Enhancing Immunity Against Melanoma using Type I Interferon

by

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Declaration

I declare this thesis is my own account of my research and contains as its main content, work which has not been previously submitted for a degree at any tertiary educational institution.

..............................................................

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Abstract

Melanoma is an aggressive cancer, which when diagnosed in the late stages has a median survival rate of 6 months. The current gold-standard for adjuvant therapy is high doses of the type I interferon (IFN)-α2, which prolongs disease free survival, but not overall survival. This high-dose therapy has many serious side-effects, resulting in doses to be lowered to sub-therapeutic levels. It is not clear how IFN-α2 acts to treat melanoma, however it is accepted that immune enhancement is involved. The two main type I IFN families, IFN-α and IFN-β, signal via the same receptor, however they have been found to be functionally different. Of the IFN-α family, 13 subtypes have been discovered and there is evidence that each is functionally unique. This project explores the therapeutic efficacy of seven type I IFN subtypes, including IFN-α2 and IFN-β, in treating melanoma. To test efficacy, a model of DNA therapy to administer plasmids encoding the IFN subtypes was combined with a melanoma model utilizing B16 F1 cells expressing the immunogenic peptide glycoprotein B. We have found that the IFN-α subtypes tested have greater efficacy than IFN-α2 in treating melanoma. The IFN subtypes delayed tumour onset, and one subtype demonstrated a significant increase in survival time. In addition, therapeutic effects could be seen when sera levels were lower than that of IFN-α2. The in vitro effects of the IFN subtypes on melanoma cells was examined, uncovering differences in potency. Furthermore, we have found evidence of anti-tumour immune enhancement is associated with IFN-α therapy but not IFN-β. This research shows that the IFN-α subtypes differ in their anti-tumour efficacy, and potency of actions. Therefore the IFN-α subtypes show potential as a superior therapy in treating melanoma, which may be administered at lower, less toxic doses.
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Abbreviations

ACT  Adoptive cell therapy
APC  Antigen presenting cell
B6   C57Bl/6J
Bcl-2 B-cell lymphoma 2 (gene)
BP   Bupivacaine
BSA  Bovine serum albumin
CFSE Carboxyfluorescein succinimidyl ester
CpG  Cytosine—phosphate—Guanine (linear sequence)
CTL  Cytotoxic T-lymphocyte
CTL-4 Cytotoxic T-lymphocyte antigen 4
DC   Dendritic cell
DNA  Deoxyribonucleic acid
dsRNA Double stranded ribonucleic acid
EDTA Ethylenediaminetetraacetic acid
ELISA enzyme-linked immunosorbent assay
FACS Fluorescence-activated cell sorting
FCS  Foetal calf serum
FSC  Forward scatter
gB   Glycoprotein B
GFP  Green fluorescent protein
HEPES hydroxyethyl piperazineethanesulfonic acid
HLA  Human leukocyte antigen (human MHC)
HSV  Herpes simplex virus
IFN  Interferon
IFN-I Type I interferon
IFNAR Type I IFN receptor
IgG  Immunoglobulin G
IL   Interleukin
IRES Internal ribosome entry site
ISGs Interferon stimulated genes
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>IU</td>
<td>International units</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenous</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus Kinase</td>
</tr>
<tr>
<td>LB</td>
<td>Lubia Broth</td>
</tr>
<tr>
<td>LN</td>
<td>Lymph node</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinases</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple Sclerosis</td>
</tr>
<tr>
<td>MU</td>
<td>Million units</td>
</tr>
<tr>
<td>NF-kB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>RAG</td>
<td>Recombination activating gene</td>
</tr>
<tr>
<td>RGP</td>
<td>Radial growth phase</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>SAV</td>
<td>Streptavidin</td>
</tr>
<tr>
<td>SN</td>
<td>Supernatant</td>
</tr>
<tr>
<td>SSC</td>
<td>Side scatter</td>
</tr>
<tr>
<td>ssRNA</td>
<td>Single stranded ribonucleic acid</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducers and activators of transcription</td>
</tr>
<tr>
<td>TA</td>
<td>Tibialis anterior</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate-EDTA</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptor</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>Tyk2</td>
<td>Tyrosine Kinase 2</td>
</tr>
<tr>
<td>VGP</td>
<td>Vertical growth phase</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet (light)</td>
</tr>
</tbody>
</table>
Acknowledgements

This year is definitely one I will remember. Thinking back to the start of the year, I can't believe how much I have grown, what I have learnt and the skills I have acquired since then. Of course, none of this would have been possible without the input of many people, of which I would like to personally thank a few.

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I am truly grateful for everything and everyone that has been involved in this amazing journey. Thank you.
Chapter 1: Introduction

1.1 MELANOMA

In Australia, exposure to high UV is common, and may result in UV induced mutations in cells of the skin. This exposure is implicated in driving malignant melanocytes, known as melanomas, to become a common disease (Berwick et al., 2005). As a result, Australia has the highest incidence and mortality rates from melanoma in the world (Garbe and Leiter, 2009; Sneyd and Cox, 2013). In addition, the incidence and mortality rates are steadily increasing worldwide. Melanoma can affect all ages, and is the most aggressive and dangerous form of skin cancer (Perlis and Herlyn, 2004). Melanoma is commonly ignored as a growth on the skin, as it can simply resemble a mole. Surgical resection is the frontline treatment, however, it often metastasises and spreads to distal sites no longer suitable for surgical removal (Perlis and Herlyn, 2004). Current cancer therapies are inadequate to treat this refractory form. Once metastases are present, the median survival rate is 6 months, and less than 5% survive after 5 years (Cummins et al., 2006). New treatment strategies are urgently needed, with the most promising results apparent from immune modulating therapies.

1.1.1 Biology of melanoma

1.1.1.1 Melanocytes

Melanocytes are specialized pigmented cells predominantly found in the skin, that can mutate in response to short intermittent UV exposure which damages the DNA before protective melanin is produced (Perlis and Herlyn, 2004). Melanocytes reside in the basal layer of the epidermis, where they are regulated by keratinocytes. Keratinocyte control is mediated via direct contact through the cell-cell adhesion receptor E-cadherin (Slominski et al., 2004). In
response to UV, keratinocytes secrete regulatory factors that alter melanocyte survival, proliferation, differentiation, motility and melanin production (Valyi-Nagy et al., 1993). The production of melanin is key in protecting our skin from the UV-elicited damage and therefore in preventing skin cancer.

1.1.1.2 Mutation

In contrast to melanocytes protecting us from UV, it is widely accepted that exposure to intermittent UV is a risk factor in melanoma development. Malignant melanoma can develop via transformation and proliferation of melanocytes, or when melanocytes no longer respond to normal cellular growth control mechanisms. Melanocytes can escape the regulation of keratinocytes due to a disruption in intracellular signaling, such as mutations in critical growth regulatory factors and loss of the adhesion receptor E-cadherin (Haass et al., 2004). This results in increased proliferation and spread, resulting in a benign mole or ‘naevus’ (Gray-Schopfer et al., 2007). Naevi can become dysplastic and if allowed to persist may spontaneously progress to the invasive ‘radial growth phase’ (RGP), which leads to the potentially metastatic ‘vertical growth phase’ (VGP) (see figure 1.1) (Gray-Schopfer et al., 2007). In addition to genetic alterations to proliferation and survival, E-cadherin down-regulation and a shift to N-cadherin coincides with melanoma progression. N-cadherin is a survival factor for melanoma cells to communicate with fibroblasts and endothelial cells as they migrate through the dermis (Le Bon et al., 2001). It is the vertical growth phase that leads directly to metastatic malignant melanoma, as vertical growth can invade the dermis into the subcutaneous tissue and infiltrate the vascular and lymphatic systems (figure 1.1). Other common mutations in melanoma results in upregulation of STAT (Signal Transducer and
Activator of Transcription) -3, which promotes tumour growth and expression of immune-suppressing factors (Kortylewski et al., 2005); aberrant activation of BRAF, which promotes survival (Tseng et al., 2013) and c-KIT upregulation, which acts as a growth factor receptor (Tseng et al., 2013).

Figure 1.1 Progression of melanocyte transformation.

(A) Normal skin containing melanocytes. (B) Accumulation of melanocytes to form a benign naevus. (C) Malignant melanocytes in the vertical growth phase (RGF). (D) Vertical growth phase (VGP) of melanoma. This phase is potentially metastatic, as melanocytes may enter the lymphatic or vascular system. This leads directly to metastatic malignant melanoma, which is deadly and has a poor prognosis. Image adapted from Gray-Schopfer et al. (2007).
1.1.2 Epidemiology and trends

Australia has the highest incident rate of melanoma in the world, and the prevalence is increasing worldwide (Garbe and Blum, 2001). In white populations, melanoma is the most rapidly increasing cancer. The risk of melanoma varies with geographic zone, gender and age. The highest incidence rates occur closer to the equator, where UV intensity is greater (Dobson and Leeder, 1982; Garbe and Leiter, 2009). Of particular risk are populations who have migrated to live closer to the equator. This increased UV intensity increases the risk of sunburn, which is one of the leading factors attributed to melanoma development. Sunburn in childhood is a particularly high risk factor, and it is hypothesized that melanoma risk is acquired in childhood (Gallagher et al., 1990). In Australia, gender also accounts for melanoma risk, with a higher prevalence in men. Accompanying this trend, melanomas tend to develop on areas of the body that receive intermittent sun exposure, which for men is the trunk and women the legs (Garbe et al., 1995). Increasing age, and hence increased accumulated damage from UV exposure, is another factor in melanoma development, with the majority of diagnoses made between 50 and 60 years (Garbe and Blum, 2001).

1.1.3 Treatment and survival

1.1.3.1 Surgical intervention

Melanoma may be thought as harmless as it grows quietly on the skin, simply resembling a mole. The tumour thickness is the most important prognostic factor in early melanoma (Buettner et al., 2005). Early detection results in approximately a 100% survival rate, as early stage disease can easily be surgically removed. However, if the
melanoma has metastasised surgery is limited to removal of the primary tumour and affected regional lymph nodes. Currently, surgery is the only effective routine method for curing melanoma. Due to the high capacity for invasion and metastasis of this cancer, late diagnosis sharply decreases chance of survival. Without further treatment, the average survival time is only 6 months (Cummins et al., 2006).

1.1.3.2 Chemotherapy and drugs

Melanoma is extremely aggressive and notoriously resistant to cytotoxic agents classically used to treat other forms of cancer. Melanoma cells display resistance to apoptosis, and hence chemotherapeutic drugs that aim to induce apoptosis in malignant cells are largely ineffective (Soengas and Lowe, 2003). The only approved chemotherapeutic for melanoma is dacarbazine, results in a 20% objective response rate, with no statistically significant increase in survival (Serrone et al., 2000).

A range of drugs have been designed to target various cellular activities that have become mutated in metastatic disease. This drug diversity for melanoma therapy makes selecting the right drug difficult, with potential for cells to develop resistance through further mutation. Many drugs block commonly mutated cellular growth pathways such as c-KIT (a growth factor receptor) (Beadling et al., 2008) and BRAF (a signal transduction protein kinase) inhibitors (Saldanha et al., 2006). However, the therapies must be mutation specific, and even then the majority have low response rates (~30% response) with marginal or no improvement in overall survival (Tarhini and Agarwala, 2006; Tseng et al., 2013).
Drugs enhancing the immune system have proven the most promising in treating melanoma. High dose IFNα2 or pegylated IFNα2 is the most commonly used immunotherapy for late stage patients. Standard IFNα therapy has a short half-life (<12 hours) that requires constant administration (at least 3 times a week) to maintain effective levels in the blood. The short half-life of IFNα has led to the development of longer lasting preparations achieved by the attachment of a large polyethylene glycol molecule (pegylation) directly to IFN-α (Glue et al., 2000). This pegylated IFNα2 has a longer half-life (40 to 80 hours) allowing once weekly administration (Glue et al., 2000). This treatment has had the most clinical success in the treatment of melanoma, used as a single agent in adjuvant therapy, resulting in decreased tumour burden and prolonged time to relapse (average of 9 months), however, only 1 out of 11 comparable trials to date have shown an increase in overall survival (Bottomley et al., 2009; Cameron et al., 2001; Cascinelli et al., 2001; Creagan et al., 1995; Eggermont et al., 2005; Eggermont et al., 2008; Grob et al., 1998; Hancock et al., 2004; Kirkwood et al., 1996; Kleeberg et al., 2004; Pehamberger et al., 1998). In rare cases, patients have had ongoing relapse-free survival. The benefits of increased survival with IFNα2 therapy appear to be confined to trials with microscopic spread to lymph nodes (Tarhini et al., 2012). The anti-tumour efficacy is considered to be due to enhancement of the immune response, and reduction of regulatory T cells (T-reg) (Ascierto and Kirkwood, 2008; Viguier et al., 2004). Mouse models of melanoma have also demonstrated that IFN-α2 therapy by plasmid DNA injection reduces tumour burden, increases survival and reduces the number of metastases in the lungs (Horton et al., 1999). To date, IFN-α2 remains the only IFN-α subtype investigated in the clinic, despite evidence each subtype has different cellular actions (Cull et al., 2003).
High dose interleukin-2 (IL-2), which is an activator of T cells and natural killer (NK) cells, is also approved for clinical treatment of melanoma. IL-2 has been reported to convert self-specific tolerant T cells to effector T cells and induce autoimmunity (Waithman et al., 2008). Autoimmunity associated with IFNα2 and IL-2 therapy has been found to correlate with increased survival (Gogas et al., 2006). IL-2 therapy involves a series of bolus injections of IL-2. Clinical trials using this agent have shown it has a lower response rate than IFN-α2 therapy (Davar et al., 2011). However, both IFNα2 and IL-2 immunotherapies require high doses that are not well tolerated and have a wide range of short-term side effects due to their toxicity (Pestka, 2007).

A new immune therapy, anti-CTLA-4, blocks the inhibitory receptor on T cells. Blocking this receptor prevents inactivation of tumour specific T cells, and hence enhances the function of effector T cells. This receptor is highly expressed on T-reg cells, and blocking this receptor inhibits their function and also targets them for depletion. Anti-CTLA-4 therapy has shown significant efficacy in treating melanoma (Prieto et al., 2012). This therapy has also shown enhancement of immunomodulating drugs in generating anti-tumour immunity (Lesterhuis et al., 2013; Prieto et al., 2012). Side-effects similar to IFNα2 and IL-2 been reported, mainly of autoimmune manifestations, which too correlates with greater treatment outcome (Attia et al., 2005; Kähler and Hauschild, 2011; Tseng et al., 2013)
1.1.4 Immune response to melanoma

1.1.4.1 Host anti-tumour mechanisms

The body has evolved many mechanisms to prevent mutation, control cell proliferation and identify and destroy mutated cells. Once a cell has mutated, the immune system possesses means to distinguish transformed cells from normal cells. Specifically, CD4+ and CD8+ T cells recognize tumor antigens in the context of MHC class II and class I proteins, respectively. T cell recognition of tumour antigen, is used to develop a specific adaptive immune response, targeting and destroying the tumour (Garcia-Lora et al., 2003). NK cell activation is regulated by the expression of MHC class I molecules on the surface of the target cell, which deliver inhibitory signals through the interaction with receptors on the surface of the NK cell (Markovic and Murasko, 1991). Therefore a loss of MHC I molecules, which may occur due to malignant transformation, results in an attack against the target cell (Drake et al., 2006; Garcia-Lora et al., 2003). In addition to tumor antigens presented on MHC molecules, transformed cells may overexpress other molecular signposts that can function as recognition targets in the immunosurveillance process (Raulet, 2004). These molecules may act as “danger” signals, aiding the development of an immune response. Alternatively, as these ‘tumour antigens’ are derived from self, they may be ignored by the immune system or cause the deletion of T cells thought to be autoreactive (Kappler et al., 1988). In addition, the ability of malignant cells to mutate further allows them to ‘evolve’ under immune pressure until they lose immunogenicity and therefore escape the immune system (Drake et al., 2006).
Chapter 1

Introduction

1.1.4.2 Immune evasion

Tumours differ from normal cells in their antigenic profile as genetic instability leaves a molecular imprint. Deletion and/or mutational inactivation of apoptosis regulatory genes results in genetic changes, and as a consequence new antigens are generated. In addition, as a result of methylation the expression of hundreds of altered genes may be increased significantly raising the concentration of proteins, resulting in alteration of a tumour's antigenicity (Jones and Baylin, 2002). There are several mechanisms by which tumour cells can evade immune responses and these are discussed below.

1.1.4.2.1 Loss of MHC class I

One mechanism of immune escape is the downregulation of antigen processing and presenting machinery, in particular the MHC class I pathway. MHC class I molecules are necessary for the presentation of peptide antigens to cytotoxic T lymphocytes (CTLs) and the immune surveillance of NK cells. The classical MHC class I molecules are the human leukocyte antigens (HLA)-A, -B and -C in humans and H-2K, D and L antigens in mice (Bjorkman et al., 1987). These classical class I molecules are present on the surface of most mammalian cells. The loss of MHC class I molecules is used to escape recognition and destruction by CTLs (Garrido et al., 1993). Individual HLA/H-2 alleles that present the most immunodominant antigens can be selectively lost. In some circumstances, overall MHC class I expression may be totally absent. Downregulation of MHC class I molecules can be mediated by mutation, deletion or epigenetic silencing, with the latter reported to be reversed with IFNα/γ treatment, which in turn leads to enhanced CTL recognition (Propper et al., 2003). Although this mechanism escapes CTL recognition, NK cells recognize and kill cells with low MHC class I levels (Lanier and Phillips, 1996).
Therefore this is not an effective strategy to hide from innate immune recognition, but is rather a strategy used in cancer cell progression to avoid CTL recognition. (Drake et al., 2006; Garcia-Lora et al., 2003). Indeed, many analyses of metastatic melanoma biopsies have found low MHC class I expression in patients where tumour specific CTLs are present (Jager et al., 1997; Wang et al., 1993).

1.1.4.2.2 Tumour-associated antigen loss

Considering immune pressure from NK cells necessitates MHC class I retention by tumour cells, a more reliable immune escape mechanism is the loss of immunogenic antigen expression. Specific CTL therapies or tumour-peptide vaccines used in melanoma treatment often results in initial objective tumour regression, followed by tumour progression despite the presence of strong CTL responses (Jager et al., 1996; Kawakami and Rosenberg, 1997). Loss of specific tumour antigen has been observed in relapsed tumours, however they can be induced to re-express the antigen. These studies highlight that a reversible downregulation of antigen expression is employed as a mechanism of immune escape (Landsberg et al., 2012).

