Manipulating the Immune Response of Tasmanian Devils to Target Devil Facial Tumour Disease

Candidate: Gabriella Kathleen Brown

Bachelor of Biotechnology with First Class Honours

(University of Tasmania, 2008)

Submitted in fulfilment of the requirements for the degree of

Doctorate of Philosophy

University of Tasmania

September 2013

Declaration of Originality

This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

_____

Gabriella Brown

April 2013

Authority of Access

This thesis may be made available for loan and limited copying and communication in accordance with the Copyright Act 1968.

_____

Gabriella Brown

April 2013

Statement regarding published work contained in thesis

The publishers of papers comprising material from Chapters 3 and 4 of this thesis hold the copyright for that content, and access to the material should be sought from the respective journals. The remaining non published content of the thesis may be made available for loan and limited copying and communication in accordance with the Copyright Act 1968.

_____

Gabriella Brown

April 2013

Statement of Co-authorship

The following people and institutions contributed to the publication of work undertaken as part of this thesis:

Author details and their roles:

Candidate: Gabriella K. Brown, Menzies Research Institute Tasmania

Author 2: Alexandre Kreiss, Menzies Research Institute Tasmania

Author 3: A. Bruce Lyons, School of Medicine, University of Tasmania

Author 4: Gregory M. Woods, Menzies Research Institute Tasmania

Paper: Natural Killer Cell Mediated Cytotoxic Responses in the Tasmanian Devil

Publisher: *PLoS One.* ***Accepted*** *August 11, 2011;* ***Published*** *September 21, 2011*

Comprising information contained in chapters 3 and 4 of this thesis

The candidate was the primary author and authors 2, 3 and 4 contributed to the idea, its formalisation and development.

The candidate and authors 2 and 4 obtained materials, reagents, tools and permissions, including cell lines and ethics approval, for the performance of the experiments presented in the paper. The candidate and author 2 performed the laboratory work presented in the paper.

All authors played roles in analysis of the data collected.

The candidate and author 4 were primarily responsible for the preparation of the manuscript

We the undersigned agree with the above stated “proportion of work undertaken” for each of the above published (or submitted) peer-reviewed manuscripts contributing to this thesis:

Signed: _____ _____

Gregory Woods	*Thomas Marwick*
Supervisor	*Director*
Menzies Research Institute Tasmania	*Menzies Research Institute Tasmania*

Date:_____

Statement of Ethical Conduct

The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University.

_____

Gabriella Brown

April 2013

Table of Contents

Abstract

The Tasmanian devil (*Sarcophilus harrisii*) is a marsupial carnivore confined to the island of Tasmania, where it is the top predator in the natural ecosystem. However, the Tasmanian devil is in danger of extinction due to the emergence of a contagious cancer. Devil Facial Tumour Disease (DFTD) is transmitted between devils by biting, a common behaviour which occurs during feeding and mating. The disease was first identified in 1996 and has since spread through over 60% of the natural distribution of the Tasmanian devil. Once contracted, the disease is invariably fatal, and has reduced Tasmanian devil population numbers by over 80%. Epidemiology estimates that the Tasmanian devil may become extinct in the wild within 20 years. Considering the importance of this animal in the natural ecosystem, its extinction in the wild must be prevented. One of the few options to achieve this is to develop an immunological intervention, a vaccine or immunotherapy capable of targeting this deadly tumour. This thesis aimed to characterise the anti-tumour immune response of the Tasmanian devil and to identify target pathways for immunological intervention.

This thesis details the first characterisation of specific anti-tumour responses in Tasmanian devils. An analysis of DFTD infected Tasmanian devils found no evidence for a natural anti-tumour response against the tumour cells. The integrity of specific anti-tumour immunity was analysed using xenogeneic tumour cell immunisation with human K562 tumour cells to induce strong responses. Cytotoxicity responses were measured using chromium release assays and antibody production was analysed using flow cytometry. This result suggested that, with a sufficiently immunogenic preparation, specific anti-tumour responses could also be induced against DFTD. However, trials using six different killed DFTD cell preparations failed to induce consistent immune responses, with only two of fourteen devils showing evidence for a response against whole cells.

During this project, investigations performed between our laboratory and collaborators verified a lack of MHC I expression on DFTD tumours. In the absence of this protein, cytotoxic T lymphocytes would not target the tumours, providing an explanation for the poor response in the vaccine trials. However, the K562 tumour cells used in the xenogeneic immunisations also lacked surface MHC I, but they were successfully recognised by the Tasmanian devil's immune system. This thesis also analysed the mechanisms of this anti-tumour response against K562 cells. Evidence was provided for the presence of functional natural killer (NK)-like cells in Tasmanian devils, which could consistently kill K562 cells by antibody dependent cell mediated cytotoxicity (ADCC). However, although Tasmanian devils can form strong ADCC responses against MHC I negative cells, antibody responses against DFTD are generally poor, and the results of initial experiments testing ADCC killing of DFTD cells were not promising.

Some immunotherapy strategies used in humans can induce MHC I independent killing of tumour cells using non-specific stimulation with cytokines, activating antibodies or mitogens. This thesis reports the discovery of a technique which can consistently activate Tasmanian devil lymphocytes to target DFTD cells. Stimulation with the mitogen Con A resulted in the generation of cytotoxic cells which had the capacity to kill up to 80% of DFTD cells *in vitro*. Activation could also be achieved using the cytokine-rich supernatant from Con A cultures, recombinant Tasmanian devil IL-2 and the toll-like receptor agonist Poly I:C. This promising result provided a basis for immunotherapy of DFTD, and this thesis also reports the use of these mitogen-activated killer cells in the first successful treatment of DFTD in a Tasmanian devil.

The work presented in this thesis provided the first evidence for functional anti-tumour responses in Tasmanian devils, and that it is possible to induce cytotoxic responses against DFTD cells in Tasmanian devils. The identification of NK-like cells and a technique to consistently activate these and other lymphocytes to kill DFTD cells was a major advance which provided a basis for an immunotherapy. The results of this preliminary trial were extremely promising and should direct the development of vaccine and immunotherapy strategies for the disease in the future.

Commonly Used Abbreviations

ADCC	Antibody Dependent Cell-mediated Cytotoxicity
APC	Antigen Presenting Cell
β_2M	Beta 2 Microglobulin
CD(3)	Cluster of Differentiation (eg. CD3)
CD (1)	Captive devil (eg. CD 1)
cDNA	Coding DNA
CIK	Cytokine-Induced Killer (Cell)
Con A	Concanavalin A
Con A sup	Cytokine rich supernatant from Con A lymphocyte cultures
CpG	Referring to a synthetic oligonucleotides which contains repeated motifs containing Cytosine and Guanine bases
CPM	Counts Per Minute (Radiation emission measure)
CTVT	Canine Transmissible Venereal Tumour
CTL	Cytotoxic T lymphocyte
DC	Dendritic Cell
DD	Diseased wild devil
devil	Tasmanian devil
DFTD	Devil Facial Tumour Disease
DNA	Deoxyribonucleic Acid
DPIPWE	Tasmanian Department of Primary Industries, Parks, Wildlife and the Environment
ECM	Extracellular Matrix
ELISA	Enzyme-Linked Immunosorbent Assay
FACS	Flow-Assisted Cell Sorting (Flow cytometry)
FBS	Foetal Bovine Serum

FcR	Fc (antibody portion) Receptor
g	Grams (unit of mass) var. Kilograms (kg), milligrams (mg)
g (Italicised)	Gravities (unit of centrifugal force)
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
Gy	Grey (radiation unit)
HCC	Hepatocellular Carcinoma
HPV	Human Papilloma Virus
HRP	Horseradish Peroxidase
HSP	Heat Shock Protein
Ig	Immunoglobulin
IFN	Interferon
IL	Interleukin
L	Litres (unit of volume) var. mililitres (mL), microlitres (µL)
LAK	Lymphokine Activated Killer (Cell)
LSAB	labelled streptavidin biotin
m	Meters (unit of measurement) var. centimetres (cm), nanometers (nm)
MAK	Mitogen-Activated Killer (Cell)
MHC	Major Histocompatibility Complex
MLR	Mixed Lymphocyte Reaction
MMP	Matrix Metalloproteinase
MNC	Mononuclear Cells
mRNA	Messenger RNA
NCAM	Neural Cell Adhesion Molecule
NK	Natural Killer (Cell)
NNAC	Nylon wool Non Adherent Cells

PAMP	Pathogen Associated Molecular Pattern
PBS	Phosphate Buffered Saline
PHA	Phytohaemagglutanin
PNAC	Plastic Non Adherent Cells
Poly I:C	Polyinosinic:polycytidylic acid
PRR	Pattern Recognition Receptor
PRX	Periaxin
RCC	Renal Cell Carcinoma
RNA	Ribonucleic Acid
TAA	Tumour Associated Antigens
TAP	Transporter associated with Antigen Processing
TCR	T cell Receptor
TGF	Transforming Growth Factor
TH	T Helper (Cell)
TIL	Tumour Infiltrating Lymphocytes
TLR	Toll-like Receptor
TNF	Tumour Necrosis Factor
Treg	T Regulatory Cell
TSA	Trichostatin A
VEGF	Vascular Endothelial Growth Factor
WD	Wild devil

Chapter 1 - Literature Review

The Tasmanian devil (*Sarcophilus harrisii*), the world's largest extant marsupial carnivore, has recently become the host of an extraordinary disease. Devil facial tumour disease (DFTD), is a contagious cancer that has emerged from a single founder female Tasmanian devil [1]. Emergence of a transmissible cancer is extremely rare, as cancer usually originates within, and only affects, one animal. For this to occur, the cancer must evade the host's immune surveillance system, avoiding an allogenic immune response. Consistent with this hypothesis, wild devils show no evidence of anti-tumour responses when infected with the disease [1] although they are otherwise capable of mounting functional immune responses [1,2] and rejecting allografts [3]. The spread of DFTD has resulted in a severe population decline and may drive this unique animal to extinction [4]. This chapter contains a literature review which will discuss current knowledge of immune responses in mammals and the Tasmanian devil, the characteristics of DFTD and possible immunological interventions for use against it.

1.1 Innate Immunity

All living creatures require protection from pathogens to survive. Organisms from all kingdoms display a wide variety of immune mechanisms designed to protect against microbial invasion, differing in specificity and complexity from the evolution of diversity-generating retroelements in bacteria [5] to the complex, multi-faceted immune systems of vertebrate animals. Within this vast diversity, this literature review will particularly concentrate on the immune system of one particular class of animals, mammals, and their responses against one disease; cancer.

Mammals have developed complex immune responses capable of protecting against acquired pathogens and environmental antigens. Broadly, the immune system is divided into two branches: innate immunity which is responsible for consistent, first line defences against all pathogens, and specific (or adaptive) immunity in which powerful responses can be developed against individual pathogens. The innate immune system comprises several levels of response, including physical barriers, a diverse symbiotic microbial flora, chemical components including proteins and cellular defences [6]. Together, these different sections of the innate immune system provide a highly successful initial defence against invading pathogens.

1.1.1 Physical, chemical and biological barriers

Initial exclusion of pathogens is accomplished at epithelial barriers. On the external surface, the skin consists of numerous constantly replenished strata cells, which provide a thick barrier from the

environment [7]. Interior surfaces including the respiratory, gastrointestinal and reproductive tracts are covered by mucous membranes, which differ in structure and complexity depending on their location. Examples of specialised structure and function include the epithelium of the lung, which consists of ciliated cells and a thick secretion of mucus allowing it to effectively trap and physically remove pathogens ([8], reviewed in [9]). Some epithelial surfaces accumulate immune proteins within mucus, such as the accumulation of immunoglobulins and cytokines in the cervical mucus in the reproductive tract [10] to further protect against pathogens by altering immune responses at the site. All types of epithelia house residual populations of innate immune cells. When the barriers are compromised, such as through burns [11], wounds [12] or menstruation [13], these components are ready to continue the innate immune response.

Several types of immune proteins are produced in the innate immune response. Firstly, epithelial and residual immune cells at the site of infection produce cytokines, including Interleukins (IL), interferons (IFN) and tumour necrosis factor alpha (TNFα), to alter the milieu in favour of inflammation as soon as the barrier is compromised [14,15]. Increased release of TNFα and IL-1 by tissue dwelling macrophages initiates the upregulation of adhesion molecules in the epithelial cells of blood vessels near the site [16]. Macrophages and endothelial cells then release IL-8 which, like TNFα, is a powerful chemoattractant for neutrophils and increases extravasation at the site of infection. In the case of endothelial cells, the IL-8 is stored in granules known as Weibel-Palade bodies, ready for rapid release [17]. Endothelial cells at the site of infection are also capable of producing IFNγ, a cytokine which acts primarily to activate Natural Killer (NK) cells to eliminate virus infected cells [18] but can also promote neutrophil phagocytosis [19] and activate cells of the adaptive immune system [20].

Complement is another group of small innate immune proteins that is present in the blood. The components of the complement system are synthesized in the liver and have several primary actions to augment innate cell responses. In situations of infection, each component in the complement pathway is activated in a set sequence, or ‘cascade’ [6]. There are three pathways for the activation of the complement system. The ‘classical’ complement activation pathway, so called because it was the first of the three mechanisms identified, is engaged following antibody-antigen interaction. This pathway therefore requires activation of responses from the adaptive immune system and provides an opportunity for interaction between the two branches of immunity (reviewed in [21]). The ‘alternative’ complement pathway is activated by direct binding to the pattern associated molecular patterns (PAMPs) of a microbe. A specific example of a potent molecular activator for the alternative complement pathway is the measles virus envelope fusion protein [22]. Activation of the ‘lectin’

complement pathway occurs through the engagement of lectin receptors, such as mannose receptors, with target molecules on the cell surface of a pathogen. This pathway often serves to enhance the activation of the alternative complement pathway [23]. The effects of complement proteins mimic and supplement the effects of cytokines in the innate immune system, including cell migration and phagocytosis. The activated complement protein C5a shows a similar effect to that of IL-8 and serves as a chemoattractant for neutrophils [24] while C3a is a powerful inducer of extravasation [25]. Other complement proteins, such as C3b, function as opsonins to increase phagocytosis [21] or, in the case of C9, mediate direct lysis of target pathogens through pore formation by multimerisation and insertion into the cell membrane [26] via attachment to C5b, C6, C7, and C8 [27].

1.1.2 Cellular responses

The cells of the innate immune system are able to respond against a variety of invading pathogens due to the expression of pathogen-associated molecular patterns (PAMPs). Examples include bacterial products such as lipopolysaccharide and flagellin, or virus associated molecules such as double-stranded RNA. The receptors for PAMPs are the pattern recognition receptors (PRR) of the mammalian innate immune system [6]. As previously discussed, plants also have PRR [28]which, although they bear many similarities to the receptors in animals, are thought to have arisen through convergent evolution [29]. There are three main classes of PRR: mannose receptors, glycosphingolipids and toll-like receptors (reviewed in [30,31]).

Mannose receptors are expressed on the surface of mononuclear phagocytes (monocytes and macrophages) [32] and subsets of dendritic cells [33]. They are glycoproteins that interact with glycoconjugates bearing terminal D-mannose, L-fucose and N-acetylglucosamine residues. These include microbial polysaccharides, glycoproteins and glycolipids (reviewed in [34]). Binding occurs through interaction of the target molecule with carbohydrate recognition domains [33] and stimulation of mannose receptors activates phagocytosis [35].

Glycosphingolipids are highly expressed on neutrophils where they serve as attachment points for many pathogen-derived molecules, including viral proteins, glycoproteins and carbohydrates [36,37]. The binding between microbes and sphingolipids occurs at terminal carbohydrate residues [37] and the signals are transduced through intramembrane domains via a PI-3K-dependent signal transduction pathway [38,39]. Glycosphingolipid binding contributes to the induction of phagocytosis in neutrophils under non-opsonised conditions [36].

Members of the toll-like receptor (TLR) family of PRR respond to a variety of common pathogenic stimuli. One example of interactive TLR responses is those against bacteria. The ligand for TLR regulated responses against gram negative bacteria is lipopolysaccharide, which activates TLR4 [40] on the cell surface [41]. Peptidoglycan, a molecule associated with gram positive bacteria, activates TLR2, as well as mannose receptors [40], on the cell surface. TLR2 is then endocytosed along with the pathogen and recruits an additional TLR (TLR6) to the phagosome membrane, where they co-operatively induce the production of TNFα [40,42] and induce oxidative destruction of the microbe [43]. However, within this study, two TLRs are of key importance: TLR3 and TLR9.

Toll-like receptor 3 is expressed internally and on the cell surface of fibroblasts and epithelial cells. However, in immune cells its expression is localised to the endosomal compartment [44]. The major role of TLR3 is thought to be the induction of immune responses against viral infection, as a major experimental ligand for its activation is the synthetic double-stranded RNA molecule, Polyinosinic:polycytidylic acid (Poly I:C) [45]. Activation of TLR3 induces the production of inflammatory cytokines including type I IFN [44], IFNγ [46], IL-6, IL-8 [47], TNF α, and IL-12 [45]. Binding of ligands such as Poly I:C to TLR3 can also stimulate the upregulation of activating receptors in some innate cell subsets, including CD69 [48], a receptor which mediates proliferation of lymphocytes such as NK cells. TLR3 activation can also induce responses from components of the adaptive immune system [49,50].

Another TLR with wide ranging effects on both innate and adaptive immune responses is TLR9 [51]. The major ligands for these receptors are single stranded DNA fragments containing repeated motifs of unmethylated cytosine and guanine nucleotides, a characteristic which is more common in bacterial DNA than eukaryotic DNA [52]. Stimulation of TLR9 induces expression of a similar milieu of inflammatory cytokines to those released in response to TLR3 activation [31,53]. Although the ability of TLR9 to activate NK cell cytotoxicity is well established [54], recent work has shown that stimulation of TLR9 may play a role in communication between the innate and adaptive immune responses through activation of dendritic cell antigen presentation [55] and activation of adaptive immune subsets including T and B lymphocytes [52,56]. As such, synthetic agonists of TLR9 and TLR3 are good candidates for adjuvant supplements in vaccines [45,56].

1.1.3 Phagocytic cells

Removal of microbes by phagocytosis is central to innate defence against pathogens. Polymorphonuclear leukocytes, commonly known as neutrophils, are the most abundant phagocytic lymphocytes in the circulation [57,58]. Neutrophils have a distinctive histological appearance, with

multilobar nuclei and cytoplasmic granules, and expression of the marker protein NIMP-R14 [59]. Eosinophils and basophils are also types of polymorphonuclear leukocytes, which function mainly in allergy and responses against parasites [60,61]. Macrophages are mononuclear cells which label positively for express CD14 and CD68 [62,63]. Neutrophils and macrophages are the main effectors of destructive phagocytosis of pathogens [58,64] and are sometimes known as 'professional phagocytes'. The process involves attachment of the microbes through interaction with PAMPS, then uptake to intracellular vesicles. Upon activation of PRR in the vesicle membrane [40], the pathogen is then destroyed using chemical degradation by reactive oxygen species and enzymes such as peroxidise and superoxide radicals [43,65]. Neutrophils and macrophages produce inflammatory cytokines which can augment the activity of other innate phagocytes and promote responses in other cell subsets, including those of the adaptive immune system [16,66].

An additional role for macrophages is to present antigens to cells of the specific immune system [30]. Following phagocytosis of a pathogen, a macrophage can process its proteins into short peptides which are then expressed on the cell surface. This process is known as antigen 'presentation', and plays a vital role in the induction of specific responses against infections. Due to their ability to perform this function, macrophages are classed as 'professional' antigen presenting cells (APC).

Dendritic cells (DC) are also innate phagocytes and APC. They have a distinctive shape *in situ*, with numerous dendrite-like processes [67] and exist in low numbers in peripheral tissues [33], where they sample peptides from their environment and phagocytose microbes then efficiently process them for antigen presentation [33,68]. Although DC are part of the innate immune system, their principal function is to present antigens to naive T cells of the adaptive immune system. They primarily interact with T lymphocytes [69], inducing production of cytokines with subsequent activation of B lymphocytes [70] and cytotoxicity from innate cells [71]. They therefore form an important bridge between the two immune branches, a role which will be discussed in detail later in this review.

Monocytes, the precursor for macrophages and DC, are capable of a wide range of innate effector functions. Their differentiation is controlled by the presence of the cytokine GM-CSF, while other cytokines drive the further specific differentiation of the final cell types. In general, macrophage development requires exposure to IL-3 [72] while transition to DC requires the presence of IL-4 [73]. Other factors can bias this development towards differentiation to DC, including the inflammatory cytokine TNFα [74]. Undifferentiated monocytes are also capable of cytotoxicity against tumour

cells in the presence of cytokines such as IFNγ [75] and TNF α [76]. Additionally, they can function as effectors of antibody-dependent cell mediated cytotoxicity responses [77].

1.1.4 Innate cytotoxicity: Natural Killer cells

The most effective cytotoxic responses of the innate immune system are mediated by NK cells. Structurally, NK cells appear as large lymphocytes with abundant cytoplasmic granules [78]. They are lymphoid derived [79] but are phenotypically and functionally different from lymphocytes of the specific immune system. NK cells are commonly characterised by the expression of the activating ligand and adhesion molecule CD56 and the Fcγ III receptor, CD16 [80]. A large proportion of NK cells also express CD8, the marker for cytotoxic T lymphocytes [81]. These identifying characteristics highlight important structural features corresponding to their major functions of NK cells: they are 'constitutively' activated for formation of rapid cytotoxicity responses, capable of mediating antibody-dependent cell mediated cytotoxicity and produce cytokines capable of augmenting both innate and specific responses.

The responses of NK cells, like other lymphocytes, are directed by interaction with the major histocompatibility complex (MHC) proteins of target cells. The targets for NK cell cytotoxicity are cells with missing, or aberrant, MHC I molecules [82]. The NK cell receptor for MHC I consists of a complex between CD94 and NKG2, known as the KLRC complex [83]. When binding to cells expressing normal MHC I molecules occurs, NK cells receive an inhibitory signal and release the cell undamaged. Upon encounter with a target cell in which cell surface MHC I is absent or aberrant, the NK cell will mount a cytotoxic response. For this reason, NK cells are termed 'unrestricted' by MHC molecules and have the capacity to kill abnormal cells which are otherwise resistant to cytotoxic lymphocyte responses.

The NK cell response against specific target cells requires the formation of a tight junction, or 'immunological synapse' between the two cells. The NCAM protein (CD56), which is highly expressed in NK cells, aids in attachment to target cells [84]. Circulating natural killer cells contain high concentrations of cytotoxic granules [80] which are released when in contact with a target cell. The events leading to granule release are characterised by rapid restructuring of the cytoskeleton [85], relocation of liposomes , which contain cytotoxic granules composed of perforin and granzymes, to the cell surface [86], and release into the 'immunological synapse' between killer and target cell. These two protein components have distinct functions for the induction of cytotoxicity. Perforin is a pore-forming protein which associates with the membrane of the target cell in a very similar manner to that of the complement protein C9 [87] and causes osmotic stress and lysis, and by forming an

entry point for other cytotoxic factors [88]. Granzymes are cytotoxic proteases which initiate apoptosis through activation of the caspase cascade beginning with the cleavage of caspase 3 [89]. The mechanisms of cytotoxicity are similar between NK cells and cytotoxic T lymphocytes (CTL) of the specific immune system, and the two cell types often serve complementary functions.

Typical target cells for NK cells include virus-infected, transformed or tumour cells with absent or abnormal MHC and xenogeneic cells with intact but foreign MHC (reviewed in [82]). NK cells are effectively activated by inflammatory cytokines, such as IL-15 from the innate immune system, IFNγ and IL-12 from both the innate and adaptive immune systems, and IL-2 from the adaptive immune system [18,90,91]. Exposure to IL-21 enhances the proliferation and cytotoxic responses of NK cells in conjunction with IL-15, an effect which can be perpetuated in vitro using non-specific agonists such as Flt3 ligand [92]. Activation by some TLRs, especially TLR3 and TLR9 which mimic viral infection, directly induce NK cell cytotoxicity [54,93]. The wide variety of activation pathways and the capacity for rapid and unrestricted cytotoxic responses make NK cells an attractive target for manipulation using vaccines and immunotherapies.

1.2 Adaptive immunity

One important characteristic of the vertebrate immune response is the capacity to form long-lasting and specific responses. The adaptive immune response, so named because it allows the host to 'adapt' and develop resistance against infections, is mediated by cells such as T lymphocytes, which recognise a target antigen, and antibody-producing B lymphocytes. Adaptive immune responses are crucial for immunity against tumours and foreign cells. However, the success of a specific response is often achieved in concert with factors from the innate immune system, and the interaction between the two branches of immunity will also be discussed.

1.2.1 Antigen presentation and costimulation

T lymphocytes require access to individual peptides in order to form specific responses. The three major pathways for antigen 'presentation' for T lymphocyte activation in mammalian cells are: MHC I restricted presentation, which occurs in all nucleated cells; MHC II restricted presentation and cross presentation, which primarily occur in DC [94,95,96,97]. B lymphocytes can also endocytose specific antigens that match their B cell receptor and present these to T lymphocytes [98].

The MHC I restricted pathway provides a mechanism to allow presentation of antigens by all cells for immune surveillance [99]. In this pathway, proteins in the cell can be tagged with ubiquitin molecules and degraded into small peptides within a proteasome [100]. The peptide fragments are

then transported to the endoplasmic reticulum via transporter associated with antigen processing (TAP) proteins [101]. The peptide is then bound to MHC I molecules which is coupled with the stabilising molecule beta 2 microglobulin (β_2M), a process in which TAP also plays a crucial role [102]. The peptide-MHC I complex is transported to the cell surface where it is bound to the T cell receptor (TCR) in combination with the MHC I-specific ligand CD8 [103,104]. Thus, the pathway that results in presentation to CD8 positive cells, most commonly CTL, is termed the 'MHC I restricted' pathway.

Like the MHC I pathway, presentation of antigen on MHC II molecules are also 'restricted' to T lymphocytes bearing a specific ligand: CD4. T cell populations positive for this molecule include T helper lymphocytes and T regulatory lymphocytes. The MHC II restricted pathway involves fusion of phagosomes with lysosomes, vesicles that contain proteolytic enzymes stored at an acidic pH, which is optimal for their function [105]. Presentation of MHC II molecules on the membrane of APC results in the exposure of pathogenic proteins to T cells via attachment of the TCR and CD4 molecules [103]. The third major antigen-presentation pathway is cross presentation, which involves the binding of peptides that have been processed through the Class II pathway onto class I MHC molecules. This confers some APC the ability to present antigen directly to CD8 positive cells, such as CTL, rather than only to CD4 positive cells [97]. This is an important pathway for cell-mediated immunity as it results in direct activation of cytotoxic lymphocytes against pathogens and non-self antigens.

A fourth antigen presentation pathway, known as cross-dressing, has recently been proposed. This pathway involves the direct transfer of preformed antigen-MHC I complexes from donor cells to professional APC without the need for further antigen processing by trogocytosis [106]. A study performed by Wakim and Bevan [107] presented evidence to support the theory of cross-dressing, and suggested a role for this pathway in the activation of $CD8^+$ T lymphocytes, particularly those previously activated by exposure to antigen.

As well as presenting antigens to specific immune cells, APC are also responsible for the co-stimulation of T lymphocytes, a process which is required to fully activate their immune responses [108]. Thus, activation of T lymphocytes requires two signals: the first is the encounter of antigen, via the TCR as described above, and the second requires a 'co-stimulatory' signal. In the case of inflammatory responses, this is typically provided through interaction of molecules expressed on the membrane of APC and the T cell [108,109]. The best known costimulatory ligand is CD28, which interacts with B7 molecules on the T cell membrane [110]. The original view that costimulation through the CD28/B7 pathway was essential for activation of T lymphocyte responses became the subject of some conflict within the literature [111], especially when analysis of CD28 deficient mice

strains showed a capacity for development of T lymphocyte-mediated responses [112]. Additionally, some responses, such as the production of IL-4, do not require co-stimulation [113]. The complexities of costimulation were subsequently explained by the discovery that a range of stimuli can co-activate T lymphocytes. Among these were inducible costimulator ICOS (otherwise known as CD278), and soluble factors such as IL-1 [114]. These factors largely influence T lymphocyte responses which augment antibody production, and the CD28/B7 pathway appears to be crucial for the induction of effective TH1-driven inflammation, through the induction of IL-2 secretion, lymphocyte proliferation and by delaying apoptosis [115].

1.2.2 Cellular immunity

As previously discussed, T lymphocytes are broadly defined in two classes: CD4 positive and CD8 positive lymphocytes. The two subsets of T lymphocytes have distinct functions in mediating antigen-specific immune responses. CD4 positive T lymphocytes are indirect effector cells which can either be capable of augmenting or diminishing and controlling immune responses. T helper (TH) lymphocytes perform the former function, whereas different types of T regulatory cells are responsible for the latter. Within the population of CD4 lymphocyte population there are a number of functionally distinct subsets, including the inflammatory TH_1, TH_2 and TH_{17} cells [116,117], and regulatory types including $FoxP3^+/CD25^+$ regulatory cells (Tregs) and TH_3 cells [118].The best characterised inflammatory CD4 cell types are TH_1 and TH_2 cells, which promote cellular immunity and inflammation or humoral responses, respectively. The most characterised T regulatory cells are the $FoxP3^+/CD25^+$ Tregs. The activity of T regs is modulated by TH_3 cells, which are believed to arise from a different lineage, are involved in mucosal immunity and produce large amounts of TGFβ, a cytokine with a complex role in tumour immunity and increases the activity of Treg cells [119,120]. In relation to tumour immunity, type 1 (TH_1) responses are the most desirable outcome of lymphocyte activity. CD8 positive cells most often mediate direct responses against target cells, particularly cytotoxicity. Conversely, inflammatory responses against tumours can be reduced through the activity of regulatory TH cell types, which can induce tolerogenic tumour microenvironments. Activation of inflammatory T lymphocyte responses and the prevention of tolerance is crucial for successful responses against virus-infected cells and tumours, and also plays a pivotal role in graft rejection.

As previously discussed, TH cells are primed to respond against specific antigens following encounter of antigen presented by professional APC, through binding of TCR in conjunction with CD4 receptors [103]. If costimulation also occurs, TH lymphocytes are able to proliferate and secrete copious amounts of cytokines to augment and direct the development of immune responses [116]. The

cytokine milieu determines the bias of TH cells towards type 1 or 2 responses. In particular, IFNγ induces TH_1 responses and IL-4 directs TH_2 responses. In a TH_1 type response, effector cells produce IL-2 and IFNγ [116], which can direct the responses of cytotoxic lymphocytes or innate immune cells, including NK cells and macrophages [121,122], and promote the release of inflammatory factors, such as IL-12, in other cell subsets [123].

Cytotoxic T lymphocytes (CTL) express CD8 receptors and are primed through encounter of specific antigen presented on MHC I molecules. This 'restriction' for MHC I, which is expressed on all nucleated cells, allows CTL to function as the primary effectors of immune surveillance [124,125] and responses are induced against cells which display abnormal antigens. As previously discussed, some cytotoxic pathways of CTL are very similar to those of NK cells and are mediated through a number of mechanisms. In particular the granule exocytosis pathway, which involves the release of cytotoxic granules into the synaptic cleft resulting in lysis of the target cell membrane through perforin insertion and the activation of apoptosis by granzymes is similar between the two types [87]. Other apoptosis inducing molecules, Fas ligand (FasL) and TNFα, can also play important roles in CTL cytotoxicity [126]. The effector responses of CTL can be perpetuated by cytokines produced by a variety of cell types. Cytotoxic cells proliferate in response to IL-2 [127] which is largely produced by TH_1 cells [128]. Potent CTL responses are also formed in the presence of IFNγ , which is produced by NK cells and macrophages as well as TH_1 cells [129]. Studies in both CTL clones and primary cells have verified that these cells release IL-2, IFNγ and GM-CSF in various models of activation, such as Concanavalin A stimulation [130,131]. Selective proliferation and increased cytotoxicity of CTL occurs in the presence of IL-21 [92,132], a cytokine which is largely produced by helper T lymphocytes. Functional studies performed using antibodies as agonists also suggest that IL-21 can enhance the effects of co-stimulation and TLR activation in CTL [92]. Thus, many sources contribute to the upregulation of CTL responses.

The capacity for T and B lymphocytes to form specific memory responses against individual diseases is an important ability of the mammalian adaptive immune response. Following the initial response against cells or pathogens bearing the target antigen, a small proportion of T lymphocytes become memory cells, which are characterised by expression of CD45RO molecules [133]. Memory T lymphocytes have the capacity to rapidly proliferate and mount specific responses upon subsequent encounter of their target antigen [134] and to recruit naive T cells to respond [135]. Memory T cells can be CD4 or CD8 positive, and give rise to mainly cytokine-producing or cytotoxic effector lymphocytes depending on their existing bias [135,136]. The persistence of memory T cells is crucial for the success of vaccines and for the development of long-term immunity against common

infections. Although CTL play crucial roles in the elimination all abnormal cell types, including virus-infected cells, their roles in graft rejection and tumour immunity are of considerable importance in this study, and will be reviewed in more detail in later sections.

1.2.3 Humoral Immunity

B lymphocytes of the adaptive immune system are responsible for the production of antibody. The B lymphocytes of mammals derive from the same precursor as T lymphocytes, but develop in the bone marrow and later migrate to the spleen [137]. The receptors of B lymphocytes (B cell receptors) are formed through random recombination of variable diverse joined (VDJ) gene segments, which produce a variety of receptors that can bind unique antigens [6]. Maturation continues in the periphery, where naive B lymphocytes encounter antigens within the spleen or lymph nodes. B cells can process environmental antigens through the MHC II restricted pathway [138], although antigen presentation is not their major role. Upon encounter of specific antigen, B cells form antigen-specific receptors, which can be synthesized for expression on the cell surface in a soluble form (antibody) [98].

As B lymphocytes mature they undergo immunoglobulin isotype switching to different isotypes, beginning with IgM and usually switching to IgG then to IgA or IgE in a subset of cells. In the final stage of maturation, some B lymphocytes become plasma cells, a transition which involves loss of the capacity to present antigen and a switch to produce IgG antibody (reviewed in [139]). Once B lymphocytes become differentiated into plasma cells they can persist in the bone marrow or in the periphery, secreting low levels of specific antibodies against their target antigen. Additionally, some B lymphocytes differentiate into 'memory cells'. The memory B lymphocytes which reside in the germinal centres of lymphoid follicles are well located to encounter their B cell receptor specific antigen. The production of specific antibodies by B lymphocytes plays an important role in the immune responses against many diseases. B cell memory is also the basis of the majority of vaccines licensed for use in humans. In disease, the effects of antibody include agglutination of virus particles (IgM), immunity at mucous membranes (IgA), responses against parasites and helminths (IgE), opsonisation of microbes and formation of attachment points for NK cell and macrophage cytotoxicity against foreign and tumour cells (IgG). Antibody also mediates the first stage of graft rejection responses, through the formation of lytic plaques in blood vessels [140].

1.2.4 Innate/Adaptive Interaction

In the past, the innate and adaptive immune systems were regarded as separate branches which were responsible for distinct immune responses. However, recent studies have shown that constant interaction between the two immune branches drives optimal responses (reviewed in [141]). In this Thesis, three specific mechanisms of interaction between the innate and adaptive immune responses have been particularly significant: stimulation of cytotoxic responses with agonists of TLR receptors, activation of innate or non-specific cytotoxic responses using a heterogeneous group of cytokines and induction of antibody-dependent cytotoxicity.

As previously discussed, activation of toll-like receptors on innate immune cells has a wide range of effects [44,54,142], including the development of adaptive responses by B and T lymphocytes [52,56,143]. Other effects of TLR9 activation include upregulation of antigen presentation in DC [95] and production of cytokines such as IFNγ , which result in increased stimulation of T lymphocyte activity [144]. Activation of TLR9 can also induce maturation of dendritic cells [145]. Exposure to certain types of CpG oligonucleotides can also directly induce B lymphocyte proliferation, activate the cells upon simultaneous encounter with specific antigen, and can 'license' them for transition to plasma cells [52,146]. The effects of CpG on both the innate and specific immune responses make them a valuable addition to vaccine adjuvants [56], a characteristic which was utilised in experiments reported in this Thesis.

Interaction between the innate and adaptive immune responses can also be controlled by cytokines. An example of an adaptive immune-system cytokine with the capacity to augment responses in innate immune cells is IL-2. Although it is synthesized primarily by T lymphocytes, IL-2 can induce cytotoxic responses in NK cells [121,147]. The mechanism for this response is an upregulation of IFNγ [148], which itself is a cytokine capable of inducing responses from both innate and specific immune subsets. IFNγ is a powerful inflammatory cytokine, and its expression requires complex mechanisms for regulation. Major sources include NK cells [46] and helper T lymphocytes [129], but the cytokine is also produced in various macrophage populations, particularly following stimulation with a combination of IL-12 and IL-18. Although IL-12, like IL-2 and IFNγ, is a cytokine with the capacity to induce cytotoxic responses from NK cells and increases the inflammatory activity of macrophages [122,147], it can upregulate antibody responses [149] and can also stimulate T lymphocyte cytokine production, including secretion of IFNγ [150]. In fact, some studies have shown the capacity of IL-12 to selectively upregulate groups of helper T lymphocytes that are responsible for IFNγ production [151]. However, optimal production of IL-12 by DC requires the presence of IFNγ as well as stimulation of the activating ligand CD40 [152]. Thus, this intricate positive feedback

interaction between cytokines such as IL-2, IL-12 and IFNγ contributes to the activation of both innate and specific immune responses.

There are also many situations in which antibody, a component of the adaptive immune response, can induce responses by cells of the innate immune system. One important role of antibody is opsonisation of pathogens for phagocytosis by innate immune cells, such as neutrophils and macrophages, which bear receptors for the Fc portion of immunoglobulin. A related pathway, antibody-dependent cell-mediated cytotoxicity (ADCC) pathway is another example of interaction between innate and specific immune responses. Mediation of ADCC requires two individual factors: formation of specific antibody through activation of the adaptive immune response and cytotoxicity responses by effector cells bearing receptors for the Fc portion of immunoglobulin. Interestingly, the cytotoxic effector cells of the adaptive immune response, namely CTL, do not bear Fc receptors (FcR). However, innate immune cells including monocytes, macrophages, neutrophils, eosinophils and NK cells express different variations of FcR and are capable of killing cells coated in specific antibody [77,153,154,155,156]. In the case of NK cells, which express FcR IIIγ (CD16) molecules [157], cytotoxicity is mediated through the same lytic pathway as for other targets. Thus, although killing in the ADCC pathway is mediated by innate immune cells, the responses are specific due to the involvement of antibody. The ADCC pathway plays a role in graft rejection [158], particularly in acute responses [140]. One example for the capacity of ADCC to induce allograft rejection is response against tolerated skin grafts in ducks following adoptive infusion of hyperimmune serum [159]. Development of ADCC is also important in the immune response against cancers, and specific antibody against tumour associated proteins can induce responses from NK cells [160,161].

1.3 Immune responses against allografts

The ability to reject foreign cells from genetically different individuals is an important characteristic of the mammalian immune response. There are three different categories of graft rejection response in mammals: hyperacute, acute and chronic rejection. Many mechanisms have been shown to participate in graft rejection responses, including both innate and specific immune cells, ADCC and the activation of complement. Each of the three types of responses are mediated by different immune mechanisms and have distinct pathological characteristics. This section will discuss the graft rejection response.

The fastest and most effective type of response against allografts is hyperacute rejection, which happens within days of exposure to the foreign cells [162]. The mechanism responsible for hyperacute rejection is the binding of pre-formed antibodies, which activate the complement

cascade, leading to cell damage. Hyperacute rejection is a prominent cause of kidney transplant rejection [163], through antibody-mediated agglutination of donor erythrocytes in the blood vessels or destruction of MHC II mismatched donor B cells within the transplant tissue [164]. Exposure to mismatched ABO blood antigens or MHC I molecules can also cause hyperacute rejection, although this effect can be abrogated in some patients using immunoadsorbtion of antibody [165].

Acute rejection is the term used to describe rapid rejection that occurs anywhere from one week to several months following transplantation. The major mechanism responsible for acute allograft rejection is through the activation of cell-mediated immunity against mismatched MHC I molecules [166]. The activity of several cytokines, most notably TNFα, IFNγ and IL-10 [167,168], are particularly implicated in the responses against allografts, with patients prone to expressing high levels of TNFα and IFNγ particularly likely to have earlier rejection. The role of antibody in acute graft rejection remains controversial [140]. Unlike grafts which undergo hyperacute responses, biopsies of allografts following acute rejection show little evidence for the accumulation of antibody or complement components within blood vessels. However, although acute rejection can be mediated in the absence of antibody, a subset of patients pre-sensitised against graft-associated proteins can form antibody, resulting in ADCC responses by NK cells [140]. NK cells may also mediate cellular cytotoxicity responses against grafts in the absence of CTL and are particularly implicated in the rejection of bone marrow transplants [169].

Chronic rejection of a graft occurs after several months or years of full establishment and sufficient function. Some cases of chronic rejection are caused by fibrosis of blood vessels, resulting in insufficient circulation and necrosis of the transplant. In the remainder of cases, immunological mechanisms are involved in the rejection of long term grafts, although the pathogenesis of chronic rejection remains incompletely understood [140]. Similar to acute rejection, the development of alloreactive antibodies after transplantation is considered a risk factor for graft failure [170]. Additionally, blockade of CD4 signalling to inhibit TH cell responses does not affect the outcome of chronic cardiac graft rejection models but blockade of CD40L, which prevents their capacity to interact with B lymphocytes and augment CTL responses, leads to indefinite graft survival [171]. Although many studies have shown similar decreases in rejection responses through blockade of the CD40/CD40L pathway [172], some studies have shown little effect in overall outcome [173]. Chronic graft rejection can also involve an alteration of dendritic cell status from tolerogenic to a non-tolerogenic state, resulting in activation of TH cells to promote rejection [174]. Thus, multiple mechanisms can contribute to chronic rejection of allografts.

1.4 Immune responses against cancer

Following a breakdown in the regulation of cell division, increased growth of normal cells leads to the development of neoplasms , masses of abnormally dividing tissue [175]. The resultant lesions are often referred to as tumours (a word which simply means 'lumps'). Tumours are initially benign but they can progress to become malignant and form cancers. The transition to cancer requires a number of changes to the cells of a neoplasm: often less differentiated, immature cells within tumours tend to selectively proliferate, causing the tumour to lose hallmarks of the primary tissue and revert to a progenitor-like cell type; the cells divide at an uncontrollable rate and begin to invade neighbouring tissues, including the bloodstream where they can metastasise to other sites.

1.4.1 Cancer development and pathogenesis

It is widely acknowledged that development of a cancer requires a number of distinct mutation events [176,177]. Mechanisms for the development of cancer have been most thoroughly studied in human subjects. Genetic analysis has resulted in the identification of alleles responsible for a heritable tendency for the development of specific types of cancers, such as Wilms' tumour locus in kidney cancer and BRCA mutations in breast and ovarian cancers [178,179]. However, epidemiological studies involving identical twins suggest that genetic predisposition accounts for only a small percentage of cancers, suggesting that environmental factors play a greater role in their development [180]. Cancer risk can be increased by exposure to environmental carcinogens, such as cigarette smoke [181], hair dyes [182], food borne mutagens, such as 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) [183], or ultraviolet radiation [184]. Recurrent infection, especially with pathogens such as *Helicobacter pylori*, can also increase cancer risk [185,186]. Environmental carcinogens can exacerbate cancer development either by increasing rates of cell growth and mutation [175] or by contributing to suppression of the immune response [187].

Defining characteristics of cancers include the capacity to invade neighbouring tissues, to divert blood supply or undergo angiogenesis, and to evade the immune system. Some factors involved in cancer pathogenesis is are derived from normal physiological processes but overexpressed in cancer cells. This section will briefly discuss several examples of proteins that may aid invasion, angiogenesis and immune escape by cancer cells and their potential importance in a contagious cancer such as DFTD.

In order to become invasive and metastasise, a cancer must first break down their surrounding basement membrane, which involves destroying the type IV collagen that is secreted in the

extracellular matrix (ECM) of the tissue of origin [188]. The major class of enzymes that aid in this breakdown are the matrix metalloproteinases (MMP), a class of zinc-atom bearing endopeptidase proteins that normally function in the remodelling of ECM [189]. The expression of MMP is usually tightly controlled via a number of mechanisms including regulation of transcription, mRNA stability and protein degradation (reviewed in [190]). However, high levels of MMP expression in cells can impart an invasive phenotype [191,192,193] and is associated with progression in several types of human malignancies, including prostate, breast and colon cancer [194,195,196]. Levels of MMP are also modified by certain hormones, including platelet-derived growth factor, cytokines such as TNFα [197] and tissue inhibitors of metalloproteinases (TIMPs) [198]. Interestingly, the activity and production of MMP is induced by some inflammatory cytokines normally associated with anti-tumour responses, including TNFα, but downregulated by cytokines that are otherwise related to tumour progression, such as TGFβ [199,200]. Thus, the control of factors that aid in metastasis of cancers may be complex and difficult to modulate. Following a rapid increase in size, tumours become limited by blood supply and require angiogenesis for continued growth and invasion [201]. The secretion of MMP, in particular MMP-9, can play a role in increasing angiogenesis. Some tumours are able to recruit host cells, such as neutrophils, to produce a source of MMP-9 for increased angiogenesis [202]. Other endothelial cell growth factors, such as VEGF, are also important for angiogenesis and are therefore produced in large amounts by some cancer cells [203].

In addition to an increased rate of growth and angiogenesis, cancers must also evade, overcome or actively manipulate the host immune response. Many tumour types overexpress stress-associated chaperonin molecules, such as heat shock proteins (HSP), to delay or reduce apoptosis and increase proliferation [204]. Expression of HSP90 (90 kDa heat shock protein) and HSP 70 is associated with poor prognosis, particularly in breast and prostate cancer [205,206], and can contribute to disease severity, even in the absence of other cancer associated factors, such as VEGF expression [207]. One proposed mechanism for this relationship is that HSP, which normally act as a ‘molecular chaperone’ to preserve essential proteins under conditions of stress, may protect certain oncogenic proteins from degradation, although this remains contentious [208,209].

There is abundant evidence that many types of tumours evade the host immune response by altering the surface expression of proteins through epigenetic mechanisms. One of the most common epigenetic adaptations of cancer cells is to downregulate expression of antigen-presentation molecules, such as MHC I, TAP or accessory proteins within immunoproteasome (reviewed in [210]). In some cancer types, such as cervical cancer, the loss of MHC I expression occurs in several stages [211,212]. Another prominent epigenetic mechanism is the loss of tumour

associated antigens (TAA). This has been particularly well characterised with melanoma, in which immunoselection promotes the loss of TAA such as the MART-1/Melan-A antigen in more progressive tumours [213,214]. The loss of TAA can also occur following immunotherapy or vaccination with specific tumour antigens [215].

In addition to manipulating their own phenotype to evade immune responses, many types of tumours are capable of manipulating the host immune response to induce a tolerant immunophenotype. Tolerance to tumours is often mediated by the localised polarisation of Treg responses [216] or through the induction of tolerogenic DC (reviewed in [217]). Additionally, some tumours can induce chronic stimulation of CTL, resulting in corrupted memory function and exhaustion of the anti-tumour response[218]. Decreased expression of MHC I or the associated β_2M protein is common in many malignancies [219] including melanoma, particularly metastases, and colorectal cancer [220,221] and often correlates with a poor prognosis. *In vitro* studies using co-culture of melanoma cells with autologous lymphocytes or utilising the B16 melanoma cell line have confirmed that decreased expression of MHC I is associated with poor *in vitro* induction of CTL responses [220,222]. Another common mechanism for immune escape is the production of immunosuppressive cytokines, such as interleukin 10 and TGFβ. IL-10 can modulate the Th1 and CTL driven cellular anti-tumour response and protect tumours against apoptosis [223,224,225]. The role of TGFβ in tumorigenesis is complex; many types of immune cells can secrete TGFβ, which normally functions to regulate their own proliferation [226]. Additionally, as the cytokine can prevent growth of tumour cells, even at very low concentrations [227], it is thought to play a role in tumour prevention. However, some tumour types can become resistant to the effects of TGFβ activity [228,229] or can produce the cytokine to prevent proliferation and activity of immune cells in the local area. Additionally, resistance to TGFβ is often indicative of a more invasive and metastatic tumour phenotype [186,228,229].

1.4.2 Anti-tumour immune responses

Anti-tumour responses broadly progress in a sequence of events involving all of the components of the mammalian immune system. Initially tumours are infiltrated by antigen presenting cells, including macrophages and DC, which sample and process tumour associated antigens (TAA). The majority of these cells are immature DC [230] which then migrate to tumour-draining lymph nodes and activate TAA specific cellular responses, particularly those of TH_1 lymphocytes. This results in the production of abundant cytokines, including IL-2, TNFα and IFNα and the migration of T lymphocytes to the tumour site.

Within the tumour site, resident APC can produce cytokines including IL-12 that attract T lymphocytes. Increased levels of Th1 cytokines attract tumour- antigen primed CTL, which can mediate direct lysis of tumour cells. The presence of high numbers of $CD8^+$ CTL in tumours is correlated with better prognosis compared to a higher abundance of $CD4^+$ T lymphocytes, which can contain sub-populations of T regulatory (Treg) cells [231]. In tumours with absent, low or aberrant MHC I expression, NK cells can mediate anti-tumour cytotoxic responses. Cytokines including IL-2, IL-12 and IFNγ can induce tumour infiltration by NK cells and augment their cytotoxicity. NK cells can also interact with DC to increase cytokine production and promote cross-presentation of TAA to drive anti-tumour responses.

Development of ADCC responses is also important in the immune response against cancers. The increased response from $CD4^+$ T lymphocytes can also induce the production of tumour-specific antibody by B lymphocytes. This can induce direct anti-tumour ADCC responses from NK cells, macrophages and neutrophils. ADCC is the basis of some new anticancer therapeutics, which are antibodies targeted at epitopes common to cancer cells. These include Herceptin, which targets the oncogene HER2 [232] that is expressed highly in some breast cancers and adenocarcinomas [233] and can induce ADCC responses from NK cells [160]. Rituximab, a similar ADCC drug used for the treatment of leukaemia and lymphoma by targeting the CD20 protein [234], also induces NK cell responses [161].

The development of an anti-tumour response can produce at least five possible outcomes: tumour tolerance, immunoselection, tumour progression, stable disease or tumour regression. Tolerance to tumours is often mediated by the polarisation of Treg and dendritic cell responses [216,235]. This can occur rapidly and early in an anti-tumour response and can be a powerful suppressor of cytotoxic anti-tumour responses [236]. Immunoselection of tumours results in the generation of a more evasive phenotype, often through the downregulation of MHC I expression or production of immunosuppressive cytokines. Both of these outcomes usually lead to tumour progression, characterised by metastasis and growth, and a worse disease prognosis. If the immune system can successfully respond against a tumour, the result would either be stable disease or tumour regression. This outcome could lead to the resolution of the disease before its symptoms occur, or in improvement if clinical disease has been identified.

1.5 The canine transmissible venereal tumour: a contagious cancer

Cancer is usually an endogenous disease that originates from genetic changes leading to a loss of normal cell proliferation. Due to the activity of normal immune responses against cancers and

allografts, the emergence of a contagious tumour is extremely rare. Apart from isolated cases of tumour transmission between related or immunocompromised individuals, there are only two known naturally occurring diseases that are caused by transmissible tumours: DFTD and the Canine Transmissible Venereal Tumour (CTVT).

CTVT is an established disease in the canine populations of many countries, and affects dogs on all continuously populated continents. The tumours of this disease are transmitted to wounds on the genitals developed during mating or in the nose and mouth following sniffing or licking of the infected area. Following infection the tumours can initially grow rapidly in the absence of an immune response. There is abundant evidence for the aetiology of this disease, as tumours can be caused only through the transmission of live cells, either naturally or by experimental implantation [237,238], but not lysates or killed cells [239]. Additionally, all CTVT cells bear gross cytogenetic anomalies, which are similar between tumours but different to cells of the host. These include an altered number of chromosomes to between 58 – 60, compared to 78 in a normal canine cell, the loss of at least one sex chromosome and pronounced nucleoli in most cells [240,241]. Although the earliest records of this disease date back approximately 200 years, it is thought to have originated in a population of wolves or Asiatic dogs up to 2500 years ago [242]. However, the chromosomal alterations of CTVT were found to be very well conserved despite geographical divergence of the disease over a long period of time [241].

Unlike its aggressive counterpart, DFTD, CTVT does not usually develop into a malignant cancer. Although the tumour is initially capable of evading the host immune system, it later becomes sensitive to the anti-tumour response and undergoes regression. The immunological factors that characterise these two disease phases have been well characterised. During the period of immune escape CTVT cells downregulate the expression of β_2M and MHC I proteins on the cell surface [242,243] and secrete TGFβ [244], which act to suppress both CTL and NK cell anti-tumour responses [239,244]. The CTVT cells can also produce factors that can induce apoptosis in monocytes, DC and B lymphocytes [245] However, after several months of tumour growth, a sudden signal switch occurs; the tumour becomes susceptible to lymphocyte infiltration, leading to abundant production of IL-6 and an upregulation of MHC I in the tumour cells [243,244]. The cytotoxic activity of infiltrating lymphocytes leads to tumour necrosis [246] and rapid regression [237,247]. Following resolution of the tumours, protection against the disease is long-lasting and recovered dogs are usually immune to rechallenge with CTVT cells [238]. Although most CTVT infections regress, some cases result in metastatic disease and fatality. This is rare, reported in less than 5% of cases [247], and appears to be more common in oral or nasal disease [248] where metastasis occurs to the tonsils and adjacent

lymph nodes, but can also involve the spleen, skin, lungs and liver [249]. In some cases CTVT can become disseminated and result in mortality [250].

Although CTVT usually regresses spontaneously it is often treated. Surgery, chemotherapy, radiotherapy and immunotherapy are all effective for treatment of CTVT. Single agent chemotherapy with vincristine sulphate is an effective and preferred option and three infusions cures most CTVT cases [251]. Some CTVT tumours, particularly large tumours, those in older animals and those with a plasmacytoid cell type, are more resistant to vincristine therapy [252,253] in which case agents such as doxorubicin, methotrexate or cyclophosphamide can be used [251,254]. Radiotherapy of CTVT usually involves 10 Gy doses, with some tumours responding after a single treatment [255].

Although chemotherapy and radiotherapy are generally effective against CTVT, both treatments are associated with stress of the animal and deleterious side-effects. Common symptoms associated with the standard chemotherapy treatment include neutropenia, leukocytosis, thrombocytopaenia and red cell-related disorders such as haemoglobanemia [251]. Consequently, several studies have aimed to develop immunotherapies as less injurious treatment options for CTVT. In the past, vaccines attempted to generate immunity against the tumours in healthy dogs using tumour homogenate preparations or via passive transfer of antibodies from immune animals [256]. However, although some trials were successful the results between studies were highly variable [247]. More recently, studies have utilised modern immunotherapy methods, including generation of LAK cells [246] and injection of dendritic cell/tumour cell hybrids during early stages of tumour infection [257]. Immunisation with hybrid DC induced a significant reduction in maximum tumour size and showed no adverse side-effects in the recipients. This treament also generated systemic effects including CTL and NK cell cytotoxicity. Additionally, as the therapy in this trial was given as fortnightly sub-cutaneous injections it could be less injurious than weekly chemotherapy doses.

The two examples of naturally occurring contagious tumours have many characteristics in common. Both CTVT and DFTD bear gross karyotypic abnormalities compared to their hosts but have persisted as stable cell lines [241,258] and both initially infect without inducing an immune response in the host [1,237]. However, there are many fundamental differences between the two contagious tumours. Firstly, CTVT rarely becomes malignant and appears to co-exist successfully with its host through its pattern of infection and spontaneous regression. In contrast, DFTD is consistently fatal and does not generate an immune response. The immunological mechanisms responsible for the immune escape of CTVT may provide a good basis for the study of DFTD infection. Additionally, the

development of immunotherapies including LAK cells and hybrid vaccines against CTVT may also provide direction for the development of an immunological intervention against DFTD.

1.6 Devil Facial Tumour Disease: the cancer and its host

1.6.1 The Tasmanian devil

The Tasmanian devil (*Sarcophilus harrisii*) is the largest extant marsupial carnivore. It is approximately 'fox terrier-sized', with a short black or black and white coat, a large head, powerful jaws, and a 'strong, root-like tail' [259]. Typically, male Tasmanian devils weigh between 7.5 kg and 13 kg, while females are usually smaller at between 4.5 and 9kg [4]. Since the death of the last known thylacine in 1936, the devil has become the top predator in the natural Tasmanian ecosystem. They are also scavengers, and play an important role in the removal of carrion from the environment. The presence of the Tasmanian devils is considered the major contributing factor to the control of harmful feral species, such as foxes and feral cats. The devil belongs to an important species with a crucial environmental niche in Tasmania.

The Tasmanian devil is confined to the island of Tasmania. It does not inhabit any adjacent islands [260] except those on which it has been artificially introduced. Historically, the devil has been a widespread native species across the main island of Tasmania, although there have been anecdotal reports of population declines in the past [260]. Devils usually inhabit the pastures, woodland and costal scrub areas across northern, central, eastern and south-eastern Tasmania [4]. The distribution of the species in the south west of Tasmania has not been extensively examined due to its rugged topography and inclement conditions.

Tasmanian devils often travel long distances at night, although males tend to move about more than females [260]. Daily movements within devil populations average 9km [4] but anecdotal evidence from Tasmanian wildlife biologists suggest that some individuals can travel over 50 kilometers in one night [261]. Movement over such large distances is often necessary in search of food. The Tasmanian devil is the top predator in the natural food chain of the Tasmanian woodlands [4].Their diet mainly consists of carrion and can be highly varied. Post-mortem dissections of devils suggest that they eat other mammals and marsupials, fish, birds, roots, berries and grasses [260]. Food is mainly consumed as carrion although devils are also capable of hunting their prey [4]. Many devils can feed cooperatively on one carcass, but compete with their companions for a share [262]. These competitions involve the devils making loud noises and locking jaws in displays of strength, a behaviour which often results in wounds from the canine fangs. During most of the year, Tasmanian

devils are non-territorial with large home ranges which are often shared by many other devils [260]. Studies using radio collars to survey devil movement showed that individual devils interact with many others in one night's movement [263], although not all encounters result in aggression. The exception to their non-territorial behaviour is in the mating season, where both male and female devils fight competitively, and the courtship behaviour also involves biting [262]. During these aggressive encounters bite wounds most frequently occur on the face and head [262] and it is not uncommon to see adult devils with extensive facial scarring [4].

1.6.2 The immune response of the Tasmanian devil

Several studies have characterised innate and adaptive immune responses in Tasmanian devils. Lymphoid tissues, including the thymus, spleen and lymph nodes, are present in all animals, including juveniles, and all structures were similar in appearance to those in placental mammals [1]. The one notable difference in Tasmanian devil lymphoid tissue compared to other species was a large number of plasma cells in the spleen, lymph nodes and bone marrow [1]. Tasmanian devils have a normal range of white blood cells [2], which are similar in size and appearance to those of other animals. Immunohistochemistry suggests that the immune cells of Tasmanian devils express characteristic proteins appropriate to the different immune cell types. The T lymphocytes of devils express CD3 and their B lymphocytes express MHC II and CD79b protein [264]. Other antigen presenting cells such as monocytes, macrophages and DC also express MHC II protein, and markers such as CD11b [265].

The neutrophils of Tasmanian devils are capable of normal phagocytosis, an anticipated result in the immune system of a scavenging animal [2]. Their lymphocytes proliferate when stimulated *in vitro* with common mitogens, and there is no significant variation in this response with sex, age or between DFTD diseased and healthy animals [2]. Tasmanian devils are capable of mounting strong humoral responses, as demonstrated through immunisation with horse red blood cells [1]. These results provide evidence for competent innate and adaptive immune responses in Tasmanian devils.

1.6.3 Devil Facial Tumour Disease

The biting behaviour of the Tasmanian devil during mating, the co-operative sharing of food and the extended daily range of movement in shared territories would contribute to the spread of Devil Facial Tumour Disease (DFTD), a contagious cancer which was first identified in 1996 [4,266]. Its spread has caused vast decreases in species numbers and has brought some populations close to local extinction [267]. DFTD is invariably fatal to affected individuals [268], and wild animals show no

immune response against the disease [1]. The disease is characterised by 'disfiguring and debilitating' tumours in and around the mouth and on the face, head and neck [269]. The cells of the tumours are poorly differentiated [269], and distinctively round or spindle-shaped with large nuclei. The cells may be collected together in follicles or nests surrounded by sheaths of connective tissue [269]. There is abundant evidence to suggest that DFTD is a transmissible neoplasm, transferred between Tasmanian devils as an 'allograft' [266,270]. Therefore, rather than the involvement of a virus or microorganism, the tumour cells themselves are the aetiological agent of the disease. The most likely route of DFTD transmission is transfer of live tumour cells through biting [266], either during fighting or other means such as acquisition of viable cells into wounds during co-operative feeding or cannibalism of dead diseased devils [262].

A number of genetic, cytogenetic and molecular studies have indicated that the cells of the disease are clonal [266,270,271] and the original tumour would have arisen in a female Tasmanian devil in the early 1990s [272]. The first identified cases of DFTD were recorded in photos of devils taken by a wildlife photographer in 1996 at Mount William National Park in north-eastern Tasmania [4]. Ten years later, the disease was confirmed in devils from 41 separate sites covering over 51% of mainland Tasmania [4]. DFTD has since spread to many previously disease-free sites, and continues to encroach on uninfected populations. Currently, the only area that may be confidently labelled disease-free is the far north west of the state. Immunohistochemistry studies initially identified DFTD as an undifferentiated neoplasm of neuroendocrine origin [273]. DFTD cells stain strongly for the marker periaxin [274] and express many genes associated with the myelination pathway, suggesting that DFTD could have arisen from a Schwann cell of the peripheral nervous system [271].

The proportion of adult devils found to be infected in any one site by trapping surveys has reached up to 83% [4]. In the area where the disease first appeared, mean spotlighting sightings have decreased by 80% in the 10 years following disease arrival [4]. The most recent analysis of Tasmanian devil numbers using spotlighting data suggest a state-wide population decline of 80% since the disease emerged [261]. Epidemiological estimates published in 2007 suggested that the species will become extinct in the wild within 25 years [268]. However, it is possible that the rate of decline has increased over the subsequent years as the disease continues to infect new populations. There is therefore an urgent need to develop and implement a conservation strategy to prevent the extinction of the Tasmanian devil in the wild.

The original analysis of DFTD pathology was performed by Loh and colleagues [269]. The majority of DFTD tumours appear as large, solid, often multicentric tissue masses inside and outside the mouth, on the face and neck regions of infected devils. Ulceration and necrosis are common features of

large DFTD tumours. The tumours are highly invasive; metastasis occurs in at least 65 percent of cases, and commonly affects organs such as the lung, liver, kidneys and regional lymph nodes. These pathological characteristics of DFTD are implicated as factors in its ability to cause mortality in infected animals and also its capacity for infection. The frequent ulceration of DFTD tumours [269], which results in large areas of exudative surface, can increase the opportunity for secondary infection or loss of protein and can increase the friability of the underlying tumour. The rapid growth of the tumour and high rate of metastasis also contribute to DFTD-associated mortality. Other theories for the pathogenesis of DFTD suggest that the growth of tumours in and around the mouth may prevent feeding or interfere with senses used in the acquisition of food [275]. The collective evidence for the pathogenesis and epidemiology of DFTD suggests that it is a debilitating and dangerous disease with the capacity to irreversibly affect the Tasmanian devil population.

1.6.4 Theories for transmission of DFTD

Observations that wild Tasmanian devils infected with DFTD inevitably succumb to the tumour within a short period of time suggest that there is no host immune response developed against the disease [1]. Histological data from DFTD tumour biopsy samples supports this assertion, with no evidence for lymphocyte infiltration in the majority of tumour samples [273]. Since Tasmanian devils are otherwise prone to developing tumours [259], one explanation for this lack of immune response is generalised immunodeficiency resulting in a failure to mount functional anti-tumour responses. However, as previously discussed, studies examining the immune system of the Tasmanian devil have shown that the species possesses a range of functional responses. One deficit in the information from previous studies on the Tasmanian devil's immune system is an analysis of specific anti-tumour responses. Importantly, the capacity for lymphocyte cytotoxicity against tumours has not been assessed. However, given the current evidence for a competent immune system it appears unlikely that immunodeficiency is the basis of DFTD transmission.

There is abundant historical evidence for previous population declines in Tasmanian devils following the geographical isolation to the island of Tasmania [276]. As low population numbers can lead to inbreeding, and other marsupial species have been found to show decreased genetic diversity [277], this was initially investigated as a factor in the transmission of DFTD. Genetic studies analysing nuclear microsatelite markers indicated a limited general genetic diversity within the Tasmanian devil population [278]. The Tasmanian devil MHC I and II genes, which are critical for responses against allografts and tumours, were sequenced following construction of a spleen cDNA library [279]. Analysis of Tasmanian devil MHC I and II genes by single-strand conformational polymorphism PCR showed a limited genetic diversity at these loci throughout the eastern Tasmanian devil

population [270]. Additionally, poor responses were formed in functional studies involving *in vitro* mixed lymphocyte reactions (MLR) between unrelated devils [1,270]. From 2006 to 2011 this was generally accepted, and the laboratories leading these genetic studies were optimistic that genetically resistant animals would be identified within the wild population [280,281].

However, despite the initial evidence for a genetic basis of DFTD transmission, recent studies of disease dynamics and the Tasmanian devil immune response has superseded this theory. Analysis of mitochondrial DNA suggested that only a limited loss of genetic diversity has occurred over an extended time period [282]. A study examining the effect of MHC allele variation in wild populations on the transmission of DFTD found no effect [283]. Another trial documented the successful rejection of allogeneic skin grafts, even among devils that were thought to have identical MHC I genes [3]. This provided functional evidence to dispute the role of low genetic diversity in DFTD. Additionally, the site located on the disease front, at which a previous study noted a reduced severity of DFTD infection attributed to increased genetic diversity [281], has recently undergone a change in disease dynamics and has suffered a severe population decline similar to the other areas included in the report [284]. This collection of data suggests that a lack of genetic diversity is not solely responsible for the transmission of DFTD and that other factors must be involved.

If immunodeficiency or genetic paucity cannot account for the transmission of DFTD, tumour associated factors are most likely involved. Since DFTD arose from an immune privileged tissue from the nervous system [271] it is not surprising that the immune response against the tumour is limited. Several studies have found evidence for the alteration of molecules associated with tumorigenesis and the production of factors capable of modulating the immune response. Genetic mapping suggests several alleles that are known tumour suppressor or oncogenes in other species are located in an area at which gross chromosomal rearrangement occurred in DFTD [272]. As previously discussed, production of anti-inflammatory cytokines is a common adaptation in tumour cells. Immunohistochemistry studies suggest that the majority of DFTD tumours produce IL-10 and that many produce TGFβ [265]. Interestingly, the number of DFTD tumours positive for TGFβ appeared to increase over time, and may represent another tumour adaptation for increased transmission and immune escape [265].

Downregulation of MHC I protein also appears to be an important adaptation to allow DFTD to evade the host immune response. Recent studies in two research groups have shown that DFTD cells fail to express this protein on the cell surface. A potential mechanism to account for this is the decreased production of essential components in the MHC I antigen processing pathway. DFTD cells express low levels of TAP1 and PSMB8 mRNA compared to normal DFTD tissues [265]. This would

result in limited transport of MHC I protein to its appropriate cellular location and therefore decreased expression and function of the MHC I antigen presentation pathway in DFTD cells, compared to normal Tasmanian devil cells such as fibroblasts [265]. The absence of surface MHC I expression on DFTD cells was recently confirmed using a combination of molecular and immunohistochemical techniques [285]. A downregulation of MHC I expression would render the DFTD cells impervious to CTL activity and provide an important mechanism of immune escape.

The mechanisms of immune escape identified in DFTD bear a resemblance to those utilised by the other natural contagious tumour, CTVT. There are many consistencies between the two diseases, with the fundamental difference being the change in immune response that occurs in CTVT [286]. Similar to CTVT, the capacity for DFTD cells to produce MHC I is not irreparably impaired [285]. Surface expression of the MHC I protein can be restored by treatment with cytokine rich supernatants prepared from Con A stimulation of Tasmanian devil lymphocytes, which are likely to contain high levels of IFNγ [285]. It is therefore possible that immunological mechanisms targeted at modulating tumour MHC I expression, or those used to overcome infection or reduce disease burden in CTVT could direct strategies for immunological intervention against DFTD.

1.7 Immunological intervention against cancers

The persistence of tumours and cancers is less likely to occur in the presence of a fully competent immune response. As previously discussed, cancers must either escape immune surveillance, induce local immune suppression or grow rapidly enough to overwhelm the immune response. Activating the immune system to target established cancers is therefore an attractive therapeutic strategy, and this goal has been pursued since the 1970s. Two major areas of interest for immunological intervention against cancer are vaccines and immunotherapies. Interestingly, these two areas often overlap, particularly regarding the use of 'cellular vaccines' to treat cancer or the use of cytokines or adjuvants in immunotherapy. This literature review will broadly define vaccines as 'therapeutics containing antigens derived from tumour cells' and immunotherapies as 'infusions containing autologous cells or recombinant cytokines'. The approaches used to achieve immune activation against established tumours, their advantages, limitations and potential for adaptation to treat and prevention DFTD will be discussed.

1.7.1 Cancer Vaccines

Vaccine development has been the focus of extensive research for approximately 200 years and has resulted in the production of immunisations against many widespread diseases. However, the

development of vaccines against cancers must overcome unique challenges. Although the majority of successful vaccines against other diseases are prophylactic, cancer vaccines are mainly therapeutic, and must induce responses against established disease [287]. Additionally, tumours often show significant variation and heterogeneity in the expression of antigens, a characteristic which makes them poor targets for specific vaccines. Cancer vaccines must also activate T lymphocyte-mediated responses such as cytotoxicity and cytokine production in preference to humoral responses, whereas many current vaccines rely on the generation of antibody. Consequently, many cancer vaccines and immunotherapies target different pathways, including antigen presentation and NK cells. Another important problem is the activation of autoimmunity, which has occurred as a side-effect in several experimental, but otherwise promising, clinical cancer vaccine trials [288,289,290]. Therefore, careful selection of vaccine components is crucial for the development of safe and effective cancer vaccines.

The most important factors in a vaccine are an immunogenic target antigen and an effective adjuvant (reviewed in [287,291]). An appropriate choice of vaccine preparation is crucial to success. Recently, a number of specific antigens associated with particular cancers have been identified as targets for vaccines. Breast cancer is an example for which many candidate antigens have been analysed. Proteins such as Her2, MAGE-3 (melanoma associated antigen 3), mammaglobulin, and CEA (carcinoembrionic antigen) have all been used as vaccine targets in animal models and have induced immune responses without activating autoimmunity. Some antigens, which are common to several types of tumours, provide potential targets for prophylactic vaccines. These include NY-ESO1, heat shock proteins (HSP) and vimentin [292,293,294,295]. Cancer vaccines are also being investigated for use in animals. An example is immunisation against canine melanoma using the surface antigen GD3, which can induce tumour site inflammation, cellular responses and antibody development in normal dogs [296]. Due to the limited number of molecular tools available to assess the presence of DFTD-specific antigens, these conserved tumour markers may provide candidates for targeting DFTD tumours. Vimentin and HSP are of particular interest, as proteomics studies have identified Tasmanian devil antibodies to vimentin following DFTD immunisation [297] and expression of HSP can be induced with factors such as heat, ultrasound and irradiation (reviewed in [204]).

Identification of specific antigens on DFTD cells is an important area for ongoing research. However, as no candidate antigens have been discovered to date, development of vaccines for DFTD will be restricted to the use of preparations containing whole tumour cells in the near future. Many methods have been evaluated to kill pathogens for vaccines, including irradiation, sonication and temperature-induced lysis. As vaccines against DFTD must aim to induce cellular responses in

preference to antibody, preparations containing live attenuated cells are likely to be most effective (reviewed in [298,299]). However, since use of live DFTD cells in vaccines would carry an unacceptable risk of disease transmission, only killed cell preparations could be used. Immunisation with cellular extracts, such as DNA, are also possible [298,300], although they are generally not as effective in the induction of cellular immunity (reviewed in [301]). Irradiation may be an appropriate method of attenuation for DFTD cells, as the ultrastructure of the cells would not be compromised, and immune responses could be targeted to surface antigens. Additionally, since irradiation can increase the expression of MHC molecules in tumour cells [302,303], this method of attenuation could potentially overcome one of the major immune escape mechanisms associated with DFTD infection. Alternatively, the use of sonication or temperature induced lysis could expose intracellular proteins which are otherwise expressed only in small amounts, or could increase the expression of HSP and other immunogenic chaperonin proteins [304]. Use of DNA or protein extracts from DFTD cells would be more likely to induce antibody formation than cellular responses, unless combined with specific adjuvants to increase cross presentation.

Many recent vaccines have incorporated cell hybrids designed to increase immune exposure to tumour antigens. Hybrids are usually created by *in vitro* fusion of tumour cells with cultured DC. This strategy aims to exploit the natural capacity of DC to activate both $CD4^+$ and $CD8^+$ T lymphocytes through their ability to express tumour antigens on both MHC I and II and provide costimulation [305]. DC hybrid vaccines have been shown to increase cytotoxic responses against tumour cells [306]. Additionally, vaccine potency can be enhanced through their ability to secrete cytokines, such as IL-12 and IFNγ [306,307]. In particular, vaccines containing patient-derived DC and tumour cell hybrids have yielded promising results in early trials. The use of patient-derived dendritic cells minimises MHC disparity between vaccine and recipient [306]. Thus, the presentation of antigens on homologous MHC I molecules is recognised efficiently leading to more effective vaccines. There is also no risk of graft-versus-host responses occurring in recipients. However, DC hybrid-based vaccines may be less effective in patients with advanced disease. Long established tumours can often bias the immune response to produce a tolerogenic environment in which the cancer is not targeted [308]. Consequently, in this situation the activity of DC would be affected and the efficacy of the vaccine would be decreased.

The choice of an appropriate adjuvant is imperative to the success of a cancer vaccine. TLR agonists, such as CpG DNA and Poly I:C, are increasingly being used as adjuvants for cancer vaccines because of their potential to induce specific responses via activation of cells in the innate immune system [52,56,143,309]. Many cancer vaccines combine TLR agonists with other immunomodulators, such

as Montanide to increase the effect of the individual adjuvants [292,310,311]. When used with specific antigens, this combination of adjuvants in cancer vaccines can induce antibody development and long-lasting TH1 and $CD8^+$ T lymphocyte responses [292,311]. The use of CpG DNA useful as an adjuvant can also activate many components of the immune response, including B lymphocytes, NK cells and DC [53,55,56,57,139]. Another TLR agonist that is commonly used as an adjuvant is Poly I:C, which can induce responses from NK cells and DC [47,93,312]. Other adjuvants that target specific pathways, such as Flt 3 ligand which promotes DC differentiation and activation [313,314,315,316] or immune stimulating complexes (ISCOMs) like ISCOMATRIX® which stimulates cross presentation of protein antigens [317] could also be good candidates for use in adjuvants in immunisations containing DFTD cell protein.

1.7.2 Immunotherapy

For many decades, immunotherapy of cancer has been an expanding area of research. Many types of immunotherapy have been trialled in human and animal medicine, including infusion or injection of cytokines [318]. Unfortunately, as the Tasmanian devil is a little-studied species, with few tools available to characterise and manipulate its cells, some of these techniques are not applicable for treatment of DFTD. Therefore, this review will concentrate on the use of intravenous cytokines and cytokine-induced cells for immunotherapy against cancers.

As previously discussed, inflammatory cytokines play a crucial role in the immune response against cancer. The use of cytokines as immunotherapeutics against cancers has therefore been extensively investigated. Many cytokines have been trialled successfully in animal models and adapted for testing in humans, including interleukins (IL)-2, IL-7, IL-12, IL-15, IL-21 [319,320,321,322,323,324,325,326] and interferons (IFN)α and IFNγ [327,328]. Although some trials have shown promising results, clinical efficacy has been variable with the majority of these cytokines, and only two are currently licensed for use in immunotherapy: IFNα and IL-2 [318].

