussenzweig, 2003). The intestinal microbiota and ingested probiotics can interact with the host's innate and adaptive immune system, regulating cell differentiation in the gut which is involved in sustaining immune tolerance. In particular, microbiota are able to activate distinct tolerogenic DCs in the gut and consequently drive Treg cell differentiation (Foligne et al., 2007). DCs also endocytose probiotics and present short antigenic peptides to T cells, stimulating Th1, Th2, Th17 and/or Treg cells. Due to these roles, in recent years considerable focus has been directed towards the anti-inflammatory properties of probiotics e.g. *L. rhamnosus and L. delbrueckii* and the induction of tolerogenic DCs and their effect on the stimulation of Treg cells (Smelt et al., 2012). In particular, significantly reduced co-stimulatory cell surface molecules (CD80, CD83, CD86), IL-12 and NF-kappa B were noted and increased expression of indoleamine 2,3-dioxygenase (IDO) and IL-10, suggesting that probiotics are able to modify properties of DCs to modulatory cells, which may contribute to immune tolerance and immune balance (Esmaeili et al., 2018). Recently, two novel *Lactobacillus* probiotics strains (s193 and s292) isolated from Funazushi (a traditional Japanese fermented food) were shown to increase beta-8 integrin on mesenchymal DCs which strongly activated $CD4^+$ T cell differentiation into Treg cells; monoclonal antibodies against beta-8 integrin are able to block the differentiation of Treg cells (Okada et al., 2018). DCs co-cultured with *L. reuteri* and *L. casei*, although displaying different cytokine profiles (IFN-gamma production), activate both Th1 and Treg cells. However, DCs stimulated with *L. reuteri*

and *L. casei* and combined with TNF-alpha stimulate only Treg cells (Sytze de Roock et al., 2011). On the other hand, DCs exposed to *L. lactis* do not stimulate Treg cells, showing the importance of the Lactobacilli strain used in the ensuing immune response. Oral administration of *B. infantis* in mice stimulates DCs to a maturation state and $CD103^+$ tolerogenic DCs accumulate in the GALT, induces Treg cells and suppresses Th2-biased responses (L. Fu, Song, Wang, Fu, & Wang, 2017). Given the roles of DCs and the intestinal residing DCs in endocytosing bacteria, it is clear that DCs play a central part in immune homeostasis in the healthy intestine and in the pathology of IBD (Farache, Zigmond, Shakhar, & Jung, 2013; A. J. Stagg, Hart, Knight, & Kamm, 2004). Thus, understanding the effect of stromal cells and microbial signals on DC function are important as the manipulating regime of DCs through probiotics, nutrition and microbiota, leading to therapeutic modalities against inflammatory diseases.

4.2. Monocytes and macrophages

Monocytes are present in peripheral blood which differentiate to tissue macrophages and myeloid lineage DCs. Intestinal microbiota and ingested probiotics can interact with macrophages for beneficial immune effects (Farache et al., 2013). In fact, IL-12 secreted by macrophages stimulate $CD4^+$ T cells and NK cells to secrete IFN-gamma, resulting in the generation of pro-inflammatory Th1 cells. Interestingly, IFN-gamma and IL-12 production seem to be controlled in a positive feedback loop, as IFN-gamma in turn, stimulates production of IL-12. This loop of IFN-gamma and IL-12 production can be deleterious, as it may result in uncontrollable expression of cytokines and possible shock. However, co-culturing peripheral blood mononuclear cells (PBMCs) with selected bacteria (LAVRI-A1, *L. rhamnosus* GG, *Bifidobacteria* and *L. acidophilus*) induce anti-inflammatory cytokines IL-4, IL-10 and TGF-beta (Donkor, Ravikumar, et al., 2012a; Donkor, Shah, Apostolopoulos, & Vasiljevic, 2010). These cytokines inhibit the production of IL-12, IFN-gamma and other pro-inflammatory cytokines which are beneficial for autoimmune and allergic responses. In addition, probiotics from dairy source such as LAB *L. gasseri* strains could induce IFN-alpha production by monocytes with *L. gasseri* DSM20243T (Kitazawa et al., 1994), and 3 strains of LAB (*L. delbrueckii* ssp. *bulgaricus*, *B. bifidum, L. acidophilus*, ; trilac) being the most potent (Gutkowski et al., 2010). In addition, $CD4^+$ T cell proliferative responses, high levels of IL-10 and TNF-alpha (not IL-12 and IFN-gamma) are induced, as well as increased expression of cell surface markers (CD14, CD80, HLA-DR, ICAM-1, IL-2 receptor) (Gutkowski et al.,

2010). The probiotic *L. paracasei* DG commonly used in commercial probiotic products, shows immunostimulatory properties by increasing the expression of TNF-alpha, IL-6, IL-8 and CCL20 by human monocyte cell line, THP-1 (Balzaretti et al., 2017) . Similarly, it has recently been demonstrated that *S. thermophilus* induced differential cytokine secretion of human monocyte U937 cell line; IL-4 and IL-10 secretion important for anti-inflammatory responses, TNF-alpha and IL-6 necessary for stimulation of the innate immune response, as well as CXCL8 (IL-8) and GM-CSF required for cell recruitement at sites of inflammation [Dargahi et al., submitted]. Hence, the use of commensal LAB results in an anti-inflammatory profile and activation of monocytes, which is beneficial in suppressing pathogenic-induced pro-inflammatory responses in microbiota (via IL-10 production) and simultaneous induction of anti-microbial effects (via TNF-alpha stimulation and monocyte induction).

4.3. NK cells

NK cells play a keyrole in innate immunity and are cytotoxic to tumors. NK cells have a regulatory role in the development of allergic respiratory disease. *L. casei* subspecies Shirota administered in mice intraperitoneally, or intravenously leads to activated splenic NK cells, peritoneal exudate cells and thoracic exudate cells, respectively, but not via oral administration. In contrast, *L. casei* Shirota administered orally to newborn mice is able to activate splenic NK cells (Matsuzaki & Chin, 2000). Thus, oral consumption of probiotics, such as *L. casei*, early in life boosts the innate immune system (D'Souza et al., 2010). In human PBMCs, *L. casei* subspecies Shirota stimulates pro-inflammatory cytokines IL-12 and TNF-alpha; the type of immune response required for a DC-NK cell interaction and defence mechanism (K. Shida, T. Suzuki, J. Kiyoshima-Shibata, S. Shimada, & M. Nanno, 2006a). Another study showed that *Lactobacillus* strains were able to stimulate IL-12 secretion by DCs, resulting in activation of NK cells and subsequent secretion of IFN-gamma, which not only leads to innate immune responses, but also activation of the adaptive immune responses (Sytze de Roock et al., 2011). Upon stimulation by *Bifidobacterium*, high levels of IL-10 is produced which neutralizes IL-12 induced by *Lactobacillus* strains (Sytze de Roock et al., 2011). This suggests that co-administration of probiotics might not be favorable when used for immunomodulatory therapeutic methods for NK-DC mediated type of immune responses.

Results from such studies suggest that *Lactobacillus* strains may initiate cellular immune responses whilst *Bifidobacterium* strains can induce polarization towards anti-inflammatory and Treg cells. Thus, *Bifidobacterium* might be the probiotic of choice to be used to manage or prevent inflammatory immune disorders such as, allergies and autoimmune conditions, although, *Lactobacillus* strains may be beneficial for anti-tumor immunity. In addition, LAB have been shown to stimulate T/NK cells via induction of IL-12/IFN-gamma, whereas, other strains can either suppress or boost Treg/Th2 responses via IL-4, IL-5 and IL-10 (K. Shida, T. Suzuki, J. Kiyoshima-Shibata, S. I. Shimada, & M. Nanno, 2006b). Understanding the cytokine profiles induced following probiotic exposure and their immune modulating properties can provide a clear path for managing immunopathologies (Dong, Rowland, & Yaqoob, 2012; Hardy et al., 2013). These studies infer that host innate immune responses are important in relation to the development of some diseases and inducing it by probiotic bacteria can help to enhance immunity and health. *L. casei* strains have established safety and functional characteristics and are extensively used in probiotic dairy products, and have long been used as staple food ingredients in many countries including Japan and Europe.

4.4. Regulatory T cells

Evidence that Treg cells play an active role in immune tolerance was established in the 1970s (Gershon & Kondo, 1970), at which time they were referred to as suppressor T cells. During the late 1980's research into the role of these cells in immune tolerance dwindled, however the identification of cell-surface markers in the mid-1990s led to a resurgence in interest (Sakaguchi, Sakaguchi, Asano, Itoh, & Toda, 1995). In 1994, Tregs were described as key cells in the establishment of oral tolerance (Y. Chen, Kuchroo, Inobe, Hafler, & Weiner, 1994), and were proposed as a potential target for the treatment/prevention of diseases that are caused by over reaction of the immune system (*i.e.* autoimmunity, inflammatory metabolic diseases, asthma and allergy) all of which have exhibited a global increase, especially in western countries (Y. Chen et al., 1994; McKenzie, Tan, Macia, & Mackay, 2017; Thorburn, Macia, & Mackay, 2014). Tregs are responsible for the lack of immune response to specific antigens (self-peptides); a process known as immune tolerance, which is critical for the maintenance of immune homeostasis (Zeng, Zhang, Jin, & Chen, 2015).

There is accumulating evidence that suggests that probiotics, are not only beneficial for the treatment of Th1 or Th2 mediated inflammatory disorders, but are also able to induce

beneficial Treg cells to modulate immune balance (Figure 2). Indeed, *L. acidophilus* suppresses IgE allergic responses to a model antigen in mice as well as downregulating the secretion of IFN-gamma, IL-4 and IL-10, and significantly increase the levels of IgA, TGF-beta and Treg cells (Torii et al., 2007). *L. plantarum* WCFS1 is also associated with $CD103^+$ DC infiltration in the intestine, increases Th2 cytokines and is involved in the generation of Treg cells in mice (Bermudez-Brito et al., 2018). More recently, the combination of probiotics, zinc, and coenzyme Q10 synergistically reduced rheumatoid inflammation in mice by significantly downregulating IgG in serum, decreased the expression of pro-inflammatory cytokines (IL-1, IL-6, IL-17, TNF-alpha) and vascular endothelial growth factor in the joint synovium and upregulated Treg cells (S. Y. Lee et al., 2018). *L. casei* administration in mice with *E. coli* induced intestinal inflammation is associated with $CD4^+CD25^+Foxp3^+$ Treg cells in the spleen and mesenteric lymph nodes with corresponding decreased $CD4^+IL\text{-}17^+$ Th17 cells and RORγt mRNA levels (K. Wang et al., 2017).

Thus, *L. casei* could have possible therapeutic implications against intestinal inflammation by modulating the Treg/Th17 balance. However, intragastric administration of *L. casei* BL23 stimulates the expression of $CD4^+Foxp3^+$ Treg cells in addition to $ROR\gamma t^+$ Th17 cells, termed type-3 Treg cells which are believed to control luminal inflammatory disorders by suppressing inflammation (Cortes-Perez, Lozano-Ojalvo, Maiga, Hazebrouck, & Adel-Patient, 2017).

LAB from *Bifidobacteria* strains, can also prime expression of TGF-beta and IL-10, and increase the number of Treg cells, which is associated with suppressive function/tolerance (L. L. Zhang et al., 2010). Additionally, administration of *B. animalis* subsp. *lactis* 420 (B420) or *L. salivarius*-33 (Ls-33) to mice show reduced levels of pro-inflammatory cytokines TNF-alpha, IL-6, IL-17 and monocyte chemoattractant protein-1 (MCP-1) and skewed towards IL-10 anti-inflammatory cytokine profile and generation of Treg cells (Danilo et al., 2017). Likewise, *B. infantis* 14.518 fed with shrimp allergies, show DC-dependent stimulation of Treg cells (L. Fu et al., 2017). It is clear that an array of probiotics exert their beneficial effects and mediate an immune balance by their stimulation of Treg cells.

SCFAs produced by commensal microbiota following fermentation of dietary fibers, have been shown to expand and differentiate intestinal Treg cells (Atarashi et al., 2013). In particular, butyrate initiates the differentiation of Tregs (Singh et al., 2014) as well as

suppressing the onset of colon cancer and reducing allergic responses in the lung of mice (Singh et al., 2014; Trompette et al., 2014). In mouse models of asthma and colitis (newborn and mature) which were orally subjected to a mixture *L. casei, L. lactis*, *L. acidophilus*, *B. bifidum* and *B. lactis* daily, showed a lower proportion of $CD4^+$ and $CD8^+$ T cells and higher Treg cells; these effects were shown to be mediated by SCFAs (acetate, propionate and butyrate) (Consonni et al., 2018). However, in other studies it was noted that butyrate induces the expression of the transcription factor T-bet resulting in IFN-gamma secreting T cells and not Treg cells. Thus, butyrate may exert either beneficial or detrimental effects on the mucosal immune system. Further studies are required to determine the dose of butyrate and its mechanism by which it induces beneficial Treg cells.

4.5. Th17 cells

Th17 cells secrete IL-17 (IL-17A), IL-17F, and IL-22 and eliminate external pathogens mainly at epithelial mucosal sites (Mangan et al., 2006; Zheng et al., 2008). At mucosal sites, secretion of anti-microbial defensins is dependent on the stimulatory activities of IL-17, IL-22 and differentiation and stimulation of neutrophils which is dependent on these cytokines. However, it is believed that excessive or persistent responses of Th17 cells can initiate/drive the onset of inflammatory diseases, and as such, Th17 cells together with Th1 cells, are generally responsible for the pathophysiology of autoimmune diseases, asthma, allergy and the development and progression of tumors (Kryczek et al., 2008; Wilke, Bishop, Fox, & Zou, 2011). Similar to human Th17 cells, murine Th17 cells also play a critical role in numerous mouse autoimmune disease models such as, experimental autoimmune encephalomyelitis (EAE) (Dargahi et al., 2017; Zhao et al., 2018).

Intestinal microflora are shown to regulate and maintain the quantity and function of DCs, through which they are able to differentially modulate naïve CD4+ T cell responses towards specific Th cell populations (Figure 2). Studies in germ free mice show that the number of $CX3CR1^+$ DCs is reduced which favorably induces naïve $CD4^+$ T cell differentiation towards Th1 and Th17 (Niess & Adler, 2010). In addition, filamentous bacteria affect the whole microflora of the GIT immune system which subsequently stimulate Th17 cells residing in lamina propria (Atarashi et al., 2008). The number of Th17 cells in the GIT lamina propria is associated with the presence of microbiota, thus, in germ free mice, the percentage of Th17 cells is very low (Ivanov et al., 2008). As such, the severity of EAE symptoms and autoimmune

arthritis are reduced due to lower levels of Th17 cells and lower IL-17 and IFN-gamma cytokines (H. S. Lee et al., 2008; H. J. Wu et al., 2010). On the contrary, colonization of segmented filamentous bacteria which induce high levels of local Th17 cells in the lamina propria, prevents diabetes in non-obese diabetic mice (Kriegel et al., 2011). Thus, Th17 cells and IL-17 play differential roles depending on the disease model. Commensal microbiota are also able to stimulate secretion of IL-25 (IL-17E) in the GIT that prevents expression of IL-23; without IL-23, pathogenic Th17 cells cannot survive (Zaph et al., 2008).

Numerous animal models as well as clinical trials have established beneficial effects of probiotics in IBD due to anti-inflammatory properties as a result of down-regulating the expression of IL-17 (Fitzpatrick, 2012; Tanabe, Kinuta, & Saito, 2008). Several studies with colitis-induced mouse models have shown that the probiotics *B. longum* subsp. *infantis*, *B. breve*, *L. acidophilus*, *L. gasseri* A5 *B. longum* and *S. thermophilus* ST28, are able to down-regulate the production of IL-17 leading to relief of colitis symptoms (Ghadimi, Helwig, Schrezenmeir, Heller, & de Vrese, 2012; Miyauchi et al., 2013; Ogita, Tanii, Morita, Suzuki, & Tanabe, 2011; Owaga et al., 2015). Two individual studies determined the effect of blocking IL-17 by using novel immunosuppressive drugs 4SC-101 (Fitzpatrick et al., 2010) and vidofludimus (Fitzpatrick, Small, Doblhofer, & Ammendola, 2012) to compare with probiotic effects; drugs similarly alleviated severe colitis in mice by targeting IL-17 and confirmed the suppressive mechanisms of probiotics. IL-23, also associated with development, maintenance and polarization of Th17, is another activator for inflammatory conditions (L. Chen et al., 2015); in fact, suppressing the IL-23/IL-17 axis, is now a promising target for probiotic treatments in the prevention and management of IBD (Owaga et al., 2015). Studies on the effects of *B. breve* and *L. rhamnosus* GG on colitis-induced mouse models revealed that LPS-induced IL-23 expression by intestinal cells, was reduced as a consequence of probiotic administration (Ghadimi et al., 2012; Owaga et al., 2015).

In *in vitro* studies using intestinal epithelial cell lines, *B. longum* subsp. *infantis* JCM 1222 was shown to suppress the expression of co-stimulatory cell surface molecules, CD40 and CD80 as well as IL-17A at mRNA and protein levels (Miyauchi et al., 2013). In another comparative study on the effect of *B. breve* and *L. rhamnosus* GG, in human intestinal HT-29/B6 or T84 cells and PBMCs, reduced CD40 and IL-17A mRNA expression induced by LPS stimulation (Ghadimi et al., 2012). Similarly, *B. longum* subsp. *infantis* (Miyauchi et al., 2013) is able to reduce the expression of RORγt and simultaneously suppress IL-17A

production following oral administration in a mouse model of colitis. Given the vital role of RORγt as a specific transcription factor for differentiation of Th17 cells, it is considered a promising candidate to target for probiotic therapeutics in order to inhibit of Th17 cells and subsequently alleviate inflammatory conditions. Furthermore, feeding of *L. acidophilus* or *Bacillus clausii* in ovariectomized mice for 6 weeks was able to skew Th17 cells towards a Treg phenotype by reducing pro-inflammatory IL-6, IL-17 and TNF-alpha and increasing expression of IL-10 (Hamid Y. Dar et al., 2018; H. Y. Dar et al., 2018). Hence, there is an increasing value of the use of probiotics as a driver of treating many inflammatory disorders by skewing Treg/Th17 balance.

5. Probiotics, allergy and autoimmune diseases

Studies in animal and in human clinical trials suggest that probiotics are able to prevent or treat allergies (atopic dermatitis and allergic rhinitis) and autoimmune diseases (IBD and MS). It is believed, based on the 'hygiene hypothesis' that any change to the human GIT and microflora results in increased risk of such diseases. Regulation of gut microbiota by the consumption of probiotics has been shown to influence the development of the mucosal and systemic immune response (Figure 1, 2) and alter immune homeostasis and immune profile for the beneficial effects allergies and autoimmune disorders.

5.1. Probiotics and allergies

Food allergy is described as the activation of mast cells or basophils and production of IgE in response to specific food proteins (Kraneveld, Sagar, Garssen, & Folkerts, 2012). This can result in life-threatening food hypersensitivity reactions with symptoms usually appearing within minutes of exposure; a condition quite distinct from food intolerances, such as Coeliac disease (Hajeb & Selamat, 2012; Lopata & Jeebhay, 2013). Around 1–2% of adults and 5–7% of children suffer from some type of food allergy, which has significantly increased in prevalence over the last 20 years (Burks et al., 2012). Although allergy symptoms are manageable with medications, no cure is available for food allergy except for strict avoidance of allergy triggering food. Interestingly, it is now widely accepted that early life exposure to bacteria such as probiotics results in activation of APCs and immune homeostasis. Probiotics are able to stimulate the immune system to express pro- and anti-inflammatory cytokines differentially in a strain-dependent manner. As previously discussed, probiotics have been

shown to modulate immune responses *in vitro* and *in vivo*, and can skew immune responses towards Th1/Th2 and Treg phenotypes and play important role in management or prevention of immune-mediated pathologies, such as allergies (Barberi et al., 2015). Oral consumption of killed *L. casei* Shirota has been reported to stimulate the production of Th1 cytokines, resulting in decreased stimulation of allergic IgE antibodies against ovalbumin (OVA) in experimental allergic mouse models with allergy to OVA (Matsuzaki & Chin, 2000). Thus, the use of probiotic bacteria to shift mucosal immunity towards Th1 responses supports their use as a viable approach for the treatment of allergic disorders (Matsuzaki & Chin, 2000). Oral feeding of *S. thermophilus*, *L. fermentum* and yeast (such as zymosan and *Saccharomyces cerevisiae*) (Fonseca et al., 2017; Sadakane, Ichinose, Nishikawa, Takano, & Shibamoto, 2016), also, differentially stimulate immune responses. In one study, mice were fed with yeast or *L. fermentum*, then fed with OVA, then vaccinated with OVA (Matsuzaki & Chin, 2000). The probiotic-exposed mice responded more efficiently to vaccination with OVA than mice with no pre-feeding of either probiotics or OVA, or the mice which were only given OVA. Nevertheless, vaccination in mice that were given either yeast or *L. fermentum* followed by yeast and OVA or *L. fermentum* and OVA showed significantly supressed antibody responses as a result of vaccination with OVA. This suggests that, although feeding the antigen alone appears to prime the onset of immune responses, co-feeding the same antigen with probiotics can repress both cellular and humoral immunity (Matsuzaki & Chin, 2000). Similarly, studies showing the effects of probiotic treatment (*L. casei, L. lactis*, *L. acidophilus*, *B. bifidum* and *B. lactis*) on the suppression in mice of experimental OVA-induced asthma, an allergic airway disease model, either at the time of birth or at later developmental stages, resulted in decreased numbers of Th1,Th2 and $CD8^{+}$T cells in the airways compared to control mice (Nunes et al., 2018). This data may provide insights into an effective treatment approach for controlling allergic reactions.

In humans, *B. longum* and *L. acidophilus* in milk or yogurt, if used as probiotics powder, capsules, or even heat-killed bacteria, showed an improvement in alleviating the severity on the clinical scales for allergic rhinitis and asthma symptoms compared to placebo (Vliagoftis, Kouranos, Betsi, & Falagas, 2008). In children with allergies and recurrent respiratory infections, *B. clausii* was shown to beneficially modulate cytokine profiles, and induce Treg cells in parallel with increased levels of IL-10 and TGF-beta (Ciprandi, Vizzaccaro, Cirilio, & Tosca, 2005). Similarly, PBMC from subjects with allergic rhinitis who consumed yogurt or skimmed milk (containing *L. delbruekii,* sub *bulgaricusand, S.*

thermophilus, *L. acidophilus* and *Bifidobacterium*) showed higher IFN-gamma responses and improved symptom score (G. Yang, Liu, & Yang, 2013). It is clear that the use of probiotics is a novel approach in allergic conditions due to their great potency in polarizing T cells in the gut towards Treg cells.

5.2. Probiotics and atopic dermatitis

Atopic dermatitis is a chronic inflammatory skin disorder which is predominantly noted in infants and toddlers. Although the acute phase of atopic dermatitis is dominated by Th2 (IL-4, IL-5, IL-13) and Th22 cells, during the chronic phase, Th1 cells (IFN-gamma and IL-12) take the lead that results in atopic dermatitis (Rø et al., 2017; Shin, Chung, & Seo, 2016). In a mouse model of atopic dermatitis, consumption of a probiotic mixture of 7 strains of *Bifidobacterium* and LAB for 8 weeks resulted in reduced atopic skin irritation and increased Treg cells (Shin et al., 2016). In another study, Treg cells were increased locally in the skin of mice following supplementation of *L. casei* (Hoeppli, Wu, Cook, & Levings, 2015). Similarly, an increase in Treg cells were noted in the spleen after *L. reuteri* supplementation in mouse models with asthma (Russell et al., 2013). In addition, Tregs have been shown to be induced from human PBMCs following exposure to probiotic species (*L. acidophilus*, *B. lactis*, and *L. plantarum*) with *L. acidophilus* being the most potent (Sytze de Roock et al., 2011; S. De Roock et al., 2010).

In humans, a randomized controlled study of probiotic consumption (*B. animalis* subsp. *lactis* Bb-12, *L. rhamnosus* GG, and *L. acidophilus* La-5) in pregnant women and their children, showed reduced incidence of atopic dermatitis and reduced Th22 cells (Rø et al., 2017). Additionally, ingestion of *L. rhamnosus* GG alone in pregnant women with strong family history of allergic rhinitis, eczema or asthma, showed significant delay in developing atopic dermatitis in their infants during the first six months of delivery, which correlated to increased levels of IL-10 cytokine (Chouraqui et al., 2008). Likewise, the combination of *L. rhamnosus* GG and *B. lactis* throughout pregnancy and breastfeeding reduces the risk of atopic eczema and allergic sensitization in children (Kalliomäki et al., 2001), whereas a mixture of probiotics (*Lactobacillus* GG, *B. breve*, *L. rhamnosus* LC705, and *Propionibacterium freudenreichii* ssp. *Shermanii* JS) failed to reduce the risk of atopic eczema, due to the strain dependency of probiotic bacteria (Kalliomäki et al., 2001). In a systematic review and meta-analysis studies of pediatric atopic dermatitis it was noted that probiotics are effective in

preventing this condition (J. Lee, Seto, & Bielory, 2008). Furthermore, cutaneous exposure to a lysate of probiotics (*Vetreoscilla filiformis*) alleviates skin inflammation and induces IL-10 secreting tolerogenic DCs and Treg cells and reduces Th1 pro-inflammatory cells and cytokines (Volz et al., 2014).

5.3. Probiotics and inflammatory bowel disease

IBD, including Crohn's disease (CD) and ulcerative colitis (UC), is a major human health problem (Vadasz et al., 2015). IBD is a group of chronic inflammatory disorders of the GIT, which is currently incurable and results in intestinal inflammation, severe diarrhoea, fatigue, pain and subsequent weight loss. A link has been established between insufficient intestinal probiotics (dysbiosis) and the development of CD and UC (Xun, Zhang, Xu, Chen, & Chen, 2018). In fact, mucosal-associated microbiota of twin individuals with CD has been shown to have lower abundance of *Faecalibacterium prausnitzii* and significantly higher levels of *E. coli,* compared to corresponding healthy twin siblings (Willing et al., 2009). Such dysbiosis of intestinal microbiota is believed to contribute to pathophysiology of CD and UC through an imbalance between intestinal microbiota and mucosal immunity, leading to inflammation in the intestine. Interestingly, experimental colitis, in mice, caused by dysbiosis, is corrected by consuming *F. prausnitzii* orally, which leads to anti-inflammatory effects and reduction of severity of colitis (Sokol, Barton, Farr, & Medzhitov, 2008). Consumption of probiotics in general has been increasingly used as a means of preventing and/or managing CD and UC by restoring damaged intestinal mucosal barrier (Oelschlaeger, 2010). In fact, suppression of effector responses and induction of Treg cells has been shown in mice, following consumption of *Clostridium* strains, and their consumption in early life is also beneficial in the protection against colitis (Atarashi et al., 2008). Consumption of *B. infantis* by mice results in increased number of Treg cells and leads to prevention of *Salmonella typhimurium* infection. Furthermore, Bifidobacteria increases gastrointestinal defense against enterohaemorrhagic pathogenic *E. coli* by generating acetate (Fukuda et al., 2011). Recently it was shown that SCFAs produced through the fermentation of indigestible dietary fibers by *Bifidobacteria* and other anaerobic bacteria (Nunes et al., 2018) resulted in the differentiation of colonic Treg cells. *L. rhamnosus* and *B. infantis* protected the host against the development of colitis via the effect of their SCFAs products on the immune system (van der Kleij, O'Mahony, Shanahan, O'Mahony, & Bienenstock, 2008). Additionally, *L. casei, L. lactis*, *L. acidophilus*, *B. bifidum* and *B. lactis* also generate SCFAs (acetate, propionate and butyrate)

which induce the differentiation of Treg cells in the colon, subsequently suppressing the development of DSS-induced experimental colitis in mice (Miyauchi et al., 2013).

Interestingly, while *Citrobacter rodentium* can cause colitis and subsequently IBD, commensal microflora of the gut can provide protection against invasive pathogens like *C. rodentium* through colonization of the host gut (Bonaz, Bazin, & Pellissier, 2018; Y. Jiang et al., 2016). In a mouse model of *C. rodentium*-induced colitis, administration of *L. plantarum* A, *L. acidophilus* ATCC 4356 and *L. rhamnosus* ATCC 53103 increased expression of $CD11c^+$ DCs and Treg cells, whilst down-regulating pro-inflammatory cytokines (IL-17, IFN-gamma and TNF-alpha) and protection against colitis (Nikoopour & Singh, 2014). Hence, individuals experiencing gut dysbiosis, probiotics and gut microbiome are considered therapeutic interventions to restore gut microbial balance to its normal state by balancing the immune system (Bäckhed et al., 2012). To this end, fecal microbiota transplantation has shown to be effective in colitis patients through rapid restoration of the composition of intestinal microbiota. The specific mechanisms are not clear, however, the intestinal microbiota is restored to be similar to that of the donor (Brown, 2014).

Bifidobacteria have also been shown to contribute to intestinal homeostasis and diminish inflammation. *Bifidobacteria* mixture was tested on DC functionality from different sources; children with IBD, DCs from PBMCs of patients with CD, UC, and healthy controls. DCs were pre-treated with probiotics and incubated with *E. coli* fluorochrome-conjugated particles or DQ-ovalbumin (DQ-OVA) (Strisciuglio et al., 2015). Following incubation with probiotics, DCs from CD children had a higher uptake of bacterial particles and DQ-OVA processing; whereas DCs from the other 2 groups showed no significant changes. DCs from CD children also showed higher TNF-alpha and no effect on IFN-gamma and IL-17 (Strisciuglio et al., 2015). This shows that *Bifidobacteria* can significantly increase antigen uptake and processing of DCs which are from patients with CD, in which DCs demonstrate a decreased autophagic function. Probiotics had a lesser effect on antigen uptake or autophagy by DCs sourced from CD/UC and healthy people. Enhanced antigen sampling/uptake and processing enhancement could be a viable approach to solving innate immunity deficiency in GIT and a method to reducing uncontrolled bacterial growth in the intestine of IBD children.

Probiotics have also shown to be effective against specific infections in infants and children, including infectious diarrhoea, traveller's diarrhoea, and infants necrotizing

enterocolitis and *Helicobacter pylori* (Ritchie & Romanuk, 2012; Wolvers et al., 2010). Neonates with extremely low birth weight due to necrotizing enterocolitis have marked reduced incidence and severity following probiotics consumption in particular combinations of, *B. infantis*, *S. thermophilus*, *B. bifidus* and *L. acidophilus* (D'Souza et al., 2010). Caveolin-1 regulates nitric oxide (NO) signaling which is responsible for the pathogenesis of necrotizing enterocolitis, and in formula-fed neonatal rats, the intestinal caveolin-1 and NO signalling are deficient (D'Souza et al., 2010). Probiotics fed to babies, improves the survival of the microorganism in the intestine and beneficially improves caveloin-1 and NO signalling and growth factors in the terminal ileum.

5.4. Probiotics and multiple sclerosis

Chronic inflammation has been generally accepted as a common hallmark of neuro-degenerative diseases, such as, MS, Alzheimer's disease and Parkinson's disease (Dargahi et al., 2017; Nemazannikova, Mikkelsen, Stojanovska, Blatch, & Apostolopoulos, 2018). Gut microbiota associate with central nervous system (CNS) (Catanzaro et al., 2015), as there is cross-talk between the GIT, the CNS and the immune system, which is known as the gut-brain axis (Catanzaro et al., 2015). It is believed that any dysfunction in the gut-brain axis is associated with the pathogenesis of a number of diseases inside and outside the GIT. Comparisons of germ free mice to mice that have been exposed to probiotics, pathogenic bacteria or antibiotics have shown that microbiota is involved in communication between GIT and brain. This further demonstrates the role of microbiota in the body's defence, immune regulation and incidence of autoimmune diseases (Catanzaro et al., 2015).

EAE, an animal model of human MS has been used to study the effects of probiotic microorganisms. One of the safe and appropriate ways to induce tolerance towards peripheral T-cell in autoimmune diseases like MS, is the oral administration of autoantigens (Buerth et al., 2016; Maassen et al., 2003). A food based probiotic yeast, *Candida utilis,* fused with an immunodominant myelin epitope (MOG_{35-55})-peptide as carrier, when administered in mice, protects mice against the development and clinical symptoms of EAE (Buerth et al., 2016). In addition, administration of heat-inactivated *C. utilis* also resulted in reduction of disease severity in animals which indicated that tolerance effect is independent of viability of the yeast. Rechallenge with MOG_{35-55} resulted in decreased disease severity associated with reduced

cellular inflammation in the spinal cord, demyelination, lower T cell proliferation and higher Treg cells in the lymph nodes. These results showed that using a food-grade fungus, *C. utilis,* with surface-infused immunogenic MOG_{35-55} peptide can successfully stimulate oral tolerance against this epitope in EAE (Buerth et al., 2016); thus *C. utilis* is effective in modulating immune responses. Similarly, oral and intranasal administration of recombinant Lactobacilli expressing myelin antigens also showed reduced EAE (Maassen et al., 2003). Furthermore, a panel of recombinant Lactobacilli were constructed to produce some of human and guinea pig myelin proteins and peptides, such as proteolipid protein peptide 139–151 ($PLP_{139–151}$) and myelin basic protein (MBP) and. In this study the effect of these *Lactobacillus* recombinants on inducing intranasal and oral tolerance in EAE animal models were studied. Soluble cell extracts of *Lactobacillus* transformants were given to Lewis rats intranasally three times prior to induction of EAE. In order to induce oral tolerance, animals were given live recombinant Lactobacilli for 20 days. Within 10 days of first oral consumption, rats were induced for EAE induction. Using extracts containing guinea pig $MBP_{72–85}$ peptide epitope intranasally inhibited EAE in Lewis rats significantly. These studies provide evidence that Lactobacilli presenting myelin infused-antigens are able to reduce EAE if intranasally and orally administered (Buerth et al., 2016; Maassen et al., 2003). This and other studies offer novel approaches for mucosal tolerance induction by mucosal administration of recombinant yeast or Lactobacilli expressing relevant autoantigens, which may be applicable in autoimmune disease such as MS.

In mice, administration of *Lactobacillus* or *Bifidobacterium* strains result in less severe EAE clinical scores, recovery of myelin content in the spinal cord, increased levels of TGF-beta and Treg cells compared to control mice (Consonni et al., 2018). Administration of a mixture of the two strains induced a more significant delay in EAE onset and clinical score which was accompanied with significant decreased mononuclear infiltration into the central nervous system, and enhanced $CD4^+CD25^+Foxp3^+$ expressing Treg cells in the spleen and lymph nodes of mice (Salehipour et al., 2017). Likewise, intraperitoneal injection of *L. helveticus* in mice significantly reduced the incidence and clinical score of EAE, which correlated with reduced Th17 cells and reduced IL-6 (an essential cytokine for Th17 differentiation) (Yamashita et al., 2018). Other probiotics which decrease disease activity, increase IL-10, TGF-beta and Treg cells include, *L. casei* Shirota, *L. casei* 393, *L. reuteri*, *L. paracasei*, *B. breve* and *B. animalis* (Y. Liu, Alookaran, & Rhoads, 2018).

Oral administration of probiotics (*Lactobacillus, Bifidobacterium* and *Steptococcus*) in 9 MS patients and 13 control subjects, twice a day for 8 weeks, resulted in decreased abundance of *Akkermansia* and *Blautia*, known to be associated with dysbiosis in MS patients and increased abundance of *Lactobacillus* and *Bifidobacterium* (Tankou et al., 2018). In addition, decreased number of inflammatory monocytes, and decreased expression of MHC-II and co-stimulatory molecule CD80 were noted. In another study which was a randomized, placebo-controlled study, 60 patients with MS were given a probiotic capsule comprising of *L. acidophilus, L. casei, B. bifidum* and *L. fermentum* for 12 weeks; expanded disability status scale was improved, as well as depression and anxiety symptoms, and reductions noted in c-reactive protein (inflammatory marker), and plasma oxidative metabolites and malondialdehyde (markers of oxidative stress) (Kouchaki et al., 2017). Thus, probiotics have been shown to modulate symptoms of disease in mice (EAE model) and in MS patients, and warrants further research into the use of probiotics as an adjunct treatment in humans with MS.

6. Conclusion

In both animal models and humans, probiotics have been shown to alter immune responses, and induce tolerance. The rapidly growing knowledge of microbiome-host interactions has revealed new avenues for understanding the immunopathological basis of many diseases. It is clear that one of the approaches for maintaining or restoring immune balance and, thereby preventing or treating diseases, is through use of probiotics in the form of fermented foods or probiotic supplements. The mechanism by which probiotics affect individual's health is multidimensional and has been the focus of many studies. The prevailing evidence suggests that probiotics keep the immune system in check, by differentially modulating cellular (Th1, Th2, Th17, Treg, Tc) and humoral (B cells) immune responses specific to the probiotic strain(s) and disease pathophysiology.

Table 1. Effects of probiotics in health and disease	
Probiotic/ probiotic strain	**Beneficial applications**
Probiotics in general	• Stimulate secretion of IFN-gamma and IL-10 • Promote effective immune responses against pathogens • Modulate host immunity
Bifidobacterium (*B.*) *Saccharomyces* (*S.*) *boulardii*	• Detoxify xenobiotics • Produce mmicrobial products, toxins, host metabolites, bile salts and food components • Improves intestinal defense against enterohaemorrhagic *E. coli* • Regulates intestinal epithelial barrier function
Lactobacilli (*L.*) species	• Biosynthesize vitamin K_1, folic acid, biotin, B12. • Absorption of calcium, magnesium, iron, fermentation of lactose • Modulation of intestinal gas production • Produce short chain fatty acids -acetate, propionate, butyrate, lactate • Ferment indigestible dietary fibres • Modulation of the immune responses • Protect against cancer and allergy development
Lactobacillus recombinants	• Inhibits experimental autoimmune encephalomyelitis (EAE) in animal models of multiple sclerosis (MS)
L. Plantarum *B.* B94	• Prevention and treatment of MS
L. acidophilus *L. bulgaricus* *B. bifidum*	• Stimulate the innate immune system (via phagocytosis, NK cell activity)
L. plantarum A *L. acidophilus* ATCC 4356 *L. rhamnosus* ATCC 53103	• Increase expression of $CD11c^+$ DCs and Treg cells and protection against colitis
L. plantarum WCFS1 *L. fermentum* AGR1485	• Upregulate CD83 and CD86 cell surface expression of antigen presenting cells (APC) • Increase IL-12p70, TNF-alpha and IL-1beta
L. bulgaricus subsp. delbrueckii 11842, *Lactococcus lactis* R704 *B. lactis* BB12 *L. acidophilus* *L. bulgaricus* *B. bifidum*	• Beneficial in allergic rhinitis and asthma
L. casei Shirota	• Enhances innate immunity
Bacteroides fragilis *Bacillus subtilis*	• Induce the development of gut-associated lymphoid tissues (GALT) • Induce pre-immune antibody repertoire
Clostridium species *L. rhamnosus* GG *B. infantis*	• Reduces the risk of respiratory tract infections • Reduces the incidence and severity of atopic dermatitis in pregnant mothers and infants

	• Reduces the risk of atopic eczema and allergic sensitization in children • Protects host against development of colitis
B. infantis	• Prevents *Salmonella typhimurium* infection
Escherichia (E.) coli *C. subterminale* *Staphylococcus epidermidis* *Bacteroides fragilis* *Bacillus subtilis*	• Promotes GALT development
F. prausnitzii	• Reduces the severity of experimental colitis in mice
S. thermophilus *L. fermentum*	• Stimulates immune responses
Seven strains of *Bifidobacteria* and LAB (G17)	• Release symptoms of skin lesions in atopic dermatitis mouse model by increasing Tregs and reducing Th1/Th2 cells and associated cytokines
E. coli	• Suppress pathogen growth • Secrete bacteriocin and microsin S • Reduce eczema • Promotes GALT development
Candida utilis yeast	• When fused with MOG_{35-55} peptide, protects mice against EAE
L. delbruekii, sub *bulgaricus* *S. thermophilus* *L. acidophilus* *Bifidobacterium*	• Improves or prevents allergic recurrences in allergic rhinitis
B. bifidum *B. lactis* *L. lactis*	• Reduce eczema

Table 2. Effects of probiotics on the immune response	
Probiotic / probiotic strain	**Immune stimulation outcome**
Microbiota, ingested probiotics	• Maintain immune tolerance • Activate tolerogenic DCs • Drive regulatory T (Treg) cell differentiation • Stimulate differential Th1, Th2, Th17, Treg cells • Improves atopic dermatitis • Improves allergies, inflammatory bowel disease and autoimmune disorders including multiple sclerosis
Lactobacillu (L.). rhamnosus *L. delbrueckii*	• Induction of tolerogenic DCs • Reduce cell surface molecules, CD80, CD83, CD86 • Reduce IL-12 and NF-kB • Increase indoleamine 2,3-dioxygense (IDO) • Increase IL-10 • Stimulate Treg cells
Combination of *L. rhamnosus* and *B. lactis*	• Injestion during pregnancy and during breast feeding reduces risk of atopic eczema • Increased IL-10
A mixture of probiotics (*L.* GG, *L. rhamnosus* LC705, B. *breve*, and *Propionibacterium freudenreichii* ssp. *Shermanii* JS)	• Fails to reduce the risk of atopic eczema
L. s193 *L.* s292	• Increase beta-8 integrin on mesenchymal DCs • Activate differentiation of CD4+ T cells into Treg cells
L. reuteri	• Activates Th1 and Treg cells
L. casei Shirota	• Activates Th1 and Treg cells • Decreases IgE antibody responses • Activate splenic NK cells • Boost innate immune system • Stimulates TNF-alpha and IL-12 on human peripheral blood mononuclear cells
Combination of *L.* reuteri, *L. casei* and TNF-alpha	• Activate only Treg cells
L. lactis	• Do not activate Treg cells
L. acidophilus	• Suppresses IgE allergic responses • Downregulates IFN-gamma, IL-4 cytokines • Upregulates IgA antibody responses • Upregulates TGF-beta and Treg cells
Combination of *L. acidophilus* and *B. longum*	• Improves allergic rhinitis
L. plantarum WCFS1	• Upregulates $CD103^+$ DCs in the intestine • Increases Th2 cytokines • Upregulates Treg cells

LAVR1-A1 *L. rhamnosus* GG *L. acidophilus*	• Induce anti-inflammatory IL-4, IL-10 and TGF-beta cytokines • Inhibit IL-12 and IFN-gamma • Increase expression of co-stimulatory cell surface markers, CD14, IL-2R, HLA-DR, ICAM-1 and CD80 on monocytes
Combination of a mixture of probiotics, zinc and the coenzyme Q10	• Down regulates IgG in serum • Decreases TNF-alpha, IL-1, IL-6 and IL-17 • Decreases vascular endothelial growth factor (VEGF)
L. gasseri	• Induces IFN-alpha of monocytes
L. paracasei DG	• Increases TNF-alpha, IL-6, IL-8, CCL20 by human monocyte cell line THP-1
L. helveticus	• Reduces IL-17 cytokine and Th-17 cells • Reduces IL-6 • Increases IL-10, TGF-beta and Treg cells
Bifidobacterium (B.) infantis	• Matures DCs • Induces $CD103^+$ tolerogenic DCs • Induces Treg cells • Suppresses Th2 • Stimulates high levels of IL-10 which neutralizes IL-12 • Stimulates high levels of TGF-beta
B. animalis	• Reduces IL-6, IL-17, TNF-alpha and monocyte chemoattactant protein-1 • Increases IL-10 • Stimulates Treg cells
B. clausii	• Increases IL-10 • Increases TGF-beta • Improves allergic responses
B. bifidum *B. lactis*	• *In vivo*, lower number of $CD4^+$ and $CD8^+$ T cells. Higher Treg cells • Mediated by short chain fatty acids
B. breve *B. longum* *L. acidophilus* *L. gasseri* *Streptococcus (S.) thermophilus*	• Downregulate IL-17A • Downregulate IL-23 • Relieves colitis in mice • Suppress CD40, CD80
Short chain fatty acids	• Expand and differentiate Treg cells

Review paper manuscript 2.

1b- Multiple sclerosis: Immunopathology and treatment update

ABSTRACT

The treatment of multiple sclerosis (MS) has changed over the last 20 years. All immunotherapeutic drugs target relapsing remitting MS (RRMS) and it still remains a medical challenge in MS to develop a treatment for progressive forms. The most common injectable disease modifying therapies in RRMS include the intramuscular or subcutaneous β-interferons 1a or 1b and glatiramer acatate, however, one of the major challenge of injectable disease modifying therapies has been the poor adherence with approximately 50% of patients discontinuing the therapy within the first year of treatment. Herein, we go back to the basics to understand the immunopathophysiology of MS which may give insights in the development of new improved drug treatments. We also present current drug treatments and new and emerging immune modulating approaches for the immunotherapy of MS.

Keywords: multiple scleroris; immunotherapy, drug delivery; vaccine

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Review

Multiple Sclerosis: Immunopathology and Treatment Update

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Abstract: The treatment of multiple sclerosis (MS) has changed over the last 20 years. All immunotherapeutic drugs target relapsing remitting MS (RRMS) and it still remains a medical challenge in MS to develop a treatment for progressive forms. The most common injectable disease-modifying therapies in RRMS include β-interferons 1a or 1b and glatiramer acetate. However, one of the major challenges of injectable disease-modifying therapies has been poor treatment adherence with approximately 50% of patients discontinuing the therapy within the first year. Herein, we go back to the basics to understand the immunopathophysiology of MS to gain insights in the development of new improved drug treatments. We present current disease-modifying therapies (interferons, glatiramer acetate, dimethyl fumarate, teriflunomide, fingolimod, mitoxantrone), humanized monoclonal antibodies (natalizumab, ofatumumab, ocrelizumab, alemtuzumab, daclizumab) and emerging immune modulating approaches (stem cells, DNA vaccines, nanoparticles, altered peptide ligands) for the treatment of MS.

Keywords: multiple sclerosis; immunotherapy; drug delivery; vaccine

1. Introduction

In the early 1900s, only a few cases of multiple sclerosis (MS) were reported, which quickly became a common occurrence for admission to neurological wards. Today, MS accounts over 2.5 million affected individuals with an estimated cost of US$2–3 billion per annum [1]. The distribution of MS varies according to geographic location. For example, the further north or south from the equator the higher the prevalence of MS; countries that lie on the equator have extremely low prevalence compared to Scotland, Norway, and Canada. The prevalence of MS has increased progressively over time with 30/100,000 diagnosed in 2008 to 33/100,000 diagnosed in 2013 globally. In fact, in a Norwegian cohort over 53 years (1961–2014), the prevalence increased from 20 to 203/100,000 and the incidence increased from 1.9 to 8/100,000 [2]. It is possible that the increase in prevalence is due to improved diagnostic procedures and reporting and changes in lifestyle (lack of vitamin D and increased smoking) [1]. MS is commonly diagnosed between 20 years and 40 years of age although it can affect younger and older individuals [3], and most commonly affects those with a genetic predisposition (major histocompatibility complex (MHC) class II phenotype, human leukocyte antigen (HLA)-DR2 and HLA-DR4 most commonly affected). In fact, the incidence of MS is increased 10-fold in monozygotic

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The *Brain Sciences* editorial team would like to congratulate the winner of the 2018 Best Paper Award. The winning paper, which was nominated by the Editor-in-Chief and Associate Editor-in-Chief, was the most cited in 2018:

Title: Multiple Sclerosis: Immunopathology and Treatment Update
Authors: Narges Dargahi, Maria Katsara, Theodore Tselios, Maria-Eleni Androutsou, Maximilian De Courten, John Matsoukas and Vasso Apostolopoulos
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We congratulate the authors and thank them for choosing *Brain Sciences* to publish their work. In recognition of their accomplishment, the winner will receive 500 Swiss Francs and a certificate. All the original research papers and review articles published between 1 January 2017 and 31 December 2017 were eligible for consideration.

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2018 BEST PAPER AWARD
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1. Introduction

In the early 1900's, only a few cases of multiple sclerosis (MS) were reported which quickly became a common occurrence for admission to neurological wards. Today, MS accounts to over 2.5 million affected individuals with an estimated expenditure of $2-3 billion per annum (Compston & Coles, 2002). The distribution of MS alters according to the geographic location of the country, for example, the further north or south from the equator the higher the prevalence of MS; regions of countries that lie on the equator have extremely low prevalence compared to Scotland, Norway, and Canada. The prevalence of MS has increased progressively over time with 30/100,000 diagnosed in 2008 to 33/100,000 diagnosed in 2013 globaly. In fact, in a Norwegian cohort over 50 years (1961-2014), the prevalence increased from 20 to 203/100,000 and the incidence increased from 1.9 to 8/100,000 (Grytten, Torkildsen, & Myhr, 2015). It is possible that the increase in prevalence is due to improved diagnostic procedures and reporting and changes in lifestyle behaviors (lack of vitamin D and increased smoking) (Compston & Coles, 2002). MS is commonly diagnosed between 20-40 years of age although it can affect younger and older individuals (Antel, Antel, Caramanos, Arnold, & Kuhlmann, 2012), and most commonly affects those with a genetic predisposition (MHC class II phenotype, HLA-DR2 and HLA-DR4 most commonly affected). In fact, the incidence of MS is increased 10-fold in monozygotic twins as compared to siblings of patients with MS (H. Dai, Ciric, Zhang, & Rostami, 2012; Hemmer, Nessler, Zhou, Kieseier, & Hartung, 2006; Sadovnick, Ebers, Dyment, & Risch, 1996). In addition, viral infections can trigger disease where parts of the virus mimics that of the myelin sheath (Hogeboom, 2015). Although usually not life shortening, MS is a chronic neurological disease often interfering with life and career plans of an individual (Rieckmann, 2004).

MS is categorized into 4 distinct types, primarily based on clinical course, which are characterized by increasing severity: (a) Relapsing/remitting MS (RRMS), the most common form, affecting 85 % of all MS patients which involves relapses followed remission, (b) secondary progressive MS (SPMS), which develops over time following diagnosis of RRMS, (c) primary progressive MS (PPMS) affecting 8-10 % of patients, noted as gradual continuous neurologic deterioration, and (d) progressive relapsing MS (PRMS) the least common form (< 5 %), which is similar to PPMS but with overlapping relapses (Deraos et al., 2008a; Eckstein & Bhatti, 2016; Lublin & Reingold, 1996). MS leads to a wide range of symptoms with various

severity involving different parts of the body. MS diagnosis is mainly clinically based however, magnetic resonance imaging (MRI) assists in diagnosis (Polman et al., 2011). As such, examination of the cerebrospinal fluid (CSF) and visual induced potentials with MRI can assist in confirming the clinical suspicions of MS (Lunde Larsen, Larsson, & Frederiksen, 2010; Polman et al., 2011). MS symptoms and disease progression are varied, with some individuals experiencing little disability whilst most (up to 60 %) require a wheelchair 20 years from diagnosis (Katsara, Deraos, Tselios, Matsoukas, & Apostolopoulos, 2008b).

Although treatments against MS are able to decrease the relapse rate in RRMS, the prevention of long-term effects remains a problem; medications for progressive forms of MS are also limited in their efficacy. Hence, new improved drugs are required to effectively treat MS. One of the major pathophysiological mechanisms of MS involves autoreactive T cells, primarily T helper (Th)-1 $CD4^+$ T cells and Th17 cells leading to cytokine secretion and activation of an inflammatory cascade resulting in demyelination within the brain and spinal cord and axonal damage; autoreactive antibodies cannot be discounted. Indeed, MS is generally known as a chronic autoimmune disorder of the central nervous system (CNS) (Gafson, Giovannoni, & Hawkes, 2012; Mahad, Trapp, & Lassmann, 2015). MS causes breakdown of the blood brain barrier (BBB) leading to migration of immune cells (macrophages, T cells, B cells) and secretion of pro-inflammatory cytokines and chemokines (Minagar & Alexander, 2003) which induces inflammation, formation of sclerotic plaques (lesions), demyelination and neurodegeneration (L. Steinman, 1996). MS lesions may form in any location of the CNS white matter or in grey matter, often leading to physical disability and sometimes, decline in cognitive ability (Bennett et al., 2010; Minagar & Alexander, 2003). It is therefore, conceivable to target immune cells and their products in order to prevent tissue damage by modulating inflammation (Farjam, Zhang, Ciric, & Rostami, 2015; Katsara, Deraos, et al., 2008b) whilst reducing potential side effects such as, global immunosuppression (Dandekar, Wu, Pewe, & Perlman, 2001; Farjam et al., 2015; Hemmer et al., 2006). The major constituents of the myelin sheath in which autoreactive T cells and antibodies recognize, include, myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) and proteolipid protein (PLP).

2. Immunopathophysiology of MS

The brain has primarily been considered to be an organ which is highly immune-advantaged, although a number of studies have challenged this (Hemmer et al., 2006). In the last 10 years an important shift has surfaced in MS research, suggesting that MS is not just a disease of the immune system, but equally involves factors contributed by the CNS (Bianchini et al., 2017b; J. Jiang & Kelly, 2011). Immune cells residing in the CNS get activated following damage to CNS tissue; notably microglial cells whereby they upregulate MHC class I and II molecules and cell surface co-stimulatory molecules and secrete cytokines and chemokines, paving entry for T (CD4 and CD8) cells, B cells, monocytes, macrophages and dendritic (DC)-like cells into CNS lesions (Hemmer et al., 2006). Infiltrating immune cells secrete pro-inflammatory cytokines, nitric oxide, and matrix metalloproteinases (Tabarkiewicz, Pogoda, Karczmarczyk, Pozarowski, & Giannopoulos, 2015; Van Hamburg et al., 2011), leading to destruction of the myelin sheath.

It has been generally accepted that chronic inflammation is the hallmark to neurodegenerative diseases, such as, MS, Alzheimer's disease and Parkinson's disease (Hemmer et al., 2006; Sospedra & Martin, 2005b). Myelin-reactive auto-T cells cross the BBB (Farjam et al., 2015) and their migration into the CNS consequently initiates an inflammatory cascade followed by demyelination of the CNS and axonal damage. These cells reside in the perivenous demyelinating lesions which generate distinct inflammatory demyelinated plaques situated within the white matter (Dolati et al., 2017). MS lesions appear in the white matter inside the visual neuron, basal ganglia, brain stem and spinal cord (Münzel & Williams, 2013). White matter cells transmit neural signals from grey matter, where information is gathered, and transferred to the rest of the body (Dolati et al., 2017; Inglese & Petracca, 2015). MS involves 2 main steps, (i) myelin sheath damage resulting in formation of lesions in the CNS and (ii) inflammation, which together destroy the neuron tissue (Dolati et al., 2017; Koriem, 2016). In MS, damage of oligodendrocytes and destruction of myelin sheath leads to breakdown of the nerve axon and loss of neuronal function (Koriem, 2016). Demyelination increases the inflammatory activation processes leading to damage of BBB and stimulation of macrophage activation and oxidative stress pathways (Kallaur et al., 2016). The white matter lesions include myelin breakdown together with infiltration of monocytes, B cells, T cells and DC (Mirshafiey & Jadidi-Niaragh, 2010). Microglia and macrophages are the main innate immune cells present

in MS lesions where they either act together with T and B cells, or directly cause neuroinflammatory tissue damage (Fischer et al., 2012). Cells involved in the inflammatory process include those that are both in the innate and adaptive immune systems and are described below (Figure 1).

2.1. Natural killer T (NKT) cells

NKT cells share properties of both T cells and NK cells and recognize glycolipid antigens presented in complex with the MHC class I-like molecule, CD1d. Two subsets of NKT cells have been identified (type I, invariant NKT (iNKT) cells and type II, variant NKT (vNKT) cells) and are implicated in the pathogenesis of MS in humans and in the murine model of MS, experimental autoimmune encephalomyelitis (EME). iNKT cells express cell surface markers characteristic of activated or memory T cells (CD25, CD44, CD69) with the majority being $CD4^+$ as well as markers characteristic of NK cells (NK1.1 or CD161, Ly49). Following activation of iNKT cells (via binding to α-GalCer-CD1d complex) an array of cytokines are secreted that are associated with both pro- and anti-inflammatory immune responses and play a role in both innate and acquired immunity. As such, iNKT cells, (i) secrete interleukin (IL)-4 and IL-13 which stimulate $CD4^+$ T cells to differentiate into anti-inflammatory Th2 cells (IL-4, IL-10 producers) which inhibit Th17, Th1, CD8+ T cells in the CNS; (ii) secrete IL-2 and tumor growth factor (TGF)-beta which stimulate the production of T regulatory (Treg) cells (IL-10, TGF-beta producers) which inhibit Th17, Th1 and $CD8^+$ T cells in the CNS, and (iii) secrete IL-4, IL-10, IL-13, interferon (IFN)-gamma and GM-CSF which activate suppressive myeloid derived suppressor cells (MDCs), DC and macrophages which in turn secrete IL-10 to activate Treg cells and supress Th17, Th1 and CD8+ T cells in the CNS (Van Kaer, Wu, & Parekh, 2015). Due to the pleitrophic properties of iNKT cells, they play a role in protecting the host against pathogens, tumors, autoimmunity and are involved in tissue rejection, ishcaemia reperfusion injury and obesity related diabetes (Van Kaer et al., 2015); deficiency or dysfunction of iNKT cells has been shown to be linked to the development of autoimmune diseases. Indeed, iNKT cell numbers are decreased in patients with MS (Van Kaer et al., 2015) and are restored in patients in remission (Gigli, Caielli, Cutuli, & Falcone, 2007). Analysis of iNKT cells in MS patients in remission showed a Th2 cytokine profile, suggesting an immunoregulatory effect of iNKT cells in MS (Araki et al., 2003).

Similarly, in the EAE mouse model, protection of EAE development is associated with high levels of iNKT cells and suppression of Th1 and Th17 cells (Mars et al., 2002). Interestingly, injection of α-Galactosylceramide (α-GalCer) and analogues thereof, have potent activities in protecting mice against, cancer, infections, inflammatory conditions and autoimmune disorders. Hence, it is possible to develop iNKT cell based modulating therapies against MS (Van Kaer, 2005; Van Kaer, Parekh, & Wu, 2011). Like iNKT cells, variant NKT (vNKT) cells also share properties of both T cells ($CD4^+$) and NK cells (NK1.1) and recognize □-linked glycolipid antigens in complex with CD1d. They are less common in mice compared to iNKT cells but are more abundant in humans. Of interest, vNKT cells recognize the self-glycolipid, sulphatide, that is abundantly expressed within the myelin sheath suggesting a role in MS although not yet established (Jahng et al., 2004). Likewise, vNKT cells recognizing suphatide self myelin ligand are present in high levels in mice with EAE suggesting their role in disease progression (Jahng et al., 2004).

2.2. Mucosal-associated invariant T (MAIT) cells

MAIT cells are a subset of T cells of the innate immune system to defend against microbial infections. They are present in the liver, lungs, mucosa and blood and make up to 25% of CD8 T cells in healthy individuals; they also support adaptive immune responses in that they have a memory like phenotype (Napier, Adams, Gold, & Lewinsohn, 2015). The MHC class I-like molecule, MRI, presents microbial antigens and vitamin B metabolites to MAIT cells, leading to their activation (Kjer-Nielsen et al., 2012). However, MAIT cells have also been implicated in autoimmune diseases such as MS, inflammatory bowel disease and rheumatoid arthritis where they are often noted at the site of autoimmune attack. Recently, it was reported that in MS, MAIT cells are highly present at the sites of demyelination and secrete pro-inflammatory Th1 cytokines (IFN-gamma and TNF-alpha) and activate Th17 cells (IL-17 and IL-22 cytokines) (Bianchini et al., 2017a); the major cytokines in the pathogenesis of chronic inflammatory and autoimmune diseases. In addition, MAIT cell have been noted in white matter inflammatory lesions (Abrahamsson et al., 2013) as well as transcription over expression of MR1 in MS lesions. Conversely, its been reported that MAIT cells are decreased in blood of patients with RRMS (Miyazaki, Miyake, Chiba, Lantz, & Yamamura, 2011). It is not clear whether MAIT cells exert a protective or a non-protective role, thus a better

understanding of how MAIT cells are involved in MS and of their interactions would aid in a better understanding of the pathogenesis of MS and development of therapeutic strategies.

2.3. Regulatory T cells (Tregs)

Regulatory T cells (Tregs; originally known as suppressor T cells) are a subset of $CD4^+$ T cells that modulate immunity, maintain tolerance against self antigens and prevent autoimmunity. Tregs are primarily characterized as $Foxp3^+CD25^+CD4^+$ and are anti-inflammatory (secrete IL-10). One of the first evidence of the role of Treg cells in MS was in mouse EAE models, where adoptive transfer of Treg cells from control mice into MOG or PLP induced EAE mice prevented the onset and progression of EAE (Kohm, Carpentier, Anger, & Miller, 2002; X. Zhang et al., 2004). Adoptive transfer of Treg cells recovering from EAE into MOG-induced active EAE mice resulted in resolution of EAE (McGeachy, Stephens, & Anderton, 2005a). In addition, induction of Treg cells by estradiol or by monocytes under glatiramer acetate treatment, reduced clinical signs of MOG-EAE (Matejuk et al., 2004; Weber et al., 2007). Furthermore, injection anti-CD28 monoclonal antibody in Lewis rats results in Treg cell expansion and reduction in EAE disease severity (Beyersdorf et al., 2005). Interestingly, injection of anti-CD25 monoclonal antibody, which blocks the effects of Treg cells into C57BL/6 mice increased susceptibility to EAE induction (McGeachy, Stephens, & Anderton, 2005b). In patients with MS however, the frequency of $Foxp3^+CD25^+CD4^+$ Treg cells does not differ to those in healthy individuals, although the function of such cells are impaired (maturation and migration) (Zozulya & Wiendl, 2008). In addition, mRNA and protein levels of Foxp3 are impaired in Treg cells of patients with MS especially in RRMS and are normalized during SPMS (Zozulya & Wiendl, 2008). Hence, impaired functionality of Treg cells is primarily observed in the early stages of MS but not in their chronic stage, suggesting a causative role (Diebold & Derfuss, 2016). Further studies of Treg cells in MS may aid in the understanding for why tolerance against self antigens is broken, leading to disease. However, it is not clear whether the impaired function of Treg cells is a direct cause of MS or whether such impairment is a general outcome for all autoimmune disorders.

2.4. Macrophages and microglia

In the last decade macrophages been divided into M1 or M2 macrophages based on their pro- or anti-inflammatory cytokine secretion phenotype (Mosser & Edwards, 2008b). The

M1 macrophage phenotype of mice ($F4/80^+CD11b^+CD11c^+iNOS^+$) and human ($CD40^+CD86^+CD64^+CD32^+$) is induced in the presence of interferon (IFN)-gamma and/or toll-like receptor (TLR) ligands such as lipopolysaccharide (LPS). M1 macrophages are pro-inflammatory and primarily secrete IL-1, IL-6, IL-12, TNF-alpha, iNOS and MCP-1 (Mosser & Edwards, 2008a). In general they stimulate adaptive immune responses. The M2 macrophage phenotype of mice ($F4/80^+CD11c^-CD301^+Arg1^+CD206^+$) and humans ($CD163^+CD206^+$) is induced in the presence of IL-4, IL-10, IL-13 and Arg1 that blocks iNOS activity (Mosser & Edwards, 2008a). M2 macrophages are anti-inflammatory and primarily secrete IL-1 receptor antagonist, IL-4, IL-10, transforming growth factor (TGF)-beta1. Macrophages play a crucial role in the pathogenesis of MS. In fact, in active demyelinating and early re-myelinating lesions, macrophages are highly present compared to inactive, demyelinated or late re-myelinated lesions (Brück et al., 1996). However, a distinction of M1 vs M2 macrophages in human brain tissues is not so clear, with both M1 macrophages and an intermediate subtype (M1/M2, $CD40^+CD206^+$) being present (Vogel et al., 2015; Vogel et al., 2013). Like macrophages, microglia cells are divided into M1- and M2-polarized microglia cells. M1 microglia cells are pro-inflammatory and express CD40, CD74, CD86 and CCR7, whereas, M2 microglia cells are anti-inflammatory and express mannose receptor (CD206) and CCL22. In MS brain lesions however, like macrophages, an intermediate microglia phenotype is present expressing CD40, CD74, CD86 and CCL22 but not CD206 markers (Peferoen et al., 2015). Interestingly, in an EAE model it was shown that suppression of CCL22 decreased M1 macrophage accumulation in the CNS, thus therapies designed to suppress CCL22 have the potential to decrease demyelination and progression of disease. In addition, in mice M1 microglia cells have been found to switch to M2 microglia cells during remyelination, hence M2 polarization is necessary for efficient remyelination (Miron et al., 2013). Indeed, fasudil (a selective Rho kinase inhibitor), injected into EAE bearing mice shifted M1 to M2 macrophages and ameliorated the clinical severity of EAE (C. Liu et al., 2013).

2.5. T helper cells

CD4 T cells or T helper (Th) cells, recognize short 9-17 amino acid peptides presented on the surface of antigen presenting cells in complex with MHC class II. CD4 T cells differentiate into distinct Th cells depending on the cytokine secretion profiles (Apostolopoulos et al., 2016). (i) Th1 cells are pro-inflammatory and produce high levels of IL-2, IL-12, TNF-alpha and IFN-gamma; (ii) Th2 cells are anti-inflammatory and secrete IL-4, IL-5, IL-6, IL-10,

IL-13, IL-25; (iii) Th17 cells are pro-inflammatory and secrete high levels of IL-17A, IL-17F, IL-21, IL-22, IL-24, IL-26 and low levels of IL-9 and IFN-gamma; (iv) Th22 cells which are a combination of Th1, Th2, Th17 phenotype and secrete IL-13, IL-22 and TNF-alpha and (v) the newest addition to the Th subset, Th9, was identified for its potent secretion of IL-9. Th1, Th9, Th17 cells are key contributors to MS by increasing inflammation within the mileau of the myelin site.

Th1 cells and their pro-inflammatory cytokine products are present in high levels within the demyelinating axon and CNS lesions of humans and in MOG, PLP or MBP induced EAE in mice. Th1 cells recognize MOG, PLP and MBP peptide epitopes presented in the context of MHC class II, HLA-DRB1*1501 (HLA-DR2, HLA-DR15) and HLA-DRB1*04 (HLA-DR4) alleles. As a result CD4 T cells become activated, cross the blood brain barrier and induce CNS autoimmunity. A number of drug therapeutics target the MHC class II-peptide-T cell receptor (TCR) complex in an attempt to modulate or divert Th1 responses to therapeutic Th2 responses. Indeed, it was recently shown that dimethyl fumurate (DMF) injection in RRMS patients reduced Th1, Th17 and CD8 T cells and increased Th2 cells; this resulted in high levels of IL-4 and decreased levels of IFN-gamma and IL-17 (Q. Wu et al., 2017). In addition, we have shown that mannan conjugation of self MBP, PLP or MOG native peptides or altered peptide ligands, are able to divert Th1 responses to Th2 responses in human PBMC from MS patients, in immunized mouse spleen cells and are able to ameliorate EAE in mice (Deraos et al., 2008a; Deraos et al., 2015b; Katsara, Deraos, et al., 2008b; Katsara et al., 2014; Katsara, Minigo, Plebanski, & Apostolopoulos, 2008a; Katsara et al., 2006; Katsara et al., 2008a, 2008b; T. Tselios et al., 2002b; T. V. Tselios et al., 2005b; Tseveleki et al., 2015a). The role of Th9 cells in MS is not as clear although in mice, IL-9 and Th9 cells induce EAE and inflammation and IL-9 knockout mice are protected from developing EAE (Deng et al., 2017). Th17 cells play a crucial role in the pathogenesis of MS in both mice and humans by inducing an inflammatory mileau. In fact, IL-17A is present at high levels in CNS lesions, cerebrospinal fluid and in the serum of patients with MS (Vogel et al., 2015; Vogel et al., 2013). Th17 cells express high levels of CCR6 which binds to the ligand CCL20 on vascular endothelial cells, enabling their entry through the blood brain barrier where they secrete pro-inflammatory cytokines including IL-17A. In addition, IL-17 interferes with the remyelination process. Of interest, anti-IL-17A humanized neutralizing monoclonal antibody (AIN457 or Secukinumab) injected in patients with MS showed reduction of lesions compared to placebo-treated control subjects (Vogel et al., 2015; Vogel et al., 2013). In addition, Th22 cells are highly present in the peripheral blood

and cerebral spinal fluid of patients with active RRMS (Rolla et al., 2014), and IL-22 mRNA and Th22 cells are increased in relapsing MS compared to remitting MS patients (Muls, Nasr, Dang, Sindic, & Van Pesch, 2017). Furthermore, Th22 cells specifically recognize MBP and are resistant to IFN-beta therapy (Rolla et al., 2014).

IL-27, a member of the IL-6/IL-12 cytokine family, is secreted by macrophages, dendritic cells and microglia cells, with pleiotropic roles in immunomodulation being either pro- or anti-inflammatory. IL-27 also stimulates or inhibits T cell differentiation. Th1 cells are induced by IL-27 whereas Th2, Th17 and Treg cells are inhibited by IL-27. In addition, Tr1 cells a specialized subset of T cells which secrete IL-10 are induced in the presence of IL-27 (Y. Iwasaki, Fujio, Okamura, & Yamamoto, 2015). In 40 patients with RRMS, circulating plasma IL-27 levels were significantly higher compared to healthy control subjects (Naderi et al., 2016). Likewise, IL-27 and IL-27R are elevated in post-mortem MS brain lesions compared to non MS control brains. Macrophages and microglia were identified to be the source of IL-27 and triggering infiltration of CD4 and CD8 T cells (Sénécal et al., 2016). In addition, the effects of IL-27 on microglia cells showed that nitric oxide, TNF-alpha and IL-6 were secreted, promoting Th1 polarization, suggestive that IL-27 enhances microglia neuroinflammation (Kawanokuchi, Takeuchi, Sonobe, Mizuno, & Suzumura, 2013). Hence, suppressing IL-27 may be a strategy to modulate inflammatory responses in patients with MS.

2.6. CD8 T cells

Classical CD8 T cells or cytotoxic T cells (Tc1 cells), recognize short antigenic 7-9-mer peptide epitopes presented on the surface of antigen presenting cells in complex with MHC class I. In MS there is a genetic association with HLA-A3 (Sawcer & Hellenthal, 2011); HLA-A2 has been shown to reduce the risk of MS in individuals that also express MHC class II, HLA-DRB1*1501. The antigen specificity of CD8 Tc1 cells isolated from patients with MS, has been suggested to be against MAG, MBP and PLP with cytolytic activity against neuronal cells *in vitro* (Dressel et al., 1997) although their pathogenic role in MS is still not clear. More recently other subsets of CD8 T cells have been identified and are grouped into different subsets based on their cytokine profile. In as such, classical Tc1 cells secrete IFN-gamma, Tc2 secrete IL-4, Tc10 secrete IL-10, Tc17 secrete IL-17, Tc21 secrete IL-21, Tc22 secrete IL-22 and another subset is characterized by secreting TNF-alpha. In MS, regardless of the stage and activity of disease CD8 T cells are noted in high numbers, much higher than CD4 T cells at a

ratio of 10:1 CD8:CD4 T cells. MHC class I is highly expressed within MS lesions and astrocytes, oligodendrocytes, neurons in addition to the classical antigen presenting cells, DCs and macrophages. In fact, CD8 T cells are found in great abundance within CNS tissues and cerebrospinal fluid of patients with MS. CD8 T cells present in both acute and chronic MS lesions secrete high levels of IL-17 - Tc17 CD8 T cells (Tzartos et al., 2008). Tc17 cells secrete IL-17 and TNF-alpha and low IFN-gamma and are negative for granzyme B, perforin and cytolytic activity unlike the classical CD8 Tc1 cells. In peripheral blood of patients with SPMS and RRMS elevated levels of Tc1 and Tc17 cells are noted as well as a high percentage of TNF-alpha secreting CD8 T cells (Salehi et al., 2016); Tc21 cells are increased in the remission phase of RRMS compared to SPMS. In addition, higher levels of $CD8^{+}IFN\text{-}gamma^{+}TNF\text{-}alpha^{+}IL\text{-}17^{+}$ T cells in the relapsing phase of RRMS compared to remission phase, SPMS and controls (Salehi et al., 2016). It is clear that CD8 T cells contribute to the pathogenesis of MS, and it is important to understand how such cells escape T cell tolerance and induce CNS autoimmunity in order to design and develop new therapeutics against MS.

2.7. B cells

Although there is a prevalence of T cells in MS plaques, B cells also contribute to the pathogenesis of MS where they secrete auto-antibodies and cytokines and being antigen presenting cells they activate T cells. In patients with MS the presence of oligoclonal bands (OCB) in cerebrospinal fluid and brain parenchyma is a consistent finding in over 95 % of patients. OCB is a product of clonally expanded B cells and IgG synthesis. In MS plaques plasma cells are noted in large numbers where antigen uptake, processing and presentation takes place as well as synthesis of IgG. Interestingly, over 50 antibodies isolated from cerbrospinal fluid from patients with MS did not react to MBP, PLP or MOG (Disanto, Morahan, Barnett, Giovannoni, & Ramagopalan, 2012) but some groups reporting that they bind to intracellular proteins such as, MKNK1/2, FAM84A, AKAP12A and glial potassium channel KIR4.1, or, against intracellular lipid determinants (Wekerle, 2017; Winger & Zamvil, 2016). Moreover, anti-MOG auto-antibodies is a hallmark of childhood MS as well as in some patients with neuromyelitis optical spectrum disorder. It is clear, that abnormal activation of B cells within the CNS of patients with MS, suggests that B cells play a role in the pathophysiology of the disease. Further studies are required to ascertain whether B cell or auto-antibody depletion is able to restore immune function in MS and hence, be used as a therapeutic target against MS.

2.8. Dendritic cells

DC are professional antigen presenting cells which process and present antigenic peptide epiopes on their surface in complex with MHC class I or class II, resulting in CD4 or CD8 T cell stimulation respectively. Even though MS is generally associated with predominant auto-reactive T cells, emerging evidence indicates that DCs play an important role in the pathophysiology of MS, primarily due to their T cell activating and cytokine secreting properties. Following activation of DCs in the periphery, T cells specific to myelin epitopes are activated inducing pro-inflammatory cytokine secretion aiding their entry through the BBB into the CNS. In the CNS resident antigen presenting cells and T cells are further activated leading to demyelination and motor deficits. In patients with MS, DCs are abundantly present within inflammed lesions, cerebrospinal fluid and in the circulation and produce high levels of TNF-alpha, IFN-gamma and IL-6 (Y. M. Huang et al., 1999). In addition, the expression of cell surface co-stimulatory molecules, CD40 and CD80 on DCs are increased in RRMS and SPMS patients, suggesting an activated pro-inflammatory state of DCs, hence their contributing role in the pathogenesis of MS.

2.9. Myeloid derived suppressor cells

Myeloid-derived suppressor cells (MDSC) are myeloid progenitors, the same lineage to that of macrophages, DC and neutrophils. However, MDSC have strong immunosuppress ive properties rather than immune-stimulatory properties as noted with macrophages, DC and neutrophils (Kong, Fuchsberger, Xiang, Apostolopoulos, & Plebanski, 2013). Their major role is in tumor development and chronic inflammation having immune suppressive effects (Kong et al., 2013). As such, it was recently shown following MBP_{1-11} peptide immunization in mice, that MDSCs were increased adopting a suppressive phenotype, inhibiting the activation of $CD4^+$ T cells via arginase-1 and inducible nitric oxide synthase; such approach inhibited the development of EAE in mice (Wegner, Verhagen, & Wraith, 2017). In addition, MDSC secrete inhibitory enzyme indoleamine 2,3-dioxygenase and Th2 cytokine, IL-10 (J. Yu et al., 2013). It is not clear whether the number of MDSCs are reduced or whether their functionality is altered in patients with MS, leading to the failure of MDSCs to suppress autoimmune T cells, as a result of disease progression. The use of *ex vivo* cultured MDSCs could be a viable strategy to develop new improved treatments against MS.

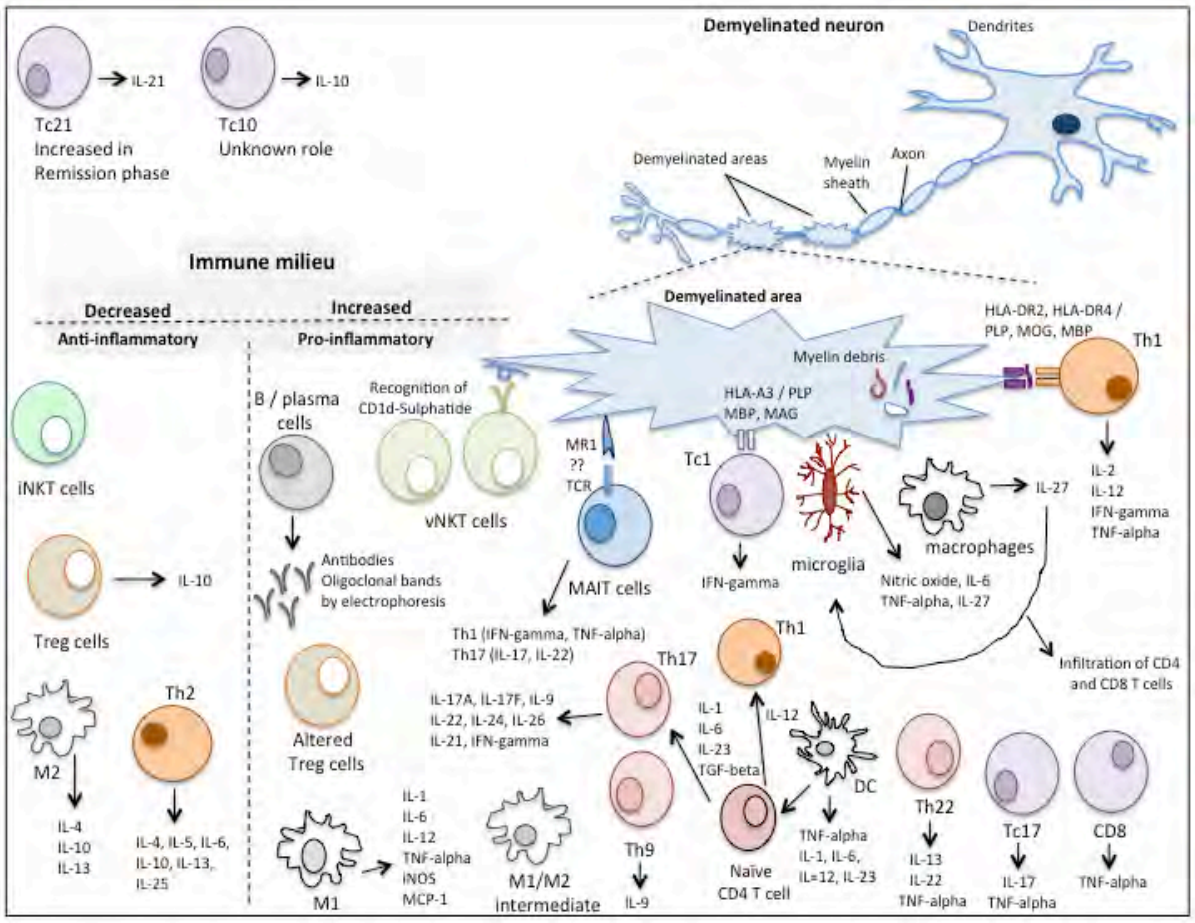

Figure 1. The immunological complexity of the immune / cytokine network in multiple sclerosis.

3. Current drug therapies for Multiple Sclerosis

The majority of the treatments for MS are long term mainly suppressing the immune system however, such immunosuppressants pose increased risks for infections and cancer (Inglese & Petracca, 2015). Alternative treatment options involve disease modifying therapies such as, interferons, glatiramer acetate, monoclonal antibodies and sphingosine-1-phosphate receptor modulators (Table 1, Figure 2). These therapies have dramatically reduced the number of attacks and decreased disease progression. In fact, interferons are effective in the early relapsing phases of MS but not in the advanced phases of the disease (Inglese & Petracca, 2015). Ultimately, induction of tolerance against self antigens and re-establishing immune homeostasis can effectively 'cure' the disease; such strategies have been the focus of recent research.

3.1. Treatment of MS relapses

Patients with MS who present with a relapse are generally treated with corticosteroids intravenously, plasma exchange or adrenocorticotropic hormone injections (Diebold & Derfuss, 2016; Filippini et al., 2000). Although effective in reducing the duration of the relapse and patients recovery faster there are no long term neuroprotective benefits (Havrdova et al., 2009; Inglese & Petracca, 2015; Morrow, Metz, & Kremenchutzky, 2009; Myhr & Mellgren, 2009; Van Der Voort et al., 2009).

3.2. Long-term treatment of MS with disease-modifying agents

The treatment of MS has been a challenge with treatment options being limited mainly to corticosteroids, the potent alkylating agent cyclophosphamide and potent immunosuppressant methotrexate (Table 1, Figure 2). But with the advent of immunomodulatory drugs in mid-1990s, a big shift was carried to treatment options for the first time (Diebold & Derfuss, 2016). The first disease-modifying drug for RRMS, interferon beta-1 (IFNβ-1) was the primary key breakthrough for the treatment of MS (Ludwig Kappos et al.; L. Kappos et al., 2006). Disease modifying agents intend to modify the course of the disease rather than improving symptoms.

Until the approval of the first oral treatment in 2010 (Eckstein & Bhatti, 2016), all MS treatments consisted of either intramuscular or subcutaneous injectable drugs. To date, 13 FDA approved disase modifying drugs are available for RRMS, and several more agents are in different developmental stages (Calabresi et al., 2014; Eckstein & Bhatti, 2016; Katsara, Matsoukas, Deraos, & Apostolopoulos, 2008a; Katsara, Minigo, et al., 2008a; Katsara et al., 2006; Katsara, Yuriev, et al., 2009b). In the last 20 years there has been an evolving trend in novel treatments for MS and the global progress of therapies for MS has been quite promising. In general treatments consist of Ampyra®, Aubagio®, Avonex®, Betaseron®, Copaxone®, Extavia®, Gilenya®, Lemtrada®, Novantrone®, Plegridy®, Rebif®, Tecfidera® and Tysabri® (D. R. Huang, 2015). Such treatment options consist of alemtuzumab (depletes lymphocytes), daclizumab (blocks the cytokine receptor IL-2), dimethylfumarate (combines features of immunomodulatory and immunosuppressive actions), fingolimod (modulates the sphingosine-receptor system), natalizumab (inhibits the migration of lymphocytes) and teriflunomide (inhibits activated T and B cells) (Diebold & Derfuss, 2016; Inglese & Petracca, 2015; Katsara, Matsoukas, et al., 2008a). Examples of current interferons include, Schering

AG's Betaferon/Betaseron (IFNβ-1b), Biogen's Avonex (IFNβ-1a) and Serono/Pfizer's Rebif (IFNβ-1a). In addition, immune modulating agents include, Teva's Copaxone® (copolymer glatiramer acetate), Amgen/Serono's (Novantrone®; mitoxantrone), azathioprine, cyclophosphamide (Endoxan®) and Natalizumab® an a_4-integrin antagonist (Fenu et al., 2015; Kipp et al., 2015). Disease modifying agents have commonly been shown to reduce the rate of relapses, reduce MRI lesions and stabilize or delay MS disability. The key therapeutic feature of disease modifying drugs are their anti-inflammatory effects in the relapsing phase of MS, although demyelination leading to chronic disability still remains a major hurdle (Greenberg et al., 2013; Inglese & Petracca, 2015; Noyes & Weinstock-Guttman, 2013; Shirani et al., 2012). Some studies, however, have shown that early intervention of diseae modifying drugs to patients with RRMS can reduce acute disability or death (Goodin, Ebers, et al., 2012; Goodin, Reder, et al., 2012; Inglese & Petracca, 2015; Rommer & Stüve, 2013; Trojano et al., 2009).

In general, disease modifying drugs main action is by suppressing or altering the immune system. Hence, based on this theory that MS is, at least in part, a result of altered or abnormal immune response that results in attack of the myelin sheath. Current available drugs and their actions are described below (Table 1, Figure 2).

3.2.1. Interferons (Avonex®, Biogen; Betaseron®, Bayer; Extavia®, Novartis Pharma Corporation; Rebif®, EMD Serono Inc; Plegridy®, Biogen)

Interferon (IFN) type 1 consist of a group of IFNs (IFN-α, -β, -ε, -κ, -τ, -δ, -ζ, -ω, -ν) which help regulate the immune system. IFN-β is primarily produced by fibroblasts but other cells such as, NK cells, B cells, T cells, macrophages also secrete IFN-β. IFN-β has anti-viral and anti-tumor activity as well as being effective in reducing the relapse rate in patients with MS (Shirani et al., 2012). The mechanism by which IFN-β acts, is that it balances the expression of pro- and anti-inflammatory cytokines in the brain and decreases the number of inflammatory cells crossing the blood brain barrier. As a consequence there is decreased inflammation of neurons, increases nerve growth factors and improves neuronal survival. Moreover, IFN-β reduces Th17 population and IL-17 cytokine which are known to be involved in the immunopathophysiology of MS (Mitsdoerffer & Kuchroo, 2009). IFN-β injection subcutaneously or intramuscularly to patients with RRMS aims to decrease the relapse rate, duration and severity, however, there is lack of efficacy to long-term disability. Avonex was

approved in 1996, the first FDA approved treatment for RRMS. To date there are 3 approaches using IFN-β; IFN-β1a low dosage (Avonex®), IFN-β1a (Rebif®) high dosage, and, IFN-β1b (Betaseron®, Extavia®) high dosage. Furthermore, pegIFN-β-1a (Plegridy®) has polyethylene glycol linked to IFN-β-1a allowing it to be active for longer in the body, hence fewer injections are required compared to Avonex®, Rebif®, Betaseron® and Extavia®. The first large scale human clinical trial in patients with RRMS using IFN-β was published in 1993 and showed that relapse rates were reduced by 34 % in high dose IFN-β1b and by 8 % in lower dose compared to placebo group and severity of relapses were also reduced ("Interferon beta-1b is effective in relapsing-remitting multiple sclerosis. I. Clinical results of a multicenter, randomized, double-blind, placebo-controlled trial. The IFNB Multiple Sclerosis Study Group," 1993). Subsequent followup 5 year data showed that IFN-β1a and IFN-β1b decreased lesions up to 30 % and reduced the formation of new lesions up to 50 %, however, the study failed to show any reduction in disability progression in patients ("Interferon beta-1b in the treatment of multiple sclerosis: final outcome of the randomized controlled trial. The IFNB Multiple Sclerosis Study Group and The University of British Columbia MS/MRI Analysis Group," 1995). IFNs have no direct neuroprotective effects, however, through their direct effect on $CD4^{+}$Th1 cells and altering their profile results in decreased demyelination of neurons, which prevents further neuronal damage (Yong, Giuliani, Xue, Bar-Or, & Metz, 2007). Despite the impact of IFN-β in disease progression in patients with RRMS there are limitations in their use, with side effects ranging from local body aches, skin reactions (swelling, redness), fever, myalgia, flu-like symptoms to more serious side effects such as suicidal thoughts, hallucinations, seizures and heart and liver problems (Katsara, Matsoukas, et al., 2008a). As a result many patients have stopped treatment and overall the benefit of using IFNs is relatively small.

3.2.2. Glatiramer acetate (Copaxone®, Teva Neuroscience Inc)

Glatiramer acetate (GA) is a synthetic 4-mer peptide (L- glutamic acid, lysine, alanine, and tyrosine) mimic of MBP that competes with short antigenic MBP peptides in complex with MHC class II. Initially, GA was designed to induce EAE but instead it suppressed EAE, which was quickly translated into human trials with MS in order to prevent disease progression, as it bound to MHC class II and inhibited the activation of enchephalitogemic T cells (Neuhaus, Farina, Wekerle, & Hohlfeld, 2001; Ragheb, Abramczyk, Lisak, & Lisak, 2001; Wolinsky, 1995; Wolinsky et al., 2007). GA diverts Th1 cells to Th2 cells that suppress

inflammatory responses as well as activating Treg cells in the periphery (Haas et al., 2009). In human clinical trials it was noted that GA significantly reduced disease symptoms and development of new lesions by up to 30 % in RRMS, although it showed no improvement in long term efficacy on progression of disability (Johnson et al., 1995). GA injection in patients results in side-effects ranging from minor (fever, chills) to more serious (cardiovascular, digestive, muscular, respiratory issues).

3.2.3. Dimethyl fumarate (Tecfidera®, Biogen)

Dimethyl fumarate (BG-12) is a methyl ester of fumaric acid that modulates immune responses and was approved by the FDA in 2013. BG-12 was shown in phase III clinical trials to reduce relapse rate and increase the time to disability progression in patients with RRMS (Gold et al.). BG-12 reduces the migration of inflammatory cells through the blood brain barrier and activates nuclear factor erythroid 2-related factor (Nrf2) (Moharregh-Khiabani, Linker, Gold, & Stangel, 2009). Nrf2 regulates anti-oxidative proteins that protect cells against oxidative damage and inflammation. In fact, BG-12 protects neuronal cells from oxidative stress by increasing glutathione levels and suppressing pro-inflammatory cytokines from splenocytes *in vitro* (Albrecht et al., 2012). Side effects of BG-12 include diarrhea, abdominal pain, nausea, abnormal liver enzymes and decreased lymphocyte counts.

3.2.4. Teriflunomide (Aubagio®, Sanofi Genzyme)

Teriflunomide is an active metabolite of leflunomide (an immunosupressive disease modifying drug used for rheumatoid arthritis) which inhibits the enzyme dihydroorotate dehydrogenase (Palmer, 2010) and inhibits the proliferation of B and T cells. In addition, teriflunomide exerts anti-inflammatory properties by inhibiting IFN-gamma producing T cells whilst IL-4 and IL-10 producing T cells are unaffected (Korn, Magnus, Toyka, & Jung, 2004). In MS, oral administration of teriflunomide reduced relapse rates, MS lesions and decreased disability progression (Confavreux et al., 2014; P. O'Connor et al., 2011; P. W. O'Connor et al., 2006; Sanvito, Constantinescu, & Gran, 2011; Vermersch et al., 2014; Yeh, 2011). Moreover, permanent discontinuation due to side effects were substantially less common in MS patients who received teriflunomide compared to IFN-β-1a. Side effects inlcude, reduced white blood cell count, numbness in hand and feet, allergic reactions, breathing issues and

increased blood pressure. Teriflunomide was approved by the FDA in 2012 and by EMA in 2013 for use in patients with RRMS.

3.2.5. Fingolimod (Gilenya®, FTY720, Novartis Pharma AG)

Fingolimod was granted FDA approval in 2010 and was the first oral therapy (0.5 mg once daily) available for patients with relapsing forms of MS. Fingolimod is a sphingosine 1-phosphate (S1P) receptor modulator, which acts as an antagonist of S1P receptor causing receptor internalization and leading to reduced infiltration of potentially auto-reactive lymphocytes into the CNS (Brinkmann et al., 2002b; Mandala et al., 2002; Matloubian et al., 2004). In addition, secondary beneficial effects of fingolimod is that it targets SIP receptors on glia cells in the CNS, activating signalling pathways within the CNS (Brinkmann et al., 2002a; Choi et al., 2011). Based on Phase III human clinical trials in patients with RRMS (TRANSFORMS, FREEDOMS and FREEDOMS II), fingolimod was more effective compared to first line treatment IFNβ-1a and placebo, in reducing the frequency of flare-ups (clinical exacerbations), disability progression, MRI outcome measures, including brain volume loss and was associated with clearly identified adverse events (Calabresi et al., 2014; Cohen et al., 2010; L. Kappos et al., 2010). More than 180,000 patients have been treated with fingolimod in clinical trials and post-marketing settings globally, and the total patient exposure now exceeds 395,000 patient-years. Side effects include blurred vision, diarrhea, difficulty breathing, joint pain, muscle pain, nervousness and vomiting. With reasonable data showing its long-term safety and disease improvement, fingolimod is a great alternative choice for patients with MS who prefer the oral treatment option.

3.2.6. Mitoxantrone (Novantrone®, Immunex/Amgen)

Mitoxantrone is primarily used to treat certain types of cancers, in particular, non-Hodgkin's lymphoma, acute myeloid leukemia, breast and prostate cancer. Mitoxantrone is a type-II topoisomerase inhibitor which disrupts DNA synthesis and DNA repair of cancer cells, however, normal cells are also affected. It is a potent immune suppressant, suppressing T cells, B cells and macrophages and reduces pro-inflammatory cytokines (IFN-γ, TNF-α, and IL-2) (B. Huang et al., 2012; Lenk, Muller, & Tanneberger, 1987). In patients with SPMS, intravenous injection of 12 mg/m^2 mitoxantrone every 3 months up to 2 years resulted in reduced disability progression by 84 % (Edan et al., 1997; H.-P. Hartung et al.). However,

several side effects are associated with mitoxantrone which range from nausea, vomiting, hair loss, to, cardiotoxicity, leukaemia, infertility, infection, leukopenia and thrombocytopenia (Eckstein & Bhatti, 2016). As a result, its use has significantly been reduced over time. Furthermore, due to the risk of cardiotoxicity and leukemia, there is a limit on the cumulative lifetime dose to be administered to patients (Eckstein & Bhatti, 2016; Martinelli, Radaelli, Straffi, Rodegher, & Comi, 2009).

3.2.7. Dalfampridine (Ampyra®, Acorda Therapeutics)

Dalfampridine is not intended to delay symptoms or change the course of disease, but rather, to improve motor symptoms such as walking. Dalfampridine, is a potassium channel blocker, resulting in improved potassium currents and nerve conductance. Dalfampridine is used in patients who have had MS for more than 3 years and it was approved by the FDA in 2010. Common side effects include nausea, nervousness and dizziness which are relatively minor compared to other MS drugs.

3.3. Treatment using humanized monoclonal antibodies

3.3.1. Natalizumab (Tysabr®, Biogen)

Natalizumab is a humanized monoclonal antibody against the cellular adhesion molecule α4- integrin. Integrins are transmembrane receptors that enable cell-extracellular matrix adhesion activating cell signaling which regulate cell growth, division, survival, differentiation and migration. Integrins are expressed on T cells, B cells, monocytes, macrophages, NK cells, DC, neutrophils and eosinophils. Interfering or blocking □4-integrin affects immune cell migration across the blood brain barrier, thus, by blocking the interaction between □4-integrin and vascular endothelial adhesion molecule-1, inhibits transendothelial migration to the CNS (Sheremata, Minagar, Alexander, & Vollmer, 2005). Natalizumab is administered intravenously once a month (Calabresi et al., 2014; Rice, Hartung, & Calabresi, 2005) which reduces activated T cells within the CNS, resulting in anti-inflammatory responses and hence, neuroprotective effects (Yong et al., 2007). In a phase III clinical trial natalizumab reduced brain lesions and the rate of disability progression up to 24 months (Klotz et al., 2011; Polman et al., 2011). In addition, natalizumab decreased by 92 % of contrast-enhancing lesions,

by 83% of new or expanding T2-weighted lesions, and by 76% in new T1-weighted hypointense lesions (Jarius, Hohlfeld, & Voltz, 2003; Miller et al., 2003). Natalizumab, was approved by the FDA in 2004, but was withdrawn due to 3 cases of rare brain infection, progressive multifocal leukoencephalopathy (PML; that usually leads to death or severe disability), but was re-introduced in 2006 under a special prescription program. However, by 2012 a further 212 cases (or 2.1/1000) of PML were reported to be attributed to natalizumab ("Natalizumab. Multiple sclerosis: Risky market approval," 2008). Despite these reports the FDA has not withdrawn natalizumab from the market as the clinical benefits outweigh the risks involved. Other side effects include, hepatotoxicity, allergic reactions and increased risks of infection. Due to the risks involved with natalizumab, there are reservations over its use as a preferred treatment option.

3.3.2. Ofatumumab (Arzerra®, Novartis Pharma Corporation)

B cells play a role in the pathogenesis of MS. B cells have essential functions in regulating immune response, by activating CD4+ T-cells and regulating T-cell responses via the secretion of cytokines and antibodies. B cells are present at demyelinating areas and in cerebrospinal fluid of patients with MS (Frohman, Racke, & Raine, 2006; Hauser et al., 2008). CD20 is a marker and present on the cell surface of all B cells. In an attempt to reduce the number of B cells including autoreactive B cells, the use of anti-CD20 antibodies would conceivably improve MS relapses and progression. In fact, there are several humanized anti-CD20 antibodies, such as rituximab, ocrelizumab (L. Kappos et al., 2011) and ofatumumab (Sorensen et al., 2014), which have shown high efficacy in patients with RRMS. In 2015, Novartis acquired the rights from GlaxoSmithKline for the development of ofatumumab in oncology and other autoimmune indications. Ofatumumab (OMB157) is the first fully human type 1 IgG1 kappa (IgG1κ) monoclonal antibody and is currently licensed for the treatment (of patients with chronic lymphocytic leukemia (intravenously (iv), Arzerra®). Ofatumumab binds to 2 unique novel epitopes on the CD20 molecule, induces B-cell depletion via complement dependent cytotoxicity and antibody-dependent cell-mediated cytotoxicity causing B cell apoptosis (Bleeker et al., 2008).

Ofatumumab has demonstrated high efficacy in hematologic malignancies and in rheumatoid arthritis. Based on 2 Phase II dosing human clinical studies, ofatumumab demonstrated high efficacy in reducing new MRI lesion activity more than 90% and was well

tolerated in patients with MS (Sorensen et al., 2014). Currently, ofatumumab is being further investigated in 2 Phase III trials (ASCLEPIOS I AND ASCLEPIOS II) and are recruiting patients with relapsing forms of MS (ofatumumab versus teriflunomide). The adaptive study design of both trials was recently presented by Hauser SL and colleagues at the American Academy of Neurology April 2017 in Boston, USA and results are highly anticipated ("Identifier: NCT02792218 and NCT02792231," 2017).

3.3.3. Ocrelizumab (Ocrevus®, Genentech Inc)

A few months ago (March 2017), the FDA approved ocrelizumab to be used in PPMS, the first drug approved by the FDA for this form of MS and phase IV clinical trials were a requirement of the FDA to be conducted in order to determine the safety of ocrelizumab in younger patients with MS, ie, risk of cancer and effects on pregnancy (study outcomes due by 2024); although clinical trials in patients with lupus and rheumatoid arthritis were halted due to high rates of infections and increased risk of progressive multifocal leukoencephalopathy (FDA, 2017). In addition, in patients with MS, there was an increased risk of breast cancer (6/781 females with MS on ocrelizumab compared to 0/668 females with MS in other trials) (FDA, 2017).

3.3.4. Alemtuzumab (Lemtrada®, Sanofi Genzyme)

Alemtuzumab is a humanized monoclonal antibody against CD52, a cell surface molecule expressed on B and T cells; mature NK cells, plasma cells, neutrophils and importantly, hematological stem cells do not express CD52. In phase III clinical trials in patines with RRMS, alemtuzumab showed significantly lower annualized relapse rates and MRI measures (gadolinium-enhancing lesions, new or enlarging T2 lesions and brain atrophy) and were free of clinical disease longer, compared to IFNα-1a (Cohen et al., 2012; Coles et al., 2012). Alemtuzumab can cause serious side effects including, immune thrombocytopenia, kidney problems, serious infusion problems (trouble breathing, swelling, chest pain, and irregular heart beat), certain cancers (blood cancers, thyroid cancer), cytopenia and serious infections. It was approved by the FDA in 2014 to be used in RRMS patients, but due to the frequent and significant adverse events of alemtuzumab, it is generally used in patients with RRMS who have used 2 or more MS drugs and have failed to work.

3.3.5. Daclizumab (Zinbryta®, Biogen)

Daclizumab is a humanized monoclonal antibody against CD25, the IL-2 receptor expressed on the surface of T cells. The mechanism by which daclizumab works is that it blocks the IL-2 receptor on T cells, preventing the activation of T cells. It was originally approved by the FDA in 1997 to prevent acute kidney transplants (together with corticosteroids and cyclosporin) however its use was halted due to low market demand. In recent years its use has re-emerged to treat patients with RRMS, it is injected subcutaneously, once a month (Lycke, 2015). In human clinical trials, daclizumab showed 45% reduced annualized relapse rates and 54% lower in the number of new lesions (Lycke, 2015). The side effects associated with daclizumab are relatively minor compared to other MS drugs, and include infections, skin rashes and liver complications.

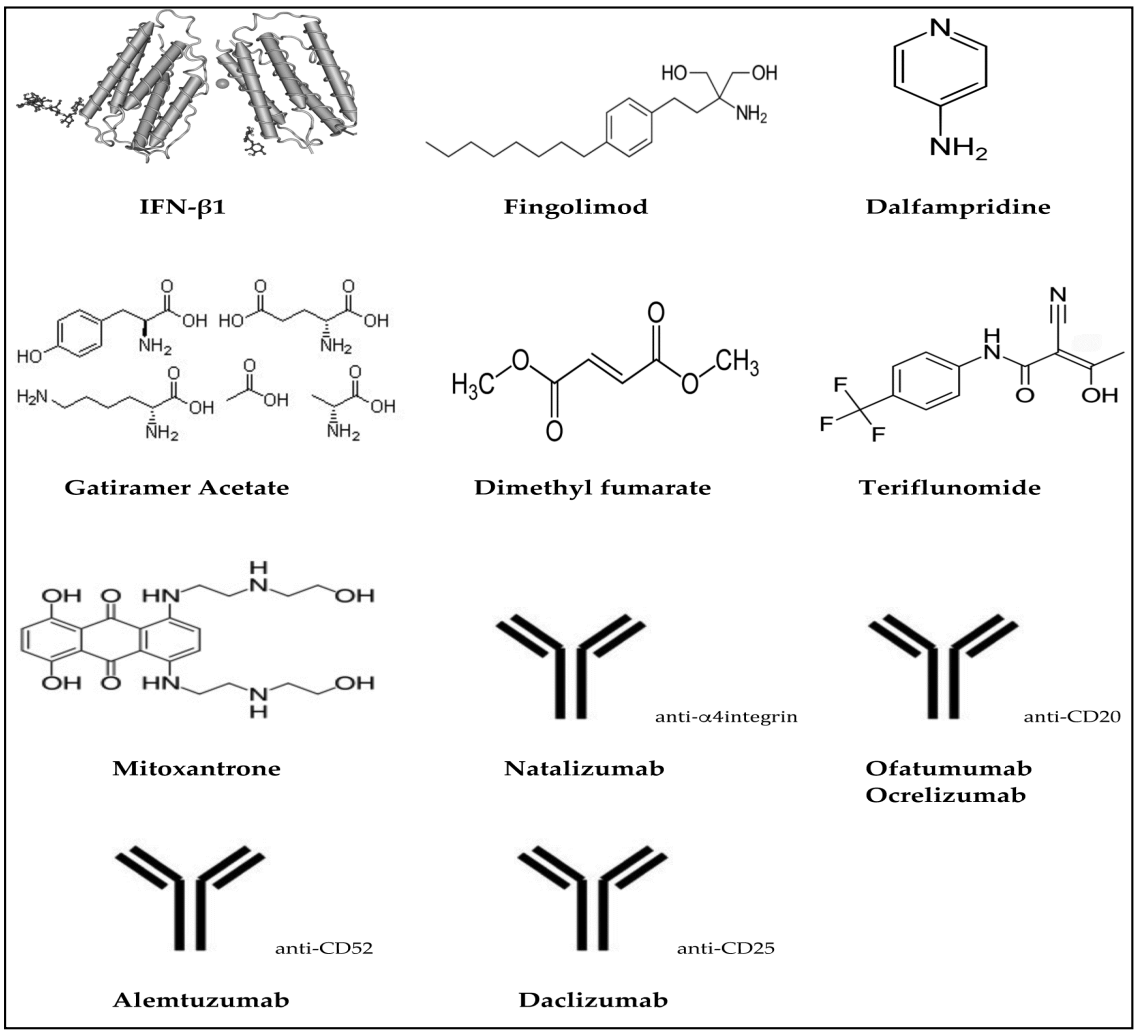

Figure 2. Chemical / schematic structures of treatments / drugs for MS

Table 1. Disease modifying drugs available to patients with RRMS

Drug	Brand	Dose	# of injections, route	Actions
IFN-β1a	Avonex®	7.5 µg 1st dose 15 µg 2nd dose 22.5 µg 3rd dose 30 µg all subsequent doses	1 / week, i.m	Balances pro- and anti-inflammatory cytokines Decreases Th17 cells Decreases IL-17
	Rebif®	22 µg or 44 µg	3 / week, s.c	
IFN-β1b	Betaseron®	62.5 µg and increase over 6 weeks to 250 µg	1 / 2 days, s.c	
	Extavia®	62.5 µg and increase over 6 weeks to 250 µg	1 / 2 days, s.c	
pegIFN-β1a	Plegridy®	63 µg 1st dose 95 µg 2nd dose 125 µg all subsequent doses	1 / 2 weeks, s.c	
Glatiramer acetate, EKAY	Copaxone®	20 µg or 40 mg	1/day, s.c 3/week, s.c	Blocks pMHC
Dimethyl fumarate	Tecfidera®	240 mg	2-3/day, oral	Anti-inflammatory Anti-oxidative stress
Teriflunomide	Aubagio®	7 or 14 mg	1/day, oral	Inhibits dihydroorotate dehydrogenase, T, B cells and IFN-γ secreting T cells
Fingolimod	Glenya®	0.5 mg	1/day, oral	Antagonist of SIP receptor Decrease T, B cells activates SIP signaling in CNS
Mitoxantrone	Novatrone®	12 mg/m^2	1/3 months up to 2 years i.v	Suppresses T, B cells and macrophages. Reduces Th1 cytokines
Dalfampridine	Ampyra®	10 mg	2/day, oral	Potassium channel blocker. Improves motor symptoms, ie. walking.
Humanized monoclonal antibody treatments				
Natalizumab	Tysabr®	300 mg	1/28 days, i.v	Humanized anti-α 4-integrin Mab. Affects cell migration, division, growth and suvival
Ofatumumab	Arzerra®	3-700 mg	1/2 weeks, i.v	Humanized anti-CD20 Mab Cytotoxic to CD20$^+$ cells via CDC and ADCC
Ocrelizumab	Ocrevus®	300-600 mg	300 mg weeks 1 and 3, then 600 mg 1/6 months, i.v	Humanized anti-CD20 Mab
Alemtuzumab	Lemtrada®	12 mg	5 days in a	Humanized anti-CD52 Mab.

			row; after 1 year, 3 days	Depletes T, B cells, increases Treg, Th2, decrease Th1 cells
Daclizumab	Zinbryta®	150 mg	1/month, s.c	Humanized anti-CD25 Mab. Blocks IL-2R, decreases T cells, increases NK cells

ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity; DC, dendritic cells; EKAY, single amino acid code for L- glutamic acid, lysine, alanine, tyrosine; IFN, interferon; IL-2R, interleukin-2 receptor; i.m, intramuscular; i.v, intravenous; Mab, monoclonal antibodies; NK, natural killer cells; pegIFN, polytheylene glycol linked to IFN; pMHC, peptide-major histocompatibility complex; RRMS, relapsing remitting multiple sclerosis; s.c, subcutaneous; SIP, sphingosine-1-phosphate; Th, helper T cells; Treg, regulatory T cells ($CD4^+CD25^+FoxP3^+$).

4. New and emerging immunotherapeutic strategies against MS

Antigen/peptide specific immunotherapy or using immune cells (*ie*. stems cells), aim to restore tolerance whilst avoiding the use of non specific immunosuppressive drugs as describe in section 3, is a promising approach to fight autoimmune diseases including MS. As such, a number of approaches have been utilized.

4.1. Stem cells

Multipotent hematopoietic stem cells (HSC) are cells isolated either from the bone marrow, umbilical cord blood or peripheral blood and are transplanted into the recipient. More commonly used for hematological malignancies (leukemia, multiple myeloma) its application has also expanded into autoimmune diseases. The first report of a bone marrow transplant in 1997 in a chronic myelogenous leukemia patient with MS which showed marked improvements in MS brain lesions (McAllister, Beatty, & Rose, 1997) quickly led to the use of HSC transplantation (HSCT) in MS patients. HSCT in patients with active RRMS, reduce progression in about 70% of patients, decrease relapses dramatically and suppresses inflammatory MRI activity (Mancardi & Saccardi, 2008). MS patients who have not responded to conventional therapy, who's disease is aggressive with relapsing-remitting course and who are not presenting with high level of disability, are considered appropriate candidates for such treatment (Sormani et al., 2017). Although the clinical efficacy of HSCT long term has not

been established. The mechanism by which HSCT works, is that HSCT 'reboots' the immune system and thus, prevents inflammation associated with the disease.

Mesenchymal stem cells (MSC) are isolated from an adult's bone marrow, are differentiated *in vitro* for 2-3 weeks and re-injected back into the patient. In recent years a vast amount of research has been conducted in MSCs to treat MS with most studies being in mice and EAE models, and more recently in human clinical trials. In fact, in a pilot study in advanced MS patients, MSC transplantation improved expanded disability scale score with stabilization in 1/7 and disease progression in 1/7 patients and vision and low contrast sensitivity test showed improvement in 5/6 patients with 1/6 showing worsening effects . In a phase II randomized double-blind, placebo-controlled crossover clinical trial showed lower mean cumulative number of lesions in patients receiving MSCs compared to placebo (Llufriu et al., 2014). No serious adverse events were reported. The mechanism of action of MSC includes immunomodulation, neuroprotection and neuroregeneration (Yamout et al., 2010). The use of MSCs which reduce MRI parameters is a new and emerging research focus to develop new improved treatments for MS.

4.2. DNA vaccine studies

BHT-3009, a DNA vaccine which encodes the full-length human MBP was developed with the aim to tolerize patients with MS against MBP (Kang et al., 2012; Katsara, Matsoukas, et al., 2008a). In fact, in 30 patients with RRMS or SPMS who received 4 injections of BHT-3009 on weeks 1, 3, 5, 9 with escalating doses of 0.5 mg, 1.5 mg or 3 mg was reported to be safe and conferred positive changes on brain MRI and reduced the number of $CD4^+$ T cells (Correale & Fiol, 2009; Kang et al., 2012; Katsara, Matsoukas, et al., 2008a). In addition, in a retrospective, randomized double blind, phase II study in 155 MS patients, BHT-3009 had no impact on the risk for persitent black holes (axonal loss and disability progression). However, there was a correlation to those who had generated high anti-IgM MBP antibodies to reduced risk of perisistent black holes(Papadopoulou et al., 2012).

4.3. Nanoparticles

Nanoparticles have extensively been characterized and used as vaccine formulations in pre-clinical models of cancer and infectious diseases (Xiang et al., 2006; Xiang, Selomulya,

Ho, Apostolopoulos, & Plebanski, 2010). Polymeric biodegradable lactic-glycolic acid (PLGA) nanoparticles loaded with MOG_{35-55} peptide together with recombinant IL-10, were partially endocytosed by dendritic cells, secreted both MOG_{35-55} peptide and IL-10 in culture media for several weeks *in vitro* (Cappellano et al., 2014). In mice, PLGA nanoparticles (MOG_{35-55} + IL-10) showed significant amelioration of EAE and reduction of IL-17 and IFN-gamma secretion by splenic T cells *in vitro* (Cappellano et al., 2014). Recently, poly (ε-caprolactone) nanoparticles loaded with recombinant human MBP reduced IFN-gamma cytokines, reduced the clinical score and showed only mild histological changes of the myelin sheath (Al-Ghobashy et al., 2017). Hence, nanoparticles as a delivery method of self antigens are a promising tool to treat MS.

4.4. Altered peptide ligands

Altered peptide ligands (APL) are peptides closely related to the native (agonist) peptide with defined 1-2 substituted amino acid residues which interact with the T cell receptor (TCR) yet retains its binding ability to the MHC (Katsara, Minigo, et al., 2008a). In phase I/II clinical trial by Neurocrine Biosciences Inc, used an APL of $MBP_{83–99}$, where L-amino acids were changed to D-amino acids at positions 83, 84, 89, 91 (NBI-5788) (Crowe, Qin, Conlon, & Antel, 2000). However, this mode of APL induced T cell cross reactivity between the APL and the wild-type / agonist $MBP_{83–99}$ peptide and adverse events in some patients resulted (H. P. Hartung, Kieseier, & Hemmer, 2005). A subsequent multi-centre double-blinded phase II clinical trial with NBI-5788 was suspended - Th2 responses were induced (IL-5, IL-13), however, 13/142 patients developed immediate-type hypersensitivity, who also generated anti-NBI-5788 antibodies which cross-reacted with native agonist MBP_{83-99} peptide (Crowe et al., 2000; L. Kappos, Comi, Panitch, Oger, Antel, Conlon, Steinman, et al., 2000). National Institute of Neurological Disorders and Stroke sponsored trial, CGP77116, was used in a MRI-controlled phase II clinical trial. CGP77116, has Ala D-amino acids of MBP_{83-99} peptide at positions 83, 84, 89, 91 (CGP77116) of MBP_{83-99} peptide, in order to enhance stability (L. Kappos, Comi, Panitch, Oger, Antel, Conlon, Steinman, et al., 2000). However, this peptide was poorly tolerated at the dose tested, and the trial had to be discontinued. Three patients showed exacerbations to disease of which two were linked to CGP77116 injection with high IFN-gamma and low IL-4 (Th1-skewing) were secreted by activated $CD4^+$ T cells. These $CD4^+$ T cells also cross reacted with the native agonist MBP_{83-99} peptide (Bielekova et al., 2000).

Accordingly, the problems noted with both NBI-5788 and CGP77116 were likely due to inadequate pre-screening of APL effects on the many clonotypes against the targeted epitopes. Thus, although the APL was highly effective at blocking or switching some clones, it activated others. Thus, further pre-clinical testing is required and new modified peptides need to be designed, or a carrier needs to be used which further changes the resulting immune response.

4.4.1. Cyclic peptides

Cyclization of peptides increases the stability, since linear peptides are sensitivity to proteolytic enzymes. In addition, cyclic peptides an important intermediate step and a useful template towards the rational design and development of a non-peptide mimetics. While mimetic strategy is a challenging perspective it is worth pursuing in particular for MBP epitope based MS therapy as it is still in its infancy. Efforts to design semi mimetics of MBP_{72-85} epitope by combining non-natural amino acids as spacers and MBP epitope immunophores (Ser, Arg, Glu, Ala, Gln), led to substances which were effective to some extent in inducing the onset of EAE. Cyclic peptides are not only as a step towards non-peptide mimetics but also as putative therapeutics in MS (Katsara et al., 2006).

Structure activity studies of the immunodominant agonist peptide MBP_{87-99}, have shown that K^{91},P^{96} are important T cell receptor contact residues. Double mutation of K^{91},P^{96} to R^{91},A^{96} or single mutation of P^{96} to A^{96} (APL) of either in their linear or cyclic forms, results in suppression of EAE and decreased inflammation in the spinal cord of Lewis rats (T. Tselios et al., 2002a). Single and double cyclic[A^{91}]MBP_{83-99} peptide and cyclic[$A^{91}A^{96}$]MBP_{83-99} peptides emulsified in CFA induced IL-4 cytokines in SJL/J mice however conjugation to reduced mannan further enhanced IL-4 cytokines with no IFN-gamma responses (Katsara, Deraos, et al., 2009a). In guinea pigs and Lewis rats, cyclic [$A^{91}A^{96}$] MBP_{83-99} showed significantly reduced mechanical pain hypersensitivity compared to cyclic MBP_{83-99} peptide. This was associated with reduced T cell and macrophage infiltration to injured nerves of the spinal cord of animals (Peferoen et al., 2015; Perera, Duffy, et al., 2015; Perera, Lees, et al., 2015; Tian, Perera, Apostolopoulos, & Moalem-Taylor, 2013). In addition, these APL decreased $CD4^+$ T cell line proliferation raised from a patient with MS, increased IL-10 cytokine secretion, bound to HLA-DR4 and were more stable to lysosomal enzymes (cathepsin B, D, H) compared to their linear counterparts . Double mutation of K^{91},P^{96} to A^{91},A^{96} in either linear or cyclic forms were also shown to be active, with suppression of EAE in SJL/J mice,

higher Th2 over Th1 cytokines produced, bound to HLA-DR4, the cyclic forms were more stable to lysosomal enzymes and induced high levels of IL-10 of peripheral blood mononuclear cells from patients with MS (Deraos et al., 2015a). Recently, cyclic native agonist MOG_{35-55} peptide was shown to ameliorate clinical and neuropathological features of EAE in mice compared to its linear counterpart (Lourbopoulos et al., 2017b). Thus, cyclic peptides, which offer greater stability and are able to modulate immune responses, are novel leads for the immunotherapy of many diseases, such as MS (Katsara et al., 2006).

4.4.2. Mannan as a carrier to modulate immune responses

Mannan, a polymannose, isolated from the wall of yeast cells has been shown to bind to the mannose receptor on dendritic cells as well as being a ligand for toll-like receptor 4 (Apostolopoulos, Pietersz, Loveland, Sandrin, & McKenzie, 1995; Apostolopoulos, Pietersz, & McKenzie, 1996). Mannan conjugated to MUC1 cancer protein induces immune responses in mice and protects mice against tumor challenge. This work was translated into human phase I, II and pilot III clinical trials; mannan-MUC1 induces protection against cancer recurrence at 18 years follow-up (Apostolopoulos et al., 2006; Apostolopoulos et al., 2014; Karanikas et al., 1997; Vassilaros et al., 2013). Furthermore, *ex vivo* cultured dendritic cells pulsed with mannan-MUC1 (CVac™) and re-injection into patients induces strong cellular and clinical responses in ovarian cancer patients (Loveland et al., 2006; P. L. R. Mitchell et al., 2014). Due to the immunomodulatory properties of mannan, its effects as a carrier to MS peptides was determined.

Mutations of MBP_{83-99} agonist native peptide to result in mutant peptides (APL) - linear $[A^{91}]MBP_{83-99}$, $[E^{91}]MBP_{83-99}$, $[F^{91}]MBP_{83-99}$, $[Y^{91}]MBP_{83-99}$ and $[R^{91}, A^{96}]MBP_{83-99}$, induced IFN-gamma albeit reduced compared to the native agonist peptide, however, only the double APL $[R^{91}, A^{96}]MBP_{83-99}$ induced IL-4 secretion by T cells and antagonized IFN-gamma production *in vitro* by T cells against the native MBP_{83-99} peptide (Katsara et al., 2008a). In addition, T cells against the native MBP_{83-99} peptide cross-reacted with all peptides except $[Y^{91}]MBP_{83-99}$ and $[R^{91},A^{96}]MBP_{83-99}$ (Katsara et al., 2008b). Conjugation of $[R^{91},A^{96}]$ MBP_{83-99}, $[A^{91},A^{96}]MBP_{83-99}$, $F^{91}]MBP_{83-99}$, $[Y^{91}]MBP_{83-99}$ peptides to mannan, completed abrogated IFN-gamma responses and elicited high IL-4 (*ie.* Th1 to Th2 switch) (Katsara, Deraos, et al., 2009b; Katsara, Yuriev, et al., 2009b). Likewise, linear double-mutant APL $[L^{144}R^{147}]$ $PLP_{139-151}$ induces high levels of IL-4, and cyclization of this analog elicited low levels of IFN-gamma.

When conjugated to mannan, [$L^{144}R^{147}$] $PLP_{139-151}$ peptide completely abrogated IFN-gamma, whilst both linear and cyclic native agonist $PLP_{139-151}$ peptides stimulated IFN-gamma secreting T cells (Katsara et al., 2014). Furthermore, mannan conjugated to the immunedominant agonist MOG_{35-55} peptide primes non-pathogenic Th1 and Th17 cells and ameliorates EAE in mice (Tseveleki et al., 2015b); a phase I human clinical trial is planned using mannan conjugated to MOG_{35-55} peptide later this year. It is clear that, mannan is able to divert immune responses from Th1 to Th2 and is a promising carrier for further studies for the development of immunetherapeutics against MS.

5. Conclusion and future prospects

MS is an autoimmune disorder of the CNS with an array of immune cells being either activated or suppressed leading to demyelination and disease progression. In addition, genetic predisposition, viral mimicry, vitamin and mineral deficiency, geographical location are also aetiological factors that contribute to disease. More recently, citrulination of myelin peptides have been shown to contribute to disease activation (Apostolopoulos et al., 2017a; Deraos et al., 2008b). A number of treatment options are available to patients with MS, in particular those with active disease, however due to side effects, limited long term effectiveness and inability to reverse disease, new improved treatment options are required. As described herein a number of new an upcoming promising therapeutic candidates are becoming available, although their effectiveness in human clinical trials remains to be determined. Recently, it was reported that non-peptide mimetics mapping the MBP_{83-96} T cell epitope can function as T cell receptor antagonists, hence such an approach may pave the way to developing alternative and improved immunetherapeutics against MS (Yannakakis et al., 2017b). With the plethora of information regarding the immunopathophysiology of MS and availability of treatment options and new upcoming treatments, the future holds promise for managing and treating the disease.

Chapter 2

Chapter 2

Immunomodulatory effects of Streptococcus thermophilus on U937 monocyte cell cultures

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ABSTRACT

Probiotics are beneficial to the host through its contribution to the development and maintenance of a healthy immune system. Some probiotics are used in the food industry as secondary starter cultures to ferment dairy products including *Streptococcus thermophilus* (ST). ST bacteria were used to determine their modulatory effects on a promonocytic cell line which exhibited differential cytokine induction, in particular, IL-4 and IL-10 which are important in injury, infection and play a central role in anti-inflammatory responses. CXCL8 and GM-CSF are also activated - important for chemotaxis and recruitment of cells at sites of inflammation, and, increased CD11c, CD86, C206, CD209, MHC-1 expression. As ST are used in the dairy industry, are well tolerated when consumed and remain viable during cold storage, their consumption might be a practical approach in modulating immune responses in the host, and be beneficial to an array of diseases, including, autoimmunity and inflammatory bowel diseases.

Keywords: Probiotics; *Streptococcus thermophilus*; Monocytes; Cytokines; Inflammation

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Immunomodulatory effects of *Streptococcus thermophilus* on U937 monocyte cell cultures

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1. Introduction

The regular consumption of probiotics has been shown to contribute to the maintenance of a healthy microbiome in the intestinal tract and associated health benefits (Ahtesh, Stojanovska, & Apostolopoulos, 2018; Hardy, Harris, Lyon, Beal, & Foey, 2013). It has been documented that there are over 1000 existing species within the microbiome – with 400 well known, which are all essential for the establishment and maintenance of a healthy and functional immune system (A. J. Stagg, Hart, Knight, & Kamm, 2004; J. Stagg et al., 2011; Jensen, Drømtorp, Axelsson, & Grimmer, 2015). Commensal strains of the human intestinal microbiota have been used as probiotic supplements, either in food or as capsules, for a variety of medical issues including diarrhoea, constipation and various infections (Di Caro et al., 2005; Isolauri, Sütas, Kankaanpää, Arvilommi, & Salminen, 2001; Ouwehand, Salminen, & Isolauri, 2002; Vliagoftis, Kouranos, Betsi, & Falagas, 2008). This is based on the role that the microbiome plays in establishing a balanced immune response during early life and maintaining it throughout adulthood (Kelly, King, & Aminov, 2007; Langhendries, 2005, 2006; Mead et al., 1999). These beneficial bacteria were termed "probiotic" by Fuller in 1989 (AFRC, 1989), which were then defined by the Food and Agriculture Organization and the World Health Organization as "live microorganisms which upon administration in adequate amounts confer a health benefit to the host" (Guarner & Schaafsma, 1998; Lebeer, Vanderleyden, & De Keersmaecker, 2008; Vasiljevic & Shah, 2008). Likewise, "ghost probiotics", i.e. non-viable microbial cells, intact or broken or crude cell extracts also confer benefits to the host (Deshpande, Athalye-Jape, & Patole, 2018).

Most probiotics today belong to the group of lactic acid bacteria (LAB) which represent gram-positive lactic acid producing microorganisms, and include several genera of lactobacilli, bifidobacteria and enterococci; LAB are abundantly present in the intestine, especially in the lower small intestinal lumen and the colon (Fink et al., 2007; Maassen et al., 2000; Michalkiewicz et al., 2003). LABs are commonly supplemented in foods as live probiotic strains and have been shown to confer health benefits to humans (Asarat, Apostolopoulos, Vasiljevic, & Donkor, 2015, 2016; Asarat, Vasiljevic, Apostolopoulos, & Donkor, 2015; Fink et al., 2007; Guarner & Schaafsma, 1998; Salazar et al., 2009). In addition, *Streptococcus* species (a member of the LAB), including exopolysaccharide-producing strains of *Streptococcus thermophilus* (ST) such as *S. thermophilus* ST1342, *S. thermophilus* ST1275 and *S. thermophilus* ST285 (Purwandari & Vasiljevic, 2009; Salazar et al., 2009) are widely used due to their functional properties such as, immunosuppressive effects in the treatment of acute ulcerative colitis,

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The regular consumption of probiotics has been shown to contribute to the maintenance of a healthy microbiome in the intestinal tract and associated health benefits (Hardy et al., 2013). It has been documented that there are over 1,000 existing species within the microbiome - with 400 well known, which are all essential for the establishment and maintenance of a healthy and functional immune system (H. Jensen, Drømtorp, Axelsson, & Grimmer, 2015; A. J. Stagg et al., 2004; J. Stagg et al., 2011). Commensal strains of the human intestinal microbiota have been used as probiotic supplements, either in food or as capsules, for a variety of medical issues including diarrhoea, constipation and various infections (Di Caro et al., 2005; Isolauri, Sütas, Kankaanpää, Arvilommi, & Salminen, 2001; Ouwehand, Salminen, & Isolauri, 2002; Vliagoftis et al., 2008). This is based on the role that the microbiome plays in establishing a balanced immune response during early life and maintaining it throughout adulthood (Kelly et al., 2007; Langhendries, 2005, 2006; Mead et al., 1999). These beneficial bacteria were termed "probiotic" by Fuller in 1989 (AFRC, 1989), which were then defined by the Food and Agriculture Organization and the World Health Organization as ''live microorganisms which upon administration in adequate amounts confer a health benefit to the host'' (Guarner & Schaafsma, 1998; Lebeer, Vanderleyden, & De Keersmaecker, 2008; Vasiljevic & Shah, 2008). Likewise, "ghost probiotics", *i.e.* non-viable microbial cells, intact or broken or crude cell extracts also confer benefits to the host (Deshpande, Athalye-Jape, & Patole, 2018).

Most probiotics today belong to the group of lactic acid bacteria (LAB) which represent gram-positive lactic acid producing microorganisms, and include several genera of lactobacilli, bifidobacteria and enterococci; LAB are abundantly present in the intestine, especially in the lower small intestinal lumen and the colon (Fink et al., 2007; Maassen et al., 2000; Michałkiewicz et al., 2003). LABs are commonly supplemented in foods as live probiotic strains and have been shown to confer health benefits to humans (Asarat, Apostolopoulos, et al., 2015; Asarat et al., 2016; Asarat, Vasiljevic, et al., 2015; Fink et al., 2007; Guarner & Schaafsma, 1998; Salazar et al., 2009). In addition, *Streptococcus* species (a member of the LAB), including exopolysaccharide-producing strains of *Streptococcus thermophilus* (ST) such as *S. thermophilus* ST1342, *S. thermophilus* ST1275 and *S. thermophilus* ST285 (Purwandari & Vasiljevic, 2009; Salazar et al., 2009) are widely used due to their functional properties such as, immunosuppressive effects in the treatment of acute ulcerative colitis, improving lactose digestion (R. Iyer, Tomar, Uma Maheswari, & Singh, 2010; Rabot, Rafter,

Rijkers, Watzl, & Antoine, 2010; Savaiano, 2014), improving the intestinal barrier function restricting adhesion and invasion of pathogens (Brigidi, Swennen, Vitali, Rossi, & Matteuzzi, 2003; Elli et al., 2006; Kebouchi et al., 2016) as well as their production of bacteriocins and vitamins (R. Iyer et al., 2010; Ng et al., 2010; Uriot et al., 2017). Furthermore, *ST* present characteristics that enable them to be used in fermented milk products (i.e. yogurt), flavoring of dairy, and is recognized as the next most important species after *Lactococcus lactis* (Hols et al., 2005). Since 2002, *ST* has been accepted to be safe and approved by the American Food and Drug Administration (FDA, 2018) and the Qualified Presumption of Safety grade/rank/status from the European Food Safety Authority (Kebouchi et al., 2016). However, in contrast with other LAB, using the term probiotic for *ST* is still a matter of debate (Mohammadi, Sohrabvandi, & Mohammad Mortazavian, 2012; Uriot et al., 2017; Vasiljevic & Shah, 2008).

In studies of human primary macrophages, ST bacteria induce the anti-inflammatory interleukin (IL)-10 cytokine, although pro-inflammatory IL-12 cytokine is also produced (Latvala, Miettinen, Kekkonen, Korpela R., & I., 2011). Furthermore, ST1275 and *Bifidobacterium longum* BL536 were shown to stimulate high levels of transforming growth factor (TGF)-beta, important for the differentiation of regulatory T cells (Treg) and T-helper (Th)-17 cells from bulk cultures of peripheral blood mononuclear cells (Donkor, Ravikumar, et al., 2012b). *S. salivarius, S. equinus* and *S. parasanguinus* have been shown to induce IL-8, tumor necrosis factor (TNF)-alpha and IL-12 in human dendritic cells (DC). *Streptococcus* and *Veillonella* often co-occur in bio-environments and can potentially have metabolic collaboration; in fact their combination collectively show immunomodulatory effects. Whilst *Veillonella parvula* was only able to stimulate IL-6 production; combinations of *Streptococcus* and *Veillonella* were able to down regulate IL-12 whilst up regulating IL-6, IL-8, IL-10 and TNF-alpha (van den Bogert, Meijerink, Zoetendal, Wells, & Kleerebezem, 2014). In mice, administration of *ST* either orally or intraperitoneally, was shown to enhance immune responses by activating phagocytic activity of macrophages and increased antibody production by B cells (Perdigon, Nader de Macias, Alvarez, Oliver, & Pesce de Ruiz Holgado, 1987). Mice with dextran sodium sulphate induced colitis showed reduced clinical signs of disease and decreased cellular infiltration (associated with inflammation) in the colon following *ST* oral administration (Bailey, Vince, Williams, & Cogan, 2017). Conversely, in a human clinical study, 20 participants with positive skin prick tests and atopic history consumed yogurt that contained live *ST* and *Lactobacillus delbrueckii* subsp *bulgaricus* did not show any

improvement in immune cell parameters; phagocytic function, antibody responses, cytokine secretion by T cells (IFN-gamma, IL-2, IL-4), number and function of natural killer (NK) cells and neutrophils (Wheeler et al., 1997). Thus, although probiotics are able to modulate host immune responses, much is still unknown regarding their direct effect on immune cells such as monocyte/macrophages (Lebeer et al., 2008). Thus, three strains of *S. thermophilus* (ST1275, ST285, ST1342) were chosen for investigation to determine their direct effects on the human pro-monocytic cell line, U937 cells that were differentiated into monocyte/macrophage cells using vitamin D_3. (Mogensen, 2009; Suresh & Mosser, 2013). Pattern recognition receptors present on monocytes and macrophages have been shown to be responsible for the recognition of bacteria, therefore these cells were used in the current study to determine the direct effect (cell surface markers and cytokine expression) of *S. thermophilus* bacteria on these cells.

