THE ROLES OF CHEMOKINES, CHEMOKINE RECEPTORS AND GLYCOLIPID ACTIVATION IN NKT CELL TUMOUR INFILTRATION

By

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ABSTRACT

Higher levels of NKT cell infiltration correlate with improved prognosis in many human cancers. Therefore, we sought to identify factors which contribute to this process. We identified three chemokine receptors, CXCR3, CXCR6, and CCR5, which mediate NKT cell tumour homing. In competitive homing experiments, wild type (WT) NKT cells homed more efficiently to tumours than $CCR5^{-/-}$, $CXCR3^{-/-}$ or $CXCR6^{-/-}$ NKT cells. We also demonstrated that combined intratumoural administration of a CXCR3 ligand (CXCL10) and an NKT cell activating glycolipid (α-GalCer) significantly enhanced NKT cell recruitment and reduced tumour growth. In contrast, treatment with CXCL16 (CXCR6 ligand) increased tumour size. As CCR5 has been suggested to regulate NKT cell activity, we compared expansion and cytokine production from WT and $CCR5^{-/-}$ NKT cells, identifying reduced serum levels of IL-4 in $CCR5^{-/-}$ mice following NKT cell stimulation. Our results demonstrate that co-treatment of tumours with exogenous chemokines and NKT cell-activating glycolipids represents a novel strategy for therapeutic development.

LIST OF ABBREVIATIONS AND SYMBOLS USED

α-GalCer	α-Galactosylceramide
β-GlcCer	β-Glucosylceramide
APC	Allophycocyanin
APC(s)	Antigen Presenting Cells
bFGF	Basic Fibroblast Growth Factor
Con A	Concanavalin A
CSC(s)	Cancer Stem Cells
CTL(s)	Cytotoxic T Lymphocytes
DC(s)	Dendritic Cells
DLN(s)	Draining Lymph Nodes
DMBA	7,12-Dimethylbenzaanthracene
DNA	Deoxyribonucleic Acid
DNMT(s)	DNA Methyltransferase
DP	Double Positive
ECM	Extracellular Matrix
EMT	Epithelial-Mesenchymal Transition
EPC	Endothelial Progenitor Cell
FasL	Fas Ligand
GAG(s)	Glycosaminoglycan
GDP	Guanosine 5'-Diphosphate
GM-CSF	Granulocyte-Monocyte Colony Stimulating Factor
GPCR(s)	G-Protein Coupled Receptor

GTP	Guanosine 5'-Triphosphate
HSA	Heat Stable Antigen
IFN-γ	Interferon γ
iGb3	Isoglobotrihexosylceramide
KRN7000	((2S, 3S, 4R)-1-O- (α-D-galactopyranosyl)-N-hexacosanoyl-2-amino-1, 3, 4- octadecanetriol)
LLC	Lewis Lung Carcinoma
MAGE I	Melanoma-associated Antigen 1
MCA	Methylcholanthrene
MDSC(s)	Myeloid Derived Suppressor Cells
MHC	Major Histocompatibility Complex
MMTV	Mouse Mammory Tumour Virus
Mult-I	Murine ULBP-Like Transcript 1
PE	Phycoerythrin
PBMC(s)	Peripheral Blood Mononuclear Cell
PI3K	Phosphatidylinositol-3-Kinase
PMN	Polymorphonuclear
NK	Natural Killer
NKT	Natural Killer T
RB	Retinoblastoma
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SLAM	Signalling Lymphocyte Activation Molecule

TAA	Tumour Associated Antigen
TAM(s)	Tumour Associated Macrophage
TCR	T Cell Receptor
TGF-β	Transforming Growth Factor β
Th1/2	T Helper 1/2
TIL(s)	Tumour Infiltrating Lymphocyte
TLR	Toll-Like Receptor
TNF	Tumour Necrosis Factor
TP53	Tumour Protein p53
TPA	12-O-Tetradecanoylphorbol-13-acetate
TRAIL	Tumor Necrosis Factor-Alpha-Related Apoptosis-Inducing Ligand
Treg(s)	T regulatory
TSA	Tumour Specific Antigen
VEGF	Vascular Endothelial Growth Factor

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CHAPTER 1. INTRODUCTION

1.1 Cancer

Cancer can be described as a group of diseases which result from the unregulated proliferation and spread of abnormal cells. In 2014, the Canadian Cancer Society estimated that approximately 2 out of every 5 Canadians will develop some form of cancer in their lifetime, and about 1 in 4 will die of cancer[1]. Out of all new cancer cases, over half will be lung, breast, colorectal and prostate cancer. Of these, lung cancer is the leading cause of death and will result in more deaths than the other three cancers combined. The overall survival for people diagnosed with cancer is 63% over 5 years. Although overall cancer rates in Canada are declining in recent years, it still remains the leading cause of death in Canada, responsible for 30% of all deaths[1].

Cancer can be classified into 6 major subtypes based on the histological site in which they arise. These are: carcinoma, sarcoma, myeloma, leukemia, lymphoma and mixed (tumours which arise from multiple tissue types)[2]. Carcinoma refers to cancers of epithelial origin, and can be further divided into adenocarcinomas, which originate in mucus secreting glands, and squamous carcinoma, with origins in squamous epithelial tissue. Sarcomas originate in cells of mesenchymal origin (bone, cartilage, fat, muscle etc.) and often occur in young adults. Myeloma occurs in the plasma cells of bone marrow. Leukemia is a cancer of myeloid or lymphoid stem cells and can result in increased populations of immature white blood cells and thus increased risk of infection[2]. Lymphoma is a cancer of developing lymphocytes which originates in the glands and nodes of the lymphatic system, and often occurs as solid tumours[3].

The genetic mutations which lead to cancer development can occur as a result of various circumstances. Some are hereditary, while others are a result of environmental factors including various carcinogens and lifestyle choices such as smoking, alcohol intake and diet. Multiple genetic mutations need to take place in order for cells to become cancerous. These include mutations which lead to the inactivation of tumour suppressor genes, mutations in normal apoptotic function, mutations in DNA repair mechanisms and mutations in the cell cycle[4]. The accumulation of such mutations will ultimately lead to

the development of cancers which are resistant to normal elimination signals. There are ten hallmarks of cancer that have been identified by Hanahan and Weinberg[4] as of 2011, including: growth signal self-sufficiency, insensitivity to anti-growth signals, tissue invasion and metastasis, limitless replicative potential, sustained angiogenesis, evasion of apoptosis, abnormal metabolic pathways, immune system evasion, DNA instability and chromosome abnormalities, and tumor-promoting inflammation.

One of the most important fundamental traits of cancer cells is their ability to maintain abnormal proliferation[5]. Unlike normal tissue, which is capable of regulating the production and release of growth signals and progression through the cell cycle, cancer cells have lost such aspects of regulation. Rather, cancer cells sustain proliferative signals through a number of mechanisms. For example, in addition to producing their own growth signals, cancer cells can send stimulatory signals to the surrounding stromal cells, resulting in further release of various growth factors[6,7]. This uncontrolled proliferation can lead to the formation of tumours, which are now understood to be very complex tissues composed of malignant cells that interact with stromal cells and other components of the tumour microenvironment to promote tumour growth[4]. Immune cells which are recruited to the tumour can also play active roles in tumourigenesis[8] as a result of chronic inflammation or the accumulation of immunosuppressive cell subsets. Tumours can also attain nutrients and oxygen required for continued growth by stimulating angiogenesis and the development of a tumour associated neovasculature. These mechanisms of cancer initiation and progression will be discussed in further detail in the following sections.

1.1.1 Cancer Initiation

The development of cancer can be referred to as carcinogenesis, a process consisting of multiple steps ultimately leading to tumour formation. A series of gene errors lead to the accumulation of mutations which result in the dysregulation of normal cellular pathways. Specifically, mechanisms which control proliferation and survival of cells are severely altered in carcinogenesis; including DNA repair mechanisms, contact with surrounding cells and extracellular matrix (ECM), blood supply, apoptosis and cell cycle checkpoints[9].

A number of mechanisms have been described which attempt to explain how cells become cancerous. These can differ greatly depending on the type of cancer being studied. For example, it has been demonstrated that inflammatory cells are a key factor in the microenvironment of carcinomas[7]. This observation was originally made by Rudolf Virchow over 150 years ago when he noticed that cancers tend to occur at sites of chronic inflammation[10]. Since then, many studies have demonstrated that an increased risk of cancer often arises in association with inflammation-causing infectious disease[10]. Inflammatory cells are capable of contributing to cancer initiation and promotion through their release of various cytokines, chemokines and growth factors which work to stimulate epithelial proliferation and create reactive oxygen species (ROS) that can cause DNA damage[7]. Pre-cancerous epithelial cells are then able to activate the associated stroma to support tumour cell growth[11].

As well, cancer cells must bypass systems that negatively regulate cell proliferation, such as tumour suppressor genes[4]. There have been numerous tumour suppressors that have been identified in recent years, although the two best characterized encode the retinoblastoma (RB) associated protein[12] and the TP53[13] protein. These suppressors act as control points with the ability to enter or halt proliferation through senescence and apoptosis[14]. Defects in tumour suppressor pathways can result in unregulated progression into the cell cycle and development into cancerous cells. An important genetic mutation which has been implicated in the loss of tumour suppressor genes is one which leads to the methylation of CpG islands. When normally unmethylated CpG islands undergo methylation in cancer cells, it results in the loss of expression of flanking genes, such as tumour suppressers[15]. It is estimated that approximately 1.4% of CpG islands in the human genome are aberrantly methylated, and this methylation continues to accumulate in approximately 10% of islands in tumour development[16]. Several studies have demonstrated that when DNA demethylation is induced by drugs, tumour suppresser genes can be re-established and tumour control can be achieved[17]. DNA methyltransferases (DNMTs) and other key enzymes that regulate DNA transcription are likely to play important roles in cancer development[9]. Indeed, the overexpression of DNMTs at the mRNA level has been demonstrated to occur in several cancers[18]. In addition to CpG island methylation, other epigenetic modifications have also

been implicated in cancer initiation. For example, histone acetyltransferases and histone deacetylases will modify histones, thus acting as gene activators or repressors[19]. Thus, abnormal acetylation of histones can also lead to the silencing of important tumour suppressor genes, leading to the development of malignant disease.

One type of cell which has a well-studied role in the process of cancer initiation are fibroblasts[20]. Fibroblasts are capable of synthesizing, remodelling and depositing much of the extracellular matrix (ECM) in the tumour stroma. They are also an important source of paracrine growth factors, thus contributing to the growth of carcinoma cells[20]. Previous studies have suggested that stromal fibroblasts play a more minor enabler role in cancer, however some data suggests that they can also act as inducers of carcinomas[7]. It has been demonstrated that tumour fibroblasts are capable of stimulating epithelial cell proliferation and malignant transformation[21]. Fibroblasts are capable of releasing various growth factors which contribute to enhanced proliferation of cells, including fibroblast growth factor (FGF) family, the IGF family, the EGF family, hepatocyte growth factor (HGF)[22]. These growth factors have been implicated in both autocrine and paracrine stromal-epithelial interactions and can enhance carcinoma initiation and progression[7]. In contrast, production of TGF-β by fibroblasts has been shown to act as a growth inhibitor of most epithelial cells[23], and it has been suggested that this growth factor plays an important role in tumour suppression. For example, it has been demonstrated that increased TGF-β signalling can lead to suppressed tumour formation while inhibition of the pathway enhances carcinogenesis[24].

1.1.2 Angiogenesis

One of the hallmarks of cancer mentioned above is the formation of new blood vessels, a process termed angiogenesis[4]. Vessels are required for tissue growth and regeneration as they supply oxygen and nutrients, and are therefore exploited by cancer cells[25]. Cancer cells will fuel the production of new vessels in order to continue proliferating[26]. It was first observed that angiogenesis occurs near tumour sites nearly a century ago[25]. Decades later, it was hypothesized that tumours produce and secrete an angiogenic substance[27], and in 1976 it was demonstrated that pre-cancerous tissues acquire the ability to induce angiogenesis as they become cancerous[25]. Since then, several

studies have demonstrated that tumour blood vessels can develop by sprouting from pre-existing vessels. In addition, circulating endothelial pre-cursers can assist with tumour angiogenesis[28,29].

There are numerous factors which have been identified to play important roles in tumour angiogenesis. Among these, the most prominent are vascular endothelial growth factor (VEGF) and the angiopoietin (Ang) family[30]. These molecules are dysregulated in tumours, leading to abnormal vessel development. Vessels formed through tumour angiogenesis differ in many aspects from those formed by normal vessel development. For example, tumour vessels lack functional perivascular cells, and thus lack protection from changes in hormonal and oxygen balance[31]. As well, the vessel walls are often heterogeneous. That is, they are often lined by a mixture of cancer cells and endothelial cells. Furthermore, the vasculature in tumour vessels is disorganized in comparison to that of normal vessels. They often have a very uneven diameter, with a high rate of branching and many shunts[25]. Tumour vessels also have many widened inter-endothelial junctions, and an unstable and sometimes absent basement membrane, making them very prone to "leaks"[25]. Moreover, tumour vessels show abnormal expression of adhesion molecules, leading to reduced leukocyte-endothelial cell interactions[32].

As the signalling molecule VEGF has been shown to play a significant role in mediating angiogenesis, there have been multiple studies and clinical trials aimed at targeting cancer through the inhibition of VEGF. There are several VEGF pathway inhibitors that are approved for the treatment of certain cancer types[33], including a humanized anti-VEGF-A monoclonal antibody, small molecules inhibiting VEGFR-2 signal transduction[34], and a VEGF receptor chimeric protein[35]. Clinical studies examining the effectiveness of VEGF inhibition in combination with chemotherapy resulted in increased survival in patients diagnosed with metastatic colorectal cancer[36]. The majority of clinical trials examining the efficacy of VEGF inhibitors have been done with patients with advanced cancers, however preclinical studies suggest that these inhibitors may be most beneficial in early stage cancers[33]. Although VEGF inhibition has been shown in numerous trials to have significant benefit, it has also resulted in significant adverse side effects, such as metastasis and bleeding complications in some patients[37]. Therefore,

more studies are required to fully elucidate the effects of such treatments and determine which patients are likely to receive the highest benefit with the lowest risk of adverse effects.

1.2 Tumour Immunosurveillence

The process through which both the innate and adaptive immune systems identify and work to eliminate transformed cells is termed tumour immunosurveillence. The concept of immunosurveillence suggests that the immune system is capable of identifying abnormal self-antigens such as those present in precancerous or cancerous cells, and is thus able to attack and kill growing tumour cells[38]. When this concept was originally proposed by Burnet and Thomas[39], it was met with a great deal of debate and skepticism. It was largely rejected by the scientific community due to lack of evidence; however, it later re-emerged when studies demonstrated that immunodeficient mice had a significantly greater risk of spontaneous tumour development in comparison to immunocompetent mice[40]. Further studies supported this finding, and immunosurveillence became a new and leading area of research. It has been demonstrated that the incidence of tumours significantly increases in mice lacking cytotoxic T lymphocytes (CTLs), CD4 helper T cells or Natural Killer (NK) cells[41]. Importantly, combined deficiencies in both T cells and NK cells results in even higher susceptibility to cancer development than with either deficiency alone[42],[39]. Thus, it is clear that both the innate and adaptive immune systems play significant roles in cancer detection and elimination. Today, there is a breadth of research demonstrating the importance of both the innate and adaptive arms of immunity in immunosurveillence. Figure 1 outlines the various activities of the innate and adaptive immune systems in tumour immunosurveillence.

1.2.1 Innate Tumour Immunosurveillence

The innate immune system plays several roles in immunosurveillence, particularly as danger signals are released from transformed cells. Innate immune cells involved in this process include polymorphonuclear cells (PMNs), macrophages, NK cells, and Natural Killer T (NKT) cells. They are able to provide the “first line of defence” against

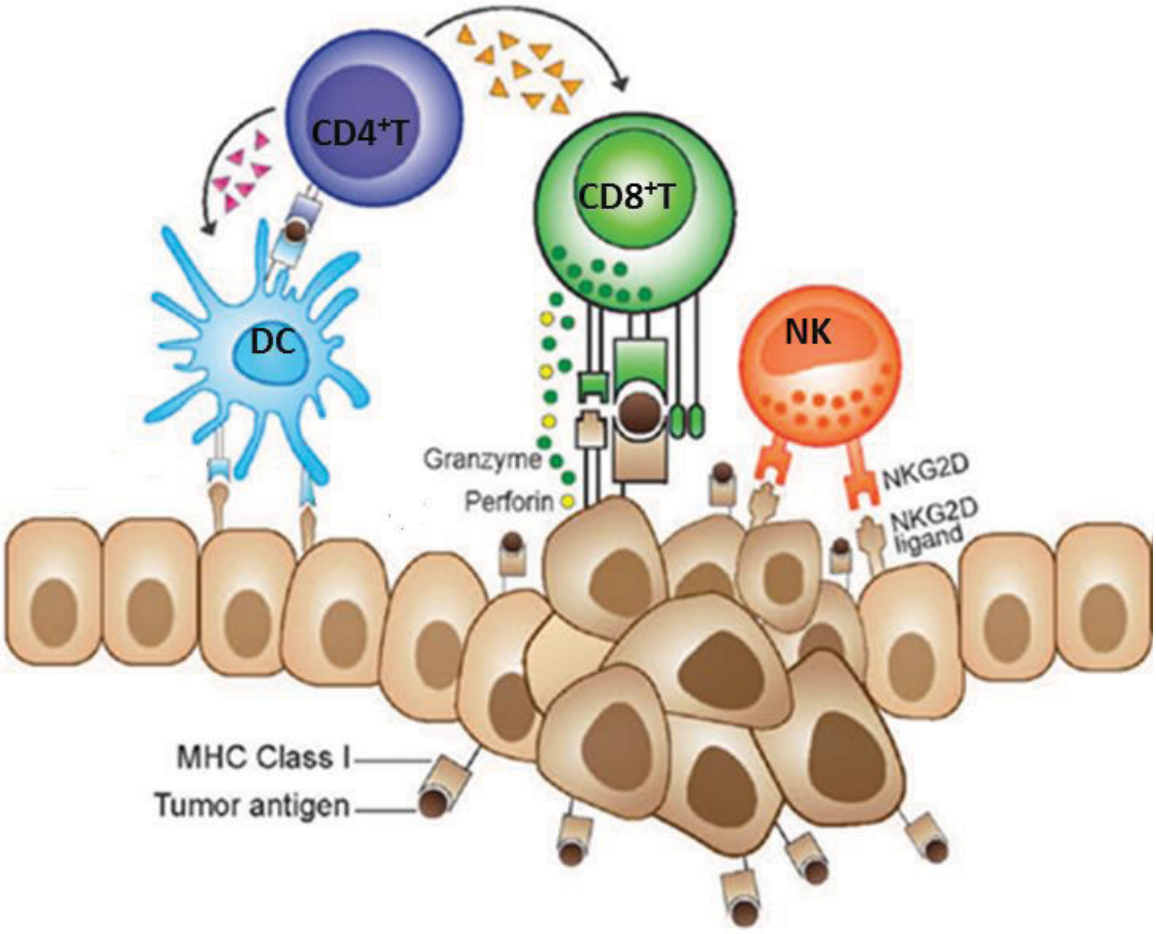


Figure 1: Tumour Immunosurveillence. Both the innate and adaptive immune system are involved in tumour immunosurveillence. Danger signals released from transformed cells can lead to activation of cells of the innate immune system, such as Natural Killer (NK) cells. Dendritic Cells (DCs) can capture and present tumour antigens to $CD4^+$ and $CD8^+$ T cells. Activated $CD8^+$ T cells and NK cells can attack and kill transformed cells through perforin or granzyme mediated cytotoxicity. These responses rely on stress ligands such as NKG2D and recognition of TAAs in the TCR-MHC complex.

Adapted from Monjazeb et al., Front. Oncol, 2013

cancer, acting in an immediate but non-specific manner. As such, the innate immune response is usually unable to provide long-lasting protective immunity. Cells of the innate immune system employ several receptors to recognize and respond to tumour danger signals, including NKG2D, NKR-P1, and NKG2/CD94 heterodimers[43]. In addition, innate cells can detect stress induced or down regulated MHC I-like molecules on tumour cells via Ly49 (mouse) or KIR (human) molecules, allowing them to attack and kill malignant cells[41,44]. They can kill tumour cells directly through FasL or tumor necrosis factor-alpha-related apoptosis-inducing ligand (TRAIL), and release perforin and granzyme to induce cancer cell death[45,46]. Importantly, it was demonstrated that in methylcholanthrene (MCA)-induced fibrosarcoma, dimethylbenzanthracene (DMBA)-induced cancers, and phorbol ester (TPA)-induced skin carcinomas, the absence of innate immune cells such as NK cells, NKT cells and γδ T cells results in much higher incidence of tumours[46].

Several studies have provided evidence to suggest that recently transformed cells directly interact with innate immune cells, resulting in their elimination. For example, it has been demonstrated that NK cells, macrophages and subsets of T cells can recognize stress induced MHC I-like molecules, which leads to tumour cell death[47]. Specifically, the activating receptor NKG2D allows subsets of NK cells, γδ T cells, macrophages, and NKT cells to detect and lyse cells which have undergone cancer-related genomic stress. NKG2D is a lectin-like homodimer which associates with the phosphatidyl inositol 3 kinase (PI3K) activator DAP10 [44]. The NKG2D-DAP10 complex can interact with several MHC class-I related ligands which are induced by cell stress[47]. The ligands for NKG2D consist of MICA, MICB, and ULBO1-6 in humans and Rae-1, Mult1, and H-60 for in mice[48]. NKG2D ligand expression is low on normal tissue, but higher expression has been reported in epithelial carcinomas[49]. When expressed by tumour cells and tumour stroma, NKG2D ligands lead to enhanced sensitivity to NK cell mediated lysis[47,50]. This has been demonstrated both in vitro and in vivo[51] . Specifically, the ectopic expression of the NKG2D ligands Rae-1 and H60 in multiple tumour cell lines results in rejection, even in tumour cells expressing normal levels of MHC I[52]. As well, macrophages will release nitric oxide and produce tumour necrosis factor (TNF) in response to these ligands [50]. When NKG2D is neutralized, wild type C57BL/6 and BALB/c mice become increasingly

sensitive to MCA-induced fibrosarcoma[46]. As well, it has been demonstrated that IL-12 therapy is largely dependent on the NKG2D pathway in preventing MCA-induced sarcomas[46]. Together, these and other findings have demonstrated that NKG2D ligands which arise under conditions of cellular transformation play a key role in stimulating the innate immune response.

NK cells are able to detect MHC class I deficient tumour cells and their metastases and kill them through perforin-mediated cytotoxicity[53]. In addition, NK cells produce inflammatory cytokines, such as IFN-γ, that contribute to immune activation and tumour cell elimination. NK cells are able to rapidly respond to a variety of cytokines, including IL-2, IL-12 and IL-15, which enhance their anti-tumour activities[38]. As well, a subset of T cells with NK cells markers, known as NKT cells, have been shown to regulate anti-tumour activity[54]. These cells recognize glycolipid tumour antigens in the context of CD1d and rapidly release a variety of both Th1 and Th2 cytokines, thus bridging the adaptive and innate immune responses[38]. Mice deficient in NKT cells develop MCA-induced sarcomas at a significantly higher frequency than wild type mice[55]. Similarly, mice lacking one copy of the tumour suppressor p53 succumb to tumour development faster if NKT cells are absent[56]. NKT cells and their functions in cancer will be discussed in further detail in the following sections.

Another major component of innate immunosurveillence are type 1 interferons. When IFNα/β is neutralized in mice, growth of transplanted syngeneic tumour cells is significantly enhanced[57].It has also been demonstrated that host IFNα/β can prevent the growth of MCA-induced sarcomas[58,59]. Importantly, it has been shown that IFNα/β exert many of their anti-tumorigenic effects by acting on host hematopoietic cells rather than on tumour cells directly[58,59]. In experiments in which exogenous IFNα/β was administered, it stimulated NK cell proliferation and enhanced NK cytotoxicity through cytokine secretion, TRAIL-mediated cytotoxicity, and increased activation and MHC expression on tumour cells and APCs[46,60]. The protective effect of IFNα/β is not completely overlapped by that of IFN-γ, suggesting that it plays a critical role in immunosurveillence[58]. It has also been demonstrated that IFNα/β is a key requirement for the rejection of highly immunogenic MCA-induced sarcomas, and is effectively able to

prevent the outgrowth of such tumours[58]. IFNα/β has thus been suggested to play an important role in preventing cellular transformation and promoting the development of anti-tumour immune cells[46]. Type I IFNs have also been shown to promote the development of $CD8^+$ T cells through IL-15 induction[61]. In addition to IFNs, many other cytokines such as IL-1β, TNF and heat shock proteins (HSP) act as danger signals which indicate cell stress.

1.2.2 Adaptive Tumour Immunosurveillence

The killing of tumour cells by NK cells or other innate immune cells will result in the release of tumour antigens, leading to adaptive immune responses[62]. These antigens can be either specific to individual tumours (tumour specific antigens-TSA) or associated with tumours (tumour associated antigens-TAA)[63]. TSAs are highly immunogenic but are very rarely shared between different individuals. TAAs have been identified in normal tissues; however they display increased expression in tumour cells, allowing them to serve as potential immunological targets[64]. They could also be proteins which are normally expressed on fetal tissues but not on adult tissues, such as carcino-embryonic antigen[65]. These antigens show broad expression within the same tumour type in different individuals, thus making them potential vaccine targets. However, because they do overlap in expression with normal tissues, there is potential for tolerance to such vaccines, thus an adjuvant would be required in order to produce a strong immune response. The potential danger of targeting TAAs is that you can enhance autoimmunity.

DCs capture and load tumour antigens into MHC molecules to prime and activate both $CD4^+$ and $CD8^+$ T cells[38]. Through the interaction of costimulatory molecules such as CD40 on APCs and CD40L on T cells, $CD4^+$T cells are activated and licensed to help $CD8^+$ T cells[66]. Activated $CD8^+$ T cells can then differentiate into cytotoxic T lymphocytes and lyse tumour cells in an antigen-specific manner[38]. NK cells participate in cross talk with DCs leading to DC maturation and migration to tumour draining lymph nodes (DLNs). This results in enhanced presentation of tumour antigens to naïve T cells, leading to expansion of CTLs[67]. Antigen specific CTLs can then be recruited to tumour sites and attack tumour cells[39]. As well, TAAs such as melanoma antigen 1 (MAGE1), which is expressed on human melanoma cells, can be recognized and processed by skin

Langerhans cells. Once the Langerhans cells are activated they will express co-stimulatory signals and migrate to DLNs where they can stimulate T cell responses[38]. There has been a great deal of effort made in identifying new tumour antigens, such as cancer testis antigens, melanocyte differentiation antigens, and normal antigens that are overexpressed in transformed tissues which can be recognized by CTLs. However, TAA presentation to T cells can also result in T cell anergy due to recognition of "self" antigen[68]. Therefore, treatments using TAAs need to counteract and overcome tolerance before they can be effective. One method used to overcome tolerance is the use of viral vectors in the combination with TAAs and costimulatory molecules such as CD40L, which ensure that strong co-stimulatory and maturation signals are present between T cells and TAA presenting DCs[69].

1.3 Tumour Infiltrating Immune Cells

Cells of both the innate and adaptive immune systems are capable of migrating to and infiltrating growing tumours[4]. These include macrophage subtypes, mast cells, neutrophils and B and T lymphocytes. Several studies have indicated that tumour-infiltrating lymphocytes (TILs) are able to attack and kill tumour cells. For example, it has been shown that a high frequency of $CD8^+$ T cells in tumours of patients with various cancer types, including melanoma[70], head and neck cancers[71], ovarian cancer[72], pancreatic cancer[73] and colorectal cancer[74] is correlated with significantly improved survival. However, conflicting reports have shown that TILs can act in both tumour antagonizing and tumour promoting ways[4]. For example, the accumulation of $CD4^+$ Tregs has been correlated with poor prognosis in patients[75,76]. Thus, the presence and function of immunostimulatory versus immunosuppressive cells within tumour environments is critically important in preventing cancer progression and promoting patient survival[77].

In addition to T cells, NK and NKT cells have been found in high numbers in some tumours. NKT cell infiltration into primary tumours has been associated with improved prognosis in neuroblastoma patients and patients with primary colorectal carcinoma[78,79]. NKT cells thus appear to play a crucial role in controlling tumour growth and development in multiple forms of cancer.

Although the majority of studies examining tumour infiltrating immune cells have focused on those with strong tumour killing ability, such as NK or $CD8^+$ T cells, some studies have also suggested that B cells can be beneficial[80]. For example, B cells have been shown to be a positive prognostic factor in breast cancer[81], and a proportion of tumour-infiltrating B cells have the ability to produce tumour antigen specific antibodies[82]. It has been suggested that B cells found within tumours can be organized into several subclasses, including those that have tumour killing ability, tumour suppressive functions, or tumour promoting functions[80]. More studies will need to be done in order to clearly elucidate the roles of tumour infiltrating B cells in cancer.

In addition to the various lymphocytes already discussed, myeloid cells constitute a significant proportion of tumour infiltrating immune cells. There are heterogeneous myeloid cell subtypes found within tumours[83] that can have differing functions in the progression of cancer[84]. Classical M1 macrophages are activated by IL-2, IFN-γ and IL-12 and display high levels of APC function, while alternative M2 macrophages display defective IL-12 production and have been associated with induction of angiogenic factors, cytokines and proteases that promote tumour growth and spread[85].Thus, macrophages which display the M1 classically activated phenotype have anti-tumour effects, while alternatively activated M2 macrophages, or Tumour Associated Macrophages (TAMs) typically have pro-tumorigenic effects[86]. New evidence has also suggested that TAMs may be a population of cells distinct from M2 cells[87]. Studies in a number of different models have indicated that high TAM infiltration is associated with enhanced vascularization and growth[88,89]. Myeloid derived suppressor cells (MDSCs), which are immature cells of myeloid origin with immunosuppressive function, have also been found in significant frequencies in tumours. MDSCs and TAMs have many overlapping functions and markers (ex. $CD11b^+Gr1^+F4/80^+Arg1^+$)[90], making it difficult to decipher and differentiate the effects of each in a tumour environment. More studies will need to be conducted in order to clearly differentiate these immunosuppressive myeloid subtypes and their functions. MDSCs and their roles in a tumour microenvironment will be discussed further in the following section.

1.4 Tumour Escape

Although the immune system has developed numerous mechanisms to detect and eliminate cancerous or transformed cells, tumours can often counteract these actions and minimize immune responses, leading to tumour escape (Fig.2). Cancer cells are capable of evading the immune system by working to disable immune components which have been sent to eliminate them[4]. This can be done through the process of immunoediting, which encompasses three phases: elimination, equilibrium and escape[91]. Elimination is the result of successful immunosurveillence on the part of the immune system, where tumour cells are destroyed by the innate or adaptive immune systems[92]. Equilibrium occurs when the immune response leads to the selection of less immunogenic tumour cells. In this case, rare cell variants survive the elimination phase but the adaptive immune system prevents further tumour cell outgrowth[39]. The immune system is thus able to contain the tumour cells in a state of dormancy, rather than eliminating them. This is the longest of the three phases of immunoediting[39]. The final phase of the immunoediting process, escape, occurs due to the outgrowth of tumour variants which are able to resist destruction by immune cells. Cells within the tumour which have the ability to avoid recognition and destruction by the immune system grow progressively and develop into tumours[39]. This occurs due to alterations such as loss of tumour antigens, which results in reduced recognition by immune effector cells and antibodies[39]. It can also occur as a result of increased resistance to the cytotoxic effects of the immune response.