Immunotherapy with IFNα has been particularly explored in the treatment of certain cancers, including renal cell carcinoma (RCC) and melanoma [327,329], but has also been trialled in the treatment of haematological malignancies and Kaposi's sarcoma [328]. Although IFNα is approved by the American FDA for treatment of hepatocellular carcinoma (HCC), there is some conflict within the literature in relation to the ideal route of administration [330,331,332]. The efficacy of IFNα treatment can often be enhanced when administered in combination with specific antibodies [333] or cytotoxic drugs [332], or modified using PEGylation [331]. IFNα is also commonly used for immunotherapy of melanoma, in which it has been shown to prolong survival time and decrease the

probability of relapse [327], although the effect of IFNα treatment on long term survival has been debated [334]. A recent meta-analysis concluded that IFNα therapy is advantageous in the treatment of melanoma, and recommended that the practice be continued [335].

Because of its effects on T lymphocyte proliferation and activation, IL-2 is also commonly used for immunotherapy. Like IFNα, infusion with IL-2 is also common in the treatment of RCC and melanoma, and the two are sometimes used in conjunction [330]. However, infusion of IL-2 alone can be effective, and has been trialled in the treatment of melanoma, RCC and malignant pleural effusion [320,336]. In treatment of melanoma, high dose IL-2 can induce clinical responses in up to 25 percent of cases (reviewed in [318,328]) and can lead to the development of durable, long lasting responses in melanoma patients [337]. Other strategies used to manipulate the immunogenicity of melanoma include the administration of IFNγ in order to upregulate MHC I expression in the tumour cells [338] which, in a small proportion of patients, can lead to complete regression [338]. Apart from this effect treatment with IFNγ, which otherwise plays such an important role in the immune response, has not yielded promising clinical results [328].

Immunotherapy can involve transfer of living immune cells. Adoptive cell transfer is based on the *in vitro* selection and expansion of tumour-reactive autologous lymphocytes. Several methods have been investigated for improved efficacy in adoptive cell transfer. Many protocols involve the injection of autologous leukocytes, following activation or priming *in vitro*. An ideal population of cells for use in adoptive transfer is tumour infiltrating lymphocytes (TIL), as they are likely to be primed against tumour-specific antigens. Infiltrating lymphocytes are isolated from tumour biopsies or resections using density gradient centrifugation, flow cytometry and properties such as adherence, then expanded *in vitro* using stimuli such as cytokines, native tumour cell antigens, irradiated allogenic cells or activated antibodies [339,340,341,342]. The technique used for *in vitro* expansion of TIL is crucial for the biological activity of the cells, as excessive stimulation can exhaust the cells, leading to reduced clinical efficacy [342]. Some human clinical trials using TIL have shown promising clinical results, with clinical response rates of 50 – 70 percent of patients [339,340,341]. Some protocols use lymphokine-activated killer (LAK) cells, T lymphocytes or NK cells which are stimulated using IL-2 [343]. However, although LAK cell killing can be efficient *in vitro* and has successfully induced responses in animal studies [344], its use as an immunotherapy has yielded inconsistent results [345]. Use of LAK cells is particularly hampered by a poor capacity to divide *in vitro* [346] and many immunotherapy protocols combine LAK cells and IL-2 infusion, with higher efficacy [345,347]. However, as the capacity to rapidly expand the activated cells *in vitro* is important for a successful immunotherapy, other techniques for lymphocyte activation have been sought.

More recently, studies have activated naive immune cells for therapy using a combination of cytokine treatment and specific antibodies capable of activating the T cell receptor, via activation of CD3. The technique usually involves initial culture with IFNγ, then a combination of an anti-CD3 activating antibody and IL-2 for further activation and expansion [348]. This produces an effector cell type known as cytokine-induced killer (CIK) cells, which exhibit potent cytotoxic capacity and show a high rate of proliferation [348,349,350,351]. Despite the fact that CIK cells are usually generated from heterogeneous lymphocyte populations, the subset of cells which shows the highest proliferation and effector characteristics are $CD3^+/CD56^+$, a phenotype usually associated with NKT cells [351,352]. CIK cells often exhibit more potent activity than LAK cells *in vitro* and in animal models [346,350,351] and have shown promising results in human clinical trials [353,354,355], including the reduction of tumour size and levels of tumour associated markers in the blood, and the capacity to induce ongoing increases in numbers of cytotoxic cells in the peripheral blood of patients [353].

Some immunotherapy strategies use antibodies to target molecules and ligands that are involved in the activation of the anti-tumour responses, particularly those of T lymphocytes. Several antibody-based therapies are now in use to treat a variety of cancers. Many antibody-based agents target specific molecules that block the immune response, resulting in the abolition of tolerance and increasing the anti-tumour immune response. Two examples are antibodies against either CTLA4 or PD-1. Such molecules deliver inhibitory signals to T lymphocytes and regulate their activity [356,357].

The use of antibodies as agonists to cause CTLA-4 blockade has recently been extensively tested in patients with metastatic melanoma, but may also be used to treat a variety of other cancers, including prostate, breast, lung, ovarian and renal-cell cancer [356,358,359]. Many trials have documented beneficial and durable effects of CTLA-4 antagonists, although like most immunotherapies the response rates among patients were generally low [360,361,362]. Overall, treatment with CTLA-4 increased patient survival in many trials [363]. One unusual characteristic of the response to CTLA-4 blockade is that disease stabilisation or regression often occurs after a delay in initial effect [356]. Treatment with CTLA-4 antagonists is often performed in conjunction with administration of cancer peptide vaccines [363,364,365], which aims to utilise the enhanced inflammatory immune response to increase the effectiveness of the vaccines. However, despite the potential benefits of CTLA-4 therapy, its use has the capacity to cause a spectrum of autoimmune side effects [366,367]. These commonly include colitis and associated gastrointestinal symptoms, dermatitis, hepatitis and endocrine disturbances [368,369]. Consequently, future trials using CTLA-4

agonists will need to customise treatment protocols to reduce the severity of side effects as well as maximising the therapeutic benefits of the agents.

The PD-1 (Programmed death 1) receptor is also found on T lymphocytes and interacts with ligands (namely PD-L1 and PD-L2) on non-hematopoietic cells. The PD-1 molecule delivers suppressive signals to T lymphocytes at normal tissues, providing an important mechanism for the control of inflammatory responses and normal tolerance [370]. Consequently, if its receptors are expressed on tumour cells they can likewise induce tolerance to cancers. By using antibodies to bind PD-1 or its ligands, the pathway, and the inhibitory signal to responding T lymphocytes, is disrupted [357]. Antibodies against PD-1 or its ligands can increase T-cell responses *in vitro* and induce anti-tumour activity in clinical trials [357,371]. One particularly promising trial showed objective responses in 18-28% of patients with lung cancer, melanoma or renal-cell cancer [357]. However, like the use of CTLA-4 antibody, agonists against PD-1 and its ligands can induce side effects linked to autoimmunity [357]. Future research in this area will concentrate on characterising, monitoring and reducing the side effects associated with these therapies.

1.7.3 Advantages and limitations of cancer vaccines and immunotherapy

A major advantage of immunotherapy over traditional cancer treatments such as radiotherapy or chemotherapy is the specificity of the resulting response. This is particularly evident when specific antigens are used for a cancer type, but may be less so if immunisation is limited to whole cell preparations [287]. Specific responses also have the advantage of being effective in situations such as 'minimal residual disease', whereas other types of therapy are not as effective when the cancers exist only at low levels. However, although many of the strategies for cancer immunisation and immunotherapy are theoretically sound, their practical application has often yielded poor results. Due to the evasive nature of cancer as a disease, the heterogeneity within cancer types and the variety of immune-evasion mechanisms available for cancer cells, there are many problems that must be overcome to consistently induce an immune response. Although some cancer therapeutics, particularly cellular immunotherapies, have shown promising success in both human and animal models [257,339,340,341], many regimes have shown conflicting results, and few immunotherapies are currently licensed for human use [328]. There may be several factors which have contributed to the poor outcome of many cancer vaccine and immunotherapy trials, most notably the subset of patients recruited for therapy. The vast majority of trials are performed in patients with advanced metastatic cancer, which may limit the capacity for effective therapy [330]. Additional complications associated with cancer immunotherapy and vaccinations include toxicity, which is particularly prevalent in cytokine immunotherapy [372,373], and the activation of autoimmune responses

following treatment. This side effect has been observed in both animal and human models, where it can affect up to 26 percent of patients [288,372].

1.7.4 Application of immunotherapy for treatment of DFTD

Although it would be preferable to design strategies for prevention of DFTD infection, immunological intervention would not be limited solely to naive animals. Indeed, given the lack of MHC I protein expression on DFTD cells, the capacity for development of functional anti-tumour responses in naive devils may be limited. Treatment of pre-existing tumours in diseased devils could be a more successful strategy. Therefore, the use of cancer vaccines and immunotherapy in DFTD may be an important intervention to preserve the species in the wild. This section will discuss how the vaccination and immunotherapy techniques above could be applied in the treatment of DFTD.

One strategy that could be used to develop preventative vaccines, as well as therapeutic vaccines, is the identification of specific antigens expressed on the surface of DFTD tumour cells. As no specific antigens have been identified, immunisation against DFTD is currently limited to whole tumour cells or purified cellular components. Although these preparations would contain a variety of tumour associated antigens, surface proteins may only be present in small amounts. This may decrease the specificity of the vaccines [287]. Less specific vaccines could also carry a higher risk of side effects, such as autoimmunity. DFTD arose from a Tasmanian devil Schwann cell, and express many similar antigens to this cell type [274]. Several types of immunotherapy can also result in autoimmunity, as a side-effect of the treatment [288,372]. Autoimmunity against Schwann cells is well known to be a major mechanism in many diseases, including multiple sclerosis and optic neuritis [374]. Given the similarity between DFTD cells and Schwann cells, development of autoimmunity is likely to have severe consequences for an affected Tasmanian devil. Consequently, vaccine and immunotherapy strategies should be selected carefully to minimise the risk of this side effect.

Immunotherapy is likely to be a more effective intervention against DFTD than chemotherapy. Previous trials performed within our laboratory [375] and by the Tasmanian Department of Primary Industries, Parks, Wildlife and the Environment (Stephen Pyecroft, personal communication) have shown that chemotherapy is not effective in the treatment of DFTD. Additional advantages of a successful immunological intervention would be the specificity of the resulting response against the tumours, and the potential for ongoing immunity. Also, there are unique traits of this disease that could make immunological intervention even more likely to succeed. Firstly, the clonal nature of the disease may mean that, if an intervention can induce a response against one tumour, it should be able to target the cancer in all animals. This is not observed in human cancer patients, as the genetic

and physiological variation makes the tumours unique to the individual. Secondly, primary DFTD tumours usually originate at easily accessible sites. The immune evasion mechanisms employed by the tumours may hamper the development of an immunological intervention. Consequently, immunotherapy techniques should be specifically designed to circumvent these issues.

The immune evasion strategies utilised by DFTD are similar to those of CTVT, a disease in which several immunotherapy options have been explored. One particular study on CTVT involved the use of cellular vaccines containing hybrids between canine dendritic cells and the tumour cells [257]. As previously described, the rationale of this strategy is to exploit the ability of dendritic cells to present antigens bound to MHC I directly to CTL. The use of these vaccines in dogs significantly inhibited tumour progression and accelerated the rate of regression compared to untreated dogs, in which the tumours underwent natural regression. Histology of the CTVT tumours in treated dogs showed that lymphocyte infiltration occurred earlier in the tumours of vaccinated dogs. The study concluded that the vaccine successfully amplified the adaptive anti-tumour immune responses, and that NK cell cytotoxicity also played an important role. At the time of inoculation, the CTVT tumours would have been in an infectious state similar to the cells in DFTD tumours. Consequently, DC hybrid vaccines may be an appropriate strategy to induce immune responses. If a similar vaccine could achieve these outcomes in DFTD, it may be possible to induce tumour regression. However, there is currently no technique defined for effective culture of Tasmanian devil DC. Refinement of techniques for the culture of Tasmanian devil DFTD cells will be an important area of future research, with the aim to produce DC fusion vaccines for use against DFTD in the future.

The mechanisms that induce CTVT to spontaneously regress could also be appropriate situations to simulate in order to target an immune response to DFTD. One important component of the response against CTVT tumours is infiltration of LAK cells into the tumours [244]. LAK cells have been used for immunotherapy in other animals, such as mice, in which their anti-tumour effects included decreased establishment of metastases [344] and reduction in existing secondary tumours [345]. In some studies, LAK therapy has been combined with chemotherapy [376,377,378]. LAK cells are known to play an important role in the immune response against CTVT. They alter the cytokine environment of the tumour, leading to inflammation, and can mediate cytotoxic responses [244]. Additionally, the presence of LAK cells coincides with the upregulation of MHC I in the tumour, which results in a T lymphocyte response [286]. These would all be desirable effects in the case of DFTD. Original experiments generated LAK cells using the cytokine-rich supernatants from lymphocytes stimulated using mitogens such as Concanavalin (Con) A [344,379], a strategy that may be useful in DFTD since large quantities of purified cytokines are not readily available. Another type of cytotoxic

cells, CIK cells, may also be useful for immunotherapy. One useful characteristic of CIK cells is their capacity for proliferation *in vitro*, as well as cytotoxicity [352]. The preparation of CIK cells normally involves activating naive leukocytes with anti-CD3 antibody, followed by stimulation with IL-2 and IFNγ. Although no specific antibody is available against CD3 in Tasmanian devils, the functional pathway, and production of the cytokines necessary for CIK cell production, may be activated using other ligands, such as Con A [380]. Therefore, generation of CIK cells in Tasmanian devils may be possible, and may provide a basis for immunotherapy against DFTD. Consequently, the use of activated killer cells could be another immunotherapy strategy for the treatment of DFTD.

Project Aims

In other mammals, antitumour immune responses are mainly mediated through the activity of cytotoxic cells such as T lymphocytes and NK cells. Other specific responses, such as the development and release of cytokines and antibody, can also play an important role. However, evidence from an increasing number of studies suggests that components of the innate immune system can influence antitumour activity. The overall aim of this project was to characterise the immune responses against tumour cells in Tasmanian devils and to identify pathways and mechanisms through which the immune system could be induced to target DFTD cells. Thus, this project was split into three basic components: analysis of the specific antitumour immune responses in Tasmanian devils, characterisation of the specific pathways involved in these antitumour responses, and manipulation of the pathways *in vivo* to target DFTD. Each of these aims formed the basis of a results chapter for this thesis.

The first aim of this thesis was to determine if Tasmanian devils could form antitumour cytotoxicity and antibody responses, against foreign cancer cells and DFTD cells. One assumption that is often made about DFTD transmission is the complete absence of a host immune response against the tumour. However, only one study has provided histological evidence for this immune ignorance [269], and none have sought functional evidence for a lack of response. Thus, a secondary aim of this chapter was to determine if DFTD diseased Tasmanian devils can form specific antitumour responses against DFTD cells. Tasmanian devils are a species particularly prone to developing cancer [259]. If Tasmanian devils did fail to produce an immune response against DFTD, one explanation could be a generalised immunodeficiency across the species. Therefore, a secondary aim of this chapter was to determine if Tasmanian devils could form cytotoxicity responses and antibody against foreign cancer cells. If the antitumour pathways were intact in Tasmanian devils, it may be possible to immunise against DFTD cells. Consequently, a secondary aim of this chapter was to determine if Tasmanian devils could be induced to form antitumour responses against DFTD cells through immunisation with killed cell preparations.

The results of recent experiments suggest that DFTD cells fail to express MHC I protein on the cell surface [285]. Induction of antitumour responses against these cells would be very difficult, as the obligatory ligand for T lymphocyte cytotoxicity is missing. Therefore, the second aim of this thesis was to characterise the antitumour cytotoxicity responses of Tasmanian devils against cancer cells in the absence of functional MHC I protein. Although several studies have previously analysed the presence of immune cell subsets in Tasmanian devils, Natural Killer (NK) cells, the innate cytotoxic cell type that is crucial for responses against MHC I negative cells, has not been characterised. Thus,

a secondary aim of this chapter was to identify NK cells in Tasmanian devils. The mechanisms for targeting MHC I negative cells could potentially be manipulated to induce responses against DFTD cells and tumours. Immunisation with xenogeneic K562 cells, which lack MHC I provided a good model to analyse these responses. In other studies assessing responses against xenogeneic cells, antibody-dependent cell mediated cytotoxicity (ADCC) has been an important pathway for responses. Consequently, a secondary aim of this chapter was to determine if Tasmanian devils can form ADCC responses against tumour cells. Cytotoxicity responses can also be activated through nonspecific stimulation with factors such as mitogens, cytokines and agonists for specific receptors. Therefore, a secondary aim of this chapter was to determine if these stimuli could activate cytotoxic responses against DFTD cells.

The third chapter of this thesis aimed to determine if the mechanisms for activation identified in the previous chapters were able to induce immune responses against DFTD *in vivo*. Strategies such as adoptive cell transfer, therapeutic immunisation and cytokine injection were available to treat one Tasmanian devil with DFTD. The secondary aims of this chapter were to assess whether each of these prospective treatments was effective or viable strategies to treat the DFTD tumours of affected Tasmanian devils in captivity.

Summary of Aims:

This project aimed to characterise immune responses against tumour cells in Tasmanian devils and to identify pathways and mechanisms through which the immune system could target DFTD cells.

- **Chapter 3: Determine if Tasmanian devils could form antitumour cytotoxicity and antibody responses, against foreign cancer cells and DFTD cells.**
  - Determine if DFTD diseased Tasmanian devils can form specific antitumour responses against DFTD cells.
  - Determine if Tasmanian devils could form cytotoxicity responses and antibody against cancer cells.
  - Determine if Tasmanian devils could be induced to form antitumour responses against DFTD cells through immunisation with killed cell preparations.
- **Chapter 4: Characterise the antitumour cytotoxicity responses against MHC I negative cells in Tasmanian devils.**
  - Identify Natural Killer cells in Tasmanian devils.
  - Determine if Tasmanian devils can form ADCC responses against tumour cells.
  - Determine if non-specific stimulation with mitogens, cytokines or agonists for specific receptors could activate cytotoxic responses against DFTD cells.
- **Chapter 5: Determine if activation mechanisms identified in the previous chapters of this thesis were able to induce immune responses against DFTD *in vivo*.**
  - Assess the *in vivo* effects of adoptive transfer of mitogen-activated immune cells on the immune response against a DFTD tumour.
  - Assess the *in vivo* effect of therapeutic vaccines containing cytokine treated, MHC I expressing, DFTD cell on the immune response against a DFTD tumour.
  - Assess the *in vivo* effect of cytokine injection on the immune response against a DFTD tumour.

Chapter 2 - Materials and Methods

2.1 Laboratory equipment and consumables

2.1.1 Reagents

Reagents	Supplier	Catalogue Number
Aminopropyltriethoxysilane (APTS)	Sigma - Aldrich	A3648
^{51}Chromium solution	PerkinElmer	NEZ030S001MC
Concanavalin A (Con A)	Sigma - Aldrich	C 7275
Dimethyl Sulfoxide solution (DMSO)	Sigma - Aldrich	D2650
Disodium Hydrogen Phosphate	Merck	1065855000
Flt 3 Ligand	CSL	Not Provided
Fetal Bovine Serum (FBS)	Bovogen	SFBS
Formaldehyde (methanol buffered)	Sigma - Aldrich	F1635
Gentamicin Sulfate	Pfizer	61022027
Giemsa solution	Fluka/Sigma Aldrich	48900
Glucose (solid)	Sigma - Aldrich	D9434
Histopaque 1077	Sigma - Aldrich	10771
ISCOMATRIX® adjuvant	CSL	Not Provided
Isofluorane (pressurised liquid)	Attane™/ Bomac Pty Ltd	APVMA 58070/250/1203
L – Glutamine	Sigma - Aldrich	9871901
α-D-Mannose (solid)	Sigma - Aldrich	M6020
Montanide gel 645101 adjuvant	Seppic	639101
Montanide ISA51 VG	Seppic	645101
Phosphate Buffered Saline (PBS) tablets	Oxoid	BR0014G
Phytohemagglutanin	Sigma - Aldrich	L2646
Polyinosinic:polycytidylic acid (Poly I:C)	Sigma - Aldrich	P0913
Potassium Chloride (solid)	Calbiochem (EMD Millipore)	529552
Potassium Dihydrogen Phosphate	Sigma - Aldrich	P9791
Propidium Iodide	Sigma	P4170

RPMI 1640 Medium	Invitrogen GIBCO	22400
Sodium Azide (NaN_3)	Sigma - Aldrich	S2002
Sodium Chloride (solid)	Sigma - Aldrich	S6191
Trichostatin A (TSA)	Sigma - Aldrich	T8552
Triton X-100	BDH	30632
Trypan Blue	Sigma - Aldrich	T6146

2.1.2 Consumables

Product	Supplier	Catalogue Number
Automatic pipette tips 50 – 1000 µL	Eppendorf	02519
Automatic pipette tips 2 – 200 µL	Eppendorf	03439
Automatic pipette tips 0.1 – 20 µL	Eppendorf	02249
3 – 4 mm Biopsy punch	Paramount Surgimed Ltd	5607
35 mm Cell culture dish	Iwaki	4000-010
25 cm^3 cell culture flask	Iwaki	3100-025
75 cm^3 cell culture flask	Iwaki	3110-075
10 mL Centrifuge tube	Schering Plough	LBSCT1203X
15 mL Centrifuge tube	Iwaki	3235-105
50 mL Centrifuge tube	Iwaki	2345-050
Coverslip	Esco	400163
Cryogenic freezing vials	Iwaki	2712-002
Disposable Pasteur pipette	Samco	225 - 15
Eppendorf tube	Quantum Scientific	LAC11514
25 mm 0.8/0.2 Filter	Pall Corporation	4187
Flow cytometry tubes	BD Falcon	350028
10 mL lithium heparin blood storage tubes	BD	367 526
Microscope slide	Esco	4951C
18 G needle	Terumo	NN-1838R
21 G needle	Terumo	NN-2125R
Nylon wool	Poly Sciences inc.	18359

2 mL Serum clot activator tubes	Greiner bio-one	454 906
10 mL Syringe	Terumo	SS+10ES
5 mL Syringe	Livingstone	DSL005MLS
1 mL Syringe	Terumo	SS-01T
96 Well round-bottom microplate with lid	Iwaki	3870-096
96 Well flat-bottom microplate with lid	Greiner	655180
6 Well flat-bottomed microplate with lid	Iwaki	3810-006
24 Well flat bottomed microplate with lid	Iwaki	3820-024

2.1.3 Laboratory Equipment

Product	Supplier	Model number
Automatic cell harvester	Skatron	Combi 11025
Benchtop centrifuge	Sorvall	RT 6000D
Benchtop microcentrifuge	Eppendorf	5415D
Class II biological safety cabinet	Gelman Sciences	BH – 204
Electric Pressure Cooker	Russel Hobbs	RHNHP401
Flow Cytometer	Becton-Dickinson	Canto II
Fluorescent Microscope	Olympus	BX 50
Gamma radiation counter	Laboratory Technologies	Genesys Genii HE
Haemocytometer	Hawksley	improved neubauer
Incubator 35ºC	Heraeus	BB15
Incubator 37ºC	Binder	142489
Inverted Microscope	Leitz	090-123.012
Microplate fluorescence detector	BIO-RAD	3550
Microscope	Olympus	246046
Microscope-mounted camera	Leica	DFC 495
“Stinger™” Anaesthetic machine	Advanced Anaesthesia Specialists	00449

2.2 Reagents

2.2.1 Complete medium (for cell culture)

RPMI liquid culture medium (GIBCO, New York, USA) was refrigerated before use. Foetal Bovine Serum (FBS) (Bovogen Biological, Victoria, Australia) was stored at -20°C, thawed when required. The serum was heat inactivated in a water bath at 56°C for 1 hour then aliquoted into 50 mL volumes under sterile conditions and stored at -20°C. L-glutamine (Sigma Aldrich, Ayrshire, UK) was stored at -20°C then dissolved using heat. Gentamicin sulfate (Pfizer, Western Australia, Australia) was refrigerated at 4°C.

To prepare complete medium, 50 mL from the contents of a 500 mL RPMI 1640 medium bottle was removed under sterile conditions and discarded and replaced with 50 mL of heat-inactivated FBS (10% V/V). L-glutamine was added for a total of 2 mM (equivalent to 5 mL). Gentamicin was added for a total of 100 IU/mL (equivalent to 375 µL). The complete medium was thoroughly mixed and refrigerated at 4°C until required, then warmed to room temperature (21°C) for use in cell culture.

2.2.2 Phosphate buffered saline (PBS)

2.2.2.1 Cell culture grade PBS

One PBS tablet (Oxoid Ltd., Hampshire, England) per 100 mL of water was mixed to dissolve using a magnetic stirrer. The solution was autoclaved at 121°C for 20 minutes and stored at 4°C until required.

2.2.2.2 Standard PBS

A stock solution of PBS was prepared by dissolving 160 g of solid sodium chloride (Sigma Aldrich, New South Wales, Australia) 4g of potassium chloride (Calbiochem/EMD Millipore, Massachusetts, USA), 23 g of disodium hydrogen phosphate (Merck, Massachusetts, USA) and 4 g of potassium dihydrogen phosphate (Sigma Aldrich, New South Wales, Australia) in 1 L of water. The pH of the solution was adjusted to 7.3 using dropwise additions of concentrated HCl and NaOH as required. The stock solution was diluted 1/20 for use.

2.2.3 FACS buffer

Foetal Bovine Serum (FBS) (Bovogen Biological, Victoria, Australia) was diluted to 5% V/V in standard PBS. The solution was either used immediately for flow cytometry or frozen at -20 °C.

2.2.4 FACS fixative

In a glass bottle, 13 mL of 37% aqueous formaldehyde solution with 10 – 15% methanol (Sigma Aldrich, New South Wales, Australia), 10 g of solid glucose (Sigma Aldrich, New South Wales, Australia), 1.1 mL of 15% NaN_3 (Sigma Aldrich, New South Wales, Australia) in PBS were combined with standard PBS to a total volume of 500 mL.

2.3 Cell culture

2.3.1 Cell line characteristics

2.3.1.1 K562 Cells

Human K562 cells were originally sourced from the American Type Culture Collection (ATCC). They appeared as large and round cells which were unattached in culture. The identity of samples we used was verified by positive labelling for Glycophorin A (an erythrocyte marker) and as appropriate target cells for NK cell cytotoxicity in 4 hour chromium release assays.

2.3.1.2 DFTD cells

DFTD cell lines were provided by A-M. Pearse and K. Swift, from the Tasmanian Department of Primary Industries, Parks, Wildlife and Environment (DPIPWE). The cell lines were established from primary tumour biopsy samples taken under the approval of the Animal Ethics Committee of Tasmania's Park and Wildlife Services (permit numbers 33/2004–5 and 32/2005–6). DFTD cells were attached to the substrate in culture and varied in shape from rounded to long and spindle-like in appearance. Three strains of DFTD cells were available for use in the laboratory; however the cell line used in the majority of experiments was C5065, a Strain 3 DFTD cultivar. The cultured DFTD cells consistently labelled strongly for periaxin when assessed by flow cytometry or immunohistochemistry of cytospins or fixed on glass coverslips.

2.3.2 Cell culture

All cell cultures, cryopreservations and thawing of cell lines were performed under sterile conditions in a type II Biohazard cabinet. As a standard procedure, all sterile solutions (such as culture medium and PBS) were warmed to room temperature and aliquoted from larger storage containers for use with individual cell lines or in primary cultures.

K562 cells were cultured in complete RPMI medium at 37 °C in a humidified atmosphere containing 5% CO_2 in 95% air. The cells grew rapidly and required sub-culture each 1 – 2 days. For regular maintenance, 75% volume from the K562 cell suspension was removed under sterile conditions and replaced with an equal volume of fresh culture medium. Harvested cells were pelleted from suspension by centrifuging at 240 *g* for 5 minutes.

DFTD cells were cultured in complete RPMI medium at 35 °C in a humidified atmosphere containing 5% CO_2 in 95% air. The cells were harvested or sub-cultured at 75% (as the cells grew at a variable rate this was required sub-culture each 1 – 2 days for fast-growing cells or up to 14 days for slow-growing cells). The cells were firmly attached to the substrate in culture and were dislodged by repeated flushing with culture medium through a pasteur pipette. For regular maintenance, 30 - 50% volume from the DFTD cell suspension was removed under sterile conditions and replaced with an equal volume of fresh culture medium. Harvested cells were pelleted from suspension by centrifuging at 240 *g* for 7 minutes.

2.3.3 Cryopreservation and thawing

Suspended cells (cultured K562 or DFTD cells, or primary cells such as lymphocytes) were harvested from culture and pelleted as described above, then placed on ice in a sterile biohazard cabinet. All supernatant was drained and the pellets were resuspended in a minimum volume. A solution of 10% DMSO (Sigma Aldrich, New South Wales, Australia) in culture medium was prepared and added, dropwise, to the cell pellet, with gentle agitation with each addition. Aliquots of 1 – 2 mL in volume were transferred to cryogenic vials and placed on ice. All samples were frozen to -80 °C in an ethanol bath, allowing a gradual temperature decrease of 1 °C per minute. If necessary, the cryopreserved samples were transferred to liquid nitrogen for long-term storage.

Frozen cells were placed in a 37 °C water bath until almost thawed. With a small amount of ice remaining, the cells were transferred to a sterile centrifuge tube and 8 mL of complete RPMI 1640 medium was added dropwise, with mixing after each addition. The solution was centrifuged at 240 *g* rpm for 5 minutes then the supernatant was aspirated, the pellet resuspended in 10 mL of complete medium and the cells were inoculated into a culture flask.

2.3.4 Cell counts and viability examination

Cell number and viability was determined using trypan blue exclusion according to the previously published protocol [381]. Briefly, 10 – 20 µL of 0.4% trypan blue solution (Sigma-Aldrich, St Louis, USA) per sample was placed into separate wells of a 96 well plate. An equal volume of cell

suspension was added and pipetted to mix. A glass coverslip was placed on the centre of a haemocytometer and a volume of the cell suspension mixture sufficient to cover the central chamber, was loaded by capillary action. At 40x magnification, dead cells could be easily distinguished due to the dark blue staining of their nuclei. The viable cells within each individual square of the central chamber were counted to a total of at least 100 cells. The number of cells was calculated using the following formula:

$$\text{Cell concentration} = \text{number of cells} \times (25/\text{number of squares}) \times 10^4 \text{ (cells/mL)}$$

The number of dead cells in the same area of the haemocytometer was also counted. When required, the percent viability of the samples was determined using the following formula:

$$\text{Viability} = (\text{viable cell count} / \text{total cell count}) \times 100$$

2.4 Animals

A full list of information about the wild and captive Tasmanian devils used in the experiments detailed in this thesis is given in Section A.1 of the Appendix (Chapter 8).

All experiments involving the use of Tasmanian devils were conducted under the approval of the University of Tasmania Animal Ethics Committee (permit number A0009215). The captive Tasmanian devils used in this study were fully adapted to captivity and housed in secure shelters under quarantine conditions at 3 locations within the greater Hobart: Fern Tree, Richmond and Taroona. Female devils could be kept in groups of up to 4 devils per pen. All male devils were housed individually in separate pens. The devils were fed on a diet of native meat from disease free areas and their health was maintained by DPIPWE keepers and vets. Wild Tasmanian devils used in this study were captured using pipe traps then transferred to hessian sacks for examination. The devils were micro chipped, if necessary, then euthanised (if infected with DFTD) or retained for collection of blood samples then released.

Anaesthesia of the Tasmanian devil is required for blood collection, and immunisation, and has been widely used by DPIPWE veterinarians. The vapour anaesthetic Isofluorane® is the agent of choice, given its short recovery period and fewer harmful side effects than other inhalation anaesthetics (reviewed in [382]). Isofluorane gas was administered in oxygen at an approximate rate of 2 L/minute via a mask. No adverse effects were recorded in the Tasmanian devils used in this study. All devils were anaesthetised and approximately 10 mL of blood was taken from the jugular vein. Up to 2 mL of blood from each sample was injected into clot activating tubes (Greiner Bio-one,

Frickenhausen, Germany). The remainder was injected into lithium heparin anticoagulant tubes (BD Biosciences, New Jersey, USA). The samples were stored at room temperature until arrival at the laboratory (< 24 hours).

2.5 Blood processing

2.5.1 Mononuclear cells

Whole blood samples were processed under sterile conditions. Peripheral blood mononuclear cells (MNC) were isolated from uncoagulated whole blood using density gradient centrifugation on Histopaque 1077 solution according to the manufacturer's protocol (Sigma Aldrich, St Louis, USA). The MNC were washed with PBS for 10 minutes at 250 *g*. The cells were diluted for assay use in culture medium.

2.5.2 Adherent cell differentiation

2.5.2.1 Nylon wool adherent cells

As no methods were available for the specific isolation of cytotoxic cells in Tasmanian devils, T lymphocytes were enriched in MNC suspensions by depleting B lymphocytes using nylon wool adherence according to the previously published method [383]. Briefly, columns containing 0.6 g of nylon wool were saturated with RPMI culture medium and equilibrated at 37 °C for 30 minutes and washed with RPMI culture medium. Suspensions of MNC were applied to the columns. Small volumes of RPMI culture medium were added gradually, over a period of approximately 10 minutes. The eluent containing enriched T cells was centrifuged at 250 *g*. The cells were diluted for assay use in RPMI culture medium.

2.5.2.2 Plastic adherent cells

Monocytes were depleted from mononuclear cell layers using plastic adherence, as described by Horowitz and colleagues [384]. MNC suspensions in RPMI culture medium were applied to the surface of 35 mm culture dishes (Iwaki, Tokyo, Japan), gently agitated to thinly cover the surface and incubated at 37 °C for 45 minutes. RPMI culture medium was added dropwise and the dish was gently agitated to loosen the plastic non adherent cells. The solution was collected and the wash repeated twice. The plastic non adherent cells were centrifuged for 10 minutes at 250 *g*. The cells were diluted for assay use in culture medium.

2.5.3 Mitogen stimulation of Tasmanian devil mononuclear cells

Previous studies have established that Tasmanian devil leukocytes proliferate following exposure to mitogens such as Concanavalin A (Con A) (Sigma Aldrich, New South Wales, Australia) and phytohaemagglutinin (PHA) (Sigma Aldrich, St Louis, USA) [385]. Con A and PHA were received as lyophilised powders and reconstituted in cell culture grade PBS. For mitogen stimulation of peripheral blood MNC from Tasmanian devils were cultured for 48 hours in complete medium containing 25 µg/mL Con A or 50 µg/mL PHA, doses which induced proliferation responses in the majority of samples in the previous study. After 48 hours, the culture was harvested and centrifuged for 15 minutes at 250 *g* then the supernatant and the cell pellet were separated.

2.5.4 Generation of Concanavalian A culture supernatant

Following a 48 hour Con A stimulation (with 5 µg/mL Con A) of Tasmanian devil MNC, the supernatant of each sample was separated from the cell pellet following centrifugation. The residual Con A was removed from the solution using chelation with 15 mg/mL α-D-Mannose (Sigma Aldrich, New South Wales, Australia) then the samples were passed through 25 mm 0.8/0.2 µm filters (Pall Corporation, New York, USA) under sterile conditions. The resulting solution contained the cytokines present after Con A culture, with little residual mitogen or mannose.

2.5.5 Separation of serum

Blood stored in clot-activating tubes was centrifuged at 1100 *g* for 10 minutes and the serum was harvested. The clot was removed and the process repeated. The serum was aliquotted in 200 µL volumes into cryogenic vials and stored at 4 °C (for short-term use within 2 months) or at -80 °C (for long-term storage).

2.6 Immunisations and adjuvants

A full list of information about the captive Tasmanian devils used in the immunisation experiments detailed in this thesis is given in Chapter 8 (Appendix Section 1).

2.6.1 Immunisation preparation

2.6.1.1 K562 cells

K562 cells were harvested, resuspended in PBS and combined with an equal volume of Montanide adjuvant under sterile conditions then incubated at 37 °C before transfer to the captive facility. Four

healthy female Tasmanian devils (CD 2, CD 3, CD 4 and CD 5) were injected with 10^8 cells in a total volume of 1 mL, containing equal parts cell suspension and adjuvant, subcutaneously into the right shoulder. A total of two doses was given at monthly intervals. Blood samples were collected 14 days (± 2 days) after each injection. Six months later, two devils (CD 2 and CD 4) were boosted with a third dose of K562 cells.

2.6.1.2 Irradiated K562 and DFTD cells in Montanide adjuvant

K562 and DFTD cells were harvested from culture then irradiated with 20 Gy of gamma radiation using a Varian Clinac 23-EX linear accelerator (Varian Medical Systems Inc., California, USA). The cells were pelleted and resuspended in PBS then combined with an equal volume of Montanide adjuvant (Seppic, Puteaux, France) under sterile conditions then incubated at 37 °C before transfer to the captive facility. Two healthy female Tasmanian devils (CD 8 and CD 9) were injected with 10^8 irradiated cells in a total volume of 1mL, containing equal parts cell suspension and adjuvant, subcutaneously into the right shoulder, limiting the number of injection sites. A total of four doses was given at monthly intervals. Blood samples were collected 14 days (± 2 days) after each injection.

2.6.1.3 Irradiated DFTD cells in CpG Montanide adjuvant

DFTD cells were irradiated as described in 2.6.1.2. Two CpG oligonucleotides were chosen based on their reported capacity to induce NK cell and CTL responses [143]. The CpG oligonucleotides suspensions were mixed with Montanide adjuvant and then an equal volume of irradiated DFTD cell suspension under sterile conditions then incubated at 37 °C before transfer to the captive facility. Two healthy devils (CD 10 and CD 13) were injected with irradiated DFTD cells and adjuvant containing CpG ODN 1585 (sequence: GGGGTCAACGTTGAGGGGGG) subcutaneously into the right shoulder. Two more devils (CD 11 and CD 12) were injected with irradiated DFTD cells and adjuvant containing CpG ODN 1826 (sequence: TCCATGACGTTCCTGACGTT) subcutaneously into the right shoulder. A total of three doses was given at monthly intervals. Blood samples were collected 14 days (± 2 days) after each injection.

2.6.1.4 Sonicated DFTD cells

DFTD cells were harvested from culture then placed on ice. The chilled samples were sonicated with 24 kHz of ultrasound energy for 4 repetitions of 60 seconds, returning to the ice in between for 60 second intervals. The cell debris was pelleted and resuspended in PBS then combined with an equal volume of Montanide adjuvant (Seppic, Puteaux, France) containing CpG ODN1585 under sterile conditions then incubated at 37 °C before transfer to the captive facility. Due to limited access to

research animals, the only available devils for this trial had previously been injected with DFTD cells but had been rested for an extended period (over 12 months). The cytotoxicity and antibody responses of the two devils (CD 10 and CD 14) were examined and found to be low, a state referred to as pre immune 2 (PI 2). The sonicated DFTD cells and CpG adjuvant were injected subcutaneously into the right shoulder. A total of two doses was given at monthly intervals. Blood samples were collected 14 days (± 2 days) after each injection.

2.6.1.5 Heat treated DFTD cell protein extracts

DFTD cells were harvested from culture then heat treated at 56 °C for 1 hour then chilled at -80 °C. The chilled samples were sonicated with 24 kHz of ultrasound energy, for 4 repetitions of 60 seconds, returning to the ice for 60 second intervals in between. The cell debris was pelleted and resuspended in PBS then the total protein was extracted using commercial RIPA buffer (Thermo Scientific, lllinois, USA) and a standard procedure for isolating protein from monolayer cultured mammalian cells. The DFTD cell protein extract was combined with an equal volume of ISCOMATRIX® adjuvant (CSL, Victoria, Australia) under sterile conditions then incubated at 37 °C before transfer to the captive facility. The immunisations were injected subcutaneously into the right shoulder in two healthy devils (CD 15 and CD 16). A total of three doses was given at monthly intervals. Blood samples were collected 14 days (± 2 days) after each injection.

In a different trial, DFTD cell protein extracts were mixed (as described above) with equal volumes of ISCOMATRIX® adjuvant supplemented with the immunostimulant Flt 3 ligand and the TLR 3 agonist Poly I:C (CSL, Victoria, Australia) then injected subcutaneously into the right shoulder in two healthy devils (CD 7 and CD 17). A total of three doses was given at monthly intervals. Blood samples were collected 14 days (± 2 days) after each injection.

2.6.1.6 Con A culture supernatant-treated DFTD cells

Culture medium was removed from flasks containing DFTD cells and replaced with medium containing 5% Con A culture supernatant (prepared as described in 2.5.4). The cells were incubated for 48 hours at 35 °C in a humidified atmosphere containing 5% CO_2. The cells were repeatedly frozen in liquid nitrogen then thawed at 37 °C in a water bath a total of 10 times then checked for viability as described in 2.3.4. The cell debris was pelleted and resuspended in PBS then combined with an equal volume of ISCOMATRIX® adjuvant (CSL, Victoria, Australia) under sterile conditions then incubated at 37 °C before transfer to the captive facility. The immunisations were injected

subcutaneously into the right shoulder in two healthy devils (CD 39 and CD 40). Two doses were given, at 0 and 35 days and blood samples were taken 14 days (± 2 days) after each dose.

2.6.1.7 Trichostatin A treated DFTD cells

Culture medium was removed from flasks containing DFTD cells and replaced with medium containing 10 nM trichostatin A (TSA) (Sigma Aldrich, New South Wales, Australia). The cells were repeatedly frozen in liquid nitrogen then thawed at 37 °C in a water bath a total of 10 times then checked for viability as described in 2.3.4. The cell debris was pelleted and resuspended in PBS then combined with an equal volume of ISCOMATRIX® adjuvant under sterile conditions then incubated at 37 °C before transfer to the captive facility. The cell debris was pelleted and resuspended in PBS then combined with an equal volume of ISCOMATRIX® adjuvant and injected subcutaneously into the right shoulder in one healthy Tasmanian devil (CD 1). Two doses were given, at 0 and 35 days and blood samples were taken 14 days (± 2 days) after each dose.

2.6.2 Live cell challenge

Cultured DFTD cells were harvested (as described in 2.3.2), pelleted at 240 *g* for 7 minutes and counted for number and viability. An aliquot containing 25 000 DFTD cells was removed to a cryogenic vial and resuspended in a total of 100 – 200 µL of PBS then the sample was immediately transferred to the captive facility, where the cells were injected sub-cutaneously into the shoulder or back of immunised Tasmanian devils. The challenged devils were anaesthetised once every 7 – 14 days and the injection site was palpated for evidence of tumour growth. At first sign of tumours, measurements were taken in 3 dimensions using a caliper and ruler. When the tumour had reached an appropriate size, a 3 - 4 mm core biopsy was taken from the tumour and immediately fixed in formalin (as some tumours were quite small at identification this was sometimes 2 – 3 weeks). The tumours were then either monitored and measured each 7 – 14 days or removed by surgery.

2.7 Cytotoxicity assays

2.7.1 Chromium release assays

Cytotoxicity assays were performed using triplicate samples in V-bottomed 96 well plates (Greiner Bio-one, Frickenhausen, Germany). Effector ratios (100:1, 50:1, 25:1, 12:1, 6:1 to 3:1) were tested against samples of 10^4 target cells. Negative and positive controls contained RPMI culture medium and 1% Triton X detergent in water, respectively. Cultured DFTD cells, from the strain 3 line C5065, were incubated with 100 µCi of radioactive ^{51}Cr solution (5 mCi/mL sodium chromate in normal

saline – PerkinElmer, Massachusetts, USA) for 2.5 hours, with frequent gentle agitation, each 10 – 15 minutes (this was essential to maintain viability of the cells and to ensure efficient radioactive labelling). Cultured K562 cells were incubated with 100 µCi of radioactive ^{51}Cr solution for 1 hour, with regular agitation. Labelled cells were washed 3 times in RPMI culture medium then diluted for assay use. The assays were incubated for 18 hours at 37 °C in a humidified atmosphere of 5% CO_2. The plates were centrifuged briefly at 170 *g* for 4 minutes then 100 µL aliquots of supernatant were harvested into polystyrene tubes and analysed for radioactivity (in counts per minute) using a Genesys gamma radiation counter (Laboratory Technologies Inc., Illinois, USA).

2.7.2 ADCC and NK cell cytotoxicity assays

The procedure for chromium release cytotoxicity assays was modified to detect antibody-dependent killing. Triplicate samples of MNC, nylon wool non adherent cells or plastic non adherent cells at ratios of 25:1, 12:1, 6:1 and 3:1 were tested against samples of 10^4 target cells. Serum from K562 immunised devils was diluted 1/10 in RPMI culture medium and 50 µL was added to the wells of test assays. Pre immune serum diluted 1/10 or RPMI culture medium was added to control assays. The assays were incubated for 18 hours before analysis as described in 2.7.1. NK cell assays were performed using standard and antibody-dependent cell-mediated cytotoxicity assay procedure but incubated for 4 hours before analysis.

2.7.3 Separation of assay culture supernatant

K562-immunised Tasmanian devil MNC were cultured in 24 well plates at ratios of 100:1 with DFTD tumour cells at 10^4 cells/mL. The samples were incubated for 18 hours at 37°C in a humidified atmosphere of 5% CO_2, after which the supernatants were harvested, centrifuged at 1200 rpm for 15 minutes and separated from the pellets. The supernatant was aliquotted in 200 µL volumes into cryogenic vials and stored at -20°C until required for use to supplement ADCC assays.

2.7.4 IL-10 block assays

Triplicate samples of Tasmanian devil MNC at ratios of 50:1, 25:1, 12:1 and 6:1 were tested against samples of 10^4 target cells. Rat anti mouse IL-10 or goat anti mouse IL-10 antibodies were diluted 1/25 in RPMI culture medium and 50 µL was added to the wells of the assays. An equivalent volume of culture medium was added to untreated control assays. The assays were incubated for 18 hours before analysis as described in 2.7.1.

2.7.5 Mitogen supplemented cytotoxicity assays

Triplicate samples of Tasmanian devil MNC at ratios of 50:1, 25:1, 12:1 and 6:1 were tested against samples of 10^4 target cells. MNC samples were prepared in culture medium containing 50 µg/mL Con A or 100 µg/mL PHA for final mitogen concentrations of 25 and 50 µg/mL, respectively. Untreated control samples were prepared without addition of the mitogen. The assays were incubated for 18 hours before analysis as described in 2.7.1.

In some assays, Tasmanian devil MNC were stimulated with Con A as described in 2.5.3. The cells were washed twice in complete medium to remove any traces of the mitogen. The stimulated cells were then used in 18 hour cytotoxicity assays, as described in 2.7.1 and incubated for 18 hours before analysis as described in 2.7.1.

2.7.6 Con A culture supernatant supplemented cytotoxicity assays

Triplicate samples of Tasmanian devil MNC at ratios of 50:1, 25:1, 12:1 and 6:1 were tested against samples of 10^4 target cells. MNC samples were prepared in culture medium containing 50% Con A culture supernatant (prepared as described in 2.5.4) for a final concentration of 25% within the assays. Untreated control samples were prepared without addition of the mitogen supernatant. The assays were incubated for 18 hours before analysis as described in 2.7.1.

2.7.7 IL-2 supplemented cytotoxicity assays

Tasmanian devil IL-2 was synthesised by our collaborators at the Walter and Eliza Hall Institute, based on the published devil genome sequence. Briefly, the cytokine was synthesised using DNA2.0 encoding the full length sequence for devil IL-2 with a 3Gly6His C-terminal tag. The gene, as a BamHI-NotI fragment was cloned into a pFastBac1 expression vector and then transformed into *E. coli.* Positive bacmid DNA was transformed into Sf21 cells according to the Bac-to-Bac protocol using the CellFectin II reagent and cultured for 4 days. The supernatant was subsequently harvested and concentrated. Triplicate samples of Tasmanian devil MNC at ratios of 50:1, 25:1, 12:1 and 6:1 were tested against samples of 10^4 target cells. MNC samples were prepared in culture medium containing cloned Tasmanian devil IL-2 diluted at 1/200 or 1/2000 for a final concentration of 1/100 or 1/1000 within the assays. Untreated control samples were prepared without addition of the mitogen supernatant. The assays were incubated for 18 hours before analysis as described in 2.7.1.

2.7.8 Poly I:C supplemented cytotoxicity assays

Polyinosinic:polycytidylic acid (Sigma Aldrich, St Louis, USA) was received as a lyophilised powder and reconstituted in cell culture grade PBS at a concentration of 5 mg/mL. The Poly I:C solution was then heated to 50 °C for 30 minutes and cooled to allow optimal annealing of the double-stranded RNA. Triplicate samples of Tasmanian devil MNC at ratios of 50:1, 25:1, 12:1 and 6:1 were tested against samples of 10^4 target cells. MNC samples were prepared in culture medium containing 20, 10, 2 and 0.2 µg/mL Poly I:C for final concentrations of 10, 5, 1 and 0.1 within the assays. Untreated control samples were prepared without addition of the mitogen supernatant. The assays were incubated for 18 hours before analysis as described in 2.7.1.

2.7.9 Formulae and statistics

Mean counts per minute (CPM) values were calculated from replicates and the percent cytotoxicity values were calculated according to the equation:

$$\frac{\text{Percent cytotoxicity} = (\text{(sample CPM} - \text{mean negative control CPM)}}{(\text{mean maximum control CPM} - \text{mean negative control CPM})) \times (100/1)}$$

Statistical significance for chromium release data was calculated using an F test of pre immune and post immune data sets for immunised devils or on serum free vs. serum supplemented samples for ADCC and 4 h NK assays. In assays involving nylon wool and plastic non adherent cells, F tests were performed between pre immune and non adherent cell data sets, then between total mononuclear cell and non adherent cell data sets to calculate statistical significance. Results were considered significant with a p value below 0.05.

2.8 Flow Cytometry

2.8.1 Cell type marker flow cytometry (Glycophorin A, Periaxin)

K562 cells were harvested in suspension and pelleted as described in 2.3.2 then diluted to 5×10^6 cells/mL and 100 µL aliquots were incubated with 10 µL of phycoerythrin (PE)-conjugated CD235a (Glycophorin A) antibody (Dako, California, USA) for 20 minutes at room temperature. The samples were washed three times in PBS, with centrifugation at 14,000 *g* (in a microcentrifuge) for 1 minute.

DFTD cells were harvested and pelleted as described in 2.3.2 then diluted to 5×10^6 cells/mL. Rabbit anti-mouse periaxin antibody (Sigma Aldrich, St Louis, USA) was diluted 1/500 in FACS buffer then 100 µL aliquots were incubated with equal volumes of DFTD cell suspension for 20 minutes at room

temperature. The samples were washed three times in PBS, with centrifugation at 14,000*g* (in a microcentrifuge) for 1 minute then incubated with an Alexa Fluor 488 conjugated goat anti rabbit IgG (Invitrogen, Oregon, USA) and washed again as above.

All samples were diluted to approximately 400 µL volume and analysed by flow cytometry on a BD Canto II (Becton Dickinson, New Jersey, USA) operating a 488 nm solid state laser. Although the parameters were adjusted for each sample, approximate voltages used on DFTD and K562 cells were 235 (FSc), 405 (SSc), 269 (Alexa Fluor 488) and 286 (PE).

2.8.2 Serum/supernatant antibody flow cytometry

Rabbit anti devil immunoglobulin (RαDIg) was purified using a protein A column (Sigma Aldrich, St Louis, USA) from the serum of rabbits immunised with ammonium sulphate precipitated Tasmanian devil serum. The specificity of this reagent for flow cytometry with devil serum was previously established and optimised by A. Kreiss [385], and appears to target an epitope in bound antibody but not IgG associated with the surface of B lymphocytes (observed using flow cytometry and immunohistochemistry but not presented in this thesis). Tasmanian devil serum was diluted 1/25 in FACS buffer. DFTD tumour cells were harvested and pelleted as described in 2.3.2 then diluted to 5×10^6 cells/mL in FACS buffer. Aliquots of 100 µL (containing approximately 5×10^5 cells) were incubated with an equal volume of diluted serum for 20 minutes at room temperature then washed three times in PBS, with centrifugation at 14,000 *g* (in a microcentrifuge) for 1 minute. The samples were incubated with RαDIg at 1/10 in FACS buffer, washed (as above) in PBS, then incubated with an Alexa Fluor 488 conjugated goat anti rabbit IgG (Invitrogen, Oregon, USA) and washed again as above. All samples were diluted to approximately 400 µL volume and analysed by flow cytometry on a BD Canto II (Becton Dickinson, New Jersey, USA) operating a 488 nm solid state laser. Although the parameters were adjusted for each sample, approximate voltages used on DFTD and K562 cells were235 (FSc), 405 (SSc) and 269 (Alexa Fluor 488).

2.9 Immunohistochemistry

2.9.1 Antibodies (primaries, secondaries, concentrations and dilutions)

Target Epitope	Host	Experimental Dilution	Supplier and catalogue Number
Polyclonal Human CD3ε (intracellular portion)	Rabbit	1/400	Dako, California USA A0452
Monoclonal Human MHC II	Mouse	1/100	Dako, California USA M0775
Polyclonal Human Periaxin	Rabbit	1/400	Sigma, Missouri, USA HPA 001868
Monoclonal Devil CD8 (hybridoma culture supernatant – not commercially tested)	Mouse	1/10	Walter and Eliza Hall Institute, Vicroria, Australia
Monoclonal Devil MHC I (intracellular portion)	Mouse	1/100	University of Southern Denmark
Monoclonal Devil β_2-Microglobulin (hybridoma culture supernatant – not commercially tested)	Rat	1/20	University of Southern Denmark
Polyclonal anti rabbit isotype control	Rabbit	1/400	Abcam, New South Wales, Australia ab27472
Monoclonal anti mouse isotype control	Mouse	1/100	Abcam, New South Wales, Australia ab18447

2.9.2 Immunohistochemistry of cytospins

Suspensions of MNC were diluted to 2×10^5 cells/mL in standard PBS. Cytospins were prepared at 55 *g* for 5 minutes then immediately fixed in acetone.

The cytospins were rehydrated in PBS and drained. Peroxidase block (3% hydrogen peroxide in PBS) was applied to each cytospin for 15 minutes. This was followed by Dako's serum free protein block solution (Dako, California, USA) for 30 minutes. Rabbit anti-human CD3 (Dako, California, USA), rabbit anti-human periaxin (Sigma, Missouri, USA) and mouse anti-human MHC II (Dako, California, USA) were diluted in commercial diluent (Dako, California, USA), as listed in Table 2.9.1, then applied for 2 hours at room temperature.

Secondary anti-rabbit and mouse HRP linked secondary antibodies (Dako, California, USA) were applied to samples labelled with single antibodies and the LSAB universal link HRP system (Dako California, USA) was applied to slides labelled with both antibodies. Finally, the samples were labelled with DAB chromogen (Dako, California, USA), counterstained in Mayer's hematoxylin (HD Scientific, New South Wales, Australia), mounted in aqueous medium (Dako, California, USA) and visualised under a light microscope (Olympus, Victoria, Australia) with mounted camera (Leica, Wetzlar, Germany).

2.9.3 Giemsa staining of cytospins

Samples were covered in a modified giemsa solution designed for staining of cellular blood components and blood parasites (Fluka/Sigma Aldrich, St Louis, USA). The solution was filtered and diluted 1:10 in phosphate buffered water (pH 6.5) prior to use. The samples were stained for 6 minutes then washed thoroughly in water.

2.9.4 Biopsy removal and processing

Core biopsies from DFTD tumours were taken using 3mm punches and immediately fixed in 10% buffered formalin (Sigma Aldrich, New South Wales, Australia) for 7 days. The biopsies were embedded in paraffin wax, sectioned longitudinally into 3µm thick slices and placed onto aminotriethoxysilane-coated slides (Sigma Aldrich, St Louis, USA).

2.9.5 Immunohistochemistry of formalin-fixed Tasmanian devil tissues

The tissue sections from Tasmanian devil tumour biopsies were deparaffinized in xylene and rehydrated through graded alcohol solutions to water. All sections were boiled in citrate buffer solution (pH 6) in an electric pressure cooker (Russel Hobbs, Greater Manchester, England) for 10 minutes at medium heat. Slides were left to cool to 35°C then quenched in water. A peroxidase block solution (3% hydrogen peroxide in PBS) was applied to each cytospin for 15 minutes, followed by Dako's serum free protein block solution (Dako, California, USA) for 30 minutes. Rabbit anti-human CD3 (Dako, California, USA), mouse anti-human MHC II (Dako, California, USA), Rabbit anti human periaxin (Sigma Aldrich, St Louis, USA) and mouse anti devil CD8 (provided by L. Corcoran and colleagues, WEHI, Victoria, Australia) primary antibodies and appropriate negative controls (Dako, California, USA) were diluted in commercial diluent (Dako, California, USA), as listed in Table 2.9.3, and applied to the sections for 2 hours at room temperature. Monoclonal rat anti devil β_2M antibody (provided by H. Siddle and colleagues, Cambridge University, England) were diluted in the same commercial diluent and applied for 18 hours at 4°C.

LSAB universal link HRP system (Dako, California, USA) antibodies were applied to slides for 30 minutes at 21 °C then the samples were washed and labelled with DAB chromogen (Dako, California, USA). The slides were counterstained in Mayer's hematoxylin (HD Scientific, New South Wales, Australia), mounted in aqueous medium (Dako, California, USA) and visualised under a light microscope (Olympus, Victoria, Australia) with mounted camera (Leica, Wetzlar, Germany).

2.10 Immunotherapy of Tasmanian devils

A full list of information about the captive Tasmanian devils used in the immunotherapy experiments detailed in this thesis is given in Chapter 8 (Appendix Section 1).

2.10.1 LAK cell immunotherapy

One DFTD diseased captive female Tasmanian devil (CD 16) was available for immunotherapy. The DFTD tumour was developed following challenge with live cells after a vaccine trial showed evidence for antibody development by ELISA and appeared as a clearly visible mass on the back at commencement of therapy. The tumour type was known to be Strain 3. The method for generation of Tasmanian devil LAK cells was chosen based on assessment of cytotoxic responses against DFTD cells following stimulation with mitogens, cytokines or the agonist Poly I:C of which stimulation with Con A induced the strongest responses.

For immunotherapy, whole blood samples, of approximately 10 mL in volume, were taken as described in 2.4. The MNC were extracted as described in 2.5.1 and the cells were stimulated with Con A as described in 2.5.3 to induce a transition to LAK cells. The samples were washed 3 times, at 250 *g* for 10 minutes, in sterile cell culture grade PBS then counted for number and viability in a total volume of 1 mL. In general, 50 - 60% of the original number of cells extracted was viable when recovered from mitogen stimulation. The LAK cells were transferred to a cryogenic vial and the original tube was washed with 1 mL of extra PBS. The cells were pelleted once more, resuspended in 100 – 200 µL of cell culture grade PBS then incubated at 35 °C before transfer to the captive facility.

Upon arrival at the facility, the candidate devil was anaesthetised as described in 2.4 and the tumour was measured in 3 dimensions using a calliper and ruler. A 3 - 4 mm core biopsy was taken from the tumour and immediately fixed in formalin. The activated MNC were then injected into several sites within the tumour mass using a 14 gauge needle.

A thorough health examination of CD 16 was performed at each visit. Injections of autologous LAK cells were given at weeks 0, 5 and 7. Tumour measurements were taken on weeks 0, 5, 7 and late in week 8 and biopsy punches were taken on weeks 0, 5, late in week 8, to monitor the effect of LAK cell injection.

2.10.2 Con A culture supernatant-treated cell immunotherapy

The treatment of cells with Con A culture supernatant upregulated surface MHC I expression [285]. MHC I positive cells were cultured as described in 2.1.2.6 and washed 3 times in cell culture grade PBS then counted for number and viability. An aliquot containing 10^8 cells was taken to a cryovial, completed to 2 mL with PBS then pelleted and resuspended in 100 – 200 µL then incubated at 35 °C before transfer to the captive facility. Upon arrival, the candidate devil was anaesthetised as described in 2.4 and the MHC I positive cells were injected subcutaneously into the shoulder of the candidate devil at week 8.5. Tumour measurements were taken on weeks 11, 12, 13 and a punch biopsy was taken on week 11 to monitor the effect of MHC I positive cell injection. A subsequent injection of MHC I positive cells was given at late in week 17 following a sudden decrease in tumour size.

Two wild DFTD diseased Tasmanian devils (DD 11 and DD 18) were trapped and relocated to the captive facility at Richmond. The devils were anaesthetised as described in 2.4 then blood samples were taken and tumour number and size was recorded and punch biopsies were taken from selected tumours. 7 days later, both devils were injected with MHC I positive cells prepared as above. The devils were examined once a week and tumour measurements and biopsy punches were taken.

2.10.3 Con A culture supernatant injection

Con A culture supernatant, which was free from activated MNC, was prepared as described in 2.5.4 and diluted 1:4 (to a final concentration of 20%) in cell culture grade PBS and incubated at 35 °C before transfer to the captive facility. Upon arrival at the facility, the devil was anaesthetised as described in 2.4 and the tumour was measured in 3 dimensions using a caliper as described in 2.6.2. The solution of Con A culture supernatant was then injected into several sites within the tumour mass using a 21 gauge needle. Tumour measurements were taken late in weeks 14, late in week 15, late in week 17, late in week 18, week 19, and late in week 24. A punch biopsy was taken late in week 17 to monitor the effect of intratumoural Con A culture supernatant injection. Blood samples were taken in weeks 19, 22 and 24 for analysis of cytotoxicity responses.

Chapter 3 - Analysis of immune responses against xenogeneic tumour cells and DFTD tumour cells in Tasmanian devils

3.1 Introduction

In order to be transmissible, DFTD must be able to evade the host immune response. Although previous studies have provided evidence for the normal development and function of many immune responses in Tasmanian devils [2], there is little evidence for immune activity against DFTD, with poor lymphocyte infiltration into the tumours [269]. However, the immune responses of infected wild devils against DFTD have never been analysed. The first aim of this thesis chapter was to determine if Tasmanian devils with DFTD can form immune responses against DFTD cells.

A potential explanation for the poor immune response against DFTD could be that, despite evidence for an otherwise competent immune system, Tasmanian devils fail to form anti-tumour responses altogether. Past observations of Tasmanian devils in zoos [259], and recent observations in our captive research populations, suggest that they are prone to developing cancers. However, the development of anti-tumour responses in the species has not been verified. In mammals, anti-tumour responses are largely mediated by the specific immune system. Therefore, it was important to examine the development of lymphocyte cytotoxicity and antibody responses against DFTD and other tumour cells. Therefore, the second aim of this thesis chapter was to determine if Tasmanian devils can mount functional cytotoxic and humoral anti-tumour responses. To test these responses in Tasmanian devils, a tumour immunisation model was developed. Immunisation with foreign cancer cells was likely to induce a strong systemic immune response, including development of cytotoxicity and antibody. Since DFTD cells lack cell surface MHC I expression human K562 [285], a cell line that is also deficient in this protein, was selected for the experiments. The radioactive chromium release assay was used to analyse cytotoxicity responses of Tasmanian devils against K562 and DFTD cells.

If the anti-tumour responses of Tasmanian devils were functional, it should be possible to induce immune recognition of DFTD through vaccination. Therefore, the third aim of this chapter was to determine if Tasmanian devils can be induced to form immune responses against DFTD cells. Immunisation strategies were designed to induce lymphocyte cytotoxicity responses, either directly or through the prior activation of innate immune responses. The non-specific immunomodulatory adjuvant Montanide provided the basis for several immunisation preparations. ISCOMATRIX®, which can increase cross presentation of protein antigens between dendritic cells and cytotoxic T

lymphocytes [317], was also tested. Other molecules used to supplement these basic adjuvants included TLR agonists such as CpG oligonucleotides and Poly I:C, which can augment specific responses against tumours [143] and a stimulatory ligand for dendritic cells, Flt 3 ligand. In addition to adjuvant choice, several strategies for killing DFTD cells were used, and their effects on immunogenicity were assessed.

3.2 Results

3.2.1 DFTD diseased Tasmanian devils do not form cytotoxicity or antibody responses against DFTD tumour cells

The transmissible nature of DFTD suggests that Tasmanian devils do not form a functional immune response against the tumour cells when exposed to the disease. Further evidence for a lack of response has been provided by immunohistochemistry studies which show limited lymphocyte infiltration into DFTD tumours. Evidence for a functional immune response directed at the tumour has not been specifically investigated. To determine if Tasmanian devils with DFTD showed evidence of functional cytotoxic responses against the tumour cells, cytotoxicity responses were measured using the chromium release cytotoxicity assay (Section 2.7.1). Eight representative responses are shown in Figure 3.1. None of the animals tested showed any evidence of cytotoxicity responses against DFTD tumour cells and the responses formed were not statistically different to healthy controls; one healthy wild and one captive Tasmanian devil (Fig. 3.1).

Another important specific immune response is antibody production. Serum collected from wild devils with DFTD was assessed using flow cytometry for the presence of antibodies against DFTD tumour cells (2.8.2). The fluorescence profiles from 12 wild devils with DFTD were compared to three healthy captive devils. None of the diseased animals showed any evidence for an antibody response to DFTD cells, with similar fluorescence profiles compared to the controls. Representative results from six devils are shown in Figure 3.2.

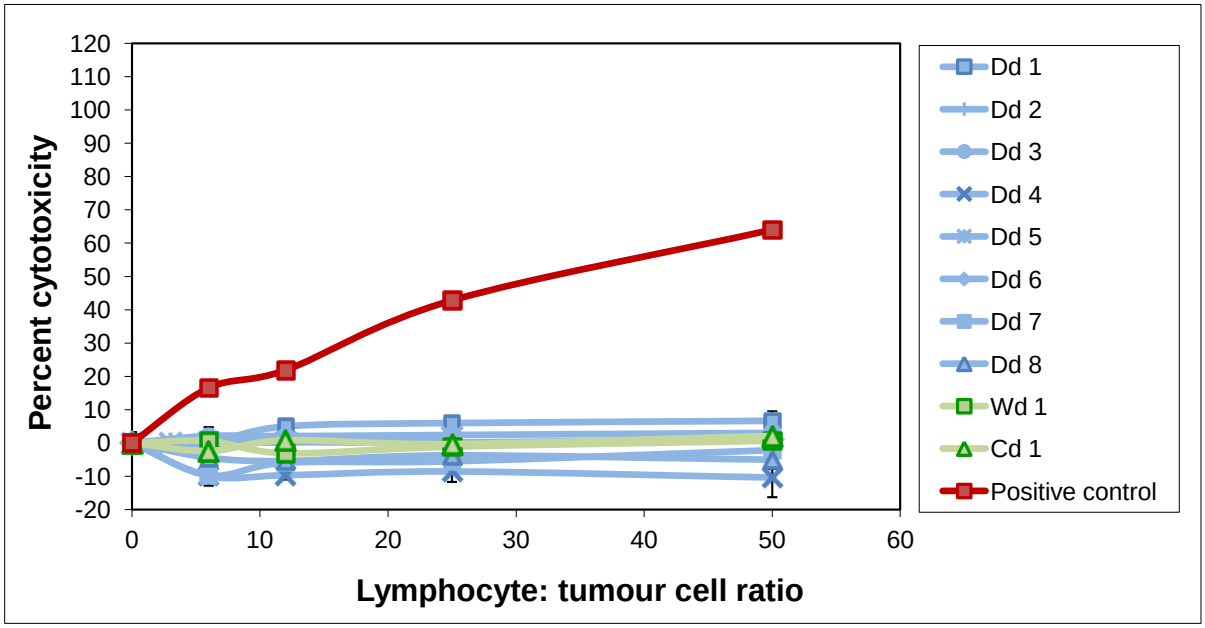

Figure 3.1. ***In vitro*** **cellular cytotoxicity responses of DFTD infected wild devils against DFTD tumour cells.**

Chromium release cytotoxicity assays were performed by culturing mononuclear cells from DFTD diseased Tasmanian devils with radioactively labelled DFTD cells for 18 hours. Percent cytotoxicity values at mononuclear cell: tumour cell ratios of 50:1 to 6:1 are presented for mononuclear cell samples from eight diseased Tasmanian devils (Dd 1 - Dd 8). The cytotoxicity responses of two healthy devils, one from a wild DFTD affected population (Wd 1) and one living in captivity (Cd 1), and one captive devil that formed a cytotoxic response against DFTD after vaccination (positive control) are also shown. The statistical difference between the responses formed by the DFTD diseased Tasmanian devils the healthy devils were assessed using F Tests, with a value of $P<0.05$ classified as significant and marked with an asterisk (*).

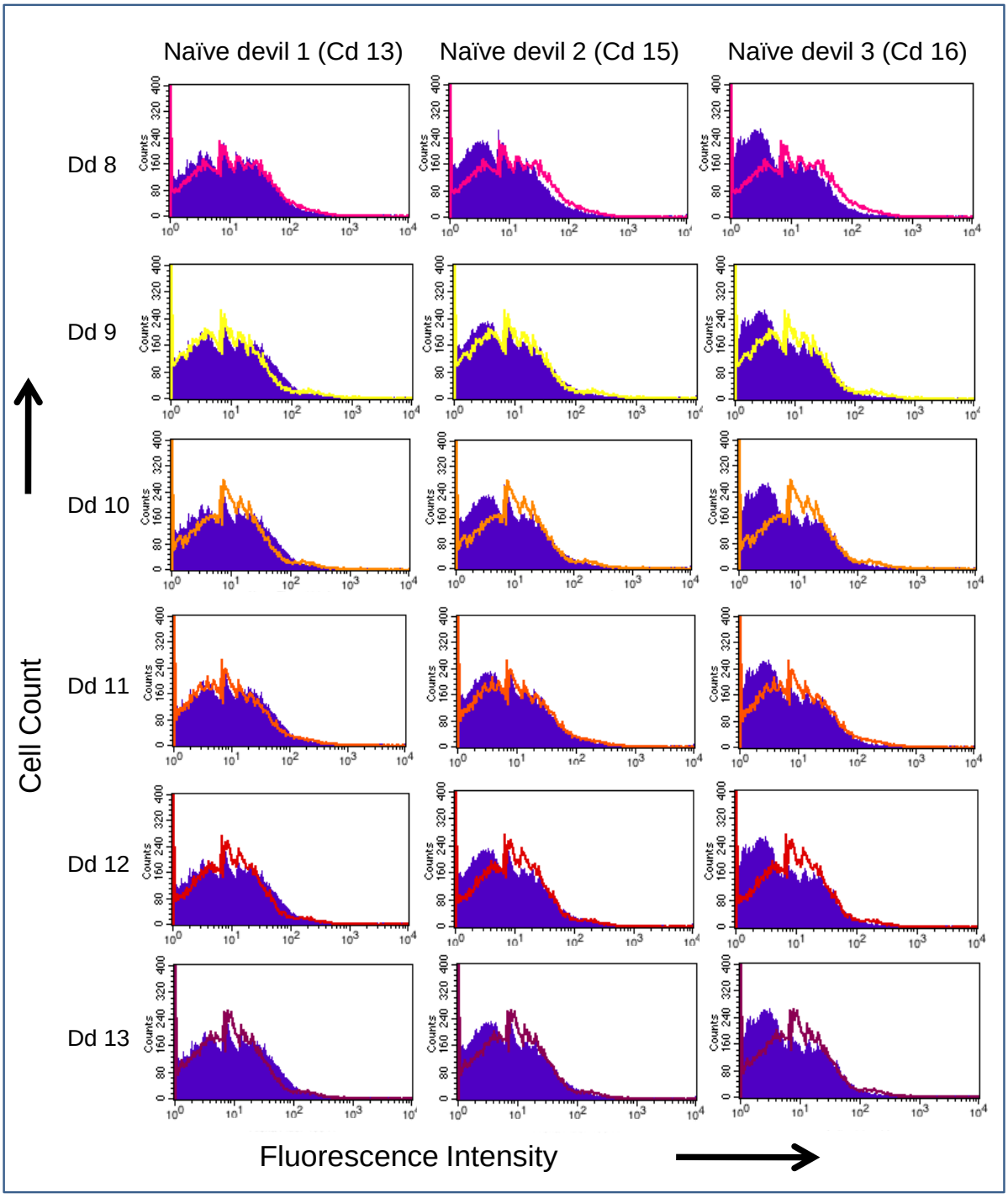

Figure 3.2. Antibody responses of DFTD infected wild devils against DFTD tumour cells.

Serum antibody levels of six wild DFTD infected Tasmanian devils (Dd 1 – Dd 13) were analysed using flow cytometry. Samples were compared to the levels of DFTD antibody in the serum of three naive captive devils (Cd 14, Cd 16 and Cd 17), which are represented by the solid purple curves in each panel. The responses of individual diseased devils are shown using coloured histogram outlines.

3.2.2 Functional cytotoxicity and antibody responses occur against foreign tumour cells in Tasmanian devils

The failure of Tasmanian devils to produce immune responses against DFTD tumour cells may be due to an intrinsic inability to develop anti-tumour immunity. To examine the ability to mount cytotoxic responses, four Tasmanian devils were immunised with a xenogeneic tumour cell line, human K562 (2.6.1.1).

Four Tasmanian devils were injected subcutaneously with human K562 cells and lymphocyte cytotoxicity was measured 14 days after each dose using the chromium release cytotoxicity assay (2.1.7). No spontaneous cytotoxicity was observed in samples from any of the four devils prior to immunisation (Fig. 3.3). One of the four devils formed a weak but statistically significant cytotoxic response after one K562 cell immunisation (Fig. 3.3a). After a second dose, three of the four devils formed clear cytotoxic responses, all of which were statistically significant compared to the levels of cytotoxicity prior to immunisation (Fig. 3.3a, e and g). One devil did not form a cytotoxic response (Fig. 3.3c).

Serum collected 14 days after each dose was assessed using flow cytometry for the presence of antibodies against K562 tumour cells and the fluorescence profile was compared to pre immune levels as a baseline (2.8.2). None of the four devils produced antibody after the first dose of K562 cells. However, all four devils formed strong antibody responses after two doses as the fuorescence profile shifted to the right along the x axis, indicating an increase in bound serum antibody (Fig. 3.3 b, d, f and h).

To provide evidence for the formation of long-lasting responses after immunisation of Tasmanian devils, the lymphocyte cytotoxicity responses of two animals that had been previously immunised against K562 cells were measured several months after the final dose (2.7.1). Four months after the final injection with K562 cells, one devil produced a strong cytotoxic response against K562 (Fig. 3.4a). This response was similar in intensity to the response after the second K562 immunisation and was statistically different to the level of cytotoxicity before immunisation. The other devil showed a low level of cytotoxicity which was detectable only at the highest effector: tumour cell ratio (Fig. 3.4b). When a boost of K562 cells was given, both devils produced strong cytotoxicity responses within seven days, which were greater than those previously formed against the second dose of K562 tumour cells (Fig. 3.4).

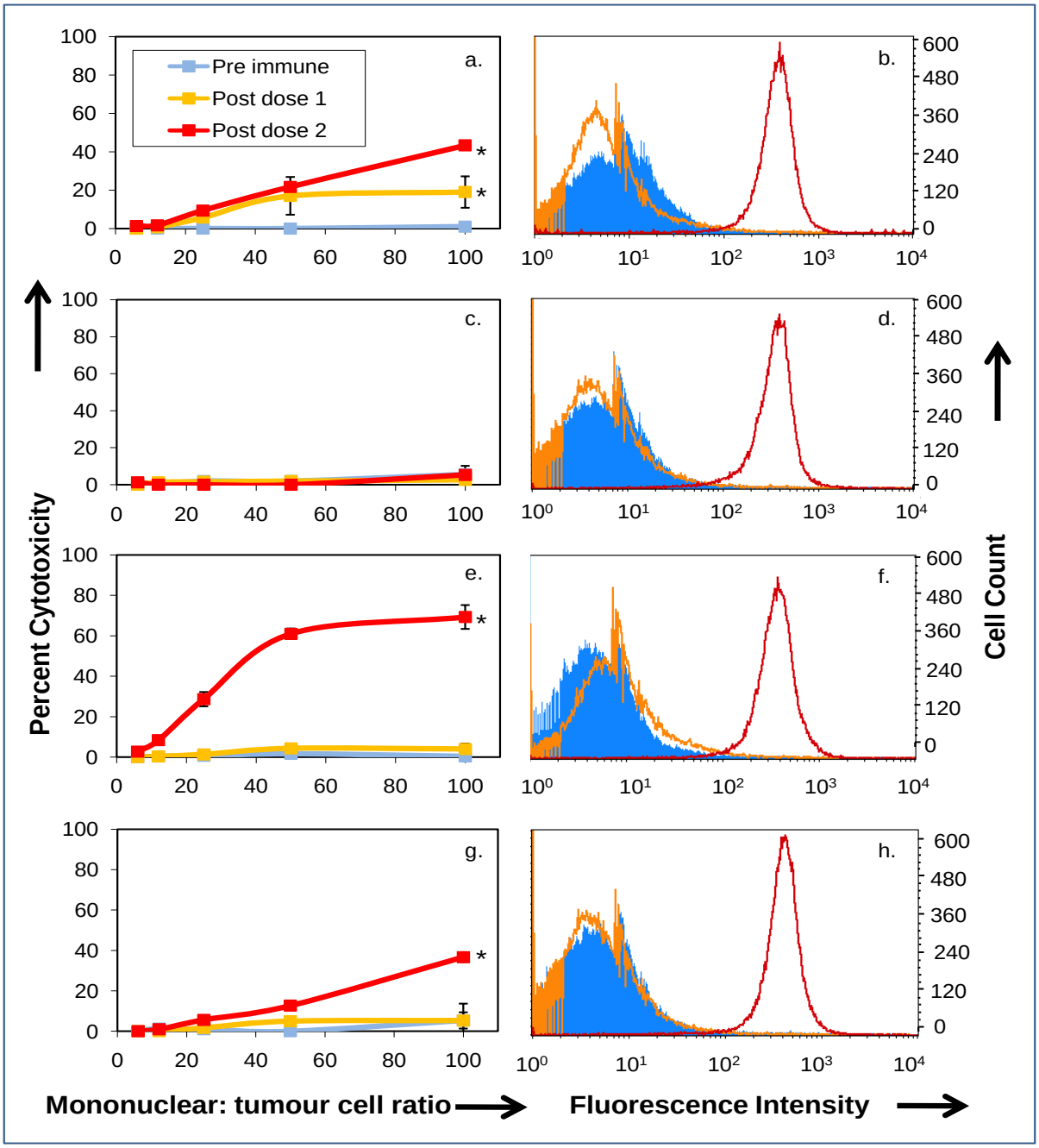

Figure 3.3. Cellular cytotoxicity and antibody responses of healthy captive devils immunised against live K562 tumour cells in Montanide adjuvant.