1.1.4.2.3 Tumour immune modulation

There is strong evidence to suggest that tumour cells are capable of actively inhibiting danger signals as well as inducing tolerance (Drake et al., 2006; Kortylewski et al., 2005). Tumours use oncogenic signaling pathways to promote their own growth and survival as well as for modulating immune cells. A common example is through manipulation of the STAT3 pathway, which is able to induce expression factors that inhibit functional development of dendritic cells (DCs), innate immune cells and destruction by CTLs.
(Drake et al., 2006; Kortylewski et al., 2005). As a result, DCs found in the tumour microenvironment are immature and inactive, and when they present self-antigen in the absence of costimulatory (danger) signals, T cell tolerance and regulatory T cells are induced (Bonifaz et al., 2002; Hawiger et al., 2001; Steinman et al., 2003). Molding the immune response to tolerance results in uninhibited tumour growth, as it is ignored by the immune system (Ochsenbein, 2005; Ochsenbein et al., 1999). Finally, tumours can impair CTL function due to persistent antigen exposure resulting in “exhausted” tumour-antigen CTLs with diminished effector function (Willimsky and Blankenstein, 2005; Zajac et al., 1998). Proinflammatory cytokines, such as IFNs or IL-2 can alter the immunological balance between tolerance and productive anti-tumour immunity (Essery et al., 1988; Waithman et al., 2008).

1.2 INTERFERONS

A phenomenon known as “viral interference” remained a mystery until the 1950s. A team of researchers noted that a virus is able to block the infection of a second virus in the same cell culture. Remarkably this inhibitory factor could be transferred to a different cell culture to provide viral resistance (Henle, 1950). Investigations into viral interference in 1957 (Isaacs and Lindenmann, 1957) discovered this interference was the action of a soluble factor from the host cells. This is where the term ‘interferon’ was derived, and the name has remained despite findings that they are not restricted to this role, having numerous functions in the immune system. These initial anti-viral results raised an interest in the therapeutic potential of the IFNs in disease treatment.
The role of type I IFN has not been fully elucidated, and since their initial discovery, the view on this family of IFNs has changed several times. It is now well known their actions extend beyond an antiviral role with many reports highlighting they are key cytokines involved in multiple innate and adaptive immune responses (Trinchieri, 2010). Their roles encompass the modulation of immune cell functions, required for tumour surveillance and defense, as well as possessing antimicrobial function. Additionally, they are implicated in the development of various autoimmune diseases that include systemic lupus erythromatosis, psoriasis, type I diabetes mellitus and hypothyroidism (Biggioggero et al., 2010)

1.2.1 Type I interferons

There are three types of interferon (I, II and III), distinguished by the receptors they utilize. This project explores only the type I family of IFNs (IFN-Is). The IFN-Is in mammals are separated into nine families; IFN-α, IFN-β, IFN-ε, IFN-κ, IFN-ω, IFN-δ*, IFN-τ*, IFN-λ, IFN-ζ (∗ indicates those not found in humans) (Pestka et al., 2004a; Pestka et al., 2004b), of which this project will focus on IFN-α and IFN-β. There is one gene encoding each of the IFN-Is and one corresponding protein, with the exception of the IFN-α family, which is divided into multiple subtypes.

1.2.1.1 Interferon –α and –β

IFN-α and -β both bind the same receptor and have similar cellular actions. However, primary sequence comparison between the -α and -β subtypes reveal approximately 50% amino acid homology, while the amino acid homologies between the IFN-α subtypes are
approximately 80% (Pestka et al., 1987). These IFNs have structurally similar α-helical cores, however they also have many structural differences (Pestka et al., 2004a) (figure 1.2). This indicates there is a clear division between IFN-α and -β subtypes. There are two chains of the IFN receptor (IFNAR), IFNAR1 and IFNAR2 (Cleary, 1994) At the level of receptor recruitment, a prominent feature of IFN-β compared to IFN-α2 is a ~50-fold higher affinity towards IFNAR1 (Jaitin et al., 2006).

The cellular actions of these IFN-Is, although similar, vary in magnitude. In particular, IFNβ elicits a markedly higher antiproliferation response in some cell types such as melanocytes than do IFN-α. The binding affinity of IFN-β towards IFNAR1 is directly related to the antiproliferative activity of IFN (Jaitin et al., 2006). IFN-α and IFN-β also vary in their cellular actions. This is due to diverse cellular signalling as a result of their different binding affinities to the IFNAR (Pestka et al., 1987; Stark et al., 1998).
1.2.1.2 Type I interferon-α subtypes

There are 14 IFN-α genes, encoding 13 distinct IFN-α subtypes (Pestka et al., 2004a; Pestka et al., 2004b). All cells are capable of producing, as well as responding to IFN-I. As aforementioned, the IFN-Is all bind to a common receptor, IFNAR. It is unknown why there are several subtypes within the IFN-I family with similar morphology. It has been postulated these cytokines are so important that immune redundancy is required, or that each IFN subtype plays a slightly different role. Evidence is accumulating to suggest

Figure 1.2 Ribbon diagrams of IFNα2 and IFN β.

(A) IFNα2 structure compared to (B) IFNβ structure. The alpha-helices are structurally similar (green). The AB loop (blue) is responsible for the high binding affinity to IFNAR2. The AB loop is conserved in the IFN-Is, however there are structural differences, resulting in different binding affinity. It is also hypothesised to differ between IFNα subtypes. Image from Peska et.al (2004).
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that each IFN subtype can induce similar courses of action, whilst signaling functionally unique mechanisms. Each subtype binds to the IFNAR with a different affinity due to both distinct and overlapping receptor binding sites, resulting in different cellular actions (Cull et al., 2003). Binding to the IFN receptor results in signal transduction via the Janus Kinase (JAK)/STAT pathway and expression of IFN-stimulated genes (ISGs) (Cull et al., 2003). The ISGs induced by each IFN subtype has great overlap, and due to this it is believed they are redundant. However each subtype is capable of inducing different ISGs, which translate to cytokines that are important mediators of inflammation and apoptosis. The ISGs induced also varies between cell type. Thus, IFNs have numerous effects on the body, and are particularly important in modulating the immune system.

1.2.2 Production of interferon

1.2.2.1 IFN producing cells

IFN production is induced in response to invading pathogens as part of the early innate immune response. Triggers of IFN-I production are a key area of study for understanding when and how IFN-Is play a role in immunity. All cells are capable of producing IFN-I, with the main trigger being viral infection. However, microbial pathogens or chemical inducers may also induce expression. Infected or phagocytotic cells possess an internal identification system that can result in IFN-I production. IFN-I production is then induced in neighbouring cells in response to activation of the IFNAR on these cells, which can be further amplified via a receptor mediated feedback loop (Beutler et al., 2003). The predominant cell type to produce IFN-I are plasmacytoid dendritic cells (pDC), which can produce up to 1000 times more than other cells, with IFNs comprising 60% of the genes these cells express (Ito et al., 2003).
Therefore, studies on the regulation and production of IFN-I have focused on the subsequent induced immune responses.

1.2.2.2 Inducers of IFN

Production of IFN-I is initiated in viral infection when double stranded RNA (dsRNA) is detected, a product of viral replication, or when viral glycoproteins are present. In bacterial infection, microbial components such as lipopolysaccharides (LPS) and unmethylated CpG motifs of bacterial DNA (CpG DNA) (not found in mammalian DNA) also initiate IFN production (Takeda and Akira, 2004). Such molecules are referred to as “pathogen-associated molecular patterns” (PAMPs). PAMPs are recognised by pattern recognition receptors (PRR), mainly toll-like receptors (TLRs) (Takeda and Akira, 2004). Treatment of cells with IFN-α/β results in the up-regulation of TLRs, suggesting there is a positive feedback loop to strengthen the innate immune response (Miettinen et al., 2001). Thus, the production of negligible amounts of IFN-I is amplified, and can lead to immune activation.

Tumour associated antigens have been reported to be ‘sensed’ by cells via PRRs, with an increase in IFN-β, which is associated with enhanced CD8+ T cell anti-tumour responses (Fuertes et al., 2011). Autoimmune diseases, which develop due to self-antigen being recognised as foreign have been associated with increased IFN-α production (Theofilopoulos et al., 2005). In turn, therapeutic IFN-α can induce autoimmune disease (Gota and Calabrese, 2003), highlighting that IFNs are key modulators in the balance between self-and non-self recognition. The induction of IFN in response to a broad range of microbial pathogen invasions emphasises the importance that these cytokines have in initiating a productive immune response.
1.2.3 Interferon signaling

1.2.3.1 Type I IFN receptor

The specific binding of IFN-I to the IFNAR determines how they exert their actions. The IFN-Is are defined as one family due to the fact they bind and activate the same receptor. This receptor, termed IFNAR, is a heterodimer consisting of two components; low affinity (IFNAR-1) and high affinity (IFNAR-2) (Pestka et al., 2004a). Both IFNAR-1 and -2 are required for complete IFN-I signaling and the downstream development of an effector immune state (Hwang et al., 1995). The different IFN-Is interact with the receptor in various ways, binding with different affinities (Pestka et al., 2004a). They bind at common sites and subtype-specific sites to activate to differentially activate downstream signaling cascades. Within the IFN-α subtypes, distinct cell signaling pathway responses have been observed (Cull et al., 2003; Hilkens et al., 2003a; Hilkens et al., 2003b). This diversity of signal pathway activation induces a gene expression profile specific to the inducing IFN subtype.

1.2.3.2 Signal transduction

Binding of IFN-I to the IFNAR-1 and IFNAR-2 leads to complex formation in which pre-associated with tyrosine kinase 2 (Tyk2) and JAK1, respectively, are activated by cross-phosphorylation. Activation of kinase activity enables phosphorylation of the receptor, allowing recruitment of STAT1 and 2. These STAT molecules are subsequently phosphorylated permitting their dimerization and translocation to the nucleus. Other transcription factors may be recruited to this multi-protein complex, such as Interferon regulatory factor-9 or p48, to further specify binding of the ‘IFN-stimulated response element’ located in the promoter region of ISGs (Pestka et al., 2004a; Pestka et al., 2004b). Notably, the different IFN-I subtypes receptor binding specificities lead to selective activation
of STAT and other signaling molecules (Cull et al., 2003). Alternative signaling pathways include the mitogen-activated protein kinases (MAPK) (Cull et al., 2003), PI3-Kinase (Platanias, 2005), NFkb (Du et al., 2007) and CRKL-dependant pathways (Grumbach et al., 2001). The signalling pathway activated by the IFN subtype determines the induction pattern of ISGs transcribed, and their subsequent cellular actions. The major pathways activated by IFN-I subtypes are the same, albeit different signalling activation between subtypes results in activation of different pathways (Cull et al., 2003).

1.2.4 Anti-tumour action of IFN

1.2.4.1 Direct anti-tumour action

Just as IFN-I inhibits proliferation and induces apoptosis of virally infected cells, it can also act upon transformed cells in a similar manner. IFN-α can directly inhibit the proliferation of tumour cells both in vitro and in vivo (Bolling et al., 2000). Other direct effects on tumour cells include the down-regulation of oncogene and antiapoptotic gene expression (eg. Bcl-2) and induction of tumour suppressor/proapoptotic genes (eg. TNF-related apoptosis-inducing ligand (TRAIL)), which may contribute to the antiproliferative activity of this cytokine (Pfeffer et al., 1998). IFN-I can prevent cellular transformation through enhancing the cellular expression of the p53 tumour suppressor gene (Takaoka et al., 2003). Furthermore, IFN-α has been reported to lower the secretion of IL-8, the major angiogenic factor produced by tumours (Lingen et al., 1998). This results in inhibition of angiogenesis induced by tumour cells (Nyberg et al., 2005).
1.2.4.2  Enhancement of anti-tumour immunity

While evidence exists for direct anti-tumour effects, IFN-I can also have an effect on host immune cells, thus supporting its central role in the overall anti-tumour response (Belardelli, 1995). The body relies on immune surveillance to identify and modulate immunity against mutated cells. However, a healthy immune system may not identify tumour growth, allowing cells to escape immune pressure. By ‘enhancing’ the immune system, IFN may initiate tumour recognition and increase tumour immunogenicity leading to a more robust anti-tumour immune response. Blocking or genetic knockout of the IFN-I receptor on host cells has been a valuable tool in elucidating the anti-tumour properties of IFN-Is. It has been reported there is no change in tumour immunogenicity of ex vivo tumours from IFNAR1 knockout mice (Dunn et al., 2005). Interestingly, IFNAR knockout mice develop more carcinogen-induced primary tumours than wild type control mice (Dunn et al., 2005; Swann and Smyth, 2007), and have an inability to reject transplanted immunogenic IFNAR+ tumours (Dunn et al., 2005). This suggests IFN-I plays a role in immune surveillance of tumours. Additionally, IFN-I therapy is equally effective on interferon-resistant leukemia cells as interferon-sensitive leukemia cells (Belardelli et al., 1983), reinforcing the suggestion that the course of action is immune enhancement rather than a direct effect of the IFN-I. These results indicate that IFN-I plays a major role in tumour rejection and that IFN-I plays a pivotal role for the initiation of a productive anti-tumour immune response. In summary, accumulating evidence indicates that the anti-tumour activity of IFN-I results from both direct action on the tumour as well as and modulation of immune responses.
1.2.4.3 Enhanced innate anti-tumour action

IFN-I is known to augment NK cell–mediated killing directly, providing further evidence for its role in helping to identify and destroy transformed cells. As previously discussed, NK cells recognize and kill cells with low MHC class I levels (Lanier and Phillips, 1996). IFN-Is increase the capacity of NK cells to produce IFN-γ and kill target cells, through both direct and indirect mechanisms via inducing chemokines (Lee et al., 2000; Nguyen et al., 2002). IFN-I encourages NK cells to produce IFN-γ that acts through an autocrine loop to induce production of other cytokines. In addition, the cytotoxicity of NK cells is enhanced by IFN-α/β through induction of TRAIL (Sato et al., 2001). A particularly important feature of TRAIL is its ability to kill cancers with mutations in the p53 gene, which is a common mutation in cancers such as melanoma (Smyth et al., 2003) Indirect actions of IFN-I such as the STAT 1–dependent production of IL-15, stimulates NK cells, which is vital for their proliferation and maintenance (Nguyen et al., 2002). Thus, the actions of IFN-I can unleash a range of cytokines that stimulate NK cells, which in turn increases their ability to identify and kill malignant cells.

IFN-I directly enhances the function of DCs, which are central to the initiation of adaptive immune responses (Steinman and Banchereau, 2007). IFN-Is are important modulators of DCs, influencing their differentiation, maturation, and migration (Ito et al., 2001). IFN-I also up-regulates their co-stimulatory activity and enhances their capacity to present or cross-present antigen (Diamond et al., 2011; Gallucci et al., 1999; Luft et al., 1998; Montoya et al., 2002). In vitro treatment of immature DCs with IFN-I upregulates the surface expression of MHC class I, MHC class II and co-stimulatory molecules CD40, CD80, CD83, and CD86.
which are required for antigen presentation and T cell activation (Montoya et al., 2002; Santodonato et al., 2003).

1.2.4.4 Adaptive anti-tumour immunity

In addition to IFN-Is’ effects on DCs, it is reported that IFN-Is act directly on T cells, and that the ability of T cells to respond to IFN-I is required to reach optimal effector function and clonal expansion (Havenar-Daughton et al., 2006; Kolumam et al., 2005; Le Bon et al., 2006a). IFN-I prolongs the survival of T cells (Havenar-Daughton et al., 2006; Kolumam et al., 2005; Marrack et al., 1999), and reduces the activity of T-reg cells which have been reported to be present at higher levels in melanoma patients (Viguier et al., 2004). Additionally, IFN-I enhances the capacity of T cells to respond to antigen (Hervas-Stubbs et al., 2012). It has also been shown that IL-15 produced by DCs in response to IFN-I is required for the persistence of CD8 T cells, and conversion/maintenance of memory T cells (Zhang et al., 1998). These traits are important to ‘wake up’ the immune system to tumour antigens, to launch a tumour specific attack.

IFN-I again acts as a link between innate and adaptive immunity for microbial infection through enhancing the primary antibody response. IFN-I stimulates the production of all subclasses of IgG, and induces long-lived antibody production by B cells. This enhanced antibody response and stimulation of isotype switching is, in part, due to the IFN-I effects on CD4+ T cells (Coro et al., 2006; Le Bon et al., 2006b), as well as DCs that stimulate CD4 T cells to activate B cells (Le Bon et al., 2001). IFN-I also has direct effects, such as enhancing B cell differentiation, antibody production and isotype class switching (Le Bon et al., 2001).
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Antitumor monoclonal antibodies have been reported to enhance cross-presentation of tumour antigen, which results in production of tumour specific CD8 T cells (Dhodapkar et al., 2002).

1.2.5 Therapeutic applications

The immune modulating capacity of the IFN-I family makes them useful therapeutics. It is well accepted that IFN-I, in particular IFN-α and IFN-β, possess powerful antiviral activity as well as perform a critical role in modulating immune responses to foreign and self-antigens. IFN-α is currently approved for human treatment of hairy cell leukaemia, malignant melanoma, follicular lymphoma, genital warts (human papillomavirus), Kaposi sarcoma, hepatitis B and C; and IFN-β for multiple sclerosis (Pestka, 2007).

1.2.5.1 Side effects

A caveat of IFN-I therapy is the high dose required for therapeutic efficacy (up to 20 MU/m²/day). Many patients cannot tolerate the high doses necessary to control their cancer or viral disease due a range of undesirable side effects. The most common described by patients are ‘flu-like’ symptoms, including headache, fatigue, fever, chills and joint pains, which occur after administration and continue for a few hours (Dusheiko, 1997; Walther and Hohlfeld, 1999). These symptoms are predictable and often short in duration. However, other adverse advents, most notably neuropsychiatric side effects including depression and irritability which decrease quality of life (Valentine et al., 1998), require the dose to be decreased or therapy to be ceased altogether. Due to these systemic effects, locally administered IFN-I could be developed as a possible future therapy.
Therapeutic administration of IFN-α during malignant or viral disease occasionally leads to development of autoimmunity, most prominently a lupus-like syndrome, (Fitzgerald-Bocarsly et al., 2008; Gilliet et al., 2008) or thyroid autoimmunity, which resolves when treatment has ceased (Burman et al., 1986). Interestingly, IFN-I induced autoimmunity is associated with improved outcome in patients with cancer, particularly melanoma (Gogas et al., 2006).