2. Material and Methods

2.1. Bacterial strains

Pure bacterial cultures of *S. thermophilus* 1342 (ST1342), *S. thermophilus* 1275 (ST1275) and *S. thermophilus* 285 (ST285) were obtained from Victoria University Culture Collection (Werribee, Victoria, Australia). Stock cultures were stored in 40 % glycerol at −80° C. Prior to each experiment the cultures were propagated in M17 broth (Oxoid, Melbourne Australia) and were incubated at 42° C. Bacteria were also cultured in M17 agar (1.5 % w/v agar) for characteristics and assessment of their purity, morphology and gram status by gram staining.

2.2. Preparation of live bacterial cell-suspensions

All media were prepared and sterilized by autoclaving at 121 °C for 15 min. Prior to actual experiments, the cultures were grown 3 times in M17 broth, at 37 °C for 18 hours with a 1 % inoculum transfer rate. *S. thermophilus* start to synthesize autolysins at the end of the exponential growth phase (Husson-Kao et al., 2000), or during or after the transition from exponential to stationary growth phase (Sandholm & Sarimo, 1981). Our cultures were obtained from Victoria University culture collection, which are cultured at 37-42° C for 24 hours (Purwandari & Vasiljevic, 2009). We kept our culture growth time consistent 18 hours

(at the end of the exponential growth phase) and before stationary growth phase to prevent cell lysis. Growth rate varies for various subspecies as well as their temperature (30-50° C) (Armin Tarrah et al., 2018). On the day of experiment, bacteria were harvested during stationary growth phase, by centrifugation (6000×g for 15 min at 4 °C, Beckman J2/HS centrifuge, JA-14 rotor, Palo Alto, CA, USA), washed twice with phosphate-buffered saline (PBS) (Gibco, Australia) and resuspended in RPMI 1640 culture media. These samples constituted the live-cell suspensions.

2.3. Enumeration of bacterial cells

Bacterial strains were scraped from M17 agar and transferred into Dulbecco's PBS (Invitrogen, Pty Ltd. Australia) adjusted to a final concentration of 10^8 cfu/ml by measuring the optical density at 600 nm, and washed twice with PBS before co-culturing with monocyte cell cultures.

2.4. Culture, differentiation and stimulation of U937 cells

U937 cells were cultured in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen, Pty Ltd. Australia), 1% antibiotic-antimycotic solution and 2 mM L-glutamine at 37 °C, 5 % CO_2. For differentiation of U937 cells into monocytes, U937 cells were adjusted to 3×10^5 cells/ml and 100 nM vitamin D_3 was added followed by incubation for 72 h. The resulting cells have characteristics of monocytes with CD14, CD11b, CD86 and MHC class II surface expression (Table 1).

Differentiated U937 cells (5×10^5 cells/ml) were stimulated with 1.5×10^8 live probiotic bacteria (ST1342, ST1275 or ST285) or lipopolysaccharide (LPS, 1 μg/ml; internal positive control) or non-stimulated as reference background control. The ratio of cells to bacteria is usually 1:10, however this ratio is usually for PBMC in which there is only 10-13 % monocytes present. Although there are only a few studies that use pure monocyte cultures, 1:300 ratio of cells to bacteria has been reported (H. Jensen et al., 2015); hence in our experiments, 1:300 ratio cells to ST bacteria was used. All cell cultures were incubated at 37 °C, 5 % CO_2 for either 24 hours or 48 hours. Supernatants were centrifuged and filtered to remove bacteria and were used for cytokine analysis and cells were used for cell surface marker

expression by flow cytometry. Similar protocols have been used for other probiotic bacteria and on epithelial cells or PBMC (Asarat, Apostolopoulos, et al., 2015; Asarat, Vasiljevic, et al., 2015; Donkor, Ravikumar, et al., 2012a).

2.5. Cytokine analysis

Cytokine concentrations of supernatants were measured by commercially available capture and detection antibodies in a Bio-Plex assay using a 9-plex kit (BioRad, Melbourne Australia) to measure IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, GM-CSF, IFNγ, and TNFα. Supernatants were collected and the assay procedures were performed according to the manufacturer's instructions. Data was collected and expressed as the mean cytokine response minus background (pg/ml) of each treatment from 4 replicate wells, plus or minus the standard error of the mean.

2.6. Flow cytometry assay for cell surface markers

Following stimulation of differentiated U937 cells with probiotics, cells were centrifuged and 5×10^5 cells were incubated with Fc block (BD Life Sciences) for 45 minutes on ice. After washing, cells were labelled with cell surface marker antibodies (Biolegend and BD Life Sciences) linked to fluorochrome and incubated on ice for 45 minutes. The antibodies were diluted in PBS/FBS at the following dilutions according to the manufacturers recommendations (CD11b-PE 1:400; CD83-Alexafluor488 1:400; CD14-BV421 1:200; CD40, CD80, CD83, CD86Alexafluor 488 1:400; CD16-PE 1:400; CD206, CD209-PE/Cy7 1:200; MHCI, MHCII-BV510 1:200). Samples were analyzed using a BD fluorescence activated cell sorter (FACS) Canto II. Data was acquired using Cell Quest program (BD Life Sciences), and analysis performed using FACS Diva software (BD Life Sciences) for percentage of expressed markers; isotype antibody controls (Biolegend and BD Life Sciences) were used as background quadrants set up.

2.7. Statistics

Significant differences between all treatment groups were tested by analysis of variance (ANOVA) followed by a comparison between treatments performed by Fisher's least significant difference (LSD) method, with a level of significance of $p < 0.05$.

3. Results and Discussion

3.1. S. thermophilus bacterial strains activate monocytes necessary for the innate immune response

The innate immune system is the first line of defence against invading pathogens which react quickly and non-specifically. Following this non-specific encounter cytokines (such as, IL-1β, IL-6, TNFα and IFNγ) and chemokines are secreted by innate cells (monocytes, macrophages, dendritic cells, NK cells, granulocytes) which play an important role in the innate immune response. This results in inflammation at the site of infection to aid in pathogen clearance (Parihar, Eubank, & Doseff, 2010). IL-1β, IL-6, TNFα and IFNγ are pro-infammatory cytokines which also aid to recruit and activate T and B cells to mount an adaptive immune response (Lacy & Stow, 2011). Secretion of IL-1β by monocytes is involved in regulating immune and inflammatory responses to infections and injury, hence its role in innate immunity (Lopez-Castejon & Brough, 2011). *S. thermophilus* ST1342 stimulated high levels of IL-1β ($p < 0.001$), whereas, ST1275 ($p < 0.05$) and ST285 ($p < 0.07$) did not induce IL-1β cytokine by differentiated U937 cells (Figure 1). IL-6 regulates both innate and adaptive immune responses and is secreted by monocytes to stimulate immune responses during infection (Jones, 2005). TNFα is a pro-inflammatory cytokine and a main trigger of the inflammatory response by causing vasodilation and vascular permeability allowing the influx of immune cells to the site of infection (Matsuki & Duling, 2000). High levels of TNFα was secreted by monocytes in the presence of ST1342, ST1275 and ST285 ($p < 0.001$) (Figure 1). It has been shown that IL-1β, LPS and TNFα induce IL-6 production by monocytes, and IL-6 is required for resistance against bacteria (Tosato & Jones, 1990). A trend towards increased levels of IL-6 was noted, although this was not significant for all probiotic strains ST1342, ST1275 and ST285 (Figure 1). In addition, all three ST1342, ST1275 and ST285 strains activated high levels of IFNγ secretion (Figure 1); a pro-inflammatory cytokine that is crucial in both innate and adaptive immune responses and has both anti-bacterial and anti-viral properties. It is clear that ST1342, ST1275 and ST285 activate cytokine secretion by monocytes, required for activation of the innate immune response and responsible for pathogen elimination. Similarly, it was noted that the probiotic *L. paracasei* DG commonly used in commercial probiotic products, has been shown to have immunostimulatory properties by

increasing expression of IL-6, TNFα and CCL20 in the human monocyte cell line, THP-1 (Balzaretti et al., 2017).

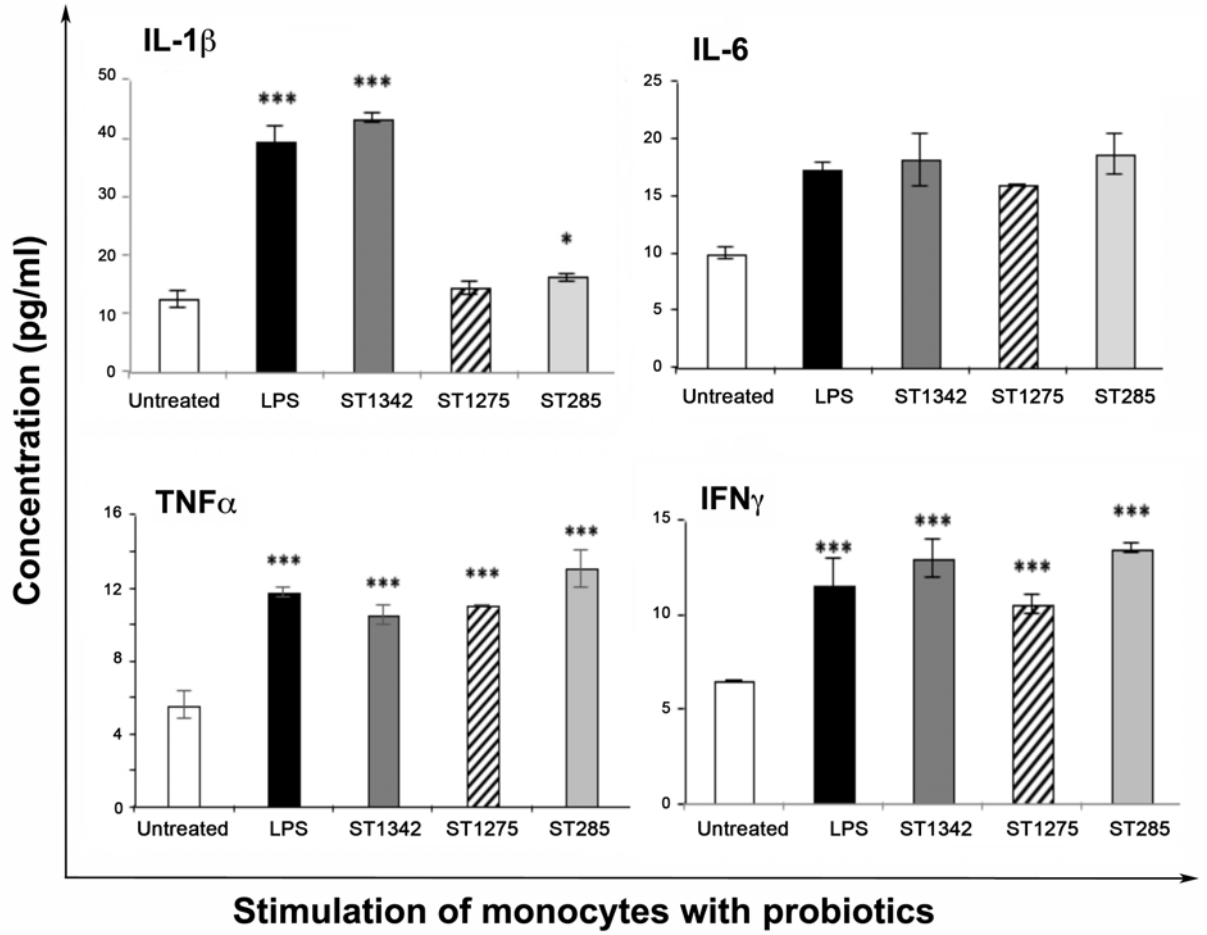

Figure 1. ***S. thermophilus*** **bacterial strains activate monocytes necessary for the innate immune response.** U937 cells were differentitated into monocytes and stimlated with *S. thermophilus* (ST) - ST1342, ST1275 or ST285 for 24 hours and secretion of IL-1β, IL-6, TNFα and IFNγ were measured. LPS was used as an internal positive control and untreated refers to differentiatied U937 cells not stimulated with ST probiotic bacteria (background control). Symbols represent *p* value for Tukey Test (One way ANOVA) where * $p < 0.05$ and *** $p < 0.001$.

3.2. S. thermophilus bacterial strains activates CXCL8 and GM-CSF: role in chemotaxis and recruitment of cells at sites of inflammation

IL-8 (also known as chemokine CXCL8) is an important cytokine of the innate immune system. IL-8 induces chemotaxis of neutrophils and other granulocytes toward the site of infection and it is a key mediator associated with inflammation; it also induces phagocytosis at

the site of infection (Baggiolini & Clark-Lewis, 1992a). The probiotic *L. paracasei* DG has been shown to increase expression of IL-8 in the human monocyte cell line, THP-1 (Balzaretti et al., 2017). In addition, short chain fatty acids, produced by probiotic bacteria, also stimulate IL-8 secretion and mRNA levels in the human epithelial cell line HT-29 (Asarat, Vasiljevic, et al., 2015). Likewise, ST1342 ($p < 0.005$), ST1275 ($p < 0.07$) and ST285 ($p < 0.001$) activated monocytes to secrete high levels of IL-8 compared to non-stimulated cells (Figure 2). GM-CSF stimulates the production of white blood cells, in particular, it rapidly increases macrophages *in vivo*, important cells necessary for fighting infections. It also enhances the anti-bacterial activity of monocytes and modulates macrophage/dendritic cell phenotypes; as such, molecular targeting of the GM-CSF pathway has recently been developed to treat a number of autoimmune disorders (Ushach & Zlotnik, 2016). Of interest, ST1275 and ST285 induced monocytes to secrete high levels of GM-CSF ($p < 0.001$) while, conversely, ST1342 stimulated lower levels of GM-CSF ($p < 0.001$) (Figure 2).

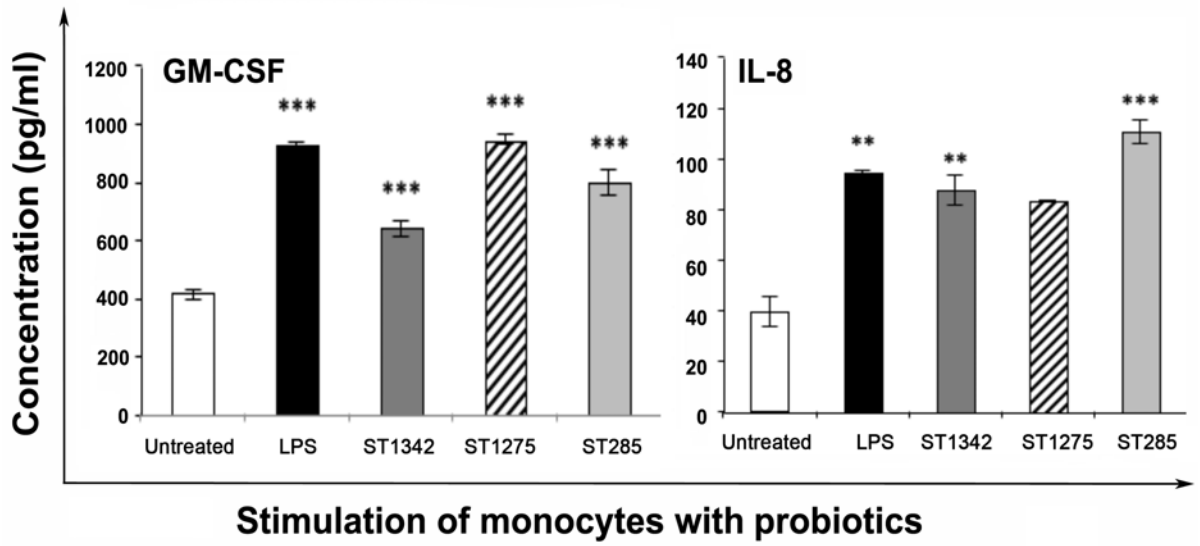

Figure 2. *S. thermophilus* bacterial strains activate CXCL8 and GM-CSF essential for recruitment of cells at sites of inflammation. U937 cells were differentitated into monocytes and stimlated with *S. thermophilus* (ST) - ST1342, ST1275 or ST285 for 24 hours and secretion of IL-8 and GM-CSF were measured. LPS was was used as an internal positive control and untreated refers to differentiatied U937 cells not stimulated with ST probiotic bacteria (background control). Significant differences between treatments were tested by analysis of variance (ANOVA). Symbols represent p value for Tukey Test (One way ANOVA) where # $p < 0.07$, ** $p < 0.005$ and *** $p < 0.001$.

3.3. S. thermophilus bacterial strains activate anti-inflammatory cytokines

IL-4 is an anti-inflammatory cytokine which differentiates naïve $CD4^+$ Th0 cells to Th2 cells. IL-4 stimulates B cells and T cells and is a key regulator of humoral and adaptive immune responses at sites of injury. IL-4 promotes M2 anti-inflammatory macrophages and inhibits classical M1 pro-inflammatory macrophages. IL-4 together with IL-10 are important at sites of injury or infection by inhibiting bacterial mediated induction of pro-inflammatory cytokines. In addition, IL-4 and IL-10 are important cytokines required for anti-inflammatory responses against inflammatory diseases such as, autoimmunity and allergies (R. E. Mitchell et al., 2017). The probiotic *Bifidobacterium (B) breve* but not *Lactobacillus (L) casei* has been shown to induce IL-10 producing intestinal Treg cells as well as intestinal $CD103^+$ IL-10/IL-27 secreting DCs in mice (Jeon et al., 2012). Oral *B. breve* administration ameliorates colitis in mice but not in IL-10 knockout mice, demonstrating preventive effect of *B. breve* on colonic inflammation (Jeon et al., 2012). Likewise, *L. reuteri* and *L. lactis* strains given in mice orally stimulates anti-inflammatory IL-10 and Treg cells (Levkovich et al., 2013; Souza et al., 2016).

Furthermore, co-culturing PBMC with selected bacteria (LAVRI-A1, *L. rhamnosus* GG, *Bifidobacteria* and *L. acidophilus*) induce anti-inflammatory cytokines IL-4, IL-10 and TGF-beta (Donkor, Ravikumar, et al., 2012a; Donkor et al., 2010). These cytokines inhibit the production of IL-12, IFNγ and other pro-inflammatory cytokines which are beneficial for autoimmune and allergic responses. Here we show that, ST1342 stimulated IL-4 production by monocytes ($p < 0.001$) and to a lesser degree ST1275 ($p < 0.07$) and ST285 ($p < 0.005$), (Figure 3). Similarly, IL-10 was secreted by monocytes in the presence of ST1342, ST1275 and ST285 ($p < 0.001$), with ST1275 and ST285 stimulating higher levels (Figure 3). It is clear that ST probiotic bacteria have potential anti-inflammatory properties which could have positive implications in chronic inflammatory diseases (autoimmunity and allergies) and warrant further investigation.

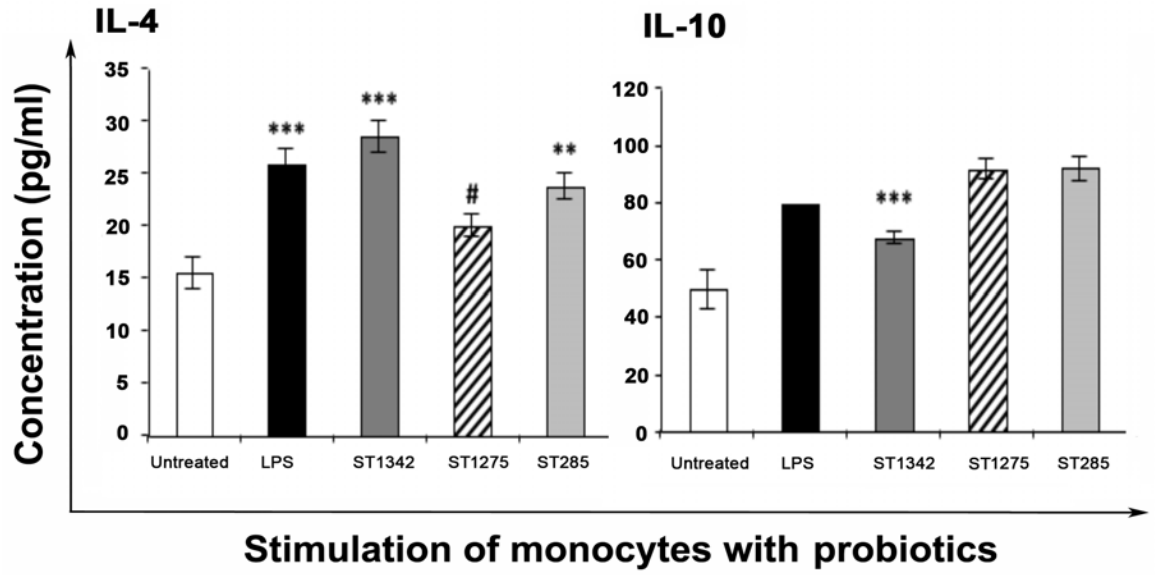

Figure 3. *S. thermophilus* bacterial strains activate anti-inflammatory cytokines. U937 cells were differentitated into monocytes and stimlated with *S. thermophilus* (ST) - ST1342, ST1275 or ST285 for 24 hours and secretion of IL-8 and GM-CSF were measured. LPS was was used as an internal positive control and untreated refers to differentiatied U937 cells not stimulated with ST probiotic bacteria (background control). Symbols represent *p* value for Tukey Test (One way ANOVA) where # $p < 0.07$, ** $p < 0.005$ and *** $p < 0.001$.

3.4. S. thermophilus bacterial strains upregulate the expression of cell surface markers on differentiated U937 cells; role in initiating innate and adaptive immune responses

Monocytes are major constituent cells of the innate immune system, which also play a role in the adaptive immune response. The expression of cell surface markers on monocytes is crucial in the ensuing immune responses. The specific markers presented on monocytes is dependent on their environment and their exposure to pathogens and/or pathogenic peptides and pathogen derived metabolites; with these factors causing alterations in the profile of monocyte markers, accordingly (Ziegler-Heitbrock, 2015). The human pro-monocytic histiocytic lymphoma cell line, U937 cells, are commonly used to study the behavior and differentiation of monocytes. They exhibit pro-monocytic characteristics by displaying monoblast morphology, produce lysozymes and have esterase activity (dos Santos et al., 2009; Sundstrom & Nilsson, 1976). They are not phagocytic, they express low levels of CD14, CD54, CD86, and major histocompatibility complex (MHC)-class II is not detectable (Azam et al., 2006). However, upon stimulation with viral or bacterial fragments, or, vitamin D_3, they express markers demonstrating monocyte/macrophage morphology, with increased expression

of CD14 (dos Santos et al., 2009; Koss, Lucero, & Koziner, 1996; Santegoets, Van Den Eertwegh, Van De Loosdrecht, Scheper, & De Gruijl, 2008).

Our data shows that U937 cells incubated with ST1342, ST1275 or ST285 results in enhanced expression of CD14, CD11c, CD86, CD206, CD209 and MHC1 cell surface markers at varying levels; CD11b, CD16, CD40, CD80 and CD83 were also up regulated, albeit at a much lower level (Table 1). In other studies, the combination of 3 probiotics (*L. acidophilus*, *L. delbrueckii* ssp. *bulgaricus* and *B. bifidum*) stimulated increased expression of cell surface markers, CD14, MHC class II and CD80 (Gutkowski et al., 2010).

CD14 is expressed on the surface of monocytes and macrophages and primarily binds to bacterial LPS; although other bacterial cell wall constituents also bind to CD14 such as, lipid A, *Staphylococcus aureus, Escherichia coli* and lipoteichoic acid (Bron, Tomita, Mercenier, & Kleerebezem, 2013; I. C. Lee, Tomita, Kleerebezem, & Bron, 2013; van Baarlen, Wells, & Kleerebezem, 2013). The interaction between CD14 and its ligands initiates the innate immune response (Bedell et al., 2018), as well as further up regulating its expression (CD14 expression) (Landmann et al., 1996). Indeed, ST1342, ST1275 and ST285 up regulated CD14 expression on U937 cells after 24 and 48 hours incubation, with ST285 being the most significant at 48 hours (Table 1).

CD11c is a type I transmembrane protein expressed by DCs, monocytes, macrophages and neutrophils (Dyer, Garcia-Crespo, Killoran, & Rosenberg, 2011). The presence of CD11c on these cells allows their adherence to endothelial cells, phagocytosis of complement positive cells (important for innate immune defence) and activates cellular immune responses. Selected strains of *Lactobacillus* (*L. reuteri*, *L. plantarum* Lb1 and *L. fermentum*) cultured with murine bone marrow cells and GM-CSF, induce high levels (85-90 %) of $CD11c^{+}$ cells (Christensen, Frøkiær, & Pestka, 2002). Basal expression levels of CD11c on U937 cells was 26-27%, which almost doubled following LPS (48-49%) and ST1342 (48-50%) stimulation; significant up regulation was also noted with ST1275 (37-43%) and ST285 (43-46%) after 24 or 48 hours respectively (Table 1). Interestingly, there were no major differences in CD11c expression, whether cells were stimulated for 24 or 48 hours.

CD86 (B7-2) expression on antigen presenting cells (DCs, macrophages, B cells) is involved in co-stimulatory signalling that is required for the priming and proliferation of T cells

(Fleischer et al., 1996). Monocytes express low levels of CD86 which is up regulated following stimulation with IFN-gamma or other ligands. In fact we showed that expression of CD86 increased significantly from 8.6% to 33.4% (ST1342), 28.1% (ST1275) and 38% (ST285) after 24 hour co-culture, which was lower than that after LPS stimulation (46.3%) (Table 1). The up regulation of CD86 was transient and after 48 hours the levels decreased significantly. It is clear that *S. thermophilus* bacteria promote CD86 expression levels, required for T cell activation and the maintenance of immune responses (Fleischer et al., 1996). Similarly, *L plantarum* WCFS1 and *L. fermentum* GR1485 have been shown to upregulate CD86 cell surface expression on monocytes, however, *L. rhamnosus* and *L. delbruekii* reduce cell surface expression of CD86 (Esmaeili et al., 2018).

CD206 (mannose receptor, MR) (Geurtsen et al., 2009), is primarily present on the surface of macrophages and immature DCs (Kerrigan & Brown, 2009), and functions to arrest antigens and pathogenic components, followed by processing and presentation to T cells (Engering et al., 2004). The MR recognizes mannose, fucose and N-acetylglucosamine residues (Kerrigan & Brown, 2009; R. E. Mitchell et al., 2017) commonly expressed on the surface of microorganisms (such as *Pneumocystis*, *Candida*, *Mycobacterium*, *Leishmania*), and capsular polysaccharides of *Streptococcus* and *Klebisella* (Geurtsen et al., 2009; Kerrigan & Brown, 2009; Zamze et al., 2002), which results in the destruction of bacteria (innate immune response) and activation of the adaptive immune response (cellular responses). Poly-mannose (mannan) linked to protein antigens as a model, targets the MR on DCs and macrophages resulting in stimulation of either pro- or anti-inflammatory responses, significant in a number of diseases from cancers to autoimmunity (Apostolopoulos, Barnes, Pietersz, & McKenzie, 2000; Apostolopoulos & McKenzie, 2001; Apostolopoulos, Pietersz, Gordon, Martinez-Pomares, & McKenzie, 2000; Apostolopoulos et al., 1995; Apostolopoulos et al., 1996; Sheng et al., 2006). Here we show that U937 cells co-cultured with ST1342, ST1275 or ST285 up regulated the expression levels of CD206 within 24 hours (ST285 inducing the highest levels) which subsided by 48 hours, but did not reach basal level expression (Table 1, Figure 4). In addition, CD209 (DC-SIGN), a C-type lectin receptor expressed on the surface of macrophages and DCs also binds to mannose residues present on bacteria, viruses and fungi. The interaction between CD209 and mannose moieties activates phagocytosis as well as endocytosis for processing and presentation to T cells (Apostolopoulos et al., 2014; Cambi et al., 2003; Proudfoot, Apostolopoulos, & Pietersz, 2007; Sheng et al., 2008; Sheng, Pietersz, Wright, & Apostolopoulos, 2005). U937 cells cultured in the presence of ST strains also up regulated the

expression of CD209 with maximal up regulation noted within 24 hours (Table 1); ST285 stimulation resulted in the highest up regulation at both 24 and 48 hours. Thus, *S. thermophilus* strains induce CD206 and CD209 expression, as a result have a positive role in activating both the innate and adaptive immune responses (Apostolopoulos et al., 2006; Apostolopoulos et al., 2014).

The major histocompatibility complex class I (MHC-I) is expressed by all nucleated cells and presents processed antigenic peptides on its surface to activate $CD8^+$ T cells (Neefjes, Jongsma, Paul, & Bakke, 2011). U937 cells express low levels of MHC-I which is up regulated within 24 hours in the presence of ST1342, ST1275 or ST285 and remains up regulated after 48 hours of stimulation (Table 1). Hence, *S. thermophilus* strains are beneficial in upregulating MHC-I molecules on monocyte/macrophage cells for enhanced $CD8^+$ T cell stimulation, required for the elimination of tumour cells and viruses.

Table 1. Proportion (%) of cell surface marker expression shown, as analyzed by flow cytometry at 24 and 48 hours of stimulation of U937 cells with *S. thermophilus* strains

	Control		LPS		ST1342		ST1275		ST285	
	24	48	24	48	24	48	24	48	24	48
CD11b	6.1	4.1	13.9	9.1	12.1	8.6	12	7.5	11.8	21
CD11c	27.3	26.2	48	49	50	48	37	43	43	47
CD14	6.6	4	13.5	19	15.6	21	19.1	16	19.8	35
CD16	3	4	7.1	6.5	7.5	6	8.9	4	9.1	12
CD40	1.6	4	6	6	6.2	5.8	8.5	5	6	13
CD80	4	4	5.5	5.5	7.5	5.7	7.5	5.2	5.5	11
CD83	1.7	4	7	6.5	6.8	4.7	13	5	7.1	10
CD86	8.6	4.5	46.3	16	33.4	13	29.8	12.5	38	16.5
CD206	17	7	40.9	30	38.5	30	36.4	34	47.8	34.5
CD209	4	4.5	37.1	20	38.7	18.8	30	16.8	39	31
MHCI	4.2	10	18.7	23	18.9	24	20.8	22.5	22.7	24

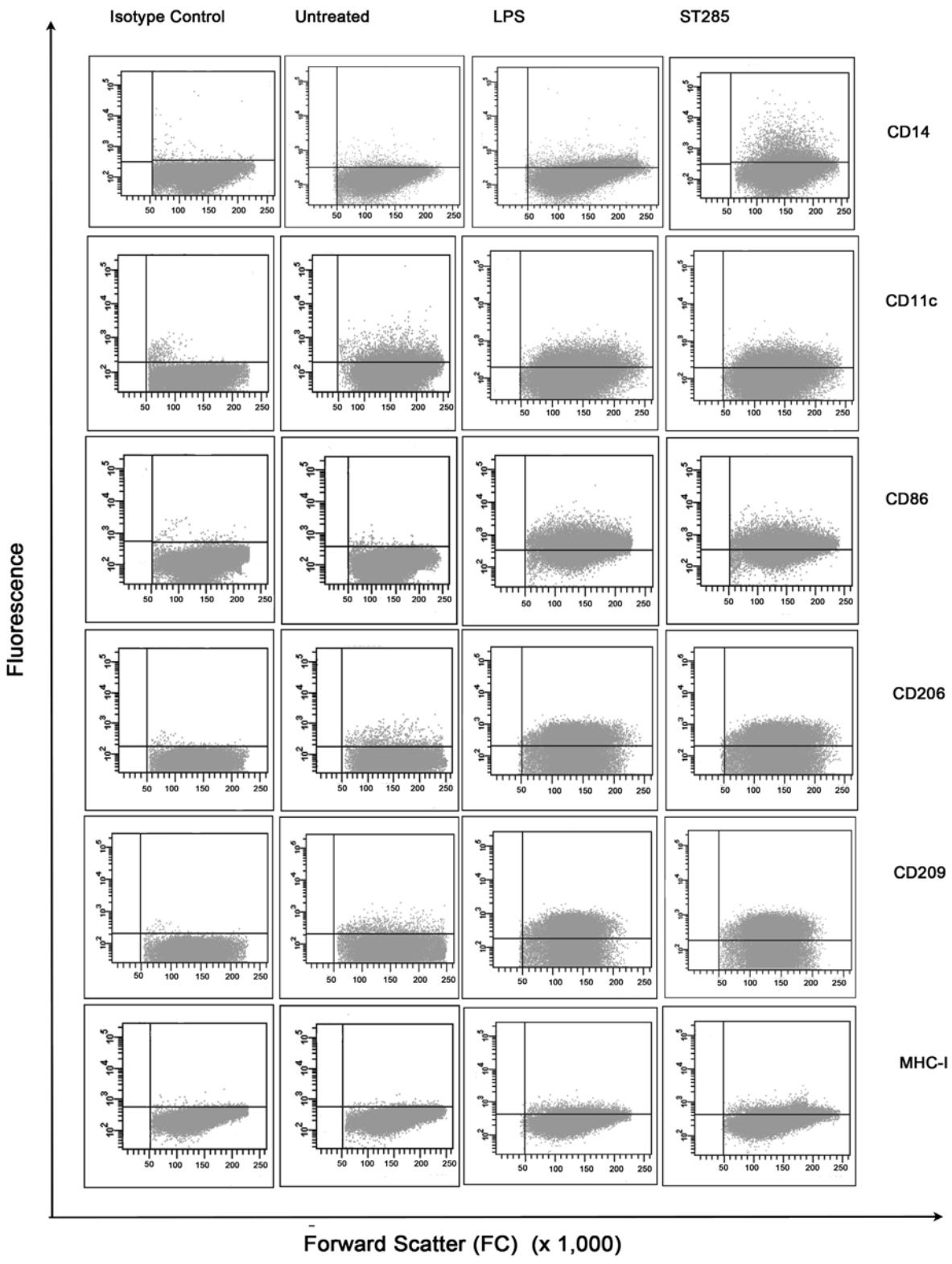

Figure 4. *S. thermophilus* (ST) bacterial strains increase cell surface marker expression. U937 cells were differentiated into monocytes and stimlated with ST1342, ST1275 or ST285 for 24 or 48 hours and cell surface marker expression assessed. Upregulation of CD14, CD11c, CD86, CD206, CD209 and MHC class I are shown at 24 hours for ST285. LPS was was used as an internal positive control and untreated refers to differentiatied U937 cells not stimulated with ST probiotic bacteria (background control).

4. Conclusion

Activation of monocyte cells with *Streptococcus thermophilus* such as *S. thermophilus* ST1342, *S. thermophilus* ST1275 and *S. thermophilus* ST285 strains, and secretion of IL-1β, IL-6, TNFα and IFN-γ suggests their role in the subsequent activation of the immune responses aiding in the elimination of pathogens. In addition, *S. thermophilus* strains, up regulated the secretion of IL-8, a chemokine involved in chemotaxis and phagocytosis, as well as up regulating the secretion of GM-CSF, a major cytokine for increasing the number of macrophages at the site of infection. Clearly, *S. thermophilus* strains up regulated cytokine levels by monocytes, required for activation of the innate immune response. Furthermore, the activation of anti-inflammatory cytokines (IL-4 and IL-10) could be beneficial in modulating chronic inflammatory conditions and allergies. Moreover, *S. thermophilus* strains up regulated monocyte cell surface markers, CD14, CD11c, CD86, CD206, CD209 and MHC-I suggestive of their potential benefit to activate innate and adaptive immune responses. These findings support a role for these probiotic strains in the healthy modulation of monocyte activity and their roles in innate and cellular immunity. The results also present a potential role for these strains in modulating the inflammatory response, which warrants further investigation. Overall, these findings are in agreement with the body of research that supports the role that the regular consumption of probiotics (including *S. thermophilus*) has in the establishment and maintenance of a healthy immune system and opens pathways to further determine the mechanisms by which these strains modulate immune responses.

Chapter 3

Chapter 3

Immunomodulatory effects of Streptococcus thermophilus on gene expression of human peripheral blood mononuclear cells

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ABSTRACT

Consumption of probiotics contributes to a healthy microbiome of the GIT leading to many health benefits. They also contribute to the modulation of the immune system and are becoming popular for the treatment of a number of immune and inflammatory diseases. The main objective of this study was to evaluate anti-inflammatory and modulatory properties of *Streptococcus thermophilus*. Peripheral blood mononuclear cells from healthy donors were used and assessed modifications in the mRNA expression of their genes related to innate and adaptive immune system. The results showed strong immune modulatory effects of *S. thermophilus* 285 to human peripheral blood mononuclear cells with an array of anti-inflammatory properties. *S. thermophilus* 285 reduced mRNA expression in a number of inflammatory immune mediators and markers, and upregulated a few of immune markers. *S. thermophilus* is used in the dairy industry, survives during cold storage, tolerates well upon ingesting, and their consumption may have beneficial effects with potential implications in inflammatory and autoimmune disorders.

Keywords: Probiotics; microbiome; Lactic acid bacteria; *Streptococcus thermophilus*; Peripheral blood mononuclear cells; Monocyte; RNA; Innate immune response; Adaptive immune response; Inflammation

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1. Introduction

The human body and, in particular, the gastrointestinal tract (GIT) hosts a variety of microbial populations referred to collectively as the microbiome (Dargahi, Johnson, Donkor, Vasiljevic, & Apostolopoulos, 2018). The microbiome of the GIT plays a key role in the maintenance of a healthy immune system (Dargahi et al., 2018; Dargahi et al., 2017), and disruptions to the microbiome composition can lead to serious effects on health (H. Jensen et al., 2015; A. J. Stagg et al., 2004; J. Stagg et al., 2011). In order to maintain a healthy microbiome, regular ingestion of probiotic supplements, or the ingestion of fermented dairy products/capsules has been suggested. These practices have led to various improved health outcomes, ranging from enhanced overall human wellbeing to the treatment of infections, constipation, diarrhoea etc (Dargahi et al., 2018).

The majority of probiotics belong to the lactic acid bacteria (LAB) family; gram positive lactic acid producing microorganisms that include several genera such as bifidobacteria, lactobacilli streptococci and enterococci (Dargahi et al., 2018). The small intestine and the colon are highly enriched with these microorganisms (Fink et al., 2007; Maassen et al., 2000; Michałkiewicz et al., 2003), which are routinely supplemented in foods as live strains due to their beneficial effects on human health (Asarat, Apostolopoulos, et al., 2015; Asarat et al., 2016; Asarat, Vasiljevic, et al., 2015; Dargahi et al., 2018, 2019; Dargahi et al., 2017; Fink et al., 2007; Salazar et al., 2009). *Streptococcus* species such as exopolysaccharide-producing strains of *Streptococcus thermophilus* (ST) (Di Caro et al., 2005; Purwandari & Vasiljevic, 2009; Salazar et al., 2009) are among those consumed. These characteristics of ST enable them to be used in fermented milk products (i.e. yogurt) including flavoring of dairy, and is recognized as the next most important species

after *Lactococcus lactis* (Hols et al., 2005; Uriot et al., 2017). ST and *L. brevis* synergistically display well established health benefits, and ST is one of the bacteria in the VSL#3 probiotic mixture, which has long been broadly applied in the treatment of inflammatory conditions (C. Dai et al., 2013; Mennigen et al., 2009). In addition, probiotics interact with the immune system leading to immunomodulation and anti-inflammatory properties (Han et al., 2008; J. Stagg et al., 2011; Vliagoftis et al., 2008).

The 'hygiene hypothesis' suggests that the positive trend in the incidence of immune-related disorders can been attributed to intestinal dysbiosis, resulting in immune dysfunction (*ie*. asthma, eczema, allergies and autoimmune diseases). Use of probiotic bacteria can increase abundance and concurrently modulate immune cells, including B, T helper (Th)-1, Th-2, Th-17 and regulatory T (Treg) cells. This in turn, directly influences human health and modulates pathologies of immune/autoimmune diseases (Dargahi et al., 2018, 2019; Dargahi et al., 2017). In fact, we previously noted that ST1342, ST1275 and ST285 modulate the U937 monocyte cell line. Specifically, we showed that interleukin (IL)-4, IL-10, GM-CSF and CXCL8 production were increased, and, cell surface marker expression CD11c, CD86, C206, CD209, MHC-1 were upregulated (Dargahi et al., 2018). In another study, ST1275 and *Bifidobacterium longum* BL536 demonstrated increased levels of transforming growth factor (TGF)-beta (a key factor in the differentiation of Treg and T-helper Th)-17 cells by bulk peripheral blood mononuclear cell (PBMC) cultures (Donkor, Ravikumar, et al., 2012a). Primary macrophages co-cultured with ST bacteria stimulate production of anti-inflammatory IL-10 and pro-inflammatory IL-12 cytokines (Latvala et al., 2008).

Herein, the changes in the expression of genes associated with innate and adaptive immunity are described including cytokines, chemokines and immune cell marker expression by human PBMC following exposure to live ST285 bacteria.

2. Material and methods

2.1. Bacterial strains

Pure bacterial cultures of ST285 were obtained from Victoria University culture collection (Werribee, VIC, Australia). Stock cultures were stored in cryobeads at −80° C. Prior to each experiment the cultures were propagated in M17 broth (Oxoid, Denmark) with 20 g/L lactose and incubated at 37° C under aerobic conditions. Bacteria were also cultured in M17 agar (1.5 % w/v agar) with 20 g/L lactose (Oxoid, Denmark), to assess characteristics, morphology, purity and gram-positive confirmation (Dargahi et al., 2018).

2.2. Preparation of live bacterial suspensions

Media were prepared and autoclaved at 121° C for 15 minutes (mins) prior to experiments. Bacterial cultures were grown 3 times in M17 broth with 20 g/L lactose, at 37° C aerobically for 18 hours (hr) with a 1 % inoculum transfer rate (Husson-Kao et al., 2000). Cultures grow optimally at 37-42° C for 24 hrs (Purwandari & Vasiljevic, 2009). The growth period of cultures were consistent at 18 hr (at the end of the exponential growth phase) and before stationary growth phase to prevent cell lysis. Bacteria were harvested during stationary growth phase on the day of experiment, centrifuged (6000×g) for 15 min at 4° C, followed by two washes with Dulbecco's phosphate-buffered saline (DPBS) (Invitrogen, Pty Ltd. Australia) and resuspended in the Roswell Park Memorial Institute (RPMI) 1640 culture media. These samples constituted the live-cell suspensions.

2.3. Enumeration of bacterial cells

Bacterial strains were scraped from M17 agar and transferred into Dulbecco's PBS (Invitrogen, Pty Ltd. Australia) adjusted to a final concentration of 10^8 colony forming units (cfu)/ml by measuring the optical density at 600 nm, and washed two times with PBS and resuspended in RPMI 1640 prior to co-culturing with PBMC (Dargahi et al., 2018).

2.4. Isolation, culture, and stimulation of PBMC

PBMC isolated from whole blood constitute a wide range of diverse immune cells (monocytes, lymphocytes and granulocytes) that collaboratively play vital roles in balancing immune homeostasis and keeping human health in check (Green, Rudolph-Stringer, Chantry, Wu, & Purton, 2019; Kaczorowski et al., 2017). In addition, PBMC contain cells which are crucial components of the innate and adaptive immune system, defend the body against bacterial, viral and parasitic infections, as well as destroying foreign antigens and cancer cells (Green et al., 2019). PBMC are predominantly made up of T cells (~70 %), with the balance comprising of B cells (~15 %), natural killer (NK) cells (~10 %) and monocytes (~5-30 %) (Saito, Shiozaki, Nakashima, Sakai, & Sasaki, 2007). In spite of variations in the fraction of subtypes of immune cells within the total PBMC isolated from different samples (Saito et al., 2007), isolation, characterization and molecular studies of these cells have benefited medical research (Corkum et al., 2015).

2.4.1. Isolation of PBMC using Ficoll-Paque

PBMC isolation from whole blood was via Ficoll-Paque density gradient centrifugation (Asarat, Apostolopoulos, et al., 2015). Buffy coats were collected from the Australian Red Cross Blood Bank on the day of experiment (Victoria University human research ethics). Calcium and magnesium free PBS, pH7.2, (Invitrogen, Pty Ltd. Australia) was used after adding 2 mM EDTA and 2% heat-inactivated fetal bovine serum (FBS) (Invitrogen, Pty Ltd. Australia); PBS buffer. SEPMATE tubes (50 ml) with inner inserts (STEMCELL technology, Canada) were used to isolate PBMC following Ficoll-Paque density gradient protocol (Betsou, Gaignaux, Ammerlaan, Norris, & Stone, 2019; Grievink, Luisman, Kluft, Moerland, & Malone, 2016). PBMCs were washed, counted and the required number of PBMC were co-cultured with ST285 and the remaining PBMC were stored in freeze mix and transferred into liquid nitrogen for future use.

2.4.2. Stimulation of PBMC with ST285

PBMC (3x 10^7 cells) were resuspended in RPMI 1640 media supplemented with 10% heat-inactivated FBS (Invitrogen, Pty Ltd. Australia), 1% antibiotic-antimycotic solution and 2 mM L-glutamine in cell culture flasks, and 3x10^8 ST285 bacteria were added. PBMC with RPMI media without the addition of ST285 bacteria were used as a control and incubated at 37° C, 5 % CO_2 for 24 hrs (Dargahi et al., 2018). We previously demonstrated that 24 hrs co-culture was optimal for stimulation of U937 monocyte/macrophage cell line, and all incubations described herein were for 24 hrs (Dargahi et al., 2018). PBMCs were snap frozen post incubation and stored at -80° C prior to RNA extraction.

2.5. RNA extraction from PBMC

Total RNA was extracted from stimulated PBMCs using the RNeasy® mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, cells were centrifuged and harvested, supernatants were removed and RNA extracted from each cell pellet and resuspended in lysis buffer supplemented with β-mercaptoethanol to disrupt the cells. PBMC were lysed and each cell lysate passed through the supplied Qia-shredder columns to homogenize and was subsequently mixed with equal volume of 70% ethanol. Cell lysates were transferred onto RNeasy mini-spin columns and DNA was removed using DNase digestion/ treatment using RNase-Free DNase Set (Qiagen, Hilden, Germany.) The RNA Integrity Number (RIN) of all RNA samples were measured using an Agilent 2100 Bioanalyzer and Agilent RNA 6000 nano kit (Agilent Technologies, Santa Clara, CA, USA); with a minimum RIN of 7.5 used as the criterion for inclusion in gene expression analysis. The concentration of each individual RNA sample was measured using a Qubit RNA BR Assay (Invitrogen) in triplicate.

2.6. Assessing changes in the expression of genes associated with innate and adaptive immunity

Aliquots of each RNA sample were reverse-transcribed to make complementary DNA (cDNA) using RT first strand kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the 'Human Innate and Adaptive immune Response' kit (Qiagen, Hilden, Germany) to evaluate gene/mRNA expression. The relative expression profiles of treated PBMC samples were analyzed in comparison with untreated PBMC cultured in RPMI using Thermo-cycler

(Biorad, Melbourne Australia). The RT^2 qPCR Primer innate and adaptive immune response arrays target a set of 84 innate and adaptive immune-related genes and five housekeeping genes, an RT control, a positive PCR control, and a human genomic DNA contamination control. The levels of the expression of these genes were calculated using the Qiagen web-based software (Qiagen, Germany) and then calculated the fold changes and analyzed data manually to compare results. Differential expression (up and down regulation) of the genes were identified using the criteria of a > 2.0-fold increase/decrease in gene expression in treated PBMCs in comparison with those genes in control PBMC cultures.

2.7. Data analysis

The Delta-Delta CT (ΔΔCT) was used to calculate fold-changes (Livak & Schmittgen, 2001). Fold-regulation represents fold-change results in a biologically meaningful way. In our RT2 profiler PCR array results, fold-change values greater than one, indicate a positive (or an up-) regulation, in fact in upregulated genes, the fold-regulation is equal to the fold-change. Fold-change values less than one specifies a negative (or a down) regulation, and in this case, the fold-regulation is the negative inverse of the fold-change (Souza et al., 2016; Z. Yang, Zhong, Zhong, Xian, & Yuan, 2015; T. Zhang et al., 2017). Data related to changes in the expression of the genes were analyzed by ΔΔCT method using Qiagen RT^2 profiler data analysis webportal that utilises the delta delta CT method in determining fold-changes. The raw CT values were uploaded to the Qiagen data analysis webportal with the lower limit of detection set for 35 cycles and 3 internal controls: PCR array reproducibility, RT efficiency and genomic DNA contamination were assessed to ensure all arrays successfully passed all of these control checks. Normalization of the raw data was performed using the included

housekeeping genes (HKG) panel. Then using the ΔΔCT method, both housekeeping gene references and untreated/ controls were assessed to calculate relative expression of mRNA.

2.8. Statistical analysis

The p values are calculated based on a Student's *t-test* of the Triplicate 2^ (- Delta CT) [(2^-ΔCT)] values for each gene in the control group and treatment groups (Biasin et al., 2017; dos Santos et al., 2009; Z. Yang et al., 2015; T. Zhang et al., 2017).

3. Results

Among 84 genes assessed, 31 genes were significantly altered > 2.0 fold up/down in PBMC samples (n=3) following exposure to ST285 compared to control PBMC (Fig 1).

A

Layout	01	02	03	04	05	06	07	08	09	10	11	12
A	APCS **-1.05** C	C3 **-3.38** A	CASP1 **-1.24**	CCL2 **-1.88**	CCL5 **-1.10**	CCR4 **-1.22**	CCR5 **-6.29** A	CCR6 **-1.29** B	CCR8 **-1.11** B	CD14 **-25.29** A	CD4 **-1.86** B	CD40 **-15.39**
B	CD40LG **-1.55** A	CD80 **-1.70** A	CD86 **-8.04** A	CD8A **-2.96** A	CRP **-1.05** C	CSF2 **130.35** A	CXCL10 **-5.30** A	CXCR3 **-1.22** B	DDX58 **-1.02**	FASLG **1.02**	FOXP3 **-1.85**	GATA3 **-22.15** A
C	HLA-A **-1.45**	HLA-E **-1.22**	ICAM1 **-1.31**	IFNA1 **-1.05** C	IFNAR1 **-1.88**	IFNB1 **-1.05** C	IFNG **8.72** A	IFNGR1 **-4.03**	IL10 **2.05**	IL13 **1.52** B	IL17A **-1.05** C	IL18 **-73.04** A
D	IL1A **2.78**	IL1B **4.82**	IL1R1 **-1.50**	IL2 **-7.27** B	IL23A **3.08** A	IL4 **-1.34** B	IL5 **-1.45** B	IL6 **25.12**	CXCL8 **11.26**	IRAK1 **-1.05** B	IRF3 **1.08**	IRF7 **-12.32**
E	ITGAM **-2.76** A	JAK2 **-1.29**	LY96 **-1.85**	LYZ **-37.91**	MAPK1 **-1.79**	MAPK8 **-1.27**	MBL2 **-1.05** C	MPO **-2.33**	MX1 **1.17**	MYD88 **-1.73**	NFKB1 **-1.24**	NFKBIA **-1.48**
F	NLRP3 **-2.11** A	NOD1 **-1.23** A	NOD2 **-1.41** B	RAG1 **1.05** B	RORC **-1.70** B	SLC11A1 **-4.72**	STAT1 **1.47**	STAT3 **-1.39**	STAT4 **-1.26**	STAT6 **1.02**	TBX21 **-1.01**	TICAM1 **-1.26**
G	TLR1 **-2.63** A	TLR2 **-2.68**	TLR3 **1.36** B	TLR4 **-5.65** A	TLR5 **-1.30** B	TLR6 **-1.73**	TLR7 **1.07** B	TLR8 **-11.41** A	TLR9 **-1.29** B	TNF **6.10**	TRAF6 **-1.18**	TYK2 **-10.03**

B

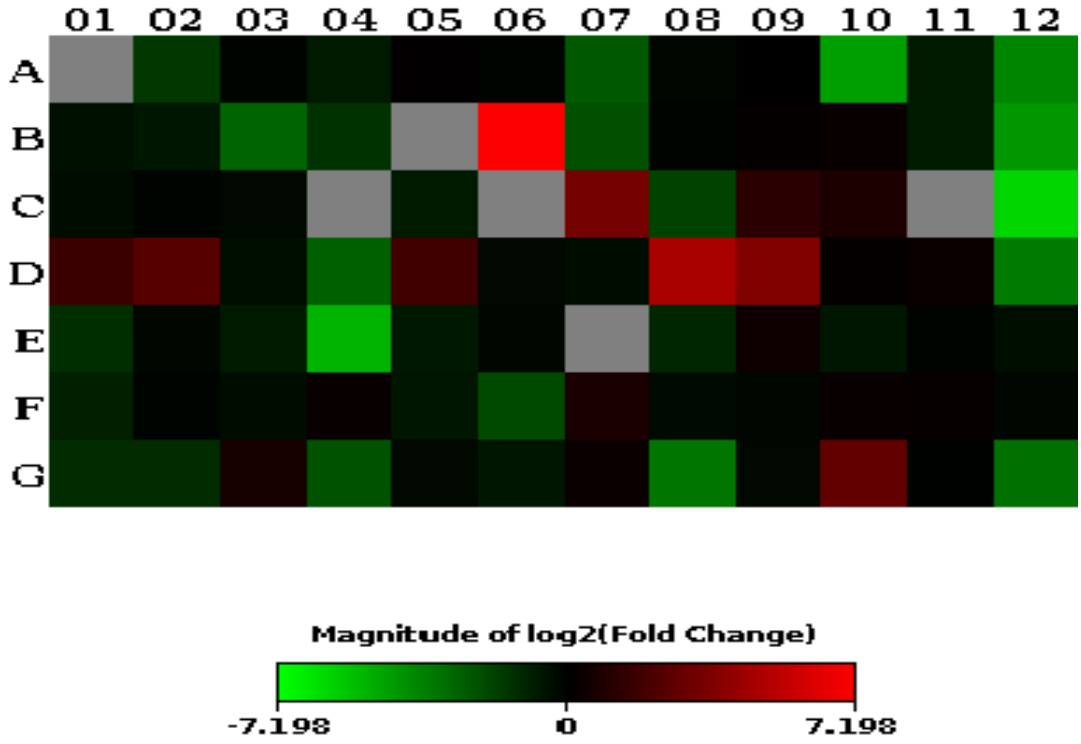

Figure 1. Effects of co-culturing ST285 with PBMCs (n=3) on gene/RNA expression compared to control PBMCs after 24 hrs. (A) All 84 genes are shown including those with significant high up/down regulated genes (more than 2-fold) and those with no significant change (less than 2-fold). The housekeeping genes (HKG) panel and other genes used for normalization of the raw data are not presented. Letter A specifies the gene's average threshold cycle to be reasonably high (> 30) in either the treated samples or the controls and relatively low (< 30) in the other/opposite sample. Thus, in case of presenting fold changes with letter A, the estimate fold change may be an underestimate. Letter B suggests a reasonably high (> 30) gene's average threshold cycle that means a low level of average expression of relevant gene, in both test/treated samples and untreated control samples, and the p-value for the fold-change might be either relatively high ($p > 0.05$). Thus, in case of presenting fold changes with letter B, the estimate fold change may be slightly overestimate or unavailable. Letter C indicates that that gene's average threshold cycle is either not determined or greater than the defined default 35 cut-off value, in both test/treated samples and control samples, suggesting that its expression was not detectable, resulting in the fold-change values being un-interpretable (Gaston et al., 2017; Goad, Ko, Kumar, Syed, & Tanwar, 2017) (Abubaker et al., 2013). (B) Presentation of data as a heatmap of average gene/RNA expressions of PBMC (n=3) co-cultured with ST285, compared to control. Green represents down regulated genes to red represents upregulated genes.

3.1. ST285 alters cytokine gene expression levels of PBMC

3.1.1. Interleukin mRNA expression levels

IL-1α and IL-6 are secreted by dendritic cells (DC), B cells and macrophages (MQ) are involved in acute phase responses, B cell maturation, macrophage differentiation, promote Th2 differentiation and inhibit Th1 polarization. IL-1α is upregulated 2.78 ± 0.6 fold and IL-6 25.12 ± 0.61 fold (Fig 2). IL-23α is secreted by $CD4^+$ T cells and aids in the stimulation of Th17 cells together with IL-6. IL-23α is highly upregulated 3.8 ± 1.0 fold (Fig 2). IL-2 has an array of functions it activates T cell proliferation and increases or decreases inflammatory responses. IL-2 is downregulated 7.27 ± 0.53 fold (Fig 2). IL-17A a pro-inflammatory cytokine secreted by Th17 cells, was not altered following PBMC co-cultured with ST285.

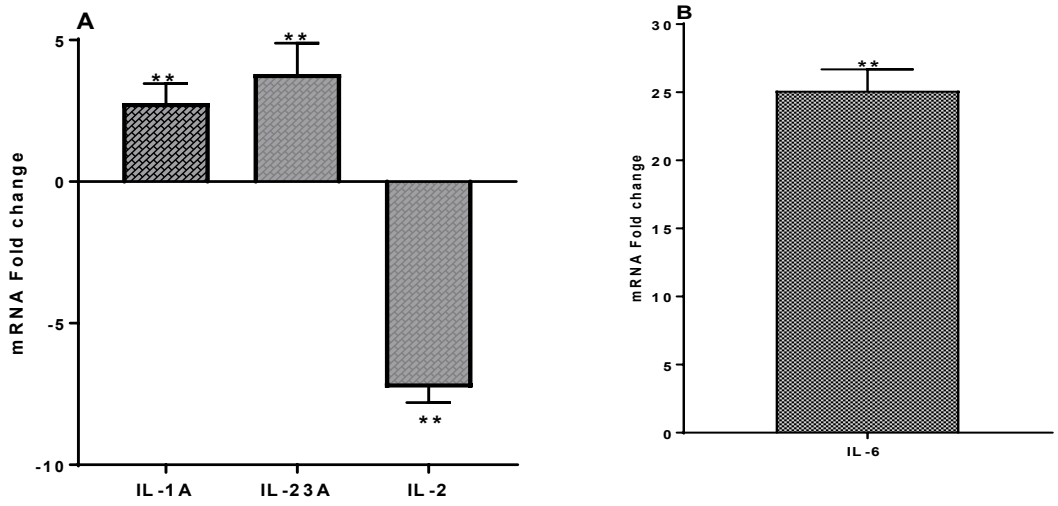

Figure 2. (A) IL-1α, IL-23α and IL-2 and (B) IL-6, mRNA fold change following 24 h co-culture of ST285 with PBMCs (n=3), compared to control PBMC. The innate and adaptive RT^2 gene profiler arrays were used to determine changes in gene expression. Symbols represent *p* value for Tukey Test (One way ANOVA) where ** $p < 0.04$.

3.1.2. Th1/Th2 mRNA expression levels

IFNγ, a Th1 cytokine important in the defense against bacterial infection is upregulated 8.73 ± 0.94 fold. Likewise, the Th1 cytokine IL-1β is upregulated 4.82 ± 0.74 fold (Fig 3). Of interest, IL-18 a Th1 inducing pro-inflammatory cytokine was vastly downregulated (75 ± 0.66 fold), in addition, IFNγR1, a transmembrane protein which interacts with IFNγ, is also downregulated 4.03 ± 0.25 fold (Fig 3). Tumor-necrosis factor-alpha (TNFα), important in the defense against bacterial infections, and in acute phase reactions is upregulated 6.10 ± 1.4 fold (Fig 3). IL-10, an anti-inflammatory cytokine secreted by Th2 and Treg cells is upregulated 2.05 ± 0.52 fold (Fig 3). Gene expressions of other cytokines, IFNB1, IL-4, IL-5 and IL-13 are not significantly altered.

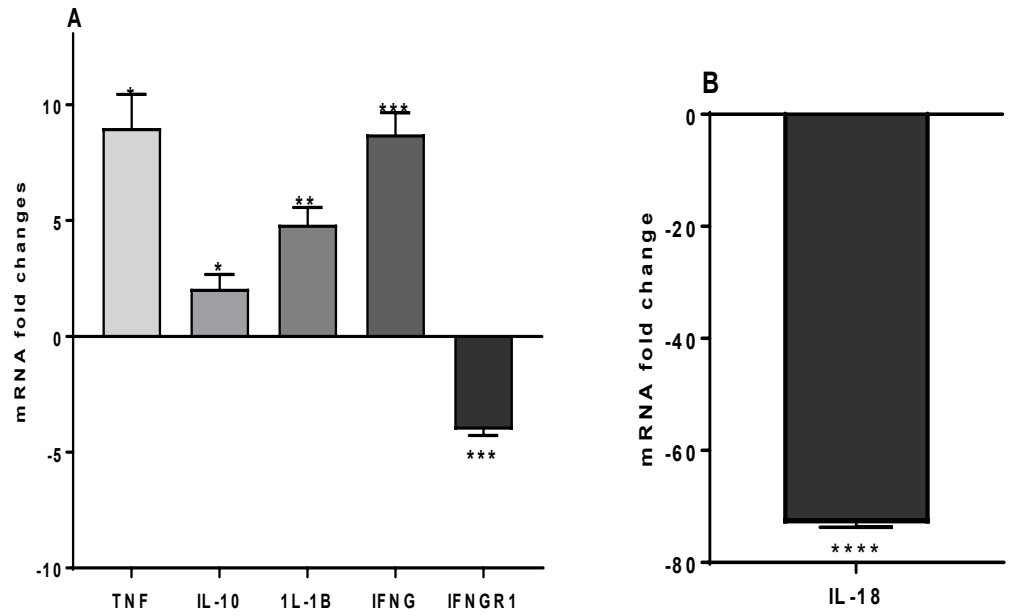

Figure 3. (A) TNF-α, IL-10, IL-1β, IFN-γ, and IFN-γ−R and (B) IL-18, mRNA fold change following 24 h co-culture of ST285 with PBMCs (n=3), compared to control PBMC. The innate and adaptive RT^2 gene profiler arrays were used to determine changes in gene expression. Symbols represent *p* value for Tukey Test (One way ANOVA) where * $p < 0.05$, ** $p < 0.04$, *** $p < 0.02$ and **** $p < 0.01$.

3.2. ST285 alters chemokine gene expression levels of PBMC

Chemokine (CXCL8, IL-8) is important in the innate immune system, it stimulates chemotaxis and is upregulated 11.26 ± 0.27 fold following ST285 co-culture with PBMC cells. However, CCR5 and CXCL10 (INP10) are down regulated 6.29 ± 0.32 and 5.30 ±1.8 fold respectively (Fig 4). No significant differences are noted for gene expressions of other chemokines, including CCL2 (MCP-1), CCL5 (RANTES), CCL8, CCR4, CCR8, CXCR3, CCL2, IFNA1.

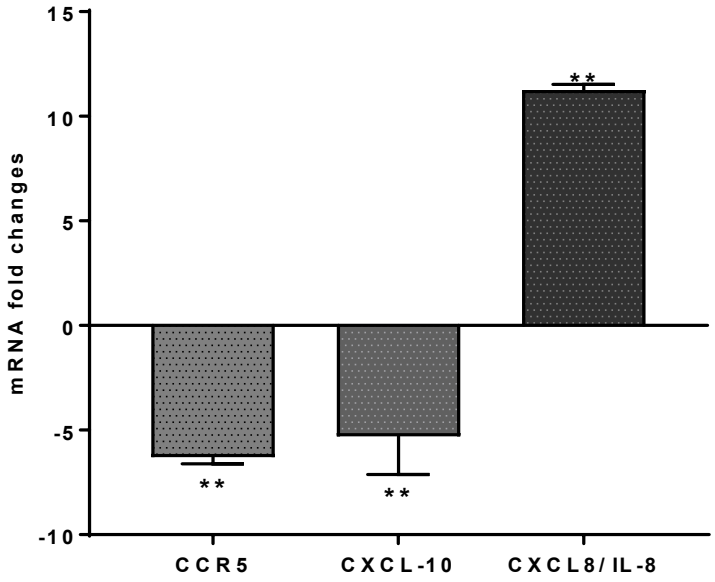

Figure 4. CCR5, CXCL10 and CXCL8 (IL-8), mRNA fold change following 24 h co-culture of ST285 with PBMCs (n=3), compared to control PBMC. The innate and adaptive RT^2 gene profiler arrays were used to determine changes in gene expression. Symbols represent *p* value for Tukey Test (One way ANOVA) where ** $p < 0.04$.

3.2.1 Colony stimulating factor mRNA expression levels

Colony-stimulating factor (CSF)-2, secreted by MQs, NK cells and T cells, enables cell proliferation and differentiation and is significantly increased by 130.35 ± 1.0 fold (Fig 5) after co-culturing PBMC with ST285 bacteria.

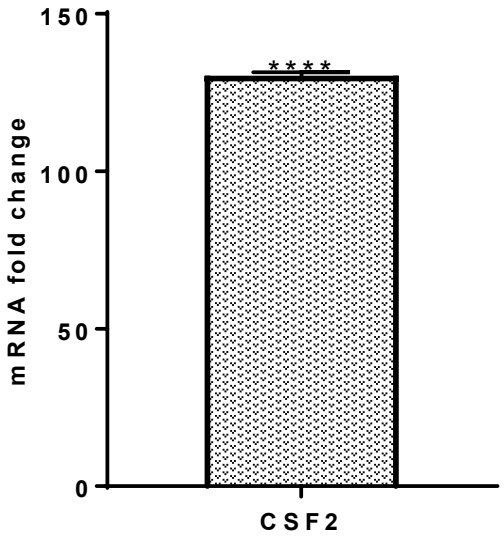

Figure 5. CSF-2, mRNA fold change following 24 h co-culture of ST285 with PBMCs (n=3), compared to control PBMC. The innate and adaptive RT^2 gene profiler arrays were used to determine changes in gene expression. Symbols represent *p* value for Tukey Test (One way ANOVA) where **** $p < 0.01$.

3.4. ST285 alters Toll like receptor gene expression levels of PBMC

TLR (toll like receptor)-1, TLR-2, TLR-4 and TLR-8 are part of the innate immune response and involved in the defense response to bacteria. PBMC co-cultured with ST285 induced downregulation of TLRs at varying levels; TLR-1 (-2.63 ± 0.43), TLR-2 (-2.69 ± 0.8 fold), TLR-4 (-5.65 ± 0.56 fold), TLR-8 (-11.41 ± 1.27 fold) (Fig 6). However, changes to other pattern recognition receptors such as, TLR-3, TLR-5, TLR-6, TLR-9 were not significant.

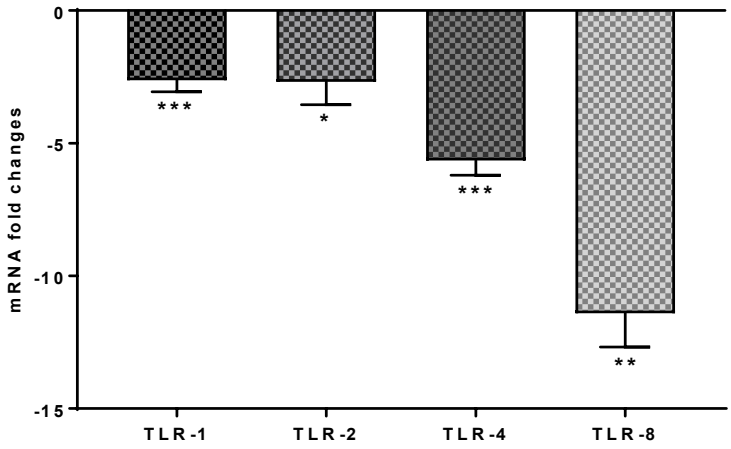

Figure 6. TLR-1, TLR-2, TLR-4 and TLR-8, mRNA fold change following 24 h co-culture of ST285 with PBMCs (n=3), compared to control PBMC. The innate and adaptive RT^2 gene profiler arrays were used to determine changes in gene expression. Symbols represent *p* value for Tukey Test (One way ANOVA) where * $p < 0.05$, ** $p < 0.04$ and *** $p < 0.02$.

3.5. Cell surface markers CD14, CD40, CD86 mRNA expression levels

Expression of the monocyte cell surface markers CD14, CD40 and CD86 significantly downregulated -25.29 ± 3.46, -15.39 ± 1.36, -8.04 ± 0.14 fold, respectively (Fig 7). Expression of the CD8A gene, which is involved in adaptive immunity and in response to defense against viruses, was downregulated by -2.96 ± 0.68 fold (Fig 7). Expression of CD4, CD80, FOXP3, STAT3, CD40LG (TNFSF5), HLA-A, HLA-E and RORC genes do not show significant changes.

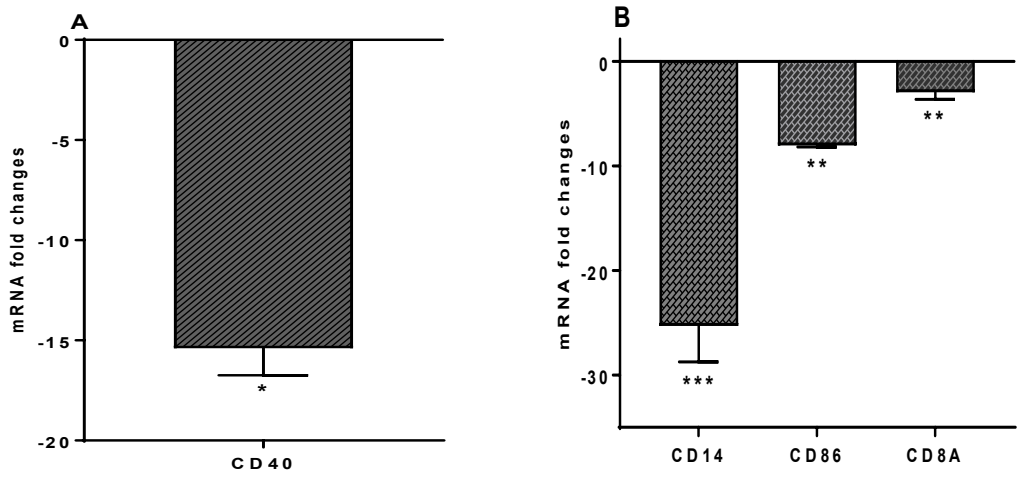

Figure 7. (A) CD40 and (B) CD14, CD86 and CD8A, mRNA fold change following 24 h co-culture of ST285 with PBMCs (n=3), compared to control PBMC. The innate and adaptive RT^2 gene profiler arrays were used to determine changes in gene expression. Symbols represent *p* value for Tukey Test (One way ANOVA) where * $p < 0.05$, ** $p < 0.04$ and *** $p < 0.02$.

3.6. Changes to other innate and adaptive molecules, mRNA expression levels

Changes to other genes were also noted following ST285 co-culture with PBMC. ACTB (-3.01 ± 1.0) fold, ITGAM (-2.76 ± 0.9) were both downregulated. Downregulated genes were noted to the following: MPO (2.33 ± 0.2), NLRP3 (2.11 ± 0.6), SLC11A1 (4.72 ± 0.23) and complement component (C)-3 (3.38 ± 1.5), TYK2 (10.03 ± 0.7), IRF7 (12.32 ± 0.4), LYZ (37.91 ± 0.4) and GATA3 (22.15 ± 1.64) (Fig 8). Other immune markers including FASLG (TNFSF6), CRP, IFNAR1, JAK2, IL-1R1, MAPK8 (JNK1), IRF3, MBL2, NFKB1, MX1, ICAM1, MBL2, MYD88, NOD1 (CARD4), NOD2, DDX58 (RIG-I), RAG1 and TICAM1 (TRIF) showed no significant mRNA gene changes in the levels of their expression.

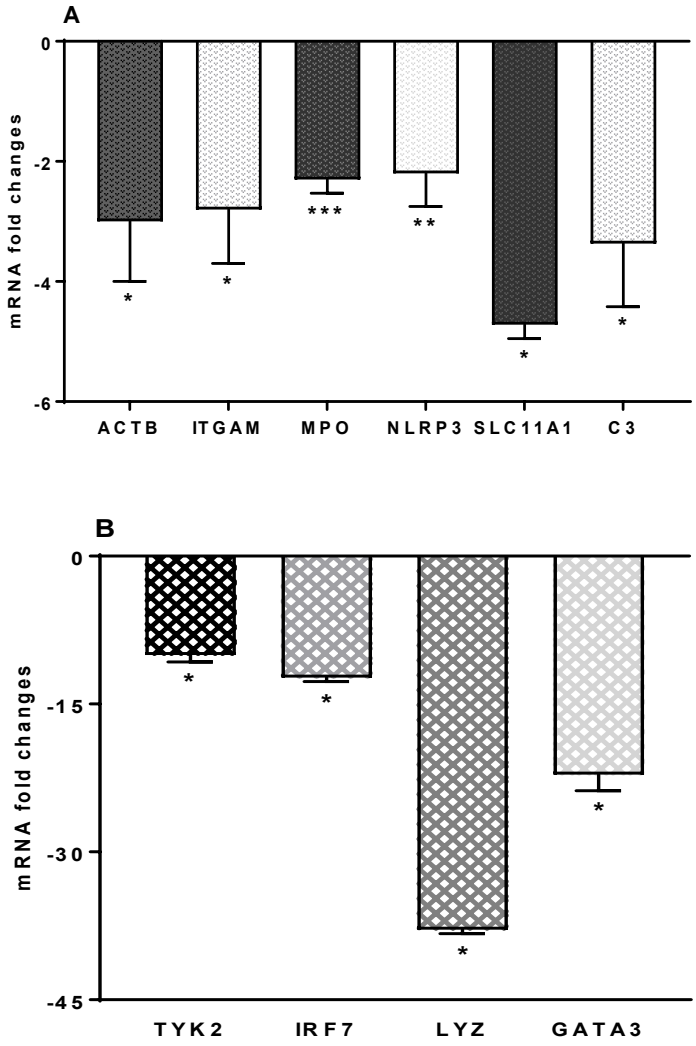

Figure 8. (A) ACTB, CCR5, ITGAM, MPO, NLRP3, SLC11A1, and C3 and (B) TYK2, IRF7, LYZ and GATA3, mRNA fold change following 24 h co-culture of ST285 with PBMCs (n=3), compared to control PBMC. The innate and adaptive RT^2 gene profiler arrays were used to determine changes in gene expression. Symbols represent *p* value for Tukey Test (One way ANOVA) where * $p < 0.05$, ** $p < 0.04$ and *** $p < 0.02$.

4. Discussion

4.1. ST285 promotes Th2 polarization

IL-1α secreted by peripheral blood DC and B cells induces Th2 differentiation and inhibits Th1 polarization (Ben-Sasson et al., 2009), is significantly upregulated. Similarly, *Enterococcus faecium* NCIMB 10415 was shown to upregulate IL-1α in porcine jejunal epithelial cells (IPEC-J2) *in vitro*, (Kern et al., 2017). IL-6 produced by Th2 cells is increased in the presence of ST285 by PBMC which was also shown previously to be upregulated by pro-monocyte cell line U937 (Dargahi et al., 2018). Others have shown that PBMC co-cultured with ST1275 also increases IL-6 (Donkor, Henriksson, Vasiljevic, & Shah, 2007). Likewise, mixed probiotics of ST, *Lactobacillus* (*L.*) *rhamnosus*, *L. casei*, *L. acidophilus, B. longum* and *B. bifidum* stimulated PBMC to produce IL-6 (Djaldetti & Bessler, 2017; Dong et al., 2012). Our study shows that IL-1α and IL-6 are increased, highlighting the role of ST285 in stimulation of immune responses involved in acute phase; B cell maturation, macrophage differentiation, promotion of Th2 differentiation and inhibiting Th1 polarization.

IL-10 is an anti-inflammatory cytokine secreted by Th2 and Treg cells and co-culture of ST285 with PBMC increased expression of IL-10. Cultured PBMC with other live ST strain (ST1275) also showed increased IL-10 (Donkor, Henriksson, Vasiljevic, & Shah, 2006; Donkor, Henriksson, et al., 2007; Donkor, Ravikumar, et al., 2012a; Donkor et al., 2010; Donkor, Stojanovska, Ginn, Ashton, & Vasiljevic, 2012; Donkor, Tsangalis, & Shah, 2007). Similarly, in a study using mixed probiotic cultures (*S. thermophiles, L. rhamnosus*, *L. casei*, *L. acidophilus, B. longum* and B. *bifidum*) high levels of IL-10 were stimulated by PBMC (Djaldetti & Bessler, 2017). Conversely, in a study using *B. breve* and ST combined to

stimulate PBMC, IL-10 was only increased in the presence of *B. breve*, whereas exposing PBMC to ST reduced the IL-10 level (Ménard et al., 2004). We also previously noted that monocyte cell line (U937), co-cultured with ST1342 stimulated production of high levels of IL-10 (Dargahi et al., 2018).

IL-18 is involved in the initiation of severe inflammatory responses, indicating the role of IL-18 in inflammatory and autoimmune disorders. Co-culture of PBMC with ST285 significantly downregulated IL-18 which indicates an anti-inflammatory role for ST285 bacteria. Likewise, a mixture of Lactobacilli species (*L. rhamnosus*, *L. paracasei*, and *L. plantarum*) was shown to supress the secretion of pro-inflammatory IL-18 gene by undifferentiated IPEC-1 intestinal porcine epithelial cell line (Taranu et al., 2018), highlighting supportive role of Lactobacilli probiotics in functioning against inflammation and suppression of immune response activities. However, other studies with other probiotics such as, *L. rhamnosus* E509, *L. rhamnosus* GG E522 (ATCC 53103), *L. bulgaricus* E585 and *S. pyogenes* serotype T1IH32030, increased IL-18 production by human PBMC (Miettinen et al., 1998). Hence, different probiotic strains induce different cytokine profiles.

IL-2 is involved in signalling of immune responses and activates proliferation of lymphocytes. We note downregulation of IL-2 gene expression in PBMC after exposure to ST285. IL-23 known to activate Th17 cells was upregulated although IL-17, the key pro-inflammatory cytokine secreted by Th17 cells was not altered. Upregulation of IL-1α, IL-6, IL-10, and downregulation of IL-2, IL-18 and an absence of change in IL-17A (despite increase in IL-23α) designates ST285 to possess anti-inflammatory effects on human PBMC.

4.2 ST285 stimulates expression of cytokines involved in the defence against bacteria

IFN-γ is an adaptive immunity cytokine secreted by Th1 cells in the defense response to microbes and viruses. IFN-γ is predominantly secreted by NK, NKT cells as part of the innate immune response, and by CD4 Th1 and $CD8^+$ T cells of the adaptive immune response (Schoenborn & Wilson, 2007a). ST285 upregulated IFN-γ gene expression by human PBMCs. This is similar to studies of a combination of probiotic strains including ST, *Lactobacillus, Bifidobacterium, Propionibacterium, E. coli* and *Leuconostoc* (Kekkonen et al., 2008), where upregulation of IFN-γ mRNA expression by PBMC was noted (Kekkonen et al., 2008). Likewise, co-cultures of pooled PBMC with ST1275 also induced upregulation of IFN-γ (Donkor, Henriksson, et al., 2007). We previously noted that monocyte cell line co-cultured with ST1342, ST1275 or ST285 strains induced high levels of IFN-γ secretion (Dargahi et al., 2018). In a study with Lactobacilli (*L. rhamnosus* E509, *L. rhamnosus* GG E522 (ATCC 53103) and *L. bulgaricus* E585), and streptococci (*S. pyogenesserotype* T1 IH32030), IFN-γ was produced by human PBMC (Miettinen et al., 1998).

IL-1β secretion by monocytes is involved in regulating immune and inflammatory responses to bacterial infections and injury, hence its role in innate immunity (Lopez-Castejon & Brough, 2011). IL-1β is upregulated by ST285 co-cultured with PBMC, which is in accord with studies of PBMC co-cultured with mixed probiotics (ST, *L. rhamnosus*, *L. casei*, *L. acidophilus, B. longum* and *B. bifidum*) (Djaldetti & Bessler, 2017). We previously noted in monocyte cell line co-cultured with three different strains of ST, only ST1342 stimulated production of high levels of IL-1β whereas, ST1275 and ST285 did not induce IL-1β cytokine (Dargahi et al., 2018). A mixture of Lactobacilli strains (*L. rhamnosus*, *L. paracasei*, and *L. plantarum*) co-cultured with intestinal porcine epithelial cell line (IPEC-1) also upregulated

IL-1β gene expression (Taranu et al., 2018). Similarly, the combination of *L. casei* Shirota, *L. rhamnosus* GG, *L. plantarum* NCIMB 8826 and *L. reuteri* NCIMB 11951, *B. bifidum* MF 20/5 and *B. longum* SP 07/3 co-cultured with PBMC, significantly augmented IL-1β production (Dong et al., 2012).

TNFα plays a key role in the defense against bacterial infections. It is a pro-inflammatory cytokine, which also supports recruitment and activation of T and B cells to promote an adaptive immune response. We previously demonstrated high levels of TNFα secretion by U937 monocyte cell line in the presence of ST1342, ST1275 and ST285 (Dargahi et al., 2018). Likewise, our current findings show that ST285 co-cultured with PBMC results in upregulation of TNFα. However, in a study using *B. breve* and ST together to stimulate PBMC, TNF-α secretion was inhibited (Ménard et al., 2004). In addition, a mixture of strains of probiotics (*L. casei* Shirota, *L. rhamnosus* GG, *L. plantarum* NCIMB 8826 and *L. reuteri* NCIMB 11951, *B. bifidum* MF 20/5 and *B. longum* SP 07/3) co-cultured with PBMC, significantly increased the production of TNFα (Dong et al., 2012). In another study of human PBMCs exposed to different probiotics (*L. mesenteroides* ssp. cremoris PIA2 (DSM 18892) *S. pyogenes* serotype T1M1, *S. thermophilus* THS, *E. coli* (DH5α), *L. rhamnosus* Lc705 (DSM 7061), *L. lactis* ssp. cremoris ARH74 (DSM 18891), *L. rhamnosus* GG (ATCC 53103), *L. helveticus* Lb 161, *L. helveticus* 1129, *B. longum* 1/10, *B. breve* Bb99 (DSM 13692), *B. animalis* ssp. lactis Bb12, and *Propionibacterium* (*P.*) *freudenreichii* ssp. shermanii JS (DSM 7067)), all induced TNF-α mRNA expression (Kekkonen et al., 2008). Given that IFNγ, IL-1β and TNFα are upregulated by PBMC following co-culture with ST285 this suggests that ST285 induces powerful defense against invading pathogens and could be beneficial against virus infection and tumours.

The unpregulation of IFNγ, IL-1β and TNFα coupled with a significant decrease in IFNγ receptor and IL-18 shows an antagonising effect of ST285 inflammatory responses and leading to an overall anti-inflammatory profile.

4.3. ST285 activates mRNA expression of CXCL8 and downregulates CCR5 and CXCL10

IL-8, also known as CXCL8 is an important chemokine of the innate immune system, involved in the recrutiment of neutrophils and other granulocytes as the first line of defense (Baggiolini & Clark-Lewis, 1992b). ST1342, ST1275 and ST285 were previously shown to activate U937 monocyte cell line to produce high levels of IL-8 (Dargahi et al., 2018). The probiotic *L. paracasei* DG also increases expression of IL-8 to the human monocyte cell line, THP-1 (Balzaretti et al., 2017). Likewise, short chain fatty acids, produced by probiotic bacteria, also stimulate IL-8 secretion and mRNA levels to the human epithelial cell line HT-29 (Asarat, Vasiljevic, et al., 2015). These studies are in accord to our current findings that ST285 upregulates CXCL8 production by human PBMC.

C-C chemokine receptor type 5 (CCR5, CD195) is involved in Th1 immune responses and its gene expression is downregulated by PBMC following ST285 co-culture. However, in mice prolonged feeding with VSL#3 probiotic mixture shows significant gene upregulation of CCR5 (Mariman, Tielen, Koning, & Nagelkerken, 2015). Differences could be attributed to one probiotic strain applied and varying effects of the strain (ST) used in current study versus a mixture of different strains and species used in mice VSL#3 (*L. delbruekii Bulgaricus*, *L. casei*, *L. plantarum*, *L. acidophilus*, *B. breve*, *B. longum*, *B. infantis* and ST).

CXC motif chemokine 10 (CXCL10), or IFN-γ-induced protein-10 (IP-10), is secreted by a number of cell types (endothelial cells, monocytes and fibroblasts). Few roles have been ascribed to CXCL10 including chemo-attraction of NK cells, monocytes/macrophages, T cells and DCs, favouring adhesion of T cells to endothelial cells, anti-cancer/tumour action, and preventing angiogenesis and bone marrow colony development. CXCL10 is downregulated in PBMC culture following ST285 exposure. Conversely, monocyte-derived DCs co-cultured with *B. breve* Bb99, *L. lactics* subsp. cremoris ARH74 and *S. thermophilus* THS increased expression of CXCL10 and ST was the most efficient probiotic in the induction of CXCL10 (Latvala et al., 2008). Additionally, microarray results of the intestines of mice prolonged administrated with VSL#3 probiotic mixture in healthy mice showed differential effects on intestinal immune parameters, including upregulation of CXC10 which contrasts with our findings (Mariman et al., 2015). The difference are most likely due to cell types, as well as bacterial strains in our study (PBMC co-cultured with ST285 bacteria) compared to using mouse cells exposed to three strains (*B. breve* Bb99, *L. lactics* subsp. cremoris ARH74 and *S. thermophilus* THS) in the other study. Also in the latter experiments, it is quite predictable to observe different results in mice intestine administered with VSL#3 due to different cells involved in mice study in contrast to PBMC cell population.

In summary, increased expression of IL-8 on its own could singularly be indicative of inflammation, but in the context of all other upregulated anti-inflammatory cytokine and mediators found in this study, this may not be interpreted as an inflammatory effect. IL-8 upregulation might also be interpreted as requirement for the initial stimulatory effect of ST285 to switch on the immune responses by initiating innate immunity, which by the progress of immune response, expression of CCR5 (which in turn influences Th1 immune responses), as

well as CXCL10 (induced by IFNγ) are reduced by ST285. This might be suggestive of modulation of immune responses by ST285 to keep the adaptive immune responses in check.

4.4. ST285 significantly upregulates mRNA expression level of colony stimulating factor

CSF (GM-CSF) is secreted by machrophages, NK cells and T cells, enables cell proliferation and differentiation, stimulates the production of various immune cells, in particular it increases the production of machrophages which are important in fighting againts infections. CSF-2, is vastly increased (130 fold) by PBMC co-cultured with ST285 which is in alignment to our previous data whereby ST1275, ST1342 and ST285 induced U937 monocyte cell line to secrete high levels of GM-CSF with ST285 being the highest inducer (Dargahi et al., 2018). Likewise, another study used RT^2 Profiler PCR Arrays for mouse cytokines and chemokines to demonstrate that *L. rhamnosus* GR-1 (GR-1) induced high levels of granulocyte CSF (G-CSF) mRNA (60-fold) to bone marrow-derived mouse macrophages (Meshkibaf, Fritz, Gottschalk, & Kim, 2015). Likewise, PBMC co-cultured with *B. infantis* 52486 significantly increases GM-CSF (You & Yaqoob, 2012).

GM-CSF is generally accepted as an inflammatory cytokine, its inflammatory activity is primarily associated with its role as granulocytes and macrophages growth and differentiation factor. GM-CSF-mediated inflammation has also been associated with certain types of autoimmune diseases such as rheumatoid arthritis and multiple sclerosis. However, in many instances GM-CSF plays anti-inflammatory/regulatory roles; GM-CSF can modulate differentiation of DC to render them into tolerogenic DCs, which, can stimulate anti-inflammatory Treg cells (Bhattacharya et al., 2015). In addition, either of pro-inflammatory or regulatory effects of GM-CSF appears to be dependent on the amount of CSF and the presence

of other relevant cytokines in the context of an immune response. There is also evidence that G-CSF induces expansion of IL-10-producing cells (Malashchenko et al., 2018). Our results show very high overexpression of CSF, which might be suggestive of anti-inflammatory effect of ST285 on PBMC.

4.5. ST285 downregulates mRNA expression levels of toll-like receptors

Toll-like receptors (TLRs) recognize pathogen-associated molecular patterns (PAMPs) that are expressed on infectious bacteria and mediate the production of cytokines necessary for the development of effective immunity (Kawai & Akira, 2010). TLRs recognize pathogens and activate the innate immune responses. TLR-1, TLR-2, TLR-4 and TLR-8 are part of the innate immune response and are involved in defense against bacteria. Co-culturing ST285 with human PBMC downregulated the expression of TLR. Similarly, *E. coli* K88 and mycotoxin zearalenone (ZEA) infection of IPEC-1 epithelial cell line was protected in the presence of mixed Lactobacillus strains (*L. acidofilus* ID11692, *L. plantarum* ID1253 and *L. paracasei* ID13239) by downregulating TLR-1, TLR-2 and TLR-4 gene expression (Taranu, Marin, Pistol, Motiu, & Pelinescu, 2015).

TLRs are critical in bacterial recognition and host defence, such as lipo-teichionic acid (LTA) and lipo-polysaccharide (LPS) from Gram-positive and Gram-negative bacteria respectively (Arce, Ramírez-Boo, Lucena, & Garrido, 2010; Kajikawa et al., 2011). Activation of some of these molecules and mediators like TLR (especially TLR-2 and TLR-4) arbitrates to pro-inflammatory actions and further defensive functions of innate immunity (Islam et al., 2013; Sugitharini, Pavani, Prema, & Berla Thangam, 2014; Sugitharini, Shahana, Prema, & Berla Thangam, 2016). The TLR-2 and TLR-4 activation and expression by LPS (pathogens)

is known as one of the most important mechanisms by which the immune system controls reactions to bacteria in particular in the activation phase, therefore, over-expression of TLR-2 and TLR-4 during any bacterial infection could cause an elevated inflammatory response in the body. While early activation of TLRs expression is reported in response to bacterial LPS from pathogenic *Salmonella typhimurium* (Arce et al., 2010) as well as *E. coli* infection in bovine intestinal epithelial cells (Takanashi et al., 2013), our results show tolerance as a result of co-culturing PBMC with ST285 by down regulation of TLRs genes.

Downregulated mRNA expression of TLRs genes, specifically TLR-1, TLR-2, TLR-4 and TLR-8 indicates anti-inflammatory characteristics for ST285. Given that TLR-1, TLR-2, TLR-4 and TLR-8 are members of the innate immune response and play key roles in the defense against bacteria, downregulation of TLRs could be suggestive of a protective mechanism to keep ST285 safe by tolerance towards ST285. Perhaps designing experiments that allow different incubation period, as well as adding pathogenic bacteria to the co-cultured ST285-PBMC can help to illustrate if lesser co-culture time and/or presence of pathogens can result in a shift towards upregulation of TLRs instead.

4.6. ST285 downregulates cell surface markers CD14, CD40, CD86

CD14, CD40 CD86 are expressed on the cell surface of monocytes, macrophages and DC. CD14 is expressed on the surface of monocytes and primarily binds to bacterial constituents (Bron et al., 2013; I. C. Lee et al., 2013; van Baarlen et al., 2013). We previously showed that U937 monocyte cell line exposed to ST1342, ST1275 or ST285 enhanced expression of CD14 after 24 and 48 hrs, and ST285 was the most potent at 48 hrs (Dargahi et al., 2018). However, in bulk PBMC cultures, CD14 expression was significantly

downregulated in the presence of ST285, which is in accordance with downregulation of TRLs in particular TRL-4. In other studies, the combination of 3 probiotics (*L. acidophilus*, *L. delbrueckii* ssp. *bulgaricus* and *B. bifidum*) stimulated increased expression of cell surface markers, CD14, CD80 and MHC class II (Dargahi et al., 2018). *E. coli* Nissle 1917, widely used as a probiotic for the treatment of inflammatory bowel disorders, expresses a K5 capsule important in *E. coli* mediating interactions with intestinal epithelial cells and chemokine expression. *E. coli* Nissle 1917 has been shown to induce mRNA expression of CD14 by intestinal Caco-2 cells (Hafez, Hayes, Goldrick, Grencis, & Roberts, 2010).

CD40 is a costimulatory protein on antigen presenting cells and is essential for their activation. CD40 is a key mediator in a wide range of inflammatory and immune responses and its gene expression was downregulated by PBMC in the presence of ST285. In previous experiments with U937 monocyte cell line, co-culture with ST1342, ST1275 or ST285, resulted in small increase in CD40 (Dargahi et al., 2018).

CD86 (B7-2) is expressed on APCs and delivers co-stimulatory signals required for the activation and survival of T cells. CD86 plays the role of the ligand for T cells external CD28, and CTLA-4 (CD28) in regulation and cell to cell dis-association. CD86 acts in conjunction with CD80 to prime Th cells, delivering opposing functions on Treg cells through CTLA-4 and T cell surface CD28 protein. Expression of CD86 by PBMC is downregulated significantly, suggesting an anti-inflammatory profile following exposure to ST285. ST bacteria promote CD86 expression required for T cell activation and the maintenance of immune responses, CD86 downregulation by ST285 suggests a regulating and damping effect of ST285 on PBMC, being interpreted as immunomodulation of adaptive immunity (Fleischer et al., 1996). We previously noted using U937 monocyte cell line in the presence of ST1342, ST1275 and ST285

increased expression of CD86 (Dargahi et al., 2018). Similarly, *L. plantarum* WCFS1 and *L. fermentum* GR1485 upregulate CD86 on monocytes, conversely, *L. rhamnosus* and *L. delbruekii* reduced its expression (Hajebi et al., 2018).

Additionally, monocytes isolated from PBMC and differentiated into immature DCs by GM-CSF and IL-4, and co-cultured with *B. breve* Bb99, *L. lactis* subsp. cremoris ARH74 and *S. thermophilus* THS also increase CD86 expression (Latvala et al., 2008) . Another study used bone marrow-derived DCs from DQ8-transgenic mice and co-culture with *L. plantarum* and *L. paracasei* and *B. lactis* increases CD86 differentially with the highest CD86 being noted in co-administration of *L. plantarum* and *L. paracasei* (D'Arienzo, Maurano, Lavermicocca, Ricca, & Rossi, 2009). The contrast between these studies to the findings herein could be due to the differences in the nature of studies; we co-cultured PBMC with ST285 bacteria only and the other studies used mouse bone marrow-derived DCs co-cultured with three different probiotics leading to predictable differences.

Given the downregulation of cell surface markers and their roles in immunity, CD14 (involved in innate immunity), CD40 (involved in innate immunity), and CD86 (T cell activation), following ST285 co-culture is suggestive of an anti-inflammatory anti-activation profile for ST285. In addition, as all these cell surface markers are interlinked with defence against bacteria either through innate or adaptive immune responses, downregulation of these markers could be suggestive of ST285 initiating self-tolerance via regulating immune responses, which in turn modulates the immune responses too.

4.7. ST285 differentially downregulates mRNA expression level of other innate and adaptive immune response markers and chemokines

Complement component 3 (C3) is associated with complement cascades in immune responses by enhancing antibody function, phagocytosis and stimulation of inflammation (Appledorn et al., 2008; Rus, Cudrici, & Niculescu, 2005; Sahu & Lambris, 2001). GATA3 transcriptome is also important in both humoral immunity and inflammatory responses. Downregulation of C3 gene expression and significant reduced expression of GATA3 transcriptome by PBMC co-cultured with ST285 in noted. Similarly, lipoteichoic acid (p-LTA) extracted from *L. plantarum* K8 inhibits C3 mRNA *in vitro* and *in vivo*. In human clinical studies, blocking GATA3 is able to control allergy responses, inflammatory diseases and asthma (Maneechotesuwan et al., 2009). C3 and GATA3 downregulation suggests that ST285 is able to lower inflammation (C3), as well as being a viable candidate for further pre-clinical and clinical studies for the management of such diseases.

Interferon regulatory factor (IRF) 7, integrin alpha M (ITGAM), Lysozyme (LYZ) and NALP3 are other innate immune response factors. IRF7, a member of IRF family and present on monocytes, macrophages, granulocytes, and NK cells, and expressed predominantly in macrophages (a component of the inflammasome) (X. Yu et al., 2018). IRF7 plays a role in the transcriptional activation of virus-inducible cellular genes, including the type I interferon genes. ITGAM is involved in a number of inflammatory responses (i.e. cell-mediated cytotoxicity, phagocytosis, and chemotaxis). LYZ acts as an antimicrobial enzyme present in neutrophils and macrophages. IRF7 gene regulation decreased considerably along with ITGAM gene expression, which is downregulated when PBMC are co-cultured with ST285. NALP3 and LYZ are downregulated markedly in co-culture of PBMC with ST285. However,

in a previous study, we showed significant upregulation of CD11b (ITGAM) by monocytic U937 cells when co-cultured with ST1342, ST1275 and ST285 bacteria (Dargahi et al., 2018). ST285-induced downregulation of IRF7, ITGAM, NALP3 and LYZ in PBMC, suggestive of an anti-inflammatory effect of ST285 on PBMC as well.

Non-receptor tyrosine-protein kinase (TYK2) is an enzyme [7] that contributes to adaptive immune responses due to its implication in IFNα, IL-6, IL-10 and IL-12 signaling, also involved in transducing signals of IL-6, IL-10 and IL-23. TYK2 gene expression is significantly downregulated in PBMC co-cultured with ST285, supporting an anti-inflammatory profile for ST285. In addition, myeloperoxidase (MPO), an enzyme abundantly expressed by neutrophils and promotes inflammation, is also involved in autoimmune disorders (multiple sclerosis, rheumatoid arthritis) (Papayannopoulos & Zychlinsky, 2009; Strzepa, Pritchard, & Dittel, 2017). A decreased expression of MPO has been suggested to manage these autoimmune disorders by decreasing the inflammatory state. ST285 co-cultured with PBMC decreased the expression of MPO, suggestive of an anti-inflammatory benefit of ST285.

IFNAR1 is a type I membrane protein which is a receptor for IFNα and IFNβ involved in defence against viruses. IFNAR1 signaling is associated with pro-inflammatory cytokine production (Goritzka et al., 2014). In fact, IFNAR1 knockout mice show decreased pro-inflammatory cytokiens and chemokines (Goritzka et al., 2014). IFNAR1 is significantly downregulated by PBMC following co-culture with ST285, supporting an anti-inflammatory role of ST285. In addition, SLC11A1 involved in T cell activation, is involved in inflammatory disorders such as autoimmune type 1 diabetes (Y. D. Dai et al., 2009; Thayer, Wilson, & Mathews, 2010), is downregulated by PBMC in the presence of ST285. Furthermore, the Beta-actin (ACTB) which stimulates eNOS and increase nitric oxide (NO) (Kondrikov et al., 2010)

involved in immunity and inflammation (Butterworth, 2011), is downregulated by PBMC following co-culture with ST285.

The immune modulatory effects of ST285 to human PBMC were determined and showed that it has an array of anti-inflammatory immune-modulatory properties. ST285 decreases mRNA expression IL-18, IFNγR1, CCR5, CXCL10, TLR-1, TLR-2, TLR-4, TLR-8, CD14, CD40, CD86, C3, GATA3, ITGAM, IRF7, NLP3, LYZ, TYK2, IFNR1, and upregulates IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-23, IFNγ, TNFα, CSF-2. No changes to mRNA expression are noted with IFNA1, IFNB1, IL-4, IL-5, IL-13, CCL2, CCL5, CCL8, CCR4, CCR8, CXCR3, TLR-3, TLR-5, TLR-6, TLR-9, CD4, CD80, FOXP3, STAT3, CD40LG, HLA-A, HLA-E, RORC. The data demonstrates a predominant anti-inflammatory profile exhibited by ST285, and further work is required to determine its effects in inflammatory disease models *in vitro* and *in vivo*, such as multiple sclerosis, inflammatory bowel disease and allergies.

5. Conclusion

Probiotics are beneficial microorganism with immunomodulatory properties, which aid the maintenance of a healthy immune system. *Streptococcus thermophilus* (ST) is often used in fermented dairy products such as cheeses and yogurts and is believed to potentially have health benefits. We determined the immune modulatory effects of ST285 to human peripheral blood mononuclear cells and show that it has an array of anti-inflammatory immune-modulatory properties. ST285 decreases mRNA expression IL-18, IFN receptor, CCR5, CXCL10, TLR-1, TLR-2, TLR-4, TLR-8, CD14, CD40, CD86, C3, GATA3, ITGAM, IRF7, NLP3, LYZ, TYK2, IFNR1, and upregulates IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-23, IFN-γ, TNF-α, CSF-

2. No changes to mRNA expression were noted with IFNA1, IFNB1, IL-4, IL-5, IL-13, CCL2, CCL5, CCL8, CCR4, CCR8, CXCR3, TLR-3, TLR-5, TLR-6, TLR-9, CD4, CD80, FOXP3, STAT3, CD40LG, HLA-A, HLA-E, RORC.

Chapter 4

Chapter 4

Immune modulatory effects of probiotic Streptococcus thermophilus in human monocytes

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ABSTRACT

Ingesting probiotics contributes to the development of a healthy microflora in the GIT with established benefits to human health. Some of these beneficial effects may be through modulating of the immune system and probiotics have become more common in the treatment of many inflammatory and immune disorders. We demonstrate a range of immune modulating effects of *Streptococcus thermophilus* by human monocytes, including, decreased mRNA expression of IL-1R, IL-18, IFNγR1, IFNαR1, CCL2, CCR5, TLR-1, TLR-2, TLR-4, TLR-5, TLR-6, TLR-8, CD14, CD86, CD4, ITGAM, LYZ, TYK2, IFNR1, IRAK-1, NOD2, MYD88, ITGAM, SLC11A1, and, increased expression IL-1α, IL-1β, IL-2, IL-6, IL-8, IL-23, IFNγ, TNFα, CSF-2. Routine administration of *Streptococcus thermophilus* in fermented dairy products, and their consumption may be beneficial to the treatment/management of inflammatory and autoimmune diseases.

Keywords: Probiotics; microbiome; Lactic acid bacteria; *Streptococcus thermophilus*; Peripheral blood mononuclear cells; Monocyte; RNA; Innate immune response; Adaptive immune response; Inflammation

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This chapter assesses changes in immune modulation caused by probiotic *Streptococcus thermophilus* in human monocytes. This paper is also one of the first to analyze 84 different genes related to human innate and adaptive immune responses simultaneously using *S. thermophilus* ST285 to modulate monocyte cells.

1. Introduction

The human body and, in particular, the gastrointestinal tract (GIT) hosts a variety of microbial populations collectively referred to as the microbiome (Dargahi et al., 2018). The GIT microbiome plays a fundamental role in the maintenance of a healthy immune system (Dargahi et al., 2018; Dargahi et al., 2017), and any disruption to the microbiome can lead to serious ill health effects (A. J. Stagg et al., 2004; J. Stagg et al., 2011). In order to maintain a healthy microbiome, regular ingestion of probiotic supplements either as capsules or in fermented dairy products has been suggested. These practices have led to various improved health outcomes and treatment of ill health, such as infections, constipation and diarrhoea (Dargahi et al., 2018; Hardy et al., 2013; Kinross, Roon, Holmes, Darzi, & Nicholson, 2008).

The majority of probiotics belong to the lactic acid bacteria (LAB) family; gram positive lactic acid producing microorganisms that include several genera such as bifidobacteria, lactobacilli streptococci and enterococci (Dargahi et al., 2018). The small and large intestines are highly populated with these microorganisms (Fink et al., 2007; Maassen et al., 2000; Michałkiewicz et al., 2003), and are routinely supplemented in foods as live strains due to their established beneficial effects to human health (Asarat, Apostolopoulos, et al., 2015; Asarat et al., 2016; Asarat, Vasiljevic, et al., 2015; Dargahi et al., 2018, 2019; Dargahi et al., 2017; Fink et al., 2007; Salazar et al., 2009). *Streptococcus* species such as exopolysaccharide-producing strains of *Streptococcus thermophilus* (ST) (Di Caro et al., 2005; Purwandari & Vasiljevic, 2009; Salazar et al., 2009) are amongst those consumed. ST is used for fermentation of milk products and is recognized as an important species for its health benefits (Hols et al., 2005; Uriot et al., 2017). In fact, ST and *L. brevis* synergistically display health benefits which are well established, also, ST is one of the bacteria in the VSL#3 probiotic mixture, which has been applied for the treatment of inflammatory conditions (C. Dai et al., 2013; Mennigen et al., 2009). Probiotics also interact with the immune system where they exhibit immunomodulatory and anti-inflammatory effects (Han et al., 2008; J. Stagg et al., 2011; Vliagoftis et al., 2008).

Use of probiotic bacteria can increase the abundance of and concurrently modulate immune cells including B, T helper (Th)-1, Th-2, Th-17 and regulatory T (Treg) cells. This in turn, directly influences human health and modulates pathologies of immune/autoimmune

diseases (Dargahi et al., 2018, 2019; Dargahi et al., 2017). In fact, primary macrophages co-cultured with ST bacteria have been shown to increase production of anti-inflammatory IL-10 and pro-inflammatory IL-12 cytokines (Latvala et al., 2008). ST1275 and *Bifidobacterium longum* BL536 induce expression of high levels of transforming growth factor (TGF)-beta, a key factor in the differentiation of Treg and Th-17 cells by bulk peripheral blood mononuclear cell (PBMC) cultures (Donkor, Ravikumar, et al., 2012a). Probiotic bacteria, however, can only confer these benefits through interaction with specific immune cells, primarily antigen presenting cells (APC), which include monocytes, as mediators between bacteria/foreign agents and the immune system's effector adaptive immune cells (Gaudino & Kumar, 2019).

In line with these findings, and as noted in chapter 2 that ST1342, ST1275 and ST285 modulated U937 monocyte cell line by increasing IL-4, IL-10, GM-CSF and CXCL8 production. In addition the cell surface marker expression of CD11c, CD86, C206, CD209, MHC-1 were upregulated, suggesting that ST bateria has an influence on the immune system (Dargahi et al., 2018). Furthermore, as shown in chapter 3, ST285 exerted an array of anti-inflammatory immune-modulatory properties to human PBMC [manuscript submitted]. In particular, ST285 decreased mRNA expression of IL-18, IFNγR1, CCR5, CXCL10, TLR-1, TLR-2, TLR-4, TLR-8, CD14, CD40, CD86, C3, GATA3, ITGAM, IRF7, NLP3, LYZ, TYK2, IFNR1, and upregulated IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-23, IFNγ, TNFα, CSF-2 [manuscript submitted]. The data demonstrated a predominant anti-inflammatory profile exhibited by ST285. Due to the role of monocytes and their progeny in initiation and maintenance of both innate and adaptive immune responses, we now show immune modulatory properties of ST285 on monocytes from healthy blood donors. The data paves the way for further work to determine the effects of ST285 in inflammatory disease models *in vitro* and *in vivo*, such as multiple sclerosis, inflammatory bowel disease and allergies.

2. Material and methods

2.1. Bacterial strains

Pure bacterial cultures of ST285 were obtained from Victoria University culture collection (Werribee, VIC, Australia). Stock cultures were stored in cryobeads at −80° C. Prior to each experiment the cultures were propagated in M17 broth (Oxoid, Denmark) with 20 g/L lactose and incubated at 37° C under aerobic conditions. In order to confirm gram-positivity and assess purity, morphology and characteristics, bacteria were cultured in M17 agar (1.5 % w/v agar) with 20 g/L lactose (Oxoid, Denmark) as well (Dargahi et al., 2018).

2.2. Preparation of live bacterial suspensions

Prior to experiments bacteria medium was prepared and autoclaved at 121° C for 15 minutes (mins) and bacterial cultures were grown 3 times in M17 broth with 20 g/L lactose, at 37° C aerobically for 18 hours (hr) with a 1 % inoculum transfer rate (Husson-Kao et al., 2000) at 37-42° C (Purwandari & Vasiljevic, 2009). Bacteria were harvested during stationary growth phase on the day of experiment, centrifuged (6000×g) for 15 min at 4° C, followed by washing twice with phosphate-buffered saline (PBS) (Invitrogen, Pty Ltd. Australia) and resuspended in the Roswell Park Memorial Institute (RPMI) 1640 culture media (Invitrogen, Pty Ltd. Australia), which constituted the live-bacteria suspensions.

2.3. Enumeration of bacterial cells

Prior to co-culturing with PBMC, bacterial strains cultured in M17 broth, were centrifuged and transferred into PBS (Invitrogen, Pty Ltd. Australia), adjusted to a final concentration of 10^8 colony forming units (cfu)/ml by measuring the optical density at 600 nm. Then washed twice with PBS and resuspended in RPMI 1640 (Invitrogen, Pty Ltd. Australia) (Dargahi et al., 2018).

2.4. Isolation of monocytes from buffy coat

Buffy coats were received from the Australian Red Cross blood bank in Melbourne, and PBMC were isolated using standard Ficoll-Paque density gradient centrifugation method as previously described (Asarat, Apostolopoulos, et al., 2015). PBMC cells were resuspended at ~5 x 10^8 cells/mL in adequate amount of Dulbecco's phosphate-buffered saline, D-PBS (D-PBS without Ca^{++} and Mg^{++}) supplemented with 2% FBS and 3 mM cell culture grade EDTA (Life Technologies; Thermofisher) prior to monocyte isolation. Monocytes were isolated using the EasySep Human Mono Isolation Kit (STEMCELL technology, Canada) (Marzaioli et al., 2017). Isolation method involved the use of immunomagnetic negative selection method targeting $CD16^+$ monocytes, excluding non-monocyte cells, and platelets, yielding highly pure $CD14^+CD16^-$ monocytes. As such the unwanted cell populations are labelled with specific cell surface marker antibodies and magnetic particles, and removed following separation by using an EasySep™ magnet (STEMCELL technology, Canada) according to manufacturer's instructions (Marzaioli et al., 2017). Monocyte cells were added into a fresh tube, checked for viability and purity.

2.5. Stimulation of monocytes with ST285

Monocytes (~3-5x 10^7 cells) isolated from three different donors were resuspended in RPMI 1640 media supplemented with 10% heat-inactivated FBS (Invitrogen, Pty Ltd. Australia), 1% antibiotic-antimycotic solution and 2 mM L-glutamine in cell culture flasks, into which 5x10^8 ST285 bacteria were added. Monocytes (~3-5x 10^7 cells) minus the ST285 bacteria were used as a control and incubated at 37° C, 5 % CO_2 for 24 hrs (Dargahi et al., 2018). In previous studies we demonstrated that 24 hrs co-culture was optimal for stimulation of the U937 monocyte cell line, and all incubations described herein were for 24 hrs (Dargahi et al., 2018). Monocytes were harvested post incubation period, snap frozen and stored at -80° C.

2.6. RNA extraction from monocytes

Total RNA was extracted from stimulated and unstimulated monocytes using the RNeasy® mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, monocytes were harvested using centrifugation, supernatants were removed and RNA

was extracted from each pellet by resuspending pellet in lysis buffer supplemented with β-mercaptoethanol for cell disruption. Monocytes were lysed and each cell lysate was homogenized by passing through Qia-shredder columns (Qiagen, Hilden, Germany). Each monocytes lysate was then mixed 1:1 with 70% ethanol (equal volume) and were transferred onto RNeasy mini-spin columns. DNA was eliminated using DNase digestion/ treatment using RNase-Free DNase Set (Qiagen, Hilden, Germany) by adding it directly onto the columns. The RNA Integrity Number (RIN) of all RNA samples were determined using an Agilent 2100 Bioanalyzer and Agilent RNA 6000 nano kit (Agilent Technologies, Santa Clara, CA, USA). A minimum RIN of 7.5 was used as the standard for inclusion in the gene expression study. Subsequently, the concentration of each individual monocyte RNA sample was quantified using a Qubit RNA BR Assay (Invitrogen, Pty Ltd. Australia).

2.7. Assessing changes in the expression of genes associated with innate and adaptive immunity

Using RT first strand kit (Qiagen, Hilden, Germany), adequate aliquots of each RNA sample was reverse-transcribed to produce complementary DNA (cDNA) according to the manufacturer's instructions. Quantitative real-time polymerase chain reaction (qRT-PCR) was carried out by using the 'Human Innate and Adaptive immune Response' kit (Qiagen, Hilden, Germany) to assess the expression of genes/mRNA. Using a CFX Real-Time touch PCR System thermo-cycler (Biorad, Melbourne Australia) and Qiagen prescribed cycle, the relative gene/mRNA expression of ST285-treated monocytes were analyzed in contrast to control untreated monocytes. The RT^2 qPCR innate and adaptive immune response arrays targeted a set of 84 innate and adaptive immune-related genes, five housekeeping genes, an RT control, a positive PCR control, and a human genomic DNA contamination control (Kaur, Casey, & Pichichero, 2016). Relative gene expression was calculated using the Qiagen webportal PCR array data analysis web-based software (Qiagen, Germany). Differential expression (up and down regulation) of the genes were identified using the criteria of a > 2.0-fold increase/decrease in gene expressions in treated monocytes in comparison with those genes in control monocyte cultures.

2.8. Data analysis

The Delta-Delta CT (ΔΔCT) method was used for calculating fold-changes (Livak & Schmittgen, 2001). Fold-regulation represents fold-change results in a biologically meaningful way. In these RT2 profiler PCR array results, fold-change values >1, indicate a positive (or an up-) regulation. Actually, in the case of genes which are upregulated, the fold-regulation is equivalent to the fold-change. Fold-change rates <1 indicate a negative (or a down) regulation. In the case of negative values, the fold-regulation is actually the negative inverse of the fold-change (Souza et al., 2016; Z. Yang et al., 2015; T. Zhang et al., 2017). Data related to changes in the expression of the genes were estimated using Qiagen RT^2 profiler data analysis webportal that uses the ΔΔCT method in calculating fold-changes. The raw CT values were uploaded to the Qiagen data analysis webportal with the lower limit of detection set for 35 cycles and 3 internal controls. For controls, RT efficiency, PCR array reproducibility, and genomic DNA contamination were assessed to ensure all arrays successfully passed all the control check-ponits. Normalization of the raw data was done by using the incorporated housekeeping genes (HKG) panel. Then using the ΔΔCT method, both housekeeping gene references and controls (untreated monocytes in RPMI) were evaluated to determine relative expression of mRNA.

2.9. Statistical analysis

The p values were calculated by the use of a Student's *t-test* of the Triplicate 2^ (- Delta CT) [(2^-ΔCT)] values for each gene in treatment groups (monocyte co-cultured with ST) and the control group (monocyte in RPMI media) (Z. Yang et al., 2015; T. Zhang et al., 2017).

3. Results

Among 84 genes evaluated, expression of 30 genes were significantly altered with over 2.0 fold up or down regulations in monocyte samples (n=3) following co-culture with ST285 compared to control (Figure 1).

A

Layout	01	02	03	04	05	06	07	08	09	10	11	12
A	APCS 1.05 C	C3 -1.67	CASP1 1.37	CCL2 -24.33	CCL5 1.72	CCR4 1.08 B	CCR5 -11.54	CCR6 1.25 B	CCR8 -1.09 B	CD14 -34.08	CD4 -7.14 B	CD40 1.08
B	CD40LG -1.01 B	CD80 -1.79	CD86 -10.16	CD8A -3.14 B	CRP 1.05 C	CSF2 63.82 A	CXCL10 1.09	CXCR3 -1.45 B	DDX58 -1.74	FASLG -1.30 A	FOXP3 -2.21 A	GATA3 -1.23 B
C	HLA-A -1.70	HLA-E -1.35	ICAM1 -1.17	IFNA1 -1.59 B	IFNAR1 -2.53	IFNB1 -1.14 B	IFNG 29.33 A	IFNGR1 -5.65	IL10 -1.12	IL13 -1.05 B	IL17A 1.05 C	IL18 -7.63 A
D	IL1A 4.66	IL1B 9.83	IL1R1 -2.11	IL2 -1.35 B	IL23A 11.79	IL4 1.33 B	IL5 1.10 B	IL6 45.23	CXCL8 9.18	IRAK1 -2.27	IRF3 -1.23	IRF7 -1.14
E	ITGAM -3.60 A	JAK2 -1.57	LY96 -1.81	LYZ -25.78	MAPK1 -1.68	MAPK8 -1.18	MBL2 1.05 C	MPO -3.71 B	MX1 -1.46	MYD88 -2.98	NFKB1 1.41	NFKBIA -1.23
F	NLRP3 -1.38	NOD1 -1.51 A	NOD2 -2.35	RAG1 1.01 B	RORC -1.29 B	SLC11A1 -4.70	STAT1 1.23	STAT3 -1.35	STAT4 1.14	STAT6 -1.31	TBX21 1.79	TICAM1 1.12
G	TLR -3.62	TLR2 -3.05	TLR3 -1.74 B	TLR4 -3.96	TLR5 -2.45 A	TLR6 -2.13 A	TLR7 -1.40 B	TLR8 -2.51	TLR9 -1.36 B	TNF 8.99	TRAF6 -1.42	TYK2 -2.19

B

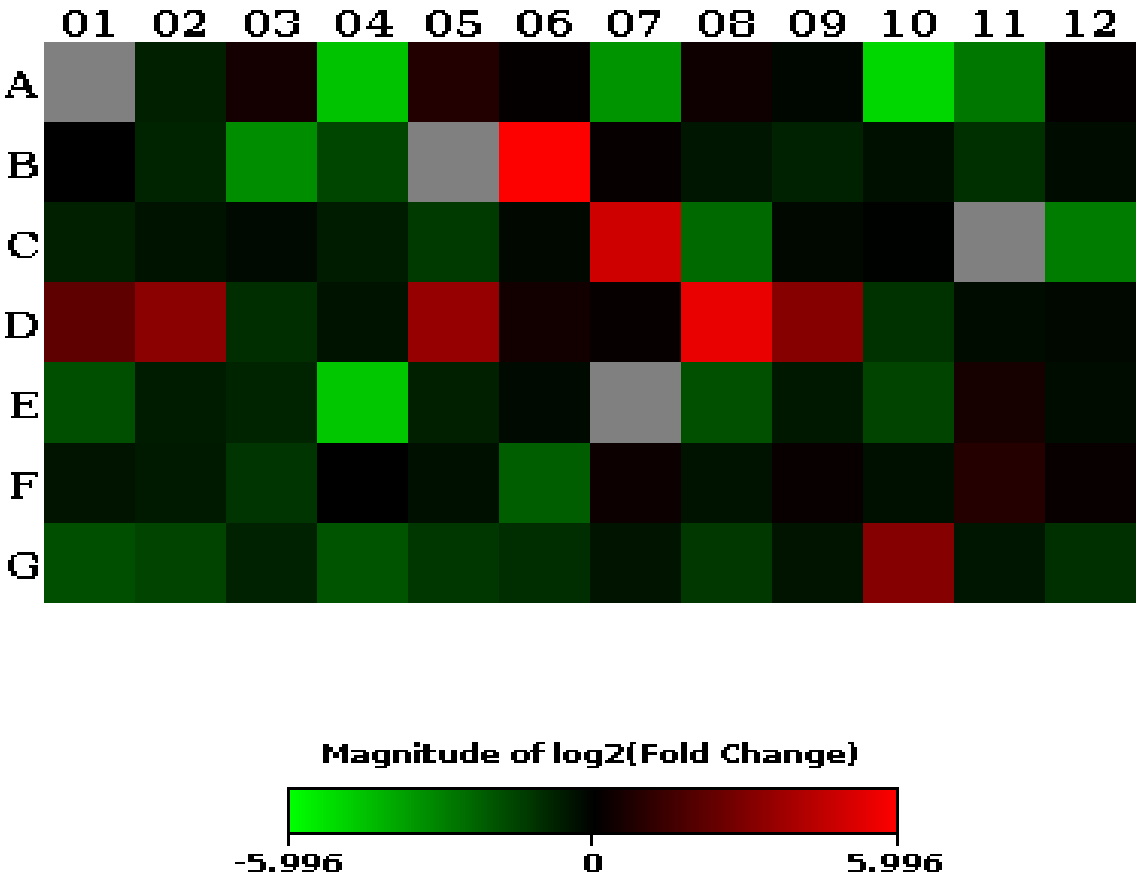

Figure 1. Effects of co-culturing ST285 with monocytes (n=3) on gene/RNA expression compared to control monocytes after 24 hrs. (A) All 84 genes are shown including those with significant high up/down regulated genes (more than 2-fold) and those with no significant change (less than 2-fold). The housekeeping genes (HKG) panel and other genes used for normalization of the raw data are not presented. In case of no letter or comments, the expression of gene/s is relatively high in both the test and control group (threshold cycle (CT) is <30). Letter A specifies the gene's average threshold cycle to be reasonably high (> 30) in either the treated samples or the controls and relatively low (< 30) in the other/opposite sample. Thus, in case of presenting fold changes with letter A, the estimate fold change may be an underestimate. Letter B suggests a reasonably high (> 30) gene's average threshold cycle that means a low level of average expression of relevant gene, in both test/treated samples and untreated control samples, and the p-value for the fold-change might be either relatively high ($p > 0.05$). Thus, in case of presenting fold changes with letter B, the estimate fold change may be slightly overestimate or unavailable. Letter C indicates that that gene's average threshold cycle is either not determined or greater than the defined default 35 cut-off value, in both test/treated samples and control samples, suggesting that its expression was not detectable, resulting in the fold-change values being un-interpretable (Abubaker et al., 2013; Gaston et al., 2017; Goad et al., 2017) . (B) Presentation of data as a heatmap of average gene/RNA expressions of monocytes (n=3) co-cultured with ST285, compared to control. Green represents down regulated genes to red represents upregulated genes.

3.1. ST285 alters cytokine gene expression levels of monocytes

3.1.1. ST285 causes upregulation of IL-1α, IL-6 and IL-23 and downregulation of IL-1R1

IL-1α was upregulated 4.66 ± 0.7 fold, IL-1β was upregulated 9.83 ± 0.49 fold, IL-6 was upregulated 42.23 ± 0.32 fold and IL-23α was upregulated 3.8 ± 1.0 fold (Figure 2). IL-1R1 was downregulated 2.11 ± 0.36 fold (Figure 2). Neither IL-17A nor IL-2, and IL-10 were altered following monocyte co-cultured with ST285.

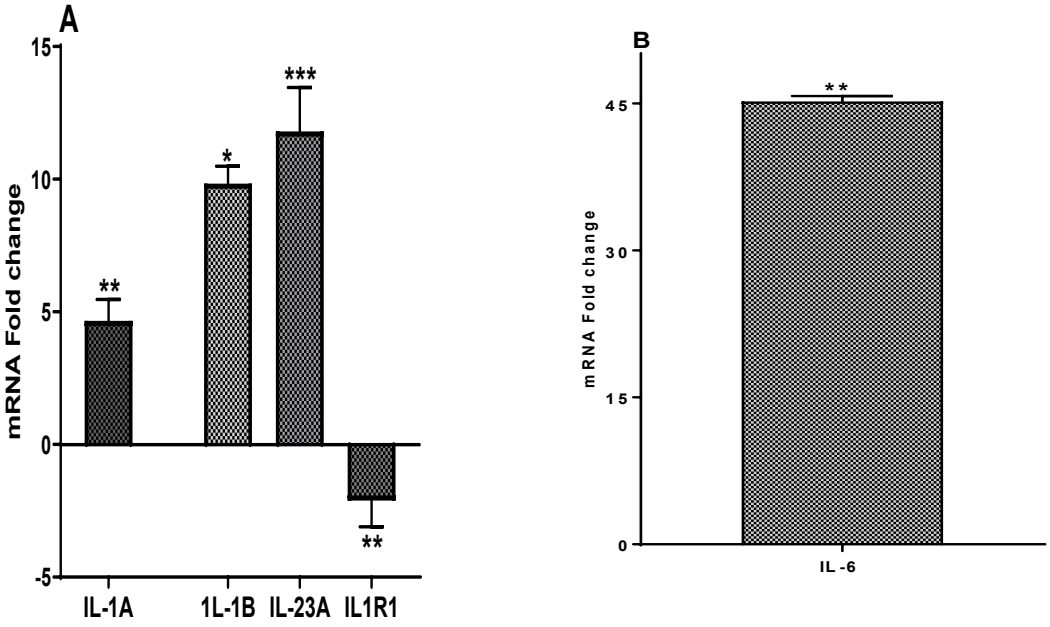

Figure 2. (A) IL-1α, IL-1β, IL-23α, IL-1R1 and (B) IL-6, mRNA fold change following 24 h co-culture of ST285 with monocytes (n=3), compared to control monocytes. The innate and adaptive RT^2 gene profiler arrays were used to determine changes in gene expression. Symbols represent *p* value for Tukey Test (One way ANOVA) where ** $p < 0.04$ and *** $p < 0.02$.

3.1.2. Modulation of pro-inflammatory cytokines

ST285 induced upregulation of IFNγ (29.33 ± 0.26 fold) (Figure 3A). IL-18 a Th1 inducing pro-inflammatory cytokine was downregulated (7.63 ± 0.37 fold) (Figure 3A). In addition, IFNγR1, a transmembrane protein which interacts with IFNγ, was also downregulated 5.65 ± 0.05 fold and IFNAR1 (involved in defence against viruses) was downregulated 2.53 ± 0.05 fold (Figure 3A). Tumor-necrosis factor-alpha (TNFα), which is important in the defense against bacterial infections, and in acute phase reactions was upregulated 8.99 ± 1.06 fold

(Figure 3). Gene expressions of other cytokines, IFNA1, IFNB1, IL-4, IL-5, IL-12 and IL-13 were not significantly altered.

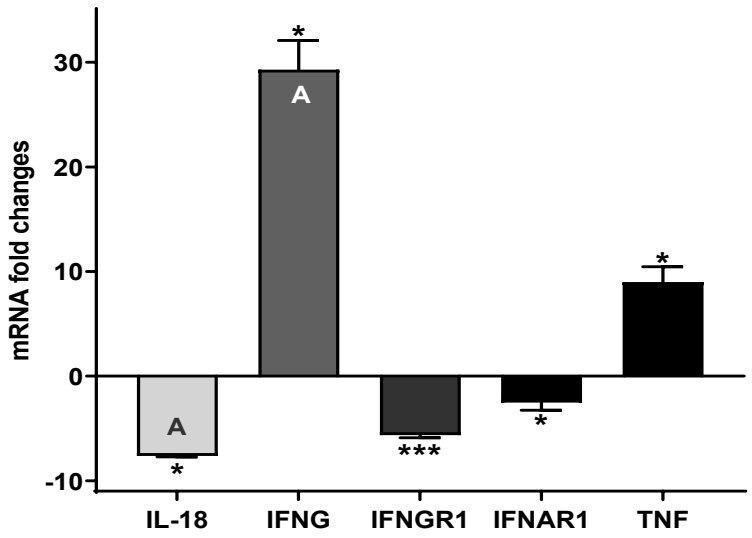

Figure 3. IL-18, IFN-γ, and IFN-γ−R1, IFN-α−R1 and TNF mRNA mRNA fold change following 24 h co-culture of ST285 with monocytes (n=3), compared to control monocytes. The innate and adaptive RT^2 gene profiler arrays were used to determine changes in gene expression. Symbols represent *p* value for Tukey Test (One way ANOVA) where * $p < 0.05$ and *** $p < 0.02$.

3.2. ST285 alters chemokine gene expression levels of monocytes

CCR5 a Th1 marker involved in immune response and CCL2 (MCP-1) involved in humoral immunity were down regulated 11.54 ± 0.23 and 24.33 ± 1.44 fold respectively (Figure 4). Chemokine (CXCL8, IL-8), important in the innate immune system, stimulates chemotaxis, was upregulated 9.18 ± 0.26 fold following ST285 co-culture with monocyte cells (Figure 4). However, no significant differences were noted for gene expressions of other chemokines, including CXCL10 (INP10), CCL5 (RANTES), CCL8, CCR4, CCR8 and CXCR3 following monocytes' exposure to ST285.

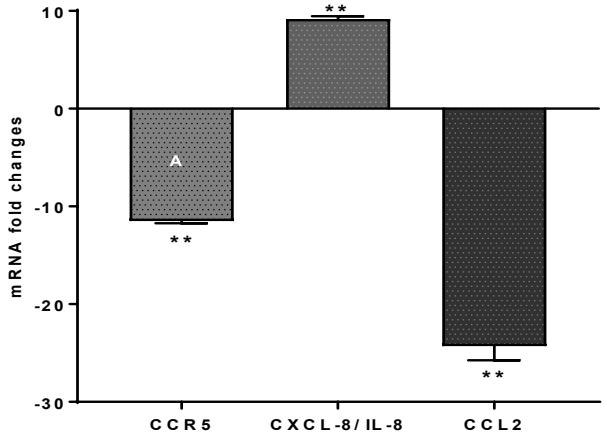

Figure 4. CCR5, CXCL8 (IL-8) and CCL2 mRNA fold change following 24 h co-culture of ST285 with monocytes (n=3), compared to control monocytes. The innate and adaptive RT^2 gene profiler arrays were used to determine changes in gene expression. Symbol represents *p* value for Tukey Test (One way ANOVA) where ** $p < 0.04$.

3.3. Significant upregulation of colony stimulating factor mRNA expression levels

Colony-stimulating factor (CSF)-2 which enables cell proliferation and differentiation of cells, was significantly increased by 63.82 ± 1.12 fold (Figure 5) after co-culturing monocytes with ST285 bacteria.

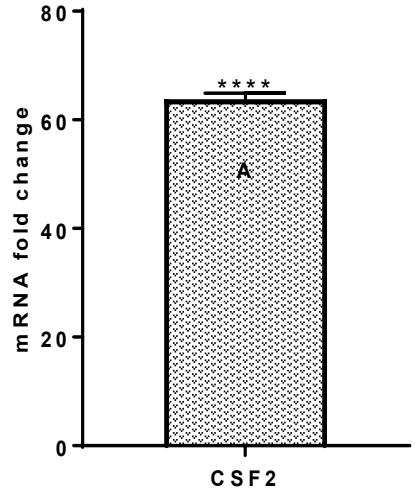

Figure 5. CSF-2, mRNA fold change following 24 h co-culture of ST285 with monocytes (n=3), compared to control monocytes. The innate and adaptive RT^2 gene profiler arrays were used to determine changes in gene expression. Symbol represents *p* value for Tukey Test (One way ANOVA) where **** $p < 0.01$.

