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*MUC1 immunotherapy against a metastatic mammary adenocarcinoma model: Importance of IFN-gamma*

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# MUC1 IMMUNOTHERAPY AGAINST A METASTATIC MAMMARY ADENOCARCINOMA MODEL: IMPORTANCE OF IFN-GAMMA

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**Abstract**

Immunotherapy using mucin 1 (MUC1) linked to oxidised mannan (MFP) was investigated in an aggressive MUC1$^+$ metastatic tumour, DA3-MUC1 because, unlike many MUC1$^+$ tumour models, DA3-MUC1 is not spontaneously rejected in mice making it an alternative model for immunotherapy studies. Further, DA3-MUC1 cells are resistant to lysis by anti-MUC1 cytotoxic T cells (CTLs). The inability of DA3-MUC1 tumours to be rejected in naïve mice as well as vaccination to MUC1 was attributed to a deficiency of expression of MHC class I molecules on the tumour cell surface. *In vitro* and *in vivo* analysis of subcutaneous tumours and lung metastases demonstrated that DA3-MUC1 tumour cells have a low expression (< 6%) of MHC class I which can be upregulated (> 90%) following culturing with IFN-γ. Results from flow cytometry analysis and immunoperoxidase staining indicated that the *in vitro* up-regulation of MHC class I could be maintained for up to seven days *in vivo*, without affecting the expression levels of MUC1 antigen. Interestingly, MUC1-specific CTL that lyse DA3-MUC1 targets *in vitro* were induced in MFP immunised mice but failed to protect mice from a DA3-MUC1 tumour challenge. These results highlight the importance of MHC class I molecules in the induction of anti-tumour immunity and the MFP immune response.

Keywords: MUC1. MHC class I, interferon-gamma, tumour, immunotherapy

## Introduction

Anti-tumour immunity and tumour eradication are induced by cell-mediated immune responses [1, 2]. The activation of tumour-specific CD8$^+$ T lymphocytes and their subsequent differentiation into cytolytic cells is dependent on 2 signals from the antigen-presenting cell. One signal is provided through the interaction of the antigenic peptide (from the tumour) pre- sented on the major histocompatibility complex (MHC) to T cells. The other is the costimulatory signals, efficiently provided by B7 [B7-1 (CD80) and B7-2 (CD86)] binding to CD28 (CD152 or CTLA-4) on T cells [3, 4]. However, malignant cells have evolved mechanisms enabling them to successfully evade the immune system, which in many cases directly affects this 2 signal process. These mechanisms

include inadequate expression of costimulatory molecules, Fas ligand, or adhesion molecules on cancer cells, antigen processing defects, the secretion of inhibitory molecules into the tumour microenvironment or absent or poorly expressed MHC molecules on the tumour cell surface [5–7]. More recently, it has been demonstrated that tumour cells have additional escape mechanisms by expressing PD-L1 (B7-H1, CD275) and/or PD-L2 on their surface, which upon binding to its ligand, PD-1, expressed by activated CD8$^+$ T cells leads to apoptosis of T cells [8].

The failure of tumours to adequately process antigens and present peptide fragments to T cells is greatly attributed to reduced expression of MHC class I molecules on the cell surface of tumour cells [5, 6, 9, 10]. In many tumour models however, this can be rectified transfecting tumour cells with MHC class I gene [11, 12]. Another approach is to transfect cytokine cDNA, in particular IFN-γ, into tumours as it directly causes an up-regulation of cell surface MHC class I expression [11, 13].

This study characterises the DA3-MUC1 metastatic tumour following the failure of mannan-MUC1 (MFP) immunisations to induce anti-tumour immunity in this MUC1$^+$ cancer model. It was demonstrated that DA3-MUC1 was non-immunogenic due to an absence of MHC class I expression on the tumour cell surface, which could be upregulated by IFN-γ but not sustained long enough *in vivo* to cause tumour eradication.

## Materials and methods
### Mice and immunisations

A MUC1-GST fusion protein containing 5 variable number of tandem repeat (VNTR) regions from the extracellular protein core of MUC1 [14] was produced in a bacterial expression system (pGEX-3X) and conjugated to oxidised mannan to form MFP as described previously [15–23]. BALB/c mice aged 6–10 weeks were given three intraperitoneal immunisations (on days 0, 7 and 14) with either MFP (containing 5μg of MUC1 fusion protein) or a control pH 9.0 phosphate buffer. BALB/c mice immunised with mannan coupled to oxidised GST (M-GST) were included as controls in the

lung metastases study. All experiments were approved by the Austin Animal Ethics Committee.

### Cell lines

DA3-MUC1 is a metastatic BALB/c DA3 mammary cell line transfected with the cDNA of the transmembrane form of human MUC1 [24, 25]; P815-MUC-1, a DBA/2 P815 mastocytoma cell lines transfected with the cDNA of the transmembrane form of human MUC1 [26, 27] were cultured in RPMI and MUC1 expression selected for every 14–20 days with 1.25 mg/ml G418-sulfate (Gibco BRL, U.S.A).