1.2.5.2 Future of type I IFN

Due to the side effects of high dose IFN, the future application of therapy may be via different routes of administration. Alternatively, inducers of endogenous IFN production may be tailored to anti-tumour therapy. Drugs or molecules which induce IFN-I production through TLR activation, such as CpG oligonucleotides and quinolinamin imiquinoids (Hemmi et al., 2002; Hemmi et al., 2000) could provide a cost effective alternative to purified IFN-I. However, the TLR signaling pathway also induces other inflammatory cytokines which may not be beneficial to tumour clearance (Krieg, 2006). Future drugs may include IFN-I agonists to produce specific immunomodulatory effects, whilst minimising unwanted side effects.

IFN-Is have immunostimulatory properties, such as inducing maturation of DCs, indicating its potential to be used as a vaccine adjuvant. This has been demonstrated in a study where co-injection of IFN-I with antigen resulted in enhanced T cell priming by DCs and improved immune response against the antigen (Cull et al., 2002; Gallucci et al., 1999; Prchal et al., 2009). IFN-I can also enhance or modulate antibody production (Cull et al., 2002; Le Bon et al., 2001) potentially optimizing the immune response.
An interesting area of research into anti-tumour immunity is the use of adoptive cell therapy (ACT). In ACT, tumour specific CD8 T cells are extracted from the patient, expanded in vitro, and re-administered. This treatment can result in significant control and remarkably, complete clearance of disease (Rosenberg and Dudley, 2009; Rosenberg et al., 1986). A study exposing tumour specific CD8 T cells to IFN-α during priming resulted in increased proliferation and enhanced functional activity against tumour antigen (Hervas-Stubbs et al., 2011). Perhaps following more rigorous testing, IFN-Is may routinely be employed to improve ACT.

1.3 DNA GENE THERAPY

1.3.1 Immuno-gene therapy

Immune gene therapy is the ability to administer DNA encoding a specific cytokine without a vehicle to generate foreign genes in vivo. This is a promising method to deliver treatments in a way that is long lasting and consistent (Levy et al., 1996). This is particularly advantageous in the administration of immunostimulatory cytokines as it is able to generate high concentrations locally. This mimics the body’s own response against foreign antigen and sustains high levels in the body that can activate the immune system.

Naked DNA vaccines consist of a protein-encoding gene, such as a viral protein or cytokine, incorporated into a self-replicating, closed circular DNA plasmid. The plasmid contains DNA sequences for control of replication in bacteria, for vaccine production, and for control of expression of the foreign gene within mammalian cells. Following manufacture in bacteria,
the plasmid is then purified to remove contaminating bacterial products prior to administration, hence the term ‘naked DNA’ (McDonnell and Askari, 1996).

1.3.2 Administration

The introduction of naked plasmid directly into cells *in vivo* was first demonstrated by Wolff et al (1990). It was discovered that skeletal muscle could take up and express the proteins encoded by naked plasmid DNA just as effectively as cells transfected *in vitro* under optimal conditions. Muscle is particularly suited to plasmid uptake and protein expression due to its structural features (Wolff et al., 1990). In addition, muscle is capable of regeneration after damage, which is when cells are most susceptible to DNA uptake.

Danko and Wolff (1994) found that inducing muscle cell damage increases DNA uptake and expression. The optimal pre-treatment to cause muscle damage is with bupivacaine, which selectively destroys myofibres. Treatment with 0.5% bupivacaine 7 days prior to plasmid injection gives up to 40 fold greater expression, which lasts up to 2 months (Bartlett et al., 2003; Danko and Wolff, 1994). In treatment of localized lesions, such as tumours, this technology may be administered into or nearby the lesion, resulting in a more local effect (Heller et al., 2002; Nabel et al., 1993) The persistence of protein expression has been investigated for DNA gene therapy, with most studies reporting a peak in expression 1-2 weeks following inoculation (Bartlett et al., 2003). However, persistent expression depends on the antigenicity of the protein (Prud'homme et al., 2001).
1.4 AIMS AND OBJECTIVES

1.4.1 Summary

Melanoma can be a highly immunogenic tumour, and enhancement of the immune response with IFN-α2 has proven a successful therapy in battling this cancer. The therapeutic efficacy of the IFN-I subtypes on melanoma has not been elucidated, despite IFN-α2 being the gold standard therapy for late stage melanoma patients. IFN-α2 has been found to modulate the immune system and it has become accepted that this action enhances anti-tumour immune responses. In addition, IFN-α2 has also demonstrated direct anti-tumour activities. However, the precise mode of action that IFN-α2 has in decreasing melanoma burden is not well documented. Finally, there is accumulating evidence for distinct and overlapping functions of the type I IFN family, in particular the IFN-α subtypes. It has been found that the IFN-α subtypes display different biological activities, and therefore may have the potential to have different therapeutic efficacies. Investigation of the IFN-α subtype cytokines other than IFNα2 may lead to development of new therapies in the treatment of melanoma. Therefore, the immediate goal of this project was to determine if the IFN-I subtypes have efficacy in treating melanoma.

1.4.2 Hypotheses

The basis of this project has arisen upon two hypotheses. There is already strong evidence to support both these hypotheses.

1. Type I IFN subtypes induce differential efficacy in the treatment of melanoma.
2. Type I IFN modulates the immune response to melanoma which is IFN subtype dependent.

1.4.3 Aims

1. Combine a melanoma model expressing immunogenic peptide (gB) with a model of DNA therapy to administer IFN subtypes.

2. Identify the type I IFN subtypes with therapeutic efficacy in treating melanoma
   
   \textit{in vivo}.

3. Identify differences in the actions exerted upon melanoma between the type I IFN subtypes.

4. Determine if immune enhancement against tumour antigen occurs as a result of type I IFN therapy.
Chapter 2: Materials and Methods

2.1 PREPARED BUFFERS AND SOLUTIONS

Table 2.1: Constitutes of prepared buffers

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Constitutes</th>
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<tbody>
<tr>
<td>Annexin V binding buffer (1x)</td>
<td>10mM HEPES; 140mM NaCl; 2.5 mM CaCl&lt;sub&gt;2&lt;/sub&gt; dissolved in ddH&lt;sub&gt;2&lt;/sub&gt;O</td>
</tr>
<tr>
<td>cRPMI</td>
<td>RPMI (Life Technologies, NY, USA) supplemented with 10% foetal calf serum (FCS) (Serana, WA, AUS), 1% M 2-mercaptoethanol (1000x)(Life Technologies, NY, USA), 1% glutaMAX (100x)(Life Technologies, NY, USA) and 1% penicillin/streptomycin (10,000 U/ml / 10,000 ug/ml) (Life Technologies, NY, USA).</td>
</tr>
<tr>
<td>FACS wash</td>
<td>D-PBS (Life Technologies, Paisley, UK) with 5 mM EDTA and 1% BSA (Sigma, USA)</td>
</tr>
<tr>
<td>High salt Neutralisation buffer</td>
<td>2.5M KOAc; 5% Formic acid (v/v)</td>
</tr>
<tr>
<td>Ketamine solution</td>
<td>Prepared by addition of 1 ml Ketamine (100 mg/ml)* and 0.75 ml Ilum Xylazil (20 mg/ml)* to 8.25 ml D-PBS (Life Technologies, Paisley, UK).</td>
</tr>
<tr>
<td>Luria broth (LB)/ agar</td>
<td>25g LB (Invitrogen, CA, USA) per 1L (≈1% Peptone 140, 0.5 % Yeast Extract, 171mM NaCl). Autoclaved. For agar, 15 g/L LE-agarose (Fisher-Biotec, AUS) added to broth before autoclaved. Kanamycin was added (10 μg/ml) and</td>
</tr>
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2.2 ANIMALS

2.2.1 Ethics

All experiments performed for this study were approved by the Telethon Institute of Child Health Research (TICHR) Animal Ethics Committee (AEC # 252).

2.2.2 Animal handling

Mice were obtained from the Animal Resources Centre (Murdoch, WA) or bred onsite and were housed in the Bioresource Centre at TICHR under strict living conditions. The temperature and lighting were controlled, with a 12-hour light/dark cycle to mimic day and night living. Mice had free access to food and water, and were provided tissue and tubes for enrichment. Mice were sacrificed at specific time-points by administration of CO\textsubscript{2} at a 20% volume/minute fill rate.

2.2.3 Mouse strains

C57Bl/6J (B6) mice were received at 6 weeks of age, and used between 8-12 weeks of age. gBT-1 x B6.CD45.1 mice, have lymphocytes that express the lymphocyte surface antigen CD45.1 (also known as Ly5.1) (C57BL/6 lymphocytes express CD45.2). The CD8 T cell

<table>
<thead>
<tr>
<th>Material</th>
<th>Description</th>
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<tbody>
<tr>
<td>LiCl solution</td>
<td>5 M LiCl; 1% MOPS (w/v), pH to 8.0</td>
</tr>
<tr>
<td>Lysis buffer</td>
<td>0.2 M NaOH: 1% SDS (made fresh in ddH\textsubscript{2}O)</td>
</tr>
<tr>
<td>TAE running buffer</td>
<td>40 mM Tris; 20mM acetic acid; 1mM EDTA</td>
</tr>
<tr>
<td>TEG swelling buffer</td>
<td>25 mM Tris, 10mM EDTA, 0.9% glucose (w/v) pH 8.0</td>
</tr>
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</table>
receptors (TCR) of gBT-I transgenic mice express V\(\alpha\)2/\(\beta\)8. These TCRs are specific for herpes simplex virus-1 glycoprotein B\(_{495-505}\) and are H-2\(K\)\(^{b}\)-resticted (Mueller et al., 2002). RAG-/- mice have no recombination activating gene, preventing maturation of T and B cells. (Mombaerts et al., 1992).

2.2.3.1 Phenotyping

The transgenic T cells of gBT.I x B6.CD45.1 mice express the markers V\(\alpha\)2 and CD45.1, which were used to phenotype offspring from heterozygous parents. To phenotype mice, blood was collected when offspring were at least 6 weeks old. Mice were first heated in a box with a heat lamp before making an incision in the tail vein with a sterile scalpel and collecting approximately 100\(\mu\)l of blood into an Eppendorf tube containing 20\(\mu\)l heparin. Pressure was applied to the wound, followed by application of ferric chloride. To lyse red blood cells, 1 ml red blood cell lysis buffer (Sigma, MO, USA) was added to the blood and incubated on ice for 5 minutes before being transferred into a FACS tube (BD, NJ, USA) and centrifuged at 1600 rpm for 5 minutes. Supernatant was poured off and the following antibody mix added: anti-mouse V\(\alpha\)2-PE, anti-mouse CD8-APC, and anti-mouse CD45.1-V450 (see section 2.5.2 for cell staining).
Figure 2.1: Phenotype of a negative and positive gBT.I x CD45.1 transgenic mouse.

Live cells were distinguished as propidium iodide (PI) negative, and lymphocytes were characteristically larger with a greater FSC (left plot). Lymphocytes expressed CD8, and samples homozygous for Vα2 receptor (larger proportion of Vα2⁺ + CD8⁺ cells) were indicative of a transgenic animal (central lower plot, upper right quadrant). These Vα2 positive lymphocytes also express CD45.1 (right plot).
2.2.4 Anaesthesia

Mice were anaesthetised either through injection of ketamine solution (see section 2.1) or by isoflurane inhalation (3%). Using the pre-prepared ketamine solution, mice were anaesthetised by intraperitoneal injection (10 µl/g body weight) using a 1 ml syringe (Terumo, Philippines) and a 27-gauge needle (Terumo, Philippines). To prevent blindness, once anaesthetised the eyes of the mice were covered with Lacri-Lube® lubricating eye ointment (Allergen, NSW, AUS). Mice were placed in cages on a heat pad until they regained consciousness to maintain a stable core body temperature. For short procedures “Attane” Isoflurane (BOMAC, NSW, AUS) was used in a specific ISOTEC 3 vaporiser (AAS, NSW, AUS) at 2.5-3% in 6L medical air/ minute. All mice were monitored until full recovery.

2.2.5 Mapping lymph node drainage

To map lymph node drainage from the tibialis anterior muscle, and two subcutaneous sites: upper and lower flank, Evan’s blue dye (0.5%) was injected into these sites. Mice were first weighed and anaesthetised, and the left side flank and leg of each mouse was then shaved with a clipper and depilated with VEET™ to remove hair. 50µl of Evan’s blue dye was injected on the left side using a 26-gauge needle, either intramuscularly into the tibialis anterior, subcutaneously into the lower flank, or subcutaneously into the upper flank (n=4 mice/group). Mice were left for 0.5 or 2 hours, in cages, on a heat pad while unconsciousness. After 0.5 or 2 hours, mice were sacrificed and the ipsilateral and contralateral brachial, axillary and inguinal lymph nodes harvested.

2.2.6 Tumour inoculation
B16 F1 melanoma cells were grown to 70% confluency, and harvested using TrypLE (Life Technologies, Denmark). Cells were then washed 3x in RPMI (Life Technologies, NY, USA) to remove any traces of FCS. Next cells were counted, and resuspended in PBS at a final concentration of $2 \times 10^6$ cells/ml. Mice were anaesthetised using Isoflurane, and clipped with a 2-speed clipper (Wahl, USA) and depilated with VEET™ (Reckitt Benckiser, NSW, AUS) to remove all hair on the left side flank. Cells in PBS (Life Technologies, Paisley, UK) were vortexed prior to loading a 1 ml syringe (Terumo, Philippines) with 26-gauge needle (Terumo, Philippines). 50 µl ($1 \times 10^5$ cells) were inoculated subcutaneously by pinching the skin up with tweezers and injecting under the dermis. The skin was wiped clean with 70% ethanol. Growth was measured by using calipers (Kincrome, USA) to determine the length and width. Tumour volume in mm$^3$ was calculated by the formula: $(\text{length} \times \text{width}^2)/2$.

### 2.2.7 DNA inoculation procedure

To induce muscle regeneration, 20 µl of 0.5% bupivacaine (BP) was injected bilaterally into the tibialis anterior muscles using a 0.5 ml insulin syringe (B.D., NJ, USA). Five days later, DNA constructs (200 µg/mouse) were inoculated in a 25 µl volume per leg into the same site.

### 2.2.8 Tail vein injection

Carboxyfluorescein succinimidyl ester (CFSE) fluorescently labelled or activated purified populations of T cells (see section 2.6.3) were resuspended in PBS at $1 \times 10^6$ cells/100 µl. Mice were warmed under a heat lamp and 200 µl injected intravenously (iv) via the tail vein using a 1 ml syringe (Terumo, Philippines) with 26 gauge needle (Terumo, Philippines)

### 2.2.9 HSV infection
Mice were anaethetised with ketamine solution. The left side of each mouse was clipped and
depililated. A 4 mm$^2$ area of skin on the lower flank was grazed using a Dremel 3000 (Dremel,
WI, USA) with a grindstone attachment, which was held on taught skin for 20 seconds. A 10
µl aliquot of HSV (10$^6$ PFU) (made in house using KOS laboratory strain) was applied to the
grazed skin and rubbed in using a cotton tip soaked in D-PBS (Life Technologies, Paisley,
UK). To contain the virus during the initial infection, a 1x2 cm piece of OpSite Flexigrid
(Smith & Nephew, Hull, UK) was placed over the inoculation site, and secured by wrapping
the flank of the mouse with Micropore tape (3M, Neuss, Germany) followed by Transpore
tape (3M, MN, USA). The tape and Flexigrid were removed 48 hours after infection.

2.1.9 Sera preparation

Whole blood was obtained from mice from the tail vein as described previously (see section
2.2.3.1) into Eppendorfs without heparin. Blood was allowed to clot for 1-2 hours at 4°C
before centrifugation at 16,000 rpm for 15 minutes to separate the sera from cells. Serum was
aliquoted and stored at -20°C until required.

2.3 MELANOMA CELLS

2.3.1 Melanoma clones

Four B16 F1_gB clones (B6F, B8F, B10M, H8M) and a K$^b$-loss variant (B16 F1 Kb$^-$) were
provided by Dr. Waithman (TICHR, WA). Clone nomenclature was derived from the clones’
co-ordinates on the plate from which it grew (first letter and number) and the last letter refers
to in vitro growth kinetics (F=fast, M=medium, S=slow). The B16 F1 cell line was originally
derived from an induced B6 murine melanoma. The gB B16 clones were engineered further to
express herpes simplex virus (HSV) glycoprotein B (gB) and a green fluorescent protein
(GFP) under the same promoter, denoted B16-gB. The B16 F1\textsuperscript{kh} variant harbours a mutation resulting in loss of MHC class I H-2K\textsuperscript{b}. The line has also lost melanin expression (Cho, Lee and Celis, 2010).

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Feature</th>
</tr>
</thead>
<tbody>
<tr>
<td>B16 F1</td>
<td>Parental murine melanoma cell line</td>
</tr>
<tr>
<td>B16 F1\textsubscript{gB} (B6F, B8F, B10M, H8M)</td>
<td>Clones of B16 F1 engineered to express HSV gB and GFP under the same promoter.</td>
</tr>
<tr>
<td>B16 F1\textsuperscript{kh}</td>
<td>B16 F1 variant which no longer expresses MHC class I H-2K\textsuperscript{b}. It is also amelanotic.</td>
</tr>
</tbody>
</table>

### 2.3.2 Thawing cells

Cells were stored in liquid nitrogen. Prior to use, cells were thawed rapidly to room temperature, diluted in cRPMI and centrifuged at 1600 rpm for 5 minutes before being
resuspended in fresh cRPMI (Life Technologies, NY, USA).

2.3.3 Culture and harvesting

Melanoma cells were grown in cRPMI medium. Cells were grown in tissue culture plates (B.D., NJ, USA) and incubated at 37°C with 5% CO², and were harvested once 70% confluent. To harvest, media was removed and 2 ml of TypI trypsin enzyme (Life Technologies, Denmark) was added and cells incubated at 37°C for 3 minutes before adding fresh complete media to deactivate the enzymes. Media containing cells was collected and centrifuged at 1600 rpm for 5 minutes. Supernatant was removed and fresh cRMPI added. Pelleted cells were resuspended by gentle pipetting and cells were split 1 in 20 prior to reseeding.

