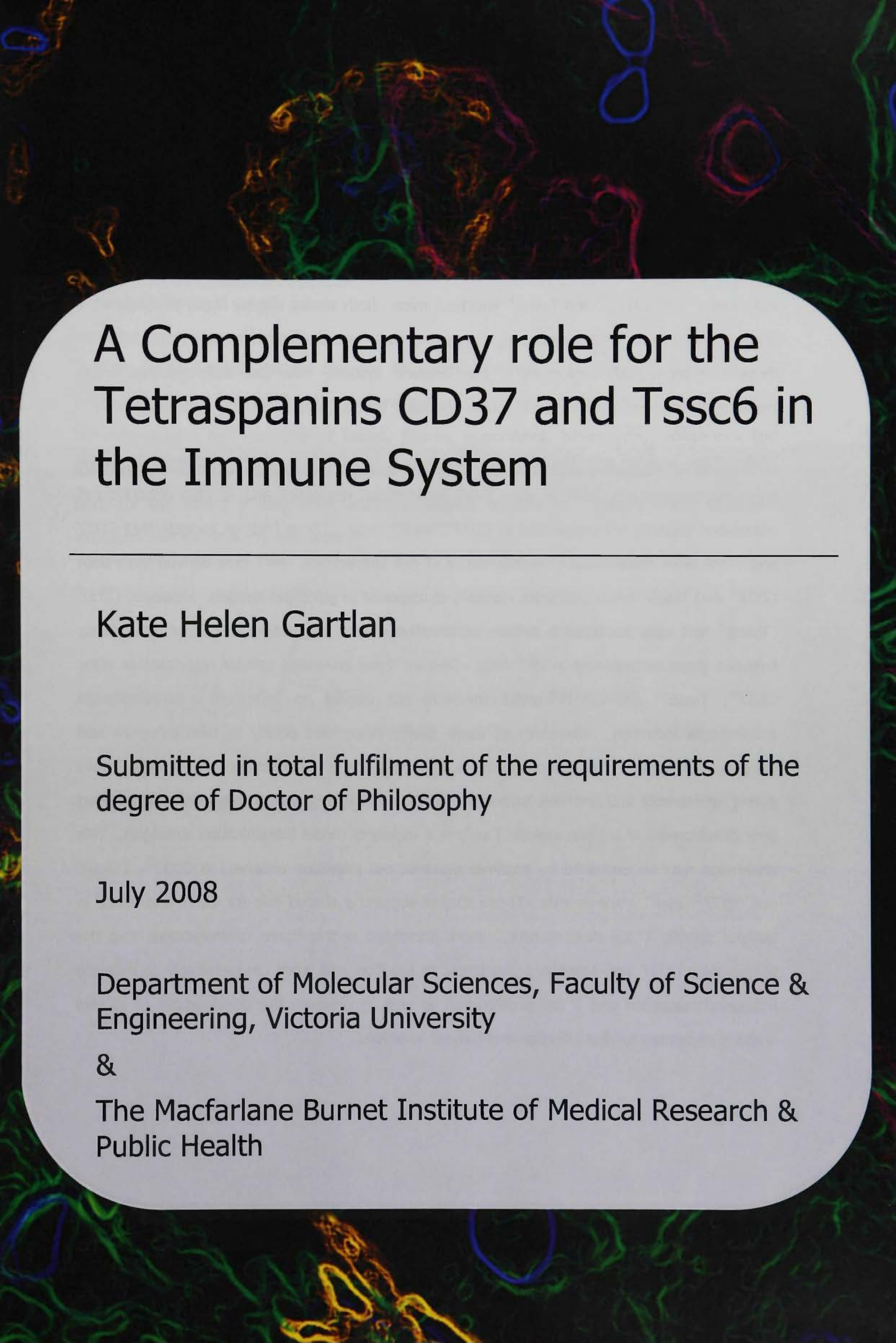
A Complementary role for the Tetraspanins CD37 and Tssc6 in the Immune System

Kate H. Gartlan



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&

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ABSTRACT

The co-operative nature of tetraspanin-tetraspanin interactions in membrane organisation suggests that functional overlap is likely to be a factor in tetraspanin biology. CD37 and Tssc6 are immune/haematopoietic restricted tetraspanins, whose function has been previously investigated with $CD37^{-/-}$ and $Tssc6^{-/-}$ knockout mice. Both strains display hyper-proliferative T cell responses to *in vitro* stimulation, suggesting that functional overlap may occur between these two tetraspanin molecules in the immune system. To test this hypothesis, the immunophenotype of a tetraspanin 'double knockout' mouse ($CD37^{-/-}Tssc6^{-/-}$) is described.

Whilst there was no role identified for Tssc6 in B cell function, when compared to single knockout counterparts, both *in vitro* T-cell proliferative responses and *in vitro* dendritic cell stimulation capacity is exaggerated in $CD37^{-/-}Tssc6^{-/-}$ mice. These findings indicate that CD37 and Tssc6 work co-operatively to regulate DC-T cell interactions. NKT cells derived from both $CD37^{-/-}$ and $Tssc6^{-/-}$ mice proliferate normally in response to glycolipid antigen. However, $CD37^{-/-}Tssc6^{-/-}$ NKT cells displayed a striking proliferative defect, suggesting functional redundancy between these tetraspanins in NKT cells. Despite these enhanced cellular responses *in vitro*, $CD37^{-/-}$, $Tssc6^{-/-}$ and $CD37^{-/-}Tssc6^{-/-}$ mice do not display an increased susceptibility to autoimmune induction. However, all three strains responded poorly to viral influenza and tumour challenges *in vivo*, demonstrating a reduced ability to produce antigen targeted T-cells during anti-tumour and anti-viral immunity. Further characterisation of these defects identified poor development of antigen specific T cells in a variety of model immunisation strategies. This phenotype may be explained by impaired dendritic cell migration observed in $CD37^{-/-}$, $Tssc6^{-/-}$ and $CD37^{-/-}Tssc6^{-/-}$ mice *in vivo*. These studies suggest a shared role for CD37 and Tssc6 in antigen specific T cell development. Work presented in this thesis, demonstrates that the tetraspanins CD37 and Tssc6 are pleiotropic in function and work co-operatively to regulate antigen presentation and T cell proliferation as well as promote the development of *in vivo* immune responses to viral infection and tumour invasion.

DECLARATION

"I, Kate Helen Gartlan, declare that the PhD thesis entitled 'A complementary role for the tetraspanins CD37 and Tssc6 in the immune system" 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: Date: Tuesday, July 8, 2008

This thesis is printed on acid free paper.

PREFACE

Pursuant to the regulations governing the degree of Doctor of Philosophy at Victoria University, I assess my contribution to each results chapter as:

Chapter 3 85%

Chapter 4 80%

Chapter 5 80%

Chapter 6 85%

Appendix I 95%

I acknowledge the important contribution of others to experiments presented here:

Chapter 3: Mariam Sofi (ELISA)

Chapter 4: Kon Kyparissoudis (NKT), Kuo-Ching Sheng (Ag presentation)

Chapter 5: Annemiek van Spriel (Tumours), Roza Nastovska (ELISpot), Fiona Chang (ELISpot), Gabrielle Belz (Influenza)

Chapter 6: Kuo-Ching Sheng (CIA), & Maria Katsara (EAE)

Appendix I: Jacqueline Tarrant (Hybridoma screening)

Therefore, the author's total contribution to the work described in this thesis was approximately 85 %.

PUBLICATIONS

Planned publications arising from the work presented in this thesis:

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Sheng KC, van Spriel A, **Gartlan KH**, Sofi M, Apostolopoulos V, Ashman, L, Wright, MD. Tetraspanins CD37 and CD151 differentially regulate antigen presentation and T cell co-stimulation by dendritic cells. In press – Eur J Imm. (2008).

Van Spriel A, Figdor CG, **Gartlan KH**, Sofi M, van der Schaaf A, Light A, Puls KL, Linssen PC, Boezeman JB, Tarlinton DM, & Wright MD. Tetraspanin CD37 is essential for long-lived plasma cell generation towards IgG1. Submitted to Plos Pathogens. (2008).

Gartlan KH, Chen F, Nastovska R, Apostolopoulos V, Pietersz GA, & Wright MD. CD37 and Tssc6 play a critical role in the development of antigen specific T cells. In preparation. (2008).

Gartlan KH, Sheng KC & Wright MD. The role of tetraspanins in antigen presentation (review article). In preparation. (2008).

Kyparissoudis K, **Gartlan KH,** Pellici D, Wright MD & Godfrey D. Functional redundancy between CD37 and Tssc6 in NKT cell development and function. In preparation. (2008).

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During my PhD, our laboratory has undergone a number of changes in affiliation and therefore I have many people who I wish to thank for their support. When I commenced my studies we formed part of the Austin Research Institute, which later merged with the Burnet Institute of Medical Research & Public Health. In my final year, we then moved to Monash University Department of Immunology. I am grateful to a number of people in these institutions and would particularly like to thank Gary Jamieson, Maria Katsara, Josh Lorimer, Charlotte Priest, Dodie Pouniotis, Vasso Apostolopoulos, Geoff Pietersz, Michael Berndt and Mark Hogarth. Important thanks must go to Victoria University for giving me the opportunity to do my PhD and generous financial support. I would also like to thank the many friends I made at all these institutions, who were always willing to listen to my woes or have a laugh over a beer.

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ABBREVIATIONS

2-ME	*2-mercaptoethanol (beta-mercaptoethanol)*
aa	*Amino Acid*
ALP	*Alkaline Phosphatase*
Amp	*Ampicillin*
APC	*Antigen Presenting Cell*
bp	*Base Pairs*
CHO	*Chinese Hamster Ovary*
CIA	*Collagen Induced Arthritis*
ConA	*Concanavalin A*
Cpm	*Counts per minute*
C-terminal	*Carboxy terminal*
DC	*Dendritic Cell*
DNA	*Deoxyribonucleic Acid*
EC1	*Extracellular Domain 1*
EC2	*Extracellular Domain 2*
EDTA	*Ethylenediaminetetraacetic acid*
ELISA	*Enzyme linked immunosorbent assay*
ELISPOT	*Enzyme linked immunospot assay*
FACS	*Fluorescence activated cell sorting*
FCS	*Foetal Calf Serum*
FITC	*Fluorescein Isothyocyanate*
FSC	*Forward Scatter*
HCV	*Hepatitis C Virus*
HRP	*Horseradish Peroxidase*
i.p.	*Intraperitoeal*
IFN	*Interferon*
Ig	*Immunoglobulin*
Il	*Interleukin*
IRES	*Internal ribosome re-entry site*
Kan	*Kanamycin*
kDa	*Kilodalton*
KLH	*Keyhole limpet haemocyanin*
mAb	*Monoclonal Antibody*
MHC	*Major Histocompatability Complex*
MTPBS	*Mouse Tonicity Phosphate Buffered Saline*
MUC-1	*Mucin-1*
Neo	*Neomycin*
NK cell	*Natural Killer Cell*
N-terminal	*Amino terminal*

PE	*Phycoerythrin*
PI3-K	*Phosphotidylinositol 3-kinase*
PI4-K	*Phosphotidylinositol 4-kinase*
PKC	*Protein Kinase C*
Puro	*Puromycin*
RNA	*Ribonucleic Acid*
RT	*Room Temperature*
TCR	*T cell Receptor*
Th1	*T helper type 1*
Th2	*T helper type 2*
TLR	*Toll-like receptor*
TM	*Transmembrane domain*
Tssc6	*Tumour-Suppressing subchromosomal transferable fragment*
UV	*Ultraviolet*
VNTR	*Variable Number of Tandem Repeats*

1 INTRODUCTION

1.1 INTRODUCTION

This study seeks to further our understanding of the immune system through research into tetraspanin biology. Tetraspanins are a superfamily of transmembrane proteins that play an important role in immune system function. Tetraspanins regulate the function of other molecules by organising partner proteins into multi-molecular complexes (microdomains) within cell membranes (Berditchevski, 2001; Hemler, 2001). These interactions are known to be important in a variety of cellular events such as adhesion, migration, proliferation and antigen presentation. Several of the most important cell surface molecules in the immune system have been shown to exist in tetraspanin microdomains (e.g. MHC Class II, CD4 & CD8) (Angelisova *et al.*, 1994; Todd *et al.*, 1996), however the immunological implications of these associations are yet to be fully established.

By using 'knockout' mice deficient in the expression of specific tetraspanins, I aim to investigate the functional and immunological consequences of disrupting tetraspanin interactions. As leucocytes can express several different tetraspanin molecules, it is possible that there is functional overlap in tetraspanin biology. Interbreeding "single" knockout mice is a strategy successfully used to identify functional overlap between related genes, whereby an obscured phenotype may be displayed in the absence of a surrogate molecule. A classical example of this is the functional redundancy identified between selectins in neutrophil migration. E-selectin and P-selectin are structurally similar adhesion molecules expressed by activated endothelium and engaged by leucocytes to enable rolling and migration. Mice deficient in either E/P-selectin accumulate neutrophils in the dermis in response to the yeast cell wall component zymosan in normal numbers, suggesting that these proteins are not required for this inflammatory response (Homeister *et al.*, 1998). However, when E-selectin$^{-/-}$P-selectin$^{-/-}$ mice were assessed, it was found that neutrophil accumulation was vastly reduced, indicating that not only are these proteins critical in neutrophil recruitment, but are functionally redundant in this model (Homeister *et al.*, 1998). This thesis concerns two tetraspanins known to regulate immune function – CD37 (Tspan26) and Tssc6 (Phemx, Tspan32). CD37 and Tssc6 show a similar pattern of tissue expression in mice, restricted to leucocytes and haematopoietic cells respectively. Moreover, whilst not identical, the phenotypes of CD37$^{-/-}$ (Knobeloch *et al.*, 2000; van Spriel *et al.*, 2004) and Tssc6$^{-/-}$ (Tarrant *et al.*, 2002) mice show significant similarities, particularly in cellular immunity. The two tetraspanin-deficient mice share a hyper-proliferative T cell phenotype, due, in both cases, to early up-regulation of the proliferation-inducing

cytokine Interleukin-2. Dendritic cells from CD37$^{-/-}$ DC demonstrated an enhanced capacity to present antigen *in vitro* (K.C. Sheng *et al.*, submitted for publication), although DC function by Tssc6$^{-/-}$ dendritic cells is yet to be described. However, there do exist some significant differences in immunological phenotype, with respect to humoral immunity, as CD37$^{-/-}$ mice show poor T cell dependent IgG responses (Knobeloch *et al.*, 2000) whereas antibody responses in Tssc6$^{-/-}$ mice are normal (Tarrant *et al.*, 2002).

This thesis continues the immunological examination of the previously described CD37 and Tssc6 deficient mouse strains (Knobeloch *et al.*, 2000; Robb *et al.*, 2001; Tarrant *et al.*, 2002; Tarrant *et al.*, 2003), and a third knockout mouse that is deficient in *both* CD37 and Tssc6 expression, i.e. a "double knockout" mouse line. This previously undescribed knockout mouse strain provides insight into functional redundancy in the immune system between members of the tetraspanin superfamily. Many studies to date have shown that tetraspanins play a significant role in immune function, which will be the focus of this review.

1.2 Immune system overview

The immune system is a complex network of cells and organs that provide a defence system against pathologies, including cancer and pathogen invasion. The primary aim of the immune system is to distinguish between 'self' and 'non self' and this distinction is critical in the destruction and removal of 'non-self' invaders whilst protecting the host i.e. 'self' (Burnet, 1959). The immune system in humans can be broadly divided into either *innate* or *adaptive* immunity. The innate immune system is the first line of defence, responding to pathogens in a broadly specific manner, whilst *adaptive* immune responses require more time to develop and can target pathogens with fine antigen specificity. These two arms of the immune system work together to enable us to respond quickly to invasion via innate immunity, while a more effective and highly specific response is developed by the adaptive immune system.

1.2.1 Innate immunity

The innate immune system is comprised of a number of mechanisms to minimise host invasion, including physical barriers, such as epithelial layers and mucosal tissue; chemical factors, such as lysozymes and complement; and cellular components such as macrophages, dendritic cells, natural killer cells and granulocytes (Janeway and Medzhitov, 2002). Whilst the innate immune system is antigen 'non-specific', it is capable of recognising a number of common danger signals that are exposed to the host upon infection. PAMPs or pathogen associated molecular patterns

are foreign antigens commonly expressed by bacteria or viruses that are not species specific. These antigens tend to be polysaccharides or polynucleotides that are recognised by pattern recognition receptors (PRRs) expressed by the host immune system (Takeda *et al.*, 2003a). Once a foreign antigen has been recognised, this can then lead to a number of anti-pathogen effector pathways, such as the activation and recruitment of phagocytes; cell lysis via the alternative complement pathway; inflammation induced by cytokine release; or the activation of the adaptive immune system (Janeway and Medzhitov, 2002). Importantly, because the innate immune system relies on non variable receptors of broad specificity, there is no immunological memory for species specific antigens. This means that immunity cannot *develop* via the innate immune system and therefore when a host is re-exposed to the same pathogen the innate immune system essentially responds as if it were the primary infection.

1.2.2 ADAPTIVE IMMUNITY

Unlike the innate immune system, the adaptive immune system is able to mount antigen specific immune responses. These two systems do not operate independently of each other; in fact, adaptive immunity is triggered by signals from the innate immune response (Medzhitov and Janeway, 1997). Adaptive immunity essentially refers to the development of antigen specific lymphocytes from the T cell and B cell lineages, and can be further broken down into *humoral immunity* (B cell responses) and *cell-mediated immunity* (T cell responses). The adaptive immune system is initiated by phagocytic cells from the innate immune system such as dendritic cells (DC), which are able to engulf and digest proteins and display portions of these antigens on their cell surface. When DC engulf proteins in the presence of danger signals i.e. PAMPs, these cells become activated and antigen presentation at the cell surface is upregulated as well as co-stimulatory molecule expression. DC then present these antigens to T and B cells which express randomly generated T cell and B cell receptors that may, or may not recognise these antigens. If an antigen *is* recognised by either cell type and danger induced co-stimulation occurs, the cell will rapidly divide and expand to form a population of cells that recognise that antigen. Unlike T cells, B cells can recognise both antigen and danger signals independently of antigen presentation and directly interact with, and engulf proteins when recognised by the B cell receptor (BCR). Antigen specific B cells go on to produce targeted antibodies that will bind the offending protein/cell leading to phagocytosis, targeting by cytotoxic cells and cell lysis via the complement pathway. Antigen specific T cells go on to either provide 'help' to B cell responses, produce cytokines to increase inflammatory responses

or provide direct cytotoxic effects against foreign or infected cells. An important part of the adaptive immune response is the development of 'memory', whereby post immune challenge a portion of the antigen specific T or B cell population will remain as long lived memory cells (Schluns and Lefrancois, 2003; Tarlinton, 2006). Upon re-exposure to antigen, the secondary or 'recall' response is very rapid and is significantly stronger than the primary response, providing protection against reinfection by pathogens (Dorner and Radbruch, 2007; Zhang *et al.*, 1998b).

The immune system however, is not a perfect system and the differentiation between 'self' and 'non-self' can be a complex process that faces multiple challenges. For example, since a random process of gene rearrangement generates both T and B cell receptors, auto-reactive T and B cells also arise (Gatzka and Walsh, 2007; Shlomchik, 2008). The immune system must maintain tolerance to self antigens by either deletion or anergising of these cells. Many pathogens have also evolved highly effective immune evasion strategies, such as epitope masking and immune suppression that result in poor responses and sometimes chronic infection (Coscoy, 2007; Lloyd *et al.*, 2007). During tumour development, abnormal cells display altered protein expression recognised by the immune system and the offending cells are removed. However, in some tumours this change in cellular phenotype is not sufficient to promote an adequate immune response due to the absence of danger signals such as PAMPS and subsequently poor co-stimulation during antigen presentation (Dunn *et al.*, 2002; Wu, 2007). Conversely, in autoimmunity inappropriate immune responses or cross-reactivity to normal tissues can lead to very severe and prolonged pathology (Davidson and Diamond, 2001; Goodnow *et al.*, 2005). It is therefore critical that we improve our understanding of immunity and particularly the molecules that regulate immunity, as this may enable us to develop the tools required to modulate these immune responses to combat immune pathology.

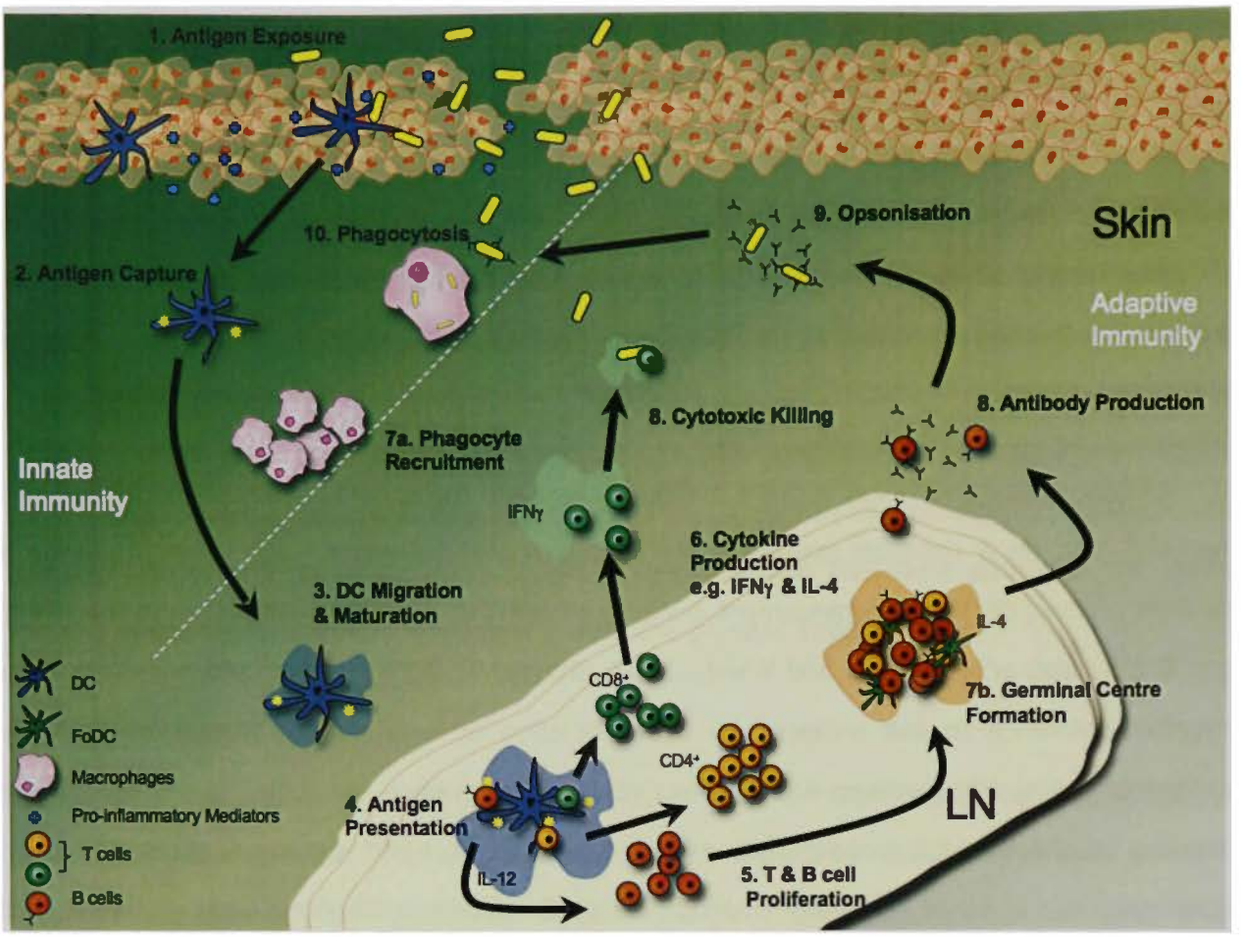

Figure 1.1 Basic overview of the immune response

Upon tissue damage and antigen exposure, pro-inflammatory mediators are released at the site of infection by the epithelium. Antigen presenting cells resident in the skin such as dermal DC and Langerhans DC capture and process antigen for presentation in the lymph node. DC mature as they migrate towards the LN by upregulating co-stimulatory molecules and the production of cytokines such as IL-12. In the LN, DC present antigen to both $CD4^+$ and $CD8^+$ T cells and antigen specific clones undergo proliferation. Antigen specific B cells exposed to antigen directly or through antigen presentation via DC, also undergo clonal expansion. Antigen specific T cells produce cytokines such as IFNγ, which activates macrophages at the site of infection; and IL-4, which promotes B cell antibody production. $CD4^+$ T cells, B cells and follicular DC form germinal centres in the LN and spleen where gene rearrangement and affinity maturation take place. $CD8^+$ T cells go on to perform cytotoxic killing of invading or infected cells. Antibodies target foreign antigen and initiate the complement cascade, leading to opsonisation and increased phagocytosis.

1.3 TETRASPANIN OVERVIEW

Tetraspanins are a distinct superfamily of proteins previously known as the Trans-Membrane-4-Super-Family (TM4SF). This group of proteins was first discovered when two research teams independently identified structural similarities between several transmembrane proteins (Oren *et al.*, 1990; Wright *et al.*, 1990). Since their identification, many more tetraspanins have been recognised and homologous proteins have been found in a wide variety of species including insects, fungi, fish and mammals (Garcia-Espana *et al.*, 2006; Hemler, 2001; Huang *et al.*, 2005). This sequence conservation during eukaryotic evolution suggests a fundamental role for tetraspanins in cell function. Tetraspanins have been most extensively studied in mammals where they have been found to associate with a variety of cell surface receptors and other tetraspanins. Through these complexes, tetraspanins regulate cellular events such as adhesion, motility, antigen presentation and co-stimulation.

1.3.1 TETRASPANIN STRUCTURE

Tetraspanins are usually between 210 – 350 amino acids in length and are characterised by the presence of four transmembrane domains, two extracellular loops, short intracellular C and N terminal regions and a series of highly conserved residues (Figure 1.2) (Maecker *et al.*, 1997; Wright and Tomlinson, 1994). The two extracellular loops differ in size and are termed the 'small extracellular loop' (SEL/EC1, 13-30 amino acids) and the 'large extracellular loop' (LEL/ EC2, 78 – 150 amino acids). Whilst the extracellular domains are considered hyper-variable, the EC2 loop contains all of the five canonical residues (see green residues in Figure 1.2), including a CCG motif and additional conserved cysteine residues. In contrast, the hydrophobic transmembrane regions are significantly conserved and contain a number of polar residues (Wright and Tomlinson, 1994). Crystallisation studies of the large extracellular loop of CD81 have confirmed the presence of disulphide bridges between the conserved cysteine residues, believed to stabilise the tertiary structure of the EC2 (Kitadokoro *et al.*, 2001a; Seigneuret *et al.*, 2001). Protein sequence analysis has revealed that in humans there are 3 possible structural sub classes of tetraspanins, based on the number of *additional* conserved cysteine residues within this domain (Seigneuret *et al.*, 2001). Whilst it is possible that these structural sub-groups have some functional significance, the relevance of these groupings is yet to be established.

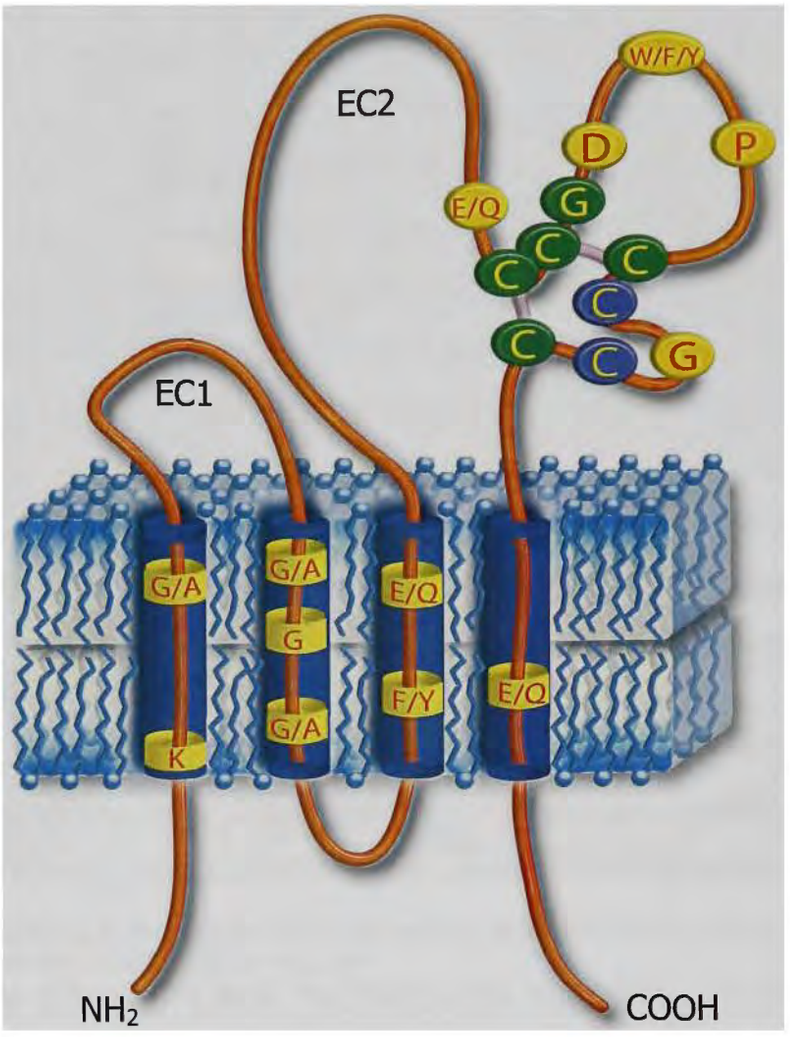

Figure 1.2 A model of tetraspanin structure

EC1 – Extracellular domain 1, EC2 – Extracellular domain 2. Green residues are 100% conserved across the tetraspanin superfamily. Purple residues indicate additional cysteine residues present in some members of the tetraspanins superfamily. Yellow residues are 80% conserved across the tetraspanin superfamily. Pink lines represent disulphide bonds predicted to occur between conserved cysteine residues (adapted from (Hemler, 2001).

1.3.2 FUNCTIONAL ASPECTS OF TETRASPANIN STRUCTURE

Tetraspanins contain multiple distinct structural domains that each potentially play a different role in protein function (Stipp *et al.*, 2003). The small extracellular loop is highly variable and has been rarely studied, although a potential role for this domain may lie in protein stabilisation at the cell surface. Mutagenesis studies have shown that the EC1 is required for cell surface EC2 expression in CD81 and CD82 (Cannon and Cresswell, 2001; Masciopinto *et al.*, 2001), suggesting a role for the EC1 in tetraspanin stabilisation or trafficking from the endoplasmic reticulum. In the large extracellular loop, both a conserved region and hypervariable region have been identified by sequence analysis of tetraspanin superfamily members. The crystallisation of this domain in CD81 led to the idea that the EC2 may serve two major functions. The hypervariable region has been proposed to play a role in protein-protein binding. Mutagenesis studies have since demonstrated the specificity of CD19-CD81 interactions to the EC2 (Shoham *et al.*, 2006) and CD151-integrin interactions have also been localised to this domain (Kazarov *et al.*, 2002). Although unconfirmed experimentally, the constant region is proposed to act as a potential dimerisation interface, enabling tetraspanin-tetraspanin interactions (Kitadokoro *et al.*, 2001a; Kitadokoro *et al.*, 2001b; Seigneuret *et al.*, 2001). The conservation of cysteine residues and the likelihood of common protein folding via disulphide bridges in the EC2, suggest a potentially universal mode of protein partner interaction for tetraspanins.

The highly conserved transmembrane domains contain a small number of conserved polar residues that have been proposed to be involved in transmembrane-transmembrane interactions (Wright and Tomlinson, 1994). Charged residues within transmembrane domains are commonly identified as necessary for the interaction of many other transmembrane protein-partner interactions (Curran and Engelman, 2003). Crystallisation and structural prediction studies have since suggested that many of these residues are involved in interchain associations between the TM domains to form a tightly packed cylindrical structure within the lipid bilayer (Figure 1.3) (Min *et al.*, 2006; Seigneuret, 2006). Sequence analysis shows that at the intracellular cytoplasmic interface of each transmembrane domain there are potential palmitoylation sites (Stipp *et al.*, 2003). In 2002, three studies demonstrated the effects of disrupting palmitoylation at these sites, each concluding palmitoylation is important for the stabilisation of tetraspanin-tetraspanin interactions, and thereby the inclusion of molecules within tetraspanin microdomains (Berditchevski *et al.*, 2002; Charrin *et al.*, 2002; Yang *et al.*,

2002). It has also been demonstrated that tetraspanin-tetraspanin interactions are not likely to be co-dependent upon the large extracellular loop, as the deletion of CD151 EC2 had little effect on CD151 association with other tetraspanins (Berditchevski *et al.*, 2001). Mutagenesis studies of CD81 have identified a role for the TM1 region of CD81 in CD19 trafficking from the ER, whereby this region alone was sufficient to enable CD19 expression at the cell surface (Shoham *et al.*, 2006).

Only a small number of studies have looked at the function of tetraspanin cytoplasmic tails. Through mutagenesis studies, it was shown that the tyrosine based sorting motif in the CD63 C-terminal tail is required for targeting of CD63 to cellular compartments (Rous *et al.*, 2002). Sequence analyses have since identified at least 17 human tetraspanins that contain potential intracellular sorting motifs in the C-terminal tail (Berditchevski and Odintsova, 2007). Mutagenesis studies of the CD81 N-terminal tail have revealed a role for this region in maturation of the CD81 partner protein CD19 in the Golgi. When constructs expressing the first transmembrane region of CD81 or the TM1 as well as the N-terminus were compared, the glycosylation of CD19 at the cell surface was altered in the absence of the N terminus (Shoham *et al.*, 2006). Since post translational modifications and glycosylation occur in the Golgi it was suggested that the CD81 N-terminus may play a role in the kinetics of CD19 trafficking. In addition, tetraspanin cytoplasmic tails play a role in cell signalling. Immuno-precipitation experiments demonstrate that intracellular signalling molecules such as PKC and PI4-K are associated with tetraspanin microdomains (Yauch and Hemler, 2000; Zhang *et al.*, 2001). Chemical cross-linking studies have also detected a potentially direct association between PKC and the tetraspanins CD9 and CD81 (Zhang *et al.*, 2001). This interaction was later shown to be independent of palmitoylation, implicating binding motifs within the tetraspanin cytoplasmic tails (Berditchevski *et al.*, 2002).

When taken together, these studies of tetraspanin structural domains identify specialised functions for a number of both conserved and non conserved regions. A detailed analysis of the sequence conservation between members of the tetraspanin superfamily may suggest functional roles which have been previously unidentified in less studied tetraspanins.

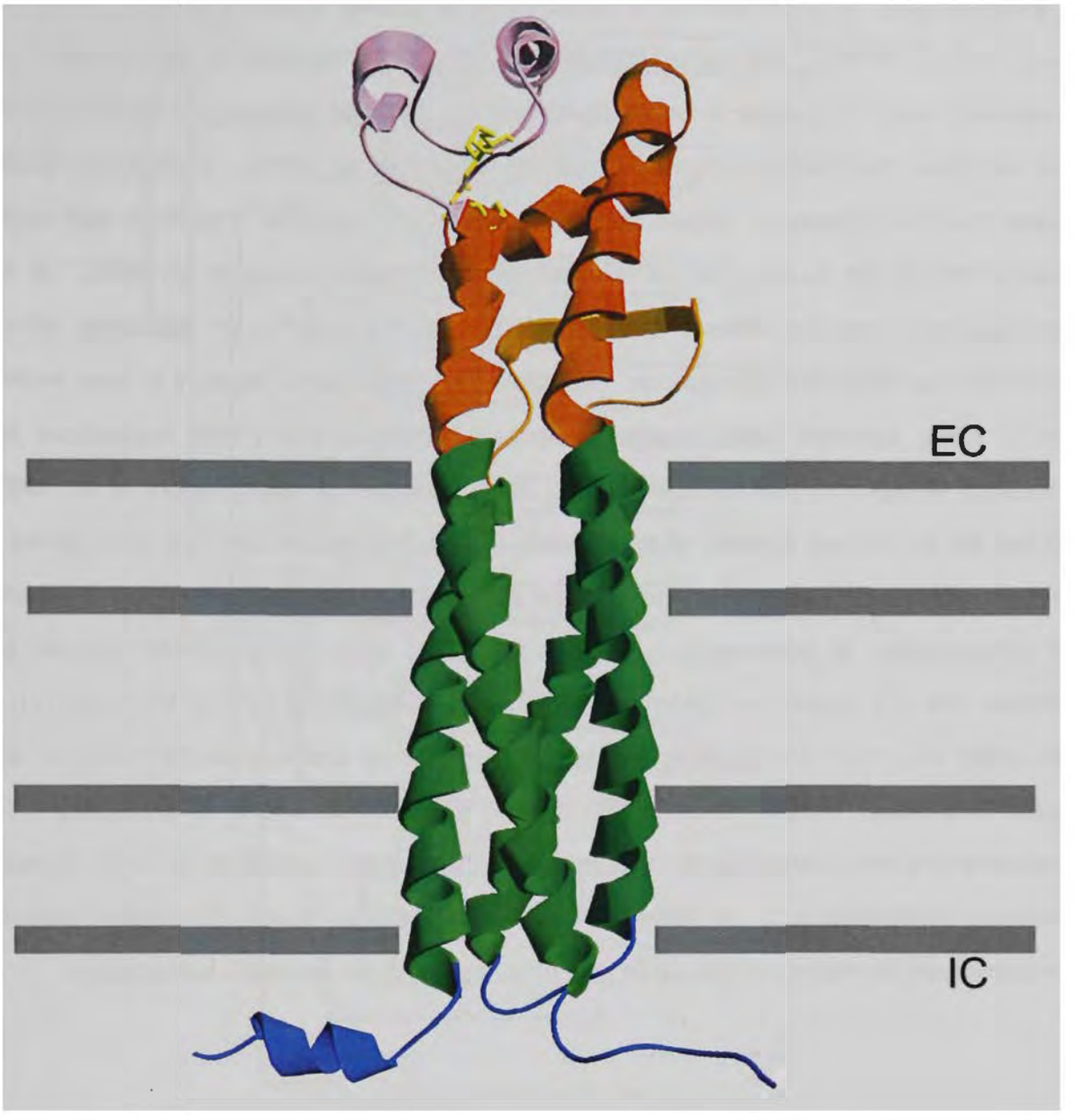

Figure 1.3 Proposed tetraspanin functional domains.

The constant EC2 region (red) is proposed to be involved in tetraspanin dimerisation. The hypervariable EC2 'mushroom like' structural region (pink) is likely to be involved in tetraspanin specific protein-protein binding. The green conserved transmembrane region may mediate protein association within the membrane, while the cytoplasmic tails (blue) have been shown in some tetraspanins to associate with signalling molecules and contain palmitoylation sites. The function of the hypervariable EC1 (orange) is poorly understood. The model shown is derived from the sequence and crystal structure of CD81 and the image generated using DeepView software (adapted from Claas et al., 2001; Stipp et al., 2003; Seigneuret et al., 2006).

1.3.3 TETRASPANIN EVOLUTION

Tetraspanins have been identified in a broad range of species including humans, mice, fish, worms, plants, insects, and fungi (Hemler, 2003). Until recently it was thought that tetraspanins were not present in unicellular organisms, however members of this superfamily have now been identified in protozoan amoebae (Huang *et al.*, 2005). Evolutionary studies suggest that the tetraspanin superfamily first evolved in unicellular organisms and rapidly expanded during the development of multicellular interactions (Huang *et al.*, 2005). It has been suggested that this reflects a broad function for tetraspanins in facilitating cell-cell interactions via membrane organisation. The tetraspanin superfamily appears to have evolved from a single ancestral gene through a series of massive and minor duplications and subsequent divergences (Garcia-Espana *et al.*, 2006; Huang *et al.*, 2005; Wright *et al.*, 1993). This has led to the high diversity of species that express tetraspanins and the large variety of members within each species (for humans refer to Figure 1.4). An example of such expansion and differentiation of tetraspanin proteins is the CD81 locus. Phylogenetic analysis has indicated that this region has undergone a series of duplications, making this area rich in tetraspanin sequences and syntenic regions between mouse and human chromosomes also support this theory (Wright *et al.*, 1993, Paulsen *et al.*, 2000, Andria *et al.*, 1991). The development of redundancy between members of this protein superfamily is to be expected when such duplications occur, however the persistence of conservation in these genes indicates that subsequent divergences have led to new functions for these emergent tetraspanins.

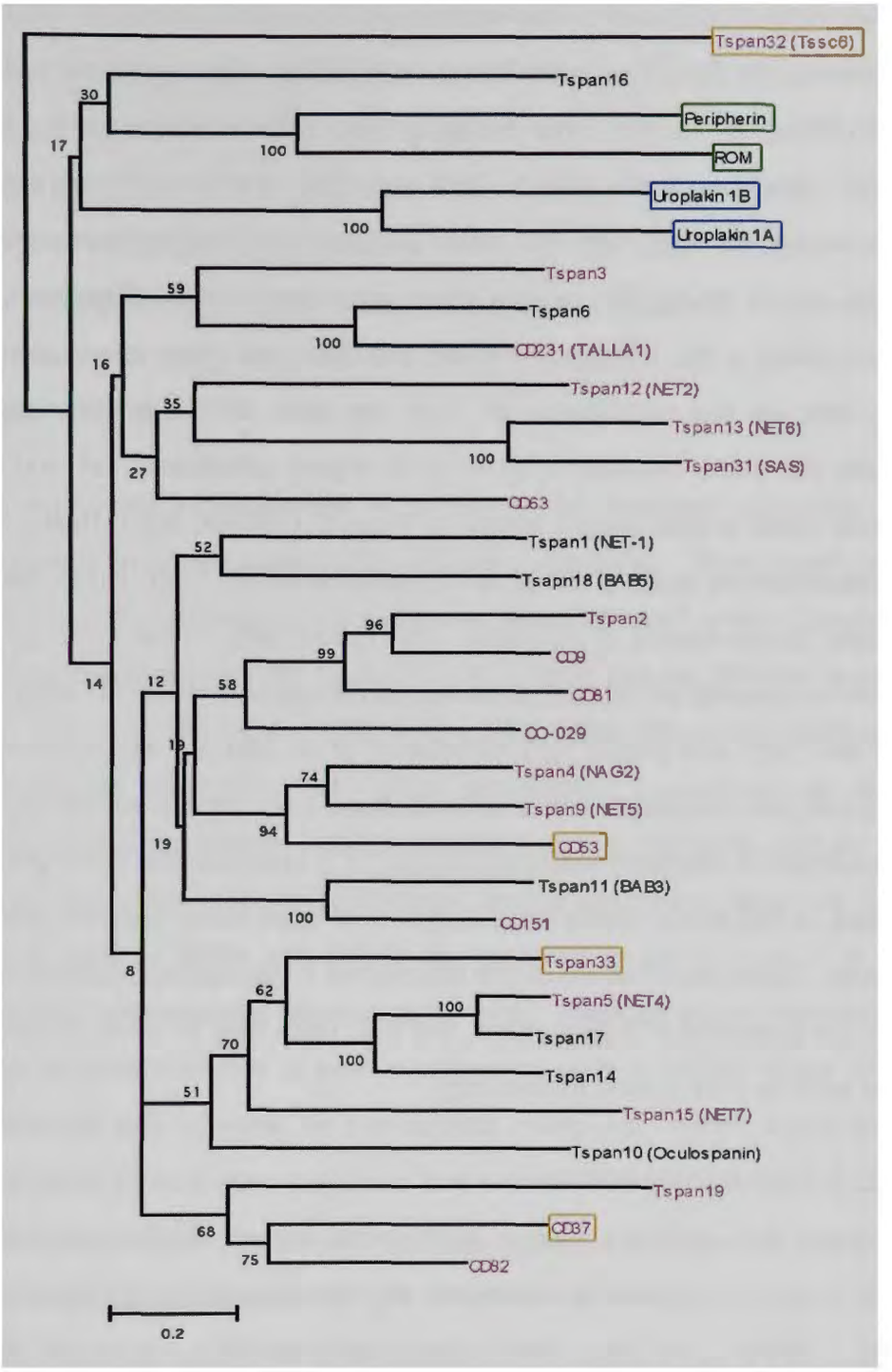

Figure 1.4 Tetraspanin Phylogram

Human tetraspanin protein sequences were aligned using the ClustalW method. Minimum evolution tree and distance analyses were generated using MEGA3 (Kumar *et al.*, 2004)*, and inferred phylogeny was tested via bootstrap calculations using 100 replications. The likelihood of the tree topology is expressed at tree branches (%), whereby higher numbers represent an increased likelihood of phylogram accuracy. To remove ambiguity in the alignment, cytoplasmic domains were removed from sequences prior to analysis. Proteins labelled in pink represent tetraspanins expressed in human leucocytes. Proteins boxed in orange, green and blue represent tetraspanins known to be restricted to blood, eye and bladder epithelial tissue respectively.*

Phylogenetic analysis of tetraspanins from a broad range of species suggests that there are four major tetraspanin gene families (Garcia-Espana *et al.*, 2008). The tetraspanins of the immune system mostly fall within one clade that evolved in two major evolutionary steps, in vertebrates and mammals. The tetraspanins CD151, CD81 and CD63 appear to be very ancient proteins estimated to have arisen more than 450 million years ago, whilst other tetraspanins expressed in the immune system are thought to have arisen approximately 100 million years ago (Garcia-Espana *et al.*, 2008). The tetraspanins CD37 and Tssc6 are more recent members of this superfamily, although the classification of Tssc6 has been difficult to determine. In some studies, Tssc6 has been excluded due to its divergent cytoplasmic tail and others have suggested that Tssc6 is most closely related to Tspan12 (Hemler, 2003; Huang *et al.*, 2005). The most comprehensive study of tetraspanin phylogeny places Tssc6 in the clade associated with Uroplakins (Garcia-Espana *et al.*, 2008). This is surprising, as the Tssc6 gene in mice as well as humans is proximal to the CD81 gene and can be partially modelled based on the crystal structure of the CD81 EC2 (Figure 1.5) (Kitadokoro *et al.*, 2001a). Furthermore, the cellular expression profile and functional studies of Tssc6 share more similarities with those of the CD clade than members of Uroplakin tetraspanin family. It is likely that the Tssc6 grouping into the Uroplakin clade in this study reflects the divergence of Tssc6 rather than its close relationship with Uroplakins. Other classifications of this superfamily in phylogenetic databases suggest that tetraspanins are composed of 6 subfamilies, and that Tssc6 may be the only representative of one of these sublines (http://www.treefam.org).

1.3.4 Tetraspanin microdomains

Through various immuno-precipitation, confocal microscopy, chemical cross-linking and mutagenesis studies, it has been demonstrated that tetraspanins can associate with signalling molecules (e.g. PI4-K, PKC), cell surface receptors (e.g. MHC Class II, CD19) and particularly integrins (e.g. $\alpha 6\beta 1$, $\alpha 4\beta 1$ & $\alpha IIb\beta 3$) (refer to Table 1.1). Notably, tetraspanins have also been repeatedly shown to associate with other tetraspanins (Charrin *et al.*, 2001; Claas *et al.*, 2001; Stipp *et al.*, 2001). These findings gave rise to the concept of a 'Tetraspanin web', where tetraspanin enriched microdomains of cell surface proteins and signalling molecules function in supra-molecular complexes which can play a regulatory role in cellular events (Boucheix and Rubinstein, 2001). In some respects, the term 'Tetraspanin web' may be misleading, as it implies a single interconnected series of tetraspanin-partner associations at the cell surface. However, it is also possible that tetraspanin microdomains are varied in their components, in

this way, more than one population of microdomain may exist at the cell surface at any one time. This theory is supported by confocal and electron micrograph analysis of tetraspanin expression at the cell surface of HeLa cells co-stained with multiple tetraspanins (Nydegger *et al.*, 2006). The analyses revealed discrete punctate staining which co-localised to demonstrate a high degree of heterogeneity between putative microdomains (Nydegger *et al.*, 2006). Furthermore, tetraspanins also vary in their subcellular localisation, for example in DC a significant proportion of CD63 is expressed intracellularly, whilst CD9 is primarily restricted to the cell surface.

Isolation of tetraspanin microdomains is performed by detergent disruption of the cell membrane followed by centrifugation and immuno-precipitation. Three levels of interaction between proteins within tetraspanin microdomains were proposed when it became apparent that variations in detergent strength could either include or exclude different molecules (Claas *et al.*, 2001). The various levels of stability among tetraspanin interactions reflect the likelihood that tetraspanins are involved in both direct and indirect associations within tetraspanin microdomains. Level 1 interactions, which are stable in strong detergents such as Triton X-100 are potentially direct, partner-partner interactions, Level 2 associations which are maintained in mild detergents such as Brij96 and Brij97, are proposed to mainly consist of tetraspanin-tetraspanin interactions sustaining the tetraspanin web. The least stable interactions are Level 3 associations demonstrated only in weaker detergents such as CHAPS, these are potentially indirect associations held together by intermediate molecules. Initial debate regarding the authenticity of Level 3 interactions suggested that such associations may be due to incomplete solubilization of the cell membrane. However, a series of studies using other methods of identifying co-localisation (FRET, mutagenesis and mAb cross-linking) have confirmed many of these findings (Imai *et al.*, 1995; Nakamura *et al.*, 1995; Szollosi *et al.*, 1996). In a multiple protein complex one could imagine partner molecules, adapter molecules and bystander molecules all interacting at varying levels of stability, whereby proteins may have partner molecules that potentially have stronger associations than those mediated by an intermediate molecule (Lammerding *et al.*, 2003).

Table 1.1 Tetraspanin Association within Haematopoietic cells

Level 1 associations (Red) are stable in 'strong' detergents such as Triton X and Digitonin. Level 2 associations (Blue) are stable in 'milder' detergents such as Brij 96 and Brij 97. Level 3 associations (Black) are stable in 'weaker' detergents such as CHAPS and Brij 99.

Cell Type	Tetraspanin Association	Reference
Primary Haematopoietic Cells		
T cells	CD81⇔ PGRL	(Clark et al., 2001)
	CD9 ⇔ CD2, CD3, CD5, CD44, CD28	(Toyo-oka et al., 1999)
	CD9, CD81, CD82 ⇔ CD4	(Imai et al., 1995; Todd et al., 1996)
	CD53 ⇔ CD2	(Bell et al., 1992)
Macrophages	CD9 ⇔ CD46, $\alpha 3\beta 1$	(Kurita-Taniguchi et al., 2002)
Neutrophils	CD63 ⇔ $\alpha L\beta 2$, Lyn, Hck	(Skubitz et al., 1996; Skubitz et al., 2000)
MoDCs	CD63 ⇔ MHC Class II	(Engering et al., 2003)
	CD63 ⇔ Dectin-1, CD11b, CD18	(Mantegazza et al., 2004)
Monocytes	CD9 ⇔ CD38, MHC Class II	(Zilber et al., 2005)
Platelets	CD63, CD151, Tssc6 ⇔ $\alpha IIb\beta 3$	(Goschnick et al., 2006; Israels et al., 2001; Lau et al., 2004)
	CD9 ⇔ CD36	(Miao et al., 2001)
	CD9 ⇔ CD47, CD42	(Longhurst et al., 1999)
	CD9 ⇔ GTP-binding protein	(Seehafer and Shaw, 1991)
	CD9 ⇔ $\alpha IIb\beta 3$	(Indig et al., 1997)
Thymocytes	CD9, CD81, CD82 ⇔CD4 & CD81, CD82 ⇔ CD8	(Imai et al., 1995; Todd et al., 1996; Toyo-oka et al., 1999)
Lymphocytes	CD53, CD63 ⇔ Unknown Tyrosine Phosphatase	(Carmo and Wright, 1995)
Haematopoietic Derived Cell Lines		
T cell lines	CD9, CD53, CD81, CD82, CD151 ⇔ PKCα, PKCβII, PKCγ	(Zhang et al., 2001)
	CD53, CD81, CD82 ⇔ GGT	(Nichols et al., 1998)
	CD9, CD63, CD81, CD151, CD231 ⇔ PI4-K	(Berditchevski et al., 1997; Yauch and Hemler, 2000)
B cell lines	CD81 ⇔ $\alpha 4\beta 1$, CD19, CD21	(Bradbury et al., 1992; Serru et al., 1999)
	CD151⇔ $\alpha 6\beta 1$	(Serru et al., 1999)
	CD53, CD81, CD82 ⇔ GGT	(Nichols et al., 1998)
	CD81 ⇔ PGRL	(Clark et al., 2001)
	CD9 ⇔ a5b1	(Rubinstein et al., 1994)
	CD9, CD37, CD53, CD63, CD81, CD82 ⇔ MHC Class II	(Angelisova et al., 1994; Rubinstein et al., 1996; Schick and Levy, 1993; Szollosi et al., 1996)
	CD9, CD63, CD81, CD151, CD231 ⇔ PI4-K	(Berditchevski et al., 1997; Yauch and Hemler, 2000)
	CD53, CD81, CD82 ⇔ MHC Class I	(Lagaudriere-Gesbert et al., 1997b; Szollosi et al., 1996)
	CD81 ⇔ $\alpha 4\beta 7$, Leu-13	(Bradbury et al., 1992)
	CD37 ⇔ Dectin-1	(Meyer-Wentrup et al., 2007)
NK Cell line	CD53 ⇔ CD2	(Bell et al., 1992)
Myeloid cell line	CD82 ⇔ CD4	(Imai et al., 1995)
Erythroleukemia cell line	CD9, CD53, CD81, CD82, CD151 ⇔ PKCα, PKCβII, PKCγ	(Zhang et al., 2001)
	CD9, CD63, CD81, CD151, CD231 ⇔ PI4-K	(Berditchevski et al., 1997; Yauch and Hemler, 2000)
	CD151 ⇔ $\alpha 5\beta 1$	(Fitter et al., 1998)
Megakaryocyte cell line	CD81 ⇔ $\alpha 4\beta 1$	(Serru et al., 1999)
	CD9, CD63 ⇔ c-kit	(Anzai et al., 2002)
	CD151 ⇔ $\alpha 5\beta 1$	(Fitter et al., 1998)

1.3.5 MEMBRANE ORGANISATION

Tetraspanin microdomains are proposed to serve one main function, to organise other molecules. This may have a number of functional consequences such as, maintaining separation between molecules, enabling the association of specific proteins by bringing partner molecules into close proximity, or by enrichment of a specific molecule within a region of the cell membrane (refer to Figure 1.5). A sequestration model suggests that microdomains may act as a reservoir for specific proteins in order to prevent possible interactions involved in signalling or cell pathway activation (Tarrant *et al.*, 2003). This may serve to keep proteins apart until cellular events signal their release from the microdomain or competitive binding may force such molecules from the microdomain (Figure 1.5A). Another separation model for tetraspanin microdomains may involve microdomain exclusion rather than inclusion. Whereby, molecules may be prevented access to binding partners within the microdomain until cellular events signal their entrance into the microdomain. Alternatively, another role of tetraspanins may be to bring molecules into close association by acting as adapter or linker molecules, which has been suggested for integrin signalling (Figure 1.5B). $\alpha 4\beta 1$ and $\alpha 6\beta 1$ integrins associate with the EC2 of CD151, while the kinase PKC interacts with the cytoplasmic regions of CD151, thus facilitating integrin signal transduction via PKC (Zhang *et al.*, 2001; Zhang *et al.*, 2002). Trafficking of partner proteins between the cell surface and intracellular compartments is also thought to enable tetraspanins to modulate cell signalling (Figure 1.5C). For example, increased expression of CD9 or CD82 in tumour cell lines results in increased internalisation of the epidermal growth factor receptor (EGFR) and dampening of the proliferative response to EGF (Murayama *et al.*, 2008; Odintsova *et al.*, 2000). Similarly, reduced expression of CD9 and CD82 correlate with increased invasiveness and tumour progression in some cancers (Huang *et al.*, 1998; Miyake *et al.*, 1996; Uchida *et al.*, 1999). During cell-cell contact, tetraspanin microdomains could also potentially cluster low affinity ligands or receptors to enhance signalling between cells (Figure 1.5D). It has been proposed that during antigen presentation tetraspanin microdomains may cluster MHC Class II molecules bearing low affinity peptides, providing enrichment of specific peptide bearing MHC in order to enhance presentation (Kropshofer *et al.*, 2002).

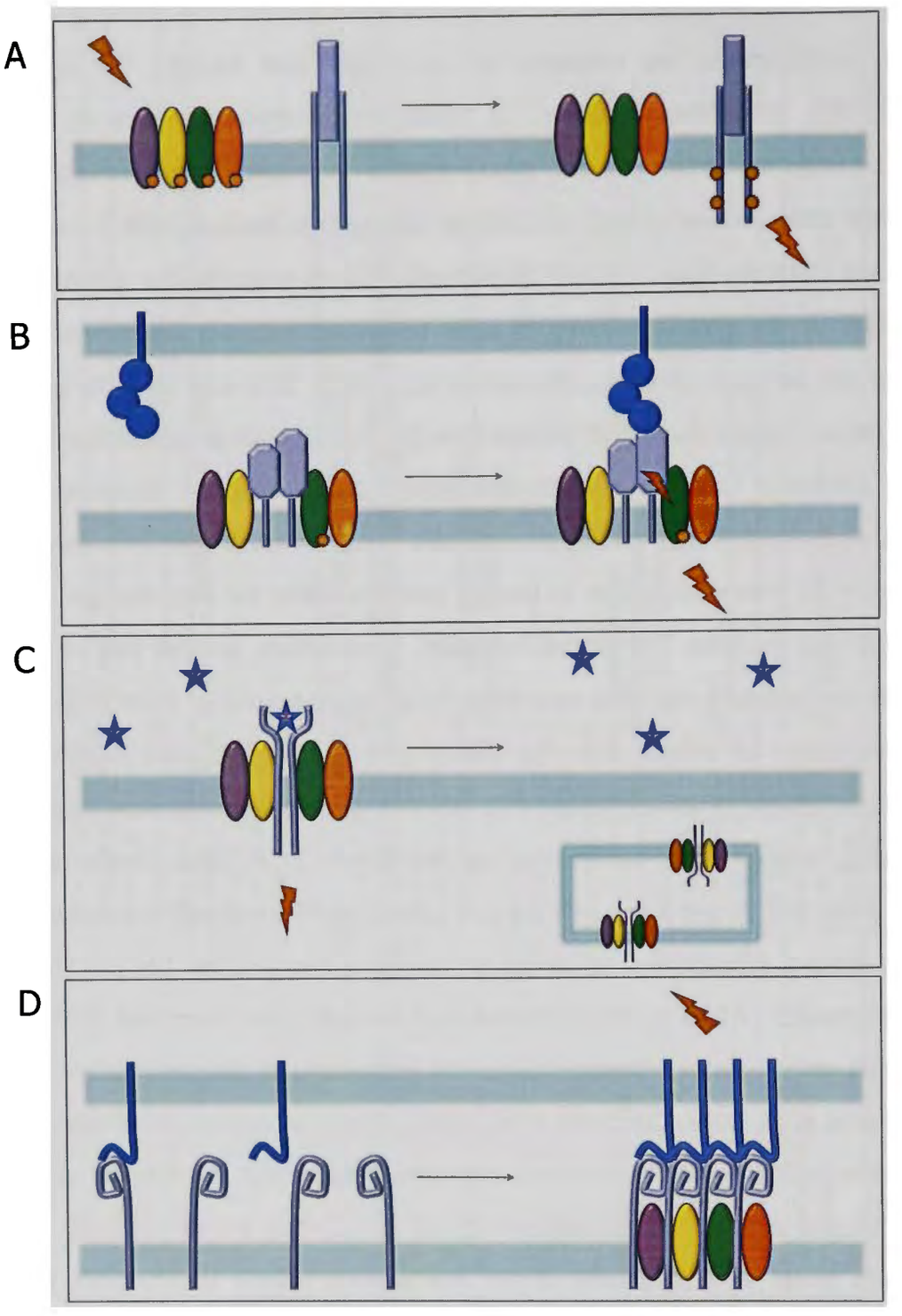

Figure 1.5 Models of tetraspanin microdomain function

Tetraspanins (coloured ovals) are thought to regulate the function of partner molecules by (A) physical disruption of protein-protein interactions; possibly through sequestration of either signalling molecules or cell surface receptors, (B) providing a physical link between proteins to enable cell surface receptor signalling, (C) regulating cell surface expression of partner molecules to modulate responses to extracellular ligands and (D) clustering of cell surface receptors to promote binding by low affinity intercellular ligands.

1.3.6 TETRASPANINS AND LIPID RAFTS

Some aspects of the tetraspanin microdomain theory show parallels with the concept of lipid rafts. Like tetraspanin microdomains, lipid rafts are also believed to organise cell surface and signalling molecules and are involved in a broad range of cellular processes. Lipid rafts are heterogeneous regions demonstrated at the cell surface enriched in cholesterol and glycosphingolipids (Pike, 2004). Notably, lipid rafts may also be identified by their incorporation of specific molecules such as Caveolin; GPI linked proteins and some members of the G protein family (Simons and Toomre, 2000), whereas such lipids and proteins are rarely if at all identified in association with tetraspanins. Solubilization and cholesterol depletion experiments have also shown that tetraspanin microdomains exist independently of lipid rafts (Berditchevski *et al.*, 1996; Israels and McMillan-Ward, 2007; Kropshofer *et al.*, 2002). Similarly, confocal studies of tetraspanin co-localisation and microdomain formation demonstrate that the bulk of tetraspanin proteins reside in distinct clusters separate from lipid raft localised proteins (Nydegger *et al.*, 2006). Importantly, tetraspanin microdomains largely consist of protein-protein interactions and can therefore be clearly characterised through immuno-precipitation studies. In comparison, lipid rafts are relatively ill-defined due to the elusive nature of lipid-protein interactions.

In the immune system lipid rafts play an important role in T cell and B cell activation by functioning as signalling reservoirs for both T cell and B cell receptors. In resting states these receptors are partitioned from signalling molecules by lipid rafts and upon activation, TCR and BCR proteins re-localise to raft domains to enable signalling. Recent studies suggest that in B cells, the transition of BCR into lipid rafts is dependent upon the tetraspanin CD81 (Cherukuri *et al.*, 2001). This suggests that these two domains co-operate in cell surface organisation. Furthermore, a similar interaction may occur between lipid rafts and tetraspanin microdomains in T cell activation, in which they have been separately shown to be involved (Lagaudriere-Gesbert *et al.*, 1997a; Simons and Toomre, 2000; Tai *et al.*, 1997). Studies into MHC clustering and organisation on APC surfaces have proposed that lipid rafts enrich MHC Class II molecules in a non-specific manner, while tetraspanin microdomains can cluster MHC Class II molecules based on peptide affinity (Kropshofer *et al.*, 2002). Such common purposes but disparate styles of interaction may also suggest interplay between these domains in antigen presentation.

1.4 TETRASPANIN FUNCTION

Antibody cross-linking studies have suggested a broad range of cellular functions associated with tetraspanins (summarised in Table 1.2). Much of the motility, aggregation and adhesion modulation abilities of anti-tetraspanin antibodies have been ascribed to a modulation of integrin function (Berditchevski, 2001). A number of studies also report co-stimulation effects of tetraspanin ligation inducing T cell proliferation and/or IL-2 production in different systems. Other reports however demonstrate that some anti-tetraspanin antibodies inhibit proliferation (Oren *et al.*, 1990, Ledbetter *et al.*, 1987, van Spriel *et al.*, 2004). It is important to note that due to the complex nature of tetraspanin microdomains and the undefined nature of the mAb-tetraspanin interactions, it is difficult to draw definitive conclusions from data generated by this approach. Anti-tetraspanin mAbs may act as agonists, antagonists, provide general physical disruption within a tetraspanin microdomain, bind to functionally irrelevant tetraspanin epitopes or cross-link inappropriate proteins. Moreover, in a group of over 30 proteins expressed in a wide range of species, only one tetraspanin has been suggested to have a candidate native ligand (i.e. PSG17-CD9) (Ellerman *et al.*, 2003). This suggests that the physiological relevance of tetraspanin ligation with mAbs is dubious. However, other methods of identifying protein specific functions are also open to criticism.

Whilst the use of knockout mice can overcome the confounding effects of antibody agonism and antagonism, the effects of cell surface disruption of protein expression cannot be separated from other potential changes in cellular function. For example, disruption of an endocytic trafficking molecule could have broad ranging effects on cellular function through impaired expression of multiple molecules (Di Pietro and Dell'Angelica, 2005). Isolating tissue specific functions can also be complicated in non-inducible knockout models. For example, the deletion of proteins involved in development may result in broad ranging phenotypes that are not directly relevant to the function of the targeted protein. However, in some circumstances these effects can be overcome with reconstitution assays. In addition, genetic variation between wild type and knockout mice due to incomplete inbreeding or carryover of target proximal genes can make it difficult to accurately compare the differences between wild type and knockout phenotypes due to genetic variation. Transcript silencing can be an effective substitute for knockout models that can avoid the consequences of protein deficiency during development, however complete abrogation of protein expression is rare (Ladunga, 2007). Ectopic expression of proteins can also be used in 'gain of function' studies, although achieving physiological

expression levels and ensuring that other partner molecules are present is difficult. Whilst this approach has been used in cell lines in tetraspanin research, it has scarcely been used in whole animal models i.e. transgenic mice. In summary, to best combat these limitations in experimental design, a series of complementary approaches is required to complete the puzzle of tetraspanin function, the findings of such studies are outlined below.

Table 1.2 Cellular functions implicated by tetraspanin targeting via monoclonal antibody treatment of immune cells.

Tetraspanin mAb	Cell Type	References
Migration Inhibition		
CD9	Pre B cell line, Megakaryocytic cell line	(Rubinstein et al., 1994)
CD9	B cell line	(Shaw et al., 1995)
CD9, CD81, CD82	Pre B cell line, Megakaryocytic cell line	(Lagaudriere-Gesbert et al., 1997a)
CD151	Neutrophils	(Yauch et al., 1998)
CD151	Endothelial cells	(Yanez-Mo et al., 1998)
Migration Stimulation		
CD9, CD63, CD81, CD82 CD151	Breast Cancer cell line	(Sugiura and Berditchevski, 1999a)
Induced Cellular Adhesion		
CD9	Pre-B cells, Bone Marrow Stromal Fibroblasts, Platelets	(Masellis-Smith and Shaw, 1994, Worthington et al., 1990)
CD81	Thymocytes	(Todd et al., 1996)
CD9, CD81, CD82	Pre B cell line, Megakaryocytic cell line	(Lagaudriere-Gesbert et al., 1997a)
CD82	Primary T cells	(Lagaudriere-Gesbert et al., 1998)
CD63	Neutrophils	(Skubitz et al., 1996; Skubitz et al., 2000)
CD151	Platelets	(Roberts et al., 1995)
Co-stimulation		
CD53	Primary T cells	(Bell et al., 1992)
CD9, CD53, CD81, CD82,	T cell line	(Lebel-Binay et al., 1995)
CD81	Thymocytes	(Todd et al., 1996)
CD82	Primary T cells	(Lagaudriere-Gesbert et al., 1998)
CD9	Primary T cells	(Tai et al., 1997)
CD81	Primary T cells	(Witherden et al., 2000)
Apoptosis		
NAG-2	Myotubules	(Tachibana and Hemler, 1999)
CD9	Endothelial cell line	(Lunardi et al., 2000)
Proliferation Inhibition		
CD37	B cells, T cells	(Ledbetter et al., 1987, van Spriel et al., 2004)
CD81	Lymphoma cell lines	(Oren et al., 1990)

1.4.1 TETRASPANIN SUBCELLULAR LOCALISATION

Whilst the bulk of this review focuses on the role of tetraspanins at the cell surface, subcellular localisation studies demonstrate that tetraspanins are also compartmentalised within endosomes, lysosomes and other secretory vesicles (Griffiths, 1996; Mantegazza *et al.*, 2004). The majority of these studies have been performed in haematopoietic cells, where the tetraspanins CD9, CD63, CD82 and CD151 have all been studied extensively, due to their localisation within either or both the endocytic system in antigen presenting cells and α–granules in platelets. Sequence analysis has revealed that many tetraspanins contain intracellular sorting motifs such as tyrosine, dileucine, and acid cluster residue patterns that are thought to induce internalisation through both clathrin dependent and independent pathways (Hunziker and Geuze, 1996, Bonifacino and Traub, 2003, Berditchevski and Odintsova, 2007).

Endosomes are a network of small sub cellular vesicles produced by the process of endocytosis. This mechanism allows the internalisation of macromolecules bound to cell surface receptors, for either degradation or use. 'Early-endosomes' are proximal to the membrane and migrate away from the cell surface becoming 'late-endosomes'. Endosomes later go on to fuse with another sub cellular compartment – lysosomes. This endosomal-lysosomal system is utilised in different ways depending on the cell type. For example, in the haematopoietic system secretory lysosomes such as azurophilic granules in neutrophils store cytotoxic factors while α–granules in platelets store clotting factors, adhesion molecules, and growth factors.