In addition to immunoediting, cancer cells can avoid elimination through the induction of immunosuppressive cytokines and immune cells. For example, immunosuppressive cytokines such as TGF-β and IL-10 can prevent CTLs and NK cells from exerting their anti-tumour effects[93,94]. The production of these cytokines by tumour cells results in a skewing of the host immune response away from a Th1 response and towards a Th2 response[95]. As well, tumour cells are able to release factors which allow for the recruitment of immunosuppressive cells, such as regulatory T cells (Tregs) and

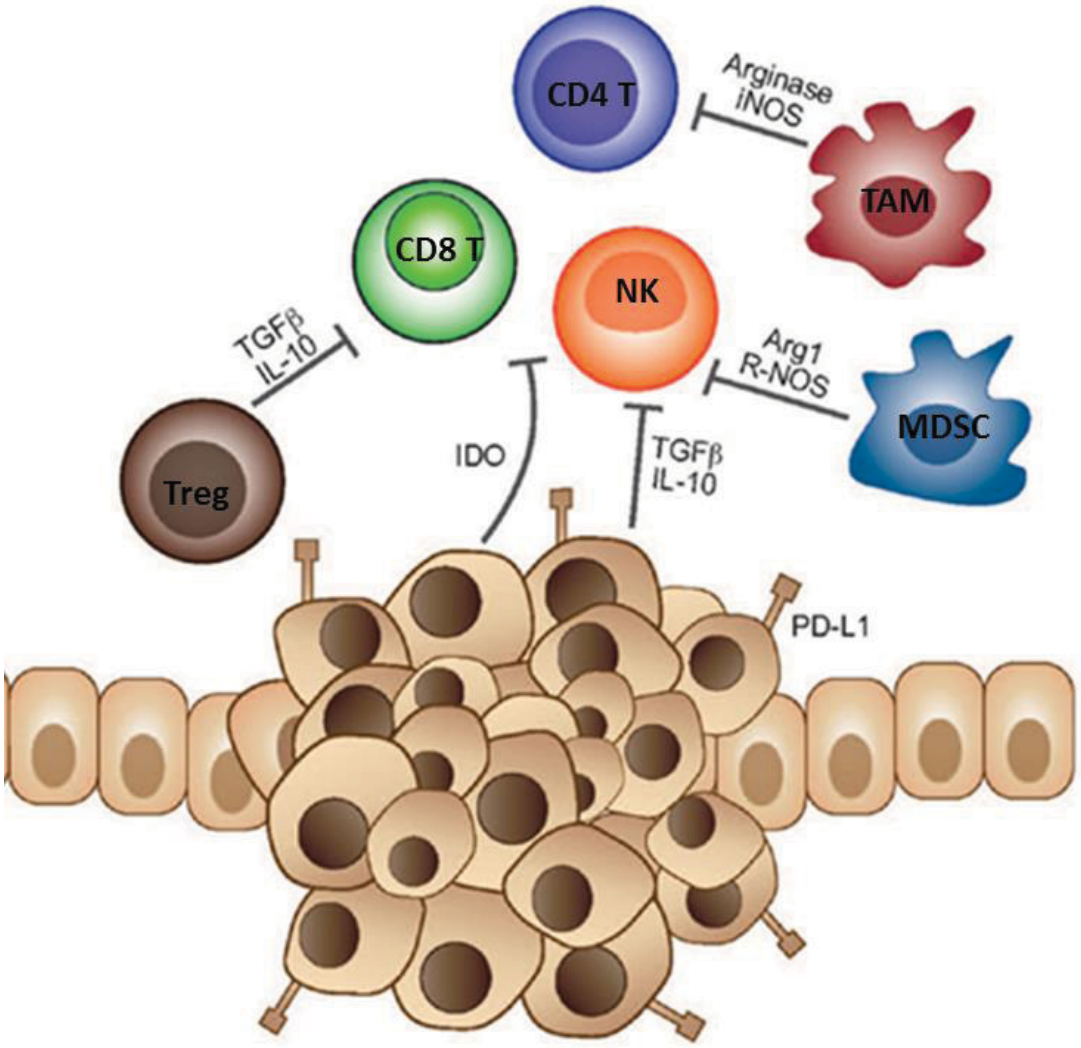


Figure 2: Tumour Escape. Tumour escape occurs when cells which are less immunogenic are selected for. Tumour cells release immunosuppressive cytokines, such as TGF-β and IL-10, which diminish the anti-tumour functions of $CD8^+$ T cells and NK cells. They are also able to attract immunosuppressive cells, such as T regulatory cells, MDSCs, and TAMs. These can in turn dampen T cell responses by sequestering nutrients using arginase, ROS generation, NO as well as by interfering with trafficking to tumour sites. Immunosuppressive enzymes such as IDO and arginase can also catabolize essential nutrients needed for proper activation of effector cells. Tumour cells downregulate expression of MHC molecules and other antigenic molecules, and increase expression of inhibitory molecules such as PD-L1.

Adapted from Monjazeb et al., Front. Oncol, 2013

MDSCs, both of which can in turn suppress the activities of cytotoxic lymphocytes[96,97]. In addition, tumour cells can directly kill attacking lymphocytes through expression of surface molecules such as FasL, which induce apoptosis[38]. Tregs which are induced and recruited by tumour cells can inhibit the function, activation and expansion of various immune cells either through direct cell to cell contact or through release of immunosuppressive cytokines, such as IL-10 and TGF-β[98]. Recruitment of Tregs to tumours has been shown to occur through several mechanisms. In breast and prostate cancers, the presence of CCL22 in tumour environments results in CCR4-dependent Treg recruitment[99,100]. In ovarian cancer, Treg recruitment is mediated by tumour derived CCL28[101]. Several studies have indicated that Treg infiltration into tumours is associated with suppression of T cell proliferation[102] and poor prognosis [103].

Tumour cells are also able to release factors that lead to the proliferation and accumulation of MDSCs[104]. These cells are able to inhibit both innate and adaptive immune responses to tumours and prevent the immune system from recognizing and attacking newly transformed cells[96]. There are two main subtypes of MDSCs in mice, granulocytic ($CD11b^{+}Ly6G^{+}Ly6C^{low}$) and monocytic ($CD11b^{+}Ly6G^{-}Ly6C^{high}$)[96]. These cells expand significantly following inoculation with transplantable tumour cells or during spontaneous tumour development[105,106]. Depending on the tumour model, the number of MDSCs can increase 5-20 fold. Their strong immunosuppressive activity allows them to inhibit the anti-tumour actions of NK cells, $CD4^{+}$ and $CD8^{+}$ T cells[107]. They inhibit pro-inflammatory cytokine production such as IFN-γ[108] and contribute to the accumulation and differentiation of Tregs within tumours via IL-10 and TGF-β production[109]. They exert their immune suppression through a number of factors, including arginase, reactive oxygen species (ROS) and reactive nitrogen species (RNS)[107,110]. When MDSCs are depleted, immune surveillance is restored, NK and T cells are activated, and the efficacy of cancer vaccines and other immunotherapies are significantly enhanced in vivo[111,112]. Recent studies have suggested that the frequency of MDSCs in tumour bearing mice can be effectively decreased through NKT cell activation[113].

In addition to the roles for immunoediting and immunosuppressive cells in tumour escape, there is mounting research in support of a subpopulation of cancer stem cells (CSCs) which are responsible for sustaining tumour growth. Evidence for the existence of CSCs originally came from a study which demonstrated that a very rare subset of cells (0.01-0.1% of the population) could induce leukaemia when transplanted into immunodeficient mice[114]. Several later studies were able to generate similar results in various tumour models. CSCs are thus defined as tumour cells which have the ability to self-renew as well as differentiate into the diverse cells that make up a tumour[115]. They vary widely in abundance in different tumours, but have found to be present in nearly all tumour types[4], indicating that CSCs have crucial roles both in the initiation and progression of cancer[115]. CSCs are often able to evade immune responses by down-regulation immunogenic markers. For example, CSCs can display lower levels of activating and/or higher levels of inhibitory NK cell receptor ligands, making them resistant to $CD8^+$ T cell and NK cell attacks[116]. In addition, CSCs contain high levels of anti-apoptotic proteins such as bcl-2, bcl-xL, or survivin, which protect CSCs from chemotherapeutic drugs[117].

1.5 Animal Tumour Models

Animal models are crucial in the study of tumour immunology as they provide a more realistic tumour environment than that found in in vitro systems. In particular, mouse models are very useful as they have extensive similarities to humans both at the physiological and molecular level[118]. The availability of gene-targeted mice also allows for the investigation of various molecular mechanisms. Three murine cancer lines were used in this body of work, the B16-F10 murine melanoma, the Lewis Lung Carcinoma (LLC), and the 4T1 mammary carcinoma.

The B16 melanoma cell line is widely used as a model in the study of tumour immunology[119]. This cell line was derived over 60 years ago from a C57BL/6 mouse carrying a chemically induced tumour. There are two subtypes of this cell line, the F1, which has low metastatic activities and the F10, which was used in this body of work, with high metastatic activities. The B16 model displays very rapid growth in-vivo, usually leading to death within 2-4 weeks following intravenous injection of cells[119]. The

subcutaneous tumour model which is used in this thesis is a very widely used model for evaluation of immunotherapy[120]. Following subcutaneous injection, a palpable tumour will develop within 10 days. A frequent problem encountered with the use of this cell line is that it has shown instability and many variants among laboratories[120]. B16 has the ability to change significantly over time, such as in its metastatic potential and antigen expression. For this reason, it is crucial to keep both culturing conditions and injection procedures constant between treatment groups.

The LLC cell line was originally derived in 1951 from a spontaneous murine lung tumour[121]. It was then passaged repeatedly in order to increase malignancy. It was demonstrated that s.c injections of 5×10^4 LLC cells leads to the eventual development of tumours in all mice, with those receiving more cells developing palpable tumours at a faster rate[121]. Examination of tumour growth rates revealed that doubling time of tumour size progressively increases as the tumour mass increases, with all mice succumbing to disease between days 35 and 45. This cell line produces tumours with more consistent phenotype and growth rate than that observed in B16 melanoma tumours; however mice need to be closely monitored as the solid and turgid nature of these tumours can lead to overgrowth and breaking of skin.

The 4T1 tumour cell line is a transplantable mammary carcinoma which was originally isolated from a spontaneous mammary tumour in a $MMTV^+$ Balb/c mouse[122]. This murine tumour cell line is highly invasive, and is able to spontaneously metastasize to multiple sites (including lymph nodes, blood, liver and lung) from a primary tumour[123]. It is also able to rapidly undergo epithelial to mesenchymal transition in vivo[124] and therefore is often used to study breast cancer metastasis[122]. Another feature which makes this a useful model for the study of metastasis is that the primary tumour can be readily removed, leaving metastatic foci intact to be studied[123]. 4T1 tumour cells express MHC class I and no MHC class II, making them potential targets of CTLs[122]. A recent study demonstrated that 4T1 tumours were more immunogenic with increased expression of many immune activation genes compared to LLC and B16 tumours[126]. This study also demonstrated increased expression of several co-stimulatory markers on TILs of 4T1 tumours compared to that of LLC or B16 tumours. In addition, the response of

tumours to immunotherapy was correlated with the level of immunogenicity of the tumour model, thus 4T1 tumours were most responsive to immunotherapy regimens.

In addition to the implantable tumour models already discussed, carcinogen-induced tumour models are often used to study the immunological responses to cancer. Two types of carcinogenesis protocols are currently in use in mice, complete and 'two-stage' carcinogenesis[127]. In complete carcinogenesis protocols, a single high dose of a carcinogen is administered. In such experiments, it can be difficult to differentiate between events related to the tumor initiation vs tumor promotion stages[127]. In two-stage skin carcinogenesis, initiation occurs after a single sub-carcinogenic dose of a carcinogen such as DMBA. This protocol requires other tumour promoting agents to be administered before tumour development can occur. As well, two stage protocols allow for tumour development to be monitored more easily[127]. In these carcinogen-induced tumour models, cancer development develops as mutations accumulate over time, thus providing conditions which are more physiologically relevant to cancer occurrence in humans compared to implantable tumour models.

In addition to carcinogen-induced and implantable models, transgenic mouse models of cancer are also incredibly useful as they can closely mimic the pathophysiology of human malignancies [118]. These models rely on mutant mice with abnormal expression of oncogenes or tumour-suppressor genes[118].

1.6 Natural Killer T cells

NKT cells are a population of T cells that express several markers of NK cells, such as NK1.1 and Ly49, in addition to a T cell receptor (TCR)[128]. Unlike conventional T cells which respond to peptide antigen presented by MHC class I or II, NKT cells respond to lipid and glycolipid antigens presented in the context of the MHC I-like molecule CD1d (Figure 3)[129]. They respond to a range of lipid antigens, including phospholipid antigens, and self and foreign glycolipids[130] . There are two major NKT cell subsets within humans and mice, classical Type I invariant (*i*NKT) cells that express a restricted TCRα rearrangement and Type II NKT cells which express a more diverse TCR repertoire[131,132]. This body of work will focus on type I *i*NKT cells and these will simply be referred to as NKT cells for the remainder of this thesis. NKT cells are defined

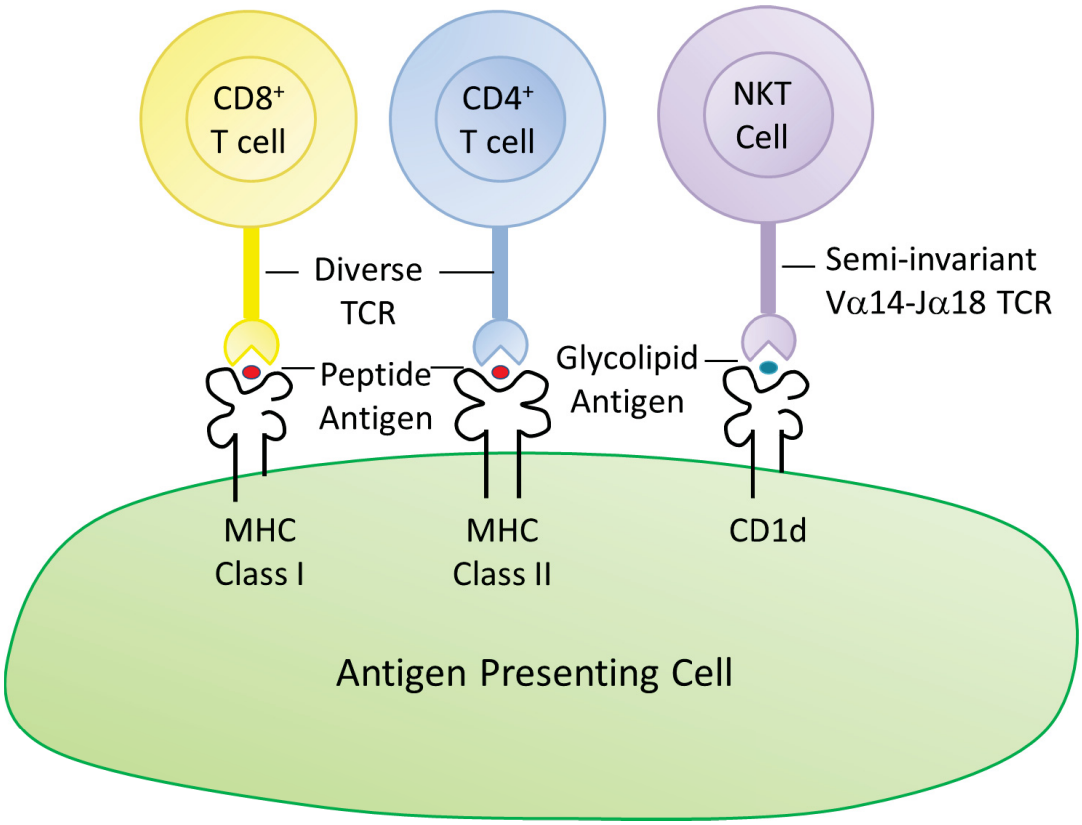


Figure 3: Natural Killer T cells. $CD8^+$ T cells express a diverse T cell receptor (TCR) which recognizes peptide antigens presented by Class I MHC. $CD4^+$ T cells express a diverse TCR which can recognize peptide antigens presented Class II MHC. In contrast, NKT cells recognize glycolipid antigens presented by CD1d on the surface of antigen presenting cells (APCs). In contrast to conventional T cells, NKT cells express an invariant TCR formed by a Vα14-Jα18 rearrangement in mice.

by their expression of an invariant Vα14-Jα18 rearrangement in mice and a Vα24-Jα18 rearrangement in humans, and their potent ability to respond to the glycolipid antigen α-GalactosylCeramide (α-GalCer)[131]. Because of this reactivity, NKT cells can be effectively identified by CD1d-tetramers loaded with α-GalCer[133].

Once activated, NKT cells respond rapidly and are able to serve as a bridge between the innate and adaptive immune responses[134]. When NKT cells are activated by glycolipid, they can kill transformed or infected cells through perforin, TRAIL and FasL mediated cytotoxicity[135]. An important feature of the NKT cell response is their ability to rapidly produce a range of different cytokines. It is well documented that activated NKT cells generate Th1 and Th2 cytokines IFN-γ and IL-4, and other cytokines including IL-2, TNF, IL-15, IL-13 and GM-CSF[136,137,138] . This rapid production of cytokines allows NKT cells to direct the immune response by activating other immune cells, including NK cells, DCs, B cells, and conventional T cells[139]. NKT cells have been demonstrated to play important roles in regulating diverse immune response pathways, including tumour clearance, host protection from infectious agents, prevention of autoimmune diseases, and the maintenance of self-tolerance[140,141].

1.6.1 NKT Cell Development

Although some early studies suggested an extra-thymic origin of NKT cells, it is clear that NKT cells develop in the thymus from common lymphoid precursors[142]. Within the thymus, NKT cell frequency is approximately 0.5% [143]. They first arise during the perinatal period, reaching significant levels in the thymus at approximately 3-4 weeks of age[144,145]. The early stages of NKT cell development are similar to that of conventional T cells. However, NKT cell development segregates from that of T cells following TCR rearrangement (Figure 4)[146]. The selection of NKT cells requires recognition of glycolipid antigens presented by CD1d on double positive (DP) cortical thymocytes[147]. Homotypic interactions between signalling lymphocyte activation molecules (SLAMs) expressed on DP thymocytes provide additional co-stimulatory signals essential for NKT cell selection[148,149]. Positive selection of NKT cells is through CD1d mediated presentation of self-lipid[150]. Many studies have focused on identifying the endogenous

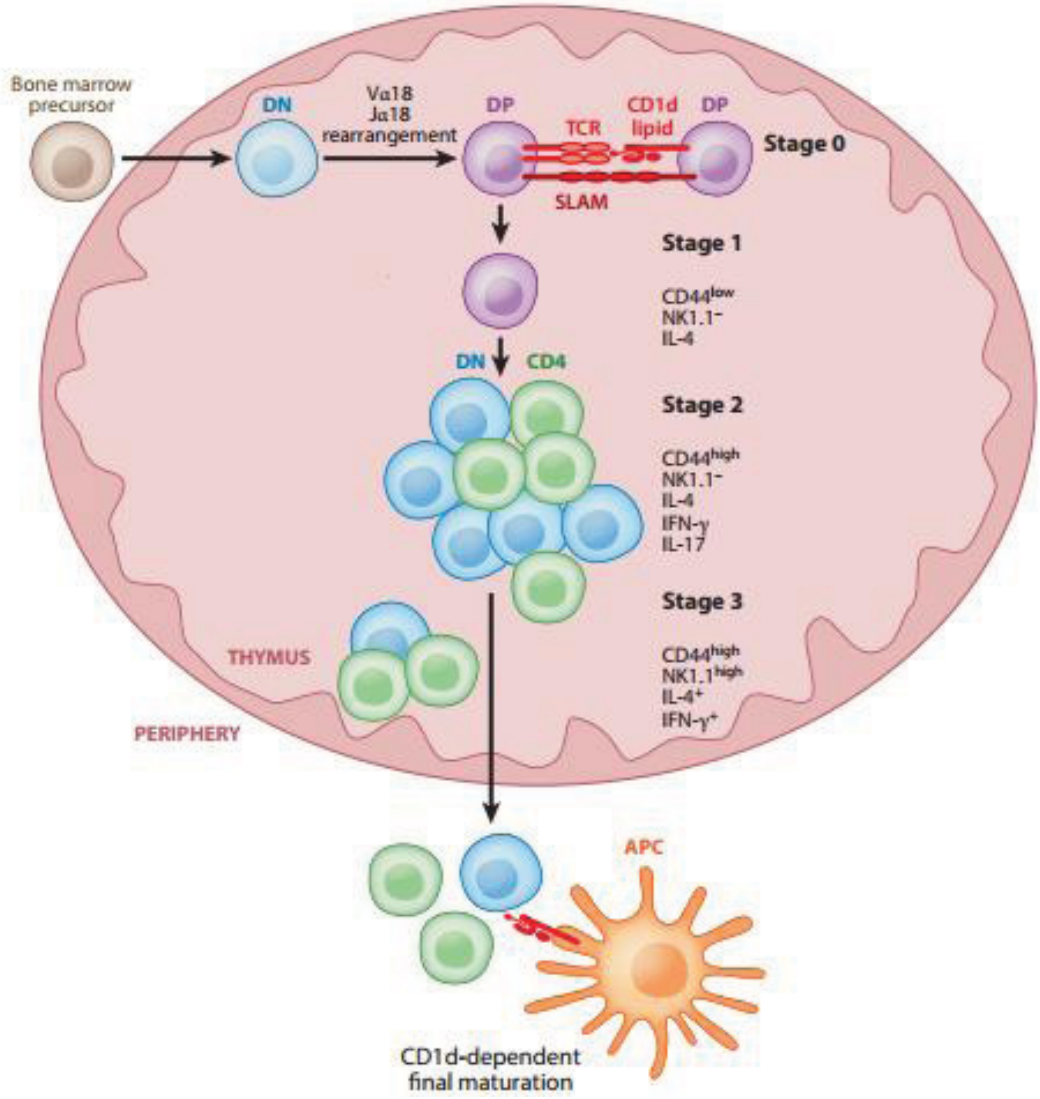


Figure 4. NKT cell development. NKT cells develop in the thymus, where their precursors differentiate from the double-positive (DP) stage. Positive selection of NKT cell precursors occurs through self-lipid presentation in the context of CD1d expressed on double positive cortical thymocytes. These precursors then progress through three stages, $(HSA)^{low}CD44^{low}NK1.1^{-}$ (stage 1), $CD44^{high}NK1.1^{-}$ (stage 2), and the final maturation to $HSA^{low}CD44^{high}NK1.1^{+}$ (stage 3). Rapid expansion occurs between stages 1 and 2. Stage 3 NKT cells produce primarily IFN-γ, and are exported to the periphery.

Adapted from: Salio et al., Annu. Rev. Immunol. 2014. 32:323‑66

glycolipids responsible for this positive selection, and these will be discussed in the subsequent section.

Following selection, there are three main stages in the development of NKT cells: heat-stable antigen (HSA)^{low}CD44^{low}NK1.1^{-} (stage 1), CD44^{high}NK1.1^{-} (stage 2), and the final maturation to HSA^{low}CD44^{high}NK1.1^{+} (stage 3)[151]. Between stage 1 and stage 2 NKT cells undergo rapid expansion[146]. NKT cells at these different developmental stages exhibit very different cytokine profiles. For example, following activation, stage 1 ((HSA)^{low}CD44^{low}NK1.1^{-}) NKT cells produce large quantities of IL-4, while stage 2 NKT cells (CD44^{high}NK1.1^{-}) are able to produce both IL-4 and IFN-γ. Once developing NKT cells transition into stage 3, they produce primarily IFN-γ[145].

During differentiation, NKT cells upregulate T-bet and other transcription factors, gain expression of various NK receptors, and upregulate CD122 which confers sensitivity to IL-15[152]. The majority of selected NKT cells in mice display the CD4^{+} phenotype (60-80%), while the remainder are DN[153,154]. Some NKT cells can acquire NK1.1 expression in the thymus; however, the majority are exported as NK1.1^{-} cells and gain expression of NK1.1 in the periphery only after interaction with CD1d[155,156]. When NKT cells emerge from the thymus, they have a memory/activated phenotype (CD69^{+} CD44^{+}) and are capable of rapidly releasing both Th1 and Th2 cytokines, depending on the type of glycolipid stimulation, following TCR engagement[157,158].

1.6.2 NKT Cell Tissue Distribution

NKT cell tissue distribution has been well characterized in mice. Of the entire T cell population in the blood and peripheral lymph nodes, NKT cells make up approximately 0.5%[159]. They are present at higher frequencies in the spleen, mesenteric and pancreatic lymph nodes, where they make up approximately 2.5% of T cells[159] However, the highest NKT cell frequencies are seen in the liver, where they make up approximately 30% of T cells[160].

NKT cell distribution is not as well characterized in humans. The frequency of NKT cells within blood of humans varies widely from one individual to another, and appears to be under genetic control[161]. In general, NKT cell frequencies appear to be

approximately 10 fold less frequent in humans than mice[160]. This is thought to be due to differences in the trafficking of CD1d, as mice expressing human CD1d have a reduced frequency of NKT cells[162]. Further differences in NKT cell phenotype, frequency and tissue distribution between mice and humans are summarized in Table 1.

1.6.3 NKT Cell Activation

As described earlier, NKT cells can be activated through glycolipid antigens presented via CD1d.Strong NKT cell immune responses are mediated by $CD1d^+$ DCs compared to other $CD1d^+$ cells. Once activated, NKT cells upregulate CD40L, which can interact with CD40 and stimulate DC release of IL-12[163,164]. IL-12 can then enhance IFN-γ production by NKT cells, allowing for the activation and recruitment of other immune cells such as NK cells or CTLs to sites of infection and inflammation. The exact type and quantity of cytokines produced by activated NKT cells depends on the kinetics of CD1d binding, strength of the antigen signal (affinity and duration), and the type of APC[160]. NKT cell activation can be mediated by exogenous microbial lipid antigens that are processed and presented via CD1d[130], such as phosphatidylinositolmannosides expressed by mycobacteria[165] and α-glucuronosylceramide derived from *Sphingomonas*[130]. Weak self- lipid antigens are upregulated in stressed or TLR-activated cells and can activate NKT cells in combination with cytokine exposure[166]. There have also been reports of NKT cell activation through cytokine stimulation alone[166]. Specifically, studies suggest that IL-12, IL-18, IL-23, IL-25 and type I IFNs may be able to stimulate and activate NKT cells in the absence of TCR engagement [167].

Recent data also show that intestinal bacteria of the genus *Bacteroides* generate α-GalCer analogs[168]. As these bacteria make up > 50% of the human intestinal microbiota[168], it is likely that these glycolipids contribute to NKT cell selection and homeostasis. However, NKT cells still develop in germ free mice, supporting the existence of endogenous ligands[169]. In the last several years, a number of studies have attempted to identify natural endogenous glycolipids which activate NKT cells. The glycosphingolipid isoglobotrihexosyceramide (iGb3) can activate the majority of both mouse and human NKT cells[170]. This lipid was proposed to be an important self-antigen

Table 1. NKT cell phenotype, frequency and tissue distribution in mice and humans. Adapted from Berzins *et al*. *Nat Rev Immunol*, 2011.

	Mouse	**Human**
TCRα	Vα14-Jα18	Vα24-Jα18
TCRβ	Vβ8.2, Vβ7, Vβ2	Vβ11
Thymus dependent	Yes	Probably
CD1d dependent	Yes	Yes
α-GalCer reactive	Yes	Yes
Immature Phenotype	$NK1.1^{low}CD4^{+}$ or DN	$CD161^{low}CD4^{+}$
Mature Phenotype	$NK1.1^{+}CD4^{+}$ or DN	$CD161^{+}CD4^{+}$ DN or $CD8^{+}$
Frequency in Thymus	0.2 – 0.5%	0.001 – 0.1%
Frequency in Blood	0.2 – 0.5%	0.001 – 1.0%
Frequency in Spleen	~1%	ND
Frequency in Liver	20 – 40%	~1-4%

in NKT cell development, as mice which were deficient for the *Hexb* gene (which is responsible for iGb4 conversion to iGb3) displayed significant defects in NKT cell development[150]. However, this conclusion was brought into question when it was discovered that iGb3 synthase deficient mice displayed normal NKT cell development[171]. It has also been questioned whether humans are able to synthesize iGb3[172]. One study screened a panel of naturally occurring glycosphingolipids (GSLs) for antigenic activity on NKT cells[173]. It was found that β-D-glucopyranosylceramide (β-GlcCer) was able to activate NKT cells through a TCR interaction in both mice and humans. This glycolipid accumulated during infection, as well as in response to Toll-like receptor agonists, which further contributed to NKT cell activation[173]. This would strongly suggest that β-GlcCer is a self-antigen for NKT cells.

Synthetic glycolipid compounds such as KRN7000, and its analogs, are extremely potent activators of NKT cells, leading to a robust immune response in many different models of infection and inflammation[174]. These glycolipid compounds are discussed in detail in the following section.

1.7 α-GalactosylCeramide and Analogues

The general designation α-GalCer is given to glycosphingolipids which consist of a galactose carbohydrate attached to a ceramide lipid by an α-linkage[175]. The ceramide lipid can have acyl and sphingosine chains of various lengths[176] (Figure 5). These compounds were originally discovered and derived from the marine sponge *Agelas mauritianus*, and the synthetic form KRN7000 ((2*S*, 3*S*, 4*R*)-1-*O*- (α-D-galactopyranosyl)-*N*-hexacosanoyl-2-amino-1, 3, 4- octadecanetriol) was developed from it. This is usually referred to simply as α-GalCer and is used in experimental studies of NKT cell activation and expansion. During NKT cell activation, the lipid portion of α-GalCer interacts within the hydrophobic antigen binding groove of CD1d. This allows accessibility of the carbohydrate portion for interaction with the NKT cell TCR[176]. In response to α-GalCer, NKT cells quickly produce a variety of cytokines and upregulate expression of several costimulatory molecules[177], induce NKT cell cytotoxicity[178] and as mentioned earlier lead to activation and recruitment of numerous other immune cell

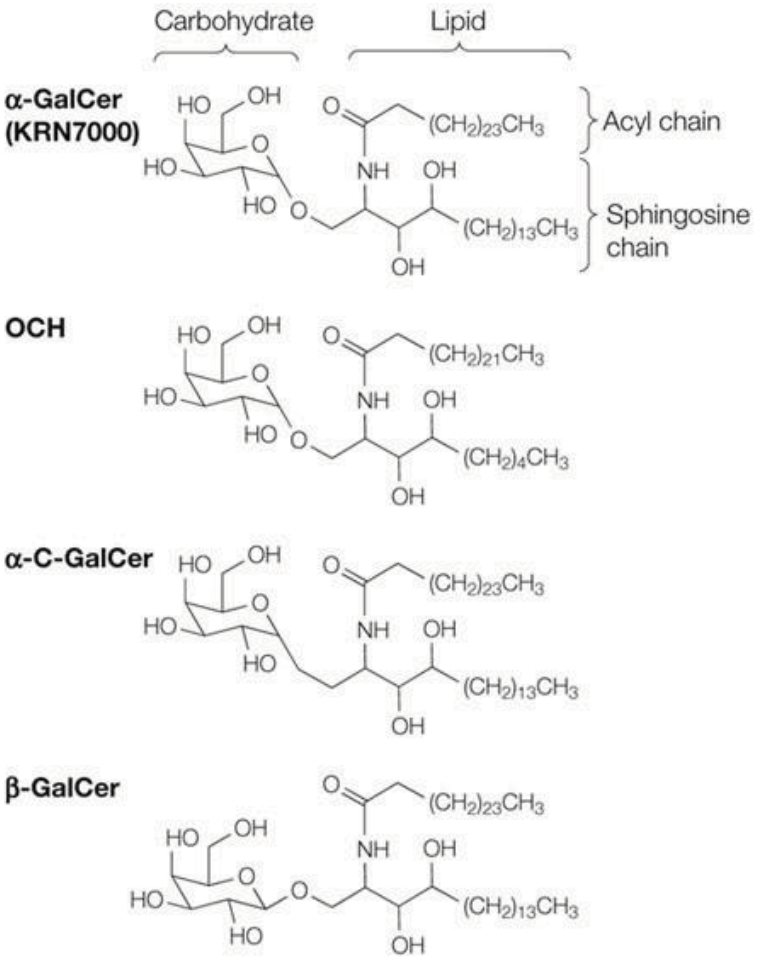


Fig. 5. Glycolipid antigens recognized by NKT cells. α-Galactosylceramide (α-GalCer) is a sphingolipid which contains a galactose carbohydrate attached to a ceramide lipid containing acyl and sphingosine chains of variable lengths through an α-linkage. The lipid portion of α-GalCer interacts with the hydrophobic antigen binding groove of CD1d. OCH is a synthetic analog of α-GalCer with truncated acyl and sphingosine chains. Activation of NKT cells through OCH results in release of large quantities of IL-4. α-C-GalCer is also a synthetic analog of α-GalCer which contains a carbon glycoside and promotes release of IFNγ by NKT cells. β-GalCer differs from α-GalCer in that its carbohydrate portion is attached to the lipid portion by a β-linkage rather than by an α-linkage. At high doses, β-GalCer is able to activate NKT cells in vivo.

Adapted from Nature Reviews Immunology, Kaer V. L., 2005.

subsets[179]. Because of its potent activating abilities, α-GalCer has been studied extensively in its ability to mediate anti-tumour responses[160,180]

An important drawback in the use of α-GalCer in tumour treatment is the resulting hypo-responsiveness of *i*NKT cells following initial activation with free glycolipid[181]. Although initial α-GalCer treatment results in robust expansion and cytokine production, repeated treatments induce NKT cell anergy characterized by defects in proliferation and cytokine production[181,182], and a reduced ability to activate other cells to prevent B16 metastasis[181]. Although anergy has been shown to be mediated by α-GalCer presentation on B cells or other APCs, administration of α-GalCer loaded DCs leaves NKT cells significantly less susceptible to anergy[183,184].

