“Son, keep your room clean or else opportunity will walk out the door”

-Dad

University of Alberta

Activation of natural killer T cells and dendritic cells with *Caulobacter crescentus*: Implications for developing tumour immunity

by

Eric Wah-Leck Loo

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Dedication

This thesis is dedicated to my grandpa, Harry Loo, and all those who have lost a loved one to cancer too early in life.

Abstract

Cancer remains a leading cause of mortality worldwide. Efforts to develop immuno-therapies to control the growth of cancer, while limiting host cell damage, have focused on targeting specific tumour associated antigens. These treatments have yielded some clinical success however; the limited targeting of tumour antigens potentially allows the tumour to escape the treatment through antigen mutation or down-regulation of expression. In this thesis, we focused on the ability of non-pathogenic, Gram negative bacteria, *Caulobacter crescentus* to stimulate innate immunity to generate a response capable of controlling the growth of syngeneic tumours. We evaluated the ability of *C.crescentus* to activate natural killer T cells (NKT) and dendritic cells (DCs) as both cell populations affect the continued development of the inflammatory process. The activation of NKT cells was determined using Jα18-/- or CD1d-/- mice which lacked either a subset or all CD1d-dependent NKT cells respectively. NKT cell activation was determined through measurements of the early activation marker CD69 and various cytokines such as IFN-γ. DC activation *by C.crescentus* was characterized through observations made with bone marrow derived DCs and their ability to express co-stimulatory markers such as CD40, CD54, CD80, and CD86. The interaction of *C.crescentus* stimulated NKT cells and DCs revealed that *C. crescentus* stimulated NKT cells through a contact dependent pathway which may not require the recognition of the CD1d-lipid complex. Additionally, the interaction of *C.crescentus* activated NKT cells and DCs resulted in an enhanced expression of factors that are known promoters of Th1 cellular

immunity such as IL-12p70 and CD40. The immunity stimulated by *C.crescentus* was shown to slow the growth of EL4 subcutaneous tumours. Interestingly, through the course of our studies we revealed a role for a subset of NKT cells, type 1 NKT cells, absent in Jα18-/- mice to support the growth of syngeneic tumours. We found that Jα18-/- mice bone marrow derived DCs expressed increased Th1 promoting factors. This novel observation indicates a role for NKT cells in the development and maintenance of DC homeostasis in the wild-type animal.

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Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
αGalCer	alpha-Galactosylceramide
APC	Antigen presenting cells
BCR	B cell receptor
BMDC	Bone marrow derived dendritic cell
Cc	*Caulobacter crescentus*
CD1d	Cluster of differentiation 1 d
CD3	Cluster of differentiation 3
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
CD11c	Cluster of differentiation 11c
CD28	Cluster of differentiation 28
CD40	Cluster of differentiation 40
CD40L	Cluster of differentiation 40 ligand
CD49b	Cluster of differentiation 49 b
CD54	Cluster of differentiation 54
CD69	Cluster of differentiation 69
CD80	Cluster of differentiation 80
CD86	Cluster of differentiation 86
CFSE	5-(and 6)-Carboxyfluorescein diacetate succinimidyl ester
cpm	Counts per minute

CFU	Colony forming units
CTLA4	Cytotoxic T-Lymphocyte Antigen 4
DC	Dendritic cell
ELISA	Enzyme-linked immunosorbent assay
EGFR	Epidermal growth factor receptor
FACS	Fluorescent activated cell sorting
HK	Heat killed
HLA	Human leukocyte antigen
IgE	Immunoglobulin E
IFN-α/β	Interferon alpha/beta
IFN-γ	Interferon gamma
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-10	Interleukin 10
IL-12p70	Interleukin 12p70
IL-17	Interleukin 17
IP	Intraperitoneum
iNKT	Invariant natural killer T cell
LPS	Lipopolysaccharaide
M1	Macrophage 1
M2	Macrophage 2
mAb	Monoclonal antibody
MAIT	Mucosal associated invariant T cell

MHC	Major histocompatibility complex
MPL	Monophosphoryl lipid A
MUC1	Mucin 1
NK	Natural killer cell
NKT	Natural killer T cell
PRR	Pattern recognition receptor
PE	Peritoneal exudate
PBS	Phosphate buffered saline
pg/mL	Picogram per millilitre
RAG	Recombination activating gene
RBC	Red blood cell
TCR	T cell receptor
Th1	T helper 1
Th2	T helper 2
TLR	Toll-like receptor
TGF-β	Transforming growth factor beta
TAA	Tumour associated antigen
TIL	Tumour infiltrating lymphocytes
TNF-α	Tumour necrosis factor alpha
VEGF	Vascular endothelial growth factor
WT	Wild-type

CHAPTER-1

General Introduction

1.1 Cancer

Cancer is a major worldwide health problem. It is estimated that in 2008 over 12.7 million new cases of cancer and 7.6 million deaths were reported worldwide (1). This translates to over 21,000 deaths per day from cancer, making cancer the second leading cause of disease related death worldwide (Table 1-1). In Canada during 2012 there will be over 186,000 deaths from cancer (2). That number is expected to grow in future years due to the overall increasing age of the Canadian population. Worldwide, the incidence of cancer is expected to rise due to the adoption of western lifestyles by developing countries. A western lifestyle includes poor diets and physical inactivity which can raise the risk factors for cancer (1).

The distribution of cancer type is related to the standard of living (Figure 1-1A and B) (1). Breast and prostate cancer prevalence is closely associated with developed economies. Continued economic growth in developing countries means we are likely to see an increase in the incidence of prostate and breast cancer in men and women respectively. The rates of both prostate and breast cancers are highest in North America affecting more than 66.8 and 72.3 people for every 100,000 (Figure 1-1B and C). Certain geographically isolated indigenous populations also have disproportionately high rates of certain types of cancers (3). Worldwide, certain types of cancers, such as non-melanoma skin cancer among Europeans, can dominate cancer reporting rates (4). Despite current advances in treatments and diagnostics to detect cancer, large segments of the population succumb to the disease each year (1, 2). The need for developing

new, effective treatments to better manage and control cancer will only continue to rise as lifestyle habits change and the overall population ages.

1.2 Genesis of cancer

Cancer is a general term used to associate the many different types of uncontrolled cell growth within body tissues. The tissue or organ of origination often determines the name applied to the cancer. Despite this diversity in tissues affected, all cancers share a common etiology. *Oncogenesis* is the process whereby normal cells gain the mutations that cause uncontrolled growth. The number of mutations seen within a tumour cell is abnormally high, considering cells have DNA repair mechanisms and other protections to prevent DNA mutation.

Loeb has suggested a "mutator phenotype" to explain this abnormally high mutation rate (5). He argues that if an essential gene, such as a gene that encodes a DNA repair protein, was mutated to give a loss of function, the cell would inherit a mutator phenotype and potentially accumulate additional mutations within its genome, enhancing its oncogenic potential. This was supported by a study on zebrafish, where progeny inheriting a recessive loss of function mutation to an essential gene, responsible for genome stability, were found to develop multiple tumours earlier in life (6).

Genes that control cell cycling, DNA repair, DNA replication, chromosome segregation and recombination have been extensively studied because they have the potential to give rise to the mutator phenotype. These

genes became known as *proto-oncogenes*. An oncogenic event may arise if a carcinogenic compound, an external chemical agent that can trigger a high rate of mutations within a cell, causes the mutation of a proto-oncogene. Radiation is another source of mutations in DNA that can cause trigger an oncogenic event.

Chronic inflammation has been identified as an internal mechanism that can increase mutations of surrounding cells (7). This has been suggested as a cause for the increase in cancer rates observed among those infected with *Helicobacter pylori* (7). The infiltrating macrophages and innate cells limit the infection by releasing toxic oxygen molecules into the immediate environment, thereby creating a highly oxidizing space. This toxic environment also affects the surrounding tissue cells and can result in normal DNA insults (8). If this insult affects an essential cell regulatory gene, then it may also generate a mutator phenotype and the cell may become cancerous or neoplastic. Another link for inflammation and cancer was also shown in people with inflammatory bowel disease. These people display a higher rate of colon cancer and it is postulated to be due to the chronic inflammation in the colonic tissue (9).

1.3 Immunity

The immune system is generally regarded as the natural defence mechanism for the body. In reality, the immune system is charged with maintaining body homeostasis and integrity by attacking foreign invading bodies and initiating tissue damage repair. In mammals, this system has evolved to include both an innate and adaptive immune components.

1.3.1 Innate immunity

Innate immunity is evolutionarily the oldest form of immunity (10). It provides protection against foreign invading organisms. The simplest organs of the innate immune system are the physical barriers designed to keep foreign elements outside of the body, such as the skin. In mice and humans, cellular components of innate immunity include neutrophils, basophils, eosinophils, mast cells, macrophages, natural killer (NK) cells, natural killer T cells (NKT) and dendritic cells (DCs). Each plays a unique role in maintaining the homeostasis of the host.

Pattern recognition receptors (PRRs), found on many innate immune cells, are an evolutionarily conserved pathway used to initiate an immune response. The most studied PRRs are the Toll-like receptors (TLRs). Toll was originally identified in the fruit fly *Drosophila melanogaster*. There are 13 identified TLRs in mammals that recognize different molecular ligands indicative of a possible infection. TLRs 1-10 are known to be expressed in humans (Figure 1-2) (11-13). TLRs are comprised of leucine-rich repeats that bind to the ligand and largely signal through the MyD88 cell signalling pathway to activate NF-kB mediated immune genes.

Neutrophils, basophils, mast cells and eosinophils collectively make up the family of granulocyte cells that are identified by their staining with the cellular dyes eosin and hematoxylin. They are easily identified by their segmented or lobed nuclei and the presence of numerous vesicles in the cell

cytoplasm. Neutrophils are often the first responders to a site of infection and attack invading pathogens by phagocytosis and releasing toxic factors to kill bacteria in the extracellular environment. These toxic elements can unfortunately, harm surrounding host tissue (14). They also secrete chemokines and cytokines that attract other immune cells to the site of inflammation (14). Eosinophils, basophils and mast cells are more commonly associated with parasitic worm infections and allergy responses (15). They attack worms by releasing their toxic substrates contained in cytoplasmic vesicles. Together, they are known to signal to the adaptive immune system to stimulate the production of IgE and promote Th2 immune responses (15).

Macrophages along with neutrophils are known as professional phagocytosing cells of the immune system. Together, they help coordinate the innate immune response, mainly against intracellular and extracellular bacterial pathogens (16). Macrophages can additionally coordinate the development of the innate immune response. M1 macrophages are known to be pro-inflammatory and send signals to promote inflammation such as TNF and IL-12. They can also set up toxic environments by releasing oxidizing molecules to prevent the growth and establishment of bacterial infections (17). The second group of macrophages, termed M2, are known to signal the end of the inflammatory process and initiate the clean up response and tissue repair mechanisms, thereby maintaining body homeostasis (17). The inhibitory cytokine, IL-10 is associated with the M2 response.

NK cells are cytotoxic killer cells initially identified by their natural ability to kill tumour cells (18). They have been subsequently found to be invaluable in developing innate immunity against viruses. Viruses are obligate intracellular pathogens and require host cell machinery to replicate and grow. When host cells become virally infected they can raise molecular flags, recognized by NKG2D present on NK cells to kill them and thereby control virus infection. Some viruses evade adaptive immunity by forcing infected cells to down-regulate their MHC class I molecules, which will be described more in the following section. NK cells can detect the down-regulation of MHC class I and kill the virally infected cell. Additionally, NK cells also mediate inflammation, in a similar fashion to macrophages, by releasing cytokines to promote cellular based immunity to target viruses, such as IFN-γ (18).

Dendritic cells are specialized antigen presenting cells which bridge the innate and adaptive immune systems together. They are principally responsible for presenting antigen on both MHC class I and class II molecules along with co-stimulatory markers required to initiate an adaptive, T cell immune response. CD80 and CD86 are the principle co-stimulatory markers on DCs (19). Co-stimulatory marker expression is dependent on pro-inflammatory signals, to prevent the development of antigen specific immunity against non-inflammatory antigens. Along with co-stimulation, their production of cytokines can influence the development of adaptive immunity.

1.3.2 Adaptive immunity

Adaptive immunity describes those immune responses where a subsequent challenge with a previously encountered foreign body results in a quick and more vigorous immune response compared to the primary immune challenge. Thymus matured cells (T cells) and bone marrow matured (B cells) are mediators of adaptive immunity. They are capable of recognizing specific antigens from foreign bodies through their T and B cell receptors (TCR/BCR). Genetic recombination, mediated by recombination activating genes (Rag) proteins, of the TCR/BCR genes gives T and B cells a diverse repertoire of antigen recognizing ability (20). To prevent their TCR/BCR from recognizing and establishing an immune response to self antigens, T and B cells must undergo positive and negative selection processes that eliminate self-reactive cells from entering the body.

B cells are responsible for establishing humoral immunity. When their BCR recognizes an antigen, their cellular morphology changes and they become either memory or plasma cells. A plasma cell generates antibodies, based on the BCR, which can bind to their antigen target. Antibodies are small proteins designed to bind to its target antigen. Antibodies can opsonize a target for cellular attack by macrophages or NK cells, neutralize the target so it can not directly bind to body surfaces, or signal the target to be destroyed by a process known as complement fixation. Before a B cell can become fully activated to produce antibodies and develop memory of the antigen, they require T cell help. This secondary signal prevents the B cell from producing potentially

inflammatory antibodies against non-inflammatory targets. Similar to DCs, B cells can also process and present antigens to T cells.