### Flow cytometry

The expression of cell surface molecules on DA3-MUC1 were measured by flow cytometry. The following monoclonal antibodies were used; a) MUC1 (BC2: supernatant) [28], b) MHC class 1 H2$^d$ (34.1.2s, 1/1000 dilution of ascites fluid) [29], c) MHC class II I-A8 (1/500 dilution of ascites fluid), d) B7.1 (4μg) (Pharmingen, San Diego, USA), e) ICAM-2 (1μg) (Pharmingen), f) CD28 (4μg) (Pharmingen), g) LFA-2 (1μg) (Pharmingen) and h) CTLA-4 (1μg) (Pharmingen). DA3-MUC1 tumour cells were prepared for FACS analysis by either a) culturing in growth media, b) culturing with 20 ng/ml vaccinia virus-IFN-γ [22] for 72 h, or c) culturing with 20 ng/ml IFN-γ for 72 h and then removing IFN-γ for subsequent culturing. In preparation for flow cytometry, tumour cells (2–5 x 10$^5$ cells/ml) were incubated with the specified antibodies for 45 min at 4$^0$C, washed with phosphate buffer and incubated with either FITC-conjugated sheep (Fab')$_2$ anti-mouse, anti-rat or anti-hamster immunoglobulin (Amersham, UK) (1/50 dilution) for a further 45 min at 4$^0$C. Cells were washed and analysed by flow cytometry.

### Immunoperoxidase staining
### of DA3-MUC1 tumour cells

Cell surface expression of MUC1 and MHC class I proteins on DA3-MUC1 tumour cells *in vivo* were analysed by immunoperoxidase staining. DA3-MUC1 tumour cells were either, a) injected subcutaneously into BALB/c

mice and grown for > 30 days to establish lung metastasis. Mice were culled and samples taken from both the subcutaneous tumour site and from lung metastasis; or b) cultured with 20 ng/ml IFN-$\gamma$ for 72 h and injected subcutaneously into BALB/c mice. Mice were culled and samples taken from the subcutaneous tumour site on days 4 and 7 and from lung metastasis > 30 days after injection.

All tissue samples were snap frozen in isopentane and sections 5–6 $\mu$m thick were cut using a Microm HM500 cryostat (MICROM Laborgerate, Strässe, Germany), mounted and fixed on silane coated slides [30]. Endogenous peroxidase activity was blocked by incubating with 0.5% $H_2O_2$ for 40 minutes at room temperature. Tissue sections were incubated for 45 min at $4^0C$ with biotinylated BC2 to detect MUC1 expression or biotinylated anti-H2$^d$ (1/1000 dilution) to detect MHC class I expression. Excess antibodies were removed by thorough washing and samples incubated with streptavidin-HRP conjugate (Amersham, UK) (1/50 dilution) for a further 45 min at $4^0C$. Antibody binding was detected with 1.5 mg/ml 3–3 diaminobenzidine (DAB, Sigma, St. Louis, USA) in phosphate buffered saline containing 0.5% $H_2O_2$ for 5 min, slides were washed and mounted.

### Tumour model

The immunogenicity of DA3-MUC1 tumour was characterised using the following tumour growth experiments and MFP immunisations.

(i) BALB/c mice (x 10) were subcutaneously injected with 5 x $10^6$ DA3-MUC1 tumour cells and tumour growth measured with electronic callipers every week for 10 weeks to establish a DA3-MUC1 growth curve. Mice were sacrificed > 30 days after the tumour challenge and lung metastasis determined by microscopically counting the number of metastasis present in random cross sections of similar sizes from formalin fixed lung samples (Anatomical Pathology Unit, Austin and Repatriation Medical Centre, VIC Australia).

(ii) BALB/c mice (x 20 per group) were immunised 3 times on days 0, 7 and 14 with either MFP (5 $\mu$g) or M-GST (5 $\mu$g) and chal-

lenged with 5 x $10^6$ subcutaneous DA3-MUC1 tumour cells. A minimum of 4 mice from each group were sacrificed each week for five weeks and the number of metastatic lesions present on each lung determined microscopically.

(iii) BALB/c mice (x 10 per group) were injected subcutaneously with 5 x $10^6$ DA3-MUC1 tumour cells until tumours of ~50 mm$^2$ were established (Day 17). Mice were then immunised intraperitoneally on days 17, 19 and 21 with 5 $\mu$g MFP. Tumour sizes were measured every 2–3 days for 30 days using electronic callipers.