Chromium release cytotoxicity assays were performed by culturing mononuclear cells from four immunised Tasmanian devils with radioactively labelled K562 cells for 18 hours. Percent cytotoxicity values at mononuclear cell: tumour cell ratios of 100:1 to 6:1 are shown. The statistical difference between the pre immune responses of each devil and the cytotoxicity formed after each dose was assessed using F Tests, with a value of $P<0.05$ classified as significant and marked with an asterisk (*). Serum K562 antibody levels of the immunised Tasmanian devils were analysed using flow cytometry.

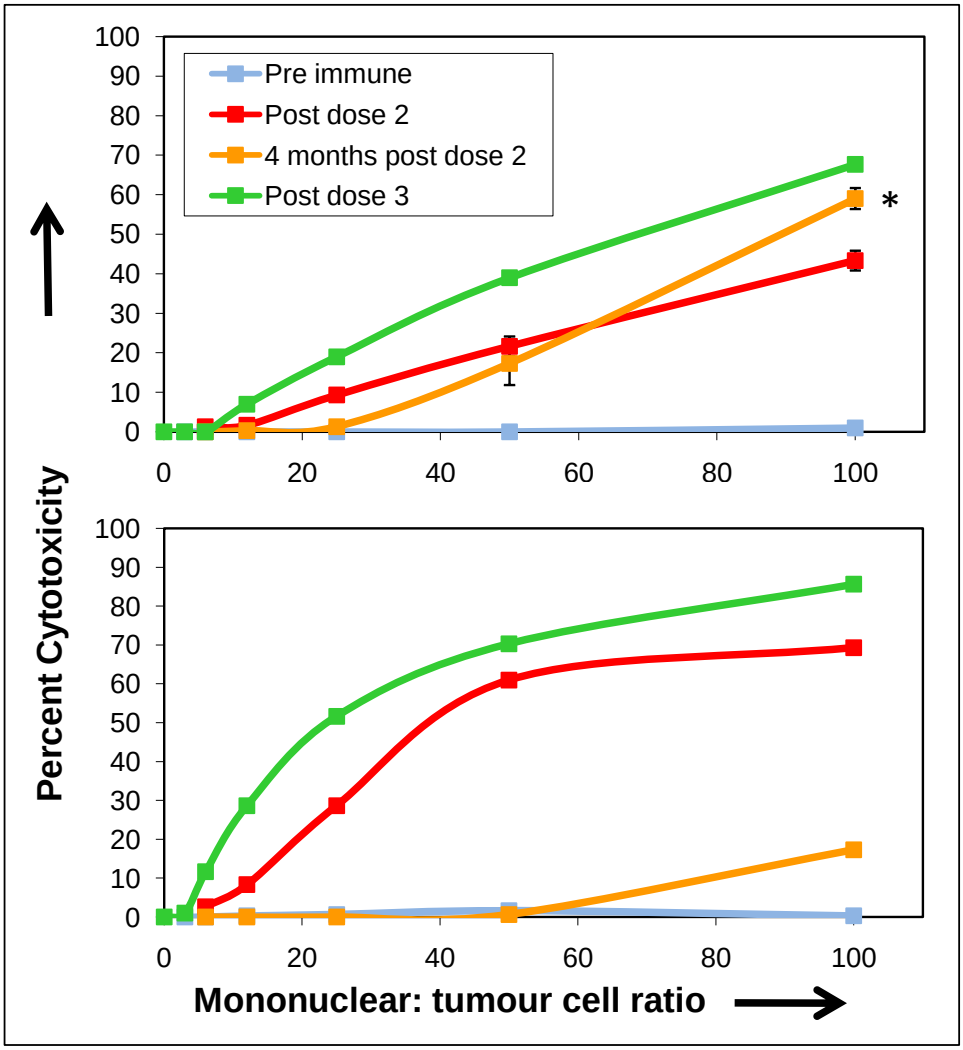

Figure 3.4. Memory cytotoxicity responses of devils previously immunised against live K562 tumour cells in Montanide adjuvant.

Chromium release cytotoxicity assays were performed by culturing mononuclear cells from two immunised Tasmanian devils with radioactively labelled K562 cells for 18 hours. Percent cytotoxicity values at mononuclear cell: tumour cell ratios of 100:1 to 6:1 are shown for pre immune, post dose 2 and four months post dose 2 samples. The statistical difference between the pre immune responses of each devil and the cytotoxicity formed four months following the second immunisation was assessed using F Tests, with a value of $P<0.05$ classified as significant and marked with an asterisk (*). Serum K562 antibody levels of the immunised Tasmanian devils were analysed using flow cytometry.

To produce an effective vaccine against DFTD, the tumour cells would have to be killed, as the presence of live cells could cause the disease in the vaccinated devil. To determine the effect of irradiation on the responses induced, two Tasmanian devils were injected subcutaneously with irradiated human K562 cells (2.6.1.2) and cytotoxicity responses were measured 14 days after each dose using the chromium release cytotoxicity assay (2.7.1). No spontaneous cytotoxicity was

observed prior to immunisation (Fig. 3.5). Neither devil formed a cytotoxic response after the first or second immunisation with K562 cells. The assay was performed at a higher ratio for the third immunisation. After the third dose of irradiated K562 cells one devil produced a cytotoxicity response at effector: tumour cell ratios of 50 and 100:1 (Fig. 3.5a) and the other at a ratio of 100:1 only (Fig. 3.5c).

Serum collected 14 days after each dose was assessed using flow cytometry for the presence of antibodies against K562 tumour cells and the fluorescence profile was compared to pre immune levels as a baseline (2.8.2). Neither devil produced an antibody response after the first dose of irradiated K562 cells. However, both devils showed evidence for an antibody response after the second dose, (Fig. 3.5b and d). These responses did not increase after a third dose of K562 cells.

3.2.3 Natural Killer cells are not directly responsible for the killing of K562 cells by Tasmanian devil mononuclear cells

The absence of MHC I on K562 cells should make them targets for NK cell cytotoxicity. Distinguishing characteristics of NK cells include innate cytotoxicity against MHC null cells and the capacity to develop rapid cytotoxic responses. Therefore, these characteristics were analysed in the mononuclear cells of K562 immunised Tasmanian devils to determine if NK cells were responsible for the killing. NK cell functional assays are classically performed over four-hour time periods to determine the capacity for rapid killing of target cells [386]. To further analyse the role of NK cells in anti K562 cytotoxicity, short length (4 hour) cytotoxicity assays were performed using K562 cells and the MNC from immunised Tasmanian devils (2.7.2). No evidence for rapid, NK like killing of K562 cells was observed within these assays (Fig 3.6a). These results suggested that the effector cells in the anti K562 responses were not behaving in the characteristic manner of NK cells.

Basic specificity assays, in the form of “Bystander Killing”, were performed using K562 cells and Yac-1 cells, a MHC I null mouse tumour line, for 18 hours. Bystander killing of radioactively labelled Yac-1 cells was examined in a mixture with unlabelled K562 cells, and specificity for each cell line was examined separately using standard chromium release assays (2.7.1). No killing was observed in the Yac-1 sample or the mixture (Fig 3.6b). However, the mononuclear cells from immunised devils formed strong specific responses against K562 cells. This result demonstrated that some degree of priming against K562 had occurred.

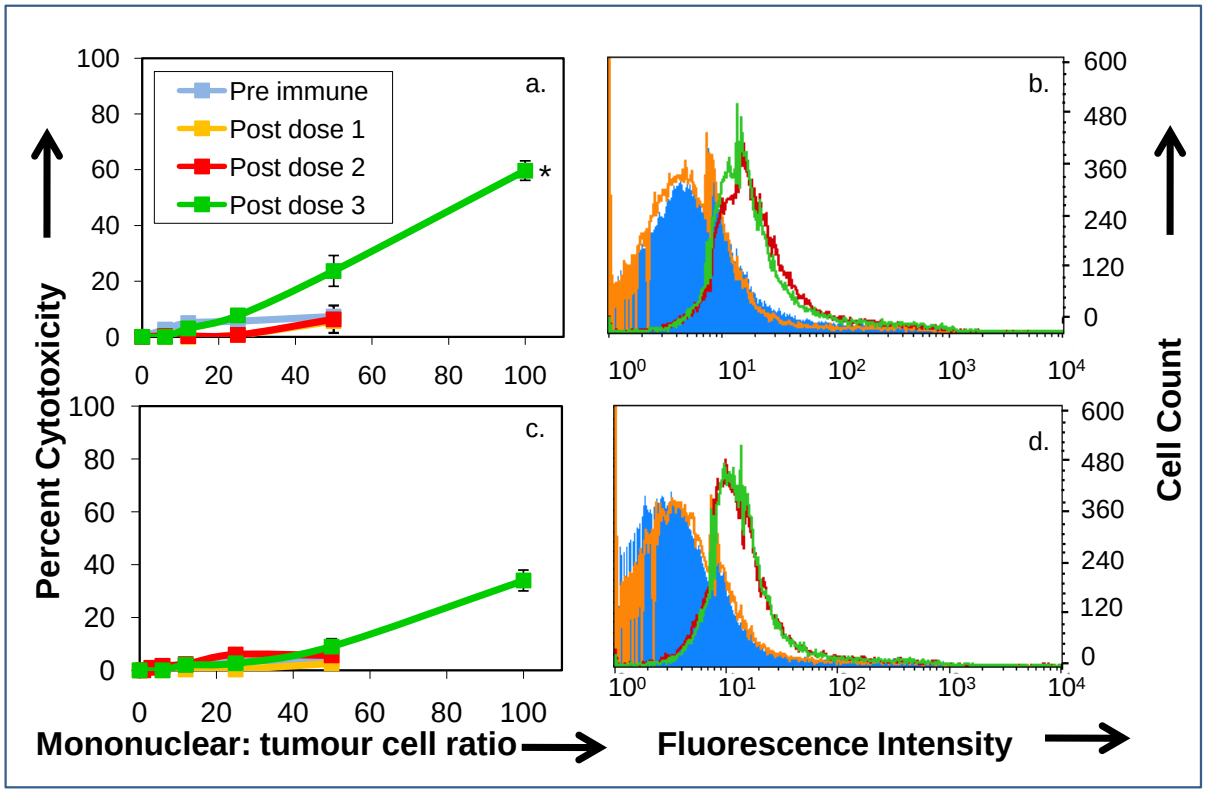

Figure 3.5. Cellular cytotoxicity and antibody responses of healthy captive devils immunised against irradiated K562 tumour cells in Montanide adjuvant.

Chromium release cytotoxicity assays were performed by culturing mononuclear cells from two immunised Tasmanian devils with radioactively labelled K562 cells for 18 hours. Percent cytotoxicity values at mononuclear cell: tumour cell ratios of 100:1 to 6:1 are shown. The statistical difference between the pre immune responses of each devil and the cytotoxicity formed after each dose was assessed using F Tests, with a value of $P<0.05$ classified as significant and marked with an asterisk (*). Serum K562 antibody levels of the immunised Tasmanian devils were analysed using flow cytometry.

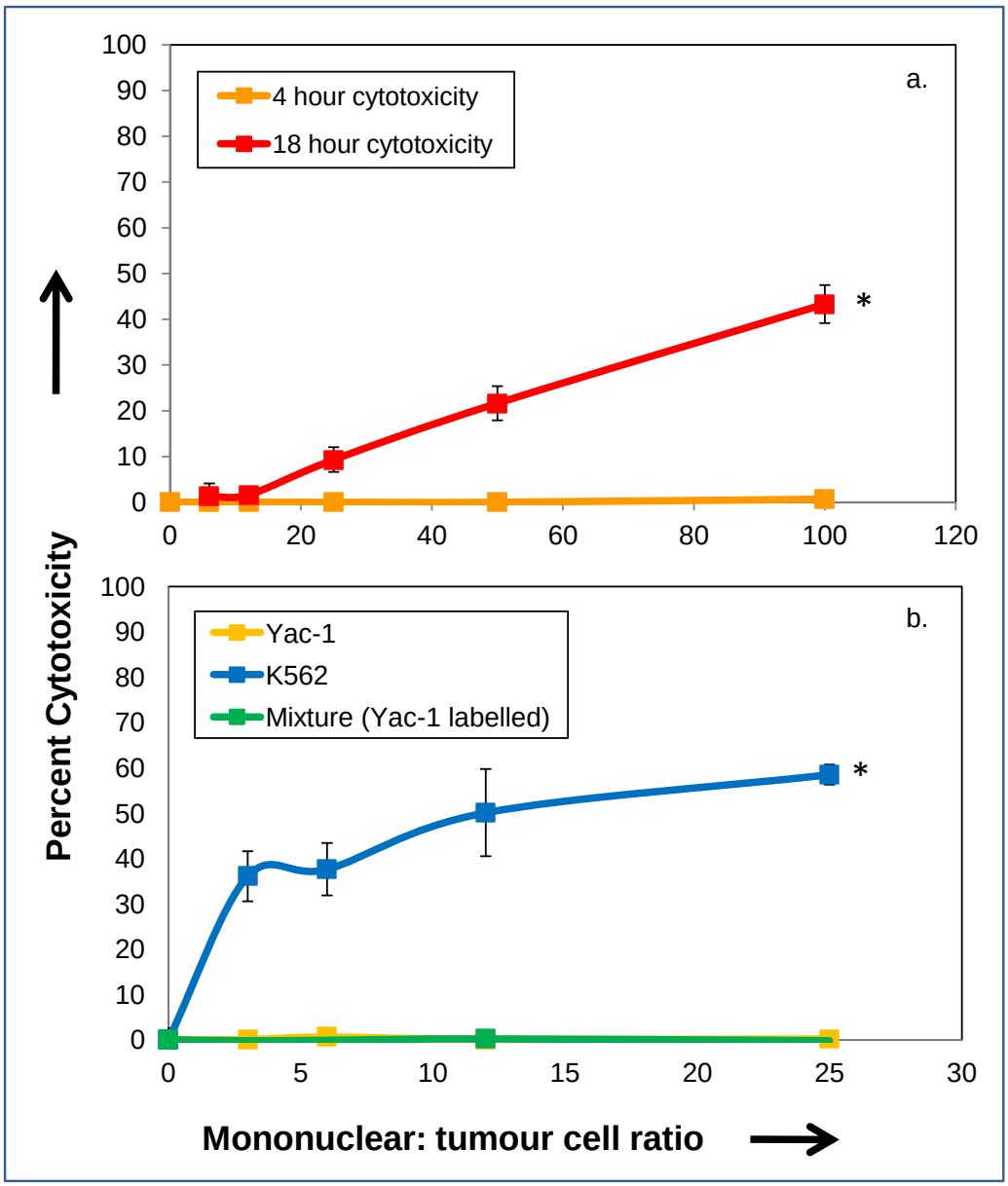

Figure 3.6. Tumour cell line specificity and short length NK cell cytotoxicity assays in an immunised Tasmanian devil.

NK cell cytotoxicity assays were performed by culturing mononuclear cells from two immunised Tasmanian devils with radioactively labelled K562 cells for 18 hours. Specificity assays (bystander killing) were performed using chromium release cytotoxicity assays containing mononuclear cells from an immunised Tasmanian devil with radioactively labelled Yac-1 cells, K562 cells or a mixture of radioactively labelled Yac-1 and unlabelled K562 cells for 18 hours. The statistical difference between the two cell populations was assessed using F Tests, with a value of $P<0.05$ classified as significant and marked with an asterisk (*).

3.2.4 Immunisation against irradiated DFTD cells does not consistently induce cytotoxicity and antibody responses in Tasmanian devils

Given the evidence that Tasmanian devils can produce functional cytotoxicity and antibody responses against tumour cells, immunisation with killed DFTD cells could potentially induce protective immune responses. Tasmanian devils were injected with sequential doses of irradiated DFTD cells (2.6.1.2) to assess the formation of anti-tumour responses against DFTD.

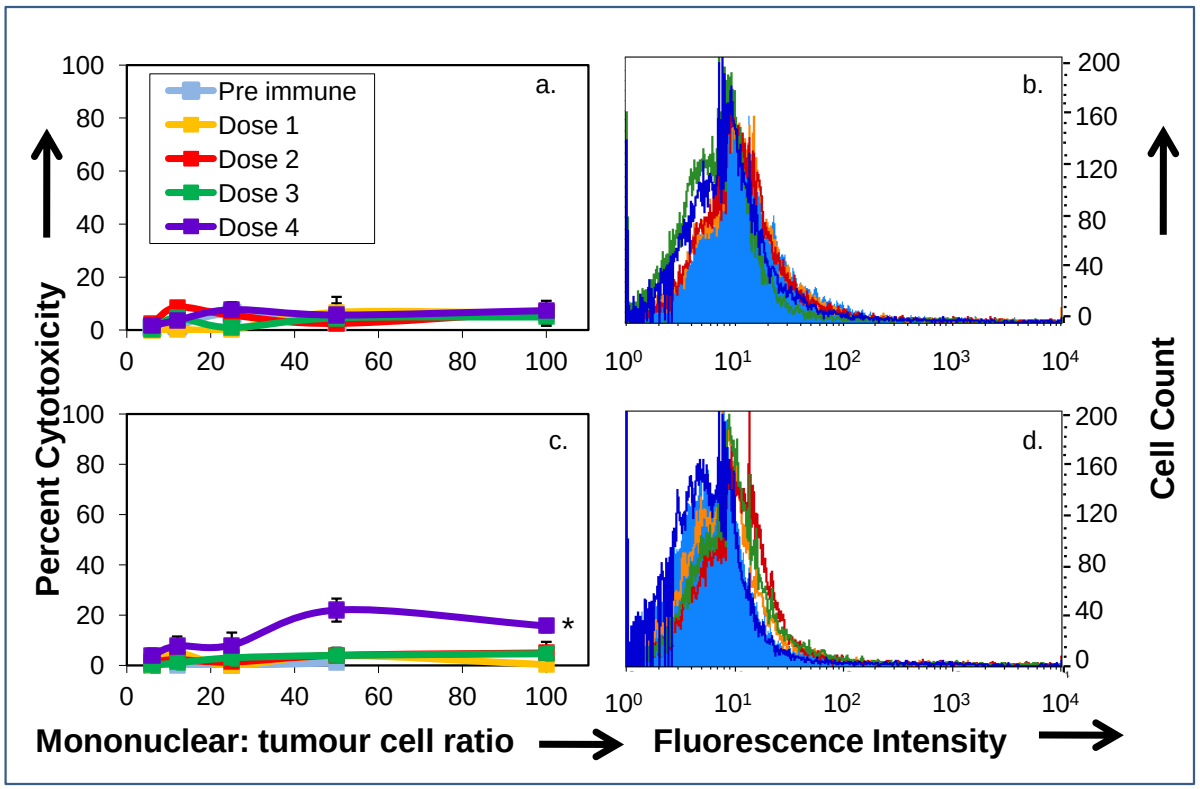

Figure 3.7. Cellular cytotoxicity and antibody responses of healthy captive devils immunised against irradiated DFTD tumour cells in Montanide adjuvant.

Chromium release cytotoxicity assays were performed by culturing mononuclear cells from two immunised Tasmanian devils with radioactively labelled DFTD cells for 18 hours. Percent cytotoxicity values at mononuclear cell: tumour cell ratios of 100:1 to 6:1 are shown. The statistical difference between the pre immune responses of each devil and the cytotoxicity formed after each dose was assessed using F Tests, with a value of $P<0.05$ classified as significant and marked with an asterisk (*). Serum DFTD antibody levels of the immunised Tasmanian devils were analysed using flow cytometry.

Two healthy captive Tasmanian devils were injected subcutaneously with irradiated DFTD cells and cytotoxicity was measured 14 days after each dose using the chromium release cytotoxicity assay (2.7.1). Prior to injection there was no evidence for spontaneous cytotoxicity against the DFTD cells (Fig. 3.7). After four injections, one devil did not produce cytotoxic responses after any dose (Fig 3.7a). The second devil produced very weak but statistically significant cytotoxic responses against DFTD cells after doses 1 and 4 (Fig 3.7c).

Serum collected 14 days after each dose was assessed using flow cytometry for the presence of antibodies against DFTD tumour cells and the fluorescence profile was compared to pre immune levels as a baseline (2.8.2). There was no evidence of antibody response in either devil after the first dose (Fig. 3.7b and d). After the second dose, one devil showed a slight increase in fluorescence intensity (Fig. 3.7d), indicating a small increase in antibody level. The second devil showed no evidence of antibody development. The weak response in the previously responsive devil remained constant after the third injection but decreased after the fourth dose. The unresponsive devil showed no change after either the third or fourth injection.

3.2.5 Immunisation against irradiated DFTD cells in Montanide adjuvant supplemented with CpG DNA induces variable levels of cytotoxicity but no antibody development

Altering the adjuvant used in irradiated DFTD cell immunisations may increase the responses produced against the tumour cells. Adjuvants supplemented with immunomodulatory molecules such as CpG oligonucleotides, immunisation preparations containing accessible MHC I molecules and heat shock proteins or the cross-presentation inducing agent ISCOMATRIX® were used to determine if repeated exposure to killed DFTD cells could induce cytotoxicity and antibody responses against the tumour cells.

Four devils were injected three times with irradiated DFTD cells in Montanide adjuvant supplemented with synthetic oligodeoxynucleotides (ODN) containing repeated CpG motifs (2.6.1.3). Cytotoxicity responses were measured 14 days after each dose using the chromium release cytotoxicity assay (2.7.1). Prior to injection there was no evidence for spontaneous cytotoxicity against DFTD cells (Fig. 3.8). After one dose, all devils showed evidence for a moderate level of cytotoxicity (Fig. 3.8a, c, e and g).

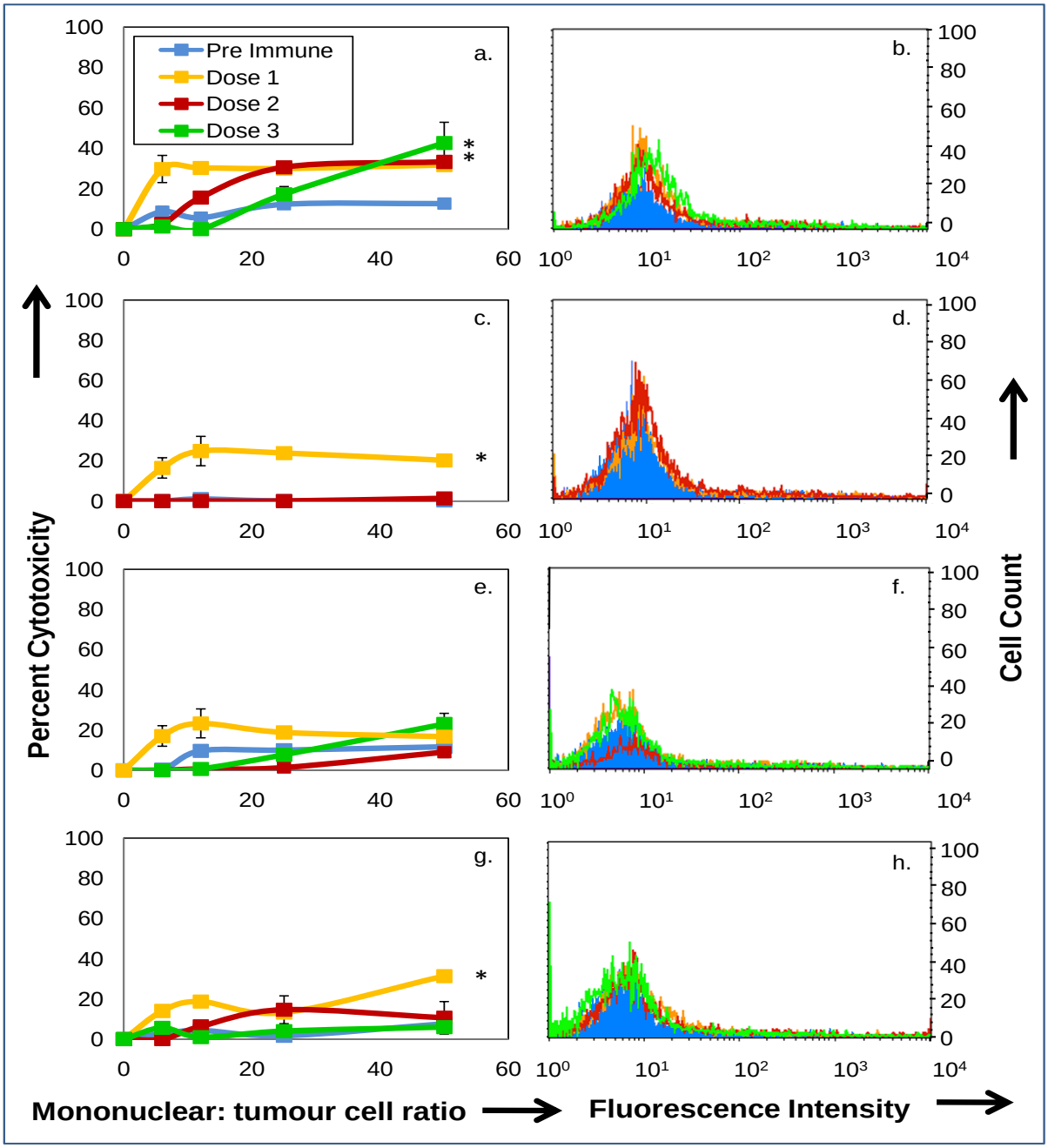

Figure 3.8. Cellular cytotoxicity and antibody responses of healthy captive devils immunised against irradiated DFTD tumour cells in CpG Montanide adjuvant.

Chromium release cytotoxicity assays were performed by culturing mononuclear cells from four immunised Tasmanian devils with radioactively labelled DFTD cells for 18 hours. Percent cytotoxicity values at mononuclear cell: tumour cell ratios of 50:1 to 6:1 are shown. The statistical difference between the pre immune responses of each devil and the cytotoxicity formed after each dose was assessed using F Tests, with a value of $P<0.05$ classified as significant and marked with an asterisk (*). Serum DFTD antibody levels of the immunised Tasmanian devils were analysed using flow cytometry.

After a second dose, only one of the four devils produced a moderate cytotoxicity response that was statistically significant compared to the level prior to injection (Fig. 3.8a). No other devil formed a clear cytotoxic response after the second dose (Fig. 3.8c, e and g). After a third injection, the responsive devil maintained the cytotoxicity response, which retained statistical significance (Fig. 3.8a). One other devil formed a weak cytotoxicity response after the second dose, although this was not statistically significant (Fig. 3.8e). The third devil that received a third dose of irradiated DFTD cells in Montanide adjuvant with CpG oligonulceotides did not produce a cytotoxic response after the third dose (Fig. 3.8g).

Serum collected 14 days after each dose was assessed using flow cytometry for the presence of antibodies against DFTD tumour cells and the fluorescence profile was compared to pre immune levels as a baseline (2.8.2). There was no clear evidence of antibody development in any of the four devils injected with DFTD cells and CpG Montanide adjuvant, even after three doses (Fig. 3.8b, d, f and h).

3.2.6 Sonication of DFTD cells increases the immunogenicity of DFTD cell preparations but does not induce antibody development

The absence of MHC I molecules on the surface of DFTD cells [285] could result in a failure to induce CTL responses. However, despite a decreased surface expression, MHC I protein may be present in the cytoplasm of DFTD cells in which case immunisation preparations containing lysed cells may be more immunogenic. The presence of intracellular MHC I protein was determined using immunohistochemistry. A mouse anti-Tasmanian devil MHC I protein IgG antibody was developed by our collaborators at the University of Southern Denmark. Immunohistochemistry with this antibody was performed on cytospins of cultured DFTD cells (2.9.2). MHC I protein staining was present in the cytoplasm of cells in DFTD cytospin samples, with a punctate pattern and little nuclear localisation, consistent with limited membrane expression (Fig. 3.9).

The effect of exposing the molecules to the immune system of Tasmanian devils was assessed using immunisations with DFTD cells lysed by sonication (2.6.1.4). An additional advantage of this of lysis technique was the potential upregulation of chaperone protein expression, including highly immunogenic heat shock proteins, in the sonicated samples. Due to limited access to research animals the following experiments were performed in two Tasmanian devils that had been previously injected with killed DFTD cells. The two devils were rested for more than 12 months and their cytotoxicity responses against DFTD cells were tested (this was called ‘Pre Immune 2’) at which time both devils showed no evidence of cytotoxicity against DFTD cells (Fig. 3.10). The devils were

injected with sonicated DFTD cells in Montanide adjuvant supplemented with CpG oligonucleotides. Cytotoxicity responses were measured 14 days after each dose using the chromium release cytotoxicity assay (2.7.1). After one dose of sonicated DFTD cells in CpG Montanide adjuvant, both devils produced evidence for moderate cytotoxicity responses, which were statistically significant to the level prior to injection (Fig. 3.10a and c). After the second dose, one devil produced a weak cytotoxic response but the response of the other increased. Both responses retained their statistical significance (Fig. 3.10c).

Serum collected 14 days after each dose was assessed using flow cytometry for the presence of antibodies against DFTD tumour cells and the fluorescence profile was compared to pre immune 2 levels as a baseline (2.8.2). There was no clear evidence for antibody development in one devil, even after three doses (Fig. 3.10b). The serum antibody level of the devil that showed evidence of cytotoxicity increased slightly after dose 1 and it remained at this level after doses 2 and 3 (Fig. 3.10d), providing evidence for a weak antibody response.

ISCOMATRIX® adjuvant can promote cytotoxicity responses through cross-presentation of protein antigens to cytotoxic T lymphocytes by dendritic cells [317]. Two Tasmanian devils were injected with a preparation containing DFTD protein extracts in ISCOMATRIX® adjuvant (2.6.1.5) and cytotoxicity was measured 14 days after each dose using the chromium release cytotoxicity assay (2.7.1). Prior to injection there was no evidence for spontaneous cytotoxicity in either devil (Fig. 3.11). Neither devil showed evidence of a cytotoxic response after any dose of DFTD protein (Fig. 3.11a and c).

3.2.7 Immunisation with DFTD protein and ISCOMATRIX® adjuvant and other immune agonists does not induce cytotoxicity responses or antibody

Serum collected 14 days after each dose was assessed using flow cytometry for the presence of antibodies against DFTD tumour cells and the fluorescence profile was compared to pre immune levels as a baseline (2.8.2). There was no evidence of antibody development in one devil after three doses (Fig. 3. 11b) but there appeared to be a slight increase in the serum antibody of the other after the second dose (Fig. 3.11d). This response increased slightly again after dose 3.

The activity of dendritic cells can be increased by stimulation with activating ligands such as Flt 3 ligand and Poly I:C. Two Tasmanian devils were injected with a preparation containing DFTD protein extracts in ISCOMATRIX® adjuvant supplemented with Flt 3 ligand and Poly I:C (2.6.1.5) and cytotoxicity was measured 14 days after each dose using the chromium release cytotoxicity assay

(2.7.1). Prior to injection there was no evidence for spontaneous cytotoxicity in either devil (Fig. 3.12). Neither devil showed evidence of a cytotoxic response after any dose (Fig. 3.12a and c).

Serum collected 14 days after each dose was assessed using flow cytometry for the presence of antibodies against DFTD tumour cells and the fluorescence profile was compared to pre immune levels as a baseline (2.8.2). There was no evidence of antibody development in either devil, even after three doses (Fig. 3.12b and d).

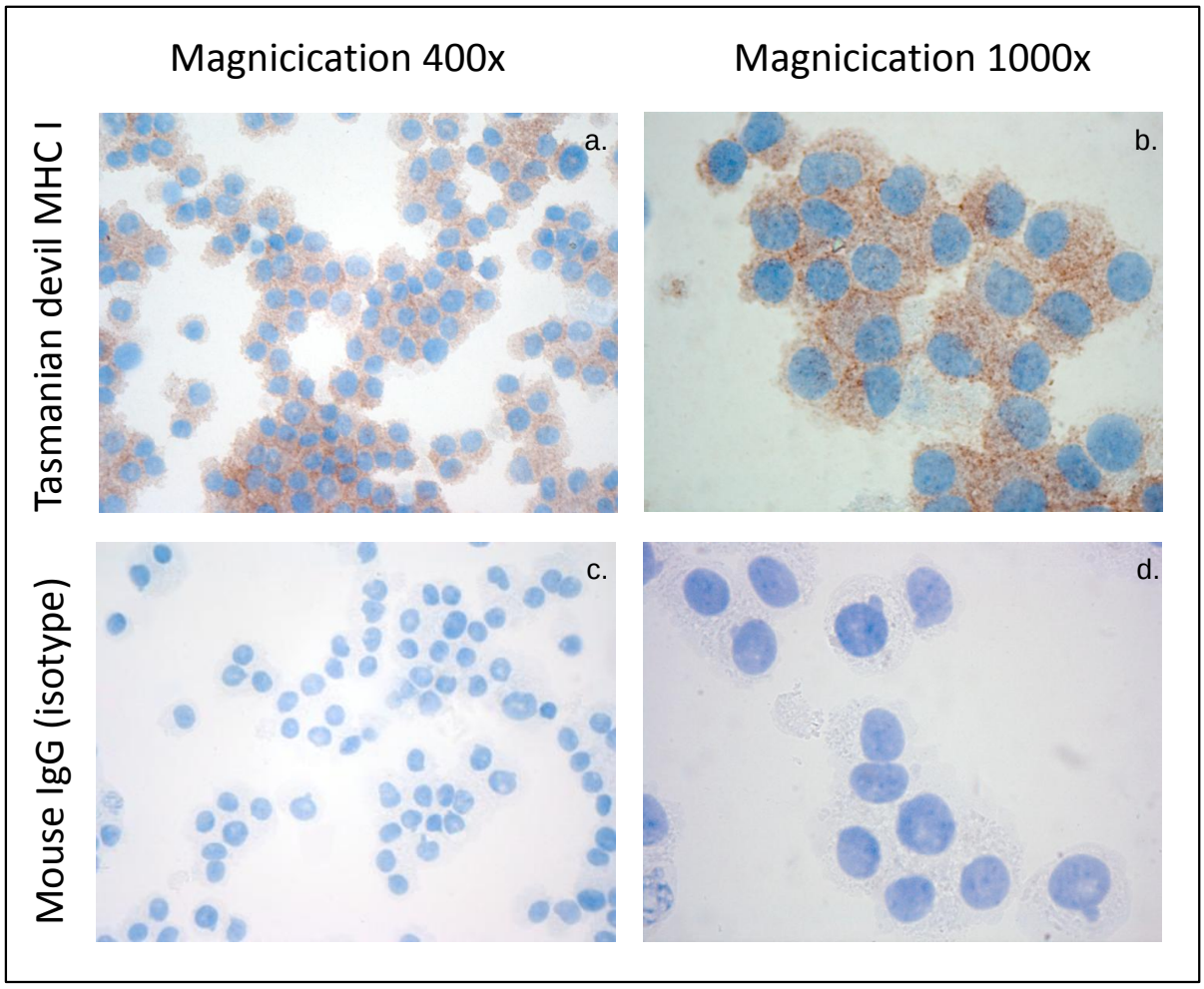

Figure 3.9. Distribution of MHC I protein in DFTD cell cytospins.

Immunohistochemistry was performed on cytospins of C5065 DFTD cells using antibodies for Tasmanian devil MHC I. Images were taken at 400x and 1000x magnification. The brown staining indicates cell regions expressing MHC I protein. The distribution of MHC I protein appeared to be intracellular, a pattern characterised by the absence of staining across the nucleus and no accumulation at cell borders. Panels a. and b. show a samples labelled with Mouse anti Tasmanian devil MHC I IgG and panels c. and d. show samples labelled with mouse IgG as a negative control.

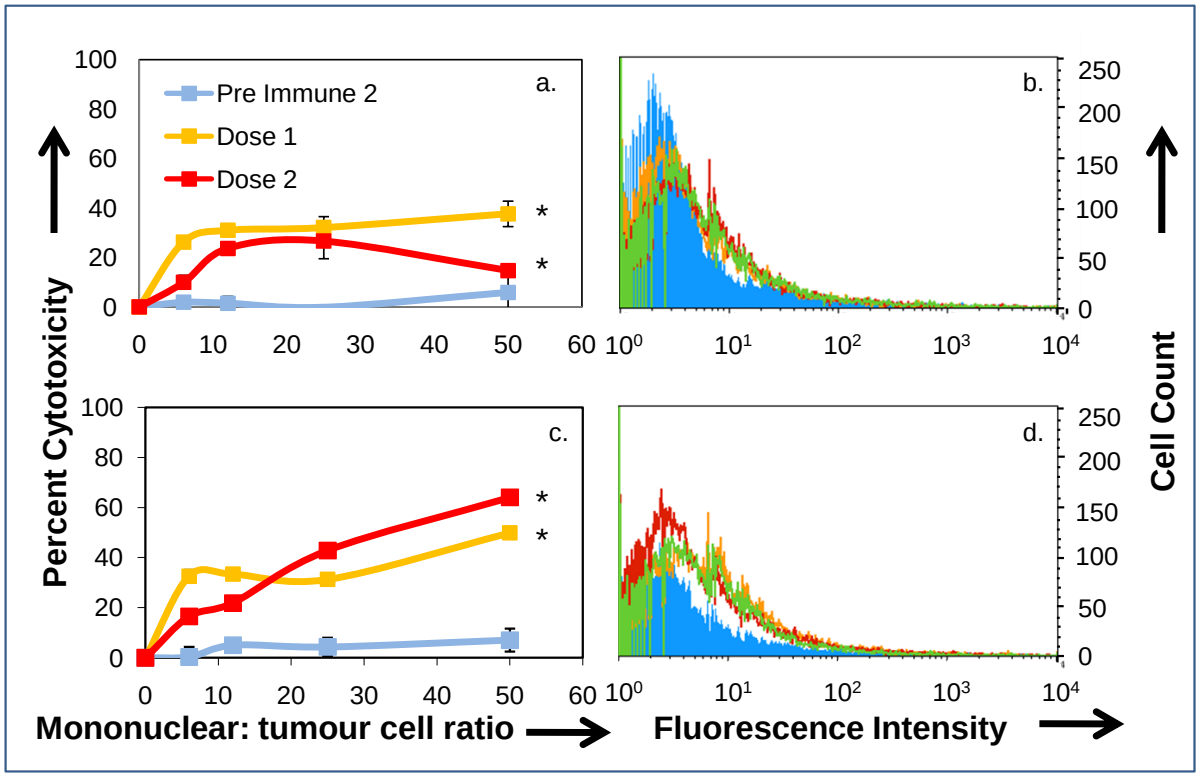

Figure 3.10. Cellular cytotoxicity and antibody responses of healthy captive devils immunised against sonicated DFTD tumour cells in CpG Montanide adjuvant.

Chromium release cytotoxicity assays were performed by culturing mononuclear cells from two immunised Tasmanian devils with radioactively labelled DFTD cells for 18 hours. Percent cytotoxicity values at mononuclear cell: tumour cell ratios of 100:1 to 6:1 are shown. The statistical difference between the pre immune responses of each devil and the cytotoxicity formed after each dose was assessed using F Tests, with a value of $P<0.05$ classified as significant and marked with an asterisk (*). Serum DFTD antibody levels of the immunised Tasmanian devils were analysed using flow cytometry.

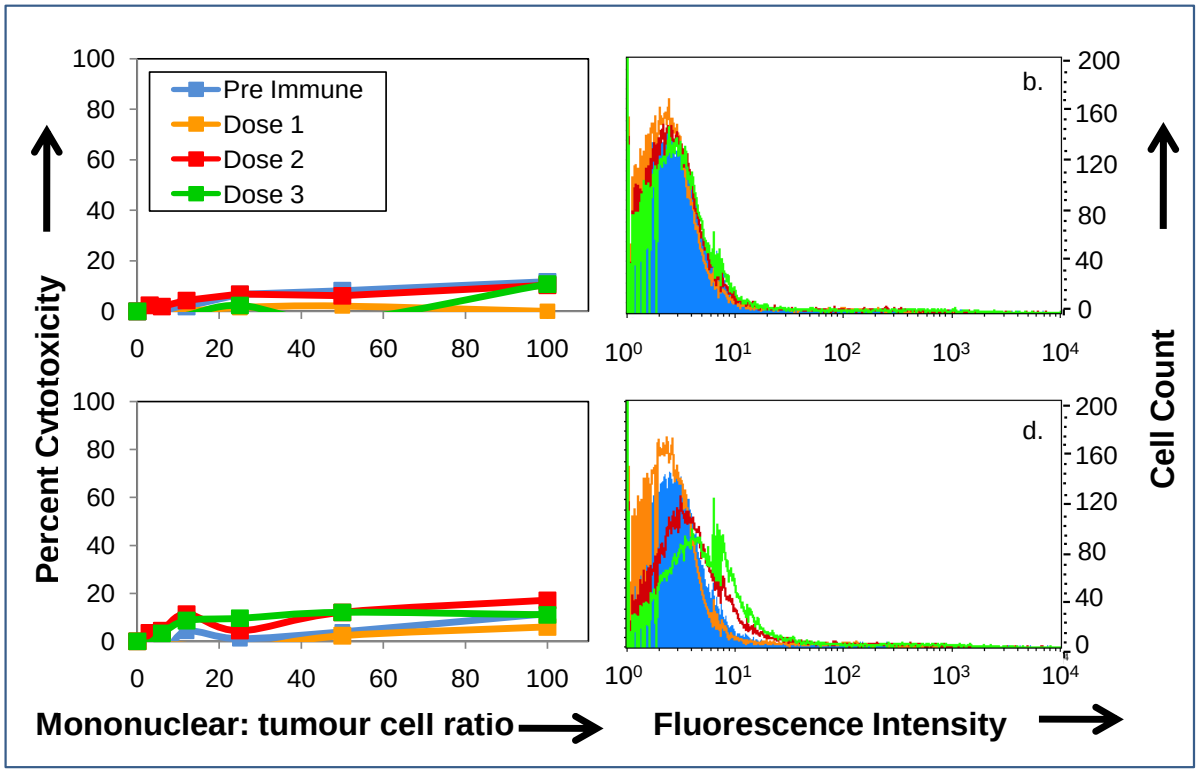

Figure 3.11. Cellular cytotoxicity and antibody responses of healthy captive devils immunised against DFTD tumour cell protein in ISCOMATRIX® adjuvant.

Chromium release cytotoxicity assays were performed by culturing mononuclear cells from two immunised Tasmanian devils with radioactively labelled DFTD cells for 18 hours. Percent cytotoxicity values at mononuclear cell: tumour cell ratios of 100:1 to 6:1 are shown. The statistical difference between the pre immune responses of each devil and the cytotoxicity formed after each dose was assessed using F Tests, with a value of $P<0.05$ classified as significant and marked with an asterisk (*). Serum DFTD antibody levels of the immunised Tasmanian devils were analysed using flow cytometry.

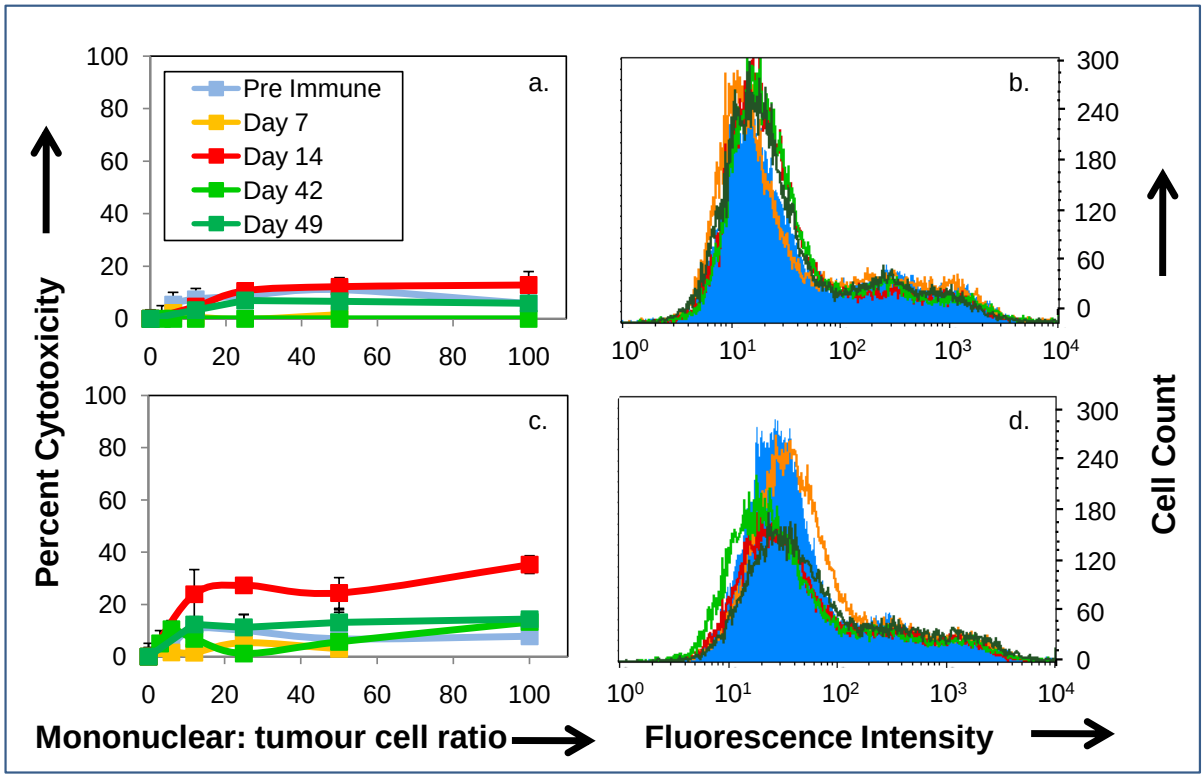

Figure 3.12. Cellular cytotoxicity and antibody responses of healthy captive devils immunised against DFTD tumour cell protein in ISCOMATRIX® adjuvant supplemented with Flt 3 ligand and Poly I:C.

Chromium release cytotoxicity assays were performed by culturing mononuclear cells from two immunised Tasmanian devils with radioactively labelled DFTD cells for 18 hours. Percent cytotoxicity values at mononuclear cell: tumour cell ratios of 100:1 to 6:1 are shown. The statistical difference between the pre immune responses of each devil and the cytotoxicity formed after each dose was assessed using F Tests, with a value of $P<0.05$ classified as significant and marked with an asterisk (*). Serum DFTD antibody levels of the immunised Tasmanian devils were analysed using flow cytometry.

3.3 Discussion

Devil Facial Tumour Disease (DFTD) is one of two examples of a naturally occurring transmissible cancer. There is no evidence for disease resistance among wild devils [1]. The disease rapidly progresses after infection, with death occurring within only a few months after tumour appearance [269]. The transmissible nature of the disease suggests that the tumour cells are capable of evading the host immune response [1]. Histology has provided evidence for a lack of anti-tumour activity against DFTD, with low levels of lymphocyte infiltration in only seven percent of samples [269]. The experiments discussed in this chapter sought to confirm the lack of immune response against DFTD using functional studies of lymphocyte cytotoxicity and antibody production against DFTD tumour cells. Additionally, the production of functional anti-tumour immune responses in Tasmanian devils was examined as their absence could provide a reason for DFTD transmission. Furthermore, the presence of functional cytotoxicity and antibody responses in Tasmanian devils would be crucial for the use of an immunological intervention, such as a immunisation, against DFTD.

The results presented in this chapter provided evidence that wild Tasmanian devils with DFTD fail to mount a specific anti-tumour immune response against cells of the disease. This suggests that, despite prolonged exposure to the disease, the immune system fails to identify and eliminate the tumour cells. This finding was in good agreement with the immunohistochemical evidence of low lymphocyte presence within DFTD tumours [269]. This low level of lymphocyte infiltration would not result in cytotoxicity. One potential explanation for the lack of immune activity against DFTD tumours in Tasmanian devils is an inability to mount specific anti-tumour responses. Work in our laboratory has previously shown that Tasmanian devils have a functional immune system, with a normal range of cells and the capability of phagocytic responses and mitogen induced lymphocyte proliferation [1,2]. However, the formation of anti-tumour responses, including cytotoxicity, against tumour cells had not been assessed.

Cytotoxic lymphocytes are important cellular mediators of anti-tumour immunity in other mammals [387] but have yet to be identified in Tasmanian devils. These cells require prior exposure to their targets, as well as co-stimulatory signals, before they form a response and have the capacity to produce lasting ‘memory’ responses [6]. Since these cells fail to form responses against DFTD under normal conditions, studies to determine their functional presence in Tasmanian devils were clearly warranted. The approach taken in this study was to immunise Tasmanian devils with xenogeneic tumour cells from the human erythroleukaemia K562 line [388] in order to induce the development of anti-tumour cytotoxic responses. Antibody and cell mediated cytotoxicity was assessed to obtain evidence of an anti-tumour response. All Tasmanian devils immunised with K562 cells developed

cytotoxic responses and specific antibody against the cell line. The responses against K562 cells occurred after the second immunisation with K562 cells, thus suggesting a requirement for prior exposure to the target cells, which is a defining characteristic of cytotoxic T lymphocyte cytotoxicity. As the assays were performed fourteen days after the first immunisation, it is possible there may have not have been a sufficient time for the responses to fully develop before a second dose was given. This may have skewed the interpretation of the data to suggest that two doses, rather than a longer time period for reaction, were required. One piece of evidence supporting this interpretation was the development of a weak response in a single devil after the first immunisation. If the trial was repeated, measurements could be taken at day 21 to further examine the timing of the responses and a requirement for a second dose of K562 cells. In addition to the requirement of multiple doses, there were no responses observed in short-length assays. There was also no evidence for spontaneous activity or by-stander killing, suggesting some specificity for the target cell type. These characteristics collectively supported the possibility that T lymphocytes were likely to be responsible for the cytotoxic responses observed against K562 cells.

The development of long-lasting cytotoxicity against K562 cells and evidence of rapid generation of responses upon rechallenge is consistent with the formation of immunological memory. Some animals produced long-lasting responses, with cytotoxicity still evident after several months, and mounting rapid responses following challenge with a boost of tumour cells. The K562 immunisation experiments therefore provided the first evidence for the development of long lasting, specific responses against tumour cells in Tasmanian devils. However, the formation of memory responses was only analysed in Tasmanian devils immunised against live cells as the two devils that received irradiated cell injections became unavailable for vaccine research soon after the completion of the trial; one died suddenly of a suspected viral infection, the other was required for breeding. Nevertheless, since the formation of memory responses in Tasmanian devils occurs, it is possible that a successful DFTD vaccine could induce the same type of response.

Considering Tasmanian devils are capable of forming cytotoxic responses, it should be possible to induce an immune response against DFTD. However, as inoculation with live DFTD tumour cells has the potential to establish the disease [275] it was necessary to use killed cells for immunisation. High-dose γ-irradiation is a simple and widely used method for inducing cell death for vaccination [389]. This method has the additional advantages of inducing the upregulation of immune proteins such as MHC I [303] and cell death molecules such as Fas [390] in tumour cells. Use of radiation would also largely preserve the ultrastructure of the tumour cell to target immune responses against the extracellular proteins found in living cells. The immunisation of Tasmanian devils with K562 cells

provided a useful model to assess the effect of this treatment on tumour cell immunogenicity. The reactions against irradiated K562 cells required one additional dose of tumour cells for induction of cytotoxicity and antibody responses. This result suggested that rather than increasing the intensity of immune responses, irradiation of DFTD cells slightly decreased the intensity of the responses formed compared to viable cells. Alternatively, the decreased immune response could be explained by the absence of actively growing cells in irradiated cell immunisations, limiting the expression of immunogenic factors associated with tumour cell proliferation. Even though irradiated cells were not as effective, an immune response was still generated, suggesting that induction of immune responses against irradiated DFTD cells may still be possible.

Following the successful induction of anti-tumour responses against irradiated K562 cells, the development of cytotoxicity and antibody responses against DFTD was assessed using multiple injections of irradiated cells in the presence of Montanide adjuvant. One of the two devils injected with irradiated DFTD cells showed evidence of a weak but statistically significant cytotoxic response but no evidence for antibody. With no prior studies assessing cytotoxicity against DFTD, it was difficult to relate statistical significance to capacity to protect against the disease. Therefore, because the one cytotoxicity response formed against these immunisations was clearly weaker than those induced against the model cell line K562 and there was no evidence of concurrent antibody development as observed in previous trials it is unlikely to be protective against DFTD. Consequently, strategies were required to increase the immunogenicity of the immunisations.

Immune responses against irradiated DFTD cells may be improved through alteration of the adjuvant used in the preparations. Many studies have reported increased cellular cytotoxicity with the use of adjuvants supplemented with synthetic nucleotides containing areas rich in cytosine and guanine motifs (CpG oligonucleotides). These are more common in the genomes of prokaryotes than in mammalian DNA [52]. They provide pathogen-associated molecular patterns (PAMPs) which contribute to the activation of innate immune responses through stimulation of Toll-like Receptor (TLR) 9 [51]. Although the primary receptors of CpG DNA occur in the innate immune system, the molecules have a remarkable range of effects on the effector cells of specific immunity. The presence of CpG oligonucleotides promotes the growth, maturation and activation of antigen-presenting cells (APC), resulting in the induction of an inflammatory, Th1 type, immune response [55,142,391]. CpG oligonucleotides are also mitogenic for T and B lymphocytes. They can stimulate antibody production, increase CTL cytotoxicity and activate Natural Killer (NK) cells [52,54,56]. The efficacy of immunisations containing CpG oligonucleotides has been demonstrated against a variety of pathogens. These include the malaria parasite [309], the hepatitis C virus [392] and the bacterium

Chlamydia trachomatis [310]. Cancer immunisation trials, where immune responses against well known cancer antigens such as NY-Eso-1 were induced, have also been promising [292]. Therefore, supplementation of killed DFTD cell immunisations with CpG oligonucleotides was an attractive strategy for increasing their effects.

The addition of CpG oligonucleotides appeared to increase the immunogenicity of the irradiated DFTD cell preparation. All devils immunised with irradiated cells in the presence of CpG adjuvant developed evidence for a cytotoxic response after one dose. The immunisation preparation was clearly not ideal for immunisation against DFTD cells, as only one of the four devils maintained a significant level of cytotoxicity after further doses of DFTD cells. This response, although it only occurred in one animal, appeared to be real as it increased in intensity with each following dose. This was an extremely important finding, which provided the first evidence that the immune systems of Tasmanian devils are able to form cytotoxic responses against DFTD cells following immunisation. However, since there was no antibody development in any devil, we were not confident that even the moderate cytotoxicity response observed would be protective against DFTD. Therefore, since induction of cytotoxicity against DFTD was possible, perhaps a more immunogenic immunisation would increase the capacity for responses. Manipulation of the cell preparation, rather than the adjuvant, could increase the immunogenicity of the immunisation preparations.

An ideal protein target for generation of an immune response against DFTD would be Major Histocompatibility Complex (MHC) class I molecules. In the majority of vertebrates these proteins are responsible for the presentation of healthy 'self' antigen for surveillance by CTL. They are highly polymorphic and the arrangement of the genes in this protein is essentially unique to each individual [393]. Since all cases of DFTD are derived from a single tumour in one devil [271], the MHC proteins of all tumours should be identical. Molecular studies of Tasmanian devil MHC I genes have provided strong evidence for an identical MHC type in all DFTD tumours which differs from that of the host [270]. As the presence of foreign MHC I in DFTD tumours should activate a CTL response in the host devil, forcing the immune system of a naive animal to identify the DFTD tumour MHC I protein could induce a lasting immune response against the disease. T lymphocytes of Tasmanian devils do not infiltrate DFTD tumours [269] nor do they respond against the tumour cells either during prolonged disease or following immunisation. Thus, there may be an abnormality in the MHC I protein. Studies performed in our lab and by our colleagues at the University of Cambridge suggest that DFTD cells fail to express MHC I protein at the cell surface [285,297]. This would prevent the induction of a CTL response against the tumour cells. However, the MHC I protein appeared to be present in the

cytoplasm of the tumour cells. Therefore, immunisations containing lysed DFTD cells could potentially induce stronger immune responses through exposure to intracellular MHC I.

A common method for lysis of cells for immunisation is treatment with ultrasound, or sonication. This technique has the additional advantage of inducing the expression of chaperone proteins associated with environmental stressors, including heat shock proteins (HSP) [304]. Many of the common HSP have been widely investigated as molecules of interest for cancer vaccination or therapy as they are upregulated in many types of cancer cells [Reviewed in [204]]. They are also highly immunogenic and can induce effective specific responses through the recruitment of the host immune system [394,395]. Consequently, with the exposure of intracellular MHC I and other immunogenic proteins and the potential for upregulation of HSP, the use of a preparation containing sonicated DFTD cells was an alternative option for immunisation of Tasmanian devils. The adjuvant chosen was Montanide supplemented with CpG oligonucleotides, as the evidence of a response after the first dose in previous trial was encouraging.

Due to a limited number of available research animals, the two devils injected with heat-treated sonicated cells in this study had been previously immunised with killed DFTD cells. They were, however, rested for a prolonged period of time and their cytotoxicity and antibody responses against DFTD cells were reassessed and found to be at low levels consistent with other pre immune responses. Both devils immunised with sonicated cells produced evidence of a cytotoxic response after the first immunisation. The intensity of responses in one devil, like the functional response observed in the previous trial, continued to increase after the subsequent doses of DFTD cells. These results provided further evidence that Tasmanian devils can be induced to form cytotoxic responses against DFTD. One substantial difference between sonicated cells and the irradiated cells used in the previous trial is exposure to intracellular proteins rather than those expressed only on the cell surface. Neither devil immunised with sonicated cells showed evidence for antibody development against intact cells, suggesting that responses were not directed against surface antigens. Such a response would be unlikely to provide protection against DFTD tumours where amounts of available intracellular protein would be low. The possibility of a bias in these results, caused by the previous DFTD cell immunisations cannot be discounted. Therefore, although the results of this trial were encouraging, immunisation of more Tasmanian devils with this preparation will be required to verify its effectiveness.

The ability to induce a lasting memory response is a vital characteristic of a successful vaccine. Several alternative immune pathways can lead to the development of cytotoxicity and memory against tumours if normal CTL responses are not activated. One such pathway is cross-presentation,

a phenomenon in which antigen-presenting cells (APC) can present foreign antigen on MHC I molecules in addition to MHC II. This response plays an important role in anti-tumour immunity because, even though tumours express foreign cellular antigens, they may lack the co-stimulatory molecules required to fully activate CTL responses. Cross presentation of antigen by APC results in direct activation of CTL through simultaneous presentation of antigen and costimulation [6]. The recently developed adjuvant ISCOMATRIX® promotes cross presentation of protein directly to CTL [317]. Therefore, we reasoned that immunising Tasmanian devils with DFTD protein extracts and ISCOMATRIX® could result in an upregulation of cross presentation and increased cytotoxicity. However, immunisation with the combination of ISCOMATRIX® adjuvant and DFTD protein did not induce cytotoxicity or antibody responses. It is also possible to increase the function of APC, especially dendritic cells (DC) through stimulation with innate activating molecules. Examples include Flt 3 ligand, a hematopoietic cytokine and growth factor, and polyriboinosinic polyribocytidylic acid (Poly I:C), a synthetic double-stranded RNA analogue, which are capable of inducing proliferation and increasing activity of DC [312,313,314]. These ligands are also capable of concurrently activating NK cell cytotoxicity, making them useful for supplementation of immunisation adjuvants. However, addition of these factors to DFTD protein and ISCOMATRIX® immunisations of Tasmanian devils still did not increase the responses formed.

The results presented in this chapter provide evidence that Tasmanian devils have functional anti-tumour responses. Some devils showed evidence for cytotoxicity against DFTD cells, although immunisation against killed DFTD cells resulted in poor immune responses in the majority of devils. There were also striking differences between the responses formed against K562 and DFTD cell immunisations. The foreign cell line appeared to require two doses to elicit a response and had all the hallmarks of CTL cytotoxicity. However, the majority of significant responses formed against DFTD cells occurred after the first dose and were not induced after later doses. These rapid, short duration responses may have been formed by an innate cell subset, potentially NK cells, and would be unlikely to result in long-lasting immunity. In addition to poor cytotoxicity responses, very few devils immunised with DFTD cells showed evidence for any development of antibody. The flow cytometry assay used detected only small increases in some devils. The potential effect of these responses could have been analysed by determining the statistical significance of the mean fluorescence intensity values of individual samples from those of the pre immune sample. However, as these immunisation experiments were limited to a small sample size, this type of analysis was likely to result in an overestimation of significance, rather than showing a limited response. Consequently, the likelihood of a functional response was assessed qualitatively in this data, and few animals formed sufficient antibody levels to consider the responses protective.

As previously discussed, DFTD cells fail to express MHC I protein on the cell surface. NK cells are innate cytotoxic lymphocytes capable of killing target cells without the presence of MHC I [6]. If DFTD tumour cells completely downregulate surface MHC I expression, NK cells should kill DFTD cells under normal conditions. However, there is no evidence for spontaneous cytotoxicity in naive or diseased Tasmanian devils, suggesting that NK cells are not functional against the tumour cells.

In summary, although Tasmanian devils do not form immune responses against DFTD when they harbour the tumours, the species is capable of producing functional anti-tumour responses against foreign cells. Additionally, immunisation with killed DFTD cells has provided the first evidence for lymphocyte cytotoxicity responses against DFTD tumour cells in some Tasmanian devils. However, the reactions to the DFTD immunisations were highly variable and only immunisation with sonicated cells and CpG oligonucleotides resulted in promising responses against the disease. Clearly, more immunisation trials with this preparation and new adjuvant combinations are warranted. An alternative strategy for developing a vaccine against DFTD would be to investigate the other immune pathways which contributed to the response against K562 cells with a view to manipulating the Tasmanian devil's immune response against DFTD.

Chapter 4 - Mechanisms of cytotoxicity and their effector cells in Tasmanian devils

4.1 Introduction

Despite the evidence for functional immune responses, immunisation to induce a response against DFTD has been unsuccessful in the majority of Tasmanian devils, even after repeated exposure to killed cells. This may have been due to the lack of MHC I on the surface of the tumour cells and the inability to induce a cytotoxic T lymphocyte (CTL) response. Since this major anti-tumour response does not appear to be present against DFTD cells in Tasmanian devils, strategies to induce immune responses should target other cell types and cytotoxicity pathways that do not rely on MHC I expression on DFTD tumour cells. The immune response against K562 cells would provide a good model to study responses against MHC I negative cells [388]. Immunisation with K562 would therefore allow the characterisation of immune responses against MHC I negative cells that could potentially be harnessed for responses against DFTD cells.

The peripheral blood mononuclear cells (MNC) used in the *in vitro* cytotoxicity assays must contain a population of cells which have the capacity to kill MHC I negative K562 cells. Previous studies performed in our laboratory have examined the different types of white blood cells in peripheral blood [2] but the cell types present in MNC suspensions isolated from Tasmanian devils has not been thoroughly analysed. Therefore, the first aim of this chapter was to analyse the composition of the MNC fractions in Tasmanian devil peripheral blood, using immunocytochemistry. This would identify the potential effector cells of the response against MHC I negative K562 cells. The cell types with the capacity to mount cytotoxic responses, such as T lymphocytes and Natural Killer (NK) cells, were of particular interest. T lymphocytes have previously been identified in Tasmanian devils using immunohistochemistry with a cross-reactive antibody to CD3ε [264]. However, although NK cells play an important role in the anti-tumour responses of other mammals, they have not been evaluated in Tasmanian devils. The second aim of this chapter was to identify NK cells in Tasmanian devils.

Identification of pathways capable of killing these MHC I negative cells may provide targets for manipulation against DFTD and direct future strategies for vaccine or therapy development. Therefore, in addition to determining the immune effector cell populations present in the experiments, it was important to analyse the mechanisms responsible for the cytotoxicity responses against K562 cells in Tasmanian devils. Therefore, the third aim of this chapter was to determine the mechanism of killing in the MHC I negative K562 cell immunisations.

The K562 cells used for immunisations described in the previous chapters were xenogeneic cells. Thus, responses to xenogeneic tissues may provide clues as to the nature of the effector cells. NK cells are usually a prominent effector type in responses against foreign cells, as the MHC I molecules involved are either disparate or, as in the case of K562 cells, missing. The cytotoxicity responses formed by the Tasmanian devils immunised with K562 cells were not typical of NK cells, but resembled those of CTL. Prior immunisation was required to induce responses and there was no evidence for spontaneous killing of the cancer cells. Previous studies using a xenogeneic model have reported similar observations, including limited spontaneous NK cell cytotoxicity against xenogeneic cells and increased cytotoxicity following priming with target-cell specific antigen [396]. Thus, NK cells may still be candidates for the cytotoxicity responses against K562 cells. The authors of this study also found that the NK cells could kill the foreign cells in the presence of target cell-specific antibody [396]. Since all devils immunised with K562 cells produced strong antibody responses, antibody-dependent cell mediated cytotoxicity (ADCC) may be one candidate pathway for the responses against these tumour cells.

A variety of innate immune cell types can form ADCC responses, including NK cells, monocytes, eosinophils and neutrophils. Only MNC types, such as monocytes and NK cells, would be present in the populations used in these experiments. Basic techniques for cell separation, such as adherence to nylon wool or plastic, could be used to enrich or deplete certain cell types from MNC suspensions for use in functional assays. The serum from immunised Tasmanian devils, which would contain anti-K562 antibody, could also be added to chromium release cytotoxicity assays to determine if ADCC responses can target MHC I negative tumour cells.

4.2 Results

4.2.1 Immunocytochemistry analysis of peripheral blood mononuclear cell populations

The fraction of T and B lymphocytes, monocytes and polymorphonuclear cells in MNC suspensions was assessed by immunocytochemistry of cytospins (2.9). As some of the procedures used involved the enrichment of cell types based on adherence, the proportions of different cell types in nylon wool non adherent cell and plastic non-adherent cell populations were also enumerated using immunohistochemsitry. In order to identify Tasmanian devil NK cells, both immunohistochemistry and Giemsa staining techniques were used (2.9).

Table 4.1. Percentages of individual cell types in Tasmanian devil peripheral blood mononuclear cell, nylon wool filtered and plastic non adherent cell populations analysed by immunohistochemistry

Cell type	Labelling	Morphology	Mean % presence ± Standard deviation		
			Mononuclear Cells	Nylon non adherent cells	Plastic non adherent cells
T lymphocyte	$CD3^+$	Round nucleus, scanty cytoplasm	55 ± 8	73 ± 7	76 ± 12
B lymphocyte	$MHCII^+$	Round nucleus, scanty cytoplasm	33 ± 8	9 ± 6	13 ± 4
Monocyte	$MHCII^+$	Large cell, bean-shaped nucleus, abundant cytoplasm	5 ± 3	4 ± 2	1 ± 1
NK like	$CD3^-/MHCII^-$	Large lymphocyte, round nucleus, scanty cytoplasm,	4 ± 1	5 ± 2	3 ± 2
Neutrophil	$CD3^-/MHCII^-$	Large cell, multi-lobar or ring-shaped nucleus	7 ± 5	13 ± 10	4 ± 3

4.2.1.1 Cell types present in total mononuclear cell suspensions

T lymphocytes were identified with an antibody against an intracellular CD3ε epitope. The T lymphocytes of Tasmanian devils were similar in appearance to their human equivalent, with large round nuclei and scant cytoplasm. They were the most abundant cell type in the majority of samples, representing an average of 55% of the total MNC population (Table 4.1). CD3ε protein was strongly expressed in T lymphocytes in a distribution consistent with membrane association as the staining was evenly spread throughout the cell, and across the nucleus, and appeared to be stronger at the cell periphery (Fig. 4.1a). Some larger lymphocytes expressed lower levels of CD3. These may have represented naive lymphoblasts or Natural Killer T cells (Fig. 4.1a).

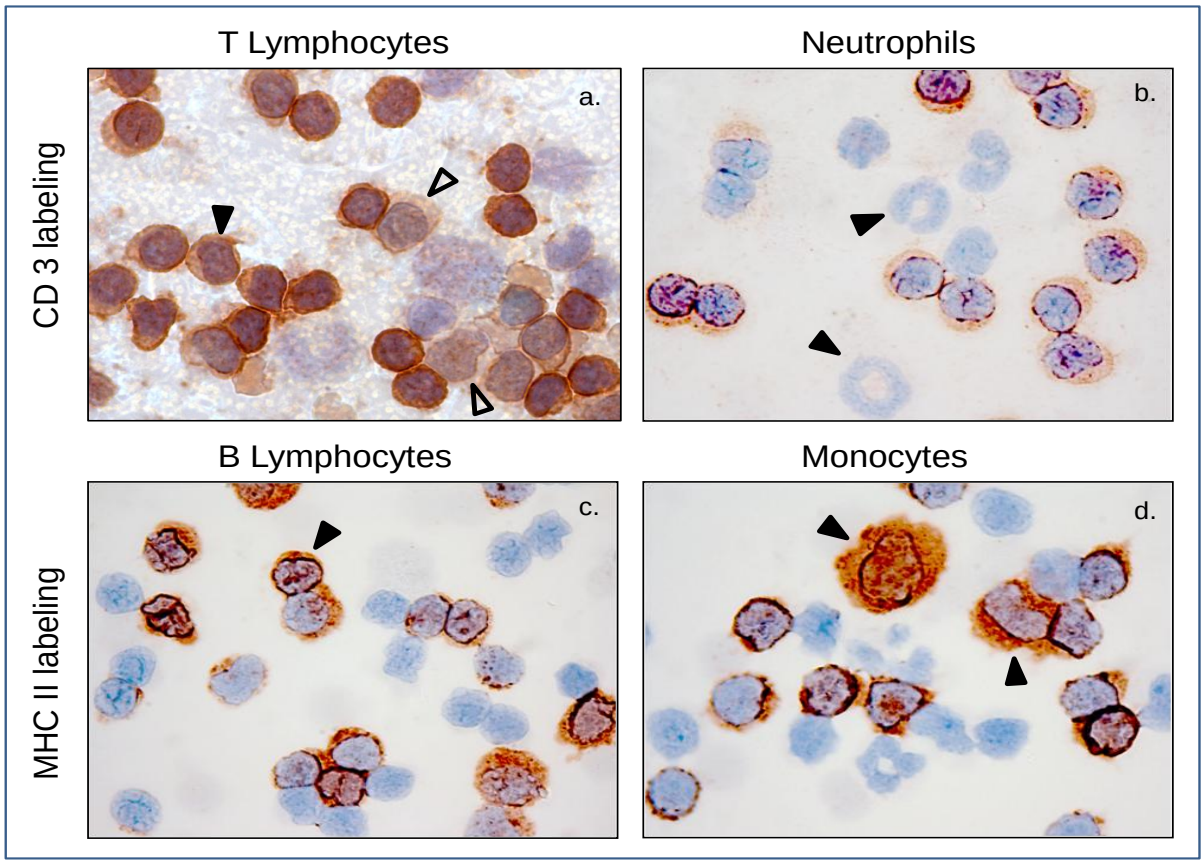

Figure 4.1. Appearance and Immunohistochemistry labelling patterns of normal peripheral blood mononuclear cells from Tasmanian devils.

Panel a: CD3 labelled MNC cytospins showing positive staining of T lymphocytes. Images were taken at 1000x magnification. Cells annotated with solid arrowheads represent T lymphocytes with normal morphology. Faintly stained cells annotated with open arrowheads are likely to be naive T lymphoblasts or NKT cells.

Panel b: CD3 labelled mononuclear cell cytospins showing negative staining of neutrophils. Images were taken at 1000x magnification. Cells annotated with solid arrowheads represent neutrophils with normal morphology as large cells characterised by a multilobar or circular nucleus.

Panel c: MHC class II labelled mononuclear cell cytospins showing positive staining of B lymphocytes. Images were taken at 1000x magnification. Cells annotated with solid arrowheads represent B lymphocytes with normal morphology.

Panel d: MHC class II labelled mononuclear cell cytospins showing positive staining of monocytes. Images were taken at 1000x magnification. Cells annotated with solid arrowheads represent monocytes with normal morphology characterised as large, MHC II positive cells with round or bean-shaped nuclei.

Polymorphonuclear cells (neutrophils) were identified as large cells which had multi-lobar nuclei and did not express CD3ε or MHC II, (Fig. 4.1b). Although contamination with neutrophils was generally low when using histopaque gradient centrifugation, this cell type was found in all Tasmanian devil MNC samples. The majority of these contaminating neutrophils in the cytospins had ring-shaped nuclei, suggesting they were likely to be immature. Neutrophils comprised on average 7% of the total cell population.

B cells were identified as lymphocytes which expressed MHC II. The distribution was consistent with a mainly cytoplasmic protein with strong expression outside, rather than across, the nucleus, and no accumulation at the cell periphery (Fig. 4.1c). This type comprised, on average, 33% of the MNC population in Tasmanian devils (Table 4.1). Monocytes in MNC samples also strongly expressed MHC II but could be distinguished from B lymphocytes as they were larger and had distinct bi-lobar nuclei (Fig. 4.1d). This cell type comprised, on average, 5% of the total MNC population (Table 4.1).

4.2.1.2 Cell types in differentiated cell suspensions

Filtration of MNC through nylon wool depleted B lymphocytes in the MNC suspensions, reducing their percentage to an average of 9% compared to 33% in total MNC suspensions. Consequently, the percentage of T lymphocytes within the nylon non-adherent fractions increased, to an average of 73% (Table 1). Nylon wool filtration did not substantially affect the proportion of monocytes present in the cell suspensions indicating that, similar to other species, Tasmanian devil monocytes are nylon wool non-adherent. Nylon wool filtration increased the percentage of contaminating neutrophils in the population to an average of 13% (Table 1). However, there was substantial variation in neutrophil numbers between samples. Several attempts were made to elute the nylon wool adherent cells from the columns. However, recovery was consistently poor, with low numbers and viability in each sample. Consequently, the cell populations present in the nylon adherent population could not be analysed.

Plastic adherence substantially decreased the monocyte fraction to an average of 1% of the total population. The effect of plastic adherence on lymphocyte populations was similar to that of nylon wool, increasing T lymphocyte presence to an average of 76% and reducing B lymphocyte numbers to 13% (Table 1). The percentage of neutrophils increased slightly in plastic adherent cells compared to total MNC, with an average of 4%.

4.2.1.1 Evidence for the physical presence of Natural Killer-like cells in the peripheral blood of Tasmanian devils

Natural Killer cells may be an important cytotoxic effector cell type in the response against both K562 cells and DFTD cells. However, the absence of specific markers has hampered efforts to identify this cell type in Tasmanian devils. In other species, NK cells can be histologically identified as large lymphocytes (compared to T or B cells) with prominent cytotoxic granules in the cytoplasm (large granular lymphocytes). Giemsa staining is commonly used to identify NK cells, as it can stain the acidic cytotoxic granules of NK cells. Therefore, the cytospins of Tasmanian devil MNC were Giemsa stained to provide further evidence for the presence of NK-like cells in Tasmanian devil peripheral blood. NK-like cells were defined as cells with a similar appearance to T or B lymphocytes but a larger diameter and the presence of azurophilic (darkly stained) granules in the cytoplasm. Large granular lymphocytes matching this description were identified in all Giemsa-stained MNC preparations examined (Fig. 4.2a and b). NK cells can also be identified as large $CD3^-$/MHC II^- using immunohistochemistry. NK-like cells were identified in Tasmanian devil MNC cytospins (Fig. 4.2c and d) and accounted for on average 4% of the total MNC population (Table 4.1). Some $CD3^-$/MHC II^- cells in the MNC cytospins had abundant cytoplasm, a typical morphological characteristic of plasma cells rather than NK cells, and were not included in this enumeration.