Tetraspanins are expressed in both platelet surface membranes and internal vesicles. CD9 and CD151 can be found on external membranes and internal α-granules, whilst CD63 is expressed at the platelet surface, internal dense body vesicles and lysosomal compartments (Cramer *et al.*, 1994; Israels and McMillan-Ward, 2005). Tssc6 can be detected at the platelet surface and internal compartments also, although the exact internal distribution is not known (Goschnick *et al.*, 2006). The platelet surface expression of each of these tetraspanins is upregulated during platelet activation and degranulation, when intracellular platelet adhesion and clotting factors are released into the platelet microenvironment to enable thrombus formation (Cramer *et al.*, 1994; Goschnick *et al.*, 2006; Israels and McMillan-Ward, 2005; Lau *et al.*, 2004). Tetraspanin function in platelets is primarily linked to modulation of the integrin αIIbβ3, which constitutively associates with CD9, CD151 and Tssc6; and also with CD63 upon its relocalisation to the platelet surface (Goschnick *et al.*, 2006; Indig *et al.*, 1997; Lau *et al.*, 2004).

In APC, the endosomal/lysosomal system is critical in exogenous antigen presentation, where antigen is processed and before vesicle fusion with MHC class II rich compartments (MIIC). Here, peptides are loaded onto MHC class II molecules before translocation to the cell surface. Multiple tetraspanins have been detected in APC MIIC compartments including CD37, CD53, CD63, CD81 and CD82, and the peptide editors DM and DO (Engering and Pieters, 2001; Escola *et al.*, 1998). Furthermore CD63, which is primarily located intracellularly in immature DC, displays altered post translational modifications after DC maturation (Engering *et al.*, 2003). It has also been reported that during DC maturation, morphological changes to the lysosomal system occur in concert with the addition of poly-*N*-lactosaminoglycans to CD63 (Engering *et al.*, 2003). However, very little is understood about the link between these two events. The role of tetraspanins in exosome function is a newly emerging area of study. Exosomes are small lipid bound compartments that are formed in the late endosome and accumulate in multi-vesicular bodies, before budding from the cell via the cell surface (Thery *et al.*, 2002). Whilst these entities are still poorly understood, a range of functions have been proposed for exosomes in the immune system where they have been most extensively studied. It has been suggested that exosomes may play a role in antigen presentation, HIV and prion pathogenesis, immune activation and immune suppression (Thery *et al.*, 2002). The bulk of these functions are due to the ability of exosomes to carry proteins away from the host cell and be endocytosed by other cells. Exosomes from antigen presenting cells express both peptide bound MHCI and MHCII, which can prime T cell responses in the absence of DC-T cell contact and can therefore act as early T cell activators. Importantly, it has been shown that exosomes express high levels of a number of late endosome derived tetraspanins including CD9, CD63 and CD82 which have been identified in DC and B cell derived exosomes (Engering and Pieters, 2001; Escola *et al.*, 1998). If exosomes do play a significant role in antigen presentation and T cell activation, it will be important to assess the effects on exosome production and function in the absence of tetraspanins in future studies.

1.4.2 Tetraspanins, adhesion & motility

One major function identified for tetraspanins and tetraspanin microdomains is a role in integrin modulation and the resultant effects on adhesion and motility. Integrins are cell surface heterodimers that bind ligands expressed by other cells (e.g. ICAM-1, VCAM-1) and extracellular matrix (ECM) proteins such as collagen, fibronectin and laminin. The binding of integrins to these substrates enables intercellular adhesion, basement membrane adhesion, and migration.

Integrin signalling is bi-directional, such that integrins undergo both 'inside-out' and 'outside-in' signalling during cellular responses (Ginsberg *et al.*, 2005). Inside-out signalling is initiated by the activation of a variety of cellular receptors other than the integrin itself, which leads to a conformational change in the integrin and subsequent binding to extracellular proteins such as ICAMs and ECMs. Upon ligand binding, multiple integrins cluster and initiate outside-in signalling, whereby the intracellular integrin domains are linked to the cytoskeleton, resulting in cytoskeletal reorganisation for cell spreading, adhesion and aggregation (Qin *et al.*, 2004).

Integrin associations are most often described for the tetraspanin CD151, although molecular associations have been described between integrins and CD9, CD37, CD53, CD63, CD81, CD82, CD151 and Tssc6 in haematopoietic cells alone (Table 1.1). The bulk of data on tetraspanin associations with integrins involves the β1 integrin subunit, although associations have been demonstrated with β2 integrins in DC, neutrophils and T cells, β3 integrins in platelets and β4 integrins in keratinocytes (Berditchevski, 2001, Hemler, 2003). Mutagenesis studies of CD151 have demonstrated that strong interactions between α3β1, α6β1 and CD151 are mediated by a short sequence within the tetraspanin large extracellular loop (Berditchevski *et al.*, 2001; Kazarov *et al.*, 2002). This interaction is not dependent on palmitoylation, although palmitoylation is required for tetraspanin microdomain formation (Yang *et al.*, 2002). Interestingly, mutations in this sequence within the CD151 EC2 did not abrogate integrin binding but significantly reduced the strength of these associations, suggesting a secondary site for CD151-integrin interactions (Kazarov *et al.*, 2002). This weakening of CD151-integrin associations also led to functional defects in cell spreading and migration on basement membrane matrices.

Previously, ECM adhesive interactions via integrins were thought to be independent of tetraspanin function, based on evidence from mAb targeting of tetraspanins (Berditchevski, 2001; Stipp *et al.*, 2003; Sugiura and Berditchevski, 1999b). However, other studies have suggested this may not be the case. For example, CD81 mAbs can induce α4β1 dependent adhesion of B cells to tonsillar stroma through binding of fibronectin (Behr and Schriver, 1995). When complexes formed between CD151 and α3β1 are disrupted, adhesion to the α3β1 substrate laminin is also impaired (Nishiuchi *et al.*, 2005). Furthermore, the function of α3β1 dependent migration on laminin 5 substrates was impaired when CD151 expression was silenced in epithelial carcinoma cells (Winterwood *et al.*, 2006). Investigations into wound

healing in CD151 deficient mice detected impaired basement membrane formation due to altered laminin deposition in the absence of CD151 (Cowin *et al.*, 2006). These data suggest a role for CD151 not only in ECM adhesion but basement membrane formation as well.

Mutagenesis studies have revealed a role for CD151 in strengthening integrin mediated adhesion. Lammerding *et al* utilised CD151 transfectants (NIH-3T3) that carried a mutation in the C-terminal cytoplasmic tail that disrupted cell spreading and the formation of cellular cables on matrigel substrates (Lammerding *et al.*, 2003, Zhang *et al.*, 2002). Whilst static adhesion of these cells was unaffected on laminin-1, over time cells transfected with CD151 mutants were prone to detachment from laminin coated beads under controlled forces (Lammerding *et al.*, 2003). Adhesion to laminin-1 in these cells was almost completely dependent upon $\alpha 6\beta 1$ since adhesion could be blocked using $\alpha 6\beta 1$ mAbs. These data suggest that $\alpha 6\beta 1$ adhesion to laminin-1 is independent of CD151 but maintenance and strengthening of this interaction is dependent upon coupling of integrins with potential signalling molecules through the C-terminal cytoplasmic tail (Lammerding *et al.*, 2003). Furthermore, the authors propose that CD151 may therefore be critical in the maintenance of cellular adhesion under force. It has since been suggested that the tyrosine based sorting motif present in the CD151 C-terminal tail may control integrin function through intracellular trafficking of integrins between the cell surface and intracellular compartments (discussed in section 1.4.4).

A large number of studies have found that tetraspanins also modulate the role of integrins in heterotypic and homotypic intercellular adhesion (Berditchevski, 2001; Hemler, 2003; Yanez-Mo *et al.*, 2001). For instance, in early work investigating the effects of tetraspanin ligation, it was demonstrated that both CD9 and CD151 mAbs induce platelet activation and aggregation (Worthington *et al.*, 1990, Roberts *et al.*, 1995). CD9, CD151, CD63 and Tssc6 have all since been linked to the platelet integrin $\alpha IIb\beta 3$ and its function in platelet activation (Goschnick *et al.*, 2006, Lau *et al.*, 2004). Platelet function has also been investigated in both CD151 and Tssc6 deficient mice, where defects in haemostasis were observed in both strains. These mice share defective platelet aggregation responses, impaired platelet spreading and delayed kinetics of clot retraction *in vitro* (Goschnick *et al.*, 2006; Lau *et al.*, 2004). These changes were attributed to a defect in outside-in signalling of $\alpha IIb\beta 3$ and similar phenotypes were observed in mice that lack functional $\alpha IIb\beta 3$ cytoplasmic signalling domains (Law *et al.*, 1999). mAbs against CD9, CD81, and CD82 all induce homotypic cellular adhesion of pre-B cell and

megakaryocyte cell lines that is likely to be mediated by integrin binding (Lagaudriere-Gesbert *et al.*, 1997a). Heterotypic adhesion also occurs during immunological synapse formation between APC and T cells. The immunological synapse (IS) is a highly organised interface composed of concentric rings enriched in MHC, TCR, co-stimulatory molecules and adhesion molecules (Dustin *et al.*, 1998; Monks *et al.*, 1998). Confocal microscopy studies have demonstrated dynamic redistribution of the tetraspanin CD81 in both T cells and APC during IS formation (Mittelbrunn *et al.*, 2002). The role of CD81 in the IS may be two-fold since the molecular association between CD81 and MHC suggest a direct role in MHC-TCR interactions, whilst CD81 ligation on T cells has also been demonstrated to activate the integrin $\alpha L\beta 2$ (LFA-1) (Todd *et al.*, 1996, VanCompernolle *et al.*, 2001).

Since the mechanics of cellular trafficking are dependent on adhesion and detachment to both the ECM and other cells, it is not surprising that tetraspanins also modulate cell motility. There is abundant data linking tetraspanin expression with cell migration (reviewed in Berditchevski, 2001, Hemler, 2003)). For example, CD9 and CD151 have been investigated in the migration of melanocytes, where these tetraspanins dynamically localise to cellular structures associated with motility, such as dendrites and cell-cell contact sites (Garcia-Lopez *et al.*, 2005). In knockdown models of these tetraspanins, melanocyte motility is significantly enhanced, suggesting a modulatory role for these tetraspanins in cell migration. Surprisingly, tetraspanins appear to regulate intercellular adhesion and motility in two ways. During leucocyte migration and extravasation across endothelial layers, tetraspanin regulation of adhesion molecules other than integrins is also important (Barreiro *et al.*, 2005). Endothelial TEMs co-localise with the integrin ligands ICAM-1 and VCAM-1 and are enriched in leucocyte contact interfaces during leucocyte migration. In addition, modulation of tetraspanin expression and tetraspanin interface blocking result in mildly altered VCAM-1 and ICAM-1 expression and significantly impaired trans-endothelial migration and leucocyte adhesion. The expression of CD81 by both monocytes and B cells has been found to significantly augment $\alpha 4\beta 1$ and $\alpha 5\beta 1$ mediated adhesion VCAM-1 in an outside-in integrin signalling manner (Feigelson *et al.*, 2003). Therefore, tetraspanin proteins may modulate interactions between both integrins and their intercellular ligands.

In summary, tetraspanins participate in integrin function in a variety of contexts including intercellular adhesion, adhesion to extracellular matrices and migration. The major role for tetraspanins in these interactions is likely to be in promoting outside-in signalling.

1.4.3 TETRASPANINS IN SIGNAL TRANSDUCTION

A variety of signalling molecules have been detected in complex with members of the tetraspanin superfamily, including phosphoinositide kinases (PI4-K), activated conventional protein kinase C isoforms (PKCα, PKCβII and PKCγ) and Src family tyrosine kinases (Lck, Hck and Lyn) (Table 1.1). These molecular associations have been demonstrated to have functional relevance in cytoskeletal reorganisation, apoptosis and proliferation. The bulk of evidence regarding the involvement of tetraspanins in signal transduction comes from their relationship with integrins. Tetraspanins are proposed to act as intermediary molecules between integrins and tetraspanin-associated signalling proteins (Figure 1.5). For example, inducible associations have been described between activated PKC isoforms and tetraspanins CD9, CD53, CD81 and CD82 (Zhang *et al.*, 2001). These associations are independent of tetraspanin-integrin complexes and are proposed to link PKC with integrins α3β1 and α6β1, which are constitutively associated with tetraspanins and are PKC signalling dependent. PKC also mediates actin polymerisation via the PKC substrate myristoylated alanine rich C-kinase substrate (MARCKS). Co-localisation of MARCKS with tetraspanins suggests that these interactions may also be linked by inducible PKC association with tetraspanins (Berditchevski and Odintsova, 1999). Ligation of tetraspanin CD151 has been found to preferentially activate the GTPase CDc42 during filopodial extension of adenocarcinoma cells on αCD151 mAb coated substrates (Shigeta *et al.*, 2003). Whilst this signalling is PKC dependent and results in actin polymerisation the detail of this signalling cascade is not yet understood.

Tetraspanins have also been repeatedly linked to Ras mediated pathways that lead to either proliferation or apoptosis. mAbs directed against CD37 inhibit proliferation of both B cells and T cells, while CD81 mAbs have been found to inhibit proliferation of a lymphoma cell line (Ledbetter *et al.*, 1987; Oren *et al.*, 1990; van Spriel *et al.*, 2004). More detailed analyses have determined that CD81 is linked to the ERK/MAPK pathway via PI4-K (Carloni *et al.*, 2004). PI4-K is constitutively associated with a large subset of tetraspanins and functions to generate membrane proximal phosphoinositides, which recruit Shc isoforms and initiate Ras activation and downstream ERK/MAPK (Yauch and Hemler, 2000). CD81 ligation resulted in proliferation

inhibition whilst CD81 over-expression led to increased proliferation (Carloni *et al.*, 2004). In other studies, CD9 ligation inhibited proliferation and induced apoptosis of multiple tumour cell lines through Shc recruitment and subsequent caspase 3 and Ras mediated apoptosis (Murayama *et al.*, 2004). Conversely, CD151 over-expression can inhibit the activation of Ras when fibroblast integrins are dissociated from their ligands (Sawada *et al.*, 2003). Whilst the mechanism of Ras inhibition has yet to be described, this function was localised to the C-terminal cytoplasmic domain of CD151, which contains a PDZ binding motif. It has since been suggested that an intracellular association with an intermediate molecule that possesses a PDZ domain may be responsible (Hemler, 2005). Tetraspanins may also link PI3 kinases to integrins since disruption of $\alpha 3\beta 1$ tetraspanin associations result in increased phosphorylation of PKB/Akt, which is likely to be through PI3-K function (Berditchevski *et al.*, 2002). The Rho activation pathway has been linked to the tetraspanin web through the interaction of CD81 C-terminal domain with ezrin-radixin-moesin (ERM) proteins (Sala-Valdes *et al.*, 2006). ERMs link cytoskeletal actin to cell surface adhesion molecules and are also associated with the Rho activation pathway and a small number of signalling molecules, including PI3-K (Louvet-Vallee, 2000). It has been suggested that ERM-CD81 associations may be functionally relevant in intercellular adhesion (Sala-Valdes *et al.*, 2006).

In addition, tetraspanins regulate cell signalling via their associations with molecules other than integrins. Modulation of epidermal growth factor receptor (EGFR) signalling has been observed in both CD82 and CD9 expressing tumour cell lines, although signal attenuation is performed by modulating cell surface expression of this receptor rather than direct association with signalling proteins (discussed in section 1.4.4). Signalling through the human growth factor (HGF) receptor c-Met is directly associated with tumour invasiveness and growth (Cramer *et al.*, 2005, Dong *et al.*, 2004). In tumour cells, CD82 and c-Met partially co-localise and can be detected in complex through immuno-precipitation of cells lysed with weak detergents (level 3 interactions) (Takahashi *et al.*, 2007). In CD82 expression systems, CD82 has been found to regulate c-Met activity by interfering with the binding of adaptor proteins required for Ras-Rac and Cdc42 signalling pathways (Takahashi *et al.*, 2007). However, signalling was not abrogated in these systems since Akt or MAPK pathways were still responsive to HGF stimulation. These data suggest that the correlation between CD82 down-regulation and tumour metastasis may be due to increased c-Met signalling in the absence of CD82 (Takahashi *et al.*, 2007).

mAbs directed against CD9 and CD151 have been reported to activate platelets in an FcR dependent manner. In these studies, it was demonstrated that CD151 and CD9 directed mAbs co-ligate FcγRIIa resulting in platelet activation (Roberts *et al.*, 1995; Worthington *et al.*, 1990). This interaction has been best studied using CD9 mAbs, which induce signalling via the tyrosine kinase - Syk in a manner distinct from the effects of signalling via mAb induced FcR clustering (Ozaki *et al.*, 2000; Ozaki *et al.*, 1995). In later studies using FcR blocking antibodies, it was demonstrated that both CD151 and CD9 also function in an FcR independent manner (Thai le *et al.*, 2003; Wu *et al.*, 2000). Whilst the likelihood of tetraspanin ligand binding is questionable, it has been proposed that these assays reflect a physical relationship between tetraspanins and FcR at the platelet surface (Moseley, 2005). If such a relationship occurs, these interactions are most likely to be indirect, since co-precipitation of FcR and tetraspanins have not been demonstrated in platelets (Moseley, 2005; Thai le *et al.*, 2003).

1.4.4 Tetraspanins and intracellular protein trafficking

Recent studies have identified an emerging role for tetraspanins in regulating cellular events through intracellular protein trafficking (Berditchevski and Odintsova, 2007). One way that tetraspanins have been found to regulate adhesion and motility, is through integrin trafficking between the cell surface and subcellular compartments. As discussed earlier, many tetraspanins possess endocytic localisation/sorting motifs in the C-terminal cytoplasmic tail (Berditchevski and Odintsova, 2007). CD151 is proposed to exert regulatory effects upon its associated integrins by regulating their cell surface expression via endocytosis. In CD151 gene silencing studies, α3β1 internalisation and motility on laminin-5 coated surfaces was impaired in carcinoma cells treated with CD151 siRNA (Winterwood *et al.*, 2006). A second study attributed this regulatory role to the endosomal targeting motif in the CD151 C-terminal cytoplasmic domain (Liu *et al.*, 2007). During integrin internalisation these molecules accumulate in CD151 positive intracellular compartments. Mutation of the CD151 sorting motif YRSL to ARSA resulted in impaired endocytosis of both CD151 and its associated integrins α3β1, α5β1 and α6β1 (Liu *et al.*, 2007). Likewise, over-expression of wild type CD151 promotes cell motility on both laminin-1 and fibronectin, whereas transfection of the mutated CD151 abrogated these effects (Liu *et al.*, 2007). The tetraspanin CD82 has also been found to regulate cell surface expression of integrins α6β1 and α6β4 (He *et al.*, 2005). Through both over-expression studies and siRNA knockdown of CD82, it was demonstrated that CD82 expression led to increased

internalisation of α6 and subsequently inhibited integrin mediated adhesion of epithelial cells to laminin-1 substrates (He *et al.*, 2005).

Tetraspanins are also known to play a role in the endocytosis of other cell surface molecules such as the C-type lectin Dectin-1 and the epidermal growth factor receptor (EGF-R). Through immuno-precipitation, both CD37 and CD63 can be detected in complex with Dectin-1 in relatively weak detergents (Meyer-Wentrup *et al.*, 2007, Mantegazza *et al.*, 2004). Dectin-1 is a yeast carbohydrate receptor that when activated, results in pro-inflammatory cytokine production by APC (Willment *et al.*, 2005). During yeast cell phagocytosis assays, both Dectin-1 and CD63 are internalised from the DC surface into intracellular compartments (Mantegazza *et al.*, 2004). This effect appears to be functionally linked to CD63 association with Dectin-1, since CD63 internalisation does not occur in similar experiments using particles whose uptake is independent of Dectin-1, such as latex beads (Mantegazza *et al.*, 2004). Dectin-1 expression, cytokine production and phagocytic capacity were investigated in CD37 deficient macrophages (Meyer-Wentrup *et al.*, 2007). Increased endocytosis of Dectin-1 was observed in activated $CD37^{-/-}$ macrophages resulting in lower overall cell surface expression of Dectin-1. Whilst phagocytosis of yeast derived particles was unaltered in the absence of CD37, IL-6 production in response to Dectin-1 binding was strikingly elevated (Meyer-Wentrup *et al.*, 2007). This increase in pro-inflammatory cytokine production could be blocked by Dectin-1 mAbs and was therefore specific to Dectin-1 activation. These data suggest that CD37 both negatively regulates Dectin-1 function and stabilises Dectin-1 on the cell surface of macrophages (Meyer-Wentrup *et al.*, 2007).

The tetraspanins CD9 and CD82 have both been proposed to play a functional role in internalisation of the EGF receptor, thereby attenuating signalling through EGF-EGFR. Decreases in expression of both CD82 and CD9 are poor prognostic markers in some cancers and coincide with increased tumour invasion capacity and growth (Huang *et al.*, 1998; Miyake *et al.*, 1996; Uchida *et al.*, 1999). It has been recently demonstrated in a gastrointestinal cancer cell line that CD9, EGFR and β1 integrins are all molecularly associated (Murayama *et al.*, 2008). Furthermore, cell surface CD9 expression levels were inversely correlated with EGFR expression in EGF treated cells, since over-expression of CD9 led to decreased internalisation of EFGR and CD9 siRNA knockdown resulted in increased cell surface EGFR (Murayama *et al.*, 2008). Similar findings have been described for CD82 internalisation of the EGFR upon EGF

stimulation of epithelial cells (Odintsova *et al.*, 2000). EGFR also molecularly associates with CD82 in similar level 3 interactions as that described for CD9 and β1 (Odintsova *et al.*, 2000, Murayama *et al.*, 2008). CD82 contains a C-terminal tyrosine-based sorting motif proposed to couple CD82 to adaptor protein molecules and allow clatherin targeting and endocytosis of CD82/EGFR complexes (Berditchevski and Odintsova, 2007). Interestingly, CD9 does not contain this sorting motif, or a dileucine motif proposed to have a similar function (Rapoport *et al.*, 1998). Mutation studies have demonstrated that CD82 mediated EGFR internalisation is dependent upon the tyrosine based motif (Berditchevski and Odintsova, 2007), however the mechanism of CD9 internalisation remains unknown. Perhaps CD9 modulation of EGFR in gastrointestinal carcinomas is due to interactions between CD9 and CD82.

In contrast to the examples described above, CD81 regulates cell surface expression of CD19 in B cells through trafficking *to* the cell membrane and not through internalisation. B cells deficient in CD81 expression display reduced levels of cell surface CD19 (Maecker and Levy, 1997; Miyazaki *et al.*, 1997). This defect has been attributed to prolonged association of CD19 with the ER before passing to the Golgi (Shoham *et al.*, 2003). Mutagenesis studies have since demonstrated that the first transmembrane region of CD81 is sufficient for CD19 trafficking to the B cell surface (Shoham *et al.*, 2006). A similar, but distinct role in trafficking from the ER has been identified for the bladder epithelial restricted tetraspanins UPIa and UPIb. These tetraspanins form ordered structures at the cell surface with their partner molecules UPIIb and UPIII (Min *et al.*, 2006; Wu *et al.*, 1995). Transfection studies have demonstrated that of these four proteins, only UPIb can be expressed at the cell surface without its partner molecule (Tu *et al.*, 2002). It has been proposed that the heterodimerisation of these proteins results in a necessary conformational change that enables translocation from the ER.

1.4.5 Lessons from targeted deletion of tetraspanins in mice

To date, there have been 33 tetraspanins identified in humans, of which, at least 20 are expressed in immune cells (Huang *et al.*, 2005; Tarrant *et al.*, 2003). Mouse orthologs have been identified for all but two human tetraspanins (Tspan19 and Tspan16) and of those that are shared, there have been no significant differences in tissue distribution observed between mice and humans. Therefore mice are thought to be suitable models to study the function of tetraspanins in the immune system. Knockout mouse models of specific tetraspanins have proved to be very informative for understanding protein function. By phenotyping these mice in

comparison to wild type mice of a matching genetic background, we can elucidate the various cellular processes a protein may be involved in and therefore come closer to understanding the function of a specific molecule. Of the tetraspanins expressed in the blood, there have been seven knockout mouse lines generated, five of which contain a targeted deletion of a single tetraspanin, while two others display deficient expression of two tetraspanins.

1.4.5.1 CD81$^{-/-}$ mice

Three laboratories separately developed and described the first tetraspanin knockout mouse, deficient in the expression of CD81. The CD81 knockout mice were reported to be developmentally normal, fertile, displayed no sign of histological abnormality and normal survival (Maecker and Levy, 1997; Miyazaki *et al.*, 1997; Tsitsikov *et al.*, 1997). Later it was shown that CD81 knockout mice have a striking defect in brain development resulting in phenotypically enlarged brains (Geisert *et al.*, 2002). However, since the severity of the phenotype was dependent upon the genetic background, there are likely to be other proteins also involved in this pathology. Although CD81 is widely expressed in a variety of tissues, studies with mAbs have implied a role for CD81 in T cell activation and co-stimulation of the BCR. Therefore immune function in CD81$^{-/-}$ mice was also investigated. In the absence of CD81 it was shown that while T cell development was normal, there was a marked decrease in B cell CD19 expression indicating a role for CD81 in CD19 stabilisation or trafficking to the cell membrane (Tsitsikov *et al.*, 1997). CD81, CD19, Leu 13 and CD21 together form the B cell co-receptor complex, which increases B cell sensitivity to antigen when cross-linked to the BCR (Carter and Fearon, 1992). CD81$^{-/-}$ B cells were deficient in antibody responses to T cell dependent antigens, displayed increased antibody responses to T independent antigens and increased basal IgM Ab serum levels (Maecker and Levy, 1997; Miyazaki *et al.*, 1997; Tsitsikov *et al.*, 1997). Like other knockout mice deficient in members of the B cell activation pathway (including CD19), the CD81$^{-/-}$ mice displayed a dramatic reduction in the B cell subset B1a, whose maturation is dependent upon B cell activation (Engel *et al.*, 1995; Tsitsikov *et al.*, 1997). Conflicting results were seen in B cell proliferation, both hyper-proliferation and hypo-proliferation of B cells was observed in response to αCD40 and IgM-BCR cross-linking respectively, by one group (Miyazaki *et al.*, 1997), and normal B cell responses seen by another (Maecker *et al.*, 2000). It is likely that any B cell defects are due at least in part to low levels of B cell CD19 surface expression. One possible explanation for the discrepancies observed between CD81 deficient B cell proliferation, may be differing genetic backgrounds between the

mice used in these studies. If so, this may also indicate the involvement of other proteins in B cell proliferative responses. In line with a role for CD81 in T cell activation, $CD81^{-/-}$ T cells showed hyper-proliferative responses to stimulation with the T cell mitogen conA, TCR engagement with αCD3 and TCR engagement in the presence of co-stimulation using αCD3 and αCD28 mAbs (Miyazaki *et al.*, 1997).

1.4.5.2 $CD9^{-/-}$ mice

The $CD9^{-/-}$ mouse strain displays a defect in sperm-egg fusion and therefore significantly reduced female fertility. As CD9 and other tetraspanins have been shown to associate with various integrins, it was proposed that a fertility defect might be due to an ensuing defect in integrin function in $CD9^{-/-}$ knockout mice. Specifically, this deficiency was attributed to α6β1, an integrin demonstrated to bind sperm fertilin and also potentially modulated by CD9 (Le Naour *et al.*, 2000). However, it was later shown that the absence α6β1 did not lead to a reduction in fertilisation efficiency (He *et al.*, 2003). Like CD81, CD9 has a wide tissue distribution although immune function of $CD9^{-/-}$ mice has only recently been reported. $CD9^{-/-}$ mouse B cells are developmentally and functionally normal. All populations were present, antibody responses to T-dependent and T-independent antigens were normal and the frequency of antibody forming cells was also unchanged in $CD9^{-/-}$ mice (Cariappa *et al.*, 2005). Pathogen resistance was demonstrated to be poor in response to hepatic infection of the bacterial pathogen *P. acnes*. Defective $CD9^{-/-}$ granuloma formation in response to the intracellular infection was attributed to altered cytokine responses (IFNγ & TNFα) (Yamane *et al.*, 2005). Recently it has been reported that cell surface expression of CD9 and its associated integrins α4 and α6 are down-regulated by peritoneal B1 cells in response to TLR activation (Ha *et al.*, 2006). This co-ordinated down-regulation was suggested to reduce the adhesive qualities of this B cell subset to allow egress of peritoneal B cells and promote B cell responses to infection. Moreover, $CD9^{-/-}$ B1 cells display an increased migration capacity *in vivo* during competitive migration assays in B cell deficient ($RAG2^{-/-}$) mice.

1.4.5.3 $CD37^{-/-}$ mice

CD37 has a leucocyte restricted pattern of expression and $CD37^{-/-}$ defective phenotypes have so far only been identified in the immune system. These mice have normal rates of fertility, survival is unaffected and tissue development and histology is normal. However, immune function in $CD37^{-/-}$ mice is altered in T cells, B cells and APC. Importantly, CD19 expression is

normal in $CD37^{-/-}$ B cells although antibody production was shown to be enhanced in response to T cell-independent antigens, and deficient in reaction to T cell-dependent antigens. Unlike $CD81^{-/-}$ mice, $CD37^{-/-}$ basal serum IgM antibody levels are normal and IgG_1 levels were reduced in non-immunised mice (Knobeloch *et al.*, 2000). Unfortunately, differing immunological backgrounds and ages make these results difficult to compare with other tetraspanin deficient strains. In recent studies, B cell responses were re-examined in $CD37^{-/-}$ mice backcrossed over 10 times onto a C57Bl/6 background. Again T cell independent responses were normal and T cell dependent responses altered. IgG_1 production was significantly impaired and IgA production significantly elevated, in both naïve and NP-KLH immunised mice (van Spriel *et al.*, submitted for publication).

T cell function is also affected in $CD37^{-/-}$ mice, which display hyper-proliferative T cell responses to conA, αCD3 and αCD3 + αCD28 stimulation. This enhanced reaction is due to early elevated production of the proliferative cytokine IL-2 and is most prominent in the absence of co-stimulation (van Spriel *et al.*, 2004). TCR expression in $CD37^{-/-}$ T cells is normal and the rate of cellular division is also comparable to wild type mice, however increased activity of the TCR signalling molecule Lck was induced by a lack of CD37 expression. A role for CD37 in sequestering molecules involved in the T cell activation pathway was proposed to explain both altered signalling Lck dynamics and enhanced T cell responses in the absence of CD37 (van Spriel *et al.*, 2004). DC function *in vitro* is also enhanced in the absence of CD37, since peptide pulsed $CD37^{-/-}$ DC are hyper-stimulatory to antigen specific T cells in comparison to wild type controls (Sheng *et al.*, submitted for publication). Recently, the C-type lectin Dectin-1 was found to be molecularly associated with CD37 (Meyer-Wentrup *et al.*, 2007). Furthermore, cell surface expression of Dectin-1 was found to be poorly upregulated by activated $CD37^{-/-}$ macrophages and cell surface stabilisation of Dectin-1 was also dependent upon CD37 (Meyer-Wentrup *et al.*, 2007). CD37 also regulated signal transduction through Dectin-1 as production of the Th2 cytokine IL-6 was elevated in $CD37^{-/-}$ macrophages (Meyer-Wentrup *et al.*, 2007).

1.4.5.4 $CD151^{-/-}$ mice

Like CD81 and CD9, CD151 has a broad tissue expression. CD151 molecular interactions have been studied to a greater extent than most tetraspanins. As mentioned earlier, a number of integrins have been shown to associate with CD151 in strong level 1 interactions such as α3β1, α5β1, α6β1 and α6β4 (Fitter *et al.*, 1998; Serru *et al.*, 1999; Sincock *et al.*, 1999; Yauch *et al.*,

1998). Since targeted deletions of integrins in mice such as α3, α6 and β4 result in lethal disruptions to kidney, lung and skin, it was expected that CD151$^{-/-}$ mice may display similar phenotypes (Georges-Labouesse *et al.*, 1996; Kreidberg *et al.*, 1996; van der Neut *et al.*, 1996). Two of three incarnations of CD151$^{-/-}$ mice are healthy, fertile and developmentally normal (Takeda *et al.*, 2007; Wright *et al.*, 2004a). Surprisingly, tissue architecture was normal in the absence of CD151, in contrast with mice deficient in the integrins CD151 is known to interact with. Integrin levels were also normal in the tissues analysed, which was unexpected since CD151 had been previously proposed to stabilise integrin expression at the cell surface (Sincock *et al.*, 1999; Yauch and Hemler, 2000). It was suggested that the presence of the closely related tetraspanin Bab22924 (Tspan11) may play a surrogate role in the absence of CD151 (Wright *et al.*, 2004b). Mild abnormalities in haemostasis, angiogenesis and tissue migration were later identified in these mice, which were attributed to altered function of the integrins αIIbβ3, α6 and β4 (Cowin *et al.*, 2006; Lau *et al.*, 2004; Takeda *et al.*, 2007). Immunologically, T cell and B cell development is normal and B cell antibody responses to T cell-dependent antigens and B cell proliferation were reported as normal. T cell proliferation was enhanced in response to conA, TCR cross-linking via mAbs, in both the presence and absence of co-stimulatory signals (Wright *et al.*, 2004a). As seen in CD37$^{-/-}$ DC, CD151$^{-/-}$ DC are hyper-stimulatory to antigen specific T cells during antigen presentation assays *in vitro* (Sheng *et al.*, submitted for publication). The third independently generated CD151$^{-/-}$ mouse line displays a progressively lethal defect in kidney function, which is similar to that observed in human patients with CD151 deficiency (Sachs *et al.*, 2006). Kidney failure in these mice is due to disorganisation of the glomerular basement membrane, tubular cystic dilation and focal glomerulosclerosis. A similar phenotype was observed in conditional α3 deficient mice that lacked α3 expression in renal podocytes (Sachs *et al.*, 2006). Therefore, altered integrin function in the absence of CD151 was proposed as the underlying mechanism of disease in CD151$^{-/-}$ mice. The differing genetic backgrounds between the previously reported CD151$^{-/-}$ mice (C57Bl/6) and those that suffer renal failure (FVB) suggest that other genetic factors must also be at play in this pathology (Sachs *et al.*, 2006; Takeda *et al.*, 2007; Wright *et al.*, 2004a).

1.4.5.5 Tssc6$^{-/-}$ mice

Tssc6 is a cell lineage specific tetraspanin also known as PHEM-X (pan-haematopoietic cellular expression marker). A relatively divergent member of the tetraspanin superfamily, the Tssc6 protein has an elongated cytoplasmic C terminal tail, approximately six times the length of

traditional tetraspanin superfamily members (Robb *et al.*, 2001). Tssc6$^{-/-}$ mouse fertility, histology and development are normal, although like other tetraspanin knockout mice, T cell function is altered. Haematopoiesis is normal and cells were shown to be present in normal numbers, although as observed in CD151$^{-/-}$ mice, mild defects were observed in haemostasis that were attributed to impaired platelet integrin function (Goschnick *et al.*, 2006). Tssc6$^{-/-}$ mice displayed defective platelet aggregation responses, impaired platelet spreading and delayed kinetics of clot retraction *in vitro*. B cell responses were tested via immunisation with T cell-dependent and independent antigens and found to be comparable with wild type mice. T cell proliferation was shown to be defective in response to mitogenic stimulus and TCR activation. Once again, hyper-proliferation was most striking in the absence of αCD28. Like CD37$^{-/-}$, Tssc6$^{-/-}$ T cell function was attributed to early production of IL-2, which could be normalised by the addition of IL-2 to stimulated wild type cultures. In contrast to CD37$^{-/-}$ T cells, it was shown that only CD8^{+} T cells were hyper-proliferative in the presence of co-stimulation. In order to determine the role of the TCR in the altered T cell phenotype, PMA and ionomycin were used to circumvent the TCR and stimulate T cells. This resulted in no difference in proliferation between wild type and knockout mice, indicating a critical role for the TCR in the tetraspanin knockout phenotype (Tarrant *et al.*, 2002). Antigen presentation is yet to be investigated in Tssc6 deficient mice.

1.4.5.6 Tspan33$^{-/-}$ mice

The most recent tetraspanin deficient mouse to be described is the Tspan33 knockout. Tspan33, also known as Penumbra, is expressed in both mouse and human haematopoietic cells and was identified through representational difference analysis (RDA) between haematopoietic and myeloid cell lines (Chen *et al.*, 2005; Heikens *et al.*, 2007). At the transcript level, Tspan33 is highly expressed by TER119^{+} erythroblasts and appears restricted to erythroid cells. When labelled fusion constructs were expressed in the pro B cell line BaF3, Tspan33 localised to the cell surface and vesicular membranes. Whilst haematopoietic development is unaffected, in the absence of Tspan33 30% of outbred mice develop anaemia and splenomegaly due to reduced frequencies of RBCs and increased frequencies of basophilic macrocytes and erythroblasts (Heikens *et al.*, 2007). However, the limited penetration of this phenotype suggests that there may be other factors at play, such as partial redundancy between other tetraspanins or hormonal differences between individuals. In the same study, a multipotent haematopoietic cell line derived from Tspan33$^{-/-}$ mice displayed an impaired

capacity to produce erythroid progenitors in comparison to wild type controls (Heikens *et al.*, 2007). This phenotype was rescued by the reintroduction of Tspan33 expression, suggesting that Tspan33 plays a critical role in erythropoiesis. Interestingly, differentiation of other cells such as neutrophils, monocytes and megakaryocytes was unaffected and perhaps it can therefore be presumed that Tspan33 is not required for general myeloid development.

1.4.6 LESSONS FROM TETRASPANINS AND DISEASE

1.4.6.1 Cancer

Tetraspanins have been implicated in a broad range of diseases including cancer, viral infection and autoimmunity. Tumour metastasis, a process that is dependent upon cell migration and motility has been strongly linked to tetraspanin expression. A reduction in cell surface CD9, CD63, and CD82 have each been correlated with an increased likelihood of metastatic tumour development in a number of carcinomas (Dong *et al.*, 1995; Hotta *et al.*, 1988; Miyake *et al.*, 1991). Transfection studies have also shown that the over expression of CD9, CD63 and CD82 can reduce metastatic potential (Dong *et al.*, 1995; Ikeyama *et al.*, 1993; Radford *et al.*, 1995). Alternatively, as with CD151, it has also been shown in some cancers that increased expression of tetraspanins such as CD151 correlates with metastatic potential (Tokohara *et al.*, 2001). Therefore tetraspanins can serve as either positive or negative regulators of tumour metastasis.

In the literature there is a plethora of clinical studies that report altered tetraspanin expression associated with tumour development, however the vast majority of these studies have been descriptive rather than functional (Wright *et al.*, 2004b, Hemler *et al.*, 1996, Boucheix *et al.*, 2001). However, an increased focus on the mechanisms behind these correlations have revealed that tetraspanins can modulate a variety of cell surface proteins involved in proliferation, adhesion, migration and angiogenesis. As discussed earlier, many of these functional associations are linked with the capacity of tetraspanins to modulate integrin function (Hemler *et al.*, 1996, Berditchevski, 2001). For example, CD151 expression is elevated in approximately one third of breast tumours and can promote breast cancer growth by modulation of the integrin $\alpha 6$ (Yang *et al.*, 2008). *In vitro*, CD151 deficient mammary tumour cells displayed impaired cell migration, invasion, spreading and reduced signalling. CD151 disruption in these cells blocked the associated of $\alpha 6\beta 4$ integrin with tetraspanin microdomains and EGFR-integrin association, resulting in delayed tumour growth in breast cancer models (Yang *et al.*, 2008). A role for tetraspanin integrin complexes in metalloproteinase production

may also be linked to these findings. Metalloproteinases are known to enhance tumour progression through degradation of basement matrices and increase tumour invasiveness (Coussens and Werb, 1996). Multiple mAbs directed against tetraspanin-integrin complexes have been found to promote the production of the metalloproteinase MMP-2 in a PI3-K dependent manner (Sugiura and Berditchevski, 1999b). This effect was specific to the tetraspanin-integrin complex since mAbs directed against other integrin associated proteins had no effect on MMP2 production. These findings suggest that reduced invasiveness of CD151 deficient tumours may be due to altered MMP2 mediated ECM proteolysis (Sugiura and Berditchevski, 1999b).

Tetraspanins are also implicated in tumour growth through their ability to regulate the function of growth factor receptors, such as the EGF receptor and the HGF receptor c-met (Murayama *et al.*, 2008; Odintsova *et al.*, 2000; Takahashi *et al.*, 2007). In these examples, tetraspanins regulate proliferative signal recognition by either down-modulating the expression of these receptors or disrupting the association of signalling molecules with the receptor. A role for tetraspanins has also been identified in regulating angiogenesis, an important requirement for tumour vascularization. In rats, the tetraspanin CO-029 has been identified as a strong promoter of angiogenesis through systemic upregulation of angiogenesis factors (Gesierich *et al.*, 2006). Ectopic expression of CO-029 by implanted tumour cells led to marked increases in vessel density in surrounding areas and was inhibited by CO-029 ligation with mAbs. Exosomes derived from these cells contained a number of tetraspanins including CO-029 and treatment of fibroblasts and endothelial cells with these exosomes was sufficient to up-regulate expression of vascular endothelial growth factor (VEGF), VEGF receptor, matrix metalloproteinases and urokinase-type plasminogen activators (Gesierich *et al.*, 2006). This role for CO-029 in angiogenesis correlates with findings in humans where CO-029 is a poor prognosis factor in gastro-intestinal and other cancers (Kanetaka *et al.*, 2003). A role for tetraspanin microdomains in angiogenesis is also supported by $CD151^{-/-}$ phenotyping (Takeda *et al.*, 2007). Whilst developmental angiogenesis was unaffected in CD151 deficient mice, tumour dependent angiogenesis was impaired. Both aortic valve ring outgrowth *in vitro* (correlated with tumour angiogenesis) and solid tumour formation by implanted lung carcinomas *in vivo* were reduced in the absence of CD151 (Takeda *et al.*, 2007).

From these data it is clear that tetraspanins can participate in tumour growth and metastasis through a variety of mechanisms. Whilst the mechanisms behind altered tetraspanin expression in these systems are rarely defined, tetraspanin down-regulation is proposed to be due to altered methylation of transcription factors controlling gene expression - a feature common in tumour cells (Drucker *et al.*, 2005).

1.4.6.2 Infection

Tetraspanins are involved in a range of pathogenic processes including pathogen entry, intracellular trafficking, secretion, syncytia formation and immune modulation (summarised in Table 1.3). The most extensively studied associations between infection and tetraspanins has been with CD81 and HCV, as well as a number of tetraspanins and HIV. Notably, the large extracellular loop of CD81 has been identified as the binding site for the HCV envelope glycoprotein E2 (Pileri *et al.*, 1998). Mapping of this interaction has identified a small number of residues in close proximity to conserved cysteine residues in the large extracellular loop (Dhanasekaran *et al.*, 2003; Higginbottom *et al.*, 2000, Drummer *et al.*, 2002). Whilst HCV infectivity is not dependent upon CD81 expression (Petracca *et al.*, 2000; Sasaki *et al.*, 2003), it has been suggested that the cross-linking of CD81 by HCV may be related to the pathogenesis of HCV infection. *In vitro* cross-linking of CD81 using purified E2 protein results in altered NK cell, T cell and B cell responses that may facilitate HCV immune evasion (Crotta *et al.*, 2002; Flint *et al.*, 1999; Nattermann *et al.*, 2004). More recently, it has been found that CD81 targeted antibodies can have a neutralising effect on *in vitro* HCV infection of human hepatocytes (Molina *et al.*, 2008) and in mice capable of HCV infection, CD81 mAbs are protective against viral challenge (Meuleman *et al.*, 2008).

A role for tetraspanins has also been identified in HIV-1 infection. Unlike HCV, multiple tetraspanins have been implicated, although CD63 has been most prominent in these findings. Increasing reports suggest that HIV-1 assembly occurs in tetraspanin rich intracellular compartments in both T cells and dendritic cells positive for expression of CD9, CD53, CD63 CD81 and CD82 (Garcia *et al.*, 2005, Ruiz-Mateos *et al.*, 2008). In DC, these compartments are likely to be MIIC compartments where peptide loading occurs in APC (Raposo *et al.*, 2002). DC play an important role in the cell-cell spread of HIV by incorporating the virus and passing on infection to T cells through synapse formation. Whilst there are conflicting reports of whether HIV-1 proteins such as Gag and HIV envelope glycoproteins associate with cell surface TEMs,

the bulk of studies suggest that this is the case in both myeloid and lymphoid lineages. It has been suggested that these discrepancies may be due to differences in cell types used and the expression of HIV-1 viral proteins such as Gag in the absence of others such as Env and Nef. During budding, tetraspanins are incorporated into the viral envelope, where CD63, CD81 and CD9 have all been detected on the surface of HIV-1 virions (Khurana *et al.*, 2007, Nydegger *et al.*, 2006, Jolly and Sattentau, 2007). By targeting these tetraspanins during HIV-1 infection it has been found that CD63 mAbs, but not CD9 or CD81 mAbs, can block the entry of HIV into macrophages (an important reservoir of HIV-1 virions) and CD9 mAbs inhibit HIV particle release by HIV-1 producing HeLa cells (Khurana *et al.*, 2007). However, ablation of CD63 expression did not impair HIV-1 infectivity of macrophages, suggesting that the physical effects of CD63 ligation are important in the previous studies (Ruiz-Mateos *et al.*, 2008).

Both CD81 and CD82 also participate in syncytia formation following infection with HTLV-1 (Imai and Yoshie, 1993). Similar to the observation in HIV-1, the HTLV-1 viral protein Gag is localised within TEMs at the surface of infected cells (Mazurov *et al.*, 2006; Pique *et al.*, 2000). This association has been isolated to the intracytoplasmic loops and membrane proximal cysteines of CD81 and CD82 and therefore it is likely that HTLV-Gag localisation within tetraspanin microdomains is palmitoylation dependent (Mazurov *et al.*, 2007). Other viruses are also thought to utilise tetraspanins in cell-cell spread such as feline immunodeficiency virus (FIV) and canine distemper virus (CDV), in which CD9 ligation or expression can alter infectivity (de Parseval *et al.*, 1997, Willett *et al.*, 1997, Loffler *et al.*, 1997). Intriguingly, it has also been shown that mice deficient in the expression of CD81 display resistance to infection by the malarial parasite strain *P.yoelii*, but are not resistant to other rodent strains of malaria (Silvie *et al.*, 2003). Contrary to this finding, $CD151^{-/-}$ and $CD37^{-/-}Tssc6^{-/-}$ mice may actually be more susceptible to non-lethal *P.yoelii* (unpublished data from G. Minigo). Other tetraspanin-pathogen interactions are outlined below.

Table 1.3 Tetraspanins and infection.

HTLV-1, Human T-Lymphotropic virus-1; FIV, Feline immunodeficiency virus; CDV, Canine distemper virus; HCV, Hepatitis C virus; HIV-1, Human immunodeficiency virus -1; PRRSV, Porcine reproductive and respiratory syndrome virus; TSE, Transmissible spongiform encephalopathy.

Tetraspanin	Pathogen	Observation	References
Infectivity/Syncytia Formation			
CD82	HTLV-1	mAb treatment blocks syncytia formation in HTLV infected cells.	(Imai and Yoshie, 1993, Fukudome et al., 1992)
CD9	FIV	CD9 mAbs inhibit viral replication in culture, while ectopic CD9 expression confers susceptibility.	(Willett et al., 1997, de Parseval et al., 1997)
CD9	CDV	CD9 mAbs inhibit viral infection in culture, whilst over-expression of CD9 increases viral production.	(Loffler et al., 1997)
Attachment/Pathogen entry			
CD81	Plasmodium	CD81 expression is required for sporozoite infection of murine hepatocytes.	(Silvie et al., 2003)03
CD81	HCV	CD81 EC2 acts as a co-receptor for HCV infection and interacts with the E2 envelope glycoprotein.	(Pileri et al., 1998, Takikawa et al., 2000)
CD63	HIV-1	CD63 mAbs can block HIV viral entry into macrophages.	(von Lindern et al., 2003)
UPIa	E. coli	UPIa identified as urinary tract receptor for an invasive E. coli strain.	(Zhou et al., 2001, Wu et al., 1996)
CD9	C. Diphtheriae	With HB-EGF acts as co-receptor for C. diphtheriae toxin entry into the cell.	(Cha et al., 2000; Collier, 2001)
CD151	PRRSV	CD151 expression is required for infection, ectopic expression can confer susceptibility and CD151 mAbs block infection.	(Shanmukhappa et al., 2007)
Secretion/Trafficking			
CD9, CD81	HIV-1	Involved in trafficking of virus to APC-T cell contact sites during infectious synapse formation.	(Nydegger et al., 2006, Wiley and Gummuluru, 2006, Garcia et al., 2005)
CD9, CD81	HIV-1	Secretion of HIV-1 via tetraspanin rich exosomes.	(Masciopinto et al., 2001)
CD9, CD63, CD81, CD82, CD231	HIV-1	Incorporation of tetraspanins into HIV viral particles	(Jolly and Sattentau, 2007, Sato et al., 2008)
CD63	C. Trachomatis	Required for intracellular trafficking of bacteria in multivesicular bodies.	(Beatty, 2006, 2008)
Immunomodulation			
CD53	Multiple	CD53 deficiency results in increased susceptibility to infection.	(Mollinedo et al., 1997)
CD81	HCV	HCV E2 protein can modulate T cell, NK cell and B cell function.. Infected patients develop cryoglobulinemia	(Crotta et al., 2002; Flint et al., 1999; Tseng and Klimpel, 2002, Pileri et al., 1998, Wack et al., 2001, Soldaini et al., 2003)
CD9	TSE	CD9 expression upregulated by infected astrocytes, auto-reactive CD9 antibodies produced.	(Shimizu et al., 2002, Doh-ura et al., 2000)

1.4.6.3 Genetic disease

A limited number of defects in tetraspanin function have been identified as the cause of genetic disease, confirming the functional roles of tetraspanins in these biological systems. As discussed earlier in mice, the genetic background was a critical factor in CD151 deficiency induced renal pathology. In humans, a truncation inducing frame shift mutation in the CD151 gene has been reported in a small group of patients suffering kidney failure (Karamatic Crew *et al.*, 2004). This defect resulted in altered glomerular basement membrane structure, epidermal disruptions, hearing loss and β thalassemia minor (Kagan *et al.*, 1988). Much of these symptoms were attributed to the disruption of functional interactions between CD151 and the integrins α3β1 and α6β4 (Karamatic Crew *et al.*, 2004). The truncated nature of the CD151 defect in these patients may explain the differences between the pathology observed in these patients in comparison to the normal kidney function in two strains of $CD151^{-/-}$ mice. Whilst the mutated protein was not predicted to reach the cell surface, perhaps this may alter the localisation of integrins such as α3β1 and α6β1 normally associated with CD151 (Karamatic Crew *et al.*, 2004). In addition, the less severe phenotypes observed in mice, may suggest the presence of a protein that can perform a compensatory role for the absence of CD151, e.g. another tetraspanin.

Altered expression of the tetraspanin CD231 in humans is associated with non-syndromic X-linked mental retardation (MRX) (Zemni *et al.*, 2000). Individuals with chromosomal translocations, missense mutations and truncation of the CD231 coding region suffer mild neurological defects (Abidi *et al.*, 2002; Maranduba *et al.*, 2004; Zemni *et al.*, 2000). Whilst there has been some debate over the causative nature of one missense mutation of CD231, this change has since been identified in an MRX patient unrelated to those in the original study (Gomot *et al.*, 2002; Maranduba *et al.*, 2004). The pathogenesis of CD231 mutations is not yet understood although CD231 expression in neural tissues is high (Zemni *et al.*, 2000). Previous links between tetraspanins and integrin function were suggested as potential factors in altered brain development, although CD231 itself has not yet been directly identified in complex with integrins. Since CD231 is broadly expressed in the body including the immune system (Takagi *et al.*, 1995), the non syndromal nature of CD231 pathology may suggest that in other tissues, functional redundancy between tetraspanins occurs. A phenotypic analysis of mice deficient in CD231 expression would be a particularly informative method of further investigating the function of CD231.

CD63 expression in platelets derived from Hermansky-Pudlak syndrome patients is significantly reduced during platelet activation (Nishibori *et al.*, 1993). These patients suffer impaired function of secretory cells, including poor platelet function and bleeding defects (Salles *et al.*, 2008). However, CD63 deficiency is not the cause of this syndrome, since CD63 gene expression is normal (Armstrong *et al.*, 1998). The genes responsible for these effects are varied, but all relate to organelle biosynthesis or protein transport from the golgi network to the endosomal/lysosomal pathway (Wei, 2006). Therefore, many other proteins are also affected and it is not yet understood what part impaired CD63 expression plays in the pathogenesis of this disease.

In mice as well as humans, mutation of the retinal tetraspanins peripherin 2 (Tspan22) and Rom-1 (Tspan23) results in retinal degeneration and subsequent blindness. These two tetraspanins are expressed in rod and cone photoreceptors and form homodimers as well as homo and heterotetrameric complexes in these cells (Goldberg *et al.*, 1995). Patients affected by impaired peripherin 2/rom function display autosomal-dominant retinitis and macular dystrophies (Pierce, 2001). An autosomal dominant pattern is also observed in peripherin 2 deficient mice ($Rds^{-/-}$). $Rds^{-/-}$ mice fail to develop photoreceptor outer segments, whilst $Rds^{-/+}$ mice display disorganised photoreceptor outer segments (Sanyal and Jansen, 1981; Travis *et al.*, 1989). In mice, Rom-1 appears to play a less critical role in photoreceptor function, as $Rom^{-/-}$ photoreceptor outer segments are only transiently altered in young mice (Clarke *et al.*, 2000). However, both $Rds^{-/-}$ and $Rom\text{-}1^{-/-}$ photoreceptors undergo premature apoptosis leading to retinal degeneration, which correlate well with human findings (Clarke *et al.*, 2000; Sanyal and Jansen, 1981).

1.4.6.4 Autoimmunity

Autoimmunity remains a relatively unstudied area in tetraspanin biology. In tetraspanin deficient mice there has been little reported on the development of auto-immunity. The hyper-proliferative *in vitro* phenotypes described in these mice suggest an increased potential for T cell mediated auto-immunity. Previous *in vitro* studies in humans found that the tetraspanin CD9 is differentially expressed on a naïve $CD4^+$ T cell subset associated with T cell auto-reactivity (Kobayashi *et al.*, 2004). Furthermore, proliferative responses to auto-antigen by this population can be abrogated by anti-CD9 mAbs. Whilst the impact of cross-linking tetraspanins is difficult to interpret, this suggests that the CD9 molecule itself may play a role in regulating this self reactive T cell response (Kobayashi *et al.*, 2004). The tetraspanin CD81 has been suggested to play a role in T cell autoimmunity in chronic HCV

sufferers. Hepatitis infection is characterised by invasion of both HCV specific and non-specific T cells into the liver, leading to widespread tissue destruction over time (He *et al.*, 1999). Secondary complications in HCV patients are created by the development of auto-reactive T and B cells leading to a range of autoimmune pathologies. CD81 binding by HCV has been demonstrated to induce T cell activation (Wack *et al.*, 2001); it has been proposed that this sub-optimal T cell stimulation may be responsible for the autoimmune component of this disease. In part, this thesis attempts to address the paucity of studies regarding the role of tetraspanins in autoimmunity.

1.5 TETRASPANIN IMMUNOBIOLOGY OVERVIEW

1.5.1 HUMORAL IMMUNITY

A large number of tetraspanins have been identified on the B cell surface, including CD9, CD37, CD63, CD53, CD81, CD82 and CD151 (Tarrant *et al.*, 2003). In the context of B cells, the most extensively studied tetraspanin is CD81, which was originally identified as the target of a B cell anti-proliferative antibody (TAPA-1) (Oren *et al.*, 1990). CD81 is part of the BCR co-receptor complex, which can co-ligate antigen bound to the BCR and provides additional signalling for B cell activation. B cells recognise antigen through the B cell receptor complex (BCR) which is composed of cell surface IgM and associated Ig family members Igα and Igβ. In resting cells, the Src family kinases required for BCR signalling, such as Lyn and Fyn, are located within lipid raft domains devoid of the BCR complex. Upon B cell activation however, antigen cross-linked BCRs transiently enter the signalling molecule rich lipid raft domains. The Src kinases phosphorylate ITAM regions within the Igα and Igβ chains and a signalling cascade is initiated by recruitment of Syk, which activates downstream signalling molecules such as phospholipase Cγ (PLCγ). The tetraspanin CD81 is a member of the BCR co-receptor complex that includes CD19, CD21, Leu13 and CD81 (Figure 1.6). When complement bound antigen is recognised by both the BCR and the complement receptor CD21, co-ligation enables CD19 phosphorylation and the activation of additional kinases such as PI3-K and Vav. This results in a second activation pathway and downstream signalling to PLCγ and other molecules (Pierce, 2002; Xu *et al.*, 2005). Combined signalling through both the BCR and the BCR co-receptor is thought to reduce the threshold for B cell activation as much as 10,000 fold (Dempsey *et al.*, 1996).

The role of CD81 within the BCR co-receptor complex appears to be two-fold. Chemical cross-linking analysis via FRET has determined that when antigen is co-ligated by both BCR

and BCR co-receptor complexes, lipid raft association is prolonged in comparison to BCR cross-linked antigen alone (Cherukuri *et al.*, 2001). Partitioning of this co-ligated antigen into lipid rafts is dependent on CD81 expression, suggesting that CD81 actively stabilises lipid raft association of the co-ligated BCR-BCR co-receptor complex (Cherukuri *et al.*, 2004b). Moreover, this function was also dependent upon inducible palmitoylation of CD81, which has been demonstrated in other proteins to promote segregation into lipid rafts (Cherukuri *et al.*, 2004a; Zhang *et al.*, 1998a). CD81 also plays a significant role in the maturation and trafficking of CD19 to the cell surface (Shoham *et al.*, 2006). Mutagenesis studies revealed that CD81 contains multiple distinct functional domains that enable physical association between CD81 and CD19 early in protein synthesis (EC2), complete glycosylation within the golgi (N-terminus) and translocation to the cell surface (TM1) (Shoham *et al.*, 2006).

Defined roles in B cell function are yet to be determined for the many other tetraspanins expressed by B cells. B cell function has been investigated in five tetraspanin deficient mouse lines to date and the effects of tetraspanin deficiency have been varied (Cariappa *et al.*, 2005; Knobeloch *et al.*, 2000; Maecker and Levy, 1997; Miyazaki *et al.*, 1997; Tarrant *et al.*, 2002; Tsitsikov *et al.*, 1997; Wright *et al.*, 2004a). As discussed earlier, both CD37 and CD81 deficient mice show some similarities in B cell function, particularly in antibody responses to T cell dependent antigens. Due to the role of CD19 in B cell co-receptor signalling, it is difficult to separate the effects of CD81 deletion from the reduced cell surface expression of CD19. It is likely that the reduced expression of CD19 contributes significantly to the B cell phenotypes described. Therefore, the $CD37^{-/-}$ model may be more suitable for investigating tetraspanin microdomain function in B cells.

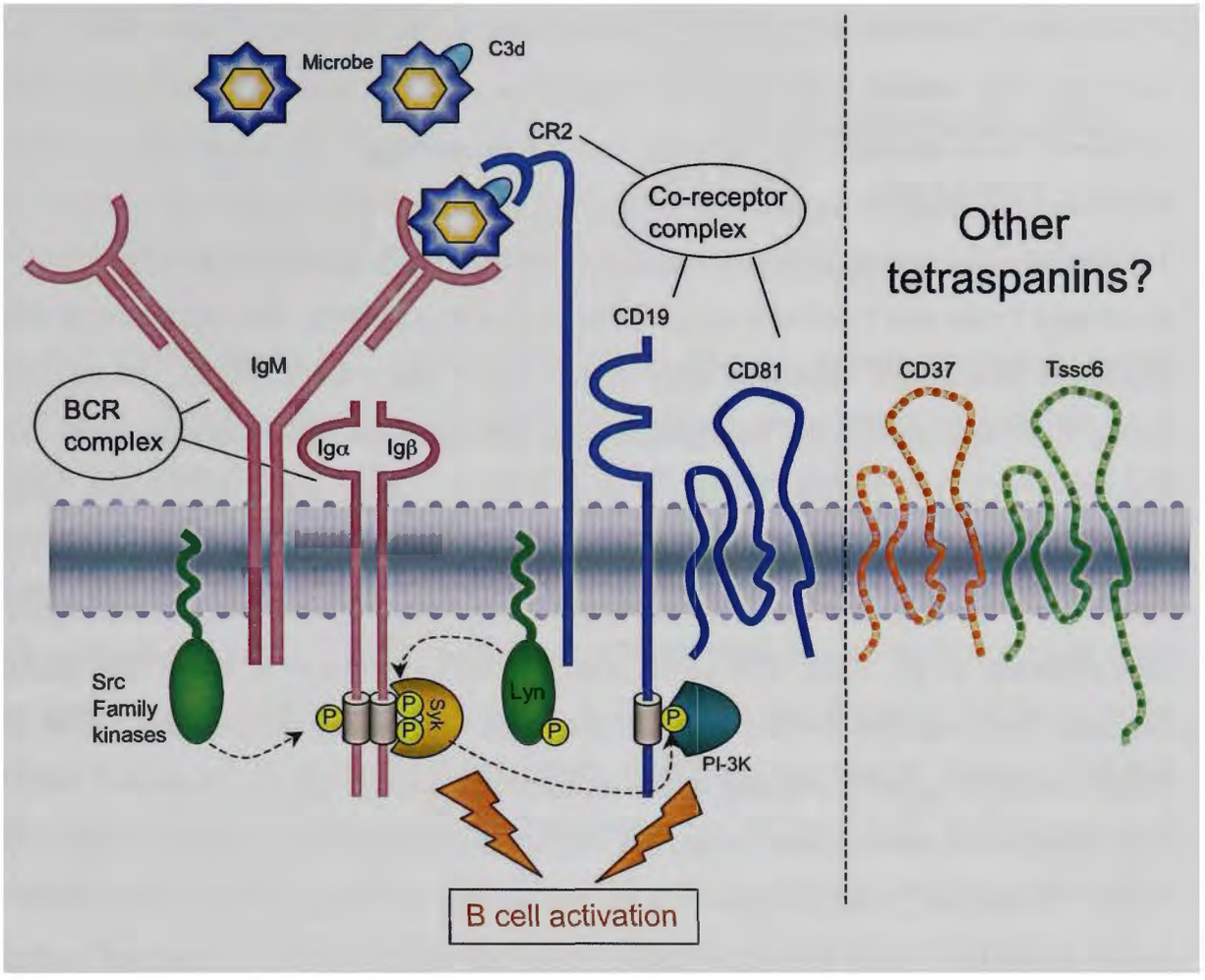

Figure 1.6 B cell signal enhancement via the BCR co-receptor complex.

B cell signalling in response to antigen recognition is strengthened by co-ligation of complement bound antigen via the BCR co-receptor complex. This is comprised of complement receptor CR2, CD19, Leu13 (not shown) and the tetraspanin CD81. Members of the Src kinase family such as Lyn, and Fyn are brought together by cross-linking of the BCR (not shown) and phosphorylate ITAM regions within the Igα and Igβ chains of the BCR complex. This begins a signalling cascade initiated by Syk binding to phosphorylated ITAM regions activating numerous downstream signalling pathways. When the B cell co-receptor complex is brought into proximity of the activated BCR complex by complement recognition, CD19 phosphorylation occurs and activation of additional kinases such as PI-3K or Vav. This second activation pathway and downstream signalling results in augmentation of the primary B cell activation signal. Adapted from Abbas et al., 2007; Pierce, 2002; Xu et al., 2005.

1.5.2 CELL MEDIATED IMMUNITY

1.5.2.1 T cell function

Tetraspanin expression in T cells is widespread, with up to 20 tetraspanin proteins identified on primary T cells and T cell lines (reviewed in Tarrant *et al.*, 2003). The expression of these proteins is variable and dependent upon developmental and activation state. For example, during T cell development the tetraspanins CD81 and CD53 are differentially expressed. In the thymus, CD81 is expressed primarily in $CD4^+CD8^+$ double positive (DP) T cells, whilst CD53 expression is high in $CD4^-CD8^-$ double negative (DN) thymocytes (Tomlinson *et al.*, 1995). Both molecules continue to be expressed at low levels in mature single positive (SP) T cells (Maecker *et al.*, 2000; Puls *et al.*, 2002). CD53 expression is linked with positive selection, which requires low affinity interactions with MHC bearing self peptides. CD69 is a similar marker of positive selection in DN thymocytes, although the two molecules differ in their upregulation kinetics when presented with various peptides of low affinity (Puls *et al.*, 2002). In contrast, CD81 expression in DP thymocytes may suggest CD81 involvement in negative selection. Since at this DP stage, T cells that recognise MHC bearing self peptides with high avidity T cells are eliminated (Bonomo and Matzinger, 1993). The exact mechanisms of CD53 and CD81 involvement in thymic selection are unknown, however these findings point to a potentially important role for tetraspanins in T cell development.

Evidence of tetraspanin involvement in T cell activation is more abundant. Initially, such evidence came from the mitogenic and co-stimulatory effects observed during CD53 ligation with mAbs (Bell *et al.*, 1992). Later, co-stimulatory effects were also demonstrated using αCD3 stimulated T cells and mAbs directed against CD9, CD63, CD81 and CD82 (Lagaudriere-Gesbert *et al.*, 1997a; Lebel-Binay *et al.*, 1995; Nojima *et al.*, 1993; Pfistershammer *et al.*, 2004; Tai *et al.*, 1997; Witherden *et al.*, 2000). However the quality and strength of these co-stimulatory effects vary between tetraspanins. For example, CD9 ligation provides potent early T cell activation, but fails to induce IL-2 production and leads to T cell apoptosis (Tai *et al.*, 1997), whereas ligation of other tetraspanins such as CD63, CD81 and CD82 were able to induce sufficient IL-2 production (Lagaudriere-Gesbert *et al.*, 1998; Pfistershammer *et al.*, 2004; Todd *et al.*, 1996). CD81 and CD82 ligation on T cells has also been shown to induce cytoskeletal rearrangement and actin co-localisation, which was demonstrated in CD81 to be dependent upon the kinase vav-1 and Rho-GTPases (Crotta *et al.*, 2006; Lagaudriere-Gesbert

et al., 1998). Despite these studies, a ligand for tetraspanin binding between T cells and APC is yet to be identified and one may not exist. It is therefore possible that these co-stimulatory effects are not physiologically relevant and are due to disruption of tetraspanin-partner interactions within the T cell membrane, potential changes in tetraspanin associated signalling molecule function and/or artefactual cross-linking of tetraspanin microdomains.

The role of tetraspanins in T cell signalling is likely to occur through tetraspanin partner interactions. The tetraspanins CD37, CD81 and CD82 are each thought to modulate T cell signalling through their effects on Lck activity. Lck is a kinase associated with CD4 and CD8 adapter molecules that is brought into contact with the TCR complex when peptide-MHC co-ligates with the CD4/CD8 and TCR molecules. This proximity allows phosphorylation of the TCR associated molecule CD3, through which TCR signalling occurs. Both CD81 and CD82 can molecularly associate with CD4 and CD8 in T cells and may therefore play a role in the regulation of T cell activation (Imai and Yoshie, 1993). Further analysis demonstrated that CD4 exists in two cellular pools, associated with either Lck or CD81/CD82. This suggests that these tetraspanins may regulate T cell activation through inhibition of Lck/CD4 coupling (Imai *et al.*, 1995). CD37 has been implicated in Lck function due to enhanced kinase activity of CD4 and CD8 associated Lck in CD37 deficient T cells (van Spriel *et al.*, 2004). One explanation may be that dysregulation of Lck de-phosphorylation occurs in the absence of CD37 due to disruption of an unidentified tetraspanin associated phosphatase, such as that detected in association with CD53 and CD63 in rat lymphocytes (Carmo and Wright, 1995).

Tetraspanins are also thought to influence T cell activation through their participation in immunological synapse formation. The immunological synapse is a highly organised structure formed between T cells and APC and is enriched in molecules associated with adhesion, co-stimulation, signalling, and antigen presentation/recognition. In T cells, the tetraspanins CD81 and CD82 have been identified within the immunological synapse (Mittelbrunn *et al.*, 2002). In simulations of immunological synapse formation with αCD3 coated beads, CD37 was also found to redistribute to T cell-bead contact sites (unpublished data A. van Spriel). In both CD81 and CD82 this is likely to be related to cytoskeletal rearrangement and CD81/CD82 actin co-localisation. The maintenance of the immunological synapse is dependent on cell-cell adhesion mediated by adhesion molecules and integrins. In T cell-B cell synapses a critical interaction occurs between the integrin αLβ2 (LFA-1) and the adhesion molecules ICAM-1. Tetraspanins are also likely to contribute to these interactions since CD81

ligation on T cells is known to activate αLβ2 and CD82 upregulation can enhance αLβ2-ICAM-1 adhesion (Levy *et al.*, 1998; VanCompernolle *et al.*, 2001).

