Devil Facial Tumour Disease: *In vivo* studies in mice

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Declaration of Originality

I declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any tertiary institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

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Statement of Co-authorship

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

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The candidate was the primary author, designed and performed the experiments, analysed and presented results.

Author two contributed towards experimental design and interpretation of results.

Author three contributed towards the editing and clarity of the manuscript as well as co-supervising the project.

Author four was the primary supervisor for the project, was responsible for ethics approval, contributed towards experimental design and interpretation of results, contributed towards the editing and clarity 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:

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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 of Tasmania.

Terry Pinfold

Conference presentations

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Abbreviations

7AAD	7-Aminoactinomycin D (fluorescent stain used to labelled dead cells)
APC	Antigen presenting cell or Fluorochrome used for flow cytometry
CFSE	Carboxyfluorescein succinimidyl ester (fluorescent stain to label cells)
Con A sup	Supernatant obtained from concanavalin A stimulation of lymphocytes
CpG	Synthetic oligonucleotides that act as TLR-9 agonists
CTL	Cytotoxic T Lymphocyte
CTVT	Canine Transmissible Venereal Tumor
DFTD	Devil Facial Tumour Disease
DPIPWE	Department of Primary Industries, Parks, Water and Environment
ELISA	Enzyme-Linked Immunosorbent assay
FACS	Fluorescence-activated cell sorting
FDA	Food and Drug Administration (USA)
FSB	FACS staining buffer (described in methods)
FSC	Forward Scatter (Flow cytometry parameter related to cell size)
IP	Intraperitoneal
LAK	Lymphokine activated killer (cell)
MHC	Major Histocompatibility Complex
MNC	Mononuclear Cell
NK	Natural Killer Cell
NKT	Natural Killer T Cell
PBMNC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline (described in methods)
PI	Propidium iodide (fluorescent stain used to labelled dead cells)
SC	Subcutaneous
SSC	Side Scatter (Flow cytometry parameter related to cell granularity)
WEHI	Walter and Eliza Hall Institute of Medical Research

Table of Contents

Abstract

Devil Facial Tumour Disease (DFTD) is an infectious cancer cell line transmitted as an allograft between Tasmanian devils. On transmission it does not evoke an immune response, is 100% fatal and is driving the Tasmanian devil towards extinction in the wild. Due to their endangered status, access to Tasmanian devils to study DFTD is limited. As a consequence this study of DFTD was undertaken in mice to complement studies being done in Tasmanian devils.

Inoculation of immunocompetent mice with DFTD cells did not result in DFTD tumour establishment. This rejection was a specific immune response because DFTD specific antibodies were produced. This provided evidence that DFTD cells are immunogenic and susceptible to killing by the immune system making them suitable targets for immunotherapy and vaccines. Immunocompetent mice also provided a model to study immunogenicity of various DFTD cell preparations and injection protocols applicable to vaccine development. For example, 14 day prime-boost intraperitoneal injections of DFTD cells resulted in enhanced antibody and cytokine responses in mice compared to subcutaneous injections. Inactivation of DFTD cells by freeze-thawing or sonication reduced the immunogenicity of DFTD cells while irradiation of DFTD cells maintained immunogenicity.

NOD/SCID mice have severe immune system defects that prevented protective immune responses against DFTD cells allowing tumours to establish. Consequently, these mice provided a xenograft model to study aspects of DFTD that could not be replicated in an *in vitro* setting. This included DFTD establishment and growth as well as efficacy of adoptive cell transfer trials. Adoptive cell transfer from immunocompetent mice conferred protection against DFTD as did adoptive transfer of Tasmanian devil lymphokine activated killer (LAK) cells. In this context, LAK cells refer to lymphocytes which have been stimulated *in vitro* with mitogens or cytokines to induce non-specific activated killer cells capable of killing DFTD cells without harming normal cells.

The xenograft model also facilitated the evaluation of the chemotherapeutic agents afatinib, withaferin A and imiquimod. The most promising results came from intratumoural injections of imiquimod which caused the upregulation of β_2-microglobulin on the surface of the DFTD cells. DFTD cells avoid immune

recognition in Tasmanian devils because they do not express MHC on the cell surface. Upregulation of β_2-microglobulin indicates that MHC was upregulated. This has important implications for the Tasmanian devil as the MHC would make the DFTD cells visible to the Tasmanian devil's immune system and this should invoke protective immune responses.

In conclusion, DFTD cells are immunogenic and can be targeted by antibodies and cytotoxic cells. This makes them suitable candidates for vaccines or immunotherapy in Tasmanian devils. They avoid the Tasmanian devils immune system by downregulating MHC. This ignorance can be overcome by non-specific activation of LAK cells capable of killing DFTD cells in a MHC independent manner. The tumour cells can be targeted by imiquimod to upregulate surface molecules including β_2-microglobulin and MHC to make them more immunogenic and potential targets for MHC dependent cytotoxic responses.

Dedication

This thesis is dedicated to my son

Matthew John Pinfold

(1991-2010)

A life too short

A son and brother much loved

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Literature Review

Literature Review

1 Literature review

1.1 Devil Facial Tumour Disease

Devil Facial Tumour Disease (DFTD) is a new and emerging disease having a devastating impact on the wild Tasmanian devil (*Sarcophilus harrisii*) population that is restricted to the island of Tasmania south of mainland Australia (Pyecroft et al 2007). The disease only occurs in the Tasmanian devil and "Epidemiology theory" advocates that a pathogen restricted to a single host species is unlikely to drive its host to extinction (Lachish et al 2007). However, the 100% mortality rates associated with this disease fuels speculation that the Tasmanian devil will face extinction in the wild (Lachish et al 2007).

To date there is no evidence of recovery or natural immunity to DFTD in any Tasmanian devil (Lachish et al 2007). Over 50% of the state of Tasmania, representing more than 85% of the original devil distributional range has been impacted by the disease. The population has declined by more than 85% with local population declines exceeding 95% (Brueniche-Olsen et al 2013, Cheng et al 2012, Lachish et al 2007). The disease front is yet to extend to the west coast and pockets in the mid-north coast of Tasmania (DPIPWE 2012, Pyecroft et al 2007).

The timeframe for the emergence of the disease is contentious. Animals with chronic scarring, acute wounds, tumours and other lesions cannot be distinguished visually from early stage DFTD. It is generally accepted that the first signs of the disease were recorded as photographs in 1996 (Hawkins et al 2006, McCallum and Jones 2006). In retrospect, the photographs revealed what appear to be facial tumours typical of the appearance of confirmed DFTD tumours (Hawkins et al 2006). The first field biopsy and necropsy from Tasmanian devils to confirm what would become known as DFTD was undertaken at Freycinet National Park in 2001 (Hawkins et al 2006). Karyotype and genetic studies of DFTD tumour cells support a recent origin of DFTD from a single Tasmanian devil (Murchison et al 2012, Pearse and Swift 2006).

DFTD pathology is consistent with an undifferentiated neoplasm resulting from atypical uncontrolled cell growth (Pyecroft et al 2007). Historically neoplasia has been a common problem observed in captive devil populations (Griner 1979). It has

been reported that 50% of Tasmanian devils necropsied (n=18) at San Diego Zoo had neoplasms or preneoplastic hyperplasia. In comparison, only 3% of other mammals and marsupials at the same zoo had neoplasms detected during necropsy (Griner 1979). This suggests that devils are predisposed to development of neoplasms. This may be as a result of increased susceptibility to carcinogens or oncogenic viruses (Griner 1979).

Archived samples of the neoplasms from San Diego Zoo are not suggestive of DFTD and previous population crashes anecdotally recorded in the literature around 1863 and 1908-1920 make no mention of a facial tumour (Loh et al 2006a). The pathology for DFTD neoplasm is inconsistent with previously described neoplasms in devils (Loh et al 2006a). DFTD cells can be described as pleomorphic and anaplastic because they bear little resemblance to normal cells and rapidly divide to form large tumourous growths that serve no physiological function (Pyecroft et al 2007). Unlike the spontaneously occurring neoplasms that the Tasmanian devils have been plagued with, DFTD is a clonal cell line that originated in a single devil as a Schwann cell tumour (Murchison et al 2010) and has been transmitted as an allograft between individuals (Pearse and Swift 2006).

Aside from DFTD the only other known naturally occurring infectious cancerous cell line is CTVT (Murgia et al 2006, Rebbeck et al 2009). DFTD and Canine Transmissible Venereal Tumor (CTVT) diseases can both be described as parasitic cancers (Siddle and Kaufman 2013). Another transmissible cancer in golden Syrian hamsters has been observed in experimental settings only (Fabrizio 1965, McCallum 2008).

1.1.1 Impact of disease on Tasmanian devil population

Once abundant throughout Australia the Tasmanian devil is now restricted to the island of Tasmania (165,000 km^2) (Jones et al 2004). The oldest fossil records for Tasmanian devils on mainland Australia are dated between 3000 to 4000 years (Brown 2006). It has been suggested that the introduction of the dingo around 3500 years ago, anthropogenic extinction by Aboriginals and susceptibility to the effects of climate variability could have all contributed to the extinction of mainland devil populations (Brown 2006).

The Tasmanian devil is regarded as a keystone species that has co-evolved with other endemic species and developed equilibrium predator-prey relationships (Jones et al 2007). This has resulted in unique biodiversity for Tasmanian wilderness areas that has significant positive impacts including the benefits for ecosystem health and financial benefits of tourism based on the natural appeal of the Tasmanian ecosystem. It has been suggested that the Tasmanian devil has a counteractive affect on invasive species such as the feral cat and red fox minimising their impact and in the case of the red fox possibly preventing its establishment in Tasmania (Lachish et al 2007, Wright and DPIPWE 2010).

If the Tasmanian devil becomes extinct in Tasmania its niche will be filled by feral species including the red fox and feral cats which have not coevolved with the native species. The predator prey relationship will not be a balanced one and some species will be preyed on to the point of extinction. The impact of foxes on mainland Australia has irrefutably resulted in the loss of many endemic species. The success of the fox invasion on mainland Australia may have been facilitated by the absence of Tasmanian devils. The fox and the cat are regarded as “super-predators” that our native animals are ill equipped to coexist with (Lachish et al 2007, Wright and DPIPWE 2010).

1.1.2 **Pathogenesis DFTD**

DFTD neoplasms have a 100% mortality rate and the actual mechanism of death is still speculative but implications include starvation, septicaemia from secondary infections, metastases and toxins release by necrosis (Deakin et al 2012).

Starvation and cachexia may occur because the tumour growth may impair the senses associated with finding food by overgrowing the eye and obscuring vision, diminishing the tactile senses of the whisker beds, or compromising the senses of smell and taste. The lesions associated with the face and mouth may preclude the acts of seizing, holding and chewing on food (Pyecroft et al 2007). The devil’s appetite may be suppressed by the tumour producing appetite suppressing cytokines (Inui 2002). Other causes of cachexia include increased catabolism, diverting calories to tumour growth and the loss of protein through the tumour surface (Bruera 1997, Pyecroft et al 2007).

There is a large amount of necrosis associated with DFTD. This has the potential to release toxins and promote secondary infections which could result in septicaemia (Pyecroft et al 2007). The disease has demonstrated a high rate of metastatic invasion of other organs and this may impact on the functioning of organs such as the respiratory organs (Loh et al 2006a). Even the factor of pain may contribute to the mortality of the disease (Loh et al 2006b, Pyecroft et al 2007).

1.1.3 **DFTD pathology**

DFTD is a malignant neoplasm producing large amounts of necrosis (73% n=91), rapid tumour growth and a high incidence of metastatic disease in infected devils (65% n=91) (Loh et al 2006a, Loh et al 2006b). DFTD presents with a singular morphology as an undifferentiated subepithelial sarcoma, presumed to arise at the site of transfer as a result of biting (Loh et al 2006b). The tumours develop into large masses that usually protrude from the face, mouth or neck region and tend to be ulcerated, exuding flat surfaces that crumble and deposit infectious cells on the canine teeth making possible further transmission of the disease (Loh et al 2006a). Post mortem examinations (n=91) detected metastases in lymph nodes (57%), lungs (47%); spleen (12%); heart, ovary and serosal surface of rib (6%); kidney, mammary, adrenal and pituitary glands (5%); and vascular invasion in 4% of cases (Loh et al 2006a).

DFTD cells are anaplastic and pleomorphic presenting as round (≈ 8 µm) or spindle shaped with no distinctive ultrastructural features. They have a single round nucleus (≈ 5.75 µm) with scattered condensed chromatin and the cytoplasm is a hazy blue colour with a high nuclear to cytoplasmic ratio of 1:1.2. Transmission electron microscopy revealed that concentration of organelles was low and included rough endoplasmic reticulum, free ribosomes, polyribosomes, cytoskeletal filaments, large vesicular mitochondria, ribosomelamella complexes, secretory granules, endocytotic vesicles, well developed Golgi apparatus, centrioles and myelin bodies. Primitive desmosome-like structures were detected at low numbers which explains their tendency to clump together in preparations (Loh et al 2006a).

The principle of immunohistochemical tests when determining origin of cells in poorly differentiated neoplasms presumes that immunophenotypes are preserved. By studying the proteins expressed by the DFTD cells it was hoped that the origin of

DFTD could be determined. This work was hindered by the lack of previous studies of immunohistochemistry (IHC) applied to the Tasmanian devil. What was seen was that the DFTD neoplasm develops as a heavily vascularised well defined dense cellular structure that is resistant to infiltrates and is often enclosed in a pseudocapsule produced by the compression of the surrounding connective tissue. Cells can be arranged in a variety of patterns including bundles, cords, palisades and sheets (Loh et al 2006a). The DFTD cells test positive for vimentin (n=50/50), S-100 (n=41/48), melan A (n=11/39), neuron specific enolase (n=35/35), chromogranin A (n=12/12) and synaptophysin (n=29/30) (Loh et al 2006b, Pyecroft et al 2007).

It should also be noted that DFTD neoplasms tested negative for cytokeratin (n= 0/48), epithelial membrane antigen (n= 0/42), von Willebrand factor (n= 0/11), smooth muscle actin (n= 0/26), desmin (n= 0/47), glial fibrillary acid protein (n= 0/13), CD16 (n= 0/13), CD57 (n= 0/43), CD3 (n= 0/18), LSP1 (n=0/16) and amyloid (n= 0/30). They were weakly argyrophilic (n= 3/40) using Grimelius histochemical stain but failed to be stained with silver using the Singh silver method (n= 0/34) (Loh et al 2006b).

The IHC results are inconsistent with DFTD originating from Ewing's sarcoma, CTVT, Merkel cell tumour, melanoma, neuroblastoma and lymphosarcoma. The negative results for desmin, smooth muscle actin and glial fibrillary acid protein suggest that it is unlikely that DFTD originated from muscle or neural cells. The lack of CD16, CD57 and LSP 1 demonstrates that DFTD is not associated with leukocytes including B cells, T cells, monocytes, dendritic cells (DCs) and macrophages. Lack of epithelial markers including cytokeratin, epithelial membrane antigen and von Willebrand factor combined with the positive results for S-100 and vimentin support DFTD being classified as a sarcoma (Loh et al 2006b).

These staining characteristics are consistent with cells of neuroectodermal origin and electron microscopy failed to contribute towards the histiogenic origins of DFTD (Loh et al 2006b). The positive staining of DFTD tumour tissues with antibody specific to Schwann cell specific myelin protein, periaxin (PRX) further narrowed down the origin of DFTD to a Schwann cell tumour (Murchison et al 2010). Periaxin was identified as a most useful molecular marker to confirm DFTD neoplasm in suspected neoplasms and associated biopsies (Tovar 2012).

The premise that DFTD arose from a Schwann cell origin is consistent with the observation of S-100 (n= 41/48) which is regarded as a marker associated with neural-derived tumours (Stroup and Pinkus 1988). Further evidence of neural origin is the consistent detection of neuron specific enolase (n= 35/35), chromogranin A (n= 12/12) and synaptophysin (n= 29/30) which are IHC stains specifically associated with neuroendocrine cells (Loh et al 2006b). The morphology, ultrastructural characteristics and weak argyrophilic differentiation of DFTD cells are consistent with a Schwann cell origin (Loh et al 2006b).

DFTD tumours are arranged in a manner comparable to neuroendocrine organs such as the thyroid, islets of Langerhans, pituitary and adrenal glands. Neuroendocrine cells are also dispersed throughout the body and are particularly concentrated in tissues associated with the sense of touch including the lips and whisker beds. These facial regions are the locations were DFTD transmission is usually observed and therefore the tissues would be conducive to the establishment of the disease after transmission by biting (Loh et al 2006b).

1.1.4 **Karyotype reveals clonal nature of DFTD**

The consistency of IHC and chromosome rearrangement of DFTD cells regardless of gender and geographic location of Tasmanian devils supports the contention that DFTD is a single tumour clone transmitted between individuals (Loh et al 2006b, Pyecroft et al 2007). The DFTD karyotype represents an extensive rearrangement of the Tasmanian devil's chromosomes that is relatively stable and consistent between hosts (Pearse and Swift 2006). There are now nine strains identified which are closely related and easily explained by evolution of the original strain (DPIPWE 2009)

A normal devil has 14 chromosomes including the sex chromosomes. DFTD cells lack the sex chromosomes, chromosome 2 pair is missing, chromosome 6 is not a pair and the long arm of chromosome 1 is deleted. DFTD has an additional four marker chromosomes present (M1-M4) giving it a total of 13 chromosomes but only eight of these are in pairs (Figure 1-1) (Pearse and Swift 2006).

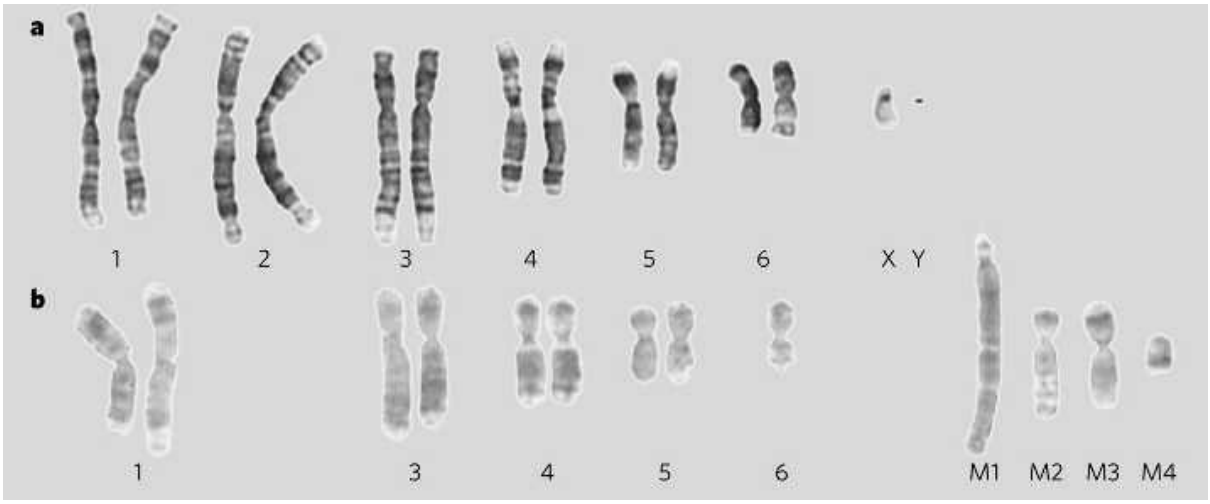

Figure 1-1 - Chromosomes of DFTD cells compared to male Tasmanian devil (Pearse and Swift 2006)

a) Typical karyotype for male Tasmanian devil
b) Karyotype of DFTD consistent between hosts

The lack of intermediate stages during disease development supports the theory that DFTD is an infectious allograft. Molecular studies reveal that DFTD cells have MHC class I and II genes that are distinct from the host's genes and Amplified Fragment Length Polymorphism have revealed considerable genetic difference between DFTD cells and those of the host (Pyecroft et al 2007). Further supporting evidence that DFTD represents a clonal cell line came from the observation of a pericentric inversion of chromosome 5 in all the tissues of a particular devil. The chromosome 5 in the DFTD cells did not have this inversion indicating the tumour had not developed from the devil's own tissues (Pearse and Swift 2006).

1.1.5 DFTD transmission

Most primary tumours develop on the face. This is consistent with the theory that DFTD is an allograft transmitted through the biting behaviour of Tasmanian devils (Pyecroft et al 2007). These tumours ulcerate, become friable and deposit contagious cells on the canines of infected devils which in turn facilitate the transmission of the allograft to the next devil (Pearse and Swift 2006). Biting is frequent in Tasmanian devils when squabbling over sex or food and penetrating bites are more frequent in adults rather juveniles (Hamede et al 2008).

Tasmanian devils have competent immune systems and on transmission should reject the DFTD cells (Kreiss et al 2008). Examination of haematoxylin and eosin

sections of DFTD tumours revealed only 7% demonstrated lymphocyte infiltration providing little evidence of cell-mediated immunological responses (Loh et al 2006b). Possible explanations for the lack of immune surveillance and rejection include alteration of MHC class I and class II antigen expression by the DFTD cells (O'Neill 2010). However, Siddle et al. (2007) stated that this form of immune escape was not occurring based on results from real-time PCR experiments. They proposed that lack of MHC diversity in the Tasmanian devils allowed DFTD cells to be seen as 'self' rather than 'non-self' thereby avoiding elimination by the immune system (Siddle et al 2007).

Research on other species, such as the African cheetah (*Acinonyx jubatus*), indicate that MHC rejection between individuals can be compromised due to limited MHC diversity (O'Brien et al 1985). In skin graft trials all allografts between unrelated cheetahs were accepted and did not cause acute graft rejection (O'Brien et al 1985). A similar trial with Tasmanian devils revealed an acute rejection of all skin grafts providing evidence for sufficient MHC diversity amongst the devil population to cause allograft rejection (Kreiss et al 2011a). Therefore MHC expression on DFTD cells should be recognised by at least some Tasmanian devils as non-self.

The Tasmanian devil skin graft results meant that a lack of allorecognition did not explain the transmissibility of DFTD cells (Kreiss et al 2011a). Despite this, in 2012 the hypothesis of MHC bottleneck in Tasmanian devils being the prime mechanism facilitating DFTD transmission was still highly regarded and cited by experts in the field of DFTD (Belov 2012). In 2013, further research by Siddle et al. revealed MHC was epigenetically downregulated and lack of surface MHC molecules was the prime mechanism facilitating transmission of DFTD cells (Siddle et al 2013).

1.1.6 **DFTD management**

When the disease was first detected in animals inhabiting the Freycinet Peninsula in 2001, it had been thought that younger animals were not susceptible to the disease or that there was a long latency period for the disease (Hawkins et al 2006). In due course diseased sub-adults were captured (Hamede et al 2008, McCallum et al 2009). The natural progression of the disease in a population was older animals first, followed by younger adults and when the majority of the adults had died out then juveniles became infected (Lachish et al 2007).

Research into the contact networks of Tasmanian devils suggest that DFTD is capable of spreading to every individual within the population once a single individual becomes infected (Hamede et al 2009). To facilitate disease management there is an urgent need for a pre-clinical diagnostic test capable of rapid detection of infected animals before they become infectious. Such a test would permit population surveillance and suppression of the disease by removal of infected animals before they have the opportunity to transmit the disease (Hamede et al 2009). The benefit of removing infected individuals from a population needs to be balanced against the reproductive input that the diseased individual contributes to the population (Lachish et al 2007, McDonald-Madden et al 2010).

1.2 **Canine Transmissible Venereal Tumor**

Besides DFTD, CTVT is the only other known transmissible tumour in real world populations. CTVT transmission occurs during coitus and results in a neoplastic disease that affects the external genitalia of both sexes (Harmelin et al 2001).

Molecular studies of the CTVT tumour genes support the theory that all cases of CTVT, despite being geographically diverse, arose from a single cellular clone that is transmitted by engraftment (Harmelin et al 2001). Characteristic marker chromosomes make CTVT genetically distinct to the host (Murgia et al 2006). The dog leukocyte antigen haplotype of CTVT is different to each host but consistent between all CTVT tumours (Murgia et al 2006).

The possibility of viral aetiology has been considered in the case of both CTVT and DFTD (Pyecroft et al 2007). CTVT can only be transmitted by viable tumour cells and not dead cells or cell free filtrates which argue against a viral aetiology and supports the suggestion of a transmittable clone (Murgia et al 2006). Additional evidence in the case of both CTVT and DFTD is that the host's individual MHC is not expressed on the cell surface of the tumours suggesting that the tumours did not arise from a virally caused transformation of the host cells (Harmelin et al 2001, Pyecroft et al 2007).

CTVT has three distinct disease phases. The first phase is the "progressive phase" immediately following infection. MHC class I expression by the tumour is relatively low in this first phase (Das and Das 2000). This protects the cells from T cell

responses while the partial expression of MHC is protective from natural killer (NK) cell responses (Fassati and Mitchison 2010). The second phase of CTVT is the “stationary phase” were the tumour neither seems to progress or regress and this may be as a result of equilibrium between immunosurveillance killing CTVT cells and proliferation of CTVT cells (Fassati and Mitchison 2010). When this equilibrium is lost the disease moves into the third phase, “regression phase”, which ultimately results in elimination of experimentally induced CTVT after 3-9 months (Fassati and Mitchison 2010, Murgia et al 2006).

In the regression phase of CTVT MHC class I is upregulated to normal levels and the immune system recognises the tumour and eliminates it (Fassati and Mitchison 2010). The ability of CTVT to regulate MHC expression differentially suggests an epigenetic mechanism is at work and pathology suggests that tumour infiltrating lymphocytes (TILs) and macrophages may be altering the tumour’s microenvironment triggering regression (Fassati and Mitchison 2010). This can be explained by the TILs producing IL-6 and IFN-γ which counteracts the TGF-β activity and induces MHC class I antigen expression (Hsiao et al 2008).

As well as suppression of MHC class I, additional elements of immune system invasion revealed by the CTVT model include suppression of MHC class II molecules, downregulation or loss of β_2-microglobulin and expression of cytokines that suppress immune system responses against the tumour (Fassati and Mitchison 2010). CTVT production of TGF-β suppresses MHC expression and inactivates IFN-γ activity (Hsiao et al 2008).

The upregulation of MHC class II expression in the regression phase may also be significant as suggested by the presence of antibodies to MHC II in recovered dogs. MHC class II is fundamentally a receptor to facilitate communications between lymphocytes and is rarely expressed in other types of cancer and tumour cells (Fassati and Mitchison 2010).

1.3 Immune system responses to tumours

1.3.1 Cancer immunosurveillance and immunoediting hypotheses

The “cancer immunoediting hypothesis” proposes that the immune system is constantly surveying the health of cells, recognising and destroying cancerous or pre-cancerous cells before they have the opportunity to proliferate. This process on one hand eliminates most tumours before they become clinically relevant but on the other hand subjects tumours to selection pressures that are responsible for the immunogenic phenotype of tumours that overwhelm, evade or hijack the immune system becoming clinically relevant (Dunn et al 2002, Finn 2012).

Mouse models have demonstrated that tumours which develop in immunocompromised mice are typically more immunogenic than those that develop within the constraints of healthy immune systems. It would be debatable if this is simply a case of “survival of the fittest” selecting the least immunogenic transformed cells for survival or if it is an active adaptation by tumours regulating expression of genes associated with immunogenic markers (Dunn et al 2002).

It has been proposed that immunoediting consists of three stages or processes: elimination, equilibrium and escape. Immunosurveillance may detect and destroy individual transformed cells before they proliferate or when a critical mass of cells is reached. A deficient immune response may allow the tumour to enter the equilibrium or escape phase. In the equilibrium phase a bed of tumour cells survives. These are presumably genetically unstable and mutating into new variants facilitating “natural selection” for tumour variants capable of escaping immunosurveillance and ultimately leading to clinical disease (Dunn et al 2002). In the escape stage the tumours have thwarted the immune system responses. This may be as a result of becoming invisible to the immune surveillance or through genetic or epigenetic changes downregulating the immune system response or proliferating at a rate that overwhelms the immune system (Dunn et al 2002).

1.3.2 Immunosurveillance by the innate and adaptive immune system

Innate and adaptive tumour suppression pathways that depend on cytokines and lymphocytes are well defined (Dunn et al 2002). The innate immune system is the first line of protection if the host has an inflammatory response to tumour cells. The

response includes activation of the complement system to label cells for destruction and migration of neutrophils and NK cells to the site of inflammation (Mroz et al 2011). The NK cells have the function of directly killing tumour cells as well as producing cytokines to attract and activate additional NK cells, macrophages and DCs to intensify the immune response (Hanna et al 2004, Kelsall and Rescigno 2004, Orange and Ballas 2006, Trinchieri 1994).

When foreign antigens are detected by macrophages the primary response is to phagocytose and destroy the antigens making them poor antigen presenting cells (APCs). Dendritic cells preserve antigenic peptides from the cells they phagocytose, migrate to the lymphoid organs and present antigens to the naïve T cells and B cells to initiate adaptive immune responses making them more effective APCs (Herr et al 2000, Savina and Amigorena 2007).

Upon presentation of tumour antigens in the lymphoid organs clonal expansion of tumour-specific $CD4^+$ T helper cells, $CD8^+$ cytotoxic T cells (CTLs) and antigen specific B cells occurs. Chemokines attract these cells to the tumour site and if the immune system response is strong enough and specific enough the tumour will be eliminated (Dunn et al 2002).

The innate immune system is characterised by the lack of immunological memory and cannot be educated to initiate stronger responses on subsequent exposures (Mroz et al 2011). The components of the adaptive immune system, such as T and B cells, are capable of being educated into a memory response that enhances subsequent responses to a previously experienced antigen. The adaptive immune system can then recruit components of the innate immune system through antibodies and cytokines to work in synergy as part of a memory response to provide lifelong immunity to some tumours (Mroz et al 2011).

1.3.3 Cells of the innate immune system involved in immunosurveillance

Granulocytes

Neutrophils, basophils and eosinophils are a group of innate immune cells that are classified as granulocytes. Their main function is releasing leukotrienes, prostaglandins and cytokines to promote inflammation. Tumour–infiltrating neutrophils are implicated in promoting anti-tumour $CD8^+$ T cell responses in mouse models (Grivennikov et al 2010, Mroz et al 2011). Chronic inflammation can promote

tumourigenesis so granulocytes can also have a pro-tumour role (Grivennikov et al 2010).

NK cells

Natural killer cells are thymus independent innate immune cells. On activation NK cells lyse tumour/virus infected cells, produce cytokines to upregulate the innate immune system and promote development of adaptive immune responses (Sivori et al 2014).

Activation is controlled by a balance of inhibitory receptors and activating receptors. Natural killer cells have killer Ig-like receptors (KIRs) that bind to self MHC class I molecules which inhibits activation to protect healthy cells. These receptors have been identified in the Tasmanian devil (Kraan et al 2012). The KIRs can induce activation if binding is to aberrant or foreign MHC. Tumour cells and virally infected cells often have compromised MHC class I expression making them NK–susceptible. Some tumours, such as CTVT, maintain a degree of MHC expression to avoid NK killing (Das and Das 2000, Sivori et al 2014).

NK cells have a number of activating receptors including natural cytotoxicity receptors (NCRs) and toll-like receptors (TLRs) with affinity for microbial antigens and the ability to detect transformed or foreign cells. In order to be NK-sensitive aberrant cells need to present ligands for these receptors. Inducing downregulation of NCR expression on NK cells is a mechanism used by some cancer cells to resist NK cells (Sivori et al 2014). Besides binding to viral ligands on infected cells natural cytotoxicity receptors (NCRs) can bind to tumour cells, which over-express self-antigens on the cell surface (Sivori et al 2014). For example, membrane bound Hsp70 is immunogenic to NK cells but not to T cells (Moser et al 2002).

Macrophages

Macrophages are phagocytes derived from monocytes circulating in the blood. As well as the secondary lymphoid organs they are located in tissues in close proximity to the external environment such as the skin and mucosa (Kelsall and Rescigno 2004, Mroz et al 2011). Macrophages express a multiplicity of receptors specific for many endogenous and exogenesis ligands as well as antibodies and complement. Microbes and other cells that have been targeted by opsonisation with antibodies or complement are more effectively phagocytosed by macrophages (Mroz et al 2011).

Macrophage phenotypes are controlled by gene expression rather than lineage or differentiation pathways (Grivennikov et al 2010). Macrophages can be either induced into M1 macrophages or M2 macrophages by the tumour microenvironment (Heusinkveld et al 2011, Qian and Pollard 2010).

M1 macrophages arise from stimulation with IFN-γ and TNF-α and produce inflammatory cytokines including IL-12 and IFN-γ, which polarises immune responses to T_H1 cells (Grivennikov et al 2010, Hao et al 2012). M1 macrophages are associated with anti-tumour activity and killing (Qian and Pollard 2010).

M2 macrophages are induced by T_H2 cytokines, such as IL-4, IL-10 and IL-13 and produce IL-10 and IL-4 cytokines, which polarise immune responses to T_H2 cells (Grivennikov et al 2010, Hao et al 2012). M2 macrophages promote tolerance to tumours by suppressing T_H1 cells (Heusinkveld et al 2011, Lindau et al 2013). These M2 macrophages also support pro-tumour functions including facilitating metastasis, angiogenesis, intravasation, immune suppression, tumour cell invasion and inflammation (Qian and Pollard 2010).

Dendritic cells

Dendritic cells derived from bone marrow are particularly efficient APCs and fundamental to promoting long-term tumour immunity (Fong and Engleman 2000, Mroz et al 2011). This is because, unlike macrophages, DCs preserve antigenic peptides from the cells they phagocytose. These mature DCs then migrate to the lymphoid organs where they present antigens to the naïve T cells and B cells to initiate adaptive immune responses (Herr et al 2000, Savina and Amigorena 2007).

Dendritic cells exist in either an immature state or are activated by environmental signals into a mature state. Tumours often evade the immune system by suppressing the necessary signals within the tumour microenvironment to activate DCs (Mroz et al 2011). When immature DCs examine the tumour environment, capture antigens, migrate to the lymph nodes and present these antigens to T cells without costimulation this leads to tolerance by deleting reactive T cells and generation of regulatory T cells (Tregs) (Mroz et al 2011).

Cytokines associated with inflammation induce maturation of DCs which then presents the phagocytosed antigens to $CD4^+$ and $CD8^+$ T cells in the lymph nodes

via surface expressed MHC molecules in conjunction with the necessary costimulatory molecules. This results in adaptive immune system responses from primed $CD4^+$ T helper cells, $CD8^+$ cytotoxic T cells and antigen specific B cells. Mature DCs express IL-12 which promotes T_H1 immune responses which are required for anti-tumour immunity (Mroz et al 2011).

Dendritic cells also express TLRs. When DCs are stimulated through TLR3 they produce cytokines including IL-12 which recruit and activate NK cells. These NK cells then target the viral infected cells and immature DCs. This 'editing' of DCs selects for mature DCs that will present to CTLs to promote a T_H1 type response (Sivori et al 2014).

1.3.4 Cells of the adaptive immune system involved in immunosurveillance

B cells

During immunosurveillance macrophages and DCs present tumour antigens to naïve B cells (Finn 2012). Naïve B cells then mature into either plasma B cells or memory B cells. Plasma cells are relatively short lived and act as antibody factories to assist the immune system's acute response to a pathogen. The memory cells are long lived and serve a surveillance role that rapidly switches to clonal expansion of new plasma cells on subsequent exposure to the specific antigen (Abbas and Lichtman 2003).

These antibodies can have direct cytostatic or cytotoxic effect on the tumour cells. Antibody binding to tumour cells can engage NK cells to directly kill the tumour cells as well as increasing the efficiency of phagocytosis by DCs and macrophages. These phagocytes can then cross-present more tumour antigens to T cells and B cells further enhancing the immune response by generating polyclonal responses against multiple tumour antigens. This would minimise antigen-negative tumour escape mechanisms (Finn 2012).

B cells can also have a pro-tumour role subverting T_H1 anti-tumour responses towards ineffective T_H2 responses. This is because B cells can produce IL-10 which counterbalances IFN-γ activity. IL-10 promotes Tregs, inhibits CTL responses and prevents maturation of DCs. When immature DCs present tumour antigens to T cells this promotes tolerance rather than elimination (Lo-Man 2011).

T cells

A suite of naïve T cells are located in the lymphoid organs and clonal expansion of the naïve T cells with the correct antigen specificity occurs on APC presentation. These differentiate into memory T cells and CD4+ effector cells or CD8+ effector T cells. Some cells remain in the lymphoid organ while others disperse into the bloodstream where they can be transported to the site of infection. Effector cells are relatively short lived and primarily serve the role of an acute response to eliminate the pathogen (Abbas and Lichtman 2003, Herr et al 2000, Savina and Amigorena 2007).

CD4 T helper cells can be T_H1, T_H2 or T_H17 phenotype (Grivennikov et al 2010). $CD4^+$ T_H2 cells recognise class II MHC associated peptides presented on the cell surface of APCs and secrete cytokines and express membrane molecules to switch M1 macrophages to M2 phenotype and direct B cells to produce antigen–specific antibodies (Abbas and Lichtman 2003, Grivennikov et al 2010). These actions tend to be ineffective against tumours and often promote tumour tolerance, growth and metastasis (Grivennikov et al 2010).

$CD4^+$ T_H1 cells also recognise MHC class II associated peptides presented on the cell surface of APCs but produce T_H1 cytokines such as IFN-γ that help CTL responses against tumours. $CD4^+$ T_H1 cells can switch M2 macrophages to M1 macrophages (Heusinkveld et al 2011). $CD4^+$ T_H17 cells promote the activation of CTL by producing appropriate cytokines (Grivennikov et al 2010).

$CD8^+$ T cells function as CTLs and kill atypical cells based on MHC I associated peptides presented on cells. The CTLs have the discerning power to ignore self and target foreign MHC I associated peptides. They directly lyse tumour cells and secrete cytotoxic T_H1 cytokines such as IFN-γ and TNF-α (Grivennikov et al 2010). They initiate a long-term cellular response (Karbach et al 2012, Stevanovic 2002).

Before exposure to a specific antigen about one in a million naïve T cells is specific to that antigen. Following presentation of the antigen by APCs clonal expansion of the antigen specific naïve T cells occurs. Then one in ten T cells may be specific for that antigen during the peak phase of immune response. Post infection the antigen-specific T cells undergo apoptosis and stabilise at about one in ten thousand T cells. Compared to naïve T cells, effector and memory T cells require lower levels of

antigen to develop strong responses. This makes B cells and macrophages efficient APCs in the effector phase of T cell responses (Abbas and Lichtman 2003). However, it has been suggested that there is impaired interactions between the T cells and the macrophages in the Tasmanian devil (Michael and Sangster 2010).

1.3.5 Immunosuppression in the tumour microenvironment

While the immune system is capable of mounting effective anti-tumour responses it can also promote tumour survival, invasiveness and metastasis. Tumour cells can attract immunosuppressive cells (Finn 2012, Freire and Osinaga 2012). The most common suppressive cells include Tregs, regulatory B cells (Bregs), M2 macrophages, type 2 NKT cells and myeloid derived suppressor cells (Bjordahl et al 2012, Lindau et al 2013, Vasievich and Huang 2011). The purpose of these cells is to prevent harm such as autoimmune diseases from an unrestrained immune response but tumours subvert these cells to their advantage (Finn 2012, Freire and Osinaga 2012).

1.3.6 Cytokines and cancer

Cytokines play a critical role in promoting or suppressing tumours. There is a fundamental concept of a balance between T_H1 and T_H2 responses that can be characterised by cytokines and the type of immune cells that become activated (Shurin et al 1999). Interferon-gamma (IFN-γ) is the dominant T_H1 cytokine that is counterbalanced by the T_H2 cytokine IL-10 (Shurin et al 1999). T_H2 cytokine responses are seen as pro-tumour and supportive of the growth and spread of cancer (Shurin et al 1999)

T_H1 cytokines: IL-2, IFN-γ and IL-12

Good anti-tumour responses are initiated and maintained by $CD4^+$ T_H1 cells producing IL-2 and IFN-γ cytokines. Lack of IL-2 and IFN-γ responses promotes allograft acceptance (Shurin et al 1999).

IL-2 promotes proliferation of CTLs and enhances the function of NK cells (Dranoff 2004, Shurin et al 1999). Downregulation of IL-2 is associated with most cancers and Tregs suppress CTL responses by consuming IL-2 which is critical to CTL function (Schreiber et al 2011, Shurin et al 1999).

IFN-γ mediates activation of CTLs and other T_H1 cells (Shurin et al 1999). It is produced by T cells, NK cells, NKT cells and to a smaller degree macrophages and DCs (Dranoff 2004). IFN-γ can increase the immunogenicity of tumour cells by upregulation of MHC class I expression (Dunn et al 2002). It increases tumour antigen presentation, promotes cytotoxicity and suppresses tumours of microbial aetiology (Dranoff 2004).

IL-12 is the foremost cytokine in promoting a T_H1 response that increases cytotoxicity and inhibits angiogenesis (Dranoff 2004, Shurin et al 1999). IL-12 along with IFN-α and IFN-γ are produced by mature DCs to overcome tolerance and switch the T cell responses from regulatory T cell responses to effective CTL responses (Yong et al 2012).

The anti-tumour activity of T_H1 inflammatory cytokines is counterbalanced by the tumourigenesis role of these cytokines during chronic infection and inflammation (Dranoff 2004).

T_H2 cytokines: IL-10 and IL-4

IL-10 is widely recognised for its anti-inflammatory properties and immunoregulatory functions (Emmerich et al 2012). It is a cytokine of particular significance to the study of cancer as it has a dominant role in suppressing anti-tumour T_H1 responses and promoting ineffective T_H2 responses (Shurin et al 1999). Activation of $CD4^+$ T cells is inhibited by IL-10 signals, preventing them from expressing cytokines (Emmerich et al 2012).

IL-10 promotes tumour survival by inhibiting tumour antigen presentation by APCs (Dranoff 2004). This is achieved by suppressing the expression of MHC class II and co-stimulatory molecules of APCs (Emmerich et al 2012). Expression of pro-inflammatory cytokines such as IFN-γ by APCs is also downregulated by IL-10 acting as an immunoregulatory cytokine (Emmerich et al 2012).

The immunoregulatory suppression role of IL-10 is most evident in the priming phase of the immune system response (Emmerich et al 2012). This results in suppressed activation of macrophages, DCs and T cells. However, previously activated $CD8^+$ T cells, because of their increased expression of IL-10 receptors become reactivated rather than suppressed by IL-10 (Emmerich et al 2012).

IL-4 is an important T_H2 cytokine that is often upregulated in the tumour microenvironment and contributes towards metastatic disease (Shurin et al 1999). IL-4 promotes T cell activation and eosinophil function but inhibits CTLs (Dranoff 2004, Shurin et al 1999). IL-4 contributes towards tumour progression but does not play a critical role when tumours are in a state of equilibrium (Teng et al 2012). Upregulation of both IL-4 and IL-10 suppresses CTLs and promote allograft acceptance by inducing antigen specific tolerance (Shurin et al 1999).

Anti-tumour cytokines: IL-15, IL-18, M-CSF, GM-CSF, IFN-α, TNF-α and TRAIL

IL-15 is produced mainly by APCs and promotes cytotoxic responses by stimulating the clonal expansion of CD8+ T cells, memory cells and to a lesser extent effector cells (Abbas and Lichtman 2003, Dranoff 2004).

IL-18 promotes a T_H1 response increasing cytotoxicity and inhibiting angiogenesis (Dranoff 2004, Shurin et al 1999). IL-18 can be generated by DCs in the tumour microenvironment and this maintains CTLs and T_H1 cells (Shurin et al 1999).

Macrophage colony-stimulating factor (M-CSF) is a cytokine produced by macrophages, endothelial cells, fibroblasts, bone-marrow stroma cells and promotes anti-tumour macrophage function (Dranoff 2004).

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a cytokine produced by T cells, NK cells, NKT cells, macrophages, endothelial cells, fibroblasts and respiratory epithelial cells. It promotes growth, differentiation and tumour antigen presentation of DCs (Dranoff 2004, Yong et al 2012). It also increases the activity of macrophages, granulocytes and NKT cells (Dranoff 2004).

IFN-α increases tumour antigen presentation and promotes cytotoxicity. Clinical trials with IFN-α have shown that some patients have prolonged survival and generate $CD8^+$ T cells that are specific to the tumour (Dranoff 2004).

TNF-α induces apoptosis in tumour cells and activates endothelium and granulocytes as well as promoting DC maturation and antigen presenting abilities to elicit CTL anti-tumour responses (Dranoff 2004, Yong et al 2012).

TNF-related apoptosis-inducing ligand (TRAIL) is a cytokine produced by most normal tissues and by binding to death receptors on tumour cells it can induce apoptosis of tumour cells (Cormier 2013, Dranoff 2004).

Pro-tumour cytokines: IL-6, IL-13 and TGF-β

The role of IL-6 in cancer is difficult to define as it has both pro- and anti-tumour activities (Dranoff 2004, Sato et al 1993). It is a regulator of Tregs and enhances T cell and B cell function (Kimura and Kishimoto 2010). Downregulation of IL-6 inhibits proliferation of lymphocytes (Dranoff 2004). IL-6 is often upregulated in the tumour microenvironment (Shurin et al 1999). IL-6 and IL-15 upregulation correlates to increased NK cell killing in CTVT (Fassati and Mitchison 2010).

IL-13 stimulates humoral responses and inhibits cytotoxic responses (Dranoff 2004, Shurin et al 1999).

Transforming growth factor beta (TGF-β) is a cytokine secreted by many cells and can be released by the tumour cells themselves as a mechanism to weaken immune responses (Dranoff 2004, Shurin et al 1999). It is one of the most potent immunosuppressive cytokines that directly inhibits DC, NK and CTL functions while promoting the activation and proliferation of Tregs which suppress effective anti-tumour immune system responses (Dandawate et al). Upregulation of TGF-β is tumour protective and worthy of investigation in the DFTD microenvironment; however, CTVT cells secrete TGF-β but levels do not significantly vary between progressive and recessive disease stages (Hsiao et al 2008).

Cytokine therapies

Intratumoural injections of cytokines such as IL-2, IL-4, IL-6, IL-7, IL-12, IFN-γ, lymphotactin and GM-CSF can promote anti-tumour immune responses. Systemic therapies involving cytokine injections are less promising causing what could be described as a cytokine storm (Dranoff 2004).

High dose IL-2 therapy results in tumour regression in only a small number of patients. Lower doses of IL-2 upregulates NK cells, however, regulatory T cells which suppress anti-tumour responses are also upregulated (Dranoff 2004). IL-2 has been given FDA approval for treatment of melanoma and renal cancer. The IL-2 can be included in vaccines to maintain dendritic cell growth (Yong et al 2012).

While the pro-tumour effects of IL-10 are widely recognised the anti-tumour roles of IL-10 are rarely reported. Localised IL-10 in the tumour micro-environment promotes tumour resident $CD8^+$ T cell activation, proliferation and anti-tumour cytotoxic responses (Emmerich et al 2012). The benefits of this IL-10 induced anti-tumour effect are dependent on the presence of $CD8^+$ resident T cells that have previously been activated by tumour antigens (Emmerich et al 2012).

Treatment with IL-10 has been shown to increase the IFN-γ expression by tumour infiltrating $CD8^+$ T cells while lacking a similar effect on $CD8^+$ lymphocytes resident in secondary lymphoid organs (Emmerich et al 2012). This observation is consistent with the fact that most lymph node resident $CD8^+$ T cells are naïve while the tumour resident $CD8^+$ T cells are more likely to be antigen activated cells which are known to have increased IL-10 receptor expression (Emmerich et al 2012).

1.3.7 **Apoptosis in cancer**

A characteristic of many cancers is the ability to resist apoptosis. This can lead to tumour development, growth and metastasis in addition to hindering anticancer therapies (Fulda 2009, Leblanc et al 1999, Lowe and Lin 2000).

It has been suggested that most tumour cells retain the machinery required for apoptosis but have pathway mutations (Lowe and Lin 2000). The apoptosis signalling pathway is a multifaceted complementary combination of pro- and anti-apoptosis signals produced within the cell and in the external tumour environment (Lowe and Lin 2000).

The intrinsic apoptotic pathway is controlled by the cell's mitochondria to facilitate programmed cell death by upregulation of pro-apoptotic molecules (Igney and Krammer 2002b). The extrinsic apoptotic pathway involves signalling via death receptors and Fas receptors by cytotoxic cells during immunosurveillance.

The Bcl-2 pathway, Fas/CD95 receptor pathway and PI-3 kinase pathway all have significant roles in apoptosis (Lowe and Lin 2000). The downregulation and mutation of pro-apoptotic molecules or the expression of anti-apoptotic molecules can inhibit apoptosis at the death receptor as well as interfering with perforin/granzyme pathway (Igney and Krammer 2002a). A number of anti-apoptotic proteins, such as FLIP, Bcl-

2, Bcl-x_1, Mcl-1, Survivin and PI-9/SPI-6 have been identified in human cancers and are predictive of a poor diagnosis.

Upregulation of soluble receptors and decoy receptors such as sCD95 and DcR3 that lack functionality and compete for apoptotic signalling ligands in human and animal models have also been identified as a tumour escape mechanism (Igney and Krammer 2002a). Downregulation of pro-apoptotic molecules such as CD95 and various TRAIL receptors may impair tumour surveillance by NK and T cells (Igney and Krammer 2002a).

Determining apoptosis

There is a number of standard means of determining apoptosis in cells. During the earliest stages of apoptosis there is a loss of asymmetry of the cell membrane. This leads to exposure of the phosphatidylserine found on the intracellular leaflet of the plasma membrane that can then be bound by annexin V. In the very earliest stages of apoptosis the membrane remains impermeable to molecules such as propidium iodide (PI) and 7AAD but these molecules pass through in late stage apoptosis. Necrotic cells pass through PI but bind very little annexin V and this allows discrimination of early and late stage apoptosis from necrosis (Vermes et al 2000).

The TUNEL assay (**T**dT-mediated d**U**tp **N**ick **E**nd **L**abelling) is another protocol to detect late stage apoptosis. The exposed 3'-hydroxyl ends of DNA breaks that occur in late stage apoptotic cells are labelled with the polymerase terminal deoxynucleotidyl transferase (TdT). TdT is an enzyme which then catalyses the incorporation of fluorochrome or biotinylated deoxyuridine triphosphate which can be detected by immunofluorescence (Obrien et al 1997, Vermes et al 2000).

Therapies to promote tumour apoptosis

Understanding the ability of a specific tumour cell line to undergo or resist apoptosis would be critical to selecting appropriate anti-cancer therapies. The genes, proteins and pathways that regulate apoptosis are suitable targets for anticancer therapies. For example; if a specific tumour upregulates expression of *Bcl-2* gene products, which inhibit apoptosis, administration of Ad-DF3-Bax to inhibit Bcl-2 may prove effective. Alternatively, the viral protein apoptin induces apoptosis and its activity is enhanced by Bcl-2 in tumour cells leaving healthy cells intact (Lowe and Lin 2000).

Atypical regulation of NF-$_K$B activity, which controls transcription of DNA, may promote tumour survival and the administration of a suitable inhibitor such as I-$_K$B may induce tumour cell death. As well, the disruption of the survival signals generated by the PI-3 kinase/Akt pathway may reinstate apoptosis in the tumour. Inhibition of the Ras-GTPase activating protein appears to selectively promote apoptosis in human cancers and not affect normal cells. Restoring lost or mutated genes such as *p53* is a strategy that has been adopted in clinical trials (Leblanc et al 1999, Lowe and Lin 2000).

Selective induction of apoptosis may be possible through TRAIL-induced apoptosis. TRAIL is a TNF related protein that binds to the DR4 and DR5 death receptors. Healthy human cells express decoy receptors for TRAIL proteins that minimise attachment of the TRAIL protein to functioning DR4 and DR5 receptors. Many human cancers demonstrate a loss of decoy receptors. This makes them more susceptible than healthy cells to induction of apoptosis by increased exposure to TRAIL protein as a cancer therapy (Lowe and Lin 2000).

Most cytotoxic anti-tumour therapies succeed by promoting apoptosis; however, adverse effects are often linked to apoptotic death of normal cells because the apoptotic signalling extends beyond the tumour cell population (Lowe and Lin 2000). It may be that with apoptosis resistance cell lines the promotion of cell senescence would be an effective anti-cancer therapy (Lowe and Lin 2000). These treatments should prove more effective in synergy with other cancer therapies such as surgery, chemotherapy and radiation therapy (Leblanc et al 1999, Lowe and Lin 2000).

1.4 Murine models for tumour immunology

1.4.1 Mouse models

Ideally tumour immunology should be studied in the host species and not a mouse model because the results from mouse models do not always correlate to the host species (Bierer 2009, Frese and Tuveson 2007). Nevertheless, it is often impractical, undesirable or unethical to conduct experiments in the host species in which case mouse models can provide valuable information and preliminary data prior to any clinical trials (Frese and Tuveson 2007).

While some would argue that promising *in vitro* trials should progress to the host species rather than an intermediary mouse model (Bailey 2011) all clinically approved agents for the treatment of human cancer have demonstrated positive activity in mice (Becher and Holland 2006). However, many agents that have therapeutic benefit in the mouse model do not translate into effective treatment in the human host (Becher and Holland 2006).

The use of transgenic animals is intended to minimise the difference between the mouse and the host species and it has been noted in some genetically engineered mouse models that tumour growth is so similar to that in the host that clinical pathologists have difficulty telling them apart using a microscope (Becher and Holland 2006). Xenografts obtained directly from patient tumours have replicated the histology and biology of the primary tumour more faithfully than cell lines, some of which may have passed through more than 100 passages (Becher and Holland 2006, Borrell 2010).

When using mouse models for the study of tumour regression or rejection it is paramount to consider the implications that the underlying rejection mechanism may be graft rejection rather than tumour specific rejection. In the case of DFTD this is less of an issue because in the wild the tumour is transmitted as an allograft which under normal circumstances should be rejected by a graft rejection mechanism (Azimzadeh et al 1996, Pearse and Swift 2006).

The shorter lifespan of mice compared to human disease development is significant (Kim et al 2003); however, this may be less of a problem in the DFTD model as the disease is particularly virulent leading to mortality of the natural host within months of infection (Lachish et al 2007).

There are usually differences in the progression of tumours between host species and mouse and this is often seen in variant cellular targets, size of tumour and metastatic disease progression. For example, the metastatic route in human breast cancer is usually lymphatic while in the mouse model the route is usually the blood vessels (Kim et al 2003).

The study of metastatic disease can be difficult in mice because most implantations are done subcutaneously rather than orthotopic (Becher and Holland 2006). This

may be less of a problem in the study of DFTD as the implantation of cells in the natural host is through biting which implants the cells close to the skin surface in the dermis or submucosal connective tissue in the mouth (Loh et al 2006a).

Mice consume higher amounts of oxygen per cell compared to larger animals and this may be significant in the tumour microenvironment were different expression of hypoxia-induced genes may occur altering proliferation and differentiation (Kim et al 2003). The development of blood supply by the process of neovascularisation is determined by the host not the tumour itself (Becher and Holland 2006). The interaction between stroma cells and cancer is artificial since the stroma is murine (Becher and Holland 2006). Species or class specific differences in the binding of proteins and metabolism can be another variable in the mouse model experiments (Becher and Holland 2006).

Mouse models for CTVT

Since the Russian veterinarian Norwinsky's first experiments in 1876, CTVT has been studied experimentally by transferring viable cells into animals (Das and Das 2000). Animal studies of CTVT have provided an understanding of how CTVT is transmitted as an allograft, accepted by the new host and ultimately regresses leaving the dog immune to re-infection (Harmelin et al 2001). Since dogs are not an endangered species most of these studies have been conducted in the host species (Das and Das 2000).

Murine xenograft models for CTVT have also been used to reduce the need for maintaining allogeneic transfer in dogs (Harmelin et al 2001). Compared to dogs the murine model is relatively low cost to house and maintain. There would also be fewer problems with maintaining animal ethics approval and greater availability of antibodies to study immune responses (Bierer 2009, Harmelin et al 2001).

CTVT has been engrafted into mice that have had their immune system suppressed with irradiation or into immunocompromised athymic nude mice and NOD/SCID mice (Harmelin et al 2001). The NOD/SCID model could be considered the model of choice as it allows CTVT to be established and progress with the typical characteristics of CTVT in the natural host (Harmelin et al 2001). An inoculation of 1 x 10^6 cells will produce tumours within 47 days in the NOD/SCID model (Harmelin et al 2001).

The mouse models primarily can be used to test hypotheses and predict responses to treatments. Complex biological problems can be examined and predictive models focus on testing treatment responses including efficacy and toxicity (Coghlan 2013). The CTVT murine model provides a precedent and justification for using murine xenograft models to study the similarly infectious cancer DFTD. It could be expected that mouse models that have been successfully exploited for the study of CTVT would prove suitable for the study of DFTD since they are both transmissible neoplasms (Bierer 2009, Harmelin et al 2001, Loh et al 2006a).

1.4.2 Xenograft tolerant mouse strains

There is a variety of mice strains which have specific immunological impairments that clarify the immunological functions imperative to tumour engraftment, rejection, or regression (Frese and Tuveson 2007, Harmelin et al 2001).

The three strains discussed in the following paragraphs all lack functional T cells. Lack of T cells would limit the protection offered by macrophages (Bancroft et al 1986). As part of the surveillance of the innate immune system macrophages detect threats and present early signals to promote T cell proliferation and differentiation. In turn the T cells provide feedback signals that enhance the activity of the macrophages (Bancroft et al 1986).

C.B-17 scid/scid mice

C.B-17 scid/scid (scid) mice are homozygous for the severe combined immunodeficiency (scid) mutation and this results in a lack of functionality of B and T cells; however, some young adults might generate a few functional B and T cells and by 10 to 14 months nearly all the older adults have developed a limited number of functional T cells (Bancroft et al 1986).

B and T cells are the only leukocytes that have impaired function in a scid mouse. NK cells, macrophages, APCs, monocytes, granulocytes and DCs are all normal in the scid mouse (Bosma and Carroll 1991). This would provide a model to study macrophage, NK cell and DC responses to DFTD cells independent of T and B cell interactions (Bancroft et al 1986).

Lymphoid tissues are underdeveloped. The thymus is typically less than 10% of the normal size and the lymph nodes are minuscule and contain few lymphocytes. The

spleen presents with an unusual histology and contains macrophages and large granular cells but only low numbers of lymphoid cells and plasmacytes. Red blood cell levels are normal and serum Ig concentrations are less than 20 ng/ml (Bancroft et al 1986, Bosma and Carroll 1991).

Xenogeneic tumours can be successfully engrafted into scid mice (Bancroft et al 1986, Bosma and Carroll 1991). The scid mutation inhibits the early development of B and T cells but does not affect the ability of the mice to support normal lymphocyte proliferation and this has allowed the reconstitution of the immune system with functioning lymphocytes from other mice and humans (Bosma and Carroll 1991, Pearson et al 2008).

NOD/SCID mice

The NOD/SCID mouse strain has been created by backcrossing mice with the scid mutation and mice that have a diabetes-susceptible non-obese diabetic (NOD) background (Prochazka et al 1992). NOD/SCID mice have a more compromised immune system than athymic nude mice and CB-17-scid mice. NOD/SCID mice lack T and B cells, lack effective levels of serum antibody, have no complement activity, impaired development and function of macrophages and other APCs but do demonstrate limited NK cell function (Harmelin et al 2001).

The lack of functioning T cells from the scid background makes the mice diabetes resistant (Prochazka et al 1992). The impaired immunity means these mice must be maintained in a pathogen free environment and have a short life expectancy of about eight months (Harmelin et al 2001). A single injection with broad spectrum antibiotics will usually prevent bacterial infection in NOD/SCID mice (Bastide et al 2002, Harmelin et al 2001).

The NOD/SCID strain has proved suitable for studying certain human cancers because there is no evident tumour immunity (Bastide et al 2002). CTVT tumours have demonstrated the ability to undergo numerous passages in these mice providing a source of tumour cell lines maintained in-vivo (Harmelin et al 2001). The NOD/SCID xenograft model preserves the cytological, histological and molecular characteristics of CTVT. This facilitates the study of engraftment, disease progression including metastasis, diagnosis and treatments for CTVT (Harmelin et al 2001).

Adoptive transfer of immune cells from competent mice to NOD/SCID mice can identify the contribution of individual components to effective immunity against challenges (Hicks et al 2006). It may be possible to apply this approach to DFTD engraftment in NOD/SCID mice to elucidate the ability and role of specific cell populations in rejection and or regression of DTFD.

Athymic nude mice

The nude mouse has an autosomal recessive mutation of the 11th chromosome that disrupts the FOXN1 gene resulting in failure to grow hair and lack of a functional thymus. The lack of a thymus means that nude mice are deficient in mature T cells including CD4 + and CD8+ cells which has negative implications for cell-mediated immune responses including the lack of CD4+ helper T cells to produce antibodies (Kim et al 2003).

The lack of effective anti-tumour immunity makes this strain suitable as a xenograft model without the need of additional immune system suppression (Kim et al 2003). Human tumours can be maintained through more than fifty passages and still demonstrate the same morphology and phenotype with no species hybridization (Spangthomsen and Visfeldt 1976).

Xenograft tumour growth is contained locally in a well defined capsule-like connective tissue that is not attached to the skin or underlying tissues. If the athymic nude mice did engraft DFTD cells they may because of their hairless nature and translucent skin make visual monitoring of tumour growth and precise intratumoural injections easier. Another advantage of the athymic nude mice is that they retain some functional components of the immune system including macrophages, DCs, B cells and NK cells (Spangthomsen and Visfeldt 1976) and these may be stimulated with therapeutic agents to target the DFTD cells.

1.5 **Evaluating immune responses**

By studying immune system responses to DFTD it is possible to infer how the immune system and the tumour cells respond to each other and this may provide the rationale for developing a cancer vaccine or immunotherapy. Antibodies are easily measured using flow cytometry. ELISA, ELISPOT, PCR and flow cytometry can be used to detect and measure cytokine production by cells in response to specific

tumour antigens or tumour cells. Cytotoxic responses against tumours can be evaluated by tumour rejection or regression *in vivo* or cytotoxic activity against the tumour cells *in vitro* (Clay et al 2001).

The type and level of antibody produced can be informative about the cells and cytokines that are directing isotype switching from IgM to specific IgG isotypes. For example IFN-γ suppresses IgG1 and promotes IgG2a (Finkelman et al 1988). A low ratio of IgG1/IgG2a indicates a T_H1-mediated antibody response while a high ratio indicates a T_H2-mediated antibody response (Kanai et al 2007). T cells are required for isotype switching (Arrenbrecht and Mitchell 1975) but other cells can influence the process such as NK cells which promote IgG1 production, which mediates hyperacute rejection of xenografts (Yin et al 2004).

Combining antibody responses with cytokine responses presents a more complete picture of the T_H1/T_H2 balance of the immune response. A T_H1 response is associated with the upregulation of IL-2, IFN-γ and TNF-α cytokines while T_H2 responses are associated with the upregulation of IL-4, IL-5, IL-6, IL-10 and IL-13 cytokines.