3.4. ST285 alters Toll like receptor gene expression levels of monocytes

TLR (toll like receptor)-1, TLR-2, TLR-4, TLR-5, TLR-6 and TLR-8 are part of the innate immune response and involved in the defense response to bacteria. Monocytes co-cultured with ST285 induced significant differential downregulation of TLRs; TLR-1 (-3.63 ± 0.14), TLR-2 (-3.05 ± 0.36 fold), TLR-4 (-3.96 ± 0.16 fold), TLR-5 (-2.45 ± 0.23 fold), TLR-6 (-2.13 ± 0.23 fold), and TLR-8 (-2.51 ± 0.12 fold) (Figure 6). However, changes to TLR-3 and TLR-9 were not significant.

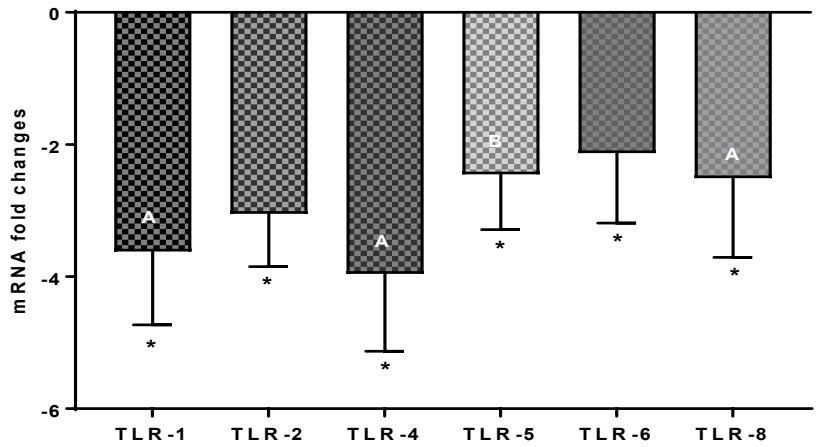

Figure 6. TLR-1, TLR-2, TLR-4, TLR-5, TLR-6 and TLR-8, mRNA fold change following 24 h co-culture of ST285 with monocytes (n=3), compared to control monocytes. The innate and adaptive RT^2 gene profiler arrays were used to determine changes in gene expression. Symbol represents *p* value for Tukey Test (One way ANOVA) where * $p < 0.05$.

3.5. Cell surface markers CD14, CD86 and CD4 mRNA expression levels

Expression of the monocyte cell surface markers CD14 and CD86 were significantly downregulated 34.08 ± 3.42 and 10.16 ± 0.14 fold, respectively (Figure 7). CD4 is expressed by Th cells, monocytes, macrophages (MQ), and dendritic cells (DCs), was downregulated 7.14 ± 0.41 fold. No significant change was observed in the expression of CD8A, CD40, CD80, GATA3, FOXP3, STAT3, CD40LG (TNFSF5), HLA-A, HLA-E and RORC genes.

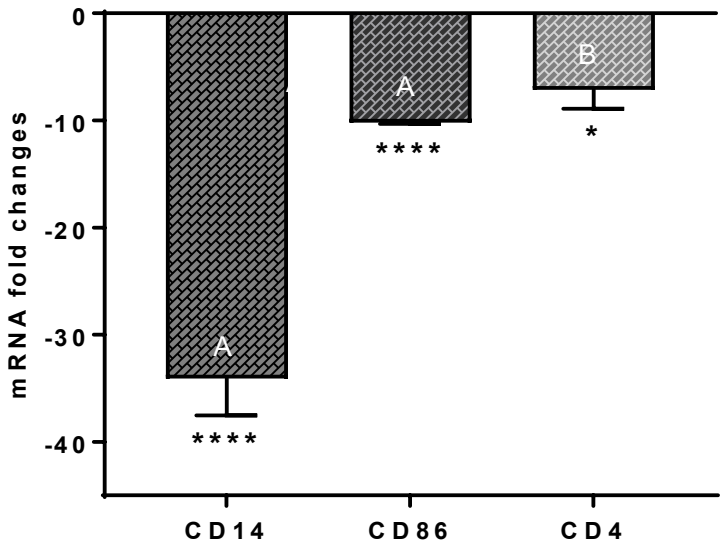

Figure 7. CD14, CD86 and CD4 mRNA fold change following 24 h co-culture of ST285 with monocytes (n=3), compared to control monocytes. The innate and adaptive RT^2 gene profiler arrays were used to determine changes in gene expression. Symbols represent *p* value for Tukey Test (One way ANOVA) where * $p < 0.05$ and *** $p < 0.02$.

3.6. Changes to other innate and adaptive molecules, mRNA expression levels

Altered expression levels are noted in other genes following ST285 co-culture with monocytes. Significant downregulation of the following genes were noted: TYK2 (-2.19 ± 0.37), IRAK-1 (-2.27 ± 0.45), NOD2 (-2.35 ± 0.04), MYD88 (-2.98 ± 0.23), ITGAM (-3.6 ± 0.23), MPO (3.71 ± 0.12), SLC11A1 (-4.7 ± 0.17) (Figure 8A), and LYZ (25.78 ± 0.36) (Figure 8B). Other immune markers including FASLG (TNFSF6), ACTB, GATA3, complement component (C)-3, CRP, IFNAR1, JAK2, IL-1R1, MAPK8 (JNK1), IRF3, MBL2, NLRP3, NFKB1, MX1, ICAM1, MBL2, NOD1 (CARD4), DDX58 (RIG-I), RAG1, TICAM1 (TRIF) and IRF7 showed no significant mRNA gene changes in the levels of their expression.

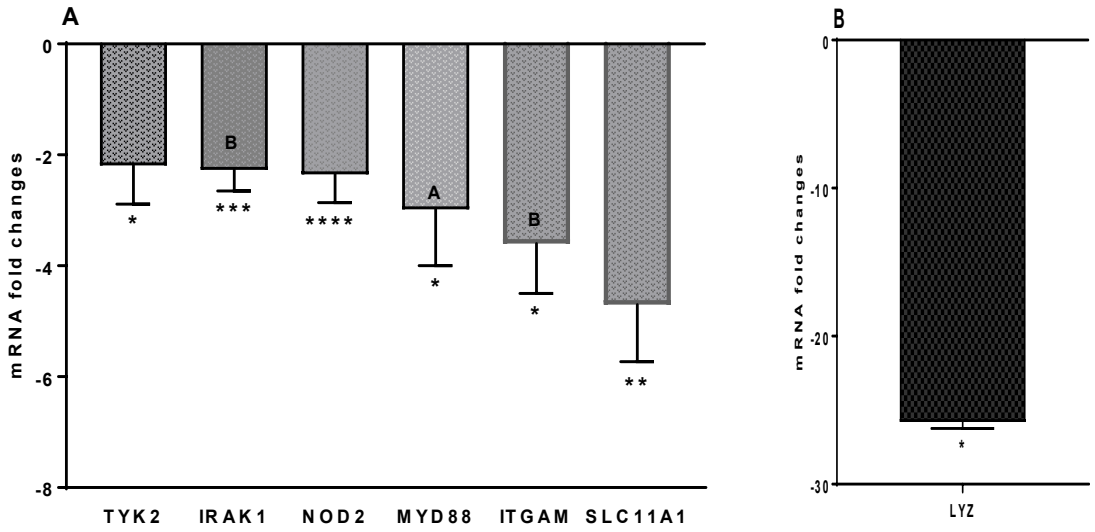

Figure 8. (A) TYK2, IRAK1, NOD2, MYD88, ITGAM, SLC11A1 and (B) LYZ and GATA3, mRNA fold change following 24 h co-culture of ST285 with monocytes (n=3), compared to control monocytes. The innate and adaptive RT^2 gene profiler arrays were used to determine changes in gene expression. Symbols represent *p* value for Tukey Test (One way ANOVA) where * $p < 0.05$, ** $p < 0.04$, *** $p < 0.02$ and **** $p < 0.01$.

4. Discussion

ST285 co-cultured with human monocytes resulted in significant changes to 30 genes associated with different immune responses of the innate and adaptive immunity compared to control. In particular, mRNA gene expression of IL-1R, IL-18, IFNγR1, IFNαR1, CCL2, CCR5, TLR-1, TLR-2, TLR-4, TLR-5, TLR-6, TLR-8, CD14, CD86, CD4, ITGAM, LYZ, TYK2, IFNR1, IRAK-1, NOD2, MYD88, ITGAM, SLC11A1 are downregulated. Whilst ST285 increases mRNA expression of IL-1α, IL-1β, IL-1α–R, IL-6, IL-8, IL-23, IFNγ, TNFα and CSF-2. These results were broadly in agreement with our previous findings showing a predominant anti-inflammatory profile by human PBMC upon co-culture with ST285 [manuscript submitted]. Likewise, our previous data showed a similar trend for a number of cytokine, chemokine and cell surface markers for three different ST bacteria to human U937 monocyte cell line, where ST285 was most effective (Dargahi et al., 2018).

4.1. ST285 induces IL-1α and IL-6 and downregulates IL-1R1

IL-1α secreted by DCs and MQs, usually initiates Th2 differentiation, while preventing polarization of Th1 cells (Ben-Sasson et al., 2009). IL-6 is produced by activated immune cells including monocytes/MQs (Choy & Rose-John, 2017). IL-1α and IL-6 are significantly upregulated, whereas IL-1R1 (CD121a), a key mediator associated with several inflammatory and immune responses is downregulated in monocytes after exposure to ST285. This is in accord to PBMCs co-cultured with ST285 [manuscript submitted] and U937 monocyte cell line co-cultured with ST285 (Dargahi et al., 2018).

IL-6 acts as both pro- and anti-inflammatory cytokine (Scheller, Chalaris, Schmidt-Arras, & Rose-John, 2011) and its anti-inflammatory roles are associated with its inhibitory effects on IL-1, TNF-α, and activation of IL-10 and IL-1Ra (Garbers et al., 2012; Scheller et al., 2011). On the other hand, the inhibitor of NF-κB kinase (IKK) governs IL-6 mRNA stability (through phosphorylation of regnase-1), in response to IL-1R/TLR stimulation (H. Iwasaki et al., 2011). As such, *Lactobacillus paracasei* has been shown to reduce IL-6 production via prevention of NF-κB activation to THP-1 cell line (Sun et al., 2017) which is in contrast with our findings. Whereas, the surface-associated exopolysaccharide (EPS) extracted from *L. paracasei* DG showed immune-stimulating properties to human monocytic cell line THP-1 by increasing TNF-α and IL-6 gene expression which is in line with current findings (Balzaretti et al., 2017). In addition, human monocytes and monocyte-derived DCs co-cultured with *Veillonella parvula*, *Escherichia (E.) coli*, *B. adolescentis* and *L. plantarum* strains, stimulated high level of IL-6 upon exposure to *V. parvula* and *E. coli* but not *B. adolescentis* and *L. plantarum* (Karlsson, Larsson, Wold, & Rudin, 2004).

IL-1β is secreted by monocytes and activated MQs, is involved in regulating immune and inflammatory responses to bacterial infections and injuries, hence its role in innate immunity (Lopez-Castejon & Brough, 2011). IL-1β is upregulated by ST285 co-cultured with monocytes, which is similar to ST285 stimulation of PBMC (Dargahi et al., 2019), although ST285 did not stimulate IL-1β in the U937 monocyte cell line (Dargahi et al., 2018). However, in other studies *L. paracasei* cultured with THP-1 cell line either before LPS treatment or together with LPS, reduced IL-1β secretion (Sun et al., 2017). Consumption of a mixed probiotic or a conventional yogurt with equal *S. thermophiles*, *L. bulgaricus* and surplus *L.*

casei DN114001, induces high IL-1β production by *ex vivo* cultured monocytes following LPS and phytohaemmaglutinin stimulation (Meyer, Elmadfa, Herbacek, & Micksche, 2007).

The increased expression of IL-1α and IL-6, suggests the role of ST285 in the induction of immune responses required for acute phase (including MQs differentiation, B cell maturation, and activation of Th2 differentiation and prevention of Th1 polarization). A decrease in IL-1R1 gene expression could highlight the role of ST285 as a brake that controls the pro-inflammatory roles of both IL-6 and IL-1α.

4.2. ST285 changes expression of cytokines involved in inflammation and defence against bacteria

IL-18 is associated with severe inflammatory responses and plays a role in inflammatory and autoimmune disorders. Monocytes co-cultured with ST285 significantly reduced the gene expression of IL-18, which is in agreement with our recent study of ST285 co-cultured with PBMC [manuscript submitted], suggesting an anti-inflammatory role for ST285 bacteria. IFN-γ is an important activator of MQs, is secreted by monocytes, NK and NKT cells, and is critical for functional innate and adaptive immune responses against viruses, some bacterial and protozoa infections (Schoenborn & Wilson, 2007b). Monocytes co-cultured with ST285 show increased gene expression of IFN-γ suggesting an anti-bacterial response. Similarly, blood monocytes from healthy individuals who ingested either a probiotic mixed of *S. thermophiles*, *L. bulgaricus* and surplus *L. casei* DN114001 or a conventional yogurt containing same probiotic mixture, showed increased production of IFN-γ upon co-culturing monocyte cells *ex vivo* with LPS and phytohaemmaglutinin (Meyer et al., 2007). In another study, the effects of *L. casei* Shirota on monocyte was shown indirectly; as depletion of monocytes from PBMC co-cultured with *L. casei* Shirota was associated with an absence of IFN-γ and other cytokines demonstrating the importance of monocytes against bacterial challenge (Shida et al., 2006b). Similarly, *L. plantarum* alone and mixed *L. plantarum* and *Helicobacter pylori* added to monocytes (and lymphocytes) resulted in the production of high levels of IFN-γ with *L. plantarum* alone, compared to the mixed cultures (Wiese et al., 2012).

TNFα, a pro-inflammatory cytokine is required against bacterial infections and is involved in activating and recruiting T and B cells in the initiation of adaptive immune responses. We show upregulation of TNFα when human monocytes are co-cultured with

ST285, in agreement with observations with PBMCs [manuscript submitted] and the U937 monocyte cell line (Dargahi et al., 2018). Isolated human monocytes and monocyte-derived DCs co-cultured with *V. parvula*, *E. coli*, *B. adolescentis* and *L. plantarum* strains, similarly showed higher levels of TNFα (Karlsson et al., 2004). In addition, EPS from *L. paracasei* DG also induced increased TNFα gene expression by THP-1 monocyte cell line (Balzaretti et al., 2017). Although, *L. paracasei* itself decreased TNF-α production by THP-1 cell line via inhibition of NF-κB activation (Sun et al., 2017). Similarly, *L. plantarum* genomic DNA reduced the production of TNFα in THP-1 monocyte cells (Hee Kim et al., 2012). Additionally, the importance of monocytes in phagocytosis was shown by using monocyte-depleted-PBMC in co-culture with *L. casei* Shirota, which led to no secretion of TNFα (Shida et al., 2006b).

IFNAR1 is a membrane protein and a receptor for both IFNα and IFNβ associated with defence against viruses. IFNAR1 signalling is involved in production of pro-inflammatory cytokines (Goritzka et al., 2014), as such that IFNAR1 knockout mice demonstrate reduced pro-inflammatory chemokines and cytokines (Goritzka et al., 2014). IFNAR1 is significantly downregulated by monocytes following co-culture with ST285 supporting an anti-inflammatory role for ST285. Upregulation of IFNγ, IL-1β and TNFα by monocytes following ST285 co-culture suggests a powerful defense against invading pathogens induced by ST285 that could be advantageous in defense against virus infection and tumours. Of interest, in spite of the upregulation of IFNγ, IL-1β and TNFα, considering collective down regulation of IFNAR1, IFNGR1, IL-18, our results might reveal an antagonistic effect of ST285 on pro-inflammatory IFNγ, IL-1β and TNFα responses which may lead to an overall downstream tolerance, and even an ultimate anti-inflammatory outcome.

4.3. ST285 activates mRNA expression of CXCL8 and downregulates CCR5 and CCL2

IL-8, also known as CXCL8 is produced by MQs; an important innate immune system chemokine which is associated with recruting neutrophils and other granulocytes of innate immune defense (Baggiolini & Clark-Lewis, 1992b). Our findings show a significant increased IL-8 gene expression by monocytes after exposure to ST285. We previously noted that ST1342, ST1275 and ST285 stimulate the U937 monocyte cell line to secrete increased levels of IL-8 (Dargahi et al., 2018). Similarly, we showed PBMC exposure to ST285 results in overexpression of IL-8 [manuscript submitted]. Correspondingly, EPS from *L. paracasei* DG

probiotic displayed immune-stimulating effects to human monocytic cell line THP-1 by increased expression of IL-8 gene (Balzaretti et al., 2017). In contrast, it was shown that dairy and soy fermented milks inoculated with *S. thermophilus* ST5 (ST5) mixed with either *L. helveticus* R0052 (R0052) or *B. longum* R0175 (R0175) added to LPS-challenged THP-1 monocyte cell line, decreased IL-8 production only when co-cultured with ST5+R0175 (Masotti, Buckley, Champagne, Tompkins, & Green-Johnson, 2010). In addition, milk fermented with ST5+R0052 or ST5+R0175 did not alter the production of IL-8 by U937 monocyte cell line, whilst soy ferment prepared with ST5+R0175 downregulated IL-8 production (Masotti et al., 2010).

C-C chemokine receptor type 5 (CCR5, CD195) and chemokine (C-C motif) ligand (CCL) 2 are mainly expressed on monocytes, DCs and MQs (Deshmane, Kremlev, Amini, & Sawaya, 2009). CCR5 is associated with Th1 immune responses and CCL2 with pathogenicity of a number of inflammatory diseases including rheumatoid arthritis and psoriasis, categorized by monocytic infiltrates through chemo-attracting monocytes (I. Lee et al., 2003). Monocytes co-cultured with ST285 significantly downregulated CCR5 and CCL2, which is similar to ST285 co-cultured with PBMCs [manuscript submitted], suggesting an anti-inflammatory influence of ST285.

Although overexpression of IL-8 exclusively, may be interpreted as an inflammatory effect, taking into account the largely upregulated anti-inflammatory cocktail of cytokines and mediators induced by ST285, can in fact modulate this effect towards an anti-inflammatory profile for ST285. Upregulated IL-8 might be an initiating function of ST285 in order to trigger immune responses in the innate immune system, which then gets controlled by ST285 through reduction in the expression of CCR5. This in turn may lead to reduced Th1 immune responses, as well as decreased CCL2 and subsequently resulting in a controlled recruitment of monocyte. These effects may again highlight immunomodulatory effects of ST285 bacteria.

4.4. ST285 significantly upregulates mRNA expression level of colony stimulating factor

Colony stimulating factor (CSF, GM-CSF) is secreted by monocyte/MQs and supports and induces propagation, differentiation and production of different immune cells, mainly monocyte/MQs which are fundamental in responses againts infections. CSF is significantly increased (63.82 fold) by monocyte cultures in the presence of ST285, which is in alignment

to our recent data showing increased CSF gene expression by PBMC co-cultured with ST285 [manuscript submitted]. In addition, ST1275, ST1342 and ST285 were also noted to induce high levels of GM-CSF production by U937 monocyte cell line (Dargahi et al., 2018). It is known that G-CSF induces the development of IL-10-producing cells (Malashchenko et al., 2018), hence, suggesting that ST285 may have an anti-inflammatory effect on the immune system.

4.5. ST285 downregulates mRNA expression levels of toll-like receptors

Toll-like receptors (TLRs) are mediators of innate immune responses primarily required in the defense against pathogens (Kawai & Akira, 2010). ST285 induced significant downregulation of TLR-1, TLR-2, TLR-4, TLR-5, TLR-6 and TLR-8, similar to our previous findings showing reduction of several TLRs by PBMC co-cultured with ST285 [manuscript submitted]. Activated TLR (especially TLR-2 and TLR-4) together with other immune system factors can facilitate pro-inflammatory responses as well as further stimulating innate immune system actions (Islam et al., 2013; Sugitharini et al., 2014; Sugitharini et al., 2016). Thus, an increased expression of TLR-2 and TLR-4 can lead to predominant inflammatory responses in the host, and their downregulation suggests reduction in such pro-inflammatory responses. Moreover, TLR-5 activation leads to stimulation of NF-κB which results in pro- inflammatory TNF-α production (Galli et al., 2010) and its reduced expression in monocyte co-cultured with ST285 may additionally signify an anti-inflammatory role for ST bacteria. Similar to our findings, another study has shown decreased expression of TLR-2, TLR-4, and TLR-9 using *L. plantarum* genomic DNA with THP-1 monocyte cells (Hee Kim et al., 2012). However, a study using human monocytes and monocyte-derived DCs exposed to UV-radiated *V. parvula*, *E. coli*, *B. adolescentis* and *L. plantarum*, showed higher expression of TLR-2 on monocytes compared to DCs, while TLR-4 was not detectable on DCs (Karlsson et al., 2004). Additionally, in the same study it was shown that TLR-4 expression on monocytes was also down regulated in response to exposure to either *E. coli* or *L. plantarum* (Karlsson et al., 2004). Downregulation in mRNA expression of TLRs genes, specifically when it occurs across a wide range including TLR-1, TLR-2, TLR-4, TLR-5, TLR-6 and TLR-8, designates anti-inflammatory properties for ST285.

4.6. ST285 downregulates cell surface markers CD14, CD86, CD4

CD14 is expressed on the cell surface of monocytes, MQs and DC and primarily binds to bacterial components (Bron et al., 2013; I. C. Lee et al., 2013; van Baarlen et al., 2013), CD14 was significantly downregulated when co-cultured with ST285 bacteria suggesting an anti-inflammatory response. CD14 together with TLR-4 bind to bacterial components and both CD14 and TLR-4 were downregulated in the presence of ST285 bacteria. Co-culture of PBMC with ST285 also led to downregulated CD14 and TLR-4 expression [manuscript submitted]. However, ST285 upregulated expression of CD14 by U937 monocyte cell line (Dargahi et al., 2018). In accord to our findings, human monocytes isolated from PBMC and exposed to *E. coli* or *L. plantarum* displayed down-regulated expression of CD14 (Karlsson et al., 2004). CD86 (B7-2), is a co-stimulatory molecule necessary for initiating and maintaining T cells. Expression of CD86 mRNA levels by monocyte is significantly downregulated following culture with ST285, in line with our previous findings where CD86 was downregulated by bulk PBMC cultures [manuscript submitted]. Therefore, ST285 seem to induce an anti-inflammatory profile. Likewise, ST5+R0052 or ST5+R0175 milk or soy ferments also reduced expression of CD86 (Masotti et al., 2010). However, *L. fermentum* GR1485 and *L. plantarum* WCFS1 increased expression of CD86 by monocytes, inversely to *L. delbruekii* and *L. rhamnosus* that reduced CD86 expression (Hajebi et al., 2018). Additionally, monocyte derived immature DCs co-cultured with *L. lactis* subsp. cremoris ARH74, *B. breve* Bb99 and *S. thermophilus* THS increased the expression of CD86 (Latvala et al., 2008). The contrast in these findings is not surprising and may be due to the dissimilarities in the nature of experiments; co-culture of monocytes with ST285 bacteria only compared to differentiated monocytes into immature DCs co-cultured with several probiotics or associated with differences in the properties of each bacteria.

CD4 is an extracellular cell surface molecule expressed by monocytes, MQ, DCs and Th cells and acts as a co-receptor between T cells and antigen presenting cells (Glatzová & Cebecauer, 2019). CD4 was significantly downregulated in monocyte cultures with ST285. In HIV-infected monocytes and MQs, CD4 is required for entry into the cell, and suggest that ST285 may have anti-viral properties.

Given the functional role of cell surface markers in immune responses, CD14 involvement in native immune responses, CD86 in T cell activation, and presence of CD4 on

many cells underpinning innate and adaptive immunity, their downregulation in the presence of ST285 indicates an anti-inflammatory and anti-stimulatory profile for ST285. Additionally, due to the role of these cell surface markers in mediating innate and/or adaptive immune responses in defence against bacteria, downregulation of such markers could be suggestive of ST285 initiating self-tolerance via its immune modulation effects.

4.7. ST285 differentially downregulates mRNA expression level of other innate and adaptive immune response markers and chemokines

Integrin alpha M (ITGAM) or CD11b is another innate immune response factor associated with several inflammatory reactions such as phagocytosis, cell-mediated cytotoxicity, and chemotaxis. Lysozyme (LYZ) is also an innate immune response mediator associated with several inflammatory actions exists in mononuclear phagocytes such as MQs and performs as an antimicrobial enzyme. ITGAM and LYZ gene expressions are vastly downregulated in monocytes co-cultured with ST285, similarly to our recent findings showing downregulation of ITGAM and LYZ by PBMC co-cultured with ST285 [manuscript submitted]. Conversely, in U937 monocyte cell line, exposure to ST285 caused significant upregulation of CD11b/ ITGAM (Dargahi et al., 2018), the contrast could be related to difference between monocytes from healthy blood donors compared to monocyte cell line. MYD88, implicated in innate immunity, is downregulated by monocytes in response to ST285 co-culture. IL1RA1 has been shown to interact with MYD88 (together with PIK3R1 and IL1RAP) (J. Huang, Gao, Li, & Cao, 1997), is also downregulated, which both additionally highlight an anti-inflammatory role for ST285. Non-receptor tyrosine-protein kinase (TYK2) is an enzyme involved in various cellular events and extensive studies of TYK2-deficient mice indicate compromised IFNα, IL-12, and IL-23 pathways (Sohn et al., 2013), and IL-12/Th1 and IL-23/Th17 axes (Ishizaki et al., 2011), but it is dispensable for the signaling pathways of IL-6 or IL-10 (Sohn et al., 2013). It is believed that TKY2 is associated with a broader cellular pathways in human and it has a role in IL-12/Th1 and IL-23/Th17 axes involved in inflammatory/ autoimmunity, highlighting TKY2 choice as an effective therapeutic approach for select autoimmune diseases (Sohn et al., 2013). TYK2 is significantly downregulated by monocytes upon co-culture with ST285, a similar trend was found in our results when PBMC co-cultured with ST285 recently [manuscript submitted], which mutually support an anti-inflammatory profile for ST285.

IL-1-receptor-associated kinase-1 (IRAK1) is involved in innate immunity, and ST285 induced a significant downregulation of IRAK1 by monocyte culture. Likewise, *L. paracasei* stimulated the expression of IRAK3, but not IRAK1 in THP-1 cell line post differentiation with PMA. IRAK4 inhibitor suppressed the expression of negative regulators (Sun et al., 2017). In contrast, THP-1 monocyte cells treated with *L. plantarum* genomic DNA induced a slight increase in IRAK-1 production (Hee Kim et al., 2012). SLC11A1 is a monocyte-MQ protein-1 involved in T cell activation and inflammatory disorders such as type 1 diabetes (Y. D. Dai et al., 2009; Thayer et al., 2010), Crohn's disease (L. C. Stewart et al., 2010) and rheumatoid arthritis (Archer, Nassif, & O'Brien, 2015), is downregulated by monocytes upon co-culture with ST285. Our previous findings using ST285 to co-culture with PBMC similarly showed a reduced expression of SLC11A1 [manuscript submitted], again suggesting an anti-inflammatory role for ST285. Induced downregulation of IRAK1, MYD88, TKY2, ITGAM, NOD2, SLC11A1 and LYZ by monocytes due to exposure to ST285 is suggestive of anti-inflammatory effects of ST285.

5. Conclusion

Commensal bacteria and probiotics have made their entry to the mainstream of healthcare and contribute to immune homeostasis in the gastrointestinal tract as well as conferring beneficial immunomodulatory properties that assist in the maintenance of a healthy immune system. ST is commonly applied in dairy products to ferment cheeses and yogurts and is thought to be beneficial to human health. The immune modulatory effects of ST285 on human monocytes were assessed and demonstrated that it delivers a range of potential immunomodulatory and anti-inflammatory properties. ST285 decreases mRNA expression of IL-1R, IL-18, IFNγR1, IFNαR1, CCL2, CCR5, TLR-1, TLR-2, TLR-4, TLR-5, TLR-6, TLR-8, CD14, CD86, CD4, ITGAM, LYZ, TYK2, IFNR1, IRAK-1, NOD2, MYD88, ITGAM, SLC11A1, and upregulates IL-1α, IL-1β, IL-2, IL-6, IL-8, IL-23, IFNγ, TNFα, CSF-2. No changes to mRNA expression were noted with IL-4, IL-5, IL-13, CCL2, CCL5, CCL8, CCR4, CCR8, CXCR3, CXCL10, TLR-3, TLR-9, CD8A, CD40, CD80, IFNB1, MPO, FOXP3, GATA3, STAT3, CD40LG, HLA-A, HLA-E, RORC. The data exhibits a predominant anti-inflammatory profile of cytokine, chemokine and cell markers induced by ST285.Therefore, the use of ST285 may be an efficacious approach for the treatment of select autoimmune diseases without using broad immunosuppression caused by currently available treatments for

autoimmune disorders. Supplementary work is required to determine whether ST bacteria displays similar anti-inflammatory effects *in vitro* and *in vivo* in compromised immune disorders/ models such as inflammatory bowel disease, multiple sclerosis and allergies.

Chapter 5

Chapter 5

5a– Immune modulation of linear and cyclic MBP_{83-99} peptide conjugated to mannan

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ABSTRACT

The immune modulatory effects of probiotics ST285, ST1275 and ST1342 on human monocyte cell line U937 were shown in chapter 2 where ST285 showed the best response. In particular IL-4 and IL-10 anti-inflammatory cytokines were induced as well as upregulation of cell surface markers CD11c, CD14, CD86, CD206, C209 and MHC-I suggestive of beneficial and immune modulating effects of ST285. In chapers 3 aned 4 it was clear that ST285 modulated immune responses to human peripheral blood mononuclear cells and to human monocytes respectively. As a consequence of these results, it is important to assess ST285 bacteria and their immune modulatory and anti-inflammatory benefits in an autoimmune setting, such as, multiple sclerosis (MS). However, before such benefits can be determined the immune modulatory effects of MS peptide MBP_{83-99} needs to be analysed in their free state and following conjugation to mannan. Mannan is a carier known to modulate immune responses.

Key words: Multiple sclerosis*;* Experimental Autoimmune Encephalomyelitis; Central Nervous System; MHC class II; T-cell receptor (TCR); peptide ligands; mannan

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1. Introduction

Experimental Autoimmune Encephalomyelitis (EAE) is an experimental model of demyelination, inflammatory processes and axonopathy within the Central Nervous System (CNS) of susceptible animals, using the triggering of various CNS antigens such as MBP, PLP and MOG proteins or their peptides (Grigoriadis, Ben-Hur, Karussis, & Milonas, 2004). Due to the similarities of EAE with multiple sclerosis (MS), this model is widely used to study pathological mechanisms as well as novel experimental treatments for the disease.

The peptides that induce EAE bear different immunogenic properties in each animal species which is largely determined by the specific properties of the MHC class II of the animal. Specific peptide motifs within the antigen-binding groove of the MHC complex determines the affinity to the antigen and subsequent T-cell receptor (TCR) recognition and activation of the cell (Degano et al., 2000). This tri-molecular complex (MHC-peptide-TCR) is imperative to induce EAE with antigenic peptides and is one of the key factors to MS immune-pathology (Kalbus et al., 2001; L. Kappos, Comi, Panitch, Oger, Antel, Conlon, & Steinman, 2000). In fact, a number of immunotherapeutic strategies are based on blocking the formation of this complex, such as, anti-MHC-II antibodies or anti-CD4 antibodies which consequently suppress EAE (Brostoff & Mason, 1984; L. Steinman, Rosenbaum, Sriram, & McDevitt, 1981; Waldor et al., 1985). In addition, any modification to the antigenic peptide, and any deviation from a tight MHC-antigen-TCR match can lead to reduced T-cell activation and different profiles of secreted cytokines.

Based on this concept, there have been several attempts to alter the trimolecular complex affinities and render autoreactive T-cells in MS and EAE inactive or eliminated. As such, altered peptide ligands with 1-2 amino acid modifications of the peptide, have led to immunomodulation of inflammatory responses. Conjugation of these altered peptide ligands to mannan completely diverts pro- to anti- inflammatory cytokines (Katsara, Deraos, Tselios, Matsoukas, & Apostolopoulos, 2008a; Katsara, Deraos, et al., 2009a; Katsara et al., 2014; Katsara, Matsoukas, Deraos, & Apostolopoulos, 2008b; Katsara, Minigo, Plebanski, & Apostolopoulos, 2008b; Katsara, Yuriev, et al., 2008a, 2008b; Katsara, Yuriev, et al., 2009a; Keramida et al., 2006). Likewise, substitution of the TCR binding amino acids at positions 144 (Tryptophan, W) and 147 (Histidine, H) to L^{144} and R^{147} was able to antagonise T cell clones

specific for $PLP_{139-151}$ epitope, block the induction of EAE and prevent progression of EAE (Katsara et al., 2014; Kuchroo et al., 1994; Kuchroo et al., 1992; Kuchroo et al., 1991). An alternative approach was recently published (chapter 5b), whereby non-peptide mimetics of $MBP_{83–96}$ T cell epitope can function as TCR antagonists by the inclusion of compounds obtained through the ZINC database (Yannakakis et al., 2017a). Hence such an approach may pave the way to developing alternative and improved immunotherapeutics against MS (Yannakakis et al., 2017a).

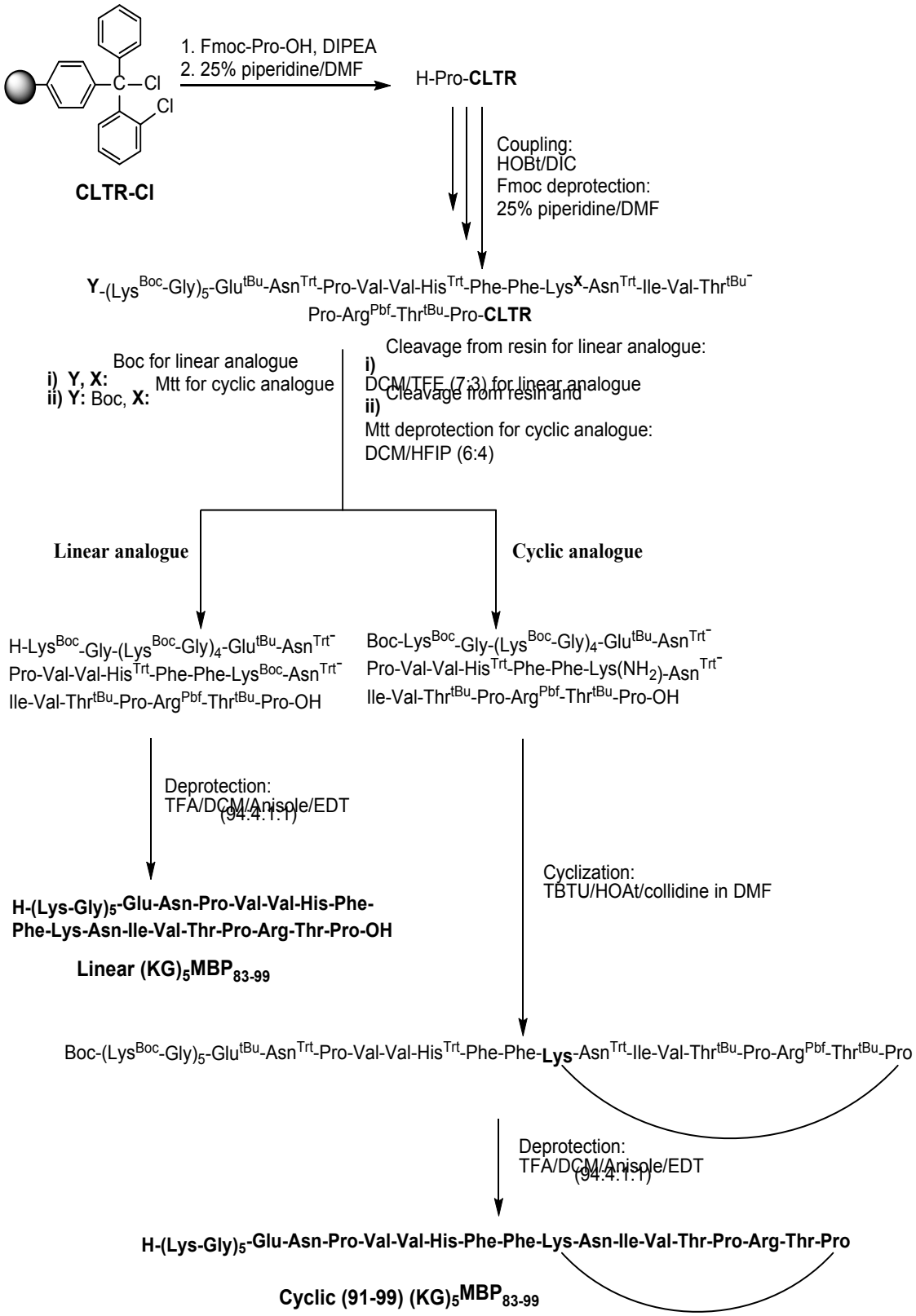
Recently, in an attempt to altering the tight interaction of the trimolecular complex, it was shown that cyclic MOG_{35-55} peptide (Lourbopoulos et al., 2017b) and cyclic $PLP_{139-151}$ peptide (Lourbopoulos et al., 2018) showed significant lower immunogenic potential in mice, due to reduced affinity to MHC-II. Therefore, herein, the effects and immune modulating properties of MBP_{83-99} peptide in its linear and cyclic forms were determined. The prophylactic effects of a cyclic MBP_{83-99} peptide (cMBP), using previous established methods were used (Lourbopoulos et al., 2017a). By keeping the same antigenic linear epitope of MBP_{83-99} and changing the sterotaxy of the molecule to a cyclic one (cMBP), the prophylactic clinical potential of the specific $cMBP_{83-99}$ peptide in mice induced with MOG EAE and analyzed the underlying inflammatory, axonopathic and demyelinating processes. Next the interactions of the cMBP peptide compared to linear MBP counterpart in complex with MHC-II (H-2 IA^s) using appropriate *in silico* binding and structural studies (docking and homology modelling). Finally, conjugation of linear MBP_{83-99} and cyclic MBP_{83-99} peptides to mannan and evaluation of their immune-modulating potential was assessed.

2. Materials and Methods

2.1. Peptide Synthesis of linear and cyclic MBP_{83-99} peptides

The synthesis of linear $(KG)_5MBP_{83-99}$ and cyclic(91-99)$(KG)_5MBP_{83-99}$ peptides (Scheme 1) was achieved step by step using the 2-chlorotrityl chloride resin (CLTR-Cl, 0.7 mmol Cl/g resin), following by the Fmoc/tBu methodology as previously described (Barlos, Chatzi, Gatos, & Stavropoulos, 1991; Ieronymaki et al., 2015; Keramida et al., 2006; Matsoukas et al., 2005a; Tapeinou et al., 2015; T. Tselios et al., 2014; T. Tselios et al., 1999). The synthesis of the peptides used in this chapter were made by the laboratory collaborators professor John

Matsoukas and professor Theodore Tselios. The first N^{α}-9-fluorenylmethyloxycarbonyl (Fmoc) protected amino acid, Fmoc-Pro-OH, was esterified to the resin in the presence of *N,N*-diisopropylethylamine (DIEA) in dichloromethane (DCM) for 1.5 h at room temperature (RT). The remaining chain of each peptide was assembled by sequential couplings of Fmoc protected amino acids using *N,N'*-diisopropylcarbodiimide (DIC) and 1-hydroxybenzotriazole (HOBt) as coupling reagents in dimethylacetamide (DMAC). Cleavage from the 2-chlorotrityl chloride resin was performed as described in Scheme 1. The cyclization of protected peptide was achieved using *O*-(Benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate (TBTU), 1-hydroxy-7-azabenzotriazole (HOAt) and 2,4,6-collidine (2,4,6-trimethylpyridine) (Katsara, Deraos, et al., 2008a; Katsara, Deraos, et al., 2009a; Katsara et al., 2014; Katsara, Yuriev, et al., 2008a, 2008b; Katsara, Yuriev, et al., 2009a; Lourbopoulos et al., 2017a). The final deprotection of each peptide was accomplished using trifluoroacetic acid (TFA) in DCM in the presence of scavengers (Scheme 1). The purification of final crude peptides was performed on a semi-preparative reverse phase high performance liquid chromatography (RP-HPLC) whereas the identification was achieved by electron spray ionization mass spectrometry (ESI-MS) (Figure 1). The purity of each peptide was verified by analytical RP-HPLC and it was observed to be more than 97% (Figure 2).



Scheme 1: Schematic representation of the synthetic procedure of linear $(KG)_5MBP_{83-99}$ and cyclic(91-99)$(KG)_5MBP_{83-99}$ peptides. The Mtt group was used for the side chain protection of Lys^{91} for the synthesis of cyclic analogue

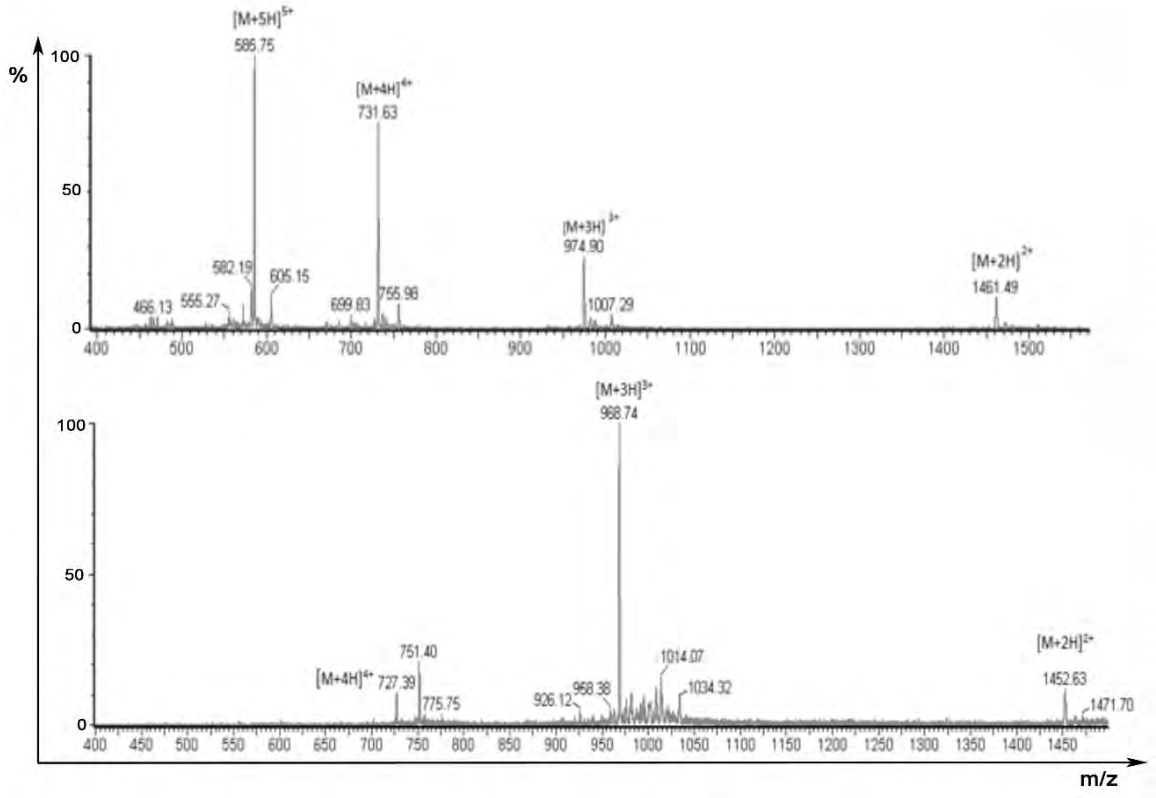


Figure 1. ESI-MS of linear $(KG)_5MBP_{83-99}$ (top, $M_{calculated}$: 2921.11) and cyclic(91-99)$(KG)_5MBP_{83-99}$ (bottom, $M_{calculated}$: 2903.39).

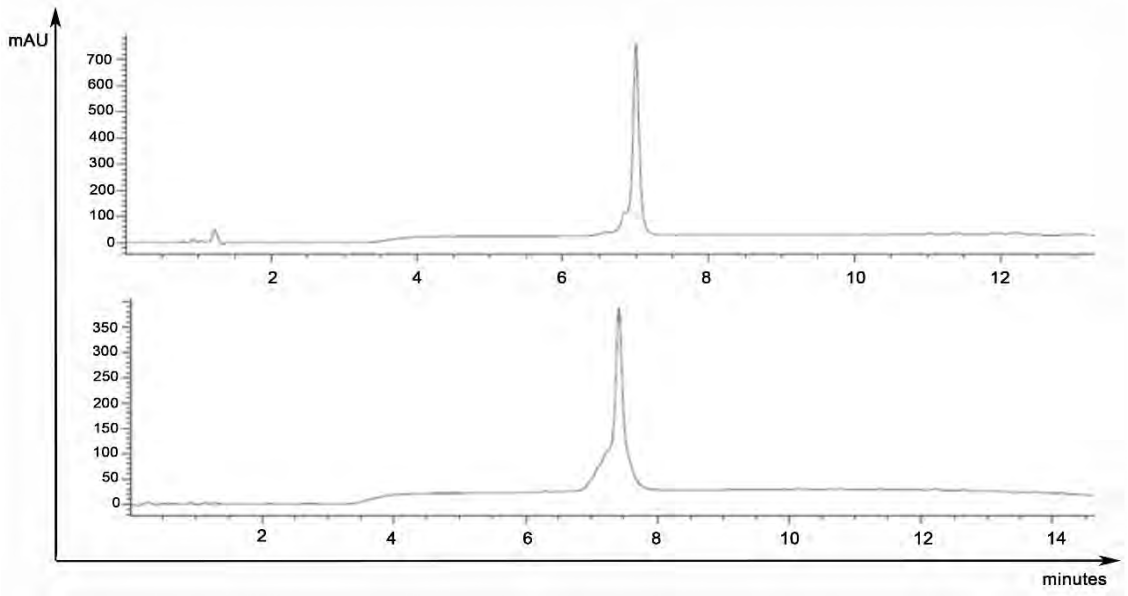


Figure 2. RP-HPLC chromatograms at 214 nm of linear $(KG)_5MBP_{83-99}$ (top, t_R: 7.00 min, Purity: 97.9%) and cyclic(91-99) $(KG)_5MBP_{83-99}$ (bottom, t_R: 7.42 min, Purity: 98.0%). RP-HPLC conditions: i) Column: Agilent ZORBAX Eclipse Plus C18 (3.5 µm, 100x4.6 mm); ii) solvents: H_2O (0.08% TFA) and acetonitrile (ACN) (0.08% TFA); iii) gradient elution: from 10% ACN to 100% ACN over 15 min.

2.2. Induction of EAE in mice and histopathological assessment

2.2.1 Induction of EAE

Female C57BL/6 mice, 8 weeks-old, were purchased from Hellenic Pasteur Institute, Athens, and housed in the P3 animal facility of the B' Neurological Department of the AHEPA University Hospital, Aristotle University Medical School, Greece. Animals were handled in accordance with the National Institute of Health guidelines, were fed a regular diet and given water without antibiotics. All animals were clinically assessed and weighted before disease induction (baseline). EAE was induced in all animals on day 0 with a linear MOG_{35-55} peptide following an established protocol as previously described in detail (Lourbopoulos et al., 2017a; Lourbopoulos et al., 2018) (each animal was injected subcutaneously (sc) with 200 μl of a 1:1 PBS-CFA emulsion containing 300 μg MOG_{35-55} and 0.2 mg Mycobacterium Tuberculosis HR37a, plus intraperitoneously (IP) 400 ng pertussis toxin dissolved in 500 μl sterile PBS). On day 2 and 7 all animals received an additional booster of pertussis (200 ng, IP) and an injection of MOG_{35-55} (300 μg, sc on day 0).

At day of disease induction, mice were randomly divided into 2 experimental groups: a) the non-treated group, which was immunized for EAE but received no additional peptide treatment (control group) and b) the treated group, which received MOG_{35-55}-CFA emulsion in addition to 300 μg of cyclic MBP_{83-99} (cMBP group, preventive treatment). In order to test the preventive effects of cyclic MBP_{83-99} we run the experimental setting twice: the first experiment (n= 6-7 per group) was designed to study the effect of cyclic MBP_{83-99} at the pathological procedures of acute phase of EAE and the second one (n=8 per group) to study the effect of the peptide up to the chronic phase of EAE.

All mice were weighted daily (body weight changes due to EAE) and blinded evaluated for clinical signs of disease using a 0-6 grade established clinical scale, as previously described (Lourbopoulos et al., 2017a; Lourbopoulos et al., 2011) (0: without clinical disease; 1: flail tail; 2: tail paralysis; 3: hind limb weakness sufficient to impair righting; 4: paraplegia; 5: paraplegia with forelimb paresis or plegia; 6: death from EAE).

2.2.2. Histopathology

On day 17 (acute phase of EAE) and day 46 post-EAE-induction (chronic phase of EAE) animals were subjected to transcardial perfusion with 4% paraformaldehyde in PBS. Brains and spinal cords were removed, post-fixed in the same fixative for approximately 20 hours, routinely processed for paraffin embedding and sectioned at 6 μm. Sections from animals of acute and chronic phases of the disease, were then stained using the following methods: a) a modified Bielschowsky silver impregnation staining method combined with haematoxylin, for the simultaneous evaluation of axonal injury, axonal loss and inflammatory processes in EAE as previously described in detail(Lourbopoulos et al., 2017a; Lourbopoulos et al., 2011) and b) Luxol fast blue staining counterstained with Nuclear fast Red for the detection of demyelinating areas within the CNS of animals, using routine histopathological protocols (Lourbopoulos et al., 2017a).

Pathological evaluation was performed under a light microscope (Olympus Axioplan-2) by two blinded investigators and photos were taken using a CCD camera (Nikon). Five randomly selected longitudinal sections per tissue (brains and spinal cords) were evaluated as follows: for each animal, each section was evaluated under 20x or 40x optical fields (depending on the object of study) so as to cover the entire area of the section. Initial study of pathology revealed that spinal cords had the majority of lesions (compared to brains) and thus further detailed study was focused on the spinal cord sections of the mice.

Depending on the object of interest we used the following scales to perform the evaluation, as previously described (Lourbopoulos et al., 2017a; Lourbopoulos et al., 2018): (a) For axonal injury (AI), we used the following scale under 40x optical fields: 0 = no AI, 1+ = scattered dystrophic injured axons without any spheroid or ovoid, 2+ = mild AI with the presence of one spheroid or ovoid, 3+ = moderate AI, 4+ = severe AI; generally injured axons were defined and identified as spheroids and ovoids (which represent axonotmesis), and dilated (dystrophic) axons which represent injured axons not yet being cut. (b) For axonal loss (AL): 0 = normal axonal density, 1+ = <25% AL, 2+ = mild AL (26-50%), 3+ = moderate to severe (51-75%) AL, 4+ = severe AL (>75%); various degrees of decreased density of axons was attributed to axonal loss and evaluated accordingly. (c) For infiltrations: number of infiltrating cells per mm^2 ("InfLoad") and number of infiltrating cells per infiltration ("InfSize"). (d) Demyelination was evaluated under 20x optical fields using a prefrontal grid: we measured the area of

demyelination and the total area of white matter in each optical field and then subtracted the % of demyelination (% Dem) present in each optical field using the formula "(demyelinating area / total white matter area)*100".

2.2.3. Statistical analysis

Statistical analysis of the data was performed using the SPSS 23.0 and GraphPad Prism 6 software. Scale clinical, histopathological and in vitro proliferation data were initially tested for normality using Shapiro-Wilk test to assess their validity for parametric analysis (Student's t-test). Histopathological data from sections' analysis of demyelination and inflammation were pooled as one respective value per each animal and these values were used for final statistical analysis. This approach removes analysis bias (Dirnagl, 2006) but stringents the analysis. For non-parametric analysis of two groups we used the Mann-Whitney U test. For comparison of nominal or ordinal data we applied a Pearson chi-square test or Fisher's exact test, depending on the tables' properties. The total disease burden (evaluated as the area under the curve, AUC), the maximal disease severity (evaluated as the mean maximal score, MMS) and the day of disease onset (mean day of disease onset with a clinical score of 1, dDO) for each group were calculated as previous described in detail (Fleming et al., 2005; Lourbopoulos et al., 2017a). Survival analysis was performed using Mantel-Cox log-rank test (Kaplan-Meier survival analysis). Values are expressed as mean±SE.

2.3. Molecular Modeling and Molecular Dynamics

2.3.1. Model complexes.

Production of the model complexes of H2-IA^{s} and MBP_{83-99} was made by homology modeling using MODELLER 9.17 (Webb & Sali, 2014), using the crystal structure of H2-IA^{u} complex, bound to the MBP_{1-11} peptide (PDB ID: 1k2d) (He et al., 2002) as template. The sequences of the alpha and beta chains were obtained from the Universal Protein Resource (UNIPROT) database (UNIPROT IDs: P14437.1 and P06345.1 respectively). The structural model involved the disulfide bond addition between C107 - C163 in chain A and C15 – C79 and C117 – C173 in chain B. Linear MBP_{83-99} peptide was aligned to the aforementioned crystalized peptides, resulting in V^{87}, H^{88}, F^{90}, N^{92} and T^{95} occupying pockets P1, P2, P4 P6 and P9. The overall stereochemical quality of the final models was evaluated by visual

inspection and the discrete optimized energy (DOPE) (Shen & Sali, 2006). Cyclization of the linear MBP_{83-99} peptide between residues K^{91} and P^{99} was performed manually by joining the side chain amide group of K^{91} and the carboxyl group of P^{99}, followed by an energy minimization of the whole complex by means of the conjugate gradient algorithm for 1000 steps in Discovery Studio v3.5. For the linear MBP_{83-99} complex, the same energy minimization parameters were used. The modles were constructed by Doctor Miros Matsoukas from New Drug, Patras Greece.

2.3.2. Molecular Dynamics (MD)

All MD simulations were performed using GROMACS 2016 (Pronk et al., 2013). After the minimization and finalization of the structural models, the complex was inserted in a pre-equilibrated box containing water and a 0.15M concentration of Na and Cl ions. The AMBER99SB-ILDN force field was used for all the dynamics simulations in conjunction with the TIP3P water model. Special force field parameters for the modified K^{91}, which was used as a cyclization point were made, in which the side chain amino group was set up with an Sp2 hybridization. New, manual amino acid entries were added to the force field parameters in order to enable cyclization. Each system consisted of the protein, the peptide, ~15.000 water molecules and ~130 ions in an 80 x 80 x 80 Å simulation box. The two model systems were subjected to a 10 ns MD equilibration, with positional restraints on protein backbone atom coordinates. These restraints were released, and 500 ns MD trajectories were produced in constant temperature of 300K using separate v-rescale thermostats for the protein, the peptide and solvent molecules. A time step of 2 fs was used and all bonds were constrained using the LINCS algorithm. Lennard-Jones interactions were computed using a cutoff of 10 Å, and the electrostatic interactions were treated using PME with the same real-space cutoff.

2.4. Immunological evaluation of linear and cMBP peptides conjugated to mannan

2.4.1. Conjugation of peptides to mannan

$MBP_{83–99}$ peptide with $(KG)_5$ at the C-terminus was conjugated to mannan via the $(KG)_5$ bridge via a method previously described (Apostolopoulos, Barnes, et al., 2000; Apostolopoulos et al., 2017b; Apostolopoulos, Pietersz, et al., 2000; Apostolopoulos et al., 1995; Apostolopoulos et al., 1996). Briefly, mannan (14 mg, poly-mannose from

Saccharomyces cerevisiae), was oxidized to aldehydes using sodium periodate ($NaIO_4$) and purified by size exclusion chromatography as previously described (Apostolopoulos, Barnes, et al., 2000; Apostolopoulos, Pietersz, et al., 2000; Apostolopoulos et al., 1995; Apostolopoulos et al., 1996). The purified oxidized mannan was mixed with 1 mg $(KG)_5MBP_{83-99}$ or cyclic (91-99) $(KG)_5MBP_{83-99}$ and incubated at RT in the dark for at least 24 h. Conjugation of each peptide to oxidized mannan occurred via Schiff base formation between the free amino groups of the peptide sequences and the aldehydes of oxidized mannan. Unreacted aldehydes and remaining Schiff bases in the oxidized mannan-peptide conjugate, were reduced to alcohols and amines respectively by sodium borohydride ($NaBH_4$), to achieve the reduced mannan-peptide conjugates (Apostolopoulos, Barnes, et al., 2000; Apostolopoulos et al., 2017b; Apostolopoulos, Pietersz, et al., 2000; Apostolopoulos et al., 1995; Apostolopoulos et al., 1996; Deraos et al., 2008a). The conjugation of peptides to oxidized or reduced mannan was verified using tricine SDS-PAGE (T. V. Tselios et al., 2005a). The resultant $MBP_{83–99}$-$(KG)_5$-mannan conjugates were used to immunize mice.

2.4.2. Immunization of mice with linear and cMBP peptide - mannan conjugates

Female SJL/J mice used in all experiments aged 6-9 weeks, were purchased from Animal Resources Centre (ARC, Perth, Australia), and accommodated at the animal house (Victoria University, Werribee campus, Melbourne, Australia). All mice ensured free access to water and food, and were housed in a temperature controlled room with 12 hr day 12 hr night cycle. All immunizations were conducted according to the guidelines of the Australian code of Practice for the care and use of animals for scientific purposes and the study was approved by the Victoria University animal ethics committee (AEC15/013) of Victoria University, Melbourne, Australia.

The $MBP_{83–99}$ mannan peptide conjugates (50 μg/mouse) were injected in SJL/J mice subcutaneously into the base of the tail, 3 times, on days (Dargahi et al., 2017) (Yannakakis et al., 2017a). This conjugate has been shown to induce T cell proliferation and IFN-gamma cytokine secretion to linear $MBP_{83–99}$ peptide in SJL/J mice. 7-10 days after the 3rd injection, spleen cells were isolated, red blood cells lysed using 0.73 % NH_4Cl and counted.

2.4.3. Isolation of spleen cells and Cytokine production analysis

Spleen cells cells were resuspended in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Pty Ltd. Australia), 1% antibiotic-antimycotic solution and 2 mM L-glutamine in T75 cm^2 cell culture flasks. Mouse spleen cells (1×10^7) in RPMI media only was used as negative control and 5 μg/ml recall $MBP_{83–99}$ peptide was used and cultured at 37 °C, 5 % CO_2 for 24 hour (Dargahi et al., 2017).

Cytokine secretion of spleen cell culture supernatants was analyzed by commercially available capture and detection antibodies in a Bioplex multiplex bead assay for a panel of 33 mouse cytokines and chemokines using a 33-plex kit (BioRad, Melbourne Australia). Cell-free supernatants were collected and the assay procedures were performed according to the manufacturer's instructions. Briefly, flat bottom 96-well plate was coated with 1×coupled beads, washed twice, followed by adding the standard serial dilutions, blank and samples to assigned wells. Post incubation at shaking at room temperature, plates were washed twice, adequate 1× detection antibody was added, incubated at room temperature at. Plates were washed three times and 1× Streptavidin Phycoerythrin (SA-PE) stop solution was added to each well, followed by incubating at room temperature at and wash. Data collection was repeated twice, data was expressed as the mean cytokine response minus background (pg/ml) of each treatment from 3 replicate wells, plus or minus the standard error of the mean.

2.4.4. Statistical analysis

Significant differences between all treatment groups were tested by analysis of variance (ANOVA) using the Statistical Package for the Social Sciences for Windows 25.0 (SPSS; IBM Corp) followed by a comparison between treatments performed by Tukey's honest significance test/degree and Fisher's least significant difference method, with a level of significance defined as $p < 0.05$.

3. Results

3.1. Co-immunization with cMBP weakly ameliorates the clinical phenotype of EAE

The effects of cMBP were evaluated in two separate experiments. In the acute experiment, (sacrifice at day 17, Figure 3a-c) the control animals developed a severe and aggressive disease with fast and steep disease onset and highly homogenous (low variability in clinical scores). The cMBP group had lower mean clinical scores at days 15, 16 and 17 ($p<0.05$, Figure 3a), indicating milder acute disease. This has been verified by lower mean Areas Under the Curve (mAUC) for the cMBP group (mAUC=13.3±0.9 for cMBP compared to mAUC=18.0±1.4 for control, $p=0.016$, Figure 3c). Mean Maximal Clinical Scores (MMS) were not calculated for this experiment since animals were not allowed to live long enough to reach their maximal scores. Mean day of disease onset (dDO) was not different between the 2 groups (dDO for control = 11.7±0.5 and cMBP = 12.0±0.3, Kaplan-Meier survival analysis, log rank $p=0.73$). Both groups experienced a similarly severe and abrupt weight loss up to day 17 (Figure 3b). Thus clinical data from acute phase indicate a mild clinical benefit from MBP_{83-99} preventive co-administration in a severe and aggressive form of MOG-EAE.

In the second experiment, animals were allowed to survive until day 46 post EAE induction (chronic phase, Figure 3d-g). During the second EAE experiment control animals developed a moderate EAE with a lower disease peak (Figure 3d). Animals of the cMBP group had a lower mean clinical score throughout the entire observation period but this failed to reach statistical significance ($p>0.05$, Figure 3d). Similarly, cMBP animals had lower but not significantly different AUC (AUC=51.1±12.9 for cMBP compared to AUC=66.5±13.9 for control group, $p>0.05$, Figure 3f) and lower but not statistically different MMSs (MMS=2.4±0.5 for cMBP compared to MMS=3.1±0.5 for control group, $p>0.05$, Figure 3g). Mean day of disease onset (dDO) was not different between the 2 groups (dDO=17.0±0.3 for cMBP compared to dDO=18.5±0.7 for control group, Kaplan-Meier survival analysis, log rank $p=0.19$). Body weight loss was not different between the 2 groups (Figure 3e). Overall, the data from chronic EAE combined with those of the acute experiment indicate a repeated trend for amelioration of the disease by the cMBP co-immunization, which did not reach statistical significance in all cases but is interesting for further experimental clarification.

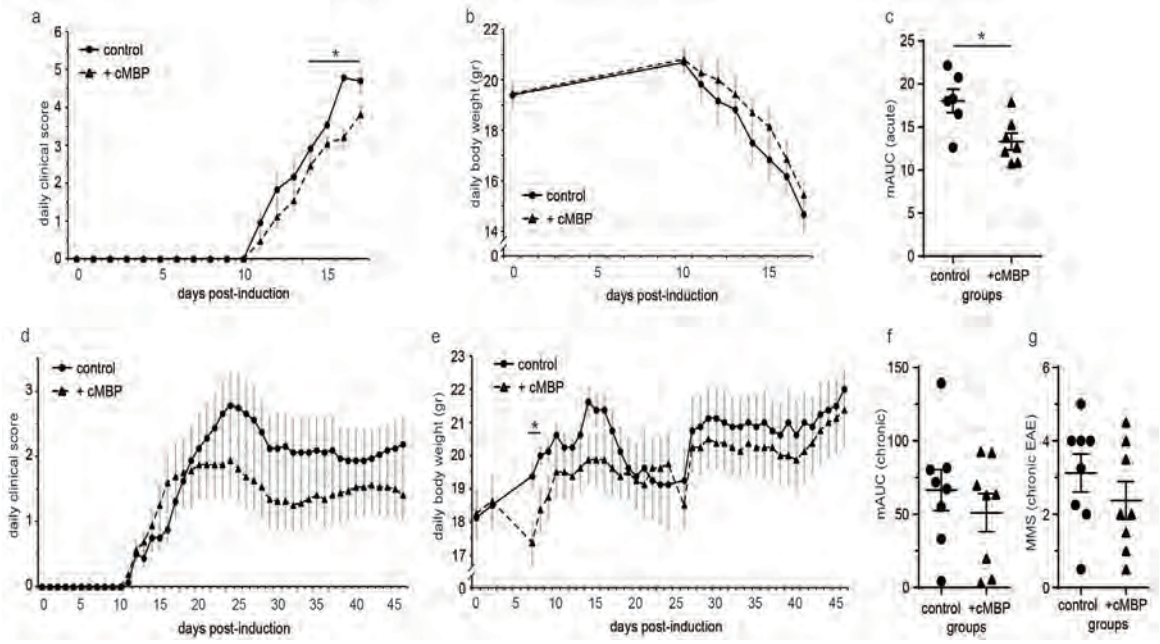


Figure 3. Clinical course and outcome parameters of EAE (control group as continuous line and +cMBP group as dotted line in a, b, d, e). Panels a-c show the acute clinical course and parameters (acute experiment) of animals: (a) the short-term clinical course of the disease (mean daily clinical score, significant differences on days 15-17, (b) the body weight loss due to EAE and (c) the overall burden of disease (mAUC). Panels (d-g) show the corresponding long-term clinical course and parameters of control and +cMBP groups (chronic experiment): (d) the long-term follow-up and clinical course of animals (mean daily clinical score), (e) the body weight loss due to EAE, (f) the overall burden of the disease by day 46 (mAUC) and (g) the maximal disease deficits of each animal (mean MMS). * denotes $p<0.05$. Data are displayed (also the horizontal lines ± error bars in c, f, g) as mean ± SEM.

3.2. cMBP partially ameliorates underlying inflammatory, demyelinating and axonopathic pathology of EAE

Study of inflammatory processes of acute phase of EAE showed no quantitative differences of inflammatory processes the between the control and cMBP groups (InfLoad 259.4±42.7 for control group and 245.8±28.5 for cMBP group, $p>0.05$; InfSize 56.0±4.0 for control and 54.1±9.5 for cMBP group, $p>0.05$, Figure 4a-b). However, in the experiment of chronic phase, the residual inflammatory processes tended to be lower in cMBP animals, indicating either lower inflammatory burden during the acute phase or a stronger resolution of the inflammation (InfLoad for control group 27.4±8.2 compared to 8.9±4.1 for cMBP group, $p=0.056$; InfSize 15.5±4.0 for control group compared to 6.4±2.7 cells/lesion for cMBP group, $p=0.098$, Figure 4a-b). In summary, data from both experiments indicate a trend for stronger

resolution of the inflammatory processes from co-immunization with cMBP up to the chronic phase of EAE, which needs to be further evaluated.

Analysis of axonopathy and demyelination showed that co-immunization with the cMBP was beneficial for these degenerating processes (Figure 4). As such, cMBP had significantly less active demyelination of the white matter tracks of spinal cords during the acute (20.9±3.4% and 9.7±1.2% demyelination for control and cMBP groups respectively, $p=0.03$, Figure 4c) and less residual demyelination during the chronic phase of EAE compared to control group (15.9±3.4% and 8.6±3.7% demyelination for control and cMBP groups respectively, $p=0.09$, Figure 4c). Similarly, cMBP group had less axonal injury (Pearson's chi-square, $p=0.006$, Figure 4d) and axonal loss (Pearson's chi-square, $p<0.001$, Figure 4e) during the acute phase. This resulted in lower axonal loss during chronic phase in cMBP group compared to control (Pearson's chi-square, $p<0.001$, Figure 4e), despite similar residual chronic axonal injury (Pearson's chi-square, $p>0.05$, Figure 4d). Collectively, our data indicate that co-immunization with the cyclic MBP_{83-99} at disease onset ameliorates the demyelinating and axonopathic processes within the spinal cords of the animals, despite similar inflammatory burden (Figure 4a-b), strongly suggesting possibly reduced degenerating capacities of the infiltrating cells in the cMBP group.

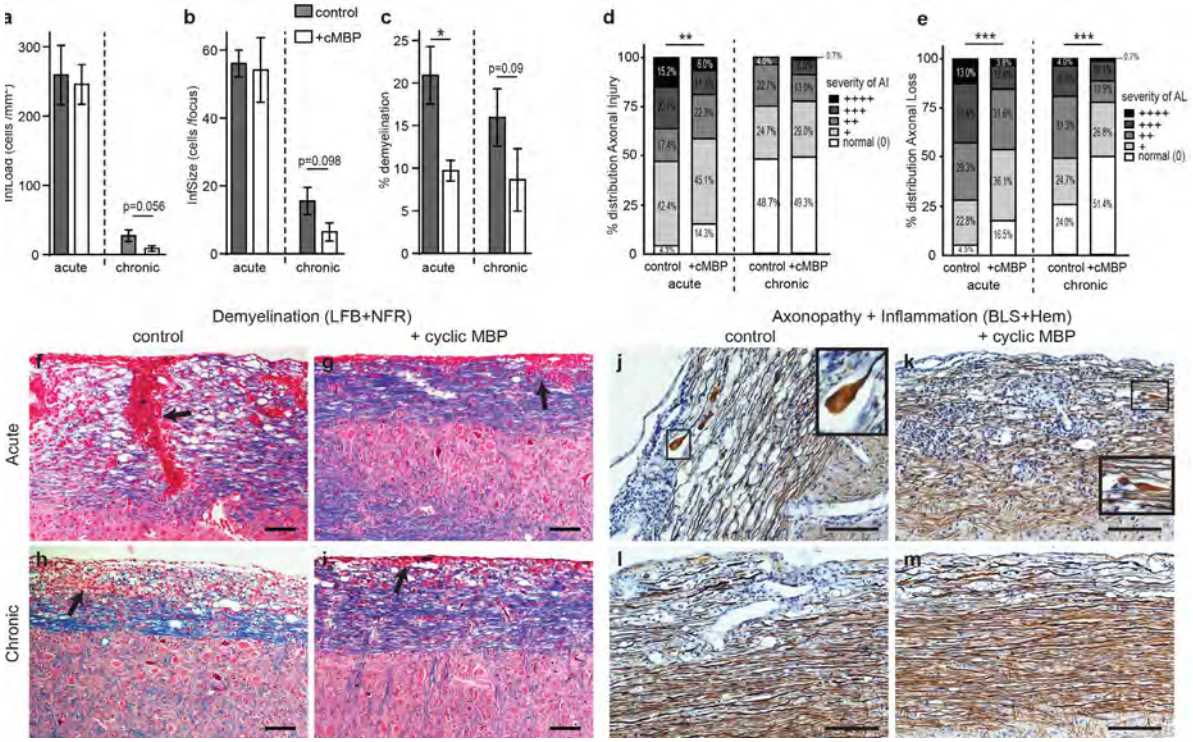


Figure 4. Neuropathological processes in the spinal cords of control and cMBP animals. Graphs show the inflammatory load (a, InfLoad), the size of inflammatory foci (b, InfSize), the % demyelination of the white matter tracks (c), the axonal injury (d, semiquantitative scale 0 to 4+ on the right side of graph, % of evaluated optical fields with corresponding scores) and the axonal loss (e, semiquantitative scale 0 to 4+ on the right side of graph, % of evaluated optical fields with corresponding scores) in control and cMBP groups, in both acute and chronic phases of EAE (scales). Photos f-i show representative longitudinal spinal cord sections (scale bar 100µm) of control and +cMBP co-immunized animals stained with LFB plus NFR staining (blue: intact myelin, red: cells, arrows point at sites of inflammatory foci and/or demyelination), in acute (f, g) and chronic (h, i) phase. Photos j-m show representative longitudinal spinal cord sections (scale bar 100µm) of control and +cMBP co-immunized animals stained with Bielschowsky and Hematoxylin (BLS+Hem) (axonopathy and inflammation, blue: cells bodies, dark brown: axons, inserts show magnifications of spheroid/ovoid in each animal) in acute (j, k) and chronic (l, m) phases. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Data in a-c are displayed as mean ± SEM.

3.3. Structural insights of linear and cMBP83-99 in complex with H2-IA^s

Several peptides containing the MBP83-99 sequence have been co-crystallized with MHC alleles in their clefts, having two different binding modes. In HLA-DR2b and HLA-DQ1, pockets P1, P4, P6 and P9 are occupied by V^{87}, F^{90}, N^{92} and T^{95} respectively (Michael Hahn, Melissa J Nicholson, Jason Pyrdol, & Kai W Wucherpfennig, 2005; Dhruv K Sethi et al., 2011) and in HLA-DR2a, the peptide register is shifted by three residues, in which the pockets are

occupied by the side chains of F^{90}, I^{93}, T^{95} and T^{98}. As noted previously, H2-IA^s possesses V86B and T71B, which result in a shallow P1 and wider P4 and respectively (Apostolopoulos et al., 2017b). Thus, binding of the two MBP peptides should occur as in the HLA-DR2b and HLA-DQ1 cavities. For obtaining the starting models of linear and cyclic MBP complexed to H2-IA^s in order to examine their stability in the cavity with molecular dynamics simulations, the linear MBP_{83-99} peptide was modelled accordingly in the H2-IA^s MHC cleft. Cyclization between the amino group of K^{91} and C-terminal group of P^{99} was made manually onto the same model and subsequently minimized to obtain a low energy complex.

The molecular dynamics simulations showed quite a stable binding for the linear and cyclic MBP_{83-99} peptides. In the case of $cMBP_{83-99}$ the backbone RMSD of the peptide was stable at a value close to 4 Å, whereas for linear MBP_{83-99} higher fluctuations occurred (Figure 5A), basically because of the movement flexibility of residues P^{96}, R^{97}, T^{98} and P^{99}, which are not forming consistent interactions with H2-IA^s. The MHC backbone is stable throughout the simulations for both complexes (Figure 5B).

As mentioned before, the binding register for the two peptides is V^{87}, F^{90}, N^{92} and T^{95} occupying P1, P4, P6 and P9 pockets respectively as shown in the molecular dynamics simulations (Figure 5C,D). During the simulations, we observed that in both peptides the aliphatic side chain of V^{87} interacts in the highly hydrophobic P1 pocket with H24α, W31α, F32α and V86β and F^{90} forms hydrophobic interactions with F11β and V78β in pocket P4. N^{92} is located in P6, forming hydrogen bonds with T11α and Y30β, whereas T95 forms a stable hydrogen bond with D59β. The overall interaction of both linear and cyclic peptides with the H2-IA^s MHC remains stable, however there are distinct differences. First of all, the C-terminal of the linear peptide is highly mobile during the most of the simulation and finally rests in a stable position after the first 300 ns (FIgure 5A). This stabilizing orientation involves a network of hydrophobic interactions with Y58α, which is sandwiched by V^{94} and P^{99}. On the other hand, cyclization between K^{91} and P^{99} (fig. XD) results in less flexible $P^{96}RTP^{99}$ region (Figure 5D). This results to a consistent R^{97} sidechain topology, totally exposed as a potential TCR contact.

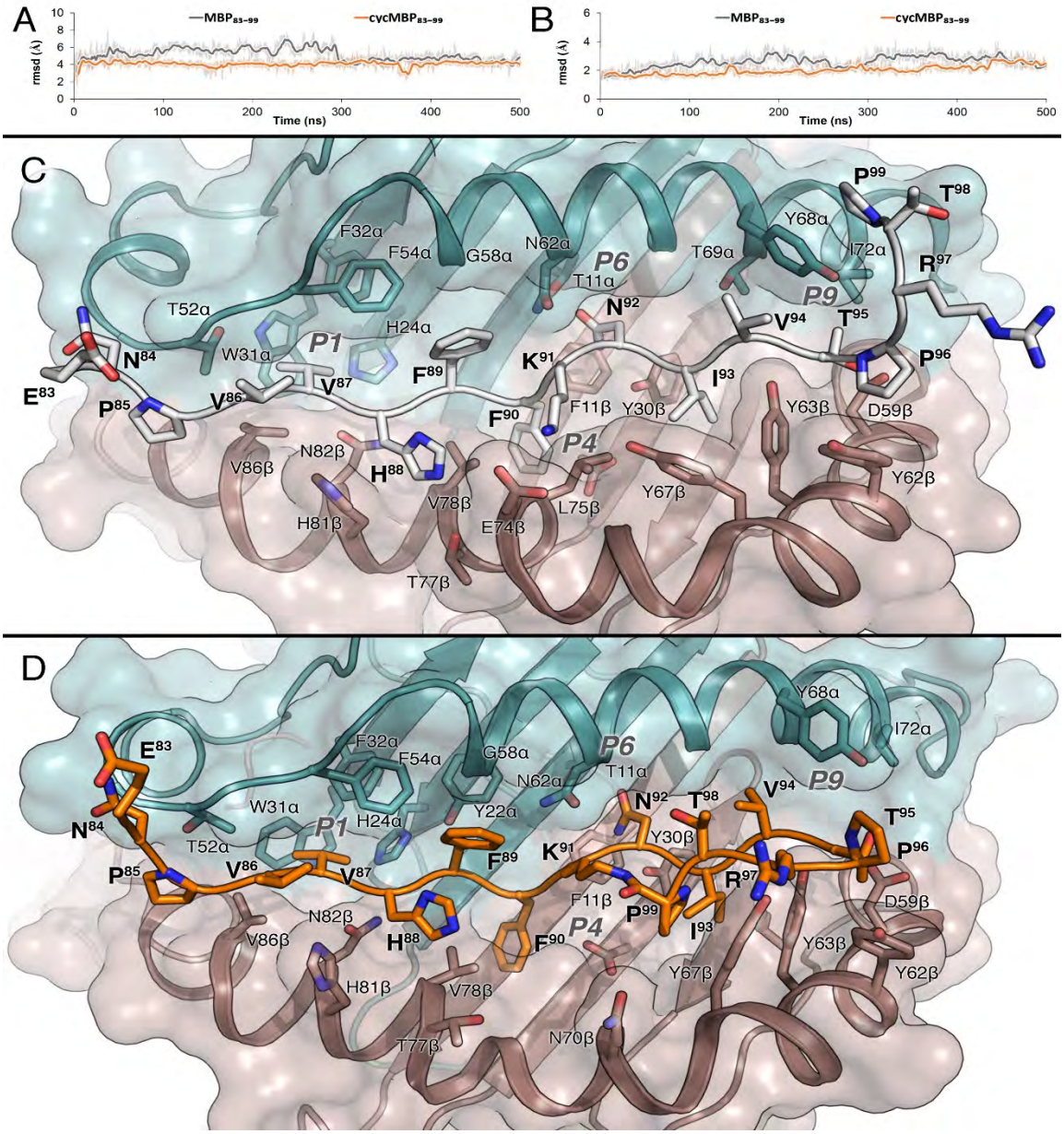
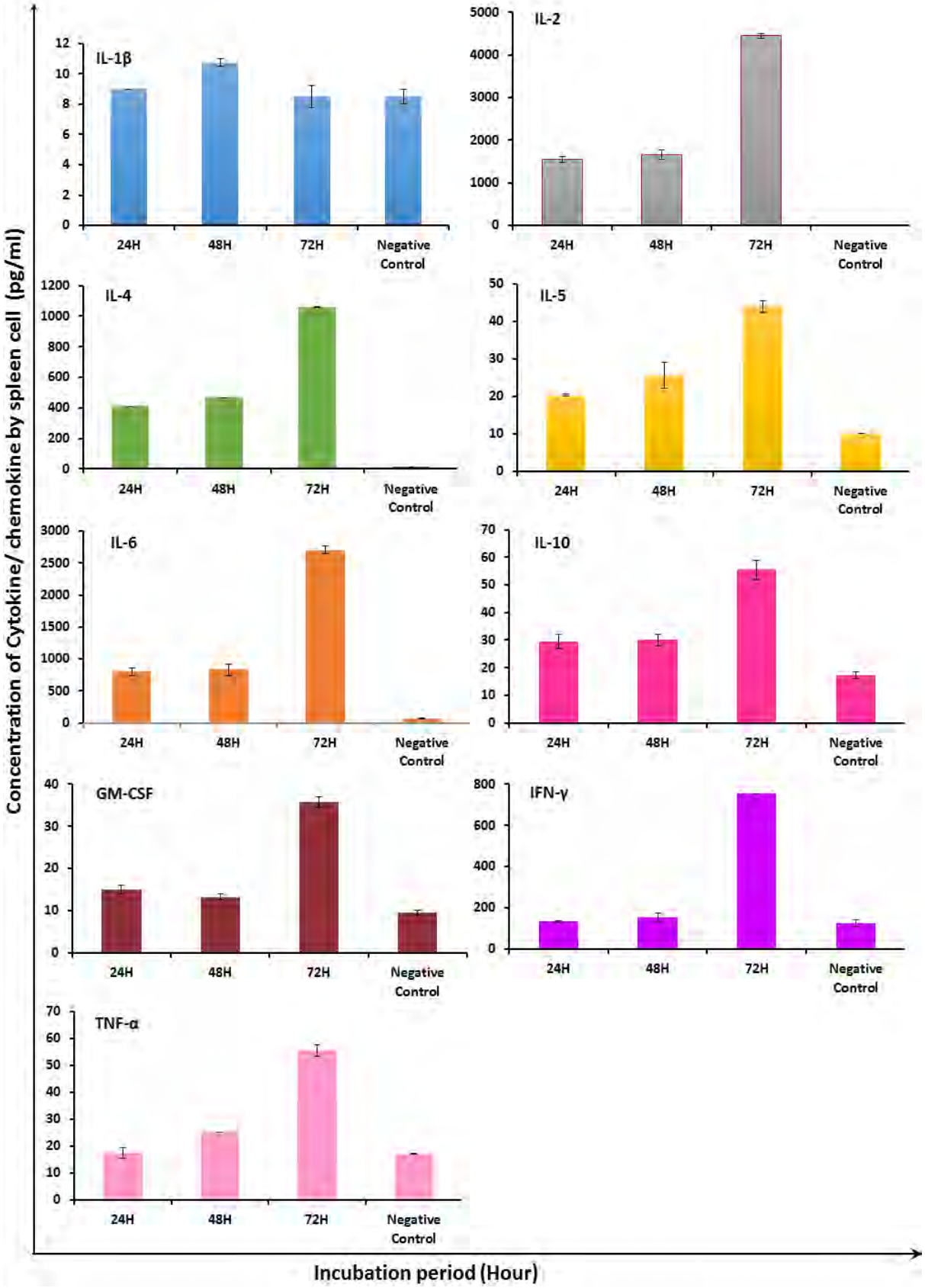
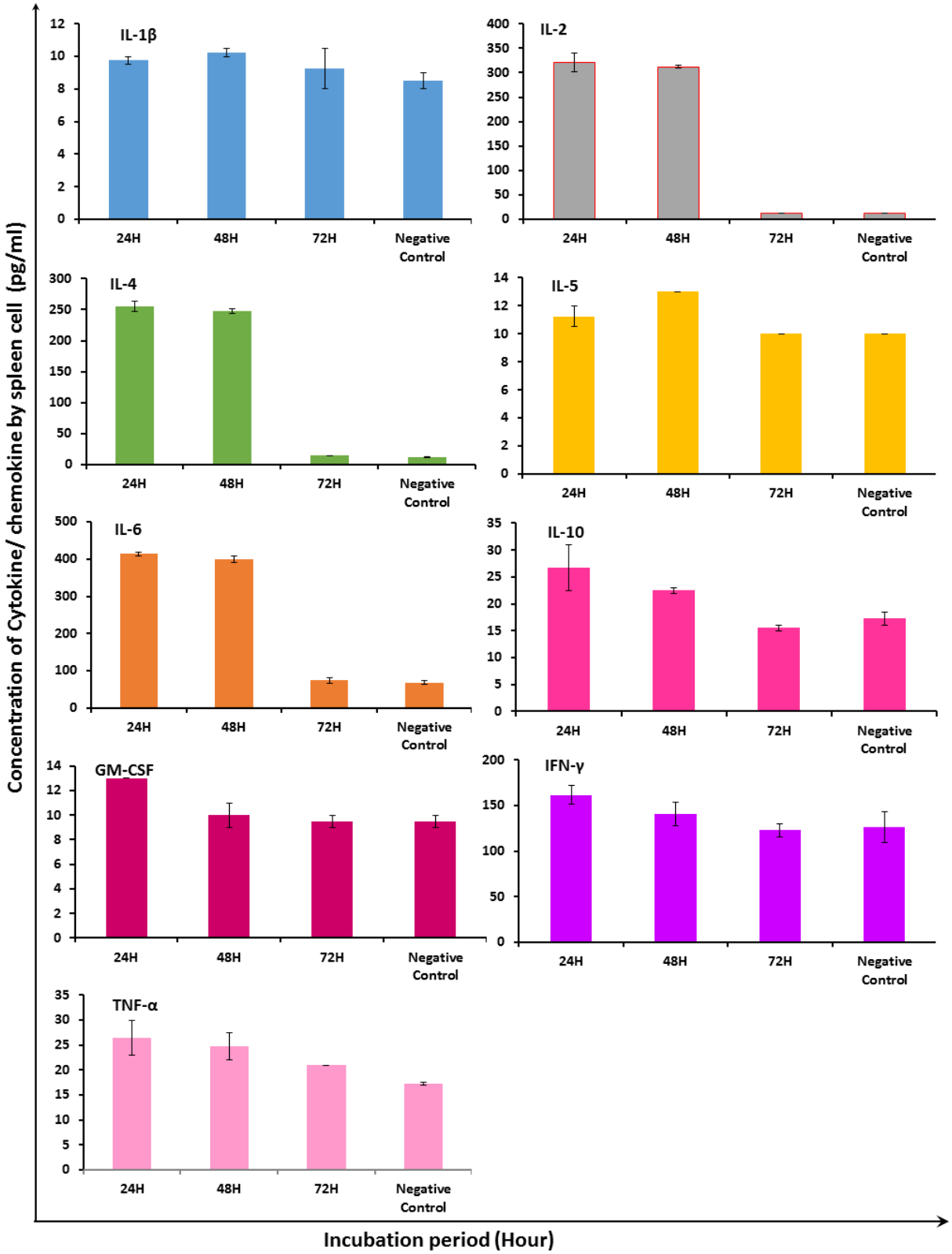


Figure 5. Root mean squared deviations from the molecular dynamics simulations of the A) linear MBP_{83-99} and cyclic MBP_{83-99} backbone atoms and B) H2-IA^s backbone atoms from the two simulations. Mean RMSD values are shown in bold lines. The C) linear and D) cyclic MBP_{83-99} representative binding orientations as extracted by the molecular dynamics simulations. MBP_{83-99} is shown in white and cyclic MBP_{83-99} in orange cartoon and stick representation respectively. Chains α and β of H2-IA^s are shown in teal and light brown colors respectively. Peptide numbering is shown in bold letters.*3.4. Immune modulation of linear and cMBP83-99 peptides conjugated to mannan.*

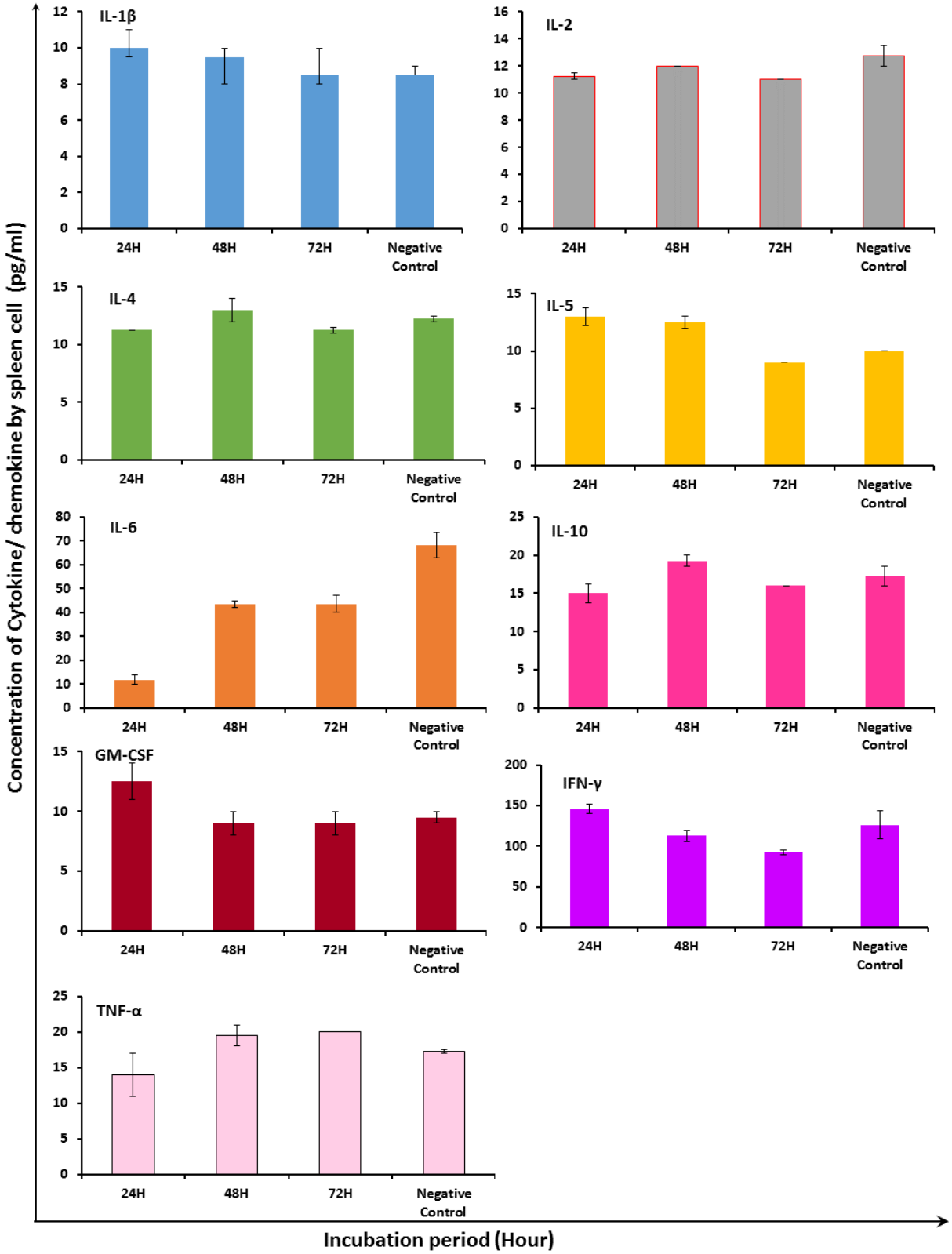
3.4.1. Immune modulation of oxidised mannan-(KG)$_5$-linear MBP$_{83-99}$



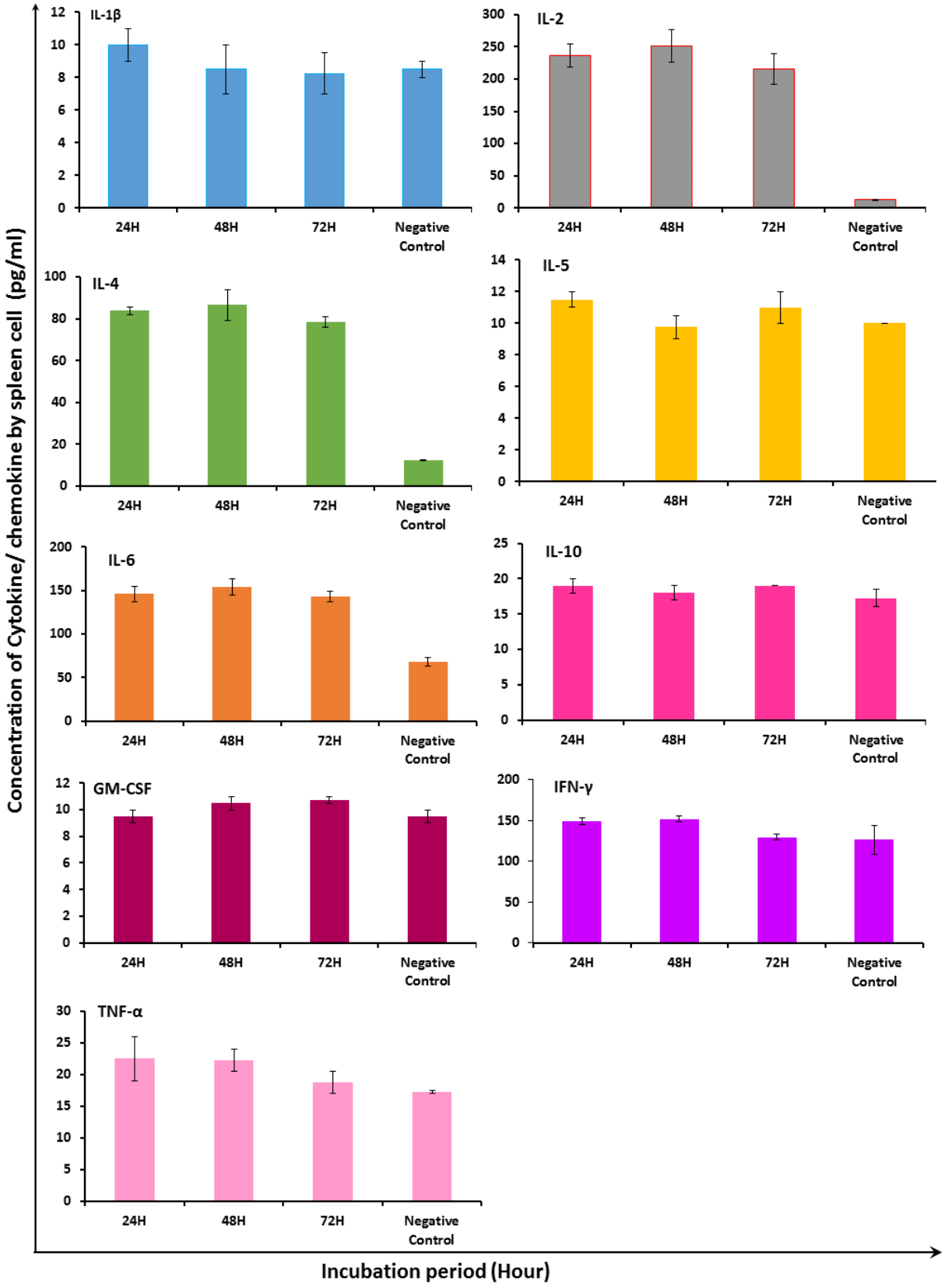
3.4.2. Immune modulation of reduced mannan-(KG)$_5$-linear MBP$_{83-99}$



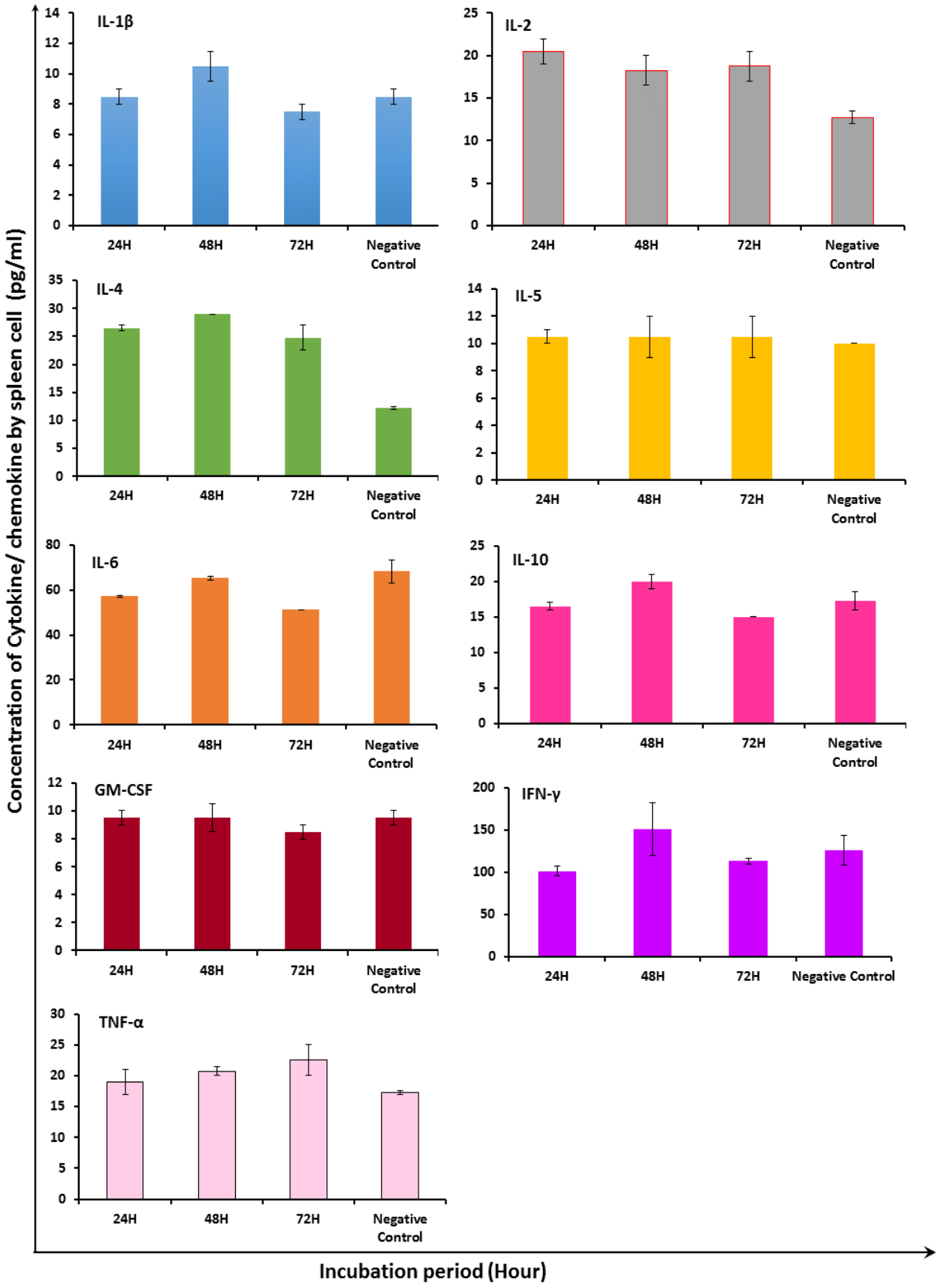
3.4.3. Vaccine 1c - Immune modulation of $(KG)_5$-cMBP$_{83-99}$



3.4.4. Vaccine 2c - Immune modulation of oxidized mannan-(KG)$_5$-cMBP$_{83-99}$

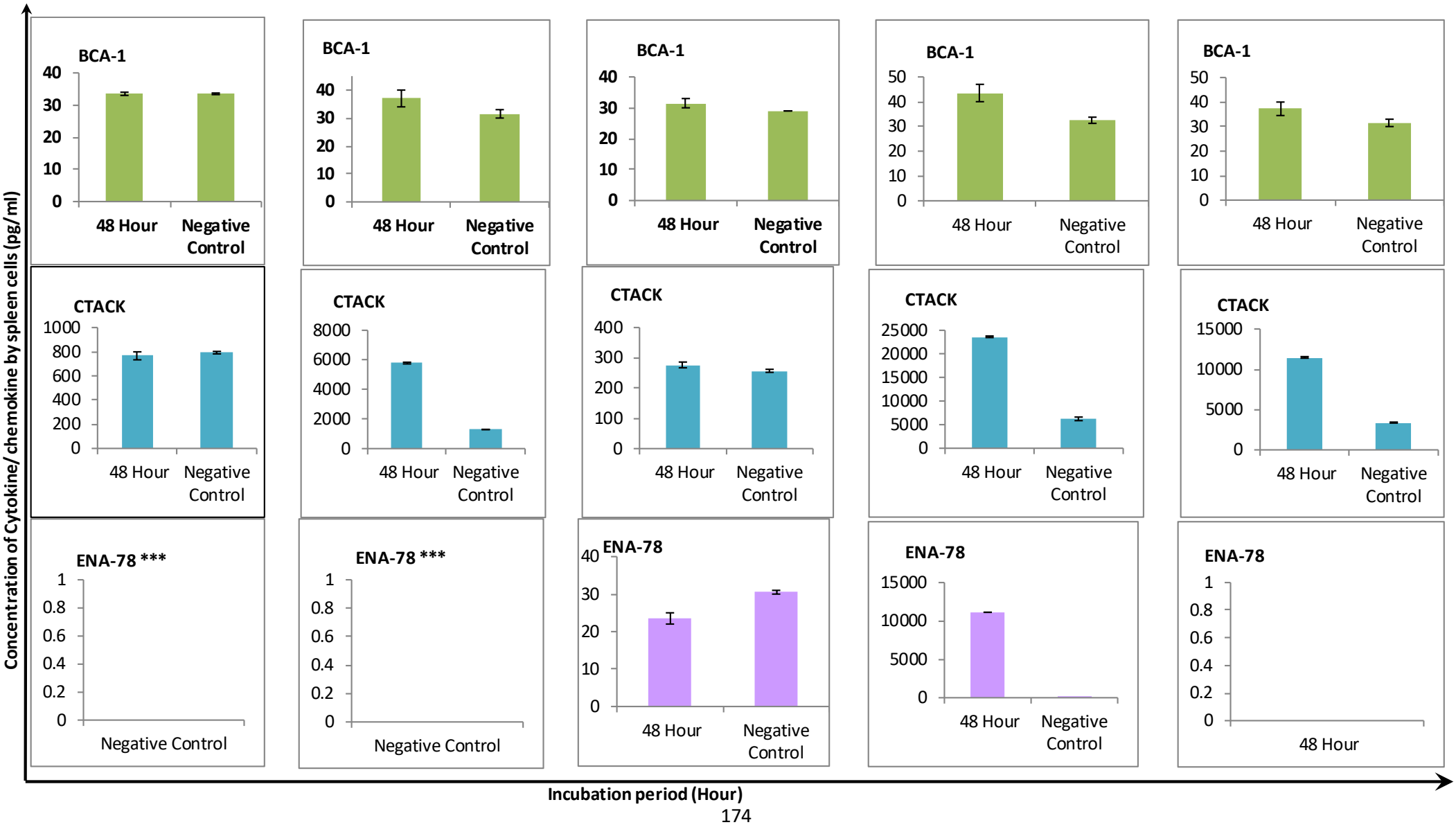


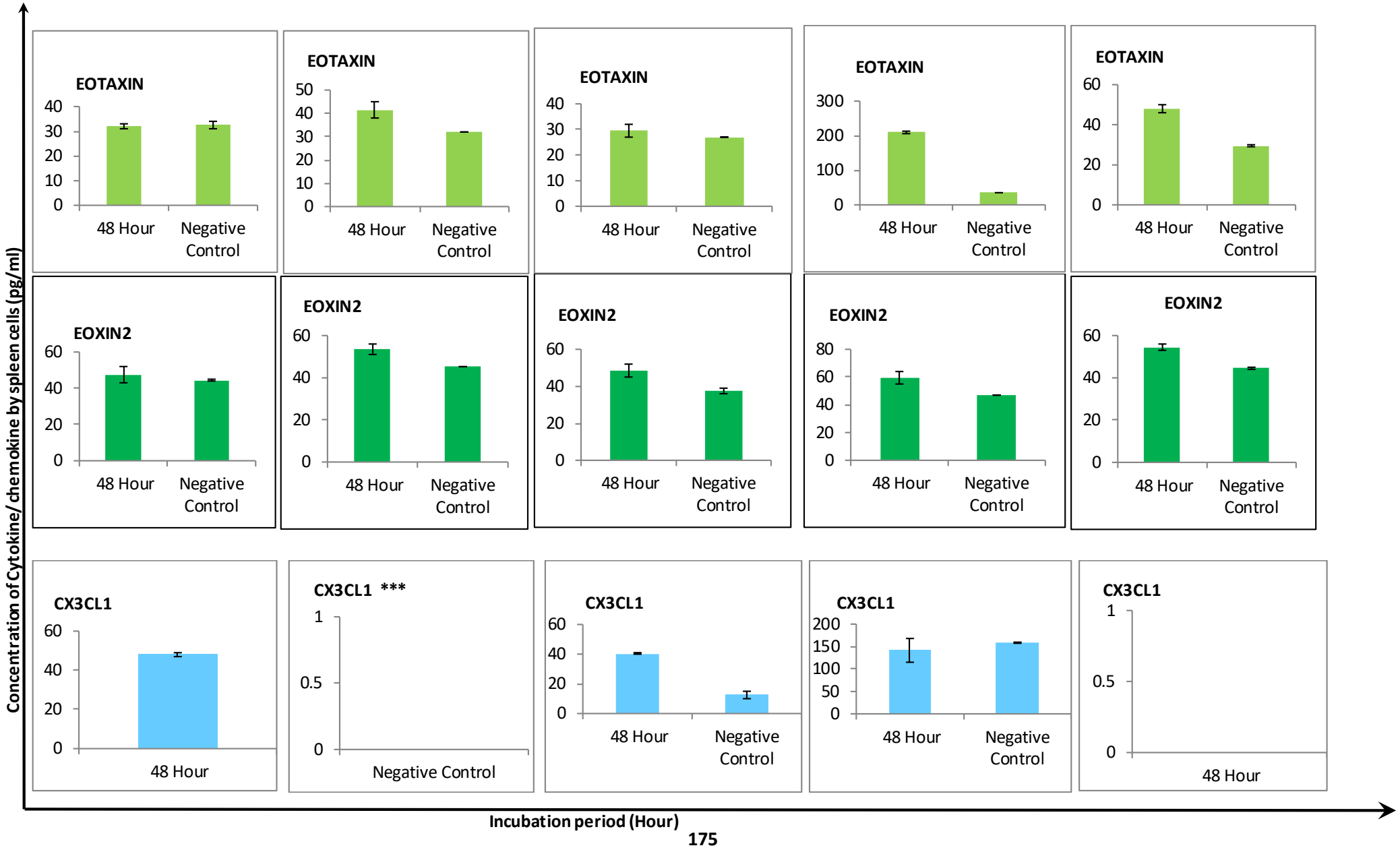
3.4.5. Vaccine 2c - Immune modulation of reduced mannan-$(KG)_5$-$cMBP_{83-99}$

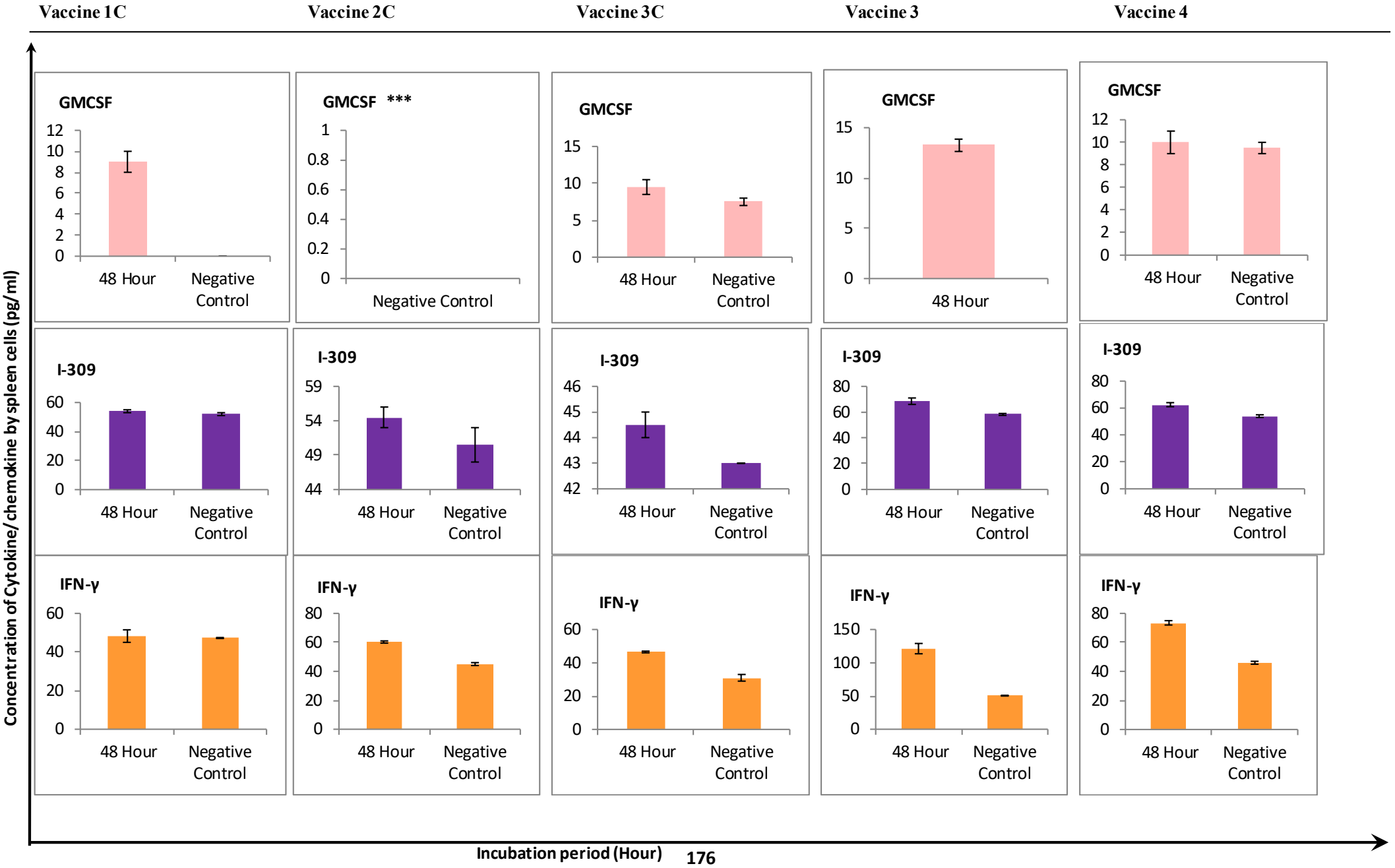


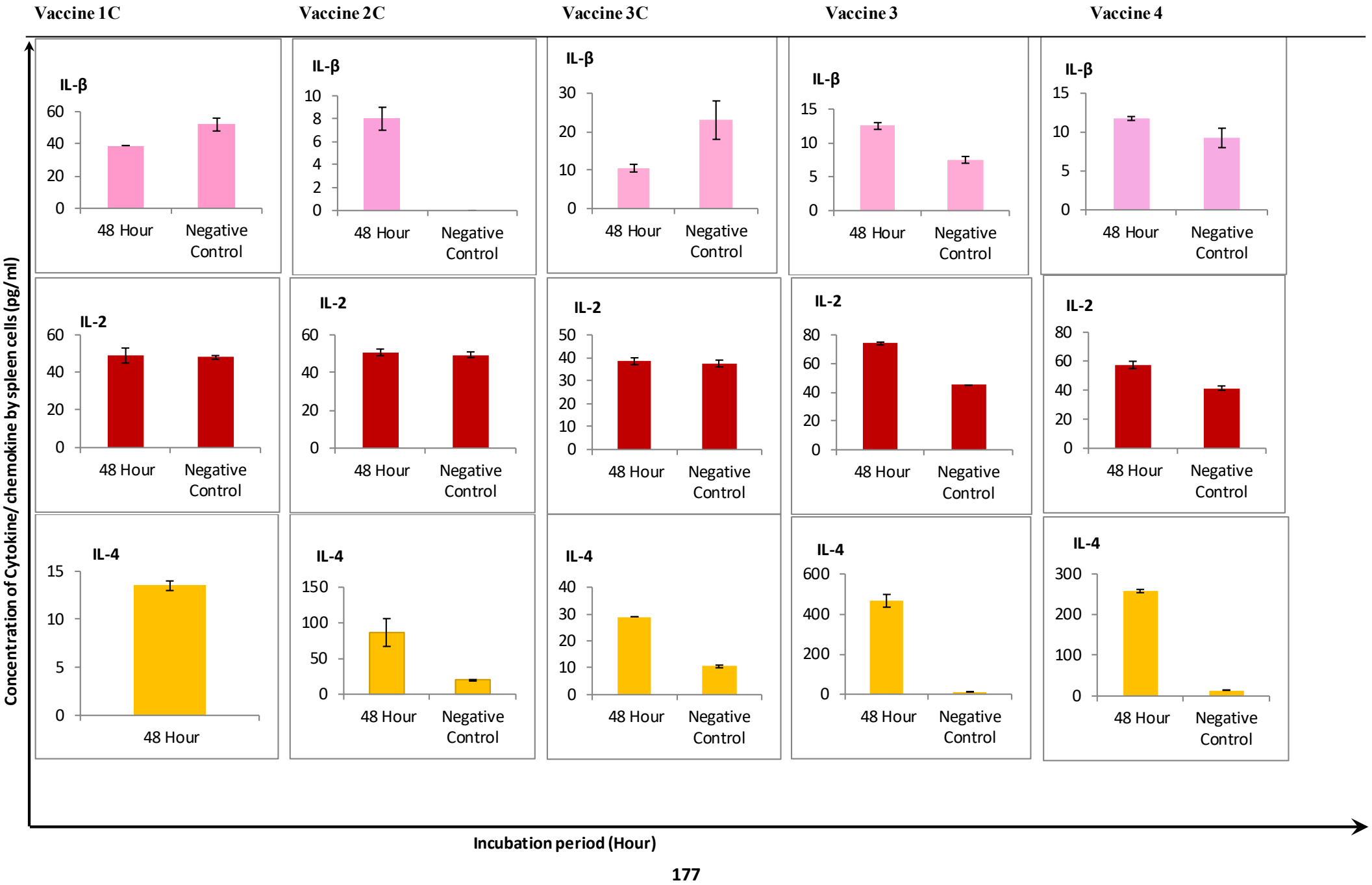
3.5. More comprehensive analysis

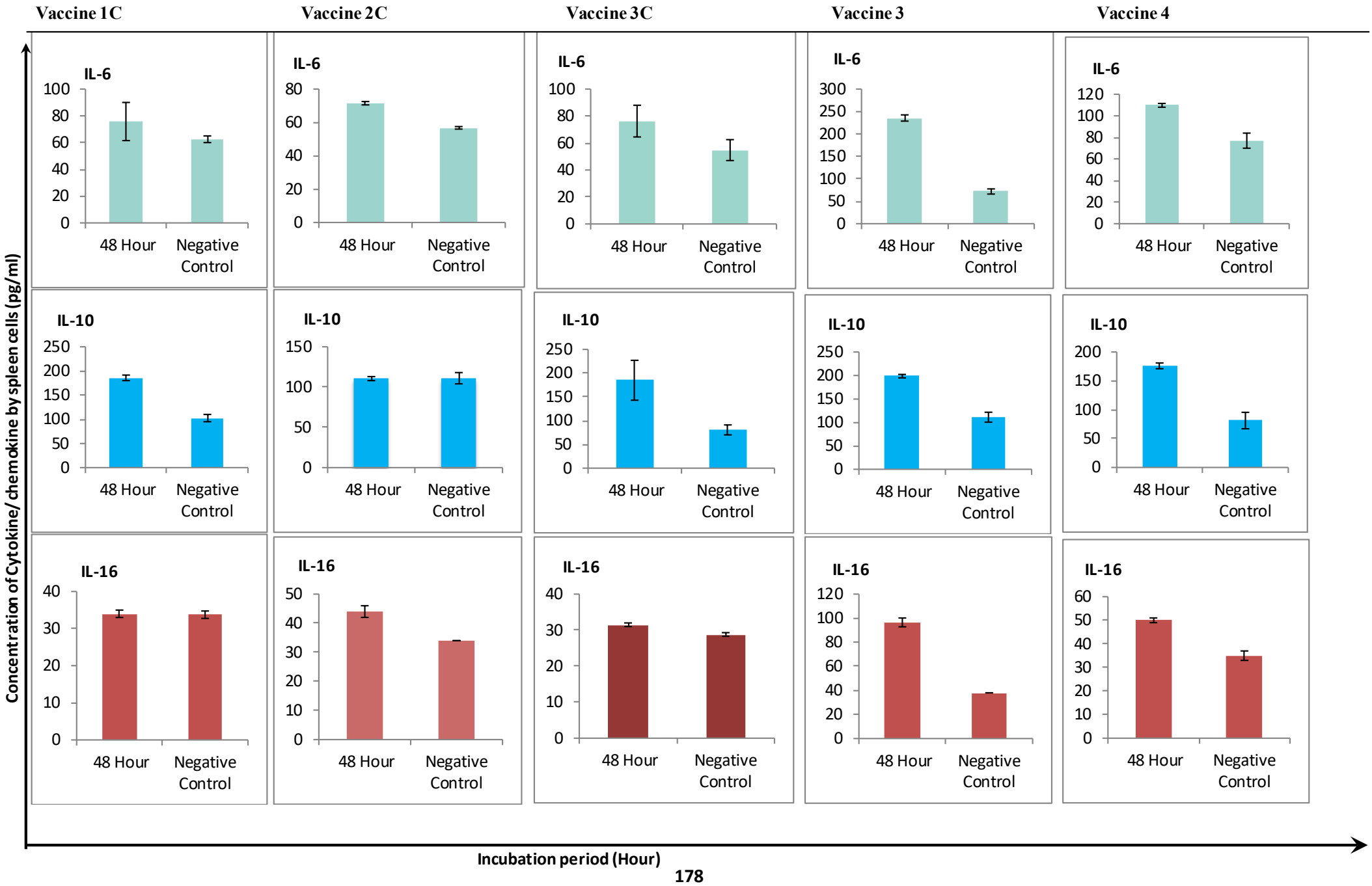
The 33-plex mouse cytokine/chemokine bioplex assay was conducted on the cyclic peptides as they showed the best differential responses

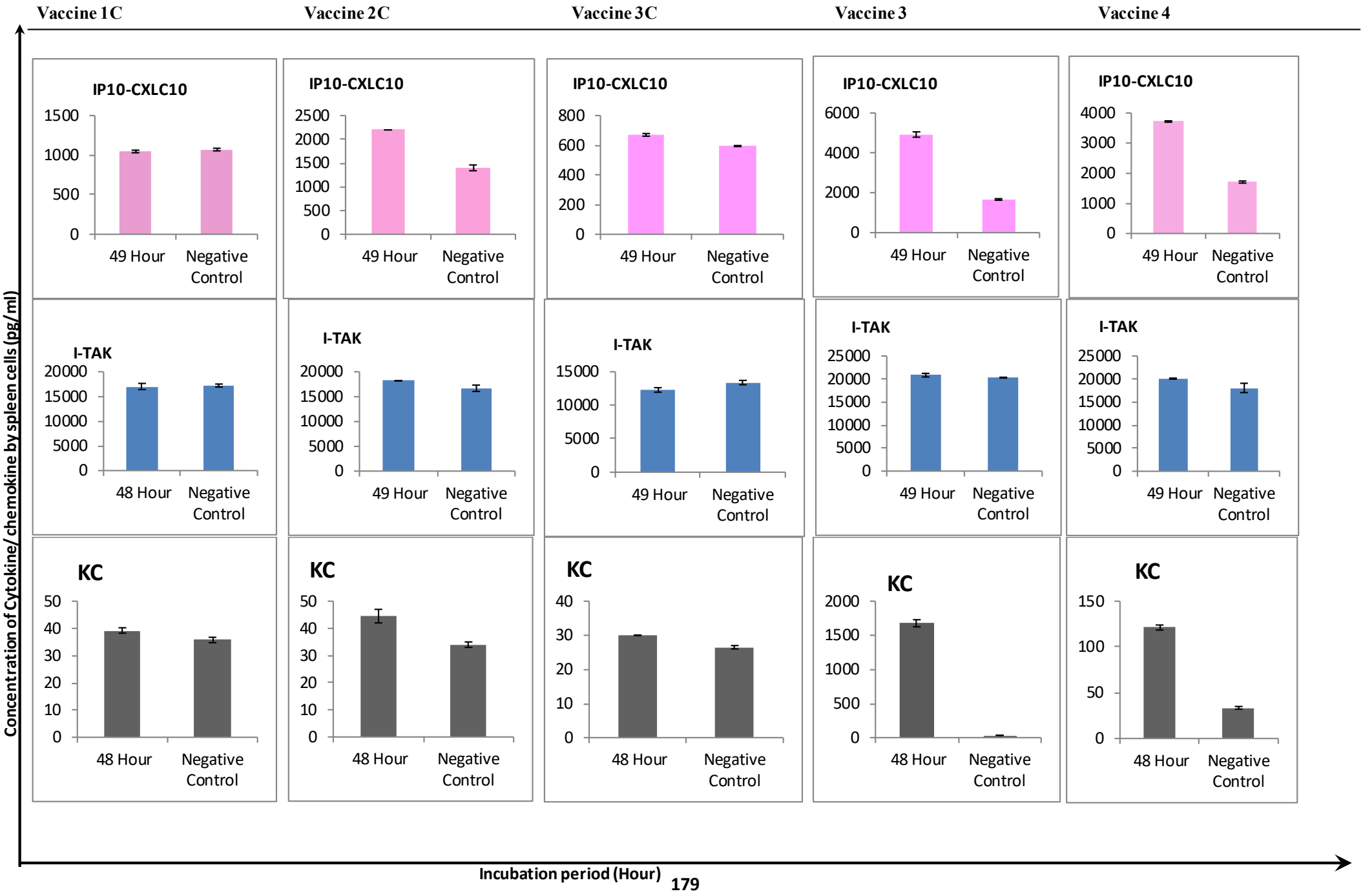

Vaccine 1C
Vaccine 2C
Vaccine 3C
Vaccine 3
Vaccine 4
Concentration of Cytokine/ chemokine by spleen cells (pg/ml)
BCA-1
CTACK
ENA-78 ***
ENA-78
48 Hour
Negative Control
Incubation period (Hour)

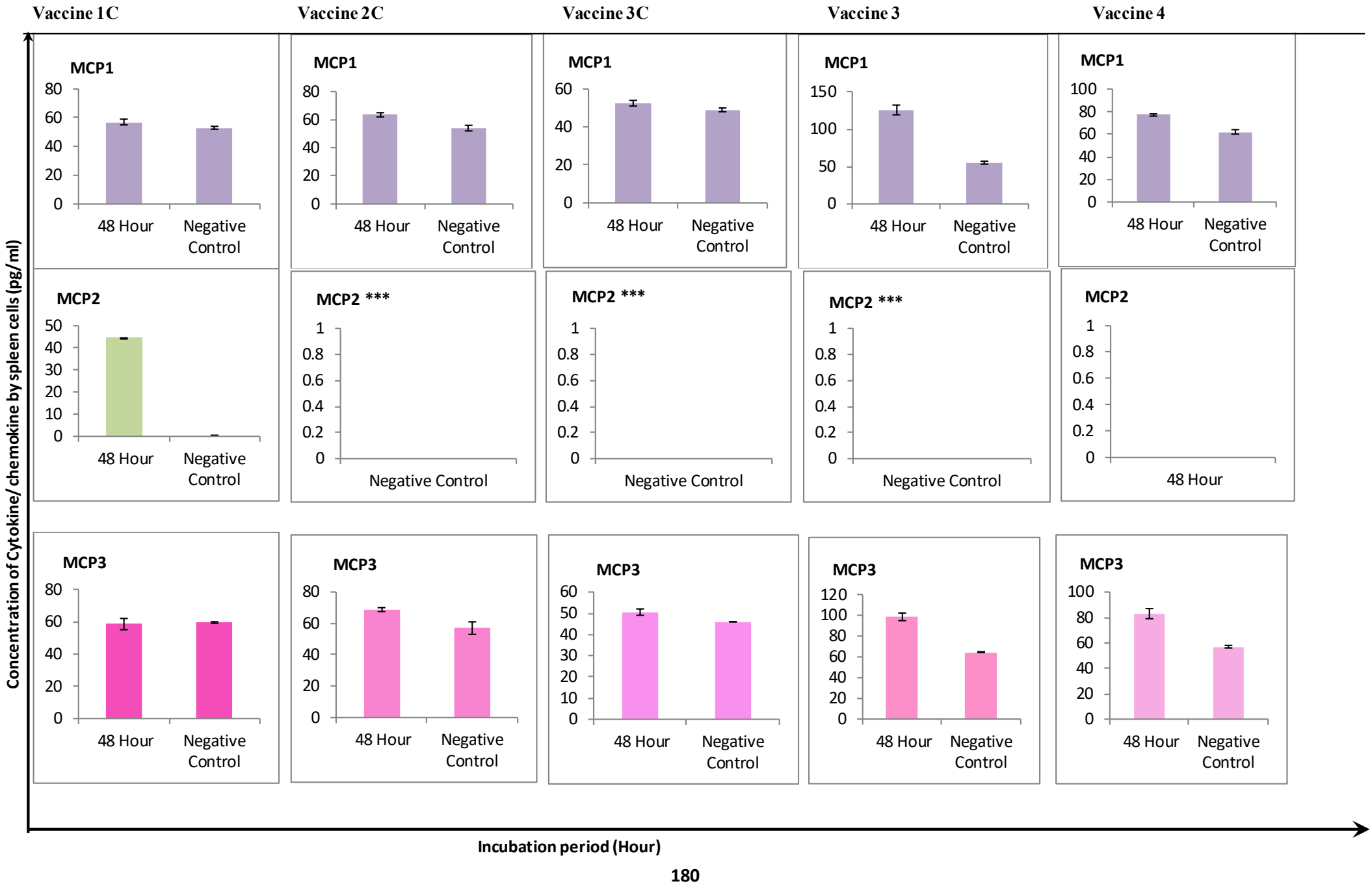

Vaccine 1C
Vaccine 2C
Vaccine 3C
Vaccine 3
Vaccine 4
EOTAXIN
EOXIN2
CX3CL1
CX3CL1 ***
48 Hour
Negative Control
Concentration of Cytokine/ chemokine by spleen cells (pg/ml)
Incubation period (Hour)

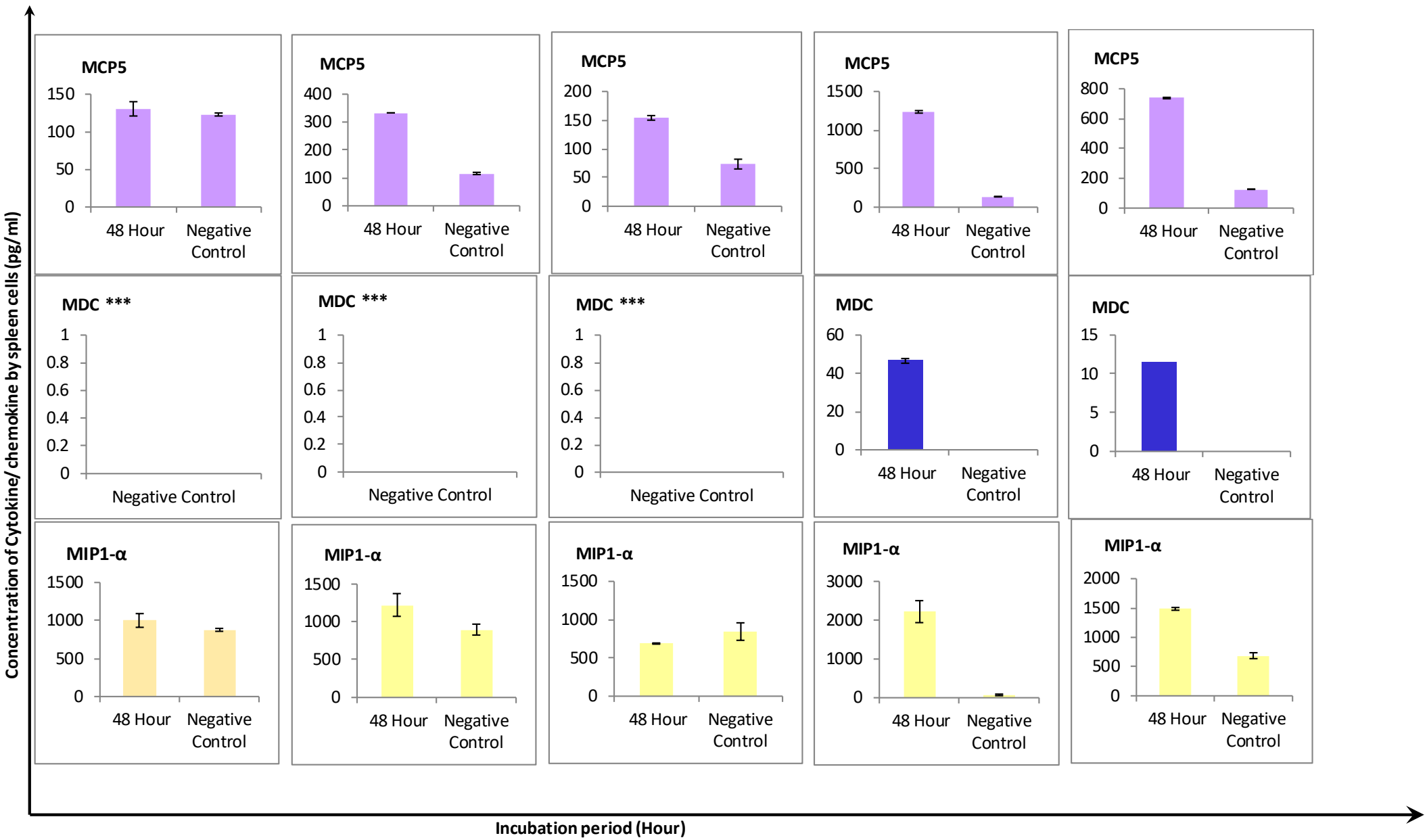

Vaccine 1C
Vaccine 2C
Vaccine 3C
Vaccine 3
Vaccine 4
GMCSF
GMCSF ***
I-309
IFN-γ
48 Hour
Negative Control
Concentration of Cytokine/ chemokine by spleen cells (pg/ml)
Incubation period (Hour)

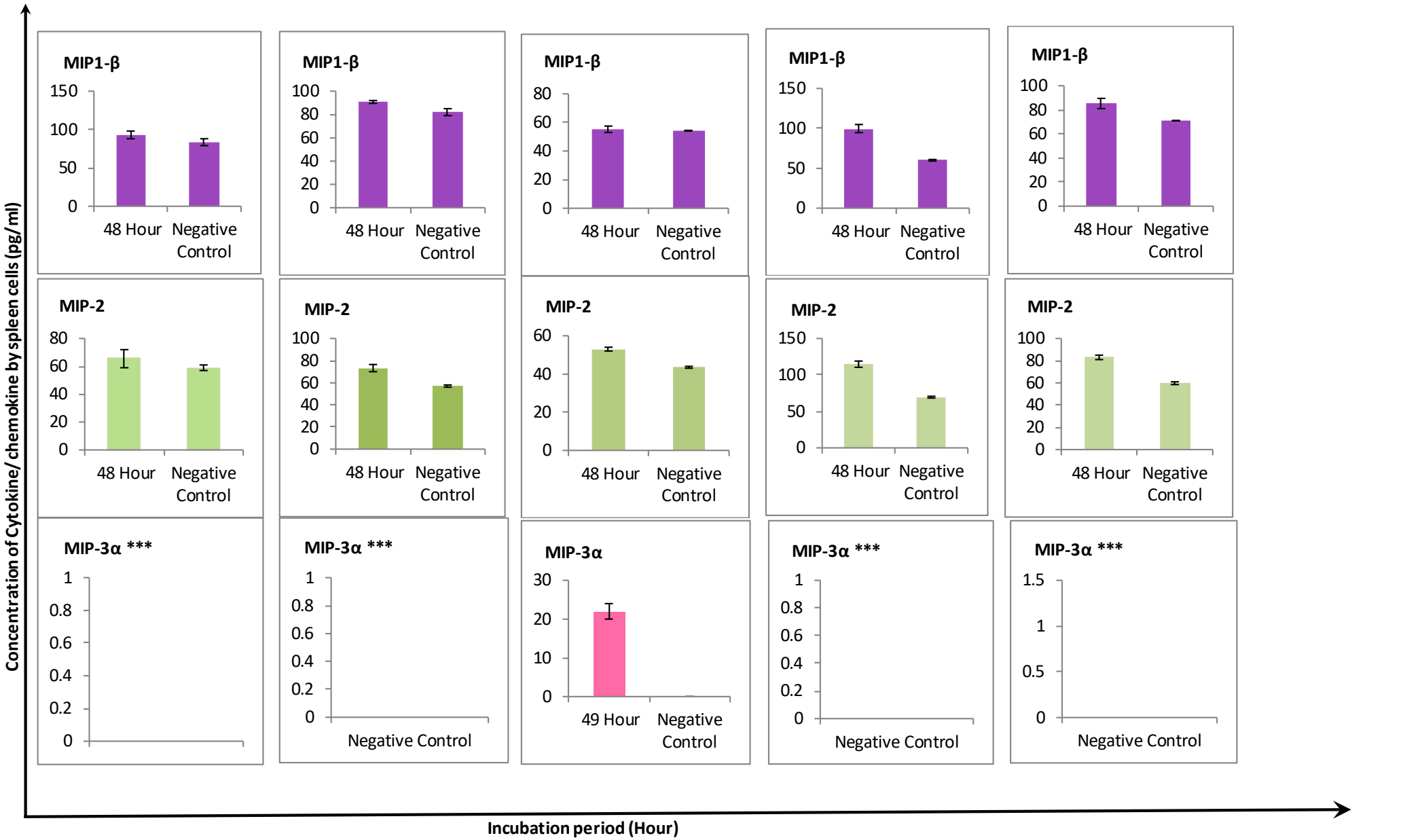

Vaccine 1C
Vaccine 2C
Vaccine 3C
Vaccine 3
Vaccine 4
IL-β
IL-2
IL-4
48 Hour
Negative Control
Concentration of Cytokine/ chemokine by spleen cells (pg/ml)
Incubation period (Hour)

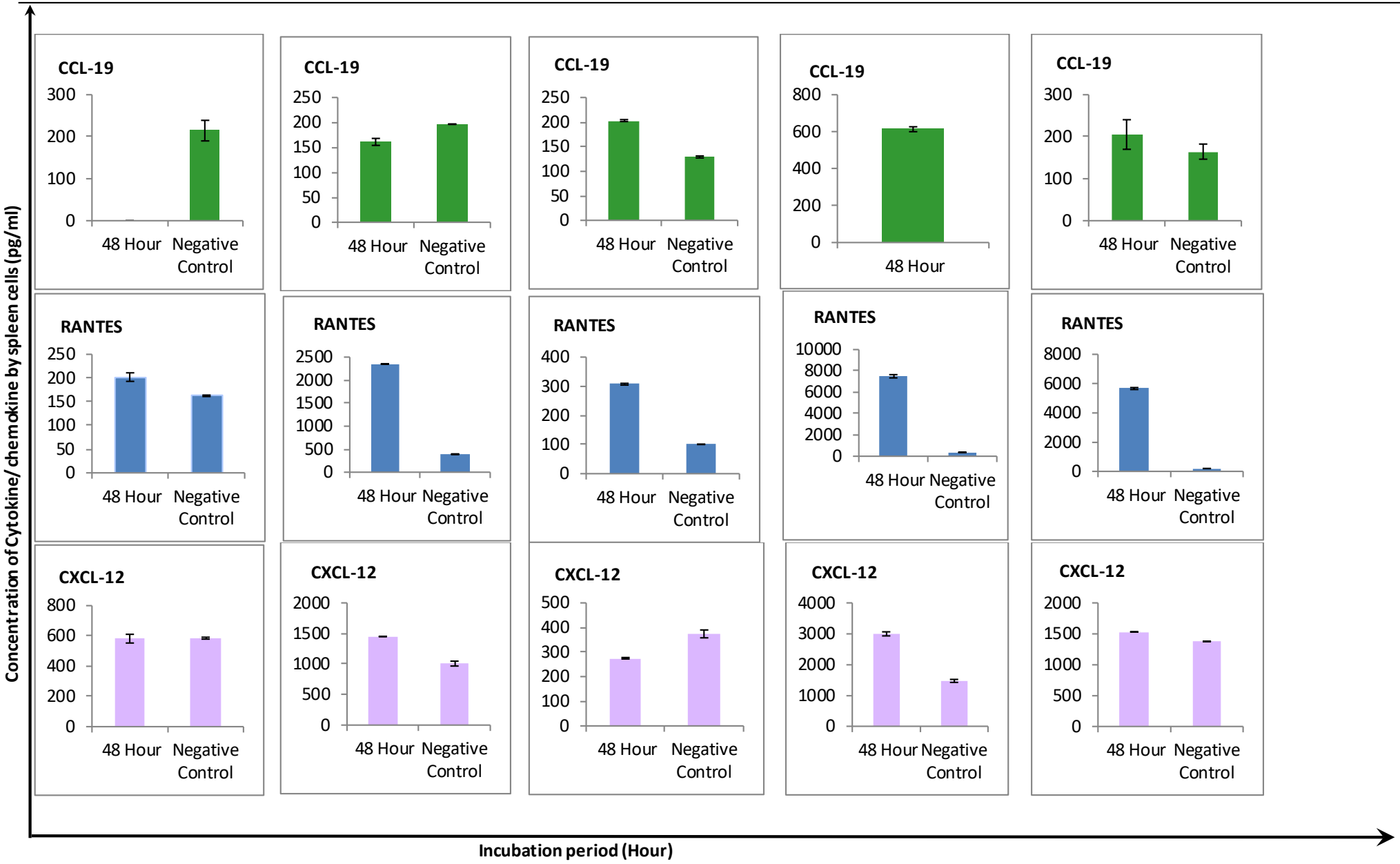

Vaccine 1C
Vaccine 2C
Vaccine 3C
Vaccine 3
Vaccine 4
IL-6
IL-10
IL-16
48 Hour
Negative Control
Concentration of Cytokine/ chemokine by spleen cells (pg/ml)
Incubation period (Hour)

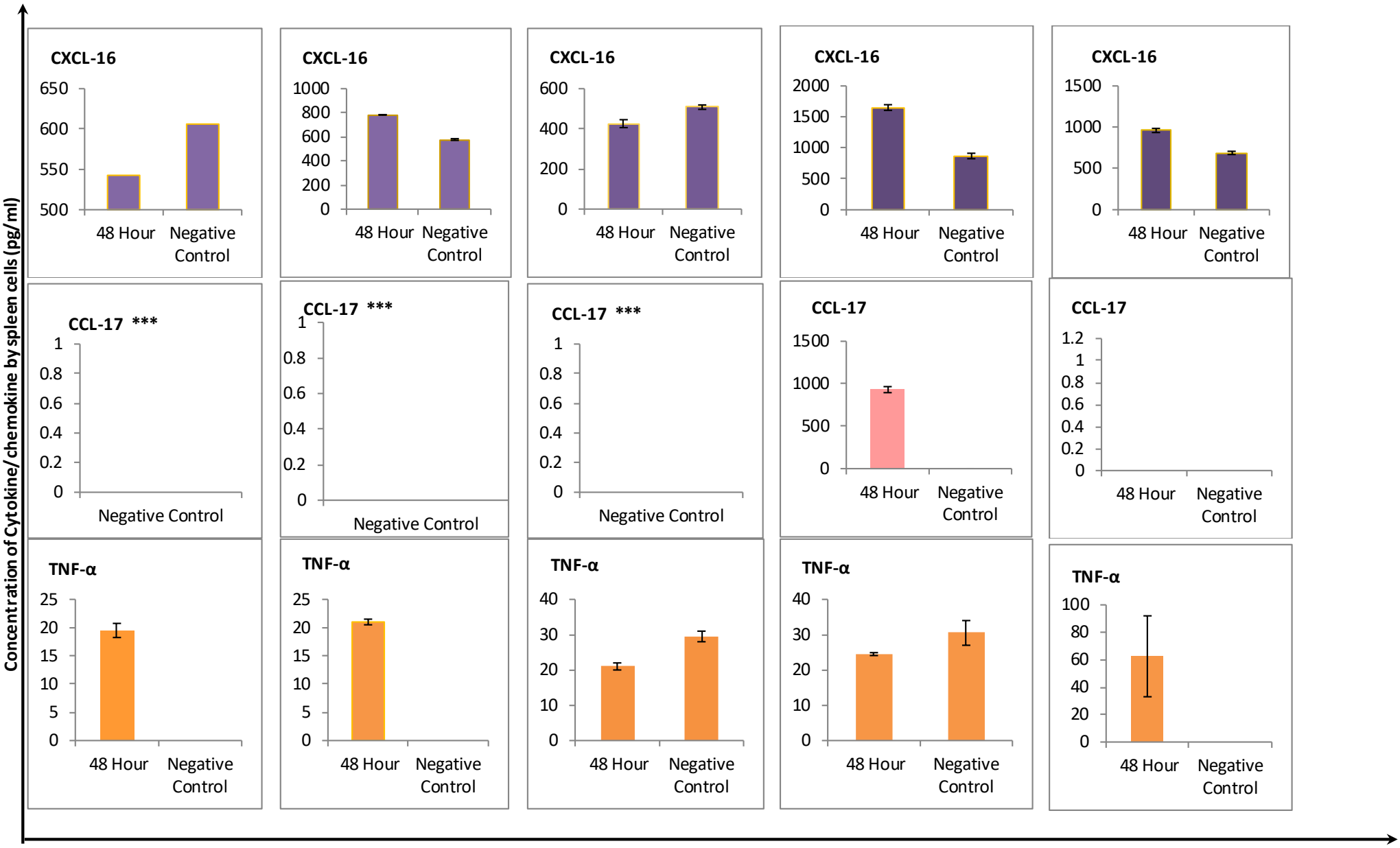

Vaccine 1C
Vaccine 2C
Vaccine 3C
Vaccine 3
Vaccine 4
IP10-CXLC10
I-TAK
KC
49 Hour
48 Hour
Negative Control
Concentration of Cytokine/ chemokine by spleen cells (pg/ml)
Incubation period (Hour)


Vaccine 1C
Vaccine 2C
Vaccine 3C
Vaccine 3
Vaccine 4
MCP1
MCP2
MCP2 ***
MCP3
48 Hour
Negative Control
Concentration of Cytokine/ chemokine by spleen cells (pg/ml)
Incubation period (Hour)


Vaccine 1C
Vaccine 2C
Vaccine 3C
Vaccine 3
Vaccine 4
MCP5
MDC ***
MDC
MIP1-α
48 Hour
Negative Control
Concentration of Cytokine/ chemokine by spleen cells (pg/ml)
Incubation period (Hour)


Vaccine 1C
Vaccine 2C
Vaccine 3C
Vaccine 3
Vaccine 4
MIP1-β
MIP-2
MIP-3α ***
48 Hour
Negative Control
49 Hour
Concentration of Cytokine/ chemokine by spleen cells (pg/ml)
Incubation period (Hour)


Vaccine 1C
Vaccine 2C
Vaccine 3C
Vaccine 3
Vaccine 4
CCL-19
RANTES
CXCL-12
48 Hour
Negative Control
Concentration of Cytokine/ chemokine by spleen cells (pg/ml)
Incubation period (Hour)


Vaccine 1C
Vaccine 2C
Vaccine 3C
Vaccine 3
Vaccine 4
Concentration of Cytokine/chemokine by spleen cells (pg/ml)
CXCL-16
650
600
550
500
48 Hour
Negative Control
CXCL-16
1000
800
600
400
200
0
48 Hour
Negative Control
CXCL-16
600
400
200
0
48 Hour
Negative Control
CXCL-16
2000
1500
1000
500
0
48 Hour
Negative Control
CXCL-16
1500
1000
500
0
48 Hour
Negative Control
CCL-17 ***
1
0.8
0.6
0.4
0.2
0
Negative Control
CCL-17 ***
1
0.8
0.6
0.4
0.2
0
Negative Control
CCL-17 ***
1
0.8
0.6
0.4
0.2
0
Negative Control
CCL-17
1500
1000
500
0
48 Hour
Negative Control
CCL-17
1.2
1
0.8
0.6
0.4
0.2
0
48 Hour
Negative Control
TNF-α
25
20
15
10
5
0
48 Hour
Negative Control
TNF-α
25
20
15
10
5
0
48 Hour
Negative Control
TNF-α
40
30
20
10
0
48 Hour
Negative Control
TNF-α
40
30
20
10
0
48 Hour
Negative Control
TNF-α
100
80
60
40
20
0
48 Hour
Negative Control
Incubation period (Hour)

4. Discusion and conclusion

Co-injection of the $cMBP_{83-99}$ peptide at disease induction at 1:1 ratio seems to offer a mild clinical benefit which depends on the severity of EAE and is not as strong as the one observed in analogous experiments in Lewis rats' MBP-EAE (T. Tselios et al., 2002a; T. Tselios et al., 2000). The data suggests that the observed benefit in C57BL/6 mice' MOG-EAE is not always statistical significant and is dependent on the clinical characteristics of the produced EAE (inter-group variability, disease severity and aggressiveness). Another explanation is that our cMBP does induce a direct 1:1 antagonist effect, as is the case with the cyclic MOG peptide (Lourbopoulos et al., 2017a), and thus does not produce a strong clinical effect.