T cells are further defined by their expression of either CD4 or CD8. $CD4^+$ T cells consist of a TCR which recognizes peptide antigens bound to MHC class II molecules on antigen presenting cells such as DCs. These T cells are termed helper T cells (Th) because of their significance in providing the co-stimulatory signals to B cells to mature into effector plasma cells and the cytokines to assist in the maturation of cytotoxic $CD8^+$ T cells. Through their activation by MHC class II on DCs, they can polarize to become either Th1 or Th2 type cells. This polarization is characterized by the types of cytokines produced (21). Th1 type cytokines (IFN-γ and TNF-α) promote cellular immunity characterized by the development of cytotoxic T cells and the promotion of other cytotoxic elements such as macrophages and NK cells. Th2 immunity is focused on the development of humoral neutralizing immunity and is characterized by the production of cytokines such as IL-4 and IL-5. Both of these responses do not function in a mutually exclusive environment. In most cases of infection, both types of immunity are required for the successful clearance of the pathogen. It is the balance of Th1/Th2 that is mobilized to the infection which determines disease outcome.

$CD8^+$ T cells are characterized as antigen specific cytotoxic T cells. They recognize peptide antigens bound to MHC class I molecules. MHC class I is present on all host cells and normally presents self antigens to the $CD8^+$ T cells, indicating normal protein development in that cell. In the case of a viral infection

of a host cell, the MHC class I can signal the intracellular infection to antigen specific $CD8^+$ T cells by presenting viral peptides rather than self peptides. These $CD8^+$ T cells can release factors to kill the infected host cell through a process known as apoptosis. In addition to being activated through recognition of its peptide antigen bound MHC class I, Th1 cytokines from $CD4^+$ T helper cells enhance the maturation of $CD8^+$ cytotoxic killer cells (22).

In addition to the classical $CD4^+$ and $CD8^+$ T cells, research has identified various other types of T cells including Th17 and regulatory T cells (Tregs). Th17 cells are T helper cells that produce the cytokine IL-17 and IL-22 (23). They have been found in sites of autoimmunity leading to the early suggestion of their role in promoting autoimmunity. More recently, they have been shown to play a role in enhancing and promoting the immune response to clear certain bacterial and fungal pathogens such as *Candida albicans* and *Mycobacterium tuberculosis* (23-25).

Tregs, as the name suggests, play a role in regulating the immune response. They are important in maintaining peripheral tolerance to self-antigens as well as controlling and preventing the development of chronic inflammatory responses (26). They largely produce cytokines that inhibit the immune response such as IL-10 and TGF-β. Others have additionally shown that Tregs can directly regulate effector T cells by triggering them into apoptosis through granzyme or TNF-related apoptosis-inducing ligand (TRAIL) mediated pathways (26, 27).

1.3.3 NKT cells

NKT cells are classically identified as T lymphocytes that express NK phenotypic markers, such as NK1.1 (28). NKT cells, much like their T cell counterparts, must go through a positive selection process in the thymus. Unlike traditional T cells, NKT cells are selected by binding to the MHC class I-like lipid binding molecule CD1d (29). Two classes of NKT cells have been commonly identified: Class 1 or invariant NKT cells (iNKT) are identified by their invariant TCR comprised of Vα14-Jα18 chains in mice and Vα24–Jα18 in humans (28); Class 2 NKT cells have a less restrictive TCR repertoire. Although having the qualities of a T lymphocyte, NKT cells are not classified as an adaptive immune cell and have more in common with other innate immune cells due to its failure to generate a long lasting memory response. Mice lacking class 1 invariant NKT cells by knocking out the Jα18 gene are useful in the study of class 2 NKT cell function while due to their retention of a small proportion of NKT cells (30).

Once NKT cells are thymus-matured they are released into the periphery, where they can be activated in a manner similar to traditional T cells, utilizing endogenous and exogenous lipid antigen presentation by CD1d interacting with their T cell receptor (TCR). Unlike traditional T cells, NKT cells do not require the presence of a foreign lipid presented by CD1d to become activated. They can be activated through recognition of endogenous lipids presented by CD1d accompanied with inflammatory cytokine signalling (Figure 1-3) (31). The presence of TLRs on NKT cells is still unknown and not suspected as co-cultures of TLR pathway deficient DCs with NKT cells stimulated with heat-killed bacteria do not show significant production of IFN-γ or IL-4 (32). Their innate

activation through antigen presenting cells is believed to play a role in defining the antigen-specific adaptive immune response by modulating antigen-presenting cells through direct cell contact and cytokine signalling (33). Additionally, similar to macrophages, NKT cell activation and cytokine signalling is believed to support the ongoing innate immune response (33).

NKT cells are not the only unconventional T cells. Mucosal associated invariant T cells (MAIT) are thymus matured, MR1 restricted T cells with an invariant T cell receptor consisting of Vα7.2 in humans and Vα19 in mice (34). MR1 has been characterized as a protein that has evolved separately from the MHC locus yet retains high homology amongst mammalian species. Similar to CD1d, it forms a heterodimer with β2 microglobulin, however its ligand for presentation is still unknown although it has been suspected to present lipids (34, 35). MAIT cells, as the name suggests are found primarily associated with mucosal tissues such as the gut. Its exact role is still unknown however, it has been shown to be important in the control of bacterial infections in their resident tissues and recognition of $MR1^{+}$ bacterial infected cells leads to IFN-γ and TNF-α production (34). Their role in immunity is suggested to be similar to NKT cells as they appear at the interface of innate and adaptive immunity.

1.4 Immunity and cancer

1.4.1 Immunity promoting cancer

The inflammation process has a complex relationship with cancer. It has been shown to be able to control the growth of tumours and in some cases promote their eradication. Yet in other cases, it can promote the generation of cancer and assist in the ongoing evolution and development of tumours.

Balkwill has suggested the chemokine cascade is responsible for the ongoing development of tumours (36). *Chemokines* are proteins and other small molecules secreted by cells to attract a target population of cells. A cross-section of tumour will contain cells that are not identifiable as tumour cells; they are leukocytes that have migrated to the tumours due to chemokines. Tumours are known to secrete their own chemokines to attract the infiltration of macrophages and dendritic cells (Figure 1-4).

Macrophages, as described earlier, are one of the primary host innate immune cells responsible for phagocytosing foreign bodies or clearing debris from tissue areas. In the case of tumours, an M2 macrophage infiltrate is often detected that can assist in tumour metastasis by secreting growth-promoting factors that stimulate *tumour angiogenesis*; development of blood vessel networks within the tumour environment (17). This gives the tumour a continued source of nourishment and a gateway for metastasis to the body. Macrophages also control tumour specific immunity by secreting high levels of TGF-β and IL-10, both anti-inflammatory cytokines (37),

Dendritic cells are normally responsible for bridging the innate and adaptive immune systems by collecting antigens from an inflammatory site and presenting them to T cells. DCs attracted to the site of the tumour are contained

there due to the chemokine cascade. They are prevented from leaving to the lymph nodes and therefore, unable to stimulate a strong T cell response (36, 38). Previous studies have also shown the ability of tumour associated DCs being able to suppress the activation of tumour specific T cells (39).

The tumour has also been shown to favour the accumulation of Th2 cells, enabling it to escape an effective Th1 cell-mediated immunity response (40). The Th1/Th2 balance is important in shaping the adaptive immune response between Th2 humoral (antibody) and Th1 cellular responses. Shifting the Th1/Th2 balance within the tumour prevents the development of an effective cellular response targeting the tumour. Along with Th2 cells, the tumour accumulates Treg cells to control the establishment of an antigen specific cytotoxic immune response (Figure 1-5)(41).

The production of anti-inflammatory factors is not limited to infiltrating type 2 macrophages and other leukocytes. The tumour cells themselves have been shown to release anti-inflammatory factors such as indoleamine 2,3-dioxygenase (IDO) (42). IDO is regularly expressed by activated macrophages and DCs and, based upon *in vitro* observations, was previously believed to be an enzymatic factor limited to catabolizing tryptophan in the environment and starving the growth of foreign bacteria. Studies later revealed that IDO expression by DCs could either directly or indirectly act as a chemical messenger, making T cells non-responsive to their specific antigen and initializing tolerance (43, 44). This has implications in tumour immunology because tumour cells have been also shown to express IDO along with the leukocytes in its micro-

environment. This would add to the immunosuppressive effects of the tumour micro-environment and would have implications in the design of immunotherapeutics utilizing a cytotoxic T cell response against tumours.

Tumours have been shown to participate actively in the destruction of T cells targeted towards them. This would help to explain why tumour-specific T cells measured from blood samples in cancer patients do not amount to a significant clinical response against the tumour (45). The Fas/Fas ligand (FasL) system is normally expressed on both activated T and B cells and acts as a control to their clonal expansion by limiting their concentration in the body. FasL is also expressed on cells within immune-privileged sites such as the eyes and testis to eliminate any autoreactive T cells that may infiltrate the tissue with the potential to cause injury (46). FasL has also been shown to be expressed by tumour cells and can interact with Fas on infiltrating T cells and signal them for destruction, thus protecting the tumour (Figure 1-6) (47).

The Fas/FasL interaction, however, can work in reverse with some activated cytotoxic T cells that enter the tumour micro-environment, able to kill tumour cells by linking to tumour-expressed Fas (48). An overexpression of FasL on a tumour can signal it for killing through neutrophil-mediated pathways (46). Killing through Fas/FasL interactions allows the T cells to kill without the traditional HLA/TCR interaction, which is beneficial since tumour cells regularly have decreased amounts of HLA on their cell surface (45).

1.4.2 Immunity controlling cancer

Host immunosurveillance prevents the development of many potential neoplastic events and can help slow, but rarely completely control, a growing tumour. Definitive evidence displaying immunosurveillance has not yet been shown, but there is much supporting evidence. Shankaran and colleagues found that both arms of the immune system were important in the theory of immunosurveillance (49).

Rag1 and Rag2 are proteins present in the nucleus of lymphocytes that are responsible for generating the recombinant events within the T cell receptor and B cell receptor genes, allowing for greater diversity in antigen recognition; both are required for successful recombination (50). A Rag2 knockout mouse lacks the ability to reorganize the BCR/TCR genes and therefore lack an effective adaptive immune system consisting of T and B cells. Shankaran and colleagues demonstrated a significant increase and earlier onset of spontaneous tumours in a Rag2-/- mouse lacking an adaptive immune response (47, 49). Stat-1, a transcription factor that normally signals for the production of IFN-α/β and IFN-γ, was also shown to be important in controlling the development of spontaneous tumours. A combined Rag2 and Stat-1 deficiency led to a synergistic increase in the number of tumours present in these mice. They concluded that an attenuated innate immune system along with the lack of adaptive immunity led to a greater increase in the number of spontaneous tumours because normal neoplastic events were not being recognized and cleared by the immune system.

When potential neoplastic cells develop an immune-escaping mutation, the result is uncontrolled tumour division. A strong post-tumour immune

response, measured through the number of tumour-infiltrating lymphocytes (TILs), has been shown in humans to lead to a positive clinical outcome with less metastasis and slower tumour growth (47, 49, 51). The cancer patients where these findings were made received no prior immunostimulatory treatment demonstrating that these TILs are the result of a natural immune response generated to the tumour.

Cho and colleagues offer evidence that both CD4 and CD8 populations of T cells are required for a positive outcome in cancer patients after treatment (52). Immunohistochemical analysis of tumour sections determined that those patients with a natural response of TILs consisting of CD8 cells within the tumour nest and CD4 in the tumour stroma resulted in the best clinical outcomes. The presence of these TILs provide further evidence of immunosurveillance: a naturally-existing population of lymphocytes can recognize certain tumour antigens and partially control the tumours.

Despite all the immunological controls over tumour growth, potential neoplastic cells can still garner enough mutations to circumvent immunosurveillance, which leads to the spread of neoplastic cells and the development of a tumour.

1.4.3 Concept of immuno-editing

Efforts to consolidate the paradoxical outcomes regarding the immune response to cancer have led to the concept of immuno-editing of tumours. Quite simply, it applies a Darwinian concept of evolution to explain the changes

observed in tumours over the course of disease (53). Schreiber and colleagues describe 3 definitive stages during the oncogenic process that highlight the role of immuno-editing; elimination, equilibrium, and escape (Figure 1-7). Elimination refers to immune responses that can remove oncogenic cells. This category is an updated version of the term immuno-surveillance. The existence of elimination is inferred from observations in mice lacking certain immune cell subsets that develop earlier tumours compared to wild-type mice. Equilibrium describes a state where the immune system is activated and recognizes the development of a tumour but is not capable of eradicating it. This stage requires the activation of adaptive immunity to control the growth of the tumour. The antigen specific control of tumour growth is what shapes the ongoing development of the tumour mass. Escape finally occurs when cells in the tumour mass evolve mutations that allow them to escape recognition by the adaptive immune system. Studies that support the concept of immuno-editing have found that transplanted tumours from a Rag2-/- mouse, deficient in T cells, were far more immunogenic than similar tumours derived in wild-type mice (53, 54). This would be due to the adaptive immune response in the wild-type mice being able to shape the tumour cells to become less immunogenic. Koebel and colleagues showed that the adaptive immune system in mice could hold a tumour in equilibrium, preventing its continued growth without actually eradicating it (55). This finding supports a central tenet in the concept of immuno-editing: tumours can exist in an equilibrium state with the immune system, neither growing nor shrinking. The tumour becomes dangerous to the patient once it evolves the means to escape this

equilibrium, usually through genetic mutations that become additive due to the mutator phenotype established.

The reported duplicity of the immune response should not be unexpected, since it has been tasked with maintaining body integrity and homeostasis from exogenous pathogens and tissue injury. Tumours have evolved, due to selection pressures from immuno-editing, towards the recruitment of the tissue repair type macrophages, which can aid in tumour development (56). Both tumour-promoting and tumour-controlling immunity concepts co-exist in the model of cancer immune-editing proposed by Schreiber and colleagues. The equilibrium that is established can allow for growth inhibition in the tumour (53, 55, 57).

1.5 Immunotherapy of cancer

Despite the evasion strategies used by tumours and the theory of immuno-editing, immunotherapy is still actively researched and proposed as a treatment for cancer. The concept of immunotherapy within the immuno-editing framework would be to alter the equilibrium in favour of tumour control and eradication. Currently, the immunotherapies being developed utilize the success of vaccines by delivering antigen-specific treatments, either through active or passive immunizations. The evolving knowledge of the immune system has also led to the development of treatments that attempt to pharmaceutically modulate the immune response in favour of tumour eradication. Other groups are investigating the relationship between bacterial infection and cancer regression in the hopes of finding a treatment option.