In vitro cytotoxicity assays can be an important step in identifying cytotoxic function of specific cell types including T cells, NK cells and phagocytes (Clay et al 2001, Niles et al 2008). Cytotoxicity can be directly measured by enumerating dead cells or can be inferred from assays that measure viability (Niles et al 2008).

Cytotoxicity assays are a direct measure of tumour cell death as a result of CD8+ T cells or NK cells lysing tumour cells. The technique can involve incubating effector cells with tumour cells that are labelled with radioactive ^{51}Cr. When the tumour cells undergo apoptosis they release the ^{51}Cr into the supernatant. The supernatant is harvested cell free and measured for ^{51}Cr and this provides a quantitative measure of cytotoxicity. However, phagocytosis can be a very efficient form of eliminating tumour cells and ^{51}Cr is retained by the phagocytes making this assay insensitive to this form of killing (Munn and Cheung 1990).

Other assays such as calcein release assay, lactate dehydrogenase assay and Adenosine 5′-triphosphate (ATP) based assays are available based on similar principles. There are also fluorescent techniques that visualise dying cells or viable

cells to estimate cytotoxicity (Clay et al 2001, Hermans et al 2004, Lappalainen et al 1994).

An *in vivo* alternative may involve CFSE labelling of DFTD cells which can be injected and tracked for cytotoxic activity in vivo. CFSE labelling is reported to affect the viability and proliferation of certain cell lines and this may present an experimental artefact in any assay involving CFSE (Black et al 2006, Clay et al 2001, Hermans et al 2004, Lastovicka et al 2009).

1.6 **Cancer vaccines**

Fundamentally a vaccine exposes the host's immune system to pathogen specific antigens in a non-infectious setting. The vaccine exploits the immune's system ability to recognise, eliminate and remember antigens in the body (Abbas and Lichtman 2003, Mackay and Rosen 2001, National Cancer Institute 2014).

Most successful preventative vaccines target the viral aetiology of the cancer rather the cancer itself (Castellsagué et al 2011, National Cancer Institute 2014). However, there is no basis to suspect a viral link to DFTD disease transmission (Pyecroft et al 2007).

Therapeutic vaccines are used to treat established tumours and can be used as an alternative to, or in synergy with, treatments such as surgery, radiation and chemotherapy (Yang et al 2012b). Development of therapeutic vaccines has proven more difficult than development of preventative vaccines against viral aetiologies. Therapeutic vaccines must target specific immune responses and these immune responses must be of a magnitude great enough to overcome the protective barriers utilised by the tumour cells to thwart the immune system (Yang et al 2012b). The vaccine must induce tumour specific $CD8^+$ T CTLs to lyse the tumours and tumour specific $CD4^+$ T cells to provide cytokines to enhance CTL activity (Schlom 2012).

Most vaccines are effective because they stimulate antibody production resulting in antigen-specific immunity based on subsequent exposure to T cells (Clay et al 2001, National Cancer Institute 2014, Waldmann 2006). Passive immunisation with sera or whole blood from CTVT convalescent animals has been trialled (Ganguly et al 2013). Antibodies can modify tumours or help identify the tumour to other components of the immune system such as CTLs, NK cells or macrophages (Clay et al 2001, Yang

et al 2012b). With antibody-dependent cellular cytotoxicity (ADCC) tumour specific antibodies bind to the tumour cells and the Fc receptor on NK cells bind to the antibody triggering the NK cell to lyse the tumour cell (Waldmann 2006, Yang et al 2012b).

Conventional vaccines

Conventional vaccines can also be manufactured from killed or weakened cancer cells obtained from the patient themselves (autologous) or another person's cancer (allogeneic) (Buonaguro et al 2011, Schlom 2012). The use of freeze-thaw lysates obtained from autologous tumour material demonstrates considerable potential to induce targeted T cell responses in cancer vaccines (Herr et al 2000). Active immunisation with CTVT tumour lysates has been used with some efficacy (Ganguly et al 2013).

Irradiating tumour cells may be more effective than freeze thaw. Studies suggest irradiated tumour cells prime dendritic cell-mediated immunity while freeze thaw appear to inhibit *in vitro* killing by CTLs (Meng et al 2012). Irradiated cells present danger signals, increased MHC class I expression and express cytokines promoting DC and CTL activation for enhanced anti-tumour responses (Meng et al 2012).

Subunit and conjugate vaccines

Subunit vaccines present tumour specific surface peptide antigens to activate B and T cells via DCs (Black et al 2010). They require the addition of adjuvants to activate the immune system as peptides on their own are usually poorly immunogenic (Black et al 2010). Conjugate vaccines induce IgG responses by fusing T cell-independent antigens with proteins easily recognised by T cells (McCormick et al 2006, National Cancer Institute 2014).

To date antigens have included proteins, carbohydrates, glycoproteins, carbohydrate-protein combinations (glycopeptides) and carbohydrate-lipid combinations (gangliosides) (Joshi et al 2012, Vigneron et al 2013). These antigens represent just part of the tumour being targeted by the vaccine and can be sourced from the tumour itself or synthesize based on sequencing data (Black et al 2010, Buonaguro et al 2011, Lakshminarayanan et al 2012). The advantages of synthetic peptide sequences include they are defined, stable and non-infectious; however, it may prove difficult to identify and mimic the correct sequences and sometimes the

epitopes recognised by the B-cells are in reality discontinuous sequences (Black et al 2010, Mackay and Rosen 2001).

Dendritic cell vaccines

Vaccines which depend on the immunogenicity of the vaccine alone without the presentation of antigens via DCs are predisposed to failure as they are unlikely to elicit a strong enough CTL response. Vaccine strategies aimed at loading DCs either *in vitro* or *in vivo* with tumour antigens are more likely to promote effective CTL responses against the tumour (Baar 1999).

Dendritic-cell vaccines can be manufactured by growing DCs *in vitro* while feeding them tumour specific or tumour associated antigens. Antigen loading techniques for DCs include pulsing DCs with antigens, loading DCs with tumour lysates or whole tumour cells, transfecting DCs with tumour DNA or RNA, or infecting DCs with bacterial, viral or yeast vectors. The DCs then manufacture and express the antigens on their surface (Baar 1999). Dendritic/tumour cell hybrid vaccines have been trialled with CTVT (Pai et al 2011).

Dendritic cells have great potential as a cancer vaccine adjuvant because they promote both CD4+ and CD8+ T cell responses by presenting the tumour antigens to naïve T cells resulting in generation of short disease response and long term memory response (Herr et al 2000, Ma et al 2012).

Transgenic vaccines

Enhanced subunit vaccines can be produced using recombinant-DNA techniques that result in both humoral and cellular immune responses (Mackay and Rosen 2001). Recombinant vaccines involve transgenic transfer of antigen expressing genes from the pathogen to another cell type. DNA can also be inserted into bacterial plasmids and this will stimulate a strong immune response because of the CpG motifs associated with bacterial DNA. Intramuscular injection of the transgenic plasmids will result in dendritic cell activation and maturation. Alternatively a gene gun could be used to blast the plasmids through the skin causing some plasmids to enter DCs directly (Mackay and Rosen 2001). This approach would have potential as a vaccine delivery method for Tasmanian devils caught through a trapping program.

1.6.1 **Adjuvants**

Early attempts at cancer vaccines depended on tumour antigen presentation but failed to recognise the importance of co-stimulatory signals required to elicit a CTL response. This is a critical requirement of the immune system response to a cancer vaccine if it is to be effective. The use of suitable adjuvants may be required to provide the maturation signal to the DCs to cause the release of cytokines including IL-12 to promote T cell differentiation and activation as CTLs (Yong et al 2012). Without the addition of adjuvants many vaccines such as subunit vaccines lack the immunogenicity to engage the immune system adequately (Black et al 2010).

Nearly every marketed vaccine utilises alum as an adjuvant. This has occurred because it was the only FDA approved adjuvant until recently (Vasievich and Huang 2011). AS04 (monophosphorylated lipid A conjugated to aluminum hydroxide) was the second adjuvant approved by the FDA and is used in the Cervarix preventative vaccine. Granulocyte macrophage-colony stimulating factor (GM-CSF) is an (Cerkovnik et al 2010) adjuvant used in Provenge (Sipuleucel-T) which is the first FDA approved therapeutic cancer vaccine.

GM-CSF is technically not classified as an adjuvant by the FDA for the purpose of approval as it is a cytokine but effectively is functioning as an adjuvant (Vasievich and Huang 2011). Other cytokines and molecules that can be employed as adjuvants include costimulatory molecules such as ICAM-1, B7-1 and LFA-3 and cytokines such as IL-2, IFN-α and GM-CSF (Vasievich and Huang 2011). These can be synthesized and added to treatment vaccines to boost the DCs and CTL responses (Vasievich and Huang 2011).

PAMPs, DAMPs and TLR ligands as adjuvants

The growing knowledge about the immune system has recognised the indispensible role of the innate immune system in presenting tumour antigens to the adaptive arm of the immune system. The innate immune system has evolved to produce strong and effective responses against bacterial, viral and fungal antigens (Mroz et al 2011, Ridnour et al 2013).

Pathogen-associated molecular patterns (PAMPs) act as signals to the immune system to mount a response against infection. Various PAMPs from bacteria and viruses bind to Toll-like receptors (TLRs) and stimulate cytokine expression, promote

inflammation and recruits innate immune responses (Ridnour et al 2013). In addition to PAMPS are DAMPs that are intracellular proteins or nucleic acids released during necrosis serving as immuno-stimulants capable of promoting pro-inflammatory responses (Mroz et al 2011, Ridnour et al 2013).

Toll-like receptors recognise PAMPs associated with microbes. Bacterial PAMPs are recognised by TLR2 (lipoprotein), TLR5 (flagellin) and TLR9 (CpG DNA motifs). Viral PAMPs are recognised by TLR3 (double-stranded RNA), TLR7/8 (single-stranded RNA) and TLR9 (CpG DNA motifs) (Sivori et al 2014).

While NK cells can be directly activated by TLRs the synergy with the microenvironment can enhance their cytotoxic activity, direct their regulatory function and lead to recruitment of CTL responses (Sivori et al 2014). Recent research has also identified PAMPs and damage-associated molecular patterns (DAMPs) as molecules which switch on dendritic cell maturation resulting in a full-blown immune system response against the presented antigens (Ridnour et al 2013).

The design of adjuvants has evolved with the understanding of the interaction of PAMPs with specific pattern recognition receptors (PRR) including toll-like receptors (TLRs) (Dubensky Jr and Reed 2010). TLR ligands are potentially adjuvants starting a cascade of events to promote anti-tumour responses (Yong et al 2012). TLR ligands induce maturation of DCs into effective APCs. At the same time TLR ligands upregulate the expression of costimulatory molecules on DCs and cytokine expression by DCs which are essential for the activation and differentiation T cells towards T_H1 or T_H2 responses (Yong et al 2012).

Tumour cells typically fail to express PAMPs allowing immune system tolerance. Administration of TLR antagonistic PAMPS in some instances promote T_H1 responses by activating DCs and promoting production of inflammatory cytokines including TNF-α and IL-12.Administration of TLR antagonistic PAMPS needs to be localised as systemic administration is highly toxic (Lu 2014). Bacillus Calmette-Guerin (BCG) and imiquimod are two FDA approved TLR agonists used as immunotherapies for treating cancer. Monophosphoryl lipid A (MPL) is a TLR agonist approved as a vaccine adjuvant (Adams et al 2012, Lu 2014).

On the occasion that TLR agonists are not effective it could be explained by the regulatory role of TLR agonists which suppress immune responses by inducing IL-10, Tregs and PD-L1 (Lu 2014). The TLR7 agonist imiquimod and the TLR9 agonist CpG have induced IL-10 and Tregs in some studies (Lu 2014). TLRs including imiquimod, CpG and poly I:C have been shown in some studies to induce PD-L1 which is a protein that inhibits T cell activation by binding the programmed death-1 (PD-1) receptor on activated T cells (Lu 2014). The use of antibodies to blockade regulatory signals such as IL-10 is being considered in many of the latest trials with TLR agonists (Lu 2014).

Necrosis of tumour cells tends to promote inflammatory responses and the chronic release of DAMPs by necrotic tumour cells can promote tumour survival through activation of TLR 1-9 receptors on tumour cells (Mroz et al 2011, Ridnour et al 2013). Activation upregulates NF-κB, inhibits apoptosis and generates pro-tumour cytokines to hijack immune system responses (Ridnour et al 2013). On the otherhand, apoptosis generally results in non-inflammatory disposal by phagocytes. Depending of the species of phagocyte recruited apoptosis can be immunogenic or non-immunogenic and this is most likely linked to DAMPs (Mroz et al 2011). Inflammatory responses are required to promote anti-tumour immunity and the discovery of DAMPs explains why apoptosis can in some instances promote anti-tumour immunity.

Cells of the immune system including DCs and B cells can be directly activated by bacterial DNA segments with 5’-Cytosine-phosphodiester-Guanin (CpG)-3’ motifs. The CpG oligodeoxynucleotide sequences are recognised by immune cells which express toll-like receptor (TLR9) and signalling through this pathway leads to innate immune responses from B cells and plasmacytoid DCs (pDCs). T cells, NK cells and monocytes express lower levels of TLR-9 in humans and are not the target of CpG immunotherapy strategies. The best CpG-ODN sequences vary between host species (Kawarai et al 2011, Vasievich and Huang 2011).

When the TLR-9 pathway is activated in pDCs and B cells their production of T_H1 and pro-inflammatory cytokines is upregulated as well as TNF. These cells then differentiate into plasma cells and/or APCs which present antigens to T cells resulting in strong CTL activity against some cancers (Dubensky Jr and Reed 2010,

Kawarai et al). The addition of emulsified oils such as montanide can further enhance the immune response by maintaining the antigens at the site of injection for a prolonged period (Black et al 2010, Cerkovnik et al 2010).

1.6.2 Vaccination protocols and immunisation routes

Vaccination protocols usually involve repeated immunisation with the same vaccine. A prime-boost protocol may be better for promoting effective and long lasting CTL responses against tumours (Mackay and Rosen 2001).

There are various immunisations routes including intramuscular, intravenous, intraperitoneal, subcutaneous, mucosal and epidermal. These are not always equal for inducing appropriate immune responses and what may be best for one pathogen may be ineffective for another (Fynan et al 1993). As an example, in one study immunity against Leishmania could be induced by immunisation with inactivated promastigotes administered intravenously or intraperitoneally (Liew et al 1985). In the same study subcutaneous or intramuscular immunisations not only failed to induce immunity but worsened the disease and resulted in earlier death (Liew et al 1985).

1.7 Potential immunotherapy options for DFTD

Immunotherapy can be passive or active. Passive immunotherapy involves the transfer of donor lymphocytes and or antibodies to provide protection. Active immunotherapy's, such as cancer vaccines, modulate the host's own immune system to recognise and overcome tolerance of tumour cells. Promotion of tumour-specific CTLs can provide life-long immunity (Yong et al 2012).

1.7.1 Immunotherapy based on Coley's toxins and bacteria

Many would regard William Coley as the "Father of Immunotherapy" (McCarthy 2005) but William Coley was ignorant of the underlying mechanisms of the immune system that made his "toxins" effective against cancer. The concept of the immune system only appears in the literature in the 1960s (Moulin 1988). He hypothesised that the bacteria produced toxins that directly attacked the tumour, believed the heat of fever was critical to the cure and even thought there was a 'cancer bacillus' responsible for the disease (Coley 1891).

William Coley was a New York surgeon frustrated by his ineffectiveness in treating cancer patients (Coley 1891). Through a search of hospital records he discovered over 40 cases of tumour remission following erysipelas infections. Erysipelas infections were a not uncommon post-operative infection caused by *Streptococcus pyogenes* in the late 19th Century (Hobohm 2009).

Coley read the 1885 case notes of a German immigrant named Fred Stein. He had inoperable recurrent sarcoma of the neck which five operations had failed to control (Coley 1891). Stein contracted erysipelas and nearly died, but when he recovered the cancer had gone. In 1891, Coley found Stein who had remained cancer free (Coley 1891, Martin 2006, Nauts et al 1953). This inspired him to deliberately induce erysipelas in cancer patients to affect a cure (Starnes 1992).

William Coley was not the first doctor to try treating cancer with deliberate erysipelas infections (Coley 1891, Van Arsdale 1886). Coley himself referred to the attempts by Bosh who tried patient to patient infection, Fehleisen who used cultured bacteria to infect patients and Bruns who reported 3 out of 5 cancer cures following inoculation with *Streptococcus pyogenes* (Coley 1891).

Coley decided to treat a late-stage cancer patient named Zola with erysipelas. Zola's case notes showed deliberate erysipelas resulted in lifelong remission (Hobohm 2009, Martin 2006). Coley's work over next two years with 12 late-stage patients demonstrated 2 full remissions, 6 partial remissions and 2 deaths (Hobohm 2009).

Working with live *Streptococcus pyogenes* was abandoned due to the fatalities. Coley switched to inactivated bacteria and in 1893 treated his first patient with "Coley's mixed toxins". The first patient was a sixteen year old with malignant inoperable cancer. He was given heat sterilised *Streptococcus pyogenes* combined with *Serratia marcescens* and had complete remission and died of unrelated cause 26 yrs later (Hobohm 2009, Nauts et al 1953).

One of the most remarkable cures of cancer by Coley's toxins took place in 1926. The patient had reticulum cell sarcoma and despite amputation of his leg at the hip metastatic disease took hold. The stump from the amputation increased to 31 inches in circumference due to tumour growth and metastases appeared above the umbilicus, in the scalp, vertebrae and cranial bones (Nauts et al 1953). The patient

was given 28 daily intratumoural injections into the stump by Dr Palmer and Dr Christian (Martin 2006, Nauts et al 1953). Sixty days later there was no signs of cancer and he died in 1959 from a heart attack and was cancer free (Martin 2006).

Despite his impressive case histories his work continues to be ridiculed and mocked to this day. The American Cancer Society declared it was “quack medicine” and the FDA outlawed its use by classifying it as a new drug even though it had been in use for 60 years (Hoffer 1992). Currently it would be difficult to get Coley’s toxins approved as a vaccine adjuvant or treatment because the regulatory authorities would require a complete understanding of the mechanism of action before approval would be granted (Black et al 2010).

It would be naïve to suggest that Coley’s toxins is a miracle cures all cancer treatment. Coley himself felt that his treatment was more effective against sarcomas than other cancers (Starnes 1992). Despite Coley’s dedication and determination he was not a methodical scientist and his treatment protocols and bacterial extracts were inconsistent and reflected in the patient outcomes (Nauts and Swift 1946). Coley recognised that he made a fundamental error in the route of administration of his toxins in many of his patients. He wanted to demonstrate the systemic benefits of his treatment to his peers and treated many of his patients with IV injections or injections at sites removed from the tumour. This was less successful than direct injections into the tumour (Nauts et al 1953).

Coley lacked modern day immunological knowledge to interpret the effects he was observing. Coley believed *Streptococcus pyogenes* produced an anti-tumour product and we now know this product was not a toxin but a “perfect storm” of TLR and other PRR agonists (Decker and Safdar 2009). Coley’s heat inactivated toxins have recently been subjected to an immunological study by Maletzki et al (2012) where they showed that the effects are caused by activating the immune system through toll-like and other pattern recognition receptors. In fact they found the mixture contained CpG, lipoteichoic acid and lipopolysaccharide (LPS) which all engage TLRs switching immune system responses from one of tolerance to one of effective anti-tumour responses (Maletzki et al 2012).

Commercial preparations such as MBV (produced by Bayer) and Vaccineurin (produced by Suedpharma) were produced in the 1960s and 70s which were similar

to Coley's toxins but not identical. These preparations had a degree of mixed success and failures because 20th Century attempts to apply Coley's toxins universally failed to recognise the importance of prolong treatment and high fever. Fever was considered undesirable and treatment has been modified to minimise fever (Hobohm 2009). Also many of the attempts to apply Coley's toxins have been undertaken after the patient's immune system had been compromised by radiation therapy and chemotherapy (Starnes 1992). Coley's toxins commercially started being produced in 2005 by MBVax and exported to countries where regulators permitted it use. The company claims that regression was observed in about 70% of patients and complete remission in about 20% of patients (DeWeerdt 2013).

Near the end of his life Coley reflected that it was not unreasonable to presume other forms of bacterial toxins could target different types of cancers (Starnes 1992). In recent years this has been shown to be true with intratumoural injection of *Salmonella typhimurium* causing tumour regression in human melanoma (Saccheri et al 2010, Yoon et al 2011) and injections of *Bacillus Calmette-Guerin* (BCG) have been shown to cause regression of CTVT (Hess et al 1977). In recent years the ability of various bacterial toxins to target and internalise within host cells has been studied as a means of eliminating cancer cells or enhancing immune responses by being included as an adjuvant in immunotherapies (Adkins et al 2012).

1.7.2 LAK cell therapy

LAK cells is the term applied to lymphokine-activated killer cells (Qian et al 2014). A similar phenomenon is observed with mitogen activated killer (MAK) cells and cytokine-induced killer (CIK) cells (Qian et al 2014). A common feature of all these activated killer cells is that they target tumours in a non-MHC restricted manner. This allows them to target cells which have downregulated MHC expression to avoid the immune system (Qian et al 2014).

LAK cell therapy involves the activation of autologous killer cells populations by culturing in cytokines such as IL-2 (Lamb et al 2013). The preparation of LAK cells is highly irregular due to variability in the source and status of the autologous lymphocytes obtained for activation and inconsistency of protocols to activate these cells (Lamb et al 2013). Besides being difficult to produce to a consistent standard

they have only a transitory effect *in vivo* which is further complicated by the toxicity effects of IL-2 on return to patient (Lamb et al 2013).

NK resistant tumours are sensitive to LAK cell lysis (Grimm et al 1983). Initially it was believed that LAK cells therefore represented a population of cytotoxic cells distinct from NK and CTLs (Grimm et al 1983) but it is now known that LAK cells are predominantly NK cells (Herberman et al 1987). The *in vivo* LAK killing phenomenon extends beyond NK cells and involves a cascade of events that recruits macrophages and CTL responses (Geldhof et al 2002). There is also evidence of a critical role of NKT cells in LAK and MAK cell activation and killing (Linn and Hui 2010, Richards 1989).

The cost of generating LAK cells *in vitro* and the short duration of anti-tumour activity following adoptive transferred to the patient has limited the clinical application of LAK cells (Ishikawa et al 2012). Inadequate cytotoxic activity against the tumour and limited effector cell numbers presents an obstacle in treating established metastatic disease (Cesano et al 1994). Both of which might be overcome by the establishment of allogeneic NK cell lines (Ishikawa et al 2012).

In a LAK cell study performed on dogs it was found some tumour types, such as squamous cell or mammary carcinoma, impaired the ability of NK cells to be activated into LAK cells while melanoma tumours could be completely regressed (Funk et al 2005).

LAK cells have been shown to not only target tumour cells but also M2 macrophages (Geldhof et al 2002). M2 macrophages express lower levels of MHC class I which inhibits NK lysis and higher levels of B7-costimulatory molecules and CD11b adhesion molecules which confer NK sensitivity (Geldhof et al 2002). By editing the macrophage population through depletion of the M2 macrophages and activation of M1 macrophages through cytokine signals CTL responses are enhanced by LAK cells (Geldhof et al 2002).

1.7.3 **Adoptive cell transfer therapy**

Tumour infiltrating lymphocytes (TILs) and CTLs recognise tumour antigens presented by MHC molecules (Qian et al 2014). These cells can be identified and

expanded *in vitro* before being reintroduced to the patient as adoptive cell transfer therapy (Butler et al 2011, Qian et al 2014). Clinical research has shown increased survival of lung cancer and gastric cancer patients through this approach (Wang et al 2014).

Several studies have shown the usefulness of mouse models to study the efficacy of adoptive transfer therapies such as LAK cell therapy and TIL therapy (Cesano et al 1994, Malkovska et al 1992, Rosenberg et al 2008, Takahashi et al 1993). The mouse model revealed the need to deplete T-regs before the administration of TIL therapy. When this was done the TIL cells were seen to proliferate *in vivo* and persist for long periods resulting in primary and metastatic tumour regression in patients (Rosenberg et al 2008).

1.7.4 *In vivo* activation of NK cells to enhance anti-tumour CTL responses

Immature DCs present tumour antigens to T cells in a manner that promotes tolerance and supports tumour survival (Morandi et al 2012). Eliminating immature DCs and maximising the presentation of antigens by mature DCs would switch the tumour surveillance paradigm towards a protective response (Morandi et al 2012). Activated NK cells have been proposed as a mechanism to editing DC populations leaving a more mature DC population capable of promoting anti-tumour CTL activity (Morandi et al 2012). These NK cells can be activated *in vivo* by injecting MHC-devoid cells as an NK target (Morandi et al 2012). In mice this has been achieved using YAC-1 cells (Morandi et al 2012). The NK cells become activated against the YAC-1 cells and as a bystander effect lyse immature DCs (Morandi et al 2012).

1.7.5 Laser immunotherapy

Laser immunotherapy has been proposed by Immunophotonics (www.immunophotonics.com) as a potential treatment for DFTD. Laser immunotherapy is an *in situ* autologous cancer vaccine (inCVAX) based on the same principle as photodynamic therapy (Li et al 2012). A near-infrared laser is used to create photothermal damage to the tumour and the injection of glycated chitosan (a proprietary immunoadjuvant) promotes systemic immune responses that can target the primary and metastatic tumours (Li et al 2012). The exact mechanisms providing the protection are still speculative but preliminary clinical trials in Peru have shown

great promise in treating breast cancer. The Peruvian Ministry of Health have requested that immunophotonics start a Phase III human breast cancer clinical trial with inCVAX (Chen 2014, Li et al 2012).

The laser induces a gradient of heat damage to the tumour ranging from total destruction to the release of heat-shock proteins (HSPs) (Li et al 2012). It is believed that the inflammation, the HSPs and release of tumour antigens invokes an immune system response which sees DCs transport tumour antigens to CTLs in the lymph node (Li et al 2012). Effectively this is an *in vivo* generation of *in situ* whole-cells cancer vaccine (Li et al 2012).

1.7.6 Plant and algae extracts

Numerous plant extracts are being explored for their immuno-stimulatory effects and possible anti-tumour activities.

Fucoidan

Fucoidans are seaweed extracts that are complex sulphated polysaccharides with biological effects on mammalian cells (Araya et al 2011). Fucoidans constitute up to 30% of the dry weight of specific seaweeds (Myers et al 2011). The structure and function of fucoidans varies between species of seaweed used to obtain the extracts (Kwak 2014).

Fucoidans are well tolerated with low toxicity and no severe side effects in human clinical trials (Kwak 2014, Myers et al 2011). Excessive dosages exceeding 900 mg/kg caused delayed blood clotting but no other signs of toxicity (Myers et al 2011). In animal studies, brown algae extracts have been shown to have anti-tumour effects when delivered intravenously, intraperitoneally or orally (Kar et al 2011, Kwak 2014, Myers et al 2011).

In Japan, where seaweed consumption forms part of the regular diet, an epidemiological study associated dietary seaweed with numerous health benefits including lowered all-cause mortality and lower mortality to some cancers (Myers et al 2011).

In a study of HTLV-1 patients treated with fucoidan there was little effect on the cells of the immune system. However, benefits were observed in patients and this was possibly the result of inhibition of cell-to-cell transmission of the virus (Araya et al

2011). In contrast, other researchers have shown fucoidans have an immuno-modulating role as evident in *vivo* lymphocyte populations (Kwak 2014, Yang et al 2013).

There is empirical evidence that *in vivo* fucoidans modulate the immune system promoting maturation of bone marrow-derived DCs and mobilisation of hemopoetic stem cells to replenish immune cells within the body's tissues and organs (Myers et al 2011). This was complemented *in vitro* with fucoidan induced changes to activation and function of lymphocyte populations (Kwak 2014).

Many researchers suggest that fucoidan is an immuno-modulatory compound that induces T_H1 cytokines including IFN-γ, TNF-α and IL-12 while suppressing T_H2 cytokines IL-10 and TGF-β (Kar et al 2011). Fucoidan also promotes the generation of nitric oxide and reactive oxygen species (Kwak 2014, Yang et al 2013). In one study, cytokine expression by monocyte-derived DCs was altered resulting in increased levels of TNF-α, IFN-γ and IL-12 while decreasing levels of IL-6 (Myers et al 2011). Fucoidan treated DCs also direct naïve T cells towards a T_H1 differentiation (Myers et al 2011). Additional studies have also shown a modulation of T_H1:T_H2 ratio towards T_H1 profiles, which are required for effective anti-tumour responses (Myers et al 2011).

In vivo effect of fucoidan has also been demonstrated with mechanisms ranging from NK cell-mediated inhibition of angiogenesis, reduced tumour growth and prevention of metastasis (Kwak 2014). It has also been shown that fucoidan treatment can enhance NK cell activity (Myers et al 2011), significantly increase CTL numbers and phagocytic capacity of monocytes (Kwak 2014, Lu and Negrin 1994). Cancer cell apoptosis has been observed *in vitro* by fucoidan but this varied between tumour cell types species of fucoidan (Kwak 2014, Yang et al 2013). In clinical trials fucoidan has been reported as a strong selectin blocker demonstrating anti-inflammatory properties with osteoarthritis (Myers et al 2010).

Fucoidan demonstrated an ability to eliminate Leishmania infection and promote extended protective response through immunomodulation (Kar et al 2011). Kar *et al* (2011) administered fucoidan orally and found 200 mg/kg/day (5 mg/mouse/day) gave maximum results *in vivo* in mice infected with Leishmania. Examination of

cytokine production revealed that the $CD4^+$ T cell T_H2 immune response was switched to a T_H1 biased response by administration of fucoidan (Kar et al 2011). With *in vitro* experiments they used 50µg/ml for maximum response. They tested up to 150 ug/ml and had no adverse effect on cell viability but failed to enhance responses (Kar et al 2011).

The difficulty with fucoidan is that, since it is a natural product, there is no standardisation between manufacturers and batches. This makes it difficult to determine the mode of action and mechanisms involved. What needs to be determined is which structural characteristics are responsible for anti-tumour activity (Kwak 2014).

Withaferin A

The traditional Indian medicine system, Ayurvedic Medicine, has used the medicinal plant *Withania somnifera* (Indian Winter Cherry) for many centuries to treat a variety of ailments. Withaferin A (WA) is a bioactive ingredient isolated from this plant which has been shown to have immuno-modulatory, anti-inflammatory, anti-angiogenic and anti-tumour properties (Yang et al 2012a).

Withaferin A reduces proteasomal activity inhibiting synthesis of DNA, RNA and various pro-tumour proteins required for tumour cell growth and cycle (Kamath et al 1999, Yang et al 2007, Yang et al 2012a). Analysis of gene expression following WA treatment showed downregulation of a number of cell growth and metastasis transducers including c-myc and vimentin (Patel et al 2013, Yang et al 2012a).

Many cancers over express the Notch oncogene which activates the signal pathways of Akt, nuclear factor-kappa B (NF-κB) and mammalian target of rapamycin (mTOR) (Koduru et al 2010). This promotes proliferation and survival by regulating cell fate decisions (Koduru et al 2010). Withaferin A suppresses the Notch gene and ultimately this suppresses the Akt and mTOR growth and proliferation signalling pathways resulting in a dose-dependent cell cycle arrest of susceptible tumours (Grogan et al 2013). Akt activation by Notch also plays a critical role in metastatic spread of cancer (Koduru et al 2010). Proliferation is halted at the G2/m phase cell cycle by modulating p53-dependent proteins (Munagala et al 2011).

The inhibition of proteasomal activity by WA also promotes apoptosis in tumour cells by disrupting mitochondrial functions (Yang et al 2007). Gene expression analysis following WA treatment showed an upregulation of Bax and downregulation of Bcl-2 (anti-apoptotic protein) which causes a shift in Bax:Bcl-2 ratio that favours apoptosis an upregulation of pro-apoptotic Bax and IκB-α proteins (Koduru et al 2010, Munagala et al 2011, Patel et al 2013, Yang et al 2012a).

STAT3 expression promotes proliferation, angiogenesis and apoptosis resistance (Munagala et al 2011). WA inhibits STAT3 activation and promotes p53-mediated apoptosis (Munagala et al 2011). Caspase-3 activation, poly (ADP-ribose) polymerase (PARP) cleavage and condensed nucleus which are characteristics of apoptosis have resulted from WA treatment of cancer cells (Yang et al 2012a). In human MPM cells WA has been shown to induce caspase-3 activation, PARP cleavage and condensed nucleus which are characteristics of apoptosis (Yang et al 2012a).

Upregulation of the Notch-1 gene results in downregulation of c-Jun and JNK resulting in suppression of apoptosis (Koduru et al 2010). Withaferin-A targets the cancer cells, downregulating Notch-1 which thereby permits JNK activation and ultimately induces c-Jun-NH_2-kinase (JNK)–mediated apoptosis (Koduru et al 2010). JNKs are activated by stress and inflammatory signals which not only induce apoptosis but also inhibit proliferation (Fuchs et al 1998, Koduru et al 2010).

Other anti-tumour activities of withaferin A include changing the architecture of the cytoskeleton by target vimentin (Grogan et al 2013, Patel et al 2013). Vimentin is a cytoskeletal protein responsible for cell shape, integrity and flexibility (Satelli and Li 2011). Over expression of vimentin indicates an aggressive cancer associated with metastatic disease and poor diagnosis (Yang et al 2012a).

Vimentin is present in the cytosol, nucleus and to a lesser degree as an extracellular protein (Satelli and Li 2011). Vimentin is a marker of epithelial–mesenchymal transition (EMT) that can be used in diagnosis (Satelli and Li 2011). During EMT the phenotype of epithelial cells changes to a mesenchymal phenotype which alters their shape and increases their motility promoting metastatic disease (Satelli and Li 2011). Withaferin A counteracts this by causing the accumulation of vimentin in the perinuclear space of cancer cells and then breaking it down (Patel et al 2013).

Vimentin has additional pro-tumour activities (Satelli and Li 2011). Secreted vimentin can protect the tumour by neutralising NK cell activity through blocking the NKp46 receptors (Satelli and Li 2011). The breaking down of vimentin by WA not only prevents metastatic disease but promotes apoptosis (Satelli and Li 2011). The fact that withaferin A preferentially targets cancer cells over-expressing vimentin could partly explain the low toxicity towards normal cells which have lower vimentin levels (Satelli and Li 2011).

The ability of WA to target tumours over expressing vimentin is relevant to DFTD since high vimentin expression is a characteristic of DFTD (Loh et al 2006b). Such expression is generally regarded as supporting a poor prognosis for cancers since it is correlated to metastatic disease. The ability of WA to selectively target vimentin suggests a mechanism to exploit against DFTD cells and warrants further investigation.

Withaferin A is a natural product and is associated with fewer side effects and lower toxicity than synthetic options (Yang et al 2007). It can be administered orally as well as injected (Kamath et al 1999, Yang et al 2007). Intraperitoneal injection of 5 mg/kg of WA for 17 days following palpation of engrafted tumours has inhibited tumour growth in an *in vivo* murine model of malignant pleural mesothelioma (MPM). 10 µM doses of WA have been shown to be effective for *in vitro* trials. Withaferin A has also been shown to have anti-tumour effects against a variety of human cancers including prostate, breast and soft tissue sarcoma (Yang et al 2012a).

The LD_{50} for withaferin A is ≈ 80 mg/kg with mortalities commencing around 60 mg/kg (Sharada et al 1996). The toxic effect of withaferin A is cumulative while the tumour killing effect may not be. Tumour cells may be able to tolerate or recover from low levels of withaferin A whereas higher acute levels of withaferin A may be required for anti-tumour activity. Investigations by other researchers have revealed that individual doses should not exceed 40 mg/kg (Kamath et al 1999, Sharada et al 1996). A protocol of three daily doses of 30 mg/kg is the maximum tolerated dose of withaferin A. Increasing the dose per fraction to 40 mg/kg was not tolerated when given twice and 30 mg/kg dose per fraction was not tolerated when given four or more times (Sharada et al 1996).

1.8 Chemotherapy option for DFTD

Afatinib

Afatinib has been shown to inhibit proliferation of DFTD cells *in vitro* by the Sanger Research Institute in Britain (Elizabeth Murchison, personal communication, 2012). Afatinib is a protein tyrosine kinase inhibitor (TKI). Tyrosine kinases are proteins which stimulate cells to grow. Afatinib inhibits ErbB1 (epidermal growth factor receptor – EGFR), ErbB2 (Her2) and ErbB4 (Her4). These receptors are often over expressed on tumours (Normanno et al 2006). Afatinib irreversibly binds to the intracellular tyrosine kinase domain of the ErbB family receptors interrupting downstream signalling inhibiting growth and promoting apoptosis (Yap et al 2010).

The manufacturer recommends fasting three hours before and one hour after taking afatinib since high fat food prevents the uptake of afatinib by as much as 50% (Boehringer Ingelheim International 2014). Afatinib treatment is rarely without side effects ranging from minor to severe. In human trials these have seen most people suffering diarrhoea and skin changes (80%), loss of appetite (30%), sore mouth (60%) and nose bleeds (20%) (Yap et al 2010).

Responders to afatinib in human trials tend to relapse after developing resistance to the treatment and methods to overcome this can be very toxic (Nanjo et al 2013). Resistance can be due to selection of resistant tumour cells or expression of hepatocyte growth factor (HGF) in the tumour microenvironment (Nanjo et al 2013). The HGF can be expressed by the tumour cells themselves or the surrounding fibroblasts (Nanjo et al 2013).

Methods

Methods

2 Methods

2.1 Laboratory equipment and consumables

2.1.1 Reagents

Reagent	Supplier	Cat #
Afatinib (BIBW2992)	Boehringer Ingelheim	Lot # 1040023
Annexin V-FITC	Miltenyi Biotec	130-092-852
Annexin V-PE	BioVision	1014-1000
Antibiotic Antimycotic solution (Anti-Anti)	Sigma Aldrich	A5955
Annexin Binding Buffer (x10)	PharMingen	66121E
BD™ CBA mouse T_H1, T_H2, T_H17 cytokine kit	BD Bioscience	560485
Bovine Serum Albumin	Invitrogen	15561-020
Camptothecin	BioVision	1039-1
CellTrace Violet™	Life Technologies	C34557
CpG 1585	GeneWorks	
CpG 1668	GeneWorks	
CpG 2395	GeneWorks	
^{51}Cr	PerkinElmer	NEZ030S001MC
Dimethyl Sulphoxide (DMSO)	Sigma Aldrich	D2650
EasySep™ Mouse CD8+ T cell Enrichment Kit	Stemcell	19753
EasySep™ Mouse CD4+ T cell Enrichment Kit	Stemcell	19752
EasySep™ Mouse NK cell Enrichment Kit	Stemcell	19755
Ethanol	Merck	4102309020
Foetal Calf Serum	Gibco	1099-141
Fucoidan (*Fucus vesiculosus*)	Marinova	Lot # SK110199A
Glutamax	Gibco	35050-079
Histopaque®-1077	Sigma-Aldrich	10771
Imiquimod	AdipoGen	AG-CRI-3569-M100
In Situ Cell Death Detection Kit, Fluorescein	Roche	11 684 795 910
Methanol	Merck	901459020
Montanide 71	Seppic	
Nuclear yellow (Hoechst S769121)	Invitrogen	N21485

Reagent	Supplier	Cat #
NuPAGE® Antioxidant	Invitrogen	NP0005
NuPAGE® LDS Sample Buffer (4X)	Invitrogen	NP0007
NuPAGE® MOPS SDS Running Buffer (20X)	Invitrogen	NP0001
NuPAGE® Novex® 4-12% BIS-Tris Gels	Invitrogen	NP0322BOX
NuPAGE® Sample Reducing Agent (10X)	Invitrogen	NP0004
NuPAGE® Transfer Buffer (20X)	Invitrogen	NP0006-1
Paraformaldehyde	Sigma-Aldrich	P6148
Phosphate Buffered Saline (PBS) tablets	Oxoid	BR0014g
Potassium chloride	Calbiochem	529552
Potassium dihydrogen phosphate	Sigma-Aldrich	P9791
Propidium iodide	Sigma-Aldrich	P4170
RPMI 1640 Medium	Gibco	224000-089
Sodium Azide (NaN_3)	Sigma-Aldrich	S2002
Sodium Chloride	Sigma-Aldrich	S6191
Sodium citrate	Sigma-Aldrich	S-4641
Spectra™ Multicolor Broad Range Protein Ladder	Fermentas	SM1841
Triton X-100	BDH	30632
Trypan Blue	Sigma-Aldrich	T6146
In Situ Cell Death Detection Kit, Fluorescein	Roche	11 684 795 910
Withaferin A	Sigma-Aldrich	89910-10MG
7AAD	Sigma-Aldrich	A-9400

2.1.2 **Antibodies**

Antibody	Supplier	Cat #
Anti-mouse IgM (2 mg/mL)	Invitrogen	A21042 (lot 898246)
Anti-mouse IgG/IgM (2 mg/mL)	Invitrogen	A10680 (lot 1008684)
Anti-mouse IgG (2 mg/mL)	Invitrogen	A31553 (lot 799225)
Anti-mouse IgG1 (2 mg/mL)	Invitrogen	A21121 (lot 845809)
Anti-mouse IgG2a (2 mg/mL)	Invitrogen	A21136 (lot 939316)
Anti-mouse IgG2b (2 mg/mL)	Invitrogen	A21146 (lot 948496)
Anti-mouse IgG3 (2 mg/mL)	Invitrogen	A21151 (lot 982318)
Anti-mouse IL-4	BD PharMingen	554434
Anti-mouse IL-10	PharMingen	554465
Anti-mouse IL-12	PharMingen	18491D
Anti-mouse IFN-γ	BD PharMingen	554410
Anti-mouse CD3e (AlexaFluor 488)	Biolegend	100321
Anti-mouse CD4 (PerCP/Cy5.5)	Biolegend	100540
Anti-mouse CD8 (Pacific Blue)	Biolegend	100725
Anti-mouse CD49b (APC)	Biolegend	108910
Anti-mouse CD69 (PE)	Biolegend	104508
Anti-mouse MHC-II I-A/I-E (APC)	BD Bioscience	557000
Anti-mouse CD19 (PE/Cy7)	Biolegend	115520

2.1.3 **Consumables**

Item	Supplier	Cat #
0.1-20 µL pipette tips	Eppendorf	022492012
2-200 µL pipette tips	Eppendorf	022492039
50-1000 µL pipette tips	Eppendorf	022492055
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
Cryogenic freezing vials	Iwaki	2712-002
Disposable Pasteur pipette	Samco	225-15
Axygen MaxyClear Microtube	Axygen Scientific	MCT-175-C
Eppendorf tube	Quantum Scientific	LAC11514
Flow cytometry tubes	BD Falcon	367 526
24 well flat-bottom microplate	Iwaki	3820-024
96 well round-bottom microplate	Iwaki	655180
40 µm Cell Strainer	BD Falcon	352340
10 mL syringe	Terumo	SS+10ES

2.1.4 **Laboratory equipment**

Equipment	Supplier	Model #
-80°C freezer	Sanyo	MDF-U32V
Centrifuge	Sorvall	RT 6000D
Microcentrifuge	Eppendorf	5415D
Centrifuge	Eppendorf	5430R
Class II biological safety cabinet	Gelman Sciences	BH-204
Class II biological safety cabinet	LAF Technologies	BCS 1200
Fume hood	Conditionaire	HC-05
Fluorescent microscope	Olympus	BX 50
Flow cytometer	BD Bioscience	FACSCanto II
FACS cell sorter	Beckman Coulter	Astrios
Gamma radiation counter	Laboratory Technologies	Genesys Genii HE
Haemocytometer	Hawksley	Improved neubauer
Incubator 35°C	Heraeus	BB15
Incubator 37°C	Binder	142489
Platform Mixer	Ratek	RPM5

2.1.5 **Software**

Software	Supplier
FCS Express 4 Research Edition	De Nova Software (USA)
Flowing Software vers. 1.6.0	Turku Centre for Biotechnology (Finland)
GraphPad 5	GraphPad Software (USA)
BD FACSArray Bioanalyzer	BD Bioscience (USA)

2.1.6 **Solutions and reagents**

FACS staining buffer (FSB)

PBS with 1% BSA w/v and Sodium azide 0.1%

PBS (pH 7.3)*

Disodium hydrogen phosphate	1.15 g/L
Sodium chloride	8.0 g/L
Potassium dihydrogen phosphate	0.2 g/L
Potassium chloride	0.2 g/L

***** Alternatively PBS tablets (Oxoid, Hampshire, England) were used

RPMI-10

500 mL RPMI medium (GIBCO, New York, USA), 50 mL Foetal Bovine Serum (Bovogen Biological, Victoria, Australia), 5 mL GlutaMax™ (GIBCO, New York, USA) and 5 mL Antibiotic-Antimycotic (GIBCO, New York, USA)

TBS (pH 7.5)

Tris base 2.42 g/L

Sodium chloride 11.7 g/L

2.1.7 **Mice**

Strain	Supplier
BALB/c	Animal Services, Menzies Research Institute Tasmania
B6.Ighm/J	Animal Services, Menzies Research Institute Tasmania
B6.raG2/J	Animal Services, Menzies Research Institute Tasmania
B6.TNF	Animal Services, Menzies Research Institute Tasmania
CBA/nu	Walter and Eliza Hall Institute of Medical Research (WEHI)
C57/BL6	Animal Services, Menzies Research Institute Tasmania
NOD/SCID	Animal Services, Menzies Research Institute Tasmania

Unless specifically stated in the protocol the mice used in the experiments were at least 5 week's old, mixed-sex and normal weight for the strain. Numbers of animals used in each trial stated in the results.

2.1.8 **Cell lines**

K562: Cell line sourced from liquid nitrogen store University of Tasmanian. They were maintained in culture in RPMI-10 medium.
YAC-1: Cell line source from liquid nitrogen store University of Tasmanian. They were maintained in culture in RPMI-10 medium.
C5065: DFTD cell line provided by A-M. Pearse and K. Swift , Tasmanian Department of Primary Industries, Parks, Wildlife and Environment (DPIPWE). They were maintained in culture in RPMI-10 medium.
Incubation of cell lines: Cells were incubated at 35°C or 37°C (as stated in experiment protocol) in humidified incubator with 5% CO_2
Injections: Cells were washed in PBS (5 minutes at 500 rcf) and resuspended in PBS. Cell viability was determined using trypan blue exclusion and injections prepared at the viable cell concentration stated in protocol. Volumes between 100-200 µl were injected as stated in protocol. BALB/c and C57/BL6 mice were injected intraperitoneal or subcutaneously into the flanks as stated in the results. NOD/SCID, B6.Ighm/J, B6.raG2/J, B6.TNF and CBA/nu mice were injected subcutaneously into the flanks as stated in the results.

2.2 **Methods**

2.2.1 **Camptothecin induced apoptosis**

1. Camptothecin stock solution: 2 mM/mL in DMSO had previously been prepared

2. 5 x 10^6 DFTD cells were harvested

3. 4 µL Camptothecin stock solution (giving final concentration 4 µM/mL) was added to 5x10^6 DFTD cells suspended in 2 mL complete media (placed in a 10 mL centrifuge tube with a round base to minimize pelleting of cells).

4. The cells were incubated for 4 hours at 22°C on a platform mixer

5. Cell suspension transferred to 10 mL centrifuge tubes and washed twice with cold PBS and then resuspend cells in 2 mL of 1x Annexin V Binding Buffer (10x Binding Buffer diluted with distilled water)

6. Transfer 100 µL of the cell suspension to each of 12 flow cytometry tubes labelled 1 to 12.Tubes 1 to 3 served as negative controls with no FITC Annexin V and Propidium Iodide stains*.

7. In tubes 4 to 6 was added 5 µL of FITC Annexin V only. In tubes 7 to 9 was added 5 µL of Propidium Iodide only. In tubes 10 to 12 was added 5 µL of FITC Annexin V and 5 µL Propidium Iodide

8. The tubes were gently vortex and incubated for 15 minutes at R/T (23°C) protected from light

9. 100 µL of 1x Annexin V Binding Buffer was added to each tube and analysed by flow cytometry within one hour.

   *PE Annexin V and 7AAD were sometimes substituted for FITC Annexin V and propidium iodide using otherwise identical protocol

2.2.2 **TUNEL assay**

TUNEL assay was performed using *In Situ* Cell Death Detection Kit, Fluorescein (Roche, Germany) as per manufacturer's instructions

https://cssportal.roche.com/LFR_PublicDocs/ras/11684795910_en_16.pdf

1. C5065 DFTD cells were harvested and suspended in RPMI-10 medium
2. Sterile cover slips were placed in the top two rows of two 24 well microplates and 500ul of the DFTD cell suspension was placed over the cover slips.
3. The plates were incubated for 48 hours in 35^0C incubator.
4. One plate was subjected to 5 minutes exposure of UV-B lamps estimated to be 8 kilojoules of radiation. The second plate was used as an untreated control to measure spontaneous apoptosis.
5. Plates were incubated for an additional 24 hours in 35^0C incubator.
6. Working in a fume hood the media was replaced with 500 µL of 4% paraformaldehyde in PBS (PFA) and incubated @ 23°C for 15minutes.
7. The PFA was replaced with PBS and the well plates were stored in fridge for a few days.
8. The cover slips were then lifted from the wells using a bent hypodermic needle and forceps and placed onto a marked out wax block.

UV treated neg control	UV treated pos control	UV treated sample 1	UV treated sample 2	UV treated sample 3	UV treated sample 4
Untreated neg control	Untreated pos control	Untreated sample 1	Untreated sample 2	Untreated sample 3	Untreated sample 4

9. The cover slips were gently dried using tissues and then 50 µL endogenous peroxidase blocking solution (3% H_2O_2 in methanol) was placed onto the cover slip and incubated for 10 minutes.
10. PBS wash x1 followed by 2 minute incubation in 4^0C room in permeabilisation solution (0.1% TritonX-100 in 0.1% sodium citrate)

11. PBS wash x2

12. Removed 100 µL label solution from vial 2 and added 50 µL per each negative control cover slip.

13. Prepared TUNEL reaction mixture by adding total volume (50 µL) of Enzyme solution (vial 1) to the remaining 450 µL Label solution in vial 2 to obtain 500 µL TUNEL reaction mixture. Nuclear yellow was added as a counter stain at 2 µM.

14. Removed 100 µL of TUNEL reaction mixture and added 0.1 µL Dnase I. 50 µL of this mixture was placed on the two positive control cover slips.

15. 50 µL of the remaining TUNEL reaction mix was added to the remaining cover slips.

16. Incubated at 37^0C in a humidified atmosphere in the dark for 1 hour.

17. Prepared slides with drops of Faramount aqueous mounting medium. Pick up cover slip; wash by dipping in milli-Q water, place cell surface down onto mounting medium, gently push cover slip down.

18. Fluorescence microscope with excitation wavelength in the range of 450-500 nm and detection in the range of 515-565 was used to detect TdT labelling. Nuclear Yellow counter stain was detected using ≈335/495 nm excitation/emission.

2.2.3 **Standard protocol to detect DFTD specific antibodies in mouse serum**

1. Harvested C5065 DFDT cells, counted cells and re-suspended in FACS Staining Buffer (FSB) typically at 10^6 cells per mL concentration

2. Added 1 µL relevant thawed mouse serum sample to the appropriate wells of a 96 well round-bottom microplate. Added 50 µL of DFTD cell suspension to each well and incubated on ice for 30 minutes. Controls included target cells only, target cells and 2nd antibody, Target cells plus naïve mouse serum and target cells and known positive serum (see diagram below for typical layout).

Row	Column 1
A	Target cells only
B	Target cells + 2nd antibody
C	Naïve mouse serum
D	Known positive serum control
E	Sample being tested
F	Sample being tested
G	Sample being tested
H	Sample being tested

3. Centrifuged 5 minutes at 1500 rcf and then perform x2 PBS washes

4. Briefly centrifuged conjugated antibody to eliminate protein aggregates which contribute towards background staining.

5. Diluted 2nd antibody
    a. IgG_1 488, $IgG2_b$ 633 and IgG/IgM 488 at 1:1000 in FSB
    b. IgM 488, IgG405, IgG488 and IgG_3 at 1:500 in FSB
    c. IgG 488 at 1:100 to 1:1000 (as stated in experiment protocol) in FSB

6. Placed 50 µL in each relevant well and incubated on ice for 30 minutes

7. Washed twice in PBS and re-suspended in 200 µL of PBS for immediate reading on flow cytometry equipment

2.2.4 Standard biotin antibody labelling protocol for mouse lymphocytes

1. Harvested mouse lymphocytes, counted cells and resuspended in FACS staining buffer (FSB) typically at 10^7 cells/mL.
2. Added biotinylated anti-mouse antibodies
    - Biotinylated rat anti-mouse CD49b (≈ 1µL/10^7 cells)
3. Incubated on ice for 30 minutes
4. Added PBS, pelleted cells and then perform two more PBS washes
5. Placed 10^7 cells in tube with 500 µL FSB with 1 µL Anti-biotin Strep-APC antibody and incubated on ice for 10minutes in dark.
6. Washed cells and re-suspended in FSB for immediate sorting

2.2.5 Conjugated antibodies for flow cytometry

1. Harvested lymphocytes, counted cells and re-suspended in FACS staining buffer (FSB) typically at 10^7 cells/mL.
2. Added conjugated anti-mouse antibody typically diluted between 1:300 to 1:500
    - Anti-mouse CD3e (AlexaFluor 488) diluted 1:300
    - Anti-mouse CD4 (PerCP/cy5.5) diluted 1:500
    - Anti-mouse CD8 (Pacific blue) diluted 1:400
    - Anti-mouse CD49 (APC) diluted 1:300
    - Anti-mouse CD69 (PE) diluted 1:300
    - Anti-mouse MHC-II I-A/I-E (APC) diluted 1:400
    - Anti-mouse CD19 (PE/Cy7) diluted 1:300
3. Incubated on ice for 30 minutes
4. Filled tube with PBS, pellet and performed second PBS wash
5. Re-suspended in PBS and run on flow cytometry equipment

2.2.6 **Anti β_2-microglobulin and anti-MHC+ DFTD cell antibodies**

To test upregulation of β2-microglobulin, DFTD cells obtained from culture or mouse xenografts (as stated in experiment protocol) were labelled with WEHI produced anti-devil β_2-microglobulin (#13-34-20). To test upregulation of MHC, DFTD cells were labelled with serum generated in a Tasmanian devil against MHC+ DFTD cells (Control serum accessed Tasmanian devil Missy's serum 11-4-12).

Protocol for testing upregulation of MHC in DFTD cells

1. DFTD cells from xenograft tumours or cultured DFTD cells were re-suspended as single cell suspension in FACS staining buffer (FSB)
2. Treated cells were transferred to five micro-tubes. This will provide four sample controls no missy serum primary antibody, no WEHI anti-devil secondary antibody, no Alexa Fluor tertiary antibody, Missy pre-immune negative control and Missy's serum (11-4-12) anti-MHC+ DFTD antibody*.
3. Two more control tubes of cells from untreated DFTD xenograft and untreated cultured cells were placed in micro-tubes as negative controls.
4. All tubes were washed in PBS (3min 750 rcf) and resuspend in 50 µL of PBS with 1:300 of Missy 4/11/12 serum. One control tube minus Missy's serum and one with Missy's pre-immune serum.
5. Incubated at R/T (23° C) for 20 minutes and then wash x3 in PBS.
6. Added 50 µL of 1:200 mouse anti-devil 42/11-A4-B1-2-1 2 mg/mL WEHI α-devil antibody. One control minus mouse anti-devil Ab. Incubate 15 minutes at R/T.
7. Washed x3 in PBS and re-suspended 1:1000 anti-mouse IgG 488 and incubated 20 minutes at R/T. One control minus anti-mouse IgG 488.
8. Washed x2 PBS and re-suspended in 200 µL PBS for reading on flow.

*To test for β2-microglobulin substituted Missy's serum with WEHI produced anti-devil β_2-microglobulin (#13-34-20) and deleted Missy's pre-immune control.

2.2.7 ^{51}Cr cytotoxicity assay

1. Target cells were incubated with 100 uCi ^{51}Cr for 2 hours, given two PBS washes and resuspended at 10^5/mL concentration

2. 96 V bottomed well plate was prepared with effector cells (lymphocytes) doing 100 µL serial halving dilutions of effector cells usually from 100:1 to 3:1. Dilution ratios varied between assays dependent on lymphocyte recovery.

3. Control wells were prepared without any lymphocytes being added. Minimum control cells were prepared with 100 µL RPMI-10FCS and maximum control cells with 100 µL of Triton X 1%.

4. 100 µL of ^{51}Cr labelled DFTD cells at 10^5/mL concentration were added to wells

5. Plate was centrifuged for 2 minutes at 1000 rcf

6. Plate was incubated for 18 hours at 37°C with 5% CO_2

7. Plate was centrifuged for 4 minutes at 1000 rcf

8. Being careful not to disturb the pellet, 100 µL of supernatant was transferred into 5 mL tubes and gamma measurements were obtained using a Genesys gamma radiation counter (Laboratory technologies Inc., Illinois, USA)

9. Data analysis was undertaken using Microsoft Excel and/or GraphPad Prism

2.2.8 **CFSE and PI cytotoxicity assay**

1. Added 2 µL of 5 mM CFSE to 5 mL of RPMI-10 then added media to target cells and incubated on a platform mixer for 10 minutes at R/T (23° C) or 30 minutes on ice. (some target cells were retained unlabelled to set PMT voltages on flow cytometry)

2. Performed two PBS washes before re-suspending in complete media and doing cell count and viability check. Re-suspended CFSE labelled target cells at 10^5/mL concentration.

3. Effector cells were prepared at 2 x 10^7 cells /mL if possible

4. 96 round bottomed well plate was prepared doing 100 µL serial halving dilutions of effector cells to typically give a range from 200:1 to 3:1 effector to target cell ratio. This was achieved by placing 200 µL of lymphocytes at 2 x10^7 cells/mL concentration in the 200:1 wells and 100 µL of RPMI media in the rest of the dilution wells. 100 µL was then transferred as a serial dilution through the relevant wells from 200:1 to 3:1.

5. Minimum control cells were prepared with addition of 100 µL RPMI and no lymphocytes

6. 100 µL of CFSE labelled target cells were added to all wells

7. Incubated for 18 hours at 37°C with 5% CO_2

8. Diluted propidium iodide (PI) stock solution 1:25 in PBS and added 25 µL of PI working solution to each well. (7AAD can be substituted for PI)

9. Place the following controls in flow cytometry tubes and set the PMT voltages on the FACSCanto II flow cytometer.
    a. Target cells unstained
    b. Target cells + PI only
    c. Target cells CFSE labelled only
    d. Target cells DFTD CFSE labelled and PI

10. Connected HST plate reader to FACSCanto II flow cytometer and analyse promptly

11. Flow cytometry data was analysed to calculate the number of dead target cells as a percentage of the total target cells. The data was then normalised by subtracting the percentage of spontaneous cell death observed in the wells with target cells only. Data analysis was undertaken using Microsoft Excel and plotted using GraphPad Prism 5

2.2.9 **CellTrace Violet and PI cytotoxicity assay**

1. Labelled target cells with CellTrace™ Violet
    a. The CellTrace Violet Cell Proliferation Kit contains single-use vials of dry dye. A 2.5 mM stock solution was prepared by dissolving the contents of a vial in 40 µL anhydrous DMSO prior to use

    b. Stock solution was used between 1:2500 and 1:250.

    c. To stain 10^6 cells in 1 mL of ***pre-warmed*** PBS added 1 µL of stock solution (1:1000). Cells were incubated for 20 minutes at R/T (23°C) with gentle agitation protected from light.

    d. Quenched any unbound dye remaining in solution by adding five times the volume of ***pre-warmed*** RPMI-10 media and incubating for 5 minutes.

    e. Pelleted cells and re-suspended in RPMI-10 media for assay at 10^5 cells/mL. Cells could be kept growing in 37°C incubated for a number of days before use.

2. Effector cells were prepared at 2 x 10^7 cells /mL if possible

3. 96 round bottomed well plate was prepared doing 100 µL serial halving dilutions of effector cells to typically give a range from 200:1 to 3:1 effector to target cell ratio. This was achieved by placing 200 µL of lymphocytes at 2 x10^7cells/mL concentration in the 200:1 wells and 100 µL of RPMI media in the rest of the dilution wells. 100 µL was then transferred as a serial dilution through the relevant wells from 200:1 to 3:1.

4. Minimum control cells were prepared with addition of 100 µL RPMI and no lymphocytes

5. 100 µL of CellTrace labelled target cells were added to all wells

6. Incubated for 18 hours at 37°C with 5% CO_2

7. Diluted propidium iodide (PI) stock solution 1:25 in PBS and added 25 µL of PI working solution to each well. (7AAD can be substituted for PI)

8. Placed the following controls in flow cytometry tubes and set the PMT voltages on the FACSCanto II flow cytometer.
    e. Target cells unstained
    f. Target cells + PI only
    g. Target cells CellTrace Violet labelled only
    h. Target cells labelled with CellTrace Violet and PI

9. Connected HST plate reader to FACSCanto II flow cytometer and analysed promptly

10. Flow cytometry data was analysed to calculate the number of dead target cells as a percentage of the total target cells. The data was then normalised by subtracting the percentage of spontaneous cell death observed in the wells with target cells only. Data analysis was undertaken using Microsoft Excel and plotted using GraphPad Prism 5

2.2.10 **Measuring cytokines by ELISA**

1. Dilute IL-4 (cat #) 1:250, IL-10 (cat #), IL-12 (cat #) and IFN-γ (cat #) capture antibodies with coating buffer to give 50 µL per well.

2. Add 50 µL per each well except for control well as per well plan which gets coating buffer instead.

3. Cover plate with parafilm and incubate 4 °C overnight

	IL-4 (or IL-12)						IL-10 (or IFN-α)					
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	Standards 20k	No #1 antibody	200:1	200:1	200:1		Standards 20k	No #1 antibody	200:1	200:1	200:1	
B	Standards 10k	No supernatant sample	100:1	100:1	100:1		Standards 10k	No supernatant sample	100:1	100:1	100:1	
C	Standards 5k	No #2 antibody	50:1	50:1	50:1		Standards 5k	No #2 antibody	50:1	50:1	50:1	
D	Standards 2.5k	No avidin	25:1	25:1	25:1		Standards 2.5k	No avidin	25:1	25:1	25:1	
E	Standards 1.2k		12:1	12:1	12:1		Standards 1.2k		12:1	12:1	12:1	
F	Standards 600		MNC	MNC	MNC		Standards 600		MNC	MNC	MNC	
G	Standards 300		DFTD				Standards 300		DFTD			
H	Standards zero						Standards zero					

4. Plate was washed twice in TBS (PBS + 250 µL Tween/500 mL)

5. Added 200 µL blocking buffer to each well (PBS/FCS/BSA…%?) and incubate at room temp for 2 hours

6. Defrosted samples and standards.

7. Wash plate three times with TBS

8. Add 200 µL of cytokine standard which has been prepared at 1:20000 dilution (Cat# and concentration) to the first standards well and then serially halving dilution with 100 µL of blocking buffer added to the next six wells. A zero standard is obtained in the eighth well by only having blocking buffer.