As far as histopathology is concerned, the results indicate that there is a quantitative similar inflammatory burden within the spinal cords of the 2 groups (control and $cMBP_{83-99}$) during the acute phase. This inflammatory load is cleared in both groups, as they progress to chronic phase, but the clearance tends to be more efficiently in cMBP group and leads to a stronger reduction of inflammatory cells within their spinal cords. At the same time, the cMBP mice had in general less demyelination and milder axonopathic processes compared to controls, suggesting a less offensive inflammatory process in these animals. Thus, it is probable that the equal numbers of infiltrating cells during acute phase of the disease do not necessary correspond to equal degenerative capacity of these cells within the parenchyma. Such a scenario could explain the reduced demyelination, reduced axonopathic processes and increased clearance of the inflammatory cells as the disease progressed to chronicity (Abromson-Leeman et al., 2004; Bauer et al., 1998; Berger et al., 1997; Lassmann, 2007; Pender & Rist, 2001; Suvannavejh, Dal Canto, Matis, & Miller, 2000).

Thus, $cMBP_{83-99}$ peptide is inducing some beneficial effects in the C57BL/6 MOG-EAE; however this effect is not as strong as in MBP-EAE in Lewis rats. More experiments are needed to clarify possible stronger or milder effects on the chronic model of MOG-EAE, testing different ratio schemes and routes of administration in the C57BL/6 mice.

Molecular modeling and. Molecular dynamics was undertaken to gain insights of the structural interactions with MHC class II, in order to understand the effects noted of cMBP peptide in EAE and histopathological experiments. It is evident that cyclization does not interfere significantly to the binding of the peptide and cyclization may contributes to the binding affinity due to conformational strain.

Conjugation of cMBP to mannan in reduced and oxidised forms shows immune modulation following mouse injections and recalling of peptide following cytokine analysis using bioplex bead arrays. cMBP in particular induces a pre-dominant anti-inflammatory profile when conjugated to oxidised mannan and warrants further studies as a vaccine immune-modulator.

In the next section (chapter 5b) the agonist peptide MBP_{83-96} (the shorter version of MBP_{83-99} peptide) is used to antagonize immune responses in paticular to antagonise T cell receptor binding. Due to the university regulation for a minimum 60% contribution by candidate as requirement for including published paper in the thesis, this paper has been removed from this chapter and included in the appendix. I am only using biological studies (animal model) from this published paper, for a full version of paper see appendix.

5 b– Design and Synthesis of Non-Peptide Mimetics Mapping the Immunodominant Myelin Basic Protein ($MBP_{83–96}$) Epitope to Function as T-Cell Receptor Antagonists

ABSTRACT

Encephalitogenic T cells are heavily implicated in the pathogenesis of multiple sclerosis (MS), an autoimmune demyelinating disease of the central nervous system. Their stimulation is triggered by the formation of a trimolecular complex between the human leukocyte antigen (HLA), an immunodominant myelin basic protein (MBP) epitope, and the T cell receptor (TCR). We detail herein our studies directed towards the rational design and synthesis of non-peptide mimetic molecules, based on the immunodominant $MBP_{83–96}$ epitope that is recognized by the TCR in complex with HLA. We focused our attention on the inhibition of the trimolecular complex formation and consequently the inhibition of proliferation of activated T cells. A structure-based pharmacophore model was generated, in view of the interactions between the TCR and the HLA-$MBP_{83–96}$ complex. As a result, new candidate molecules were designed based on lead compounds obtained through the ZINC database. Moreover, semi-empirical and density functional theory methods were applied for the prediction of the binding energy between the proposed non-peptide mimetics and the TCR. We synthesized six molecules that were further evaluated *in vitro* as TCR antagonists. Analogues **15** and **16** were able to inhibit to some extent the stimulation of T cells by the immunodominant $MBP_{83–99}$ peptide from immunized mice. Inhibition was followed to a lesser degree by analogues **17** and **18** and then by analogue **19**. These studies show that lead compounds **15** and **16** may be used for immunotherapy against MS.

Keywords: multiple sclerosis; trimolecular complex; rational drug design; non-peptide mimetics; molecular modeling; cell proliferation; T cell antagonism

1. Introduction

Multiple sclerosis (MS) is an immunologically controlled, inflammatory, demyelinating disease, described as the destruction of the myelin sheath of the central nervous system, which can lead to paralysis (Sospedra & Martin, 2005a; L. Steinman, 1996). Although evidence suggests the important role of B-cells (auto-antibodies), T helper (Th)-17 cells, and $CD8^+$ T cells in disease pathogenesis (Mouzaki et al., 2015), it is well regarded that $CD4^+$ Th1 cells contribute to initiation and progression of disease. Indeed, $CD4^+$ T cells have been identified in patients with MS to react to self-peptide epitopes within the myelin sheath, including that of myelin basic protein (MBP), proteolipid protein, myelin oligodendrocyte glycoprotein, and myelin associated glycoprotein (Ben-Nun et al., 2006; Wucherpfennig et al., 1997). In the context of MS, encephalitogenic T cells are activated through the formation of a trimolecular complex between the T cell receptor (TCR), a short 14–18 amino acid myelin peptide (epitope), and the major histocompatibility complex (MHC) class II. In fact, the MHC class II, human leukocyte antigen (HLA) locus is the most closely correlated genetic locus to the development of MS, in particular HLA-DR1, HLA-DR2, and HLA-DR4 (International Multiple Sclerosis Genetics et al., 2007; Moise et al., 2015; Shahrizaila & Yuki, 2011). In humans, the MHC class II (HLA) consists of dimers (the α chain and the β chain) (Adams & Luoma, 2013; Madden, 1995), which present short antigenic peptide epitopes to $CD4^+$ Th cells, resulting in the formation of the trimolecular complex (HLA-peptide-TCR). The TCR is also composed of two different polypeptide chains (α and β chains) that consist of variable domains (complementarity determining regions; CDRs). CDRs are implicated in the recognition of the TCR to HLA-peptide complex, and their structural diversity plays a crucial role in the recognition of the different antigens presented to T cells by antigen presenting cells (Feng et al., 2015; X. Yang et al., 2015). In fact, there are more than 2.5×10^7 unique TCR (CDRs) structures in humans (X. Yang et al., 2015). In addition, the rigorous positive and negative selection process of T cells in the thymus does not prevent auto-reactive T cells from escaping thymic deletion (Buckley et al., 2015; Hesnard et al., 2015; Lessard et al., 2012), thus initiating the development of autoimmune disorders such as MS.

In patients with MS, T cell responses are primarily associated with recognition of the 81–105 region of MBP (QDENPVVHFFKNIVTPRTPPPSQGK) (Valli et al., 1993), with the $MBP_{83–99}$ (ENPVVHFFKNIVTPRTP) peptide epitope displaying the strongest binding to HLA-DR2 (Martin et al., 1991; Ota et al., 1990), $MBP_{83–96}$ being the minimal recognized epitope. T cell recognition of $MBP_{83–96}$ has also been shown in healthy individuals, albeit at relatively low precursor frequencies (Bieganowska et al., 1997). Hence, the immunodominant $MBP_{83–96}$ epitope plays an important role at inducing $CD4^+$ T cells, which contribute to the demyelination process, and it is therefore considered one of the main targets for developing molecular therapeutics (Mantzourani, Platts, Brancale, Mavromoustakos, & Tselios, 2007; Spyranti et al., 2007). The primary binding residues of $MBP_{83–96}$ to HLA-DR2 are via hydrophobic V^{87} and F^{90}, which anchor the peptide into pockets P1 and P4, respectively, as noted in the HLA-DR2-peptide-TCR crystal structure (M. Hahn, M. J. Nicholson, J. Pyrdol, & K. W. Wucherpfennig, 2005); albeit at a low resolution of 3.5 A, this structure served as the basis of all future studies of MBP peptides interacting with HLA-DR2. Additionally, other crystal structures reported in the RCSB databank (D. K. Sethi et al., 2011; Yin, Li, Kerzic, Martin, & Mariuzza, 2011) that address the role of MBP immunodominant epitopes in MS induction contain the same TCR sequence. Furthermore, it was noted that a second step in the T cell activation process involves the recognition of His^{88} and Phe^{89}, which are placed in pockets P2 and P3 of the TCR (M. Hahn et al., 2005), with secondary binding residues being Val^{86} and Lys^{91}, which are oriented in pockets P-1 and P5 of the TCR (M. Hahn et al., 2005). Thus, a detailed analysis of the interactions between HLA-$MBP_{83–96}$-TCR complexes would lead to valuable information towards rational design of non-peptide mimetics with inhibitory activity. Indeed, a number of studies have shown that using antagonist peptides (1–2 amino acid mutations to TCR contact residues), or altered peptide ligands, can effectively modulate T cell responses and switch from pro- to anti-inflammatory responses (Apostolopoulos et al., 2017a; Katsara, Deraos, et al., 2008b; Katsara, Matsoukas, et al., 2008a; Katsara, Minigo, et al., 2008a; Katsara et al., 2006; Katsara et al., 2008a, 2008b; Katsara, Yuriev, et al., 2009b; Matsoukas et al., 2005b; Tapeinou et al., 2015; T. Tselios et al., 1999). In addition, using a computational structure-based approach, non-peptide mimetics of small organic compounds that were able to bind to MHC class II and block the presentation of $MBP_{152-185}$ to auto-reactive T cells were identified (Koehler et al., 2004).

The principal goal of this study was the rational design of non-peptide mimetic molecules that could bind to the TCR with increased affinity and not to the MHC–peptide complex. Such potential inhibitors would prevent the formation of the trimolecular complex and consequently the stimulation of T cells. To this end, robust computational techniques, such as molecular docking, pharmacophore modeling, and molecular dynamics, were utilized for the design of novel TCR inhibitors. The application of pharmacophore modeling in the trimolecular complex (HLA-$MBP_{83–96}$-TCR) allows the differentiation between the different contributions (e.g., electrostatic and van der Waals interactions, hydrogen donors and acceptors) involved in the epitope recognition process. By analyzing the variations in these aspects, it is possible to search through diverse chemical databases and filter the results for the identification of potential lead TCR antagonists (hits). Furthermore, molecular docking methodologies can be implemented in order to identify and isolate common substructures of the top ranking hits. Subsequently, the analogue with the best docking score (lead molecule) and preferable structural orientation over the TCR is selected for further optimization and this optimized structure then opts for increased interactions with the TCR. Molecular dynamics (MD) simulations and molecular orbital calculations were carried out in the optimized hits in order to evaluate their binding to the TCR. Finally, the proposed analogues were synthesized to evaluate their biological activity against $MBP_{83–}$99 primed mouse T cells and to human peripheral blood T cells.

2. Materials and Methods

2.1 to 2.7. Structure Preparation

Structure preparation, structure preparation, virtual screening, molecular docking, lead optimization, molecular dynamics (md) simulation, chemistry were conducted using different procedures (see appendix).

2.8. In Vitro Evaluation of the Analogues Using Human PBMC

Peripheral blood samples (PBMCs) were used for *in vitro* biological studies (see appendix).

2.9. In Vitro Evaluation of the Analogues Using Mouse-Specific $MBP_{83–99}$ T Cells

Mice, SJL/J females, aged 4–9 weeks were purchased from the Animal Resource Centre (Perth Australia). All mice had free access to food and water, and were housed in a temperature-controlled environment with 12-h day/night cycles at the animal holding room Werribee Campus Animal Facility (Melbourne, Australia). They were allowed to acclimatize for at least 7 days before immunizations. All experiments were completed according to the guidelines of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and were approved by Victoria University Animal Experimentation Ethics Committee (AEC15/013). Mice were subcutaneously injected with 50 μg/100 μL reduced mannan conjugated to $MBP_{83–99}$ via a 10 amino acid linker $(KG)_5$ as previously described (Day et al., 2015; Tapeinou et al., 2015). This conjugate has been shown to induce T cell proliferation to native peptide $MBP_{83–99}$ (Day et al., 2015; Katsara, Deraos, et al., 2008b; Katsara, Matsoukas, et al., 2008a; Katsara et al., 2008a, 2008b; Katsara, Yuriev, et al., 2009b; Tapeinou et al., 2015). Spleen cells from 3 immunized SJL/J mice were isolated 10 days after immunization and assessed by T cell proliferation assay. As we have previously shown that the native peptide $MBP_{83–99}$ conjugated to mannan induces strong proliferative T cells to recall $MBP_{83–99}$ peptide, we used 3 mice/group to test each of the compounds' ability to inhibit this T cell proliferation. Hence, 3 mice/group in this screening process are adequate for determining the optimal compound for inhibiting T cell proliferation. Spleen cells at 2×10^5 in 100 μL of culture media were seeded into 96 well U-bottom plates and incubated for 1–6 days at 37 °C in the presence of recall $MBP_{83–99}$ peptide (10 nM) with or without 100x molar excess of compounds **15**–**19** or **AMB**. Proliferation was assessed by the addition of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) for 6 hours and proliferation assessed via spectrophotometry (Biorad microplate reader, 6.0) using a wavelength of 570 nm. All experiments were conducted in triplicate. The percentage of inhibition of cell proliferation in the presence of compound was calculated and plotted.

3. Results and Discussion

3.1. to 3.5.Pharmacophore Modeling and Virtual Screening

Potential T cell receptor (TCR) antagonist compounds were designed using pharmacophore moleculaer modeling and docking (see appendix).

3.6. Biological Assays

3.6.1. Human Peripheral Mononuclear Cells

These were biological assays using peripheral blood mononuclear cells (PBMCs) isolated from the blood samples (see appendix).

3.6.2. Mouse $MBP_{83–99}$ Specific T Cell Assays

Autoimmune $CD4^+$ T cells can be stimulated in mice following immunization with $MBP_{83–99}$ peptide together with *Mycobacterium*, which results in experimental autoimmune encephalomyelitis (EAE), an animal model for MS (Zamvil et al., 1985). Characteristics of EAE are comparable to those of MS in humans where Th1 phenotype (IFN-γ) and Th17 cells contribute to pathogenesis of disease in mice. Similar to MS, EAE susceptibility is dependent on the mouse (MHC class II background) and diverse peptides are immunogenic in different mouse strains. The SJL/J mouse strain (MHC class II $H\text{-}2^s$ haplotype) is commonly used for EAE, since numerous histopathological, clinical, and immunological features resemble those of human MS (Kalbus et al., 2001). In the SJL/J mouse strain, the peptide $MBP_{81\text{-}100}$ has been shown to bind with high affinity to MHC class II, $H\text{-}2^s$. In fact, the minimum epitope required for binding is $MBP_{83–99}$ (Kalbus et al., 2001), similar to human HLA-DR2 binding. Hence, the epitope $MBP_{83–99}$ could be used as an agonist peptide to immunize mice to activate $CD4^+$ T cells, as we previously demonstrated(Katsara, Deraos, et al., 2008b; Katsara et al., 2008a, 2008b; Katsara, Yuriev, et al., 2009b).. Here, mice were immunized with $MBP_{83–99}$ peptide conjugated to the carrier reduced mannan. Following three immunizations, spleen cells were isolated and mixed with recall peptide $MBP_{83–99}$ for six days in vitro. In addition, compounds **15–19** or **AMB** (lead compound **10**) were added at 100× molar excess to each well in order to determine whether T cell proliferation to the recall peptide $MBP_{83–99}$ could be inhibited. The

particular compounds (**15–19**), due to their increased calculated binding affinity (Table 1) to TCR, were employed in order to assess the potency in mouse $MBP_{83–99}$ specific T cell assays. Compound **15** and **16** showed the greatest % inhibition of $MBP_{83–99}$-specific T cell proliferation, followed by compound **17** and **18**; compound **19** showed the least inhibition, and lead compound AMB was able to inhibit proliferation comparable to that of the other compounds (Figure 8). Compounds **15** and **16** have simpler structures compared to **17–19** and **AMB**. It is likely that the reduced activity of **17–19** analogues, compared to **15** and **16**, may be due to an inappropriate position of the acidic/esteric group. Even though complete inhibition of T cell proliferation is not noted, compounds **15** and **16**, based on in silico conformational studies, show promise for further optimization studies in order to develop new improved TCR antagonists with improved activity.

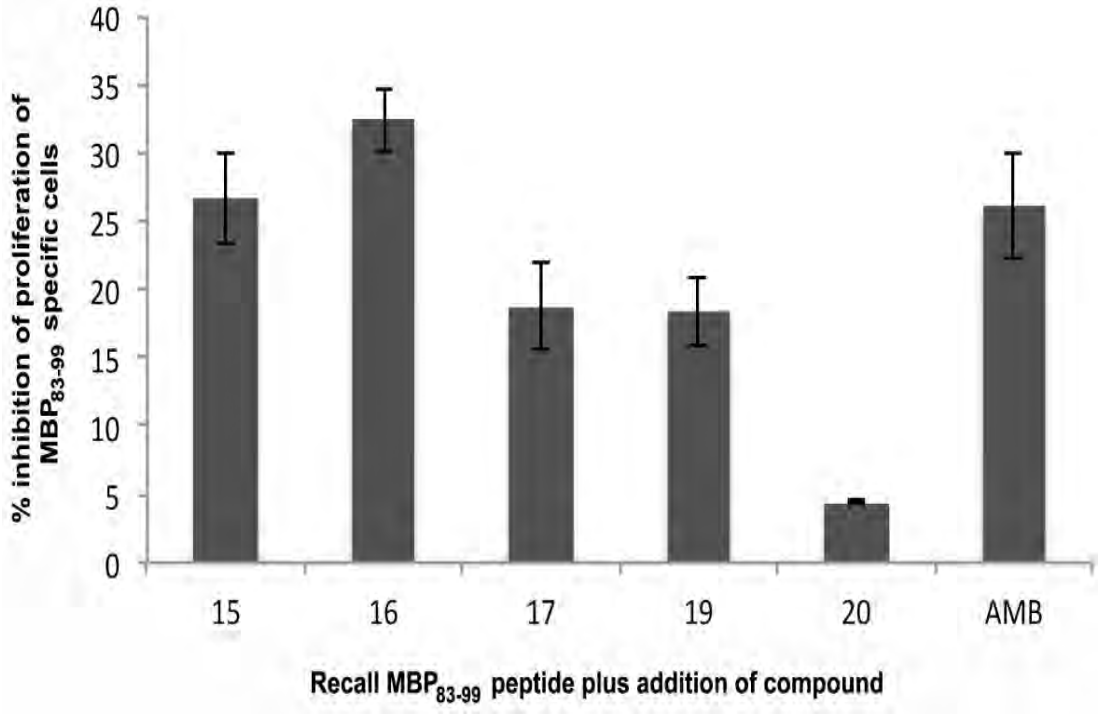


Figure 8. Specific $MBP_{83–99}$ epitope T cell proliferation using MTT as a readout. Mice were immunized three times with reduced mannan conjugated to $MBP_{83–99}$ peptide. Ten days following the last immunization, mice were sacrificed and spleen cells isolated, and $MBP_{83–99}$ peptide was added for 6 days. In addition, compounds **15–19** and lead compound **AMB** (lead compound **10**) were added at 100× molar excess. The percent inhibition of T cell proliferation to $MBP_{83–99}$ of each compound is shown. The mean of three individual mice are shown in triplicate wells ± standard error of the mean.

4. Conclusions

A ligand-based pharmacophore model was developed based on the conformational properties of the dominant $MBP_{83–96}$ epitope in complex with the TCR. The resulting model was employed for the virtual screening of the ZINC database for potential hits. A subset of the database, containing 500,000 all clean/ commercially available compounds, were screened, and the search yielded 13 hits. The potential inhibitors were ranked according to their inhibitory activity against TCR with the employment of molecular docking simulations. The compound with the highest docking score (compound **10**) was selected as lead and was subjected to optimization via chemical modifications. The resulting optimized molecule (compound **14**) presented increased docking score to the TCR and improved chemical properties such as TPSA and logP (Table 2).

The conformational analysis and the positioning of compound **14** in the TCR binding pocket led to the further modification with the addition of a methylene group and the organic synthesis of two isomers (compounds **15** and **16**). The analysis of the conformational properties of the three analogues via MD simulation experiments showed that analogue **15** has the most optimal positioning inside the TCR binding cavity and is better tethered within the receptor (Figure 5a). Extensive MD simulations may offer a deeper understanding of the interactions between the designed analogues and the receptor, and prove to be a valuable tool in drug design. Furthermore, the interaction energy between the potential inhibitor (compound **15**) and the TCR was explored by employing a variety of molecular orbital approaches. DFT and SE methodologies were used in order to calculate the interaction energy between selected residues of the TCR, as well as the entire TCR, and the proposed inhibitor **15**. The combination of the two methodologies allows us to identify whether only certain residues have the greatest impact in the binding of compound **15** or other conformational aspects of the TCR are important in its binding. The agreement between the DFT and the SE methods show that the binding of the potential inhibitor to the TCR is attributed only to the residues surrounding the binding cavity and not to other conformational changes observed in the TCR. The results of the in vitro evaluation (Figure 8) suggest that both analogues **15** and **16** may serve as good candidate antagonists to be developed further for the inhibition of proliferation of T-cells that recognize the $MBP_{83–96}$ antigen.

Supplementary Materials: Supplementary materials can be found at www.mdpi.com/link.

Chapter 6

Chapter 6

Streptococcus thermophilus ST285 alters pro-inflammatory to anti-inflammatory cytokine secretion against myelin basic protein ($MBP_{83–99}$) peptide in mice

ABSTRACT

Probiotic bacteria have beneficial effects to the development and maintenance of a healthy microflora that subsequently has health benefits to humans. Some of the health benefits attributed to probiotics have been noted to be via their immune modulatory properties supressing inflammatory conditions. Hence, probiotics have become prominent in recent years of investigation in regards to their health benefits. As such, in the current study, we determined the effects of *Streptococcus thermophilus* to agonist MBP_{83-99} peptide immunized mouse spleen cells. It was noted that *Streptococcus thermophilus* induced significant increase in the expression of anti-inflammatory IL-4, IL-5, IL-10 cytokines, and decreased the secretion of pro-inflammatory IL-1β and IFN-γ. Regular consumption of *Streptococcus thermophilus* may therefore be beneficial in the management and treatment of autoimmune diseases such as multiple sclerosis.

Keywords: Probiotics; *Streptococcus thermophilus*; ST285; $MBP_{83–99}$ peptide; mannan; immune modulation; multiple sclerosis; agonist peptide

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1. Introduction

There is an increasing trend in immune-mediated disorders across the world which is believed to be in part, a result of intestinal dysbiosis. The imbalance in the intestinal ecosystem can lead to a dysfunctional immune system that consequently causes immune disorders including autoimmune diseases (multiple sclerosis, MS) and other inflammatory disorders (Adamczyk-Sowa, Medrek, Madej, Michlicka, & Dobrakowski, 2017; De Palma, Collins, Bercik, & Verdu, 2014). Probiotics have long been implicated for the overall improvement of health and the management of a number of health conditions including, infection, constipation, allergies and autoimmune diseases, and are either consumed in the form of different fermented foods and dairy products or taken as capsules. In either case, there is strong evidence that suggests the ingestion of probiotics can alter the intestinal dysbiosis and relieve dysfunctionality complications, with subsequent improvements to health (Dargahi et al., 2019).

Probiotic bacteria have been evolved inside the human intestinal tract (GIT), and through this co-evolution the gut and its microbiome have developed a symbiotic relationship that is of mutual benefit. While the GIT microflora relies on gut's warm habitat and food content, in return it not only provides numerous unique bioactive components such as vitamin B and K, minerals, short chain fatty acids (SCF) (Asarat, Apostolopoulos, et al., 2015) and miosins to the host, but it also assists in modulating the immune system (Dargahi et al., 2019). In fact, probiotics are able to modulate monocytes, macrophages, B cells, T helper (h)1, Th2, Th17, regulatory T cells (Treg), natural killer (NK) cells and dendritic cells (DC) (Dargahi et al., 2019; Hardy et al., 2013; Wolvers et al., 2010).

Chronic inflammation is the pathophysiological condition involved in neuro-degenerative disorders, including MS, Parkinson's disease and Alzheimer's disease (Dargahi et al., 2017; Mukherjee, Biswas, & Das, 2016). There is a cross-talk between gut microbiota and the central nervous system (CNS) (Catanzaro et al., 2015; Mukherjee et al., 2016; Russo et al., 2018), known as the gut-brain axis. An insufficient or imbalanced GIT microflora can also lead to dysfunctions in the gut-brain axis and the pathogenesis of a number of diseases inside the GIT (IBD) and outside the GIT (such as the CNS) (Russo et al., 2018). Experimental autoimmune encephalomyelitis (EAE) is an animal model of

human MS that has been used to study the effects of probiotic bacteria on CNS (Kwon et al., 2013; Lavasani et al., 2010). One of the safe and appropriate ways to modulate T cells in MS is to orally administer specific autoantigens (Buerth et al., 2016; Maassen et al., 2003). Administration of *Bifidobacteria* or *Lactobacteria* probiotic strains to mice, has been shown to increase Treg cells and tumor growth factor (TGF)-β levels and reduce the severity of EAE clinical symptoms, in parallel with improvement in the regeneration of myelin in the spinal cord compared to control (Consonni et al., 2018). Administration of both *Bifidobacteria* and *Lactobacteria* strains induce additional significant delay in the onset of EAE and related clinical symptoms, together with substantial reduction of mononuclear infiltration into the CNS, and increased level of Treg cells of $CD4^+CD25^+Foxp3^+$ phenotype in mouse spleen and lymph nodes (Salehipour et al., 2017).

In SJL/J mice, immunization with MBP_{83-99} peptide mixed with mycobacterium stimulates autoimmune $CD4^+$ T cells in mice, and induces EAE (Dargahi et al., 2017; Yannakakis et al., 2017b). MHC class II H-2^s haplotype in the SJL/J mouse strain resembles many clinical, histopathological and immunological characteristics of human MS, thus SJL/J mouse is regularly used for immunization studies (Dargahi et al., 2017). Different peptides are immunogenic in different mouse strains however, in the SJL/J mouse strain, the peptide $MBP_{81–100}$ binds to MHC class II H-2^s with high affinity with the minimum epitope being $MBP_{83–99}$ (Dargahi et al., 2017; Yannakakis et al., 2017b). As such, the $MBP_{83–99}$ epitope has been used as an agonist peptide to immunize mice for activation of $CD4^+$ T cells (Dargahi et al., 2017; Yannakakis et al., 2017b). We have shown that injection of MBP_{83-99} peptide conjugated to the carrier mannan or mixed in complete Freund's adjuvant induces Th1 pro-inflammatory interferon-gamma (IFN-γ) secreting $CD4^+$ T cells (Katsara & Apostolopoulos, 2018; Katsara, Deraos, et al., 2008b; Katsara, Deraos, et al., 2009a; Katsara et al., 2014; Katsara, Matsoukas, et al., 2008b; Katsara et al., 2008a, 2008b; Katsara, Yuriev, et al., 2009a).

Streptococcus genus constitutes over 100 species, amongst which *S. thermophilus* (ST) are non-pathogenic and food related bacteria that represent outstanding technological features in the food industry (A. Tarrah, L. Treu, et al., 2018). ST are commonly used as secondary starter cultures in dairy products to transform lactose into lactic acid and to acidify the pH of milk (A. Tarrah, V. Noal, et al., 2018; A. Tarrah, L. Treu, et al., 2018);

contributing to both fermentation and flavouring of dairy products (Dargahi et al., 2018). Most probiotics belong to lactic acid bacteria (LAB); gram-positive lactic acid producing bacteria which include lactobacilli, bifidobacteria and enterococci (Dargahi et al., 2019). As such, live LABs are not only used in foods for their health benefits, but exopolysaccharide-producing strains of ST such as, ST1342, ST1275 and ST285 are generally used due to their beneficial properties (i.e. relieving lactose intolerance and suppressing acute conditions such as acute ulcerative colitis) (Dargahi et al., 2018).

It was recently shown that ST bacteria have anti-inflammatory properties (Dargahi et al., 2018). U937 pro-monocytic cell line co-cultured with three ST bacteria (ST1342, ST1275 and ST285), induced an anti-inflammatory profile (chapter 2) (Dargahi et al., 2018). ST285 was further shown to have immune modulating effects via gene arrays to human peripheral blood mononuclear cells (PBMC) and monocyte cells isolated from PBMC (chapter 3,4) [Dargahi et al., manuscripts submitted]. Herein, SJL/J mice were immunized with agonist $MBP_{83–99}$ peptide conjugated to mannan 3 times, spleen cells were isolated and after re-stimulation of spleen cells with $MBP_{83–99}$ peptide, IFN-γ was secreted by spleen cells. Re-stimulation of spleen cells with $MBP_{83–99}$ peptide in the presence of ST285 probiotics was able to downregulate IFN-γ responses and stimulate Th2, IL-4, IL-5, IL-10 cytokine profile. These studies show that probiotics are able to modulate and alter the immune profile of $MBP_{83–99}$ specific cells to anti-inflammatory, which warrant *in vivo* EAE mouse experiments and holds promise as a therapeutic alternative approach to MS in human clinical trials.

2. Material and method

2.1. Bacterial strains

Pure bacterial cultures of *S. thermophilus* 285 (ST285) were obtained from Victoria University culture collection (Werribee, VIC, Australia). Stock cultures were stored in cryobeads at −80°C. Prior to each experiment the cultures were propagated in M17 broth (Oxoid, Denmark) with 20 g/L lactose and incubated at 37°C under aerobic conditions. Bacteria was also cultured on M17 agar (1.5 % w/v agar) with 20 g/L lactose (Oxoid,

Denmark) to assess characteristics, morphology, purity and gram-positive confirmation [1].

2.2. Preparation of live bacterial suspensions

Media were prepared and autoclaved at 121° C for 15 minutes (min) prior to experiments. Bacterial cultures were grown 3 times in M17 broth with 20 g/L lactose, at 37° C aerobically for 18 hours (hr) with a 1 % inoculum transfer rate [39]. Cultures grow optimally at 37-42° C for 24 hours (Dargahi et al., 2018). The growth period of cultures were consistent at 18 hr (at the end of the exponential growth phase) and before stationary growth phase to prevent cell lysis.

2.3. Enumeration of bacterial cells

For the actual experiment, bacteria were grown in broth media to stationary phase at 37°C aerobically, pelleted by centrifugation (6000×g) for 15 min at 4°C, transferred and resuspended in Dulbecco's phosphate-buffered saline, pH 7.4 (Invitrogen, Pty Ltd. Australia). The bacterial density in suspension was adjusted to 10^8 colony forming units (cfu)/ml for final concentration by determining the optical density at 600 nm, followed by two washes with Dulbecco's phosphate-buffered saline. These samples constituted the live-cell suspensions, were resuspended in the Roswell Park Memorial Institute (RPMI) 1640 culture media prior to co-culturing with spleen cells [1].

2.4. Mouse experimental procedures

2.4.1. Mice, conjugates and immunization schedule

Female SJL/J mice used in all experiments aged 6-9 weeks, were purchased from Animal Resources Centre (ARC, Perth, Australia), and accommodated at the animal house (Victoria University, Werribee campus, Melbourne, Australia). All mice ensured free access to water and food, and were housed in a temperature controlled room with 12 hr day 12 hr night cycle. All immunizations were conducted according to the guidelines of the Australian code of Practice for the care and use of animals for scientific purposes and

the study was approved by the Victoria University animal ethics committee (AEC15/013) of Victoria University, Melbourne, Australia.

$MBP_{83–99}$ agonist peptide was synthesized by ELDrug SA Patras Science Park, Greece, of over 99% purity with $(KG)_5$ at the C-terminus. $MBP_{83–99}$ peptide was conjugated to mannan via the $(KG)_5$ bridge via a method previously described (Apostolopoulos, Barnes, et al., 2000; Apostolopoulos, Pietersz, et al., 2000; Apostolopoulos et al., 1995; Apostolopoulos et al., 1996; T. V. Tselios et al., 2005a). Briefly, mannan (Sigma, VIC Australia) 14 mg was oxidised in sodium carbonate buffer and 0.1M sodium periodate at 4°C after which ethylene glycol was added and incubated for 30 minutes at 4°C. Oxidised mannan comprising aldehyde groups was passed through a PD-10 column (Sigma, VIC Australia) pre-equilibrated in carbonate-bicarbonate buffer pH9.0 and 2 ml of oxidised mannan collected and 1 mg of $MBP_{83–99}$-$(KG)_5$ peptide added and allowed to react overnight at room temperature in the dark. The resultant $MBP_{83–99}$-$(KG)_5$-mannan conjugate was used to immunize mice.

The $MBP_{83–99}$ mannan peptide conjugate (50 μg/mouse) were injected in SJL/J mice subcutaneously into the base of the tail, 3 times, every 2 weeks (Yannakakis et al., 2017b). This conjugate has been shown to induce T cell proliferation and IFN-γ cytokine secretion to agonist $MBP_{83–99}$ peptide in SJL/J mice (Katsara, Deraos, et al., 2008b; Katsara et al., 2008a, 2008b; Yannakakis et al., 2017b). 10-14 days after the 3 injection, spleen cells were isolated, red blood cells lysed using 0.73 % NH_4Cl and counted.

2.4.2. Isolation of spleen cells and in vitro stimulation with ST285

Spleen cells cells were resuspended in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Pty Ltd. Australia), 1% antibiotic-antimycotic solution and 2 mM L-glutamine in T75 cm^2 cell culture flasks. Mouse spleen cells (1×10^7) in RPMI media only was used as negative control, 5 μg/ml recall agonist $MBP_{83–99}$ peptide was used as a recall control, or, 1×10^8 ST285 bacteria were added together with $MBP_{83–99}$ peptide, and cultured at 37 °C, 5 % CO_2 for 24 hours (Dargahi et al., 2018). We previously showed that 24 hour co-culture was adequate for stimulation of monocyte/macrophage cell line, human peripheral blood mononuclear cells and human

monocytes isolated from peripheral blood mononuclear cells, (Dargahi et al., 2018) (Dargahi et al., manuscripts submitted). At the end of the culture period, cells were transferred into falcon tubes, centrifuged for 5 minutes at 1200 rpm to pellet the cells. All cell-free supernatants were collected and frozen at -20 °C until cytokine analysis.

2.5. Cytokine production analysis

Cytokine secretion of spleen cell culture supernatants was analyzed by commercially available capture and detection antibodies in a Bioplex multiplex bead assay for a panel of 9 mouse cytokines and chemokines using a 9-plex kit (BioRad, Melbourne Australia) to measure Interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-10, GM-CSF, TNF-α and IFN-γ. Cell-free supernatants were collected and the assay procedures were performed according to the manufacturer's instructions. Briefly, flat bottom 96-well plate was coated with 1×coupled beads, washed twice, followed by adding the standard serial dilutions, blank and samples to assigned wells. Post incubation at shaking at room temperature, plates were washed twice, adequate 1× detection antibody was added, incubated at room temperature. Plates were washed three times and 1× Streptavidin Phycoerythrin (SA-PE) stop solution was added to each well, followed by incubating at room temperature and wash. Data collection was repeated twice, data was expressed as the mean cytokine response minus background (pg/ml) of each treatment from 3 replicate wells, plus or minus the standard error of the mean.

2.6. Statistical analysis

Significant differences between all treatment groups were tested by analysis of variance (ANOVA) using the Statistical Package for the Social Sciences for Windows 25.0 (SPSS; IBM Corp) followed by a comparison between treatments performed by Tukey's honest significance test/degree and Fisher's least significant difference method, with a level of significance defined as $p < 0.05$.

3. Results

3.1. ST285 reduces pro-inflammatory TNF-α and IFN-γ production by $MBP_{83–99}$ primed mouse splenocytes

TNF-α, a Th1 cytokine was not secreted by spleen cells from immunized mice wither by re-stimulation of MBP_{83-99} peptide or MBP_{83-99} peptide plus ST285 (Figure 1A). Interferon gamma (IFN-γ) is pro-inflammatory Th1 cytokine involved in macrophage activation and cellular immunity. IFN-γ promotes Th1 cells and inhibits Th2 anti-inflammatory cells. In MS IFN-γ is induced following $CD4^+$ T cell activation by agonist peptide MBP_{83-99}. In SJL/J mice immunized with MBP_{83-99} – mannan conjugates induce IFN-γ responses by spleen cells following overnight MBP_{83-99} peptide re-stimulation (Figure 1B, $p < 0.01$). Spleen cells re-stimulated with agonist MBP_{83-99} peptide in the presence of ST285 reduced IFN-γ cytokine secretion (Figure 1B, $p < 0.05$).

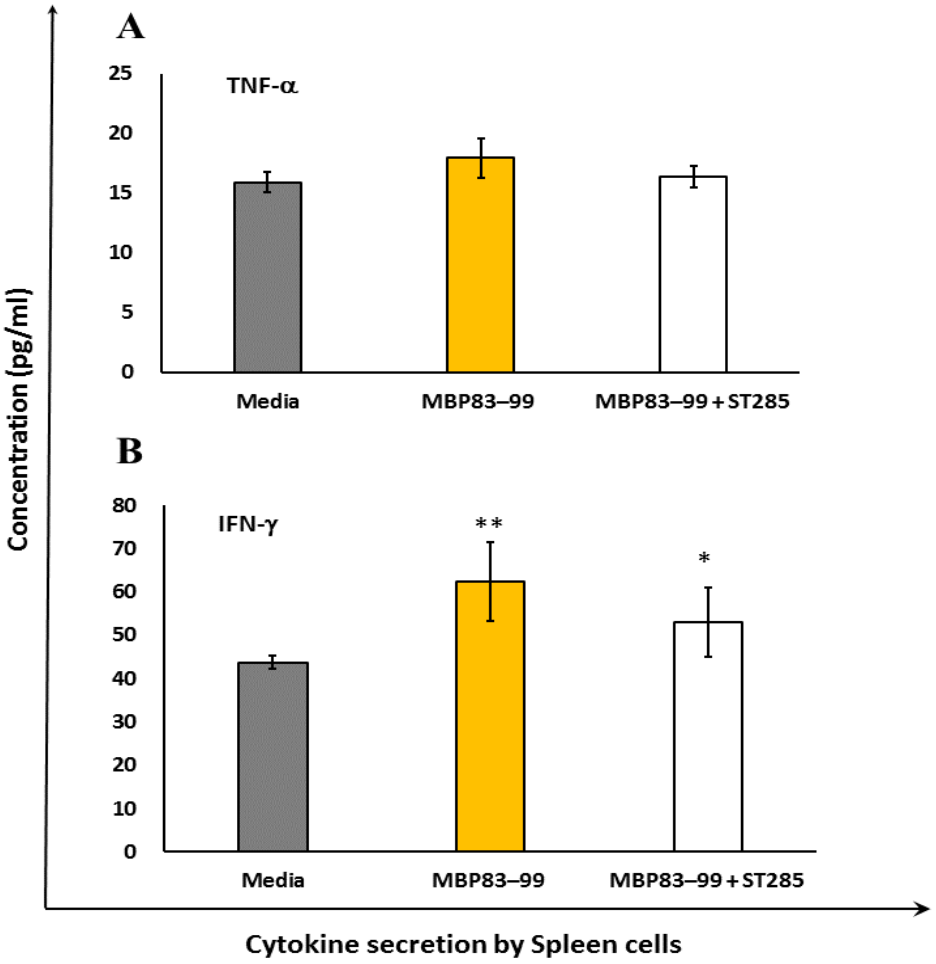

Figure 1. ***S. thermophilus*** **285 reduces pro-inflammatory cytokine production by mouse splenocytes.** Spleen cells isolated from immunized mice (n=3) were stimulated with *S. thermophilus* (ST) ST285 and recall agonist MBP_{83-99} peptide for 24 hours and secretion of TNF-α and IFN-γ were measured. Recall MBP_{83-99} peptide was used as an internal positive control, and media refers to spleen cells from immunized mice (n=3) without any additional recall peptide, or ST285 probiotic bacteria plus MBP_{83-99} peptide. Means of 2 different readings of 3 replicate experiments were measured and analyzed. The means of readings for n=3 mice are calculated and presented as plus or minus (±) the standard error of the mean. Symbols represent *p* value for Tukey Test (One way ANOVA) where * $p < 0.05$ and ** $p < 0.01$.

3.2. ST285 decreases secretion of IL-1β, IL-2 and IL-6 by mouse spleen cells

Secretion of IL-1β was slightly but significantly reduced in immunized mouse spleen cells re-stimulated with MBP_{83-99} peptide and ST285 compared to no re-stimulation, or MBP_{83-99} peptide re-stimulation without ST285 ($p < 0.05$) (Figure 2A). IL-2 production was significantly increased in immunized spleen cells re-stimulated with MBP_{83-99} peptide ($p < 0.01$) which was weakly but significantly decreased as a result of co-stimulation of mouse spleen cells with MBP_{83-99} peptide plus ST285 ($p < 0.05$) (Figure 2B). The production of IL-6 was profoundly increased by immunized mouse spleen cells upon co-culture of ST285 and recall MBP_{83-99} peptide compared to control media or MBP_{83-99} recall peptide ($p < 0.001$) (Figure 2C); spleen cells recalled with MBP_{83-99} peptide alone also increased IL-6 secretion.

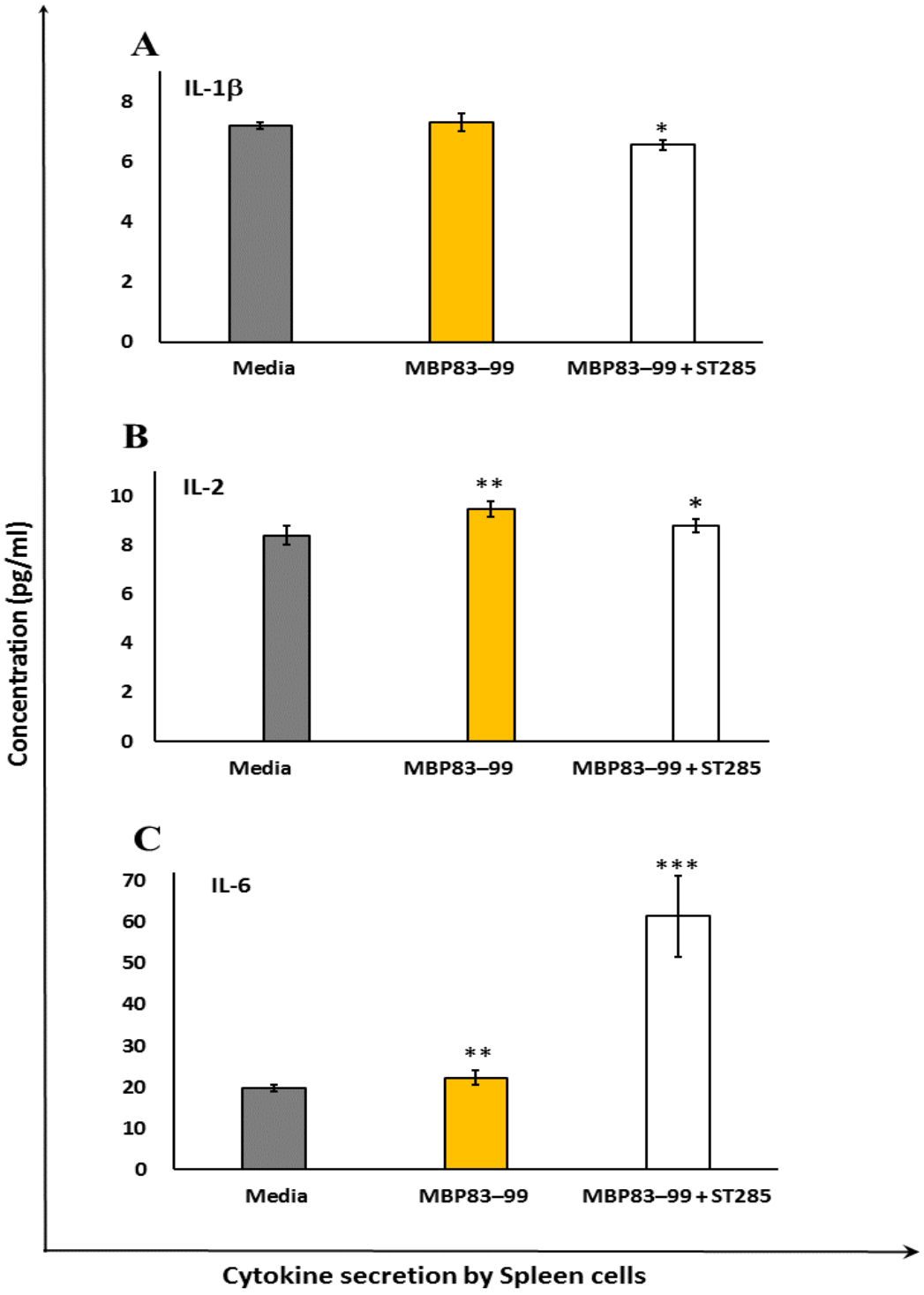

Figure 2. *S. thermophilus* 285 decreases expression of IL-1β, IL-2 and increases IL-6 by mouse spleen cells. Spleen cells isolated from immunized mice (n=3) were stimlated with *S. thermophilus* (ST) ST285 and recall agonist $MBP_{83–99}$ peptide for 24 hours and secretion of IL-1β, IL-2 and IL-6 were measured. Recall $MBP_{83–99}$ peptide was used as the reference peptide, and media refers to spleen cells from immunized mice (n=3) without any additional recall peptide, or ST285 probiotic bacteria plus $MBP_{83–99}$ peptide. Means are shown as plus or minus (±) standard error of the means. Symbols represent p value for Tukey Test (One way ANOVA) where * $p < 0.05$ and ** $p < 0.01$ and *** $p < 0.001$.

3.3. ST285 induces anti-inflammatory cytokine profile by mouse splenocytes

Mice immunized with $MBP_{83–99}$ agonist peptide do not induce IL-4, IL-5 and IL-10 anti-inflammatory cytokines in control (media alone) and recall agonist peptide $MBP_{83–99}$ (Figure 3). However, Th2 anti-inflammatory cytokine IL-4 was significantly ($p < 0.001$) increased by immunized mouse spleen cells when $MBP_{83–99}$ recall peptide was co-cultured with ST285 probiotic bacteria (Figure 3A). IL-5 was also increased by immunized spleen cells following co-culture with ST285 and recall agonist $MBP_{83–99}$ peptide (Figure 3B) ($p < 0.01$). The anti-inflammatory IL-10 cytokine was also significantly increased by immunized mouse spleen cells when co-cultured with ST285 and agonist recall $MBP_{83–99}$ peptide compared to $MBP_{83–99}$ peptide alone or media control ($p < 0.001$) (Figure 3C).

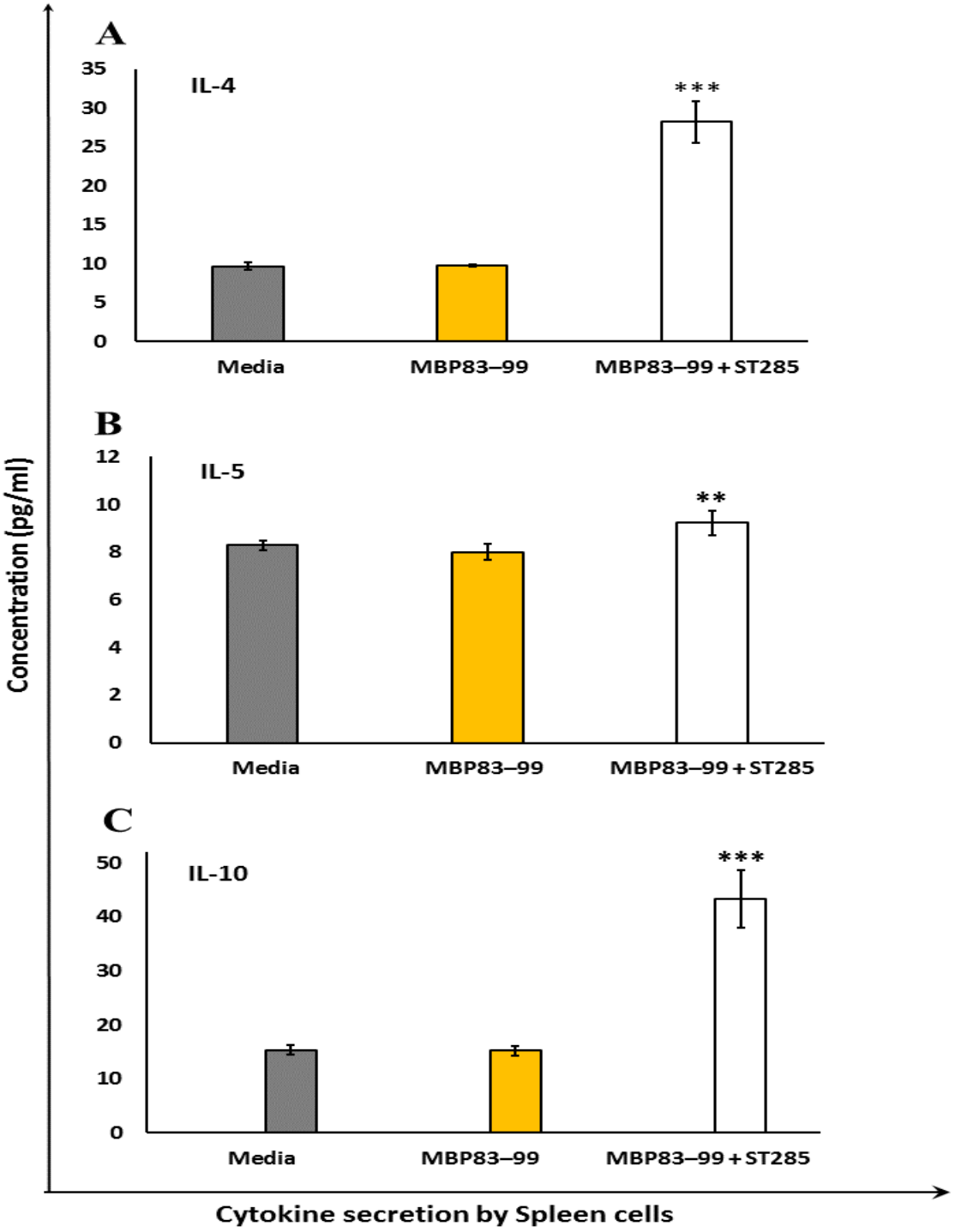

Figure 3. *S. thermophilus* 285 induces anti-inflammatory cytokine profile by immunized mouse splenocytes. Spleen cells isolated from immunized mice (n=3) were stimlated with *S. thermophilus* (ST) ST285 and recall agonist $MBP_{83–99}$ peptide for 24 hours and secretion of IL-4, IL-5 and IL-10 were measured. Recall $MBP_{83–99}$ peptide, media alone, or recall $MBP_{83–99}$ peptide plus ST285 are shown from immunized mice (n=3). The means of readings for n=3 mice are calculated and presented as plus or minus (±) the standard error of the mean. Symbols represent p value for Tukey Test (One way ANOVA) where ** $p < 0.01$ and *** $p < 0.001$.

3.4. ST285 does not alter the secression of GM-CSF by mouse spleen cells

Secretion of granulocyte-macrophage colony-stimulating factor (GM-CSF) did not show any change by immunized mouse spleen cells upon co-culture with ST285 and agonist recall $MBP_{83–99}$ peptide compared to negative control or $MBP_{83–99}$ peptide (Figure 4), despite significant upregulation of GM-CSF by ST285 on monocytes/macrophage cells (Dargahi et al., 2018).

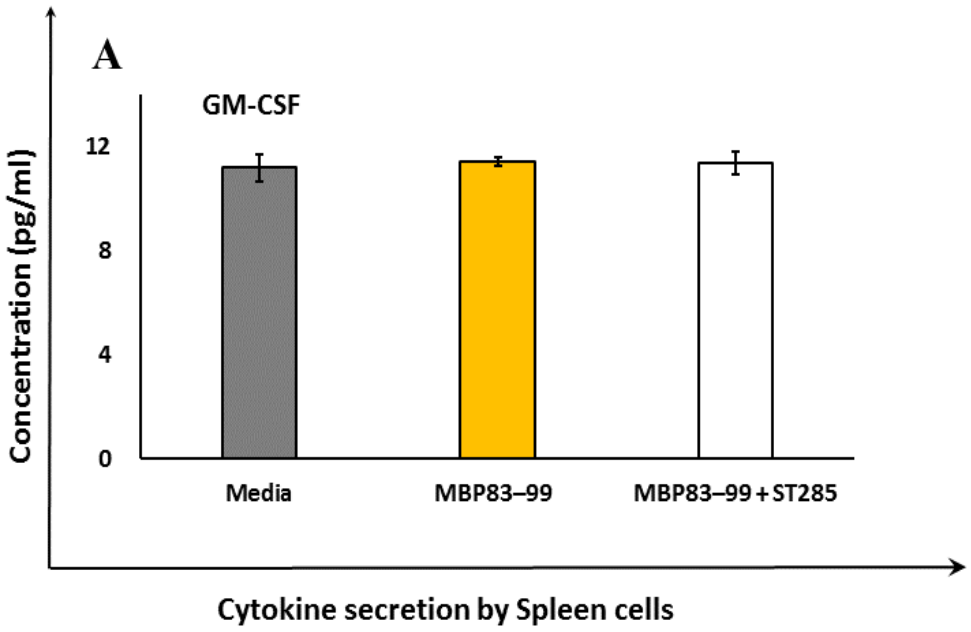

Figure 4. *S. thermophilus* 285 does not alter secression of GM-CSF by mouse spleen cells. Spleen cells isolated from immunized mice (n=3) were stimlated with *S. thermophilus* (ST) ST285 and recall reference peptide for 24 hours and secretion of GM-CSF was measured. Recall $MBP_{83–99}$ peptide, media alone, or recall $MBP_{83–99}$ peptide plus ST285 are shown from immunized mice (n=3). The means of readings for n=3 mice are calculated and presented as plus or minus (±) the standard error of the mean.

4. Discussion

The Th1 pro-inflammatory cytokines TNF-α and IFN-γ are both involved in the defense against bacterial infections, and in acute phase reactions. In MS, these two cytokines are implicated in the pathogenesis of disease by stimulating $CD4^+$ T cells against the myelin sheath. Mice immunized with mannan $MBP_{83–99}$ peptide stimulated IFN-γ secretion which was reduced in the presence of ST285. This reduction is very important in the context of inflamed situations such as autoimmune and inflammatory diseases, as

any reduction in the amount of mediators that cause inflammation is imperative in the relief of symptoms. We previously noted that high levels of TNF-α and IFN-γ was secreted by U937 monocytic cell line in the presence of ST285 (Dargahi et al., 2018). However, the addition of ST285 to $MBP_{83–99}$ recall peptide reduced IFN-γ secretion by mouse splenocytes. Spleen cells are populated with B, T, NK cells, macrophages and monocytes, while U937 cell line that we previously used, are purely monocytic/macrophage cells. Also, the polarized inflammatory state of cytokines as a result of the immunization regimen and further exposure of spleen cells to recall $MBP_{83–99}$ peptide that operate as inflammatory stimuli, compared to U937 monoclonal cells only being exposed to ST285 bacteria, might give a clue to the ability of ST285 probiotics to dampen the inflammatory immune response in the instance of exposure to polyclonal spleen cells.

Secretion of IL-1β by monocytes is involved in regulating the immune and inflammatory responses to infections and injuries; therefore, it has a role in innate immunity. IL-1β is also a major mediator in inflammatory responses associated with various cellular activities such as differentiation, proliferation and apoptosis (Masters, Simon, Aksentijevich, & Kastner, 2009). In addition, IL-1β is a regulator of inflammatory reactions and is involved in the stimulation of the central nervous system through cyclooxygenase-2 (PTGS2/COX2) which is involved in neurodegenerative disorders such as MS (McGuinness et al., 1997; Paré et al., 2018), Down's Syndrome, Alzheimer's disease (Griffin et al., 1989) and HIV-associated dementia (Shaftel, Griffin, & Kerry, 2008).

It was noted the secretion of IL-1β by immunized mouse spleen cells was marginally, but significantly reduced in the presence of ST285 with recall $MBP_{83–99}$ peptide. We previously noted that ST285 did not induce IL-1β cytokine to U937 cell lines (Dargahi et al., 2018), however, significant upregulation of IL-1β mRNA was induced by human PBMC and monocytes post co-culture with ST285 [Dargahi et al., manuscripts submitted]. It is therefore, clear that in immunized mouse spleens and recall of T cells with $MBP_{83–99}$ peptide in the presence of ST285, cause reduction of IL-1β secretion. Likewise, IL-2 was marginally decreased in the presence of ST285, compared to the increased secretion caused by $MBP_{83–99}$ peptide in positive control. Co-culturing human PBMC with ST285 also downregulates IL-2 mRNA expression [manuscript submitted].

IL-6 is produced by activated immune cells including DC, B cells and macrophages. Although IL-6 is associated with acute phase responses, it is also associated with reduction of Th1 polarization, while promoting Th2 differentiation, B cell maturation and macrophage differentiation. Proliferation and differentiation of Th2 cells changes the polarized Th1 environment and skews the Th1/Th2 balance towards Th2, which is beneficial in relieving autoimmune conditions such as MS. IL-6 production was significantly higher (three times) in mouse splenocytes cultured with ST285 compared to control, hence, it is likely that ST285 bacteria may potentially change the balance towards a healthier state in MS. We previously noted significant upregulation of IL-6 to human monocytes [manuscript submitted] and to bulk PBMC co-cultures [manuscript submitted] with ST285, which are also in accord to the increase in IL-6 levels by U937 promonocytic cell line co-cultured with ST285 (Dargahi et al., 2018). Likewise, the commercially used probiotic *L. paracasei* DG induces IL-6 cytokine to THP-1 human monocyte cell line (Balzaretti et al., 2017). In contrast, ingestion of *B. bifidum* by mice did not increase in IL-6 levels, though, it boosted anti-oxidation activities in spleen and thymus of mice and improved other immune functions by changing the gene expression of immune mediators (Y. R. Fu, Yi, Pei, & Guan, 2010).

It is likely that the constant-shifting in the equilibrium and the dynamics that exsits between pro- and anti-inflammatory cytokines leads to some controversy in research findings regarding IL-6. As on one hand IL-6 may ease the autoimmune condition due to its donwstrean immunological effects. But on the other hand, elevated levels of pro-inflammatory effector T cell cytokines such as IFN-γ, IL-17, as well as IL-6 are noted in patients with autoimmune myasthenia gravis and MS (Danikowski, Jayaraman, & Prabhakar, 2017). Thus, it might be likely that the role that cytokines such as IL-6 play, may depend on their bio-environment and may be advantageous to the body if probiotics such as ST285 are used for nuetralization and/or reversing from a pro- to an anti-inflammatory state in the body.

IL-4 is one of the important cytokines required for anti-inflammatory responses against inflammatory conditions such as, MS and allergies (Dargahi et al., 2018). IL-4 production is significantly increased by mouse spleen cells in the presence of recall $MBP_{83–99}$ peptide and ST285 compared to either $MBP_{83–99}$ peptide alone or negative

control (media). Likewise, it was previously noted that ST285 induced U937 monocytic cells to produce IL-4 (Dargahi et al., 2018), although no changes to mRNA expression levels of IL-4 were noted to human monocytes or to bulk human PBMC following co-cultures with ST285 [Dargahi et al., manuscripts submitted]. In contrast, feeding BALB/c mice with *L. paracasei* BEJ01 alone or combined with aflatoxins B1 (AFB1) and fumonisin B1 (FB1) (known foodborne mycotoxins with immunomycotoxic effects on human health) was used to evaluate *L. paracasei* BEJ01 detoxification effects that happen through bindage and degradation of the AFB1 and FB1 toxins (Abbès, Ben Salah-Abbès, Jebali, Younes, & Oueslati, 2016). Assessing different splenic immunological factors indicated that exposure to these mycotoxins led to increased IL-4 mRNA levels, oxidative stress and immunotoxicity in the spleen (Abbès et al., 2016). Whereas, combined LAB treatment with AFB1 or FB1 suppressed and normalized mRNA levels of IL-4 showing protective effects induced by LAB against AFB1 and FB1 via diminishing toxins adhesion and bioavalability (Abbès et al., 2016). In contrast, spleen cells isolated from BALB/c mice *in vitro* co-cultured individually with LAB strains (*L. casei* Lc2w (Lc), *L. plantarum* CCFM47 (Lp) and *L. acidophilus* CCFM137 (La)) showed reduced IL-4 production by spleen cells exposed to La only, while parallel animal studies displayed LAB-induced alleviation of inflammation post airway allergy for all strains through increased Treg cells and modulation of Th1/Th2 balance (Ai et al., 2016).

The anti-inflammatory cytokine IL-5 is produced by Th2 cells and mast cells. In the event of infection with helminth parasites, IL-5 leads to a lesser risk of autoimmune disorders (Finlay et al., 2016), which is indirectly acredited to some therapeutic charactristics of IL-5 in autoimmune disorders. We noted a slight increase in the IL-5 production by spleen cells in response to ST285, whereas no changes to the mRNA expression levels of IL-5 were previously noted in ST285 co-cultures with human PBMC, or human monocyte cells [manuscripts submitted]. A study showed that treating mice with *Fasciola hepatica* excretion/ secretions (FHES) reduced EAE clinical signs, due to a significant decrease in infiltration of Th1 and Th17 cells into the brain, and an increase in IL-5 (and IL-23) response, with subsequent increase in eosinophils (Finlay et al., 2016). It is likely that the small but significant increase of IL-5 may be beneficial to MS.

IL-10, an anti-inflammatory cytokine is secreted by Th2 and Treg cells. Amongst all the anti-inflammatory cytokines and chemokines, anti-inflammatory properties of IL-10

are the most potent in suppressing inflammatory mediators by other activated immune cells (TNF-α, IFN-γ, IL-1, IL-17 and IL-23 cytokines) (S. S. Iyer & Cheng, 2012). A significant amplification in IL-10 levels secreted by the spleen cells in the presence of ST285 was noted, which was similarly shown for U937 monocytic cell line in the presence of ST285 (Dargahi et al., 2018), and to human PBMC, although no significant changes was shown to human monocyte cells [manuscripts submitted]. Likewise, oral administration of *L. reuteri* and *L. lactis* strains to mice stimulates production of anti-inflammatory IL-10 and Treg cells (Levkovich et al., 2013; Souza et al., 2016). In addition, sub-clinical studies of *L. salivarius* UCC118, *L. lactis* MG1363 and *L. plantarum* WCFS1 administered to mice and re-exposure of their isolated bone marrow cells to the 3 bacterial co-cultures showed all 3 strains differentially stimulated IL-10 production (Smelt et al., 2012). Correspondingly, when DC from spleen and mesenteric lymph nodes of mice were matured using *L. acidophilus* X37, and exposed to commensal gut *Bifidobacterium longum* Q46, *L. acidophilus* X37 and *Escherichia coli* Nissle 1917, increased IL-10 levels were noted (Fink & Frøkiær, 2008). Similarly, after BALB/c mice were fed with *L. paracasei* BEJ01 alone or combined with aflatoxins B1 and fumonisin B1, high IL-10 mRNA levels were incduced (Abbès et al., 2016). In addition, mice fed with kefir-derived *Lactobacillus kefiri* CIDCA 8348 also increase IL-10 gene expression (Carasi et al., 2015). In the context of MS the use of ST285 is shown to downregulate Th1 responses and up regulate Th2 responses, something of utmost importance to patients with MS to aleviate MS symptons and/or reversal of the disease.

5. Conclusion

Immunization of SJL/J mice with agonist $MBP_{83–99}$ peptide conjugated to mannan induces Th1 pro-inflammatory IFN-γ responses and no Th2 anti-inflammatory responses when spleen cells are co-cultured *in vitro* in the presence of agonist recall $MBP_{83–99}$ peptide. However, stimulation of spleen cells with recall $MBP_{83–99}$ peptide in the presence of ST285 significantly increased the secretion of IL-4, IL-6 and IL-10 along with mild upregulation in IL-2 and IL-5, suggesting a role for ST285 in activation of immune response phenotyes towards a predominant anti-inflammatory profile, tolerance and suppression of inflammation. In addition, ST285, down regulated the secretion of IL-1β and IFN-γ; the immune mediators involved in Th1 type responses, collectively pointing to

a shift in immune responses from Th1 to a Th2 phenotype. More importantly, the significant increase of IL-10 can further contribute by differentiation of naïve $CD4^+$ T cells and proliferation of Tregs, which can also drive the immune balance further towards a dominant anti-inflammatory phenotype. Additionally, given the drastic increase of GM-CSF in our previous studies of ST285 co-cultured with U937 monocytic cell line, human PBMC and human monocyte cells, and no change to the secretion of GM-CSF in spleen cells, and GM-CSF being a major cytokine for proliferation and recruitment of the immune cells, it might indicate a deliberate and purposeful neutralization of GM-CSF by ST285. The effects of ST285 on the immune response could be used as a novel approach in modulating chronic inflammatory and autoimmune conditions such as MS. Further studies should involve effects of ST285 in mice with EAE or be used to prevent EAE induction, which will pave the way for new modalities for the treatment of MS in human clinical trials.

Chapter 7

Chapter 7

General discussion and future prospects

Intestinal microflora can regulate and balance many health condition and return the body into a normal status through tolerating to food and allergens and helping the body to resist diverse health threats and overcome many existing health issues (Dargahi et al., 2019). Microbiota in the GIT do all of these and perform many more beneficiary activities through a broad range of known and mostly unknown mechanisms that exist in the GUT flora. In the event of any imbalance in the GIT microbiome; such as taking antibiotics, or an unhealthy diet, it leads to lesser microbiota and prevention of their thriving. As such, the body undergoes extreme risk of bacterial infection which poses serious ill health.

For years, probiotics have been used to manage different infections as a replacement of antibiotics and for the treatment of a number of diseases, including allergies and asthma (Dargahi et al., 2019; Fijan et al., 2019; Freedman et al., 2020; McFarland, Ship, Auclair, & Millette, 2018; Sikorska & Smoragiewicz, 2013). Regular ingestion of probiotics either in the form of fermented foods, dairy products, or capsules recolonize the gut and results in the dominance of the gut ecosystem by probiotics. This new ecosystem can repopulate and reconstitute the gut healthy microflora, and contribute to our health by controlling infections, also microbiome provide moderate stimuli to the immune system that controls different allergic and inflammatory conditions. Gut microflora do all these, together with the production of SCFA (Consonni et al., 2018), vitamins (K1, B12, biotin, folic acid), and many more substances only by consuming indigestible fibres, which otherwise would go to waste (Dargahi et al., 2019; Meganathan & Kwon, 2009). When the gut microbiome alters, a number of changes to the body are followed; how and why these changes are yet to be discovered. Nonetheless, ultimately, human health is determined by the GIT bacterial composition and its diversity, together with the balance between probiotic populations and the infectious pathogens. Hence, gut microbes affect the body both physically and psychologically in fundamental, yet inexplicable ways.

Myasthenia gravis (MG) and MS are the most common neurodegenerative autoimmune diseases that happen outside the GUT and set the patients to be depended on the first-line therapies by using regular drug medications. Although MS does not kill the person, it has a heavy impact on everyday life that reduces the quality of life dramatically. In addition, the current medication for MS cannot be simply administered to the majority of the patients and must be tailored for individuals for minimisation of adverse side effects (Consonni et al., 2018). The medications are mainly non-antigen specific and general immune inflammatory suppressants that cause side effects due to the long-term administration; therefore, new, novel and improved therapies can assist in the enhancement of life quality among MS patients.

In MS the demyelination of the CNS happen in a Th cell-dependent manner as a result of chronic inflammation; the interactions between inflammatory factors such as IL-17, IFN-γ and TNF-α cytokines and neuro-degenerative features cause neural relapses, which further accumulate and proceed to progressive debility (Consonni et al., 2018). The role of probiotic bacteria become more prominent in view of these bacteria interplay with the host microbiome to provide health benefits via various mechanisms, including regulation of local, mucosal and systemic immune responses, restoring gastrointestinal barrier and balancing the gut microscopic homeostasis without any major risks (Consonni et al., 2018). Thus, our microbiome has a key role in determining immune homeostasis in the gut and the periphery, and in regulating host predisposition to autoimmune disorders (Consonni et al., 2018), hence, it has implication in a number of autoimmune diseases including IBD, RA and MS (Tankou et al., 2018). Therapeutic effects of probiotics have been shown in numerous animal models of autoimmune diseases, such as experimental autoimmune myasthenia gravis (EAMG), RA, type 1 diabetes and EAE (Consonni et al., 2018).

The EAE animal model resembles several common immunological, clinical and pathogenic characteristics with the human MS disease, which makes it suitable for therapeutic studies [31–34]. In some EAE studies, the disease can be induced with low dosages of guinea pig MBP; the monophasic EAE shows characteristic severe paralysis of hind limb after immunization followed by a recovery phase (Consonni et al., 2018). These studies show that perturbation of the integrity of gut microflora changes proneness to EAE,

but colonization of mice with *Bacteroides fragilis* improves EAE symptoms, while colonization of mice with a Th17 inducing segmented-filamentous-bacteria aggravates EAE (Tankou et al., 2018). The gut commensals produce aryl hydrocarbon receptor (AHR) ligands derived from tryptophan which can approach the CNS to regulate the functions of astrocytes and suppress inflammation and neurodegeneration (Tankou et al., 2018). Likewise, a study of the human HLA class II transgenic EAE mouse model shows that commensal Prevotella derived from the human gut represses EAE in mouse (Tankou et al., 2018). It has similarly been shown that MS patients experience alterations in their gut microbiome; recent reports not only show reduced Butyricimonas and increased Methanobrevi-bacter in MS subjects (Tankou et al., 2018), but recent findings also show dysbiosis in the microbiome of MS subjects (Tankou et al., 2018). In addition, others have shown parallel alterations in the gut microbiota composition along with higher rate of intestinal effector Th17 cells concomitantly with increased MS symptoms (Tankou et al., 2018). Therefore, we determined the effects of probiotics to immune cells (monocyte cell line, peripheral blood mononuclear cells (PBMC) and human blood monocytes isolated from PBMC) as well as its effects to pro-inflammatory induced cells isolated from agonist peptide immunized mice.

In this thesis the immunomodulatory effects of three different ST bacteria were assessed on U937 human monocytic cell line. It was noted that ST strains could induce differential expression of cell surface markers (CD11c, CD14, CD86, CD206, CD209 and MHC-I), cytokines and chemokines suggestive of ST potential benefit in activation of innate and adaptive immune responses to U937 monocytes. These findings showed predominantly anti-inflammatory profile for ST285, with significant up regulation of the GM-CSF secretion, as a major cytokine for the increase of macrophages at the site of infection, and also up regulation of cytokines by monocytes, necessary for activation of the innate immune response. ST also induced increased production of anti-inflammatory IL-4 and IL-10 that delivered clear clues regarding ST beneficial influence in modulating chronic inflammatory conditions, allergies and autoimmune disorders. These results led the further assessment of the effects of ST on the immune system by using ST bacteria to stimulate human bulk PBMC cultures and determining the expression of 84 genes related to innate and adaptive immunity. Interestingly, an array of anti-inflammatory immune-modulatory properties for ST285 was noted, with significant decreases in the mRNA expression of IL-18, IFN receptor, CCR5, CXCL10, TLR-1, TLR-2, TLR-4, TLR-8,

CD14, CD40, CD86, C3, GATA3, ITGAM, IRF7, NLP3, LYZ, TYK2, IFNR1, and upregulation in the mRNA expression of IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-23, IFN-γ, TNF-α, CSF-2. Following this, the immune-modulatory effects of ST to human monocyte cells isolated from PBMC was assessed for mRNA gene expression of 84 of the innate and adaptive immune response related-genes, and further confirmed a dominant anti-inflammatory profile. In fact, highly significant downregulation of mRNA expressions for TLR-1, TLR-2, TLR-4, TLR-5, TLR-6, TLR-8, CD4, CD14, CD86, IL-1R, IL-18, IFNR1, IFNαR1, IFNγR1, CCL2, CCR5, ITGAM, SLC11A1, ITGAM, LYZ, TYK2, IRAK-1, NOD2, MYD88 were noted, and, significant upregulation in IL-1α, IL-1β, IL-2, IL-6, IL-8, IL-23, IFNγ, TNFα and CSF-2 mRNA expression was noted. Given the immune functions mediated by these factors and the general and specific interactions between theses mediators, their receptors and their role in different immunological pathways, once more the overall data exhibited a somewhat an anti-inflammatory profile of ST285. Therefore, through three consecutive experiments with ST285 bacteria, using different techniques and different cells, ST was determined as a potential candidate and an effective approach to assess in an autoimmune setting. As such, SJL/J mice were immunized with the agonist $MBP_{83–99}$ peptide conjugated to the carrier mannan. Such conjugate induces a Th1 profile (secretion of IL-1β and IFN-γ) with no Th2 cytokines. When spleen cells were cultured *ex vivo* with recall $MBP_{83–99}$ peptide a Th1 profile was induced with no Th2 cytokines, however, when spleen cells were cultured *ex vivo* with recall $MBP_{83–99}$ peptide and ST285, a predominant Th2 profile resulted with high IL-4, IL-6 and IL-10 and moderate IL-2 and IL-5 cytokine secretion with no IL-1β and IFN-γ. Additionally, immune modulating effects of a number of $MBP_{83–99}$ peptide peptides either in their linear or cyclic forms or as 1-2 amino acid mutations were assessed and it was noted that cyclic $MBP_{83–99}$ peptide in particular conjugated to mannan was able to modulate immune responses in mice and upregulate Th2 cytokine profile and down regulate Th1 profile. This peptide as shown to decrease EAE in mice and molecular modeling gave insights into its immune modulatory activity.

Further experiments using EAE mouse models, and administering ST285 orally or intraperitoneally, and assessing EAE symptoms as well as spinal cord for demyelination and cell infiltration, spleen cells/tissues and brain tissue to evaluate changes in inflammatory damage are required to understand the *in vivo* effects of ST285. In addition,

isolation of blood PBMC from patients with MS and stimulating with ST285 are required prior to any human clinical studies in MS patients. MS patients could be used to determine the effects of ST285 to disease outcome.

In this thesis, the immune modulatory effects of MBP peptide analogs and ST285 probiotic bacteria were assessed. ST285 probiotics show promise and paves the way for further testing and future developments of ST285 in human clinical trials in patients with MS.

Chapter 8

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Chapter 9

Chapter 9

Appendices

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5 b– Design and Synthesis of Non-Peptide Mimetics Mapping the Immunodominant Myelin Basic Protein ($MBP_{83–96}$) Epitope to Function as T-Cell Receptor Antagonists

ABSTRACT

Encephalitogenic T cells are heavily implicated in the pathogenesis of multiple sclerosis (MS), an autoimmune demyelinating disease of the central nervous system. Their stimulation is triggered by the formation of a trimolecular complex between the human leukocyte antigen (HLA), an immunodominant myelin basic protein (MBP) epitope, and the T cell receptor (TCR). We detail herein our studies directed towards the rational design and synthesis of non-peptide mimetic molecules, based on the immunodominant $MBP_{83–96}$ epitope that is recognized by the TCR in complex with HLA. We focused our attention on the inhibition of the trimolecular complex formation and consequently the inhibition of proliferation of activated T cells. A structure-based pharmacophore model was generated, in view of the interactions between the TCR and the HLA-$MBP_{83–96}$ complex. As a result, new candidate molecules were designed based on lead compounds obtained through the ZINC database. Moreover, semi-empirical and density functional theory methods were applied for the prediction of the binding energy between the proposed non-peptide mimetics and the TCR. We synthesized six molecules that were further evaluated *in vitro* as TCR antagonists. Analogues **15** and **16** were able to inhibit to some extent the stimulation of T cells by the immunodominant $MBP_{83–99}$ peptide from immunized mice. Inhibition was followed to a lesser degree by analogues **17** and **18** and then by analogue **19**. These studies show that lead compounds **15** and **16** may be used for immunotherapy against MS.

Keywords: multiple sclerosis; trimolecular complex; rational drug design; non-peptide mimetics; molecular modeling; cell proliferation; T cell antagonism

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Design and Synthesis of Non-Peptide Mimetics Mapping the Immunodominant Myelin Basic Protein ($MBP_{83–96}$) Epitope to Function as T-Cell Receptor Antagonists

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Abstract: Encephalitogenic T cells are heavily implicated in the pathogenesis of multiple sclerosis (MS), an autoimmune demyelinating disease of the central nervous system. Their stimulation is triggered by the formation of a trimolecular complex between the human leukocyte antigen (HLA), an immunodominant myelin basic protein (MBP) epitope, and the T cell receptor (TCR). We detail herein our studies directed towards the rational design and synthesis of non-peptide mimetic molecules, based on the immunodominant $MBP_{83–96}$ epitope that is recognized by the TCR in complex with HLA. We focused our attention on the inhibition of the trimolecular complex formation and consequently the inhibition of proliferation of activated T cells. A structure-based pharmacophore model was generated, in view of the interactions between the TCR and the HLA-$MBP_{83–96}$ complex. As a result, new candidate molecules were designed based on lead compounds obtained through the ZINC database. Moreover, semi-empirical and density functional theory methods were applied for the prediction of the binding energy between the proposed non-peptide mimetics and the TCR. We synthesized six molecules that were further evaluated in vitro as TCR antagonists. Analogues **15** and **16** were able to inhibit to some extent the stimulation of T cells by the immunodominant $MBP_{83–99}$ peptide from immunized mice. Inhibition was followed to a lesser degree by analogues **17** and **18** and then by analogue **19**. These studies show that lead compounds **15** and **16** may be used for immunotherapy against MS.

Keywords: multiple sclerosis; trimolecular complex; rational drug design; non-peptide mimetics; molecular modeling; cell proliferation; T cell antagonism

1. Introduction

Multiple sclerosis (MS) is an immunologically controlled, inflammatory, demyelinating disease, described as the destruction of the myelin sheath of the central nervous system, which can lead to

1. Introduction

Multiple sclerosis (MS) is an immunologically controlled, inflammatory, demyelinating disease, described as the destruction of the myelin sheath of the central nervous system, which can lead to paralysis (Sospedra & Martin, 2005a; L. Steinman, 1996). Although evidence suggests the important role of B-cells (auto-antibodies), T helper (Th)-17 cells, and $CD8^+$ T cells in disease pathogenesis (Mouzaki et al., 2015), it is well regarded that $CD4^+$ Th1 cells contribute to initiation and progression of disease. Indeed, $CD4^+$ T cells have been identified in patients with MS to react to self-peptide epitopes within the myelin sheath, including that of myelin basic protein (MBP), proteolipid protein, myelin oligodendrocyte glycoprotein, and myelin associated glycoprotein (Ben-Nun et al., 2006; Wucherpfennig et al., 1997). In the context of MS, encephalitogenic T cells are activated through the formation of a trimolecular complex between the T cell receptor (TCR), a short 14–18 amino acid myelin peptide (epitope), and the major histocompatibility complex (MHC) class II. In fact, the MHC class II, human leukocyte antigen (HLA) locus is the most closely correlated genetic locus to the development of MS, in particular HLA-DR1, HLA-DR2, and HLA-DR4 (International Multiple Sclerosis Genetics et al., 2007; Moise et al., 2015; Shahrizaila & Yuki, 2011). In humans, the MHC class II (HLA) consists of dimers (the α chain and the β chain) (Adams & Luoma, 2013; Madden, 1995), which present short antigenic peptide epitopes to $CD4^+$ Th cells, resulting in the formation of the trimolecular complex (HLA-peptide-TCR). The TCR is also composed of two different polypeptide chains (α and β chains) that consist of variable domains (complementarity determining regions; CDRs). CDRs are implicated in the recognition of the TCR to HLA-peptide complex, and their structural diversity plays a crucial role in the recognition of the different antigens presented to T cells by antigen presenting cells (Feng et al., 2015; X. Yang et al., 2015). In fact, there are more than 2.5×10^7 unique TCR (CDRs) structures in humans (X. Yang et al., 2015). In addition, the rigorous positive and negative selection process of T cells in the thymus does not prevent auto-reactive T cells from escaping thymic deletion (Buckley et al., 2015; Hesnard et al., 2015; Lessard et al., 2012), thus initiating the development of autoimmune disorders such as MS.

In patients with MS, T cell responses are primarily associated with recognition of the 81–105 region of MBP (QDENPVVHFFKNIVTPRTPPPSQGK) (Valli et al., 1993), with the $MBP_{83–99}$ (ENPVVHFFKNIVTPRTP) peptide epitope displaying the strongest binding to HLA-DR2 (Martin et al., 1991; Ota et al., 1990), $MBP_{83–96}$ being the minimal recognized epitope. T cell recognition of $MBP_{83–96}$ has also been shown in healthy individuals, albeit at relatively low precursor frequencies (Bieganowska et al., 1997). Hence, the immunodominant $MBP_{83–96}$ epitope plays an important role at inducing $CD4^+$ T cells, which contribute to the demyelination process, and it is therefore considered one of the main targets for developing molecular therapeutics **(Mantzourani et al., 2007; Spyranti et al., 2007)**. The primary binding residues of $MBP_{83–96}$ to HLA-DR2 are via hydrophobic V^{87} and F^{90}, which anchor the peptide into pockets P1 and P4, respectively, as noted in the HLA-DR2-peptide-TCR crystal structure (M. Hahn et al., 2005); albeit at a low resolution of 3.5 A, this structure served as the basis of all future studies of MBP peptides interacting with HLA-DR2. Additionally, other crystal structures reported in the RCSB databank (D. K. Sethi et al., 2011; Yin et al., 2011) that address the role of MBP immunodominant epitopes in MS induction contain the same TCR sequence. Furthermore, it was noted that a second step in the T cell activation process involves the recognition of His^{88} and Phe^{89}, which are placed in pockets P2 and P3 of the TCR (M. Hahn et al., 2005), with secondary binding residues being Val^{86} and Lys^{91}, which are oriented in pockets P-1 and P5 of the TCR (M. Hahn et al., 2005). Thus, a detailed analysis of the interactions between HLA-$MBP_{83–96}$-TCR complexes would lead to valuable information towards rational design of non-peptide mimetics with inhibitory activity. Indeed, a number of studies have shown that using antagonist peptides (1–2 amino acid mutations to TCR contact residues), or altered peptide ligands, can effectively modulate T cell responses and switch from pro- to anti-inflammatory responses (Apostolopoulos et al., 2017a; Katsara, Deraos, et al., 2008b; Katsara, Matsoukas, et al., 2008a; Katsara, Minigo, et al., 2008a; Katsara et al., 2006; Katsara et al., 2008a, 2008b; Katsara, Yuriev, et al., 2009b; Matsoukas et al., 2005b; Tapeinou et al., 2015; T. Tselios et al., 1999). In addition, using a computational structure-based approach, non-peptide mimetics of small organic compounds that were able to bind to MHC class II and block the presentation of $MBP_{152-185}$ to auto-reactive T cells were identified (Koehler et al., 2004).

The principal goal of this study was the rational design of non-peptide mimetic molecules that could bind to the TCR with increased affinity and not to the MHC–peptide

complex. Such potential inhibitors would prevent the formation of the trimolecular complex and consequently the stimulation of T cells. To this end, robust computational techniques, such as molecular docking, pharmacophore modeling, and molecular dynamics, were utilized for the design of novel TCR inhibitors. The application of pharmacophore modeling in the trimolecular complex (HLA-$MBP_{83–96}$-TCR) allows the differentiation between the different contributions (e.g., electrostatic and van der Waals interactions, hydrogen donors and acceptors) involved in the epitope recognition process. By analyzing the variations in these aspects, it is possible to search through diverse chemical databases and filter the results for the identification of potential lead TCR antagonists (hits). Furthermore, molecular docking methodologies can be implemented in order to identify and isolate common substructures of the top ranking hits. Subsequently, the analogue with the best docking score (lead molecule) and preferable structural orientation over the TCR is selected for further optimization and this optimized structure then opts for increased interactions with the TCR. Molecular dynamics (MD) simulations and molecular orbital calculations were carried out in the optimized hits in order to evaluate their binding to the TCR. Finally, the proposed analogues were synthesized to evaluate their biological activity against $MBP_{83–}$99 primed mouse T cells and to human peripheral blood T cells.

2. Materials and Methods

2.1. Structure Preparation

The X-ray crystallographic coordinates contained in PDB entry 1YMM were obtained from the Protein Data Bank (M. Hahn et al., 2005). The particular PDB entry was selected because it contains the main immunodominant epitope $MBP_{83–96}$ involved in MS, as well as a human TCR from a patient with MS. The Molecular Operating Environment (MOE2010) software (Inc., 2016) was utilized for the preparation of the complex. The peptide–TCR complex was isolated, and the residues were protonated accordingly with all hydrogen positions optimized using the AMBER94 force field (Cornell WD, 1995). All the possible protonation states for the histidine (His) residues were explored and evaluated with the use of the PROpKa (J. H. Jensen, Li, Robertson, & Molina, 2005; Li, Robertson, & Jensen, 2005) and AMBER94 tools in MOE2010. The analysis supports the prevalence

of neutral His in all cases, in agreement with the results reported by Wucherpfenning et al. (Wucherpfennig, Gagnon, Call, Huseby, & Call, 2010; Wucherpfennig & Sethi, 2011).

2.2. Pharmacophore Modeling

The pharmacophore model was designed based on the $MBP_{83–96}$ key residues (Y. Li et al., 2005) involved in the binding with the HLA receptor and the TCR. A combination of features from structure– and ligand-based pharmacophore models was utilized in the development of the model presented in this study. According to the crystal structure of the binding cavity of the TCR, an analysis of its chemical features was carried out using the MOE2010 software (Inc., 2016). The development of the ligand-based pharmacophore model relied on features such as aromaticity (Aro), a hydrogen bond cation (Cat) and donor, and hydrophobic groups (Hyd). The Aro motifs were modeled on the His^{88} and Phe^{89}residues of the epitope, the Hyd feature on Val^{86}, and the Cat feature on Pro^{85}, respectively, all residues that interact with the TCR. The volume exclusion (V) features of the pharmacophore model were developed based on Val^{87} and Phe^{90} that interact with the HLA.

2.2.1. Virtual Screening

The pharmacophore hypothesis based on the TCR active site as well as the $MBP_{83–96}$ epitope were utilized to scan 500,000 compounds from the ZINC database (Irwin, Sterling, Mysinger, Bolstad, & Coleman, 2012). The compounds were filtered according to Lipinski's rule of five (Lipinski, Lombardo, Dominy, & Feeney, 2001) and their commercial availability. Finally, the molecules were sorted based on their fitness to the selected hypothesis.

2.3. Molecular Docking

Molecular docking simulations were performed on the TCR using MOE2010 (Inc., 2016). The ligand, as well as the TCR residues in a radius of 4.5 Å surrounding the ligand, was considered flexible. The definition of the TCR binding site was performed manually by selecting the area including the residues involved in the main binding pockets. The ligands were allowed to move freely in the vicinity of the active site. The top 500 poses

for each ligand were ranked using the London ΔG scoring function (Inc., 2016). Subsequently, a maximum of 10 poses were retained based on their docking scoring function, and the poses were rescored using the GBVI/WSA (Generalized-Born Volume Integral/Weighted Surface Area) scoring function (Corbeil, Williams, & Labute, 2012).

2.4. Lead Optimization

Thirteen potential inhibitors (hits) were directly purchased for additional in vitro biological evaluation, as TCR antagonists. Based on their properties and binding scores with the TCR, compound **10** was selected as a lead compound for further optimization. Chemical groups were modified to improve the binding properties, such as orientation of the molecule inside the TCR. Additionally, new chemical groups were added to lengthen the carbon chain and optimize the pocket fit.

2.5. Molecular Dynamics (MD) Simulation

The construction of the TCR parameters was performed using the AMBER force field ff14SB (Maier et al., 2015), while the parameters for the organic molecules were constructed using the general Amber force field (GAFF) (J. Wang, Wolf, Caldwell, Kollman, & Case, 2004). The TIP3P water model (Jorgensen, Chandrasekhar, Madura, Impey, & Klein, 1983) was utilized for the solvation of the system, and the total charge was neutralized by the addition of Cl^- ions. Truncated octahedral periodic boundary conditions were applied to the system with a cutoff distance of 10 Å. The next step involved minimization, followed by the heating of the system, under a constant volume, to 300 K for 100 ps using the Langevin dynamics temperature scaling (Izaguirre, Catarello, Wozniak, & Skeel, 2001). This was followed by equilibration for another 100 ps under constant pressure. Both heating and pressure equilibration were carried out using a 10 $kcal \cdot mol^{-1} \cdot Å^{-2}$ restraint on the solute. The equilibration step under constant pressure was prolonged for a further 200 ps, after removing all restraints. The MD production run was performed under constant pressure and temperature conditions (NPT ensemble) for 50 ns. The temperature was kept constant with the use of the Langevin thermostat (using a collision frequency of 2 ps^{-1}). All bonds involving hydrogen atoms were kept to their equilibrium distance with the SHAKE algorithm (allowing for a 2 fs time step to be used) (Ryckaert, Ciccotti, & Berendsen, 1977). The long range electrostatic interactions were

calculated with the Particle Mesh Ewald (PME) method (Darden, York, & Pedersen, 1993). The different systems were subjected to all-atom unrestrained MD simulations in explicit solvent using AMBER12 (D.A. Case et al., 2012). The cpptraj module (Roe & Cheatham, 2013) of AMBER12 was implemented for the trajectory analysis (clustering, RMSD, hydrogen bonds).

2.6. Chemistry

Reactions involving moisture sensitive reagents were carried out under an argon atmosphere in addition to oven-drying glassware and anhydrous solvents. Room temperature (rt) refers to 20–25 °C. Crude products were purified by flash chromatography on 230–400 mesh silica gel in the solvents system stated. Analytical thin layer chromatography was performed on pre-coated aluminium plates (Merck 60G F254 silica). TLC visualization was performed out with ultraviolet light (254 nm). The yields were calculated in w/w. ^{1}H and ^{13}C nuclear magnetic resonance (^{1}H NMR, ^{13}C NMR) spectra (Figures S2–S14) were acquired on Bruker Avance 400 and Bruker Ascend 600 spectrometer at ambient temperature in the deuterated solvent stated. All chemical shifts are quoted in parts per million (ppm) relative to the internal standard (TMS). All coupling constants, *J*, are quoted in Hz. Multiplicities are indicated by s (singlet), d (doublet), t (triplet), q (quartet), and m (multiplet). The abbreviation Ar is used to denote aromatic, br to denote broad, and app to denote apparent. Mass spectrometry (*m*/*z*) data were acquired on an Electrospray Ionization Platform spectrometer (ESI-MS) by Micromass and a MassLynx NT 2.3 operating system (Waters Corporation).

2.6.1. General Procedure A: N-alkylation of Pyrroles

To a solution of 1*H*-pyrrole analogue (1.00 equiv) in DMF (5–10 mL/mmol), under argon at 0 °C was added sodium hydride 60% (1.50–2.50 equiv), and the resulting mixture was stirred at the same temperature for 10–20 min. Then, a solution of the corresponding alkyl bromide (1.00–1.50 equiv) in DMF (5–10 mL/mmol) was added dropwise, and the reaction mixture warmed to rt over ~2 h (monitored by TLC). It was quenched with water (20 mL) and extracted with EtOAc (3 × 20 mL). The combined organics were washed with brine (20 mL), dried (Na_2SO_4), filtered, and concentrated in vacuo. Purification of the

residue by column chromatography on silica gel (using the appropriate mixture of eluents) allowed pyrroles *N*-protected **15a**, **16a**, and **17b–19b**.

2.6.2. General Procedure B: Hydrolysis of Methyl Pyrrole-2/3-Carboxylates

To a solution of methyl *N*-benzyl pyrrole 3- or 2-carboxylate **15a** or **16a** (1.00 equiv) in MeOH–H_2O 3:1 *v*:*v* (15.0 mL/mmol), an aq solution of KOH 30% (15.0 mL/mmol) was added. The resulting reaction mixture was refluxed and monitored by TLC (10% MeOH–DCM) until completion (~2 h). Then, it was allowed to attain rt and acidified pH = 1 via the addition of 6.0 M HCl (until cloudiness persisted). The white precipitate was filtered off and washed with ice-water to give the crude of **15b** or **16b**, respectively, which was used in the next step without further purification.

2.6.3. General Procedure C: Amidation Reaction

To a solution of the required pyrrole 3- or 2-carboxylic acid, **15a** or **16a** (1.00 equiv) in dichloromethane (DCM) (20.0 mL/mmol), 4-dimethylaminopyridine (DMAP) (20 mol %), *N*-Boc-ethylenediamine (1.00 equiv), and then *N,N′*-dicyclohexylcarbodiimide (DCC) (1.50 equiv) at 0 °C were added. The resulting mixture was warmed to rt and stirred for a further 16 h (monitored by TLC, 10% MeOH–DCM). After completion of the reaction, dicyclohexylurea (DCU) formed was filtered off and washed with DCM (5 mL) at 0 °C. The organic layer was quenched with 0.1 M HCl (20 mL) and extracted with EtOAc (3 × 20 mL). The combined organics were washed with brine (20 mL), dried (Na_2SO_4), filtered, and concentrated in vacuo. Purification of the residue by column chromatography on silica gel (using the appropriate mixture of eluents) allowed pyrrole 3-/2-carboxamides **15c** or **16c**.

2.6.4. General Procedure D: Removal of the Boc-group

The corresponding *N*-Boc analogue (1.00 equiv) was dissolved in trifluoroacetic acid (TFA)–DCM 95:5 *v*/*v* DCM (20–30 mL/mmol) (and added triethylsilane (TES, 1.00 equiv) if required). The reaction mixture was stirred at rt, and the progress was monitored by TLC (10% MeOH–DCM) until complete consumption of the starting material.

2.6.5. General Procedure E: Guanylation Reaction

The amine salt **15c'** or **16c'** (as crude derived from *N*-Boc deprotection of **15c** or **16c**) was dissolved in a mixture of MeOH–DCM 4:1 *v*:*v* (20.0 mL/mmol), under argon. Then, *N*,*N*′-di-(*tert*-butoxycarbonyl)thiourea (1.50 equiv), *N*,*N*-diisopropylethylamine (DIPEA) (4.00 equiv) and *N*-iodosuccinimide (1.50 equiv) in one portion were added at rt. The reaction mixture was stirred at rt under argon, and monitored by thin layer chromatography (TLC) (20% MeOH–DCM) until completion (~24 h). It was next quenched with an aq solution of 1 M sodium thiosulfate solution (20 mL), and the resulting solution was then diluted in water (20 mL) and extracted with EtOAc (3 × 20 mL). The combined organic layer was washed with brine (20 mL), dried (Na_2SO_4), filtered, and concentrated in vacuo. Purification of the residue by column chromatography on silica gel (using the appropriate mixture of eluents) allowed the corresponding di-Boc-guanidino derivatives **15d** or **16d**.

2.6.6. Synthesis of Methyl 1-Benzyl-1H-Pyrrole-3-Carboxylate 15a (Padwa, 1985)

From methyl 1*H*-pyrrole-3-carboxylate (98.4 mg, 0.79 mmol) and NaH 60% (38.0 mg, 1.58 mmol) in dimethylformamide (DMF) (4.0 mL), and a solution of benzyl bromide (0.14 mL, 1.18 mmol) in DMF (6.0 mL), following the general procedure A (2 h) and after chromatographic purification (DCM), **15a** (144 mg, 85%) was obtained as a clear gum. Data for **15a**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.31–7.37 (m, 4 H, Ph, Ar), 7.13–7.15 (m, 2 H, Ph), 6.60–6.63 (m, 2 H, Ar), 5.06 (s, 2 H, CH_2Ph), 3.79 (s, 3 H, OMe); ESI-MS *m/z* found for $C_{13}H_{13}NO_2$: 216.32 $[M+H]^+$; RP-HPLC gradient separation from 30% to 100% acetonitrile at 30 min, flow rate: 1 mL/min, t_R = 10.8 min.

2.6.7. Synthesis of 1-Benzyl-1H-Pyrrole-3-Carboxylic Acid 15b (Villarreal & Martínez, 2010)

From methyl 1-benzyl-1*H*-pyrrole-3-carboxylate **15a** (144 mg, 0.67 mmol) in MeOH–H_2O (10.0 mL) and an aq solution of KOH 30% (10.0 mL), following the general procedure B (2 h) and after precipitation, the crude of **15b** (90.0 mg, 67%) was used in the next step without further purification. Data for **15b**: proton nuclear magnetic resonance (^{1}H NMR) (400 MHz, $CDCl_3$) δ 7.29–7.40 (m, 4 H, Ph, Ar), 7.15 (d, 2 H, J = 7.2 Hz, Ph), 6.63-6.66 (m, 2 H, Ar), 5.07 (s, 2 H, CH_2Ph); electrospray ionization mass spectrometry (ESI-MS) *m/z* found for $C_{12}H_{11}NO_2$: 425.41 $[2M+Na]^+$, 202.25 $[M+H]^+$; RP-HPLC

gradient separation from 5% to 100% acetonitrile at 30 min, flow rate: 1 mL/min, t_R = 14.2 min.

2.6.8. Synthesis of 1-Benzyl-1H-N-[2-(Tert-Butoxycarbonyl)Aminoethyl]Pyrrole-3-Carboxamide 15c

From 1-benzyl-1*H*-pyrrole-3-carboxylic acid **15b** (90.0 mg, 0.45 mmol) in DCM (9.0 mL), DMAP (10.9 mg, 0.09 mmol), *N*-Boc-ethylenediamine (0.07 mL, 0.45 mmol), and then DCC (138 mg, 0.67 mmol), following the general procedure C (16 h) and after chromatographic purification (20% MeOH–DCM), **15c** (126 mg, 82%) was obtained as a clear gum. Data for **15c**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.29–7.35 (m, 3 H, Ph), 7.25–7.26 (m, 1 H, Ar), 7.12–7.14 (m, 2 H, Ph), 6.62 (app t, 1 H, J = 2.4 Hz, Ar), 6.48 (br s, 1 H, NH), 6.41 (br s, 1 H, Ar), 5.04 (s, 2 H, CH_2Ph), 4.98 (br s, 1 H, NH), 3.51–3.45 (m, 2 H, CH_2), 3.32–3.35 (m, 2 H, CH_2), 1.41 (s, 9 H, 3 × CH_3*t*-Bu); ESI-MS *m/z* found for $C_{19}H_{25}N_3O_3$: 344.33 $[M+H]^+$, 288.32 $[(M–Ph)+Na]^+$; reversed phase high-performance liquid chromatography (RP-HPLC) gradient separation from 5% to 100% acetonitrile at 30 min, flow rate: 1 mL/min, t_R = 17.0 min.

2.6.9. Synthesis of 1-Benzyl-1H-N-[2-(2,3-Di-Tert Butoxycarbonyl) Guanidinoethyl] Pyrrole-3-Carboxamide 15d

From *N*-Boc analogue **15c** (120 mg, 0.35 mmol) in TFA–DCM 95:5 (7.0 mL), following the general procedure D (1 h), the crude of 2-(1-benzyl-1*H*-pyrrole-3-carboxamido)ethanaminium 2,2,2-trifluoroacetate **15c'** was dissolved in MeOH–DCM (7.0 mL). Then, *N*,*N*′-di-(*tert*-butoxycarbonyl)thiourea (145 mg, 0.52 mmol), DIPEA (0.24 mL, 1.40 mmol, 4.00 equiv), and *N*-iodosuccinimide (118 mg, 0.52 mmol), following the general procedure E (24 h) and after chromatographic purification (20% MeOH–DCM), **15d** (56.6 mg, 33%) was obtained as a clear gum. Partial data for **15c'**: ESI-MS *m/z* found for $C_{14}H_{17}N_3O$: 244 $[M]^+$; RP-HPLC gradient separation from 5% to 100% acetonitrile at 30 min, flow rate: 1 mL/min, t_R = 10.3 min. Data for **15d**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.50 (s, 1 H, NH), 8.71 (s, 1 H, NH), 7.28–7.41 (m, 5 H, Ph, Ar, NH), 7.11–7.13 (m, 2 H, Ph), 6.56–6.59 (m, 2 H, Ar), 5.04 (s, 2 H, CH_2Ph), 3.67–3.71 (m, 2 H, CH_2), 3.53–3.57 (m, 2 H, CH_2), 1.51 (s, 9 H, 3 × CH_3*t*-Bu), 1.49 (s, 9 H, 3 × CH_3*t*-Bu); ESI-MS *m/z* found

for $C_{25}H_{35}N_5O_5$: 486.34 $[M+H]^+$; RP-HPLC gradient separation from 30% to 100% acetonitrile at 30 min, flow rate: 1 mL/min, t_R = 13.4 min.

2.6.10. Synthesis of 1-Benzyl-1H-N-(2-Guanidinoethyl)Pyrrole-3-Carboxamide 15

From di-Boc guanidine analogue **15d** (50.0 mg, 0.10 mmol) in TFA–DCM 95:5 (3.0 mL), following the general procedure D (1 h) and after chromatographic purification (0.5% NH_4OH, 19.5% MeOH, 80% DCM), final product **15** (26.5 mg, 91%) was obtained as a white solid. Data for 15: ^1H NMR (400 MHz, CD_3OD) δ 7.27–7.36 (m, 4 H, Ph, Ar), 7.20–7.22 (m, 2 H, Ph), 6.78 (dd, 1 H, J = 2.8, 2.4 Hz, Ar), 6.52 (dd, 1 H, J = 2.8, 2.0 Hz, Ar), 5.13 (s, 2 H, CH_2Ph), 3.46 (t, 2 H, J = 6.3 Hz, CH_2), 3.35 (t, 2 H, J = 6.3 Hz, CH_2); ^{13}C NMR (100 MHz, CD_3OD) δ 168.6 (C=O), 159.0 (C=NH), 139.0 (C Ph), 129.8 (2 × CH Ph), 129.0 (CH Ph), 128.5 (2 × CH Ph), 125.4 (CH Ar), 123.6 (CH Ar), 120.1 (C Ar), 109.0 (CH Ar), 54.5 (CH_2Ph), 42.4 (CH_2), 39.4 (CH_2); ESI-MS *m/z* found for $C_{15}H_{19}N_5O$: 286.66 $[M+H]^+$, 243.21 $[M–(C(NH)NH_2)+H]^+$, 214.16 $[M–(CH_2NHC(NH)NH_2)+H]^+$; RP-HPLC gradient separation from 10% to 100% acetonitrile at 30 min, flow rate: 1 mL/min, t_R = 19.3 min, $R_{f=}$ 0.46 (MeOH–DCM 2:8).

2.6.11. Synthesis of Methyl 1-Benzyl-1H-Pyrrole-2-Carboxylate 16a (Padwa, 1985)

From methyl 1*H*-pyrrole-2-carboxylate (151 mg, 1.21 mmol) and NaH 60% (58.1 mg, 2.42 mmol) in DMF (6.0 mL), and a solution of benzyl bromide (0.21 mL, 1.80 mmol) in DMF (9.0 mL), following the general procedure A (3 h) and after chromatographic purification (DCM), **16a** (234 mg, 90%) was obtained as a pale yellow oil. Data for **16a**: ^1H NMR (400 MHz, $CDCl_3$) δ 7.23–7.34 (m, 3 H, Ph), 7.11 (d, 2 H, J = 7.2 Hz, Ph), 7.02 (dd, 1 H, J = 3.4, 1.6 Hz, Ar), 6.89 (app t, 1 H, J = 1.6 Hz, Ar), 6.19 (app t, 1 H, J = 3.4 Hz, Ar), 5.57 (s, 2 H, CH_2Ph), 3.77 (s, 3 H, OMe); ESI-MS *m/z* found for $C_{13}H_{13}NO_2$: 216 $[M+H]^+$, 138 $[(M–Ph)+H]^+$; RP-HPLC gradient separation from 30 to 100% acetonitrile at 30 min, flow rate: 1 mL/min, t_R = 13.9 min.

2.6.12. Synthesis of 1-Benzyl-1H-Pyrrol-2-Carboxylic Acid 16b

From methyl 1-benzyl-1*H*-pyrrole-2-carboxylate **16a** (230 mg, 1.07 mmol) in MeOH–H_2O (16.0 mL) and an aq solution of KOH 30% (16.0 mL), following the general

procedure B (3 h) and after precipitation, the crude of **16b** (172 mg, 80%) was used in the next step without further purification. Data for **16b**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.24–7.33 (m, 3 H, Ph), 7.14 (dd, 1 H, *J* = 3.8, 2.0 Hz, Ar), 7.11 (d, 2 H, *J* = 6.8 Hz, Ph), 6.93 (app t, 1 H, *J* = 2.0 Hz, Ar), 6.21 (dd, 1 H, *J* = 3.8, 2.8 Hz, Ar), 5.56 (s, 2 H, CH_2Ph); ESI-MS (EI) *m/z* found for $C_{12}H_{11}NO_2$: 240 $[M+K]^+$, 224 $[M+Na]^+$, 202 $[M+H]^+$; RP-HPLC gradient separation from 5% to 100% acetonitrile at 30 min, flow rate: 1 mL/min, t_R = 16.2 min.

2.6.13. Synthesis of 1-Benzyl-1H-N-[2-(Tert-Butoxycarbonyl)Aminoethyl]Pyrrole-2-Carboxamide 16c

From 1-benzyl-1*H*-pyrrole-2-carboxylic acid **16b** (172 mg, 0.86 mmol) in DCM (17 mL), DMAP (21.0 mg, 0.17 mmol), *N*-Boc-ethylenediamine (0.13 mL, 0.86 mmol), and then DCC (266 mg, 1.29 mmol), following the general procedure C (16 h) and after chromatographic purification (20% MeOH-CH_2Cl_2), **16c** (248 mg, 84%) was obtained as a clear gum. Data for **16c**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.20-7.30 (m, 5 H, Ph, Ar, NH), 7.11 (d, 2 H, *J* = 7.2 Hz, Ph), 6.79 (br s, 1 H, Ar), 6.64 (br d, 1 H, *J* = 2.0 Hz, NH), 6.13 (app t, 1 H, *J* = 3.2 Hz, Ar), 5.60 (s, 2 H, CH_2Ph), 3.42 (t, 2 H, *J* = 5.6 Hz, CH_2), 3.29 (app t, 2 H, *J* = 5.6 Hz, CH_2), 1.43 (s, 9 H, 3 × CH_3*t*-Bu); ESI MS *m/z* found for $C_{19}H_{25}N_3O_3$: 367 $[M+Na]^+$, 344 $[M+H]^+$, 288 $[(M–Ph)+Na]^+$, 244 $[(M–Boc)+H]^+$; RP-HPLC gradient separation from 30% to 100% acetonitrile at 30 min, flow rate: 1 mL/min, t_R = 13.3 min.

2.6.14. Synthesis of 1-Benzyl-1H-N-[2-(2,3-Di-Tert-Butoxycarbonyl)Guanidinoethyl] Pyrrole-2-Carboxamide 16d

From *N*-Boc analogue **16c** (248 mg, 0.72 mmol) in TFA–DCM 95:5 (14.4 mL), following the general procedure D (1 h), the crude of 2-(1-benzyl-1*H*-pyrrole-2-carboxamido) ethanaminium 2,2,2-trifluoroacetate **16c'** was dissolved in MeOH–DCM (14.4 mL). Then, from *N*,*N*′-di-(*tert*-butoxycarbonyl)thiourea (299 mg, 1.08 mmol), DIPEA (0.50 mL, 2.88 mmol), and *N*-iodosuccinimide (243 mg, 1.08 mmol), following the general procedure E (~24 h) and after chromatographic purification (20% MeOH-CH_2Cl_2), **16d** (136 mg, 39%) was obtained as a clear gum. Partial data for **16c'**: ESI-MS *m/z* found for $C_{14}H_{17}N_3O$: 267 $[M+Na]^+$, 244 $[M]^+$, 227 $[M–NH_2]^+$, 184 $[M–HN(CH_2)NH_2]^+$, 158 $[M–(CO)HN(CH_2)NH_2]^+$; RP-HPLC gradient separation from 5% to

100% acetonitrile at 30 min, flow rate: 1 mL/min, t_R = 11.1 min. Data for **16d**: ^{1}H NMR (400 MHz, CD_3OD) δ 7.28–7.18 (m, 3 H, Ph), 7.07 (d, 2 H, *J* = 6.8 Hz, Ph), 6.92 (dd, 1 H, *J* = 2.4, 1.6 Hz, Ar), 6.76 (dd, 1 H, *J* = 3.6, 1.6 Hz, Ar), 6.12 (dd, 1 H, *J* = 3.6, 2.4 Hz, Ar), 5.57 (s, 2 H, CH_2Ph), 3.29–3.32 (m, 2 H, CH_2), 3.16 (t, 2 H, *J* = 6.2 Hz, CH_2), 1.52 (s, 9 H, 3 × CH_3*t*-Bu), 1.42 (s, 9 H, 3 × CH_3*t*-Bu); ESI-MS *m/z* found for $C_{25}H_{35}N_5O_5$: 486 $[M+H]^+$; RP-HPLC gradient separation from 5% to 100% acetonitrile at 30 min, flow rate: 1 mL/min, t_R = 21.9 min.

2.6.15. Synthesis of 1-Benzyl-1H-N-(2-Guanidinoethyl)-Pyrrole-2-Carboxamide 16

From di-Boc guanidine analogue **16d** (114 mg, 0.23 mmol) in TFA–DCM 95:5 (7.0 mL), following the general procedure E (1 h) and after chromatographic purification (0.5% NH_4OH, 19.5% MeOH, 80% DCM), final product **16** (58.6 mg, 88%) was obtained as a white solid. Data for **16**: ^{1}H NMR (600 MHz, CD_3OD) δ 7.19–7.27 (m, 3 H, Ph), 7.07 (d, 2 H, *J* = 7.8 Hz, Ph), 6.97–6.98 (m, 1 H, Ar), 6.79–6.80 (m, 1 H, Ar), 6.14–6.15 (m, 1 H, Ar), 5.59 (s, 2 H, CH_2Ph), 3.40 (t, 2 H, *J* = 6.3 Hz, CH_2), 3.26 (t, 2 H, *J* = 6.3 Hz, CH_2); ^{13}C NMR (100 MHz, CD_3OD) δ 165.2 (C=O), 159.9 (C=NH), 140.6 (C Ph), 129.5 (2 × CH Ph), 129.3 (CH Ph), 128.3 (CH Ar), 127.9 (2 × CH Ph), 126.0 (C Ar), 114.9 (CH Ar), 109.0 (CH Ar), 52.7 (CH_2Ph), 42.4 (CH_2), 39.3 (CH_2); ESI-MS *m/z* found for $C_{15}H_{19}N_5O$: 286 $[M+H]^+$; RP-HPLC gradient separation from 5% to 100% acetonitrile at 30 min, flow rate: 1 mL/mi, t_R = 20.2 min, R_f = 0.46 (MeOH–DCM 2:8).

2.6.16. Synthesis of N-[2-(2,3-Di-Tert-Butoxycarbonyl)Guanidinoethyl]-1-(m-(1-Trityl-Tetrazol-5-yl)Benzyl)-1H-Pyrrole-3-Carboxamide 17b [24]

From 1*H*-pyrrole **21** (65.1 mg, 0.26 mmol), NaH 60% (15.4 mg, 0.39 mmol) in DMF (2.6 mL), and a solution of **17a** (Agelis et al., 2011) (124 mg, 0.26 mmol) in DMF (2.6 mL), following the general procedure A (2 h) and after chromatographic purification (10–100% AcOEt-Et_2O), **17b** (81.8 mg, 40%) was obtained as a white solid. Data for **17b**: ^{1}H NMR (600 MHz, $CDCl_3$) δ 11.48 (s, 1 H, NH), 8.06 (d, 1 H, *J* = 7.8 Hz, Ar'), 7.99 (s, 1 H, Ar'), 7.32–7.42 (m, 13 H, Ar, Ar', Trt, NH), 7.13–7.16 (m, 8 H, Ar, Ar', Trt), 6.60 (d, 1 H, *J* = 1.8 Hz, Ar), 5.09 (s, 2 H, CH_2Ar), 3.76 (br s, 2 H, CH_2), 3.59 (br s, 2 H, CH_2), 1.51 (s, 9 H, 3 × CH_3 *t*-Bu), 1.49 (s, 9 H, 3 × CH_3 *t*-Bu); ESI-MS *m/z* found for $C_{45}H_{49}N_9O_5$: 796.30 $[M+H]^+$, 696.27 $[(M–Boc)+H]^+$, 341.71 $[(17a–Trt)+Boc+H]^+$, 243 $[Trt]^+$; RP-

HPLC gradient separation from 60% to 100% acetonitrile at 30 min, flow rate: 1 mL/min, t_R = 15.8 min.

2.6.17. Synthesis of 1-(2-(1-(m-(1H-Tetrazol-5-Yl)Benzyl)-1H-Pyrrole-3-Carboxamido)Ethyl) Guanidinium 2,2,2-Trifluoroacetate 17

From **17b** (20 mg, 0.025 mmol) and TES (0.004 mL, 0.025 mmol) in TFA–DCM 95:5 (0.75 mL), following the general procedure D (5 h) and after purification by semi-preparative HPLC (10–60% ACN, 45 min), then lyophilization, final product **17** (7.89 mg, 68%) was obtained as a white solidwith 99% purity. Data for **17**: ^{1}H NMR (600 MHz, CD_3OD) δ 7.95 (d, 1 H, *J* = 7.8 Hz, Ar'), 7.90 (s, 1 H, Ar'), 7.57 (t, 1 H, *J* = 7.8 Hz, Ar'), 7.43 (d, 1 H, *J* = 7.8 Hz, Ar'), 7.41 (app dd, 1 H, *J* = 2.4, 1.8 Hz, Ar), 6.85 (dd, 1 H, *J* = 3.0, 2.4 Hz, Ar), 6.56 (dd, 1 H, *J* = 3.0, 1.8 Hz, Ar), 5.26 (s, 2 H, CH_2Ar'), 3.47 (t, 2 H, *J* = 6.0 Hz, CH_2), 3.36 (t, 2 H, *J* = 6.0 Hz, CH_2); ^{13}C NMR (100 MHz, CD_3OD) δ 168.6 (C=O), 159.0 (2 × C=NH), 140.8 (C Ar'), 131.5 (CH), 131.1 (CH), 127.7 (CH), 127.2 (CH), 126.3 (C Ar'), 125.4 (CH), 123.7 (CH), 120.5 (C Ar), 109.4 (CH Ar), 54.0 (CH_2Ar'), 42.4 (CH_2), 39.5 (CH_2); ESI-MS *m/z* found for $C_{16}H_{19}N_9O$: 354.53 $[M+H]^+$; RP-HPLC gradient separation from 10% to 100% acetonitrile at 30 min, flow rate: 1 mL/min, t_R = 17.7 min.

2.6.18. Synthesis of N-[2-(2,3-Di-Tert-Butoxycarbonyl)Guanidinoethyl]-1-(p-Tert-Butoxycarbonyl Methyl)Benzyl-1H-Pyrrole-3-Carboxamide 18b

From 1*H*-pyrrole **21** (168 mg, 0.66 mmol), NaH 60% (39.8 mg, 1.66 mmol) in DMF (6.6 mL), and a solution of **18a**, (189 mg, 0.66 mmol) in DMF (6.6 mL), following the general procedure A (2 h) and after chromatographic purification (50–100% AcOEt-Et_2O), **18b** (87.6 mg, 22%) was obtained as a white solid). Data for **18b**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.49 (s, 1 H, NH), 8.78 (s, 1 H, NH), 7.46 (s, 1 H, NH), 7.31 (s, 1 H, Ar), 7.22 (d, 2 H, *J* = 8.0 Hz, Ar'), 7.07 (d, 2 H, *J* = 8.0 Hz, Ar'), 6.57 (d, 2 H, *J* = 2.0 Hz, Ar), 5.02 (s, 2 H, CH_2Ar), 3.67–3.71 (m, 2 H, CH_2), 3.56 (br s, 2 H, CH_2), 3.50 (s, 2 H, CH_2CO_2t-Bu), 1.51 (s, 9 H, 3 × CH_3t-Bu),1.49 (s, 9 H, 3 × CH_3t-Bu), 1.43 (s, 9 H, 3 × CH_3t-Bu).

2.6.19. Synthesis of 1-(2-(1-(p-(Carboxymethyl)Benzyl)-1H-Pyrrole-3-Carboxamido)Ethyl) Guanidinium 2,2,2-Trifluoroacetate 18

From **18b** (50 mg, 0.083 mmol) and TES (0.01 mL, 0.083 mmol) in TFA–DCM 95:5 (2.50 mL), following the general procedure D (5 h) and after purification by semi-preparative HPLC (10–60% ACN, 45 min), then lyophilization, final product **18** (23.2 mg, 61%) was obtained as a white solidwith 98% purity. Data for **18**: ^{1}H NMR (600 MHz, CD_3OD) δ 7.33 (app dd, 1 H, J = 2.4, 1.8 Hz, Ar), 7.27 (d, 2 H, J = 8.1 Hz, Ar'), 7.17 (d, 2 H, J = 8.1 Hz, Ar'), 6.78 (dd, 1 H, J = 3.0, 2.4 Hz, Ar), 6.52 (dd, 1 H, J = 3.0, 1.8 Hz, Ar), 5.11 (s, 2 H, CH_2Ar'), 3.59 (s, 2 H, CH_2CO_2H), 3.46 (t, 2 H, J = 6.0 Hz, CH_2), 3.35 (t, 2 H, J = 6.0 Hz, CH_2); ESI-MS *m/z* found for $C_{17}H_{21}N_5O_3$: 344.66 $[M+H]^+$; RP-HPLC gradient separation from 10% to 100% acetonitrile at 30 min, flow rate: 1 mL/min, t_R = 16.6 min.