Two other commonly used α-GalCer analogues are OCH and α-C-GalCer. α-C-GalCer is a Th1-biasing antigen that contains a CH_2-based glycosidic linkage in place of the oxygen based glycosidic linkage found in α-GalCer[185]. This altered linkage may change the position of the glycolipid in the CD1d antigen binding domain, thus causing alterations in NKT-TCR interactions and affinity[186]. The Th1 biased response following NKT cell activation with α-C-GalCer is likely due to improved stability of α-C-GalCer leading to enhanced presentation by APCs, more efficient NKT cell activation, and prolonged NK production of IFN-γ[175]. It has also been demonstrated that α-C-GalCer allows for enhanced protection from melanoma metastasis and malaria infection in mice [186,187]. OCH, in contrast, results in a skewing towards production of Th2 type responses, favouring increased production of IL-4 compared to IFN-γ following NKT cell engagement[188]. Studies have demonstrated that shorter side chains in OCH result in unstable binding in the CD1d antigen groove and shorter interactions with the NKT TCR[189]. Glycolipids that induce Th2 skewed cytokine production, such as OCH, can be presented to NKT cells without a need for intracellular loading of CD1d, as they were able to rapidly associate with CD1d at the cell surface[189]. This was in contrast to α-GalCer and α-C-GalCer interactions, which required intracellular loading that resulted in CD1d distribution within lipid rafts[189]. Thus, a mechanism is suggested whereby intracellular loading of Th1 glycolipids leads to the transport of glyclipid-CD1d complexes to areas with lipid rafts, which mediate recruitment into immunological

synapses[189,190]. In contrast, the shorter side chains glycolipids such as OCH can exchange at the cell surface, resulting in shorter signaling and a Th2 skewed cytokine release.

1.8 Chemokines and Chemokine Receptors

Chemokines are a family of approximately 50 low molecular weight proteins (8-10 kDA) which can direct the migration of structural or immune cells during homeostasis or inflammation[191]. They contain an N-terminal unstructured domain which is required for proper signalling, a three stranded β-sheet and a C terminal helix[84]. Although there are 50 identified chemokines thus far, there are only 20 functional chemokine receptors. Therefore, many chemokines have the ability to bind to more than one receptor, and the majority of chemokine receptors have several chemokine ligands[192]. Chemokine receptors are composed of seven transmembrane domains coupled to G-proteins (GPCRs), and they thus signal through the heterotrimeric G proteins to induce activation and migration[193]. When chemokine receptors are activated by ligand, they function as exchange factors for $G\alpha_i$ subunits, resulting in the substitution of GTP for GDP. This leads to the dissociation of $G\alpha$ subunits from $\beta\gamma$ heterodimers[194]. Activation of downstream effectors by $G\alpha_i$ and $\beta\gamma$ subunits then leads to directed cell migration and other effector functions[195], dependent on the cell subset, signaling machinery, and ligand concentration.

Chemokines can be divided into subgroups based on the number and location of cysteine residues in their N' terminus, creating the four major structural subgroups : CXC,CC,C and CX_3C[192]. Chemokines can also be divided into two major groups based on their functionality: homeostatic and inflammatory[191]. Homeostatic chemokines are expressed at a basal level in lymphoid organs and other tissues to mediate homeostatic immune cell trafficking and hematopoiesis[191]. In contrast, inflammatory chemokines are induced following infection or injury, and are thus able to recruit immune cells to damaged sites[196,197]. Thus, chemokines are crucial regulators of leukocyte migration, and different immune cells are recruited to specific sites based on their expression of specific chemokine receptors[198].

It has been demonstrated that chemokines localize to glycosoaminoglycans (GAGs) or other extracellular matrix (ECM) components[199]. This is likely critical in the formation of gradients, but may also facilitate conformation changes. In addition to mediating migration, chemokines play important roles in cellular proliferation, survival, differentiation/polarization (Th1 vs Th2), and effector functions (oxidative burst, degranulation). In the context of injury and cancer, chemokines also have an important role in regulating angiogenesis[200].

1.8.1 Chemokines in Cancer

Chemokines present within tumours can be expressed by cancer cells, stromal cells, or infiltrating leukocytes[197,201]. Some chemokines can have strong anti-tumorigenic effects, while others can contribute to malignancy and cancer spread[202]. Chemokines and their receptors have three major roles in cancer; these are: shaping the tumour microenvironment, providing directional cues for migration and metastasis, and providing growth and survival signals[84].

In conditions where chemokines and their receptors lead to recruitment of lymphocytes such as Th1 cells or NK cells to tumours, tumour growth and spread is often inhibited[84]. For example, it was demonstrated that the chemokines that bind receptors CCR5 and CXCR3 recruit lymphocyte subsets into human liver tumours[203]. The expression of these receptors on $CD4^+$ and $CD8^+$ TILs was also associated with significantly higher expression of the activation marker CD69[198]. In contrast, malignant cells can often have increased or abnormal expression of certain chemokine receptors such as CXCR4, CCR7, and CCR10[204,205], which have pro-tumourigenic effects[201]. For example, CXCR4 on tumour cells protected them from apoptosis in culture and increased survival under suboptimal conditions[206]. Chemokines are able to mediate tumour cell invasion and migration, resulting in site directed metastasis[207].The receptor CXCR4 is expressed very highly in breast cancer cells compared to normal breast epithelial tissue cells. When antibodies against CXCR4 are used in mouse models of breast cancer, metastasis is reduced. In addition, the expression of certain chemokines in tumour environments can also result in the recruitment of immunosuppressive cells such as

Tregs, MDSCs or TAMs[208–210], leading to dampened immune responses and enhanced tumour development.

1.8.2 CXCR3 and CXCL9, CXCL10, CXCL11

CXCR3 is a CXC chemokine receptor expressed on activated T-lymphocytes[211]. It can recognize and respond to three chemokine ligands, CXCL9, CXCL10 and CXCL11 to induce calcium flux and directed chemotaxis[212] . The expression of CXCR3 on activated T lymphocytes is required for efficient responses to parasitic, viral, bacterial and fungal infections[213]. Because CXCR3 is highly expressed on NK cells, NKT cells, CTLs, and Th1 cells, it is likely important for their recruitment to tumours or other inflamed or infected tissues[214,215]. All three ligands have been demonstrated to have angiostatic properties through inhibition of endothelial cell migration, VEGF and basic fibroblast growth factor (bFGF) [216]. Mice deficient for the CXCR3 ligand CXCL10 have impaired recruitment of Th1 cells into inflamed tissue[217].

Importantly, CXCR3 and its ligands have been demonstrated to induce strong anti-tumour activities. For example, in patients with stage III melanoma, the expression of CXCR3 on T cells is correlated with improved survival[218]. In patients with high risk cutaneous melanoma, IFN-α can be used to induce CXCL9 and CXCL10[219]. As well, the expression of CXCL10 by peripheral blood mononuclear cells (PBMCs) in cancer patients is correlated with improved tumour control[220]. Interestingly, it has been shown that the expression of CXCR3 on cutaneous melanoma cells is correlated with decreased infiltration of lymphocytes[221]. Although the mechanism for this observation is not known, it may suggest that CXCR3 expressing cancer cells can compete for chemokine ligands attracting T lymphocytes, thus escaping the immune response.

A recent study demonstrated that the presence of CXCL10 within tumours results in significantly reduced tumour size and increased infiltration of $NK1.1^+$ cells[222]. In $CD4^+$ cells, high expression levels of CXCR3 is correlated with a memory ($CD45RO^+$) phenotype in all tissues[198]. In contrast, within the $CD8^+$ T cell population this correlation was observed only in TILs, not on lymphocytes in other sites, suggesting that CXCR3 directs memory $CD8^+$ T cells specifically to tumours[223].

1.8.3 CXCR6 and CXCL16

The chemokine receptor CXCR6 is expressed by NKT cells, $CD8^+$ T cells, and subsets of $CD4^+$ T cells and NK cells[224,225]. The chemokine ligand for CXCR6 is CXCL16, which is generated in both soluble and transmembrane forms[224,226]. The transmembrane form is expressed on macrophages, dendritic cells, monocytes and B cells [224,226] and functions as a costimulatory molecule that enhances IFN-γ production from NKT cells[227]. The soluble form of CXCL16 is able to direct the trafficking of activated $CXCR6^+$ lymphocytes[224,226]. In CXCR6 deficient mice, the number of intrahepatic NKT cells is significantly reduced in both normal conditions and in models of chronic liver injury[128,182,228]. Cytokine production is impaired in NKT cells from $CXCR6^{-/-}$ and $CXCL16^{-/-}$ mice consistent with a role for CXCR6/CXCL16 interactions in co-stimulation[182,227]. Studies have also demonstrated that $CXCR6^+$ NKT cells mediate fibrosis in models of toxic and metabolic liver injury[128].

Certain studies have suggested an anti-tumorigenic role of CXCR6-CXCL16 in cancer. For example, metastasis of transplanted tumours is increased in $CXCR6^{-/-}$ mice[136], implicating a role for CXCR6 in immunosurveillance. In contrast, several studies have also provided evidence for a significant pro-tumorigenic role of CXCR6-CXCL16. It has been demonstrated that CXCR6 and CXCL16 are over-expressed in several human cancers[229,230], and that the CXCR6-CXCL16 interaction has roles in the stimulation of growth, invasion and survival of cancer cells[231]. In lung cancer cell lines, the addition of exogenous CXCL16 increased in vitro invasiveness, which could be effectively reversed though the use of CXCL16 neutralizing antibody or down-regulation of CXCR6[232]. The same study also showed that CXCL16 is spontaneously secreted by human adenocarcinoma cells, and that blockade of the CXCR6-CXCL16 interaction results in decreased viability and invasiveness of the cells in vitro[232]. As well, in human prostate cancers, CXCL16/CXCR6 expression is significantly higher than that in non-cancerous prostate disease[233]. More aggressive forms of prostate cancer express higher amounts of CXCR6[233]. Furthermore, it has been shown that CXCR6 expressing T cells can stimulate CXCL16 production by tumour cells, which can in turn enhance growth of tumour cells[234]. Together, these data indicate that the CXCR6-CXCL16 signalling axis have multiple differing roles in tumour control and development.

1.8.4 CCR5 and CCL3, CCL4, CCL5

The chemokine receptor CCR5 is expressed on Th1 cells, $CD8^+$ T cells, monocytes and macrophages, but can also be expressed by tumour cells and endothelial cells[235]. CCR5 has three chemokine ligands to which it can bind, CCL5, CCL4 and CCL3 [191]. These chemokines are primarily involved in the recruitment of leukocytes to sites of injury and infection[193]; however, studies have also identified roles for CCR5 in tumour cell migration and survival in cancer[191,236]. For example , after malignant transformation, CCL5 is produced by breast cancer cells[237]. High levels of CCL5 within tumours is correlated with enhanced disease stage[238]. As well, both CCR5 and CCL5 have enhanced expression in metastatic lymph nodes compared to primary tumour sites[239], suggesting that CCR5-CCL5 signalling may play a role in promoting cancer cell migration. Both primary tumour growth and metastasis formation can be enhanced through high expression of CCR5 ligands CCL3 and CCL5[240,241]. As well, CCL5 has been shown to act directly on cancer cells, resulting in enhanced proliferation.

Furthermore, CCL5 has been shown to inhibit certain anti-tumorigenic activities of $CD8^+$ T cells, and can cause apoptosis of these cells[237]. In line with this, it was demonstrated that NKT cells from Con-A treated CCR5-deficient mice were resistant to apoptosis[242]. This was evidenced by reduced expression of annexin V, which is used to detect cells undergoing early stages of apoptosis. This would suggest that CCR5 expression on NKT cells has a regulatory role in their survival, activation and immune activity.

CCL5 has also been demonstrated to induce MDSC generation[243]. The tumour infiltrating MDSCs can then in turn produce more CCR5 ligands and attract CCR5 expressing Tregs[244]. T regulatory cells have been shown to express CCR5, and can thus migrate to primary tumours and inhibit anti-tumour responses[245]. In mice deficient for CCR5, tumour growth was delayed and the frequency of Tregs was reduced[244]. Thus, although there has been evidence for possible anti-tumorigenic effects of CCR5 and its chemokine ligands (migration of CCR5 expressing lymphocytes to tumours), much of the emerging data suggest a primarily pro-tumorigenic function.

1.9 Objective

The infiltration of NKT cells into tumours has been correlated with improved prognosis in cancer patients. However, the mechanisms involved in NKT cell tumour infiltration are not well understood. Therefore, the first objective of this study was to identify which chemokines and chemokine receptors mediate NKT cell migration to tumours. The chemokine receptors CXCR3 and CXCR6 are highly expressed on NKT cells, and it was hypothesized that they could contribute to NKT cell tumour homing. This was tested using $CXCR3^{-/-}$ and $CXCR6^{-/-}$ mice, and confirmed by competitive adoptive transfer assays in tumour bearing mice. The second objective was to determine whether treatment of tumours with CXCR3 or CXCR6 ligands would increase NKT cell tumour infiltration, and impact tumour growth. We also sought to investigate whether activation of NKT cells through the administration of α-GalCer would enhance the migration and anti-tumour function of NKT cells alone and in combination with chemokine treatments. It was hypothesized that activation of NKT cells through exogenous α-GalCer would further enhance the anti-tumour functions of NKT cells and improve tumour control. The third objective was to investigate whether these treatments altered recruitment of regulatory immune cells, such as Tregs and TAMs. It was hypothesized that activation and recruitment of NKT cells would enhance the anti-tumour immunity and reduce immunosuppressive cells.

The last objective was to determine the role of CCR5 in NKT cell activation, immune function and migration. Several studies have suggested that CCR5 may have an immune-regulatory role, thus we wanted to determine whether it affects NKT cell activation, cytokine production and migration to tumour sites. It was hypothesized that CCR5 plays a role in the migration of NKT cells to tumours but may also regulate NKT cell activation and cytokine production.

CHAPTER 2. MATERIALS AND METHODS

2.1 Media and Solutions

Phosphate buffered saline (PBS) 10X was purchased from Invitrogen-Life Technologies (Burlington, ON) and diluted to 1X using Millipore filtered distilled water. Hanks Balanced Salt Solution (HBSS) without calcium or magnesium, RPMI 1640 medium containing L-glutamine, fetal bovine serum (FBS) and high glucose Dulbecco's Modified Enriched Medium (DMEM) containing L-glutamine and pyridoxine hydrochloride, 10 000U/ml Penicillin-10,000µg/ml Streptomycin (P/S), and 0.25% Trypsin-EDTA were all purchased from Invitrogen-Life Technologies. Saline solution (0.9% sodium chloride) was purchased from B. Braun Medical Inc. (Irvine, CA). DC medium was prepared using 500µL of a 10mM stock of β2-Mercaptoethanol (Sigma Aldrich, Oakville, ON), 1mL of 100x MEM non-essential amino acids (Invitrogen-Life Technologies) and 1mL of 100x sodium pyruvate (Invitrogen-Life Technologies) in 98mL of RPMI-1640 containing 10% FBS and 1% P/S. All media and solutions were stored at 4°C with the exception of FBS, P/S and Trypsin-EDTA (-20°C), and methylene blue (20°C).

2.2 Glycolipid Compounds

α-GalCer ((2S,3S,4R)-1-O-(α-D-galactipyranosyl)-2-(N-hexacosanoylamino)-1,3,4-octadecanetriol) was purchased from Toronto Research Chemicals (Toronto, ON). It was reconstituted to a concentration of 1 mg/ml in saline solution containing 0.5% Tween-20 (Sigma Aldrich, St Louis, MO) and stored at 4ºC once dissolved. Prior to use, α-GalCer was sonicated at 50°C for 15 minutes.

2.3 Mice

$CCR5^{-/-}$, CD45.1 and $CXCR3^{-/-}$ mice were purchased from Jackson Laboratories (Bar Harbor, MA). $CXCR6^{-/-}$ mice were provided by Dr. Dan Littman (New York University Medical Centre, New York, NY) and backcrossed on the C57BL/6 background for >10 generations[246]. The CXCR6 gene in these mice is replaced by an enhanced green fluorescent protein (eGFP). $J\alpha18^{-/-}$ mice on the C57BL/6 background were provided by Dr. Masaru Taniguchi (RIKEN Research Center for Allergy and Immunology, Kanagawa, Japan). These mice have a deletion in the TCR Jα18 segment,

which is critical for invariant type I NKT cell development[247]. C57BL/6 and BALB/c wild type mice were acquired form Charles River (Lasalle, QC) or Jackson Laboratories (Bar Harbour, ME). FoxP3-GFP mice were provided by Dr. Jean Marshall (Dalhousie University). Mice were housed in the Carleton Animal Care facility (Dalhousie University) and experimental procedures were approved by the University Committee on Animal Care. All experiments were performed using female mice 8-12 weeks of age.

2.4 Cell Lines and Culture

B16-F10 melanoma cells and Lewis Lung Carcinoma (LLC) cell lines were purchased from the American Tissue Culture Collection (Manassas, VA). 4T1 tumour cells were obtained from Dr. Jean Marshall (Dalhousie University). The cells were cultured in 20mL of DMEM supplemented with 1% penicillin/streptomycin and 10% FBS. They were incubated in 75cm^2 vented tissue flasks at 36°C in 6% CO_2. Cells were passaged every 3 days at a ratio of 1:10. All cells collected for tumor experiments were passaged 24 hours before use in order to ensure they were in the logarithmic stage of growth. Once confluence was reached, tumour cells were collected by washing with complete DMEM, and releasing cells by incubation with 4 mL of 0.25% trypsin-EDTA at 37°C (6.5% CO_2) for 5 minutes. The trypsin was neutralized with 5 mL complete DMEM and cells were collected into a 50 mL conical tube. Cells were then centrifuged for 7 minutes at 300 g, aspirated, and resuspended in 5 mL of HBSS. The cells were then passed through a 40μm filter and viability was examined by trypan blue staining. All experiments were performed with cells that exhibited >90% viability.

To test the effect of CXCL16 on proliferation of B16 and LLC cells in vitro, B16 F10 melanoma and LLC tumour cells were cultured in 5.0 mL DMEM supplemented with 1% PS and 10% FBS. Half of the cultures were also supplemented with 5.0 μg CXCL16. Cells were collected and counted at 24 hours, 48 hours, 72 hours and 5 days. Following each count, cells were re-plated with or without added CXCL16.

2.5 Flow Cytometry

An allophycocyanin (APC) labelled CD1d tetramer loaded with the α-GalCer analog PBS57 was obtained from the National Institute of Allergy and Infectious Disease

(NIAID) Tetramer facility, Emory University Vaccine Centre (Atlanta, GA). CXCR6 expression was detected using a CXCL16-Fc chimera fusion protein[224]. The Fc portion of the CXCL16-Fc fusion protein was detected using a phycoerythrin (PE)-conjugated goat anti-human Fcγ polyclonal antibody, purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). All other antibodies used in this body work are listed in Table 2. Intracellular cytokine staining was performed using the Cytofix/Cytoperm Plus kit (eBioscience, San Diego CA).

Samples of $2x10^6$ cells were centrifuged at 300g for 10 minutes. Pellets were resuspended in 100 µl 1XPBS. Each sample was incubated for 10 minutes at 4°C with anti-CD16/32. Prior to use, each antibody was titred to an optimal concentration. Following incubation, cells were stained with antibody cocktails and incubated at 4º C for 30 minutes. All cells were then centrifuged at 300 x g for 10 minutes and fixed in 200 µL Paraformaldahyde (PFA) (Fisher Scientific, Mississauga, ON). Cells were stored at 4°C until FACS analysis.

2.6 Chemokine Receptor Expression

C57BL/6 mice between 8-12 weeks old were treated with intraperitoneal injections of saline or 4 µg α-GalCer in saline and sacrificed 4 hours or 72 hours later by cervical dislocation under anesthesia. Tissues were harvested and cells from the spleen, liver and bone marrow were labelled with α-GalCer loaded CD1d tetramer, TCRβ and antibodies for the various chemokine receptors (see Table 2). CXCR6 expression was determined via a CXCL16-Fc chimera. CXCL16- Fc (100 µL) was added directly to 2 x 10^6 cells following incubation with CD16/32. Cells were incubated at 4°C for 30 min, washed with 1XPBS and centrifuged at 300 x g for 7 min. Cells were then resuspended in 100 µL 1XPBS and stained with the secondary detection antibody goat anti-human Fcγ at 4°C for 20 minutes. Cells were again washed in 1XPBS, centrifuged at 300 x g for 10 minutes, fixed in 200 µL PBS and stored at 4°C until flow cytometry analysis.

2.7 Experimental Tumourigenesis

B16-F10, LLC and 4T1 cells were harvested from tissue culture flasks and their viability was assessed as described above. On day 0, mice of matching age and sex were

Table 2. Antibodies Used. All antibodies used in this body of work are listed alphabetically. The flourochrome, clone number and source of each antibody is as listed. All antibodies used were first titred to determine the optimal staining concentration.

	Flourochrome	Clone	Source
CCR5	PE	7A4	eBioscience (San Diego, CA)
CD11b	APC	M1/70	eBioscience (San Diego, CA)
CD11b	PE	M1/70	eBioscience (San Diego, CA)
CD16/32	N/A	93	eBioscience (San Diego, CA)
CD25	APC	PC61	eBioscience (San Diego, CA)
CD45.2	PerCP	104	BD Biosciences (Mississauga, ON)
CXCR3	PE	220803	BD Biosciences (Mississauga, ON)
IFN-γ	PE	XMG1.2	eBioscience (San Diego, CA)
IL-4	PE	11B11	Biolegend (San Diego, CA)
Ly6C	FitC	AL-21	BD Biosciences (Mississauga, ON)
Ly6G	PE	1A8	BD Biosciences (Mississauga, ON)
rigG1	PE	eBRG1	eBioscience (San Diego, CA)
TCRβ	FitC	H57-597	eBioscience (San Diego, CA)
TCRβ	PE	H57-597	eBioscience (San Diego, CA)
TCRβ	PerCP	H57-597	eBioscience (San Diego, CA)

anesthetized via inhaled isoflurane (purchased from PPC, Richmond Hill, ON) and injected subcutaneously in the hind flank with 2.5 x 10^5 B16 or LLC cells, or 1 x 10^5 4T1 cells in 100 µl 1XPBS. Intratumoural treatments (see below)were given on days 11 and/or 14, and all tumours were harvested by day 17.

2.8 Intratumoural Treatments

On day 11 following tumour cell inoculation, wild type C57Bl/6 mice containing s.c B16 melanoma or LLC tumours, or Balb/c mice containing 4T1 tumours, were anesthetized through isofluorune inhalation. Mice received intratumoural injections (40 µL) of 4µg α-GalCer in 1X PBS, 2.0µg of CXCL10, or 2.0µg CXCL16 in 1X PBS, or a combination treatment of α-GalCer with CXCL10 or CXCL16 . Treatments with CXCL10 or CXCL16 were repeated on day 14. All tumours were harvested on Day 17. Once tumours became palpable, tumour area was measured every 1-2 days using calipers.

2.9 Cell Isolations

Mice were anesthetized via inhaled isoflurane and sacrificed by cervical dislocation. Livers, spleens, tumours, and lymph nodes were harvested from each mouse and single cell suspensions were generated by dispersing the tissue through a wire mesh using 1XPBS supplemented with 2% FBS. 4T1 tumours were sliced into small pieces and placed into 0. 15 mg/mL Liberase TL (Roche Diagnostics, Laval, QC) in 1 mL RPMI at 37°C for 45 minutes to aid digestion. Cells were centrifuged at 500 x g for 7 minutes, washed with 1XPBS containing 2% FBS solution, and centrifuged again under the same conditions. Liver and tumour lymphocytes were isolated by centrifugation at 700g for 16 minutes through 20 mL of a 33% Percoll gradient (GE Healthcare, Baie d'Urfe, QC). Red blood cells within the pellet were then lysed by immersion in ammonium chloride for 5 minutes, and then neutralized with 1XPBS 2% FBS solution. The resulting suspension was centrifuged at 300g for 10 minutes. The pellet was resuspended in 1XPBS containing 2% FBS. Spleen and lung lymphocytes were isolated in the same manner without the Percoll enrichment step. Leukocyte samples were stained with PE-TCRβ, PerCP-CD45.2 and APC-CD1d tetramer to identify NKT cell populations; PerCP CD45.2, APC CD11b, FITC Ly6C and PE Ly6G to identify TAMs; and PE CD4, PerCP TCRβ and APC CD25

to identify Treg cells in FoxP3-GFP mice. All cell populations were examined by flow cytometry.

2.10 NKT Cell Expansion with α-GalCer-Loaded DCs

Wild type C57BL/6 mice were anesthetized with inhaled isoflurane and sacrificed via cervical dislocation. Under sterile conditions, femurs and tibias from mice were removed and stripped of muscle and skin tissue. A syringe containing DC medium and a 30 gauge needle was used to flush bone marrow onto a 40 μm nylon cell strainer. The marrow was washed with DC media and pushed through the strainer using a syringe plunger. The cell suspension was then centrifuged at 300 x g for 10 minutes. The supernatant was aspirated and an erythrocyte lysis was performed using 3 mL ammonium chloride for 30 seconds. The cells were washed and resuspended in 15 mL DC medium supplemented with 15 μL GM-CSF (0.1μg/ml) and 15 μL IL-4. The resulting solution was distributed evenly into 3 wells of a 6 well culture plate and incubated at 37°C with 5% CO_2 for 3 days. On day 3, 5 μL GM-CSF and 5 μL IL-4 were added to each of the three wells and the plate was again incubated at 37°C in 5% CO_2 for 3 days. On day 6, non-adherent cells were removed from each well and placed in a 50 mL conical tube. The cell suspension was centrifuged at 300 x g for 10 minutes and aspirated. Cells were then resuspended in 15 mL DC medium supplemented with 2.5 μL GM-CSF and 2.5 μL IL-4 and 5 mL was placed in each of 3 wells in a 6 well culture plate and incubated again under the same conditions. On day 7, 10 μL of sonicated α-GalCer was added to each of the three wells. Twenty-four hours later, both non-adherent and adherent cells were collected from each well and combined. The cell suspension was centrifuged at 300 x g for 10 minutes. Cells were then aspirated and resuspended in saline solution for counting with a haemocytometer. Cells were resuspended at a concentration of 6 x 10^6 cells/mL and 100 μL was injected into the tail vein of recipient mice to allow for in-vivo expansion of NKT cells to be used for adoptive transfers.

2.11 Co-Adoptive Transfers

$CXCR3^{-/-}$, $CXCR6^{-/-}$, $CCR5^{-/-}$ and wild type CD45.1 congenic mice received intravenous tail vein injections of 6 x 10^5 α-GalCer-loaded DCs in 100 μL saline to expand NKT cells. Liver lymphocytes were isolated from each mouse 72 hours later, as

described above. Cells were pooled from 6-8 mice per group and resuspended in 1-2mL 1XPBS. Anti-CD16/32 was added to each staining tube and cells were incubated at 4°C for 10 minutes. All cells were stained with FITC TCRβ and APC CD1d tetramer and incubated at 4°C for 20 minutes. Cells were then washed with 1XPBS and centrifuged at 500 x g for 7 minutes and resuspended in 2 mL 1X PBS. Cells were then sorted according to expression of both TCRβ and APC CD1d tetramer using the FACSAria cell sorter (BD Biosciences). Sorted NKT cells were counted using a haemocytometer. An equal number of CD45.1 congenic NKT cells and chemokine receptor-deficient NKT cells ($CXCR3^{-/-}$, $CXCR6^{-/-}$, or $CCR5^{-/-}$) were combined in saline (~5 x 10^6 cells of each in 100 μL). Cells were then transferred intravenously into $Ja18^{-/-}$ mice bearing sc. B16 melanoma tumours on day 11 following tumour cell injection. All mice were humanely sacrificed 3 days after adoptive transfer. Tumours, livers, spleens and tumour draining lymph nodes were harvested, processed, and stained as described above.

2.12 Intracellular Cytokine Staining

WT and $CCR5^{-/-}$ mice were injected intraperitoneally with 4 μg α-GalCer to examine NKT cell responsiveness in these mice. At 2 hour, 24 hours and 3 days post injection, mice were sacrificed and cells from various tissues were processed and labelled with CD1d tetramer and TCRβ to identify NKT cells as described above. Following surface staining, all cells were fixed and permeabilized for intracellular cytokine staining. PE-labelled anti IFN-γ, IL-4, and $rIgG_{2a}$ were each diluted 50 fold in permeabilization buffer and 50 μL of this diluted antibody mixture was added to the appropriate tubes for staining. The cells were then incubated in the dark at 20°C for 20 minutes. Following incubation, 1 mL of permeabilization buffer was added to each staining tube and the cells were centrifuged for 5 minutes at 300 x g. The supernatant was aspirated and the cells were resuspended in 200 μL 1xPBS. Cells were stored at 4°C in the dark until flow cytometry analysis.

2.13 Serum Cytokine Analysis

Blood samples were collected by submandibular venipuncture 2 hr, 24 hr, and 72 hrs post α-GalCer treatment (ip. 4 μg). All samples were collected into 1.5 mL micro

centrifuge tubes containing 10 μL of 10 000U/ml heparin (Sigma Aldrich). Samples were then centrifuged at 5000 x g for 5 minutes. The serum was collected from each tube and stored at -20°C. Cytokine levels of IFN-γ and IL-4 were determined using IFN-γ and IL-4 ELISA Ready-SET-Go kits (eBioscience, San Diego, CA) according to manufacturer instructions. ELISA plates were read using a BioTeck Epoch microplate Spectrophotometer with BioTeck Gen 5 data analysis software (Winooski, VT) and analyzed using SoftMax Pro software from Molecular Devices (Sunnyvale, CA).

2.14 Chemotaxis Assay

Approximately $1x10^6$ liver lymphocytes were placed in the upper chamber of transwell inserts (5 μm pore size). Inserts were then placed in wells containing 600 μL RPMI supplemented with 10% FBS alone (basal) or 10% RPMI with 5 nM CCL4 (purchased from R&D Systems, Minneapolis, MN). After 2 hours of migration at 37ºC, inserts were removed and polystyrene beads (Polysciences, Warrington, PA) were added to each well as an internal standard. Three wells were pooled for each condition. Migrated NKT cell populations were stained with PE-TCRβ, APC-CD1d tetramer and PerCP-NK1.1 and quantified by flow cytometry. Chemotaxis was calculated as the percent of input cells migrated into the lower chamber, using the polystyrene beads as an internal control.

2.15 Statistical Analysis

All data are expressed as mean ± standard error of mean (SEM) of pooled data sets. All statistical analysis was performed using GraphPad Prism 5 software (Graphpad Software Inc.; La Jolla, CA). Statistical analysis for multiple comparisons was performed by non-parametric one-way analysis of variance (ANOVA) Kruskal Wallis test and Dunn's post-test. Comparisons between two groups were made using a non-parametric two tailed Mann Whitney test. Statistical significance was set at p values less than 0.05. Significant differences are indicated in each figure.

CHAPTER 3. RESULTS

3.1 Mice deficient for CXCR3, CXCR6 and CCR5 exhibit decreased NKT cell tumour infiltration

NKT cell infiltration into tumours has been correlated with improved prognosis in patients with multiple cancer types[79,248]. Therefore, it is important to elucidate the factors which contribute to NKT cell homing to tumours, such as chemokines and chemokine receptors. We examined the expression of chemokine receptors on resting NKT cells and NKT cells from mice activated with 4.0 μg α-GalCer. The chemokine receptors CXCR3 and CXCR6 were very highly expressed on both resting and activated liver NKT cells (~80-100%) (Fig.6). These receptors have previously been shown to play important roles in the recruitment of immune cells to sites of inflammation and infection[214,215]. CCR5 displayed moderate expression on resting NKT cells (~30%), and increased to ~90% by 3 days after stimulation with α-GalCer (Fig. 6). Therefore, we examined the roles of these receptors in their ability to mediate NKT cell migration to tumours.

Chemokine receptor deficient mice were used to determine the role for each chemokine receptor in NKT homing to tumours. Mice deficient in CXCR3, CXCR6 or CCR5 were injected subcutaneously in their hind right flank with 2.5 x 10^5 B16 F10 melanoma cells. Tumours were allowed to develop for 14 days, at which point all tumours were removed. TILs were enriched using a 33% percoll gradient and stained with CD45.2, TCRβ and CD1d tetramer to identify the NKT cell population. Flow cytometry analysis revealed that the NKT cell population infiltrating the tumour was significantly reduced in CXCR3, CXCR6 and CCR5 deficient mice compared to WT mice (Fig.7). This suggests that these chemokine receptors play a role in mediating migration of NKT cells into tumour sites.

