The Therapeutic Role of NK Cells in DC Immunotherapy

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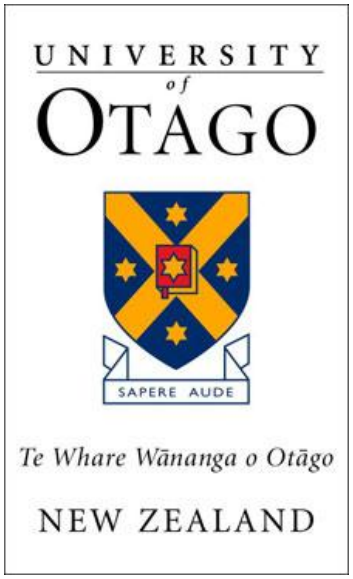

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I would like to dedicate this thesis to my Mum, Jenny Caldwell, who died in 1999 after fighting against cancer. I will never stop believing in your words "You can do whatever you want to do, and you can!"

Abstract

Dendritic cell (DC) immunotherapy is a promising treatment for cancer. Despite now being used in the clinic, the role and mechanisms of lymphocyte subsets stimulated by DC immunotherapy are not completely understood. Potential mechanisms for anti-tumour responses include cytokine (e.g. interferon-gamma; IFN-γ) or direct cell-killing mechanisms (e.g. perforin) performed by natural killer (NK) cells, NKT cells or $CD4^+$ and $CD8^+$ T cells. This current study used a B16/OVA-melanoma tumour model with bacteria-stimulated dendritic cells, coupled with antibody-mediated depletions (NK cells, $CD4^+$ and $CD8^+$ T cells and IFN-γ) and mice genetically deficient in IFN-γ and perforin to determine the mechanism of successful DC immunotherapy. In agreement with previous work, $CD4^+$ T cell, $CD8^+$ T cell and NK cell depletion resulted in a loss in efficacy of DC immunotherapy, although in this study, this effect failed to reach statistical significance for all lymphocytes. We found that DC immunotherapy induced an IFN-γ, not perforin, mediated anti-tumour response against B16/OVA melanoma tumour. Determining the role and mechanism of killing tumour cells in DC immunotherapy is of great importance and could lead to improvements in DC immunotherapy strategies. Showing particular promise is optimisation of IFN-γ release to enhance a potent anti-tumour response.

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List of Abbreviations

APC	Antigen presenting cell
ASGM1	Rabbit anti-asialo GM1
BMDC	Bone marrow-derived dendritic cells
BSA	Bovine serum albumin
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
HBSS	Hanks buffered salt solution
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
LN	Lymph node
MHC	Major histocompatibility complex
NK cells	Natural killer cells
NKT cells	Natural killer T cells
OVA	Ovalbumin
PBS	Phosphate buffered saline
TCR	T cell receptor
THB	Todd-Hewitt Broth
TLR	Toll-like receptor

1 INTRODUCTION

1.1 Melanoma

Melanoma is considered to be one of the most aggressive and treatment-resistant cancers [1]. It is caused by malignant transformation of melanocytes and is associated with reoccurring exposure to ultraviolet light or mutations which are either inherited or induced [2, 3]. Mutations within oncogenes, such as BRAF or NRAS members of the Ras gene family are associated with melanoma [3]. Mutations in the tumour suppressor gene, cyclin-dependent kinase inhibitor 2A (CDKN2A), have accounted for 35-40% of familial melanomas [2]. This gene encodes two proteins that inhibit cellular senescence, $p16^{INIK4a}$ and $p16^{ARF}$. New Zealand has the highest incidence of melanoma in the world, with more than 2,000 cases reported each year [4]. Melanoma is ranked as New Zealand's fourth most common cancer. If melanoma is detected early, surgical excision may be sufficient [3], however if the spread is extensive or the tumour has metastasised, then radiation and chemotherapy may also be required [1]. Melanoma has proved difficult to treat due to the ability of melanoma cells to easily metastasise and resist destruction by radiation and chemotherapy [3]. Traditional treatment strategies for cancer, such as surgery, radiation and chemotherapy, have shown poor efficacy for complete eradication of cancer [5-7], therefore the demand for effective new cancer therapeutics is high [8].

1.2 Immunotherapy

Immunotherapy is a promising new approach for the treatment of tumours. There are many different types of immunotherapy; however the general procedure involves stimulating patient immune cells *ex vivo* to enhance an anti-tumour response when administered back into the patient. Strategies are being investigated to improve anti-tumour responses to immunogenic tumours, such as melanoma, by enhancing tumour antigen presentation to naive or memory T cells and activating other effector cells, such as natural killer (NK) cells [9]. Ultimately, effective immunotherapy should protect patients from future tumour relapses by inducing a long-term memory response, while delaying or inhibiting tumour growth [10].

Currently there are three different types of immunotherapy; non-specific stimulation, active immunotherapy and adoptive transfer. Non-specific stimulation, such as interleukin-2 (IL-2) or interferon-α (IFN-α) treatment, aims to enhance the immune

system and as a result attack cancer, however this treatment alone has shown to be ineffective [11]. Active immunotherapy stimulates the host's own immune system to generate a response against a disease, such as cancer, with the use of vaccination. It has many advantageous features, such as low toxicity and potential for cellular memory, but the effectiveness of vaccinations has been varied [12]. The last type of immunotherapy, adoptive transfer, involves isolating a patient's immune cells which are capable of reacting to cancer antigens, growing and modifying these cells *ex vivo* before 'adoptively' transferring them back into the patient [13]. Adoptive transfer immunotherapy is an attractive treatment due to its specificity and effectiveness, however it is limited in use in that it is inherently patient-specific and very expensive [12].

Combinations of different types of immunotherapies, or a combination of immunotherapy and traditional treatments could prove to be an effective treatment strategy for cancer. For example, combinations of monoclonal antibodies and IL-2 provide antibody-dependent cellular cytotoxicity, which enhance an anti-tumour response [12]. Monoclonal antibodies directly activate targeted cells by interacting with specific surface molecules. Current immunotherapeutic treatments for melanoma include Tremelimumab and Ipilimumab, which are anti-cytotoxic T-lymphocyte antigen 4 (anti-CTLA-4) monoclonal antibodies, and tasisulam sodium, an antitumor agent that inhibits the mitotic progression characteristic of cancerous cells [14, 15]. In 2013 a clinical trial showed that Dabrafenib, an inhibitor of BRAF, was well tolerated, but not clinically active in patients with BRAF(V600E/K) mutation-positive metastatic melanoma [16].

1.3 Dendritic cells

Dendritic cells (DC) in the skin were initially identified as Langerhans cells in the late nineteenth century by Paul Langerhans [17]. However it was not until 1973 that the term dendritic cell was coined by Ralph M Steinman and Zanvil A Cohn [18]. They identified DC as a small subset of cells from mouse peripheral lymphoid organs which had distinct morphological features, including a large contorted, refractile nucleus and cytoplasmic processes [18]. Steinman continued researching and discovered that DC had a vital role in activating naive and memory T cells- work that awarded him one-half of the Nobel Prize in Physiology or Medicine three days after his death' [19].

Dendritic cells are specialised at taking up antigen, processing it and presenting it on major histocompatibility complex (MHC) molecules, hence are known as antigen presenting cells (APC). Dendritic cells present intracellular antigen on MHC class I (MHC-I) molecules and extracellular antigen on MHC-II and also on MHC-I through cross-presentation [20]. For naive T cells to become activated there is an absolute requirement of interaction with DC [21].

Bacteria contain pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide, that are conserved microbial structures. PAMPs are recognised by pathogen recognition receptors (PRRs), such as Toll-like receptors. The recognition of PAMPs by PRRs provides a danger signal that is required for DC to become activated and present antigen in order to mount an immune response. While there is probably no relevance for PAMPs in a normal anti-tumour response, there is potential for the use of PAMPs to stimulate DC *ex vivo*; however the ability of PAMPs to enhance anti-tumour responses is not completely understood [22].

Physiologically, DC are capable of inducing anti-tumour responses by stimulating antigen-specific T cells with three required signals; 1) recognition of antigen expressed on DC-derived MHC-I molecules to T cell receptors [23], 2) signal interaction between co-stimulatory ligands, and lastly 3) cytokine signalling for T cells to activate, proliferate and differentiate in to effector and memory T cells [10]. It is highly critical that T cells recognise the difference between healthy cells and tumour cells to ensure specificity and keep toxicity to healthy cells at a minimum [24].

1.3.1 DC immunotherapy

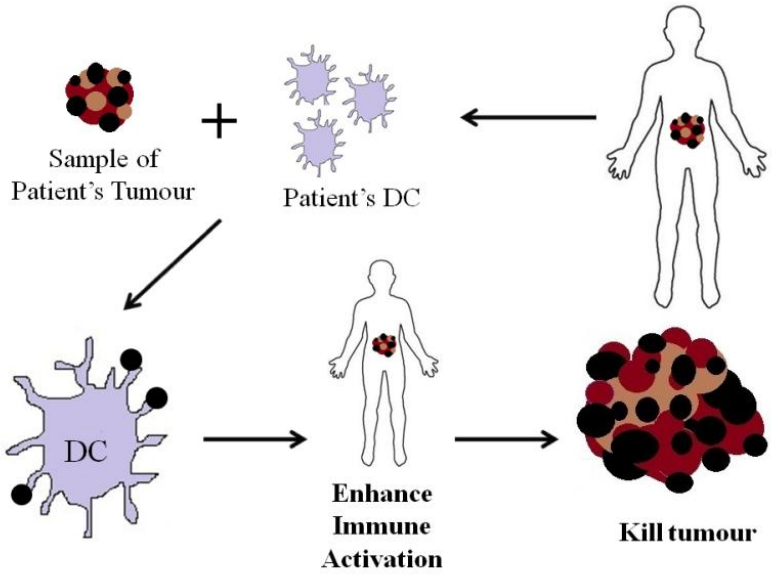


Figure 1: Dendritic cell immunotherapy in the clinic.

In DC immunotherapy clinical trials, DC and a tumour sample, as a source of antigen, are taken from the patient. The DC are optimally activated *ex vivo* so that they induce a potent anti-tumour response to eliminate cancer once administered back in to the patient.

A schematic diagram of DC immunotherapy is shown in Figure 1. The potential for DC to promote potent anti-tumour responses in patients holds great promise as it could lead to effective cancer treatment strategies. Dendritic cells are capable of inducing various anti-tumour responses; this is due to the ability of DC to activate different immune cells, including $CD4^+$ T cells, $CD8^+$ T cells and NK cells [25]. Dendritic cells are co-incubated with a sample of tumour extract, which the DC process and present on MHC-I/II molecules, and an adjuvant, such as lipopolysaccharide, thereby stimulating a tumour antigen-specific response once administered into the patient. Dendritic cell immunotherapy is now used in the clinic and has proven to be nontoxic, feasible and effective in some patients, especially with appropriate activation and maturation of DC [26, 27].

Edgar Engleman and Ronald Levy were the first to use DC as a therapeutic intervention to patients with non-Hodgkins lymphoma who presented with no clinical response to chemotherapy [28]. In this trial they isolated patient DC and 'loaded' the cells with

immunoglobulin ‘idiotype’ from the patient’s lymphoma to then re-administer back into patients. Of 23 patients who received DC immunotherapy after chemotherapy 65% successfully mounted T cell or humoral anti-tumour responses. In another group of patients with residual tumours, DC immunotherapy prevented tumour progression at a median of 43 months after chemotherapy. Since then, adoptively transferred DC have provided anti-tumour responses in multiple types of cancer models, such as melanoma [23, 25, 29] and B cell lymphoma [28]. As it is still unclear how DC immunotherapy provides anti-tumour immunity in only some patients, the killing mechanisms induced by DC immunotherapy need to be further elucidated. A better understanding of these mechanisms will allow the design of DC immunotherapy strategies to be improved in order to elicit an optimal anti-tumour response.

1.4 Natural killer cells

Natural killer (NK) cells were first discovered by Kiessling *et al* in 1975 as large granular lymphocytes that were distinct from T and B cells in mice [30]. Subsequently, NK cells were shown to be important effector cells in innate immunity by the ability to directly lyse cells without prior sensitisation [31]. Natural killer cells do this by surveying the body and destroying cells that are virally infected or have the potential to become cancerous in a process called ‘immunosurveillance’ [32]. Natural killer cells comprise approximately 10% of peripheral blood mononuclear cells [33]. Cytotoxic and immunoregulatory NK cell subsets have been identified in humans and 90% of NK cells found in the blood and spleen are of the cytotoxic subset [34], which implies that most circulating NK cells are capable of inducing cell death.

1.4.1 NK cell activation

Natural killer cells have inhibitory and activating receptors from two different families, the immunoglobulin superfamily (killer-cell immunoglobulin-like receptors (KIR) and natural cytotoxicity receptors (NCR)) and the C-type lectin superfamily [34]. In basic terms, the balance of signals that NK cells receive will determine whether or not NK cells become activated [35]. NK cells receive an activated signal when ligands, expressed only on stressed cells, are recognised and an inhibitory signal when MHC-I is recognised [34]. In virally infected cells or tumour cells, the expression of MHC-I is down-regulated, therefore the NK cells do not receive the inhibitory signal and become

activated by the activating signals. Natural killer cells also express Toll-like receptors which bind to foreign conserved microbial structures, causing NK cells to increase the production of cytokines and become cytotoxic. Generally, NK cells become activated by the presence of activating signals, Toll-like receptor signalling, cytokine signalling and lack of inhibitory signals, resulting in cytokine production, as well as perforin and granzyme release for cytotoxic killing [31, 34].

1.4.2 NK cells and cytokines

Once activated, NK cells require cellular interactions to further develop and maintain function, most commonly in the form of cytokines. Type one interferons and other cytokines, such as IL-2, IL-12, IL-15, IL-18, and IL-23, are capable of modulating NK cell activity [31]. In particular, the membrane-bound form of IL-15 has been shown to have an important role in NK activation, proliferation and survival [31]. Evidence has also shown that IL-15 deficient mice lack NK cells and IL-2 deficient mice do not, despite sharing the common γ-chain receptor, thereby indicating that certain cytokines are required for NK development and maintenance [33].

Natural killer cells release interferon gamma (IFN-γ), an important pro-inflammatory cytokine [36, 37]. Interferon gamma and tumour necrosis factor alpha (TNF-α) are important in undertaking NK functions, such as committing DC to maturation, stimulating anti-tumour responses and controlling T cell-dependent responses [38].

1.5 Role of NK cells in anti-tumour immunity

Cytotoxic $CD8^+$ T cells (CTL) were classically thought be the key immune cells to enhance in cancer therapeutics due to the ability of CTLs to directly kill tumour cells that expressed specific tumour antigen [25]. In spite of this, it was found that tumour cells, like virally infected cells, may down-regulate MHC-I molecules, thereby preventing the MHC-I/tumour antigen and T cell receptor (TCR) interaction and escaping CTL-mediated cytotoxic killing. It has been reported that cytotoxic $CD8^+$ T cell responses alone are not sufficient to destroy tumours after tumour challenge [24]. This suggested that other effector cells were required to complement CTL-mediated anti-tumour immunity or have a synergistic effect for optimal anti-tumour responses [39, 40]. As NK cells have the capacity to become activated by recognising low levels of MHC-I molecules [41] and binding to tumour ligands via activating receptors, the

potential to induce anti-tumour immunity in immunotherapeutic strategies is theoretically possible [42].

Kiessling *et al.* first described anti-tumour effects of NK cells *in vitro* in 1975 using mouse Moloney leukemia cells [30]. By 1980 these effects were also shown *in vivo* by Talmadge *et al* [43]. The potential for activated NK cells to enhance anti-tumour immunity is of great importance for the development of future cancer therapeutics, therefore the ability to enhance NK cell activity in different vaccination strategies and tumour models is under current investigation [22, 44-46].

1.5.1 NK cell based immunotherapy

Adoptive transfer of NK cells is also being investigated as an immunotherapy. A clinical trial by Parkhurst *et al.* found high levels of NK cells circulating in the blood several months after administration of autologous NK cells, but there was no anti-tumour response [47]. Using adoptively transferred NK cells as a treatment for cancer has not been ruled out, but needs to be further studied, especially in combination with other therapeutic strategies [34, 46].