2.3.4 Cryopreservation

Cryopreservation was performed by pelleting harvested cells and resuspending in 10%DMSO (Merck, Darmstadt, Germany), 90% FCS (Serana, WA, AUS) to a final concentration of 2x10⁶ cells/ml. Samples were aliquoted into cryovials (Fisher-Biotech, QC, Canada), placed into a CoolCell (Biocision, USA) cryo-freezing container and placed in a -80°C freezer overnight. Cryovials were transferred to liquid nitrogen the following day.

2.3.5 Centrifugation and cell counting

Cells were pelleted by centrifugation at 1600 rpm for 5 minutes unless otherwise stated. Cell counts were performed by vortexing cells and taking a 20 μl aliquot, which was mixed with 20 μl trypan blue (0.4% in PBS)(Life Technologies, NY, USA) and counted using a haemocytometer (Neubauer, Germany) viewed under a microscope (Olympus, Japan). Cells
counted in one grid were entered into the following formula:

\[ \text{#cells/ml} = \text{#cells counted} \times \text{dilution factor} \times 10^4. \]

## 2.4 MOLECULAR BIOLOGY

### 2.4.1 In vitro interferon assays

In house murine type I IFN subtypes were provided by Dr. Fear (TICHR, WA). Commercial IFN-α and IFN-β preparations were used as comparative controls. The in house IFN-I subtypes were derived from the supernatant of COS-7 monkey kidney cells transiently transfected with a vector encoding each subtype.

Melanoma cells were grown in a 6 well plate (B.D., NJ, USA) at 5x10⁴ cells/ well for 24 hours prior to stimulation. IFN-I subtypes were diluted in cRPMI to a concentration of 100 IU/ml. Recombinant murine IFN-γ (Shenandoah Biotec, PA, USA) was diluted to 10 ng/ml in cRPMI. Old media was aspirated from the plate and 2 ml of fresh media containing IFN was added to each well (1 well per subtype). A well with only media was used as a control. For IFN-I assays, supernatant from COS-7 cells infected with a blank plasmid was used as an additional control. Cells were incubated for 48 hours prior to harvest unless otherwise stated.

### 2.4.2 Interferon DNA

The IFN subtypes were incorporated into the mammalian expression vector pkCMVint (Vical, Inc.). DH5-α E. coli cultures containing plasmids encoding the Type I IFN subtypes were provided by Dr. Fear (TICHR, WA).
2.4.3 Plasmid preparation

Bacterial cultures containing the required plasmid were prepared by inoculating a loopful of glycerol stock plasmid onto LB agar plate containing 10 g/mL Kanamycin and incubated overnight at 37°C. A single colony was taken from the plate, placed in 1L of LB broth with 10 μg/ml Kanamycin and incubated overnight at 37°C with shaking. The bacterial cells were harvested by centrifugation at 3500 rpm for 10 minutes at 4°C. The pellet was resuspended in TEG swelling buffer at 4°C followed by the addition of fresh lysis buffer. The cells were left on ice for 10 minutes before the addition of 0.5 volumes high salt neutralisation buffer. This mixture was centrifuged at 3500 rpm for 10 minutes at 4°C and the supernatant then filtered through sterile gauze and an equal volume of isopropanol added prior to re-centrifugation. The pellet was then resuspended in H₂O, an equal volume LiCl solution added and left on ice for 10 minutes before centrifugation at 3500 rpm, 4°C for 15 minutes. The supernatant was transferred to a new tube and an equal volume of isopropanol added prior to re-centrifugation. The pellet was briefly dried and dissolved in ddH₂O (Baxter, NSW, AUS) before treatment with 2 μl RNase A (100 mg/ml) (Qiagen, Germany) per 100 μl, then incubated at 37°C for 15 minutes. The supernatant was then extracted with phenol: chloroform: IAA (25:24:1) and centrifuged at 10000rpm for 5 minutes at 20°C. The aqueous phase was transferred to a fresh tube. The DNA was precipitated with 1/10 volume 3M sodium acetate, pH 4.8, and 2.5 volumes ethanol at -20°C for 30 minutes. DNA was pelleted by and centrifugation at 16000 for 20 minutes. The pellet was washed in 70% ethanol, re-centrifuged for 5 minutes and resuspended in ddH₂O. DNA concentration was determined by spectrophotometric analysis using a Nanodrop (NanoDrop Technologies Inc., DE, USA) at 260 nm. DNA purity was assessed by 260/280 ratio >1.8.
2.4.3.1  *Restriction enzyme digest*

1μg DNA was digested with 1U of each restriction endonuclease in the corresponding 1x Reaction Buffer with BSA at 37°C overnight.

<table>
<thead>
<tr>
<th>R.E. (Promega)</th>
<th>Buffer range</th>
</tr>
</thead>
<tbody>
<tr>
<td>XbaI/BamHI</td>
<td>NEB 3 (NEB Inc., MA, USA)</td>
</tr>
<tr>
<td>SalI/XbaI</td>
<td>D (Promega, WI, USA)</td>
</tr>
<tr>
<td>XbaI/BglII</td>
<td>D</td>
</tr>
<tr>
<td>BamHI/BglII</td>
<td>NEB 3</td>
</tr>
<tr>
<td>SalI/BglII</td>
<td>D</td>
</tr>
<tr>
<td>PstI/SalI</td>
<td>D</td>
</tr>
<tr>
<td>PstI/BglII</td>
<td>D</td>
</tr>
</tbody>
</table>

2.4.3.2  *Gel electrophoresis*

1% Agarose gel was prepared by addition of 1g agarose (Fisher-biotec, AUS) in 100 ml 1 x
TAE (see section 2.1) which was then heated and cooled before the addition of 5 μl Midori Green Advanced DNA stain (Nippon Genetics, Duren, Germany) poured into gel tray and allowed to set. The gel was placed in 1 x TAE running buffer containing Midori Green Advanced DNA stain. DNA was mixed with 6x loading dye (Promega, WI, USA) and loaded into gel wells. The gel was run at 100 V for 30-40 minutes alongside Lambda/ Hind III standards (Promega, WI, USA) and 10 kb ladder (Promega, WI, USA).

2.5 IMMUNOLOGICAL METHODS

2.5.1 ELISA

Murine Type I IFN levels in sera and tissue supernatant was quantified using a VeriKine mouse IFN-α ELISA kit (PBL interferon source, NJ, USA) designed to detect all murine type I IFN-α subtypes in the range of 15-1000 pg/ml. To detect murine IFN-β, a pre-coated VeriKine mouse IFN-β ELISA kit (PBL interferon source, NJ, USA) which detects in the range 12.5-2000 pg/ml was used. The kits contained all required solutions and the procedure was performed according to the manufacturers instructions. Standards and samples were diluted 1:2 in dilution buffer and added to the plate (100 μl/well). The plates were read using a WALLAC Victor 3 spectrophotometer (PerkinElmer, MA, USA) at absorbance of 450 nm within 5 minutes of the stop solution.
2.5.2 T cell harvesting and activation

T cells were harvested from the lymphoid organs of one gBT.I x CD45.1 mouse and one B6 mouse for adoptive transfer. Lymphoid organs were harvested and made into a single cell suspension by passing through a sterile metal mesh using the blunt end of a 3 ml syringe, and filtered through 70 μm nylon. B6 cells were pelleted and resuspended in 4 ml RPMI, to which 4 μl of $10^{-3}$ M gB peptide was added, to make a final concentration of approx. $200 \times 10^7$ cells/ml. gBT.I x CD45.1 and B6 cells were incubated at 37°C for one hour with vortexing every 15 minutes. Cells were washed twice in RPMI (Life Technologies, NY, USA) and resuspended in 4 ml cRPMI with the addition of 3 μg/ml LPS (Sigma, USA). gBT.I x CD45.1 cells were pelleted and resuspended in 4 ml cRPMI, of which 2 ml was added to 2 ml of B6 cells with the addition of 36 ml cRPMI in a T175 culture flask (Greiner Bio-one, Germany). Cells were cultured upright at 37°C. After 2 days the cells were split 1:2, topped up to 40 ml with cRPMI and 40 IU/ml recombinant IL-2 added (Peprotech, USA). Cells were incubated upright at 37°C for one day before being collected.

2.6 FLOW CYTOMETRY

2.6.1 Sample preparation

For single cell preparations from tumours, lymph nodes and spleens, whole organs were collected in cRPMI, pushed through a metal mesh and passed through 70 μm nylon mesh. Viable cell counts were performed using trypan blue (0.4% in PBS)(Life Technologies, NY, USA) exclusion. For blood analysis see section 2.2.3.1. Cells grown in vitro were made into a single cell suspension using TryplE (Life Technologies, Denmark) and passed through a 70
\( \mu m \) nylon mesh before analysis by flow cytometry.

### 2.6.2 Cell staining

Flow cytometry antibody staining for extracellular markers was typically conducted in round-bottom FACS tubes. Cells were pelleted by centrifugation and washed with FACSwash before staining with an excess of antibody for 30 minutes in the dark at 4\(^\circ\)C. Cells were washed with FACSwash following each stain. If subsequent rounds of staining were necessary (eg. SAV conjugates), this process was repeated. Prior to analysing the cells by flow cytometry, cells were resuspended in 100-200 \( \mu l \) FACSwash and 10 \( \mu l \) of propidium iodide (50 \( \mu g/ml \))(Sigma, USA) was added to each sample prior to analysis to discriminate between live (PI-) and dead (PI+) populations, unless otherwise stated. Sample data was collected by flow cytometry using a LSRFortessa\textsuperscript{TM} (B.D., USA) with FACSDIVA\textsuperscript{TM} (v.6.2) software, and subsequent analysis was performed using FlowJo\textsuperscript{TM} (TreeStar, USA) software. Table 2.1 summarises the antibodies used:

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-mouse CD8-APC</td>
<td>1:200</td>
<td>BD Pharmingen, CA, USA</td>
</tr>
<tr>
<td>Anti-mouse Va2-PE</td>
<td>1:200</td>
<td>BD Pharmingen, CA, USA</td>
</tr>
<tr>
<td>Anti-mouse CD45.1-V450</td>
<td>1:200</td>
<td>e-bioscience, CA, USA</td>
</tr>
<tr>
<td>Anti-mouse MHC class I (H-2K\textsuperscript{b})-biotin</td>
<td>1:200</td>
<td>In-house</td>
</tr>
<tr>
<td>Anti-mouse MHC class II (I-A\textsuperscript{b})-PE</td>
<td>1:200</td>
<td>BD Pharmingen, CA, USA</td>
</tr>
</tbody>
</table>
2.6.3 CFSE labeling

2.6.3.1 CD8+ T cell purification via negative selection

T cells were first purified from harvested lymphoid organs before labelling. Lymphoid organs were harvested as outlined in section 2.5.1. A T cell enrichment antibody cocktail containing anti-Mac-1; anti-F4/80; anti-erythrocyte; anti-GR-1; anti-I-A/I-E; and anti-CD4 was added to the cells for 30 minutes. “Biomag” goat anti-rat IgG-coupled magnetic beads (Qiagen, Hilden) were washed 3x in cRPMI and magnetized with a magnetic column. Excess T cell enrichment antibody cocktail was washed off with cRPMI before addition of Biomag beads at a ratio of 7.5:1 to bind to antibody coated cells. The cell and bead mixture was incubated on a rotor at 4°C for 20 minutes before placing on a magnetic column to remove antibody coated cells, and harvesting the T cells in the supernatant. An aliquot was taken to check cell purity (see section 2.1.4.1).

2.6.3.2 CFSE labelling of T cells and B16 F1 tumour cells

To CFSE label purified T cells or melanoma cells, the cells were spun down and resuspended in 10 ml PBS + 0.1% BSA (Sigma, USA) to which 2.5 μl of 10 mM CFSE (Molecular Probes, USA) was added and quickly vortexed. Cells were incubated at 37°C for 10 minutes before being washed with RPMI.
To determine GFP expression levels in cell lines, cells were harvested and flow analysed without staining using the FITC channel.

2.6.5 **Biotin-conjugation of monoclonal antibody**

Anti-mouse MHC class I (H-2K\(^b\)) monoclonal IgG was biotinylated using Sulfo-NHS-SS-Biotin. Sulfo-NHS-SS-Biotin was equilibrated to room temperature before opening. 2.2 mg of Sulfo-NHS-SS-Biotin was weighed and added to 360 μl of ddH\(_2\)O to make a 10 mM solution. The amount of Sulfo-NHS-SS-Biotin required for a 20-fold molar excess to 1 ml of 9.84 mg/ml was calculated to be 0.001312 mmol, and hence calculated 130.3 μl of 10 mM solution was required. 130.3 μl of 10 mM Sulfo-NHS-SS-Biotin was added to 1 ml of 9.84 mg/ml IgG. Ab-biotin solution was incubated at room temperature for 1 hour. The Ab-biotin solution was added to a Slide-A-Lyser Dialysis Cassette (Thermo Scientific, USA) according to manufacturer’s instructions using a 3 ml syringe with 23-gauge needle. The cassette was dialysed in 5 L PBS overnight at 4°C. The resulting anti-mouse MHC class I (H-2K\(^b\))-biotin was removed from the cassette as per the manufacturer’s instructions and diluted with PBS to 2 mg/ml with the addition of 1% FCS and 1% NaAzide.

2.6.6 **Analysis of adoptively transferred gBT.I T cells**

Adoptively transferred, CFSE labelled gBT.I T cells were analysed for cell division in the spleen and lymph nodes 60 hours after transfer. Single cell suspensions were prepared as stated previously (section 2.5.1). Cells were stained using anti-mouse CD8-APC, anti-mouse V\(\alpha2\)-PE and anti-mouse CD45.1-V450 to identify the transferred CSFE labeled cell population. Following acquisition, analysis was performed by gating on lymphocytes using PI and FSC followed by further gating the CD8\(^+\) population, and gBT.I T cells discriminated
from the B6 T cells as Vα2+ and CD45.1+ (see section 2.1.4.1 phenotyping).

Cell division was determined via dilution of CFSE dye in Vα2+ CD45.1+ CD8+ T cells via histogram analysis.

2.6.7 MHC Class I and II

Cells were stained first with anti-mouse MHC class II (I-A\(^b\))-PE and anti-mouse MHC class I (H-2K\(^b\))-biotin, followed by SAV-APC. Melanoma cells were gated using PI and FSC, then MHC class I or MHC class II expression was assessed, and mean fluorescent intensity calculated.

2.6.8 Apoptosis

Apoptotic cells were discriminated using anti-mouse Annexin V-biotin in Annexin binding buffer which was incubated at room temperature. Cells were washed and then incubated with SAV-APC which was not washed after and 5 μl/sample of PI (50 μg/ml) which was incubated at room temperature for 15 minutes before addition of 1 ml binding buffer. Early apoptotic cells were classified as PI-, Annexin V+, and late apoptotic/necrotic cells as PI+, Annexin V+.

2.7 SOFTWARE AND STATISTICS

Flow cytometry analysis, tables and graphs were produced using FlowJo software (Tree star,
USA). Survival (Kaplan-Meier) curves were produced in GraphPad Prism, and analysed using a Log-rank (Mantel-Cox) test. Tumour growth curves were analysed in Prism using a Mann-Whitney U Test with Dunn’s multiple comparison test. Bar graphs displaying parametric data were produced using Prism software and analysed by T-test. Statistical significance was reached when \( p < 0.05 \).
Chapter 3: Results

3.1 LYMPHATIC DRAINAGE OF THE SKIN AND MUSCLES

To obtain constitutive and systemic production of IFN and overcome the requirement for serial injections, IFN DNA therapy was selected as the mode of delivery. It is hypothesized that IFN DNA gene therapy more closely resembles pegylated IFN therapy used in the clinical setting, where a small dose is available systemically at a constant rate. For IFN DNA therapy, inoculation into skeletal muscle was required. The TA muscle was chosen as the site of inoculation as this is a large skeletal muscle that is easily accessible and can withstand up to a 25 µl volume of DNA per bilateral injection.

The melanoma tumour model employed for this study involved subcutaneous inoculation of $10^5$ B16 melanoma cells. As the site of DNA therapy was the TA muscle, the site of melanoma injection was optimised so as to share the same lymph node drainage site. Two subcutaneous sites (the upper and lower flank) were tested in parallel with the TA muscle (figure 3.1A). To map the lymphatic drainage of these areas, 50 µl of Evan’s blue dye was injected into each site, and mice sacrificed after 0.5 or 2 hours to examine drainage. The axillary, brachial and inguinal lymph nodes were examined macroscopically for intensity of blue dye on both the ipsilateral and contralateral sides. Two mice were used per group for the three sites, for each time point (figure 3.1B).

After 0.5 hours, there was limited drainage to the lymph nodes. After 2 hours, drainage was clearly visible by the presence of the blue dye. The drainage was consistent between mice for each site of injection (n=2 per site, per time point). No drainage to the contralateral side was
observed. The TA muscle drained to the axillary and inguinal lymph nodes (figure 3.1B and C left), the upper flank drained to the brachial and to a lesser extent the axillary lymph nodes (figure 3.1B and C centre), and the lower flank drained to the axillary and inguinal lymph nodes (figure 3.1B and C right). The drainage from the TA muscle and the lower flank aligned to the inguinal and axillary lymph node, and therefore the lower flank was selected as the site for tumour inoculations in all subsequent experiments.
Figure 3.1: Mapping lymph node drainage using Evan’s Blue dye.

(A) Locations of 50µl dye injection- A-Subcutaneous into upper flank, B-Subcutaneous into lower flank, C- Intramuscularly into TA muscle. (B) Photographs of drainage of dye from site of injection to lymph nodes. (C) The removed brachial, axillary and inguinal lymph nodes from the contralateral side of injection ‘C’ and ipsilateral side of injection ‘I’.
3.2 SELECTION OF A CLONAL MELANOMA LINE

3.2.1 In vivo growth

The B16 F1 cell line was engineered previously via retroviral transduction to express GFP and HSV-derived gB under the same promoter. The introduced proteins are separated by an internal ribosome entry site (IRES) and the line is termed B16 F1-gB. Four clones of B16 F1-gB that had not been characterised previously were investigated. To determine which of these clones would best suit the melanoma model, clones were assessed for in vivo growth, expression of MHC class I and II, level of gB expression, and immunogenicity to gB specific T cells in vivo.