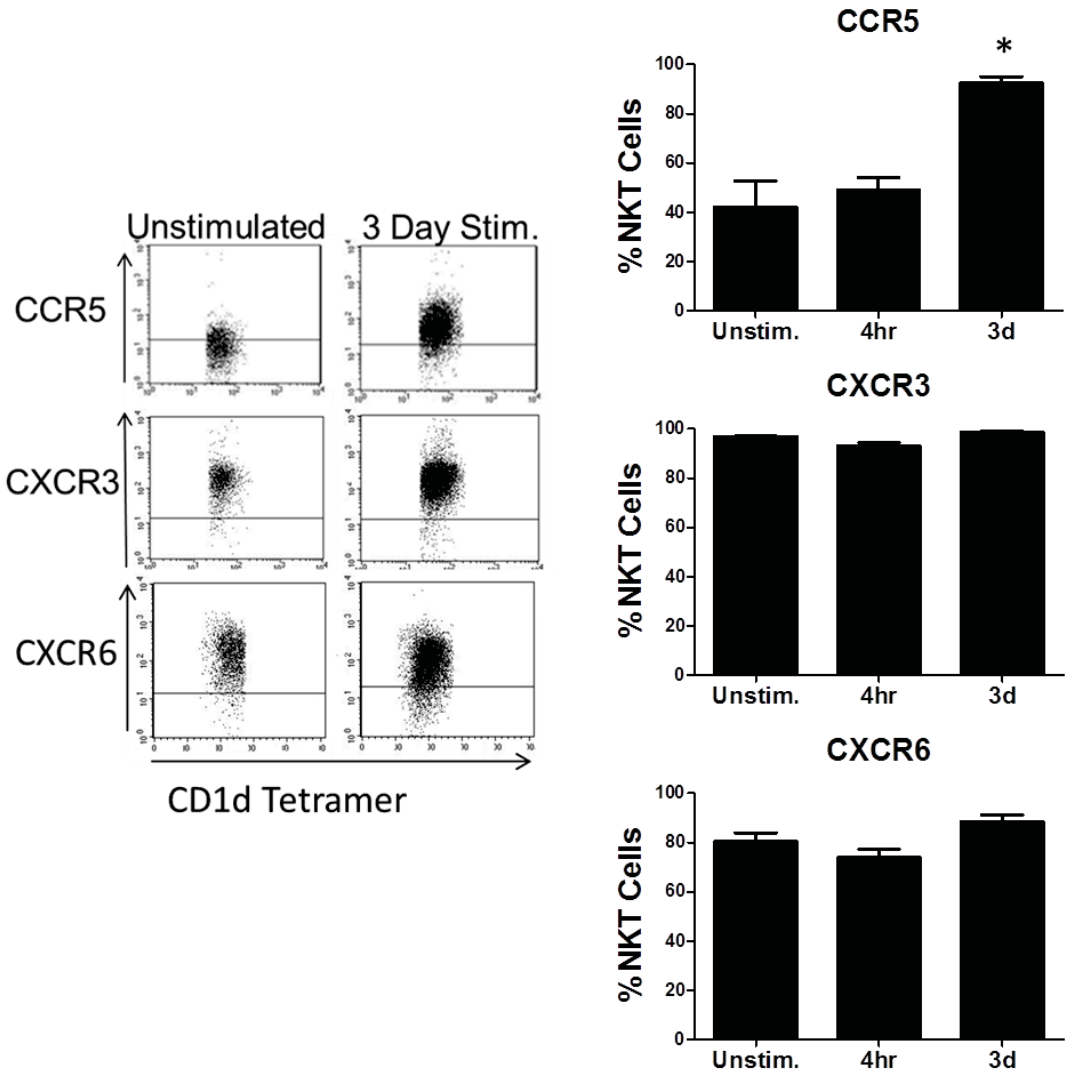


Figure 6. Expression of CXCR3, CXCR6 and CCR5 on resting and α-GalCer activated NKT cells. Lymphocytes isolated form the liver of untreated and α-GalCer treated (ip. 4 μg) mice were stained with FitC TCRβ, APC α-GalCer loaded CD1d tetramer and with PE antibodies for CXCR3, CXCR6 or CCR5. The frequency of NKT cells within each tissue expressing each chemokine receptor was determined by flow cytometry. FACS plots are gated on the $TCR\beta^{+}CD1d\text{-}tet^{+}$ population. *$P < 0.05$ compared to unstimulated, n=3 per group.

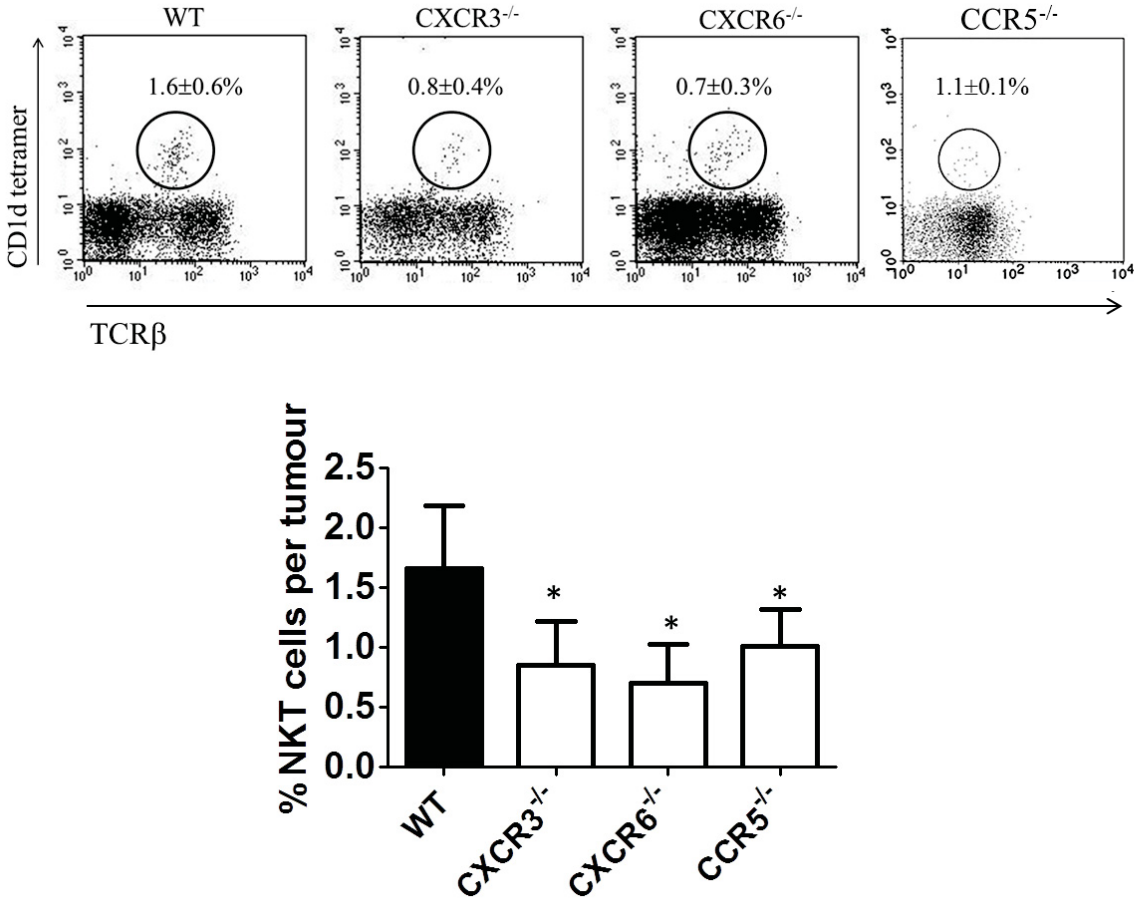


Figure 7. CCR5, CXCR3 and CXCR6 deficient mice have reduced NKT cell tumour infiltration. Subcutaneous B16 melanoma tumours were harvested 14 days post injection from WT, CXCR3$^{-/-}$, and CXCR6 $^{-/-}$ mice and cells were labelled with PerCP CD45.2 , APC CD1d tetramer and PE TCRβ. Data is gated on the CD45.2^{+} population. CCR5$^{-/-}$, CXCR3$^{-/-}$ and CXCR6$^{-/-}$ tumours had a lower frequency of NKT cell infiltration (0.1±0.1%, 0.8±.04% and 0.7±.3%, respectively) in comparison to wild type mice (1.6±.6%). tumours. *P< 0.05 compared to wild type. n = 12 WT, 9 CXCR3$^{-/-}$, 8 CXCR6$^{-/-}$, 8 CCR5$^{-/-}$.

3.2 NKT cells homing into tumours following co-adoptive transfers

The reduced frequency of tumour infiltrating NKT cells within tumours of CCR5, CXCR3 and CXCR6 deficient mice strongly suggests that these receptors play roles in the recruitment of NKT cells to tumour sites. However, NKT cell recruitment in knockout mice could be influenced by the absence of these chemokine receptors on other cell populations. In order to conclude that reduced NKT cell frequency within tumours was due to the specific lack of chemokine receptors on NKT cells, co-adoptive transfer experiments were conducted using Jα18$^{-/-}$ (NKT cell deficient) mice bearing B16 tumours. Liver lymphocytes were collected from C57Bl/6 WT and chemokine receptor deficient mice for transfer. WT mice with the CD45.1 congenic marker were used to differentiate them from chemokine receptor deficient NKT cells (CD45.2). In order to obtain sufficient NKT cells for adoptive transfer, all donor mice were treated with α-GalCer loaded DCs 72 hours prior to adoptive transfer to expand the NKT cell populations in vivo (Figure 8). Liver NKT cells were sorted by FACS, and WT and receptor-deficient NKT cells were combined in equal numbers for injection into recipient mice (total cell number ranged from 6 - 10 x 10^6 NKT cells). Seventy-two hours later, tumours, tumour-DLNs, spleens and livers were harvested and stained with PerCp CD45.1, APC CD1d tetramer and FitC TCRβ to identify WT and chemokine receptor-deficient NKT cells.

3.2.1 Reduced frequency of CXCR3$^{-/-}$ NKT cells in tumours following co-adoptive transfers

As CXCR3 is expressed on endothelium and plays a role in inhibiting angiogenesis[249], this could impact tumour growth in CXCR3$^{-/-}$ mice. Adoptive transfer of NKT cells circumvents this potential issue. Following the adoptive transfers described above, the frequency of WT NKT cells within tumours was significantly higher than that of CXCR3$^{-/-}$ NKT cells, suggesting that CXCR3 plays a role in directing NKT cells to the tumour (Figure 9). In order to confirm that this was not a result of reduced survival or other deficiencies of receptor knock-out cells, the frequency of each donor cell population within the liver, spleen and tumour draining lymph nodes was examined. The frequency

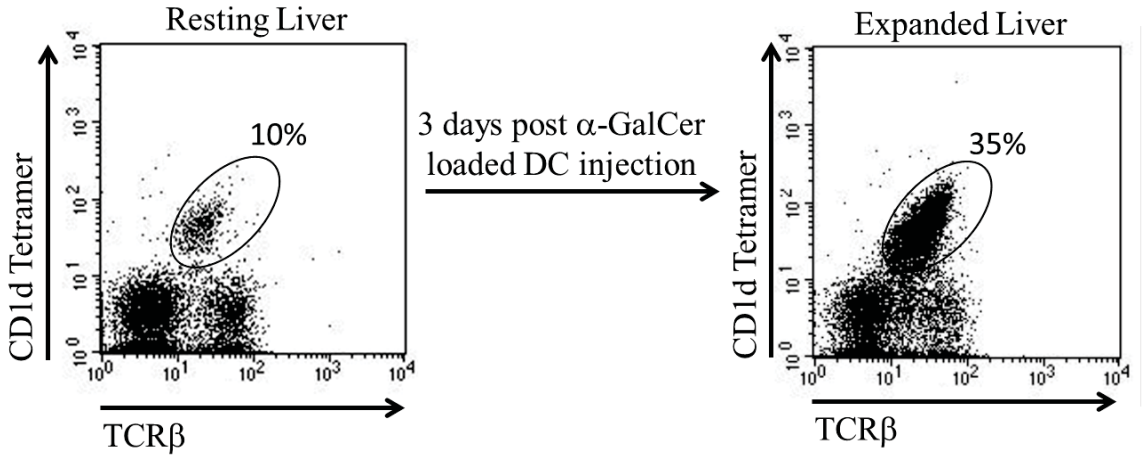


Figure 8. NKT cell expansion through α-GalCer loaded DCs. All donor mice used in adoptive transfer experiments first received i.v injections of 6 x 10^5 α-GalCer loaded DCs 72 hours prior to harvest. This allowed for significant expansion of the NKT cell population, as illustrated by the representative flow cytometry plots. Liver lymphocytes were labelled with FitC TCRβ and APC CD1d tetramer to identify NKT cell populations.

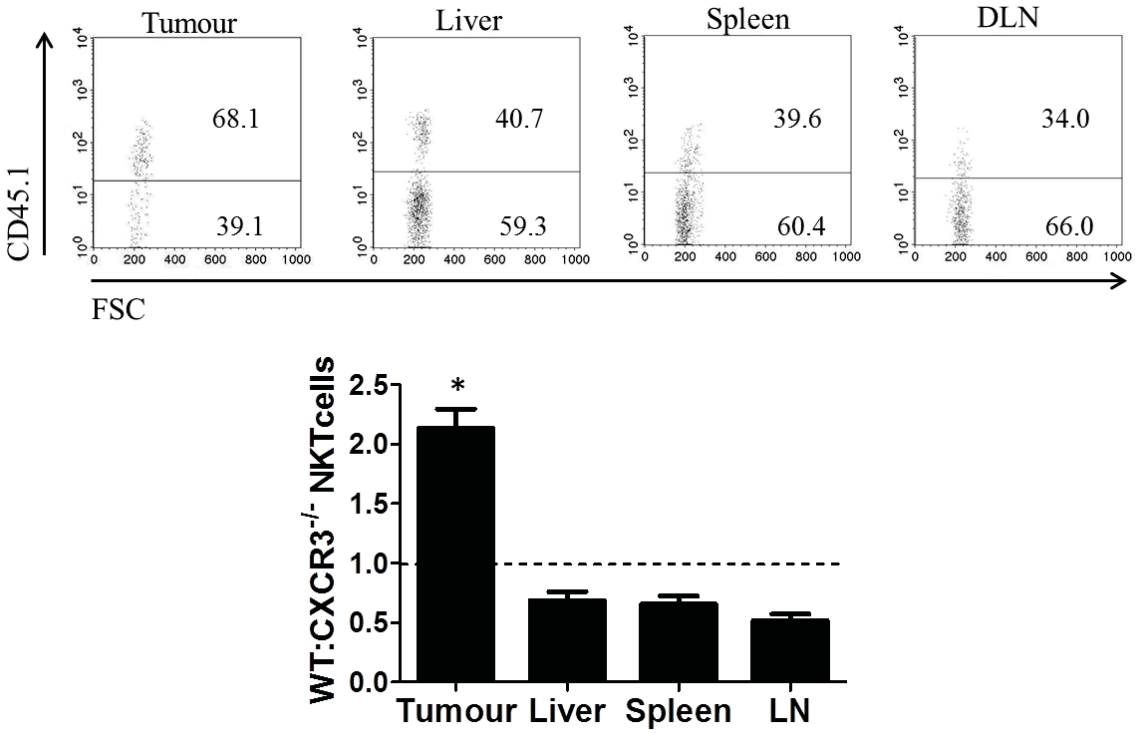


Figure 9. CXCR3$^{-/-}$ NKT cells display reduced homing to B16 melanoma tumours. On day 0, Jα18$^{-/-}$ C57BL/6 mice were injected subcutaneously into their hind right flank with 2.5 x 10^5 B16 F10 melanoma cells. On day 11, mice were treated intravenously with equal numbers of CXCR3$^{-/-}$ (CD45.2) and WT (CD45.1) sorted liver NKT cells (total of 6-10 x 10^6 cells). On day 14, all tumours, livers, spleens and draining lymph nodes were harvested and stained with TCRβ, α-GalCer-loaded CD1d tetramer and CD45.1 to identify donor cells. FACS plots are gated on the TCRβ$^+$CD1d-tetramer$^+$ population. Percent displayed is the mean. Data is represented as ratio of WT donor cells to CXCR3$^{-/-}$ donor cells within each tissue. (n=6 per tissue). *P<0.05 compared to non-tumour tissue.

of CXCR3$^{-/-}$ NKT cells was significantly higher in these tissues than WT NKT cells (Figure 9). Thus, our data suggests that CXCR3 plays a role in directing NKT cell recruitment directly to tumour sites.

3.2.2 Reduced frequency of CXCR6$^{-/-}$ NKT cells in tumours following co-adoptive transfers

Previous studies have demonstrated that mice deficient in CXCR6 have reduced numbers of NKT cells in the liver and lungs[182,228]. Therefore, in order to determine that the reduced frequency of tumour infiltrating NKT cells within CXCR6 deficient mice was not a result of reduced NKT cell numbers, we repeated the above co-adoptive transfer experiments using CD45.1 WT and CXCR6 deficient NK cells. We again observed that the frequency of WT NKT cells within tumours of recipient mice was significantly higher than that of CXCR6$^{-/-}$ donor NKT cells (Fig.10). Homing of CXCR6$^{-/-}$ NKT cells was also impaired in the liver, consistent with the role for CXCR6 in NKT cell accumulation in the liver[182]. There were no significant differences in donor cell frequency observed in the spleen, and the frequency of CXCR6$^{-/-}$ NKT cells was higher in the DLNs compared to CD45.1 NKT cells (Fig.10).

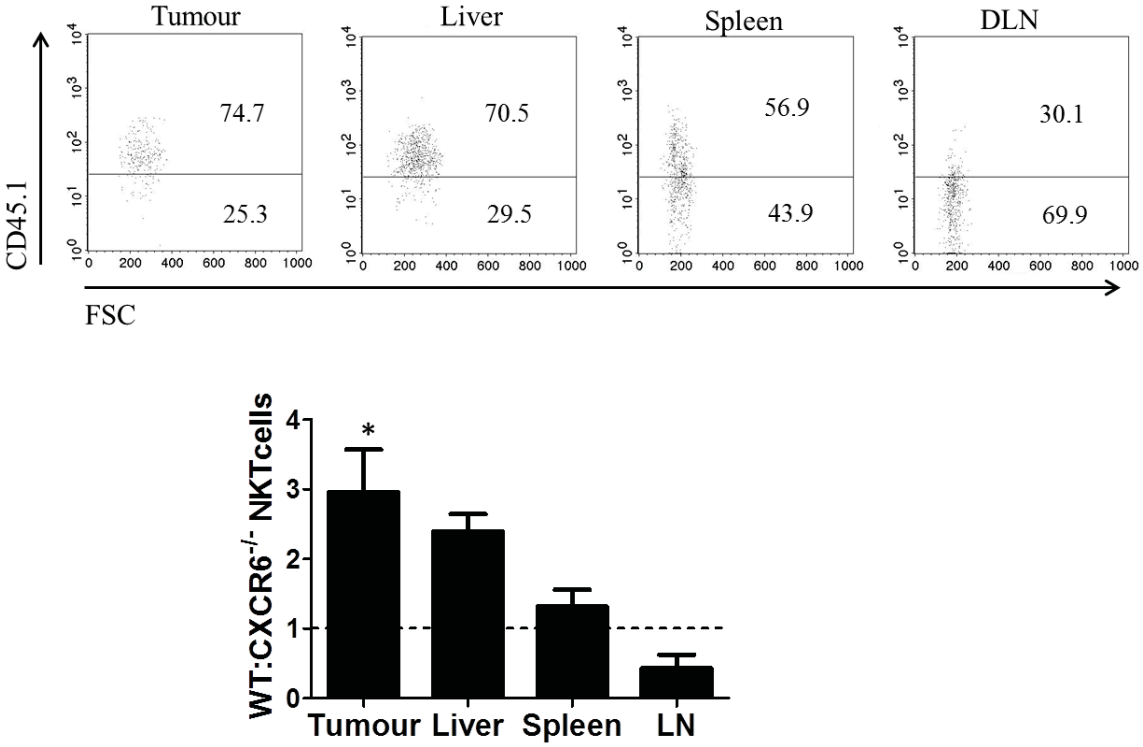


Figure 10. CXCR6$^{-/-}$ NKT cells display reduced homing to B16 melanoma tumours. On day 0, Jα18$^{-/-}$ C57BL/6 mice were injected subcutaneously into their hind right flank with 2.5 x 10^5 B16 F10 melanoma cells. On day 11, mice were treated intravenously with equal numbers of CXCR6$^{-/-}$ (CD45.2) and WT (CD45.1) sorted liver NKT cells (total of 6-10 x 10^6 cells). On day 14, all tumours, livers, spleens and draining lymph nodes were harvested and stained with TCRβ, α-GalCer-loaded CD1d tetramer and CD45.1 to identify donor cells. FACS plots are gated on the TCRβ^+CD1d-tetramer$^+$ population. Percent displayed is the mean. Data is represented as ratio of WT donor cells to CXCR3$^{-/-}$ donor cells within each tissue. (n=10 per tissue). *P<0.05 compared to non-tumour tissue.

3.2.3 Reduced frequency of CCR5$^{-/-}$ NKT cells in tumours following co-adoptive transfers

Adoptive transfer experiments were repeated using NKT cells from CCR5 deficient mice. The frequency of WT NKT cells within tumours of recipient mice was significantly higher than that of CCR5$^{-/-}$ NKT cells (Fig.11). Lower levels of receptor deficient NKT cells were also observed in the livers of these mice, suggesting a novel role for CCR5 in homing or retention of NKT cells in the liver. There were no significant differences in donor cell frequency observed in the spleen or DLN (Fig.11).

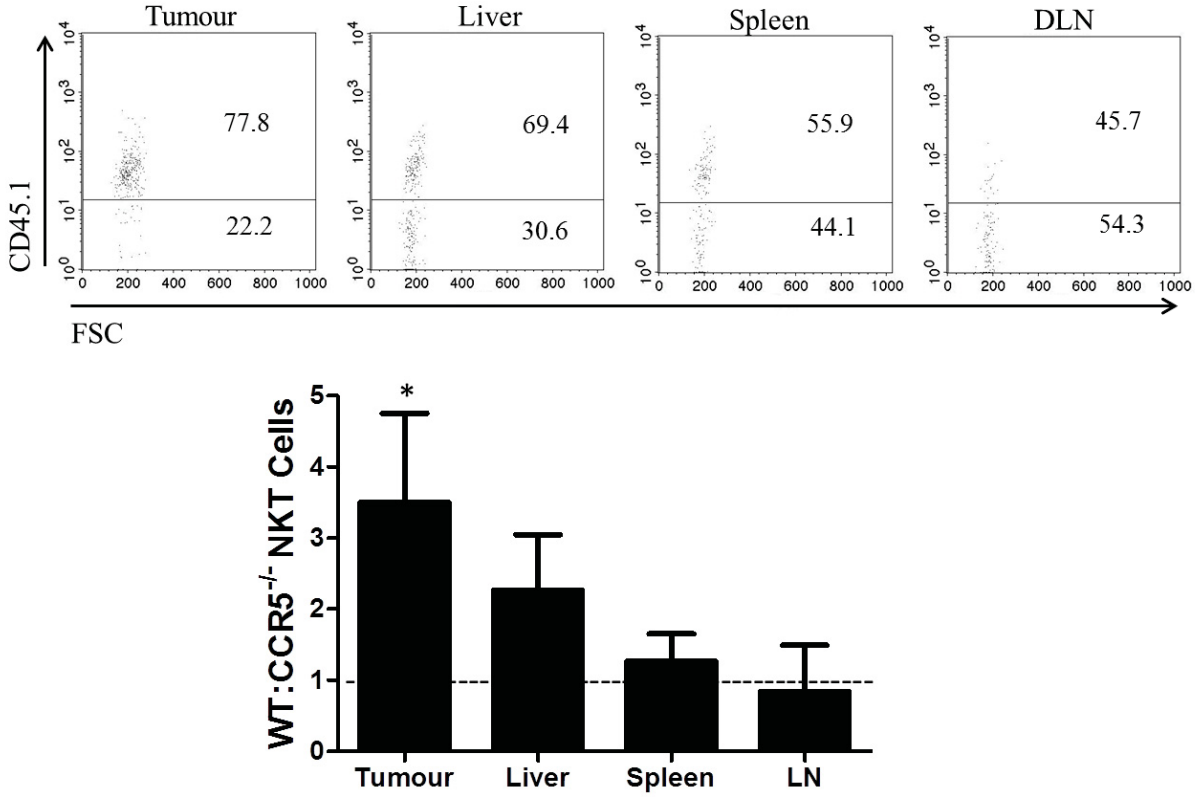


Figure 11. CCR5$^{-/-}$ NKT cells display reduced homing to B16 melanoma tumours. On day 0, Jα18$^{-/-}$ C57BL/6 mice were injected subcutaneously into their hind right flank with 2.5 x 10^5 B16 F10 melanoma cells. On day 11, mice were treated intravenously with equal numbers of CCR5$^{-/-}$ (CD45.2) and WT (CD45.1) sorted liver NKT cells (total of 6-10 x 10^6 cells). On day 14, all tumours, livers, spleens and draining lymph nodes were harvested and stained with TCRβ, α-GalCer loaded CD1d tetramer and CD45.1 to identify donor cells. FACS plots are gated on the TCRβ$^+$CD1d-tetramer$^+$ population. Percent displayed is the mean. Data is represented as ratio of WT donor cells to CXCR3$^{-/-}$ donor cells within each tissue. (n=6). *P<0.05 compared to non-tumour tissue.

3.3 Treatment with CXCL10 and α-GalCer enhances NKT cell tumour infiltration and slows tumour growth

Our adoptive transfer experiments demonstrated that CXCR3 is able to directly mediate recruitment of NKT cells to tumours. Therefore, we sought to identify whether the CXCR3-ligand, CXCL10, would enhance NKT cell recruitment. We also wished to determine whether glycolipid activation of NKT cells enhanced homing to tumours. To test this, WT mice bearing subcutaneous B16 F10 melanoma tumours were injected intratumourally with CXCL10, α-GalCer, or both in combination. Tumours were harvested 3 days later to examine NKT cell infiltration. In mice that received CXCL10 or α-GalCer alone, there was no increase in NKT cell infiltration compared to untreated tumours (Fig.12). In contrast, those that received a combined treatment with both CXCL10 and α-GalCer exhibited a significant increase in the frequency and number of infiltrating NKT cells (Fig.12). Similar results were also observed in the tumour draining lymph nodes of these mice, with significantly increased frequency of NKT cells in the glycolipid and chemokine treatment conditions (Fig.13). This would suggest a synergistic effect of NKT cell activation and recruitment through CXCL10 on NKT cell tumour infiltration. Tumour size was measured with calipers throughout the experiment (Fig.14a). Mice receiving a combination treatment exhibited significantly reduced tumour size at day 17 compared to untreated mice or mice treated with CXCL10 or α-GalCer alone (Fig.14b,c). These experiments were repeated in NKT cell deficient $J\alpha18^{-/-}$ mice to determine whether the reduced tumour size was indeed NKT cell dependent. There were no differences in tumour weight in any of the treatment groups in NKT cell deficient mice (Fig.14d), suggesting that increased NKT cell recruitment mediates the reduction in tumour growth. To ensure that this effect was not limited to the B16 melanoma model, experiments were repeated using LLC tumour bearing mice. As illustrated in Figure 15a, LLC tumours receiving α-GalCer and CXCL10 in combination exhibited a significant increase in NKT cell infiltration, and very similar results were again observed in the tumour-DLNs (Fig.15b). Mice that received intratumoural injections of both CXCL10 and α-GalCer also exhibited significantly reduced tumour growth at day 17 in comparison to untreated mice or mice treated with CXCL10 or α-GalCer alone (Fig. 16a-

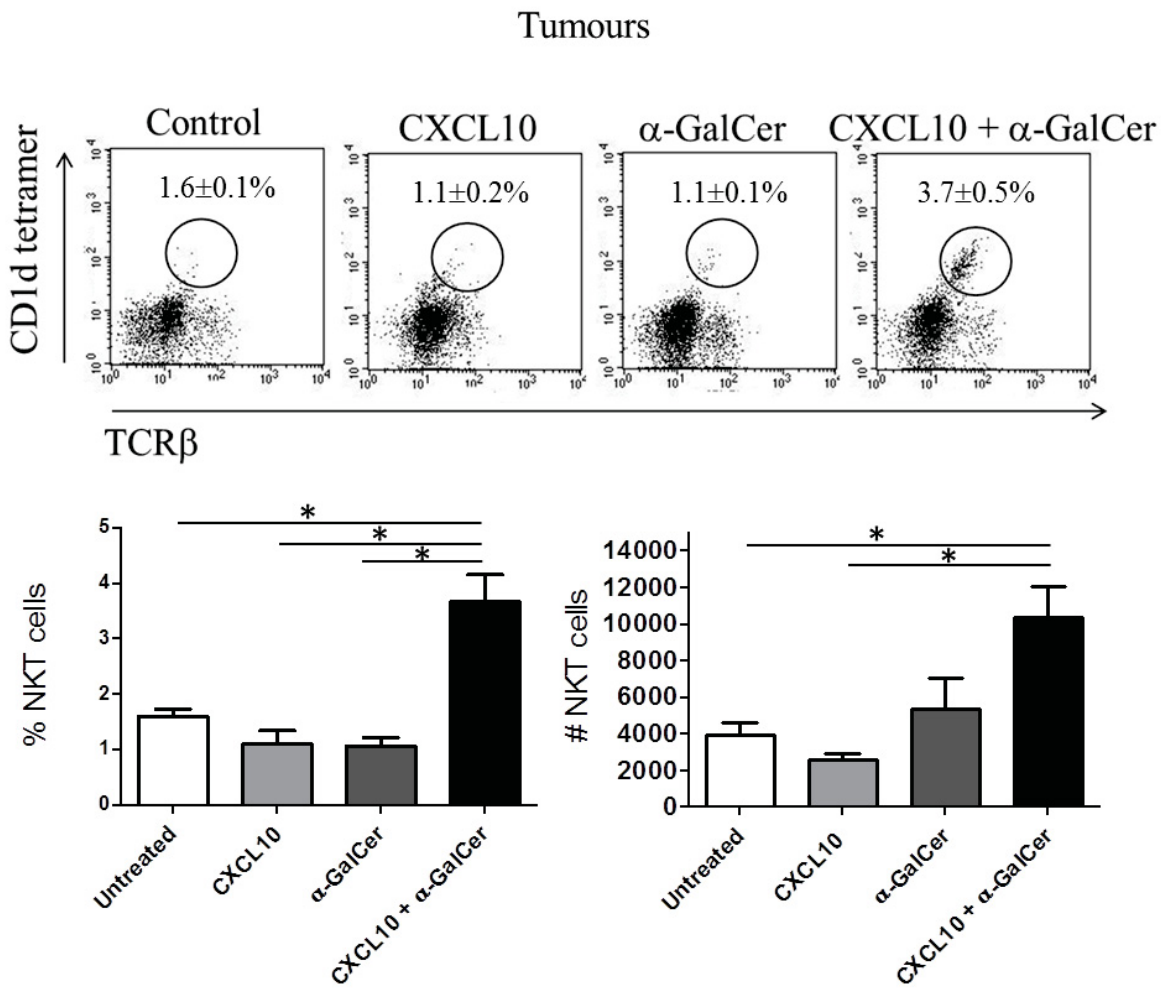


Figure 12. Intratumoural injection of CXCL10 and α-GalCer synergistically enhance NKT cell tumour infiltration in B16 tumours. C57BL/6 mice bearing subcutaneous B16F10 melanoma tumours received intratumoural injections of either 2.0 μg CXCL10, 4.0 μg α-GalCer or a combination of both on day 11 following tumour inoculation, with repeat injections of CXCL10 on day 14. All tumours were harvested on day 17 and cells were stained with PerCP CD45.2, FITC-TCRβ and APC-CD1d tetramer. The frequency of NKT cells in the tumour was determined through flow cytometry analysis. (n= 56 Untreated, 13 CXCL10, 6 α-GalCer, 38 CXCL10 + α-GalCer) *P<0.05.