In vitro studies of tetraspanin deficient primary T cells have revealed a common role for some tetraspanins in the regulation of T cell proliferation. CD37, CD81, CD151 and Tssc6 deficient T cells are all hyper-proliferative when stimulated *in vitro* with αCD3 mAbs (Miyazaki *et al.*, 1997; Tarrant *et al.*, 2002; van Spriel *et al.*, 2004; Wright *et al.*, 2004a). In at least CD37 and Tssc6 deficient T cells, this has been attributed to early elevated IL-2 production. Taken together, these phenotypes suggest a fundamental role for tetraspanins in the regulation of T cell proliferation and cell mediated immunity, however it is yet to be determined if these phenotypes translate to functional defects in cellular immunity *in vivo*.

1.5.2.2 Antigen presentation

Very little is known about the role tetraspanins play in the development of APC or APC heterogeneity. There have been no reports of developmental defects of APC in tetraspanin deficient mice, although as mentioned earlier, reduced numbers of a B cell subset was reported in $CD81^{-/-}$ mice (Tsitsikov *et al.*, 1997). This phenotype is most likely to be related to CD19 deficiency rather than CD81 deficiency *per se*, as similar B cell developmental changes are observed in the absence of CD19 (Engel *et al.*, 1995). A number of tetraspanins such as CD9, CD37, CD53, CD63, CD81, CD82 and CD151 have been detected by immunofluorescence at the cell surface of APC including B cells, monocyte derived DC and immature DC (Mantegazza *et al.*, 2004; Rubinstein *et al.*, 1996; Schwartz-Albiez *et al.*, 1988; Tohami *et al.*, 2004; Woodhead *et al.*, 1998). DC play a critical role in the development of cellular immunity and are the most potent antigen presenting cells of the immune system. DC function requires a complex series of events such as antigen capture, processing, DC relocation to the sites of naïve T lymphocytes and antigen presentation. Tetraspanins are associated with many key molecules involved in these processes and therefore clarifying tetraspanin involvement in antigen presentation may significantly improve our understanding of DC function.

A role for tetraspanins in antigen uptake by immature DC is suggested by CD63 and CD82 internalisation upon mAb ligation (Mantegazza *et al.*, 2004). In the same study, a specific role for tetraspanins in phagocytosis of the yeast *Saccharomyces cerevisiae* was also proposed. During yeast cell uptake, cell surface CD63 is also translocated into intracellular compartments containing the foreign cells. This correlates with the finding that the yeast

protein receptor Dectin-1 is molecularly associated with CD63 (Mantegazza *et al.*, 2004). A similar Level 3 molecular association between Dectin-1 and CD37 has also been demonstrated where CD37 was shown to stabilise Dectin-1 expression on the cell surface (Meyer-Wentrup *et al.*, 2007). Tetraspanins are also likely to play an important role in antigen processing. Intracellularly, the tetraspanins CD63, CD82 are highly expressed in MIIC and endocytic compartments of immature DC and to a lesser extent CD81 is also expressed (Mantegazza *et al.*, 2004). MIIC compartments are the site of peptide loading onto MHC class II and here both CD82 and CD63 molecularly associate with MHC class II and the peptide editors DM and DO (Hammond *et al.*, 1998). Upon DC maturation, post translational modifications of CD63 are apparent; this coincides with morphological changes in MIIC compartments (Engering *et al.*, 2003). Tetraspanins such as CD37, CD63, CD81 and CD82 have also been identified in APC derived exosomes (Escola *et al.*, 1998). Exosomes originate from the late endocytic pathway and are thought to play a functional role in antigen presentation and T cell activation (Thery *et al.*, 2002). Together, these data suggest an important role for tetraspanins in antigen uptake and processing by APC.

In APC, tetraspanins have been identified in complex with both MHC class I and MHC class II (Angelisova *et al.*, 1994; Schick and Levy, 1993; Szollosi *et al.*, 1996). Whilst very little is known about the functional consequences of this relationship with MHC class I, a number of studies have investigated the effect of MHC class II association with tetraspanins, where there has recently been some controversy generated. Kropshofer *et al* published a study describing a significant role for tetraspanins in the organisation of MHC class II, resulting in clustering of peptide-MHC complexes. This study utilised CDw78 as a marker of MHC class II molecules clustered by tetraspanin microdomains, based on the observation that the CDw78 determinant is found within only a portion of HLA-DR molecules associated with tetraspanins such as CD37, CD81, CD82 and CD9 (Drbal *et al.*, 1999; Kropshofer *et al.*, 2002). The bound peptide repertoire was found to be restricted within CDw78 associated MHC class II pools and the peptide sequence determined CDw78 localisation. The peptide editor HLA-DM was also precipitated within $CDw78^{+}$ MHC class II fractions derived from endosomal compartments and its function as a peptide editor was proposed to explain the restricted repertoire of peptides within $CDw78^{+}$ microdomains. Since MHC class II multimerisation can enhance presentation of low affinity antigens (Schafer and Pierce, 1994), these authors suggested that tetraspanins may function to cluster MHC bearing peptides of low affinity (Vogt *et al.*, 2002).

Poloso *et al* challenged the specificity of the CDw78 determinant as a marker of tetraspanin microdomain associated MHC II (Poloso *et al.*, 2006). This study concluded that CDw78 expression was dependent on association of MHC class II with the invariant chain (Ii) chaperone molecule, suggesting that CDw78 is merely a marker of MHC recently derived from the lysosomal antigen processing compartments (Poloso *et al.*, 2006). A third group examined the differences between cell surface MHC clustering between B cells and DC to explain the significantly enhanced capacity of DC to present antigen (Unternaehrer *et al.*, 2007). From this study it was concluded that the tetraspanin CD9 played a functional role in MHC II clustering on the cell surface resulting in improved antigen presentation in DC. Unfortunately, an assessment of MHC class II presentation in CD9 deficient APC was not used to confirm this finding (Unternaehrer *et al.*, 2007). Despite the conflicting opinions currently represented in the literature, it remains likely that tetraspanins play a role in antigen presentation via MHC organisation on the cell surface.

The tetraspanin CD9 has also been proposed to participate in antigen presentation through its presence in CD9/CD38/MHC class II complexes. CD38 is an ectoenzyme molecularly associated with MHC class II involved in monocyte and dendritic cell activation (Zilber *et al.*, 2005). CD38 ligation on both monocytes and DC results in the upregulation of co-stimulatory molecules, MHC class II expression and cytokine release consistent with APC activation (Fedele *et al.*, 2004). Since both CD38 and MHC class II lack cytoplasmic signalling motifs, it has been suggested that an intermediary transmembrane protein facilitates signalling through CD38 and MHC class II. Immuno-precipitation experiments have revealed a Level 3 interaction between CD9 and these two proteins, although MHCII/CD9 interactions also occur independently of CD38 (Zilber *et al.*, 2005). A shared signalling pathway through the Src family kinases Hck and Fgr was determined by mAb ligation of each of the three proteins. MHC class II and CD38 ligation-induced Ca^{++} flux responses were dependent upon raft integrity, whilst CD9 signalling was unaffected by raft disruption (Zilber *et al.*, 2005). Though the authors of this study do not suggest that CD9 is the only facilitator of CD38/MHC class II signalling, it would be of great interest to reinvestigate these signalling pathways in CD9 deficient mice. This would also enable the exclusion of any potentially confounding effects of mAb ligation.

As a general comment in relation to the investigation of tetraspanin involvement in antigen presentation, very little work has been performed using conventional *ex vivo* dendritic cells. This is likely to be related to the paucity of mAbs directed against murine tetraspanins and

the impracticality of acquiring differentiated human DC. However, tetraspanin deficient mice provide a valuable tool for rectifying this dearth in the literature, in particular those tetraspanins identified in complex with MHC.

1.6 EVIDENCE OF FUNCTIONAL OVERLAP BETWEEN TETRASPANINS

Previous studies on tetraspanin redundancy have demonstrated some degree of functional overlap between different members of the tetraspanin superfamily. As discussed, the $CD9^{-/-}$ mice display a sperm-egg fusion defect. By introducing excess CD81 mRNA into the CD9 deficient eggs, fusion rates can be restored by up to 50% (Kaji *et al.*, 2002). This finding suggests that there is some level of compensation offered by over-expression of the CD81 protein. A $CD9^{-/-}CD81^{-/-}$ mouse has also been described, produced in order to investigate functional similarities between the two molecules. Phenotypically, these mice were completely infertile confirming partial functional redundancy between CD9 and CD81 in sperm egg fusion (Rubinstein *et al.*, 2006). These mice also demonstrated high numbers of multinuclear phagocytes in the absence of infection, resulting in severe osteoporosis and emphysema, a phenotype absent in the respective single knockout mouse strains (Takeda *et al.*, 2003b). In *Drosophila*, three tetraspanins have been studied for their involvement in motoneuron function. Whilst the deletion of one protein (Lbm) leads to a delay in synapse formation between neurons, the absence of all three proteins leads to a more severe phenotype. This may indicate partial overlap of function between these proteins (Fradkin *et al.*, 2002).

When taken together, the results of the tetraspanin targeted deletions of CD37, CD81, CD151 and Tssc6 indicate a common role for tetraspanin involvement in T cell activation. B cell data is less concordant indicating that not all tetraspanins are involved in B cell function. Both CD81 and CD37 knockout mice exhibited B cell defects, although importantly CD19 expression was altered on CD81 deficient B cells and not on $CD37^{-/-}$ B cells. Whilst it is difficult to separate the effects of CD81 deletion and CD19 reduced expression, the similarities between $CD19^{-/-}$ mice suggest that many of the B cell defects in $CD81^{-/-}$ mice may be due to altered CD19 function. This difference is worth noting as it may indicate a separate mechanism for the B cell phenotype in $CD37^{-/-}$ mice. T cell phenotypes on the other hand are very consistent. On all tetraspanin knockout mice tested, T cell hyper-proliferation was seen in response to various TCR stimuli and a particularly sharp distinction was seen in suboptimal co-stimulatory conditions. In both $CD37^{-/-}$ and $Tssc6^{-/-}$ mice it was shown that this hyper-proliferative defect was due to early IL-2 production.

What are the implications of these findings on functional overlap between members of the tetraspanin superfamily? A model for sequestration of components of the TCR signalling pathway may suit these group findings. As tetraspanins have been shown to co-associate to form cell surface microdomains, perhaps in the absence of a microdomain member and therefore suboptimal microdomain formation, sequestration is less efficient? Perhaps suboptimal microdomain formation may lead to a reduced ability to bring molecules together that rely on indirect association or adapter molecules? In the absence of two tetraspanins would we see a more striking T cell phenotype, and could this therefore lead to potential autoimmunity? Functional overlap is also apparent in between the tetraspanins CD151 and Tssc6 in platelet function. It is possible that underlying redundancy between these molecules masks other roles for these tetraspanins in haemostasis and immune function. Again, in the absence of both tetraspanins novel functions in platelets may also be revealed.

1.7 THE TETRASPANIN CD37

Human CD37 is a heavily glycosylated tetraspanin composed of 281 a.a. It was first identified when a series of anti-human antibodies against B cells in various stages of development were produced (Schwartz-Albiez *et al.*, 1988). Expressed in leucocytes only, CD37 can be detected at high levels in pre-B and resting B cells and is down-regulated upon activation. It is also seen on both resting and activated T cells, resting dendritic cells, neutrophils, granulocytes and is found intracellularly on macrophages (Knobeloch *et al.*, 2000; Schwartz-Albiez *et al.*, 1988). Two additional cysteine residues are located in the large extracellular loop suggesting a structure similar to CD82 (Seigneuret *et al.*, 2001). Due to divergence from CD81 - the only tetraspanin to be partially crystallized at high resolution, a structure for CD37 is unable to be accurately predicted. The CD37 gene is located on human chromosome 19 and mouse chromosome 7 and shares closest protein sequence with CD82 (Figure 1.4). Homologues have also been identified in a number of species including mouse, rat and frog. Studies using anti human antibodies have demonstrated that in B cell lines, CD37 associates with CD53, MHC Class II, CD81 and CD82 (Angelisova *et al.*, 1994; Szollosi *et al.*, 1996). More recently, CD37 has been detected in complex with the C-type lectin Dectin-1 (Meyer-Wentrup *et al.*, 2007). It is assumed that these molecular interactions would be replicated in the mouse, however there have been no anti-mouse CD37 mAbs produced to date.

1.8 THE TETRASPANIN TSSC6

Tumour suppressing sub-chromosomal transferable fragment (Tssc6) is a relatively divergent member of the tetraspanin superfamily, proposed to be one of a small number of tetraspanins produced from independent duplications in vertebrate evolution (Huang *et al.*, 2005). The mouse protein encoded by 256 amino acids is most highly related to the tetraspanin CD81, however it contains an extended C terminal cytoplasmic tail. This notable structural feature is not shared with other known tetraspanins, being more than six times the normal length (Robb *et al.*, 2001). Containing only 4 cysteine residues in the EC2, it is a member of the subgroup of tetraspanin proteins that contains the lowest number of cysteine residues. This feature enables it to be partially modelled using the known structure of CD81 (Figure 1.7). Tssc6 was first identified in humans as one of two non-imprinted genes within a large imprinted domain on chromosome 11 (Lee *et al.*, 1999). This region is associated with a number of human genetic abnormalities related to altered imprinting of genes, most of which result in tumour growth. It was shown that DNA fragments from within this domain could suppress the growth of a rhabdocarcinoma cell line, implicating these genes in disease associated with this region (Paulsen *et al.*, 2000). At one stage renamed PhemX (pan haematological expression transcript), Tssc6 was identified at the mRNA transcript level as being exclusively expressed in hemopoietic cells (Nicholson *et al.*, 2000). Tssc6 later became of interest again as the product of a gene trap study aimed at identifying genes involved in early hemopoietic development, from which the $Tssc6^{-/-}$ mouse strain was developed (Robb *et al.*, 2001). Recently an anti-human Tssc6 polyclonal antibody has become available that was successfully used to demonstrate a molecular interaction between the integrin $\alpha IIb\beta 3$ and Tssc6 in human platelets (Goschnick *et al.*, 2006).

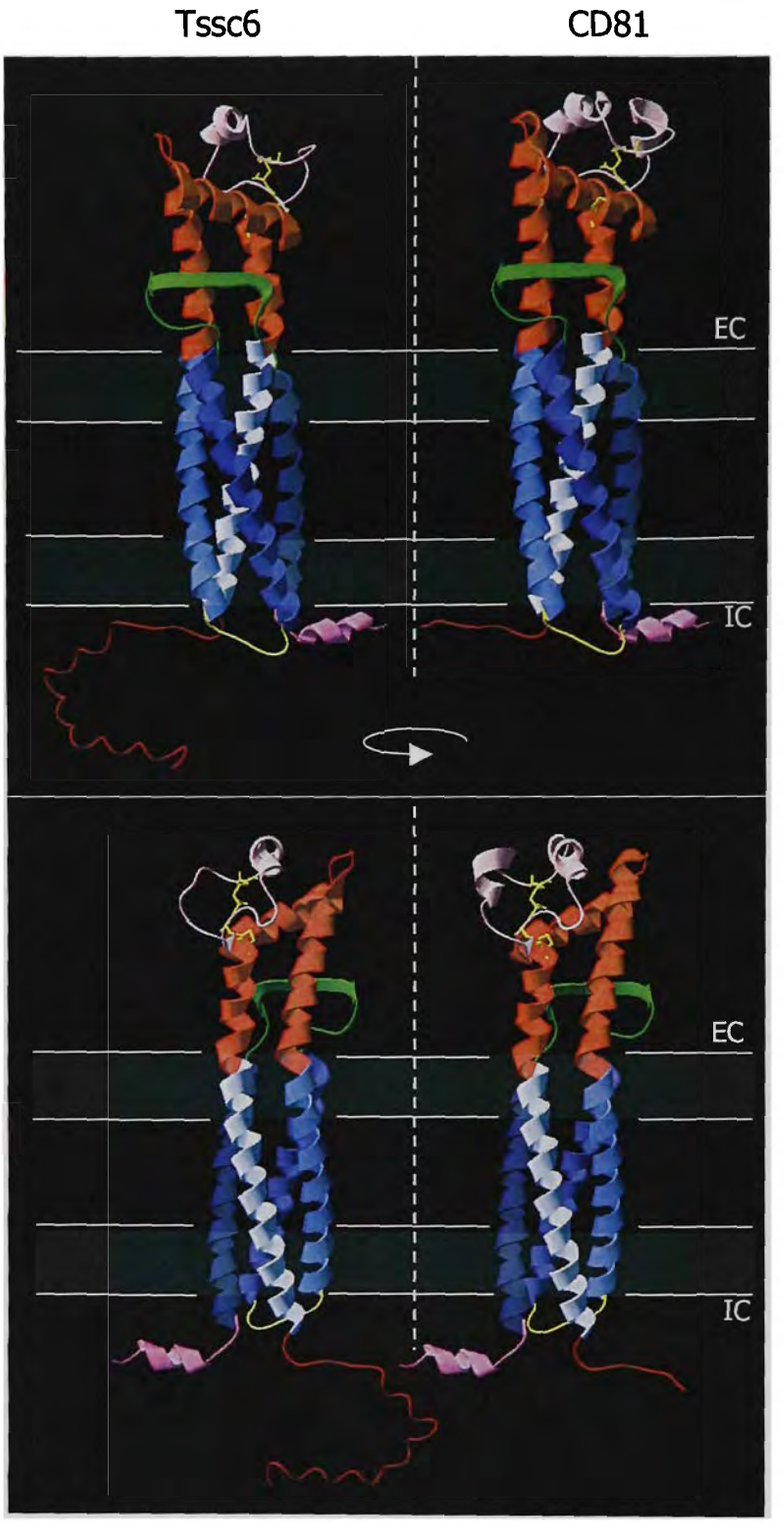

Figure 1.7 Complete three-dimensional predicted structure of mouse Tssc6

Mouse Tssc6 protein model based on the human CD81 crystal structure and a complete three-dimensional predicted structure of hCD81 (Kitadokoro *et al.*, 2001a; Kitadokoro *et al.*, 2001b; Seigneuret, 2006; Seigneuret *et al.*, 2001). *C-terminus, N-terminus, TM1-4, EC1, conserved EC2, variable EC2 and the intracellular loop are labelled respectively as follows: magenta, brown, dark blue, royal blue, blue, light blue, green, red and pink. Disulphide bonds are drawn in the variable EC2 domain between C156-C173 and C155-C183 in Tssc6 or C157-C175 and C156-C190 in CD81 in yellow. This model was generated using Swiss-MODEL and DeepView software. The secondary structure of the Tssc6 C-terminal tail was predicted using APSSP, whilst the C-terminal tertiary structure is unpredicted* (Raghava, 2000).

1.9 SUMMARY

The aim of this project was to investigate the potential for functional overlap between the tetraspanins CD37 and Tssc6 in the immune system. These two molecules were chosen due to the similar restricted expression profile in leucocytes and haematopoietic cells, respectively. The approach taken was to immunophenotype mice deficient in the expression of both CD37 and Tssc6. Given the role of both CD37 and Tssc6 in the regulation of T cell proliferation, the proliferative responses of $CD37^{-/-}Tssc6^{-/-}$ T cells were investigated and this work was extended to the T cell subset NKT cells. Since B cell function was altered in $CD37^{-/-}$ mice and normal in $Tssc6^{-/-}$ mice, the potential for functional redundancy between CD37 and Tssc6, that may result in normal B cell function in $Tssc6^{-/-}$ mice, was also examined. $CD37^{-/-}$ mice were also known to be hyper-stimulatory to antigen specific T cells, whereas DC function in $Tssc6^{-/-}$ was unexplored. Therefore the stimulatory capacity of $Tssc6^{-/-}$ and $CD37^{-/-}$ $Tssc6^{-/-}$ DC was compared to wild type and $CD37^{-/-}$ mice. Preliminary studies of anti-viral immunity in all three tetraspanin deficient strains found poor development of antigen specific T cell responses. This finding was confirmed in model antigen immunisation studies and the mechanisms behind these phenotypes investigated. The role of tetraspanins in the development of autoimmunity remains largely unstudied; to rectify this, ageing in tetraspanin deficient mice and the susceptibility of CD37/Tssc6 null mice to active autoimmune induction were studied.

This project focussed on the initial characterisation of a tetraspanin double knockout mouse. Whilst mechanism is partially explored in both poor development of antigen specific T cells in $CD37^{-/-}$ and $Tssc6^{-/-}$ mice and altered antibody responses in $CD37^{-/-}$ B cells, the mechanisms behind many of the phenotypes described are as yet undetermined. This will be the focus of future work. This thesis describes the identification of both functional redundancy and a complementary role for CD37 and Tssc6 in the immune system. These findings will be described in the following chapters.

2 MATERIALS & METHODS

2.1 MICE

CD37$^{-/-}$ mice were generated by homologous recombination (Knobeloch *et al.*, 2000) and backcrossed 10 times to C57BL/6 background. Tssc6$^{-/-}$ mice were generated by genetrap technology on a C57Bl/6 background (Tarrant *et al.*, 2002). These mice were interbred to create CD37$^{-/-}$Tssc6$^{-/-}$ mice. Age and sex-matched C57Bl/6 wild type (WT), DBA/1, μMT, C57Bl/6-OTI and C57Bl/6-OT2 and Balb/c mice were obtained from the Walter and Eliza Hall Institute (Melbourne, Victoria, Australia). CD37$^{-/-}$, Tssc6$^{-/-}$, CD37$^{-/-}$Tssc6$^{-/-}$ and FcγRIIa.SJL.Tg mice were bred at the Burnet Institute animal facility (Heidelberg, Victoria, Australia). Mice were used at 8-12 weeks of age. The animal ethics committees of the Austin and Repatriation Medical Centre, the Walter and Eliza Hall Institute and Burnet Institute approved all animal studies presented in this thesis. The genotypes of all mouse lines bred 'in-house' were monitored regularly, i.e. each breeding pair was genotyped before being matched, and the first litter of each new pair was genotyped. All mice used in comparative assays were aged between 6 and 12 weeks and both age and sex matched prior to experiments.

2.1.1 GENOTYPING - PCR

Mouse genomic DNA was prepared using the Wizard® Genomic DNA Purification Kit (Promega, WI, USA). Mice were tailed upon weaning, and genomic DNA isolated using the Animal Tissue (Mouse Tail) protocol outlined in the accompanying Wizard® Genomic Technical Manual. All oligonucleotides used in genotyping PCR were purchased from Proligo Australia (SA, Australia) and HotStart-Taq DNA polymerase purchased from Qiagen (CA, USA). The oligonucleotides used are listed in Table 2.1. PCR genotyping was performed using an Eppendorf Master Gradient Thermal Cycler (Hamburg, Germany) under the following conditions; an initial denaturation step of 94°C for 15 mins; followed by 30 cycles of 94°C for 30 secs, 55°C for 30 secs, and 72°C for 30 secs; and a final 4°C soak.

Table 2.1 Tetraspanin knockout genotyping primers

Primer	*Primer Sequence*
Tssc6	
Tssc6KO F	*5′ AGCGTATTCAACAAGG 3′*
Tssc6KO R	*5′ CAAGGCGATTAAGTTGGGT 3′*
Tssc6WT F	*5′ TCTGAGCACCATAGCCACTG 3′*
Tssc6WT R	*5′ ATGGAGAACTGGATACGGCA 3′*
CD37	
CD37KO F	*5′ CTGCACGAGACTAGTGAG 3′*
CD37KO R	*5′ GGACCCTTCGCTCCAG 3′*
CD37WT F	*5′ AGGTCAGATAGGCCCATACA 3′*
CD37WT R	*5′ CATCTGGTCTGGTACTCCTGA 3′*

2.1.2 Genotyping – Flow Cytometry

Peripheral blood (PB) was obtained by retro-orbital bleeding of mice aged between 5-6 weeks. PB was collected into tubes containing Alsever's Solution (75 mM NaCl, 25 mM sodium citrate, 110 mM glucose, pH 6.1) to prevent coagulation. Cells were centrifuged and washed in m-PBS/2% FCS and red cell lysis performed using ACK lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.2) for 5 mins at 37°C. Cells were then washed and red cell lysis repeated to remove residual erythrocytes. The remaining cells were then stained with αCD3 (KT3-1.1) and Vα2 (B20.1) mAbs and resuspended in propidium iodide (0.2% final concentration). Flow cytometry was used to identify the frequency of $V\alpha2^+$ T cells after exclusion of remaining erythrocytes and non viable cells via gating.

2.2 Cell Lines

The following cell lines have been used throughout this study: RMA, RMA-Muc1, B16-OVA, EL4, EG7, MD45, Jurkat and E3-Thymoma. All cells were cultured using RPMI Complete Medium, composed of RPMI 1640 medium (Gibco-BRL, NY, USA) supplemented with 10% (v/v) Foetal Calf Serum, 100μg/ml Streptomycin, 100U/ml Penicillin, 4mM L-glutamine and 100μM 2-mercaptoethanol. Cells were housed in 37°C incubators supplemented with 5% CO_2. MD45, Jurkat and subsequent transfected cell lines were treated with Mycoplasma Removal Agent (Serotec, UK) and housed in Mycoplasma free incubators.

2.3 Lymphocyte Isolation

2.3.1 Splenocytes

Spleens were removed from freshly culled mice and single cell suspensions were made by perfusing the spleens with 3ml of RPMI Complete Medium followed by mechanical disruption of the tissue. Cells were treated with red cell removal buffer, washed and counted using a haemocytometer while diluted in 0.4% Trypan Blue (Sigma, MO, USA).

2.3.2 Dendritic Cells

Dendritic cells were isolated from spleen by density cut followed by bead depletion. Briefly, spleens were removed from freshly culled mice and a single cell suspension was created by digestion at room temperature with 0.02mg/ml Bovine pancreatic DNase (Roche, IN, USA) and 1mg/ml Collagenase Type III (Worthington Biochemical Corp, NJ, USA) in the presence of 1640 RPMI containing 2% FCS. To disrupt T cell-DC rosettes, $Mg2^+$ was chelated using 0.1M EDTA (pH 7.2) for 5 mins at room temperature. The 5% light fraction of buoyant cells were isolated using 1.077g/ml Nycodenz (Axis-Shield, Oslo, Norway) density cut at 3100rpm

for 10 mins. Cells were washed in EDTA-BSS-FCS to remove Nycodenz and then further purified by either negative or positive selection. In negative selection protocols, cells were incubated with a negative depletion cocktail containing the following rat-α-mouse mAbs; KT3-1.1, T24/31.7, TER119, RB68C5 and RA36B2 for 30 mins at 4°C. Cells were washed and then incubated with α-rat Ig coated beads (Qiagen) at a 10:1 bead to cell ratio for 30 mins at 4° while rotating. Two rounds of magnet selection removed contaminating cells and beads and the remaining highly enriched DC washed and resuspended in mPBS/FCS. During positive selection assays, after density centrifugation cells were incubated with αCD11c coated magnetic bead particles (Miltenyi Biotech) and incubated at ~ 8°C for 20 minutes. Cells were then washed and resuspended in mPBS/FCS and magnetic bead selection performed by AutoMACS instruments (Miltenyi Biotech) using the program Posseld. Purity was determined by mAb staining with rat-α-mouse CD11c PE or APC. All centrifugation steps other than density cut were performed at 1700 rpm for 7 mins. EDTA-BSS-FCS was made using NaCl and KCl in a 40:1 ratio, pH 7.2 HEPES Buffer, 5mM EDTA with a final osmolarity of 308m. Negative selection methods yielded an average purity of 70% $CD11c^+$ cells, whilst positive selection protocols yielded a purity of >95% $CD11c^+$ cells.

2.3.3 T CELLS

T cells were isolated from either spleen or lymph node using an antibody-bead depletion method. Briefly, organs were removed from freshly culled mice and a single cell suspension was created by either teasing apart the lymph nodes or spleen perfusion. For total T cell isolation, cells were incubated with a negative depletion cocktail containing the following rat-α-mouse mAbs: M1/70, RB68C5, RA36B2, TER119 and M5/114. For $CD4^+$ T cell isolation, cells were incubated with a negative depletion cocktail containing the following rat-α-mouse mAbs: M1/70, RB68C5, RA36B2, TER119, M5/114 and YTS169.4. For $CD8^+$ T cell isolation, cells were incubated with a negative depletion cocktail containing the following rat-α-mouse mAbs; M1/70, RB68C5, RA36B2, TER119, M5/114 and GK1.5. Cells were washed and then incubated with α-rat Ig coated beads (Qiagen, Vic, Australia) at an 8:1 bead to cell ratio for 30 mins at 4° while rotating. Contaminating cells and beads were removed by magnet and purity was determined by Ab staining with rat-α-mCD3 Cy5. This T cell isolation method yields approximately 90-95% purity.

2.3.4 NKT CELLS

NKT cells were investigated from spleen, thymus and liver. Organs were macerated to form single cell suspensions and red cells lysed at 37°C. To remove excess erythrocytes from hepatic preparations, livers were perfused with mPBS prior to maceration. Splenic NKT cells were enriched by B cell depletion. Splenocytes were stained with αB220 (RA3.6B2) at 4°C for 30 mins, washed, and incubated with sheep αrat-IgG-conjugated immunomagnetic beads (Dynal Biotech). Bead-labeled cells were magnetically removed according to the supplier's instructions and the remaining cells were surface labelled with αGal-Cer loaded CD1d tetramers generated as previously described (Matsuda *et al.*, 2000).

2.3.5 PERIPHERAL BLOOD LYMPHOCYTES

Peripheral blood was collected from retro-orbital bleeds into tubes containing Alsever's solution (75 mM NaCl, 25 mM sodium citrate, 110 mM glucose, pH 6.1) to prevent coagulation. Cells were centrifuged and washed in mPBS/2% FCS and red cell lysis performed using ACK lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.2) for 5 mins at 37°C. Cells were then washed and red cell lysis performed again.

2.3.6 BONE MARROW ISOLATION AND RECONSTITUTION

Bone marrow lymphocytes were isolated from WT, $CD37^{-/-}$ and μMT mouse femur and tibia by perfusion with mPBS/FCS. Cells were washed twice and resuspended in mPBS before adoptive transfer. Bone marrow chimeras were generated as previously described (Huntington *et al.*, 2006). Briefly, chimeras were generated by intravenous transfer of either 1×10^7 $CD37^{-/-}$ and μMT ($CD37^{+/+}$) bone marrow cells, or 1×10^7 wild type and μMT ($CD37^{+/+}$) bone marrow cells, into lethally irradiated C57Bl/6 recipients at a ratio of 1:4. Six weeks after reconstitution, heparinized blood was obtained from chimeric mice and reconstitution of all major leucocyte populations monitored by flow cytometry, using mAbs against: CD19 (1D3), GR-1 (RB6-8C5), CD3 (KT3-1.1), F4/80 (F4/80), and CD11b (M1/70).

2.4 *IN VITRO* ANALYSIS OF TETRASPANIN DEFICIENT LYMPHOCYTES

2.4.1 CELL LABELLING

2.4.1.1 Cell surface Flow Cytometry

Cells were surface labelled with monoclonal antibodies at 2×10^5 cells per tube. Cells were aliquoted and incubated with titrated concentrations of the required antibody in mPBS containing 2% FCS for 30 mins at 4°C. Cells were then washed in 2 ml of mPBS-FCS and

resuspended in 200μl of mPBS-FCS either with or without 0.2% propidium iodide. After staining, cells were kept at 4°C until being analysed via FACScalibur (Becton-Dickinson, CA, USA), FACSCanto II (Becton Dickinson, CA, USA) or sorted by FACS Diva (Becton-Dickinson, CA, USA). Antibodies against the following leucocyte surface markers were used: CD3 (KT3-1.1), CD4 (GK1.5), CD8α (YTS169.4), CD11b (M1/70), CD11c (N418), CD19 (1D3), CD25 (7D4), CD28 (37.51), CD40 (FGK45.5), CD45R (RA36B2), CD45RA (14.8), CD49e (5H10-27), CD80, CD86, CD90 (T24/31.7), CD62L ((MEL-14), DEC205 (NLDC145), F4/80, MHC-II (M5/114), NK1.1 (P136), TER119 (TER-119). These antibodies were purified, biotinylated or conjugated to fluorescein isothyocyanate, phycoerythrin, APC or Cy5 (refer to Appendix II). All profiles were gated on cells that excluded propidium iodide, autofluorescence and were inclusive of a broad range of forward and side scatter properties. Analysis was performed using the following software: Weasel 2.4.1. (WEHI), CellQuest Pro (BD) or Diva Analysis software (BD).

2.4.1.2 Intracellular Flow Cytometry

2 x 10^5 B16-OVA cells were aliquoted per FACS tube and fixed with 2% paraformaldehyde for 15 mins at room temperature. 0.5% (v/v) saponin/FCS mix was used for blocking and permeabilising cells at 4°C for 30 mins. Rabbit αchicken ovalbumin (ICN Pharmaceuticals, Ohio, USA) was diluted in 0.05% (v/v) saponin/FCS mix. Normal rabbit serum was included as the isotype control, and unstained cells as a negative control. Cells were incubated for 30 mins at 4°C and centrifuged at 1700 rpm for 7 mins. FITC conjugated anti-rabbit Ig F(ab")2 fragment (Silenus, Australia) was added to the cells and incubated for 30 mins at 4°C. Cells were washed and resuspended in mPBS/FCS. Intracellular FoxP3 staining was performed in accordance with the manufacturer's protocol (eBioscience, San Diego, USA). Splenocytes were stained for surface markers CD4, CD3 and CD25 with the respective antibodies before fixing. Fixation/permeabilisation solution was prepared by diluting the concentrate into the diluent solution provided, at a ratio of 1:4. Cells were incubated at 4°C for 30 mins and blocked with 1 x permeabilisation buffer. FoxP3 Cy5 diluted in 1 x permeabilisation buffer was then added and incubated for a further 30 mins at 4°C.

2.4.1.3 CFSE Labelling

Cells were labelled with Carboxy Fluoroscein Succinimidyl Ester (Invitrogen) in order to track cellular division. Prior to staining, cells were washed twice in mPBS and resuspended in pre-warmed (37°C) mPBS containing 0.1% BSA. CFSE was added to the cell suspension to make

a final concentration of 10μM and vortexed immediately. Cells were then incubated at 37°C for 10 minutes and ice cold heat inactivated FCS added to make a 20% FCS solution. Cells were then washed in 10ml of cold complete RPMI twice and resuspended before counting. CFSE labelling was assessed by flow cytometry based on forward scatter and CFSE intensity.

2.4.2 B CELL PROLIFERATION ASSAYS

Splenocytes were aliquoted onto 96 well plates at $1x10^5$ cells/well in 100μl of RPMI Complete Medium and stimulated with the B cell mitogen lipopolysaccharide (LPS), purified αCD40 (FGK 45.5) or the Fab region of αIgM. Cells were cultured for 5 days and unstimulated splenocytes were included as a control. Splenocyte proliferation was assayed at 24 hour intervals on days 1 – 5 by ^{3}H-thymidine incorporation.

2.4.3 T CELL PROLIFERATION ASSAYS – TRITIATED THYMIDINE

Splenocytes were aliquoted onto 96 well plates at $1x10^5$ cells/well in 100μl of RPMI Complete Medium and stimulated with the T cell mitogen concanavalin A (conA) at between 0.1 – 3μg/ml for 5 days. For purified T cell assays, 96 well plates (Falcon) were coated with αCD3 mAb (KT3-1.1) at 0.1 – 10μg/ml overnight at 4°C or for 3 hours at 37°C, then washed using mPBS. Total T cells, $CD4^+$ T cells or $CD8^+$ T cells were aliquoted in RPMI Complete Medium at 1×10^5 cells per well in the presence or absence of 1μg/ml αCD28 mAb (37.51). Plates were incubated at 37°C for 1 – 5 days. Unstimulated splenocytes or T cells were included as controls and T cell proliferation was assayed at 24 hour intervals on days 1 – 5 by ^{3}H-thymidine incorporation.

2.4.4 T CELL PROLIFERATION ASSAYS – CFSE

24 well plates (BD Falcon) were coated with αCD3 mAb (KT3-1.1) at 0.1 – 2.5μg/ml overnight at 4°C or for 3 hours at 37°C, then washed using mPBS. CFSE labelled (10μM) $CD4^+$ T cells or $CD8^+$ T cells were aliquoted in 500 μl RPMI Complete Medium at 5×10^5 cells per well. Plates were then incubated at 37°C 5% CO_2 for 24 – 72 hours. As controls, unstimulated T cells and PMA (100ng/ml) + ionomycin (1μg/ml) stimulated T cells were included in each experiment. T cell proliferation was assayed at various time-points by flow cytometry. Supernatants were reserved for cytokine measurement while cells were harvested and resuspended in mPBS containing 2% FCS. CFSE staining was assessed at 488nm by FACSCanto II (Becton Dickinson, CA, USA) on cells displaying a broad forward and side scatter profile.

2.4.5 NKT CELL PROLIFERATION ASSAYS

After isolation, 4 x 10^7 spleen lymphocytes from naïve wild type, $CD37^{-/-}$, $Tssc6^{-/-}$ and $CD37^{-/-}$ $Tssc6^{-/-}$ mice were CFSE-labelled in 1 ml of mPBS with 16 μl of CFSE at 0.1 mM in 2 ml of mPBS with 0.1% BSA for 10 min at 37°C with gentle mixing. Cells were washed twice with 5 ml of culture medium containing 20% FCS, resuspended in culture medium, and cultured in the presence of α-GalCer (ranging from 5 to 50 ng/ml). After either 2.5 or 3.5 days of culture, cells were harvested, counted, and analysed by flow cytometry. CFSE positive NKT cells were identified as TCR^+CD1d-αGal-Cer $tetramer^+$ double positive.

2.4.6 ANTIGEN PRESENTATION ASSAYS – OT-I

Dendritic cells and $CD8^+$ T cells were isolated as described above. OT-I T cells were counted and aliquoted onto 96 well plates at 2 x 10^4 T cells/well. Dendritic cells were pulsed with SIINFEKL peptide at between 0.01 – 1.0mg/ml for one hour. DC's were then washed 3 times in 10ml complete RPMI and incubated at 2 x 10^3 DC's/well in a final volume of 200μl of RPMI complete medium for 7 days. Unstimulated and T cell and DC only controls were included on each plate. T cell proliferation was assayed at 24 hour intervals on days 2-6 by ^{3}H-thymidine incorporation.

2.4.7 ANTIGEN PRESENTATION ASSAYS – OT-II

Dendritic cells and $CD4^+$ T cells were isolated as above, OT-II T cells were counted and aliquoted onto 96 well plates at 2 x 10^4 T cells/well in 200μl of RPMI Complete Medium. Dendritic cells were pulsed with Helper peptide (ISQAVHAAHAEINEAGR) at between 10 – 50μg/ml for one hour. DC's were then washed 3 times in 10ml complete RPMI and incubated at 2 x 10^3 DC's/well with pre-plated T cells for 6 days. Unstimulated and T cell and DC only controls were included on each plate and background readings for these wells remained below 1500 cpm. T cell proliferation was assayed at 24 hour intervals on days 2-6 by ^{3}H-thymidine incorporation.

2.4.8 DENDRITIC CELL CO-STIMULATION ASSAYS

96 well plates were coated with 0.5 or 1μg/ml of αCD3 mAb (KT3-1.1) overnight at 4°C. 1 × 10^5 magnetic bead purified total T cells and either 2 × 10^3 or 1 x 10^4 Automacs purified dendritic cells per well were co-incubated for 3 days with T cell only and DC only wells included as controls. T cell proliferation was assayed on day 3 by ^{3}H-thymidine incorporation.

2.4.9 Dendritic cell activation assays

Dendritic cells were purified by positive selection via AutoMACS and resuspended in RPMI complete medium. Cells were plated into 96 well round bottom plates at 1 x 10^5 cells per well in 100μl in the presence of 10 ng/ml of GM-CSF and IFNγ. Stimulants were added in 100ml aliquots onto pre-plated DC to generate the final concentrations as follows; CpG (10μg/ml), LPS (1μg/ml), heat killed SAC Pansorbin® (20μg/ml) and αCD40 mAbs (5μg/ml or 25μg/ml), and unstimulated wells were included as controls. DC were incubated overnight at 37°C 5% CO_2 and supernatants collected for further analysis.

2.4.10 Co-stimulation of T cells using activated DC supernatants

96 well flat bottom plates were coated overnight at 4°C with 1.0μg/ml αCD3 (KT3-1.1) in sterile mPBS. Wild type total T cells were purified and 1 x 10^5 cells plated into αCD3 coated plates in 100μl of complete RPMI medium. 100μl of DC supernatants from αCD40 stimulations were added to the pre-plated T cells to make a final volume of 200μl and incubated at 37°C 5% CO_2 for 3 days. T cell proliferation was monitored at 24 hour intervals via ^3H Thymidine incorporation.

2.4.11 T cell and Dendritic cell soluble cytokine detection

Cytokine secretion in stimulated T cell and dendritic cell supernatants were assessed by CBA array (BD Pharmingen). aCD3 stimulated T cell supernatants were assayed for the cytokines; IL-2, IL-4, IL-5, IFNg and TNFa using the Th1/Th2 cytokine detection kit, whilst CpG, LPS, SAC and aCD40 stimulated DC supernatants were analysed for the cytokines IFNg, IL-6, IL-10, IL-12p70, MCP-1 and TNF. CBA array analysis was performed as per the instructions of the supplier. Briefly, The CBA kit is a multiplex of enzyme linked immunosorbent assays (ELISA) that allows the quantification of multiple different cytokines. Similar to a conventional sandwich ELISA, cytokine capturing antibodies of discrete fluorescence intensities were mixed with PE-conjugated detecting antibodies. Standards were provided for determining the relative concentration of each cytokine present in the supernatant. Flow cytometric analyses were performed with FACSCanto and quantitation of cytokines determined by FCAP™ cytometric bead array software.

2.5 *In vivo* Analysis of Tetraspanin Deficient Mouse lines

2.5.1 Basal Immunoglobulin Assessment

Basal immunoglobulin levels were tested by sandwich ELISA. Plates were coated with anti-mouse Ig (H+L) (Chemicon) and naïve mouse sera diluted in 1% BSA/PBS. Anti-mouse IgG_1, IgG_{2a}, IgG_{2b+c}, IgG_3, IgM, IgE and IgA antibodies (PharMingen) detected bound antibody. TMB peroxidase substrate solution (Invitrogen) and 0.1M HCl were used for colorimetric detection. Optical density was measured at 450 nm with the BioLumin 960 microassay reader (Molecular Dynamics).

2.5.2 Immunisation Studies – B cells

Groups of 6 mice were given a 100μl intraperitoneal injection of 25μg/ml NP-KLH precipitated in 10% alum. After 21 days mice were boosted with 100μl of 25μg/ml of NP-KLH in mPBS via peritoneal injection. Serum was collected each week for ELISA from each mouse.

2.5.3 Antigen Specific Immunoglobulin Assessment

2.5.3.1 ELISA

Mice were immunised i.p. with 100μg $NP_{(20)}$-KLH (Biosearch) precipitated in alum or 50μg $NP_{(3)}$-LPS (Biosearch) and after 21 days boosted with same. Mice were bled retro-orbitally over 35 days and serum used in ELISA. 96 well immunosorbent plates (Costar) were coated with 5μg /ml $NP_{(20)}$-BSA or $NP_{(3)}$-BSA (Biosearch) overnight at 4°C. Plates were blocked with 3% BSA/PBS and serum diluted in 1% BSA/PBS. NP specific antibodies were detected by anti-mouse IgG_1 and IgM HRP conjugated antibodies (PharMingen).

2.5.3.2 B cell ELISPOTs

96 well Mutliscreen HA plates (Millipore) were pre coated with either NP-BSA (10μg/ml), αmouse IgA (1μg/ml) or αmouse total Ig (1μg/ml) in sterile mPBS overnight at 4°C. Plates were washed and 5 x 10^5 splenocytes or bone marrow cells isolated from naïve and NP-KLH immunised mice were plated per well in complete RPMI. Plates were incubated at 37°C 5% CO_2 for 24 hours and then developed. Between each of the following procedures, plates were washed 6 times in mPBS-0.05% Tween-20 and 6 times in mPBS. Cells and media were removed after incubation by washing and 1μg/ml of biotinylated αmouse IgG_1 (BD) or biotinylated αmouse IgA (BD) was added for 2 hours at RT. Plates were washed and 1μg/ml Streptavidin-ALP-PQ was added for a further 2 hours at RT. Finally plates were washed again and developed using an AP Conjugate Substrate Kit (Biorad, CA, USA) by following the

manufacturer's instructions. Results were quantified by AID ELISPOT Reader System (Autoimmun Diagnostika GmbH).

2.5.4 Immunisation Studies – T cells

2.5.4.1 Cell associated antigens

Single cell suspensions of B16-OVA or RMA-Muc1 cells were generated by the addition of 5 mM EDTA for 5 mins at 37°C. Cells were then washed three times in mPBS before being irradiated at approximately 4,000 rads (Gammacell 100 ELITE, Norton International). Each mouse was injected intradermally with 5 x 10^6 cells in 100ml of mPBS.

2.5.4.2 Antennapedia peptide conjugated Antigens

ANTp-OVA and ANTp-SIINFEKL were a kind gift from Prof. Geoff Pietersz (Burnet Institute). ANTp-SIINFEKL was synthesised to include the 16-amino-acid Antennapedia peptide and SIIN (RQIKIWFQNRRMKWKKSIINFEKL). ANTp-OVA was formed by conjugating the Antennapedia peptide (RQIKIWFQNRRMKWKK) to chicken egg protein ovalbumin as previously described (Pietersz *et al.*, 2001). 25 μg of the specified antigens were resuspended in sterile mPBS and injected intradermally in a final volume of 100μl.

2.5.4.3 Adjuvant emulsified antigens

Chicken Egg Ovalbumin, Grade V (Sigma-Aldrich, Steinheim, Germany) was emulsified in complete Freund's adjuvant (Sigma-Aldrich, Steinheim, Germany) at a 1:1 ratio, to make up a final concentration of 1 mg/ml. Each mouse was injected intradermally at the base of the tail with 50 μg of ovalbumin in 100 μl.

2.5.5 Antigen specific Cytokine responses

Mice were immunised with a variety of antigens via base of tail intradermal injection. After 2 weeks, the mice received a boost injection at the same site or were culled for ELISPOT analysis, while boosted mice were later culled at 5 weeks for ELISPOT analysis. Serum was collected via eye bleed or cardiac puncture for later use. 96 well MultiScreen-HTS ELISPOT plates (Millipore, UK) were coated at 5μg/ml of αIFNγ mAb (RA-642) or αIl-4 mAb (11B11) overnight at 4°C. Plates were then washed using mPBS and blocked for 2 hours in RPMI including 2.5% FCS. 5 x 10^5/well splenocytes were added in Complete RPMI Medium and stimulated with either; 2.5μg/ml ConA, 20μg SIINFEKL peptide, 20μg Helper peptide, or 2 x 10^5 irradiated RMA-Muc1 cells. Unstimulated controls were also included on each plate. Cells

were then incubated for 18 (IFNγ) or 24 (Il-4) hours at 37°C. Between each of the following procedures, plates were washed 6 times in mPBS-0.05% Tween and then washed 6 times in mPBS. Cells and media were removed after incubation by washing and 1mg/ml of biotinylated αIFNγ mAb (XMG1.2) or αIl-4 mAb (BVD6-24G2) was added for 2 hours at RT. Plates were washed and 1μg/ml Streptavidin-ALP-PQ was added for a further 2 hours at RT. Finally plates were washed again and developed using an AP Conjugate Substrate Kit (Biorad, CA, USA) by following the manufacturer's instructions. Results were quantified by AID ELISPOT Reader System (Autoimmun Diagnostika GmbH).

2.5.6 *In vivo Dendritic Cell Migration Assays*

Flurescein-5-isothiocyanate (FITC 'Isomer I') (Invitrogen, Oregon, USA) was dissolved in dimethyl sulphoxide (DMSO) at 10% w/v. Acetone and dibutyl-phtalate were added at a 1:1 ratio to make up a final 1% (w/v) FITC solution. 20μl or 100 μL was applied onto the dorsal side of the ear or a shaved abdominal region of each mouse respectively. After 2-3 days mice were culled and subauricular (ear painted) or inguinal (abdominal painted) lymph nodes were removed. DC were purified from lymph nodes as described in the CD11c positive selection protocol above. DC were stained with CD11c (N418), CD8a (YTS.169) and DEC205 (NLDC-145) and gated on FITC positive cells. Brachial LNs were removed as negative controls in painted mice to determine autofluorescence and drain site specificity.

2.5.7 *In vivo Killing Assays*

Mice were immunised with 25μg/ml of ANTp-SIINFEKL intradermally at the base of the tail. 8 days later, splenocytes from WT mice were either; labelled with 0.5μM CFSE and pulsed with 250ng/ml Muc1 peptide ($CFSE^{lo}$) or labelled with 5μM CFSE and pulsed with 250ng of SIINFEKL peptide ($CFSE^{hi}$). Splenocytes were then washed and mixed at a 1:1 ratio and 2 x 10^7 cells were injected intravenously into immunised mice in sterile mPBS. 20 hours post iv injection. Mice were culled and splenocytes prepared for flow cytometry. Cells were stained with propidium iodide, live CFSE positive cells were gated for analysis and the ratio of remaining $CFSE^{lo}$ versus $CFSE^{hi}$ cells calculated.

2.6 Disease Models

2.6.1 *Influenza Challenges*

Mice were anaesthetized with methoxyfluorane and then intranasally infected with a non-lethal challenge of $10^{4.5}$ PFU of A/HKx31 (H3N2, X31) influenza virus diluted in 25 μl sterile

PBS. Virus-specific $CD8^+$ T cells were identified using MHC class I/peptide tetrameric complexes of the H-$2D^b$ glycoprotein and peptides derived from the nucleoprotein (ASNENMETM) (Townsend *et al.*, 1986) and acid polymerase (PA) (SSLENFRAYV) (Belz *et al.*, 2000) referred to as D^bNP_{366} and D^bPA_{224}, respectively. Recombinant H-$2D^b$ molecules with a BirA biotinylation motif substituted for the carboxyl-terminal transmembrane domain were refolded with human β_2-microglobulin plus the appropriate viral peptide, biotinylated with BirA, and complexed at a 4:1 molar ratio with neutravidin-phycoerythrin (Molecular Probes, Eugene, Oreg.). Lymphocytes were stained for 60 min at room temperature with the tetrameric complexes in PBS-bovine serum albumin-azide and then were stained with CD8a-FITC for 30 minutes on ice, washed twice, and analyzed by flow cytometry.

2.6.2 Collagen II Induced Arthritis

Mice between aged between 6 – 8 weeks were given an emulsion of Collagen II (Chicken sternal cartilage, Sigma-Aldrich, MO, USA) in Complete Freunds Adjuvant (CFA). Collagen II was dissolved in 10mM Acetic Acid (2mg/ml) and mixed thoroughly. CFA was made using Mycobacterium in Incomplete Freunds Adjuvant (Sigma-Aldrich, MO, USA) at 5mg/ml. Collagen solution and CFA were mixed at a 1.1:1 ratio and mixed thoroughly to form an emulsion. Mice were inoculated with 100µl of the injection mix subcutaneously at the base of the tail. At Day 21 mice were boosted with 100µl of the injection mix subcutaneously at the base of the tail. Mice were scored for arthritis on Day 7, 14, 21 and then every 3 – 4 days until Day 60. Mice exhibiting severe arthritis and/or distress are culled from the experiment. Mice were scored as follows; Score 0 = normal mouse, Score 1 = Minor swelling or redness in a single knuckle or digit, Score 2 = Severe swelling and redness, but no loss of joint function, Score = 3 Severe swelling and redness, joint dysfunction and stiffness. Majority of limb affected by disease, distorted, claw-like feet. Serum samples were taken by eye-bleed pre-inoculation and every 2 weeks during the experiment.

2.6.3 Experimental Autoimmune Encephalomyelitis (EAE)

EAE was induced in C57BL/6 mice using a standard protocol. 200µg of MOG_{35-55} was dissolved in PBS and emulsified in an equal volume of complete Freund's adjuvant containing 1 mg/ml of heat killed *Mycobacterium Tuberculosis* HR37a (Sigma). On Day 0 (day of disease induction), one subcutaneous injection was given per mouse in both of the flanks. Pertussis toxin (Sigma, P2980) (400 ng/mouse) was injected intraperitoneally on the day of the induction of EAE, followed 48 hours later by a second dose (200 ng/mouse). Animals

were daily evaluated for clinical signs of disease, starting from day 1 post immunisation, using a 6-grade clinical scale: 0, normal animal; 0.5, weight loss; 1, inability to elevate the tail, tail weakness; 2, tail paralysis; 3, tail paralysis/hind limb paresis; 4, hind limb paralysis/forelimb weakness; 5, quadriplegia/moribund; 6, death from EAE.

2.6.4 Tumour Challenge

Mice were challenged with 5 – 10 x 10^6 cells of the following Tumour cell lines; RMA-Muc1, E3 Thymoma, EG7, & B16-OVA. Cells were grown in RPMI Complete Medium and harvested during log phase growth. Before injection, cells were washed three times in MT-PBS to remove excess foreign proteins and injected subcutaneously into the mouse abdomen. Tumour growth was monitored between days 3 – 14 and measured by calliper. Mice with Tumours in excess of 1.5cm^2 were culled from experiments for ethical reasons.

2.6.5 Pathology of Ageing Mice

Groups of 10 age matched female wild type, CD37$^{-/-}$, Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ mice were housed under germ free conditions over a period of 24 months at the Burnet Institute animal facility. Mice were cared for by animal staff during this period and monitored for the development of autoimmunity. Mice were weighed and urine analysis performed by dipstick to assess glucose and ketone excretion. After 24 months, peripheral blood was collected and full blood analysis performed by Coulter Counter instruments. Peripheral blood was also collected to perform biochemical analysis of serum. Mouse sera was analysed on Unicell Dxi 800 instruments. Mice were culled at the 24 month time point and major organs weighed and splenic lymphocytes analysed by flow cytometry.

2.6.6 Statistical Analyses

Statistical analyses were performed using the computer program Microsoft Excel via students t-test. P values less than 0.05 were considered significant.

2.7 Molecular Biology

2.7.1 Electrophoresis

Agarose gels (0.75 – 1.0% w/v agarose in TBE with 25μg/ml Ethidium Bromide) were run at 100V in TBE Buffer and DNA was visualised under UV Transilluminator. Gels were documented using the Eagle Eye System (Stratagene, CA, USA). 500ng of Bacillus subtilis SPP-1 Bacteriophage DNA Markers restricted with EcoRI (GeneWorks, SA, Australia), or

Lambda DNA Markers restricted with EcoRI + Hind III (Promega, WI, USA) were used to determine DNA fragment sizes and yields.

2.7.2 PLASMIDS

The plasmid pGMET-easy (Promega, WI, USA) was used as the primary cloning vector in all molecular biology throughout this project. Fluorescent contructs were generated using the vectors pEGFP-N1, pECFP-C1 and pEYFP-C1 (Clonetech). To convert the vectors containing C-terminal fluorescent tags to N-terminal fluorescent tags, the fluorescent proteins CFP and YFP were isolated from their original vectors by PCR with the addition of a NotI restriction site at the C terminus. These products were then sequenced and digested using the restriction enzymes AgeI and NotI. The GFP component of the PEGFP-N1 vector was then excised via AgeI and NotI and replaced with either CFP or YFP fragments by standard cloning techniques. These plasmids were then sequenced and primers specific to each fluorescent protein sequence used as diagnostic markers. Tetraspanin proteins were inserted into the MCS of each plasmid and sequencing was performed to confirm the integrity of the final expression constructs.

2.7.3 LIGATION

Ligation of DNA was performed by incubating vector and insert at 4°C for 4-24 hours with Rapid Ligation Buffer (Promega, WI, USA) and Ligase (New England Bioloabs, MA, USA) to a total volume of 10μl. Insert and vector were compatible either due to complementary sticky ends created by restriction digestion, or by A-tailed insert with a T-tailed vector. Ligations were set at both 1:1 and 3:1 molar ratios with insert only and vector only controls at all times. For transformation, 5μl of ligation and 45μl of H_2O were precipitated by addition of 500μl of Butanol and centrifugation at 13,000 rpm for 15 minutes, Pellets were dried and resuspended in 2μl of H_2O before electroporation. 20μl of competent bacteria were pulsed with 1μl of precipitated ligation at 1.25kV, 25mF using a Gene Pulser (Bioroad, CA, USA). Cells were resuspended in 1ml of LB (3.5% (w/v) tryptone, 2% (w/v) yeast extract, 0.5% (w/v) NaCl, pH 7.0) and incubated at 37°C for 1 hour before being grown on agar plates containing 100μg/ml Ampicillin or 50μg/ml Kanamycin.

2.7.4 DNA PURIFICATION

Plasmid DNA from bacterial cultures was purified using the Qiagen Miniprep kit as per the manufacturers instructions, and cDNA plasmid inserts were purified using the Qiaquick Gel

purification Kit also using standard protocols outlined in the accompanying manual (Qiagen, Vic., Australia).

2.7.5 PREPARATION OF ELECTRO-COMPETENT DH10β E.COLI

A starter culture was grown of DH10b E. coli bacteria by inoculation of 2mls of LB and incubation at 37°C, 225rpm shaking for 16-18 hours. 500μl of starter culture was used to inoculate 800ml of LB and grown at 37°C, 225rpm shaking for 3-6 hours until culture reached an OD_{600} between 0.5 – 1.0. Culture was chilled for 30 mins at 4°C for 30 mins and then centrifuged at 7000rpm for 10 mins (Beckman Avanti Centrifuge). The cell pellet was then aspirated and resuspended in 200ml of ice-cold H_2O/1mM HEPES solution. This process was repeated at 200ml, 50ml, 10ml, and then cells were resuspended in 2-3 x the volume of the cell pellet, finally the bacteria was aliquoted and frozen in Liquid N_2.

2.7.6 SEQUENCING

DNA plasmid inserts were sequenced with the Prism Big Dye Terminator Cycle Sequencing ready Reaction kit 3.1 (Applied Biosystems, CA, USA). Sequencing reactions comprised 250 – 500ng plasmid DNA, 1μl Big Dye 3.1, 3.5μl Sequencing Buffer, 1μl of appropriate sequencing primer (3.2pmols/μl and H_2O to a final volume of 20μl. PCR conditions consisted of 96°C for 2 mins, then 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes. Following PCR samples were transferred to a tube containing 80ml 75% Isopropanol and were incubated for 15 minutes at room temperature. Tubes DNA was then pelleted by centrifugation for 20 minutes at 13,000rpm. Pellets were aspirated and dried for 15 minutes at room temperature. All sequencing was resolved at the Australian Genome Research Facility (Vic., Australia).

2.7.7 STABLE CELLULAR EXPRESSION OF TETRASPANINS

MD45 and Jurkat cell lines were transfected via electroporation during log phase growth. 5 x 10^6 cells were pulsed with 30mg purified plasmid DNA at 0.52kV and 25mF in a Gene Pulser (Bioroad, CA, USA). Cells were incubated at 4°C for 10 mins and 1ml of RPMI Complete Medium was added. After a further 10 mins at RT 9 ml of RPMI Complete Medium was added and then cells were placed at 37°C 5%CO2 for 48 hours. Either G418 or Puromycin was added to select for successfully transfected cells. Transfectants expressing YFP, CFP or GFP were selected for fluorescent intensity from the top 5% range by FACS DiVa (Becton-Dickinson, CA, USA) and recultured.

3 HUMORAL IMMUNITY IN CD37$^{-/-}$TSSC6$^{-/-}$ MICE

3.1 INTRODUCTION

In order to investigate the effect of CD37 and Tssc6 deficiency in the immune system, both humoral and cellular immunity were examined. This chapter describes *in vitro* and *in vivo* analyses of the humoral immune system in CD37$^{-/-}$, Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ mice.

Effective humoral immunity develops over a series of stages including B cell differentiation, activation and antibody secretion. However, depending upon the antigen encountered, can also include B cell antigen presentation, T cell help, affinity maturation and the development of either, long lived antibody secreting cells (ASC) or memory B cells (Figure 3.1). B cell antigens are often classified as either T cell *independent* (TI) or T cell *dependent* (TD). TD antigens are processed by B cells and derived peptides presented to CD4^{+} T cells (Th) in the context of MHC class II. Cognate Th cells form germinal centres (GC) with activated B cells, where B cells proliferate and T cell cytokines induce antibody class switching from the IgM subclass to other longer lasting isotypes such as IgG_1 and IgA (Cozine *et al.*, 2005, Kelsoe, 1996). Conversely, TI antigens are often highly repetitive multimers, such as the bacterial cell wall component lipopolysaccharide, which can cross-link the B cell receptor and also activate pattern recognition receptors (Mond *et al.*, 1995). Since these antigens do not require T cell help to induce B cell antibody production, antibody responses to TI antigens are restricted to the IgM isotype and do not undergo class switching.

Affinity maturation also occurs in the GC, such that somatic hyper-mutation of variable Ig domains enables minor changes in the affinity of antibody to antigen (MacLennan, 1994). A third cell type prominent in the GC is the Follicular dendritic cell (FoDC), which despite being of non-haematopoietic origin shares a similar role to conventional DC by presenting antigen (Kosco-Vilbois *et al.*, 1993). FoDC are stromal cells that retain antigen for extended periods of time and participate in the GC reaction by providing anti-apoptotic signals to B cells producing high affinity antibodies to FoDC presented antigen (Tew *et al.*, 1979; Tew and Mandel, 1979). Here, B cells are positively selected based on antibody affinity, since higher affinity antibodies result in improved signalling between B cells and FoDC, leading to increased survival of these cells (Shih *et al.*, 2002). Conversely, low antibody-antigen affinity leads to B cell death through apoptosis. Whilst the effect on B cell survival from FoDC interaction in the GC is clear, the exact mechanisms by which these signals are provided remain difficult to decipher. The results presented in this chapter suggest that tetraspanins play a significant role in these interactions.

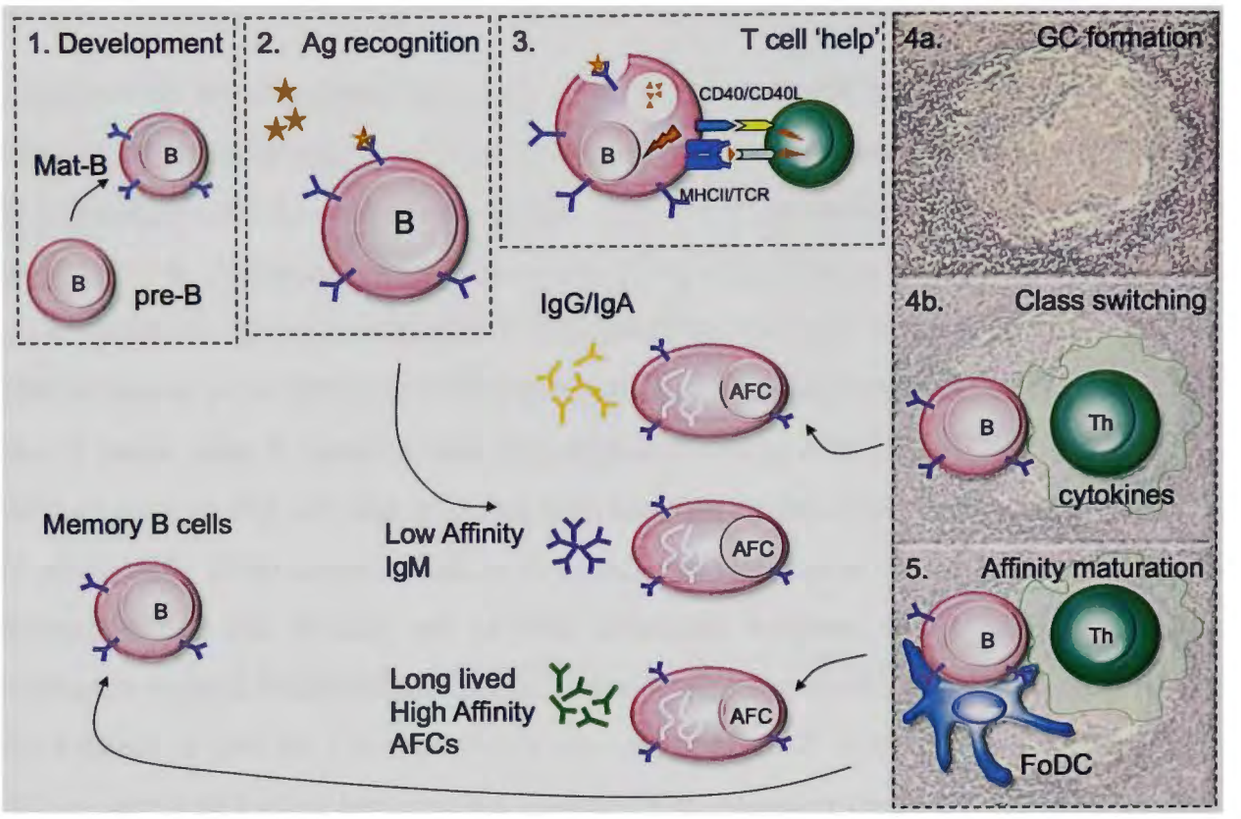

Figure 3.1 Overview of humoral immunity

B cell development is initiated in the bone marrow, followed by a series of maturation stages during migration to the follicular regions of the lymphoid tissue. During this process the cell undergoes multiple positive and negative selection phases to prevent auto-reactivity and ensure functional expression of cell surface receptors (Hardy and Hayakawa, 2001). *B cells are activated by antigen binding and depending on the antigen, either produce low affinity IgM antibodies or receive T cell help and produce high affinity antibodies. T cell help is provided by antigen specific T helper (Th) cell populations previously exposed to antigen by APC. Activated B cells that present cognate antigen to Th cells receive additional signals from the Th cell to enable the production of high affinity antibodies. B cells and Th cells form germinal centres and T cell cytokines induce class switching to other isotypes such as IgG and IgA. B cells also undergo affinity maturation in the GC where they compete for antigen presented by Follicular dendritic cells (FoDC). FoDC interactions promote the survival of B cells producing higher affinity antibodies and thus, are positively selected.*

3.1.1 Study Rationale

B cells express a variety of tetraspanins including CD9, CD37, CD53, CD63, CD81, CD82, CD151 and Tssc6 (reviewed by Tarrant *et al.*, 2003). In B cells, CD81 is molecularly associated with CD19 and forms part of the BCR co-receptor complex, which plays an important role in increasing B cell responsiveness to antigen (Levy *et al.*, 1998). In the absence of CD81, CD19 expression is reduced and association of the BCR co-receptor with signalling molecules is altered and B cell signalling impaired (Shoham *et al.*, 2003). Whilst an important role for the tetraspanin CD81 has been described in B cell activation, the function of other tetraspanins expressed at high levels in B cells such as CD9 and CD37 remain poorly understood. In two previous studies on the effect of tetraspanin deficiency in B cell function, it was demonstrated that CD81 and CD37 play important roles in the generation of TD immune responses and basal Ig production in B cells (Miyazaki *et al.*, 1997, Knobeloch *et al.*, 2000). However, other studies characterising B cells deficient in either Tssc6, CD151 or CD9 expression showed no change in humoral immunity (Cariappa *et al.*, 2005; Tarrant *et al.*, 2002; Wright *et al.*, 2004a). Given the potential for co-operation between tetraspanins in cellular function, I investigated the possibility of functional redundancy between the tetraspanins CD37 and Tssc6 in humoral immunity. As discussed earlier, tetraspanins exist at the cell surface in multimolecular complexes that include multiple members of the tetraspanin superfamily. It is possible that the absence of a single member of the tetraspanin superfamily may only partially disrupt the formation of functional microdomains at the B cell surface and therefore yield limited evidence of tetraspanin function in B cells. By investigating humoral immunity in the absence of multiple tetraspanins this may lead to previously undescribed phenotypes and potentially reveal an obscured role for Tssc6 in B cell function.

This chapter aims to:

(1) Determine if functional redundancy exists between CD37 and Tssc6 that may lead to the normal B cell function observed in Tssc6$^{-/-}$ mice.

(2) Examine the potential for novel B cell phenotypes in the absence of both CD37 and Tssc6.

(3) Investigate the mechanism behind altered serum Ig responses to TD antigens in the absence of CD37.