2.6.20. Synthesis of N-[2-(2,3-Di-Tert-Butoxycarbonyl)Guanidinoethyl]-1-(P-Methoxycarbonyl) Benzyl-1H-Pyrrole-3-Carboxamide 19b

From 1*H*-pyrrole **21** (109 mg, 0.43 mmol), NaH 60% (25.8 mg, 0.65 mmol) in DMF (4.3 mL), and a solution of **19a**, (98.5 mg, 0.43 mmol) in DMF (4.3 mL), following the general procedure A (2 h) and after chromatographic purification (50–100% AcOEt-Et_2O), **19b** (103 mg, 44%) was obtained as a white solid. Data for **19b**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.49 (s, 1 H, NH), 8.73 (s, 1 H, NH), 7.98 (d, 2 H, J = 8.2 Hz, Ar'), 7.49 (s, 1 H, NH), 7.31 (t, 1 H, J = 1.8 Hz, Ar), 7.15 (d, 2 H, J = 8.2 Hz, Ar'), 6.56–6.60 (m, 2 H, Ar), 5.10 (s, 2 H, CH_2Ar), 3.90 (s, 3 H, OCH_3), 3.67–3.71 (m, 2 H, CH_2), 3.55–3.57 (m, 2 H, CH_2), 1.50 (s, 9 H, 3 × CH_3*t*-Bu), 1.49 (s, 9 H, 3 × CH_3*t*-Bu); ESI-MS *m/z* found for $C_{27}H_{37}N_5O_7$: 344.60 $[(M–2\times Boc)+H]^+$; RP-HPLC gradient separation from 5% to 100% acetonitrile at 30 min, flow rate: 1 mL/min, t_R = 25.7 min.

2.6.21. Synthesis of 1-(2-(1-(p-(Methoxycarbonyl)Benzyl)-1H-Pyrrole-3-Carboxamido)Ethyl) Guanidinium 2,2,2-Trifluoroacetate 19

From **19b** (20 mg, 0.037 mmol) in TFA–DCM 95:5 (0.74 mL), following the general procedure D (5 h) and after purification by semi-preparative HPLC (10–60% ACN, 45 min), then lyophilization, final product **19** (13.3 mg, 79%) was obtained as a white solid

with 99% purity. Data for **19**: ^{1}H NMR (400 MHz, CD_3OD) δ 7.98 (d, 2 H, J = 8.0 Hz, Ar'), 7.36 (app t, 1 H, J = 2.0 Hz, Ar), 7.28 (d, 2 H, J = 8.0 Hz, Ar'), 6.81–6.82 (m, 1 H, Ar), 6.55 (dd, 1 H, J = 2.8, 2.0 Hz, Ar), 5.23 (s, 2 H, CH_2Ar'), 3.89 (s, 3 H, OCH_3), 3.47 (t, 2 H, J = 6.4 Hz, CH_2), 3.35 (t, 2 H, J = 6.4 Hz, CH_2); ESI-MS *m/z* found for $C_{17}H_{21}N_5O_3$: 344.68 $[M+H]^+$; RP-HPLC gradient separation from 5% to 100% acetonitrile at 30 min, flow rate: 1 mL/min, t_R = 18.9 min.

2.6.22. Synthesis of N-(2,3-Di-(Tert-Butyloxycarbonyl)Guanidinoethyl)Pyrrole-3-Carboxamide 21

To a solution of 1*H*-pyrrole-3-carboxylic acid (85.5 mg, 0.77 mmol, 1.00 equiv) in DCM (5 mL) and DMF (1 mL), at 0 °C, HOBt (178 mg, 1.16 mmol, 1.50 equiv) and DCC (239 mg, 1.16 mmol, 1.50 equiv) were added. The mixture was stirred at the same temperature for 10 min, and was then supplemented with a solution of **20** (Exposito, Fernandez-Suarez, Iglesias, Munoz, & Riguera, 2001) (350 mg, 1.16 mmol, 1.50 equiv) in DCM (13 mL) followed by DIPEA (0.20 mL, 1.16 mmol, 1.50 equiv). The reaction mixture warmed to rt over 3 h and monitored by TLC (10% MeOH in DCM). The solvents were removed in vacuo, and the remaining residue was purified by column chromatography (5% EtOH in Et_2O) to yield **21** (170 mg, 0.67 mmol, 87%) as a beige solid. Data for **21**:^{1}H NMR (400 MHz, $CDCl_3$) δ 11.51 (s, 1 H, NH), 8.82 (s, 2 H, NH), 7.57 (s, 1 H, NH), 7.43–7.44 (m, 1 H, Ar), 6.73–6.75 (m, 1 H, Ar), 6.63–6.65 (m, 1 H, Ar), 3.71–3.75 (m, 2 H, CH_2), 3.57–3.60 (m, 2 H, CH_2), 1.55 (s, 9 H, 3 × CH_3*t*-Bu), 1.51 (s, 9 H, 3 × CH_3*t*-Bu); ESI-MS *m/z* found for $C_{18}H_{29}N_5O_5$: 341 $[M–tBu+H]^+$, 381 $[M–tBu+K+H]^{2+}$; RP-HPLC gradient separation from 5% to 100% acetonitrile at 30 min, flow rate: 1 mL/min, t_R = 21.4 min.

2.7. Molecular Orbital Calculations

Two different approaches were applied in order to calculate the binding energy of the compounds inside TCR, namely density functional theory (DFT) (Kohn & Sham, 1965) and semi-empirical (SE) methods (J. J. Stewart, 2013). For the application of DFT, several variants (McNamara & Hillier, 2007) differing in choice of functional (McNamara, Sharma, Vincent, Hillier, & Morgado, 2008) and basis set were implemented in order to calculate the interaction. This procedure was followed to select the most appropriate

method for our complex (see Supporting Information). The self-consistent reaction field (SCRF) was used with DFT energies, optimizations, and frequency calculations to model the system in solution (H_2O). All DFT calculations were performed with Gaussian09 (M. J. Frisch, 2009). A similar protocol was applied for the calculation of the interaction energy including the whole TCR with SE methodologies. The MOPAC2012 (J. J. Stewart, 2013) software was used for the SE calculations. Due to the large size of the protein–ligand systems, the keyword MOZYME (J.J.P., 2007) was employed to accelerate the calculations, and the COSMO (A. Klamt, 1993) function was used to estimate the effect of the solvent. For the methods including dispersion (D), the optimized parameters for H, N, C, and O, as reported by McNamara and Hillier (McNamara & Hillier, 2007; McNamara et al., 2008), were used. Semi-empirical calculations were performed on the whole complex (ligand–TCR), while DFT on the ligand and selected TCR residues.

2.8. In Vitro Evaluation of the Analogues Using Human PBMC

Peripheral blood samples (10 mL) were drawn from two healthy volunteers (one 24-year-old male and one 35-year-old female) and were analyzed in a CELL-DYN Sapphire hematology analyzer (Abbot Diagnostics) to determine the absolute numbers and percentages of leukocytes, in particular lymphocytes and monocytes. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation over a Ficoll–Paque gradient (Biochrom AG) and washed ×3 with ice-cold RPMI1640 culture medium (Gibco BRL). The cells were stained with CellTrace CFSE for flow cytometry (Invitrogen-Thermo Fisher Scientific Inc.) as described and cultured in RPMI1640 (with 10% Fetal Bovine Serum, 50 IU/mL penicillin, 100 μg/mL streptomycin, and 5×10^{-5} mol/L mercaptoethanol) (Invitrogen) at a concentration of 10^6 cells/mL. PBMCs were cultured for three days in the presence of an anti–CD28 antibody (5 μg/mL) (BD Biosciences/Pharmingen) and different concentrations of peptide $MBP_{83–96}$ (0.01 nM, 0.1 nM, 1 nM, 10 nM, and 100 nM) to estimate the optimal concentration that induces T cell proliferation. When the optimal $MBP_{83–96}$ concentration was determined, the cultures were repeated as previously with the addition of the same concentration of each of the studied analogues per point, in triplicate. T cell proliferation was monitored and quantified by flow cytometry. Flow cytometric acquisition and analysis were performed on at least 10,000 acquired events per sample using the BD FACSCalibur™ platform.

2.9. In Vitro Evaluation of the Analogues Using Mouse-Specific $MBP_{83–99}$ T Cells

Mice, SJL/J females, aged 4–9 weeks were purchased from the Animal Resource Centre (Perth Australia). All mice had free access to food and water, and were housed in a temperature-controlled environment with 12-h day/night cycles at the animal holding room Werribee Campus Animal Facility (Melbourne, Australia). They were allowed to acclimatize for at least 7 days before immunizations. All experiments were completed according to the guidelines of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and were approved by Victoria University Animal Experimentation Ethics Committee (AEC15/013). Mice were subcutaneously injected with 50 μg/100 μL reduced mannan conjugated to $MBP_{83–99}$ via a 10 amino acid linker $(KG)_5$ as previously described (Day et al., 2015; Tapeinou et al., 2015). This conjugate has been shown to induce T cell proliferation to native peptide $MBP_{83–99}$ (Day et al., 2015; Katsara, Deraos, et al., 2008b; Katsara, Matsoukas, et al., 2008a; Katsara et al., 2008a, 2008b; Katsara, Yuriev, et al., 2009b; Tapeinou et al., 2015). Spleen cells from 3 immunized SJL/J mice were isolated 10 days after immunization and assessed by T cell proliferation assay. As we have previously shown that the native peptide $MBP_{83–99}$ conjugated to mannan induces strong proliferative T cells to recall $MBP_{83–99}$ peptide, we used 3 mice/group to test each of the compounds' ability to inhibit this T cell proliferation. Hence, 3 mice/group in this screening process are adequate for determining the optimal compound for inhibiting T cell proliferation. Spleen cells at 2×10^5 in 100 μL of culture media were seeded into 96 well U-bottom plates and incubated for 1–6 days at 37 °C in the presence of recall $MBP_{83–99}$ peptide (10 nM) with or without 100x molar excess of compounds **15**–**19** or **AMB**. Proliferation was assessed by the addition of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) for 6 hours and proliferation assessed via spectrophotometry (Biorad microplate reader, 6.0) using a wavelength of 570 nm. All experiments were conducted in triplicate. The percentage of inhibition of cell proliferation in the presence of compound was calculated and plotted.

3. *Results and Discussion*

3.1. Pharmacophore Modeling and Virtual Screening

In computational drug discovery, screening of large databases with chemical property information obtained from relatively small data is essential. The combination of results from structure- and ligand-based pharmacophore models allows a thorough search in order to discover potential antagonists. The proposed pharmacophore model (Figure 1) is based on features such as an aromatic ring (Aro, green), a hydrogen bond (HB), cation and donor (Cat, magenta), hydrophobic groups (Hyd, orange), and volume exclusion (V, gray). The detailed parameters utilized for the construction of the model are described in the Materials and Methods section. The key features are based on residues His^{88} and Phe^{89} (Aro, Figure 1, green sphere), Val^{86} (Hyd, Figure 1 orange sphere), and Pro^{85} (Cat, Figure 1, magenta sphere). The grey spheres in Figure 1 represent residues with bulky side chains, such as Val^{87} and Phe^{90}, that do not interact with the TCR. These residues are employed to define the Volume Exclusion (V) feature of the pharmacophore model. This information is important for excluding residues that interact with the HLA receptor and consequently are not involved in key interactions with the TCR.

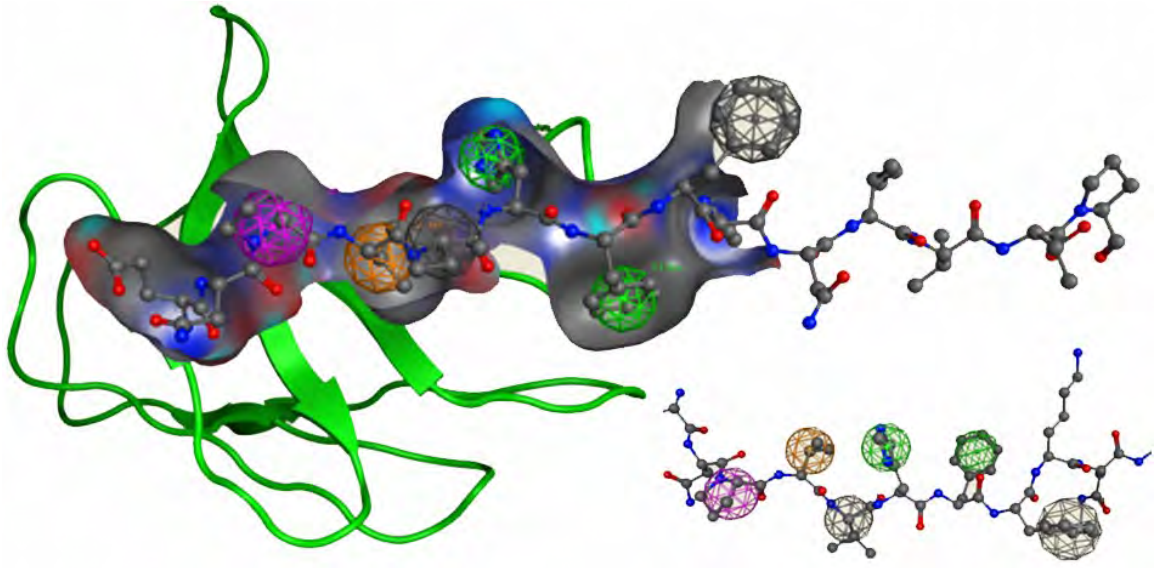
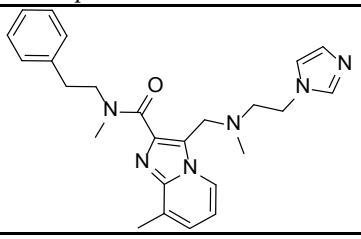
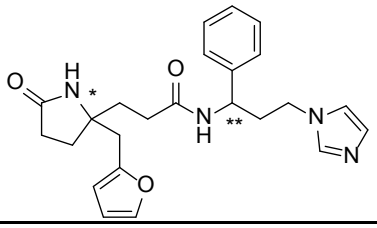
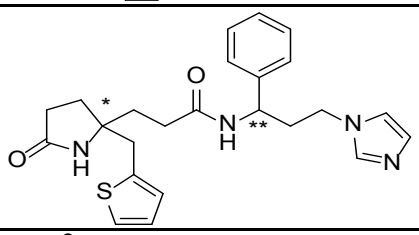
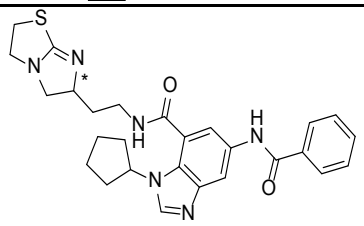
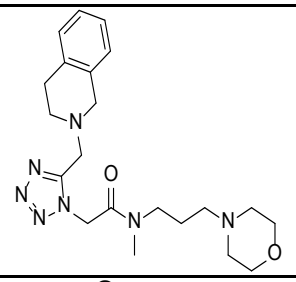
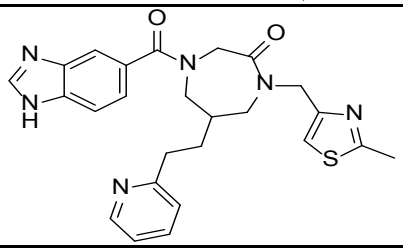
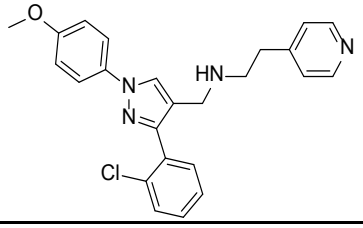
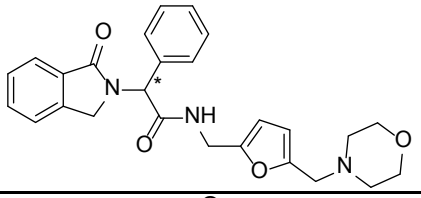
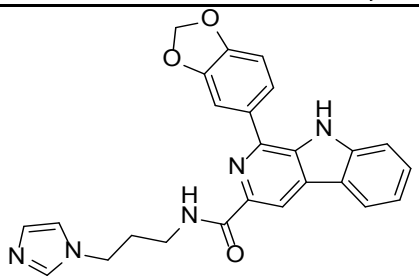
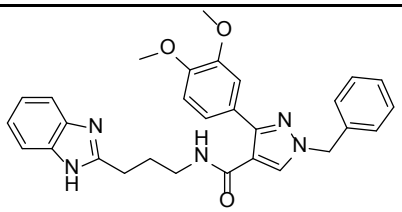
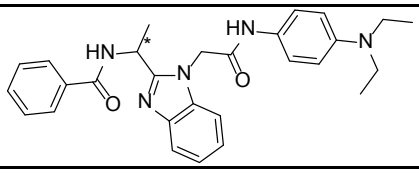
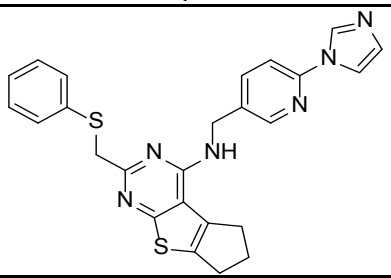


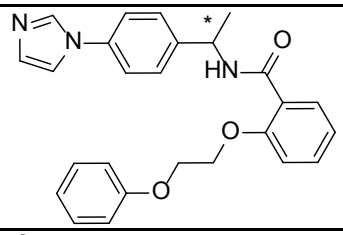
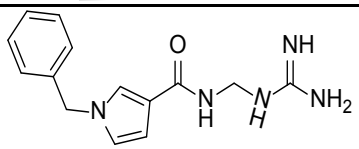
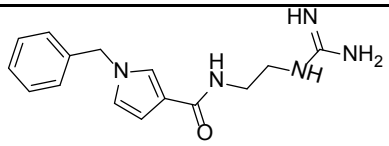
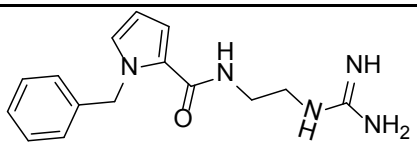
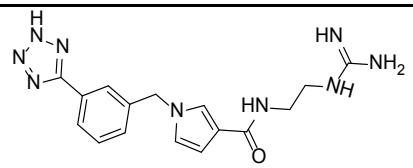
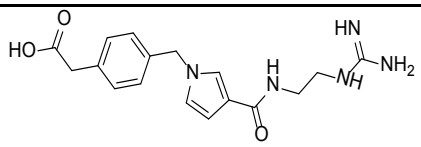
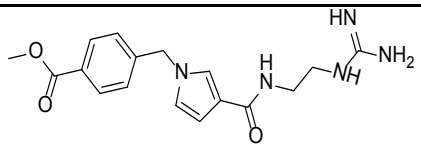
Figure 1. The proposed pharmacophore model, based on the myelin basic protein MBP_{83-96} epitope, with the relevant features depicted as spheres (Aro: green; Cat: magenta; Hyd: orange; V: gray). Only the binding cavity of the T cell receptor (TCR) is presented in the figure as surface and ribbons. The residues of the MBP_{83-96} are depicted as sticks.

The next step was the implementation of the pharmacophore model for the virtual screening of chemical databases. As described in the Materials and Methods section; the ZINC database was employed in the virtual screening process. The combinatorial information yielded from the pharmacophore model was employed as the starting point of our search. The examination of compounds in databases yielded a total of 340 potential inhibitors (hits). A subsequent visual analysis revealed 13 molecules (compounds **1**–**13**, Table 1) with binding conformations that closely resembled the positioning of the MBP_{83-96} epitope inside the TCR binding cavity (Tables 1 and S1).

Table 1. Chemical structure and docking scores of the proposed potential T cell receptor (TCR) antagonists (compounds 1–19).

Compound Number	Structure	ΔG [d] (kcal/mol)
MBP_{83-96}	Seq: ENPVVHFFKNIVTP	−11.89
1 [a]		−15.87
2 (* S/** R)		−19.71
3 (* S/** S)		−14.46
4 (* R)		−14.43

5		−10.32
6		−15.34
7		−16.38
8 (* S)		−13.26
9		−15.86
10 *(Lead Compound)*		−21.56
11 (* R)		−20.85
12		−16.05

13 (* S)		−20.65
14 [b]		−23.76
15		−18.13
16		−18.03
17 [c]		−18.49
18		−20.70
19		−21.32

[a] Compounds **1**–**13** were obtained from the pharmacophore model. [b] Compounds **14**–**16** were derived through modifications of the lead compound **10**. [c] Compounds **17**–**19** were modified analogues of compound **15**. [d] Docking score as calculated by the MOE2016 software at 298 K.

3.2. Lead Optimization and Molecular Docking Calculations

All the selected molecules were visualized in MOE2010, while their structural orientation and binding with the TCR were assessed. Each of the potential hits was subjected to molecular docking calculations, and the results are presented in Table 1. The analysis of the docking experiments showed that, of the 13 compounds obtained from the pharmacophore screen, compound **10** presented with the highest docking score (−21.56 kcal/mol) inside the TCR binding cavity, while the lowest docking score was reported for

compound **5** (−10.32 kcal/mol). This suggests that compound **10** may be considered the best candidate for lead optimization. The formation of only 2 hydrogen bonds with residues AspA92 and GlyA96 of the TCR is noted along with the existence of a π-stacking interaction between the aromatic rings of the compound and the side chain of TyrA98 in the TCR. Despite the favorable interactions between analogue **10** and the TCR, the bulky nature of the lead compound prevents the better positioning of the molecule inside the binding cavity.

The optimization process for target compound **10** included the removal and addition of functional groups in order to improve the placement of the molecule inside the selected TCR pockets and subsequently increase the interactions (Figure 2a). As depicted in Figure 2a, the substituted aromatic ring was removed to decrease the bulky nature of the potential inhibitor. The benzimidazole was replaced by a guanidino group (Figure 2a) to enhance the hydrogen bonding potential of the designed inhibitor. This preliminary study led to the identification of compound **14** as drug-target (Table 1 and Figure 2b). The next step was the setup of a molecular docking simulation for compound **14** in the TCR. The results of the docking experiments show that the alterations in compound **14** increase its binding affinity inside the TCR compared to the lead compound **10** (−23.76 to −21.56 kcal/mol, Table 1). The ligand pose with the best docking score for compound **14** (Figure 2b) presented the formation of six hydrogen bonds with residues of the TCR. In addition to the hydrogen bond interactions with amino acids AspA92 and GlyA96, the optimized compound further interacts with residues AsnA30 and ThrA97 (Figure 2b). The improved interaction, via the increased number of hydrogen bonds, may further explain the better binding affinity of compound **14**, due to the more favorable positioning inside the binding cavity of the receptor. As expected the π-stacking interaction with TyrA98 in the TCR is retained in the new optimized compound, further enhancing its binding.

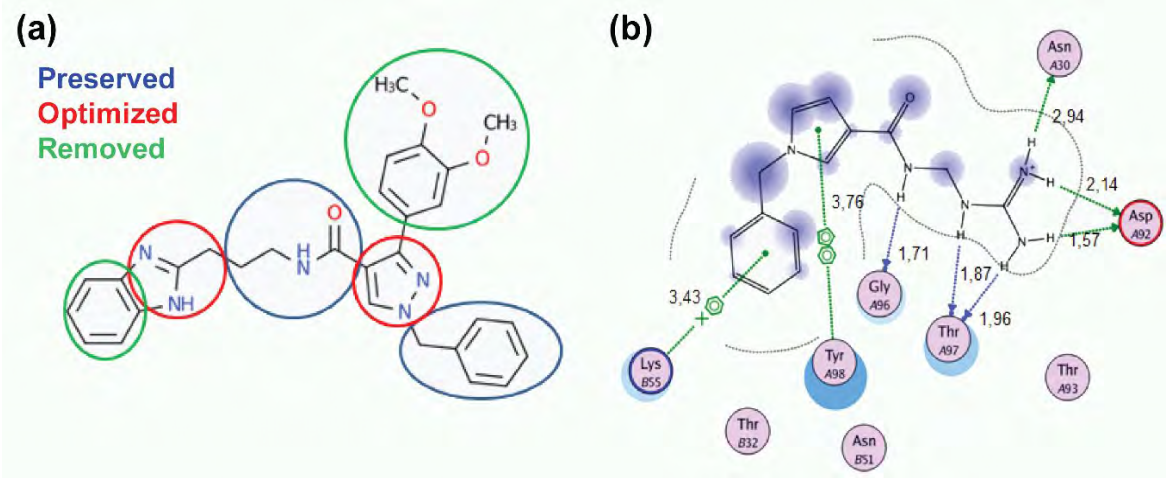


Figure 2. (a) Optimization process for the lead compound 10. The preserved groups are presented in blue, while the optimized groups are shown in red and the removed ones in green; **(b) best docking pose of compound 14 inside the TCR binding site, showing the different interactions.** Green arrow: Hydrogen Bond (HB) formed with the side chain of the residue; Blue arrow: HB formed with the backbone; Blue shade: Solvent accessible surface area (SASA) of the ligand; Turquoise halo: SASA of the receptor residues; Green dotted lines: π-π interactions between two phenyl rings or interaction of a cation (+) with a π system (phenyl ring).

As stated in the Materials and Methods section, the filtering process of the pharmacophore search was based on Lipinski's rule of five. Properties such as size (molecular weight, MW), hydrophobicity content (logP), and Total Polar Surface Area (TPSA) were recorded for the potential candidates (Table S1). The lead compound (compound **10**) was selected due to its high binding affinity (Table 2) and its better positioning inside the TCR binding cavity. The optimization process that led to the design of compound **14** aimed to enhance the binding affinity as well as to improve its positioning deeper within the TCR. Additionally, the modifications in the lead compound were intended to reduce its hydrophobic content (logP) and increase the polar surface area of the proposed inhibitor (Table 2). The smaller size of optimized compound **14** (MW = 272.33, Table 2), showed a notable decrease in its hydrophobic content (−0.84 from 5.25 of compound **10**, Table 2) and an increase in its TPSA (Table 2). Both of these chemical properties are indicators of compound's membrane/cell permeability. Compound **14** proved better potential absorption properties than the lead compound, as indicated by the logP and TPSA values.

Table 2. Properties of lead compound 10 and optimized analogues 14–19.

Compound	MW (g/mol)	TPSA (Å^2)	logP	Docking Score (kcal/mol)

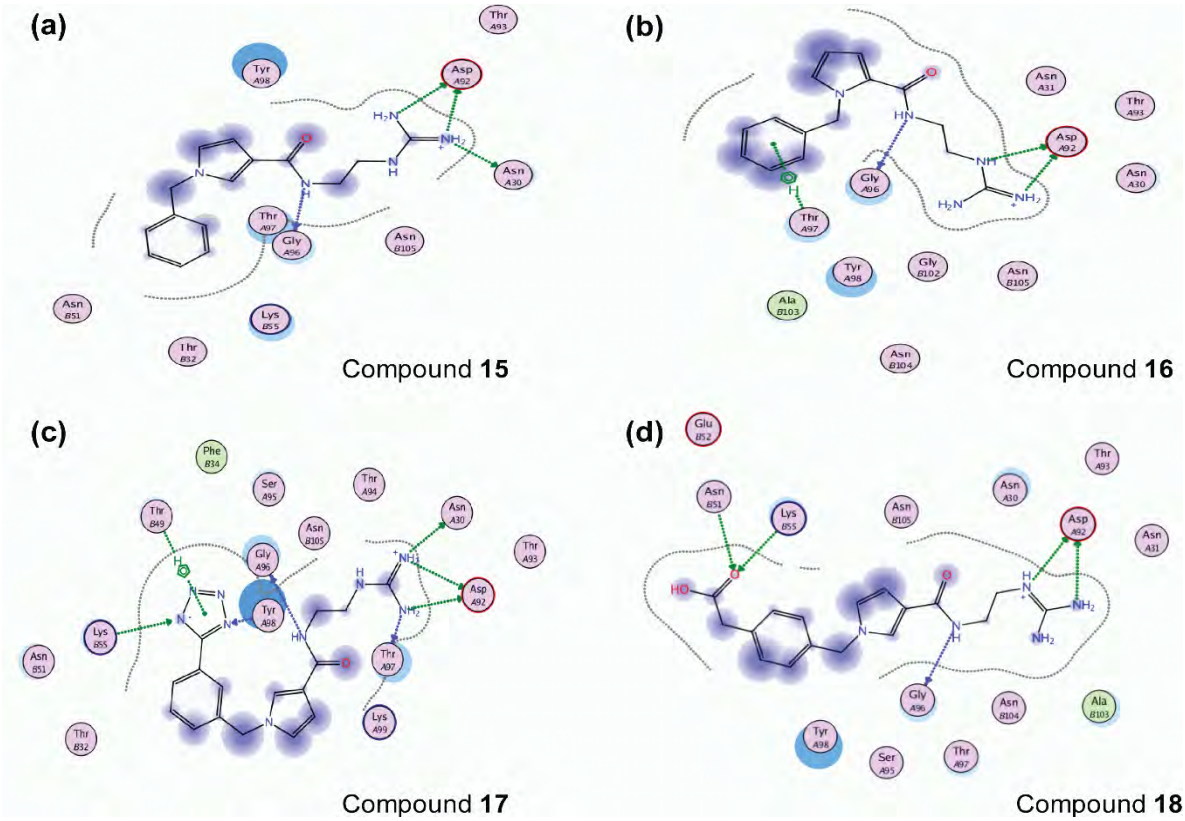
10	495.57	94.06 [a]	5.25 [b]	−21.56
14	272.33	97.67	−0.84	−23.76
15	286.36	97.67	−0.84	−18.13
16	286.36	97.67	−0.71	−18.03
17 [c]	354.40	152.13	−1.62	−18.49
18	344.39	137.34	−1.49	−20.70
19	344.39	126.34	−1.42	−21.32

[a,b] Total Polar Surface Area (TPSA) and hydrophobicity content (logP) values are reported as shown on Ambinter Chemicals catalogue: http://www.ambinter.com/. [c] Compounds **17**–**19** are modified analogues of compound **15**.

Based on the calculated properties of the compounds **10** and **14** (logP and TPSA, Table 2), we aimed to further optimize analogue **14** through small changes in the compound's backbone to explore whether an additional increase in binding affinity is possible. Thus, two new target molecules **15** and **16** were obtained (Table 2, Figure 3); the 3–substituted pyrrole ring with an additional methylene group (–CH_2–) between the amide bond and the guanidino group, compound **15** (Figure 3a), and its 2–substituted pyrrole ring isomer, compound **16** (Figure 3b). The addition of the methylene group aimed to improve the positioning of the guanidino group in the P2 pocket of TCR. As expected, this variation increases the molecular weight but does not affect the hydrophobicity content, and the TPSA values of the two derivatives in comparison to compound **14** (Table 2). Molecular docking simulations were also carried out for analogues **15** and **16** in complex with the TCR, and the results are reported in Table 2 and Figure 3. The reported interactions for compounds **15** and **16** show the retention of the hydrogen bonds with AspA92 and GlyA96 (Figure 3a,b), while the addition of the methylene group prevents the interactions with residues AsnA30 and ThrA97 reported for compound **14** (Figures 2b and 3a,b). The absence of these interactions, compared to compound **14**, may explain the differences observed for the binding affinities of the two derivatives **15** and **16** (Table 2).

The abolition of interactions with residues AsnA30 and ThrA97 for analogue **15** and the subsequent decrease in the binding affinity compared to compound **14** (Table 2) led to the design of derivatives **17**–**19** (Figures 3c–e). The analogues include meta– (compound **17**) and para– (compounds **18** and **19**) substitutions of the aromatic ring in compound **15**. The meta– substitution with the tetrazole group in compound **17** restores the hydrogen bond with ThrA97 (Figure 3c). Furthermore, the tetrazole interacts via the formation of a

hydrogen bond with TyrA98 (Figure 3c). The new interactions between the compound and TCR residues are mirrored in the increased docking score of the molecule as reported in Table 2. The para $-CH_2COOH$ substitute (compound **18**) retains the interactions of analogue **15** with AspA92 and GlyA96, while creating hydrogen bonds with residues AsnB51 and LysB55 (Figure 3d). The amino acids AsnB51 and LysB55 are located in the TCR binding site, opposite to AspA92 and GlyA96, thus enhancing the positioning of derivative **18** in the TCR binding cavity. A similar pattern of interactions inside the TCR cavity is observed for the para– methyl ester substituent (compound **19**, Figure 3e). Again, the methyl ester group allows the compound to be better oriented inside the binding site. The possible advantageous positioning of compounds **18** and **19** is mirrored in their docking scores (−20.70 and −21.32 kcal/mol, respectively, Table 2).



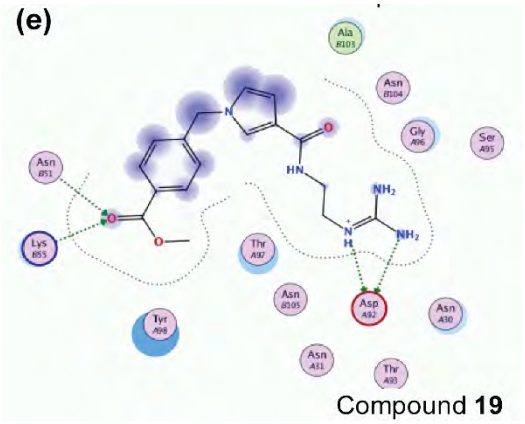


Figure 3. Best docking poses inside the TCR binding site, showing the different interactions for compounds: (**a**) **15**; (**b**) **16**; (**c**) **17**, (**d**) **18**; (**e**) **19**. Green arrow: Hydrogen Bond (HB) formed with the side chain of the residue; Blue arrow: HB formed with the backbone; Blue shade: Solvent accessible surface area (SASA) of the ligand; Turquoise halo: SASA of the receptor residues; Green dotted lines: interaction of C-H with a π system (phenyl ring).

3.3. Molecular Dynamics Simulations

Molecular dynamics (MD) simulation experiments were performed on the optimized compounds (**14**–**19**). The best docking poses were utilized as the starting conformations in the different MD simulation runs. The conformational changes observed for the TCR are similar in the different MD simulation runs (Figure S1a). This pattern is also observed in the atomic positional fluctuations of the residues of the TCR (Figure S1b). The different amino acids of the receptor show an identical pattern of deviation from their original position in the complexes with different analogues. Furthermore, the conformational analysis of the ligands showed that there are no extensive conformational changes (Figure S1c) during the simulation time. The average RMS value (1.97 Å ± 0.1) for compound **14** presents the greatest deviation from its starting conformation compared to compounds **15** (1.90 Å ± 0.37), **16** (1.72 Å ± 0.20), **17** (1.82 Å ± 0.63), and **18** (1.01 Å ± 0.13). Only analogue **19** presents a higher average RMS value (2.01 Å ± 0.47) to all the other derivatives (Figure S1c). These deviations in the RMS values for the designed analogues reflect very small changes in their conformation during MD simulations.

The clustering analysis for the different MD simulations showed that compound **14** presents two dominant conformational groups throughout the simulation (Figure 4a, blue and yellow). The difference between the two conformations is in the positioning of the aromatic ring inside the P3 pocket of the TCR (Figure 4b). In one instance, the aromatic ring is facing towards TyrA98 (Figure 4b, green) and in the other it faces away from TyrA98 and towards PheB34 (Figure 4b, yellow). In both cases, though, the docking pose is not retained throughout the MD simulation and the guanidino group is facing away from the binding cavity of the TCR (Figure 4b). The modification of compound **14** in which an additional methylene group (analogues **15** and **16**) is introduced might lead to a better positioning inside the TCR binding cavity. The clustering analysis for the two modified analogues **15** and **16** revealed the presence of only one dominant conformation for both compounds (Figure 5a, black and salmon respectively). The positioning of the two analogues **15** and **16** inside the binding cavity of the TCR is very similar (Figure 5a, black and salmon, respectively). The most pronounced difference between them is the positioning of the aromatic ring. In 2–substituted pyrrole analogue **16**, the aromatic ring during the MD simulations points away from the binding pockets (Figure 5a, salmon). On the other hand, 3–substituted pyrrole analogue **15** adopts a more optimal conformation inside the binding pockets of TCR (Figure 5a, black). While analogues **14** and **16** have a portion of their structure pointing away from the TCR receptor (Figures 4b and 5a), the addition of the methylene group in compound **15** allows for the conformation of the molecule to create a bent, thus optimizing the orientation inside pockets P–1, P2, and P3 of the TCR (Figure 5a, black).

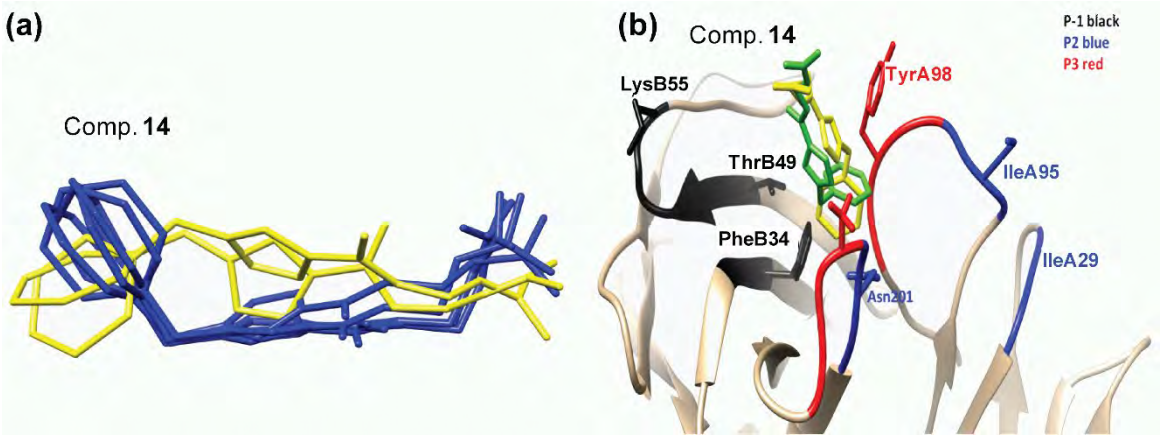


Figure 4. (a) Representative conformations of compound 14 inside the TCR as reported by the clustering analysis in the molecular dynamics (MD) simulations and (b) the positioning of analogue 14 representative conformations (yellow and green) inside the TCR binding pockets.

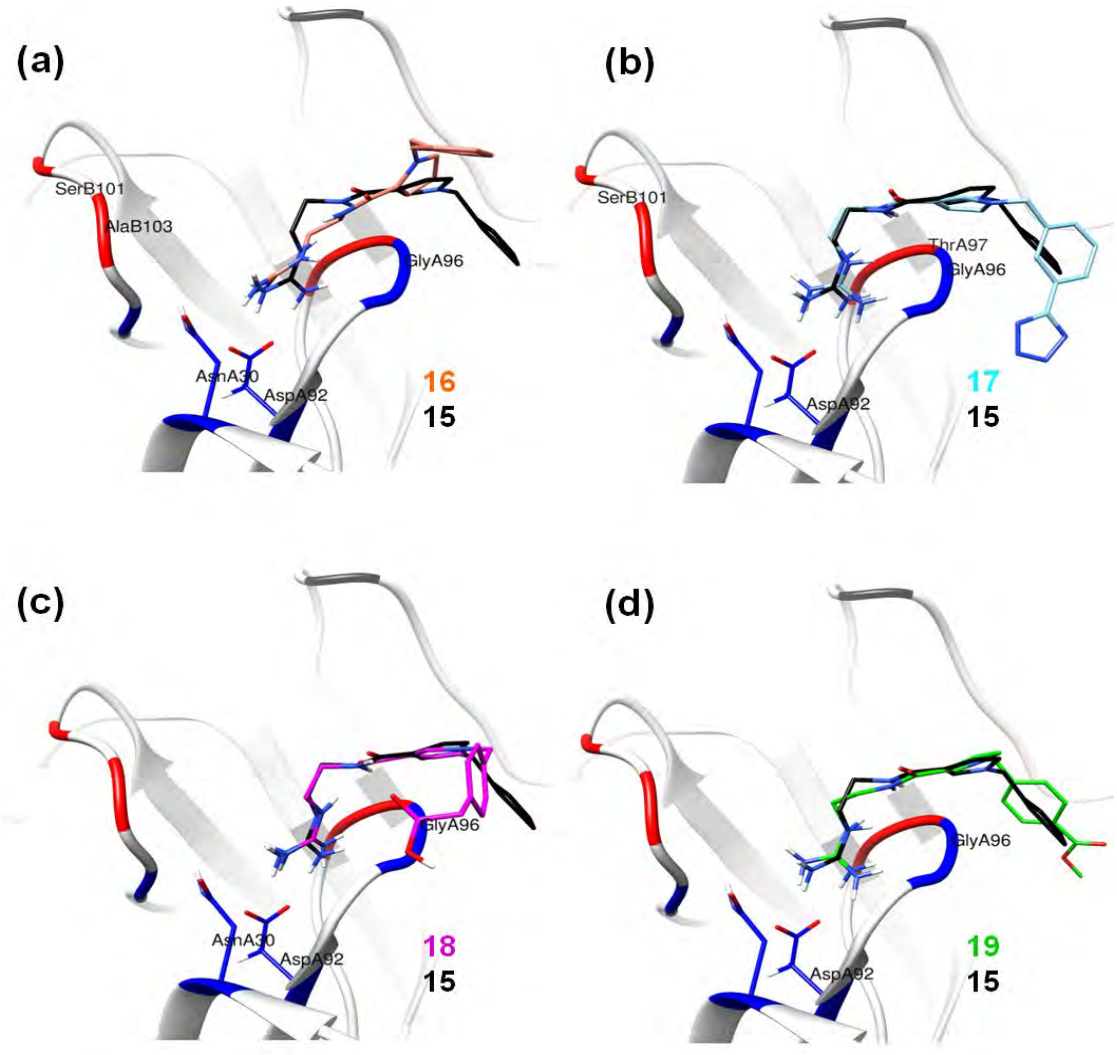


Figure 5. Conformations of compounds 15–19 inside the TCR binding pockets. The common backbone features have been implemented for the superimposition of the derivatives on analogue **15** (black); (**a**) with **16** (salmon); (**b**) with **17** (cyan); (**c**) with **18** (magenta); (**d**) with **19** (green).

As previously mentioned, the best possible positioning of compound **15** inside the binding pocket observed in the docking experiments led to the design of derivatives **17**–**19**. The clustering analysis of the particular simulations confirmed the results obtained from the RMS analysis (Figure S1c). Likewise, with compounds **15** and **16**, the derivatives **17**–**19** present only one dominant conformation throughout the MD simulations. The structural similarities of analogue **15** with compounds **17**–**19** led to the supposition that the derivatives would adopt a similar positioning inside the TCR. The superimposition of the representative conformations with that of compound **15** (Figure 5) confirmed the above supposition. Compounds **17** and **18** present identical positioning inside pockets P2 and P3 with that of compound **15** (Figures 5b,c), suggesting that the guanidino group firmly

anchors the analogues inside the receptor. At the opposite end of the derivatives though the substitutions with the tetrazole (compound **17**) and the $-CH_2COOH$ (compound **18**) groups do not greatly improve the conformational positioning of the designed analogues in the binding cavity. Additionally, the $-CH_2COOH$ substituent in compound **18** orients the aromatic ring of the derivative away from the pockets of TCR (Figure 5c). On the other hand, the positioning of compound **19**, which has a para– methyl ester substitution, in the binding site of the receptor closely resembles that of analogue **15** (Figure 5d, green). The substitution seems to position the analogue inside the TCR between pockets P3 and P–1 in an even better way (Figure 5d, red and black).

3.3. Hydrogen Bond Interactions

Analysis of the hydrogen bond interactions for all compounds (**14–19**) was performed during the MD simulations. The results are outlined in Table 3 and compared with the interactions reported from the molecular docking experiments. The changes in the orientation of the molecules inside the TCR are mirrored in the observed differences of the interactions for each molecule. As mentioned above, compound **14** creates hydrogen bonds with residues in pockets P2 and P3 (Figure 2b and Table 3) with the guanidino group anchoring the compound in pocket P2 (AsnA30) and pocket P3 (ThrA97). During the MD simulation time, these interactions are not retained, and the terminal nitrogens of the guanidino group do not create stable interactions with the TCR. Instead the only interactions are those with residues of P2 pocket of TCR (AsnB104 and GluB106). The same pattern is observed for compound **16**, where the interactions with residues AspA92 and GlyA96 (P2 pocket of TCR) are not retained during the MD simulations. Instead, analogue **16** is involved in hydrogen bonding interactions with residues TyrA98 and AlaB103, both in the P3 pocket of the receptor (Table 3).

Table 3. Hydrogen bonds for all optimized analogues (14–19) as reported in the docking and MD simulation experiments.

TCR Residues	Compounds											
	14		15		16		17		18		19	
	Dock	MD	Dock	MD	Dock	MD	Dock	MD	Dock	MD	Dock	MD
AsnA30	✓[α]		✓	✓			✓			✓		✓
AspA92	✓		✓	✓	✓		✓	✓	✓	✓	✓	✓
ThrA93												✓
GlyA96	✓		✓	✓	✓	✓	✓	✓	✓	✓		✓
ThrA97	✓			✓			✓	✓				

TyrA98				✓		✓	
TyrA100	✓						
AsnB51					✓		✓
LysB55					✓		✓
SerB101			✓	✓			
AlaB103			✓				
AsnB104	✓						
GluB106	✓						

[a] presence of hydrogen bonds

In contrast to the previous two analogues, compound **15** retains the hydrogen interactions reported in the molecular docking experiments (Figure 3a, Table 3). The hydrogen bonds with residues AsnA30 and GlyA96 in the P2 pocket of the TCR are conserved, while the orientation of the molecule inside the cavity allows for interaction with ThrA97 in the P3 pocket (Table 3). Furthermore, the anchoring of the compound **15** inside the two pockets (P2 and P3, Figure 5a), in combination with the bent conformation of the molecule, allows better positioning of the aromatic ring inside the P–1 pocket (Figure 5a). This may lead to increased interactions between the potential inhibitor and the receptor. Similarly to compound **15**, the three derivatives (**17**–**19**) present comparative interaction patterns (Table 3). The guanidino group of these analogues retains the interaction with AspA30 and GlyA96 in the P2 pocket of the TCR observed for compound **15** (Table 3), while there are small changes in the interaction patterns with the neighboring amino acids. Compound **17** further interacts with ThrA97 and SerB101 in the P2 pocket, while compound **18** further interacts with AsnA30 in the P3 pocket and TyrA98 in the P2 pocket of the receptor. Finally, the very similar positioning of compounds **15** and **19** (Figure 5d) points to the conservation of the interactions between the two designed analogues (Table 3). The only difference is the hydrogen bond of compound **19** with ThrA93 instead of GlyA96 in the P2 pocket of the receptor.

3.4. Chemistry

Initial studies on the synthesis of pyrrole-based TCR antagonists provided candidates **15** and **16** via a six-step synthetic procedure with a total yield of 14% and 21%, respectively (Route A, Scheme 1). *N*-alkylation of commercially available 3- or 2-methyl pyrrolecarboxylates, with benzyl bromide in the presence of sodium hydride, afforded the 3-/2-substituted *N*-benzylpyrroles **15a**/**16a** (Mochona et al., 2010). Subsequent hydrolysis of the methyl ester, followed by standard procedure for DCC/DMAP amide coupling with

N-Boc-ethylenediamine, gave the corresponding pyrrole carboxamides **15c**/**16c**. *N*-Boc-deprotection with TFA followed by *N*-iodosuccinimide-mediated guanylation reaction (Ohara, 2009) with di-Boc-thiourea furnished the di-Boc-guanidino derivatives **15d**/**16d**, which allowed final molecules **15**/**16**, after Boc cleavage.

Upon further investigations, a rapid and simple three-step protocol (Route B, Scheme 1) was developed to expand the scope and utility of this synthetic methodology and readily prepare diverse pyrrole analogues. Thus, the guanidine moiety **20** (Exposito et al., 2001) was first synthesized and then reacted with pyrrole-3-carboxylic acid to provide a common structural core **21**, after amidation reaction. Subsequent pyrrole-*N*-protection (Mochona et al., 2010) with primary alkyl bromides **17a**–**19a**, followed by removal of the Boc-groups, produced target compounds **17**–**19** in a shorter sequence and an 11–27% overall yield.

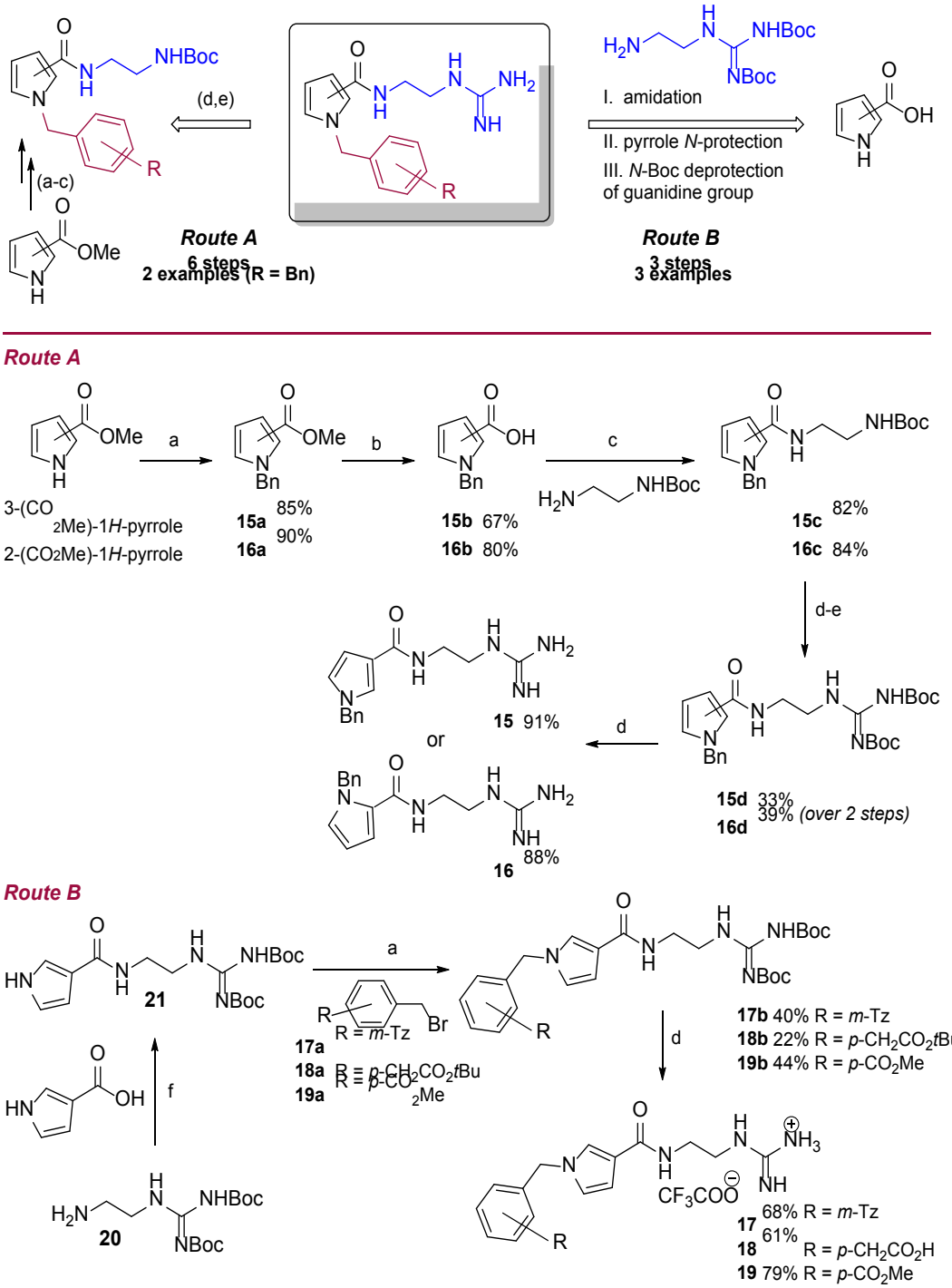
3.5. Molecular Orbital Calculations

From the three analogues (**14**–**16**) reported in this study, compound **15** presents a high docking score (−18.13 kcal/mol) coupled with a preferred orientation inside the binding cavity of the TCR (Figure 5a). This, in combination with the compound's favorable pharmacokinetic properties (TPSA and logP, Table 2), inspired us to explore the analogue **15**/TCR complex by employing molecular orbital methods.

2.5.1. Semi-Empirical Simulation Method

In order to better estimate the interaction energy of the system, a number of different approaches were employed. The results (Table S2) show that the PM7 (parameterization method 7) (Aldulaijan & Platts, 2010) approach best reproduces the density functional theory (DFT) calculations for the selected residues. All other semi-empirical (SE) methods tested present considerable errors compared to PM7 despite the inclusion of dispersion correction. Based on these observations, the PM7 method was used as the most appropriate for further calculations on the entire receptor–ligand complex (Table S2). Two protocols were utilized for our calculations. In the first one, the ligand along with the same residues used in the DFT calculations was preferred. The interaction energy of the particular system was calculated to −24.09 (kcal/mol). The larger value compared to the DFT calculations

(−31.63/−42.85 kcal/mol) may be attributed to the level of accuracy for the SE methodologies and the treatment of the electron density of the various atoms in the system.



Scheme 1. Synthesis of 2-/3-substituted pyrrole analogues **15**–**19**. Reagents and conditions: Route A: (**a**) BnBr, NaH, DMF; (**b**) i. KOH 30%, MeOH–H_2O, reflux; ii. 6 M HCl; (**c**) DCC, DMAP, DCM; (**d**) TFA, DCM; (**e**) BocNHC (S) NHBoc, NIS, DIPEA, MeOH–DCM; then (**d**) TFA, DCM. Route B: (**a**) BnBr, NaH, DMF; (**f**) DCC/HOBt, DIPEA, DMF–DCM; (**d**) TFA, DCM.

The second approach employed in our calculations involved the ligand with the whole receptor. In order to explore the effect of the different TCR residues, amino acids within a cutoff distance of 4.5 Å from the ligand were initially elected. Subsequent rounds of interaction energy calculations followed, by augmenting the selected area per 4.5 Å each time until the entire receptor was included in our calculations (Figure 6a). The interaction energy calculated for the TCR in complex with compound **15** is −34.39 kcal/mol. In order to further study the interaction energy of compound **15**, different snapshots were taken from the MD simulation run (the last 20 ns of the simulation). For each snapshot, the interaction energy was calculated with the PM7 method to monitor the fluctuations in the energy (Figure 6b and Table S3). The mean value over the 20 snapshots for the interaction energy was −47.26 kcal/mol. The low interaction energy calculated from both the best docking pose and the different MD snapshots (Tables S2 and S3) suggests that derivative **15** interacts strongly with the TCR and thus may be competitive with native ligands. The interaction energy calculated for compounds **17**–**19** with the SE methodology are reported in Table S5. The values range between −35.39 and −37.20 kcal/mol, higher than the value reported for analogue **15** (−47.26 kcal/mol).

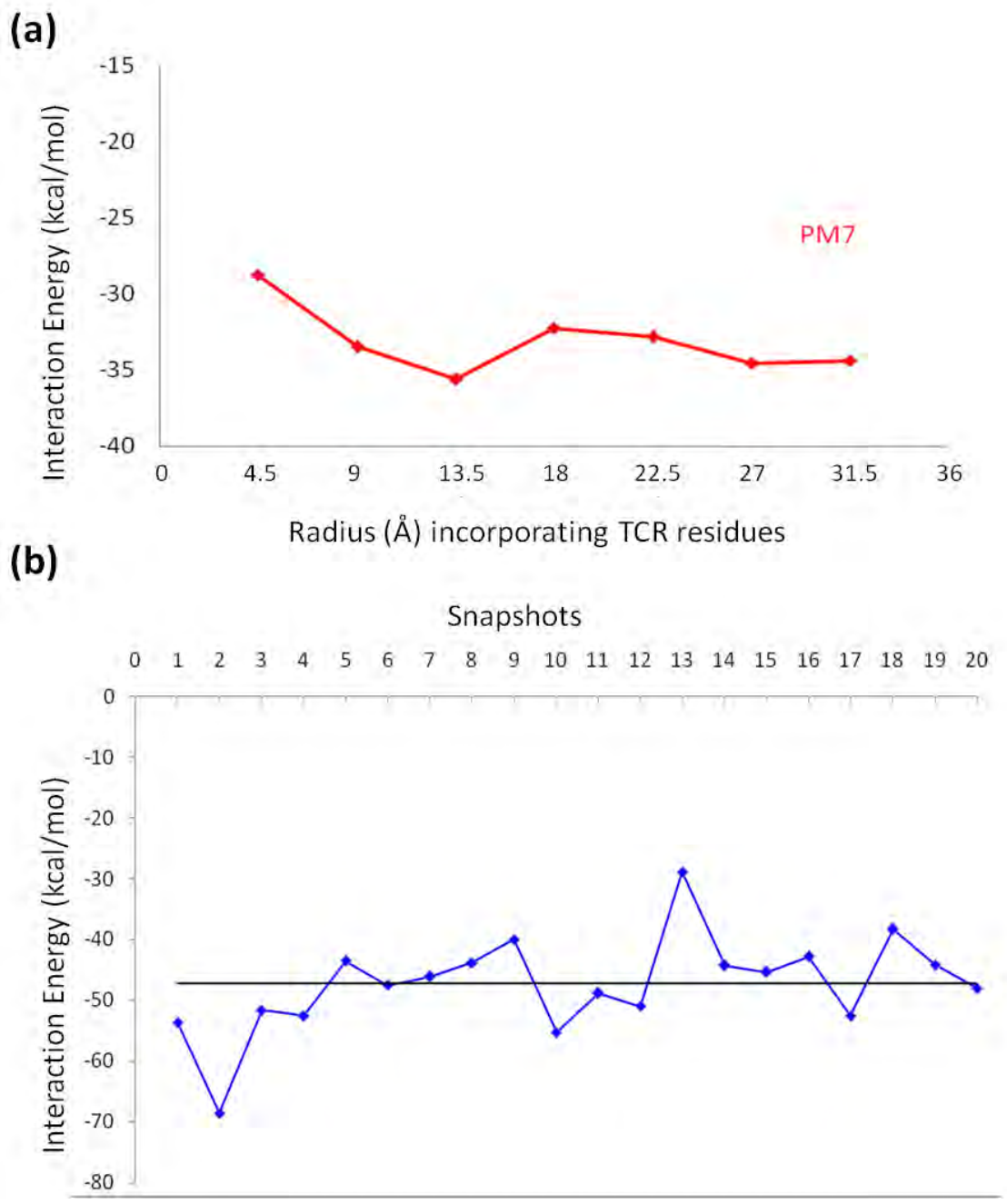


Figure 6. (**a**) Interaction energies for derivative **15** and the residues of the binding cavity, extended by 4.5 Å until the entire TCR is included, calculated by the PM7 method (red) in solvent and (**b**) graphical representation of the interaction energy calculated via the PM7 method for the different MD snapshots.

3.5.2. DFT Calculations

The large size of the TCR (341 amino acids) hinders the use of DFT methodologies on the entire complex (Friesner, 2005; Orio, Pantazis, & Neese, 2009). Thus, to calculate the interaction energy for the complex, the best docking pose was selected. The selection

of the receptor residues (total of nine amino acids) was based on the interactions formed with compound **15** and their distance from it (<3.5 Å). As reported in the Materials and Methods section, Section 4.6, different methodologies were employed, and the results are outlined in Table S2. The analysis of the calculations showed variability depending on the method and the basis set selected. In fact, the methods that include dispersion either explicitly or implicitly (e.g., M06, M06-2X, B97D, BHandH, and B3LYP-D) calculate more negative interaction energies (Table S4) (Goerigk & Grimme, 2011; Miertuš, Scrocco, & Tomasi, 1981). In contrast, the choice of basis set does not have such an extensive impact in the calculation of the interaction energy. Therefore, in order to obtain a more accurate result, the inclusion of dispersion functions was considered in our calculations (Carvalho et al., 2015). Based on this, the interaction energy between compound **15** and the selected residues of the TCR was calculated between −31.63 and −42.85 kcal/mol (Table S4). Compared to the SE methodologies, DFT techniques allow for a more accurate prediction of interaction energy between the ligand and the residues that are directly involved in the binding to TCR. The application of DFT incorporates the effect of all atoms, without any of the approximations (empirical data) applied during the SE calculations.

3.6. Biological Assays

3.6.1. Human Peripheral Mononuclear Cells

Blood samples were drawn from two healthy subjects for biological assays and contained: 1st person: 2.84×10^3 lymphocytes/μL of blood (42.9% of total leukocytes) and 410 monocytes/μL of blood (6.2% of total leukocytes); 2nd person: 1.83×10^3 lymphocytes/μL of blood (34.6% of total leukocytes) and 330 monocytes/μL of blood (6.2% of total leukocytes). The peripheral blood mononuclear cells (PBMCs) isolated from the blood samples were cultured in the presence of various concentrations of the $MBP_{83–96}$ peptide to estimate the optimal concentration for inducing T-cell proliferation. It is noteworthy that the specific culture conditions used in this work, i.e., allo-peptidic antigens and anti-CD28 antibody, target T-cell responses (Raulf-Heimsoth, 2008). T-cell proliferation was measured by flow cytometry. The highest T-cell proliferation was noted at 0.1 nM $MBP_{83–96}$ (Figure 7a). PBMC cultures were then repeated with 0.1 nM $MBP_{83–96}$ and 0.1 mM of each of the fifteen analogues (Figure 7, Table 1: compounds **1**–**13**, **15**,

and **16**) per experimental point, in triplicate. The results show that analogue **15** was the most effective TCR antagonist, i.e., it conferred the highest inhibition of T cell proliferation (Figure 7b).

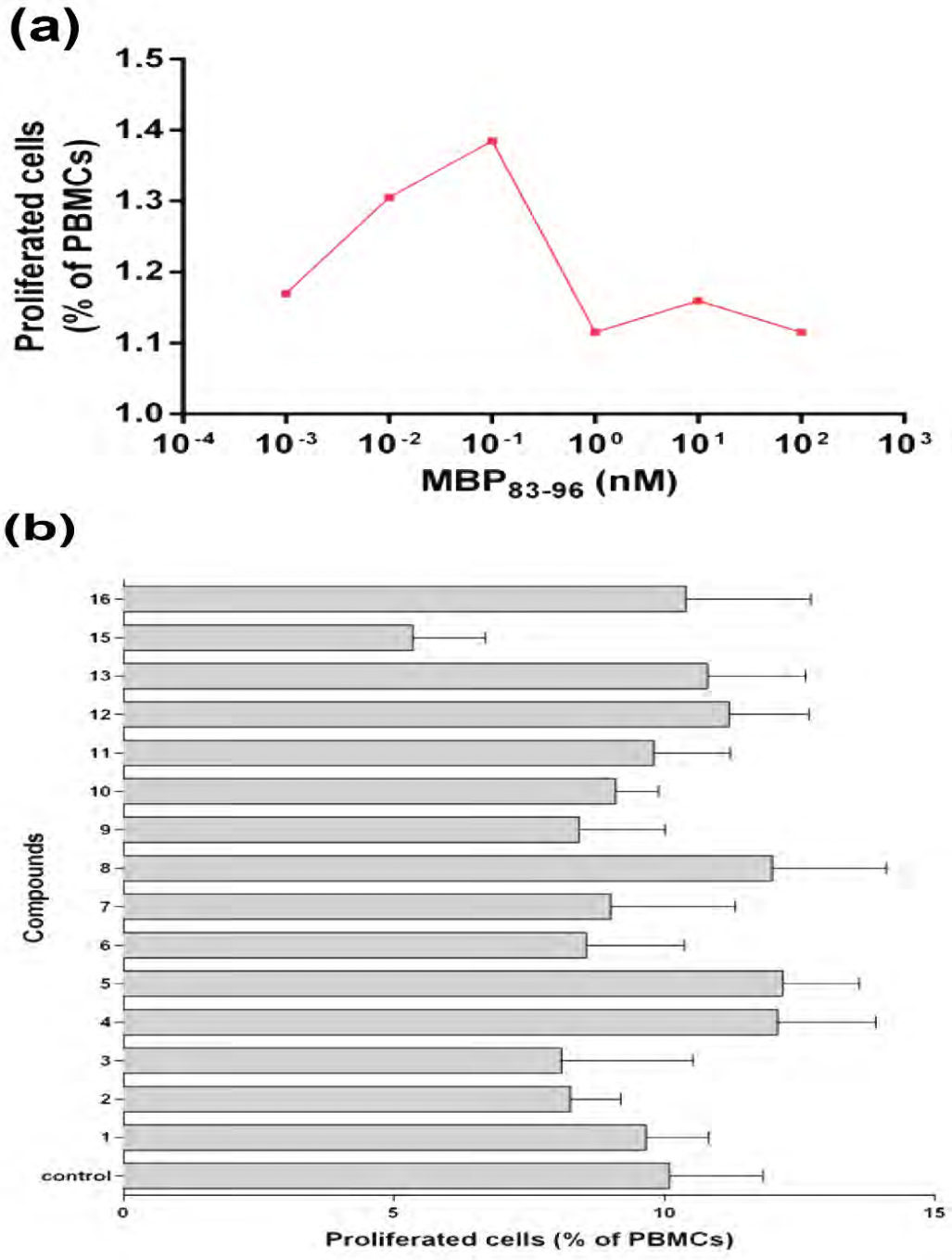


Figure 7. (a) T cell proliferation in the presence of $MBP_{83–96}$. Peripheral blood mononuclear cells (PBMC) were cultured with several concentrations of the $MBP_{83–96}$ peptide for 3 days; cell proliferation was measured by flow cytometry. Data are shown as median of triplicate measurements. (**b**) Proliferation of PBMC in the presence of 0.1 nM $MBP_{83–96}$ and 0.1 mM of each of the 15 analogues/point, in triplicate. Control: $MBP_{83–96}$ peptide alone. Data are shown as mean ± standard error of the mean.

3.6.2. Mouse $MBP_{83–99}$ Specific T Cell Assays

Autoimmune $CD4^+$ T cells can be stimulated in mice following immunization with $MBP_{83–99}$ peptide together with *Mycobacterium*, which results in experimental autoimmune encephalomyelitis (EAE), an animal model for MS (Zamvil et al., 1985). Characteristics of EAE are comparable to those of MS in humans where Th1 phenotype (IFN-γ) and Th17 cells contribute to pathogenesis of disease in mice. Similar to MS, EAE susceptibility is dependent on the mouse (MHC class II background) and diverse peptides are immunogenic in different mouse strains. The SJL/J mouse strain (MHC class II $H\text{-}2^s$ haplotype) is commonly used for EAE, since numerous histopathological, clinical, and immunological features resemble those of human MS (Kalbus et al., 2001). In the SJL/J mouse strain, the peptide $MBP_{81\text{-}100}$ has been shown to bind with high affinity to MHC class II, $H\text{-}2^s$. In fact, the minimum epitope required for binding is $MBP_{83–99}$ (Kalbus et al., 2001), similar to human HLA-DR2 binding. Hence, the epitope $MBP_{83–99}$ could be used as an agonist peptide to immunize mice to activate $CD4^+$ T cells, as we previously demonstrated(Katsara, Deraos, et al., 2008b; Katsara et al., 2008a, 2008b; Katsara, Yuriev, et al., 2009b).. Here, mice were immunized with $MBP_{83–99}$ peptide conjugated to the carrier reduced mannan. Following three immunizations, spleen cells were isolated and mixed with recall peptide $MBP_{83–99}$ for six days in vitro. In addition, compounds **15–19** or **AMB** (lead compound **10**) were added at 100× molar excess to each well in order to determine whether T cell proliferation to the recall peptide $MBP_{83–99}$ could be inhibited. The particular compounds (**15–19**), due to their increased calculated binding affinity (Table 1) to TCR, were employed in order to assess the potency in mouse $MBP_{83–99}$ specific T cell assays. Compound **15** and **16** showed the greatest % inhibition of $MBP_{83–99}$-specific T cell proliferation, followed by compound **17** and **18**; compound **19** showed the least inhibition, and lead compound AMB was able to inhibit proliferation comparable to that of the other compounds (Figure 8). Compounds **15** and **16** have simpler structures compared to **17–19** and **AMB**. It is likely that the reduced activity of **17–19** analogues, compared to **15** and **16**, may be due to an inappropriate position of the acidic/esteric group. Even though complete inhibition of T cell proliferation is not noted, compounds **15** and **16**, based on in silico conformational studies, show promise for further optimization studies in order to develop new improved TCR antagonists with improved activity.

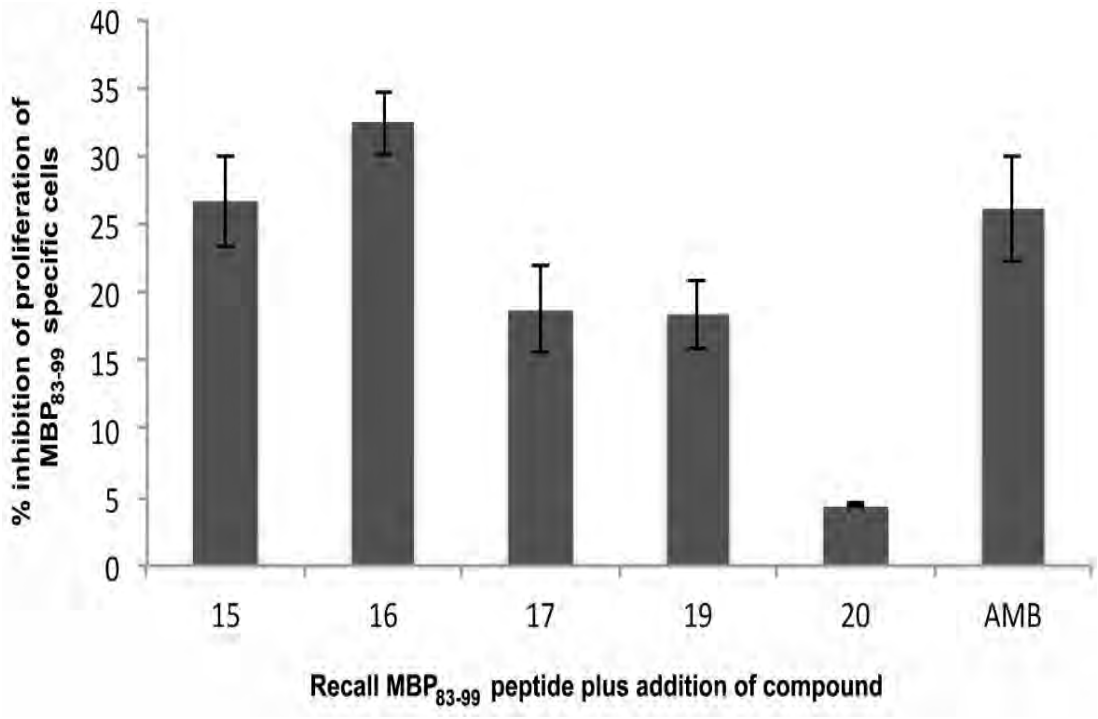


Figure 8. Specific $MBP_{83–99}$ epitope T cell proliferation using MTT as a readout. Mice were immunized three times with reduced mannan conjugated to $MBP_{83–99}$ peptide. Ten days following the last immunization, mice were sacrificed and spleen cells isolated, and $MBP_{83–99}$ peptide was added for 6 days. In addition, compounds **15**–**19** and lead compound **AMB** (lead compound **10**) were added at 100× molar excess. The percent inhibition of T cell proliferation to $MBP_{83–99}$ of each compound is shown. The mean of three individual mice are shown in triplicate wells ± standard error of the mean.

4. Conclusions

A ligand-based pharmacophore model was developed based on the conformational properties of the dominant $MBP_{83–96}$ epitope in complex with the TCR. The resulting model was employed for the virtual screening of the ZINC database for potential hits. A subset of the database, containing 500,000 all clean/ commercially available compounds, were screened, and the search yielded 13 hits. The potential inhibitors were ranked according to their inhibitory activity against TCR with the employment of molecular docking simulations. The compound with the highest docking score (compound **10**) was selected as lead and was subjected to optimization via chemical modifications. The resulting optimized molecule (compound **14**) presented increased docking score to the TCR and improved chemical properties such as TPSA and logP (Table 2).

The conformational analysis and the positioning of compound **14** in the TCR binding pocket led to the further modification with the addition of a methylene group and the organic synthesis of two isomers (compounds **15** and **16**). The analysis of the conformational properties of the three analogues via MD simulation experiments showed that analogue **15** has the most optimal positioning inside the TCR binding cavity and is better tethered within the receptor (Figure 5a). Extensive MD simulations may offer a deeper understanding of the interactions between the designed analogues and the receptor, and prove to be a valuable tool in drug design. Furthermore, the interaction energy between the potential inhibitor (compound **15**) and the TCR was explored by employing a variety of molecular orbital approaches. DFT and SE methodologies were used in order to calculate the interaction energy between selected residues of the TCR, as well as the entire TCR, and the proposed inhibitor **15**. The combination of the two methodologies allows us to identify whether only certain residues have the greatest impact in the binding of compound **15** or other conformational aspects of the TCR are important in its binding. The agreement between the DFT and the SE methods show that the binding of the potential inhibitor to the TCR is attributed only to the residues surrounding the binding cavity and not to other conformational changes observed in the TCR. The results of the in vitro evaluation (Figure 8) suggest that both analogues **15** and **16** may serve as good candidate antagonists to be developed further for the inhibition of proliferation of T-cells that recognize the $MBP_{83–96}$ antigen.

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Immunomodulatory effects of probiotics: Can they be used to treat allergies and autoimmune diseases?

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ABSTRACT

As a person ages, physiological, immunological and gut microbiome changes collectively result in an array of chronic conditions. According to the 'hygiene hypothesis' the increasing prevalence of immune-mediated disorders may be related to intestinal dysbiosis, leading to immune dysfunction and associated conditions such as eczema, asthma, allergies and autoimmune diseases. Beneficial probiotic bacteria can be utilized by increasing their abundance within the gastrointestinal lumen, which in turn will modulate immune cells, such as, T helper (Th)-1, Th2, Th17, regulatory T (Treg) cells and B cells, which have direct relevance to human health and the pathogenesis of immune disorders. Here, we describe the cross-talk between probiotics and the gastrointestinal immune system, and their effects in relation to inflammatory bowel disease, multiple sclerosis, allergies and atopic dermatitis.

1. Introduction

Regular ingestion of probiotic bacteria, has been used for tapping into the health benefits exerted by microbiota of the gastrointestinal tract (GIT) within a healthy subject. Use of probiotics in the form of fermented milk dates back to ancient times and in Middle Eastern traditions, with claims that Abraham's longevity was due to the consumption of fermented milk [1,2]. The physiological changes of the GIT and alterations in the gut microbiome associated with age, along with changes in life style and dietary behaviors, leads to changes to the immune system [3]. Crosstalk between gut microflora and the immune system enables a balanced gut homeostasis in healthy individuals, however, alterations in the gut ecosystem due to the aged gut or diseases, causes changes in GIT microflora homeostatic equilibrium, resulting in a number of chronic diseases [3]. In fact, there are decreased anaerobic and *Bifidobacteria* populations, and increased *Enterobacter* species in the intestine of ageing individuals [4]. Advances in the gut microbiota probiotics and its synbiotics may be beneficial to the ageing population [5]. Probiotics consumed by humans must be non-pathogenic and survive GI transit to render their health benefits [6]. Whether or not probiotic strains should be of human origin is a matter of debate; although, it is accepted that if a strain can survive and colonize the human large intestine, its origin is not important [6,7]. The survival of probiotics is crucial within the gastric acid environment, and as such, new genes are activated to encode a number of stress proteins for their survival. Once in the lower small intestine and colon, probiotics confer health benefits (Tables 1 and 2), although there is evidence that dead cells can also induce beneficial outcomes [7].

Probiotic bacteria, mainly belong to the lactic acid bacteria (LAB) family, which are commonly found in decomposing milk products and secrete lactic acid, fermentating carbohydrates. In fact, LAB have been used in fermentation and storage of certain foods (milk, vegetables, meat) for thousands of years [8]. LAB have also been shown to contribute to the healthy microflora of the human gut [7,9]. As such, the genera *Lactobacilli* (L.) (L. *rhamnosus*, *L. helveticus*), *Bifidobacterium (B.)*, *Streptococcus* (*S.*) and *Enterococcus* species have been used as probiotic strains, i.e. supplementing foods with live microorganisms, and subsequent improvements in a number of human health conditions [9,10]. The basis for these benefits include, the detoxification of xenobiotics [11], microbial toxins, host metabolites i.e. bile salts and food components [12], biosynthesis of vitamin K_1, folic acid, biotin, vitamin B12, an increase in the absorption of calcium, iron and magnesium, fermentation of lactose, modulation of intestinal gas production [13,14] and production of short-chain fatty acids (SCFAs; acetate, propionate, butyrate, lactate) [15–17]. SCFAs are used as a source of energy to favour the growth and differentiation of GI epithelial cells, in addition to, modulating the immune system by regulating proliferation and cytokines of T cells, T helper (Th)-17 cells and T regulatory (Treg) cells of

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Table 1
Effects of probiotics in health and disease.

Probiotic/ probiotic strain	Beneficial applications
Probiotics in general	• Stimulate secretion of IFN-gamma and IL-10 • Promote effective immune responses against pathogens • Modulate host immunity
Bifidobacterium (B.) Saccharomyces (S.) boulardii	• Detoxify xenobiotics • Produce mimicrobial products, toxins, host metabolites, bile salts and food components • Improves intestinal defense against enterohaemorrhagic *E. coli* • Regulates intestinal epithelial barrier function
Lactobacilli (L.) species	• Biosynthesize vitamin K_2, folic acid, biotin, B12. • Absorption of calcium, magnesium, iron, fermentation of lactose • Modulation of intestinal gas production • Produce short chain fatty acids -acetate, propionate, butyrate, lactate • Ferment indigestible dietary fibres • Modulation of the immune responses • Protect against cancer and allergy development
Lactobacillus recombinants	• Inhibits experimental autoimmune encephalomyelitis (EAE) in animal models of multiple sclerosis (MS)
L. Plantarim *B.* B94	• Prevention and treatment of MS
L. acidophilus *L. bulgaricus* *B. bifidum*	• Stimulate the innate immune system (via phagocytosis, NK cell activity)
L. plantarim A *L. acidophilus* ATCC 4356 *L. rhamnosus* ATCC 53,103	• Increase expression of $CD11c^+$ DCs and Treg cells and protection against colitis
L. plantarum WCFS1 *L. fermentum* AGR1485	• Upregulate CD83 and CD86 cell surface expression of antigen presenting cells (APC) • Increase IL-12p70, TNF-alpha and IL-1beta
L. bulgaricus subsp. delbrueckii LB43, *Lactococcus lactis* R704 *B. lactis* BB12 *L. acidophilus* *L. bulgaricus* *B. bifidum*	• Beneficial in allergic rhinitis and asthma
L. casei Shirota *Bacteroides fragilis* *Bacillus subtilis* *Clostridium* species *L. rhamnosus* GG *B. infantis*	• Enhances innate immunity • Induce the development of gut-associated lymphoid tissues (GALT) • Induce pre-immune antibody repertoire • Reduces the risk of respiratory tract infections • Reduces the incidence and severity of atopic dermatitis in pregnant mothers and infants • Reduces the risk of atopic eczema and allergic sensitization in children • Protects host against development of colitis
B. infantis *Escherichia (E.) coli* *C. subterminale* *Staphylococcus epidermidis* *Bacteroides fragilis* *Bacillus subtilis*	• Prevents *Salmonella typhimurium* infection • Promotes GALT development
F. prausnitzii *S. thermophilus* *L. fermentum*	• Reduces the severity of experimental colitis in mice • Stimulates immune responses
Seven strains of *Bifidobacteria* and LAB (G17)	• Release symptoms of skin lesions in atopic dermatitis mouse model by increasing Tregs and reducing Th1/Th2 cells and associated cytokines
E. coli *Candida utilis* yeast *L. delbrueckii, sub bulgaricus* *S. thermophilus* *L. acidophilus* *Bifidobacterium*	• Suppress pathogen growth • Secrete bacteriocin and microsin S • Reduce eczema • Promotes GALT development • When fused with MOG_{35-55} peptide, protects mice against EAE • Improves or prevents allergic recurrences in allergic rhinitis
B. bifidum *B. lactis* *L. lactis*	• Reduce eczema

peripheral blood [12,15–17]. A non-pathogenic (probiotic) strain of *Escherichia coli* (G3/10) has been shown to suppress pathogenic bacterial growth, and secrete potent antimicrobial peptides (bacteriocin, microsin S) which are harmful to gastroenteric pathogens (i.e. *Helicobacter pylori, Campylobacter jejuni, Clostridium difficile* and *rotavirus*) [18] (Fig. 1). LAB compete with enteric pathogenic bacteria for binding to mucin (primarily MUC2) sites on the surface of epithelial cells, which may be a mechanism for inhibiting pathogenic bacteria translocating from the gut to different organs including the liver; identified as one of the benefits of some probiotic strains [19]. The impact of LAB on the immune system has also drawn much attention in the last decade leading to their use in several pre-clinical and clinical studies in allergic and autoimmune disorders [7,20]. In 2001, the first randomized placebo controlled clinical study was published on the use of *L. rhamnosus*, to modulate immune responses in late stages of pregnancy or to newborns, (Table 3) and showed a significant decrease in the prevalence of atopic eczema [21]. In addition, using probiotic supplements alone, or in combination with vitamins and minerals have been recommended for the prevention and treatment of multiple sclerosis (MS) (Table 3) [20]; in particular, *L. plantarum* and *Bifidobacterium* B94 (BB94) were shown

Table 2
Effects of probiotics on the immune response.

Probiotic / probiotic strain	Immune stimulation outcome
Microbiota, ingested probiotics	• Maintain immune tolerance • Activate tolerogenic DCs • Drive regulatory T (Treg) cell differentiation • Stimulate differential Th1, Th2, Th17, Treg cells • Improves atopic dermatitis • Improves allergies, inflammatory bowel disease and autoimmune disorders including multiple sclerosis
Lactobacillu (L.). rhamnosus *L. delbrueckii*	• Induction of tolerogenic DCs • Reduce cell surface molecules, CD80, CD83, CD86 • Reduce IL-12 and NF-kB • Increase indoleamine 2,3-dioxygense (IDO) • Increase IL-10 • Stimulate Treg cells
Combination of *L. rhamnosus* and *B. lactis*	• Injestion during pregnancy and during breast feeding reduces risk of atopic eczema • Increased IL-10
A mixture of probiotics (*L.* GG, *L. rhamnosus* LC705, B. *breve*, and *Propionibacterium freudenreichii* ssp. *Shermanii* JS)	• Falls to reduce the risk of atopic eczema
L. s193 *L.* s292	• Increase beta-8 integrin on mesenchymal DCs • Activate differentiation of CD4 + T cells into Treg cells
L. reuteri	• Activates Th1 and Treg cells
L. casei Shirota	• Activates Th1 and Treg cells • Decreases IgE antibody responses • Activate splenic NK cells • Boost innate immune system • Stimulates TNF-alpha and IL-12 on human peripheral blood mononuclear cells
Combination of *L.* reuteri, *L. casei* and TNF-alpha	• Activate only Treg cells
L. lactis	• Do not activate Treg cells
L. acidophilus	• Suppresses IgE allergic responses • Downregulates IFN-gamma, IL-4 cytokines • Upregulates IgA antibody responses • Upregulates TGF-beta and Treg cells
Combination of *L. acidophilus* and *B. longum*	• Improves allergic rhinitis
L. plantarum WCFS1	• Upregulates $CD103^+$ DCs in the intestine • Increases Th2 cytokines • Upregulates Treg cells
LAVR1-A1 *L. rhamnosus* GG *L. acidophilus*	• Induce anti-inflammatory IL-4, IL-10 and TGF-beta cytokines • Inhibit IL-12 and IFN-gamma • Increase expression of co-stimulatory cell surface markers, CD14, IL-2R, HLA-DR, ICAM-1 and CD80 on monocytes
Combination of a mixture of probiotics, zinc and the coenzyme Q10	• Down regulates IgG in serum • Decreases TNF-alpha, IL-1, IL-6 and IL-17 • Decreases vascular endothelial growth factor (VEGF)
L. gasseri	• Induces IFN-alpha of monocytes
L. paracasei DG	• Increases TNF-alpha, IL-6, IL-8, CCL20 by human monocyte cell line THP-1
L. helveticus	• Reduces IL-17 cytokine and Th-17 cells • Reduces IL-6 • Increases IL-10, TGF-beta and Treg cells
Bifidobacterium (B.) infantis	• Matures DCs • Induces $CD103^+$ tolerogenic DCs • Induces Treg cells • Suppresses Th2 • Stimulates high levels of IL-10 which neutralizes IL-12 • Stimulates high levels of TGF-beta
B. animalis	• Reduces IL-6, IL-17, TNF-alpha and monocyte chemoattactant protein-1 • Increases IL-10 • Stimulates Treg cells
B. clausii	• Increases IL-10 • Increases TGF-beta • Improves allergic responses
B. bifidum *B. lactis*	• *In vivo*, lower number of $CD4^+$ and $CD8^+$ T cells. Higher Treg cells • Mediated by short chain fatty acids
B. breve *B. longum* *L. acidophilus* *L. gasseri* *Streptococcus (S.) thermophilus*	• Downregulate IL-17A • Downregulate IL-23 • Relieves colitis in mice • Suppress CD40, CD80
Short chain fatty acids	• Expand and differentiate Treg cells

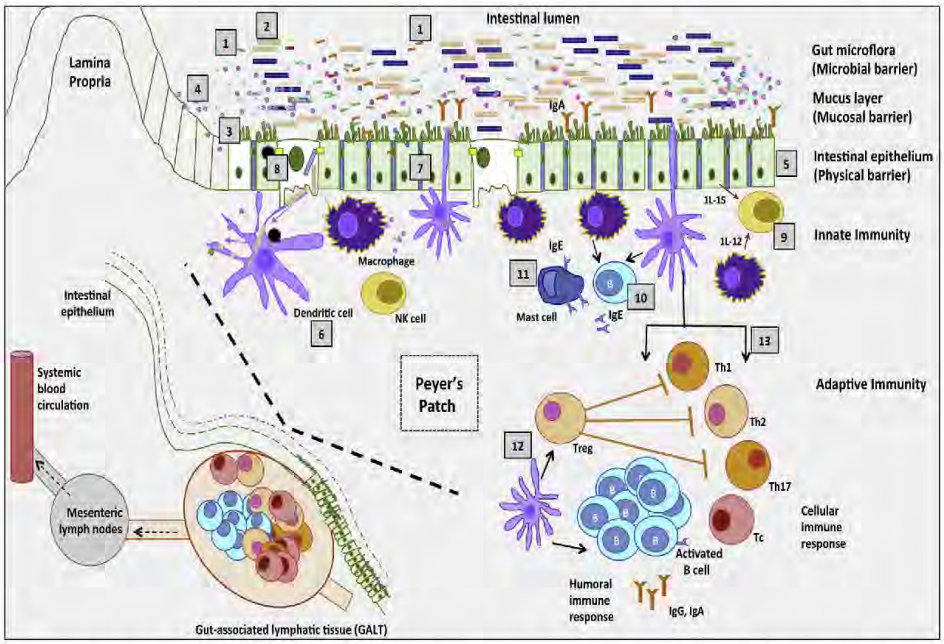

Fig. 1. The immunological complexity of the gut associated lymphoid tissue (GALT) and its interaction and activation with probiotics and gut microbiota. Probiotic and commensal microflora (1) collaboratively perform barrier function and compete with microbial pathogens (2) for nutrients and adhesion to the epithelial surface (3). Probiotics stimulate mucin secretion by goblet cells which protects the mucosal barrier. Antimicrobial peptides (4) such as, bacteriocin and microsin S by probiotics have antagonistic effects against pathogens. Probiotics can also induce mucosal epithelial cells (5) to secrete defensins. Dendritic cells (DCs) (6) endocytose bacterial products either via extending into the enteric lumen throughout epithelial tight junctions (7), or via bacterial transit through microfold M cells (8), or via pinocytosis of probiotics/microflora by epithelial cells (5). The innate immune response (natural killer (NK) cells), are triggered by IL-12 secreted by macrophage/DCs and IL-15 by epithelial cells (9). Immune response to extracellular pathogens via B cells are activated by macrophage/DC and secrete IgE (10) which stimulate mast cells (11) contributing to innate immunity. Adaptive immune responses are triggered by DCs, macrophages, and epithelial cells which process and present probiotics that stimulate regulatory T (Treg) cells (12) and tolerogenic TGFβ, IL-10 cytokines leading to suppression of IgA secretion and effector T helper (Th)-1, Th2, Th17 and cytotoxic T (Tc) cell responses (13). If pathogens bypass the epithelial barrier, pathogenic peptides presented by DC/macrophages trigger a series of immune responses; Th1 and Th17 pro-inflammatory and Th2 anti-inflammatory responses which can lead to pathogenicity of the immune system such as inflammation, allergic reactions, autoimmune disorders, and cancer. Such disorders can be modulated with probiotics supplementation. Thus, probiotics confer an array of benefits on epithelial barrier activities and the ensuing responses of the underlying mucosal immune and GALT systems.

to positively impact the spatial memory and learning of rats [20]. Herein, we describe the effects of probiotics to immune cells and their use in allergic and autoimmune disorders.

2. Methodology

Searches were conducted using PubMed, Google Scholar and Medline using the following key terms: Probiotics OR lactic acid bacteria AND human health, probiotics OR lactic acid bacteria AND immune disorders OR allergies AND therapy, probiotics OR lactic acid bacteria AND immune cells, probiotics OR lactic acid bacteria AND immune cells differentiation, probiotics OR lactic acid bacteria AND inflammation, probiotics AND inflammatory diseases, probiotics AND Inflammatory bowel disease, probiotics OR lactic acid bacteria AND multiple sclerosis, probiotics AND atopic dermatitis. Studies from all years were included, specifically those published within the last 10 years. Additionally, terms such as therapeutic, health benefits, and specific immune cells (i.i. monocytes, T cells, natural killer cells, and dendritic cells) were searched in the context of probiotic effects and gastrointestinal microflora. Some review articles and their reference lists were also searched to identify related articles. Over 1000 papers were retrieved and the most relevant were included in this article.

3. Cross talk between probiotics, intestinal epithelium and immune development

The beneficial effects of probiotics was first explained by the 'hygiene hypothesis', suggesting a lack of exposure to microbial stimuli early in childhood was the major factor behind allergic reactions [22,23]. Probiotics and intestinal microflora interact and confer an array of positive effects on the epithelial layer to maintain gastrointestinal and systemic health, by interacting with the gut-associated lymphoid tissues (GALT) which mediate oral tolerance and mucosal immunity [24]. Within the GALT are Peyer's patches, specialized areas of the intestinal immune system consisting of numerous lymphoid follicles surrounded by specialized epithelial cells of the mucosa-associated lymphoid tissues (M-cells), which are involved in the translocation of most antigens and bacteria, including probiotics, from the intestinal lumen into the patches [25] (Fig. 1, Table 1). Interestingly, specific bacteria in the intestinal microflora, such as combination of *Bacteroides fragilis* and *Bacillus subtilis,* can induce the development of GALT and pre-immune antibody repertoire [24]. Germ-free mice, that lack intestinal bacteria, display major immune deficiencies, such as, structural lymphoid deficiencies in the spleen and Peyer's patches and lymph nodes, with no germinal zones [26]. Yet, after exposure to microbial antigenic stimuli such as, *Salmonella paratyphi*, germinal zones are developed, and antibodies are present in the circulation [26]. In addition, germ-free mice show abnormal T cells, intraepithelial lymphocytes, macrophages and dendritic cells (DCs). However, colonization with commensal flora early in life allows proper development of immune cells [27–29]. Thus, establishing a balanced gut microflora and maintaining it by adequate exposure to commensal bacteria at birth and early in childhood provides challenges to the immune system by infections or other immune insults resulting in impacts on the

Table 3
Effects of probiotics on gastrointestinal tract, allergies and autoimmune diseases.

Type of disease	Probiotic bacteria	Form of used bacteria	Study model: Animal / Human trial *In vitro / In vivo*	Outcome (primary and secondary effects)	Ref
Gastroenteric infections	Non-pathogenic *Escherichia (E.) coli (G3/10)*	Oral injection of live bacteria	Human trial: Probiotic treatment of irritable bowel syndrome in children	Stops gastroenteric pathogens *H. pylori*, *C. jejuni*, *C. difficile* and rotavirus) by producing microcin	[18]
Functional gastrointestinal disorders	*E. coli* G3/10 *E. coli* G4/9 *E. coli* G5 *E. coli* G6/7 *E. coli* G8 *E. coli* MDS42 genomotypes present in probiotic drug	Live wild type in the form of probiotic drug Symbioflor 2 (DSM17252)	Human study: Treats functional gastrointestinal disorders; particularly IBS in adults and children (298 patients with IBS -lower abdominal symptoms)	Reduces abdominal pain score (APS) and general symptom score (GSS)	
Atopic eczema	*L. rhamnosus*	Oral injection of live bacteria	Clinical studies: Pregnant mothers and their newborn infants	Reduces the incidence of atopic eczema in newborns at high-risk	[21]
Multiple sclerosis (MS)	*L. plantarum* and *Bifidobacterium* B94 (BB94)	Live with/ without vitamins	Animal study: rat model	Improved spatial memory and learning	[20]
Allergy to Albumin	*Lactobacillus (L.) acidophilus* strain L-92	Oral administration of heat-killed lyophilized bacteria	Mouse model of Ovalbumin Allergy Splenocytes and	Modified total and OVA-specific serum IgE levels total IgA	[61]
Allergic asthma	*S. thermophilus*, *L. fermentum* and yeast (*zymosan* and *Saccharomyces cerevisiae*)	Oral feeding administration	Animal study: mice	Prevents allergic asthma by differential stimulation of immune responses	[101,102]
Allergies and recurrent	*Bacillus clausii*	Oral feeding *B. clausii* spores	Children (fluids collected from nasal lavage)	Modulates cytokine profiles (Increases IL-10 and TGF-beta), induces Treg cells	[105]
Respiratory infections	*Bacillus clausii*	Oral feeding *B. clausii* spores	Children	Prevents recurrent respiratory infections and shortens duration	
Atopic dermatitis	*B. animalis* subsp. *lactis* Bb12 *L. rhamnosus* GG *L. acidophilus* La-5	Oral injection of live bacteria as probiotic milk	Pregnant women and their children	Reduced incidence of atopic dermatitis and reduced Th22 cells	[107]
Allergic rhinitis, atopic dermatitis, eczema or asthma	*L. rhamnosus* GG	Oral ingestion of live bacteria	Pregnant women with strong family history of allergic rhinitis, eczema or asthma	Significant delay in atopic dermatitis in infants within 1 st six months of life due to increased levels of IL-10	[112]
Atopic eczema atopic eczema, allergic rhinitis, or asthma	*L. rhamnosus* GG and *B. lactis*	Oral ingestion of bacteria as capsules/ capsules content dissolved in water (for infants)	Pregnant and breastfeeding mothers or infants	Reduces risk of atopic eczema and allergic sensitization in children	[21]
Experimental colitis and dysbiosis	*F. prausnitzii*	Oral consumption of live bacteria	Mouse model	Anti-inflammatory effects and reduction of severity of colitis	[118]
Crohn's disease (CD) and ulcerative colitis (UC)	*Clostridium* strains	Oral consumption of live bacteria	Mouse model	Protects against colitis by suppressing effector responses and inducing Tregs	[79] Delete 120
Colitis	*Clostridium* strains	Colonization of GF mice with fecal material from mice colonized with 46 Clostridium strains	Gnotobiotic mice model	Protects against colitis and resistance to systemic IgE responses	[120] Delete 79
Colitis and subsequent IBD caused by *Citrobacter rodentium*	*L. plantarum* A, *L. acidophilus* ATCC 4356 and *L. rhamnosus* ATCC 53,103	Oral feeding of live bacteria	Mouse model of *C. rodentium*-induced colitis	Colonize the gut and protects against invasive *C. rodentium*	[123]
Colitis	*Bacteroides fragilis*	Oral feeding of live bacteria	Mouse germ free model	Protects against colitis by over expression of Treg cells mediated by bacterial capsular polysaccharide antigen (PSA) to TLR2	[124]
Colitis	All microflora in fecal microbiota	Live bacteria fecal microbiota transplantation	Human colitis patients	Relieves colitis symptoms by restoring composition of intestinal microbiota	[126]
Chrohn's disease (CD)and ulcerative colitis (UC)	*Bifidobacteria*	Live bacteria co-cultured with PBMC or dendritic cells			[127]

(*continued on next page*)

Table 3 (*continued*)

Type of disease	Probiotic bacteria	Form of used bacteria	Study model: Animal / Human trial *In vitro / In vivo*	Outcome (primary and secondary effects)	Ref
			Peripheral blood mononuclear cells (PBMC)-dendritic cells (DCs) from Chrohn's disease (CD) children	DCs showed high uptake of bacterial particles, higher TNF-alpha, no effect on IFN-gamma and IL-17, UC	
CD and UC	*Bifidobacteria*	Live bacteria co-cultured with PBMC or DCs	PBMCs of healthy and adult patients with CD, UC	DCs from healthy and CD, UC adults did not show significant changes	[127]
Necrotizing enterocolitis	Mixture of *B. infantis*, *S. thermophilus*, *B. bifidus* and *L. acidophilus*	Oral feeding of live bacteria	Extremely low birth weight neonatal rats	Beneficially improves caveloin-1 and nitric oxide signalling and growth factors in the terminal ileum	[27]
Experimental allergic encephalomyelitis (EAE) in mice	*Candida utilis*	Oral feeding of live bacteria-fused with MOG35-55-peptide as carrier	EAE mouse model	Protects mice against the development and clinical symptoms of EAE	[131]
EAE in mice	Recombinant *Lactobacilli* expressing myelin antigens	Oral feeding and intranasal administration of $PLP_{139-151}$ and MBP	EAE in Lewis rats	Inhibited EAE	[132]
EAE in mice	*Lactobacillus* and *Bifidobacterium*	Oral feeding	EAE mouse model	Delays EAE onset and clinical score significantly	[134]
EAE in mice	*L. helveticus*	Intraperitoneal injection	EAE mouse model	Reduces the incidence and clinical score of EAE	[135]
EAE in mice	VSL#3 *L. casei* Shirota, *L. casei* 393, *L. reuteri*, *L. paracasei*, *B. breve* and *B. animalis*	Bfdbm breve strain Yakult; Lacto-mix: Lctb paracasei, Lctb plantarum, Lctb delbrueckii; LGG: Lctb GG	EAE mouse model	Reduces the clinical score and EAE activity by, increasing IL-10, TGF-beta and Treg cells	[71]
EAE-induced with guinea pig- spinal cord homogenate	*L. casei* Shirota	Oral feeding of live bacteria (started before EAE)	Infant Lewis rats with EAE	Represses EAE neurological symptoms development (not statistically significant)	[71]
EAE-induced with guinea pig-MBP	*L. casei* Shirota	Oral feeding of live bacteria (started before EAE)	Infant Lewis rats with EAE	Represses EAE neurological symptoms development (statistically significant)	[71]
EAE-induced with guinea pig-MBP	*L. casei* Shirota and *B. breve* strain Yakult (BbY)	Oral feeding of live bacteria (started before EAE)	Infant Lewis rats with EAE	Represses EAE neurological symptoms development (statistically significant)	[71]
Multiple Sclerosis (MS)	*Lactobacillus, Bifidobacterium* and *Steptococcus*	Oral feeding of live bacteria	Human: MS patients	Decrease abundance of *Akkermansia* and *Blautia* bacteria, Decreases inflammatory monocytes, MHC-II and CD80	[136]
MS	*L. acidophilus, L. casei, B. bifidum* and *L. fermentum*	Oral feeding of live bacteria in capsules	Human: MS patients	Improve disability status scale, depression and anxiety symptoms and reduced C-reactive protein, plasma oxidative and malondialdehyde	[137]

microbiome population, colonization, and host health.

Both probiotics and commensal bacteria enforce the functions of the mucosal barrier of the GIT epithelia, induce mucus secretion, and stimulate secretion of IgA which neutralizes pathogens inside the lumen (Fig. 1) [13]. Correspondingly, cross talk between epithelial cells and residing epithelial immune cells are mediated and enforced by probiotics, and contribute to their effector functions [6]. Probiotics are also able to induce expression of adhesion molecules, similarly important for regulatory function of residing immune cells. Probiotics stimulate the innate immune system, antigen presenting cells (APC) and natural killer (NK) cells in both mice and humans [30]. Adaptive immunity is stimulated by probiotics, such that IgG and IgA antibodies are produced in response to probiotic consumption, as demonstrated with *L. acidophilus, L. bulgaricus and B. bifidum* (Fig. 1) [31,32]. In addition, macrophages, $CD8^+$ T cells, Treg cells and cytokines (i.e. interferon (IFN)-gamma and interleukin (IL)-10) are stimulated by probiotics [33]. APCs exposed to probiotics or commensal bacteria, present harmless peptides to T cells and subsequently induce Treg cells, to produce anti-inflammatory cytokines including, transforming growth factor beta (TGF-beta), IL-10 and retinoic acid. In addition, Treg cells suppress effector Th1, Th17 and cytotoxic T (Tc) cells and IgA secretion [34]. However, if APCs present invasive pathogenic peptides, it leads to the initiation of effector, pro-inflammatory Th1/Th17 responses. Thus, probiotic bacteria can control the 'on/off switch' of immune responses in a strain-dependant manner, modulating the host immune system at the mucosal level. Lack of sufficient probiotic bacteria and their subsequent stimulatory impact on the immune system, leads to inadequate or inappropriate immune modulation. Insufficient probiotic bacteria alone, or inadequacy, together with stimulation of immune system by invasive pathogens (which bypass the mucosal barrier), mediate a range of immunopathogenic disorders such as, allergies, asthma, atopic disorders, inflammatory bowel diseases (IBD), cancer, type-2 diabetes and autoimmune disorders (Table 1) [6,35–37].

4. Probiotic organisms and immune cells

Probiotic bacteria have numerous beneficial immune and health effects. They not only enhance the bioavailability of nutrients and moderate health, they also aid in regulating the gastrointestinal ecosystem and stimulate immunomodulatory properties to a number of immune cells (Table 1, Figs. 1 and 2).

4.1. Dendritic cells

DCs play a key role in directing immune responses to self and non-self antigens. Upon endocytosis of antigens, DCs mature functionally and phenotypically. As mature cells, they prime T cells towards Th1 or Th2 phenotypes, however, in their immature state (tolerogenic DCs) they cause the deletion of T cells or stimulation of Treg cells (Fig. 2, Table 2) [38]. The intestinal microbiota and ingested probiotics can interact with the host's innate and adaptive immune system, regulating cell differentiation in the gut which is involved in sustaining immune tolerance. In particular, microbiota are able to activate distinct tolerogenic DCs in the gut and consequently drive Treg cell differentiation [39]. DCs also endocytose probiotics and present short antigenic peptides to T cells, stimulating Th1, Th2, Th17 and/or Treg cells. Due to these roles, in recent years considerable focus has been directed towards the anti-inflammatory properties of probiotics e.g. *L. rhamnosus and L. delbrueckii* and the induction of tolerogenic DCs and their effect on the stimulation of Treg cells [40]. In particular, significantly reduced co-stimulatory cell surface molecules (CD80, CD83, CD86), IL-12 and NF-kappa B were noted and increased expression of indoleamine 2,3-dioxygenase (IDO) and IL-10, suggesting that probiotics are able to modify properties of DCs to modulatory cells, which may contribute to immune tolerance and immune balance [41]. Recently, two novel *Lactobacillus* probiotics strains (s193 and s292) isolated from Funazushi (a traditional Japanese fermented food) were shown to increase beta-8 integrin on mesenchymal DCs which strongly activated $CD4^+$ T cell differentiation into Treg cells; monoclonal antibodies against beta-8 integrin are able to block the differentiation of Treg cells [42]. DCs co-cultured with *L. reuteri* and *L. casei*, although displaying different cytokine profiles (IFN-gamma production), activate both Th1 and Treg cells. However, DCs stimulated with *L. reuteri* and *L. casei* and combined with TNF-alpha stimulate only Treg cells [9]. On the other hand, DCs exposed to *L. lactis* do not stimulate Treg cells, showing the importance of the *Lactobacilli* strain used in the ensuing immune response. Oral administration of *B. infantis* in mice stimulates DCs to a maturation state and $CD103^+$ tolerogenic DCs accumulate in the GALT, induce Treg cells and suppress Th2-biased responses [43]. Given the roles of DCs and the intestinal residing DCs in endocytosing bacteria, it is clear that DCs play a central part in immune homeostasis in the healthy intestine and in the pathology of IBD [44,45]. Thus, understanding the effect of stromal cells and microbial signals on DC function are important as the manipulating regime of DCs through probiotics, nutrition and microbiota, leading to therapeutic modalities against inflammatory diseases, which still require further research

4.2. Monocytes and macrophages

Monocytes are present in the peripheral blood which then differentiate to tissue macrophages and myeloid lineage DCs. Intestinal microbiota and ingested probiotics can interact with macrophages for beneficial immune effects [44]. In fact, IL-12 secreted by macrophages stimulates $CD4^+$ T cells and NK cells to secrete IFN-gamma, resulting in the generation of pro-inflammatory Th1 cells. Interestingly, IFN-gamma and IL-12 production seem to be controlled in a positive feedback loop, as IFN-gamma in turn, stimulates production of IL-12. This loop of IFN-gamma and IL-12 production can be deleterious, as it may result in uncontrollable expression of cytokines and possible shock. However, co-culturing peripheral blood mononuclear cells (PBMCs) with selected bacteria (LAVRI-A1, *L. rhamnosus* GG, *Bifidobacteria* and *L. acidophilus*) induces anti-inflammatory cytokines IL-4, IL-10 and TGF-beta [46,47]. These cytokines inhibit the production of IL-12, IFN-gamma and other pro-inflammatory cytokines which are beneficial for autoimmune and allergic responses. In addition, probiotics from dairy source such as LAB *L. gasseri* strains could induce IFN-alpha production by monocytes with *L. gasseri* DSM20243 T [48], and 3 strains of LAB (*L. delbrueckii* ssp. *bulgaricus*, *B. bifidum, L. acidophilus*, trilac) being the most potent [49]. In addition, $CD4^+$ T cell proliferative responses, high levels of IL-10 and TNF-alpha (not IL-12 and IFN-gamma) are induced, as well as increased expression of cell surface markers (CD14, CD80, HLA-DR, ICAM-1, IL-2 receptor) [49]. The probiotic *L. paracasei* DG commonly used in commercial probiotic products, shows immunostimulatory properties by increasing the expression of TNF-alpha, IL-6, IL-8 and CCL20 by human monocyte cell line, THP-1 [50]. Similarly, it has recently been demonstrated that *S. thermophilus* induced differential cytokine secretion of human monocyte U937 cell line; IL-4 and IL-10 secretion important for anti-inflammatory responses, TNF-alpha and IL-6 necessary for stimulation of the innate immune response, as well as CXCL8 (IL-8) and GM-CSF required for cell recruitement at sites of inflammation [Ref.]. Hence, the use of commensal LAB results in an anti-inflammatory profile and activation of monocytes, which is beneficial in suppressing pathogenic-induced pro-inflammatory responses in microbiota (via IL-10 production) and simultaneous induction of anti-microbial effects (via TNF-alpha stimulation and monocyte induction).

4.3. NK cells

NK cells play a keyrole in innate immunity and are cytotoxic to tumors. NK cells have a regulatory role in the development of allergic respiratory disease. *L. casei* subspecies Shirota (S) administered in mice intraperitoneally, or intravenously leads to activated splenic NK cells,

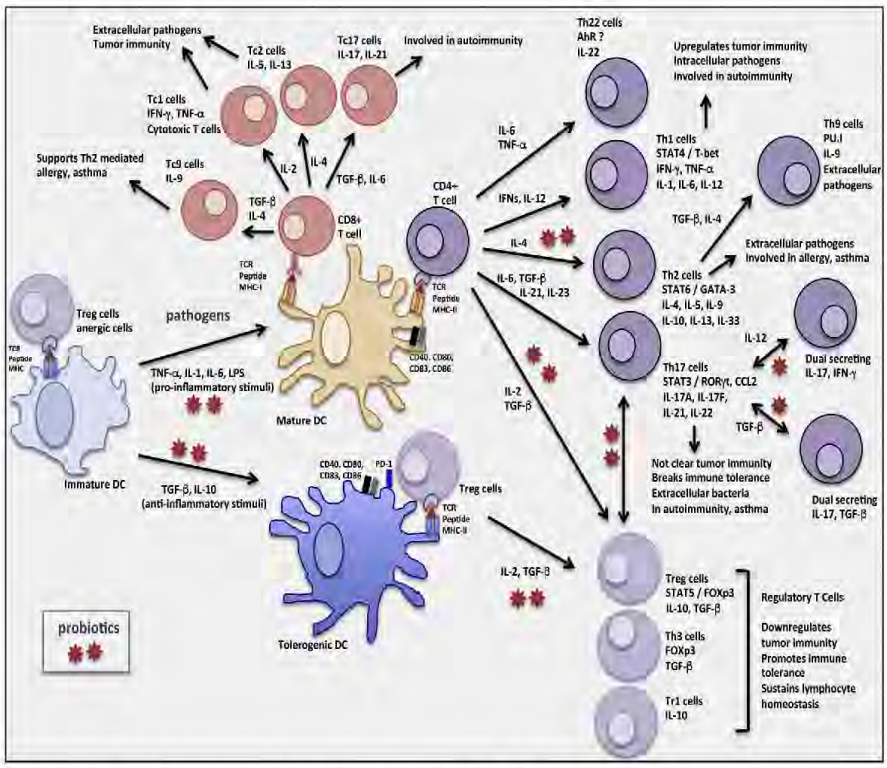

Fig. 2. Immunomodulatory effects of probiotics stimulating T helper (Th)-1, Th2, Th17 cells or T regulatory (Treg) cells. Imature dendritic cells (DC) either mature following pro-inflammatory stimuli (IL-1, IL-6, lipopolysacharride (LPS), TNF-alpha or pathogens) or become tolerogenic DCs in the presence of anti-inflammatory stimuli TGF-beta and IL-10. The differentiation of immature DCs into mature or tolerogenic DCs is also regulated in the presence of probiotics. Mature DCs confer an array of stimulatory functions whereby they stimulate Th or Treg cells depending on the cytokine produced. Mature DCs interact with naïve CD4 + T cells and depending on the resulting cytokine produced by the CD4 + T cells they differentiated into either, (i) inflammatory Th1 (secrete IL-1, IL-6, IL-12, IFN-gamma, TNF-alpha) or Th17 (secrete IL-17 A, IL-17 F, IL-21, IL-22) cells; (ii) anti-inflammatory Th2 (secrete IL-4, IL-5, IL-9, IL-10, IL-13, IL-33) or Th9 (secrete IL-9) cells; (iii) regulatory Treg (secrete IL-10, TGF-beta), Th3 (secrete TGF-beta) or Tr1 (secrete IL-10) cells or (iv) Th22 cells which secrete IL-22. In addition, Th17 cells can be further divided into dual secreting cells of either IL-17, IFN-gamma or IL-17, TGF-beta. Probiotics have been shown to regulate Treg cells and/or Th2 cells depending on the strain and disease. Tolerogenic DCs interact with T cells and can directly stimulate Treg cells in the presence of IL-2 and TGF-beta (or probiotics). Furthermore, mature DCs stimulate CD8 + T cells into Tc1, Tc2, Tc9 or Tc17 cells however, it is not clear whether probiotics are involved in their differentiation / stimulation.

peritoneal exudate cells and thoracic exudate cells, respectively, but not via oral administration. In contrast, *L. casei* S administered orally to newborn mice is able to activate splenic NK cells [51]. Thus, oral consumption of probiotics, such as *L. casei*, early in life boosts the innate immune system [27]. In human PBMCs, *L. casei* S stimulates pro-inflammatory cytokines IL-12 and TNF-alpha; the type of immune response required for a DC-NK cell interaction and defence mechanism [52]. Another study showed that *Lactobacillus* strains were able to stimulate IL-12 secretion by DCs, resulting in activation of NK cells and subsequent secretion of IFN-gamma, which not only leads to innate immune responses, but also activation of the adaptive immune responses [9]. Upon stimulation by *Bifidobacterium*, high levels of IL-10 is produced which neutralizes IL-12 induced by *Lactobacillus* strains [9]. This suggests that co-administration of probiotics might not be favorable when used for immunomodulatory therapeutic methods for NK-DC mediated type of immune responses. Results from such studies suggest that *Lactobacillus* strains may initiate cellular immune responses whilst *Bifidobacterium* strains can induce polarization towards anti-inflammatory and Treg cells. Thus, *Bifidobacterium* might be the probiotic of choice to be used to manage or prevent inflammatory immune disorders such as, allergies and autoimmune conditions, although, *Lactobacillus* strains may be beneficial for anti-tumor immunity. In addition, LAB have been shown to stimulate T/NK cells via induction of IL-12/IFN-gamma, whereas, other strains can either suppress or boost Treg/Th2 responses via IL-4, IL-5 and IL-10 [53]. Understanding the cytokine profiles induced following probiotic exposure and their immune modulating properties can provide a clear path for managing immunopathologies [6,54]. These studies infer that host innate immune responses are important in relation to the development of some diseases and inducing it by probiotic bacteria can help to enhance immunity and health. Therefore, *L. casei* strains have established safety and functional characteristics and are extensively used in probiotic dairy products, and have long been used as staple food ingredients in many countries including Japan and Europe.

4.4. Regulatory T cells

Evidence that Treg cells play an active role in immune tolerance was established in the 1970s [55], at the time they were referred to as suppressor T cells. During the late 1980's research into the role of these cells in immune tolerance dwindled, however the identification of cell-surface markers in the mid-1990s led to a resurgence in interest [56]. In 1994, Tregs were described as key cells in the establishment of oral tolerance [57], and were proposed as a potential target for the treatment/prevention of diseases that are caused by over reaction of the immune system (i.e. autoimmunity, inflammatory metabolic diseases, asthma and allergy) all of which have exhibited a global increase, especially in western countries [58,59]. Tregs are responsible for the

state of unresponsiveness of the immune system to specific antigens (self-peptides); a process known as immune tolerance, which is critical for the maintenance of immune homeostasis [60].

There is accumulating evidence which suggests probiotics are not only beneficial for the treatment of Th1 or Th2 mediated inflammatory disorders, but are also able to induce beneficial Treg cells to modulate immune balance (Fig. 2).`For instance, *L. acidophilus* strain L-92 suppresses IgE allergic responses to an antigen model in mice, as well as downregulating the secretion of IFN-gamma, IL-4 and IL-10, and significantly increase the levels of IgA, TGF-beta and Treg cells [61]. *L. plantarum* WCFS1 is also associated with $CD103^+$ DC infiltration in the intestine, increases Th2 cytokines and is involved in the generation of Treg cells in mice [62]. More recently, the combination of probiotics, zinc, and coenzyme Q10 synergistically reduced rheumatoid inflammation in mice by significantly downregulating IgG in serum, decreased the expression of pro-inflammatory cytokines (IL-1, IL-6, IL-17, TNF-alpha) and vascular endothelial growth factor in the joint synovium and upregulated Treg cells [63]. *L. casei* administration in mice with *E. coli* induced intestinal inflammation is associated with $CD4^+CD25^+Foxp3^+$ Treg cells in the spleen and mesenteric lymph nodes with corresponding decreased $CD4^+IL\text{-}17^+$ Th17 cells and RORγt mRNA levels [64]. Thus, *L. casei* could have possible therapeutic implications against intestinal inflammation by modulating the Treg/Th17 balance. However, intragastric administration of *L. casei* BL23 stimulates the expression of $CD4^+Foxp3^+$ Treg cells and $ROR\gamma t^+$ Th17 cells, termed type-3 Treg cells which are believed to control luminal inflammatory disorders by suppressing inflammation [65].

LAB from *Bifidobacteria* strains, can also prime expression of TGF-beta and IL-10, and increase the number of Treg cells, which is associated with suppressive function/tolerance [66]. Similarly, administration of *B. animalis* subsp. *lactis* 420 (B420) or *L. salivarius*-33 (Ls-33) to mice shows reduced levels of pro-inflammatory cytokines TNF-alpha, IL-6, IL-17 and monocyte chemoattractant protein-1 (MCP-1) and skews towards IL-10 anti-inflammatory cytokine profile and generation of Treg cells [67]. Likewise, *B. infantis* 14.518 fed with shrimp allergies, show DC-dependent stimulation of Treg cells [43]. It is clear that an array of probiotics exert their beneficial effects and mediate an immune balance by their stimulation of Treg cells.

SCFAs produced by commensal microbiota following fermentation of dietary fibers, have also been shown to expand and differentiate intestinal Treg cells [68]. In particular, butyrate initiates the differentiation of Tregs [69] as well as suppressing the onset of colon cancer and reducing allergic responses in the lung of mice [69,70]. In mouse models of asthma and colitis (newborn and mature) which were orally subjected to a mixture *L. casei, L. lactis, L. acidophilus, B. bifidum* and *B. lactis* daily, showed a lower proportion of $CD4^+$ and $CD8^+$ T cells and higher Treg cells; these effects were shown to be mediated by SCFAs (acetate, propionate and butyrate) [71]. However, in other studies it was noted that butyrate induces the expression of the transcription factor T-bet resulting in IFN-gamma secreting T cells and not Treg cells. Thus, butyrate may exert either beneficial or detrimental effects on the mucosal immune system. Further studies are required to determine the dose of butyrate and its mechanism by which it induces beneficial Treg cells.

4.5. Th17 cells

Th17 cells secrete IL-17 (IL-17 A), IL-17 F, and IL-22 and eliminate external pathogens mainly at epithelial mucosal sites [72,73]. At mucosal sites, secretion of anti-microbial defensins is dependent on the stimulatory activities of IL-17, IL-22 and differentiation and stimulation of neutrophils which is dependent on these cytokines. However, it is believed that excessive or persistent responses of Th17 cells can drive the onset of inflammatory diseases, and as such, Th17 cells together with Th1 cells, are generally responsible for the pathophysiology of autoimmune diseases, asthma, allergy and the development and progression of tumors [74,75]. Similar to human Th17 cells, murine Th17 cells also play a critical role in numerous mouse autoimmune disease models such as, experimental autoimmune encephalomyelitis (EAE) [76,77].

Intestinal microflora are shown to regulate and maintain the quantity and function of DCs, through which they are able to differentially modulate naïve $CD4^+$ T cell responses towards specific Th cell populations (Fig. 2). Studies in germ free mice show that the number of $CX3CR1^+$ DCs is reduced which favorably induces naïve $CD4^+$ T cell differentiation towards Th1 and Th17 [78]. In addition, filamentous bacteria affect the whole microflora of the GIT immune system which subsequently stimulate Th17 cells residing in lamina propria [79]. The number of Th17 cells in the GIT lamina propria is associated with the presence of microbiota, thus, in germ free mice, the percentage of Th17 cells is very low [80]. As such, the severity of EAE symptoms and autoimmune arthritis are reduced due to lower levels of Th17 cells and lower IL-17 and IFN-gamma [81,82]. On the contrary, colonization of segmented filamentous bacteria which induce high levels of local Th17 cells in the lamina propria, prevents diabetes in non-obese diabetic mice [83]. Thus, Th17 cells and IL-17 play differential roles depending on the disease model. Commensal microbiota are also able to stimulate secretion of IL-25 (IL-17E) in the GIT that prevents expression of IL-23; without IL-23, pathogenic Th17 cells cannot survive [84].

Numerous animal models as well as clinical trials have established beneficial effects of probiotics in IBD due to anti-inflammatory properties as a result of down-regulating the expression of IL-17 [85,86]. Several studies with colitis-induced mouse models have shown that the probiotics *B. longum* subsp. *infantis*, *B. breve*, *L. acidophilus*, *L. gasseri* A5, *B. longum* and *S. thermophilus* ST28, are able to down-regulate the production of IL-17 leading to relief of colitis symptoms [87–90]. Two individual studies determined the effect of blocking IL-17 by using novel immunosuppressive drugs 4SC-101 [91] and vidofludimus [92] to compare with probiotic effects; drugs similarly alleviated severe colitis in mice by targeting IL-17 and confirmed the suppressive mechanisms of probiotics. IL-23 which is also associated with development, maintenance and polarization of Th17, is another activator for inflammatory conditions [93]; in fact, suppressing the IL-23/IL-17 axis, is now a promising target for probiotic treatments in the prevention and management of IBD [90]. Studies on the effects of *B. breve* and *L. rhamnosus* GG on colitis-induced mouse models revealed that LPS-induced IL-23 expression by intestinal cells, was reduced by these probiotics [87,90].

In in vitro studies using intestinal epithelial cell lines, *B. longum* subsp. *infantis* JCM 1222 was shown to suppress the expression of co-stimulatory cell surface molecules, CD40 and CD80 as well as IL-17 A at mRNA and protein levels [88]. In another comparative study on the effect of *B. breve* and *L. rhamnosus* GG, in human intestinal HT-29/B6 or T84 cells and PBMCs, reduced CD40 and IL-17 A mRNA expression induced by LPS stimulation [87]. Similarly, *B. longum* subsp. *infantis* [88] is able to reduce the expression of RORγt and simultaneously suppress IL-17 A production following oral administration in a mouse model of colitis. Due to the vital role of RORγt as a specific transcription factor for differentiation of Th17 cells, it can be a promising candidate to target for probiotic therapeutics in inhibiting Th17 cells and subsequent alleviation of inflammatory conditions. Furthermore, feeding of *L. acidophilus* or *B. clausii* in ovariectomized mice for 6 weeks was able to skew Th17 cells towards a Treg phenotype by reducing pro-inflammatory IL-6, IL-17 and TNF-alpha and increasing expression of IL-10 [94,95]. Hence, there is an increasing value of the use of probiotics as a driver of treating many inflammatory disorders by skewing Treg/Th17 balance.

5. Probiotics, allergy and autoimmune diseases

Studies in animal and in human clinical trials suggest that probiotics have the potential to prevent or treat allergies (atopic dermatitis and

allergic rhinitis) and autoimmune diseases (IBD and MS). Based on the 'hygiene hypothesis', it is believed that any change to the human GIT and microflora results in increased risk of such diseases. Regulation of gut microbiota by the consumption of probiotics has been shown to influence the development of the mucosal and systemic immune response (Figs. 1 and 2) that alter immune homeostasis and immune profile and positively affect allergies and autoimmune disorders.

5.1. Probiotics and allergies

Food allergy is described as the activation of mast cells or basophils and production of IgE in response to specific food proteins [96]. This can be in the fomr of life-threatening food hypersensitivity reactions with symptoms usually appearing within minutes of exposure; a condition quite distinct from food intolerances, such as Coeliac disease [97,98]. Around 1–2% of adults and 5–7% of children suffer from some type of food allergy, which its prevalence has significantly increased over the last 20 years [99]. Although allergy symptoms are manageable with medications, no cure is available for food allergy except for strictly avoiding allergy triggering food. Interestingly, it is now widely accepted that early life exposure to bacteria such as probiotics, results in activation of APCs that leads toimmune homeostasis and subsequent reduction in allergies. Probiotics are able to differentially stimulate the immune system to express pro- and anti-inflammatory cytokines in a strain-dependent manner. As previously discussed, probiotics have been shown to modulate immune responses *in vitro* and *in vivo* to skew immune responses towards Th1/Th2 and Treg phenotypes, which is essential in managing and preventing immune-mediated pathologies, including allergies [100]. Oral consumption of killed L. *casei* Shirota has been reported to stimulate the production of Th1 cytokines, resulting in decreased stimulation of allergic IgE antibodies against ovalbumin (OVA) in experimental allergic mouse models with allergy to OVA [51]. Thus, the use of probiotic bacteria to shift mucosal immunity towards Th1 responses supports their use as a viable approach for the treatment of allergic disorders [51]. Oral feeding of *S. thermophilus*, *L. fermentum* and yeast (such as zymosan and *Saccharomyces cerevisiae*) [101,102], also, differentially stimulate immune responses. In one study for example, mice were fed with yeast or *L. fermentum*, then fed with OVA, and vaccinated with OVA [51]. The probiotic-exposed mice responded more efficiently to vaccination with OVA than mice with no pre-feeding of either probiotics or OVA, or the mice which were only given OVA. Nevertheless, vaccination in mice that were given either yeast or *L. fermentum* followed by yeast and OVA or *L. fermentum* and OVA showed significantly suppressed antibody responses as a result of vaccination with OVA. This suggests that, although feeding the antigen alone appears to prime the onset of immune responses, co-feeding the same antigen with probiotics can repress allergic responses due to suppressing both cellular and humoral immunity [51]. Similarly, studies showing the effects of probiotic treatment (*L. casei, L. lactis*, *L. acidophilus*, *B. bifidum* and *B. lactis*) on the suppression in mice of experimental OVA-induced asthma, an allergic airway disease model, either at the time of birth or at later developmental stages, resulted in decreased numbers of Th1,Th2 and $CD8^+$T cells in the airways compared to control mice [103]. This data may provide insights into an effective treatment approach for controlling allergic reactions.

In humans, *B. longum* and *L. acidophilus* in milk or yogurt, if used as probiotics powder, capsules, or even heat-killed bacteria, can alleviate the severity of the clinical scales of allergic rhinitis and asthma symptoms compared to placebo [104]. In children with allergies and recurrent respiratory infections, *B. clausii* was shown to beneficially modulate cytokine profiles, and induce Treg cells in parallel with increased levels of IL-10 and TGF-beta [105]. Similarly, PBMC from subjects with allergic rhinitis who consumed yogurt or skimmed milk (containing *L. delbruekii,* sub *bulgaricusand, S. thermophilus*, *L. acidophilus* and *Bifidobacterium*) showed higher IFN-gamma responses and improved symptom score [106]. All thes suggest the use of probiotics as a novel approach in allergic conditions due to their great potency in polarizing T cells in the gut towards Treg cells.

5.2. Probiotics and atopic dermatitis

Atopic dermatitis is a chronic inflammatory skin disorder which is predominantly noted in infants and toddlers. Although the acute phase of atopic dermatitis is dominated by Th2 (IL-4, IL-5, IL-13) and Th22 cells, during the chronic phase, Th1 cells (IFN-gamma and IL-12) take the lead, resulting in atopic dermatitis [107,108]. In a mouse model of atopic dermatitis, consumption of a mixed of 7 strains of *Bifidobacterium* and LAB for 8 weeks resulted in reduced atopic skin irritation and increased Treg cells [108]. In another study, Treg cells were increased locally in the skin of mice following supplementation of *L. casei* [109]. Similarly, an increase in Treg cells were noted in the spleen after *L. reuteri* supplementation in mouse models with asthma [110]. In addition, Tregs have been shown to be induced from human PBMCs following exposure to probiotic species (*L. acidophilus*, *B. lactis*, and *L. plantarum*) with *L. acidophilus* being the most potent [9,111].

In humans, a randomized controlled study of probiotic consumption (*B. animalis* subsp. *lactis* Bb-12, *L. rhamnosus* GG, and *L. acidophilus* La-5) in pregnant women and their children, showed reduced incidence of atopic dermatitis and reduced Th22 cells [107]. Additionally, ingestion of *L. rhamnosus* GG alone in pregnant women with strong family history of allergic rhinitis, eczema or asthma, showed significant delay in developing atopic dermatitis in their infants during the first six months of delivery, which correlated to increased levels of IL-10 [112]. Likewise, the combination of *L. rhamnosus* GG and *B. lactis* throughout pregnancy and breastfeeding reduces the risk of atopic eczema and allergic sensitization in children [21], whereas a mixture of probiotics (*Lactobacillus* GG, *B. breve*, *L. rhamnosus* LC705, and *Propionibacterium freudenreichii* ssp. *Shermanii* JS) failed to reduce the risk of atopic eczema, due to the strain dependency of probiotic bacteria [21]. In a systematic review and meta-analysis studies of pediatric atopic dermatitis it was noted that probiotics are effective in preventing this condition [113]. Furthermore, cutaneous exposure to a lysate of probiotics (*Vetreoscilla filiformis*) alleviates skin inflammation and induces IL-10 secreting tolerogenic DCs and Treg cells and reduces Th1 pro-inflammatory cells and their cytokines [114].

5.3. Probiotics and inflammatory bowel disease

IBD, including Crohn's disease (CD) and ulcerative colitis (UC), is a major human health problem [115]. IBD is a group of chronic inflammatory disorders of the GIT, which is currently incurable and results in intestinal inflammation, severe diarrhoea, fatigue, pain and subsequent weight loss. A link has been established between insufficient intestinal probiotics (dysbiosis) and the development of CD and UC [116]. In fact, mucosal-associated microbiota of twin individuals with CD has been shown to have lower abundance of *Faecalibacterium prausnitzii* and significantly higher levels of *E. coli,* compared to corresponding healthy twin siblings [117]. Such dysbiosis of intestinal microbiota is believed to contribute to pathophysiology of CD and UC through an imbalance between intestinal microbiota and mucosal immunity, leading to inflammation in the intestine. Interestingly, experimental colitis, in mice, caused by dysbiosis, is corrected by consuming *F. prausnitzii* orally, which leads to anti-inflammatory effects and reduction of severity of colitis [118]. Consumption of probiotics in general has been increasingly used as a means of preventing and/or managing CD and UC by restoring damaged intestinal mucosal barrier [119]. In fact, suppression of effector responses and induction of Treg cells has been shown in mice, following consumption of *Clostridium* strains, and their consumption in early life is also beneficial in the protection against colitis [79,120]. Consumption of *B. infantis* by mice results in increased number of Treg cells and leads to prevention of *Salmonella typhimurium* infection. Furthermore, Bifidobacteria increases

gastrointestinal defense against enterohaemorrhagic pathogenic *E. coli* by generating acetate [121]. Recently it was shown that SCFAs produced through the fermentation of indigestible dietary fibers by *Bifidobacteria* and other anaerobic bacteria [103] resulted in the differentiation of colonic Treg cells. *L. rhamnosus* and *B. infantis* protected the host against the development of colitis via the effect of their SCFAs products on the immune system [122]. Additionally, *L. casei, L. lactis*, *L. acidophilus*, *B. bifidum* and *B. lactis* also generate SCFAs (acetate, propionate and butyrate) which induce the differentiation of Treg cells in the colon, subsequently suppressing the development of DSS-induced experimental colitis in mice [88].