(iv) BALB/c mice (x 10 per group) were immunised intraperitoneally on days 0, 7 and 14 with either 5 $\mu$g MFP or pH 9.0 buffer, and challenged subcutaneously on day 21 with 3 x $10^6$ DA3-MUC1 tumour cells. Prior to challenge, the tumour cells were cultured with 20 ng/ml vaccinia virus-IFN$\gamma$ supernatant (UV inactivated) [22, 23, 25, 26] for 72 h to increase cell surface MHC class I expression. Tumour growth was measured every 2–3 days for 2 weeks using electronic callipers.

### Cytotoxic T cell $^{51}$Cr release assay

BALB/c mice immunised (x 3) with MFP (5 $\mu$g) were culled and their spleen cells collected and treated with 0.83% $NH_4Cl$. Two-fold serial dilutions of effector spleen cells from the immunised mice were plated into a 96 well plate beginning at a concentration of 1 x $10^6$ cells per well in duplicate. 1 x $10^4$ $^{51}$Cr labelled DA3-MUC1 cells cultured with IFN-$\gamma$ (20 ng/ml) for 72 h, DA3-MUC1, P815-MUC1 or P815 target cells were added to the effectors. The spontaneous release of $^{51}$Cr from the labelled cells was determined by incubating target cells in RPMIM media and the maximum release was determined by incubation with 10% SDS (BDH Chemicals, Dorset, England). Cultures were incubated for 4 h before transferring 100 $\mu$l of supernatant to 96 well flat Optiplates (Disposable Products, Australia) containing 100 $\mu$l of Microscint 40 (Packard, USA) for analysis on the microplate scintillation counter (Packard USA). The specific percentage lysis of target cells was determined by; [(experimental-spontaneous) cpm / (maximum-spontaneous) cpm] x 100%.

## Results
### Immunisation which does not protect against DA3-MUC1 tumour growth

To examine the anti-tumour effects of MFP immunisations on the DA3-MUC1 tumour *in vivo*, 2 immunotherapy models were used.

In the first model, BALB/c mice with an established DA3-MUC1 tumour (~50 mm$^2$) were immunised 3 times (days 17, 19 and 21) with either MFP or control pH 9.0 buffer, and, tumour growth and lung metastases measured for 30 days. Unlike other tumour models [30] in the DA3-MUC1 model, there was no difference in tumour growth (Figure 1A) or the number of lung metastases (as determine by lung weight) (data not shown). Therefore, therapy with MFP was not effective at treating established DA3-MUC1 tumours.
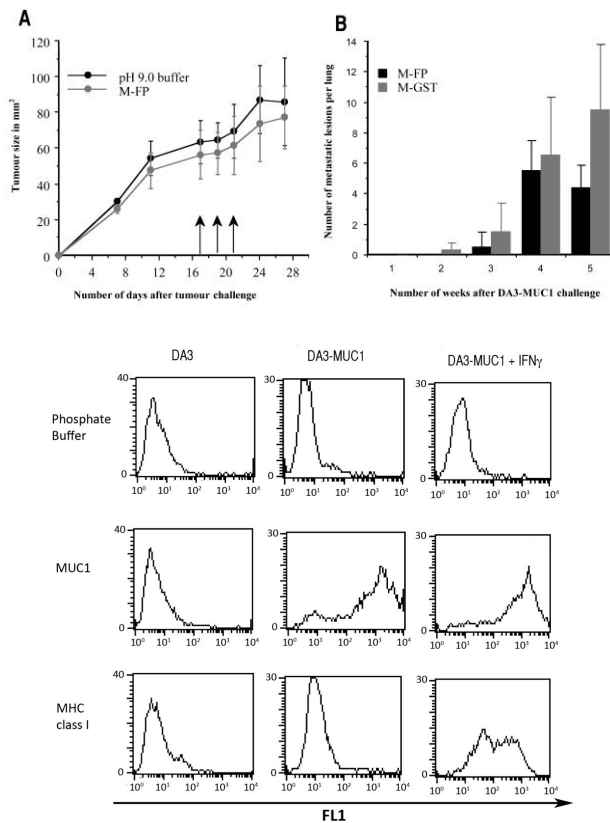


*Figure 1. A – Subcutaneous DA3-MUC1 (5 x 10$^6$ cells/mouse) tumour growth in BALB/c mice immunised with MFP. Mice with an established 17 day tumour were immunised intraperitoneally on days 17, 19 and 21 with 5 μg MFP or pH 9.0 buffer and tumour growth measured. B. Lung metastases in BALB/c mice immunised with either MFP or a control M-GST 3 times (days 0, 7 and 14), then challenged with DA3-MUC1 (5 x 10$^6$ cells/mouse). 4–5 mice were culled each week for 5 weeks and microscopic lung metastasis counted. Data is presented as mean +/- standard error of the mean*

In the second model, BALB/c mice were immunised 3 times with either MFP or a control preparation, M-GST, and challenged with 5 x 10$^6$ DA3-MUC1 tumour cells subcutaneously. Metastatic lung nodules from 4–6 mice per week were examined microscopically for five weeks (Figure 1B). Immunisation (prophylactic model) with MFP did not protect mice challenged with DA3-MUC1 from developing lung metastases as assessed by the number of lung metastases per lung compared to immunised control mice.