9. Added 100 µL of samples to relevant wells.
10. Covered plate with parafilm and incubate 4°C overnight
11. Washed plate four times with TBS
12. Diluted IL-4 and IL-12 secondary antibodies 1:2000 with blocking buffer; and diluted IL-10 and IFN-γ secondary antibodies 1:1000 with blocking buffer. 100 µL per well was required.
13. Added 100 µL per each well except for control well as per well plan.
14. Covered plate with parafilm and incubated for 1 hour at 22°C
15. Washed plate six times in TBS
16. Added 100 µL of avidin-HRP conjugate, which has been diluted 1:1000 from 1mg/mL stock kept on bench fridge door shelf.
17. Covered plate with parafilm and incubated for 30minutes at 22 °
18. Washed eight times in TBS
19. Added 100 µL of TMB pre-warmed to 22°C to each well and allowed colour to develop for 5-10 minutes.
20. Stop reaction by adding 100 µL ELISA stop solution (weak acid solution 1M HCl)
21. Read within 30 minutes at OD 450nm

2.2.11 Cytokine assay workflow for CBA T_H1, T_H2, T_H17 micro bead array

Step 1: Preparing mouse cytokine standards

a) Fresh cytokine standards were reconstituted and serially diluted immediately before mixing the capture beads and PE detection reagent.

b) Transferred the ball of each lyophilized cytokine standard to flow tube (labelled 1:1), added 2 mL of Assay Diluent and allowed to sit for at least 15 minutes at R/T (23° C) before mixing gently with a pipette.

c) Added 300 µL assay diluent to each of 8 flow tubes (labelled 1:2 to 1:256) and performed a halving serial dilution by transferring 300 µL from the 1:1 tube to tube 1:2 and so on until tube 1:256.

d) Prepare a 9^{th} tube with 300 µL of diluent only as a zero value control.

Step 2: Mixing mouse cytokine capture beads

The capture beads were bottled individually (blue caps) and all bead reagents needed to be pooled immediately before using them in the assay. 1 µL of each concentrate was pooled with capture bead diluent to provide 50 µL mixed capture beads per test (see table for calculations).

Capture Bead and Detection Reagent Diluent Calculations

Number of flex sets used	Volume of each Capture Bead or PE Detection Reagent/test	Total Capture Bead volume/test	Volume of Capture Bead or Detection Reagent Diluent/test	Total volume of mixed capture Bead or PE Detection Reagent/test
1	1 µL	1 µL	49 µL	50 µL
2	1 µL	2 µL	48 µL	50 µL
3	1 µL	3 µL	47 µL	50 µL
4	1 µL	4 µL	46 µL	50 µL
5	1 µL	5 µL	45 µL	50 µL
6	1 µL	6 µL	44 µL	50 µL
7	1 µL	7 µL	43 µL	50 µL
8	1 µL	8 µL	42 µL	50 µL
9	1 µL	9 µL	41 µL	50 µL
10	1 µL	10 µL	40 µL	50 µL

a) Calculated number of flex kits, number of tests (unknowns + 10 standards + 3 extra) and added required volume of Capture Bead diluent to labelled tube.

b) Vigorously vortex each capture bead suspension for 15 seconds before use and added 1µL per test as calculated above.
c) Vortex bead mixture thoroughly.

Step 3: Performing mouse cytokine assay

a) Added 50 µL of mixed capture beads to each assay tube
b) Added 50 µL cytokine standards or unknowns to relevant tubes

Tube label	Concentration (pg/mL)	Cytokine Standard dilution
1	0	Assay Diluent only
2	20	1:256
3	40	1:128
4	80	1:64
5	156	1:32
6	312.5	1:16
7	625	1:8
8	1250	1:4
9	2500	1:2
10	5000	1:1

c) Mixed tubes gently and incubate for 1 hour at R/T
d) Prepared Mixed PE Detection reagent in a similar manner to the mixed capture beads and stored protected from light in fridge.
e) Added 50 µL of the PE Detection Reagent to all tubes and incubate for 1 hour at R/T protected from light (performed cytometer setup during this incubation period)
f) Added 1 mL of wash buffer to each assay tube and centrifuged 200 rcf for 5 minutes
g) Gently tipped out supernatant and re-suspended bead pellet in 300 µL of wash buffer
h) Run the samples on the flow cytometer. To assist analysis, started with zero standard to highest standard followed by unknowns.
i) Data was analysed with manufacturer's software BD FACSArray Bioanalyzer, Excel and GraphPad 5.

2.2.12 **EasySep NK enrichment per mouse spleen**

1. Harvested spleen and run over histopaque.
2. Pelleted and re-suspended in 1000 µL PBS + 2% FCS + 1 mM EDTA and transfer to a 5 mL capped tube.
3. Centrifuged EasySep Negative Selection Mouse NK Cell Enrichment Cocktail and added 50 µL to cells, mixed well and incubated 15 minutes at R/T (23° C)
4. Added EasySep Biotin Selection Cocktail 100 µL to cells, mixed well and incubated for 15 minutes at R/T
5. Vortex EasySep D Magnetic particles for 30 seconds to ensure they were uniformly suspended and had no aggregates. Added 100 µL particles to cells, mixed well and incubated for 10 minutes at R/T
6. Brought cell suspension to a total volume of 2.5 mL by adding PBS + 2% FCS + 1 mM EDTA. Mixed gently with pipette x3. Placed tube without cap into magnet and stood for 5 mins.
7. Picked up magnet and gently poured cell suspension into new tube without shaking magnet. Held inverted for 2 to 3 seconds.
8. Removed the old tube which was NK depleted and placed the new tube into magnet for 5 mins and repeated the separation step 7.

2.2.13 **EasySep CD4+ or CD8+ enrichment per mouse spleen**

1. Harvested spleen and run over histopaque.
2. Pelleted and re-suspended in 1000 µL PBS + 2% FCS + 1 mM EDTA and transferred to a 5 mL capped tube.
3. Added 50 µL of Normal Rat Serum (code # 13551)
4. Added 50 µL EasySep Mouse CD4+ or CD8+ Cell Enrichment Cocktail to cells, mixed well and incubated 15 minutes at 2 to 8° C
5. Added 100 µL EasySep Biotin Selection Cocktail 2 to cells, mixed well and incubated for 15 minutes at 2 to 8° C (time was exceeded due to multiple samples)
6. Vortex EasySep D Magnetic particles for 30 seconds to ensure they were uniformly suspended and there were no aggregates. Added 100 µL particles to cells, mixed well and incubated for 5 to 10 minutes at 2 to 8° C
7. Brought cell suspension to a total volume of 2.5 mL by adding PBS + 2% FCS + 1 mM EDTA. Mixed gently with pipette x3. Placed tube without cap into magnet and stood for 5 minutes.
8. Picked up magnet and gently poured cell suspension into new tube without shaking magnet. Held inverted for 2 to 3 secs.
9. Removed the old tube which was CD4+ or CD8+ depleted and placed the new tube into magnet for 5 mins and repeated the separation step 7.

2.2.14 **Stimulation of Tasmanian devil monocytes with concanavalin A**

Cytotoxic cells (LAK cells) could be generated by 48 hours stimulation with concanavalin A. As stated in the experiment protocols between 5 µg/mL and 20 µg/mL concanavalin A in RPMI-10 media was used, however, 5 µg/ mL was sufficient. After 48 hours, the culture was harvested and centrifuged for 5 minutes at 500 rcf then the cell pellet and supernatant were separated.

2.2.15 **Generation of concanavalin A supernatant (Con A sup)**

Following stimulation of Tasmanian devil monocytes with concanavalin A as described previously, the supernatant minus cells was collected. The residual concanavalin a was removed from solution by chelation with 15 mg/mL α--D-Mannose (Sigma Aldrich, New South Wales, Australia). Samples were then passed through 2 mm 0.8/0.2 µm filters (Pall Corporation, New York, USA) under sterile conditions. The resulting solution contained cytokines present after concanavalin A culture, with little residual mitogen or mannose.

2.2.16 **Separation of serum**

Blood was stored in microtubes and allowed to clot at R/T for at least 2 hours or overnight at 4°C. Sample was then spun down for 20 minutes at 10,000 rpm in Ependorf Microcentrifuge 5415D. Serum was aliquotted into microtubes and stored at -20°C or -80°C.

2.2.17 **Afatinib therapy**

Afatinib stock solution was prepared at 293 mg/ml concentration by dissolving in DMSO. A working solution of afatinib was prepared by diluting stock solution 1:10 with sterile water. 10 µL of working solution, which equivalent to 293 µg, was concealed in peanut butter and fed to the mice.

2.2.18 **Withaferin A therapy**

Withaferin A stock solution was prepared at 50 mg/ml concentration by dissolving in DMSO. Working solution was prepared by diluting stock solution 1:200 in PBS and 200 µL of working solution was injected in each mouse per dose.

2.2.19 **Fucoidan therapy**

Fucoidan working-stock solution was prepared at 10 mg/ml concentration by dissolving fucoidan (*Fucus vesiculosus* species from Marinova, Tasmania) in sterile water. Mice were injected with 1.25 mg intraperitoneally as stated in protocol.

2.2.20 **Imiquimod therapy**

Imiquimod working-stock solution was prepared at 1 mg/ml concentration by dissolving initially in DMSO and then adding required sterile water. Mice were treated with 100 µg of imiquimod intratumoural injections as stated in protocol.

DFTD investigation using *in vitro* assays and a murine DFTD model

DFTD investigation using *in vitro* assays and a murine DFTD model

3 DFTD investigation using in vitro assays and a murine DFTD model

3.3 Introduction

The Tasmanian devil (*Sarcophilus harrisii*) faces possible extinction in the wild due to a transmissible cancer known as Devil Facial Tumour Disease. The unique and conserved chromosomal rearrangements of DFTD cells compared to the host's, negate the possibility of transmission from a viral or bacterial agent, pollutants or toxins in the environment (Pearse and Swift 2006). The cancer cells are transmitted through facial biting of successive hosts (Murchison et al 2010, Murchison et al 2012, Pearse and Swift 2006) and alongside the CTVT it is described as a clonal cell line immortalised as a parasitic infectious allograft (Murchison 2008). Genetic and chromosomal research has provided convincing evidence that the malignant neoplasm originated in an individual female Tasmanian devil (Murchison et al 2012). Not only did the DFTD cancer cells evade the original host's immune system but the immune systems of subsequent devils.

It was first considered that the limited MHC diversity of the population of devils, from which the DFTD founder was derived, enabled the establishment of DFTD throughout subsequent devil populations (Jones et al 2004, Siddle et al 2007). It had been hypothesised that since the tumour arose in a Tasmanian devil the tumour itself expressed normal MHC and was not perceived as a threat within the original host. It was further hypothesised that following transmission to another devil the MHC was so similar between hosts that it was not perceived as a foreign cell. Nevertheless, recent evidence reveals that genetically diverse animals are prone to the disease (Siddle and Kaufman 2013) while skin graft experiments showed MHC diversity was sufficient between individuals to result in graft rejection (Kreiss et al 2011a). It is important to note that skin is one of the most immunogenic organs of the body and this skin graft rejection does not disprove the hypothesis of MHC bottleneck within the species contributing towards the transmission of DFTD.

Siddle et al. (Siddle et al 2013) revealed that DFTD cells do not express cell surface MHC molecules *in vitro* or *in vivo*. The genes contributing to the essential

components of the antigen-processing pathway, such as β_2-microglobulin and transporters associated with antigen processing are downregulated (Siddle et al 2013). The loss of gene expression is not due to structural mutations, but to regulatory changes including epigenetic deacetylation of histones (Siddle et al 2013). By downregulating MHC, the tumour cells remain invisible to parts of the devil's immune system. However, lack of surface MHC class I molecules should make DFTD cells targets for NK cells (Das and Das 2000) unless DFTD are non-immunogenic.

When tumours spontaneously occur in any animal they undergo random mutations that result in certain traits that promote escape from tumour surveillance. The lack of any identified resistant or immune wild Tasmanian devils suggests the pro-tumour mechanisms may hide the cells from the recognition of the immune system. One possible explanation could be that the DFTD cells have evolved to be non-immunogenic and are therefore invisible to any immune system. The lack of interspecies transmission contradicts this possibility.

To determine if DFTD cells are immunogenic and therefore potential targets for immunotherapy a xenograft mouse model was used to look for immune responses to DFTD. DFTD tumours successfully implant in immunocompromised NOD/SCID mice but do not implant in immunocompetent BALB/c mice (Kreiss et al 2011b).The aim was to determine if this rejection by immunocompetent mice was an active immunological response and not due to other factors such as preformed antibodies commonly associated with xenogeneic graft rejection. This was performed by examining specific antibody, cytokine and cell mediated cytotoxicity responses to the DFTD xenograft.

In addition this xenograft model was used to investigate the possibility that DFTD evolved certain traits that modify appropriate immune responses to inappropriate responses which protect the tumour. Polarising the immune system towards a T_H2 response is a mechanism exploited by tumour cells to suppress anti-tumour CTL activity and promote ineffective humoral antibody responses (Singh et al 2011). This could be evaluated in the mouse model with the caution that results obtained may not directly translate to the Tasmanian devil.

Ideally an immunological study of an allograft tumour in the host species is necessary, but in the case of the Tasmanian devil conducting large scale immunological experiments are not possible due to the endangered species status. Therefore, since it is widely accepted that mouse models provide valuable insights into the study of human cancers this research exploits a mouse model to study DFTD. The particular advantage of a mouse model is the readily available antibodies to detect mouse immune responses while there is a paucity of equivalent antibodies currently available for the Tasmanian devil immune system. Developing an understanding of the mechanism and pathways used by immunocompetent mice to reject DFTD xenografts may provide insight into targeting DFTD cells for immunotherapy within the wild Tasmanian devil population.

To complement the *in vivo* studies of DFTD in the mouse model *in vitro* studies could explore the question of whether or not DFTD cells can undergo apoptosis. Avoidance of apoptosis is known to contribute towards carcinogenesis, progression and resistance to treatment (Fulda 2009).

3.4 Results

3.4.1 The intrinsic apoptosis pathway in DFTD cells

Resistance to apoptosis is one mechanism employed by certain tumours to avoid killing by immune systems and drugs (Igney and Krammer 2002). Tasmanian devils with DFTD lack the ability to kill DFTD cells. This may be due to a lack of recognition of DFTD cells by the host. But there is also the possibility that DFTD cells can avoid immune destruction due to resistance of the DFTD cells to apoptosis.

In this chapter flow cytometry was used to identify apoptosis in DFTD cells by Annexin V, propidium iodide (PI) and scatter analysis. Annexin V binds to the cell membrane of cells from the earliest stages of apoptosis. DNA labelling by PI occurs in later stages of apoptosis when the membranes become permeable and also as a result of necrosis.

In late stage apoptosis the cellular contents are being condensed causing a more granular characteristic to the cell resulting in increased side scatter (SSC) of the laser. The condensing of the cellular contents during apoptosis also reduces the size of the cells resulting in a lower forward scatter (FSC). In comparison necrotic cells have both low FSC and SSC as the cellular contents are released (Darzynkiewicz et al 1997).

Apoptosis can be induced by the cytotoxic drug camptothecin. Therefore, to evaluate the ability of DFTD cells to undergo apoptosis C5065 DFTD cells were incubated with camptothecin for four hours. The cells were then labelled with Annexin V-FITC and propidium iodide before being analysed by flow cytometry.

Based on cell morphology two populations were identified by flow cytometry (Figure 3-1). Population 1, gated in blue, represents cells with lower FSC and relatively high SSC whereas Population 2, gated in red, represents cells with higher FSC and normal SSC. Population 1 had a strong correlation to PI^+ cells whereas Population 2 correlated to PI^- cells. Within Population 1, as analysed by Annexin V and PI, the late apoptotic cells were identified as Annexin V^+ PI^+ and in this example represented 77% of the total cells. Within Population 2, Annexin V^+PI^- identified early stage apoptotic cells and represented 5% of the total cells. Annexin V^-PI^- identified viable cells and represented 16% of the total cells. Only 2% of the cells could be classified as necrotic based on being Annexin V^-PI^+. Therefore, following four hours exposure to the cytotoxic drug, DFTD cells showed obvious signs of dying via apoptosis.

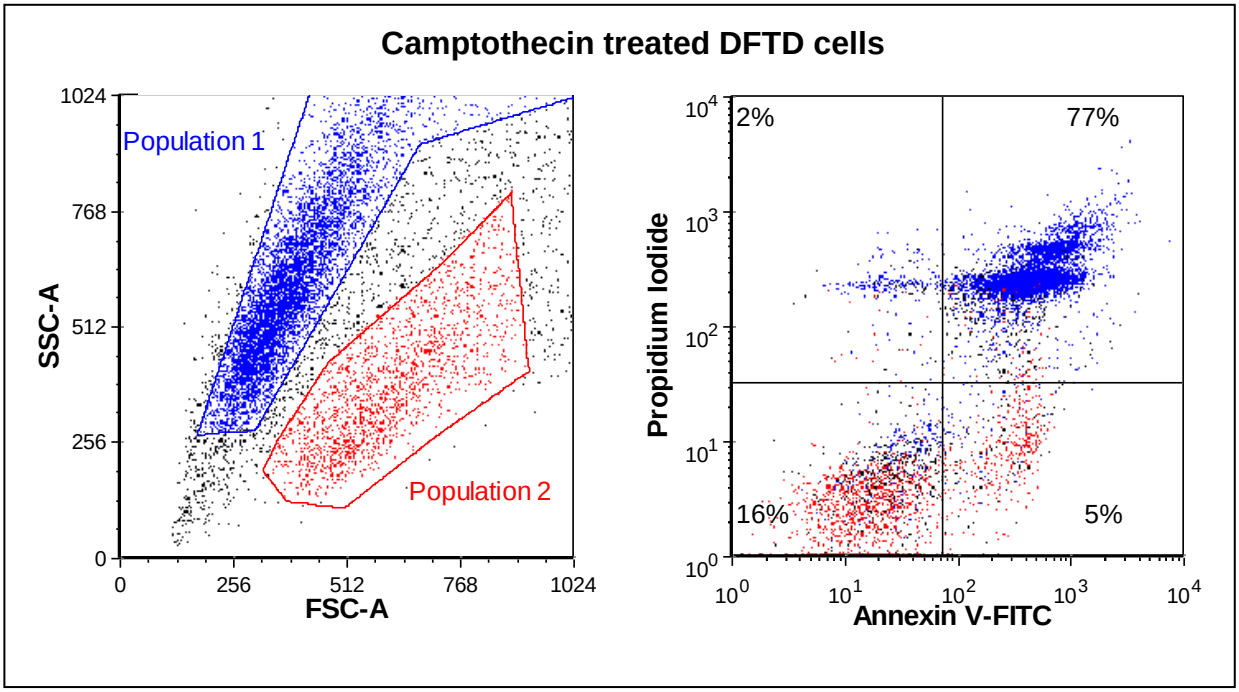

Figure 3-1. Flow cytometry analysis of DFTD cells incubated with 4 µM camptothecin for four hours. Based on forward-scatter (FSC) and side-scatter (SSC) late apoptotic cells were identified as lower FSC and higher SSC values (represented by population 1 shown in blue). Population 2 (shown in red) represents viable and early stage apoptotic cells. 82% of the cells are Annexin V positive and 77% of the cells were positive for both Annexin V and propidium iodide (right panel).

Necrosis is a non-apoptotic cell death that disrupts the membrane in a way that makes it permeable to dead cell markers such as PI but does not expose the membrane's phosphatidylserine for Annexin V binding. As camptothecin only produced 2% necrotic cells, C5065 DFTD cells were induced to undergo necrosis by rapid freeze thawing. This was used to confirm that the previously described Annexin V binding to DFTD cells was specific for apoptosis and not resulting from non-specific binding to necrotic cells.

There were two distinct populations, Annexin V^- PI^- and Annexin V^- PI^+. There was no evidence of Annexin V^+ cells. Necrotic cells incorporated PI while viable cells excluded PI (Figure 3-2). As none of the necrotic cells, induced by freeze thawing, were Annexin V^+ this provides support that the assay is specific for detecting apoptosis in DFTD cells and that DFTD cells have the ability to die via apoptosis.

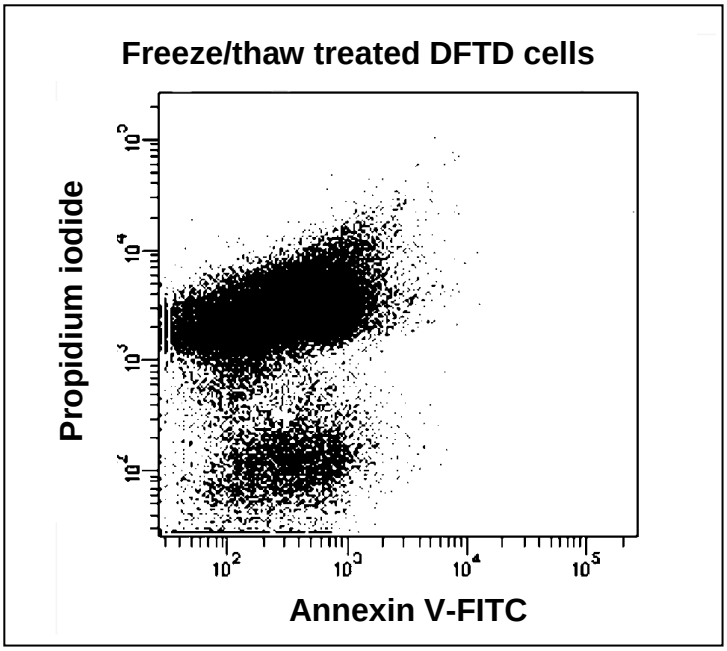

Figure 3-2. Annexin V-FITC and PI were used to label DFTD cells following freeze-thaw treatment. Dead cells have labelled positive for PI and negative for Annexin V-FITC.

A further verification of specificity of the assay for apoptosis was performed with K562 cells and resting lymphocytes. These cells are resistant to camptothecin induced apoptosis (Ferraro et al 2000, Tian et al 2011) and were selected as negative controls to further validate the use of Annexin V. K562 cells, devil lymphocytes and DFTD cells were incubated with camptothecin, labelled with Annexin V-PE and 7AAD and then analysed by flow cytometry.

Following exposure to camptothecin, K562 cells and devil lymphocytes were not positive for Annexin V. In contrast, 42% of the DFTD cells were Annexin V^+ of which 16% were Annexin V^+ $7AAD^+$ (late apoptosis) and 26% of the cells were Annexin V^+ $7AAD^-$ (early apoptosis) (Figure 3-3). The lack of Annexin V binding in the K562 cells and devil lymphocytes confirms that the Annexin V binding to DFTD cells was specific for camptothecin mediated apoptosis.

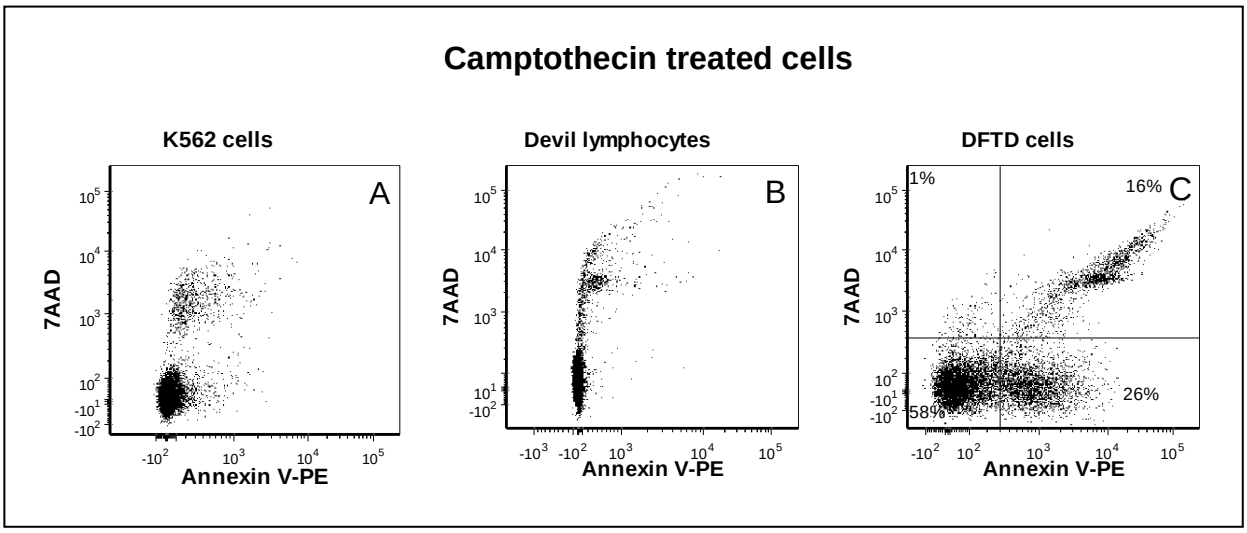

Figure 3-3. Following two hours incubation with camptothecin there is only limited binding of Annexin V to K562 cells (A) and no significant binding to resting lymphocytes (B) while there was binding of Annexin V to C5065 DFTD cells (C). 7AAD has bound to dead cells (A, B & C).

UV-radiation (UV-R) was used as an alternative treatment to induce apoptosis to further corroborate apoptosis in DFTD cells. Since K562 cells are sensitive to UV induced apoptosis (Ujvarosi et al 2007) they were used as a positive control cell line. The cells were exposed to 4 kj/m^2 of UV-R and Annexin V-FITC and PI were used to detect apoptosis following 24 hours in culture.

Following UV-R exposure apoptosis was detected in both K562 and C5065 DFTD cells. Various stages from viable cells to apoptotic cells are evident for both cell lines. The bottom left hand corner of each panel shows viable cells, which are Annexin V$^-$ and PI$^-$. To the right of this population are Annexin V$^+$ cells ranging from PI$^-$ to PI^{dim} to PI^{bright} as they progress from early to late stage apoptosis (Figure 3-4). In the example shown 59% of the K562 cells and 26% of the DFTD cells have undergone apoptosis.

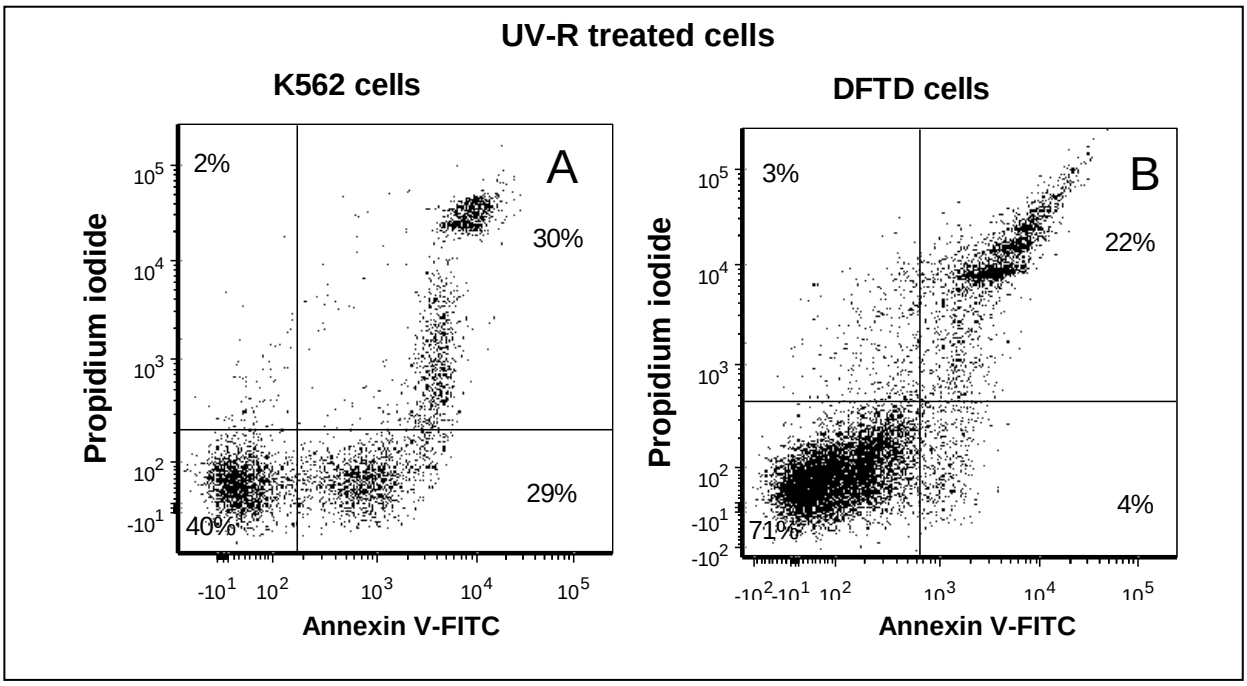

Figure 3-4. Following exposure to 4 kj/m^2 of UV-R radiation apoptosis was evaluated using Annexin V-FITC and PI. Bottom left quadrant contains viable cells, bottom right quadrant contains cells in early stage apoptosis and top right quadrant contains cells in late stage apoptosis.

Another indicator of apoptosis is DNA fragmentation. DFTD cells were exposed to 4 kj/m^2 of UV-R and cultured for 24 hours. DNA fragmentation was then evaluated with TUNEL assay, which labels the exposed 3'-hydroxyl ends of DNA breaks that occur in late stage cell apoptosis. Nuclear yellow staining was included to identify all cells.

Cells positive for nuclear yellow and TUNEL appear yellow in the overlaid images. In the representative images shown (Figure 3-5) 26% and 43% of the DFTD cells were TUNEL positive confirming apoptotic damage is occurring to the DNA of C5065 DFTD cells following UV-R exposure. This result correlates with the 26% apoptotic cells evaluated by Annexin V following similar UV-R irradiation (Figure 3-4).

From these combined observations it can be concluded that DFTD cells can undergo apoptosis. This verifies the intrinsic apoptosis pathway in DFTD cells has remained functional and this does not explain the lack of immune response to DFTD cells in infected Tasmanian devils.

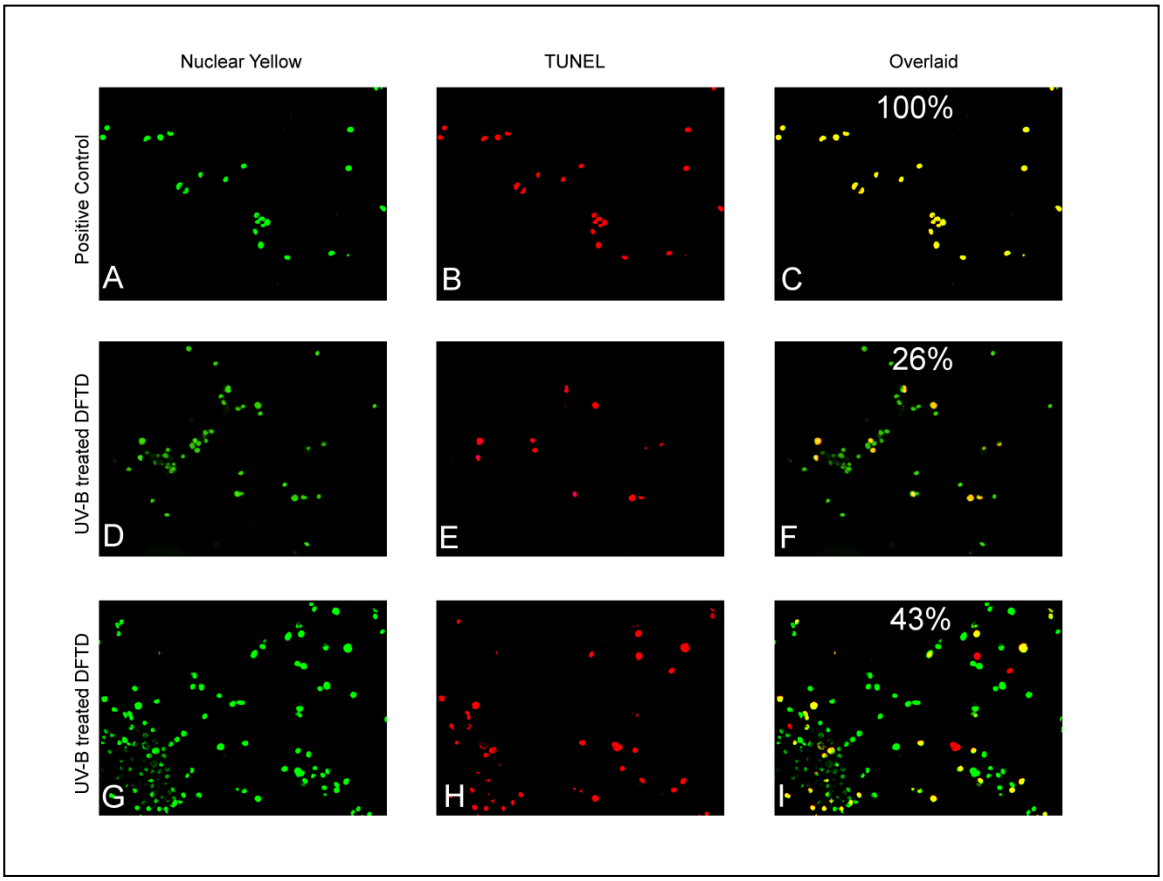

Figure 3-5. Following exposure to 4 kj/m^2 of UV-R radiation apoptosis in DFTD cells was evaluated using TUNEL assay. Positive control was treated with DNase and Nuclear Yellow labelling shown in A, TUNEL labelling shown in B and overlay shown in C. Two replicates of C5065 cells (D to F and G to I) demonstrate apoptosis in 26 and 43% in these examples.

3.4.2 Antibody responses by mice following immunisation with DFTD cells

In the wild Tasmanian devil population there is no evidence of immune responses or resistance to DFTD. Having shown that DFTD cells have the capacity to undertake apoptosis this leads to the hypothesis that DFTD cells are not immunogenic and therefore invisible to any immune system. To test this hypothesis, immunocompetent C57/BL6 mice were subcutaneously immunised one to four times with 10^6 viable DFTD cells into the flanks. This cell number was selected based on observations of DFTD tumour establishment in BALB/c mice by Kreiss et al (2011b).These injections included 50 µg CpG1668 and 50 µl Montanide 71 as adjuvants designed to enhance immune responses.

Indirect immunofluorescence was used to identify IgG anti-DFTD antibodies in the serum. As shown in Figure 3-6, six of eight immunised mice produced varying levels of DFTD specific antibody. Of the two non-responders one had received three immunisations. This result established that DFTD cells can induce an immune response but individual responses are variable. Multiple subcutaneous immunisations did not guarantee that an IgG immune response would be detected.

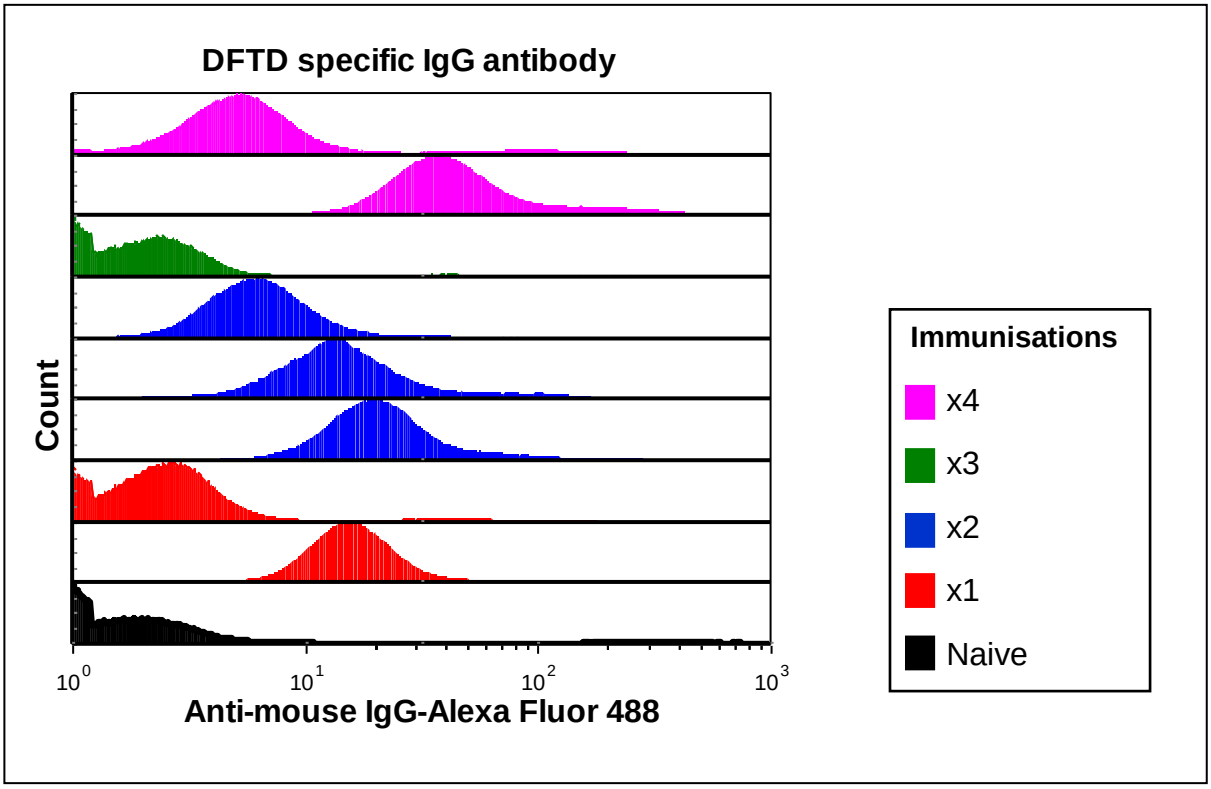

Figure 3-6. Goat anti-mouse IgG (H+L) Alexa-fluor 488 conjugated antibodies were used to test mouse serum for DFTD specific antibodies. Naïve mouse serum shown in black was used as a negative baseline control. Mice were immunised subcutaneously and received one (shown in red), two (shown in blue), three (shown in green) or four (shown in pink) immunisations.

To determine which antibody isotypes were produced subsequent to subcutaneous immunisations with DFTD cells, sera from C57/BL6 mice were screened for IgM, IgG1, IgG2a, IgG2b and IgG3 isotype production following immunisation. Comparing mean fluorescence intensity level of sample against serum from naïve mice facilitated calculation of relative anti-DFTD antibody responses as a fold increase (Table 3-1).

IgM anti-DFTD was detected in 4/15 mice whereas IgG anti-DFTD was detected in 14/15 mice at varying levels. IgG1 was the isotype detected in all responders. IgG1 alone was detected in 5/15 while in 2/15 mice IgG1 and IgG2b were detected and all IgG isotypes in 7/15 mice. When IgM was detected all isotypes were identified.

These results indicate that IgM/IgG isotype switching is occurring in C57/BL6 mice following immunisation with DFTD cells and that IgG1 is the dominant response.

Table 3-1. Relative anti-DFTD antibody levels in serum from C57/BL6 mice that had been immunised by subcutaneous injection with DFTD cells (ND represents no change from naïve serum)

Relative anti-DFTD isotype antibody levels					
Mouse ID#	**IgM**	**IgG1**	**IgG2a**	**IgG2b**	**IgG3**
100219-g	ND	5	ND	2	ND
100219-h	ND	5	ND	ND	ND
100219-i	ND	2	ND	ND	ND
100219-j	ND	7	ND	ND	ND
10419-a-38	2	4	2	3	2
10419-b-39	ND	3	ND	ND	ND
10419-c-10	ND	3	ND	ND	ND
10419-d-12	ND	11	ND	ND	ND
10419-e-11	2	52	2	5	2
10419-f-18	2	67	7	51	6
10419-g-19	ND	9	ND	2	ND
10419-h-17	4	112	12	44	7
100823-207	ND	10	5	30	8
100823-208	ND	15	5	20	10
100823-209	ND	6	5	10	5

BALB/c mice were injected subcutaneously with 10^6 DFTD cells from the same culture either into a single site or divided between multiple sites in the flanks. This was performed to investigate if distributing the same number of DFTD cells between multiple injection sites rather than concentrating the cells into a single site enhanced responses to subcutaneous injections. The serum were screened for IgG and IgM anti-DFTD isotypes.

Multiple site subcutaneous injections produced higher relative levels of immunoglobulins compared to single site subcutaneous injections (Figure 3-7). IgM was not detected in any of the serum samples screened. IgG1 was detected following 4/4 single site injections and 4/4 multi-site injections. IgG2a was detected following 3/4 single site injections and 4/4 multi-site injections. IgG2b was detected following 1/4 single site injections and 3/4 multi-site injections. IgG3 was detected following 1/4 single site injections and 2/4 multi-site injections. These results indicate that distributing the same number of cells over multiple sites significantly enhances the anti-DFTD antibody response in BALB/c mice.

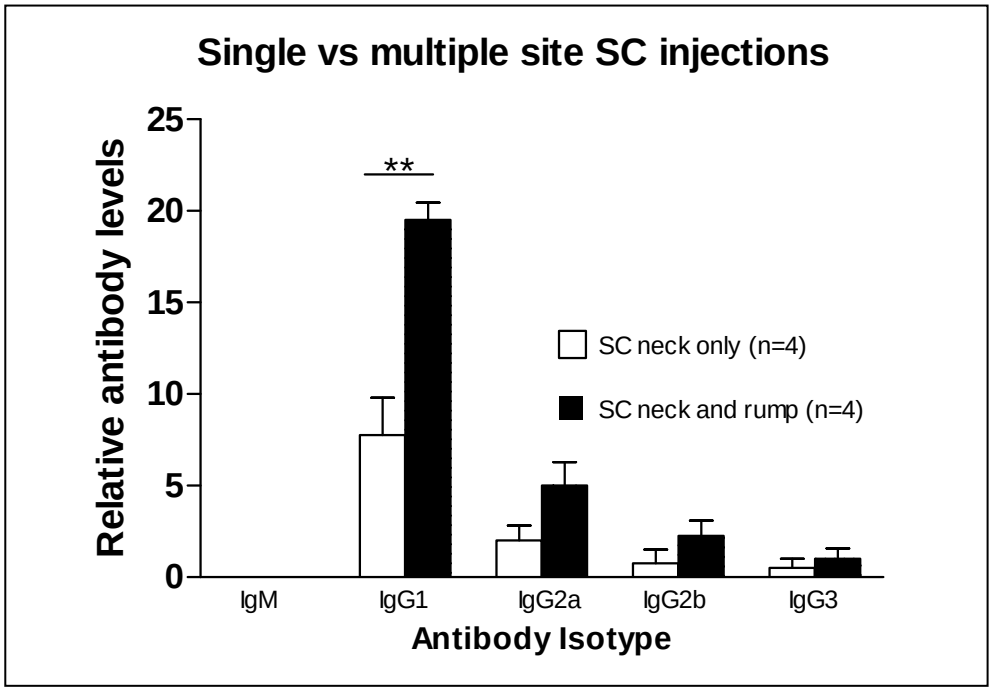

Figure 3-7. Comparison of antibody responses of BALB/c mice inoculated subcutaneously (SC) with DFTD cells on days 0 and 16. Serum was collected day 25. IgM was not detected. Higher levels of IgG1 were detected for mice immunised by injections into multiple sites. (Data are expressed as mean of four mice ± SEM, probability calculated by Student's unpaired t-test * P < 0.05, ** P < 0.01)

Intraperitoneal injections were compared to subcutaneous immunisations. C57/BL6 mice were injected with 10^6 DFTD cells from the same culture either into a single intraperitoneal site or divided between two subcutaneous locations by injections to the neck and rump. The mice were immunised on day 0 and day 16 and serum collected on day 25. The serum was then analysed by indirect immunofluorescence for relative anti-DFTD antibody levels.

Intraperitoneal injections produced significantly higher levels of immunoglobulins compared to multi-site subcutaneous injections (Figure 3-8). For the intraperitoneally immunised mice all tested isotypes were detected in all three mice. For multi-site subcutaneous immunised mice IgG, IgG1 and IgG2b were detected in all four mice while IgM was detected in two of the four mice and IgG3 in one of the four mice.

These results indicate that intraperitoneal immunisations produced stronger and more consistent anti-DFTD antibody responses following immunisation.

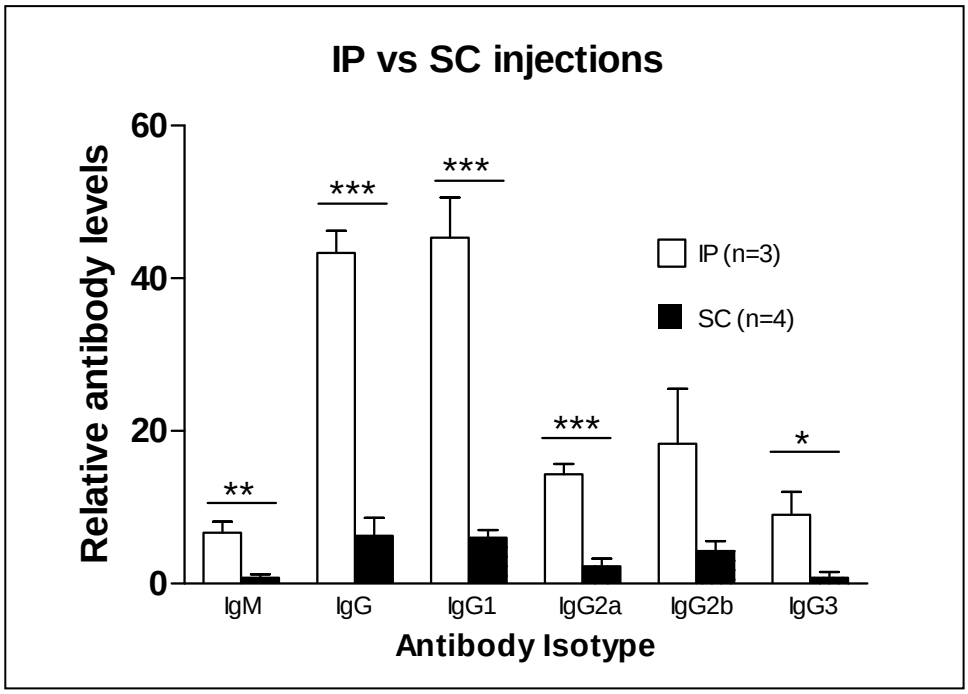

Figure 3-8. Comparison of antibody responses for intraperitoneal (IP) and subcutaneous (SC) injections of DFTD cells. Relative antibody levels were determined in serum samples obtained from C57BL/6 mice immunised with DFTD cells on day 0 and 16. Serum was collected on day 25. The SC immunisations were given as two injections divided between the neck and rump region to target multiple draining lymph nodes. (Data are expressed as mean of three IP and four SC immunised mice ± SEM, probability calculated by Student's unpaired t-test * P < 0.05, ** P < 0.01, *** P < 0.001)

Having determined that intraperitoneal immunisations were more effective than subcutaneous immunisations, a protocol described by Elsawa et al (2003) was evaluated to determine if two intraperitoneal injections spaced 14 days apart would enhance antibody responses against DFTD cells. A similar procedure produced rapid and reliable responses against viruses in mice within 21 days (Elsawa et al 2003). 10^6 viable DFTD cells were intraperitoneally injected into mice with a second injection 14 days later. Serum was collected 7 days after the second injection and IgG antibody levels analysed. All the immunised mice produced detectable antibody responses with variability between individuals (Figure 3-9).

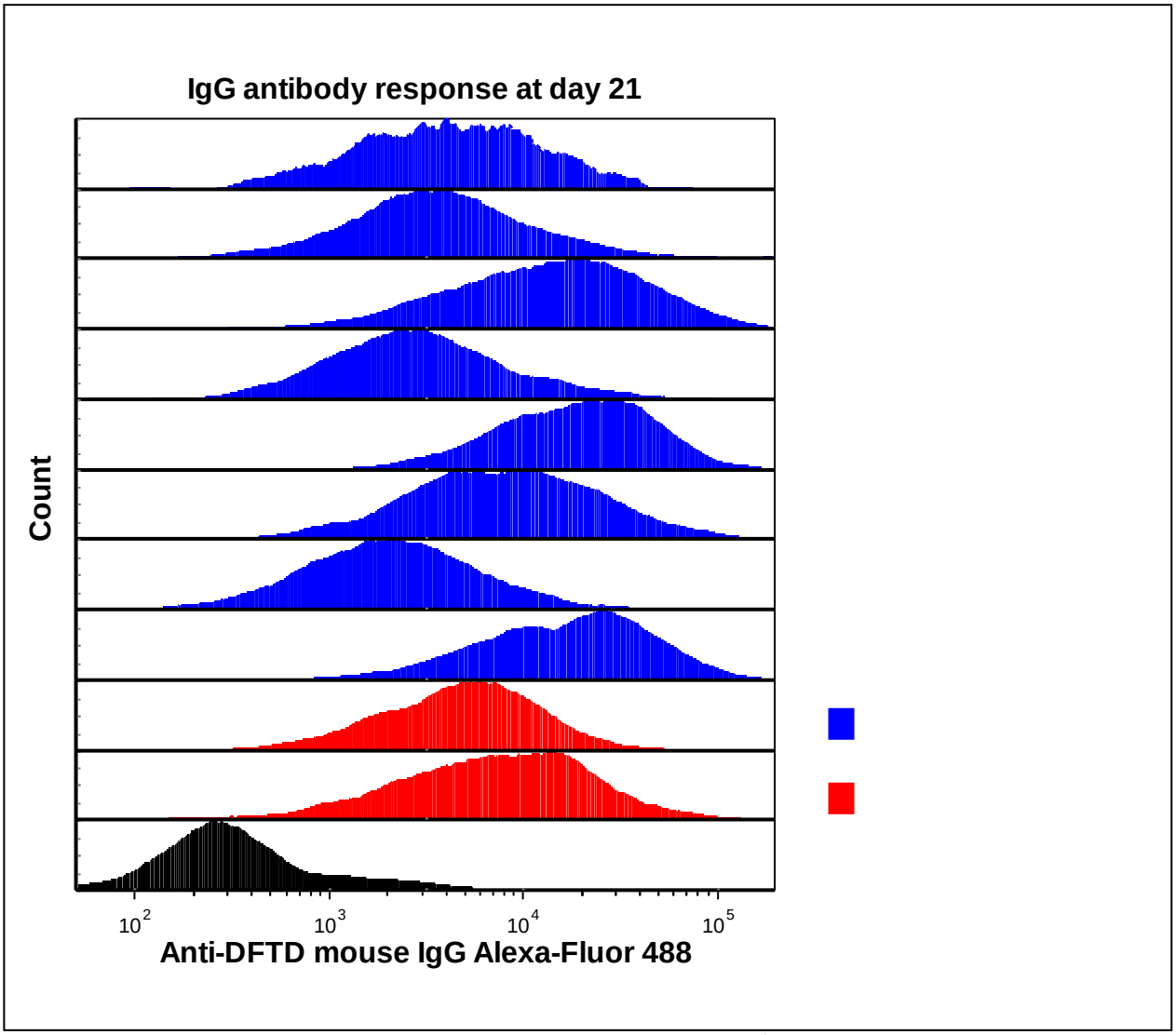

Figure 3-9. Mice were given two intraperitoneal injections of 10^6 C5065 DFTD cells 14 days apart and serum collected 7 days after the second immunisation. Naive C57/BL6 mouse serum (shown in black) was used as a negative baseline control.

To determine if antibody responses could be enhanced further and variability between individual mice minimised the relative IgG levels were compared for C57/BL6 mice given two versus three intraperitoneal 10^6 DFTD immunisations. All mice produced IgG antibody responses following immunisation with DFTD cells. The mice immunised three times had less variability in antibody levels but not higher antibody levels than those with two immunisations. There would be little advantage gained by prolonging the immunisation protocol.

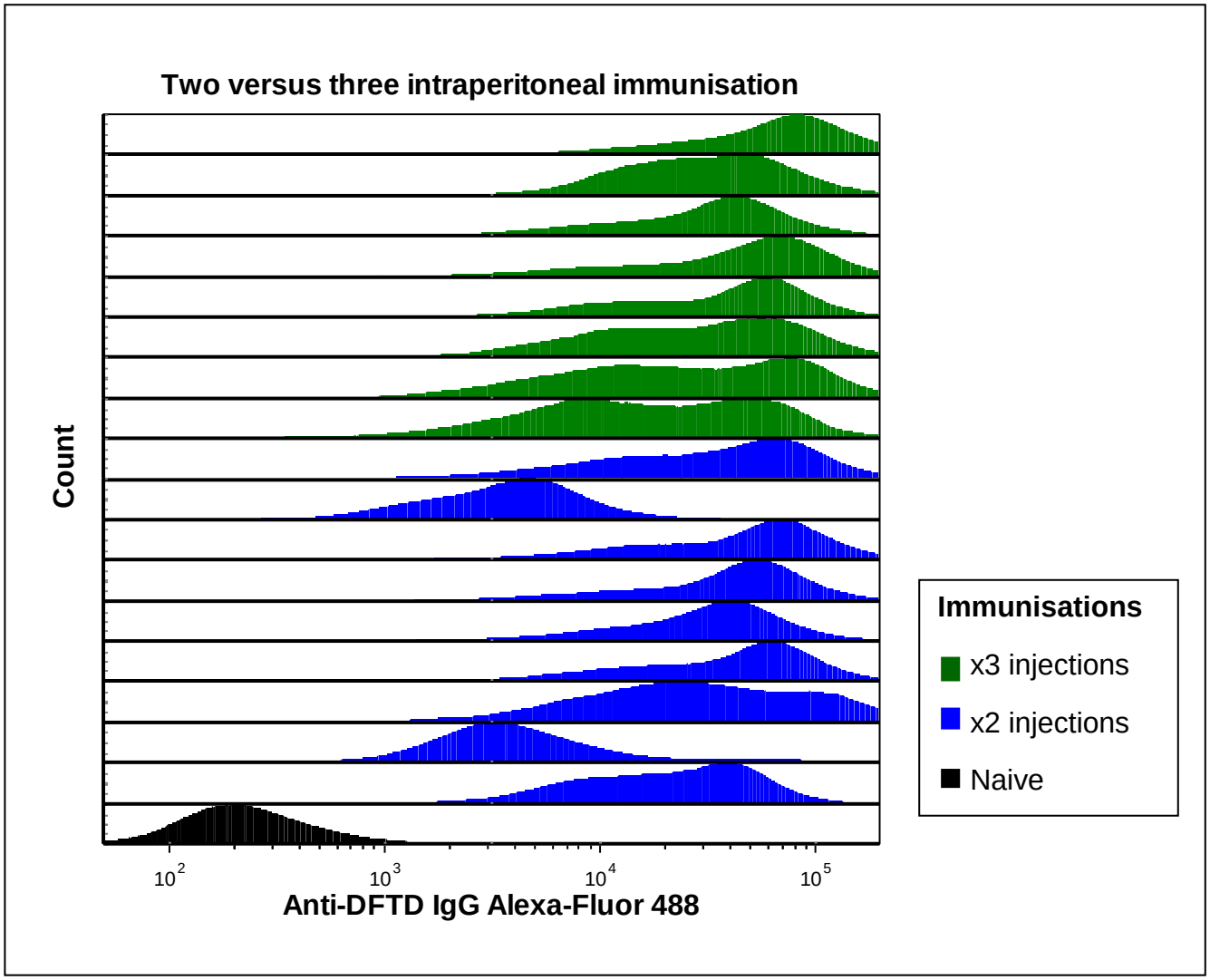

Figure 3-10. Mice were given two (shown in blue) or three (shown in green) intraperitoneal injections of C5065 DFTD cells. Serum were collected 7 days after the last immunisation. Pooled naïve C57/BL6 mouse serum (shown in black) was used as a negative baseline control.

Two strains of immunocompetent mice were analysed because of their reportedly opposing T_H1 (C57/BL6) or T_H2 (BALB/c) dominated immune responses (Mills et al 2000, Reiner and Locksley 1995). Since T_H1 and T_H2 responses can be discriminated based on IgG isotype polarisation, C57/BL6 and BALB/c mice that were immunised against 10^6 DFTD cells with a second injection on day 14 and serum collected on day 21 were compared for IgG and IgM profiles.

Both strains of mice produced all of the tested antibody isotypes. Levels of IgM, IgG1 (T_H2 antibodies) and IgG3 (T_H1 antibody) were similar for the two strains (Figure 3-11). BALB/c mice produced significantly higher levels of IgG2a (T_H1 antibody) and significantly lower levels of IgG2b (T_H1 antibody) compared to C57/BL6 mice. The relevance of these findings is that immunisation with DFTD cells did not polarise the immune system of either strain towards a T_H1 or T_H2 response. The difference in IgG2 isotypes between the two strains suggests a disparity in the immune response between the strains.

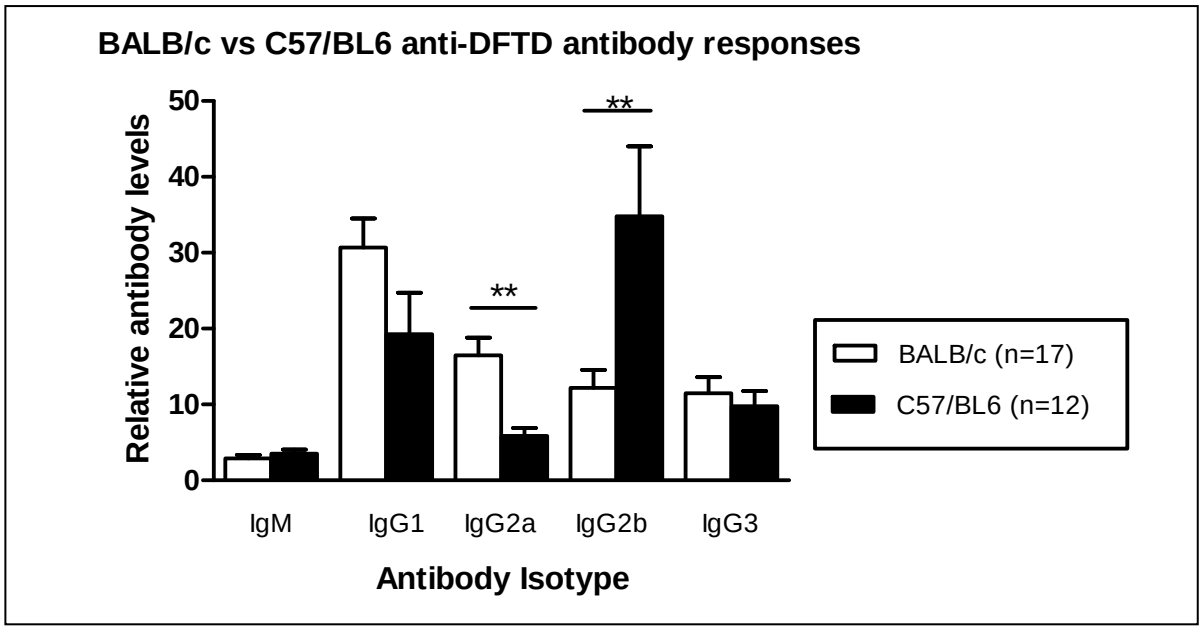

Figure 3-11. Cohorts of BALB/c and C57/BL6 mice were injected with 10^6 DFTD cells by intraperitoneal injection on day 0, with a second injection day 14 or 16; serum was collected 7 or 8 days later. While anti-DFTD antibody expression varied between individuals there was a consistent trend that BALB/c mice were skewed towards higher levels of IgG2a and lower levels of IgG2b compared to C57/BL6 mice. (Data are expressed as mean ± SEM, probability calculated by Student's unpaired t-test ** P < 0.01)

Although DFTD transmissibility in the Tasmanian devil population did not show any gender bias it was unknown if this was the case for DFTD responses in the mouse model. Therefore to investigate if there was any significant gender bias, serum from male and female BALB/c siblings were compared for antibody levels following immunisation with the same preparation of 10^6 DFTD cells. All tested isotypes were produced by both genders with no skewing of the antibody isotypes.

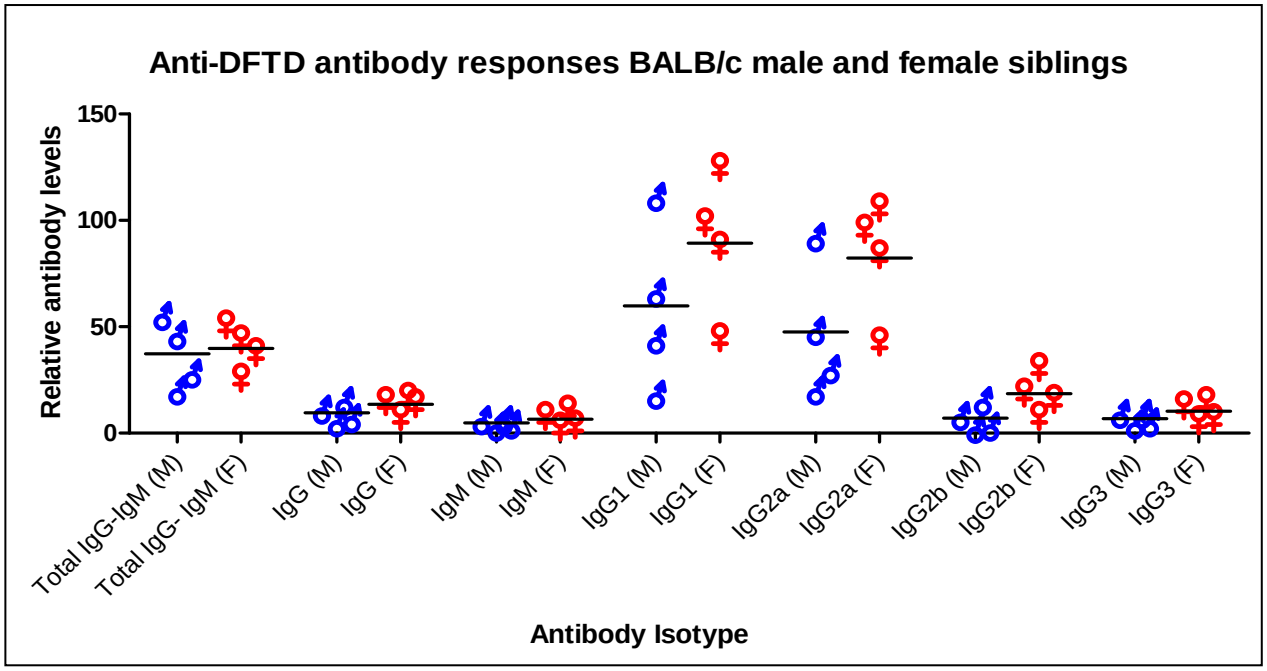

Figure 3-12. Four male and five female BALB/c mice were injected with the same DFTD cell preparation and protocol. Unpaired Student's two tailed t-test revealed that there was no statistically significant difference between genders.

To investigate how long after immunisation it took to induce detectable antibody responses and timing of IgG isotype switching, C57/BL6 mice were injected intraperitoneally with 10^6 DFTD cells and bled four and seven days post immunisation. Four days following immunisation with DFTD cells two mice had produced detectable levels of IgM and the mouse with the highest IgM level (shown in red) was the only mouse with detectable IgG at this time. Seven days following immunisation all four mice produced both IgM and IgG. The mouse producing the highest level of IgM at day seven (shown in green) also had the highest level of IgG at this time (Figure 3-13). The significance of this result is that four days is insufficient to produce reliable antibody responses and seven days will produce both IgM and IgG antibodies following DFTD immunisation.