1.6 Role of NK cells in DC Immunotherapy

Natural killer cells have been demonstrated to play an important role in the anti-tumour response induced by DC immunotherapy [23, 25, 36]. In multiple studies the depletion of NK cells has impaired anti-tumour immunity [25, 41-43, 48-50]. The cellular interactions of NK cells in anti-tumour immunity in DC immunotherapy is not completely understood, but various roles have been uncovered which include; bi-directional activation with DC [48], tumour lysis to release antigen [50], DC editing [51], interaction with Natural Killer T (NKT) cells [23] and co-operation with $CD4^+$ T cells and/or $CD8^+$ T cells [49, 50, 52].

1.6.1 Bidirectional activation

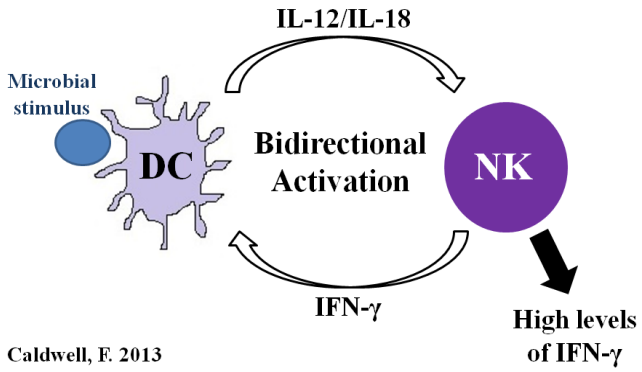


Figure 2: Bi-directional activation between dendritic cells and natural killer cells

Dendritic cells and NK cells have been demonstrated to interact and become activated through a positive feedback loop, known as bi-directional activation. In bi-directional activation the DC activate and sustain proliferation of NK cells and the NK cells promote DC maturation [38].

As shown in Figure 2, a microbial stimulus activates DC to produce IL-12 and IL-18 which stimulate NK cells to release IFN-γ and, in turn, induce higher levels of IL-12 and IL-18 from DC [21]. Bi-directional activation causes vast amounts of IFN-γ to be produced which activates Th1 responses and inhibits Th2 responses, overall promoting the activation of tumour-specific CTL responses [21, 25, 49, 51]. Other cytokines produced by DC that activate NK cells include IL-2, IL-15 and type I IFN [23].

Despite remarkable efforts showing that bi-directional activation stimulates NK cells involved in anti-tumour responses, it has been shown that tumour cells can actively interfere with this cross-signalling interaction [38]. Capobianco et al. found that the interaction between DC and NK/LAK cells elicited high levels of TNF-α and IFN-γ from NK/LAK cells, but MHC-II low B16 melanoma tumour cells interfered with this bi-directional interaction, thereby decreasing the production of the cross-signalling cytokines, IL-18 and IFN-γ cytokines. Tumours have also been shown to suppress the growth, maturation and activity of NK cells through manipulation of the cellular microenvironment [53]. This dramatically affects the potential of DC immunotherapy to provide anti-tumour responses through NK cell-mediated cytotoxicity. However, in

conjunction with other treatments to modulate the tumour microenvironment, NK cells in DC immunotherapy still hold promise to mounting strong anti-tumour responses.

1.6.2 Tumour lysis

NK cells may stimulate anti-tumour immunity by direct lysis of tumour cells to release tumour antigen for uptake of antigen presenting cells. Evidence from Liu *et al*. showed that after NK cell-mediated tumour lysis, there was enhanced presentation of tumour antigen on DC [50], which may imply that NK cells are capable of increasing the amount of available tumour antigen to be presented by DC to elicit a tumour-specific T cell response.

1.6.3 DC editing

Morandi *et al.* have provided evidence that activated NK cells kill immature DC that have low immunogenicity, selecting for highly immunogenic DC which are more capable of stimulating a strong tumour-specific CTL response. Studies using perforin knockout mice demonstrated that this NK cell-mediated DC killing is perforin-dependent [51]. This theory of NK cells editing DC was based upon results from draining and contralateral lymph nodes. Other areas of the body which harbour DC and NK cells, such as blood, spleen and liver should also be investigated to ensure this role of NK cells is consistent.

1.6.4 Interactions with Natural Killer T cells

Shimizu and Fujii provided evidence that NK and NKT cells provide anti-tumour protection for up to twelve months and that their activation required vaccinated DC, host DC and $CD4^+$ T cells [23]. Their hypothesis was that DC activates $CD4^+$ T cells, which in turn trigger DC to produce T helper 1 cytokines that induce NK cell activation, such as IL-12. They believed that $CD8^+$ T cells were not involved in the NK and NKT cell anti-tumour response, which contrasts other findings that state $CD8^+$ T cells are involved in anti-tumour responses [23, 41]. $V_\alpha 14$ NKT cells have shown to become activated by alpha galactosylceramide (α-GalCer) and produce vast amounts of IL-4 and IFN-γ, which not only exerts an anti-tumour response but also activates NK cells to produce IFN-γ [54-56].

1.6.5 Interaction with T cells

Evidence from various sources agree that both NK cells and $CD8^+$ T cells are both involved in producing high levels of IFN-γ and tumour-specific CTL-mediated immunity [41, 49, 52]. Another example showing the importance of both CTL and NK cells can be explained by Zhou *et al.* This group activated CTL and NK cells, via the NKG2D receptor, in a DNA vaccine and found the anti-tumour response was greatly enhanced in both therapeutic and prophylactic settings [57]. Kim *et al.* suggested that the initial innate responses from NK cells may facilitate the development of CTL responses, because NK depletion *in vivo* significantly reduced the activity of tumour-specific CTL immunity [49]. It has been suggested that NK cells do not provide anti-tumour protection alone, but instead co-operate with T cells after administration of DC immunotherapy to produce a tumour antigen-specific cytotoxic response with various cytokines, thus providing anti-tumour immunity [48]. The McLellan laboratory showed that DC immunotherapy was antigen-dependent and suggested that NK cells co-operate with T cells to elicit an anti-tumour response [36].

1.7 Mechanism of NK cell-mediated tumour killing in DC immunotherapy

As discussed, NK cells have been demonstrated to have an important role in anti-tumour immunity, but the mechanism by which the NK cells kill tumour cells in DC immunotherapy is poorly understood. Natural killer cells are capable of killing tumour cells via three different mechanisms. These are perforin/granzyme-mediated cytotoxic killing, death receptor-mediated apoptosis and indirect killing via IFN-γ.

1.7.1 Perforin/granzyme-mediated cytotoxic killing

Like other cytotoxic cells, NK cells can kill tumour cells in a perforin/granzyme B-mediated mechanism. Natural killer cells form cell-to-cell synapses with targeted tumour cells before releasing perforin and granzyme. Perforin is a membrane-disrupting protein which allows entry of granzymes into the targeted tumour cell. Granzymes, such as Granzyme B, are serine proteases which have various substrate specificities that induce apoptotic cell death of the targeted cell [58]. Perforin/granzyme–mediated killing is rapid and potent due to the cytotoxic nature of granzyme B.

1.7.2 Death receptor mediated apoptosis

NK cells can kill tumour cells via death receptor-mediated mechanisms. Death receptors, such as Fas (CD95), are expressed on targeted tumour cells and are recognised by tumour necrosis factor (TNF) family ligands expressed on NK cells [58]. These include Fas ligand (CD178), TNF, and tumour-necrosis factor-related apoptosis inducing ligand (TRAIL) and induce caspase-dependent apoptosis when engaged with Fas on the targeted tumour cell [59].

1.7.3 Interferon-γ anti-tumour mechanisms

Activated NK cells secrete various cytokines, such as tumour necrosis factor (TNF), granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin-10 (IL-10), IL-13 and most importantly IFN-γ. As IFN-γ is a pro-inflammatory cytokine it can signal to other effector cells to upregulate the expression of IFN-γ or other pro-inflammatory cytokines. IFN-γ can also suppress anti-inflammatory cytokines produced by tumour cells. IFN-γ upregulates the expression of MHC-I, which increases the level of antigen presented, thus enhancing the ability of antigen presenting cells to present tumour antigen and mount a potent anti-tumour response [60].

Although IFN-γ has been demonstrated to inhibit tumour growth in various models [54, 59, 61, 62], the power of IFN-γ derived from NK cells in a therapeutic setting, such as DC immunotherapy, remains poorly understood.

1.8 Therapeutic role and mechanism of NK cells in DC immunotherapy against B16/OVA-F10 melanoma.

Initially, NK cells were thought to be important immune cells at the time of priming (time of DC immunisation) [25, 49]. Using a prophylactic model the McLellan laboratory has demonstrated that murine NK cells have a role at the time of priming, but exert a more profound effect at the time of challenge [37]. However; these findings assume that NK cells, depleted at the time of priming, had regenerated back to baseline control levels by the time of challenge, which was within a sixty day time frame.

This study in mice used a B16/ovalbumin (OVA) melanoma and DC immunotherapy model. B16/OVA melanoma cells are engineered to express OVA, making it an ideal model for DC immunotherapy as OVA can be targeted as a model tumour associated

antigen. Ovalbumin is loaded on to MHC molecules and,- along with adjuvants, co-stimulatory molecules and cytokines, generates an anti-OVA tumour response [36]. Heat killed (HK) *Streptococcus salivarius* (Ssa) K12, used as an adjuvant, has shown to effectively stimulate an anti-tumour response in human and murine systems [63] and is also a potent stimulator of inducing human peripheral blood mononuclear cells to release IL-12 [64]. Bouwer *et al.* showed that HK Ssa K12 activated DC to produce IL-12 which stimulated NK cells to produce large amounts of IFN-γ, but did not encourage the NK cells to become cytotoxic [37].

1.8.1 Aims and Hypothesis

The role of individual cell subsets in the anti-tumour response stimulated by DC immunotherapy is varied and not well understood. In order to elucidate the role and mechanisms of NK cells in DC immunotherapy this study will investigate two main aims:

1) Determine the therapeutic role of NK cells in DC immunotherapy
2) Determine if DC immunotherapy induces an IFN-γ, or perforin, mediated anti-tumour response

To determine the therapeutic role of NK cells, relative to $CD4^+$ and $CD8^+$ T cells, in DC immunotherapy, an antibody-mediated depletion strategy, which depletes mice of either NK cells, $CD4^+$ or $CD8^+$ T cells, was used to determine the role of these lymphocyte subsets in DC immunotherapy.

To determine the second aim an antibody-mediated IFN-γ depletion strategy and IFN-$\gamma^{-/-}$ and Perforin$^{-/-}$ (Pfp$^{-/-}$) mice will be used to determine whether an IFN-γ, or perforin, mediated anti-tumour response is induced by DC immunotherapy. Interferon gamma is produced by multiple cells and perforin is produced in CTL killing, therefore this study was not completely subjective to NK cells, but still demonstrates the importance of all mechanisms associated with IFN-γ and perforin in DC immunotherapy.

This study hypothesises that NK cells play an important role in DC immunotherapy and kill tumour indirectly by producing IFN-γ.

1.9 Summary

Determining the role and mechanism of NK cells in DC immunotherapy is of great importance. If NK cells prove to have an important role in DC immunotherapy, future DC immunotherapy strategies could be designed to favour a specific NK cell mechanism, such as recombinant IL-12 or IL-18 to enhance production of IFN-γ by NK cells, thus stimulating an optimal anti-tumour response.

2 MATERIALS AND METHODS

2.1 Mice

C57BL/6 mice were obtained from Jackson Laboratories and bred in specific pathogen free conditions at the Hercus Taieri Resource Unit (HTRU), before being housed at the Microbiology and Immunology Animal Facility (University of Otago). C57BL/6 and Ptp mice were also obtained from Animal Resources Centre (Canning Vale 6970, Western Australia) and housed at the Microbiology and Immunology Animal Facility (University of Otago). Perforin knockout ($Pfp^{-/-}$) and interferon gamma knockout (IFN-$\gamma^{-/-}$) mice were obtained from Malaghan Institute (Victoria, University of Wellington, New Zealand) and bred and housed at the HTRU. Mice were euthanised by CO_2 asphyxiation. The University of Otago Animal Ethics committee approved these animal studies (AEC #74/12).

2.2 *In vitro* techniques

2.2.1 Antibody quantification

To determine the concentration of antibody stocks to be used in the depletion experiment, an enzyme-linked immunosorbent assay (ELISA) was performed. A 96 well MaxiSorp® (NUNC) plate was coated with goat anti-rat IgG capture antibody at 1 mg/ml in Gibco phosphate buffered saline (PBS) and left overnight at 4°C. The plate was washed three times with wash buffer (Media and buffers) and blocked for five minutes with 200 µl of 1% fetal calf serum (FCS)/PBS per well. Antibodies to be quantified were diluted in 1% FCS/PBS and tripling dilutions were performed from 1 µg/ml to 0.00045 µg/ml in a total volume of 100 µl per well. These antibodies included 53-5.8, anti-IFN-γ XMG.D6, rat immunoglobulin G_{2a} (IgG_{2a}) isotype control and anti-CD4 YTS191.12. The plate was incubated overnight at 4°C before being washed three times with wash buffer. Rabbit anti-rat IgG-horse radish peroxidase (HRP) was diluted to 1/400 in 1% FCS/PBS and added at 100 µl per well. The plate was incubated for one hour at 37°C + 5% CO_2. After three washes with wash buffer, 100 µl of 3,3',5,5'-Tetramethylbenzidine (TMB) was added per well and the plate incubated in the dark. Once a blue colour change (indicative of a complete reaction) was observed, 50 µl of 2N H_2SO_4 was added to stop the reaction and the plate was read at 450 nm with a Bio-RAD model 550 plate reader.

2.2.2 Interferon-gamma enzyme-linked immunosorbent assay (IFN-γ ELISA)

A 96 well MaxiSorp® plate was coated with 100 µl/well of purified anti-mouse IFN-γ (clone R4-6A2; BD #551216) at a final concentration of 2 µg/ml in PBS and incubated overnight at 4°C. The plate was washed three times with wash buffer and blocked for ten minutes at room temperature with 200 µl/well of 1% BSA/PBS. The standard was 60 ng/ml and diluted in doubling dilutions. IFN-γ was added at 60 ng/ml at 200 µl to first two wells and doubling dilutions were performed from 60 ng/ml to 0.06 ng/ml. Samples were added at 100 µl to each well and if necessary diluted in 1% BSA/PBS and the plate incubated overnight at 4°C. After three washes with wash buffer, 100 µl of 1 µg/ml biotinylated anti-IFN-γ monoclonal antibody (rat anti-mouse IFN-γ (XMG-1.2; BD #554410)) in 1% BSA/PBS was added to each well. Plates were incubated at

room temperature for 45 minutes, then washed again three times with wash buffer. Streptavidin horseradish peroxidase complex (Dako 0.77 g/L) was diluted in 1% BSA/PBS (1/5000) and 100 µl added to each well and incubated at 37°C for 60 minutes. After the plate was washed three times with wash buffer, 100 µl of TMB was added to each well and kept in the dark. Once a blue colour change was observed, 50 µl of 2N H_2SO_4 was added to stop the reaction and the plate was read at 450 nm with a Bio-RAD model 550 plate reader.

2.2.3 Preparation of heat killed *Streptococcus salivarius* K12 (Ssa K12)

Streptococcus salivarius K12 was obtained from BLIS Technologies Ltd (Dunedin). Ssa K12 was streak plated on sheep blood agar and incubated at 37°C for 16 hours to obtain single colonies. A single colony was then plated on selective Mitis salavarius agar for confirmation of bacterial strain. From the blood agar plate, 1/3, 1/9 and 1/27 dilutions in Todd Hewitt broth (THB) were made and optical density measured at 600 nm using blank THB to zero (volume in dilutions measuring one ml). To calculate the total number of Ssa K12 in one ml of THB using the most appropriate dilution (1/9) the following equation was used: number of bacteria per ml = $(e((\ln(OD/3\times10^{-8})0.8416)) \times$ dilution factor. SSa K12 was then heat killed at 56°C for 45 minutes before centrifuging for five minutes at 320 × *g* and supernatant was discarded. Cells were washed in PBS then resuspended at 5×10^9 bacteria/ml in PBS and frozen at -20°C.