3.2 RESULTS

3.2.1 NORMAL B CELL DEVELOPMENT AND PROLIFERATION IN CD37$^{-/-}$TSSC6$^{-/-}$ MICE

Defects in B cell development have been previously described in CD81$^{-/-}$ mice, which display reduced cell surface expression of CD19 and a reduced frequency of the peritoneal B cell subset B1a (Miyazaki *et al.*, 1997). Conversely, B cell development is normal in CD37$^{-/-}$ and Tssc6$^{-/-}$ mice (Tarrant *et al.*, 2002; van Spriel *et al.*, 2004). To confirm these findings in CD37$^{-/-}$Tssc6$^{-/-}$ mice, FACS analyses of lymphoid tissue were performed. Pro-B/pre-B, immature and mature B cells can be delineated in the bone marrow based on the expression of CD45R (B220) and surface IgM (Hardy *et al.*, 1991). Similarly, the transition of immature to mature B cells can be visualised in peripheral blood by the upregulation of cell surface IgD. In the absence of CD37 and Tssc6 these developmental populations were present and in normal numbers (Figure 3.2A-B). Unlike CD81 deficient mice, cell surface CD19 expression remained unchanged (Figure 3.2C). The tetraspanin CD9 is expressed at high levels in some B cell subsets and when co-stained with αCD21 can be used as a marker of marginal zone (MZ) B cells (Won and Kearney, 2002). There were no significant differences between CD9 expression and the frequency of MZ B cells in the absence of CD37 and/or Tssc6 (Figure 3.2D). Likewise, the frequency of the B cell subset B1a (also characterised by high CD9 expression) in peritoneal exudate cells (PEC) of CD37/Tssc6 deficient mice was normal when stained with αCD5 and αCD19 mAbs (data not shown). Total B cell numbers in the spleen were also unaffected in CD37$^{-/-}$Tssc6$^{-/-}$ mice (Figure 3.2E). Together these data provide strong evidence that CD37 and Tssc6 are not required for normal B cell development.

To assess B cell proliferative responses in the absence of both CD37 and Tssc6, splenic B cells were isolated from CD37$^{-/-}$, Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ mice and proliferation was assessed in comparison to wild type controls. Cells were stimulated in two ways, first with the B cell mitogen lipopolysaccharide (LPS) and later, by cross-linking the BCR with αIgM mAbs in the presence and absence of co-stimulation (αCD40 mAbs) (Figure 3.3). These stimulants were titrated to investigate potential differences in dose responses and cell division was tracked over a period of 3-5 days. As previously demonstrated in both CD37 and Tssc6 deficient mice, B cell proliferation in CD37$^{-/-}$ and Tssc6$^{-/-}$ mice was normal in response to all stimulants (Tarrant *et al.*, 2002; van Spriel *et al.*, 2004). CD37$^{-/-}$Tssc6$^{-/-}$ B cell peak proliferation and dose response kinetics were also normal, indicating that there is no cryptic role for CD37 or Tssc6 in B cell proliferation.

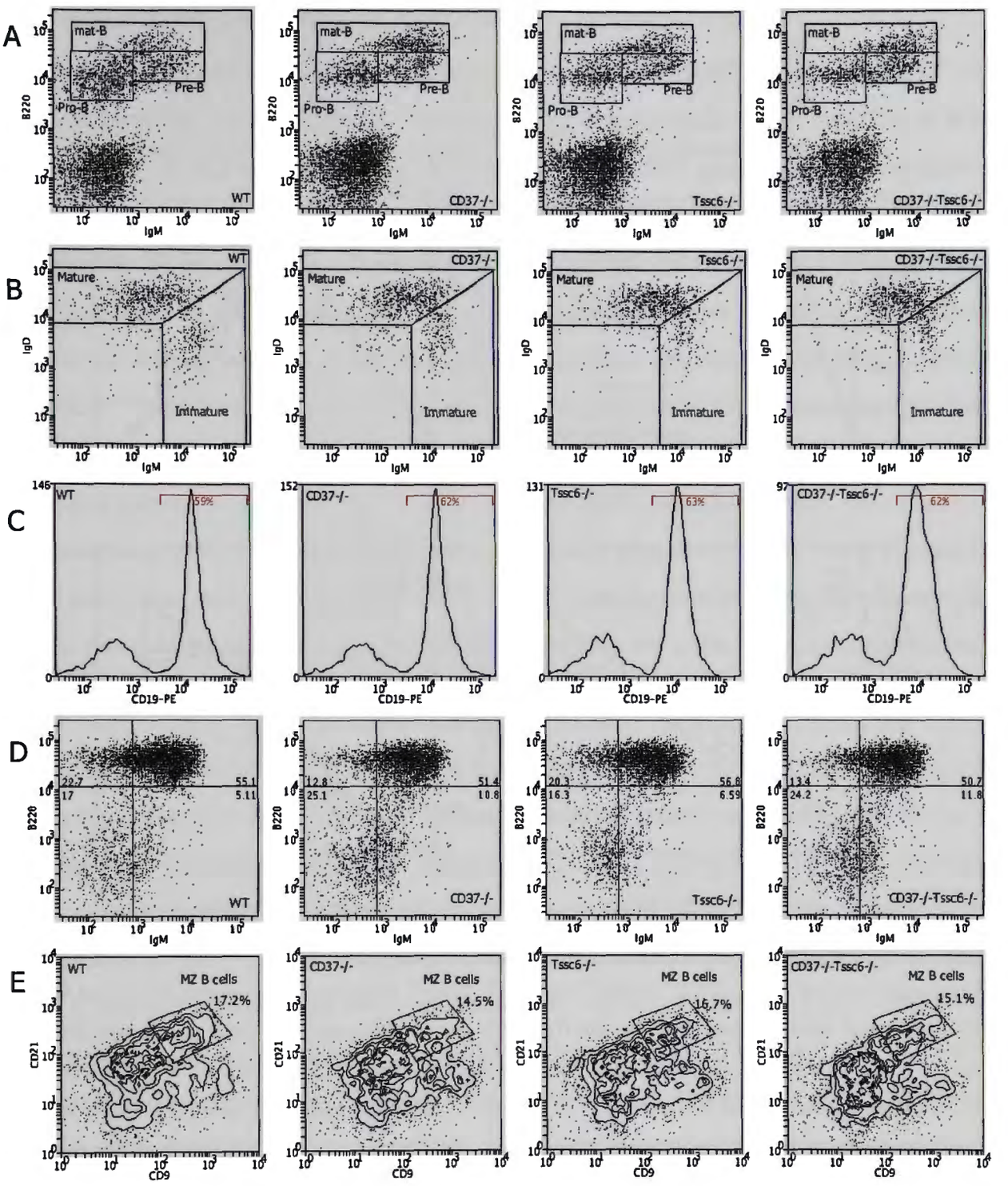

Figure 3.2 CD37 and/or Tssc6 deficient mice display normal B cell development.

(A) Bone marrow cells were stained with αB220 and αIgM, pro-B, pre-B and mature B cells are highlighted. (B) Immature and mature B cells in the peripheral blood were delineated by IgD and IgM expression. Splenic B cells were stained with (C) αCD19, (D) αB220 and αIgM and to separate marginal zone subsets; (E) αCD9 and αCD21. Lymphocytes were gated based on forward scatter and side scatter properties and the exclusion of propidium iodide (n=2, 4 mice/group).

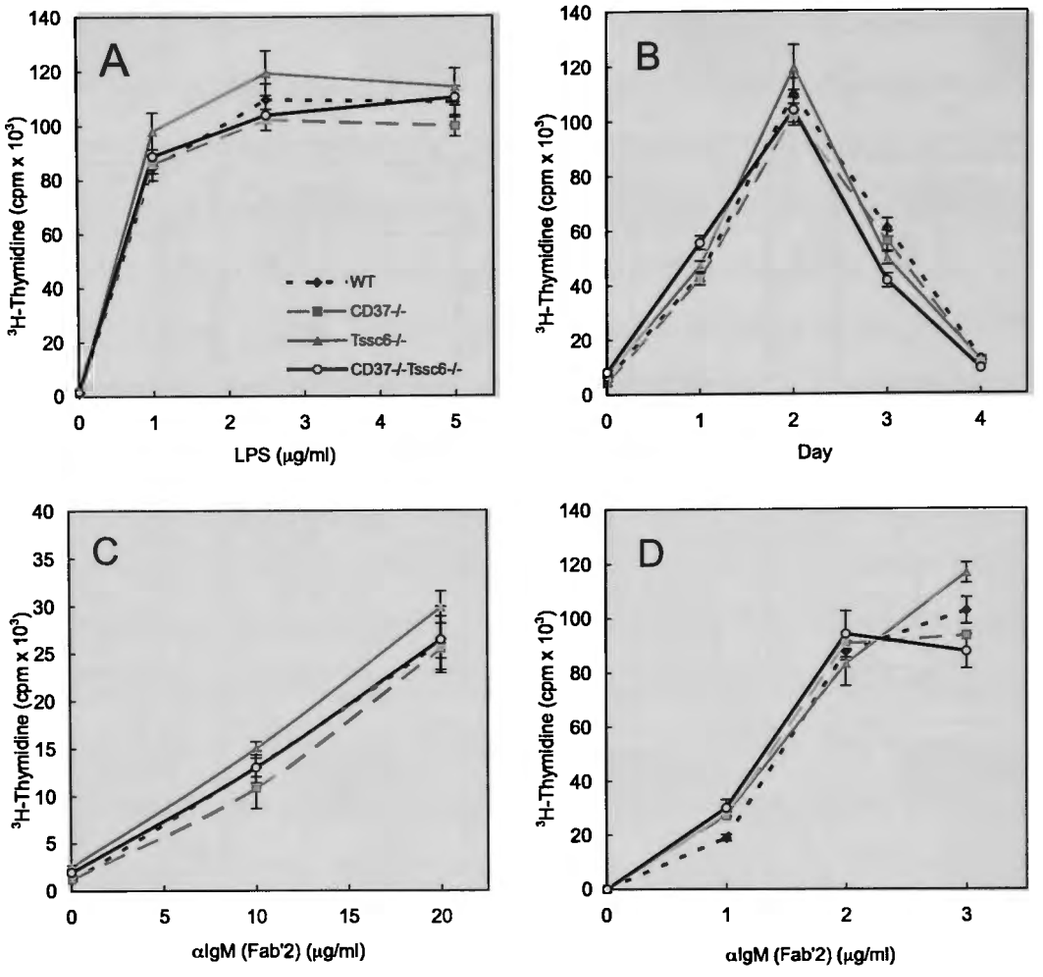

Figure 3.3 Normal in vitro proliferation of CD37$^{-/-}$Tssc6$^{-/-}$ B cells.

Splenocytes from wild type, CD37$^{-/-}$, Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ mice were isolated and stimulated in vitro with (A) titrated doses of lipopolysaccharide (LPS), (B) 2μg/ml LPS over four days, (C) titrated doses of αIgM (Fab'2) or (D) titrated doses of αIgM (Fab'2) in the presence of 50μg/ml αCD40. B cell proliferative responses were measured at 2 days unless otherwise stated. Data points represent the mean tritiated thymidine incorporation across quadruplicate wells. There were no significant differences between tetraspanin deficient mice and wild type controls when data points were tested by students t test (n=4, 2 mice/group).

3.2.2 Altered Immunoglobulin Production in CD37$^{-/-}$Tssc6$^{-/-}$ Mice

Basal immunoglobulin levels were quantified in naïve CD37$^{-/-}$, Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ mice and compared to wild type controls. Resting immunoglobulin levels are an indication of B cell homeostasis. It has been previously demonstrated that CD37$^{-/-}$ mice display altered basal Ig levels, whereby basal IgG_1 levels in naïve mice were low in comparison to wild type mice (Knobeloch *et al.*, 2000). Conversely, Tssc6 deficient mice display normal levels of immunoglobulin in unimmunised mice across all isotypes (Tarrant *et al.*, 2002). In this study, the earlier findings were confirmed and a broadly similar phenotype was identified in CD37$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ mice (Figure 3.4). In both strains deficient in CD37 expression, IgG_1 levels were low in sera from 8 week old mice. There were no significant differences between wild type and tetraspanin deficient mice in other isotypes tested. CD37$^{-/-}$ deficient mice housed in the laboratory of collaborators have recently been found to produce excess levels of basal IgA (van Spriel *et al.*, submitted for publication). Whilst these findings were not reproduced in this study, this does not exclude the possibility that under different animal housing conditions, basal IgA secretion may be altered in the absence of CD37.

In order to measure antigen specific B cell responses in the absence of CD37 and Tssc6, mice were immunised with both TI and TD antigens. Humoral responses to the hapten NP can be measured when conjugated to carrier antigens such as LPS (TI) and KLH (TD). NP can also be used to detect high and low affinity antibodies in serum by modifying the ratio of the hapten to the antigen in ELISA. When measuring TI immune responses, NP specific IgM was quantified over a period of 4 weeks post NP-LPS immunisation. CD37$^{-/-}$, Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ mice all responded with normal IgM production over the four week period (Figure 3.5A). In order to look at TD immune responses the development of serum anti-NP antibodies of the IgG_1 isotype were measured after NP-KLH immunisation. As described previously, IgG_1 responses to NP-KLH were normal in Tssc6$^{-/-}$ mice and poor in CD37$^{-/-}$ mice (Knobeloch *et al.*, 2000; Tarrant *et al.*, 2002). In this study, poor IgG_1 responses to NP-KLH were also observed in CD37$^{-/-}$Tssc6$^{-/-}$ mice (Figure 3.5C). However, this phenotype was not exaggerated in the absence of both CD37 and Tssc6 when compared to CD37$^{-/-}$ mice.

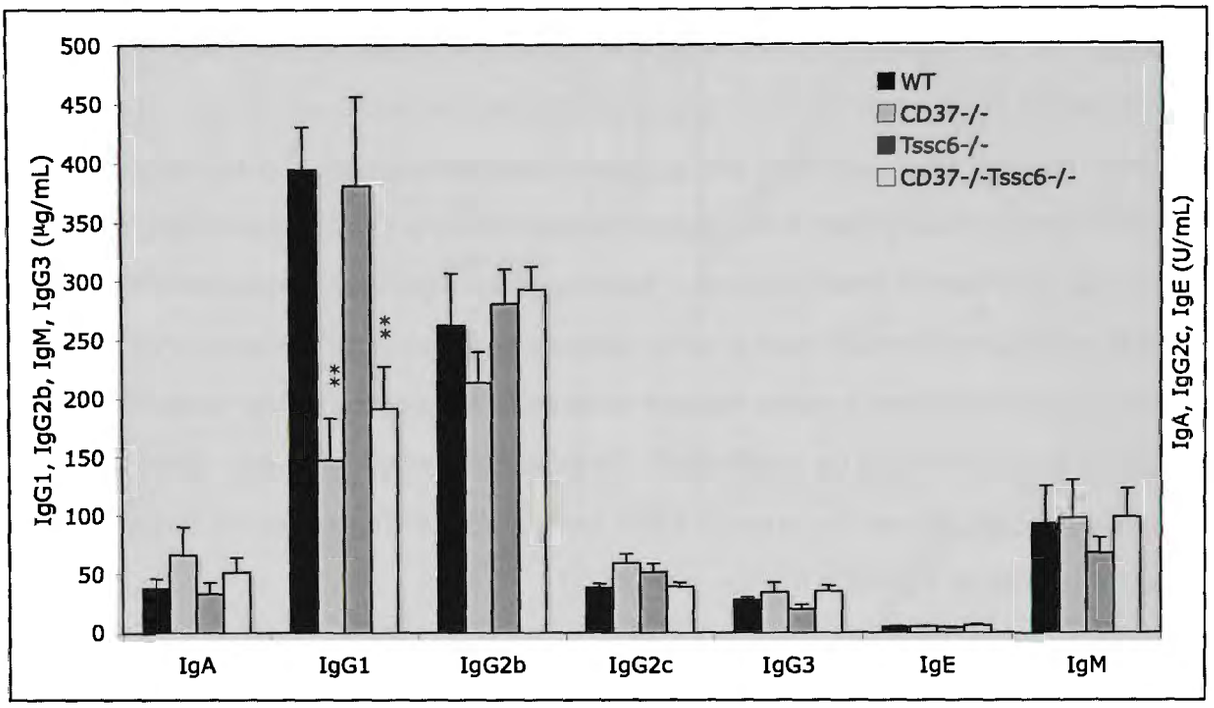

Figure 3.4 Altered basal immunoglobulin levels in CD37$^{-/-}$Tssc6$^{-/-}$ mice.

*Naïve serum immunoglobulin levels from 8 week old wild type, CD37$^{-/-}$, Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ mice were measured by isotype specific ELISA (8 mice per group). Histogram bars represent the Ig isotype concentration of individual mice and horizontal bars represent the mean of each group. Significance was tested by students t test in comparison to wild type controls (IgG$_1$, IgA and IgM n=2, 6 mice/group, IgE, IgG$_{2a+c}$, IgG$_{2b}$, 6 mice/group). *P<0.05; **, P<0.005.*

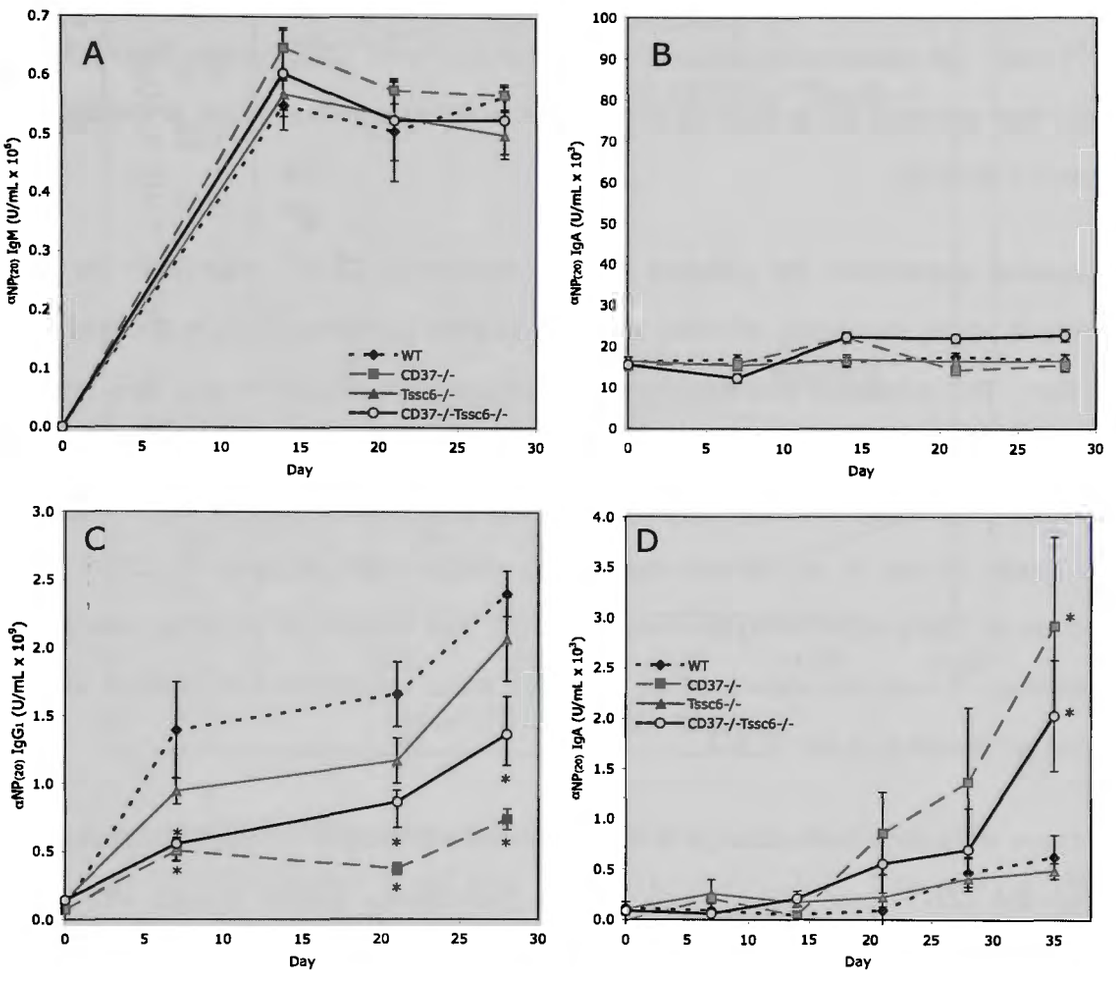

Figure 3.5 Poor humoral responses to the T cell dependent antigen NP-KLH in CD37$^{-/-}$Tssc6$^{-/-}$ mice.
*Serum immunoglobulin was assessed in wild type, CD37$^{-/-}$, Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ mice post immunisation. (A) NP specific IgM and (B) IgA responses to NP-LPS immunisation. (C) NP specific IgG$_1$ and (D) IgA responses to NP-KLH immunisation. Data points represent the mean immunoglobulin production and significance was tested by students t test in comparison to wild type controls (n-2, 6 mice/group). *, P<0.05, **, P<0.005.*

From collaborative studies, CD37$^{-/-}$ mice were known to also display elevated serum IgA responses to NP-KLH (van Spriel *et al.*, submitted for publication), whilst Tssc6$^{-/-}$ IgA responses were untested. To investigate the possibility of dysregulated IgA production in response to TI antigens, IgA levels were assessed in NP-LPS immunised mice. These assays found that IgA responses to NP-LPS are normal in all tetraspanin deficient strains tested (Figure 3.5B). IgA production post TD antigen immunisation (NP-KLH) was also normal in Tssc6$^{-/-}$ mice, however CD37$^{-/-}$Tssc6$^{-/-}$ IgA production was elevated, similar to that of CD37$^{-/-}$ mice (Figure 3.5D). This indicates that elevated IgA production in response to NP-KLH immunisation is specifically due to the absence of CD37.

One possible explanation for reduced IgG_1 production in CD37$^{-/-}$ mice may be that class switching is poorly regulated, whereby B cells switch to IgA production in preference to IgG_1 production. This possibility was examined by comparing IgA production and IgG_1 production in individual mice five weeks after NP-KLH immunisation (Figure 3.6). From this analysis, two clear trends were apparent: CD37$^{+/+}$ mice produced high levels of IgG_1 and low levels IgA post immunisation (Trend A or normal response), whilst mice deficient in CD37 expression (regardless of Tssc6 expression) produced low IgG_1 and excess IgA post immunisation (Trend B). However, it was also apparent that there are some individuals that produce low levels of IgG_1, yet no elevation of serum IgA.

Since there is no detectable change in Tssc6$^{-/-}$ B cell phenotype and no significant differences between the CD37$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ B cell phenotypes, further studies into the role of tetraspanins in humoral immunity were restricted to CD37 deficient mice.

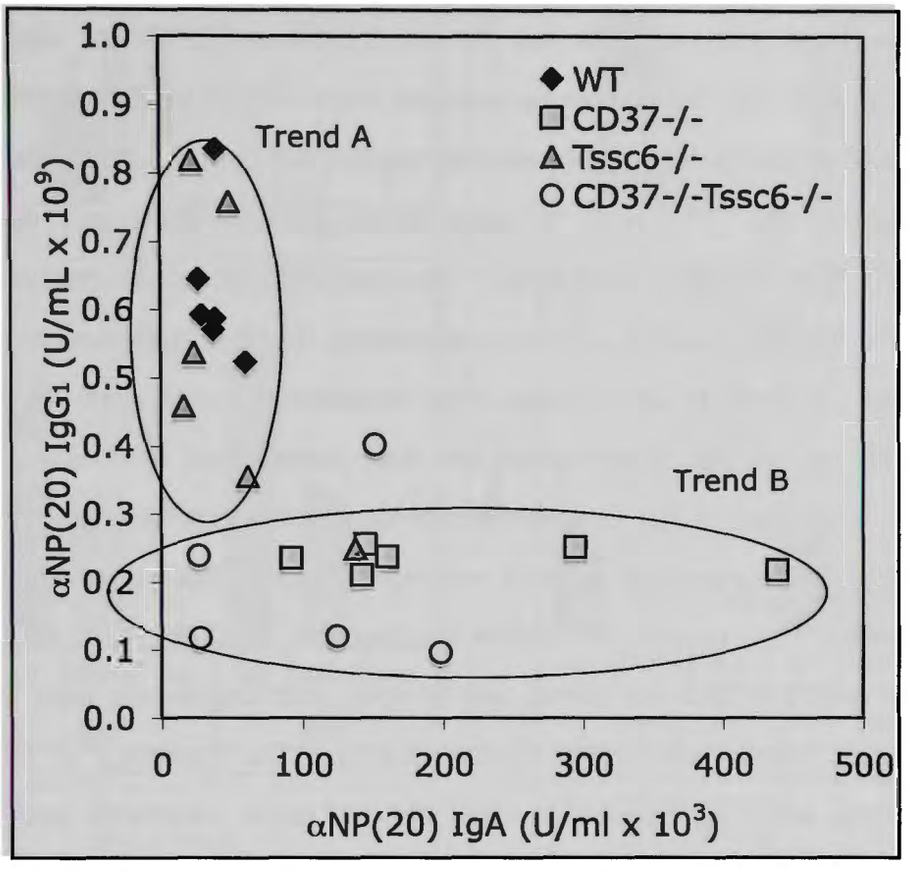

Figure 3.6 Poor correlation between excess IgA and reduced IgG_1 in the absence of CD37

Serum immunoglobulin was assessed in wild type, CD37$^{-/-}$, Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ mice 35 days post NP-KLH immunisation. NP specific IgG_1 and IgA responses to NP-KLH immunisation were compared. Trend A: high IgG_1 production and low IgA production, Trend B: low IgG_1 production and variable to high levels of IgA. Data points represent individual immunoglobulin production (6 mice/group).

The reduced IgG_1 and elevated IgA production seen in CD37$^{-/-}$ mice may be the result of two possible factors, the total frequency of NP specific ASC numbers may be reduced, or the immunoglobulin output of individual NP specific ASC may be poor. To investigate these possibilities, the frequency of ASC was determined by ELISPOT. In naïve mice, the frequency of total IgG_1 and IgA producing cells was compared between CD37$^{-/-}$ and wild type controls (Figure 3.7A-B). From this data, it can be seen that there were fewer IgG_1 secreting B cells in CD37 deficient bone marrow and slightly elevated numbers of IgA producing cells in the spleen. This suggests that the differences in basal serum immunoglobulin are due to altered frequencies of ASC in the absence of CD37. This data also hints at the reason for increased basal IgA levels detected in our collaborator's laboratory. In NP-KLH immunised mice a similar trend was seen. At both 14 and 35 days after immunisation, mice were sacrificed and the frequency of NP specific ASC in the spleen and bone marrow was determined by binding to either NP_{20} or NP_3 (Figure 3.7C-F). Antibodies binding to NP_{20} coated membranes represent both high and low affinity antibodies, whilst only high affinity antibodies are detected with NP_3. From the two week and five week time points it is clear that in CD37$^{-/-}$ mice, significantly lower frequencies of both high and low affinity anti-NP IgG_1 producing B cells were present in the spleen and bone marrow after NP-KLH immunisation. The frequency of NP-specific IgA producing B cells was also elevated in CD37 deficient bone marrow at both time points, however this difference was not significant.

The paucity of IgA producing cells in the spleen and bone marrow make these assays difficult to draw definitive conclusions from. To further refine these experiments, and determine if the trend seen in IgA ELISpot data thus far is significant, future experiments will require B cell purification to increase the sensitivity of these assays. These data correlate well with the reduced levels of NP specific IgG_1 and elevated IgA observed in CD37$^{-/-}$ mouse sera. ELISpot assays can also be used to give an indication of individual cell secretion of the immunoglobulin of interest. During these analyses it was clear that whilst the immunoglobulin output varied widely from cell to cell, there were no obvious differences in the size or intensities of the spots generated by either wild type or CD37$^{-/-}$ B cells. Together, this data suggest that the defects seen in NP-KLH responses between wild type and CD37$^{-/-}$ mice are due to altered frequencies in ASC cells and not ASC Ig secretion capacity.

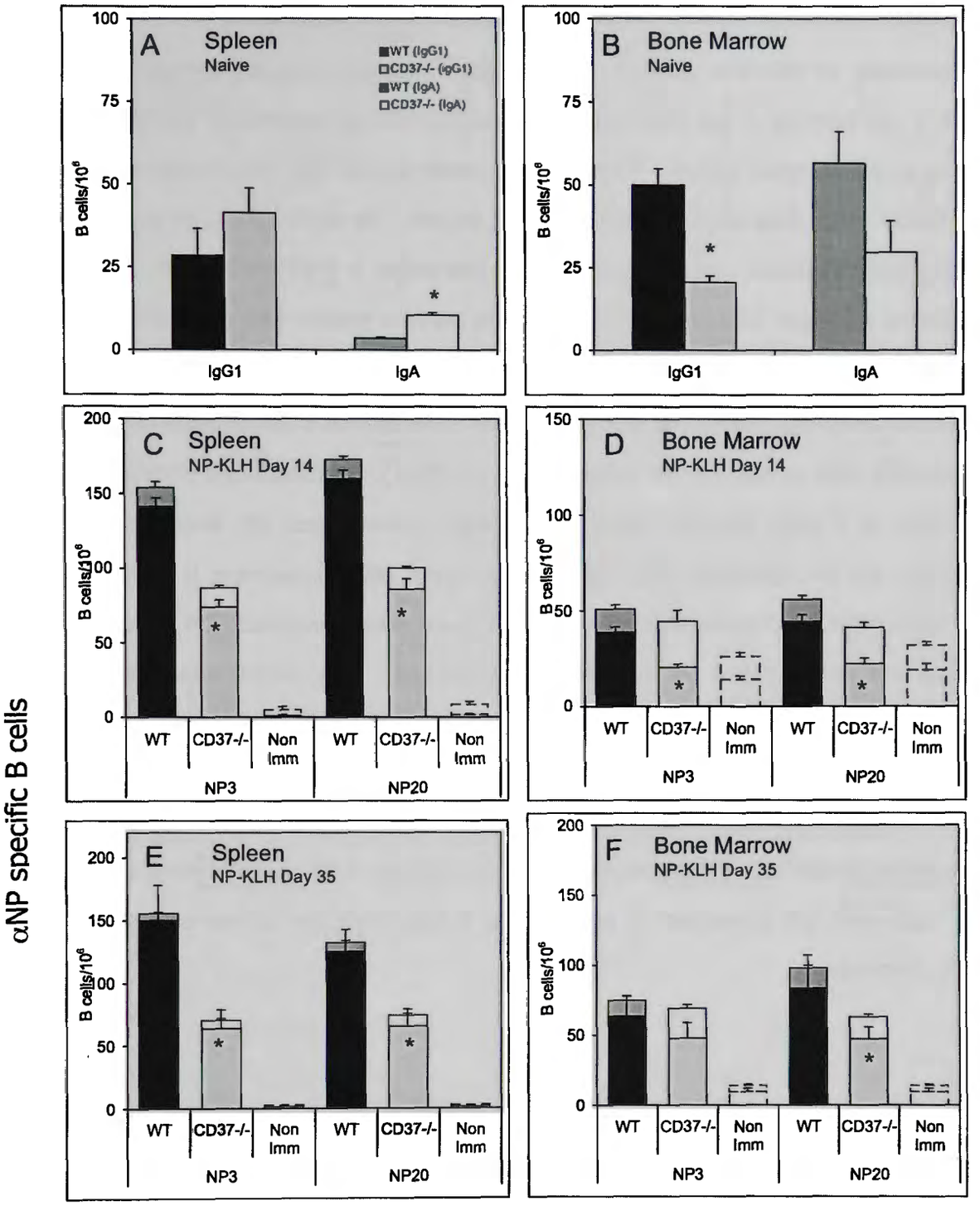

Figure 3.7 Reduced frequency of IgG$_1$ producing B cells in CD37$^{-/-}$ mice.

*(A-B) Spleen and bone marrow cells derived from naïve wild type and CD37$^{-/-}$ mice were cultured in anti-mouse Ig coated ELISpot plates to determine the basal frequency of IgG$_1$ and IgA producing B cells. Spleen and bone marrow cells derived from wild type and CD37$^{-/-}$ mice were cultured in NP-BSA-coated ELISpot plates to measure the frequency of high affinity (NP$_3$) and total (NP$_{20}$) NP-specific IgG$_1$ and IgA secreting cells after 14 days (C-D) and 35 days (E-F) post NP-KLH immunisation. Results are expressed as the mean frequency of IgG$_1$ or IgA producing cells across triplicate wells. Non-immunised mice and antigen negative wells were included as controls for NP specific assays. Significance was tested by students t test in comparison to wild type controls. *, P< 0.05 (n=3, 4 mice per group).*

3.2.3 A B CELL INTRINSIC DEFECT IN IgG_1 PRODUCTION BY CD37$^{-/-}$ B CELLS

The generation of effective antigen specific IgG_1 responses requires antigen recognition, efficient B cell homing, T cell help, isotype switching, affinity maturation, and anti-apoptotic signalling in the germinal centre. Therefore the cause of poor IgG_1 responses in CD37$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ mice may be due to a number of factors. To dissect the phenotype, chimeric mice were generated that were deficient in CD37 expression in B cells only. This was achieved by irradiating wild type mice and reconstituting the immune system with either WT (20%):μMT (80%) bone marrow or CD37$^{-/-}$ (20%):μMT (80%) bone marrow. μMT mice carry a targeted mutation in the IgM μ chain gene leading to a complete lack of B cell development, whilst all other immune cells remain normal (Kitamura *et al.*, 1991). Reconstituted mice in this study derive 80% of T cells, dendritic cells, macrophages, granulocytes etc. from the μMT bone marrow and are phenotypically wild type, whereas the B cell compartment is reconstituted by either wild type or CD37 deficient B cells. Chimeric mice were immunised with NP-KLH antigens as before and sera analysed for IgG_1 and IgA production. Mice reconstituted with CD37$^{-/-}$ B cells shared a similar phenotype to intact CD37$^{-/-}$ mice. High affinity and total NP specific IgG_1 was reduced in reconstituted CD37$^{-/-}$ sera (Figure 3.8A-B) and NP specific IgA was elevated (Figure 3.8C-D). B cell ELISpots also revealed significantly reduced frequencies of NP specific IgG_1 secreting B cells in the bone marrow and spleen (Figure 3.8E-F). Therefore, the defect in CD37$^{-/-}$ IgG_1 and IgA production in response to T cell dependent antigens is due a B cell intrinsic phenotype.

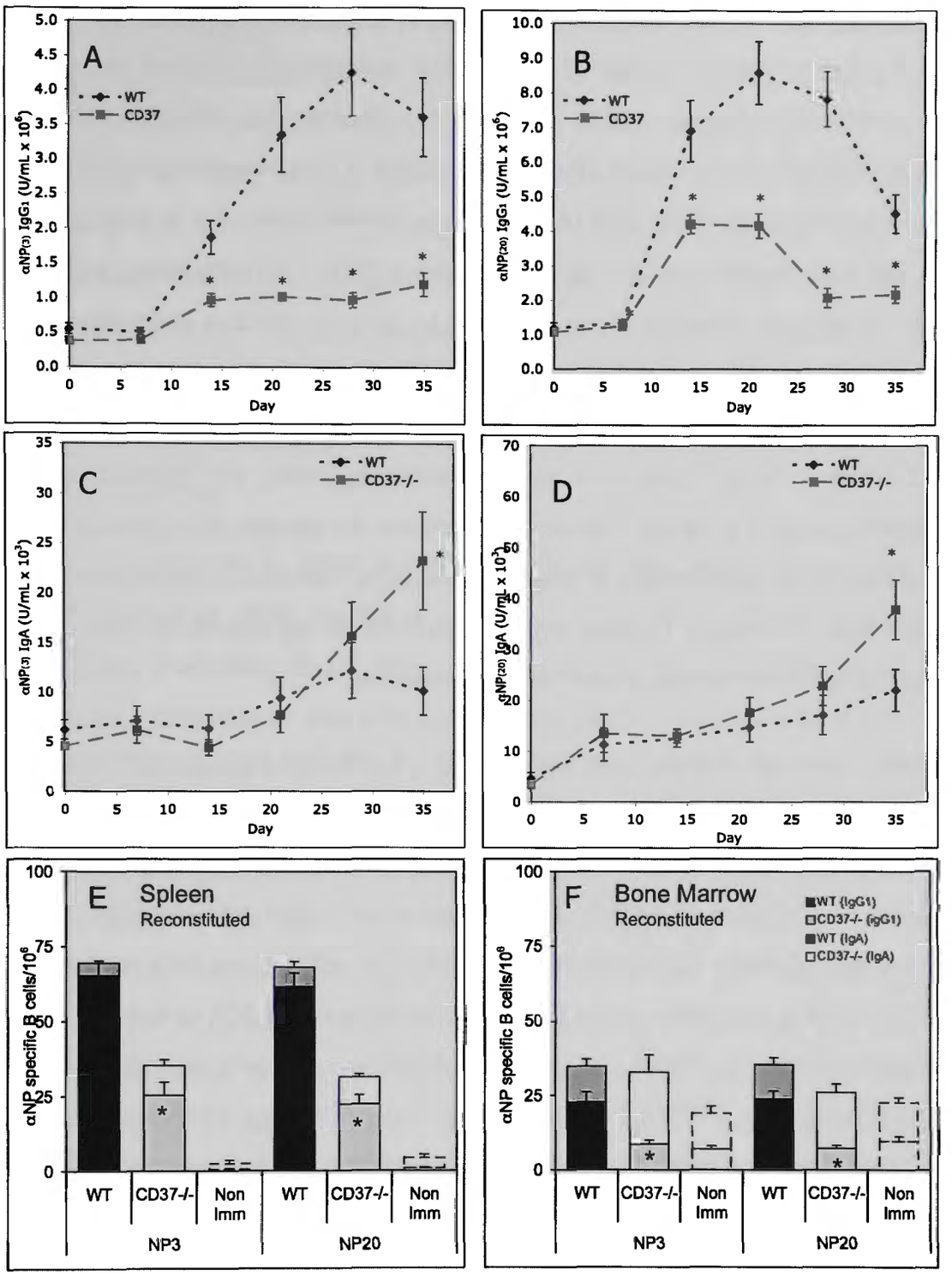

Figure 3.8 Altered humoral responses in CD37$^{-/-}$ B cell reconstituted mice.

*Wild type mice were irradiated and later reconstituted with a 1:4 mixture of either WT:μMT or CD37$^{-/-}$:μMT bone marrow cells. After recovery, mice were immunised with NP-KLH and serum collected over a 5 week period. High affinity (NP3) and total (NP20) αNP production was measured by ELISA for both IgG$_1$ (A-B) and IgA (C-D) isotypes. Data points represent the mean humoral responses of 7 mice. Spleen and bone marrow cells from reconstituted mice were cultured in NP-BSA-coated ELISpot plates 35 days (E-F) post NP-KLH immunisation. Results are expressed as the mean frequency of IgG$_1$ or IgA producing cells across triplicate wells and antigen negative wells were included as controls. Significance was tested by students t test in comparison to wild type controls. *, P< 0.05 (n=2, 7 mice per group).*

3.3 DISCUSSION

Normal B cell development is initiated by lymphoid precursors in the bone marrow. CD45R (B220) positive cells acquire surface IgM and are disseminated throughout the body via peripheral blood and lymph vessels. During this process, B cells pass through a series of stages that can be identified by their level of gene rearrangement within the Ig locus and to some degree, cell surface expression (Hardy and Hayakawa, 2001). At each stage, B cells undergo positive and negative selection events, required to eliminate inactive and autoreactive cells. From this study, we can confirm that B cells develop normally from precursors to pro-B, pre-B, immature-B to mature naïve B cells, in the absence of CD37 and Tssc6. Total B cell numbers in adult CD37$^{-/-}$ and Tsssc6$^{-/-}$ mice were also unaffected suggesting that homeostatic regulation and apoptosis occur in a normal manner. Furthermore, the absence of *both* molecules had no effect on naïve B cell development or total B cell numbers. Therefore, it is concluded that there is no functional redundancy between these tetraspanins during early B cell development that may have compensated for the absence of either molecule.

It has been previously demonstrated that in CD81$^{-/-}$ B cells, cell surface expression of CD19 is reduced by approximately 30-50% (Maecker and Levy, 1997; Miyazaki *et al.*, 1997; Tsitsikov *et al.*, 1997). A lack of CD81 expression in B cells was also shown to significantly alter the localisation of co-ligated BCR-CD19/CD21 complexes into lipid and signalling protein rich membrane microdomains (Cherukuri *et al.*, 2004b). Together these data suggest that in B cells, CD81 plays a dual role in CD19 stabilisation and regulating BCR co-receptor interactions with signalling molecules. Whilst CD37 has been shown to molecularly associate with CD81 in B cells (Angelisova *et al.*, 1994), there is no effect on cell surface CD19 expression in CD37 and/or Tssc6 deficient B cells. Therefore, it can be inferred that the absence of either or both CD37 and Tssc6 is unlikely to affect CD81 expression on the B cell surface or significantly disrupt the function of CD81 in these cells.

Both CD81 and CD37 ligation with mAbs produces an anti-proliferative effect on B cells *in vitro* (Ledbetter *et al.*, 1987; Oren *et al.*, 1990). However, the absence of either CD37 or CD81 has no effect on B cell proliferation (Knobeloch *et al.*, 2000; Miyazaki *et al.*, 1997). Due to a lack of antibodies, the effects of Tssc6 ligation in B cells are unknown, although normal Tssc6$^{-/-}$ B cell proliferation has been demonstrated. From this study, it was seen that CD37$^{-/-}$Tssc6$^{-/-}$ B cell proliferative responses were also unaffected. This data suggests that there is unlikely to be a role for either CD37 or Tssc6 in B cell proliferation obscured in the single knockout phenotypes

by functional redundancy between these two molecules. It is possible that the anti-proliferative effects induced by CD37 ligation are due to a number of factors such as cross-linking of the tetraspanin with other molecules, forcing an unusual conformation of CD37 or its natural binding partners, or even altering the localisation of molecules to either tetraspanin microdomains or lipid rafts. The stimuli used in this study were chosen to activate the B cell signalling pathways associated with either TI antigens such as LPS, which cross-link the BCR and bind pattern recognition receptors such as TLR4; or TD antigens, which cross-link the BCR and require CD40 stimulation via T cells. The normal proliferation responses demonstrated in CD37/Tssc6 deficient B cells suggest that there is no effect on B cell signalling by the absence of these two tetraspanins.

Immunoglobulin production in CD37 and Tssc6 mice has been previously reported in two studies (Knobeloch *et al.*, 2000; Tarrant *et al.*, 2002). Whilst Tssc6 deficient mice display no defect in antibody production, CD37$^{-/-}$ mice respond poorly to TD antigens and display altered basal serum Ig. Discrepancies also exist between the initial characterisation of CD37$^{-/-}$ mice and the more recent studies of CD37 null B cell function; these are likely to reflect the differing genetic backgrounds of the animals used in these studies. When backcrossed over ten times onto the C57Bl/6 background, the immunoglobulin profiles of CD37$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ mice are strikingly similar in this study. Both strains display decreased basal IgG_1, normal IgM responses to NP-LPS and poor IgG_1 responses as well as elevated IgA responses to NP-KLH (Figure 3.5). IL-6 is a cytokine produced by macrophages, B cells and Th2 cells, which promotes IgA production *in vivo* (Mora *et al.*, 2006). It has been recently shown that stimulation of the CD37 associated molecule Dectin-1 in CD37$^{-/-}$ macrophages leads to a dramatic increase in IL-6 production. This reflects a role for CD37 in regulating IL-6 production (Meyer-Wentrup *et al.*, 2007). Class switching experiments performed with CD37$^{-/-}$ B cells *in vitro* also indicated an increased capacity of CD37$^{-/-}$ B cells to produce IgA, which was mimicked in wild type B cells by the addition of exogenous IL-6 (van Spriel *et al.*, submitted for publication). It is possible that elevated IL-6 production in these mice is the primary cause of excess serum IgA in CD37$^{-/-}$ mice immunised with NP-KLH. This was confirmed by immuno-histochemical staining of IL-6 in immunised wild type and CD37$^{-/-}$ spleens, which showed excess IL-6 in CD37$^{-/-}$ GC. IL-6 neutralisation studies led to reduced IgA titres in NP-KLH immunised CD37$^{-/-}$ mice (van Spriel *et al.*, submitted for publication). The lack of elevated serum IgA in

immunised Tssc6 mice suggests that IL-6 production in Tssc6 macrophages and B cells is likely to be normal.

By investigating the frequency of IgA secreting B cells in comparison to IgG_1 secreting B cells after TD antigen immunisation it was confirmed that the lack of IgG_1 secreting cells was not due to a class switching defect from IgG_1 to IgA (Figure 3.7). Similarly, the comparison of IgG_1 and IgA titres in individual tetraspanin deficient mice post NP-KLH immunisation did not support this possibility. ELISpot data also confirmed that the poor IgG_1 titre was not due to a reduced capacity for CD37$^{-/-}$ B cells to secrete Ig. Further investigations in to the CD37$^{-/-}$ phenotype found that altered IgG_1 and IgA responses to TD antigens were B cell intrinsic.

In a collaborative study, it was demonstrated that the formation of GC in CD37$^{-/-}$ spleen was normal and that the generation of short lived IgG_1 producing cells was therefore unaffected (van Spriel *et al.*, submitted for publication). However, from immuno-histochemical staining it was clear that apoptosis within the GC was increased in CD37$^{-/-}$ B cell follicles. Since anti-apoptotic signals are derived from FoDC, which promote survival of long lived ASC, it is likely that CD37 present on B cells can enhance this interaction. The apparent similarities to the GC-competent, but long lived ASC-defective, VCAM-1 knockout strain led to the investigation of adhesion molecules in CD37$^{-/-}$ B cells (Leuker *et al.*, 2001). Likewise the wealth of documented interactions between tetraspanins and integrins also suggested a potential dysregulation of integrin function in CD37$^{-/-}$ B cells (Berditchevski, 2001). In subsequent analyses, it was established that the function of the VCAM-1 ligand $\alpha4\beta1$ was impaired in CD37$^{-/-}$ B cells, leading to dysfunctional interactions between B cells and FoDC in the germinal centre and poor B cell migration (van Spriel *et al.*, submitted for publication). Whilst the mechanism of CD37 regulation of $\alpha4\beta1$ function is yet to be determined, it is possible that intermediary molecules, including other tetraspanins, may be involved in this interaction.

This chapter confirms an important role for CD37 in humoral immunity and excludes the possibility of functional redundancy between CD37 and Tssc6 in B cell function. By inference, the phenotypes described in CD37$^{-/-}$ in B cells, such as excess IL-6 production and $\alpha4\beta1$ dysregulation, are unlikely to be shared by Tssc6 in B cells. It still remains a strong possibility that CD37 and CD81 work co-operatively in the development of humoral immune responses, since the B cell phenotypes in CD81 and CD37 deficient mice show some similarities in altered Ig responses to TD antigens. However the confounding effects of CD81 deficiency on CD19

expression will make this interaction difficult to investigate via tetraspanin double knockout mice. The reduced expression of CD19 in the absence of CD81 may also mask other roles for this tetraspanin in humoral immunity. Since the absence of CD37 does not affect CD19 expression, perhaps further characterisation of CD37$^{-/-}$ mice will provide a better approach to understanding tetraspanin microdomain function in B cells that is not confounded by altered CD19 expression.

4 *IN VITRO* CELLULAR IMMUNITY IN CD37$^{-/-}$TSSC6$^{-/-}$ MICE

4.1 INTRODUCTION

Cell mediated immunity plays a key role in defence against intracellular pathogens such as viruses and the provision of anti-tumour immunity. Effective cellular immunity is dependent upon antigen capture, processing, maturation and antigen presentation by DC; and antigen recognition, receipt of co-stimulation, cytokine production and proliferation by T cells. Therefore, interactions between DC and T cells are critical to the development of effective cellular responses (Figure 4.1). Despite the volume of literature on DC-T cell interactions, many of the regulatory processes behind receptor organisation, signalling and immunological synapse formation remain poorly defined. Increasing reports suggest that tetraspanins may play an important role in these processes.

Functional roles for multiple tetraspanins have been described in antigen processing, immunological synapse formation, MHC-TCR interactions and T cell proliferative responses (Tarrant *et al.*, 2003; Wright *et al.*, 2004b). However, little is known about the mechanisms behind these tetraspanin functions or, the level of co-operation between tetraspanins in these roles. Through *in vitro* analyses of tetraspanin deficient mice, CD37, CD81, CD151 and Tssc6 were each found to regulate T cell proliferative responses (Wright *et al.*, 2004a, Tarrant *et al.*, 2002; van Spriel *et al.*, 2004, Miyazaki *et al.*, 1997). T cells deficient in any one of these tetraspanins are hyper-proliferative and appear to have a reduced requirement for co-stimulation. A common role has also been revealed for CD37 and CD151 in regulating DC antigen presentation *in vitro* (Sheng *et al.*, submitted for publication), however DC function in other tetraspanin null mice is yet to be explored. These findings suggest that tetraspanins may display complementary roles or even functional redundancy in regulating these processes. This chapter describes the investigation of *in vitro* cellular immunity in the absence of both CD37 and Tssc6.

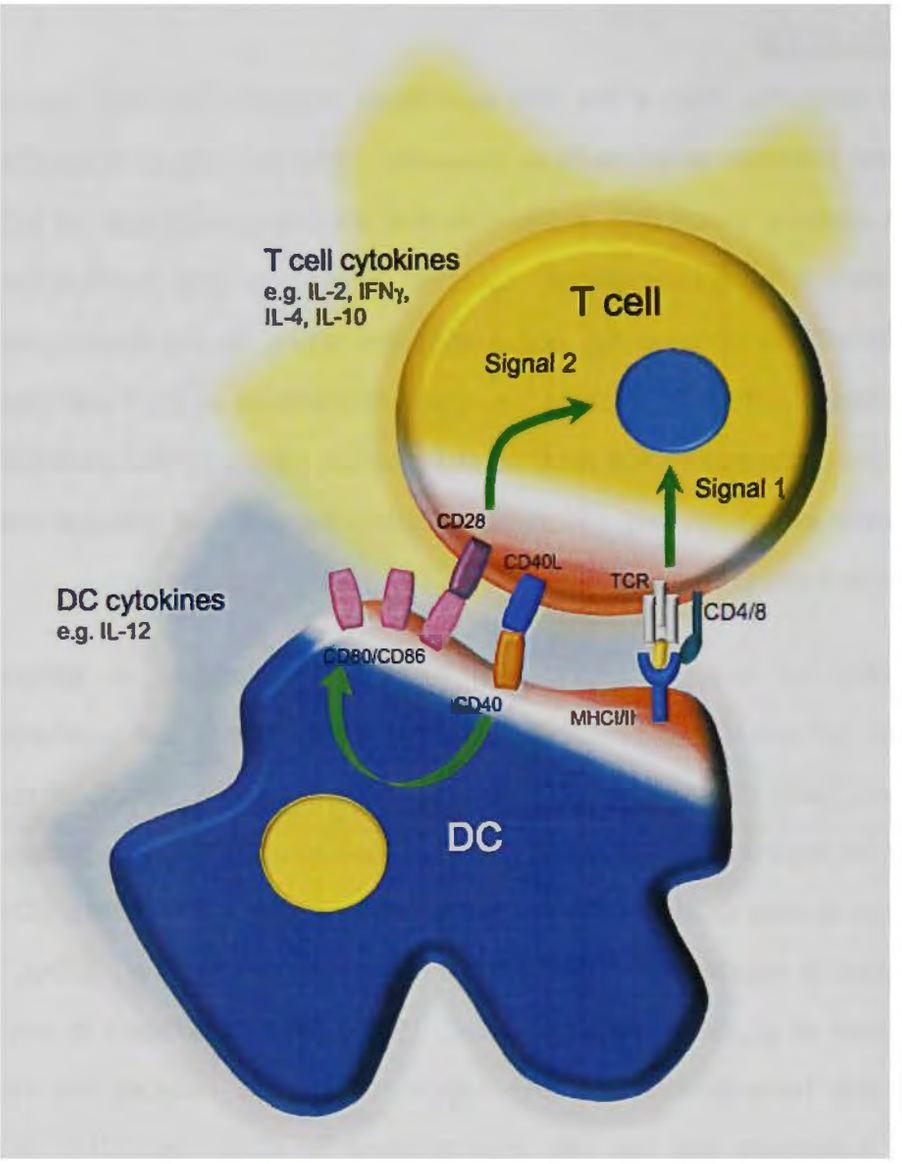

Figure 4.1 DC-T cell antigen presentation

The classic paradigm of T cell activation describes a process controlled by two signals (Janeway, 2005). Signal 1 is provided by MHC-peptide-TCR interactions and requires antigen recognition by the T cell. Signal 2 is provided by co-stimulatory molecules expressed by dendritic cells, primarily through CD40 and members of the B7 family such as CD80 and CD86. These molecules are upregulated on the DC surface after antigen capture and CD40-CD40L interactions further stimulate CD80/CD86 expression and subsequent signalling through CD28 on T cells (Cantrell, 1996). A critical event that enables these interactions to occur is the formation of the immunological synapse (IS). The IS is a highly ordered structure that is formed at the cell-cell contact site between T cells and APC. A variety of molecules, including some tetraspanins, are known to dynamically re-localise to the IS, forming two concentric rings (Delaguillaumie et al., 2004, Mittelbrunn et al., 2002, Lin et al., 2005). These are termed the central supramolecular activation complex or c-SMAC (red) and the peripheral supramolecular activation complex or p-SMAC (white). The c-SMAC is enriched in MHC/TCR and co-stimulatory molecules/receptors, whereas the p-SMAC is enriched in adhesion molecules, particularly LFA-1 ($\alpha L\beta 2$)/ ICAM-1 (Lin et al., 2005). Cytokines expressed by DC and T cells also contribute to antigen presentation by promoting T cell activation, proliferation and further cytokine secretion (Janeway, 2005).

4.1.1 Study Rationale

The similar phenotypes observed in four tetraspanin deficient mice in T cell proliferation (CD37$^{-/-}$, CD81$^{-/-}$, CD151$^{-/-}$ and Tssc6$^{-/-}$) and two tetraspanin deficient mice in antigen presentation (CD37$^{-/-}$ and CD151$^{-/-}$), suggest a co-operative role or possibly functional redundancy between tetraspanins in DC-T cell interactions. To investigate this, T cell and DC function was examined in the absence of both CD37 and Tssc6 in comparison to CD37$^{-/-}$, Tssc6$^{-/-}$ and wild type counterparts. NKT cells are thought to be important in infection and anti-tumour immunity due to their ability to respond very rapidly to antigen via cytokine release (Kronenberg and Gapin, 2002). In comparison to conventional T cells, NKT cells are restricted in their TCR repertoire and antigen reactivity, however they share many of the same pathways for activation and proliferation. Therefore, NKT cell proliferation was also examined in CD37$^{-/-}$, Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ mice. To simplify these studies, *in vitro* assays were used to characterise DC, T cell and NKT cell function in controlled conditions.

This chapter aims to:

(1) Investigate the potential for a complementary role between CD37 and Tssc6 in regulating T cell proliferation.

(2) Examine the role of CD37 and Tssc6 in regulating NKT cell proliferation.

(3) Determine if the hyper-stimulatory phenotype observed in CD37$^{-/-}$ DC is shared by Tssc6$^{-/-}$ DC.

(4) Compare the capacity of CD37 and/or Tssc6 deficient DC to co-stimulate T cells.

4.2 RESULTS

4.2.1 NORMAL T CELL DEVELOPMENT IN CD37$^{-/-}$Tssc6$^{-/-}$ MICE

T cell numbers were assessed by flow cytometry in naïve CD37$^{-/-}$Tssc6$^{-/-}$ mice in comparison to single knockout counterparts and wild type controls. Cell surface expression markers known to be associated with T cell activation and function were assessed, as well as the presence of major cell populations and subpopulations. CD37$^{-/-}$Tssc6$^{-/-}$ T cells express normal levels of TCR and CD4^{+} and CD8^{+} T cell populations were present in normal numbers (Figure 4.2A-B). Naïve CD37/Tssc6 null mice displayed no change in T cell expression of the activation markers CD25 and CD62L (Figure 4.2C-D). T cell development in the thymus was also normal, with double negative, CD4^{+}, CD8^{+} and CD4^{+}CD8^{+} double positive cells present in normal ratios (data not shown). T regulatory cells were identified via cell surface CD3 and intracellular FoxP3 staining, which revealed no significant differences in the absence of CD37 or Tssc6 (Figure 4.2E).

4.2.2 HYPER-PROLIFERATIVE T CELL RESPONSES IN TETRASPANIN DEFICIENT MICE

Previous studies have demonstrated a T cell hyper-proliferative defect in CD37$^{-/-}$ CD4^{+} and CD8^{+} T cells in the presence (αCD3+αCD28) or absence (αCD3) of co-stimulation (van Spriel *et al.*, 2004). Tssc6 knockout mice have a similar phenotype in that CD4^{+} and CD8^{+} T cells are hyper-proliferative in the absence of co-stimulation, however in the presence of co-stimulation, only CD8^{+} Tssc6$^{-/-}$ T cells are hyper-proliferative (Tarrant *et al.*, 2002). In order to investigate a potential co-operative role for CD37 and Tssc6 in T cell function, we compared wild type, CD37$^{-/-}$, Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ T cell proliferative responses. The T cell mitogen concanavalin A (conA) was used at multiple concentrations to stimulate splenocytes *in vitro*. Proliferative responses of groups of 2-3 mice from each strain were tested, which confirmed that upon stimulation with conA, CD37$^{-/-}$ and Tssc6$^{-/-}$ T cells were hyper-proliferative (Figure 4.3A-B). As suspected, CD37$^{-/-}$Tssc6$^{-/-}$ T cells were also hyper-proliferative. This difference was seen at concentrations above 1μg/ml between days 1 and 3 of culture.

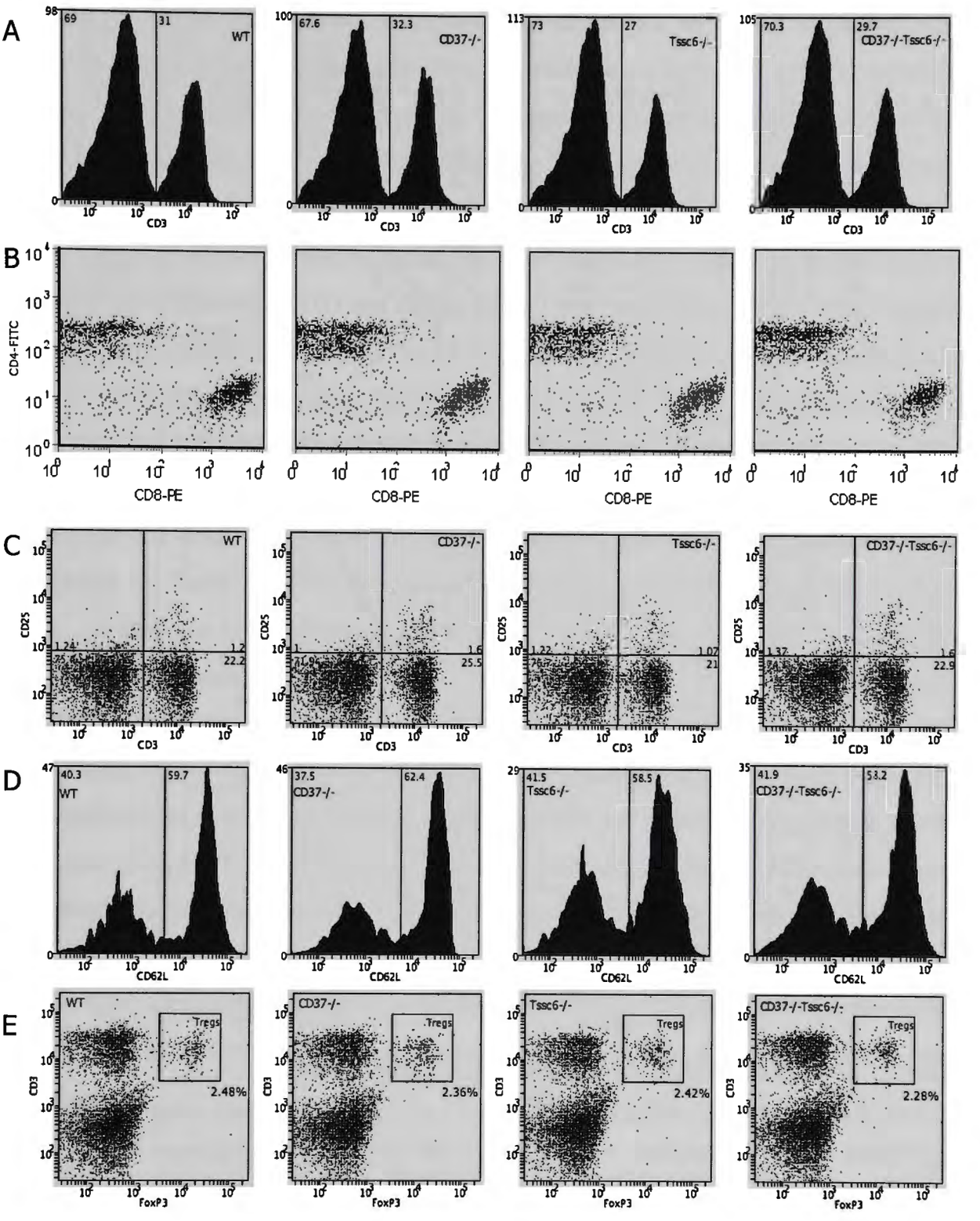

Figure 4.2 Normal T cell surface expression and population ratios in tetraspanin deficient mice.

Naïve wild type, CD37$^{-/-}$, Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ splenocytes were stained with (A) αCD3 mAbs, (B) αCD3, αCD4 and αCD8α mAbs (gated on CD3^{+} cells), (C) αCD3 and αCD25 mAbs, (D) αCD3 and αCD62L (gated on CD3^{+} cells). (E) Splenocytes were stained with αCD3 mAbs before permeabilisation and staining with FoxP3 mAbs. Autofluorescent cells were excluded via gating and in non permeabilised cell analyses stained with propidium iodide to exclude non viable cells.

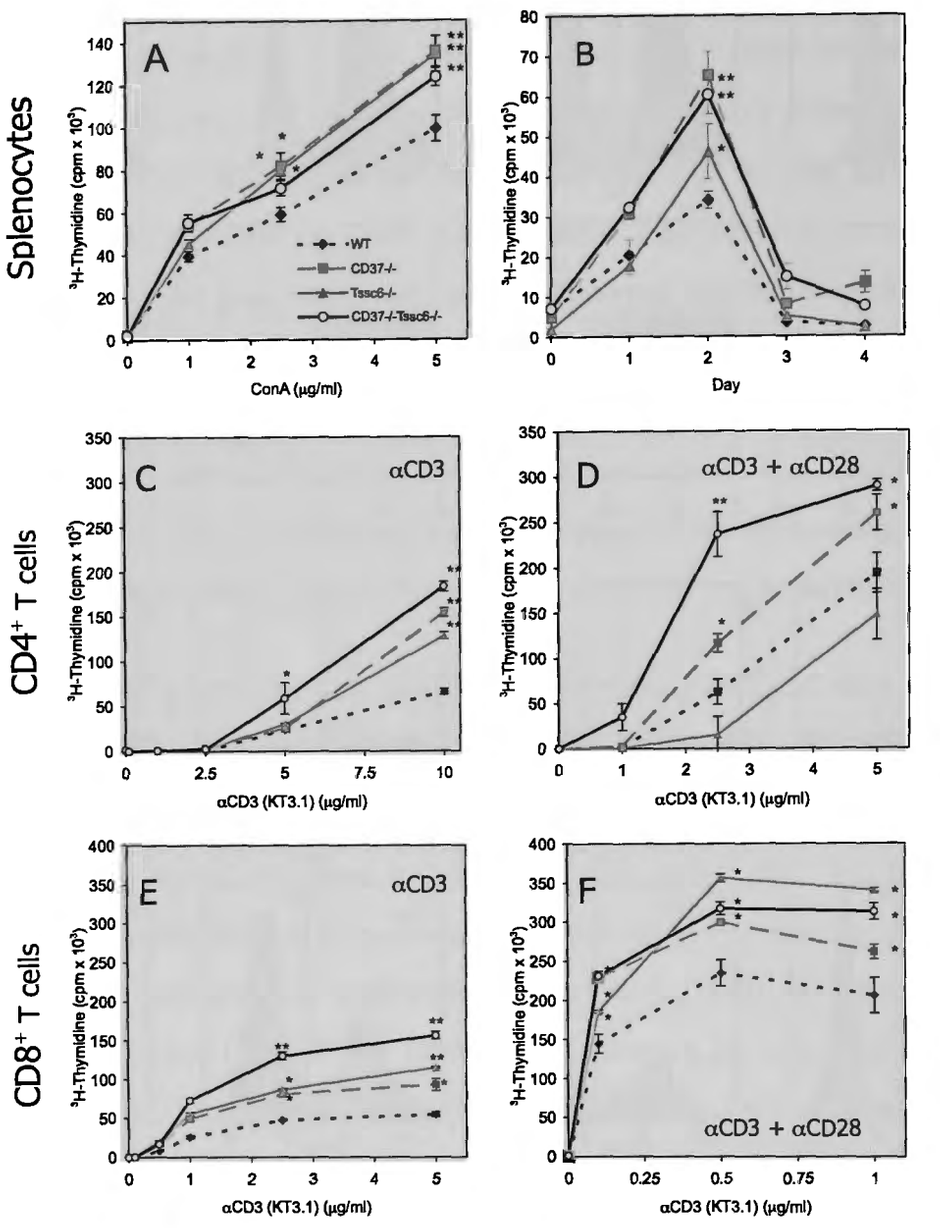

Figure 4.3 Exaggerated T cell hyper-proliferation in CD37$^{-/-}$Tssc6$^{-/-}$ in vitro.

*In separate experiments, (A) Wild type, CD37$^{-/-}$, Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ splenocytes were stimulated with titrated doses of concanavalin A over 2 days or (B) with 2.5μg/ml concanavalin-A over 4 days. Purified CD4^{+} or CD8^{+} T cells from wild type, CD37$^{-/-}$, Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ mice were stimulated over 3 days with varying concentrations of surface adsorbed αCD3 mAbs, in the presence or absence of soluble αCD28 mAbs (1μg/ml). (C) CD4^{+} T cells (αCD3), (D) CD4^{+} T cells (αCD3 + αCD28), (E) CD8^{+} T cells (αCD3), (F) CD8^{+} T cells (αCD3 + αCD28). Data points represent the mean tritiated thymidine incorporation across quadruplicate wells (splenocytes n=2, 2 mice/group; T cells n=5, 4 mice/group). Significance was tested by students t test in comparison to wild type controls. *, P< 0.05; **, P< 0.005.*

In order to further refine these experiments, T cell subsets were purified by antibody – magnetic bead depletion to reduce the effect of contaminating cells, and antigen presentation was simulated by direct engagement of the T cell receptor complex. The αCD3 mAb KT3-1.1 acts as a TCR agonist and induces signalling via the T cell activation pathway. In the absence of co-stimulation this response is subdued in wild type mice; however the addition of αCD28 mAbs mimics co-stimulation and enhances the T cell response. It has been previously shown that upon stimulation with αCD3 and αCD28 mAbs, CD37$^{-/-}$ and Tssc6$^{-/-}$ total T cells are hyper-proliferative (Knobeloch *et al.*, 2000; Tarrant *et al.*, 2002). This defect was most striking in the absence of co-stimulation. When CD37$^{-/-}$ and Tssc6$^{-/-}$ T cells were separated into CD4^{+} and CD8^{+} subsets, CD8^{+} T cell populations were hyper-proliferative with and without co-stimulation. In CD37$^{-/-}$ and Tssc6$^{-/-}$ CD4^{+} T cells, hyper-proliferation was also observed in the absence of co-stimulation but only CD37$^{-/-}$ CD4^{+} T cells were hyper-proliferative in the presence of co-stimulation i.e. Tssc6$^{-/-}$ CD4^{+} T cell responded normally to αCD3+αCD28 stimulation. In this study, we identified hyper-proliferation in both CD4^{+} and CD8^{+} CD37$^{-/-}$Tssc6$^{-/-}$ T cells in both the presence and absence of co-stimulation (Figure 4.3C-F). This hyper-proliferative defect is significantly exaggerated in CD4^{+} and CD8^{+} double knockout T cells in the absence of co-stimulation, although this exaggeration could was only observed in CD4^{+} T cells in the presence of co-stimulation. Together these data suggest that CD37 and Tssc6 play a complementary role in regulating murine T cell proliferation. To confirm the hyper-proliferation observed in tritiated thymidine cultures, wild type and CD37$^{-/-}$Tssc6$^{-/-}$ T cell subsets were purified and stained with carboxyfluorescein succinimidyl ester (CFSE) and incubated with surface adsorbed αCD3 mAbs (Figure 4.4). As observed in thymidine cultures, CD37$^{-/-}$Tssc6$^{-/-}$ T cells were hyper-proliferative when compared to wild type T cells and supernatants from these assays were used in future experiments.