From these studies, it was concluded that immunisation with MFP could not induce tumour protection in mice challenged with DA3-MUC1 tumour cells, nor could it offer protection against an established DA3-MUC1 tumour. These results were in contrast to findings in all other MUC1$^+$ tumour models investigated, where immunisation with MFP was able to successfully induce anti-tumour immunity and tumour protection *in vivo* [14–23, 26, 31]. It was hypothesised that DA3-MUC1 tumours were not immunogenic due to a decrease in either costimulatory or MHC molecules on their surface. To test these hypotheses, the DA3-MUC1 tumour was characterised for cell surface molecule expression *in vitro* and *in vivo*.

### DA3-MUC1 tumour cells express high levels of MUC1 but do not express MHC class I
*In vitro* characterisation of DA3-MUC1. The DA3-MUC1 metastatic cell line was analysed for expression of human MUC1, MHC class I and other cell surface markers by flow cytometry (Table 1 and Figure 2). MUC1 is highly expressed on the surface (> 85%) of DA3-MUC1 cells compared to < 2% on non-transfected parental DA3 cells. In contrast, MHC class I expression was considerably decreased in both DA3-MUC1 cells (6%) and non transfected DA3 cells (< 3%). There was no detectable MHC class II, B7.1, ICAM-2, CD28, LFA-2 or CTLA-4 on DA3 or DA3-MUC1 tumour cells (Table 1). Phosphate buffer was used as a control for non-specific (Fab')$_2$ FITC-conjugate binding .

Table 1

*In vitro expression of cell surface markers on DA3 and DA3-MUC1 tumour cells cultured with or without 20 ng/ml IFN-γ for 72 h. Values represent the percentage of cells positive for each antibody determined by flow cytometry*

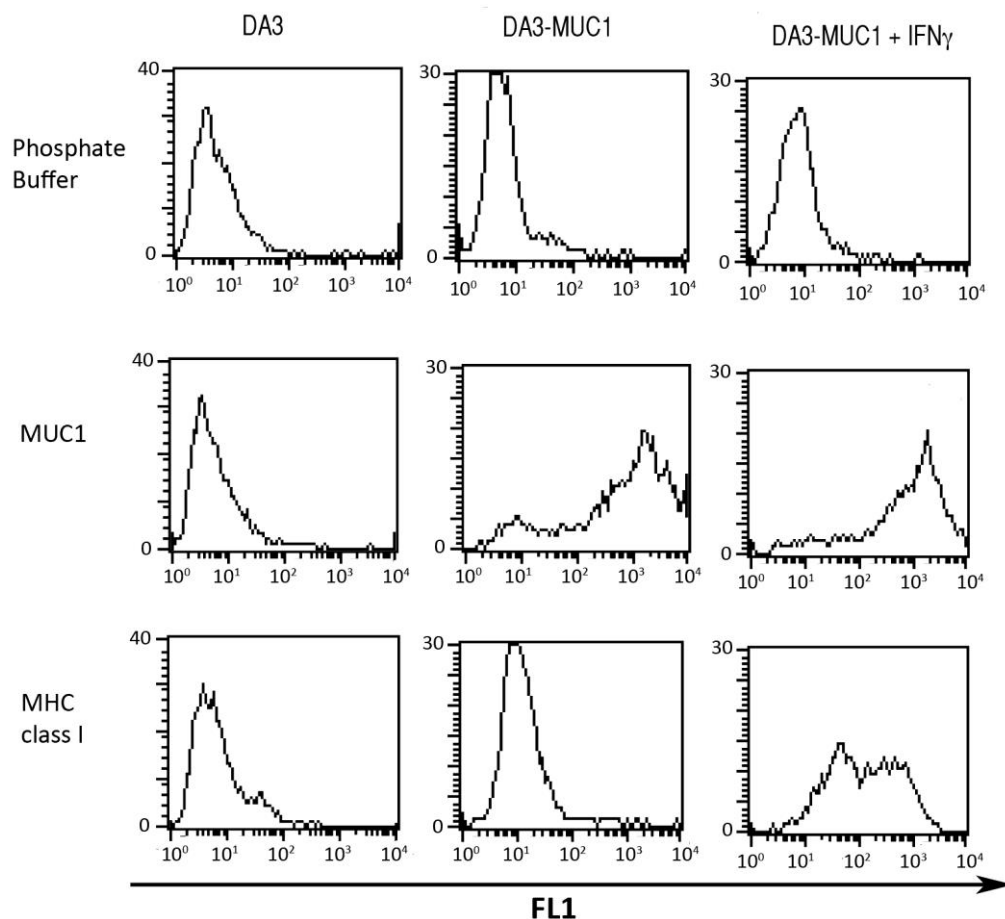| Cell Surface Markers | DA3 (%) | DA3-MUC1 (%) | DA3-MUC1+IFN-γ (%) |
|---|---|---|---|
| negative control | 1.94 | 3.12 | 2.71 |
| MUC1 | 3.36 | 87.73 | 93.6 |
| MHC class I | 2.98 | 5.67 | 76.85 |
| MHC class II | 3.81 | 3.72 | 2.01 |
| B7.1 | 1.49 | 3.03 | 1.67 |
| ICAM 2 | 3.70 | 4.42 | 2.75 |
| CD28 | 4.13 | 5.22 | 4.03 |
| LFA-2 | 2.27 | 3.09 | 2.12 |
| CTLA-4 | 4.81 | 6.09 | 3.70 |