1.5.1 Active immunizations

The goal of active immunizations is to stimulate the adaptive immune system to target a tumour in an antigen-specific manner. An effective vaccine design would be an immunotherapeutic product that requires minimal introduction and provides long-lasting protection against the tumour. The design of a tumour-associated antigen (TAA) targeted vaccine would likely take the form of a subunit-type vaccine. Whole killed tumour cell vaccines or attenuated tumour cells would be too dangerous to administer in an already immuno-compromised cancer patient. Additional problems arise when considering a prophylactic immunization strategy since live, killed, or attenuated tumour cells would be poorly immunogenic due to immuno-editing (58). TAAs are antigens that are found to be overexpressed or unique to a tumour (59). Many TAAs have been discovered by studying the reactivity of T cells to cultured tumours *in vitro* (59). The existence of naturally occurring TAA-targeting T cells supports the plausibility of a cancer vaccine.

TAAs fall into 4 main categories, as described by Paschen and colleagues (60):

1) proteins/molecules specific to cancers within the same lineage of tissue;
2) proteins/molecules not normally expressed in the inflicted tissue;
3) tumour-specific proteins/molecules resulting from a gene mutation in that tumour only; and

4) proteins over-expressed on tumour cells, but normally expressed at lower levels on normal tissue.

Effective vaccine designs have therefore centered on developing a subunit formulation. Potential TAAs must be isolated and delivered with an immune stimulator or adjuvant to direct the immune response in favour of tumour-specific rejection. The goal is to design a formulation that can generate an immune response capable of overcoming the immuno-suppressive environment of the tumour.

Antigen choice is the most important aspect to consider when designing a cancer vaccine (Figure 1-8) (61). This is followed by the choice of adjuvant, which concerns the vaccine delivery vehicle and the inclusion of immune modulators. In the case of the only cancer vaccine approved by the U.S. Food and Drug Administration (FDA), the targeted antigen is prostate antigen and the adjuvant is granulocyte–macrophage colony-stimulating factor (62). The delivery of this vaccine requires the need for *ex vivo* maturation of antigen-loaded autologous DCs. Despite being the only cancer treatment vaccine currently approved for use in humans, it is reported to extend life by only four months (62).

One way that vaccine researchers hope to enhance the efficacy of their vaccines is by utilizing different immune-potentiating factors. Attention has been focused on identifying ligands for PRRs that can initiate an innate immune response.

Utilizing the knowledge regarding PRRs, such as TLRs, and their corresponding ligands, vaccine researchers have been devising formulations with adjuvants that can directly activate a specific type of immunological response. Lipopolysaccharide (LPS) found on the outer cell membrane of Gram-negative bacteria, for example, is known to activate an immune response through the TLR4 signalling pathway. Vaccine researchers have developed detoxified forms of LPS, termed monophosphoryl lipid A (MPL), which can signal an immune response through the CD14/TLR4 pathway (63). TLR9 agonists, such as unmethylated bacterial CpG DNA, have also been synthetically developed and suggested as adjuvants for vaccine development (64).

Despite efforts to develop an antigen-specific cancer vaccine, the limitations suggested through the theory of immuno-editing of tumours may impede progress. The use of an antigen that is survival-dependent for the tumour, one that could not be altered or down-regulated by the tumour, would be a key to success.

1.5.2 Passive immunotherapy

Compared to active immunization strategies, passive strategies have found more success in the clinic. The concept of passive immunotherapy is focused on the development and use of monoclonal antibodies (mAb) that are specific for TAAs. These antibodies require repeated administration, since the host B cells are not the source of these antibodies, hence the term passive immunity. There are more than 12 different monoclonal antibody therapies to cancer approved for human use by the FDA (Table 1-2) (65). Many others are in clinical trials.

The most successful mAb treatments developed all target the epidermal growth factor family of receptors that include epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF) (65). Rather than triggering tumour killing through antibody-dependent cellular cytotoxicity (ADCC), it is believed that these mAbs may be interfering with growth signalling within the tumour (66).

Another consideration for the development of mAbs is their use in cancer diagnosis and treatment monitoring. A successful mAb of this type is B27.29, which was raised by injecting ovary tumor cells into mice and selecting out a plasma cell population that presented anti-MUC1 properties (67). The epitope recognized by B27.29 is a section of glycosylated MUC1 peptide 'PDTRP', which would closely match the form found on tumours (67). Its radiolabelled form proved successful in identifying shed MUC1 in the sera of cancer patients. The success came when it was determined that the amount of shed MUC1 measured in the sera of patients was found to have a prognostic correlation to the severity and eventual outcome of the disease (68). The antibody was eventually commercialized into a diagnostic kit and sold to clinics internationally. B27.29 has also been routinely used in MUC1 functional research because it binds well to both tumour-expressed and T cell-expressed MUC1 (69, 70). Unfortunately, further research into clinical treatment options have ended because MUC1 is shed into the tumour periphery and prevents B27.29 from directly binding to the tumour in sufficient quantities to stimulate ADCC.

One of the major downfalls attributed to passive immunotherapy is the shear cost of treatment. The treatment must be routinely administered and, due to the passive nature of immunity, does not result in long-lasting protection against the tumour (71).

1.5.3 Immune modulation as treatment for cancer

Efforts to treat cancer have progressed along with our evolving knowledge of the immune system. Discovery of cellular inhibitors of co-stimulatory pathways, such as CTLA4, have led researchers to propose pharmaceutical interventions to increase anti-tumour immunity through immune modulation. This concept was demonstrated effective when antibodies targeting CTLA4, a molecule that is known to competitively bind to CD80 and CD86 on DCs to inhibit costimulatory signals between DCs and T cells, were found to enhance anti-tumour immunity in mice (65, 72). This began the prospect of developing novel methods of blocking or enhancing co-stimulation to increase tumour immunity, either indirectly or to a designated antigen.

A recent development in attempting to modulate immunity to treat cancer was the discovery of natural killer T cells (NKT). NKT cell activation has been shown to enhance DC activity and promote the development of Th1 immunity through the secretion of IL-12p70 and IFN-γ (Figure 1-9) (73, 74). IL-12p70, derived from DCs or myeloid cells, is an important mediator in stimulating effective immunity to the tumour. Direct delivery of IL-12p70 to the tumour has been shown to reverse the tolerogenic conditions of the micro-environment (75,

76), which can promote the control of tumour growth and a return to the equilibrium phase of immuno-editing. NKT cells have been suggested to play a role in the rapid recruitment of other immune effector cells to combat infection or cancer (77). Additionally, activated NKT cells have been suggested as direct killers of tumour cells through granzyme and receptor mediated pathways (Figure 1-9).

Efforts to modulate the activation of NKT cells have led to the use of αGalactosylceramide (αGalCer), a glycolipid isolated from the sea sponge *Agelas mauritianus* (78). αGalCer is known to be recognized and bound for antigen presentation by CD1d, after which it is an activator of iNKT cells. NKT cell activation by αGalCer has been shown to control tumour growth in mice (78, 79). Human clinical trials have progressed from the initial results observed in mice. Unfortunately, results have been unfavourable, as solid tumour patients receiving αGalCer showed a decrease of circulating NKT cells after treatment with unbound αGalCer (78). However, antigen presenting cells loaded *ex vivo* with αGalCer have been shown to expand the number of NKT cells in patients suffering from metastatic cancer that had failed initial treatment (80).

Definitive survival benefit in the human population has yet to be determined. The search for other methods to specifically activate NKT cells has led researchers to discover that non-pathogenic *Sphingomonas* bacteria are activators of NKT cells (81).

1.5.4 Surrogate infection to control cancer

Spontaneous regression of tumours has been hypothesized to be induced during a concurrent bacterial infection (82-84). Evidence of this phenomenon originally dates back hundreds of years, when it was observed that some cancer patients who develop concurrent bacterial and viral infections experience tumour regression (85).

Dr. William Coley initially experimented with purposefully infecting cancer patients with *Streptococcus pyogenes* (86, 87). This treatment yielded great results against the inoperable sarcomas he was targeting; however, complications due to bacterial infections ultimately led him to the use of heat-killed bacteria (86). This treatment, called Coley's Toxins, consisted of heat-killed *Streptococcus pyogenes* and *Serratia marcescens* (86), and was used successfully to treat many inoperable sarcoma patients.

Since the use of Coley's Toxins ended in 1963, others have proposed using different bacteria, such as *Salmonella, Bifidobacteria* and other anaerobic bacteria, as broad immune activators to treat to cancer (88-91). These bacteria are suggested because, aside from activating the immune system, they preferentially grow within the tumour micro-environment. The immunity stimulated from these bacterial treatments could be due to NK and NKT cell populations; both have been shown to be important for the immune response to control tumours and bacteria (92). Although theoretical benefit and success in non-humans for treating cancer has been established, these modified pathogenic bacteria are only now entering early clinical trials in humans.

1.6 *Caulobacter crescentus*

Caulobacter crescentus (*Cc*) is a non-pathogenic, freshwater, Gram-negative bacterial organism that has been widely studied due to its dimorphic lifecycle. It has a protein S-layer positioned outside its outer membrane. Studies have demonstrated that proteins of interest can be easily incorporated into the S-layer, providing a platform for antigen delivery (93). Depending on the location of incorporation into the S-layer subunit protein RsaA, the protein of interest could be made to be secreted by the bacteria or retained on the bacterial surface (93). Due to the ability to insert antigens of interest onto the bacteria, it has been proposed as an immunizing vector for new vaccine formulations against the pilin protein in *Pseudomonas aeruginosa.* The non-pathogenic nature of *Cc* has led to the disregard of its ability to directly stimulate the immune system. However, it was determined that the lipopolysaccharide isolated from *Cc* has a biological potency 100 times less than *E. coli* (94).

One previous study by Bhatnagar and colleagues investigated the use of *Cc* transgenic for the TAA MUC1 and its ability to raise an antigen-specific tumour response (95). The immunizations unexpectedly limited the growth of syngeneic tumours, independent of antigen specificity (95). The biology and mechanism of the response was not determined.

1.7 Rationale and hypothesis

Cancer is a devastating disease with a limited number of treatment options, and these treatments have varying degrees of success. The use of

immunotherapy to treat tumours could take advantage of the long-observed phenomenon of spontaneous tumour regression associated with infection by modulating the immune system to target the tumour.

Many immunotherapies currently being developed focus on the specific targeting of TAAs. Due to their dependence on the expression of TAAs on tumour cells, these therapies are all susceptible to tumour immuno-editing. In order to overcome immuno-editing, I propose the use of a bacteria to stimulate a broad-acting, antigen non-specific, innate immune response to control the growth of the cancer. Unlike previous studies that rely on developing attenuated bacterial strains, I have chosen to evaluate the use of a non-pathogenic bacteria, *Caulobacter crescentus* which has previously shown antigen non-specific anti-tumour potential in a previous study (95). In this thesis, I will examine the different mechanisms by which *Cc* could control tumour growth. I hypothesize:

1. Based on the elimination pathway presented in the theory of cancer immuno-editing and the ability of *Caulobacter crescentus* immunizations to prevent syngeneic tumour growth, that *Caulobacter crescentus* can stimulate the activation of innate lymphocytes, particularly, NK/NKT cells to control the growth of a tumour cell line.

2. *Caulobacter crescentus* can mature bone marrow-derived DCs to express co-stimulatory proteins and allow enhanced interaction of NK/NKT cells with DCs

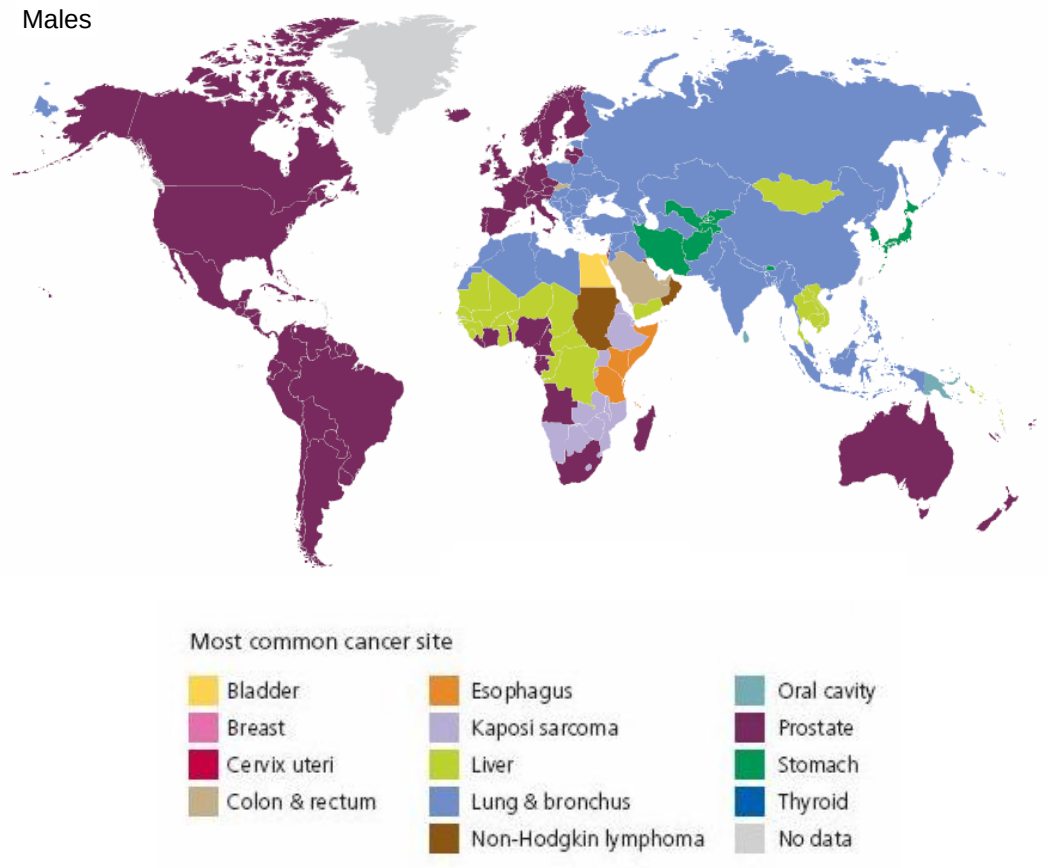
3. *Caulobacter crescentus* immunization can slow the growth of $CD1d^+$ tumours through NKT cell-mediated pathways.