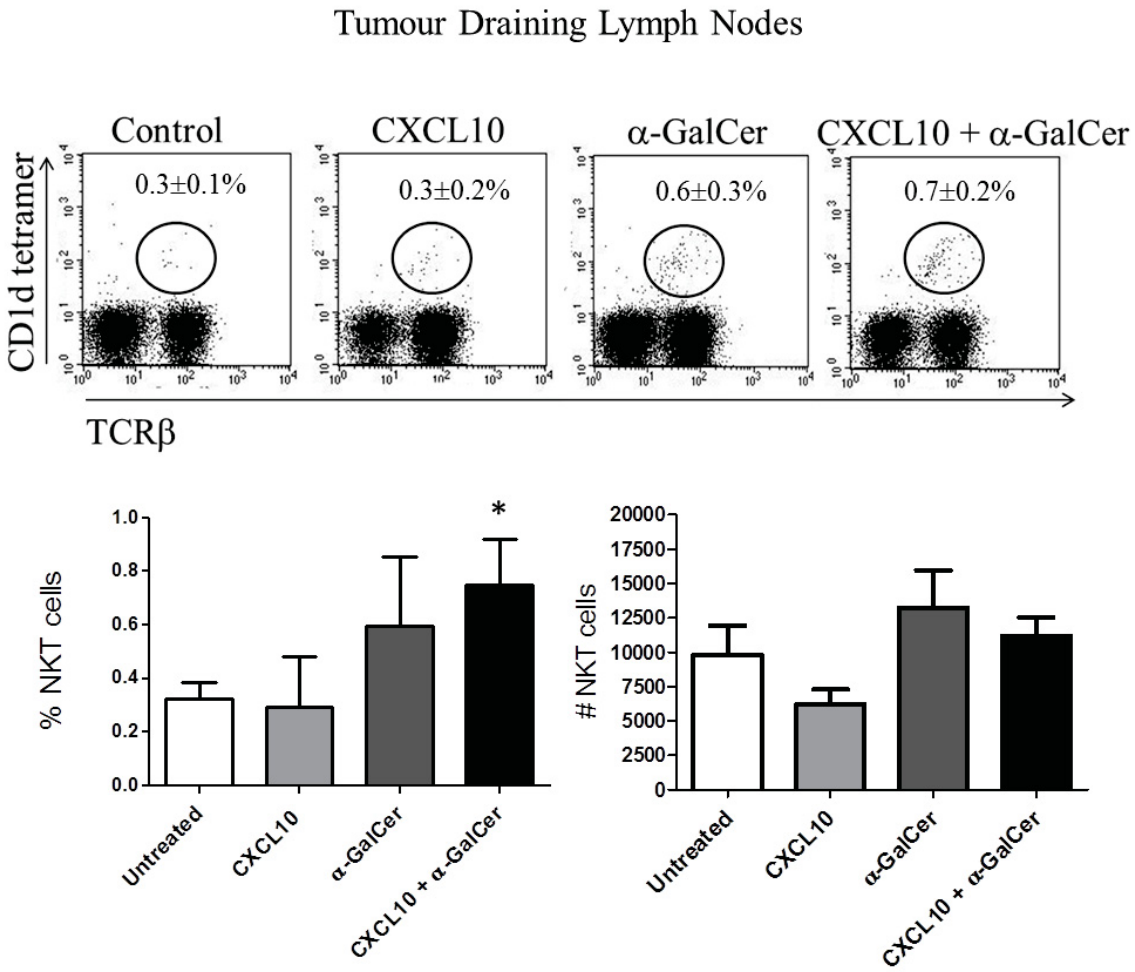


Figure 13. Intratumoural injection of CXCL10 and α-GalCer synergistically enhance NKT cell populations in B16 tumour-DLN. C57BL/6 mice bearing subcutaneous B16F10 melanoma tumours received intratumoural injections of either 2.0 μg CXCL10, 4.0 μg α-GalCer or a combination of both on day 11 following tumour inoculation, with repeat injections of CXCL10 on day 14. Tumour-DLNs were harvested on day 17 and cells were stained with PerCP CD45.2, FITC-TCRβ and APC-CD1d tetramer. The frequency of NKT cells in the DLNs was determined through flow cytometry analysis. (n= 56 Untreated, 13 CXCL10, 6 α-GalCer, 38 CXCL10 + α-GalCer) *P<0.05 compared to untreated.

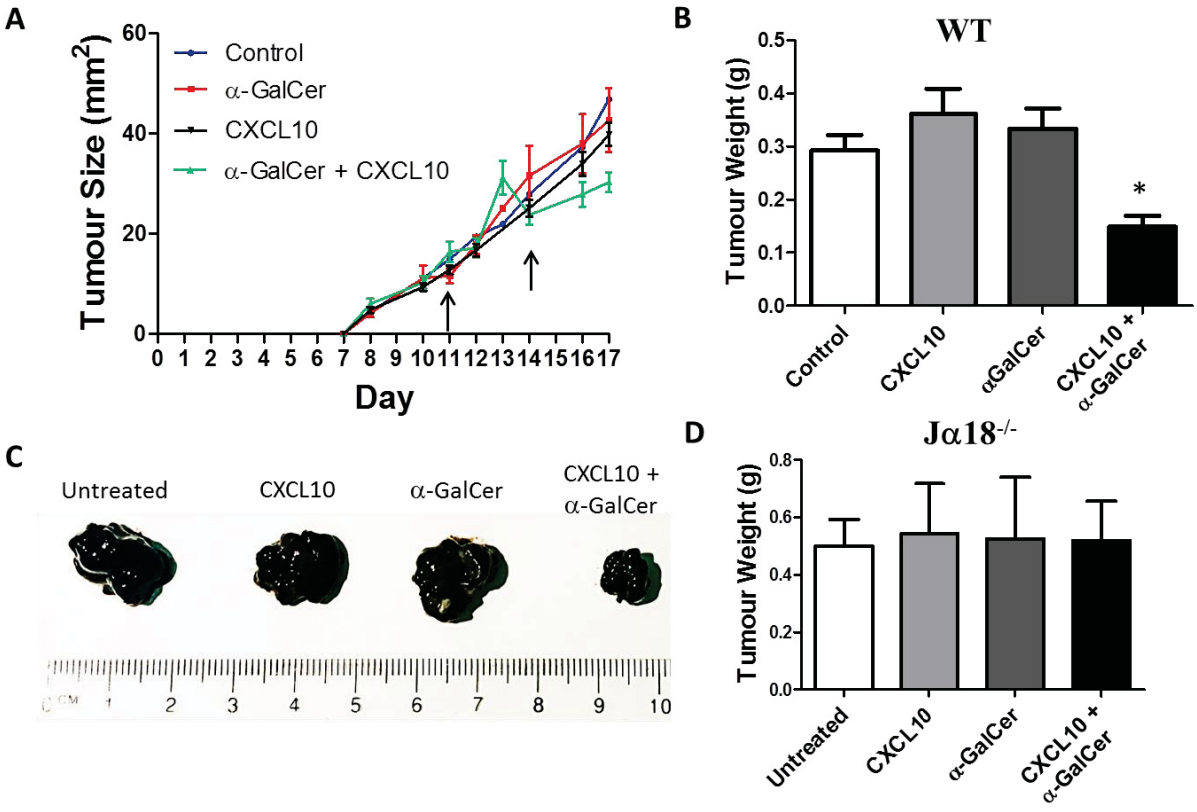


Figure 14. Intratumoural treatment with CXCL10 and α-GalCer results in slowed B16 melanoma tumour growth. C57BL/6 mice bearing B16 F10 tumours received intratumoural treatments of 2.0 µg CXCL10, 4.0 µg α-GalCer or a combination of both. Tumour development was assessed through caliper measurements (A). Arrows indicate the day of α-GalCer and CXCL10 injection. Second injections were of CXCL10 alone. Final tumour weight was measured on day 17 (B, C) n=29 untreated, 9 α-GalCer, 8 CXCL10, 31 α-GalCer + CXCL10. Experiments were repeated in Jα18$^{-/-}$ mice and tumour weight was measured on day 17 (D). n=3 per group. *P<0.05 compared to untreated.

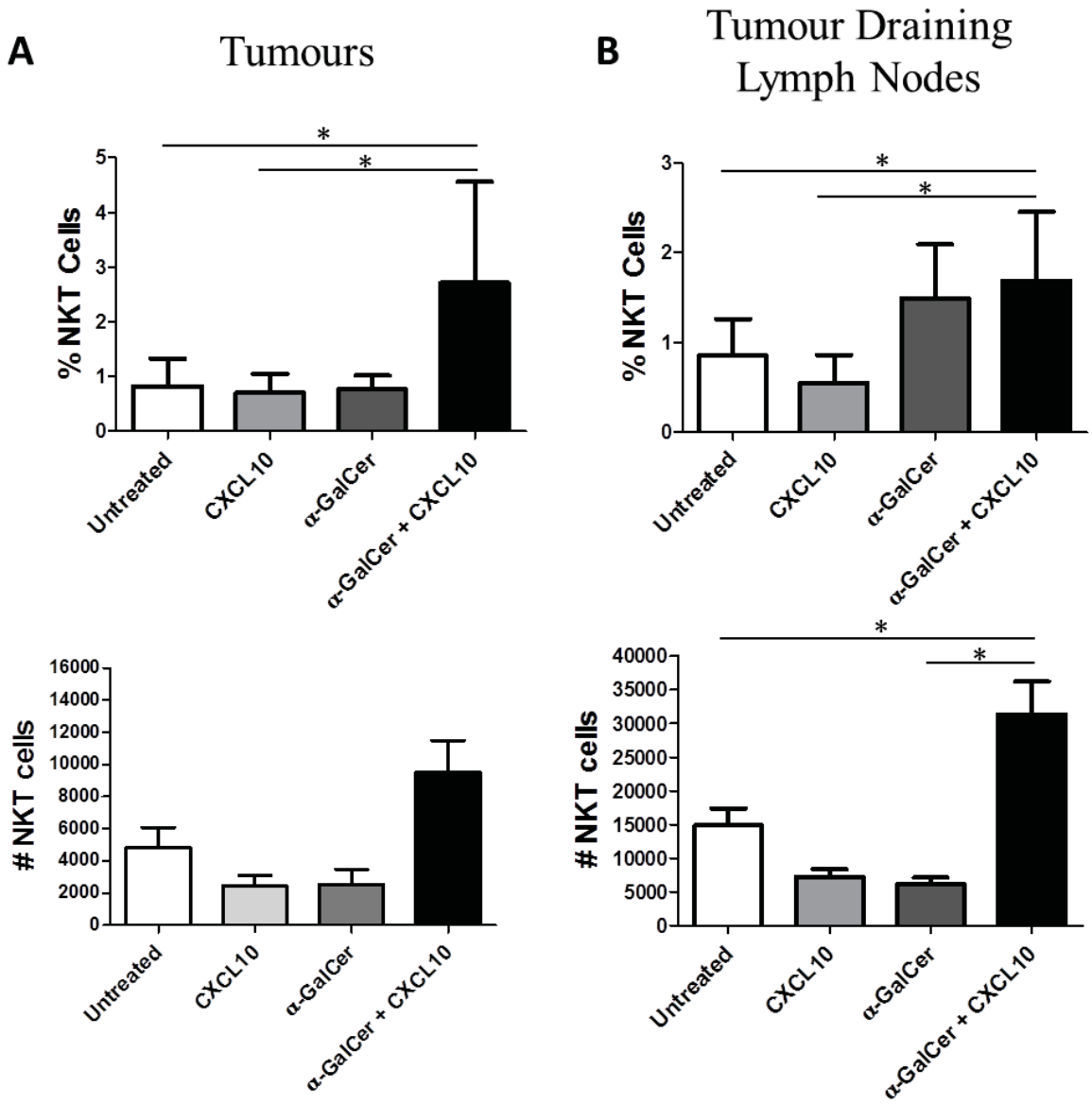


Figure 15. Intratumoural injection of CXCL10 and α-GalCer synergistically enhance NKT cell infiltration in LLC tumours and Tumour-DLNs. C57BL/6 mice bearing subcutaneous LLC tumours received intratumoural injections of either 2.0 μg CXCL10, 4.0 μg α-GalCer or a combination of both on day 11 and 14 following tumour inoculation. Tumours (A) and their DLNs (B) were harvested on day 17 and cells were stained with PerCP CD45.2, FITC-TCRβ and APC-CD1d tetramer. The frequency of NKT cells in each was determined through flow cytometry analysis. (n= 15 Untreated, 4 CXCL10, 6 α-GalCer, 19 CXCL10 + α-GalCer). *P<0.05.

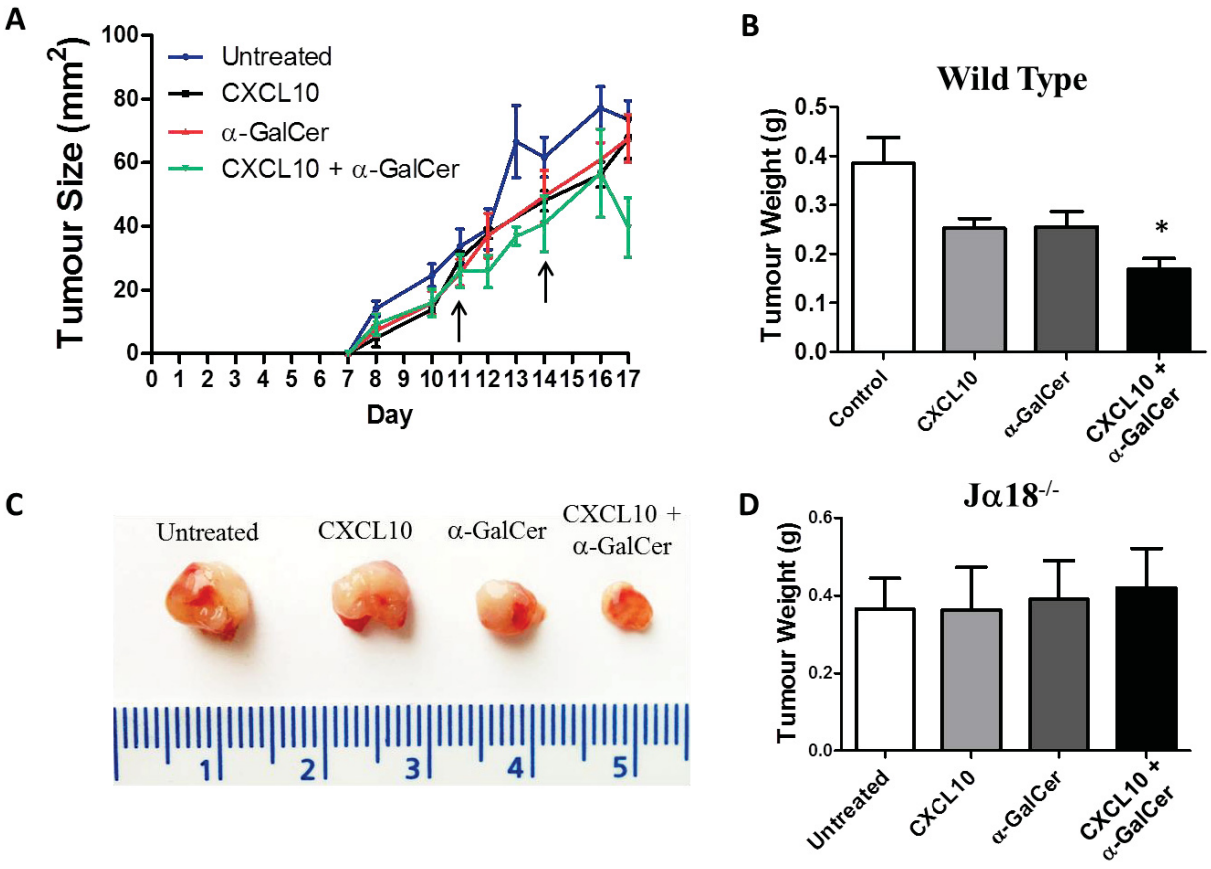
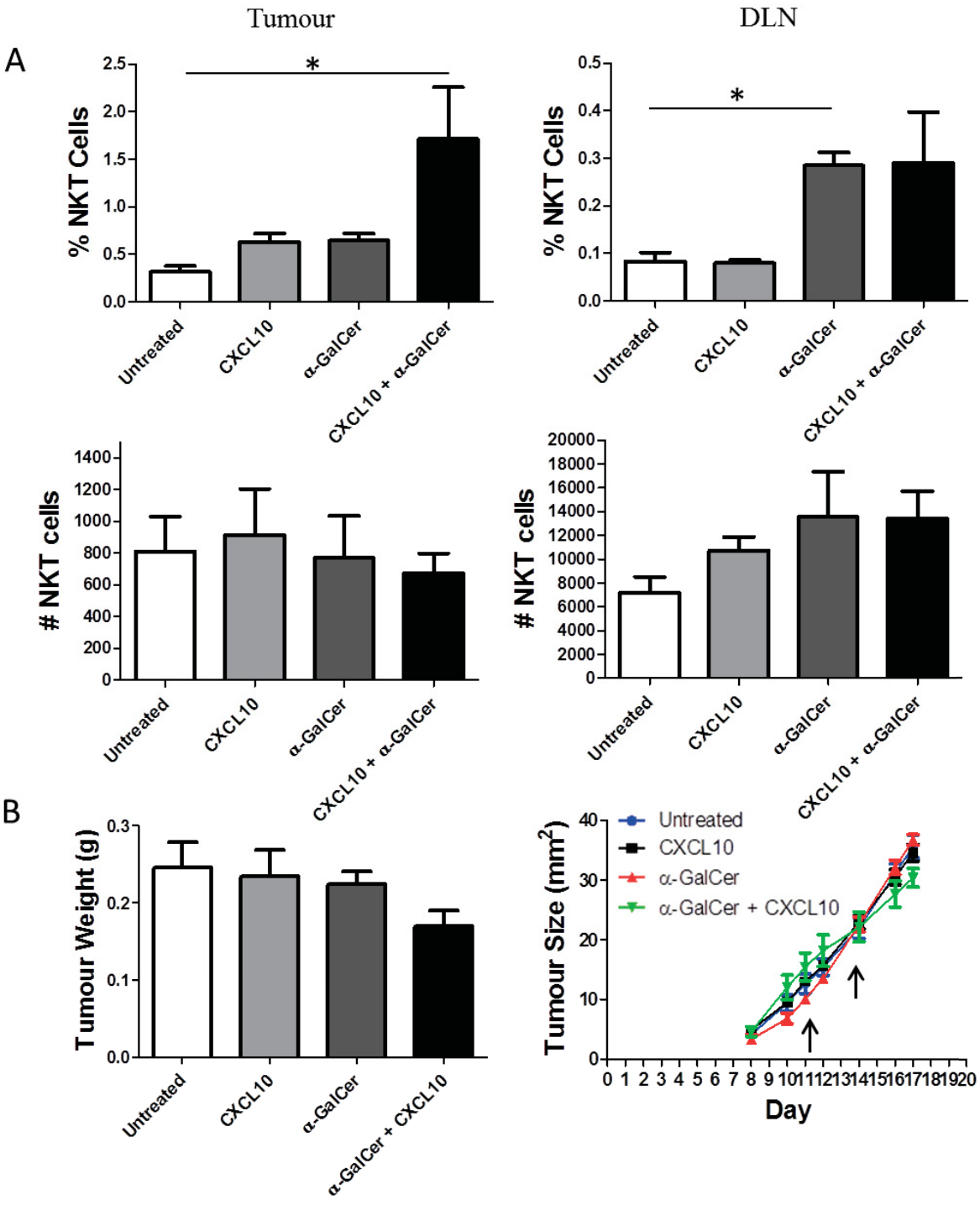


Figure 16. Intratumoural treatment with CXCL10 and α-GalCer results in slowed LLC tumour growth. C57BL/6 mice bearing LLC tumours were treated with intratumoural 2.0 µg CXCL10, 4.0 µg α-GalCer or a combination of both. Tumour development was assessed through caliper measurements (A). Arrows indicate the day of α-GalCer and/or CXCL10 injection. Second injections were of CXCL10 alone. Final tumour weight was measured on day 17 (B, C). n= 15 untreated, 6 α-GalCer, 4 CXCL10, 19 α-GalCer + CXCL10. Experiments were repeated in Jα18$^{-/-}$ mice and tumour weight was measured on day 17 (D). *P<0.05 compared to untreated.

c). No such differences in tumour size were observed in NKT cell deficient mice (Fig 16d), indicating that this was an NKT-cell dependent effect.

Both LLC tumours and B16 tumours were developed and modelled in mice with a C57BL/6 background. We wanted to determine whether these results could also be repeated in mice with a different genetic background. We therefore repeated the above experiments using the 4T1 mammary carcinoma model in BALB/c mice. Mice were inoculated with 1.0×10^5 4T1 cells as these tumours grew at much faster rates. Mice were treated with CXCL10, α-GalCer, or a combination of both. On day 17 all mice were sacrificed and tumour size and NKT cell infiltration was examined. As observed in the LLC and B16 models, animals receiving the combination treatment had a significantly higher frequency of NKT cells within tumours and their DLNs compared to untreated mice or mice treated with CXCL10 or α-GalCer alone (Fig.17a). We also observed a slight decrease in tumour size in those that received a combination treatment (Fig.17b); however these experiments will need to be repeated before statistical significance can be obtained.

Figure 17. CXCL10 and α-GalCer intratumoural treatment in s.c 4T1 tumours. Balb/c received s.c injections of 1.0 x 10^5 4T1 tumour cells on day 0. Tumours received injections of 2.0 μg CXCL10, 4.0μg α-GalCer or a combination of both on day 11 and repeat injections of CXCL10 on day 14. All tumours were harvested on day 17 and stained with CD45.2, TCRβ and α-GalCer-loaded CD1d tetramer to identify the NKT cell population. Mice which received intratumoural injections of CXCL10 in combination with α-GalCer showed significantly increased NKT cell frequency in tumours and tumour DLNs (A). There were no significant differences in tumour size, although those which received a combination treatment showed slightly reduced growth. Arrows indicate days of injection. (B). n= 5 untreated, 6 α-GalCer, 4 CXCL10, 3 CXCL10 + α-GalCer. *$P<0.05$.


Tumour
DLN
A
% NKT Cells
# NKT cells
Untreated
CXCL10
α-GalCer
CXCL10 + α-GalCer
B
Tumour Weight (g)
α-GalCer + CXCL10
Tumour Size (mm²)
Day

3.4 CXCL16/α-GalCer treatment enhances NKT cell recruitment but does not reduce tumour size

Our co-adoptive transfer experiments strongly indicated a role for CXCR6 in mediating NKT cell recruitment directly to tumours. Therefore, we examined whether the CXCR6 ligand, CXCL16, could also work to enhance NKT cell recruitment and modify tumour growth. C57BL/6 WT mice bearing subcutaneous B16 F10 melanoma tumours were injected intratumourally with CXCL16, α-GalCer, or both in combination day 11. Repeat injections of CXCL16 were performed at day 14. Tumours were harvested on day 17 to examine NKT cell recruitment. Flow cytometry analysis revealed significant increases in NKT cell infiltration within tumours that received combination treatments (Fig.18).There was no increase in NKT cell recruitment to the draining lymph nodes (Fig. 18). In contrast to tumours that received CXCL10 and α-GalCer, mice treated with CXCL16 and α-GalCer did not exhibit reductions in tumour size. Instead, mice that received CXCL16 injections exhibited significantly enhanced tumour development (Fig.19a-c). This was not dependent on NKT cells as CXCL16 increased tumour growth in Jα18$^{-/-}$ mice (Fig. 19d). Once again these experiments were repeated using a LLC tumour model. LLC tumours which received injections of both CXCL16 and α-GalCer displayed significantly increased NKT cell frequencies in both the tumours and tumour – DLNs (Fig.20). There was no statistically significant difference in tumour size between the different treatment groups; however, we observed a trend towards enhanced tumour size in those that received CXCL16 injections alone (Fig.21a-c). There were no significant differences in tumour size when these experiments were repeated in NKT cell deficient mice (Fig 21d).

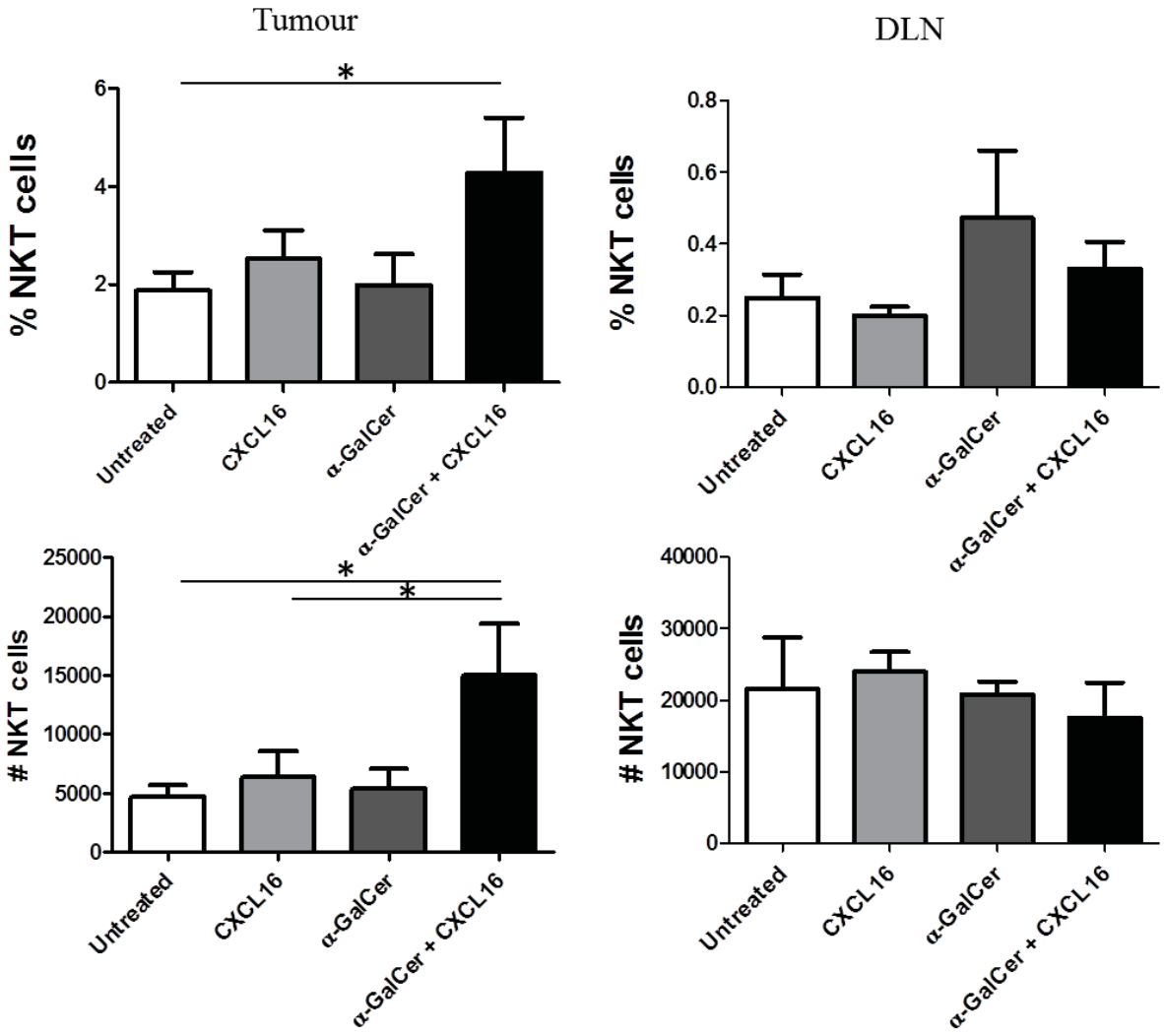


Figure 18. Treatment of B16 Melanoma tumours with CXCL16 and α-GalCer enhances NKT cell tumour infiltration. C57BL/6 WT mice bearing subcutaneous B16F10 melanoma tumours received intratumoural injections of either 2.0 μg CXCL16, 4.0 μg α-GalCer or a combination of both on day 11 following tumour inoculation, with repeat injections of CXCL16 on day 14. All tumours and tumour-DLNs were harvested on day 17 and cells were stained with PerCP CD45.2, FITC-TCRβ and APC-CD1d tetramer. The frequency of NKT cells in each was determined through flow cytometry analysis. (n= 28 Untreated, 36 CXCL16, 4 α-GalCer, 16 CXCL16 + α-GalCer). *P<0.05

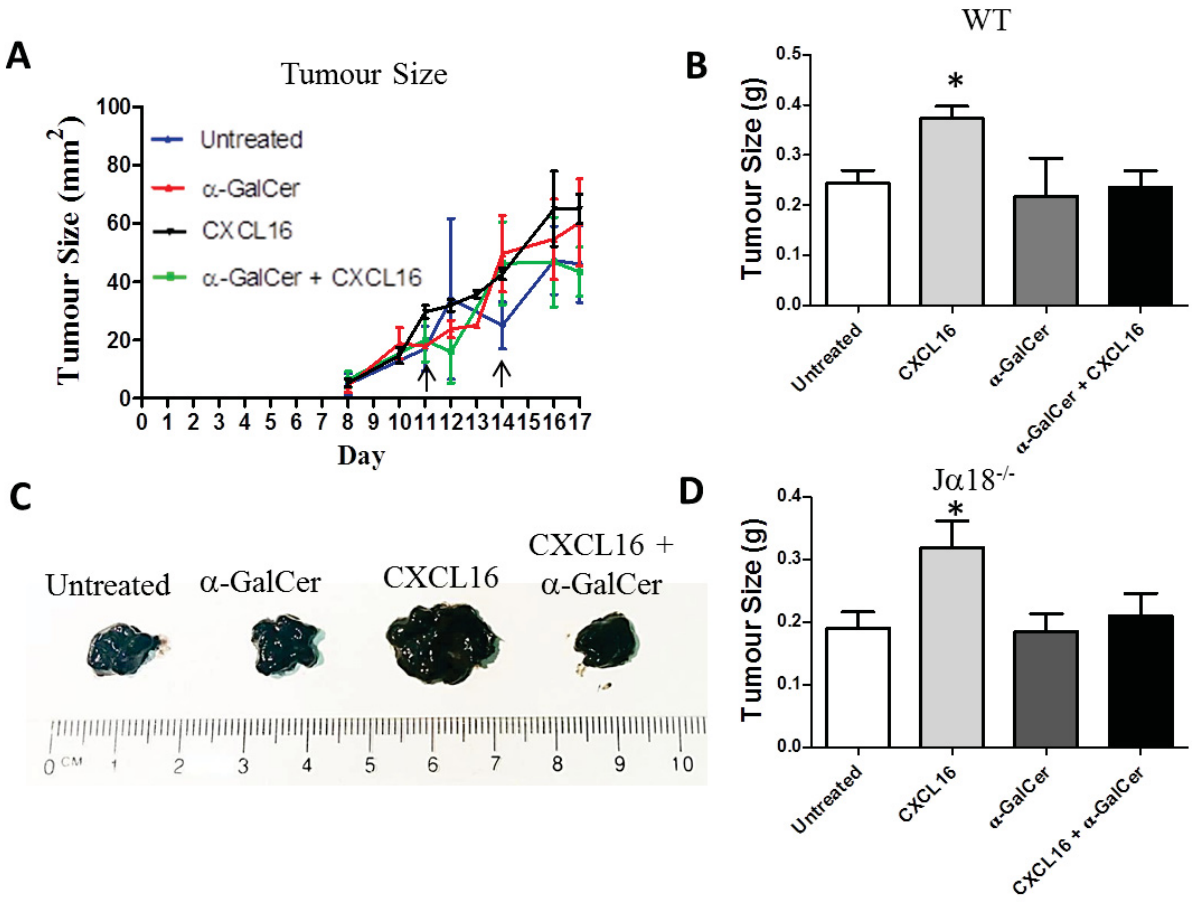


Figure 19. Intratumoural treatment with CXCL16 results in enhanced tumour growth. C57BL/6 WT mice bearing B16 F10 melanoma tumours received intratumoural treatments of either 4.0 µg α-GalCer, 2.0 µg CXCL16 or a combination of both. Tumour development was assessed through caliper measurements (A). Arrows indicate day of injections. Final tumour weight was measured on day 17. (n= 40 Untreated, 36 CXCL16, 5 α-GalCer, 19 CXCL16 + α-GalCer) (B, C). Experiments were repeated in Jα18$^{-/-}$ mice and tumour weight was measured on day 17 (D). n= 6 per group. *P<0.05 compared to untreated.