Interestingly, while *Citrobacter rodentium* can cause colitis and subsequently IBD, commensal microflora of the gut can provide protection against invasive pathogens like *C. rodentium* through colonization of the host gut [123]. In a mouse model of *C. rodentium*-induced colitis, administration of *L. plantarum* A, *L. acidophilus* ATCC 4356 and *L. rhamnosus* ATCC 53103 increased expression of $CD11c^+$ DCs and Treg cells, whilst down-regulating pro-inflammatory cytokines (IL-17, IFN-gamma and TNF-alpha) and protection against colitis [124]. Hence, for individuals experiencing gut dysbiosis, probiotics and gut microbiome are therapeutic interventions for restoring gut microbial equilibrium and balancing the immune system for better health [125]. To this end, fecal microbiota transplantation has shown to be effective in colitis patients through rapid restoration of intestinal microbiota composition. The specific mechanisms are not clear, however, the intestinal microbiota is restored to be similar to that of the donor [126].

Bifidobacteria have also been shown to contribute to intestinal homeostasis and help to diminish inflammation. *Bifidobacteria* mixture was tested on DC functionality from different sources; children with IBD, DCs from PBMCs of patients with CD, UC, and healthy controls. DCs were pre-treated with probiotics and incubated with *E. coli* fluorochrome-conjugated particles or DQ-ovalbumin (DQ-OVA) [127]. Following incubation with probiotics, DCs from CD children had a higher uptake of bacterial particles and DQ-OVA processing; whereas DCs from the other 2 groups showed no significant changes. DCs from CD children also showed higher TNF-alpha and no effect on IFN-gamma and IL-17 [127]. This shows that *Bifidobacteria* can significantly increase antigen uptake and processing of DCs which are from patients with CD, in whome DCs demonstrate a decreased autophagic function. Probiotics had a lesser effect on antigen uptake or autophagy by DCs sourced from CD/UC and healthy people. Enhanced antigen sampling and processing could be a viable approach to solving innate immunity deficiency in GIT and a method to reducing uncontrolled bacterial growth in the intestine of IBD children.

Probiotics have also shown to be effective against specific infections in infants and children, including infectious diarrhoea, traveller's diarrhoea, and infants necrotizing enterocolitis and *Helicobacter pylori* [54]. Neonates with extremely low birth weight due to necrotizing enterocolitis have marked reduced incidence and severity following probiotics consumption, in particular combinations of, *B. infantis*, *S. thermophilus*, *B. bifidus* and *L. acidophilus* [27]. Caveolin-1 regulates nitric oxide (NO) signaling which is responsible for the pathogenesis of necrotizing enterocolitis, and in formula-fed neonatal rats, the intestinal caveolin-1 and NO signalling are deficient [27]. Probiotics fed to babies, improves the survival of the microorganism in the intestine and beneficially improves caveloin-1 and NO signalling and growth factors in the terminal ileum.

5.4. Probiotics and multiple sclerosis

Chronic inflammation has been generally accepted as a common hallmark of neuro-degenerative diseases, such as, MS, Alzheimer's disease and Parkinson's disease [76,128,129]. Gut microbiota associate with central nervous system (CNS) [130], as there is cross-talk between the GIT, the CNS and the immune system, known as the gut-brain axis. Any dysfunction in the gut-brain axis is associated with the pathogenesis of a number of diseases inside and outside the GIT.

EAE, an animal model of human MS has been used to study the effects of probiotic microorganisms on CNS. One of the safe and appropriate ways to induce tolerance towards peripheral T-cell in autoimmune diseases like MS, is the oral administration of specific autoantigens [131,132]. A food based probiotic yeast, *Candida utilis,* fused with an immunodominant myelin epitope (MOG_{35-55})-peptide as carrier, when administered in mice, protects mice against the development and clinical symptoms of EAE [131]. In addition, administration of heat-inactivated *C. utilis* also resulted in reduction of disease severity in animals which indicated that tolerance effect is independent of viability of the yeast. Rechallenge with MOG_{35-55} resulted in decreased disease severity associated with reduced cellular inflammation in the spinal cord, demyelination, lower T cell proliferation and higher Treg cells in the lymph nodes. These results showed that using a food-grade fungus, *C. utilis,* with surface-infused immunogenic MOG_{35-55} peptide is effective in modulating immune responses and can successfully stimulate oral tolerance against this epitope in EAE [131]. Similarly, oral and intranasal administration of recombinant *Lactobacilli* expressing myelin antigens also showed reduced EAE [132]. Furthermore, a panel of recombinant *Lactobacilli* were constructed to produce some of human and guinea pig myelin proteins and peptides, such as proteolipid protein peptide 139–151 ($PLP_{139-151}$) and myelin basic protein (MBP) and. The effect of these *Lactobacillus* recombinants on inducing intranasal and oral tolerance in EAE animal models were studied. Soluble cell extracts of *Lactobacillus* transformants were given to Lewis rats intranasally three times prior to induction of EAE. In order to induce oral tolerance, animals were given live recombinant *Lactobacilli* for 20 days. Within 10 days of first oral consumption, rats were induced for EAE induction. Using extracts containing guinea pig MBP_{72-85} peptide epitope intranasally inhibited EAE in Lewis rats significantly. It seems that *Lactobacilli* with myelin infused-antigens are able to reduce EAE if intranasally and orally administered [131,132]. This and other studies offer novel approaches for mucosal tolerance induction by mucosal administration of recombinant yeast or *Lactobacilli* expressing relevant autoantigens, which may be applicable in autoimmune disease such as MS.

In mice, administration of *Lactobacillus* or *Bifidobacterium* strains result in less severe EAE clinical scores, recovery of myelin content in the spinal cord, increased levels of TGF-beta and Treg cells compared to control mice [133]. Administration of a mixture of the two strains induced a more significant delay in EAE onset and clinical score, along with significant decreased mononuclear infiltration into the central nervous system, as well as elevated $CD4^+CD25^+Foxp3^+$ expressing Treg cells in the spleen and lymph nodes of mice [134]. Likewise, intraperitoneal injection of *L. helveticus* in mice significantly reduced the incidence and clinical score of EAE, which correlated with reduced Th17 cells and IL-6 (an essential cytokine for Th17 differentiation) [135]. *L. casei* Shirota, *L. casei* 393, *L. reuteri*, *L. paracasei*, *B. breve* and *B. animalis* also reduce disease activity by increasing IL-10, TGF-beta and Treg cells. [71].

Oral administration of probiotics (*Lactobacillus, Bifidobacterium* and *Steptococcus*) in 9 MS patients and 13 control subjects, twice a day for 8 weeks, resulted in decreased abundance of *Akkermansia* and *Blautia*, known to be associated with dysbiosis in MS patients, and increased abundance of *Lactobacillus* and *Bifidobacterium* [136]. In addition, decreased number of inflammatory monocytes, and decreased expression of MHC-II and co-stimulatory molecule CD80 were noted. In a randomized, placebo-controlled study, 60 patients with MS were also given a probiotic capsule containing *L. acidophilus, L. casei, B. bifidum* and *L. fermentum* for 12 weeks. Subsequently, expanded disability status scale, as well as depression and anxiety symptoms were improved, c-reactive protein (inflammatory marker), plasma oxidative metabolites and malondialdehyde (markers of oxidative stress) were reduced [137]. Thus, probiotics have been shown to modulate symptoms of disease in mice (EAE model) and in MS patients, and warrants further research

into the use of probiotics as an adjunct treatment in humans with MS, however, there is a need for further animal and human studies to understand the effects of probiotics to human health.

6. Conclusion

Probiotics have been shown to alter immune responses, and induce tolerance in both animal models and humans. The rapidly growing knowledge of microbiome-host interactions has revealed new avenues for understanding the immunopathological basis of many diseases. Evidence is slowly emerging, that one of the approaches for maintaining or restoring immune balance and, thereby preventing or treating diseases, is through the use of probiotics in the form of fermented foods or probiotic supplements. The mechanism by which probiotics affect individual's health is multidimensional and has been the focus of many studies. The prevailing evidence suggests that probiotics keep the immune system in check, by differentially modulating cellular (Th1, Th2, Th17, Treg, Tc) and humoral (B cells) immune responses specific to the probiotic strain(s) and disease pathophysiology. However, current knowledge still lacks most pieces of the puzzle for a complete picture that explains the mechanisms by which probiotics improve general health and diseases, specifically in regards to treating or preventing allergies, gastrointestinal and immune disorders. There seems to be a lack of adequate research on the effects of probiotics on the central nervous system, such as autoimmune diseases, specifically MS, and additional *in vivo* and *in vitro* research on MS is required to provide sound evidence and detailed information for the beneficial effects of probiotics.

Contributors

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Review

Multiple Sclerosis: Immunopathology and Treatment Update

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Abstract: The treatment of multiple sclerosis (MS) has changed over the last 20 years. All immunotherapeutic drugs target relapsing remitting MS (RRMS) and it still remains a medical challenge in MS to develop a treatment for progressive forms. The most common injectable disease-modifying therapies in RRMS include β-interferons 1a or 1b and glatiramer acetate. However, one of the major challenges of injectable disease-modifying therapies has been poor treatment adherence with approximately 50% of patients discontinuing the therapy within the first year. Herein, we go back to the basics to understand the immunopathophysiology of MS to gain insights in the development of new improved drug treatments. We present current disease-modifying therapies (interferons, glatiramer acetate, dimethyl fumarate, teriflunomide, fingolimod, mitoxantrone), humanized monoclonal antibodies (natalizumab, ofatumumab, ocrelizumab, alemtuzumab, daclizumab) and emerging immune modulating approaches (stem cells, DNA vaccines, nanoparticles, altered peptide ligands) for the treatment of MS.

Keywords: multiple sclerosis; immunotherapy; drug delivery; vaccine

1. Introduction

In the early 1900s, only a few cases of multiple sclerosis (MS) were reported, which quickly became a common occurrence for admission to neurological wards. Today, MS accounts over 2.5 million affected individuals with an estimated cost of US$2–3 billion per annum [1]. The distribution of MS varies according to geographic location. For example, the further north or south from the equator the higher the prevalence of MS; countries that lie on the equator have extremely low prevalence compared to Scotland, Norway, and Canada. The prevalence of MS has increased progressively over time with 30/100,000 diagnosed in 2008 to 33/100,000 diagnosed in 2013 globally. In fact, in a Norwegian cohort over 53 years (1961–2014), the prevalence increased from 20 to 203/100,000 and the incidence increased from 1.9 to 8/100,000 [2]. It is possible that the increase in prevalence is due to improved diagnostic procedures and reporting and changes in lifestyle (lack of vitamin D and increased smoking) [1]. MS is commonly diagnosed between 20 years and 40 years of age although it can affect younger and older individuals [3], and most commonly affects those with a genetic predisposition (major histocompatibility complex (MHC) class II phenotype, human leukocyte antigen (HLA)-DR2 and HLA-DR4 most commonly affected). In fact, the incidence of MS is increased 10-fold in monozygotic

twins as compared to siblings of patients with MS [4–6]. In addition, viral infections can trigger disease where parts of the virus mimics that of the myelin sheath [7]. Although usually not life-shortening, MS is a chronic neurological disease often interfering with life and career plans of an individual [8].

MS is categorized into 4 distinct types, primarily based on its clinical course, which are characterized by increasing severity: (a) Relapsing/remitting MS (RRMS), the most common form, affecting 85% of all MS patients which involves relapses followed by remission; (b) secondary progressive MS (SPMS), which develops over time following diagnosis of RRMS; (c) primary progressive MS (PPMS) affecting 8–10% of patients, noted as gradual continuous neurologic deterioration; and (d) progressive relapsing MS (PRMS) the least common form (<5%), which is similar to PPMS but with overlapping relapses [9–11]. MS leads to a wide range of symptoms with various severity involving different parts of the body. MS diagnosis is mainly clinically based however, magnetic resonance imaging (MRI) assists in diagnosis [12]. As such, examination of the cerebrospinal fluid (CSF) and visual induced potentials with MRI can assist in confirming the clinical suspicion of MS [12,13]. MS symptoms and disease progression are varied, with some individuals experiencing little disability while most (up to 60%) require a wheelchair 20 years from diagnosis [9].

Although treatments against MS are able to decrease the relapse rate in RRMS, the prevention of long-term effects remains a problem; medications for progressive forms of MS are also limited in their efficacy. Hence, new improved drugs are required to effectively treat MS. One of the major pathophysiological mechanisms of MS involves autoreactive T cells, primarily T helper (Th)-1 $CD4^+$ T cells and Th17 cells leading to cytokine secretion and activation of an inflammatory cascade resulting in demyelination within the brain and spinal cord and axonal damage; autoreactive antibodies cannot be discounted. Indeed, MS is generally known as a chronic autoimmune disorder of the central nervous system (CNS) [14,15]. MS causes breakdown of the blood brain barrier (BBB) leading to migration of immune cells (macrophages, T cells, B cells) and secretion of pro-inflammatory cytokines and chemokines [16] which induces inflammation, formation of sclerotic plaques (lesions), demyelination and neurodegeneration [17]. MS lesions may form in any location of the CNS white matter or in grey matter, often leading to physical disability and sometimes, decline in cognitive ability [16,18]. It is therefore, conceivable to target immune cells and their products in order to prevent tissue damage by modulating inflammation [9,19] while reducing potential side effects such as global immunosuppression [6,19,20]. The major constituents of the myelin sheath in which autoreactive T cells and antibodies recognize, include, myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) and proteolipid protein (PLP).

2. Immunopathophysiology of MS

The brain has primarily been considered to be an organ which is highly immune-advantaged, although a number of studies have challenged this [6]. In the last 10 years an important shift has surfaced in MS research, suggesting that MS is not just a disease of the immune system, but equally involves factors contributed by the CNS [21,22]. Immune cells residing in the CNS get activated following damage to CNS tissue; notably microglial cells whereby they upregulate MHC class I and II molecules and cell surface co-stimulatory molecules and secrete cytokines and chemokines, paving entry for T (CD4 and CD8) cells, B cells, monocytes, macrophages and dendritic (DC)-like cells into CNS lesions [6]. Infiltrating immune cells secrete pro-inflammatory cytokines, nitric oxide, and matrix metalloproteinases [23,24], leading to destruction of the myelin sheath.

It has been generally accepted that chronic inflammation is the hallmark of neurodegenerative diseases, such as MS, Alzheimer's disease and Parkinson's disease [6,7]. Myelin-reactive auto-T cells cross the BBB [19] and their migration into the CNS consequently initiates an inflammatory cascade followed by demyelination of the CNS and axonal damage. These cells reside in the perivenous demyelinating lesions which generate distinct inflammatory demyelinated plaques situated within the white matter [25]. MS lesions appear in the white matter inside the visual neuron, basal ganglia, brain stem and spinal cord [26]. White matter cells transmit neural signals from grey matter, where

information is gathered, and transferred to the rest of the body [25,27]. MS involves 2 main steps, (i) myelin sheath damage resulting in formation of lesions in the CNS and (ii) inflammation, which together destroy the neuron tissue [25,28]. In MS, damage of oligodendrocytes and destruction of myelin sheath leads to breakdown of the nerve axon and loss of neuronal function [28]. Demyelination increases the inflammatory activation processes leading to damage of BBB and stimulation of macrophage activation and oxidative stress pathways [29]. The white matter lesions include myelin breakdown together with infiltration of monocytes, B cells, T cells and DC [30]. Microglia and macrophages are the main innate immune cells present in MS lesions where they either act together with T and B cells, or directly cause neuroinflammatory tissue damage [31]. Cells involved in the inflammatory process include those that are both in the innate and adaptive immune systems and are described below (Figure 1).

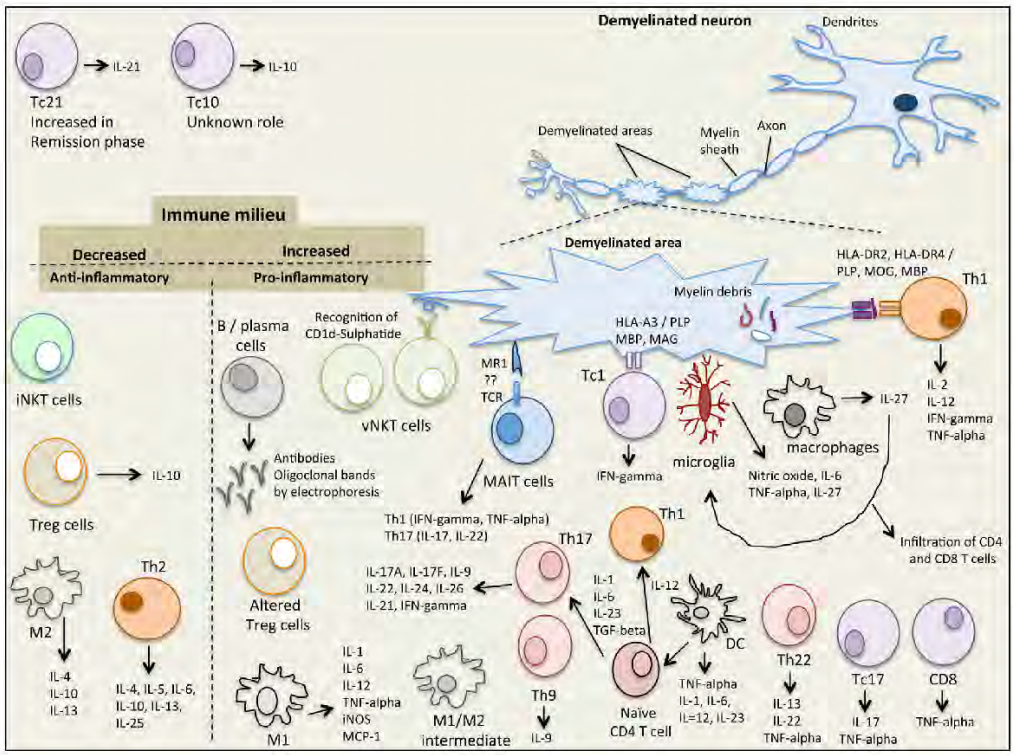

Figure 1. The immunological complexity of the immune/cytokine network in multiple sclerosis.

2.1. Natural Killer T (NKT) Cells

NKT cells share properties of both T cells and NK cells and recognize glycolipid antigens presented in complex with the MHC class I-like molecule, CD1d. Two subsets of NKT cells have been identified (type I, invariant NKT (iNKT) cells and type II, variant NKT (vNKT) cells) and are implicated in the pathogenesis of MS in humans and in the murine model of MS, experimental autoimmune encephalomyelitis (EME). iNKT cells express cell surface markers characteristic of activated or memory T cells (CD25, CD44, CD69) with the majority being $CD4^+$ as well as markers characteristic of NK cells (NK1.1 or CD161, Ly49). Following activation of iNKT cells (via binding to α-GalCer-CD1d complex) an array of cytokines is secreted that are associated with both pro- and anti-inflammatory immune responses and play a role in both innate and acquired immunity. As such, iNKT cells, (i) secrete interleukin (IL)-4 and IL-13 which stimulate $CD4^+$ T cells to differentiate into anti-inflammatory Th2 cells (IL-4, IL-10 producers) which inhibit Th17, Th1, $CD8^+$ T cells in the CNS; (ii) secrete IL-2

and tumor growth factor (TGF)-beta which stimulate the production of T regulatory (Treg) cells (IL-10, TGF-beta producers) which inhibit Th17, Th1 and $CD8^+$ T cells in the CNS; and (iii) secrete IL-4, IL-10, IL-13, interferon (IFN)-gamma and GM-CSF which activate suppressive myeloid derived suppressor cells (MDCs), DC and macrophages which in turn secrete IL-10 to activate Treg cells and suppress Th17, Th1 and $CD8^+$ T cells in the CNS [32]. Due to the pleiotropic properties of iNKT cells, they play a role in protecting the host against pathogens, tumors, autoimmunity and are involved in tissue rejection, ischemia reperfusion injury and obesity related diabetes [32]; deficiency or dysfunction of iNKT cells has been shown to be linked to the development of autoimmune diseases. Indeed, iNKT cell numbers are decreased in patients with MS [32] and are restored in patients in remission [33]. Analysis of iNKT cells in MS patients in remission showed a Th2 cytokine profile, suggesting an immunoregulatory effect of iNKT cells in MS [34]. Similarly, in the EAE mouse model, protection of EAE development is associated with high levels of iNKT cells and suppression of Th1 and Th17 cells [35]. Interestingly, injections of α-Galactosylceramide (α-GalCer), and analogues thereof, have potent activities in protecting mice against, cancer, infections, inflammatory conditions and autoimmune disorders. Hence, it is possible to develop iNKT cell based modulating therapies against MS [36,37]. Like iNKT cells, variant NKT (vNKT) cells also share properties of both T cells ($CD4^+$) and NK cells (NK1.1) and recognize β-linked glycolipid antigens in complex with CD1d. They are less common in mice compared to iNKT cells but are more abundant in humans. Of interest, vNKT cells recognize the self-glycolipid, sulphatide, which is abundantly expressed within the myelin sheath suggesting a role in MS although not yet established [38]. Likewise, vNKT cells recognizing sulphatide self-myelin ligand are present in high levels in mice with EAE suggesting their role in disease progression [38].

2.2. *Mucosal-Associated Invariant T (MAIT) Cells*

MAIT cells are a subset of T cells of the innate immune system to defend against microbial infections. They are present in the liver, lungs, mucosa and blood and make up to 25% of CD8 T cells in healthy individuals; they also support adaptive immune responses in that they have a memory like phenotype [39]. The MHC class I-like molecule, MRI, presents microbial antigens and vitamin B metabolites to MAIT cells, leading to their activation [39,40]. However, MAIT cells have also been implicated in autoimmune diseases such as MS, inflammatory bowel disease and rheumatoid arthritis where they are often noted at the site of autoimmune attack. Recently, it was reported that in MS, MAIT cells are highly present at the sites of demyelination and secrete pro-inflammatory Th1 cytokines (IFN-gamma and TNF-alpha) and activate Th17 cells (IL-17 and IL-22 cytokines) [22]; the major cytokines in the pathogenesis of chronic inflammatory and autoimmune diseases. In addition, MAIT cell have been noted in white matter inflammatory lesions [41] as well as transcription over expression of MR1 in MS lesions. Conversely, it has been reported that MAIT cells are decreased in blood of patients with RRMS [42]. It is not clear whether MAIT cells exert a protective or a non-protective role, thus a better understanding of how MAIT cells are involved in MS and of their interactions would aid in a better understanding of the pathogenesis of MS and development of therapeutic strategies.

2.3. *Regulatory T Cells (Tregs)*

Regulatory T cells (Tregs; originally known as suppressor T cells) are a subset of $CD4^+$ T cells that modulate immunity, maintain tolerance against self-antigens and prevent autoimmunity. Tregs are primarily characterized as $Foxp3^+CD25^+CD4^+$ and are anti-inflammatory (secrete IL-10). One of the first evidence of the role of Treg cells in MS was in mouse EAE models, where adoptive transfer of Treg cells from control mice into MOG or PLP induced EAE mice prevented the onset and progression of EAE [43,44]. Adoptive transfer of Treg cells recovering from EAE into MOG-induced active EAE mice resulted in resolution of EAE [45]. In addition, induction of Treg cells by estradiol or by monocytes under glatiramer acetate treatment reduced clinical signs of MOG-EAE [46,47]. Furthermore, injection

anti-CD28 monoclonal antibody in Lewis rats results in Treg cell expansion and reduction in EAE disease severity [48]. Interestingly, injection of anti-CD25 monoclonal antibody, which blocks the effects of Treg cells into C57BL/6 mice increased susceptibility to EAE induction [45]. In patients with MS however, the frequency of $Foxp3^+CD25^+CD4^+$ Treg cells does not differ to those in healthy individuals, although the function of such cells are impaired (maturation and migration) [49]. In addition, mRNA and protein levels of Foxp3 are impaired in Treg cells of patients with MS especially in RRMS and are normalized during SPMS [49]. Hence, impaired functionality of Treg cells is primarily observed in the early stages of MS but not in their chronic stage, suggesting a causative role [50]. Further studies of Treg cells in MS may aid in the understanding for why tolerance against self-antigens is broken, leading to disease. However, it is not clear whether the impaired function of Treg cells is a direct cause of MS or whether such impairment is a general outcome for all autoimmune disorders.

2.4. Macrophages and Microglia

Macrophages are divided into M1 or M2 based on their pro- or anti-inflammatory cytokine secretion phenotype [51]. M1 macrophage phenotype of mice ($F4/80^+CD11b^+CD11c^+iNOS^+$) and human ($CD40^+CD86^+CD64^+CD32^+$) is induced in the presence of interferon (IFN)-gamma and/or toll-like receptor (TLR) ligands such as lipopolysaccharide (LPS). M1 macrophages are pro-inflammatory and primarily secrete IL-1, IL-6, IL-12, TNF-alpha, iNOS and MCP-1 [51]. In general, they stimulate adaptive immune responses. The M2 macrophage phenotype of mice ($F4/80^+CD11c^-CD301^+Arg1^+CD206^+$) and humans ($CD163^+CD206^+$) is induced in the presence of IL-4, IL-10, IL-13 and Arg1 that blocks iNOS activity [51]. M2 macrophages are anti-inflammatory and primarily secrete IL-1 receptor antagonist, IL-4, IL-10, transforming growth factor (TGF)-beta1. Macrophages play a crucial role in the pathogenesis of MS. In fact, in active demyelinating and early re-myelinating lesions, macrophages are highly present compared to inactive, demyelinated or late re-myelinated lesions [52]. However, a distinction of M1 vs M2 macrophages in human brain tissues is not so clear, with both M1 macrophages and an intermediate subtype (M1/M2, $CD40^+CD206^+$) being present [53]. Like macrophages, microglia cells are divided into M1- and M2-polarized microglia cells. M1 microglia cells are pro-inflammatory and express CD40, CD74, CD86 and CCR7, whereas, M2 microglia cells are anti-inflammatory and express mannose receptor (CD206) and CCL22. In MS brain lesions however, like macrophages, an intermediate microglia phenotype is present expressing CD40, CD74, CD86 and CCL22 but not CD206 markers [54]. Interestingly, in an EAE model it was shown that suppression of CCL22 decreased M1 macrophage accumulation in the CNS, thus therapies designed to suppress CCL22 have the potential to decrease demyelination and progression of disease. In addition, in mice M1 microglia cells have been found to switch to M2 microglia cells during remyelination, hence M2 polarization is necessary for efficient remyelination [55]. Indeed, fasudil (a selective Rho kinase inhibitor), injected into EAE bearing mice shifted M1 to M2 macrophages and ameliorated the clinical severity of EAE [56].

2.5. T Helper Cells

CD4 T cells or T helper (Th) cells, recognize short 9–17 amino acid peptides presented on the surface of antigen presenting cells (APC) in complex with MHC class II. CD4 T cells differentiate into distinct Th cells depending on the cytokine secretion profiles [57]. (i) Th1 cells are pro-inflammatory and produce high levels of IL-2, IL-12, TNF-alpha and IFN-gamma; (ii) Th2 cells are anti-inflammatory and secrete IL-4, IL-5, IL-6, IL-10, IL-13, IL-25; (iii) Th17 cells are pro-inflammatory and secrete high levels of IL-17A, IL-17F, IL-21, IL-22, IL-24, IL-26 and low levels of IL-9 and IFN-gamma; (iv) Th22 cells which are a combination of Th1, Th2, Th17 phenotype and secrete IL-13, IL-22 and TNF-alpha and (v) the newest addition to the Th subset, Th9, was identified for its potent secretion of IL-9. Th1, Th9, Th17 cells are key contributors to MS by increasing inflammation within the milieu of the myelin site.

Th1 cells and their pro-inflammatory cytokine products are present in high levels within the demyelinating axon and CNS lesions of humans and in MOG, PLP or MBP induced EAE in mice.

Th1 cells recognize MOG, PLP and MBP peptide epitopes presented in the context of MHC class II, HLA-DRB1*1501 (HLA-DR2, HLA-DR15) and HLA-DRB1*04 (HLA-DR4) alleles. As a result CD4 T cells become activated, cross the blood brain barrier and induce CNS autoimmunity. Some drug therapeutics target the MHC class II-peptide-T cell receptor (TCR) complex in an attempt to modulate or divert Th1 responses to therapeutic Th2 responses. Indeed, it was recently shown that dimethyl fumarate (DMF) injection in RRMS patients reduced Th1, Th17 and CD8 T cells and increased Th2 cells; this resulted in high levels of IL-4 and decreased levels of IFN-gamma and IL-17 [58]. In addition, we have shown that mannan conjugation of self-MBP, PLP or MOG native peptides or altered peptide ligands, are able to divert Th1 responses to Th2 responses in human PBMC from MS patients, in immunized mouse spleen cells and are able to ameliorate EAE in mice [59–73]. The role of Th9 cells in MS is not as clear although in mice, IL-9 and Th9 cells induce EAE and inflammation and IL-9 knockout mice are protected from developing EAE [74]. Th17 cells play a crucial role in the pathogenesis of MS in both mice and humans by inducing an inflammatory milieu. In fact, IL-17A is present at high levels in CNS lesions, cerebrospinal fluid and in the serum of patients with MS [75]. Th17 cells express high levels of CCR6 which binds to the ligand CCL20 on vascular endothelial cells, enabling their entry through the blood brain barrier where they secrete pro-inflammatory cytokines including IL-17A. In addition, IL-17 interferes with the remyelination process. Of interest, anti-IL-17A humanized neutralizing monoclonal antibody (AIN457 or Secukinumab) injected in patients with MS showed reduction of lesions compared to placebo-treated control subjects [75]. In addition, Th22 cells are highly present in the peripheral blood and cerebral spinal fluid of patients with active RRMS [76], and IL-22 mRNA and Th22 cells are increased in relapsing MS compared to remitting MS patients [77]. Furthermore, Th22 cells specifically recognize MBP and are resistant to IFN-beta therapy [76].

IL-27, a member of the IL-6/IL-12 cytokine family, is secreted by macrophages, dendritic cells and microglia cells, with pleiotropic roles in immunomodulation being either pro- or anti-inflammatory. IL-27 also stimulates or inhibits T cell differentiation. Th1 cells are induced by IL-27 whereas Th2, Th17 and Treg cells are inhibited by IL-27. In addition, Tr1 cells a specialized subset of T cells which secrete IL-10 are induced in the presence of IL-27 [78]. In 40 patients with RRMS, circulating plasma IL-27 levels were significantly higher compared to healthy control subjects [79]. Likewise, IL-27 and IL-27R are elevated in post-mortem MS brain lesions compared to non-MS control brains. Macrophages and microglia were identified to be the source of IL-27 and triggering infiltration of CD4 and CD8 T cells [80]. In addition, the effects of IL-27 on microglia cells showed that nitric oxide, TNF-alpha and IL-6 were secreted, promoting Th1 polarization, suggestive that IL-27 enhances microglia neuroinflammation [81]. Hence, suppressing IL-27 may be a strategy to modulate inflammatory responses in patients with MS.

2.6. CD8 T Cells

Classical CD8 T cells or cytotoxic T cells (Tc1 cells), recognize short antigenic 7-9-mer peptide epitopes presented on the surface of APC in complex with MHC class I. In MS there is a genetic association with HLA-A3 [82]; HLA-A2 has been shown to reduce the risk of MS in individuals that also express MHC class II, HLA-DRB1*1501. The antigen specificity of CD8 Tc1 cells isolated from patients with MS, has been suggested to be against MOG, MBP and PLP with cytolytic activity against neuronal cells in vitro [83] although their pathogenic role in MS is still not clear. More recently other subsets of CD8 T cells have been identified and are grouped into different subsets based on their cytokine profile. In as such, classical Tc1 cells secrete IFN-gamma, Tc2 secrete IL-4, Tc10 secrete IL-10, Tc17 secrete IL-17, Tc21 secrete IL-21, Tc22 secrete IL-22 and another subset is characterized by secreting TNF-alpha. In MS, regardless of the stage and activity of disease CD8 T cells are noted in high numbers, much higher than CD4 T cells at a ratio of 10:1 CD8:CD4 T cells. MHC class I is highly expressed within MS lesions and astrocytes, oligodendrocytes, neurons in addition to the classical APC, DCs and macrophages. In fact, CD8 T cells are found in great abundance within CNS tissues and cerebrospinal fluid of patients with MS. CD8 T cells present in both acute and chronic

MS lesions secrete high levels of IL-17 (classed as, Tc17 CD8 T cells) [84]. Tc17 cells secrete IL-17 and TNF-alpha and low IFN-gamma and are negative for granzyme B, perforin and cytolytic activity unlike the classical CD8 Tc1 cells. In peripheral blood of patients with SPMS and RRMS elevated levels of Tc1 and Tc17 cells are noted as well as a high percentage of TNF-alpha secreting CD8 T cells [85]; Tc21 cells are increased in the remission phase of RRMS compared to SPMS. In addition, higher levels of $CD8^{+}$IFN-$gamma^{+}$TNF-$alpha^{+}$$IL\text{-}17^{+}$ T cells in the relapsing phase of RRMS compared to remission phase, SPMS and controls [85]. It is clear that CD8 T cells contribute to the pathogenesis of MS, and it is important to understand how such cells escape T cell tolerance and induce CNS autoimmunity in order to design and develop new therapeutics against MS.

2.7. B Cells

Although there is a presence of T cells in MS plaques, B cells also contribute to the pathogenesis of MS where they secrete autoantibodies and cytokines and being APC they activate T cells. In patients with MS the presence of oligoclonal bands (OCB) in cerebrospinal fluid and brain parenchyma is a consistent finding in over 95% of patients. OCB is a product of clonally expanded B cells and IgG synthesis. In MS plaques plasma cells are noted in large numbers where antigen uptake, processing and presentation takes place as well as synthesis of IgG. Interestingly, over 50 antibodies isolated from cerebrospinal fluid from patients with MS did not react to MBP, PLP or MOG [86] but some groups reporting that they bind to intracellular proteins such as, MKNK1/2, FAM84A, AKAP12A and glial potassium channel KIR4.1, or, against intracellular lipid determinants [87,88]. Moreover, anti-MOG autoantibodies is a hallmark of childhood MS as well as in some patients with neuromyelitis optical spectrum disorder. It is clear, that abnormal activation of B cells within the CNS of patients with MS, suggests that B cells play a role in the pathophysiology of the disease. Further studies are required to ascertain whether B cell depletion is able to restore immune function and hence, be used as a therapeutic target against MS.

2.8. Dendritic Cells

DC are professional APC which process and present antigenic peptide epitopes on their surface in complex with MHC class I or class II, resulting in CD4 or CD8 T cell stimulation respectively. Even though MS is generally associated with predominant auto-reactive T cells, emerging evidence indicates that DCs play an important role in the pathophysiology of MS, primarily due to their T cell activating and cytokine secreting properties. Following activation of DCs in the periphery, T cells specific to myelin epitopes are activated inducing pro-inflammatory cytokines aiding their entry through the BBB into the CNS. In the CNS resident APC and T cells are further activated leading to demyelination and motor deficits. In patients with MS, DCs are abundantly present within inflamed lesions, cerebrospinal fluid and in the circulation and produce high levels of TNF-alpha, IFN-gamma and IL-6 [89]. In addition, the expression of co-stimulatory molecules, CD40 and CD80 on DCs are increased in RRMS and SPMS patients, suggesting an activated pro-inflammatory state of DCs, hence their contributing role in the pathogenesis of MS.

2.9. Myeloid Derived Suppressor Cells

Myeloid-derived suppressor cells (MDSC) are myeloid progenitors, the same lineage to that of macrophages, DC and neutrophils. However, MDSC have strong immunosuppressive properties rather than immune-stimulatory properties as noted with macrophages, DC and neutrophils [90]. Their major role is in tumor development and chronic inflammation having immune suppressive effects [90]. As such, it was recently shown following MBP_{1-11} peptide immunization in mice, that MDSCs were increased adopting a suppressive phenotype, inhibiting the activation of $CD4^{+}$ T cells via arginase-1 and inducible nitric oxide synthase; such approach inhibited the development of EAE in mice [91]. In addition, MDSC secrete inhibitory enzyme indoleamine 2,3-dioxygenase and Th2 cytokine, IL-10 [92]. It is not clear whether the number of MDSCs are reduced or whether their functionality is altered

in patients with MS, leading to the failure of MDSCs to suppress autoimmune T cells, as a result of disease progression. The use of ex vivo cultured MDSCs could be a viable strategy to develop new improved treatments against MS.

3. Current Drug Therapies for Multiple Sclerosis

The majority of the treatments for MS are long term mainly suppressing the immune system however, such immune-suppressants pose increased risks for infections and cancer [27]. Alternative treatment options involve disease-modifying therapies such as, interferons, glatiramer acetate, monoclonal antibodies and sphingosine-1-phosphate receptor modulators (Table 1, Figure 2). These therapies have dramatically reduced the number of attacks and decreased disease progression. In fact, interferons are effective in the early relapsing phases of MS but not in the advanced phases of the disease [27]. Ultimately, induction of tolerance against self-antigens and re-establishing immune homeostasis can effectively "cure" the disease; such strategies have been the focus of recent research.

Table 1. Disease-modifying drugs available to patients with RRMS.

Drug	Brand	Dose	Number of of Injections, Route	Actions
IFN-β1a	Avonex®	7.5 mg 1st dose 15 mg 2nd dose 22.5 mg 3rd dose 30 mg all subsequent doses	1/week, i.m	Balances pro- and anti-inflammatory cytokines Decreases Th17 cells Decreases IL-17
	Rebif®	22 mg or 44 mg	3/week, s.c	
IFN-β1b	Betaseron®	62.5 mg and increase over 6 weeks to 250 mg	1/2 days, s.c	
	Extavia®	62.5 mg and increase over 6 weeks to 250 mg	1/2 days, s.c	
pegIFN-β1a	Plegridy®	63 mg 1st dose 95 mg 2nd dose 125 mg all subsequent doses	1/2 weeks, s.c	
Glatiramer acetate, EKAY	Copaxone®	20 mg or 40 mg	1/day, s.c 3/week, s.c	Blocks pMHC
Dimethyl fumarate	Tecfidera®	240 mg	2–3/day, oral	Anti-inflammatory Anti-oxidative stress
Teriflunomide	Aubagio®	7 or 14 mg	1/day, oral	Inhibits dihydroorotate dehydrogenase, T, B cells and IFN-γ secreting T cells
Fingolimod	Glenya®	0.5 mg	1/day, oral	Antagonist of SIP receptor Decrease T, B cells activates SIP signaling in CNS
Mitoxantrone	Novatrone®	12 mg/m^2	1/3 months up to 2 years	Suppresses T, B cells and macrophages. Reduces Th1 cytokines
Dalfampridine	Ampyra®	10 mg	2/day, oral	Potassium channel blocker Improves motor symptoms, i.e., walking
Humanized Monoclonal Antibody Treatments				
Natalizumab	Tysabr®	300 mg	1/28 days, i.v	Humanized anti-α4-integrin Mab. Affects cell migration, division, growth and survival
Ofatumumab	Arzerra®	3–700 mg	1/2 weeks, i.v	Humanized anti-CD20 Mab. Cytotoxic to CD20+ cells via CDC and ADCC
Ocrelizumab	Ocrevus®	300–600 mg	300 mg weeks 1 and 3, then 600 mg 1/6 months, i.v	Humanized anti-CD20 Mab
Alemtuzumab	Lemtrada®	12 mg	5 days in a row; after 1 year, 3 days	Humanized anti-CD52 Mab. Depletes T, B cells, increases Treg, Th2, decrease Th1 cells
Daclizumab	Zinbryta®	150 mg	1/month, s.c	Humanized anti-CD25 Mab.Blocks IL-2R, decreases T cells, increases NK cells

ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity; DC, dendritic cells; EKAY, single amino acid code for L-glutamic acid, lysine, alanine, tyrosine; IFN, interferon; IL-2R, interleukin-2 receptor; i.m, intramuscular; i.v, intravenous; Mab, monoclonal antibodies; NK, natural killer cells; pegIFN, polyethylene glycol linked to IFN; pMHC, peptide-major histocompatibility complex; RRMS, relapsing remitting multiple sclerosis; s.c, subcutaneous; SIP, sphingosine-1-phosphate; Th, helper T cells; Treg, regulatory T cells ($CD4^+CD25^+FoxP3^+$).

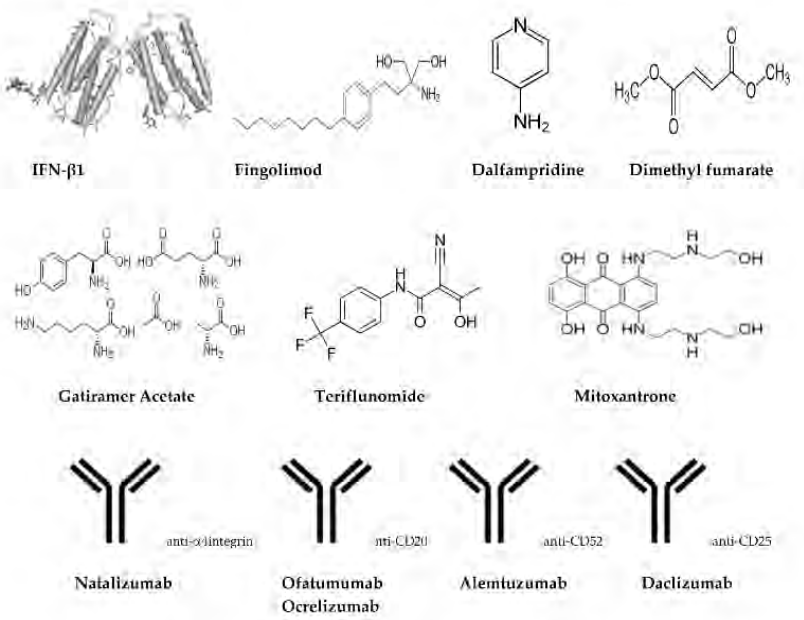

Figure 2. Chemical/schematic structures of treatments/drugs for MS.

3.1. Treatment of MS Relapses

Patients with MS who present with a relapse are generally treated with corticosteroids intravenously, plasma exchange or adrenocorticotropic hormone injections [50,93]. Although effective in reducing the duration of the relapse and patients recovery faster there are no long-term neuroprotective benefits [27,94–97].

3.2. Long-Term Treatment of MS with Disease-Modifying Agents

The treatment of MS has been a challenge with treatment options being limited mainly to corticosteroids, the potent alkylating agent cyclophosphamide and potent immunosuppressant methotrexate (Table 1, Figure 2). However, with the advent of immunomodulatory drugs in mid-1990s, a big shift was carried to treatment options for the first time [50]. The first disease-modifying drug for RRMS, interferon beta-1(IFNβ-1) was the primary key breakthrough for the treatment of MS [98,99]. Disease-modifying agents intend to modify the course of the disease rather than improving symptoms.

Until the approval of the first oral treatment in 2010 [11], all MS treatments consisted of either intramuscular or subcutaneous injectable drugs. To date, 13 FDA approved disease-modifying drugs are available for RRMS, and several more agents are in different developmental stages [9,11,65,66,69]. In the last 20 years there has been an evolving trend in novel treatments for MS and the global progress of therapies for MS has been quite promising. In general treatments consist of Ampyra®, Aubagio®, Avonex®, Betaseron®, Copaxone®, Extavia®, Gilenya®, Lemtrada®, Novantrone®, Plegridy®, Rebif®, Tecfidera® and Tysabri® [100]. Such treatment options consist of alemtuzumab (depletes lymphocytes), daclizumab (blocks the cytokine receptor IL-2), dimethylfumarate (combines features of immunomodulatory and immunosuppressive actions), fingolimod (modulates the sphingosine-receptor system), natalizumab (inhibits the migration of lymphocytes) and teriflunomide (inhibits activated T and B cells) [9,27,50]. Examples of current interferons include, Schering AG's Betaferon/Betaseron (IFNβ-1b), Biogen's Avonex (IFNβ-1a) and Serono/Pfizer's Rebif (IFNβ-1a). In addition, immune modulating agents include, Teva's Copaxone® (copolymer glatiramer acetate), Amgen/Serono's (Novantrone®; mitoxantrone), azathioprine, cyclophosphamide (Endoxan®) and Natalizumab® an a_4-integrin antagonist [101–103]. Disease-modifying agents have commonly been shown to reduce the rate of relapses, reduce MRI lesions and stabilize or delay MS disability.

The key therapeutic features of disease-modifying drugs are their anti-inflammatory effects in the relapsing phase of MS, although demyelination leading to chronic disability still remains a major hurdle [27,104–106]. Some studies, however, have shown that early intervention of disease-modifying drugs to patients with RRMS can reduce acute disability or death [27,107–110].

In general, disease-modifying drugs main action is by suppressing or altering the immune system. Hence, based on this theory that MS is, at least in part, a result of altered or abnormal immune response that results in attack of the myelin sheath. Current available drugs and their actions are described below (Table 1, Figure 2).

3.2.1. Interferons (Avonex®, Biogen, Cambridge, MA, USA; Betaseron®, Bayer, Leverkusen, Germany; Extavia®, Novartis Pharma AG, Basel, Switzerland; Rebif®, EMD Serono Inc., Darmstadt, Germany; Plegridy®, Biogen, Cambridge, MA, USA)

Interferon (IFN) type 1 consist of a group of IFNs (IFN-α, -β, -ε, -κ, -τ, -δ, -ζ, -ω, -ν) which help regulate the immune system. IFN-β is primarily produced by fibroblasts but other cells such as NK cells, B cells, T cells, macrophages also secrete IFN-β. IFN-β has anti-viral and anti-tumor activity as well as being effective in reducing the relapse rate in patients with MS [106]. The mechanism by which IFN-β acts, is that it balances the expression of pro- and anti-inflammatory cytokines in the brain and decreases the number of inflammatory cells crossing the blood brain barrier. As a consequence, there is decreased inflammation of neurons, increases nerve growth factors and improves neuronal survival. Moreover, IFN-β reduces Th17 population and IL-17 cytokine which are known to be involved in the immunopathophysiology of MS [111]. IFN-β injection subcutaneously or intramuscularly to patients with RRMS aims to decrease the relapse rate, duration and severity, however, there is lack of efficacy to long-term disability. Avonex was approved in 1996, the first FDA approved treatment for RRMS. To date there are 3 approaches using IFN-β; IFN-β1a low dosage (Avonex®), IFN-β1a (Rebif®) high dosage, and, IFN-β1b (Betaseron®, Extavia®) high dosage. Furthermore, pegIFN-β-1a (Plegridy®) has polyethylene glycol linked to IFN-β-1a allowing it to be active for longer in the body, hence fewer injections are required compared to Avonex®, Rebif®, Betaseron® and Extavia®. The first large scale human clinical trial in patients with RRMS using IFN-β was published in 1993 and showed that relapse rates were reduced by 34% in high dose IFN-β1b and by 8% in lower dose compared to placebo group and severity of relapses were also reduced [112]. Subsequent 5 year follow-up data showed that IFN-β1a and IFN-β1b decreased lesions up to 30% and reduced the formation of new lesions up to 50%, however, the study failed to show any reduction in disability progression in patients [113]. IFNs have no direct neuroprotective effects, however, through their direct effect on $CD4^+$Th1 cells and altering their profile results in decreased demyelination of neurons, which prevents further neuronal damage [114]. Despite the impact of IFN-β in disease progression in patients with RRMS there are limitations in their use, with side effects ranging from local body aches, skin reactions (swelling, redness), fever, myalgia, flu-like symptoms to more serious side effects such as suicidal thoughts, hallucinations, seizures and heart and liver problems [9]. As a result, many patients have stopped treatment and overall the benefit of using IFNs is relatively small.

3.2.2. Glatiramer Acetate (Copaxone®, Inc., Petah Tikva, Israel)

Glatiramer acetate (GA) is a synthetic 4-mer peptide (L-glutamic acid, lysine, alanine, and tyrosine) mimic of MBP, which competes with short antigenic MBP peptides in complex with MHC class II. Initially, GA was designed to induce EAE but instead it suppressed EAE, which was quickly translated into human trials with MS in order to prevent disease progression, as it bound to MHC class II and inhibited the activation of encephalitogenic T cells [115–118]. GA diverts Th1 cells to Th2 cells that suppress inflammatory responses and activate Tregs in the periphery [119]. In patients, GA significantly reduced disease symptoms and development of new lesions by up to 30% in RRMS, although it showed no improvement in long-term efficacy on progression of disability [120]. GA injection in patients

results in side effects ranging from minor (fever, chills) to more serious (cardiovascular, digestive, muscular, respiratory issues).

3.2.3. Dimethyl Fumarate (Tecfidera®, Biogen, Cambridge, MA, USA)

Dimethyl fumarate (BG-12) is a methyl ester of fumaric acid that modulates immune responses and was approved by the FDA in 2013. BG-12 was shown in phase III clinical trials to reduce relapse rate and increase the time to disability progression in patients with RRMS [121]. BG-12 reduces the migration of inflammatory cells through the blood brain barrier and activates nuclear factor erythroid 2-related factor (Nrf2) [122]. Nrf2 regulates anti-oxidative proteins that protect cells against oxidative damage and inflammation. In fact, BG-12 protects neuronal cells from oxidative stress by increasing glutathione levels and suppressing pro-inflammatory cytokines from splenocytes in vitro [123]. Side effects of BG-12 include diarrhea, abdominal pain, nausea, abnormal liver enzymes and decreased lymphocyte counts.

3.2.4. Teriflunomide (Aubagio®, Sanofi Genzyme, Cambridge, MA, USA)

Teriflunomide is an active metabolite of leflunomide (an immunosuppressive disease-modifying drug used for rheumatoid arthritis) which inhibits the enzyme dihydroorotate dehydrogenase [124] and inhibits the proliferation of B and T cells. In addition, teriflunomide exerts anti-inflammatory properties by inhibiting IFN-gamma producing T cells while IL-4 and IL-10 producing T cells are unaffected [125]. In MS, oral administration of teriflunomide reduced relapse rates, MS lesions and decreased disability progression [126–131]. Moreover, permanent discontinuation due to side effects was substantially less common in MS patients who received teriflunomide compared to IFN-β-1a. Side effects include, reduced white blood cell count, alopecia, hepatic effects, nausea, diarrhea, numbness in hand and feet, allergic reactions, breathing issues and increased blood pressure. Teriflunomide was approved by the FDA in 2012 and by EMA in 2013 for use in patients with RRMS.

3.2.5. Fingolimod (Gilenya®, FTY720, Novartis Pharma AG, Basel, Switzerland)

Fingolimod was granted FDA approval in 2010 and was the first oral therapy (0.5 mg once daily) available for patients with relapsing forms of MS. Fingolimod is a sphingosine 1-phosphate (S1P) receptor modulator, which acts as a super agonist of S1P receptor causing receptor internalization and leading to reduced infiltration of potentially auto-reactive lymphocytes into the CNS, and as such, they remain localized in the lymph nodes [132–134]. In addition, a secondary beneficial effects of fingolimod is that it targets SIP receptors on glia cells in the CNS, activating signaling pathways within the CNS [132,135]. Based on Phase III human clinical trials in patients with RRMS (TRANSFORMS, FREEDOMS and FREEDOMS II), fingolimod was more effective compared to first line treatment IFNβ-1a and placebo, in reducing the frequency of flare-ups (clinical exacerbations), disability progression, MRI outcome measures, including brain volume loss and was associated with clearly identified adverse events [103,136,137]. More than 180,000 patients have been treated with fingolimod in clinical trials and post-marketing settings globally, and the total patient exposure now exceeds 395,000 patient-years. Side effects include bradycardia (within 6 h after treatment initiation), blurred vision, diarrhea, back pain, headache, cough and vomiting. With reasonable data showing its long-term safety and disease improvement, fingolimod is a great alternative choice for patients with highly active RRMS and who prefer the oral treatment option.

3.2.6. Mitoxantrone (Novantrone®, Immunex/Amgen, Thousand Oaks, CA, USA)

Mitoxantrone is primarily used to treat certain types of cancers, in particular, non-Hodgkin's lymphoma, acute myeloid leukemia, breast and prostate cancer. Mitoxantrone is a type-II topoisomerase inhibitor, which disrupts DNA synthesis and DNA repair of cancer cells, however, normal cells are also affected. It is a potent immune suppressant, suppressing T cells, B cells and

macrophages and reduces pro-inflammatory cytokines (IFN-γ, TNF-α, and IL-2) [138,139]. In patients with SPMS, intravenous injection of 12 mg/m^2 mitoxantrone every 3 months up to 2 years resulted in reduced disability progression by 84% [140,141]. However, several side effects are associated with mitoxantrone which range from nausea, vomiting, hair loss, to, cardiotoxicity, leukemia, infertility, infection, leukopenia and thrombocytopenia [11]. As a result, its use has significantly been reduced over time. Furthermore, due to the risk of cardiotoxicity and leukemia, there is a limit on the cumulative lifetime dose to be administered to patients [11,142].

3.3. Treatment Using Humanized Monoclonal Antibodies

3.3.1. Natalizumab (Tysabr®, Biogen, Cambridge, MA, USA)

Natalizumab is a humanized monoclonal antibody against the cellular adhesion molecule α4- integrin. Integrins are transmembrane receptors that enable cell-extracellular matrix adhesion activating cell signaling which regulate cell growth, division, survival, differentiation and migration. Integrins are expressed on T cells, B cells, monocytes, macrophages, NK cells, DC, neutrophils and eosinophils. Interfering or blocking α4-integrin affects immune cell migration across the blood brain barrier, thus, by blocking the interaction between α4-integrin and vascular endothelial adhesion molecule-1, inhibits transendothelial migration to the CNS [143]. Natalizumab is administered intravenously once a month [144] which reduces activated T cells within the CNS, resulting in anti-inflammatory responses and hence, neuroprotective effects [114]. In a phase III clinical trial natalizumab reduced brain lesions and the rate of disability progression up to 24 months [12,145]. In addition, natalizumab decreased by 92% of contrast-enhancing lesions, by 83% of new or expanding T2-weighted lesions, and by 76% in new T1-weighted hypointense lesions [146,147]. Natalizumab, was approved by the FDA in 2004, but was withdrawn due to 3 cases of rare brain infection, progressive multifocal leukoencephalopathy (PML; that usually leads to death or severe disability), but was re-introduced in 2006 under a special prescription program. However, by 2012 a further 212 cases (or 2.1/1000) of PML were reported to be attributed to natalizumab [148]. Despite these reports the FDA has not withdrawn natalizumab from the market as the clinical benefits outweigh the risks involved. Other side effects include, hepatotoxicity, allergic reactions and increased risks of infection. Due to the risks involved with natalizumab, there are reservations over its use as a preferred treatment option.

3.3.2. Ofatumumab (Arzerra®, Novartis Pharma AG, Basel, Switzerland)

Ofatumumab (OMB157) is the first fully human type 1 IgG1 kappa (IgG1κ) monoclonal antibody and is currently licensed for the treatment (of patients with chronic lymphocytic leukemia (intravenously (iv), Arzerra®). It has also been shown to be beneficial to patients with rheumatoid arthritis, follicular non-Hodgkin's lymphoma, diffuse B cell lymphoma and MS. B cells play a role in the pathogenesis of MS. B cells have essential functions in regulating immune response, by activating CD4$^+$ T-cells and regulating T-cell responses via the secretion of cytokines and antibodies. B cells are present at demyelinating areas and in cerebrospinal fluid of patients with MS [149]. CD20 is a marker and present on the cell surface of all B cells. In an attempt to reduce the number of B cells including autoreactive B cells, the use of anti-CD20 antibodies would conceivably improve MS relapses and progression. In fact, there are several humanized anti-CD20 antibodies, such as rituximab [150], ocrelizumab [151] and ofatumumab [152], which have shown high efficacy in patients with RRMS. In 2015, Novartis acquired the rights from GlaxoSmithKline for the development of ofatumumab in oncology and other autoimmune indications. Ofatumumab binds to 2 unique novel epitopes on the CD20 molecule, induces B-cell depletion via complement dependent cytotoxicity and antibody-dependent cell-mediated cytotoxicity causing B cell apoptosis [153]. Ofatumumab has demonstrated high efficacy in hematologic malignancies and in rheumatoid arthritis. Based on 2 Phase II dosing human clinical studies, ofatumumab demonstrated high efficacy in reducing new MRI lesion activity more than 90% and was well tolerated in patients with MS [152]. Currently, ofatumumab is

being further investigated in 2 Phase III trials (ASCLEPIOS I AND ASCLEPIOS II) and are recruiting patients with relapsing forms of MS (ofatumumab versus teriflunomide). The adaptive study design of both trials was recently presented by Hauser SL and colleagues at the American Academy of Neurology April 2017 in Boston, USA and results are highly anticipated [154].

3.3.3. Ocrelizumab (Ocrevus®, Genentech Inc., San Fransisco, CA, USA)

A few months ago (March 2017), the FDA approved ocrelizumab to be used in PPMS, the first drug approved by the FDA for this form of MS and phase IV clinical trials were a requirement of the FDA to be conducted in order to determine the safety of ocrelizumab in younger patients with MS, ie, risk of cancer and effects on pregnancy (study outcomes due by 2024); although clinical trials in patients with lupus and rheumatoid arthritis were halted due to high rates of infections and increased risk of progressive multifocal leukoencephalopathy [155]. In addition, in patients with MS, there was an increased risk of breast cancer (6/781 females with MS on ocrelizumab compared to 0/668 females with MS in other trials) [155].

3.3.4. Alemtuzumab (Lemtrada®, Sanofi Genzyme, Cambridge, MA, USA)

Alemtuzumab is a humanized monoclonal antibody against CD52, a cell surface molecule expressed on B and T cells; mature NK cells, plasma cells, neutrophils and importantly, hematological stem cells do not express CD52. In phase III clinical trials in patients with RRMS, alemtuzumab showed significantly lower annualized relapse rates and MRI measures (gadolinium-enhancing lesions, new or enlarging T2 lesions and brain atrophy) and were free of clinical disease longer, compared to IFNβ-1a [156,157]. Alemtuzumab can cause serious side effects including, immune thrombocytopenia, kidney problems, serious infusion problems (trouble breathing, swelling, chest pain, irregular heart beat), certain cancers (blood cancers, thyroid cancer), cytopenia and serious infections. It was approved by the FDA in 2014 to be used in RRMS patients, but due to the frequent and significant adverse events of alemtuzumab, it is generally used in patients with RRMS who have used 2 or more MS drugs and have failed to work.

3.3.5. Daclizumab (Zinbryta®, Biogen, Cambridge, MA, USA)

Daclizumab is a humanized monoclonal antibody against CD25, the IL-2 receptor expressed on the surface of T cells. The mechanism by which daclizumab works is that it blocks the IL-2 receptor on T cells, preventing the activation of T cells. It was originally approved by the FDA in 1997 to prevent acute kidney transplants (together with corticosteroids and cyclosporine) however its use was halted due to low market demand. In recent years its use has re-emerged to treat patients with RRMS, it is injected subcutaneously, once a month [158]. In human clinical trials, daclizumab showed 45% reduced annualized relapse rates and 54% lower in the number of new lesions [158]. The side effects associated with daclizumab are relatively minor compared to other MS drugs, and include infections, skin rashes and liver complications.

4. New and Emerging Immunotherapeutic Strategies against MS

Antigen/peptide specific immunotherapy or using immune cells (i.e., stems cells), aim to restore tolerance while avoiding the use of non-specific immunosuppressive drugs as describe in Section 3, is a promising approach to fight autoimmune diseases including MS. As such, a number of approaches have been utilized.

4.1. Stem Cells

Multipotent hematopoietic stem cells (HSC) are cells isolated either from the bone marrow, umbilical cord blood or peripheral blood and are transplanted into the recipient. More commonly used for hematological malignancies (leukemia, multiple myeloma) its application has also expanded into

autoimmune diseases. The first report of a bone marrow transplant in 1997 in a chronic myelogenous leukemia patient with MS which showed marked improvements in MS brain lesions [159] quickly led to the use of HSC transplantation (HSCT) in MS patients. HSCT in patients with active RRMS, reduce progression in about 70% of patients, decrease relapses dramatically and suppresses inflammatory MRI activity [160]. MS patients who have not responded to conventional therapy, who's disease is aggressive with relapsing-remitting course and who are not presenting with high level of disability, are considered appropriate candidates for such treatment [161]. Although the clinical efficacy of HSCT long term has not been established. The mechanism by which HSCT works is that HSCT "reboots" the immune system and thus, prevents inflammation associated with the disease.

Mesenchymal stem cells (MSC) are isolated from an adult's bone marrow, are differentiated in vitro for 2–3 weeks and re-injected back into the patient. In recent years a vast amount of research has been conducted in MSCs to treat MS with most studies being in mice and EAE models, and more recently in human clinical trials. In fact, in a pilot study in advanced MS patients, MSC transplantation improved expanded disability scale score with stabilization in 1/7 and disease progression in 1/7 patients and vision and low contrast sensitivity test showed improvement in 5/6 patients with 1/6 showing worsening effects [162]. In a phase II randomized double-blind, placebo-controlled crossover clinical trial showed lower mean cumulative number of lesions in patients receiving MSCs compared to placebo [163]. No serious adverse events were reported. The mechanism of action of MSC includes immunomodulation, neuroprotection and neuroregeneration [162]. The use of MSCs that reduce MRI parameters is a new and emerging research focus to develop new improved treatments for MS.

4.2. DNA Vaccine Studies

BHT-3009, a DNA vaccine that encodes the full-length human MBP, was developed with the aim to tolerize patients with MS against MBP [9,164,165]. In fact, in 30 patients with RRMS or SPMS who received 4 injections of BHT-3009 on weeks 1, 3, 5, 9 with escalating doses of 0.5 mg, 1.5 mg or 3 mg was reported to be safe and conferred positive changes on brain MRI and reduced the number of $CD4^+$ T cells [9,164,166]. In addition, in a retrospective, randomized double blind, phase II study in 155 MS patients, BHT-3009 had no impact on the risk for persistent black holes (axonal loss and disability progression). However, there was a correlation to those who had generated high anti-IgM MBP antibodies to reduced risk of persistent black holes [167].

4.3. Nanoparticles

Nanoparticles have extensively been characterized and used as vaccine formulations in pre-clinical models of cancer and infectious diseases [168,169]. Polymeric biodegradable lactic-glycolic acid (PLGA) nanoparticles loaded with $MOG_{35–55}$ peptide together with recombinant IL-10, were partially endocytozed by dendritic cells, secreted both $MOG_{35–55}$ peptide and IL-10 in culture media for several weeks in vitro [170]. In mice, PLGA nanoparticles ($MOG_{35–55}$ + IL-10) showed significant amelioration of EAE and reduction of IL-17 and IFN-gamma secretion by splenic T cells in vitro [170]. Recently, poly(ε-caprolactone) nanoparticles loaded with recombinant human MBP reduced IFN-gamma cytokines, reduced the clinical score and showed only mild histological changes of the myelin sheath [171]. Hence, nanoparticles as a delivery method of self-antigens are a promising tool to treat MS.

4.4. Altered Peptide Ligands

Altered peptide ligands (APL) are peptides closely related to the native (agonist) peptide with defined 1–2 substituted amino acid residues which interact with the T cell receptor (TCR) yet retains its binding ability to the MHC [65]. In phase I/II clinical trial by Neurocrine Biosciences Inc, used an APL of $MBP_{83–99}$, where L-amino acids were changed to D-amino acids at positions 83, 84, 89, 91 (NBI-5788) [172]. However, this mode of APL induced T cell cross reactivity between the APL and the wild-type/agonist $MBP_{83–99}$ peptide and adverse events in some patients resulted [173]. A subsequent

multi-center double-blinded phase II clinical trial with NBI-5788 was suspended—Th2 responses were induced (IL-5, IL-13), however, 13/142 patients developed immediate-type hypersensitivity, who also generated anti-NBI-5788 antibodies which cross-reacted with native agonist $MBP_{83–99}$ peptide [172,174]. National Institute of Neurological Disorders and Stroke sponsored trial, CGP77116, was used in a MRI-controlled phase II clinical trial. CGP77116, has Ala D-amino acids of $MBP_{83–99}$ peptide at positions 83, 84, 89, 91 (CGP77116) of $MBP_{83–99}$ peptide, in order to enhance stability [174]. However, this peptide was poorly tolerated at the dose tested, and the trial had to be discontinued. Three patients showed exacerbations to disease of which two were linked to CGP77116 injection with high IFN-gamma and low IL-4 (Th1-skewing) were secreted by activated $CD4^+$ T cells. These $CD4^+$ T cells also cross reacted with the native agonist $MBP_{83–99}$ peptide [175]. Accordingly, the problems noted with both NBI-5788 and CGP77116 were likely due to inadequate pre-screening of APL effects on the many clonotypes against the targeted epitopes. Thus, although the APL was highly effective at blocking or switching some clones, it activated others. Thus, further pre-clinical testing is required and new modified peptides need to be designed, or a carrier needs to be used which further changes the resulting immune response.

4.4.1. Cyclic Peptides

Cyclization of peptides increases the stability, since linear peptides are sensitive to proteolytic enzymes. In addition, cyclic peptides are an important intermediate step and a useful template towards the rational design and development of non-peptide mimetics. While mimetic strategy is a challenging perspective it is worth pursuing in particular for MBP epitope-based MS therapy as it is still in its infancy. Efforts to design semi-mimetics of $MBP_{72–85}$ epitope by combining non-natural amino acids as spacers and MBP epitope immunophores (Ser, Arg, Glu, Ala, Gln), led to substances that were effective to some extent in inducing the onset of EAE. Cyclic peptides are not only as a step towards non-peptide mimetics but also as putative therapeutics in MS [66].

Structure activity studies of the immunodominant agonist peptide $MBP_{87–99}$, have shown that K^{91}, P^{96} are important T cell receptor contact residues. Double mutation of K^{91}, P^{96} to R^{91},A^{96} or single mutation of P^{96} to A^{96} (APL) of either in their linear or cyclic forms, results in suppression of EAE and decreased inflammation in the spinal cord of Lewis rats [71]. Single and double cyclic[A^{91}]$MBP_{83–99}$ peptide and cyclic[$A^{91}A^{96}$]$MBP_{83–99}$ peptides emulsified in CFA induced IL-4 cytokines in SJL/J mice [62] however conjugation to reduced mannan further enhanced IL-4 cytokines with no IFN-gamma responses [63]. In guinea pigs and Lewis rats, cyclic[$A^{91}A^{96}$]$MBP_{83–99}$ showed significantly reduced mechanical pain hypersensitivity compared to cyclic $MBP_{83–99}$ peptide. This was associated with reduced T cell and macrophage infiltration to injured nerves of the spinal cord of animals [176–178]. In addition, these APL decreased $CD4^+$ T cell line proliferation raised from a patient with MS, increased IL-10 cytokine secretion, bound to HLA-DR4 and were more stable to lysosomal enzymes (cathepsin B, D, H) compared to their linear counterparts [70]. Double mutation of K^{91}, P^{96} to A^{91}, A^{96} in either linear or cyclic forms were also shown to be active, with suppression of EAE in SJL/J mice, higher Th2 over Th1 cytokines produced, bound to HLA-DR4, the cyclic forms were more stable to lysosomal enzymes and induced high levels of IL-10 of peripheral blood mononuclear cells from patients with MS [61]. Recently, cyclic native agonist $MOG_{35–55}$ peptide was shown to ameliorate clinical and neuropathological features of EAE in mice compared to its linear counterpart [179]. Thus, cyclic peptides, which offer greater stability and are able to modulate immune responses, are novel leads for the immunotherapy of many diseases, such as MS [66].

4.4.2. Mannan as a Carrier to Modulate Immune Responses

Mannan, a polymannose, isolated from the wall of yeast cells has been shown to bind to the mannose receptor on dendritic cells as well as being a ligand for toll-like receptor 4 [180,181]. Mannan conjugated to MUC1 cancer protein induces immune responses in mice and protects mice against tumor challenge. This work was translated into human phase I, II and pilot III clinical

trials; mannan-MUC1 induces protection against cancer recurrence at 18 years follow-up [182–185]. Furthermore, ex vivo cultured dendritic cells pulsed with mannan-MUC1 (CVac^{TM}) and re-injection into patients induces strong cellular and clinical responses in ovarian cancer patients [186,187]. Due to the immunomodulatory properties of mannan, its effects as a carrier to MS peptides were determined.

Mutations of $MBP_{83–99}$ agonist native peptide to result in mutant peptides (APL)—linear [A^{91}]$MBP_{83–99}$, [E^{91}]$MBP_{83–99}$, [F^{91}]$MBP_{83–99}$, [Y^{91}]$MBP_{83–99}$ and [R^{91}, A^{96}]$MBP_{83–99}$, induced IFN-gamma albeit reduced compared to the native agonist peptide, however, only the double APL [R^{91}, A^{96}]$MBP_{83–99}$ induced IL-4 secretion by T cells and antagonized IFN-gamma production in vitro by T cells against the native $MBP_{83–99}$ peptide [67]. In addition, T cells against the native $MBP_{83–99}$ peptide cross-reacted with all peptides except [Y^{91}]$MBP_{83–99}$ and [R^{91},A^{96}]$MBP_{83–99}$ [68]. Conjugation of [R^{91},A^{96}]$MBP_{83–99}$, [A^{91},A^{96}]$MBP_{83–99}$, F^{91}]$MBP_{83–99}$, [Y^{91}]$MBP_{83–99}$ peptides to mannan, completed abrogated IFN-gamma responses and elicited high IL-4 (i.e., Th1 to Th2 switch) [63,69,188]. Likewise, linear double-mutant APL [$L^{144}R^{147}$]$PLP_{139–151}$ induces high levels of IL-4, and cyclization of this analog elicited low levels of IFN-gamma. When conjugated to mannan, [$L^{144}R^{147}$]$PLP_{139–151}$ peptide completely abrogated IFN-gamma, while both linear and cyclic native agonist $PLP_{139–151}$ peptides stimulated IFN-gamma secreting T cells [64]. Furthermore, mannan conjugated to the immunodominant agonist $MOG_{35–55}$ peptide primes non-pathogenic Th1 and Th17 cells and ameliorates EAE in mice [73]; a phase I human clinical trial is planned using mannan conjugated to $MOG_{35–55}$ peptide later this year. It is clear that, mannan is able to divert immune responses from Th1 to Th2 and is a promising carrier for further studies for the development of immunotherapeutics against MS.

5. Symptomatic Medication

Dalfampridine (Ampyra/Fampyra®, Acorda Therapeutics)

Dalfampridine is not intended to delay symptoms or change the course of disease, but rather, to improve motor symptoms such as walking. Dalfampridine, is a potassium channel blocker, resulting in improved potassium currents and nerve conductance. Dalfampridine is used in patients who have had MS for more than 3 years and it was approved by the FDA in 2010. Common side effects include nausea, nervousness and dizziness, which are relatively minor compared to other MS drugs.

6. Conclusions and Future Prospects

MS is an autoimmune disorder of the CNS with an array of immune cells being either activated or suppressed leading to demyelination and disease progression. In addition, genetic predisposition, viral mimicry, vitamin and mineral deficiency, geographical location are also etiological factors that contribute to disease. More recently, citrulination of myelin peptides have been shown to contribute to disease activation [59,60]. A number of treatment options are available to patients with MS, in particular those with active disease, however due to side effects, limited long term effectiveness and inability to reverse disease, new improved treatment options are required. As described here a number of new and upcoming promising therapeutic candidates are becoming available, although their effectiveness in human clinical trials remains to be determined. Recently, it was reported that non-peptide mimetics mapping the $MBP_{83–96}$ T cell epitope can function as T cell receptor antagonists, hence such an approach may pave the way to developing alternative and improved immunotherapeutics against MS [189]. With the plethora of information regarding the immunopathophysiology of MS and availability of treatment options and new upcoming treatments, the future holds promise for managing and treating the disease.

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Immunomodulatory effects of *Streptococcus thermophilus* on U937 monocyte cell cultures

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ABSTRACT

Probiotics are beneficial to the host through its contribution to the development and maintenance of a healthy immune system. Some probiotics are used in the food industry as secondary starter cultures to ferment dairy products including *Streptococcus thermophilus* (ST). ST bacteria were used to determine their modulatory effects on a promonocytic cell line which exhibited differential cytokine induction, in particular, IL-4 and IL-10 which are important in injury, infection and play a central role in anti-inflammatory responses. CXCL8 and GM-CSF are also activated – important for chemotaxis and recruitment of cells at sites of inflammation, and, increased CD11c, CD86, C206, CD209, MHC-1 expression. As ST are used in the dairy industry, are well tolerated when consumed and remain viable during cold storage, their consumption might be a practical approach in modulating immune responses in the host, and be beneficial to an array of diseases, including, autoimmunity and inflammatory bowel diseases.

1. Introduction

The regular consumption of probiotics has been shown to contribute to the maintenance of a healthy microbiome in the intestinal tract and associated health benefits (Ahtesh, Stojanovska, & Apostolopoulos, 2018; Hardy, Harris, Lyon, Beal, & Foey, 2013). It has been documented that there are over 1000 existing species within the microbiome – with 400 well known, which are all essential for the establishment and maintenance of a healthy and functional immune system (A. J. Stagg, Hart, Knight, & Kamm, 2004; J. Stagg et al., 2011; Jensen, Drømtorp, Axelsson, & Grimmer, 2015). Commensal strains of the human intestinal microbiota have been used as probiotic supplements, either in food or as capsules, for a variety of medical issues including diarrhoea, constipation and various infections (Di Caro et al., 2005; Isolauri, Sütas, Kankaanpää, Arvilommi, & Salminen, 2001; Ouwehand, Salminen, & Isolauri, 2002; Vliagoftis, Kouranos, Betsi, & Falagas, 2008). This is based on the role that the microbiome plays in establishing a balanced immune response during early life and maintaining it throughout adulthood (Kelly, King, & Aminov, 2007; Langhendries, 2005, 2006; Mead et al., 1999). These beneficial bacteria were termed "probiotic" by Fuller in 1989 (AFRC, 1989), which were then defined by the Food and Agriculture Organization and the World Health Organization as "live microorganisms which upon administration in adequate amounts confer a health benefit to the host" (Guarner & Schaafsma, 1998; Lebeer, Vanderleyden, & De Keersmaecker, 2008; Vasiljevic & Shah, 2008). Likewise, "ghost probiotics", i.e. non-viable microbial cells, intact or broken or crude cell extracts also confer benefits to the host (Deshpande, Athalye-Jape, & Patole, 2018).

Most probiotics today belong to the group of lactic acid bacteria (LAB) which represent gram-positive lactic acid producing microorganisms, and include several genera of lactobacilli, bifidobacteria and enterococci; LAB are abundantly present in the intestine, especially in the lower small intestinal lumen and the colon (Fink et al., 2007; Maassen et al., 2000; Michalkiewicz et al., 2003). LABs are commonly supplemented in foods as live probiotic strains and have been shown to confer health benefits to humans (Asarat, Apostolopoulos, Vasiljevic, & Donkor, 2015, 2016; Asarat, Vasiljevic, Apostolopoulos, & Donkor, 2015; Fink et al., 2007; Guarner & Schaafsma, 1998; Salazar et al., 2009). In addition, *Streptococcus* species (a member of the LAB), including exopolysaccharide-producing strains of *Streptococcus thermophilus* (ST) such as *S. thermophilus* ST1342, *S. thermophilus* ST1275 and *S. thermophilus* ST285 (Purwandari & Vasiljevic, 2009; Salazar et al., 2009) are widely used due to their functional properties such as, immunosuppressive effects in the treatment of acute ulcerative colitis,

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improving lactose digestion (Iyer, Tomar, Uma Maheswari, & Singh, 2010; Rabot, Rafter, Rijkers, Watzl, & Antoine, 2010; Savaiano, 2014), improving the intestinal barrier function restricting adhesion and invasion of pathogens (Brigidi, Swennen, Vitali, Rossi, & Matteuzzi, 2003; Elli et al., 2006; Kebouchi et al., 2016) as well as their production of bacteriocins and vitamins (Iyer et al., 2010; Ng et al., 2010; Uriot et al., 2017). Furthermore, ST present characteristics that enable them to be used in fermented milk products (i.e. yogurt), flavoring of dairy, and is recognized as the next most important species after *Lactococcus lactis* (Hols et al., 2005). Since 2002, ST has been accepted to be safe and approved by the American Food and Drug Administration (FDA, 2018) and the Qualified Presumption of Safety grade/rank/status from the European Food Safety Authority (Kebouchi et al., 2016). However, in contrast with other LAB, using the term probiotic for ST is still a matter of debate (Mohammadi, Sohrabvandi, & Mohammad Mortazavian, 2012; Uriot et al., 2017; Vasiljevic & Shah, 2008).

In studies of human primary macrophages, ST bacteria induce the anti-inflammatory interleukin (IL)-10 cytokine, although pro-inflammatory IL-12 cytokine is also produced (Latvala, Miettinen, Kekkonen, Korpela, & Julkunen, 2011). Furthermore, ST1275 and *Bifidobacterium longum* BL536 were shown to stimulate high levels of transforming growth factor (TGF)-beta, important for the differentiation of regulatory T cells (Treg) and T-helper (Th)-17 cells from bulk cultures of peripheral blood mononuclear cells (Donkor et al., 2012). *S. salivarius, S. equinus* and *S. parasanguinus* have been shown to induce IL-8, tumor necrosis factor (TNF)-alpha and IL-12 in human dendritic cells (DC). *Streptococcus* and *Veillonella* often co-occur in bio-environments and can potentially have metabolic collaboration; in fact their combination collectively show immunomodulatory effects. Whilst *Veillonella parvula* was only able to stimulate IL-6 production; combinations of *Streptococcus* and *Veillonella* were able to down regulate IL-12 whilst up regulating IL-6, IL-8, IL-10 and TNF-alpha (van den Bogert, Meijerink, Zoetendal, Wells, & Kleerebezem, 2014). In mice, administration of ST either orally or intraperitoneally, was shown to enhance immune responses by activating phagocytic activity of macrophages and increased antibody production by B cells (Perdigon, et al., 1987). Mice with dextran sodium sulphate induced colitis showed reduced clinical signs of disease and decreased cellular infiltration (associated with inflammation) in the colon following ST oral administration (Bailey, Vince, Williams, & Cogan, 2017). Conversely, in a human clinical study, 20 participants with positive skin prick tests and atopic history consumed yogurt that contained live ST and *Lactobacillus bulgaricus* did not show any improvement in immune cell parameters; phagocytic function, antibody responses, cytokine secretion by T cells (IFN-gamma, IL-2, IL-4), number and function of natural killer (NK) cells and neutrophils (Wheeler et al., 1997). Thus, although probiotics are able to modulate host immune responses, much is still unknown regarding their direct effect on immune cells such as monocyte/macrophages (Lebeer et al., 2008). Thus, we chose to investigate three strains of *S. thermophilus* (ST1275, ST285, ST1342), to determine their direct effects on the human pro-monocytic cell line, U937 cells that were differentiated into monocyte/macrophage cells using vitamin D_3. (Mogensen, 2009; Suresh & Mosser, 2013). Pattern recognition receptors present on monocytes and macrophages have been shown to be responsible for the recognition of bacteria, therefore these cells were used in the current study to determine the direct effect (cell surface markers and cytokine expression) of *S. thermophilus* bacteria on these cells.

2. Material and methods

2.1. Bacterial strains

Pure bacterial cultures of *S. thermophilus* 1342 (ST1342), *S. thermophilus* 1275 (ST1275) and *S. thermophilus* 285 (ST285) were obtained from Victoria University Culture Collection (Werribee, Victoria, Australia). Stock cultures were stored in 40% glycerol at −80 °C. Prior to each experiment the cultures were propagated in M17 broth (Oxoid, Melbourne Australia) and were incubated at 42 °C. Bacteria were also cultured in M17 agar (1.5% w/v agar) for characteristics and assessment of their purity, morphology and gram status by gram staining.

2.2. Preparation of live bacterial cell-suspensions

All media were prepared and sterilized by autoclaving at 121 °C for 15 min. Prior to actual experiments, the cultures were grown 3 times in M17 broth, at 37 °C for 18 h with a 1% inoculum transfer rate. *S. thermophilus* start to synthesize autolysins at the end of the exponential growth phase (Husson-Kao et al., 2000), or during or after the transition from exponential to stationary growth phase (Sandholm & Sarimo, 1981). Our cultures were obtained from Victoria University culture collection, which are cultured at 37–42 °C for 24 h (Purwandari & Vasiljevic, 2009). We kept our culture growth time consistent 18 h (at the end of the exponential growth phase) and before stationary growth phase to prevent cell lysis. Growth rate varies for various subspecies as well as their temperature (30–50 °C) (Tarrah et al., 2018). On the day of experiment, bacteria were harvested during stationary growth phase, by centrifugation (6000× for 15 min at 4 °C, Beckman J2/HS centrifuge, JA-14 rotor, Palo Alto, CA, USA), washed twice with phosphate-buffered saline (PBS) (Gibco, Australia) and resuspended in RPMI 1640 culture media. These samples constituted the live-cell suspensions.

2.3. Enumeration of bacterial cells

Bacterial strains were scraped from M17 agar and transferred into Dulbecco's PBS (Invitrogen, Pty Ltd. Australia) adjusted to a final concentration of 10^8 cfu/ml by measuring the optical density at 600 nm, and washed twice with PBS before co-culturing with monocyte cell cultures.

2.4. Culture, differentiation and stimulation of U937 cells

U937 cells were cultured in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen, Pty Ltd. Australia), 1% antibiotic-antimycotic solution and 2 mM L-glutamine at 37 °C, 5% CO_2. For differentiation of U937 cells into monocytes, U937 cells were adjusted to 3×10^5 cells/ml and 100 nM vitamin D_3 was added followed by incubation for 72 h. The resulting cells have characteristics of monocytes with CD14, CD11b, CD86 and MHC class II surface expression (Table 1).

Differentiated U937 cells (5×10^5 cells/ml) were stimulated with 1.5×10^8 live probiotic bacteria (ST1342, ST1275 or ST285) or lipopolysaccharide (LPS, 1 μg/ml; internal positive control) or non-stimulated as reference background control. The ratio of cells to bacteria is

Table 1
Proportion (%) of cell surface marker expression shown, as analyzed by flow cytometry at 24 and 48 h of stimulation of U937 cells with *S. thermophilus* strains.

Control			LPS		ST1342		ST1275		ST285	
	24	**48**	**24**	**48**	**24**	**48**	**24**	**48**	**24**	**48**
CD11b	6.1	4.1	13.9	9.1	12.1	8.6	12	7.5	11.8	21
CD11c	27.3	26.2	48	49	50	48	37	43	43	47
CD14	6.6	4	13.5	19	15.6	21	19.1	16	19.8	35
CD16	3	4	7.1	6.5	7.5	6	8.9	4	9.1	12
CD40	1.6	4	6	6	6.2	5.8	8.5	5	6	13
CD80	4	4	5.5	5.5	7.5	5.7	7.5	5.2	5.5	11
CD83	1.7	4	7	6.5	6.8	4.7	13	5	7.1	10
CD86	8.6	4.5	46.3	16	33.4	13	29.8	12.5	38	16.5
CD206	17	7	40.9	30	38.5	30	36.4	34	47.8	34.5
CD209	4	4.5	37.1	20	38.7	18.8	30	16.8	39	31
MHCI	4.2	10	18.7	23	18.9	24	20.8	22.5	22.7	24

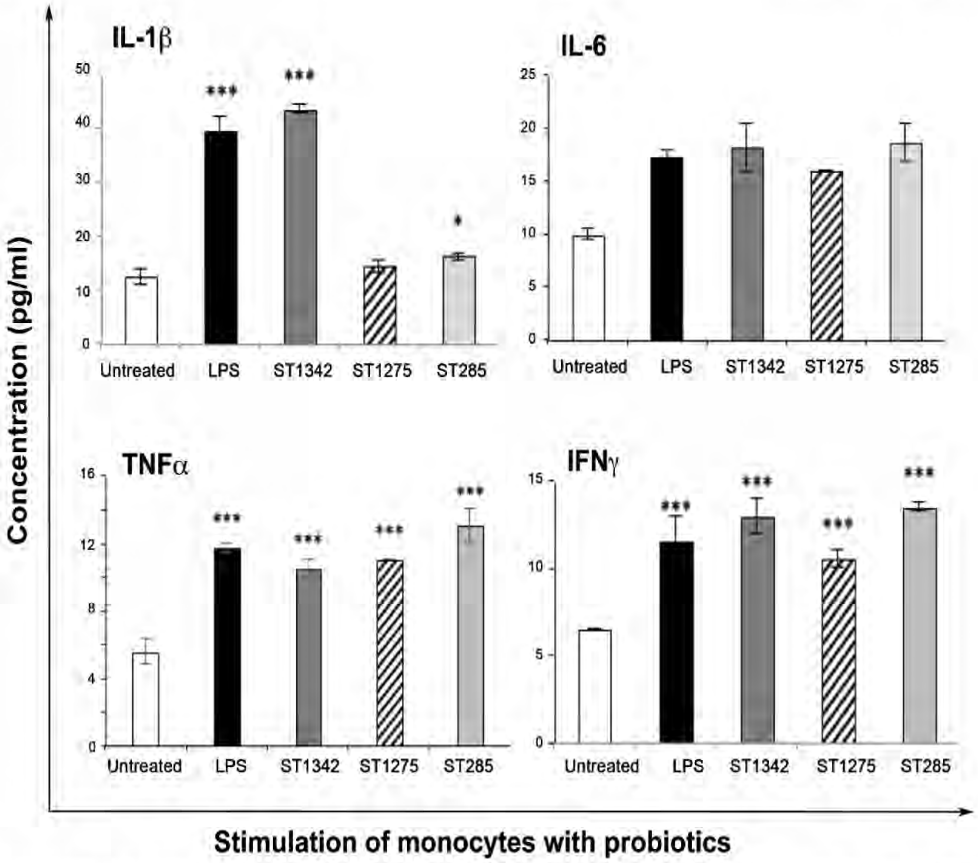

Fig. 1. *S. thermophilus* bacterial strains activate monocytes necessary for the innate immune response. U937 cells were differentitated into monocytes and stimlated with *S. thermophilus* (ST) – ST1342, ST1275 or ST285 for 24 h and secretion of IL-1β, IL-6, TNFα and IFNγ were measured. LPS was used as an internal positive control and untreated refers to differentiatied U937 cells not stimulated with ST probiotic bacteria (background control). Symbols represent p value for Tukey Test (One way ANOVA) where $^{*}p < 0.05$ and $^{***}p < 0.001$.

usually 1:10, however this ratio is usually for PBMC in which there is only 10–13% monocytes present. Although there are only a few studies that use pure monocyte cultures, 1:300 ratio of cells to bacteria has been reported (Jensen et al., 2015); hence in our experiments, 1:300 ratio cells to ST bacteria was used. All cell cultures were incubated at 37 °C, 5% CO_2 for either 24 h or 48 h. Supernatants were centrifuged and filtered to remove bacteria and were used for cytokine analysis and cells were used for cell surface marker expression by flow cytometry. Similar protocols have been used for other probiotic bacteria and on epithelial cells or PBMC (Asarat, Apostolopoulos, et al., 2015; Asarat, Vasiljevic, et al., 2015; Donkor et al., 2012).

2.5. Cytokine analysis

Cytokine concentrations of supernatants were measured by commercially available capture and detection antibodies in a Bio-Plex assay using a 9-plex kit (BioRad, Melbourne Australia) to measure IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, GM-CSF, IFNγ, and TNFα. Supernatants were collected and the assay procedures were performed according to the manufacturer's instructions. Data was collected and expressed as the mean cytokine response minus background (pg/ml) of each treatment from 4 replicate wells, plus or minus the standard error of the mean.

2.6. Flow cytometry assay for cell surface markers

Following stimulation of differentiated U937 cells with probiotics, cells were centrifuged and 5×10^5 cells were incubated with Fc block (BD Life Sciences) for 45 min on ice. After washing, cells were labelled with cell surface marker antibodies (Biolegend and BD Life Sciences) linked to fluorochrome and incubated on ice for 45 min. The antibodies were diluted in PBS/FBS at the following dilutions according to the manufacturers recommendations (CD11b-PE 1:400; CD83-Alexafluor488 1:400; CD14-BV421 1:200; CD40, CD80, CD83, CD86Alexafluor 488 1:400; CD16-PE 1:400; CD206, CD209-PE/Cy7 1:200; MHCI, MHCII-BV510 1:200). Samples were analyzed using a BD fluorescence activated cell sorter (FACS) Canto II. Data was acquired using Cell Quest program (BD Life Sciences), and analysis performed using FACS Diva software (BD Life Sciences) for percentage of expressed markers; isotype antibody controls (Biolegend and BD Life Sciences) were used as background quadrants set up.

2.7. Statistics

Significant differences between all treatment groups were tested by analysis of variance (ANOVA) followed by a comparison between treatments performed by Fisher's least significant difference (LSD) method, with a level of significance of $p < 0.05$.

3. Results and discussion

3.1. S. thermophilus bacterial strains activate monocytes necessary for the innate immune response

The innate immune system is the first line of defence against invading pathogens which react quickly and non-specifically. Following this non-specific encounter cytokines (such as, IL-1β, IL-6, TNFα and IFNγ) and chemokines are secreted by innate cells (monocytes, macrophages, dendritic cells, NK cells, granulocytes) which play an important role in the innate immune response. This results in inflammation at the site of infection to aid in pathogen clearance (Parihar, Eubank, & Doseff, 2010). IL-1β, IL-6, TNFα and IFNγ are pro-inflammatory cytokines which also aid to recruit and activate T and B cells to mount an adaptive immune response {Lacy, 2011 #104}. Secretion of IL-1β by monocytes is involved in regulating immune and inflammatory responses to infections and injury, hence its role in innate immunity (Lopez-Castejon & Brough, 2011). *S. thermophilus* ST1342 stimulated high levels of IL-1β ($p < 0.001$), whereas, ST1275 ($p < 0.05$) and ST285 ($p < 0.07$) did not induce IL-1β cytokine by differentiated U937 cells (Fig. 1). IL-6 regulates both innate and adaptive immune responses and is secreted by monocytes to stimulate immune responses during infection (Jones, 2005). TNFα is a pro-inflammatory cytokine and a main trigger of the inflammatory response by causing vasodilation and vascular permeability allowing the influx of immune cells to the site of infection (Matsuki & Duling, 2000). High levels of TNFα was secreted by monocytes in the presence of ST1342, ST1275 and ST285 ($p < 0.001$) (Fig. 1). It has been shown that IL-1β,LPS and TNFα induce IL-6 production by monocytes, and IL-6 is required for resistance against bacteria (Tosato & Jones, 1990). A trend towards increased levels of IL-6 was noted, although this was not significant for all probiotic strains ST1342, ST1275 and ST285 (Fig. 1). In addition, all three ST1342, ST1275 and ST285 strains activated high levels of IFNγ secretion (Fig. 1); a pro-inflammatory cytokine that is crucial in both innate and adaptive immune responses and has both anti-bacterial and anti-viral properties. It is clear that ST1342, ST1275 and ST285 activate cytokine secretion by monocytes, required for activation of the innate immune response and responsible for pathogen elimination. Similarly, it was noted that the probiotic *L. paracasei* DG commonly used in commercial probiotic products, has been shown to have immunostimulatory properties by increasing expression of IL-6, TNFα and CCL20 in the human monocyte cell line, THP-1 (Balzaretti et al., 2017).

3.2. S. thermophilus bacterial strains activates CXCL8 and GM-CSF: role in chemotaxis and recruitment of cells at sites of inflammation

IL-8 (also known as chemokine CXCL8) is an important cytokine of the innate immune system. IL-8 induces chemotaxis of neutrophils and other granulocytes toward the site of infection and it is a key mediator associated with inflammation; it also induces phagocytosis at the site of infection (Baggiolini & Clark-Lewis, 1992). The probiotic *L. paracasei* DG has been shown to increase expression of IL-8 in the human monocyte cell line, THP-1 (Balzaretti et al., 2017). In addition, short chain fatty acids, produced by probiotic bacteria, also stimulate IL-8 secretion and mRNA levels in the human epithelial cell line HT-29 (Asarat, Vasiljevic, et al., 2015). Likewise, ST1342 ($p < 0.005$), ST1275 ($p < 0.07$) and ST285 ($p < 0.001$) activated monocytes to secrete high levels of IL-8 compared to non-stimulated cells (Fig. 2). GM-CSF stimulates the production of white blood cells, in particular, it rapidly increases macrophages *in vivo*, important cells necessary for fighting infections. It also enhances the anti-bacterial activity of monocytes and modulates macrophage/dendritic cell phenotypes; as such, molecular targeting of the GM-CSF pathway has recently been developed to treat a number of autoimmune disorders (Ushach & Zlotnik, 2016). Of interest, ST1275 and ST285 induced monocytes to secrete high levels of GM-CSF ($p < 0.001$) while, conversely, ST1342 stimulated lower levels of GM-CSF ($p < 0.001$) (Fig. 2).

3.3. S. thermophilus bacterial strains activate anti-inflammatory cytokines

IL-4 is an anti-inflammatory cytokine which differentiates naïve $CD4^+$ Th0 cells to Th2 cells. IL-4 stimulates B cells and T cells and is a key regulator of humoral and adaptive immune responses at sites of injury. IL-4 promotes M2 anti-inflammatory macrophages and inhibits classical M1 pro-inflammatory macrophages. IL-4 together with IL-10 are important at sites of injury or infection by inhibiting bacterial mediated induction of pro-inflammatory cytokines. In addition, IL-4 and IL-10 are important cytokines required for anti-inflammatory responses against inflammatory diseases such as, autoimmunity and allergies (Mitchell et al., 2017). The probiotic *Bifidobacterium (B) breve* but not *Lactobacillus (L) casei* has been shown to induce IL-10 producing intestinal Treg cells as well as intestinal $CD103^+$ IL-10/IL-27 secreting DCs in mice (Jeon et al., 2012). Oral *B. breve* administration ameliorates colitis in mice but not in IL-10 knockout mice, demonstrating preventive effect of *B. breve* on colonic inflammation (Jeon et al., 2012). Likewise, *L. reuteri* and *L. lactis* strains given in mice orally stimulates anti-inflammatory IL-10 and Treg cells (Levkovich et al., 2013; Souza et al., 2016). Furthermore, co-culturing PBMC with selected bacteria (LAVRI-A1, *L. rhamnosus* GG, *Bifidobacteria* and *L. acidophilus*) induce anti-inflammatory cytokines IL-4, IL-10 and TGF-beta (Donkor et al., 2012; Donkor, Shah, Apostolopoulos, & Vasiljevic, 2010). These cytokines inhibit the production of IL-12, IFNγ and other pro-inflammatory cytokines which are beneficial for autoimmune and allergic responses. Here we show that, ST1342 stimulated IL-4 production by monocytes ($p < 0.001$) and to a lesser degree ST1275 ($p < 0.07$) and ST285 ($p < 0.005$), (Fig. 3). Similarly, IL-10 was secreted by monocytes in the presence of ST1342, ST1275 and ST285 ($p < 0.001$), with ST1275 and ST285 stimulating higher levels (Fig. 3). It is clear that ST probiotic bacteria have potential anti-inflammatory properties which could have positive implications in chronic inflammatory diseases (autoimmunity and allergies) and warrant further investigation.

3.4. S. thermophilus bacterial strains upregulate the expression of cell surface markers on differentiated U937 cells; role in initiating innate and adaptive immune responses

Monocytes are major constituent cells of the innate immune system, which also play a role in the adaptive immune response. The expression of cell surface markers on monocytes is crucial in the ensuing immune responses. The specific markers presented on monocytes is dependent on their environment and their exposure to pathogens and/or pathogenic peptides and pathogen derived metabolites; with these factors causing alterations in the profile of monocyte markers, accordingly (Ziegler-Heitbrock, 2015). The human pro-monocytic histiocytic lymphoma cell line, U937 cells, are commonly used to study the behavior and differentiation of monocytes. They exhibit pro-monocytic characteristics by displaying monoblast morphology, produce lysozymes and have esterase activity (dos Santos et al., 2009; Sundstrom & Nilsson, 1976). They are not phagocytic, they express low levels of CD14, CD54, CD86, and major histocompatibility complex (MHC)-class II is not detectable (Azam et al., 2006). However, upon stimulation with viral or bacterial fragments, or, vitamin D_3, they express markers demonstrating monocyte/macrophage morphology, with increased expression of CD14 (dos Santos et al., 2009; Koss, Lucero, & Koziner, 1996; Santegoets, Van Den Eertwegh, Van De Loosdrecht, Scheper, & De Gruijl, 2008).

Our data shows that U937 cells incubated with ST1342, ST1275 or ST285 results in enhanced expression of CD14, CD11c, CD86, CD206, CD209 and MHC1 cell surface markers at varying levels; CD11b, CD16, CD40, CD80 and CD83 were also up regulated, albeit at a much lower level (Table 1). In other studies, the combination of 3 probiotics (*L.*

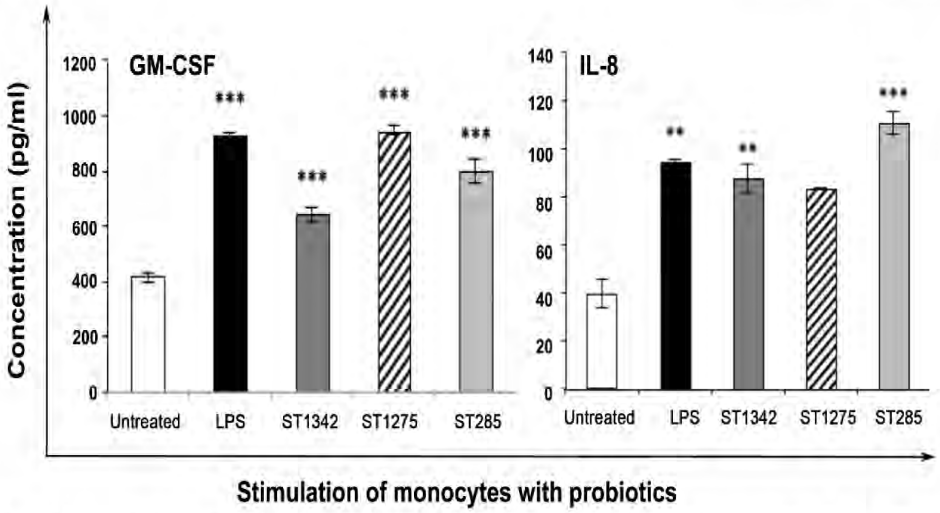

Fig. 2. *S. thermophilus* bacterial strains activate CXCL8 and GM-CSF essential for recruitment of cells at sites of inflammation. U937 cells were differentitated into monocytes and stimlated with *S. thermophilus* (ST) - ST1342, ST1275 or ST285 for 24 h and secretion of IL-8 and GM-CSF were measured. LPS was used as an internal positive control and untreated refers to differentiatied U937 cells not stimulated with ST probiotic bacteria (background control). Significant differences between treatments were tested by analysis of variance (ANOVA). Symbols represent p value for Tukey Test (One way ANOVA) where # $p < 0.07$, ** $p < 0.005$ and *** $p < 0.001$.

acidophilus, *L. delbrueckii* ssp. *bulgaricus* and *B. bifidum*) stimulated increased expression of cell surface markers, CD14, MHC class II and CD80 (Gutkowski et al., 2010).

CD14 is expressed on the surface of monocytes and macrophages and primarily binds to bacterial LPS; although other bacterial cell wall constituents also bind to CD14 such as, lipid A, *Staphylococcus aureus, Escherichia coli* and lipoteichoic acid (Bron, Tomita, Mercenier, & Kleerebezem, 2013; Lee, Tomita, Kleerebezem, & Bron, 2013; van Baarlen, Wells, & Kleerebezem, 2013). The interaction between CD14 and its ligands initiates the innate immune response (Bedell et al., 2018), as well as further up regulating its expression (CD14 expression) (Landmann et al., 1996). Indeed, ST1342, ST1275 and ST285 up regulated CD14 expression on U937 cells after 24 and 48 h incubation, with ST285 being the most significant at 48 h (Table 1).

CD11c is a type I transmembrane protein expressed by DCs, monocytes, macrophages and neutrophils (Dyer, Garcia-Crespo, Killoran, & Rosenberg, 2011). The presence of CD11c on these cells allows their adherence to endothelial cells, phagocytosis of complement positive cells (important for innate immune defence) and activates cellular immune responses. Selected strains of *Lactobacillus* (*L. reuteri, L. plantarum* Lb1 and *L. fermentum*) cultured with murine bone marrow cells and GM-CSF, induce high levels (85–90%) of $CD11c^+$ cells (Christensen, Frøkiær, & Pestka, 2002). Basal expression levels of CD11c on U937 cells was 26–27%, which almost doubled following LPS

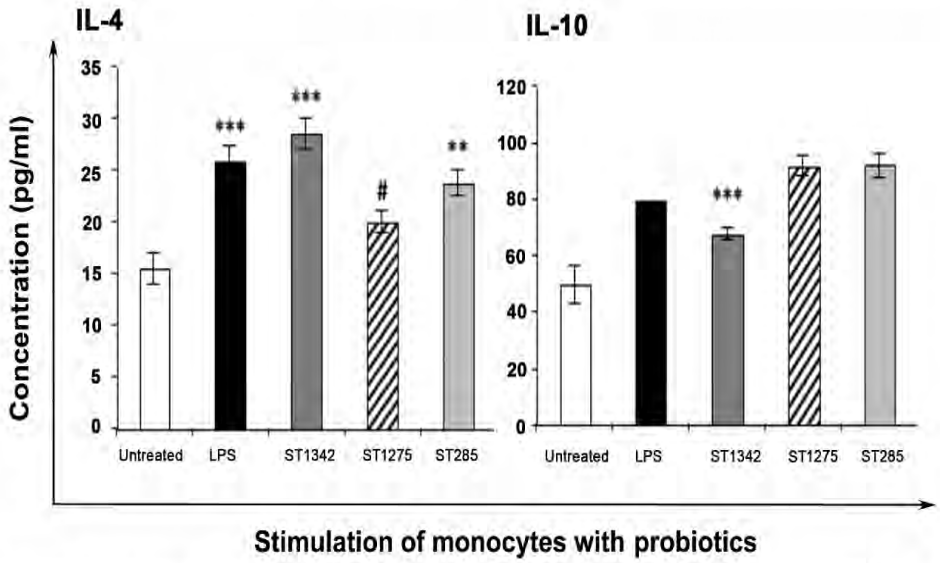

Fig. 3. *S. thermophilus* bacterial strains activate anti-inflammatory cytokines. U937 cells were differentitated into monocytes and stimlated with *S. thermophilus* (ST) – ST1342, ST1275 or ST285 for 24 h and secretion of IL-8 and GM-CSF were measured. LPS was used as an internal positive control and untreated refers to differentiatied U937 cells not stimulated with ST probiotic bacteria (background control). Symbols represent p value for Tukey Test (One way ANOVA) where # $p < 0.07$, ** $p < 0.005$ and *** $p < 0.001$.

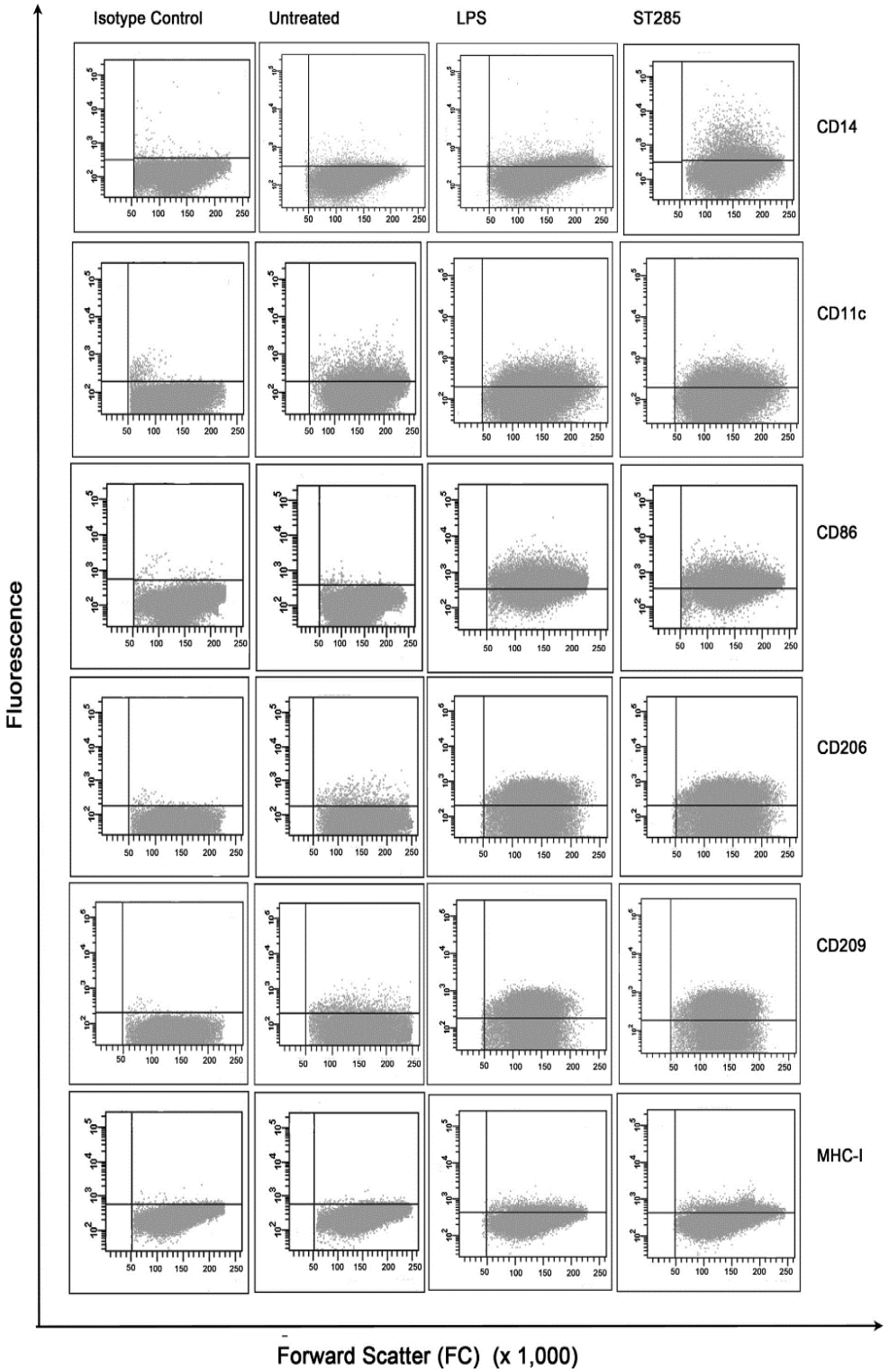

Fig. 4. *S. thermophilus* (ST) bacterial strains increase cell surface marker expression. U937 cells were differentiated into monocytes and stimlated with ST1342, ST1275 or ST285 for 24 or 48 h and cell surface marker expression assessed. Upregulation of CD14, CD11c, CD86, CD206, CD209 and MHC class I are shown at 24 h for ST285. LPS was used as an internal positive control and untreated refers to differentiatied U937 cells not stimulated with ST probiotic bacteria (background control).

(48–49%) and ST1342 (48–50%) stimulation; significant up regulation was also noted with ST1275 (37–43%) and ST285 (43–46%) after 24 or 48 h respectively (Table 1). Interestingly, there were no major differences in CD11c expression, whether cells were stimulated for 24 or 48 h.

CD86 (B7-2) expression on antigen presenting cells (DCs, macrophages, B cells) is involved in co-stimulatory signalling that is required for the priming and proliferation of T cells (Fleischer et al., 1996). Monocytes express low levels of CD86 which is up regulated following stimulation with IFN-gamma or other ligands. In fact we showed that expression of CD86 increased significantly from 8.6% to 33.4% (ST1342), 28.1% (ST1275) and 38% (ST285) after 24 h co-culture, which was lower than that after LPS stimulation (46.3%) (Table 1). The up regulation of CD86 was transient and after 48 h the levels decreased significantly. It is clear that *S. thermophilus* bacteria promote CD86 expression levels, required for T cell activation and the maintenance of immune responses (Fleischer et al., 1996). Similarly, *L plantarum* WCFS1 and *L. fermentum* GR1485 have been shown to upregulate CD86 cell surface expression on monocytes, however, *L. rhamnosus* and *L. delbruekii* reduce cell surface expression of CD86 (Esmaeili et al., 2018).

CD206 (mannose receptor, MR) (Geurtsen et al., 2009), is primarily present on the surface of macrophages and immature DCs (Kerrigan & Brown, 2009), and functions to arrest antigens and pathogenic components, followed by processing and presentation to T cells (Engering et al., 2004). The MR recognizes mannose, fucose and N-acetylglucosamine residues (Kerrigan & Brown, 2009; Mitchell et al., 2017) commonly expressed on the surface of microorganisms (such as *Pneumocystis*, *Candida*, *Mycobacterium*, *Leishmania*), and capsular polysaccharides of *Streptococcus* and *Klebisella* (Geurtsen et al., 2009; Kerrigan & Brown, 2009; Zamze et al., 2002), which results in the destruction of bacteria (innate immune response) and activation of the adaptive immune response (cellular responses). Poly-mannose (mannan) linked to protein antigens as a model, targets the MR on DCs and macrophages resulting in stimulation of either pro- or anti-inflammatory responses, significant in a number of diseases from cancers to autoimmunity (Apostolopoulos & McKenzie, 2001; Apostolopoulos, Barnes, Pietersz, & McKenzie, 2000; Apostolopoulos, Pietersz, & McKenzie, 1996; Apostolopoulos, Pietersz, Gordon, Martinez-Pomares, & McKenzie, 2000; Apostolopoulos, Pietersz, Loveland, Sandrin, & McKenzie, 1995; Sheng et al., 2006). Here we show that U937 cells co-cultured with ST1342, ST1275 or ST285 up regulated the expression levels of CD206 within 24 h (ST285 inducing the highest levels) which subsided by 48 h, but did not reach basal level expression (Table 1, Fig. 4). In addition, CD209 (DC-SIGN), a C-type lectin receptor expressed on the surface of macrophages and DCs also binds to mannose residues present on bacteria, viruses and fungi. The interaction between CD209 and mannose moieties activates phagocytosis as well as endocytosis for processing and presentation to T cells (Apostolopoulos et al., 2014; Cambi et al., 2003; Proudfoot, Apostolopoulos, & Pietersz, 2007; Sheng et al., 2008; Sheng, Pietersz, Wright, & Apostolopoulos, 2005). U937 cells cultured in the presence of ST strains also up regulated the expression of CD209 with maximal up regulation noted within 24 h (Table 1); ST285 stimulation resulted in the highest up regulation at both 24 and 48 h. Thus, *S. thermophilus* strains induce CD206 and CD209 expression, as a result have a positive role in activating both the innate and adaptive immune responses (Apostolopoulos et al., 2006, 2014).

The major histocompatibility complex class I (MHC-I) is expressed by all nucleated cells and presents processed antigenic peptides on its surface to activate CD8+ T cells (Neefjes, Jongsma, Paul, & Bakke, 2011). U937 cells express low levels of MHC-I which is up regulated within 24 h in the presence of ST1342, ST1275 or ST285 and remains up regulated after 48 h of stimulation (Table 1). Hence, *S. thermophilus* strains are beneficial in upregulating MHC-I molecules on monocyte/macrophage cells for enhanced CD8+ T cell stimulation, required for the elimination of tumour cells and viruses.

3.5. Conclusion

Activation of monocyte cells with *Streptococcus thermophilus* such as *S. thermophilus* ST1342, *S. thermophilus* ST1275 and *S. thermophilus* ST285 strains, and secretion of IL-1β, IL-6, TNFα and IFN-γ suggests their role in the subsequent activation of the immune responses aiding in the elimination of pathogens. In addition, *S. thermophilus* strains, up regulated the secretion of IL-8, a chemokine involved in chemotaxis and phagocytosis, as well as up regulating the secretion of GM-CSF, a major cytokine for increasing the number of macrophages at the site of infection. Clearly, *S. thermophilus* strains up regulated cytokine levels by monocytes, required for activation of the innate immune response. Furthermore, the activation of anti-inflammatory cytokines (IL-4 and IL-10) could be beneficial in modulating chronic inflammatory conditions and allergies. Moreover, *S. thermophilus* strains up regulated monocyte cell surface markers, CD14, CD11c, CD86, CD206, CD209 and MHC-I suggestive of their potential benefit to activate innate and adaptive immune responses. These findings support a role for these probiotic strains in the healthy modulation of monocyte activity and their roles in innate and cellular immunity. The results also present a potential role for these strains in modulating the inflammatory response, which warrants further investigation. Overall, these findings are in agreement with the body of research that supports the role that the regular consumption of probiotics (including *S. thermophilus*) has in the establishment and maintenance of a healthy immune system and opens pathways to further determine the mechanisms by which these strains modulate immune responses.

Ethics statement

No ethics were required for this research paper.

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Conflict of interest

The authors declare no conflicts of interest.

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RESEARCH ARTICLE

Streptococcus thermophilus alters the expression of genes associated with innate and adaptive immunity in human peripheral blood mononuclear cells

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Abstract

Consumption of probiotics contributes to a healthy microbiome of the GIT leading to many health benefits. They also contribute to the modulation of the immune system and are becoming popular for the treatment of a number of immune and inflammatory diseases. The main objective of this study was to evaluate anti-inflammatory and modulatory properties of *Streptococcus thermophilus*. We used peripheral blood mononuclear cells from healthy donors and assessed modifications in the mRNA expression of their genes related to innate and adaptive immune system. Our results showed strong immune modulatory effects of *S. thermophilus* 285 to human peripheral blood mononuclear cells with an array of anti-inflammatory properties. *S. thermophilus* 285 reduced mRNA expression in a number of inflammatory immune mediators and markers, and upregulated a few of immune markers. *S. thermophilus* is used in the dairy industry, survives during cold storage, tolerates well upon ingesting, and their consumption may have beneficial effects with potential implications in inflammatory and autoimmune disorders.

1. Introduction

The human body and, in particular, the gastrointestinal tract (GIT) hosts a variety of microbial populations referred to collectively as the microbiome [1]. The microbiome of the GIT plays a key role in the maintenance of a healthy immune system [1, 2], and disruptions to the microbiome composition can lead to serious effects on health [3–5]. In order to maintain a healthy microbiome, regular ingestion of probiotic supplements, or the ingestion of fermented dairy products/capsules has been suggested. These practices have led to various improved health outcomes, ranging from enhanced overall human wellbeing to the treatment of infections, constipation, diarrhoea etc [1].

The majority of probiotics belong to the lactic acid bacteria (LAB) family; gram positive lactic acid producing microorganisms that include several genera such as bifidobacteria, lactobacilli streptococci and enterococci [1]. The small intestine and the colon are highly

2.2. Preparation of live bacterial suspensions

Media were prepared and autoclaved at 121˚C for 15 minutes (mins) prior to experiments. Bacterial cultures were grown 3 times in M17 broth with 20 g/L lactose, at 37˚C aerobically for 18 hours (hr) with a 1% inoculum transfer rate [28]. Cultures grow optimally at 37–42˚C for 24 hrs [15]. The growth period of cultures were consistent at 18 hr (at the end of the exponential growth phase) and before stationary growth phase to prevent cell lysis. Bacteria were harvested during stationary growth phase on the day of experiment, centrifuged (6000×g) for 15 min at 4˚C, followed by two washes with Dulbecco's phosphate-buffered saline (DPBS) (Invitrogen, Pty Ltd. Australia) and resuspended in the Roswell Park Memorial Institute (RPMI) 1640 culture media. These samples constituted the live-cell suspensions.

2.3. Enumeration of bacterial cells

Bacterial strains were scraped from M17 agar and transferred into Dulbecco's PBS (Invitrogen, Pty Ltd. Australia) adjusted to a final concentration of 10^8 colony forming units (cfu)/ml by measuring the optical density at 600 nm, and washed two times with PBS and resuspended in RPMI 1640 prior to co-culturing with PBMC [1].

2.4. Isolation, culture, and stimulation of PBMC

2.4.1. Isolation of PBMC using Ficoll-Paque. PBMC isolation from whole blood was via Ficoll-Paque density gradient centrifugation [9]. Three buffy coats were collected from the Australian Red Cross Blood Bank on the day of experiment (Victoria University human research ethics). Calcium and magnesium free PBS, pH7.2, (Invitrogen, Pty Ltd. Australia) was used after adding 2 mM EDTA and 2% heat-inactivated fetal bovine serum (FBS) (Invitrogen, Pty Ltd. Australia); PBS buffer. SEPMATE tubes (50 ml) with inner inserts (STEMCELL technology, Canada) were used to isolate PBMC following Ficoll-Paque density gradient protocol [29, 30]. PBMCs were washed, counted and the required number of PBMC were co-cultured with *S. thermophilus* 285 and the remaining PBMC were stored in freeze mix and transferred into liquid nitrogen for future use.

2.4.2. Stimulation of PBMC with *S. thermophilus* 285. PBMC (3x 10^7 cells) were resuspended in RPMI 1640 media supplemented with 10% heat-inactivated FBS (Invitrogen, Pty Ltd. Australia), 1% antibiotic-antimycotic solution and 2 mM L-glutamine in cell culture flasks, and 3x10^8 *S. thermophilus* 285 bacteria were added. PBMC with RPMI media without the addition of ST285 bacteria were used as a control and incubated at 37˚C, 5% CO_2 for 24 hrs [1]. We previously demonstrated that 24 hrs co-culture was optimal for stimulation of U937 monocyte/macrophage cell line, and all incubations described herein were for 24 hrs [1]. PBMCs were snap frozen post incubation and stored at -80˚C prior to RNA extraction.

2.5. RNA extraction from PBMC

Total RNA was extracted from stimulated PBMCs using the RNeasy® mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, cells were centrifuged and harvested, supernatants were removed and RNA extracted from each cell pellet and resuspended in lysis buffer supplemented with β-mercaptoethanol to disrupt the cells. PBMC were lysed and each cell lysate passed through the supplied Qia-shredder columns to homogenize and was subsequently mixed with equal volume of 70% ethanol. Cell lysates were transferred onto RNeasy mini-spin columns and DNA was removed using DNase digestion/ treatment using RNase-Free DNase Set (Qiagen, Hilden, Germany.) The RNA Integrity Number (RIN) of all RNA samples were measured using an Agilent 2100 Bioanalyzer and Agilent RNA 6000

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enriched with these microorganisms [6–8], which are routinely supplemented in foods as live strains due to their beneficial effects on human health [1, 2, 8–13]. *Streptococcus* species such as exopolysaccharide-producing strains of *Streptococcus thermophilus* (ST) [12, 14, 15] are among those consumed. These characteristics of *S. thermophiles* enable them to be used in fermented milk products (i.e. yogurt) including flavoring of dairy, and is recognized as the next most important species after *Lactococcus lactis* [16, 17]. ST and *L. brevis* synergistically display well established health benefits, and *S. thermophilus* is one of the bacteria in the VSL#3 probiotic mixture, which has long been broadly applied in the treatment of inflammatory conditions [18, 19]. In addition, probiotics interact with the immune system leading to immunomodulation and anti-inflammatory properties [4, 20, 21].

The 'hygiene hypothesis' suggests that the positive trend in the incidence of immune-related disorders can been attributed to intestinal dysbiosis, resulting in immune dysfunction (ie. asthma, eczema, allergies and autoimmune diseases). Use of probiotic bacteria can increase abundance and concurrently modulate immune cells, including B, T helper (Th)-1, Th-2, Th-17 and regulatory T (Treg) cells. This in turn, directly influences human health and modulates pathologies of immune/autoimmune diseases [1, 2, 13]. In fact, we previously noted that *S. thermophilus* 1342, *S. thermophilus* 1275 and *S. thermophilus* 285 modulate the U937 monocyte cell line. Specifically, we showed that interleukin (IL)-4, IL-10, GM-CSF and CXCL8 production were increased, and, cell surface marker expression CD11c, CD86, C206, CD209, MHC-1 were upregulated [1]. In another study, *S. thermophilus* 1275 and *Bifidobacterium longum* BL536 demonstrated increased levels of transforming growth factor (TGF)-beta (a key factor in the differentiation of Treg and T-helper Th)-17 cells by bulk peripheral blood mononuclear cell (PBMC) cultures [22]. Primary macrophages co-cultured with ST bacteria stimulate production of anti-inflammatory IL-10 and pro-inflammatory IL-12 cytokines [23].

Peripheral blood mononuclear cells (PBMC) isolated from whole blood constitute a wide range of diverse immune cells that play vital roles in balancing immune homeostasis and keeping human health in check [24, 25]. These cells are crucial components of the innate and adaptive immune system, defend the body against bacterial, viral and parasitic infections, as well as destroying foreign antigens and cancer cells [25]. PBMC are predominantly made up of lymphocytes (~70–90%), monocytes (~10–20%) and other cells such as dendritic cells comprise less than 1–2% [26]. In spite of variations in the fraction of subtypes of immune cells within the total PBMC isolated from different samples [26], isolation, characterization and molecular studies of these cells have benefited medical research [27].

Herein, we describe changes in the expression of genes associated with innate and adaptive immunity including cytokines, chemokines and immune cell marker expression by human PBMC following exposure to live *S. thermophilus* 285 bacteria.

2. Material and methods

2.1. Bacterial strains

Pure bacterial cultures of *S. thermophilus* 285 were obtained from Victoria University culture collection (Werribee, VIC, Australia). Stock cultures were stored in cryobeads at −80˚C. Prior to each experiment the cultures were propagated in M17 broth (Oxoid, Denmark) with 20 g/L lactose and incubated at 37˚C under aerobic conditions. Bacteria were also cultured in M17 agar (1.5% w/v agar) with 20 g/L lactose (Oxoid, Denmark), to assess characteristics, morphology, purity and gram-positive confirmation [1].

nano kit (Agilent Technologies, Santa Clara, CA, USA); with a minimum RIN of 7.5 used as the criterion for inclusion in gene expression analysis. The concentration of each individual RNA sample was measured using a Qubit RNA BR Assay (Invitrogen) in triplicate. Several blood samples were collected for PBMC isolation, treatment and extraction of RNA and only RNA samples with the highest RIN numbers (all above 8) were included for PCR.

2.6. Assessing changes in the expression of genes associated with innate and adaptive immunity

Aliquots of each RNA sample were reverse-transcribed to make complementary DNA (cDNA) using RT^2 first strand kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the 'Human Innate and Adaptive immune Response' kit (Qiagen, Hilden, Germany) to evaluate gene/mRNA expression. The relative expression profiles of treated PBMC samples were analyzed in comparison with untreated PBMC cultured in RPMI using Thermo-cycler (Biorad, Melbourne Australia). The RT^2 qPCR Primer innate and adaptive immune response arrays target a set of 84 innate and adaptive immune-related genes and five housekeeping genes, an RT control, a positive PCR control, and a human genomic DNA contamination control. The levels of the expression of these genes were calculated using the Qiagen web-based software (Qiagen, Germany) and then calculated the fold changes and analyzed data manually to compare results. Differential expression (up and down regulation) of the genes were identified using the criteria of a $>$ 2.0-fold increase/decrease in gene expression in treated PBMCs in comparison with those genes in control PBMC cultures.

2.7. Data analysis

The Delta-Delta CT (ΔΔCT) was used to calculate fold-changes [31]. Fold-regulation represents fold-change results in a biologically meaningful way. In our RT2 profiler PCR array results, fold-change values greater than one, indicate a positive (or an up-) regulation, in fact in upregulated genes, the fold-regulation is equal to the fold-change. Fold-change values less than one specifies a negative (or a down) regulation, and in this case, the fold-regulation is the negative inverse of the fold-change [32–34]. Data related to changes in the expression of the genes were analyzed by ΔΔCT method using Qiagen RT^2 profiler data analysis webportal that utilises the delta delta CT method in determining fold-changes. The raw CT values were uploaded to the Qiagen data analysis webportal with the lower limit of detection set for 35 cycles and 3 internal controls: PCR array reproducibility, RT efficiency and genomic DNA contamination were assessed to ensure all arrays successfully passed all of these control checks. Normalization of the raw data was performed using the included housekeeping genes (HKG) panel. Then using the ΔΔCT method, both housekeeping gene references and untreated/ controls were assessed to calculate relative expression of mRNA.

2.8. Statistical analysis

The p values are calculated based on a Student's *t-test* of the Triplicate 2^ (- Delta CT) [(2^-ΔCT)] values for each gene in the treatment group vs. the control group [32, 33, 35, 36].

3. Results

Among 84 genes assessed, 31 genes were significantly altered $>$ 2.0 fold up/down in PBMC samples (n = 3) following exposure to *S. thermophilus* 285 compared to control PBMC (Fig 1, Table in S1 Table and S1 Fig).

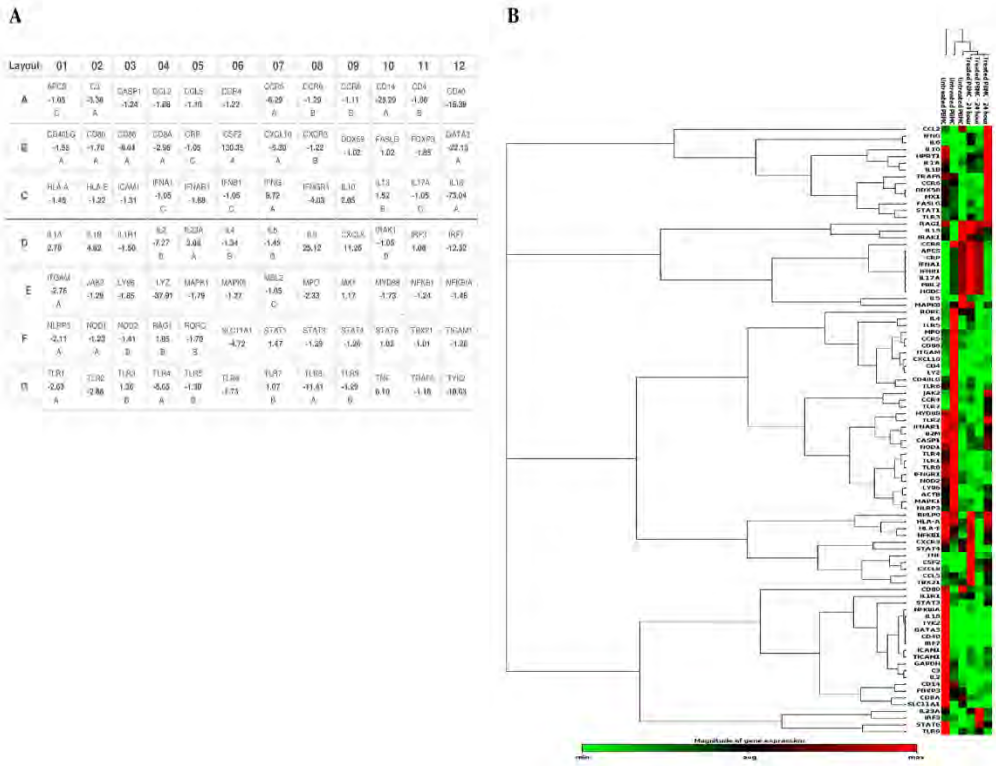

Fig 1. Effects of co-culturing *S. thermophilus* 285 with PBMCs (n = 3) on gene/RNA expression compared to control PBMCs after 24 hrs. (A) All 84 genes are shown including those with significant high up/down regulated genes (more than 2-fold) and those with no significant change (less than 2-fold). The housekeeping genes (HKG) panel and other genes used for normalization of the raw data are not presented. Letter A specifies the gene's average threshold cycle to be reasonably high (> 30) in either the treated samples or the controls and relatively low (< 30) in the other/opposite sample. Thus, in case of presenting fold changes with letter A, the estimate fold change may be an underestimate. Letter B suggests a reasonably high (> 30) gene's average threshold cycle that means a low level of average expression of relevant gene, in both test/treated samples and untreated control samples, and the p-value for the fold-change might be either relatively high ($p > 0.05$). Thus, in case of presenting fold changes with letter B, the estimate fold change may be slightly overestimate or unavailable. Letter C indicates that that gene's average threshold cycle is either not determined or greater than the defined default 35 cut-off value, in both test/treated samples and control samples, suggesting that its expression was not detectable, resulting in the fold-change values being un-interpretable [86, 87] [88]. (B) Presentation of data as a hierarchical clusters of average gene/RNA expressions of PBMC (n = 3) co-cultured with *S. thermophilus* 285, compared to control. Green represents down regulated genes to red represents upregulated genes.

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3.1. *S. thermophilus* 285 alters cytokine gene expression levels of PBMC

3.1.1. Interleukin mRNA expression levels. IL-1α and IL-6 are secreted by dendritic cells (DC), B cells and macrophages (MQ) are involved in acute phase responses, B cell maturation, macrophage differentiation, promote Th2 differentiation and inhibit Th1 polarization. IL-1α is upregulated 2.78 ± 0.6 fold and IL-6 25.12 ± 0.61 fold (Fig 2). IL- 23α is secreted by $CD4^+$ T cells and aids in the stimulation of Th17 cells together with IL-6. IL-23α is highly upregulated 3.8 ± 1.0 fold (Fig 2). IL-2 has an array of functions it activates T cell proliferation and increases or decreases inflammatory responses. IL-2 is downregulated 7.27 ± 0.53 fold (Fig 2). IL-17A a pro-inflammatory cytokine secreted by Th17 cells, was not altered following PBMC co-cultured with *S. thermophilus* 285.

3.1.2. Th1/Th2 mRNA expression levels. IFNγ, a Th1 cytokine important in the defense against bacterial infection is upregulated 8.73 ± 0.94 fold. Likewise, the Th1 cytokine IL-1β is upregulated 4.82 ± 0.74 fold (Fig 3). Of interest, IL-18 a Th1 inducing pro-inflammatory

cytokine secreted by Th2 and Treg cells is upregulated 2.05 ± 0.52 fold (Fig 3). Gene expressions of other cytokines, IFNB1, IL-4, IL-5 and IL-13 are not significantly altered.

3.2. *S. thermophilus* 285 alters chemokine gene expression levels of PBMC

Chemokine (CXCL8, IL-8) is important in the innate immune system, it stimulates chemotaxis and is upregulated 11.26 ± 0.27 fold following *S. thermophilus* 285 co-culture with PBMC cells. However, CCR5 and CXCL10 (INP10) are down regulated 6.29 ± 0.32 and 5.30 ±1.8 fold respectively (Fig 4). No significant differences are noted for gene expressions of other chemokines, including CCL2 (MCP-1), CCL5 (RANTES), CCL8, CCR4, CCR8, CXCR3, CCL2, IFNA1.

3.3. Colony stimulating factor mRNA expression levels

Colony-stimulating factor (CSF)-2, secreted by MQs, NK cells and T cells, enables cell proliferation and differentiation and is significantly increased by 130.35 ± 1.0 fold (Fig 5) after co-culturing PBMC with *S. thermophilus* 285 bacteria.