Each of these three hypotheses will be tested in the following three chapters.

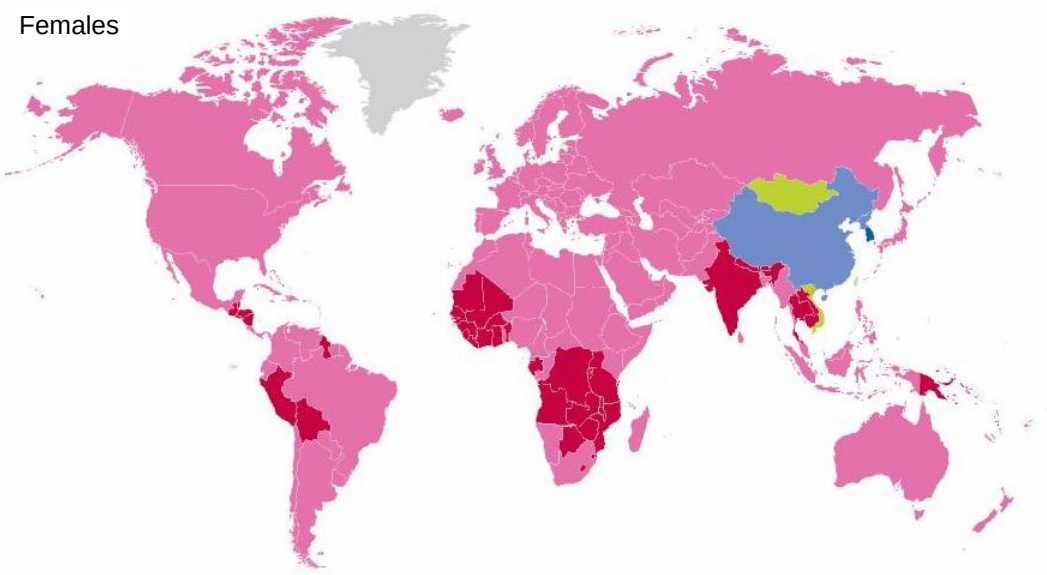
1.8 Figures

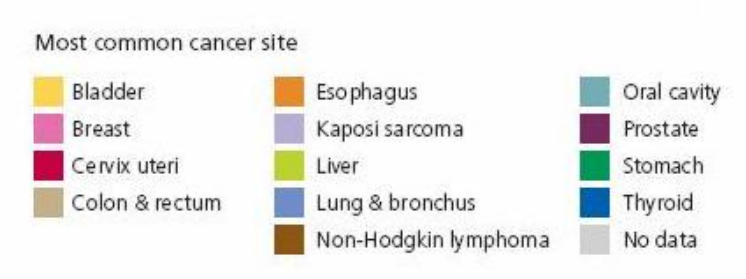
Figure 1-1

A)

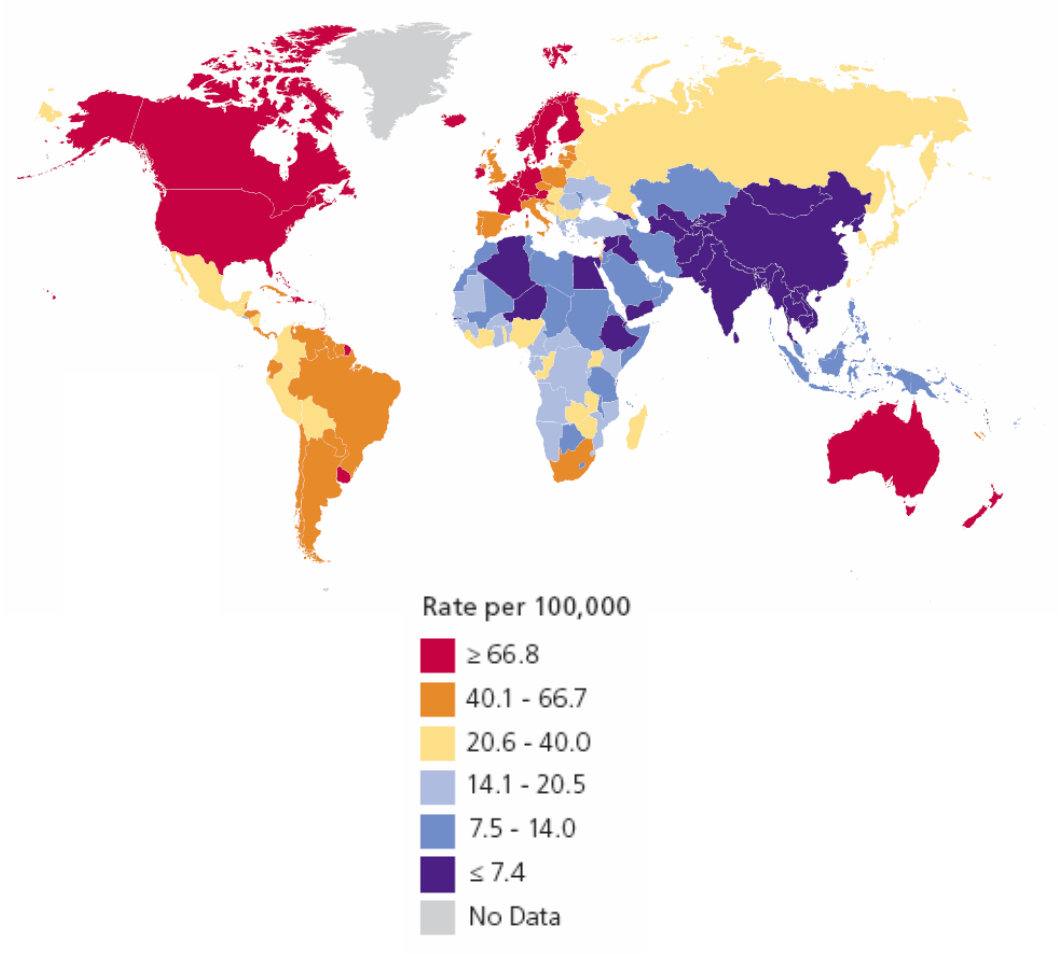


Females

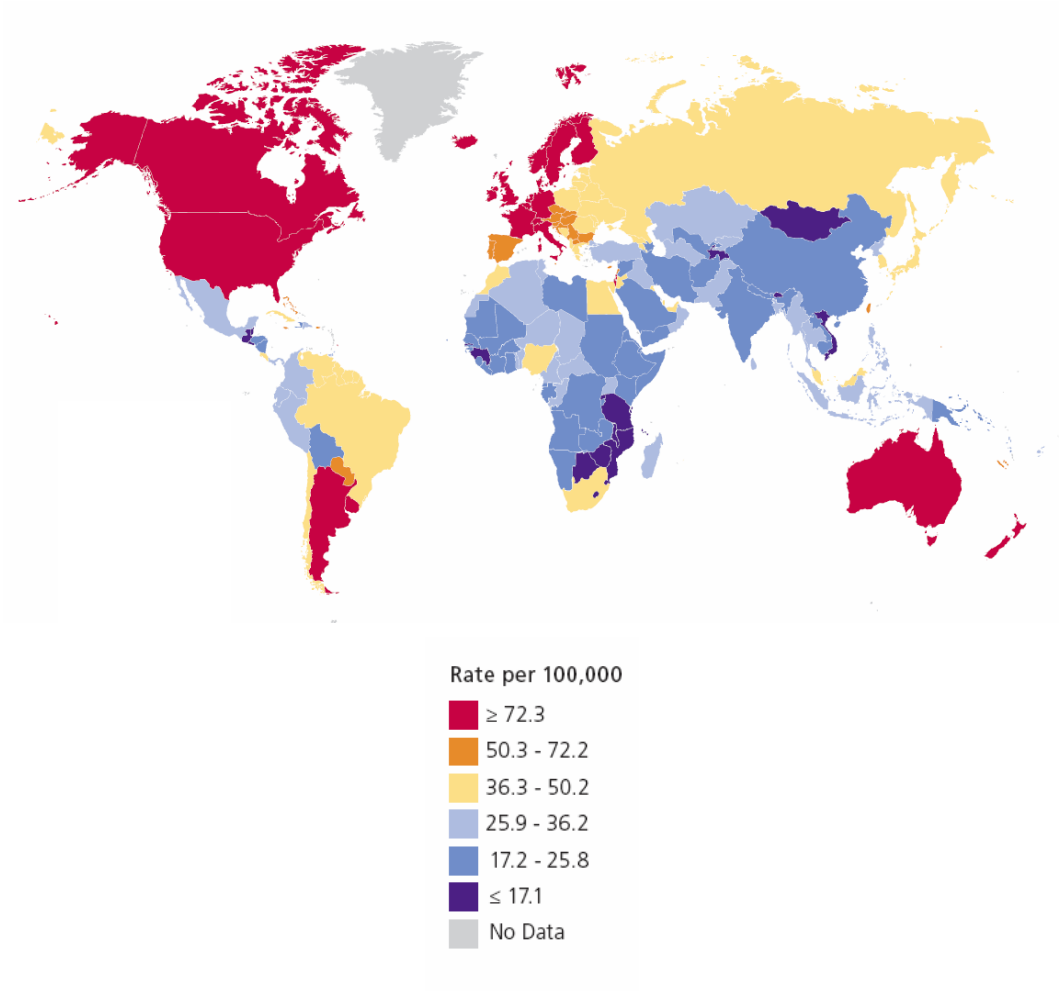



Most common cancer site
Bladder
Breast
Cervix uteri
Colon & rectum
Esophagus
Kaposi sarcoma
Liver
Lung & bronchus
Non-Hodgkin lymphoma
Oral cavity
Prostate
Stomach
Thyroid
No data

B)

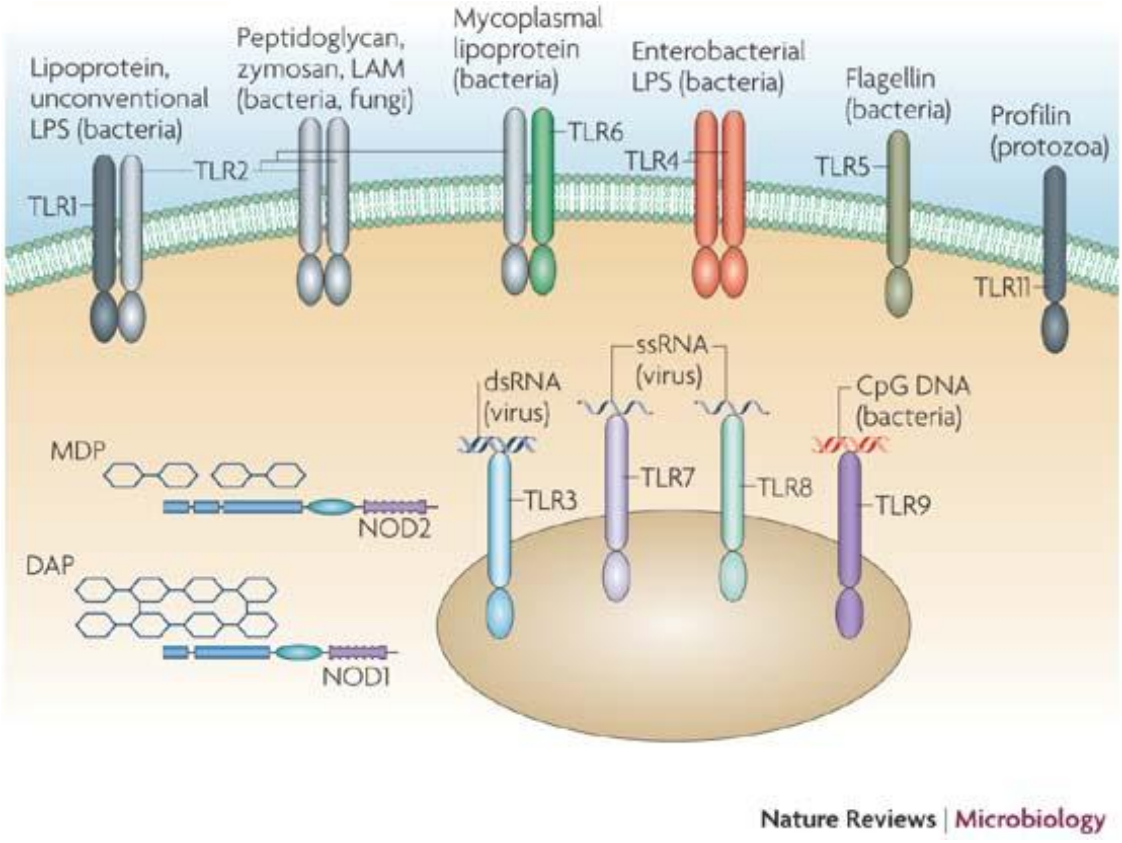


C)