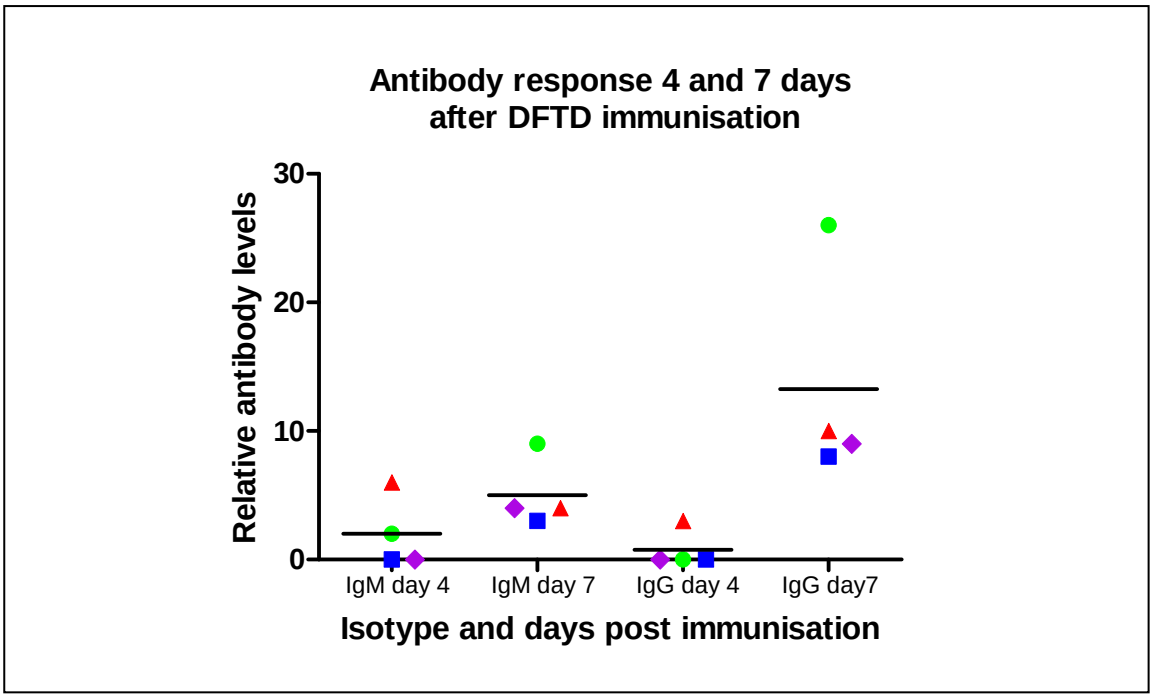

Figure 3-13. C57/BL6 mice (shown in individual colours) were immunised intraperitoneally with 10^6 DFTD cells and serum collected 4 and 7 days post injection. All samples were analysed in the same flow cytometry experiment to permit direct comparison of mouse anti-DFTD IgM and IgG levels in the serum.

Primary and secondary responses were compared to determine if secondary responses were enhanced as a result of recall or memory. C57/BL6 mice were immunised once intraperitoneally with 10^6 DFTD cells and serum samples collected 4 (n=4), 7 (n=4), 16 (n=3) or 24 (n=3) days later to evaluate the fluctuation of antibody levels following primary immunisation. Other C57/BL6 mice (n=3) were given a second injection on day 95 and serum collected 7 days later to evaluate secondary responses.

Figure 3-14 shows that for the primary response peak detection levels for IgM were at day 7 and 16 following immunisation and subsided by day 24. IgM levels were restored to levels similar to the primary response 7 days following a second immunisation at day 95.

IgG1, IgG2a and IgG2b primary response levels were highest at day 16 and had subsided by day 24. IgG1 had higher levels 7 days after the second immunisation compared to seven days after the first immunisation. The error bars for the secondary response at day 7 were large indicating variability in individuals but the response was not significantly higher than the peak primary response observed on day 16. The enhanced speed of the secondary response is consistent with a memory response.

Ig2a primary response peak levels were also the highest at day 16 and had subsided by day 24. Seven days after the second immunisation IgG2a was higher than 7 days after the first immunisation. This was equivalent to the peak primary response observed on day 16. The enhanced speed of this secondary response is consistent with a memory response.

IgG2b primary response levels also peaked at day 16 and subsided by day 24. Seven days after the second immunisation IgG2b was higher than 7 days after the first immunisation. This was significantly higher than the peak primary response observed on day 16. The secondary response was both more rapid and stronger suggesting a memory response had occurred.

IgG3 primary response levels were the highest at days 7 and 16 and subsided by day 24. The response 7 days after the second immunisation was higher than 7 days

after the first immunisation. The secondary response was both more rapid and stronger than the primary suggesting a memory response had occurred.

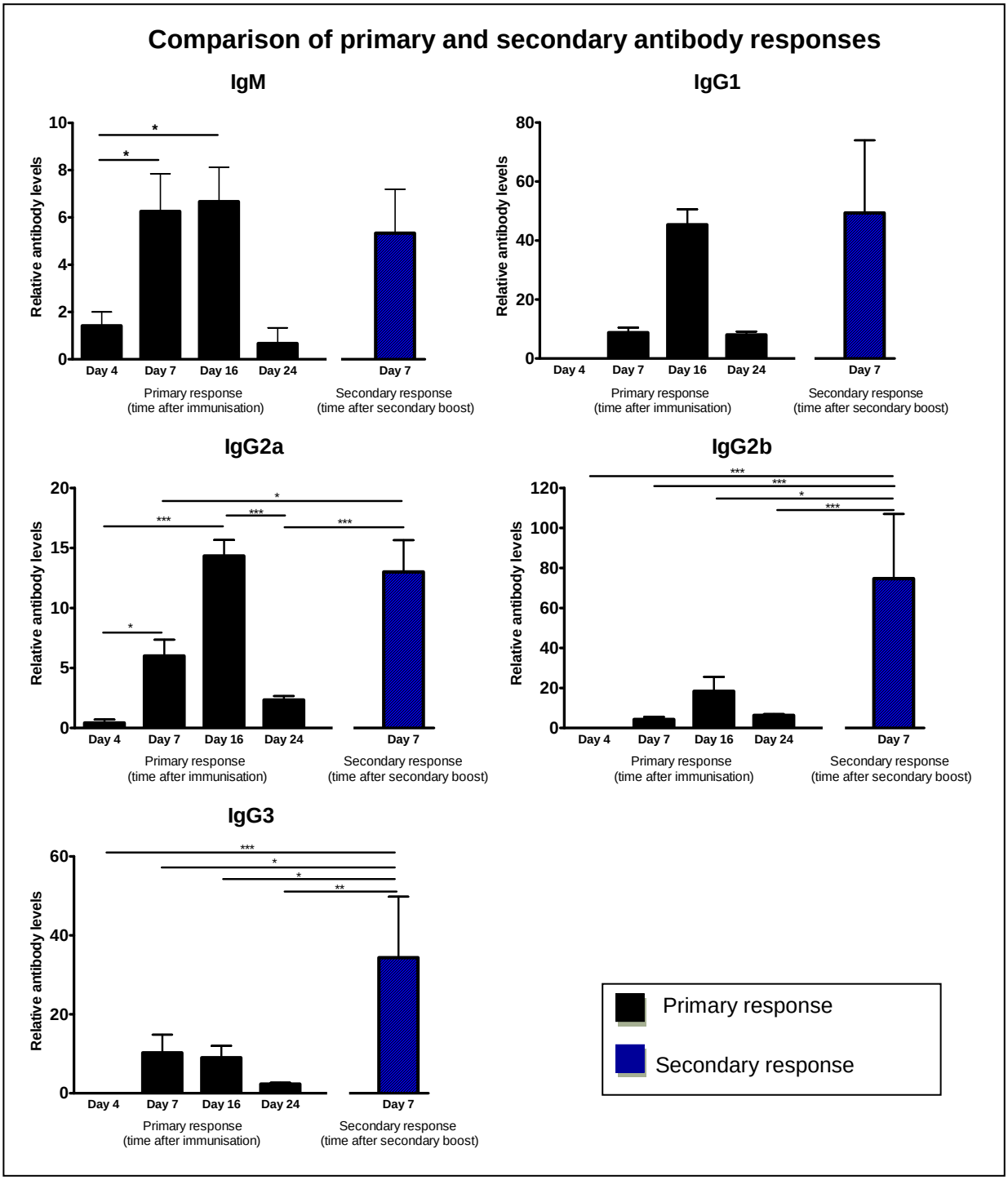

Figure 3-14. Comparison of primary and secondary antibody responses following immunisation with DFTD cells. Primary antibody responses were measured as MFI fold increase compared to naïve serum. Serum samples were collected on days 4, 7, 16 and 24 (results shown in black). Secondary immunisation occurred on day 95 and serum was collected 7 days later (results shown in blue). (Data are expressed as mean ± SEM, probability calculated by Student's unpaired t-test * P < 0.05, ** P < 0.01, *** P < 0.001)

3.4.3 **Development of a cytotoxicity assay for DFTD**

One of the aims of the research into DFTD is to induce cell-mediated cytotoxic responses. This research requires a sensitive, robust and repeatable *in vitro* cytotoxicity assay. The ^{51}Cr assay is one of the most commonly used cytotoxicity assays but it is expensive and has inherent safety concerns associated with radioactive isotopes. For this reason a non-radioactive assay needed to be developed and validated specifically for DFTD cells.

The non-radioactive cytotoxic assay involved labelling the target cells with CFSE or CellTrace Violet™. This allowed discrimination of target cells from unlabelled effector cells. Then following the required incubation period a dead cell marker such as propidium iodide (PI) or 7AAD was used. Flow cytometry was used to analyse the percentage of dead target cells.

Validation for this assay was undertaken using human lymphocytes as effector cells against K562 target cells and also mouse lymphocytes against YAC-1 target cells. The human leukaemia cell line K562 is a known cytotoxic target for human NK cells while YAC-1 cells are a known target for murine NK cell-mediated cytotoxicity. The non-radioactive assay technique detected cytotoxicity for both of these effector target cell combinations as a dose response curve was evident. Human lymphocytes showed greater cytotoxicity than mouse lymphocytes, hence the use of lower effector to target cells ratios (Figure 3-15).

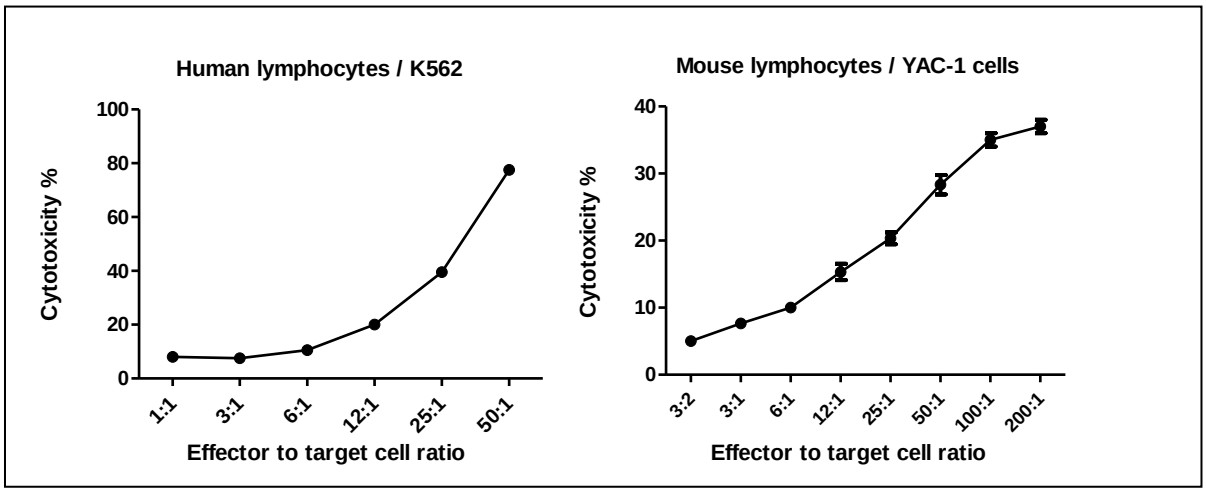

Figure 3-15. To test the cytotoxicity assay K562 and YAC-1 cytotoxicity was calculated following 18 hours incubation with effector cells. (Data points represent the mean of duplicates (K562) or triplicates (YAC-1) and error bars represent SEM)

The ^{51}Cr radioactive assay has long been regarded as the gold standard of cytotoxicity assays. To compare the sensitivity and reproducibility of the non-radioactive assay a direct comparison of the ^{51}Cr assay to the non-radioactive cytotoxicity assay was undertaken. YAC-1 tumour cells from the same culture and mouse lymphocytes from the same mouse were used in these parallel assays to minimise variability.

The ^{51}Cr and CellTrace Violet assay results closely correlated (Figure 3-16). The main difference being the CellTrace Violet plot had smaller error bars indicating smaller variance between replicate wells than ^{51}Cr. The exception was the 3:1 E:T ratio when CellTrace Violet had the highest variability in replicate wells which included negative values. CellTrace Violet assay is cheaper, easier and safer than the ^{51}Cr assay and is not inferior to ^{51}Cr for repeatability and sensitivity. Consequently, the non-radioactive assay was deemed as a suitable replacement for the ^{51}Cr assay.

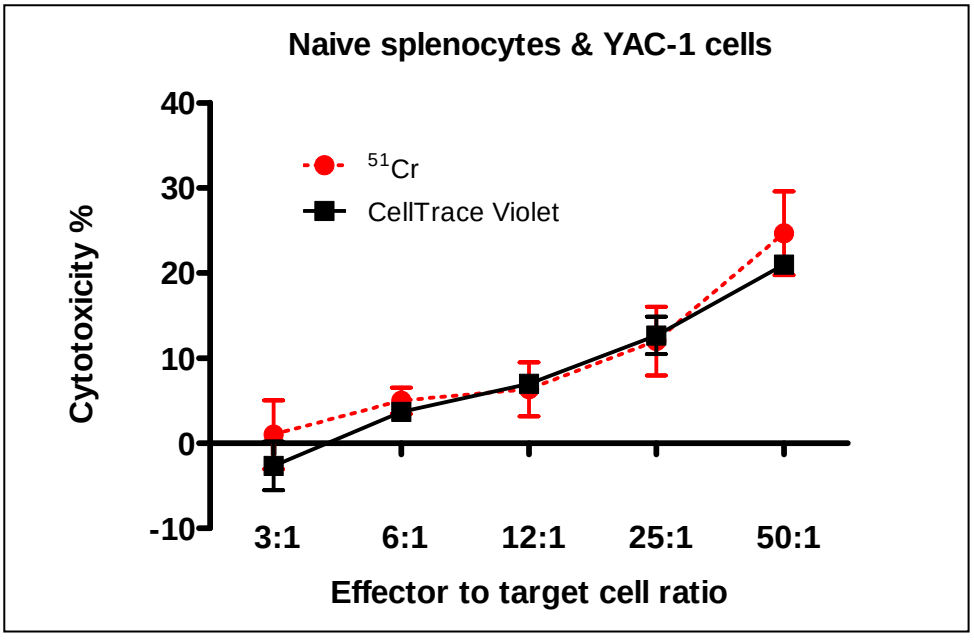

Figure 3-16. Splenocytes from a C57/BL6 mouse were used as effector cells against ^{51}Cr labelled YAC-1 target cells and CellTrace Violet labelled YAC-1 target cells in 18 hour *in vitro* cytotoxicity assays. (Data points represent mean of three replicate wells and error bars represent SEM)

3.4.4 *In vitro* murine cell-mediated cytotoxic responses to DFTD

Splenocytes from naïve and immunised C57/BL6 mice were compared for cytotoxicity responses against DFTD cells following 18 hours *in vitro* incubation. The immunised mice had been injected intraperitoneally with 2 x 10^6 C5065 DFTD on days 0 and 25 with the splenocytes harvested on day 33.

The magnitude of killing from naïve mice was equivalent to that of immunised mice. This indicated that the killing was mediated by unprimed cells of the immune system (Figure 3-17). There were varying amounts of background killing at low effector to target cell ratios. All mice in this experiment demonstrated a dose response cytotoxicity against DFTD cells.

However it should be noted that cytotoxic responses by mouse splenocytes were not always observed in other experiments. This is evaluated in more detail in Chapter 6.

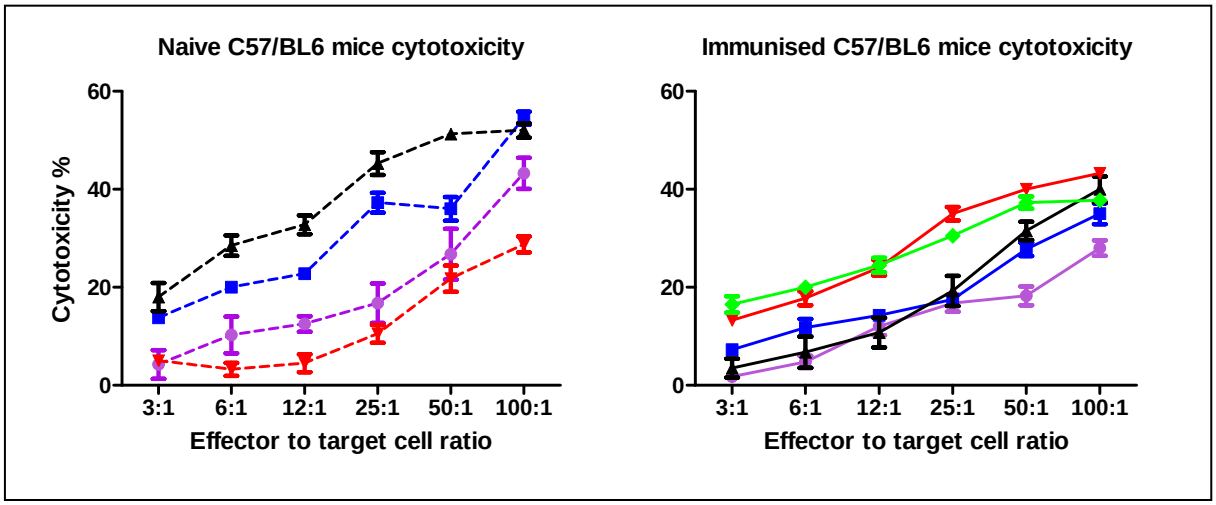

Figure 3-17. Splenocytes from immunised and naïve C57/BL6 were compared for cytotoxicity against DFTD cells following 18 hours *in vitro* co-incubation. (Data points represent mean of four replicate wells with error bars representing SEM)

3.4.5 *In vitro* cytokine responses to DFTD cells by murine lymphocytes

As upregulation of certain cytokines in the tumour microenvironment can enhance or suppress tumour rejection, cytokines from immunised C57/BL6 mice were evaluated to provide an additional assessment of immune responses to DFTD cells. Two C57/BL6 mice were subcutaneously immunised with DFTD cells and lymphocytes from the lymph nodes were co-cultured with DFTD cells for five days to allow time for cytokines to be produced. Five days culturing in 96 well plates proved problematic in terms of nutrient exhaustion and evaporation from the plate edges. The supernatant was analysed in an ELISA assay for IFN-γ, IL-4, IL-10 and IL-12 cytokines as these would allow discrimination between T_H1 or T_H2 dominated immune responses.

Lymphocytes from both immunised mice produced IL-4, IL-10 and IFN-γ but IL-12 was not detected (Figure 3-18). IFN-γ is a principal T_H1 cytokine and IL-10 is the most important T_H2 cytokine to downregulate T_H1 responses. The presence of both of these cytokines suggests the immune system is not polarised to either a T_H1 or T_H2 response.

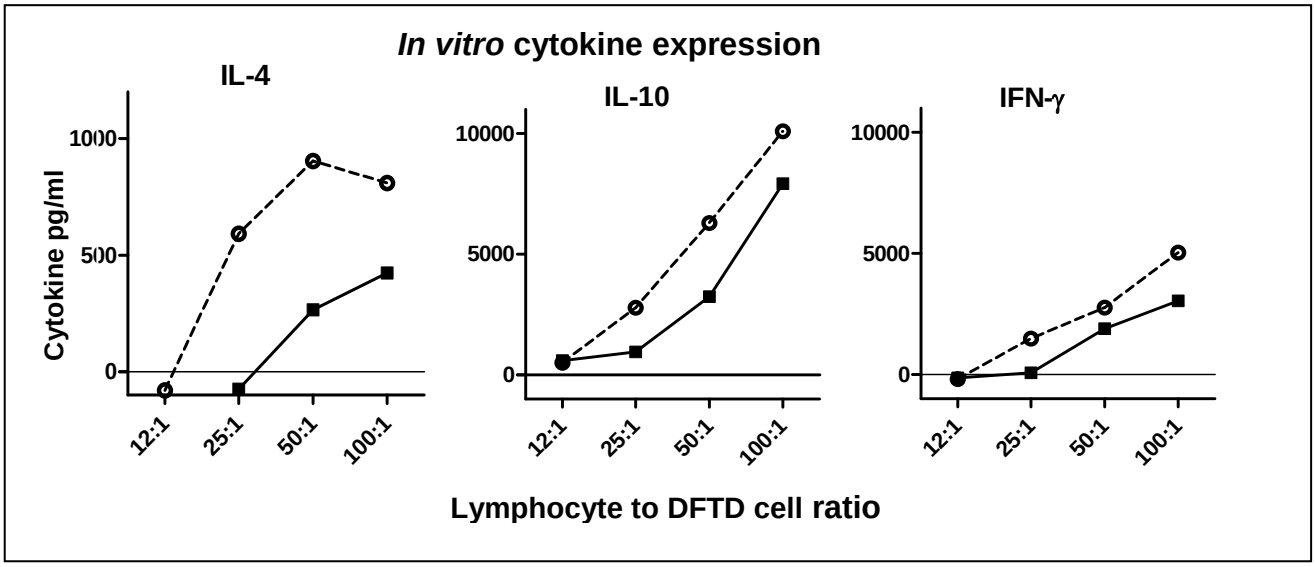

Figure 3-18. Cytokine levels detected by ELISA assay following five days co-culturing of DFTD cells and lymphocytes from two immunised C57BL/6 mice. (Data points represent the mean of duplicates)

Since five days incubation was problematic when incubating lymphocytes with DFTD cells to produce cytokines an investigation was undertaken to determine an optimal incubation period. As a consequence, lymphocytes and DFTD cells were cultured together for 72 hours at a ratio of 50:1 and supernatant sampled at 24 hour time points to measure cytokine production.

There was increasing IFN-γ and IL-10 levels in the culture supernatant as culture time was extended to 72 hours. Shorter culturing times of 48 hours or less significantly reduced the cytokine levels in the supernatant (Figure 3-19). IL-4 and IL-12 was not detected at any time point (data not shown). Based on these results 72 hours was selected for the incubation period for future cytokine assays.

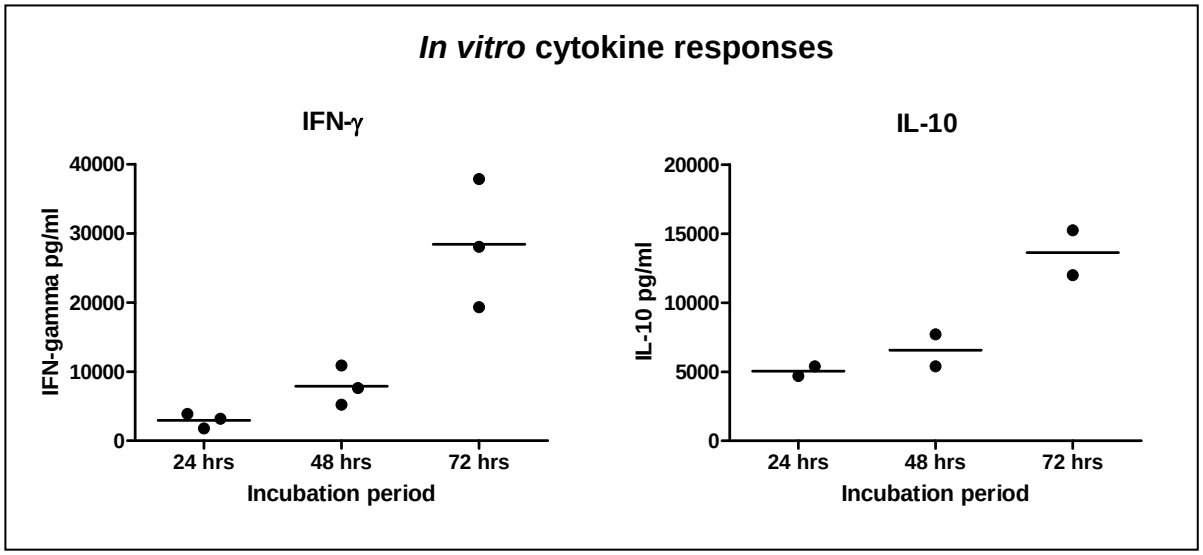

Figure 3-19. Evaluation of cytokine levels following various incubation periods for lymphocytes and DFTD cells in 96 well plates. (Horizontal bars represent the mean)

Having determined 72 hours as a suitable incubation time for detecting cytokine levels in the supernatant the best effector to target cell ratios for cytokine production needed to be determined. One of the limiting factors was the availability of effector cells as these were also required for other experiments conducted concurrently. For this reason the maximum ratio of effector to target cells tested was 100:1 and a series of doubling dilutions to 3:1 were cultured together for 72 hours.

An effector to target cell ratio of 100:1 produced the highest level of IFN-γ and IL-10 while 12:1 was ineffective at producing detectable levels of cytokines (Figure 3-20). IL-4 and IL-12 was not detected at any ratio (data not shown). Based on these results an effector to target cell ratio of 100:1 with 72 hours incubation would become standard protocol for future cytokine assays.

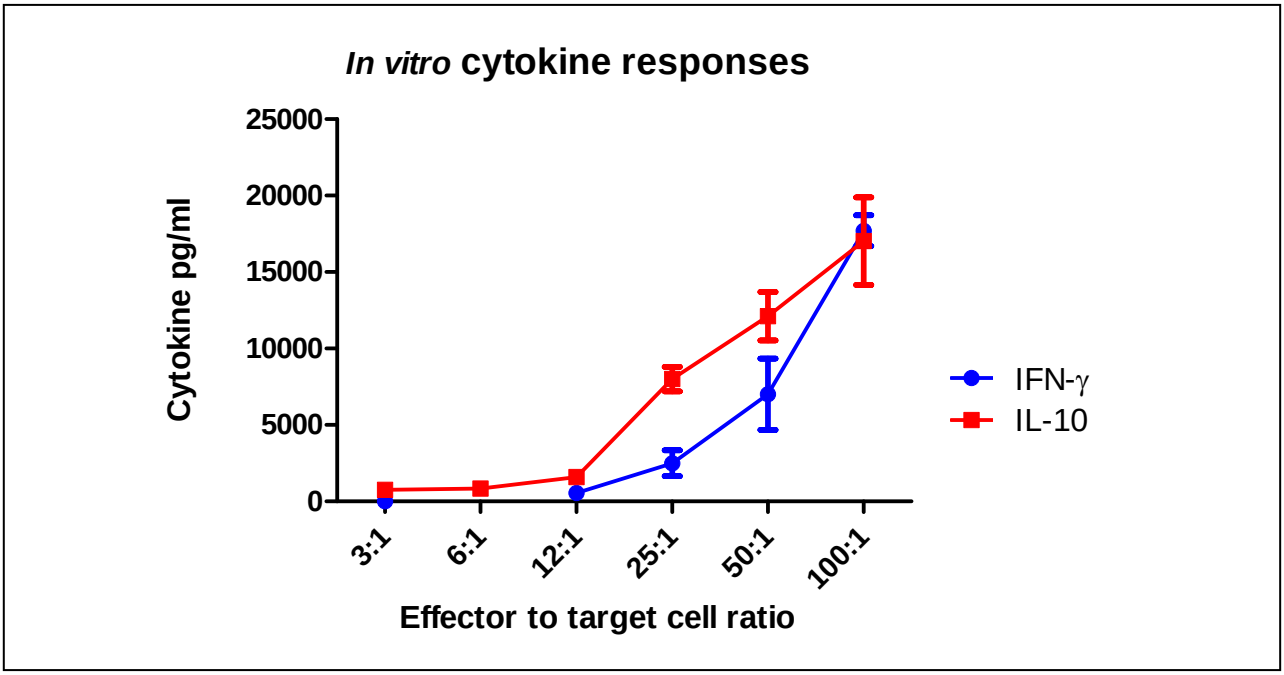

Figure 3-20. Evaluation of cytokines levels following 72 hours incubation at various effector to target cell ratios. (Data points represents mean of three immunised C57/BL6 mice and error bars SEM)

To evaluate cytokine responses in more detail the cytokine analysis was switched from an ELISA based technique to a CBA T_H1, T_H2, T_H17 micro-bead array kit. The micro-bead assays have the advantage of being able to evaluate multiple cytokines in a single sample. Also, by using 96 well plates multiple samples can be analysed in the same experiment using high throughput analysis on flow cytometry equipment. This allows direct comparison of relative cytokine levels without experimental variability between ELISA plates confounding the results.

The micro-bead assay was used to compare splenocytes from naïve and immunised mice to see if the cytokine response varied depending on previous exposure to DFTD cells. BALB/c and C57/BL6 mice were injected intraperitoneally with viable DFTD cells on day 0 and 15 with splenocytes harvested on day 22. Splenocytes from naïve mice were harvested at the same time.

Immunisation altered the cytokine responses in BALB/c and C57/BL6 mice. Splenocytes from naïve BALB/c and C57/BL6 mice produced higher levels of the inflammatory cytokine IL-6, the anti-tumour cytokine TNF-α and the T_H1 cytokine IFN-γ. Splenocytes from immunised mice produced higher levels of the T_H2 regulatory cytokine IL-10 (Figure 3-21).

Naïve NOD/SCID mice had been included in the experiment because it was predicted that they would be non-responders to the DFTD cells. However, the NOD/SCID mice produced levels of TNF-α and IL-6 equivalent to the levels produced by naïve BALB/c and C57/BL6 mice. There was no significant production of IL-10 and IFN-γ from the NOD/SCID mice (Figure 3-21).

IL-2, IL-4 and IL-17a were not detected in any of the samples (data not shown). DFTD cells alone and splenocytes alone did not produce detectable levels of cytokines (data not shown).

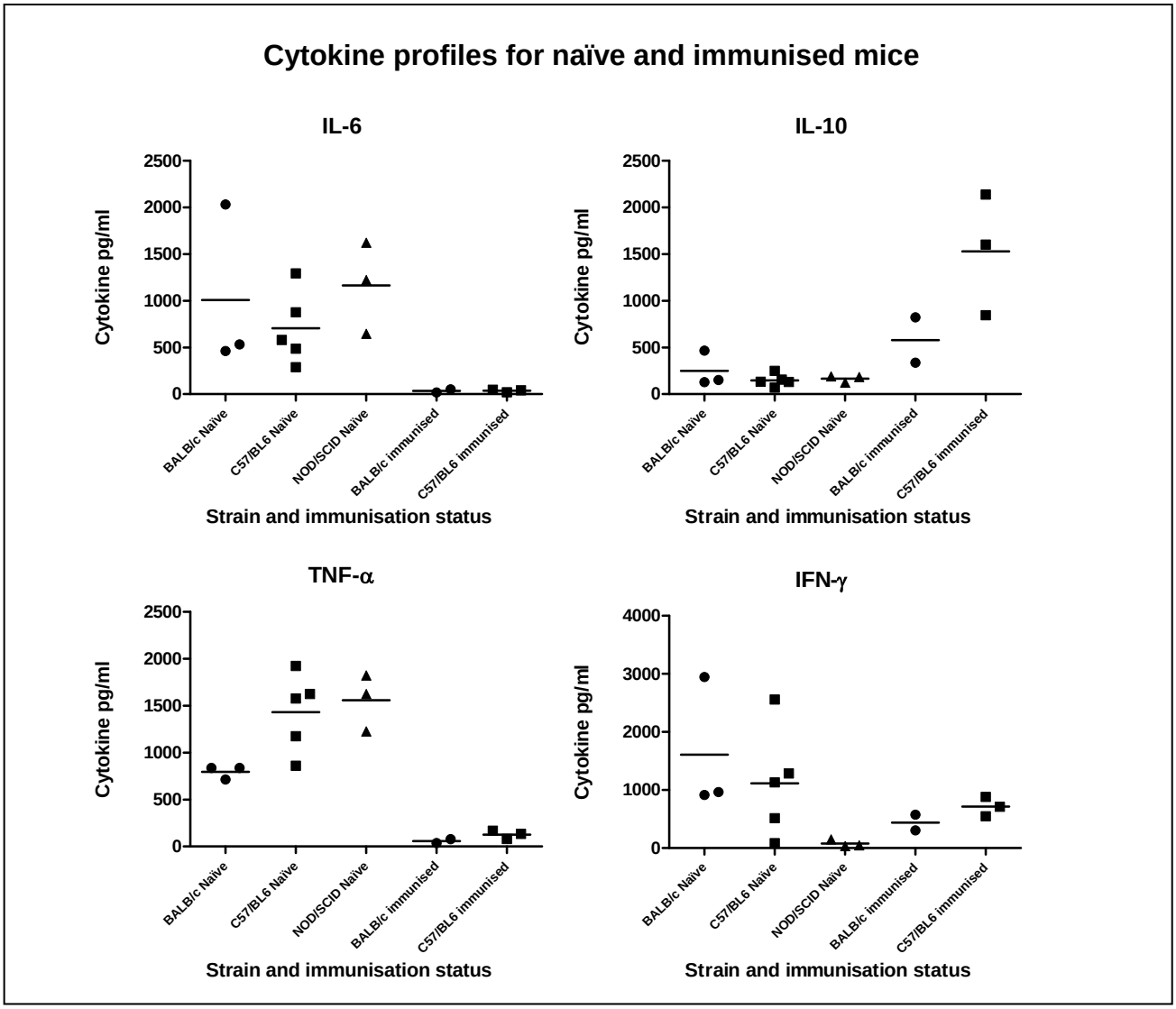

Figure 3-21. Evaluation of cytokine levels obtained in culture supernatants of splenocytes from naïve and immunised mouse cultured *in vitro* for 72 hours with C5065 DFTD cells. Supernatant analysed using CBA T_H1, T_H2, Th17 micro-bead array kit. (Horizontal bars represent mean and individual points raw data)

To further assess the effect of priming the immune system with DFTD cells, various intraperitoneal immunisation strategies were compared in C57/BL6 mice and cytokine responses analysed. The first involved injections on day 0 and 15 with the splenocytes harvested on day 22. This protocol was chosen to replicate the previously described work in Figure 3-21. The next involved a series of three immunisations on day 0, 47, 75 and splenocytes harvested on day 81. This protocol was chosen to see if repetitive exposure modified responses. The third involved injections on day 0 and 95 with the splenocytes harvested on day 102. This protocol was to evaluate if a memory immune response to DFTD cells developed in the C57/BL6 mice following primary exposure.

The cytokine production by mice immunised day 0, day 15 and splenocytes harvested on day 22 were equivalent to the previous results for comparable immunised C57/BL6 mice as shown in Figure 3-21. This immunisation schedule resulted in cytokine production dominated by the T_H2 regulatory cytokine IL-10 (Figure 3-22).

Immunisation on days 0, 47 and 75 resulted in the highest levels of IL-6, IL-10, TNF-α and IFN-γ. There was no skewing towards a T_H1 or T_H2 profile (Figure 3-22).

Compared to two injections 15 days apart, two injections 95 days apart produced higher levels of IL-6 and TNF-α, lower levels of IL-10 and similar levels of IFN-γ (Figure 3-22).

IL-2, IL-4 and IL-17a were not detected in any of the samples (data not shown).

Priming the immune system altered the cytokine responses to subsequent DFTD exposure. Repetitive exposure increased all detected cytokine levels however there is no evidence that this effect is maintained over prolong periods as a 95 day interval between DFTD exposure did not produce enhance cytokine responses compared to naïve mice (Figure 3-21,Figure 3-22)

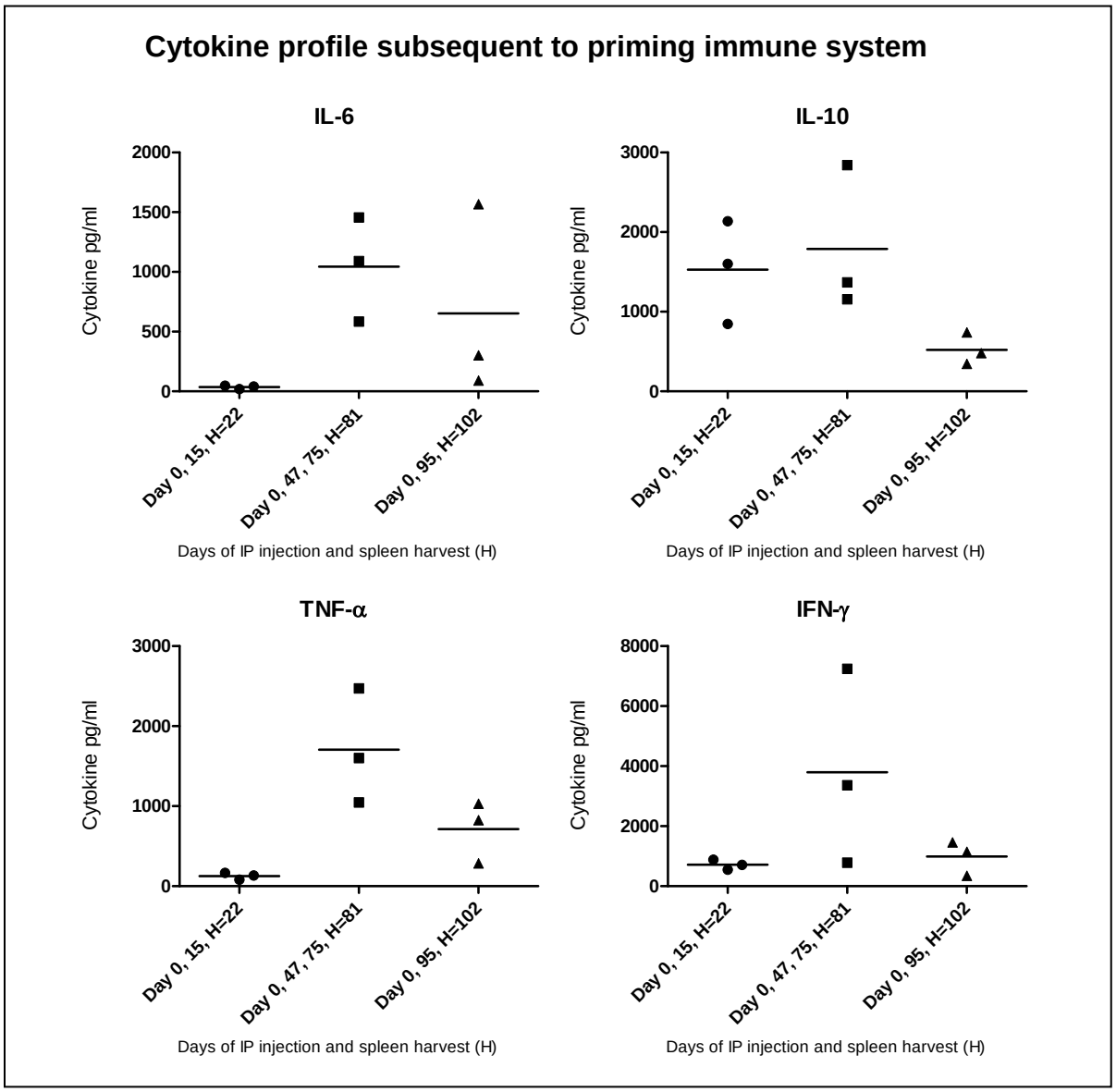

Figure 3-22. C57/BL6 mice were injected intraperitoneally with 10^6 C5065 DFTD cells and splenocytes harvested 6 or 7 days after the last injection. The splenocytes were cultured for 72 hours *in vitro* with the DFTD cell line C5065. The supernatant was analysed using CBA T_H1, T_H2, Th17 micro-bead array kit. (Horizontal bars represent mean and individual points raw data)

As shown in Figure 3-21 immunised mice produced higher levels of IL-10 while naïve mice produced higher levels of IFN-γ. This lead to the hypothesis that early immune responses were T_H1 dominated and later responses switched to T_H2 dominated responses. To test this hypothesis, cytokine responses were compared for C57/BL6 mice four and 21 days after a primary immunisation, as well as C57/BL6 mice given a secondary immunisation 57 days after the primary.

The primary responses for IL-6, IL-10, TNF-α and IFN-γ increased from day 4 until day 21. IFN-γ dominated IL-10 primary responses while this was reversed as a secondary response. The secondary response for IL-10 was more rapid and stronger than the primary response but IFN-γ secondary and primary responses were equivalent. Secondary responses for IL-6 and TNF-α were enhanced compared to the primary response (Figure 3-23). IL-2, IL-4 and IL-17a were not detected in any of the samples (data not shown). The greatest variation in the cytokine response was the domination of IL-10 as a secondary response supporting the hypothesis that later responses switch towards a T_H2 response.

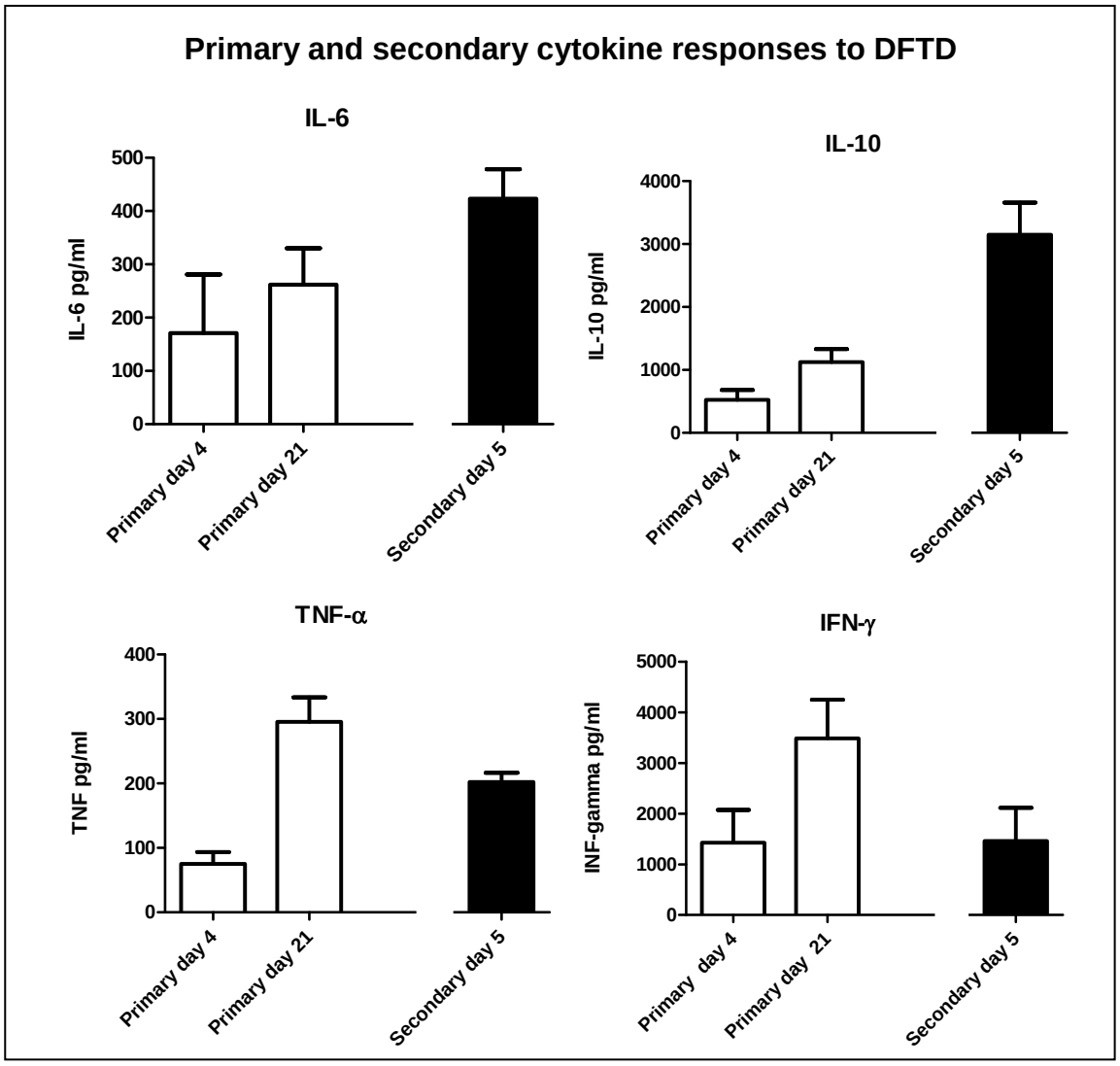

Figure 3-23. Cytokine production by splenocytes from DFTD immunised C57/BL6 mice, cultured *in vitro* with DFTD cells for 72 hours. Primary responses refer to mice given a single injection of 2 x 10^6 DFTD cells and day refers to time post immunisation. Secondary responses refer to mice immunised with 2 x10^6 DFTD cells, rested 57 days, given a second immunisation of 2 x 10^6 DFTD cells and splenocytes collected 5 days later. (Data expressed as mean of five mice and error bars SEM)

To further test the hypothesis that early immune responses were T_H1 dominated and later responses switched to T_H2 dominated responses IL-10 and IFN-γ levels were compared 4 days after DFTD primary immunisation and either 5 or 7 days after secondary DFTD immunisations. The secondary immunisations were performed on either day 27, day 57 or day 95. Serum was collected 5 days after the day 26 or 57 immunisations and 7 days after the day 95 immunisation.

IFN-γ production was unaltered between primary and secondary responses; however, IL-10 response was greatly enhanced as a secondary response on day 27 and diminished to similar levels as a primary response by day 95 (Figure 3-24).

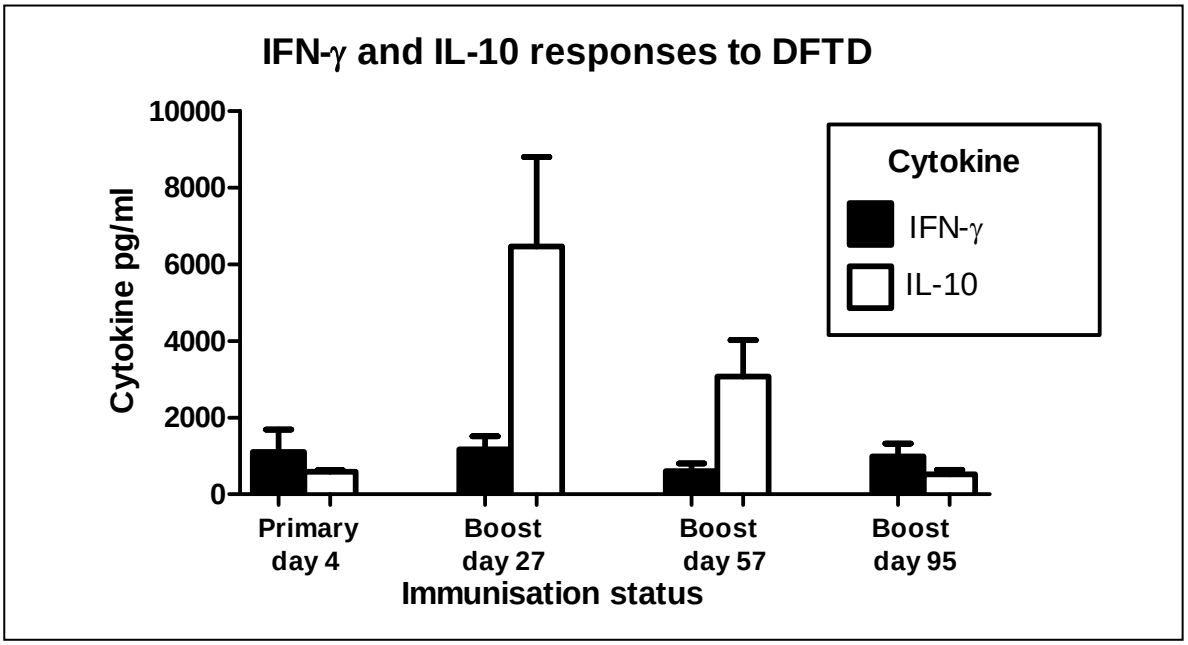

Figure 3-24. IFN-γ and IL-10 responses compared for primary responses four days post DFTD immunisation and responses five days post-secondary DFTD immunisations given at day 27 or 57 and seven days post-secondary DFTD immunisations given at day 95. (Columns represent mean of five mice except for day 95 is mean of three mice and error bars SEM)

It was important to determine if any gender bias occurred in the murine immune responses to DFTD as this variable would need to be factored for in future experimental designs. Following identical immunisations on days 0 and 15 the splenocytes were harvested and cultured with DFTD cells for 72 hours. The supernatant was then analysed for cytokine levels and no significant gender biases were detected (Figure 3-25).

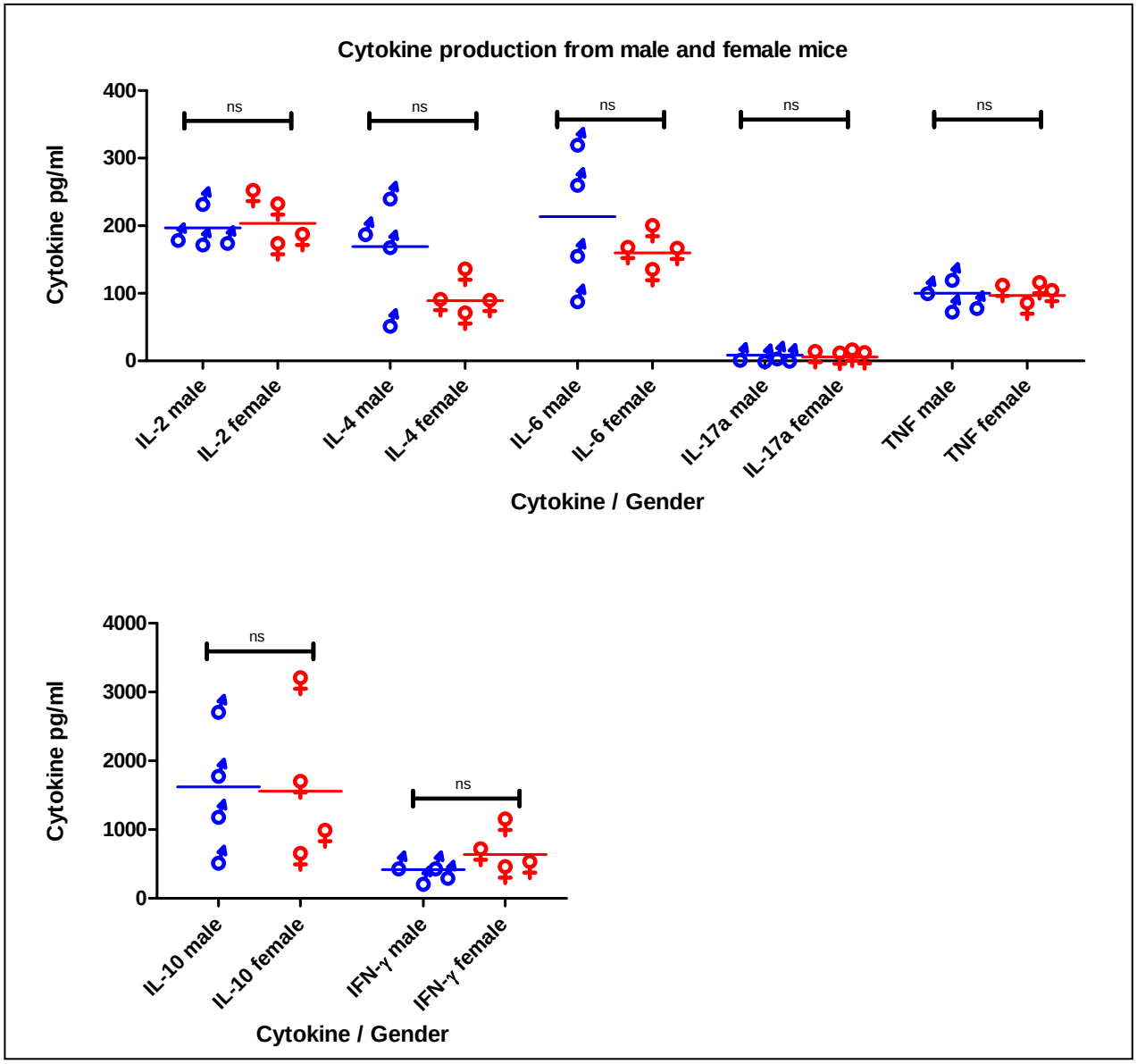

Figure 3-25. Cytokine levels were compared for male and female BALB/c mice immunised twice with 2 x 10^6 DFTD cells. (One way ANOVA with Bonferroni's multiple comparison tests used to analyse significance. Horizontal bars represent mean and individual points raw data).

3.4.6 Maintaining immunogenicity while inactivating DFTD cells

Vaccine trials and immunotherapy experiments with Tasmanian devils require the injection of inactivated DFTD cells to induce immune responses without the risk of infection. To evaluate how inactivating DFTD cells affected immunogenicity the BALB/c mouse model was used. Relative antibody responses were compared between mice immunised with viable cells, irradiated cells, sonicated cells or freeze/thawed cells.

Both IgG and IgM antibody responses were reduced when BALB/c mice were immunised with sonicated and freeze/thawed inactivated cells. Using irradiated cells for immunisations maintained similar levels of antibody responses compared to viable cells (Figure 3-26).

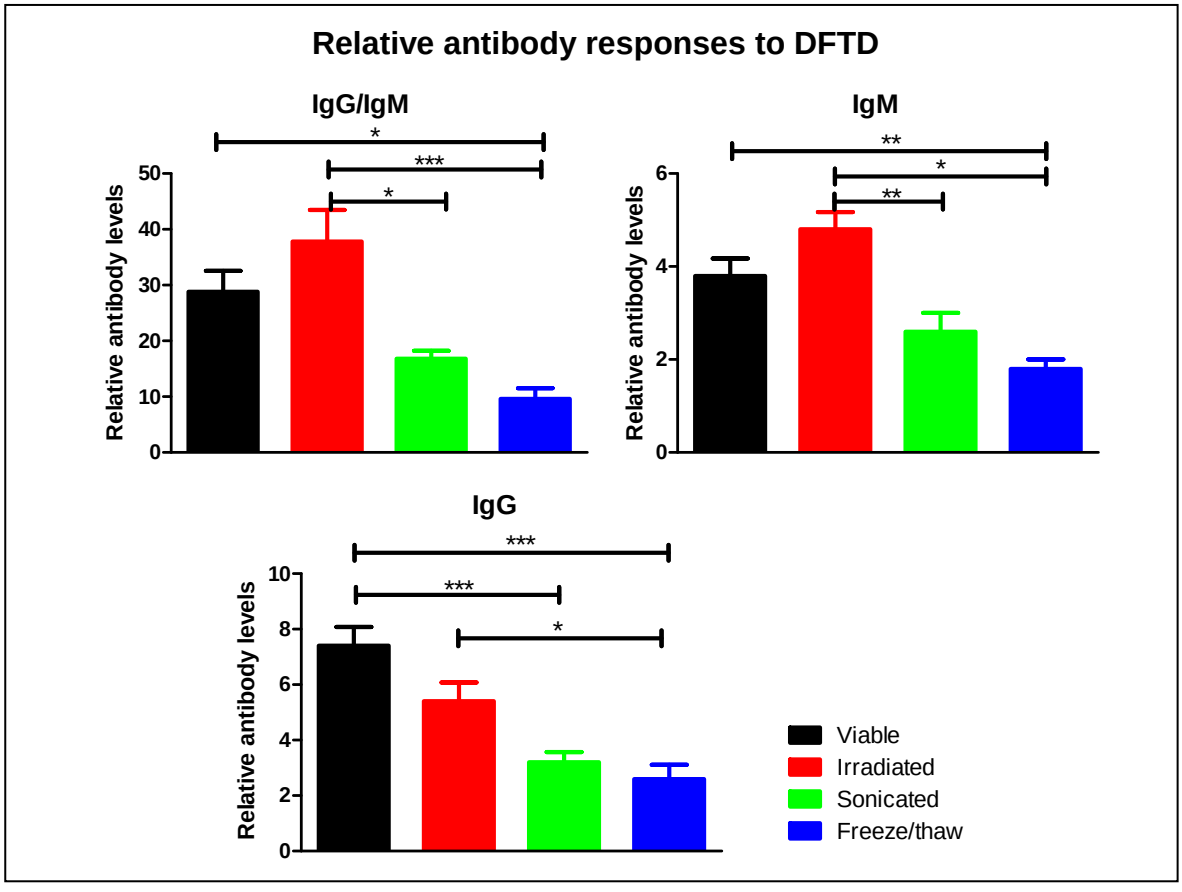

Figure 3-26. BALB/c mice were injected intraperitoneally with 10^6 viable, irradiated, sonicated or freeze/thaw inactivated C5065 DFTD cells on day 0, given a second injection day 14 and serum collected day 21. (Columns represent mean of five mice and error bars SEM. Statistical analysis by one way ANOVA with Bonferroni's multiple comparison test * P < 0.05, ** P < 0.01 and *** P < 0.001.)

To examine in more detail the IgG responses following immunisations with inactivated DFTD cells the mouse sera were analysed for IgG1, IgG2a, IgG2b and IgG3 isotypes levels. Following immunisation with irradiated cells all tested IgG isotype responses remained similar to viable cell immunisations. There was a trend for all tested IgG isotypes to be lower following immunisations with sonicated or freeze/thaw cells and this was statistically significant in the case of IgG1 and IgG2a (Figure 3-27). This provided further evidence that sonication and freeze/thawing cells for immunisations reduces the subsequent immune responses while irradiation of cells has no significant effect on subsequent immune responses.

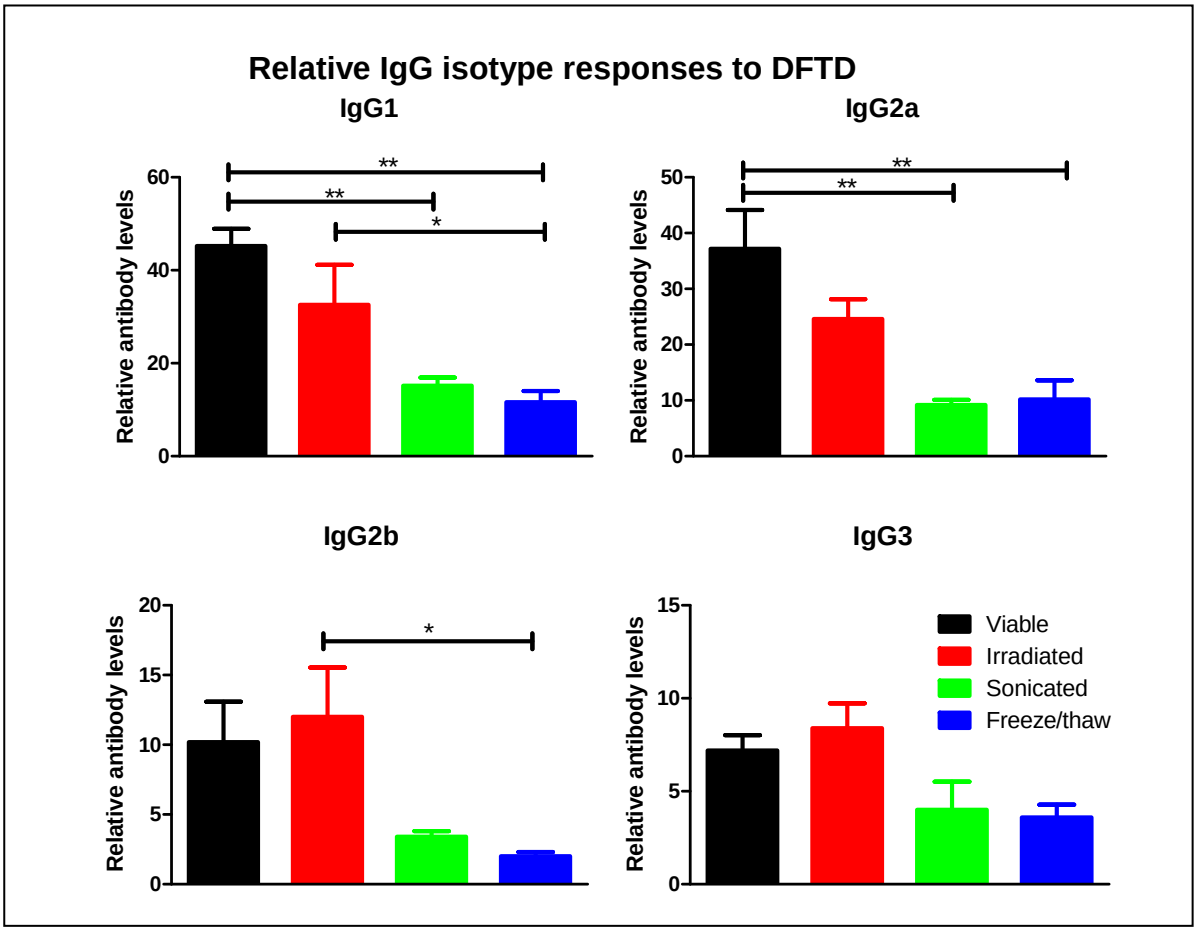

Figure 3-27. BALB/c mice were injected intraperitoneally with 10^6 viable, irradiated, sonicated or freeze/thaw inactivated C5065 DFTD cells on day 0, given a second injection day 14 and serum collected day 21. (Columns represent mean of five mice and error bars SEM. Statistical analysis by one way ANOVA with Bonferroni's multiple comparison test * P < 0.05.)

In vitro cytokine levels were examined following immunisation with inactivated cells as cytokine levels are informative about the efficacy of immune responses against tumours. The anti-tumour cytokines IFN-γ and TNF-α response were suppressed when DFTD cells were inactivated by sonication or freeze/thawing. IFN-γ production was maintained at normal levels when DFTD cells were inactivated by irradiation. IL-2 and IL-4 cytokines were also suppressed when DFTD cells were inactivated by sonication or freeze/thawing. Results for IL-6 and IL-10 were highly variable and not statistically significant but responses from irradiated cells tended to be higher than those from sonicated or freeze/thaw inactivated cells (Figure 3-28). The cytokine responses are consistent with the antibody responses providing further evidence that immunisation with sonicated or freeze/thaw cell preparations produced lower immune responses compared to viable or irradiated cells.