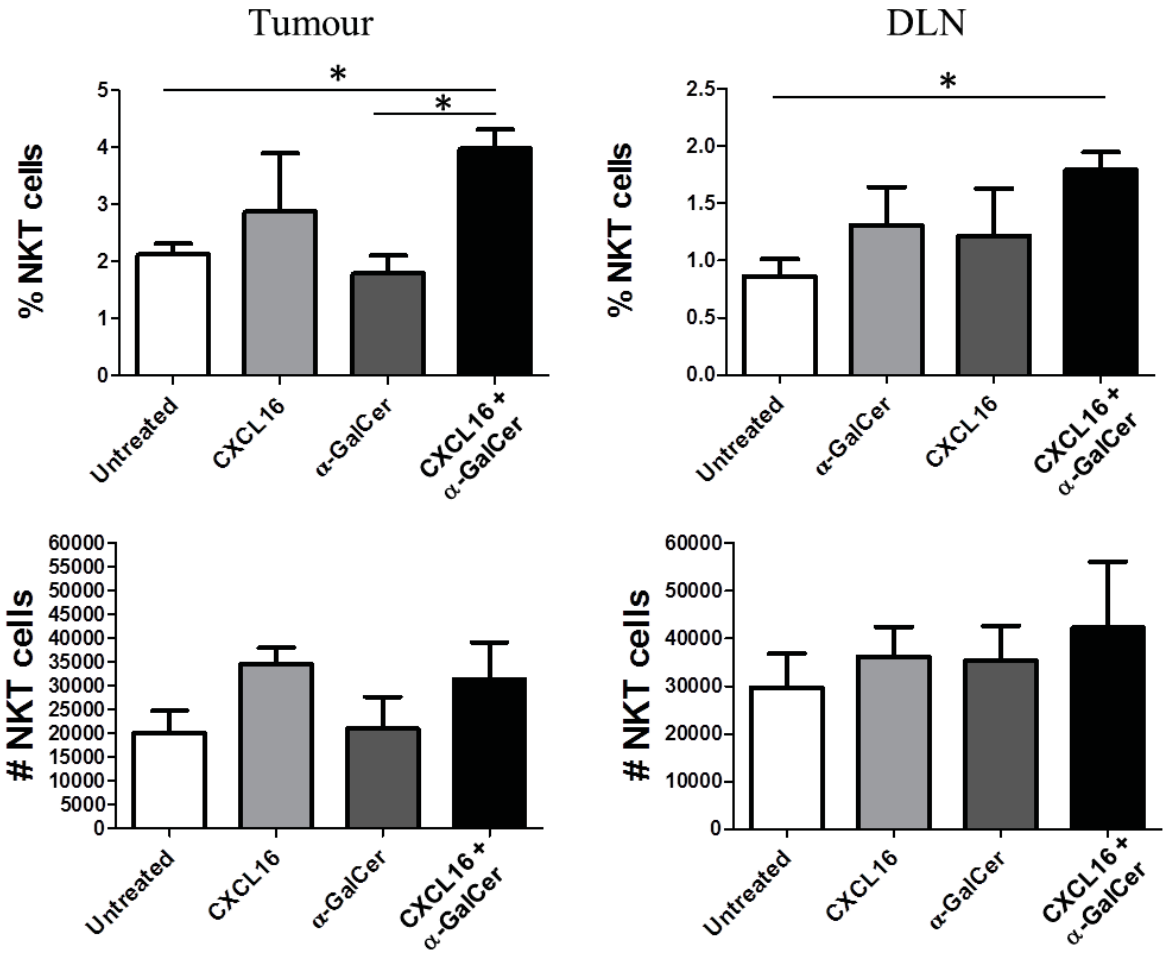


Figure 20. Treatment of LLC tumours with CXCL16 and α-GalCer enhances NKT cell tumour infiltration. C57BL/6 WT mice bearing subcutaneous LLC tumours received intratumoural injections of either 2.0 μg CXCL16, 4.0 μg α-GalCer or a combination of both on day 11 following tumour inoculation, with repeat injections of CXCL16 on day 14. All tumours and tumour-DLNs were harvested on day 17 and cells were stained with PerCP CD45.2, FITC-TCRβ and APC-CD1d tetramer. The frequency of NKT cells in each was determined through flow cytometry analysis. (n= 14 untreated, 4 CXCL16, 6 α-GalCer, 10 CXCL16 + α-GalCer). *P<0.05.

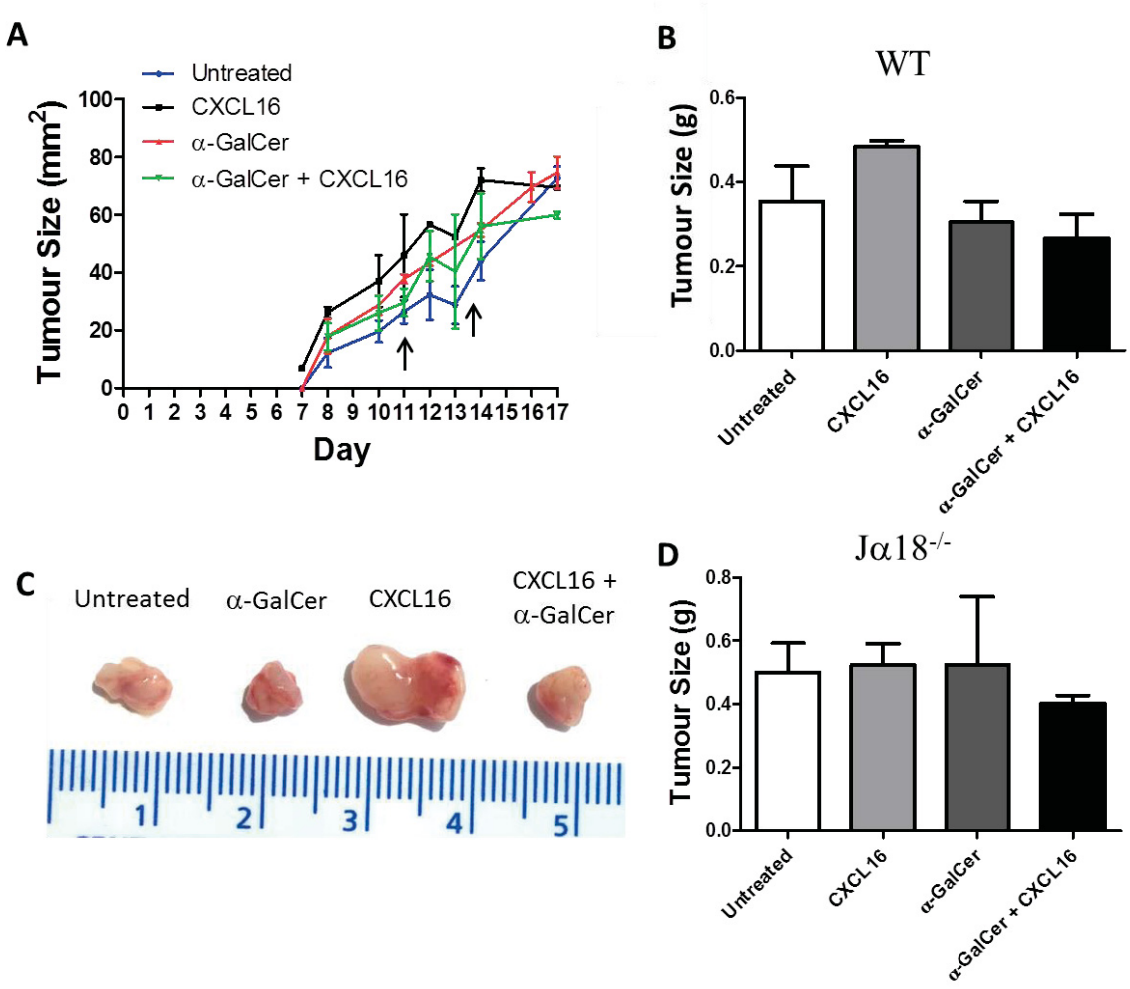


Figure 21. Effect of intratumoural CXCL16 and α-GalCer treatments on LLC tumour size. WT mice bearing s.c LLC tumours received intratumoural treatments of either 4.0 µg α-GalCer, 2.0 µg CXCL16 or a combination of both. Tumour development was assessed through caliper measurements (A).Arrows indicate day of tumour injections. Final tumour weight was measured on day 17 (B, C) (n= 14 Untreated, 4 CXCL16, 6 α-GalCer, 10 CXCL16 + α-GalCer)**.** Experiments were repeated in Jα18$^{-/-}$ mice and tumour weight was measured on day 17.(n=3 per group). (D) *P<0.05 compared to untreated.

3.5 Effect of intratumoural treatments on macrophage tumour infiltration

Intratumoural injections with CXCL10 and CXCL16 in combination with α-GalCer enhanced NKT cell recruitment into tumours. Interestingly, injection of CXCL16 alone resulted in a trend towards increased tumour size, despite its ability to recruit NKT cells. Therefore, we hypothesized that CXCL16 may recruit other immune cells which counteract the tumour killing functions of NKT cells. We therefore examined the frequency of tumour associated macrophages (TAMs). Cells were isolated on day 17 and stained with PerCP CD45.2, APC CD11b, FitC Ly6C and PE Ly6G. Cells were gated on the $CD45.2^{+}$ population and examined for their expression of Ly6G and CD11b. Although there were no significant differences observed in macrophage frequency between the different treatment groups, tumours that received combined treatments of CXCL10 or CXCL16 with α-GalCer had significantly increased absolute macrophage number compared to untreated controls (Fig.22). This could be explained by increased overall infiltration in the combined treatments. There also appeared to be a trend towards increased TAM frequency within tumours that received treatment with CXCL16 alone (Fig. 22). No significant differences were observed in macrophage frequency or number between the various treatment groups in tumour-DLNs (Fig.23).

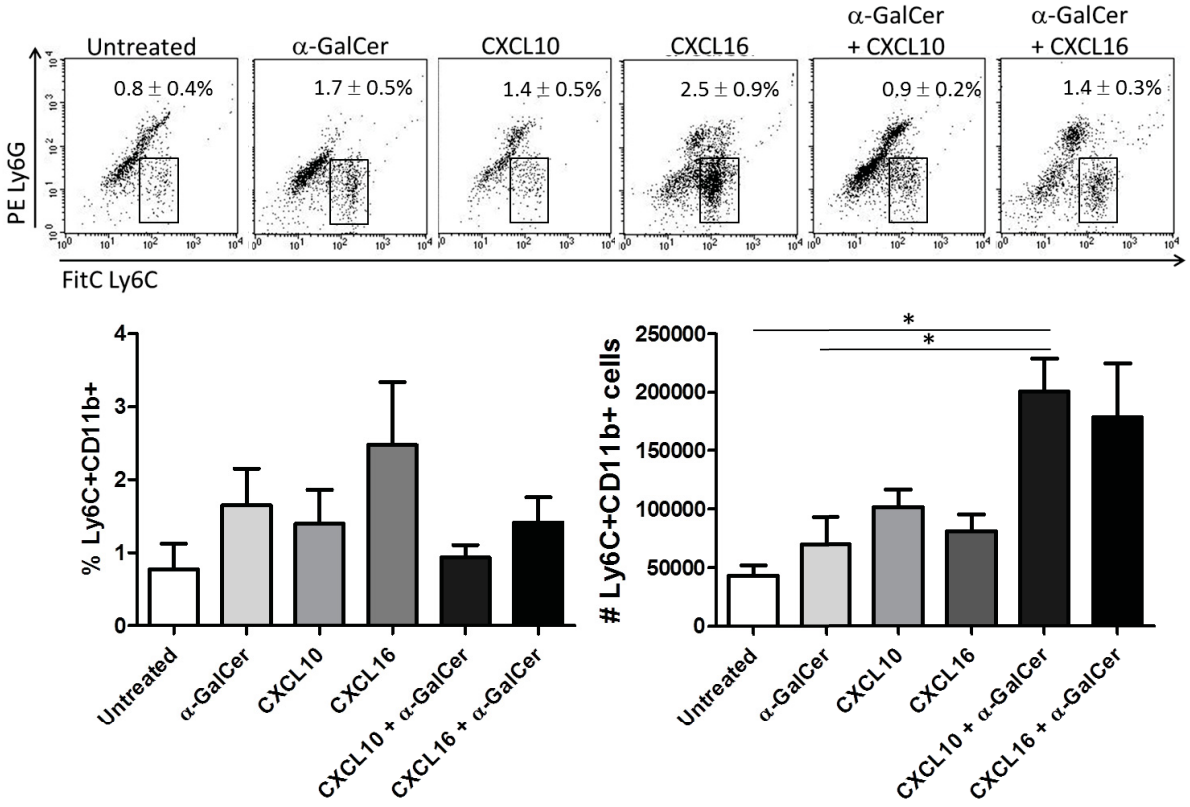


Figure 22. Frequency and number of macrophages within tumours treated with α-GalCer and/or chemokine. B16 F10 melanoma subcutaneous tumours were treated with either 2.0 μg CXCL10, 2.0 μg CXCL16, 4.0 μg α-GalCer or a combination on day 11, with repeat injections of CXCL10 or CXCL16 on day 14. Tumours were harvested on day 17. Cells were stained with CD45.2, CD11b, Ly6C and Ly6G and analyzed through flow cytometry. (n= 5 untreated, 10 α-GalCer, 9 CXCL10, 9 CXCL16, 7 α-GalCer + CXCL10, 9 CXCL16 + α-GalCer). *P<0.05.

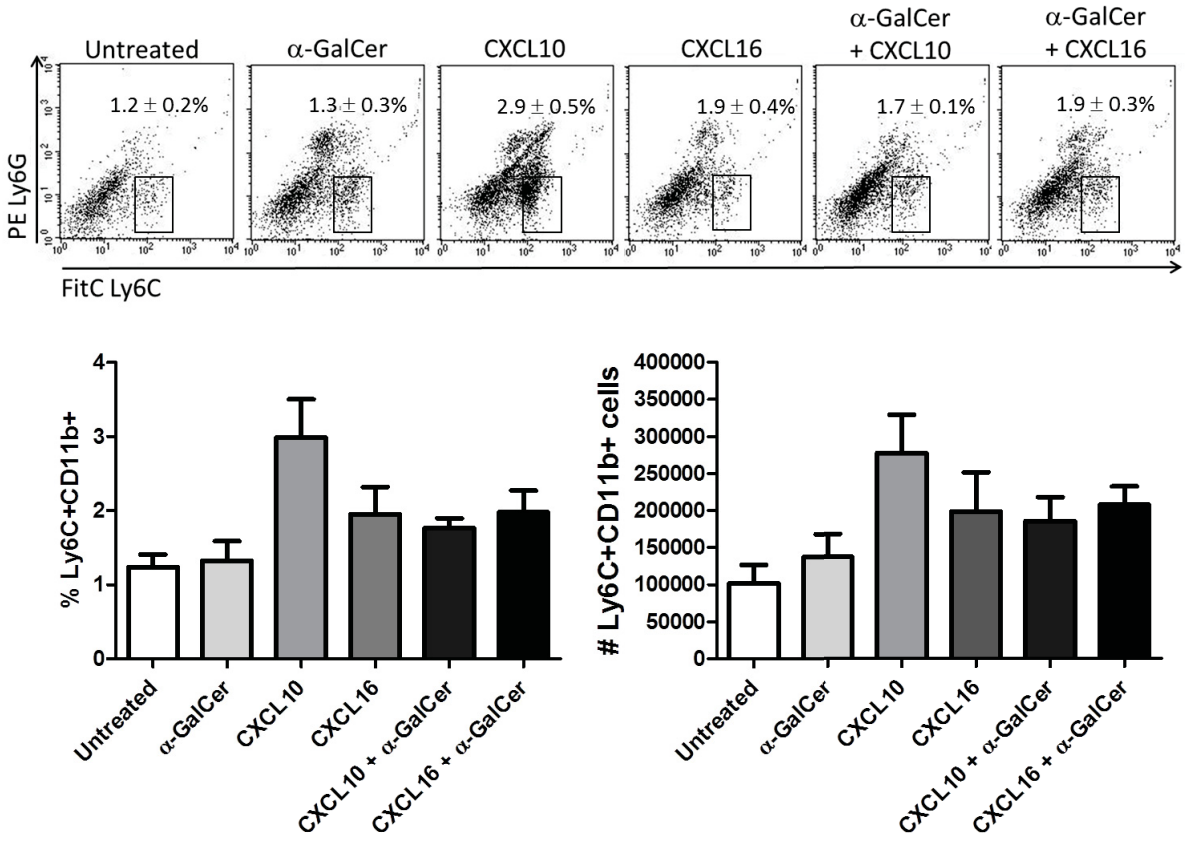


Figure 23. Frequency of macrophages within tumour DLNs treated with α-GalCer and/or chemokine. B16F10 melanoma subcutaneous tumours were were treated with either 2.0 μg CXCL10, 2.0 μg CXCL16, 4.0 μg α-GalCer or a combination on day 11, with repeat injections of CXCL10 or CXCL16 on day 14. Tumour-DLNs were harvested on day 17. Cells were stained with CD45.2, CD11b, Ly6C and Ly6G and analyzed through flow cytometry. (n= 5 Untreated, 10 α-GalCer, 9 CXCL10, 9 CXCL16, 7 α-GalCer + CXCL10, 9 CXCL16 + α-GalCer).

3.6 Effect of intratumoural treatments on T-regulatory cell recruitment to tumours

Our data has demonstrated that treatment of tumours with CXCL16 can lead to increased tumour size. The possibility of this being due to enhanced accumulation of TAMs was examined, and no significant differences in recruitment between the treatment groups was observed that would correlate with increased tumour growth. Therefore, we investigated whether CXCL16 treatment enhanced recruitment of Tregs to tumours. To examine this, C57BL/6 mice expressing a GFP-Foxp3 reporter gene were subcutaneously injected with 2.5 x 10^5 B16 melanoma cells into their hind right flanks. Mice were treated with α-GalCer, CXCL10, or CXCL16 alone or in combinations. All tumours were harvested by day 17 and stained with CD4, TCRβ, and CD25 to examine the Treg population by flow cytometry. There were no significant differences in the Treg frequencies between the different treatment groups (Fig.23-24). The frequency of Tregs was also examined within the tumour draining lymph nodes, with no significant differences observed. Therefore, it appears that the increase in tumour growth following CXCL16 injection is not due to an increase in the population of Tregs within tumours.

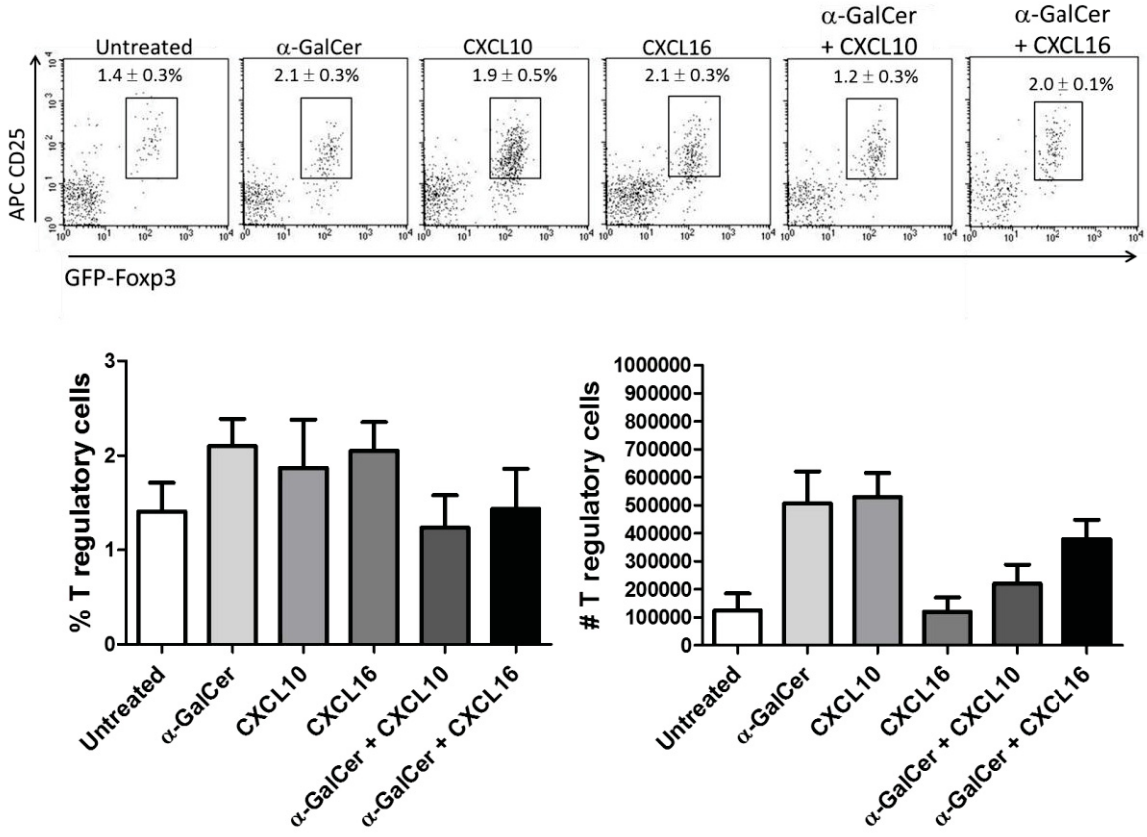


Figure 24. Frequency of T-regulatory cells within B16 tumours of Foxp3-GFP expressing mice. Mice received s.c injections of 2.5 x 10^5 B16 melanoma cells on day 0. On day 11, they received intratumoural injections of 2.0 μg CXCL10, 2.0 μg CXCL16, 4.0 μg α-GalCer or a combination of α-GalCer and chemokine. Repeat injections of chemokine were administered on day 14. All tumours were harvested on day 17. Cells were stained with APC CD25, CD4 and TCRβ to identify Treg populations. FACS plots are gated on the $CD4^+TCR\beta^+$ population. There were no significant differences observed in Treg populations within the different treatment groups. (n= 8 Untreated, 10 α-GalCer, 6 CXCL10, 9 CXCL16, 4 α-GalCer + CXCL10, 4 CXCL16 + α-GalCer).

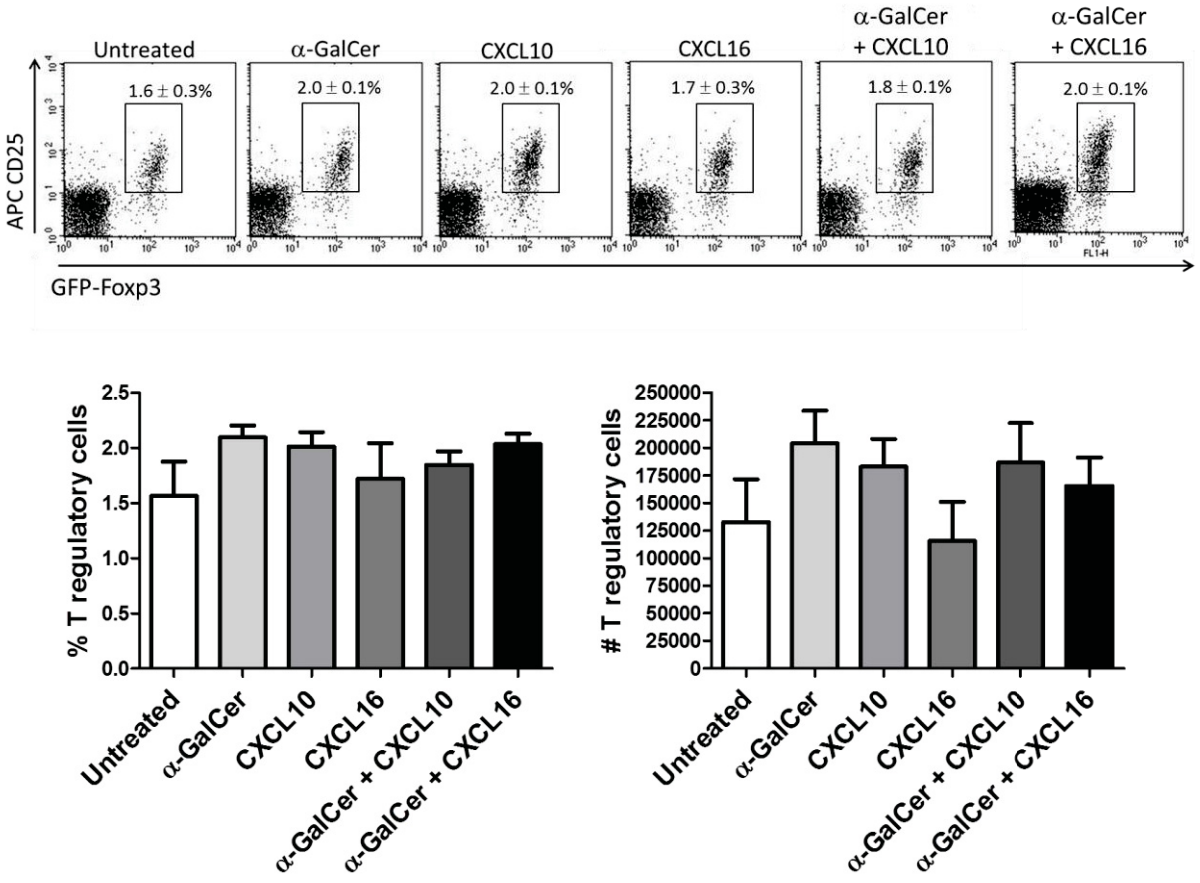


Figure 25. Frequency of Tregulatory cells within DLNs of B16 tumours of Foxp3-GFP expressing mice. Mice received s.c injections of 2.5 x 10^5 B16 melanoma cells on day 0. On day 11, they received intratumoural injections of 10.0 µg CXCL10, 10.0 µg CXCL16, 4.0 µg α-GalCer or a combination of α-GalCer and chemokine. Repeat injections of chemokine were administered on day 14. All tumour-DLNs were harvested on day 17. Cells were stained with APC CD25, CD4 and TCRβ to identify Treg populations. FACS plots are gated on the $CD4^+TCR\beta^+$ population. There were no significant differences observed in Treg populations within the different treatment groups. (n= 8 Untreated, 10 α-GalCer, 6 CXCL10, 9 CXCL16, 4 α-GalCer + CXCL10, 4 CXCL16 + α-GalCer).

3.7 Effect of CXCL16 on tumour cell proliferation

We demonstrated that intratumoural treatment with CXCL16 can enhance tumour growth. This result was not dependent on NKT cells in B16 tumours, and there were no significant increases in either TAMs or Treg cells in CXCL16-treated tumours that could account for such an increase in tumour growth. Previously, it was found that LLC and B16 cells express mRNA for CXCR6 (Germanov 2006 MSc Thesis). We therefore hypothesized that CXCL16 could directly affect tumour cell growth. Tumour cell growth was examined in vitro in cultures supplemented with CXCL16. B16 and LLC cells exhibited slight increases in growth that were not statistically significant (Fig.25).

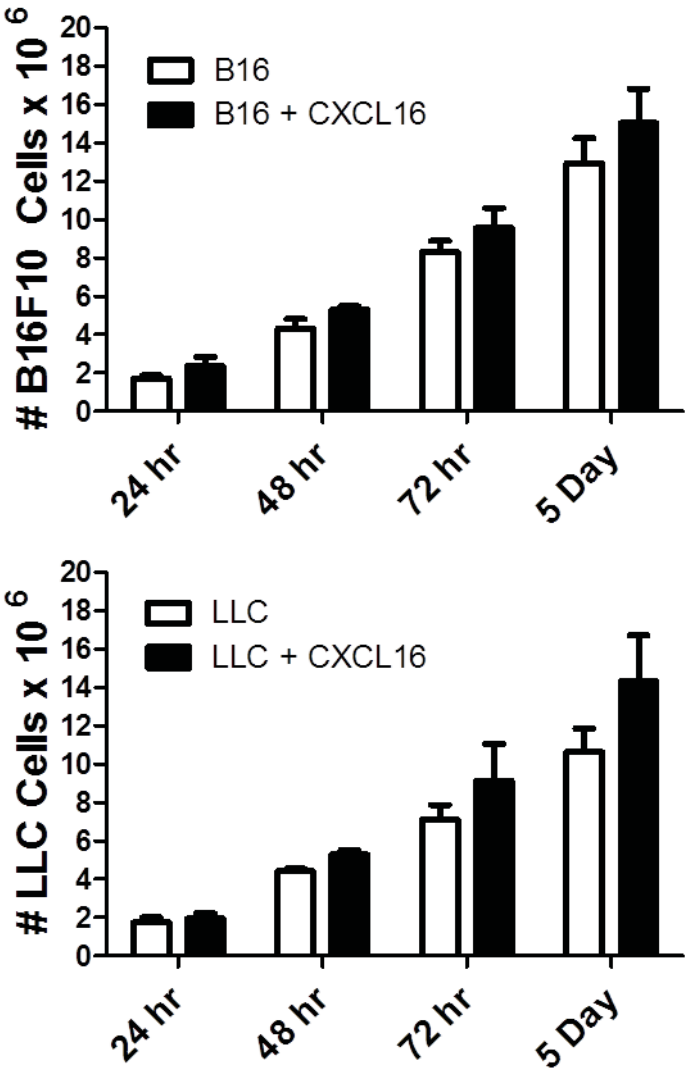


Figure 26. Effect of CXCL16 on in-vitro tumour cell growth rate. B16 F10 Melanoma and LLC tumour cells were cultured in DMEM supplemented with 1% PS and 10% FBS. Half of the cultures were also supplemented with 5.0 µg CXCL16. Cells were collected and counted at 24 hours, 48 hours, 72 hours and 5 days. Following each count, cells were re-plated with fresh media with or without CXCL16. n = 5 per group.

3.8 Expansion and cytokine production of CCR5 deficient mice

Our data demonstrated that α-GalCer-induced NKT cell activation, dramatically enhanced expression of CCR5 (Fig.6). Interestingly, NKT cell chemotaxis towards CCR5 ligands was not significantly increased following α-GalCer activation (Fig 27). However, studies have demonstrated a possible role for CCR5 in regulating NKT cell apoptosis and effector function[242]. Therefore, we wanted to investigate whether CCR5 on NKT cells affects their expansion and cytokine production following α-GalCer activation. To do this, 4.0 μg α-GalCer was injected intraperitaneally into C57BL/6 WT mice and $CCR5^{-/-}$ mice. Mice were sacrificed and their livers and spleens harvested at 2 hours, 24 hours, and 72 hours post-treatment. Cells were stained with TCRβ Ab and CD1d tetramer to identify NKT cell populations. Flow cytometry analysis revealed significant expansion of NKT cells in WT and $CCR5^{-/-}$ mice at the 3 day time point. NKT cells within the liver (Fig. 28) and spleen (Fig. 29) exhibited WT and CCR5 deficient mice exhibited no significant differences in expansion. Intracellular cytokine staining for IL-4 and IFN-γ was also examined at each time point for liver and spleen NKT cells, with no significant differences observed (Fig.30). Serum samples were also analyzed for levels of circulating IL-4 and IFN-γ (Fig.31). Although no differences were observed in serum concentration of IFN-γ, $CCR5^{-/-}$ mice had significantly lower concentrations of IL-4 2 hours post α-GalCer treatment (Fig.31). This suggests that $CCR5^{-/-}$ cells generate less IL-4 per cell than WT cells.

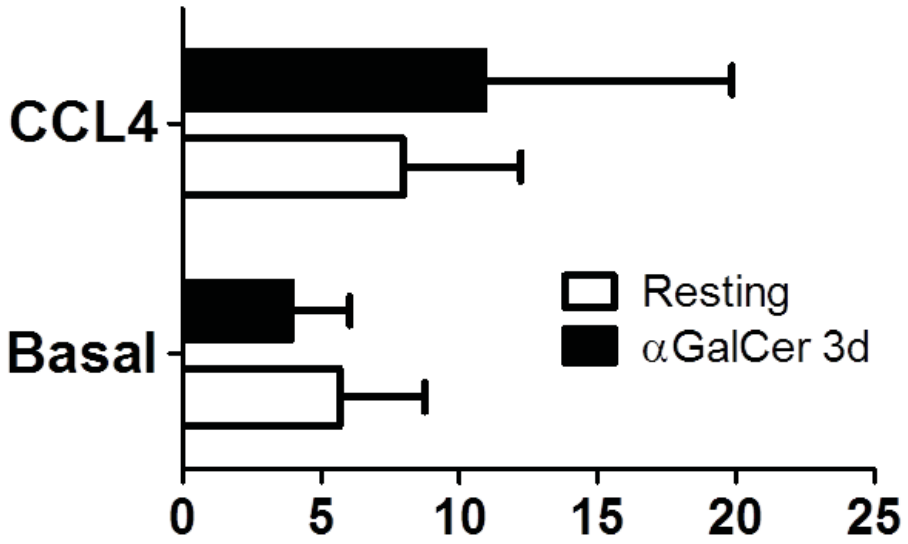


Figure 27. Migration of NKT cells to CCL4 is not affected by activation state. Chemotaxis assays were conducted using resting and 3 day α-GalCer activated lymphocytes from the liver of C57Bl/6 WT mice. Lymphocytes were stained with APC α-GalCer loaded C1d tetramer and FITC-TCRβ to determine the frequency of migrating NKT cells by flow cytometry. (n = 4-5 per group).

Figure 28. Liver NKT cell expansion following systemic activation with α-GalCer in WT and CCR5$^{-/-}$ mice. WT and CCR5$^{-/-}$ mice received intraperitoneal injections of 4.0 ug α-GalCer. Mice were harvested at 2 hours, 24 hours, and 3 days following injections. Livers and spleens were harvested from each mouse and stained with TCRβ Ab and α-GalCer loaded CD1d tetramer to identify NKT cell populations through flow cytometry. n = 3-4 per group. *P<0.05 compared to resting.