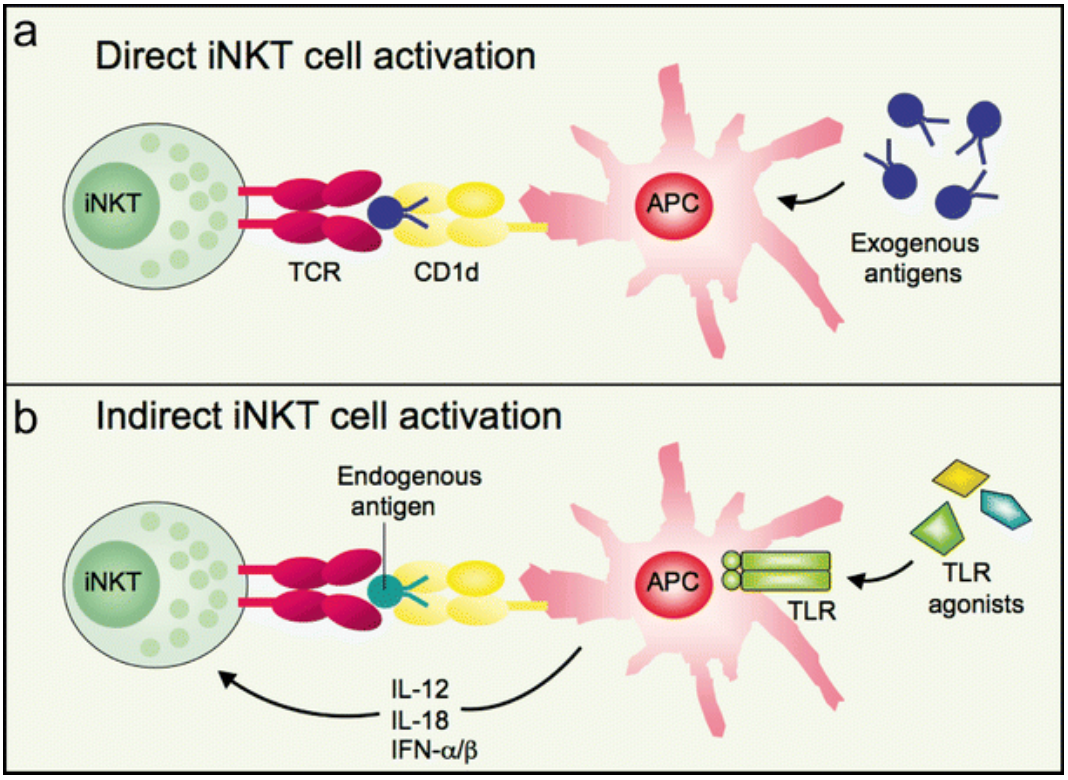
A) The worldwide distribution of the most common forms of cancer in relation to country. B) The incidence rate of prostate cancer. C) The incidence rate of breast cancer. Figure is adapted from (1).

Figure 1-2



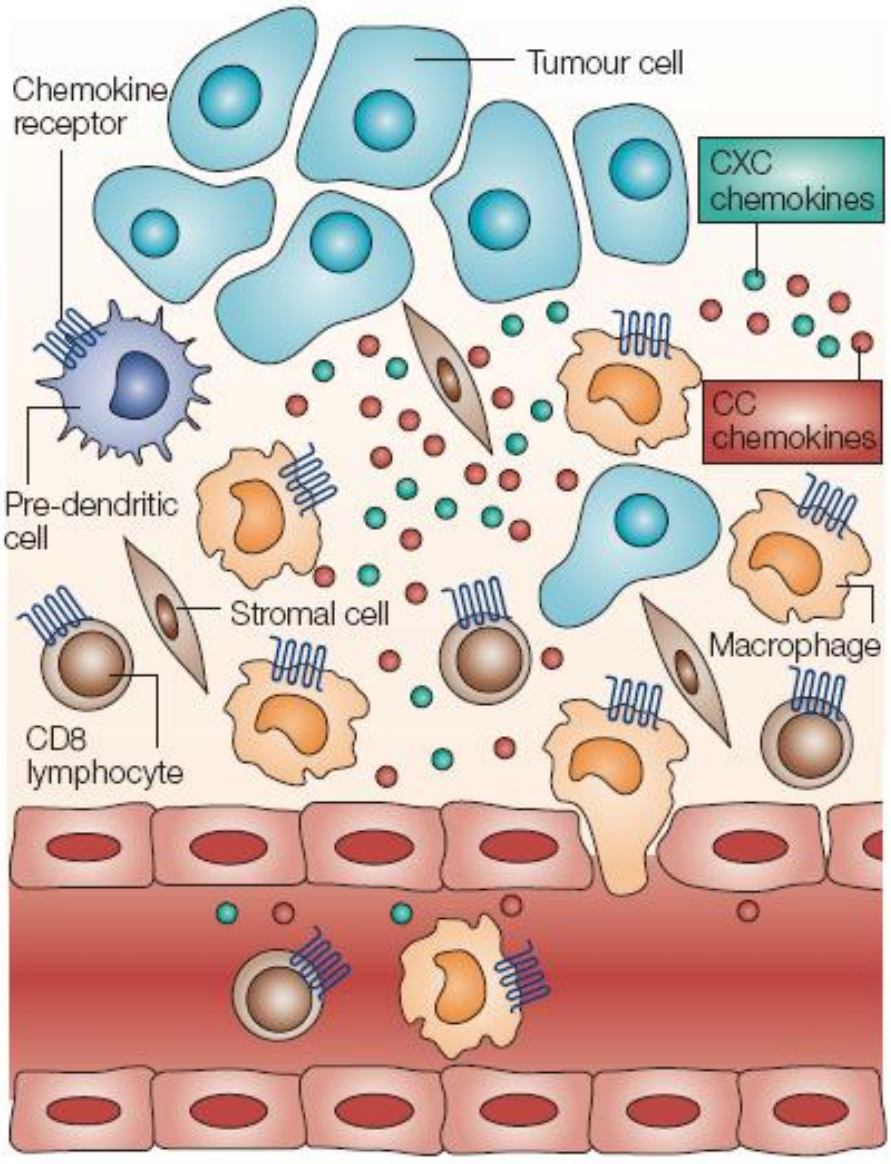
Toll-like receptors function as pathogen pattern recognition receptors in an effort to detect and alert the immune system to the possibility of an infection. Their location in the cells can help determine their molecular ligand. TLR10 is not included in this figure due to its relatively new discovery and its distribution and biology has not yet been fully determined. Figure has been adapted from (12).

Figure 1-3



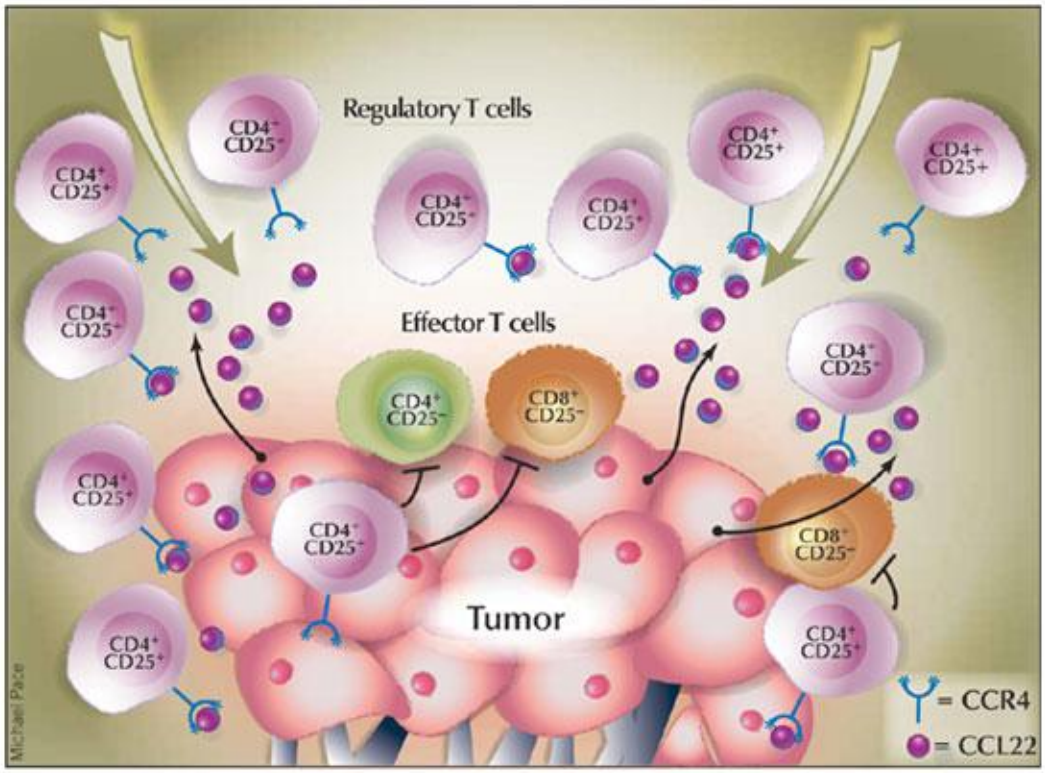
Mechanisms of iNKT cell activation through antigen presenting cells. A) Direct method of iNKT cell activation utilizing an exogenous lipid antigen presented by CD1d. B) Indirect method of activation utilizing cytokines secreted by antigen presenting cells due to TLR sensing danger signals. Figure is adapted from (31).

Figure 1-4



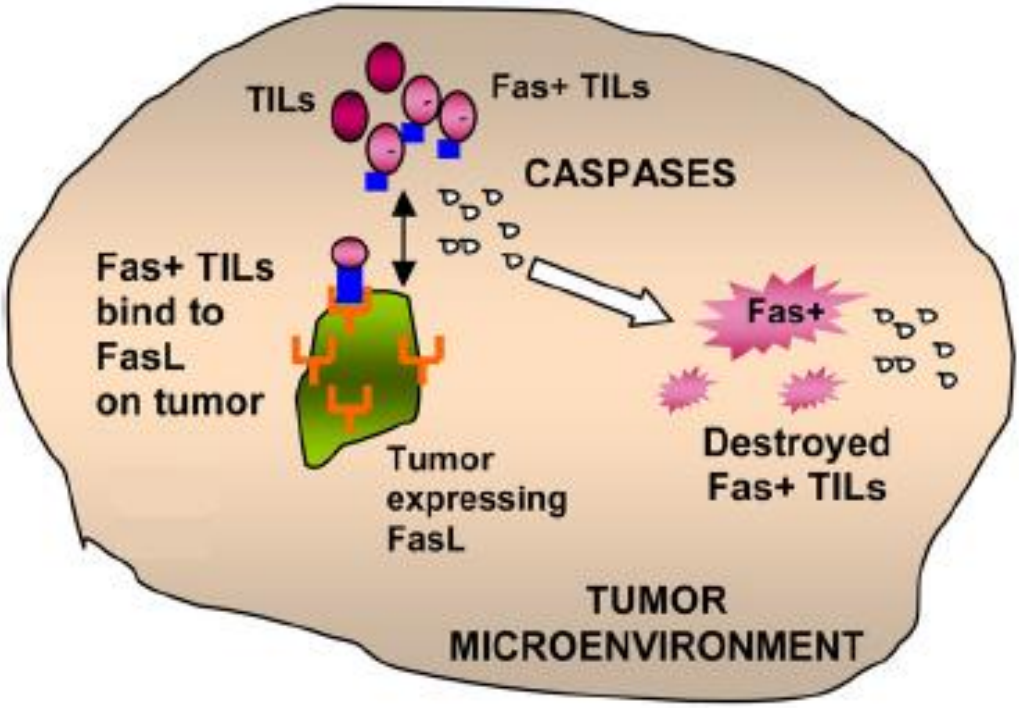
A diagram depicting the chemokine cascade initiated by the growing tumour cells to attract leukocytes such as macrophages and DC which in turn secrete more chemokines to attract Th2 favouring cells to the tumour micro-environment. Picture adopted from (36).

Figure 1-5



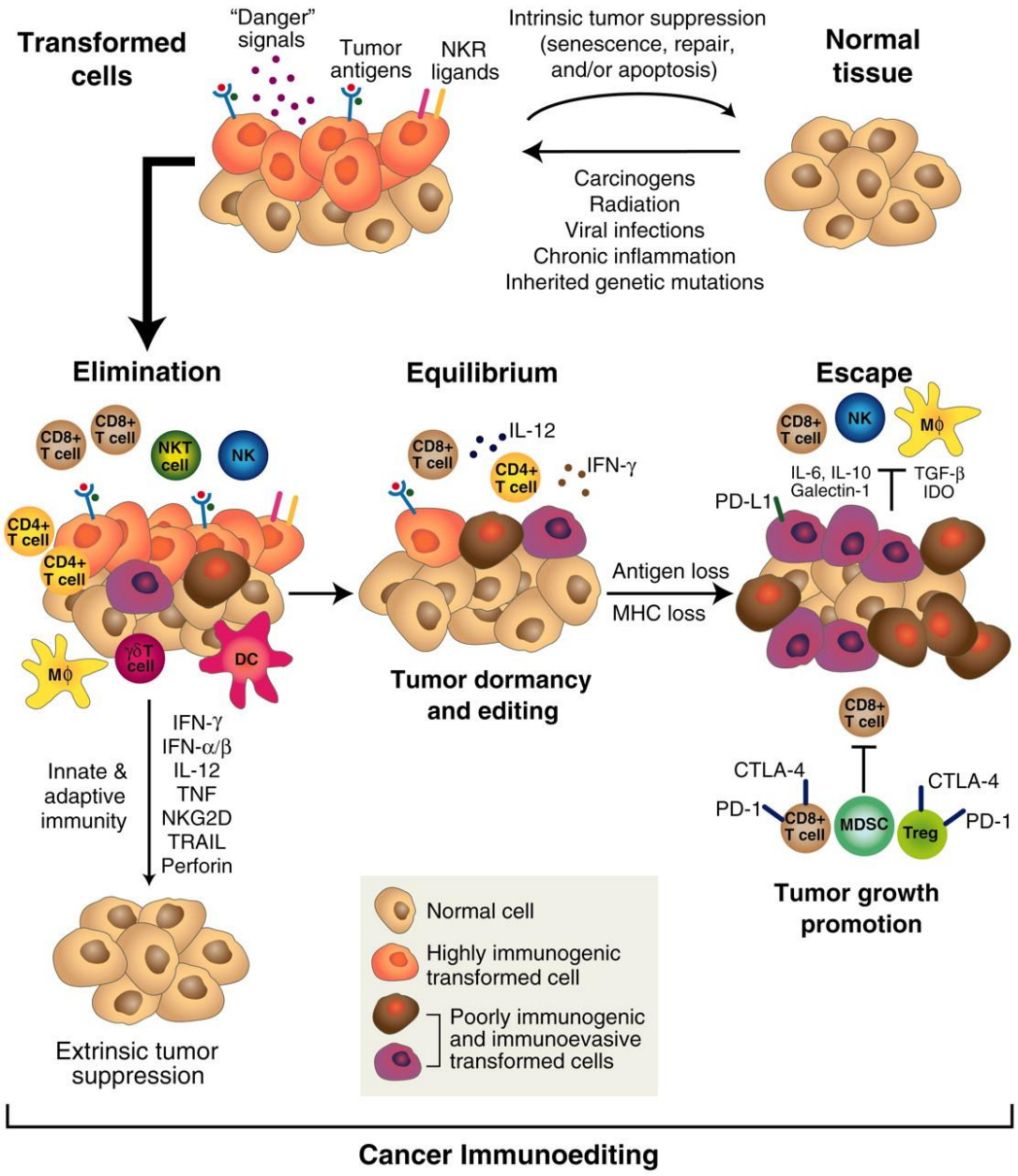
The tumour mass can attract the accumulation of Treg cells into the tumour micro-environment to control the activity of effector immune responses. Figure was taken from (41).