*Figure 2 – Flow cytometric analysis of in vitro expression of cell surface MUC1 and MHC class I on DA3-MUC1 cells cultured with or without 20 ng/ml IFNγ for 72 h. The non transfected parental cell line, DA3 was used as a control. Phosphate buffer represents negative control binding of FITC-conjugated sheep (Fab')₂ anti-mouse (1/50) to the tumour cell lines*

***In vivo* characterisation of DA3-MUC1.** To characterise the DA3-MUC1 tumour *in vivo*, BALB/c mice were challenged with $5 \times 10^6$ metastatic cells and tumour growth monitored for 10 weeks (Figure 3A). Mice had palpable subcutaneous tumours after 2–3 weeks and were culled after 10 weeks and their subcutaneous tumours and lungs removed for tumour analysis.
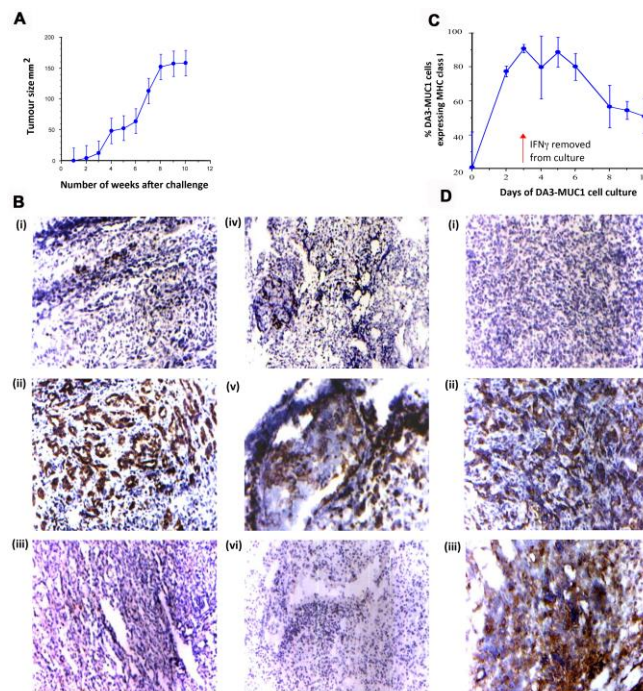
*Figure 3ABCD – Tumour growth in BALB/c mice challenged with 5 x 10⁶ DA3-MUC1 cells. **B.** MUC1 and class I (H2ᵈ) surface expression on DA3-MUC1 tumour cells determined by immunoperoxidase staining; data is presented as mean +/- standard error of the mean. DA3-MUC1 tumour cells from the site of a BALB/c subcutaneous tumour were stained for, **B(i)** negative control, **B(ii)** MUC1 expression using biotinylated-BC2 and **B(iii)** MHC class I expression using biotinylated-34.1.2s. DA3-MUC1 lung metastases were stained for, **B(iv)** negative control, **B(v)** MUC1 expression using biotinylated-BC2 and **B(vi)** MHC class I expression using biotinylated-34.1.2s. All images are shown at 200x magnification. Non-specific binding of (Fab')₂ conjugate was blocked with 10% BSA in DME and control samples were incubated with 10% BSA in DME. **C.** FACS analysis determining the length of time MHC class I expression remained elevated on DA3-MUC1 cells cultured with IFN-γ in vitro. Tumour cells were cultured for 72 h with 20 ng/ml IFN-γ to increase MHC class I expression, IFN-γ removed from culture and MHC class I expression measured daily. **D.** Immunoperoxidase staining of DA3-MUC1 cells pre-cultured with IFN-γ and grown in vivo. Mice were sacrificed every three days for 10 days, tumours removed, formalin fixed and stained for, **D(i)** negative control, **D(ii)** MUC1 expression using biotinylated BC2 and **D(iii)** MHC class I expression using anti-H2ᵈ. Phosphate buffer was used as a negative control – binding of FITC-conjugated sheep (Fab')₂ anti-mouse (1/50) to the tumour cells*