3.4. *S. thermophilus* 285 alters Toll like receptor gene expression levels of PBMC

TLR (toll like receptor)-1, TLR-2, TLR-4 and TLR-8 are part of the innate immune response and involved in the defense response to bacteria. PBMC co-cultured with *S. thermophilus*

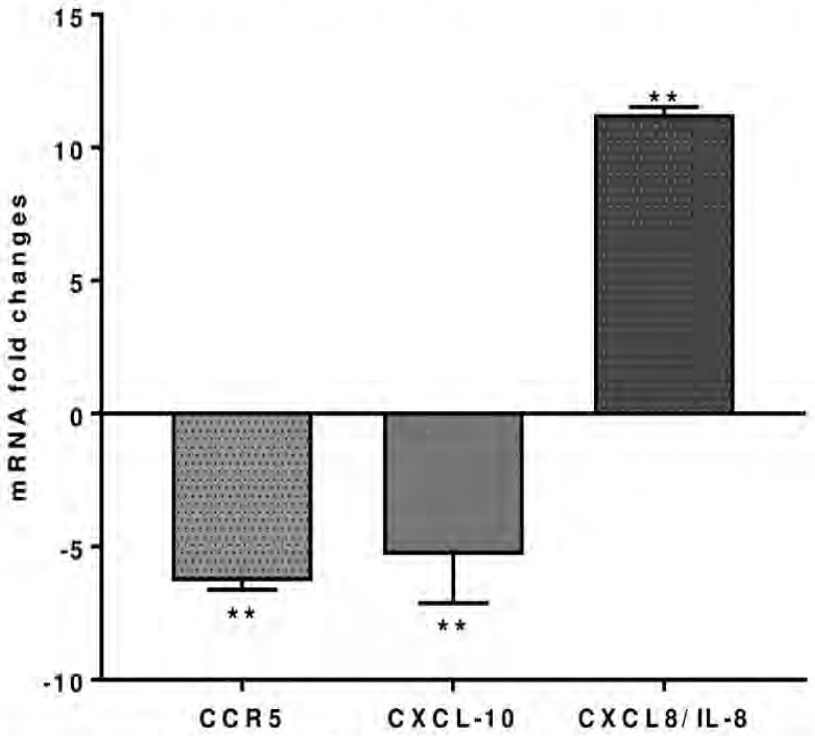

Fig 4. CCR5, CXCL10 and CXCL8 (IL-8), mRNA fold change following 24 h co-culture of *S. thermophilus* 285 with PBMCs (n = 3), compared to control PBMC. The innate and adaptive RT^2 gene profiler arrays were used to determine changes in gene expression. Symbols represent p value for Tukey Test (One way ANOVA) where ** $p < 0.04$.

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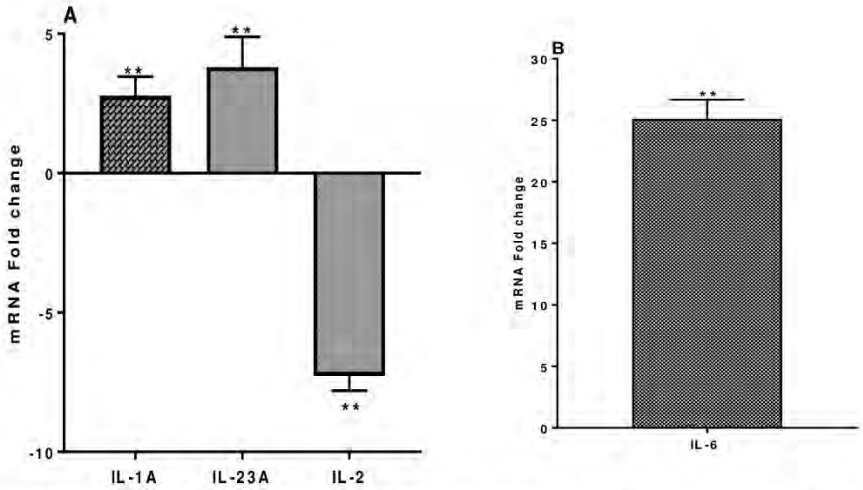

Fig 2. (A) IL-1α, IL-23α and IL-2 and (B) IL-6, mRNA fold change following 24 h co-culture of *S. thermophilus* 285 with PBMCs (n = 3), compared to control PBMC. The innate and adaptive RT^2 gene profiler arrays were used to determine changes in gene expression. Symbols represent *p* value for Tukey Test (One way ANOVA) where ** $p < 0.04$.

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cytokine was vastly downregulated (75 ± 0.66 fold), in addition, IFNγR1, a transmembrane protein which interacts with IFNγ, is also downregulated 4.03 ± 0.25 fold (Fig 3). Tumor-necrosis factor-alpha (TNFα), important in the defense against bacterial infections, and in acute phase reactions is upregulated 6.10 ± 1.4 fold (Fig 3). IL-10, an anti-inflammatory

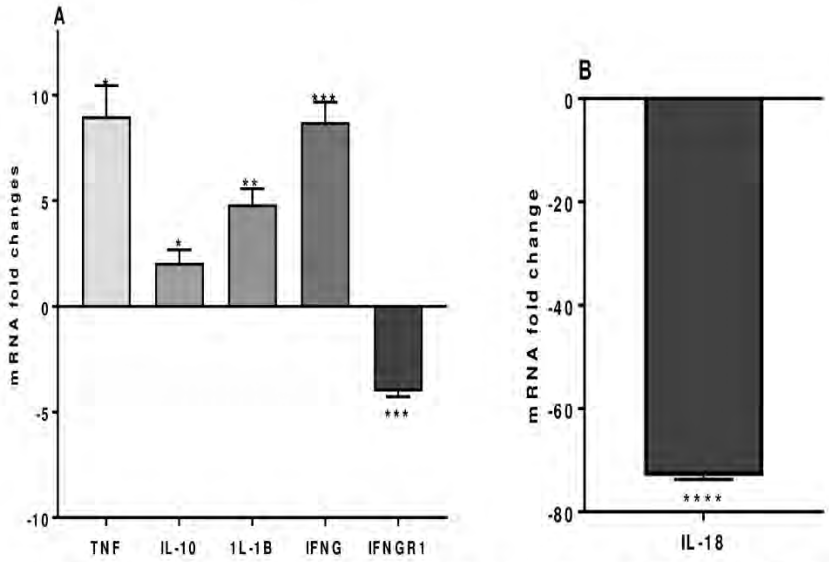

Fig 3. A) TNF-α, IL-10, IL-1β, IFN-γ, and IFN- γ–R and (B) IL-18, mRNA fold change following 24 h co-culture of *S. thermophilus* 285 with PBMCs (n = 3), compared to control PBMC. (The innate and adaptive RT^2 gene profiler arrays were used to determine changes in gene expression. Symbols represent *p* value for Tukey Test (One way ANOVA) where * $p < 0.05$, ** $p < 0.04$, *** $p < 0.02$ and **** $p < 0.01$.

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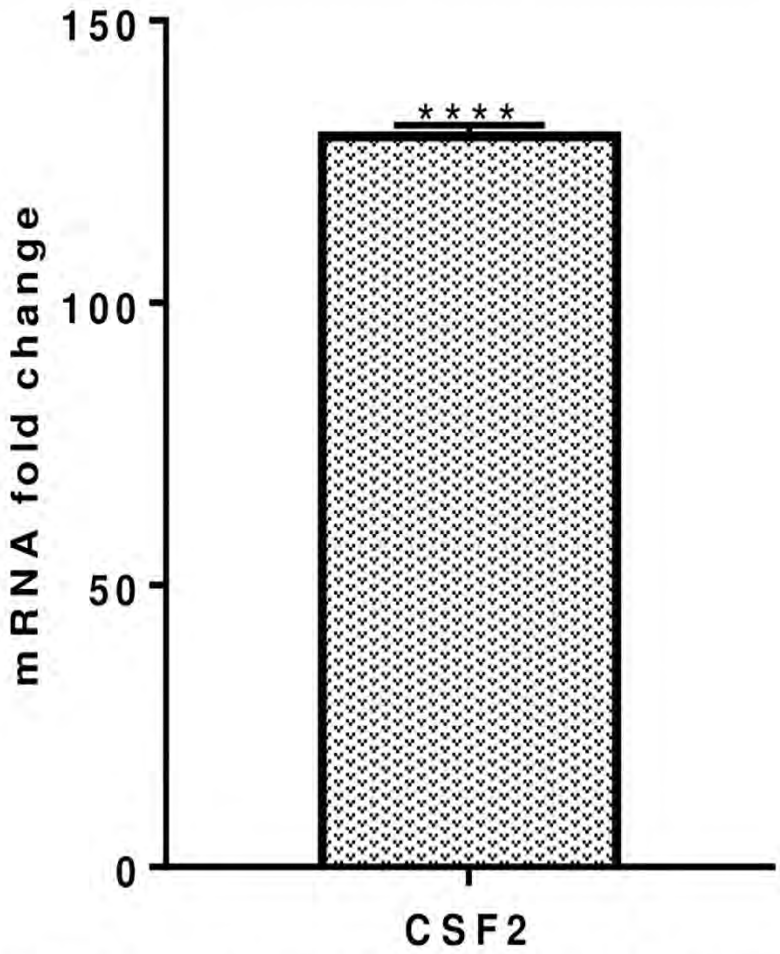

Fig 5. CSF-2, mRNA fold change following 24 h co-culture of *S. thermophilus* 285 with PBMCs (n = 3), compared to control PBMC. The innate and adaptive RT^2 gene profiler arrays were used to determine changes in gene expression. Symbols represent *p* value for Tukey Test (One way ANOVA) where **** $p < 0.01$.

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285 induced downregulation of TLRs at varying levels; TLR-1 (-2.63 ± 0.43), TLR-2 (-2.69 ± 0.8 fold), TLR-4 (-5.65 ± 0.56 fold), TLR-8 (-11.41 ± 1.27 fold) (Fig 6). However, changes to other pattern recognition receptors such as, TLR-3, TLR-5, TLR-6, TLR-9 were not significant.

3.5. Cell surface markers CD14, CD40, CD86 mRNA expression levels

Expression of the monocyte cell surface markers CD14, CD40 and CD86 significantly downregulated -25.29 ± 3.46, -15.39 ± 1.36, -8.04 ± 0.14 fold, respectively (Fig 7). Expression of the CD8A gene, which is involved in adaptive immunity and in response to defense against viruses, was downregulated by -2.96 ± 0.68 fold (Fig 7). Expression of CD4, CD80, FOXP3, STAT3, CD40LG (TNFSF5), HLA-A, HLA-E and RORC genes do not show significant changes.

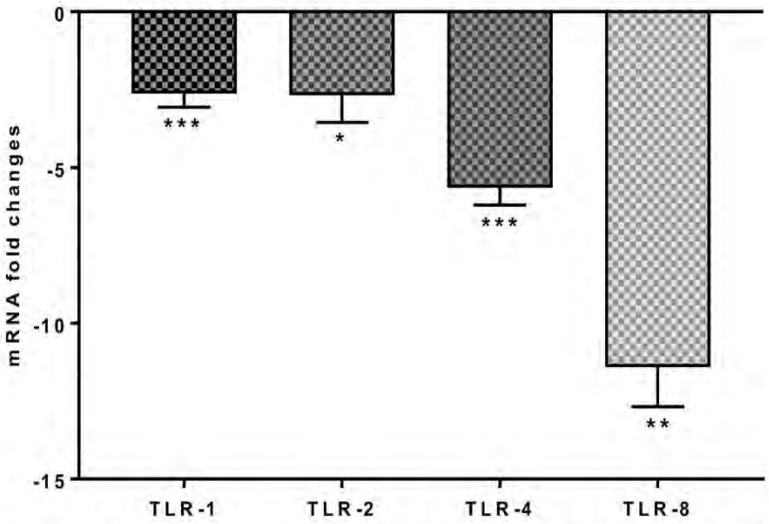

Fig 6. TLR-1, TLR-2, TLR-4 and TLR-8, mRNA fold change following 24 h co-culture of *S. thermophilus* 285 with PBMCs (n = 3), compared to control PBMC. The innate and adaptive RT^2 gene profiler arrays were used to determine changes in gene expression. Symbols represent *p* value for Tukey Test (One way ANOVA) where * $p < 0.05$, ** $p < 0.04$ and *** $p < 0.02$.

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3.6. Changes to other innate and adaptive molecules, mRNA expression levels

Changes to other genes were also noted following *S. thermophilus* 285 co-culture with PBMC. ACTB (-3.01 ± 1.0) fold, ITGAM (-2.76 ± 0.9) were both downregulated. Downregulated genes were noted to the following: MPO (2.33 ± 0.2), NLRP3 (2.11 ± 0.6), SLC11A1 (4.72 ± 0.23) and complement component (C)-3 (3.38 ± 1.5), TYK2 (10.03 ± 0.7), IRF7

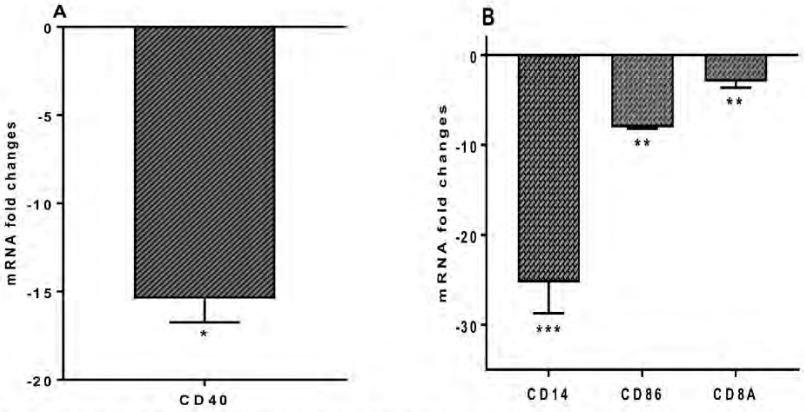

Fig 7. (A) CD40 and (B) CD14, CD86 and CD8A, mRNA fold change following 24 h co-culture of *S. thermophilus* 285 with PBMCs (n = 3), compared to control PBMC. The innate and adaptive RT^2 gene profiler arrays were used to determine changes in gene expression. Symbols represent *p* value for Tukey Test (One way ANOVA) where * $p < 0.05$, ** $p < 0.04$ and *** $p < 0.02$.

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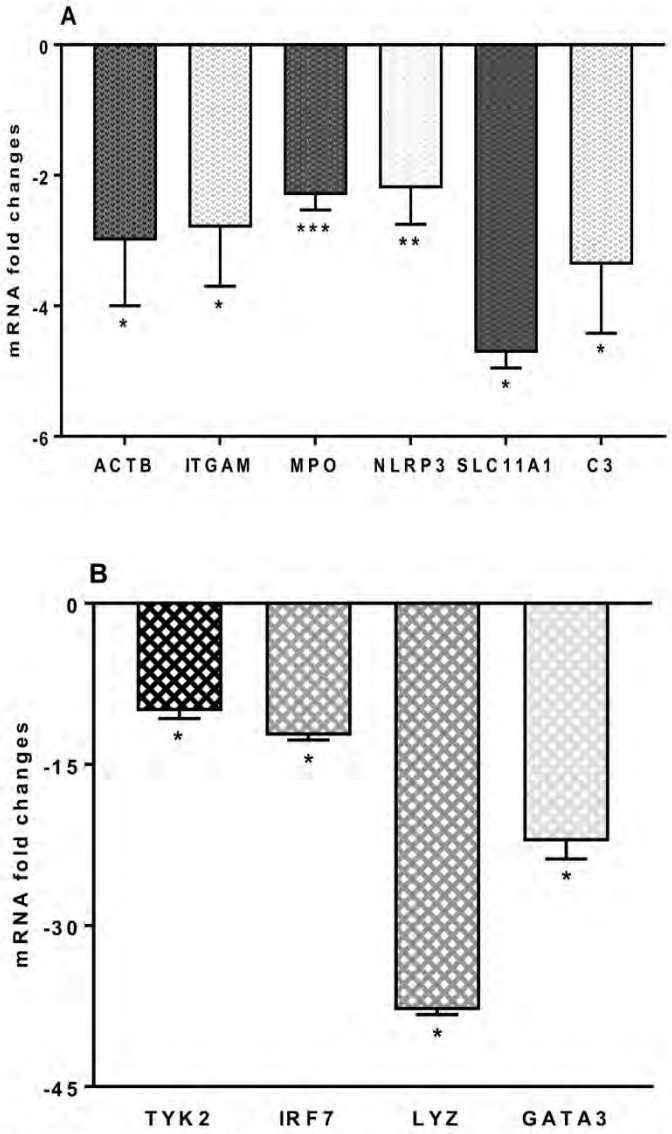

Fig 8. (A) ACTB, CCR5, ITGAM, MPO, NLRP3, SLC11A1, and C3 and (B) TYK2, IRF7, LYZ and GATA3, mRNA fold change following 24 h co-culture of *S. thermophilus* 285 with PBMCs (n = 3), compared to control PBMC. The innate and adaptive RT^2 gene profiler arrays were used to determine changes in gene expression. Symbols represent p value for Tukey Test (One way ANOVA) where * $p < 0.05$, ** $p < 0.04$ and *** $p < 0.02$.

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(12.32 ± 0.4), LYZ (37.91 ± 0.4) and GATA3 (22.15 ± 1.64) (Fig 8). Other immune markers including FASLG (TNFSF6), CRP, IFNAR1, JAK2, IL-1R1, MAPK8 (JNK1), IRF3, MBL2, NFKB1, MX1, ICAM1, MBL2, MYD88, NOD1 (CARD4), NOD2, DDX58 (RIG-I), RAG1 and TICAM1 (TRIF) showed no significant mRNA gene changes in the levels of their expression.

4. Discussion

Our previous publications illustrated immune modulatory effects of *S. thermophilus* 285, *S. thermophilus* 1275 and *S. thermophilus* 1342 on U937 monocytic cell line and human monocytes by using secreted cytokines for bioplex assays, as well as flow cytometry of immune cell surface marker changes. The current study, aimed to get a more comprehensive overview of the data, by undertaking an in depth gene array analysis of the effects of probiotics to human PBMC.

4.1. *S. thermophilus* 285 promotes Th2 polarization

IL-1α secreted by peripheral blood DC and B cells induces Th2 differentiation and inhibits Th1 polarization [37], is significantly upregulated. Similarly, *Enterococcus faecium* NCIMB 10415 was shown to upregulate IL-1α in porcine jejunal epithelial cells (IPEC-J2) *in vitro*, [38]. IL-6 produced by Th2 cells is increased in the presence of *S. thermophilus* 285 by PBMC which was also shown previously to be upregulated by pro-monocyte cell line U937 [1]. Others have shown that PBMC co-cultured with *S. thermophilus* 1275 also increases IL-6 [39]. Likewise, mixed probiotics of *S. thermophilus*, *Lactobacillus* (*L.*) *rhamnosus*, *L. casei*, *L. acidophilus*, *B. longum* and *B. bifidum* stimulated PBMC to produce IL-6 [40, 41]. Our study shows that IL-1α and IL-6 are increased, highlighting the role of *S. thermophilus* 285 in stimulation of immune responses involved in acute phase; B cell maturation, macrophage differentiation, promotion of Th2 differentiation and inhibiting Th1 polarization.

IL-10 is an anti-inflammatory cytokine secreted by Th2 and Treg cells and co-culture of *S. thermophilus* 285 with PBMC increased expression of IL-10. Cultured PBMC with other live *S. thermophilus* strain (*S. thermophilus* 1275) also showed increased IL-10 [22, 39, 42–45]. Similarly, in a study using mixed probiotic cultures (*S. thermophilus*, *L. rhamnosus*, *L. casei*, *L. acidophilus*, *B. longum* and B. *bifidum*) high levels of IL-10 were stimulated by PBMC [40]. Conversely, in a study using *B. breve* and *S. thermophilus* combined to stimulate PBMC, IL-10 was only increased in the presence of *B. breve*, whereas exposing PBMC to *S. thermophilus* reduced the IL-10 level [46]. We also previously noted that monocyte cell line (U937), co-cultured with *S. thermophilus* 1342 stimulated production of high levels of IL-10 [1].

IL-18 is involved in the initiation of severe inflammatory responses, indicating the role of IL-18 in inflammatory and autoimmune disorders. Co-culture of PBMC with *S. thermophilus* 285 significantly downregulated IL-18 which indicates an anti-inflammatory role for *S. thermophilus* 285 bacteria. Likewise, a mixture of Lactobacilli species (*L. rhamnosus*, *L. paracasei*, and *L. plantarum*) was shown to supress the secretion of pro-inflammatory IL-18 gene by undifferentiated IPEC-1 intestinal porcine epithelial cell line [47], highlighting supportive role of lactobacilli probiotics in functioning against inflammation and suppression of immune response activities. However, other studies with other probiotics such as, *L. rhamnosus* E509, *L. rhamnosus* GG E522 (ATCC 53103), *L. bulgaricus* E585 and *S. pyogenes* serotype T1IH32030, increased IL-18 production by human PBMC [48]. Hence, different probiotic strains induce different cytokine profiles.

IL-2 is involved in signalling of immune responses and activates proliferation of lymphocytes. We note downregulation of IL-2 gene expression in PBMC after exposure to ST285. IL-23 known to activate Th17 cells was upregulated although IL-17, the key pro-inflammatory cytokine secreted by Th17 cells was not altered. Upregulation of IL-1α, IL-6, IL-10, and downregulation of IL-2, IL-18 and an absence of change in IL-17A (despite increase in IL-23α) designates ST285 to possess anti-inflammatory effects on human PBMC.

4.2 *S. thermophilus* 285 stimulates expression of cytokines involved in the defence against bacteria

IFN-γ is an adaptive immunity cytokine secreted by Th1 cells in the defense response to microbes and viruses. IFN-γ is predominantly secreted by NK, NKT cells as part of the innate immune response, and by CD4 Th1 and $CD8^+$ T cells of the adaptive immune response [49]. *S. thermophilus* 285 upregulated IFN-γ gene expression by human PBMCs. This is similar to studies of a combination of probiotic strains including *S. thermophilus*, *Lactobacillus*, *Bifidobacterium*, *Propionibacterium*, *E. coli* and *Leuconostoc* [50], where upregulation of IFN-γ mRNA expression by PBMC was noted [50]. Likewise, co-cultures of pooled PBMC with ST1275 also induced upregulation of IFN-γ [39]. We previously noted that monocyte cell line co-cultured with *S. thermophilus* 1342, *S. thermophilus* 1275 or *S. thermophilus* 285 strains induced high levels of IFN-γ secretion [1]. In a study with Lactobacilli (*L. rhamnosus* E509, *L. rhamnosus* GG E522 (ATCC 53103) and *L. bulgaricus* E585), and streptococci (*S. pyogenes* serotype T1 IH32030), IFN-γ was produced by human PBMC [48].

IL-1β secretion by monocytes is involved in regulating immune and inflammatory responses to bacterial infections and injury, hence its role in innate immunity [51]. IL-1β is upregulated by *S. thermophilus* 285 co-cultured with PBMC, which is in accord with studies of PBMC co-cultured with mixed probiotics (*S. thermophilus*, *L. rhamnosus*, *L. casei*, *L. acidophilus*, *B. longum* and *B. bifidum*) [40]. We previously noted in monocyte cell line co-cultured with three different strains of *S. thermophilus*, only *S. thermophilus* 1342 stimulated production of high levels of IL-1β whereas, *S. thermophilus* 1275 and *S. thermophilus* 285 did not induce IL-1β cytokine [1]. A mixture of Lactobacilli strains (*L. rhamnosus*, *L. paracasei*, and *L. plantarum*) co-cultured with intestinal porcine epithelial cell line (IPEC-1) also upregulated IL-1β gene expression [47]. Similarly, the combination of *L. casei* Shirota, *L. rhamnosus* GG, *L. plantarum* NCIMB 8826 and *L. reuteri* NCIMB 11951, *B. bifidum* MF 20/5 and *B. longum* SP 07/3 co-cultured with PBMC, significantly augmented IL-1β production [41].

TNFα plays a key role in the defense against bacterial infections. It is a pro-inflammatory cytokine, which also supports recruitment and activation of T and B cells to promote an adaptive immune response. We previously demonstrated high levels of TNFα secretion by U937 monocyte cell line in the presence of *S. thermophilus* 1342, *S. thermophilus* 1275 and *S. thermophilus* 285 [1]. Likewise, our current findings show that ST285 co-cultured with PBMC results in upregulation of TNFα. However, in a study using *B. breve* and ST together to stimulate PBMC, TNF-α secretion was inhibited [46]. In addition, a mixture of strains of probiotics (*L. casei* Shirota, *L. rhamnosus* GG, *L. plantarum* NCIMB 8826 and *L. reuteri* NCIMB 11951, *B. bifidum* MF 20/5 and *B. longum* SP 07/3) co-cultured with PBMC, significantly increased the production of TNFα [41]. In another study of human PBMCs exposed to different probiotics (*L. mesenteroides* ssp. cremoris PIA2 (DSM 18892) *S. pyogenes* serotype T1M1, *S. thermophilus* THS, *E. coli* (DH5α), *L. rhamnosus* Lc705 (DSM 7061), *L. lactis* ssp. cremoris ARH74 (DSM 18891), *L. rhamnosus* GG (ATCC 53103), *L. helveticus* Lb 161, *L. helveticus* 1129, *B. longum* 1/10, *B. breve* Bb99 (DSM 13692), *B. animalis* ssp. lactis Bb12, and *Propionibacterium* (*P.*) *freudenreichii* ssp. shermanii JS (DSM 7067)), all induced TNF-α mRNA expression [50]. Given that IFNγ, IL-1β and TNFα are upregulated by PBMC following co-culture with *S. thermophilus* 285 this suggests that *S. thermophilus* 285 induces powerful defense against invading pathogens and could be beneficial against virus infection and tumours.

The upregulation of IFNγ, IL-1β and TNFα coupled with a significant decrease in IFNγ receptor and IL-18 shows an antagonising effect of *S. thermophilus* 285 inflammatory responses and leading to an overall anti-inflammatory profile.

4.3. *S. thermophilus* 285 activates mRNA expression of CXCL8 and downregulates CCR5 and CXCL10

IL-8, also known as CXCL8 is an important chemokine of the innate immune system, involved in the recrutiment of neutrophils and other granulocytes as the first line of defense [52]. *S. thermophilus* 1342, *S. thermophilus* 1275 and *S. thermophilus* 285 were previously shown to activate U937 monocyte cell line to produce high levels of IL-8 [1]. The probiotic *L. paracasei* DG also increases expression of IL-8 to the human monocyte cell line, THP-1 [53]. Likewise, short chain fatty acids, produced by probiotic bacteria, also stimulate IL-8 secretion and mRNA levels to the human epithelial cell line HT-29 [11]. These studies are in accord to our current findings that *S. thermophilus* 285 upregulates CXCL8 production by human PBMC.

C-C chemokine receptor type 5 (CCR5, CD195) is involved in Th1 immune responses and its gene expression is downregulated by PBMC following *S. thermophilus* 285 co-culture. However, in mice prolonged feeding with VSL#3 probiotic mixture shows significant gene upregulation of CCR5 [54]. Differences could be attributed to one probiotic strain applied and varying effects of the strain (*S. thermophilus*) used in current study versus a mixture of different strains and species used in mice VSL#3 (*L. delbruekii Bulgaricus*, *L. casei*, *L. plantarum*, *L. acidophilus*, *B. breve*, *B. longum*, *B. infantis* and *S. thermophilus*).

CXC motif chemokine 10 (CXCL10), or IFN-γ-induced protein-10 (IP-10), is secreted by a number of cell types (endothelial cells, monocytes and fibroblasts). Few roles have been ascribed to CXCL10 including chemo-attraction of NK cells, monocytes/macrophages, T cells and DCs, favouring adhesion of T cells to endothelial cells, anti-cancer/tumour action, and preventing angiogenesis and bone marrow colony development. CXCL10 is downregulated in PBMC culture following *S. thermophilus* 285 exposure. Conversely, monocyte-derived DCs co-cultured with *B. breve* Bb99, *L. lactics* subsp. cremoris ARH74 and *S. thermophilus* THS increased expression of CXCL10 and *S. thermophilus* was the most efficient probiotic in the induction of CXCL10 [23]. Additionally, microarray results of the intestines of mice prolonged administrated with VSL#3 probiotic mixture in healthy mice showed differential effects on intestinal immune parameters, including upregulation of CXC10 which contrasts with our findings [54]. The difference are most likely due to cell types, as well as bacterial strains in our study (PBMC co-cultured with *S. thermophilus* 285 bacteria) compared to using mouse cells exposed to three strains (*B. breve* Bb99, *L. lactics* subsp. cremoris ARH74 and *S. thermophilus* THS) in the other study. Also in the latter experiments, it is quite predictable to observe different results in mice intestine administered with VSL#3 due to different cells involved in mice study in contrast to PBMC cell population.

In summary, increased expression of IL-8 on its own could singularly be indicative of inflammation, but in the context of all other upregulated anti-inflammatory cytokine and mediators found in this study, this may not be interpreted as an inflammatory effect. IL-8 upregulation might also be interpreted as requirement for the initial stimulatory effect of *S. thermophilus* 285 to switch on the immune responses by initiating innate immunity, which by the progress of immune response, expression of CCR5 (which in turn influences Th1 immune responses), as well as CXCL10 (induced by IFNγ) are reduced by *S. thermophilus* 285. This might be suggestive of modulation of immune responses by *S. thermophilus* 285 to keep the adaptive immune responses in check.

4.4. *S. thermophilus* 285 significantly upregulates mRNA expression level of colony stimulating factor

CSF (GM-CSF) is secreted by machrophages, NK cells and T cells, enables cell proliferation and differentiation, stimulates the production of various immune cells, in particular it

increases the production of machrophages which are important in fighting againts infections. CSF-2, is vastly increased (130 fold) by PBMC co-cultured with *S. thermophilus* 285 which is in alignment to our previous data whereby *S. thermophilus* 1275, *S. thermophilus* 1342 and *S. thermophilus* 285 induced U937 monocyte cell line to secrete high levels of GM-CSF with ST285 being the highest inducer [1]. Likewise, another study used RT^2 Profiler PCR Arrays for mouse cytokines and chemokines to demonstrate that *L. rhamnosus* GR-1 (GR-1) induced high levels of granulocyte CSF (G-CSF) mRNA (60-fold) to bone marrow-derived mouse macrophages [55]. Likewise, PBMC co-cultured with *B. infantis* 52486 significantly increases GM-CSF [56].

GM-CSF is generally accepted as an inflammatory cytokine, its inflammatory activity is primarily associated with its role as granulocytes and macrophages growth and differentiation factor. GM-CSF-mediated inflammation has also been associated with certain types of autoimmune diseases such as rheumatoid arthritis and multiple sclerosis. However, in many instances GM-CSF plays anti-inflammatory/regulatory roles; GM-CSF can modulate differentiation of DC to render them into tolerogenic DCs, which, can stimulate anti-inflammatory Treg cells [57]. In addition, either of pro-inflammatory or regulatory effects of GM-CSF appears to be dependent on the amount of CSF and the presence of other relevant cytokines in the context of an immune response. There is also evidence that G-CSF induces expansion of IL-10-producing cells [58]. Our results show very high overexpression of CSF, which might be suggestive of anti-inflammatory effect of *S. thermophilus* 285 on PBMC.

4.5. *S. thermophilus* 285 downregulates mRNA expression levels of toll-like receptors

Toll-like receptors (TLRs) recognize pathogen-associated molecular patterns (PAMPs) that are expressed on infectious bacteria and mediate the production of cytokines necessary for the development of effective immunity [59]. TLRs recognize pathogens and activate the innate immune responses. TLR-1, TLR-2, TLR-4 and TLR-8 are part of the innate immune response and are involved in defense against bacteria. Co-culturing *S. thermophilus* 285 with human PBMC downregulated the expression of TLR. Similarly, *E. coli* K88 and mycotoxin zearalenone (ZEA) infection of IPEC-1 epithelial cell line was protected in the presence of mixed Lactobacillus strains (*L. acidofilus* ID11692, *L. plantarum* ID1253 and *L. paracasei* ID13239) by downregulating TLR-1, TLR-2 and TLR-4 gene expression [60].

TLRs are critical in bacterial recognition and host defence, such as lipo-teichionic acid (LTA) and lipo-polysaccharide (LPS) from Gram-positive and Gram-negative bacteria respectively [61, 62]. Activation of some of these molecules and mediators like TLR (especially TLR-2 and TLR-4) arbitrates to pro-inflammatory actions and further defensive functions of innate immunity [63–65]. The TLR-2 and TLR-4 activation and expression by LPS (pathogens) is known as one of the most important mechanisms by which the immune system controls reactions to bacteria in particular in the activation phase, therefore, over-expression of TLR-2 and TLR-4 during any bacterial infection could cause an elevated inflammatory response in the body. While early activation of TLRs expression is reported in response to bacterial LPS from pathogenic *Salmonella typhimurium* [61] as well as *E. coli* infection in bovine intestinal epithelial cells [66], our results show tolerance as a result of co-culturing PBMC with *S. thermophilus* 285 by down regulation of TLRs genes.

Downregulated mRNA expression of TLRs genes, specifically TLR-1, TLR-2, TLR-4 and TLR-8 indicates anti-inflammatory characteristics for *S. thermophilus* 285. Given that TLR-1, TLR-2, TLR-4 and TLR-8 are members of the innate immune response and play key roles in the defense against bacteria, downregulation of TLRs could be suggestive of a protective

mechanism to keep *S. thermophilus* 285 safe by tolerance towards *S. thermophilus* 285. Perhaps designing experiments that allow different incubation period, as well as adding pathogenic bacteria to the co-cultured *S. thermophilus* 285-PBMC can help to illustrate if lesser co-culture time and/or presence of pathogens can result in a shift towards upregulation of TLRs instead.

4.6. *S. thermophilus* 285 downregulates cell surface markers CD14, CD40, CD86

CD14, CD40 CD86 are expressed on the cell surface of monocytes, macrophages and DC. CD14 is expressed on the surface of monocytes and primarily binds to bacterial constituents [67–69]. We previously showed that U937 monocyte cell line exposed to *S. thermophilus* 1342, *S. thermophilus* 1275 or *S. thermophilus* 285 enhanced expression of CD14 after 24 and 48 hrs, and *S. thermophilus* 285 was the most potent at 48 hrs [1]. However, in bulk PBMC cultures, CD14 expression was significantly downregulated in the presence of *S. thermophilus* 285, which is in accordance with downregulation of TRLs in particular TRL-4. In other studies, the combination of 3 probiotics (*L. acidophilus*, *L. delbrueckii* ssp. *bulgaricus* and *B. bifidum*) stimulated increased expression of cell surface markers, CD14, CD80 and MHC class II [1]. *E. coli* Nissle 1917, widely used as a probiotic for the treatment of inflammatory bowel disorders, expresses a K5 capsule important in *E. coli* mediating interactions with intestinal epithelial cells and chemokine expression. *E. coli* Nissle 1917 has been shown to induce mRNA expression of CD14 by intestinal Caco-2 cells [70].

CD40 is a costimulatory protein on antigen presenting cells and is essential for their activation. CD40 is a key mediator in a wide range of inflammatory and immune responses and its gene expression was downregulated by PBMC in the presence of *S. thermophilus* 285. In previous experiments with U937 monocyte cell line, co-culture with *S. thermophilus* 1342, *S. thermophilus* 1275 or *S. thermophilus* 285, resulted in small increase in CD40 [1].

CD86 (B7-2) is expressed on APCs and delivers co-stimulatory signals required for the activation and survival of T cells. CD86 plays the role of the ligand for T cells external CD28, and CTLA-4 (CD28) in regulation and cell to cell dis-association. CD86 acts in conjunction with CD80 to prime Th cells, delivering opposing functions on Treg cells through CTLA-4 and T cell surface CD28 protein. Expression of CD86 by PBMC is downregulated significantly, suggesting an anti-inflammatory profile following exposure to *S. thermophilus* 285. *S. thermophilus* bacteria promote CD86 expression required for T cell activation and the maintenance of immune responses, CD86 downregulation by *S. thermophilus* 285 suggests a regulating and damping effect of *S. thermophilus* 285 on PBMC, being interpreted as immunomodulation of adaptive immunity [71]. We previously noted using U937 monocyte cell line in the presence of *S. thermophilus* 1342, *S. thermophilus* 1275 and *S. thermophilus* ST285 increased expression of CD86 [1]. Similarly, *L. plantarum* WCFS1 and *L. fermentum* GR1485 upregulate CD86 on monocytes, conversely, *L. rhamnosus* and *L. delbruekii* reduced its expression [72].

Additionally, monocytes isolated from PBMC and differentiated into immature DCs by GM-CSF and IL-4, and co-cultured with *B. breve* Bb99, *L. lactis* subsp. cremoris ARH74 and *S. thermophilus* THS also increase CD86 expression [23]. Another study used bone marrow-derived DCs from DQ8-transgenic mice and co-culture with *L. plantarum* and *L. paracasei* and *B. lactis* increases CD86 differentially with the highest CD86 being noted in co-administration of *L. plantarum* and *L. paracasei* [73]. The contrast between these studies to the findings herein could be due to the differences in the nature of studies; we co-cultured PBMC with *S. thermophilus* 285 bacteria only and the other studies used mouse bone marrow-derived DCs co-cultured with three different probiotics leading to predictable differences.

Given the downregulation of cell surface markers and their roles in immunity, CD14 (involved in innate immunity), CD40 (involved in innate immunity), and CD86 (T cell activation), following *S. thermophilus* 285 co-culture is suggestive of an anti-inflammatory anti-activation profile for *S. thermophilus* 285. In addition, as all these cell surface markers are interlinked with defence against bacteria either through innate or adaptive immune responses, downregulation of these markers could be suggestive of *S. thermophilus* 285 initiating self-tolerance via regulating immune responses, which in turn modulates the immune responses too.

4.7. *S. thermophilus* 285 differentially downregulates mRNA expression level of other innate and adaptive immune response markers and chemokines

Complement component 3 (C3) is associated with complement cascades in immune responses by enhancing antibody function, phagocytosis and stimulation of inflammation [74–76]. GATA3 transcriptome is also important in both humoral immunity and inflammatory responses. Downregulation of C3 gene expression and significant reduced expression of GATA3 transcriptome by PBMC co-cultured with *S. thermophilus* 285 in noted. Similarly, lipoteichoic acid (p-LTA) extracted from *L. plantarum* K8 inhibits C3 mRNA *in vitro* and *in vivo*. In human clinical studies, blocking GATA3 is able to control allergy responses, inflammatory diseases and asthma [77]. C3 and GATA3 downregulation suggests that *S. thermophilus* 285 is able to lower inflammation (C3), as well as being a viable candidate for further pre-clinical and clinical studies for the management of such diseases.

Interferon regulatory factor (IRF) 7, integrin alpha M (ITGAM), Lysozyme (LYZ) and NALP3 are other innate immune response factors. IRF7, a member of IRF family and present on monocytes, macrophages, granulocytes, and NK cells, and expressed predominantly in macrophages (a component of the inflammasome) [78]. IRF7 plays a role in the transcriptional activation of virus-inducible cellular genes, including the type I interferon genes. ITGAM is involved in a number of inflammatory responses (i.e. cell-mediated cytotoxicity, phagocytosis, and chemotaxis). LYZ acts as an antimicrobial enzyme present in neutrophils and macrophages. IRF7 gene regulation decreased considerably along with ITGAM gene expression, which is downregulated when PBMC are co-cultured with *S. thermophilus* 285. NALP3 and LYZ are downregulated markedly in co-culture of PBMC with *S. thermophilus* 285. However, in a previous study, we showed significant upregulation of CD11b (ITGAM) by monocytic U937 cells when co-cultured with *S. thermophilus* 1342, *S. thermophilus* 1275 and *S. thermophilus* 285 bacteria [1]. *S. thermophilus* 285-induced downregulation of IRF7, ITGAM, NALP3 and LYZ in PBMC, suggestive of an anti-inflammatory effect of *S. thermophilus* 285 on PBMC as well.

Non-receptor tyrosine-protein kinase (TYK2) is an enzyme [7] that contributes to adaptive immune responses due to its implication in IFNα, IL-6, IL-10 and IL-12 signaling, also involved in transducing signals of IL-6, IL-10 and IL-23. TYK2 gene expression is significantly downregulated in PBMC co-cultured with *S. thermophilus* 285, supporting an anti-inflammatory profile for *S. thermophilus* 285. In addition, myeloperoxidase (MPO), an enzyme abundantly expressed by neutrophils and promotes inflammation, is also involved in autoimmune disorders (multiple sclerosis, rheumatoid arthritis) [79, 80]. A decreased expression of MPO has been suggested to manage these autoimmune disorders by decreasing the inflammatory state. *S. thermophilus* 285 co-cultured with PBMC decreased the expression of MPO, suggestive of an anti-inflammatory benefit of *S. thermophilus* 285.

IFNAR1 is a type I membrane protein which is a receptor for IFNα and IFNβ involved in defence against viruses. IFNAR1 signaling is associated with pro-inflammatory cytokine

Given the downregulation of cell surface markers and their roles in immunity, CD14 (involved in innate immunity), CD40 (involved in innate immunity), and CD86 (T cell activation), following *S. thermophilus* 285 co-culture is suggestive of an anti-inflammatory anti-activation profile for *S. thermophilus* 285. In addition, as all these cell surface markers are interlinked with defence against bacteria either through innate or adaptive immune responses, downregulation of these markers could be suggestive of *S. thermophilus* 285 initiating self-tolerance via regulating immune responses, which in turn modulates the immune responses too.

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Interferon regulatory factor (IRF) 7, integrin alpha M (ITGAM), Lysozyme (LYZ) and NALP3 are other innate immune response factors. IRF7, a member of IRF family and present on monocytes, macrophages, granulocytes, and NK cells, and expressed predominantly in macrophages (a component of the inflammasome) [78]. IRF7 plays a role in the transcriptional activation of virus-inducible cellular genes, including the type I interferon genes. ITGAM is involved in a number of inflammatory responses (i.e. cell-mediated cytotoxicity, phagocytosis, and chemotaxis). LYZ acts as an antimicrobial enzyme present in neutrophils and macrophages. IRF7 gene regulation decreased considerably along with ITGAM gene expression, which is downregulated when PBMC are co-cultured with *S. thermophilus* 285. NALP3 and LYZ are downregulated markedly in co-culture of PBMC with *S. thermophilus* 285. However, in a previous study, we showed significant upregulation of CD11b (ITGAM) by monocytic U937 cells when co-cultured with *S. thermophilus* 1342, *S. thermophilus* 1275 and *S. thermophilus* 285 bacteria [1]. *S. thermophilus* 285-induced downregulation of IRF7, ITGAM, NALP3 and LYZ in PBMC, suggestive of an anti-inflammatory effect of *S. thermophilus* 285 on PBMC as well.

Non-receptor tyrosine-protein kinase (TYK2) is an enzyme [7] that contributes to adaptive immune responses due to its implication in IFNα, IL-6, IL-10 and IL-12 signaling, also involved in transducing signals of IL-6, IL-10 and IL-23. TYK2 gene expression is significantly downregulated in PBMC co-cultured with *S. thermophilus* 285, supporting an anti-inflammatory profile for *S. thermophilus* 285. In addition, myeloperoxidase (MPO), an enzyme abundantly expressed by neutrophils and promotes inflammation, is also involved in autoimmune disorders (multiple sclerosis, rheumatoid arthritis) [79, 80]. A decreased expression of MPO has been suggested to manage these autoimmune disorders by decreasing the inflammatory state. *S. thermophilus* 285 co-cultured with PBMC decreased the expression of MPO, suggestive of an anti-inflammatory benefit of *S. thermophilus* 285.

IFNAR1 is a type I membrane protein which is a receptor for IFNα and IFNβ involved in defence against viruses. IFNAR1 signaling is associated with pro-inflammatory cytokine

production [81]. In fact, IFNAR1 knockout mice show decreased pro-inflammatory cytokiens and chemokines [81]. IFNAR1 is significantly downregulated by PBMC following co-culture with ST285, supporting an anti-inflammatory role of *S. thermophilus* 285. In addition, SLC11A1 involved in T cell activation, is involved in inflammatory disorders such as autoimmune type 1 diabetes [82, 83], is downregulated by PBMC in the presence of *S. thermophilus* 285. Furthermore, the Beta-actin (ACTB) which stimulates eNOS and increase nitric oxide (NO) [84] involved in immunity and inflammation [85], is downregulated by PBMC following co-culture with *S. thermophilus* 285.

We determined the immune modulatory effects of *S. thermophilus* 285 to human PBMC and show that it has an array of anti-inflammatory immune-modulatory properties. *S. thermophilus* 285 decreases mRNA expression IL-18, IFNγR1, CCR5, CXCL10, TLR-1, TLR-2, TLR-4, TLR-8, CD14, CD40, CD86, C3, GATA3, ITGAM, IRF7, NLP3, LYZ, TYK2, IFNR1, and upregulates IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-23, IFNγ, TNFα, CSF-2. No changes to mRNA expression are noted with IFNA1, IFNB1, IL-4, IL-5, IL-13, CCL2, CCL5, CCL8, CCR4, CCR8, CXCR3, TLR-3, TLR-5, TLR-6, TLR-9, CD4, CD80, FOXP3, STAT3, CD40LG, HLA-A, HLA-E, RORC. The data demonstrates a predominant anti-inflammatory profile exhibited by *S. thermophilus* 285, and further work is required to determine its effects in inflammatory disease models *in vitro* and *in vivo*, such as multiple sclerosis, inflammatory bowel disease and allergies. Future investigations using RNA-Seq and Western blots are some of the next logical steps to confirm and further investigate these results.

5. Conclusion

Probiotics are beneficial microorganism with immunomodulatory properties, which aid the maintenance of a healthy immune system. *S. thermophilus* is often used in fermented dairy products such as cheeses and yogurts and is believed to potentially have health benefits. We determined the immune modulatory effects of *S. thermophilus* 285 to human peripheral blood mononuclear cells and show that it has an array of anti-inflammatory immune-modulatory properties. *S. thermophilus* 285 decreases mRNA expression IL-18, IFN receptor, CCR5, CXCL10, TLR-1, TLR-2, TLR-4, TLR-8, CD14, CD40, CD86, C3, GATA3, ITGAM, IRF7, NLP3, LYZ, TYK2, IFNR1, and upregulates IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-23, IFN-γ, TNF-α, CSF-2. No changes to mRNA expression were noted with IFNA1, IFNB1, IL-4, IL-5, IL-13, CCL2, CCL5, CCL8, CCR4, CCR8, CXCR3, TLR-3, TLR-5, TLR-6, TLR-9, CD4, CD80, FOXP3, STAT3, CD40LG, HLA-A, HLA-E, RORC.

Supporting information

S1 Table. List of the innate or adaptive genes and housekeeping genes; their symbols, full name and description that are investigated in in this study.
(DOCX)

S1 Fig. Presentation of data as a heatmap of average gene/RNA expressions of PBMC (n = 3) co-cultured with *S. thermophilus* ST285, compared to control. Green represents down regulated genes to red represents upregulated genes.
(DOCX)

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Design and Synthesis of Non-Peptide Mimetics Mapping the Immunodominant Myelin Basic Protein ($MBP_{83–96}$) Epitope to Function as T-Cell Receptor Antagonists

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Abstract: Encephalitogenic T cells are heavily implicated in the pathogenesis of multiple sclerosis (MS), an autoimmune demyelinating disease of the central nervous system. Their stimulation is triggered by the formation of a trimolecular complex between the human leukocyte antigen (HLA), an immunodominant myelin basic protein (MBP) epitope, and the T cell receptor (TCR). We detail herein our studies directed towards the rational design and synthesis of non-peptide mimetic molecules, based on the immunodominant $MBP_{83–96}$ epitope that is recognized by the TCR in complex with HLA. We focused our attention on the inhibition of the trimolecular complex formation and consequently the inhibition of proliferation of activated T cells. A structure-based pharmacophore model was generated, in view of the interactions between the TCR and the HLA-$MBP_{83–96}$ complex. As a result, new candidate molecules were designed based on lead compounds obtained through the ZINC database. Moreover, semi-empirical and density functional theory methods were applied for the prediction of the binding energy between the proposed non-peptide mimetics and the TCR. We synthesized six molecules that were further evaluated in vitro as TCR antagonists. Analogues **15** and **16** were able to inhibit to some extent the stimulation of T cells by the immunodominant $MBP_{83–99}$ peptide from immunized mice. Inhibition was followed to a lesser degree by analogues **17** and **18** and then by analogue **19**. These studies show that lead compounds **15** and **16** may be used for immunotherapy against MS.

Keywords: multiple sclerosis; trimolecular complex; rational drug design; non-peptide mimetics; molecular modeling; cell proliferation; T cell antagonism

1. Introduction

Multiple sclerosis (MS) is an immunologically controlled, inflammatory, demyelinating disease, described as the destruction of the myelin sheath of the central nervous system, which can lead to

paralysis [1,2]. Although evidence suggests the important role of B-cells (auto-antibodies), T helper (Th)-17 cells, and $CD8^+$ T cells in disease pathogenesis [3], it is well regarded that $CD4^+$ Th1 cells contribute to initiation and progression of disease. Indeed, $CD4^+$ T cells have been identified in patients with MS to react to self-peptide epitopes within the myelin sheath, including that of myelin basic protein (MBP), proteolipid protein, myelin oligodendrocyte glycoprotein, and myelin associated glycoprotein [4,5]. In the context of MS, encephalitogenic T cells are activated through the formation of a trimolecular complex between the T cell receptor (TCR), a short 14–18 amino acid myelin peptide (epitope), and the major histocompatibility complex (MHC) class II. In fact, the MHC class II, human leukocyte antigen (HLA) locus is the most closely correlated genetic locus to the development of MS, in particular HLA-DR1, HLA-DR2, and HLA-DR4 [6–8]. In humans, the MHC class II (HLA) consists of dimers (the α chain and the β chain) [9,10], which present short antigenic peptide epitopes to $CD4^+$ Th cells, resulting in the formation of the trimolecular complex (HLA-peptide-TCR). The TCR is also composed of two different polypeptide chains (α and β chains) that consist of variable domains (complementarity determining regions; CDRs). CDRs are implicated in the recognition of the TCR to HLA-peptide complex, and their structural diversity plays a crucial role in the recognition of the different antigens presented to T cells by antigen presenting cells [11,12]. In fact, there are more than 2.5×10^7 unique TCR (CDRs) structures in humans [12]. In addition, the rigorous positive and negative selection process of T cells in the thymus does not prevent auto-reactive T cells from escaping thymic deletion [13–15], thus initiating the development of autoimmune disorders such as MS.

In patients with MS, T cell responses are primarily associated with recognition of the 81–105 region of MBP (QDENPVVHFFKNIVTPRTPPPSQGK) [16], with the $MBP_{83–99}$ (ENPVVHFFKNIVTPRTP) peptide epitope displaying the strongest binding to HLA-DR2 [17,18], $MBP_{83–96}$ being the minimal recognized epitope. T cell recognition of $MBP_{83–96}$ has also been shown in healthy individuals, albeit at relatively low precursor frequencies [19]. Hence, the immunodominant $MBP_{83–96}$ epitope plays an important role at inducing $CD4^+$ T cells, which contribute to the demyelination process, and it is therefore considered one of the main targets for developing molecular therapeutics [20,21]. The primary binding residues of $MBP_{83–96}$ to HLA-DR2 are via hydrophobic V^{87} and F^{90}, which anchor the peptide into pockets P1 and P4, respectively, as noted in the HLA-DR2-peptide-TCR crystal structure [22]; albeit at a low resolution of 3.5 A, this structure served as the basis of all future studies of MBP peptides interacting with HLA-DR2. Additionally, other crystal structures reported in the RCSB databank [23,24] that address the role of MBP immunodominant epitopes in MS induction contain the same TCR sequence. Furthermore, it was noted that a second step in the T cell activation process involves the recognition of His^{88} and Phe^{89}, which are placed in pockets P2 and P3 of the TCR [22], with secondary binding residues being Val^{86} and Lys^{91}, which are oriented in pockets P-1 and P5 of the TCR [22]. Thus, a detailed analysis of the interactions between HLA-$MBP_{83–96}$-TCR complexes would lead to valuable information towards rational design of non-peptide mimetics with inhibitory activity. Indeed, a number of studies have shown that using antagonist peptides (1–2 amino acid mutations to TCR contact residues), or altered peptide ligands, can effectively modulate T cell responses and switch from pro- to anti-inflammatory responses [25–35]. In addition, using a computational structure-based approach, non-peptide mimetics of small organic compounds that were able to bind to MHC class II and block the presentation of $MBP_{152–185}$ to auto-reactive T cells were identified [36].

The principal goal of this study was the rational design of non-peptide mimetic molecules that could bind to the TCR with increased affinity and not to the MHC–peptide complex. Such potential inhibitors would prevent the formation of the trimolecular complex and consequently the stimulation of T cells. To this end, robust computational techniques, such as molecular docking, pharmacophore modeling, and molecular dynamics, were utilized for the design of novel TCR inhibitors. The application of pharmacophore modeling in the trimolecular complex (HLA-$MBP_{83–96}$-TCR) allows the differentiation between the different contributions (e.g., electrostatic and van der Waals interactions, hydrogen donors and acceptors) involved in the epitope recognition process. By analyzing the variations in these aspects, it is possible to search through diverse chemical databases and filter the results for the identification of

potential lead TCR antagonists (hits). Furthermore, molecular docking methodologies can be implemented in order to identify and isolate common substructures of the top ranking hits. Subsequently, the analogue with the best docking score (lead molecule) and preferable structural orientation over the TCR is selected for further optimization and this optimized structure then opts for increased interactions with the TCR. Molecular dynamics (MD) simulations and molecular orbital calculations were carried out in the optimized hits in order to evaluate their binding to the TCR. Finally, the proposed analogues were synthesized to evaluate their biological activity against MBP_{83-99} primed mouse T cells and to human peripheral blood T cells.

2. Results and Discussion

2.1. Pharmacophore Modeling and Virtual Screening

In computational drug discovery, screening of large databases with chemical property information obtained from relatively small data is essential. The combination of results from structure- and ligand-based pharmacophore models allows a thorough search in order to discover potential antagonists. The proposed pharmacophore model (Figure 1) is based on features such as an aromatic ring (Aro, green), a hydrogen bond (HB), cation and donor (Cat, magenta), hydrophobic groups (Hyd, orange), and volume exclusion (V, gray). The detailed parameters utilized for the construction of the model are described in the Materials and Methods section. The key features are based on residues His^{88} and Phe^{89} (Aro, Figure 1, green sphere), Val^{86} (Hyd, Figure 1 orange sphere), and Pro^{85} (Cat, Figure 1, magenta sphere). The grey spheres in Figure 1 represent residues with bulky side chains, such as Val^{87} and Phe^{90}, that do not interact with the TCR. These residues are employed to define the Volume Exclusion (V) feature of the pharmacophore model. This information is important for excluding residues that interact with the HLA receptor and consequently are not involved in key interactions with the TCR.

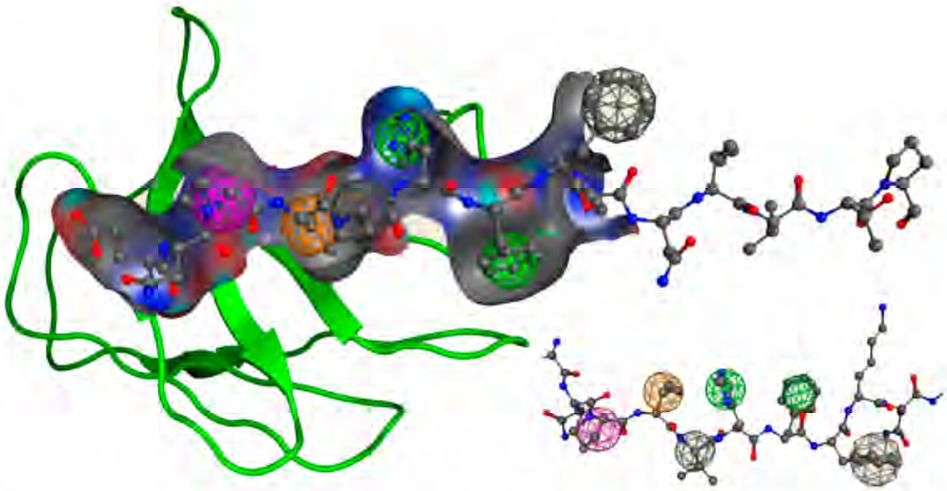


Figure 1. The proposed pharmacophore model, based on the myelin basic protein MBP_{83-96} epitope, with the relevant features depicted as spheres (Aro: green; Cat: magenta; Hyd: orange; V: gray). Only the binding cavity of the T cell receptor (TCR) is presented in the figure as surface and ribbons. The residues of the MBP_{83-96} are depicted as sticks.

The next step was the implementation of the pharmacophore model for the virtual screening of chemical databases. As described in the Materials and Methods section; the ZINC database was employed in the virtual screening process. The combinatorial information yielded from the pharmacophore model was employed as the starting point of our search. The examination of

compounds in databases yielded a total of 340 potential inhibitors (hits). A subsequent visual analysis revealed 13 molecules (compounds **1**–**13**, Table 1) with binding conformations that closely resembled the positioning of the $MBP_{83–96}$ epitope inside the TCR binding cavity (Tables 1 and S1).

Table 1. Chemical structure and docking scores of the proposed potential T cell receptor (TCR) antagonists (compounds **1**–**19**).

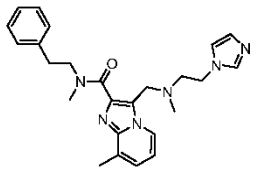
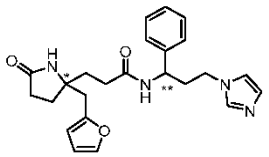
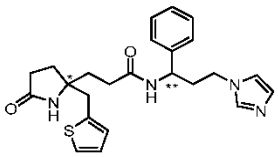
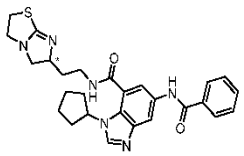
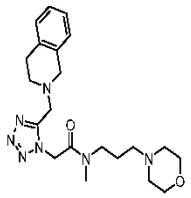
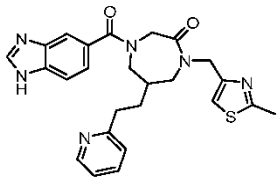
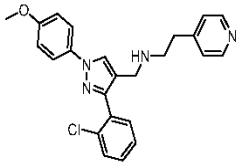
Compound Number	Structure	ΔG [d] (kcal/mol)
$MBP_{83–96}$	Seq: ENPVVHFFKNIVTP	−11.89
1 [a]		−15.87
2 (* S/** R)		−19.71
3 (* S/** S)		−14.46
4 (* R)		−14.43
5		−10.32
6		−15.34
7		−16.38

Table 1. *Cont.*

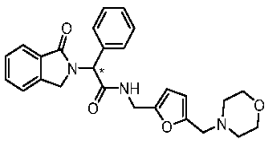
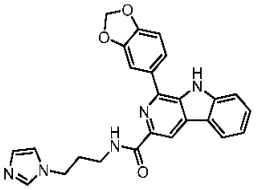
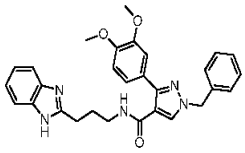
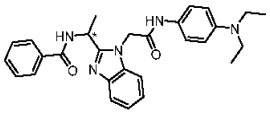
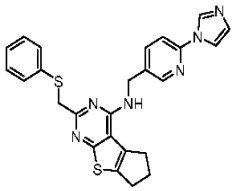
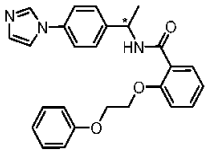
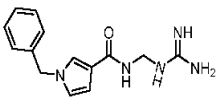
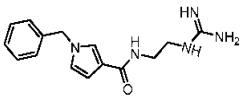
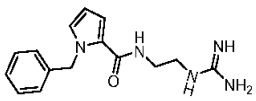
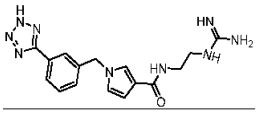
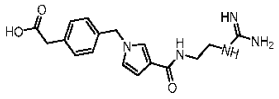
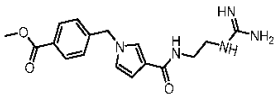
Compound Number	Structure	ΔG [d] (kcal/mol)
8 (* S)		−13.26
9		−15.86
10 (*Lead Compound*)		−21.56
11 (* R)		−20.85
12		−16.05
13 (* S)		−20.65
14 [b]		−23.76
15		−18.13
16		−18.03
17 [c]		−18.49

Table 1. *Cont.*

Compound Number	Structure	ΔG [d] (kcal/mol)
18		−20.70
19		−21.32

[a] Compounds **1**–**13** were obtained from the pharmacophore model. [b] Compounds **14**–**16** were derived through modifications of the lead compound **10**. [c] Compounds **17**–**19** were modified analogues of compound **15**. [d] Docking score as calculated by the MOE2016 software at 298 K.

2.2. *Lead Optimization and Molecular Docking Calculations*

All the selected molecules were visualized in MOE2010, while their structural orientation and binding with the TCR were assessed. Each of the potential hits was subjected to molecular docking calculations, and the results are presented in Table 1. The analysis of the docking experiments showed that, of the 13 compounds obtained from the pharmacophore screen, compound **10** presented with the highest docking score (−21.56 kcal/mol) inside the TCR binding cavity, while the lowest docking score was reported for compound **5** (−10.32 kcal/mol). This suggests that compound **10** may be considered the best candidate for lead optimization. The formation of only 2 hydrogen bonds with residues AspA92 and GlyA96 of the TCR is noted along with the existence of a π-stacking interaction between the aromatic rings of the compound and the side chain of TyrA98 in the TCR. Despite the favorable interactions between analogue **10** and the TCR, the bulky nature of the lead compound prevents the better positioning of the molecule inside the binding cavity.

The optimization process for target compound **10** included the removal and addition of functional groups in order to improve the placement of the molecule inside the selected TCR pockets and subsequently increase the interactions (Figure 2a). As depicted in Figure 2a, the substituted aromatic ring was removed to decrease the bulky nature of the potential inhibitor. The benzimidazole was replaced by a guanidino group (Figure 2a) to enhance the hydrogen bonding potential of the designed inhibitor. This preliminary study led to the identification of compound **14** as drug-target (Table 1 and Figure 2b). The next step was the setup of a molecular docking simulation for compound **14** in the TCR. The results of the docking experiments show that the alterations in compound **14** increase its binding affinity inside the TCR compared to the lead compound **10** (−23.76 to −21.56 kcal/mol, Table 1). The ligand pose with the best docking score for compound **14** (Figure 2b) presented the formation of six hydrogen bonds with residues of the TCR. In addition to the hydrogen bond interactions with amino acids AspA92 and GlyA96, the optimized compound further interacts with residues AsnA30 and ThrA97 (Figure 2b). The improved interaction, via the increased number of hydrogen bonds, may further explain the better binding affinity of compound **14**, due to the more favorable positioning inside the binding cavity of the receptor. As expected the π-stacking interaction with TyrA98 in the TCR is retained in the new optimized compound, further enhancing its binding.

As stated in the Materials and Methods section, the filtering process of the pharmacophore search was based on Lipinski's rule of five. Properties such as size (molecular weight, MW), hydrophobicity content (logP), and Total Polar Surface Area (TPSA) were recorded for the potential candidates (Table S1). The lead compound (compound **10**) was selected due to its high binding affinity (Table 2) and its better positioning inside the TCR binding cavity. The optimization process that led to the design of compound **14** aimed to enhance the binding affinity as well as to improve its positioning deeper within the TCR. Additionally, the modifications in the lead compound were intended to reduce its hydrophobic content (logP) and increase the polar surface area of the proposed inhibitor (Table 2). The smaller size of optimized compound **14** (MW = 272.33, Table 2), showed a notable decrease

in its hydrophobic content (−0.84 from 5.25 of compound **10**, Table 2) and an increase in its TPSA (Table 2). Both of these chemical properties are indicators of compound's membrane/cell permeability. Compound **14** proved better potential absorption properties than the lead compound, as indicated by the logP and TPSA values.

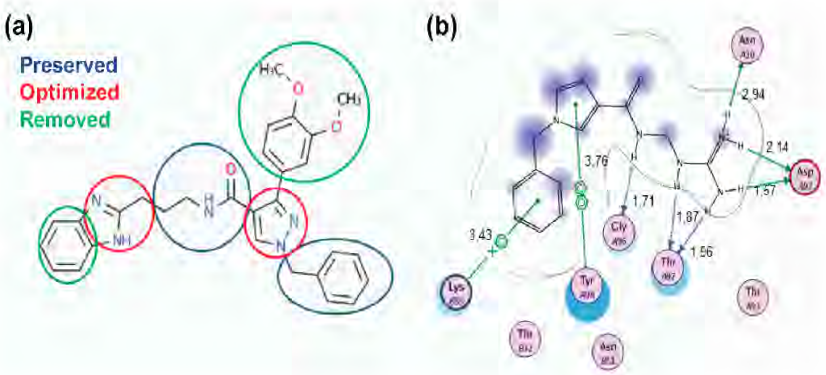


Figure 2. (**a**) Optimization process for the lead compound **10**. The preserved groups are presented in blue, while the optimized groups are shown in red and the removed ones in green; (**b**) best docking pose of compound **14** inside the TCR binding site, showing the different interactions. Green arrow: Hydrogen Bond (HB) formed with the side chain of the residue; Blue arrow: HB formed with the backbone; Blue shade: Solvent accessible surface area (SASA) of the ligand; Turquoise halo: SASA of the receptor residues; Green dotted lines: π-π interactions between two phenyl rings or interaction of a cation (+) with a π system (phenyl ring).

Table 2. Properties of lead compound **10** and optimized analogues **14**–**19**.

Compound	MW (g/mol)	TPSA (Å^2)	logP	Docking Score (kcal/mol)
10	495.57	94.06 [a]	5.25 [b]	−21.56
14	272.33	97.67	−0.84	−23.76
15	286.36	97.67	−0.84	−18.13
16	286.36	97.67	−0.71	−18.03
17 [c]	354.40	152.13	−1.62	−18.49
18	344.39	137.34	−1.49	−20.70
19	344.39	126.34	−1.42	−21.32

[a,b] Total Polar Surface Area (TPSA) and hydrophobicity content (logP) values are reported as shown on Ambinter Chemicals catalogue: http://www.ambinter.com/. [c] Compounds **17**–**19** are modified analogues of compound **15**.

Based on the calculated properties of the compounds **10** and **14** (logP and TPSA, Table 2), we aimed to further optimize analogue **14** through small changes in the compound's backbone to explore whether an additional increase in binding affinity is possible. Thus, two new target molecules **15** and **16** were obtained (Table 2, Figure 3); the 3–substituted pyrrole ring with an additional methylene group (–CH_2–) between the amide bond and the guanidino group, compound **15** (Figure 3a), and its 2–substituted pyrrole ring isomer, compound **16** (Figure 3b). The addition of the methylene group aimed to improve the positioning of the guanidino group in the P2 pocket of TCR. As expected, this variation increases the molecular weight but does not affect the hydrophobicity content, and the TPSA values of the two derivatives in comparison to compound **14** (Table 2). Molecular docking simulations were also carried out for analogues **15** and **16** in complex with the TCR, and the results are reported in Table 2 and Figure 3. The reported interactions for compounds **15** and **16** show the retention of the hydrogen bonds with AspA92 and GlyA96 (Figure 3a,b), while the addition of the methylene group prevents the interactions with residues AsnA30 and ThrA97 reported for compound **14** (Figures 2b and 3a,b). The absence of these interactions, compared to compound **14**, may explain the differences observed for the binding affinities of the two derivatives **15** and **16** (Table 2).

The abolition of interactions with residues AsnA30 and ThrA97 for analogue **15** and the subsequent decrease in the binding affinity compared to compound **14** (Table 2) led to the design of derivatives **17**–**19** (Figure 3c–e). The analogues include meta– (compound **17**) and para– (compounds **18** and **19**) substitutions of the aromatic ring in compound **15**. The meta– substitution with the tetrazole group in compound **17** restores the hydrogen bond with ThrA97 (Figure 3c). Furthermore, the tetrazole interacts via the formation of a hydrogen bond with TyrA98 (Figure 3c). The new interactions between the compound and TCR residues are mirrored in the increased docking score of the molecule as reported in Table 2. The para $-CH_2COOH$ substitute (compound **18**) retains the interactions of analogue **15** with AspA92 and GlyA96, while creating hydrogen bonds with residues AsnB51 and LysB55 (Figure 3d). The amino acids AsnB51 and LysB55 are located in the TCR binding site, opposite to AspA92 and GlyA96, thus enhancing the positioning of derivative **18** in the TCR binding cavity. A similar pattern of interactions inside the TCR cavity is observed for the para– methyl ester substituent (compound **19**, Figure 3e). Again, the methyl ester group allows the compound to be better oriented inside the binding site. The possible advantageous positioning of compounds **18** and **19** is mirrored in their docking scores (−20.70 and −21.32 kcal/mol, respectively, Table 2).

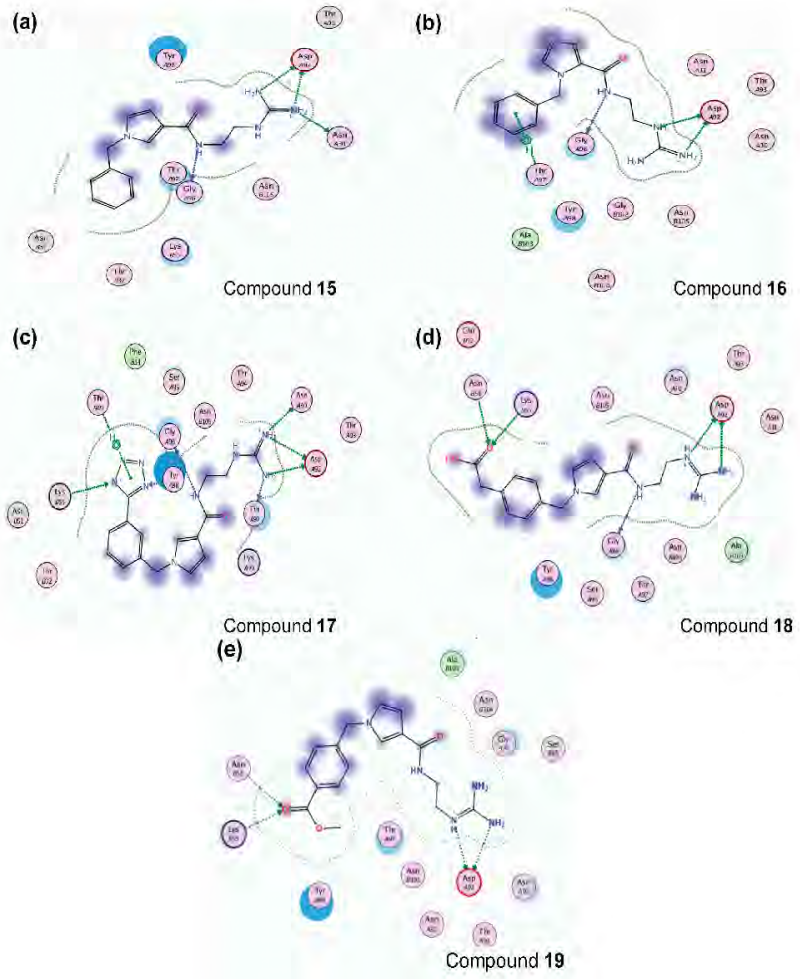


Figure 3. Best docking poses inside the TCR binding site, showing the different interactions for compounds: (**a**) **15**; (**b**) **16**; (**c**) **17**, (**d**) **18**; (**e**) **19**. Green arrow: Hydrogen Bond (HB) formed with the side chain of the residue; Blue arrow: HB formed with the backbone; Blue shade: Solvent accessible surface area (SASA) of the ligand; Turquoise halo: SASA of the receptor residues; Green dotted lines: interaction of C-H with a π system (phenyl ring).

2.3. Molecular Dynamics Simulations

Molecular dynamics (MD) simulation experiments were performed on the optimized compounds (**14**–**19**). The best docking poses were utilized as the starting conformations in the different MD simulation runs. The conformational changes observed for the TCR are similar in the different MD simulation runs (Figure S1a). This pattern is also observed in the atomic positional fluctuations of the residues of the TCR (Figure S1b). The different amino acids of the receptor show an identical pattern of deviation from their original position in the complexes with different analogues. Furthermore, the conformational analysis of the ligands showed that there are no extensive conformational changes (Figure S1c) during the simulation time. The average RMS value (1.97 Å ± 0.1) for compound **14** presents the greatest deviation from its starting conformation compared to compounds **15** (1.90 Å ± 0.37), **16** (1.72 Å ± 0.20), **17** (1.82 Å ± 0.63), and **18** (1.01 Å ± 0.13). Only analogue **19** presents a higher average RMS value (2.01 Å ± 0.47) to all the other derivatives (Figure S1c). These deviations in the RMS values for the designed analogues reflect very small changes in their conformation during MD simulations.

The clustering analysis for the different MD simulations showed that compound **14** presents two dominant conformational groups throughout the simulation (Figure 4a, blue and yellow). The difference between the two conformations is in the positioning of the aromatic ring inside the P3 pocket of the TCR (Figure 4b). In one instance, the aromatic ring is facing towards TyrA98 (Figure 4b, green) and in the other it faces away from TyrA98 and towards PheB34 (Figure 4b, yellow). In both cases, though, the docking pose is not retained throughout the MD simulation and the guanidino group is facing away from the binding cavity of the TCR (Figure 4b). The modification of compound **14** in which an additional methylene group (analogues **15** and **16**) is introduced might lead to a better positioning inside the TCR binding cavity. The clustering analysis for the two modified analogues **15** and **16** revealed the presence of only one dominant conformation for both compounds (Figure 5a, black and salmon respectively). The positioning of the two analogues **15** and **16** inside the binding cavity of the TCR is very similar (Figure 5a, black and salmon, respectively). The most pronounced difference between them is the positioning of the aromatic ring. In 2–substituted pyrrole analogue **16**, the aromatic ring during the MD simulations points away from the binding pockets (Figure 5a, salmon). On the other hand, 3–substituted pyrrole analogue **15** adopts a more optimal conformation inside the binding pockets of TCR (Figure 5a, black). While analogues **14** and **16** have a portion of their structure pointing away from the TCR receptor (Figures 4b and 5a), the addition of the methylene group in compound **15** allows for the conformation of the molecule to create a bent, thus optimizing the orientation inside pockets P–1, P2, and P3 of the TCR (Figure 5a, black).

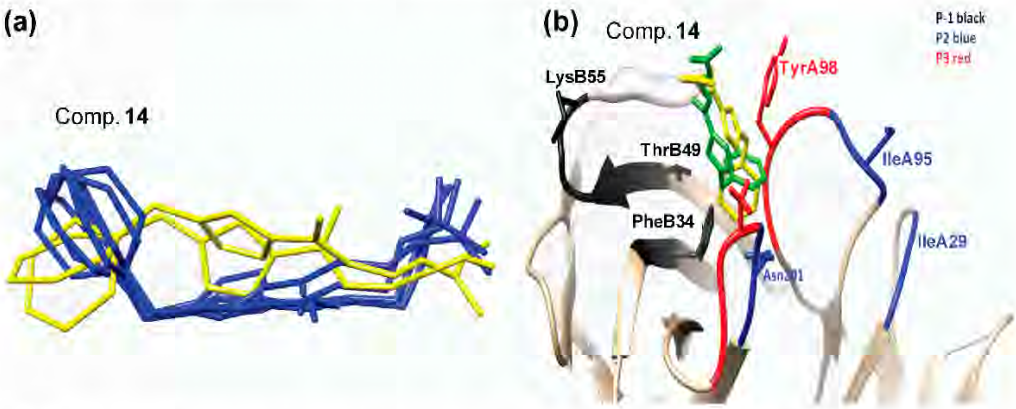


Figure 4. (**a**) Representative conformations of compound **14** inside the TCR as reported by the clustering analysis in the molecular dynamics (MD) simulations and (**b**) the positioning of analogue **14** representative conformations (yellow and green) inside the TCR binding pockets.

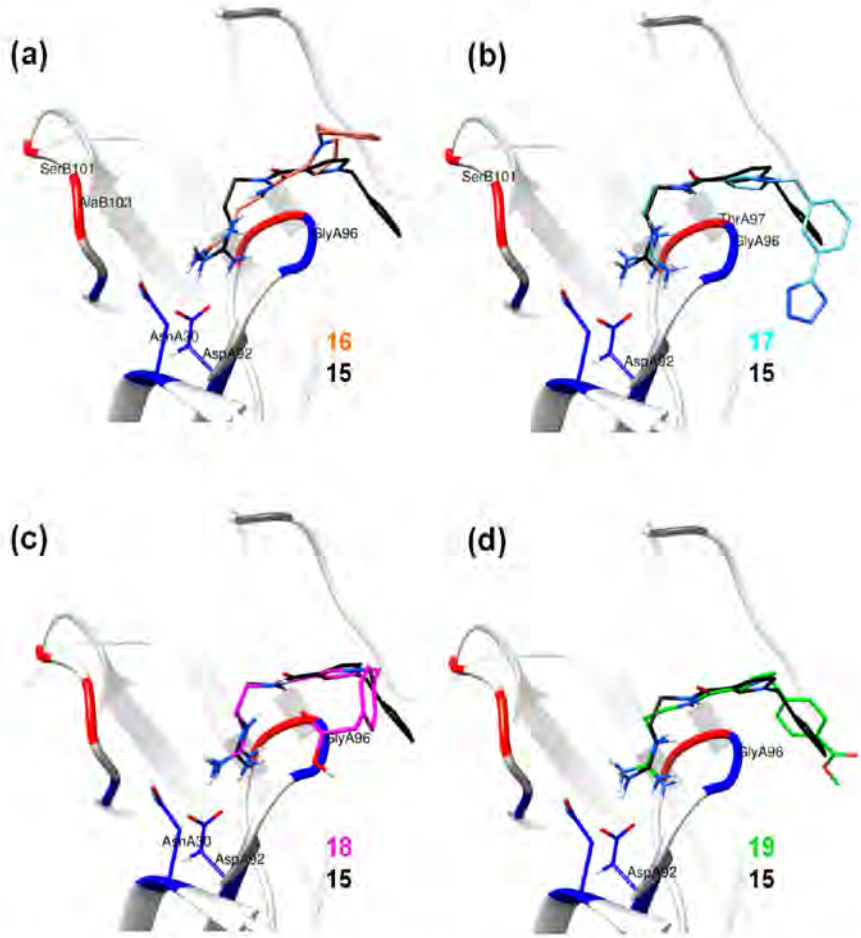


Figure 5. Conformations of compounds **15–19** inside the TCR binding pockets. The common backbone features have been implemented for the superimposition of the derivatives on analogue **15** (black); (**a**) with **16** (salmon); (**b**) with **17** (cyan); (**c**) with **18** (magenta); (**d**) with **19** (green).

As previously mentioned, the best possible positioning of compound **15** inside the binding pocket observed in the docking experiments led to the design of derivatives **17–19**. The clustering analysis of the particular simulations confirmed the results obtained from the RMS analysis (Figure S1c). Likewise, with compounds **15** and **16**, the derivatives **17–19** present only one dominant conformation throughout the MD simulations. The structural similarities of analogue **15** with compounds **17–19** led to the supposition that the derivatives would adopt a similar positioning inside the TCR. The superimposition of the representative conformations with that of compound **15** (Figure 5) confirmed the above supposition. Compounds **17** and **18** present identical positioning inside pockets P2 and P3 with that of compound **15** (Figure 5b,c), suggesting that the guanidino group firmly anchors the analogues inside the receptor. At the opposite end of the derivatives though the substitutions with the tetrazole (compound **17**) and the $-CH_2COOH$ (compound **18**) groups do not greatly improve the conformational positioning of the designed analogues in the binding cavity. Additionally, the $-CH_2COOH$ substituent in compound **18** orients the aromatic ring of the derivative away from the pockets of TCR (Figure 5c). On the other hand, the positioning of compound **19**, which has a para– methyl ester substitution, in the binding site of the receptor closely resembles that of analogue **15** (Figure 5d, green). The substitution seems to position the analogue inside the TCR between pockets P3 and P–1 in an even better way (Figure 5d, red and black).

Hydrogen Bond Interactions

Analysis of the hydrogen bond interactions for all compounds (**14**–**19**) was performed during the MD simulations. The results are outlined in Table 3 and compared with the interactions reported from the molecular docking experiments. The changes in the orientation of the molecules inside the TCR are mirrored in the observed differences of the interactions for each molecule. As mentioned above, compound **14** creates hydrogen bonds with residues in pockets P2 and P3 (Figure 2b and Table 3) with the guanidino group anchoring the compound in pocket P2 (AsnA30) and pocket P3 (ThrA97). During the MD simulation time, these interactions are not retained, and the terminal nitrogens of the guanidino group do not create stable interactions with the TCR. Instead the only interactions are those with residues of P2 pocket of TCR (AsnB104 and GluB106). The same pattern is observed for compound **16**, where the interactions with residues AspA92 and GlyA96 (P2 pocket of TCR) are not retained during the MD simulations. Instead, analogue **16** is involved in hydrogen bonding interactions with residues TyrA98 and AlaB103, both in the P3 pocket of the receptor (Table 3).

Table 3. Hydrogen bonds for all optimized analogues (**14**–**19**) as reported in the docking and MD simulation experiments.

TCR Residues	Compounds											
	14		15		16		17		18		19	
	Dock	MD	Dock	MD	Dock	MD	Dock	MD	Dock	MD	Dock	MD
AsnA30	✓[a]		✓	✓			✓			✓		✓
AspA92	✓		✓	✓	✓		✓	✓	✓	✓	✓	✓
ThrA93												✓
GlyA96	✓		✓	✓	✓	✓	✓	✓	✓	✓		✓
ThrA97	✓			✓			✓	✓				
TyrA98							✓			✓		
TyrA100		✓										
AsnB51									✓		✓	
LysB55									✓		✓	
SerB101						✓		✓				
AlaB103						✓						
AsnB104		✓										
GluB106		✓										

[a] presence of hydrogen bonds.

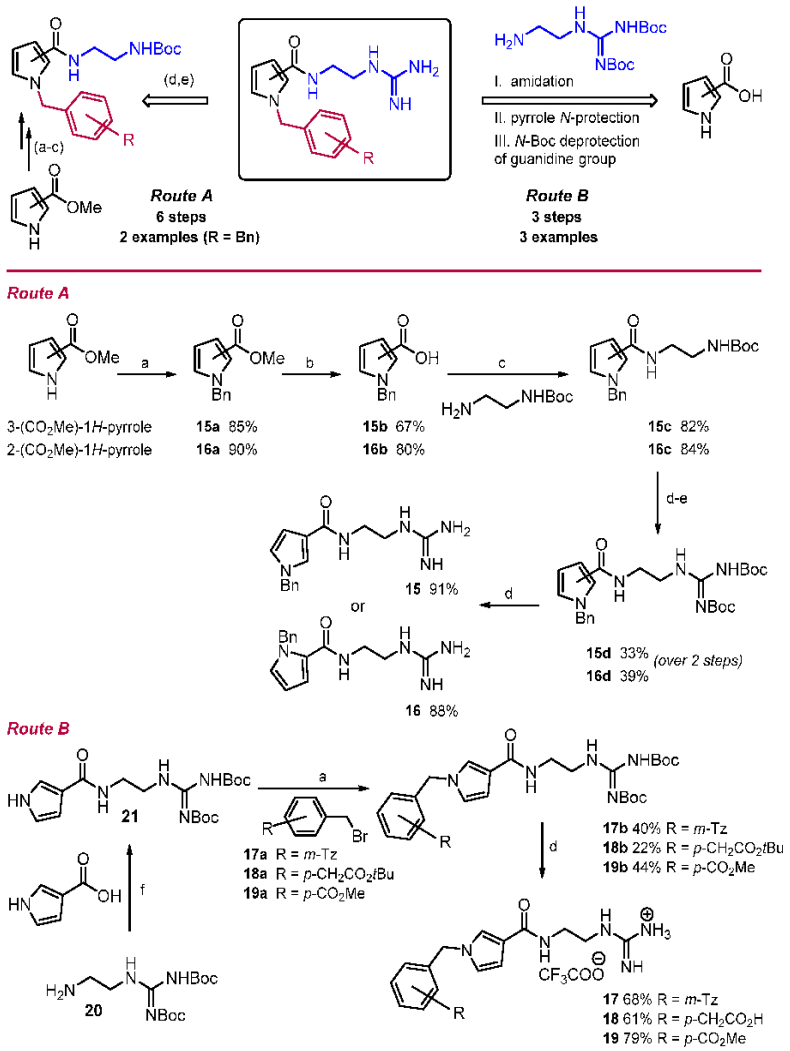
In contrast to the previous two analogues, compound **15** retains the hydrogen interactions reported in the molecular docking experiments (Figure 3a, Table 3). The hydrogen bonds with residues AsnA30 and GlyA96 in the P2 pocket of the TCR are conserved, while the orientation of the molecule inside the cavity allows for interaction with ThrA97 in the P3 pocket (Table 3). Furthermore, the anchoring of the compound **15** inside the two pockets (P2 and P3, Figure 5a), in combination with the bent conformation of the molecule, allows better positioning of the aromatic ring inside the P–1 pocket (Figure 5a). This may lead to increased interactions between the potential inhibitor and the receptor. Similarly to compound **15**, the three derivatives (**17**–**19**) present comparative interaction patterns (Table 3). The guanidino group of these analogues retains the interaction with AspA30 and GlyA96 in the P2 pocket of the TCR observed for compound **15** (Table 3), while there are small changes in the interaction patterns with the neighboring amino acids. Compound **17** further interacts with ThrA97 and SerB101 in the P2 pocket, while compound **18** further interacts with AsnA30 in the P3 pocket and TyrA98 in the P2 pocket of the receptor. Finally, the very similar positioning of compounds **15** and **19** (Figure 5d) points to the conservation of the interactions between the two designed analogues (Table 3). The only difference is the hydrogen bond of compound **19** with ThrA93 instead of GlyA96 in the P2 pocket of the receptor.

2.4. Chemistry

Initial studies on the synthesis of pyrrole-based TCR antagonists provided candidates **15** and **16** via a six-step synthetic procedure with a total yield of 14% and 21%, respectively (Route A, Scheme 1).

N-alkylation of commercially available 3- or 2-methyl pyrrolecarboxylates, with benzyl bromide in the presence of sodium hydride, afforded the 3-/2-substituted *N*-benzylpyrroles **15a**/**16a** [37]. Subsequent hydrolysis of the methyl ester, followed by standard procedure for DCC/DMAP amide coupling with *N*-Boc-ethylenediamine, gave the corresponding pyrrole carboxamides **15c**/**16c**. *N*-Boc-deprotection with TFA followed by *N*-iodosuccinimide-mediated guanylation reaction [38] with di-Boc-thiourea furnished the di-Boc-guanidino derivatives **15d**/**16d**, which allowed final molecules **15**/**16**, after Boc cleavage.

Upon further investigations, a rapid and simple three-step protocol (Route B, Scheme 1) was developed to expand the scope and utility of this synthetic methodology and readily prepare diverse pyrrole analogues. Thus, the guanidine moiety **20** [39] was first synthesized and then reacted with pyrrole-3-carboxylic acid to provide a common structural core **21**, after amidation reaction. Subsequent pyrrole-*N*-protection [37] with primary alkyl bromides **17a**–**19a**, followed by removal of the Boc-groups, produced target compounds **17**–**19** in a shorter sequence and an 11–27% overall yield.



Scheme 1. Synthesis of 2-/3-substituted pyrrole analogues **15**–**19**. Reagents and conditions: Route A: (**a**) BnBr, NaH, DMF; (**b**) i. KOH 30%, MeOH–H_2O, reflux; ii. 6 M HCl; (**c**) DCC, DMAP, DCM; (**d**) TFA, DCM; (**e**) BocNHC (S) NHBoc, NIS, DIPEA, MeOH–DCM; then (**d**) TFA, DCM. Route B: (**a**) BnBr, NaH, DMF; (**f**) DCC/HOBt, DIPEA, DMF–DCM; (**d**) TFA, DCM.

2.5. Molecular Orbital Calculations

From the three analogues (**14**–**16**) reported in this study, compound **15** presents a high docking score (−18.13 kcal/mol) coupled with a preferred orientation inside the binding cavity of the TCR (Figure 5a). This, in combination with the compound's favorable pharmacokinetic properties (TPSA and logP, Table 2), inspired us to explore the analogue **15**/TCR complex by employing molecular orbital methods.

2.5.1. Semi-Empirical Simulation Method

In order to better estimate the interaction energy of the system, a number of different approaches were employed. The results (Table S2) show that the PM7 (parameterization method 7) [40] approach best reproduces the density functional theory (DFT) calculations for the selected residues. All other semi-empirical (SE) methods tested present considerable errors compared to PM7 despite the inclusion of dispersion correction. Based on these observations, the PM7 method was used as the most appropriate for further calculations on the entire receptor–ligand complex (Table S2). Two protocols were utilized for our calculations. In the first one, the ligand along with the same residues used in the DFT calculations was preferred. The interaction energy of the particular system was calculated to −24.09 (kcal/mol). The larger value compared to the DFT calculations (−31.63/−42.85 kcal/mol) may be attributed to the level of accuracy for the SE methodologies and the treatment of the electron density of the various atoms in the system.

The second approach employed in our calculations involved the ligand with the whole receptor. In order to explore the effect of the different TCR residues, amino acids within a cutoff distance of 4.5 Å from the ligand were initially elected. Subsequent rounds of interaction energy calculations followed, by augmenting the selected area per 4.5 Å each time until the entire receptor was included in our calculations (Figure 6a). The interaction energy calculated for the TCR in complex with compound **15** is −34.39 kcal/mol. In order to further study the interaction energy of compound **15**, different snapshots were taken from the MD simulation run (the last 20 ns of the simulation). For each snapshot, the interaction energy was calculated with the PM7 method to monitor the fluctuations in the energy (Figure 6b and Table S3). The mean value over the 20 snapshots for the interaction energy was −47.26 kcal/mol. The low interaction energy calculated from both the best docking pose and the different MD snapshots (Tables S2 and S3) suggests that derivative **15** interacts strongly with the TCR and thus may be competitive with native ligands. The interaction energy calculated for compounds **17**–**19** with the SE methodology are reported in Table S5. The values range between −35.39 and −37.20 kcal/mol, higher than the value reported for analogue **15** (−47.26 kcal/mol).

2.5.2. DFT Calculations

The large size of the TCR (341 amino acids) hinders the use of DFT methodologies on the entire complex [41,42]. Thus, to calculate the interaction energy for the complex, the best docking pose was selected. The selection of the receptor residues (total of nine amino acids) was based on the interactions formed with compound **15** and their distance from it (<3.5 Å). As reported in the Materials and Methods section, Section 3.6, different methodologies were employed, and the results are outlined in Table S2. The analysis of the calculations showed variability depending on the method and the basis set selected. In fact, the methods that include dispersion either explicitly or implicitly (e.g., M06, M06-2X, B97D, BHandH, and B3LYP-D) calculate more negative interaction energies (Table S4) [43,44]. In contrast, the choice of basis set does not have such an extensive impact in the calculation of the interaction energy. Therefore, in order to obtain a more accurate result, the inclusion of dispersion functions was considered in our calculations [45]. Based on this, the interaction energy between compound **15** and the selected residues of the TCR was calculated between −31.63 and −42.85 kcal/mol (Table S4). Compared to the SE methodologies, DFT techniques allow for a more accurate prediction of interaction energy between the ligand and the residues that are directly involved

in the binding to TCR. The application of DFT incorporates the effect of all atoms, without any of the approximations (empirical data) applied during the SE calculations.

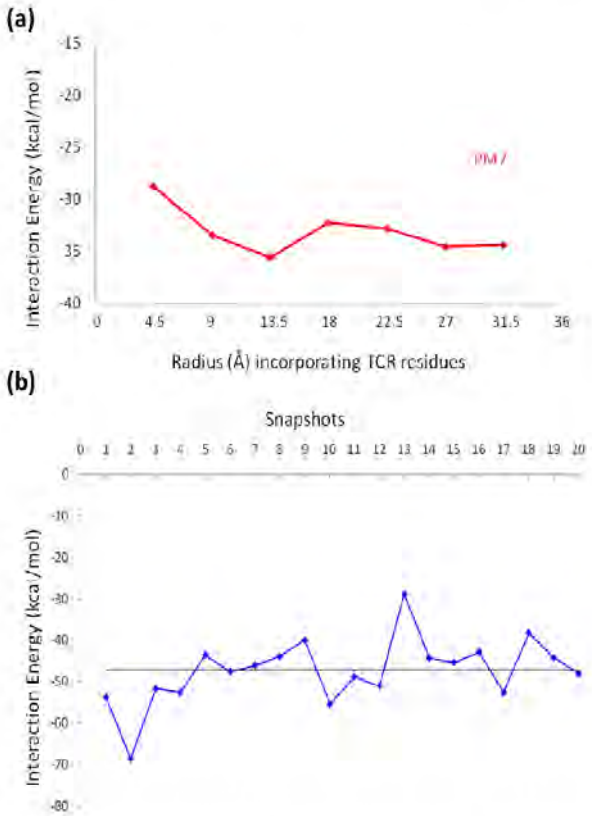


Figure 6. (**a**) Interaction energies for derivative **15** and the residues of the binding cavity, extended by 4.5 Å until the entire TCR is included, calculated by the PM7 method (red) in solvent and (**b**) graphical representation of the interaction energy calculated via the PM7 method for the different MD snapshots.

2.6. Biological Assays

2.6.1. Human Peripheral Mononuclear Cells

Blood samples were drawn from two healthy subjects for biological assays and contained: 1st person: 2.84 × 10^3 lymphocytes/μL of blood (42.9% of total leukocytes) and 410 monocytes/μL of blood (6.2% of total leukocytes); 2nd person: 1.83 × 10^3 lymphocytes/μL of blood (34.6% of total leukocytes) and 330 monocytes/μL of blood (6.2% of total leukocytes). The peripheral blood mononuclear cells (PBMCs) isolated from the blood samples were cultured in the presence of various concentrations of the $MBP_{83–96}$ peptide to estimate the optimal concentration for inducing T-cell proliferation. It is noteworthy that the specific culture conditions used in this work, i.e., allo-peptidic antigens and anti-CD28 antibody, target T-cell responses [46]. T-cell proliferation was measured by flow cytometry. The highest T-cell proliferation was noted at 0.1 nM $MBP_{83–96}$ (Figure 7a). PBMC cultures were then repeated with 0.1 nM $MBP_{83–96}$ and 0.1 mM of each of the fifteen analogues (Figure 7, Table 1: compounds **1**–**13**, **15**, and **16**) per experimental point, in triplicate. The results show that analogue **15** was the most effective TCR antagonist, i.e., it conferred the highest inhibition of T cell proliferation (Figure 7b).

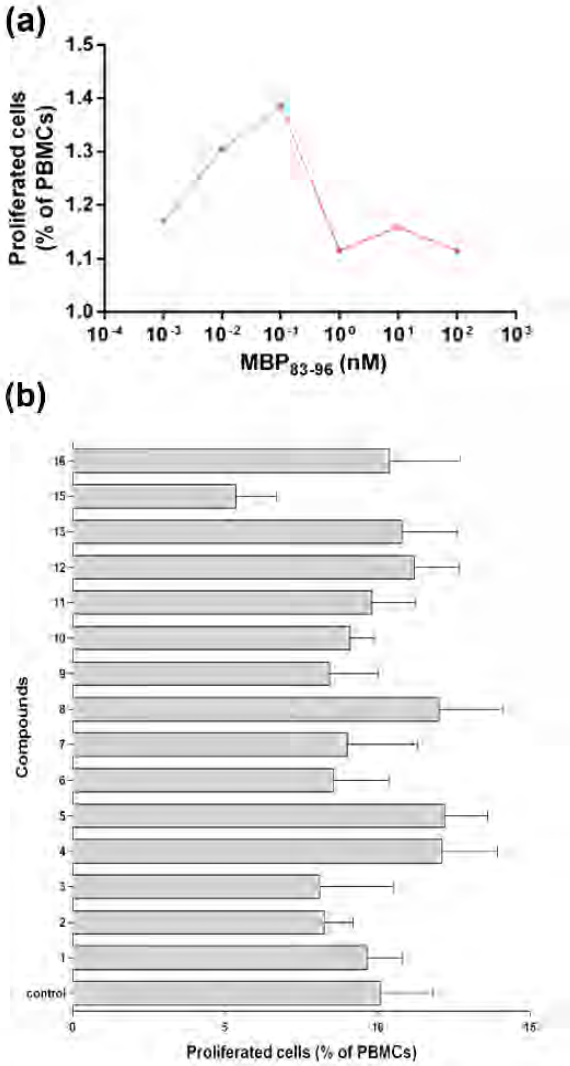


Figure 7. (**a**) T cell proliferation in the presence of $MBP_{83–96}$. Peripheral blood mononuclear cells (PBMC) were cultured with several concentrations of the $MBP_{83–96}$ peptide for 3 days; cell proliferation was measured by flow cytometry. Data are shown as median of triplicate measurements. (**b**) Proliferation of PBMC in the presence of 0.1 nM $MBP_{83–96}$ and 0.1 mM of each of the 15 analogues/point, in triplicate. Control: $MBP_{83–96}$ peptide alone. Data are shown as mean = standard error of the mean.

2.6.2. Mouse $MBP_{83–99}$ Specific T Cell Assays

Autoimmune $CD4^+$ T cells can be stimulated in mice following immunization with $MBP_{83–99}$ peptide together with *Mycobacterium*, which results in experimental autoimmune encephalomyelitis (EAE), an animal model for MS [47]. Characteristics of EAE are comparable to those of MS in humans where Th1 phenotype (IFN-γ) and Th17 cells contribute to pathogenesis of disease in mice. Similar to MS, EAE susceptibility is dependent on the mouse (MHC class II background) and diverse peptides are immunogenic in different mouse strains. The SJL/J mouse strain (MHC class II $H\text{-}2^s$ haplotype) is commonly used for EAE, since numerous histopathological, clinical, and immunological features resemble those of human MS [48]. In the SJL/J mouse strain, the peptide $MBP_{81–100}$ has been shown to bind with high affinity to MHC class II, $H\text{-}2^s$. In fact, the minimum epitope required for binding is $MBP_{83–99}$ [48], similar to human HLA-DR2 binding. Hence, the epitope $MBP_{83–99}$ could be used as an

agonist peptide to immunize mice to activate $CD4^+$ T cells, as we previously demonstrated [26,30–32]. Here, mice were immunized with $MBP_{83–99}$ peptide conjugated to the carrier reduced mannan. Following three immunizations, spleen cells were isolated and mixed with recall peptide $MBP_{83–99}$ for six days in vitro. In addition, compounds **15**–**19** or **AMB** (lead compound **10**) were added at 100× molar excess to each well in order to determine whether T cell proliferation to the recall peptide $MBP_{83–99}$ could be inhibited. The particular compounds (**15**–**19**), due to their increased calculated binding affinity (Table 1) to TCR, were employed in order to assess the potency in mouse $MBP_{83–99}$ specific T cell assays. Compound **15** and **16** showed the greatest % inhibition of $MBP_{83–99}$-specific T cell proliferation, followed by compound **17** and **18**; compound **19** showed the least inhibition, and lead compound AMB was able to inhibit proliferation comparable to that of the other compounds (Figure 8). Compounds **15** and **16** have simpler structures compared to **17**–**19** and **AMB**. It is likely that the reduced activity of **17**–**19** analogues, compared to **15** and **16**, may be due to an inappropriate position of the acidic/esteric group. Even though complete inhibition of T cell proliferation is not noted, compounds **15** and **16**, based on in silico conformational studies, show promise for further optimization studies in order to develop new improved TCR antagonists with improved activity.

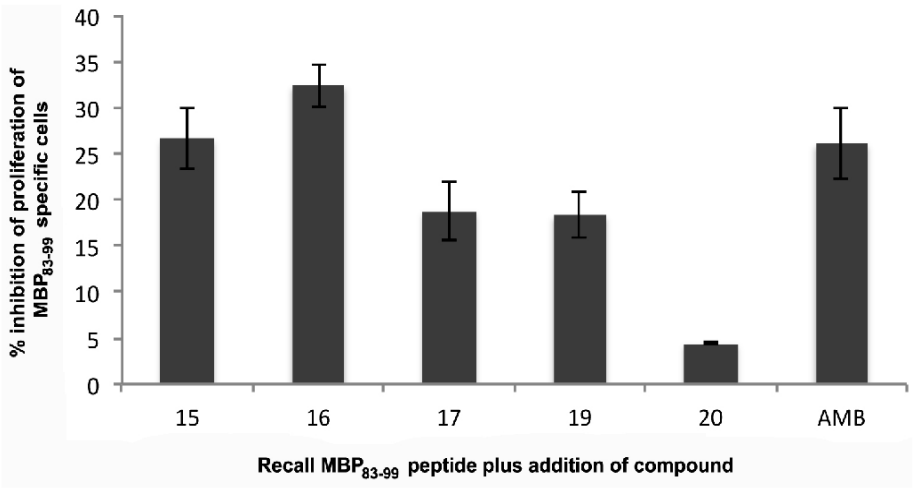


Figure 8. Specific $MBP_{83–99}$ epitope T cell proliferation using MTT as a readout. Mice were immunized three times with reduced mannan conjugated to $MBP_{83–99}$ peptide. Ten days following the last immunization, mice were sacrificed and spleen cells isolated, and $MBP_{83–99}$ peptide was added for 6 days. In addition, compounds **15**–**19** and lead compound **AMB** (lead compound **10**) were added at 100× molar excess. The percent inhibition of T cell proliferation to $MBP_{83–99}$ of each compound is shown. The mean of three individual mice are shown in triplicate wells ± standard error of the mean.

3. Materials and Methods

3.1. Structure Preparation

The X-ray crystallographic coordinates contained in PDB entry 1YMM were obtained from the Protein Data Bank [22]. The particular PDB entry was selected because it contains the main immunodominant epitope $MBP_{83–96}$ involved in MS, as well as a human TCR from a patient with MS. The Molecular Operating Environment (MOE2010) software [49] was utilized for the preparation of the complex. The peptide–TCR complex was isolated, and the residues were protonated accordingly with all hydrogen positions optimized using the AMBER94 force field [50]. All the possible protonation states for the histidine (His) residues were explored and evaluated with the use of the PROpKa [51,52] and AMBER94 tools in MOE2010. The analysis supports the prevalence of neutral His in all cases, in agreement with the results reported by Wucherpfenning et al. [53,54].

3.2. Pharmacophore Modeling

The pharmacophore model was designed based on the $MBP_{83–96}$ key residues [55] involved in the binding with the HLA receptor and the TCR. A combination of features from structure- and ligand-based pharmacophore models was utilized in the development of the model presented in this study. According to the crystal structure of the binding cavity of the TCR, an analysis of its chemical features was carried out using the MOE2010 software [49]. The development of the ligand-based pharmacophore model relied on features such as aromaticity (Aro), a hydrogen bond cation (Cat) and donor, and hydrophobic groups (Hyd). The Aro motifs were modeled on the His^{88} and Phe^{89}residues of the epitope, the Hyd feature on Val^{86}, and the Cat feature on Pro^{85}, respectively, all residues that interact with the TCR. The volume exclusion (V) features of the pharmacophore model were developed based on Val^{87} and Phe^{90} that interact with the HLA.

Virtual Screening

The pharmacophore hypothesis based on the TCR active site as well as the $MBP_{83–96}$ epitope were utilized to scan 500,000 compounds from the ZINC database [56]. The compounds were filtered according to Lipinski's rule of five [57] and their commercial availability. Finally, the molecules were sorted based on their fitness to the selected hypothesis.

3.3. Molecular Docking

Molecular docking simulations were performed on the TCR using MOE2010 [49]. The ligand, as well as the TCR residues in a radius of 4.5 Å surrounding the ligand, was considered flexible. The definition of the TCR binding site was performed manually by selecting the area including the residues involved in the main binding pockets. The ligands were allowed to move freely in the vicinity of the active site. The top 500 poses for each ligand were ranked using the London ΔG scoring function [49]. Subsequently, a maximum of 10 poses were retained based on their docking scoring function, and the poses were rescored using the GBVI/WSA (Generalized-Born Volume Integral/Weighted Surface Area) scoring function [58].

3.4. Lead Optimization

Thirteen potential inhibitors (hits) were directly purchased for additional in vitro biological evaluation, as TCR antagonists. Based on their properties and binding scores with the TCR, compound **10** was selected as a lead compound for further optimization. Chemical groups were modified to improve the binding properties, such as orientation of the molecule inside the TCR. Additionally, new chemical groups were added to lengthen the carbon chain and optimize the pocket fit.

3.5. Molecular Dynamics (MD) Simulation

The construction of the TCR parameters was performed using the AMBER force field ff14SB [59], while the parameters for the organic molecules were constructed using the general Amber force field (GAFF) [60]. The TIP3P water model [61] was utilized for the solvation of the system, and the total charge was neutralized by the addition of Cl^- ions. Truncated octahedral periodic boundary conditions were applied to the system with a cutoff distance of 10 Å. The next step involved minimization, followed by the heating of the system, under a constant volume, to 300 K for 100 ps using the Langevin dynamics temperature scaling [62]. This was followed by equilibration for another 100 ps under constant pressure. Both heating and pressure equilibration were carried out using a 10 $kcal \cdot mol^{-1} \cdot Å^{-2}$ restraint on the solute. The equilibration step under constant pressure was prolonged for a further 200 ps, after removing all restraints. The MD production run was performed under constant pressure and temperature conditions (NPT ensemble) for 50 ns. The temperature was kept constant with the use of the Langevin thermostat (using a collision frequency of 2 ps^{-1}). All bonds involving hydrogen atoms were kept to their equilibrium distance with the SHAKE algorithm (allowing for a 2 fs time step

to be used) [63]. The long range electrostatic interactions were calculated with the Particle Mesh Ewald (PME) method [64]. The different systems were subjected to all-atom unrestrained MD simulations in explicit solvent using AMBER12 [65]. The cpptraj module [66] of AMBER12 was implemented for the trajectory analysis (clustering, RMSD, hydrogen bonds).

3.6. Chemistry

Reactions involving moisture sensitive reagents were carried out under an argon atmosphere in addition to oven-drying glassware and anhydrous solvents. Room temperature (rt) refers to 20–25 °C. Crude products were purified by flash chromatography on 230–400 mesh silica gel in the solvents system stated. Analytical thin layer chromatography was performed on pre-coated aluminium plates (Merck 60G F254 silica). TLC visualization was performed out with ultraviolet light (254 nm). The yields were calculated in *w*/*w*. ^{1}H and ^{13}C nuclear magnetic resonance (^{1}H NMR, ^{13}C NMR) spectra (Figures S2–S14) were acquired on Bruker Avance 400 and Bruker Ascend 600 spectrometer at ambient temperature in the deuterated solvent stated. All chemical shifts are quoted in parts per million (ppm) relative to the internal standard (TMS). All coupling constants, *J*, are quoted in Hz. Multiplicities are indicated by s (singlet), d (doublet), t (triplet), q (quartet), and m (multiplet). The abbreviation Ar is used to denote aromatic, br to denote broad, and app to denote apparent. Mass spectrometry (*m*/*z*) data were acquired on an Electrospray Ionization Platform spectrometer (ESI-MS) by Micromass and a MassLynx NT 2.3 operating system (Waters Corporation, Milford, MA, USA).

3.6.1. General Procedure A: *N*-Alkylation of Pyrroles

To a solution of 1*H*-pyrrole analogue (1.00 equiv) in DMF (5–10 mL/mmol), under argon at 0 °C was added sodium hydride 60% (1.50–2.50 equiv), and the resulting mixture was stirred at the same temperature for 10–20 min. Then, a solution of the corresponding alkyl bromide (1.00–1.50 equiv) in DMF (5–10 mL/mmol) was added dropwise, and the reaction mixture warmed to rt over ~2 h (monitored by TLC). It was quenched with water (20 mL) and extracted with EtOAc (3 × 20 mL). The combined organics were washed with brine (20 mL), dried (Na_2SO_4), filtered, and concentrated in vacuo. Purification of the residue by column chromatography on silica gel (using the appropriate mixture of eluents) allowed pyrroles *N*-protected **15a**, **16a**, and **17b**–**19b**.

3.6.2. General Procedure B: Hydrolysis of Methyl Pyrrole-2/3-Carboxylates

To a solution of methyl *N*-benzyl pyrrole 3- or 2-carboxylate **15a** or **16a** (1.00 equiv) in MeOH–H_2O 3:1 *v*:*v* (15.0 mL/mmol), an aq solution of KOH 30% (15.0 mL/mmol) was added. The resulting reaction mixture was refluxed and monitored by TLC (10% MeOH–DCM) until completion (~2 h). Then, it was allowed to attain rt and acidified pH = 1 via the addition of 6.0 M HCl (until cloudiness persisted). The white precipitate was filtered off and washed with ice-water to give the crude of **15b** or **16b**, respectively, which was used in the next step without further purification.

3.6.3. General Procedure C: Amidation Reaction

To a solution of the required pyrrole 3- or 2-carboxylic acid, **15a** or **16a** (1.00 equiv) in dichloromethane (DCM) (20.0 mL/mmol), 4-dimethylaminopyridine (DMAP) (20 mol %), *N*-Boc-ethylenediamine (1.00 equiv), and then *N*,*N*′-dicyclohexylcarbodiimide (DCC) (1.50 equiv) at 0 °C were added. The resulting mixture was warmed to rt and stirred for a further 16 h (monitored by TLC, 10% MeOH–DCM). After completion of the reaction, dicyclohexylurea (DCU) formed was filtered off and washed with DCM (5 mL) at 0 °C. The organic layer was quenched with 0.1 M HCl (20 mL) and extracted with EtOAc (3 × 20 mL). The combined organics were washed with brine (20 mL), dried (Na_2SO_4), filtered, and concentrated in vacuo. Purification of the residue by column chromatography on silica gel (using the appropriate mixture of eluents) allowed pyrrole 3-/2-carboxamides **15c** or **16c**.

3.6.4. General Procedure D: Removal of the Boc-Group

The corresponding *N*-Boc analogue (1.00 equiv) was dissolved in trifluoroacetic acid (TFA)–DCM 95:5 *v*/*v* DCM (20–30 mL/mmol) (and added triethylsilane (TES, 1.00 equiv) if required). The reaction mixture was stirred at rt, and the progress was monitored by TLC (10% MeOH–DCM) until complete consumption of the starting material.

3.6.5. General Procedure E: Guanylation Reaction

The amine salt **15c′** or **16c′** (as crude derived from *N*-Boc deprotection of **15c** or **16c**) was dissolved in a mixture of MeOH–DCM 4:1 *v*:*v* (20.0 mL/mmol), under argon. Then, *N*,*N*′-di-(*tert*-butoxycarbonyl)thiourea (1.50 equiv), *N*,*N*-diisopropylethylamine (DIPEA) (4.00 equiv) and *N*-iodosuccinimide (1.50 equiv) in one portion were added at rt. The reaction mixture was stirred at rt under argon, and monitored by thin layer chromatography (TLC) (20% MeOH–DCM) until completion (~24 h). It was next quenched with an aq solution of 1 M sodium thiosulfate solution (20 mL), and the resulting solution was then diluted in water (20 mL) and extracted with EtOAc (3 × 20 mL). The combined organic layer was washed with brine (20 mL), dried (Na_2SO_4), filtered, and concentrated in vacuo. Purification of the residue by column chromatography on silica gel (using the appropriate mixture of eluents) allowed the corresponding di-Boc-guanidino derivatives **15d** or **16d**.

3.6.6. Synthesis of Methyl 1-Benzyl-1*H*-Pyrrole-3-Carboxylate **15a** [67]

From methyl 1*H*-pyrrole-3-carboxylate (98.4 mg, 0.79 mmol) and NaH 60% (38.0 mg, 1.58 mmol) in dimethylformamide (DMF) (4.0 mL), and a solution of benzyl bromide (0.14 mL, 1.18 mmol) in DMF (6.0 mL), following the general procedure A (2 h) and after chromatographic purification (DCM), **15a** (144 mg, 85%) was obtained as a clear gum. Data for **15a**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.31–7.37 (m, 4H, Ph, Ar), 7.13–7.15 (m, 2H, Ph), 6.60–6.63 (m, 2H, Ar), 5.06 (s, 2H, CH_2Ph), 3.79 (s, 3H, OMe); ESI-MS *m*/*z* found for $C_{13}H_{13}NO_2$: 216.32 $[M + H]^+$; RP-HPLC gradient separation from 30% to 100% acetonitrile at 30 min, flow rate: 1 mL/min, t_R = 10.8 min.

3.6.7. Synthesis of 1-Benzyl-1*H*-Pyrrole-3-Carboxylic Acid **15b** [68]

From methyl 1-benzyl-1*H*-pyrrole-3-carboxylate **15a** (144 mg, 0.67 mmol) in MeOH–H_2O (10.0 mL) and an aq solution of KOH 30% (10.0 mL), following the general procedure B (2 h) and after precipitation, the crude of **15b** (90.0 mg, 67%) was used in the next step without further purification. Data for **15b**: proton nuclear magnetic resonance (^{1}H NMR) (400 MHz, $CDCl_3$) δ 7.29–7.40 (m, 4H, Ph, Ar), 7.15 (d, 2H, *J* = 7.2 Hz, Ph), 6.63-6.66 (m, 2H, Ar), 5.07 (s, 2H, CH_2Ph); electrospray ionization mass spectrometry (ESI-MS) *m*/*z* found for $C_{12}H_{11}NO_2$: 425.41 $[2M + Na]^+$, 202.25 $[M + H]^+$; RP-HPLC gradient separation from 5% to 100% acetonitrile at 30 min, flow rate: 1 mL/min, t_R = 14.2 min.

3.6.8. Synthesis of 1-Benzyl-1*H*-*N*-[2-(Tert-Butoxycarbonyl)Aminoethyl]Pyrrole-3-Carboxamide **15c**

From 1-benzyl-1*H*-pyrrole-3-carboxylic acid **15b** (90.0 mg, 0.45 mmol) in DCM (9.0 mL), DMAP (10.9 mg, 0.09 mmol), *N*-Boc-ethylenediamine (0.07 mL, 0.45 mmol), and then DCC (138 mg, 0.67 mmol), following the general procedure C (16 h) and after chromatographic purification (20% MeOH–DCM), **15c** (126 mg, 82%) was obtained as a clear gum. Data for **15c**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.29–7.35 (m, 3H, Ph), 7.25–7.26 (m, 1H, Ar), 7.12–7.14 (m, 2H, Ph), 6.62 (app t, 1H, *J* = 2.4 Hz, Ar), 6.48 (br s, 1H, NH), 6.41 (br s, 1H, Ar), 5.04 (s, 2H, CH_2Ph), 4.98 (br s, 1H, NH), 3.51–3.45 (m, 2H, CH_2), 3.32–3.35 (m, 2H, CH_2), 1.41 (s, 9H, 3× CH_3*t*-Bu); ESI-MS *m*/*z* found for $C_{19}H_{25}N_3O_3$: 344.33 $[M + H]^+$, 288.32 $[(M–Ph) + Na]^+$; reversed phase high-performance liquid chromatography (RP-HPLC) gradient separation from 5% to 100% acetonitrile at 30 min, flow rate: 1 mL/min, t_R = 17.0 min.

3.6.9. Synthesis of 1-Benzyl-1*H*-*N*-[2-(2,3-Di-Tert-Butoxycarbonyl)Guanidinoethyl]Pyrrole-3-Carboxamide **15d**

From *N*-Boc analogue **15c** (120 mg, 0.35 mmol) in TFA–DCM 95:5 (7.0 mL), following the general procedure D (1 h), the crude of 2-(1-benzyl-1*H*-pyrrole-3-carboxamido)ethanaminium 2,2,2-trifluoroacetate **15c'** was dissolved in MeOH–DCM (7.0 mL). Then, *N*,*N'*-di-(*tert*-butoxycarbonyl) thiourea (145 mg, 0.52 mmol), DIPEA (0.24 mL, 1.40 mmol, 4.00 equiv), and *N*-iodosuccinimide (118 mg, 0.52 mmol), following the general procedure E (24 h) and after chromatographic purification (20% MeOH–DCM), **15d** (56.6 mg, 33%) was obtained as a clear gum. Partial data for **15c'**: ESI-MS *m/z* found for $C_{14}H_{17}N_3O$: 244 $[M]^+$; RP-HPLC gradient separation from 5% to 100% acetonitrile at 30 min, flow rate: 1 mL/min, t_R = 10.3 min. Data for **15d**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.50 (s, 1H, NH), 8.71 (s, 1H, NH), 7.28–7.41 (m, 5H, Ph, Ar, NH), 7.11–7.13 (m, 2H, Ph), 6.56–6.59 (m, 2H, Ar), 5.04 (s, 2H, CH_2Ph), 3.67–3.71 (m, 2H, CH_2), 3.53–3.57 (m, 2H, CH_2), 1.51 (s, 9H, 3 × CH_3*t*-Bu), 1.49 (s, 9H, 3× CH_3*t*-Bu); ESI-MS *m/z* found for $C_{25}H_{35}N_5O_5$: 486.34 $[M + H]^+$; RP-HPLC gradient separation from 30% to 100% acetonitrile at 30 min, flow rate: 1 mL/min, t_R = 13.4 min.

3.6.10. Synthesis of 1-Benzyl-1*H*-*N*-(2-Guanidinoethyl)Pyrrole-3-Carboxamide **15**

From di-Boc guanidine analogue **15d** (50.0 mg, 0.10 mmol) in TFA–DCM 95:5 (3.0 mL), following the general procedure D (1 h) and after chromatographic purification (0.5% NH_4OH, 19.5% MeOH, 80% DCM), final product **15** (26.5 mg, 91%) was obtained as a white solid. Data for 15: ^{1}H NMR (400 MHz, CD_3OD) δ 7.27–7.36 (m, 4H, Ph, Ar), 7.20–7.22 (m, 2H, Ph), 6.78 (dd, 1H, *J* = 2.8, 2.4 Hz, Ar), 6.52 (dd, 1H, *J* = 2.8, 2.0 Hz, Ar), 5.13 (s, 2H, CH_2Ph), 3.46 (t, 2H, *J* = 6.3 Hz, CH_2), 3.35 (t, 2H, *J* = 6.3 Hz, CH_2); ^{13}C NMR (100 MHz, CD_3OD) δ 168.6 (C=O), 159.0 (C=NH), 139.0 (C Ph), 129.8 (2× CH Ph), 129.0 (CH Ph), 128.5 (2× CH Ph), 125.4 (CH Ar), 123.6 (CH Ar), 120.1 (C Ar), 109.0 (CH Ar), 54.5 (CH_2Ph), 42.4 (CH_2), 39.4 (CH_2); ESI-MS *m/z* found for $C_{15}H_{19}N_5O$: 286.66 $[M + H]^+$, 243.21 $[M–(C(NH)NH_2) + H]^+$, 214.16 $[M–(CH_2NHC(NH)NH_2) + H]^+$; RP-HPLC gradient separation from 10% to 100% acetonitrile at 30 min, flow rate: 1 mL/min, t_R = 19.3 min, R_f = 0.46 (MeOH–DCM 2:8).

3.6.11. Synthesis of Methyl 1-Benzyl-1*H*-Pyrrole-2-Carboxylate **16a** [67]

From methyl 1*H*-pyrrole-2-carboxylate (151 mg, 1.21 mmol) and NaH 60% (58.1 mg, 2.42 mmol) in DMF (6.0 mL), and a solution of benzyl bromide (0.21 mL, 1.80 mmol) in DMF (9.0 mL), following the general procedure A (3 h) and after chromatographic purification (DCM), **16a** (234 mg, 90%) was obtained as a pale yellow oil. Data for **16a**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.23–7.34 (m, 3H, Ph), 7.11 (d, 2H, *J* = 7.2 Hz, Ph), 7.02 (dd, 1H, *J* = 3.4, 1.6 Hz, Ar), 6.89 (app t, 1 H, *J* = 1.6 Hz, Ar), 6.19 (app t, 1H, *J* = 3.4 Hz, Ar), 5.57 (s, 2H, CH_2Ph), 3.77 (s, 3H, OMe); ESI-MS *m/z* found for $C_{13}H_{13}NO_2$: 216 $[M + H]^+$, 138 $[(M–Ph) + H]^+$; RP-HPLC gradient separation from 30 to 100% acetonitrile at 30 min, flow rate: 1 mL/min, t_R = 13.9 min.

3.6.12. Synthesis of 1-Benzyl-1*H*-Pyrrol-2-Carboxylic Acid **16b**

From methyl 1-benzyl-1*H*-pyrrole-2-carboxylate **16a** (230 mg, 1.07 mmol) in MeOH–H_2O (16.0 mL) and an aq solution of KOH 30% (16.0 mL), following the general procedure B (3 h) and after precipitation, the crude of **16b** (172 mg, 80%) was used in the next step without further purification. Data for **16b**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.24–7.33 (m, 3 H, Ph), 7.14 (dd, 1H, *J* = 3.8, 2.0 Hz, Ar), 7.11 (d, 2H, *J* = 6.8 Hz, Ph), 6.93 (app t, 1H, *J* = 2.0 Hz, Ar), 6.21 (dd, 1H, *J* = 3.8, 2.8 Hz, Ar), 5.56 (s, 2H, CH_2Ph); ESI-MS (EI) *m/z* found for $C_{12}H_{11}NO_2$: 240 $[M + K]^+$, 224 $[M + Na]^+$, 202 $[M + H]^+$; RP-HPLC gradient separation from 5% to 100% acetonitrile at 30 min, flow rate: 1 mL/min, t_R = 16.2 min.

3.6.13. Synthesis of 1-Benzyl-1*H*-*N*-[2-(Tert-Butoxycarbonyl)Aminoethyl]Pyrrole-2-Carboxamide **16c**

From 1-benzyl-1*H*-pyrrole-2-carboxylic acid **16b** (172 mg, 0.86 mmol) in DCM (17 mL), DMAP (21.0 mg, 0.17 mmol), *N*-Boc-ethylenediamine (0.13 mL, 0.86 mmol), and then DCC (266 mg, 1.29 mmol),

following the general procedure C (16 h) and after chromatographic purification (20% MeOH-CH_2Cl_2), **16c** (248 mg, 84%) was obtained as a clear gum. Data for **16c**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.20–7.30 (m, 5H, Ph, Ar, NH), 7.11 (d, 2H, *J* = 7.2 Hz, Ph), 6.79 (br s, 1H, Ar), 6.64 (br d, 1H, *J* = 2.0 Hz, NH), 6.13 (app t, 1H, *J* = 3.2 Hz, Ar), 5.60 (s, 2H, CH_2Ph), 3.42 (t, 2H, *J* = 5.6 Hz, CH_2), 3.29 (app t, 2H, *J* = 5.6 Hz, CH_2), 1.43 (s, 9H, 3× CH_3*t*-Bu); ESI MS *m/z* found for $C_{19}H_{25}N_3O_3$: 367 $[M + Na]^+$, 344 $[M + H]^+$, 288 $[(M–Ph) + Na]^+$, 244 $[(M–Boc) + H]^+$; RP-HPLC gradient separation from 30% to 100% acetonitrile at 30 min, flow rate: 1 mL/min, t_R = 13.3 min.

3.6.14. Synthesis of 1-Benzyl-1*H*-*N*-[2-(2,3-Di-Tert-Butoxycarbonyl)Guanidinoethyl]Pyrrole-2-Carboxamide **16d**

From *N*-Boc analogue **16c** (248 mg, 0.72 mmol) in TFA–DCM 95:5 (14.4 mL), following the general procedure D (1 h), the crude of 2-(1-benzyl-1*H*-pyrrole-2-carboxamido)ethanaminium 2,2,2-trifluoroacetate **16c′** was dissolved in MeOH–DCM (14.4 mL). Then, from *N*,*N*′-di-(*tert*-butoxycarbonyl)thiourea (299 mg, 1.08 mmol), DIPEA (0.50 mL, 2.88 mmol), and *N*-iodosuccinimide (243 mg, 1.08 mmol), following the general procedure E (~24 h) and after chromatographic purification (20% MeOH-CH_2Cl_2), **16d** (136 mg, 39%) was obtained as a clear gum. Partial data for **16c′**: ESI-MS *m/z* found for $C_{14}H_{17}N_3O$: 267 $[M + Na]^+$, 244 $[M]^+$, 227 $[M–NH_2]^+$, 184 $[M–HN(CH_2)NH_2]^+$, 158 $[M–(CO)HN(CH_2)NH_2]^+$; RP-HPLC gradient separation from 5% to 100% acetonitrile at 30 min, flow rate: 1 mL/min, t_R = 11.1 min. Data for **16d**: ^{1}H NMR (400 MHz, CD_3OD) δ 7.28–7.18 (m, 3H, Ph), 7.07 (d, 2H, *J* = 6.8 Hz, Ph), 6.92 (dd, 1 H, *J* = 2.4, 1.6 Hz, Ar), 6.76 (dd, 1H, *J* = 3.6, 1.6 Hz, Ar), 6.12 (dd, 1H, *J* = 3.6, 2.4 Hz, Ar), 5.57 (s, 2H, CH_2Ph), 3.29–3.32 (m, 2H, CH_2), 3.16 (t, 2H, *J* = 6.2 Hz, CH_2), 1.52 (s, 9 H, 3× CH_3*t*-Bu), 1.42 (s, 9H, 3× CH_3*t*-Bu); ESI-MS *m/z* found for $C_{25}H_{35}N_5O_5$: 486 $[M + H]^+$; RP-HPLC gradient separation from 5% to 100% acetonitrile at 30 min, flow rate: 1 mL/min ,t_R = 21.9 min.

3.6.15. Synthesis of 1-Benzyl-1*H*-*N*-(2-Guanidinoethyl)-Pyrrole-2-Carboxamide **16**

From di-Boc guanidine analogue **16d** (114 mg, 0.23 mmol) in TFA–DCM 95:5 (7.0 mL), following the general procedure E (1 h) and after chromatographic purification (0.5% NH_4OH, 19.5% MeOH, 80% DCM), final product **16** (58.6 mg, 88%) was obtained as a white solid. Data for **16**: ^{1}H NMR (600 MHz, CD_3OD) δ 7.19–7.27 (m, 3 H, Ph), 7.07 (d, 2H, *J* = 7.8 Hz, Ph), 6.97–6.98 (m, 1H, Ar), 6.79–6.80 (m, 1H, Ar), 6.14–6.15 (m, 1 H, Ar), 5.59 (s, 2H, CH_2Ph), 3.40 (t, 2 H, *J* = 6.3 Hz, CH_2), 3.26 (t, 2H, *J* = 6.3 Hz, CH_2); ^{13}C NMR (100 MHz, CD_3OD) δ 165.2 (C=O), 159.9 (C=NH), 140.6 (C Ph), 129.5 (2× CH Ph), 129.3 (CH Ph), 128.3 (CH Ar), 127.9 (2× CH Ph), 126.0 (C Ar), 114.9 (CH Ar), 109.0 (CH Ar), 52.7 (CH_2Ph), 42.4 (CH_2), 39.3 (CH_2); ESI-MS *m/z* found for $C_{15}H_{19}N_5O$: 286 $[M + H]^+$; RP-HPLC gradient separation from 5% to 100% acetonitrile at 30 min, flow rate: 1 mL/mi, t_R = 20.2 min, R_f = 0.46 (MeOH–DCM 2:8).

3.6.16. Synthesis of *N*-[2-(2,3-Di-Tert-Butoxycarbonyl)Guanidinoethyl]-1-(*m*-(1-Trityl-Tetrazol-5-yl)Benzyl)-1*H*-Pyrrole-3-Carboxamide **17b** [24]

From 1*H*-pyrrole **21** (65.1 mg, 0.26 mmol), NaH 60% (15.4 mg, 0.39 mmol) in DMF (2.6 mL), and a solution of **17a** [69] (124 mg, 0.26 mmol) in DMF (2.6 mL), following the general procedure A (2 h) and after chromatographic purification (10–100% AcOEt-Et_2O), **17b** (81.8 mg, 40%) was obtained as a white solid. Data for **17b**: ^{1}H NMR (600 MHz, $CDCl_3$) δ 11.48 (s, 1H, NH), 8.06 (d, 1H, *J* = 7.8 Hz, Ar′), 7.99 (s, 1H, Ar′), 7.32–7.42 (m, 13H, Ar, Ar′, Trt, NH), 7.13–7.16 (m, 8H, Ar, Ar′, Trt), 6.60 (d, 1H, *J* = 1.8 Hz, Ar), 5.09 (s, 2H, CH_2Ar), 3.76 (br s, 2H, CH_2), 3.59 (br s, 2H, CH_2), 1.51 (s, 9H, 3× CH_3 *t*-Bu), 1.49 (s, 9H, 3× CH_3 *t*-Bu); ESI-MS *m/z* found for $C_{45}H_{49}N_9O_5$: 796.30 $[M + H]^+$, 696.27 $[(M–Boc) + H]^+$, 341.71 $[(17a–Trt) + Boc + H]^+$, 243 $[Trt]^+$; RP-HPLC gradient separation from 60% to 100% acetonitrile at 30 min, flow rate: 1 mL/min, t_R = 15.8 min.

3.6.17. Synthesis of 1-(2-(1-(*m*-(1*H*-Tetrazol-5-Yl)Benzyl)-1*H*-Pyrrole-3-Carboxamido)Ethyl) Guanidinium 2,2,2-Trifluoroacetate **17**

From **17b** (20 mg, 0.025 mmol) and TES (0.004 mL, 0.025 mmol) in TFA–DCM 95:5 (0.75 mL), following the general procedure D (5 h) and after purification by semi-preparative HPLC (10–60% ACN, 45 min), then lyophilization, final product **17** (7.89 mg, 68%) was obtained as a white solidwith 99% purity. Data for **17**: ^{1}H NMR (600 MHz, CD_3OD) δ 7.95 (d, 1H, *J* = 7.8 Hz, Ar'), 7.90 (s, 1H, Ar'), 7.57 (t, 1H, *J* = 7.8 Hz, Ar'), 7.43 (d, 1H, *J* = 7.8 Hz, Ar'), 7.41 (app dd, 1H, *J* = 2.4, 1.8 Hz, Ar), 6.85 (dd, 1 H, *J* = 3.0, 2.4 Hz, Ar), 6.56 (dd, 1H, *J* = 3.0, 1.8 Hz, Ar), 5.26 (s, 2H, CH_2Ar'), 3.47 (t, 2H, *J* = 6.0 Hz, CH_2), 3.36 (t, 2H, *J* = 6.0 Hz, CH_2); ^{13}C NMR (100 MHz, CD_3OD) δ 168.6 (C=O), 159.0 (2× C=NH), 140.8 (C Ar'), 131.5 (CH), 131.1 (CH), 127.7 (CH), 127.2 (CH), 126.3 (C Ar'), 125.4 (CH), 123.7 (CH), 120.5 (C Ar), 109.4 (CH Ar), 54.0 (CH_2Ar'), 42.4 (CH_2), 39.5 (CH_2); ESI-MS *m/z* found for $C_{16}H_{19}N_9O$: 354.53 $[M + H]^+$; RP-HPLC gradient separation from 10% to 100% acetonitrile at 30 min, flow rate: 1 mL/min, t_R = 17.7 min.