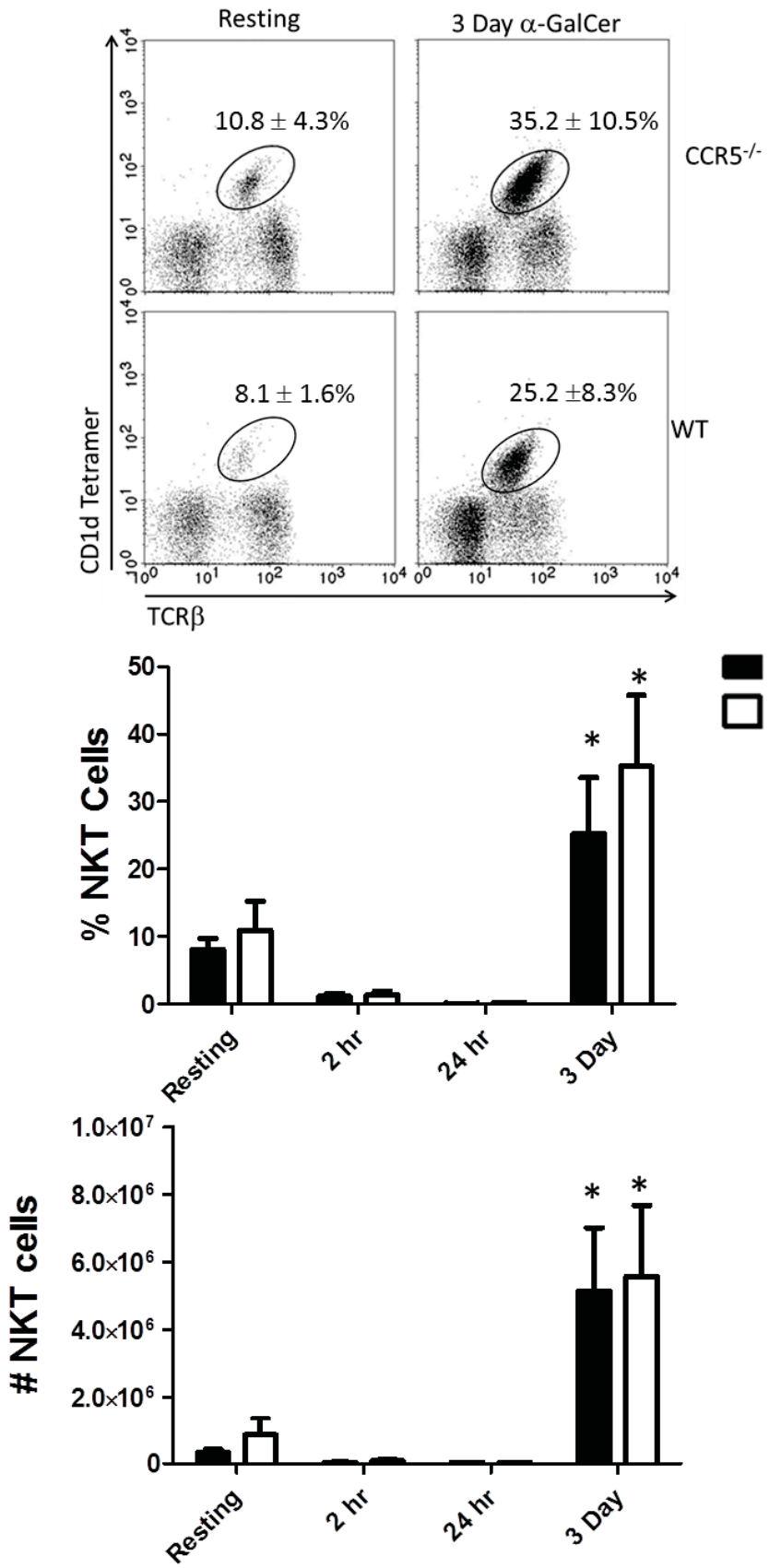
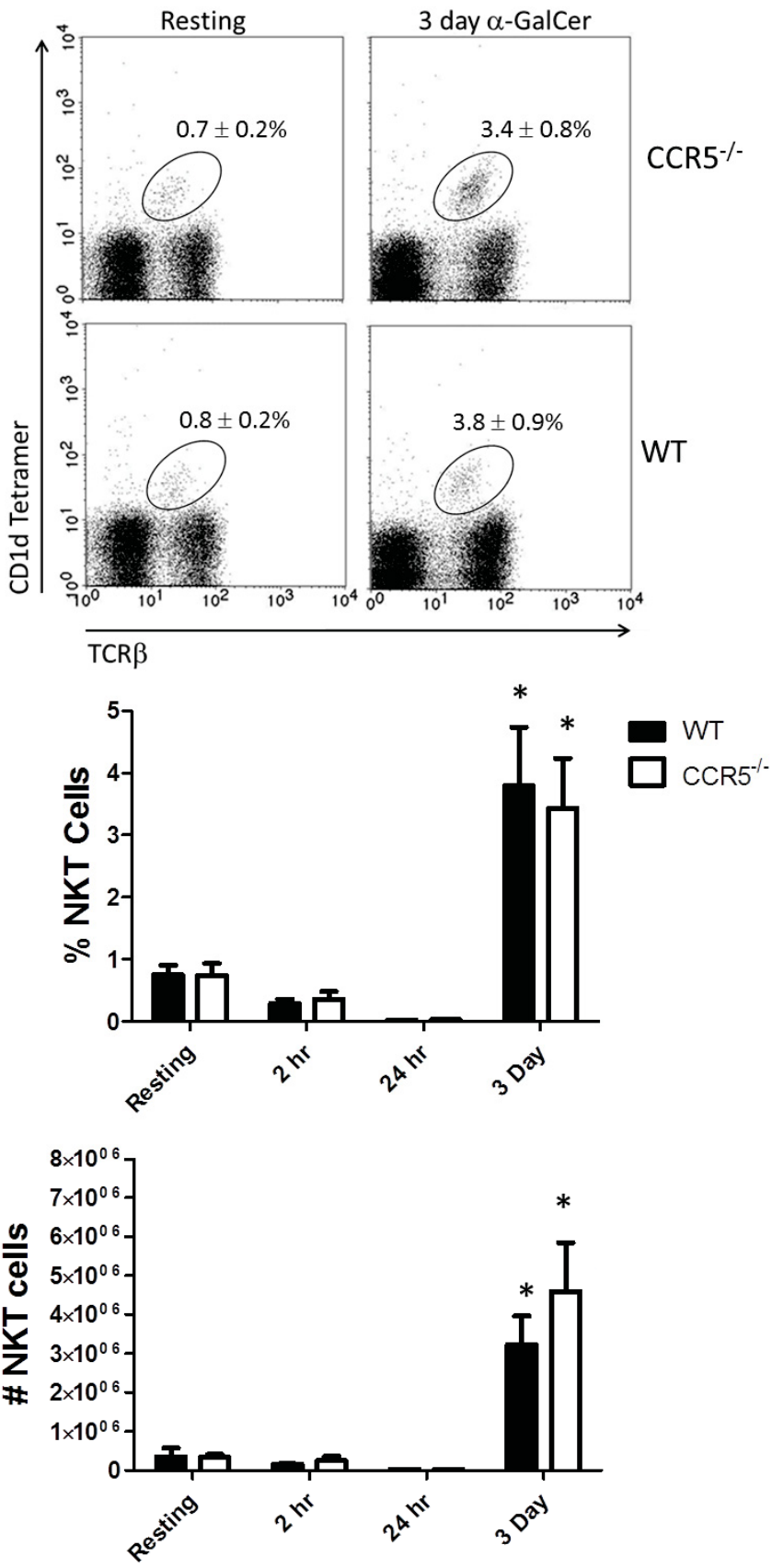

Resting
3 Day α-GalCer
10.8 ± 4.3%
35.2 ± 10.5%
CCR5-/-
8.1 ± 1.6%
25.2 ±8.3%
WT
CD1d Tetramer
TCRβ
WT
CCR5-/-
% NKT Cells
# NKT cells
Resting
2 hr
24 hr
3 Day

Figure 29. Spleen NKT cell expansion following systemic activation with α-GalCer in WT and CCR5$^{-/-}$ mice. WT and CCR5$^{-/-}$ mice received intraperitoneal injections of 4.0 ug α-GalCer. Mice were harvested at 2 hour, 24 hour, and 3 days following injections. Spleens were harvested from each mouse and stained with TCRβ Ab and α-GalCer loaded CD1d tetramer to identify NKT cell populations through flow cytometry. n=3-4 per group. *P<0.05 compared to resting.


Resting
3 day α-GalCer
0.7 ± 0.2%
3.4 ± 0.8%
CCR5-/-
0.8 ± 0.2%
3.8 ± 0.9%
WT
CD1d Tetramer
TCRβ
% NKT Cells
WT
CCR5-/-
Resting
2 hr
24 hr
3 Day
NKT cells
Resting
2 hr
24 hr
3 Day

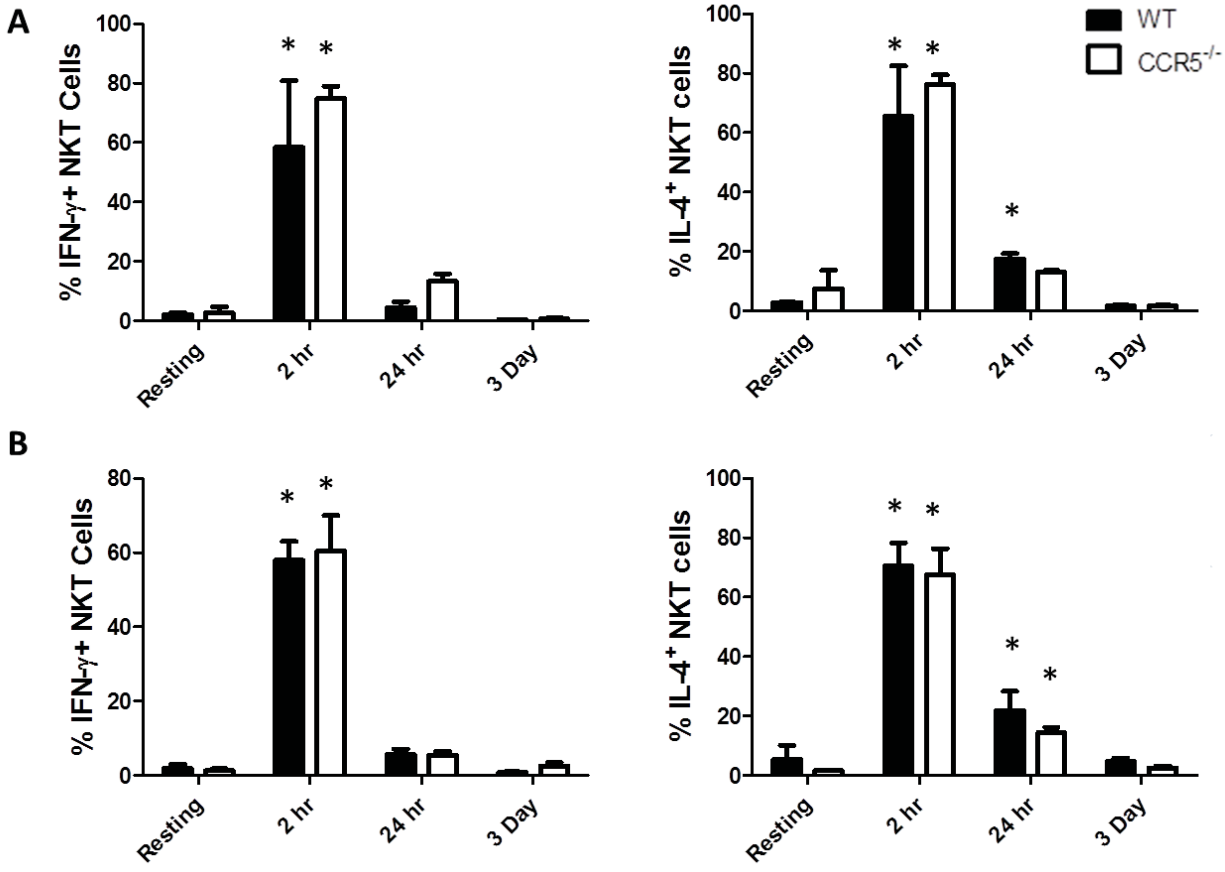


Figure 30. NKT intracellular cytokine production following systemic activation with α-GalCer. WT and CCR5$^{-/-}$ mice received intraperitoneal injections of 4.0 ug α-GalCer. Mice were harvested at 2 hour, 24 hour, and 3 days following injections. Livers (A) and spleens (B) were harvested from each mouse and stained with TCRβ Ab and α-GalCer-loaded CD1d tetramer to identify NKT cell populations, and for intracellular IL-4 and IFN-γ. n=3-4 per group. *P<0.05 compared to resting.

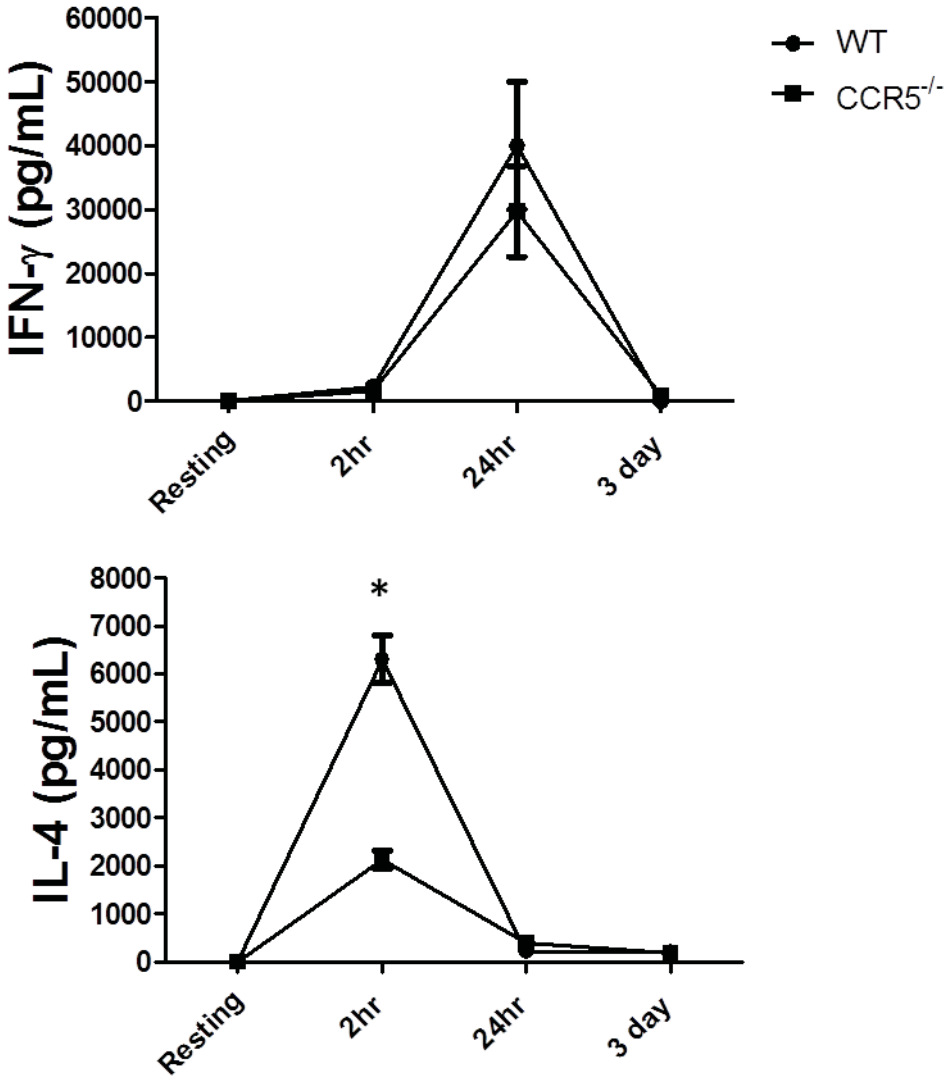


Figure 31. IFN-γ and IL-4 within serum of WT and CCR5$^{-/-}$ mice following systemic α-GalCer injection. Blood samples were collected from the submandibular vein of WT and CCR5$^{-/-}$ mice 2 hours, 24 hours and 3 day following I.P α-GalCer injection. Serum was collected from each and IL-4 and IFN-γ concentration was quantified. n= 2 resting, 3-7 per other groups. *P<0.05 compared to WT.

CHAPTER 4. DISCUSSION

4.1 CXCR3/CXCL10 in NKT cell tumour homing and tumour growth

The accumulation of NKT cells within tumours has been associated with enhanced tumour control and improved prognosis in multiple cancer types[78,79]. Therefore, the objective of this project was to elucidate which chemokines and chemokine receptors contribute to NKT cell recruitment to tumours, and how they can be used to enhance tumour control. The chemokine receptor CXCR3 is expressed on several T cell subsets and is responsible for T cell recruitment to sites of infection and inflammation[250]. Several studies have also indicated that the loss of CXCR3 or one of its ligands can significantly inhibit Th1 and CTL infiltration in several Th1 driven disease models[250]. Therefore, we hypothesized that this receptor could also play a significant role in the recruitment of NKT cells to tumour sites. Analysis of CXCR3 expression on NKT cells revealed that it is very highly expressed; both on resting and α-GalCer activated cells (Fig.6). Therefore, we investigated the role of this receptor further in a tumour model.

We demonstrated that mice deficient in CXCR3 have significantly reduced NKT cell frequency within tumours compared to WT mice (Fig.7). This result is in line with our hypothesis that CXCR3 contributes to the recruitment of NKT cells to tumour sites. As well, this adds to a recent study in which reduced frequencies of $CD4^+$ and $CD8^+$ T cells were found within mammary tumours of $CXCR3^{-/-}$ mice[251]. This study also demonstrated that tumours in $CXCR3^{-/-}$ mice develop faster and were significantly larger by day 14[251]. Furthermore, CXCR3 and its ligands have been shown to be involved in the inhibition of angiogenesis[249]. Therefore, in order to control for the effects of CXCR3 deficiency on other cell types and conclude that the reduction in we observed in NKT cell tumour infiltration is directly due to the loss of CXCR3 on these cells, we conducted co-adoptive transfer experiments. We found that the frequency of $CXCR3^{-/-}$ NKT cells within tumours was significantly lower than that of the WT donor NKT cells, suggesting that this receptor indeed does mediate migration of NKT cells to tumours (Fig. 9). This same pattern was not observed in the other tissues examined (liver, spleen and tumour-DLNs), making it unlikely that the reduced homing is due to impaired cell survival. This is also in accordance with a recent study which demonstrated that the majority of TILs

found within tumour sites expressed CXCR3[252]. Because CXCR3 mediated NKT cell homing to tumours, we next investigated whether the CXCR3 ligands could enhance NKT cell recruitment and tumour control. The three known ligands for CXCR3 are CXCL9, CXCL10 and CXCL11. Previous studies examining the role of these ligands in various inflammatory models have suggested that the requirement for CXCL10 dominates[253]. For example, in dengue virus infection[254] and West Nile virus infection[255] the presence of CXCL9 and CXCL11 could not compensate for a deficiency in CXCL10. As well, in lymphocytic choriomeningitis virus infection it was demonstrated that when mice were treated with CXCL10-neutralizing antibodies, disease progression was abrogated, whereas those treated with CXCL9 neutralizing antibodies showed no change in disease progression[256]. Furthermore, previous studies have shown that high expression of CXCL10 at the tumour site is correlated with increased infiltration of CTLs[257,258]. Therefore, for these experiments we chose to use CXCL10.

Intratumoural injection of CXCL10 or α-GalCer alone did not result in any increases in NKT cell infiltration. However, simultaneous intratumoural injection with CXCL10 and α-GalCer significantly increased NKT cell frequency within tumours (Fig.12). This was replicated in both a LLC tumour model as well as in a 4T1 tumour model using Balb/c mice (Fig.15 and 17). This suggests that both NKT cell activation and expansion through α-GalCer and a strong chemotactic gradient (CXCL10) are required for enhanced NKT cell recruitment. It is possible that CXCL10 injection alone was not sufficient for enhanced NKT cell recruitment due to quick diffusion away from the tumour site. However, the immediate chemokine gradient in combination with rapid NKT cell activation and expansion through α-GalCer could counteract such diffusion. That is, a large number of NKT cells are rapidly recruited to the tumour site, where they are presented with α-GalCer and quickly expand in number. Once in the tumour site, NKT cells are also exposed to tumour glycolipid antigens, which can also result in activation and direct cytotoxic activity (Fig.32).

In addition to increased NKT cell tumour infiltration, CXCL10 and α-GalCer treatment significantly reduced tumour size in both B16 and LLC models (Fig.14 and 16). A slight decrease in tumour size was also observed in the 4T1 model, albeit not

statistically significant (Fig.17). In order to confirm that this reduction in tumour size was the result of enhanced NKT cell tumour infiltration, these experiments were repeated in NKT cell deficient Jα18$^{-/-}$ mice. In both the LLC and B16 tumour models, no differences in tumour size were observed, indicating that the reduction in growth was indeed a result of enhanced NKT cell frequency and activation within the tumour (Fig.14d and 16d). This would suggest that although CXCL10 treatment may result in increased recruitment of other immune cell subsets, these cells on their own are not able to mediate a reduction in tumour growth.

Tumour infiltrating NKT cells likely secrete IFN-γ locally, resulting in the activation and infiltration of other effector cells, such as CTLs and NK cells. Previous studies have shown that enhanced recruitment of CTLs to tumours can mediate tumour regression[259,260] . Moreover, NKT cells can directly attack and kill tumour cells through perforin, TRAIL or FasL[261]. We demonstrated that the absence of CXCR3 on NKT cells significantly decreased recruitment (Fig.7). Reduced NKT cell recruitment will likely result in reduced production of pro-inflammatory cytokines at the tumour site (such as IFN-γ), leading to lower numbers of TILs, reduced cytotoxicity and poor tumour control.

4.2 CXCR6/CXCL16 in NKT cell tumour homing

Several studies have indicated multiple roles for CXCL16/CXCR6 in cancer. This chemokine-receptor pair have conflicting and bidirectional effects on tumour development, growth, and control. The expression of CXCR6 on immune cells can enhance recruitment to tumour sites, where they can exert powerful anti-tumorigenic effects[262]. However, several studies have shown that CXCL16 within tumour sites can also enhance tumour growth[263]. There have been no studies examining what role the CXCR6-CXCL16 chemokine axis plays in NKT cell recruitment to tumours and few studies examining CXCR6-CXCL16 in NKT cell anti-tumour activity[136]. We therefore investigated the possible role of this chemokine/receptor pair in NKT cell tumour infiltration.

Similar to CXCR3, CXCR6 was highly expressed on NKT cells both in resting and activated states (Fig.6). NKT cell infiltration into tumours was also significantly reduced in CXCR6 deficient mice (Fig.7). Previous studies in our lab have demonstrated

that CXCR6 deficient mice have significantly reduced NKT cell numbers in the liver and lungs[182], therefore it was important to control for any differences in NKT cell number using the co-adoptive transfer experiments. Our results revealed that the infiltration of $CXCR6^{-/-}$ NKT cells into tumours was significantly lower than infiltration of WT NKT cells (Fig.10). In order to confirm that this reduced frequency was not a result of reduced survival of receptor deficient cells, cell frequencies were examined within the liver, spleen and DLNs. The liver also had an increased frequency of WT donor cells compared to $CXCR6^{-/-}$, in agreement with previous studies demonstrating that CXCR6 is required for NKT cell accumulation in the liver[182]. There were no significant differences in the spleen. In contrast, DLNs showed a higher proportion of $CXCR6^{-/-}$ cells compared to WT. It is unclear how these cells entered the DLN as NKT cells generally have low expression of L-selection and CCR7 activity required to enter the lymph nodes[225]. Taken together, these results strongly imply that CXCR6 can mediate NKT cell recruitment to tumour sites. Studies have previously demonstrated that CXCR6 is more highly expressed on $CD8^+$ and $CD4^+$ T cells within the tumour compared to in other tissues [264], supporting a role for this receptor in the homing of immune cells to tumour sites.

We then investigated whether intratumoural administration of CXCL16 could enhance NKT cell recruitment to tumours. Treatment with CXCL16 alone did not affect the accumulation of NKT cells in B16 or LLC tumours (Fig.18 and 20). However, when tumours were treated simultaneously with CXCL16 and α-GalCer, the frequency of NKT cells dramatically increased. This phenomenon is likely due to a combination of expanded NKT cells in response to α-GalCer presentation, and a strong chemokine gradient provided by CXCL16. In contrast to our observations with CXCL10 and α-GalCer, a combination treatment with CXCL16 and α-GalCer did not result in reduced tumour size. This implies that there is another mechanism at work which counteracts the protective functions provided by the increased NKT cell infiltration. Most interestingly, treatment of tumours with CXCL16 alone resulted in a trend towards enhanced tumour development (Fig. 19). This is in line with a study in patients with colorectal cancer, which demonstrated that high serum levels of CXCL16 are associated with tumour cell proliferation, invasive growth and poor prognosis [265]. Interestingly, it has also been

demonstrated that CXCL16 in colorectal cancer patients leads to increased frequency of $CD4^+$ and $CD8^+$ TILs[266]. These studies mirror what was observed in our experiments, where it appears that intratumoural injection of CXCL16 leads to enhanced tumour growth despite increased accumulation of anti-tumour immune cells. In accordance with this, it has been found that although CXCL16 in serum of ovarian cancer patients is an indicator of poor prognosis, this is not correlated with immune cell tumour infiltration[263]. Thus it appears that two independent pathways are at work. One in which CXCL16 and α-GalCer provide a chemotactic gradient and NKT cell expansion required for increased tumour infiltration, and another involving direct signalling of CXCL16 on tumour cells and/or CXCL16-mediated angiogenesis, resulting in enhanced growth (Fig.30). In studies where intratumoural CXCL16 has been associated with prolonged survival, the benefits were a result of enhanced T lymphocyte recruitment to tumours[266]. In our study, we demonstrated that injection of CXCL16 in B16 melanoma tumours of NKT cell deficient mice also resulted in an increased rate of tumour growth, indicating that the effect is not NKT cell dependent. Rather, the increased tumour growth may be a result of direct CXCL16 signalling on tumour cells or a result of increased angiogenesis mediated by CXCL16. These possibilities will be discussed further in the following section.

The CXCR6-CXCL16 signalling axis also plays important roles in NKT-DC co-stimulation. DCs presenting glycolipid antigen to NKT cells upregulate expression of transmembrane CXCL16, which then interacts with CXCR6 on NKT cells to enhance IFN-γ production [267]. Intratumoural CXCL16 in our treatment models may have various effects on this co-stimulation. Soluble CXCL16 may signal on NKT cells in addition to transmembrane (Tm)-CXCL16 signalling from DCs, thus resulting in enhanced co-stimulation and increased NKT cell activation and cytokine production. Indeed, previous studies in our lab have shown that when CXCL16 protein is added to cultures of liver mononuclear cells plated with αCD3, IFN-γ production by NKT cells significantly increases (our unpublished data). This suggests that CXCL16 is able to polarize NKT cells toward a Th1 immune response. This is supported by a previous study which demonstrated that mice deficient for CXCL16 had reduced IFN-γ production following stimulation with α-GalCer[268]. These data may partially explain why we observed

significant increases in NKT cell frequency in mice receiving a combination treatment of α-GalCer and CXCL16, but not in those receiving CXCL16 alone. In the combination treatment, α-GalCer is loaded onto DCs and presented to NKT cells, resulting in TCR ligation. Co-stimulation between CXCR6 on NKT cells and CXCL16 on DCS will then be further amplified by the injected CXCL16, which in addition to enhancing IFN-γ production could also result in enhanced NKT cell activation, expansion, and recruitment.

4.3 CXCL16 in tumour growth and proliferation

Intratumoural treatment of B16 and LLC tumours with CXCL16 alone resulted in increased tumour size. This observation could be explained by the results of several recent studies which have suggested that CXCL16 has several pro-tumorigenic functions, resulting in enhanced tumour growth. For example, in studies examining CXCL16 in prostate cancer, it was found that high expression of CXCR6 and CXCL16 was correlated with more aggressive tumours [229,269,270]. In addition, in patients with colorectal cancer, high serum levels of CXCL16 are associated with tumour cell proliferation, invasive growth and poor prognosis[265]. Furthermore, recent studies have identified a role for CXCL16 in angiogenesis[271,272]. In a model of rheumatoid arthritis, it was demonstrated that CXCL16 was an important factor in endothelial cell (EC) angiogenesis[272]. CXCL16 acts as a strong agonist of angiogenesis by mediating human dermal microvascular endothelial cell (HMVEC) recruitment and incorporation, as well as vasculogenesis through its ability to mediate endothelial progenitor cell (EPC) migration[272]. Thus, the increased tumour size that we observed in our tumour models may be due to enhanced angiogenesis mediated by the intratumoural CXCL16. There is also evidence that CXCL16 can lead to upregulation of CXCL16 mRNA expression, thus any effects of the injected CXCL16 could be further enhanced by this amplification[272].

In contrast to B16 melanoma tumours, treatment with CXCL16 in LLC tumours from Jα18$^{-/-}$ (NKT cell deficient mice) did not result in any significant changes in tumour growth (Fig.21). This is likely due to differences between tumour microenvironments of B16 and LLC tumours. Our data suggests that CXCL16 treatment contributes to a pro-tumorigenic role of NKT cells in LLC tumours, whereas the CXCL16-mediated tumour growth observed in B16 melanoma is not NKT cell dependent. Future experiments will

focus on determining what effects intratumourally injected CXCL16 has on NKT cells and how these can lead to enhanced tumour development.

Evidence for a role of CXCR6/CXCL16 in cancer cell proliferation and tumour progression also comes from a study by Wang et al which demonstrated that expression of mRNA for this chemokine receptor pair is higher in more aggressive cancers[206,273]. Moreover, it has been demonstrated that soluble CXCL16 can enhance proliferation of cancer cells expressing CXCR6 in vitro[229]. In renal cell carcinoma[274], colorectal cancer[266], pancreatic ductal adenocarcinoma[275], nasopharyngeal carcinoma[276], and melanoma[277], the expression of CXCR6 and CXCL16 was found to be consistently higher than that on their respective non cancer tissue cells[231]. When tumours are established with cells expressing reduced levels of CXCR6, tumour growth was significantly decreased[273]. To investigate whether CXCL16 can act directly on tumour cells to promote growth and expansion, we performed in vitro experiments in which LLC and B16 tumour cells were cultured with CXCL16. Although we observed no significant differences in cell number between those cultured with CXCL16 and those without, there was a consistent trend for slightly higher numbers of cells in cultures supplemented with CXCL16 (Fig.25). Production of CXCL16 can also occur naturally in in vivo tumour models. In glioblastomas, proliferating tumour cells produce significant quantities of CXCL16[278], suggesting that CXCL16 and CXCR6 on tumour cells may function in an autocrine loop, providing signals for growth and development. In support of this, CXCL16 and/or CXCR6 have been found to be constitutively expressed on several tumour cell lines, including prostate[279], breast[280], nasopharyngeal[276], and pancreatic cancer [275]. Thus, it would be expected that supplementation of culture media with CXCL16 may result in enhanced tumour cell proliferation. It is possible that a higher concentration of CXCL16 is needed in order to significantly enhance cell proliferation of the B16 and LLC lines in vitro. In the future, several concentrations will need to be tested to determine the optimal amount of CXCL16 needed to induce tumour cell proliferation. It may also be possible that CXCL16 is not directly affecting proliferation of tumour cells, but rather playing a pro-angiogenic role in the tumour microenvironment. Therefore, it will be important to determine whether direct CXCR6-CXCL16 signalling on tumour cells or CXCL16-mediated angiogenesis is responsible for the observed

increase in tumour growth. One way of determining the role of CXCL16 in angiogenesis is through the use of vascular tube formation assays. In these assays, endothelial cells (ECs) would be cultured at sub-confluent densities with appropriate ECM with or without added CXCL16. We would then measure the growth of any capillary-like structures (tubes) in order to determine pro-angiogenic functions mediated by CXCL16.

4.4 Treg and TAM accumulation following glycolipid activation and CXCL10/CXCL16 treatment

Enhanced tumour growth in CXCL16 treated tumours may also be a result of the recruitment of immunosuppressive cell subtypes. Therefore, we examined the recruitment of Tregs and TAMs to tumours which received each treatment. The accumulation of immunosuppressive cell subsets, such as Tregs or M2 TAMs within tumours has been associated with enhanced tumour growth and poor prognosis[281,97]. We examined the accumulation of Treg cells and TAMs within B16 tumours following treatments with CXCL10, CXCL16, and α-GalCer alone or in combination. Although there were no significant differences in TAM accumulation between the different treatment groups, there was a slight increase in TAM frequency within CXCL10 treated tumours (Fig.22). CXCR3 has been shown to be expressed on macrophages, and some animal disease models have demonstrated that CXCR3 is required for macrophage recruitment and activation[282,283]. This would explain why we observed slightly higher TAM frequency within CXCL10 treated mice. Once infiltrated into the tumours, TAMs often differentiate into the alternatively activated M2 phenotype. This is in response to signals derived from the tumour microenvironment, including IL-4, IL-10, IL-13 and TGF-β[281,284]. This process in turn can lead to enhanced tumour progression. TAMs can also promote tumour growth and progression through other mechanisms, including through facilitating angiogenesis[285] and promoting tumour cell invasion and metastasis [286].