2.2.4 Culture of murine B16/OVA melanoma cell line

Frozen B16/OVA melanoma cells were taken from culture collection in liquid nitrogen. In a class II biological safety cabinet, cells were thawed with 14 ml of RPMI Medium 1640 + 10% fetal calf serum (R10) warmed to 37°C, then centrifuged at 20°C, at 453 × *g* for five minutes and supernatant removed. Cells were resuspended in R10 and the cell line maintained at 1-2 × 10^5 cells/ml in 175 cm^2 vented cap flasks (NUNC). To maintain the expression of OVA, Geneticin G418 was added at a final concentration of 50 µg/ml on the first day of culture and two days prior to tumour challenge.

2.2.5 Generation of XMG.D6 antibodies

XMG.D6 antibodies were produced by growing an XMG.D6 hybridoma cell line (Malaghan Institute) in RPMI Medium 1640 in a CELLine^{TM} bioreactor (INTEGRA Biosciences). The cells were removed by centrifugation at 453 × *g* for five minutes and the supernatant clarified by centrifugation at 3220 × *g* for ten minutes then filtered through a 0.2 µm filter. Ammonium sulphate, $[NH_4]_2[SO_4]$, was added to give 45% saturation (27.7 g/100 ml). The solution was stirred for 15 minutes then left at 4°C overnight before centrifugation at 10,000 × *g* for 30 minutes. The supernatant was discarded and the antibody pellet was resuspended in 40 ml of PBS then transferred to a new microfuge tube. Precipitation was repeated by adding ammonium sulphate and centrifuging at 10,000 ×g for two hours at 4°C. Following precipitation the supernatant was removed by aspiration and the antibody pellet resuspended in less than 1 ml PBS then dialysed extensively against PBS. The buffer was changed twice daily for at least two days. The antibodies were then filtered using a 0.22 µm filter and stored at 4°C.

2.3 *Ex vivo* techniques

2.3.1 Surgical excision of spleen

Spleens were removed aseptically and washed with 0.1 % BSA/PBS/2 mM Ethylenediaminetetraacetic acid (EDTA). Splenocytes were pushed through a 70 µm sieve with a plunger and 0.1% BSA/PBS/2 mM EDTA and subseqeuently resuspended in 0.1 % BSA/PBS/ 2 mM EDTA.

2.3.2 IFN-γ production by heat killed Ssa K12-stimulated Splenocytes

Splenocytes were counted, centrifuged at 20°C at 453 × *g* for five minutes and the supernatant removed. Splenocytes were then resuspended at 4×10^6 cells/ml in R10 and 1 ml aliquots were added to a 24 well plate. Splenocytes were stimulated with heat killed Ssa K12 either at a 10:1 or 1:1 bacteria:splenocyte ratio (n = 2). No heat killed Ssa K12 was added to unstimulated control splenocytes (n = 2). The plate was incubated at 37°C with 5% CO_2. After 20 hours of incubation, 0.8 ml of cell-free

supernatant was removed and frozen at -80°C until analysed by IFN-γ ELISA as previously described.

2.3.3 Generation of bone marrow derived dendritic cells and culture

Tibias and femurs were removed aseptically from the mice and sterilised with 70% ethanol. Bone marrow was flushed with R10 using a 10 ml syringe with a 27 gauge needle. The cell suspension was filtered through a 70 µm sieve and centrifuged for five minutes at 20°C at 453 × *g*. Cells were then counted using a Neubauer haemocytometer slide (1:1 ratio cell suspension to trypan blue dye) and plated at 5×10^6 in 10 ml of R10 + 200 U/ml Granulocyte macrophage colony stimulating factor (GM-CSF) in Petri dishes. On day three an additional 10 ml of R10 + 5% GM-CSF was added, which was then replaced on day six. In the IFN-γ release experiments (see section 2.3.6), DC were stimulated with heat killed Ssa K12 for four hours prior to DC harvest and injection. In tumour experiments, OVA was added to a concentration of 200 µg/ml on day seven. Heat killed Ssa K12 was added to DC at a 1:1 ratio for either 16 hours (overnight) or four hours before DC harvest and immunisation.

2.4 *In vivo* techniques

2.4.1 Tumour challenge

On the day of tumour challenge, adherent B16/OVA melanoma cells were scraped to resuspend and centrifuged at 20°C at 453 × *g* for five minutes. The cells were washed with PBS and centrifuged before being resuspended in PBS to 1×10^6 cells/ml. Mice were subcutaneously injected with 100 µl of 1×10^5 B16/OVA cells in the left hip/flank area.

2.4.2 Dendritic cell harvest and immunisation

DC were washed twice with 50 ml PBS and centrifuged as before for 5 minutes at 453 × *g*. DC were then counted on the last wash and resuspended in PBS at 2×10^6 cell/ml.

One Petri dish was harvested prior to Ssa K12 stimulation to determine the amount of Ssa K12 to be added for a 1:1 DC to Ssa K12 ratio. Mice were subcutaneously injected with 100 µl of 2×10^5 DC in the right hip/flank area (opposite flank to that of tumour) with an insulin syringe (BD Ref # 32882).

2.4.3 Antibody-mediated depletions

Mice were intravenously injected in the lateral tail vein with the appropriate antibody to deplete either a subset of lymphocyte or IFN-γ. To deplete NK cells, mice were injected with 20 µl (± 0.54 mg) of Wako anti-asialoGM1 plus 80 µl sterile PBS (anti-asialo GM1 treatment). In tumour challenge experiments, mice were injected with anti-asialo GM1 treatment on day -1 and +2, relative to tumour challenge (d0). Mice depleted of either CD4+ or CD8+ T cells were injected on day -3, -1 and +2 with either YTS191.12 (anti-CD4) or 53-5.8 (anti-CD8) at 100 µg in 100 µl PBS. Mice depleted of IFN-γ were injected with XMG.D6 on day -1, +2 and +4 with 100 µg in 100 µl PBS. Control mice were injected with $RatIgG_{2a}$ for mock depletion on day -3, -1 and +2 with 100 µg in 100 µl.

2.4.4 Monitoring tumour growth

Seven days after tumour injection tumours were measured daily with vernier callipers until at least day 40. The tumour size was calculated as the product of the width and length measurements; 2 mm was subtracted from these values due to the thickness of skin accounting for 1mm either side. Mice were euthanised once the tumour size reached the ethical boundary of 150 mm^2.

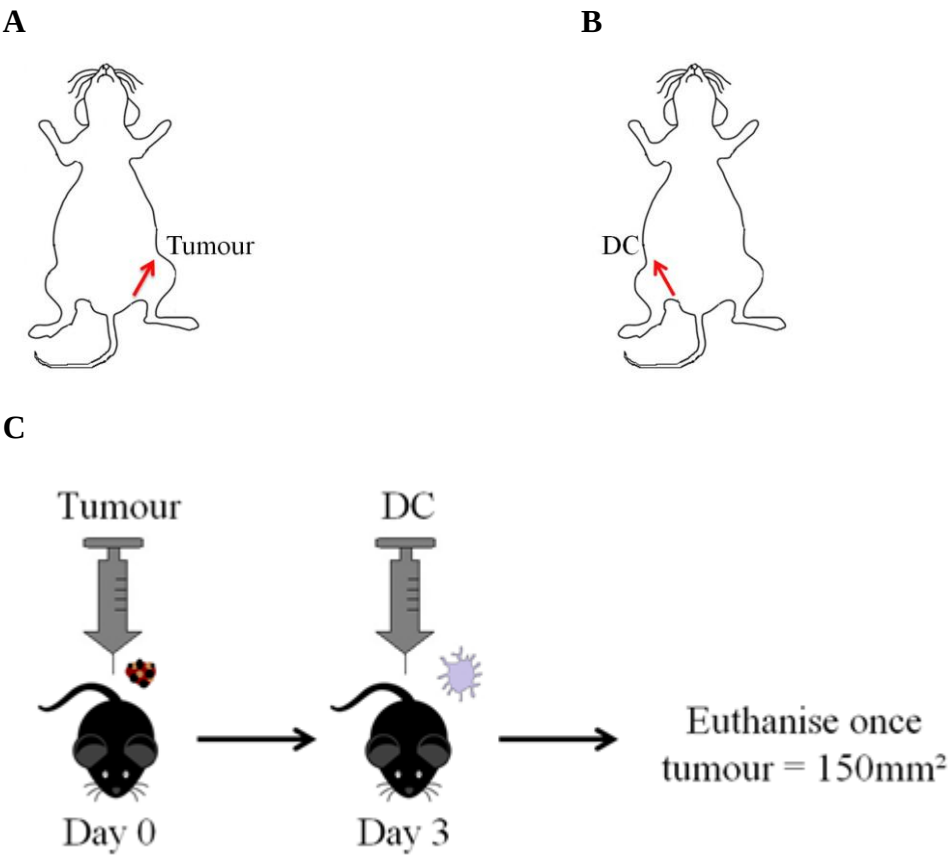


Figure 3: Schematic diagram of tumour experiment protocol

Site of subcutaneous injections for B16/OVA tumour immunisation (A) and DC immunisation (B). Strategy for administration of tumour, DC and antibody-mediated lymphocyte/IFN-γ depletions (C)

2.4.5 Tail bleeding

Approximately 100 µl of blood (3-5 drops) was taken by making a small incision at the tip of the tail. Blood was left overnight to allow the serum to separate from the blood for serum IFN-γ detection experiments. For NK cell processing and staining, blood was transferred into a 1.5 ml microfuge tube with 400 µl of Alsevier's solution and left overnight at 4°C.

2.4.6 IFN-γ release into serum after DC immunotherapy

Dendritic cells were isolated and cultured for seven days as described previously (see section Generation of bone marrow derived DC and culture). Dendritic cells were then washed twice in PBS, counted on the last wash, and resuspended at 2×10^7 cells/ml in PBS. At t = 0 hr C57BL/6 mice were intravenously injected with 100 µl of either 2×10^5 DC (10 µl of 2×10^7 cells/ml DC suspension + 90 µl PBS), 2×10^6 DC or PBS (n=2). At t = 6hr mice were tail bled and the blood transferred into a 1.5 ml microfuge tube. Blood samples were left overnight at room temperature to separate the serum from the blood clot. At t = 24hr mice were euthanised and bled via cardiac puncture. Blood was also taken at 48 hrs via puncture from a replicate group. Blood samples were then centrifuged at 320 × *g* for five minutes and the serum removed for further analysis. The serum was analysed by IFN-γ ELISA as described above.

2.4.7 Blood NK cell processing

Blood was centrifuged for five minutes at 500 × *g*. The supernatant was discarded to remove the Alsevier's solution and 400 µl of ammonium-Chloride-Potassium Lysing Buffer (ACK) was added for three minutes to lyse the red blood cells. Subsequently, 1 ml of 0.1% BSA/PBS/2 mM EDTA was added and cells centrifuged as previous and supernatant discarded. Cells were kept on ice and resuspended in 1 ml of 0.1% BSA/PBS/2 mM EDTA, transferred to 1.2 ml micro titertube, centrifuged for five minutes at 452 ×g at 4°C and supernatant removed.

2.4.8 Splenic NK cell processing

Splenocytes were centrifuged for five minutes at 453 × *g* at 20°C and supernatant discarded. Red blood cells were lysed using 10 ml of ACK for three minutes then 0.1% BSA/PBS/2 mM EDTA was added to 50 ml and centrifuged as previous. Splenocytes were resuspended in 1 ml of 0.1% BSA/PBS/2 mM EDTA and 80 µl transferred to 1.2 ml micro titertube then 1 ml of 0.1% BSA/PBS/2 mM EDTA added. Cells were centrifuged for five minutes at 453 × *g* at 4°C and supernatant discarded.

2.4.9 NK cell staining

NK cells were defined as $CD49b^{+}TCR\beta^{-}$. A stock of 2 µg/ml DX5-R-phycoerythrin (DX5-PE) and 0.25 µg/ml TCRβ-Brilliant Violet 421 (TCRβ-BV421) or 2 µg/ml TCRβ-fluorescein isothiocyanate (TCRβ-FITC) in 0.1% BSA/PBS/2 mM EDTA was made and 50 µl added to cells. The cells were kept on ice for 30 minutes. The tubes were then topped up with 1 ml of 0.1% BSA/PBS/2 mM EDTA and centrifuged for five minutes at 453 × *g* at 4°C and supernatant discarded. The cells were then resuspended in 100 µl of fixing buffer (1% PFA/1% BSA/PBS/0.02% azide) and kept at 4°C until analysed by flow cytometry. Unstained, DX5-PE, TCRβ-BV421 or TCRβ-FITC cell controls were made for compensation.

2.4.10 Flow cytometry and FlowJo

Flow cytometry was performed using a BD Fortessa. Unstained and DX5-PE, TCRβ-BV421 or TCRβ-FITC cell controls were used to set the voltages of antibody fluorescence channels and for compensation. The total events recorded was 100,000. NK cell percentages were analysed by FlowJo (version 9.6.4) according to the gating strategy (Results; Figure 4).

2.5 Statistical analysis

GraphPad Prism 6 was used to analyse all data. Data from tumour experiments are represented as the mean tumour size until the first mouse reached a tumour size of 150 mm^2. Kaplan-Meier survival plots were used and analysed by Mantel-Cox test. NK cell depletion and regeneration experiments were presented as flow cytometry plots, individual and mean + standard deviation of NK cells for each group analysed. For statistical analysis a one-way ANOVA was used with a Tukey's post-correction test.

2.6 Antibodies

Table 1: Monoclonal and polyclonal antibodies used in this study

mAb/Ab	Clone	Species/Isotype	Source	Cat #.
Flow cytometry				
CD49b/Pan NK-PE	DX5	Rat IgM	BD Pharmingen	553858
TCRβ-FITC	H57-597	Armenian Hamster $IgG_{2,\lambda1}$	BD Pharmingen	553170
TCRβ-BV421	H57-597	Hamster $IgG_{2,11}$	Biolegend	109229
Depletions				
Anti-CD4 for depletion of CD4 T cells	YTS191.12	Rat Ig	Dr. Ulf Dittner	In house
Anti-CD8 for depletion of CD8 T cells	53.5-8	Rat Ig	Prof. Geoff Hill	In house
Anti-IFN-γ	XMG.D6	XMG.D6 Hybridoma	Prof. Franca Ronchese	In house
Anti-asialo GM1 for depletion of NK cells	Polyclonal	Rabbit	Wako Technologies	986-10001
Rat $IgG_{2a,\kappa}$	R35-95	Rat $IgG_{2a,\kappa}$	BD Pharmingen	553926
ELISA				
Anti-mouse IFN-γ	R4-6A2	Goat IgG	BD Pharmingen	551216
Biotinylated anti-IFN-γ	XMG-1.2	553926	BD Pharmingen	554410
Anti-IgG	Polyclonal	Goat anti-rat IgG	Southern Biotechnology Associates INC	3010-01

2.7 Media and buffers

Wash buffer

For 2 L:

200 ml 10 x PBS

1 ml Tween 20

Made up to a total of 2 L with deionised water

Ammonium-Chloride-Potassium Lysing Buffer

For 1 L:

0.79 g NH_4HCO_3

7.72 g NH_4CL

0.74 g KCL

0.037 g EDTA

Made up to a total of 1 L with milliQ H_2O

pH 7.2

Filter sterilised (0.22µm)

Dulbecco's Phosphate Buffered Saline (PBS)

For 1 L:

1 sachet GIBCO PBS (Gibco cat. #21600-010)

Made up to a total of 1 L with milliQ H_2O

pH 7.3

Filter sterilised

RPMI Medium 1640

For 1 L:

1 sachet RPMI Medium 1640 (Gibco cat. # 31800-022)

2 g $NaHCO_3$

100 U/ml penicillin

100 µg/ml streptomycin (Gibco cat. # 15140-122)

55 mM β-mercaptoethanol (Gibco cat. # 21985-023)

Made up to a total of 1 L with milliQ H_2O

pH 7.3

Filter sterilised

R5 / 10

95% / 90% RPMI Medium 1640

5% / 10% Foetal calf serum

Fixing buffer

For 100 ml:

1 g PFA (Paraformaldehyde)

90 ml PBS

0.1 10% BSA/PBS

0.02% Azide

3 RESULTS

3.1 NK cell depletion and regeneration

To demonstrate the importance of NK cells in tumour experiments, NK cells were depleted using antibody-mediated depletion. This allowed any differences in tumour growth and survival of mice with or without NK cells to then be analysed. A NK cell detection experiment was first conducted to ensure the NK cells were depleted by anti-asialo GM1 and that the levels of NK cells remained low over a substantial period of time (Figure 4 and 5).