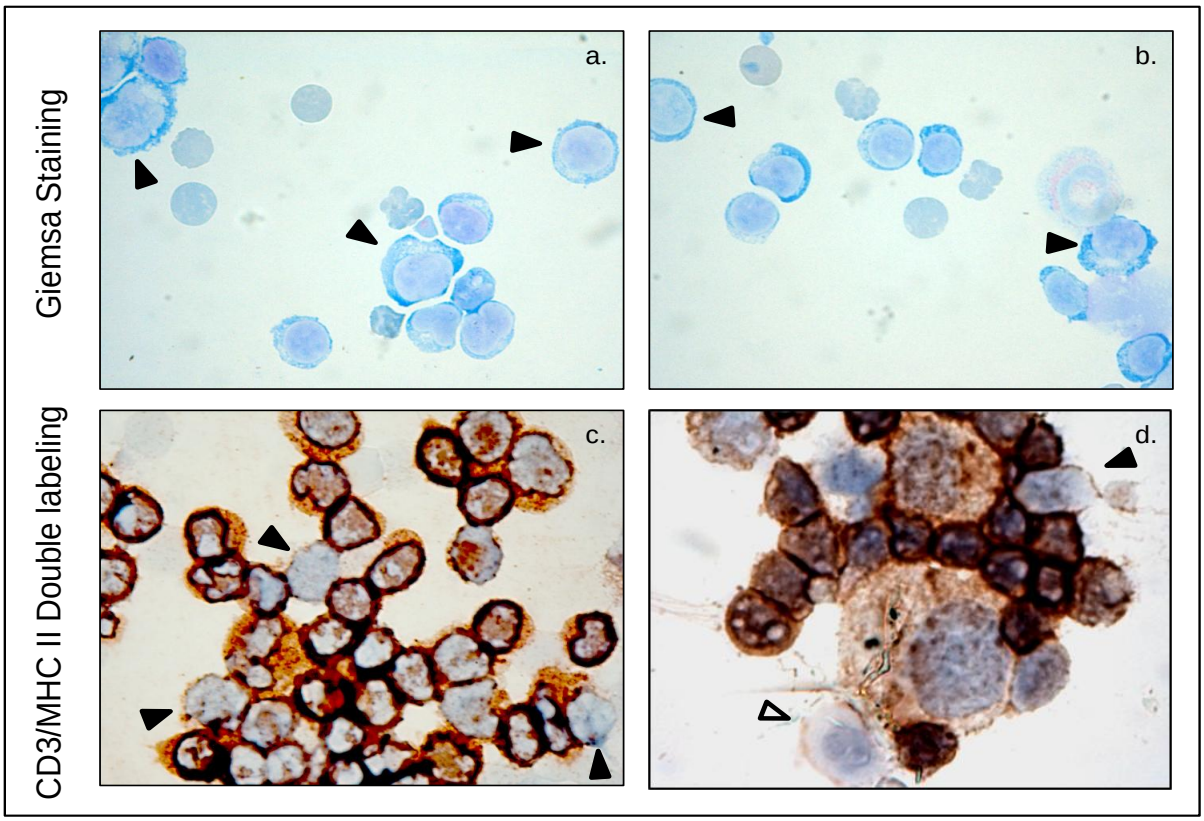

Figure 4.2. Identification of Natural Killer-like cells in Tasmanian devils.

Panels a and b show Giemsa stained MNC cytospins. The presence of large granular lymphocytes consistent with the morphological appearance of NK cells is indicated with solid black arrowheads. Panels c and d show immunohistochemistry double-staining with CD3 and MHC II. The presence of negatively stained large lymphocytes consistent with the phenotype of NK like cells is indicated with solid black arrowheads. The typical morphology of a plasma cell (also negative for both markers) is indicated with an open arrowhead.

4.2.2 Evidence for antibody-dependent cell mediated cytotoxicity (ADCC) against K562 cells in Tasmanian devils

The results of Chapter 3 show that devils are capable of producing cytotoxicity and antibody responses against K562 tumour cells. Thus, the failure of Tasmanian devils to mount immune responses against DFTD cells cannot be attributed to a lack of capacity to form anti-tumour responses. Further study of the K562 immunisation model may provide some insight into the effector cell types and pathways of cytotoxic responses in Tasmanian devils. With increased understanding of these responses, it may be possible to manipulate their components to target DFTD.

4.2.2.1 Exclusion of B lymphocytes by nylon wool filtration and activation of ADCC by addition of serum to cytotoxicity assays

Nylon wool non adherent cell suspensions were used in 18 hour cytotoxicity assays to determine if an increased proportion of T lymphocytes would enhance cytotoxicity against K562 cells (2.7.1). When the anti-K562 cytotoxic activity of nylon wool non adherent cells from two immunised devils was evaluated in 18 hour assays, no response occurred (Fig. 4.3). The total MNC layers of these samples formed strong responses. Thus, an essential component of the cytotoxicity response against K562 cells was removed during nylon wool filtration.

Depleting B lymphocytes and plasma cells by nylon wool filtration would deplete the potential for antibody formation within the assay, which could facilitate antibody dependent cell-mediated cytotoxicity (ADCC) against the tumour cells. The capacity for ADCC responses was assessed by adding serum from K562 immunised devils to cytotoxicity assays containing MNC from naive devils. The MNC formed cytotoxic responses against K562 cells in the presence of immune serum (Fig. 4.4a-d). There were no cytotoxicity responses in the absence of serum or with pre immune serum. Consequently, MNC from Tasmanian devil peripheral blood contain cells that are capable of ADCC.

Despite the presence of functional cytotoxic responses, devils with DFTD do not show evidence of cytotoxicity against the tumours. One explanation is a systemic suppression of the host immune system in devils with DFTD. In order to determine if DFTD diseased devils are capable of forming cytotoxic responses, their ability to mount ADCC against K562 cells was assessed. Standard ADCC assays were performed with the MNC from two DFTD diseased devils and serum from a K562 immunised Tasmanian devil (2.7.2). Both diseased devils formed cytotoxic responses against the K562 tumour cells in the presence of immune serum (Fig. 4.4e and f), indicating that the presence of DFTD does not alter the activity of cells responsible for ADCC. There was no response detected in assays containing MNC and pre immune serum.

Nylon wool adherence (2.5.2.1) may have depleted effector or helper cells that mediated the ADCC responses against K562 (Table 1). Serum from immunised devils was added to cytotoxicity assays performed with nylon wool non-adherent cells (NNAC) to determine if the effector cells were removed following adherence to nylon wool. In the presence of immune serum, NNAC cells formed cytotoxic responses in all samples (Fig. 4.5). Consequently, the effector cells of ADCC responses against K562 cells are not removed from the MNC suspensions following nylon wool filtration, although the process may have removed helper cells.

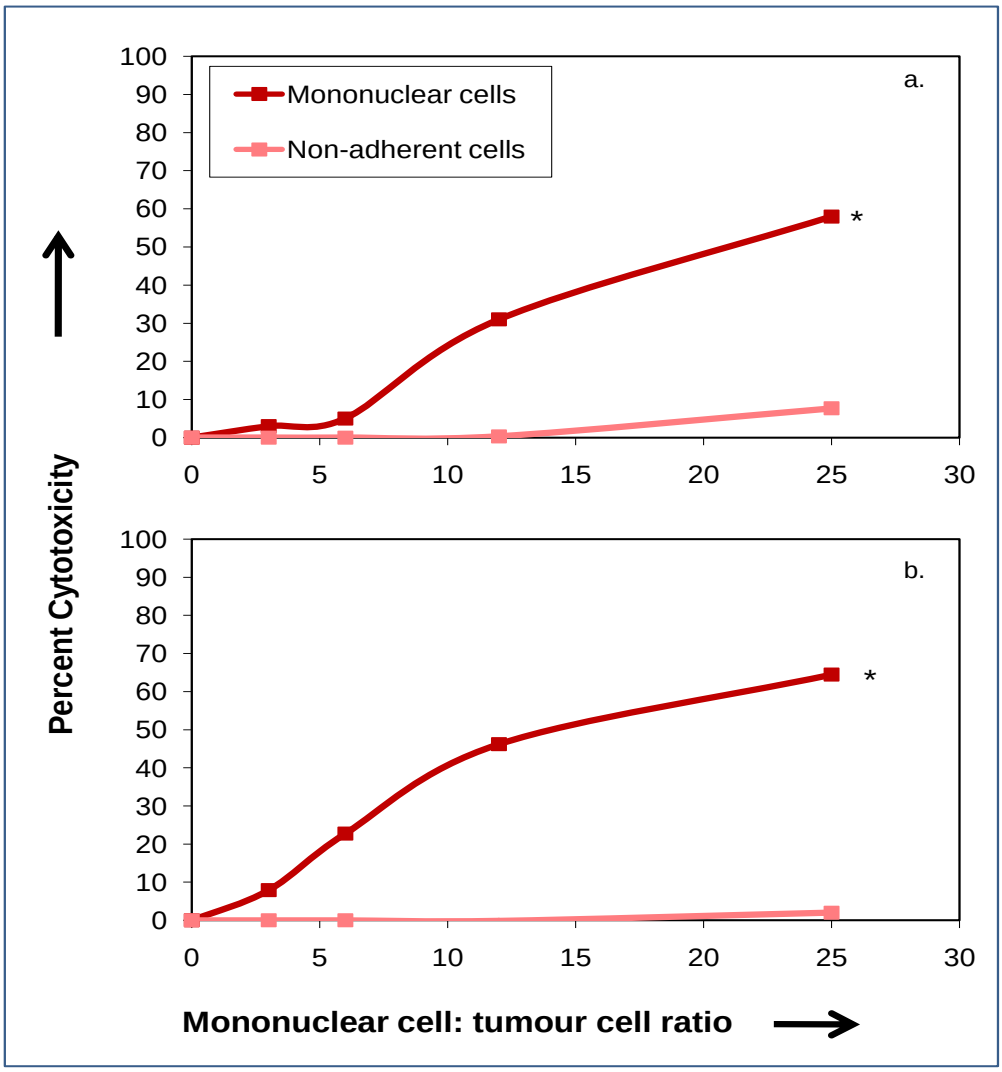

Figure 4.3. Cellular cytotoxicity responses of nylon wool non adherent cells and total mononuclear cells from two K562 immunised Tasmanian devils.

Chromium release cytotoxicity assays were performed by culturing MNC from two immunised Tasmanian devils with radioactively labelled K562 cells for 18 hours. Percent cytotoxicity values ± 1 standard deviation at MNC: tumour cell ratios of 25:1 to 3:1 are shown for nylon wool non adherent cells and total MNC. The statistical difference between the two cell populations was assessed using F Tests, with a value of $P<0.05$ classified as significant and marked with an asterisk (*).

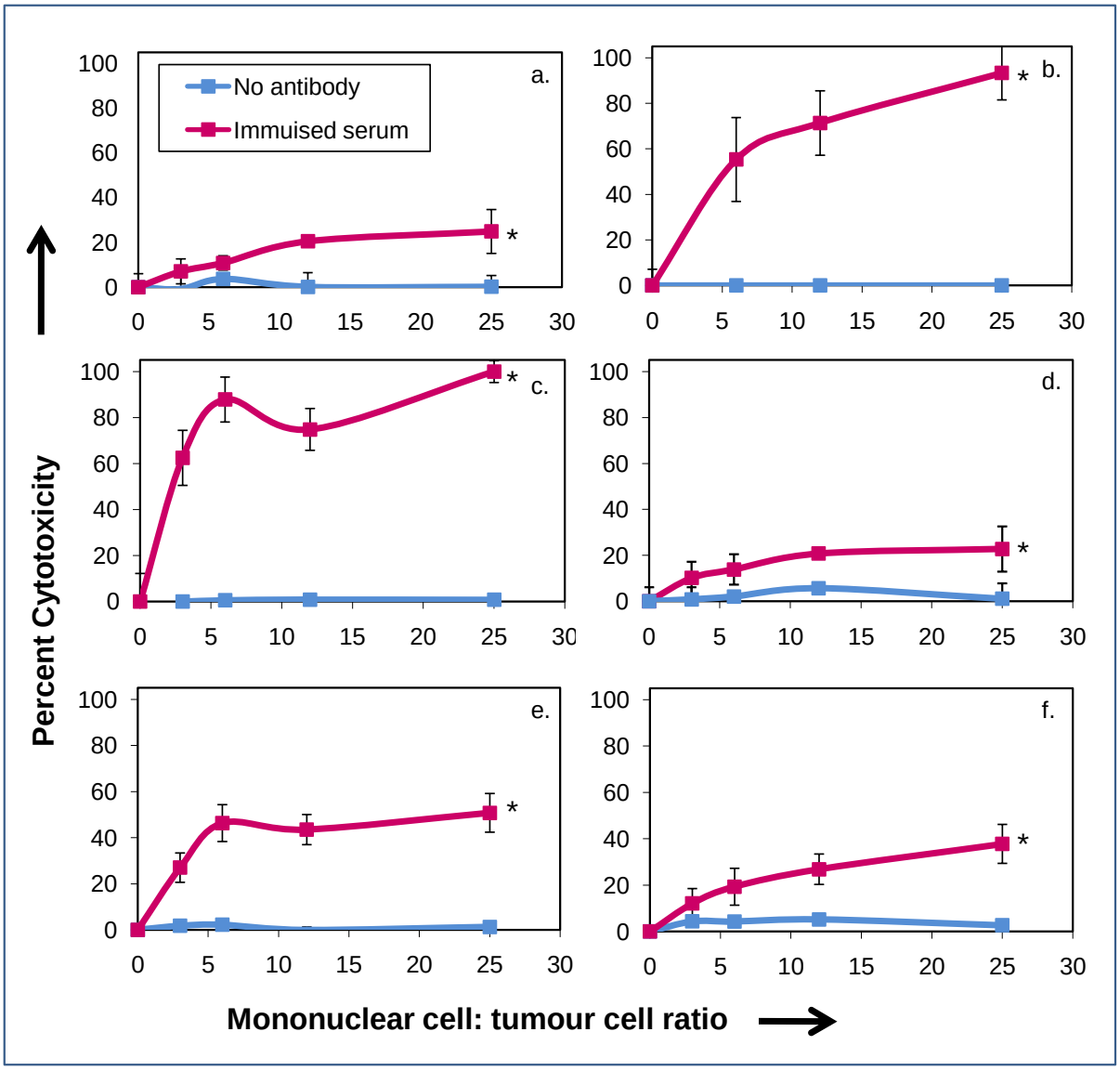

Figure 4.4. Antibody-dependent cell-mediated cytotoxicity responses of mononuclear cells from four healthy, K562 naive Tasmanian devils and two DFTD diseased devils.

Chromium release cytotoxicity assays were performed by culturing MNC from six Tasmanian devils with radioactively labelled K562 cells for 18 hours in the presence of antibody in the form of K562 immunised devil whole serum or pre immune serum (no antibody) as a control. Percent cytotoxicity values ± 1 standard deviation at mononuclear cell: tumour cell ratios of 25:1 to 3:1 are shown for serum free and serum supplemented samples. The statistical difference between responses in the presence and absence of antibody was assessed using F Tests, with a value of $P<0.05$ classified as significant and marked with an asterisk (*). The ADCC responses from healthy devils are displayed in panels a – d, and the responses of two wild diseased devils are displayed in panels e and f.

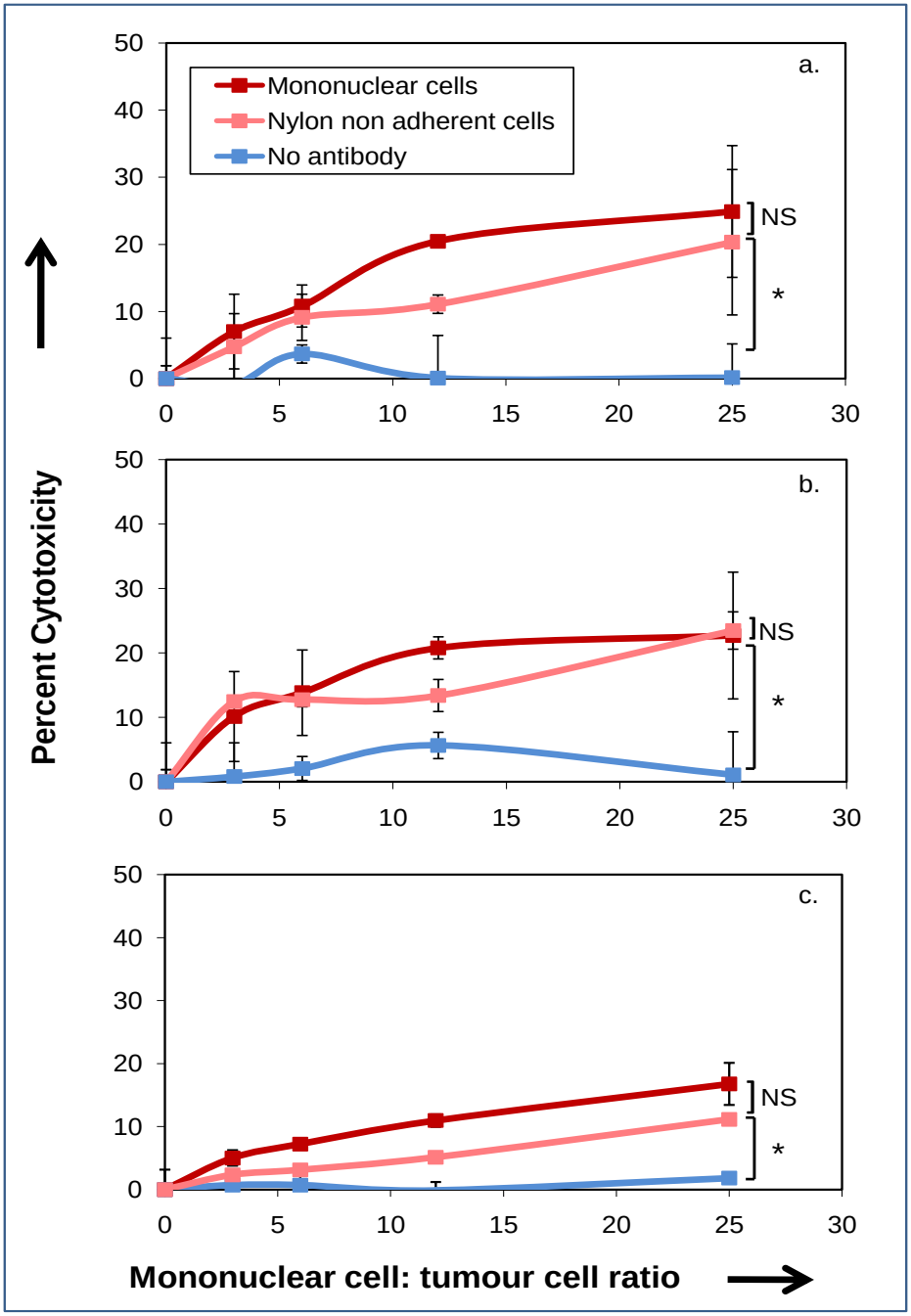

Figure 4.5. Antibody-dependent cell-mediated cytotoxicity responses of nylon wool non adherent cells and total mononuclear cells from two healthy, K562 naive Tasmanian devils and one DFTD diseased devil.

Chromium release cytotoxicity assays were performed by culturing radioactively labelled K562 cells for 18 hours in the presence of Tasmanian devil MNC, MNC plus K562 specific antibody (from immunised serum), or nylon wool non adherent cells plus K562 specific antibody. Percent cytotoxicity values ± 1 standard deviation at mononuclear cell: tumour cell ratios of 25:1 to 3:1 are shown for serum free samples and serum supplemented samples containing nylon wool non adherent cells or total MNC. The statistical difference between responses in the presence and absence of antibody was assessed using F Tests, with a value of $P<0.05$ classified as significant and marked with an asterisk (*).

4.2.2.2 Analysis of *in vitro* antibody formation

In order for ADCC responses to occur in 18 hour cytotoxicity assays containing MNC from immunised Tasmanian devils and K562 cells, specific antibody must be produced within the assays. The supernatant from cytotoxicity assays containing immunised Tasmanian devil MNC and K562 cells (K562 culture supernatant) was added to the cytotoxicity assays containing naive MNC to determine if it contained sufficient antibody to induce ADCC responses (2.8.2). None of the devils tested showed any evidence for cytotoxicity responses in the presence of immunised devil culture supernatant (Fig. 4.6). Thus, there was not sufficient anti-K562 antibody released into the culture supernatant to induce ADCC responses.

One explanation for the absence of ADCC responses in samples containing culture supernatant was that all antibody produced within the assay was bound to the target (K562) cells. The presence of antibody in the supernatant was analysed using flow cytometry labelling of K562 cells and compared to the immune and pre immune serum of the same devil as positive and negative controls, respectively. There was no evidence for the presence of antibody attached to the K562 cells in these assays, with similar fluorescence intensity to the pre immune sample (Fig. 4.7). This suggested that the culture supernatant did not contain anti-K562 antibody. The presence of antibody bound to the K562 cells in the 18 hour cultures was also analysed using flow cytometry. To accomplish this, K562 cells from these cultures were cultured with rabbit anti-devil IgG as a secondary reagent to detect devil anti K562 antibody. When the K562 cells from these cultures were examined using flow cytometry, the mean fluorescence intensity of the assay pellet cells was greater than that of untreated K562 cells (Fig. 4.8). The increase in fluorescence suggested that antibody was bound to the tumour cells in the samples, rather than in the supernatant.

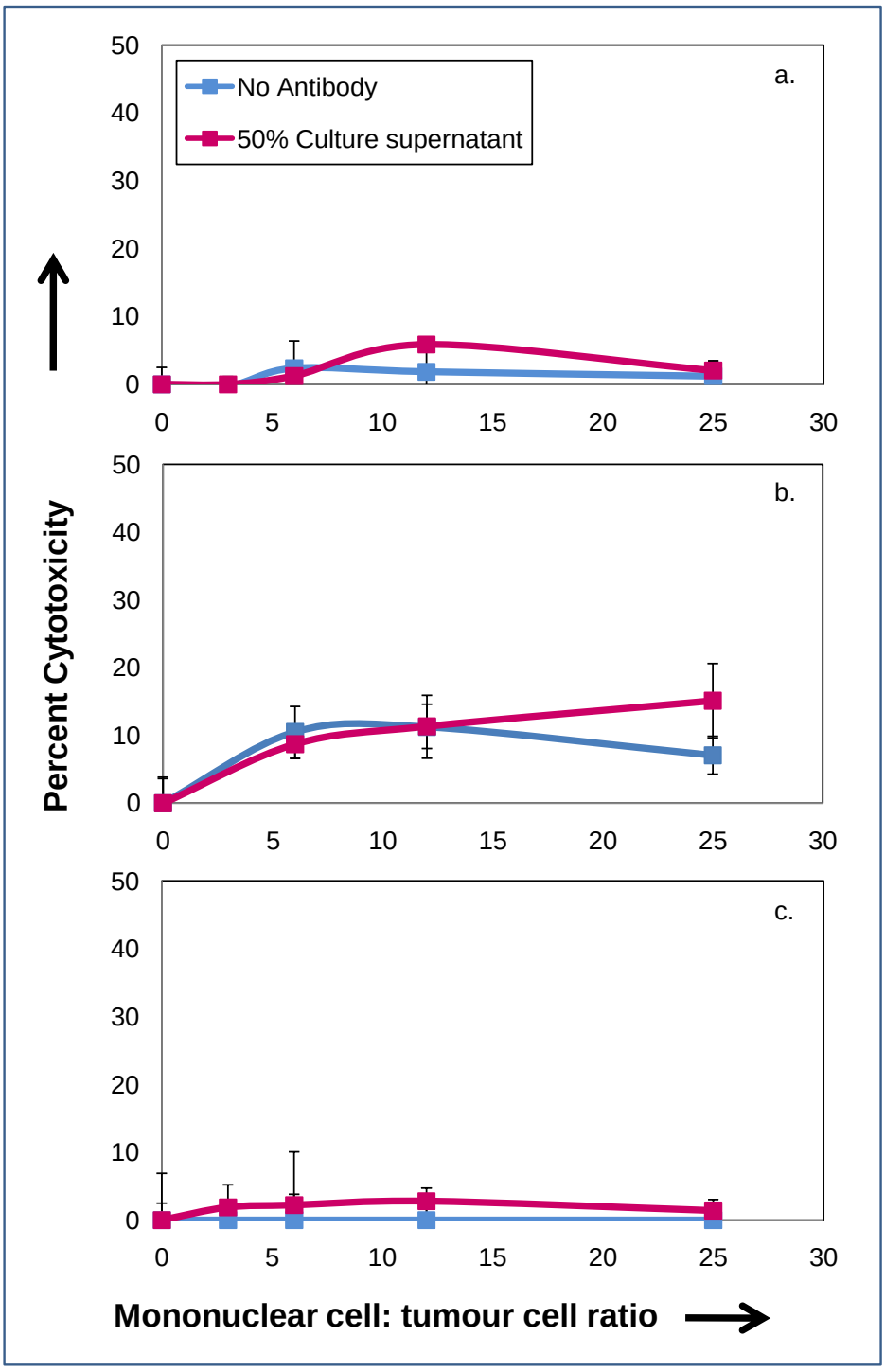

Figure 4.6. Antibody-dependent cell-mediated cytotoxicity responses in the presence of supernatant from K562 cell and immunised Tasmanian devil cultures.

Chromium release cytotoxicity assays were performed by culturing MNC from three Tasmanian devils with radioactively labelled K562 cells for 18 hours in the presence or absence of culture supernatant from cultures containing K562 cells and MNC from immunised Tasmanian devils. Percent cytotoxicity values ± 1 standard deviation at mononuclear cell: tumour cell ratios of 25:1 to 3:1 are shown for supernatant free and supernatant supplemented samples. The statistical difference between responses in the presence and absence of antibody was assessed using F Tests, with a value of P<0.05 classified as significant and marked with an asterisk (*).

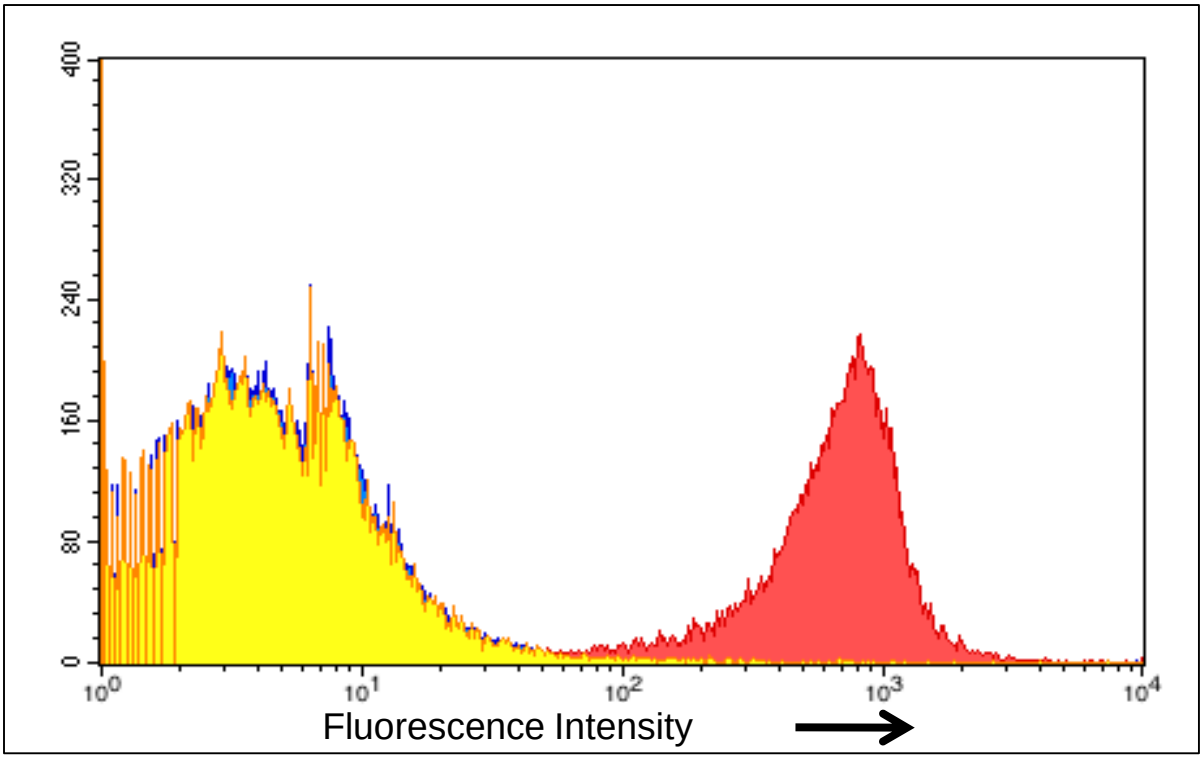

Figure 4.7. Flow cytometry profile of K562 positive serum and K562 culture supernatant.

Antibody content of known positive serum from a K562 immunised Tasmanian devil (red curve), the supernatant from K562 culture with MNC from immunised devils (yellow curve) and pre immune serum from the same devil (blue curve) were analysed using flow cytometry, where the fluorescence intensity is directly proportional to the amount of bound antibody in the primary sample.

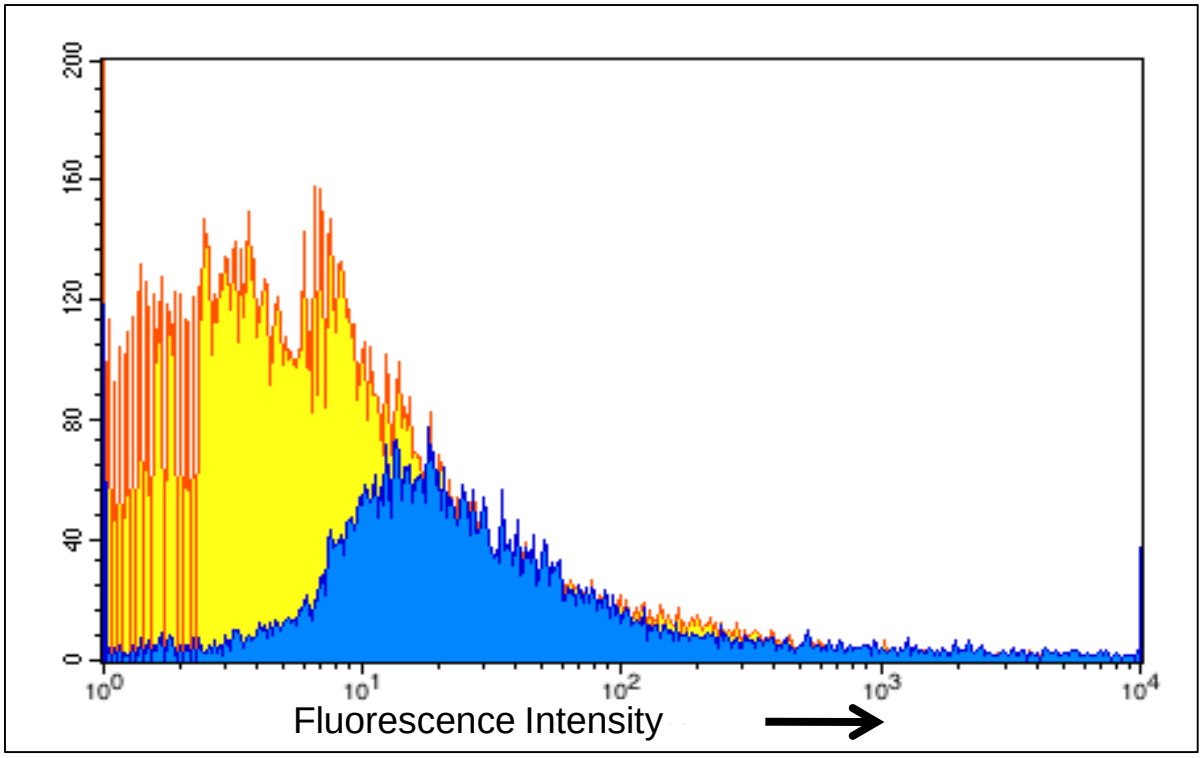

Figure 4.8. Flow cytometry profile of untreated K562 cells and cell pellets from cultures containing mononuclear cells from immunised devils.

Antibody content of the cell pellet (blue curve) and untreated K562 cells (yellow curve) were compared using flow cytometry. The fluorescence intensity of the curves is directly proportional to the amount of bound antibody in the primary sample.

4.2.3 Analysis of ADCC effector cells by selective depletion and functional assays

The ability to activate Tasmanian devil ADCC responses against MHC I negative tumour cells may provide a mechanism to target DFTD cells. Further analysis of the effector cell types involved in the ADCC responses against K562 cells could identify a cell population to target for the induction of cytotoxic responses against DFTD. The MNC suspensions contained four cell types with the capacity for cytotoxicity or ADCC: T lymphocytes, monocytes, neutrophils and NK cells.

T lymphocyte responses can be differentiated from those of innate cells by the length of time in which they occur. Monocytes and NK cells can mediate cytotoxicity responses within 4 hours [397,398], whereas those of T lymphocytes require longer periods of time. Short length (4 hour) cytotoxicity assays were performed with MNC from non-immunised devils and anti-K562 antibody (2.7.2). Cytotoxic responses were consistently formed within this time period (Fig. 4.9). One devil

was tested twice, on different days, and formed clear responses in both assays (Fig 4.9 a and d). This suggests that innate cells are more likely to be responsible for the ADCC responses against K562 cells than T lymphocytes.

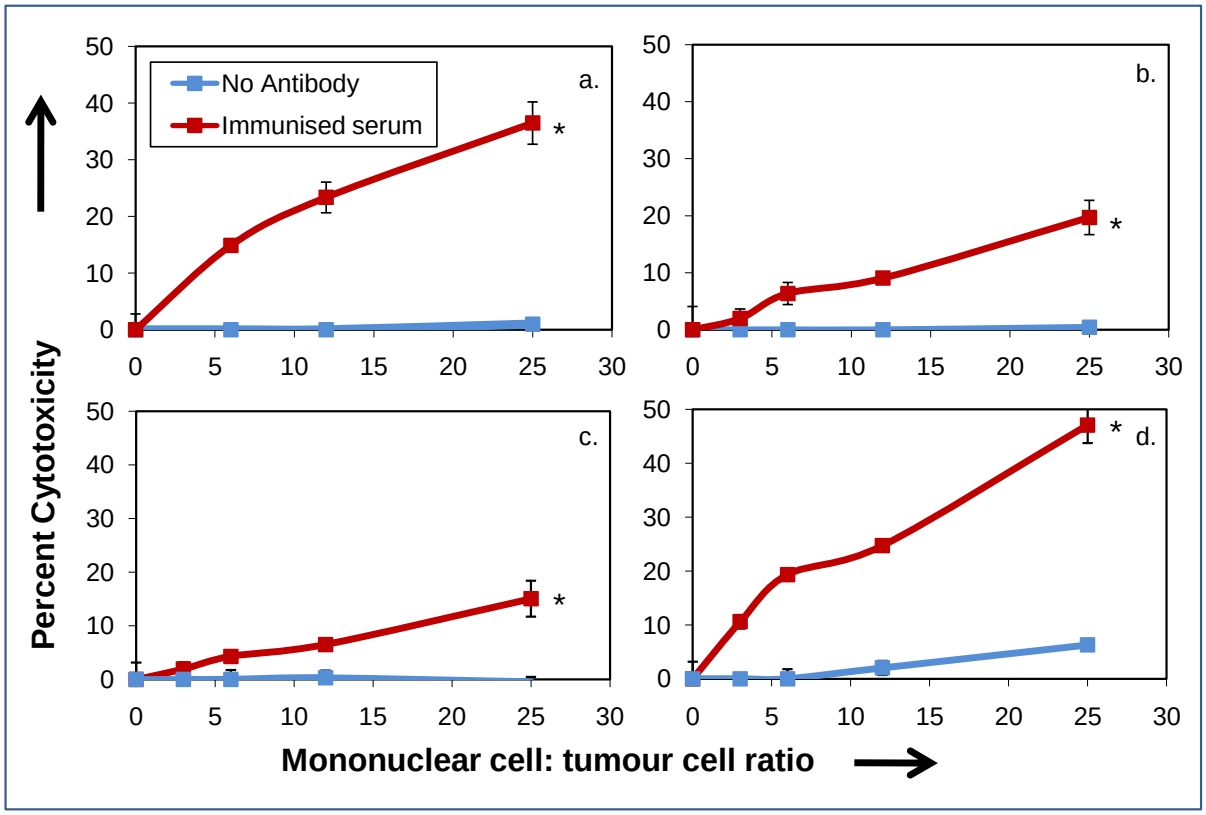

Figure 4.9. Short duration antibody-dependent cell-mediated cytotoxicity responses of mononuclear cells from three Tasmanian devils.

Chromium release cytotoxicity assays were performed by culturing MNC from three Tasmanian devils with radioactively labelled K562 cells for 4 hours in the presence of antibody in the form of K562 immunised devil whole serum or pre immune serum (no antibody). The assay was performed twice on different days using MNC from one Tasmanian devil (panels a and d). Percent cytotoxicity values ± 1 standard deviation at mononuclear cell: tumour cell ratios of 25:1 to 3:1 are shown for serum free samples and serum supplemented samples. The statistical difference between responses in the presence and absence of antibody was assessed using F Tests, with a value of $P<0.05$ classified as significant and marked with an asterisk (*).

Monocytes, neutrophils and NK cells were therefore candidates for the effectors of the ADCC responses. The majority of neutrophils in the MNC samples appeared to be immature when examined by microscopy, as their nuclei were ring-shaped rather than the characteristic multi-lobar appearance of the mature cells and were thus unlikely to participate in ADCC responses against

serum. Monocytes are strongly adherent to plastic and were removed from MNC suspensions by incubating in plastic vessels (2.5.2.2) to leave NK cells as the major population of cells with the capacity for ADCC. Cytotoxicity assays were performed with plastic non-adherent cells in the presence and absence of immune serum (2.7.1). In all assays, monocyte depleted cells formed cytotoxic responses which were not significantly different from those of total MNC but were significantly different from samples without antibody (Fig. 4.10). Therefore, monocytes are not major effectors of ADCC against MHC I negative tumour cells and, by elimination, the responses are most likely mediated by NK cells.

4.2.4 Serum from DFTD immunised mouse or devil serum does not induce ADCC responses

Given that Tasmanian devils can form ADCC responses against tumour cells, it is possible that cytotoxicity responses could occur against DFTD cells in the presence of specific anti-DFTD antibody. Tasmanian devils generally produce only weak antibody responses against whole DFTD cells (refer to Figures 3.7, 3.8 and 3.10 – 12). However, in samples from some devils, antibody can be detected against total DFTD cell protein using an ELISA (A. Kreiss, personal communication). These serum samples may contain sufficient antibody against surface epitopes on DFTD cells to induce ADCC responses. Additionally, immunised mice develop strong antibody responses against DFTD cells and serum from these mice could also be used as a source of anti DFTD antibody.

Standard ADCC assays were performed with MNC from naive Tasmanian devils and serum from a DFTD immunised Tasmanian devil that had received two doses of sonicated DFTD cells (2.7.2). None of the devils tested formed evidence for functional ADCC responses in either the presence or absence of serum (Fig. 4.11). The responses in assays containing immune serum were not significantly different to pre immune samples ($p = 0.095$). Cytotoxicity assays were performed with MNC from naive Tasmanian devils and serum from a DFTD immunised mouse. None of the devils tested formed cytotoxicity responses that were significantly higher than levels of cytotoxicity against DFTD cells in samples containing serum from a non-immunised mouse (Fig. 4.12; $p = 0.595$). Therefore, neither devil nor mouse anti DFTD serum induced ADCC responses against the tumour cells.

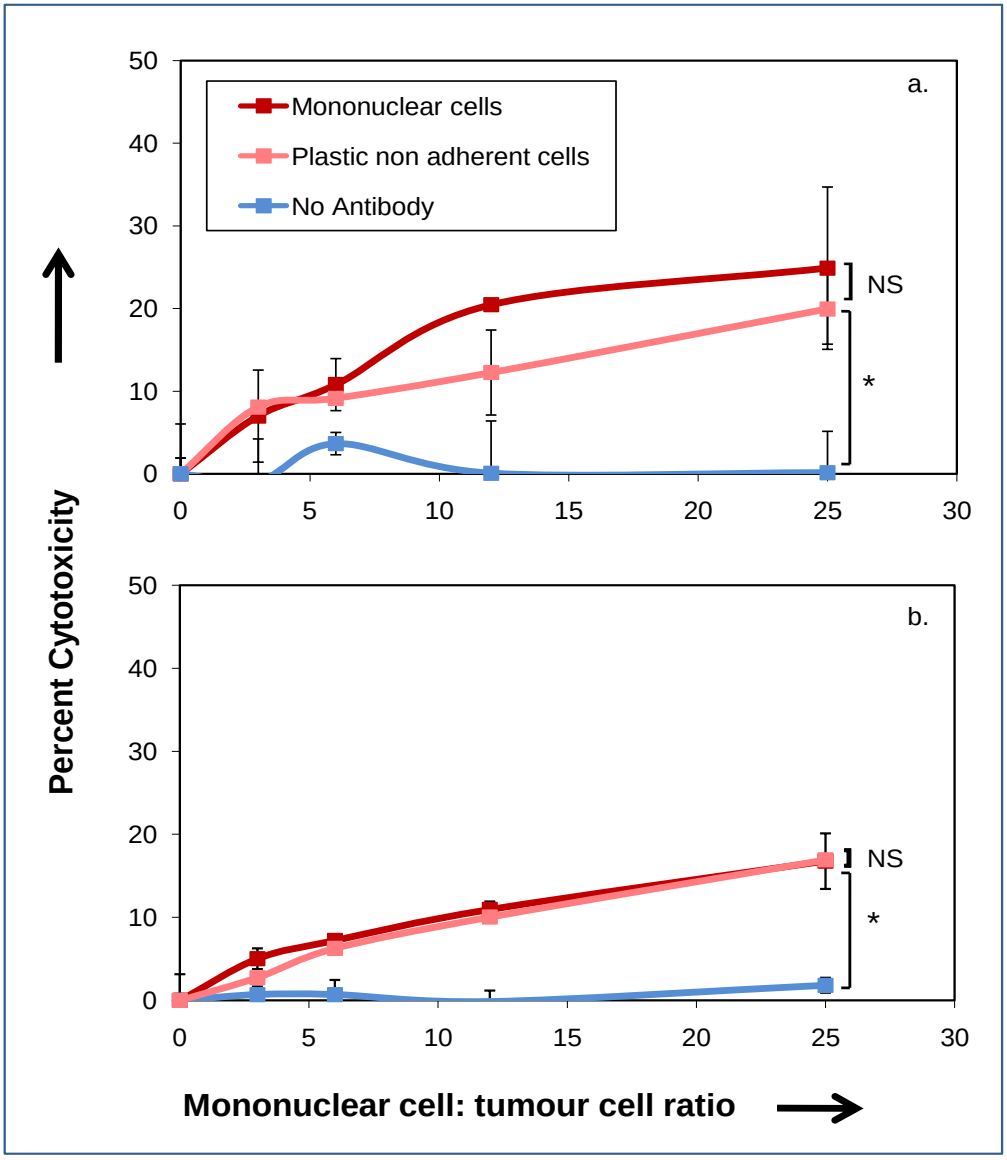

Figure 4.10. Antibody-dependent cell-mediated cytotoxicity responses of plastic non adherent cells and total mononuclear cells from two healthy, K562 naive Tasmanian devils and one DFTD diseased devil.

Chromium release cytotoxicity assays were performed by culturing radioactively labelled K562 cells for 18 hours in the presence of Tasmanian devil MNC, MNC plus K562 specific antibody (from immunised serum), or plastic non adherent cells plus K562 specific antibody. Percent cytotoxicity values ± 1 standard deviation at mononuclear cell: tumour cell ratios of 25:1 to 3:1 are shown for serum free samples and serum supplemented samples containing plastic non adherent cells or total MNC. The statistical difference between responses in the presence and absence of antibody was assessed using F Tests, with a value of $P<0.05$ classified as significant and marked with an asterisk (*).

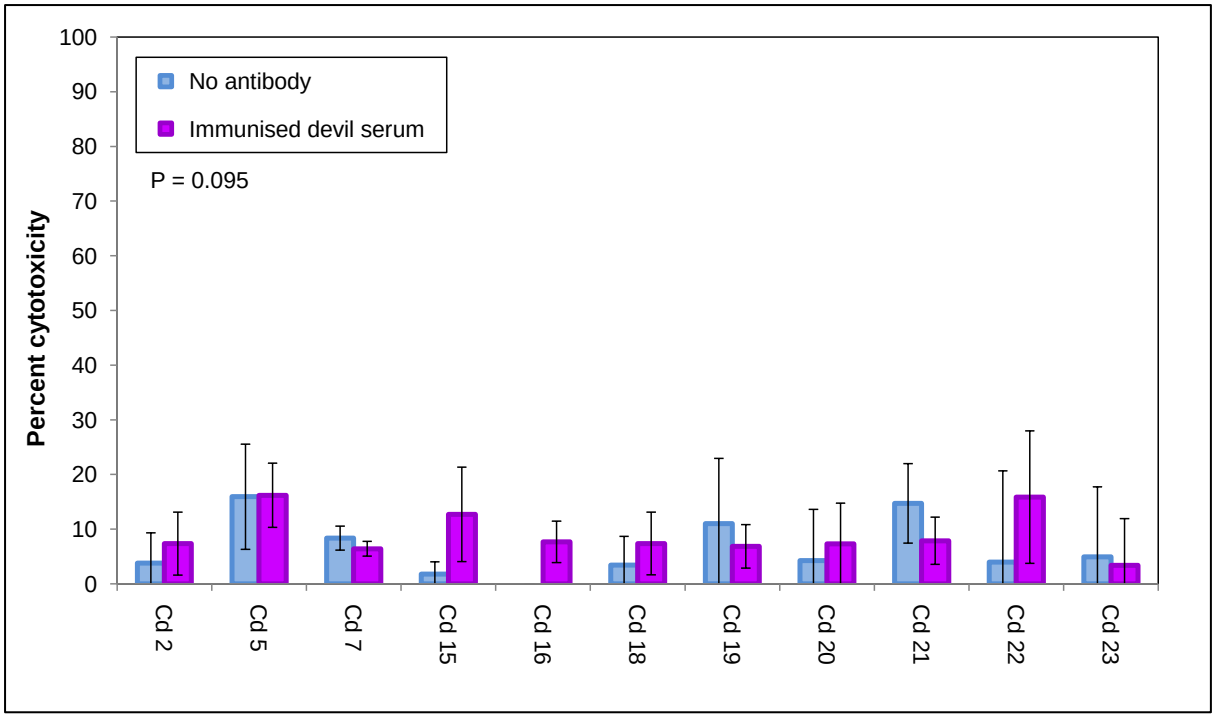

Figure 4.11. Antibody-dependent cell-mediated cytotoxicity responses of mononuclear cells from eleven Tasmanian devils in the presence of serum from DFTD immunised mice.

Chromium release cytotoxicity assays were performed by culturing MNC from eleven Tasmanian devils (Cd 2 – Cd 34) with radioactively labelled DFTD cells for 18 hours in the presence or absence of antibody from DFTD immunised devil serum. Percent cytotoxicity values ± 1 standard deviation at mononuclear cell: tumour cell ratios of 25:1 are shown for serum free samples and serum supplemented samples. The statistical difference between responses between untreated and serum supplemented groups was assessed using F Tests, with a value of $P<0.05$ classified as significant.

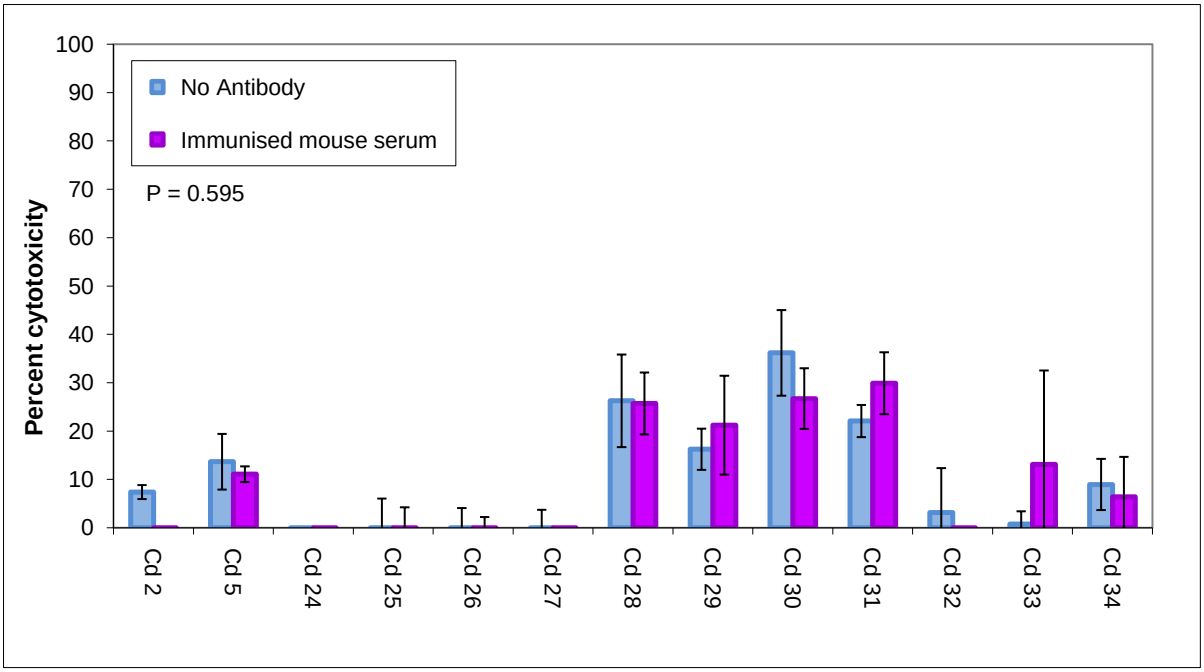

Figure 4.12. Antibody-dependent cell-mediated cytotoxicity responses of mononuclear cells from thirteen Tasmanian devils in the presence of serum from DFTD immunised mice.

Chromium release cytotoxicity assays were performed by culturing MNC from thirteen Tasmanian devils (Cd 2 – Cd 23) with radioactively labelled DFTD cells for 18 hours in the presence or absence of antibody from DFTD immunised mouse serum. Percent cytotoxicity values ± 1 standard deviation at mononuclear cell: tumour cell ratios of 25:1 are shown for serum free samples and serum supplemented samples. The statistical difference between responses between untreated and serum supplemented groups was assessed using F Tests, with a value of P<0.05 classified as significant.

4.2.5 Activation with Concanavalin A, cytokines and Poly I:C induces Tasmanian devil mononuclear cells to form cytotoxicity against DFTD cells

Another approach to activate an immune response against DFTD cells is to non-specifically activate cytotoxic cells to produce anti-tumour responses. Stimulation with mitogens, toll-like receptor agonists or cytokines can non-specifically activate T lymphocytes and NK cells in other species [18,47,147,399,400,401]. A previous study has demonstrated successful induction of proliferation responses in Tasmanian devil MNC using mitogens such as concanavalin A (Con A) [2] but the induction of cytotoxicity has not been tested. As mitogen-stimulated mononuclear cells also produce inflammatory cytokines *in vitro*, the supernatants of these cultures may provide a source of cytokines for analysis of their effects. To determine if mitogens could stimulate the MNC of Tasmanian devils to become cytotoxic to DFTD cells, Concanavalin (Con) A or Phytohemaggluanin (PHA) was added to standard 18 hour chromium release cytotoxicity assays containing MNC from

naive Tasmanian devils (2.7.5). Neither Con A nor PHA induced significant cytotoxicity responses against DFTD cells (Fig. 4.13a and b).

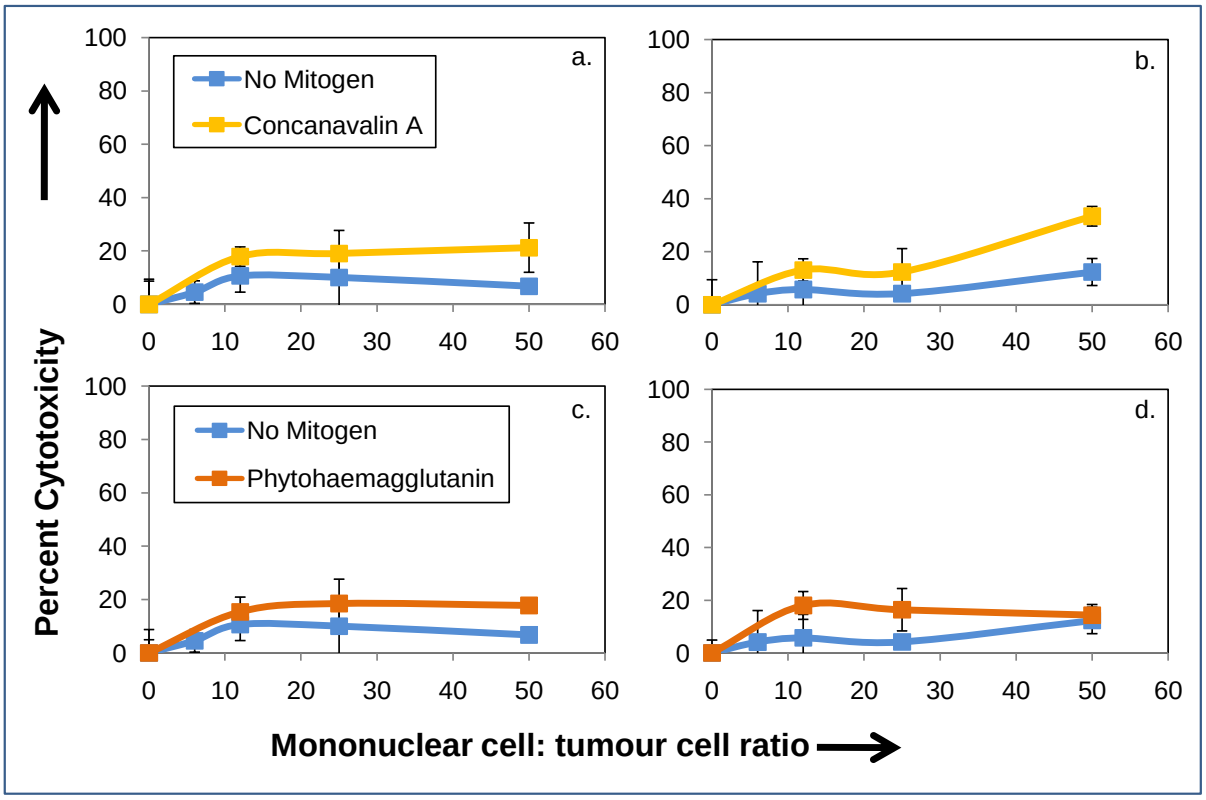

Figure 4.13. Effect of mitogen supplementation on cytotoxicity responses against DFTD.

Chromium release cytotoxicity assays were performed by culturing MNC from two naive Tasmanian devils with radioactively labelled DFTD cells and concanavalin A (Con A) or phytohaemagglutanin (PHA) for 18 hours. Percent cytotoxicity values ± 1 standard deviation at mononuclear cell: tumour cell ratios of 50:1 to 6:1 are shown for each treatment. The statistical difference between the two treatments was assessed using F Tests, with a value of $P<0.05$ classified as significant and marked with an asterisk (*).

As supplementation with mitogens did not induce responses within the duration of a standard cytotoxicity assay, a longer period of time may be required for activation. Therefore, Tasmanian devil MNC were cultured with Con A for 48 hours and then cytotoxicity assays were performed against DFTD cells (2.7.5). In all samples tested, the Con A stimulated MNC formed cytotoxic responses, which were statistically different from those of untreated samples (Fig. 4.14). Therefore, Con A stimulation is a method capable of consistently activating cytotoxic responses against DFTD cells.

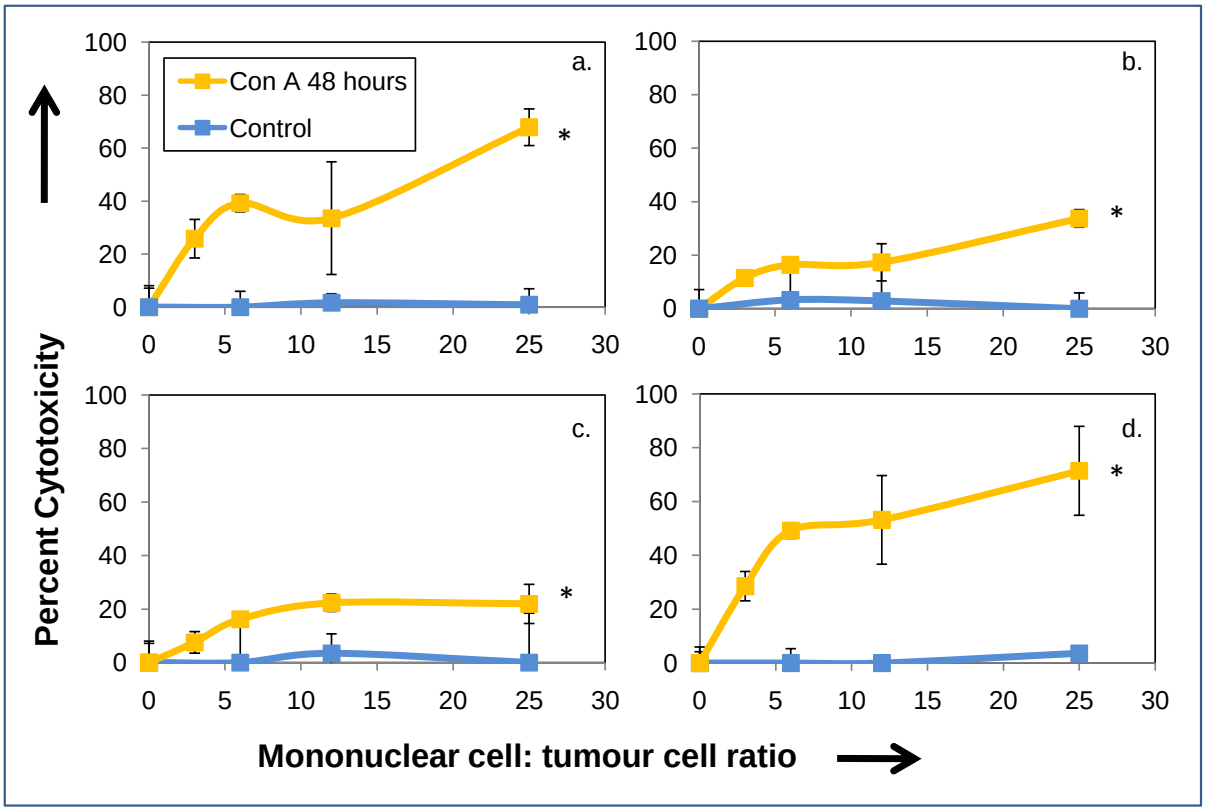

Figure 4.14. Effect of 48 hour Con A culture on cytotoxicity responses against DFTD.

Chromium release cytotoxicity assays were performed by culturing 48 hour Con A activated MNC from four Tasmanian devils with radioactively labelled DFTD cells for 18 hours. Percent cytotoxicity values ± 1 standard deviation at mononuclear cell: tumour cell ratios of 25:1 to 3:1 are shown for each treatment. The statistical difference between the treatments was assessed using F Tests, with a value of P<0.05 classified as significant and marked with an asterisk (*).

The cytokine rich supernatant from cultures containing MNC stimulated with Con A (Con A culture supernatant) can be used as a source of cytokines for induction of mononuclear cell responses [402,403]. Therefore, Con A culture supernatant was added to cytotoxicity assays containing MNC from Tasmanian devils in order to test its ability to activate cytotoxic cells (2.7.6). Tasmanian devil MNC consistently formed clear cytotoxic responses in all assays containing Con A culture supernatant (Fig. 4.15). The sample from one devil showed some evidence for a weak response in the untreated sample, and this cytotoxicity response increased when the sample was supplemented with Con A culture supernatant (Fig. 4.15f). Seven of eight samples supplemented with Con A culture supernatant showed significantly higher cytotoxicity responses than the untreated control samples (Fig. 4.15a – e, g and h). Thus, Tasmanian devil MNC form cytotoxic responses in the presence of cytokine-rich Con A culture supernatant.

The Con A culture supernatant prepared from Tasmanian devil MNC was likely to contain a range of cytokines that are capable of inducing cytotoxicity responses. Interleukin 2 (IL-2) was one candidate as it induces cytotoxicity responses in CTL and NK cells. Tasmanian devil IL-2 was added to cytotoxicity assays containing naive Tasmanian devil MNC at dilutions of 1/100 or 1/1000 (2.7.7). All samples formed cytotoxic responses in the presence of IL-2 (Fig. 4.16). Statistically significant differences between treated and control samples were only observed at dilutions of 1/100 (Fig. 4.16a and b). IL-2 can therefore induce Tasmanian devil MNC to produce cytotoxicity responses against DFTD cells.

Potential effector cells of the mitogen-induced cytotoxic responses against DFTD cells were Tasmanian devil NK cells. Poly I:C, a common agonist of NK cell cytotoxicity, was used to supplement cytotoxicity assays to determine if NK cells could be specifically activated to kill DFTD cells. A range of concentrations between 10 and 0.1 μg/mL Poly I:C was used to examine the cytotoxicity response (2.7.8). The strongest cytotoxicity response was formed at a concentration of 5μg/mL, although responses at any dose were significantly stronger than in untreated controls (Fig. 4.17). All subsequent assays were performed at a concentration of 5 μg/mL and showed an increase in cytotoxicity in the presence of poly I:C compared to untreated samples (Fig. 4.18). Two of the four reactions showed a statistically significant difference between untreated and Poly I:C treated samples (Fig. 4.18a and c). The other two reactions showed raised levels of cytotoxicity, particularly at ratios of 25:1 and 12:1, but the responses were not significant compared to the untreated samples (Fig. 4.18b). Therefore, Poly I:C can activate Tasmanian devil cells to form cytotoxicity responses against DFTD cells.

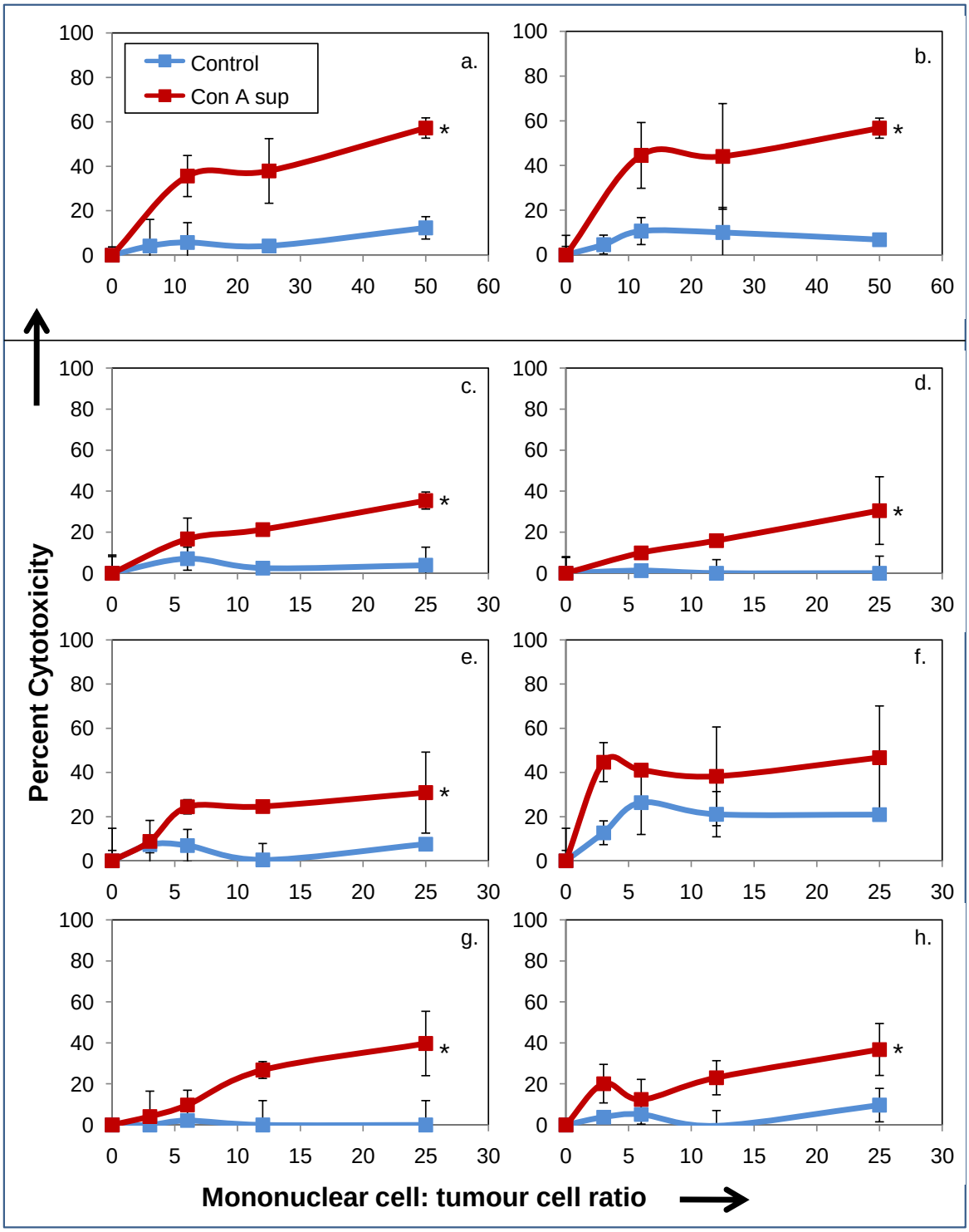

Figure 4.15. Effect of Con A culture supernatant on cytotoxicity responses against DFTD.

Chromium release cytotoxicity assays were performed by culturing MNC from eight Tasmanian devils with radioactively labelled DFTD cells for 18 hours in 25% Con A culture supernatant. Percent cytotoxicity values ± 1 standard deviation at mononuclear cell: tumour cell ratios of 50:1 to 6:1 or 25:1 – 3:1 are shown for each treatment. The statistical difference between the treatments was assessed using F Tests, with a value of $P<0.05$ classified as significant and marked with an asterisk (*).

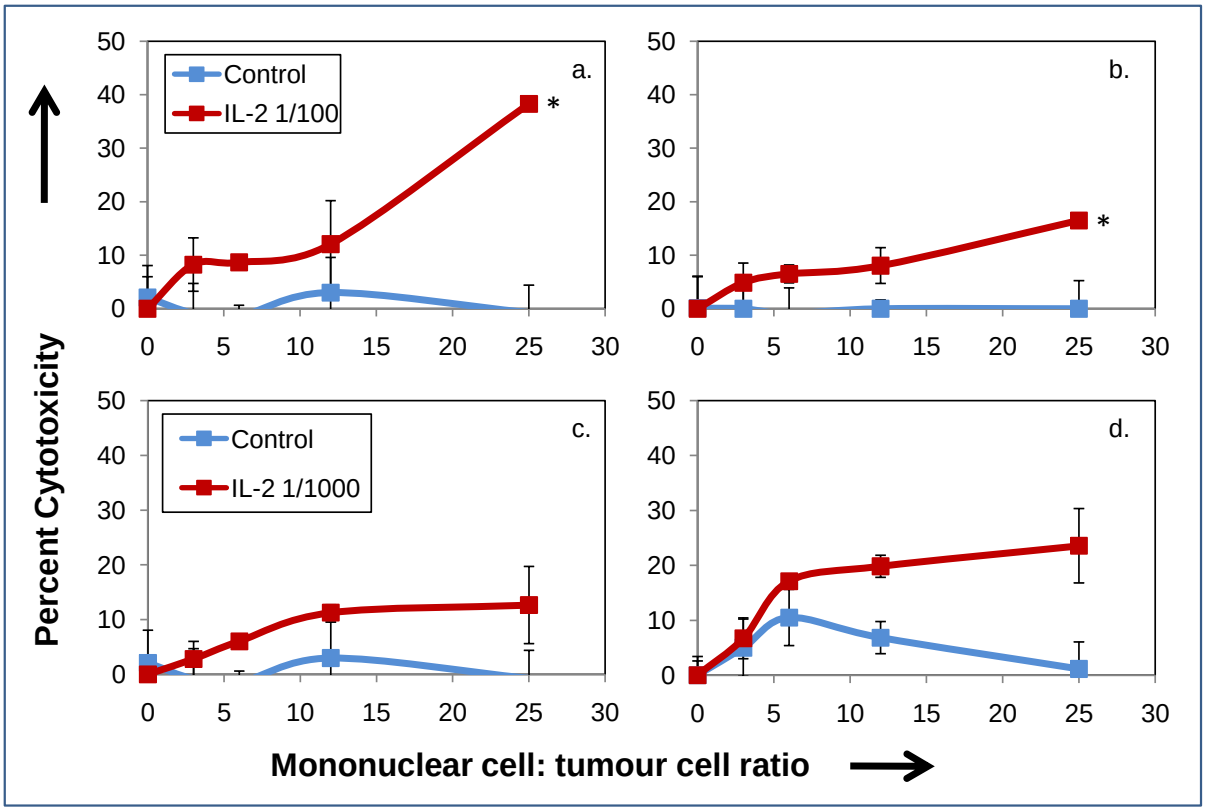

Figure 4.16. Effect of Interleukin (IL)-2 on cytotoxicity responses against DFTD.

Chromium release cytotoxicity assays were performed by culturing MNC from four Tasmanian devils with radioactively labelled DFTD cells for 18 hours. Cloned Tasmanian devil IL-2 was added at dilutions of 1/100 or 1/1000. Percent cytotoxicity values ± 1 standard deviation at mononuclear cell: tumour cell ratios of 25:1 – 3:1 are shown for each treatment. The statistical difference between the treatments was assessed using F Tests, with a value of $P<0.05$ classified as significant and marked with an asterisk (*).

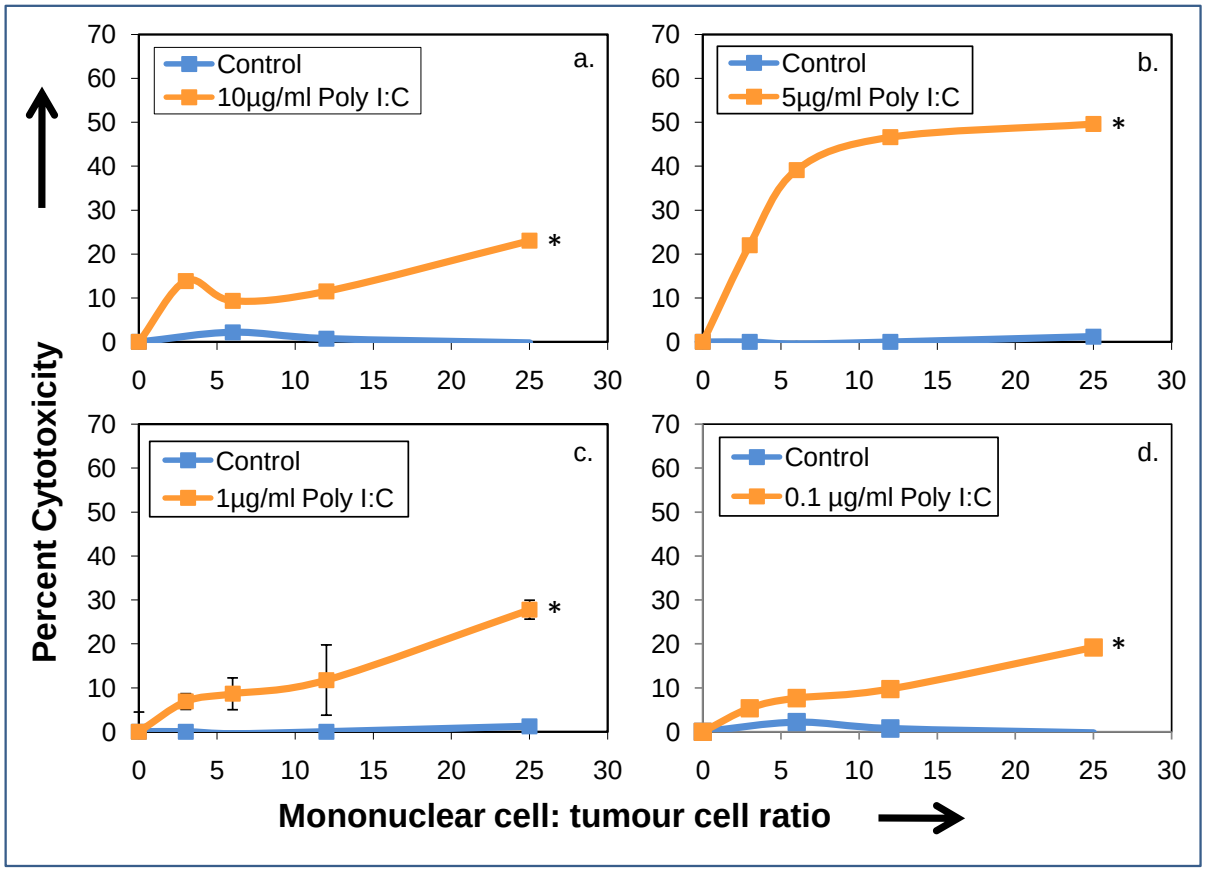

Figure 4.17. Effect of Poly I:C concentration on cytotoxicity responses against DFTD.

Chromium release cytotoxicity assays were performed by culturing MNC from two Tasmanian devils with radioactively labelled DFTD cells for 18 hour, with Poly I:C added at concentrations of 10, 5, 1 and 0.1 µg/mL. Percent cytotoxicity values ± 1 standard deviation at mononuclear cell: tumour cell ratios of 25:1 – 3:1 are shown for each treatment. The statistical difference between the treatments was assessed using F Tests, with a value of $P<0.05$ classified as significant and marked with an asterisk (*).

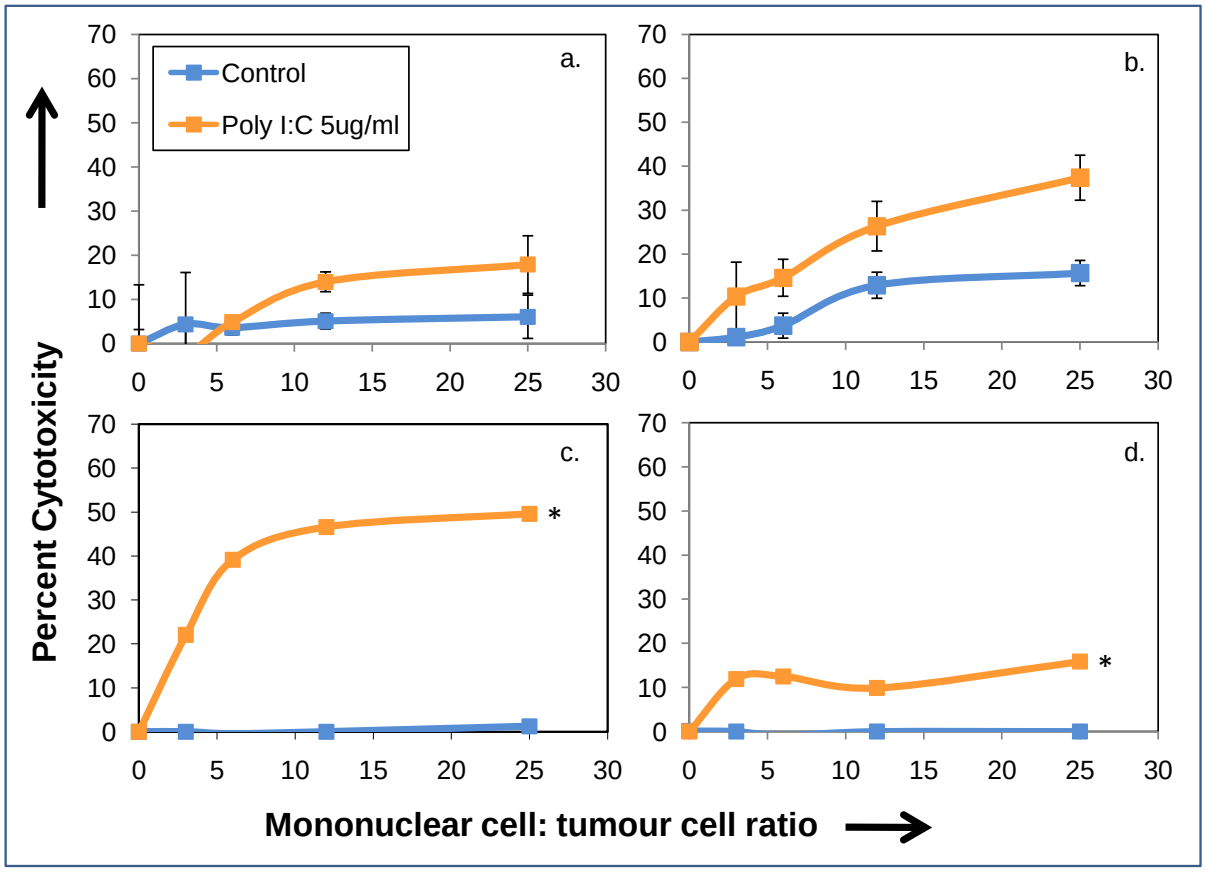

Figure 4.18. Effect of 5 µg/mL Poly I:C on cytotoxicity responses against DFTD.