Transplanted clones B6F, B10M and H8M consistently developed tumours when $10^5$ cells were administered subcutaneously into the lower flank and grew over time, however the clone B8F did not graft (figure 3.2 top right). The clone H8M displayed delayed growth (figure 3.2 bottom right) compared to the parental control (data not shown). Clones B6F and B10M consistently grew well between mice (figure 3.2 left) and shared similar kinetics to the parental cell line and previously reported B16 tumour growth. Thus, clones B6F and B10M were chosen for further experimental analysis.
3.2.2 MHC Class I and II

B16 F1 cells have been reported to suppress their MHC class I H-2 expression *in vitro* by clonal selection (Nanni 1983). The expression of MHC molecules by the B16 F1-gB clones is required to present the gB peptide and other tumour antigens. Therefore, the expression of these molecules was examined. Cultured cells were exposed to IFNγ for 48 hours, as it has been reported that this cytokine can upregulate the expression of MHC molecules (Propper et al., 2003). A melanoma variant known to have lost H-2Kb, termed B16 F1kb, was used as a negative control when staining for MHC class I (Cho et al., 2011). The parental cell line B16 F1 was used as a positive control, as it is documented this cell line expresses MHC class I and

**Figure 3.2** Tumour growth kinetics of B16 F1-gB clones *in vivo.*
1x10⁵ cells were injected subcutaneously into the lower flank to determine growth kinetics *in vivo.* Each line is representative of one mouse (n=3 per group)
II upon IFNγ exposure (Propper et al., 2003). Cells were stained using antibodies for H-2Kb and I-Ab, to detect MHC class I and MHC class II expression respectively, and analysed by flow cytometry. Cells exposed to IFNγ were compared to cells without IFNγ stimulation. The clones B6F and B10M upregulated both MHC class I and II following IFNγ exposure in a similar fashion to that of the parental cell line (Figure 3.3). The clone B6F had slightly higher expression of MHC class I after IFNγ treatment than the B10M clone which was higher than the MHC class I expression profile of the B16 F1 cells (figure 3.3A). The B6F clone had lower MHC class II expression as compared to B10M, while the B10M histogram more closely paralleled the B16 F1 control (figure 3.3B). As both the B16 F1-gB B6F and B10M clones could be induced to express these molecules, they remained potential candidates for the downstream experimentation.

Figure 3.3 Upregulation of MHC class I and II in melanoma cell lines after culture with IFNγ.

B16 F1 variants were cultured with 10 μg/ml IFNγ for 48 hours Flow cytometry of cell surface expression of MHC molecules before (shaded) and after (unshaded) IFNγ treatment. (A) Expression of MHC class I allelic form H-2kb and (B) class II allelic form I-Ab by the cell lines B16 F1 H-2kb loss, B16 F1 and clones B6F and B10M after IFNγ treatment. The B16 F1 H-2kb loss mutant was a negative control for H-2kb expression.
3.2.3 gB expression

Expression of the GFP is used as a surrogate marker for the expression of gB. As GFP emits colour in the green wavelength, expression can be analysed by flow cytometry in the FITC channel. The parental cell line B16 F1 does not express this protein, and was used as a negative comparative control. Both the clones B6F and B10M expressed GFP (Figure 3.4A). The clone B6F had higher expression than the B10M clone, and both were detectable against the background histogram plot of the parental line. Since each clone expressed GFP, they both were considered suitable for further investigations.

**Figure 3.4:** Expression of green fluorescent protein in the B16 F1 parental and B16 F1 clones B6F and B10M.

(A) The expression of GFP by clones B16 F1-gB B6F and B10M compared to the parental line B16 F1. (B) Illustration of the construct the clones B6F and B10M have been engineered to express containing gB and GFP linked by an IRES.
3.2.4 Antigen presentation

Next, we screened whether effector T cells could reject the B16 F1-gB melanoma clones B6F and B10M \textit{in vivo}. $10^5$ tumour cells were injected subcutaneously into the lower flank and allowed to grow until all tumours became visible ($>4 \text{ mm}^3$). As confirmed previously by GFP expression, these tumour clones carried the gene for gB (Figure 3.4A). Transgenic T cells specific to gB (termed gBT.I) were pre-activated before their administration to mice harbouring B6F or B10M tumours. Activated gBT.I T cells were administered via tail vein injection 5 days after B6F or B10M tumour inoculation, when tumours were palpable, typically $4 \text{ mm}^3$ (figure 3.5A). Tumour growth was measured over time and a decrease in tumour size was used to indicate that the T cells were actively recognizing and destroying the tumour cells. B10M tumours decreased in size until the tumour was no longer visible. These mice remained tumour-free for 10 to 12 days before tumour re-emergence. In contrast, B6F tumours were unresponsive to adoptively transferred T cells and continued to grow exponentially (figure 3.5A). However, one mouse, which developed a small ($4 \text{ mm}^3$) B6F tumour, displayed limited growth for 10 days post T cell administration. These results show that activated gBT.I T cells were able to identify and destroy B10M tumours, but not B6F tumours. The re-emergence of B10M tumours after therapy suggested that strong immune pressure on the tumour caused down regulation of target genes to escape immune attack.

As the B6F tumours did not respond to treatment with activated gBT.I T cells, the T cells either could not detect the presence of gB on the melanoma cells, or the melanoma cells were able to mutate quickly in response to immune pressure. We next performed an experiment that would determine if these clones contained enough antigen to elicit an immune response to gB \textit{in vivo}. If gB was present at a high enough threshold within the tumour cells, antigen
presenting cells (APCs) would acquire and present the antigen to T cells in the lymphoid organs. To this end, $10^6$ CFSE labelled naive gBT.I T cells were injected iv when tumours were visible, and lymph nodes harvested 60 hours after transfer. As a positive control, mice were infected with HSV (a natural carrier of gB) in the same location as the tumour inoculations. The gBT.I T cells were identified by flow cytometry (see section 2.2.3.1). T cells were gated by large FSC, followed by CD8$^+$ staining. To identify transferred gBT.I T cells from the endogenous T cells, the CD45.I$^+$, V$\alpha_2^+$ population was gated. CD45.I expression is restricted to transgenic cells, and the V$\alpha_2$ marker confirms specificity of these cells to gB. From this population, CFSE dilution was used as an indicator of T cell proliferation.

Proliferation is associated with T cell activation in response to antigen recognised on antigen presenting cells. The far right peak showing the highest intensity of CFSE represented cells that did not divide. As cells divided, the amount of CFSE was halved per division. Cell division was determined by gating right to left, after the highest intensity peak, with the area under each peak representative of the proportion of cells within each cycle (Lyons and Parish, 1994). T cells in mice infected with HSV showed the greatest amount of proliferation, with the majority of cells dividing more than 7 times (figure 3.5B left). In contrast, T cells in mice harbouring B6F tumours did not divide (figure 3.5B centre), indicating the T cells were not presented with gB from the B6F tumour. T cells did respond to B10M tumours with an average of 55.1% proliferation and up to 7 cell divisions (figure 3.5B right). In light of the above experiments, B10M was selected for further experiments.

(Lyons and Parish, 1994)
Figure 3.5: Response of tumour specific T cells (gBT.I) to B16 F1-gB clones *in vivo*. (A) Mice harbouring B6F or B10M tumours (~4 mm\(^3\)) were administered 10\(^7\) activated gBT.I T cells i.v. Activated gBT.I T cell mediated destruction the tumour was determined by decrease in size. The B10M tumours re-emerged after ~10 days. (B) Mice harbouring B6F or B10M tumours, infected with HSV were administered 10\(^6\) naïve, CFSE labeled gBT.I T cells i.v. Proliferation of gBT.I T cells was measured in the draining lymph nodes 60 hours after transfer by dilution of CFSE dye. HSV was used as a positive control. Percent proliferation average of 3 mice +/- SD
3.3 IFN DNA VACCINE

DNA constructs containing individual murine IFN-I subtype genes were provided in bacterial glycerol stocks. IFN-α2, IFN-β and five other experimental IFNα subtypes were utilised. The latter five, labelled ‘V’, ‘W’, ‘X’, ‘Y’ and ‘Z’ were coded for intellectual property and potential patent protection. Each IFN gene had been cloned previously into the mammalian expression vector kCMVintPolyli (figure 3.6A) each with unique restriction endonuclease sites so that each IFN gene (figure 3.6B) could be identified by a specific restriction enzyme endonuclease digestion.

Extracted plasmid DNA was cut with the specific restriction enzymes and analysed by agarose gel electrophoresis to confirm presence and size of the gene fragments (figure 3.6C). The IFN fragment sizes range from 546 to 620 bp, and this was confirmed by gel electrophoresis in 1% agarose, 1 x TAE gel and fragment length compared to Lambda/Hind III standards and 10kb ladder. The bands from the IFN-subtype gene fragments were sitting between the 750 bp and 500 bp markers or just under the 500 bp marker of the 10 kb ladder, confirming correct sizes of each fragment (Figure 3.6C). The amount of DNA was further quantitated using a nanodrop spectrophotometer, with DNA purity as measured by A260/280 ratio in the range 1.8 – 2.1 (data not shown).
Figure 3.6: The type I IFN constructs.

The IFN transgenes have been cloned into the poly linker of (A) the mammillarian expression vector pKCM-Vint (Vical), each with unique restriction enzyme cloning sites. (B) Diagram of IFN-α2 and -β transgenes, representative of the gene fragments. (C) IFN gene fragments cut from the vector using restriction enzymes were stained with Midori Green DNA stain and run on an agarose gel. A 10 kb ladder (lane 9) was used to confirm fragment sizes. The 10000, 3000 1000, 750 and 500 bp fragments of the 10 kb ladder are labelled.
3.4 EFFICACY OF TYPE I IFN SUBTYPES TO TREAT MELANOMA IN VIVO

3.4.1 Experimental plan

The panel of individual IFN subtypes was examined for an inhibitory effect on melanoma tumour growth in vivo. The ability of skeletal muscle to uptake naked DNA can be enhanced through inducing muscle regeneration, with best results via administration of bupivacaine 5 days prior to plasmid (Bartlett et al., 2003; Wolff et al., 1990). At day -12, mice were administered bupivacaine into the TA muscle and at day -7, mice were inoculated with individual IFN-I expressing DNA constructs in the same location. It has been reported that IFN can be detected as early as seven days after DNA injection, although, elevated serum levels have not yet peaked (Bartlett et al., 2002). Therefore seven days after DNA administration, at day 0, mice were inoculated with $10^5$ B10M tumour cells subcutaneously into the lower flank. Tumour growth was measured over time and mice were culled before tumours ulcerated or when they reached a maximal size of 1000 mm$^3$. To determine the systemic level of IFN in individual mice, sera were collected on day 14 (21 days after DNA injection), as it was expected that systemic levels would be at their peak at this time (Bartlett et al., 2002) (figure 3.7).
3.4.2 Progression of tumour during IFN treatment

Tumour progression in response to IFN-I treatment was mapped using growth curves (figure 3.8). It was predicted that IFN levels declined by day 21 (1 month after DNA injection) as the vector degrades (Bartlett et al., 2002). Control groups included mice administered with saline alone (vehicle), bupivacaine alone (BP), or bupivacaine and an empty vector (vector) (figure 3.8 top row, respectively). Tumours arose in all control groups by 7-12 days after inoculation with the average time to onset 9.6, 9 and 9.4 days, respectively. The average survival of each
control was 19.8, 17 and 19.4 days, respectively. The majority of mice in the bupivacaine control group were culled before maximum size as many of the tumours showed signs they would become ulcerated.

The IFN-α2 group displayed similar growth and survival to the control cohorts, although tumours arose later, arising on average after 11.4 days. Surprisingly, survival time was worse compared to controls with an average of 18.2 days (figure 3.8 second row, left). IFN-β showed some efficacy, with delayed tumour growth to 12 days and increased survival to 24.8 days (figure 3.8 second row, right). It was evident that one mouse responded better to treatment, with the tumour arising on day 27, around when IFN levels began to decline. This trend was also evident in the other IFN treatment groups, where a proportion of mice had objective responses. Mice with tumours that arose after day 14 and survived past day 28 have been classified as ‘evident responders’ due to the fact that all mice in the control groups had a tumour before day 12 and none survived past 28 days. The IFN-α2 group did not have any ‘evident responders’, and the IFN β group had only one (summarised in table 3.2).

The tumour growth of the treatment groups IFN ‘V’, ‘W’, ‘X’, ‘Y’ and ‘Z’ are shown in figure 3.8 rows 3 and 4. Each of the treatment groups showed delayed tumour onset and increased survival. IFN ‘X’ had one complete responder, as the transplanted tumour failed to grow to palpable size. All these treatment groups showed greater efficacy than both IFNα2 and IFN β. Of the ‘evident responders’, tumour growth was delayed and significantly greater survival was observed. The tumour growth kinetics were altered, taking the tumour a greater amount of time to reach full size from the day of onset. The averages of each treatment can be seen in table 3.2.
Figure 3.8 Growth kinetics of B16 F1-gB B10M tumours 7 days after IFN DNA therapy.

$10^5$ cells were inoculated subcutaneously on day 0, 7 days after therapy, when IFN levels were just below detectable levels in the sera. Each line represents one mouse (n=5 per group). Mice were euthanased before reaching $1000\text{mm}^3$, or before ulceration occurred. Dashed line represents approximate decline in IFN levels. Row 1: controls. Row2: IFN $\alpha_2$ and IFN $\beta$. Rows 3 and 4: IFN$\alpha_2$ subtypes.
3.4.3 Survival of treated mice

The survival for each treatment group is summarized in figure 3.9. The upper left graph compares the control groups, showing minimal variation between the groups, with no significant difference. The control group receiving the blank vector showed greatest survival of the controls and was used as the comparative control for the IFN treatment groups. This control was selected for comparison as the presence of the vector alone may induce endogenous IFN production due to the vector consisting of bacterial DNA. Therefore, any difference seen in the treatment groups in comparison to this control is due to the expression of the introduced IFN. Mice receiving IFNα2, figure 3.9 top centre, showed a slight decrease in survival (-4%) compared to the vector, indicating IFNα2 had no significant efficacy against melanoma. IFNβ, figure 3.9 top right, showed a trend in longer survival, with the average survival 24.8 days (28% increase), but this was not statistically significant. Groups receiving the IFNα treatments ‘V’, ‘W’, ‘X’, ‘Y’ and ‘Z’ showed an increase in survival time (figure 3.9 rows 2 and 3). The average survival time of IFNs ‘V’, ‘W’, ‘X’, ‘Y’ and ‘Z’ had 38, 23, 21, 28 and 53% longer survival compared to the vector control, respectively. However this increase in survival was only significant for IFN ‘Z’ (figure 3.9 bottom centre).

Notably, in the IFN ‘V’ treatment group, shown in figure 3.9 middle left, there was one mouse surviving at day 42. However, the mouse did have a tumour and only survived a further 48 h. The survival of the IFN ‘X’ treatment group, shown in figure 3.9 middle right, had one complete responder mouse where the tumour did not arise, and the mouse remained melanoma free for several months.
The results show there is different efficacy of the IFN subtypes in the treatment of melanoma. Treatment with IFN ‘Z’ was the only group which had a significant increase in survival compared to all controls (figure 3.9 bottom centre). Significance with 95% confidence could not be obtained for the other treatment groups and expansion of the group size (n=5) will be required to further determine their efficacy in melanoma survival rate.

**Figure 3.9 IFN therapy increases survival time**

Time to death as depicted by survival curves. The survival curves of the controls were compared against each other (top left). Survival curves of the treatment groups are in comparison to the blank vector. The dashed line represents approximated decline of IFN levels. Statistical significant in survival over the control vector is indicated by * (*=p<0.05)
3.4.4 Tumour onset

Tumour onset was determined by the day that a tumour became clearly visible, i.e. 4 mm$^3$. Tumour onset was the growth variable most affected by IFN treatment. Comparison of tumour onset has been summarized by waterfall curves (figure 3.10). Control groups had similar times to palpable tumour development, with all control mice developing a tumour by 12 days post inoculation. Thus, significant differences between control groups was not observed (figure 3.10 top left). IFN$\alpha$2 and IFN$\beta$, figure 3.10 top centre and right respectively, delayed tumour onset significantly ($p<0.05$), delaying average tumour development to 13 and 15 days respectively. The remaining IFN treatment groups further delayed tumour onset ($p<0.01$). IFN W tumour development was delayed to day 16, while administration of IFN ‘Y’ and ‘Z’ delayed onset to day 18 and 19 respectively. IFN ‘V’ was even better, and tumour onset was delayed to 28 days. Strikingly, one complete responder in the IFN ‘X’ treatment group did not develop a tumour. These data illustrated that there are clear anti-tumour responses to IFN treatment of melanoma. 90% of mice treated with IFN showed an objective response in delaying tumour onset. Many IFN subtypes had enhanced efficacy compared to the IFN-$\alpha$2 therapy. In addition the IFN$\alpha$ subtypes varied in their ability to suppress tumour establishment.
Figure 3.10 IFN therapy delays tumour onset

Day of tumour onset was defined as when tumours were palpable (~4 mm$^3$). Tumour onset of each mouse over time represented as an event (decrease in % without palpable tumour). The time to tumour onset of the controls were compared against each other (top left). Time to tumour onset of the treatment groups are in comparison to the blank vector. Statistical significant in delay to tumour onset over the control vector is indicated by * (*=p<0.05, **=p<0.01) N.S.= not significant,
Table 3.2 Comparison of tumour onset, survival and growth between treatment groups.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Average day of tumour onset</th>
<th>Average Survival (days)</th>
<th>Average duration of tumour growth (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>9.6</td>
<td>19.8</td>
<td>10.2</td>
</tr>
<tr>
<td>Bupivacaine</td>
<td>9</td>
<td>17</td>
<td>8</td>
</tr>
<tr>
<td>Vector</td>
<td>9.4</td>
<td>19.4</td>
<td>10</td>
</tr>
<tr>
<td>IFN α2</td>
<td>11.4</td>
<td>18.2</td>
<td>6.8</td>
</tr>
<tr>
<td>IFN β</td>
<td>12</td>
<td>24.8</td>
<td>12.8</td>
</tr>
<tr>
<td>IFN ‘V’</td>
<td>16.2</td>
<td>26.8</td>
<td>10.4</td>
</tr>
<tr>
<td>IFN ‘W’</td>
<td>13.8</td>
<td>23.8</td>
<td>10</td>
</tr>
<tr>
<td>IFN ‘X’*</td>
<td>13</td>
<td>23.5</td>
<td>10.5</td>
</tr>
<tr>
<td>IFN ‘Y’</td>
<td>14.6</td>
<td>24.8</td>
<td>10</td>
</tr>
<tr>
<td>IFN ‘Z’</td>
<td>18.4</td>
<td>29.6</td>
<td>11</td>
</tr>
</tbody>
</table>

*Data not included for the 1 mouse where the tumour did not arise

3.4.5 Evident responders

The evident responders, defined in 3.4.2 as responding to IFN treatment resulting in a delay in tumour growth past day 14 and surviving past day 28 have been identified for further analyses. This group excluded the complete responder in the IFN ‘X’ treatment group. The number of responders per group, average day of tumour onset, average survival and average duration of tumour growth have been summarised in table 3.3. IFN-α2 had no evident responders, and the IFN-β and ‘X’ treatment groups had only one evident responder. Compared to table 3.2, all evident responders had a longer duration of tumour growth, except IFN-β which had a shorter duration from 12.8 to 10 days of growth (n=1). This shows that the evident responders not only survived longer due to a longer delay in tumour onset, but also
due to slowed tumour growth. 40-60% of mice treated with IFN ‘V’, ‘W’, ‘X’, ‘Y’ or ‘Z’ had an objective response on tumour progression.