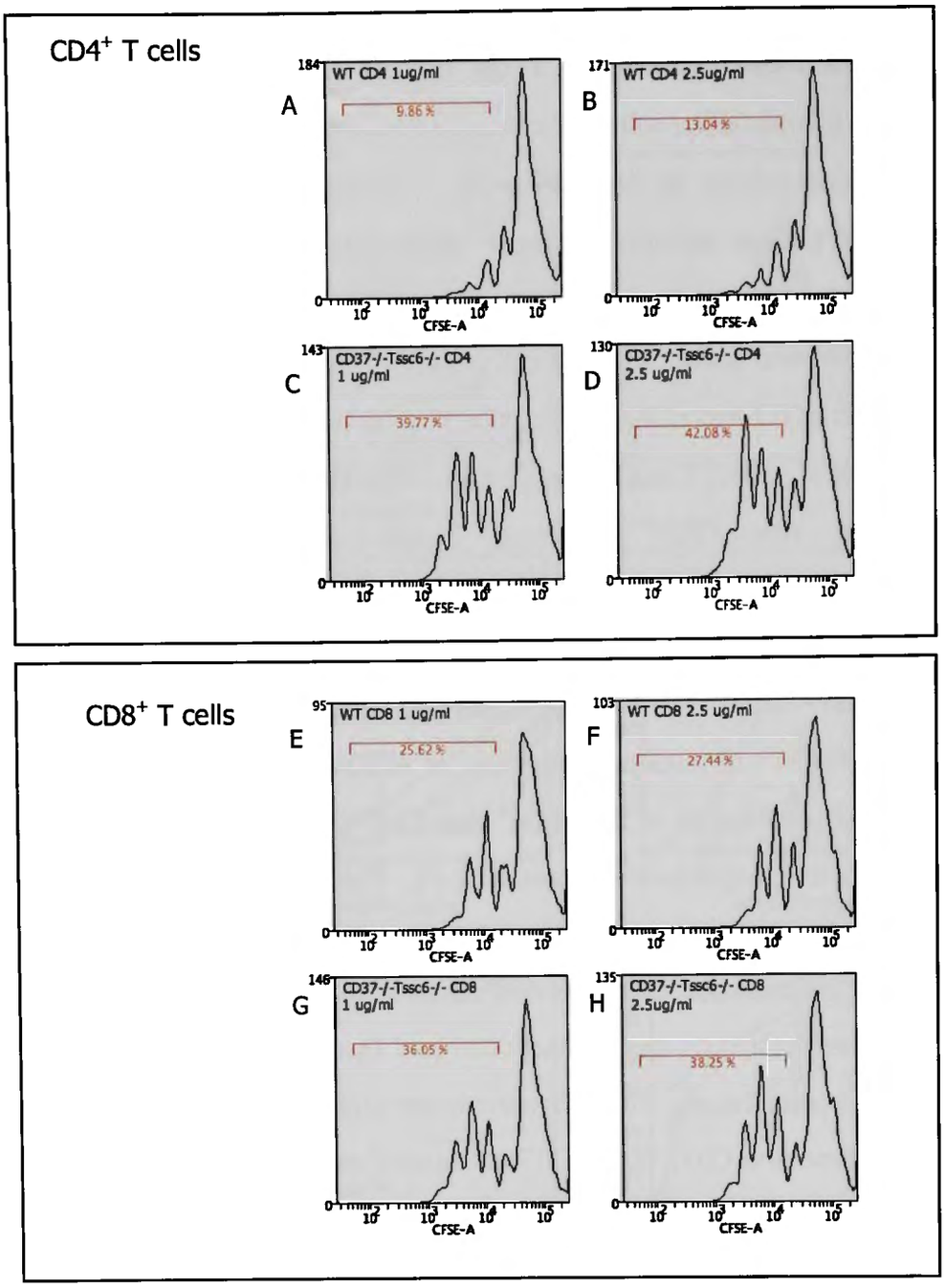

Figure 4.4 Exaggerated hyper-proliferation in CD37$^{-/-}$Tssc6$^{-/-}$ T cells – in vitro

CFSE labelled, purified T cells from wild type and CD37$^{-/-}$Tssc6$^{-/-}$ mice were stimulated with 1.0μg/ml and 2.5 μg/ml surface adsorbed αCD3 mAbs. (A-B) WT CD4^{+} T cells, (C-D) CD37$^{-/-}$Tssc6$^{-/-}$ CD4^{+} T cells, (E-F) WT CD8^{+} T cells, (G-H) CD37$^{-/-}$Tssc6$^{-/-}$ CD8^{+} T cells. After 3 days, cell division was assessed by flow cytometry. Unstimulated T cells were used as negative controls in these assays. Data points represent the percentage of propidium iodide negative cells which have undergone >2 divisions (4 mice/group).

4.2.3 T CELL CYTOKINE PRODUCTION – IN VITRO

The defect in CD37$^{-/-}$ and Tssc6$^{-/-}$ T cell proliferation is attributed to early upregulation of the T cell cytokine IL-2 (Tarrant *et al.*, 2002; van Spriel *et al.*, 2004). It is possible that cytokine production is further dysregulated in the absence of both CD37 and Tssc6, which may not only contribute to hyper-proliferative responses but also induce potential defects in T cell effector functions. Using supernatants derived from purified CFSE labelled T cells stimulated with αCD3 (Figure 4.4), cytometric bead array (CBA) analysis was used to measure production of the following cytokines: IL-2, IL-4, IL-5, IFNγ and TNFα. From this data it is clear that there is no difference in naïve T cell cytokine production between CD37$^{-/-}$, Tssc6$^{-/-}$, CD37$^{-/-}$Tssc6$^{-/-}$ and wild type supernatants in the absence of stimulation (Figure 4.5A-B). Therefore there is no constitutive upregulation of cytokines in the absence of CD37 and Tssc6 contributing to the hyper-proliferative T cell responses detected (Figure 4.5A-B). However, when stimulated with αCD3 mAbs, there was a clear difference in CD37$^{-/-}$Tssc6$^{-/-}$ T cell cytokine output in comparison to wild type controls. Not surprisingly, little difference was observed between CD37 and Tssc6 single knockout and wild type T cell supernatants, as previous studies have shown that early IL-2 upregulation was only detectable in the presence of co-stimulation. However, excess IL-2 production was clear in the CD8^{+} double knockout counterparts even in the absence of αCD28 (Figure 4.5C). Furthermore, in CD37$^{-/-}$Tssc6$^{-/-}$ CD8^{+} T cell cultures, IL-4 production was also elevated. IFNγ was in excess in both CD4^{+} and CD8^{+} double knockout stimulated T cell supernatants (Figure 4.5C-D). Due to the proliferation inducing effect of IL-4 on T cells (Yokota *et al.*, 1986), it can be concluded that both IL-2 and IL-4 contribute to the exaggerated CD37$^{-/-}$ Tssc6$^{-/-}$ hyper-proliferative phenotype seen in CD8^{+} cultures *in vitro*.

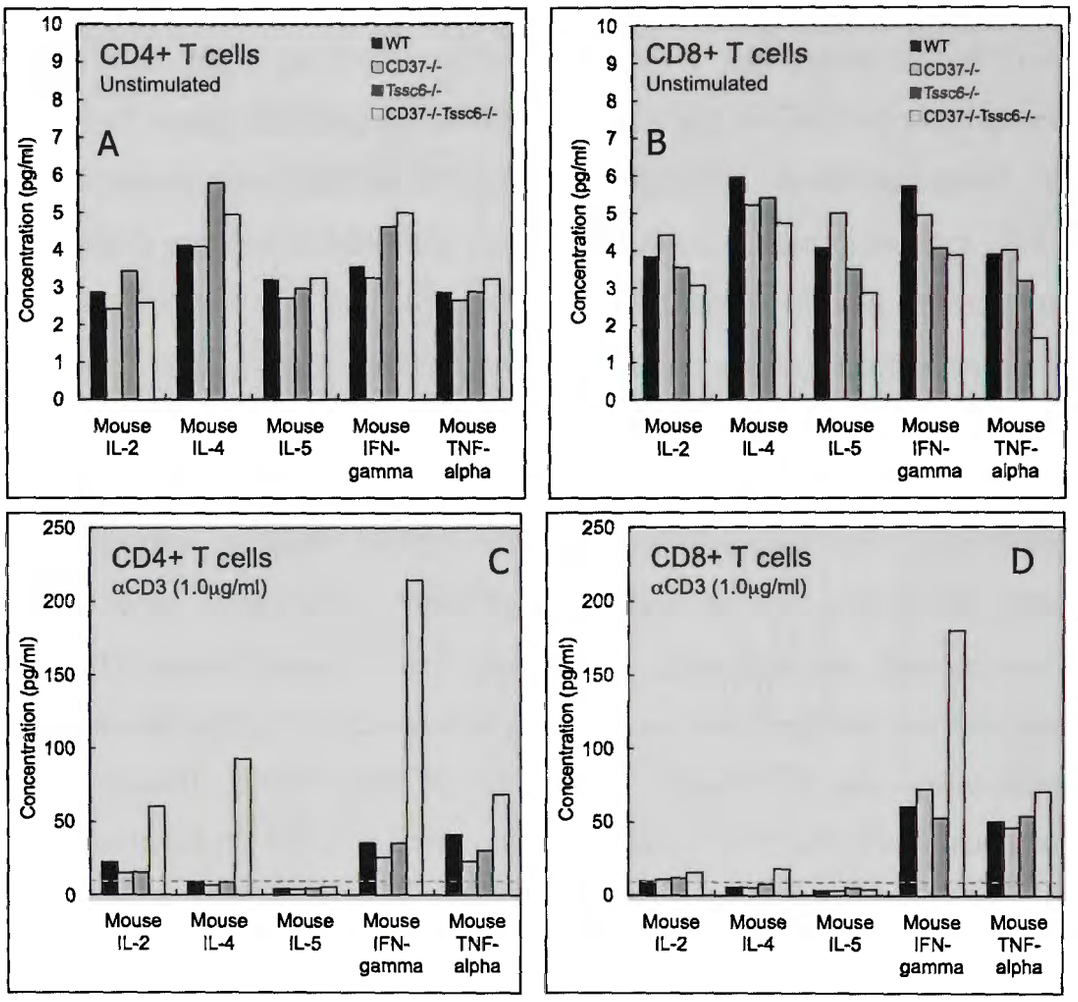

Figure 4.5 Altered cytokine production by CD37$^{-/-}$Tssc6$^{-/-}$ T cells – in vitro

Purified T cells from wild type, CD37$^{-/-}$, Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ mice were stimulated with 1μg/ml of surface adsorbed αCD3 mAbs. After 3 days, supernatants were collected and a selection of Th1/Th2 cytokines measured via CBA array. (A) Unstimulated CD8^{+} T cells, (B) Unstimulated CD4^{+} T cells (C) αCD3 stimulated CD8^{+} T cells and (D) αCD3 stimulated CD4^{+} T cells. Cytokine detection below 10pg/ml was dismissed as below the level of accurate detection in these assays (dotted lines) (4 mice/group).

4.2.4 NKT CELLS ARE HYPER-PROLIFERATIVE IN CD37$^{-/-}$TSSC6$^{-/-}$ MICE

The initial characterisation of NKT cells in CD37/Tssc6 null mice was performed using αCD3 and αNK1.1 FACS analyses. Since NK1.1 is not expressed on all NKT cells and given the functional defect demonstrated below, this analyses was repeated using αCD3 mAbs and the CD1d-αGal-Cer tetramer. From these results it was clear that the number of splenic NKT cells in CD37$^{-/-}$ Tssc6$^{-/-}$ naïve mice was significantly elevated when compared to single knockout and wild type controls (Figure 4.6). High levels of both IL-4 and IFNγ secretion are hallmarks of NKT cell effector function (Kronenberg and Gapin, 2002). To determine if CD37/Tssc6 deficient NKT cells were hyper-proliferative, splenic NKT cells were enriched and stimulated with varying concentrations of the NKT cell antigen alpha-galactosyl-ceramide (αGal-Cer). NKT cell division was tracked by CFSE labelling and FACS analyses of CD1d-αGal-Cer tetramer positive cells. These experiments demonstrated that at both 5ng and 50ng doses of αGal-Cer, there is a novel hyper-proliferative phenotype in NKT cells derived from CD37$^{-/-}$Tssc6$^{-/-}$ double knockout mice, which was not shared by CD37$^{-/-}$ or Tssc6$^{-/-}$ single knockout counterparts (Figure 4.7). This suggests that there is functional redundancy between the tetraspanins CD37 and Tssc6 in regulating NKT cell development and proliferation.

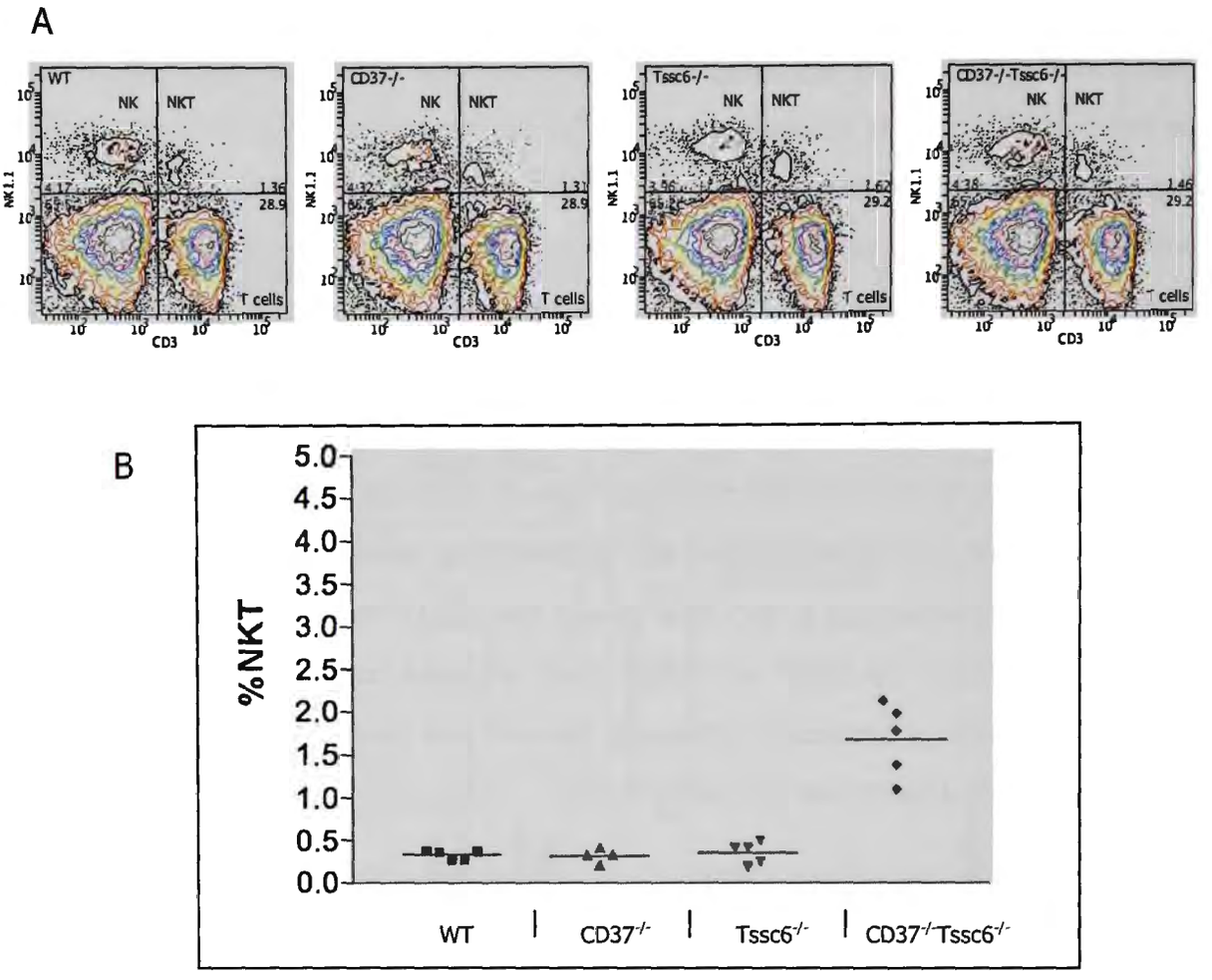

Figure 4.6 Increased numbers of NKT cells in CD37$^{-/-}$Tssc6$^{-/-}$ spleens.

Splenocytes were prepared from wild type, CD37$^{-/-}$, Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ mice and NKT cell numbers assessed by flow cytometry. (A) NK1.1^{+} NKT cells were identified by αCD3 and αNK1.1 mAb staining (n=2, 2 mice/group). (B) αGal-Cer reactive NKT cells were identified by αTCR and CD1d/αGal-Cer tetramer staining (n=2, 5 mice/group).

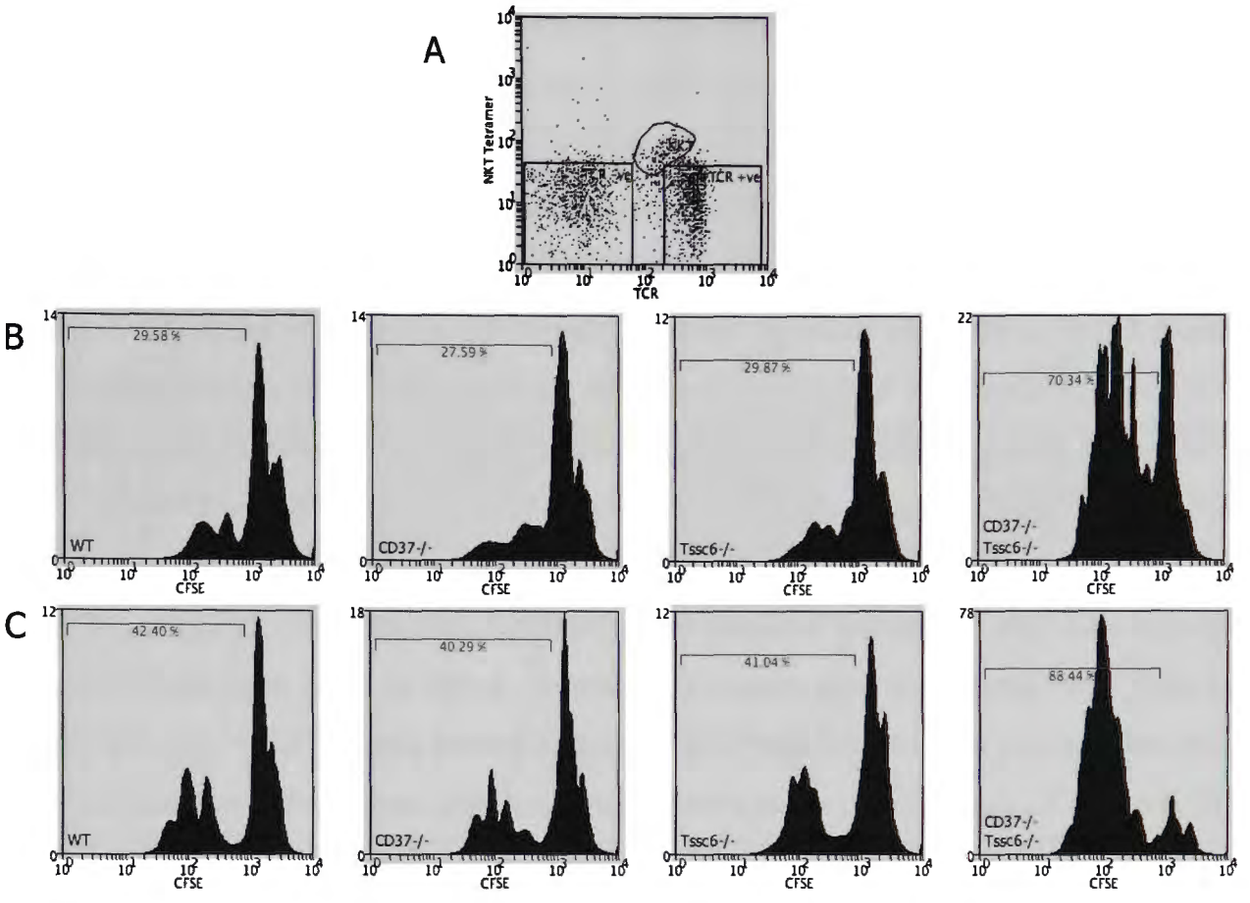

Figure 4.7 CD37$^{-/-}$Tssc6$^{-/-}$ NKT cells are hyper-proliferative when stimulated with αGal-Cer

NKT cell enriched splenocyte preparations from wild type, CD37$^{-/-}$, Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ mice were CFSE labelled and cultured in the presence of αGal-Cer. After 2.5 (data not shown) or 3.5 days, NKT cells were identified by FACS analysis as αGal-Cer/CD1d tetramer^{+}/TCR^{+} as shown in (A). NKT were gated and cell division was assessed by CFSE. The percentage of dividing NKT cells are indicated in response to (B) 5ng/ml and (C) 50ng/ml of αGal-Cer. Unstimulated controls were used for all strains tested and no cell division was detected. This result was seen in two independent experiments (n=2, 4 mice/group).

4.2.5 DC DEVELOPMENT IS NORMAL IN CD37/TSSC6 DEFICIENT MICE

To investigate a potential role for CD37 and Tssc6 in dendritic cell development, knockout lymphoid organs were harvested, DC isolated and the DC surface characterised to delineate the major subpopulations (Figure 4.8). The integrin αXβ2 (CD11c) is the primary marker of murine DC and equal cell surface expression was confirmed across all tetraspanin deficient strains to ensure future comparisons would be unbiased. Splenic DC are typically subdivided based on CD4 and CD8 expression, whilst lymph node (LN) DC are divided based on the expression of CD8 and the C-type lectin CD205 (DEC205). Splenic CD11c^{+} cells were isolated via density cut and magnetic bead depletion and normal numbers of CD4^{+}CD8^{-}, CD4^{-}CD8^{+} and CD4^{-}CD8^{-} (DN) populations were present. Similarly, CD11c^{+} cells were isolated from mesenteric and subcutaneous LNs and normal numbers of DEC205^{-}CD8^{-}, DEC205^{lo}CD8^{lo}, DEC205^{hi}CD8^{int} and DEC205^{int}CD8^{hi} populations were present in all strains. In the LN these populations represent both resident (DEC205^{-}CD8^{-}, DEC205^{int}CD8^{hi}) and skin derived (DEC205^{lo}CD8^{lo}, DEC205^{hi}CD8^{int}) DC, including Langerhans cells. This provides some indication also that the cellularity of these peripheral populations is likely to be normal in CD37/Tssc6 knockout mice. The key antigen-presentation molecules MHC class I (data not shown) and MHC class II were also expressed at normal levels within CD37/Tssc6 null mice (Figure 4.9). The expression of MHC class II and co-stimulatory molecules on DC membranes can be used as a marker of cell maturity and activation state. Naïve DC populations present in lymphoid organs display low to intermediate levels of CD40, CD80 and CD86 expression and high levels of MHC class II, which are all upregulated upon DC activation. These co-stimulatory and MHC molecules were all expressed at comparable levels in wild type and CD37/Tssc6 deficient DC (Figure 4.9).

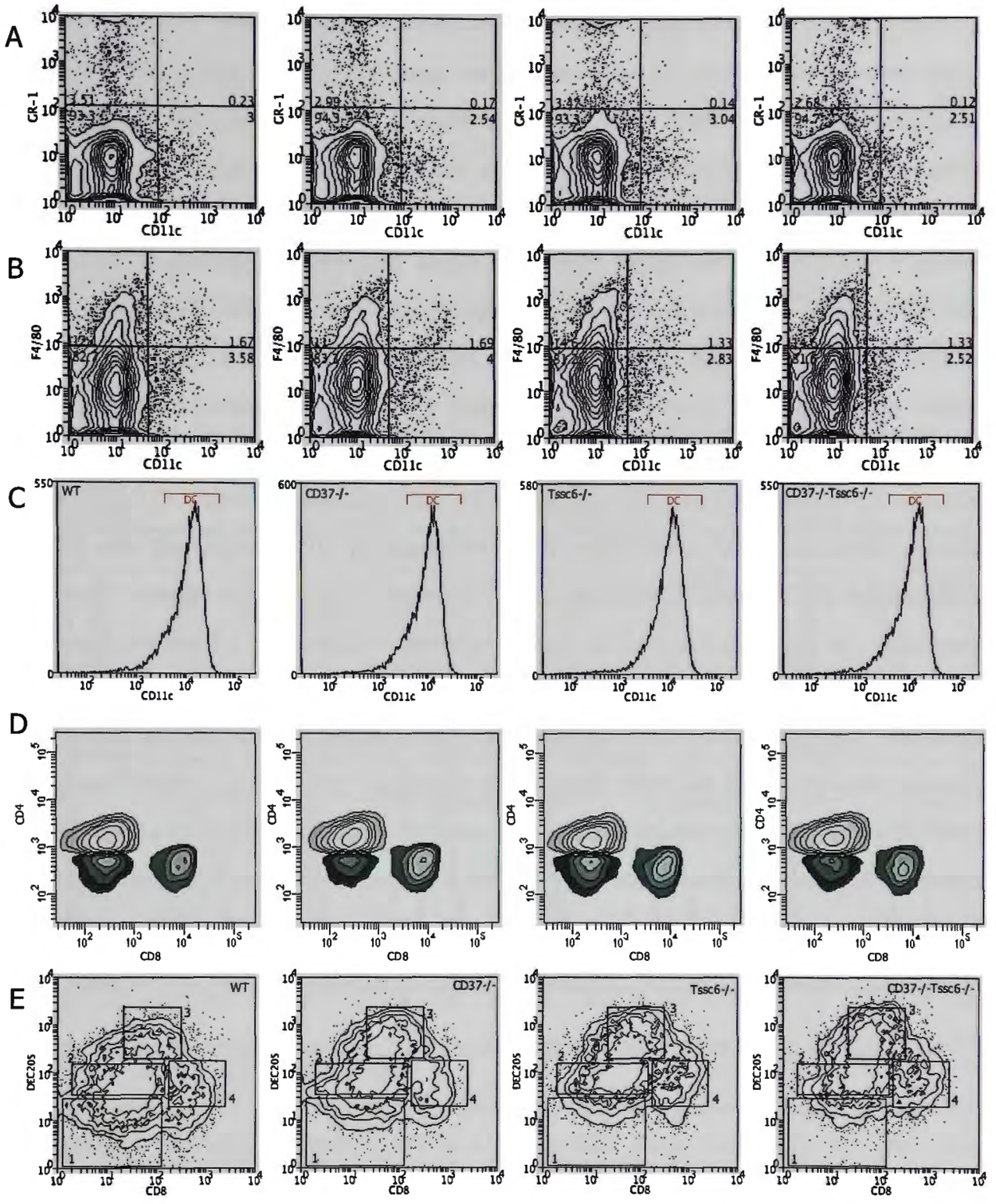

Figure 4.8 Dendritic cell Surface Markers on Tetraspanin Knockout Mice

(A-B) Splenocytes from wild type, CD37$^{-/-}$, Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ mice were isolated and dendritic cells identified by CD11c expression. Dendritic cells were then purified by density centrifugation and magnetic bead selection. (C) CD11c mean fluorescence intensity was normal in CD37/Tssc6 null mice. (D) Splenic DC subsets were identified as CD4^{+}CD8^{-}, CD4^{-}CD8^{+} and CD4^{-}CD8^{-} and (E) Lymph node DC subsets identified as (1) DEC205^{-}CD8^{-}, (2) DEC205^{lo}CD8^{lo}, (3) DEC205^{hi}CD8^{int} and (4) DEC205^{int}CD8^{hi} (n=>4, 2 mice/group).

4.2.6 In vitro antigen presentation by CD37$^{-/-}$Tssc6$^{-/-}$ dendritic cells

Recent data has demonstrated a hyper-stimulatory capacity for CD37$^{-/-}$ and CD151$^{-/-}$ dendritic cells (Sheng *et al.*, submitted for publication), whereas Tssc6 deficient DC had not been previously described. To assess DC function in Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ mice, the same experimental design was used to compare DC function with CD37$^{-/-}$ and wild type DC. By utilising T cells from the transgenic OT-I/OT-II mouse lines, T cell responses to tetraspanin knockout dendritic cells were assessed in an antigen specific manner. Knockout and wild type DC were isolated and pulsed with the ovalbumin peptides SIINFEKL and ISQAVHAAHAEINAGR (Helper peptide). These cells were then incubated with T cells expressing transgenic T cell receptors specific for each of these peptides. Ignoring cross-presentation, SIINFEKL is presented by DC on MHC Class I and recognised by CD8^{+} transgenic T cells (OT-I) and Helper peptide is presented by DC on MHC Class II and recognised by CD4^{+} transgenic T cells (OT-II). In this model, MHC presentation can be isolated while excluding potential variation in antigen processing. As shown in Figure 4.10, it was confirmed that transgenic T cell responses are higher when peptide is presented by CD37 deficient DC, and a similar but less striking phenotype was identified in Tssc6 deficient DC (Figure 4.10). These findings indicate an important regulatory role for these tetraspanins in antigen presentation. Furthermore, this hyper-stimulatory capacity was enhanced in DC deficient in *both* CD37 and Tssc6 expression, suggesting that these molecules are likely to have a complementary role in regulating antigen presentation.

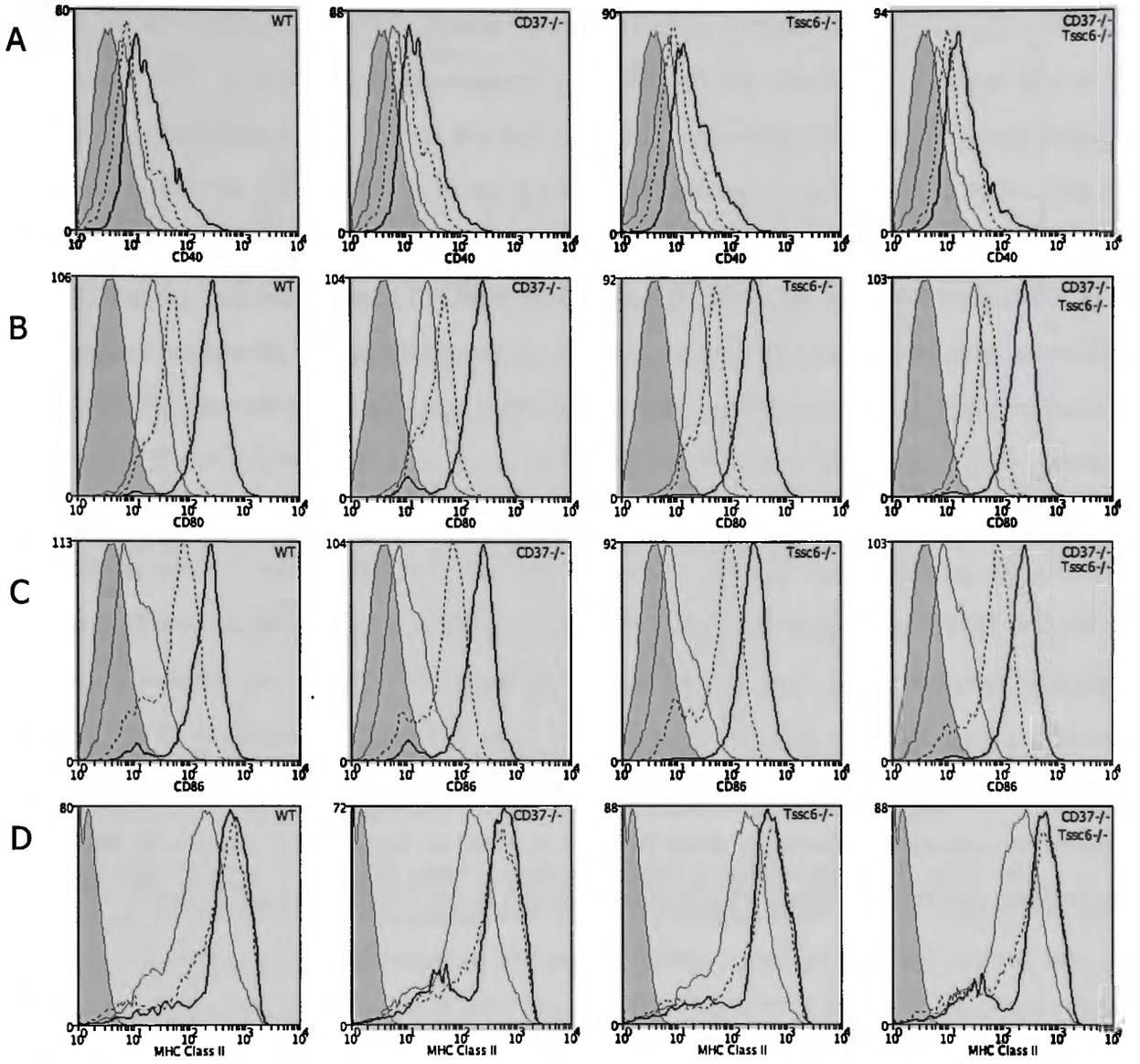

Figure 4.9 Normal expression and up-regulation of co-stimulatory molecules in vitro by CD37$^{-/-}$, Tssc6$^{-/-}$, CD37$^{-/-}$Tssc6$^{-/-}$ dendritic cells.

Wild type and knockout naïve dendritic cells were purified and cultured over 24 hours. Up-regulation of (A) CD40, (B) CD80, (C) CD86, MHC Class I[§] and (D) MHC Class II was assessed at 0 (light grey solid), 4 (dark grey dotted), 6[§] and 24 (black solid) hours by flow cytometry (unstained cells are shown filled in grey). There was no significant difference between strains in the basal expression of dendritic cell co-stimulatory molecules, and no difference in up-regulation of these molecules during culture ([§] data not shown, basal analysis n=2, 2 mice/group; maturation analysis 4 mice/group).

4.2.7 Normal CD37$^{-/-}$Tssc6$^{-/-}$ DC Maturation and Co-stimulation

T cell activation and proliferation in response to antigen presentation is a result of both MHC-TCR interactions and co-stimulatory signals provided by APC. To further understand the role of CD37 and Tssc6 in dendritic cell function, the co-stimulatory capacity of the tetraspanin knockout dendritic cells were assessed. Dendritic cells are known to spontaneously up-regulate the expression of co-stimulatory molecules and MHC class II (MHC-II) *in vitro* (Wilson *et al.*, 2003). Therefore, the basal expression and subsequent increase in expression of CD40, CD80, CD86, MHC class I (MHC-I) and MHC-II over a period of 24 hours *in vitro* was compared. No differences were observed in co-stimulatory molecule expression or any differences in their rate of up-regulation between wild type and CD37/Tssc6 knockout dendritic cells (Figure 4.9). Similarly, MHC-I and MHC-II expression was normal in all strains, with MHC-II expression increasing over the incubation period. However, there are several additional molecules that can also provide co-stimulatory signals. Consequently, co-stimulation assays were performed, where wild type T cells were stimulated with αCD3 mAbs and co-stimulation provided by co-incubated syngeneic wild type or tetraspanin deficient DC. Again, no differences in co-stimulatory capacity were detected between wild type and CD37$^{-/-}$, Tssc6$^{-/-}$ or CD37$^{-/-}$Tssc6$^{-/-}$ dendritic cells (Figure 4.11). This data suggests that the increased antigen presentation capacity in tetraspanin knockout dendritic cells is likely to be due to changes in MHC-TCR interactions, rather than altered co-stimulatory signals in the absence of CD37/Tssc6.

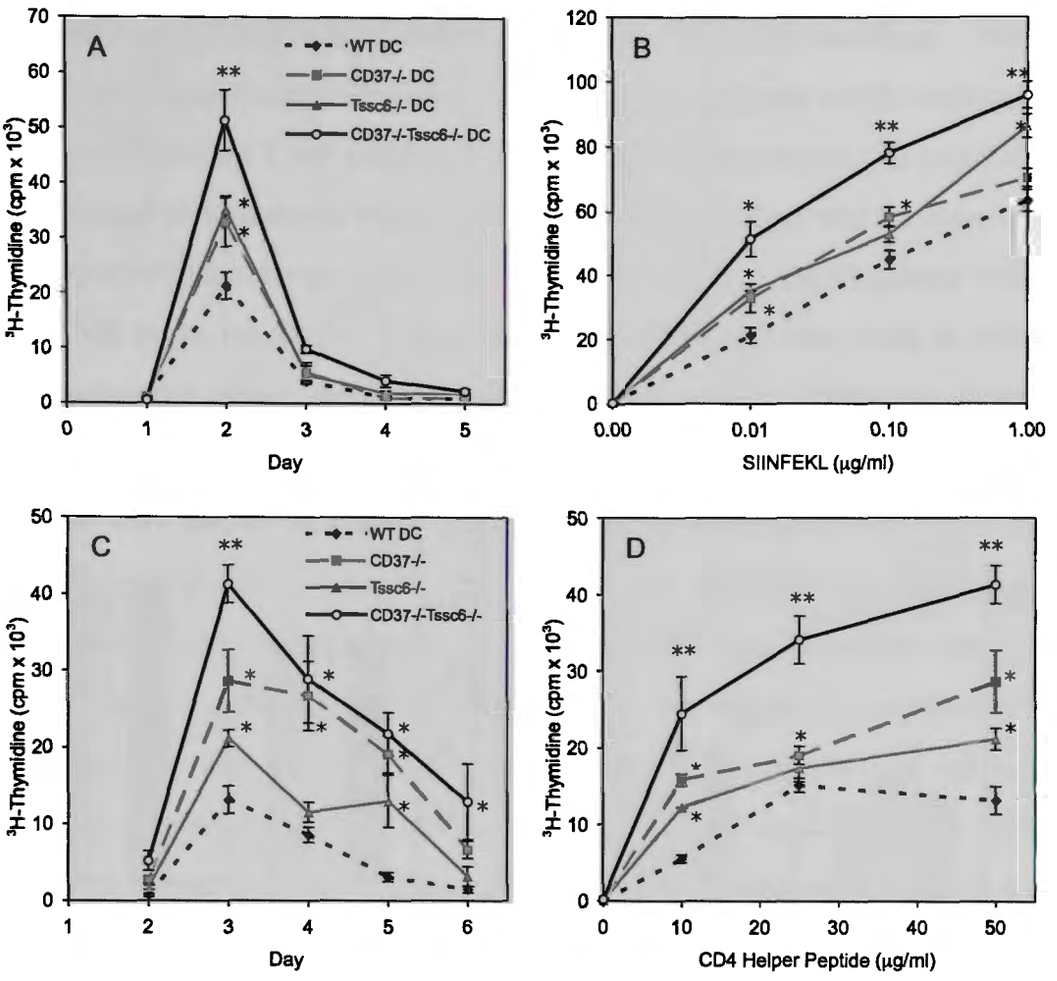

Figure 4.10 CD37$^{-/-}$Tssc6$^{-/-}$ dendritic cells are hyper-stimulatory to antigen specific T cells in vitro.

*Purified splenic dendritic cells were isolated from wild type (C57Bl/6), CD37$^{-/-}$, Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ mice, pulsed with peptide and co-incubated with antigen specific T cells. (A) 0.01 µg SIINFEKL with OT-I T cells over 5 days; (B) titrated doses of SIINFEKL, with OT-I T cells for 2 days; (C) 25µg Helper peptide with OT-II T cells over 6 days; or (D) titrated doses of Helper peptide with OT-II T cells for 3 days. Data points represent the mean tritiated thymidine incorporation across triplicate wells (n=6, 4 mice/group). Significance was tested by students t test in comparison to wild type controls (n=4, 4 mice/group, *, P< 0.05; **, P< 0.01).*

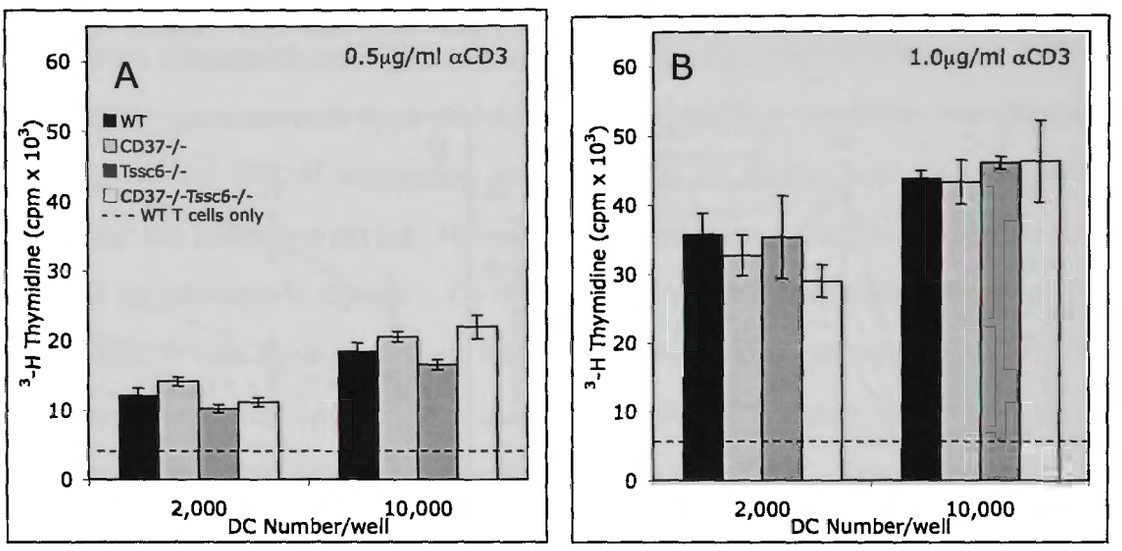

Figure 4.11 Normal in vitro co-stimulation by CD37$^{-/-}$, Tssc6$^{-/-}$, CD37$^{-/-}$Tssc6$^{-/-}$ dendritic cells.

Purified wild type T cells were stimulated in vitro with titrated doses of surface adsorbed αCD3 mAbs. Titrated numbers of purified naïve DC from wild type or tetraspanin knockout mice were co-incubated to co-stimulatory signals. T cell proliferation in response to (A) 0.5μg/ml αCD3 and either 2,000 or 10,000 DC and (B) 1.0μg/ml αCD3 and either 2,000 or 10,000 DC. T cell proliferation in the absence of DC is indicated by dashed lines. There was no significant difference between the co-stimulatory capacity of wild type and knockout dendritic cells. Data points represent the mean tritiated thymidine incorporation across triplicate wells (n=2, 4 mice/group). Significance was tested by students t test in comparison to wild type controls.

Soluble mediators secreted by tetraspanin deficient DC such as cytokines may explain a fundamental change in antigen presentation capacity in the assays described. Inflammatory cytokines or unidentified co-stimulatory factors produced by activated CD37/Tssc6 deficient DC may have some impact on T cell proliferative responses. To investigate this possibility and to confirm the normal co-stimulatory findings in section 4.2.7, wild type and tetraspanin deficient DC were cultured with the pro-survival cytokines GM-CSF and IL-4 and stimulated with multiple doses of αCD40 mAbs overnight. Supernatants from these DC were used to measure DC cytokine secretion and culture wild type T cells in the presence of TCR ligation (αCD3 mAbs). The cytokines IL-6, IL-10, IFNγ, TNFα, IL-12p70 and MCP-1 were tested via CBA cytokine array, however these were below the level of detection in either unstimulated or αCD40 stimulated cultures (data not shown). Despite the absence of detectable secreted cytokines in unstimulated and αCD40 mAb stimulated cultures, low level T cell proliferation was induced by these supernatants (Figure 4.12). However, this did not appear to be CD40 stimulation dependent and there was no significant difference seen between wild type and tetraspanin knockout strains. These T cell responses are most likely to be a result of IL-4 in the dendritic cell cultures and unrelated to DC stimulation. This preliminary assessment suggests that at the doses tested, there was no evidence for enhanced secretion of soluble co-stimulatory factors by tetraspanin deficient DC. However, to confirm these findings further testing with both higher doses of αCD40 and increased DC numbers will also be investigated.

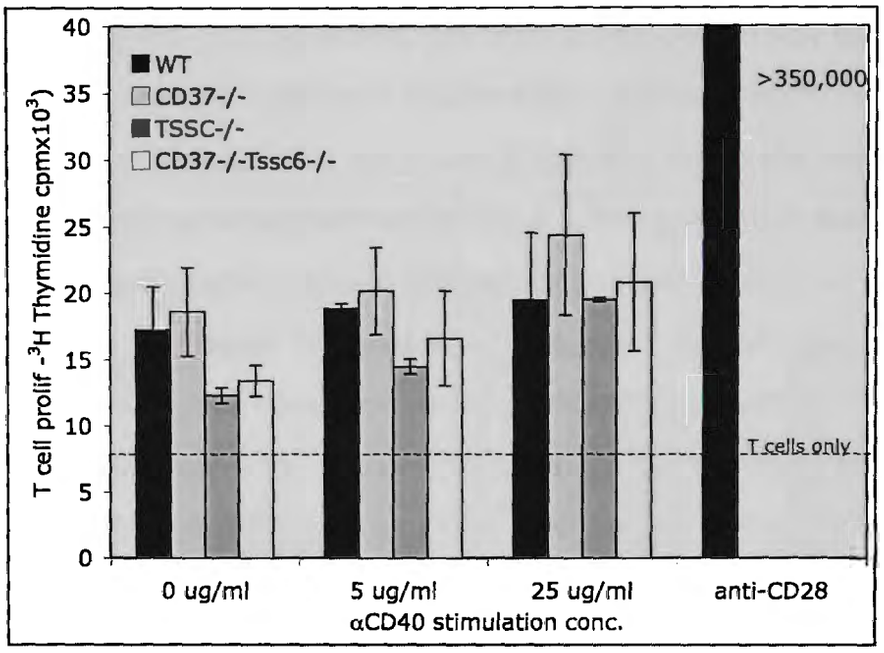

Figure 4.12 In vitro T cell co-stimulation by activated wild type and tetraspanin knockout DC supernatants
Purified DC from wild type (C57Bl/6), CD37$^{-/-}$, Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ mice were stimulated overnight with titrated doses of αCD40 mAbs. Supernatants were harvested and wild type purified T cells incubated for 3 days with 1.0μg/ml of surface adsorbed αCD3 mAbs and 1:2 dilution of the stimulated DC supernatants. Histogram bars represent the mean tritiated thymidine incorporation across duplicate wells. 1μg/ml soluble αCD28 mAbs were used as positive controls, whilst T cell only wells were included as negative controls for T cell proliferation (WT & CD37 n=4, Tssc6 & 2xKO, 3 mice/group). Significance was tested by students t test in comparison to wild type controls.

4.3 Discussion

Whilst there are no significant changes in conventional T cell development in the absence of CD37 and Tssc6, basal NKT cell numbers are significantly increased. NKT cells undergo a similar developmental process as conventional αβ T cells, until the DP stage in the thymus. Here, conventional T cells undergo positive selection determined by weak recognition of self antigens presented on MHC expressed by cortical epithelium. Conversely, NKT cells are positively selected via recognition of glycolipids presented on CD1d molecules expressed by other DP cortical thymocytes (Bendelac, 1995, Coles and Raulet, 2000). Negative selection of NKT cells is poorly understood, although there are some similarities with conventional T cells, whereby the presence of TCR agonists such as αGal-Cer in the thymus can induce NKT cell deletion (Pellicci *et al.*, 2003, Chun *et al.*, 2003). Glycolipid antigen recognition is restricted to a limited repertoire of TCR chains. In mice, these are predominantly combinations of Vα14 – Vα18 and Vβ2, Vβ7 and Vβ8.2 chains. These TCR αβ chain combinations appear to be randomly generated rather than positively regulated, but then go on to be positively selected in the thymus by glycolipid recognition (Gapin *et al.*, 2001, Godfrey and Berzins, 2007). It is possible that CD37$^{-/-}$Tssc6$^{-/-}$ T cells display altered levels of gene rearrangement leading to a reduced repertoire of TCR chains expressed in the thymus. However, since there is no evidence for tetraspanin function in transcriptional or recombination regulation, this is unlikely. Another possibility is that the absence of CD37 and Tssc6 may alter CD1d organisation and antigen presentation by cortical thymocytes. However, in one study where CD1d was over-expressed on cortical thymocytes, it was demonstrated that increased presentation of native glycolipids led to reduced NKT cell numbers (Chun *et al.*, 2003). This data suggests that an improved capacity for antigen presentation of glycolipids via CD1d, may have a *negative* effect on NKT cell development by resulting in increased negative selection in the thymus.

The defect described in CD37$^{-/-}$Tssc6$^{-/-}$ NKT cell development may be due to altered homeostatic regulation of NKT cell numbers in the periphery. In mice, NKT cell numbers are maintained in the periphery by IL-15 and IL-7 induced cell division, which occurs every ~0-2 days (Matsuda *et al.*, 2002). IL-15 and IL-7 are also responsible for the maintenance of memory CD8^{+} and naïve CD4^{+} populations respectively (Tan *et al.*, 2001, Tan *et al.*, 2002). Whilst these cytokines are as yet unmeasured in CD37$^{-/-}$Tssc6$^{-/-}$ mice, it may be worth investigating both IL-7 and IL-15 secretion as well as cytokine receptor expression in tetraspanin deficient NKT cells. A further point of interest arising from the data presented in

this chapter is the failure to identify a defect in NKT development based on CD3^{+}NK1.1^{+} cellularity. NKT cells are thought to increase expression of NK1.1 during maturation, although NK1.1^{-} cells are still responsive to the TCR agonist αGal-Cer (Pellicci *et al.*, 2002). It is possible that the increased numbers of αGal-Cer reactive NKT cells detected in CD37$^{-/-}$Tssc6$^{-/-}$ mice may reflect an increase in NK1.1^{-} NKT cells i.e. immature NKT cells, in the absence of both CD37 and Tssc6.

Functional overlap in the regulation of T cell proliferation was demonstrated in the initial studies on CD37$^{-/-}$ and Tssc6$^{-/-}$ mice (Tarrant *et al.*, 2002; van Spriel *et al.*, 2004). In this study, the *in vitro* hyper-proliferative phenotypes seen in CD37$^{-/-}$ and Tssc6$^{-/-}$ T cells were exaggerated in CD37$^{-/-}$Tssc6$^{-/-}$ T cells, indicating that CD37 and Tssc6 play a complementary role in regulating T cell proliferation. The differences between CD37 and Tssc6 deficient CD4^{+} T cell proliferation in the presence of co-stimulation remain unexplained. Surprisingly, CD37$^{-/-}$Tssc6$^{-/-}$ CD4^{+} T cell proliferative responses were exaggerated in the presence of co-stimulation. Perhaps there is potential for CD37 to compensate for the absence of Tssc6 in CD4^{+} T cells during αCD3 + αCD28 stimulation? Unfortunately, it has not been determined if there is increased CD4/CD8 associated Lck activity in stimulated Tssc6$^{-/-}$ or CD37$^{-/-}$Tssc6$^{-/-}$ T cells. By comparing Lck activity between αCD3 stimulated CD37$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ T cells for example, this may give some further indication of the causative nature of Lck activity on tetraspanin deficient T cell hyper-proliferation.

When Th1/Th2 cytokines were measured in activated T cell cultures, it was apparent in CD4^{+} supernatants in particular that increased levels of IL-2, IL-4 and IFNγ are produced by the dividing CD37$^{-/-}$Tssc6$^{-/-}$ T cells. In CD8^{+} cultures this was true only for IFNγ production. Both IL-4 and IFNγ are secreted in high quantities by activated NKT cells, which are also capable of responding to αCD3/αCD28 stimulation. This therefore raises the possibility that the exaggerated hyper-proliferative phenotype described above may be due to increased NKT cell proliferation in CD37$^{-/-}$Tssc6$^{-/-}$ mice. In mice, NKT cells exist in the periphery as either CD4^{+} or DN (CD8^{-}CD4^{-}) populations. In the T cell experiments described, we used a negative selection method to isolate T cell subsets to avoid inadvertently activating them by TCR ligation. As a result, CD8^{+} T cell isolations included DN NKT populations, whilst CD4^{+} T cell isolations contained both CD4^{+} and DN NKT cell populations.

To investigate the potential contribution of NKT cells in CD37$^{-/-}$Tssc6$^{-/-}$ T cell hyper-proliferation, preliminary studies into NKT cell proliferation in CD37 and Tssc6 deficient mice were performed. αGal-Cer stimulations of splenocyte cultures enriched in NKT cells, demonstrated a striking hyper-proliferative phenotype in CD37$^{-/-}$Tssc6$^{-/-}$ NKT cells not shared by single knockout counterparts. This is the first description of a hyper-proliferative phenotype in NKT cells and the first link identified between tetraspanin function and NKT cells. NKT cell signalling in response to glycolipid antigens is somewhat less defined than that of conventional T cells. CD1d ligation does not require adaptor molecules such as CD4 or CD8, although it has been demonstrated that CD4 expressed by NKT cells can still contribute to NKT cell activation (Thedrez *et al.*, 2007). NK receptors expressed by NKT cells such as SLAM family (signalling lymphocytic activation molecules) receptors are also thought to contribute to NKT cell signalling through the recruitment of SAP (SLAM associated protein) (Pasquier *et al.*, 2005, Nichols *et al.*, 2005, Chung *et al.*, 2005). Since tetraspanins have been previously shown to provide a functional link between cell surface receptors and signalling molecules (e.g. integrins) (Zhang *et al.*, 2001; Zhang *et al.*, 2002), CD37 and Tssc6 may be involved in SLAM signalling in NKT cells in a similar manner.

To complement these investigations into T cell function, the antigen presentation capacity of tetraspanin deficient DC was also assessed. As mentioned earlier, recent findings in our laboratory have identified a hyper-stimulatory capacity in CD37$^{-/-}$ and CD151$^{-/-}$ DC. During this current study, this phenotype was also observed in Tssc6$^{-/-}$ DC but to a lesser degree. Therefore, these tetraspanins appear to display functional overlap in the regulation of T cell stimulation by DC. When CD37$^{-/-}$Tssc6$^{-/-}$ DC were compared to wild type and 'single knockout' DC this phenotype was further exaggerated, suggesting that CD37 and Tssc6 play complementary roles in antigen presentation. T cell responses to antigen are dependent on a range of factors, such as antigen processing, co-stimulation, cytokine signalling, MHC expression and MHC-TCR interactions. Given the abundance of data on tetraspanin localisation to endosomal/lysosomal pathways it would not be surprising if tetraspanin deficiency induced some changes in antigen processing (Engering *et al.*, 2003; Engering and Pieters, 2001, Escola *et al.*, 1998). However, in the model described in this chapter, the DC of interest present synthetic peptides to antigen specific T cells. Therefore, the phenotype observed is independent of antigen processing.

Characterisation of tetraspanin deficient DC was unable to reveal any significant differences in cell surface expression of co-stimulatory molecules. Furthermore, when tetraspanin knockout DC were activated *in vitro*, these molecules were upregulated at a similar rate in comparison to wild type controls. Co-stimulation assays also confirmed that the capacity of CD37/Tssc6 deficient DC to provide a second signal to TCR stimulated T cells was normal. This is in contrast to the findings for DC deficient in the tetraspanin CD151, suggesting that tetraspanin function in DC is pleiotropic (Sheng *et al.*, submitted for publication). Soluble factors secreted by DC that may contribute to T cell activation were also investigated, however there was no evidence of enhanced T cell responses to tetraspanin deficient DC supernatants. This may also have implications in the context of exosome secretion by tetraspanin deficient DC. One explanation for an increased capacity to stimulate antigen specific T cells may be increased secretion of MHC-peptide bearing exosomes, also known to bear multiple tetraspanin proteins (Engering and Pieters, 2001; Escola *et al.*, 1998). Whilst the experiments described in this chapter were aimed at comparing cytokine secretion, these findings may also imply normal secretion of exosomes by tetraspanin deficient DC. To confirm this however, exosome purification and quantitation may also be required.

In the absence of any changes in co-stimulation in CD37/Tssc6 deficient DC, we concluded that the enhanced capacity to present antigen was likely to be due to changes in MHC-TCR interactions. Whilst there were no differences observed in the cell surface expression of MHC I or MHC II, this does not eliminate the possibility that changes in cell surface organisation of these molecules may occur in CD37$^{-/-}$ and Tssc6$^{-/-}$ DC. In part, these findings support a role for tetraspanin microdomains in MHC clustering suggested by previous studies (Kropshofer *et al.*, 2002; Unternaehrer *et al.*, 2007). However, one would predict from these earlier findings that the disruption of tetraspanin microdomains in DC would have a *negative* effect on antigen presentation. This may reflect opposing roles for some tetraspanins in MHC organisation, or the potential regulation of tetraspanins that promote MHC clustering (e.g. CD9) by other tetraspanins (e.g. CD37). However, until potentially 'pro-MHC clustering' tetraspanin deficient APC are described, this can only be speculation. These investigations into MHC organisation in tetraspanin deficient DC and CD37-MHC dynamics could also be furthered by confocal analysis of MHC organisation in activated versus resting DC.

An enhanced capacity for CD37$^{-/-}$Tssc6$^{-/-}$ DC to present antigen to T cells introduces new questions in regard to NKT cell hyper-proliferation in CD37$^{-/-}$Tssc6$^{-/-}$ mice. In the assays described in this chapter, αGal-Cer was presented to NKT cells by DC derived from the same mice, i.e. tetraspanin deficient DC presented glycolipids to tetraspanin deficient NKT cells. Therefore, there may be some contribution from CD37$^{-/-}$Tssc6$^{-/-}$ DC to the NKT cell phenotype described. To separate the effects of antigen presentation from NKT cell responses, future experiments will isolate NKT and DC via flow cytometry and NKT cell responses will be assessed when glycolipids are presented by wild type DC. Similarly, reciprocal experiments will be performed where WT NKT cell responses are compared when antigen is presented by either CD37$^{-/-}$Tssc6$^{-/-}$ or wild type DC.

This chapter outlines a complementary role for CD37 and Tssc6 in T cell proliferation *in vitro* whereby CD37$^{-/-}$Tssc6$^{-/-}$ T cell hyper-proliferation is exaggerated in comparison to single knockout counterparts. A novel NKT cell developmental and hyper-proliferative defect in response to αGal-Cer was also identified in the absence of both CD37 and Tssc6, a phenotype not shared by CD37$^{-/-}$ or Tssc6$^{-/-}$ mice. This proves that functional redundancy exists between CD37 and Tssc6 in the immune system. A complementary role for CD37 and Tssc6 was identified in *in vitro* antigen presentation assays that are attributed to changes in MHC-TCR interactions and not co-stimulation or cytokine secretion. Together these findings demonstrate important roles for CD37 and Tssc6 in the immune system and support earlier findings that CD37 and Tssc6 contribute to the regulation of T cell activation and antigen presentation.

5 *IN VIVO* CELLULAR IMMUNITY IN CD37$^{-/-}$TSSC6$^{-/-}$ MICE

5.1 INTRODUCTION

The *in vitro* studies described in the previous chapter have provided insight into the role of CD37 and Tssc6 in cell mediated immunity. These tetraspanins work together to regulate both T cell proliferation and antigen presentation *in vitro*. To complement these studies and to determine if the tetraspanin deficient phenotypes observed *in vitro* translate to altered responses to pathogen and tumour invasion, *in vivo* cellular immunity was investigated in CD37$^{-/-}$, Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ mice.

Given the hyper-stimulatory capacity of CD37$^{-/-}$, Tssc6$^{-/-}$ DC, combined with the hyper-proliferative CD37$^{-/-}$, Tssc6$^{-/-}$ T cell responses observed *in vitro*, strong cellular immunity *in vivo* is suggested in these mice (Tarrant *et al.*, 2002; van Spriel *et al.*, 2004). Furthermore, in CD37$^{-/-}$Tssc6$^{-/-}$ mice where both of these phenotypes are exaggerated and an additional NKT hyper-proliferative response is present this may further enhance *in vivo* cellular immune responses. However, *in vivo* cellular immunity is significantly more complex than the DC-T cell interactions examined in the previous chapter. For example in a tumour model, danger signals must be recognised by DC in the periphery, antigen captured and processed, DC must become activated as they migrate toward the lymphoid tissue before antigen presentation and co-stimulation to T cells. T cells resident in the lymphoid organ must recognise the antigen, receive co-stimulatory signals, become activated and proliferate. CD4$^+$ T cell help and CD8$^+$ effector function must also be successful in promoting phagocytosis and tumour eradication. Innate immune cells such as macrophages and NK cells are also important in tumour rejection. Since tetraspanins display pleiotropism in their function, the effects of tetraspanin deficiency may result in enhanced cellular responses in one system and but impaired cellular responses in another. For example, in Tssc6 deficient mice, T cell and DC function is enhanced *in vitro* but platelet function is impaired both *in vitro* and *in vivo* (Goschnick *et al.*, 2006). This chapter describes *in vivo* cellular immunity in CD37$^{-/-}$, Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ mice, in response to tumour challenge, viral infection and model immunisation strategies.

5.1.1 STUDY RATIONALE

When taken together, the *in vitro* cellular immune responses suggest that CD37 and Tssc6 play a fundamental role in the generation of effective cellular immunity.

This chapter aims to:

(1) Determine if functional redundancy, or even synergism exists between CD37 and Tssc6 in anti-tumour immunity.

(2) Establish if a reduced frequency of antigen specific T cells is responsible for poor anti-tumour immunity.

(3) Investigate the potential mechanism for reduced frequencies of antigen specific T cells in CD37/Tssc6 knockout mice.

5.2 RESULTS

5.2.1 IMPAIRED ANTI-VIRAL IMMUNITY IN THE ABSENCE OF CD37 AND/OR TSSC6.

Due to the enhanced T cell proliferative responses and antigen presentation capacity in the CD37/Tssc6 null mice *in vitro*, the possibility of enhanced cellular responses was investigated. CD37$^{-/-}$, Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ mice were given a variety of immune challenges, including viral infection and tumour growth. Initial experiments performed by collaborators found that cellular immune responses were surprisingly poor in these mice. Using the Influenza A model of transient respiratory infection, wild type, CD37$^{-/-}$, Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ mice were infected via intranasal inoculation with A/HKx31 virus. The generation of antigen specific CD8^{+} T cells in response to influenza infection can be detected by tetramer staining for TCR specific to immuno-dominant viral epitopes (Belz *et al.*, 2000; Townsend *et al.*, 1986). From these studies it was clear that eight days post infection, the frequency of antigen specific CD8^{+} T cells in the spleen was lower in CD37$^{-/-}$ and Tssc6$^{-/-}$ mice (Figure 5.1). In CD37$^{-/-}$Tssc6$^{-/-}$ mice this reduction was most striking, with a surprising increase in animal death induced by the non-lethal influenza strain (G. Belz personal communication). These data suggest that both CD37 and Tssc6 play a complementary role in the development of antigen specific CD8^{+} T cells in response to influenza infection.

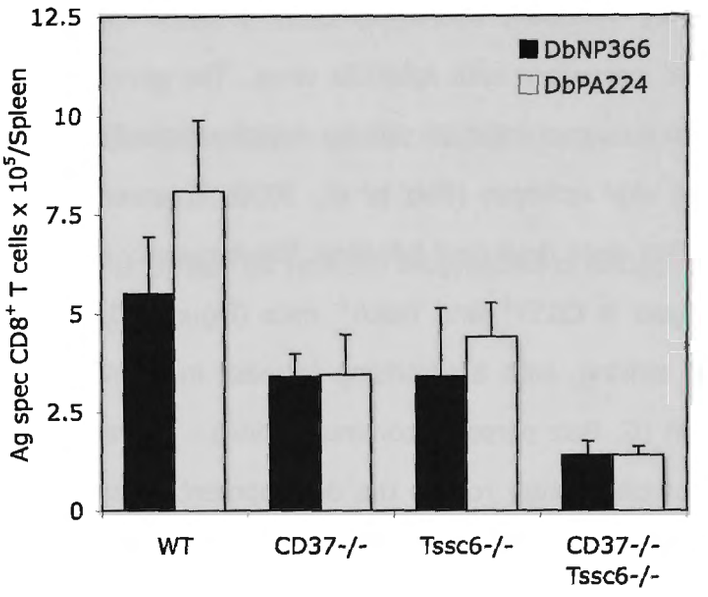

Figure 5.1 In vivo influenza challenge and tumour challenge of tetraspanin deficient mice.

(A) Groups of 5 mice were infected intra-nasally with a non-lethal dose of A/HKx31 influenza virus. After 8 days lymphoid organs were harvested and virus-specific CD8$^+$ T cells were identified by flow cytometry using D^bNP_{366} and D^bPA_{224} tetramers (courtesy G Belz WEHI).

5.2.2 Impaired tumour rejection capacity in the absence of CD37 and/or Tssc6.

Preliminary studies into the capacity for anti-tumour immunity in tetraspanin deficient mice indicated a more broad role for CD37 and Tssc6 in the development of antigen specific T cell responses. During tumour rejection, the development of antigen specific effector T cells is a critical requirement for the targeting and removal of invading cells (Shankaran *et al.*, 2001). The syngeneic thymoma derived cell line RMA, transfected with the human cancer antigen Mucin 1 (Muc1), was used in this study. Muc1 is a human mucin glycoprotein over-expressed in the majority of lung, breast, prostate, colon and ovary adenocarcinomas (Taylor-Papadimitriou *et al.*, 1999). Whilst untransfected RMA cells grow progressively in mice derived from a C57Bl/6 background, Muc1 transfected RMA cells (RMA-Muc1) are recognised and antigen specific T cell responses lead to tumour rejection.

Preliminary experiments showed that RMA-Muc1 tumours grow progressively in CD37$^{-/-}$ mice, whilst wild type mice reject these tumours two weeks after challenge. This indicated that CD37 has a role in anti-tumour responses and induction of antigen specific effector T cells. The functional overlap and complementary roles for CD37 and Tssc6 in cellular immunity suggest that this phenotype may be shared by Tssc6 deficient mice. To investigate this, preliminary tumour challenges were also performed on Tssc6$^{-/-}$ and CD37$^{-/-}$Tsssc6$^{-/-}$ mice. Muc1 expression by transfected RMA cells was confirmed by flow cytometry and log phase growth established (Figure 5.2A). Groups of 5 wild type, CD37$^{-/-}$, Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ mice were subcutaneously injected with 5 x 10^6 cells and abdominal tumour growth monitored over a period of 14 days. In these experiments, tumour rejection was apparent in wild type mice and poor in CD37$^{-/-}$ and Tssc6$^{-/-}$ mice (Figure 5.2C-D). Whilst the trend was similar in CD37$^{-/-}$Tssc6$^{-/-}$ mice, tumour clearance was more variable (Figure 5.2E). These data suggest that both CD37 and Tssc6 proteins are required for successful development of anti-tumour immunity, although there was no evidence for a complementary role for these proteins in tumour rejection.

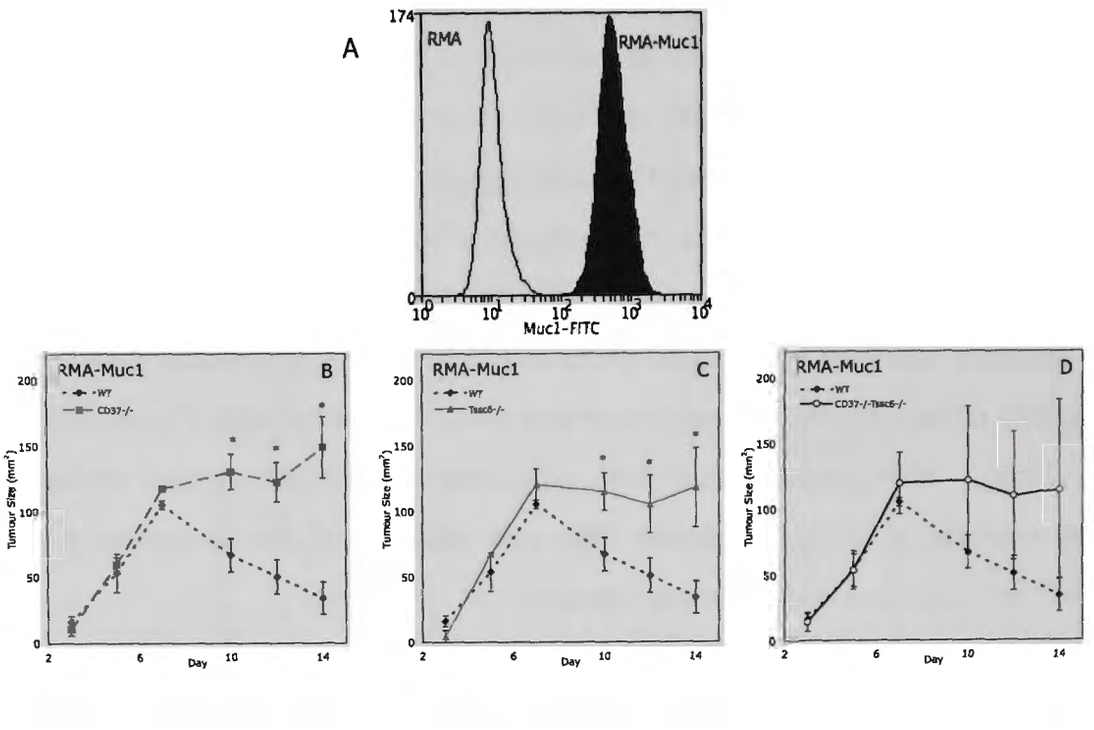

Figure 5.2 Tumour susceptibility in CD37 and Tssc6 deficient mice.