As expected, immunoperoxidase staining for MUC1 and MHC class I expression on subcutaneous established DA3-MUC1 tumours were similar to that observed *in vitro* studies. DA3-MUC1 tumour cells express high levels of MUC1 (75–100% of cells) (Figures 3B) and very little, if any, MHC class I (0–15% of cells) (Figure 3B i-iii) on their cell surface. Lung metastases (macroscopic and microscopic) were observed 30–35 days after a subcutaneous injection of 5 x 10⁶ DA3-MUC1 cells, however there was no evidence of metastases to the liver (data not shown). Immunoperoxidase staining for MUC1 and MHC class I expression on lung metastases showed MUC1 expression on ~ 50% of tumour cells in the lung but no evidence of MHC class I expression (Figure 3B iv-vi).

### Elevation of MHC class I expression on DA3-MUC1 with IFN-γ

It is clear that one of the factors hindering the immunogenicity of the DA3-MUC1 tumour was a decrease in the expression of MHC class I. Numerous studies have shown that MHC class I expression, and therefore tumour immunogencity, can be increased by culturing the tumour with recombinant IFN-γ. To determine whether MHC class I expression could be up-regulated on DA3-MUC1 tumour cells, cells were cultured with 20 ng/ml vaccinia virus-IFN-γ for 72 h and MHC class I expression determined using flow cytometry.

DA3-MUC1 expression of MHC class I molecules on the tumour surface could be greatly increased *in vitro* by culturing the DA3-MUC1 cells with IFN-γ (Table 1 and Figure 2).

Prior to *in vitro* culturing with IFN-γ, only 6% of DA3-MUC1 cells expressed MHC class I on their cell surface (Figure 2), however, after culturing cells with IFN-γ for 72 h, 77% of DA3-MUC1 tumour cells expressed MHC class I (Figure 2) and MUC1 expression still remained high (Figure 2).

To determine the length of time MHC class I expression remained elevated on DA3-MUC1, tumour cells were cultured with IFN-γ for 72 h, removed, and then examined daily for MHC class I expression (Figure 3C). MHC class I expression at 72 h (~ 90%) remained elevated (> 70%) for 3 days after the removal of the cytokine, after which time the expression dropped constantly to plateau at ~ 55% by day 10.

To ensure the elevated class I levels observed *in vitro* could be sustained *in vivo,* DA3-MUC1 cells were cultured with IFN-γ and injected subcutaneously into BALB/c mice. Tumours were examined on days 4 and 7 for MHC class I expression by immunoperoxidase staining (Figure 3D). DA3-MUC1 cells cultured with IFN-γ expressed high levels of MHC class I molecules on 75% of tumour cells removed from the subcutaneous site on day 4 (data not shown), with 50% of tumour cells still remaining positive on day 7 (Figure 3D i, ii). *In vivo* expression of MUC1 on the subcutaneous tumour was not altered after culturing with IFN-γ (Figure 3D iii). Thus, culturing DA3-MUC1 cells with IFN-γ increases the expression of MHC class I molecules on the cell surface for at least 7 days after removal of IFN-γ both *in vitro* and *in vivo*.

### T cells from MFP immunised mice lyse IFN-γ treated DA3-MUC1 tumour cell targets

From the data so far, it would appear that the DA3-MUC1 tumour is not immunogenic as it does not express MHC class I on its surface. However, culturing DA3-MUC1 cells with IFN-γ increases MHC class I expression, both *in vitro* and *in vivo* for at least 7 days following removal of IFN-γ. We determined whether the level of increase in MHC class I on DA3-MUC1 cells was adequate to be susceptible to MUC1 specific cytotoxic T cells (CTL) lysis.

BALB/c mice were therefore immunised with MFP and 7–10 days following the final immunisation, spleens were isolated and CTL were able to lyse DA3-MUC1 tumour cells (Figure 4A). Lysis of DA3-MUC1 tumour cells (treated with IFN-γ) was similar to that of P815-MUC1 tumour cells (H-2d$^+$ MUC1$^+$ MHC class I$^+$); non MUC1 transfected P815 cells (H-2d$^+$ MUC1$^-$ MHC class I$^+$) were used as a negative control. Interestingly, without culturing with IFN-γ, DA3-MUC1 were lysed by MUC1 CTL but the response was considerably weaker. Therefore, *in vitro* MUC1 T cell cytotoxicity to DA3-MUC1 tumours increases substantially with elevated MHC class I expression.