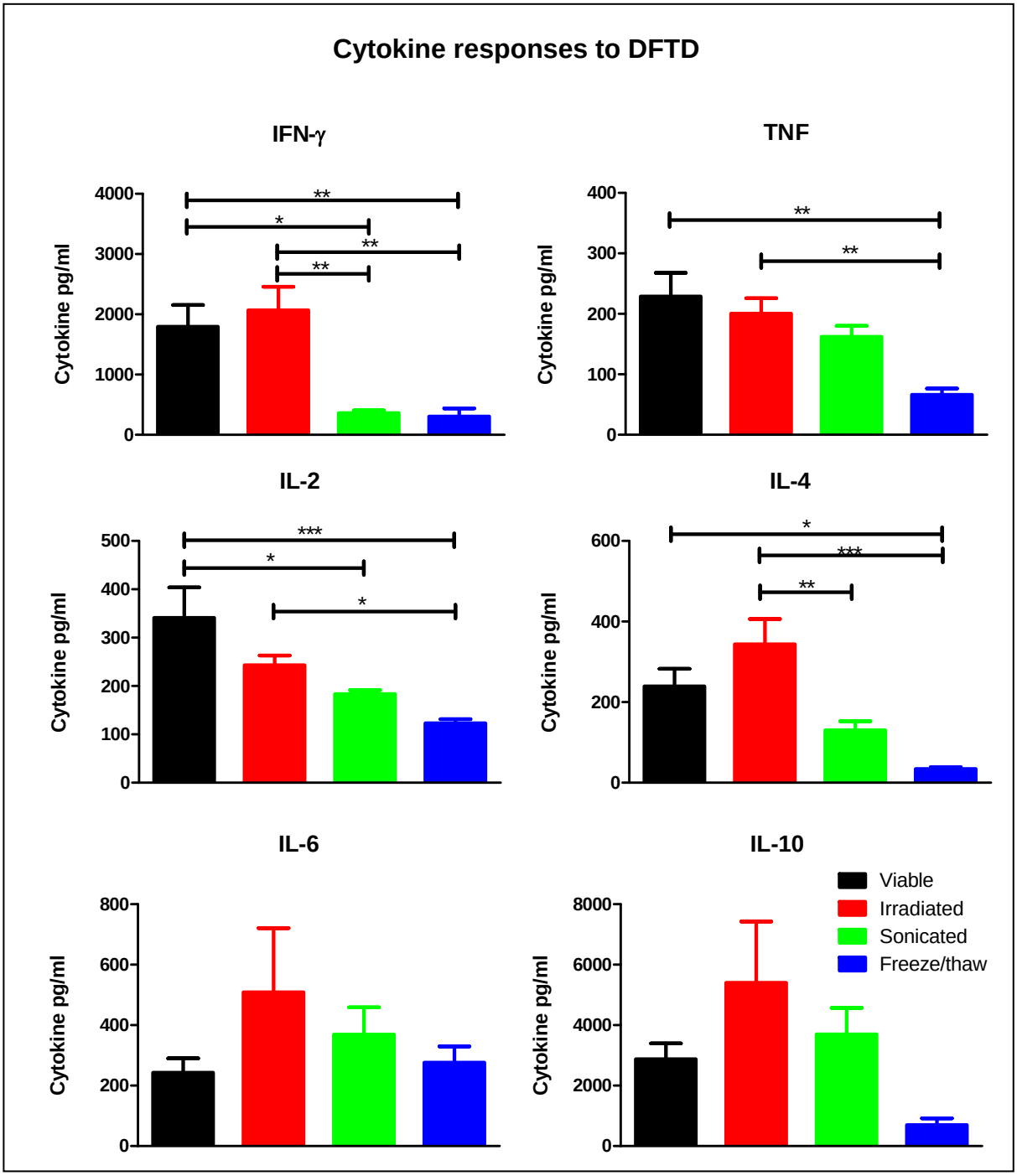

Figure 3-28. BALB/c mice were injected intraperitoneally with 10^6 viable, irradiated, sonicated or freeze/thaw inactivated C5065 DFTD cells on day 0, given a second injection day 14 and serum collected day 21. (Columns represent mean five mice and error bars SEM. Statistical analysis by one way ANOVA with Bonferroni's multiple comparison test * P < 0.05, ** P < 0.01 and *** P < 0.001.)

During the experiments described in this results chapter and the experiments described in the proceeding chapters BALB/c and C57/BL6 were injected with DFTD cells. The DFTD xenograft development kinetics in NOD/SCID mice are described in chapter four (Figure 4-2). In consideration of the time to tumour development in relationship to number of DFDT cells injected at least 71 BALB/c or C57/BL6 were injected with DFTD cells of sufficient numbers and given sufficient time to establish DFTD xenografts and none developed tumours.

Table 3-2. Summary table of DFTD cell injections into BALB/c and C57/BL6 mice

Mouse Strain	Injected cells	Number of mice	
		Tumour	No Tumour
BALB/c mice	$\geq 10^6$ DFTD cells	0	35
C57/BL6	5×10^5 DFTD cells	0	8
C57/BL6	$\geq 10^6$ DFTD cells	0	28
Overall		0	71

3.5 Discussion

Devil Facial Tumour Disease is a cancer that is transmitted from host to host with a lack of allo-recognition. An inability to trigger an immune response suggests that DFTD cells are non-immunogenic and could be imperceptible to any immune system. Using a mouse model this chapter analysed whether DFTD cells can induce an immune response and if so can they be targets for cytotoxic cells and be killed by apoptosis.

3.5.1 Apoptosis

The ability of DFTD cells to undergo apoptosis was investigated because if an immune response could be generated against DFTD, it would be ineffective if DFTD cells could not be killed. Some tumours can undermine immune responses by the inactivation of the apoptotic pathway. Apoptosis resistance is not only relevant to immunosurveillance but could impact on possible interventions including chemotherapy, radiotherapy and immunotherapy. These therapies primarily depend on inducing apoptosis (Igney and Krammer 2002). Resistance to apoptosis would present a major hurdle in developing effective treatments and would have implications for protocols to inactivate the DFTD cells for potential vaccine development. For these reasons, very early priorities in this project aimed to determine if DFTD cells could undergo apoptosis.

Apoptosis had not been shown in DFTD cells. A well established protocol for assessing apoptosis in mammalian cells involves Annexin V, which labels the membrane of apoptotic cells and propidium iodide (PI), which labels the DNA of cells at late stages of apoptosis and necrosis. In both the early and late stages of apoptosis the intracellular leaflet in the cell membrane becomes exposed facilitating Annexin V binding to the phosphatidylserine located there. This does not occur if cells undergo necrosis. Cells that have undergone necrosis, or have entered late stage apoptosis, will be labelled with DNA stains such as PI or 7AAD because the cell membranes have become permeable to these molecules while viable cells and cells in early stage apoptosis exclude these molecules.

Following four hours exposure to the cytotoxic drug, camptothecin, 82% of DFTD cells showed obvious signs of dying via apoptosis and only 2% by necrosis based on Annexin V and PI analysis. K562 cells and lymphocytes, which are known to be

resistance to camptothecin mediated apoptosis, were Annexin V^- when incubated with camptothecin while DFTD cells were positive. As camptothecin only produced evidence for 2% necrotic cells DFTD cells were induced to undergo necrosis by rapid freeze thaw. None of the necrotic cells, induced by freeze thawing had Annexin V binding. This provides support that the assay is specific for detecting apoptosis rather than necrosis in DFTD cells and that DFTD cells have the ability to die via apoptosis.

Further evidence of apoptosis in DFTD cells was provided by scatter analysis in flow cytometry. In late stage apoptosis the DFTD cellular contents were condensed causing a more granular characteristic to the cell resulting in increased side scatter (SSC) of the laser. The size of the cells also decreased resulting in a lower forward scatter (FSC). In comparison, necrotic DFTD cells released their cellular contents and the SSC was low, consistent with necrosis as described by Darzynkiewicz et al. (1997). Scatter analysis, which evaluates cell morphology should complement Annexin V binding which is less ambiguous and detects early and late stage apoptosis.

UV-radiation (UV-R) was used as an alternative treatment to induce apoptosis to further corroborate apoptosis in DFTD cells. Since K562 cells are sensitive to UV induced apoptosis (Ujvarosi et al 2007) they were used as a positive control cell line. Following UV-R exposure, apoptosis was detected in DFTD cells. The TUNEL assay was another means to evaluate apoptosis by labelling DNA breaks, which occur during late stage apoptosis. Following UV-R exposure DFTD cells were TUNEL positive confirming apoptotic damage was occurring to the DNA of the DFTD cells. These results further validated the ability of DFTD cells to undergo apoptosis.

From these combined observations it can be concluded that DFTD cells undergo apoptosis. This suggests that DFTD cells do not avoid immune responses by inactivating the apoptotic pathway. Further investigations are required to determine if the appropriate signalling receptors are present on the cell surface to initiate apoptosis by cellular ligands, including cytotoxic cells.

3.5.2 **Antibody responses**

Having shown that DFTD cells have the capacity to undertake apoptosis led to the hypothesis that DFTD cells are not immunogenic and therefore invisible to any immune system. However, the ability to establish DFTD xenografts in immunocompromised NOD/SCID mice but not in immunocompetent BALB/c and C57BL/6 mice (Kreiss et al 2011b) suggests that the DFTD cells are immunogenic. To confirm that the failure to establish DFTD xenografts in immunocompetent mice was a specific immune response, BALB/c and C57BL/6 mice were challenged with viable DFTD cells and antibody responses were evaluated.

Both BALB/c and C57BL/6 mice consistently rejected the DFTD cells as no tumours developed. Naïve mice lacked anti-DFTD antibodies while immunisation resulted in production of both IgM and IgG anti-DFTD antibodies. The significance of this is two-fold. Firstly, since naïve mice lacked DFTD specific antibodies rejection was not as a result of hyperacute xenograft rejection, which depends on preformed antibodies. Secondly, since immunisation induced DFTD specific antibody production this provided evidence that viable DFTD cells are immunogenic.

Having demonstrated that DFTD cells were immunogenic and therefore could be targeted by the immune system the next stage of this project evaluated means of enhancing immune responses following immunisation. Since access to Tasmanian devils is limited due to their endangered status and there are limited reagents such as monoclonal antibodies, this work continued in the DFTD mouse model.

Detection by flow cytometry of antibodies specific to DFTD cell surface antigens in the serum of BALB/c and C57BL/6 mice provided the most robust method for evaluating immune responses following immunisation with DFTD cells. The subcutaneous injection route for immunisations had originally been selected because of its similarity to the transfer of DFTD cells in the Tasmanian devil population. Adjuvants such as CpG and Montanide were also added to the immunisations. Following a single injection of DFTD cells immunocompetent mice did not develop tumours. This did not always induce a detectable antibody response. Three consecutive immunisations always produced a detectable antibody response but further immunisations did not enhance antibody levels.

The use of adjuvants was discontinued and future immunisations were with viable DFTD cells, which is what Tasmanian devils are infected with and therefore more biologically relevant. Adjuvants might alter the cells from their natural state or cause cell death independent of immune responses.

Single site versus multiple sites subcutaneous injections were compared and it was established that multiple site injections produced a higher relative antibody response. This was most likely due to the targeting of an increased number of draining lymph nodes and thereby establishing more germinal centres for the production of antibodies. Although implantations at a single site may occur frequently in wild Tasmanian devils, multiple site injections would be more effective in a vaccination program.

When intraperitoneal injections were compared to multiple site subcutaneous injections, the result was even greater and more reliable antibody response with all of the mice responding. The enhanced immunological response via the intraperitoneal route in the mouse model may have implications for the induction of a protective immune response in Tasmanian devils. Vaccination of Tasmanian devils with intraperitoneal may prove more effective than subcutaneous injections.

Intraperitoneal immunisations generated both IgM and IgG responses to the DFTD cells with the switch from IgM to IgG detected between four and seven days after immunisation. The isotype switching of B cell antibody production is T cell dependent and directed by T cell derived cytokines resulting in antibody isotypes characteristic of either a T_H1 or T_H2 profile (Isakson et al 1982, Kanai et al 2007, Tangye et al 2002). Immunisation with DFTD cells induced all tested IgG isotypes and did not polarise the immune response to a T_H1 or T_H2 antibody response.

To further optimise the immunisation of mice a protocol known to produce rapid and reliable responses against viruses in mice within 21 days was trialled (Elsawa et al 2003). This protocol involved a primary immunisation with a second immunisation two weeks later when IgG antibodies would be established and cytotoxic T cells would be activated. When mice were intraperitoneally immunised with DFTD cells using this two week prime-boost protocol antibody responses were consistently observed by the third week. This was not significantly enhanced by further immunisations. Evaluation of secondary versus primary responses in the mouse

DFTD model confirmed a T cell dependent immunological memory response with rapid production of high levels of IgG on subsequent exposure to DFTD cells. This memory response was long lasting and still present 95 days after the first immunisation.

3.5.3 **Cytokine responses**

The cytokine profile developed by the two week prime-boost protocol saw an upregulation of the T_H2 cytokine IL-10, which dominated the immune response at day 21. In comparison, a single immunisation induced an immune response at day 21 dominated by IFN-γ, which is a T_H1 cytokine. However, since the two week prime-boost protocol produced all tested IgG isotypes which included both T_H1 and T_H2 associated isotypes this protocol did not polarise the immune response.

To further understand cytokine responses, primary and secondary responses to DFTD cells were analysed. This was achieved by co-culturing splenocyte effector cells obtained from mice and DFTD cells at a 100:1 ratio for 72 hours and measuring the cytokines produced. When MNC effector cells were obtained from mice immunised once these profiles were regarded as primary responses and from mice immunised twice as secondary responses.

A number of cytokines were not detected in the assays including IL-2, IL-4 and IL-17a. These may have been produced and consumed within the assay. IL-2 is a cytokine used by cells for proliferation and it is likely that this was consumed within the assay. IL-6, TNF-α and IFN-γ were produced at similar levels for both the primary and secondary responses. The expression of these cytokines indicates an inflammatory response typical of anti-tumour activity.

IL-6 is a pro-inflammatory cytokine which enhances T cell, B cell and NK cell activity and promotes lymphocyte proliferation (Dranoff 2004, Fassati and Mitchison 2010). TNF-α induces apoptosis in tumour cells, promotes maturation of DCs increasing their antigen presenting abilities to elicit CTL anti-tumour responses (Dranoff 2004, Yong et al 2012). IFN-γ is produced by T cells, NK cells, NKT cells, macrophages and DCs. It can increase immunogenicity of tumour cells by upregulating MHC expression. IFN-γ is the principal cytokine to define a T_H1 response leading to cellular immune responses by $CD8^+$ T cells, macrophages and NK cells. It induces

the production of T_H1 antibodies IgG2a and IgG3 (Dranoff 2004, Finkelman et al 1988, Snapper et al 1992).

IL-10 was the only cytokine observed to significantly alter as a secondary response. IL-10 is the principle cytokine to define a T_H2 response and its upregulation is a mechanism employed by some tumours to permit tumour surveillance escape by suppressing T_H1 anti-tumour responses (Salazar-Onfray 1999, Schulte et al 2008, Singh et al). However, the pro-tumour immunoregulatory suppression role of IL-10 is most evident in the priming phase of the immune response. This results in suppressed activation of macrophages, DCs and T cells. IL-10 responses to DFTD cells peaked as a secondary response rather than during the priming phase of the immune response. However, the significance may be that previously activated CTLs increase their IL-10 receptors and become reactivated rather than suppressed by IL-10 in secondary responses and this can function against the tumour (Emmerich et al 2012).

IL-10 also promotes the activation and proliferation of antigen-specific B cells (Singh et al 2011). The timing of IL-10 upregulation in the primary response was consistent with the production of IgG antibodies against DFTD cells in the mice. It can therefore be concluded that the primary response is a balance between T_H1 and T_H2 cytokines with dominance by IFN-γ. However, secondary responses showed a strong upregulation of IL-10 that dominated the IFN-γ responses which had remained at similar levels to the primary response. This observation held true for secondary immunisations up to 57 days after the primary but was not observed in secondary immunisations 95 days after the primary. Secondary responses 95 days after the primary had returned to cytokine levels equivalent the primary responses. The relevance of this is the immune system is being primed by primary exposure to DFTD cells and the initial T_H1 response is being directed towards a T_H2 response which remains enhanced for at least 57 days but has subsided by 95 days.

The exact roles of IFN-γ and IL-10 in DFTD cell rejection by immunocompetent mice are not fully understood. Nevertheless, the significance of these observations in the mouse is to suggest that the study of IFN-γ and IL-10 responses in the Tasmanian devil should become a priority. This would require the production of anti-devil IFN-γ and anti-devil IL-10 antibodies which are currently not available.

3.5.4 **Cytotoxic responses**

Cytotoxicity responses were evaluated using *in vitro* cytotoxicity assays to see if cytotoxic responses against DFTD cells could be detected and if primed splenocytes were more effective than naïve splenocytes. This required the optimisation and validation of a non-radioactive assay suitable for DFTD cells since DFTD cells label poorly with ^{51}Cr. This meant the ^{51}Cr assay, which is regarded as the gold standard cytotoxicity assay (Kane et al 1996), was not optimal for studying DFTD cells. As well ^{51}Cr is expensive, has a short half life and has inherent safety concerns associated with the use and disposal of radioactive isotopes.

The use of a fluorometric assay based on propidium iodide to determine NK cell function has been validated for clinical use and has been found to be a viable alternative to ^{51}Cr assay. Fluorometric assays have the advantages that they can be standardised between laboratories and since not all research facilities are licensed to for radioactive isotope use of fluorometric assays have more universal application (Kane et al 1996). Labelling target cells with CFSE or CellTrace Violet™ allowed discrimination from unlabelled effector cells. Then following the required incubation period a dead cell marker such as propidium iodide (PI) or 7AAD discriminated viable and dead cells and flow cytometry facilitated evaluation of cytotoxicity.

In our laboratory, further validation for this fluorometric cytotoxicity assay was undertaken using the human leukaemia cell line K562, a target for human NK cells and YAC-1 cells, a target for murine NK cells. There was a dose-response curve for both these cell lines. Then using YAC-1 cells a direct comparison of ^{51}Cr assay and the fluorometric assay revealed that the fluorometric assay was not inferior to the ^{51}Cr assay and would be a sensitive and robust cytotoxicity assay that could be adapted to DFTD cells.

Cytotoxic responses against DFTD cells by mouse splenocytes did not provide evidence for CTL activation as splenocytes from DFTD immunised mice produced the same level of cytotoxicity as splenocytes from naïve mice. This is not unexpected as CTL responses are MHC dependent and Tasmanian devil MHC would be too foreign to bind mouse CD8. It is more likely that the observed cytotoxicity may have been mediated by a combination of APCs, NK cells, NKT cells or unprimed T cells responding to xenogeneic determinants (Fox et al 2001). In light of these results it is

unlikely that an immunisation protocol in mice would enhance the cytotoxic responses against DFTD.

3.5.5 Comparison of BALB/c and C57/BL6 mouse strains

Two strains of immunocompetent mice were selected for the DFTD mouse model based on their reportedly opposing T_H1 (C57/BL6) and T_H2 (BALB/c) dominated immune responses (Mills et al 2000, Reiner and Locksley 1995) . The comparison of both immunised and naïve BALB/c and C57/BL6 mouse cytokine responses revealed no significant difference between the strains.

Further evaluation of the T_H1 T_H2 responses in BALB/c and C57/BL6 mice involved the analysis of IgG isotypes, which discriminate between T_H1 and T_H2 responses (Schulte et al 2008). Both strains of mice expressed high levels of IgG1, which is regarded as a T_H2 response. They also expressed IgG2a, IgG2b and IgG3 which are regarded as T_H1 cytokines. There was some skewing towards IgG2a in the BALB/c mice and towards IgG2b in the C57BL/6 mice, but this is a strain specific observation and not necessarily due to exposure to DFTD cells. The relevance of these findings is that the DFTD cells do not polarise either mouse strain towards a T_H1 or T_H2 immune response.

The antibody and cytokine responses for both genders were compared in BALB/c mice and there was no gender bias in the responses. The conclusions that can be drawn from these observations is that both strains and genders are equally suitable for experiments studying immune responses to DFTD cells.

3.5.6 Inactivating DFTD cells for vaccine trials

Vaccine trials and immunotherapy experiments with Tasmanian devils require the injection of inactivated DFTD cells to induce immune responses without the risk of infection. The BALB/c DFTD mouse model facilitated direct comparison of the immunogenicity of DFTD cells inactivated by gamma radiation, sonication or freeze-thawing and compared these with viable DFTD cells.

Freeze-thawing and sonication significantly reduced the immunogenicity of DFTD cells. This was evident in reduced levels of IgG and IgM antibodies and suppression of the anti-tumour cytokines IFN-γ and TNF-α. Inactivation of DFTD cells by

irradiation did not reduce antibody or cytokines responses and therefore would the better method of inactivating DFTD cells for vaccine and immunotherapy trials.

3.5.7 **Conclusions**

The main findings of this chapter are that DFTD cells are immunogenic, undergo apoptosis, can be targeted and killed by immune systems. Both BALB/c and C57/BL6 mice of either gender can be used similarly for studying immune responses against DFTD. Cytotoxic responses against DFTD cells by mice did not reveal evidence for CTL activation and were most likely mediated by unprimed cells. The most robust method of detecting immune responses in mice was by serum antibody levels specific for DFTD. The use of intraperitoneal prime-boost immunisations in the mice produced the most reliable immune responses and may prove more effective than subcutaneous immunisations in the Tasmanian devil.

Cytokine and antibody responses against DFTD demonstrated a balance of T_H1 and T_H2 responses showing the mouse immune system is not being polarised following immunisation with DFTD cells. The development of anti-devil IFN-γ and IL-10 antibodies to study the role of T_H1 and T_H2 immune response in Tasmanian devils should be a priority. The inactivation of DFTD cells for vaccine and immunotherapy trials would be best done using gamma radiation as it has no significant impact on the immunogenicity of the cells.

Immunocompromised mouse models to evaluate DFTD establishment, adoptive protection and xenograft rejection

Immunocompromised mouse models to evaluate DFTD establishment, adoptive protection and xenograft rejection

4 Immunocompromised mouse models to evaluate DFTD establishment, adoptive protection and xenograft rejection

4.1 Introduction

Devil Facial Tumour Disease is an infectious cancer. It could be described as an infectious parasitic clonal cell line that survives as an allograft transmitted between Tasmanian devils. There is only one other infectious cancer cell line known to exist in nature and that is CTVT. There have been very few laboratory models used to study these transmissible cancers. A NOD/SCID murine xenograft model has previously been used to study CTVT (Harmelin et al 2001). Models of this kind are essential to further understand the pathology or epidemiology of such diseases.

The NOD/SCID model is useful for studies concerning anti-tumour immunity and tumour progression in CTVT. The model provides a means to test treatments, chemotherapy and immunotherapy in a biologically relevant setting. Advantages include cost, reproducibility, analogy to natural disease and decreased need for dogs to study CTVT (Harmelin et al 2001, Rivera et al 2005).

NOD/SCID mice were developed by crossing the SCID mutation into the NOD background. Due to genetic mutations SCID mice lack functional B and T cells and do not produce functional immunoglobulin and T cell receptors. NOD mice have immune defects including low NK cell activity, defective macrophages and a deficiency in the C5 component of complement system (Shultz et al 1995, Vormoor et al 2001). These combined defects in the NOD/SCID mouse prevented any immunity to CTVT and facilitated an *in vivo* model that reproduced the main features of tumour establishment, progression and metastasis (Harmelin et al 2001).

In this chapter the NOD/SCID mouse model was applied to the study of DFTD because of DFTD similarities to CTVT. There were three main objectives in this chapter. The first was to study DFTD establishment in NOD/SCID mice as an alternative to using Tasmanian devils. The second was to evaluate adoptive protection of NOD/SCID mice by transfer from immunocompetent mice. The third was to identify cells and mechanisms of rejection by immunocompetent mice through adoptive transfer to genetically modified mice.

DFTD is highly infectious but the number of cells needed to be transmitted between Tasmanian devils to establish tumours in the new host is unknown. The period of disease latency between an individual becoming infected to them being infectious is also uncertain. This information is important to biologists trying to model and manage the spread of the disease in Tasmanian devils (Hamede et al 2012a). However, it is not practical to comprehensively investigate this in the Tasmanian devil due to their endangered status. Such questions can be investigated using the NOD/SCID mouse model.

The inability of Tasmanian devils to mount an immune response against DFTD is not fully understood. The previous chapter of this thesis used immunocompetent mice to demonstrate that DFTD cells are immunogenic and can be killed by the immune system. Further understanding the mechanisms by which the mice rejected the DFTD cells may lead to a revelation of mechanisms which can be exploited in the Tasmanian devil.

To study biological mechanisms associated with tumour rejection, the NOD/SCID mouse has been widely used because of its ability to accept adoptive transfer of immune cells from mice and other species including humans (Belizário 2009, Feuerer et al 2001, Xue et al 2005) .This chapter explored the possibility of co-transplanting lymphocytes from immunocompetent mice and DFTD cells into NOD/SCID mice to study the effect of competent immune cells in rejecting DFTD cells. The adoptive transfer experiments were augmented by evaluation of the ability of genetically modified mice with specific defects to reject or engraft DFTD tumours.

4.2 **Results**

4.2.1 **DFTD xenograft model to study disease establishment**

In the previous chapter, immunocompetent BALB/c and C57/BL6 mice were shown to consistently reject DFTD cells (Table 3-2). This rejection was a specific immune response that produced anti-DFTD antibodies and cytokines such as IFN-γ, TNF-α, IL-6 and Il-10. *In vitro* analyses revealed killing by unprimed cells. In comparison, the work presented in this chapter demonstrates NOD/SCID mice did not reject the tumour. This is most likely because of their compromised immune system, which is absent of T cells, B cells, macrophages, DCs and functional NK cells (Belizário 2009, Lapidot et al 1997). NOD/SCID mice therefore provide an *in vivo* platform to study DFTD in a xenograft setting. This mouse model will provide an alternative to *in vitro* assays to advance our understanding of the kinetics of infection including disease latency. The model would also facilitate determination of the minimum number of cells required to infect a new host, if there is a direct dose response and provide indication to the shortest and longest time to clinical manifestation following implantation. Such information is not possible with *in vitro* assays. The DFTD cells can be implanted subcutaneously and typically develops as a nodule that can be palpated and measured non-invasively in a living mouse (Figure 4-1). The DFTD tumour can be removed at necropsy for measurement of size, mass or fluid displacement.

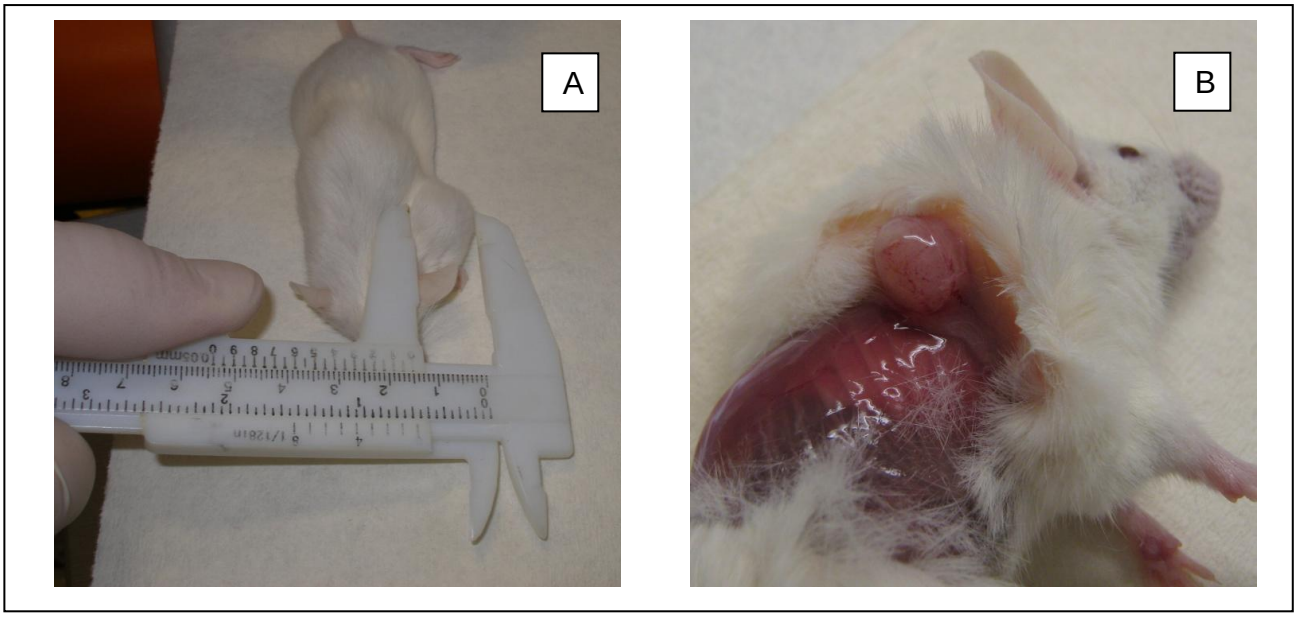

Figure 4-1. Subcutaneous DFTD xenograft in NOD/SCID mouse being measured with Vernier calipers (panel A). Typical subcutaneous DFTD xenograft growing as a nodule (panel B).

4.2.2 Kinetics of DFTD xenograft establishment

The period to detection and the minimum number of cells required to consistently establish DFTD xenografts in NOD/SCID mice was unknown. As a consequence, NOD/SCID mice were injected with DFTD cells ranging from 2.5 x 10^3 to 1 x10^6 cells and for up to 20 weeks the mice were monitored by palpation for the first sign of tumour development.

Palpation of tumours as small as 2mm diameter was the first evidence of DFTD xenograft establishment. In the absence of any pre-clinical marker this was the best available method to measure earliest sign to detection. There was a direct correlation between the number of DFTD cells injected and the period latency. The number of days to detection increased as the number of cells injected decreased. Implantation of 10^6 cells resulted in the establishment of DFTD xenografts within 32 days and when 10^4 cells were implanted this was up to 99 days (Figure 4-2).

To determine the minimum number of cells required to ensure xenograft establishment all mice that failed to develop DFTD tumours were carefully examined in autopsy to confirm that the tumour had not established in cryptic locations. Xenograft failure was observed in four of 28 mice injected with 10^4 DFTD cells or less. Xenografts were established in all mice implanted with 5 x 10^4 DFTD cells (n=5) and 2.5 x 10^4 DFTD cells (n=17). Xenografts also established in 30 of 31 mice implanted with 1 x10^5 DFTD cells and all mice implanted with 10^6 DFTD cells (n=18) (Figure 4-2).

The more cells injected the more consistent the time to tumour development. Decreasing the number of cells injected increased the chance of xenograft failure. Time to detection ranged, in a dose dependent response, from 17 days with 10^6 cells to 130 days with 10^5 cells. From this it was determined that 10^6 cells should be injected to ensure tumour engraftment and fast tumour establishment.

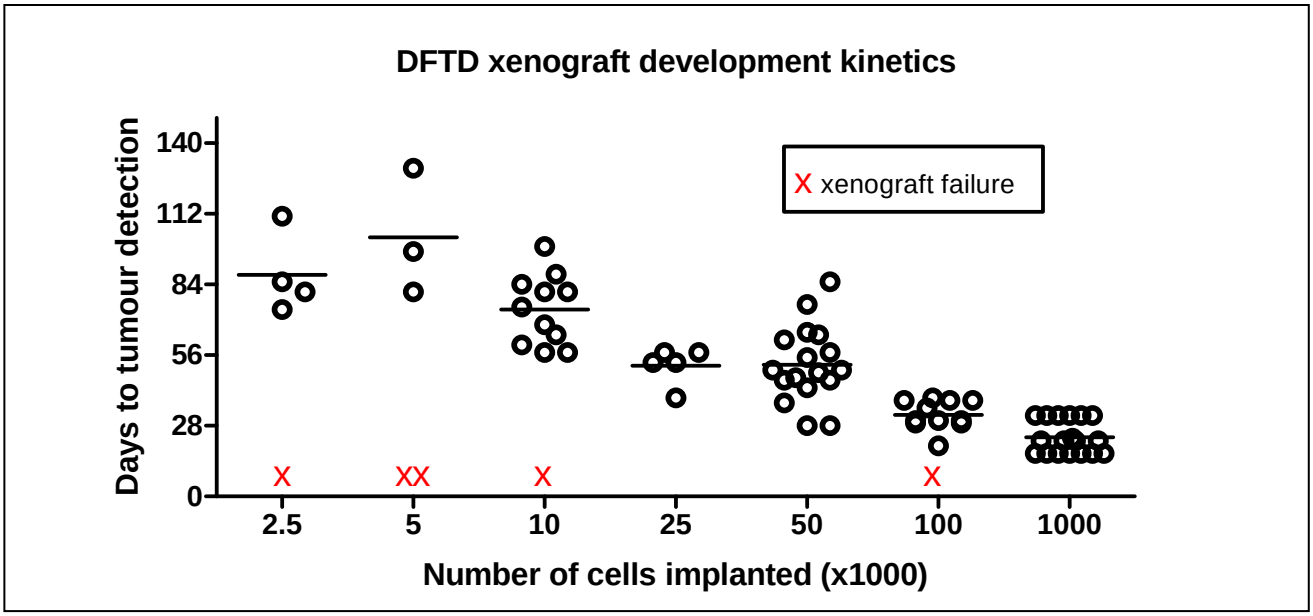

Figure 4-2. Varying quantities from 2.5 x 10^3 to 1 x 10^6 C5065 DFTD cells were SC injected into NOD/SCID mice. The mice were monitored by visualisation and palpation for tumour development. The day of first detection was recorded. Horizontal lines represent mean and red crosses failure to establish xenograft.

4.2.3 C57/BL6 splenocyte adoptive transfer to NOD/SCID mice

To extend our understanding of DFTD rejection by immunocompetent mice, splenocytes from DFTD immunised C57/BL6 mice were adoptively transferred to NOD/SCID mice to determine if the immune system could be partially reconstituted and an immune response against DFTD established. The splenocytes obtained from whole spleens were combined with 10^6 DFTD cells and injected subcutaneously into eleven NOD/SCID mice. Within 11 days the recipient mice showed symptoms consistent with graft versus host (GVH) rejection. As a consequence all mice were euthanised within 11 to 18 days of rejection; serum was collected and tested for DFTD specific antibodies.

Eight of the 10 NOD/SCID mice had varying levels of DFTD specific IgG antibodies detectable in their serum (Figure 4-3). The only IgG isotype detected was IgG1; IgG2a, IgG2b and IgG3 were not detected (Figure 4-4).

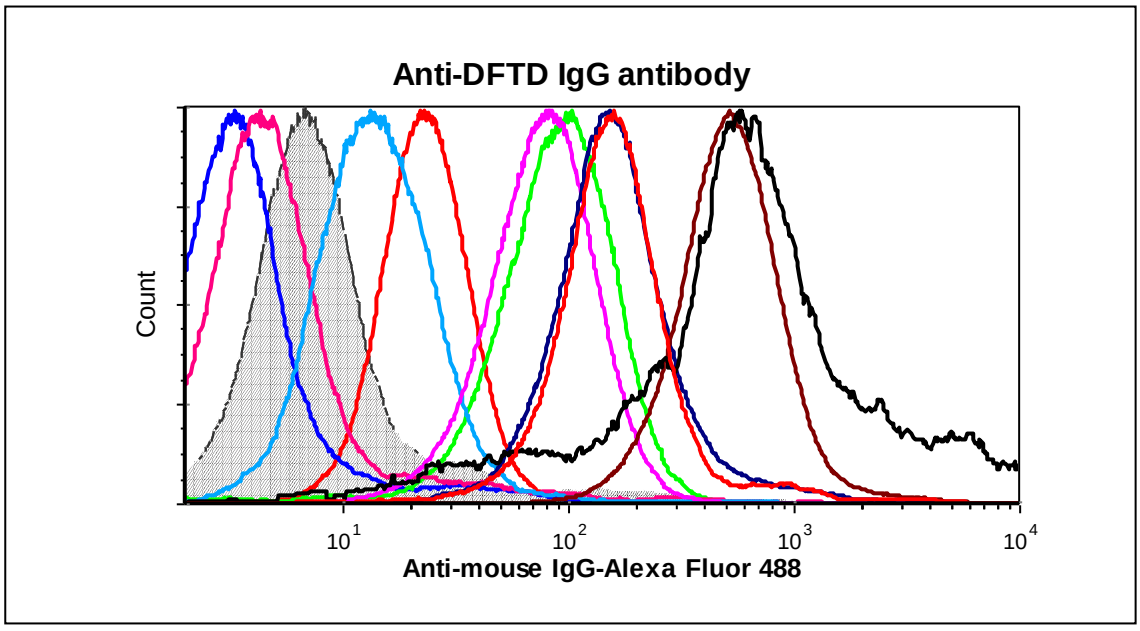

Figure 4-3. NOD/SCID mice were injected with splenocytes from immunised C57/BL6 mice and 10^6 C5065 DFTD cells. Serum was collected between day 11 and day 18 after reconstitution and compared to serum from a naïve C57/BL6 mouse (indicated in grey shading). Eight of 10 mice produced DFTD specific IgG antibodies.

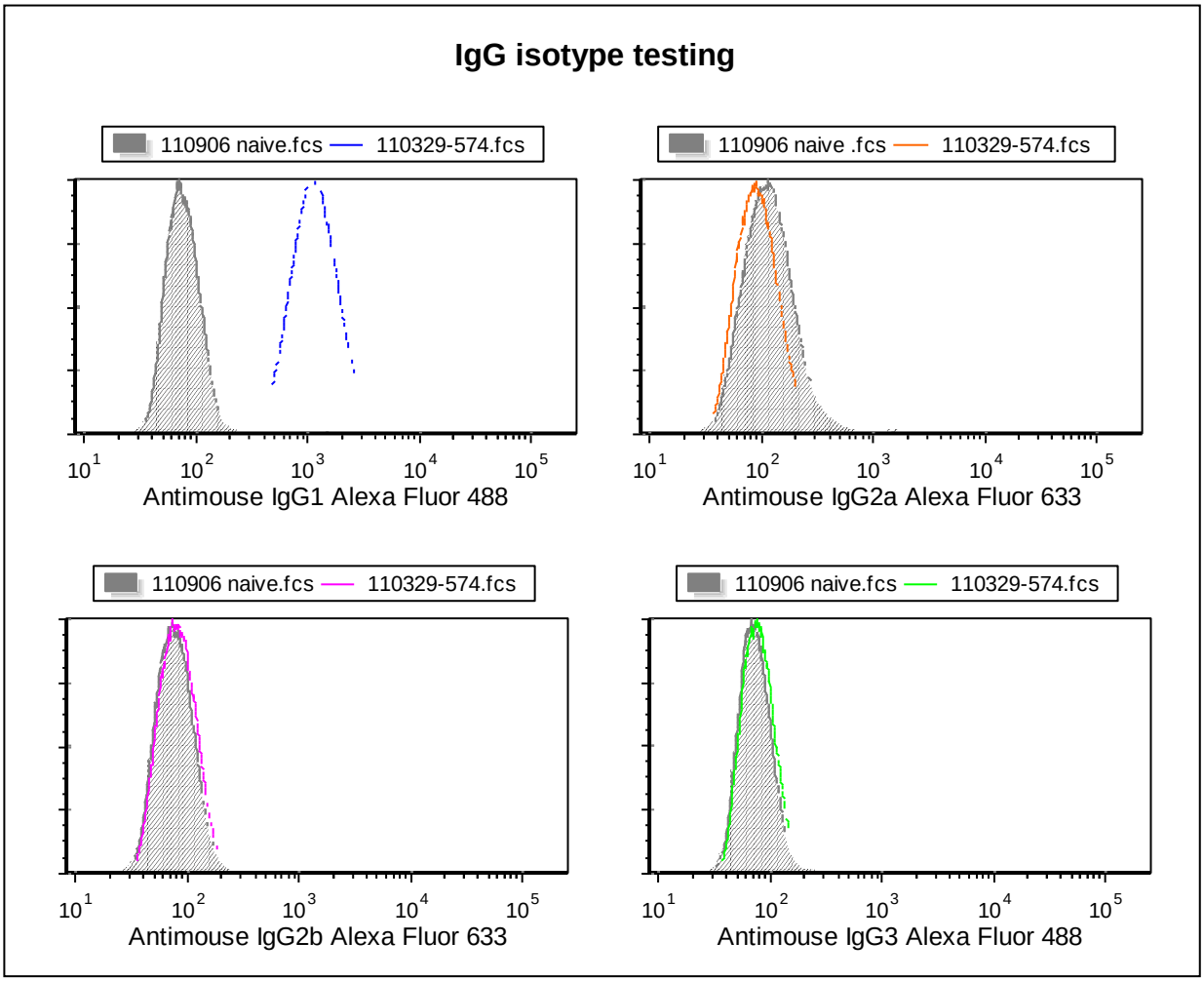

Figure 4-4. Alexa Fluor conjugated anti-mouse antibodies were used to screen serum collected from NOD/SCID mice, which had been reconstituted with splenocytes from an DFTD immunised C57/BL6 mouse. Plots shown are representative of responses showing a serum that tested positive for IgG_1 isotypes but negative for IgG2a, IgG2b and IgG3 isotypes.

4.2.4 **BALB/c splenocyte adoptive transfer to NOD/SCID mice**

C57/BL6 donor mice had proven unsuitable for adoptive transference of splenocytes to NOD/SCID mice because of GVH rejection. BALB/c mice splenocytes were subsequently used and did not promote GVH rejection in NOD/SCID mice. Both naïve and immunised BALB/c splenocytes were compared. Furthermore, splenocytes from immunised mice were used with and without the addition of autologous serum and CpG 2395.

Splenocytes from 14 naïve BALB/c mice were used for adoptive transfer to NOD/SCID mice. Firstly this involved adoptive transfer to four NOD/SCID mice and 3 of 4 developed tumours. Tumours were not detected before 88 days which suggests delayed tumour growth compared to the DFTD xenograft development kinetics discussed in Figure 4-2. In a second experiment transferring naïve splenocytes from BALB/c mice to NOD/SCID mice 10 of 10 mice did not develop tumours. Splenocytes from immunised BALB/c mice prevented tumour establishment in 15 of 15 mice. This included cells alone or cells plus either immune serum or CpG. All control mice that received only DFTD cells established tumours (n=10) (Table 4-1).

Table 4-1. Adoptive transfer of splenocytes from BALB/c to NOD/SCID mice. NOD/SCID mice received splenocytes from naïve or immunised BALB/c mice as indicated co-injected with 10^6 DFTD cells. As indicated some received 50 µl serum from the same immunised BALB/c mice and some mice also received the adjuvant CpG 2395. Mice were monitored up to 20 weeks or until tumours were observed.

NOD/SCID mice injected with 10^6 DFTD cells plus	Challenged mice	Tumour development
Splenocytes from naïve BALB/c mice	n=14	Tumours n=3/14 No tumours n=11/14
Splenocytes from immunised BALB/c mice	n=5	No tumours n=5
Splenocytes and serum from immunised BALB/c mice	n=5	No tumours n=5
Splenocytes and serum from immunised BALB/c mice plus CpG 2395	n=5	No tumours n=5
DFTD cells only with no serum, splenocytes or CpG	n=10	Tumours n=10

To demonstrate that rejection of DFTD cells in NOD/SCID mice following adoptive transfer of donor splenocytes from BALB/c mice was a specific immune response two NOD/SCID mice were subcutaneously co-injected with splenocytes from immunised BALB/c mice and 10^6 DFTD cells. After 33 days the mice were sacrificed to collect the spleens and serum. The mice had no detectable tumours. The splenocytes were used for an *in vitro* cytotoxicity assay against DFTD cells. The serum was analysed for IgM, IgG1, IgG2a, IgG2b and IgG3 DFTD specific antibodies.

The cytotoxicity assay demonstrated a dose response curve following 18 hours *in vitro* incubation and anti-DFTD IgG1 antibody was detected in both mice (Figure 4-5) but IgM, IgG2a, IgG2b and IgG3 isotypes were not detected (data not shown). These results demonstrate a DFTD specific immune response following adoptive transfer of BALB/c splenocytes.

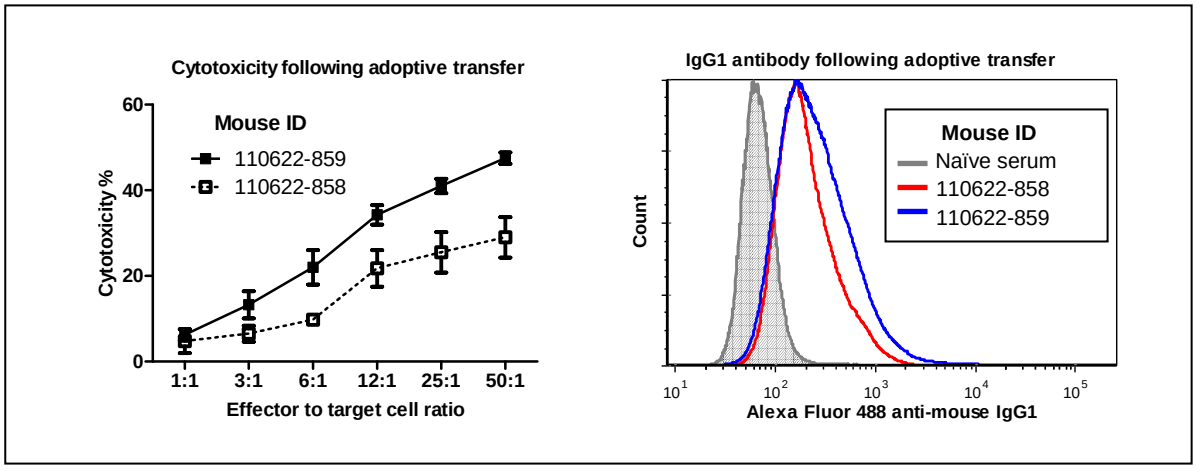

Figure 4-5. Two NOD/SCID mice received splenocytes from DFTD immunised BALB/c mice co-injected subcutaneously with 10^6 C5065 DFTD cells. The mice were sacrificed after 33 days and had no detectable tumours. The splenocytes were recovered and incubated for 18 hrs *in vitro* with CFSE labelled C5065 DFTD cells. (Each data point represents the mean of four replicate wells and error bars the SEM). Serum was collected and screened for IgG1 antibodies specific to DFTD. The same serum had no detectable IgM, IgG2a, IgG2b or IgG3 (data not shown).

Adoptive transference of protection to NOD/SCID mice could be achieved when splenocytes were co-transplanted with DFTD cells. However, it was unknown if naïve splenocytes could be induced *in vivo* to produce anti-DFTD immune responses following transfer to NOD/SCID mice if they were not co-injected with the DFTD cells. Consequently, NOD/SCID mice were injected with 2×10^6 DFTD cells either seven days prior to or seven days after adoptive transfer of naïve BALB/c splenocytes. There was no evidence of an immune response to DFTD cells injected at a different time point. Tumours developed in all eight mice and no antibodies were detected in their serum (Figure 4-6).

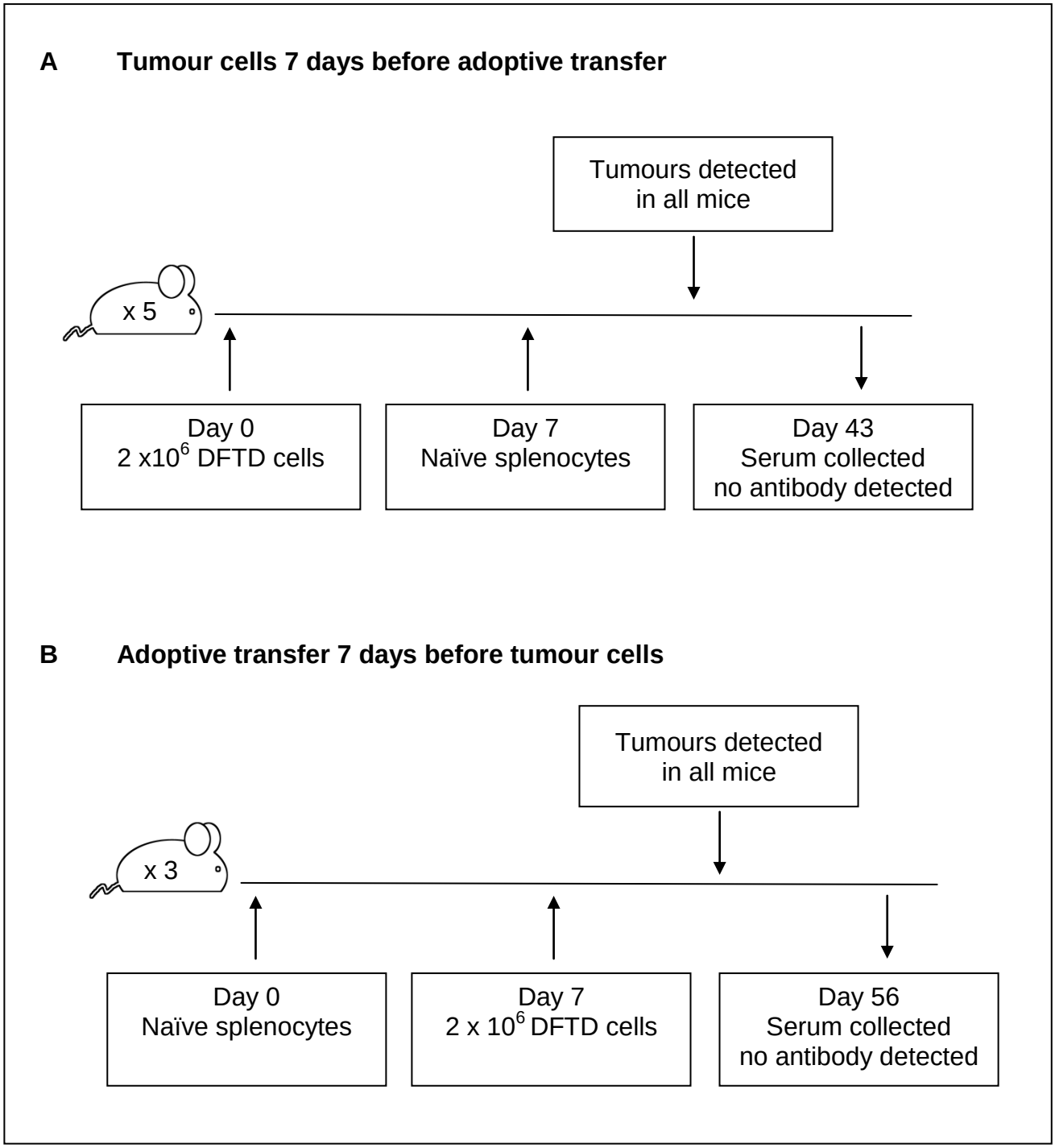

Figure 4-6. Adoptive transfer of splenocytes from naive BALB/c mice to NOD/SCID mice (A) schema of five NOD/SCID mice which were injected subcutaneously with 10^6 DFTD cells followed seven days later with adoptive transfer of splenocytes from naïve BALB/c mice . (B) schema of three NOD/SCID mice which had adoptive transfer of splenocytes from naïve BALB/c mice followed seven days later by subcutaneous injection of 2 x 10^6 DFTD cells. All mice grew tumours and no anti-DFTD specific antibodies were detected in their serum which was collected at necropsy.

4.2.5 **Adoptive enrichment and depletion splenocyte transfer trials**

Splenocytes transferred from DFTD immunised BALB/c mice to NOD/SCID mice consistently protect against DFTD tumour engraftment. To investigate if a specific population of cells such as NK, NKT, $CD4^+$ or $CD8^+$ cells was responsible for protection these populations were either enriched or depleted using either magnetic bead separation or flow cytometric cell sorting. The enriched or depleted populations were then co-injected subcutaneously into NOD/SCID mice with 5×10^4 DFTD cells. Control mice were injected with DFTD cells of the same number from the same culture.

EasySep™ magnetic bead sorting kits were used to establish enriched and depleted populations of CD4, CD8 or NK cells as stated in methods. Purity was at least 85% and checked using flow cytometry detecting CD4, CD8 or CD49b (NK cells) for the respective cell types being enriched or depleted. Both the enriched and depleted populations from magnetic bead sorting prevented establishment of DFTD tumours in treated mice while all the controls established tumours (Table 4-2).

Flow cytometry sorting was used to establish enriched and depleted populations of CD4, CD8, CD49b or NKT cells. CD4 and CD8 cells were positively selected based on their respective CD marker combined with CD3e T cell marker. NK cells were positively selected for using CD49b and NKT cell subset was selected for using CD49b and CD3e.

Flow cytometry provided at least 95 % purity. Populations depleted of just CD49b (n=8), $CD4^+$ T cells (n=4) or $CD8^+$ T cells (n=4) all rejected the DFTD cells. Enriched populations of $CD8^+$ T cells (n=6) and NKT cells (n=3) all rejected the DFTD cells. However, 3 of 16 CD49b enriched and 1 of 5 $CD4^+$ T cell enriched populations was not protective against the DFTD cells and tumours established. This result suggests that CD49b and $CD4^+$ T cells are less effective than $CD8^+$ T cells at protecting the recipient mice from DFTD cells.

Table 4-2. Adoptive transference of enriched and depleted splenocyte populations. Immunised splenocytes from BALB/c mice had specific populations enriched or depleted by magnetic bead separation or using an Astrios cell sorter. These cells were then co-injected with 5 x 10^4 DFTD cells. Control mice were also injected with the same number of DFTD cells only.

	Donor splenocytes	Observation
	Magnetic bead enrichment/depletion	
NK	NK enriched (>85% purity)	Rejected DFTD cells (n= 5/5)
	NK depleted (>85% purity)	Rejected DFTD cells (n=5/5)
	Controls	Tumours (n= 3/3)
	Magnetic bead enrichment/depletion	
CD4	CD4 enriched (>85% purity)	Rejected DFTD cells (n= 5/5)
	CD4 depleted (>85% purity)	Rejected DFTD cells (n=5/5)
	Controls	Tumours (n= 4/4)
	Magnetic bead enrichment/depletion	
CD8	CD8 enriched (>85% purity)	Rejected DFTD cells (n= 5/5)
	CD8 depleted (>85% purity)	Rejected DFTD cells (n=5/5)
	Controls	Tumours (n= 5/5)
	Flow cytometry cell sorter enrichment/depletion	
CD49b	CD49b enriched (>95% purity)	Rejected DFTD cells (n= 13/16) Tumour (n=3/16)
	CD49b depleted (>95% purity)	Rejected DFTD cells (n=8/8)
	Controls	Tumours (n= 16/16)
	Flow cytometry cell sorter enrichment/depletion	
CD4	CD4 enriched (>95% purity)	Rejected DFTD cells (n= 4/5) Tumour (n=1/5)
	CD4 depleted (>95% purity)	Rejected DFTD cells (n=4/4)
	Controls	Tumours (n= 5/5)
	Flow cytometry cell sorter enrichment/depletion	
CD8	CD8 enriched (>95% purity)	Rejected DFTD cells (n= 6/6)
	CD8 depleted (>95% purity)	Rejected DFTD cells (n=4/4)
	Controls	Tumours (n= 5/5)
	Flow cytometry cell sorter enrichment	
NKT	NKT cell enriched (>95% purity)	Rejected DFTD cells (n=3/3)
	Controls	Tumours (n= 3/3)

4.2.6 **Evaluating DFTD engraftment in congenic and knockout mouse strains**

To further investigate which murine immune cells protect against the DFTD cells congenic and knockout mice were challenged with DFTD cells and monitored for tumour growth or rejection. Ighm/J, a B cell knockout strain, rejected 10^6 DFTD cells (Table 4-3). A TNF knockout strain, B6.TNF, rejected 10^6 DFTD cells (Table 4-3). DFTD tumours established in the B and T cell knockout strain, Rag/2 (n= 8/10) and the congenic T cell deficient strain, CBA/nu mice (n=9/9) following a challenge with 10^6 DFTD cells (Table 4-3). Significantly, the tumours in the Rag/2 mice grew to one-tenth the size of the tumours of the athymic CBA/nu mice within the same time period (Figure 4-7). Both strains of mice had been injected with the same number of cells from the same cell culture on the same day.

Table 4-3. DFTD challenge of knockout mice strains. Genetically modified mice were injected subcutaneously with 10^6 DFTD cells and monitored for up to 20 weeks or until tumours developed.

Strain	Deficiency	Observation
Ighm/J	B cell knockout	Rejected 10^6 DFTD cells (n=5/5)
B6.TNF	TNF knockout	Rejected 10^6 DFTD cells (n=10/10)
Rag/2	B cell and T cell knockout	Small tumours in 8/10 mice at day 52
CBA/nu	T cell deficient	Tumours in 10/10 mice at day 53

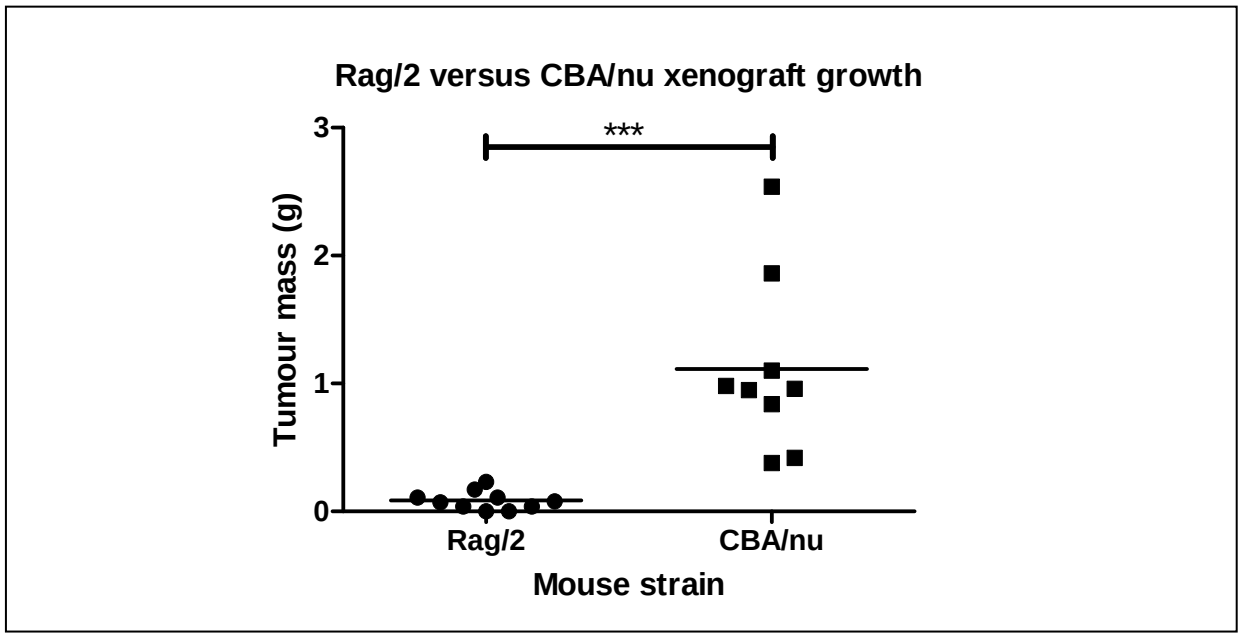

Figure 4-7. 10 Rag/2 and 9 CBA/nu mice were injected with 10^6 identical C5065 cells at the same time. They were sacrificed on day 52 and 53 respectively. The xenograft established in 9/9 Nude mice but only 8/10 Rag mice. Furthermore the Nude mice grew tumours more than tenfold larger. (Horizontal bars represent mean and probability calculated by unpaired two-tailed t test *** P < 0.001)

4.2.7 **Rag/2 versus CBA/nu mice immuno-phenotyping**

The significant ($P < 0.001$) size difference between DFTD xenografts grown in Rag/2 and CBA/nu mice over the same time period suggested that the Rag/2 mice have an immune response that inhibits tumour growth more than CBA/nu mice. Understanding the difference between the two strains would provide insight into the protective mechanisms used by immunocompetent mice to reject DFTD cells. Consequently, the immunophenotypes of the splenocytes obtained from these two strains were evaluated using flow cytometry. FITC anti-mouse CD3e antibodies confirmed that mature T cells were absent in both strains (Figure 4-8). APC anti-mouse CD49b antibodies confirmed both strains had NK cells but the percentage of NK cells was more than twice as high in the Rag/2 mice compared to the CBA/nu mice (Figure 4-8). PE/Cy7 anti-mouse CD19 antibodies confirmed that only the CBA/nu mice had mature B cells (Figure 4-9). PE anti-mouse MHC II antibodies confirmed both strains had APCs. However, the Rag/2 mice had more than twice the percentage of APCs compared to the CBA/nu mice (Figure 4-9).

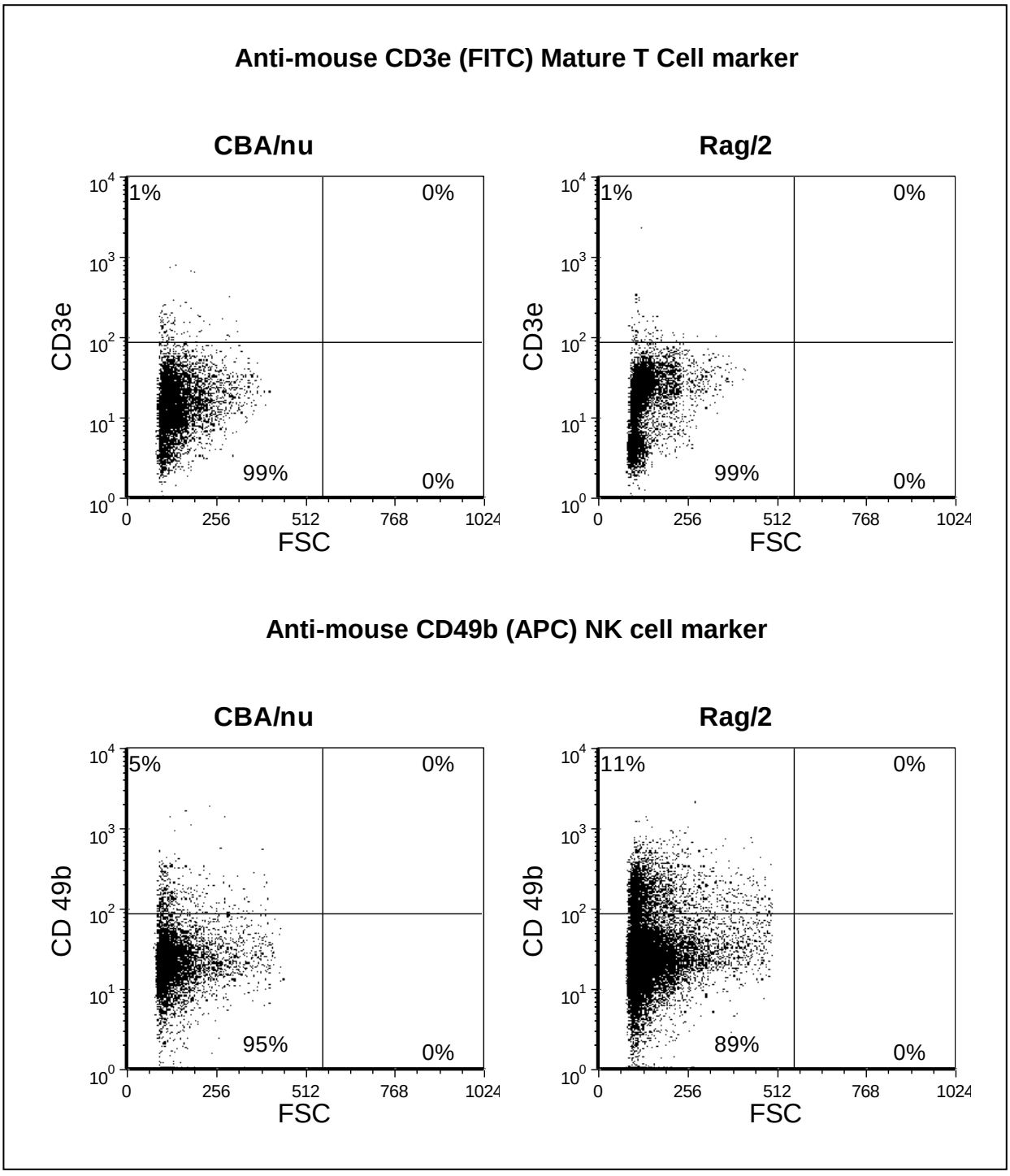

Figure 4-8. Splenocytes from CBA/nu and Rag/2 mice were labelled with FITC anti-mouse CD3e and APC anti-mouse CD49b and analysed by flow cytometry.