Mapping of tumour growth kinetics of evident responders demonstrated the rate of growth slowed, and tumours took a longer time to reach maximum size (1000mm$^3$) (figure 3.11A). This was statistically significant compared to the weaker responders and control groups. Interestingly, weaker responders to IFN treatment, although significantly delayed time to onset, showed no significant effect in the rate of tumour progression (figure 3.11A). The weak responders showed a trend for more rapid growth than the controls, although tumour growth was delayed, which could explain why survival was not significantly increased in most IFN treatment groups. The greater delay of tumour onset (figure 3.11B) and slowed progression of tumour growth (figure 3.11A) demonstrated by the evident responders resulted in a statistically significant increase in survival (p<0.01) (data not illustrated).

It was observed that in the weak responders, IFN did not slow tumour progression. To analyse whether the weak responders delayed tumour onset in response to IFN treatment, without the evident responders influencing group results, they were analysed separately (figure 3.11B). Compared to the controls tumour onset was a significantly delayed in the weak responders (p<0.05) (figure 3.11B). This was more pronounced in the evident responders (p<0.01). These data revealed a correlation between delayed onset and delayed tumour growth after administration of IFN-$\alpha$ subtypes, but not IFN-$\beta$. There was also individual variation in response to treatment, resulting in either a ‘weak’ or ‘evident’ response.
Figure 3.11 ‘Evident responders’ display both delayed onset and slower tumour progression.

Mice have been characterised as evident responders if their tumours became palpable after day 14 and they survived to or beyond day 28. (A) Average growth kinetics (pooled mean tumour volume +/- SE) of the ‘evident responders’, ‘weak responders’ and controls, starting from each individual’s day of tumour onset. (B) Comparison of the day of tumour onset between the ‘evident responders’, ‘weak responders’ and controls. (*=p<0.05 **=p<0.01, ****=p<0.0001)
Table 3.3 Summary of the evident responders for each treatment group

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Evident responders per group (n)</th>
<th>Average day of tumour onset</th>
<th>Average Survival (days)</th>
<th>Average duration of tumour growth (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>N/A</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IFN α2</td>
<td>(n=0)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IFN β</td>
<td>(n=1)</td>
<td>27</td>
<td>37</td>
<td>10</td>
</tr>
<tr>
<td>IFN ‘V’</td>
<td>(n=2)</td>
<td>24</td>
<td>37</td>
<td>13</td>
</tr>
<tr>
<td>IFN ‘W’</td>
<td>(n=2)</td>
<td>16.5</td>
<td>33.5</td>
<td>17</td>
</tr>
<tr>
<td>IFN ‘X’</td>
<td>(n=1)*</td>
<td>15</td>
<td>28</td>
<td>13</td>
</tr>
<tr>
<td>IFN ‘Y’</td>
<td>(n=2)</td>
<td>17.5</td>
<td>34</td>
<td>16.5</td>
</tr>
<tr>
<td>IFN ‘Z’</td>
<td>(n=3)</td>
<td>20</td>
<td>35</td>
<td>15</td>
</tr>
</tbody>
</table>

*data not included for the 1 mouse where the tumour did not arise

3.4.6 IFN levels within the sera

The administration of DNA constructs results in constitutive production of the protein; however, the amount of DNA taken up by the TA muscle is a source of variation. Additionally, the degradation and dissemination of each IFN subtype may vary, affecting the time and distribution of IFN in the body. Thus, there may be variation between individuals and IFN subtype. Therefore a number of mice may have higher levels of IFN, which may be a factor in the anti-tumour efficacy. To account for variation, serum was taken from mice at day 14 post tumour injection, when IFN levels reached a peak level systemically. Sera were analysed with a murine multi IFN-α or IFN-β ELISA to determine IFN levels. The results displayed in figure 3.12A show the IFN levels of individual mice in each treatment group.
Animals that received the vehicle only or bupivacaine only showed negligible levels of IFN, as endogenous IFN levels were likely below the detection level of the ELISA (12.5 pg/ml). The control group that received the vector alone showed slightly greater levels of IFN, indicating that the vector itself induced production of endogenous IFN, however this was not significant compared to the vehicle and bupivacaine controls. The IFNα2 treatment group showed the highest levels of IFN, despite having the lowest efficacy. IFN ‘V’ also had high levels of IFN, and both treatment groups IFN-α2 and IFN ‘V’ had significantly higher levels compared to the controls without DNA (vehicle and bupivacaine), and the blank vector control. The level of IFN was also significantly higher than the controls without DNA in groups receiving IFN W and IFN ‘Z’. IFN ‘X’ and IFN ‘Y’ did not have significantly higher levels than the controls, with the level of IFN ‘Y’ detected lower than the control levels (figure 3.12A). We speculate that the assay failed to detect this IFN subtype. The level of IFN did not correlate to the magnitude of efficacy of the subtypes. The murine IFN-β ELISA was unsuccessful in producing a standard curve and therefore data were not shown.

When the level of IFN-α was correlated with the survival of each treatment group, no correlation was found for any IFNα subtype (data not shown). The overall IFN level compared to survival is summarised in figure 3.12B, showing no correlation (r =0.00015). There was also no correlation between IFN level and evident responders. As the level of IFN does not influence survival, the different efficacy of each of the subtypes is likely due to their different anti-tumour effects.

In summary, this in vivo data revealed that unique, previously untested IFN subtypes showed enhanced efficacy in treating melanoma, and this was dependent on the subtype administered.
IFN ‘V’, ‘W’, ‘X’, ‘Y’ and ‘Z’ showed greater efficacy than IFNα2, the current gold standard treatment for late stage melanoma patients. Notably, these subtypes demonstrated greater efficacy with lower levels of IFN and may provide a better treatment option to the current high-dose IFN-α2 therapy.

**Figure 3.12 IFN levels in the sera at day 14 (21 days post DNA therapy).**

(A) The IFNα levels in the sera for each of the controls and IFN treatment groups. * denotes significance compared to the vehicle. # denotes significance compared to the vector. (*=p<0.05 **=p<0.01) (B) Correlation between IFNα level in the sera and length of survival. Goodness of fit $r^2=0.000153$ indicates no correlation.
3.5 DIRECT ANTI-TUMOUR EFFECTS OF IFN IN VITRO

3.5.1 MHC molecules

To elucidate how these IFN-I subtypes exert their anti-tumour effect, direct in vitro effects were analysed. The protein model antigen gB and other tumour antigens are presented on MHC class I and II, and therefore an effect on the presentation of these molecules would alter the immune response (e.g., enhance CD8+ and CD4+ T cell recognition). B10M cells were cultured with the different IFN-I subtypes for 48 hours, similar to the aforementioned IFNγ protocol. Cells were then stained for MHC Class I H-2Kb and Class II I-A b and analysed by flow cytometry. Cells cultured with each of the IFN subtypes all upregulated MHC class I, however with different intensities (figure 3.13A). Cells cultured with media or blank COS-7 cell supernatant (SN) showed no change in MHC expression. This demonstrated that the IFN subtypes, not the SN that the IFN was collected in, exerted these effects. In addition, each IFN subtype upregulated MHC class I to varying degrees, potentially indicating each subtype exerts a differential effect when binding to the IFNAR receptor. The results are summarised in figure 3.13C, showing all the IFN subtypes significantly upregulated MHC class I by mean fluorescence intensity (MFI). All the IFN-I treated cells significantly upregulated MHC class I compared to the controls. Among the IFN-α subtypes, IFN W was significantly lower and the other IFN-α subtypes were significantly higher when compared to IFN-α2. There was no significant difference between IFN-α2 and -β (figure 3.13C).

The IFN subtypes showed marginal or no upregulation of MHC class II (figure 3.13B). This was in contrast to IFNγ (data shown previously figure 3.3B right), which upregulated both class I and class II. As gB is presented on H-2Kb, upregulation of this molecule with the IFN-I
subtypes may result in increased presentation of this antigen, enabling enhanced recognition by effector CD8$^+$ T cells specific for the tumour \textit{in vivo}. 
Figure 3.13 Expression of MHC class I and II after culture with IFN-I subtypes.

B16 F1-gB B10M cells were cultured with 100IU IFN-I for 48 hours. (A-B) Histograms are representative of MHC expression without (filled) and with (unfilled) IFN-I treatment. (A) Upregulation of MHC class I H-2Kb (B) Upregulation of MHC class II. (C) Average MHC class I upregulation by mean fluorescent intensity (+/- SE). *= significant to controls. #= significant to IFN-α2. (*=p<0.05)
3.5.2 Inhibition of tumour growth by IFN subtypes

B10M cells were cultured in the presence of each IFN subtype to determine their impact on apoptosis. Culture with IFN-1 for 48 hours resulted in decreased confluency, altered morphology and an increase in floating cells. Microscopic images of the cells in figure 3.14 show the different IFN subtypes compared to the control (blank SN) (figure 3.14 A). The control depicted classical growth in vitro, when cell reached >90% confluency after 48 hours and grew in a spindle-like shape. Cells cultured with IFN reached 50-70% confluency, and displayed a more rounded shape. Clumps of floating cells (shown by the black arrows figure 3.14) were also observed in all the IFN cultured cell plates, most notably IFN-β, IFN ‘V’, IFN W and IFN ‘Z’.
Figure 3.14 Culture with IFN-I alters tumour cell morphology and confluency.

Light microscopy images of B16 F1-gB B10M cells after culture with 100IU of IFN-I subtypes for 48 hours. Floating cell clumps, indicative of cell death, is indicated by black arrows. (A) Cells cultured with blank supernatant with typical spindle like growth and ~90% confluency. (B-H) Altered morphology and decreased confluency after culture with IFN, which varies with subtype: (B) IFN-α2, (C) IFN-β, (D) IFN ‘V’ (E) IFN ‘W’ (F) IFN ‘X’ (G) IFN ‘Y’ (H) IFN ‘Z’.
3.5.3 Induction of apoptosis by IFN subtypes

To determine if IFN-I was causing tumour cell death, cells cultured with the IFN subtypes were stained for the apoptotic marker Annexin V. This molecule is present on the surface of cells undergoing apoptosis and staining for this marker was used in combination with propidium iodine (PI). The latter binds intracellularly and was used to discriminate cells that have disrupted membranes, indicative of necrosis or cell death. Non-adherent cells in the S/N were also collected and stained in order to include the cell population that had detached from the plate. Figure 3.15B top row shows the gating strategy and populations in each quadrant. Using FSC, larger melanoma cells and smaller cellular debris were included in analyses and high FSC anomalies were excluded. The resulting PI vs Annexin graph have 4 distinct populations; Q:I = PI+ annexin- (dead/remnants), Q:II = PI+ annexin+ (late apoptotic/necrotic), Q:III = PI- annexin- (live), Q:IV = PI- annexin+ (early apoptotic). Cells cultured in media or SN had very few cells in quadrants I, II and IV, indicating that few cells were undergoing apoptosis and cell death when cultured in vitro. There was no significant difference between these controls, indicating there was nothing present in the SN causing apoptosis. This is in contrast to cells cultured in the presence of IFN-Is, where significantly larger populations of cells in quadrants I, II and IV were observed (figure 3.15B rows 2-4). Interestingly, apoptotic death is variable between the different subtypes, indicative that the IFN subtypes exert different magnitudes of effect on the apoptotic pathway. IFN ‘V’, ‘X’, ‘Y’, ‘Z’ and -β had a significantly greater number of dead cells (Q:I) than the control groups (figure 3.15E), and IFN W and -β had a significantly greater necrotic/late apoptotic cell population (figure 3.15D). All IFN-I subtypes, with the exception of IFN-α2, had significantly more early apoptotic cells, with IFN ‘V’ and IFN-β having the greatest effect (p<0.01) (Figure 3.15C). IFN-α2 had increased cells in quadrants I, II and IV, however this
was not significantly different. The late apoptotic and necrotic populations cannot be distinguished, so it cannot be determined if the IFNs are also causing necrotic death.

In summary, IFN-α2 had the lowest percent of early apoptotic cells (2.14%) (figure 3.15C), and dead cells (5.35%) (figure 3.15E) at 48 hours compared to the other IFN subtypes. IFN ‘Y’ had the greatest percent of dead cells (10.7%), IFN W had the greatest percent of necrotic/late apoptotic cells (4.59%) and IFN-β had the greatest percent of early apoptotic cells (4.42%) (figure 3.15C). Overall, IFN ‘Y’ had greatest potency, with 18% of cells at 48 hours to be at a stage of apoptosis, and IFN-α2 the weakest potency, with only 9% of cells.

The results showed that different IFN subtypes have direct anti-tumour effects, and their ability to induce apoptotic cell death was IFN subtype dependent. The amount of apoptosis was ranged from 0.9-5.5 % of cells, and therefore does not explain the overall anti-tumour effect observed \textit{in vivo}, especially since serum IFN levels were lower than 100 IU/ml. Although the induction of apoptosis could be a potential mechanism of the IFN-Is’ anti-tumour effect \textit{in vivo}, it may not be the sole contributing factor. Apoptosis was also measured after 48 hours with 1000 IU/ml of IFN, resulting in similar results to 100 IU (data not shown), suggesting that 100 IU/ml may be at a saturating level.
Chapter 3

Results

A

Q I = Dead / Remnants
Q II = Necrotic / Late Apoptotic
Q III = Live
Q IV = Early Apoptotic

B

Annexin V

IFN 'V'

IFN 'W'

IFN 'X'

IFN 'Y'

IFN 'Z'

Annexin V
Figure 3.15 Induction of apoptosis in tumour cells after culture with IFN-I.

B16 F1-gB B10M cells were cultured with 100IU IFN-I subtype for 48 hours. Annexin V staining was used to discriminate apoptotic populations. (A) Gating on cells to include larger cell debris, and exclude abnormal FSC/SSC (left). Subsequent plot (right) showing 4 quadrants gated on 4 populations (Q I, Q II, Q III and Q IV) determined by presence of annexin (apoptotic cells) and PI staining (damaged membrane). (B) Representative contour plots of each the IFN subtypes compared to the control (SN, top left). (C-E) Average percent of cells from quadrants I, II and IV. (+/- SE). (C) Percent early apoptotic cells, (D) percent late apoptotic cells and (E) percent dead cells. * denotes significance compared to controls (both media and SN). (*=p<0.05 **=p<0.01)
3.5.4 Inhibition of proliferation by IFN subtypes

Microscopic analysis of the tumour cells in section 3.5.2 showed a clear inhibition in growth and evidence of cell death of melanoma cells cultured with IFN-I. The results in section 3.5.3 demonstrated that the decreased tumour growth observed could not be accounted for by the proportion of apoptotic cells (section 3.5.3). To determine if the IFN-I subtypes had an effect on proliferation, tumour cells were CFSE labelled prior to addition of IFN. Since B10M cells express GFP, which emits light at the same wavelength as CFSE, these cells could not be analysed by flow cytometry due to spectral overlap. Therefore, the parental cell line B16 F1 was used in this experiment. CFSE labelled B16 F1 tumour cells were analysed by flow cytometry after 48 hours of culture with 100 IU/ml IFN. Cell division results in a halving of the dye, and therefore reduced intensity. Gates on the graphs in figure 3.16A show the percent of the population that had undergone cell division compared to that which had not. Cells in media or blank SN had over 90% of the cells undergo cell division after 48 hours (3.16A top left), whilst cells cultured with IFN-α had 78.8 to 83.1% proliferation, indicating IFN had reduced tumour cell division (figure 3.16A top row centre, and rows 2 and 3). IFN-β had the greatest anti-proliferative effect, with 67.6% of cells remaining undivided (figure 3.16A top right). This shows a clear difference in the antiproliferative properties of IFN-α subtypes compared to IFN-β. The percent of undivided cells (right gates on figure 3.16A) has been summarised in figure 3.16B. Figure 3.16B highlights the similarity of the IFN-α subtypes, of which treatment with all the subtypes resulted in ~20% undivided cells, which was ~10% greater than the controls. A large percent of cells (68%) did not proliferate when cultured with IFN-β (figure 3.16B).
Figure 3.16 IFN-I inhibits tumour cell proliferation.

B16 F1 cells were CFSE labelled prior to culture with 100IU IFN-I for 48 hours. (A) proliferation as measured by CFSE dilution. Gates represent proliferated (left) and unproliferated (right) percent of the population. Control (SN) shown in top left graph. (B) Percent of undivided cells of each IFN subtype in comparison to controls.
3.6 IMMUNE ENHANCEMENT

IFN-I has been reported to alter the immune response, therefore we hypothesised that immune enhancement may also account for the anti-tumour effect observed with each of the IFN subtypes. The B10M cell line expresses GFP in conjunction with gB, and therefore a decrease in GFP expression may be indicative of downregulation of gB expression due to gene suppression to avoid immune recognition. In this model, if the expression of gB results in immune pressure via CD8\(^+\) T cell recognition of the tumour, expression of that gene may be downregulated. Potentially upregulation of MHC class I by the IFN-I would increase gB expression on the tumour cell surface and aid with immune recognition. To counter this, tumour cells often decrease the expression of MHC class I (Jager et al., 1997). In this way, suppression of specific antigens enable tumours to escape immune recognition.