Initially, levels of NK cells in the blood were analysed after anti-asialo GM1 treatment. These results were highly variable and the levels of NK cells in control PBS-treated mice were inconsistent (data not shown) over different days. The standard approach of analysing NK cells in the spleen was subsequently undertaken [37].

Mice were depleted of NK cells on day zero and the levels of splenic NK cells analysed at two, seven, fifteen, thirty and sixty days after anti-asialo GM1 (n = 4; Figure 5). Splenic NK cells were analysed by the gating strategy in Figure 4. The levels of NK cells in the spleen of control mice were 4.61% (± 0.39 SD, range 4.13-5.04%). Compared to control, the levels of NK cells significantly reduced to 18.7% (0.88% ± 0.07 SD, range 0.78-0.96%) two days after depletion and 57.4% (2.64% ± 0.40 SD, range 2.20-3.15%) sixty days after anti-asialo GM1 (**** $P < 0.0001$, one-way ANOVA, Tukey's multiple comparisons test). Despite NK cell levels beginning to rise two days after depletion, NK cell levels remained well below control levels. Even after sixty days the NK cells had not regenerated back to baseline NK cell levels seen in control mice. Individual data of NK cell levels in control and NK cell depleted mice are shown in the Appendix.

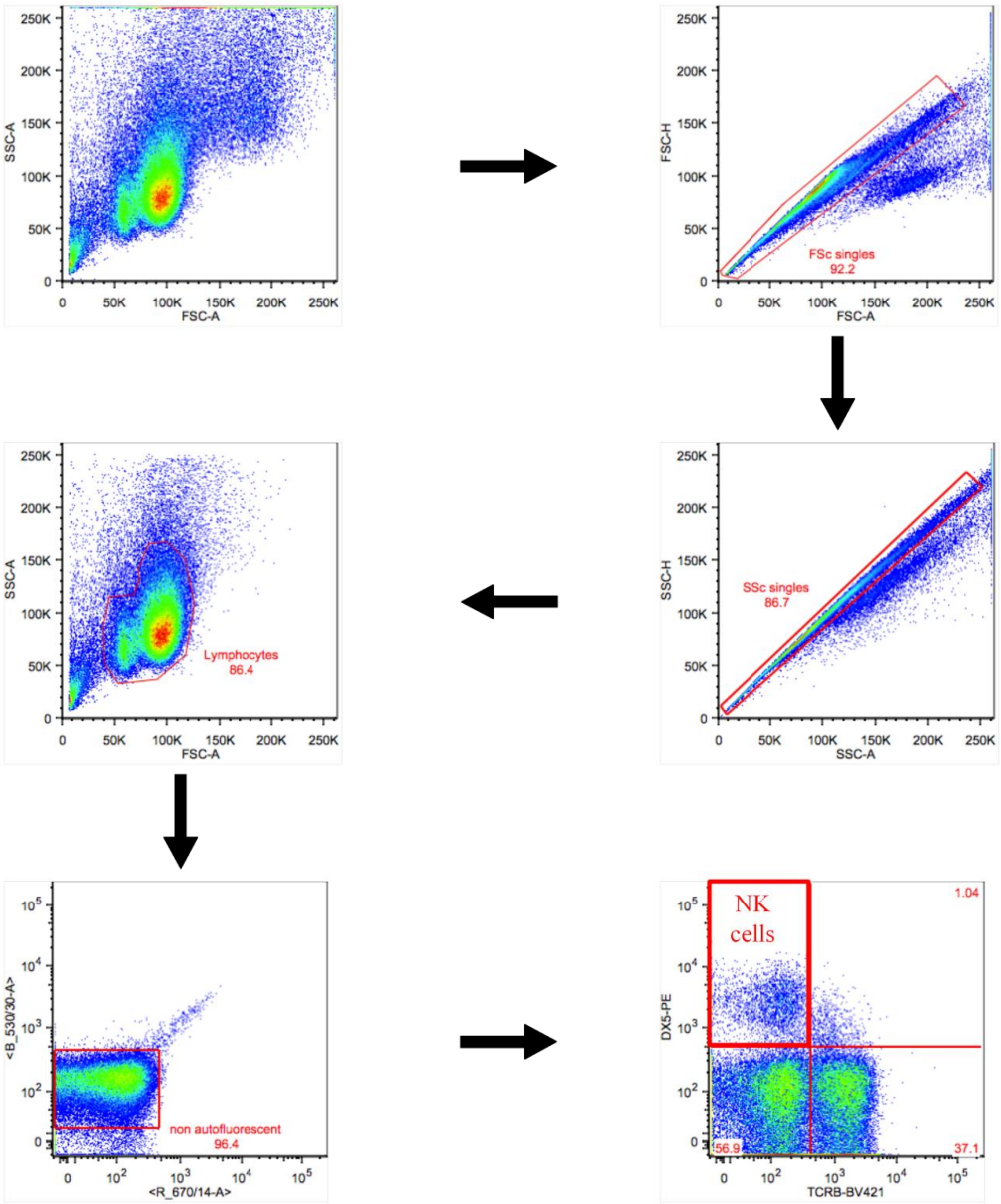


Figure 4: NK cell gating strategy.

Unstained and single stained DX5-PE and TCRβ-BV421 splenocyte controls were used to set the voltages of antibody fluorescent channels and compensate for antibodies fluorescing into other channels. Forward and side-scatter doublets were removed and the cells gated on the lymphocyte population. Auto-fluorescent cells were gated out by using two empty channels; channels in which no fluorochromes should be excited. The percentage of $CD49b^{+}TCR\beta^{-}$ splenic NK cells were determined by using DX5-PE and TCRβ-BV421 antibodies.

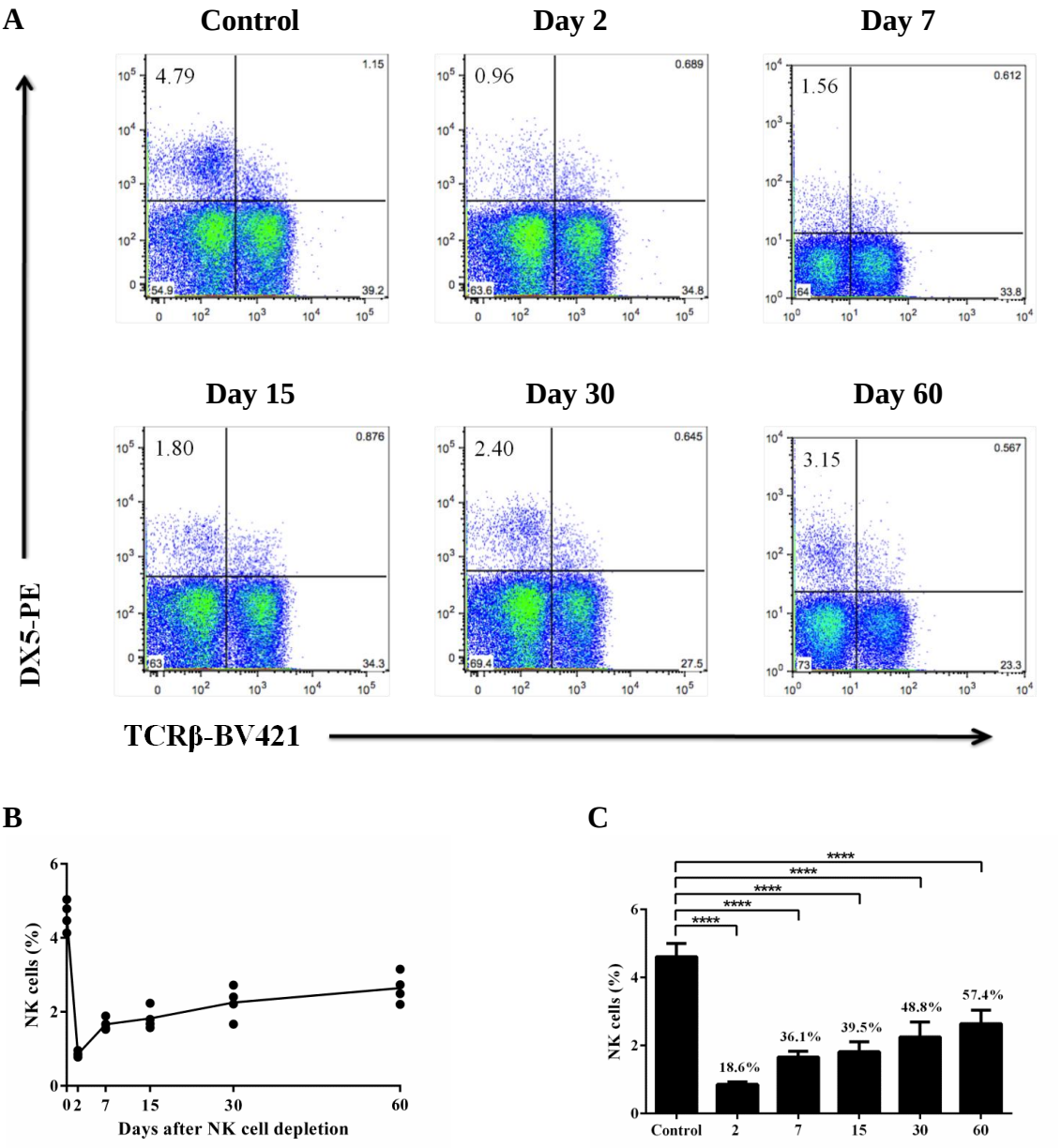


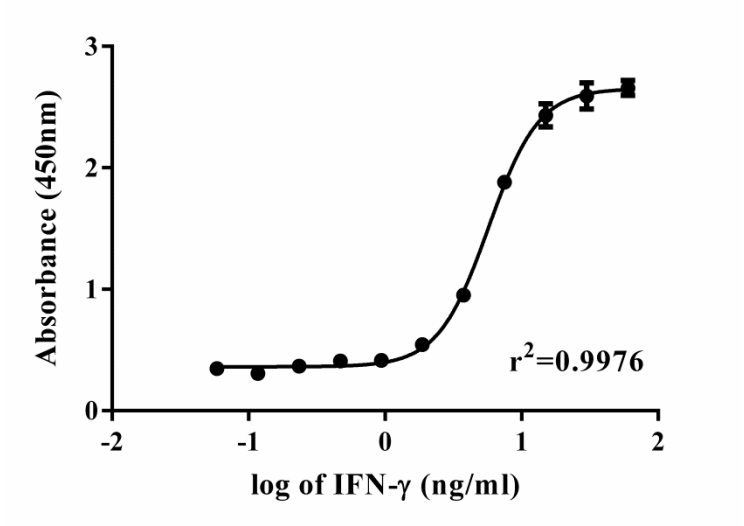
Figure 5: Anti-asialo GM1 treatment depletes natural killer cells.

Representative flow cytometry plots detecting $CD49b^{+}TCR\beta^{-}$ splenic NK cells in control mice and NK cell depleted mice at 2, 7, 15, 30 and 60 days after anti-asialo GM1 (A) (n = 4 per group). Splenic NK cells, within the gated lymphocyte population, in individual mice from control and NK cell-depleted mice at specified days after depletion (B). Mean splenic NK cells ± SD in control and NK cell-depleted mice at specified days after depletion (C). Percentage of NK cells relative to control recorded above each column (mean NK cell level in control set at 100%). **** $P < 0.0001$, One-way ANOVA, Tukey's multiple comparisons test.

3.2 Production of IFN-γ by Ssa K12-stimulated splenocytes

The ability of NK cells to function and produce IFN-γ was analysed in parallel to the detection of NK cell levels after anti-asialo GM1. Previous experiments from this laboratory showed that $CD49b^+$ NK cells, not $TCR\beta^+$ T cells, produced high levels of IFN-γ in response to HK Ssa K12 stimulation [37]. Therefore, the production of IFN-γ by HK Ssa K12-stimulated splenocytes was analysed as these levels were thought to represent the levels of NK cells in the spleen. This current study showed that IFN-γ was still produced in NK cell depleted mice at levels similar to control (Figure 6), which did not correlate with these previous studies that showed NK cells were the major producers of IFN-γ. There was no IFN-γ gamma produced by splenocytes stimulated without HK Ssa K12 (0 ng/ml) and no significant decrease in IFN-γ production after anti-asialo GM1 treatment.

A



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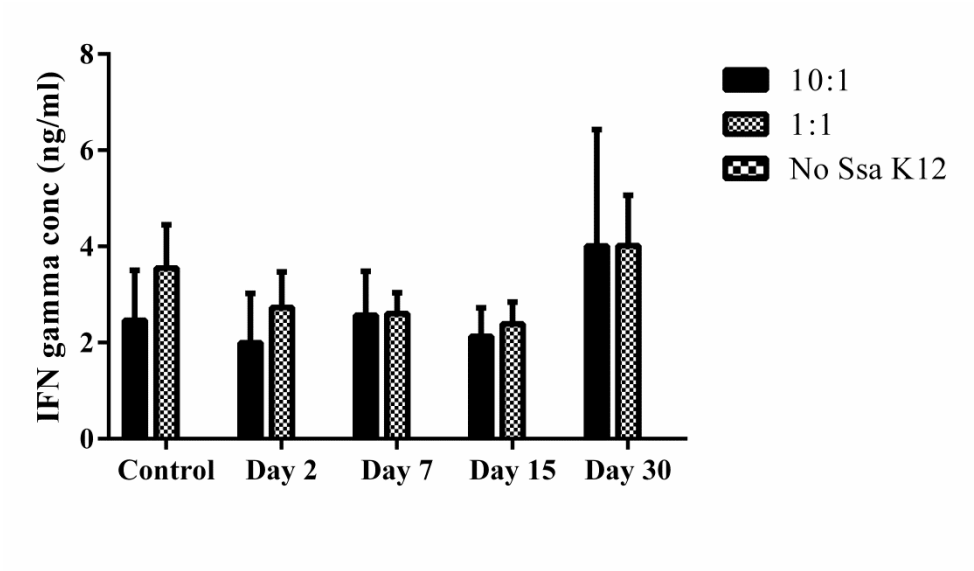


Figure 6: Splenocytes continue to produce IFN-γ after anti-asialo GM1 treatment.

Transformed standard curve, r^2 = 0.9976 (A). Mean concentration + SEM of IFN-γ produced by 4 × 10^6 splenocytes from control and NK cell-depleted mice analysed at 2, 7, 15 or 30 days after anti-asialo GM1 (n = 4 per group) that were stimulated with HK Ssa K12, at either a 10:1 or 1:1 bacteria:leukocyte ratio. Splenocytes that had no HK Ssa K12 stimulation did not produce any detectable IFN-γ.

3.3 Optimisation of bacterial-stimulation of dendritic cells

The DC immunotherapy protocol employed in this laboratory stimulated DC with HK Ssa K12 for four hours prior to DC immunisation. This study investigated different time periods of HK Ssa K12 stimulation to optimise the activation of NK cells stimulated by DC immunotherapy.