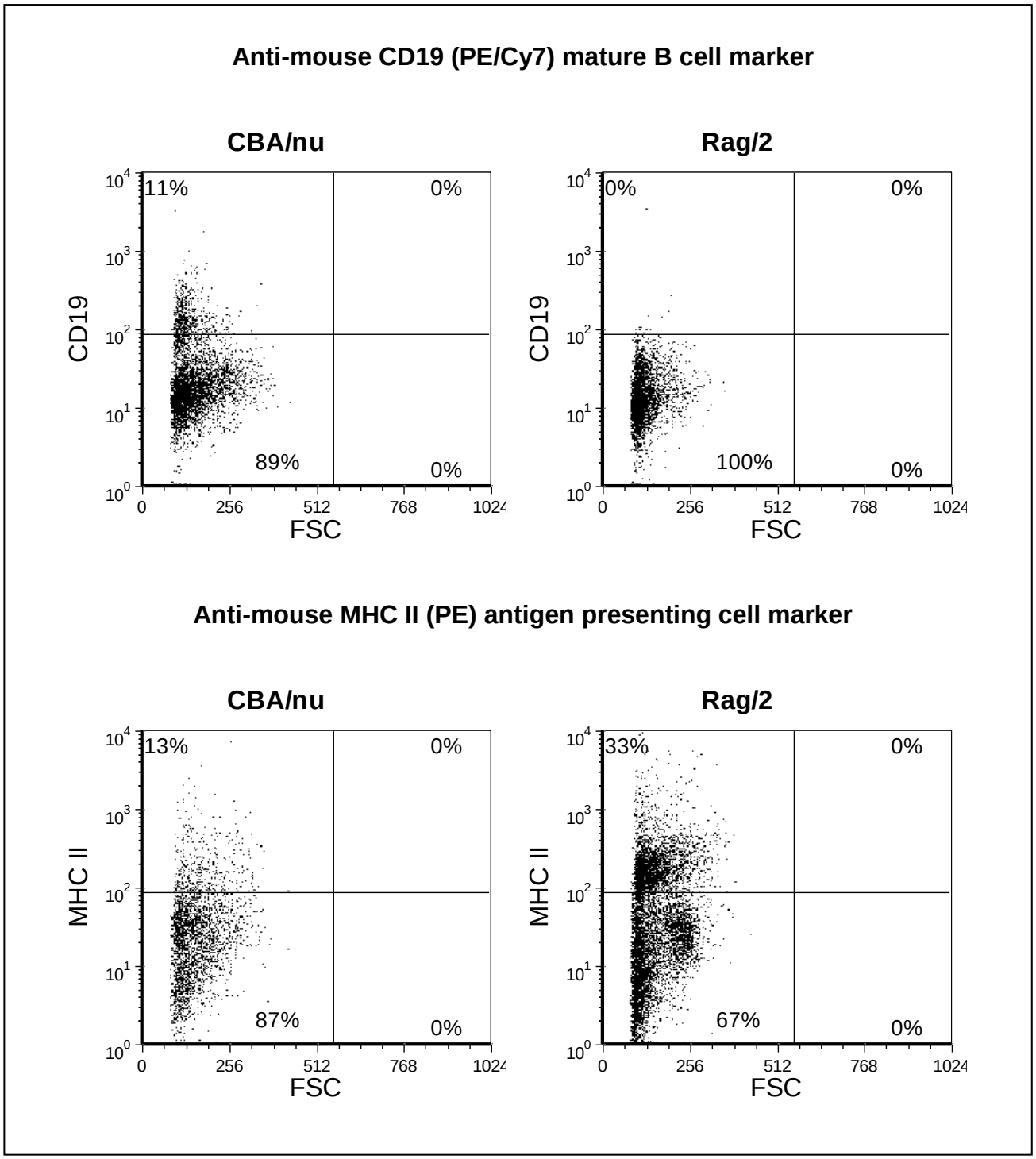

Figure 4-9. Splenocytes from CBA/nu and Rag/2 mice were labelled with PE/Cy7 anti-mouse CD19 and PE anti-mouse MHC II antibodies and analysed by flow cytometry.

4.3 **Discussion**

The previous chapter used BALB/c and C57/BL6 immunocompetent mice to demonstrate that DFTD cells are immunogenic and can be rejected by murine immune systems. In this chapter, NOD/SCID immunocompromised mice were used to evaluate various aspects of DFTD establishment to avoid the risk of harming endangered Tasmanian devils. An evaluation of adoptive transfer of protection from immunocompetent mice to the NOD/SCID mice was undertaken to reveal the components of the immune system affording protection to mice. Fluorescence-activated cell sorting (FACS) combined with genetically modified mice was used to further investigate which components of the immune system contribute towards the protection against DFTD in mice.

4.3.1 **DFTD establishment**

In vitro assays are indispensible tools for the study of cancer but some investigations require an *in vivo* setting. There is a wealth of evidence that many critical clinical developments such as drugs, treatments and cures for human diseases and cancer have been developed with the use of animal models (Sausville and Burger 2006, Suggitt and Bibby 2005). There are many aspects of disease that cannot be recapitulated in an *in vitro* setting and in this instance the establishment and progression of DFTD in the Tasmanian devil is one aspect. The environment of cells grown as a monolayer on a plastic substrate lacks the complexity of a dynamic three-dimensional *in vivo* environment, which includes epigenetic changes to gene expression that can alter tumour growth and influence results (Luca et al 2013). The limited access to Tasmanian devils and difficulties associated with housing them in a biosecure environment hindered *in vivo* studies in the host species. As a consequence mouse models were used as substitutes for Tasmanian devils to minimise the use of Tasmanian devils and complement *in vitro* studies.

NOD/SCID mice can be used as surrogates for Tasmanian devils as both have no known immune response to DFTD cells (Kreiss et al 2011, Siddle and Kaufman 2013). The Tasmanian devil immune system fails to recognise the DFTD cells while NOD/SCID mice lack an effective immune system to generate an immune response and also fail to recognise DFTD cells. NOD/SCID mice engraft DFTD tumours and provide a physiological microenvironment that preserves the three-dimensional

tumour structure with cell to cell interactions and angiogenesis that is biologically relevant for the study of DFTD establishment and growth.

Following subcutaneous injection of DFTD cells into NOD/SCID mice the first evidence of tumour development was the identification of a small nodule about the size of a pinhead (1-2 mm diameter) near the injection site. In some mice two or more nodules established near the site of injection. Metastatic disease was not observed in any of the mice injected, which suggests that the DFTD cells do not migrate from the injection site and proliferating cells clump together to create the nodules.

It was unknown how many DFTD cells are required to establish a tumour in NOD/SCID mice and if DFTD tumour development is cell dose-dependent. To evaluate this, DFTD cells ranging from 2.5×10^3 to 1×10^6 were subcutaneously injected into NOD/SCID mice. Tumours always established when 10^6 DFTD cells were injected but below these levels there was an increasing rate of mice without tumour establishment. This ranged from 3% when 10^5 cells were injected to 30% when 5×10^3 or fewer cells were injected. When 10^6 DFTD cells were injected tumours of approximately 2mm diameter were observed within 32 days in all mice. Reducing the number of cells implanted extended the period to detection and increased the probability of not establishing a DFTD tumour in the mouse.

One possible explanation for lack of tumour establishment at low cell numbers could relate to the cancer stem cell hypothesis. This hypothesis suggests that not all cancer cells are capable of self renewal and only a limited number of cancer stem cells drive tumour growth and development (Clevers 2011). The results in the mouse model suggest only a small percentage of the cells transferred were responsible for tumour establishment. While this result is consistent with the hypothesis of cancer stem cells, this has not been investigated in the cultured DFTD cell lines used in these trials.

An alternative explanation to account for the small percentage of cells that establish as tumours may be that all DFTD cells are capable of proliferation but there is a reduced rate of engraftment as a consequence of the xenogeneic environment. Tumour cell growth requires synergy with supporting cells such as fibroblasts, endothelial cells, mesenchymal cells and tumour infiltrating macrophages (Rahman

et al 2011). CTVT cells are known to express TGFβ1 which suppresses the immune system, induces proliferation of the surrounding stromal cells and promotes angiogenesis (Morris and Belov 2013). If a similar scenario occurs with DFTD it is unlikely that the cytokines and receptors that promote these interactions are compatible between mouse and Tasmanian devil. Increased DFTD cell densities may overcome these barriers by promoting cell to cell signalling between the DFTD cells.

It is unknown if the observations concerning the cell numbers associated with DFTD establishment and period of latency until detection in mice reflects the situation with the Tasmanian devils. In the wild the disease appears to be spread by biting between devils with cells being transmitted into cuts and open wounds in the oral cavity (Hamede et al 2013). There have been no published data on the number of DFTD cells transferred between individuals as a result of biting. It would be reasonable to presume that just hundreds or at most a few thousand cells are transferred. It therefore appears likely that just a few cells in a Tasmanian devil are sufficient to establish a DFTD tumour.

While there is a paucity of evidence to support this statement there is a single anecdotal case of a wild devil in captivity developing DFTD more than 40 weeks after capture (Hamede et al 2013). In preliminary transmission trials undertaken by Department Primary Industries Parks Water Environment (DPIPWE) it was shown that 1 x10^6 DFTD cells established tumours between 2 and 4 weeks (Kreiss 2009). In trials utilising 25,000 DFTD cells tumour establishment took between 12 to 38 weeks (Kreiss 2009). Explanations for the long latency period could be a slow replicating strain of DFTD or host resistance. But in light of the observation that tumour development is cell dose-dependent in mice it is likely that the wild devil was infected with a very low number of cells and following engraftment cell replication was exponential and it took ten months for the tumour mass to be large enough for detection. The seasonality of disease detection in the wild combine with field observations has been extrapolated to suggest a latency period of 6 to 9 months in the wild population subsequent to transmission (Hamede et al 2013).

To guarantee the maximum number of mice that develop tumours this study revealed that at least 10^5 or 10^6 DFTD cells need to be injected. Cell numbers of 10^5

or less were transferred with the intention of establishing tumours with a small number of cells that could be targeted effectively in cell transfer experiments. This resulted in 3% to 60% of NOD/SCID mice not establishing tumours while 10^6 DFTD cells always resulted in tumour establishment. Too high a number of cells will overwhelm the immune system and prevent cell transfer experiments efficacy. Conversely too low a number will see an increasing number of failures to engraft tumours which will confound the results of cell transfer treatments. To overcome this, the number of replicate mice for each experiment would have to be substantially increased to ensure statistical significance between control and treatment groups.

Another negative aspect of reducing the number of DFTD cells injected is the increased time to tumour detection and which extends the duration of the experiments. In the mouse model a one hundred-fold decrease in cell numbers extended the time to detection by three-fold. The longest time to detection in the mice was 81 to 130 days when 5000 cells were transferred. Such long latency times are detrimental with NOD/SCID mice since they are prone to develop metastatic thymic lymphomas by 20 weeks of age (Shultz et al 1995). The implications for experimental design are that young mice 5 to 8 weeks old need to be selected at the commencement of experiments and duration of experiments should be no greater than 12 weeks.

There is an unknown period when disease is undetectable because of a lack of pre-clinical markers or antibody responses to identify infected Tasmanian devils. For the purpose of disease management and monitoring in the wild defining this period between exposure until detection would be useful. Understanding the period of latency would benefit decisions about how often diseased animals need to be culled from the populations to interrupt transmission. The latency period combined with seasonality of transmission has implications on scheduling monitoring trips. The best evidence so far suggests a seasonal pattern associated with transmission that means infrequent sampling of sites may be skewing the data between different populations of Tasmanian devils.

Given that Tasmanian devils and the NOD/SCID mice generate no immune response to inhibit the establishment and growth of DFTD it is not unreasonable to expect that the results obtained in the mouse model would translate to the

Tasmanian devil. As a consequence, period from exposure to detection of DFTD was assessed in NOD/SCID mice with the intention of extrapolating this to what might be occurring in the Tasmanian devil. It was found that when 5 x 10^3 DFTD cells were transferred time to detection was between 81 and 130 days. However, this was in a mouse where injection site was known and detection was by palpation of a 2 mm diameter tumour. Therefore detection for a Tasmanian devil in the field would require extended growth of the tumour. The current estimate for disease latency by field biologist is 6 to 9 months (Hamede et al 2013) and extrapolation of results from the mouse model agrees with this estimate.

4.3.2 **Adoptive protection**

The next stage of the project involved taking splenocytes from immunocompetent mice and transferring them to NOD/SCID mice to see if protection could be adoptively transferred. This would facilitate analysis of immune responses in mice to identify if components such as antibodies, primed cells or unprimed cells of the immune system were critical to protection in mice. Evaluating the pathways and mechanisms used by immunocompetent mice to reject DFTD cells would reveal suitable targets of DFTD cells that could be exploited by the immune system in developing a vaccine or treatment for DFTD in Tasmanian devils.

C57BL/6 mice were immunised with DFTD cells and their spleens harvested for splenocytes which were then subcutaneously co-transplanted with DFTD cells into NOD/SCID mice. Blood was collected between 11 and 18 days post transfer and antibody responses against DFTD were detected in eight of ten (80%) of the NOD/SCID mice. This result is evidence that transferred cells were functional against DFTD targets within the new host.

NOD/SCID mice lack B cells and do not produce antibody responses against DFTD. That means the source of the antibody had to be B cells from the C57/BL6 mice that had survived and maintained function following adoptive transfer. Furthermore, since only IgG1 was detected in the absence of IgM this suggests memory B cells had previously been generated following the immunisation of the C57BL/6 donor mice prior to adoptive transfer and were responsible for the antibody response in the NOD/SCID mice.

Following adoptive transfer the mice became unwell, developed puffy eyes, body shakes and respiratory problems. This was most likely as a consequence of graft versus host (GVH) rejection as NOD/SCID mice are H-2^{g7} MHC class and C57BL/6 are H-^{2d} MHC class (Hu et al 2012). This meant the experiments had to be terminated within 18 days which was insufficient time to evaluate DFTD engraftment or rejection. But the observation of GVH like symptoms and detection of antibody responses provided proof of concept that immune cells could be adoptively transferred and retain function.

To overcome the GVH limitation the adoptive transfer model was switched to BALB/c donors for the NOD/SCID recipient mice. BALB/c mice have immune responses biased more towards T_H2 and for this reason were not the first choice for adoptive transfer trial. However, BALB/c mice did not promote GVH rejection in NOD/SCID mice and the mice could be monitored for prolonged periods following adoptive transfer. Splenocytes from both immunised and naive BALB/c mice were compared in the adoptive transfer trial to determine if primed cells from immunised mice were superior to unprimed cells for adoptive protection. The addition of autologus serum from the immunised BALB/c donor and inclusion of CpG adjuvant was also assessed to see they were necessary for protection.

Adoptively transferred splenocytes from immunised BALB/c donors protected 15 of 15 (100%) recipient mice from DFTD. Five of these mice had received splenocytes only, five received splenocytes and autologus serum and five received splenocytes, autologous serum and CpG 2395. When splenocytes from naïve BALB/c mice were transferred to NOD/SCID mice only 11of 14 mice were protected from DFTD. This suggests that immunised cells benefit from a priming effect that enhances the protection when the cells are adoptively transferred. This is in contrast to the *in vitro* cytotoxicity assay result which found equal killing by naïve and immunised splenocytes. This suggests that *in vitro* assay is not as informative as the *in vivo* challenge.

There are various primed cells that may have worked in synergy to produce the enhanced result in the *in vivo* challenge. Primed B cells may have produced antibodies to opsonise the DFTD cells *in vivo* where the overnight *in vitro* assay would be too short to generate such a response. CTL cells and other cytotoxic cells

targeting the DFTD cells may have had the opportunity to clonally expand *in vivo* where *in vitro* assay was too short a time to induce this enhanced activity. Another factor would be the contribution to the response by the NOD/SCID mouse itself. Even though it is severely compromised it is capable of producing reduced levels of cytokines and other factors which may have contributed to the synergy of the response by primed cells.

The failure to establish DFTD tumours was evidence of successful adoptive protection in the NOD/SCID mice. Further evidence that the transferred cells had survived and retained function was evident in detection of IgG1 antibodies in the serum of NOD/SCID recipient mice. As well, the splenocytes harvested from NOD/SCID recipient mice used in *in vitro* cytotoxicity assays against DFTD cells demonstrated dose dependent killing of DFTD cells.

Adoptive protection is enhanced when DFTD cells are co-transplanted with the splenocytes. Adoptive transfer of naïve splenocytes prior to or after DFTD cells did not induce antibody responses and tumours established. This suggests that if the tumour cells are transplanted at a different time the transferred splenocytes have limited opportunity to interact with the DFTD cells. Future experiments will require co-transplantation for analysis of cell types. This could be viewed as the equivalent of *in vitro* responses in an *in vivo* setting which has many advantages over *in vitro* assays. The environment within the mouse is a complex environment in which tumour growth is not restricted and angiogenesis can supply nutrients and oxygen as the tumour expands in a biologically relevant setting. As previously alluded to, the NOD/SCID mouse to a limited degree can also contribute to the response by producing low levels of cytokines and other stimulatory factors.

The next stage of the project evaluated which specific immune cells were responsible for the protection. The rationale being that by understanding which specific cells of the immune system induced protection following adoptive transfer it may be possible to identify targets of DFTD cells that could be exploited in the development of a vaccine or treatment for DFTD in Tasmanian devils. To evaluate this, spleens from BALB/c mice were divided into enriched or depleted populations of NK, CD4, CD8 and NKT (enriched only) populations. These cells were then co-transplanted with DFTD cells into NOD/SCID mice.

With the CD4, CD8 and NK cells sorted by magnetic beads both the enriched and depleted populations rejected the DFTD cells while all the control mice grew tumours. The purity by magnetic bead sorting was greater than 85%. This result suggests that even a small percentage of contaminating cells was protective or the individual populations tested were not the sole population providing protection and were therefore redundant.

Fluorescence activated cell sorting (FACS) was an alternative method of enriching and depleting populations of cells. When this was used the purity was at least 95% which was higher than magnetic beads. While the results were not conclusive 3 of 16 (19%) NOD/SCID mice with NK enriched cells and 1 of 5 (20%) NOD/SCID mice with CD4 enriched cells established DFTD tumours following co-transplantation. This result suggests that that NK cells and CD4 cells may not be critical to rejection of DFTD cells *in vivo*. In contrast to this result co-transplantation of even small numbers of NKT cells proved protective in 3 of 3 (100%) mice suggesting that that NKT cells may be capable of killing DFTD cells.

With regards to NKT cells, they are known to be activated by glycolipid antigens presented by CD1d molecules on the target cell (Godfrey and Rossjohn 2011, Joyce et al 2011). However DFTD cells have β_2-microglobulin downregulated which is an obligatory molecule associated CD1d molecules making NKT cells unlikely effector cells. However, as β_2-microglobulin expression can be restored in the presence of cytokines (Siddle and Kaufman 2013) NKT cells could have contributed to the immune response. Another possibly could be non-classical NKT cells which are CD1-independent and have been implicated in anti-tumour responses in human cancers (Konishi et al 2004).

Additional evidence of which cells contributed to a protective response to DFTD cells was undertaken using congenic and knockout mice. Ighm/J, a B cell knockout strain, rejected DFTD cells revealing B cells are not critical to DFTD tumour rejection in the mouse model. This is important since antibody responses against DFTD have been the principal method of detecting and measuring immune responses to DFTD in both mice and Tasmanian devils thus far. So while antibodies are produced by fully competent mice such as BALB/c and C57BL/6 they are not essential for the killing of

DFTD cells but may still contribute towards the response by opsonising the targets cells.

A TNF knockout strain, B6.TNF, rejected DFTD cells indicating TNF expression by cells was not essential to DFTD rejection in the mouse model. TNF was one of the cytokines produced in response to DFTD cells by C57BL/6 and BALB/c mice splenocytes in *in vitro* cultures. The role of TNF in DFTD rejection in mice, while not apparently critical, may still contribute to DFTD rejection. Inflammatory responses are promoted by TNF and this may contribute towards recruiting effective anti-DFTD immune cells to the site of infection in fully competent mice.

To assess the role of T cells in DFTD rejection two strains of T cell deficient mice were evaluated. CBA/nu is an athymic mouse that lacks T cells but maintains B cell, macrophage, dendritic and NK cell activity. The Rag/2 mouse has a thymus but lacks T cell receptors and therefore does not have functioning T cells or B cells but maintains macrophage, dendritic and NK cell activity. The DFTD tumour established in 80 % of the Rag/2 mice (n= 8/10) and 100% of CBA/nu mice (n=9/9). Significantly, the tumours in the Rag/2 mice grew to one-tenth the size of the tumours of the CBA/nu mice within the same time period following injection with the same number of cells from the same cell culture on the same day.

Understanding the differences in the immune responses by these two strains would provide insight into the protective mechanisms used by immunocompetent mice to reject DFTD cells. Consequently, the immunophenotype of the splenocytes obtained from these two strains was evaluated. The lack of T cells in both strains was confirmed using antibodies for CD3e. This means that T cells had not contributed to the rejection in 20% of the Rag/2 mice or slower growth in the 80% of Rag/2 mice which grew tumours. The use of CD19 antibodies confirmed the CBA/nu had mature B cells and these were absent in the RAG/2 mice. This result combined with the previous observation in the Ighm/J mice confirms B cells are not critical in anti-DFTD responses in mice.

CD49b antibody confirmed both strains had NK cells. The rag/2 mice had over 10% of the spleen being NK cells while the CBA/nu had less than 5% NK cells. Using MHC II antibody to discriminate APCs such as macrophages and DCs the RAG/2 mice had over 30% of the spleen composition as APCs while the CBA/nu had just

13%. This suggests that the increased percentage of NK cells and APCs may contribute towards anti-DFTD responses in the Rag/2 mice.

Conclusions

The NOD/SCID mice provided an informative model to facilitate study of DFTD tumour transplantation and growth kinetics, which required an *in vivo* setting. The use of mice as a substitute for Tasmanian devils protects a rare and endangered species and has the benefit of reduced husbandry costs and readily available antibodies to study the mouse immune system.

Biologists concerned with the management of DFTD in the wild population need information about the latency of the disease. This information is hard to obtain in the wild so the NOD/SCID mouse provides a biologically relevant platform to conduct experiments to determine latency and growth rates of DFTD. In the future this may prove particularly valuable as the tumour evolves into different strains. It has been hypothesized that DFTD will evolve into a less virulent strain which will facilitate co-existence of the host species and the pathogen. The growth kinetics of evolving strains could be evaluated in the reproducible setting of NOD/SCID mice and provide evidence of changes to virulence of DFTD in the wild.

It is difficult to know in the wild how many cells are transferred between devils and what the threshold number of cells to induce infection is. The NOD/SCID mice revealed that as few as 2.5×10^3 DFTD cells could induce tumours. The model also revealed tumour latency till detection was cell dose-dependent and extrapolation of the data agrees with the best estimates from the field of a latency period of 6 to 9 months in the wild and suggests that this is due to inoculation with a very small number of cells, possibly a few hundred.

There was a negative correlation of tumour engraftment as injected DFTD cell numbers declined and time to detection increased. In the NOD/SCID mice 10^6 DFTD cells always resulted in engraftment which could be detected within five weeks. This information should be used to guide immunisations of Tasmanian devil for vaccine trials. If too low a number are used tumours may fail to engraft and this could confound the results.

Elucidating the mechanisms of rejection and killing of DFTD cells in mice is ongoing research to identify targets for DFTD in vaccine and immunotherapy trials. This

chapter has shown adoptive transfer of protection from BALB/c mice to NOD/SCID mice is practical and can contribute towards our understanding of anti-DFTD responses. Caution should be applied if alternative strains of mice are used in the future due to GVH rejection and MHC matching would minimise this risk.

The results from the current adoptive transfer study suggested $CD4^+$ T cells and NK cells did not have a critical role in killing DFTD cells. NKT cells are implicated, despite difficulties understanding how they could be activated, as even a small number transferred appeared to be protective. The role of $CD8^+$ T cells was not able to be determined but primed splenocytes from immunised BALB/c donors were more protective than unprimed splenocytes suggesting $CD8^+$ T cells could be involved.

Further evidence that T cells were implicit in DFTD rejection came from two different strains of T cell knockout mice which engrafted the tumour. The response difference between these strains, CBA/nu and Rag/2, demonstrated that another cell type was augmenting the T cell protection. Ighm/J, a B cell knock strain, had already revealed B cells were not critical and the addition of B cells to the CBA/nu mouse repertoire had not enhanced its protective response. It was significant that Rag/2 mice had a higher percentage of NK cells and APCs than CBA/nu and grew tumours just 10% the size of CBA/nu mice. This suggests that NK cell activity and or antigen presenting cell activity levels may have been higher in the Rag/2 mice and slowed the initial growth of the DFTD tumour and prevented DFTD establishment in 20% of the Rag/2 mice while all CBA/nu mice grew DFTD tumours. This suggests that both innate and adaptive immune responses can kill DFTD cells and ongoing research will continue to study the different responses in these strains.

Evaluation of Tasmanian devil LAK cells

Evaluation of Tasmanian devil LAK cells

5 Evaluation of Tasmanian devil LAK cells

5.1 Introduction

In the wild, the Tasmanian devil faces extinction because of the lack of effective immune rejection of DFTD cells on transmission. The disease, which is 100% fatal, fails to engage any cells of the immune system. What is indicated from recent research is that the lack of MHC expression by DFTD cells explains the absence of T cell recognition but fails to explain why NK cells have not targeted the DFTD cells for destruction. There are three possible explanations; DFTD cells are not immunogenic, are resistant to apoptosis or are resistant to NK cells.

In the previous chapters of this work, it was noted that immunocompetent mice provided evidence that DFTD cells are immunogenic and can be targeted and killed by the immune system. *In vitro* studies in these chapters also revealed camptothecin and UV-radiation induced apoptosis in DFTD cells. Therefore DFTD cells are immunogenic and can undergo apoptosis but still fail to be recognised and killed by the Tasmanian devil immune system. This highlights the third possibility above, that DFTD tumours are NK cell resistant.

One of the promising therapies with human and animal tumours that are NK-resistant is the generation of autologus lymphokine activated killer (LAK) cells. This was first described using recombinant IL-2 to stimulate cytotoxic activity in human PBMNC and mouse splenocytes. In those studies, LAK killing targeted autologous, allogeneic and cultured tumour cell lines (Herberman et al 1987). In a similar fashion, culturing with concanavalin A also promoted cytotoxic activity equivalent to LAK cells (Miyagi et al 2004).

When lymphocytes are cultured with concanavalin A lymphokines (cytokines) are released into the culture medium (Fidler et al 1976). The culture medium retains lymphokines which act as activation factors for numerous cell types including macrophages, NK and NKT cells in the same manner achieved by recombinant cytokines such as IL-2 (Fidler et al 1976, Funk et al 2005). Once the lymphokines have been produced there is no continuing need for concanavalin A and the

supernatant from the culture medium (Con A sup) can also promote activation, even with the remaining concanavalin A inactivated (Fidler et al 1976, Palacios 1982).

LAK cells from humans and mice have been shown to be cytotoxic against tumour cells *in vitro* (Funk et al 2005). This cytotoxicity was predominantly the result of NK and NKT cells (Herberman et al 1987, Ishikawa et al 2012, Miyagi et al 2004). Concanavalin A does not directly activate NK cells but induces IFN-γ production by NKT cells, which in turn promotes activation of NK cells (Miyagi et al 2004). Macrophages are also not directly activated by concanavalin A (Palacios 1982) but Con A sup contains macrophage-activating factor (MAF) that induces cytotoxic responses by macrophages that target syngeneic, allogeneic and xenogeneic tumours while leaving normal tissues alone (Fidler et al 1976).

LAK cell therapy has been used with some efficacy in the treatment of human patients with tumours (Rosenberg et al 1985). LAK cells preferentially target cytotoxicity against tumour cells and are not harmful to normal cells (Linn and Hui 2010, Richards 1989). While the mode of action is not fully understood, the evidence is consistent with innate immune cells including NK, NKT and macrophages targeting the tumours. This sort of therapy has never previously been applied to Tasmanian devils.

In this chapter, the efficacy of LAK cell therapy against DFTD cells was evaluated in *in vitro* and *in vivo*. Because of the endangered status of the Tasmanian devil the *in vivo* work was conducted in NOD/SCID mice. This chapter evaluated if it was possible to confer protection in NOD/SCID mice by adoptive cell transfer of Tasmanian devil LAK cells. The most important purpose of these *in vivo* trials was to prove that these *in vitro* activated LAK cells can kill DFTD tumours *in vivo*.

5.2 **Results**

5.2.1 **Activation of Tasmanian devil lymphocytes**

There is no evidence in nature for Tasmanian devil immune cells targeting or killing DFTD cells. There is currently no way to activate Tasmanian devil lymphocytes to induce specific immune responses against DFTD cells. In our laboratory we have demonstrated Tasmanian devil lymphocytes can be non-specifically activated to induce cytotoxic cells capable of killing DFTD cells (Brown 2013). This was demonstrated using an *in vitro* ^{51}Cr cytotoxicity assay. In this chapter these results were confirmed using the non-radioactive CellTrace Violet and propidium iodide fluorometric assay. The activation protocol was optimised prior to *in vivo* trials of adoptively transferring activated Tasmanian devil lymphocytes into NOD/SCID mice.

Concanavalin A has the ability to activate peripheral blood mononuclear cells (PBMNC) and causes them to secrete cytokines that promotes further activation (Qian et al 2014). These cells could be called mitogen activated killer cells (MAK) but in this thesis they will be referred to as LAK cells. Cells that are activated by the cytokines secreted into the culture medium during concanavalin A stimulation will also be referred to as LAK cells. This study revealed these LAK cells are capable of killing DFTD cells. To evaluate cytotoxicity and the requirement for activation, fresh PBMNC were obtained from five Tasmanian devils and used as effector cells in an 18 hour cytotoxicity assay against DFTD cells. Non-activated PBMNC had no cytotoxicity against DFTD cells (Figure 5-1panel A). When PBMNC were stimulated for 48 hours in RPMI-10 medium supplemented with 20 µg/ml concanavalin A and used as effector cells in an 18 hour cytotoxicity assay, cytotoxicity against DFTD cells was observed (Figure 5-1panel B). At the highest effector to target cell ratios the cytotoxic response against the DFTD target cells ranged from 20% to greater than 50%. Cytotoxicity appeared to plateau at the 25:1 effector to target cell ratio.

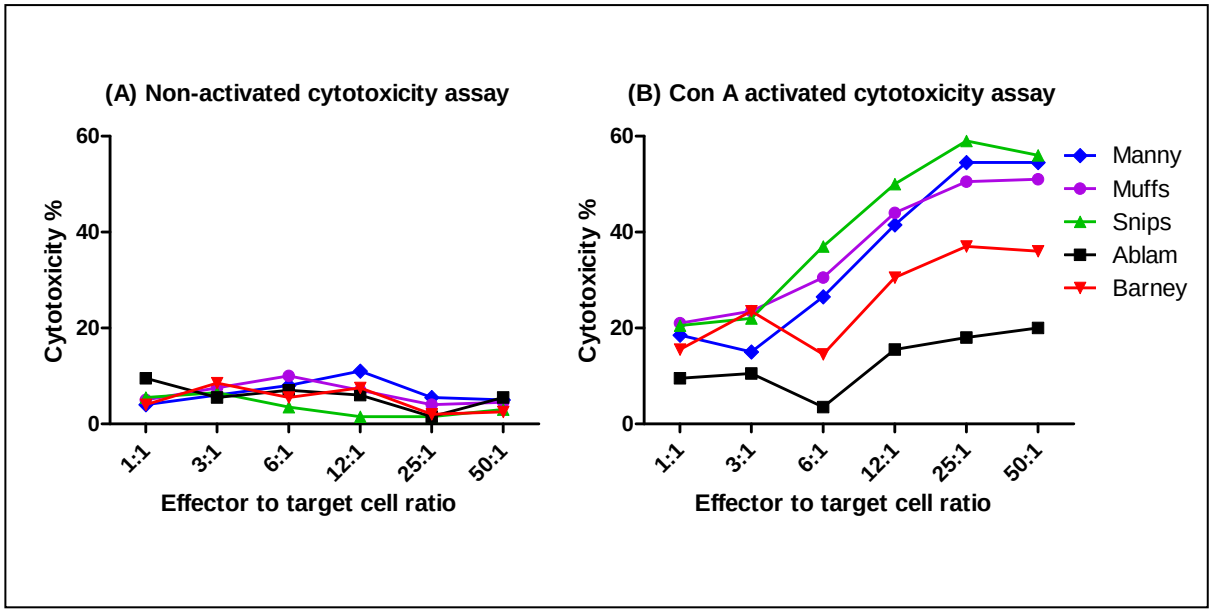

Figure 5-1. PBMNCs were obtained from five Tasmanian devils. Cytotoxicity was evaluated using propidium iodide detection by flow cytometry. (A) In an 18 hour cytotoxicity assay there was no evidence for cytotoxicity by non-activated cells. (B) Following stimulation for 48 hours with 20 µg/ml concanavalin A there was evidence for dose-dependent cytotoxicity. (Results represent mean of duplicate wells)

To determine if cytotoxic cells were also present in the spleen and if 24 hour stimulation could induce cytotoxicity equivalent to 48 hours, fresh splenocytes and PBMNC obtained at necropsy were stimulated with 25 µg/ml of concanavalin A for 24 and 48 hours. It was also hypothesised that the cytotoxicity was due to activated NK cells. Consequently the assay was performed over 4 hours to distinguish from cytotoxic T lymphocytes (CTLs), which usually require 18 hours.

After 24 hours stimulation with concanavalin A, no cytotoxicity was observed with either the splenocytes or PBMNC. Instead, the 24 hour stimulated PBMNC effector cells appeared to protect the DFTD cells from cell death resulting in an apparent inverted cytotoxic dose-curve response. This was not observed with the 24 hour stimulated splenocytes which showed no response (Figure 5-2).

After 48 hours stimulation with concanavalin A both the PBMNC and the splenocytes demonstrated dose-dependent cytotoxic responses against DFTD cells. The gradients of the dose response curves were similar but the splenocytes had a lower level of cytotoxicity. The key findings were that cytotoxic cells are present in the spleen, the efficacy was lower than PBMNC and that cytotoxicity was observed at 4 hours.

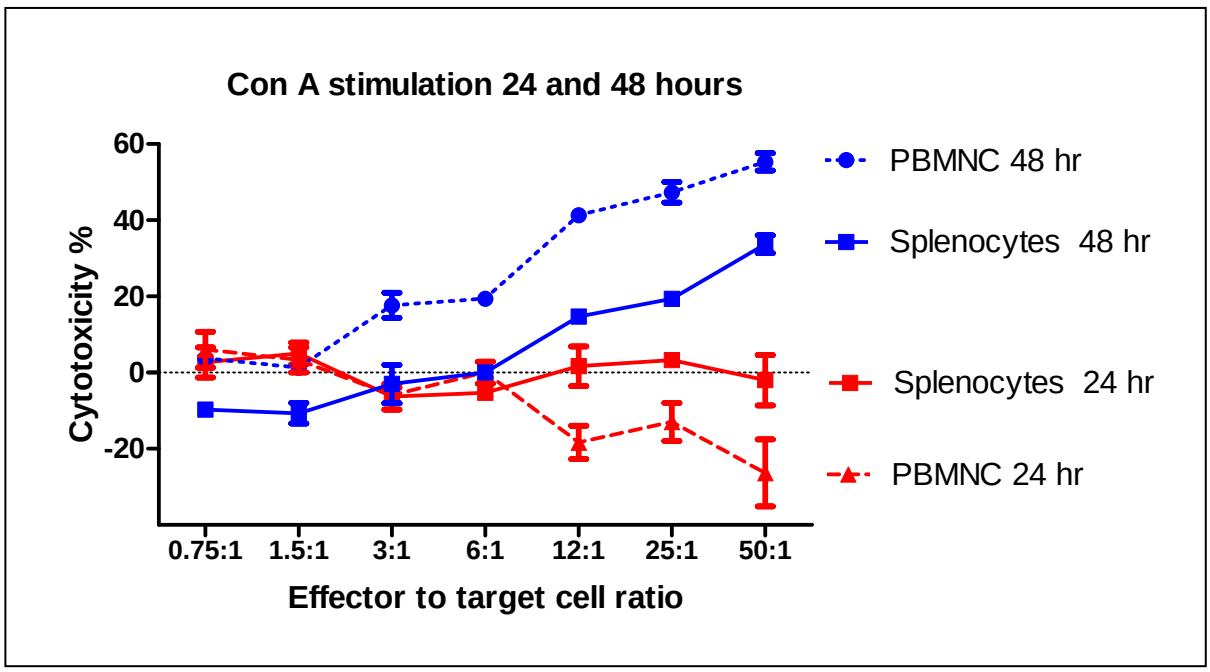

Figure 5-2. PBMNC and splenocytes were stimulated with 25 µg/ml concanavalin A for 24 or 48 hours. After treatment the splenocytes and PBMNC were used as effector cells against CellTrace Violet labelled C5065 DFTD cells in a 4 hour *in vitro* cytotoxicity assay. (Results represent mean of triplicate wells and error bars SEM)

The next section of work addressed four questions aimed at increasing the available sources of cytotoxic cells and optimising the activation of these cells into LAK cells. Firstly, can lower concentrations of concanavalin A be used to activate LAK cells? Secondly, could cytokines alone activate LAK cells? Thirdly, could cryopreserved lymphocytes be thawed and activated as LAK cells? Fourthly, could lymph nodes be used as an additional source of cytotoxic cells?

While concanavalin A has a stimulatory role on lymphocytes, too high a concentration is cytotoxic and reduces the viability (Leist and Wendel 1996). For this reason it may prove advantageous to use a lower concentration of concanavalin A to generate LAK cells. Stimulation with 5 µg/ml concanavalin A in RPMI-10 medium for 72 hours was sufficient to activate Tasmanian devil lymphocytes and resulted in dose-dependent cytotoxicity against DFTD cells in an 18 hour *in vitro* assay (Figure 5-3 panel A). There was some variability in killing responses between animals with the cytotoxicity at the maximum effector to target cell ratio of 100:1 ranging from 40 to 60%.

Concanavalin A stimulation of lymphocytes promotes production of cytokines including IFN-γ (Miyagi et al 2004, Palacios 1982). To confirm that cytokines would activate LAK cells in the absence of concanavalin A, the supernatant from Tasmanian devil lymphocytes stimulated with concanavalin A (Con A sup) was collected and the concanavalin A inactivated by the addition of mannose. Fresh splenocytes obtained during necropsy of a Tasmanian devil were cultured for 48 hours in RPMI-10 medium supplemented with 10% Con A sup. This resulted in activation of cytotoxic cells as evidenced by an 18 hour cytotoxicity assay. The cytotoxicity at 100:1 effector to target cell ratio was equivalent to the previously observed cytotoxicity for PBMNC stimulated with 5 µg/ml concanavalin A (Figure 5-3 panels A and B).

Since access to Tasmanian devils for fresh samples is limited, cryopreservation of cytotoxic cells would allow access to more samples including those obtained at necropsy. PBMNC and spleens were two sources known to contain lymphocytes that could be activated into LAK cells. It was likely that lymph nodes would be an additional source available at necropsy. The feasibility of thawing cryopreserved PBMNC and lymph node derived lymphocytes and activating them into LAK cells

was evaluated. Cells were thawed and cultured for 48 hours in RPMI-10 medium supplemented with 10% Con A sup. The freeze thawing process resulted in poor cell recovery and therefore the maximum PBMNC effector to target cell ratio was 12:1 and the maximum lymph node lymphocyte effector to target cell ratio was 50:1 in the 18 hour cytotoxicity assay. Both cell populations revealed dose-dependent cytotoxicity against DFTD target cells (Figure 5-3 panels C and D). The levels of cytotoxicity were similar for fresh cell sources (Figure 5-3 panels A and B) and thawed cryopreserved cells (Figure 5-3 panels C and D).

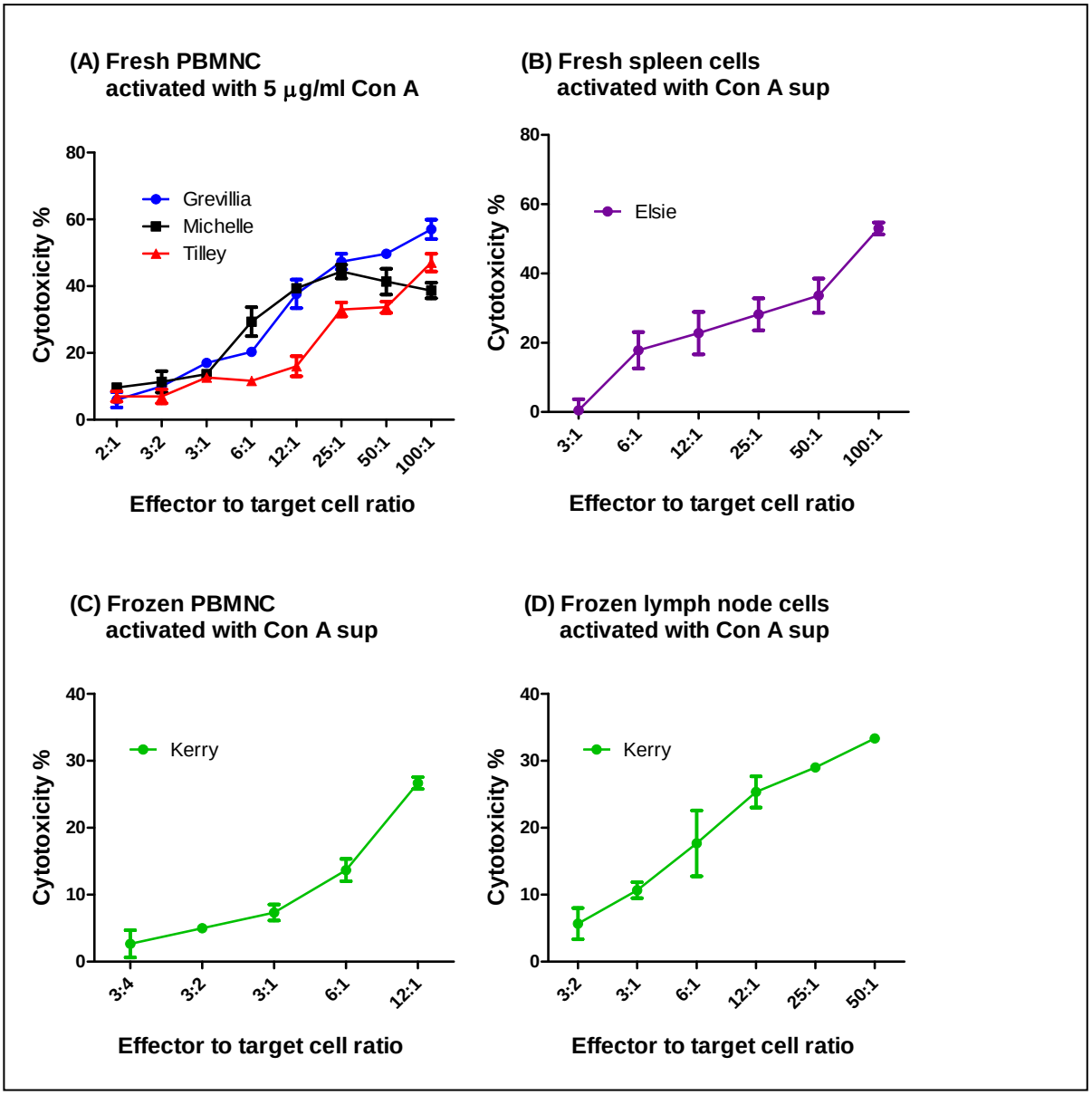

Figure 5-3. Effector cells from Tasmanian devils were activated using Con A sup or concanavalin A (as described) and then incubated with CellTrace Violet labelled C5065 DFTD cells for 18 hours. Cytotoxicity was evaluated using propidium iodide detection by flow cytometry. (A) Following stimulation for 72 hours in RPMI-10 media supplemented with 5 µg/ml concanavalin A, fresh peripheral blood mononuclear cells (PBMNC) from three Tasmanian devils showed evidence for dose-dependent cytotoxicity. (B) Following stimulation for 48 hours with 10% Con A sup, fresh splenocytes obtained from a Tasmanian devil and showed evidence for dose-dependent cytotoxicity. (C) Cryopreserved PBMNC obtained from a Tasmanian devil were thawed, stimulated for 48hours with 10% Con A sup and showed evidence for dose-dependent cytotoxicity. (D) Cryopreserved lymph node derived lymphocytes obtained from a Tasmanian devil were thawed, stimulated for 48hours with 10% Con A sup and showed evidence for dose-dependent cytotoxicity. (Data points represent mean of triplicate wells and error bars SEM)

5.2.2 **Adoptive transfer of LAK cells *in vivo* trials in NOD/SCID mice**

The previous section demonstrated Tasmanian devil lymphocytes could be activated into LAK cells that kill DFTD cells. As a preliminary trial to test if these LAK cells could be protective *in vivo* three NOD/SCID mice were injected with 5 x 10^5 DFTD cells. Two of these mice were co-transplanted with 5 x 10^6 PBMNC activated with Con A sup. After 47 days, when the control mouse developed a tumour that approached the maximum permissible size, all the mice were euthanised and tumours collected. The tumours in the LAK cell treated mice were only 3% and 9% the size of the control mouse (Figure 5-4). The LAK cells appeared to restrict the tumour growth but did not prevent establishment.

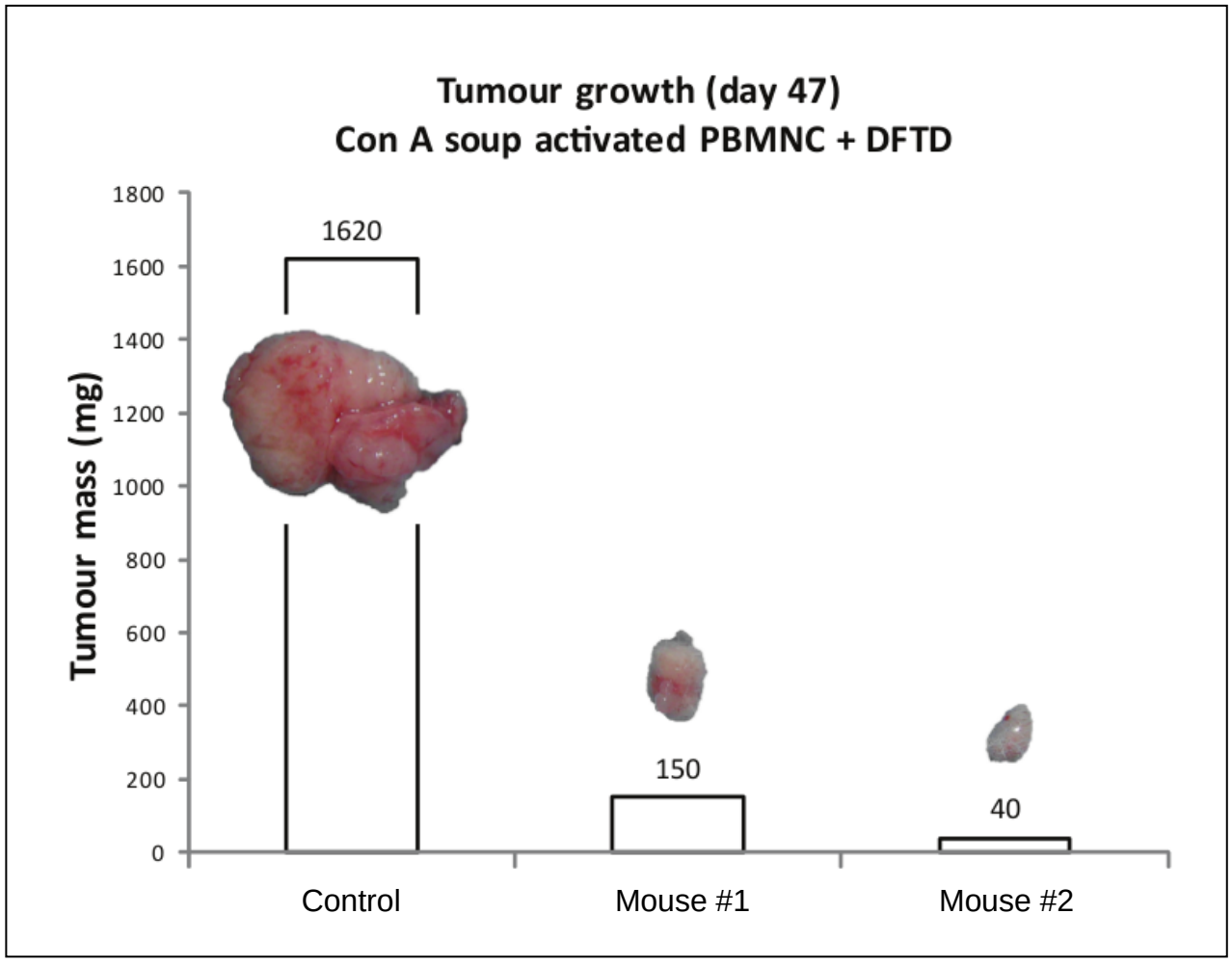

Figure 5-4. Two NOD/SCID mice (#1 and #2) were co-injected subcutaneously with 5 x 10^5 DFTD cells and 5 x 10^6 LAK cells from a Tasmanian devil which had been activated with Con A sup. A third mouse (Control) was injected with the same quantity of DFTD cells only. At 47 days the tumours were then weighed and the mice receiving the LAK cells had tumours which were 3% or 9% the size of the control mouse.

To improve the efficiency of the adoptive transfer experiments the effector to target ratio was increased by reducing the number of DFTD cells to 1x 10^4 or 2 x 10^4. This was used in preference to increasing the number of effector cells due to the limited access to peripheral blood.

PBMNC from six devils were stimulated with Con A sup for 48 hours and the resulting LAK cell populations were co-injected with DFTD cells. Because of the variable cell yields the ratio of LAK cell population to DFTD cells ranged from 74:1 to 490:1. These mice were monitored between 46 to 136 days when they were euthanised due to ethical reasons. Increasing the effector to target cell ratio prevented DFTD establishment in all seven LAK cell treated mice. However, lowering the number of DFTD cells resulted in failure of DFTD establishment in two of the four control mice and prolonged the time for tumour development (Table 5-1).

Table 5-1. Adoptive transfer into NOD/SCID mice of relatively high Tasmanian devil LAK cell to DFTD ratios

Tasmanian devil donor	Injection (number of LAK cells and DFTD cells)	LAK : DFTD cell ratio	Observation
Betty	1.7 $x10^6$ LAK + 10^4 DFTD	170:1	no tumour (day 112)
Grommit	2.4 $x10^6$ LAK + 10^4 DFTD	240:1	no tumour (day 103)
Lotti	4.9 $x10^6$ LAK + 10^4 DFTD	490:1	no tumour (day 136)
Maydin	7.4 $x10^5$ LAK + 10^4 DFTD	74:1	no tumour (day 106)
Phil	2.2 $x10^6$ LAK + 10^4 DFTD	220:1	no tumour (day 93)
Elsie	2 $x10^6$ LAK + 2 x 10^4 DFTD	100:1	no tumour (day 46)
Elsie	2 $x10^6$ LAK + 2 x 10^4 DFTD	100:1	no tumour (day 53)
Control	10^4 DFTD cells only		DFTD tumour (day 75)
Control	10^4 DFTD cells only		no tumour (day 99)
Control	2 x 10^4 DFTD cells only		DFTD tumour (day 53)
Control	2 x 10^4 DFTD cells only		no tumour (day 48)

Two problems hindering the *in vivo* LAK cell experiments were the health issues associated with the ageing NOD/SCID mice and the failure to reliably establish DFTD tumours in the control mice. To overcome this, the number of DFTD cells injected was increased to 5×10^4. This would shorten the duration to tumour development and consequently the age of the mice. But it also reduced the LAK cell to DFTD cell ratio to 20:1.

PBMNC from a single Tasmanian devil were activated with Con A sup for 48 hours and co-injected with DFTD cells into three NOD/SCID mice. Seven control mice received DFTD cells only. The mice were monitored for up to 52 days at which time all three LAK cell treated mice had developed tumours. At necropsy the control mice were examined and five of seven had developed tumours (Table 5-2).

Increasing the number of injected DFTD cells increased the proportion of control mice developing tumours within a reasonable timeframe but the LAK cells failed to provide protection (Table 5-2). This may have been the result of using a 20:1 ratio, a failure to activate the cells during stimulation with Con A sup, or biological variability associated with the Tasmanian devil PBMNC source.

Table 5-2. Adoptive transfer into NOD/SCID mice of LAK cells at 20:1 Tasmanian devil LAK cell to DFTD cell ratio

Tasmanian devil donor	Injection (number of LAK cells and DFTD cells)	LAK : DFTD cell ratio	Observation
Carlotta	10^6 LAK cells + $5x10^4$ DFTD cells	20:1	Tumour (Day 52)
Carlotta	10^6 LAK cells + $5x10^4$ DFTD cells	20:1	Tumour (Day 52)
Carlotta	10^6 LAK cells + $5x10^4$ DFTD cells	20:1	Tumour (Day 52)
Control	$5x10^4$ DFTD cells only		Tumour (Day 52)
Control	$5x10^4$ DFTD cells only		Tumour (Day 52)
Control	$5x10^4$ DFTD cells only		Tumour (Day 39)
Control	$5x10^4$ DFTD cells only		Tumour (day 42)
Control	$5x10^4$ DFTD cells only		Tumour (day 46)
Control	$5x10^4$ DFTD cells only		No tumour (Day 52)
Control	$5x10^4$ DFTD cells only		No tumour (Day 52)

Since 20:1 LAK cell to DFTD cell ratio did not appear to be protective the ratio was increased. PBMNC from four devils were activated with Con A sup for 48 hours and co-injected with 5 x 10^4 DFTD cells at LAK cell to DFTD cell ratios ranging from 50:1 to 80:1. By day 74 none of the six LAK cell treated mice had established DFTD tumours but two of four control mice had developed tumours (Table 5-3).

Table 5-3. Adoptive transfer into NOD/SCID mice of LAK cells at 50:1 to 80:1 Tasmanian devil LAK cell to DFTD cell ratios

Tasmanian devil donor	Injection (number of LAK cells and DFTD cells)	LAK : DFTD cell ratio	Observation
Bangles	4.1 x 10^6 LAK cells + 5x10^4 DFTD cells	80:1	No tumour (Day 68)
Bangles	4.1 x 10^6 LAK cells + 5x10^4 DFTD cells	80:1	No tumour (Day 74)
Floyd	3.3 x 10^6 LAK cells + 5x10^4 DFTD cells	60:1	No tumour (Day 74)
Cory	3 x 10^6 LAK cells + 5x10^4 DFTD cells	60:1	No tumour (Day 74)
Cory	3 x 10^6 LAK cells + 5x10^4 DFTD cells	60:1	No tumour (Day 74)
Andrea	2.5 x 10^6 LAK cells + 5x10^4 DFTD cells	50:1	No tumour (Day 59)
Control	5x10^4 DFTD cells only		Tumour (Day 74)
Control	5x10^4 DFTD cells only		Tumour (Day 74)
Control	5x10^4 DFTD cells only		No tumour (Day 74)
Control	5x10^4 DFTD cells only		No tumour (Day 74)

As two of the four control mice had developed tumours the next trial used 10^5 DFTD cells and the LAK cells were used at ratios ranging from 7:1 to 22:1. After 45 days two of the four LAK cell treated mice had established DFTD tumours compared to five of the six control mice. Within the LAK cell treated group of mice there was no clear evidence of LAK cell dose-dependent response as 13:1 did not develop a tumour but 20:1 did (Table 5-4).

Table 5-4. Adoptive transfer into NOD/SCID mice of LAK cells at 7:1 to 22:1 Tasmanian devil LAK cell to DFTD cell ratios

Tasmanian devil donor	Injection (number of LAK cells and DFTD cells)	LAK : DFTD cell ratio	Observation
Sedate Ed	2 x 10^6 LAK cells + 10^5 DFTD cells	20:1	Tumour (day 45)
Sedate Ed	7 x 10^5 LAK cells + 10^5 DFTD cells	7:1	Tumour (day 45)
Sedate Ed	2.2 x 10^6 LAK cells + 10^5 DFTD cells	22:1	No tumour (day 45)
Sedate Ed	1.3 x 10^6 LAK cells + 10^5 DFTD cells	13:1	No tumour (day 45)
Control	10^5 DFTD cells		Tumour (day 45)
Control	10^5 DFTD cells		Tumour (day 45)
Control	10^5 DFTD cells		Tumour (day 45)
Control	10^5 DFTD cells		Tumour (day 45)
Control	10^5 DFTD cells		Tumour (day 45)
Control	10^5 DFTD cells		No tumour (day 45)

Combining all the data from Tables 5-1 to 5-4 into a summary table highlighted the need for high LAK cell to DFTD cell ratios to protect mice from tumour development as well as the need for sufficient DFTD target cells to establish tumours in control mice. Evaluation of the combined LAK cell adoptive transfer trials revealed a 50:1 LAK cell to DFTD ratio was protective in 13 of 13 mice while a ratio of 20:1 was not protective with 5 of 6 mice developing tumours. Lowering the number of DFTD target cells appeared to decrease the proportion of control mice which established DFTD tumours in a dose-dependent manner (Table 5-5).

Table 5-5. Summary table of LAK cell adoptive transfer trials

Summary Table	
LAK cell : DFTD cell ratio	**Tumour development**
≥ 50:1	0 of 13 mice developed tumours
≤ 20:1	5 of 6 mice developed tumours
1 x 10^4 DFTD cells only controls	2 of 4 mice developed tumours
5 x 10^4 DFTD cells only controls	7 of 11 mice developed tumours
1 x 10^6 DFTD cells only controls	5 of 6 mice developed tumours

5.3 **Discussion**

The first chapter revealed that DFTD cells are immunogenic and can be killed by the murine immune system. This chapter evaluates ways of stimulating Tasmanian devil lymphocytes into cytotoxic cells capable of killing DFTD cells. Adoptive cell transfer experiments were then used to evaluate if *in vitro* activated Tasmanian devil lymphocytes could provide adoptive protection *in vivo*. However, the endangered status of the Tasmanian devil limited access for research purposes. As a consequence a suitable alternative was required; hence the *in vivo* work was conducted in NOD/SCID mice. As discussed in the second chapter, these mice had proven suitable for adoptive transfer using immune cells from BALB/c mice. In this chapter adoptive cell transfer from Tasmanian devils into NOD/SCID mice is evaluated.

5.3.1 **Overcoming NK resistance of DFTD through LAK cell activation**

Recent research has revealed that DFTD cells downregulate MHC expression (Siddle and Kaufman 2013) and thereby avoid immunosurveillance and destruction by MHC-restricted lymphocytes. The lack of MHC should make the DFTD cells targets for killing by non-MHC restricted lymphocytes such as NK cells (Siddle and Kaufman 2013). The development of tumours indicates that NK cells do not kill DFTD cells *in vivo.* It is likely that DFTD tumours are NK-resistant.

NK-resistant cancers are well documented with human patients. One way to overcome NK-resistance in humans involves *in vitro* activation of the patient's lymphocytes through stimulation with cytokines or mitogens. This activates the cells to become lymphokine activated killer (LAK) cells, cytokine induced killer (CIK) cells or mitogen activated killer (MAK) cells respectively (Qian et al 2014). These cells have the capacity to kill NK resistant tumours *in vivo* when reintroduced to the patient.

The division into the three categories of LAK, CIK and MAK cells is artificial and not without some overlap. The term LAK cells is the original term from the 1980's used to describe lymphocytes activated by cytokines (at that time referred to as lymphokines) and cytotoxicity was attributed to NK cells activated by IL-2 (Grimm et al 1982, Herberman et al 1987). CIK cells is a term first appearing in the literature in

the 1990's and then recently identified population of cells with both T cell and NK cell markers (NKT cells) were attributed as the main cytotoxic cells (Lu and Negrin 1994). MAK cells refer to lymphocytes activated by mitogens such as concanavalin A (Qian et al 2014). MAK cells include NKT cells, NK cells and monocytes activated by cytokines produced in response to mitogen stimulation converting them into killer cells (Qian et al 2014). Considering that the lymphocytes activated by these three methods are usually a mixed population sourced from PBMNC the effector cells should not be looked at in isolation but rather in synergy. It is possible to enrich specific populations of cells to ascertain the specific role of each. This could mislead rather than enlighten since each cell type produces cytokines that either promote or inhibit other cell types as a cascade of events. In this thesis the term LAK cell has been used to describe the population of Tasmanian devil lymphocytes stimulated with cytokines or mitogens.

When lymphocytes from peripheral blood were cultured with DFTD cells there was no evidence for cytotoxicity. As there is evidence for NK cells in peripheral blood (Brown et al 2011) the lack of cytotoxicity supports the concept that DFTD cells are NK resistant. But when peripheral blood lymphocytes were stimulated with either concanavalin A or Con A sup, the activated cells demonstrated a dose-dependent cytotoxic response. The significance of this observation is that activated cytotoxic cells have the capacity to kill DFTD cells. In chapter three DFTD cells were shown not to be resistant to apoptosis. Consequently NK resistance is due to a failure of recognition and subsequent activation of cytotoxic cells.

The stimulation of Tasmanian devil lymphocytes to become LAK cells was tested with different incubation times and concanavalin A concentrations. Stimulation with concanavalin A for 48 hours consistently induced cytotoxicity and 5 µg/ml was as equally effective as 25 µg/ml of concanavalin A. The lower concentration had the advantage that it had less toxic effects on lymphocytes. Hence 5 µg/ml was used in future experiments.