Tumours were cultured from mice responsive to IFN therapy that displayed tumour control beyond 21 days. These tumour explants were taken from a separate experiment with the same setup to that described in section 3.4.1. Explants were cultured from the longest surviving mice in the control groups, as well as from a RAG\(^{-/-}\) mouse that received the blank vector. Contaminated explant cultures were discarded. Tumour cells from explants were then analysed by flow cytometry for GFP expression on the same day and compared the parental B16 F1 and B10M cells grown in vitro.

The parental cell line is GFP negative (figure 3.17A left). The B10M cell line grown in vitro shows maximal levels of GFP expression, with no GFP negative cells (figure 3.17A right). Explant B10M tumour cells from a RAG\(^{-/-}\) mouse which received the blank vector stands as a negative control. RAG\(^{-/-}\) mice lack B and T cells, and therefore could not mount an adaptive
immune response against the tumour in vivo. Tumour explants from the RAG−/− had reduced GFP (6.85%), indicative of reduced gB expression (figure 3.17B top left). This ‘background’ suppression of GFP may be due activation of innate natural killer (NK) cells, which are present in RAG−/− mice. Alternatively, reduced GFP expression may be a result of growth in vivo, independent of adaptive immune pressure.

Mice from the control groups (figure 3.17B top row) showed a low percentage of tumour cells with deceased GFP. Mice that received the vehicle or bupivacaine had 4.71 and 6.91% of cells with decreased GFP, respectively. Treatment with the blank vector resulted in slightly higher percentage (8.39%) of explanted tumour cells with decreased GFP. Treatment with IFN-α2 resulted in 67.6% of ex vivo tumour cells suppressing GFP expression (figure 3.17B row 2 left). In contrast, treatment with IFN-β (figure 3.17B row 2 right) resulted in only 1.73% decrease in GFP. Treatment with the different IFN-α subtypes ‘V’, ‘W’, ‘X’ and ‘Z’ also resulted in decreased GFP (figure 3.8B rows 3 and 4), with variation between and within each subtype. Combined, the controls showed that on average, 6.7% cells lost GFP (MFI 567 +/- 44) (figure 3.17C). Ex-vivo tumour cells from the IFN ‘Z’ treatment group showed the lowest average expression of GFP in the tumour cells (MFI 307 +/- 89) (figure 3.17C) with an average of 51% loss of GFP expression. IFN ‘W’ treatment was the weakest IFN-α at reducing GFP (MFI 458 +/- 56)(figure 3.17C) with an average of 15% cells lacking GFP.

As B10M cells cultured in vitro with IFN showed no change in GFP expression (data not shown), it could be speculated that in vivo the pressure exhibited on antigen expression, resulting in decreased GFP expression, is mediated by IFN-I enhanced immunity. In summary, these data show IFN-α exerts anti-tumour effects via immune enhancement, whilst
IFN-β did not enhance immunity, but rather increased expression of GFP. In contrast, IFN-β has demonstrated greater direct anti-tumour activities than IFN-α, *in vitro*, and this may be a significant difference between these type I IFNs.
Figure 3.17 GFP expression of ex-vivo tumours from mice treated with IFN-I

B16 F1-gB B10M tumours removed after day 21 in vivo, at 1000mm³, and analysed by flow cytometry for GFP expression. (A) The parental cell line (left) does not express GFP. The B10M cells grown in vitro (right) express the original level of GFP. (B) ex vivo tumours (black) compared to parental (light grey) and B10M in vitro (filled grey). (C) Average GFP expression (+/- SE) from treatment groups. Dotted line shows upper and lower limits.
3.7 RESULTS SUMMARY

We effectively established DNA therapy in a murine model of melanoma. This approach allowed us to test the efficacy of different IFN-I subtypes against melanoma. The B16 F1 melanoma cell line employed allowed us to elucidate antigen presentation and antigen-specific T cell therapy. The clonal line used throughout was rigorously tested for growth in vivo, responsiveness to T cell killing and antigen presentation to naïve tumour-specific T cells. The DNA therapy technique used to administer IFN resulted in consistent systemic levels, similar to the pegylated IFN-α2 regime used in the clinic. The location of tumour in relation to the site DNA therapy (TA muscle) has been adapted to accommodate drainage to the same lymph node.

This project has shown a distinct difference between IFN-α and IFN-β, as well as highlighting functional diversity within the IFN-α subtypes in terms of anti-melanoma activity. The therapeutic efficacy of the different IFN-I subtypes against melanoma has been demonstrated. Our results showed that IFN-α2 had the lowest efficacy, despite the fact that subjects with IFN-α2 therapy had the highest serum levels and that IFN-α2 is the standard immunotherapy for treatment of late stage melanoma patients in the clinic. IFN-β also demonstrated low therapeutic efficacy. The IFN subtypes ‘V’, ‘W’, ‘X’, ‘Y’ and ‘Z’ showed greatest therapeutic efficacy, specifically IFN ‘Z’, which significantly increased survival. All IFN-I subtypes significantly delayed tumour onset, particularly the IFN subtypes ‘V’ to ‘Z’.

Direct effects of IFN-I on melanoma cells were upregulation of MHC class I, induced apoptosis, and decreased tumour cell proliferation in vitro. Each of the subtypes exerted these effects at different magnitudes. IFN-β was distinct from IFN-α, with the highest apoptotic effect and greatest inhibition of proliferation.
This study indicates that the mechanism via which effective IFN subtypes suppress tumour development are both direct and indirect. Direct action on tumour cells via increased apoptosis were observed with all IFNs tested except IFN-α2 *in vitro*. Furthermore IFN-α, and to a greater extent IFN-β, exerted direct anti-proliferative effects *in vitro*. In addition IFN *in vivo* effects observed on melanoma growth are likely to include indirect process, such as the enhancement of immune responses. A decrease in tumour antigen expression was observed with IFN-α therapy, but not with IFN-β potentially indicating selective immune pressure on tumour cells. Our results demonstrated the therapeutic efficacy of IFN-α subtypes in particular IFN ‘V’, ‘X’ and ‘Z’. In addition IFN-α and IFN-β displayed different mechanisms for controlling tumour cell growth.
Chapter 4: Discussion

4.1 PROJECT SUMMARY

The aim of this project was to investigate the efficacy of the type I IFN subtypes in treating melanoma. As melanoma may be considered an ‘immunogenic’ cancer, the best treatment options to date are those with immune enhancing properties. IFN-α2 is the current standard treatment for late stage melanoma, yet its efficacy has been questioned (Janku and Kurzrock, 2010). IFN-α2 is also the only IFN-α subtype whose efficacy has been tested in many disease settings, largely due to this being the first recombinant IFN-α, allowing it to be made to high purity and high concentration. The purified, high concentration of IFN-α2 was able to demonstrate efficacy in the clinic. There are also limitations to IFN-I subtype research due to a lack of specific antibodies for the individual subtypes. In addition, endogenous IFN-α subtypes are produced at levels below microarray detection. Despite this, evidence for differential activities of the IFN subtypes has been shown via different binding affinity to the type I IFN receptor (IFNAR), resulting in altered activation of the JAK/STAT pathway (Cull et al., 2003) and thereby activation of different cellular pathways and stimulation of different genes. The mechanism by which IFN-I reduces tumour burden in melanoma is of debate, but believed to be via immune enhancement in addition to direct anti-tumour effects.

This project has combined a model of DNA therapy with a subcutaneous model of melanoma to test the efficacy of the IFN-I subtypes. We found that the IFN-α subtypes significantly delayed onset of melanoma growth, with IFN ‘Z’ significantly increasing survival time. It is of particular interest that all the IFN-α subtypes tested were more efficacious than both IFN-α2 and IFN-β, and therefore shown promise as superior anti-tumour agents. The IFN-α
subtypes ‘V’, ‘W’, ‘X’, ‘Y’ and ‘Z’ were also found to have efficacy, despite having low levels in the sera, indicating they may be more potent than IFN-α2. *In vitro* assays of the IFN-α subtypes also showed evidence of different potencies of anti-tumour actions, some of which were greater than IFN-β. Finally, the IFN-α subtypes have shown evidence that they act via an immune enhancing mechanism *in vivo*, whilst IFN-β did not show any evidence of immune enhancement.

4.2 COMBINATION OF DNA IFN THERAPY WITH A MURINE MELANOMA MODEL

As IFN-I have demonstrated enhancement of immune cells, such as promoting T cell activation (Le Bon et al., 2006a) and B cell isotype switching (Le Bon et al., 2001), it is thought this is the primary mechanism by which IFN-α2 works in the treatment of melanoma. When establishing the melanoma model combined with IFN therapy, this immune mechanism of action was considered for optimisation of immune activation in the common draining lymph nodes. In the context of a viral infection, when high levels of IFN are produced, T cell activation has been reported to occur exclusively within the lymph nodes draining the site of infection (Stock et al., 2004). Therefore the optimal administration of IFN would reach the tumour draining lymph node, with local, sustained levels of IFN-I for the activation of the immune system (Li et al., 2005).

IFN is rapidly cleared from the body by the liver and kidneys, and when administered intravenously at high doses, shorter intervals are required. High dose pegylated IFN-α2 becomes available to the body in low doses and, therefore, lasts longer in the body requiring longer intervals between doses. However pegylated IFN-α2 has no superiority in efficacy to
bolus dose IFN-α2 (Grob et al., 2012). A single administration of DNA encoding IFN-α2 mimics the pharmacokinetics of pegylated IFN-α2, without the requirement of continuous administration (Glue et al., 2000). This method allows IFN-I to be present in the body long enough to mobilise the immune system (Li et al., 2005). Additionally, DNA therapy results in a gradual build up of the specified protein, avoiding the fluctuation in dose that occurs after bolus IFN-α2 injection, which causes side effects such as headache, nausea and fatigue (Pestka, 2007).

The TA muscle is commonly used for administration of DNA in murine models of DNA therapy (Bartlett et al., 2003; Cull et al., 2002; Kessler et al., 1996). An alternative site for DNA therapy is direct injection into the tumour or periphery, however this does not replicate the systemic delivery of IFN-α2 used in the clinic. As this project aimed to compare the other IFN subtypes to IFN-α2 for therapeutic use, the TA muscle was chosen for injection of plasmids encoding the IFN-I subtypes.

As the site of DNA therapy was the TA muscle, the site of melanoma injection was manipulated to share the same lymph node drainage. Our results found that the TA muscle drained to the inguinal and axillary LNs, which corresponds with other research into the lymph node drainage of Evan’s blue from the hind limbs (Harrell et al., 2008). Harrell et al (2008) observed drainage to the axillary LN only occasionally, which parallels with our data that showed only mild drainage to the axillary LN after 0.5 hours, but distinct drainage after 2 hours. Subcutaneous injection into the upper flank drained to the brachial LN, and the lower flank to the axillary and inguinal LNs. The subcutaneous drainage of the flank is not well documented, however the cutaneous area of skin on the upper trunk has been reported to drain
to the axillary or brachial LN and the lower trunk to the inguinal LN (Tilney, 1971). This study found that the TA muscle and lower flank drained to the inguinal and axillary LNs.

As the lower flank and TA muscle share the same draining lymph nodes, the lower flank was selected as the site of tumour inoculation. Tumour associated antigen captured by APCs travel to draining lymph nodes to present antigen to immune cells. With IFN-I draining to the same lymph nodes, this permitted presentation to occur in an environment with a high concentration of IFN-I. IFN-I is documented to enhance antigen presentation, particularly in a tumour setting (Diamond et al., 2011; Le Bon and Tough, 2008; Longhi et al., 2009), increase survival and expansion of effector T cells (Curtsinger et al., 2005; Marrack et al., 1999), as well as improve CD8 T cell responsiveness to antigen (Hervas-Stubbs et al., 2012). Therefore, tumour antigen and IFN-I draining to the same lymph nodes potentially optimises the response, if the mechanism of therapeutic action of IFN-I is via immune modulation.

4.3 SELECTION OF A CLONAL MELANOMA LINE

In vivo growth of B16 F1-gB melanoma cell clones B6F, B8F, B10M and H8M were first tested. An ideal model would have consistent growth between subjects. A fast growth rate of the tumour cell line would lead to ulceration before reaching maximum size (1000mm³), and a slow growth rate would not be suitable as the IFN therapy would have declined by day 21. The cell lines were tested for GFP expression as an indicator of gB expression, as this tool would be utilised for later experiments.

Additionally MHC class I and II was examined as gB and other tumour antigen is presented on these molecules. The expression of MHC class I is crucial for the presentation of antigenic
peptides to CD8 T cells. The upregulation of MHC class I and II by IFN-γ correlates with data by Propper et al (2003).

ACT was used as a final discriminator to test the melanoma clones for appropriate antigen presentation of the model antigen gB on MHC class I. The gBT.I T cells utilised are restricted to gB presented on H-2Kb (Mueller et al., 2002). The cell line B6F tumour grew unimpeded in the presence of activated gBT.I T cells, suggesting that gB was not presented at sufficient levels on H-2Kb by the B6F tumour and therefore remained undetected. Additionally, the gBT.I T cells did not proliferate in tumour draining lymph nodes of mice harbouring B6F tumours. This indicates that an insufficient level of gB antigen was not acquired and presented by APCs in the lymph nodes. The B10M tumour cells did respond and regress, but came back after approximately 10 days, due to outgrowth of remaining tumour cells which may have suppressed antigen expression in response to immune pressure. Other studies using ACT demonstrated that strong immune pressure resulted in tumour regression, however genetic and epigenetic changes hide the target antigen resulting in immune escape (Jager et al., 1996; Landsberg et al., 2012; Yee et al., 2002). As the activated gBT.I T cells are restricted to gB presented on H-2Kb, the B10M cell line must have presented detectable levels of gB on this molecule. Naïve gBT.I T cells proliferated in the presence of the B10M tumour indicating that gB was captured and presented by APCs on H-2Kb in the tumour draining lymph nodes. Thus, transfer of activated gBT.I T cells into B10M bearing mice resulted in tumour regression. Tumour re-emergence was likely related to this immune pressure, which may have resulted in decreased gB and/or MHC class I expression. Published studies have also observed tumours regressing and re-arising in response to tumour-specific activated T
cells (Kaluza et al., 2012). B16 F1 cells have been reported to downregulate or lose expression of H-2 antigens in vivo in response to immune pressure (Nanni et al., 1983).

4.4 IFN-I THERAPY AFFECTS MELANOMA GROWTH IN VIVO

4.4.1 Administration of IFN

In this project, IFN was administered via DNA therapy, which most closely resembles pegylated IFN-α2 therapy in the clinic. By this method IFN becomes available constitutively, rather than in a bolus dose which is rapidly cleared and repeatedly administered to maintain levels. The administration technique used in this study mimics the clinical pegylated IFN-α2 therapy setting. Comparison between pegylated IFN-α2b and low dose IFN-α2b demonstrated there is no advantage between therapies (Grob et al., 2012). There was also no advantage observed between intramuscular or intravenous administration (Kirkwood et al., 1985). Therefore findings from this study may be compared to clinical trials of IFN-α2.

4.4.2 IFN-α2 and β have low efficacy in melanoma treatment

Currently IFN-α2 is used in the clinic to treat melanoma, however, until now the efficacy of the other type I IFN subtypes had not been elucidated. This project identified the efficacy of other IFN-α subtypes in relation to IFN-α2 and IFN-β in a murine model of melanoma. Improvement in survival was not observed after DNA therapy with IFN-α2. This directly compares to a study by Horton et al (1999) that demonstrated administration of 50μg IFN-α2 DNA into the TA muscle every 2 weeks resulted in no significant increase in survival from subcutaneous B16F10 melanoma. However, survival time lengthed when IFN-α2 DNA was administered every week or twice a week (Horton et al., 1999). Murine models using IFN-α2 and IFN-β have demonstrated anti-tumour activity against other tumour types in vivo (Cull et
al., 2003). The IFN-α subtypes have been tested previously via DNA therapy on J2E erythroid cells (erytholeukemia model) by Cull et al. (2003), who also found varying efficacy of the IFN subtypes. Similar to this project, IFN-α2 was found to slightly decrease survival, whilst the other subtypes increased survival. Interestingly, their study also found IFN-β had no effect on survival. To date there are no data published on the anti-melanoma efficacy of the IFN-α subtypes.

4.4.3 IFN-α subtypes have greater efficacy in treating melanoma

Administration of all the type I IFNs tested resulted in a significant delay in time to tumour onset, but only IFN ‘Z’ demonstrated significance in overall survival. This delay in tumour onset correlates to data in clinical studies, with all studies to date (comparing adjuvant therapy to observation) (Bottomley et al., 2009; Cameron et al., 2001; Cascinelli et al., 2001; Creagan et al., 1995; Eggermont et al., 2005; Eggermont et al., 2008; Grob et al., 1998; Hancock et al., 2004; Kirkwood et al., 1996; Kleeberg et al., 2004; Pehamberger et al., 1998), demonstrating a significant improvement in relapse-free survival time. Although IFN-α2 treatment has been found to delay recurrence, this delay time is less than 1 year, and the overall risk of recurrence is not reduced. In our study, no improvement in survival time was observed in animals that received IFN-α2 treatment, and significantly increased survival time was only observed in one IFN-α subtype. In clinical trials, significance in overall survival has only been found in 1 out of 11 studies to date (Trial E1684) (Kirkwood et al., 1996). The E1684 trial has been the most successful to date, which involved the highest doses of IFN-α2 (20 million units/day) and resulted in only 10% increase of survival at 5 years (Kirkwood et al., 1996; Terando et al., 2003).
Studies have also shown single cases of complete responses, without the melanoma reoccurring (Kirkwood et al., 2000). This correlates with data from our study, as out of 35 treated mice, we observed a single complete responder with IFN ‘X’ treatment. We also observed that IFN-β showed no improvement on survival. Similarly, a phase 2 trial using IFN-β therapy reported no significant effect on patient outcome (Voelter- Mahlknecht et al., 2006).