Mice were injected with B16/OVA on day zero and DC immunotherapy on day three, which was either stimulated with HK Ssa K12 overnight (16 hours) or for four hours (n = 3 per group). Previous results from this laboratory have shown that mice treated with B16/OVA melanoma tumour cells on day zero and HK Ssa K12-DC/OVA on days three and ten displayed exponential tumour growth between 20 and 41 days, by which all mice had tumours of 150 mm^2 in size. On day 35 of this current study two overnight HK Ssa K12 stimulated mice had developed tumours, which were 158 mm^2 and 62 mm^2 is size. The third overnight HK Ssa K12 stimulated mouse and all three 4 hr HK Ssa K12 stimulated mice appeared to have small tumours; on palpation these felt like small grains of rice (less than 5 mm^2). The timing of mice to grow tumours was seemingly different to previous experiments, thereby led to some concern over the exact location of the tumour. With this in mind, and the wellbeing of the mice paramount, mice were culled on day 35, dissected and the location of the tumours analysed.

Table 1: Day 35 dissection and tumour analysis

Overnight (16 hour)	4 hour
158 mm^2	Small clumped tumours
62 mm^2	Black dots
No tumour	No tumour

Tumour angiogenesis was observed in the overnight HK Ssa K12 stimulation mouse with a tumour of 62mm^2, shown by blood vessel formation between the tumour and left femoral artery. Dissection revealed no tumours in the peritoneal cavity or lungs. In the 4 hr HK Ssa K12 stimulation group; one mouse had particularly small tumours grouped together and another with small black dots on the peritoneal cavity, which were likely

to be tumour cells due to the black melanin pigment of B16/OVA. To the best ability of the eye, there were no tumours in two mice, one from each group.

3.4 Antibody quantification

To initiate this project antibodies were produced in house using hybridomas in a CellLine1000. The commercial Rat IgG was stated as being purified by affinity chromatography. The in house prepared antibodies were precipitated by ammonium sulphate and the concentrations determined by nanodrop. As this method is not specific for antibody, a Sandwich ELISA was performed to determine the concentration of antibodies in these preparations. Figure 7 shows that antibodies were detected and that there was no response in buffer-only control. However; due to differing response curves between the commercial Rat IgG and in house prepared antibodies in the ELISA, it was decided to use the nanodrop obtained concentrations. Nevertheless, the ELISA did demonstrate the presence of antibody.

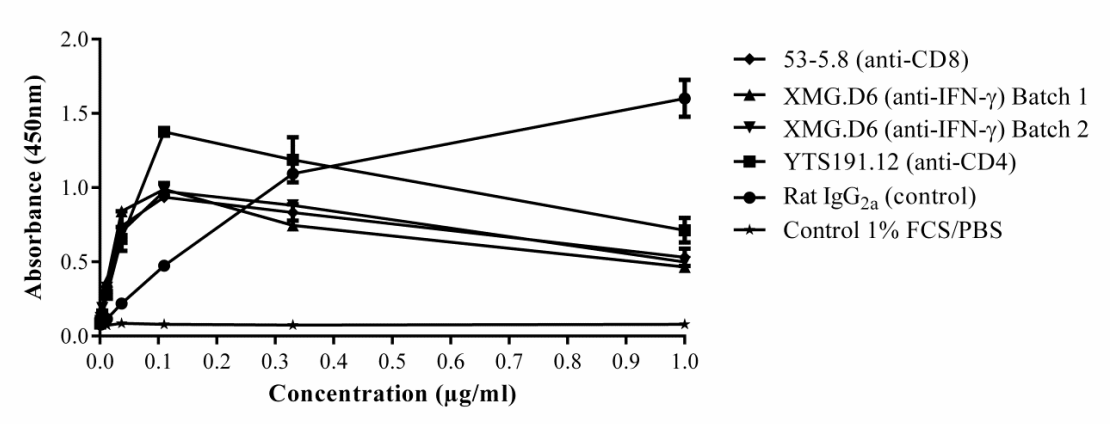


Figure 7: Antibody Quantification.

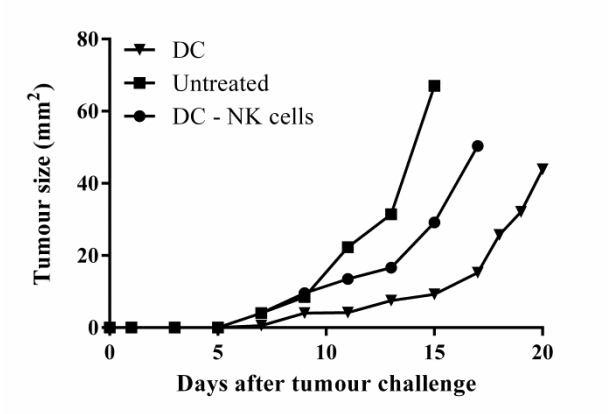
The mean + SD absorbance of diluted concentrations of antibody analysed by ELISA (n = 2).

3.5 Therapeutic role of NK cells in DC immunotherapy

In the clinic a patient is not given treatment until the cancer is found, whether the tumour is primary or has metastasised. In the following tumour experiments mice were injected with melanoma tumour prior to DC immunotherapy to model therapeutic treatments. DC immunotherapy was initiated three days after tumour injection and the delay of tumour growth was indicative of its protective response. To demonstrate the importance of NK cells in DC immunotherapy, mice were depleted of NK cells to determine the differences in tumour growth and survival between those with or without NK cells (Figure 8 and 10).

Dendritic cell immunotherapy delayed B16/OVA tumour growth and significantly increased survival compared to untreated mice ($P < 0.01$; Figure 8). NK cell depleted mice, injected with DC immunotherapy, had an increase in tumour growth and decreased survival compared to mice with NK cells, but this was not significant (Figure 8).

A



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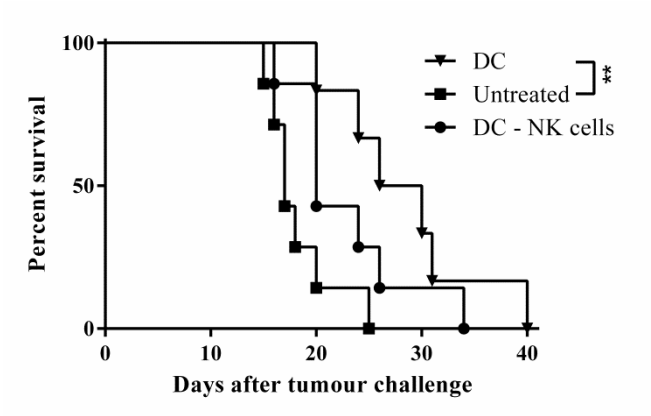

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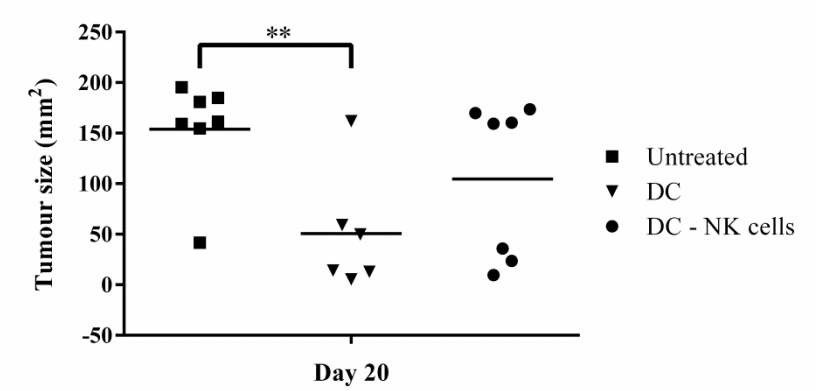


Figure 8: NK cell role in DC immunotherapy.

Mice (n = 6-7) were injected with B16/OVA on day zero with or without DC immunotherapy on day three and one group depleted of NK cells. Tumours were measured daily and mice euthanised once tumour size had reached 150 mm^2. Growth curve showing the mean tumour size until the first mouse reached a tumour size of 150mm^2 (A). Kaplan-Meier plot of the survival of mice (B). ** $P < 0.01$ Mantel-Cox test. Mean and individual tumour size of mice as at day 20 (C). ** $P < 0.01$ Two-way ANOVA Sidak's multiple comparisons test.

3.6 Release of IFN-γ in serum following DC immunotherapy

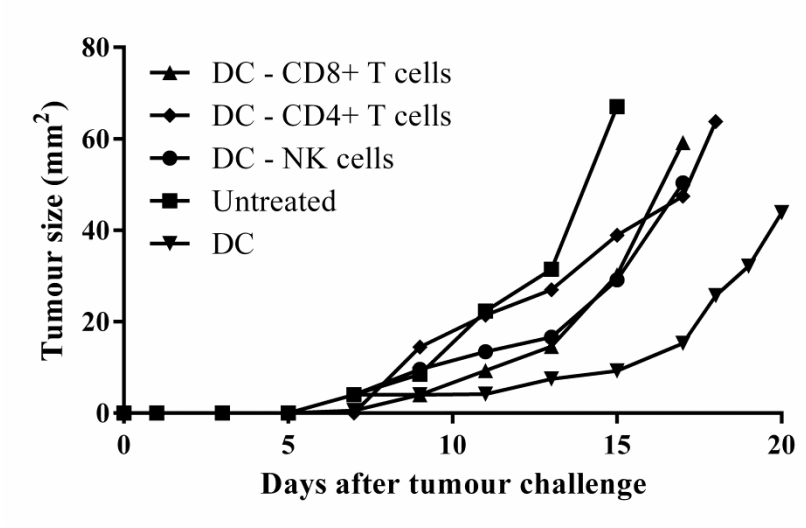
The laboratory has previously demonstrated that NK cells become activated and release IFN-γ in response to IL-12 and IL-18 produced by HK Ssa K12-stimulated DC. Blood samples were taken from mice injected with DC immunotherapy to determine if IFN-γ levels increased in response to DC immunotherapy. There was no IFN-γ detected in the blood 6, 24 and 48 hours after DC immunotherapy was administered at either 2×10^5 or 2×10^6 DC compared to PBS control (n = 2 per group; data not shown).

3.7 Role of $CD4^+$ and $CD8^+$ T cells in DC immunotherapy

To demonstrate the role of $CD4^+$ and $CD8^+$ T cells, relative to NK cells, in HK Ssa K12-stimulated DC immunotherapy, mice were depleted of either of these lymphocyte subsets and the differences in tumour growth and survival were compared to mice not depleted of these cells (Figure 9 and 10).

Tumour growth was delayed in mice injected with DC immunotherapy, but this delay was impaired in mice depleted of either $CD4^+$ T cells, $CD8^+$ T cells and NK cells (Figure 9). Dendritic cell immunotherapy increased survival in mice depleted of $CD4^+$ T cells, but not $CD8^+$ T cells, compared to untreated mice (* $P< 0.05$; Figure 9B)

A



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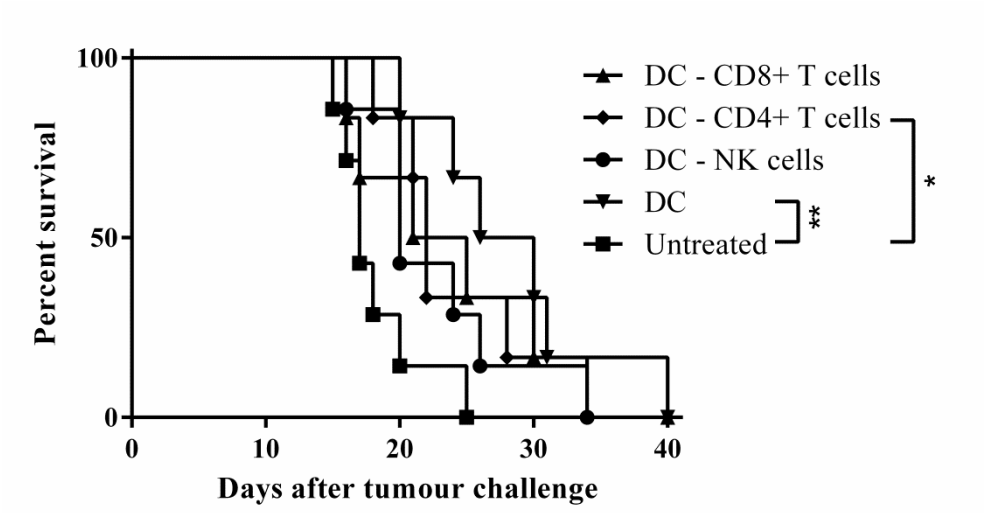


Figure 9: T cell roles in DC immunotherapy.

Mice (n = 6-7) were injected with B16/OVA on day zero with or without DC immunotherapy on day three. Selected groups were depleted of NK cells, $CD4^+$ or $CD8^+$ T cells as indicated. Tumours were measured daily and mice euthanised once tumour size had reached 150 mm^2. Growth curves showing the mean tumour size until the first mouse reached a tumour size of 150 mm^2 (A). Kaplan-Meier plot of the survival of mice (B). * $P < 0.05$ ** $P < 0.01$ Mantel-Cox test.

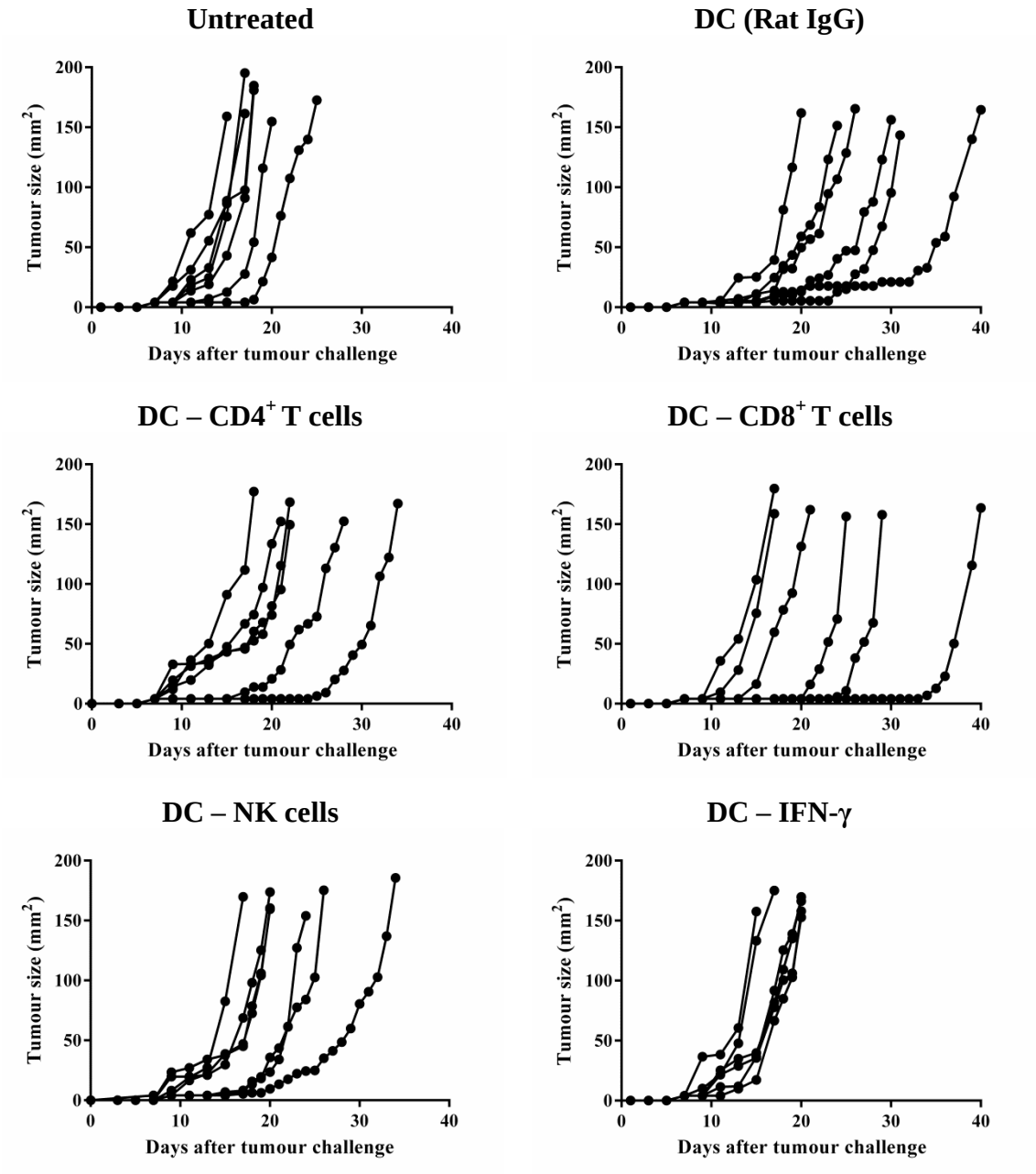


Figure 10: Individual growth curves of lymphocytes subsets or IFN-γ depleted mice.