Lymphocytes can be directly activated by cytokines (Choi et al 2012, Qian et al 2014) such as those produced during concanavalin A stimulation. Once the cytokines have been produced there is no continuing need for concanavalin A and the supernatant from the culture medium (Con A sup) can also promote activation,

even with the remaining concanavalin A inactivated (Fidler et al 1976, Palacios 1982). The addition of supernatant obtained from concanavalin A stimulated lymphocytes (Con A sup) at a final concentration of 10% to the culture medium was sufficient to activate Tasmanian devil lymphocytes into LAK cells.

Consequently there were two means of activating Tasmanian devil lymphocytes into cytotoxic cells capable of killing DFTD cells. Concanavalin A allowed precise conditions to be reproduced whereas different preparations of Con A sup varied between batches, most likely due to different levels of the cytokines produced. This source of variability added to inter-devil variability in the levels of cytotoxicity with the lymphocytes obtained from different Tasmanian devils. Inter-patient and inter-experimental variability with LAK cells trials have also been reported in human trials (Qian et al 2014).

The ability to activate Tasmanian devil lymphocytes to kill DFTD cells is a significant milestone towards development of a treatment or vaccine against DFTD. It reveals that DFTD cells can be killed by *in vitro* activated PBMNC cells. It also highlights that effective cytotoxic cells can be extracted from blood. For devils to induce cytotoxicity following vaccination it would require cytotoxic cells to be present in secondary lymphoid organs such as spleen and lymph nodes. This was investigated with devils that had been euthanised for ethical reasons. Following activation, cytotoxicity was identified in cells extracted from lymph nodes and the spleen. This important finding reveals that Tasmanian devils have a competent immune system that contains cells with the capacity to kill DFTD cells in secondary lymphoid organs.

The failure of their immune recognition (due to MHC downregulation) can be overcome by activated lymphocytes *in vitro*. LAK and MAK cell therapy is when *in vitro* activated lymphocytes are introduced into a patient to target NK resistant tumours. This may be a suitable approach to overcome the lack of recognition of DFTD cells by Tasmanian devils. To evaluate the efficacy of these approaches a reliable supply of lymphocytes was required for experiments. As a consequence, evaluation of different lymphocyte sources was undertaken. The level of killing by PBMNC was as effective as those obtained from the spleen and lymph nodes. Any of these sources would be suitable to perform further cytotoxicity experiments. For ethical reasons spleens and lymph nodes could only be obtained at necropsy.

Therefore PBMNC are the preferred source for lymphocytes but spleens and lymph nodes can be collected opportunistically at necropsy to augment supply.

A large number of lymphocytes can be obtained at necropsy from the blood, spleen and lymph nodes. It is not practical to use all of these cells at time of harvest. A possible solution would be cryopreserving cells for later use. It was unknown if Tasmanian devil lymphocytes could be cryopreserved, thawed and remain functional. As a consequence this was evaluated using lymphocytes sourced from blood, spleen and lymph nodes. Tasmanian devil lymphocytes samples were cryopreserved, thawed and tested for viability with dye exclusion. Other laboratories report between 50 to 70% recovery rate of frozen cells from humans (Jewett et al 1976, Kleeberger et al 1999) and similar results were obtained with the Tasmanian devil lymphocytes. Noteworthy is that platelet contamination negatively impacts human lymphocyte cryopreservation (Strong et al 1975) and Tasmanian devil lymphocytes have proven difficult to isolate without red blood cell and platelet contamination.

Cryopreservation of Tasmanian devil lymphocytes was possible and would provide a readily accessible source for experiments. Cryopreserved samples can be best for longitudinal studies (Jewett et al 1976) and frozen human lymphocytes demonstrate all the characteristics of fresh cells (Strong et al 1975) and can be preserved for at least 12 years (Kleeberger et al 1999). This has facilitated the application of newly developed assays to specimens in repositories to measure markers not available at the time of collection (Kleeberger et al 1999). Of important significance to the LAK and MAK cell therapy with DFTD is the observation that cryopreserved human cells have decreased responses to some specific antigens but no significant difference in response to concanavalin A (Jewett et al 1976).

When human samples are cryopreserved there is some shift in the subpopulations recovered (Jewett et al 1976, Strong et al 1975). The impact this would have on cytotoxic cells in Tasmanian devil lymphocytes was unknown. Consequently, functional cytotoxicity assays were used to compare thawed cells and fresh cells for their killing of DFTD cells. The observation that following activation thawed Tasmanian devil lymphocytes killed DFTD cells equally well as fresh lymphocytes reveals the cytotoxic cells and their function were not impacted by cryopreservation.

This is therefore a suitable means of ensuring continuous supply of cytotoxic cells for experiments.

Fresh PBMNC is the best source of viable lymphocytes. But access to Tasmanian devils is limited and therefore fresh samples are not always available for experiments. The ability to cryopreserve Tasmanian devil lymphocytes is significant because sample collection is often opportunistic rather than in response to researchers' experimental plans. Cryopreservation allows blood, spleen and lymph node derived lymphocytes to be obtained at necropsy which maximises the contribution of each Tasmanian devil to the research of DFTD. PBMNC obtained during routine veterinary checks can also be cryopreserved to augment supply.

The necessary reagents to elucidate the cytotoxic cells within the LAK cell population are not currently available for Tasmanian devils. Tasmanian devil LAK cell cytotoxicity was detected in 4 hour cytotoxicity assays consistent with NK cells being the major contributor to the cytotoxicity. But it is almost certainly not just NK cells responsible for the events leading to LAK cell killing. Human PBMNC contain approximately 15% NK cells and 0.05% NKT cells (Shimizu et al 2006) and the synergy of these two cell types is required for LAK killing (Kaneko et al 2000, Miyagi et al 2004, Palacios 1982). Despite only a small percentage of the cells being NKT cells these cells have a critical role in concanavalin A stimulation. Concanavalin A does not directly activate human or mouse NK cells or monocytes but induces IFN-γ production by NKT cells which in turn promotes activation of NK cells (Kaneko et al 2000, Miyagi et al 2004, Palacios 1982). Con A sup also contains macrophage-activating factor (MAF) and other cytokines that activate macrophages (Fidler et al 1976).

It is unknown if Tasmanian devils have NKT cells to undertake this function. What is known is that the bandicoot *Isoodon macrourus*, an Australian marsupial has a functioning CD1 gene that would permit lipid antigen presentation to NKT cells if they exist in the marsupial (Baker and Miller 2007). The opossum *Monodelphis domestica*, an American marsupial, lacks a functioning CD1 gene and therefore cannot present lipid antigens to NKT cells (Baker and Miller 2007). The loss of this CD1 gene is believed to have occurred after divergence from Australian marsupials (Baker and Miller 2007) making it more likely that the Tasmanian devil has a

functioning CD1 gene. The observation that concanavalin A induces cytotoxicity in Tasmanian devil lymphocytes is consistent with them have functioning CD1 and NKT cells or an alternative pathway to activate NK cells following concanavalin A stimulation. This is an important observation as there is a paucity of understanding about the Tasmanian devils immune responses and this knowledge contributes to the development of a vaccine or treatment for DFTD.

While LAK cells kill by non-specific activation of NK cells that target NK-resistant tumours such as DFTD cells, they also act as a bridge between the innate an adaptive immune system. In mice it has been shown that NK-LAK cells express IFN-γ which sensitises T cells to IL-2 to promote T_H1 cell development and activation of macrophages (Geldhof et al 2002). The NK-LAK cells not only kill the NK-resistant tumour but also kill M2 macrophages promoting a M1 macrophage response (Geldhof et al 2002). The M1 macrophages engage $CD8^+$ T cells generating a CTL response against tumours (Geldhof et al 2002). This indicates that LAK cell therapy in Tasmanian devils may induce a cascade of immune responses extending beyond non-specific targeting of DFTD cells to a specific DFTD response by the adaptive immune system.

In summary, Tasmanian devil lymphocytes can kill DFTD cells *in vitro* following activation by concanavalin A or cytokines. The effector cells can be found in blood, spleen or lymph nodes. Both fresh and cryopreserved cells were suitable sources for cytotoxic cells.

5.3.2 **Adoptive cell transfer of Tasmanian devil LAK cells**

The *in vitro* trials showed that activated Tasmanian devil cytotoxic cells could kill DFTD cells *in vitro* but it was unknown if this would translate to *in vivo* protection. The dynamic *in vivo* environment is different to the plastic substrate environment of *in vitro* cytotoxicity cultures. The *in vivo* environment provides a complex three-dimensional structure with supporting cells such as fibroblasts, endothelial cells and mesenchymal cells that could support the survival of the DFTD cells against cytotoxic LAK cells. The host animal may also provide a source of cytokines, growth factors and nutrients not available in the *in vitro* environment. Tumour cells are typically heterogenic and the *in vitro* cytotoxicity did not reveal 100% killing, accordingly some cells survived. It is possible that these surviving cells had the

ability to resist LAK killing and in an *in vivo* setting they would overwhelm the LAK cell response and establish a tumour.

Limited access to Tasmanian devils facilitated the need for an *in vivo* model to evaluate *in vivo* protection by LAK cells. In the previous chapter NOD/SCID mice proved a suitable model to study adoptive cell transfer protection against DFTD. Immune cells from immunocompetent BALB/c mice were co-injected with DFTD cells into NOD/SCID mice. This not only prevented the establishment of DFTD tumours but the transferred murine cells remained functional producing DFTD specific antibodies in the new host. The *in vivo* results complemented the *in vitro* results and revealed information not detectable by *in vitro* assays alone.

The protection from adoptive BALB/c cell transfer to NOD/SCID mice required co-injection of the DFTD cells and the lymphocytes. Protection was less effective if the cells were injected into separate subcutaneous sites on the body or at different time points. In this chapter, activated Tasmanian devil lymphocytes (LAK cells) were co-injected with DFTD cells to “devilise” mice. This partial reconstitution of NOD/SCID mice with Tasmanian devil lymphocytes facilitated the study of immune responses by Tasmanian devil cells in an *in vivo* setting.

LAK cell cytotoxicity was evaluated by monitoring tumour growth. LAK cells preferentially target their cytotoxicity against tumour cells and are not harmful to normal cells in humans (Linn and Hui 2010, Richards 1989) and adoptive transfer of Tasmanian devil LAK cells into NOD/SCID mice targeted the DFTD cells and showed no adverse response against normal cells as there was no evidence for graft versus host disease. Furthermore, even without an appropriate immune system to recruit as reinforcement the LAK cells on their own effectively inhibited or prevented the establishment of DFTD tumours in the NOD/SCID mice. Most importantly this ‘devilised mouse model’ provided proof of concept for adoptive cell transfer therapies such as LAK cell therapy against DFTD.

The adoptive transfers were trialled at various ratios of effector (LAK) to target (DFTD) cell ratios. It should be noted that the termed effector cell here refers to the mixed population of lymphocytes that have been stimulated but not all of these cells would be cytotoxic. Ratios of 20:1 or less failed to prevent DFTD tumour establishment but did result in smaller tumour growth in the treated mice compared

to the controls. In contrast, ratios of 50:1 or greater prevented DFTD tumour establishment in all treated mice. *In vitro*, 50:1 did not result in 100% cell death but in *in vivo* there were no surviving cells to establish DFTD tumours providing a more biologically relevant test for LAK cell activity. This means that for effective treatment in Tasmanian devils sufficient number of LAK cells will be required or the protective effect of LAK killing will be overwhelmed.

In human therapies acquiring sufficient number of activated cells to maintain LAK killing to achieve full regression has been an obstacle (Cesano et al 1994). This may also prove to be the case with Tasmanian devils but since LAK killing is presumed to be predominately NK cell mediated establishing a Tasmanian devil NK cell line could overcome this obstacle. If a Tasmanian devil NK cell line could be established this would supply sufficient cells for *in vitro* and *in vivo* trials. This has been the case with human trials where NK cell lines have been established and FDA approved (Qian et al 2014).

As previously mentioned concanavalin A will not directly activate NK cells but recombinant cytokines could be used as an alternative means of activation for an NK cell line. Cultured NK cell lines are effectively allogeneic NK cells that have enhanced efficacy in human trials against cancers such as acute myeloid leukaemia (AML) (Moretta et al , Murphy and Longo 1997). If DFTD cells upregulate MHC class I, which they can do under cytokine stimulation (Siddle et al 2013), then autologous LAK cells will see the tumour as self and not target them. On the other hand, allogeneic LAK cells would overcome this obstacle and kill the tumours without risk of graft-versus-host disease (Moretta et al , Murphy and Longo 1997, Qian et al 2014). This occurs because the NK cells have KIR receptors that bind to self MHC ligands and this acts an inhibitory signal. Allogeneic NK cells often have a KIR mismatch with tumour MHC and therefore this leads to greater tumour killing (Miller et al 2005).

The cytotoxic ability of human LAK cells has been clearly demonstrated *in vitro* against tumour cells and *in vivo* clinical trials (Funk et al 2005, Rosenberg et al 1985). In this chapter killing of DFTD cells was demonstrated both *in vitro* and *in vivo* and therefore LAK cell therapy in Tasmanian devils could be possible. While the mode of LAK killing is not fully understood, the evidence is consistent with innate

immune cells including NK, NKT and macrophages non-specifically targeting tumours in a non-MHC restricted manner (Herberman et al 1987, Ishikawa et al 2012, Miyagi et al 2004). The presence of NK and NKT cells in the Tasmanian devil is yet to be confirmed but these results provide evidence of LAK cell functions associated with these cell types.

5.3.3 **Conclusions**

The findings in this chapter reveal the Tasmanian devil has a competent immune system capable of killing DFTD cells *in vivo*. This was achieved in all the DFTD challenged NOD/SCID mice that received at least a 50:1 LAK cell to DFTD cell ratio. The inability to kill DFTD on transmission is either due to a failure to recognise the DFTD cells or failure to identify them as non-self. This can be overcome by *in vitro* activation of Tasmanian devil lymphocytes to induce LAK cells which are capable of killing DFTD cells both *in vitro* and *in vivo*. This killing targeted DFTD cells and showed no evidence of harm to normal cells.

The current study has been in a mouse model which was suitable for studying the direct LAK killing of DFTD cells. This model is not suitable to evaluating the recruitment of the adaptive immune system. LAK cell therapy has the potential to direct the Tasmanian devils resident immune system towards an adaptive DFTD specific immune response. This is yet to be shown and will require trials in Tasmanian devils which are now justified by the results shown in this chapter.

LAK cell therapy requires a reliable source of lymphocytes that can be activated. In this chapter blood, spleen and lymph nodes have been shown to be suitable sources. These cells can be cryopreserved, thawed and the cytotoxic potential is not diminished. However, possibly a better source of cells for LAK cell therapy would be the establishment of NK cell lines for Tasmanian devils. The cell lines should have KIR receptors that are mismatched to the MHC ligands that DFTD cells are capable of expressing. This would provide NK cells which would not be inactivated if DFTD cells upregulated MHC during treatment.

Utilisation of the murine model for therapy trials

6 Utilisation of the murine model for therapy trials

6.1 Introduction

One of the purposes for developing the mouse model to study DFTD was to undertake preliminary studies to evaluate treatment options that could be applied to the Tasmanian devil. In this chapter four treatments were evaluated for their efficacy against DFTD. Two involved the chemotherapeutic drugs afatinib and withaferin A that induce apoptosis and promote anti-cancer immunity (Yap et al 2010, Yang et al 2012). The two other drugs were fucoidan and imiquimod which demonstrate anti-cancer immunomodulatory activities (Yang et al 2013, Schön et al 2003).

Afatinib

The Sanger Research Institute (Elizabeth Murchison, personal communication, 2012) identified afatinib as a potential treatment for DFTD in an *in vitro* drug sensitivity trial conducted against various cancer cell lines including DFTD cells. Afatinib, also known as BIBW2992, is known to decrease proliferation and increase apoptosis in some human cancers (Yap et al 2010). They observed decreased proliferation of DFTD cells when incubated with afatinib *in vitro*.
Afatinib functions as an epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) that irreversibly binds to epidermal growth factor receptor (EGFR) as well as human epidermal growth factor receptor 2 (HER2) (Nanjo et al 2013, Yap et al 2010). These receptors become activated by EGF-like peptides that act as growth factors. To promote tumour growth and survival many cancers upregulate expression of both EGFR and EGF-like peptides (Normanno et al 2006). These receptors are activated by tyrosine kinase phosphorylation and afatinib prevents phosphorylation.

First generation drugs, such as erlotinib, gefitinib and lapatinib, had reversible binding that did not sustain suppression of phosphorylation while afatinib maintains suppression because its covalent bonding to the receptors is irreversible (Yap et al 2010). EGFR mutations in tumours including T790M point mutations enhanced resistance to the first generation drugs but are inhibited by afatinib (Yap et al 2010).

Withaferin A

The traditional Indian medicine system, Ayurvedic Medicine, has used the medicinal plant *Withania somnifera* (Indian Winter Cherry) for centuries to treat a variety of

ailments. Withaferin A is the bioactive ingredient isolated from this plant that has been shown to have immunomodulatory, anti-inflammatory, anti-angiogenic and anti-tumour properties (Yang et al 2012). The anti-tumour properties of withaferin A can inhibit proliferation, induce apoptosis and directly cause necrotic cancer cell death while normal cells are unaffected (Grogan et al 2013). Withaferin A preferentially targets cancer cells that over-express vimentin resulting in the accumulation of vimentin in the perinuclear space (Satelli and Li 2011). It binds to the vimentin, degrades it and facilitates increased apoptosis (Patel et al 2013, Satelli and Li 2011). DFTD cells are strongly positive for vimentin (Loh et al 2006a) indicating a critical role for vimentin in DFTD cells. Vimentin is an intermediate filament protein contributing towards structural and functional integrity of quiescent cells. In activated cells vimentin promotes adhesion, migration, survival and cell signalling mechanisms (Lahat et al 2010). Over expression of vimentin is generally regarded as supporting a poor prognosis for cancers since it is correlated to metastatic disease (Yang et al 2012).

Withaferin A selectively targets cancer cells and induces vimentin disassembly (Thaiparambil et al 2011, Yang et al 2012). It is effective in the treatment of many types of cancer including prostate cancer (Srinivasan et al 2007), breast cancer (Stan et al 2008), colon cancer (Koduru et al 2010), cervical cancer (Munagala et al 2011), glioblastomas (Grogan et al 2013) and head/neck squamous cell cancer (Cohen et al 2009). It is a natural product extracted from the plant *Withania somnifera* and is associated with fewer side effects and demonstrates lower toxicity than synthetic options (Yang et al 2012).

Recent pharmacological studies have identified a number of anti-cancer mechanisms in addition to targeting vimentin. These include changing the architecture of the cytoskeleton, upregulating production of reactive oxygen species (ROS) (Grogan et al 2013, Patel et al 2013), inhibiting proteasomal activity and contributing towards mitochondrial dysfunction (Yang et al 2012). Gene expression analysis following withaferin A treatment showed an upregulation of pro-apoptotic Bax and IκB-α proteins and downregulation of a number of cell growth and metastasis transducers including c-myc and vimentin (Patel et al 2013, Yang et al 2012). In human malignant pleural mesothelioma (MPM) cells withaferin A has been

shown to induce caspase-3 activation, PARP cleavage and condensed nuclei which are characteristics of apoptosis (Yang et al 2012).

Fucoidan

Fucoidan is a polysaccharide extracted from seaweed with reported anticancer and immunomodulatory activities (Kwak 2014, Yang et al 2013). It is known to target numerous receptors and signalling molecules in both tumour cells and immune cells (Kwak 2014). Studies have revealed mechanisms of activity that include activation and mobilisation of immune cells as well as altering cytokine expression. In this chapter fucoidan was evaluated for its potential to alter or enhance immune responses against DFTD cells in mice.

Fucoidan as an immunomodulatory compound is capable of inducing T_H1 cytokines including IFN-γ, TNF-α and IL-12 while suppressing T_H2 cytokines IL-10 and TGF-β (Kar et al 2011). Fucoidan treated DCs also direct naïve T cells towards a T_H1 differentiation. In an animal study of Leishmania infection, a blood parasite in humans, fucoidan switched the $CD4^+$ T cell T_H2 immune response to a T_H1 biased response resulting in elimination of the parasite (Kar et al 2011). This type of T_H1:T_H2 modulation ratio towards T_H1 profiles are required for effective anti-tumour responses and hence our interest in fucoidan's potential role against DFTD.

Imiquimod

Imiquimod is a TLR-7 agonist that was being evaluated within our laboratory in *in vitro* studies for its potential to activate innate immune cells. During these trials it was observed that *in vitro* imiquimod treatment stimulated the DFTD cells to upregulate MHC class I expression (Patchett 2013). One of the main mechanisms exploited by DFTD cells to avoid detection and subsequent destruction on transmission is the downregulation of MHC class I molecules (Siddle et al 2013). This led to the hypothesis that intratumoural injections of imiquimod could be used to upregulate MHC class I in DFTD tumours *in vivo.*

In addition to upregulation of MHC class I there are other anti-tumour properties of imiquimod that are relevant to induce an immune response against DFTD. Imiquimod is an established treatment against cancers including basal cell carcinomas (BCCs) (Schön et al 2003). Imiquimod directly induces tumour selective apoptosis independent of membrane-bound death receptors (Schön et al 2003). It

also promotes cytokine mediated cellular immune responses (Schön et al 2003). Of significant relevance to the development of a DFTD vaccine is the ability to use imiquimod as an adjuvant in vaccines to promote CD8+ T cell responses (Shackleton et al 2004).

6.2 **Results**

6.2.1 **Treatment of DFTD tumours with afatinib**

Afatinib had been shown to kill DFTD cells in an *in vitro* drug screening trial conducted by the Sanger Research Institute (Elizabeth Murchison, personal communication, 2012). For this reason it was decided to evaluate the efficacy of afatinib on established tumours. Consequently 12 NOD/SCID mice were injected with 10^5 DFTD cells subcutaneously.

The DFTD cells were given 35 days to form small tumours. This was based on results in Chapter four, which determined 1 to 2 mm DFTD tumours would be established in most mice that received 10^5 DFTD cells by this time point. On day 35, six mice commenced a series of 21 treatments over 26 days with afatinib (293 µg/mouse) concealed in peanut butter. This was equivalent to 12.5 mg/kg per dose of afatinib, which was the recommendation from the Sanger Research Institute (Elizabeth Murchison, personal communication, 2012). The drug was well tolerated and apparently palatable in the peanut butter despite being suspended in 10µl DMSO per dose. The peanut butter baits were consistently eaten by the mice and this monitored by housing the animals individually. Six control mice were not fed peanut butter or afatinib.

Two of the treated mice had to be euthanised for ethical reasons during the trial. This was not related to the treatment so they were removed from the results. The last treatment was given 60 days after the injection of 10^5 DFTD cells. The day after the final afatinib treatment all mice were sacrificed and tumour mass determined.

There was significant heterogeneity in tumour size particularly in the control group. One of the untreated controls failed to develop a tumour within the 62 days of the trial. Three of the six control mice had relatively small tumours ranging from 4 to 9 mg. Two of the control mice had considerably larger tumours of 55 and 79 mg, which caused most of the variation in results. All four afatinib treated mice grew relatively small tumours ranging from 4 to 19 mg (Figure 6-1). There was no statistical significance between the afatinib treated and untreated groups.

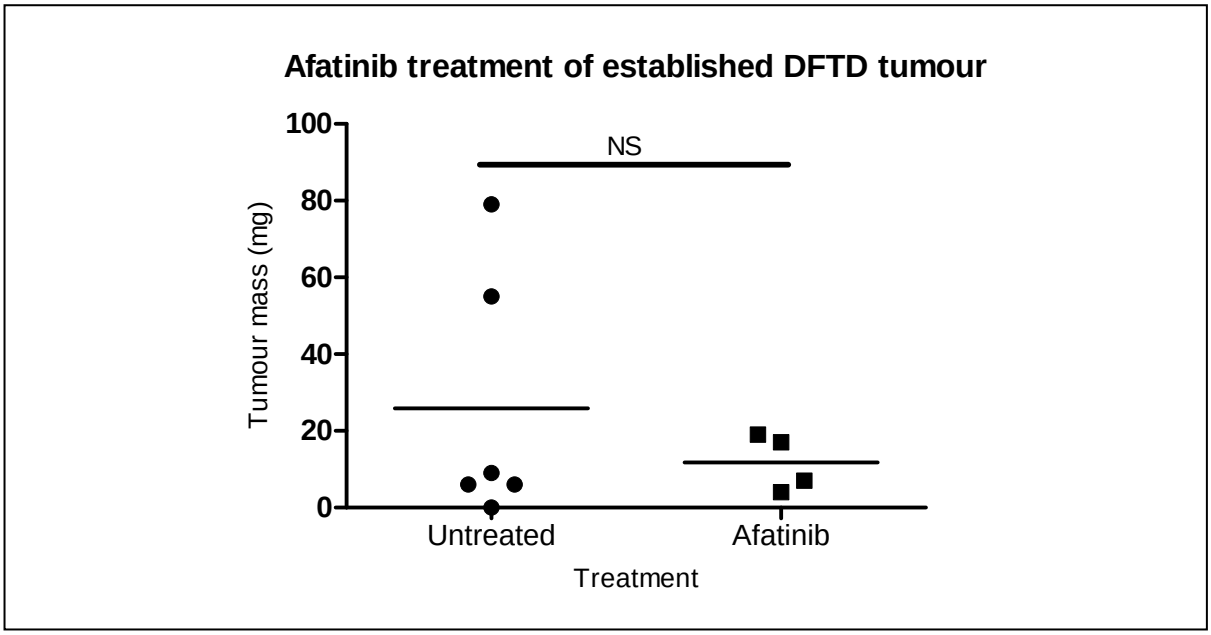

Figure 6-1. NOD/SCID mice were injected with 10^5 C5065 DFTD cells and 35 days later commenced 21 daily afatinib treatments (293 µg) over 26 days. The day after completing afatinib treatments tumour mass was measured at necropsy. (Horizontal bars represent mean. Statistical analysis involved an unpaired two-tailed t-test with 95% CI and revealed there was no significant difference between the treated and untreated cohorts)

Afatinib treatment of established tumours had not demonstrated any inhibition of DFTD growth compared to the controls. This may have been because the effects of the afatinib were overwhelmed once the tumour was established. To maximise the chance of detecting an *in vivo* response to afatinib a prophylactic trial was undertaken to evaluate if commencing the afatinib prior to DFTD inoculation could prevent DFTD establishment.

Ten NOD/SCID mice were treated with six doses of 293 µg of afatinib concealed in peanut butter commencing seven days prior to inoculation with 10^6 DFTD cells. Ten control mice were fed an equivalent amount of peanut butter. The peanut butter and baits were consistently eaten by the mice and this monitored by housing the animals individually. For a further 15 days post DFTD inoculation, the treated mice received daily doses of 293 µg afatinib and the controls peanut butter alone. Palpation of the injection site on the final day of treatment revealed three afatinib treated mice and one control mouse showed the first signs of tumour establishment. Periodic monitoring for tumours occurred over the next 18 days and the experiment was terminated 34 days following inoculation with DFTD cells. At this time all but one control mouse had palpable tumours.

Three of the treated mice and four of the control mice had to be euthanised for ethical reasons and their results are not included as they did not complete the full course of treatment.

Necropsy revealed that all treated and untreated mice developed tumours. The mean mass of tumours was 50 mg for untreated mice and 53 mg for afatinib treated mice. There was significant heterogeneity in tumour mass for both the treated and untreated cohorts of mice. The range for untreated mice was 8 to 74 mg and for afatinib treated mice 20 to 97 mg. There was no significant reduction in tumour mass of afatinib treated mice compared to the control mice (Figure 6-2).

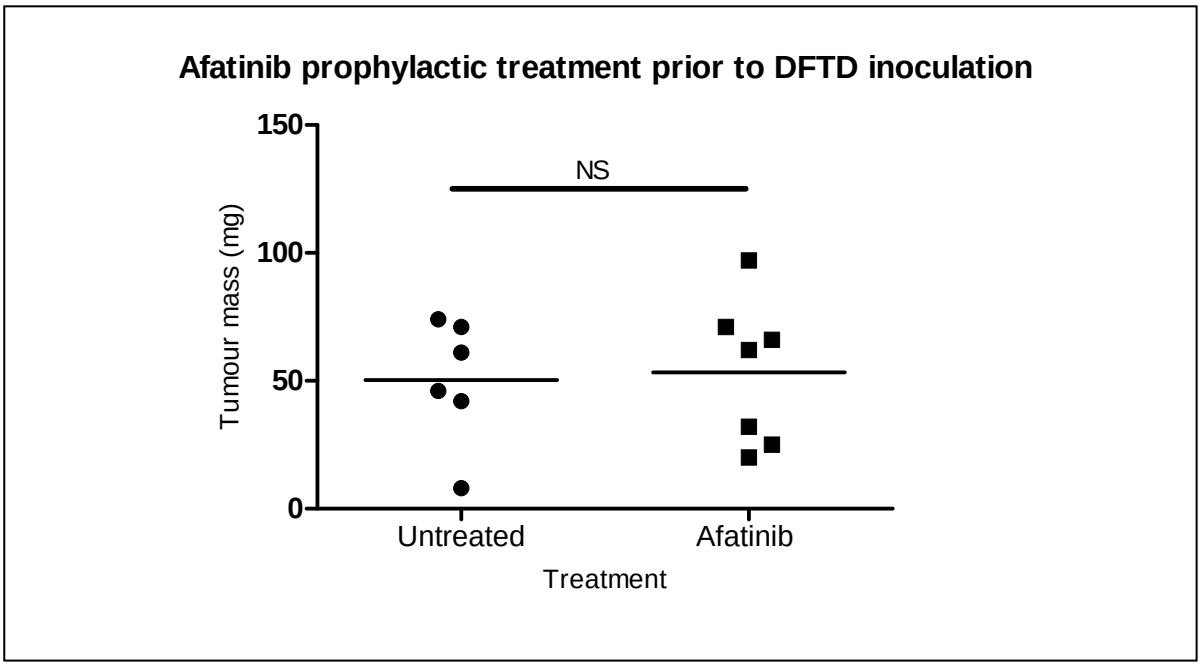

Figure 6-2. Mice were treated with daily doses of afatinib (293 µg) for 6 of 7 days prior to inoculation with 10^6 C5065 DFTD. For a further 15 days following DFTD immunisation the treated mice received daily doses of afatinib. Tumour mass was measured at necropsy 34 days after DFTD inoculation. (Horizontal bars represent mean. Statistical analysis involved an unpaired two-tailed t-test with 95% CI and revealed there was no significant difference between the treated and untreated mice)

Despite promising *in vitro* results for afatinib in the drug screening trial conducted by the Sanger Research Institute (Elizabeth Murchison, personal communication, 2012) this did not translate into *in vivo* protection that would prevent the engraftment of DFTD in the NOD/SCID mice. A further trial was undertaken to evaluate if the growth kinetics were being impacted by afatinib treatment.

Mice were fed peanut butter for seven days prior to and 21 days after, injection with 10^6 DFTD cells. Treated mice received 293 µg of afatinib concealed in the peanut butter and the control mice peanut butter alone. Tumour growth was monitored from day 22 when three treated mice and one control mouse had tumours that could be detected by palpation. There was a high degree of heterogeneity in tumour growth that did not correlate to treatment protocol. All afatinib treated mice established tumours within 40 days and there was no evidence of tumour growth rate inhibition or reduced final tumour volume as a consequence of afatinib treatment (Figure 6-3).

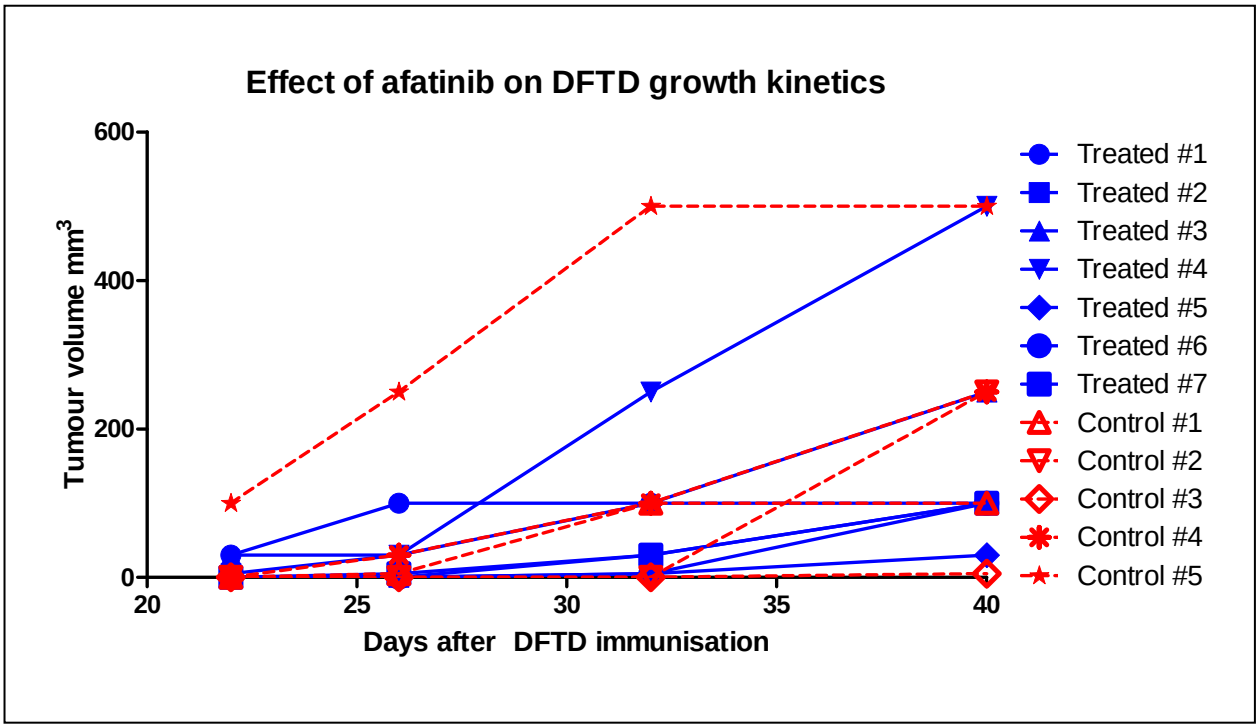

Figure 6-3. Growth kinetics for mice fed with 293 µg afatinib 7 days prior and 21 days after being subcutaneously injected with 10^6 C5065 DFTD cells. Treated mice shown in solid blue lines and untreated controls shown in dashed red lines. Afatinib did not retard tumour growth or prevent establishment of DFTD xenograft.

As there was no evidence for anti-DFTD activity an *in vitro* analysis was performed to check that the afatinib had retained its activity. DFTD cells were cultured for 72 hours and the effect of afatinib on proliferation of DFTD cells was evaluated with an MTT assay. Proliferation was inhibited in a dose-dependent response, with maximum inhibition at 1 x 10^{-5} M. These results showed that the afatinib was biologically active but that 1 x 10^{-5} M was required to prevent DFTD proliferation (Figure 6-4).

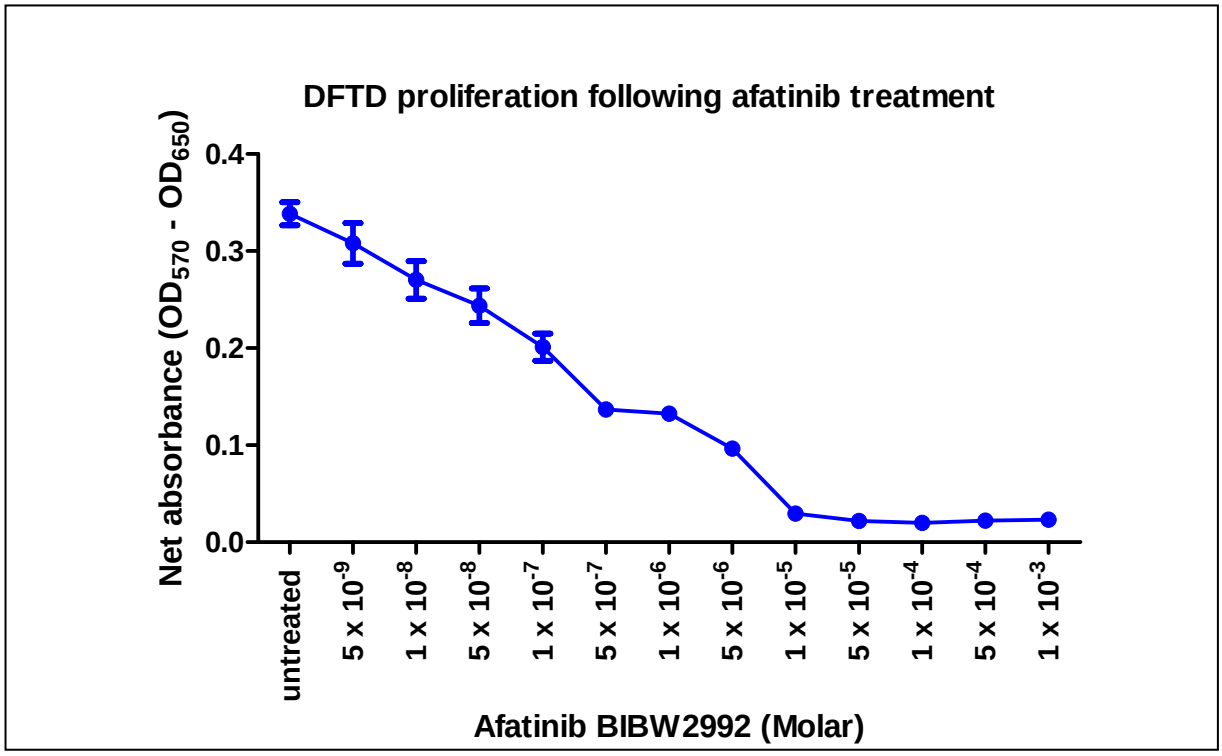

Figure 6-4. C5065 DFTD cells were subjected to 72 hours incubation in flat bottom 96 well plates with BIBW2992 at various concentrations of afatinib. (Data points represent mean of triplicate wells and error bars SEM)

6.2.2 **Treatment of DFTD tumours with withaferin A**

DFTD tumours have a high expression of vimentin (Loh et al 2006a). The over-expression of vimentin in cancer promotes faster tumour growth and metastasis. Withaferin A has the ability to induce vimentin disassembly (Thaiparambil et al 2011) and this might provide a mechanism to target DFTD cells. Consequently, a pilot study was undertaken to see if withaferin A treatment merited further investigation.

Ten NOD/SCID mice were injected with 10^6 viable DFTD cells. On day 14 half of the mice commenced daily withaferin treatments (50 µg/mouse/day as an IP injection) five days per week. They received a total of 21 treatments with the last being given on day 40. The mice were sacrificed on day 41 and tumour mass determined at necropsy.

Necropsy revealed that all treated and untreated mice developed tumours. The mean size of tumours was 33 mg for untreated mice and 64 mg for withaferin treated mice. There was significant heterogeneity in tumour size for both the treated and untreated cohorts of mice. The range for untreated mice was 4 to 61 mg and for withaferin treated mice 14 to 133 mg. There was no significant reduction in tumour mass of withaferin treated mice compared to the control mice (Figure 6-5).

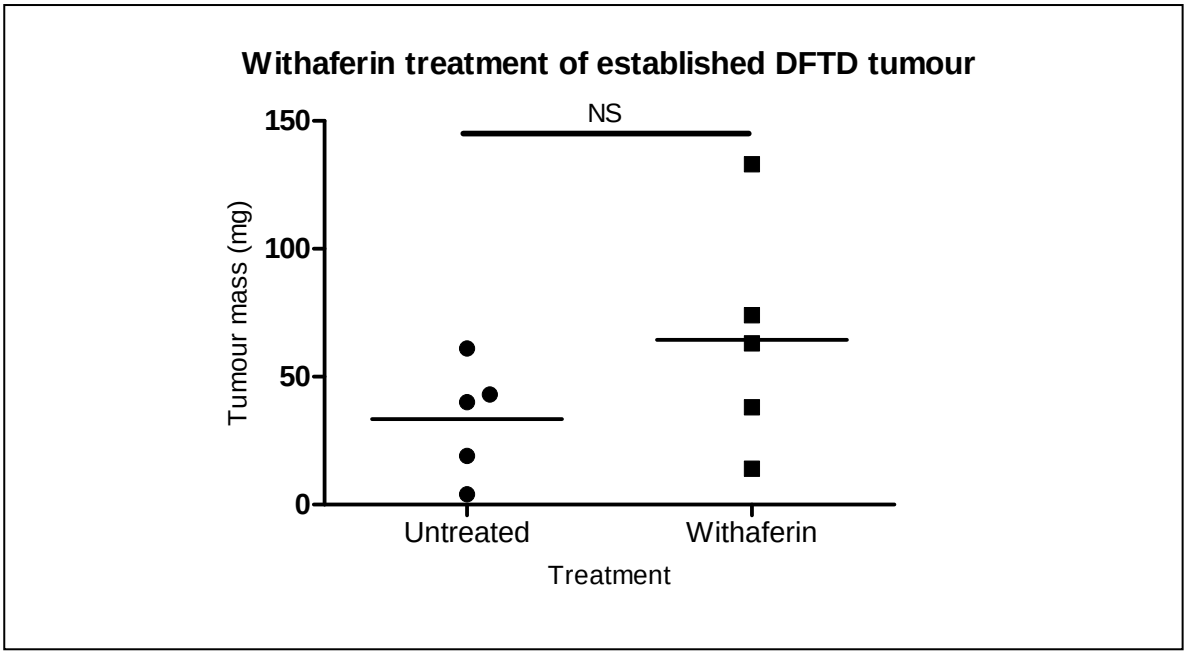

Figure 6-5. 10 NOD/SCID mice were injected with 10^6 C5065 DFTD cells. On day 14 five of these mice commenced 21 daily IP injections of 50 µg withaferin. The other five remained as untreated controls. On day 40 the last injection was given. Tumour mass was measured at necropsy 41 days after DFTD inoculation. (Horizontal bars represent mean and SEM. Statistical analysis involved a unpaired two-tailed t-test with 95% CI and revealed there was no significant difference between the treated and untreated mice)

6.2.3 **Immunomodulation following fucoidan treatment**

Studies have shown that fucoidans can mobilise and activate immune cells as well as alter cytokine expressions (Kwak 2014). In chapter three it was shown that C57/BL6 mice generate immune responses against DFTD cells. This chapter evaluated if fucoidan can alter or enhance this immune response.

Five C57BL/6 mice were conditioned with weekly IP injections of 1.25 mg Fucoidan (*Fucus vesiculosus*) for four weeks. The fucoidan was co-injected with 10^6 viable DFTD cells at the 2nd and 4th week. Five C57BL/6 control mice were injected IP with DFTD cells but no fucoidan at the 2nd and 4th week. The mice were sacrificed at the 5th week and spleens harvested for *in vitro* assays and serum collected for antibody analysis.

During necropsy splenomegaly was visibly obvious in the fucoidan treated mice and consequently the spleens were weighed. Figure 6-6 confirmed that IP injection of fucoidan significantly increased spleen mass by an average of 77% compared to the control mice ($P < 0.05$, Student's unpaired t-test). This correlated with a significant increase in the number of splenocytes recovered from the spleens of the fucoidan treated mice compared to the controls ($P < 0.01$, Student's unpaired t-test) (Figure 6-6).

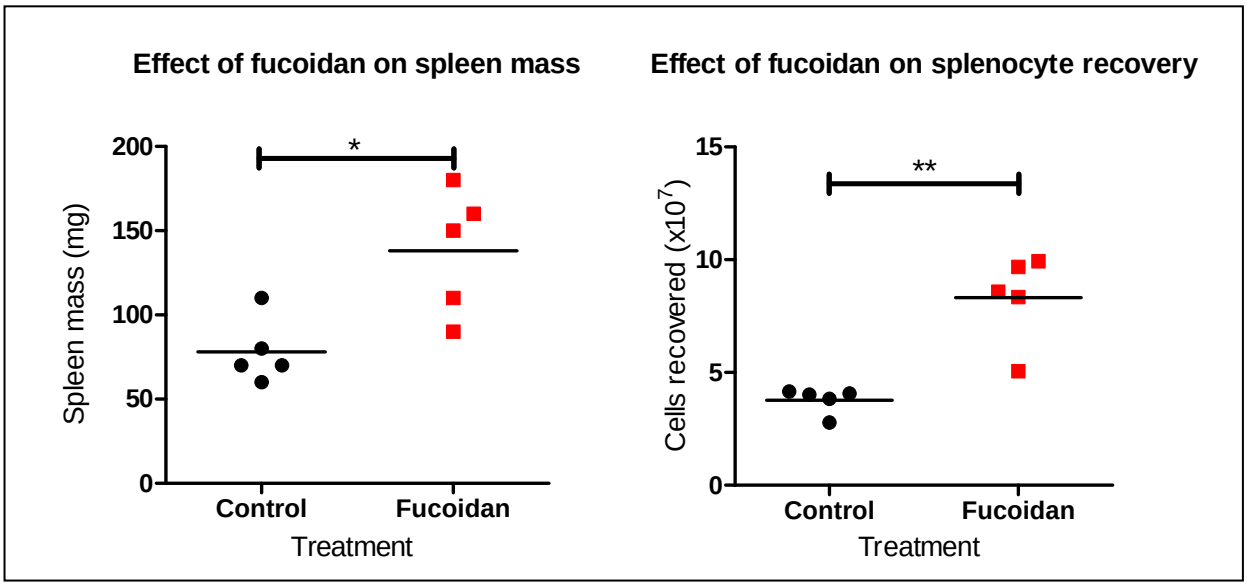

Figure 6-6. The spleens from fucoidan treated mice and untreated controls were weighed and the mean weight for fucoidan treated mice was 138 mg and the controls 78 mg. The splenocytes were recovered using histopaque separation. The cell recovery for fucoidan treated mice had a mean value of 8.3 x 10^7 cells per spleen compared to the control mice which had a mean value of 3.8 x 10^7 cells per spleen. Horizontal bars represent mean. (Probability calculated by Student's unpaired t-test * P < 0.05, ** P < 0.01)

Splenomegaly induced by fucoidan was due to increased cell number (Figure 6-6). To evaluate if the splenomegaly was the result of the expansion or migration of a specific population of lymphocytes the splenocytes were immunophenotyped with anti-mouse antibodies specific to CD8, CD4, NK cells and the activation marker CD69. There was no significant difference between the treated and untreated mice. This indicates that fucoidan treatment caused a non-specific increase in the number of cells and did not target a specific population. Less than 1% of the splenocytes expressed the T cell/NK cell activation marker CD69 and this was not significantly increased by fucoidan treatment but the total number of activated cells would have been higher in the fucoidan treated mice due to splenomegaly (Figure 6-7).

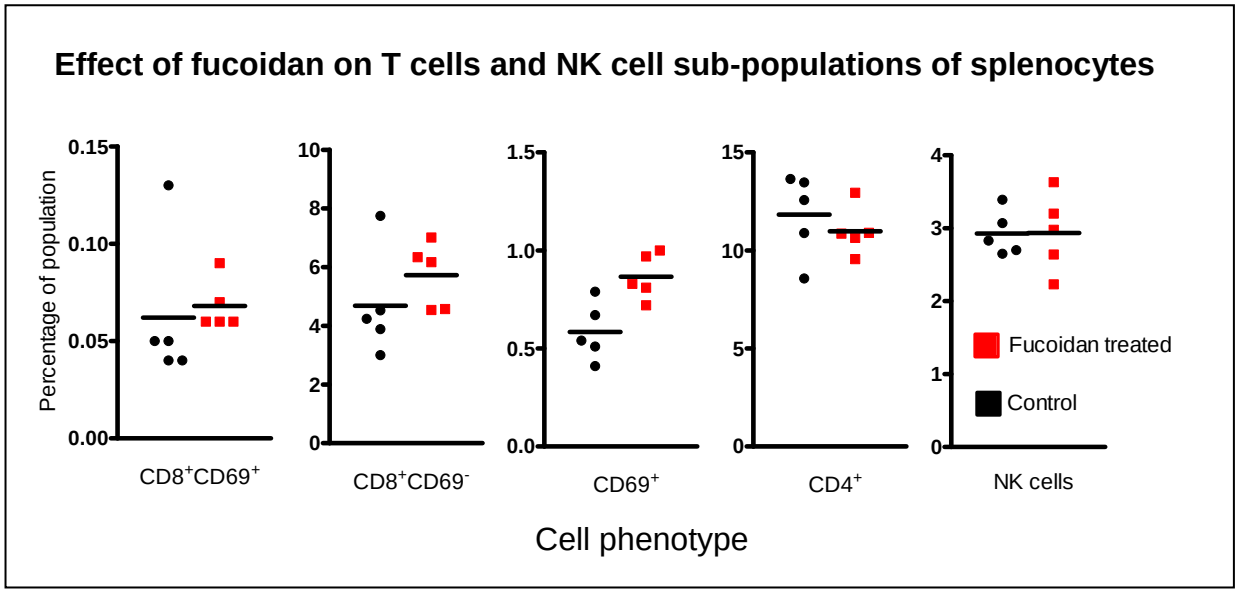

Figure 6-7. Splenocytes from the fucoidan treated mice and the control mice were labelled with anti-mouse antibodies specific to CD8, CD4, NK cells and the activation marker CD69. A one way ANOVA with Bonferroni's multiple comparison test was performed revealing no significant difference in the population of cells obtained from the spleens of fucoidan treated C57/BL6 mice compared to the control C57/BL6 mice. (Horizontal bars represent mean)

The most sensitive indicator of immune response against DFTD cells in C57/BL6 mice has been serum antibody levels. Consequently, DFTD specific antibody levels for fucoidan treated mice were evaluated to see if the immune response had been altered by fucoidan treatment.

The fucoidan treated mice demonstrated increased levels of IgG antibody responses specific to DFTD cells compared to the control mice. The fucoidan treated mice had statistically significant higher levels of IgG1 compared to the untreated controls. Three of five fucoidan treated mice had higher levels of IgM than the five controls but this was not statistically significant due to the variability in the response. There was no significant change to the IgG2a, IgG2b or IgG3 antibody responses (Figure 6-8).

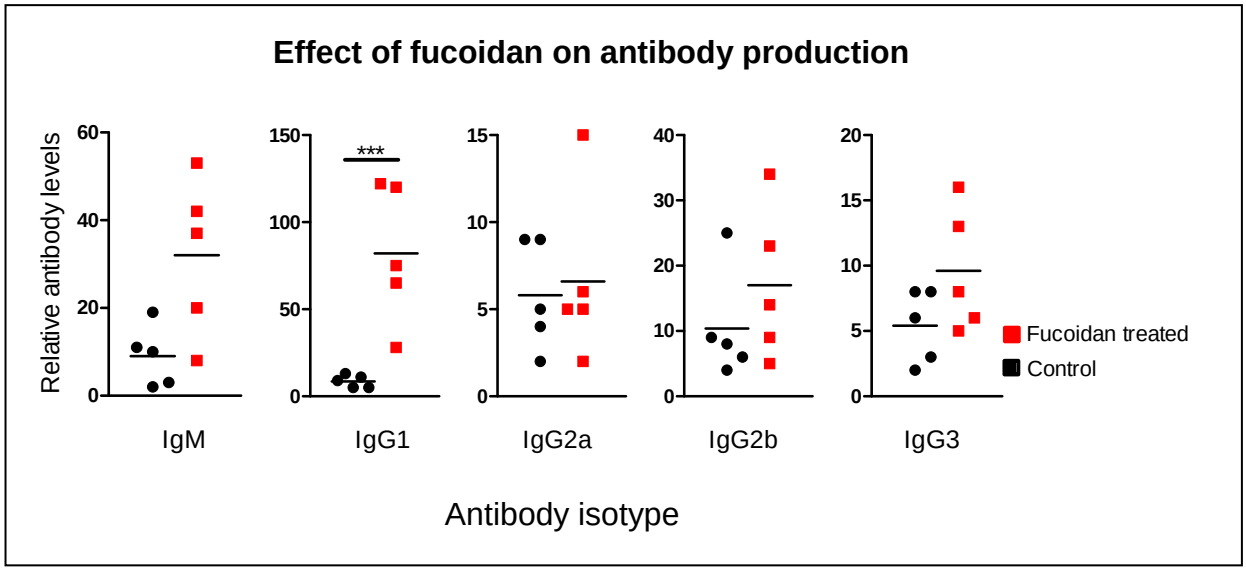

Figure 6-8. Serum was collected from the fucoidan treated mice and control mice at necropsy. Horizontal bars represents mean of five mice. A one way ANOVA with Bonferroni's multiple comparison test was performed revealing a significant difference (* P < 0.001) in the expression of IgG1 by the fucoidan treated C57/BL6 mice compared to the control C57/BL6 mice. (Horizontal bars represent mean)**

Fucoidan treatment had resulted in splenomegaly, enhanced antibody levels and elevated cytokine production. It was uncertain if these changes to the immune system were pro-tumour or anti-tumour responses. Effective anti-tumour responses would promote cell-mediated cytotoxic responses against DFTD cells. For this reason, the splenocytes of the fucoidan treated and untreated mice were compared for their cytotoxicity against DFTD.

Rather than observing a cytotoxic response against DFTD cells with the splenocytes from the control mice and the fucoidan treated mice revealed less cell death as splenocyte ratio increased (Figure 6-9).

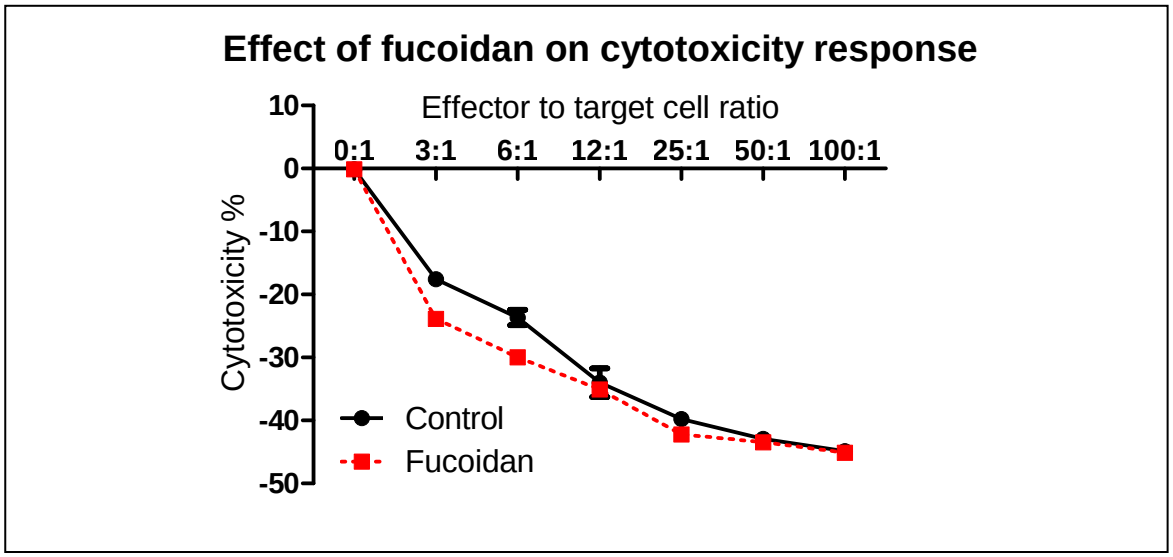

Figure 6-9. The splenocytes from the five fucoidan treated and the untreated control mice were used as effector cells in an 18 hour cytotoxicity assay against CellTrace Violet labelled C5065 DFTD cells. Propidium iodide was used to determine viability.

An apparent inverse cytotoxicity-response was observed that was similar for both the fucoidan treated and control mice splenocytes. (Data points represent mean of five mice and triplicate technical replicates of each. Error bars represent SEM.)

The apparent inverse cytotoxicity-response against DFTD cells may have been due to the splenocytes inducing proliferation or preventing death of the DFTD cells. To evaluate this, the flow cytometry data were analysed as a viability assay to measure proliferation. The viable cells were gated and based on fluorescent intensity of CellTrace Violet divided cells were identified. These daughter cells were counted as a percentage of the viable cells and recorded as proliferation.

Proliferation of DFTD cells was induced by even low ratios of splenocyte to DFTD cells. Proliferation increased in a cell dose-dependent response and was continuing to increase at 100:1 splenocyte to DFTD cell ratio. Splenocytes from fucoidan treated and untreated control mice produced the same result (Figure 6-10).

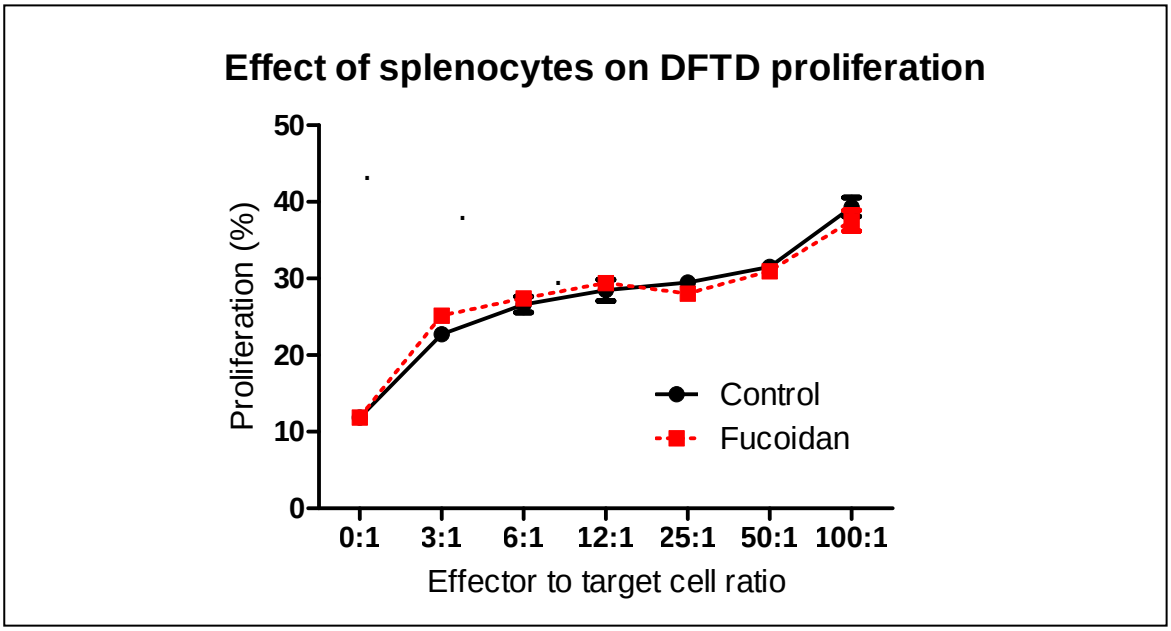

Figure 6-10. CellTrace Violet fluorescence levels of DFTD cells were used to identify DFTD cells which had divided during the 18 hours incubation period of the cytotoxicity assay. These daughter cells were counted as a percentage of the viable cells and recorded as proliferation. (Data points represent mean of five mice and triplicate technical replicates of each. Error bars represent SEM)

Fucoidan had increased the number of splenocytes but did not appear to increase their cytotoxicity against DFTD cells but did enhance the production of DFTD specific antibodies in the serum of treated mice. Fucoidan treatment may increase the sensitivity to non-specific stimuli such as concanavalin A. To investigate this, splenocytes from fucoidan treated mice were stimulated with concanavalin A to induce LAK cells.

The LAK cells produced from splenocytes of the control mice induced an apparent inverse cytotoxicity-response against DFTD cells consistent with inhibited spontaneous death and/or promotion of proliferation. In contrast, the LAK cells from the fucoidan treated mice produced an inverse cytotoxicity response until the 3:1 LAK cell to DFTD cell ratio. This level of inverse cytotoxicity did not continue in a cell dose-dependent response and remained relatively stable. There was a highly significant difference between the LAK cell killing response of the fucoidan treated and the control mouse splenocytes at the highest effector to target cell ratios (Figure 6-11).

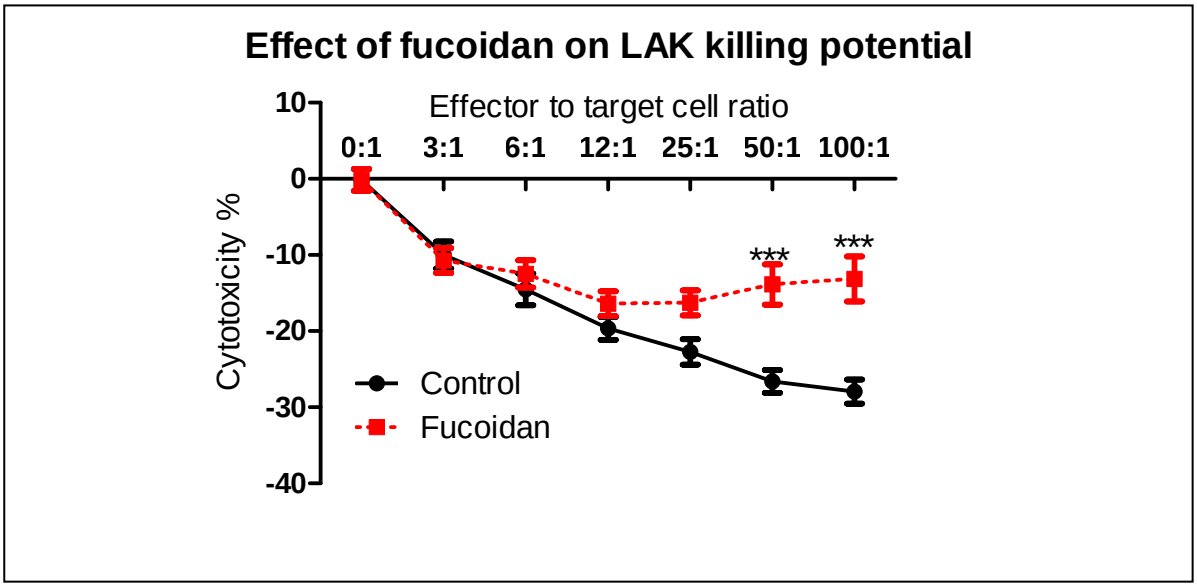

Figure 6-11. Splenocytes from fucoidan treated and untreated mice were cultured in RPMI-10 media supplemented with 5 µg/ml Con A to produce LAK cells. The LAK cells were then used as effector cells in an 18 hour cytotoxicity assay against CellTrace Violet labelled C5065 DFTD cells. Propidium iodide was used to determine viability.

An apparent inverse cytotoxicity-response that increased in a cell dose-dependent manner was observed for LAK cells from the control mice. In contrast an apparent inverse cytotoxicity response that plateaued was observed from the LAK cells from fucoidan treated mice. (Data points represent mean of five mice and triplicate technical replicates of each. Error bars represent SEM. Two way ANOVA with Bonferroni post-tests were performed ** P < 0.01 and *** P < 0.001)

The splenocytes from these fucoidan treated and untreated control mice had induced proliferation of DFTD cells rather than cytotoxicity (Figure 6-10). The splenocytes had been activated into LAK cells and produced an inverse cytotoxic response against DFTD cells. The LAK cells from the fucoidan treated mice and the untreated controls responded differently. It was unknown if this was due to different abilities to induce proliferation in DFTD cells. The flow cytometry data were re-analysed as a viability assay to determine the percentage of viable cells that resulted from proliferation during the 18 hour incubation period.