Figure 1-6



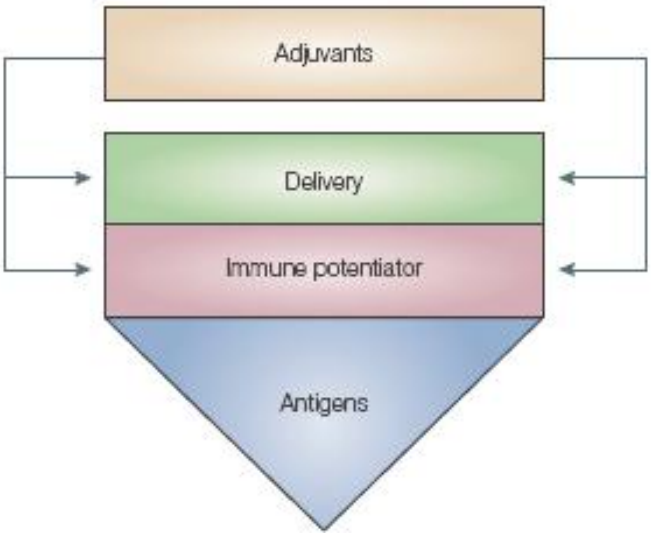
Fas ligand on tumour cells interact with Fas on TILs signalling for their destruction. This is one of many strategies that the growing tumour can use to escape an effective immune response against it. Figure was taken from (47).

Figure 1-7



Three definitive stages of cancer immuno-editing once an oncogenic event has been initiated from the normal tissue. Figure was taken from (53).

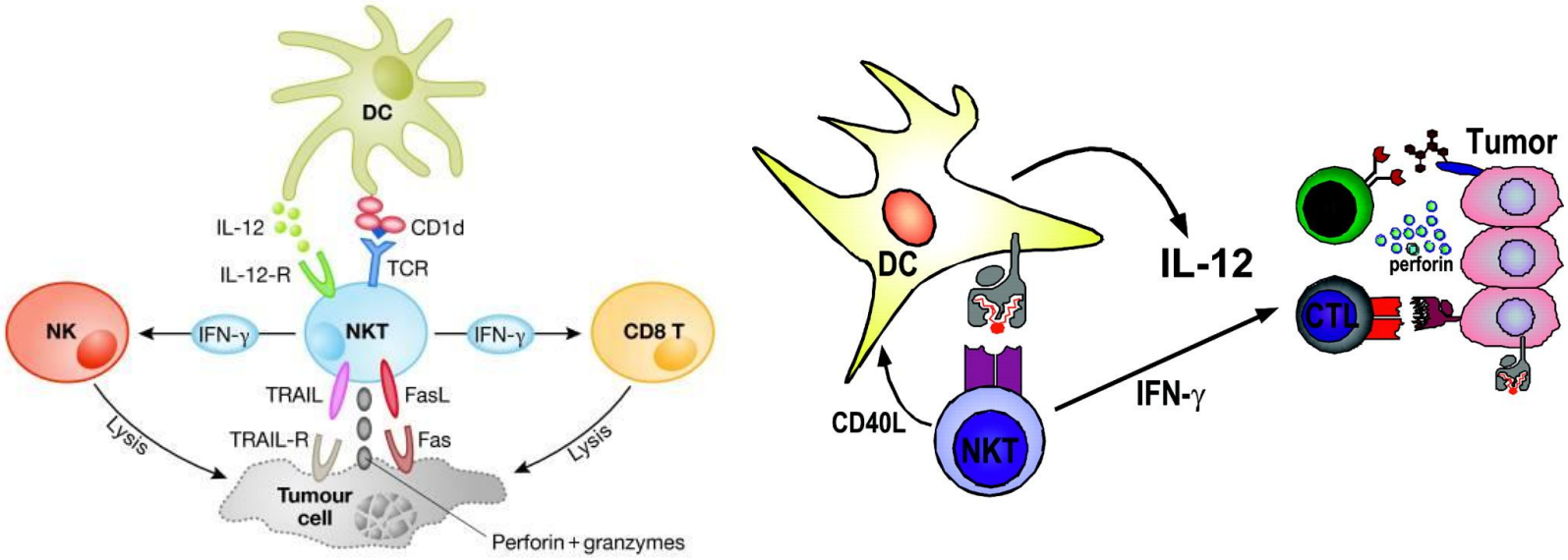
Figure 1-8



Antigens form the foundation of a subunit vaccine. The additional layers that add to the effectiveness of the vaccine are its adjuvant properties which include the delivery vehicle and any immune potentiators or in some cases specific immune inhibitors. Figure was taken from (61)

Figure 1-9

A) **B)**



A) NKT activation can modulate cellular immunity responses through $CD8^+$ T cells and NK cells to ultimately target for tumour cell death. Figure is adapted from (74). B) NKT cell interaction with DCs promote the production of IL-12 to aid in the activation of tumour targeting cellular immunity. Figure is adapted from (73).

1.9 Tables

Table 1-1

	Worldwide		
	Rank	**Deaths**	**%**
Heart diseases	1	8,923	15.1
Malignant neoplasms	2	7,424	12.6
Cerebrovascular diseases	3	5,712	9.7
Lower respiratory infections	4	4,177	7.1
Perinatal conditions*	5	3,180	5.4
Chronic obstructive pulmonary disease	6	3,025	5.1
Diarrhoeal diseases	7	2,163	3.7
HIV/AIDS	8	2,040	3.5
Tuberculosis	9	1,464	2.5
Road traffic accidents	10	1,275	2.2
Diabetes mellitus	11	1,141	1.9
Malaria	12	889	1.5
Suicide	13	844	1.4
Cirrohsis of the liver	14	772	1.3
Nephritis and nephrosis	15	739	1.3
All causes		58,772	100.0

Cancer is the second leading causing of mortality worldwide in 2004. Table is adapted from (1).

Table 1-2

Antibody	Target	FDA-approved indication	Approval in Europe*	Mechanisms of action
Naked antibodies: solid malignancies				
Trastuzumab (Herceptin; Genentech): humanized IgG1	ERBB2	ERBB2-positive breast cancer, as a single agent or in combination with chemotherapy for adjuvant or palliative treatment	Similar	Inhibition of ERBB2 signalling and ADCC
		ERBB2-positive gastric or gastro-oesophageal junction carcinoma as first-line treatment in combination with cisplatin and capecitabine or 5-fluorouracil		
Bevacizumab (Avastin; Genentech/Roche): humanized IgG1	VEGF	For first-line and second-line treatment of metastatic colon cancer, in conjunction with 5-fluorouracil-based chemotherapy; for first-line treatment of advanced NSCLC, in combination with carboplatin and paclitaxel, in patients who have not yet received chemotherapy; as a single agent in adult patients with glioblastoma whose tumour has progressed after initial treatment; and in conjunction with IFNα to treat metastatic kidney cancer	Similar	Inhibition of VEGF signalling
Cetuximab (Erbitux; Bristol-Myers Squibb)‡: chimeric human–murine IgG1	EGFR	In combination with radiation therapy for the initial treatment of locally or regionally advanced SCCHN; as a single agent for patients with SCCHN for whom prior platinum-based therapy has failed; and palliative treatment of pretreated metastatic EGFR-positive colorectal cancer	Similar	Inhibition of EGFR signalling and ADCC
Panitumumab (Vectibix; Amgen)‡: human IgG2	EGFR	As a single agent for the treatment of pretreated EGFR-expressing, metastatic colorectal carcinoma	Similar	Inhibition of EGFR signalling
Ipilimumab (Yervoy; Bristol-Myers Squibb): IgG1	CTLA4	For the treatment of unresectable or metastatic melanoma	Similar	Inhibition of CTLA4 signalling
Naked antibodies: haematological malignancies				
Rituximab	CD20	For the treatment of CD20-	Similar	ADCC, direct

(Mabthera; Roche): chimeric human–murine IgG1		positive B cell NHL and CLL, and for maintenance therapy for untreated follicular CD20-positive NHL		induction of apoptosis and CDC
Alemtuzumab (Campath; Genzyme): humanized IgG1	CD52	As a single agent for the treatment of B cell chronic lymphocytic leukaemia	Similar	Direct induction of apoptosis and CDC
Ofatumumab (Arzerra; Genmab): human IgG1	CD20	Treatment of patients with CLL refractory to fludarabine and alemtuzumab	Similar	ADCC and CDC
Conjugated antibodies: haematological malignancies				
Gemtuzumab ozogamicin (Mylotarg; Wyeth): humanized IgG4	CD33	For the treatment of patients with CD33-positive acute myeloid leukaemia in first relapse who are 60 years of age or older and who are not considered candidates for other cytotoxic chemotherapy; withdrawn from use in June 2010	Not approved in the European Union	Delivery of toxic payload, calicheamicin toxin
Brentuximab vedotin (Adcetris; Seattle Genetics): chimeric IgG1	CD30	For the treatment of relapsed or refractory Hodgkin's lymphoma and systemic anaplastic lymphoma	Not approved in the European Union	Delivery of toxic payload, auristatin toxin
^{90}Y-labelled ibritumomab tiuxetan (Zevalin; IDEC Pharmaceuticals): murine IgG1	CD20	Treatment of relapsed or refractory, low-grade or follicular B cell NHL	Similar	Delivery of the radioisotope ^{90}Y
		Previously untreated follicular NHL in patients who achieve a partial or complete response to first-line chemotherapy		
^{131}I-labelled tositumomab (Bexxar; GlaxoSmithKline): murine IgG2	CD20	Treatment of patients with CD20 antigen-expressing relapsed or refractory, low-grade, follicular or transformed NHL	Granted orphan status drug in 2003 in the European Union	Delivery of the radioisotope ^{131}I, ADCC and direct induction of apoptosis
ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity; CLL, chronic lymphocytic leukaemia; CTLA4, cytotoxic T lymphocyte-associated antigen 4; EGFR, epidermal growth factor receptor; FDA, US Food and Drug Administration; IgG, immunoglobulin G; INFa; interferon-a; NHL, non-Hodgkin's lymphoma; NSCLC, non-small-cell lung cancer; SCCHN, squamous cell carcinoma of the head and neck; VEGF, vascular endothelial growth factor.				
*Based on information from the European Medicines Agency. ‡Not recommended for patients with colorectal cancer whose tumours express mutated KRAS.				

FDA approved monoclonal antibodies for use in treatment of human cancers. Table is adapted from (65).

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CHAPTER-2

Caulobacter crescentus stimulates NKT cells to control the growth of EL4 tumour cell line

A version of this chapter is being prepared and will be submitted for peer reviewed publication

2.1 Introduction

Cancer remains a leading cause of death in the world today. Research into cancer has led to the development of many new interesting avenues for treatment. One promising avenue of treatment is the introduction of immunotherapy. Immunotherapy is designed to treat the tumour and prevent the recurrence of tumours through the activation of the immune system. The ability of the immune system to control the growth of cancer is well documented in medical literature, as cases of spontaneous regression of various tumours have been reported (1-3). Historically, it has also been recognized for hundreds of years that some cancer patients who develop concurrent bacterial and viral infections can induce their own tumour regression (4). These observations led to the first recorded use of immunotherapy by William Coley with his introduction of Coley's Toxins, which were a blend of heat-killed *S. pyogenes and S. marcescens*. The treatment was successful in controlling the growth of sarcomas in many patients; however, the overwhelming side effects from Coley's Toxins prevented wide-spread acceptance and the treatment was last used in 1963.

Current immunotherapies being developed to treat cancer focus on either directly targeting tumour-associated antigens (TAAs) through vaccination or directly controlling different arms of the immune system through the administration of exogenous factors. The recently introduced theory on immunoediting of tumours would suggest a limited amount of success through targeting single TAAs alone (5) . This theory applies Darwinian controls on the evolution of tumours over the course of disease. Targeting a single TAA could

potentially allow tumour escape and the eventual decrease of immunogenicity of the tumour. Broad activation of immunity could allow the immune system the ability to re-establish immune equilibrium with the tumour by endogenously targeting multiple TAAs (6).

Using bacteria such as *Salmonella*, *Bifidobacteria* and other anaerobic bacteria as broad immune activators has been proposed as a potential treatment to cancer (7-9). These bacteria also preferentially occupy the tumour microenvironment and attempt to induce broad immunity from the inside. Natural killer (NK) and natural killer T (NKT) cells have both been shown to be important for the immune response to control tumours and certain bacteria (10).