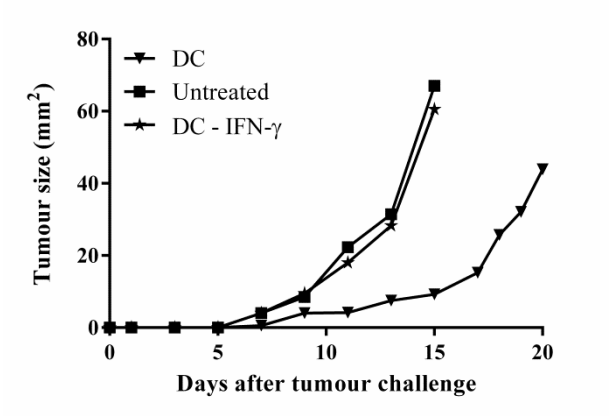
Mice were injected with B16/OVA on day zero and DC Immunotherapy on day three, except for untreated mice. Groups of mice were also depleted of NK cells, $CD4^+$ T cells, $CD8^+$ T cells, IFN-γ or mock depleted with Rat IgG (n = 6-7). Tumours were measured daily and mice euthanised once tumour size had reached 150 mm^2. Tumour growth of individual mice in the different groups are shown.

3.8 Critical role of IFN-γ in DC immunotherapy

To demonstrate the importance of IFN-γ in DC immunotherapy, mice were depleted of IFN-γ using a monoclonal antibody (XMG.D6). The tumour growth and survival of these mice were then compared to mice not depleted of IFN-γ (Figure 10 and 11).

In mice depleted of IFN-γ, DC immunotherapy did not delay B16/OVA tumour growth or increase survival (Figure 11). IFN-γ depleted mice, injected with DC immunotherapy, had significantly larger tumours on day 20 and decreased survival compared to non-depleted mice (** $P < 0.01$). Tumour growth and survival were similar between untreated mice and IFN-γ depleted mice injected with DC immunotherapy (Figure 11).

A



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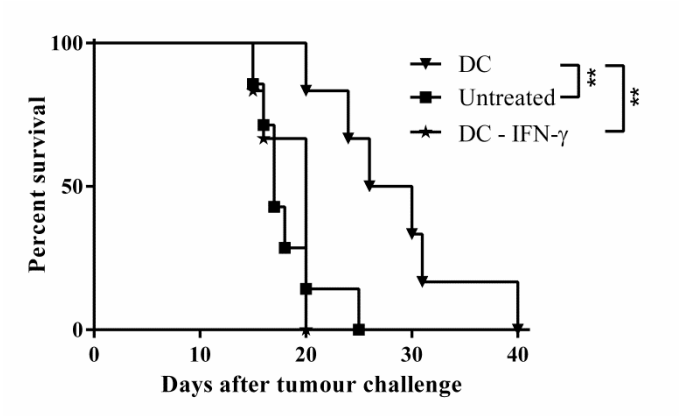

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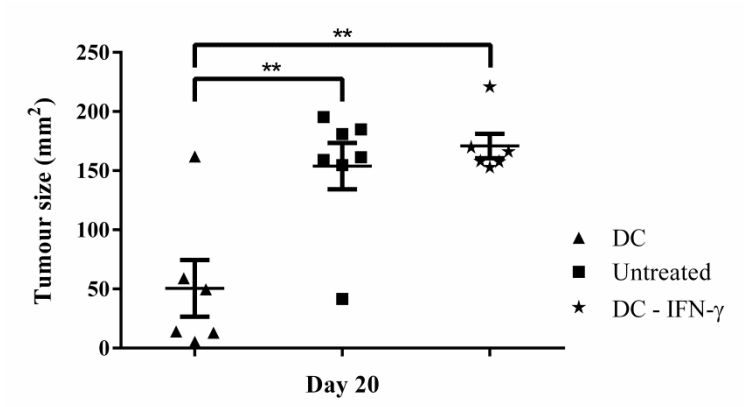


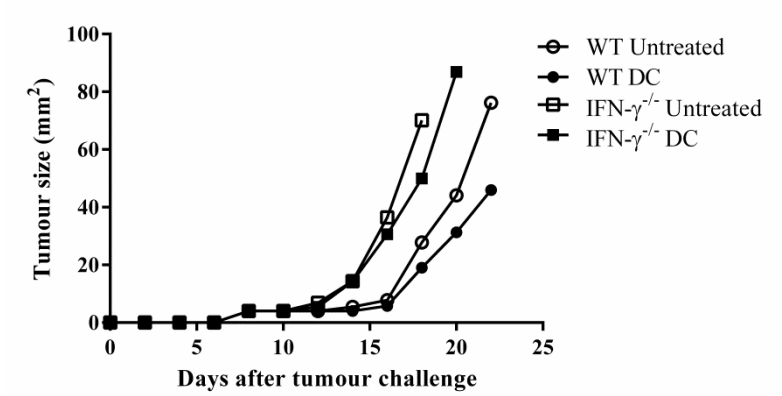
Figure 11: Interferon-γ is critical in the anti-tumour response following DC immunotherapy.

Survival curve of mice (n = 6-7) injected with B16/OVA on day zero with or without DC immunotherapy on day three. One group was depleted of IFN-γ. Growth curve showing the mean tumour size until the first mouse reached a tumour size of 150 mm^2 (A). Kaplan-Meier plot of the survival of mice (B). ** $P < 0.01$ Mantel-Cox test. Mean tumour size of mice as at day 20 (C). ** $P < 0.01$ Two-way ANOVA Sidak's multiple comparisons test.

In order to confirm the findings of the anti-IFN-γ antibody-mediated depletion strategy, IFN-$\gamma^{-/-}$ mice were utilised in a B16/OVA tumour and DC immunotherapy experiment.

DC immunotherapy delayed tumour growth and increased survival in wild-type mice, but not in IFN-$\gamma^{-/-}$ mice (Figure 12 and 15). Wild-type mice, treated with DC immunotherapy, survived significantly longer than untreated IFN-$\gamma^{-/-}$ mice ($P < 0.01$). The difference is survival between wild-type and IFN-$\gamma^{-/-}$ mice, both treated with DC immunotherapy, was close to statistical significance ($P = 0.0548$). Both the treated and untreated groups of IFN-$\gamma^{-/-}$ mice displayed very similar tumour growth and survival.

A



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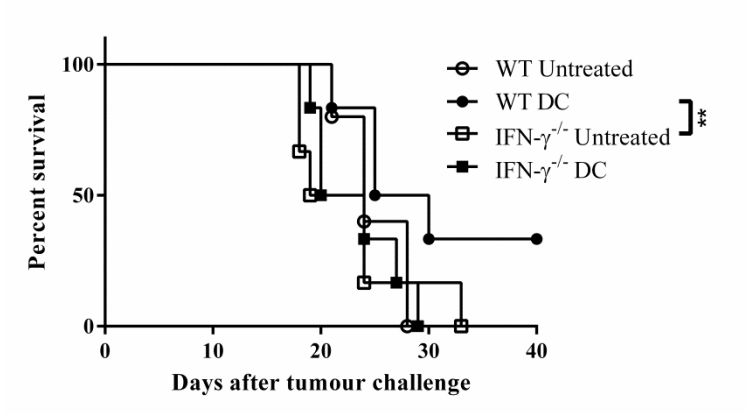


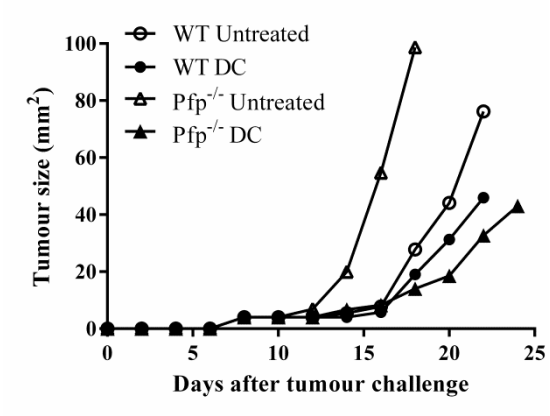
Figure 12: Interferon-γ has a critical role in the anti-tumour response following DC immunotherapy.

Wild-type and IFN-$\gamma^{-/-}$ mice were injected with B16/OVA on day zero and with or without DC immunotherapy on day three (n = 5-6). Tumour growth curves showing the mean tumour size until the first mouse reached a tumour size of 150 mm^2 (A). Kaplan-Meier plot of the survival of mice (B). ** $P < 0.01$ Mantel-Cox test.

3.9 Non-essential role of perforin in DC immunotherapy.

Natural killer cells and other cytotoxic effecter cells produce perforin. To determine if perforin is important in DC immunotherapy, tumour growth and surivial of $Pfp^{-/-}$ mice and wild-type mice, injected with B16/OVA and DC immunotherapy, were compared. Dendritic cell immunotherapy delayed tumour growth and significantly increased survival in $Pfp^{-/-}$ and wild-type mice compared to untreated $Pfp^{-/-}$ mice (*** $P < 0.001$ and ** $P < 0.01$, respectively) (Figure 13). Forty days after tumour challenge, 33% (2/6) of $Pfp^{-/-}$ and wild-type mice, injected with DC immunotherapy, had survived, but all untreated $Pfp^{-/-}$ mice did not survive the human endpoint criteria.

A



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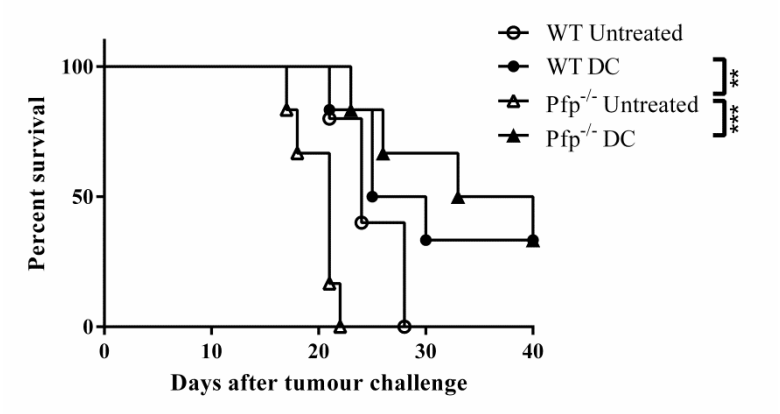


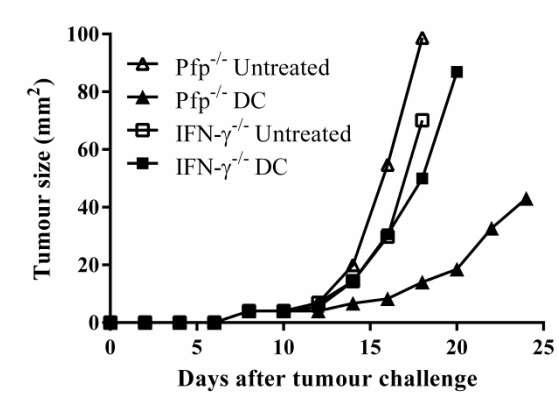
Figure 13: Perforin has a non-essential role in DC immunotherapy.

Wild-type and $Pfp^{-/-}$ mice were injected with B16/OVA on day zero and with or without DC immunotherapy on day three (n = 5-6). Tumour growth curves showing the mean tumour size until the first mouse reached a tumour size of 150 mm^2 (A). Kaplan-Meier plot of the survival of mice (B). ** $P < 0.01$ *** $P < 0.001$ Mantel-Cox test.

3.10 DC immunotherapy induces an IFN-γ mediated, not perforin-mediated, anti-tumour response

Interferon-gamma and perforin knockout mice were compared to determine if the protective anti-tumour response from DC immunotherapy was IFN-γ or perforin mediated. Dendritic cell immunotherapy delayed tumour growth in $Pfp^{-/-}$, but not IFN-$\gamma^{-/-}$ mice (Figure 14). $Perforin^{-/-}$ mice, injected with DC immunotherapy, were the only mice still surviving 40 days after tumour challenge (33%) of all $Pfp^{-/-}$ and IFN-$\gamma^{-/-}$ mice. Individual growth curves of $Pfp^{-/-}$ and IFN-$\gamma^{-/-}$ mice are shown in Figure 15.

A



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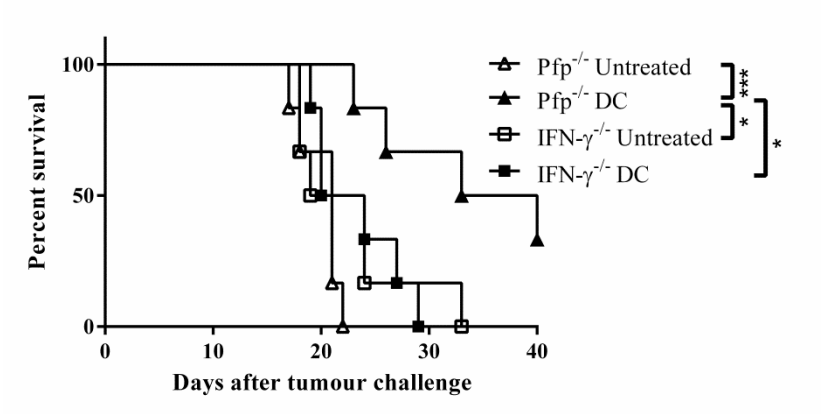

Figure 14: DC immunotherapy induces an IFN-γ mediated, not perforin-mediated, anti-tumour response.

Perforin$^{-/-}$ and IFN-$\gamma^{-/-}$ mice were injected with B16/OVA on day zero and with or without DC immunotherapy on day three (n = 5-6). Tumour growth curves showing the mean tumour size until the first mouse reached a tumour size of 150 mm^2 (A). Kaplan-Meier plot of the survival of mice (B). * $P < 0.05$ *** $P < 0.001$ Mantel-Cox test.