### MHC class I is not sustained to induce tumour protection despite induction of CTL

As IFN-γ treated tumours express elevated levels of MHC class I, and *in vitro*, MFP can stimulate MUC1$^+$ CTL capable of lysing DA3-MUC1 target cells (Figure 4A), the antitumour effects of MFP immunisation on DA3-MUC1 tumours expressing MHC class I was investigated *in vivo*. Mice were immunised 3 times with either MFP or a control pH 9.0 phosphate buffer and challenged with DA3-MUC1 tumour cells pre-cultured for 72 h with IFN-γ to increase MHC class I expression. BALB/c mice were challenged with 5 x 10$^6$ DA3-MUC1 cells with elevated MHC class I expression (Figure 4B). Interestingly, a small reduction in tumour growth, which correlated with an increase in MHC class I expression (Figures 2 and 3), was evident between days 2 and 5 in both MFP and control pH 9.0 immunised mice (Figure 4B). A significant ($p < 0.05$) decrease in tumour burden was evident in mice immunised with MFP compared to control mice, on day 3, suggesting that elevated levels (90–95%) of MHC class I expression on DA3-MUC1 tumour cells may increase their susceptibility to CTL lysis. However, no differences in tumour size were noted between MFP and control mice on any other days, and from day 6 onwards, DA3-MUC1 tumours continued to grow steadily which corresponded to a steady drop in surface MHC class I levels (Figure 3C) as the

positive tumour cells lost MHC class I expression. Thus, elevated class I expression could not be sustained *in vivo* to induce anti-tumour CTL responses.
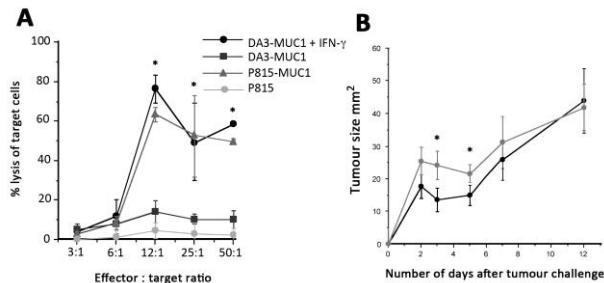


*Figure 4AB – CTL assay of spleen cells from effector BALB/c mice immunised with 5 μg MFP, on $^{51}Cr$-labelled DA3-MUC1 cells cultured with IFN-γ for 72 h, DA3-MUC1, P815-MUC1 and P815 tumour cell targets. B. Subcutaneous tumour growth of DA3-MUC1 cultured with IFN-γ in BALB/c mice. Mice (x 10 per group) were immunised intraperitoneally on days 0, 7 and 14 with either 5 μg MFP (- ●-) or pH 9.0 buffer (- ●-) and challenged with 3 x $10^6$ DA3-MUC1 tumour cells previously cultured with 20 ng/ml vaccinia virus-IFN-γ supernatant (UV inactivated) for 72 h. Data is presented as mean +/- standard error of the mean, * p < 0.05*

## Discussion

Tumour immunotherapy with mannan MUC1 fusion protein (MFP) induces CD8+ cellular immunity and tumour protection in several immunogenic MUC1+ tumour models (MUC1+ P815, MUC1+ RMA, MUC1+ 3T3) ([14–23, 26, 31–34]. In these models, the transfection of the tumour cell lines with human MUC1 results in the spontaneous rejection of the tumours after approximately 15–20 days [34]. Yet despite this, there is still a window of between 0 and 11 days in which to observe either accelerated rejection or an absence of tumour growth in immunised mice – the basic models with which the MFP anti-tumour immune responses have been described.

In this study, the aggressive MUC1+ metastatic DA3-MUC1 tumour, was investigated as a model to study MFP immunotherapy as it is not spontaneously rejected in mice. However, in contrast to other MUC1+ tumour models where MFP immunisation protected mice from a tumour challenge, DA3-MUC1 tumours grew. This resulted to determined the expression of various cell surface markers on DA3-

MUC1, required to induce cell mediated immunity. Both *in vitro* and *in vivo* studies confirmed that in contrast to other MUC1+ tumour models, DA3-MUC1 has a low expression of cell-surface MHC class I which results in reduced immunogenicity *in vivo*. However, treatment with IFN-γ *in vitro* upregulates MHC class I expression which can be sustained for several days in the absence of IFN-γ

Initial immunotherapy studies demonstrated that mice immunised with MFP and then challenged with DA3-MUC1 tumours were not protected from tumour growth. Similarly in a therapy experiment, 3 injections with MFP was also inadequate in decreasing tumour burden in mice with established DA3-MUC1 tumours. These findings were unlike other studies with MFP, whereby mice immunised with MFP were totally protected against a challenge of MUC1+ 3T3 tumours [20, 21] and the induction of a CD8+ cellular immune response caused the regression of established 15 day-old MUC1+ P815 tumours in DBA/2 mice [31].