NKT cells have become a target for immunotherapy due to their relationship with tumours and ability to act as an enhancer of adaptive immunity (11). Specifically, NKT cells have a positive prognostic effect on the clinical outcomes of adeno and squamous cell carcinomas (12, 13). It is believed that NKT cells can help generate strong cytotoxic-based responses due to their ability to produce large amounts of IFN-γ upon activation (14) and aid in the recruitment of other immune effector cells (15). This type of response from NKT cells would be beneficial in controlling cancer growth. Work to harness the power of NKT cells has led many researchers to the formulation of a specific activator of Vα24Jα18 invariant NKT cells (iNKT), αGalactosylceramide (αGalCer), which was initially isolated from a marine sponge (16). NKT cells can be classified into two specific populations: type 1 iNKT, which recognize lipid antigens bound to CD1d through an invariant receptor; and type 2 NKT cells, which have a less

restrictive TCR but also require CD1d antigen presentation (17). αGalCer has been shown to stimulate iNKT cells and provide positive effects on tumour regression in mice (18, 19). However, a limitation to this treatment is that strong activation of iNKT cells by suspended αGalCer results in the cellular depletion of the population after treatment in humans (20). Along with αGalCer, other ways of stimulating NKT cells have emerged, such as non-pathogenic *Sphingomonas* bacteria (21).

In our study, we evaluate the ability of a non-pathogenic, Gram-negative bacteria, *Caulobacter crescentus (Cc),* to activate NKT cells and mediate NKT-dependent tumour cell growth inhibition and killing. Here, we report that *Cc* has an ability to stimulate NK and NKT cells to proliferate and produce IFN-γ *in vitro*. We also observed that *in vivo Cc* immunizations stimulated both NK and NKT cell activation at the injection site and that these cells were capable of killing and inhibiting the growth of EL4 tumour cells. It is, however, unclear whether the *Cc*-stimulated activation of NKT cells is based on a *Cc*-specific lipid or non-lipid product.

2.2 Materials and Methods

2.2.1 Animals

Wild-type C57BL/6 (B6) male and female mice were purchased from Charles River, USA. They were housed by Health Sciences Laboratory Animal Services at the University of Alberta in their conventional environment facility.

Jα18-/- on B6 background mice were received as a generous gift from Dr. Kronenberg (La Jolla, California, USA). CD1d-/- B6 background breeding pairs were purchased from Jackson Laboratories (Bar Harbor, USA) and bred in our animal facility (22).

2.2.2 Bacterial growth and maintenance

Caulobacter crescentus (strain CB15), transfected with a plasmid containing a chloramphenicol resistance gene, was kindly supplied by Dr. John Smit (University of British Columbia) and grown on solid PYE agar media plates containing chloramphenicol as a selection antibiotic. An individual colony was harvested from the plate and transferred to liquid PYE media supplemented with chloramphenicol. Bacteria were grown at room temperature and the concentration of the bacteria was determined using a spectrophotometer set to measure the absorbance at the wavelength of 600nm. The following formula was used to determine bacterial colony forming units per mL (A_{600}=1.000($3x10^9$)CFU/mL). Bacterial cultures were then refrigerated until use and diluted as required.

2.2.3 *Ex Vivo* cell culture

C57BL/6 mouse tissue was collected quickly after euthanization. Spleens were obtained and ground between two sterile, frosted glass slides. Single-cell suspension of splenocytes were washed with PBS followed by red blood cells (RBC) lysis with double distilled and filtered water (ddH_2O) for 10 seconds. In

magnetic cell separation assays, the splenocytes were treated with the panNK cell isolation kit (Stemcell Technologies; Vancouver, Canada) that positively selects for NK cells based on CD49b expression (clone: DX5), before being prepared for plating. $5x10^5$ splenocytes were cultured directly in 10% heat-inactivated Fetal Bovine Serum, penicillin-streptomycin-supplemented RPMI (mouse media). $20x10^6$ CFU/mL of *Caulobacter crescentus* was prepared by pelleting and resuspending the bacteria in PBS. *Cc* was diluted in mouse media before plating in 96-well plates for all cell culture assays. Heat killing of *Cc* was completed at 80^oC for 30 minutes in a pre-heated water bath. Heat-killed *Escherichia coli* treatment groups were included in most assays to act as a bacteria comparison to *Cc* treatment. Supernatant was collected from splenocyte-*Cc* co-cultures and frozen until analysis with cytokine ELISA kits (BioSource; eBioscience, San Diego, USA). To measure proliferation from *ex vivo* cell cultures, ^{3}H-methyl-thymidine was added to the cell cultures for 18 hours then harvested onto nitrocellulose filter paper to determine proliferation using a Wallac Trilux Liquid Scintillation Counter (Perkin Elmer; Waltham, USA).

2.2.4 Cell line

EL4 mouse (C57BL/6) lymphoma cell line was obtained from Biomira Inc. in 2002. It originally came from the American Type Culture Collection (ATCC; Manassas, USA). EL4 was maintained in 5%FBS RPMI media supplemented with 2mM L-glutamine, penicillin/streptomycin and 2-mercaptoethanol. Cells were passed every 2-3 days to maintain optimum growth

conditions. Cell line morphology was confirmed through microscopic observation.

2.2.5 Cytokine ELISAs

Cytokine ELISAs were performed as per manufacturer instructions. ELISA kits for, TNF-α, IL-10 and IFN-γ were purchased from both Invitrogen BioSource (Carlsbad, USA) and eBioscience (San Diego, USA). Supernatants were diluted with assay diluent and plated in duplicate. The absorbance measured at 450nm. Absorbance values were compared to an assay specific standard curve to determine cytokine concentration. The average cytokine concentrations from duplicate samples were determined along with the standard deviation.

2.2.6 CFSE proliferation assay

C57BL/6 spleens were processed as previously mentioned, then separated using a positive panNK cell isolation kit (Stemcell Technologies; Vancouver, Canada) utilizing the antibody to CD49b (clone DX5). The isolated NK cells were washed and resuspended at $1.0x10^6$ cells/mL in PBS containing 2%FBS. 5μM CFSE (Invitrogen; Carlsbad, USA) was added to the solution for 20-30 minutes at room temperature followed by the addition of 10mL PBS to end the reaction. The CFSE stained cells were washed twice followed by reconstitution with the non-NK cells from magnetic separation in mouse media. The mix was co-cultured onto 96-well plates at $5x10^5$ cells/well. After 3 days, the cells were harvested and stained for CD69 (H1.2F3), NK1.1 (PK136), CD3ε (145-2C11)

(eBioscience; San Diego, USA) and analyzed on a FACSCanto. Cells identified as CFSE-reduced were gated as the proliferating population.

2.2.7 Immunizations

Mice were immunized intraperitoneally with 100μL of $20x10^6$ CFU of *Cc* suspended in PBS. Heat-killed *Cc* was prepared by incubating the *Cc* in PBS at 80^oC for 30 minutes. For mice receiving KRN7000 (αGalCer; Enzo LifeSciences; Farmingdale, USA), injections were prepared as suggested on the product handout and injected at 2μg/mouse.

2.2.8 Peritoneal wash

Peritoneal washes were performed with 10mL of PBS 1 day after immunization. PBS was injected into the peritoneum after CO_2 euthanization. Mouse was agitated to suspend cells into the PBS. The peritoneum was cut and the PBS was collected in 50mL conical tubes. Wash was collected in 50mL conical tubes and pelleted. The pellet was then lysed of RBCs as previously mentioned and run through a 70μm nylon filter to remove cellular debris.

2.2.9 Flow Cytometry

Cells were stained using antibodies purchased from eBioscience (San Diego, USA) for NK1.1 (clone PK136), CD3e (clone 145-2C11) and CD69 (clone H1.2F3). Granzyme B (clone GB11) antibodies were purchased from BioLegend (San Diego, USA). Intracellular staining of granzyme B was performed utilizing

permeabilization buffer consisting of 0.1% saponin with FACS buffer (2% FBS in PBS). Cells were fixed in 2% paraformaldehyde and 20,000 events were collected on a FACSCanto flow cytometer (BD Bioscience; San Jose, USA) and later analyzed using FlowJo software (Treestar; Ashland, USA).

2.2.10 EL4 killing assay

EL4 cells were harvested and stained with 1-3μM of CFSE in PBS for 30 minutes. $1x10^4$ cells were transferred into 5mL falcon tubes with IP-washed cells and left overnight in a 37^oC 5% CO_2 incubator. The next morning, 20 000 CountBright beads (Invitrogen; Carlsbad, USA) were added to each tube and vortexed to ensure a homogenized mixture. Using FACSDiva (BD Bioscience; San Jose, USA) to gate the beads the FACSCanto was used to collect 10 000 beads. This was done to normalize the amount of cells tested in each tube. A difference in CFSE stained cells between treatment tubes and control tubes indicate the number of cells that were killed in those samples. This assay was adapted from a previously reported CFSE target cell killing assay (23).

2.2.11 EL4 growth inhibition assay

IP cells were harvested and plated with irradiated and non-irradiated EL4 tumour cells at different effector-to-target ratios. Irradiated EL4 cells were prepared by irradiating the cells at 2500cGy. Irradiation of the cell line results in DNA damage and preventing proper DNA which inhibits their proliferative ability. ^{3}H-methyl-thymidine was added one hour after culture to detect proliferation over an

18hr incubation period. Cultures were harvested onto nitrocellulose filter paper to determine proliferation using a Wallac Trilux Liquid Scintillation Counter (Perkin Elmer; Waltham, USA). Irradiated EL4 culture counts per minute (cpm) was subtracted from the whole cultures to eliminate any effector cell background proliferation. Inhibition was calculated by:

$$\frac{\text{EL4 only group - (Whole sample – irradiated EL4 sample)}}{\text{EL4 only group}} \text{ X } 100$$

2.2.12 Lipid Extraction

Lipid and non-lipid fractions of *Caulobacter crescentus* were prepared by an adapted version of the Bligh and Dyer method of lipid extraction (24). The lipid (chloroform layer) and the non-lipid (methanol water) layers were dried after extraction at 40^{o}C overnight. The non-lipid fraction was resuspended in PBS while the lipid fraction was resuspended in DMSO for use in *in vitro* cell culture experiments to determine activation of NKT cells.

2.2.13 Statistics

Statistics were performed with the statistical software SPSS v.13 (IBM; New York, USA). One-way ANOVAs were performed and significance is indicated at $p<0.05$ on post hoc LSD analysis comparing to assay controls.

2.3 Results

2.3.1 *Caulobacter crescentus* leads to NK/NKT cell activation

To determine if *Caulobacter crescentus* could stimulate immune cells in an *in vitro* culture, we plated naïve murine splenocytes with *Cc* and measured the proliferation and cytokines produced after 3 days. When heat-killed *Cc* was introduced to naïve murine splenocyte cells directly in culture, we detected a marked dose-dependent proliferation of cells on day 3 of culture (Figure 2-1A and B). This proliferation is accompanied by a strong IFN-γ and TNF-α response from the tested supernatant of the proliferating cells at day 3 (Figure 2-1A). The early nature of the IFN-γ response in naïve mice and the presence of TNFα led us to speculate that the response was being generated by either NK or NKT cells.

To determine if NK or NKT cells were involved in the IFN-γ production we performed a NK cell depletion using magnetic beads coupled to an anti-CD49b antibody (clone DX5) (Figure 2-1C). The separation procedure did not completely remove all $CD49b^+$ cells however, the process did reduce the population by a considerable amount (Δ3-4%). Using the NK depleted splenocytes, we were able to detect significantly lower proliferation and IFN-γ generated in response to heat-killed *Cc* (Figure 2-1D). This would indicate that the *Cc*-responding population was either NK or NKT cells.

2.3.2 *Caulobacter crescentus* responding cells are NKT cells

To determine if the responding cells were either NK or NKT cells, we performed a CFSE-labelled cell proliferation assay where the $CD49b^+$ isolated NK cells were stained with CFSE, then re-added to the non-stained splenocytes and stimulated with *Cc*. Proliferation was indicated when there was an observed

loss of CFSE in the stained cell population because the CFSE would be split evenly amongst the newly-formed daughter cells. Here, we find further proof that both live and heat-killed *Cc* can stimulate the proliferation of $CD49^+$ NK cells (Figure 2-2A), as the loss of CFSE was greater in the *Cc*-stimulated cell culture at 10.7% compared to media which lost only 6.7%. By further analyzing the *Cc* stimulated proliferating cells, we saw that they were comprised of 73.8% $CD3\varepsilon^+$ $NK1.1^+$ cells, indicating that they are NKT cells (Figure 2-2B). The proliferating cells also had higher levels of CD69 on their cell surface, which is a marker for early lymphocyte activation. With this data, we were curious to determine if NKT cell knockout mice would lack a response to *Cc*. We performed a cell culture assay using Jα18-/- knockout mice lacking iNKT cells and CD1d-/- mice which lack all CD1d-dependent NKT cells. Here, we found that splenocyte cultures from both mice had significantly reduced levels of IFN-γ after days 1 and 3 post-culture with heat-killed *Cc* (Figure 2-3) compared to the WT splenocyte response. Differences were also observed in the levels of IL-10 produced between the Jα18-/- mice and CD1d-/- mice. IL-10 levels were significantly lower in the CD1d-/- splenocyte cultures compared to both WT and Jα18-/-, indicating the presence of a CD1d-dependent Jα18-/- NKT cell population present in the Jα18-/- mouse which is capable of secreting IL-10 upon activation with heat-killed *Cc*.

2.3.3 *In vivo* response to *Caulobacter crescentus*

To determine if *Cc* has an affect on immune cells at the site of injection, we analyzed the peritoneal exudate (PE) cells of immunized mice. *Cc* was injected intraperitoneally (IP) into C57BL/6 wild type mice and, after 24 hours, we collected PE cells and stained them to determine their phenotype. We detected an increase in the frequency of $NK1.1^+$ $CD3\varepsilon^+$ cells 24 hours after immunization (Figure 2-4A). An increase of 25% of the stained cells from *Cc* immunization compared to 12% in the saline injection group was noted. These NKT cells were also becoming activated, as indicated by their CD69 expression levels (Figure 2-4B). To a lesser degree, $NK1.1^+CD3\varepsilon$- cells were also being activated and accumulating in the IP compartment after immunization with *Cc*. αGalCer, a known activator of iNKT cells, is also capable of generating a similar response from immunization.