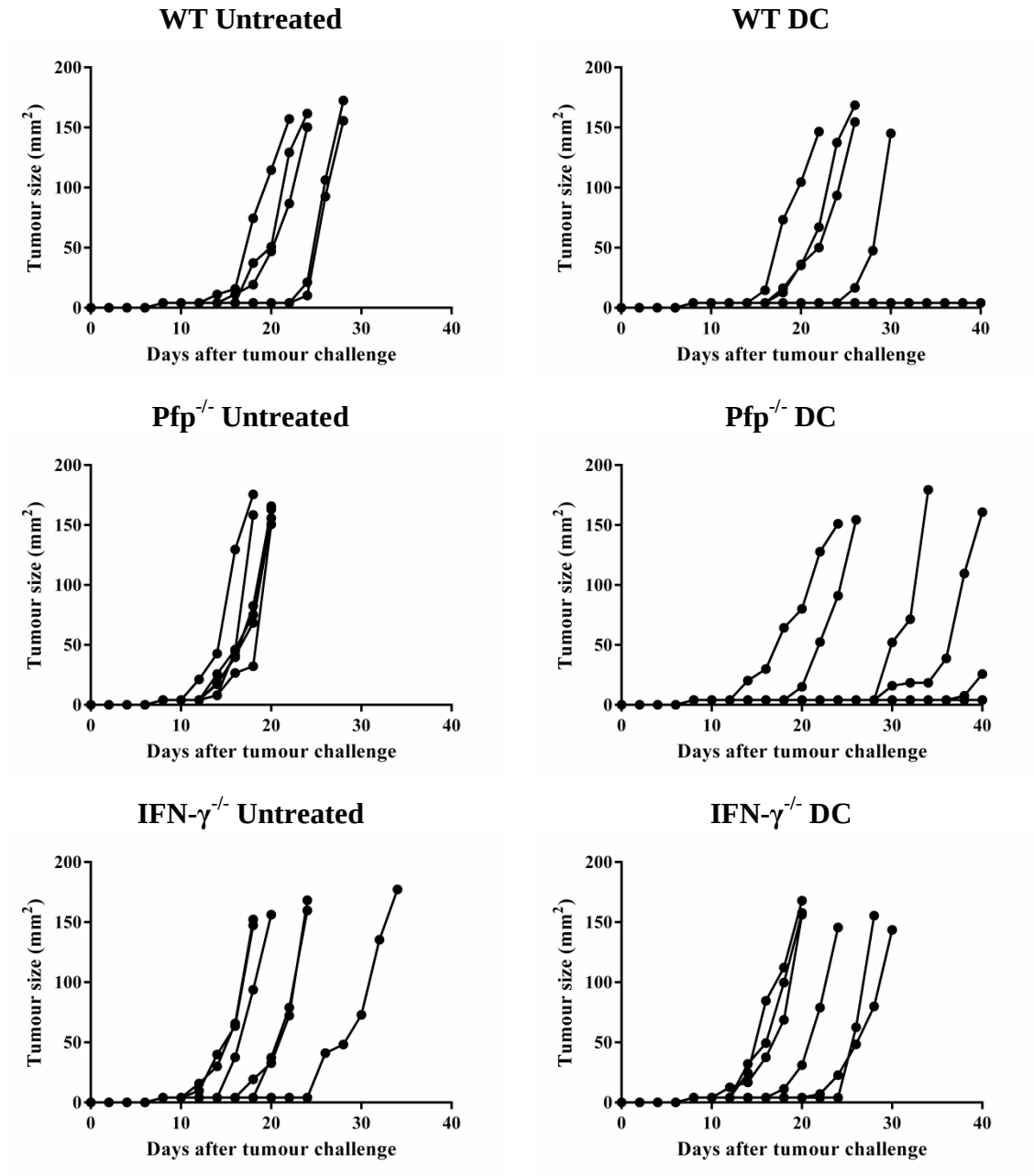

Figure 15: Individual growth curves of wild-type, Pfp$^{-/-}$ and IFN-γ$^{-/-}$ mice.

Wild-type, Pfp$^{-/-}$ and IFN-γ$^{-/-}$ mice (n = 5-6) were injected with B16/OVA on day zero and with or without DC immunotherapy on day three. Tumours were measured every two days until day 40. Individual growth curves of tumour from each mouse are shown.

4 DISCUSSION

4.1 NK cell depletion

Dendritic cell immunotherapy is currently used in the clinic as a treatment for cancer and has been demonstrated to be a feasible, non-toxic and effective treatment in some cancer patients. In spite of this, the efficacy of DC immunotherapy is varied as it does not provide anti-tumour immunity in all patients. In this study, DC immunotherapy significantly delayed the growth of subcutaneous B16/OVA melanoma tumour and increased survival in mice compared to untreated (Figure 8). Twenty days after tumour challenge, the mean tumour size was significantly smaller in mice treated with DC immunotherapy compared to those untreated. Strategies to improve DC immunotherapy rely on understanding the role and tumour killing mechanisms of lymphocytes stimulated once DC are administered. In this study, the potential role and mechanism of the anti-tumour response of NK cells was investigated.

In order to monitor NK cell depletion, this study initially attempted to monitor $CD49b^{+}TCR\beta^{-}$ NK cell levels in the blood. The monoclonal antibody anti-CD49b (DX5-PE) detects cells expressing relatively high, but not low, CD49b and is relatively non-specific [65]. Interestingly, within the lymphocyte population an unusually high percentage of $DX5^{+}TCR\beta^{-}$ cells (3-35%) were identified, which were unlikely to represent NK cells, since NK cells in mouse blood have been reported as a mean of 3.18% in C57BL/6 mice [66]. CD49b is also expressed on platelets and fibroblasts so the appearance of high levels of a $CD49b^{+}TCR\beta^{-}$ population may have represented platelets and fibroblasts in the blood [67], even though the gating strategy used should have prevented this (Figure 4). Another potential explanation for the varied percentages in the blood may be due to DX5 binding to basophils, with Gomez *et al.* having shown that approximately 10% of $DX5^{+}$ cells in the bone marrow are basophils [68]. Overall, the difficulty of quantifying NK cells in the blood is reflected by the paucity of studies demonstrating successful NK cell identification in mouse blood.

On the other hand, analysing splenic NK cells, as shown by other groups, was successful. A single anti-asialo GM1 treatment reduced the percentage of NK cells to 18.7% of control levels two days after depletion (Figure 5). The percentage of NK cells remained low even after sixty days where NK cells had only regenerated to 57.4% of baseline NK cell percentages seen in control mice (Figure 5). For the amount used, anti-asialo GM1 antibody is reported to only target NK cells [69], unlike PK136 (anti-

NK1.1) which is also commonly used for NK cell depletion, but targets both NK and NKT cells. The levels of splenic NK cells in control mice of this current study were consistent with Yoshida *et al.* who also analysed NK cells in the spleen [70]. To ensure complete reduction in NK cell numbers over the time course of tumour growth and DC immunotherapy, two treatments of anti-asialo GM1 were injected into mice for tumour studies – as per protocols used in previous tumour studies in this laboratory.

As shown in Figure 6, splenocytes from NK cell depleted mice were capable of producing IFN-γ in response to *S. salivarius*. These results conflict with other laboratory findings that showed NK cells ($CD49b^{+}TCR\beta^{-}$), not T cells ($CD49b^{-}TCR\beta^{+}$), were responsible for producing IFN-γ [37]. One treatment of anti-asialo GM1 did not deplete all NK cells (Figure 5), therefore residual asialo $GM1^{+}$ NK cells may have elicited the levels of IFN-γ produced. Additionally, a small proportion of asialo $GM1^{-}$ NK cells may also contributed to this response. In summary, this present study has highlighted that other cells are capable of producing IFN-γ when stimulated by *S. salivarius,* such as $CD4^{+}$ and $CD8^{+}$ T cells.

4.2 Optimisation of DC immunotherapy with bacteria

The variation of B16/OVA tumour growth rates in mice treated with either overnight or four hour HK *S. salivarius* K12 stimulated DC immunotherapy was surprising and raised various questions. The inguinal lymph node, which was in close proximity to tumour injection, showed signs of localised lymphadenitis (inflammation of the lymph node), as it was palpable after seven days. Confirming lymphadenitis would require analysing the cellular interactions within the lymph node; however in this present study it was not feasible to confirm that lymphadenitis had occurred in these early stages of the anti-tumour response as mice were euthanised in the late stage.

Overnight, frozen *S. salivarius* stimulation was used for proceeding experiments based on other optimisation studies of NK cells in this laboratory. These studies demonstrated higher levels of activated NK cells in the brachial and axilliary lymph nodes when DC had overnight stimulation with frozen *S. salivarius*. Although this present study did not optimise stimulation, it did highlight the importance of injecting tumour into the exact same location to reduce inconsistency in tumour growth between mice.

4.3 Role of NK cells and T cells in DC immunotherapy

This study has demonstrated that NK cells appear to have a therapeutic role in this model of HK Ssa K12-stimulated DC immunotherapy, however it was not significant. This was shown in Figure 8, where a strong anti-tumour response with DC immunotherapy was impaired in mice depleted of NK cells. The anti-tumour response was represented by a delay in tumour growth and increased survival. Repetition of this experiment is required to confirm if the therapeutic role of NK cells is statistically significant in this model of DC immunotherapy. NK cells may have a potential negative role in tumour elimination, as shown by Soderquest *et al.* where the deletion of NK cells led to an enhanced memory antigen-specific $CD8^+$ T cell response. It is unlikely that this negative role outweighs the positive anti-tumour properties of NK cells, which are seen in studies carried out by other various groups [25, 51]. In addition, Riccardi *et al.* have showed that suppression of NK cells increased growth of artificial and newly spread metastases in lung models [71], further indicating the requirement of NK cells in tumour inhibition. The present study suggests that NK cells do have a role in DC immunotherapy; however NK cells are not solely responsible for producing the anti-tumour response observed in this therapeutic setting.

Previous work from our laboratory has demonstrated NK cells are required in an antigen-dependent anti-tumour response in DC immunotherapy. Natural killer cells have been demonstrated to interact with T cells by stimulating DC to secrete Th1 cytokines, such as IL-12 and IL-15, to enhance T cell priming [72]. This implies that NK cells are likely to be interacting with T cells that require antigen presentation to become activated and subsequently produce cytokines.

Anti-tumour responses in DC immunotherapy have demonstrated that interactions between NK cells and $CD4^+$ T cells are required [23, 73]. $CD4^+$ T cells appear to be required in this model of DC immunotherapy, shown by increased tumour growth in $CD4^+$ T cell depleted mice in the initial days after tumour challenge (Figure 9A). This suggests that NK cells may require initial interactions with $CD4^+$ T cells to subsequently produce an anti-tumour response. Within the scope of this investigation, the exact interactions between NK cells and $CD4^+$ T cells could not be defined due to the high variation between mice (Figure 10). Therefore, further investigation is required

to determine these interactions and uncover the role of $CD4^+$ T cells in this model of DC immunotherapy.

The interactions between $CD8^+$ T cells and NK cells in DC immunotherapy is a topic of debate. This present study does not define a statistically significant therapeutic role of $CD8^+$ T cells in this model of DC immunotherapy; however the survival of $CD8^+$ T cell depleted mice appears to show that $CD8^+$ T cells are not required in DC immunotherapy. This was shown if Figure 9B, by the similar survival outcomes between $CD8^+$ T cell depleted and non-depleted mice. Futhermore, Ribas *et al.* showed that DC administration enhanced a protective anti-tumour response in $CD8^+$ knockout mice, thus correlating with this present study. Kuhn *et al.* showed NK cells and $CD8^+$ T cells were required to delay the growth of subcutaneous B16 melanoma, however they used immune activating treatments, such as polyinosinic-polycytidylic acid or a combination of monosodium urate crystals and *Mycobacterium smegmatis*, instead of DC immunotherapy. The DC immunotherapy model used in the current study has demonstrated NK cells are stimulated by DC-derived IL-12 and IL-18 in bidirectional activation. This suggests that NK cells may still provide an anti-tumour response, in spite of by-passing interactions with $CD8^+$ T cells. Further investigation is required to determine if NK cells and $CD8^+$ T cells interact in this model of DC immunotherapy.

4.4 Possible anti-tumour mechanisms by NK cells in DC immunotherapy.

DC immunotherapy is known to stimulate the production of IFN-γ [25, 36, 49], thus it was important to confirm IFN-γ was actually being produced in response to this model of DC immunotherapy. Interestingly, this study did not detect IFN-γ in the serum of mice intravenously injected with DC immunotherapy; however increases in IFN-γ levels in other locations should not be ruled out, such as lymph nodes where DC localise and interact with NK cells. Xu *et al.* found levels of IFN-γ were high in the serum and tumour tissue after combination of IL-12 gene therapy and 4-1BB costimulation in a subcutaneous B16-F10 melanoma model in mice [52]. The key difference between their study and this present study was the presence of tumour, thus highlighting that the presence of target tissue is most definitely required for full activation of DC immunotherapy. This further supports evidence from Bouwer *et al.* that stated the DC immunotherapy was antigen-dependent [36].

Since NK cells appear to play a therapeutic role in DC immunotherapy, the anti-tumour mechanisms of NK cells was further investigated. This was carried out using IFN-$\gamma^{-/-}$, $Pfp^{-/-}$ and IFN-γ depleted mice. This study showed that perforin has a non-essential role in DC immunotherapy. This model of DC immunotherapy induced an anti-tumour response independent of perforin. $Pfp^{-/-}$ mice, treated with DC immunotherapy, still reacted with a protective response, indicated by a delay in tumour growth and a significant increase in survival compared to untreated $Pfp^{-/-}$ mice (Figure 13 and 15). Antigen-specific CTLs kill both tumour and tumour antigen presenting DC in perforin mediated killing [49]. Therefore, DC administered in $Pfp^{-/-}$ mice can persist and present antigen longer as they are incapable of perforin mediated killing, thus enhancing the stimulated anti-tumour response. This suggests that other mechanisms by NK cells are responsible for inducing a protective anti-tumour response in this model.

This model of DC immunotherapy uses heat killed *S. salivarius* as an adjuvant which stimulates DC to produce IL-12 and IL-18. This subsequently leads to bi-directional activation between DC and NK cells, resulting in vast amounts of NK cell-derived IFN-γ [36]. Hayakawa *et al*. demonstrated that NK cell-derived IFN-γ inhibits tumour angiogenesis by preventing endothelial cell proliferation. Furthermore, studies from Shankaran *et al.* showed IFN-γ up-regulates the expression of MHC-I on tumour cells, thereby increasing the susceptibility of tumour cells to CTL killing [61]. In addition to this the production of IFN-γ from NK cells induces the production of Th1 cytokines from DC, therefore enhancing the T cell-mediated anti-tumour responses [59]. IFN-γ has demonstrated a critical role in this model of DC immunotherapy, which had HK *S. salivarius* K12 as an adjuvant. This was shown in Figures 11 and 12 where the protective anti-tumour response from DC immunotherapy was lost in IFN-γ deficient and IFN-γ knockout mice. This suggests NK cell-derived IFN-γ, in this model of DC immunotherapy, could inhibit tumour angiogenesis, stimulate DC to produce IL-12 and IL-18 Th1 cytokines to enhance the initiation and maintenance of T cell-mediated responses and ultimately, stimulate a potent anti-B16/OVA tumour response. It is possible that IFN-γ could also induce death receptor mechanisms to enhance tumour killing, such as TRAIL expression on NK cells [74]. Other IFN-γ producing cells, such as $CD4^+$ and $CD8^+$ T cells, may have also contributed to the observed IFN-γ mediated anti-tumour response induced by DC immunotherapy. Further investigation is required

to determine which lymphocyte subset(s) is responsible for producing an IFN-γ mediated anti-tumour response following HK Ssa K12-stimulated DC immunotherapy.

The anti-tumour response observed in this model of DC immunotherapy was impaired without NK cells, but this was not significant. It is possible this may have been due to NKT cells, which are not depleted by anti-asialo GM1 treatment [75]. Hayakawa *et al.* demonstrated that IFN-γ secreted primarily by NKT cells and secondarily by NK cells was critically involved in an anti-angiogenic response by alpha-galactosylceramide. They also demonstrated the overall anti-tumour response was dependent on IFN-γ and not perforin-mediated cytotoxicity, which is consistent with this present study. This suggests that NKT cells may also contribute to the IFN-γ mediated anti-tumour response in this model of DC immunotherapy by producing IFN-γ and stimulating NK cells; however it is unknown whether NKT cells are also activated in bi-directional activation with DC. Using PK136 antibody, which depletes both NK and NKT cells via NK1.1 receptor [75], in this model, the involvement of NKT cells in the anti-tumour response could be determined.

In summary, this model of DC immunotherapy, stimulated with heat killed *S.salivarius* K12, requires NK cells and induces an IFN-γ, not perforin, mediated anti-tumour response against B16/OVA melanoma tumour.

4.5 Conclusion

This study has demonstrated that NK cells appear to have a therapeutic role in DC immunotherapy. This model of DC immunotherapy, stimulated with heat killed *S.salivarius* K12, has clearly shown an IFN-γ, not perforin, mediated anti-tumour response is elicited. Further studies still need to be performed to determine how NK cell-derived IFN-γ contributes to the anti-tumour response as this is not completely understood. By including parameters which monitor NK cells in clinical trials and continued investigations in the laboratory, a better understanding of NK cell mechanisms in DC immunotherapy would be of great significance. Future DC immunotherapy strategies could be improved to enhance NK cell activation or modulate the production of IFN-γ by NK cells in order to promote an optimal anti-tumour response and ultimately eliminate cancer in patients.