(A) RMA-Muc1 cells were grown to log phase and tested for Muc1 expression via flow cytometry in comparison to untransfected RMA controls. (B-D) Tumour challenges were performed on wild type, CD37 $^{-/-}$, Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ mice by injection of 5x10^{6} RMA-Muc1 cells subcutaneously into the abdominal region. Tumour sizes were determined by calliper measurements and monitored over 14 days (p=< 0.5, 5 mice/group).*

5.2.3 IMPAIRED DEVELOPMENT OF ANTIGEN SPECIFIC T CELLS IN TETRASPANIN DEFICIENT MICE

To determine if the poor capacity for tumour rejection in the tetraspanin deficient strains was due to a similar failure to develop antigen specific T cell responses as seen in influenza challenges, the development of these T cell responses were investigated via ELISPOT.

5.2.3.1 Cell associated antigens

RMA-Muc1 cells were γ-irradiated to prevent tumour growth and injected intradermally into wild type, CD37$^{-/-}$, Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ mice. Mice were divided into two groups that received either one dose or two doses of RMA-Muc1 cells, separated by a 2 week period. After 14 or 28 days, mice were culled and splenocytes restimulated *in vitro* to determine the frequency of antigen specific cells via ELISPOT. During re-stimulation, splenocytes were re-exposed to γ-irradiated RMA-Muc1 cells. After one immunisation (14 days), the frequency of splenic Muc1 specific IFNγ producing cells was significantly lower in tetraspanin deficient mice (Figure 5.3A). This correlated well with the impaired cellular responses observed after RMA-Muc1 tumour challenges.

Th1 (e.g. IFNγ) and Th2 (e.g. IL-4) cytokine pathways are known to play cross-inhibitory roles (Mosmann and Coffman, 1989). Potentially, the cellular responses in CD37/Tssc6 deficient mice may be driven down a Th2 type pathway rather than the Th1 type response seen in wild type mice. To eliminate this possibility, the frequencies of antigen specific splenocytes secreting the Th2 cytokine IL-4 were also assessed. In these assays, the frequency of IL-4 production was very low in all strains and was therefore unlikely to be the cause of impaired development of IFNγ producing cells in tetraspanin deficient mice. Whilst antigen specific IL-4 responses remained consistently low in all assays, in some experiments IL-4 was elevated in specific groups of individuals e.g. CD37$^{-/-}$Tssc6$^{-/-}$ after 14 days (Figure 5.3B). However, these results were not consistent between experiments or groups and did not correlate with the Th1 frequencies generated in the same mice. These variations are likely to be due to stochastic effects and reflect the proximity of the data to the threshold of detection. When mice received two doses of RMA-Muc1 cells an apparent difference was observed in the CD37$^{-/-}$ IFNγ responses when compared to Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ splenocytes. This second exposure to antigen in CD37 deficient mice enabled the development of normal frequencies of Muc1 specific IFNγ producing T cells after 28 days.

In order to determine if the defect in IFNγ production was restricted to either CD4$^+$ or CD8$^+$ T cells the model antigen ovalbumin (OVA) was used. The dominant CD4$^+$ and CD8$^+$ T cell epitopes of OVA are well defined. Therefore, individual subpopulations of T cells can be restimulated with OVA peptides *in vitro* post exposure to whole OVA protein. In these studies, mice were immunised with the syngeneic tumour cell line B16-OVA. This cell line is derived from C57Bl/6 melanoma and is transfected with the chicken ovalbumin protein. Cell surface expression of ovalbumin was assessed in log phase cultures (data not shown) and as described earlier, γ-irradiated cells were injected into wild type, CD37$^{-/-}$ Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ mice. As in RMA-Muc1 challenges, antigen specific T cells were assessed after both single and boosted immunisations, although CD37$^{-/-}$Tssc6$^{-/-}$ mice were unavailable for the B16-OVA boosted immunisation schedule. 14 days after a single immunisation the frequency of IFNγ producing OVA specific CD4$^+$ and CD8$^+$ T cells is low in CD37$^{-/-}$, Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ mice (Figure 5.3C). Unlike RMA-Muc1 immunisations, a similar result was seen in both CD4$^+$ and CD8$^+$ T cell populations in all tetraspanin deficient strains after a second exposure to antigen (Figure 5.3D). These data suggest that the defect in the development of antigen specific T cells in response to cell associated antigen immunisations is not restricted to either CD4$^+$ or CD8$^+$ T cells.

In all assays, unstimulated and conA stimulated wells were included as controls (Figure 5.3E-F). In addition, splenocytes derived from mice immunised with RMA-Muc1 were exposed to untransfected RMA cells and data compensated for background responses. These results demonstrate that there is no intrinsic defect in either IFNγ production or IL-4 production in T cells derived from immunised CD37 and/or Tssc6 deficient mice. Therefore, the low frequencies of IFNγ secreting cells observed are likely to reflect a defect in the development of antigen specific T cells in tetraspanin deficient mice. The discrepancies observed between CD37$^{-/-}$ IFNγ responses after re-exposure to antigen were further investigated in the following models.

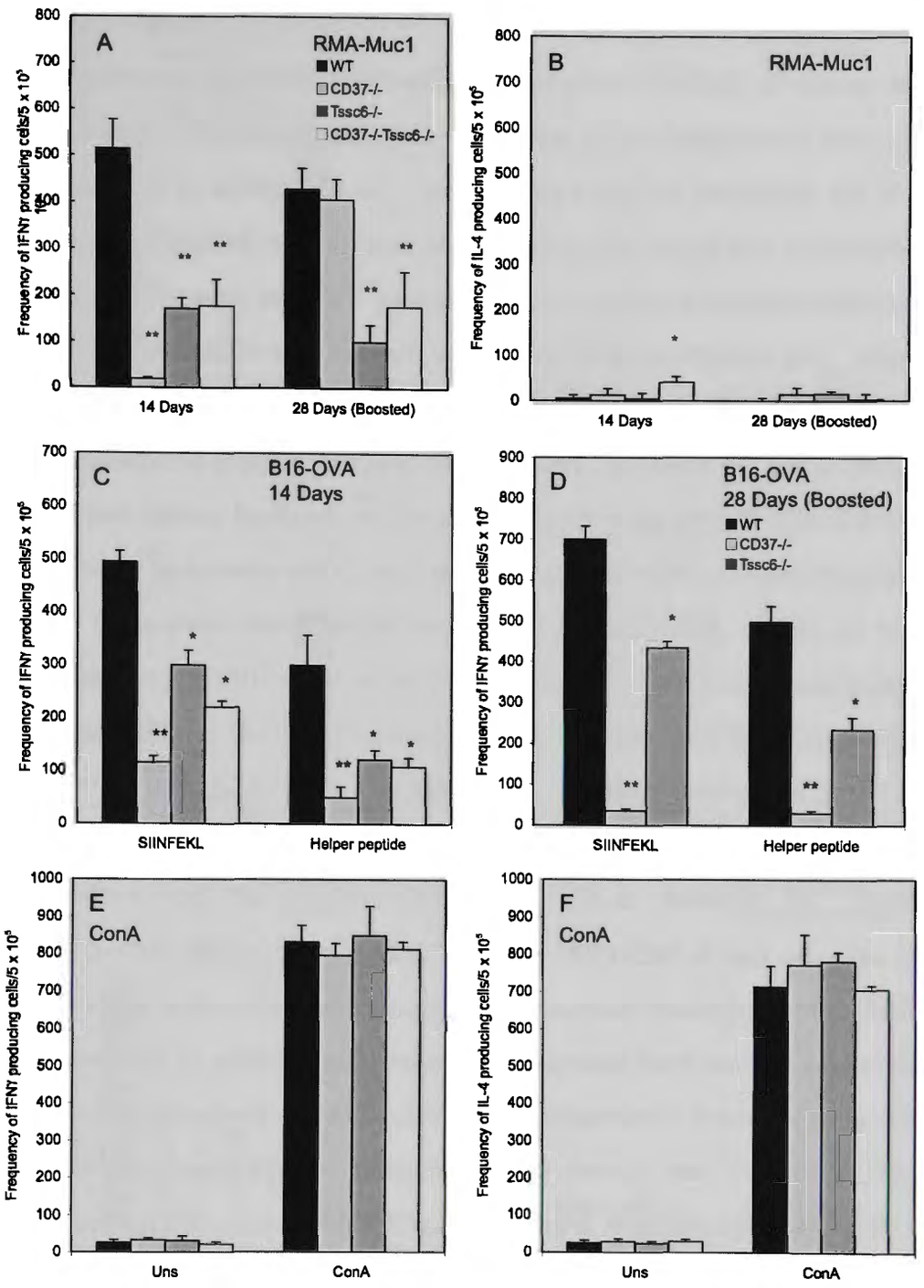

Figure 5.3 Poor IFNγ responses to cell-associated antigen in tetraspanin deficient mice.

*Groups of 8 wild type, CD37$^{-/-}$, Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ mice were immunised with γ-irradiated cells, half of which received a repeat immunisation 14 days later. After 14 or 28 days, splenocytes from immunised mice were re-exposed to antigen and ELISpots performed. In mice immunised with RMA-Muc1 cells, the frequencies of Muc1 specific (A) IFNγ^{+} and (B) IL-4^{+} cells were measured on days 14 and 28. In mice immunised with B16-OVA cells, the frequencies of CD4^{+} (Helper peptide) and CD8^{+} (SIINFEKL) ovalbumin specific IFNγ^{+} T cells were measured on day 14 (C) and day 28 (D). In all assays conA stimulated splenocytes were used as positive controls, representative assays are demonstrated for IFNγ (E) and IL-4 (F) from RMA-Muc1 immunised mice (RMA-Muc1, n=2, 4 mice/group; B16-OVA n=3, 4 mice/group; *, p<0.01; **, p<0.001)*

5.2.4 PENETRATIN CONJUGATED ANTIGENS

In a third series of ELISPOT experiments, potential differences in antigen capture and processing were investigated using penetratin conjugated antigens. Similar to the trends observed in cell associated antigen immunisations, low frequencies of antigen specific T cells were generated by tetraspanin knockout mice in response to ANTp-OVA or ANTp-SIINFEKL. ANTp (penetratin peptide) is a 16mer peptide derived from the *Drosophila* transcription factor Antennapedia. This peptide has been mapped as the sequence facilitating rapid cellular uptake of the 60 a.a. Antennapedia protein, a process demonstrated to be receptor independent (Derossi *et al.*, 1996; Derossi *et al.*, 1994). When selected antigens are chemically cross-linked or fused to the ANT peptide prior to immunisation, the resultant uptake and presentation by APCs leads to enhanced immunogenicity to the antigen in the absence of strong danger signals (Pietersz *et al.*, 2001). Wild type and tetraspanin deficient mice were again divided into two cohorts and given either a single dose of ANTp-OVA or ANTp-SIINFEKL, or given an additional booster immunisation of the same. At both 14 days (single dose) and 28 days (boosted) mice were culled and splenocytes restimulated *in vitro* with either CD4 or CD8 OVA epitopes. A striking difference was seen in tetraspanin deficient mice in comparison to wild type controls in these assays. As expected, at day 14 the frequency of wild type antigen specific IFNγ producing cells was high in both $CD4^+$ and $CD8^+$ subpopulations post ANTp-OVA immunisation (Figure 5.4A). In comparison however, this frequency was extremely low in both $CD4^+$ and $CD8^+$ populations derived from tetraspanin deficient mice. Similar to the trend seen in RMA-Muc1 cells, after a second immunisation with ANTp-OVA the frequency of OVA specific $CD8^+$ CD37 deficient T cells was normal when compared to wild type controls (Figure 5.4C). However, this effect was not seen in antigen specific $CD4^+$ T cells, which remained at very low frequencies. To examine $CD8^+$ T cell responses in the absence of $CD4^+$ T cell help, mice were immunised with ANTp-SIINFEKL and boosted with same at day 14. Again, $CD8^+$ T cell IFNγ responses were higher in wild type and CD37$^{-/-}$ mice compared to Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ mice (Figure 5.4E). IL-4 was assessed in parallel in each of these assays, where there was no evidence suggesting that upregulated IL-4 was responsible for the suppression of IFNγ (Figure 5.4B, D & F).

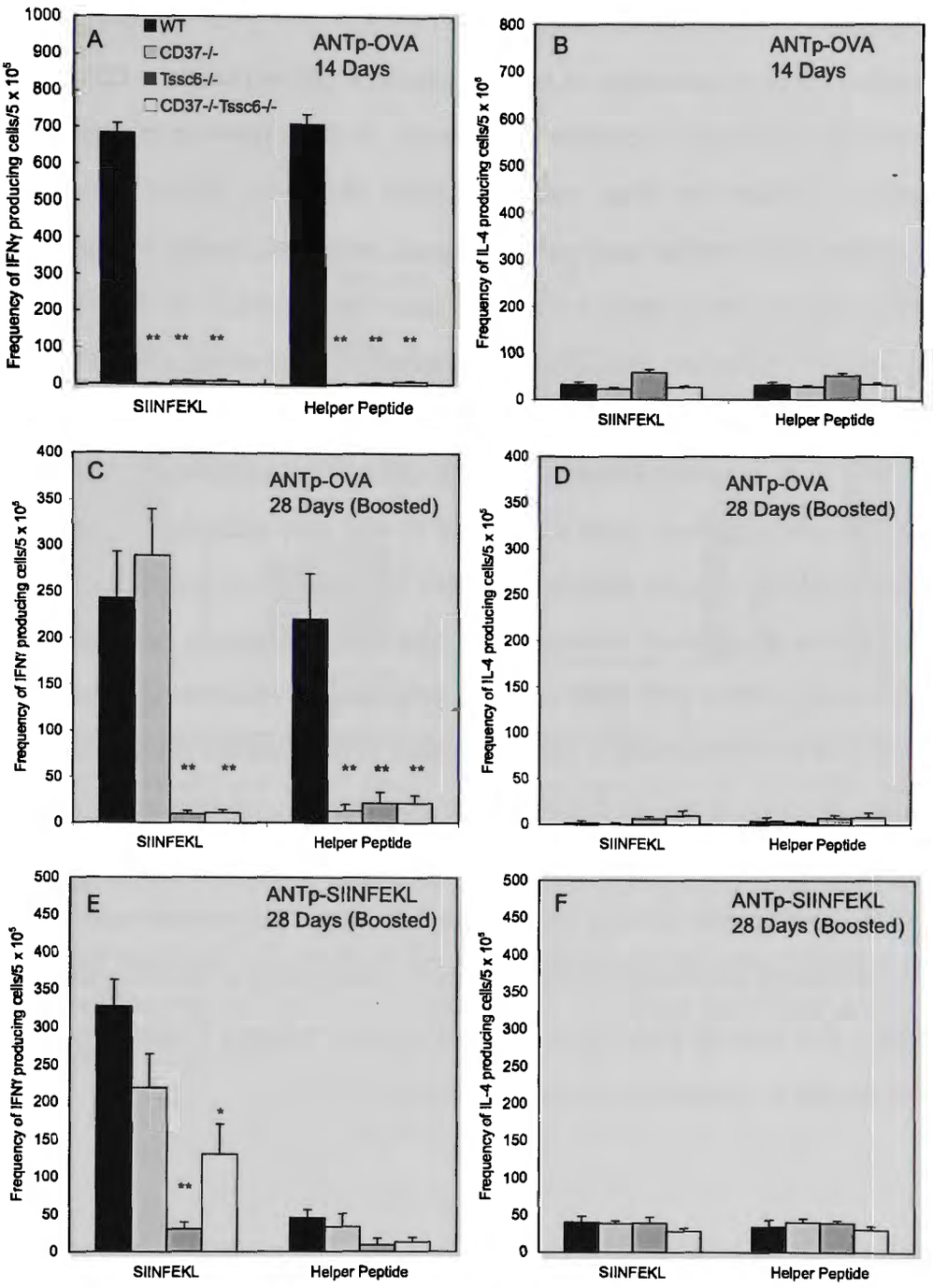

Figure 5.4 Poor IFNγ responses to penetratin peptide conjugated antigen in tetraspanin deficient mice.

*Wild type, CD37$^{-/-}$, Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ mice were immunised with (A-D) ANTp-OVA or (E-F) ANTp-SIINFEKL, half of which received a repeat immunisation on day 14. After 14 or 28 days, splenocytes from immunised mice were re-exposed to antigen and ovalbumin specific CD4$^+$ (Helper peptide) and CD8$^+$ (SIINFEKL) ELISpots performed. In mice immunised with ANTp-OVA, the frequencies of IFNγ$^+$and IL-4$^+$ cells were measured on either day 14 (A-B) or day 28 (C-D). In mice immunised with ANTp-SIINFEKL, the frequencies of IFNγ$^+$ and IL-4$^+$ cells were measured on day 28 (E-F). In all assays conA stimulated splenocytes were used as positive controls and all strains responded equally well (representative data in Figure 5.3E-F) (n=2, 4 mice/group; *, p<0.01; **, p<0.001).*

5.2.5 ADJUVANT EMULSIFIED ANTIGEN

One explanation for poor generation of antigen specific T cell responses in CD37/Tssc6 deficient mice may be that *in vivo* DC activation is impaired. In the experiments described above, we have utilised γ-irradiated cell lines; which are likely to provide danger stimuli via apoptotic signals, and penetratin peptide conjugated antigens; which are considered to be very poor at inducing DC activation (unpublished data D Pouniotis & GA Pietersz). To further investigate this phenotype we also immunised mice with OVA emulsified in the strong adjuvant CFA (Complete Freunds Adjuvant). CFA is composed of inactivated mycobacterium suspended in mineral oils, which is known to be a potent stimulator of both cellular and humoral immunity. Surprisingly, the cellular immune responses were less potent in wild type mice in comparison to previous antigen immunisation strategies described earlier. However, from these assays there were no significant differences apparent between wild type and tetraspanin deficient strains (Figure 5.5A). These data suggest that when a strong inflammatory response is generated during the immunisation, the defect observed in previous assays in the absence of CD37 and/or Tssc6 may be overcome. From these results it is also clear that the reduced frequency of IFNγ producing cells in cell-associated and ANTp conjugated antigen immunisations, is not due to an intrinsic defect in IFNγ production. To confirm this finding, naïve splenocytes were stimulated with varying concentrations of conA and IFNγ production measured in a non-antigen specific manner (Figure 5.5B). The intrinsic capacity of naïve tetraspanin deficient T cells to produce IFNγ was found to be normal in comparison to wild type controls.

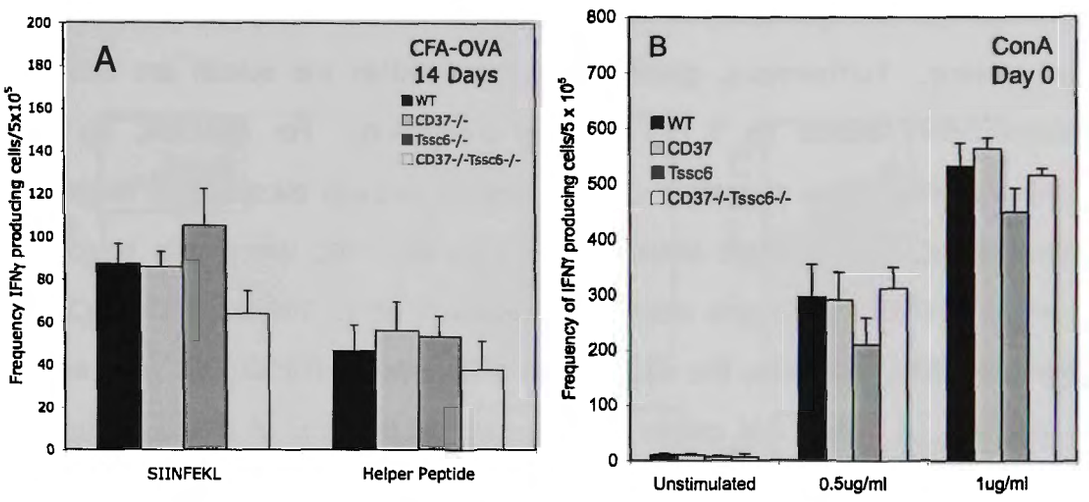

Figure 5.5 Normal IFNγ production in response CFA emulsified antigen and conA stimulation.

*(A) Wild type, CD37$^{-/-}$, Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ mice were immunised with CFA-OVA. After 14 days, splenocytes from immunised mice were re-exposed to antigen and ovalbumin specific CD4$^+$ (Helper peptide) and CD8$^+$ (SIINFEKL) ELISpots performed. In all assays conA stimulated splenocytes were used as positive controls and all strains responded equally well (representative data in Figure 5.3E-F). Splenocytes derived from naïve wild type and tetraspanin deficient mice were stimulated with titrated doses of conA and the frequency of IFNγ producing cells determined by ELISpot analyses (n=3, 4 mice/group; *, p<0.01; **, p<0.001).*

5.2.6 SPLENOCYTE POPULATION DISTRIBUTION IN NAÏVE AND IMMUNISED MICE

A reasonable explanation for the striking differences seen in antigen specific T cell development may also be that T cell frequencies in tetraspanin deficient mice during cellular immune responses are in fact lower. Similarly, an increased frequency of other cell types such as B cells, macrophages and granulocytes may also reduce the percentage of T cells in tetraspanin deficient spleens. Furthermore, specific populations within the spleen are known to have immunomodulatory effects on T cell cytokine production. For example, an increase in macrophage numbers may suggest T cell suppression through excess nitric oxide production (Eisenstein *et al.*, 1994), whilst altered frequencies of T regulatory cells could inhibit the development of Th1 type immune responses (Sakaguchi *et al.*, 2008, Jiang and Chess, 2004). To eliminate these possibilities, the distributions of splenocyte populations were assessed in a variety of states i.e. naïve, mid cellular response and at the time of IFNγ assessment (i.e. 14 days post B16-OVA immunisation). Cell surface markers for B cells, granulocytes, macrophages, DC and natural killer cells were used to separate the major splenic populations (Figure 5.6). T cell populations were further divided into $CD4^+$, $CD8^+$ and NKT cell subsets, and the activation marker CD62L was also used to compare the levels of naïve versus activated T cells (Figure 5.6). Intracellular staining for the transcription factor FoxP3 and cell surface TCR expression were used to identify potential T regulatory cells (Figure 5.6). From these assays, there were no significant differences detected in the numbers of T cells, B cells, APC, NK cells or granulocytes in naïve mice (Figure 5.7), during the primary cellular response (Figure 5.8) or prior to re-stimulation *in vitro* (Figure 5.9). Likewise, there were no abnormalities seen in the various T cell subpopulations delineated in tetraspanin deficient mice at days 0 and 10. A significant difference was observed however in the NKT cell population derived from CD37$^{-/-}$ Tssc6$^{-/-}$ mice at day 14, which correlates with the hyper-proliferative phenotype observed in these mice *in vitro*. This data suggests that the poor IFNγ responses observed are not due to an overall reduction in T cells or T cell subsets, or an increase in immunomodulatory splenic subpopulations, but the failure to expand and develop antigen specific T cell subpopulations *in vivo*.

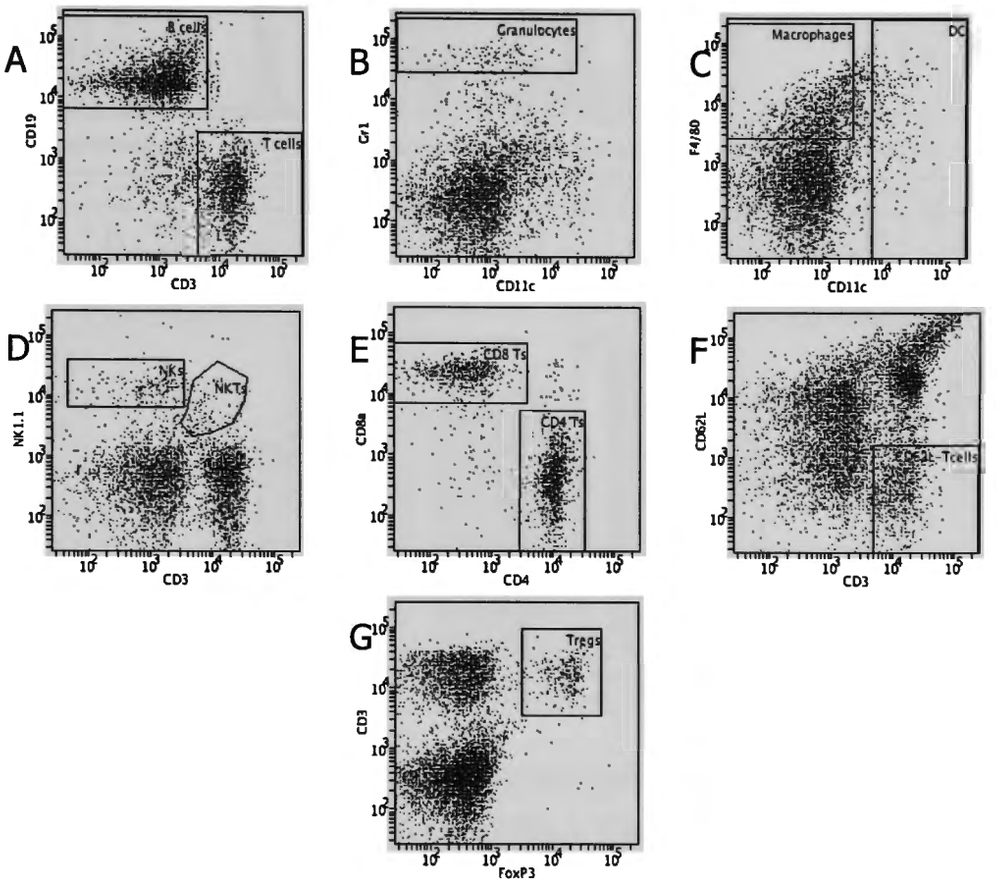

Figure 5.6 Splenocyte distribution in tetraspanin deficient mice

Naïve wild type, CD37$^{-/-}$, Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ splenocytes were stained with the following mAbs (A) αCD3 and aCD19, (B) αGR-1 and αCD11c, (C) αF4/80 and αCD11c (D) αCD3 and αNK1.1, (E) αCD3, αCD4 and αCD8α (gated on CD3^{+} cells), (F) αCD62L and αCD3 and (G) αCD3 and αFoxP3. Splenocytes were stained with αCD3 mAbs before permeabilisation and staining with FoxP3 mAbs. Autofluorescent cells were excluded via gating and non permeabilised cells were stained with propidium iodide to exclude non viable cells. Representative plots from wild type mice are demonstrated only.

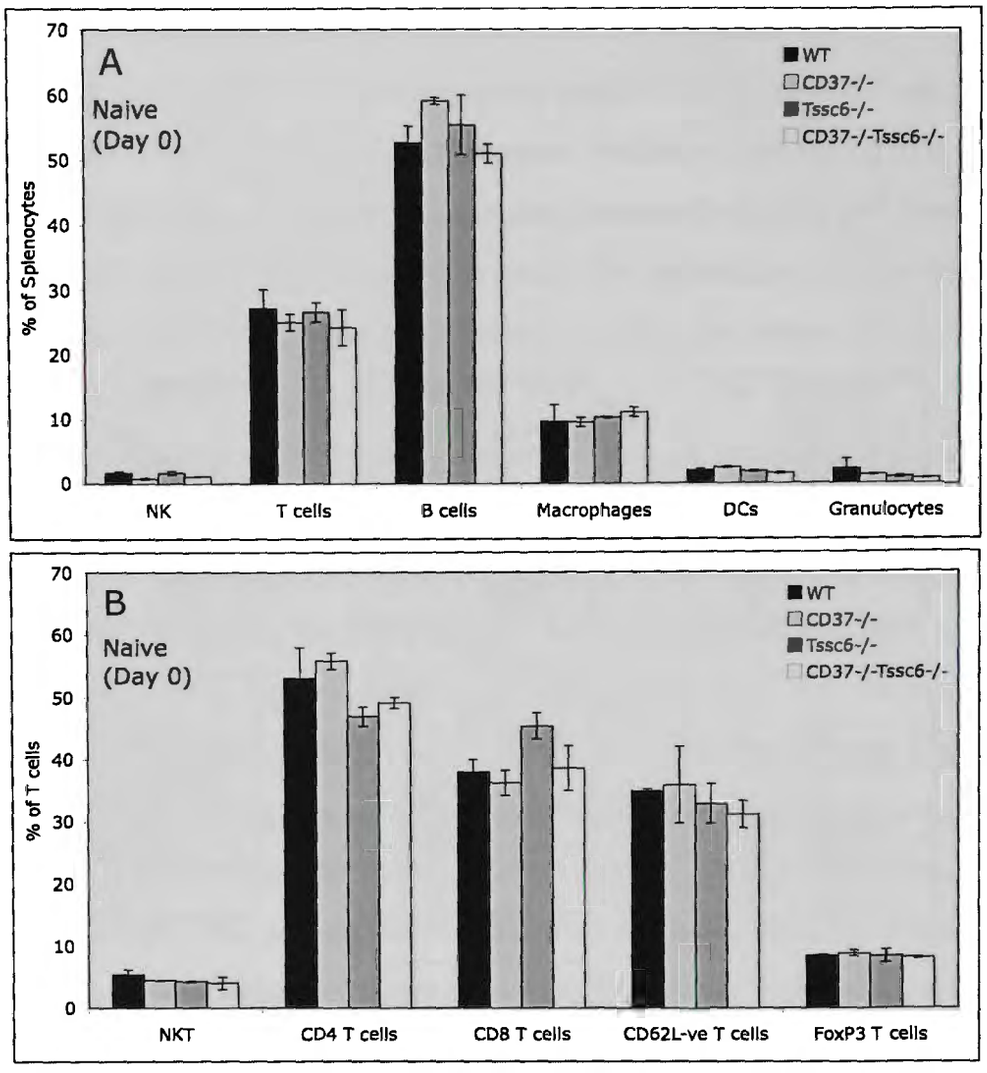

Figure 5.7 Splenocyte distribution in naïve tetraspanin deficient mice

FACS analyses of the major splenocyte and T lymphocyte populations in naïve wild-type and tetraspanin deficient mice. (A) The frequencies of major splenocyte populations such as NK cells, CD3^{+} T cells, B cells, DC, Granulocytes (Gr) and Macrophages (Mac) are expressed as a percentage relative to the total number of viable cells. (B) The frequencies of T cell subpopulations including NKT, CD4^{+}, CD8^{+} and Foxp3^{+} T_{reg} cells are expressed as a percentage relative to the total number of viable CD3^{+} T cells. Histogram bars represent the mean frequency of the given population, error bars represent the standard error of mean and significance determined by students t test. Population gating is demonstrated in Figure 5.6 (n=2, 4 mice/group).

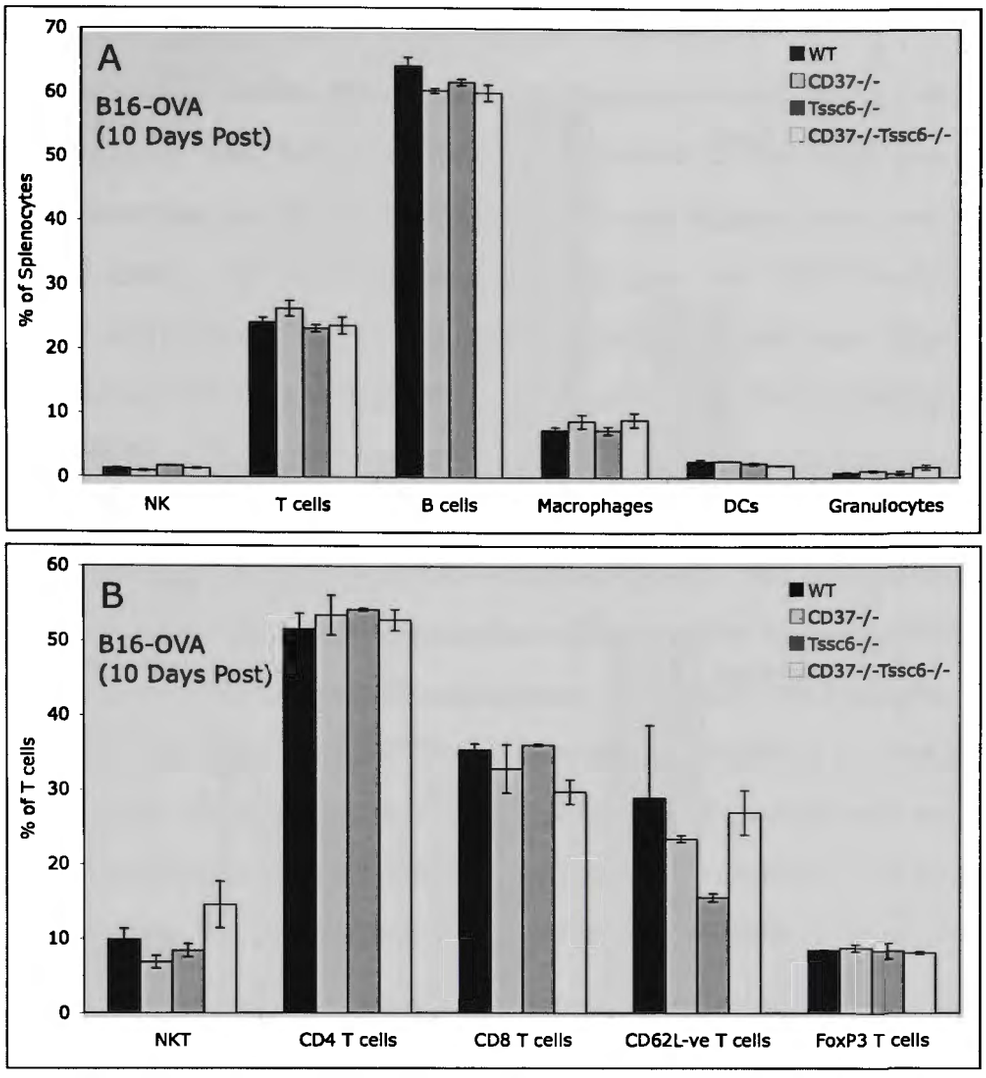

Figure 5.8 Splenocyte distribution in tetraspanin deficient mice on day 10 post B16-OVA challenge

FACS analyses of the major splenocyte and T lymphocyte populations in wild-type and tetraspanin deficient mice 10 days post γ-irradiated B16-OVA immunisation. (A) The frequencies of major splenocyte populations such as NK cells, CD3$^+$ T cells, B cells, DC, Granulocytes (Gr) and Macrophages (Mac) are expressed as a percentage relative to the total number of viable cells. (B) The frequencies of T cell subpopulations including NKT, CD4$^+$, CD8$^+$ and Foxp3$^+$ T_{reg} cells are expressed as a percentage relative to the total number of viable CD3$^+$ T cells. Histogram bars represent the mean frequency of the given population, error bars represent the standard error of mean and significance determined by students t test. Population gating is demonstrated in Figure 5.6 (n=2, 4 mice/group).

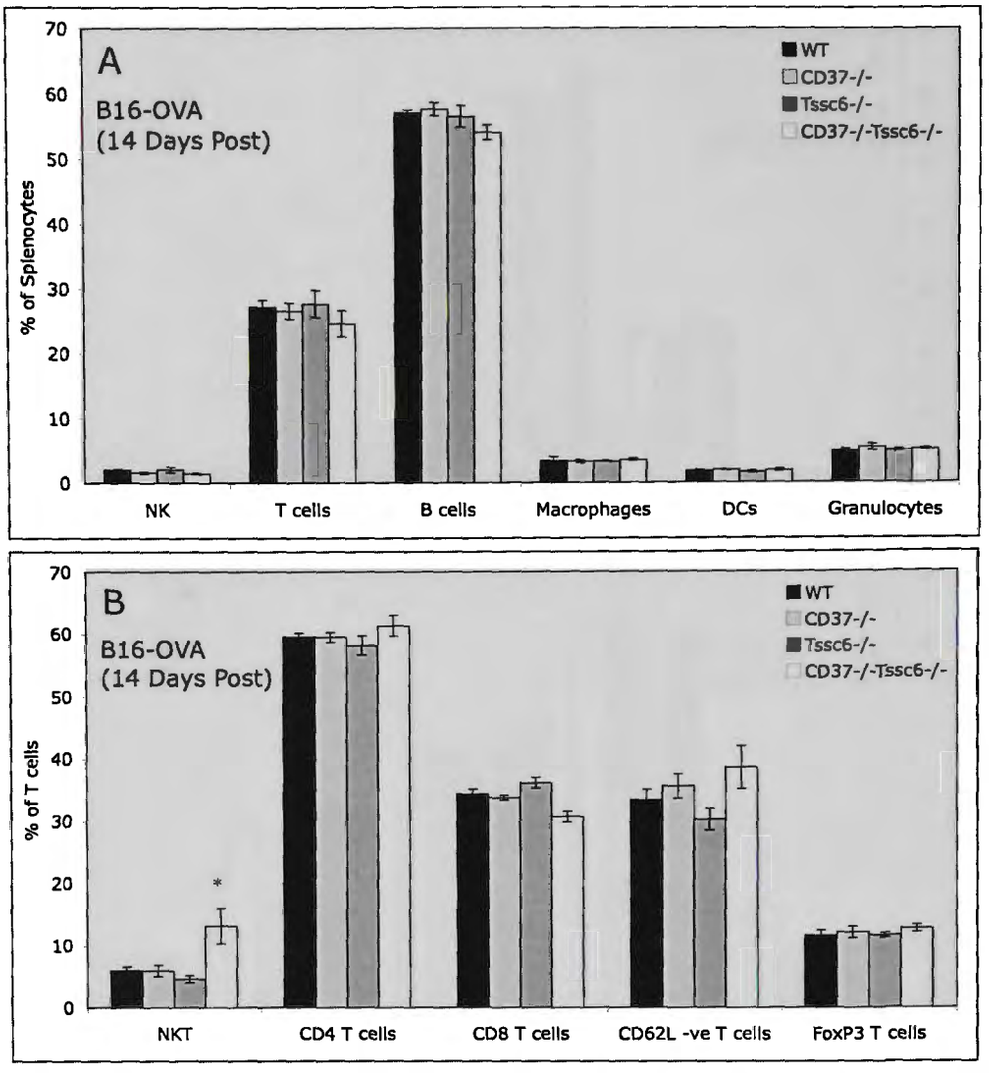

Figure 5.9 Splenocyte distribution in tetraspanin deficient mice on day 14 post B16-OVA challenge

FACS analyses of the major splenocyte and T lymphocyte populations in wild-type and tetraspanin deficient mice 14 days post γ-irradiated B16-OVA immunisation. (A) The frequencies of major splenocyte populations such as NK cells, CD3$^+$ T cells, B cells, DC, Granulocytes (Gr) and Macrophages (Mac) are expressed as a percentage relative to the total number of viable cells. (B) The frequencies of T cell subpopulations including NKT, CD4$^+$, CD8$^+$ and Foxp3$^+$ T_{reg} cells are expressed as a percentage relative to the total number of viable CD3$^+$ T cells. Histogram bars represent the mean frequency of the given population, error bars represent the standard error of mean and significance determined by students t test. Population gating is demonstrated in Figure 5.6 (n=2, 4 mice/group).

5.2.7 POOR IN VIVO KILLING CAPACITY IN TETRASPANIN DEFICIENT MICE.

Since there is a strong correlation between poor anti-tumour immunity and a deficit in antigen specific IFNγ producing cells, further analysis was performed to link these two phenotypes. Therefore, *in vivo* antigen specific T cell effector function was assessed via *in vivo* killing assays (Barchet *et al.*, 2000). In these experiments, wild type and CD37$^{-/-}$Tssc6$^{-/-}$ mice were immunised with ANTp-SIINFEKL. 8 days post immunisation, wild type splenocytes were prepared and stained with CFSE at two different concentrations. Brightly labelled splenocytes were pulsed with SIINFEKL peptide and weakly labelled splenocytes were pulsed with an irrelevant peptide derived from the Muc1 protein. These cells were then mixed in equal proportions and adoptively transferred to the immunised groups. The development and action of effector T cell responses was determined by flow cytometry after 24 hours, by comparing the frequency of remaining CFSE^{hi} and CFSE^{lo} splenocytes. As expected, the frequency of SIINFEKL pulsed splenocytes was higher in CD37$^{-/-}$Tssc6$^{-/-}$ spleens in comparison to wild type controls (Figure 5.10). These assays demonstrate poor effector T cell development and function in CD37$^{-/-}$Tssc6$^{-/-}$ mice and provide a link between the poor antigen specific T cell responses seen in ELISpot assays and the poor tumour rejection capacity observed in tetraspanin deficient mice.

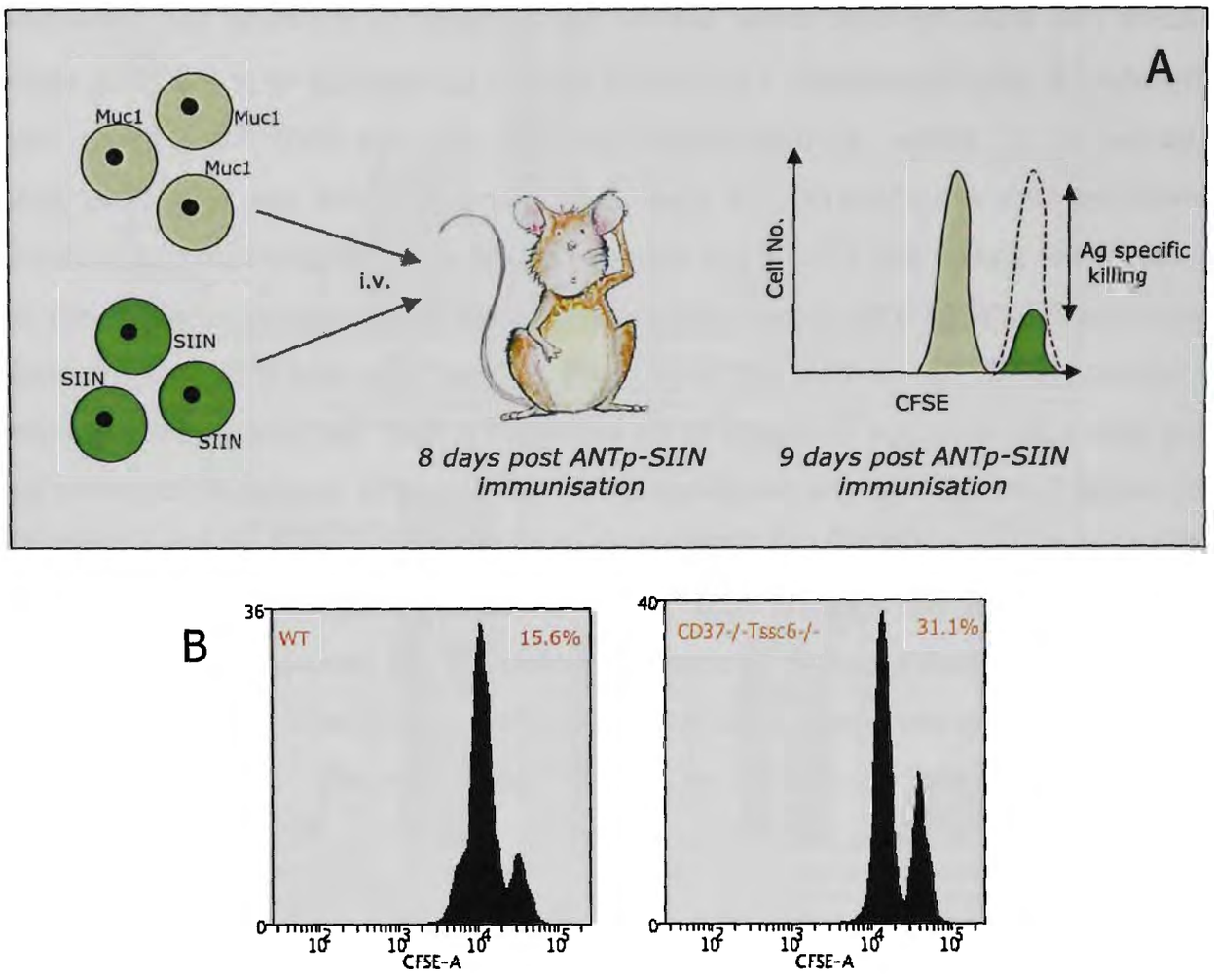

Figure 5.10 Antigen specific in vivo killing is impaired in CD37$^{-/-}$Tssc6$^{-/-}$ mice.

(A) In vivo killing assays were performed on mice 8 days post ANTp-SIIN immunisation. Splenocytes were labelled with either 0.5μM or 5 μM CFSE and pulsed with either Muc1 or SIINFEKL peptide. Labelled cells were injected i.v. into immunised mice in a 1:1 ratio. After 24 hours, spleens were removed and the frequency of recovered CFSE labelled splenocytes was determined. (B) T cell in vivo effector function was measured in wild type and CD37$^{-/-}$Tssc6$^{-/-}$ mice. Percentages displayed denote the frequency of CFSE^{hi} cells recovered from wild type and CD37$^{-/-}$Tssc6$^{-/-}$ spleens (n=2, 2 mice/group).

5.2.8 Normal IL-12 production by tetraspanin deficient DC

As discussed in Chapter 4, CD37/Tssc6 deficient T cells display altered cytokine production *in vitro*. Perhaps the failure to develop effective antigen specific T cell responses is due to further cytokine dysregulation in tetraspanin deficient DC? The secretion of IL-12 during antigen presentation is known to play an important role in promoting IFNγ production by T cells (reviewed in Trinchieri, 2003). Conversely, the presence of IL-4 is thought to drive T cells towards Th2 type cytokine production (Le Gros *et al.*, 1990). Since DC are potent producers of IL-12, secretion of this cytokine by tetraspanin deficient DC was quantified. Bioactive IL-12 is a dimer composed of the subunits p35 and p40. Previous studies have described an inhibitory form of IL-12 formed by p40 subunit homodimers and therefore in the assays described below IL-12 was quantified as the p70 heterodimer. Unfortunately, it is not feasible to accurately measure the secretion of IL-12 by DC during antigen presentation *in vivo* and therefore these assays were performed *in vitro*. Purified DC derived from wild type and tetraspanin deficient mice were cultured in the presence of the microbial stimulants CpG, LPS and inactivated *staphylococcus aureus* (SAC), in medium supplemented with recombinant DC survival cytokines GM-CSF, IFNγ and IL-4. After 24 hours, supernatants were harvested and IL-12 detected via cytometric bead array. IL-12 was secreted in large quantities in response to CpG and to a lesser extent by LPS and SAC, and was not detected in unstimulated DC wells (Figure 5.11). From these assays it was clear that there are no significant differences in IL-12 secretion in the absence of CD37 and/or Tssc6 in response to any of the stimuli tested. In a second series of experiments, αCD40 mAbs were used to stimulate DC *in vitro* to generate a less bacterially biased DC activation. However, sufficient IL-12 was not detected in these supernatants to enable conclusions to be drawn (data not shown). Similarly, IL-12 was not detected in supernatants derived from ELISpot re-stimulations of splenocytes derived from B16-OVA immunised mice (data not shown).

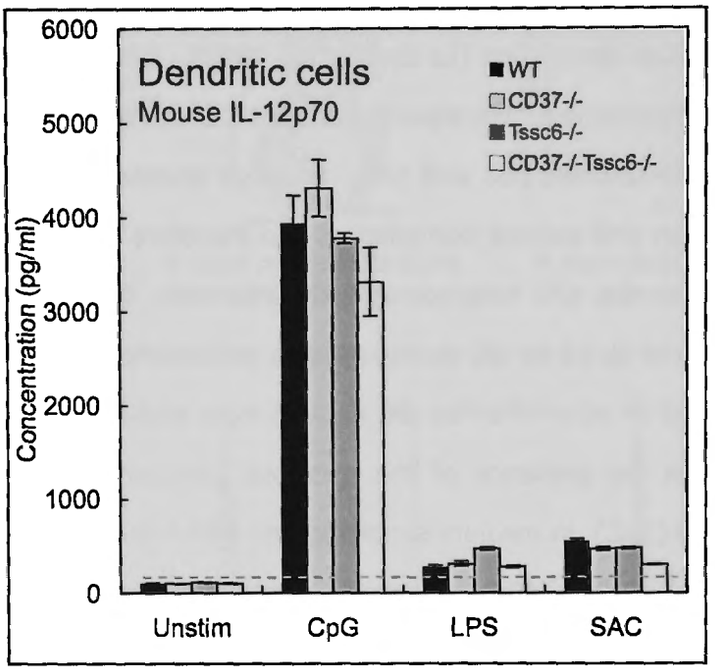

Figure 5.11 IL-12 production from naïve DC

Purified naïve splenic DC were cultured with various bacterial derived stimuli (CpG, SAC and LPS) and all cultures were supplemented with GM-CSF, IL-4 and IFNγ. After 24 hours, IL-12p70 was assessed in supernatants via CBA array. Histogram bars represent the mean concentration of IL-12p70 derived from triplicate wells, error bars represent the standard error of the mean and significance was determined by students t test. Cytokine detection below 10pg/ml was dismissed as below the level of accurate detection in these assays (dotted line) (n=2, 4 mice/group).

5.2.9 IN VIVO DC MIGRATION IS IMPAIRED IN THE ABSENCE OF CD37/TSSC6

The large volume of literature on the interactions between tetraspanins and integrins and the potential for tetraspanins to modulate motility through these interactions led to the investigation of lymphocyte migration in the CD37$^{-/-}$, Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ mice. Whilst neither CD37 nor Tssc6 have been described as molecularly associated with integrins, CD37 is known to co-localise with the integrin $\alpha4\beta1$ in B cells, and defects in CD37$^{-/-}$ B cell adhesion and rolling under flow are also apparent on the $\alpha4\beta1$ ligands fibronectin and VCAM-1 (van Spriel *et al.*, submitted for publication). One possible explanation for the defects observed in antigen specific T cell development is that antigen captured in the periphery by APC is not delivered efficiently to the T cell zones of lymphoid tissue. To investigate this possibility, DC migration assays were performed *in vivo* to monitor the transition of skin derived DC to the draining lymphoid tissue. In these assays, the cell dye FITC was suspended in a mild irritant and painted onto the skin of wild type and tetraspanin deficient mice (Czeloth *et al.*, 2005). Three days after painting, skin draining lymph nodes were removed from the corresponding sites and DC purified and assessed for FITC staining (Figure 5.12A). Results were expressed as the frequency of migrating DC relative to wild type controls, to enable the pooling of multiple experiments. Skin draining lymph nodes were removed from unpainted areas as negative controls to exclude autofluorescent contaminations (data not shown). From these data it was clear that the frequency of FITC^{+} DC in tetraspanin deficient draining lymph nodes was significantly reduced in comparison to wild type mice (Figure 5.12B). To investigate the possibility that the FITC positive skin derived DC were passing on antigen or being engulfed by lymph node resident DC, cells were co-stained with the markers CD8α and DEC205 (Figure 5.12C). These analyses showed that the FITC^{+} cells were in fact Langerhans and dermal derived DC. The reduced number in FITC^{+} DC in the tetraspanin deficient lymph nodes did not appear to be restricted to either of these subpopulations.

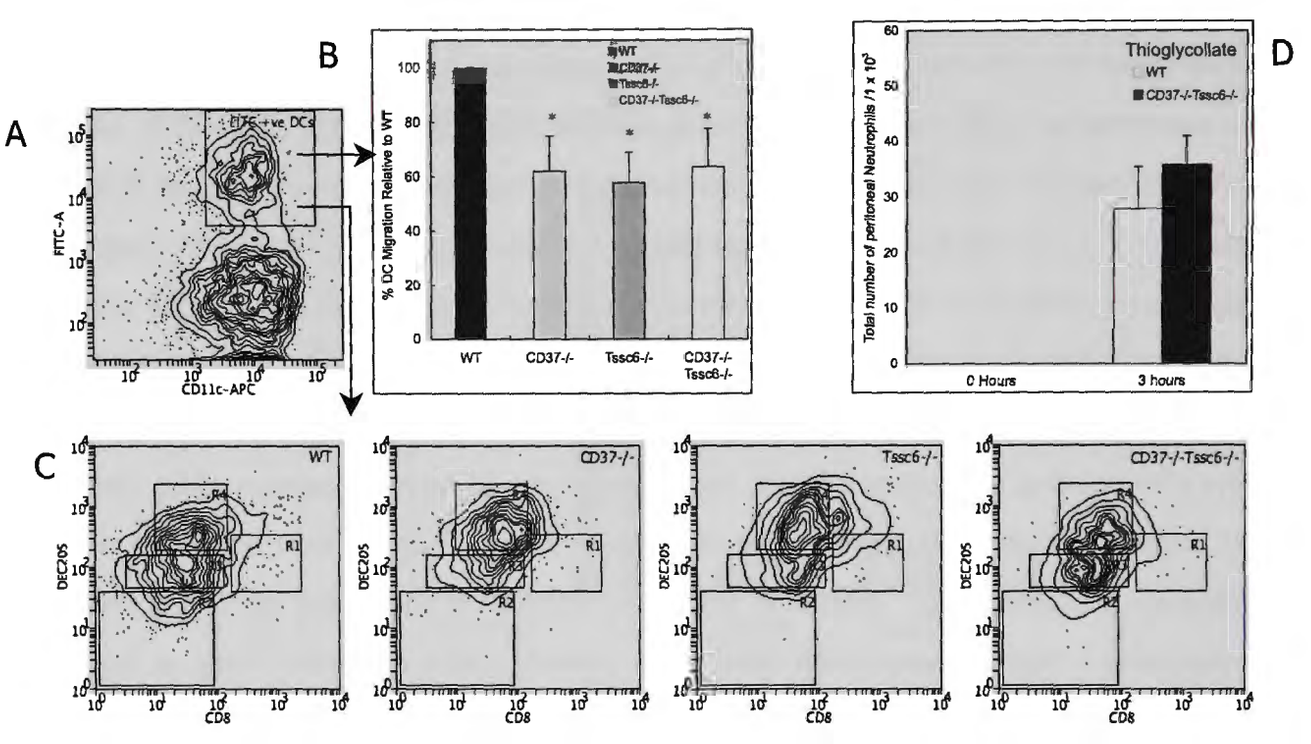

Figure 5.12 In vivo dendritic cell migration is poor in tetraspanin deficient mice.

*(A) Purified dendritic cells from inguinal or subauricular lymph nodes were assessed for FITC staining 3 days after FITC skin painting of the abdomen. Cells were stained with CD11c, post purification and gated as described (wild type example shown). (B) The percentage of migrated dendritic cells was based on the ratio of CD11c$^+$ FITC$^+$ cells found in tissue draining lymph nodes in comparison to wild type controls. (C) FITC positive DC were co-stained with αCD8α and αDEC205 to delineate skin derived DC. Gates R3 and R4 contain 'classical' CD8α^{int}DEC205^{hi} Langerhans DC and CD8α^{lo}DEC205^{int} dermal DC respectively. (D) Neutrophil migration was measured as the influx of GR1$^+$ peritoneal neutrophils 3 hours post intraperitoneal thioglycollate injection. Error bars represent the standard error of mean and significance was tested by students t test (DC migration: n=5 (pooled data), 2 mice/group; neutrophil migration: n=2, 5 mice/group; *, p<0.05).*

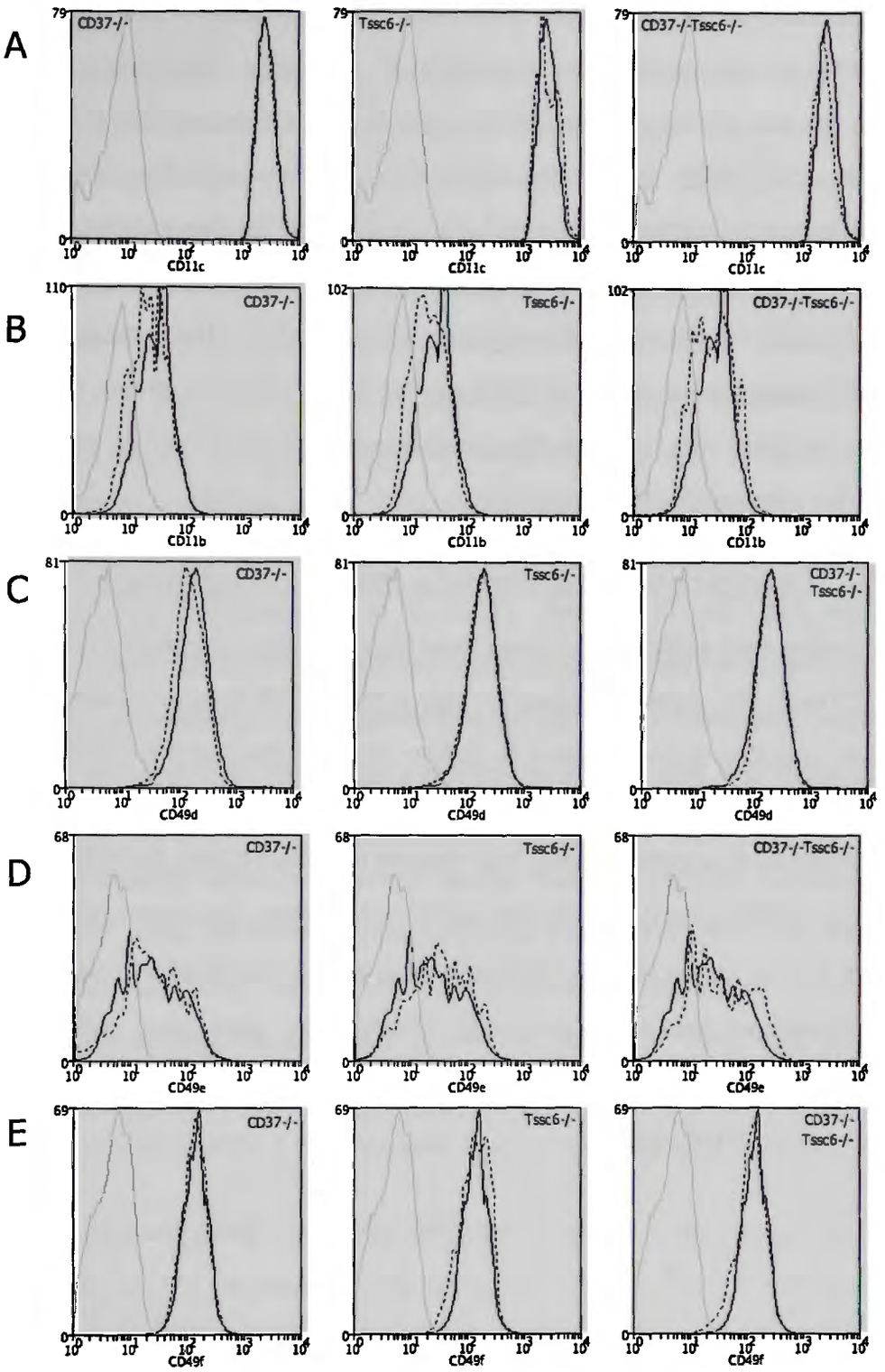

Figure 5.13 Cell surface integrin expression by naïve CD37/Tssc6 deficient DC

Dendritic cells derived from wild type, CD37$^{-/-}$, Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ mice were assessed for cell surface expression of integrins. Dendritic cells were enriched from spleens by density centrifugation and stained with (A) αCD11c and co-stained with the following antibodies (B) αCD11b, (C) αCD49d, (D) αCD49e and (E) αCD49f. Wild type (unbroken line) cell surface staining is shown in comparison to tetraspanin deficient (broken line) staining and controls (grey unbroken line). All histograms shown were gated on CD11c^{hi} cells and the exclusion of propidium idodie (2 mice/group).

Neutrophil migration assays were also performed in CD37$^{-/-}$Tssc6$^{-/-}$ mice, to investigate the possibility of a pan-leucocyte defect in migration *in vivo*. Intraperitoneal injection of thioglycollate in mice results in marked changes in the cellular composition of peritoneal exudate (Barth *et al.*, 1995). Early in the inflammatory response, an influx of neutrophils in the peritoneal cavity occurs within 3 hours and can be detected by flow cytometry of peritoneal exudate. From this study, there were no significant differences observed between the rate of wild type and CD37$^{-/-}$Tssc6$^{-/-}$ neutrophil migration (Figure 5.12D). These data suggest that the changes in DC migration observed in CD37 and/or Tssc6 deficient strains is not a general phenomena, but does not rule out the possibility of altered migratory capacity in other leucocytes in the absence of these tetraspanins.

5.2.10 Cell surface expression of integrins in CD37/Tssc6 deficient mice

Preliminary studies were performed to investigate the expression of integrins on CD37$^{-/-}$, Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ DC surfaces. Integrins play a critical role in DC migration by interacting with adhesion molecules on endothelium to enable migration towards the lymphoid tissue upon activation. Dendritic cells were partially enriched by density centrifugation and the cell surface was characterised by flow cytometry for the following integrins: CD11c ($\alpha X\beta 2$), CD11b ($\alpha M\beta 2$), CD49d ($\alpha 4\beta 1$), CD49e ($\alpha 5\beta 1$) and CD49f ($\alpha 6\beta 1$) for integrin expression (Figure 5.13). As demonstrated in Chapter 4, CD11c ($\alpha X\beta 2$) expression was normal in all strains tested and used to gate on subsequent analyses (Figure 4.8). Whilst there were some variations in CD11b ($\alpha M\beta 2$), CD49d ($\alpha 4\beta 1$) and CD49e ($\alpha 5\beta 1$) expression between strains, no strikingly altered trends have been observed to date.

5.3 DISCUSSION

From the data presented in Chapter 4, it was hypothesised that enhanced *in vivo* cellular immunity would be observed in the absence of CD37 and Tssc6. However, initial studies utilising a viral infection model suggested this was not the case. CD37$^{-/-}$, Tssc6$^{-/-}$ and CD37$^{-/-}$ Tssc6$^{-/-}$ mice all developed low frequencies of antigen specific T cells reactive to dominant Influenza epitopes in comparison to wild type controls. Tetraspanins have been implicated in viral pathogenesis in previous studies (Martin *et al.*, 2005). A number of viruses exploit the role of tetraspanins in a variety of cellular processes to enhance viral attachment, intracellular trafficking, secretion of viral particles, immunosuppression, and syncytia formation via intercellular adhesion (Fukudome *et al.*, 1992, Pique *et al.*, 2000, Flint *et al.*, 1999, Tseng and Klimpel, 2002, Crotta *et al.*, 2002, Garcia *et al.*, 2005, Masciopinto *et al.*, 2001, Higginbottom *et al.*, 2000, Pileri *et al.*, 1998, Drummer *et al.*, 2002). From CD37 and Tssc6 deficient murine responses, it is unlikely that CD37 or Tssc6 are receptors for influenza virus, since their absence led to poor anti-viral immunity. It is possible that the absence of these proteins disrupts other tetraspanins or their partner molecules, resulting in altered viral trafficking or secretion. Although reduced frequencies of antigen specific T cells may suggest immune suppression. Furthermore, preliminary tumour challenge studies found a reduced capacity for tumour rejection in CD37$^{-/-}$ mice. In this study, we confirmed this finding and demonstrated a similar defect in Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ anti-tumour immunity, suggesting functional overlap between CD37 and Tssc6 in the generation of effective cellular immune responses. To further investigate these phenotypes, we used model antigen immunisation strategies to follow the development of antigen specific T cell responses in CD37 and Tssc6 deficient mice *in vivo*.

From initial experiments using γ-irradiated RMA-Muc1 cells to immunise tetraspanin deficient mice, it was clear that the frequency of IFNγ producing antigen specific T cells was significantly lower than that of wild type mice after a single exposure to antigen. This effect was similar in CD37$^{-/-}$ and Tssc6$^{-/-}$ mice and not exaggerated in the absence of both molecules. After a second immunisation, CD37$^{-/-}$ mice were able to develop normal frequencies of antigen specific T cells, although responses remained poor in Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ mice. B16-OVA immunisations demonstrated that this effect was not restricted to either CD4^{+} or CD8^{+} T cells. However after re-exposure to antigen, the defect was observed in both populations of Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ T cells and only the CD8^{+} population of CD37$^{-/-}$ T cells. Therefore, the

normal responses seen in CD37$^{-/-}$ mice during RMA-Muc1 boosted immunisation schedules, are likely to be due to normal CD8^{+} T cell responses after re-exposure to antigen.

Flow cytometric analysis confirmed that these phenotypes could not be attributed to simple changes in the leucocyte composition of tetraspanin deficient mice, resulting in reduced T cell frequencies or increased numbers of immunomodulatory lymphocytes such as regulatory T cells. Reduced frequencies of antigen specific effector T cells were confirmed by immunisation with model antigens, followed by adoptive transfer of antigen loaded splenocytes. As expected, *in vivo* killing of the target cells was poor in CD37$^{-/-}$Tssc6$^{-/-}$ mice. When taken together, a significant defect in the development of antigen specific T cell responses is apparent in the absence of CD37 and/or Tssc6, and explains the reduced ability to ward off viral infection and reject transfected tumour growth in these mice. To explore these phenomena, several aspects of the cellular immune response were investigated.

Altered cytokine production has been previously reported in both CD37 and Tssc6 deficient T cells (Tarrant *et al.*, 2002; van Spriel *et al.*, 2004), therefore it is possible that the poor cellular immune responses to tumour challenge may reflect a more general role for CD37 and Tssc6 in the regulation of T cell cytokines. Antigen specific cytokine responses have also been investigated in CD81 knockout mice, where altered frequencies of IL-4 producing Th2 cells were detected after model antigen immunisation (Maecker *et al.*, 1998). Since Th2 type cytokines such as IL-4 can have a suppressive effect on the generation of Th1 cells (Mosmann and Coffman, 1989), the potential suppressive effect of Th2 type cytokines on Th1 cytokine secretion was investigated in parallel during model antigen immunisations. Whilst IL-4 production was very low in all strains tested, no consistent elevation was detected that may explain the reduced frequency of antigen specific IFNγ producing T cells in this study. Similarly, the IL-17 producing Th17 subset were also been investigated via T cell ELISPOTs performed on mice immunised with cell associated antigens. Whilst IL-17 secreting T cells were detected in response to conA stimulation, there was no evidence of antigen specific Th17 development in these assays (personal communication T.M Chang). In an attempt to investigate the presence of anti-inflammatory cytokines produced by immuno-regulatory cells such as Tregs, IL-10 levels were also measured via CBA array. However IL-10 protein if present was below the level of detection in this assay (data not shown).

ConA stimulations of naïve and immunised splenocytes evidenced that the intrinsic capacity for IFNγ production is not impaired by the absence of CD37 and Tssc6. It is also important to note that whilst antigen specific T cell development is required in both anti-tumour immunity and Influenza A responses (Taylor and Askonas, 1986, Kaplan *et al.*, 1998), IFNγ secretion by these cells is critical in tumour responses only (Graham *et al.*, 1993). Therefore whilst IFNγ responses to specific antigens have been used to quantify antigen specific T cell responses in the model immunisation experiments, an intrinsic defect in IFNγ production would not explain the poor development of cellular immunity post Influenza challenge. This suggests that the defect is more likely to lie in the development of tetraspanin deficient antigen specific T cell populations and not their specific cytokine secretion.

To further investigate the mechanisms behind poor IFNγ responses in the tetraspanin knockout mice, other modes of antigen delivery were explored. Using ANTp conjugated ovalbumin (ANTp-OVA) strikingly similar results were observed in CD37$^{-/-}$, Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ mice as those seen with cell associated antigen immunisations. To examine these responses in the absence of CD4^{+} T cell help and confirm the observation that CD37$^{-/-}$ T cell responses are normal after repeat antigen exposure, mice were immunised with ANTp-SIINFEKL. As seen in previous immunisations, CD37$^{-/-}$ mice responded poorly to re-stimulation at 14 days (data not shown) and normally after 28 days, whilst Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ responses remained poor after 28 days. These results suggest that not only is the defect independent of CD4^{+} T cell help, but is also unlikely to be related to poor antigen processing. Internalising peptide sequences are considered to be an antigen delivery system that does not contain the typical danger signals thought to be important when generating effective cellular responses. To test antigen specific T cell development in the presence of strong inflammatory signals, mice were also immunised with ovalbumin protein in the presence of the adjuvant CFA. In these assays, the development of antigen specific T cells was reduced in wild type mice in comparison to cell associated or ANTp conjugated antigens. However, there were no apparent differences in the frequency of IFNγ producing ovalbumin specific T cells between wild type and tetraspanin knockout mice. This is an important finding in this study, as it demonstrates that whilst the level of inflammation generated upon antigen delivery is important, antigen specific T cell populations can be elicited in the absence of CD37 and or Tssc6. Similar experiments performed in CD81$^{-/-}$ mice, utilising ovalbumin protein precipitated in the adjuvant alum, found normal IFNγ and IL-4 production after a boosted immunisation schedule (Maecker and Levy,

1997). From the data presented in this study, this previous finding does not rule out the possibility of a reduced capacity to generate antigen specific T cells in CD81$^{-/-}$ mice, since the immunisation boost and the adjuvant alum may mask an effect apparent under less inflammatory conditions.