Chromium release cytotoxicity assays were performed by culturing MNC from four Tasmanian devils with radioactively labelled DFTD cells for 18 hour with and without 5µg/mL Poly I:C. Percent cytotoxicity values ± 1 standard deviation at mononuclear cell: tumour cell ratios of 25:1 – 3:1 are shown for each treatment. The statistical difference between the treatments was assessed using F Tests, with a value of P<0.05 classified as significant and marked with an asterisk (*).

4.3 Discussion

The results of Chapter 3 provide evidence that Tasmanian devils are capable of both cytotoxic and antibody responses against cancer cells. These data, along with previous evidence of allogeneic skin graft rejection [3] suggest that immunisation against a cancer such as DFTD should be possible. However, Tasmanian devils with DFTD show no evidence for either cytotoxic or antibody responses. The use of several different DFTD immunisation preparations has also been largely unsuccessful in producing consistent immune responses against the tumour cells. The successful cytotoxic responses against the foreign cancer cell line K562 were analysed to gain insight into a mechanism for killing of MHC I negative tumour cells, which could be manipulated to target DFTD cells.

Several types of mononuclear cells, including lymphocytes and monocytes, have previously been identified in the blood of Tasmanian devils [2]. However, the cell types comprising the peripheral

blood MNC suspensions used in the cytotoxicity assays have not been thoroughly analysed. The MNC of Tasmanian devils were obtained using density gradient centrifugation and included lymphocytes and monocytes, with minimal numbers of neutrophils and erythrocytes [404]. The MNC of Tasmanian devils could also contain NK cells, which have not previously been identified in this species. As NK cells in other species are able to mediate cytotoxicity against MHC I negative cells, they may be important effector cells in the responses against the K562 cells. In order to determine if NK cells were present in the peripheral blood of Tasmanian devils, the cell types present in MNC suspensions were analysed.

Two cross-species reactive antibodies against CD3 and MHC II have previously been used to identify cell types in Tasmanian devil lymphoid tissues [264]. These antibodies were used separately or together for immunohistochemistry to distinguish individual cell populations in the MNC of Tasmanian devils. In the MNC cytospins, 'null' lymphocytes [404] which did not express CD3 or MHC were also identified. These cells were typically larger than T and B lymphocytes, and the majority had an appearance consistent with the NK cells of other species. Further evidence for the presence of NK-like cells among the MNC of Tasmanian devils was the presence of large granular lymphocytes in Giemsa-stained cytospins. Giemsa staining is commonly used to identify NK cells as it can highlight the cytoplasm. The perforin granules of NK cells are enclosed in highly acidic endosomes within the cytoplasm [405], and are stained darkly with the Azure component of the Giemsa stain [406]. Consequently, the combination of immunochemistry and Giemsa staining used in this study provided the first evidence of NK-like cells in Tasmanian devils.

Although Tasmanian devils have NK-like cells, the responses against MHC I negative K562 cells were not typical of NK cell cytotoxicity and had the hallmarks of a CTL response. There was no spontaneous killing prior to immunisation, the killing was specific, and two doses of vaccine were required, suggesting that prior exposure to the target antigen was necessary, and the responses were long-lasting (refer to Figures 3.3 – 3.6). However, despite these characteristics, it is unlikely that CTL mediated this killing of K562 cells as they were not allogeneic and do not express MHC I protein [388], the obligatory ligand for CTL responses. Many other studies have shown the capacity for NK cells to specifically reject foreign cells, although some have suggested the involvement of factors such as cytokines and complement [158,169,407]. For example, NK cells can behave in a manner consistent with these responses, especially when target cell specific antibody is present [396].Thus, antibody-dependent cell-mediated cytotoxicity (ADCC) was a potential mechanism to use for the identification of NK cell responses in Tasmanian devils.

When devil anti-K562 antibody was added to short length (4 hour) cytotoxicity assays, the MNC of naive Tasmanian devils formed cytotoxicity responses against K562 tumour cells. This provided evidence for the development of ADCC responses by innate immune cells, rather than CTL which require longer time periods for cytotoxicity. Some of the responses produced in 4 hour assays reached higher levels of cytotoxicity than those formed by other samples in 18 hour assays. The *in vitro* responses of Tasmanian devil MNC are known to vary considerably between assays and between devils [385]. Consequently, this variation may be attributable to normal variation between samples. Apart from NK-like cells, the MNC suspensions contained other cells that could mediate cytotoxicity and ADCC responses. The activity of different cell types with the capacity for ADCC were excluded based on evidence from other studies or removed from the Tasmanian devil MNC populations by adherence. Eosinophils, which mainly form ADCC responses against parasites rather than tumour cells [408] were not present in the MNC suspensions and were therefore excluded as potential effector cells. Some MNC suspensions contained contaminating neutrophils, most of which appeared to be immature, with ring-shaped nuclei rather than the characteristic multi-lobar nuclei of mature neutrophils. The immature neutrophils in the samples were also unlikely to act as the effector cells as high effector ratios are required for neutrophil ADCC responses [409] and the cell type was only present in low proportions in the MNC suspensions. Additionally, immature neutrophils form ADCC responses in the presence of IgA [410], which was not likely to be the most abundant isotype in the serum. Cytotoxicity responses were retained when monocytes were depleted by plastic adherence, suggesting that these were not the effector cells. The only remaining candidate was NK cells. Therefore, the results from these functional assays, together with the histological evidence for the presence of NK-like cells, provide evidence that functional NK cells exist in Tasmanian devils.

As well as being the effectors of ADCC responses against xenogeneic tumour cells in Tasmanian devils, NK cells may be crucial effector cells to target the induction of anti-tumour responses against DFTD. Recent research from our laboratory and collaborators at the University of Cambridge suggests that DFTD cells downregulate the expression of MHC I protein at the plasma membrane [285,297]. This would render them impervious to CTL responses in the absence of this obligatory ligand for cytotoxicity. However, as MHC I also acts as an inhibitory ligand for NK cells its absence would make DFTD cells candidates for NK cell cytotoxicity. No studies have sought to determine why Tasmanian devil NK cells do not directly recognise DFTD cells under normal conditions. Potential explanations for the absence of NK cell responses include the expression of non-classical MHC I molecules on the surface of DFTD cells, the production of immunosuppressive cytokines capable of decreasing NK cell activity or the absence of activating ligands on the tumour cells, all of which could

restrict the activity of NK cells. Thus, the interaction between NK cells and DFTD tumours provides an important area for future research.

The results supporting the development of ADCC responses of NK cells in Tasmanian devils provided an explanation for the mechanism for the responses against the MHC I negative K562 cells. As the cytotoxicity responses directed against this cell type may be harnessed to target DFTD cells, the development of ADCC against K562 cells were more thoroughly analysed. In order for ADCC to occur in the 18 hour cytotoxicity assays against K562 cells, there would need to be a source of antibody production within the assays. The most likely candidates for this were B lymphocytes and plasma cells, which can be depleted using nylon wool [383]. This B lymphocyte-depleted population did not kill K562 cells, suggesting that the effector cells, or an essential helper cell type, were removed by nylon wool filtration. As the cytotoxicity responses of nylon wool non adherent cells were restored by addition of devil anti-K562 cell antibody, the effector cells were still present in nylon-wool filtered cells. Thus, it was most likely that a helper cell population was removed by nylon wool filtration. One way to determine if antibody production was occurring within the 18 hour assays was determine if supernatant from these cultures could replace antibody in ADCC assays. There was no evidence for ADCC responses supplemented with supernatant from the assays containing K562 cells and immunised devil MNC, and no evidence of antibody presence in the culture supernatants tested. However, flow cytometry analysis of cell pellets from cytotoxicity assays containing K562 cells and mononuclear cells from Tasmanian devils suggested that there was anti-K562 antibody bound to the K562 cells. This result suggested that formation of antibody does occur within the 18 hour cytotoxicity assays. Therefore, since ADCC responses may have been occurring in the *in vitro* assays against K562 cells and they are a likely mechanism to account for the cytotoxicity against K562 cells.

Another potential pathway for the cytotoxicity against K562 cells was through activation of cytotoxic cells by cytokines formed within the assay. In addition to removing B lymphocytes, nylon wool filtration could also have removed activated T lymphocytes, which are nylon wool adherent [411]. As the obligatory ligand for CTL cytotoxicity was absent on the K562 cells, it is unlikely that CTL would form cytotoxic responses. However activated cells may have produced cytokines that augmented the responses of other cell types, including NK cells or monocytes, within the 18 hour assays. The inability to effectively elute the adherent cells from the nylon wool columns prevented a full analysis of these cells. The production of cytokines within the 18 hour cytotoxicity assays could be analysed using techniques such as ELISA. This was not possible for this project as specific antibodies were not available. If the nylon-wool adherent cells could be separated from the columns, functional assays

using supernatant from 18 hour cultures with K562 cells could have been performed to indirectly assess cytokine production.

Other mechanisms of non-specific stimulation could activate Tasmanian devil MNC and provide an avenue to analyse the production of cytokines and cytotoxicity. Mitogens such as Concanavalin (Con) A can induce direct activation of T lymphocytes and NK cells [400,412]. Since Con A stimulation can induce proliferation in Tasmanian devil MNC [2], it may also function to induce cytotoxicity and cytokine production. Stimulation with Con A for 48 hours promoted cytotoxicity responses in Tasmanian devil MNC. This was an important finding, as it provided evidence that the MNC of Tasmanian devils could be activated to kill DFTD cells. These experiments also described a method for consistent activation of cytotoxicity responses against DFTD. In addition to activating cytotoxicity responses, Con A culture can induce the production of cytokines, including IL-2 and IFNγ [400,413]. Although no specific antibodies were available for analysis of cytokines, their presence could be analysed indirectly using functional assays. In other species, the supernatant from Con A cultures (Con A culture supernatant) can activate a number of cell types, including B lymphocytes, monocytes and macrophages [402,403,414]. Thus, if cytokines were present in Con A culture supernatant it could activate responses such as cytotoxicity in naive Tasmanian devil MNC in cytotoxicity assays. The killing of DFTD cells in assays supplemented with Con A culture supernatant provided evidence for the production of cytokines during mitogen activation. The effector population of these cytotoxic responses was difficult to define. As previously discussed, the Tasmanian devil MNC used for the cytotoxicity experiments are heterogeneous populations in which Con A may induce a variety of responses in different cells, particularly T lymphocytes and NK cells [380,400,412]. It is also possible that the cytokines produced during Con A culture assisted in the mitogen activation of the MNC. Thus, the activity of cytokines may play an important role in the activation of the Tasmanian devil immune system to target the tumours.

Two cytokines which could be present in the Con A culture supernatant and capable of inducing cytotoxicity responses like those observed are IL-2 [415] and IFNγ [402]. Analysis of the individual effects of these cytokines could provide information to distinguish which is the most likely candidate for induction of the responses. Cloned Tasmanian devil IL-2 protein was available for use in functional assays, and its presence consistently induced cytotoxic responses against DFTD cells. Therefore, IL-2 in the Con A culture supernatant is one possible candidate for the active cytokine involved in the cytotoxicity responses. However, there is evidence in the literature to suggest that a major mechanism for activation of NK cytotoxicity by IL-2 is the induction of IFNγ production [148]. If this occurred in the IL-2 cytotoxicity assays, supplementation with IFNγ alone should show a similar

effect. However, cloned Tasmanian devil IFNγ protein was not available for assessment of its effect on lymphocyte function. The agonist polyriboinosinic polyribocytidylic acid (Poly I:C), which targets toll-like receptor (TLR) 3, is a potent inducer of IFNγ production [416]. This agonist can target MNC such as monocytes and NK cells [47,417]. Thus, Poly I:C activation was used as a surrogate measure of the effect of IFNγ on Tasmanian devil MNC. Addition of Poly I:C to cytotoxicity assays induced responses in all samples tested, however the strength of the response was highly variable and some samples did not show significantly greater responses than untreated MNC. Since the MNC used in the cytotoxicity assays contained both T lymphocytes and NK cells, it is difficult to distinguish the contributions of each effector population to cytotoxicity against DFTD cells. Poly I:C has well-characterised effects on NK cells, including activation of cytotoxicity against tumour cells [47], which is often attributed to the production of IFNγ. Stimulation with IFNγ can also induce T lymphocyte cytotoxicity [418], thus production of this cytokine during the 18 hour may have also activated T lymphocytes. Consequently, it is not possible to distinguish the activity from each these cytotoxic cell types using the cytokines and agonists available. Specific antibodies for labelling and sorting viable Tasmanian devil cells would be required for isolation of the individual populations to test their separate responses against DFTD cells.

The results presented in this chapter have discussed two processes through which Tasmanian devil MNC can be induced to target MHC I negative tumour cells. The activation of NK cell ADCC responses in Tasmanian devils provided a potential mechanism for targeting immune responses against DFTD cells. In the field of human medicine, NK cells can contribute to ADCC anti-tumour responses, such as those induced by monoclonal antibody based cancer therapies. Drugs like Herceptin, for targeting breast cancer, and Rituximab, for targeting chronic lymphocytic leukaemia and non-Hodgkins lymphoma, are able to induce NK cytotoxic responses by binding to FcRIII receptors [160,161]. However, attempts to use this pathway to target DFTD have been hampered by the lack of strong antibodies against DFTD. Previous studies have reported successful induction ADCC responses by transfer of antibody between closely related species. One example of such a reaction is transfer of antibodies between closely related species of ducks, which can successfully induce ADCC-mediated rejection of tolerated skin grafts [159,419]. Therefore, if functional levels of DFTD antibody could be induced in a closely related marsupial transfer to Tasmanian devils may induce ADCC responses against DFTD cells. Ideally, the species used would be directly related to Tasmanian devils, in the Dasyurid family. However, many Dasyurid marsupials are endangered or difficult to maintain in captivity and would therefore be unsuitable for these experiments. An alternative would be to use Brushtail possums (*Trichosurus vulpecula*), an abundant marsupial species which are frequently kept in captivity. Future studies of ADCC in Tasmanian devils may pursue this approach. However, the

most promising strategy for targeting cytotoxicity responses against DFTD cells appeared to be through activation with mitogens or cytokines.

The *in vitro* experiments examining non-specific activation of MNC provided the first methods for consistent activation of cytotoxicity against DFTD cells. Antibody dependent cell-mediated cytotoxicity responses appear to be functional in Tasmanian devils, and can be mediated by NK cells. The ADCC pathway could potentially be exploited to induce responses against DFTD cells for vaccination or therapy. The cytotoxic cells induced *in vitro* by stimulation with mitogens, Con A culture supernatant or Tasmanian devil IL-2 have similar qualities to lymphokine-activated killer (LAK) cells in mice and humans [343]. The activation of Tasmanian devil cells with Con A culture supernatant or cloned IL-2 is thus a similar mode of activation to that of human and mouse LAK cells, and the cells possess a similar capacity for cytotoxicity. In other species, particularly mice, LAK cells have been used for adoptive immunotherapy of tumours [344,345,347]. It is therefore possible that the activated cytotoxic cells from Tasmanian devil may also be capable of inducing responses *in vivo* and may provide the basis for an immunotherapy against DFTD. This chapter also described the first identification of NK cells in Tasmanian devils. NK cells should play an important role in immune responses against DFTD cells, in the absence of surface MHC I. Thus, the characterisation of Tasmanian devil NK cells and their interaction with DFTD cells will provide a significant area for future research.

Chapter 5 - Manipulation of Tasmanian devil immune response to target DFTD cells

5.1 Introduction

Regardless of MHC genotype [283], wild Tasmanian devils with Devil Facial Tumour Disease (DFTD) show no evidence of an immune response against the tumour [1]. Studies performed in our laboratory in collaboration with other groups suggest that DFTD cells downregulate MHC I expression on the cell surface [285,297]. This would allow them to escape the host immune response. The absence of MHC I expression presents a challenge for development of an effective immunisation strategy against DFTD. Without expression of MHC I, which is the obligatory ligand for cytotoxic T lymphocyte (CTL) activity, the cells are protected from this major anti-tumour response. The analysis of Tasmanian devil immune responses against MHC I negative tumour cells and mechanisms for cytotoxicity has identified a number of methods that can induce Tasmanian devil mononuclear cells to produce cytotoxicity responses against DFTD cells. The ability to consistently activate cytotoxicity against DFTD, in experiments reported in Chapter 4, was an extremely promising result as it provided the basis for an immunotherapy, which may be a potential intervention against DFTD.

Chapter 4 discussed the ability of several agents to induce anti-tumour immune responses against DFTD cells in peripheral blood mononuclear cells. The strongest cytotoxicity against DFTD cells was induced following activation with the mitogen Concanavalin (Con) A. This technique resulted in mitogen-activated killer (MAK) cells that could produce both cytokines and cytotoxicity responses *in vitro*. These MAK cells also had the potential to mediate direct cytotoxicity against the DFTD cells within solid tumours and provide a source of cytokines, such as interleukin (IL)-2, IL-15 and interferon gamma (IFNγ). If the cells were capable of producing IFNγ within the tumours, this could induce the upregulation of surface MHC I protein and make the DFTD cells targets for CTL activity. Therefore, the use of MAK cells as an immunotherapy was worthy of investigation.

The cytokine rich Con A culture supernatant contained the appropriate cytokines to induce the upregulation of MHC I protein on the surface of DFTD cells [285]. Flow cytometry analysis for cell surface expression of beta-2-microglobulin (B_2M) showed that 48 hours of culture in 10% Con A culture supernatant consistently induced surface MHC I expression in cultured DFTD cells [285]. Subsequently, when MHC I positive DFTD cells were required for experiments they were treated using this method, and are hereafter referred to as 'treated' cells. The Histone deacetylase inhibitor trichostatin A (TSA), which can also induce MHC I protein in tumour cells by increasing the

expression of genes associated with the antigen presentation pathway [420], was also used to treat DFTD cells [285]. However, although TSA treatment upregulated MHC I mRNA levels in DFTD cells, it induced only limited surface expression of the protein [285]. Immunisation with treated DFTD cells was therefore another potential immunotherapy strategy. The presence of the allogeneic MHC I proteins could induce responses against established tumours if a small amount of MHC I protein was present on the DFTD tumour cells. Immunisation with treated DFTD cells could also work in synergy with MAK cells to increase the effects of immunotherapy. Immunisation with treated DFTD cells would prime the Tasmanian devil's specific immune system to respond against any MHC I positive DFTD cells within the tumours. MAK cell therapy would provide a local source of cytokines, such as IFNγ, which could induce the expression of MHC I on DFTD cells within the tumours. Direct injection of the Con A culture supernatant into DFTD tumours may also be a potential strategy to augment expression of IFNγ and MHC I. The experiments in this chapter aimed to assess the effect of three immunotherapy strategies: adoptive MAK cell therapy, immunisation with treated DFTD cells and intra-tumoural injection of 10% Con A culture supernatant. One female Tasmanian devil, CD 15, with a DFTD tumour that had developed following challenge with live cultured DFTD tumour cells, was available for this immunotherapy.

5.2 Results

5.2.1 Immunotherapy with autologous MAK cells, Con A culture supernatant treated DFTD cells and Con A culture supernatant

Autologous peripheral blood mononuclear cells (MNC) from CD 15 were induced to become mitogen-activated killer (MAK) cells by culturing in Con A culture supernatant for 48 hours (2.10.1). Tumour measurements were taken over a period of 24 weeks and biopsy samples were taken to examine the effect of each treatment (2.9.5). Each biopsy was analysed using immunohistochemistry with antibodies against CD3 (T lymphocytes), CD8 (cytotoxic T cells), MHC II (APC including B lymphocytes, dendritic cells and macrophages) (2.9.5). Immune cells observed were collectively referred to as 'leukocytes'. Neutrophils, which do not express these markers, may also have been present, as part of the inflammatory response. The biopsies were also stained for periaxin to label DFTD cells. Prior to commencement of therapy, the tumour was small (Fig. 5.1) and immunohistochemistry of biopsy samples showed no evidence for the presence of $CD3^+$ or $CD8^+$ cells in or around the tumour and limited numbers of infiltrating MHC II^+ leukocytes. The DFTD tumour tissue labelled positively for periaxin (PRX), while the surrounding stromal regions immediately surrounding the tumours were composed of fibrous PRX^- tissue (Fig. 5.2). Thus, there was no evidence for an immune response against the DFTD tumour prior to the commencement of therapy.

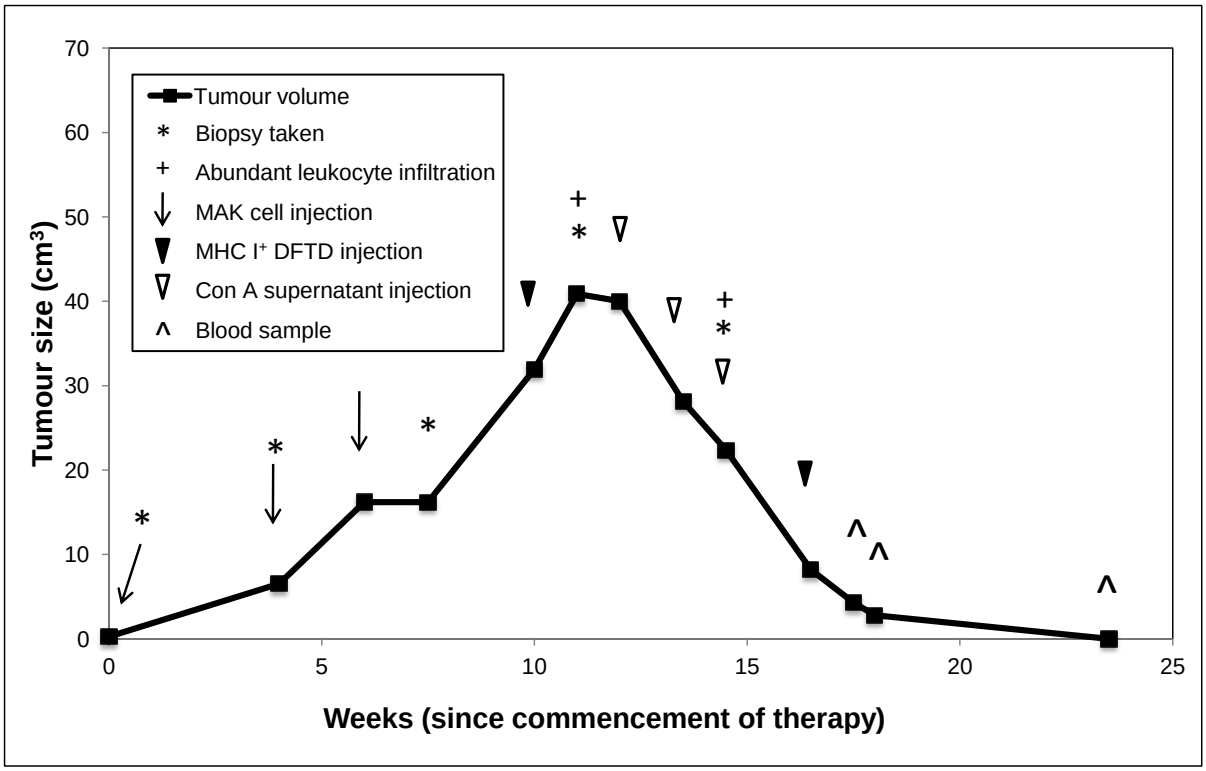

Figure 5.1. DFTD tumour volume in a Tasmanian devil during immunotherapy with mitogen-activated killer cells, MHC I positive DFTD cells and Con A culture supernatant

A Tasmanian devil, with a DFTD tumour caused by experimental inoculation with live cells, was treated with three different immunotherapy techniques over a period of 16 weeks. Doses of mitogen-activated killer (MAK) cells were given at timepoints indicated with arrows. Doses of MHC I positive (Con A culture supernatant-treated) DFTD cells were given at timepoints indicated with solid black arrowheads. Doses of 20% Con A culture supernatant solution were given at timepoints indicated with open arrowheads. Biopsy samples were taken at timepoints indicated with asterisks (*) and samples in which substantial immune cell infiltration was observed are marked with crosses (+). Time points at which blood samples were taken are marked with circumflex accents (^).

During the MAK cell therapy, the tumour steadily increased in size for the next six weeks, from 0.2 to 16.2 cm^3, plateaued until week 8 then increased sharply to 31.9 cm^3 by week 10 (Fig. 5.1). After the first injection of MAK cells there was evidence for infiltration of MHC II^+ cells to the stromal region at the periphery of the DFTD tumour (Fig. 5.3). Some $CD3^+$ cells were present in the same area, but few were $CD8^+$ cells (Fig. 5.3). A small number of leukocytes were present within areas corresponding to regions of densely packed PRX^+ DFTD tumour in the biopsy, suggesting that the infiltration was limited to the periphery of the tumour (Fig. 5.3). In a biopsy taken after all three MAK cell injections, there were fewer cells located at the tumour periphery (Fig. 5.4). However, MHC II^+ cells were scattered at low density throughout the tumour and $CD3^+$ and $CD8^+$ lymphocytes were clustered in some areas (Fig. 5.4). Large areas of DFTD tumour stained strongly for PRX (Fig. 5.4).

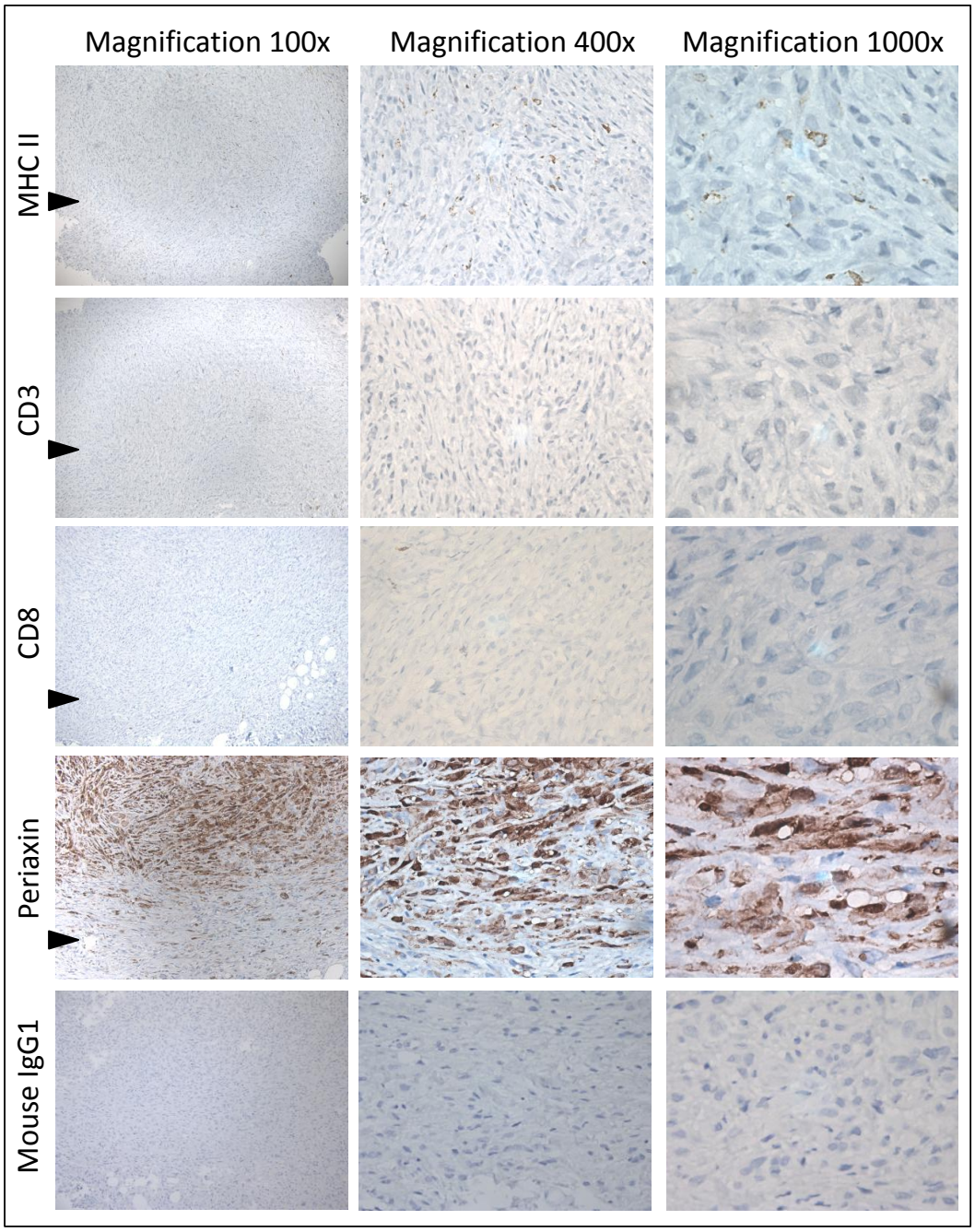

Figure 5.2. Immunohistochemistry of MHC II, CD3, CD8 and PRX in a pre-immunotherapy DFTD tumour biopsy (week 0)

Sections from a biopsy sample taken from the DFTD tumour of a Tasmanian devil prior to immunotherapy were analysed by immunohistochemistry for MHC II, CD3, CD8, PRX or Mouse IgG1 as an isotype control. Sections stained for MHC II expression are displayed in row 1. Sections stained for CD3 expression are displayed in row 2. Sections stained for CD8 expression are displayed in row 3. Sections stained for PRX expression are displayed in row 4. The stromal tissue region around the DFTD tumour is indicated with a solid black arrowhead. The immunohistochemistry shows an absence of leukocyte infiltration into the tumour prior to immunotherapy.

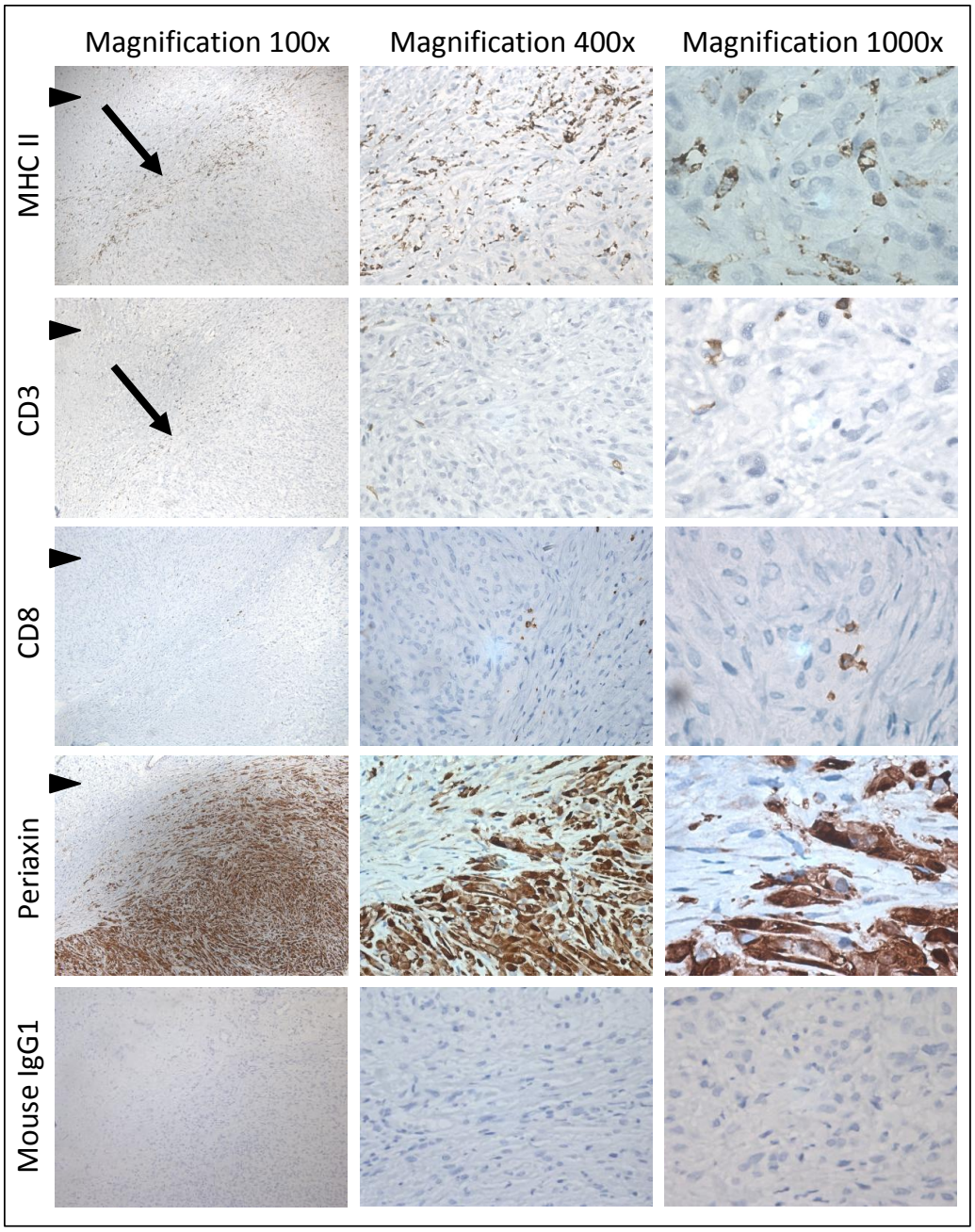

Figure 5.3. Immunohistochemistry analysis of CD3, CD8, MHC II and PRX in a DFTD tumour biopsy taken following one dose of mitogen-activated killer cells (week 5)

Sections from a biopsy sample taken from the DFTD tumour of a Tasmanian devil after one dose of mitogen-activated killer (MAK) cells were analysed by immunohistochemistry for MHC II, CD3, CD8, PRX or Mouse IgG1 as an isotype control. Sections stained for MHC II expression are displayed in row 1. Sections stained for CD3 expression are displayed in row 2. Sections stained for CD8 expression are displayed in row 3. Sections stained for PRX expression are displayed in row 4. The stromal tissue region around the DFTD tumour is indicated with a solid black arrowhead. The regions in which large numbers of MHC II^+ and $CD3^+$ cells were infiltrating are indicated with black arrows. The immunohistochemistry shows an accumulation of leukocytes at the tumour periphery but no infiltration into the tumour following one injection of MAK cells.

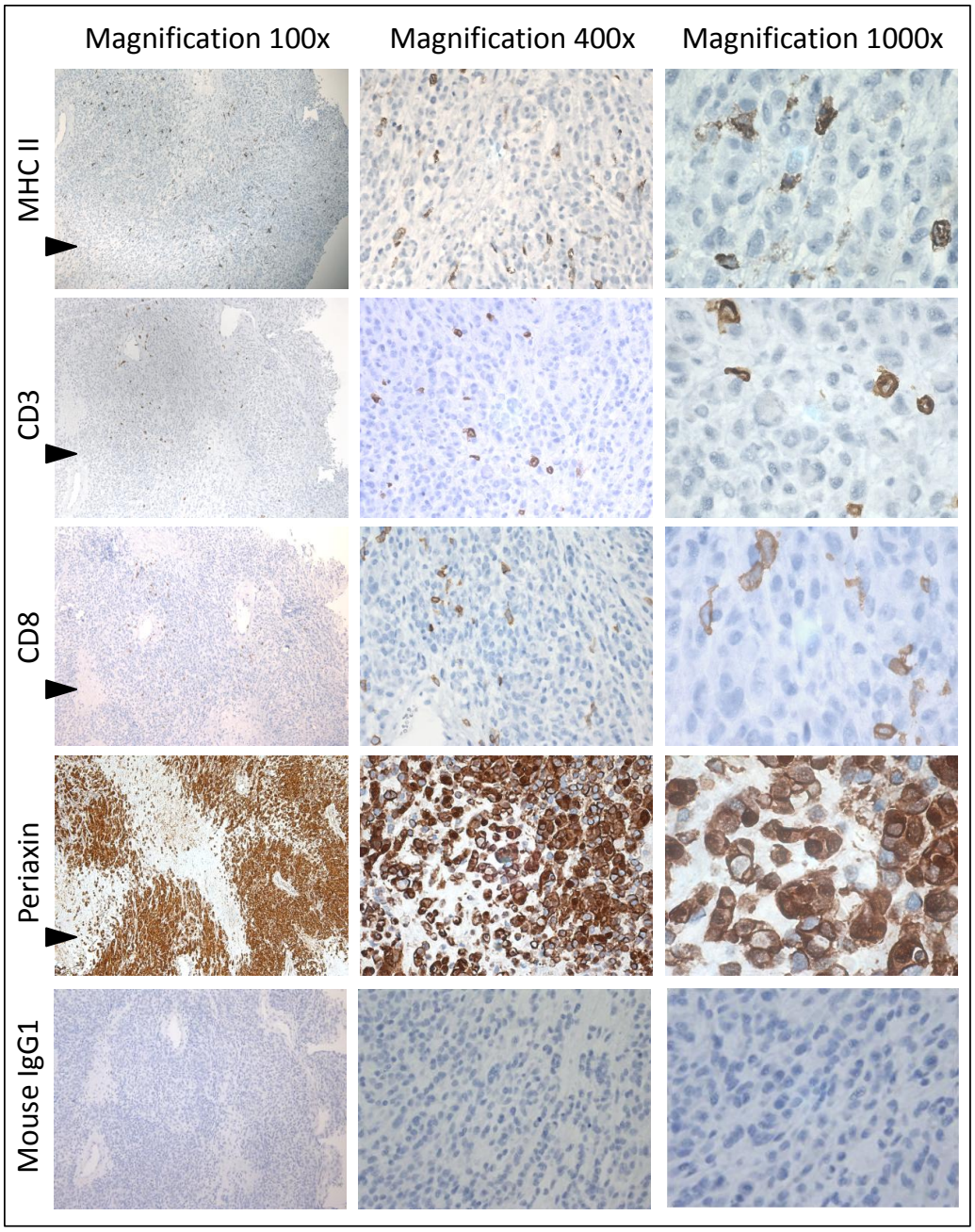

Figure 5.4. Immunohistochemistry analysis of MHC II, CD3, CD8 and PRX in a DFTD tumour biopsy taken after three doses of MAK cells (week 8)

Sections from a biopsy sample taken from the DFTD tumour of a Tasmanian devil after three doses of mitogen-activated killer (MAK) cells were analysed by immunohistochemistry for MHC II, CD3, CD8, PRX or Mouse IgG1 as an isotype control. Sections stained for MHC II expression are displayed in row 1. Sections stained for CD3 expression are displayed in row 2. Sections stained for CD8 expression are displayed in row 3. Sections stained for PRX expression are displayed in row 4. The stromal tissue region around the DFTD tumour is indicated with a solid black arrowhead. The immunohistochemistry shows a slight infiltration of $CD3^+$, $CD8^+$ and $MHC\ II^+$ leukocyte numbers within the tumour following three injections of MAK cells.

Following MAK cell therapy, an immunisation with viable Con A culture supernatant-treated DFTD cells was given to induce an allogeneic response against any MHC I positive DFTD cells present in the tumours (2.10.2). The cells were injected subcutaneously at a site near the tumour at week 10. The tumour continued to increase in size at week 11, then the growth plateaued until week 12 (Fig. 5.1). Immunohistochemistry of a biopsy taken at week 11 showed the presence of abundant MHC II^+ cells within tumour areas, suggesting an infiltration of macrophages and dendritic cells (Fig. 5.5). A large number of $CD3^+$ and $CD8^+$ lymphocytes were also present in the tumour (Fig. 5.5). Staining for PRX showed a reduction in DFTD cell numbers within this sample compared to previous biopsies and a decreased intensity of PRX staining in many tumour cells (Fig. 5.5). This pronounced change in immune response occurred within 7 days of the treated DFTD cell injection.

The third treatment strategy for immunotherapy of CD 15 was intra-tumoural injection of Con A culture supernatant, which could potentially provide a source of IL-2 and IFNγ to augment the immune response. Three intra-tumoural injections of a solution containing 20% Con A culture supernatant in phosphate-buffered saline were given at weeks 12, 13 and 14 (2.10.3). During this time, the tumour rapidly regressed in size (Fig. 5.1) and immunohistochemistry of a biopsy taken on week 14.5 showed a level of MHC II^+ and $CD3^+$ cell infiltration similar to the biopsy taken in week 11 (Fig. 5.6). The biopsy stained for CD8 showed that more cells were present in the biopsy taken in week 14.5 than at week 11. Consequently, injection with cytokine-rich supernatant may have increased the cytotoxic responses against the DFTD tumour. There were few strongly stained PRX^+ DFTD cells present in the biopsy sample, and some cells with DFTD-like morphology showed more diffuse PRX staining than in previous samples (Fig. 5.6). This suggested that the immune response may have altered the antigen expression of the tumour cells. After the third intra-tumoural dose of Con A culture supernatant, another subcutaneous dose of treated DFTD cells was given late in week 16.

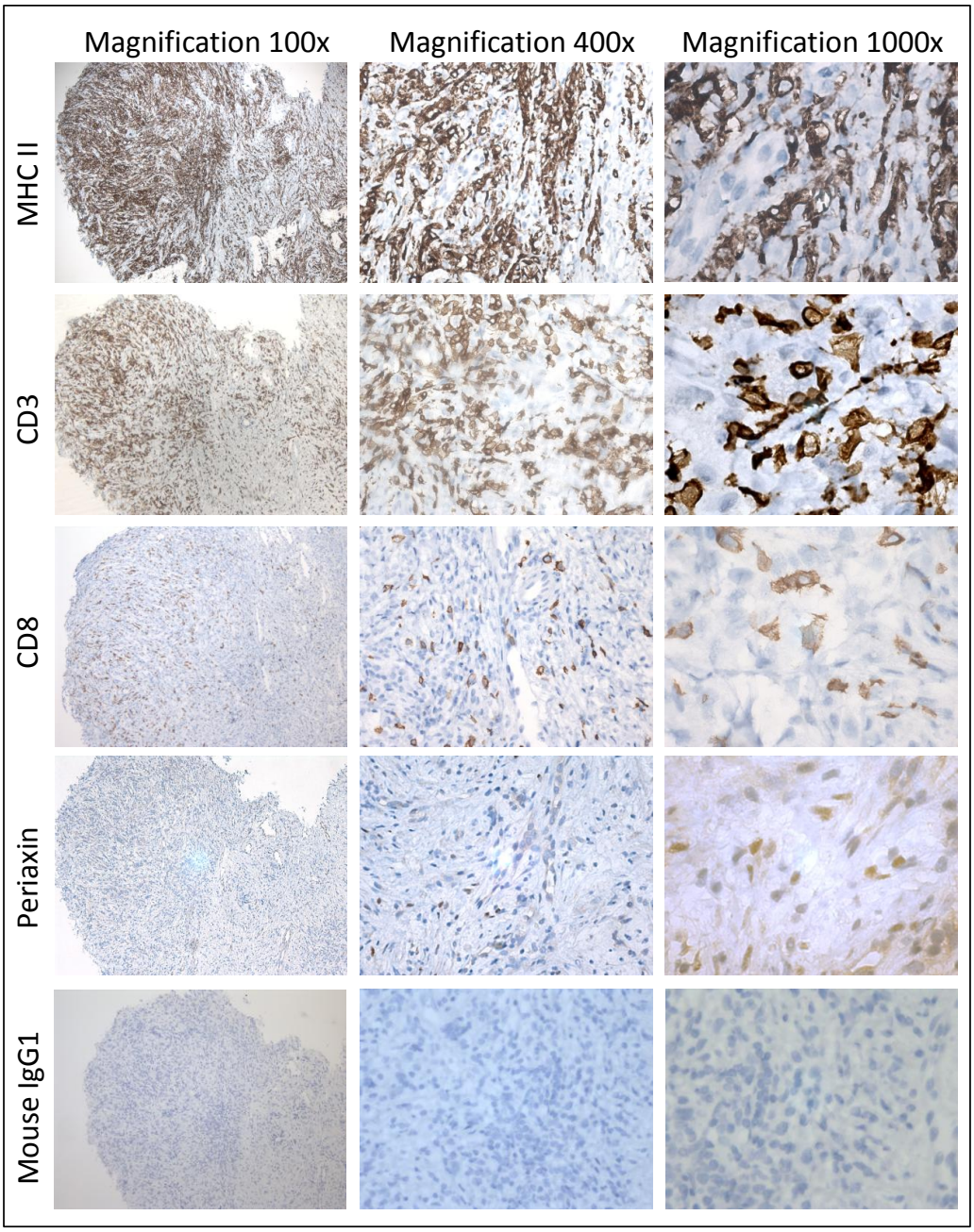

Figure 5.5. Immunohistochemistry analysis of MHC II, CD3, CD8 and PRX in a DFTD tumour biopsy taken after three doses of MAK cells and one dose of treated DFTD cells (week 11)

Sections from a biopsy sample taken from the DFTD tumour of a Tasmanian devil after three doses of mitogen-activated killer MAK cells and one dose of Con A culture supernatant-treated cells were analysed by immunohistochemistry for MHC II, CD3, CD8, PRX or Mouse IgG1 as an isotype control. Sections stained for MHC II expression are displayed in row 1. Sections stained for CD3 expression are displayed in row 2. Sections stained for CD8 expression are displayed in row 3. Sections stained for PRX expression are displayed in row 4. The immunohistochemistry shows a substantial infiltration of $CD3^+$, $CD8^+$ and $MHC\ II^+$ leukocytes into the tumour and a decrease in PRX expression in DFTD cells following one injection of treated DFTD cells.

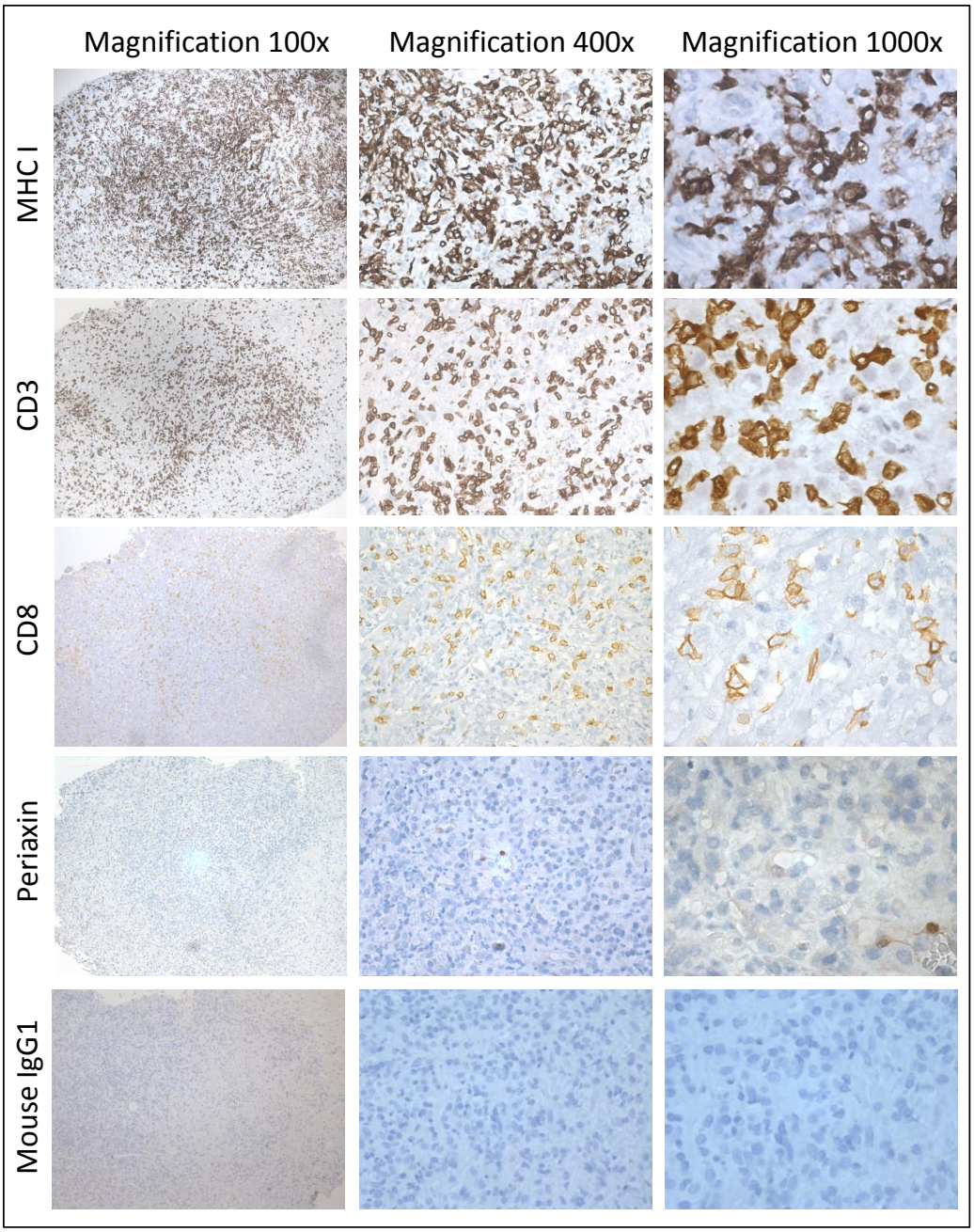

Figure 5.6. Immunohistochemistry analysis of MHC II, CD3, CD8 and PRX in a DFTD tumour biopsy taken during the regression of a DFTD tumour (week 14)

Sections from a biopsy sample taken from the regressing DFTD tumour of a Tasmanian devil which had immunotherapy treatment with mitogen-activated killer MAK cells, treated DFTD cells and intra-tumoural injections of Con A culture supernatant were analysed by immunohistochemistry for MHC II, CD3, CD8, PRX or Mouse IgG1 as an isotype control. Sections stained for MHC II expression are displayed in row 1. Sections stained for CD3 expression are displayed in row 2. Sections stained for CD8 expression are displayed in row 3. Sections stained for PRX expression are displayed in row 4. The immunohistochemistry shows that high numbers of $CD3^+$, $CD8^+$ and MHC II^+ leukocytes remained in the tumour following injection with Con A culture supernatant. The periaxin expression decreased further in this sample.

To provide evidence for the presence of a functional cellular response during regression and to elucidate potential mechanisms for the observed anti-tumour activity, blood and serum was collected late in week 17, in week 18 and late in week 23 and analysed for cytotoxicity and antibody. The first blood sample at week 17 was tested for cytotoxicity responses against both untreated and Con A culture supernatant-treated DFTD cells (2.7.1). Because of limited sample availability, the remaining assays were performed on untreated DFTD cells only because a successful vaccine would need to induce responses against DFTD cells without surface MHC I.

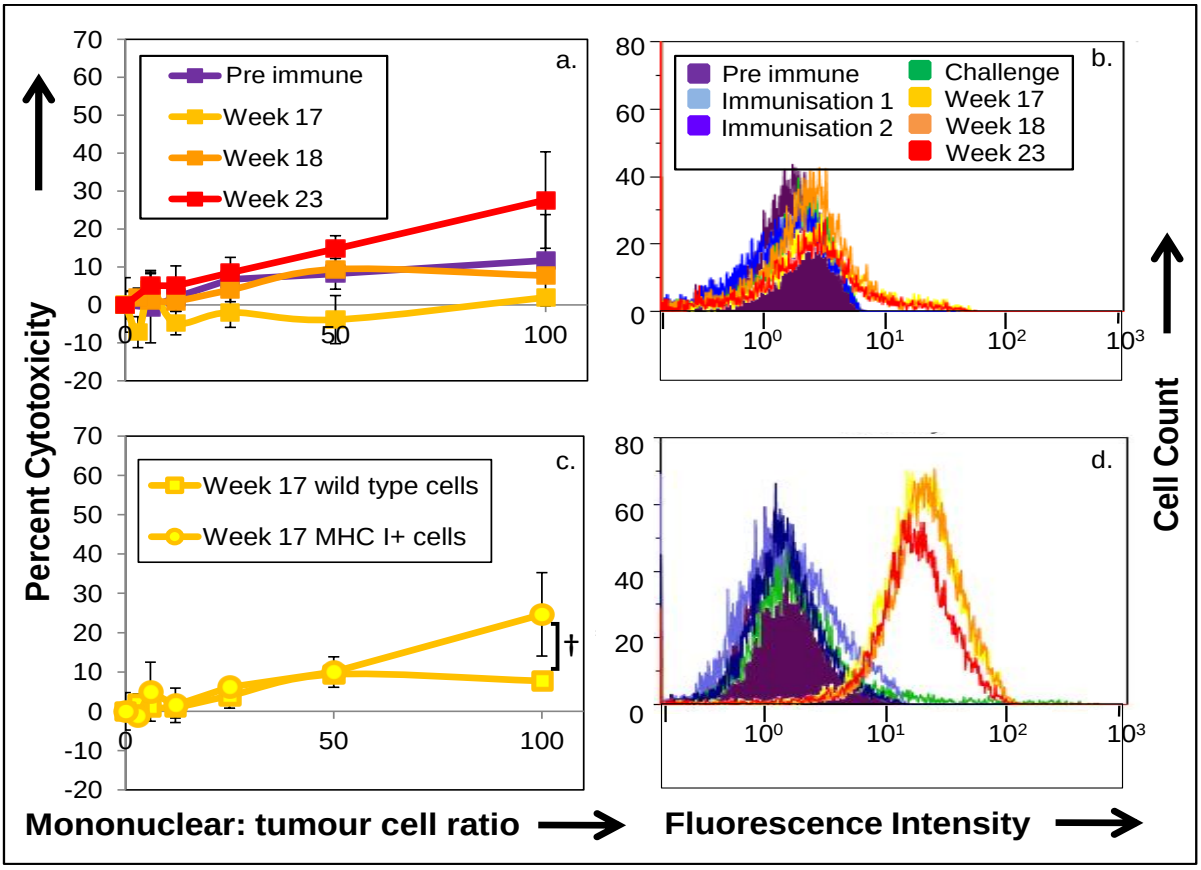

Figure 5.7. Cytotoxicity and antibody responses of a Tasmanian devil during regression of a DFTD tumour after immunotherapy

Three blood samples were taken from a Tasmanian devil that had been given immunotherapy to induce an immune response against a DFTD tumour. The samples were taken at weeks 17, 18 and 23. During this period, the DFTD tumour was regressing. Chromium release cytotoxicity assays were performed by culturing the Tasmanian devil's mononuclear cells with radioactively labelled DFTD cells for 18 hours. Percent cytotoxicity values at leukocyte: tumour cell ratios of 100:1 to 6:1 are shown for all assays (panels a. and c.). Panel a. shows the cytotoxicity responses of mononuclear cells from each blood sample against untreated (wild type) DFTD cells. The statistical difference between the samples taken during immunotherapy for DFTD cells was assessed using F Tests, with a value of P<0.05 classified as significant and marked with an asterisk (*) when significantly different from the pre immune sample. Since this Tasmanian devil had also been immunised with DFTD cell protein preparations, the responses were compared to the Pre immune

sample taken before any immunological intervention was given. Panel c. shows the responses of mononuclear cells from a single blood sample, at week 17, against Con A culture supernatant-treated DFTD cells, which would have expressed surface MHC I protein, and untreated cells, which do not express the protein. The statistical difference between MHC I positive and wild type DFTD cells was assessed using F Tests, with a value of $P<0.05$ classified as significant and marked with an asymmetrical cross (†) when a significant difference was observed between samples.

Serum antibody against untreated and Con A culture supernatant-treated DFTD cells was analysed using flow cytometry (panels b and d, respectively). The solid purple curve represents the pre immune antibody level in both panels. Pale and dark blue curves respectively represent antibody levels after the first and third protein immunisations performed on the same devil in an immunisation trial prior to immunotherapy and show an absence of antibody response against whole cells during immunisation. The green curve represents the antibody level during challenge with live DFTD tumour cells following the DFTD protein vaccines and shows an absence of response during challenge and tumour development. The yellow, orange and red curves represent the first, second and third blood samples taken in weeks 17, 18 and 23 during tumour regression in response to immunotherapy treatment and show an immune response against Con A culture supernatant-treated (surface MHC I positive) DFTD cells but not untreated DFTD cells.

In the samples taken at week 17, 18 and 23 there was no significant level of cytotoxicity against untreated DFTD cells (Fig. 5.7a.) The samples were also analysed for anti-DFTD antibody presence (2.8.2). All three samples taken during tumour regression contained antibodies which bound treated DFTD cells but not untreated cells (Fig. 5.7b and d), suggesting that there was a response against MHC I^+ cells but not untreated cells. When the cytotoxicity responses against untreated and Con A culture supernatant-treated DFTD cells was compared, there was a significant difference between the two assays (Fig. 5.7c). However, due to the limited numbers of mononuclear cells available, simultaneous assays using MHC I^+ and MHC I^- cells were only performed in the sample taken in week 17. The tumour continued to regress and appeared to be completely resolved when checked late in week 23 (Fig. 5.1). Regular (monthly) examinations of the site following tumour regression showed no recurrence of the tumour, and subsequent biopsies confirmed that only scar tissue remained at the site. The devil remained tumour free for approximately 8 months and then was euthanised due to unrelated health issues.

5.2.2 Evidence for morphological changes consistent with the redistribution of beta-2 microglobulin (β2M) protein in DFTD cells in response to MAK cell injection

The *in vitro* evidence for antibody production in CD 15 occurred only against DFTD cells which were positive for MHC I. This suggests that MHC I expression may have been important in the immune response against the DFTD tumour. An antibody specific for Tasmanian devil β2M was used for

immunohistochemistry on tumour biopsies taken during immunotherapy. In the pre-treatment biopsy, the β2M staining was strongly associated with the nucleus of DFTD cells, with some staining of the cytoplasm (Fig. 5.8). The similar areas in a biopsy section stained with PRX confirmed that the tissue observed was composed largely of DFTD cells with few stromal cells. Following a single dose of MAK cells, a morphological change occurred in the DFTD cells; the nuclei of some cells became enlarged, rounded and appeared vacuolated (Fig. 5.8). The amount of β2M expression within the cytoplasm also increased in some areas of the biopsy (the nuclei were still strongly positive). Staining with PRX confirmed that the tissue observed was composed largely of DFTD cells. In the biopsy sample taken late in week 7 (after two MAK cell doses) more cells had enlarged, vacuoled nuclei. A second morphological change occurred in this sample, with some cells showing distinct rings of β2M staining at the edges of their nuclei, and less intense staining at the centre (Fig. 5.8). This accumulation of β2M protein may be consistent with its redistribution within the cell. Two more biopsies were taken during the immunotherapy of CD 15, after Con A culture supernatant-treated DFTD cell injection and Con A culture supernatant injection, respectively. As the tumour was heavily infiltrated with immune cells, these sections stained strongly for β2M and there were few PRX^+ DFTD cells (Fig 5.8 Rows 4 and 5). The DFTD cells that were visible in these sections stained showed the ring-like staining pattern when labelled with β2M. Many DFTD also stained less intensely for PRX.

5.2.3 Immunisation of DFTD diseased and healthy Tasmanian devils with Con A culture supernatant treated DFTD cells

The earliest evidence for a response against the DFTD tumour during the immunotherapy occurred after injection with treated DFTD tumour cells. Two wild Tasmanian devils with DFTD tumours were injected with the same number of live treated DFTD cells as were used in the first immunotherapy trial to replicate the technique and examine the efficacy of treated DFTD as an immunotherapy. In blood samples taken before injection of treated cells, there was no evidence of cytotoxicity responses in either devil (Fig. 5.9). Following treatment, there was no evidence of cytotoxicity against untreated DFTD cells at 7 and 14 days after vaccination, nor was there evidence for antibody responses against treated or untreated DFTD cells was observed in any serum sample from either devil (Fig. 5.9). The original tumours in both devils continued to grow steadily. At the site of treated cell injection, both devils developed new tumours, which grew rapidly, within 14 days. One devil was euthanised 33 days after the commencement of the trial. The second devil received 2 intra-tumoural injections of Con A culture supernatant and one dose of killed treated DFTD cells. These treatments did not result in tumour regression. This devil was euthanized 47 days after commencement of the trial.

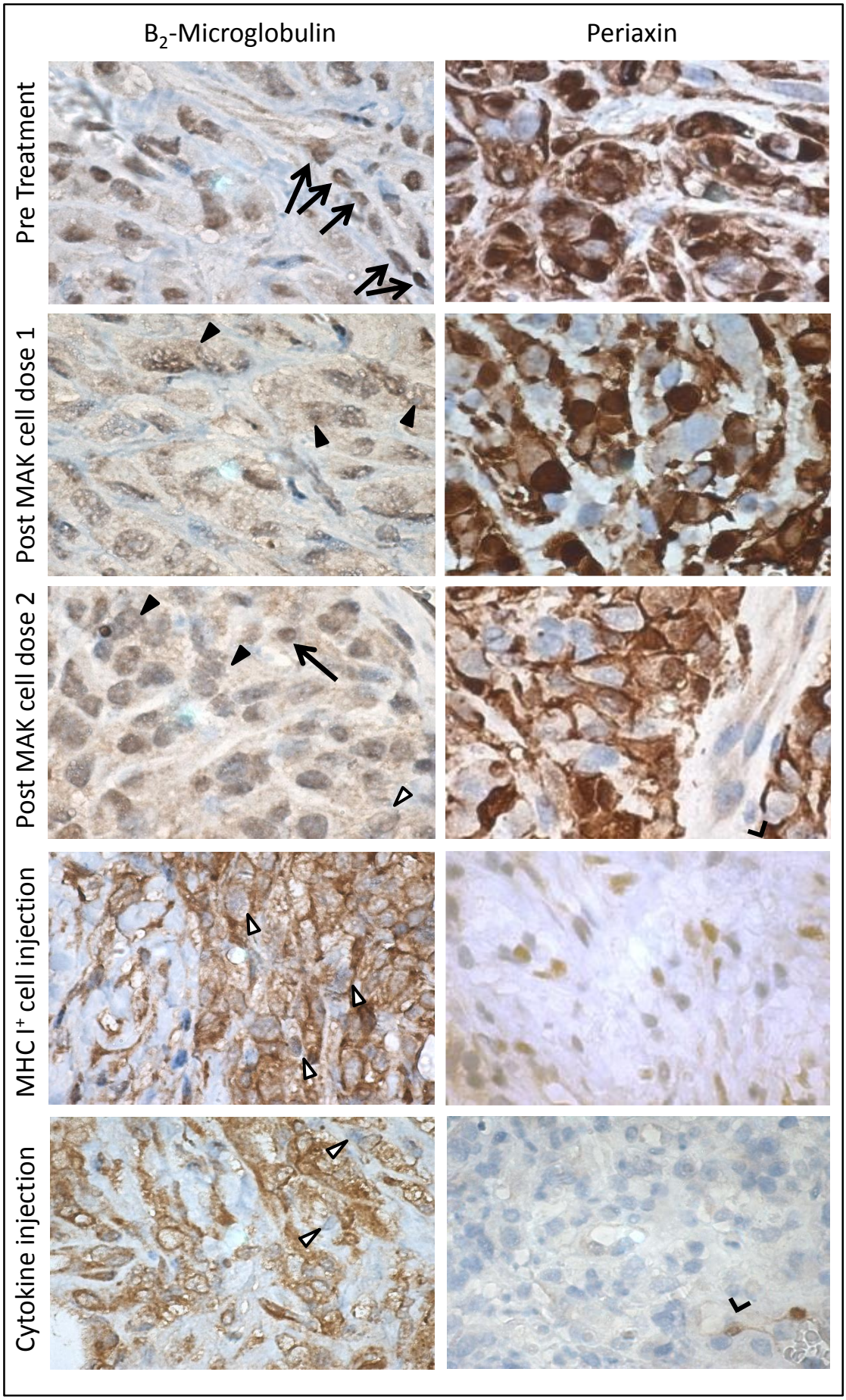
B2-Microglobulin
Periaxin
Pre Treatment
Post MAK cell dose 1
Post MAK cell dose 2
MHC I+ cell injection
Cytokine injection

Figure 5.8. Immunohistochemistry analysis of β_2M^+ expression in a DFTD tumour during immunotherapy of a Tasmanian devil

Sections from five biopsy sample taken from the DFTD tumour of a Tasmanian devil before and during immunotherapy with mitogen-activated killer (MAK) cells, MHC I positive DFTD cells and Con A culture supernatant injection were analysed by immunohistochemistry for β_2-Microglobulin (β_2M) and periaxin (PRX) expression. Photographs were taken under 1000x magnification. Cells with small, defined nuclei with strong β_2M expression, and little cytoplasmic staining, which were characteristically observed in the pre-treatment sample, are indicated with black arrows. Cells with enlarged, vacuoled nuclei and stronger cytoplasmic β_2M expression, characteristic of those observed during MAK cell threrapy, are indicated with solid black arrowheads. Cells with 'ring-like' β_2M staining at the periphery of the nucleus and weak nuclear staining, which were observed after 3 doses of MAK cell immunotherapy, Con A culture supernatant-treated cell injection and intratumoural injection of Con A culture supernatant (cytokine injection) are indicated with open arrowheads in rows. DFTD cells with weak PRX staining were observed in samples taken after Con A culture supernatant-treated cell injection and cytokine injection; examples are indicated with circumflex accents (^). The changes identified in the biopsy sections suggest that the DFTD cells in the tumour may have altered their expression of β_2M, and potentially MHC I during immunotherapy.

Three healthy Tasmanian devils were injected subcutaneously at day 0 with DFTD cells which had been modified in culture then irradiated (2.6.1.2). Two devils received the Con A culture supernatant-treated cells, which would have expressed surface MHC I. One devil received cells that had been treated with trichostatin A (TSA), which increased the levels of MHC I RNA, and potentially several immunogenic surface proteins, but induced little expression of surface protein [285]. Cytotoxicity assays were performed using untreated DFTD cells, as responses would be required against untreated cells to prevent transmission of normal tumour cells. After one dose, neither of the devils immunised with Con A culture supernatant-treated cells formed any evidence of cytotoxicity against untreated DFTD cells (Fig. 5.10a and b). The devil immunised with TSA-treated cells showed evidence for a weak but statistically significant cytotoxic response against the untreated cells at 6 days after injection (Fig. 5.10c). However, this response had receded at 13 days. None of the three devils injected formed cytotoxicity responses 7 days after a second dose (Fig. 5.10). One devil that was injected with Con A culture supernatant treated cells showed evidence for a weak, but statistically significant, cytotoxicity response after the second dose (Fig. 5.10a). There was no evidence for a response in the two remaining devils (Fig. 5.10b and c). Antibody levels in the serum samples were analysed by flow cytometry (2.8.2). In order to assess the responses against all types of cells used in the immunisation, replicate experiments were performed for untreated, Con A culture supernatant-treated and TSA-treated DFTD cells. None of the immunised devils showed any evidence for antibody development against either untreated, Con A culture supernatant-treated or

TSA-treated DFTD cells after any dose (Fig. 5.11). As the *in vitro* results after vaccination may not accurately represent responses *in vivo*, the three immunised devils were challenged with live, untreated DFTD cells 47 days after the second dose of treated DFTD cells to determine if any protective immune response had been formed. All three devils developed DFTD tumours 37 days after this challenge. Therefore, the immunisation of these healthy devils with treated DFTD cells did not induce protective responses against the disease in these devils.

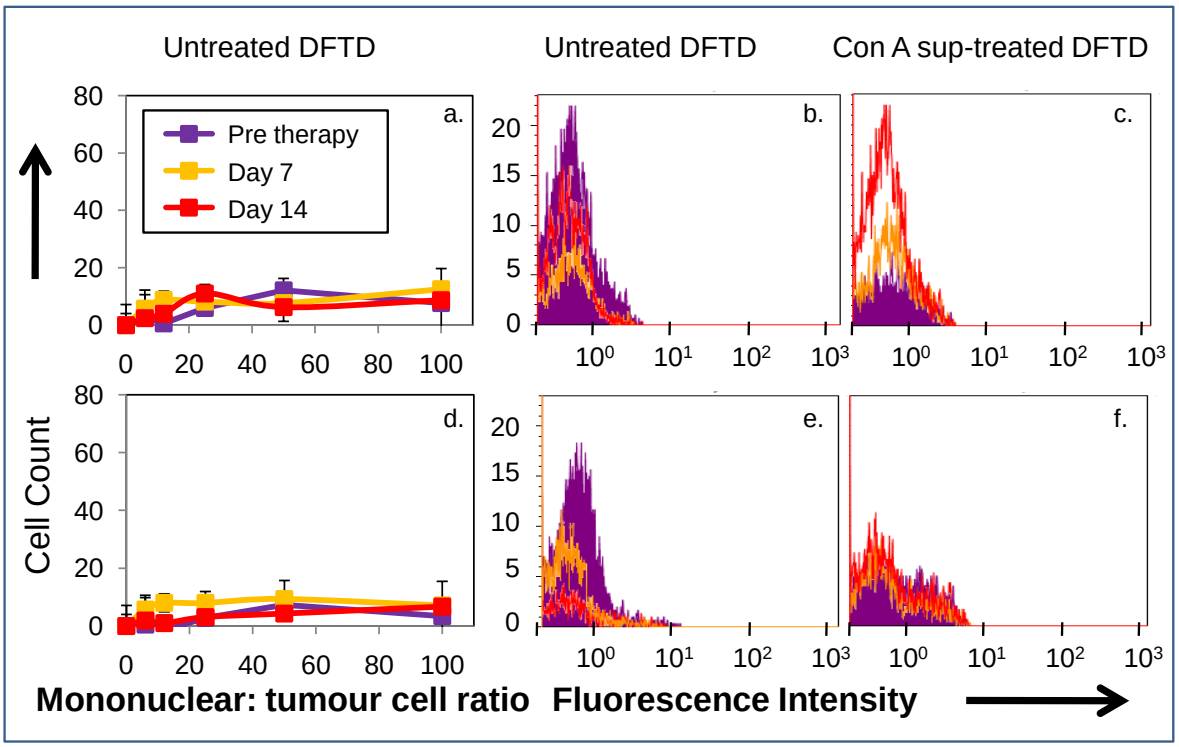

Figure 5.9. Cytotoxicity and antibody responses of two diseased Tasmanian devils following immunisation with two doses of live MHC I positive DFTD cells

Two Tasmanian devils with DFTD tumours were immunised with MHC I positive (Con A culture supernatant-treated) DFTD cells. Chromium release cytotoxicity assays were performed by culturing mononuclear cells from the immunised Tasmanian devils with radioactively labelled wild type DFTD cells for 18 hours. Percent cytotoxicity values at mononuclear cell: tumour cell ratios of 100:1 to 6:1 are shown in panels a, c and e. The statistical difference between the pre immune responses of each devil and the cytotoxicity formed after each dose was assessed using F Tests, with a value of $P<0.05$ classified as significant and marked with an asterisk (*). Serum DFTD antibody levels of the immunised Tasmanian devils were analysed using flow cytometry and are displayed in panels b, d, and f. The levels of cytotoxicity and antibody observed suggest that there was no cellular immune response against DFTD following injection of live MHC I positive DFTD cells alone.

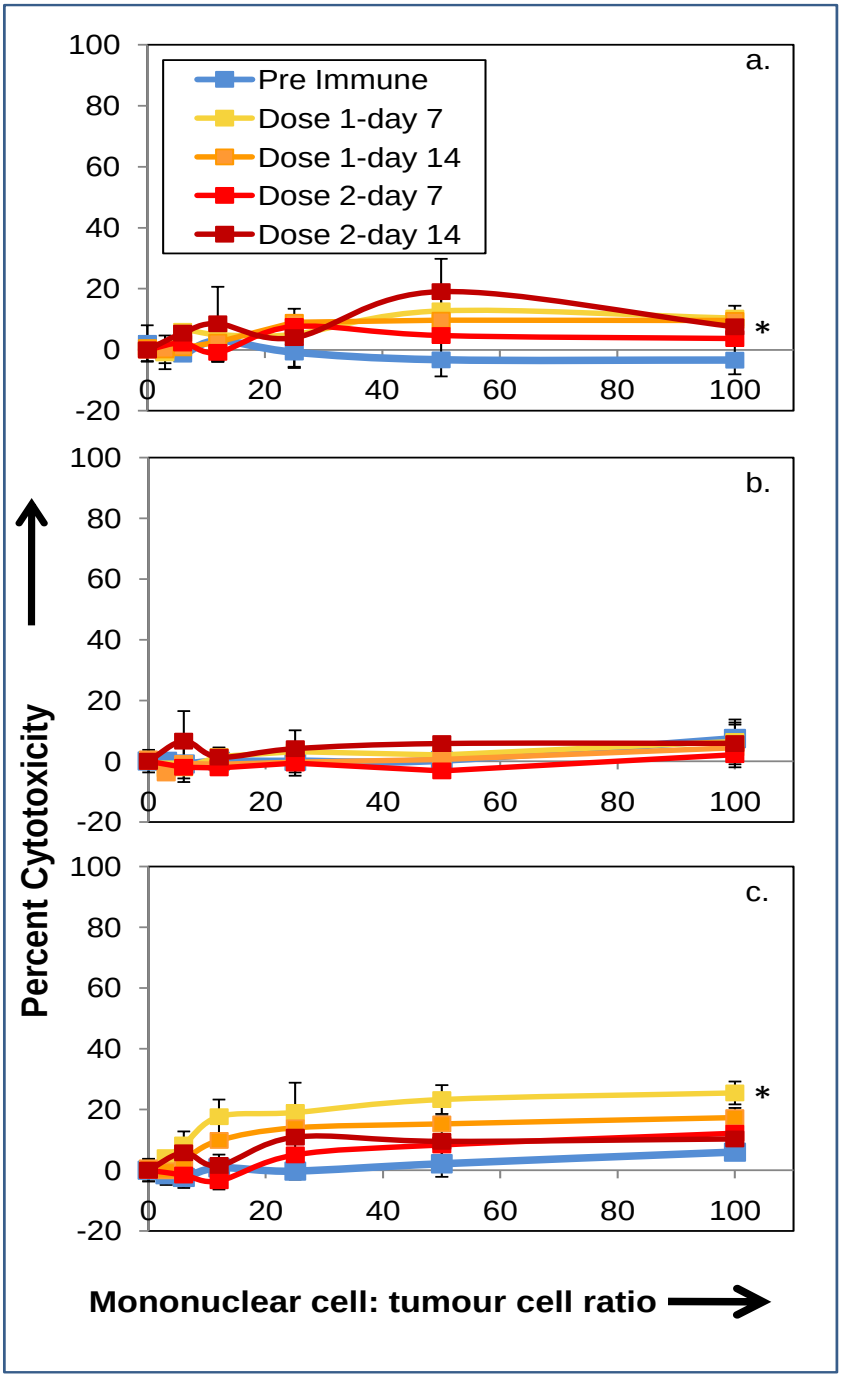

Figure 5.10. Cytotoxicity responses of three healthy Tasmanian devils following immunisation with two doses of killed MHC I positive DFTD cells

Two healthy Tasmanian devils were immunised with MHC I positive (Con A culture supernatant-treated) DFTD cells and one Tasmanian devil was immunised with Trichostatin A (TSA)-treated DFTD cells. Chromium release cytotoxicity assays were performed by culturing mononuclear cells from the immunised Tasmanian devils with radioactively labelled DFTD cells for 18 hours. Two assays were performed after each dose; day 7 and day 14. Percent cytotoxicity values at mononuclear cell: tumour cell ratios of 100:1 to 6:1 are shown. The cytotoxicity responses of the two devils immunised with irradiated MHC I positive DFTD cells are shown in panels a and b. The cytotoxicity response of one devil immunised with irradiated TSA-treated DFTD cells are shown in

panel c. The statistical difference between the pre immune responses of each devil and the cytotoxicity formed after each dose was assessed using F Tests, with a value of P<0.05 classified as significant and marked with an asterisk (*). The samples that showed statistically significant responses were was dose 2 day 14 in panel a. and dose 1 day 1 in panel c. The levels of cytotoxicity in these three devils suggest that there was no cytotoxic response against DFTD following injection of killed MHC I positive DFTD cells alone.