IFN ‘Z’ showed a statistically significant increase in survival, even with the low numbers of mice for this study. IFN ‘V’ and IFN ‘X’ also showed promise of increasing survival, although statistical significance was not reached, possibly due to small treatment group number. Clinical trials have required large cohorts to demonstrate marginal significant increases in survival with IFN-α2 therapy (Kirkwood et al., 2001; Kirkwood et al., 1996), with many trials demonstrating no statistically significant increase in survival. The IFN-α subtypes ‘V’, ‘W’, ‘X’, ‘Y’ and ‘Z’ therefore show promise as a treatment option for melanoma.

### 4.4.4 Variation within treatment groups

In this project individual variation was observed, as a proportion of mice responded better than others, termed ‘evident responders’. This is also observed in the clinic, where a proportion of patients respond to treatment, whilst others do not. Although this phenomenon has been observed, the response rates in inbred mice cannot be compared to the great diversity of the human population. It has been theorised that there is some unknown immunological status at the time of treatment that results in a greater response (Håkansson et al., 1996). This response has been found to correlate with increased STAT1 signalling, as STAT1 is involved
in IFN-I signalling and has a role in developing the effector immune response (Messina et al., 2008). In addition, responders to high dose IFN-\(\alpha\)2 treatment have increased number of lymphocytes and DCs at the tumour site (Moschos et al., 2006). Future research into the ability of the different IFN-I subtypes to elicit this response should be explored, as the subtypes have been reported to differ in their activation of STAT (Cull et al., 2003).

Another trend observed was the arising of tumours in the evident responders around day 21 (28 days after IFN treatment), when IFN-I administered by DNA therapy begins to degrade and IFN levels have been reported to decline (Bartlett et al., 2002). This has also been observed in the clinic, as during the course of IFN-\(\alpha\)2 therapy for melanoma a decrease in tumour burden is seen, however this disappears after therapy is discontinued (Terando et al., 2003).

### 4.4.5 Level of IFN in the sera does not correlate with survival

Our data also showed that there was no correlation between the IFN-I level and length of survival that correlates with findings from clinical studies. An overview of all clinical studies using IFN-\(\alpha\)2 to treat melanoma to date surprisingly showed no significant difference attributed to dosage (from 3 to 20 MU/day/m\(^2\)) in prolonging relapse free survival, or duration of IFN-\(\alpha\)2 therapy (1 month to 5 years) in prolonging survival (Janku and Kurzrock, 2010). Only the highest dose trialled (20 MU/day/m\(^2\)) has shown significance in prolonging survival (Kirkwood et al., 1996), yet there is no difference attributed to dosage when it below this level. The IFN-\(\alpha\) subtypes tested demonstrated a delay in tumour onset, with low levels in the sera. Of the IFN-\(\alpha\) subtypes tested, all had sera IFN levels lower than the IFN-\(\alpha\)2 treated group, yet had greater efficacy in delaying tumour onset. In addition, an increase in survival
could be observed, albeit at these low levels. This suggests the IFN-α subtypes tested may be more potent than IFN-α2 and β which are produced at higher levels in the body (Kadowaki and Liu, 2002). This shows promise for lower dose IFN-I therapy, which would avoid the undesirable side effects associated with high dose IFN-α2 therapy.

4.5 IFN-I HAVE DIRECT ANTI-TUMOUR ACTIONS IN VITRO

4.5.1 IFN-I upregulates MHC class I

Tumour cells have the ability to reduce antigen presentation via downregulation of MHC molecules, which can be reversed by treatment with IFN (Propper et al., 2003; Yang et al., 2004), and hence aid the immune response to identify tumour antigen. Our data show that IFN-Is have the ability to upregulate MHC class I, but only weakly upregulate MHC class II, distinguishing them from the type II IFN (IFN-γ). This relates to research by Garbe et al. (1990), who demonstrated that IFN-α and IFN-β upregulate MHC class I on melanoma cell lines. Our study found IFN-β was more effective at upregulating MHC class I than IFN-α2, although this was not significant. Published data show the same trend where IFN-β treatment resulted in higher MHC class I upregulation (Garbe et al., 1990).

Results from this experiment show that the IFN-α subtypes also share this feature, however at different potencies (eg. IFN W had the weakest upregulation, and IFN ‘X’ the greatest upregulation of MHC class I, which was greater than IFN-β). Studies to date have shown that the IFN-α2 subtype has less potency than IFN-β in MHC class I upregulation (Garbe et al., 1990). Our results show that subtypes, other than IFN-α2 were able to increase MHC class I to a greater extent than IFN-β, which has not been shown previously. The upregulation of
MHC class I may aid the immune system in identifying tumour antigens and consequently the tumour (Garcia-Lora et al., 2003). However, MHC class I upregulation did not correlate with survival or tumour onset.

4.5.2 IFN-I induces apoptosis of melanoma cells

All IFN subtypes tested induced apoptosis of melanoma cells. IFN-α2 had the weakest ability to induce apoptosis, which was not significantly greater than the controls. IFN-β induced the highest and most statistically significant increase in apoptosis of tumour cells. This is similar to a study by Chawla-Sarkar et al. (2001), that also used annexin V staining to determine amount of apoptosis after 48 hours with 100IU of IFN-α2 or IFN-β, albeit on WM9 melanoma cells. In this study, IFN-α2 did not increase apoptosis, whilst IFN-β induced apoptosis at similar proportions to that observed in our study (Chawla-Sarkar et al., 2001).

Differences in the tumour cell lines could account for the capacity of IFN-I subtypes to induce apoptosis, as other studies have found that IFN-α2 induced apoptosis in various cancer cell lines (Yano et al., 2006). In our project, the IFN-α subtypes tested induced a greater amount of apoptosis than IFN-α2. This was also observed by Yano et al (2006), who observed various IFN-I subtypes induced greater amounts of apoptosis than IFN-α2 on various tumour cell lines. The differential effects observed by the subtypes could be due to their binding affinity to the IFN receptor. Different cell lines act differently to various subtypes, indicating the subtypes may be unique in their induction of apoptosis.
In this project, the relative amount of apoptotic cells was determined at a single time point. At this time point, the induction of apoptosis observed varied by degrees of magnitude depending on the subtype. However, this does not take into account that the subtypes may vary in time taken to induce apoptosis. The order of apoptosis by the annexin V stain goes from early apoptotic to late apoptotic/necrotic to dead. Therefore a greater dead population may be indicative of a greater number of cells that have already undergone apoptosis, and may indicate these subtypes induce apoptosis at a greater rate. However, necrotic death cannot be distinguished from late apoptotic and dead cells, and so this is not an accurate representation of a time course. Bolling et al. (2000) observed that apoptosis was increased with IFN-β with increasing time, however they did not observe the same effect for IFN-α. Further research into the apoptosis induction of the IFN subtypes should examine different time points to determine if some are greater at inducing apoptosis or rather that some are faster, perhaps by signalling through a different pathway. This supports our hypothesis that the different IFN-I subtypes are unique in their actions upon melanoma cells.

The level of apoptosis observed was relatively low, and growth was still observed in vitro. Therefore, the induction of apoptosis by IFN-I could not eradicate a tumour, especially in vivo where IFN levels were less than 100 IU/ml, but may be responsible for the delay in growth observed in vivo.

### 4.5.3 IFN-I decreases proliferation of melanoma cells

The proliferation assay was performed using CFSE dye to analyse cell division. Our results showed that the IFN-α subtypes induced a small amount of apoptosis (~10% inhibition over control), whilst IFN-β had a strong anti-proliferative effect. This has been reported by a
number of studies (Bolling et al., 2000; Garbe et al., 1990; Johns et al., 1992), which have showed that IFN-β had a greater growth inhibitory effect than IFN-α in various melanoma cell lines. Data from Noguchi et al (2008) found similar results at 100 IU/ml on a carcinoma cell line, with a small decrease by IFN-α and ~60% inhibition by IFN-β. This effect of IFN-β has also been extended to in vivo studies, where IFN-β can inhibit growth of tumours in nude mice (Damdinsuren et al., 2007). Johns et al. (1992) tested other IFN-α subtypes in comparison to IFN-α2 and IFN-β, and found that IFN-α2 had a greater anti-proliferative potency than other IFN-α subtypes. In our study, the IFN-α subtypes had relatively equal effect on proliferation, however the effect may vary with different cell lines (Johns et al., 1992; Yano et al., 2006).

4.6 IFN-α ENHANCES IMMUNE PRESSURE ON MELANOMA

The ‘cancer immunoediting’ hypothesis emphasises the function of the immune system to not only prevent, but to shape the phenotype of neoplastic cells. When a tumour is identified by the immune system, it may mutate to control the immune response through either dampening the immune system or hiding any immunogenic features (Drake et al., 2006). The cell line used, B16 F1-gB B10M, expresses both GFP and gB, both of which are foreign and may elicit an immune response, resulting in silencing of the promoter, and hence decreasing expression of these proteins. In this project, it was observed that tumour explants from mice with IFN-α therapy decreased GFP expression, but IFN-β therapy did not, indicating that IFN-α enhanced the anti-tumour immune response.

In the control groups, a small amount of GFP expression was lost, including in the RAG−/− control. As the level of GFP was equal in the B6 and RAG−/− controls, this indicates there was
ignorance of the tumour by B and T cells in B6 mice, and that the small decrease of GFP was due to adaptation to \textit{in vivo} growth or possibly selection by NK cells. The decrease of GFP observed in \textit{ex-vivo} tumours from IFN-\(\text{\(\alpha\)}\)-treated mice was greater than controls, indicating that IFN-\(\text{\(\alpha\)}\) therapy resulted in enhanced immune pressure against the tumour. On the other hand, it was observed that IFN-\(\beta\) therapy resulted in less change of GFP expression than the controls, suggesting that IFN-\(\beta\) did not enhance immune pressure. IFN ‘\(V\)’ and IFN ‘\(Z\)’ showed the greatest reduction in GFP, which correlated with their efficacy \textit{in vivo}, demonstrating that an adaptive immune response is responsible for prolonging survival.

IFN-\(\beta\) has been reported to enhance the immune system, however by different mechanisms than IFN-\(\alpha\) (Harle et al., 2002). IFN-\(\alpha\) and \(\beta\) have been implicated in the adaptive immune response against cancer by acting on the CD8\(^+\) DC subset to enhance cross-presentation of tumor-derived antigens (Fuertes et al., 2011). The ability of IFN-\(\alpha\) to enhance the immune response to cancer is supported by a large body of literature; IFN-\(\alpha\) has been reported to increase activity, responsiveness and survival of T cells (Belardelli, 1995; Curtsinger et al., 2005; Hervas-Stubbs et al., 2012; Le Bon et al., 2006a; Marrack et al., 1999) and directly enhance the functions of DCs (Diamond et al., 2011; Le Bon et al., 2001; Steinman and Banchereau, 2007), which are crucial to initiate an adaptive immune response. In addition, the development of autoimmunity during IFN-\(\alpha\)2 correlates with a survival benefit (Gogas et al., 2006), demonstrating IFN-\(\alpha\) has an effect on the immune system.

As discussed previously, IFN-\(\beta\) has greater anti-tumour efficacy \textit{in vitro}, however this is not reflected \textit{in vivo} where IFN-\(\alpha\) was more efficacious. In the clinic, IFN-\(\beta\) is used to treat the autoimmune disorder multiple sclerosis (Noronha et al., 1993; Paty and Li, 1993), implying
IFN-β’s immune modulating actions differ significantly from IFN-α. Therefore it is probable that IFN-α has a greater immune enhancing effect than IFN-β. These data also correlate with IFN-I studies on immune deficient and IFNAR−/− mouse models harbouring tumours. These studies demonstrated that IFN-α loses its effects in vivo when there is no adaptive immune response or if the host cells cannot respond to interferon (Dunn et al., 2005). This confirms the ability of action of IFN-α to evoke an anti-tumour response requires host immune cells to be responsive, rather than the tumour (Dunn et al., 2005). IFN-β on the other hand has been found to be efficacious in immune deficient mice (Damdinsuren et al., 2007; Johns et al., 1992). This supports our hypothesis that IFN-α is immune enhancing, while IFN-β has direct anti-tumour effects on cancer cells (Noguchi et al., 2008).

Data obtained on the immune pressure from each of the IFN subtypes was limited, and therefore more research is needed to determine which IFN-α subtypes evoke the greatest anti-tumour response. Additionally, in vitro activation of immune cells in the presence of IFN-I subtypes should be performed to determine which IFN subtypes best improve responsiveness to tumour antigens.

4.7 LIMITATIONS

The potential for clinical use of type I IFN subtype gene therapy has been demonstrated, however, further research into the potential limitations of this type of treatment is needed. Specific limitations with this model concern the extrapolation of the experimental murine model to the clinical human system. There are distinct differences in the physiology between mice and humans. This project has demonstrated that IFN ‘Z’ is most efficacious in the
murine system to treat melanoma, however the murine type I IFNs do not directly correspond to their human counterpart (Weber et al., 1987). In addition, the diversity of human disease was not represented in this study, which used inbred mice harbouring tumours from the same cell line.

Studies have also found that the successful DNA transfer demonstrated in mice is not always verified in primates (McCluskie et al., 1999). Moreover, increasing animal size results in lower efficacy of DNA vaccines (Mölling, 1997). Therefore, extensive studies in the human IFN system are needed to gain the full clinical potential of type I IFN subtype gene therapy. Alternatively, clinical trials using low levels of purified human IFN-α subtypes should be performed to determine the IFN-α subtypes with greatest efficacy in humans.

Furthermore, the subcutaneous model of melanoma employed in this project does not reflect the true development of melanoma, which arises in the epidermis (Gray-Schopfer et al., 2007). Therefore, in our model the tumour was in a different anatomical position than the primary tumour in humans. In addition, melanoma patients typically are prescribed IFN-α2 as an adjuvant therapy, after the removal of the primary tumour. The tumours in our model were not resected, and therefore do not replicate residual disease. Another limitation to this model is that it does not metastasise and therefore, does not reflect the late stage disease that IFN-α2 is usually prescribed for. Finally, this cell line expresses the highly immunogenic gB peptide, which does not reflect the over-expressed or modified self-antigens presented by tumours.
4.8 AIMS ACHIEVED

Within completing this project, all aims had been achieved. To begin the project required the first aim to be completed. To develop our model, we tested B16 F1-gB clones to identify B10M as a suitable cell line. We then identified the tumour inoculation site to use in parallel with DNA therapy to maximise IFN levels in the draining lymph nodes. We used this model to identify the type I IFN subtypes with therapeutic efficacy in treating melanoma, which was the main goal of the project. IFN ‘V’, ‘X’ and ‘Z’ were identified as the most efficacious, with all IFN-α subtypes tested demonstrating greater efficacy than IFN-α2. In vitro studies found that the IFN-Is exerted the same effects on melanoma cells, however at different potencies. More research would be required to find if the differences in potency was due to the activation of different cellular pathways. Finally, through the use of our gB-GFP-expressing melanoma line, evidence was found which indicated enhancement against tumour antigen occurs as a result IFN-α therapy. Interestingly this was not observed for IFN-β, however more data would be required to confirm this result. This area could be further explored, such as dissecting the immune responses that take place with IFN-I therapy.
Chapter 5: Conclusion

We investigated the therapeutic efficacy of seven type I IFN subtypes, including IFN-α2, five other IFN-α subtypes (‘V’, ‘W’, ‘X’, ‘Y’ and ‘Z’), and IFN-β, against melanoma. IFN-α2 is currently used in the clinic to treat melanoma, however the efficacy of the other IFN-α subtypes had not been tested until now. In this study, the IFN-α subtypes ‘V’, ‘W’, ‘X’, ‘Y’ and ‘Z’ tested resulted in a greater anti-tumour response than IFN-α2 and IFN-β. IFN-β showed the greatest anti-tumour effect in vitro, but did not show signs of immune enhancement, resulting in low efficacy in vivo. Each of the IFN-α subtypes demonstrated the same anti-tumour effect in vitro, however at different potencies and also showed evidence of immune enhancement, resulting in increased efficacy in treating melanoma. IFN ‘Z’ showed greatest efficacy in treating melanoma, in both delaying the time to onset and increasing survival time.

The results of these experiments reflect those in clinical trials of IFN-α2, showing variable response rates and increasing disease free survival. Most notably, IFN ‘Z’ demonstrated a statistically significant increase in survival time in a small trial group. This is a significant finding which has demonstrated that the IFN-α subtypes show promise as an alternative immunotherapy with greater survival time and therefore improving the low survival statistic for patients with melanoma.

The method for IFN-I administration, DNA therapy, results in constant production of IFN-I, which overcomes the problem of the IFN-Is’ short half-life and avoids the toxicity associated
with bolus high dosage. This method resulted in low levels of IFN-I in the sera and strikingly, anti-tumour efficacy was still observed. This is also a significant finding, as this demonstrates that these IFN-α subtypes are more potent than IFN-α 2, and hence can be administered at lower doses, avoiding the side effects of IFN-I therapy but still achieving a therapeutic outcome. This is important, as many patients on IFN-α2 therapy have their dose lowered to below therapeutic levels due to toxicity, and although therapy may marginally prolong survival, the side effects often severely decrease quality of life during this time. Future research using greater levels of IFN may determine if higher doses correlate with greater therapeutic outcome.

The anti-tumour in vitro effects distinctly separate IFN-α and IFN-β, although they signal via the same receptor. The IFN-α subtypes displayed different potencies, giving further evidence that the subtypes are unique. There has been evidence for synergy between an IFN-α subtype and IFN-β in viral infection (Bartlett et al., 2002). The direct anti-tumour mechanisms of IFN-β and the immune enhancing action of IFN-α could potentially be combined, and further research should look into which IFN-α subtypes work synergistically with IFN-β in treating melanoma.

In conclusion, this study demonstrated differential efficacy of IFN-α subtypes in melanoma therapy, supporting our first hypothesis. Importantly, IFN ‘Z’ increased survival, and IFN ‘V’ and IFN ‘X’ each had a complete responder at the end of the experiment. These exciting results indicate there is a strong potential for IFN-α subtype therapy in human melanoma. IFN-β was more anti-tumourigenic than IFN-α in vitro, whilst IFN-α subtypes were generally more efficacious in vivo. Our data suggests that IFN-α but not IFN-β modulates the immune
response to melanoma, however the variation within the IFN-α subtypes to modulate anti-tumour immunity is yet to be uncovered. This provides a strong backing for our second hypothesis, however more research is needed. Future studies combining IFN-I therapy, such as with other immune therapies, may further enhance IFN-I efficacy in the treatment of melanoma.
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