In vitro studies of DC activation demonstrated that CD37$^{-/-}$, Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ DC surface expression of co-stimulatory molecules is upregulated in a normal manner. Therefore, other requirements for antigen presentation *in vitro* were investigated. The relationship between IL-12 cytokine signalling by DC and the induction of IFNγ production by T cells has been previously described (Trinchieri, 2003). IL-12 secretion is induced by DC activation and results in subsequent binding of IL-12 receptors expressed by T cells. Stimulation of the T cell IL-12 receptor leads to secretion of IFNγ, which in turn induces upregulation of cell surface IL-12 receptor expression, creating a positive feedback loop. When IL-12 secretion was compared between activated dendritic cells derived from CD37 and Tssc6 deficient strains, there were no significant differences observed. To reach the threshold of detection in these assays, strong microbial stimuli were required. It may be that differences in IL-12 secretion may manifest when tetraspanin deficient DC are stimulated in a more subtle manner, however this will require some further refining of our current method of IL-12 detection. A second possibility that remains unexplored is the potential for excess IL-12p40 homodimers to play an inhibitory role in IL-12p70 heterodimer signalling (Gillessen *et al.*, 1995). Conversely, in conjunction with IL-12, IL-18 is known to play a synergistic role in the stimulation of IFNγ production by T cells (Okamura *et al.*, 1995). Therefore, altered frequencies of these molecules may also lead to poor promotion of IFNγ secreting T cells. To exclude these factors in future experiments, IL-18 and IL-12p40 specific mAbs will be used to measure these cytokines in activated tetraspanin deficient DC supernatants.

The delivery of antigen to the T cell zones of lymphoid tissue is critically dependent upon the migration of DC between the periphery and lymph nodes. Migration defects have been observed in tetraspanin deficient cells and molecular associations between tetraspanins and integrins have been observed in a number of studies (Takeda *et al.*, 2007, Cowin *et al.*, 2006; Feigelson *et al.*, 2003; Goschnick *et al.*, 2006; Lammerding *et al.*, 2003; Lau *et al.*, 2004). Given these data, a reasonable explanation for the poor development of antigen specific T cells in CD37/Tssc6 deficient mice may simply be a failure to efficiently deliver antigen to naïve T

cells. In this study, *in vivo* DC migration studies supported this theory, whereby the frequency of skin derived DC carrying FITC label to draining lymph nodes was significantly lower in tetraspanin deficient mice. This effect was not exaggerated in the absence of both CD37 and Tssc6, indicating that whilst these tetraspanins share a similar function in modulating DC migration, there is no complementary role in their action. There was no evidence for altered motility in tetraspanin deficient neutrophils, excluding a potential pan-leucocyte migration defect.

In future experiments, a mechanism for poor DC migration in the absence of CD37 and Tssc6 must be investigated. A preliminary assessment of integrin expression in CD37$^{-/-}$, Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ mice was unable to identify major changes at the dendritic cell surface, however some subtle changes were apparent. This does not rule out the possibility that upon DC activation the expression of these integrins may be affected. The integrin $\alpha L\beta 2$ is known to participate in DC migration through its interactions with ICAM-1 on lymphatic endothelial cells. Whereby, DC trafficking from the periphery to the lymph node is dramatically impaired in the absence of endothelial ICAM-1 expression (Xu *et al.*, 2001). Similarly, blocking mAbs directed against both $\alpha L\beta 2$ and $\alpha 4$ integrins have both been found to impair DC migration to the lymph node and thymus respectively (Bonasio *et al.*, 2006; Ma *et al.*, 1994). In addition, tetraspanins have also been suggested to play a role in DC migration, whereby mAbs directed at CD9, CD63, CD81 and CD82 can be used to alter the migratory responses of human moDC to chemokine attractants (Mantegazza *et al.*, 2004). Again however, these results are very difficult to interpret since the effect of Ig binding may be misleading.

The absence of quantitative changes in integrin expression by tetraspanin deficient DC does not necessarily rule out potential changes in integrin function. For example, CD37$^{-/-}$ B cells express normal levels of $\alpha 4\beta 1$, although $\alpha 4\beta 1$ function appears to be altered when B cell adhesion was tested by fibronectin and VCAM-1 binding assays *in vitro* (van Spriel *et al.*, submitted for publication). Haptotactic migration and cellular adhesion assays on a variety of substrates will be used to further investigate the potential for integrin dysregulation in tetraspanin deficient DC. As discussed earlier, increased danger signals during antigen delivery appear to play an important role in promoting normal antigen specific immune responses in CD37 and Tssc6 deficient mice. This finding suggests that the addition of strong inflammatory stimuli during *in vivo* DC migration assays may result in improved DC motility.

Whilst impaired *in vivo* DC migration correlates well with the poor cellular immune responses observed in tumour rejection models and ELISpot re-stimulations, this is not direct proof of a link between these two phenomena. To confirm the causative nature of this defect a number of future experiments are proposed. To determine if the phenotype observed is due to aberrant DC or T cell function, CFSE labelled antigen specific T cells will be adoptively transferred into wild type and tetraspanin deficient mice. These mice will then be immunised with antigen and the proliferative response of the antigen specific T cells monitored i.e. *in vivo* antigen presentation (Li *et al.*, 2001). In reciprocal experiments, wild type and tetraspanin deficient transgenic T cells will be CFSE labelled and adoptively transferred into wild type mice. These mice will then be immunised and the antigen specific T cell responses monitored i.e. *in vivo* T cell proliferation. If the absence of CD37 and/or Tssc6 results in ineffective transport of antigen from the periphery, this should be clear in the antigen presentation assays. Otherwise, if the defect lies in generating effective T cell responses *in vivo* this should become apparent in the T cell proliferation experiments.

This chapter describes a critical role for CD37 and Tssc6 in the development of antigen specific T cells. To explain this phenotype, several requirements for the development of these cells were examined. Splenocyte composition and cytokine output by DC and Th2 cells remained comparable to wild type controls. However, DC migration between the skin and lymph nodes was significantly poorer in the absence of CD37 and/or Tssc6. This motility defect was observed in all three strains and is possibly the underlying cause of poor cellular immune responses observed in tumour and viral challenges. Another potential explanation for reduced frequencies of antigen specific T cells may be that the development of tolerance in tetraspanin deficient T cells is altered. T cell tolerance can occur due to partial activation of T cells by antigen *in vivo* resulting in either T cell deletion or anergy (Lechler *et al.*, 2001). One way to investigate this would be to immunise mice using ANTp conjugated antigen followed by an adjuvant emulsified antigen. Presumably, the initial immunisation (e.g. ANTp-OVA) would generate anergic T cells in the tetraspanin deficient mice and minimise antigen specific T cell responses. Therefore IFNγ responses to an immunisation boost (e.g. CFA-OVA) may also be poor, unlike those observed in primary CFA-OVA immunisations.

Since some T regulatory cells that secrete the suppressive cytokine IL-10 do not express FoxP3 (Weaver *et al.*, 2006), the potential for suppressive or regulatory T cells could also be further

investigated in tetraspanin deficient mice. For example, the production of IL-10 could be measured in ELISpots of B16-OVA immunised tetraspanin deficient mice. In addition, wild type IFNγ production post immunisation with ANTp-OVA or B16-OVA could be measured in ELISPots that also contain T cells derived from immunised tetraspanin deficient mice. These could be compared to co-cultured wild type T cells derived from immunised and naive mice. If the poor T cell responses observed in tetraspanin deficient mice are due to T cell suppression/regulation, IFNγ production by wild type T cells incubated in the presence of tetraspanin deficient T cells would be impaired.

There was little evidence for an exaggerated phenotype in the absence of CD37 *and* Tssc6, which indicates that these molecules display a shared role, but not functional redundancy or synergism, in the promotion of cellular immunity. This is somewhat contrasted by the exaggerated impairment of anti-viral immunity observed in CD37$^{-/-}$Tssc6$^{-/-}$ mice. It remains possible that the absence of both tetraspanins induces some immunosuppressive effect in viral infection that is not apparent in tumour challenges or antigen immunisation models.

6 AGEING & AUTOIMMUNITY IN TETRASPANIN DEFICIENT MICE

6.1 INTRODUCTION

Ageing of the immune system is typified by increased susceptibility to infection, autoimmunity and cancer in the elderly. One effect of immune ageing is a shift in haematopoietic stem cell (HSC) output from predominantly lymphoid progenitors to increasing numbers of myeloid progenitors (Rossi *et al.*, 2005). This shifting haematopoietic profile results in altered inflammatory responses and reduced adaptive immune responses (De Martinis *et al.*, 2005; Licastro *et al.*, 2005; Wang *et al.*, 1995). The reduction in adaptive immune responses due to ageing occurs in both T cell and B cell compartments. Gene rearrangement in B cells is impaired due to decreased expression of RAG genes, leading to reduced frequencies of naïve B cells (Ben-Yehuda *et al.*, 1994; Szabo *et al.*, 1998). Furthermore, B-T cell interactions are impaired due to poor germinal centre formation resulting in increased production of lower affinity antibodies and T cell independent immune responses (Lazuardi *et al.*, 2005).

Thymic involution is a natural process of thymic degeneration that occurs over an extended period of time spanning early and late adulthood. As the thymus atrophies, thymic output is reduced and peripheral T cell numbers are maintained through reduced apoptosis of memory and naïve T cells (Ernst *et al.*, 1990; Hulstaert *et al.*, 1994). These cells have diminished replicative potential based on reduced telomere length, reduced IL-2 production, Th2 biased cytokine production and altered signalling capacity (Chakravarti and Abraham, 1999). This process significantly alters cellular immune responses through an accumulation of poorly reactive naïve T cells and memory T cells in the lymphoid tissue (Saule *et al.*, 2006). Collectively, this decline in immune function is termed 'immuno-senescence' and is thought to be the underlying cause of a reduced capacity to ward off bacterial and viral infections and poor anti-tumour surveillance in the elderly.

Despite these reductions in both cellular and humoral immune responses, the incidence of autoimmunity increases with age. One consequence of the HSC shift towards myeloid progenitors is an increase in macrophage and neutrophil numbers. However, these cells are also adversely affected by ageing, displaying altered cytokine output and reduced phagocytic capacity (Khare *et al.*, 1996, Plowden *et al.*, 2004, Higashimoto *et al.*, 1993). In humans, an increase in pro-inflammatory cytokine production has been linked with diseases such as osteoporosis and rheumatoid arthritis, which are a result of chronic inflammation (Ginaldi *et al.*, 2005; Hussein *et al.*, 2008). There is also evidence that age related changes in both T and B cell function have consequences for the development of autoimmunity. Autoantibody production and auto-reactive T cells can be detected in all age groups, however tolerance

and deletion of these cells are thought to diminish over time (Falcone *et al.*, 2001; Hsu *et al.*, 2001; Wakabayashi *et al.*, 1999). Furthermore, the diminished capacity for GC formation leads to in impaired antibody affinity maturation and reduced specificity of Ig (Han *et al.*, 2003). Mechanical and age related deterioration of joints and tissue are also thought to expose antigens previously unseen by the adaptive immune system – 'neo-self antigens', and therefore the frequency of autoantibodies increases over time (Candore *et al.*, 1997).

Age related changes to the immune system such as those described above, form a complex recipe for autoimmunity and immunodeficiency. One way of combating these challenges in the ageing population is to identify molecules that may act as potential targets for immune modulation. The findings described in previous chapters of this study suggest that some tetraspanins may be potential candidates for immunotherapy development. To explore this possibility, ageing and autoimmunity in the absence of CD37 and Tssc6 are examined in this chapter.

6.1.1 Study Rationale

Although tetraspanin knockout mice display a variety of immunological defects, autoimmune disease in these mice remains largely unstudied. In this study, the *in vitro* immunological phenotypes discussed in previous chapters have suggested that the potential for autoimmune development in tetraspanin deficient mice may be altered. Similarly, these mice may display increased infection and tumour susceptibility.

This chapter aims to:

(1) Determine if the absence of CD37 and/or Tssc6 results in increased susceptibility to autoimmune induction.

(2) Establish if the absence of CD37 and/or Tssc6 in ageing mice results in increased susceptibility to spontaneous autoimmune development or mortality.

(3) Investigate a potential role for CD37/Tssc6 in the development of immuno-senescence in geriatric mice.

6.2 RESULTS

6.2.1 AUTOIMMUNE INDUCTION VIA COLLAGEN INDUCED ARTHRITIS.

Two protocols of CIA were utilised, in which C57Bl/6 mice display either a low incidence of disease, i.e. two immunisations of chicken type II collagen emulsified in complete Freund's adjuvant (CFA); or are resistant to disease, i.e. two immunisations of bovine type II collagen emulsified in incomplete Freund's adjuvant (IFA). When using the CFA/chicken collagen protocol, a higher arthritis index and an increased incidence of disease was observed in CD37$^{-/-}$Tssc6$^{-/-}$ mice compared to wild type controls (Figure 6.1A-D), however these differences were not significant. In order to test the strength of this observation, we also used the IFA protocol whereby mice on a *H-2*^{b} background show no signs of disease. In this way, any increased susceptibility may be seen in more striking contrast to wild type controls. As a positive control in these experiments we used DBA/1 (*H-2*^{q}) mice that are highly susceptible to this model of arthritis induction. From Figure 6.1E-F it can be clearly seen that there was no increased susceptibility to arthritis in any of the tetraspanin deficient mice tested in this model.

6.2.2 AUTOIMMUNE INDUCTION VIA EAE.

Autoimmune encephalomyelitis (EAE) is a mouse model of Multiple Sclerosis generated by exposure to myelin sheath peptides in the presence of adjuvant and *Bordetella pertussis* toxin (Bt). Like the collagen induced arthritis model, the exposure of strong adjuvant in the presence of self-like antigen leads to a cross-reactivity of both the cellular and humoral immune system leading to autoimmune attack. The target in the EAE model is a murine myelin protein found in the lining of nervous tissue. By inducing an immune response against the nervous system, this model has been used successfully to understand the role of innate and adaptive immunity in multiple sclerosis research (Baxter, 2007). The immune responses in the EAE model are mostly at the cellular level, where auto-reactive T cells and macrophages produce cytokines that target the central nervous system (CNS). Over a period of 2 weeks symptoms of the disease including muscle weakness and eventually paralysis develop. In the third and fourth weeks of these experiments, as cellular responses die down, there is a recovery period where gain of function can be seen in the lower limbs and tail. From these analyses, a significantly earlier onset of tail weakness and weight loss was observed in CD37$^{-/-}$Tssc6$^{-/-}$ mice, although the onset of tail paralysis occurred at a similar time in comparison to wild type controls (Figure 6.2).

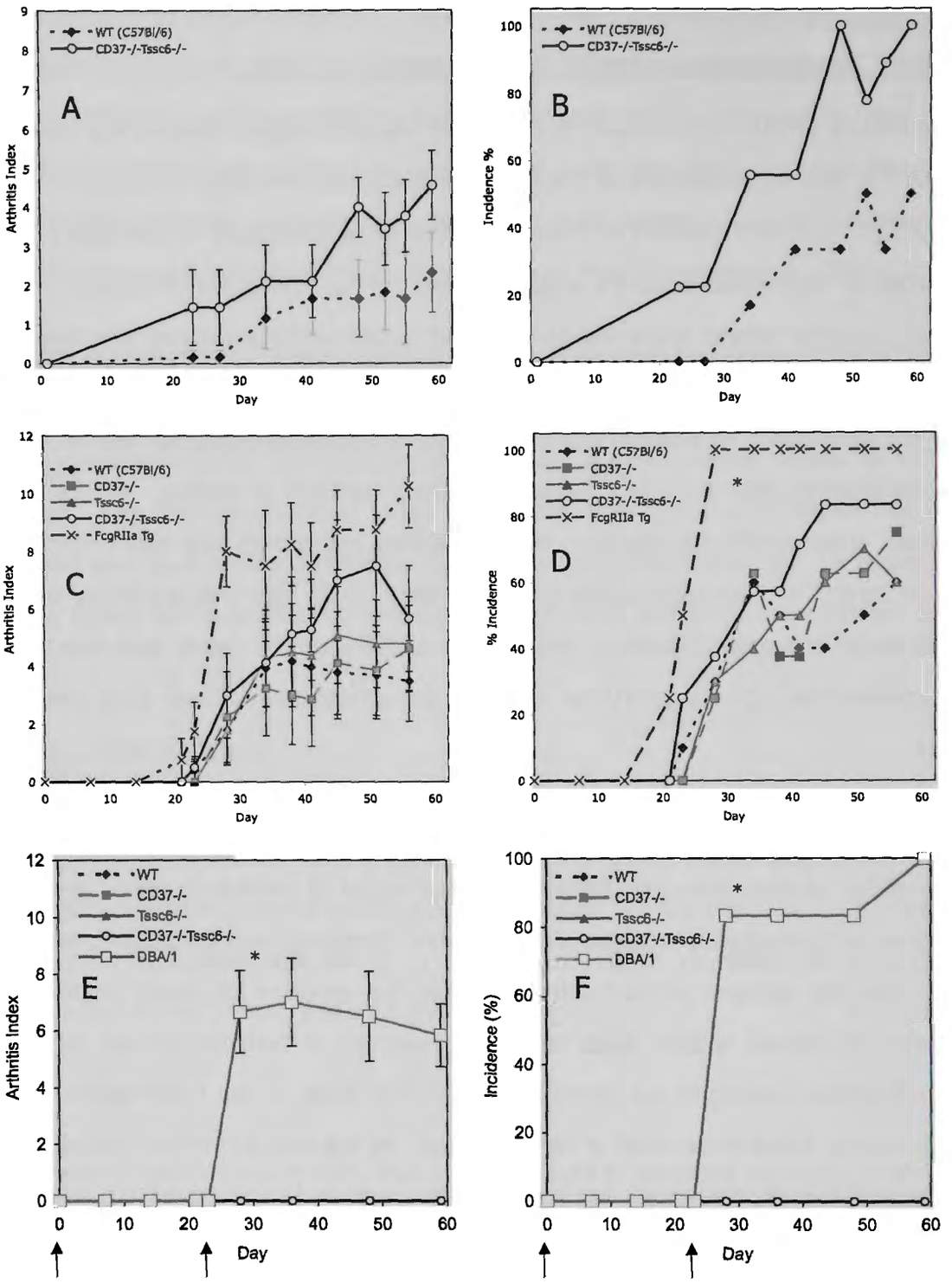

Figure 6.1 Tetraspanin knockout mice and Collagen Induced Arthritis.

Groups of 5-10 mice were immunised with 200μg chicken type II collagen emulsified in CFA (A-D) or 200μg bovine type II collagen emulsified in IFA (E-F) on days 0 and 21. Arthritis index was monitored in these mice on a weekly basis over a period of up to 9 weeks. (A, C & E) Results are expressed as a clinical score based upon the mean arthritic score for all individuals within each group. Error bars represent the standard error of the mean for each time-point and group. (B , D & F) Incidence of arthritis expressed as a percentage of animals within each group displaying clinical scores ≥ 1. Statistical analysis was performed by students t test to assess differences in mean arthritis index and log rank test to assess the rates of disease incidence (=p<0.05).*

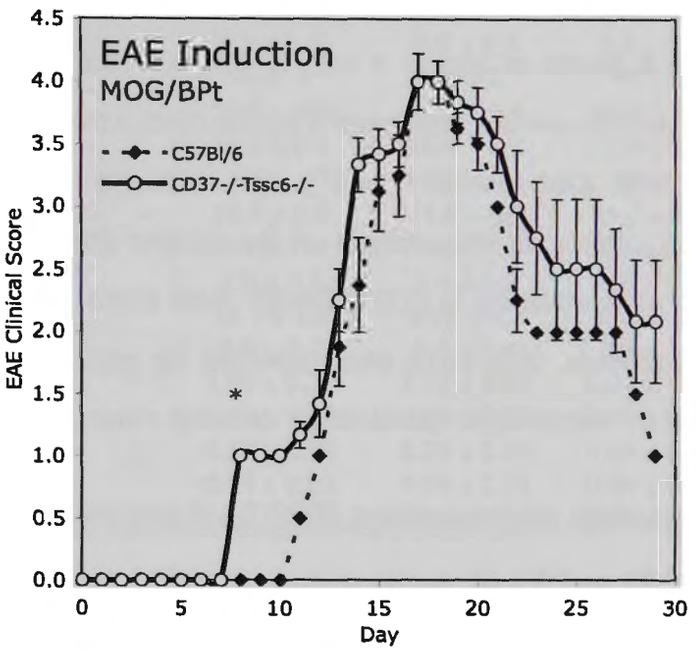

Figure 6.2 The absence of CD37 and Tssc6 confers a mild change in the kinetics of EAE

*Experimental autoimmune encephalomyelitis (EAE) was induced by immunisation of peptide derived from murine myelin sheath protein emulsified in CFA. Additional inflammatory responses were induced by co-immunisation with Bt on Day 0 and a boost injection of Bt on Day 2. Disease severity was monitored in wild type and CD37-/-Tssc6-/- mice daily over 30 days and data points represent the mean clinical score as described in materials and methods. Significance was tested by students t test in comparison to wild type controls (n=2, 6 mice/group). *, P< 0.05.*

6.2.3 Pathology of ageing CD37/Tssc6 deficient mice

To investigate potential changes in ageing and age related pathologies, groups of 10 wild type, $CD37^{-/-}$, $Tssc6^{-/-}$ and $CD37^{-/-}Tssc6^{-/-}$ female mice were housed in germ free conditions at the Burnet Institute animal research facility for a period of 18 months. This represents approximately nine tenths of the mean C57Bl/6 female mouse lifespan (Coleman *et al.*, 1966). Mice were monitored for general health on a weekly basis and during the course of this study 6 mice were culled due to ethical or experimental reasons. Two mice developed hydrocephaly before 8 weeks of age (1 x wild type, 1 x $Tssc6^{-/-}$), one mouse developed peritonitis ($CD37^{-/-}$), another mouse developed a kidney obstruction ($CD37^{-/-}$) and one mouse developed an aggressive axial tumour ($Tssc6^{-/-}$). An additional wild type mouse was also culled to serve as a control for the analysis of the tumour affected $Tssc6^{-/-}$ mouse. No obvious pathologies were observed in $CD37^{-/-}Tssc6^{-/-}$ mice that required premature removal from the ageing population. Mice were also inspected for gross neurological defects and arthritis development by macroscopic examination, of which none were detected.

6.2.4 Haematological profile in ageing CD37/Tssc6 deficient mice.

The tetraspanins CD37 and Tssc6 are expressed in leucocytes and haematopoietic cells respectively. To investigate a potential role for these molecules in long term steady state hemopoiesis, full blood examinations were performed on the tetraspanin deficient mice in comparison to age and sex matched wild type controls (Table 6.1 & Figure 6.3). From these studies, there was no evidence of thrombocytopenia, anaemia, or polycythemia. However, significantly elevated numbers of white blood cells (WBCs) were detected in $CD37^{-/-}Tssc6^{-/-}$ mice. These elevated indices correlated with an increased number of lymphocytes and basophils detected in these analyses. A similar but less striking increase was also observed in the cellularities of $CD37^{-/-}$ peripheral blood lymphocytes.

Table 6.1 Full blood examination of ageing tetraspanin deficient mice.

C57Bl/6, CD37$^{-/-}$, Tssc6$^{-/-}$ & CD37$^{-/-}$Tssc6$^{-/-}$ mice were housed under routine animal housing conditions for a period of 18 months. Mice were then weighed and peripheral blood collected for full blood examination. Values represent the mean haematological parameters of 5 female mice +/- the standard error of mean.

Full blood Examination				
	Strain			
Haematological Parameter	C57Bl/6	CD37$^{-/-}$	Tssc6$^{-/-}$	CD37$^{-/-}$Tssc6$^{-/-}$
Haemoglobin (g/L)	131 ± 9	149 ± 4	126 ± 10	144 ± 3
Red Cell Count (x 10^9/mL)	8.5 ± 0.5	9.0 ± 0.3	8.6 ± 0.8	9.2 ± 0.2
Haematocrit (%)	41 ± 3	46 ± 2	40 ± 4	46 ± 1
Mean Cell Volume (fL)	48.0 ± 0.6	50.8 ± 0.6	46.8 ± 0.8	50.2 ± 0.4
Mean Cell Haemoglobin (pg)	15.2 ± 0.4	16.4 ± 0.1	15.9 ± 2.1	15.5 ± 0.1
Mean Cell Haemoglobin Conc. (g/L)	318 ± 4	322 ± 1	341 ± 49	311 ± 1
Red Cell Distribution Width (%)	15.4 ± 0.8	14.8 ± 0.2	15.6 ± 0.7	15.3 ± 0.2
Platelets (x 10^6/mL)	926 ± 143	886 ± 57	919 ± 101	1250 ± 182
Mean Platelet Volume (fL)	3.2 ± 0.2	3.4 ± 0.2	3.6 ± 0.2	3.0 ± 0.07
Platelet Distribution Width	12.7 ± 1.2	14.9 ± 0.4	15.0 ± 0.6	13.3 ± 1.4
White Blood Cells (x 10^6/mL)	3.8 ± 0.7	5.6 ± 0.9	4.6 ± 0.9	8.2 ± 1.2
Neutrophils (x 10^6/mL)	1.06 ± 0.20	1.23 ± 0.33	0.92 ± 0.20	1.43 ± 0.32
Lymphocytes (x 10^6/mL)	2.48 ± 0.50	4.06 ± 0.59	3.51 ± 0.76	6.33 ± 1.06
Monocytes (x 10^6/mL)	0.10 ± 0.04	0.20 ± 0.09	0.06 ± 0.02	0.11 ± 0.07
Eosinophils (x 10^6/mL)	0.04 ± 0.02	0.04 ± 0.01	0.04 ± 0.01	0.07 ± 0.01
Basophils (x 10^6/mL)	0.11 ± 0.01	0.08 ± 0.03	0.08 ± 0.03	0.22 ± 0.06

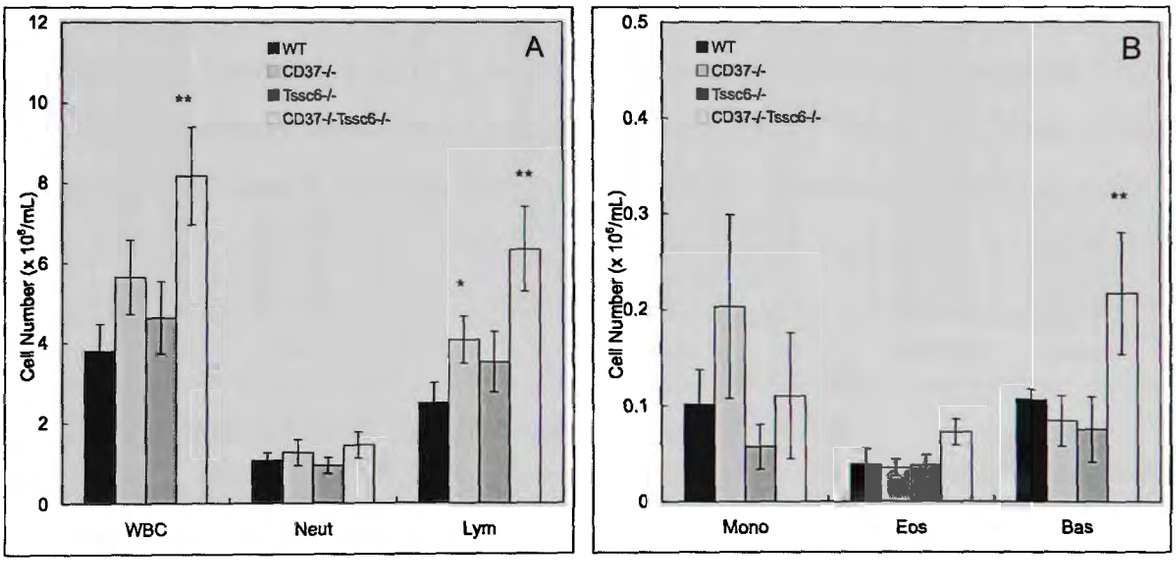

Figure 6.3 Elevated numbers of leucocytes in ageing CD37$^{-/-}$Tssc6$^{-/-}$ mice

*C57Bl/6, CD37$^{-/-}$, Tssc6$^{-/-}$ & CD37$^{-/-}$Tssc6$^{-/-}$ mice were housed under routine animal housing conditions for a period of 18 months. Mice were then weighed and peripheral blood collected for full blood examination. Histogram bars represent the mean cell number of 5 female mice and error bars represent the standard error of mean. Significance was tested by students two-tailed t test in comparison to wild type controls. *, P< 0.05, **, P< 0.005.*

6.2.5 Serum and urine biochemistry in ageing CD37/Tssc6 deficient mice

Biochemical analysis of serum was used to assess renal and hepatic function. Renal failure is characterised by abnormal levels of serum electrolytes, urea and creatinine due to poor filtration and elimination by the kidneys. Glomeruli damage is also characterised by reduced protein levels in the blood and the subsequent loss of protein through urinary excretion. There was no evidence of acute or chronic renal failure or nephrotic syndrome in the absence of CD37 and/or Tssc6, these data also suggest the absence of nephritis in the ageing tetraspanin deficient populations (Figure 6.4A). Poor liver function is characterised by the abnormal levels of protein, albumin and bilirubin in the serum. These changes are accompanied by the accumulation of liver enzymes such as alanine aminotransferase (ALT) and alkaline phosphatase (ALP) in the serum that are indicative of liver damage (Roe, 1993). Whilst there were broader variations in liver function between individual ageing mice, there were no significant differences observed in $CD37^{-/-}$, $Tssc6^{-/-}$ and $CD37^{-/-}Tssc6^{-/-}$ mice and controls (Figure 6.4B-D).

Biochemical analyses of urine specimens were used to investigate the onset of age related diabetes. High levels of glucose and normal ketone excretion were detected in one $Tssc6^{-/-}$ mouse in this study (Figure 6.5). This mouse corresponded to the heaviest mouse in the $Tssc6^{-/-}$ group and together these data are indicative of Type 2 diabetes. Surprisingly, the $CD37^{-/-}$, $Tssc6^{-/-}$ and $CD37^{-/-}Tssc6^{-/-}$ strains all displayed significantly increased weight gain in comparison to wild type controls, although no other cases of Type II diabetes were detected.

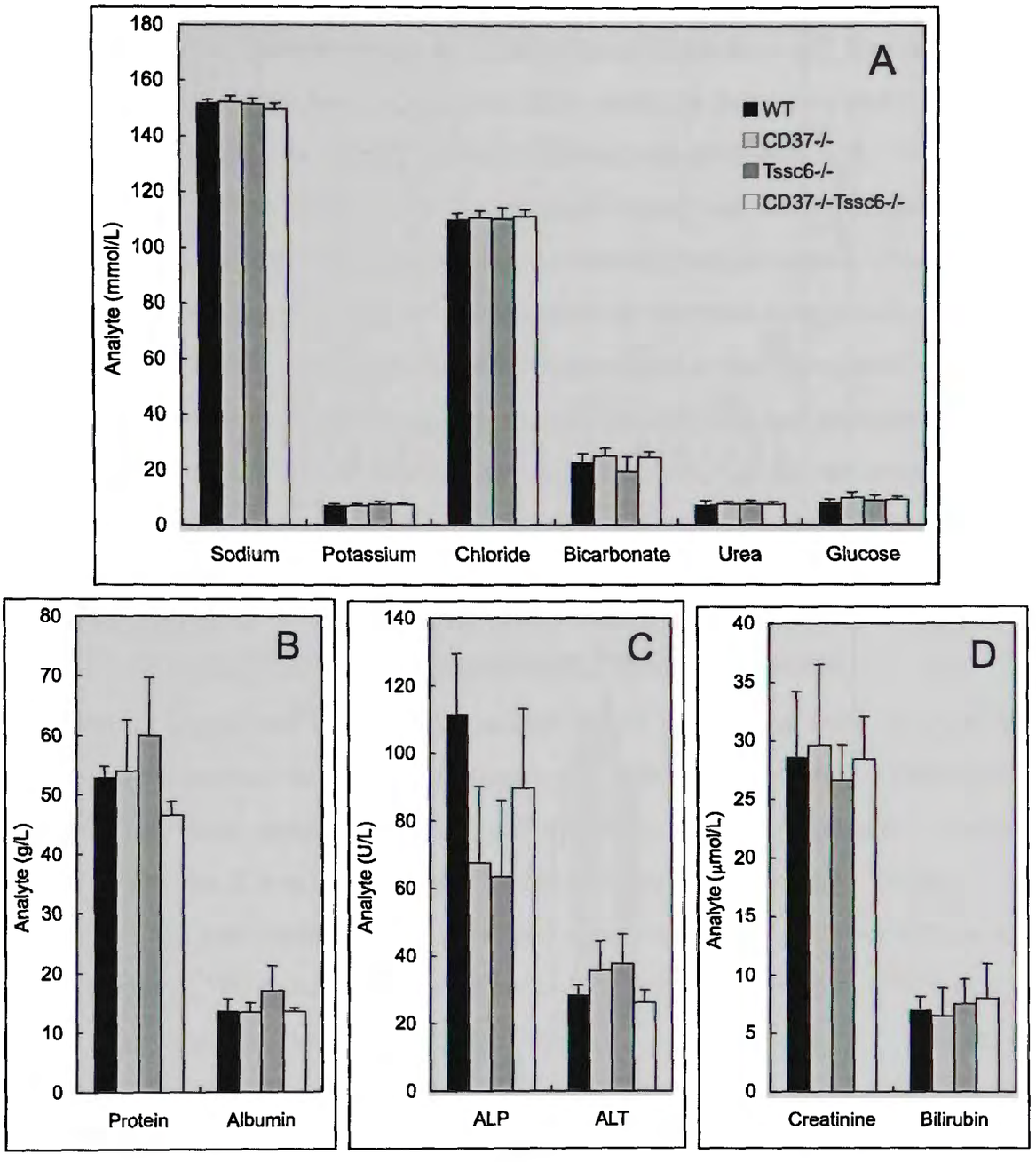

Figure 6.4 Normal liver & kidney function in ageing Tetraspanin deficient.

*C57Bl/6, CD37$^{-/-}$, Tssc6$^{-/-}$ & CD37$^{-/-}$Tssc6$^{-/-}$ mice were housed under routine animal housing conditions for a period of 18 months. Mice were then weighed and peripheral blood collected for serum biochemistry. Histogram bars represent the mean chemistry of 5 female mice and error bars represent the standard error of mean. Significance was tested by students two-tailed t test in comparison to wild type controls. *, P< 0.05, **, P< 0.005.*

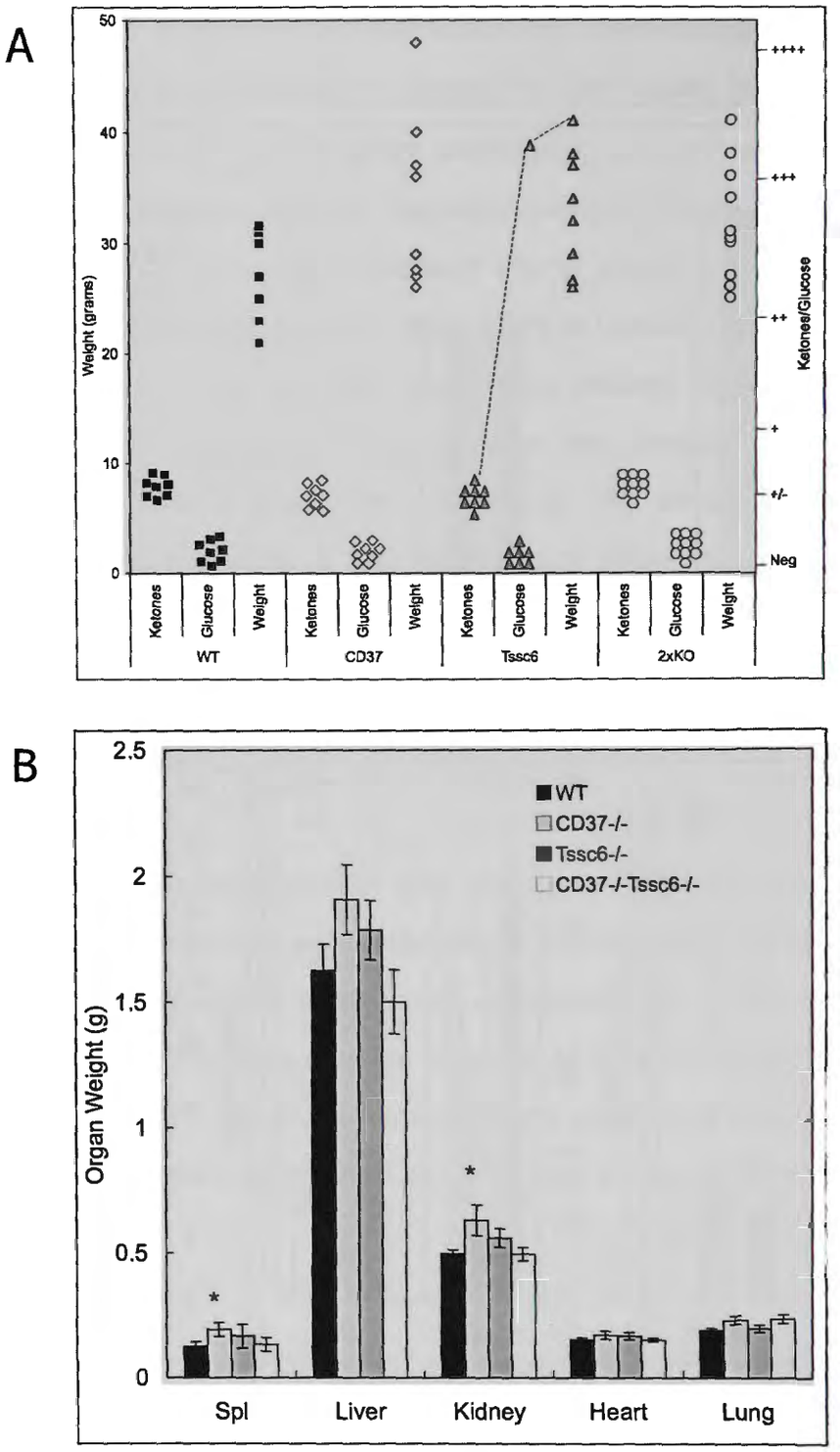

Figure 6.5 Urine biochemistry, body and organ weight in ageing tetraspanin deficient mice.

Wild type, CD37$^{-/-}$, Tssc6$^{-/-}$ & CD37$^{-/-}$Tssc6$^{-/-}$ mice were housed under routine animal housing conditions for a period of 18 months. (A) Mice were weighed and urine collected for biochemical analysis. The corresponding results for one Tssc6$^{-/-}$ mouse are linked by dashed line. (B) Mice were sacrificed and spleen, liver, kidney, heart and lung were removed and weighed. Histogram bars represent the mean organ weight of 5 mice per group and error bars represent the standard error of the mean. Statistical significance was determined by students t test (=p<0.05).*

6.2.6 Organ weights and splenocyte composition in ageing CD37/Tssc6 deficient mice

Organ weights were measured as a broad indicator of tissue pathology and inflammation. In $CD37^{-/-}$ mice, kidney and spleen weights were significantly higher than wild type counterparts. As discussed earlier, kidney function appeared to be normal in these mice and therefore it is unlikely that nephropathy or nephritis were a significant factor (Figure 6.5). One potential cause for increased organ weights may stem from the higher overall body weights in $CD37^{-/-}$ mice in comparison to both wild type and other tetraspanin deficient strains. Flow cytometry was used to further assess the composition of spleens derived from ageing mice. In contrast to the differences seen in the peripheral blood compositions of the ageing mice, there were no significant differences observed between the geriatric wild type and tetraspanin deficient splenic populations or T cell subsets (Figure 6.6). However, the splenic composition was significantly different from those of 8 week old naïve mice investigated in Chapter 5, suggesting the onset of immuno-senescence.

6.2.7 Spontaneous Tumour development in one Tssc6 deficient mouse.

The ageing wild type and tetraspanin knockout mouse populations were observed on a weekly basis to monitor for general ill health and disease susceptibility. One potential outcome of the poor antigen specific T cell responses and poor anti-tumour immunity observed in Chapter 5 may be increased susceptibility to tumourigenesis. Of the 40 mice observed in total, one $Tssc6^{-/-}$ mouse developed a macroscopic axial tumour (Figure 6.7A) after 16 months. The tumour became visible and grew rapidly over a 1 week period and histological examination determined the carcinoma to be comprised of "...spindle cells arranged in fascicles. The cells were variably sized with granular eosinophilic cytoplasm, enlarged nuclei and prominent nucleoli. The nuclei were enlarged, vesicular and cigar shaped..." (Figure 6.7B). Flow cytometric analysis of the spleen and peripheral blood demonstrated increased numbers of macrophages in the tumour affected mouse in comparison to the age matched wild type control (data not shown).

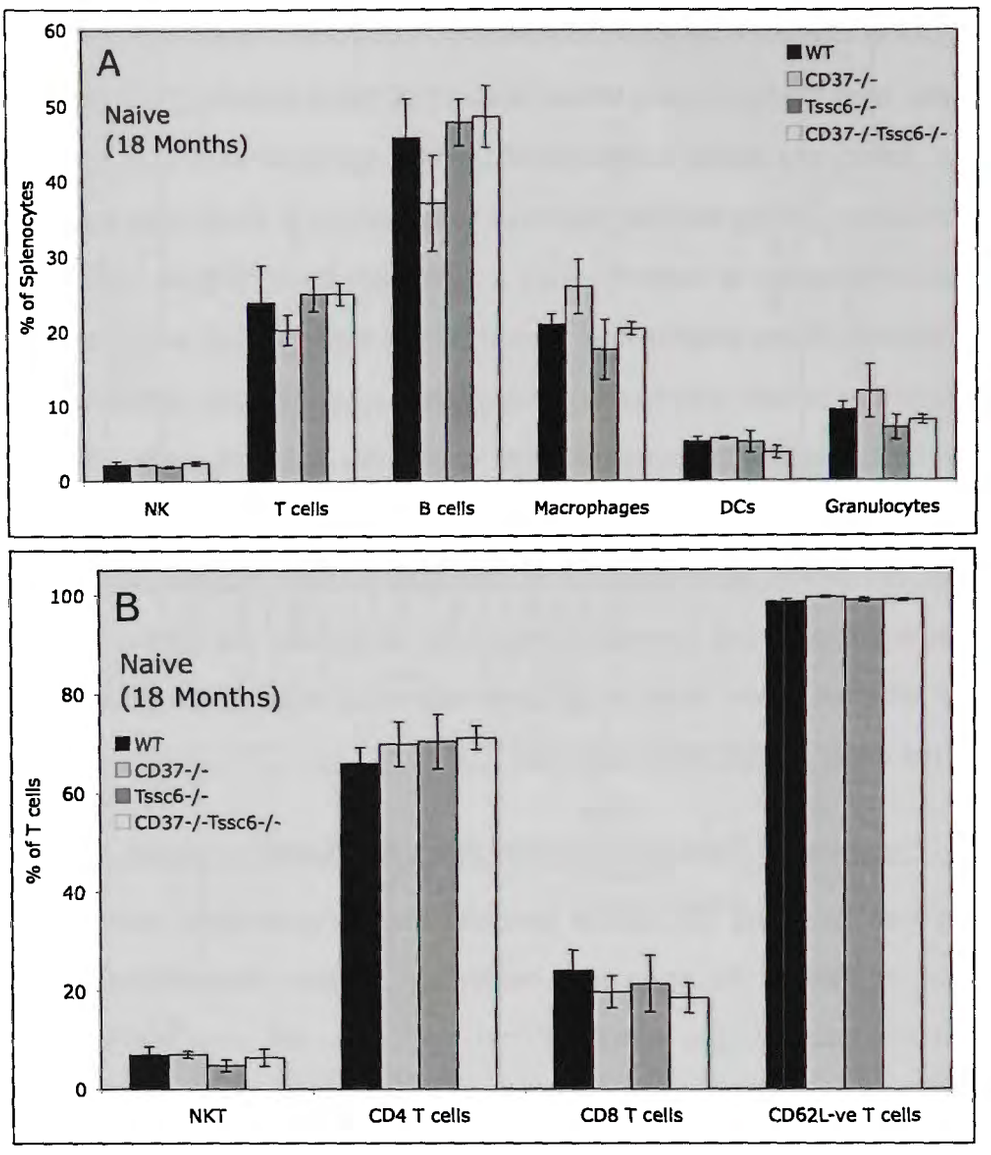

Figure 6.6 Splenocyte distribution in ageing tetraspanin deficient mice

FACS analyses were performed on splenocyte preparations from wild-type and tetraspanin deficient mice. Histogram bars represent the mean frequency of the given populations of splenic lymphocytes derived from six mice per group. Error bars represent the standard error of the mean and significance was determined by students t test. (A) The percentages of major splenocytes populations such as NK cells, CD3+ T cells, B cells, DC, Granulocytes (Gr) and Macrophages (Mac) were expressed as relative percentage to the total viable cells. (B) T cell subpopulations including NKT, CD4+, CD8+ and CD62L- cells were analysed. (=p<0.05).*

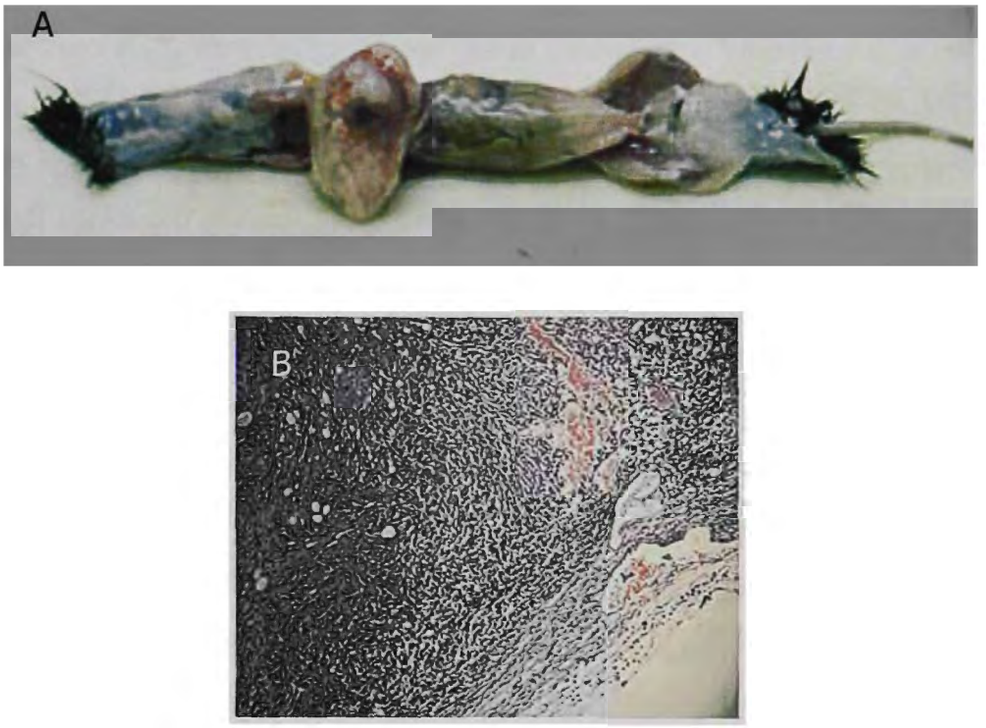

Figure 6.7 Spontaneous tumour development in one Tssc6 deficient mouse.

Groups of 10 C57Bl/6, CD37$^{-/-}$, Tssc6$^{-/-}$ & CD37$^{-/-}$Tssc6$^{-/-}$ mice were housed under routine animal housing conditions for a period of 18 months. After 16 months, a spontaneous tumour developed in one Tssc6$^{-/-}$ mouse located adjacent to the axial lymph node. (A) This mouse was culled immediately after the pathology was detected and the tumour removed for histological examination (B).

6.3 DISCUSSION

Collagen induced arthritis (CIA) is a commonly used model in mice for the study of Rheumatoid Arthritis (RA) in which autoantibody production is induced by immunisation with collagen and adjuvant (Trentham *et al.*, 1977). Upon boosting after 21 days, polyarthritic joint destruction develops over a period of 80 days. A combination of T cell activation, autoantibody and cytokine production are believed to be the underlying mechanisms for this disease (Brand *et al.*, 2003). In mice as well as humans, MHC Class II allelic expression can confer disease susceptibility or resistance in RA, notably C57Bl/6 *(H-2^b)* is considered a CIA resistant mouse strain, whilst DBA/1 (*H-2^q*) mice are highly susceptible to this model (Campbell *et al.*, 2000; Courtenay *et al.*, 1980). In this study, C57Bl/6 mice that express the human Fc receptor FcγRIIa transgene were used as a positive control. This receptor has been strongly linked to rheumatoid arthritis in humans, but is not natively expressed in mice (Ierino *et al.*, 1993). However, transgenic expression of human FcγRIIa confers susceptibility on C57Bl/6 mice to CIA (Tan Sardjono *et al.*, 2005). This was not the case for the tetraspanin deficient mice used in this study. Despite the enhanced capacity for T cell proliferation and antigen presentation *in vitro*, CD37$^{-/-}$, Tssc6$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ mice all remained resistant to collagen induced arthritis induction in the IFA immunisation model and shared similar degrees of incidence and severity with wild type mice in the more aggressive CFA model. Therefore the absence of CD37 and or Tssc6 is not sufficient to convert a genetically resistant strain to that of a susceptible one.

To further investigate the potential for increased susceptibility to autoimmune induction in CD37/Tssc6 deficient mice, experimental autoimmune encephalomyelitis (EAE) was used. EAE is a pro-inflammatory autoimmune disorder targeting the central nervous system (CNS) commonly used as an animal model for the human disease multiple sclerosis (MS) (Baxter, 2007). EAE is characterised by auto-reactive CD4$^+$ and CD8$^+$ T cells that infiltrate the CNS and induce demyelination and nervous tissue destruction (Zamvil and Steinman, 1990, Willenborg *et al.*, 1996). EAE was chosen due to the large component of T cell induced pathology, which may be altered by the absence of complementary regulation of T cell proliferation by CD37 and Tssc6. In this model, auto-reactive T cells are generated by immunisation of a peptide derived from the CNS sheath protein – myelin oligodendrite glycoprotein (MOG) emulsified in adjuvant and further inflammatory stimuli are provided by co-immunisation of pertussis toxin. Whilst there were some differences observed in the kinetics of the disease, there were no overall changes in the severity or incidence of EAE in

the absence of CD37 and Tssc6. Furthermore, as cellular responses diminished with time the reduction in disease severity was also unchanged in $CD37^{-/-}Tssc6^{-/-}$ mice.

Suppressor T cells are thought to play a significant role in the reduction of disease severity during the early stages of induction and the recovery phase of this model (Bynoe *et al.*, 2007; Lee *et al.*, 2008). However the broad and variable definitions of suppressor T cells in these studies make these data difficult to draw conclusions from. The role of IFNγ in EAE models is also complex. Whilst Th1 type $CD4^+$ T cell clones appear to be the predominant auto-reactive T cells found infiltrating the CNS, mice deficient in IFNγ expression are equally susceptible to EAE induction to wild type controls (Ferber *et al.*, 1996). Furthermore, these mice appear to recover poorly during down regulation of the effector phase that suggests IFNγ can actually play a protective role in EAE recovery (Wensky *et al.*, 2005). It is now apparent that the cytokine responsible for promoting IFNγ production - IL-12, shares one heterodimeric subunit with the cytokine IL-23 (Oppmann *et al.*, 2000). This recently identified cytokine plays a role in generating a third Th subset termed Th17, which secrete the cytokine IL-17 (Abbas *et al.*, 2007). Adoptive transfer of CNS antigen specific IL-17 secreting $CD4^+$ T cells has since been demonstrated to induce complete EAE disease pathology in non-immunised wild type mice (Langrish *et al.*, 2005). Not surprisingly, mice deficient in IL-23 expression are completely resistant to EAE induction. Prior findings of altered cytokine secretion by tetraspanin deficient T cells suggest that altered frequencies of IL-17 secreting $CD4^+$ T cells may provide one explanation for altered $CD37^{-/-}Tssc6^{-/-}$ EAE disease kinetics. To investigate this possibility in future experiments, the frequency of antigen specific IL-17 producing T cells could be assessed via ELISPOT during the early stages of EAE induction.

Ageing is associated with a decline in the quality of immune responses and the regulation of both cellular and humoral immune responses. Together these factors lead to increased susceptibility to infection, cancer and autoimmunity with age. To investigate these changes in the absence of CD37 and Tssc6, mice were housed over an extended period of time and assessed for obvious signs of autoimmune development, cancer and organ function. Whilst a small percentage of animals were culled from the experiment due to illness, no significant differences were observed in the mortality rates of these mice. The overall health of geriatric tetraspanin deficient mice was assessed at 18 months through routine pathology testing. Serum biochemistry analyses demonstrated comparable levels of renal and hepatic function between wild type and tetraspanin deficient mice.

Altered renal function has been previously described in one of three separate incarnations of CD151 knockout mice, due to progressive disruption of the glomerular basement membrane (GBM) (Sachs *et al.*, 2006). This defect is thought to be induced by defective α3 integrin function in the absence of CD151, although the absence of renal defects in $CD151^{-/-}$ mice produced by two other laboratories suggest that other genetic factors may also be in play (Takeda *et al.*, 2007; Wright *et al.*, 2004a). In this study, physical disruption of the GBM was not expected in $CD37^{-/-}Tssc6^{-/-}$ mice due to the more restricted pattern of haematopoietic expression by these tetraspanins. IgA nephropathy (IgAN) however, was a potential risk in CD37 deficient mice. IgA nephropathy occurs when excess mucosal IgA producing ASC take up residence in other sites of Ig production, e.g. bone marrow (Barratt and Feehally, 2005). The accumulation of IgA in the mesangium leads to chronic inflammation and destruction of the glomeruli. As discussed in Chapter 3, $CD37^{-/-}$ mice were found to display increased IgA production in response to T cell dependent antigens (van Spriel *et al.*, submitted for publication). The normal renal function presented in this chapter suggests IgAN did not develop in these mice and is supported by protein analysis of $CD37^{-/-}$ serum IgA, which was not found to share the glycosylation pattern of mucosally derived IgA (van Spriel *et al.*, submitted for publication).

Urinalysis of the ageing tetraspanin deficient mice detected one case of Type II diabetes in a single $Tssc6^{-/-}$ mouse. Since the incidence of excess glucose secretion amongst other $Tssc6^{-/-}$ mice was low, and mice derived from a C57Bl/6 background are known to display some susceptibility to obesity induced diabetes (Freeman *et al.*, 2006), a link between Tssc6 and Type 2 diabetes was not established in this study. Surprisingly, the $CD37^{-/-}$, $Tssc6^{-/-}$ and $CD37^{-/-}Tssc6^{-/-}$ strains all displayed significantly increased weight gain in comparison to wild type controls. This data is difficult to interpret however as published reports of weight gain in C57Bl/6 mice indicate that the tetraspanin deficient mice are below other reported ranges (45.1 ± 2.6 g) and furthermore, the wild type mice in this study fall even further below these ranges (Jeon *et al.*, 2006). Unfortunately, data is not currently available from the animal facility in which this study was performed, as these variations are likely to reflect differences in animal housing and diet between facilities. To draw conclusions from this phenotype, a second study with larger group numbers is required to investigate the potential for obesity development in tetraspanin deficient mice.

Full blood examination and analysis of splenocyte composition were used to investigate the potential for chronic inflammatory pathologies in the tetraspanin deficient mice. In peripheral

blood analyses increased numbers of leucocytes were detected in CD37 and CD37$^{-/-}$Tssc6$^{-/-}$ mice. However, these increases were not apparent in flow cytometric analyses of leucocyte populations in the spleen. There were multiple indicators of age related disruptions to the leucocyte populations of both wild type and tetraspanin deficient spleens. A HSC shift towards the production of myeloid progenitors was evidenced by increased numbers of macrophages and granulocytes and reduced frequencies of lymphoid T cells and B cells in the ageing wild type and tetraspanin deficient mice. Similarly, the CD4/CD8 T cell ratio was reduced and a striking accumulation of CD62L^{+} memory T cells was also apparent. These changes suggest that the absence of CD37 and/or Tssc6 was not able to prevent the development of immuno-senescence in these mice.

The onset of immuno-senescence is a risk factor for many diseases such as autoimmunity, viral and bacterial infection and cancer (Bender, 2003, Ginaldi *et al.*, 2005, Gavazzi and Krause, 2002). In this study, ageing tetraspanin deficient mice housed in clean animal housing conditions were not found to be more susceptible to these factors. Whilst one Tssc6$^{-/-}$ mouse did develop a carcinoma during this study, this was not sufficient to conclude that these mice were susceptible to spontaneous tumour development. Autoantibody production was not investigated in this study, although serum was collected from aged wild type and tetraspanin knockout mice prior to the conclusion of these experiments. Given the altered Ig production in CD37$^{-/-}$ and CD37$^{-/-}$Tssc6$^{-/-}$ mice (Chapter 3), the presence of anti-nuclear antibodies and anti-histone antibodies will be measured in future assays. To further investigate potential changes in disease susceptibility in aged tetraspanin deficient mice, active induction of these disease states will be required. However, altered immune function and susceptibility to viral infection and tumour challenge must be taken into account when comparing these mice in an aged state.

The potential for autoimmunity is determined by the ability to regulate autoreactive cellular and humoral responses. Despite the hyper-stimulatory DC and hyper-proliferative T cell phenotypes in the absence of CD37 and/or Tssc6 *in vitro* and altered humoral responses observed in the absence of CD37 *in vivo*, there was no evidence of an increased susceptibility to autoimmunity or age related pathologies in CD37$^{-/-}$, Tssc6$^{-/-}$ or CD37$^{-/-}$Tssc6$^{-/-}$ mice. Whilst the question of tetraspanin involvement in autoimmunity still remains largely unexplored, from this study we can conclude that the tetraspanins CD37 and Tssc6 do not play a major role in the suppression of autoimmunity or the development of immuno-senescence in mice.

7 General Discussion

7.1 INTRODUCTION

This chapter reviews the major findings of this thesis and their significance in our understanding of immune system function. After reviewing the bulk of the literature on tetraspanin function, it is clear that the co-operative nature of tetraspanin function suggests functional redundancy between these molecules is highly likely and is expected to be quite complex. If for example, tetraspanins are to be used as targets to modulate the immune system, it will be necessary to understand the implications of tetraspanin disruption on other interacting members of this family. Furthermore, it may be necessary to understand that a multiple target approach will be required in some cellular systems.

7.2 OUTCOMES FROM ADDRESSING FUNCTIONAL REDUNDANCY BETWEEN TETRASPANIN PROTEINS

In this study, the term functional overlap was used to describe examples where tetraspanins played apparently similar roles in cellular function. For example, both CD37 and CD81 display functional overlap in promoting immunoglobulin responses (Knobeloch *et al.*, 2000; Maecker and Levy, 1997). This term is not intended to imply that the mechanisms for regulating B cell function are the same, since many of the exact mechanisms of tetraspanin function remain unknown. It was hypothesised that if functional redundancy were to exist between CD37 and Tssc6 there may be roles for these tetraspanins in the immune system that were yet to be identified. Therefore, in the absence of both CD37 and Tssc6 functional redundancy may be ablated, revealing potentially novel phenotypes in $CD37^{-/-}Tssc6^{-/-}$ mice that were not apparent in single knockout counterparts. In addition, since tetraspanins are known to form functional microdomains, by disrupting multiple members of a tetraspanin microdomain, this may lead to fundamental changes in microdomain formation and reveal more dramatic phenotypes than those observed in tetraspanin 'single' knockouts (Figure 7.1). Whilst the phenotyping of tetraspanin deficient cells at the functional level cannot identify the physical effects of TEM disruption at the molecular level, in the approach used, four possible outcomes were predicted and identified as described below.

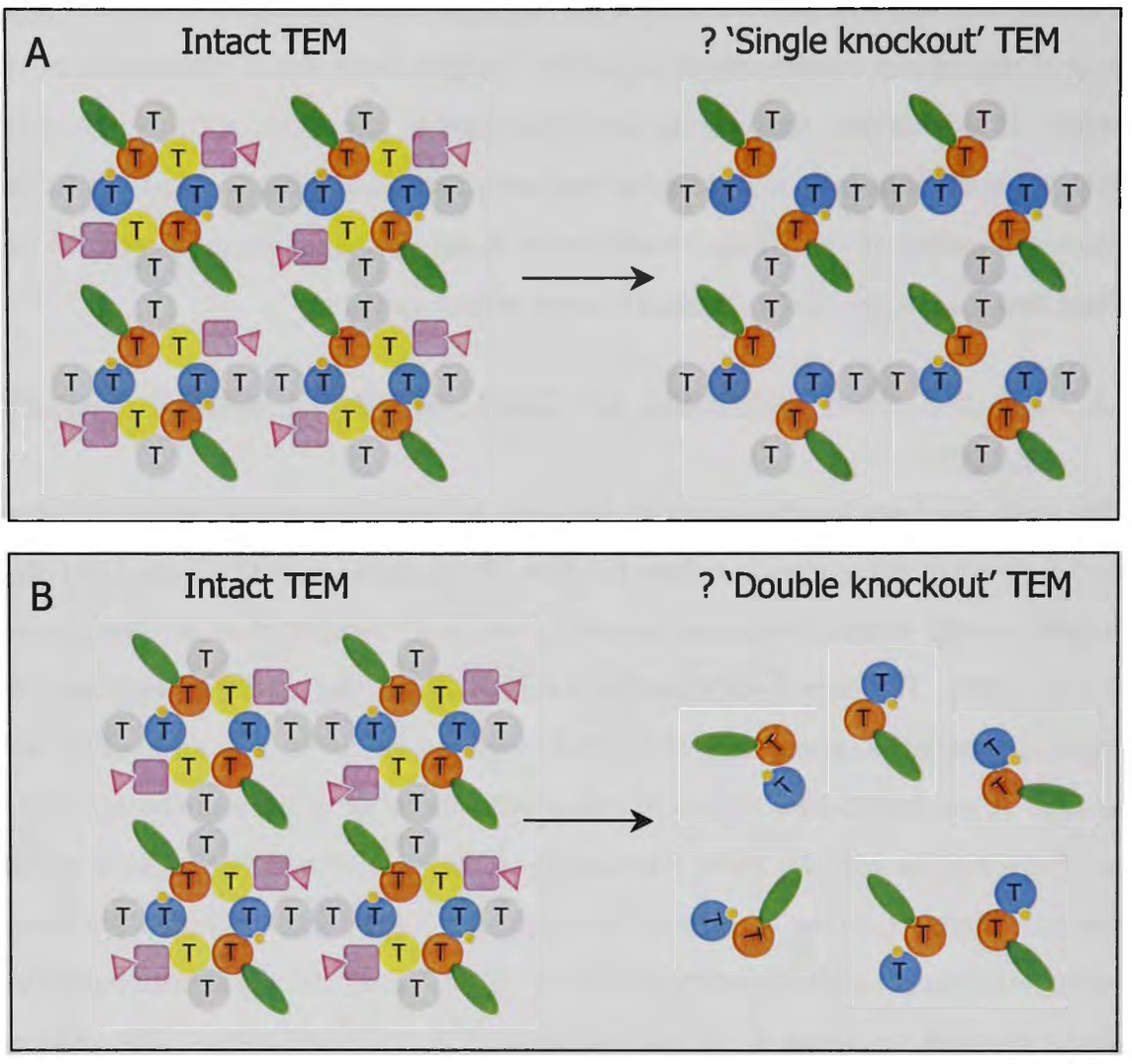

Figure 7.1 Theoretical models of tetraspanin microdomains and the effects of tetraspanin deletion.
Tetraspanins are known to exist in multi-molecular complexes composed of multiple members of the tetraspanin superfamily (marked with T), as well as various cell surface receptors and signalling molecules. Interactions within TEMs can be both direct (e.g. green oval with red tetraspanin) and indirect (e.g. pink triangle with green oval). The structural effects of tetraspanin deletion on tetraspanin enriched microdomains are poorly understood. (A) The absence of a single tetraspanin may prevent indirect associations between specific proteins but functional TEMs may still occur. (B) Perhaps the disruption of multiple tetraspanins may result in further disruption of tetraspanin interactions and the failure of TEM formation? If so, this may lead to more dramatic or even novel phenotypes in tetraspanin 'double knockout' mice.

Outcome 1. No functional overlap between CD37 and Tssc6 in humoral immunity.

Initial characterisation of $CD37^{-/-}$ and $Tssc6^{-/-}$ mice demonstrated a fundamental difference between CD37 and Tssc6 functions in B cells. These analyses suggested that whilst Tssc6 was not required for normal B cell function, CD37 expression was necessary for normal immunoglobulin responses to T cell dependent antigens (Knobeloch *et al.*, 2000; Tarrant *et al.*, 2002). If redundancy were to exist between CD37 and Tssc6 in B cells, the absence of both molecules may reveal a role for these tetraspanins in other B cell functions e.g. B cell proliferation. However, this was not the case when B cell proliferative responses were investigated in $CD37^{-/-}Tssc6^{-/-}$ mice (Figure 3.3). Moreover, there were no changes in the immunoglobulin defect observed in $CD37^{-/-}$ mice when compared to $CD37^{-/-}Tssc6^{-/-}$ mice (Figure 3.4 & Figure 3.5). These findings suggest that there is no shared role between CD37 and Tssc6 in B cell function. It is important to note that whilst this study rules out the likelihood of functional redundancy between CD37 and Tssc6 in B cells, it is eminently possible that functional redundancy exists between other tetraspanins in humoral immunity. Perhaps future studies may be aimed at investigating co-operative roles between other tetraspanins such as CD81 and CD37, both of which have been demonstrated to be important in B cell function (Levy *et al.*, 1998).

Outcome 2. Complementary roles for CD37 and Tssc6 in cellular immunity

Complementary roles for CD37 and Tssc6 were observed in both T cell proliferation and DC antigen presentation *in vitro*. As described in earlier characterisations of $CD37^{-/-}$ and $Tssc6^{-/-}$ mice, in the absence of either molecule there is an increase in T cell proliferative responses (Figure 4.4). An increased antigen presentation capacity was confirmed in $CD37^{-/-}$ mice and a similar phenotype identified in the absence of Tssc6 (Figure 4.10). When both molecules are absent these phenotypes are further exaggerated, demonstrating a complementary role for CD37 and Tssc6 in the regulation of T cell proliferation and antigen presentation. This result may also be interpreted as partial functional redundancy between these tetraspanins, whereby in the absence of one tetraspanin this can be partially compensated for by the presence of another tetraspanin. However, in the case where both single knockout phenotypes are similar this would suggest that this compensation is reciprocal between these tetraspanins. Therefore, a simpler explanation may be that both proteins work together to perform a function that is partially disrupted by the absence of one tetraspanin and more severely disrupted in the absence of both (Figure 7.2A).

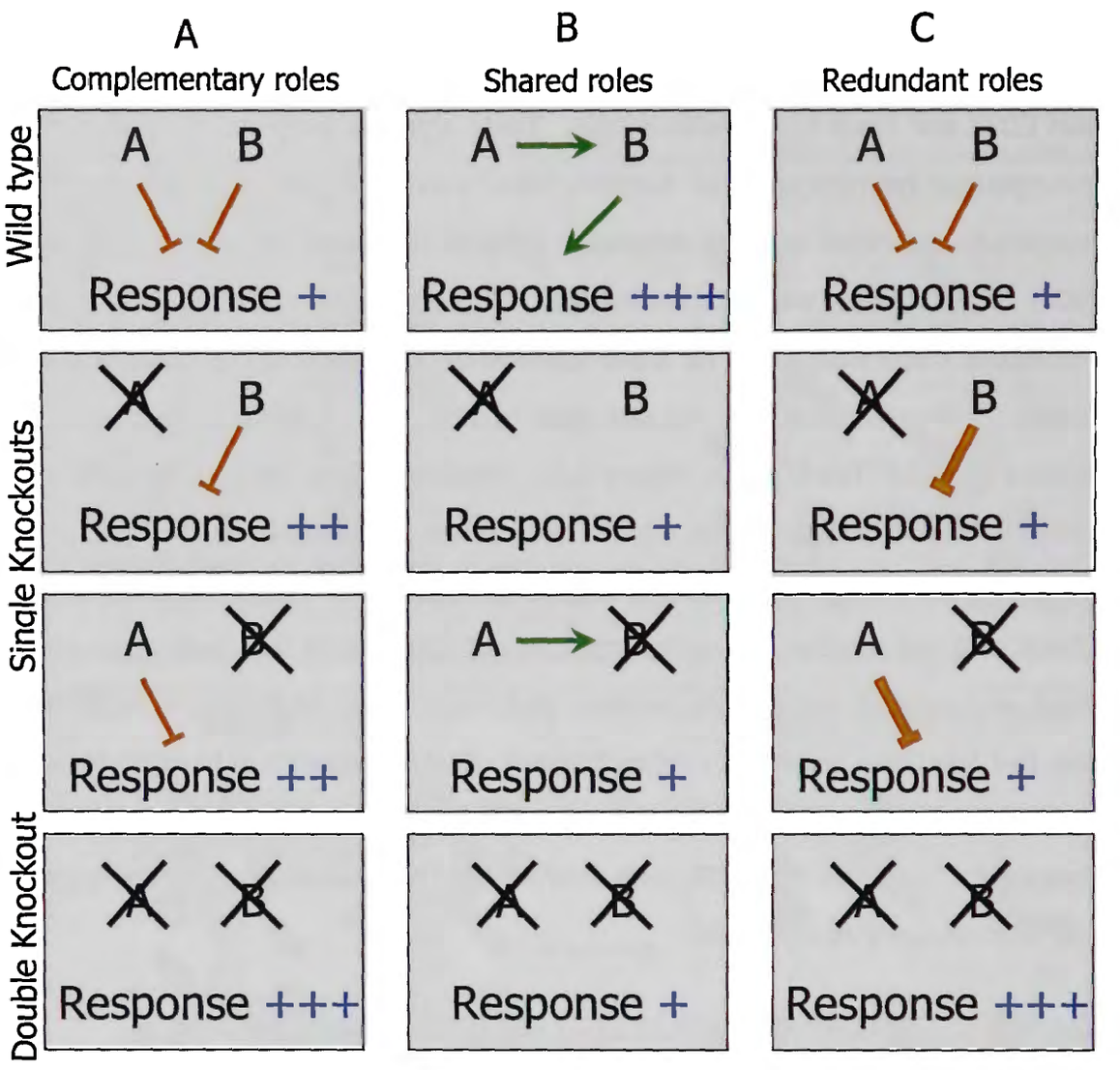

Figure 7.2 Theoretical models of the effects of tetraspanin deletion on cellular responses.