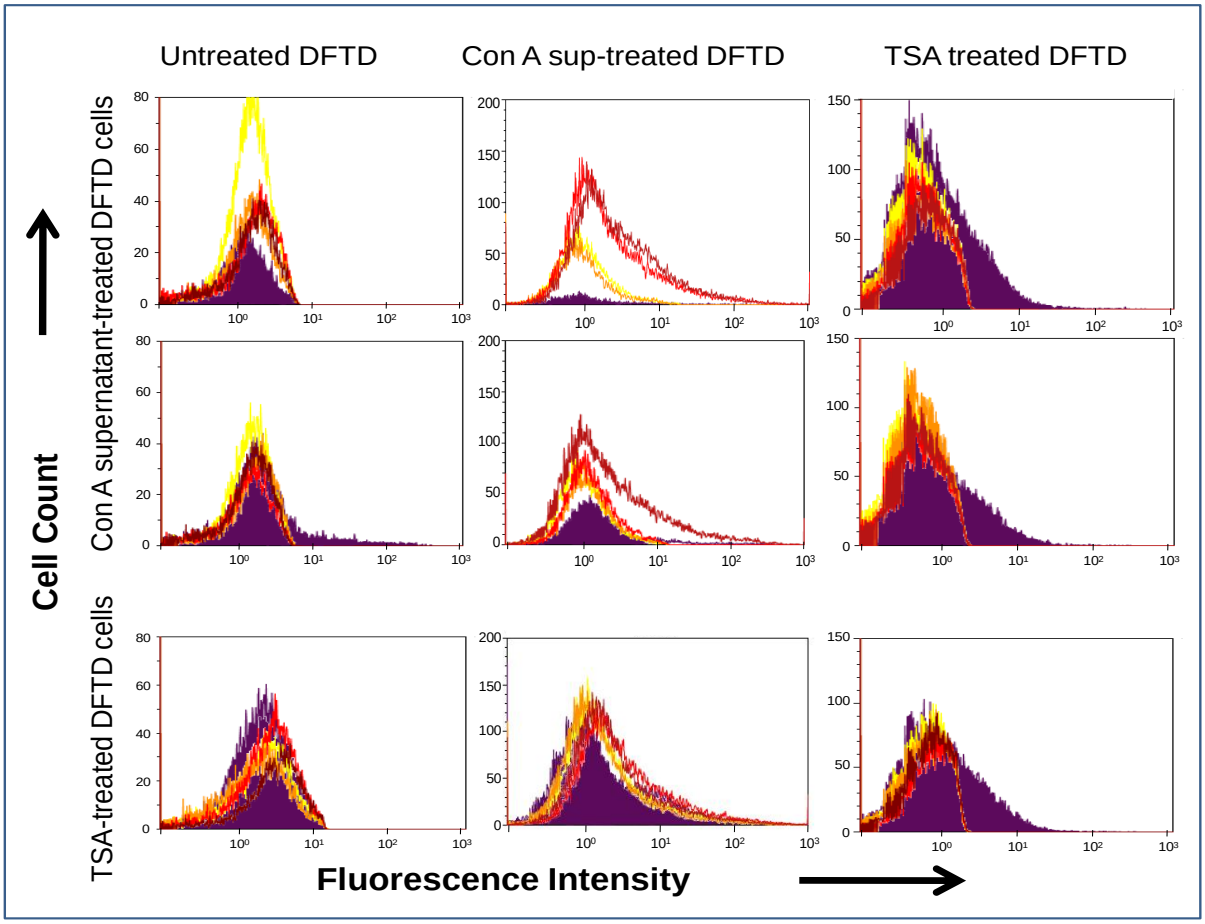

Figure 5.11. Antibody responses of three healthy Tasmanian devils following immunisation with two doses of killed MHC I positive DFTD cells

Two healthy Tasmanian devils were immunised with MHC I positive (Con A culture supernatant-treated) DFTD cells and one Tasmanian devil was immunised with Trichostatin A (TSA)-treated DFTD cells. Serum DFTD antibody levels of the immunised Tasmanian devils against wild type, Con A culture supernatant-treated and TSA treated DFTD cells were analysed using flow cytometry. Responses against wild type cells are displayed in the panels of column 1, Con A culture supernatant-treated cells are displayed in the panels of column 2 and TSA treated cells are displayed in the panels of column 3. The solid purple curves represent the pre immune antibody levels. The yellow and orange curves represent responses at 7 and 14 days post dose 1, respectively. The red and crimson curves represent responses at 7 and 14 days post dose 2, respectively. The levels of antibody in these three devils suggest that there was no humoral response against DFTD following injection of killed MHC I positive DFTD cells alone.

5.3 Discussion

Successful induction of an anti-tumour response against DFTD needs to overcome immune barriers established in the disease. The previous chapter discussed cytotoxic cells as potential effector cells against DFTD tumour cells. An important finding of this thesis was that stimulation with mitogens or cytokines could consistently induce mononuclear cells to produce cytotoxicity responses against DFTD. These cytotoxic cells have similar qualities to lymphokine-activated killer cells from mice and humans, including their potential for cytotoxicity responses and mode of activation [343]. The best responses out of the five stimuli tested against DFTD tumour cells were formed after a 48 hour culture with the mitogen Con A. Potentially, these mitogen-activated killer (MAK) cells could also mediate anti-DFTD responses *in vivo*. One anticipated mechanism for *in vivo* immune responses would be direct cytotoxicity against the tumour cells. Mitogen stimulation would also induce cytokine production, including IFNγ, which could induce upregulation of surface MHC I protein. Experiments were undertaken to assess the effect of activated Tasmanian devil cytotoxic cells as an immunotherapy for the treatment of a devil with an established DFTD tumour.

The results of this *in vivo* immunotherapy experiment were promising. The use of three techniques in sequence, MAK cell therapy, Con A culture supernatant-treated DFTD cell immunisation and intra-tumoural cytokine injection, resulted in activation of the devil's immune response and complete regression of the tumour. This experiment provided the first evidence that a Tasmanian devil's immune response has the capacity to eliminate DFTD tumours *in vivo*. As separate treatments were used, it was difficult to determine which, if not all, caused the regression. The first treatment used in the diseased devil was the injection of MAK cells directly into the tumour. Initially, there were an increased number of leukocytes present in the stroma around the tumour following the first dose of MAK cell therapy. Further MAK cell injections did not induce substantial leukocyte infiltration into the tumour and the tumour continued to grow. The lack of response to the MAK cell injections may have been due to insufficient MAK cells reaching the tumour, as only low numbers of MAK cells could be injected due to difficulty in obtaining sufficient cells for treatment. Under anaesthesia the devil had a low heart rate and slow blood flow, resulting in low sample volume and lengthy collection periods which contributed to clotting, poor mononuclear cell recovery and low viability. Compounding this effect of the low yields was the characteristic clumping caused by Con A. Larger numbers of MAK cells per injection may improve the responses. Con A induced aggregation could be avoided by stimulating the cells with IL-2. In addition to the loss of cells during stimulation, not all of the activated cells remained in the tumour following injection. As biopsy samples were taken prior to the immunotherapy injections, the biopsy site would bleed when the injection was given.

Consequently, loss of cells via bleeding from the puncture wound would have been another factor which limited the response. Future MAK cell therapies would benefit from biopsies given time to heal prior to injection of cells.

As the MAK cell immunotherapy did not produce a strong immune response and the tumour was increasing in size, Con A culture supernatant-treated DFTD cells were injected sub-cutaneously near the tumour to boost the immune response. Within seven days of injection, large numbers of immune cells colonised the tumour. The presence of $CD3^+$ cells was consistent with an infiltration of T lymphocytes. Staining for CD8 suggested that cytotoxic cells were present among the $CD3^+$ lymphocytes, which would have been directly killing MHC I positive DFTD cells within the tumour. Thus, in order for the devil's immune system to respond against the tumour following an immunisation with treated cells, they must have expressed at least trace amounts of surface MHC I at this stage. Under normal circumstances, DFTD tumours do not express surface MHC I [285]. There are two lines of evidence that suggest the MHC I expression changed in the DFTD tumour of CD 15 during the immunotherapy. Analysis of serum samples taken during tumour regression contained antibody that bound to Con A culture supernatant-treated DFTD cells but not untreated cells. This suggested that upregulation of immunogenic antigens, potentially MHC I or β_2M, occurred during the immunotherapy of CD 15 and was crucial for the immune response against the tumour. Additionally, immunohistochemistry staining with antibodies to β_2M showed changes in distribution of the protein during MAK cell immunotherapy and following injection with treated cells. Immunohistochemistry of biopsies taken throughout the immunotherapy trial showed a re-distribution of β_2M from patterns consistent with nuclear expression to cytoplasmic expression in later samples. Thus, although MAK cell treatment may not have had an obvious effect on the immune response, it may have subtly altered the tumour cells or their microenvironment to facilitate a response to the immunisation with Con A culture supernatant-treated DFTD cells. Consequently, It is possible that the combination of augmented MHC I expression and exposure to treated DFTD cells was a more effective immunotherapy than the two techniques alone. Since the MHC I protein appeared to be redistributed to the DFTD cell surface and provide an epitope for antibody production, ADCC responses may have occurred against the tumour. As discussed in Chapter 4, NK cells would have been potential effector cells in this response. Although this response could not be directly assessed in CD 15, future immunotherapy experiments may examine ADCC responses against DFTD cells *in vitro*.

Although cytotoxic T lymphocytes were likely to be major effectors against the tumour cells, other cell types may have contributed to the anti-tumour response. The presence of $CD8^+$ cytotoxic T

lymphocytes strongly suggested that there was some level of MHC I expression within the DFTD tumour at this stage of the response. The remaining $CD3^+CD8^-$ T cells were most likely $CD4^+$ cells, potentially T helper lymphocytes which would have contributed to the specific anti-tumour response. At this stage there was no antibody to CD4 to confirm their presence. The $CD3^+CD8^-$ population could have included rarer lymphocyte phenotypes such as NKT cells and γδT cells. Both of these cell types can act as cytotoxic effectors and could have promoted anti-tumour responses in other $CD3^+$ cells [421,422]. As no antibodies were available to analyse these populations, they could not be identified among the $CD3^+$ population in the DFTD tumour. Some NK cells could also have been present in the tumour along with the other leukocytes and acted as effector cells to augment the cytotoxic response against the tumour. However, as there were no antibodies to label Tasmanian devil NK cells, their presence within the tumour could not be confirmed. There was also an abundance of infiltrating MHC II^+ cells within the tumour. Morphologically, many of the MHC II^+ cells had features consistent with macrophages and dendritic cells. Such an increase in T cells and antigen presenting cells would be expected in an effective anti-tumour response [423].

The third treatment given as part of the immunotherapy of CD 15 was intra-tumoural injection with a solution containing Con A culture supernatant. As the supernatant would have contained inflammatory cytokines, such as IFNγ and IL-2, it may have amplified the existing rejection response. The use of multiple treatments and the lack of reagents to examine interactions such as cytokine production, cytotoxic cell activation and apoptosis within the tumour made it difficult to interpret the effect of intra-tumoural injection with Con A culture supernatant. Examining the outcomes of the immunotherapy treatments individually, or in combination, in other devils gave some insight into the mechanisms of activation in the first trial. Another aspect of immunity that could have been tested in this experiment was the development of long-lasting immunity against DFTD cells after tumour regression. The duration of the immune response in the devil with the regressed DFTD tumour could have been assessed by challenge with live cells. This was not done in the original experiment, but could have provided more insight into the extent of the immune response formed against the tumour cells. Additionally, the contribution of ADCC as a pathway to facilitate the anti-tumour responses observed in the treated devil could have been examined following tumour regression, using a similar process to the characterisation of the cytotoxic responses against K562 cells. Future DFTD immunotherapy trials may involve challenge after treatment to test the duration of the anti-tumour response and to determine if long-term immunity develops against the disease. Nonetheless, this was the first successful immunotherapy performed in a Tasmanian devil, and provided valuable lessons to direct the use of similar techniques in the future.

Since the most pronounced response against the DFTD tumour occurred after injection with treated DFTD cells, two more DFTD infected devils were immunised with Con A culture supernatant-treated cells. This made it possible to determine if injection with treated DFTD cells alone would induce an effective response against the DFTD tumour. Both of the devils developed DFTD tumours at the site of injection with treated cells and there was no evidence for immune responses in either devil. Thus, injection of treated cells alone did not appear to have the pronounced effect as that of the first immunotherapy experiment. It is possible that the development of tumours at the injection sites was due to a transient expression of their MHC I. Although flow cytometry confirmed surface MHC I expression, it may have been a short-lived *in vivo.* This may not have provided sufficient time to induce an immune response against the treated cells as well as a failure to eliminate the viable cells. Experiments analysing the duration of MHC I expression induced by IFNγ and Con A culture supernatant suggest that the protein remains on the cell surface for only 72 hours (H. Siddle, personal communication). Therefore, in order to reject the immunised tumour cells, effector cells of the immune response would need to be functional within 72 hours, a period of time which may have been insufficient for full activation. This also infers that there is not sufficient expression of MHC I expressed on untreated DFTD cells for responses to occur. Consequently, there would be no capacity for responses against untreated cells, even when the animals have been 'immunised' against MHC I. Injection with treated DFTD cells alone was therefore unlikely to have been the sole cause of the successful immune response against DFTD in CD 15.

Both devils used in these experiments had multiple extensively developed DFTD tumours when they were first injected with the treated cells. Consequently, the limited immune response against the treated DFTD cells could have been due to a generalised immunosuppression as a result of the relatively late stage of DFTD. To examine the effect of treated cells on the immune responses of animals in better condition, three healthy devils were immunised with treated DFTD cells that had been irradiated to prevent growth of tumours at the injection site. The presence of surface MHC I on these cells was verified using flow cytometry. None of the devils immunised with irradiated treated cells showed evidence for antibody or cytotoxic responses against untreated DFTD cells and all three devils developed tumours upon challenge with live DFTD cells. The decision to challenge the devils referred to in this comment was complex as the evidence from the two different trials reported in this chapter was conflicting. While the cytotoxicity and antibody assays performed did not detect strong immune responses in these animals, CD 15 showed a similar level of *in vitro* response against untreated DFTD cells and yet was induced to completely reject an established DFTD tumour. Consequently, the only way to conclusively establish the effect of the immunisations against the tumours was to perform a challenge with viable DFTD cells. This was undertaken in controlled

conditions, with the maximum amount of care provided for the challenged devils; the injections were made in locations on the back and shoulders where any resultuing tumours could be easily removed and the devils were monitored daily for signs of tumours or poor condition. Although the conditions for live challenge were not ideal, we believe that it was warranted to determine the outcome of a vaccine strategy that arose from such a promising result as that presented in figures 5.1 – 5.7 of this thesis.

This result supported the other evidence that DFTD cells are highly inert to the immune response of a healthy devil. There was no evidence for antibody responses against treated cells in the devils tested. Thus, it would appear that the treated cells were not immunogenic to healthy devils as well as DFTD affected devils. The development of DFTD tumours in all three devils following challenge with live, untreated cells, supported the conclusion that immunisation with killed MHC I positive DFTD cells did not induce protective responses in healthy devils. A possible explanation could be that the irradiated cells may have lost MHC I expression *in vivo* and thus removed the target molecule for a cytotoxicity response. Further studies may investigate the development of a DFTD tumour cell line with stable MHC I expression. This would be useful to confirm the effect of MHC I presence on the immune response against DFTD.

Consideration of the ineffective immune responses against treated cells alone raises the possibility that there must have been other factors in the successful treatment of the tumour of devil CD 15. One possible explanation is that the intra-tumoural injection of MAK cells in the weeks prior to the treated cells may have altered MHC I expression in the tumour. The MAK cells used for immunotherapy were treated in the same way as those that produce the Con A culture supernatant used as a source of IFNγ to upregulate MHC I. Consequently, the MAK cells could also have produced a source of cytokines in the tumour site. Another difference between the first experiment and subsequent immunotherapies was that CD 15 had been immunised several times with DFTD cell protein before the tumour developed. It is possible that these immunisations increased the potential for an immune response against tumour proteins in the form of the treated DFTD cells. However, CD 15 did not form any evidence for immune responses against intact DFTD cells in any cytotoxicity or antibody assay performed during the immunisations or challenge. There was also no evidence for an antibody response against treated DFTD cells in samples taken before the anti-tumour response was formed in the immunotherapy experiment. This suggested that there was no recognition of DFTD surface proteins prior to the immunotherapy, and implies that the immunisations given prior to challenge and tumour development did not play a significant role in the immunotherapy response against the established tumour.

Another possible explanation for the success of the first immunotherapy experiment was that all three components of the treatment were necessary for effective anti-tumour responses. The requirement for more than one factor in immunotherapy has been demonstrated in at least one other study. Overwijk and colleagues [424] used a combination of activated T lymphocytes, tumour antigen immunisations and administration of a combination of cytokines chosen to promote T cell division and activation to treat B16 melanomas in mice. Only the combination of all three therapies, not each individually or paired treatments, induced an appropriate response. In addition to tumour regression, most mice treated successfully with immunotherapy suffered vitiligo. This autoimmune condition has been correlated to tumour protection and a successful anti-tumour response, particularly in immunisations against melanoma [425,426]. There are many common factors in the protocol used in the mouse immunotherapy trial and the initial DFTD immunotherapy experiment. Both trials began with adoptive transfer of activated immune cells. In both cases, the second step was vaccination with tumour antigens. The final step in both trials was injection with a mixture of cytokines which contained both proliferative and activating factors. The therapy used in the mouse trial required a higher number of cells for adoptive transfer and the treatment regimen was more intensive than is realistic for a large animal like a captive Tasmanian devil. Future trials for DFTD immunotherapy may therefore seek to replicate the first experiment to determine if the combination of therapies, rather than individual treatments was crucial in the development of an anti-tumour immune response.

In summary, immunotherapy using a combination of mitogen activated killer cells, treated DFTD cells and intra-tumoral cytokine injection has provided evidence that activation of a Tasmanian devil's immune response can promote the rejection of a DFTD tumour. Activation of Tasmanian devil mononuclear cells with Concanavalin A produced cells that appeared to be capable of altering the β_2M expression of DFTD cells. This change in β_2M expression may have contributed to the development of strong immune responses formed against a DFTD tumour in one Tasmanian devil. Injection with treated DFTD cells could have provided a crucial stimulus to escalate an immune response against the tumour. When these treatments were used individually, there was little or no evidence for an immune response against DFTD tumours. The potential immunotherapy strategy outlined in this chapter could be refined to provide an intervention for the protection of wild Tasmanian devils against DFTD.

Chapter 6 - Final Discussion

Devil Facial Tumour Disease (DFTD) poses an unacceptable risk of extinction to the Tasmanian devil. This species occupies an important environmental niche as the top predator in the Tasmanian ecosystem. Tasmanian devils are also credited with a crucial role in controlling feral pests [427]. Consequently, its preservation is important for the protection of other endemic species. Current strategies for its conservation include establishing captive populations, which are isolated from diseased wild animals. However, the benefit of these captive populations is limited to the small areas of land they cover, and will not ensure the survival of the Tasmanian devil in its wild habitat. Immunological intervention such as a vaccine or immunotherapy could provide an option for protection of the Tasmanian devil in the wild. The studies presented in this thesis investigated the potential for an immunological intervention against DFTD. This task would not be possible without additional knowledge of the immune response of the host. As Tasmanian devils are marsupials, differences from the classical immune responses of eutherian mammals were anticipated and a variety of immune pathways were examined. The aims of this thesis were to characterise the devil's anti-tumour immune response, to identify pathways capable of killing DFTD cells and to determine the effectiveness of several immunotherapeutic strategies against DFTD.

At the commencement of this study, the mechanism of DFTD transmission was unknown. A potential mechanism was a generalised immununodeficiency within the species. One histological study provided evidence for poor lymphocyte infiltration into DFTD tumours [269] suggesting a lack of immune response to DFTD. However, other studies had shown that both healthy and DFTD-diseased Tasmanian devils had phenotypically normal immune cells that can form a range of normal immune responses [1,2,264], although none of these studies evaluated specific anti-tumour responses. An alternative mechanism for transmission was a limited diversity within the MHC genes of the Tasmanian devil population [270]. This may have decreased the capacity to respond against a transmitted tumour. This evidence included low response levels in mixed lymphocyte reactions (MLR) and the lack of diversity verified by genotyping [270]. Previous studies of immunity, which used MLR in another marsupial, the gray short tailed South American opossum *Monodelphis domestica*, also showed low response levels. Rather than interpreting this result as a lack of genetic diversity, the authors hypothesised that the low response could be due to T cells with a different ontogeny from those of eutherian mammals [428]. Further functional evidence for the lack of genetic diversity in Tasmanian devils was sought using skin grafting experiments [3]. The results of this trial showed that healthy Tasmanian devils were capable of mounting successful allogeneic

responses against foreign tissue. Consequently, it is unlikely that the low genetic diversity within the species is potentially responsible for the transmission of DFTD tumour cells between devils.

Another possible explanation for the survival of DFTD cells following transfer could be a disruption of normal apoptosis pathways. The cytotoxic cells of the immune system initiate cell death in their targets by inducing apoptosis. If normal apoptosis pathways were disrupted in DFTD cells, the tumour cells could divide unimpeded by cytotoxic signals from the host immune response. Apoptosis pathways can be experimentally activated by cytotoxic drugs. Results from work performed in our laboratory suggested that DFTD cells could undergo normal cell death responses when treated with a variety of chemotherapeutic agents. These included vincristine, which affects microtubules in rapidly growing cells, the DNA-intercallating agent doxorubicin, the antimetabolite methotrexate and the alkylating agent carmustine [375]. As these four drugs induce apoptosis through different mechanisms, it is unlikely that a failure of apoptosis accounts for the survival of transmitted DFTD cells.

The results reported in this thesis showed that DFTD cancer cells fail to induce a response from the immune system of the host devil. In order to determine if there was a consistent lack of specific immune responses against DFTD, the cytotoxicity and antibody responses of healthy and DFTD-diseased affected wild devils were tested. The data presented in Chapter 3 provided functional evidence for a lack of anti-DFTD-tumour responses in healthy devils. This was also evident in animals with established DFTD. A potential way to induce immune responses against DFTD was through vaccination with killed DFTD cells. Several trials were performed using a variety of killed DFTD cell preparations and adjuvants, all of which were chosen to stimulate cytotoxicity responses. Irradiated cells were combined with the non-specific immunomodulator Montanide, with or without supplementation with CpG oligonucleotides to increase the potential for downstream activation of anti-tumour immune responses [52,54,143,145]. Other trials utilised ISCOMATRIX®, an adjuvant designed to promote cross presentation of tumour cell antigens to cytotoxic T lymphocytes (CTL). However, when antibody and cytotoxic anti-tumour responses were assessed, only a minority of vaccinated devils showed evidence of a response. The lack of immune response in most devils following vaccinations suggests that the DFTD cells can go undetected after exposure of a healthy animal. Thus, there must be a characteristic that allows the cells to grow without activating the host's immune response.

One mechanism that would contribute to the lack of immune response induced against DFTD cells, even during extended periods of infection or after repeated vaccination, is that the tumour cells downregulate their MHC I molecules [285]. Although this is a common immune evasion mechanism

in many cancers, it had initially been discounted in DFTD as there was evidence for the expression of MHC genes in the tumour cells [270]. Recent evidence suggests that, although the gene is transcribed, the protein is not expressed on the cell surface [285,297]. Consequently, there would be no capacity for CTL responses from the host devil against the tumour cells. In the absence of a functional CTL response, the development of a vaccine would be challenging. The ideal response to protect against DFTD would be cellular cytotoxicity. In the trials documented in this thesis, all adjuvants chosen were designed to induce cytotoxicity responses. If a CTL response was induced there would be no capacity to target DFTD cells as MHC I was missing. Consequently, non-MHC I restricted cytotoxicity may need to be targeted.

One intriguing result was that, where responses were induced after immunisation, they were strongest at seven days after the first dose. In most cases, these responses were not evident at 14 days and after further doses. This situation occurred in several animals and in different trials. An interpretation of this result is that natural killer (NK) cells were initially activated and mediated the cytotoxicity. NK cells have been shown to be rapidly activated in response to immunisations, after which they drive dendritic cell maturation and interaction with T cells, resulting in cytotoxicity, cytokine production and an increased adaptive response [429,430,431]. The numbers of activated NK cells generated following immunisation with protein antigens, such as hepatitus B virus envelope proteins, can influence the strength of specific immune responses generated, particularly those of IFNγ-producing T lymphocytes [432]. In other mammals, such as mice, NK cells are capable of augmenting the activation and response of $CD4^+$ cells, which increases CTL-mediated immune responses [433]. Additionally, NK cell killing of infected cells in early disease can also provide a crucial source of antigens to $CD8^+$ T lymphocytes to generate a specific immune response [434]. Thus, NK cells can drive responses that are crucial in the development of cytotoxicity after immunisation. In the case of DFTD, although the NK cells could have been activated to increase the maturation of dendritic cells, augment the development of $CD4^+$ and $CD8^+$ T lymphocyte responses and provide a source of tumour antigen, any subsequent cytotoxicity response would not be able to target DFTD cells due to the lack of MHC I protein on the cell surface. Thus, one possible explanation for the presence of cytotoxic responses after the first immunisation only was that NK cell-mediated cytotoxicity occurred initially. As the normal switch to a $CD8^+$ T cell-mediated response occurred, the inability to target DFTD cells because of the absence of MHC I resulted in a lack of cytotoxicity after the following doses.

Since MHC I protein is absent on DFTD cancer cells, NK cells should theoretically play an important role in an immune response against the disease. However, in DFTD affected devils, there is no

evidence for NK cell killing. Prior to the commencement of this project, no studies had investigated NK cells in Tasmanian devils, although the presence of other types of immune cells had been determined [2,264]. Thus, an aim of this study was to identify NK cells in Tasmanian devils and determine if they were able to function normally against tumour cells. Despite the absence of a specific marker for Tasmanian devil NK cells, histological and immunohistochemical experiments provided the first evidence for NK-like cells in the peripheral blood. Further support for NK cells was the genetic identification of several characteristic NK cell receptors. A study by van der Kraan and colleagues [435] showed the presence of the activating receptors KLRK1/NKG-2 and another CLEC4E. No analogues for common inhibitory receptors were identified. The gene for CD69, a protein which is expressed on activated T lymphocytes and NK cells [436] was also identified in Tasmanian devils [435]. The identification of these receptors, combined with the histological evidence presented in this thesis, strongly suggest the presence of NK-like immune cells. Analysis of protein expression and function of these receptors will require future experiments. The data presented in Chapters 3 and 4 provide evidence for the functional presence of NK-like cells in Tasmanian devils. Immunisation with MHC I negative human K562 tumour cells resulted in the induction of cytotoxic responses, which were attributed to NK or NK-like cells. The NK-like cytotoxicity responses against K562 cells were dissimilar to classical NK cell responses, as there was no spontaneous killing and the reactions required up to 18 hours. Similar NK cell responses have been observed against xenogeneic cells [396]. This non-classical NK cell killing is thought to occur through alternate cytotoxicity pathways such as antibody-dependent cell mediated cytotoxicity (ADCC). The parallels observed between the results presented in this thesis and those of similar killing in other studies prompted experiments to explore the involvement of ADCC in the anti-K562 cytotoxicity responses of Tasmanian devils.

Experiments presented in Chapter 4 of this thesis identified two mechanisms through which the peripheral blood lymphocytes of Tasmanian devils could be activated to form NK-like activity: ADCC and non-specific activation. The ADCC pathway could successfully induce *in vitro* cytotoxic responses against MHC I negative K562 tumour cells following immunisation. Additionally, supplementation with serum containing K562-specific antibody could induce ADCC responses in the lymphocytes of naive Tasmanian devils. As wild Tasmanian devils do not produce antibodies against surface molecules of DFTD cells there is no opportunity for infected animals to develop ADCC responses. Immunisation of Tasmanian devils with killed DFTD cells also induced low levels of antibody, which could not induce effective ADCC responses. However, given the evidence for ADCC killing of other MHC I negative tumour cells the possibility remains that similar responses could occur against DFTD tumour cells in the presence of sufficiently strong levels of surface antibody. Studies in other models, such as species of ducks, have shown that passive transfer of antibody can induce rejection

of foreign cells and tolerated grafts through ADCC killing [159,419]. ADCC responses could thus provide a potential mechanism to induce rejection of established DFTD tumours in Tasmanian devils.

The formation of an ADCC response was one potential mechanism to explain the immune response formed against a DFTD tumour during the successful immunotherapy of one devil. Following injection with Con A culture supernatant-treated DFTD cells, the immunised devil formed antibody responses which could target the treated but not untreated cells *in vitro*. Among the molecules that could be induced on the cell surface by Con A culture supernatant treatment were β_2M and MHC I. Immunohistochemistry analysis of β_2M expression in the biopsies during the immune response following immunotherapy showed changes consistent with a potential upregulation of β_2M protein, and therefore presumably MHC I protein, on the surface of DFTD cells within the tumours. Alternatively, other molecules that are associated with β_2M could also have been upregulated in response to treatment with Con A culture supernatant. There is evidence that treatment with cytokines in Con A culture supernatant, particularly IFNγ, can upregulate the activity of the TAP pathway [285], which would potentially result in the expression of non-classical MHC I proteins. An example of a TAP associated protein which associates with β_2M is the Qa-2 molecule[437]. Consequently, if both changes in DFTD cell surface protein expression and development of a surface-reactive antibody had occurred, ADCC may have been one cytotoxicity pathway activated in the immune response following immunotherapy. The activation of the host NK cells through ADCC may also have led to production of cytokines and an increase in free tumour antigen through apoptosis [434]. Thus, an ADCC response may also have provided a stimulus to increase the infiltration and activity of T lymphocytes, particularly cytotoxic cells, within the tumour. In addition to its implications for responses in infected devils, ADCC may provide an important pathway to induce protective immune responses against DFTD in naive devils. If immunisations against DFTD cell surface antigens could induce antibody production in naive Tasmanian devils, and if this could lead to ADCC responses against the tumours, some Tasmanian devils could develop long-lasting immunity against tumours. The results presented in this thesis suggest that ADCC may be an appropriate target for future immunisation strategies to induce responses against DFTD. Specific protein antigens or DNA are often efficient inducers of antibody responses and may thus present good options for inclusion in DFTD immunisations.

The ADCC cytotoxicity data presented in this thesis are also the first experiments to provide physical evidence for the presence of functional NK cells in any marsupial species. Many studies have identified genes associated with NK cell activity in other marsupials [438,439,440,441], and the assessed various cellular responses such as proliferation and antibody formation [442,443].

However, no former studies have provided histological evidence for the presence of NK cells or functional cytotoxicity responses in marsupials. Consequently, if future studies sought to analyse whether NK cells were present in other marsupial species, they may use similar techniques and strategies to those described in this thesis. The analyses performed in the Tasmanian devil may therefore inform future strategies for the characterisation of NK cell activity in other marsupial species.

In addition to activation via the ADCC pathway, cytotoxicity responses can be induced in peripheral blood mononuclear cells of Tasmanian devils using non-specific stimulation with cytokines, mitogens and the Toll-like receptor (TLR) agonist Poly I:C. Data presented in Chapter 4 provided evidence that peripheral blood mononuclear cells activated with non-specific stimuli could kill DFTD cells. Experiments performed in our laboratory, in conjunction with collaborators from Cambridge University, showed that the cytokine rich supernatants from Con A stimulated mononuclear cells induced the upregulation of surface MHC I molecules in DFTD cells [285]. The identification of methods that could induce immune responses against DFTD and alter the antigen expression of the tumour cells, provide two potential directions for immunotherapy: adoptive cell transfer or therapeutic vaccination. After demonstrating the success of the activation techniques *in vitro*, an immunotherapy experiment was undertaken in a Tasmanian devil with DFTD. This trial incorporated adoptive transfer of activated peripheral blood lymphocytes, injection of MHC I positive DFTD cells and injection of the cytokine rich supernatant from mitogen activated lymphocyte cultures. The results of the immunotherapy experiment were encouraging, as the tumour was completely eliminated. However, the contribution of each of the three immunotherapy techniques in the trial was difficult to interpret, as discussed in Chapter 5. Thus, the mechanisms responsible for inducing the competent anti-tumour response in the initial experiment have not been defined. Additionally, there were several limitations to the extrapolation of the results of the treatments from this trial to other cases of DFTD. Firstly, the treatment was performed on only one DFTD infected Tasmanian devil that had been injected with DFTD cells in captivity as an immunisation challenge rather than being a naturally acquired tumour. Therefore the tumour was of a known strain and age. Secondly, the tumour was very small at the commencement of therapy, compared to the majority of DFTD tumours identified in the field. Thirdly, the tumour in this animal was a single mass. Adaptation of this technique to wild devils may therefore be more difficult, as their tumours may be multicentric, metastatic, advanced or a different strain to the cells used in immunotherapy.

Chemotherapy has been explored as potential methods to treat DFTD [375], with all results suggesting DFTD cells were found to be highly resistant to chemotherapy *in vivo*. Thus,

chemotherapy may not provide a reliable option for treatment of DFTD. Additionally, there is *in vitro* evidence that DFTD cells are radioresistant [375]. The results presented in this thesis suggest that the use of immunotherapy may be a more promising direction. The success of a treatment in one animal provides a 'proof of concept' for the use of immunotherapy against DFTD. With refinement of the protocols used, it is possible that other devils may also be induced to form an immune response against DFTD in future. A particular advantage of immunotherapy, compared to chemotherapy, is the specificity of the response and the possibility that a successful treatment may also induce long-lasting immunity. The development of such immunity occurs against CTVT, the other transmissible tumour which utilises similar immune evasion mechanisms to DFTD. Following regression of the MHC I negative tumour after activation of the immune response, the host dog develops lifelong immunity against the disease, even in its MHC I negative, infectious form [247]. Some immunotherapeutic approaches have also been trialled against CTVT. One strategy which has been used is therapeutic immunisation against this tumour using hybrids formed between dendritic cells and tumour cells [257]. The principle behind this technique is that the hybrids have the capacity to express tumour cell associated antigens on both MHC I and MHC II, allowing them to directly stimulate anti-tumour responses from both helper and cytotoxic T lymphocytes. This technique cannot currently be used to produce DFTD immunisations, as there is no effective method for culture of Tasmanian devil dendritic cells. However, future studies may seek to culture dendritic cell lines from Tasmanian devils for use in hybrid vaccines similar to those that can successfully induce responses against CTVT. Methods used to culture of human dendritic cells may provide direction in such studies, and direct the selection and development of reagents for Tasmanian devil dendritic cell culture.

Based on the successful immunotherapy of the DFTD diseased Tasmanian devil, therapeutic immunisation of Tasmanian devils with established DFTD tumours is worthy of further investigation. As discussed in Chapter 5, treatments that provide a source of IFNγ offer a potential strategy as this could upregulate MHC I expression within the tumours. One technique to achieve this objective would involve transfection of IFNγ genes into DFTD cells. These transfected cells could be inoculated directly into DFTD tumours to augment the immune response via MHC upregulation. This strategy has previously been used in other intra-tumoural therapeutic immunisations [444,445,446]. These methods involve transfecting tumour cells with the genes for production of lymphocyte-activating cytokines, such as IL-12, IFNγ or GM-CSF. An advantage of this technique is that production of IFNγ by transfected cells could overcome the notoriously short half life of the cytokine *in vivo*. The limited activity of IFNγ usually necessitates frequent injection for successful immunotherapy [447]. Injection with modified cells would provide a longer-lasting source of IFNγ to stimulate a continued immune

response. DFTD cells capable of secreting IFNγ would also express MHC I molecules. This is the objective of other studies assessing the effects of MHC I positive cells [445]. The expression of allogeneic MHC I would be an advantageous immunogen in therapeutic immunisations for DFTD. One possible issue with injection of live IFNγ producing DFTD cells would be their potential to revert to an infectious phenotype. This may allow them to establish new tumours or add to the bulk of existing tumours, or could provoke the development of additional immune escape mechanisms. However, previous studies have shown that transfection of IFNγ into tumour cells can activate potent anti-tumour responses and abrogate tumourigenicity [448].

Another method for therapeutic vaccination involves the isolation of tumour cell membranes. The cells used are often transfected with gene constructs that code for highly immunogenic molecules such as co-stimulatory molecules [449]. Alternatively, immunogenic proteins could be attached to the cell surface using protein transfer onto the cells surface using specific glycolipids as anchor points [450]. The membranes are then isolated to create a safe and immunogenic vaccine [451]. The membrane liposomes created using this technique can induce T lymphocyte proliferation, secretion of IFNγ and anti-tumour immune responses in mice [450]. One advantage of this method is that the immunisations would contain higher concentrations of membrane proteins, rather than intracellular proteins which are not easily accessible in a tumour mass. Use of cell membrane extracts is a similar strategy to the recently licensed vaccine against human papilloma virus (HPV), where the immunogenic agents are the capsid proteins assembled into a virus-like structure, without viral DNA [452]. Analogous to the HPV capsid structures, the membrane liposomes from tumour cells would provide safer preparations than those containing whole DFTD cells with intact membranes, such as irradiated cells. This would mean the immunisations are more likely to induce responses that can target whole cells. Consequently, DFTD cell membrane-based immunisations may also provide a potential technique for prophylactic vaccines in naive devils if they could induce responses against wild-type DFTD cells.

Choice of adjuvant is also important for the success of a cancer vaccine. Since immunisations against DFTD aimed to induce a cellular cytotoxic response, the adjuvants used reflected this strategy. TLR agonists, such as CpG DNA and Poly I:C, are increasingly being used as adjuvants [52,56,143,309] and are often combined with other immunomodulators, such as Montanide [292,310,311]. This combination of adjuvants can be used with specific antigens in cancer vaccines to induce antibody development and long-lasting Th1 and $CD8^+$ T lymphocyte responses [292,311]. Another attractive quality of CpG adjuvant supplementation is its capacity to activate many components of the immune response, including B lymphocytes, NK cells and DC [53,55,56,57,139]. This effect has been

demonstrated in cancer vaccines in animals [296]. Agonists of more specific cell subsets, such as Poly I:C and Flt3 Ligand which induce responses from NK cells and DC *in vitro* and *in vivo* could also be used [47,93,312,313,314,315,316]. Other adjuvants that target specific pathways, such as immune stimulating complexes (ISCOMs) such as ISCOMATRIX® which stimulates cross presentation of protein antigens [317], was most likely to be useful when incorporated into immunisations containing DFTD cell protein. The use of these adjuvants to induce immune responses against DFTD cells has not yet been successful. However, the DFTD cell preparations used to date have been crude, and the capacity to purify the antigens included has been limited. Many of these adjuvants may still provide a good basis for the development of DFTD immunisations, when used with more refined cellular preparations or specific antigens.

The development of an effective immunotherapy or vaccine against DFTD would be extremely valuable in the conservation effort to protect Tasmanian devils in the wild. Certain animals within wild populations would present key targets for immunotherapy. Since increased incidence of biting and, therefore, disease spread occurs during the mating season [262], female devils infected during this time may be unable to wean pouch young under normal circumstances. However, with an immunotherapy their survival may be prolonged, resulting in successful weaning. Recent results of epidemiology and behavioural studies suggest that submissive male devils are most likely to spread DFTD, and aggressive males contract tumours within the oral cavity [453]. Thus, submissive male devils may be appropriate targets for immunotherapy, if such individuals could be identified through observation with remote cameras, microchipping and tracking. The availability of an immunotherapy would also provide additional security for captive insurance populations of Tasmanian devils, and serve as an alternative to euthanasia if an outbreak of infection or breach of quarantine conditions occurs. Consequently, future research in this area will be a crucial undertaking in the effort to save the Tasmanian devil from extinction in the wild. The results of this thesis, which have outlined potential strategies for inducing immune responses against established DFTD tumours in Tasmanian devils, are significant as a basis for future research in this field.

Despite the potential for the use of adoptive cell transfer or therapeutic immunisation immunotherapy for established DFTD, the challenge remains to produce a vaccine capable of protecting naive devils from the disease. In the absence of MHC I expression, NK cells may provide a good target for induction of immune responses against DFTD, and may form early responses against DFTD cells in the first seven days following vaccination with DFTD cells. Consequently, the characterisation of this cell type and mechanisms for its activation in this thesis will provide crucial information for future vaccine development. As previously discussed, one result with particular

significance for vaccine development is the capacity for NK cells to mediate ADCC responses. The simultaneous formation of antibody against MHC I positive DFTD cells and rejection of a DFTD tumour, in which there was some evidence of increased surface MHC I protein expression, in an immunotherapy-treated devil implies that ADCC may be a potential mechanism for the regression of the tumour. Thus, the ADCC pathway could be one strategy to manipulate immune responses against DFTD tumours *in vivo*. Development of a vaccine against DFTD may therefore target antibody formation in order to induce ADCC responses against MHC I negative DFTD cells. This could potentially require development of an antibody against 'self' antigens, a process which could be very difficult. However, if a sufficiently immunogenic preparation was used to induce antibody production this could lead to ADCC against DFTD cells. Antibody responses can often be induced using vaccination against tumour specific antigens and, consequently, the identification of candidate antigens associated with DFTD cells is an important area of vaccine research. In 2012, C. Tovar produced preliminary evidence for the identification of specific DFTD antigens from the serum of some devils that are capable of forming antibody responses against DFTD cells. In this study, candidate antigens were identified using immunoproteomic techniques to separate proteins that were bound by antibodies formed in the serum for structural characterisation and identification [297]. Among the antigens identified in this study was vimentin, a protein that is abundant in other tumour types and a candidate for tumour vaccines [294]. Another potential molecule for use as a target in DFTD immunisation is periaxin, which is expressed in high amounts in DFTD cells [274]. A limitation for the use of both periaxin and vimentin in vaccines against DFTD is that both proteins are expressed intracellularly. An ideal antigen for immunisations against DFTD would be expressed on the cell surface. A possible issue with the use of periaxin as a vaccine target would be the potential for activation of autoimmunity, as periaxin is also expressed on Tasmanian devil Schwann cells, as would many cell surface antigens on DFTD tumour cells. However, the development of an autoimmune response is unlikely to occur rapidly following immunisation. As Tasmanian devils have a short lifespan, and often naturally develop neurological problems late in life (S. Peck, personal communication), the risk of autoimmunity may be outweighed by the benefit of using an immunisation to prevent infection with DFTD. To date, none of the identified antigens have been assessed for their ability to induce an immune response.

The identification of other specific antigens associated with DFTD will be an important area for further research. Methods to specifically isolate membrane associated proteins from DFTD tumour cells are also worthy of exploration. One such process could be the conjugation of DFTD cell surface proteins to a carrier protein such as ovalbumin or to haptens to increase their immunogenicity and the potential for antibody formation. This could be achieved on DFTD cells by biotinylating cell

surface molecules, using streptavidin binding to attach the selected carrier or hapten, then isolation using a specific fractionating column. In addition to increasing the immunogenicity of immunisation preparations, this technique would both provide a basis for the isolation of DFTD surface proteins. The isolated fractions could then be used to immunised Tasmanian devils, with ovalbumin acting as a carrier protein to induce antibody responses and increase the immunogenicity of the surface proteins for induction of antibodies.

The objective of this thesis was to increase knowledge of the interaction between DFTD and the immune response of a host devil. This study verified that wild Tasmanian devils do not produce immune responses against DFTD in wild Tasmanian devils under normal conditions. Additionally, healthy devils immunised with DFTD cells do not consistently form cytotoxic responses against the tumour cells. This is most likely a result of the lack of surface MHC I expression on DFTD cells, which would restrict the capacity for the cytotoxic T lymphocytes of healthy devils to form responses against the tumour cells. However, without specific molecular tools the absence of $CD8^+$ cytotoxic cell activity against DFTD cells cannot be thoroughly examined. Despite the absence of a response under normal conditions, cytotoxicity responses can be activated against DFTD cells using non-specific stimulation using the mitogen Concanavalin A, cytokines such as IL-2 and the TLR agonist Poly I:C. Thus, this study provided evidence that the immune systems of Tasmanian devils can kill DFTD cells. Tasmanian devils also possess functional NK cell responses, which are capable of targeting MHC I negative foreign tumour cells through the ADCC pathway. This response may be an attractive approach for future vaccination strategies. A crucial finding of this project was that, with sufficient stimulation, the immune system of a Tasmanian devil can form an immune response capable of rejecting an established DFTD tumour. The findings presented in this thesis will direct future immunotherapy strategies for use against DFTD. The knowledge base established through the experiments reported in this thesis will provide a basis for future efforts to develop immunological interventions against DFTD.

Conclusions and future directions

The work presented in this thesis has increased the understanding of the Tasmanian devil immune response and its interaction with DFTD. At the commencement of this study, several immune responses in Tasmanian devils had been examined, including phagocytosis, antibody development and lymphocyte proliferation, both in response to mitogens and in mixed-lymphocyte reactions. However, there had been no examination of anti-tumour responses in Tasmanian devils, and no investigations had used functional approaches to determine the reason for the observed absence of immune response against DFTD in wild Tasmanian devils. In addition to analysing the basic anti-tumour immune responses in Tasmanian devils, this study also aimed to activate immune responses against DFTD cells through the use of vaccines and immunotherapies.

The first important finding from this thesis was the confirmation that Tasmanian devils show no evidence for the formation of two major immune responses, cytotoxicity and antibody, against DFTD cells, even during long-term infection with the disease. This finding supported the previous evidence from pathological studies which did not provide a functional perspective. In addition to the lack of response in affected wild devils, immunisation with killed DFTD cells fails to induce a protective immune response in the majority of animals. The *in vitro* demonstration of the consistent lack of immune responses against DFTD in Tasmanian devils also complements the recent finding of the absence of surface MHC I protein expression on the tumour cells, as this situation would explain the absence of a response.

The experiments in this study also demonstrated the presence of functional anti-tumour responses in Tasmanian devils. Immunisation with human K562 tumour cells had the capacity to induce both cytotoxicity responses and antibody formation. Additionally, this study provided the first evidence for the presence and function of NK cells in Tasmanian devils. The finding that these responses are intact in Tasmanian devils has positive implications for the prospect of developing a vaccine against DFTD. The presence of these responses provides a basis to target in future vaccine trials.

Another result of significance in this thesis was the discovery of two mechanisms that can induce cytotoxic responses against MHC I negative tumour cells: ADCC and non-specific lymphocyte activation. The capacity for ADCC responses was examined against K562 cells. Although preliminary experiments have not successfully induced ADCC responses against DFTD cells, this pathway may provide a target to induce cytotoxicity against DFTD cells. Some results from this thesis also indicate that if antibody responses can be activated against DFTD in infected devils, ADCC may occur against DFTD tumours under certain conditions. Non-specific stimulation of mononuclear cells from

Tasmanian devils with the mitogen Concanavalin A, inflammatory cytokines such as IL-2 and TLR agonists such as Poly I:C can induce the killing of DFTD cells *in vitro*. The mitogen stimulation of DFTD cells provided the basis for an immunotherapy.

This thesis also reported the first successful induction of an anti-tumour immune response in a DFTD-affected Tasmanian devil. The use of three immunotherapeutic techniques resulted in the total regression of a DFTD tumour. In this trial it was difficult to attribute each treatment an individual effect, and it was possible that each of the three treatments played an important role in the regression response. This thesis has discussed the potential importance of ADCC responses, and evidence for changes in surface expression of MHC I within the DFTD tumour during the immunotherapy. However, regardless of the mechanism, the activation of an effective *in vivo* immune response against DFTD in one devil was a highly significant result, which urgently warrants further immunotherapy trials in Tasmanian devils.

The results of this thesis have implications for future immunotherapy and immunisation trials against DFTD. Immunotherapy experiments may seek to repeat the same protocol, with the same sequence and timing, as the first immunotherapy trial. It is probable that the presence of interferon-γ (IFNγ) in the tumour microenvironment was an important factor in the regression of the DFTD tumour in the first trial, both for its capacity to induce anti-tumour cytotoxic responses and in the upregulation of MHC I within the tumours. As such, this cytokine would make a good target for immunotherapy. Other therapeutic vaccine methods used in cancer immunotherapy studies, particularly the use of dendritic cell hybrids to induce immune responses against CTVT, may also provide good options for immunotherapy trials. This technique is not currently applicable to DFTD, as Tasmanian devil dendritic cells have not previously been cultured. Future experiments may seek to adapt techniques used for the culture of human dendritic cells, and make use of stimulating agents such as ionomycin and phytohaemagglutanin. With the adaptation and refinement of immunotherapy protocols, it is possible that other devils may be induced to form immune responses capable of rejecting established DFTD tumours. Future development of an immunological intervention could be crucial in the effort to save the Tasmanian devil from extinction in the wild.

Despite the potential for success in immunotherapy, the challenge remains to induce a protective immune response against DFTD in naive Tasmanian devils. The lack of surface MHC I protein poses an impediment to the induction of immune responses in naive Tasmanian devils, as no cytotoxic T lymphocyte response could be formed in the absence of this molecule. This thesis suggests that ADCC may be a potential pathway to target for the induction of responses against DFTD in naive Tasmanian devils. If a sufficiently strong antibody could be induced against wild-type DFTD cells

cytotoxicity could be formed against the tumour cells, in a similar way to the responses against K562 cells. Consequently, future immunisation strategies could aim to induce antibody formation rather than targeting cytotoxicity responses.

The identification of competent anti-tumour responses and methods to activate the killing of DFTD cells in this project were major advances in understanding the immune system of the Tasmanian devil and its role in this disease. This thesis has provided evidence that the Tasmanian devil's immune response can eliminate DFTD and has developed and discussed strategies to overcome the tumour's immune evasion mechanisms. And with over 80 percent decline in the species since the emergence of DFTD in 1996, the promising results and strategies developed in this thesis could not have been come at a more important time. However, our knowledge of the cancer and its interaction with the host is still limited by the inability to study the specific components of the immune response. A deeper knowledge of the processes involved in the anti-tumour responses of Tasmanian devils will be crucial to develop a successful intervention against DFTD.

Acknowledgements and Author's Note

I have many people to thank for their contribution to the researching and writing of this thesis. First and foremost I wish to thank my primary supervisor, Professor Greg Woods. From you, Greg, I have learned so much: how to address research problems, how to give a reasonable talk at a conference, how to write scientifically enough to submit a thesis (I know it hasn't been easy to teach me!) I can never fully acknowledge all the advice, guidance, training and support you have given me throughout the last four years and how much it has meant to have such a reliable mentor in a task like this. No, even I don't have enough descriptive words to do that. All I can say is: thank you.

Thank you to Dr Bruce Lyons and Dr Silvana Bettiol, my two associate supervisors. Bruce: at the very first meeting we had with you in our research group, you made a suggestion which completely changed the direction of this entire study. Your experience, skills and knowledge have been invaluable to me, and you have always been ready to help in any situation. Silvana: although you have been away overseas for large parts of this study, I could always rely on you for guidance. Thank you for your support.

This project would not have been possible without the help of Dr Alexandre Kreiss. Alex supplied the majority of samples for the experiments in this thesis, performed most of the immunisations in our trials and taught me many basic techniques in cell culture and blood processing. In addition to the provision of samples, I must thank Alex for all the discussions of the science in this thesis, and I greatly admire his dedication to the devils he tends. Many thanks, Alex (and now you may use my pink marker pen in the lab whenever you want).

There are also many other members of our research group, past and present who have guided me throughout this project. Dr Heather McGee and Dr Cesar Tovar, Jess Collins and Terry Pinfold have been instrumental in assisting me with laboratory techniques and provided important sources of knowledge and perspective for this project. Ros Malley, Lauren Howson, Takumi Kobayashi, Anne Cooray and the other students who, along with our supervisors, formed the 'Devils Advocates' have been supportive, entertaining and helpful throughout my project – I thank you all.

Several members of staff from the University of Tasmania have made important contributions to the work in this study. I thank Dr Barrie Wells for his dedication to our devils, for assisting Alex with the field work collecting the blood samples required, for his help explaining aspects of animal ethics and for teaching me "devil fieldwork 101". Another source of Tasmanian devil tissue samples from the field was provided by Rodrigo Hamede and Menna Jones, from the University Of Tasmania School Of Zoology. Menna, Rodrigo and their team have been invaluable collaborators throughout this project.

This project has also received support from several members of staff at the Tasmanian Department of Primary Industries, Parks, Wildlife and the Environment. The “Devil Vets”, in particular, Dr Sarah Peck, Dr Colette Harmsen and Dr Judy Clarke have all generously contributed to collecting samples for this project, and many dedicated devil keepers have been responsible for housing our animals over the last four years. The DFTD cell lines used in my experiments were provided by Dr Anne-Maree Pearse and Kate Swift from the Department. Anne-Maree in particular has also been a source of discussion and perspective throughout this project.

I wish to thank the staff of the Endocrinology Unit in the Royal Hobart Hospital pathology department, particularly Dr Venkat Parameswaran for allowing and assisting me to use their gamma radiation counter for a large portion of this study. I would like to thank the staff of the WP Holman Clinic, and in particular Dr Michael Young, Hobart for their assistance in irradiating our DFTD vaccines. I also wish to thank Dr Scott Ragg, Royal Hobart Hospital Pathology, for the provision of reagent samples and advice on verifying the characteristics of cell types for this thesis.

Many staff members from the University Of Tasmania School Of Medicine have assisted with this project, providing instruction and expertise on techniques I have used. Thank you to Mark Cozens, who assisted me in learning how to use a flow cytometer, how to analyse the results from this technique and how to use the software programs designed for analysis of flow cytometry data. Thank you to Narelle Phillips for preparing the histology samples used in this project; Tasmanian devil tissues can be tricky to work with but your expertise in this area is unrivalled and we were so lucky to have your help with our research. I also thank Steve Weston for his advice and help with troubleshooting our immunohistochemistry techniques. Some satellite aspects of this project, although they do not appear in this thesis, required the use of *in-silico* technology, the use of microbiology techniques and the design of PCR primers. For help and expertise in this area I must thank Roger Latham. I wish to thank Leigh Blizzard, David Gell and Tracy Dickson who have been the Graduate Research Co-ordinators for PhD candidates at the Menzies Research Institute throughout my project; you all provided me with much needed support at crucial times. In addition to those I have mentioned the Menzies Research Institute and the School of Medicine are full of supportive staff and students; I am so lucky to work around people from whom a friendly word, encouragement and discussion are never far away.

And last, but certainly not least, I wish to thank my family and friends for their unwavering support during my project. It is no secret to those who know us that the last few years have been a very trying time for my family, in many ways. I thank my parents, Sue and Greg Brown, who have always been interested in and proud of my work. My brother Dan (who suggested he moved away to South

Australia in 2012 to avoid the tempest of the last year of my PhD) and his partner Courtney (who has a PhD project of her own), my two grandmothers, aunts, uncles, cousins and family friends have likewise been supportive. To my friends, my Scouts, my Rovers and my team mates from various sports, I must apologise for all the missed dinners, games, boots and Birthday celebrations that I missed because experiments ran over-time or samples came late. Whoever said "a PhD takes charge of your life" was not, in my case, far wrong. But, at least, I can safely say that it won't happen from now on! Your support has been never ending; thank you all so much.

And now I end by acknowledging my reasons for doing this study. I find it difficult to communicate the full horror of Devil Facial Tumour Disease. A cancer is scary enough when it affects one person, to have one form a contagious disease is a terrible prospect. Since its emergence, DFTD has killed more than 8 of every 10 Tasmanian devils; this is a startling number. If a disease like this affected humans, it would have devastating consequences across the world. If a disease like this had spread to the same extent in the human population, the biggest area of research would be in treatments and vaccines for use against it. And yet, in DFTD therapy and vaccine studies make up only a tiny part of the research. The vast majority of work and funding for DFTD contributes to the establishment of isolated populations of Tasmanian devils. The strategies pursued do not contribute to preserving the role of devils in the natural ecosystem across Tasmania, and will require a long-term, expensive investment to protect devils for an 'unspecified' period of time until wild extinction occurs (if, we assume, it does occur). However, invariably, you do not solve a 'problem' like the decrease in Tasmanian devil numbers unless you solve what causes it.

In this project, I wanted to contribute to what I believe is the only reliable option to save the Tasmanian devil: creating a vaccine or immunotherapy that could deal with the disease, not just the 'problem' it has caused. Working in this area on Tasmanian devils is not easy. These little animals become our (un) willing subjects; they never question, they suffer discomfort and, sometimes, dreadful disease as part of our experiments. My only hope is that in future the work of our research group will be able to help these struggling little animals, before the unthinkable happens and we lose them, irreplaceably, in the wild. I hope that my research can, in some way, help to truly 'Save the Tasmanian Devil'.

Chapter 7 - References

1. Woods GM, Kreiss A, Belov K, Siddle H, Obendorf DL, et al. (2007) The immune response of the Tasmanian devil (*Sarcophilus harrisii*) and devil facial tumour disease. EcoHealth 4: 338-345.

2. Kreiss A, Fox N, Bergfeld J, Quinn SJ, Pyecroft S, et al. (2008) Assessment of cellular immune responses of healthy and diseased Tasmanian devils (*Sarcophilus harrisii*). Developmental & Comparative Immunology 32: 544-553.

3. Kreiss A, Cheng Y, Kimble F, Wells B, Donovan S, et al. (2011) Allorecognition in the Tasmanian devil (Sarcophilus harrisii), an endangered marsupial species with limited genetic diversity. PLoS One 6: e22402.

4. Hawkins CE, Baars C, Hesterman H, Hocking GJ, Jones ME, et al. (2006) Emerging disease and population decline of an island endemic, the Tasmanian devil *Sarcophilus harrisii*. Biological Conservation 131: 307-324.

5. Bikard D, Marraffini LA (2012) Innate and adaptive immunity in bacteria: mechanisms of programmed genetic variation to fight bacteriophages. Current Opinion in Immunology 24: 15-20.

6. Abbas AK, Lichtman AH (2005) Cellular and Molecular Immunology. Fifth Eddition. Philadelphia: Elsevier Saunders.

7. Kanitakis J (2002) Anatomy, histology and immunohistochemistry of normal human skin. European Journal of Dermatology 12: 390-401.

8. Kilburn KH (1968) A hypothesis for pulmonary clearance and its implications. American Review of Respiratory Disease 98: 449-463.

9. Wanner A, Salathé M, O'Riordan TG (1996) Mucociliary clearance in the airways. American Journal of Respiratory and Critical Care Medicine 154: 1868-1902.

10. Kutteh WH, Moldoveanu Z, Mestecky J (1998) Mucosal immunity in the female reproductive tract: correlation of immunoglobulins, cytokines, and reproductive hormones in human cervical mucus around the time of ovulation. AIDS Research and Human Retroviruses 14: S51-55.

11. Barber RC, Chang L-YE, Arnoldo BD, Purdue GF, Hunt JL, et al. (2006) Innate immunity SNPs are associated with risk for severe sepsis after burn injury. Clinical Medicine and Research 4: 250-255.

12. Dellinger EP, Miller SD, Wertz MJ, Grypma M, Droppert B, et al. (1988) Risk of infection after open fracture of the arm or leg. Archives of Surgery 123: 1320-1327.

13. Sweet RL, Blankfort-Doyl M, Robbie MO, Schacter J (1986) The occurrence of chlamydial and gonococcal salpingitis during the menstrual cycle. Journal of the American Medical Association 255: 2062-2064.

14. Nickoloff BJ, Naidu Y (1994) Perturbation of epidermal barrier function correlates with initiation of cytokine cascade in human skin. Journal of the American Academy of Dermatology 30: 535-546.

15. Wood LC, Jackson SM, Elias PM, Grunfeld C, Feingold KR (1992) Cutaneous barrier perturbation stimulates cytokine production in the epidermis of mice. The Journal of Clinical Investigation 90: 482-487.

16. Nathan CF (1987) Secretory products of macrophages. The Journal of Clinical Investigation 79: 319-326.

17. Utgaard JO, Jahnsen FL, Bakka A, Brandtzaeg P, Haraldsen G (1998) Rapid secretion of prestored interleukin 8 from Weibel-Palade bodies of microvascular endothelial cells. The Journal of Experimental Medicine 188: 1751-1756.

18. Carson WE, Giri JG, Lindemann MJ, Linett ML, Ahdieh M, et al. (1994) Interleukin (IL) 15 is a novel cytokine that activates human natural killer cells via components of the IL-2 receptor. The Journal of Experimental Medicine 180: 1395-1403.

19. Ratthé C, Girard D (2004) Interleukin-15 enhances human neutrophil phagocytosis by a Syk-dependent mechanism: importance of the IL-15Rα chain. Journal of Leukocyte Biology 76: 162-168.

20. Agnello D, Lankford CSR, Bream J, Morinobu A, Gadina M, et al. (2003) Cytokines and transcription factors that regulate T helper cell differentiation: New players and new insights. The Journal of Clinical Immunology 23: 147-161.

21. Walport MJ (2001) Complement. New England Journal of Medicine 344: 1058-1066.

22. Devaux P, Christiansen D, Plumet S, Gerlier D (2004) Cell surface activation of the alternative complement pathway by the fusion protein of measles virus. The Journal of General Virology 85: 1665-1673.

23. Neth O, Jack DL, Johnson M, Klein NJ, Turner MW (2002) Enhancement of complement activation and opsonophagocytosis by complexes of mannose-binding lectin with mannose-binding lectin-associated serine protease after binding to *Staphylococcus aureus*. The Journal of Immunology 169: 4430-4436.

24. Fernandez HN, Henson PM, Otani A, Hugli TE (1978) Chemotactic response to human C3a and C5a anaphylatoxins. The Journal of Immunology 120: 109-115.

25. Bjork J, Hugli TE, Smedegard G (1985) Microvascular effects of anaphylatoxins C3a and C5a. The Journal of Immunology 134: 1115-1119.

26. Podack ER, Tschopp J (1982) Polymerization of the ninth component of complement (C9): formation of poly(C9) with a tubular ultrastructure resembling the membrane attack complex of complement. Proceedings of the National Academy of Sciences USA 79: 574-578.

27. Götze O, Müller-Eberhard HJ (1970) Lysis of erythrocytes by complement in the absence of antibody. The Journal of Experimental Medicine 132: 898-915.

28. Spoel SH, Dong XN (2012) How do plants achieve immunity? Defence without specialized immune cells. Nature Reviews Immunology 12: 89-100.

29. Ausubel FM (2005) Are innate immune signaling pathways in plants and animals conserved? Nature Immunology 6: 973-979.

30. Gordon S (2002) Pattern recognition receptors: Doubling up for the innate immune response. Cell 111: 927-930.

31. Janeway CAJ, Medzhitov R (2002) Innate Immune Recognition. Annual Reviews of Immunology 20: 197–216.

32. Stahl PD (1992) The mannose receptor and other macrophage lectins. Current Opinion in Immunology 4: 49-52.

33. Linehan SA (2005) The mannose receptor is expressed by subsets of APC in non-lymphoid organs. BMC Immunology 6: 1-11.

34. Linehan SA, Martínez-Pomares L, Gordon S (2000) Macrophage lectins in host defence. Microbes and Infection 2: 279-288.

35. Ezekowitz RAB, Williams DJ, Koziel H, Armstrong MYK, Warner A, et al. (1991) Uptake of *Pneumocystis Carinii* mediated by the macrophage mannose receptor. Nature 351: 155-158.

36. Nakayama H, Iwahara C, Takamori K, Ogawa H, Iwabuchi K (2008) Lactosylceramide is a pattern recognition receptor that forms lyn-coupled membrane microdomains on neutrophils. Immunology, Endocrine & Metabolic Agents in Medicinal Chemistry 8: 327-335.

37. Schengrund C-L (2003) "Multivalent" saccharides: development of new approaches for inhibiting the effects of glycosphingolipid-binding pathogens. Biochemical Pharmacology 65: 699-707.

38. Sato T, Iwabuchi K, Nagaoka I, Adachi Y, Ohno N, et al. (2006) Induction of human neutrophil chemotaxis by Candida albicans-derived β-1,6-long glycoside side-chain-branched β-glucan. The Journal of Leukocyte Biology 80: 204-211.

39. Iwabuchi K (2005) Structure and functions of glycosignaling domain. Trends in Glycoscience and Glycotechnology 17: 1-14.

40. Underhill DM, Ozinsky A, Hajjar AM, Stevens A, Wilson CB, et al. (1999) The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. Nature 401: 811-815.

41. Lauriello M, Micera A, Muzi P, Di Rienzo Businco L, Bonini S (2012) TLR4 and TLR9 expression in different phenotypes of rhinitis. International Journal of Otolaryngology 2012: 1-7.

42. Ozinsky A, Underhill DM, Fontenot JD, Hajjar AM, Smith KD, et al. (2000) The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between Toll-like receptors. Proceedings of the National Academy of Sciences USA 97: 13766-13771.

43. Roilides E, Uhlig K, Venzon D, Pizzo PA, Walsh TJ (1992) Neutrophil oxidative burst in response to blastoconidia and pseudohyphae of *Candida albicans*: Augmentation by granulocyte colony-stimulating factor and interferon-γ. The Journal of Infectious Diseases 166: 668-673.

44. Matsumoto M, Seya T (2008) TLR3: Interferon induction by double-stranded RNA including poly(I:C). Advanced Drug Delivery Reviews 60: 805-812.

45. Huang CC, Duffy KE, San Mateo LR, Amegadzie BY, Sarisky RT, et al. (2006) A pathway analysis of poly(I:C)-induced global gene expression change in human peripheral blood mononuclear cells. Physiological Genomics 26: 125-133.

46. Lion E, Smits ELJM, Berneman ZN, Van Tendeloo VFI (2009) Acute myeloid leukemic cell lines loaded with synthetic dsRNA trigger IFN-γ secretion by human NK cells. Leukemia Research 33: 539-546.

47. Schmidt KN, Leung B, Kwong M, Zarember KA, Satyal S, et al. (2004) APC-independent activation of NK cells by the toll-like receptor 3 agonist double-stranded RNA. The Journal of Immunology 172: 138-143.

48. Yasuda K, Rutz M, Schlatter B, Metzger J, Luppa PB, et al. (2006) CpG motif-independent activation of TLR9 upon endosomal translocation of "natural" phosphodiester DNA. European Journal of Immunology 36: 431-436.

49. Pasare C, Medzhitov R (2005) Toll-like Receptors: Linking Innate and Adaptive Immunity. In: Gupta S, Paul WE, Steinman RM, editors. Mechanisms of Lymphocyte Activation and Immune Regulation: X: Springer, USA.

50. Liu G, Zhang L, Zhao Y (2010) Modulation of immune responses through direct activation of toll-like receptors of T cells. Clinical and Experimental Immunology 160: 168-175.

51. Takeshita F, Leifer CA, Gursel I, Ishii KJ, Takeshita S, et al. (2001) Cutting edge: Role of toll-like receptor 9 in CpG DNA-induced activation of human cells. The Journal of Immunology 167: 3555-3558.

52. Krieg AM, Yi A-K, Matson S, Waldschmidt TJ, Bishop GA, et al. (1995) CpG motifs in bacterial DNA trigger direct B-cell activation. Nature 374: 546-549.

53. Kato A, Homma T, Batchelor J, Hashimoto N, Imai S, et al. (2003) Interferon-α/β receptor-mediated selective induction of a gene cluster by CpG oligodeoxynucleotide 2006. BMC Immunology 4: 1-10.

54. Ballas ZK, Rasmussen WL, Krieg AM (1996) Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. The Journal of Immunology 157: 1840-1845.

55. Jakob T, Walker PS, Krieg AM, Udey MC, Vogel JC (1998) Activation of cutaneous dendritic cells by CpG-containing oligodeoxynucleotides: A role for dendritic cells in the augmentation of Th1 responses by immunostimulatory DNA. The Journal of Immunology 161: 3042-3049.

56. Lipford GB, Bauer M, Blank C, Reiter R, Wagner H, et al. (1997) CpG-containing synthetic oligonucleotides promote B and cytotoxic T cell responses to protein antigen: A new class of vaccine adjuvants. European Journal of Immunology 27: 2340-2344.

57. Di Carlo E, Forni G, Lollini PL, Colombo MP, Modesti A, et al. (2001) The intriguing role of polymorphonuclear neutrophils in antitumour reactions. Blood 97: 339-345.

58. Nauseef WM (2007) How human neutrophils kill and degrade microbes: an integrated view. Immunological Reviews 219: 88-102.

59. Dongari-Bagtzoglou A, Kashleva H, Dwivedi P, Diaz P, Vasilakos J (2009) Characterization of mucosal *Candida albicans* biofilms. PLoS One 4: e7967.

60. Brown SJ, Askenase PW (1982) Blood eosinophil and basophil responses in guinea pigs parasitized by *Amblyomma americanum* ticks. The American Journal of Tropical Medicine and Hygiene 31: 593-598.

61. Rumbley CA, Sugaya H, Zekavat SA, El Refaei M, Perrin PJ, et al. (1999) Activated eosinophils are the major source of Th2-associated cytokines in the schistosome granuloma. The Journal of Immunology 162: 1003-1009.

62. Khazen W, M'Bika J-P, Tomkiewicz C, Benelli C, Chany C, et al. (2005) Expression of macrophage-selective markers in human and rodent adipocytes. Federation of European Biochemical Societies Letters 579: 5631-5634.

63. Taylor PR, Martinez-Pomares L, Stacey M, Lin H-H, Brown GD, et al. (2005) Macrophage receptors and immune recognition. Annual Review of Immunology 23: 901-944.

64. Aderem A, Underhill DM (1999) Mechanisms of phagocytosis in macrophages. Annual Review of Immunology 17: 593-623.

65. Root RK, Metcalf J, Oshino N, Chance B (1975) H_2O_2 release from human granulocytes during phagocytosis I. Documentation, quantitation, and some regulating factors. The Journal of Clinical Investigation 55: 945–955.

66. Musiani P, Allione A, Modica A, Lollini PL, Giovarelli M, et al. (1996) Role of neutrophils and lymphocytes in inhibition of a mouse mammary adenocarcinoma engineered to release IL-2, IL-4, Il-7, IL-10, IFN-α, IFN-γ, and TNF-α. Laboratory Investigation 74: 146-157.

67. Steinman RM, Cohn ZA (1974) Identification of a novel cell type in peripheral lymphoid organs of mice. The Journal of Experimental Medicine 139: 380-397.

68. Mellman I, Steinman RM (2001) Dendritic cells: Specialized and regulated antigen processing machines. Cell 106: 255-258.

69. Steinman RM (1991) The dendritic cell system and its role in immunogenicity. Annual Review of Immunology 9: 271-296.

70. Dubois B, Vanbervliet B, Fayette J, Massacrier C, Kooten CV, et al. (1997) Dendritic cells enhance growth and differentiation of CD40-activated B lymphocytes. The Journal of Experimental Medicine 185: 941-952.

71. Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, et al. (2000) Immunobiology of dendritic cells. Annual Review of Immunology 18: 767–811.

72. Valledor AF, Borràs FE, Cullell-Young M, Celada A (1998) Transcription factors that regulate monocyte/macrophage differentiation. The Journal of Leukocyte Biology 63: 405-417.

73. Palucka KA, Taquet N, Sanchez-Chapuis F, Gluckman JC (1998) Dendritic cells as the terminal stage of monocyte differentiation. The Journal of Immunology 160: 4587-4595.

74. Chomarat P, Dantin C, Bennett L, Banchereau J, Palucka AK (2003) TNF skews monocyte differentiation from macrophages to dendritic cells. The Journal of Immunology 171: 2262-2269.

75. Le J, Prensky W, Yip YK, Chang Z, Hoffman T, et al. (1983) Activation of human monocyte cytotoxicity by natural and recombinant immune interferon. The Journal of Immunology 131: 2821-2826.

76. Feinman R, Henriksen-DeStefano D, Tsujimoto M, Vilcek J (1987) Tumor necrosis factor is an important mediator of tumor cell killing by human monocytes. The Journal of Immunology 138: 635-640.

77. Banerjee D, Fernando L, Sklar S, Richter M (1981) The antibody-independent cytotoxic activity of normal circulating human leucocytes. I. Lysis of target cells by monocytes and neutrophils in a non-phagocytic pathway. Immunology 44: 97–107.

78. Timonen T, Ortaldo JR, Herberman RB (1981) Characteristics of human large granular lymphocytes and relationship to natural killer and K cells. The Journal of Experimental Medicine 153: 569-582.

79. Aiba Y, Ogawa M (1997) Development of natural killer cells, B lymphocytes, macrophages and mast cells from single hematopoietic progenitors in culture of murine fetal liver cells. Blood 90: 3923-3930.

80. Cooper MA, Fehniger TA, Caligiuri MA (2001) The biology of human natural killer-cell subsets. Trends in Immunology 22: 633-640.

81. Contini P, Ghio M, Poggi A, Filaci G, Indiveri F, et al. (2003) Soluble HLA-A,-B,-C and -G molecules induce apoptosis in T and NK CD8+ cells and inhibit cytotoxic T cell activity through CD8 ligation. European Journal of Immunology 33: 125-134.

82. Sun JC, Lanier LL (2011) NK cells demonstrate unexpected specificity and longevity: Versatility in NK cell memory. Immunology and Cell Biology 89: 327–329.

83. Phillips JH, Chang C, Mattson J, Gumperz JE, Parham P, et al. (1996) CD94 and a novel associated protein (94AP) form a NK cell receptor involved in the recognition of HLA-A, HLA-B, and HLA-C allotypes. Immunity 5: 163-172.

84. Nitta T, Yagita H, Sato K, Okumura K (1989) Involvement of CD56 (NKH-1/Leu-19 antigen) as an adhesion molecule in natural killer-target cell interaction. The Journal of Experimental Medicine 170: 1757-1761.

85. Vicente-Manzanares M, Sanchez-Madrid F (2004) Role of the cytoskeleton during leukocyte responses. Nature Reviews Immunology 4: 110-122.

86. Aktas E, Kucuksezer UC, Bilgic S, Erten G, Deniz G (2009) Relationship between CD107a expression and cytotoxic activity. Cellular Immunology 254 149–154.

87. Zalman LS, Martin DE, Jung G, Müller-Eberhard HJ (1987) The cytolytic protein of human lymphocytes related to the ninth component (C9) of human complement: isolation from anti-CD3-activated peripheral blood mononuclear cells. Proceedings of the National Academy of Sciences USA 84: 2426-2429.

88. Law RHP, Lukoyanova N, Voskoboinik I, Caradoc-Davies TT, Baran K, et al. (2010) The structural basis for membrane binding and pore formation by lymphocyte perforin. Nature 468: 447-451.

89. Pinkoski MJ, Heibein JA, Barry M, Bleackley RC (2000) Nuclear translocation of granzyme B in target cell apoptosis. Cell Death and Differentiation 7: 17-24.

90. Gillgrass A, Ashkar A (2011) Stimulating natural killer cells to protect against cancer: recent developments. Expert Reviews of Clinical Immunology 7: 367–382.

91. Lehmann C, Zeis M, Uharek L (2001) Activation of natural killer cells with interleukin 2 (IL-2) and IL-12 increases perforin binding and subsequent lysis of tumour cells. British Journal of Haematology 114: 660-665.

92. Parrish-Novak J, Dillon SR, Nelson A, Hammond A, Sprecher C, et al. (2000) Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. Nature 408: 57-63.

93. Lion E, Anguille S, Berneman ZN, Smits ELJM, Van Tendeloo VFI (2011) Poly(I:C) Enhances the Susceptibility of Leukemic Cells to NK cell Cytotoxicity and Phagocytosis by DC. PLoS One 6: e20952.

94. Rock KL, Shen L (2005) Cross-presentation: underlying mechanisms and role in immune surveillance. Immunological Reviews 207: 166-183.

95. Guermonprez P, Valladeau J, Zitvogel L, Théry C, Amigorena S (2002) Antigen presentation and T cell stimulation by dendritic cells. Annual Review of Immunology 20: 621-667.

96. McDonnell AM, Robinson BWS, Currie AJ (2010) Tumor antigen cross-presentation and the dendritic cell: Where it all begins? Clinical and Developmental Immunology 2010: Article ID 539519.

97. Bevan MJ (1976) Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. The Journal of Experimental Medicine 143: 1283-1288.

98. DeFranco AL (1987) Molecular aspects of B-lymphocyte activation. Annual Review of Cell Biology 3: 143-178.

99. Groscurth P, Filgueira L (1998) Killing mechanisms of cytotoxic T lymphocytes. News in Physiological Sciences 13: 17-21.

100. Lecker SH, Goldberg AL, Mitch WE (2006) Protein degradation by the ubiquitin–proteasome pathway in normal and disease states. Journal of the American Society of Nephrology 17: 1807-1819.

101. Suh WK, Cohen-Doyle MF, Fruh K, Wang K, Peterson PA, et al. (1994) Interaction of MHC class I molecules with the transporter associated with antigen processing. Science 264: 1322-1326.

102. Ortmann B, Androlewicz MJ, Cresswell P (1994) MHC class I/β_2-microglobulin complexes associate with TAP transporters before peptide binding. Nature 368: 864-867.

103. Gay D, Maddon P, Sekaly R, Talle MA, Godfrey M, et al. (1987) Functional interaction between human T-cell protein CD4 and the major histocompatibility complex HLA-DR antigen. Nature 328: 626-629.

104. Arcaro A, Grégoire C, Bakker TR, Baldi L, Jordan M, et al. (2001) CD8β endows CD8 with efficient coreceptor function by coupling T cell receptor/CD3 to raft-associated CD8/p56lck complexes. The Journal of Experimental Medicine 194: 1485-1495.