3.6.18. Synthesis of *N*-[2-(2,3-Di-Tert-Butoxycarbonyl)Guanidinoethyl]-1-(*p*-Tert-Butoxycarbonyl Methyl)Benzyl-1*H*-Pyrrole-3-Carboxamide **18b**

From 1*H*-pyrrole **21** (168 mg, 0.66 mmol), NaH 60% (39.8 mg, 1.66 mmol) in DMF (6.6 mL), and a solution of **18a**, (189 mg, 0.66 mmol) in DMF (6.6 mL), following the general procedure A (2 h) and after chromatographic purification (50–100% AcOEt-Et_2O), **18b** (87.6 mg, 22%) was obtained as a white solid). Data for **18b**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.49 (s, 1H, NH), 8.78 (s, 1H, NH), 7.46 (s, 1H, NH), 7.31 (s, 1H, Ar), 7.22 (d, 2H, *J* = 8.0 Hz, Ar'), 7.07 (d, 2H, *J* = 8.0 Hz, Ar'), 6.57 (d, 2H, *J* = 2.0 Hz, Ar), 5.02 (s, 2H, CH_2Ar), 3.67–3.71 (m, 2 H, CH_2), 3.56 (br s, 2H, CH_2), 3.50 (s, 2H, CH_2CO_2t-Bu), 1.51 (s, 9H, 3× CH_3t-Bu),1.49 (s, 9H, 3× CH_3t-Bu), 1.43 (s, 9H, 3× CH_3t-Bu).

3.6.19. Synthesis of 1-(2-(1-(*p*-(Carboxymethyl)Benzyl)-1*H*-Pyrrole-3-Carboxamido)Ethyl) Guanidinium 2,2,2-Trifluoroacetate **18**

From **18b** (50 mg, 0.083 mmol) and TES (0.01 mL, 0.083 mmol) in TFA–DCM 95:5 (2.50 mL), following the general procedure D (5 h) and after purification by semi-preparative HPLC (10–60% ACN, 45 min), then lyophilization, final product **18** (23.2 mg, 61%) was obtained as a white solidwith 98% purity. Data for **18**: ^{1}H NMR (600 MHz, CD_3OD) δ 7.33 (app dd, 1H, *J* = 2.4, 1.8 Hz, Ar), 7.27 (d, 2H, *J* = 8.1 Hz, Ar'), 7.17 (d, 2H, *J* = 8.1 Hz, Ar'), 6.78 (dd, 1H, *J* = 3.0, 2.4 Hz, Ar), 6.52 (dd, 1H, *J* = 3.0, 1.8 Hz, Ar), 5.11 (s, 2H, CH_2Ar'), 3.59 (s, 2H, CH_2CO_2H), 3.46 (t, 2H, *J* = 6.0 Hz, CH_2), 3.35 (t, 2H, *J* = 6.0 Hz, CH_2); ESI-MS *m/z* found for $C_{17}H_{21}N_5O_3$: 344.66 $[M + H]^+$; RP-HPLC gradient separation from 10% to 100% acetonitrile at 30 min, flow rate: 1 mL/min, t_R = 16.6 min.

3.6.20. Synthesis of *N*-[2-(2,3-Di-Tert-Butoxycarbonyl)Guanidinoethyl]-1-(*P*-Methoxycarbonyl) Benzyl-1*H*-Pyrrole-3-Carboxamide **19b**

From 1*H*-pyrrole **21** (109 mg, 0.43 mmol), NaH 60% (25.8 mg, 0.65 mmol) in DMF (4.3 mL), and a solution of **19a**, (98.5 mg, 0.43 mmol) in DMF (4.3 mL), following the general procedure A (2 h) and after chromatographic purification (50–100% AcOEt-Et_2O), **19b** (103 mg, 44%) was obtained as a white solid. Data for **19b**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.49 (s, 1H, NH), 8.73 (s, 1H, NH), 7.98 (d, 2H, *J* = 8.2 Hz, Ar'), 7.49 (s, 1H, NH), 7.31 (t, 1H, *J* = 1.8 Hz, Ar), 7.15 (d, 2H, *J* = 8.2 Hz, Ar'), 6.56–6.60 (m, 2 H, Ar), 5.10 (s, 2H, CH_2Ar), 3.90 (s, 3H, OCH_3), 3.67–3.71 (m, 2H, CH_2), 3.55–3.57 (m, 2 H, CH_2), 1.50 (s, 9 H, 3× CH_3t-Bu), 1.49 (s, 9H, 3× CH_3t-Bu); ESI-MS *m/z* found for $C_{27}H_{37}N_5O_7$: 344.60 [(M–2×Boc) + H]$^+$; RP-HPLC gradient separation from 5% to 100% acetonitrile at 30 min, flow rate: 1 mL/min, t_R = 25.7 min.

3.6.21. Synthesis of 1-(2-(1-(*p*-(Methoxycarbonyl)Benzyl)-1*H*-Pyrrole-3-Carboxamido)Ethyl) Guanidinium 2,2,2-Trifluoroacetate **19**

From **19b** (20 mg, 0.037 mmol) in TFA–DCM 95:5 (0.74 mL), following the general procedure D (5 h) and after purification by semi-preparative HPLC (10–60% ACN, 45 min), then lyophilization, final product **19** (13.3 mg, 79%) was obtained as a white solid with 99% purity. Data for **19**: ^{1}H NMR (400 MHz, CD_3OD) δ 7.98 (d, 2H, J = 8.0 Hz, Ar'), 7.36 (app t, 1H, J = 2.0 Hz, Ar), 7.28 (d, 2H, J = 8.0 Hz, Ar'), 6.81–6.82 (m, 1H, Ar), 6.55 (dd, 1H, J = 2.8, 2.0 Hz, Ar), 5.23 (s, 2H, CH_2Ar'), 3.89 (s, 3H, OCH_3), 3.47 (t, 2H, J = 6.4 Hz, CH_2), 3.35 (t, 2H, J = 6.4 Hz, CH_2); ESI-MS *m/z* found for $C_{17}H_{21}N_5O_3$: 344.68 $[M + H]^+$; RP-HPLC gradient separation from 5% to 100% acetonitrile at 30 min, flow rate: 1 mL/min, t_R = 18.9 min.

3.6.22. Synthesis of *N*-(2,3-Di-(Tert-Butyloxycarbonyl)Guanidinoethyl)Pyrrole-3-Carboxamide **21**

To a solution of 1*H*-pyrrole-3-carboxylic acid (85.5 mg, 0.77 mmol, 1.00 equiv) in DCM (5 mL) and DMF (1 mL), at 0 °C, HOBt (178 mg, 1.16 mmol, 1.50 equiv) and DCC (239 mg, 1.16 mmol, 1.50 equiv) were added. The mixture was stirred at the same temperature for 10 min, and was then supplemented with a solution of **20** [39] (350 mg, 1.16 mmol, 1.50 equiv) in DCM (13 mL) followed by DIPEA (0.20 mL, 1.16 mmol, 1.50 equiv). The reaction mixture warmed to rt over 3 h and monitored by TLC (10% MeOH in DCM). The solvents were removed in vacuo, and the remaining residue was purified by column chromatography (5% EtOH in Et_2O) to yield **21** (170 mg, 0.67 mmol, 87%) as a beige solid. Data for **21**:^{1}H NMR (400 MHz, $CDCl_3$) δ 11.51 (s, 1H, NH), 8.82 (s, 2H, NH), 7.57 (s, 1H, NH), 7.43–7.44 (m, 1H, Ar), 6.73–6.75 (m, 1H, Ar), 6.63–6.65 (m, 1H, Ar), 3.71–3.75 (m, 2H, CH_2), 3.57–3.60 (m, 2H, CH_2), 1.55 (s, 9 H, 3× CH_3*t*-Bu), 1.51 (s, 9 H, 3× CH_3*t*-Bu); ESI-MS *m/z* found for $C_{18}H_{29}N_5O_5$: 341 $[M-tBu + H]^+$, 381 $[M–tBu + K + H]^{2+}$; RP-HPLC gradient separation from 5% to 100% acetonitrile at 30 min, flow rate: 1 mL/min, t_R = 21.4 min.

3.7. Molecular Orbital Calculations

Two different approaches were applied in order to calculate the binding energy of the compounds inside TCR, namely density functional theory (DFT) [70] and semi-empirical (SE) methods [71]. For the application of DFT, several variants [72] differing in choice of functional [73] and basis set were implemented in order to calculate the interaction. This procedure was followed to select the most appropriate method for our complex (see Supporting Information). The self-consistent reaction field (SCRF) was used with DFT energies, optimizations, and frequency calculations to model the system in solution (H_2O). All DFT calculations were performed with Gaussian09 [74]. A similar protocol was applied for the calculation of the interaction energy including the whole TCR with SE methodologies. The MOPAC2012 [71] software was used for the SE calculations. Due to the large size of the protein–ligand systems, the keyword MOZYME [75] was employed to accelerate the calculations, and the COSMO [76] function was used to estimate the effect of the solvent. For the methods including dispersion (D), the optimized parameters for H, N, C, and O, as reported by McNamara and Hillier [72,73], were used. Semi-empirical calculations were performed on the whole complex (ligand–TCR), while DFT on the ligand and selected TCR residues.

3.8. In Vitro Evaluation of the Analogues Using Human PBMC

Peripheral blood samples (10 mL) were drawn from two healthy volunteers (one 24-year-old male and one 35-year-old female) and were analyzed in a CELL-DYN Sapphire hematology analyzer (Abbot Diagnostics, Lake Forest, IL, USA) to determine the absolute numbers and percentages of leukocytes, in particular lymphocytes and monocytes. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation over a Ficoll–Paque gradient (Biochrom AG, Berlin, Germany) and washed ×3 with ice-cold RPMI1640 culture medium (Gibco BRL, Waltham, MA, USA). The cells were stained with CellTrace CFSE for flow cytometry (Invitrogen-Thermo Fisher Scientific Inc., Waltham,

MA, USA) as described and cultured in RPMI1640 (with 10% Fetal Bovine Serum, 50 IU/mL penicillin, 100 μg/mL streptomycin, and 5×10^{-5} mol/L mercaptoethanol) (Invitrogen-Thermo Fisher Scientific Inc., Waltham, MA, USA) at a concentration of 10^6 cells/mL. PBMCs were cultured for three days in the presence of an anti–CD28 antibody (5 μg/mL) (BD Biosciences/Pharmingen, San Diego, CA, USA) and different concentrations of peptide $MBP_{83–96}$ (0.01 nM, 0.1 nM, 1 nM, 10 nM, and 100 nM) to estimate the optimal concentration that induces T cell proliferation. When the optimal $MBP_{83–96}$ concentration was determined, the cultures were repeated as previously with the addition of the same concentration of each of the studied analogues per point, in triplicate. T cell proliferation was monitored and quantified by flow cytometry. Flow cytometric acquisition and analysis were performed on at least 10,000 acquired events per sample using the BD FACSCalibur™ platform.

3.9. In Vitro Evaluation of the Analogues Using Mouse-Specific $MBP_{83–99}$ T Cells

Mice, SJL/J females, aged 4–9 weeks were purchased from the Animal Resource Centre (Perth Australia). All mice had free access to food and water, and were housed in a temperature-controlled environment with 12-h day/night cycles at the animal holding room Werribee Campus Animal Facility (Melbourne, Australia). They were allowed to acclimatize for at least 7 days before immunizations. All experiments were completed according to the guidelines of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and were approved by Victoria University Animal Experimentation Ethics Committee (AEC15/013). Mice were subcutaneously injected with 50 μg/100 μL reduced mannan conjugated to $MBP_{83–99}$ via a 10 amino acid linker $(KG)_5$ as previously described [34,77]. This conjugate has been shown to induce T cell proliferation to native peptide $MBP_{83–99}$ [26,27,30–32,34,77]. Spleen cells from 3 immunized SJL/J mice were isolated 10 days after immunization and assessed by T cell proliferation assay. As we have previously shown that the native peptide $MBP_{83–99}$ conjugated to mannan induces strong proliferative T cells to recall $MBP_{83–99}$ peptide, we used 3 mice/group to test each of the compounds' ability to inhibit this T cell proliferation. Hence, 3 mice/group in this screening process are adequate for determining the optimal compound for inhibiting T cell proliferation. Spleen cells at 2×10^5 in 100 μL of culture media were seeded into 96 well U-bottom plates and incubated for 1–6 days at 37 °C in the presence of recall $MBP_{83–99}$ peptide (10 nM) with or without 100x molar excess of compounds **15**–**19** or **AMB**. Proliferation was assessed by the addition of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) for 6 hours and proliferation assessed via spectrophotometry (Biorad microplate reader, 6.0) using a wavelength of 570 nm. All experiments were conducted in triplicate. The percentage of inhibition of cell proliferation in the presence of compound was calculated and plotted.

4. Conclusions

A ligand-based pharmacophore model was developed based on the conformational properties of the dominant $MBP_{83–96}$ epitope in complex with the TCR. The resulting model was employed for the virtual screening of the ZINC database for potential hits. A subset of the database, containing 500,000 all clean/ commercially available compounds, were screened, and the search yielded 13 hits. The potential inhibitors were ranked according to their inhibitory activity against TCR with the employment of molecular docking simulations. The compound with the highest docking score (compound **10**) was selected as lead and was subjected to optimization via chemical modifications. The resulting optimized molecule (compound **14**) presented increased docking score to the TCR and improved chemical properties such as TPSA and logP (Table 2).

The conformational analysis and the positioning of compound **14** in the TCR binding pocket led to the further modification with the addition of a methylene group and the organic synthesis of two isomers (compounds **15** and **16**). The analysis of the conformational properties of the three analogues via MD simulation experiments showed that analogue **15** has the most optimal positioning inside the TCR binding cavity and is better tethered within the receptor (Figure 5a). Extensive MD simulations may offer a deeper understanding of the interactions between the designed analogues

and the receptor, and prove to be a valuable tool in drug design. Furthermore, the interaction energy between the potential inhibitor (compound **15**) and the TCR was explored by employing a variety of molecular orbital approaches. DFT and SE methodologies were used in order to calculate the interaction energy between selected residues of the TCR, as well as the entire TCR, and the proposed inhibitor **15**. The combination of the two methodologies allows us to identify whether only certain residues have the greatest impact in the binding of compound **15** or other conformational aspects of the TCR are important in its binding. The agreement between the DFT and the SE methods show that the binding of the potential inhibitor to the TCR is attributed only to the residues surrounding the binding cavity and not to other conformational changes observed in the TCR. The results of the in vitro evaluation (Figure 8) suggest that both analogues **15** and **16** may serve as good candidate antagonists to be developed further for the inhibition of proliferation of T-cells that recognize the $MBP_{83–96}$ antigen.

Supplementary Materials: Supplementary materials can be found at www.mdpi.com/1422-0067/18/6/1215/s1.

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Abbreviations

CDRs	complementarity determining regions
DCC	*N*,*N*′-dicyclohexylcarbodiimide
DCM	dichloromethane
DIPEA	*N*,*N*-diisopropylethylamine
DCU	dicyclohexylurea
DMAP	4-dimethylaminopyridine
DMF	dimethylformamide
ESI MS	electrospray ionization mass spectrometry
HLA	human leukocyte antigen
HOBt	1-hydroxybenzotriazole
MBP	myelin basic protein
MD	molecular dynamics
MHC	major histocompatibility complex
MS	multiple sclerosis
MW	molecular weight
^{1}H NMR	proton nuclear magnetic resonance
^{13}C NMR	carbon-13 nuclear magnetic resonance
PBMC	peripheral blood mononuclear cells
RP-HPLC	reversed phase high-performance liquid chromatography
TCR	T cell receptor
TES	triethylsilane
TFA	trifluoroacetic acid
Th	T helper
TLC	thin layer chromatography
TPSA	total polar surface area

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Article

Streptococcus thermophilus ST285 Alters Pro-Inflammatory to Anti-Inflammatory Cytokine Secretion against Multiple Sclerosis Peptide in Mice

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Abstract: Probiotic bacteria have beneficial effects to the development and maintenance of a healthy microflora that subsequently has health benefits to humans. Some of the health benefits attributed to probiotics have been noted to be via their immune modulatory properties suppressing inflammatory conditions. Hence, probiotics have become prominent in recent years of investigation with regard to their health benefits. As such, in the current study, we determined the effects of *Streptococcus thermophilus* to agonist MBP_{83-99} peptide immunized mouse spleen cells. It was noted that *Streptococcus thermophilus* induced a significant increase in the expression of anti-inflammatory IL-4, IL-5, IL-10 cytokines, and decreased the secretion of pro-inflammatory IL-1β and IFN-γ. Regular consumption of *Streptococcus thermophilus* may therefore be beneficial in the management and treatment of autoimmune diseases such as multiple sclerosis.

Keywords: probiotics; *Streptococcus thermophilus*; ST285; MBP_{83-99} peptide; mannan; immune modulation; multiple sclerosis; agonist peptide

1. Introduction

There is an increasing trend in immune-mediated disorders across the world that is believed to be in part, a result of intestinal dysbiosis. The imbalance in the intestinal ecosystem can lead to a dysfunctional immune system that consequently causes immune disorders including autoimmune diseases (multiple sclerosis, MS) and other inflammatory disorders [1,2]. Probiotics have long been implicated for the overall improvement of health and the management of a number of health conditions including infection, constipation, allergies, and autoimmune diseases, and are either consumed in the form of different fermented foods and dairy products or taken as capsules. In either case, there is strong evidence that suggests that the ingestion of probiotics can alter intestinal dysbiosis and relieve dysfunctionality complications, with subsequent improvements to health [3].

Probiotic bacteria have been evolved inside the human intestinal tract (GIT), and through this co-evolution, the gut and its microbiome have developed a symbiotic relationship that is of mutual benefit. While the GIT microflora relies on the gut's warm habitat and food content, in return, it not only provides numerous unique bioactive components such as vitamins B and K, minerals, short chain fatty acids (SCF), and miosins to the host, but it also assists in modulating the immune system [3]. In fact, probiotics are able to modulate monocytes, macrophages, B cells, T helper (h)1, Th2, Th17, regulatory T cells (Treg), natural killer (NK) cells, and dendritic cells (DC) [3–6].

Chronic inflammation is the pathophysiological condition involved in neuro-degenerative disorders including MS, Parkinson's disease, and Alzheimer's disease [7,8]. There is cross-talk

between the gut microbiota and the central nervous system (CNS) [8–10], known as the gut–brain axis. An insufficient or imbalanced GIT microflora can also lead to dysfunctions in the gut–brain axis and the pathogenesis of a number of diseases inside the GIT (such as inflammatory bowel disease, IBD) and outside the GIT (such as the CNS). Experimental autoimmune encephalomyelitis (EAE) is an animal model of human MS that has been used to study the effects of probiotic bacteria on CNS [11,12]. One of the safe and appropriate ways to modulate T cells in MS is to orally administer specific autoantigens [13,14]. Administration of Bifidobacteria or Lactobacteria probiotic strains to mice has been shown to increase Treg cells and tumor growth factor (TGF)-β levels and reduce the severity of EAE clinical symptoms, in parallel with improvement in the regeneration of myelin in the spinal cord compared to the control [15,16]. Administration of both Bifidobacteria and Lactobacteria strains induce an additional significant delay in the onset of EAE and related clinical symptoms, together with a substantial reduction of mononuclear infiltration into the CNS, and increased level of Treg cells of the $CD4^+CD25^+Foxp3^+$ phenotype in mouse spleen and lymph nodes.

In SJL/J mice, immunization with the $MBP_{83–99}$ peptide mixed with mycobacterium stimulates autoimmune $CD4^+$ T cells in mice, and induces EAE [7,17]. Major histocompatibility complex (MHC) class II H-2^s haplotype in the SJL/J mouse strain resembles many clinical, histopathological, and immunological characteristics of human MS, thus the SJL/J mouse is regularly used for immunization studies. Different peptides are immunogenic in different mouse strains however, in the SJL/J mouse strain, the peptide $MBP_{81–100}$ binds to MHC class II H-2^s with high affinity with the minimum epitope being $MBP_{83–99}$ [7,17]. As such, the $MBP_{83–99}$ epitope has been used as an agonist peptide to immunize mice for the activation of $CD4^+$ T cells [7,17]. We have shown that injection of the MBP_{83-99} peptide conjugated to the carrier mannan or mixed in complete Freund's adjuvant induces Th1 pro-inflammatory interferon-gamma (IFN γ-g) secreting $CD4^+$ T cells [18–25]. Studies have shown that there is a cross-reactivity between the MBP self-peptide and some microbial peptides (i.e., UL15, PMM) for Hy.1B11 T cell receptor (TCR), which has been isolated from a patient with MS. It has been highlighted that there are chemical interactions underlying the recognition mechanisms between TCR and the peptides presented by MHC proteins, as a critical constituent in adaptive immune responses to foreign antigens [26].

The Streptococcus genus constitutes over 100 species, amongst which *S. thermophilus* (ST) are non-pathogenic and food related bacteria that represent outstanding technological features in the food industry [27]. ST are commonly used as secondary starter cultures in dairy products to transform lactose into lactic acid and to acidify the pH of milk [27,28], contributing to both the fermentation and flavoring of dairy products [29]. Most probiotics belong to lactic acid bacteria (LAB); Gram-positive lactic acid producing bacteria that include lactobacilli, bifidobacterial, and enterococci [3]. As such, live LABs are not only used in foods for their health benefits, but exopolysaccharide-producing strains of ST such as ST1342, ST1275, and ST285 are generally used due to their beneficial properties (i.e., relieving lactose intolerance and suppressing acute conditions such as acute ulcerative colitis) [29]. Additionally, experimental studies designed to investigate the effect of VSL3 (Streptococcus, Bifidobacterium, and Lactobacillus species) on the peripheral immune system and the GIT microbiota in MS patients and healthy subjects showed improved abundance of many taxa with enriched taxa mainly consisting of Lactobacillus, Streptococcus, and Bifidobacterium. VSL3 also induced peripheral anti-inflammatory immune responses [30].

We recently showed that ST bacteria have anti-inflammatory properties [29]. U937 pro-monocytic cell line co-cultured with three ST bacteria (ST1342, ST1275 and ST285) induced an anti-inflammatory profile [29]. ST285 was further shown to have immune modulating effects via gene arrays to human peripheral blood mononuclear cells (PBMC) [31] and monocyte cells isolated from PBMC [32]. Herein, we immunized SJL/J mice with agonist $MBP_{83–99}$ peptide conjugated to mannan three times, isolated spleen cells, and after re-stimulation of spleen cells with the $MBP_{83–99}$ peptide, IFN-γ was secreted by spleen cells. Re-stimulation of spleen cells with the $MBP_{83–99}$ peptide in the presence of ST285 probiotics was able to downregulate IFN-γ responses and stimulate the Th2, IL-4, IL-5, and IL-10

cytokine profile. These studies show that probiotics are able to modulate and alter the immune profile of $MBP_{83–99}$ specific cells to anti-inflammatory, which warrant in vivo EAE mouse experiments and hold promise as a therapeutic alternative approach to MS in human clinical trials.

2. Materials and Methods

2.1. Bacterial Strains

Pure bacterial cultures of *S. thermophilus* 285 (ST285) were obtained from the Victoria University culture collection (Werribee, Vic, Australia). Stock cultures were stored in cryobeads at −80 °C. Prior to each experiment, the cultures were propagated in M17 broth (Oxoid, Denmark) with 20 g/L lactose and incubated at 37 °C under aerobic conditions. Bacteria were also cultured on M17 agar (1.5% *w/v* agar) with 20 g/L lactose (Oxoid, Denmark) to assess the characteristics, morphology, purity, and Gram-positive confirmation [1].

2.2. Preparation of Live Bacterial Suspensions

Media were prepared and autoclaved at 121 °C for 15 min prior to the experiments. Bacterial cultures were grown three times in M17 broth with 20 g/L lactose at 37 °C aerobically for 18 h with a 1% inoculum transfer rate [33]. Cultures grow optimally at 37–42 °C for 24 h [29]. The growth period of cultures were consistent at 18 h (at the end of the exponential growth phase) and before the stationary growth phase to prevent cell lysis.

2.3. Enumeration of Bacterial Cells

For the actual experiment, bacteria were grown in broth media to the stationary phase at 37 °C aerobically, pelleted by centrifugation (6000× g) for 15 min at 4 °C, transferred, and resuspended in Dulbecco's phosphate-buffered saline, pH 7.4 (Invitrogen, Pty Ltd. Australia). The bacterial density in suspension was adjusted to 10^8 colony forming units (cfu)/mL for final concentration by determining the optical density at 600 nm, followed by two washes with Dulbecco's phosphate-buffered saline. These samples constituted the live-cell suspensions and were resuspended in the Roswell Park Memorial Institute (RPMI) 1640 culture media prior to co-culturing with spleen cells [1].

2.4. Mouse Experimental Procedures

2.4.1. Mice, Conjugates, and Immunization Schedule

Female SJL/J mice, aged 6–9 weeks, used in all experiments were purchased from the Animal Resources Center (ARC, Perth, Australia), and accommodated at the animal house (Victoria University, Werribee campus, Melbourne, Australia). All mice were ensured free access to water and food, and were housed in a temperature controlled room with a 12 h day 12 h night cycle. All immunizations were conducted according to the guidelines of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and the study was approved by the Victoria University Animal Ethics Committee (AEC15/013) of Victoria University, Melbourne, Australia.

The $MBP_{83–99}$ agonist peptide of over 99% purity with $(KG)_5$ at the C-terminus was conjugated to mannan via a method previously described [34–38]. Briefly, 14 mg of mannan (Sigma, VIC Australia) was oxidized in sodium carbonate buffer and 0.1 M sodium periodate at 4 °C after which ethylene glycol was added and incubated for 30 min at 4 °C. Oxidized mannan comprising aldehyde groups was passed through a PD-10 column (Sigma, VIC Australia) pre-equilibrated in carbonate-bicarbonate buffer pH 9.0 and 2 mL of oxidized mannan was collected and 1 mg of $MBP_{83–99}$-$(KG)_5$ peptide was added and allowed to react overnight at room temperature in the dark. The resultant $MBP_{83–99}$-$(KG)_5$-mannan conjugate was used to immunize the mice.

The $MBP_{83–99}$ mannan peptide conjugate (50 µg/mouse) was injected in the SJL/J mice subcutaneously into the base of the tail, three times, every two weeks [17]. This conjugate has

been shown to induce T cell proliferation and IFN-γ cytokine secretion to the agonist $MBP_{83–99}$ peptide in SJL/J mice [17,19,23,24]. Ten to fourteen days after the three injections, spleen cells were isolated, red blood cells were lysed using 0.73% NH_4Cl, and counted.

2.4.2. Isolation of Spleen Cells and In Vitro Stimulation with ST285

Spleen cells were resuspended in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Pty Ltd. Australia), 1% antibiotic-antimycotic solution, and 2 mM L-glutamine in T75 cm^2 cell culture flasks. Mouse spleen cells (1×10^7) in RPMI media only was used as the negative control, 5 µg/mL recall agonist $MBP_{83–99}$ peptide was used as the recall control, or 1×10^8 ST285 bacteria were added together with the $MBP_{83–99}$ peptide, and cultured at 37 °C, 5% CO_2 for 24 h [29]. We previously showed that 24 h co-culture was adequate for the stimulation of the monocyte/macrophage cell line, human peripheral blood mononuclear cells, and human monocytes isolated from peripheral blood mononuclear cells [29,32]. At the end of the culture period, cells were transferred into falcon tubes, and centrifuged for 5 min at 1200 rpm to pellet the cells. All cell-free supernatants were collected and frozen at −20 °C until cytokine analysis.

2.5. Cytokine Production Analysis

Cytokine secretion of the spleen cell culture supernatants was analyzed by commercially available capture and detection antibodies in a Bioplex multiplex bead assay for a panel of nine mouse cytokines and chemokines using a 9-plex kit (BioRad, Melbourne Australia) to measure Interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-10, GM-CSF, TNF-α, and IFN-γ. Cell-free supernatants were collected and the assay procedures were performed according to the manufacturer's instructions. Briefly, a flat bottom 96-well plate was coated with 1× coupled beads and washed twice, followed by adding the standard serial dilutions, blanks, and samples to assigned wells. Post incubation at shaking at room temperature, plates were washed twice, adequate 1× detection antibody was added, and incubated at room temperature. Plates were washed three times and 1× Streptavidin Phycoerythrin (SA-PE) stop solution was added to each well, followed by incubating at room temperature and washing. Data collection was repeated twice, data were expressed as the mean cytokine response minus background (pg/mL) of each treatment from three replicate wells, plus or minus the standard error of the mean.

2.6. Statistical Analysis

Significant differences between all treatment groups were tested by analysis of variance (ANOVA) using the Statistical Package for the Social Sciences for Windows 25.0 (SPSS; IBM Corp), followed by a comparison between treatments performed by Tukey's honest significance test/degree and Fisher's least significant difference method, with a level of significance defined as $p < 0.05$.

3. Results

3.1. ST285 Reduces Pro-Inflammatory TNF-α and IFN-γ Production by $MBP_{83–99}$ Primed Mouse Splenocytes

Interferon gamma (IFN-γ) is a pro-inflammatory Th1 cytokine involved in macrophage activation and cellular immunity. IFN-γ promotes Th1 cells and inhibits Th2 anti-inflammatory cells. In MS, IFN-γ is induced following $CD4^+$ T cell activation by agonist peptide $MBP_{83–99}$. SJL/J mice immunized with $MBP_{83–99}$–mannan conjugates induced IFN-γ responses by spleen cells, following overnight $MBP_{83–99}$ peptide re-stimulation (Figure 1A, $p < 0.01$). Spleen cells re-stimulated with the agonist $MBP_{83–99}$ peptide in the presence of ST285 reduced IFN-γ cytokine secretion (Figure 1A, $p < 0.05$). TNF-α, a Th1 cytokine, was not secreted by spleen cells from immunized mice wither by re-stimulation of the MBP_{83-99} peptide or MBP_{83-99} peptide plus ST285 (Figure 1B).

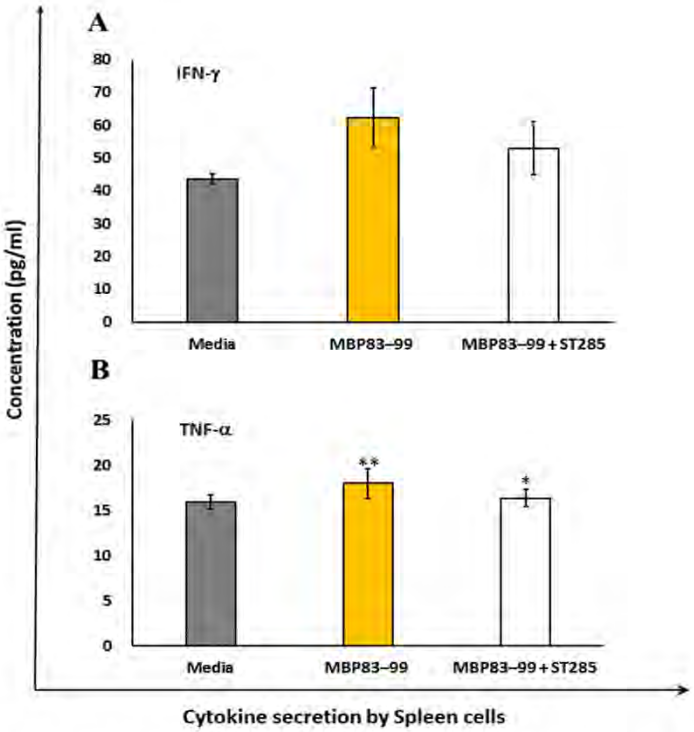

Figure 1. *S. thermophilus* 285 reduces pro-inflammatory cytokine production by mouse splenocytes. Spleen cells isolated from immunized mice ($n = 3$) were stimulated with *S. thermophilus* (ST) ST285 and recall agonist $MBP_{83–99}$ peptide for 24 h and secretion of (**A**) IFN-γ and (**B**) TNF-α were measured. Recall $MBP_{83–99}$ peptide was used as an internal positive control, and media refers to spleen cells from immunized mice ($n = 3$) without any additional recall peptide, or ST285 probiotic bacteria plus the $MBP_{83–99}$ peptide. Means of two different readings of three replicate experiments were measured and analyzed. The means of readings for $n = 3$ mice were calculated and presented as plus or minus (±) the standard error of the mean. Symbols represent the p value for the Tukey test (one way ANOVA) where * $p < 0.05$ and ** $p < 0.01$.

3.2. ST285 Decreases Secretion of IL-1β, IL-2, and IL-6 by Mouse Spleen Cells

Secretion of IL-1β was slightly, but significantly reduced in immunized mouse spleen cells re-stimulated with the $MBP_{83–99}$ peptide and ST285 compared to no re-stimulation, or the $MBP_{83–99}$ peptide re-stimulation without ST285 ($p < 0.05$) (Figure 2A). IL-2 production was significantly increased in immunized spleen cells re-stimulated with the $MBP_{83–99}$ peptide ($p < 0.01$), which was weakly but significantly decreased as a result of the co-stimulation of mouse spleen cells with the $MBP_{83–99}$ peptide plus ST285 ($p < 0.05$) (Figure 2B). The production of IL-6 was profoundly increased by immunized mouse spleen cells upon co-culture of ST285 and the recall $MBP_{83–99}$ peptide compared to the control media or $MBP_{83–99}$ recall peptide ($p < 0.001$) (Figure 2C); spleen cells recalled with the $MBP_{83–99}$ peptide alone also increased IL-6 secretion.

3.3. ST285 Induces Anti-Inflammatory Cytokine Profile by Mouse Splenocytes

Mice immunized with the $MBP_{83–99}$ agonist peptide did not induce IL-4, IL-5, and IL-10 anti-inflammatory cytokines in the control (media alone) and recall agonist peptide $MBP_{83–99}$ (Figure 3). However, the Th2 anti-inflammatory cytokine IL-4 was significantly ($p < 0.001$) increased by immunized mouse spleen cells when the $MBP_{83–99}$ recall peptide was co-cultured with ST285 probiotic bacteria (Figure 3A). IL-5 was also increased by immunized spleen cells following co-culture with ST285 and the recall agonist $MBP_{83–99}$ peptide (Figure 3B) ($p < 0.01$). The anti-inflammatory IL-10 cytokine was also significantly increased by immunized mouse spleen cells when co-cultured with ST285 and

agonist recall MBP_{83-99} peptide compared to the MBP_{83-99} peptide alone or media control ($p < 0.001$) (Figure 3C).

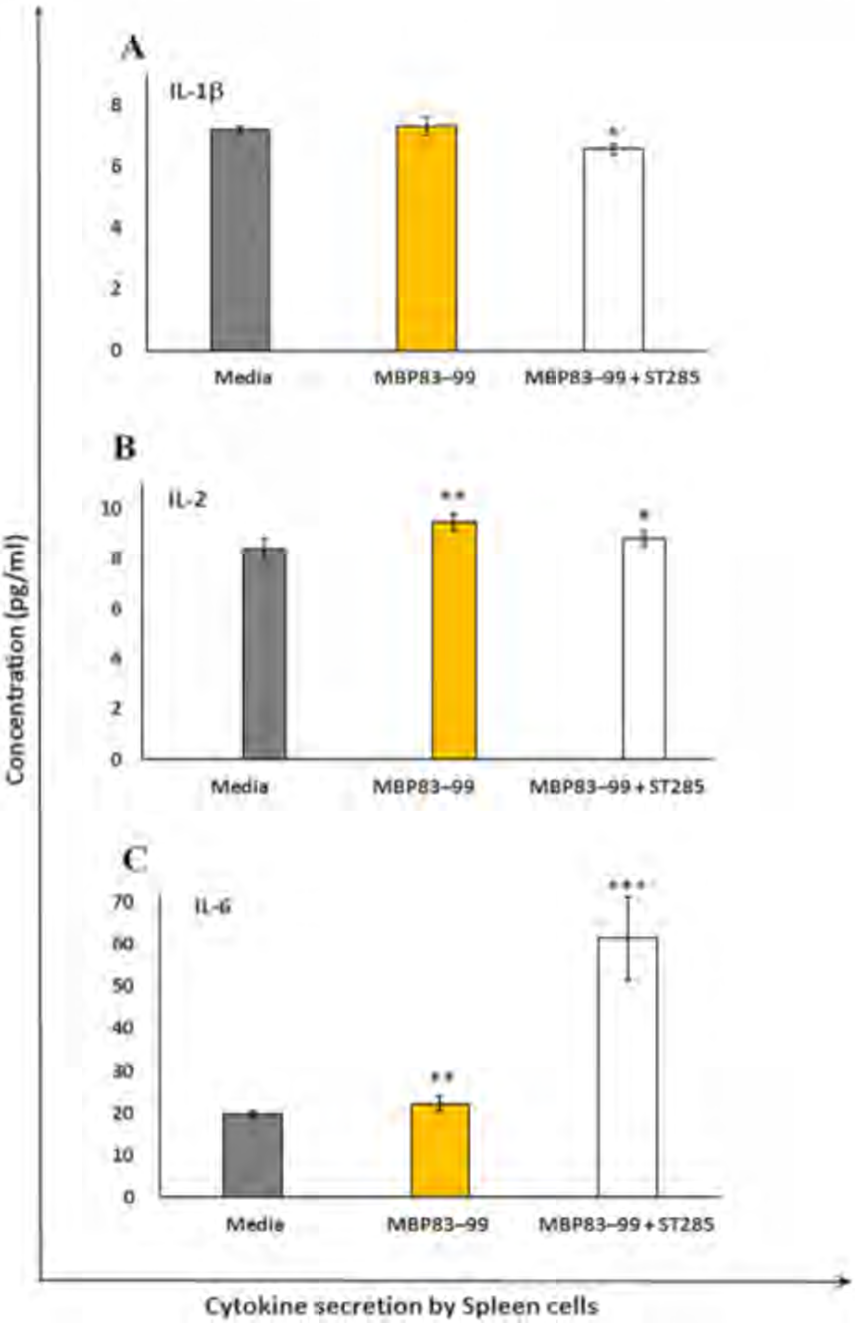

Figure 2. *S. thermophilus* 285 decreases expression of IL-1β, IL-2, and increases IL-6 by mouse spleen cells. Spleen cells isolated from immunized mice ($n = 3$) were stimulated with *S. thermophilus* (ST) ST285 and recall agonist MBP_{83-99} peptide for 24 h and secretion of (**A**) IL-1β, (**B**) IL-2, and (**C**) IL-6 were measured. Recall MBP_{83-99} peptide was used as the reference peptide, and media refers to spleen cells from immunized mice ($n = 3$) without any additional recall peptide or ST285 probiotic bacteria plus MBP_{83-99} peptide. Means are shown as plus or minus (±) standard error of the means. Symbols represent the p value for the Tukey test (one way ANOVA) where * $p < 0.05$ and ** $p < 0.01$ and *** $p < 0.001$.

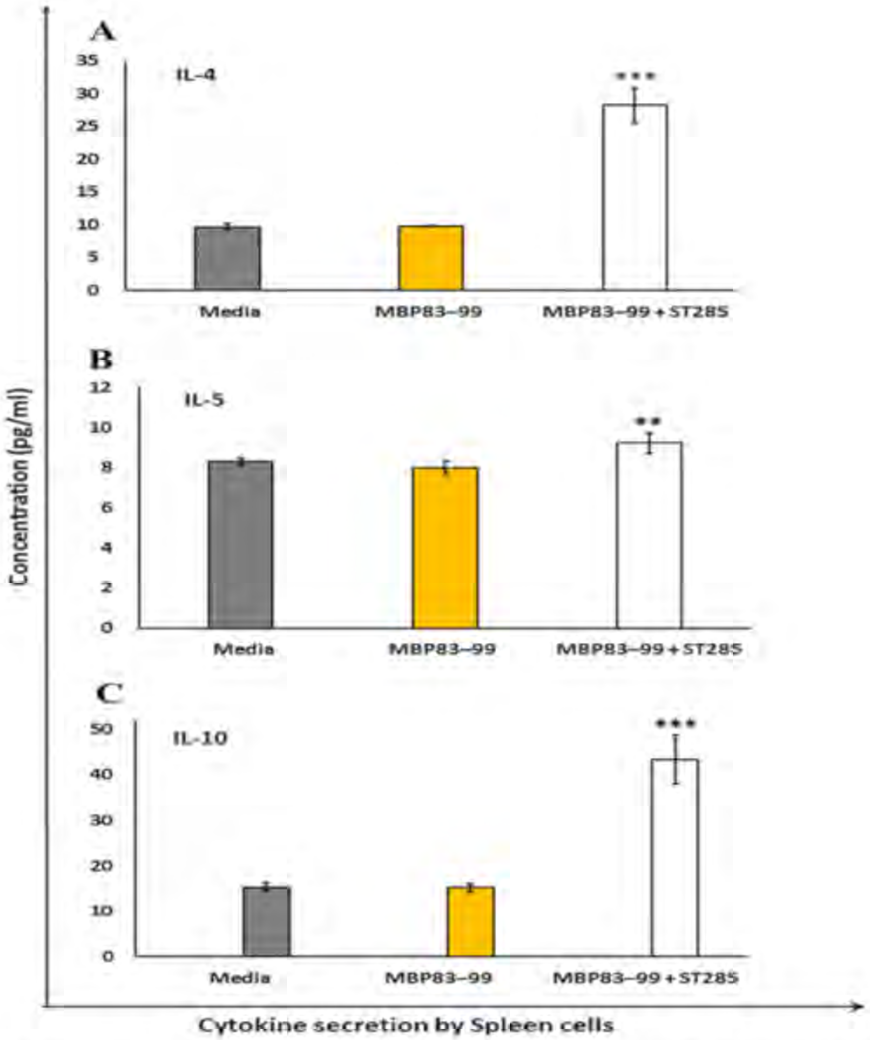

Figure 3. *S. thermophilus* 285 induces the anti-inflammatory cytokine profile by immunized mouse splenocytes. Spleen cells isolated from immunized mice ($n = 3$) were stimulated with *S. thermophilus* (ST) ST285 and the recall agonist $MBP_{83–99}$ peptide for 24 h and the secretion of (**A**) IL-4, (**B**) IL-5, and (**C**) IL-10 were measured. Recall $MBP_{83–99}$ peptide, media alone, or recall $MBP_{83–99}$ peptide plus ST285 are shown from immunized mice ($n = 3$). The means of readings for $n = 3$ mice were calculated and presented as plus or minus (±) the standard error of the mean. Symbols represent the p value for the Tukey test (one way ANOVA) where ** $p < 0.01$ and *** $p < 0.001$.

3.4. ST285 Does Not Alter the Secretion of Granulocyte-macrophage Colony-stimulating Factor by Mouse Spleen Cells

Secretion of granulocyte-macrophage colony-stimulating factor (GM-CSF) did not show any change by immunized mouse spleen cells upon co-culture with ST285 and agonist recall $MBP_{83–99}$ peptide compared to the negative control or $MBP_{83–99}$ peptide (Figure 4), despite significant upregulation of GM-CSF by ST285 on monocytes/macrophage cells [29].

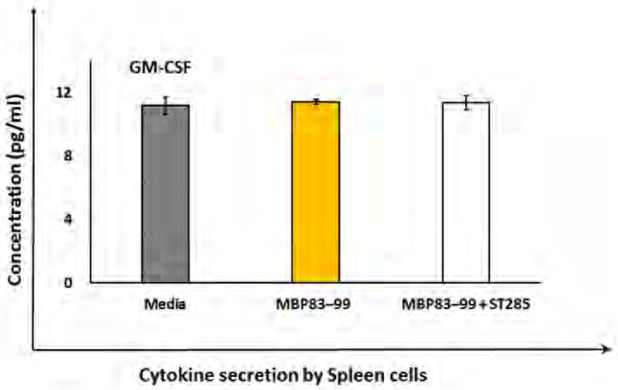

Figure 4. *S. thermophilus* 285 does not alter secretion of GM-CSF by mouse spleen cells. Spleen cells isolated from immunized mice ($n = 3$) were stimulated with *S. thermophilus* (ST) ST285 and the recall reference peptide for 24 hours and secretion of GM-CSF was measured. Recall $MBP_{83–99}$ peptide, media alone, or recall $MBP_{83–99}$ peptide plus ST285 are shown from immunized mice ($n = 3$). The means of readings for $n = 3$ mice were calculated and presented as plus or minus (±) the standard error of the mean.

4. Discussion

The Th1 pro-inflammatory cytokines IFN-γ and TNF-α are both involved in the defense against bacterial infections and in acute phase reactions. In MS, these two cytokines are implicated in the pathogenesis of disease by stimulating $CD4^+$ T cells against the myelin sheath. Mice immunized with the mannan $MBP_{83–99}$ peptide stimulated IFN-γ secretion, which was reduced in the presence of ST285. This reduction is very important in the context of inflamed situations such as autoimmune and inflammatory diseases, as any reduction in the amount of mediators that cause inflammation is imperative in the relief of symptoms. We previously noted that high levels of TNF-α and IFN-γ was secreted by the U937 monocytic cell line in the presence of ST285 [29]. However, the addition of ST285 to the $MBP_{83–99}$ recall peptide reduced IFN-γ secretion by mouse splenocytes. Spleen cells were populated with B, T, NK cells, macrophages, and monocytes, while the U937 cell line that we previously used were purely monocytic/macrophage cells. Additionally, the polarized inflammatory state of cytokines as a result of the immunization regimen and further exposure of spleen cells to the recall $MBP_{83–99}$ peptide that operate as inflammatory stimuli, compared to the U937 monoclonal cells only being exposed to ST285 bacteria, might give a clue as to the ability of ST285 probiotics to dampen the inflammatory immune response in the instance of exposure to polyclonal spleen cells.

Secretion of IL-1β by monocytes is involved in regulating the immune and inflammatory responses to infections and injuries; therefore, it has a role in innate immunity. IL-1β is also a major mediator in inflammatory responses associated with various cellular activities such as differentiation, proliferation, and apoptosis [39]. In addition, IL-1β is a regulator of inflammatory reactions and is involved in the stimulation of the central nervous system through cyclooxygenase-2 (PTGS2/COX2), which is involved in neurodegenerative disorders such as MS [33,40], Down's Syndrome, Alzheimer's disease, and HIV-associated dementia [41,42].

We noted the secretion of IL-1β by immunized mouse spleen cells was marginally, but significantly reduced in the presence of ST285 with the recall $MBP_{83–99}$ peptide. We previously noted that ST285 did not induce IL-1β cytokine to the U937 cell lines, however, significant upregulation of IL-1β mRNA was induced by human PBMC [31] and monocytes post co-culture with ST285 [32]. It is therefore clear that the immunized mouse spleens and the recall of T cells with the $MBP_{83–99}$ peptide in the presence of ST285 caused a reduction in IL-1β secretion. Likewise, IL-2 was marginally decreased in the presence of ST285 compared to the increased secretion caused by the $MBP_{83–99}$ peptide in the positive control. Co-culturing human PBMC with ST285 also downregulates IL-2 mRNA expression [31].

IL-6 is produced by activated immune cells including DC, B cells, and macrophages. Although IL-6 is associated with acute phase responses, it is also associated with a reduction of Th1 polarization, while promoting Th2 differentiation, B cell maturation, and macrophage differentiation. Proliferation and differentiation of Th2 cells changed the polarized Th1 environment and skewed the Th1/Th2 balance toward Th2, which is beneficial in relieving autoimmune conditions such as MS. IL-6 production was significantly higher (three times) in mouse splenocytes cultured with ST285 compared to the control, hence, it is likely that ST285 bacteria may potentially change the balance toward a healthier state in MS. We previously noted significant upregulation of IL-6 to human monocytes [manuscript submitted] and to bulk PBMC co-cultures [31] with ST285, which are also in accord with the increase in IL-6 levels by the U937 promonocytic cell line co-cultured with ST285 [29]. Likewise, the commercially used probiotic *L. paracasei* DG induces IL-6 cytokines to the THP-1 human monocyte cell line [43]. In contrast, ingestion of *B. bifidum* by mice did not increase the IL-6 levels, but boosted anti-oxidation activities in the spleen and thymus of mice and improved other immune functions by changing the gene expression of immune mediators [44].

It is likely that the constant-shifting in the equilibrium and the dynamics that exist between pro- and anti-inflammatory cytokines will lead to some controversy in the research findings regarding IL-6. On one hand, IL-6 may ease the autoimmune condition due to its downstream immunological effects. On the other hand, elevated levels of pro-inflammatory effector T cell cytokines such as IFN-γ, IL-17 as well as IL-6 are noted in patients with autoimmune myasthenia gravis and MS [45]. Thus, it might be likely that the role that cytokines such as IL-6 play may depend on their bio-environment and may be advantageous to the body, if probiotics such as ST285 are used for neutralization and/or reversing from a pro- to an anti-inflammatory state in the body.

IL-4 is one of the important cytokines required for anti-inflammatory responses against inflammatory conditions such as MS and allergies [29]. IL-4 production was significantly increased by mouse spleen cells in the presence of the recall $MBP_{83–99}$ peptide and ST285 compared to either the $MBP_{83–99}$ peptide alone or the negative control (media). Likewise, it was previously noted that ST285 induced U937 monocytic cells to produce IL-4, although no changes to mRNA expression levels of IL-4 were noted to human monocytes or to bulk human PBMC following co-cultures with ST285 [31]. In contrast, feeding BALB/c mice with *L. paracasei* BEJ01 alone or combined with aflatoxins B1 (AFB1) and fumonisin B1 (FB1) (known foodborne mycotoxins with immunomycotoxic effects on human health) was used to evaluate *L. paracasei* BEJ01 detoxification [46]. Assessing different splenic immunological factors indicated that exposure to these mycotoxins led to increased IL-4 mRNA levels, oxidative stress, and immunotoxicity in the spleen [46] whereas the combined LAB treatment with AFB1 or FB1 suppressed and normalized mRNA levels of IL-4, showing protective effects induced by LAB against AFB1 and FB1 via diminishing toxin adhesion and bioavailability [46]. In contrast, spleen cells isolated from BALB/c mice in vitro co-cultured individually with LAB strains (*L. casei* Lc2w (Lc), *L. plantarum* CCFM47 (Lp), and *L. acidophilus* CCFM137 (La)) showed reduced IL-4 production by spleen cells exposed to La only, while parallel animal studies displayed LAB-induced alleviation of inflammation post airway allergy for all strains through increased Treg cells and modulation of Th1/Th2 balance [47].

The anti-inflammatory cytokine IL-5 is produced by Th2 cells and mast cells. In the event of infection with helminth parasites, IL-5 leads to a lesser risk of autoimmune disorders, which is indirectly accredited to some therapeutic characteristics of IL-5 in autoimmune disorders. We noted a slight increase in the IL-5 production by spleen cells in response to ST285, whereas no changes to the mRNA expression levels of IL-5 were previously noted in ST285 co-cultures with human PBMC or human monocyte cells [manuscripts submitted]. A study showed that treating mice with *Fasciola hepatica* excretion/secretions (FHES) reduced EAE clinical signs due to a significant decrease in the infiltration of Th1 and Th17 cells into the brain and an increase in IL-5 (and IL-23) response, with subsequent increase in eosinophils [48]. It is likely that the small but significant increase of IL-5 may be beneficial to MS.

IL-10, an anti-inflammatory cytokine, is secreted by Th2 and Treg cells. Amongst all the anti-inflammatory cytokines and chemokines, anti-inflammatory properties of IL-10 are the most potent in suppressing inflammatory mediators by other activated immune cells (TNF-α, IFN-γ, IL-1, IL-17, and IL-23 cytokines) [49]. A significant amplification in the IL-10 levels secreted by the spleen cells in the presence of ST285 was noted, which was similarly shown for the U937 monocytic cell line in the presence of ST285 and to human PBMC, although no significant changes were shown in human monocyte cells [manuscripts submitted]. Likewise, oral administration of *L. reuteri* and *L. lactis* strains to mice stimulated the production of anti-inflammatory IL-10 and Treg cells [50,51]. In addition, sub-clinical studies of *L. salivarius* UCC118, *L. lactis* MG1363, and *L. plantarum* WCFS1 administered to mice and re-exposure of their isolated bone marrow cells to the three bacterial co-cultures showed all three strains differentially stimulated IL-10 production [52]. Correspondingly, when DC from spleen and mesenteric lymph nodes of mice were matured using *L. acidophilus* X37 and exposed to commensal gut *Bifidobacterium longum* Q46, *L. acidophilus* X37, and *Escherichia coli* Nissle 1917, increased IL-10 levels were noted [53]. Similarly, after BALB/c mice were fed with *L. paracasei* BEJ01 alone or combined with aflatoxins B1 and fumonisin B1, high IL-10 mRNA levels were induced [46]. In addition, mice fed with kefir-derived *Lactobacillus kefiri* CIDCA 8348 also increased IL-10 gene expression [54]. In the context of MS, the use of ST285 was shown to downregulate Th1 responses and upregulate Th2 responses, something of the utmost importance to patients with MS to alleviate MS symptoms and/or reversal of the disease [31].

5. Conclusions

Immunization of SJL/J mice with agonist $MBP_{83–99}$ peptide conjugated to mannan induces Th1 pro-inflammatory IFN-γ responses and no Th2 anti-inflammatory responses when spleen cells are co-cultured in vitro in the presence of the agonist recall $MBP_{83–99}$ peptide. However, stimulation of spleen cells with the recall $MBP_{83–99}$ peptide in the presence of ST285 significantly increased the secretion of IL-4, IL-6, and IL-10, along with mild upregulation in IL-2 and IL-5, suggesting a role for ST285 in the activation of immune response phenotypes toward a predominant anti-inflammatory profile, tolerance, and suppression of inflammation. In addition, ST285, downregulated the secretion of IL-1a and IFN-γ—the immune mediators involved in Th1 type responses—collectively pointing to a shift in immune responses from Th1 to a Th2 phenotype. More importantly, the significant increase of IL-10 could further contribute by the differentiation of naïve $CD4^+$ T cells and proliferation of Tregs, which can also drive the immune balance further toward a dominant anti-inflammatory phenotype. Additionally, given the drastic increase of GM-CSF in our previous studies of ST285 co-cultured with the U937 monocytic cell line, human PBMC, and human monocyte cells, and no change to the secretion of GM-CSF in spleen cells with GM-CSF being a major cytokine for proliferation and recruitment of the immune cells, this might indicate a deliberate and purposeful neutralization of GM-CSF by ST285. The effects of ST285 on the immune response could be used as a novel approach in modulating chronic inflammatory and autoimmune conditions such as MS. Further studies should involve the effects of ST285 in mice with EAE or be used to prevent EAE induction, which will pave the way for new modalities for the treatment of MS in human clinical trials.

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Original article

Immunomodulatory properties of selectively processed prawn protein fractions assessed using human peripheral blood mononuclear cells

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Summary Prawn muscles were treated with acetic acid and high-pressure processing (600 MPa) separately to analyse their antigenicity and immunogenicity. The protein fractions were separated and isolated using preparative HPLC, and their antigenicity was analysed using Immunoglobulin G (IgG) ELISA kit. Out of thirty-nine protein fractions, only four (A10, A11, B10 and C9) were detected with antigenic potentials. The immunogenicity of these protein fractions was analysed using human PBMCs, and supernatants were collected at multiple times from 0 to 144 h. The treated fractions (B10 and C9) analysed using Immunoglobulin E (IgE) ELISA kit showed significantly ($P < 0.05$) lower pro- and anti-inflammatory cytokine production compared with control (A10). The allergenic fractions were characterised using an LC/MS/MS, which identified nine proteins. Among these, six proteins (tropomyosin, arginine kinase, haemocyanin, enolase, vitellogenin and 14-3-3 zeta) have been established as allergenic in prawn muscle and ovaries. Other three proteins (beta-1,3-glucan-binding protein, translationally controlled tumour protein and farnesoic acid O-methyltransferase short isoform protein) identified in this study need further investigation for their immunogenic properties.

Keywords Antigenicity, immunogenicity, interleukin cytokine, LC/MS/MS, PBMCs, prawn allergy.

Introduction

Prawn allergy is one of the major causes of food-borne allergies, responsible for most severe food allergy-related emergency department visits (Sicherer *et al.*, 2004; Liu *et al.*, 2008). The upward trend of prawn-induced allergic incidents has now become an alarming issue in global food safety (Sicherer & Sampson, 2006). The major allergen in prawn protein, known as tropomyosin, is responsible for over 80% prawn allergy-related incidents. It is a 37 kDa heat-stable muscle protein having an α-helical structure associated with actin filaments (Troiano, 2016; Faisal *et al.*, 2019c). Beside this protein, arginine kinase, myosin light chain, actin, troponin, haemocyanin and sarcoplasmic calcium-binding protein are also known as minor allergen in prawns (Rahman *et al.*, 2013; Kamath *et al.*, 2014; Khanaruksombat *et al.*, 2014). Prawn allergy is an IgE-mediated type 1 allergy showing symptoms of severe mucocutaneous, respiratory, gastrointestinal, anaphylactic and cardiovascular (95.7%, 23.9%, 16.3%, 11.9% and 3.3%, respectively) disorders (Sicherer, 2011; Pedrosa *et al.*, 2015). In previous studies, Ayuso *et al.* (2002) and Zheng *et al.* (2011) reported eight IgE-binding epitopes, whereas Wang *et al.* (2012) reported 17 IgE-binding epitopes on tropomyosin. As per literature, the hypersensitivity reactions due to binding of protein epitopes with IgE antibodies are termed as allergenicity, whereas binding with IgG antibodies is known as antigenicity (Verhoeckx *et al.*, 2015; Bogahawaththa *et al.*, 2017). Moreover, when allergic components stimulate the immune system of the human body involving generation of specific IgE antibodies, the resulting stimulation is known as immunogenicity (Actor, 2014).

Wang *et al.* (2012) and Ravkov *et al.* (2013) reported the ability of allergenic protein to activate and proliferate T-helper (Th) cells in human peripheral blood mononuclear cells (PBMCs). PBMCs have been extensively studied in immunological research due to the presence of highly sophisticated immune cells lending their application in *in vitro* studies. PBMCs have often been co-cultured with various immune stimulants *in vitro*, to determine their efficacy considering various parameters of immune responses, such as cytokine production

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(Ramachandran *et al.*, 2012). The Th cells, including Th1 and Th2 subsets, play important role in interfering with the immune defence through the antibody or cell-mediated immune responses. In addition, the balance between Th1 and Th2 maintained by secretion of certain types of interleukin (IL) (such as IL-2, IL-4, IL-10 and IFN-γ) is believed to maintain the homeostasis of immune response (Donkor *et al.*, 2012; Wang *et al.*, 2012). In addition, Th17 cells release pro-inflammatory IL-17 cytokines and may differentiate into Th1 or Treg (T regulatory) cells to regulate the balance between Th1 and Th2 cells (Korn *et al.*, 2009; Gálvez, 2014).

To minimise human health risk due to allergic reactions, several researchers endeavoured to prepare hypoallergenic prawn products through structural modification of tropomyosin using various processing technologies (Kamath *et al.*, 2014; Lasekan & Nayak, 2016; Lv *et al.*, 2017; Yuan *et al.*, 2017; Faisal *et al.*, 2019c). In previous studies, Faisal *et al.* (2019a,c) reported a significant reduction of antigenicity (IgG binding) of tropomyosin in high-pressure-processed (600 MPa) and acetic acid-treated prawn samples using immunoblotting and ELISA kits. Considering that the *in vitro* immunoassays often use crude protein extracts to measure the changes of the specific IgE sensitivity based on patient serum, the affectability and explicitness of the test are not constantly acceptable in distinguishing the actual allergenicity (Morita *et al.*, 2013; Leung *et al.*, 2014; Abramovitch *et al.*, 2017). Moreover, the crude extract contains a great deal of different types of protein matrices, which often creates difficulties to point out the role of specific proteins for IgE reactivity (Faisal *et al.*, 2019c). To resolve this problem, the use of purified allergenic proteins becomes indispensable to diagnose the specific IgE sensitivity more accurately (Morita *et al.*, 2013; Leung *et al.*, 2014). Beside this, the study on cellular immune reactivity of isolated protein fractions is limited to reveal the role of Th cells completely. Therefore, the present study aimed to examine the immunogenicity (Th cell-mediated immune response *in vitro*) of isolate protein fractions in native and processed (acetic acid and high pressure treated in combination with high temperature) banana prawn samples up to certain time using human PBMC, as well as identify and characterise the protein fractions using liquid chromatography with tandem mass spectrometry (LC/MS/MS).

Materials and methods

Treatment and extraction of proteins from prawn

Fresh banana prawns (*Fenneropenaeus merguiensis*) were purchased from a local supermarket in Melbourne, Australia. Prawn samples were washed with distilled water, deshelled and deveined prior to all treatments.

High-pressure processing (HPP) of prawn muscle was performed using a Stansted ISO-LAB FPG11501 High Pressure 3.6 L unit (Stansted Fluid Power Ltd., Stansted, Essex, UK). The pressure vessel has a permitted initial temperature range from −20 to 110 °C for pressures up to 800 MPa. The maximum temperature within the vessel during pressure holding was 130 °C. A deionised water/propylene-glycol mixture (40% glycol) was used as the pressure-transmitting medium (Knoerzer *et al.*, 2010). The processing of prawn sample with high pressure (600 MPa) at 120 °C for 10 min was executed as described by Faisal *et al.* (2019a). In brief during high-pressure treatment, the vessel was conditioned to an initial temperature (90 °C), which then attained the target temperature after compression heating. Conditioning times for samples were short (<2 min) but varied slightly depending on the applied temperature. The compression and decompression rates were set to 600 and 1200 MPa min^{-1}, respectively. The temperature of the compression fluid and sample were monitored using a type T thermocouple attached to the sample carrier. HPP treatment was replicated on different days. For acetic acid-treated samples, prawn muscles were submerged in acetic acid (commercially available white vinegar) at pH 2.5 for 15 min. The prawn muscles without any processing were used as control.

The method described by Faisal *et al.* (2019c) was implemented for the extraction of proteins from the control, HPP and acetic acid-treated samples. In brief, fresh prawn muscles were homogenised using a laboratory blender (Waring 8011ES blender, NJ, USA) in phosphate-buffered saline solution (pH 7.4) at 1:3 ratio for 5 min. The protein slurry was agitated at 4 °C for 3 h, followed by centrifugation (Beckman Coulter Avanti J-26S XPI centrifuge, CA, USA) at 4 °C and speed of 29 400 ***g*** for 15 min. The supernatant of the protein mixture was separated utilising micropipette and stored in properly labelled sealed containers at −80 °C until further analysis.

Determination of protein content

The total protein content of each sample was determined by the Kjeldahl method and Bradford Assay kit (Bio-Rad Laboratories, Sydney, NSW, Australia), following the manufacturer's instructions. For Kjeldahl method, a Foss 2020 Digester Unit DS20 and Foss 2012 Distilling Unit (Hillerod, Sweden) were used for sample digestion and distillation, respectively. Bovine serum albumin (BSA) was used as the protein standard for the Bradford method (Kamath *et al.*, 2013).

Protein profiling by reversed-phase high-performance liquid chromatography (HPLC)

The proteins in control and treated (acetic acid and HPP) samples were analysed using a reversed-phase

HPLC (SHIMADZU, Prominence-i, LC-2030C, Tokyo, Japan) and a Jupiter analytical column (250 × 4.6 mm, particle size 5 μM, pore size 300 Å, connected to a security guard cartridge, wide-pore C18, 4 × 3.0 mm, Phenomenex, Torrance, CA, USA). The mobile phase consisted of the following: (i) HPLC-grade water with 0.1% TFA (trifluoroacetic acid) and (ii) 0.1% TFA in acetonitrile with the following gradient: 5% to 90% B from 0.1 min to 50 min, then 90% B for 5 min after which it reverted to 5% B for least 5 min (total run time: 60 min) at room temperature. A 10 μL sample was injected at each run, and the flow rate was maintained at 0.2 mL min^{-1}. Protein elution was monitored at 280 nm with UV detector.

Large scale protein isolation by preparative HPLC

The isolation of protein fractions in a sustainable content from different samples was conducted using a Varian HPLC system (Varian Analytical Instruments, Walnut Creek, CA, USA) equipped with a C18 Jupiter preparative column (250 × 21.2 mm, particle size 5 μM, pore size 300 Å, connected to a security guard prep cartridge, C18-300A, 250 × 21.2 mm, Phenomenex). Exactly, 1 mL sample was injected in each run and the mobile phase consisted of the following: (i) HPLC-grade water with 0.1% TFA and (ii) 0.1% TFA in acetonitrile with flow rate 4.28 mL min^{-1}. The following gradient was maintained for the separation of proteins: 5% to 90% B from 0.1 min to 50 min, then 90% B for 5 min after which it reverted to 5% B for least 5 min (total run time: 60 min) at room temperature. Proteins elution was monitored at 280 nm with a UV detector. To collect separated protein fractions, at least 25 runs/samples were performed. Total thirteen protein fractions from each sample were collected separately, and same fractions were pooled together in Falcon tubes. The eluted protein fractions in the control sample were marked as A1 to A13, whereas for acid and HPP samples marked as B1 to B13 and C1 to C13, respectively. Eluted protein fractions were frozen separately followed by freeze-drying (Dynavac FD 300 Freeze Drier, Melbourne, VIC, Australia) to concentrate the protein fractions. The freeze-dried fractions were resuspended in 2.5 mL RPMI-1640 medium to perform the following analysis.

Detection of allergenic protein fractions from separated samples using ELISA kit

ELISA is a widely accepted immunological based technique, used for fast detection and quantification of antigenicity (Faisal *et al.*, 2019b). Detection of allergenic protein fractions was performed using a Sandwich ELISA kit (RIDASCREEN®FAST Crustacean, R-Biopharm, Darmstadt, Hessen, Germany). Samples were analysed following manufacturer's instructions as described by Faisal *et al.* (2019c). In brief, extracted protein fractions (250 μg mL^{-1}) were each diluted 20-fold with extraction buffer, respectively, followed by centrifugation at 2500 *g* (Eppendorf centrifuge 5810 R, Hamburg, Germany) for 10 min. Exactly, 100 μL/well/sample was added into antibody pre-coated 96-microwell plate and incubated at room temperature for 10 min. The 96-microwell plate was washed three times with washed buffer followed by addition of 100 μL conjugate solution to each well and again incubated at room temperature for 10 min. After subsequent washing, 100 μL of chromogen was added/well and incubated in the dark at room temperature for 10 min. Finally, 100 μL of stop solution was added to each well and absorbance was measured within 10 min using ELISA plate reader (iMark microplate absorbance reader, Bio-Rad, Tokyo, Japan) at 450 nm.

Isolation of human peripheral blood mononuclear cells (PBMCs)

Peripheral blood mononuclear cells used in the current study have been obtained by meeting requirements of the National Health and Medical Research Council (National Health and Medical Research Council, 2007). Ethics application (ID: HRE16-058) has been approved by the Victoria University Human Research Ethics Committee. The Australian Red Cross Blood Services (Melbourne, Australia) supplied buffy coats from healthy donors. Isolation of PBMCs from buffy coat was performed using an established protocol (Donkor *et al.*, 2012; Bogahawaththa *et al.*, 2018) with slight modifications. In brief, 60 mL of buffy coat was diluted with equal amount of phosphate-buffered saline (pH 7.4) and layered gently on Ficoll-Paque Plus (GE Healthcare Pty Ltd., Silverwater, NSW, Australia). After centrifugation (Sorvall-RT7 centrifuge, DuPont, Newtown, USA) at 400 *g* for 25 min at 18 °C with no break, the separated layer of PBMCs was washed twice with 50 mL of RPMI-1640 immediately and centrifuged at 400 *g*/wash (18 °C for 10 min with break). The cell pellet was resuspended in 10 mL of RPMI-1640, and the cell concentration was calculated to be 3.5×10^7 cells mL^{-1}.

Stimulation of PBMCs with isolated prawn protein fractions

Stimulation of PBMCs with the isolated nine prawn protein fractions, namely A9 to A11, control; B9 to B11, acetic acid; C9 to C11, HPP, was executed as described by Bogahawaththa *et al.* (2018) with some modifications. Freshly prepared PBMCs (3.5×10^7 cells mL^{-1}) were resuspended in RPMI-1640

supplemented with 10% foetal bovine serum and 1% antibiotic–antimycotic solution (Sigma Aldrich Pty Ltd., Castle Hill, NSW, Australia). The cells, 1.66×10^6 mL^{-1}, were then co-cultured in each well with 10 μg mL^{-1} of selected prawn protein fractions in cell culture flasks and incubated at 37 °C in 5% CO_2 for 144 h. For a positive control, 1 μg mL^{-1} of lipopolysaccharide (LPS) from Escherichia coli O111: B4 (Sigma–Aldrich Pty Ltd.) was co-cultured with PBMCs, whereas unstimulated PBMCs in RPMI-1640 were used as negative control for quantifying basal cytokine production. Supernatants were collected at 0-, 4-, 8-, 12-, 24-, 48-, 72-, 96-, 120- and 144-h interval from the flasks and were stored at −80 °C for cytokine analysis.

Cytokine assays by IgE ELISA

Concentration of different cytokines, including interleukin (IL)-4, IL-10, IL-17A and interferon (IFN)-γ, produced by stimulated PBMCs at different time periods in the presence of selected protein stimulants were quantified using enzyme-linked immunosorbent assay (ELISA) (Thermo Fisher Scientific Australia Pty Ltd.) according to the manufacturer's guideline. In brief, ninety-six well-uncoated ELISA plates (Coaster 9018 ELISA plate) were coated with captured antibody and incubated at 4 °C overnight under continuous shaking. After three consecutive washes with 250 μL wash buffer per well, ELISA plate wells were blocked with 200 μL ELISTOP diluent and incubated for 1 h. Exactly, 100 μL sample/well was added and incubated at 4 °C overnight under continuous shaking to achieve maximum sensitivity. The microwells were washed three times with wash buffer followed by addition of 100 μL/well of diluted detection antibody. Streptavidin-HRP (100 μL) for detection of IL-4 and IFN-γ or avidin-HRP for IL-10 and IL-17A was added to each well and incubated at room temperature for 30 min. Following this step, 100 μL of TMB solution was added to each well and incubated for 15 min at room temperature. 100 μL of stop solution (2 N H_2SO_4) was then added to each well, and absorbance was measured within 10 min using ELISA plate reader (xMark microplate spectrophotometer, Bio-Rad, Tokyo, Japan) at 450 nm.

Characterisation of allergenic protein fractions

Fractions that showed significant allergenic reaction with PBMCs were analysed by a LC/MS/MS using the QExactive mass spectrometer (Thermo Scientific, Bremen, Germany) coupled online with a RSLC nano HPLC (Ultimate 3000, Thermo Scientific). Samples were concentrated on a 100 μm, 2 cm nanoviper pepmap100 trap column with 97.5% buffer A (0.1% TFA) at a flow rate of 15 μL min^{-1}. The peptides then eluted and separated with a ¿Thermo RSLC pepmap100, 75 μm × 50 cm, 100 Å pore size, reversed-phase nano-column with a 30 min gradient of 92.5% buffer A (0.1% formic acid) to 42.5% B (80% acetonitrile 0.1% formic acid), at a flow rate of 250 nL min^{-1}. The eluant is nebulised and ionised using the Thermo nano electrospray source with a distal coated fused silica emitter (New Objective, Woburn, MA, USA) with a capillary voltage of 1900V. Peptides were selected for MSMS analysis in Full MS/dd-MS^2 (TopN) mode with the following parameter settings: TopN 10, resolution 70000, MSMS AGC target 5e5, 118 ms Max IT, NCE 27, 1.8 m/z isolation window, and dynamic exclusion was set to 10 s.

Data from LC/MS/MS analysis were exported to Mascot generic file format (*.mgf) using proteowizard 3.0.3631 (open source software, http://proteowizard.sourceforge.net) and searched against the Uniprot Triticumaestivium databases using the MASCOT search engine (version 2.4, Matrix Science Inc., London, UK) with all taxonomy selected. The following search parameters were used: missed cleavages, 1; peptide mass tolerance, ±10 ppm Da; peptide fragment tolerance, ±0.02 Da; peptide charge, 2+, 3+ and 4+; fixed modifications, carbamidomethyl; and variable modification, oxidation (Met). Data from LC/MS/MS run were processed using Byonic (ProteinMetrics) V 3.1-19 with no specific cleavage sites specified and a precursor and fragment mass tolerance of 20 ppm. Modifications specified were Carbamidomethyl @C fixed and Oxidation @M Variable common 1. The protein output was set to 1% FDR. Moreover, data from LC/MS/MS run were processed using Peaks studio version 8 (Bioinformatics solutions using default settings for data refinement and a parent mass tolerance of 15 ppm and fragment tolerance at 0.5 Da with a max of five peptide candidates per spectrum).

Statistical analysis

Statistical analysis of results was performed using the general linear model procedure of the Statistical Analysis System (SAS v.9.2) with the treatment and replications as the main factors. The effect of selected prawn protein fraction on immunogenicity of PBMCs at various times was considered significant at $P \leq 0.05$.

Results and discussion

Profiling and isolation of prawn protein by HPLC

The HPLC protein profiles for supernatant mixture of control and treated (acetic acid and HPP) prawn samples are shown in Fig. 1(a, b and c), and retention times of eluted protein fractions are also reported in

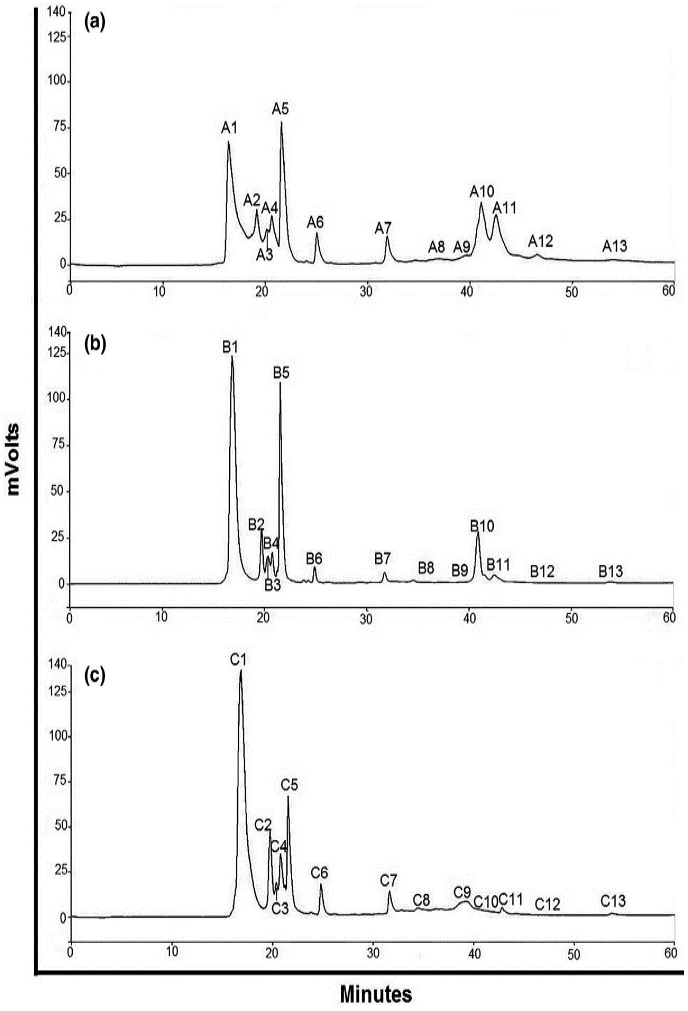

Figure 1 HPLC protein profile of untreated (a); acetic acid treated (b); and HPP at 120 °C (c) prawn protein extracts.

Table 1. In the control sample, two protein fractions (A10 and A11) showed significantly higher concentration than similar protein fractions (B10, B11, C10 and C11) eluted from the treated samples. However, the protein fractions C9 derived from HPP showed significantly higher concentration than A9 and B9. This was an indication that treatment had an effect on prawn proteins due to structural changes as a result of treatment of the proteins (Faisal *et al.*, 2019a,c). The combined effect of HPP and heat induced changes in primary and secondary structures through altering inter- and intramolecular bonds especially ionic, hydrogen and hydrophobic interactions (Chatterjee *et al.*, 2006; Wang *et al.*, 2013). On the other hand, Xu *et al.* (2012) reported that structural modification and aggregation of acid-treated proteins take place due to noncovalent, especially hydrophobic, attractions and formation of hydrogen bonds.

Antigenicity determination in isolated protein fractions by enzyme-linked immunosorbent assay (ELISA)

A sandwich ELISA test confirming antigenicity of protein fractions in control and treated prawn samples determined positive antigenicity in four out of thirty-nine eluted fractions, reported in Table 1. Out of the four antigens, two (A10 and A11) were from the control sample, whereas one each (B10 and C9) were from acetic acid- and HPP-treated samples, respectively. However, A10 showed the highest antigenicity (32 μg mL^{-1}) followed by C9 and B10. These results indicate that antigenicity was reduced by 81.25% and

Table 1 Antigenicity (IgG binding) analysis of eluted proteins assessed by ELISA kit

Control (Untreated) Protein fractions with retention time (min)	Antigenicity (IgG)	Acetic acid treated for 15 min Protein fractions with retention time (min)	Antigenicity (IgG)	HPP at 120 °C for 10 min Protein fractions with retention time (min)	Antigenicity (IgG)
A1 (16.00–18.00)	–	B1 (16.00–18.00)	–	C1 (16.00–18.40)	–
A2 (18.50–19.50)	–	B2 (19.40–20.00)	–	C2 (19.30–20.00)	–
A3 (19.55–20.30)	–	B3 (20.05–20.50)	–	C3 (20.10–20.40)	–
A4 (20.40–21.20)	–	B4 (20.55–21.20)	–	C4 (20.50–21.20)	–
A5 (21.30–22.50)	–	B5 (21.30–22.50)	–	C5 (21.30–22.50)	–
A6 (24.50–26.00)	–	B6 (24.55–25.30)	–	C6 (24.55–25.50)	–
A7 (31.40–32.50)	–	B7 (31.40–32.20)	–	C7 (31.40–32.30)	–
A8 (34.20–38.20)	–	B8 (34.00–38.20)	–	C8 (34.00–38.00)	–
A9 (38.50–40.00)	–	B9 (38.50–40.00)	–	C9 (38.50–40.00)	26 μg mL^{-1}
A10 (40.20–41.55)	32 μg mL^{-1}	B10 (40.20–41.50)	6 μg mL^{-1}	C10 (40.20–42.00)	–
A11 (42.10–44.00)	9 μg mL^{-1}	B11 (42.10–43.50)	–	C11 (42.40–43.50)	–
A12 (45.50–47.40)	–	B12 (45.50–47.40)	–	C12 (45.50–47.40)	–
A13 (53.20–55.10)	–	B13 (53.20–54.50)	–	C13 (53.20–54.50)	–

18.75% of C9 and B10, respectively, from a 100% of A10. Jin *et al.* (2015) stated that HPP in combination with temperature converted α-helix proteins into β-sheets and random coils, which likely masked or destroyed the antigen-binding epitopes. Furthermore, the partial solubility of proteins due to acid treatment likely reduced the active epitopes sites of antigens (Mohan *et al.*, 2007; Xu *et al.*, 2012) similarly observed in the current studies. Thus, processing likely masked or destroyed epitopes binding sites on antigens, resulting in less antigen–antibody binding, as previously reported (Faisal *et al.*, 2019a,c).

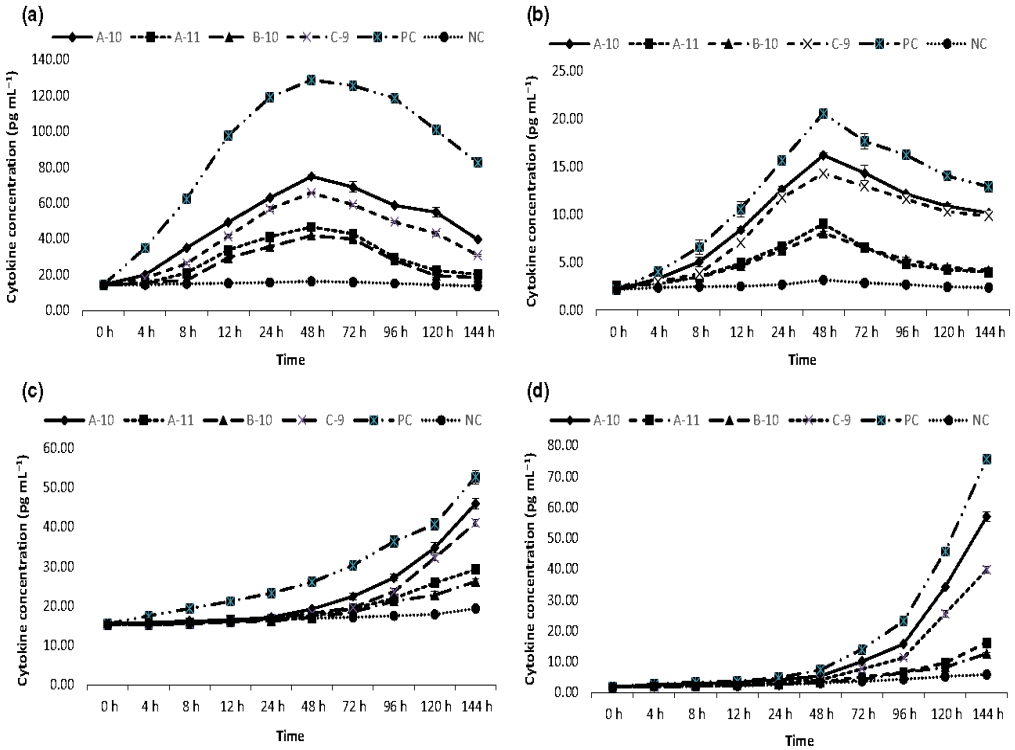

Figure 2 Changes in concentrations of cytokines in treated and untreated fractions with time – anti-inflammatory IL-10 (a) and IL-4 (b); pro-inflammatory cytokines – IFN-γ (c) and IL-17A (d). A10 and A11 from control; B10 from acetic acid treated; C9 from HPP at 120 °C treated; PC means positive control and NC means negative control.

In vitro Immunogenicity analysis of selected isolated protein fractions

Antigenicity analysis of isolated protein fractions showed that potential allergenic proteins were eluted between 38.50 and 44.00 min for both control and treated samples. Based on these findings, three protein fractions from each treatment were used to challenge human PBMCs and supernatants collected at different time intervals were analysed for cytokine production (Fig. 2). Again, four protein fractions (A10, A11, B10 and C9) showed significantly ($P < 0.05$) higher mean cytokine production in comparison with negative control. On the other hand, other protein fractions tested (A9 from control; B9 and B11 from acetic acid; C10 and C11 from HPP) showed no significant ($P > 0.05$) difference in cytokine concentration to negative control (data not shown). Two types of cytokines, anti-inflammatory (Fig. 2a and b) and pro-inflammatory (Fig. 2c and d), were produced in varying concentrations. The anti-inflammatory cytokine (IL-4 and IL-10) production increased in concentration with increasing time up to 48 h, whereas the pro-inflammatory cytokines (IFN-γ and IL-17A) did not significantly increase in concentration until after 48 h up to 144 h when IL-4 and IL-10 consistently declined (Fig. 2). Prawn protein is classified

Table 2 List of identified proteins in protein fractions by LC/MS/MS

Fractions	Protein code	Identified proteins by LC/MS/MS	Concentration of protein in fractions (%)	% of Coverage
A10 (Control)	C3VUU0	Arginine kinase OS=*Fenneropenaeus merguiensis* OX=71412 PE=2 SV=1	40.65	69.38
	D3XNS0	Tropomyosin OS=*Fenneropenaeus merguiensis* OX=71412 PE=2 SV=4	37.00	93.66
	G3GDS2	Enolase (Fragment) OS=*Fenneropenaeus merguiensis* OX=71412 GN=eno PE=4 SV=1	11.88	66.67
	Q5QD40	Translationally controlled tumour protein OS=*Fenneropenaeus merguiensis* OX=71412 PE=2 SV=1	5.00	58.33
	S5ZHH2	Haemocyanin OS=*Fenneropenaeus merguiensis* OX=71412 GN=HC PE=2 SV=1	3.33	19.52
	Q07DN6	Farnesoic acid O-methyltransferase short isoform (Fragment) OS=*Fenneropenaeus merguiensis* OX=71412 PE=2 SV=1	1.62	28.82
	D2SSM3	Vitellogenin OS=*Fenneropenaeus merguiensis* OX=71412 PE=2 SV=1	0.52	10.71
A11 (Control)	S5ZHH2	Haemocyanin OS=*Fenneropenaeus merguiensis* OX=71412 GN=HC PE=2 SV=1	35.00	88.05
	C3VUU0	Arginine kinase OS=*Fenneropenaeus merguiensis* OX=71412 PE=2 SV=1	34.58	77.25
	D3XNS0	Tropomyosin OS=*Fenneropenaeus merguiensis* OX=71412 PE=2 SV=4	20.31	81.69
	G3GDS2	Enolase (Fragment) OS=*Fenneropenaeus merguiensis* OX=71412 GN=eno PE=4 SV=1	8.06	70.08
	Q07DN6	Farnesoic acid O-methyltransferase short isoform (Fragment) OS=*Fenneropenaeus merguiensis* OX=71412 PE=2 SV=1	1.18	30.13
	Q5QD40	Translationally controlled tumour protein OS=*Fenneropenaeus merguiensis* OX=71412 PE=2 SV=1	0.70	32.74
	D2SSM3	Vitellogenin OS=*Fenneropenaeus merguiensis* OX=71412 PE=2 SV=1	0.16	19.56
B10 (Acetic acid treated)	D3XNS0	Tropomyosin OS=*Fenneropenaeus merguiensis* OX=71412 PE=2 SV=4	27.90	67.25
	S5ZHH2	Haemocyanin OS=*Fenneropenaeus merguiensis* OX=71412 GN=HC PE=2 SV=1	26.25	52.50
	C3VUU0	Arginine kinase OS=*Fenneropenaeus merguiensis* OX=71412 PE=2 SV=1	18.51	53.37
	G3GDS2	Enolase (Fragment) OS=*Fenneropenaeus merguiensis* OX=71412 GN=eno PE=4 SV=1	15.68	64.10
	Q5QD40	Translationally controlled tumour protein OS=*Fenneropenaeus merguiensis* OX=71412 PE=2 SV=1	7.80	76.19
	D9I8L2	14-3-3 zeta OS=*Fenneropenaeus merguiensis* OX=71412 PE=2 SV=1	2.11	8.94
	A0A0A7D6G0	Beta-1,3-glucan-binding protein OS=*Fenneropenaeus merguiensis* OX=71412 PE=2 SV=1	1.25	5.98
	D2SSM3	Vitellogenin OS=*Fenneropenaeus merguiensis* OX=71412 PE=2 SV=1	0.50	3.13
C9 (HPP)	D3XNS0	Tropomyosin OS=*Fenneropenaeus merguiensis* OX=71412 PE=2 SV=4	57.92	94.72
	C3VUU0	Arginine kinase OS=*Fenneropenaeus* merguiensis OX=71412 PE=2 SV=1	18.96	67.70
	S5ZHH2	Haemocyanin OS=*Fenneropenaeus merguiensis* OX=71412 GN=HC PE=2 SV=1	15.07	54.01
	G3GDS2	Enolase (Fragment) OS=*Fenneropenaeus merguiensis* OX=71412 GN=eno PE=4 SV=1	3.90	71.79
	Q5QD40	Translationally controlled tumour protein OS=*Fenneropenaeus merguiensis* OX=71412 PE=2 SV=1	2.16	30.95
	D2SSM3	Vitellogenin OS=*Fenneropenaeus merguiensis* OX=71412 PE=2 SV=1	1.15	29.96
	D9I8L2	14-3-3 zeta OS=*Fenneropenaeus merguiensis* OX=71412 PE=2 SV=1	0.84	39.43

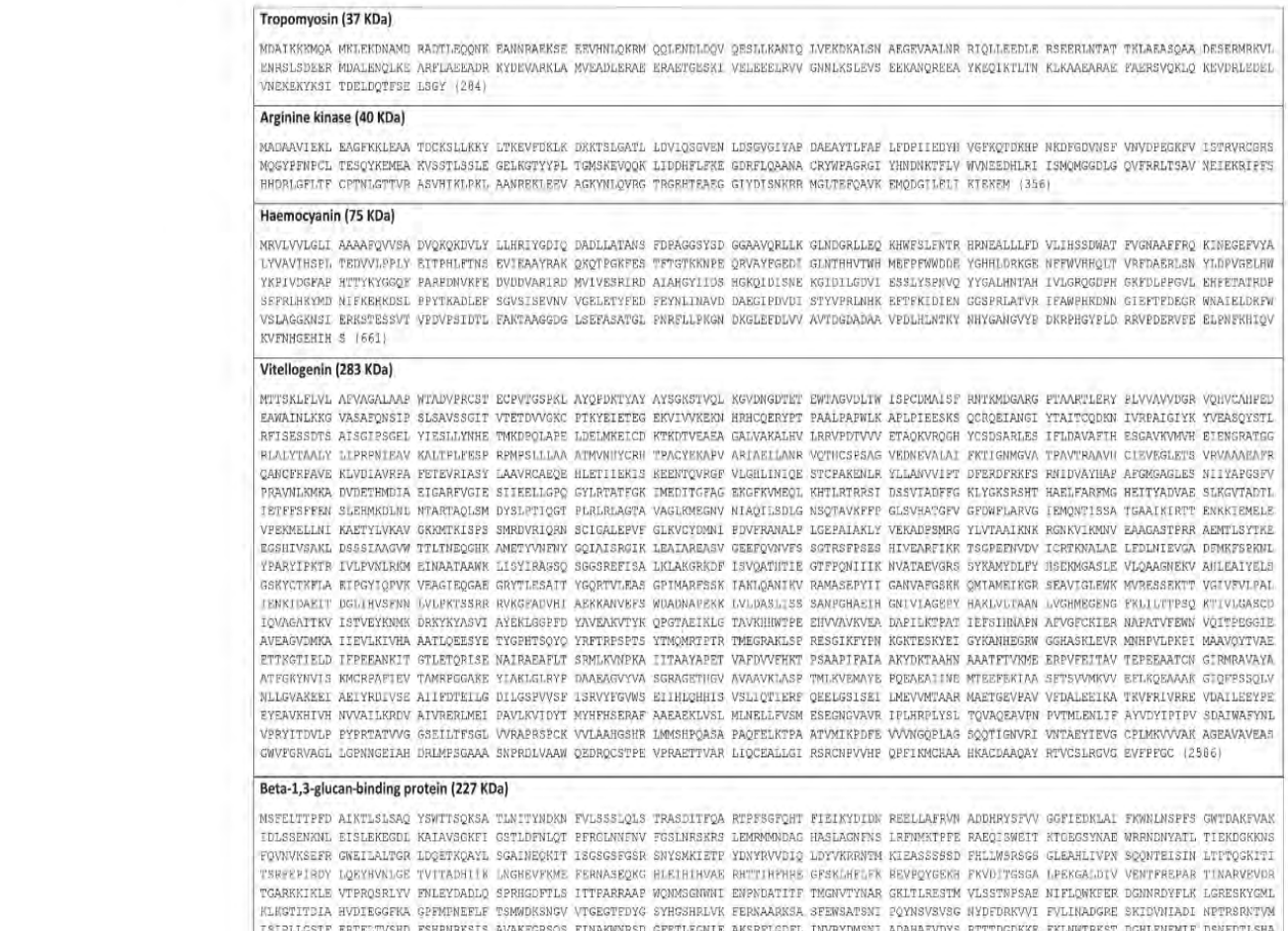

Figure 3 Characterisation of protein fractions by LC/MS/MS.

as a type 1 IgE-mediated allergy; hence, protein fractions A10, A11, B10 and C9 stimulated PBMCs to release Th2-type cytokines IL-4 and IL-10 (Untersmayr & Jensen-Jarolim, 2006; Barnes, 2011). However, the presence of antigens continued to stimulate the production of pro-inflammatory cytokines, which likely maintained homeostasis in the culture medium (Wang *et al.*, 2012; Gálvez, 2014). In general, fraction A10 showed significantly

higher cytokine production compared with fractions C9 and B10, indicative of effect of treatment on prawn proteins resulting in reduced immunogenicity. In another study, Abramovitch *et al.* (2017) also reported lower cytokine production for treated prawn muscle extract.

Characterisation of prawn protein fractions by LC/MS/MS

The allergenic protein fractions characterised by LC/MS/MS are listed in Table 2, and the amino acid sequences of nine identified proteins are represented in Fig. 3. In the control sample (A10, A11), the following proteins were identified: tropomyosin (37 KDa), arginine kinase (40 KDa), haemocyanin (75 KDa), enolase (12 KDa), translationally controlled tumour protein (19 KDa), vitellogenin (283 KDa) and farnesoic acid O-methyltransferase short isoform (26 KDa), as well as lesser proteins with lower molecular masses. On the other hand, with the exception of farnesoic acid O-methyltransferase short isoform (26 KDa) protein and some lesser molecular mass proteins in A10, B10 and C9 contained similar proteins in addition 14-3-3 zeta (27 KDa) identified. Khanaruksombat *et al.* (2014) identified vitellogenin and 14-3-3 zeta as minor allergens in Banana prawn ovaries and therefore are likely to be allergenic in muscle. In addition, translationally controlled tumour protein was identified in all fractions, and however, its impact on immunogenicity needs further investigation. Furthermore, in acetic acid-treated fraction B10, beta-1,3-glucan-binding (227 KDa) protein was identified (not in control and C9). Structural changes due to acid treatment (Mohan *et al.*, 2007; Faisal *et al.*, 2019a,c) and the presence of identified protein likely resulted in the lowest antigenicity and immunogenicity observed. As a consequence of treatment, LC/MS/MS identification showed the per cent of coverage (% of protein match with Database) significantly changed, compared with control. Thus, the disappearance of proteins from A10 and appearance of new proteins in B10 and C9 fractions are indications of processing effect on changes in prawn proteins; however, their impact on immunogenicity need further investigation. Tropomyosin is a major allergenic protein of Banana prawn (Faisal *et al.*, 2019a,c), whereas arginine kinase has been reported as a minor allergen in different species of prawns (Kamath *et al.*, 2013, 2014; Rahman *et al.*, 2013), and haemocyanin and enolase fragment have also been reported as minor allergens in Banana prawn muscle (Khanaruksombat *et al.*, 2014).

Conclusion

Processing of prawn muscle with HPP and acetic acid separately showed significant lower pro- and anti-inflammatory cytokine production resulting in decreased immunogenicity compared to control. Characterization of treated and untreated fractions showed four common proteins (tropomyosin, arginine kinase, hemocyanin and enolase) having allergenic properties in prawn muscle. Whereas, other two proteins (vitellogenin and 14-3-3 zeta) identified are likely to have minor allergenic properties in prawn overies. The remaining three proteins (beta-1,3-glucan-binding protein, translationally controlled tumour protein and farnesoic acid O-methyltransferase short isoform protein) isolated from treated and untreated fractions need further studies into their immunogenic properties.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

Author contributions

Md Faisal: concept development; experimental design and execution; data collection, analysis and interpretation; and manuscript writing. Narges Dargahi: experimental design and technical support. Dr Osaana N. Donkor: concept development; experimental design; technical support; and reviewing manuscript. Professor Todor Vasiljevic: concept development; technical support; and revising and editing manuscript.

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Can dietary ginger (*Zingiber officinale*) alter biochemical and immunological parameters and gene expression related to growth, immunity and antioxidant system in zebrafish (*Danio rerio*)?

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ABSTRACT

Effects of the dietary ginger (*Zingiber officinale*) on growth, some immunological and biochemical parameters and gene expression related to growth, immunity and antioxidant systems in zebrafish were studied. Immune parameters such as immunoglobulin level, alternative complement activity, and lysozyme activity were significantly higher in treatment groups fed with 2% and 3% ginger than the control fish. Amylase activity also increased significantly in the same pattern as the immunological parameters. Catalase and lysozyme mRNA expression levels representatives of antioxidant and immune genes respectively, were up-regulated in the fish fed with 3% ginger. Conversely, expression of gene encoding ghrelin was down-regulated in all treatment groups compared with the control group. Present study suggests that powdered ginger especially at 3% might augment some immunological and biochemical responses as well as expression of genes relevant to antioxidant and immune systems in zebrafish.

1. Introduction

Fast development of aquaculture and intensification of fish culture render the fish species highly sensitive to different diseases. Bacterial diseases have been of great concern in aquaculture, mostly because bacterial infections are the main cause of severe loss of production leading to high negative economic impact (Bondad-Reantaso et al., 2005; Plumb and Hanson, 2011; Lafferty et al., 2015). Instead of chemotherapeutics, the use of natural prophylactic supplements is considered a promising preventive practice which assists in maintaining fish welfare, and a healthy environment. Such preventive products include food additives which are either synthetic substances or are natural with herbal or animal origins (Pohlenz and Gatlin III, 2014; Guardiola et al., 2016; Bruce and Brown, 2017). There has been shown that a wide range of immunostimulants such as animal and plant extracts, vitamins, chitin, glucans, microorganisms and other available by-products have positive effects on aquaculture (Martinez-Alvarez et al., 2005; Chakraborty and Hancz, 2011; Oliva-Teles, 2012; Pohlenz and Gatlin III, 2014; Reverter et al., 2014). Plant products have been extensively used in aquaculture in order to stimulate growth rate, immune system and to render antioxidant effects, due to their active molecules such as alkaloids, terpenoids, saponins and flavonoid components (Reverter et al., 2017). However, little is known about the long-term effects of these plant extracts on fish physiology and different health related factors (Reverter et al., 2014).

Ginger (*Zingiber officinale* Roscoe) rhizome (ginger root) is widely used as a spice or condiment and potent medicinal plant according the "folk medicines" of cultures worldwide. Ginger contains several compounds such as gingerol, gingerdiol, and gingerdione that possess anti-inflammatory, anti-diabetic, anti-cancer, chemopreventive, and chemotherapeutic effects (Kikuzaki and Nakatani, 1996). Some studies have demonstrated the effect of ginger or its compounds on growth performance, antioxidant status, immune system and serum metabolites in fish species (Talpur et al., 2013; Shakya, 2015; Sukumaran et al.,

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2016). But the information regarding the effects of ginger on fish health at molecular level is scarce to understand about mechanism of action of this herb. In just one study on *Labeo rohita* fingerlings, it was shown that the dietary ginger had beneficial effects on the gene expressions related to some immune and antioxidant parameters (Sukumaran et al., 2016).

Given the various beneficial effects attributed to ginger, and the great developments achieved through studies on fish species, especially zebrafish (*Danio rerio*), research on this fish species can help to enhance biological sciences. Therefore, in this study we assessed the effects of dietary ginger on growth performance, digestive enzyme activities, several immunological and biochemical factors and gene expression related to growth, immunity and antioxidant systems in zebrafish, as s research model.

2. Materials and methods

2.1. Experimental conditions

Healthy 35-day old zebrafish (Tuebingen strain) were used in this study. Fish were reared in a private aquarium fish farm in Marand, Iran. Initially, a total number of 240 fish (mean bodyweight 54.1 ± 0.3 mg) were randomly distributed among 12 tanks (40 l), where four experimental groups (with 20 fish in each tank) were designed. The culture system was static with constant aeration using air stone and exchanged (70%) with fresh water daily. During the experimental period, basic chemical and physical parameters of the water including temperature, pH and dissolved oxygen were maintained at 25.2 ± 0.8 °C, 7.6 ± 0.3 and 7.22 ± 0.19 mg l^{-1}, respectively. During a 14-day acclimation period fish were fed basal diet, following this period fish were fed each of the experimental diets as triplicate groups for eight weeks.

2.2. Experimental diets

Ginger (*Zingiber officinale* Roscoe) rhizomes were purchased locally. This medical herb was authenticated at the botany department of Tehran University, Iran based on numerous starch grains with characteristic beak and oil secretion cells with suberized walls and yellowish content. Ginger was washed, peeled, sliced, and shade-dried for 24 h at room temperature. The dried ginger was then oven-dried at 50 °C, powdered mechanically using a grinder, and sieved using a sifter. Four incremental levels (0, 1, 2 and 3%) of ginger powder were added to the ground powder of a commercial fish diet (BioMar SAS, Nersac, France) to prepare four experimental diets. All ingredients were well-mixed and pelletized. The composition of the commercial diet was 38.9% crude protein, 15.1% crude fat, 93.6% dry matter, and 11% ash. The modified feed was stored in sealed bags at 4 °C until used in the feeding experiments. Each tanks hosted 20 fish, and fish accommodated in first, second, third and fourth groups of triplicate tanks were fed diets containing 0%, 1, 2 and 3% ginger. In this study, fish were initially fed at a feeding rate of 30 g kg^{-1} of body weight daily. Till the end of the feeding trial, fish were weighed once every 2 weeks to adjust the feeding during the study.

2.3. Growth performance

At the beginning and the end of the feeding trial, all fish in each tank were taken and after anaesthetizing in clove oil bath (50 μL l^{-1}) were weighed individually. Survival rate, thermal growth coefficient (TGC) and specific growth rate (SGR) were determined for each tank using the following formula:

$$SGR\ (\%) = 100 \times (\ln W2 - \ln W1)/T2 - T1$$

$$TGC = 100 \times [W2^{1/3} - W1^{1/3}/(T2 - T1) \times {}^{\circ}C]$$

where W1 and W2 are the initial and final weight (mg), respectively; T2 – T1 is the number of days in the feeding period and °C is the mean daily temperature (Jobling, 2003).

2.4. Preparation of fish homogenates

After biometrical measurement, 13 anaesthetized fish samples were taken and the heads and fins were removed. Fish samples were frozen in liquid nitrogen, homogenized individually with a hand homogenizer. Three fish homogenates were used for Real-time PCR. The remaining ten fish homogenates were suspended in 25 mM Tris-HCl buffer (pH 7.2) (Sheikhzadeh et al., 2017) for digestive enzyme and immunological analysis.

2.5. Digestive enzyme activities

The amylase activity was determined following the method of Bernfeld (1995). Soluble starch (1%) (250 μl) was incubated with fish homogenate (50 μl) at 37 °C for 30 min. The reaction was terminated by adding 0.5 ml of 1% dinitrosalicylic acid and boiling for 5 min. After cooling, 5 ml of distilled water was added to the mixture and thereafter absorbance was read at 540 nm. Maltose was used to draw the standard curve.

The lipase activity was determined following the method of Bulow and Mosbach (1987) with slight modifications. The reaction mixture consisted of 1 ml reaction buffer (50 mM Tris-HCl, pH 8.0 containing 4% ethanol), 20 μl of fish homogenate and 60 μl of *p*-nitrophenyl butyrate (p-NPB) (50 mM) in ethanol. Using spectrophotometer the rate of p-NPB hydrolysis was measured against the blank at 405 nm for a period of 5 min with 30 s interval.

2.6. Immunological parameters

Immunoglobulin level was determined following the method of Siwicki et al. (1994). The difference in total protein content prior to and after precipitation of the immunoglobulin component with 12% polyethylene glycol (PEG; Sigma) was determined by the Bradford method (Bradford, 1976).

Activity related to the alternative pathway of complement system was determined following the method of Andani et al. (2012). Rabbit red blood cells (RaRBC) were washed in ethylene glycol tetra acetic acid–magnesium–gelatin veronal buffer (0.01 M EGTA–Mg–GVB, pH 7) three times and were resuspended in the same buffer and adjusted to a concentration of 2×10^8 cells ml^{-1}. A 100-μl of RaRBC suspension was added to 250 μl of serially diluted homogenate prepared in veronal buffer. Samples were incubated at 20 °C for 90 min and regularly shaken. Then 0.85% NaCl solution (3.15 μl) was added to the tubes and centrifuged at 1600*g* for 10 min at 4 °C. Using a spectrophotometer the optical density of the haemolysis supernatant was measured at 414 nm (Awarness, Palm, FA). Alternative complement activity 50 (ACH50) unit was defined as the concentration of body homogenate causing 50% haemolysis of RaRBC.

Alkaline phosphatase activity was determined following the method of Sheikhzadeh et al., 2017. Briefly, fish homogenate samples (20 μl) were added to 1 ml of alkaline buffer solution (Sigma-Aldrich) individually, and incubated at 37 °C for 5 min. One ml of 0.05 N NaOH was added to the mixture to stop the reaction and absorbance was read at 410 nm using a spectrophotometer.

Lysozyme activity was measured following the method of Sheikhzadeh et al. (2012a, 2012b). Briefly, 75 μl of *Micrococcus lysodeikticus* bacterial suspension (75 μg ml^{-1}) was prepared in 0.1 M phosphate citrate buffer (pH 5.8) and aliquoted in a 96-well plate (in triplicate). Then, 25 μl of each individual homogenate was added and mixed. After vigorous mixing, turbidity changes were measured every 30 s for 5 min at 450 nm at approximately 20 °C using a microplate reader (Hiperion, Germany). The unit of lysozyme activity was defined by the amount of enzyme causing a decrease in absorbance of 0.001 per minute and reported as U g^{-1} of each sample.

Table 1
Primers selected for the expression of selected genes in zebrafish.

Target gene	Primer sequence	Primer length (bp)
Insulin-like growth factor 1	F: 5′ TTCTCTCCAAATCCGTCTCC 3′	20
	R: 5′ AGAAAGAGGATGAGATGCGG 3′	20
Ghrelin	F: 5′ TTTGTTACTAAGGAATTGCCA 3′	21
	R: 5′ TGTGTGACTTAAAGAGGGTGA 3′	21
Growth hormone 1	F: 5′ TCTATCTTGGACAAAATGGC 3′	20
	R: 5′ ATGACTGCGTTGTTGAAGAG 3′	20
Glutathione peroxidase 8	F: 5′ GACACGCCCACAAAAACTT 3′	19
	R: 5′ CTTTGAACAGTCCTGCTCGT 3′	20
Catalase	F: 5′ ACAAGTTAAATGCGATGACG 3′	20
	R: 5′ CGTCTTTCCAATATGCTCAA 3′	20
Superoxide dismutase 1	F: 5′ GTCGGTTTCTTTCACTCTCTC 3′	21
	R: 5′ ACTGGCTTCTTTTCACCCT 3′	19
Glutathione S-reductase	F: 5′ TAAACAAAACCGAAGATGGG 3′	20
	R: 5′ TTAAACCTGTAGCCGAAAGC 3′	20
Lysozyme	F: 5′ AATATGAGGCTGGCAGTGG 3′	19
	R: 5′ AGTCCTTCCCCGTATCAGC 3′	19
Interleukin 1, beta	F: 5′ CGGGCAATATGAAGTCACC 3′	19
	R: 5′ GTCCACATCTCCAGCCTGA 3′	19
Interleukin 8a	F: 5′ ACTTAGGCAAAATGACCAGC 3′	20
	R: 5′ TATGTGTTTTCCAATGCGTC 3′	20
Tumor necrosis factor alfa	F: 5′ TAGAACAACCCAGCAAACTC 3′	20
	R: 5′ TCTCCTTCTTCAACATCCAA 3′	20
Glyceraldehyde-3-phosphate dehydrogenase	F: 5′ TCACAAACGAGGACACAACC 3′	20
	R: 5′ GCCACGATCTCCACTTTCTT 3′	20

2.7. Real-time PCR and gene expression analysis

Total RNA was extracted from the whole body of three zebrafish in each tank using GeneAll Kit (GeneAll Biotechnology, Seoul, Korea). Total concentration and purity of the RNA samples were determined using Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific,Wilmington, USA) at 260/280 nm. The RNA samples quality and integrity were verified using 0.5 μg mL^{-1} ethidium bromide staining of 28S and 18S ribosomal RNA bands running on 1% agarose gel. Complementary DNA (cDNA) was synthesized using 2 μg of total RNA and random hexamer primer (HyperScript™ RT premix, GeneAll Biotechnology, Seoul, Korea). The primers of selected genes (Table 1) were designed using oligo 5 primer analysis software. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was also used as a house keeping gene to normalize the expression of the target genes. The expression of selected genes were assessed by real-time PCR using SYBR Green PCR master mixes (Real Q plus 2× master Mix Green, AMPLIQON, Denmark). PCR was performed using 10 μl SYBR green mix, 2 μl cDNA, 0.5 μl PCR forward primer, 0.5 μl PCR reverse primer in a final volume of 20 μl. RNA samples were concurrently run in parallel with the housekeeping gene for normalization of cDNA loading. The real-time PCR program is presented in supplementary Table. The obtained data were analyzed using the Rotorgene®6000 software. The fold change of mRNA expressions in selected genes was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). In all cases, each PCR reaction was repeated three times to minimize the experimental error.

2.8. Statistical analysis

The results were expressed as mean ± standard error of mean (SEM). After confirming the normality and homogeneity of variance, the statistical significance of data was evaluated by one-way analysis of variance (ANOVA), using the statistical package for social sciences (SPSS) software version 19.0. The data was analyzed by Tukey HSD analysis with the least square difference to compare the means. Differences were considered significant if $P < .05$.

3. Results

3.1. Growth performance

In this study, no fish death was observed in any of the groups. Results for eight weeks ginger dietary supplementation showed no significant effect on growth performance in any of treatment groups as compared to the control group (Table 2).

3.2. Digestive enzyme activities

Results from enzyme activities showed that lipase activity was not affected by ginger feeding in any of the groups, however, amylase activity showed significant increase in groups fed with 2% and 3% ginger compared with the control group. (Table 3).

3.3. Immunological parameters

Fish fed with 2% and 3% ginger showed higher total protein level (55.96% increase for 2% and 89.90% increase for 3% ginger). Feeding ginger at 3% could increase the immunoglobulin level (33.23% increase) but alternative complement activity was higher in fish fed with 2% ginger (4.58% increase). Alkaline phosphatase activity increased in all treatment groups (15.19%, 22.09% and 26.11% for 1%, 2% and 3% ginger respectively), whereas lysozyme activity was higher in fish that received 2% and 3% ginger compared to the control group (13.33% increase for 2% and 18.82% increase for 3% ginger feeding) (Table 4).

3.4. Real-time PCR and gene expression analysis

The gene expression levels of immune-related parameters, growth and antioxidant system are illustrated in Figs. 1–3. The results showed that among different enzymes related to immune system, the expression of lysozyme gene was up-regulated in fish fed 3% ginger in comparison to control group. A similar pattern was noticed in results for antioxidant enzymes with an average fold change of 0.2 overexpression in catalase gene in fish receiving 3% ginger. In addition, a down-regulation pattern in the expression of ghrelin gene in fish fed with different ginger supplementation compared to the control was shown (0.07, 0.24 and 0.24 fold changes for 1, 2 and 3% ginger respectively).

4. Discussion

Supplementation with ginger powder did not enhance the growth rate of zebrafish at concentrations between 1, 2 and 3% for eight weeks.

Table 2
Growth performance in zebrafish (*Danio rerio*) after feeding different doses of ginger (*Zingiber officinale*) for eight weeks.

Ginger in diet (%)	Final weight (mg)	Weight gain (mg)	Thermal growth coefficient (%)	Specific growth rate (%)	Survival rate (%)
0	166.87 ± 5.71	108.92 ± 4.24	0.737 ± 0.029	1.822 ± 0.208	100
1	163.20 ± 7.14	109.43 ± 8.29	0.759 ± 0.052	1.952 ± 0.131	100
2	170.12 ± 4.92	113.21 ± 7.88	0.763 ± 0.057	1.956 ± 0.157	100
3	169.32 ± 5.87	111.6 ± 4.21	0.751 ± 0.032	1.924 ± 0.103	100

Data are mean ± SEM. Those within a column superscripted by different letters are significantly different ($P < .05$).

Table 3
Some digestive enzyme activities in zebrafish (*Danio rerio)* after feeding different doses of ginger (*Zingiber officinale)* for eight weeks.

Ginger in diet (%)	Lipase (U mg^{-1} protein)	Amylase (U mg^{-1} protein)
0	1.33 ± 0.30	4.37 ± 0.54^{a}
1	1.41 ± 0.31	4.42 ± 0.48^{a}
2	1.49 ± 0.24	5.22 ± 0.73^{b}
3	1.51 ± 0.22	5.67 ± 0.51^{b}

Data are mean ± SEM. Those within a column superscripted by different letters are significantly different ($P < .05$).

In accordance with our findings, sturgeons (*Huso huso*) supplemented with 1% powdered ginger for 60 days did not show any changes in the growth rate and feeding efficiency (Kanani et al., 2014). In addition, supplementation with 1.0% and 1.5% ginger essential oil in another study did not enhance the growth parameters in Nile tilapia (*Oreochromis niloticus*) (Brum et al., 2017). However, the present results differ from the previous studies reporting growth promoting effects of ginger in several fish species (Shakya, 2015; Punitha et al., 2008; Nya and Austin, 2009). It looks as if variation in ginger type, variety used, agronomic, curing methods, drying and storage conditions can result in variations in ginger composition which cause various beneficial effects on the overall health of fish. It is very likely that the health benefits are also partially dependent on the fish species (Shakya, 2015). Even though growth promoting effect of powdered ginger was not noted in the present study, an increase in amylase activity was shown in fish that received 2% and 3% ginger. The digestive enzymes play a key role in the overall digestion process; therefore they have an impact on the general wellbeing of fish. Similar to our study, digestive enzyme activity significantly increased with ginger feeding in results from studies done on humans, animal and aquatic animals trials (Platel and Srinivasan, 2000; Platel and Srinivasan, 2004; Venkataramalingam et al., 2007).

Certain nutrient elements can enhance the innate cellular and humoral immunity that play important roles in maintaining optimal health and protecting the body against a wide range of diseases. Ginger is known as a natural antibiotic and effective natural immunostimulants. Previous studies have shown that ginger powder and ginger essential oil were able to enhance innate immune responses in a number of fish species (Talpur et al., 2013; Shakya, 2015; Sukumaran et al., 2016). The total immunoglobulin and total protein level (an indirect antibody level measurement) are among the immune parameters evaluated in immunostimulant supplemented diets (Vallejos-Vidal et al., 2016). In this study, similar increase of total immunoglobulin and total protein levels in fish fed ginger was noted. Alkaline phosphatase is an important antibacterial enzyme which plays protective roles during stress, parasitic infection and wound healing process in fish species (Sheikhzadeh et al., 2012a,b). In the current study, alkaline phosphatase activity was also influenced by ginger feeding. The alternative pathway of complement system plays an essential role in innate immune system to protect fish against potential pathogens including bacteria, viruses, fungi, and parasites (Andani et al., 2012). In the present study, higher complement activity in the fish supplemented with 2% ginger was shown. Lysozyme, another important index of innate immunity, shows antibacterial activity against both Gram-positive and Gram-negative bacteria. Meanwhile, activation of the complement system and phagocytes by lysozyme has been observed (Choi et al., 2008; Saurabh and Sahoo, 2008). Current study showed that dietary supplement of ginger at 2% and 3% could increase the lysozyme activity. In the fish species, two major distinct lysozyme types have been identified; the c-type (chicken or conventional type) and the g-type (goose-type) lysozymes (Saurabh and Sahoo, 2008). In the current study, the mRNA abundance of c-type lysozyme in zebrafish fed 3% ginger was altered, whereas 2% ginger supplementation could not change the c-type lysozyme gene expression compared to the control. The inconsistency in findings related to lysozyme activity and lysozyme gene expression analysis in 2% ginger group might be due to the differences in the expression of the gene related to g-type lysozyme which warrants the needs for more detailed studies. In line with this study, lysozyme activity in the plasma of Asian sea bass (*Lates calcarifer*) increased significantly when ginger was added to fish diet (Guardiola et al., 2016). Moreover, dietary supplementation of ginger has been reported to augment the skin mucosal lysozyme level of *Labeo rohita* fingerlings (Sukumaran et al., 2016). In this study, the gene expression related to other immune parameters, namely interleukin (IL-)1 beta, IL-8a and tumor necrosis factor (TNF) alpha did not change compared to the control group.

Ghrelin, a multifunctional hormone, is involved in the regulation of a number of physiological functions, including regulation of pituitary hormone release, feed intake, energy balance, growth-hormone secretion, and intestinal motility (Unniappan and Peter, 2005). According to our results, feeding ginger at 1–3% caused a reduction in ghrelin mRNA levels compared to the control group. Similarly, down-regulation of ghrelin gene expression in gold fish (*Carassius auratus* gibelio) fed on probiotic *Lactobacillus acidophilus* was shown in a previous study (Hosseini et al., 2016). In fact, the mechanism behind the ghrelin gene down-regulation upon administration of some immunostimulants is not clear, once more indicating the need for more studies to illustrate the mechanism of action. From this study, it can be assumed that these concentrations of ginger were not the optimum doses to enhance the growth performance and the expression of the genes related to appetite in zebrafish.

In this study, the possible antioxidant effect of dietary ginger demonstrated that ginger at 3% level could cause a higher expression level for catalase gene in zebrafish. The protective action of antioxidant enzymes may be due to an inhibition of reactive oxygen species (ROS) which can induce a chain reaction mediated by various antioxidant enzymes (such as superoxide dismutase, glutathione peroxidase and catalase). Similarly, ginger administration significantly up-regulated the expression of superoxide dismutase 1 and glutathione peroxidase enzymes as well as increased mRNA expression of nuclear factor erythroid 2-related factor 2 (Nrf2) in *Labeo rohita* fingerlings (Sukumaran et al., 2016). In zebrafish, Nrf2 is a key regulator that primarily regulates the expression of antioxidant enzyme (Sukumaran et al., 2016). In fact, the antioxidant activity implicated by ginger is attributed to the presence of tocopherols, such as atocopherol and d-tocopherol (Sukumaran et al., 2016). In addition, phenols and flavonoids present in ginger are known to have antioxidant properties. Hence, the observed catalase gene expression may be mediated by these compounds present

Table 4
Immunological parameters in zebrafish (*Danio rerio)* after feeding different doses of ginger (*Zingiber officinale)* for eight weeks.

Ginger in diet (%)	Total protein (g dl^{-1})	Immunoglobulin level (U g^{-1} protein)	Complement titer (U g^{-1} protein)	Alkaline phosphatase activity (U g^{-1} protein)	Lysozyme activity (U g^{-1} protein)
0	1.09 ± 0.12^{a}	0.458 ± 0.005^{a}	6.68 ± 0.15^{a}	30.14 ± 0.40^{a}	0.255 ± 0.004^{a}
1	1.23 ± 0.10^{a}	0.468 ± 0.006^{ab}	7.48 ± 0.45^{a}	34.72 ± 0.78^{b}	0.270 ± 0.008^{a}
2	1.70 ± 0.23^{b}	0.463 ± 0.005^{ab}	8.90 ± 0.26^{b}	36.80 ± 0.13^{c}	0.289 ± 0.007^{b}
3	2.07 ± 0.41^{c}	0.479 ± 0.009^{b}	6.90 ± 0.18^{a}	38.01 ± 0.50^{c}	0.303 ± 0.003^{b}

Data are mean ± SEM. Those within a column superscripted by different letters are significantly different (P < .05).

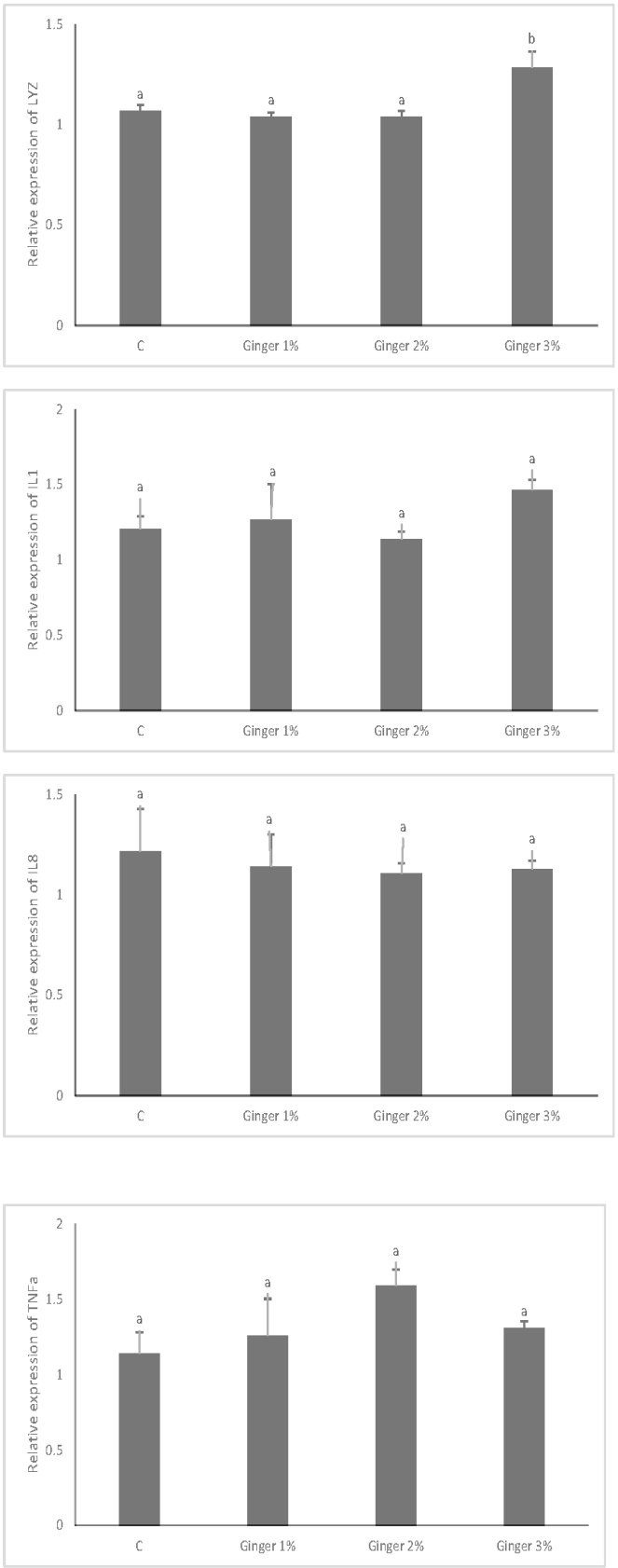

Fig. 1. Effects of the dietary ginger (*Zingiber officinale)* on immune related genes including lysozyme (LYZ), interleukin 1, beta (IL1), interleukin 8a (IL8) and tumor necrosis factor alfa (TNFa) in zebrafish at the end of the feeding trial. Different letters above bars indicate the significant difference among treatments ($P < .05$).

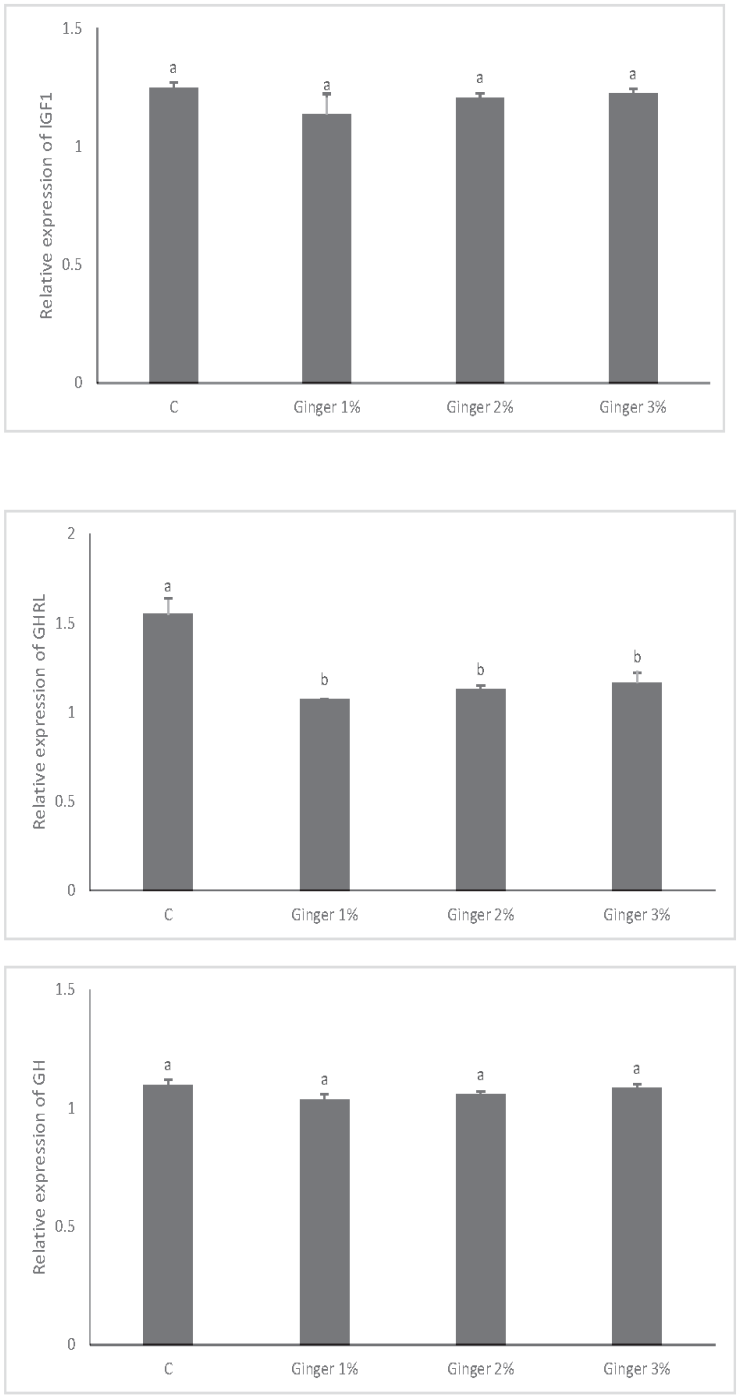

Fig. 2. Effects of the dietary ginger (*Zingiber officinale)* on growth related genes including insulin-like growth factor 1 (GF1), ghrelin (GHRL) and growth hormone 1 (GH) in zebrafish at the end of the feeding trial. Different letters above bars indicate the significant difference among treatments ($P < .05$).

in dried ginger (Sukumaran et al., 2016). Further studies are needed to identify the molecules present in ginger that are involved in the antioxidant activity in fish, as well as to ascertain their absorption, metabolism and biological activity in relation to their effects on fish antioxidant system.

To the best of our knowledge, in aquaculture no study has been conducted to examine the effects of ginger on fish health, growth and antioxidant system especially at molecular level at doses higher than 1%. The present study showed that ginger especially at 3% level increased immunological and biochemical responses, also up-regulated the immune and antioxidant related gene expression in zebrafish. More studies are needed on various aspects of ginger administration, especially to determine the mode of action and optimum ingestion level in different fish species, especially at molecular level.

5. Conclusions

In this study, powdered ginger could alter different digestive,

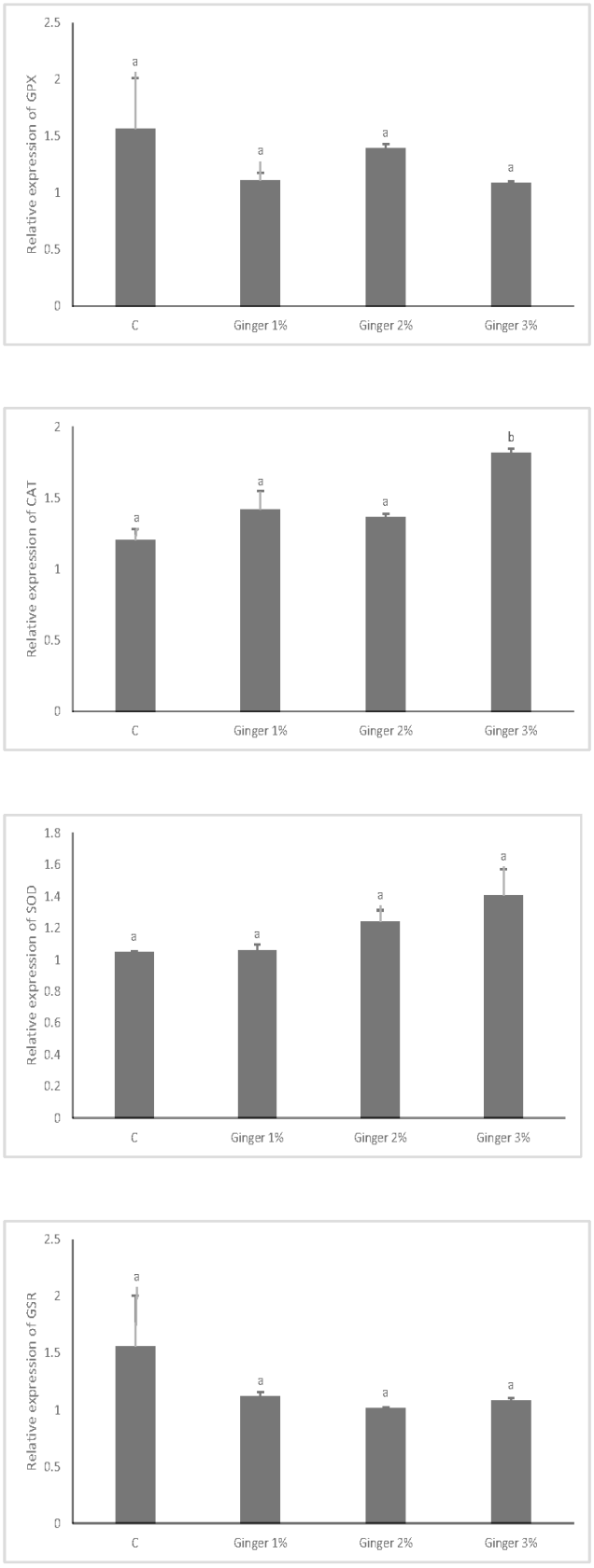

Fig. 3. Effects of the dietary ginger (*Zingiber officinale)* on antioxidant related genes including glutathione peroxidase 8 (GPX), catalase (CAT), superoxide dismutase (SOD) and glutathione S-reductase (GSR) in zebrafish at the end of the feeding trial. Different letters above bars indicate the significant difference among treatments

immunological and biological factors. These demonstrated effects were more pronounced for 3% ginger concentration to change some immunological and biochemical parameters, as well as gene expression related antioxidant and immune systems in zebrafish. Further studies are needed to prove the beneficial effects of ginger especially at these doses on immunological and antioxidant systems in zebrafish.

Conflict of interest

There are no conflicts to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2019.04.049.

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