Proliferation of DFTD cells was induced by even low ratios of LAK cells to DFTD cells from both the control and fucoidan mice. Proliferation increased in a cell dose-dependent response for the LAK cells from control mice but remained relatively level for the LAK cells from fucoidan treated mice. At 100:1 LAK cell to DFTD cell ratio the difference between the proliferation induced by the control mice and the fucoidan treated mice was very significant (Figure 6-12).

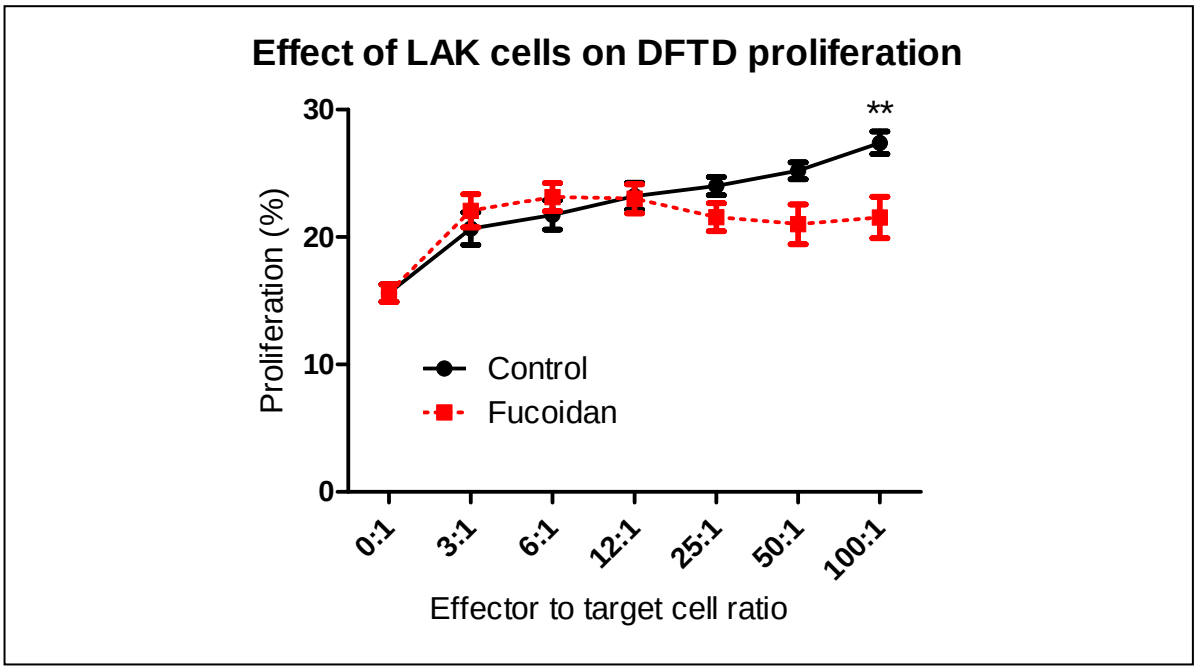

Figure 6-12. LAK cells were generated from the splenocytes of fucoidan treated and untreated mice. The LAK cells were incubated with DFTD cells for 18 hours and DFTD cell proliferation was calculated.

A significantly higher level of proliferation was observed by the LAK cells produced from control mice at 100:1 LAK cell to DFTD cell ratio. (Data points represent mean of five mice and triplicate technical replicates of each. Error bars represent SEM. Two way ANOVA with Bonferroni post-tests were performed ** P < 0.01 and *** P < 0.001)

The observation of different responses to DFTD cells by the LAK cells of the fucoidan treated mice to the controls indicated functional changes had been induced by fucoidan. To investigate this, the flow cytometry data were analysed to determine proliferation and cell death.

When DFTD cells labelled with CellTrace Violet divide the fluorescence is shared between the daughter cells and this facilitates evaluation of cell proliferation. Scatter plots at 6:1 LAK to DFTD cell ratio revealed that the DFTD cells which had divided were dying. This was similar for DFTD cells cultured with LAK cells from control and fucoidan treated mice (Figure 6-13 plots A and C). At 100:1 LAK to DFTD cell ratio dividing DFTD cell death occurred in the cultures containing LAK cells from fucoidan treated mice (Figure 6-13 plot D). In contrast, the dividing DFTD cells in the cultures containing LAK cells from control mice were viable (Figure 6-13 plot B).

The balance between cytotoxicity and protection of dividing cells shifted towards increased cytotoxicity in LAK cells sourced from fucoidan treated mice. In contrast, LAK cells sourced from control mice had increasing protective effect that overwhelmed any cytotoxic activity as E:T ratios increased (Figure 6-13).

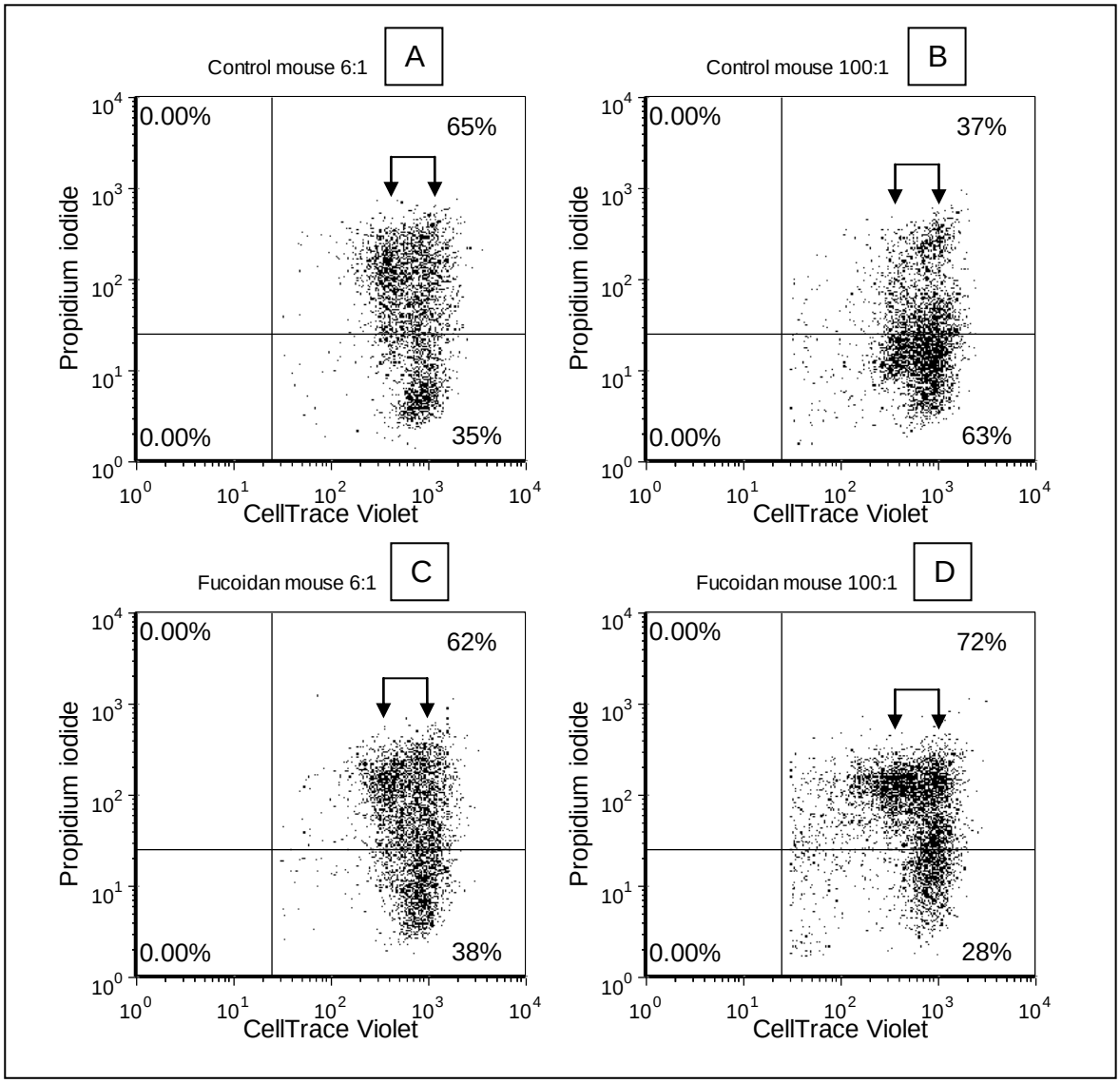

Figure 6-13. The scatter plots are representative of the typical responses observed for fucoidan treated and untreated mice at 6:1 and 100:1 effector to target (E:T) cell ratios. There are two main intensity levels of CellTrace Violet representing cell division indicated by arrows. The fucoidan treated mice LAK cells have killed the dividing DFTD cells while the control mice LAK cells have protected the dividing DFTD cells in a E:T cell dose-response.

Fucoidan demonstrated an immunomodulatory effect on treated mice. This was evident in the splenomegaly (Figure 6-6), enhanced antibody responses (Figure 6-8). To further evaluate the immunomodulation effect of fucoidan the splenocytes from treated and untreated mice were cultured *in vitro* with DFTD cells and supernatant analysed for cytokine levels after 72 hours.

There was some variation in the cytokine responses. Splenocyte cultures from the fucoidan treated mice showed elevated levels of IL-4, IL-6, IL-10 and TNF-α. There was no difference in IFN-γ levels. Splenocyte cultures from four of five control mice did not contain detectable IL-10 while one culture had relatively low levels. In contrast IL-10 was highly expressed by the splenocytes from the fucoidan treated mice.

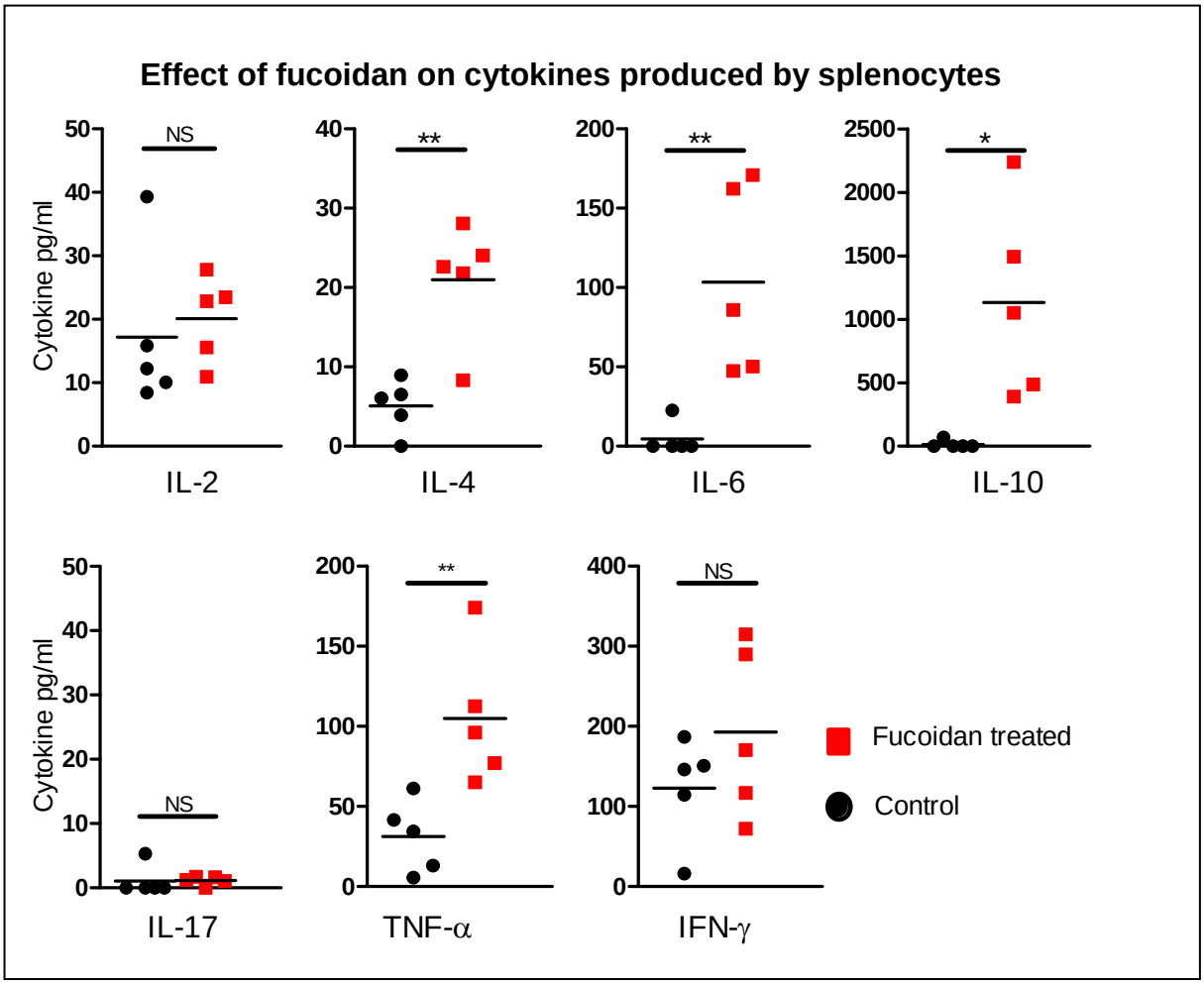

Figure 6-14. Splenocytes from fucoidan treated C57/BL6 mice and control mice were incubated at 100:1 ratio with C5065 DFTD cells for 72 hours. A CBA T_H1/ T_H2/T_H17 microbead cytokine kit was used to evaluate the cytokine levels in the culture supernatant. (Horizontal bars represent the mean of five mice. Statistical analysis involved a Student's unpaired two-tailed t-test with 95% CI. ** P < 0.01 and * P < 0.05)

Splenocytes from fucoidan treated mice and control mice stimulated with concanavalin A produced LAK cells that had significantly different activity against DFTD cell survival (Figure 6-12). To further evaluate the DFTD specific activity of these LAK cells they were cultured *in vitro* for 72 hours with DFTD cells and the supernatant was analysed for cytokine production.

Supernatants from concanavalin A stimulated splenocytes (LAK cells) from the fucoidan treated mice contained significantly lower levels of IL-2, IL-4 TNF-α and IFN-γ than the supernatants from concanavalin A stimulated splenocytes (LAK cells) from the untreated mice. The other tested cytokines IL-6, IL-17a and IL-10 appeared to be lower but this was not statistically significant (Figure 6-15).

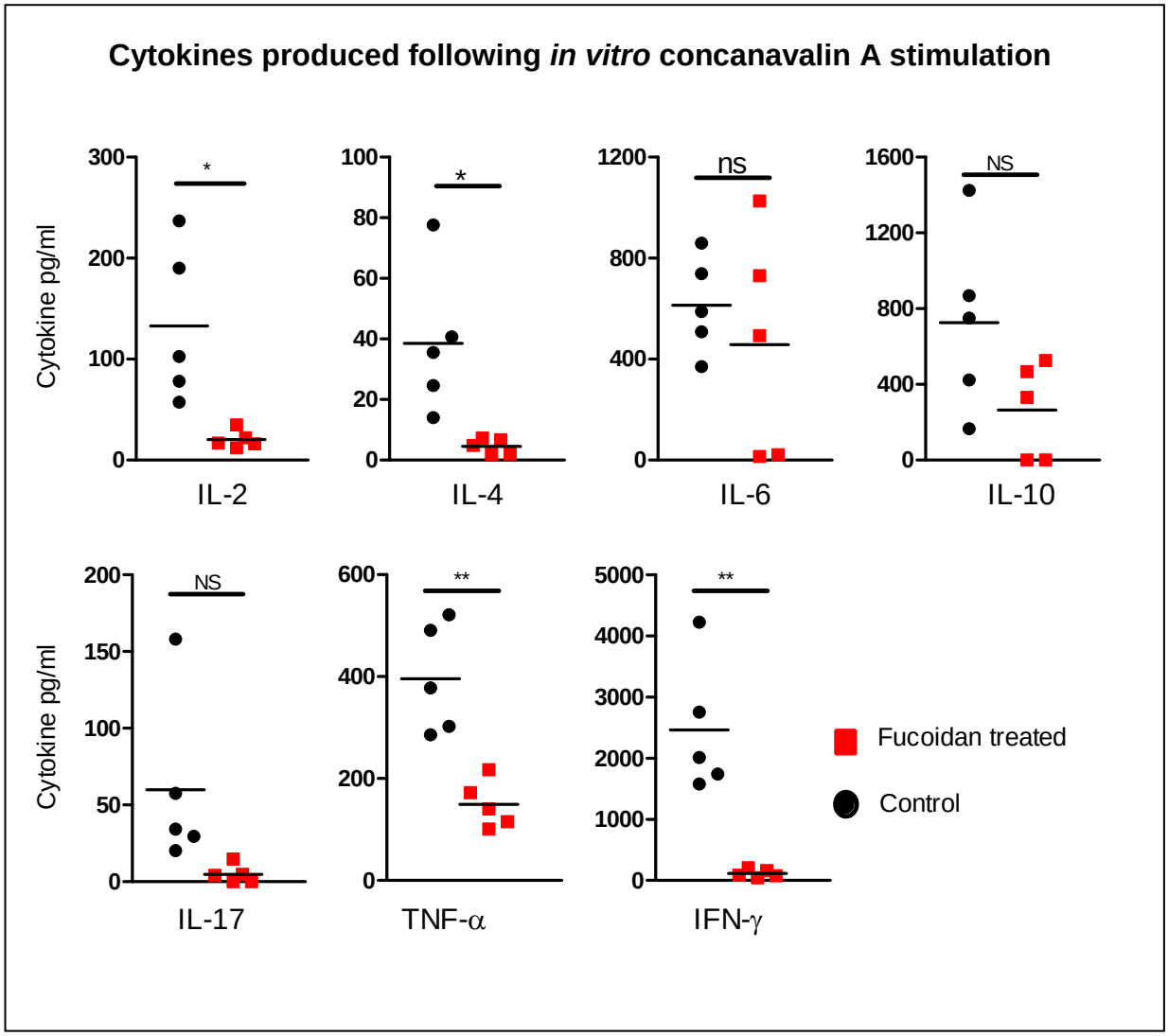

Figure 6-15. Splenocytes from fucoidan treated C57/BL6 mice and control mice were incubated for 48 hours in RPMI-10 medium supplemented with 5 µg/ml Con A. These splenocytes were then incubated at 100:1 ratio with C5065 DFTD cells for 72 hours. A CBA T_H1/ T_H2/T_H17 microbead cytokine kit was used to evaluate the cytokine levels in the culture supernatant. (Horizontal bars represent mean of five mice. Statistical analysis involved a Student's unpaired two-tailed t-test with 95% CI. ** P < 0.01 and * P < 0.05)

6.2.4 **Intratumoural injections of imiquimod**

Imiquimod had been shown to induce upregulation of MHC class I expression of DFTD cells when added to the culture medium (Patchett 2013). The ability to upregulate MHC class I expression in vivo would be an important step towards inducing immune responses by Tasmanian devils against DFTD cells. To test if direct intratumoural injections of imiquimod could upregulate MHC class I expression of DFTD tumour cells *in situ*, six NOD/SCID mice were engrafted with DFTD tumours. When the tumours had grown to a size that was clearly visible, five of the mice received 100 µg of imiquimod in 100 µl daily intratumoural injections for three days. One mouse was the untreated control. One day after the third imiquimod injection the tumours were harvested and disassociated into single cell suspension. The cells were then labelled with serum collected from a Tasmanian devil (Missy serum # 11-4-12) which reacts against IFN-γ treated cultured DFTD cells but not un-treated cultured cells. It has been presumed that the serum binds to MHC class I but this is not verified. There was no monoclonal antibody for surface MHC class I on Tasmanian devil cells at this point of time so the serum from Missy was the best available indicator of upregulation of MHC class I.

As shown in Figure 6-16 nearly 100% of the cells obtained from the tumours treated with imiquimod labelled positive with Missy serum. In comparison the untreated tumour had bi-modal labelling with Missy serum and approximately 50% of the cells obtained from the untreated tumour labelled positive.

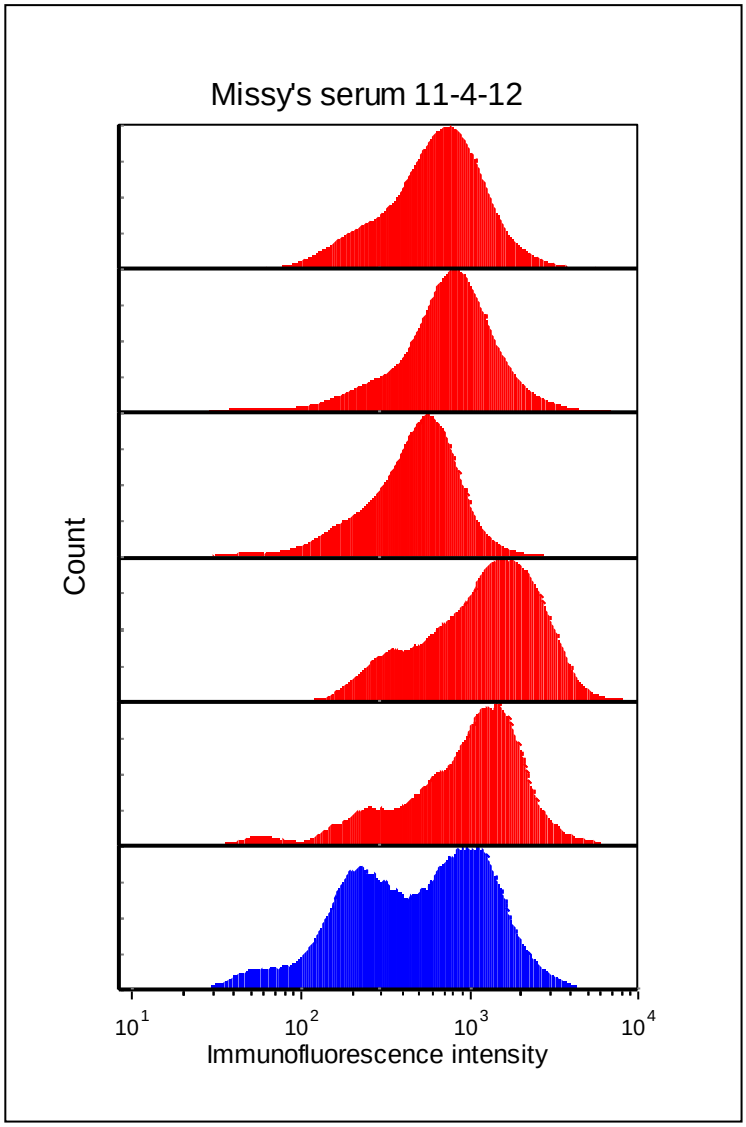

Figure 6-16. DFTD tumour cells were labelled with serum from a Tasmanian devil that contained antibodies specific for IFN-γ treated DFTD cells. DFTD xenograft cells obtained from the untreated mouse (shown in blue) demonstrated bi-modal labelling with Missy serum # 11-4-12. DFTD xenograft cells obtained from the five imiquimod treated mice (shown in red) had nearly 100% of the cells label positive.

When an antibody specific for Tasmanian devil β_2-microglobulin became available, four NOD/SCID mice were engrafted with DFTD tumours. When the tumours had grown to a size that was clearly visible, two of the mice received 100 µg of imiquimod in 100 µl daily intratumoural injections for three days. The other two mice remained untreated. The day after the third imiquimod injection the tumours were harvested and disassociated into single cell suspension.

Tumours that were treated with imiquimod had an increased expression of β2-microglobulin compared to the untreated tumours. This is consistent with the results of the previous paragraph using devil serum which reacts against IFN-γ treated DFTD cells. These results provide evidence that imiquimod upregulates MHC because β_2-microglobulin is transported to the surface of cells as part of the MHC molecule (Figure 6-17 panel A). Missy's serum 11-4-12 differentiated between the imiquimod treated and untreated tumours which is consistent with the premise that MHC^+ DFTD are targeted by this serum (Figure 6-17 panel B).

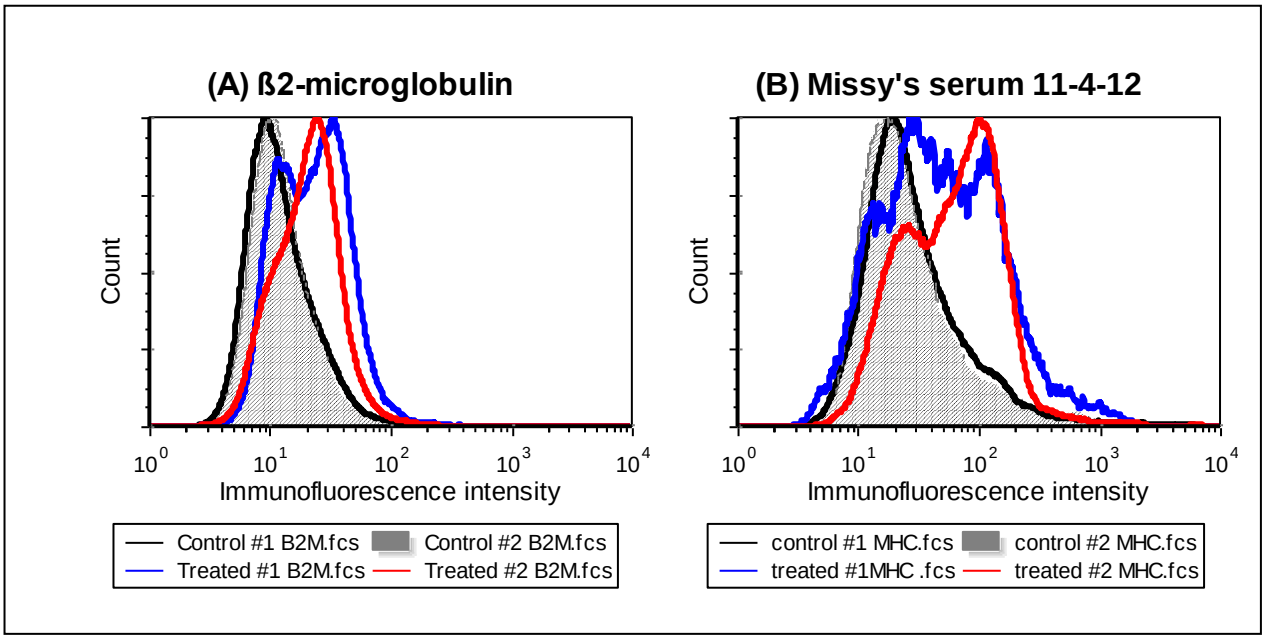

Figure 6-17. Murine DFTD tumour cells were labelled with Missy's serum or anti-β_2-microglobulin. Cells obtained from the untreated mice (shown in black or grey) had low fluorescence intensity. Cells obtained from the two imiquimod treated mice (shown in blue or red) showed a positive shift in fluorescence levels for both Missy's serum and anti-β_2-microglobulin.

6.3 **Discussion**

A major impediment to developing an effective vaccine or immunotherapy against DFTD is the lack of access to Tasmanian devils for clinical trials. Any immunotherapy trial should be conducted in the host species but since this is not always possible we developed an informative mouse model. This will allow us to undertake preliminary studies to evaluate treatment options that could be applied to the Tasmanian devil.

In this chapter, four treatments were evaluated for their efficacy against DFTD. Two of these were chemotherapy drugs that targeted specific pathways within the DFTD cells. The other two were compounds that modulated the immune response of the host. One of these, imiquimod, also had a direct effect on the DFTD cells.

6.3.1 **Afatinib**

It was unknown if the anti-DFTD activity of afatinib observed *in vitro* by the Sanger Research Institute (Elizabeth Murchison, personal communication, 2012) would translate to protection against DFTD *in vivo*. To evaluate the efficacy of afatinib *in vivo*, NOD/SCID mice were fed daily with afatinib concealed in peanut butter. This was to represent the use of meat baits that was envisioned for Tasmanian devils in the wild if afatinib was effective. This was an efficient drug delivery method for the mice as they preferentially consumed the peanut butter over their normal diet.

In the first afatinib trial, DFTD cells were given sufficient time to establish as small tumours before afatinib treatment commenced. This represented the effect of afatinib consumed by diseased Tasmanian devils in the wild. The day after the last dose the treated and untreated mice were sacrificed and tumour growth determined at necropsy. There was no evidence of any inhibition of DFTD growth *in vivo* as a result of afatinib treatment.

In the second afatinib trial, prophylactic therapy was assessed that represented what would occur in the wild if a healthy Tasmanian devil consumed afatinib baits prior to disease exposure. Afatinib treatment was commenced 7 days prior to DFTD inoculation and continued for a further 15 days afterwards. When the mice were examined at necropsy there was no evidence of any protection against DFTD

engraftment or inhibition of DFTD growth as a result of afatinib prophylactic treatment.

The third afatinib trial evaluated if the growth kinetics of DFTD were altered by afatinib treatment. Mice were fed afatinib 7 days prior to and 21 days after, injection with DFTD cells. Afatinib treatment had no effect on DFTD establishment or rate of growth.

A fourth afatinib experiment was undertaken *in vitro*. This confirmed the afatinib used in our *in vivo* trials had a comparable level of biological activity as that used by the Sanger Research Institute. To determine the concentration of afatinib required to completely prevent DFTD proliferation our *in vitro* experiment extended the upper range of afatinib concentration beyond that tested by the Sanger Institute (Elizabeth Murchison, personal communication, 2012). It required fiftyfold increase in afatinib compared to the highest levels tested at the Sanger Research Institute to stop proliferation.

The promising *in vitro* results did not translate *in vivo*. Both as a prophylactic treatment and as a treatment against established DFTD tumours afatinib did not protect the NOD/SCID mice or cause regression of the tumours. Possible explanations could be the mode of drug delivery, dosage, or the *in vivo* environment itself.

If Afatinib proved to be protective against DFTD the intention was to lace baits of meat with the drug for distribution in the wild since the drug can be taken orally. This approach has a long history of being used successfully to deliver vaccines, drugs and poisons to free roaming wild populations of carnivores (Knobel 2001, Linhart et al 1993). In Australia, aerial distribution of strychnine baits were used in the 1940'S to control predators of livestock (Linhart et al 1993). Currently 1080 predator-baiting is practised in Australia to protect native fauna from introduced species such as the red fox (*Vulpes vulpes*) (Kinnear et al 2010).

Placing afatinib concealed in food is not recommended by the manufacturer who advises a fasting period of 3 hours before and one hour after taking afatinib (Boehringer Ingelheim International 2014). In human trials the consumption of afatinib with high fat food decreases exposure to the drug between 39 and 50%

(Boehringer Ingelheim International 2014). Since food baits would be the only practical option for Tasmanian devils the use of food baits for the mouse trial was valid despite reducing efficiency of drug delivery.

Even if 50% of the drug was unavailable because of the high fat content, the dose given to the mice based on the animal's weight was 10 to 20 times the dose recommended by the manufacturer for human trials. (Boehringer Ingelheim International 2014, Yap et al 2010). The dose of 12.5 mg/kg per day of afatinib was recommended by the Sanger Research Institute (Elizabeth Murchison, personal communication, 2012).

The lack of *in vivo* response to afatinib may be due to the *in vivo* environment itself. Responders to afatinib in human trials tend to relapse after developing resistance (Nanjo et al 2013). This resistance can be due to tumour heterogeneity or hepatocyte growth factor (HGF) (Nanjo et al 2013). HGF can be over expressed by tumours as an autocrine resistance mechanism (Nanjo et al 2013). Another significant source of HGF can be the stromal fibroblasts that change the tumour microenvironment in a paracrine fashion (Nanjo et al 2013). If DFTD cells were the source of HGF then resistance to afatinib in the *in vitro* setting would have been observed. The observation of afatinib activity against DFTD cells *in vitro* but not *in vivo* could be explained by HGF. Resistance to HGF can be overcome by combining afatinib treatment with 10 mg/kg crizotinib. This is a toxic therapy that causes severe intestinal mucosal damage and can be lethal if the dosage is not carefully monitored (Nanjo et al 2013). Although combined afatinib and crizotinib therapy may prove effective against DFTD, it could be too toxic to distribute by baits in the wild.

Afatinib is not a treatment that warrants further *in vivo* trials. Monotherapy with afatinib has shown minimal benefits in human clinical trials with severe adverse effects (Nanjo et al 2013). There may be value in studying the effects of afatinib on DFTD cells *in vitro* to further our knowledge about the functional pathways and mechanisms in DFTD cells.

6.3.2 **Withaferin A**

Withaferin A is associated with fewer side effects and lower toxicity compared to afatinib (Yang et al 2012). Of the many anti-cancer mechanisms of withaferin A, the one pertinent to this work was the ability of withaferin A to disassemble vimentin (Thaiparambil et al 2011, Yang et al 2012). DFTD cells express high levels of vimentin (Loh et al 2006a, Tovar 2012). Many studies have revealed regulatory functions of vimentin in cancers that promote cell migration, enhanced invasive capacity and stimulate proliferation (Satelli and Li 2011). Therefore, over-expression of vimentin indicates an aggressive cancer associated with metastatic disease and poor prognosis (Yang et al 2012). As well, secreted vimentin can protect tumours by neutralising NK cell activity through blocking the NKp46 receptors (Satelli and Li 2011).

Despite DFTD cells expressing vimentin and the promising human trials, withaferin A did not appear to have any effect on DFTD cells in NOD/SCID mice. All mice developed tumours, growth rates were the same and there was no reduction in the size of established tumours. There are several possibilities for this result. It may be due to the dose and number of fractions of withaferin A. The withaferin A dose in this study approached the maximum tolerated dose but was divided into 21 fractions. Excessive levels of withaferin A can be toxic and the toxic effect of withaferin A is cumulative while the tumour killing effect is not (Kamath et al 1999, Sharada et al 1996).Tumour cells may be able to tolerate, or recover from, low levels of withaferin A whereas high acute levels of withaferin A may be required for anti-tumour activity (Kamath et al 1999, Sharada et al 1996). Investigations by other researchers have revealed that three daily doses of 30 mg/kg or two daily doses of 40 mg/kg is the maximum tolerated dose of withaferin A. This may be more effective than a similar dose divided in more fractions (Kamath et al 1999, Sharada et al 1996).

This pilot study was conducted in NOD/SCID mice with the aim of determining if withaferin A had anti-DFTD activity *in vivo*. *In vitro* studies could guide the choice of concentration but the dose rates *in vivo* can only be determined *in vivo*. Due to the long-term nature of this study, time did not permit further *in vivo* studies. Such studies would have included two or three intraperitoneal injections with higher doses. Established tumours could be directly injected with withaferin A to determine if intratumoural injections target DFTD cells more efficiently than systemic treatment.

6.3.3 **Fucoidan**

Fucoidan treatment resulted in splenomegaly due to an overall increase in cell numbers. Analysis of the T cell/NK cell activation marker CD69 did not provide any evidence for an increase in the activation of these cells. As it is unlikely that proliferation caused the splenomegaly, mobilisation of cells to the spleen may have occurred. The significant increase in DFTD-specific IgG1 antibody in the serum of fucoidan treated mice implicates B cell activation with CD4 helper T cell mediated isotype switching.

Fucoidan has been shown to be an immunomodulatory compound that modulates the ratio of T_H1:T_H2 immune responses towards T_H1 profiles (Kar et al 2011). However, in this study fucoidan polarised the immune response against DFTD cells towards a T_H2 response. This was evident in the significantly high level of IgG1 in the serum of fucoidan treated mice that were challenged with DFTD cells compared to mice challenged with DFTD cells only. Splenocytes from the fucoidan treated mice produced very high levels of IL-10 (T_H2 cytokine) and elevated levels of IL-6 when cultured with DFTD cells *in vitro*. This is explains the IgG1expression observed in the mice since IL-6 and IL-10 promote the switch to the IgG1 isotype by B cells. Such T_H2 responses are very effective against bacterial infections but less so against cancers which are better targeted by T_H1 responses.

Fucoidan treatment of mice did not increase cytotoxic activity by their splenocytes against DFTD cells. What was observed was an apparent inverse cytotoxicity-response curve by both the treated and untreated mouse splenocytes. This indicated that the splenocytes, rather than being cytotoxic, were protecting DFTD cells from spontaneous cell death or promoting proliferation of DFTD cells. This function was not altered by fucoidan treatment.

However, fucoidan treatment did alter function of splenocytes stimulated by concanavalin A into LAK cells. Concanavalin A stimulation of splenocytes from fucoidan treated mice resulted in different effects on DFTD cell survival *in vitro* and different cytokine production when cultured with DFTD cells. LAK cells from the fucoidan treated mice killed dividing cells preventing proliferation. The LAK cells from the control mice did not kill dividing cells promoting proliferation.

Further evidence that fucoidan treatment transformed the potential of splenocytes to react to concanavalin A stimulation was evident in the cytokines detected in supernatant when these LAK cells where cultured *in vitro* with DFTD cells. The supernatants from control mice sourced LAK cells had significantly higher levels of IFN-γ, IL-2 and TNF-α compared to the supernatants from LAK cells sourced from fucoidan treated mice. Supernatants from fucoidan treated mice had barely detectable levels of IFN-γ and significantly lower levels of IL-2 and TNF-α. This suggests that the cytokines were either being produced at lower levels or were being consumed at higher levels.

These cytokine results are perplexing because they appear to contradict the increased cytotoxicity of dividing DFTD cells by LAK cells sourced from fucoidan treated mice. LAK cells secrete IFN-γ and TNF-α when stimulated with tumour cells (Naganuma et al 1996) yet the fucoidan LAK cells did not appear to produce IFN-γ and had lower levels of TNF compared to the LAK cells from the control mice. One possible explanation could be that the fucoidan LAK cells promoted expression of suppressive cytokines such as TGF-β which inhibits secretion of IFN-γ and TNF-α. This TGF-β could be produced by the LAK cells themselves as a negative feedback mechanism or certain tumours are known to express TGF-β to promote survival (Naganuma et al 1996). TGF-β was not included in the cytokine profile in this pilot study but should be included in any further studies. The cytokines could also have been consumed within the culture and may reflect greater activation.

The conclusions that can be drawn from these experiments are that fucoidan is a potent immunomodulating agent. However there was no evidence that it promoted anti-DFTD responses that were worth further investigation. Fucoidan has been described as an immunomodulatory compound that induces T_H1 cytokines including IFN-γ while suppressing TH2 cytokines like IL-10 (Kar et al 2011). In these experiments fucoidan had no influence on IFN-γ levels and in stark contrast to the observation by Kar *et al.* (2011) it induced IL-10 production to very significant levels. Fucoidan treatment of DFTD inoculated mice resulted in a T_H2 polarisation of immune response to DFTD cells. This was evident in the induction of T_H2 cytokines such as IL-10 and IL-6 and the isotype switching to the T_H2 antibody IgG1. This switching of immune response by fucoidan towards a T_H2 response appears to be DFTD specific but it is a compound worthy of further study by immunologists.

6.3.1 **Imiquimod**

One of the main mechanisms exploited by DFTD cells to avoid detection and subsequent destruction following transmission is the absence of MHC class I molecules on the surface of the cells (Siddle et al 2013). This is due to downregulation of genes responsible for the antigen processing pathway. These include β_2-microglobulin, TAP1, TAP2, CIITA, DMB as well as Class II B and A (Siddle et al 2013).

Importantly this downregulation is not due to structural mutations but rather to epigenetic mechanisms that can be reversed. These genes can be turned back on by stimulating DFTD cells *in vitro* with IFN-γ (Siddle et al 2013). Stimulation with imiquimod also induces the upregulation of surface MHC class I *in vitro* (Patchett 2013). This led to the hypothesis that intratumoural injections of imiquimod could be used to upregulate surface MHC class I within established DFTD tumours in vivo. If this hypothesis proved true then it was envisioned that successful immunotherapy could be developed based on intratumoural injections of imiquimod to make the DFTD tumour visible to the host's immune system.

To test this hypothesis, DFTD tumours in NOD/SCID mice were treated with imiquimod. This resulted in the upregulation of MHC on the cell surface indicated by the detection of β2-microglobulin. Imiquimod has the capacity to upregulate the genes required for DFTD cells to express MHC. The NOD/SCID mouse model provided the proof of concept that this could be achieved *in vivo* through intratumoural injections.

Canine Transmissible Venereal Tumor is the only other known naturally occurring infectious cancer and provides the exemplar for the importance of MHC in tumour regression. Transmission between dogs is facilitated by downregulation of MHC and disease resolution occurs following naturally occurring upregulation of MHC expression in the new host leading to lifelong immunity (Das and Das 2000, Hsiao et al 2008).

By expressing MHC the DFTD cells should be killed by the immune system preventing development of DFTD tumours and curing established disease. This would contribute towards development of a vaccine to protect healthy Tasmanian

devils. It is also the most promising option to date to treat DFTD in diseased devils. However, the NOD/SCID mice lack the appropriate immune system components to determine if this immunotherapy would ultimately achieve tumour regression and lifelong immunity. This work needs to progress to Tasmanian devils and the mouse model will provide the opportunity to optimise the treatment protocol before this occurs.

In addition to upregulation of MHC class I there are other anti-tumour properties of imiquimod that are relevant to induce an immune response against DFTD. Imiquimod directly induces tumour selective apoptosis independent of membrane-bound death receptors (Schön et al 2003). This should result in enhanced presentation to the immune system of DFTD antigens via phagocytes. It also promotes production of cytokines through agonistic stimulation of TLR7 in immune cells that mediate cellular immune responses (Broomfield et al 2009, Schön et al 2003).

The results of this chapter also provided evidence that the antibodies produced by a Tasmanian devil following immunisation with IFN-γ treated DFTD cells targeted MHC molecules or other surface antigens associated with the upregulation of MHC by IFN-γ. This is important knowledge for the team working on a vaccine against DFTD. It reveals that upregulation of MHC makes the cells immunogenic. The Tasmanian devils immune system can target the DFTD cells and may become educated to tumour associated antigens that are unique or overexpressed on naturally occurring DFTD cells.

Final Discussion

7 Final discussion

The Tasmanian devil is being driven to extinction by Devil Facial Tumour Disease. DFTD is a malignant tumour cell line transmitted as an allograft through social interaction, especially biting, when devils squabble over food, sex and territory (Obendorf and McGlashan 2008). The most devastating aspects of the disease are that it has proven 100% fatal, is readily transmitted between Tasmanian devils and there is no evidence of immune or resistant Tasmanian devils in the wild (Belov 2012, McCallum and Jones 2006). To overcome the difficulty of working with an endangered species, this project developed an informative mouse model to study DFTD.

At the commencement of this thesis in 2010 it was assumed that MHC was expressed by DFTD cells and therefore how DFTD was transmissible was a matter of conjecture (Siddle et al 2007). Two possibilities were that the DFTD cells had unique features which prevented them from being immunogenic or that, as a consequence of substantial chromosome alterations (Pearse and Swift 2006), the cells could not be killed because the apoptotic pathway had been disrupted. The mouse model and associated work from this thesis discounted both of these possibilities. Firstly, apoptosis of DFTD cells was detected *in vitro* following exposure to camptothecin or UV radiation revealing the cells had competent apoptotic pathways. Secondly, the work in chapters three and four revealed that DFTD cells induce an immune response. Initially this was demonstrated through mice rejecting the DFTD cells through xenogeneic mechanisms. Immune competent mice produced DFTD specific antibodies and rejected tumours while immunocompromised mice did not. Protection could be transferred from immune competent BALB/c mice to immunocompromised NOD/SCID mice by adoptive transfer of splenocytes.

The demonstration that DFTD cells were immunogenic in mice did not explain why the cells were not targeted by the Tasmanian devil's immune system. The thrust of the research at this point of time into DFTD was testing the widely accepted hypothesis that transmission was facilitated by limited MHC diversity within the Tasmanian devil population causing DFTD cells to be perceived as self (Siddle et al 2007). This hypothesis was based on the fact that MHC was expressed at the mRNA

level (Belov 2012, Siddle et al 2007). In 2012 this was still cited by experts in the field of DFTD (Belov 2012). By late 2012, newly developed antibodies revealed that MHC and β_2-microglobulin were not present on the DFTD cell surface and this hypothesis lost credibility (Siddle et al 2013). Genes essential to the antigen-processing pathway, including β_2-microglobulin and TAP genes associated with antigen presentation were shown to be epigenetically downregulated resulting in the failure of functional MHC class I molecules being transported to the cell surface (Siddle et al 2013).

The lack of MHC expression on the cell surface provides the best explanation to date for how DFTD cells avoid allogeneic recognition. This mechanism is also exploited by the other naturally occurring transmissible tumour, CTVT (Chiang et al 2013).The lack of MHC class I makes the cells invisible to the new host's immune system and prevents them being targeted by $CD8^+$ T cells (Das and Das 2000, Siddle et al 2013). The absence of MHC class I should make the cells targets for NK cells. CTVT cells maintain very low levels of MHC expression to resist NK cell killing (Das and Das 2000). It is possible that DFTD cells also express low levels of MHC but the lack of suitable antibodies against surface epitopes for MHC hinders investigations of this question.

The work in chapter five provided evidence that DFTD cells are NK cell resistant as there was no spontaneous killing of DFTD cells by Tasmanian devil lymphocytes, despite evidence of functional NK cells (Brown et al 2011). Further evidence of NK resistance comes from studies of xenograft rejection in chapter four. Splenocytes from DFTD immunised BALB/c mice but not splenocytes from naïve mice always protected NOD/SCID mice from DFTD tumour cells. This provided evidence for a primed adaptive immune response. Furthermore, the use of genetically modified mice including athymic CBA/nu mice, which have functional NK cells, indicated that NK cells alone did not protect against DFTD cells. Even though priming is necessary it could be possible that activated NK cells kill or antibodies generated by primed B cells facilitate ADCC killing by NK cells but spontaneous NK killing is not occurring.

NK-resistance in human cancers can be overcome by harvesting a patient's lymphocytes and activating them *in vitro* to generate lymphokine activated killer (LAK) cells. These LAK cells are then reintroduced into the patient and have the

capacity to kill NK-resistant tumour cells while leaving healthy cells unharmed (Linn and Hui 2010, Richards 1989). The mitogen concanavalin A or the supernatant from concanavalin A stimulated lymphocytes (Con A sup) activated Tasmanian devil lymphocytes into LAK cells capable of killing DFTD cells *in vitro* (Brown 2013). Before proceeding to trials on Tasmanian devils it was necessary to demonstrate *in vivo* efficacy in mice to collect more information about the dynamics of LAK killing.

This model was established in chapter five when it was initially shown that NOD/SCID mice can accept Tasmanian devil lymphocytes without graft versus host or host versus graft rejection. LAK cells were generated *in vitro* using concanavalin A or Con A sup stimulation. When they were co-injected with DFTD cells it provided an *in vivo* setting to study tumour rejection. Depending on the ratio of LAK cells to DFTD cells, protection for the NOD/SCID mice ranged from reduced tumour formation to complete protection. Notably this demonstrated allograft killing by Tasmanian devil lymphocytes *in vivo* without risking DFTD infection of healthy Tasmanian devils.

The key findings from these experiments provided further support that the Tasmanian devil has a competent immune system capable of killing DFTD cells. This presumably required tolerance to DFTD being surmounted. LAK cells overcome this tolerance and target DFTD cells *in vitro* and *in vivo*. This would be achieved because LAK cells are activated NK cells with increased expression of receptors to promote cytotoxicity including TRAIL, NKG2D and the natural cytotoxicity receptors NKp30, NKp44 and NKp46 (Childs and Berg 2013). Normal cells are not targeted by LAK cells because they express MHC that inhibits NK activity (Linn and Hui 2010, Richards 1989). This work provides evidence that the immune system of Tasmanian devils can eliminate DFTD and that a vaccine or immunotherapy against DFTD in Tasmanian devils is achievable.

The ability to generate LAK cells from Tasmanian devil lymphocytes provides insight into the composition of their immune system. Lack of appropriate antibodies hinders investigations into the Tasmanian devil's immune system and functional analysis is often used to envisage what is occurring in their immune system. Recent evidence has indicated Tasmanian devils have cells that function like NK cells (Brown 2013, Brown et al 2011) but a lack of appropriate antibodies has prevented their isolation. NK cells on their own are not activated by concanavalin A and stimulation of LAK

cells in humans requires concanavalin A binding to NKT cells which then express IFN-γ leading to activation of NK cells (Kaneko et al 2000, Miyagi et al 2004, Palacios 1982). The observation that concanavalin A stimulates Tasmanian devil lymphocytes into LAK cells indicates that Tasmanian devils not only have NK cells but also NKT cells. Consequently, this work provides supporting evidence for NK cells in Tasmanian devils. The presence of functional NKT cells is also suggested, but requires confirmation. This contributes to an expansion of our knowledge of the Tasmanian devil immune system.

The existence of NKT cells in the Tasmanian devil increases potential immunotherapy approaches to trial against DFTD. NKT cells make potent targets for immunotherapy because they have a significant role in promoting anti-tumour responses (Bassiri et al 2014). The NKT cells directly kill some tumours but they also have an indirect effect by producing IFN-α, IL-4, IL-13, IL-17, TNF-α and GM-CSF which in turn activates DCs, NK cells, $CD8^+$ T cells and induces enhanced Ig production by B cells (Bassiri et al 2014, O'Konek et al 2012).

A thorough investigation of Tasmanian devil LAK cell activity at this point in time is not possible due to lack of suitable antibodies. However, knowledge from mouse and human studies suggest a conserved mode of function in LAK cell activity between species (Rosenberg et al 1986). This knowledge provides insight into how LAK cell therapy could be beneficial to developing immunotherapy against DFTD. The possible chain of events for LAK cells activation and function follows. The LAK cells that are generated by the NKT cells directly kill NK-resistant tumours while generating IFN-γ in the tumour microenvironment (Diefenbach et al 2000). The IFN-γ then upregulates β_2-microglobulin and MHC class I in tumours with downregulated MHC (De Fries and Golub 1988, Siddle et al 2013). The newly expressed MHC then makes the tumours vulnerable to direct CTL killing (De Fries and Golub 1988). The LAK cells amplify the CTL response by lysing M2 macrophages that promote tolerance and activate M1 macrophages which present tumour antigens to $CD8^+$ T cells to generate CTLs (Geldhof et al 2002). These functions have been observed in mice and humans and if they also were to occur in Tasmanian devils these may provide lifelong immunity.

The limitation of the NOD/SCID mouse for LAK cell trials is restricted to evaluation of direct LAK cell killing activity. The generation of CTLs and editing of macrophages cannot be evaluated in NOD/SCID mice because they lack an immune system to work in synergy with the LAK cells. However, the mouse model has been invaluable in providing the first evidence of *in vivo* activity of Tasmanian devil LAK cells.

The mouse model provides the intermediate step between identifying agents *in vitro* and testing them with the Tasmanian devil. Studies *in vitro* can be used to screen a range of agents that have the potential to target DFTD. But to obtain more complete understanding studies need to be undertaken *in vivo.* The *in vitro* cultures test the therapeutic agent on DFTD cells in isolation. This is not the same as the *in vivo* tumour microenvironment which provides a complex multi-cellular niche, ongoing nutrients and the production of cytokines and growth factors which influence tumour survival. In this thesis the mouse model was used to test afatinib, withaferin A, fucoidan and imiquimod.

Afatinib promotes apoptosis and inhibits proliferation in some cancers (Yap et al 2010) and when tested *in vitro* against DFTD cells was shown to inhibit proliferation. Because afatinib directly targets tumour cells and does not require involvement of an immune system, NOD/SCID mice engrafted with DFTD tumours provided a suitable *in vivo* setting to further evaluate the function of afatinib against DFTD cells. Despite anti-DFTD activity by afatinib *in vitro* this did not translate *in vivo*. At the dose used there was no evidence of prophylactic protection, regression of established DFTD tumours or altered DFTD growth kinetics. Possible explanations for the lack of protection include mode of delivery, dosage, bioavailability or the *in vivo* environment itself. The mouse model could be used further to determine dosage, alternative administration route or combined therapies to provide effective afatinib based treatment. The lack of protection in the mouse model and the foreseeable difficulties of treating a wild population with such an agent meant there was no immediate justification to proceed to trials on Tasmanian devils. The mouse model provided the preliminary data prior to any potential trial with Tasmanian devils and thus avoided using Tasmanian devils prior to determining efficacy *in vivo*.

Withaferin A is another therapeutic agent that was evaluated using the NOD/SCID mouse model to provide preliminary data to inform potential treatment of Tasmanian

devils. Withaferin A is a plant extract known to target cancer cells over-expressing vimentin (Thaiparambil et al 2011). DFTD cells over-express vimentin which promotes aggressive tumour growth and metastatic disease. A pilot study did not provide evidence for prophylactic protection or inhibition of DFTD by withaferin A at the given dose. The mouse model will be used to determine effective dose and dose fractions before any treatment proceeds to Tasmanian devils. If efficacy is not demonstrated in mice then Tasmanian devils will be spared unnecessary trials.

Immunocompetent C57/BL6 mice were used to evaluate the immunomodulating activity of fucoidan, a seaweed extract, on mice injected with DFTD cells. Fucoidan was shown to be a potent immunomodulating agent that altered both cytokine and antibody production. Fucoidan polarised the murine response to a T_H2 response against DFTD and did not enhance cytotoxicity. While showing no particular promise against DFTD these results add to the evidence that fucoidan alters the immune system and may be beneficial in other disease states.

DFTD tumour cells avoid immune recognition because they do not express MHC. Consequently upregulation of MHC should overcome this failure of recognition as demonstrated with CTVT. When CTVT cells are transplanted in the experimental setting, within weeks CTVT progresses to a stage that triggers epigenetic upregulation of MHC on the surface of the cells (Das and Das 2000, Hsiao et al 2008). This makes the cells visible to the host's immune system resulting in tumour regression and lifelong immunity (Belov 2012). Consequently, the new knowledge that DFTD transmission and avoidance of immunosurveillance is facilitated by epigenetic downregulation of MHC directed researchers attention towards exploring means of inducing MHC expression in DFTD cells (Siddle et al 2013).

Research focused on the idea of upregulating MHC in DFTD cells *in vitro*. This was achieved through stimulation with recombinant devil IFN-γ (Siddle et al 2013). The expectation of researchers included that DFTD cells expressing MHC may provide the starting point for an effective vaccine against DFTD (Siddle et al 2013). As well, DFTD cells expressing MHC may provide a means of immunotherapy. This could be achieved by injecting existing tumours with MHC^+ DFTD cells that would attract immune cells to the tumour site and induce the production of IFN-γ. This IFN-γ would stimulate wild-type DFTD cells to express MHC molecules (Siddle et al 2013) and in

turn this could lead to a cascade of responses that would educate the immune system to DFTD specific antigens and promote regression and lifelong immunity.

As a safeguard these MHC^+ cells would require inactivation before use as a vaccine for Tasmanian devils or immunotherapy for diseased Tasmanian devils. The mouse model provided insight into the best means of inactivating DFTD cells without reducing their immunogenicity. As shown in chapter three, irradiation of DFTD cells was preferable to freeze-thawing or sonicating DFTD cells. Irradiation maintained the integrity of the cells and the surface antigens while preventing their proliferation.

The mouse studies in chapter three revealed a primary intraperitoneal immunisation with a second intraperitoneal immunisation two weeks later produced the highest antibody responses. The subcutaneous route is favoured in Tasmanian devils to mimic infection route but this may not induce the most beneficial immune response and may not be appropriate for the development of a vaccine. The prime-boost intraperitoneal immunisation protocol could boost antibody responses in Tasmanian devils enhancing the efficacy of the treatment.

An alternative means to induce upregulation of MHC class I on DFTD cells could involve intratumoural injections of a therapeutic agent. One such agent, imiquimod, when tested *in vitro* against DFTD cells appeared to alter gene expression of DFTD cells resulting in upregulation of β_2-microglobulin and MHC class I (Patchett 2013). The NOD/SCID and athymic nude mouse models were used in chapter six to test the ability of imiquimod *in vivo* to upregulate β_2-microglobulin and MHC. Imiquimod injected directly into established DFTD tumours in mice upregulated expression of MHC and β_2-microglobulin. It is reasonable to assume that the same outcome would occur if the protocol was repeated in diseased Tasmanian devils. Considering the role of MHC in CTVT this would make DFTD cells visible to the Tasmanian devil's immune system (Belov 2012). Furthermore, if DFTD followed the same path as CTVT (Murchison 2008) then lifelong immunity would be predicted.

Potentially there are many more anti-tumour activities of imiquimod that would be relevant to DFTD. Imiquimod is a synthetic imidazoquinoline that targets TLR7 and induces innate and adaptive immunity, selectively induces apoptosis in tumour cells and counteracts immunosuppression in the tumour microenvironment by inducing pro-inflammatory cytokines such as IFN-γ,TNF-α, IL-12 and macrophage

inflammatory protein (MIP)-1 (Clark et al 2008, Gorden et al 2005, Schön et al 2003, Wolf et al 2007). Evaluation of imiquimod's immunomodulatory functions will require trials in Tasmanian devils since the NOD/SCID and athymic nude mice lack appropriate immune system capabilities.

Imiquimod is a drug already approved and widely used for topical application against various human skin cancers (Adams 2009). Oral administration to provide systemic treatment has proven ineffective in human trials (Witt et al 1993) and there is a paucity of published accounts where imiquimod was administered via intratumoural injections. This thesis extended the use of imiquimod to a novel veterinary application and administration by intratumoural injection. The use of intratumoural injections against DFTD would be of great interest to the treatment of many human and veterinary cancers if it is shown to be effective.

There are other potential applications for imiquimod that could be evaluated using immunocompetent mice. Imiquimod also has potential to be used as an adjuvant in vaccines to promote CD8+ T cell responses (Shackleton et al 2004). This could initially be evaluated by immunising immunocompetent mice with DFTD cells and seeing if the inclusion of imiquimod enhances the immune responses. Particular attention should also be given to dendritic cell responses since imiquimod is also known to activate DCs *in vivo* through the TLR7 (Shackleton et al 2004). This may provide a means of generating dendritic cell based immunotherapy avoiding the *in vitro* activation step.

Conclusions

Much of the early knowledge of DFTD resulted from establishment of DFTD cell lines (Pearse and Swift 2006). These established cell lines continue to provide the foundations for research into DFTD ranging from genetic analyses, testing therapeutic agents and vaccine development. Mouse models provide a bridge between *in vitro* cell cultures and *in vivo* work in Tasmanian devils. Mouse models should be viewed as animal cultures that overcome some of the limitations of cell culture and assume advantages of the *in vivo* environment (Frese and Tuveson 2007). The mouse model allows the study of DFTD establishment and growth in a three dimensional substrate of stromal cells that provide nutrients, angiogenesis and

paracrine growth factors that are absent in a two-dimensional colony of cells adhering to a plastic substrate.

Mice represent an invaluable resource to evaluate treatment options for DFTD by providing a substitute to Tasmanian devils. Specific treatments identified *in vitro* can be tested *in vivo* before proceeding to trials in Tasmanian devils. The advantages include easier ethics approval, lower husbandry costs, no need to maintain animals after experiments have concluded and veterinary assistance is not required for treatments.

In this thesis the first mouse models were based on xenograft rejection mechanisms. This provided valuable evidence that DFTD cells were immunogenic and could be eliminated by immune system responses *in vivo*. The use of immunocompromised mice allowed the implantation of xenografts to study the establishment and growth of DFTD cells in a permissive *in vivo* environment. A significant advance in the mouse model was when adoptive transfer of Tasmanian devil lymphocytes and DFTD cells to NOD/SCID mice provided protection against development of DFTD tumours. This facilitated the study of allograft responses against the implanted xenograft DFTD tumours.

In this thesis a number of treatments were tested in various strains of mice. Some promising treatments such as afatinib, withaferin A, LAK cells and imiquimod were discovered in the *in vitro* setting of cell cultures. These therapies were then evaluated *in vivo* with the mouse model. In the cases of afatinib and withaferin A the *in vivo* studies did not provide supporting evidence to proceed to trials in the Tasmanian devil. In contrast, the *in vivo* studies provided compelling evidence to prioritise further development of both LAK cells and imiquimod treatments towards trials in Tasmanian devils.

Through DFTD, nature has provided an ideal model to study cancer, vaccine development, immunotherapy and chemotherapy in a naturally occurring cancer in an animal with a competent immune system. The clonal nature of the disease allows replicate trials in multiple animals and multiple generations.

DFTD is not just a Tasmanian problem but a global opportunity and by producing an immunological solution for DFTD we will extend our knowledge and treatment

options for many human cancers. The costs associated with immunotherapy trials would be substantial. But put in the global context, millions of dollars are spent developing animal models to study cancer in an artificial setting. DFTD provides an excellent opportunity to study cancer in a natural setting if sufficient funding is available.

Future directions

This thesis provided an informative mouse model to undertake preliminary studies to evaluate treatment options against DFTD. Two promising treatments that were identified in the mouse model were LAK cells and imiquimod therapy.

LAK cell studies could be extended by evaluating the cascade of immune events associated with LAK cell killing of DFTD cells. This may require imaging studies to identify and describe the cell phenotypes that engage and kill the DFTD cells *in vitro*. Reconstitution of mice with adoptive transfer of Tasmanian devil lymphocytes may facilitate evaluating if direct LAK cell killing is augmented by engagement of the adaptive immune system especially CTLs. Identifying different means of activating LAK cells and establishing allogeneic Tasmanian devil NK cell lines would also advance the LAK cell studies. These could all be tested in the mouse model before proceeding to Tasmanian devil trials.

This thesis provided the first evidence that we have identified a means of modifying DFTD tumours *in vivo* to express MHC. This was achieved by intratumoural injection of imiquimod. The potent anti-DFTD effects of imiquimod justify further evaluation of how best to utilise TLR agonists in the treatment of Tasmanian devils and if this should be as a monotherapy or a combined therapy. The mouse model provides the opportunity to study TLR agonists *in vivo*.

Future studies with withaferin A should be undertaken in the athymic nude or Rag/2 mice because they have functional NK cells. Withaferin A causes vimentin disassembly. Over expression of vimentin by cancer cells blocks NK receptors preventing them from targeting malignant cells. It is possible that withaferin A will remove this protection from DFTD cells allowing them to become targets for NK cells. This can only be assessed *in vivo* with competent NK cells.

It is important that this work is translated to Tasmanian devils. Diseased Tasmanian devils could be treated with autologous and allogeneic LAK cells and tumour

biopsies could be examined by IHC for infiltrating cytotoxic cells. The use of allogeneic LAK cells as a vaccine adjuvant to enhance protection should also be trialled in Tasmanian devils. Establishment of an allogeneic NK cell line would support these trials.

Imiquimod is the most promising option to date to treat DFTD in diseased devils. The mouse model provides the means of optimising the treatment protocol before proceeding to trials on Tasmanian devils. However, it is necessary to confirm that up-regulation of MHC makes the DFTD cells targets for cytotoxic Tasmanian devil lymphocytes. This would involve *in vitro* assays to detect proliferation of cytotoxic T cells when cultured with MHC^{+} DFTD cells. This would provide the rationale for proceeding to trials in Tasmanian devils.

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