It has been demonstrated that activated NKT cells have the ability to significantly reduce the frequency and function of immunosuppressive cells such as MDSCs in multiple models[113,287,288]. In an influenza A virus infection model, NKT cells were able to abolish the suppressive activity of MDSCs[287]. Gebremeskel at al. demonstrated that NKT cell activation results in significantly decreased MDSCs in the circulation of tumour

bearing mice[113]. Furthermore, stimulation with activated NKTs cells has previously been shown to induce changes in phenotypic and maturation markers on MDSCs, suggesting that NKT cells can mediate MDSC maturation into DCs[288]. Thus, it is possible that the increased frequency of activated NKT cells within treated tumours may be altering the phenotype and immunosuppressive function of TAMs. Because of overlapping phenotypic and functional markers of M2 TAMs and MDSCs, it is difficult to conclude whether the populations reported here consist of TAMS, MDSCs, or a combination of both. In the future, it will be important to determine whether treatment with glycolipid and/or chemokine changes the proportion of macrophages with an M1 vs M2 phenotype. TAMs will also be isolated from tumours that received various treatments (CXCL10, CXCL16, α-GalCer and combinations) and tested to measure their immunosuppressive ability. This can be done by co-culturing harvested TAMs with naïve T cells and anti-CD3/CD28-coated beads to examine suppressive effects on T cell activation and proliferation. We will also examine the cytokine production of TAMs. High levels of IL-12 are indicative of a M1 phenotype, while M2 macrophages are unable to produce IL-12 and can instead release IL-10[289]. Such experiments will allow us to conclude whether our intratumoural treatments are able to alter the frequency and function of immunosuppressive myeloid cell subtypes in tumour environments.

Treatment with CXCL16 or α-GalCer did not have any significant effects on macrophage accumulation in tumours. We showed that CXCL16 intratumoural treatment leads to enhanced tumour growth. Since we could not explain this observation through enhanced TAM recruitment, we also examined Treg accumulation into tumours. We observed no differences in Treg frequency within either the tumours or tumour-DLNs within the different treatment groups, making it unlikely that the increased tumour size observed in CXCL16-treated tumours is a result of Treg recruitment (Fig.23 and 24). This observation is in contrast to a previous study implicating a role for CXCR6 in Treg recruitment to tumours[264]; however, this may be due to differing tumour models used. While our experiments examine Treg infiltration into murine B16 melanoma tumours, theirs used samples from patients with nasopharyngeal carcinoma.

Recent studies have indicated that NKT cells can influence and modulate Tregs, and Tregs in turn can modulate NKT cell activity[290]. In certain models, $CD4^+$ NKT cells have been demonstrated to be important promoters of Treg differentiation[291,292]. Following Treg differentiation, NKT cells no longer have an effect on their immunosuppressive ability[291]. This was found to be a result of IL-2 production by activated NKT cells. That is, stimulation through α-GalCer-loaded DCs led to NKT cell secretion of IL-2, which in turn promotes Treg proliferation[292]. Given this, it is expected that the increased frequency of NKT cells within chemokine and α-GalCer treated tumours may also lead to a proportional increase in Treg frequency. However, no such increase was observed. It is possible that an increase may have been observed at a different time point. All of our studies examined NKT cell tumour infiltration at 3 and 6 day time points following tumour treatment. If increased NKT cell frequency is still present at later time points, it is possible that Treg frequency is also increased. Extending time points was difficult using our experimental model, as initial injections were performed when tumours were palpable, and the fast rate of growth of B16 and LLC subcutaneous tumours limited the experimental run following treatment. As well, other studies have provided evidence that Treg development is actually impeded by NKT cells. It has been demonstrated that in α-GalCer treated mice the Treg population is significantly decreased, with no changes reported in NKT cell-deficient CD1d deficient mice[293]. Therefore, further studies are needed to determine conclusively what role the interactions of Tregs and NKT cells have in tumour microenvironments.

In addition to the modulation reported between Treg cells and NKT cells, there is also modulation occurring between MDSCs and Tregs. MDSCs have the ability to promote the expansion of Treg cells through production of IL-10, TGF-β and CD40-CD40L interactions[109]. Despite this, we did not observe any obvious patterns linking NKT cell and immunosuppressive cell accumulation within tumours. It is possible that a correlation between these different cell types may be observed at extended time points, which could not be examined in our experimental model.

4.5 CCR5 in NKT cell tumour infiltration

The receptor CCR5 is expressed on a multitude of immune cells, including Th1 cells, $CD8^+$T cells, monocytes and macrophages[235]. In addition, it is expressed by certain tumour cell lines[235]. The three chemokines to which it can bind, CCL3, CCL4 and CCL5, all have extensive involvement in the recruitment of immune cells to sites of injury or infection[294]. Therefore, we wished to examine the role of this receptor in NKT cell homing to tumours. We demonstrated that CCR5 is upregulated on NKT cells following 3 day stimulation with α-GalCer (Fig.6). In comparison to WT mice, CCR5-deficient mice had significantly decreased frequencies of NKT cells within tumours (Fig.7). In order to control for the absence of CCR5 on other cells, we again performed co-adoptive transfer experiments, where equal numbers of sorted NKT cells from WT and CCR5 KO mice were co-injected into tumour bearing NKT cell-deficient mice. Examination of the frequency of each donor cell within the tumours of recipient mice revealed a very high ratio of WT to $CCR5^{-/-}$ NKT cells (Fig.10). This strongly suggests that CCR5 plays an important role in mediating NKT cell recruitment to tumours. Examination of the donor cell ratio in the liver, spleen and tumour draining lymph nodes revealed higher proportions of WT donor cells in the liver, with approximately equal ratios in the spleen and DLN. Thus, CCR5 also plays a novel role in mediating NKT cell accumulation in the liver. A role for CCR5 in homing of immune cells to tumours is supported by a recent study demonstrating that CCR5 expression was significantly increased on tumour infiltrating $CD8^+$ T cells[244]. As well, Parsonage et al. demonstrated that CCR5 is expressed consistently on infiltrating $CD8^+$ and $CD4^+$ T cells, resulting in enrichment of $CD8^+$ $CCR5^+$ and $CD4^+CCR5^+$ (non- regulatory) T cells at tumour sites[264]. These studies, in combination with our results showing decreased tumour homing of $CCR5^{-/-}$ NKT cells to tumours, would suggest that the presence of the CCR5 ligands CCL3, CCL4 or CCL5 within tumour sites may lead to increased recruitment of NKT cells and therefore enhanced tumour control. However, Schlecker et al.[244] demonstrated that tumour infiltrating MDSCs are capable of producing large quantities of these ligands, which leads to the recruitment of Tregs in a CCR5 dependent manner. Indeed, CCR5 deficient mice had significantly decreased levels of Treg accumulation within tumours and this was associated with reduced tumour growth. Therefore the presence of CCR5 ligands

within tumours likely has two major diverging effects, both pro-tumorigenic and anti-tumorigenic. That is, production of these chemokines will likely lead to enhanced migration of NKT cells, which can have strong anti-tumorigenic functions. However, large quantities of immunosuppressive CCR5 expressing Treg cells could also be recruited, and may counteract the anti-tumorigenic functions of recruited NKT cells. Indeed, in a study using murine mammary adenocarcinoma model, it was found that inhibition of CCR5 signalling through a peptide antagonist resulted in reduced frequencies of TILs in tumours, supporting a role for this receptor in lymphocyte tumour infiltration. However, the same mice also showed overall reduced tumour growth, suggesting that the blockade also affected infiltration of immunosuppressive subsets, such as Tregs, resulting in slowed tumour progression[295].

4.6 CCR5 in NKT cell activation and cytokine production

Our experiments demonstrated that systemic activation of NKT cells using α-GalCer increased the expression of CCR5, from approximately 30% to over 90% in the liver (Fig.6). In-vitro chemotaxis assays, however, showed no significant difference in the ability of activated and resting NKT cells to migrate toward the CCR5 ligand CCL4 (Fig.27). This may be due to the type of Gα subunit coupling involved in CCR5 signalling. Previous studies have demonstrated that ligand binding to CCR5 resulted in to G_q and/or G_{11} coupling[296]. Coupling of chemokine receptors to G_q and/or G_{11} is associated with chemokine-induced cell adhesiveness more so than cell chemotaxis[297], therefore this may explain why we observed no difference in NKT cell migration in spite of increased CCR5 expression. Further studies examining Gα protein coupling in response to CCR5 ligation will need to be conducted to confirm if this is also true in NKT cells. We also hypothesized that CCR5 may play a regulatory role on NKT cells. Indeed, a study conducted by Ajuebor et al.[242] supported this hypothesis. They observed that in a hepatitis model, CD1d restricted NKT cells deficient for CCR5 were resistant to apoptosis following treatment with Concanavalin A (con A). We therefore examined whether CCR5 expression on NKT cells can also influence their expansion and cytokine production following activation with α-GalCer.

WT and CCR5 deficient mice received intraperitoneal injections of α-GalCer. NKT cell populations were examined within their livers and spleens 2 hours, 24 hours and 72 hours later. Dramatic decreases in NKT cell frequency were observed at the 24 hour time point in both WT and CCR5 deficient mice. This is not surprising, as it has been demonstrated that NKT cell markers including TCRβ are internalized from approximately 4 hours to 48 hours following activation[298]. At the 3 day time point, both CCR5 and WT mice exhibited significant increases in both the frequency and absolute number of NKT cells within the liver and spleen. In the liver, CCR5 KO NKT cells showed slightly increased expansion, although not statistically significant. This is consistent with the similar NKT cell frequencies observed in CCR5 KO and WT naïve mice by Ajuebor et al[299].

Cytokine production by WT and $CCR5^{-/-}$ NKT cells was also examined at each time point. Intracellular staining of NKT cells for IL-4 and IFN-γ within the liver and spleen showed no differences between WT and $CCR5^{-/-}$ NKT cells. Ajuebor et al[299] previously found that $CCR5^{-/-}$ NKT cells produced higher amounts of IL-4 21 hours post α-GalCer treatment; however, this was not shown in an in vivo system. Although our data does not show any increase in intracellular IL-4 on NKT cells in CCR5 KO mice, serum analysis revealed dramatically lower levels of IL-4 from CCR5 deficient mice following 2 hour α-GalCer activation (Fig.31). This suggests reduced levels of IL-4 generation by $CCR5^{-/-}$ NKT cells. Alternatively, this result may be due to increased utilization of IL-4 by CCR5 deficient cells. That is, it is possible that CCR5 deficient mice have increased expression of IL-4 receptor (IL-4R) or increased frequency of cells which have high expression of IL-4R, , leading to increased IL-4 uptake and reduced levels in the serum. These experiments will need to be repeated to confirm these results. It will also be important to examine the intracellular expression of these cytokines in other immune cell subsets of $CCR5^{-/-}$ vs WT mice, in order to determine the source of the low IL-4 levels in CCR5 deficient mice. If the reduced concentration of serum IL-4 in $CCR5^{-/-}$ mice is due to a deficiency in IL-4 production in NKT cells, then it is possible that the recruitment of $CCR5^{-/-}$ NKT cells into tumours (through CXCR3 or CXCR6) could lead to a Th1-skewed immune response and enhanced anti-tumour activity. One

method of determining the contribution of NKT cells to the change in IL-4 production is through the adoptive transfer of IL-$4^{-/-}$ NKT cells into double knockout $CCR5^{-/-}J\alpha18^{-/-}$ and $J\alpha18^{-/-}$ mice. Following adoptive transfer, both $CCR5^{-/-}J\alpha18^{-/-}$ and $J\alpha18^{-/-}$ mice would be activated through intraperitoneal injection of α-GalCer. If there are still significant differences in the serum IL-4 concentration, it can be concluded that these differences are not due to NKT cells, but cells activated indirectly following α-GalCer NKT cell activation.

4.7 Models

In order to confirm that our results were not specific to only a single tumour model or mouse strain, we extended our experimental design to three models, LLC and B16 in C57BL/6 mice, and 4T1 in Balb/c mice. Although we observed fairly consistent results between these models, each presented specific challenges in experimental design and data interpretation.

The B16 melanoma model was used for the majority of experiments presented in this body of work. Out of the three models used, B16 melanoma tumours were the most simple from which to extract different lymphocyte subsets. These tumours are soft and malleable, and the use of a simple Percoll gradient allows for significant enrichment of tumour infiltrating-leukocytes. One of the most significant challenges in working with this model is that the tumour growth was often inconsistent, even within the same treatment groups. For this reason, experiments were repeated multiple times to confirm significance of the results. In order to limit inconsistencies in tumour growth, care was taken to only use cells from cultures that were passaged no more than 10 times, and all cells were injected 24 hours post passaging, to ensure that cells were at the logarithmic growth stage. Another challenge encountered with this model is that although significant differences between NKT cells were observed in the different treatment groups, the overall number of NKT cells isolated from each tumour was very low. This proved to be a challenge, especially for the co-adoptive transfer experiments. Although 6-10 x 10^6 cells were transferred into each recipient mouse, only a small fraction of these cells were recovered from each tissue, with particularly low numbers recovered from the tumours. However, it has been demonstrated that very few NKT cells are required to provide

significant tumour control[79]. In future experiments, it may be possible to reconstitute NKT cell deficient mice with an equal number of receptor KO cells and WT NKT cells prior to tumour cell injection. In this case, cancer cells will only be injected after the NKT cell deficient mice have reached near WT levels of NKT cell reconstitution, and larger numbers of NKT cells can be isolated from the tumours. However, such an experimental design may also lead to uneven cell numbers, as WT cells may show improved survival long-term compared to receptor deficient cells. Viability of adoptively transferred WT and receptor deficient NKT cells will therefore have to be determined prior to such experiments.

The LLC and 4T1 tumour models exhibited much more consistent tumour development in comparison to B16 tumours. However, these tumours are very rigid and it was difficult to isolate significant numbers of lymphocytes from them. Due to this, very low numbers of NKT cells were detected in each, making data interpretation difficult. The Balb/c mice used for the 4T1 tumour model also showed great sensitivity following intratumoural injection, and many had to be sacrificed prior to experiment completion due to distress. A reduction in the injection volume in future experiments may help to alleviate this. The 4T1 tumour model also did not result in a significantly reduced tumour size following CXCL10 and α-GalCer treatments, which was in contrast to the B16 and LLC tumour models. However, a slight decrease in tumour size was observed. Therefore, experiments will need to be repeated, or the time course altered in order to establish the statistical significance of this effect. Previous experiments in our lab have demonstrated that treatment with α-GalCer loaded DCs following resection of primary 4T1 mammory tumours resulted in significant protection from metastasis[113], supporting a role for activation and expansion of NKT cells in tumour control in our model.

A general concern when using any murine model to study NKT cell biology is the differences in NKT cell frequency between mice and humans. Mice have significantly higher frequencies of NKT cells compared to humans[300], which can make it difficult to relate murine studies to the clinical situation in humans. However, despite low levels of NKT cells in humans, several clinical studies have demonstrated that they can contribute significantly to tumour control and improved prognosis[78,79].

4.8 Clinical Applications

The presence of NKT cells within tumours of cancer patients has been shown in multiple studies to be correlated within increased cancer control and prolonged survival[78,79]. For this reason, it is important to elucidate the ways in which NKT cell infiltration can be enhanced in cancer sites. In this body of work, we identified three receptors, CXCR3, CXCR6 and CCR5 which can contribute to NKT cell recruitment into tumours. Most significantly, we demonstrated that in a murine model, CXCL10 treatment in combination with α-GalCer activation effectively slowed tumour growth. It has been previously demonstrated that CXCL10 within tumour sites is associated with favorable prognosis in patients with colorectal cancer[258]. Several studies have also indicated that treatment of patients with α-GalCer can lead to favorable results. For example, in phase 1 clinical trials it was demonstrated in patients with head and neck cancer, treatment with α-GalCer loaded DCs could induce significant NKT cell anti-tumour activity and reductions in tumour size[301]. Similarly, NKT cell activation in a subset of patients with non-small cell lung carcinoma lead to a significant increase in survival time[302]. Investigation of these treatments in combination therapies has not yet been conducted, and our results would suggest that a combined therapy may provide the most beneficial results for patients. Such a therapy would be most beneficial to patients with non-resectable or recurrent solid tumours. It would also be important to determine whether the increased tumour control observed in CXCL10 and α-GalCer treated tumours is a result of local or systemic effects. One way of determining this would be to observe two s.c tumours on a single mouse, treating one with CXCL10 and α-GalCer and leaving the other untreated, and determine any differences in control of the untreated tumour. This would allow us to resolve whether patients with multiple tumours could benefit from such a treatment, or if multiple injection sites would be necessary and realistic.

Our experiments also examined the role of CXCL16/CXCR6 in NKT cell tumour homing and revealed that treatments enhanced NKT cell tumour infiltration; however, this will not necessarily lead to beneficial outcomes. Because CXCR6 and CXCL16 are also expressed on many tumour cells, there is danger that such a treatment would lead to tumour proliferation and growth, counteracting any beneficial effects of immune cell

tumour infiltration. Therefore, extensive studies will need to be performed examining the dual roles of CXCL16 in tumours. It is likely that CXCL16 treatment in combination with α-GalCer would be most beneficial to patients with tumours that do not express CXCR6 or CXCL16. Such a treatment would lead to enhanced NKT cell tumour infiltration, while unwanted signalling through tumour cells would not occur. However, if the increased tumour growth observed in CXCL16 treated tumours is a result of angiogenesis rather than direct signalling, then this could enhance tumour growth. More studies will need to be conducted in order to elucidate specific cancer types where such a treatment could be most beneficial.

We demonstrated that CCR5 is also able to mediate NKT cell recruitment to tumour sites, suggesting that this receptor and its ligands have the potential to promote anti-tumorigenic functions. However, previous reports have also identified a role for this receptor and its ligands in the accumulation of immunosuppressive MDSCs and Tregs within tumours, leading to enhanced tumour growth[243,244] . Thus it may be possible that any benefits derived from increased NKT cell recruitment could be counteracted by simultaneous recruitment of immunosuppressive cell subsets. It is therefore vital that the role of this receptor and its ligands is examined thoroughly in order to elucidate both possible therapeutic functions as well as unintended side effects.

4.9 Future Directions

We have demonstrated that multiple chemokines and chemokine receptors contribute to NKT cell accumulation in tumours. However, our experiments do not reveal whether receptors contribute to accumulation in an additive or overlapping way. To address this, we have begun to generate $CXCR3^{-/-}CXCR6^{-/-}$ double knockout mice. Adoptive transfer experiments will allow us to determine whether the homing abilities provided by these two receptors are additive. In addition, our experiments cannot differentiate between receptors that mediate homing into tumours versus receptors that may retain NKT cells within the tumour site. Spinning disk intravital confocal microscopy may be a useful technique to examine recruitment of WT and chemokine receptor-deficient NKT cells from the blood. We will need to further explore the specific mechanism(s) behind the synergistic action of chemokine in combination with glycolipid.

Enhanced migration of NKT cells was observed both when CXCL10 or CXCL16 were used in combination with glycolipid. Therefore, it is important to understand the signalling processes occurring at the tumour site which are contributing to this effect. We also observed significantly higher proportions of NKT cells within lymph nodes of α-GalCer and CXCL10/CXCL16 treated mice. This may be due to DCs which load α-GalCer and travel to the DLN where they present the glycolipid to NKT cells. NKT cells then rapidly expand in number and can migrate to the tumour in response to the chemokine gradient. Further experiments will also examine the other CXCR3 ligands, CXCL9 and CXCL11, and determine whether these could also mediate NKT cell tumour homing, with or without NKT cell activation with α-GalCer.

We were able to demonstrate that the increased NKT cell accumulation in CXCL10/α-GalCer treated tumours was not limited to C57BL/6 mouse models as similar observations were made in Balb/c mice bearing 4T1 tumours. In the future it will be important to repeat these experiments and determine whether this increase in NKT cell frequency also leads to a significant decrease in tumour size. As well, we will repeat these experiments in NKT cell deficient Balb/c mice and determine whether any differences in tumour size are NKT cell dependent. Tumour treatments using CXCL16 and α-GalCer will also need to be conducted using this model to determine whether the results observed in our LLC and B16 models can be extended beyond the C57BL/6 mouse models.

Intratumoural treatment with CXCL16 resulted in significant increases in tumour size, and similar results have been reported in multiple tumour models. Future studies will focus on identifying the specific mechanisms causing this increase in tumour development. We attempted to determine differences in tumour cell proliferation between CXCL16 and untreated mice through staining with the proliferation marker Ki67; however difficulties in staining and inconsistent data lead to unreliable results. Optimizing the staining procedure will be important for future studies. As well, previous work in our group has shown that CXCR6 mRNA is present in both B16 and LLC tumour cell lines. It will be important to determine whether higher mRNA expression of this receptor translates to protein expression and leads to increased cell proliferation,

particularly in the presence of CXCL16. It will also be important to identify whether exogenous CXCL16 contributes to angiogenesis, as this could explain the observed increase in tumour size. This can be done through both in vitro (tube formation assays) and in vivo through examining tumour vascularity following CXCL16 treatment.

Neither CXCL10 nor CXCL16 injection alone lead to significant increases in NKT cell migration to tumours, likely due to diffusion of the chemokines from the injection site. In the future, these injections will be attempted using Matrigel to limit the dispersal of the chemokine. Matrigel is a liquid which solidifies at body temperature, limiting chemokine diffusion. This will allow us to determine whether intratumoural treatment with chemokine alone is sufficient to enhance NKT cell recruitment and thus decrease tumour growth.

It will also be important to further elucidate the role of CCR5 in NKT cell homing, expansion and cytokine production. Our adoptive transfer experiments showed decreased frequencies of $CCR5^{-/-}$ NKT cells in the tumours and livers of recipient mice. As homing was equivalent in the spleen and DLN it is unclear whether CCR5 is important for homeostatic NKT cell localization. In future experiments, lungs, bone marrow and other tissues will also be examined in these mice in order to determine whether CCR5 mediates homing to these tissues. As well, although our preliminary experiments did not show any significant differences in NKT cell frequency within either resting or activated WT and $CCR5^{-/-}$ mice, we did observe a slight increase in NKT cell frequency within livers of mice 3 days post α-GalCer treatment. These experiments will need to be repeated in order to determine whether this pattern continues. We observed dramatically lower IL-4 production in the serum of $CCR5^{-/-}$ mice, but no difference in intracellular staining within NKT cells after glycolipid activation. This is in contrast to a previous study showing that $CCR5^{-/-}$ NKT cells had higher levels of intracellular IL-4 in comparison to WT NKT cells following Con A stimulation[242]. These experiments will need to be repeated in parallel in the future to reconcile potential stimulus dependent differences in cytokine production.

4.10 Closing Remarks

NKT cells have been shown to be important mediators of anti-tumour activity, and their presence in tumours is correlated with improved prognosis in multiple animal tumour models as well as in cancer patients. In this study we sought to identify factors responsible for mediating NKT cell migration to tumours, such that their beneficial activities can be exploited even further. We identified three receptors which mediate NKT cell homing to tumours, CCR5, CXCR3 and CXCR6. We demonstrated that chemokine ligands can be used in combination with activating glycolipid to effectively increase NKT cell tumour homing, and in the case of CXCL10 lead to significant reduction in tumour growth. Our ability to replicate these results in multiple tumour models and mouse strains strengthens our results. The CXCR6-CXCL16 axis appears to have two diverging roles in tumour development, both anti-tumorigenic through recruitment of NKT cells and pro-tumorigenic, possibly through tumour cell proliferation and/or angiogenesis. We provide evidence for a role for CCR5 in recruitment of NKT cells to tumour sites. We also demonstrate that $CCR5^{-/-}$ have significantly reduced serum IL-4 levels following α-GalCer activation, which will need to be explored further. If $CCR5^{-/-}$ NKT cells could be recruited to the tumour via CXCR3 or CXCR6 they may exhibit superior anti-tumour activity due to reduced IL-4 production. Although we have demonstrated many roles of CXCR3, CXCR6 and CCR5 in NKT cell tumour infiltration and tumour control, future studies will need to be conducted in order to clearly elucidate the roles of these receptors, their ligands and glycolipid treatment in NKT cell function in tumour environments. We are confident that the data presented in this study has the potential to aid in the development of effective NKT cell based cancer therapies.

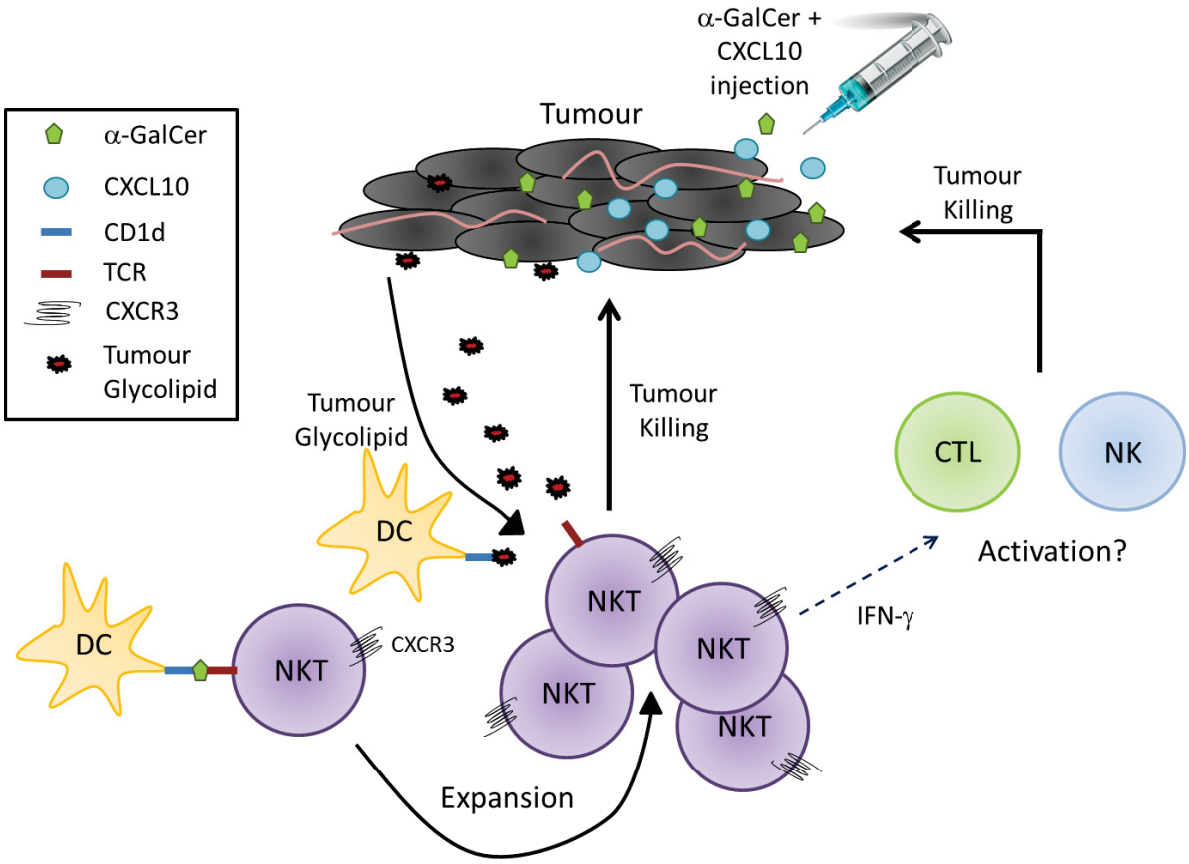


Figure 32. CXCL10 and α-GalCer treatment in tumours. Intratumoural treatment with α-GalCer and CXCL10 will lead to a cascade of responses. Dendritic cells will load α-GalCer and present it to NKT cells, which then become activated and expand. The CXCL10 within the tumour environment results in migration of expanded NKT cells to tumour sites, where they can directly kill tumours. NKT cells can also be activated by endogenous tumour glycolipid antigens. Activated NKT cells will also release pro-inflammatory cytokines such as IFN-γ, leading to the activation and recruitment of CTLs and NK cells, which can contribute to tumour eradication.

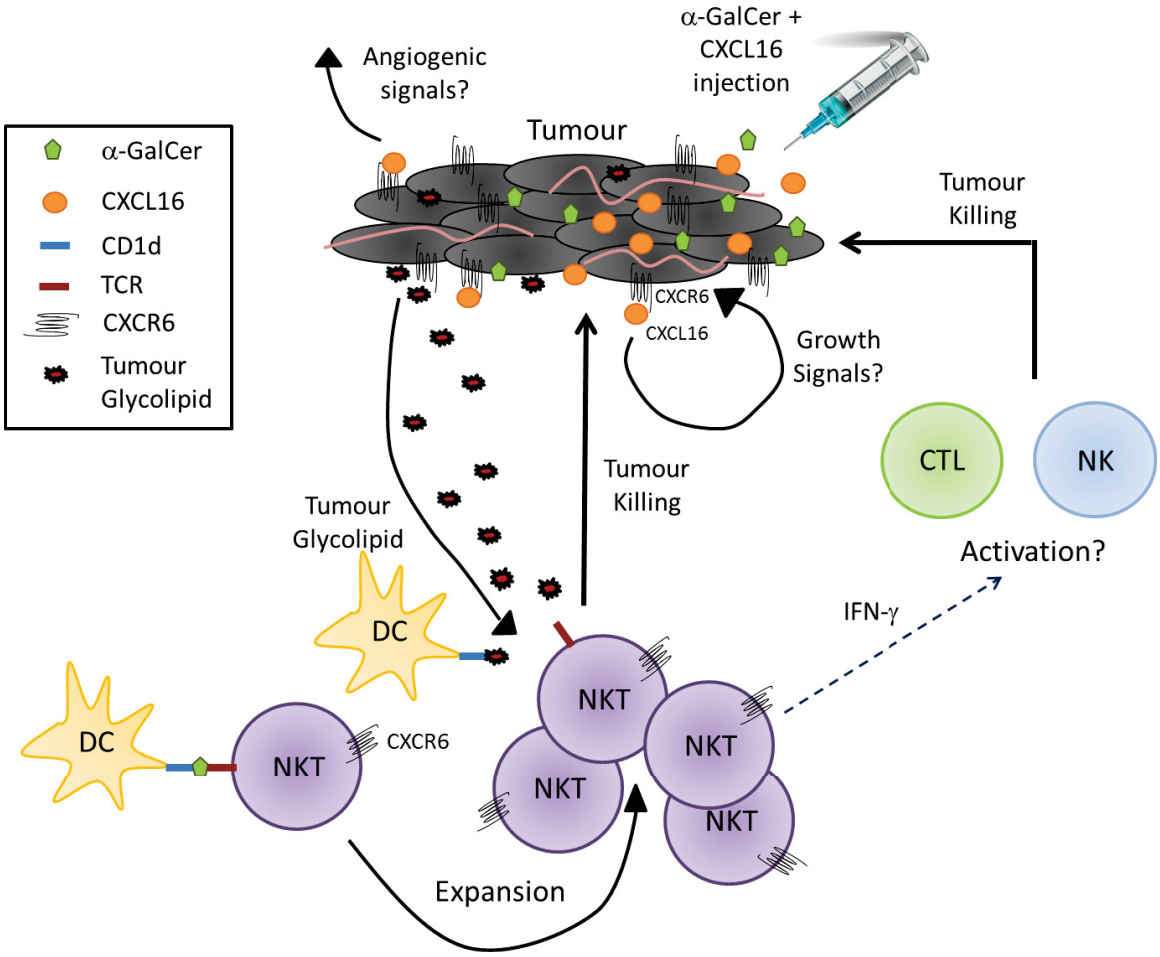


Figure 33. CXCL16 and α-GalCer treatment in tumours. Intratumoural treatment with α-GalCer and CXCL16 will lead to multiple different outcomes. Dendritic cells will load α-GalCer and present it to NKT cells, which then become activated and expand. The CXCL16 within the tumour environment results in migration of expanded NKT cells to tumour sites, where they can directly kill tumours. NKT cells can also be activated by endogenous tumour glycolipid antigens. Activated NKT cells will also release pro-inflammatory cytokines such as IFN-γ, leading to the activation and recruitment of CTLs and NK cells, which can contribute to tumour eradication. Simultaneously, CXCR6 expressed on the surface of tumour cells can directly respond to the injected CXCL16, providing growth and proliferation signals to tumour cells.

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