(A) Complementary roles between proteins suggest that both molecules perform a similar role that when combined have a reciprocal effect on function. Therefore when either protein is disrupted this partially impairs function, but when both are impaired the cellular response is also further disrupted. (B) When two proteins display a shared role in a specific cellular function this may imply some level of interdependency between these proteins (i.e. A+B leads to C) or a sequential pathway that both molecules participate in (depicted above). Therefore, if either or both proteins are disrupted the result is the same. (C) Redundant roles between proteins occur when two proteins can perform similar roles and in the absence of one of these proteins, the other can upregulate its function and compensate for the absent molecule. Therefore, in the absence of one molecule the phenotype can be completely undetectable, however when both proteins are disrupted the phenotype is observed. Note that cellular responses in either resting or impaired states are not represented as null, since other proteins may also regulate/activate the cell response e.g. other tetraspanins.

Outcome 3. A shared role between CD37 and Tssc6 in cellular immunity

Shared roles were observed between CD37 and Tssc6 in the development of antigen specific T cell responses and DC migration *in vivo* (Chapter 5). $CD37^{-/-}$, $Tssc6^{-/-}$ and $CD37^{-/-}Tssc6^{-/-}$ mice all displayed a similar impairment in these functions, which was not exaggerated in the absence of both tetraspanins. One theoretical model of how the disruption of two proteins could lead to a similar phenotype as that observed with the disruption of each molecule individually, may be that there is some degree of interdependency between these tetraspanins. For example, if molecule A is required for molecule B to function, the absence of either or both molecules would result in the same effect on the cellular response (Figure 7.2B). This may suggest a series of events similar to that of a molecular pathway which does not necessarily require any interaction between these two molecules, or a direct association between the two proteins for example by bringing two distinct partner molecules into close proximity.

Outcome 4. Functional redundancy between CD37 and Tssc6 in NKT cell proliferation

Functional redundancy was observed in NKT cell proliferation in $CD37^{-/-}$ and $Tssc6^{-/-}$ mice, where NKT cell proliferative responses were normal in the absence of either tetraspanin but poorly regulated in the absence of both molecules (Figure 4.7). This suggests, that not only are both molecules involved in regulating NKT cell proliferation, the presence of either molecule can completely compensate for the absence of the other tetraspanin (Figure 7.2C). This finding validates the approach of using double knockout mice to investigate functional redundancy, since without examination of $CD37^{-/-}Tssc6^{-/-}$ mice, the role for these tetraspanins in NKT cell proliferation would not have been detected.

7.3 Functional roles for CD37 and Tssc6 in the immune system

7.3.1 Humoral Immunity

At the beginning of this study it was known that tetraspanins contribute to humoral immunity in both B cell activation as well as downstream immunoglobulin responses to T cell dependent antigens (Knobeloch *et al.*, 2000, Tsitsikov *et al.*, 1997, Miyazaki *et al.*, 1997, Maecker and Levy, 1997). CD81 has been identified as an integral part of the CD19/CD21/Leu13 B cell co-receptor complex, which can reduce the threshold of B cell activation in response to antigen. CD81 expression is required for CD19 trafficking and stabilisation at the cell surface and consequently optimal B cell co-receptor function (Shoham *et al.*, 2006). The redistribution of

the BCR-co-receptor complex to signalling rich lipid raft domains has also been found to be dependent upon CD81 palmitoylation (Cherukuri *et al.*, 2004a). Despite a number of tetraspanins having been identified in complex with CD81 in B cells, there is no evidence to date that other tetraspanins are required for these processes. Certainly from this study, it was found that expression of CD37 and/or Tssc6 was not required for B cell activation since B cell proliferation was normal in all three strains tested (Figure 3.3). Furthermore, there was no evidence for a role for Tssc6 in B cells identified at all when $CD37^{-/-}$ and $CD37^{-/-}Tssc6^{-/-}$ mice were compared, since there was no difference between the B cell phenotype in these mice. However, it was determined that CD37 expression by B cells is required for normal germinal centre reactions. In conjunction with collaborators, it was shown that excess IL-6 production in the GC resulted in an increased frequency of IgA producing cells in $CD37^{-/-}$ mice. IgG_1 production was impaired in $CD37^{-/-}$ mice due to increased apoptosis of IgG_1 producing cells in GC. This was attributed to altered $\alpha4\beta1$-VCAM-1 interactions between $CD37^{-/-}$ B cells and FoDC (van Spriel *et al.*, submitted for publication).

In light of these results, it is interesting to consider the implications of altered integrin function in the context of other tetraspanin deficient B cells. Since CD81 has been found to directly associate with $\alpha4\beta1$ in B cells and CD81 expression and targeting by monoclonal antibodies can modulate integrin function (Feigelson *et al.*, 2003, Mannion *et al.*, 1996), it is likely that impaired immunoglobulin responses to T cell dependent antigens in $CD81^{-/-}$ mice are also linked with $\alpha4\beta1$ disruption. Therefore, the impaired humoral immune responses observed in $CD81^{-/-}$ mice may be due to two distinct mechanisms, i.e. poor B cell activation due to low CD19 expression (Maecker and Levy, 1997; Miyazaki *et al.*, 1997), and altered B cell interactions in the GC due to dysregulated $\alpha4\beta1$ function. Consequently, $CD37^{-/-}$ mice may provide an important tool for investigating the role of tetraspanin microdomains and $\alpha4\beta1$ in B cell function, in the absence of CD19 deficiency.

7.3.2 In vitro cellular immunity – T cells

Multiple lines of evidence have suggested that tetraspanins regulate T cell proliferation (summarised in section 1.5.2.1). CD37 and Tssc6 were implicated when tetraspanin deficient mice were found to have poorly regulated T cell proliferation *in vitro* due to a reduced requirement for co-stimulation (Tarrant *et al.*, 2002; van Spriel *et al.*, 2004). To establish if these molecules work co-operatively to regulate T cell responses, T cell function was investigated in $CD37^{-/-}Tssc6^{-/-}$ mice. These studies identified a complementary role for CD37

and Tssc6 in regulating of T cell proliferation, since in the absence of both proteins the phenotypes observed in $CD37^{-/-}$ and $Tssc6^{-/-}$ were exaggerated (Chapter 4). During T cell activation, reorganisation of the T cell surface occurs to form the immunological synapse, enriched in TCR, co-receptors such as CD4 and CD8 and adhesion molecules (Lin *et al.*, 2005). T cell signalling occurs through TCR redistribution to signalling rich lipid raft domains and phosphorylation of the TCR complex through the kinases Lck and Fyn and subsequent recruitment of ZAP-70 (Cantrell, 1996). Co-stimulatory signals are also required from APC to T cell through CD28, activating a second pathway through signalling molecules including PI3-K and Lck. Since CD37 and/or Tssc6 disruption leads to altered T cell responses, these tetraspanins must therefore be involved in either direct regulation of T cell signalling or cell surface organisation of T cell signalling receptors. Analyses to date suggest that tetraspanins are involved in both. CD81 and CD82 redistribute to the immunological synapse in T cells and conceivably other tetraspanins expressed at the T cell surface may also take part (Mittelbrunn *et al.*, 2002). Components of the T cell signalling pathway were examined in $CD37^{-/-}$ mice, where increased kinase activity in CD4/CD8 associated Lck was detected in resting $CD37^{-/-}$ T cells (van Spriel *et al.*, 2004). It was suggested that CD37 might therefore play a role in regulating Lck association with the TCR complex through cell surface organisation of CD4/CD8. Perhaps if the mechanism for CD37 involvement in T cell responses is indeed related to Lck sequestration, this may be borne out by similar analyses of $Tssc6^{-/-}$ or $CD37^{-/-}Tssc6^{-/-}$ mice. Whereby, exaggerated Lck kinase activity in $CD37^{-/-}Tssc6^{-/-}$ mice may provide strong support for this hypothesis.

One key question arising from the work presented in this thesis is, what do the differences in T cell and NKT cell requirements for CD37 and Tssc6 during development and proliferation, say about the differences between signalling events in these cells? Much of the research on NKT cell signalling appears to be focused on the differences between NKT cell and conventional T cell development. The SLAM family of receptors have been found to play a critical role in the differentiation of NKT cells, since mice deficient in the SLAM associated protein (SAP) completely lack NKT cells (Chung *et al.*, 2005; Nichols *et al.*, 2005; Pasquier *et al.*, 2005). SLAM activation induces SAP recruitment of the kinase Fyn and activation of the NF-κB pathway through PKCθ and also inhibits the MAPK pathway, resulting in reduced signalling through the TCR. Together, this is thought to enable positive selection during NKT cell development in the thymus (Kunisaki *et al.*, 2006, Godfrey and Berzins, 2007). Since $CD37^{-/-}Tssc6^{-/-}$ mice display increased numbers of NKT cells in naïve mice, perhaps there is

some link between these tetraspanins and SLAM receptor organisation and signalling. Interestingly, there are a number of other genes known to be critical to the development of normal NKT cell numbers including CD1d, NKT cell restricted TCR chains and the cytokines IL-2 and IL-15 summarised in (Kronenberg and Gapin, 2002). However, this is the first report of either enhanced NKT cell development or hyper-proliferative NKT responses due to genetic mutation.

Unfortunately, there is little data regarding the differences between T cell signalling in response to peptide presentation and NKT cell signalling in response to glycolipid recognition. Since the expression of SLAM family receptors is down regulated in mature NKT cells with only weak expression of SLAM and NTB-A (Ly108), it is less likely that SLAM molecules are involved in mature NKT cell responses (Veillette *et al.*, 2007). NKT cells display a more rapid capacity to respond to antigen than conventional T cells, such that NKT cells up-regulate IFNγ production within 2 hours of antigen contact, whereas T cells take up to 8 hours (Zhou, 2007). Therefore the signalling pathway is likely to be significantly different between these cells. NKT cells are known to share the T cell requirements of TCR ligation, co-stimulation and DC cytokine secretion for activation. Although, interactions between OX40 and OX40L have also been proposed as a third pathway that stimulates NF-AT, NF-kB and T bet activation in NKT cells contributing to NKT proliferation and rapid cytokine production (Zaini *et al.*, 2007; Zhou, 2007). Again, this may suggest a role for CD37 and Tssc6 in regulating OX40 signalling or organisation at the NKT cell surface. Despite the many unanswered questions on the function of tetraspanins in NKT cells, this will be an interesting area for future research that may contribute to the development of NKT cells as anti-tumour therapies, for which they have already been suggested.

7.3.3 IN VITRO CELLULAR IMMUNITY – DC

There is currently ongoing discussion within the literature about the role of tetraspanins in MHC organisation at the APC surface and their subsequent effect on antigen presentation. Tetraspanins were first proposed to be involved in antigen presentation when a number of tetraspanins (including CD37) were identified in complex with MHC in APC (Angelisova *et al.*, 1994; Lagaudriere-Gesbert *et al.*, 1997b; Rubinstein *et al.*, 1996; Schick and Levy, 1993; Szollosi *et al.*, 1996). Later studies found that multiple tetraspanins are enriched in the MIIC peptide loading compartment in APC, suggesting involvement in antigen processing (Engering and Pieters, 2001; Escola *et al.*, 1998; Hammond *et al.*, 1998; Mantegazza *et al.*, 2004). Tetraspanins are also thought to participate in immunological synapse formation where both

CD81 and CD82 have been found to relocalise to the IS in response to APC-T cell interactions (Mittelbrunn *et al.*, 2002). In addition, tetraspanins and MHC are expressed by APC derived exosomes, thought to be functionally relevant in antigen delivery prior to, or in the absence of, direct APC contact (Escola *et al.*, 1998). Studies by Kropshofer *et al* have suggested that tetraspanins play an active role in MHC clustering that results in an improved capacity to present antigen (Kropshofer *et al.*, 2002). This is supported by the work of Unternaehrer and others who argue that the exquisite antigen presentation capacity of DC, in comparison to the relatively poor capacity of B cell blasts, is due to CD9 clustering of MHC at the DC surface (Unternaehrer *et al.*, 2007). One would therefore expect that in the absence of specific tetraspanins, antigen presentation would be negatively affected.

The data presented in this thesis demonstrates that *in vitro* antigen presentation by DC is in fact promoted by the absence of tetraspanins CD37 and Tssc6. This enhancement was specifically linked to altered MHC-TCR interactions, since co-stimulatory capacity was normal in these cells. Therefore, in contrast to expected findings, CD37 and Tssc6 are likely to be *negative* regulators of MHC antigen presentation. One way to reconcile these observations may be that some tetraspanins, such as CD37 and Tssc6, regulate or block the function of other tetraspanins that promote MHC clustering. Preliminary experiments show that cell surface CD37 is down-regulated upon DC activation (personal communication A. van Spriel), whereas CD9 is expressed at relatively high levels in both mature and immature DC (Unternaehrer *et al.*, 2007). Microarray data also suggests that both CD37 and Tssc6 mRNA transcript levels decline upon DC activation. While data drawn from mRNA analysis can be misleading in terms of actual protein expression, from the crude analysis performed in Figure 7.3 and Table 7.1, it appears that mRNA levels of some tetraspanins are upregulated in activated DC and others downregulated (Edwards *et al.*, 2003). Conceivably, tetraspanins such as CD37 and Tssc6 function to sequester MHC away from CD9, or block CD9-MHC interactions in resting DC. Therefore, upon DC activation and subsequent CD37 and Tssc6 downregulation, CD9 is able to gain better access to MHC and perform clustering functions to promote antigen presentation.

However, perhaps there are other conclusions to be drawn from the previously published studies of tetraspanin MHC-clustering. Poloso and others have challenged the concept of tetraspanin clustering of MHC by suggesting that the conclusions of Kropshofer may be flawed by inappropriate interpretation of putative MHC-tetraspanin complex markers (Poloso *et al.*, 2006). Kropshofer's study utilised CDw78 as a marker of tetraspanin clustered MHC based on the observation that CD9, CD81 and CD82 associated MHC could be detected by CDw78 mAbs. When the peptide repertoire of $CDw78^+$ MHC was compared to that of $CDw78^-$ MHC, it was selectively enriched in specific peptides and antigen presenting capacity was poorer in $CDw78^-$ cells (Kropshofer *et al.*, 2002). However, Poloso argued that CDw78 was not a marker of tetraspanin clustered MHC but was dependent on association of MHC class II with the invariant chain (Ii) chaperone molecule, concluding that CDw78 is actually a marker of MHC recently derived from the MIIC compartments (Poloso *et al.*, 2006). Untenaehrer's link between CD9-MHC complexes and enhanced antigen presentation capacity could also be questioned since there is no data demonstrating the link between CD9 expression and antigen presentation in this study (Unternaehrer *et al.*, 2007). Examination of the *in vitro* presentation capacity of CD9 deficient DC may add significantly more weight to the conclusions from that research.

Table 7.1 Summary of tetraspanin mRNA levels in resting and activated DC

Tetraspanin	Resting	Activated	Trend
CD9	*++*	*++++*	↑
CD37	*++*	*+*	↓
CD53	*++*	*+++*	↑
CD63	*+*	*++*	↑
CD81	*++*	*+ / +++++ ($CD8^+$)*	↓/↑ *(CD8+)*
CD82	*+++*	*++++*	↑
CD151	*+*	*- / + (DN)*	↓
Tssc6	*±*	*-*	↓

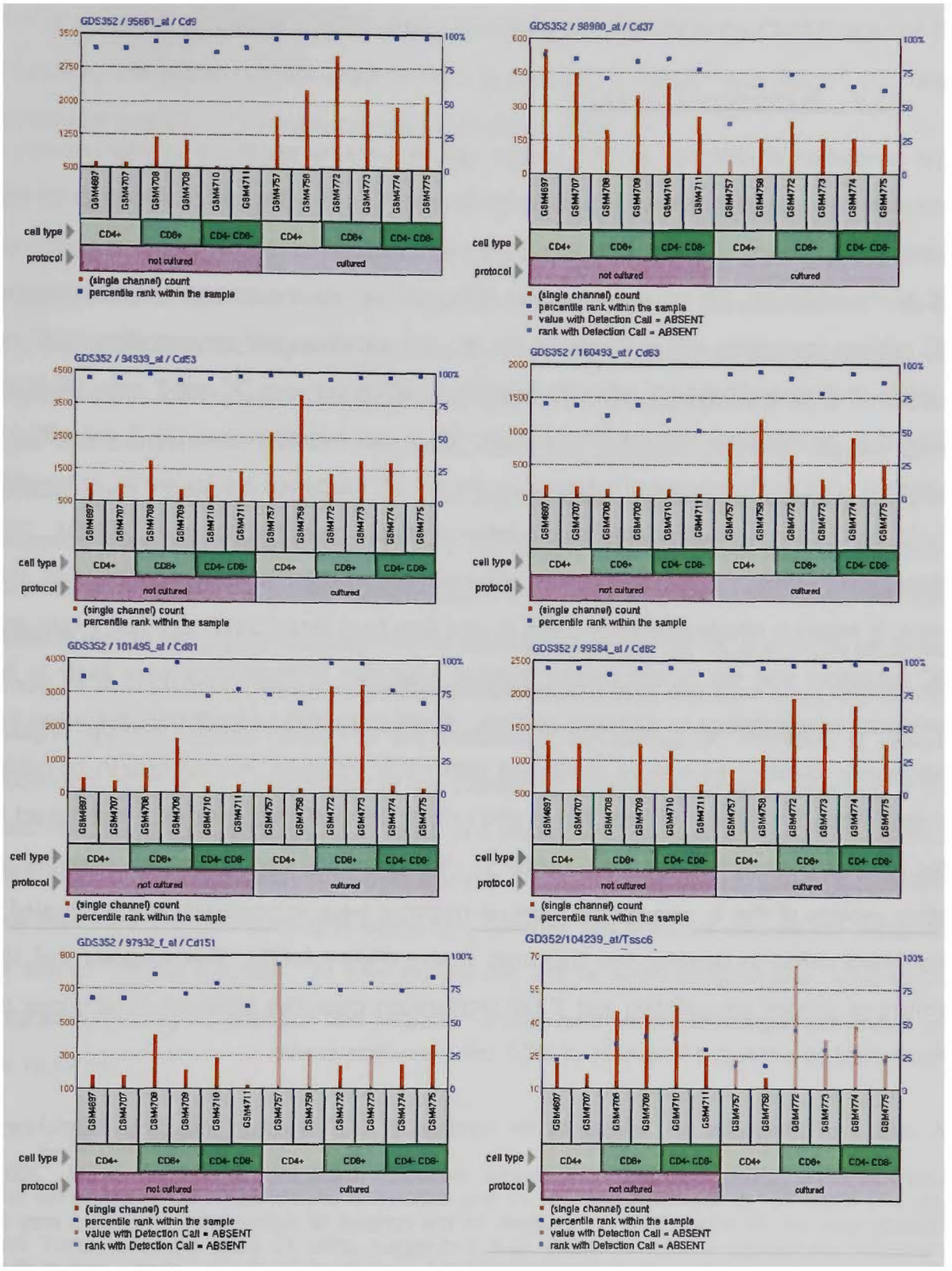

Figure 7.3 Tetraspanin mRNA expression profiles in resting and activated DC

Microarray data was obtained from the data record GDS352 housed in the Gene Expression Omnibus (GEO) (publicly available at www.ncbi.nlm.nih.gov/geo/). Data was generated from both cultured (2 hours) and uncultured C57Bl/6 purified DC, extracted mRNA was then hybridised to the Affymetrix GeneChip Murine Genome U74 Version 2 Set MG-U74A (Edwards *et al.*, 2003). *Red bars indicate the abundance of the specific transcript and blue boxes describe the rank order for the sample in the whole data set. Affymetrix detection call is used to eliminate readings deemed to be either below the level of detection or due to stray cross-hybridisation.*

7.3.4 *IN VIVO CELLULAR IMMUNITY*

The generation of effective antigen specific cellular immune responses *in vivo* requires a complex series of events involving primarily DC and T cells. This process can be broken down into a number of stages including (1) danger signal recognition and antigen uptake by DC in the periphery, (2) DC activation and migration towards the primary lymphoid tissues, (3) antigen processing and presentation by DC, (4) immunological synapse formation and peptide-MHC recognition by T cells, (5) cytokine signalling between DC and T cells, (6) T cell activation, proliferation and cytokine release, (7) T cell migration and (8) T cell effector functions such as the release of cytotoxic mediators. Tetraspanins are known to, or possibly could, participate in a surprisingly large number of these stages (Wright *et al.*, 2004b). This pleiotropism means that positive effects of tetraspanin modulation in one system may also result in negative effects in another. The *in vitro* data from both CD37/Tssc6 null T cells and DC suggested that the *in vivo* cellular immune responses in these mice were likely to be enhanced in comparison to wild type controls. Surprisingly, this was not the case when the tetraspanin deficient mice were challenged with either influenza, tumour growth or model immunisation strategies. In fact, the *in vivo* cellular immune responses were diminished in the absence of these tetraspanins (Chapter 5). To understand these divergent observations, other aspects of the *in vivo* cellular immune response were investigated, which revealed a significant defect in dendritic cell migration *in vivo* (Figure 5.12). This suggests that the enhanced antigen presentation and T cell proliferation capacities observed *in vitro* may be neutralised by a reduced frequency of DC-T cell interaction *in vivo*.

A variety of molecules are known to be involved in DC migration including chemokines, chemokine receptors, selectins and cellular adhesion molecules (Randolph *et al.*, 2005, Bonasio and von Andrian, 2006). However, in the context of tetraspanins, integrins may be the most relevant. Interactions between ICAM-1 expressed by the lymphatic endothelium and $\alpha L\beta 2$ by DC are important in skin derived DC migration between the periphery and the LN (Randolph *et al.*, 2005). Mice deficient in ICAM-1 display reduced DC trafficking from the skin and to the draining LN after exposure to antigen. Adoptive transfer studies showed that this was not an intrinsic DC phenotype and was therefore due to impaired ICAM-1 expression by the endothelium (Xu *et al.*, 2001). In a separate study, mice treated with mAbs targeted to either $\alpha L\beta 2$ or ICAM-1 also displayed impaired DC migration between skin and draining LN

(Ma *et al.*, 1994). If altered ICAM-1-αLβ2 interactions are a factor in the CD37/Tssc6 null DC migration, this would suggest αLβ2 function is impaired in $CD37^{-/-}$ and $Tssc6^{-/-}$ DC, since endothelial cells do not normally express these tetraspanins.

The integrin α4β1 is also known to be required for DC homing of circulating DC to the thymus (Bonasio *et al.*, 2006). This is thought to be an important mechanism for the presentation of self antigens during thymic selection to enable central tolerance. In mice treated with α4 blocking antibodies, DC migration between the skin and thymus was significantly reduced (Bonasio *et al.*, 2006). Unfortunately, DC migration to the LN was not reported in α4 treated mice and therefore the role of α4β1 in DC trafficking between skin and draining LN is undetermined. Given the altered function of α4β1 in $CD37^{-/-}$ B cells, perhaps α4β1-VCAM-1 interactions are also impaired in $CD37^{-/-}$ DC and this contributes to the poor DC migratory capacity in these mice. Whether Tssc6 regulates α4β1 function is yet to be determined. One possible way to investigate the link between these integrins and the DC phenotype observed, would be to measure DC migration in tetraspanin deficient mice that have been treated with integrin specific blocking mAbs. If the mechanism for altered DC migration is integrin dependent, little or no exaggeration of the phenotype would be expected. However, if the mechanisms were different, this may result in a much more severe phenotype in these mice compared to untreated controls. A brief analysis of CD37/Tssc6 deficient DC did not identify any significant changes in integrin expression, however this does not exclude the possibility of altered integrin function. To fully explore the link between integrins and impaired DC migration, *in vitro* DC adhesion and migration assays on a variety of integrin substrates will be required.

7.3.5 Autoimmunity

The enhanced T cell proliferative responses and DC stimulatory capacity identified in CD37 and Tssc6 deficient mice *in vitro,* suggested that these mice may display an increased susceptibility to autoimmune induction and spontaneous disease development. This concept was investigated in two models of active autoimmune induction, i.e. collagen induced arthritis (CIA) and experimental autoimmune encephalomyelitis (EAE). Surprisingly, there was no significant increase in susceptibility to CIA observed in the tetraspanin deficient mice when compared to wild type controls. Similarly, when EAE susceptibility was compared between wild type and $CD37^{-/-}Tssc6^{-/-}$ mice, the absence of these tetraspanins resulted in only a small change in the time of disease onset. These findings appear similar to that of the normal

antigen specific T cell development observed in CFA-OVA immunisation strategies. Whilst these experiments are very different, all utilised strong adjuvants and yielded surprisingly normal responses in all three tetraspanin deficient strains. One possible explanation may be that the defects observed in tetraspanin knockout DC are maturation dependent, and that the presence of strong inflammatory signals induces some compensatory effect through the upregulation of other molecules. Since most models of active autoimmune induction require the use of strong adjuvants including both CIA and EAE, this may not be the best system to examine the effects of tetraspanin deficiency on autoimmunity. One model that may be a more suitable option is autoimmune diabetes. 60-80% of female non-obese diabetic (NOD) mice develop a pathology very similar to that of human Type 1 Diabetes (T1D) between 20-30 weeks of age (Anderson and Bluestone, 2005). Insulitis begins in NOD mice at approximately 10 weeks of age, due primarily to the development of autoreactive $CD4^+$ and $CD8^+$ T cells, and poor immuno-regulation through central tolerance mechanisms. The phenotypes of $CD37^{-/-}$-NOD, $Tssc6^{-/-}$-NOD and $CD37^{-/-}Tssc6^{-/-}$-NOD mice are very difficult to predict but may shed some light on the role of the tetraspanins in immune regulation. For example, $CD37^{-/-}$ and $Tssc6^{-/-}$ mice might be more susceptible to T1D due to enhanced T cell proliferation and antigen presentation capacity. However, this may be counteracted by the defect in DC migration in these mice, resulting in decreased susceptibility to T1D. It has recently been published that IL-2 concentrations may be critical in promoting tolerance by T regulatory cells, such that the addition of IL-2 in low concentrations can rescue T regulatory functions and protect mice from diabetes onset (Tang *et al.*, 2008). Perhaps the altered IL-2 production by $CD37^{-/-}$ and $Tssc6^{-/-}$ T cells observed *in vitro* may also lead to a reduced susceptibility to T1D in tetraspanin deficient NOD mice (Tarrant *et al.*, 2002; van Spriel *et al.*, 2004). Of particular interest will also be the effect of the increased basal NKT numbers and their hyper-proliferative responses in the absence of both CD37 and Tssc6. NKT cells are thought to be important in protecting NOD mice from T1D onset, since $CD1d^{-/-}$ mice (NKT cell deficient) display increased susceptibility to the disease and artificial boosting of NKT numbers is protective (Novak *et al.*, 2007). Therefore $CD37^{-/-}Tssc6^{-/-}$-NOD mice may be more resistant to autoimmune diabetes.

Interestingly, the ageing study in this thesis demonstrated that in the absence of CD37 and/or Tssc6 mice were generally healthy. These mice were housed in SPF conditions but not in micro-isolator chambers during the study and were therefore exposed to low levels of pathogens in their environment. Significantly, despite the defects observed in both humoral

and cellular immunity, these mice retain sufficient immunity to survive well into old age. From this it is concluded that the tetraspanins CD37 and Tssc6 are not critical to immune function, but are likely to optimise immune responses through both positive and negative regulation. In many cases however, when the immune system/cells of these mice were challenged, e.g. viral infection and tumour challenge, the detrimental effects of CD37 and/or Tssc6 deficiency became apparent.

7.4 Future Directions

The findings described in this thesis significantly contribute to our understanding of tetraspanin function in the immune system, however many questions remain unanswered. The phenotypes observed in these mice directly suggest a number of studies are required to fully understand the specific roles of CD37 and Tssc6 in the immune system. These include dissecting the molecular differences behind T cell and NKT cell signalling in the absence of CD37 and Tssc6, examining the potential positive and negative regulators of MHC-TCR interactions in other tetraspanin deficient DC and defining the mechanism behind both poor development of antigen specific T cells and impaired DC migration in $CD37^{-/-}$, $Tssc6^{-/-}$ and $CD37^{-/-}Tssc6^{-/-}$ mice.

This study has focussed primarily on the adaptive immune system rather than innate immunity as suggested by the previous characterisations of $CD37^{-/-}$ and $Tssc6^{-/-}$ mice. However, the effect of tetraspanin deficiency on many other cell types, including macrophages, granulocytes and NK cells remain largely unexplored. Tetraspanin function is not restricted to the adaptive immune system. Examples include, altered motility in neutrophils targeted by anti-tetraspanin mAbs (Forsyth, 1991, Skubitz *et al.*, 1996; Skubitz *et al.*, 2000, Yauch *et al.*, 1998), impaired granuloma formation in $CD9^{-/-}$ macrophages and spontaneous fusion of $CD9^{-/-}CD81^{-/-}$ mononuclear phagocytes in the lung (Takeda *et al.*, 2003b, Yamane *et al.*, 2005). Due to the absence of mAbs targeted towards murine CD37 and Tssc6, this study also lacks a molecular dissection of the interactions between these tetraspanins and other molecules. Preliminary work was performed in this study to develop fusion constructs that may in part, rectify this lack of reagents (Appendix I). In future studies, it is hoped that fluorescently labelled tetraspanins will enable the subcellular localisation of both CD37 and Tssc6 during antigen presentation and immunological synapse formation.

7.5 Concluding Comments

The question of functional redundancy between tetraspanins was addressed in this study through the examination of the murine immune system in the absence of both CD37 and Tssc6. Through this work, it was found that (1) CD37 but not Tssc6 expression by B cells is required for normal humoral responses, (2) CD37 and Tssc6 have a complementary role in regulating T cell proliferation and antigen presentation, (3) CD37 and Tssc6 share a role in the development of antigen specific T cells and *in vivo* DC migration, (4) CD37 and Tssc6 display functional redundancy in regulating NKT cell proliferation and (5) despite these effects, CD37 and/or Tssc6 deficiency did not lead to increased susceptibility to autoimmune induction. These findings describe important roles for both CD37 and Tssc6, and confirm that co-operative roles between tetraspanins are widespread throughout in the immune system.

8 APPENDIX I - SUBCELLULAR LOCALISATION OF TETRASPANIN PROTEINS

8.1 INTRODUCTION

The immunological synapse is a dynamic multi-molecular junction that forms between T cells and antigen presenting cells to facilitate T cell activation (Davis and Dustin, 2004). Since the primary role of tetraspanins is to organise and maintain other molecules in multi-molecular complexes, it is not surprising that tetraspanins may play a role in the formation and stabilisation of such a structure. A T cell hyper-proliferative defect has been observed in four separate tetraspanin deficient mouse strains, which may be attributed to altered synapse formation (Miyazaki *et al.*, 1997; Tarrant *et al.*, 2002; van Spriel *et al.*, 2004, Wright *et al.*, 2004a). Combined with preliminary data from our laboratory and others that suggest tetraspanins migrate to the cell-cell contact site during synapse formation, these similar phenotypes indicate a common biochemical basis and a potential for functional overlap between tetraspanin molecules (Delaguillaumie *et al.*, 2002; Mittelbrunn *et al.*, 2002). Due to the lack of anti-mouse CD37, Tssc6 and CD151 mAbs, new tools are required to investigate subcellular localisation of these proteins. This chapter outlines the validation of four potential mAbs directed at mouse Tssc6 and the generation of fluorescently labelled mouse CD37, Tssc6 and CD151 fusion constructs.

The aims of these studies were to:

(1) Validate potential mAbs targeted towards mouse Tssc6 protein and characterise the expression of Tssc6 in murine haematopoietic cells.

(2) Generate fluorescently labelled expression constructs of murine CD37, Tssc6 and CD151 for transfection and develop stable transfected and co-transfected cell lines as tools for subcellular localisation of these tetraspanins.

8.1.1 Anti-Tssc6 Monoclonal Antibodies

In previous studies, immunisation of Wistar rats with murine myeloid FDC-P1 and erythroid DP16 cell lines generated a panel of potential anti-mouse Tssc6 mAbs (Goschnick *et al.*, 2006). Primary screening of hybridoma supernatants was performed by flow cytometry of a mixture of COS-7 cells engineered to express the FLAG epitope in the small extracellular loop of Tssc6 (COS-7 TSSC6/EC1 FLAG) and parental COS-7 cells. Selected supernatants were re-screened against COS-7 TSSC6/EC1 FLAG cells and a panel of murine hematopoietic-cell lines. Of the thirty six parental hybridoma lines that recognised COS-7 TSSC6/EC1, four that also recognised multiple haematopoietic cell lines were further investigated – 15G3 (IgG1), 14A6 (IgG2b), 20C10 (IgG1) and 16F4 (IgM).

	COS-T6	CHO-T6	Erythroid		T cell		B cell	M	
Parent			TS5	F4N	YAC	EL4	BAF/3	FDCP/1	Isotype
15G3	+	+	++	++	+	+	++	++	IgG1
14A6	+	+	++	+	-	-	++	++	IgG2b
20C10	+	+	++	-	++	++	++	++	IgG1
16F4	+	+	++	+	+	+	++	++	IgM

Table 8.1 Table of hybridoma supernatant reactivity

Potential hybridoma supernatants were screened against non haematopoietic cell lines transfected with mTssc6 and a series of blood derived cell lines. Parent, hybridoma parental line; COS-T6, COS-Tssc6; CHO-T6, CHO-Tssc6, Eryth, Erythroid; M, Monocyte; +, immuno-positivity by FACS; ++ immuno-positive with high mean fluorescence by FACS; -, no immuno-positivity by FACS (results curtesy of J Tarrant).

mAbs were purified from 14A6, 15G3, 16F4 and 20C10 hybridoma supernatants, biotinylated and tested against primary haematopoietic cells. In this study, cells from both wild type and Tssc6$^{-/-}$ derived mice were used to determine the specificity of detection. Initially thymocytes and splenocytes were stained and examined via flow cytometry (Figure 8.1). Whilst some reactivity was demonstrated by each of the clones to thymocytes, splenocytes were immuno-positive only to 16F4 and 20C10. However, there appeared to be no difference between the reactivity of wild type and Tssc6$^{-/-}$ deficient cells, suggesting that these mAbs were reactive to proteins other than Tssc6. The integrity of the Tssc6$^{-/-}$ mouse line was not in question since genotyping of the Tssc6$^{-/-}$ mice determined the presence of the genetrap vector. Previously, both northern and southern blot analyses have demonstrated that the genetrap insertion in Tssc6$^{-/-}$ mice completely abrogated Tssc6 expression (Tarrant *et al.*, 2002).

To eliminate the possibility of FcR binding as the cause for positive staining in Tssc6$^{-/-}$ mice, splenocytes and purified dendritic cells were incubated with heat aggregated immunoglobulin at high concentrations prior to staining (Figure 8.2). Unfortunately, after FcR blocking there were still no significant differences in reactivity observed between wild type and Tssc6$^{-/-}$ cells. Finally, the mAbs were used to stain permeabilised splenocytes and dendritic cells to investigate the potential for the more poorly reactive clones 14A6 and 15G3 to be targeted towards intracellular Tssc6 epitopes (Figure 8.3). Interestingly, whilst there were no changes in 14A6 and 15G3 reactivity, the staining pattern of 16F4 was significantly brighter in both Tssc6$^{-/-}$ and wild type permeabilised cells. This was particularly clear in whole splenocytes and this change in staining pattern suggests that the target of 16F4 is expressed at both the cell surface and held within intracellular pools. From these analyses it was concluded that the four potential αTssc6 mAbs were not likely to be specific for the tetraspanin Tssc6. However, since these mAbs are specifically reactive to Tssc6 transfected cell lines, perhaps they may be used to investigate Tssc6 partner proteins that may be upregulated due to Tssc6 over-expression.

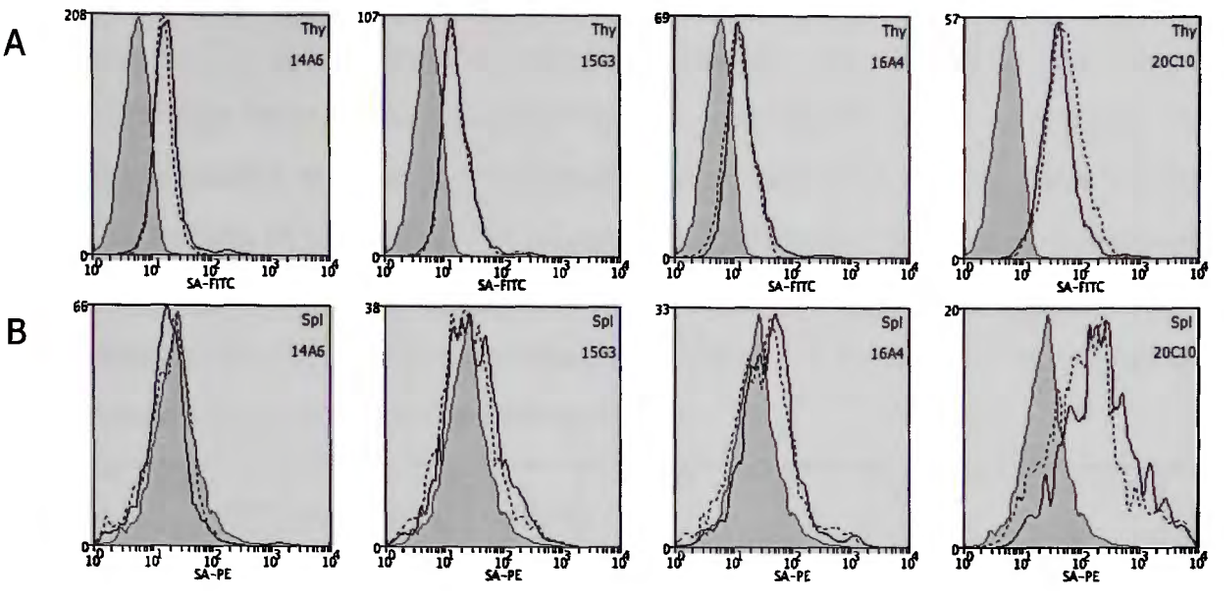

Figure 8.1 Screening of potential αTssc6 mAbs on wild type and Tssc6$^{-/-}$ primary cells.

Potential hybridoma supernatants were screened against primary haematopoietic cells derived wild type (solid lines) and Tssc6$^{-/-}$ (broken lines) mice. (A) Thymocytes and (B) splenocytes were isolated from naïve 8 week old mice and stained with biotinylated mAbs 14A5, 15G3, 16F4 and 20C10. SA-FITC was used as both secondary stain and negative controls (filled lines) (n=2, 2 mice/group).

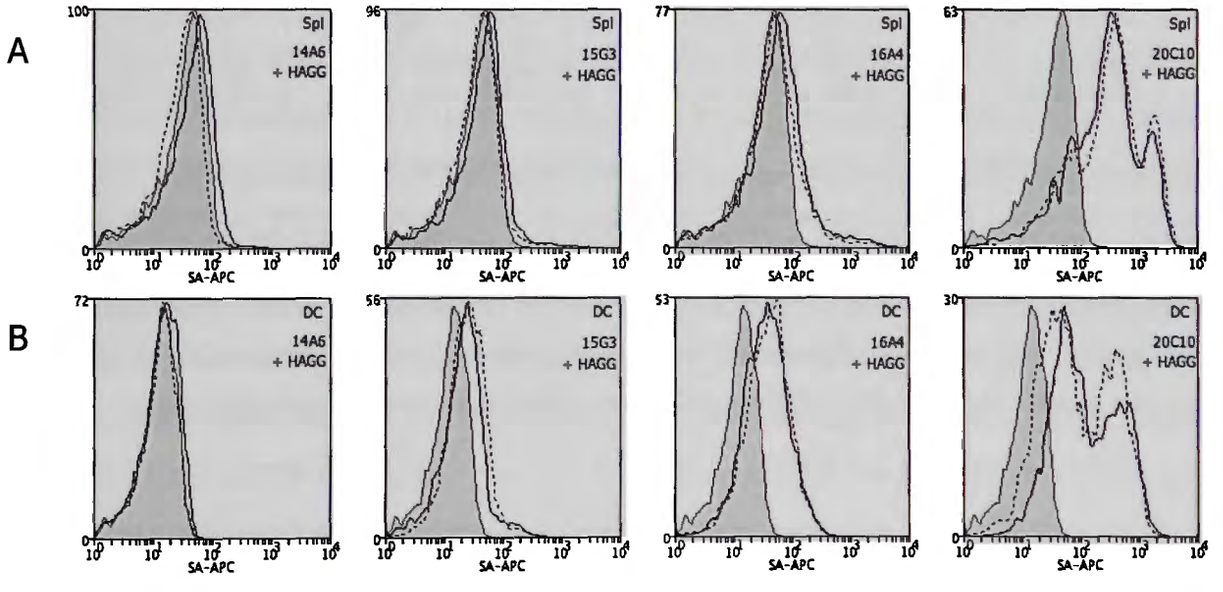

Figure 8.2 Screening of potential αTssc6 mAbs in the presence of blocking Abs.

Potential hybridoma supernatants were screened against primary haematopoietic cells derived wild type (solid lines) and Tssc6$^{-/-}$ (broken lines) mice. (A) Thymocytes and (B) splenocytes were isolated from naïve 8 week old mice and incubated with heat aggregated immunoglobulin (HAGG). Cells were then stained with biotinylated mAbs 14A5, 15G3, 16F4 and 20C10. SA-FITC was used as both secondary stain and negative controls as shown in filled lines (2 mice/group).

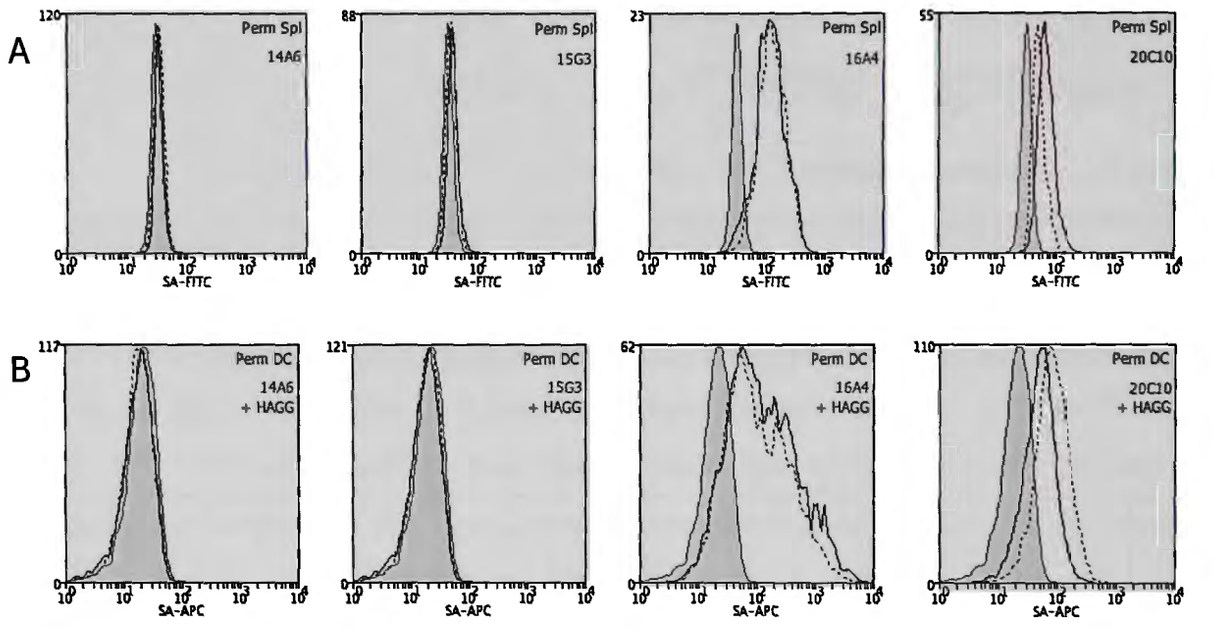

Figure 8.3 Screening of conjugated mAbs on permeabilised wild type and Tssc6$^{-/-}$ splenocytes and DC

Potential hybridoma supernatants were screened against permeabilised primary haematopoietic cells derived wild type (solid lines) and Tssc6$^{-/-}$ (broken lines) mice. (A) Thymocytes and (B) splenocytes were isolated from naïve 8 week old mice and fixed with 2% paraformaldehyde before permeabilisation with saponin. Cells were then stained with biotinylated mAbs 14A5, 15G3, 16F4 and 20C10. SA-FITC was used as both secondary stain and negative controls (filled lines) (n=2, 1 mouse/group).

8.1.2 Subcellular localisation of CD37, Tssc6 & CD151

To examine subcellular localisation and investigate the role of tetraspanins in the immunological synapse a series of tetraspanin-fluorescent fusion proteins were designed. Constructs were structured to produce tetraspanin proteins fused with green, yellow or blue fluorescent proteins at either C or N termini as described in Figure 8.4. Since the Tssc6 protein possesses an unusually long C-terminal tail, Tssc6 fusion constructs were designed to place the fluorescent protein at the N terminus. This was aimed at minimising potential disruptions to molecular associations between the Tssc6 C-terminal region or other possible functions such as protein trafficking and localisation.

8.1.3 Expression Construct Assembly

The tetraspanin fluorescent constructs were designed to produce the proteins described in Figure 8.4. Construct G i.e. CD37-GFP had been previously constructed within the Wright Laboratory. Primers containing restriction enzyme linkers were designed to amplify the cDNA coding sequence of CD151 and CD37 lacking the final three bases corresponding to the stop codon. The PCR product of CD151 was digested and cloned into the pre-digested and de-phosphorylated expression vector pEGFP-N1. pEGFP is a mammalian expression plasmid containing the gene encoding green fluorescence protein at the 3' end of the multiple cloning site. Due to the lack of tetraspanin stop codons in the cloned sequences, after successful transfection, transcription of CD151 continued into the GFP sequence generating a C-terminal fusion protein transcript. pECFP-N1 and pEYFP-N1 vectors were constructed by cloning CFP and YFP transcripts from pECFP-C1 and pEYFP-C1 vectors into pEGFP-N1 vectors that had been previously digested to remove the GFP sequence (i.e. pExxx-N1). CD37 and CD151 were then cloned into the newly constructed pECFP-N1 and pEYFP-N1 vectors. Tssc6 cDNA was amplified with the start codon removed and cloned into pECFP-C1 and pEYFP-C1 and a newly created pEGFP-C1 vector. Thereby the start codon of the fluorescent protein would begin translation and continue through to the Tssc6 stop codon.

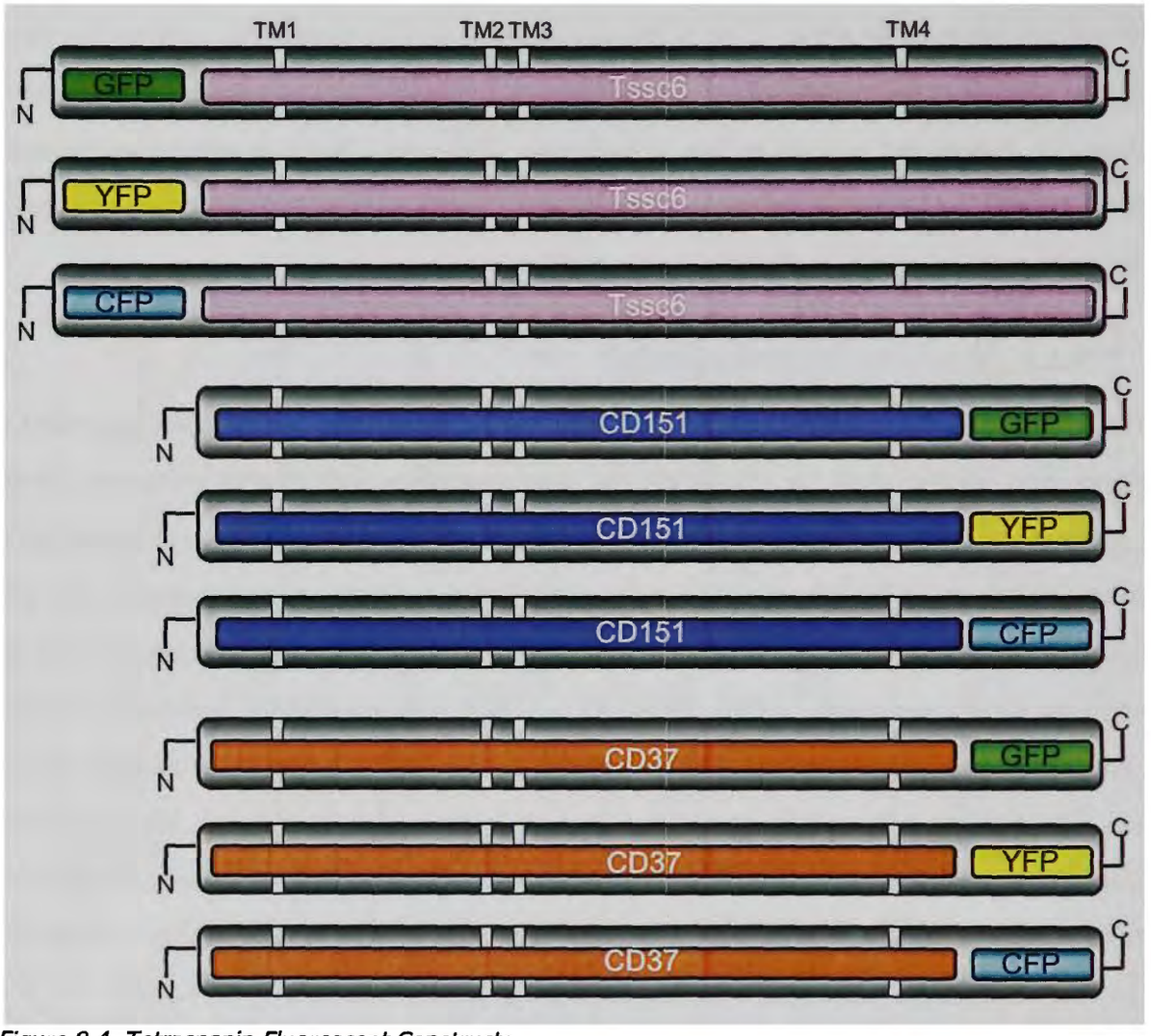

Figure 8.4 Tetraspanin Fluorescent Constructs.

(A-C) N terminal fusion proteins were designed for Tssc6. (D-I) C terminal fusion proteins were designed for CD37 and CD151.

8.1.4 GENERATION OF STABLE FLUORESCENTLY LABELLED TETRASPANIN CELL LINES

At this point due to time constraints only the CD151-GFP was electroporated into the human T cell line Jurkat and the murine T cell line MD-45 and treated with the selection agent neomycin. Since CD37, Tssc6 and CD151 have all been found to participate in the regulation of T cell proliferation, these cells were chosen to investigate the localisation of these tetraspanins during immunological synapse formation. Jurkat cells were given two rounds of cell sorting via flow cytometry to select for the brightest 10% of cells expressing the fusion construct (Figure 8.5A). After cell sorting fluorescent expression was improved dramatically and remained constant after a period of weeks of culture, indicating that the expression was stable. Next, cells were visualised under fluorescent microscopy to ensure the pattern of expression matched previous findings (Figure 8.5D-E). The CD151-GFP expression was as expected, with strong cell surface association. Interestingly, there was also bright intracellular staining in a single cluster that is likely to represent CD151 localisation in either the Golgi complex or the centrosome. In addition, confocal analysis of COS-7 transfected cells demonstrated a streaky pattern of cytoplasmic staining consistent with microtubules (Figure 8.5E). Cells were treated with Nocodazol to disrupt microtubules, which led to an increased vesicular pattern of expression and the appearance of nuclear rim staining (Figure 8.5F).

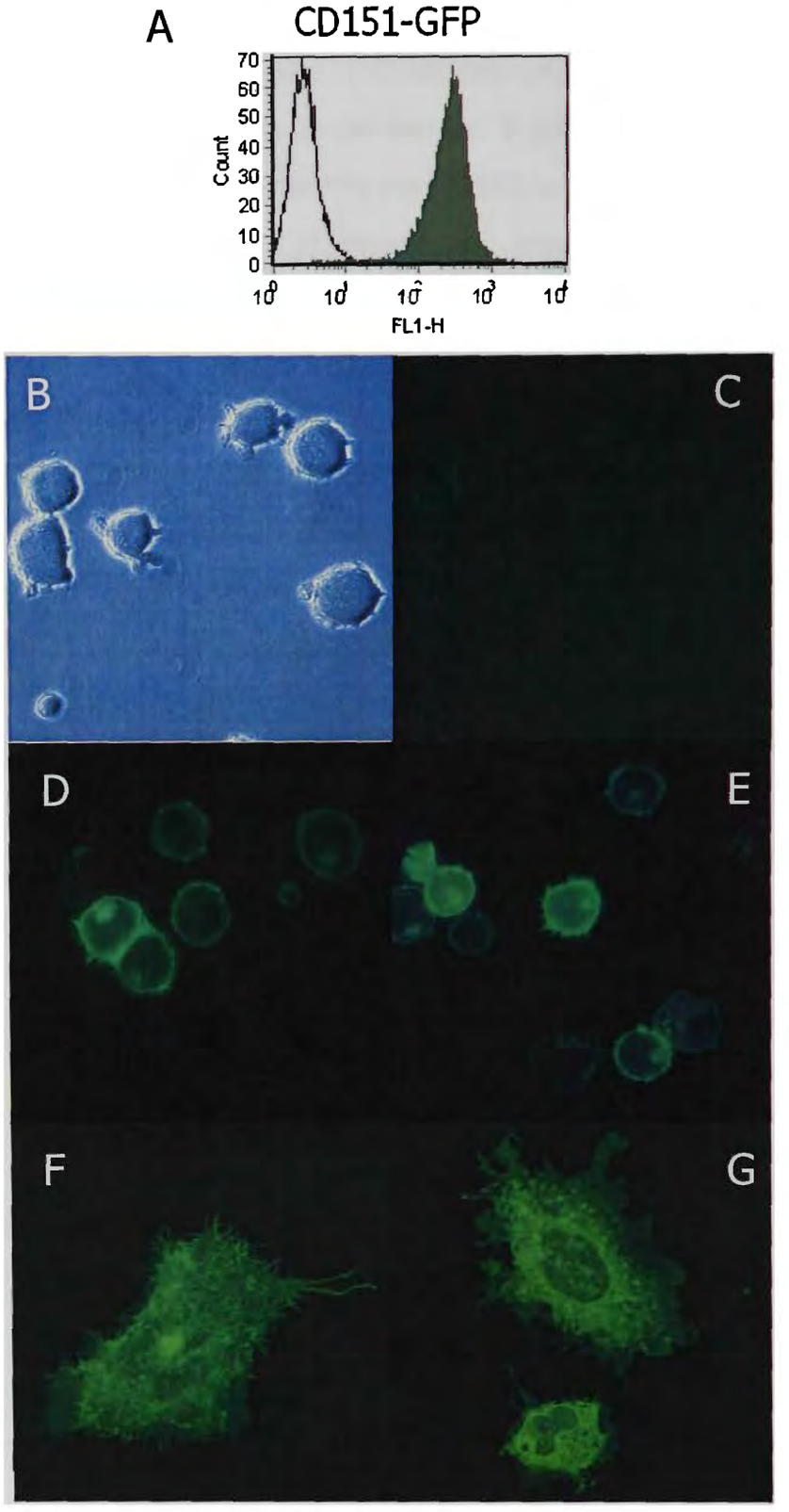

Figure 8.5 Tetraspanin Fluorescent cell lines

(A) Flow cytometry analysis of stable CD151-GFP expression in transfected Jurkats (grey) and untransfected Jurkats. (B) Bright field image and (C) green fluorescent image of untransfected Jurkat stock cells. (D-E) Green fluorescent images of CD151-GFP-Jurkat stable transfectants. Confocal images of COS-7 cells transiently transfected with CD151-GFP (F) untreated (resting) and (G) Nocodazol treated (confocal images curtesy of GW Moseley).

8.2 DISCUSSION

Confocal microscopy has been successfully used to investigate the interactions of various proteins during cellular events such as adhesion, antigen presentation and intercellular signalling. To develop our understanding of CD37 and Tssc6 in the immune system a molecular dissection of their interactions with other proteins is required. Prior to this study, collaborators had developed a panel of potential αmurine-Tssc6 hybridomas. Supernatants were initially screened on non-haematopoietic cells transfected with murine Tssc6 and later, based on reactivity to a panel of blood derived cell lines, four were chosen for further validation. Surprisingly, despite strong reactivity of some clones to primary leucocytes these mAbs were not specific to Tssc6, since Tssc6$^{-/-}$ leucocytes displayed similar immuno-positivity. An interesting question that therefore arises is: if these mAbs react specifically with Tssc6 transfected cells, what are the targets of these mAbs? Perhaps these clones may be used to detect potential Tssc6 binding partners upregulated due to Tssc6 over-expression? Given the conserved tertiary and secondary structures observed between members of the tetraspanin family, there may also be some degree of cross reactivity between other tetraspanins.

Since monoclonal antibodies directed against mouse CD37 and Tssc6 proteins are unavailable, constructs were designed to express fluorochrome conjugated tetraspanins. The tetraspanin CD151 was also included in this process due to its similar function in both T cells and DC (Wright *et al.*, 2004, Sheng *et al.*, submitted for publication). Due to time constraints CD151-GFP was the only construct that reached the stage of stable high expression in T cell lines. Fluorescent microscopy demonstrated bright CD151 expression in Jurkat transfectants at both the cell surface and within a large intracellular cluster likely to be either the Golgi complex or the centrosome. Preliminary studies to further characterise CD151 expression using COS-7 transfected cells under confocal microscopy indicated that there may be some degree of CD151-GFP co-localisation with cytoplasmic microtubules. If so, this would suggest that the large intracellular cluster could be the centrosome, which is also known as the microtubule organising centre (MTOC). Whilst these studies are very preliminary, if CD151 positive vesicles do co-localise with microtubules, this may correlate with findings in CD81 and CD82, which are thought to traffic to the immunological synapse in an actin dependent manner (Delaguillaumie *et al.*, 2004, Delaguillaumie *et al.*, 2002; Lagaudriere-Gesbert *et al.*, 1998; Poenie *et al.*, 2004). It is possible that tetraspanin subcellular localisation as well as protein-partner interactions, may be disrupted by the addition of the fluorescent proteins described in this chapter. This may also result in changes to protein trafficking, cell surface

stability and by disrupting the formation of some tetraspanin microdomains, potentially interfere with cell signalling. To combat this, the constructs designed in Figure 8.4 will also be generated with the fluorescent protein fused to the opposing terminal i.e. eventually both C-terminal and N-terminal constructs will be required. However it could be argued that since functional studies (such as those performed on CD81), demonstrate distinct functions for multiple tetraspanin protein domains (Shoham *et al.*, 2006), more than one method of protein labelling will be important in completing these studies. Therefore, the development of mAbs targeted to CD37 and Tssc6 will improve this work significantly.

9 Appendix II – Suppliers and Reagents

Table 9.1 Table of reagent and equipment suppliers

Reagent	Supplier
Cell Culture	
RPMI	Gibco-BRL (NY, USA)
FCS	
96 well plates	BD Falcon (NJ, USA)
24 well plates	BD Falcon (NJ, USA)
Multiscreen ELISPOT Plates	Millipore
G418 (Neomycin)	Invitrogen (NJ, USA)
DMSO	Sigma-Aldrich (MO, USA)
β-mercapto-Ethanol	Sigma-Aldrich (MO, USA)
Penicillin/Streptomycin Solution	Sigma-Aldrich (MO, USA)
L-Glutamine	Sigma-Aldrich (MO, USA)
Cell Separation Reagents	
DNase	Roche (IN, USA)
Collagenase	Worthington Biochemical Corp (NJ, USA)
Nycodenz	Axis Shield (Oslo, Norway)
αCD11c Magnetic Beads	Miltenyi Biotec (Bergisch Gladbach, Germany)
Sheep-αrat Ig Magnetic Beads	Qiagen (CA, USA)
Sheep-αrat Ig Magnetic Beads	Dynal Biotech (Oslo, Norway)
In vitro Cell Stimulants	
conA	Sigma-Aldrich (MO, USA)
LPS	Sigma-Aldrich (MO, USA)
Thioglycollate	Sigma-Aldrich (MO, USA)
CpG	Geneworks (Australia)
SIINFEKL	Genscript Corp (N.J., USA)
CD4 Helper Peptide	Genscript Corp (N.J., USA)
Staphylococcus aureus (SAC)	Sigma-Aldrich (MO, USA)
αGalCer	Prof Dale Godfrey (Melb Uni, Australia)
In vivo Stimulants/Adjuvants/Treatments	
CFA	Sigma-Aldrich (MO, USA)
IFA	Sigma-Aldrich (MO, USA)
MOG peptides	Gift from Prof. John Matsoukas (Greece)
Bordetella Purtussis Toxin	Sigma-Aldrich (MO, USA)
Collagen Type II (Chicken)	Sigma-Aldrich (MO, USA)
Collagen Type II (Bovine)	Chondrex (WA, USA)
ANTp-OVA	Gift from Prof Geoff Pietersz (Burnet Institute, Aus)
ANTp-SIINFEKL	Gift from Prof Geoff Pietersz (Burnet Institute, Aus)
NP-KLH	Biosearch Technologies (CA, USA)
Mycobacterium Tuberculosis HR37a	Sigma-Aldrich (MO, USA)
Ketamine-HCL	Sigma-Aldrich (MO, USA
Xylazine-HCL	Sigma-Aldrich (MO, USA)
Aluminium Salts	Sigma-Aldrich (MO, USA)
Cell/Protein Labelling and Detection	
NP-BSA	Biosearch Technologies (CA, USA)
Cell Permeablisation Kit	eBiosciences (CA, USA)
CFSE	Invitrogen (Oregon, USA)
3-H Thymidine	Amersham Pharmacia (Sweden)
Glass fibre filters	Perkin Elmer (Ma, USA)
SA-HRP	BD Pharmingen (NJ, USA)
SA-ALP-PQ	MabTech (Nacka, Sweden)
Tween 20	Sigma-Aldrich (MO, USA)
AP conjugate substrate Kit	Qiagen (CA, USA)
FITC Isomer I	Invitrogen (Oregon, USA)
Dibutyl-phtalate	Sigma-Aldrich (MO, USA)
TMB	eBiosciences (CA, USA)
Propidium Iodide	Sigma-Aldrich (MO, USA)
Trypan Blue	Sigma-Aldrich (MO, USA)
Th1/Th2 Cytokine Detection Kit	BD Pharmingen (NJ, USA)
Inflammatory Cytokine Detection Kit	BD Pharmingen (NJ, USA)

Reagent	Supplier
Instruments	
MoFlow	Cytomation (CO, USA)
FACScalibur	Becton Dickinson (CA, USA)
FACScanto II	Becton Dickinson (CA, USA)
FACS Diva	Becton Dickinson (CA, USA)
AutoMACS	Miltenyi Biotec (Bergisch Gladbach, Germany)
ELISPOT Reader	Autoimmun Diagnostika GmbH
Beta Counter (NXT TopCount)	Perkin Elmer (MA, USA)
ELISA Reader	Biorad (
Cell Dyn 1400 (Coulter Counter)	Abbot Scientific (IL, USA)
Gel Documentation System (EpiChemi II Darkroom)	Ultraviolet Products (CA, USA)
Electroporator	Biorad (
Gradient Thermal Cycler	Eppendorf (
Gammacell 100 ELITE	Norton International (
Molecular Biology Reagents	
HotStarTaq Polymerase (Genotyping)	Qiagen (CA, USA)
Proteinase K	Worthington Biochemical (NJ, USA)
Pwo Polymerase (Cloning)	Roche (NSW, AU)
Big Dye v3.1	Applied Biosystems (CA, USA)
Ampicillin	Sigma-Aldrich (MO, USA)
Kanamycin	Sigma-Aldrich (MO, USA)
Primers	Proligo (CO, USA)/ Sigma-Aldrich (MO, USA)
Restriction Enzymes	New England BioLabs (MA, USA)
SAP	Qiagen (CA, USA)
T4 DNA Ligase	New England BioLabs (MA, USA)
Plasmid DNA Miniprep Kit	Qiagen (CA, USA
Plasmid DNA Maxiprep Kit	Qiagen (CA, USA)
DNA Purification Kit	Qiagen (CA, USA))

Table 9.2 Table of Antibody Reagents and Suppliers

In house – hybridomas were grown in serum free medium and supernatants concentrated, purified and antibodies conjugated to various fluorochromes. Kate Gartlan, Mariam Sofi, Carmel Daunt, KC Sheng & Eliada Lazoura.

Target Protein	Clone	Conjugate	Source
Lymphocyte Markers			
CD3	KT3-1.1	Cy5	In house
CD3		Biotin	BD-Pharmingen (NJ, USA)
CD4	GK1.5	FITC	BD-Pharmingen (NJ, USA)
CD5	53-7.3	FITC	BD-Pharmingen (NJ, USA)
CD8α	53-6.7	PE	BD-Pharmingen (NJ, USA)
CD9	KMC8	Biotin	BD-Pharmingen (NJ, USA)
CD11b	M1/70	Biotin	In house
CD11c	N418	APC	BD-Pharmingen (NJ, USA)
CD19	1D3	PE	BD-Pharmingen (NJ, USA)
CD21/35	7G6	PE	BD-Pharmingen (NJ, USA)
CD23	B3B4	FITC	BD-Pharmingen (NJ, USA)
CD25	3C7	FITC	BD-Pharmingen (NJ, USA)
CD28	37.51	Biotin	In house
CD40	FGK45.5	FITC	In house
CD45	30-F11	FITC	BD-Pharmingen (NJ, USA)
CD45RA	RA3-6B2	Biotin	In house
CD49d	9C10	Purified	BD-Pharmingen (NJ, USA)
CD49e	5H10-27	PE	ImmunoTools (Friesoythe, Germany)
CD49f	GoH3	Purified	BD-Pharmingen (NJ, USA)
CD62L	MEL-14	PE	ImmunoTools (Friesoythe, Germany)
CD80	16-10A1	FITC	BD-Pharmingen (NJ, USA)
CD81	Eat2	Purified	
CD86	GL1	FITC	In house
CD90	T24/31.7	Biotin	In house
CD161c (NK1.1)	PK136	PE	BD-Pharmingen (NJ, USA)
CD205	NLDC145	PE	In house
MHC Class I		Biotin	In house
MHC Class II	M5/114	Cy5	In house
F4/80	F4/80	PE	BD-Pharmingen (NJ, USA)
Ly-76	TER119	Purified	In house
Gr1	RB68C5	Purified	In house
IgM	II/41	FITC	BD-Pharmingen (NJ, USA)
IgD	11-26c.2a	PE	BD-Pharmingen (NJ, USA)
IgM Fab'2 Fragment			Abcam
Secondary Reagents			
Streptavidin		FITC	BD-Pharmingen (NJ, USA)
Streptavidin		PE	BD-Pharmingen (NJ, USA)
Streptavidin		APC	BD-Pharmingen (NJ, USA)
Streptavidin		HRP	BD-Pharmingen (NJ, USA)
αrat-Ig	MRK-1	PE	BD-Pharmingen (NJ, USA)
αrabbit-Ig	Polyclonal	FITC	Silenus (Australia)
Miscellaneous			
RabαChick-OVA	Polyclonal	FITC	Millipore (MA, USA)

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