105. Bright NA, Reaves BJ, Mullock BM, Luzio JP (1997) Dense core lysosomes can fuse with late endosomes and are re-formed from the resultant hybrid organelles. The Journal of Cell Science 110: 2027-2040.

106. Leavy O (2011) Antigen presentation: Cross-dress to impress. Nature Reviews Immunology 11: 302 - 303.

107. Wakim LM, Bevan MJ (2011) Cross-dressed dendritic cells drive memory $CD8^+$ T-cell activation after viral infection. Nature 471: 629 - 632.

108. Frauwirth KA, Thompson CB (2002) Activation and inhibition of lymphocytes by costimulation. The Journal of Clinical Investigation 109: 295-299.

109. Ledbetter JA, Imboden JB, Schieven GL, Grosmaire LS, Rabinovitch PS, et al. (1990) CD28 ligation in T-cell activation: evidence for two signal transduction pathways. Blood 75: 1531-1539.

110. Linsley PS, Brady W, Grosmaire L, Aruffo A, Damle NK, et al. (1991) Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin 2 mRNA accumulation. The Journal of Experimental Medicine 173: 721-730.

111. Pardigon N, Bercovici N, Calbo S, Santos-Lima EC, Liblau R, et al. (1998) Role of co-stimulation in CD8+ T cell activation. International Immunology 10: 619-630.

112. Shahinian A, Pfeffer K, Lee KP, Kundig TM, Kishihara K, et al. (1993) Differential T cell costimulatory requirements in CD28-deficient mice. Science 261: 609-612.

113. Muñoz E, Zubiaga A, Olson D, Huber BT (1989) Control of lymphokine expression in T helper 2 cells. Proceedings of the National Academy of Sciences USA 86: 9461-9464.

114. Huber M, Beuscher HU, Rohwer P, Kurrle R, Röllinghoff M, et al. (1998) Costimulation via TCR and IL-1 receptor reveals a novel IL-1α-mediated autocrine pathway of Th2 cell proliferation. The Journal of Immunology 160: 4242-4247.

115. Bluestone JA (1995) New perspectives of CD28-B7-mediated T cell costimulation. Immunity 2: 555-559.

116. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL (1986) Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. The Journal of Immunology 136: 2348-2357.

117. Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, et al. (2005) Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nature Immunology 6: 1123-1132.

118. Carrier Y, Yuan J, Kuchroo VK, Weiner HL (2007) Th3 cells in peripheral tolerance. I. Induction of Foxp3-positive regulatory T cells by Th3 cells derived from TGF-β T cell-transgenic mice. The Journal of Immunology 178: 179-185.

119. Carrier Y, Yuan J., Kuchroo V. K, L. WH (2007) Th3 cells in peripheral tolerance. II. TGF-beta-transgenic Th3 cells rescue IL-2-deficient mice from autoimmunity. The Journal of Immunology 178: 172 - 178.

120. Weiner HL (2001) Induction and mechanism of action of transforming growth factor-β-secreting Th3 regulatory cells. Immunological Reviews 182: 207 - 214.

121. Henney CS, Kuribayashi K, Kern DE, Gillis S (1981) Interleukin-2 augments natural killer cell activity. Nature 291: 335-338.

122. Abbas AK, Murphy KM, Sher A (1996) Functional diversity of helper T lymphocytes. Nature 383: 787-793.

123. Trinchieri G, Wysocka M, D'Andrea A, Rengaraju M, Aste-Amezaga M, et al. (1992) Natural killer cell stimulatory factor (NKSF) or interleukin-12 is a key regulator of immune response and inflammation. Progress in Growth Factor Research 4: 355-368.

124. Aptsiauri N, Cabrera T, Garcia-Lora A, Lopez-Nevot MA, Ruiz-Cabello F, et al. (2007) MHC class I antigens and immune surveillance in transformed cells. International Review of Cytology 256: 139-189.

125. Young KG, MacLean S, Dudani R, Krishnan L, Sad S (2011) CD8+ T cells primed in the periphery provide time-bound immune-surveillance to the central nervous system. The Journal of Immunology 187: 1192-1200.

126. Ando K, Hiroishi K, Kaneko T, Moriyama T, Muto Y, et al. (1997) Perforin, Fas/Fas ligand, and TNF-alpha pathways as specific and bystander killing mechanisms of hepatitis C virus-specific human CTL. The Journal of Immunology 158: 5283-5291.

127. Gillis S, Smith KA (1977) Long term culture of tumour-specific cytotoxic T cells. Nature 268: 154-156.

128. Nelson BH (2004) IL-2, regulatory T cells, and tolerance. The Journal of Immunology 172: 3983-3988.

129. Carahera EM, Parenteaub M, Gruberc H, Scotta FW (2000) Flow cytometric analysis of intracellular IFN-g, IL-4 and IL-10 in CD3 4 T-cells from rat spleen. The Journal of Immunological Methods 244: 29-40.

130. Fong TAT, Mosmann TR (1990) Alloreactive murine CD8+ T cell clones secrete the Th1 pattern of cytokines. The Journal of Immunology 144: 1744-1752.

131. Kelso A, Glasebrook AL (1984) Secretion of interleukin 2, macrophage-activating factor, interferon, and colony-stimulating factor by alloreactive T lymphocyte clones. The Journal of Immunology 132: 2924-2931.

132. Wendt K, Wilk E, Buyny S, Schmidt RE, Jacobs R (2007) Interleukin-21 differentially affects human natural killer cell subsets. Immunology 122: 486-495.

133. Akbar AN, Terry L, Timms A, Beverley PC, Janossy G (1988) Loss of CD45R and gain of UCHL1 reactivity is a feature of primed T cells. The Journal of Immunology 140: 2171-2178.

134. Veiga-Fernandes H, Walter U, Bourgeois C, McLean A, Rocha B (2000) Response of naïve and memory CD8+ T cells to antigen stimulation in vivo. Nature Immunology 1: 47-53.

135. Wallace DL, Beverley PC (1990) Phenotypic changes associated with activation of CD45RA+ and CD45RO+ T cells. Immunology 69: 460-467.

136. Merkenschlager M, Beverley PCL (1989) Evidence for differential expression of CD45 isoforms by precursors for memory-dependent and independent cytotoxic responses: human CD8 memory CTLp selectively express CD45R0 (UCHL1). International Immunology 1: 450-459.

137. Loder BF, Mutschler B, Ray RJ, Paige CJ, Sideras P, et al. (1999) B cell development in the spleen takes place in discrete steps and Is determined by the quality of B cell receptor–Derived signals. The Journal of Experimental Medicine 190: 75-90.

138. West MA, Lucocq JM, Watts C (1994) Antigen processing and class II MHC peptide-loading compartments in human B-lymphoblastoid cells. Nature 369: 147-151.

139. Shapiro-Shelef M, Calame K (2005) Regulation of plasma-cell development. Nature Reviews Immunology 5: 230-242.

140. Rocha PN, Plumb TJ, Crowley SD, Coffman TM (2003) Effector mechanisms in transplant rejection. Immunological Reviews 196: 51-64.

141. Hoebe K, Janssen E, Beutler B (2004) The interface between innate and adaptive immunity. Nature Immunology 5: 171-174.

142. Hartmann G, Weiner GJ, Krieg AM (1999) CpG DNA: A potent signal for growth, activation, and maturation of human dendritic cells. Proceedings of the National Academy of Sciences USA 96: 9305-9310.

143. Ballas ZK, Krieg AM, Warren T, Rasmussen W, Davis HL, et al. (2001) Divergent therapeutic and immunologic effects of oligodeoxynucleotides with distinct CpG motifs. The Journal of Immunology 167: 4878-4886.

144. Zhang X, Munegowda MA, Yuan J, Wei Y, Xiang J (2010) Optimal TLR9 signal converts tolerogenic CD4–8– DCs into immunogenic ones capable of stimulating antitumor immunity via activating CD4+ Th1/Th17 and NK cell responses. The Journal of Leukocyte Biology 88: 393-403.

145. Bauer M, Redecke V, Ellwart JW, Scherer B, Kremer J-P, et al. (2001) Bacterial CpG-DNA triggers activation and maturation of human CD11c−, CD123+ dendritic cells. The Journal of Immunology 166: 5000-5007.

146. Poeck H, Wagner M, Battiany J, Rothenfusser S, Wellisch D, et al. (2004) Plasmacytoid dendritic cells, antigen, and CpG-C license human B cells for plasma cell differentiation and immunoglobulin production in the absence of T-cell help. Blood 103: 3058-3064.

147. Bonnema JD, Rivlin KA, Ting AT, Schoon RA, Abraham RT, et al. (1994) Cytokine-enhanced NK cell-mediated cytotoxicity. Positive modulatory effects of IL-2 and IL-12 on stimulus-dependent granule exocytosis. The Journal of Immunology 152: 2098-2104.

148. Weigent DA, Stanton GJ, Johnson HM (1983) Interleukin 2 enhances natural killer cell activity through induction of gamma interferon. Infection and Immunity 41: 992-997.

149. Metzger DW, Buchanan JM, Collins JT, Lester TL, Murray KS, et al. (1996) Enhancement of humoral immunity by interleukin-12. Annals of the New York Academy of Sciences 795: 100-115.

150. Wu CY, Demeure C, Kiniwa M, Gately M, Delespesse G (1993) IL-12 induces the production of IFN-gamma by neonatal human CD4 T cells. The Journal of Immunology 151: 1938-1949.

151. Alzona M, Jäck H-M, Simms PE, Ellis TM (1996) Interleukin-12 activates interferon-γ production by targeted activation of CD30+ T cells. Annals of the New York Academy of Sciences 795: 127-136.

152. Snijders A, Kalinski P, Hilkens CM, Kapsenberg ML (1998) High-level IL-12 production by human dendritic cells requires two signals. International Immunology 10: 1593-1598.

153. Fuson EW, Hubbard RA, Sugantharaj DG, Andrews RB, Beard MR, et al. (1983) Antibody-dependent cell-mediated cytotoxicity. Immunological Research 2: 327-340.

154. Keaney MA, Hirte H, McPhail S, Fernando L, Belanger R, et al. (1979) The antibody-dependent cell-mediated cytotoxic reaction. I. The morphological and functional heterogeneity of the rabbit cytotoxic cells. Immunology 38: 665-676.

155. Grewal AS, Rouse BT, Babiuk LA (1977) Mechanisms of resistant of herpesviruses: comparison of the effectiveness of different cell types in mediating antibody-dependent cell-mediated cytotoxicity. Infection and Immunity 15: 698-703.

156. Fukui H, Overton WR, Herberman RB, Reynolds CW (1987) Natural killer cell activity in the rat. VI. Characterization of rat large granular lymphocytes as effector cells in natural killer and antibody-dependent cellular cytotoxic activities. The Journal of Leukocyte Biology 41: 130-142.

157. Perussia B, Trinchieri G, Jackson A, Warner NL, Faust J, et al. (1984) The Fc receptor for IgG on human natural killer cells: phenotypic, functional, and comparative studies with monoclonal antibodies. The Journal of Immunology 133: 180-189.

158. Warner JF, Dennert G (1985) Bone marrow graft rejection as a function of antibody-directed natural killer cells. The Journal of Experimental Medicine 161: 563-576.

159. Hasek M, Skamene E, Karakoz I, Chutna J, Nouza K, et al. (1968) Studies on mechanisms of rejection of tolerated skin homografts and abrogation of immunological tolerance by hyperimmune serum. Folia Biologica 14: 411-424.

160. Carson WE, Parihar R, Lindemann MJ, Personeni N, Dierksheide J, et al. (2001) Interleukin-2 enhances the natural killer cell response to Herceptin-coated Her2/neu-positive breast cancer cells. European Journal of Immunology 31: 3016-3025.

161. Dall'Ozzo S, Tartas S, Paintaud G, Cartron G, Colombat P, et al. (2004) Rituximab-dependent cytotoxicity by natural killer cells: Influence of FCGR3A polymorphism on the concentration-effect relationship. Cancer Research 64: 4664-4669.

162. Shin M, Kim SJ (2011) ABO incompatible kidney transplantation—Current status and uncertainties. Journal of Transplantation 2011: Article ID 970421.

163. Williams GM, Hume DM, Hudson RP, Morris PJ, Kano K, et al. (1968) Hyperacute renal-homograft rejection in man. New England Journal of Medicine 279: 611-618.

164. Scornik JC, LeFor WM, Cicciarelli JC, Brunson ME, Bogaard T, et al. (1992) Hyperacute and acute kidney graft rejection due to antibodies against B cells. Transplantation 54: 61-64.

165. Higgins RM, J. BD (1996) Prevention of hyperacute rejection by removal of antibodies to HLA immediately before renal. Lancet 348: 1208-1211.

166. Ayala García MA, Gonzáles Yebra B, López Flores AL, Guaní Guerra E (2012) The major histocompatibility complex in transplantation. The Journal of Transplantation 2012: Article ID 842141.

167. Sankaran D, Asderakis A, Ashraf S, Roberts ISD, Short CD, et al. (1999) Cytokine gene polymorphisms predict acute graft rejection following renal transplantation. Kidney International 56: 281-288.

168. Asderakis A, Sankaran D, Dyer P, G. Johnson R, Pravica V, et al. (2001) Association of polymorphisms in the human interferon -γ and interleukin-10 gene with acute and chronic kidney transplant outcome: The cytokine effect on transplantation. Transplantation 71: 674-678.

169. Murphy WJ, Kumar V, Bennett M (1987) Rejection of bone marrow allografts by mice with severe combined immune deficiency (SCID). Evidence that natural killer cells can mediate the specificity of marrow graft rejection. The Journal of Experimental Medicine 165: 1212-1217.

170. Pelletier RP, Hennessy PK, Adams PW, Orosz CG (2002) High incidence of donor-reactive delayed-type hypersensitivity reactivity in transplant patients. American Journal of Transplantation 2: 926-933.

171. Hancock WW, Buelow R, Sayegh MH, Turka LA (1998) Antibody-induced transplant arteriosclerosis is prevented by graft expression of anti-oxidant and anti-apoptotic genes. Nature Medicine 4: 1392-1396.

172. Zhai Y, Meng L, Busuttil RW, Sayegh MH, Kupiec-Weglinski JW (2003) Activation of alloreactive CD8+ T cells operates via CD4-dependent and CD4-independent mechanisms and is CD154 blockade sensitive. The Journal of Immunology 170: 3024-3028.

173. Jones ND, Van Maurik A, Hara M, Spriewald BM, Witzke O, et al. (2000) CD40-CD40 ligand-independent activation of CD8+ T cells can trigger allograft rejection. The Journal of Immunology 165: 1111-1118.

174. Nicod LP (2006) Mechanisms of airway obliteration after lung transplantation. Proceedings of the American Thoracic Society 3: 444-449.

175. Preston-Martin S, Pike MC, Ross RK, Jones PA, Henderson BE (1990) Increased cell division as a cause of human cancer. Cancer Research 50: 7415-7421.

176. Hanahan D, Weinberg RA (2000) The hallmarks of cancer. Cell 100: 57-70.

177. Armitage P, Doll R (1954) The age distribution of cancer and a multi-stage theory of carcinogenesis. British Journal of Cancer 8: 1-12.

178. Call KM, Glaser T, Ito CY, Buckler AJ, Pelletier J, et al. (1990) Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus. Cell 60: 509-520.

179. Peto J, Collins N, Barfoot R, Seal S, Warren W, et al. (1999) Prevalence of BRCA1 and BRCA2 gene mutations in patients with early-onset breast cancer. Journal of the National Cancer Institute 91: 943-949.

180. Lichtenstein P, Holm NV, Verkasalo PK, Iliadou A, Kaprio J, et al. (2000) Environmental and heritable factors in the causation of cancer — analyses of cohorts of twins from Sweden, Denmark, and Finland. New England Journal of Medicine 343: 78-85.

181. Doll R, Peto R, Boreham J, Sutherland I (2004) Mortality in relation to smoking: 50 years' observations on male British doctors. British Medical Journal 328: 1519.

182. Ames BN, Kammen HO, Yamasaki E (1975) Hair dyes are mutagenic: identification of a variety of mutagenic ingredients. Proceedings of the National Academy of Sciences USA 72: 2423-2427.

183. Nakagama H, Nakanishi M, Ochiai M (2005) Modeling human colon cancer in rodents using a food-borne carcinogen, PhIP. Cancer Science 96: 627-636.

184. Ananthaswamy HN, Pierceall WE (1990) Molecular mechanisms of ultraviolet radiation carcinogenesis. Photochemistry and Photobiology 52: 1119-1136.

185. Coussens LM, Werb Z (2002) Inflammation and cancer. Nature 420: 860-867.

186. Hatakeyama M (2009) *Helicobacter pylori* and gastric carcinogenesis. The Journal of Gastroenterology 44: 239-248.

187. Poppe WAJ, Ide PS, Drijkoningen MPG, Lauweryns JM, van Assche FA (1995) Tobacco smoking impairs the local immunosurveillance in the uterine cervix. Gynecologic and Obstetric Investigation 39: 34-38.

188. Ray JM, Stetler-Stevenson WG (1994) The role of matrix metalloproteases and their inhibitors in tumour invasion, metastasis and angiogenesis. European Respiratory Journal 7: 2062–2072.

189. Visse R, Nagase H (2003) Matrix metalloproteinases and tissue inhibitors of metalloproteinases. Circulation Research 92: 827-839.

190. Stetler-Stevenson WG, Liotta LA, Kleiner DE (1993) Extracellular matrix 6: role of matrix metalloproteinases in tumor invasion and metastasis. The Federation of the American Societies for Experimental Biology Journal 7: 1434 -1441.

191. Bonfil RD, Reddel RR, Ura H, Reich R, Fridman R, et al. (1989) Invasive and metastatic potential of a v-Ha-ras-transformed human bronchial epithelial cell line. Journal of the National Cancer Institute 81: 587-594.

192. Sreenath T, Matrisian LM, Stetler-Stevenson WG, Gattoni-Celli S, Pozzatti RO (1992) Expression of matrix metalloproteinase genes in transformed rat cell lines of high and low metastatic potential. Cancer Research 52: 4942-4947.

193. Powell WC, Knox JD, Navre M, Grogan TM, Kittelson J, et al. (1993) Expression of the metalloproteinase matrilysin in DU-145 cells increases their invasive potential in severe combined immunodeficient mice. Cancer Research 53: 417-422.

194. Stearns ME, Wang M (1993) Type IV collagenase (Mr 72,000) expression in human prostate: Benign and malignant tissue. Cancer Research 53: 878-883.

195. Lyons JG, Birkedal-Hansen B, Moore WGI, O'Grady RL, Birkedal-Hansen H (1991) Characteristics of a 95-kDa matrix metalloproteinase produced by mammary carcinoma cells. Biochemistry 30: 1449-1456.

196. Levy AT, Cioce V, Sobel ME, Garbisa S, Grigioni WF, et al. (1991) Increased expression of the Mr 72,000 type IV collagenase in human colonic adenocarcinoma. Cancer Research 51: 439-444.

197. Jones CB, Sane DC, Herrington DM (2003) Matrix metalloproteinases: A review of their structure and role in acute coronary syndrome. Cardiovascular Research 59: 812-823.

198. Sakata K, Shigemasa K, Nagai N, Ohama K (2000) Expression of matrix metalloproteinases (MMP-2, MMP-9, MT1-MMP) and their inhibitors (TIMP-1, TIMP-2) in common epithelial tumors of the ovary. The International Journal of Oncology 17: 673-681.

199. Han YP, Tuan TL, Wu H, Hughes M, Garner WL (2001) TNF-alpha stimulates activation of pro-MMP2 in human skin through NF-(kappa)B mediated induction of MT1-MMP. The Journal of Cell Science 114: 131-139.

200. Rao VH, Singh RK, Delimont DC, Finnell RH, Bridge JA, et al. (1999) Transcriptional regulation of MMP-9 expression in stromal cells of human giant cell tumor of bone by tumor necrosis factor-alpha. The International Journal of Oncology 14: 291-300.

201. Weidner N, Semple JP, Welch WR, Folkman J (1991) Tumor angiogenesis and metastasis - Correlation in invasive breast carcinoma. New England Journal of Medicine 324: 1-8.

202. Ardi VC, Kupriyanova TA, Deryugina EI, Quigley JP (2007) Human neutrophils uniquely release TIMP-free MMP-9 to provide a potent catalytic stimulator of angiogenesis. Proceedings of the National Academy of Sciences USA 104: 20262-20267.

203. Kerbel RS (2000) Tumor angiogenesis: past, present and the near future. Carcinogenesis 21: 505-515.

204. Ciocca DR, Calderwood SK (2005) Heat shock proteins in cancer: diagnostic, prognostic, predictive, and treatment implications. Cell Stress & Chaperones 10: 86–103.

205. Ciocca DR, Clark GM, Tandon AK, Fuqua SAW, Welch WJ, et al. (1993) Heat shock protein hsp70 in patients with axillary lymph node-negative breast cancer: Prognostic implications. Journal of the National Cancer Institute 85: 570-574.

206. Cornford PA, Dodson AR, Parsons KF, Desmond AD, Woolfenden A, et al. (2000) Heat shock protein expression independently predicts clinical outcome in prostate cancer. Cancer Research 60: 7099-7105.

207. Hacihanefioglu A, Gonullu E, Mehtap O, Keski H, Yavuz M, et al. (2011) Effect of heat shock protein-90 (HSP90) and vascular endothelial growth factor (VEGF) on survival in acute lymphoblastic leukemia: an immunohistochemical study. Medical Oncology 28: 846-851.

208. Jolly C, Morimoto RI (2000) Role of the heat shock response and molecular chaperones in oncogenesis and cell death. Journal of the National Cancer Institute 92: 1564-1572.

209. Blagosklonny MV (2001) Re: Role of the heat shock response and molecular chaperones in oncogenesis and cell death. Journal of the National Cancer Institute 93: 239-240.

210. Drake CG, Jaffee E, Pardoll DM (2006) Mechanisms of Immune Evasion by Tumors. In: Allison JP, Dranoff G, Frederick WA, editors. Advances in Immunology: Academic Press. pp. 51 - 81.

211. Koopman LA, Corver WE, van der Slik AR, Giphart MJ, Fleuren GJ (2000) Multiple Genetic Alterations Cause Frequent and Heterogeneous Human Histocompatibility Leukocyte Antigen Class I Loss in Cervical Cancer. Journal of Experimental Medicine 191: 961 – 975.

212. Clarke B, Chetty R (2002) Postmodern cancer: the role of human immunodeficiency virus in uterine cervical cancer. Journal of Clinical Pathology: Molecular Pathology 55: 19 – 24.

213. Maeurer MJ, Gollin SM, Martin D, Swaney W, Bryant J, et al. (1996) Tumor Escape from Immune Recognition Lethal Recurrent Melanoma in a Patient Associated with Downregulation of the Peptide Transporter Protein TAP-1 and Loss of Expression of the Immunodominant MART-1/Melan-A Antigen. The Journal of Clinical Investigation 98: 1633 – 1641.

214. Jäger E, Ringhoffer M, Altmannsberger M, Arand M, Karbach J, et al. (1997) Immunoselection *in vivo*: independent loss of MHC class I and melanocyte differentiation antigen expression in metastatic melanoma. The International Journal of Cancer 71: 142 – 147.

215. Riker A, Cormier J, Panelli M, Kammula U, Wang E, et al. (1999) Immune selection after antigen-specific immunotherapy of melanoma Surgery 126: 112 – 120.

216. Beyer M, Schultze JL (2006) Regulatory T cells in cancer. Blood 108: 804-811.

217. Gray A, Raff AB, Chiriva-Internati M, Chen S-Y, Kast WM (2008) A paradigm shift in therapeutic vaccination of cancer patients: the need to apply therapeutic vaccination strategies in the preventive setting. Immunological Reviews 222: 316-327.

218. Klebanoff CA, Gattinoni L, Restifo NP (2006) CD8+ T-cell memory in tumor immunology and immunotherapy. Immunological Reviews 211: 214-224.

219. Bernal M, Ruiz-Cabello F, Concha A, Paschen A, Garrido F Implication of the β2-microglobulin gene in the generation of tumor escape phenotypes. Cancer Immunology, Immunotherapy: 1-13.

220. Fossati G, Taramelli D, Balsari A, Bogdanovich G, Andreola S, et al. (1984) Primary but not metastatic human melanomas expressing dr antigens stimulate autologous lymphocytes. The International Journal of Cancer 33: 591-597.

221. Watson NFS, Ramage JM, Madjd Z, Spendlove I, Ellis IO, et al. (2006) Immunosurveillance is active in colorectal cancer as downregulation but not complete loss of MHC class I expression correlates with a poor prognosis. The International Journal of Cancer 118: 6-10.

222. Seliger B, Wollscheid U, Momburg F, Blankenstein T, Huber C (2001) Characterization of the major histocompatibility complex class I deficiencies in B16 melanoma cells. Cancer Research 61: 1095-1099.

223. Hishii M, Nitta T, Ishida H, Ebato M, Kurosu A, et al. (1995) Human glioma-derived interleukin-10 inhibits antitumor immune responses *in vitro*. Neurosurgery 37: 1160-1167.

224. Alas S, Emmanouilides C, Bonavida B (2001) Inhibition of interleukin 10 by rituximab results in down-regulation of Bcl-2 and sensitization of B-cell non-hodgkin's lymphoma to apoptosis. Clinical Cancer Research 7: 709-723.

225. Sredni B, Weil M, Khomenok G, Lebenthal I, Teitz S, et al. (2004) Ammonium trichloro(dioxoethylene-o,o')tellurate (AS101) sensitizes tumors to chemotherapy by inhibiting the tumor interleukin 10 autocrine loop. Cancer Research 64: 1843-1852.

226. Pardali K, Moustakas A (2007) Actions of TGF-β as tumor suppressor and pro-metastatic factor in human cancer. Biochimica et Biophysica Acta - Reviews on Cancer 1775: 21-62.

227. Roberts AB, Anzano MA, Wakefield LM, Roche NS, Stern DF, et al. (1985) Type beta transforming growth factor: a bifunctional regulator of cellular growth. Proceedings of the National Academy of Sciences USA 82: 119-123.

228. Tang B, de Castro K, Barnes HE, Parks WT, Stewart L, et al. (1999) Loss of responsiveness to transforming growth factor β induces malignant transformation of nontumorigenic rat prostate epithelial cells. Cancer Research 59: 4834-4842.

229. McEarchern JA, Kobie JJ, Mack V, Wu RS, Meade-Tollin L, et al. (2001) Invasion and metastasis of a mammary tumor involves TGF-β signaling. The International Journal of Cancer 91: 76-82.

230. Nagorsen D, Voigt S, Berg E, Stein H, Thiel E, et al. (2007) Tumor-infiltrating macrophages and dendritic cells in human colorectal cancer: relation to local regulatory T cells, systemic T-cell response against tumor-associated antigens and survival. Journal of Translational Medicine 5: 1-8.

231. Gooden MJM, de Bock GH, Leffers N, Daemen T, Nijman HW (2011) The prognostic influence of tumour-infiltrating lymphocytes in cancer: a systematic review with meta-analysis. British Journal of Cancer 105: 93-103.

232. Coussens L, Yang-Feng TL, Liao YC, Chen E, Gray A, et al. (1985) Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. Science 230: 1132-1139.

233. Koeppen HKW, Wright BD, Burt AD, Quirke P, McNicol AM, et al. (2001) Overexpression of HER2/neu in solid tumours: an immunohistochemical survey. Histopathology 38: 96-104.

234. Plosker GL, Figgitt DP (2003) Rituximab: A review of its use in non-hodgkin's lymphoma and chronic lymphocytic leukaemia. Drugs 63: 803-843.

235. Zou W (2005) Immunosuppressive networks in the tumour environment and their therapeutic relevance. Nature Reviews Cancer 5: 263-274.

236. Peng L, Kjaergäard J, Plautz GE, Awad M, Drazba JA, et al. (2002) Tumor-induced L-selectin^{high} suppressor T cells mediate potent effector T cell blockade and cause failure of otherwise curative adoptive immunotherapy. The Journal of Immunology 169: 4811-4821.

237. Cohen D (1985) The Canine Transmissible Venereal Tumour: a unique result of tumour progression. Advances in Cancer Research 43: 75-112.

238. Epstein RB, Bennett BT (1974) Histocompatibility typing and course of canine venereal tumors transplanted into unmodified random dogs. Cancer Research 34: 788-793.

239. Dingli D, Nowak M (2006) Cancer biology: infectious tumour cells. pp. 35-36.

240. Thorburn MJ, Gwynn RVR, Ragbeer MS, Lee BI (1968) Pathological and cytogenetic observations on the naturally occurring canine venereal tumour in Jamaica (Sticker's tumour). British Journal of Cancer 22: 720-727.

241. Thomas R, Rebbeck C, Leroi A, Burt A, Breen M (2009) Extensive conservation of genomic imbalances in canine transmissible venereal tumors (CTVT) detected by microarray-based CGH analysis. Chromosome Research 17: 927-934.

242. Murgia C, Pritchard JK, Kim SY, Fassati A, Weiss RA (2006) Clonal origin and evolution of a transmissible cancer. Cell 126: 477-487.

243. Yang TJ, Chandler JP, Dunne-Anway S (1987) Growth stage dependent expression of MHC antigens on the canine transmissible venereal sarcoma. The British Journal of Cancer 55: 131-134.

244. Hsiao Y-W, Liao K-W, Hung S-W, Chu R-M (2004) Tumor-infiltrating lymphocyte secretion of IL-6 antagonizes tumor-derived TGF-beta 1 and restores the lymphokine-activated killing activity. The Journal of Immunology 172: 1508-1514.

245. Liu C-C, Wang Y-S, Lin C-Y, Chuang T-F, Liao K-W, et al. (2008) Transient downregulation of monocyte-derived dendritic-cell differentiation, function, and survival during tumoral progression and regression in an in vivo canine model of transmissible venereal tumor. Cancer Immunology, Immunotherapy 57: 479-491.

246. Mizuno S, Fujinaga T, Hagio M (1994) Role of lymphocytes in spontaneous regression of experimentally transplanted canine transmissible venereal sarcoma. Journal of Veterinary Medical Science 56: 15-20.

247. Das U, Das A (2000) Review of canine transmissible venereal sarcoma. Veterinary Research Communications 24 545-556.

248. Rogers KS, Walker MA, Dillon HB (1998) Transmissible venereal tumor: a retrospective study of 29 cases. Journal of the American Animal Hospital Association 34: 463-470.

249. Oduye OO, Ikede BO, Esuruoso GO, Akpokodje JU (1973) Metastatic transmissible venereal tumour in dogs. Journal of Small Animal Practice 14: 625-649.

250. Park M-S, Kim Y, Kang M-S, Oh S-Y, Cho D-Y, et al. (2006) Disseminated transmissible venereal tumor in a dog. Journal of Veterinary Diagnostic Investigation 18: 130–133.

251. Nak D, Nak Y, Cangul IT, Tuna B (2005) A Clinico-pathological study on the effect of vincristine on transmissible venereal tumour in dogs. Journal of Veterinary Medicine Series A 52: 366-370.

252. Scarpelli KC, Valladão ML, Metze K (2008) Predictive factors for the regression of canine transmissible venereal tumor during vincristine therapy. The Veterinary Journal.

253. Gaspar FJ, Ferreira I, Moleta Colodel M, Seullner Brandäo CV, Rocha NS (2010) Spontaneous canine transmissible venereal tumor: cell morphology and influence on P-glycoprotein expression. Turkish Journal of Veterinary and Animal Sciences 34: 447-454.

254. Amber EI, Henderson RA, Adeyanju JB, Gyang EO (1992) Single-drug chemotherapy of canine transmissible venereal tumor with cyclophosphamide, methotrexate, or vincristine. The Journal of Veterinary Internal Medicine 4: 144-147.

255. Thrall D (1982) Orthovoltage radiotherapy of canine transmissible venereal tumors. Veterinary Radiology & Ultrasound 23: 217-219.

256. Powers RD (1968) Immunologic properties of canine transmissible venereal sarcoma. American Journal of Veterinary Research 29: 1637-1645.

257. Pai C-C, Kuo T-F, Mao SJT, Chuang T-F, Lin C-S, et al. (2011) Immunopathogenic behaviours of canine transmissible venereal tumour in dogs following an immunotherapy using dendritic/tumour cell hybrid. Veterinary Immunology and Immunopathology 139: 187-199.

258. Pearse A-M, Swift K, Hodson P, Hua B, McCallum H, et al. (2012) Evolution in a transmissible cancer: a study of the chromosomal changes in devil facial tumor (DFT) as it spreads through the wild Tasmanian devil population. Cancer Genetics 205: 101-112.

259. Griner LA (1979) Neoplasms in Tasmanian devils (*Sarcophilus harrisii*). Journal of the National Cancer Institute 62: 589-593.

260. Guiler E (1970a) Observations on the Tasmanian devil, *Sarcophilus harrisii* (Marsupialia : Dasyuridae) I. Numbers, home range, movements, and food in two populations. Australian Journal of Zoology 18: 49-62.

261. www.tassiedevil.com.au (2011): Department of Primary Industries, Parks, Water and the Environment , Tasmania.

262. Hamede R, McCallum H, Jones ME (2008) Seasonal, demographic and density-related patterns of contact between Tasmanian devils (*Sarcophilus harrisii*): Implications for transmission of devil facial tumour disease. Australian Ecology 33: 614-622.

263. Hamede RK, Bashford J, McCallum H, Jones M (2009) Contact networks in a wild Tasmanian devil (Sarcophilus harrisii) population: using social network analysis to reveal seasonal variability in social behaviour and its implications for transmission of devil facial tumour disease. Ecology Letters 12: 1147-1157.

264. Kreiss A, Obendorf DL, Hemsley S, Canfield PJ, Woods GM (2009) A histological and immunohistochemical analysis of lymphoid tissues of the Tasmanian devil. The Anatomical Record: Advances in Integrative Anatomy and Evolutionary Biology 292: 611-620.

265. Howson LJ (2011) Tumour immune escape mechanisms in DFTD [Honours Thesis] BMedRes [Honours Thesis]. Hobart: University of Tasmania.

266. Pearse A-M, Swift K (2006) Allograft Theory: transmission of devil facial-tumour disease. Nature 439: 549.

267. Lachish S, McCallum H, Jones ME (2009) Demography, disease and the devil: life-history changes in a disease-affected population of Tasmanian devils (*Sarcophilus harrisii*). Journal of Animal Ecology 78: 427-436.

268. McCallum H, Tompkins DM, Jones ME, Lachish S, Marvanek S, et al. (2007) Distribution and impacts of Tasmanian devil facial tumor disease. EcoHealth 4: 318-325.

269. Loh R, Bergfeld J, Hayes D, O'Hara A, Pyecroft S, et al. (2006 a) The Pathology of Devil Facial Tumor Disease (DFTD) in Tasmanian Devils (Sarcophilus harrisii). Veterinary Pathology 43: 890-895.

270. Siddle H, Kreiss A, Eldridge MD, Noonan E, Clarke CJ, et al. (2007) Transmission of a fatal clonal tumour by biting occurs due to depleted MHC diversity in a threatened carnivorous marsupial. Proceedings of the National Academy of Sciences USA 104: 16221-16226.

271. Murchison EP, Tovar C, Hsu A, Bender HS, Kheradpour P, et al. (2010) The Tasmanian devil transcriptome reveals schwann cell origins of a clonally transmissible cancer. Science 327: 84-87.

272. Murchison EP, Schulz-Trieglaff OB, Ning Z, Alexandrov LB, Bauer MJ, et al. (2012) Genome sequencing and analysis of the Tasmanian devil and its transmissible cancer. Cell 148: 780-791.

273. Loh R, Hayes D, Mahjoor A, O'Hara A, Pyecroft S, et al. (2006 b) The immunohistochemical characterization of devil facial tumor disease (DFTD) in the Tasmanian devil (*Sarcophilus harrisii*). Veterinary Pathology 43: 896-903.

274. Tovar C, Obendorf D, Murchison EP, Papenfuss AT, Kreiss A, et al. (2011) Tumor-specific diagnostic marker for transmissible facial tumours of Tasmanian devils: Immunohistochemistry studies. Veterinary Pathology 48: 1195-1203.

275. Pyecroft SB, Pearse AM, Loh R, Swift K, Belov K (2007) Towards a case definition for devil facial tumour disease: What is it? EcoHealth 4: 346-351

276. Bradshaw CJA, Brook BW (2005) Disease and the devil: density-dependent epidemiological processes explain historical population fluctuations in the Tasmanian devil. Ecography 28: 181-190.

277. McKenzie LM, Cooper DW (1994) Low MHC class-Ii variability in a marsupial. Reproduction Fertility and Development 6: 721-726.

278. Jones ME, Patekau D, Geffen E, Moritz C (2004) Genetic diversity and population structure of Tasmanian devils, the largest marsupial carnivore. Molecular Ecology 13: 2197-2209.

279. Siddle H, Sanderson C, Belov K (2007) Characterization of major histocompatibility complex class I and class II genes from the Tasmanian devil (Sarcophilus harrisii). Immunogenetics 59: 753-760.

280. Siddle HV, Marzec J, Cheng Y, Jones ME, Belov K (2010) MHC gene copy number variation in Tasmanian devils: implications for the spread of a contagious cancer. Proceedings of the Royal Society B: Biological Sciences 277: 2001-2006.

281. Hamede R, Lachish S, Belov K, Woods GM, Kreiss A, et al. (2012) Reduced effect of Tasmanian devil facial tumor disease at the disease front. Conservation Biology 26: 124-134.

282. Miller W, Hayes VM, Ratan A, Petersen DC, Wittekindt NE, et al. (2011) Genetic diversity and population structure of the endangered marsupial *Sarcophilus harrisii* (Tasmanian devil). Proceedings of the National Academy of Sciences USA 109: 30.

283. Lane A, Cheng Y, Wright B, Hamede R, Levan L, et al. (2012) New insights into the role of MHC diversity in devil facial tumour disease. PLoS One 7: e36955.

284. Jones ME (2012) Will Tasmanian devils follow Tasmanian tigers to extinction? Royal Society of Tasmania Winter Lecture Series 2012: Royal Society of Tasmania.

285. Siddle HV, Kreiss A, Tovar C, Cheng YY, Belov K, et al. (2013) Reversible epigenetic down-regulation of MHC molecules by devil facial tumour disease illustrates immune escape by a contagious cancer. Proceedings of the National Academy of Sciences USA March 11, 2013.

286. Hsiao Y-W, Liao K-W, Hung S-W, Chu R-M (2002) Effect of tumor infiltrating lymphocytes on the expression of MHC molecules in canine transmissible venereal tumor cells. Veterinary Immunology and Immunopathology 87: 19-27.

287. Finn OJ (2003) Cancer vaccines: Between the idea and the reality. Nature Reviews Immunology 3: 630-641.

288. Ludewig B, Ochsenbein AF, Odermatt B, Paulin D, Hengartner H, et al. (2000) Immunotherapy with dendritic cells directed against tumor antigens shared with normal host cells results in severe autoimmune disease. The Journal of Experimental Medicine 191: 795-804.

289. van Elsas A, Sutmuller RPM, Hurwitz AA, Ziskin J, Villasenor J, et al. (2001) Elucidating the autoimmune and antitumor effector mechanisms of a treatment based on cytotoxic T lymphocyte antigen-4 blockade in combination with a B16 melanoma vaccine. The Journal of Experimental Medicine 194: 481-490.

290. Dudley ME, Wunderlich JR, Robbins PF, Yang JC, Hwu P, et al. (2002) Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. Science 298: 850-854.

291. Pashine A, Valiante NM, Ulmer JB (2005) Targeting the innate immune response with improved vaccine adjuvants. Nature Medicine 11: S63-S68.

292. Valmori D, Souleimanian NE, Tosello V, Bhardwaj N, Adams S, et al. (2007) Vaccination with NY-ESO-1 protein and CpG in Montanide induces integrated antibody/Th1 responses and CD8 T cells through cross-priming. Proceedings of the National Academy of Sciences USA 104: 8947-8952.

293. Janetzki S, Palla D, Rosenhauer V, Lochs H, Lewis JJ, et al. (2000) Immunization of cancer patients with autologous cancer-derived heat shock protein gp96 preparations: A pilot study. The International Journal of Cancer 88: 232-238.

294. Azumi N, Battifora H (1987) The distribution of vimentin and keratin in epithelial and nonepithelial neoplasms. A comprehensive immunohistochemical study on formalin- and alcohol-fixed tumors. American journal of clinical pathology 88: 286-296.

295. Amato DO (2003) Vaccine therapy for renal cell carcinoma. Reviews in Urology 5: 65-71.

296. Milner RJ, Salute M, Crawford C, Abbot JR, Farese J (2006) The immune response to disialoganglioside GD3 vaccination in normal dogs: A melanoma surface antigen vaccine. Veterinary Immunology and Immunopathology 114: 273-284.

297. Tovar CD (2012) Characterisation of the origin of a transmissible neoplasm, DFTD [PhD Thesis]. Hobart: University of Tasmania.

298. Gurunathan S, Klinman DM, Seder RA (2000) DNA vaccines: Immunology, application and optimization. Annual Review of Immunology 18: 927-974.

299. Siegrist C-A (2008) General aspects of vaccination. In: Plotkin S, Orenstein W, Offit P, editors. Vaccine Immunology. 5 ed. Philadelphia: Saunders Elsevier. pp. 17-36.

300. Bergman PJ, McKnight J, Novosad A, Charney S, Farrelly J, et al. (2003) Long-term survival of dogs with advanced malignant melanoma after DNA vaccination with xenogeneic human tyrosinase. Clinical Cancer Research 9: 1284-1290.

301. Chiang CL-L (2011) Adjuvants for enhancing the immunogrnicity of whole tumour cell vaccines. International Reviews of Immunology 30: 150-182.

302. Garnett CT, Palena C, Chakarborty M, Tsang K-Y, Schlom J, et al. (2004) Sublethal irradiation of human tumor cells modulates phenotype resulting in enhanced killing by cytotoxic T lymphocytes. Cancer Research 64: 7985-7994.

303. Reits EA, Hodge JW, Herberts CA, Groothuis TA, Chakraborty M, et al. (2006) Radiation modulates the peptide repertoire, enhances MHC class I expression, and induces successful antitumor immunotherapy. The Journal of Experimental Medicine 203: 1259-1271.

304. Sontag W, Kruglikov IL (2009) Expression of heat shock proteins after ultrasound exposure in HL-60 cells. Ultrasound in Medicine and Biology 35: 1032-1041.

305. Zhou J, Weng D, Zhou F, Pan K, Song H, et al. (2009) Patient-derived renal cell carcinoma cells fused with allogeneic dendritic cells elicit anti-tumor activity: *in vitro* results and clinical responses. Cancer Immunology and Immunotherapy 58: 1587-1597.

306. Cao D-Y, Yang J-Y, Yue S-Q, Tao K-S, Song Z-S, et al. (2009) Comparative analysis of DC fused with allogeneic hepatocellular carcinoma cell line HepG2 and autologous tumor cells as potential cancer vaccines against hepatocellular carcinoma. Cellular Immunology 259: 13-20.

307. Hu Z, Liu S, Mai X, Hu Z, Liu C (2010) Anti-tumor effects of fusion vaccine prepared by renal cell carcinoma 786-O cell line and peripheral blood dendritic cells of healthy volunteers in vitro and in human immune reconstituted SCID mice. Cellular Immunology 262: 112-119.

308. Landuzzi L, Antognoli A, Nicoletti G, Croci S, Palladini A, et al. (2011) HER-2/neu tolerant and non-tolerant mice for fine assessment of antimetastatic potency of dendritic cell-tumor cell hybrid vaccines. Vaccine 29: 4690-4697.

309. Kumar S, Jones TR, Oakley MS, Zheng H, Kuppusamy SP, et al. (2004) CpG oligodeoxynucleotide and montanide ISA 51 adjuvant combination enhanced the protective efficacy of a subunit malaria vaccine. Infection and Immunity 72: 949-957.

310. Pal S, Peterson EM, de la Maza LM (2005) Vaccination with the *Chlamydia trachomatis* major outer membrane protein can elicit an immune response as protective as that resulting from inoculation with live bacteria. Infection and Immunity 73: 8153-8160.

311. Fourcade J, Kudela P, Andrade Filho PA, Janjic B, Land SR, et al. (2008) Immunization with analog peptide in combination with CpG and montanide expands tumor antigen-specific CD8+ T cells in melanoma patients. The Journal of Immunotherapy 31: 781-791.

312. Verdijk RM, Mutis T, Esendam B, Kamp J, Melief CJM, et al. (1999) Polyriboinosinic polyribocytidylic acid (poly(I:C)) induces stable maturation of functionally active human dendritic cells. The Journal of Immunology 163: 57-61.

313. Drakes ML, Lu L, Subbotin VM, Thomson AW (1997) *In vivo* administration of flt3 ligand markedly stimulates generation of dendritic cell progenitors from mouse liver. The Journal of Immunology 159: 4268-4278.

314. Maraskovsky E, Brasel K, Teepe M, Roux ER, Lyman SD, et al. (1996) Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 ligand-treated mice: multiple dendritic cell subpopulations identified. The Journal of Experimental Medicine 184: 1953-1962.

315. Vollstedt S, Franchini M, Hefti HP, Odermatt B, O'Keeffe M, et al. (2003) Flt3 ligand–treated neonatal mice have increased innate immunity against intracellular pathogens and efficiently control virus infections. The Journal of Experimental Medicine 197: 575-584.

316. Shaw SG, Maung AA, Steptoe RJ, Thomson AW, Vujanovic3 NL (1998) Expansion of functional NK cells in multiple tissue compartments of mice treated with Flt3-ligand: Implications for anti-cancer and anti-viral therapy. The Journal of Immunology 161: 2817-2824.

317. Schnurr M, Orban M, Robson NC, Shin A, Braley H, et al. (2009) ISCOMATRIX adjuvant induces efficient cross-presentation of tumor antigen by dendritic cells via rapid cytosolic antigen delivery and processing via tripeptidyl peptidase II. The Journal of Immunology 182: 1253-1259.

318. Atkins MB, Regan M, McDermott D (2004) Update on the role of interleukin 2 and other cytokines in the treatment of patients with stage IV renal carcinoma. Clinical Cancer Research 10: 6342S-6346S.

319. Rosenberg SA, Mulé JJ, Spiess PJ, Reichert CM, Schwarz SL (1985) Regression of established pulmonary metastases and subcutaneous tumor mediated by the systemic administration of high-dose recombinant interleukin 2. The Journal of Experimental Medicine 161: 1169-1188.

320. Astoul P, Viallat JR, Laurent JC, Brandely M, Boutin C (1993) Intrapleural recombinant IL-2 in passive immunotherapy for malignant pleural effusion. Chest 103: 209-213.

321. Mackall CL, Fry TJ, Gress RE (2011) Harnessing the biology of IL-7 for therapeutic application. Nature Reviews Immunology 11: 330-342.

322. Colombetti S, Lévy F, Chapatt L (2009) IL-7 adjuvant treatment enhances long-term tumor antigen-specific CD8+ T-cell responses after immunization with recombinant lentivector. Blood 113: 6629-6637.

323. Melchionda F, Fry TJ, Milliron MJ, McKirdy MA, Tagaya Y, et al. (2005) Adjuvant IL-7 or IL-15 overcomes immunodominance and improves survival of the CD8+ memory cell pool. The Journal of Clinical Investigation 115: 1177-1187.

324. Di Carlo E, Comes A, Basso S, De Ambrosis A, Meazza R, et al. (2000) The combined action of IL-15 and IL-12 gene transfer can induce tumor cell rejection without T and NK cell involvement. The Journal of Immunology 165: 3111-3118.

325. He H, Wisner P, Yang G, Hu H-M, Haley E, et al. (2006) Combined IL-21 and low-dose IL-2 therapy induces anti-tumor immunity and long-term curative effects in a murine melanoma tumor model. Journal of Translational Medicine 4: 1-16.

326. Curti BD, Redman BG, Thompson JA, Sievers EL (2005) Preliminary tolerability and anti-tumor activity of intravenous recombinant human Interleukin-21 (IL-21) in patients with metastatic melanoma and metastatic renal cell carcinoma. Journal of Clinical Oncology, 2005 ASCO Annual Meeting Proceedings 23: 2502.

327. Kirkwood JM, Strawderman MH, Ernstoff MS, Smith TJ, Borden EC, et al. (1996) Interferon alfa-2b adjuvant therapy of high-risk resected cutaneous melanoma: the Eastern Cooperative Oncology Group Trial EST 1684. The Journal of Clinical Oncology 14: 7-17.

328. Lee S, Margolin K (2011) Cytokines in cancer immunotherapy. Cancers 3: 3856-3893.

329. Muss HB, Costanzi JJ, Leavitt R, Williams RD, Kempf RA, et al. (1987) Recombinant alfa interferon in renal cell carcinoma: a randomized trial of two routes of administration. The Journal of Clinical Oncology 5: 286-291.

330. Clark JI, Gaynor ER, Martone B, Budds SC, Manjunath R, et al. (1999) Daily subcutaneous ultra-low-dose interleukin 2 with daily low-dose interferon-α in patients with advanced renal cell carcinoma. Clinical Cancer Research 5: 2374-2380.

331. Bukowski R, Ernstoff MS, Gore ME, Nemunaitis JJ, Amato R, et al. (2002) Pegylated interferon alfa-2b treatment for patients with solid tumors: A phase I/II study. The Journal of Clinical Oncology 20: 3841-3949.

332. Pyrhönen S, Salminen E, Ruutu M, Lehtonen T, Nurmi M, et al. (1999) Prospective randomized trial of interferon alfa-2a plus vinblastine versus vinblastine alone in patients with advanced renal cell cancer. The Journal of Clinical Oncology 17: 2859-2867.

333. Melichar B, Koralewski P, Ravaud A, Pluzanska A, Bracarda S, et al. (2008) First-line bevacizumab combined with reduced dose interferon-α2a is active in patients with metastatic renal cell carcinoma. Annals of Oncology 19: 1470-1476.

334. Kirkwood JM, Manola J, Ibrahim J, Sondak V, Ernstoff MS, et al. (2004) A pooled analysis of eastern cooperative oncology group and intergroup trials of adjuvant high-dose interferon for melanoma. Clinical Cancer Research 10: 1670-1677.

335. Mocellin S, Pasquali S, Rossi CR, Nitti D (2010) Interferon alpha adjuvant therapy in patients with high-risk melanoma: A systematic review and meta-analysis. Journal of the National Cancer Institute 102: 493-501.

336. Rosenberg SA, Yang JC, Topalian SL, Schwartzentruber DJ, Weber JS, et al. (1994) Treatment of 283 consecutive patients with metastatic melanoma or renal cell cancer using high-dose bolus interleukin 2. Journal of the American Medical Association 271: 907-913.

337. Fisher RI, Rosenberg SA, Fyfe G (2000) Long-term survival update for high-dose recombinant interleukin-2 in patients with renal cell carcinoma. The Cancer Journal from the Scientific American 6: S55-S57.

338. Propper DJ, Chao D, Braybrooke JP, Bahl P, Thavasu P, et al. (2003) Low-dose IFN-γ induces tumor MHC expression in metastatic malignant melanoma. Clinical Cancer Research 9: 84-92.

339. Dudley ME, Wunderlich JR, Shelton TE, Even J, Rosenberg SA (2003) Generation of tumor-infiltrating lymphocyte cultures for use in adoptive transfer therapy for melanoma patients. The Journal of Immunotherapy 26: 332-342.

340. Dudley ME, Wunderlich JR, Yang JC, Sherry RM, Topalian SL, et al. (2005) Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma. The Journal of Clinical Oncology 23: 2346-2357.

341. Besser MJ, Shapira-Frommer R, Treves AJ, Zippel D, Itzhaki O, et al. (2010) Clinical responses in a phase II study using adoptive transfer of short-term cultured tumor infiltration lymphocytes in metastatic melanoma patients. Clinical Cancer Research 16: 2646-2655.

342. Tran KQ, Zhou J, Durflinger KH, Langhan MM, Shelton TE, et al. (2008) Minimally cultured tumor-infiltrating lymphocytes display optimal characteristics for adoptive cell therapy. The Journal of Immunotherapy 31: 742-751.

343. Grimm EA, Mazumder A, Zhang HZ, Rosenberg SA (1982) Lymphokine-activated killer cell phenomenon. Lysis of natural killer-resistant fresh solid tumor cells by interleukin 2-activated autologous human peripheral blood lymphocytes. The Journal of Experimental Medicine 155: 1823-1841.

344. Mazumder A, Rosenberg SA (1984) Successful immunotherapy of natural killer-resistant established pulmonary melanoma metastases by the intravenous adoptive transfer of syngeneic lymphocytes activated in vitro by interleukin 2. The Journal of Experimental Medicine 159: 495-507.

345. Lafreniere R, Rosenberg SA (1985) Adoptive immunotherapy of murine hepatic metastases with lymphokine activated killer (LAK) cells and recombinant interleukin 2 (RIL 2) can mediate the regression of both immunogenic and nonimmunogenic sarcomas and an adenocarcinoma. The Journal of Immunology 135: 4273-4280.

346. Lu P-H, Negrin RS (1994) A novel population of expanded human CD3+CD56+ cells derived from T cells with potent *in vivo* antitumor activity in mice with severe combined immunodeficiency'. The Journal of Immunology 153 1687-1696.

347. Mulé JJ, Shu S, Schwarz SL, Rosenberg SA (1984) Adoptive immunotherapy of established pulmonary metastases with LAK cells and recombinant interleukin-2. Science 225: 1487-1489.

348. Helms MW, Prescher JA, Cao Y-A, Schaffert S, Contag CH (2010) IL-12 enhances efficacy and shortens enrichment time in cytokine-induced killer cell immunotherapy. Cancer Immunology and Immunotherapy 59: 1325-1334.

349. Kim YJ, Lim J, Kang JS, Kim HM, Lee HK, et al. (2010) Adoptive immunotherapy of human gastric cancer with ex vivo expanded T cells. Archives of Pharmacal Research 33: 1789-1795.

350. Wang FS, Liu MX, Zhang B, Shi M, Lei ZY, et al. (2002) Antitumor activities of human autologous cytokine-induced killer (CIK) cells against hepatocellular carcinoma cells in vitro and in vivo. World Journal of Gastroenterology 8: 464-468.

351. Wang Y, Dai H, Li H, Lv H, Wang T, et al. (2011) Growth of human colorectal cancer SW1116 cells is inhibited by cytokine-induced killer cells. Clinical and Developmental Immunology 2011: 1-9.

352. Schmidt-Wolf IG, Lefterova P, Mehta BA, Fernandez LP, Huhn D, et al. (1993) Phenotypic characterization and identification of effector cells involved in tumor cell recognition of cytokine-induced killer cells. Experimental Hematology 21: 1673-1679.

353. Lu X, Yang B, Yu R, Chi X, Tuo S, et al. (2011) Clinical study of autologous cytokine-induced killer cells for the treatment of elderly patients with diffuse large B-cell lymphoma. Cell Biochemistry and Biophysics 62: 257-265.

354. Weng D-S, Zhou J, Zhou Q-M, Zhao M, Wang Q-J, et al. (2008) Minimally invasive treatment combined with cytokine-induced killer cells therapy lower the short-term recurrence rates of hepatocellular carcinomas. The Journal of Immunotherapy 31: 63-71.

355. Shi M, Zhang B-Z, Tang Z-R, Lei Z-Y, Wang H-F, et al. (2004) Autologous cytokine-induced killer cell therapy in clinical trial phase I is safe in patients with primary hepatocellular carcinoma. World Journal of Gastroenterology 10: 1146 -1151.

356. Callahan MK, Wolchok JD, Allison JP (2010) Anti–CTLA-4 Antibody Therapy: Immune Monitoring During Clinical Development of a Novel Immunotherapy. Seminars in Oncology 37: 473 - 484.

357. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, et al. (2012) Safety, Activity, and Immune Correlates of Anti–PD-1 Antibody in Cancer. New England Journal of Medicine 366: 2443 - 2454.

358. Small EJ, Tchekmedyian S, Rini BI, Fong L, Lowy I, et al. (2007) A pilot trial of CTLA-4 blockade with human anti-CTLA-4 in patients with hormone-refractory prostate cancer. Clinical Cancer Research 13.

359. Yang JC, Hughes M, Kammula U, Royal R, Sherry RM, et al. (2007) Ipilimumab (anti-CTLA4 antibody) causes regression of metastatic renal cell cancer associated with enteritis and hypophysitis. The Journal of Immunotherapy 30: 825 – 830.

360. Weber JS, Thompson JA, Hamid O, Minor D, Amin A, et al. (2009) A Randomized, Double-Blind, Placebo-Controlled, Phase II Study Comparing the Tolerability and Efficacy of Ipilimumab Administered with or without Prophylactic Budesonide in Patients with Unresectable Stage III or IV Melanoma. Clinical Cancer Research 15: 5591 – 5598.

361. Wolchok JD, Neyns B, Linette G, Negrier S, Lutzky J, et al. (2010) Ipilimumab monotherapy in patients with pretreated advanced melanoma: a randomised, double-blind, multicentre, phase 2, dose-ranging study. Lancet Oncology 11: 155 – 164.

362. O'Day SJ, Maio M, Chiarion-Sileni V, Gajewski TF, Pehamberger H, et al. (2010) Efficacy and safety of ipilimumab monotherapy in patients with pretreated advanced melanoma: a multicenter single-arm phase II study. Annals of Oncology 21: 1712 – 1717.

363. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, et al. (2010) Improved survival with ipilimumab in patients with metastatic melanoma. New England Journal of Medicine 363: 711 - 723.

364. Webber J (2007) Anti–CTLA-4 antibody Ipilimumab: case studies of clinical response and immune-related adverse events. The Oncologist 12: 864 – 872.

365. Gulley JL, Madan RA, Arlen PM (2007) Enhancing efficacy of therapeutic vaccinations by combination with other modalities. Vaccine 25: B89 – B96.

366. Hodi FS, Butler M, Oble DA, Seiden MV, Haluska FG, et al. (2008) Immunologic and clinical effects of antibody blockade of cytotoxic T lymphocyte-associated antigen 4 in previously vaccinated cancer patients. Proceedings of the National Academy of Sciences 105: 3005 – 3010.

367. Phan GQ, Yang JC, Sherry RM, Hwu P, Topalian SL, et al. (2003) Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. Proceedings of the National Academy of Sciences 100: 8372 – 8377.

368. Di Giacomo AM, Biagioli M, Maioa M (2010) The Emerging Toxicity Profiles of Anti–CTLA-4 Antibodies Across Clinical Indications. Seminars in Oncology 37: 499 - 507.

369. Beck KE, Blansfield JA, Tran KQ, Feldman AL, Hughes MS, et al. (2006) Enterocolitis in patients with cancer after antibody blockade of cytotoxic T-lymphocyte–associated antigen 4. The Journal of Clinical Oncology 24: 2283 - 2289.

370. Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, et al. (2000) Engagement of the PD-1 Immunoinhibitory Receptor by a Novel B7 Family Member Leads to Negative Regulation of Lymphocyte Activation. The Journal of Experimental Medicine 192: 1027 – 1034.

371. Iwai Y, Ishida M, Tanaka Y, Okazaki T, Honjo T, et al. (2002) Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. Proceedings of the National Academy of Sciences 99: 12293 – 12297.

372. Gogas H, Ioannovich J, Dafni U, Stavropoulou-Giokas C, Frangia K, et al. (2006) Prognostic significance of autoimmunity during treatment of melanoma with interferon. New England Journal of Medicine 354: 709-718.

373. Schwartzentruber DJ (2001) Guidelines for the safe administration of high-dose interleukin-2. The Journal of Immunotherapy 24: 287-293.

374. Storch MK, Stefferl, Brehm U, Weissert R, Wallström E, et al. (1998) Autoimmunity to myelin oligodendrocyte glycoprotein in rats mimics the spectrum of multiple sclerosis pathology. Brain Pathology 8: 681-694.

375. Brown GK (2008) Genetic Diversity, Cytotoxic T Lymphocyte Responses and Anti-Cancer Therapy in Tasmanian devils [Honours Thesis] BBiotech. Hobart: University of Tasmania.

376. Zhong R, Teng J, Han B, Zhong H (2011) Dendritic cells combining with cytokine-induced killer cells synergize chemotherapy in patients with late-stage non-small cell lung cancer. Cancer Immunology and Immunotherapy 60: 1497–1502.

377. Jiang J, Xu N, Wu C, Deng H, Lu M, et al. (2006) Treatment of advanced gastric cancer by chemotherapy combined with autologous cytokine-induced killer cells. Anticancer Research 26: 2237-2242.

378. Wu C, Jiang J, Shi L, Xu N (2008) Prospective study of chemotherapy in combination with cytokine-induced killer cells in patients suffering from advanced non-small cell lung cancer. Anticancer Research 28: 3997-4002.

379. Yron I, Wood TA, Spiess PJ, Rosenberg SA (1980) *In vitro* growth of murine T cells. V. The isolation and growth of lymphoid cells infiltrating syngeneic solid tumors. The Journal of Immunology 125: 238-245.

380. Tanaka S, Masuda Y, Honma C, Hosaka K, Takahashi K, et al. (2012) Manganese promotes phorbol ester-induced interleukin-2 production via AP-1 activation in Jurkat T-cells. Toxicology Letters 211: 312-318.

381. Strober W (2001) Trypan Blue Exclusion Test of Cell Viability. Current Protocols in Immunology 7: A.3B.1-2.

382. Torri G (2010) Inhalation anesthetics: a review. Minerva Anestesiologica 76: 215-228.

383. Hathcock KS, Coligan JE, Kruisbeek AM, Narguiles DH, Schevach EM, et al. (2001) T cell enrichment by nonadherence to nylon. Current Protocols in Immunology 3: 3.2.1 - 3.2.4.

384. Horwitz DA, Bakke AC, Abo W, Nishiya K (1984) Monocyte and NK cell cytotoxic activity in human adherent cell preparations: discriminating effects of interferon and lactoferrin. Journal of Immunology 132: 2370-2374.

385. Kreiss A (2009) The immune response of the Tasmanian devil (*Sarcophilus harrisii*) and the devil facial tumour disease [PhD Thesis]. Hobart: University of Tasmania.

386. Avril T, Jarousseau A-C, Watier H, Boucraut J, Le Bouteiller P, et al. (1999) Trophoblast cell line resistance to NK lysis mainly involves an HLA class I-independent mechanism. The Journal of Immunology 162: 5902-5909.

387. Weigelin B, Krause M, Friedl P (2011) Cytotoxic T lymphocyte migration and effector function in the tumor microenvironment. Immunology Letters 138: 19-21.

388. Lozzio BB, Lozzio CB (1979) Properties and usefulness of the original K-562 Human myelogenous leukemia cell line. Leukemia Research 3: 363-370.

389. Santin AD, Rose GS, Hiserodt JC, Fruehauf J, Eck LM, et al. (1996) Effects of cytokines combined with high-dose gamma irradiation on the expression of major histocompatibility complex molecules and intercellular adhesion molecule-1 in human ovarian cancers. The International Journal of Cancer 65: 688-694.

390. Chakraborty M, Abrams SI, Camphausen K, Liu K, Scott T, et al. (2003) Irradiation of tumor cells up-regulates fas and enhances CTL lytic activity and CTL adoptive immunotherapy. The Journal of Immunology 170: 6338-6347.

391. Sparwasser T, Koch E-S, Vabulas RM, Heeg K, Lipford GB, et al. (1998) Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. European Journal of Immunology 28: 2045-2054.

392. Roohvand F, Aghasadeghi M-R, Sadat SM, Budkowska A, Khabiri A-R (2007) HCV core protein immunization with Montanide/CpG elicits strong Th1/Th2 and long-lived CTL responses. Biochemical and Biophysical Research Communications 354: 641-649.

393. Sommer S (2005) The importance of immune gene variability (MHC) in evolutionary ecology and conservation. Frontiers in Zoology 2:16: 1-18.

394. Srivastava PK, Amato RJ (2001) Heat shock proteins: the 'Swiss Army Knife' vaccines against cancers and infectious agents. Vaccine 19: 2590-2597.

395. Wang H-H, Mao C-Y, Teng L-S, Jiang C (2006) Recent advances in heat shock protein-based cancer vaccines. Hepatobiliary and Pancreatic Diseases International 5: 22-27.

396. Seebach JD, Yamada K, McMorrow IM, Sachs DH, DerSimonian H (1996) Xenogenic human anti-pig cytotoxicity mediated by activated natural killer cells. Xenotransplantation 3: 188-197.

397. Shaw GM, Levy PC, Lobuglio AF (1978) Human monocyte antibody-dependent cell-mediated cytotoxicity to tumor cells. The Journal of Clinical Investigation 62: 1172-1180.

398. Ortaldo JR, Bonnard GD, Herberman RB (1977) Cytotoxic reactivity of human lymphocytes cultured *in vitro*. The Journal of Immunology 119: 1351-1357.

399. Lei H-Y, Chang C-P (2009) Lectin of Concanavalin A as an anti-hepatoma therapeutic agent. The Journal of Biomedical Science 16: 1-12.

400. Palacios R (1982) Concanavalin A triggers T lymphocytes by directly interacting with their receptors for activation. The Journal of Immunology 128: 337-342.

401. Weigent DA, Stanton GJ, Johnson HM (1983) Recombinant gamma interferon enhances natural killer cell activity similar to natural gama interferon. Biochemical and Biophysical Research Communications 111: 525-529.

402. Fidler IJ, Darnell JH, Budmen MB (1976) Tumoricidal properties of mouse macrophages activated with mediators from rat lymphocytes stimulated with Concanavalin A. Cancer Research 36: 3608-3615.

403. Schmidt ME, Douglas SD, Rubin AD (1973) Human monocyte activation by supernatants from Concanavalin A (Con A) stimulated lymphocytes. Cellular Immunology 9: 45-59.

404. Winchester RJ (1976) Methods for enumerating lymphocyte populations; Rose NR, Friedman H, editors: American Society for Microbiology. 64-66 p.

405. Uellner R, Zvelebil MJ, Hopkins J, Jones J, MacDougall LK, et al. (1997) Perforin is activated by a proteolytic cleavage during biosynthesis which reveals a phospholipid-binding C2 domain. The European Molecular Biology Organization Journal 16: 7287-7296.

406. Griffin JD, Hercend T, Beveridge R, Schlossman SF (1983) Characterization of an antigen expressed by human natural killer cells. The Journal of Immunology 130: 2947-2951.

407. Kroemer A, Xiao X, Degauque N, Edtinger K, Wei H, et al. (2008) The innate NK Cells, allograft rejection, and a key role for IL-15. The Journal of Immunology 180: 7818-7826.

408. Butterworth AE, Sturrock RF, Houba V, Mahmoud AAF, Sher A, et al. (1975) Eosinophils as mediators of antibody-dependent damage to schistosomula. Nature 256: 727-729.

409. Stockmeyer B, Beyer T, Neuhuber W, Repp R, Kalden JR, et al. (2003) Polymorphonuclear granulocytes induce antibody-dependent apoptosis in human breast cancer cells. The Journal of Immunology 171: 5124-5129.

410. Otten MA, Rudolph E, Dechant M, Tuk CW, Reijmers RM, et al. (2005) Immature neutrophils mediate tumor cell killing via IgA but not IgG Fc receptors. The Journal of Immunology 174: 5472-5480.

411. Koyama S, Fukao K (1994) Phenotypic analysis of nylon-wool-adherent suppressor cells that inhibit the effector process of tumor cell lysis by lymphokine-activated killer cells in patients with advanced gastric carcinoma. The Journal of Cancer Research and Clinical Oncology 120: 240-247.

412. Miyagi T, Takehara T, Tatsumi T, Suzuki T, Jinushi M, et al. (2004) Concanavalin A injection activates intrahepatic innate immune cells to provoke an antitumor effect in murine liver. Hepatology 40: 1190-1196.

413. Marcucci F, Klein B, Altevogt P, Landolfo S, Kirchner H (1984) Concanavalin A-Induced Interferon Gamma Production by Murine Spleen Cells and T Cell Lines. Lack of Correlation with Lyt 1, 2 Phenotype. Immunobiology 166: 219-227.

414. Kaattari SL, Rittenberg MB (1982) Concanavalin A supernatant recruits antigen-insensitive IgG memory B lymphocyte precursors into an antigen-sensitive precursor pool. The Journal of Immunology 128: 720-725.

415. Gillis S, Smith KA, Watson J (1980) Biochemical characterization of lymphocyte regulatory molecules. II. Purification of a class of rat and human lymphokines. The Journal of Immunology 124: 1954-1962.

416. Longhi MP, Trumpfheller C, Idoyaga J, Caskey M, Matos I, et al. (2009) Dendritic cells require a systemic type I interferon response to mature and induce CD4+ Th1 immunity with poly IC as adjuvant. The Journal of Experimental Medicine 206: 1589-1602.

417. Djeu JY, Heinbaugh JA, Holden HT, Herberman RB (1979) Role of macrophages in the augmentation of mouse natural killer cell activity by poly I:C and interferon. The Journal of Immunology 122: 182-188.

418. Masucci MG, Masucci G, Klein E, Berthold W (1980) Target selectivity of interferon-induced human killer lymphocytes related to their Fc receptor expression. Proceedings of the National Academy of Sciences USA 77: 3620-3624.

419. Bubeník J, Perlmann P, Hasek M (1970) Induction of cytotoxicity of lymphocytes from tolerant donors by antibodies to target cell alloantigens. Transplantation 10: 290-296

420. Khan A, Gregorie C, Tomasi T (2008) Histone deacetylase inhibitors induce TAP, LMP, Tapasin genes and MHC class I antigen presentation by melanoma cells. Cancer Immunology, Immunotherapy 57: 647-654.

421. Shimizu K, Kurosawa Y, Taniguchi M, Steinman RM, Fujii S-i (2007) Cross-presentation of glycolipid from tumor cells loaded with α-galactosylceramide leads to potent and long-lived T cell–mediated immunity via dendritic cells. The Journal of Experimental Medicine 204: 2641-2653.

422. Kabelitz D, Wesch D, He W (2007) Perspectives of γδ T cells in tumor immunology. Cancer Research 67: 5-8.

423. Hung K, Hayashi R, Lafond-Walker A, Lowenstein C, Pardoll D, et al. (1998) The central role of CD4+ T cells in the antitumor immune response. The Journal of Experimental Medicine 188: 2357-2368.

424. Overwijk WW, Theoret MR, Finkelstein SE, Surman DR, de Jong LA, et al. (2003) Tumor regression and autoimmunity after reversal of a functionally tolerant state of self-reactive CD8+ T cells. The Journal of Experimental Medicine 198: 569-580.

425. Bowne WB, Srinivasan R, Wolchok JD, Hawkins WG, Blachere NE, et al. (1999) Coupling and uncoupling of tumor immunity and autoimmunity. The Journal of Experimental Medicine 190: 1717-1722.

426. Quaglino P, Marenco F, Osella-Abate S, Cappello N, Ortoncelli M, et al. (2010) Vitiligo is an independent favourable prognostic factor in stage III and IV metastatic melanoma patients: results from a single-institution hospital-based observational cohort study. Annals of Oncology 21: 409-414.

427. Lachish S, Jones ME, McCallum H (2007) The impact of disease on the survival and population growth rate of the Tasmanian devil. The Journal of Animal Ecology 76: 926-936.

428. Stone WH, Bruun DA, Foster EB, Manis GS, Hoffman ES, et al. (1998) Absence of a significant mixed lymphocyte reaction in a marsupial (*Monodelphis domestica*). Comparative Medicine 48: 184-189.

429. Long BR, Michaelsson J, Loo CP, Ballan WM, Vu B-AN, et al. (2008) Elevated frequency of gamma interferon-producing NK cells in healthy adults vaccinated against influenza virus. Clinical and Vaccine Immunology 15: 120-130.

430. Horowitz A, Behrens RH, Okell L, Fooks AR, Riley EM (2010) NK cells as effectors of acquired immune responses: Effector CD4+ T cell-dependent activation of NK cells following vaccination. The Journal of Immunology 185: 2808-2818.

431. Vargas-Inchaustegui DA, Xiao P, Tuero I, Patterson LJ, Robert-Guroff M (2012) NK and CD4+ T cell cooperative immune responses correlate with control of disease in a macaque simian immunodeficiency virus infection model. The Journal of Immunology 189: 1878-1885.

432. Scott-Algara D, Mancini-Bourgine M, Fontaine H, Pol S, Michel M-L (2010) Changes to the natural killer cell repertoire after therapeutic hepatitis B DNA vaccination. PLoS One 5: e8761.

433. Bajénoff M, Breart B, Huang AYC, Qi H, Cazareth J, et al. (2006) Natural killer cell behavior in lymph nodes revealed by static and real-time imaging. The Journal of Experimental Medicine 203: 619-631.

434. Krebs P, Barnes MJ, Lampe K, Whitley K, Bahjat KS, et al. (2009) NK cell–mediated killing of target cells triggers robust antigen-specific T cell–mediated and humoral responses. Blood 113: 6593-6602.

435. van der Kraan LE, Wong ESW, Lo N, Ujvari B, Belov K (2012) Identification of natural killer cell receptor genes in the genome of the marsupial Tasmanian devil (Sarcophilus harrisii). Immunogenetics: 1-11.

436. Lindsey WB, Lowdell MW, Marti GE, Abbasi F, Zenger V, et al. (2007) CD69 expression as an index of T-cell function: assay standardization, validation and use in monitoring immune recovery. Cytotherapy 9: 123-132.

437. Tabaczewski P, Stroynowski I (1994) Expression of secreted and glycosylphosphatidylinositol-bound Qa-2 molecules is dependent on functional TAP-2 peptide transporter. The Journal of Immunology 152: 5268 - 5274.

438. Belov K, Deakin JE, Papenfuss AT, Baker ML, Melman SD, et al. (2006) Reconstructing an ancestral mammalian immune supercomplex from a marsupial major histocompatibility complex. PLoS Biology 4: e46.

439. Belov K, Sanderson CE, Deakin JE, Wong ESW, Assange D, et al. (2007) Characterization of the opossum immune genome provides insights into the evolution of the mammalian immune system. Genome Research 17: 982-991.

440. Daly K, Church WB, Nicholas K, Williamson P (2007) Comparative modeling of marsupial MHC class I molecules identifies structural polymorphisms affecting functional motifs. Journal of Experimental Zoology Part A: Ecological Genetics and Physiology 307A: 611-624.

441. Kondo M, Maruoka T, Otsuka N, Kasamatsu J, Fugo K, et al. (2010) Comparative genomic analysis of mammalian NKG2D ligand family genes provides insights into their origin and evolution. Immunogenetics 62: 441-450.

442. Croix DA, Samples NK, Vandeberg JL, Stone WH (1989) Immune response of a marsupial (Monodelphis domestica) to sheep red blood cells. Developmental & Comparative Immunology 13: 73-78.

443. Infante AJ, Samples NK, Croix DA, Redding TS, VandeBerg JL, et al. (1991) Cellular immune response of a marsupial, *Monodelphis domestica*. Developmental & Comparative Immunology 15: 189-199.

444. Tahara H, Zitvogel L, Storkus WJ, Zeh HJ, McKinney TG, et al. (1995) Effective eradication of established murine tumors with IL-12 gene therapy using a polycistronic retroviral vector. The Journal of Immunology 154: 6466-6474.

445. Watanabe Y, Kuribayashi K, Miyatake S, Nishihara K, Nakayama E, et al. (1989) Exogenous expression of mouse interferon gamma cDNA in mouse neuroblastoma C1300 cells results in reduced tumorigenicity by augmented anti-tumor immunity. Proceedings of the National Academy of Sciences USA 86: 9456-9460.

446. Dranoff G, Jaffee E, Lazenby A, Golumbek P, Levitsky H, et al. (1993) Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. Proceedings of the National Academy of Sciences USA 90: 3539-3543.

447. Gutterman JU, Rosenblum MG, Rios A, Fritsche HA, Quesada JR (1984) Pharmacokinetic study of partially pure γ-interferon in cancer patients. Cancer Research 44: 4164-4171.

448. Gansbacher B, Bannerji R, Daniels B, Zier K, Cronin K, et al. (1990) Retroviral vector-mediated γ-interferon gene transfer into tumor cells generates potent and long lasting antitumor immunity. Cancer Research 50: 7820-7825.

449. McHugh RS, Nagarajan S, Wang Y-C, Sell KW, Selvaraj P (1999) Protein transfer of glycosyl-phosphatidylinositol-B7-1 into tumor cell membranes. Cancer Research 59: 2433-2437.

450. Nagarajan S, Selvaraj P (2002) Glycolipid-anchored IL-12 expressed on tumor cell surface induces antitumor immune response. Cancer Research 62: 2869-2874.