4.6 Future directions

Assessing whether NK cells infiltrated the tumour would be useful as it would justify the importance of NK cells in DC immunotherapy. This could be performed by examining extracted tumour for NK cells by flow cytometry. If NK cells had infiltrated the tumour it would be interesting to then analyse the intracellular IFN-γ cytokine levels of these NK cells. This could be performed by sorting NK cells by FACS and the level of IFN-γ expression analysed by intracellular staining of IFN-γ by flow cytometry or stimulation assays where the production of IFN-γ could be detected by an ELISA.

Levels of IL-12 and IL-18 in the lymph node or serum could be analysed to confirm DCs are producing these cytokines which are known to stimulate NK cells to produce IFN-γ in the bi-directional model previously described.

Repeating these experiments with mice specifically deficient in IFN-γ or perforin in NK cells would allow the importance of IFN-γ and perforin in NK cell mediated killing to be determined. This could be achieved by flanking either IFN-γ or perforin genes with loxP sites in a Cre-Lox system where only NK cells express cre-recombinase to then excise either IFN-γ or perforin. Alternatively, $RAG1^{-/-}$ mice, which have no mature T or B cells, could be used to determine the efficacy of NK cells alone in DC immunotherapy. Adoptively transferred DC into tumour- bearing $RAG1^{-/-}$ mice would allow only activated NK cells to mount an anti-tumour response. This would clearly demonstrate whether NK cells require interactions with T cells to mount an anti-tumour response. $CD4^{+}$ or $CD8^{+}$ T cells could also be adoptively transferred into $RAG1^{-/-}$ mice, thereby determining the importance of NK cell and $CD4^{+}$ or $CD8^{+}$ T cell interactions in the anti-tumour response stimulated by DC immunotherapy.

This field of research is directing towards the use of immune activating agents that enhance the anti-tumour response induced by DC immunotherapy. NKT cells not only produce IFN-γ in response to alpha-galactosylceramide, but also stimulate NK cells to produce IFN-γ [76], thereby eliciting a strong IFN-γ mediated anti-tumour response. Activating both NK and NKT cells is likely to be a further step in enhancing DC immunotherapy strategies and could be achieved by the addition of monoclonal antibodies to receptors expressed on these cells, such as NKG2D, FcR and the aryl hydrocarbon receptor. Other investigations to enhance NK cells could include IL-12/IL-

18 cytokine-receptor complexes and bi-specific antibodies which target tumour antigen and NK cell receptors, therefore enhancing direct NK cell mediated tumour killing.

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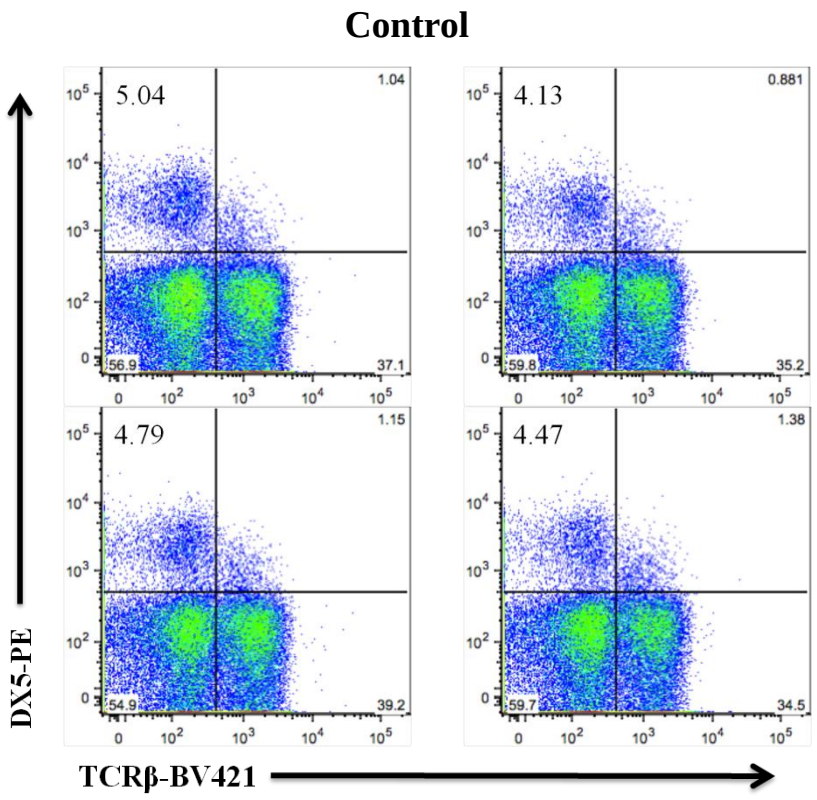
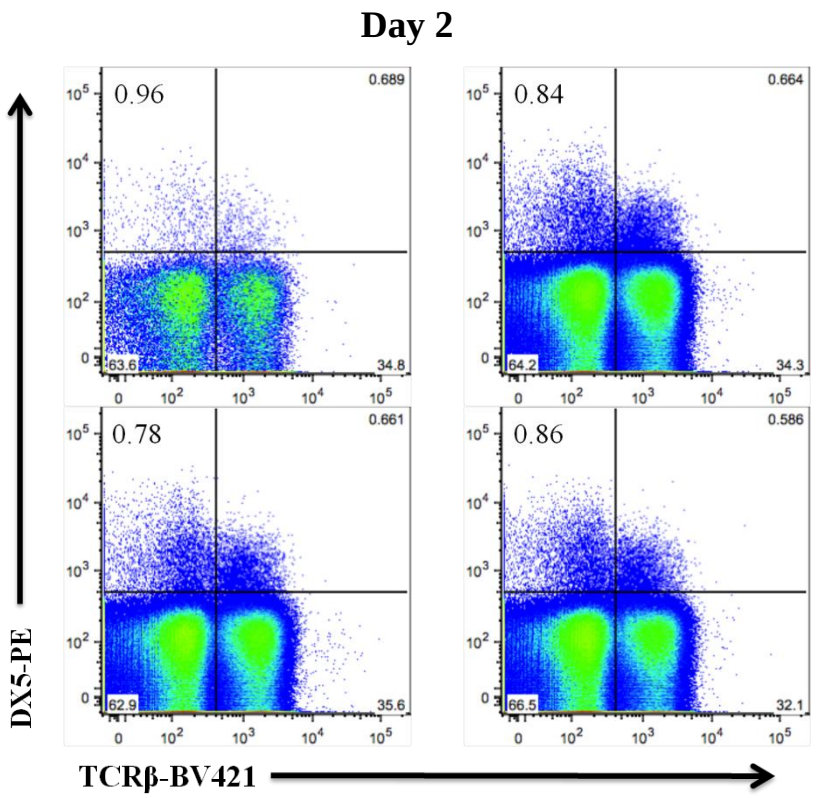
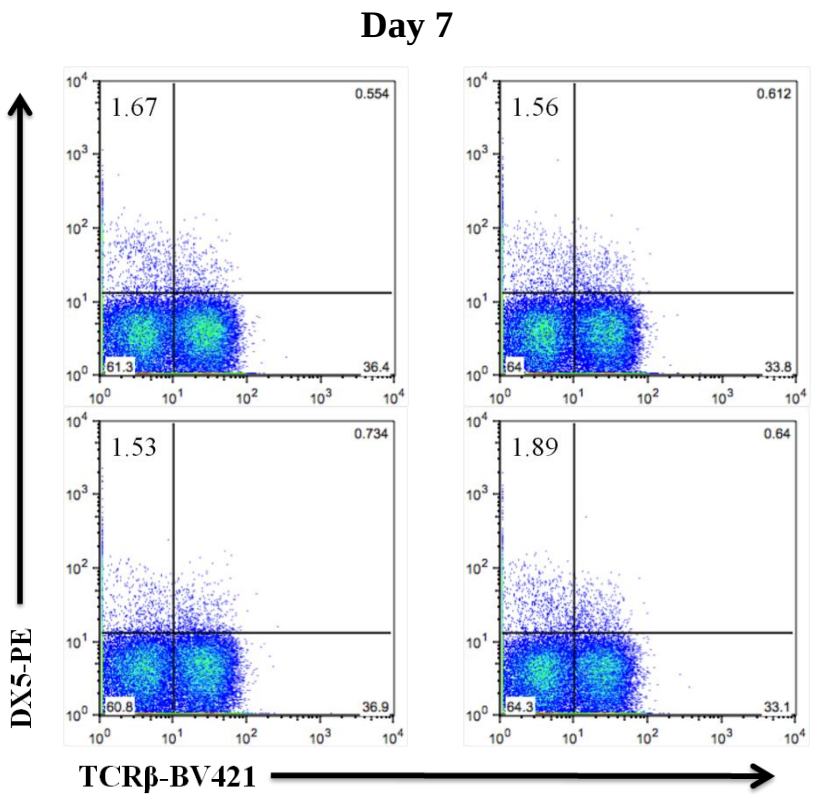
6 Appendix



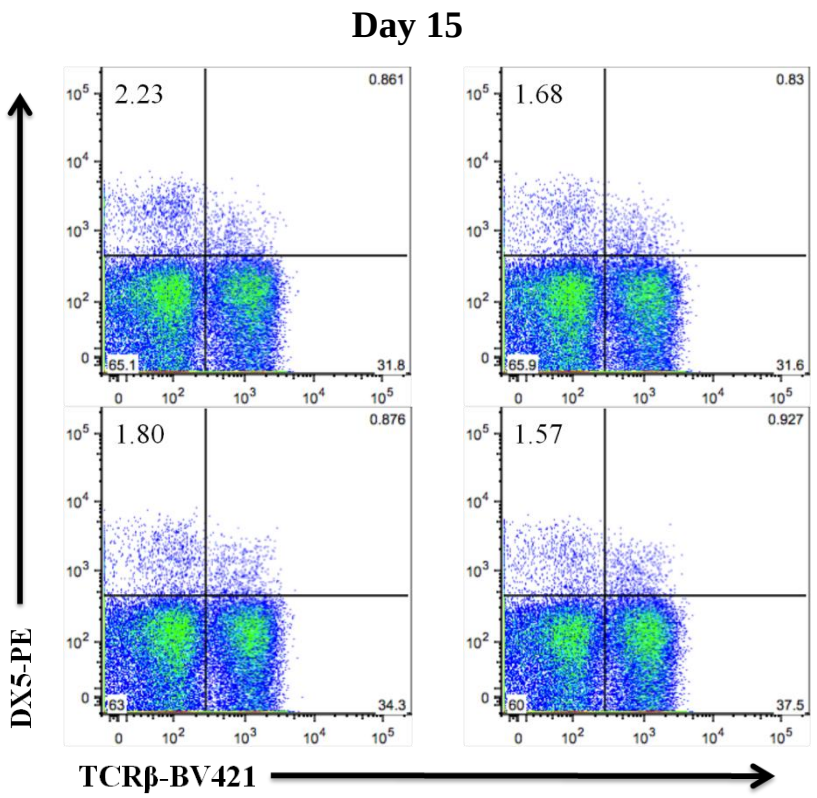
Appendix 1: Splenic DX5^{+}TCRβ^{-} NK cells from C57BL/6 mice.



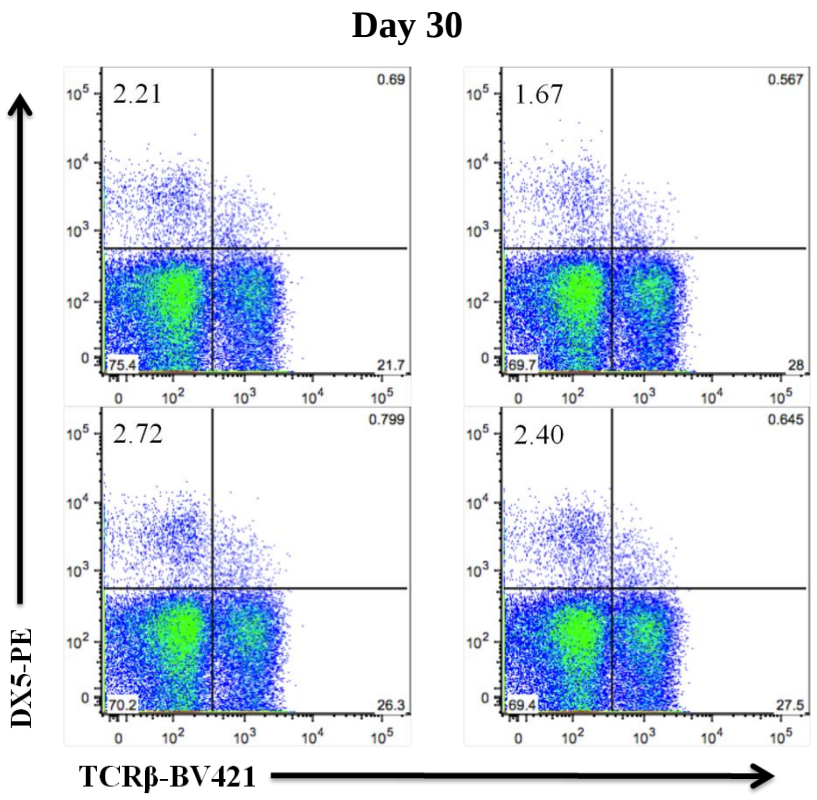
Appendix 2: Splenic DX5^{+}TCRβ^{-} NK cells from C57BL/6 mice injected with anti-asialo GM1 on day 0 and spleens analysed on day 2.



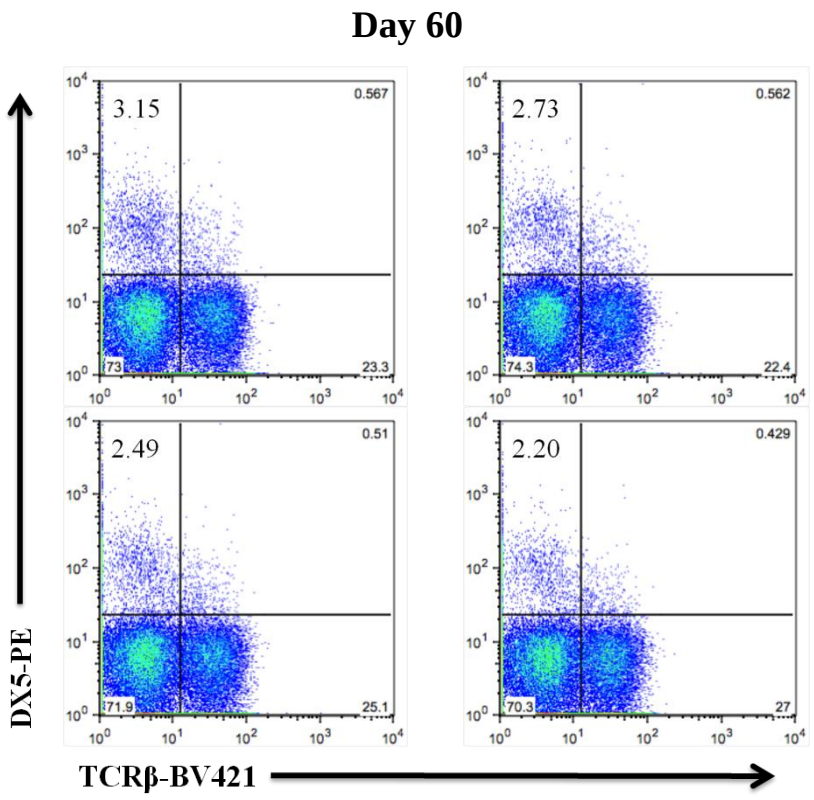
Appendix 3: Splenic DX5^{+}TCRβ^{-} NK cells from C57BL/6 mice injected with anti-asialo GM1 on day 0 and spleens analysed on day 7.



Appendix 4: Splenic DX5^{+}TCRβ^{-} NK cells from C57BL/6 mice injected with anti-asialo GM1 on day 0 and spleens analysed on day 15.



Appendix 5 Splenic DX5^{+}TCRβ^{-} NK cells from C57BL/6 mice injected with anti-asialo GM1 on day 0 and spleens analysed on day 30.



Appendix 6: Splenic DX5^{+}TCRβ^{-} NK cells from C57BL/6 mice injected with anti-asialo GM1 on day 0 and spleens analysed on day 60.