*In vitro* and *in vivo* characterisation of DA3-MUC1 indicated that the tumour was weakly immunogenic because even though high surface levels of MUC1 were expressed (> 85%), there were low levels of all other cell surface molecules needed for T cell activation including MHC class I (< 6%), MHC class II, CD80, ICAM-2, CD28, LFA-2 and CTLA-4. Similarly, metastatic lung nodules induced by DA3-MUC1 again demonstrated MUC1 expression to be present on 50% of metastatic cells but there was no MHC class I expression. The absence, or relatively low expression of these molecules on the tumour cell surface causes anergy in any activated T cells and is an effective mechanism many tumours have evolved to evade the immune system [7].

However, tumour immunogenicity can be successfully increased by up-regulating the expression of these molecules (particularly MHC and costimulatory molecules) by either gene transfection or culturing with cytokines – specifically IFN-γ [5, 6, 9, 12]. Therefore, to increase the expression of MHC class I on DA3-MUC1 cells, cells were cultured with IFN-γ. Culturing DA3-MUC1 with IFN-γ increased expression of MHC class I from < 16% to > 90% after 72 h. The MHC class I expres-

sion remained elevated for several days before declining to 50% one week after the cytokine was removed from culture. *In vivo* studies of MHC class I expression on DA3-MUC1 after IFN-γ culturing, revealed a similar pattern whereby levels previously not detected in a subcutaneous tumour, were elevated to 50–75% of cells expressing MHC class I four days later, and still present on day 7. Interestingly, culturing DA3-MUC1 with IFN-γ did not increase cell surface expression of MHC class II, CD80, ICAM-2, CD28, LFA-2 or CTLA-4 as had been previously reported in other tumour models [35].

Following the up-regulation of MHC class I on the surface of DA3-MUC1 tumours, MUC1 specific CTL isolated from the spleen of MFP immunised mice could lyse DA3-MUC1 tumour cells cultured with IFN-γ, but not DA3-MUC1 cells which were not cultured with IFN-γ. This result was considerably higher than untreated tumour cells, demonstrating that DA3-MUC1 immunogenicity is increased in the presence of MHC class I, and can be lysed by MUC1 restricted CTL *in vitro*.

The lack of an effective anti-tumour response in DA3-MUC1 tumours is, in part, a result of the down-regulation in MHC class I expression which can be overcome by culturing the tumour with IFN-γ. As culturing with IFN-γ only temporarily increases MHC class I expression, it is suggested that future studies in this model would focus on the transfection of the IFN-γ gene into DA3-MUC1 cells. Alternatively, the decrease in DA3-MUC1 immunogenicity may also be a result of tumour-reactive T cells receiving inadequate costimulation through the absence of the costimulatory molecules B7-1 and B7-2. This again can be over come through transfection with these molecules [36, 37] and is also suggested for future immunotherapy studies with MFP. Furthermore, we have not investigated whether other relevant receptors may not be expressed by DA3-MUC1 cells. Finally, DA3-MUC1 cells could be used as a model to study other mechanisms and lysis where MHC class I is not required, such as, NK cell lysis.

## Acknowledgments

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Резиме

## ИМУНОТЕРАПИЈАТА MUC1 НАСПРЕМА МЕТАСТАТСКИОТ МОДЕЛ НА АДЕНОКАРЦИНОМ НА ГРАДИТЕ: ВАЖНОСТА НА IFN-ГАМА

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Имунотерапија која користи муцин 1 (MUC1) поврзан со оксидиран манан (MFP) беше испитувана кај агресивен метастатски тумор MUC1^{+}, DA3-MUC1, затоа што, за разлика од многу туморски модели на MUC1^{+}, DA3-MUC1 не е спонтано одбиен кај глувците, што го прави алтернативен модел за студии на имунотерапија. Исто така, клетките DA3-MUC1 се отпорни на лизирање од анти-MUC1 цитотоксични Т-клетки (CTLs). Неможноста туморите DA3-MUC1 да бидат отфрлени кај глувците како и вакцинацијата за MUC1 беше припишана на недостатокот на експресија на молекулите на MHC од класа I на површината на туморските клетки. Ин витро и ин виво анализата на поткожните тумори и метастази на белите дробови покажа дека туморските клетки DA3-MUC1 имаат ниска експресија ($< 6\%$) на MHC класа I, кои може да се регулираат ($> 90\%$) по култивирање со IFN-$\gamma$. Резултатите од анализата на проточната цитометрија и боењето со имунопероксидаза посочи дека ин витро нагорната регулација на MHC класа I може да се одржува до седум дена ин виво, без засегање на нивоата на експресија на MUC1 антигенот. Интересно, MUC1 специфичните CTL кои ин витро ги лизираат DA3-MUC1 целите беа воведени кај вакцинирани глувци од MFP, но не успеаја да ги заштитат глувците од туморскиот предизвик на DA3-MUC1. Овие резултати ја нагласуваат важноста на молекулите на MHC од класа I во поттикнувањето на антитуморскиот имунитет и имниот одговор на MFP.

**Клучни зборови:** MUC1, MHC класа I, интерферон-гама, тумор, имунотерапија