451. Palena C, Schlom J (2010) Vaccines against human carcinomas: Strategies to improve antitumor immune responses. Journal of Biomedicine and Biotechnology 2010: 1-12.

452. Zhou J, Sun XY, Stenzel DJ, Frazer IH (1991) Expression of vaccinia recombinant HPV 16 L1 and L2 ORF proteins in epithelial cells is sufficient for assembly of HPV virion-like particles. Virology 185: 251-257.

453. Hamede RK, McCallum H, Jones ME (2012) Biting injuries and transmission of Tasmanian devil facial tumour disease. The Journal of Animal Ecology 82: 182-190.

Chapter 8 - Appendices

Section A.1 - Additional Tables – Materials and Methods

Table 8.1. Wild Tasmanian devils

Tasmanian devil number	Sex	Disease status	Reference Name	Capture location
Dd 1	Female	DFTD infected	Ada	West Pencil Pine
Dd 2	Female	DFTD infected	Nancy	West Pencil Pine
Dd 3	Male	DFTD infected	Swampy	Forestier Peninsula
Dd 4	Male	DFTD infected	Puma	West Pencil Pine
Dd 5	Male	DFTD infected	Jas	Forestier Peninsula
Dd 6	Male	DFTD infected	Scabby Scoota	Forestier Peninsula
Dd 7	Female	DFTD infected	Pink Peregrine	Forestier Peninsula
Dd 8	Male	DFTD infected	Gavin Dainty	Forestier Peninsula
Dd 9	Male	DFTD infected	Black Jack	Forestier Peninsula
Dd 10	Male	DFTD infected	Donkey Kong	Forestier Peninsula
Dd 11	Male	DFTD infected	Ed	Forestier Peninsula
Dd 12	Male	DFTD infected	Eenie Teeny Mikey Mo	Forestier Peninsula
Dd 13	Female	DFTD infected	Flame	Forestier Peninsula
Dd 14	Male	DFTD infected	Pussy Gums	Forestier Peninsula
Dd 15	Female	DFTD infected	Tsarina	Forestier Peninsula
Dd 16	Male	DFTD infected	Pete	Forestier Peninsula
Dd 17	Male	DFTD infected	Tank	Forestier Peninsula
Dd 18	Male	DFTD infected	Kerry	Forestier Peninsula
Wd 1	Male	Healthy	Panthazar	West Pencil Pine
Wd 2	Male	Unknown	Flynn	Forestier Peninsula
Wd 3	Female	Unknown	Esquivela	West Pencil Pine
Wd 4	Male	Unknown	Okapi	West Pencil Pine
Wd 5	Male	Unknown	Azael	West Pencil Pine
Wd 6	Male	Unknown	Pomaire	West Pencil Pine
Wd 7	Female	Unknown	Unknown (Neo)	Freycinet Peninsula

Table 8.2. Captive Tasmanian devils

Tasmanian devil number	Sex	Age (at first use in experiments)	Reference Name	Captive facility
Cd 1	Female	3	Grevillia	Fern Tree
Cd 2	Female	5	8444	Richmond
Cd 3	Female	5	6356	Richmond
Cd 4	Female	5	7277	Richmond
Cd 5	Female	5	8130	Richmond
Cd 6	Male	3	Catman	Fern Tree
Cd 7	Female	2	Betty	Fern Tree
Cd 8	Female	3	Mel	Fern Tree
Cd 9	Female	4	Candy	Fern Tree
Cd 10	Male	3	Grommit	Fern Tree
Cd 11	Female	3	Estrella	West Pencil Pine (relocated to Fern Tree)
Cd 12	Male	3	Wazza	Fern Tree
Cd 13	Female	3	Tiarna	Fern Tree
Cd 14	Male	5	Cedric	Fern Tree
Cd 15	Female	4	Missy	Fern Tree
Cd 16	Male	4	Tom	Fern Tree
Cd 17	Female	3	Carlotta	Fern Tree
Cd 18	Male	4	Bob	Fern Tree
Cd 19	Male	1	Bailey	Taroona
Cd 20	Male	1	Leo	Taroona
Cd 21	Female	1	Leila	Taroona
Cd 22	Female	1	Lolita	Taroona
Cd 23	Female	1	Storm	Taroona
Cd 24	Male	1	Mather	Taroona
Cd 25	Male	1	Muffs	Taroona
Cd 26	Male	1	Chaps	Taroona
Cd 27	Female	1	Weenie	Taroona

Cd 28	Female	1	Wizzie	Taroona
Cd 29	Male	1	Axl	Taroona
Cd 30	Female	1	Elsie	Taroona
Cd 31	Female	1	Lottie	Taroona
Cd 32	Female	1	Mildred	Taroona
Cd 33	Female	1	November Rain	Taroona
Cd 34	Female	1	Pilsner	Taroona
Cd 35	Male	1	Aggy	Fern Tree
Cd 36	Female	1	Poppy	Fern Tree
Cd 37	Male	1	Toby	Fern Tree
Cd 38	Male	2	Phil	Fern Tree
Cd 39	Female	5	Michelle	Fern Tree
Cd 40	Female	5	Tilly	Fern Tree
Cd 41	Male	5	Felix	Fern Tree
Cd 42	Female	5	Maydeem	Fern Tree

Table 8.3. K562 Immunised Tasmanian devils

Tasmanian devil number	Immunogen	Doses	Adjuvant	Reference Name	Captive facility
Cd 2	Untreated K562	2	Montanide Gel 645101	8444	Richmond
Cd 3	Untreated K562	2	Montanide Gel 645101	6356	Richmond
Cd 4	Untreated K562	2	Montanide Gel 645101	8130	Richmond
Cd 5	Untreated K562	2	Montanide Gel 645101	7277	Richmond
Cd 6	Irradiated K562	3	Montanide ISA51 VG	Catman	Fern Tree
Cd 7	Irradiated K562	3	Montanide ISA51 VG	Betty	Fern Tree

Table 8.4. DFTD Immunised Tasmanian devils

Tasmanian devil number	Immunogen	Doses	Adjuvant	Reference Name	Captive facility
Cd 8	Irradiated DFTD	4	Montanide ISA51 VG	Mel	Fern Tree
Cd 9	Irradiated DFTD	4	Montanide ISA51 VG	Candy	Fern Tree
Cd 10	Irradiated DFTD Sonicated DFTD	3 2	Montanide Gel 645101 CpG ODN 1585	Grommit	Fern Tree
Cd 11	Irradiated DFTD	3	Montanide Gel 645101 CpG ODN 1668	Estrella	Fern Tree
Cd 12	Irradiated DFTD	3	Montanide Gel 645101 CpG ODN 1668	Wazza	Fern Tree
Cd 13	Irradiated DFTD	3	Montanide Gel 645101 CpG ODN 1585	Tiarna	Fern Tree
Cd 14	Sonicated DFTD (previously immunised)	2	Montanide Gel 645101 CpG ODN	Cedric	Fern Tree
Cd 15	DFTD cell extract Live DFTD cell challenge	3	ISCOMATRIX®	Tom	Fern Tree
Cd 16	DFTD cell extract Live DFTD cell challenge	3	ISCOMATRIX®	Missy	Fern Tree

Cd 39	Frozen-thawed MHC I positive DFTD Live DFTD cell challenge	2	ISCOMATRIX®	Michelle	Fern Tree
Cd 40	Frozen-thawed MHC I positive DFTD Live DFTD cell challenge	2	ISCOMATRIX®	Tilly	Fern Tree
Cd 1	Trichostatin A treated DFTD Live DFTD cell challenge	3	ISCOMATRIX®	Grevillia	Fern Tree
Cd 7	DFTD protein,	3	ISCOMATRIX®, Flt 3 Ligand, Poly I:C	Betty	Fern Tree
Cd 17	DFTD protein,	2	ISCOMATRIX®, Flt3 and Poly I:C	Carlotta	Fern Tree

Table 8.5. Immunotherapy devils

Tasmanian devil number	Sex	Treatment	Doses	Reference Name	Location
Cd 15	Female	LAK cell immunotherapy MHC I positive cell immunotherapy Intratumoural cytokine injection	3 2 3	Missy	Fern Tree (captive devil)
Dd 11	Male	MHC I positive cell injection	1	Ed	Forestier Peninsula (relocated to Richmond)
Dd 18	Male	MHC I positive cell injection	1	Kerry	Forestier Peninsula (relocated to Richmond)

Section A.2 - Additional Tables – Chapter 3 Cytotoxicity assay data

Table 8.6. Cytotoxicity responses against DFTD cells in infected Tasmanian devils and healthy controls

Devil	Ratio	Mean (CPM)	SD (CPM)	Cytotoxicity (%)			Mean (%)	SD (%)	Significance (F test)	
Wd 1	50	794.7	48.7	4	0	-2	1	3		
	25	769.5	37.1	-3	2	-2	-1	3		
	12	741.8	62.6	0	-1	-8	-3	4		
	6	793.4	14.8	0	2	0	1	1		
Cd 1	50	964.3	102.9	-3	5	4	2	4		
	25	906.4	65.8	-3	0	2	0	3		
	12	933.0	103.9	3	4	-4	1	4		
	6	857.2	84.1	0	-6	-1	-2	4		
Dd 1	50	2213.4	71.2	5	10	5	7	3	0.3887	
	25	2196.0	45.1	5	5	8	6	2	0.4742	
	12	2176.3	43.6	6	3	6	5	2		
	6	2020.7	56.0	1	0	0	0	1		
Dd 2	50	640	12	2	1	1	1	0	0.1734	
	25	587	3	0	0	0	0	0	0.1369	
	12	591	29	1	0	0	0	1		
	6	567	68	1	-1	-1	0	2		
Dd 3	50	717	48	4	4	2	3	1	0.5884	
	25	689	11	2	2	3	2	0	0.5037	
	12	674	80	0	3	3	2	2		
	6	676	67	2	3	1	2	2		
Dd 4	50	636.6	84.9	-4	-11	-16	-10	6	0.2118	
	25	662.6	45.2	-5	-10	-10	-9	3	0.1683	
	12	646.2	18.3	-11	-9	-9	-10	1		
	6	643.8	43.0	-11	-12	-7	-10	3		
Dd 5	50	283.3	54.6	1	4	0	2	2	0.2188	
	25	244.7	13.9	0	1	0	0	1	0.1741	
	12	248.7	22.7	1	-1	1	0	1		
	6	239.7	10.5	0	0	0	0	0		
Dd 6	50	801.9	20.1	1	3	0	1	1	0.9969	
	25	782.9	23.4	1	-2	1	0	2	0.8785	
	12	804.4	69.5	3	-4	5	1	5		
	6	750.6	31.1	-5	-1	-2	-2	2		
Dd 7	50	754.1	58.8	2	-3	-6	-2	4	0.3639	
	25	705.7	37.6	-3	-7	-7	-6	3	0.4426	
	12	699.8	4.4	-6	-6	-6	-6	0		
	6	645.1	24.9	-10	-11	-8	-10	2		
Dd 8	50	713.1	39.9	-2	-5	-8	-5	3	0.1953	
	25	731.8	9.7	-3	-4	-4	-4	1	0.1547	
	12	707.5	23.4	-4	-4	-7	-5	2		
	6	721.9	9.6	-5	-4	-5	-4	1		

* Significantly different to healthy wild devil sample, # Significantly different to captive devil sample

Table 8.7. Cytotoxicity responses against K562 cells in Tasmanian devils

Devil	Assay	Ratio	Mean (CPM)	SD (CPM)	Cytotoxicity (%)			Mean (%)	SD (%)	Significance (F test)	
Cd 2	PI	100	886.6	70.4	1	0	2	1	1		
		50	838.3	12.9	0	0	0	0	0		
		25	782.7	38.4	0	0	0	0	0		
		12	766.1	33.4	0	0	0	0	0		
		6	779.2	77.5	0	0	0	0	0		
	Dose 1	100	884.7	185.7	26	10	21	19	8	0.0000	*
		50	838.1	218.5	25	6	20	17	10		
		25	602.1	45.5	4	5	8	6	2		
		12	489.4	9.6	0	1	1	1	1		
		6	427.5	12.5	0	0	0	0	0		
	Dose 2	100	1377.5	63.7	46	43	41	43	3	0.0000	*
		50	915.7	54.0	22	19	24	22	3		
		25	642.1	22.1	10	8	10	9	1		
		12	480.4	16.5	2	2	1	2	1		
		6	467.8	34.6	3	0	1	1	2		
Cd3	PI	100	1133.6	55.2	5	7	5	6	1		
		50	949.1	82.9	4	0	2	2	2		
		25	954.4	53.1	2	1	3	2	1		
		12	797.4	83.2	1	0	0	0	1		
		6	743.8	23.7	0	0	0	0	0		
	Dose 1	100	529.7	41.3	3	1	4	3	2	0.1406	
		50	510.2	23.7	2	1	3	2	1		
		25	506.3	4.9	2	1	2	2	1		
		12	471.9	68.3	3	0	1	1	2		
		6	455.3	4.1	0	0	0	0	0		
	Dose 2	50	286.8	56.9	2	11	3	5	5	0.9599	
		25	216.4	13.2	0	0	0	0	0		
		12	191.8	10.7	0	0	0	0	0		
		6	153.5	108.5	0	0	0	0	0		
		3	219.1	50.8	0	4	0	1	2		
Cd 4	PI	100	843.9	21.9	0	0	1	0	1		
		50	911.7	16.1	1	2	2	2	1		
		25	891.7	34.6	1	1	0	1	1		
		12	765.0	148.5	1	0	0	0	1		
		6	796.5	29.1	0	0	0	0	0		
	Dose 1	100	559.4	57.8	7	2	3	4	3	0.0548	
		50	565.9	8.1	4	4	5	4	1		
		25	494.2	25.6	0	2	2	1	1		
		12	471.2	23.3	0	0	1	0	1		
		6	434.5	20.1	0	0	0	0	0		
	Dose 2	100	1933.8	130.4	65	67	76	69	6	0.0000	*
		50	1759.8	65.3	58	63	62	61	3		
		25	1057.5	80.3	32	25	29	29	4		
		12	623.7	32.4	10	8	7	8	2		
		6	501.8	24.5	2	4	2	3	1		

Cd 5	PI	100	1073.3	443.9	15	1	0	5	8		
		50	799.4	65.2	0	0	0	0	0		
		25	863.3	2.4	1	1	1	1	0		
		12	904.5	120.9	0	0	4	1	2		
		6	762.1	23.3	0	0	0	0	0		
	Dose 1	100	588.0	90.9	10	3	3	5	4	0.7479	
		50	582.2	12.3	4	5	6	5	1		
		25	503.0	45.5	0	1	4	2	2		
		12	446.7	11.5	0	0	0	0	0		
		6	441.0	49.0	0	0	0	0	0		
	Dose 2	100	1235.4	45.7	35	39	36	37	2	0.0026	*
		50	719.9	47.9	11	15	12	13	2		
		25	568.5	21.6	7	5	5	6	1		
		12	456.8	49.4	3	0	0	1	2		
		6	406.8	12.0	0	0	0	0	0		

* Significantly different to the Pre Immune sample

Table 8.8. Memory cytotoxicity responses against K562 cells

Devil	Assay	Ratio	Mean (CPM)	SD (CPM)	Cytotoxicity (%)			Mean (%)	SD (%)	Significance (F test)	
Cd 2	PI	100	886.6	70.4	1	0	2	1	1		
		50	838.3	12.9	0	0	0	0	0		
		25	782.7	38.4	0	0	0	0	0		
		12	766.1	33.4	0	0	0	0	0		
		6	779.2	77.5	0	0	0	0	0		
		3	721.2	27.8	0	0	0	0	0		
	Dose 2	100	1377.5	63.7	46	43	41	43	3	0.0000	*
		50	915.7	54.0	22	19	24	22	3		
		25	642.1	22.1	10	8	10	9	1		
		12	480.4	16.5	2	2	1	2	1		
		6	467.8	34.6	3	0	1	1	2		
	4 Months Post Dose 2	100	3146.6	91.6	61	60	56	59	3	0.0000	*
		50	1595.5	195.3	23	12	17	17	6	0.4997	
		25	1001.8	15.2	1	2	1	1	1		
		12	909.6	66.3	0	1	0	0	1		
		6	799.5	65.6	0	0	0	0	0		
	Challenge	100	5895.3	201.5	71	66	66	68	3	0.0000	*
		50	3929.5	536.0	45	42	30	39	8	0.4161	
		25	2516.0	89.0	20	19	18	19	1		
		12	1711.9	75.7	7	6	8	7	1		
		6	1176.4	37.2	0	0	0	0	0		
Cd 4	PI	100	843.9	21.9	0	0	1	0	1		
		50	911.7	16.1	1	2	2	2	1		
		25	891.7	34.6	1	1	0	1	1		
		12	765.0	148.5	1	0	0	0	1		
		6	796.5	29.1	0	0	0	0	0		
		3	778.8	88.7	0	0	0	0	0		
	Post Dose 2	100	1933.8	130.4	65	67	76	69	6	0.0000	*
		50	1759.8	65.3	58	63	62	61	3		
		25	1057.5	80.3	32	25	29	29	4		
		12	623.7	32.4	10	8	7	8	2		
		6	501.8	24.5	2	4	2	3	1		
	4 Months Post Dose 2	100	1606.2	89.2	20	15	17	17	3	0.0003	*
		50	982.4	28.7	0	1	1	1	1	0.0213	#
		25	782.3	45.0	0	0	0	0	0		
		12	787.9	20.1	0	0	0	0	0		
		6	807.6	26.6	0	0	0	0	0		
	Challenge	100	5175.7	3265.5	80	88	89	86	5	0.0000	*
		50	6086.6	96.4	71	69	71	70	1	0.9929	
		25	4793.7	124.4	51	54	50	52	2		
		12	3188.0	30.0	28	29	29	29	1		
		6	2022.7	44.7	12	12	11	12	1		

* Significantly different to the Pre Immune sample, # Significantly different to the previous sample

Table 8.9. Cytotoxicity responses against irradiated K562 cells

Devil	Assay	Ratio	Mean (CPM)	SD (CPM)	Cytotoxicity (%)			Mean (%)	SD (%)	Significance (F test)	
Cd 6	PI	100	465.2	33.0	9	6	7	7	2		
		50	420.4	33.4	7	5	5	6	1		
		25	396.2	46.3	3	7	5	5	2		
		12	347.4	18.3	2	3	3	3	1		
	Dose 1	50	551.5	73.6	10	4	5	6	3	0.9135	
		25	403.8	26.2	1	1	0	1	1		
		12	395.8	27.8	0	0	1	0	1		
		6	394.2	19.5	0	0	1	0	1		
	Dose 2	50	3885.7	190.1	4	6	4	5	1	0.4884	
		25	3400.2	546.7	6	1	1	3	3		
		12	3249.7	144.5	2	1	2	2	1		
		6	2808.6	158.8	0	0	0	0	0		
		3	2637.4	46.8	0	0	0	0	0		
	Dose 3	100	1033.5	38.8	63	60	56	60	4	0.0335	*
		50	616.3	62.7	30	21	20	24	6		
		25	433.4	5.0	8	8	7	8	1		
		12	379.8	3.9	3	3	3	3	0		
		6	328.3	6.1	0	0	0	0	0		
Cd 7	PI	100	382.9	33.2	4	3	5	4	1		
		50	366.5	13.3	3	4	3	3	1		
		25	335.7	67.4	4	3	0	2	2		
		12	327.0	5.2	2	2	1	2	1		
	Dose 1	50	455.5	40.3	4	3	1	3	2	0.5537	
		25	380.1	27.6	1	0	0	0	1		
		12	379.4	32.4	1	0	0	0	1		
		6	359.3	5.5	0	0	0	0	0		
	Dose 2	50	4009.1	53.5	6	6	5	6	1	0.4496	
		25	4107.1	208.3	7	5	6	6	1		
		12	3413.7	52.8	3	2	2	2	1		
		6	3290.7	122.3	1	2	2	2	1		
		3	3129.7	130.0	1	2	0	1	1		
	Dose 3	100	738.6	43.2	34	30	38	34	4	0.1211	
		50	449.4	38.2	12	9	6	9	3		
		25	374.8	4.2	2	3	3	3	1		
		12	368.5	12.4	1	2	3	2	1		
		6	332.2	8.9	0	0	0	0	0		

* Significantly different to the Pre Immune sample

Table 8.10. Innate cytotoxic cell assays: cell line specificity and 4 hour reactions

Devil	Assay	Ratio	Mean (CPM)	SD (CPM)	Cytotoxicity (%)			Mean (%)	SD (%)	Significance (F test)	
Cd 14	Naive devil	50	665.0	31.7	2	1	1	1	1		
		25	588.3	15.8	0	0	0	0	0		
		12	537.4	32.8	0	0	0	0	0		
		6	527.5	34.5	0	0	0	0	0		
Cd 2	Immunised devil	100	925.9	105.1	0	2	0	1	1	0.2042	
		50	455.4	23.2	0	0	0	0	0		
		25	342.1	22.9	0	0	0	0	0		
		12	330.4	29.6	0	0	0	0	0		
		6	391.8	45.8	0	0	0	0	0		
Cd 3	YAC1 tumour cells	50	1267.7	162.0	0	0	0	0	0		
		25	1164.1	415.2	0	0	0	0	0		
		12	1712.8	677.9	0	0	1	1	1		
		6	1131.1	109.4	0	0	0	0	0		
	K562 tumour cells	50	522.0	47.0	0	0	0	0	0	0.5709	
		25	746.0	220.6	0	1	0	0	0		
		12	578.3	23.0	0	0	0	0	0		
		6	514.6	91.3	0	0	0	0	0		
	1:1 Mixture (YAC1 labelled)	50	7504.1	243.2	58	57	61	58	2	0.0000	#
		25	6593.4	1044.3	46	61	44	50	10		
		12	5238.0	625.7	36	44	33	38	6		
		6	5069.8	598.8	31	42	35	36	6		

* Significantly different to the naive sample, # significantly different to the YAC-1 cell sample

Table 8.11. Cytotoxicity against irradiated DFTD cells in Montanide adjuvant

Devil	Assay	Ratio	Mean (CPM)	SD (CPM)	Cytotoxicity (%)			Mean (%)	SD (%)	Significance (F test)	
Cd 8	PI	50	399.0	19.7	7	0	2	3	4		
		25	406.8	53.3	14	5	0	6	7		
		12	368.0	38.9	0	0	5	2	3		
		6	375.2	26.2	3	0	0	1	2		
	Dose 1	100	3261.0	179.3	8	10	1	6	5	0.6264	
		50	3268.8	232.2	0	11	9	7	6		
		25	2983.5	124.1	0	0	1	0	1		
		12	2998.7	51.2	0	1	0	0	1		
		6	2939.4	42.9	0	0	0	0	0		
	Dose 2	100	2677.4	102.3	10	6	5	7	3	0.7076	
		50	2893.4	659.3	3	2	33	3	1		
		25	2627.7	61.0	8	5	4	6	2		
		12	2740.8	69.9	8	7	11	9	2		
		6	2472.2	106.9	3	0	4	2	2		

	Dose 3	100	1561.9	76.9	8	4	3	5	3	0.9744	
		50	1550.9	20.0	5	4	5	5	1		
		25	1449.4	10.6	1	1	1	1	0		
		12	1546.1	20.5	4	5	5	5	1		
		6	1386.1	71.5	0	1	0	0	1		
	Dose 4	100	620.5	29.9	10	6	6	7	2	0.8927	
		50	601.5	20.3	8	5	4	6	2		
		25	623.4	36.5	6	6	11	8	3		
		12	576.2	26.5	1	4	6	4	3		
		6	550.4	5.2	2	2	1	2	1		
Cd 9	PI	50	357.0	34.9	3	0	0	1	2		
		25	333.2	23.3	0	0	0	0	0		
		12	345.2	4.5	0	0	0	0	0		
		6	341.2	20.5	0	0	0	0	0		
	Dose 1	100	3010.9	47.3	1	0	0	0	1	0.0264	*
		50	3186.0	25.5	3	5	4	4	1		
		25	2881.7	101.9	0	0	0	0	0		
		12	3213.4	112.2	4	3	8	5	3		
		6	2931.8	65.9	0	0	0	0	0		
	Dose 2	100	2600.7	149.3	10	2	3	5	4	0.1638	
		50	2567.4	110.9	7	4	1	4	3		
		25	2466.5	71.3	3	0	1	1	2		
		12	2484.1	148.5	1	0	6	2	3		
		6	2444.9	112.0	4	0	0	1	2		
	Dose 3	100	1554.7	43.9	5	6	3	5	2	0.0612	
		50	1539.7	53.1	3	6	3	4	2		
		25	1493.9	118.6	6	0	3	3	3		
		12	1442.6	23.7	0	2	1	1	1		
		6	1374.6	56.1	0	0	0	0	0		
	Dose 4	100	723.7	34.9	18	16	13	16	3	0.0008	*
		50	808.1	53.1	26	23	17	22	5		
		25	629.9	60.3	8	13	3	8	5		
		12	621.6	46.3	6	12	5	8	4		
		6	581.0	30.8	6	5	1	4	3		

* Significantly different to the Pre Immune sample

Table 8.12. Cytotoxicity responses against irradiated DFTD cells in Montanide adjuvant supplemented with CpG oligonucleotides

Devil	Assay	Ratio	Mean (CPM)	SD (CPM)	Cytotoxicity (%)			Mean (%)	SD (%)	Significance (F test)	
Cd 10	PI	50	1768.5	41.2	11	12	15	13	2		
		25	1758.4	35.5	11	13	13	12	1		
		12	1606.5	14.5	5	6	6	6	1		
		6	1675.9	49.0	11	6	9	9	3		
	Dose 1	50	1926.7	39.5	34	30	32	32	2	0.0911	
		25	1891.9	40.7	31	31	28	30	2		
		12	1897.7	6.3	30	31	30	30	0		
		6	1886.6	132.8	25	37	27	30	7		
	Dose 2	50	1037.0	6.3	33	34	33	33	1	0.0444	*
		25	998.7	30.3	29	33	30	31	2		
		12	806.6	22.0	16	14	17	16	2		
		6	635.9	22.3	4	3	1	3	2		
	Dose 3	50	2169.2	237.9	54	34	40	43	10	0.0151	*
		25	1564.7	92.1	20	13	19	17	4		
		12	1069.7	76.6	0	0	0	0	0		
		6	1155.4	72.2	1	3	0	1	2		
Cd 11	PI	50	1827.8	99.2	1	0	0	0	1		
		25	1816.0	45.7	0	0	0	0	0		
		12	1885.0	84.5	0	0	3	1	2		
		6	1816.7	30.0	0	0	0	0	0		
	Dose 1	50	1698.9	54.8	18	19	23	20	3	0.0000	*
		25	1770.4	42.0	26	25	22	24	2		
		12	1790.4	144.9	33	19	22	25	7		
		6	1624.9	99.6	18	11	21	17	5		
	Dose 2	50	592.0	58.9	4	0	0	1	2	0.5552	
		25	543.8	26.0	0	0	0	0	0		
		12	561.0	50.9	0	0	0	0	0		
		6	538.1	48.6	0	0	0	0	0		
Cd 12	PI	50	1741.9	87.3	15	8	12	12	4		
		25	1702.7	32.9	9	11	10	10	1		
		12	1697.1	29.2	9	9	11	10	1		
		6	1475.4	21.9	0	1	0	0	1		
	Dose 1	50	1628.2	56.7	20	15	15	17	3	0.4088	
		25	1670.5	39.5	17	18	21	19	2		
		12	1758.7	141.6	24	16	30	23	7		
		6	1634.7	101.1	23	15	13	17	5		
	Dose 2	50	720.9	36.1	6	10	11	9	3	0.4670	
		25	615.8	31.2	4	0	0	1	2		
		12	610.0	12.1	0	1	1	1	1		
		6	579.2	1.1	0	0	0	0	0		

	Dose 3	50	1691.1	125.6	29	19	21	23	5	0.3076	
		25	1336.9	95.6	7	4	12	8	4		
		12	1135.1	57.5	0	0	2	1	1		
		6	1105.0	28.1	0	0	0	0	0		
Cd 13	PI	50	2060.4	58.3	6	6	11	8	3		
		25	1932.2	45.4	0	4	1	2	2		
		12	1987.5	16.4	5	5	3	4	1		
		6	1940.2	79.1	4	4	0	3	2		
	Dose 1	50	1918.0	45.3	34	30	31	31	2	0.0226	*
		25	1559.7	20.5	12	14	13	13	1		
		12	1667.8	34.5	20	17	19	19	2		
		6	1576.2	12.4	14	13	15	14	1		
	Dose 2	50	743.9	25.2	9	10	13	11	2	0.1509	
		25	793.8	21.4	13	16	15	15	2		
		12	686.9	23.4	7	8	4	6	2		
		6	557.5	36.5	0	0	0	0	0		
	Dose 3	50	670.0	105.1	6	16	0	6	8	0.8933	
		25	640.1	83.7	0	0	12	4	7		
		12	570.3	47.6	0	0	3	1	2		
		6	661.5	35.2	8	2	7	6	3		

* Significantly different to the Pre Immune sample

Table 8.13. Cytotoxicity responses against sonicated DFTD cells in Montanide adjuvant supplemented with CpG oligonucleotides

Devil	Assay	Ratio	Mean (CPM)	SD (CPM)	Cytotoxicity (%)			Mean (%)	SD (%)	Significance (F test)	
Cd 10	PI	50	599.2	68.2	13	5	0	6	6		
		25	530.2	18.4	0	0	0	0	0		
		12	551.2	42.2	0	5	0	2	3		
		6	566.7	23.1	4	1	0	2	2		
	Dose 1	50	446.2	28.9	33	36	43	38	5	0.0041	*
		25	415.0	24.7	31	37	28	32	4		
		12	408.9	14.7	32	33	28	31	3		
		6	381.8	6.0	26	27	26	26	1		
	Dose 2	50	1243.0	52.7	16	16	12	15	2	0.0139	*
		25	1501.8	153.8	18	31	31	27	7		
		12	1437.7	47.4	22	23	26	24	2		
		6	1140.2	61.6	13	7	10	10	3		
Cd 14	PI	50	598.2	9.6	4	6	6	5	1		
		25	607.7	23.0	6	8	4	6	2		
		12	547.3	20.8	2	0	0	1	1		
		6	521.7	33.1	0	0	0	0	0		
	Dose 1	50	515.2	26.2	53	45	52	50	5	0.0040	*
		25	410.3	32.4	33	25	36	31	6		
		12	422.1	6.5	34	34	32	33	1		
		6	417.6	5.0	32	33	33	33	1		
	Dose 2	50	2312.8	174.8	71	55	66	64	8	0.0012	*
		25	1852.5	43.1	44	41	44	43	2		
		12	1397.5	36.0	22	24	20	22	2		
		6	1281.9	88.6	18	12	20	17	4		

* Significantly different to the Pre Immune sample

Table 8.14. Cytotoxicity responses against DFTD cell total protein in ISCOMATRIX® adjuvant

Devil	Assay	Ratio	Mean (CPM)	SD (CPM)	Cytotoxicity (%)			Mean (%)	SD (%)	Significance (F test)	
Cd 15	PI	50	288.2	19.4	8	4	12	8	4		
		25	280.9	10.0	8	8	4	7	2		
		12	258.3	17.2	-2	4	4	2	4		
		6	245.6	44.7	4	5	-11	0	9		
	Dose 1	50	1606.3	120.6	0	1	6	2	3	0.0290	*
		25	1583.7	30.2	1	2	1	2	1		
		12	1518.3	35.6	-1	1	-1	0	1		
		6	1413.3	84.3	-3	-5	-1	0	2		
	Dose 2	50	941.5	75.9	2	9	7	6	3	0.5822	
		25	956.0	45.9	5	7	9	7	2		
		12	900.4	62.9	7	4	2	4	3		
		6	847.2	71.3	-2	4	4	2	3		
	Dose 3	50	479.2	61.7	-8	-7	5	0	7	0.0281	*
		25	531.2	82.2	-7	2	12	2	9		
		12	496.5	26.8	-1	-5	1	0	3		
		6	491.4	28.7	0	-6	-1	0	3		
Cd 16	PI	50	267.7	4.3	5	4	3	4	1		
		25	253.8	37.2	-8	7	4	1	8		
		12	268.4	12.9	2	6		4	3		
		6	222.6	12.3	-5	-3	-8	0	3		
	Dose 1	50	1634.7	183.0	-3	5	6	2	5	0.2138	
		25	1418.7	121.9	-4	-6	0	0	3		
		12	1485.3	48.8	0	-3	-2	0	1		
		6	1492.7	60.8	-1	-3	0	0	2		
	Dose 2	50	1072.1	86.1	8	14	14	12	4	0.1047	
		25	902.5	16.6	5	4	4	4	1		
		12	1056.6	213.6	8	4	22	11	10		
		6	903.5	153.7	-2	12	4	4	7		
	Dose 3	50	616.6	49.2	8	10	18	12	6	0.1180	
		25	594.7	50.1	3	12	13	10	6		
		12	586.4	10.5	8	10	8	9	1		
		6	539.5	50.1	6	-3	7	3	6		

* Significantly different to the Pre Immune sample

Table 8.15. Cytotoxicity responses against DFTD cell total protein in ISCOMATRIX® adjuvant supplemented with Flt 3 ligand and Poly I:C

Devil	Assay	Ratio	Mean (CPM)	SD (CPM)	Cytotoxicity (%)			Mean (%)	SD (%)	Significance (F test)	
Cd 7	PI	100	419.5	31.4	10	2	5	6	4		
		50	458.6	34.6	9	16	8	11	5		
		25	441.3	21.7	12	6	8	9	3		
		12	431.7	30.3	12	7	3	7	4		
	Day 7 (Dose 1)	50	1070	35	2	1	2	2	1	0.0188	*
		25	985	89	-1	-1	2	0	2		
		12	1031	12	1	1	0	1	0		
		6	1100	162	1	-1	6	2	3		
	Day 14 (Dose 2)	100	1306	84	11	9	19	13	5	0.5919	
		50	1295	25	13	10	13	12	2		
		25	1268	5	11	11	10	11	0		
		12	1170	88	-1	10	5	5	5		
	Day 42 (Dose 3)	100	820	50	-1	-6	0	0	3	0.1976	
		50	830	52	2	-3	-3	0	3		
		25	810	31	-2	-1	-5	0	2		
		12	795	68	-8	-4	1	0	4		
	Day 49 (Dose 3)	100	1124	93	7	1	10	6	4	0.5111	
		50	1141	42	9	5	7	7	2		
		25	1148	24	8	6	6	7	1		
		12	1068	82	-1	6	5	3	4		
Cd 17	PI	100	1633.1	29.9	7	7	9	8	1		
		50	1609.5	273.2	-5	9	16	7	11		
		25	1689.9	148.7	3	11	15	10	6		
		12	1704.7	104.0	15	10	7	11	4		
	Day 7 (Dose 1)	100	1137	109	1	3	5	3	2	0.1580	
		50	1245	59	5	6	4	5	1		
		25	1067	111	4	1	0	1	2		
		12	1070	38	2	2	1	2	1		
	Day 14 (Dose 2)	100	1677	56	37	37	31	35	3	0.0498	*
		50	1497	97	26	29	18	24	6		
		25	1546	18	28	26	27	27	1		
		12	1488	157	21	16	34	24	9		
	Day 42 (Dose 3)	100	1060	19	14	14	12	13	1	0.1377	
		50	941	61	2	10	5	6	4		
		25	870	78	6	-4	2	1	5		
		12	959	46	3	9	8	7	3		
	Day 49 (Dose 3)	100	1312	72	12	18	13	14	3	0.5594	
		50	1284	160	15	19	5	13	7		
		25	1242	24	12	10	11	11	1		
		12	1267	58	14	14	9	12	3		

* Significantly different to the Pre Immune sample

Section A.3 - Additional Tables – Chapter 3 Cytotoxicity assay data

Table 8.16. Cytotoxicity responses of MNC and nylon non-adherent cells from K562 immunised devils against K562 cells

Devil	Sample	Ratio	Mean (CPM)	SD (CPM)	Cytotoxicity (%)			Mean (%)	SD (%)	Significance (F test)	
Cd 2	MNC	25	2584.7	43.8	57	60	57	58	2		
		12	1783.9	54.9	33	30	30	31	2		
		6	1015.9	27.3	4	6	5	5	1		
		3	962.7	65.6	1	5	3	3	2		
	Nylon wool non adherent cells	25	1106.0	94.6	9	10	4	8	3	0.0102	*
		12	872.3	40.7	1	0	0	0	1		
		6	779.3	7.6	0	0	0	0	0		
		3	808.1	18.9	0	0	0	0	0		
Cd 4	MNC	25	9168.9	262.9	63	67	64	64	2		
		12	7080.6	503.7	49	48	41	46	4		
		6	4393.4	130.6	23	22	24	23	1		
		3	2697.8	716.4	3	5	15	8	6		
	Nylon wool non adherent cells	25	2024.7	82.0	1	2	3	2	1	0.0002	*
		12	1778.8	80.5	0	1	0	0	1		
		6	1580.7	97.2	0	0	0	0	0		
		3	1671.5	133.1	0	0	0	0	0		

* Statistically significant compared to response of MNC.

Table 8.17. ADCC responses of MNC from naive devils against K562 cells

Devil	Sample	Ratio	Mean (CPM)	SD (CPM)	Cytotoxicity (%)			Mean (%)	SD (%)	Significance (F test)	
Cd 1	No Antibody	25	636.3	116.6	3	-6	3	0	5		
		12	635.0	146.7	2	-7	5	0	6		
		6	718.0	31.6	5	3	3	4	1		
		3	613.0	118.0	0	4	-6	-1	5		
	Immune Serum	25	1066.7	247.0	32	29	14	25	10	0.0051	*
		12	955.3	10.4	20	20	21	20	0		
		6	712.3	79.1	14	8	10	11	3		
		3	616.7	140.1	1	11	9	7	6		
Cd 16	No Antibody	25	880.7	38.6	-1	-2	-3	0	1		
		12	893.5	55.1	-3	-1	0	0	1		
		6	833.7	37.1	-2	-4	-4	0	1		
	Immune Serum	25	4415.1	697.8	98	109	73	93	18	0.0000	*
		12	3600.9	259.1	65	79	70	71	7		
		6	3018.9	718.4	64	69	33	55	20		

Cd 15	No Antibody	25	2015.4	103.3	1	1	0	1	1		
		12	2024.3	46.7	1	1	1	1	0		
		6	1987.3	135.3	0	0	2	1	1		
		3	1923.8	112.9	-1	0	1	0	1		
	Immune Serum	25	13452.0	515.6	92	110	101	100	9	0.0000	*
		12	11975.8	553.7	70	86	68	75	10		
		6	12715.7	681.1	74	92	97	88	12		
		3	11276.3	694.7	63	74	50	62	12		
Cd 7	No Antibody	25	658.0	155.1	4	6	-7	1	7		
		12	764.3	47.5	8	4	6	6	2		
		6	681.0	43.0	3	0	4	2	2		
		3	652.0	121.3	-5	4	4	1	5		
	Immune Serum	25	1012.0	247.2	11	30	27	23	10	0.0180	*
		12	963.3	42.9	23	19	21	21	2		
		6	788.7	166.9	18	6	18	14	7		
		3	695.7	175.7	13	2	15	10	7		
Dd 2	No Antibody	25	730	79	-1	1	3	1	2		
		12	678	86	-2	-1	2	0	2		
		6	769	26	3	2	2	2	1		
		3	751	62	2	0	3	2	2		
	Immune Serum	25	2784	112	52	52	48	51	3	0.0000	*
		12	2493	113	46	45	40	43	3		
		6	2606	243	53	43	43	46	6		
		3	1829	165	24	32	25	27	4		
Cd 3	No Antibody	25	789	45	2	4	3	3	1		
		12	892	165	10	3	3	5	4		
		6	855	38	3	5	5	4	1		
		3	857	111	2	7	5	4	3		
	Immune Serum	25	32	35	47	38	8	32	35	0.0024	
		12	27	29	25	27	2	27	29		
		6	20	20	18	19	1	20	20		
		3	10	12	14	12	2	10	12		

* Statistically significant compared to untreated sample.

Table 8.18. ADCC responses of MNC and nylon wool non-adherent cells from naive devils against K562 cells

Devil	Sample	Ratio	Mean (CPM)	SD (CPM)	Cytotoxicity (%)			Mean (%)	SD (%)	Significance (F test)	
Cd 1	MNC No Antibody	25	636.3	116.6	3	-6	3	0	5		
		12	635.0	146.7	2	-7	5	0	6		
		6	718.0	31.6	5	3	3	4	1		
		3	613.0	118.0	0	4	-6	-1	5		
	MNC Immune Serum	25	1066.7	247.0	32	29	14	25	10	0.0051	*
		12	955.3	10.4	20	20	21	20	0		
		6	712.3	79.1	14	8	10	11	3		
		3	616.7	140.1	1	11	9	7	6		
	Nylon non adherent cells No Antibody	25	951.7	272.6	27	26	8	20	11	0.0148	*
		12	719.3	34.6	13	11	10	11	1	0.6000	
		6	670.0	86.5	10	5	12	9	3		
		3	559.3	124.9	7	-1	9	5	5		
Cd 7	MNC No Antibody	25	658.0	155.1	4	6	-7	1	7		
		12	764.3	47.5	8	4	6	6	2		
		6	681.0	43.0	3	0	4	2	2		
		3	652.0	121.3	-5	4	4	1	5		
	MNC Immune Serum	25	1012.0	247.2	11	30	27	23	10	0.0180	*
		12	963.3	42.9	23	19	21	21	2		
		6	788.7	166.9	18	6	18	14	7		
		3	695.7	175.7	13	2	15	10	7		
	Nylon non adherent cells No Antibody	25	1031.0	72.9	27	22	22	23	3	0.0249	*
		12	699.3	142.5	12	15	4	13	3	0.8670	
		6	677.7	146.5	14	12	3	13	1		
		3	754.3	22.1	13	13	12	12	1		
Dd 5	MNC No Antibody	25	455.7	70.2	5	1	-1	2	3		
		12	409.0	21.0	-1	0	1	0	1		
		6	429.0	25.2	1	1	-1	1	1		
		3	429.0	16.5	1	0	1	1	1		
	MNC Immune Serum	25	795.0	60.8	18	14	18	17	3	0.0123	*
		12	654.3	40.7	9	12	11	11	2		
		6	564.3	27.6	7	6	8	7	1		
		3	511.0	33.5	6	4	5	5	1		
	Nylon non adherent cells No Antibody	25	659.0	12.1	11	12	11	11	1	0.0256	*
		12	513.7	19.7	6	4	6	5	1	0.6851	
		6	465.3	20.6	3	4	3	3	1		
		3	446.3	22.0	1	3	3	2	1		

* Statistically significant compared to untreated sample, # statistically significant compared to MNC plus antibody.

Table 8.19. ADCC responses of MNC against K562 cells in the presence of supernatant from immune MNC and K562 culture

Devil	Sample	Ratio	Mean (CPM)	SD (CPM)	Cytotoxicity (%)			Mean (%)	SD (%)	Significance (F test)	
Cd 38	No Antibody	25	1060.1	251.9	1	-1	4	1	2		
		12	1129.5	353.2	0	0	6	2	3		
		6	1189.6	442.4	5	4	-2	2	4		
		3	892.3	157.5	1	0	-2	0	1		
	K562 culture Supernatant	25	1048.4	146.4	3	2	1	2	1	0.1818	
		12	1477.5	107.7	6	7	5	6	1		
		6	958.8	115.6	2	1	1	1	1		
		3	737.9	96.6	0	-1	-2	0	1		
Cd 5	No Antibody	25	485.0	45.1	6	10	5	7	3		
		12	552.8	75.0	11	16	7	11	5		
		6	540.7	60.5	10	14	7	11	4		
	K562 culture Supernatant	25	645.2	86.9	15	10	21	15	6	0.6515	
		12	585.7	51.7	8	12	14	11	3		
		6	544.1	33.3	6	9	10	9	2		
Cd 1	No Antibody	25	854.6	43.0	-8	-2	-4	0	3		
		12	863.0	25.4	-3	-6	-3	0	2		
		6	795.2	143.5	-2			0	10		
		3	882.2	33.2	-1	-6	-2	0	2		
	K562 culture Supernatant	25	978.4	43.9	2	1	2	1	0	0.2602	
		12	1132.9	213.0	4	1	4	3	2		
		6	1068.5	177.1	4	1	1	2	2		
		3	1031.7	371.9	2	5	-1	2	3		

* Statistically significant compared to untreated sample.

Table 8.20. 4 hour ADCC responses of MNC from naive devils against K562 cells

Devil	Sample	Ratio	Mean (CPM)	SD (CPM)	Cytotoxicity (%)			Mean (%)	SD (%)	Significance (F test)	
Cd 14 (test 1)	No Antibody	25	588.3	15.8	0	0	-1	1	1		
		12	537.4	32.8	-1	-2	-1	0	1		
		6	527.5	34.5	-2	-1	-2	0	1		
	Immune Serum	25	2242.6	177.9	36	41	33	36	4	0.0001	*
		12	1615.5	128.8	24	21	26	23	3		
		6	1209.4	19.2	15	15	14	15	0		
Cd 10	No Antibody	25	292.4	1.7	0	0	1	0	0		
		12	256.5	16.0	-3	-1	-2	0	1		
		6	276.9	6.3	0	-1	0	0	0		
		3	246.0	51.8	-7	-2	0	0	4		
	Immune Serum	25	555.5	44.4	17	19	23	20	3	0.0000	*
		12	399.4	9.7	9	10	9	9	1		
		6	359.8	28.6	7	8	4	6	2		
		3	295.7	24.2	2	0	4	2	2		
Dd 5	No Antibody	25	225.7	19.8	-1	0	-1	0	1		
		12	245.0	20.4	1	0	0	0	1		
		6	237.3	9.5	0	0	0	0	0		
		3	202.0	26.7	-2	0	-2	0	1		
	Immune Serum	25	615.3	24.0	16	14	15	15	1	0.0000	*
		12	395.7	23.3	5	7	7	7	1		
		6	338.3	51.5	2	5	6	4	2		
		3	278.0	15.6	2	2	1	2	1		
Cd 14 (test 2)	No Antibody	25	357.8	20.4	4	6	5	5	1		
		12	327.1	17.3	4	2	3	3	1		
		6	295.5	20.2	1	-1	2	1	1		
		3	266.0	27.7	0	-4	0	0	2		
	Immune Serum	25	959.8	48.9	51	47	44	47	3	0.0032	*
		12	634.1	14.1	25	25	24	25	1		
		6	556.0	3.0	19	20	19	19	0		
		3	428.7	18.2	12	10	10	11	1		

* Statistically significant compared to untreated sample.

Table 8.21. ADCC responses of MNC and plastic non-adherent cells from naive devils against K562 cells

Devil	Sample	Ratio	Mean (CPM)	SD (CPM)	Cytotoxicity (%)			Mean (%)	SD (%)	Significance (F test)	
Cd 1	MNC No Antibody	25	636.3	116.6	3	-6	3	0	5		
		12	635.0	146.7	2	-7	5	0	6		
		6	718.0	31.6	5	3	3	4	1		
		3	613.0	118.0	0	4	-6	-1	5		
	MNC Immune Serum	25	1066.7	247.0	32	29	14	25	10	0.0051	*
		12	955.3	10.4	20	20	21	20	0		
		6	712.3	79.1	14	8	10	11	3		
		3	616.7	140.1	1	11	9	7	6		
	Plastic non adherent cells No Antibody	25	942.3	106.7	24	20	16	20	4	0.0180	*
		12	678.7	129.2	11	14	4	12	5	0.5338	
		6	594.7	131.3	9	9	0	9	0		
		3	644.0	13.0	7	8	8	8	1		
Dd 5	MNC No Antibody	25	455.7	70.2	5	1	-1	2	3		
		12	409.0	21.0	-1	0	1	0	1		
		6	429.0	25.2	1	1	-1	1	1		
		3	429.0	16.5	1	0	1	1	1		
	MNC Immune Serum	25	795.0	60.8	18	14	18	17	3	0.0178	*
		12	654.3	40.7	9	12	11	11	2		
		6	564.3	27.6	7	6	8	7	1		
		3	511.0	33.5	6	4	5	5	1		
	Plastic non adherent cells No Antibody	25	798.7	89.5	21	13	16	17	4	0.0075	*
		12	632.3	47.4	8	12	10	10	2	0.7917	
		6	541.3	63.8	9	5	5	6	3		
		3	456.0	29.3	4	2	2	3	1		

* Statistically significant compared to untreated sample, # statistically significant compared to MNC plus antibody.

Table 8.22. ADCC responses of MNC from naive devils against DFTD cells in the presence of serum from DFTD immunised mice

Devil	Sample	Ratio	Mean (CPM)	SD (CPM)	Cytotoxicity (%)			Mean (%)	SD (%)
Cd 2	No Antibody	25	464.8	39.8	3	-3	4	7	6
	Mouse serum	25	371.6	36.8	-6	-14	-10	-10	4
Cd 5	No Antibody	25	591.6	32.2	10	16	15	14	3
	Mouse serum	25	589.6	16.9	11	10	13	11	2
Cd 24	No Antibody	25	380.6	120.5	6	-17	-8	-6	11
	Mouse serum	25	369.6	8.3	-10	-11	-10	-10	1
Cd 25	No Antibody	25	390.4	84.0	-4	2	-14	-5	8
	Mouse serum	25	446.4	72.7	-9	-4	5	-3	7
Cd 26	No Antibody	25	406.2	75.0	-5	-11	4	-4	7
	Mouse serum	25	463.8	34.5	-3	3	-3	-1	3
Cd 27	No Antibody	25	411.6	101.1	-7	7	-11	-3	10
	Mouse serum	25	401.6	65.3	-14	-5	-3	-7	6
Cd 28	No Antibody	25	724.6	45.1	26	31	22	26	4
	Mouse serum	25	740.0	66.0	30	29	18	26	6
Cd 29	No Antibody	25	618.7	93.5	17	25	7	16	9
	Mouse serum	25	693.8	105.0	11	22	31	21	10
Cd 30	No Antibody	25	829.3	35.1	36	40	33	36	3
	Mouse serum	25	750.2	64.6	20	33	27	27	6
Cd 31	No Antibody	25	680.5	97.2	11	28	26	22	9
	Mouse serum	25	783.0	65.8	35	23	32	30	6

Cd 32	No Antibody	25	480.3	27.7	2	6	2	3	3
	Mouse serum	25	371.5	52.2	-8	-6	-16	-10	5
Cd 33	No Antibody	25	455.4	56.0	2	-5	5	1	5
	Mouse serum	25	610.8	199.2	2	2	35	13	19
Cd 34	No Antibody	25	541.7	85.0	0	10	17	9	8
	Mouse serum	25	541.7	85.0	-2	7	14	6	8

Statistical significance between untreated and serum supplemented sample groups was p=0.5951.

Table 8.23. ADCC responses of MNC from naive devils against DFTD cells in the presence of serum from DFTD immunised devils

Devil	Sample	Ratio	Mean (CPM)	SD (CPM)	Cytotoxicity (%)			Mean (%)	SD (%)
Cd 2	No Antibody	25	440.1	38.4	2	0	10	4	6
	Mouse serum	25	464.8	39.8	10	1	11	7	6
Cd 5	No Antibody	25	1833.0	107.3	5	19	24	16	10
	Mouse serum	25	1835.9	65.7	23	15	11	16	6
Cd 7	No Antibody	25	645.0	19.3	7	11	7	8	2
	Mouse serum	25	583.2	12.7	5	8	6	6	1
Cd 15	No Antibody	25	397.8	22.2	1	4	0	2	2
	Mouse serum	25	1797.2	96.4	21	4	14	13	9
Cd 16	No Antibody	25	1623.5	19.1	0	0	142	0	0
	Mouse serum	25	456.2	37.3	8	11	4	8	4
Cd 18	No Antibody	25	1664.1	97.2	0	1	9	3	5
	Mouse serum	25	1737.4	64.0	141	3	11	7	6
Cd 19	No Antibody	25	763.0	138.3	11	23	-1	11	12
	Mouse serum	25	694.5	47.0	5	11	4	7	4
Cd 20	No Antibody	25	684.4	108.9	9	10	-7	4	9
	Mouse serum	25	699.9	88.3	-1	10	13	7	7
Cd 21	No Antibody	25	805.9	84.3	22	14	8	15	7
	Mouse serum	25	706.7	51.1	4	12	7	8	4
Cd 22	No Antibody	25	681.3	194.0	17	10	-15	4	17
	Mouse serum	25	801.3	143.3	9	9	30	16	12
Cd 23	No Antibody	25	692.6	148.3	8	16	-9	5	13
	Mouse serum	25	653.6	100.8	0	13	-3	3	9

Statistical significance between untreated and serum supplemented sample groups was p=0.0953. Shaded cells represent samples with suspected contamination by radioactively labelled cells.

Table 8.24. Cytotoxicity responses of MNC from naive devils against DFTD cells in the presence of mitogens

Devil	Sample	Ratio	Mean (CPM)	SD (CPM)	Cytotoxicity (%)			Mean (%)	SD (%)	Significance (F test)	
Cd 18	No Mitogen	50	1609.5	273.2	-5	9	16	7	11		
		25	1689.9	148.7	3	11	15	10	6		
		12	1704.7	104.0	15	10	7	11	4		
	Con A	50	1883.4	235.4	29	11	24	21	9	0.2833	
		25	1827.4	222.1	9	24	24	19	9		
		12	1798.3	91.9	14	19	21	18	4		
	PHA	50	1644.0	54.6	16	19	18	18	2	0.3655	
		25	1663.8	249.1	8	23	24	19	9		
		12	1577.9	151.7	9	20	17	15	6		
Cd 38	No Mitogen	50	1745.5	58.7	10	13	14	12	2		
		25	1546.0	218.2	1	14	-3	4	9		
		12	1584.1	293.7	13	12	-8	6	12		
	Con A	50	2193.1	94.3	37	30	33	33	4	0.1370	
		25	1656.5	225.4	2	17	18	12	9		
		12	1675.0	107.1	8	14	17	13	4		
	PHA	50	1548.0	111.7	19	10	14	14	4	0.4514	
		25	1604.9	221.0	7	22	20	16	8		
		12	1650.1	144.8	12	22	20	18	5		

* Statistically significant compared to untreated sample.

Table 8.25. Cytotoxicity responses of MNC from naive devils against DFTD cells following 48h activation with Concanavalin A

Devil	Sample	Ratio	Mean (CPM)	SD (CPM)	Cytotoxicity (%)			Mean (%)	SD (%)	Significance (F test)	
Cd 23	Untreated MNC	25	889.3	48.2	-6	2	6	1	6		
		12	895.3	27.1	4	3	-2	2	3		
		6	837.3	48.4	-12	-1	-3	0	6		
	48h Con A treated MNC	25	2160.3	120.7	74	70	60	68	7	0.0000	*
		12	1560.7	371.1	49	9	42	34	21		
		6	1660.0	57.0	36	43	39	39	3		
		3	1425.3	127.0	32	28	18	26	7		
Cd 36	Untreated MNC	25	875.0	120.0	14	-1	-16	0	15		
		12	905.0	107.7	17	0	-9	3	13		
		6	908.7	21.6	4	6	0	3	3		
	48h Con A treated MNC	25	1563.3	57.2	31	37	33	34	3	0.0099	*
		12	1277.3	121.5	23	20	10	17	7		
		6	1261.0	31.5	17	18	14	16	2		
		3	1175.3	37.8	10	14	10	12	2		
Cd 37	Untreated MNC	25	863.0	149.0	8	9	-24	0	18		
		12	910.0	58.8	11	-3	3	3	7		
		6	854.7	138.7	-23	4	9	0	17		
	48h Con A treated MNC	25	1357.7	127.1	20	16	30	22	7	0.0178	*
		12	1364.3	56.6	24	19	24	22	3		
		6	1258.0	6.1	16	16	16	16	0		
		3	1106.3	69.8	3	11	8	8	4		
Wd 7	Untreated MNC	25	916.2	386.2	35	4	-5	0	6		
		12	771.2	14.5	4	3	3	4	1		
		6	705.1	18.9	1	-1	0	0	1		
		3	652.5	98.6	-9	-1	1	0	5		
	48h Con A treated MNC	25	2090.6	289.1	54	87	73	71	17	0.0009	*
		12	1771.6	287.8	34	64	62	53	16		
		6	1701.8	50.8	46	51	51	49	3		
		3	1340.7	95.8	28	23	34	29	5		

* Statistically significant compared to untreated sample. Shaded cells represent samples with suspected contamination by radioactively labelled cells.

Table 8.26. Cytotoxicity responses of MNC from naive devils against DFTD cells in the presence of Concanavalin A culture supernatant

Devil	Sample	Ratio	Mean (CPM)	SD (CPM)	Cytotoxicity (%)			Mean (%)	SD (%)	Significance (F test)	
Cd 18	Untreated MNC	50	1609.5	273.2	-5	9	16	7	11		
		25	1689.9	148.7	3	11	15	10	6		
		12	1704.7	104.0	15	10	7	11	4		
		6	1555.6	214.2	11	-5	9	5	9		
	25% Con A supernatant	50	2921.5	99.9	61	52	57	57	4	0.0240	*
		25	2638.1	529.9	17	57	59	44	24		
		12	2648.5	330.8	28	50	56	45	15		
Cd 38	Untreated MNC	50	1745.5	58.7	10	13	14	12	2		
		25	1546.0	218.2	1	14	-3	4	9		
		12	1584.1	293.7	13	12	-8	6	12		
		6	1544.9	42.7	4	6	3	4	2		
	25% Con A supernatant	50	2934.0	101.7	59	52	61	57	5	0.0309	*
		25	2501.0	326.7	22	42	50	38	15		
		12	2448.7	207.8	25	38	43	36	9		
Cd 20	Untreated MNC	25	913.3	71.2	11	6	-6	4	9		
		12	902.0	22.6	0	6	2	2	3		
		6	939.0	45.1	3	14	4	7	6		
	25% Con A supernatant	25	1139.7	35.6	31	35	40	35	4	0.0260	*
		12	1019.7	10.7	23	21	21	21	1		
		6	980.7	86.7	6	17	26	17	10		
Cd 19	Untreated MNC	25	849.0	67.1	5	-6	-11	0	8		
		12	876.3	53.6	-8	2	4	0	7		
		6	891.7	19.6	4	-1	1	1	2		
	25% Con A supernatant	25	1098.0	7.9	32	30	30	30	1	0.0004	*
		12	973.3	19.3	14	18	15	16	2		
		6	922.7	65.0	1	15	13	10	8		
Wd 3	Untreated MNC	25	977.2	9.3	7	7	8	7	1		
		12	888.6	92.2	-8	7	2	0	7		
		6	969.3	90.8	-1	9	13	7	7		
		3	974.3	45.3	4	11	7	7	4		
	25% Con A supernatant	25	1427.0	185.9	42	41	10	31	18	0.0388	*
		12	1363.1	8.6	24	25	25	25	1		
		6	1361.8	32.4	28	24	22	24	3		
		3	1203.1	96.5	-2	15	14	9	10		
Wd 4	Untreated MNC	25	1143.9	25.5	23	21	19	21	2		
		12	1145.8	127.4	10	30	24	21	10		
		6	1211.6	180.4	15	43	22	26	14		
		3	1041.1	66.6	13	18	7	13	5		
	25% Con A supernatant	25	1588.0	236.6	69	50	22	47	23	0.2483	
		12	1501.7	227.0	53	12	49	38	22		
		6	1530.6	18.3	42	42	39	41	2		
		3	1566.7	89.5	42	37	54	45	9		

Wd 5	Untreated MNC	25	873.0	156.5	4	-15	7	0	12		
		12	845.5	156.6	2	-17	5	0	12		
		6	920.1	30.9	4	0	3	2	2		
		3	799.4	90.5	-6	0	-14	0	7		
	25% Con A supernatant	25	1417.5	208.3	52	45	22	40	16	0.0001	*
		12	1246.1	54.9	30	29	22	27	4		
		6	1020.2	96.0	18	7	4	10	7		
		3	946.0	164.2	-10	8	14	4	12		
Wd 6	Untreated MNC	25	1019.3	108.4	19	4	6	10	8		
		12	885.3	99.7	0	7	-8	0	8		
		6	959.1	62.4	8	7	0	5	5		
		3	941.1	36.0	2	2	7	4	3		
	25% Con A supernatant	25	1378.6	168.3	49	23	38	37	13	0.0409	*
		12	1196.1	110.7	14	26	29	23	8		
		6	1055.6	129.8	1	16	20	12	10		
		3	1157.8	124.2	18	30	12	20	9		

* Statistically significant compared to untreated sample.

Table 8.27. Cytotoxicity responses of MNC from naive devils against DFTD cells in the presence of cloned Tasmanian devil IL-2

Devil	Sample	Ratio	Mean (CPM)	SD (CPM)	Cytotoxicity (%)			Mean (%)	SD (%)	Significance (F test)	
Cd 1	Untreated	25	1469	114	6	1	-5	-1	5		
		12	1490	145	-11	0	1	3	7		
		6	1501	46	-1	3	3	-1	2		
		3	1485	123	6	2	-5	-1	6		
	1/100 IL-2	25	2314	6	38	39	38	38	0	0.0020	*
		12	1736	179	20	13	4	12	8		
		6	1660	24	10	8	8	9	1		
		3	1651	110	5	6	14	8	5		
	1/1000 IL-2	25	1993	194	29	14	29	24	9	0.0077	*
		12	1721	66	15	10	9	11	3		
		6	1509	26	1	3	1	2	1		
		3	1427	110	-8	1	1	0	5		
Dd 5	Untreated	25	1393	115	0	-9	0	0	5		
		12	1435	36	0	-3	-1	0	2		
		6	1449	106	5	-3	-5	-1	5		
		3	1369	87	-9	-1	-4	0	4		
	1/100 IL-2	25	1833	23	15	17	17	16	1	0.0001	*
		12	1647	74	11	9	4	8	3		
		6	1613	37	7	8	5	7	2		
		3	1576	82	5	8	1	5	4		
	1/1000 IL-2	25	1554	102	6	-1	7	4	5	0.0003	*
		12	1688	71	12	6	12	10	3		
		6	1743	27	12	14	11	12	1		
		3	1648	116	2	13	9	8	5		

* Statistically significant compared to untreated sample.

Table 8.28. Cytotoxicity responses of MNC from naive devils against DFTD cells in the presence of the TLR 3 ligand Poly I:C at different concentrations

Devil	Sample	Ratio	Mean (CPM)	SD (CPM)	Cytotoxicity (%)			Mean (%)	SD (%)	Significance (F test)	
Cd 7	Untreated	25	985	89	-1	-1	2	0	2		
		12	1031	12	1	1	0	1	0		
		6	1100	162	1	-1	6	2	3		
Cd 38	Untreated	25	1054	49	1	2	0	1	1		
		12	915	64	-1	-3	-1	0	1		
		6	974	18	0	-1	0	0	0		
		3	869	227	2	-2	-8	0	5		
Poly I:C	10 µg/mL	25	1986	58	23	21	25	23	2	0.0073	*
		12	1697	71	15	11	9	12	3		
		6	1643	73	7	13	9	9	3		
		3	1756	75	11	17	14	14	3		
	5 µg/mL	25	2655	226	57	40	52	50	9	0.0000	*
		12	2579	156	53	47	40	47	6		
		6	2391	77	43	38	37	39	3		
		3	1961	252	14	19	33	22	10		
	1 µg/mL	25	2105	54	26	30	27	28	2	0.0001	*
		12	1703	201	19	12	3	12	8		
		6	1625	91	6	13	7	9	4		
		3	1580	45	6	9	6	7	2		
	0.1 µg/mL	25	1890	143	14	25	19	19	6	0.0004	*
		12	1651	54	8	9	12	10	2		
		6	1600	69	5	10	8	8	3		
		3	1541	18	5	6	5	5	1		

* Statistically significant compared to the relevant untreated sample.

Table 8.29. Replicates of cytotoxicity assays containing Poly I:C at 5 µg/mL

Devil	Sample	Ratio	Mean (CPM)	SD (CPM)	Cytotoxicity (%)			Mean (%)	SD (%)	Significance (F test)	
Cd 7	Untreated	25	468	57	6	1	11	6	5		
		12	456	21	5	7	4	5	2		
		6	439	19	3	5	3	4	2		
		3	448	136	-5	1	17	4	12		
	5 µg/mL Poly I:C	25	830	58	25	12	17	18	7	0.4009	
		12	796	20	13	12	16	14	2		
		6	715	10	6	5	4	5	1		
		3	653	47	-8	1	1	-2	5		
Cd 17	Untreated	25	579	33	13	19	15	16	3		
		12	547	34	14	15	10	13	3		
		6	440	33	1	7	3	4	3		
		3	410	16	3	0	1	1	1		
	5 µg/mL Poly I:C	25	1003	45	32	38	42	37	5	0.2000	
		12	905	50	31	28	20	26	6		
		6	801	37	17	17	10	15	4		
		3	763	70	5	19	7	10	8		
Cd 38	Untreated	25	1054	49	1	2	0	1	1		
		12	915	64	-1	-3	-1	0	1		
		6	974	18	0	-1	0	0	0		
		3	869	227	2	-2	-8	0	5		
	5 µg/mL Poly I:C	25	2655	226	57	40	52	50	9	0.0000	*
		12	2579	156	53	47	40	47	6		
		6	2391	77	43	38	37	39	3		
		3	1961	252	14	19	33	22	10		
Cd 40	Untreated	25	1393	115	0	-9	0	0	5		
		12	1435	36	0	-3	-1	0	2		
		6	1449	106	5	-3	-5	-1	5		
		3	1369	87	-9	-1	-4	0	4		
	5 µg/mL Poly I:C	25	1819	47	15	14	18	16	2	0.0001	*
		12	1687	164	16	12	2	10	7		
		6	1745	58	11	16	11	13	3		
		3	1732	97	16	12	8	12	4		

* Statistically significant compared to untreated sample.

Section A.4 - Additional Tables – Chapter 5 Cytotoxicity assay data

Table 8.30. Tumour volume measurements in a DFTD diseased Tasmanian devil undergoing immunotherapy

Devil	Week (w)	Tumour Measurements			Tumour Volume (cm^3)	Fold Change (from w=0)	Regression (from maximum volume)	
	0	**0.7**	**0.7**	**1**	**0.26**			
	4	3.3	2.1	1.8	6.52	25.46		
	6	4.1	2.8	2.7	16.21	63.26		
	7.5	3.7	3.1	2.7	16.20	63.20		
	10	5	4.7	2.6	31.96	124.69		
	11	**5.5**	**4.9**	**2.9**	**40.88**	**159.50**	Fold change	Percent change
Cd 15	12	5	4.5	3.4	40.01	156.12	0.98	2.1
	13.5	4	4.2	3.2	28.12	109.71	0.69	31.2
	14.5	4.4	3.6	2.7	22.37	87.28	0.55	45.3
	16.5	3	2.9	1.8	8.19	31.96	0.20	80.0
	17.5	2.2	2.2	1.7	4.30	16.79	0.11	89.5
	18	1.7	2.1	1.5	2.80	10.93	0.07	93.1
	23.5	**0**	**0**	**0**	**0**	**25.46**		**100.0**

Table 8.31. Cytotoxicity responses of a DFTD diseased Tasmanian devil during tumour regression following immunotherapy

Devil	Sample	Ratio	Mean (CPM)	SD (CPM)	Cytotoxicity (%)			Mean (%)	SD (%)	Significance (F test)	
Cd 15	Pre Immune	100	305.0	57.5	23	-1	13	12	12		
		50	288.2	19.4	8	4	12	8	4		
		25	280.9	10.0	8	8	4	7	2		
		12	258.3	17.2	-2	4	4	2	4		
		6	245.6	44.7	4	5	-11	-1	9		
	Draw 1	100	751.3	12.7	8	6	8	8	1	0.5983	
		50	771.3	20.1	10	11	8	9	2		
		25	706.3	37.4	6	0	5	4	3		
		12	671.3	32.3	0	-1	4	1	3		
		6	673.0	24.2	3	-1	1	1	2		
		3	681.7	30.4	3	4	-1	2	3		
	Draw 1 (MHC I positive cells)	100	1132.7	154.6	36	16	22	25	11	0.2298	
		50	919.3	56.4	6	13	11	10	4	0.0478	#
		25	863.3	25.8	6	5	8	6	2		
		12	796.0	63.5	5	3	-3	2	4		
		6	846.0	109.5	7	11	-3	5	8		
		3	757.7	16.6	-2	0	-1	-1	1		
	Draw 2	50	820.0	155.6	-6	-9	3	-4	6	0.2960	
		25	796.5	23.3	2	-5	-3	-2	4		
		12	770.5	68.6	-3	-3	-8	-5	3		
		6	981.5	89.8	0	3	10	4	5		
		3	775.5	67.2	-11	-3	-8	-7	4		
	Draw 3	50	684.7	118.2	21	20	42	28	684.7	0.0853	
		25	565.3	32.1	11	15	18	15	565.3		
		12	507.0	37.5	10	4	11	8	507.0		
		6	475.7	48.8	-1	9	7	5	475.7		
		3	475.7	28.9	4	3	9	5	475.7		

*Significantly different to pre immune sample, # significantly different to untreated DFTD cells.

Table 8.32. Cytotoxicity responses of three healthy Tasmanian devils following immunisation with MHC I positive DFTD cells

Devil	Sample	Ratio	Mean (CPM)	SD (CPM)	Cytotoxicity (%)			Mean (%)	SD (%)	Significance (F test)	
Cd 39	Pre Immune	100	1483	102	7	-2	5	-3	5		
		50	1491	121	9	-1	2	-3	5		
		25	1469	114	6	1	-5	-1	5		
		12	1490	145	-11	0	1	3	7		
		6	1501	46	-1	3	3	-1	2		
		3	1485	123	6	2	-5	-1	6		
	Dose 1 Assay 1	100	783.3	48.1	9	15	7	10	4	0.1039	
		50	811.7	68.7	7	19	12	13	6		
		25	720.0	58.7	10	5	0	5	5		
		12	718.7	20.8	4	7	4	5	2		
		6	732.0	26.2	5	4	8	6	2		
		3	647.0	40.0	3	-3	-3	-1	3		
	Dose 1 Assay 2	100	931.7	23.8	9	9	11	10	1	0.1388	
		50	933.7	12.7	10	10	9	10	1		
		25	919.3	20.0	8	10	9	9	1		
		12	788.0	19.5	3	2	4	3	1		
		6	748.7	47.8	-2	1	3	1	2		
		3	734.7	31.0	1	0	-2	0	2		
	Dose 2 Assay 1	100	1907.7	73.5	5	2	4	4	2	0.5612	
		50	1947.3	68.1	7	4	3	5	2		
		25	2066.0	29.6	7	8	7	8	1		
		12	1726.3	123.4	0	-4	2	-1	3		
		6	1851.0	91.8	5	1	0	2	2		
	Dose 2 Assay 2	100	498.3	44.7	11	2	10	8	5	0.0346	*
		50	605.3	100.0	31	15	11	19	11		
		25	465.0	88.1	14	-5	2	4	9		
		12	507.7	112.4	-2	6	22	9	12		
		6	478.7	20.1	4	5	8	5	2		
Cd 40	Pre Immune	100	1634	81	5	12	6	7	4		
		50	1476	45	2	1	-2	0	2		
		25	1393	115	0	-9	0	0	5		
		12	1435	36	0	-3	-1	0	2		
		6	1449	106	5	-3	-5	-1	5		
		3	1369	87	-9	-1	-4	0	4		
	Dose 1 Assay 1	100	737.0	66.1	12	1	7	6	737.0	0.8381	
		50	684.0	50.1	2	6	-2	2	684.0		
		25	697.0	16.1	2	4	3	3	697.0		
		12	678.0	35.3	-2	4	3	2	678.0		
		6	633.3	41.6	2	-5	-3	-2	633.3		
		3	629.3	20.0	-3	-1	-4	-3	629.3		

Cd 40	Dose 1 Assay 2	100	827.0	63.7	1	5	7	4	3	0.7485	
		50	748.0	49.4	-2	2	2	1	2		
		25	730.7	47.3	2	-1	-2	0	2		
		12	715.7	13.2	-2	-1	0	-1	1		
		6	722.0	27.6	-1	-2	1	-1	1		
		3	657.0	32.9	-5	-2	-4	-4	2		
	Dose 2 Assay 1	100	1849.7	127.0	2	6	-1	2	3	0.4018	
		50	1633.0	68.1	-5	-3	-2	-3	2		
		25	1736.7	122.3	3	-3	-1	-1	3		
		12	1672.7	48.0	-3	-2	-1	-2	1		
		6	1679.3	97.9	1	-4	-2	-2	2		
	Dose 2 Assay 2	100	482.7	72.8	15	4	-1	6	8	0.9078	
		50	482.0	6.6	5	6	6	6	1		
		25	466.7	56.5	3	11	-1	4	6		
		12	441.0	25.5	0	5	-1	1	3		
		6	490.0	92.1	0	18	2	7	10		
Cd 1	Pre Immune	100	1056.0	29.7	5	6	7	6	1		
		50	964.3	102.9	-3	5	4	2	4		
		25	906.4	65.8	-3	0	2	0	3		
		12	933.0	103.9	3	4	-4	1	4		
		6	857.2	84.1	0	-6	-1	-2	4		
		3	878.9	80.8	0	-5	1	-1	3		
	Dose 1 Assay 1	100	963.0	44.8	28	27	21	25	4	0.0068	*
		50	937.0	56.5	29	20	21	23	5		
		25	886.3	116.6	11	16	30	19	10		
		12	869.7	67.9	24	15	14	18	6		
		6	758.0	53.9	5	6	13	8	5		
		3	708.7	11.6	4	5	3	4	1		
	Dose 1 Assay 2	100	1086.0	106.0	11	21	20	17	5	0.0177	*
		50	1045.3	145.1	13	9	23	15	7		
		25	1017.7	87.2	10	19	13	14	4		
		12	933.3	37.6	11	11	8	10	2		
		6	804.3	45.2	6	2	3	3	2		
		3	731.0	20.0	0	1	-1	0	1		
	Dose 2 Assay 1	100	2247.7	208.3	16	6	14	12	5	0.0762	
		50	2094.0	88.5	9	6	10	8	2		
		25	1962.3	62.6	6	6	3	5	2		
		12	1620.0	113.0	-4	0	-6	-4	3		
		6	1697.3	49.0	0	-3	-2	-2	1		
	Dose 2 Assay 2	100	523.3	36.9	13	12	6	10	4	0.2256	
		50	516.7	58.3	10	15	3	10	6		
		25	529.3	53.7	15	13	4	11	6		
		12	442.3	16.5	1	3	0	2	2		
		6	481.7	28.3	3	5	9	6	3		

*Significantly different to pre immune sample.

Table 8.33. Cytotoxicity responses of two DFTD diseased Tasmanian devil during immunotherapy with MHC I positive cells

Devil	Sample	Ratio	Mean (CPM)	SD (CPM)	Cytotoxicity (%)			Mean (%)	SD (%)	Significance (F test)	
Dd 11	Pre Therapy	100	250.3	15.7	12	7	5	8	4		
		50	269.7	23.2	17	14	6	12	5		
		25	242.7	15.5	10	5	3	6	4		
		12	219.7	22.5	-5	2	5	1	5		
		6	229.3	35.6	11	-6	4	3	8		
	Draw 1 (day 7)	100	1183.0	258.5	21	4	12	13	9	0.8131	
		50	1035.3	100.6	4	10	9	8	3		
		25	1051.7	81.0	10	9	5	8	3		
		12	1067.7	57.0	11	8	7	9	2		
		6	976.0	176.6	-1	8	10	6	6		
	Draw 2 (day 14)	100	871.7	77.2	13	6	7	9	4	0.9426	
		50	820.3	47.3	7	8	4	6	2		
		25	919.7	101.4	12	15	6	11	5		
		12	773.3	58.1	5	6	1	4	3		
		6	742.7	41.5	0	3	4	2	2		
Dd 18	Pre Therapy	100	231.7	24.8	10	-1	1	3	6		
		50	248.7	29.1	4	15	3	7	7		
		25	229.3	28.9	7	7	-5	3	7		
		12	210.3	37.6	-11	-1	7	-2	9		
		6	219.3	9.0	-2	2	2	1	2		
	Draw 1 (day 7)	100	1020.3	29.4	8	7	7	7	1	0.8912	
		50	1087.0	24.6	10	10	9	9	1		
		25	1045.3	170.7	2	10	13	8	6		
		12	1045.0	59.4	6	9	9	8	2		
		6	977.0	36.9	5	5	7	6	1		
	Draw 2 (day 14)	100	830.7	30.0	8	8	5	7	1	0.5834	
		50	781.0	34.6	6	3	4	4	2		
		25	756.3	30.1	5	2	2	3	1		
		12	713.7	54.5	-1	0	4	1	3		
		6	732.7	23.0	3	2	1	2	1		

*Significantly different to pre therapy sample.