Three days after the initial immunization, we analyzed the splenocytes to determine if activated NKT cells could be detected as a surrogate marker for systemic immune activation. Based on the levels of CD69 from both $NK1.1^+$ and $NK1.1^+CD3\varepsilon^+$ dual-positive cells in the PE after immunization with *Cc*, we determined that there was an increase in the levels of activated NK and NKT cells present in the spleen (Figure 2-5A and B); however, their overall distribution did not appear to change. When the mice were immunized 3 times in succession, 5 days between injections, we were able to detect higher levels of granzyme B-expressing NK and NKT cells from the splenocytes of *Cc* immunized mice (Figure 2-5C). The response was greatest in $NK1.1^+CD3\varepsilon$- ($p<0.05$), while

$NK1.1^{+}CD3\varepsilon^{+}$ dual-positive cells showed a strong trend ($p<0.09$) in their increased expression of granzyme B after immunization.

2.3.4 *Caulobacter crescentus* is capable of stimulating PE cells to inhibit and kill EL4 cells

Activated, granzyme B-expressing NK and NKT cells are a good indication that *Cc* immunization may have an ability to control tumour cell growth. We sought to determine if *Cc* could directly stimulate PE cells to kill or inhibit the growth of EL4 cells. We adapted a flow cytometry-based cell killing protocol that counted the number of CFSE labelled EL4 cells remaining after co-culture with PE cells (Figure 2-6A). The difference in EL4 cells between PBS-immunized PE cells compared to *Cc* stimulated PE cells was calculated based on the number of dead cells. Mice PE cells stimulated *in vivo* with *Cc* 18 hrs prior to isolation displayed an ability to kill EL4 target cells (Figure 2-6B). The calculated number of dead cells corresponded to the effector-to-target ratio that was plated, revealing that PE cells from *Cc*-immunized mice were able to kill EL4 tumour cells (Figure 2-6C).

Utilizing a cellular proliferation assay that measured the uptake of ^{3}H-methyl-thymidine by growing EL4 tumour cells, we also showed that the *Cc*-stimulated PE cells are capable of controlling the proliferation of the EL4 tumour cell line (Figure 2-7A). An interesting finding from this was that *Cc*-stimulated PE cells obtained from CD1d-/- mice displayed a significantly lower ability to inhibit the growth of EL4 tumour cells (Figure 2-7B). Taken together, the

observation of both EL4 killing and inhibition of EL4 proliferation indicate an ability of *Caulobacter crescentus* to control the growth of EL4 tumours through stimulation of NKT cells.

2.3.5 Both lipids and non-lipids can activate NKT cells

The activation of NKT cells can be dependent on lipid recognition through the lipid antigen-presenting molecule, CD1d. To determine if *Cc* is activating NKT cells through the lipid recognition pathway, we prepared crude lipid and non-lipid fractions from whole *Cc* cultures. These fractions were then plated with WT murine splenocytes to determine their ability to stimulate proliferation and IFN-γ production. Unfortunately, after many separate preparations of lipids and non-lipids, a conclusive determination of the requirement of lipids remains elusive, having detected proliferation in both the lipid and non-lipid fractions (Figure 2-8A). The supernatant collected failed to show convincing evidence of IFN-γ production in either the lipid or non-lipid fractions (Figure 2-8B).

2.4 Discussion

NKT cell tumour infiltrate can play a role as a positive prognostic indicator for cancer patients. Their activation has been suggested to be important for establishing tumour immunity. For the first time, we have shown that *Caulobacter crescentus,* a Gram-negative, non-pathogenic bacteria, is capable of activating NKT cells. As shown previously by Bhatnager et al., *Cc* has the ability

to prevent the establishment of syngeneic lung tumours in mice (25). We believe that this response was due to NKT cell activation in these mice.

In vitro cultures combining heat-killed *Cc* with murine splenocytes stimulated strong IFN-γ and TNF-α production, followed by cellular proliferation. Compared to both LPS and heat-killed *E. coli* bacteria, *Cc* is capable of stimulating a strong response. This production was shown to be NK/NKT cell-dependent after applying a NK/NKT cell-depleting antibody. Interestingly, the lack of response from live bacteria *in vitro* was also observed by others working with *Burkholderia pseudomallei* bacteria, which are also capable of stimulating higher levels of IFN-γ in a naïve splenocyte cell culture system compared to live bacteria (26). This is believed to be due to the isolated nature of *in vitro* cell cultures lacking any migrating peripheral immune modulating cells (26). Further studies would need to be completed to determine the exact mechanism of differing responses to heat-killed and live bacteria *in vitro*.

Through flow cytometry, we were able to determine that the proliferating cells are $CD3\varepsilon^+$ $NK1.1^+$ NKT cells. To confirm this finding, we used Jα18-/- and CD1d-/- mice. These mice lack type 1 invariant NKT cells and all CD1d-dependent NKT cells, respectively. Jα18-/- mice have been shown recently to have a large reduction in the number of NKT cells, however the NKT cells that remain are shown to be type 2 NKT cells (27) . The production of IFN-γ in response to heat-killed *Cc* is, thereby, confirmed to be from type 1 NKT cells. The observed production of IFN-γ from NKT cells would support the hypothesis that *Cc* is capable of controlling the growth of tumours. This is based on previous

studies where IFN-γ from NKT cells is capable of enhancing the production of tumour specific cytotoxic T cells (28, 29). Interestingly, we also showed that *Cc* is capable of activating a type 2 NKT cell population found in Jα18-/- mice and lacking in the CD1d-/- mice. This observed population is observed to be responsible for IL-10 production and could be related to previously-reported NKT cells isolated from WT mice that are capable of controlling aberrant inflammation in autoimmune mouse models (30).

To determine if our *in vitro* observations translated to *in vivo* activation of NKT cells, we immunized mice with *Cc* and measured the presence of NKT cells, as well as the expression of the activation marker CD69 and granzyme B content. We determined that live *Cc* immunization was capable of activating NKT cells *in vivo*. Although these results are different from the *in vitro* requirement of heat-killed *Cc*, they are supported from previous research that showed heat-killed *Escherichia coli* and *Yersinia enterocolitica* delivered *in vivo* respectively, have a decreased ability to stimulate immunity and promoted regulatory immune responses (31, 32). We speculate that the immune system may determine the viability of infecting bacteria in order to limit a potentially damaging inflammatory immune response to live bacteria.

The ability of *Cc* to stimulate NKT cells to mediate EL4 tumour cell killing was speculated after observing the increase in granzyme B expression from PE cells. We showed that the *in vivo* stimulation of NKT cells using live *Cc* is capable of both killing and preventing the growth of EL4 cell line. PE from CD1d-/- mice had a decreased ability to control the growth of EL4 cells.

However, PE cells obtained from Jα18-/- displayed an ability to control the growth of EL4 cell line. This was in contrast to the lack of IFN-γ production from *in vitro*-stimulated Jα18-/- splenocytes. This would indicate that the iNKT cells, despite being potent producers of IFN-γ *in vitro*, are not primarily responsible for controlling the growth of EL4 tumour cells. However, the importance of IFN-γ in controlling tumour growth should not be dismissed for all tumour types (33).

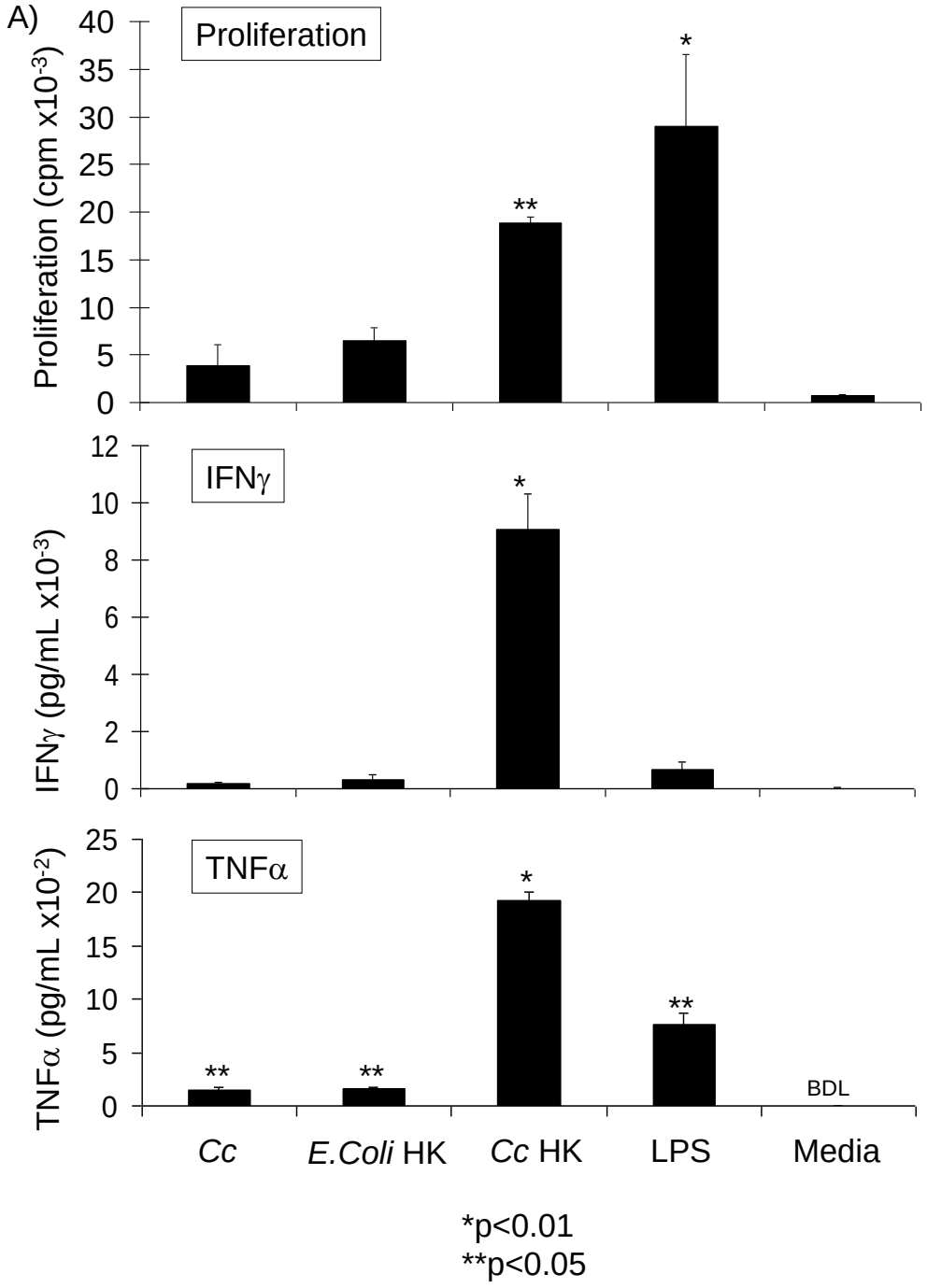
Due to the requirement of CD1d-dependent NKT cells to respond to *Cc* immunization, we speculate that the molecular fraction of *Cc* responsible for generating NKT cell responses are lipids (34). When we performed a lipid extraction of *Cc* and plated the lipid and non-lipid extracts onto murine splenocytes, we continually yielded ambiguous results. We cannot conclude from this isolation technique whether an exogenous lipid from *Cc* is responsible for activating NKT cells. However, the need to determine the agent responsible for immune stimulation by *Cc* would be an important step to avoid the differential effects observed through *in vitro* versus *in vivo* stimulation by heat-killed or live *Cc*, respectively. It would also be important in the future to determine if *Cc*-induced NKT cell activation has an effect on other cell populations of the innate immune system, particularly dendritic cells.

We are interested in evaluating the ability of *Cc* to stimulate a pro-inflammatory population of NKT cells. NKT cell activation is believed to be beneficial in the clinical outcome of cancer patients. Studies are currently ongoing to determine if pharmaceutical activation of NKT cells could provide a direct benefit in tumour-bearing patients (35, 36).

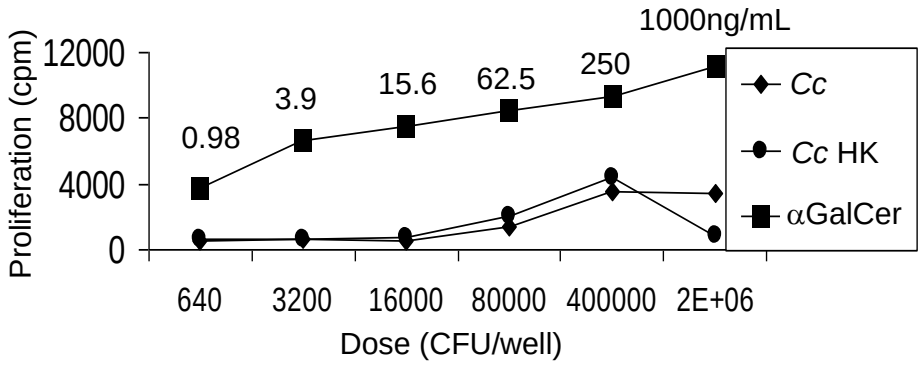
We have shown for the first time that a non-pathogenic, Gram-negative bacteria is capable of stimulating NKT cells in mice. NKT cell-mediated responses are then capable of inhibiting and killing EL4 tumour cells. Our work introduces a novel immune-stimulating therapy in the hopes of inducing tumour immunity. We provide a basis for support in the evaluation of non-pathogenic bacteria as immune stimulating agents which could be used as a treatment for cancer.

2.5 Figures

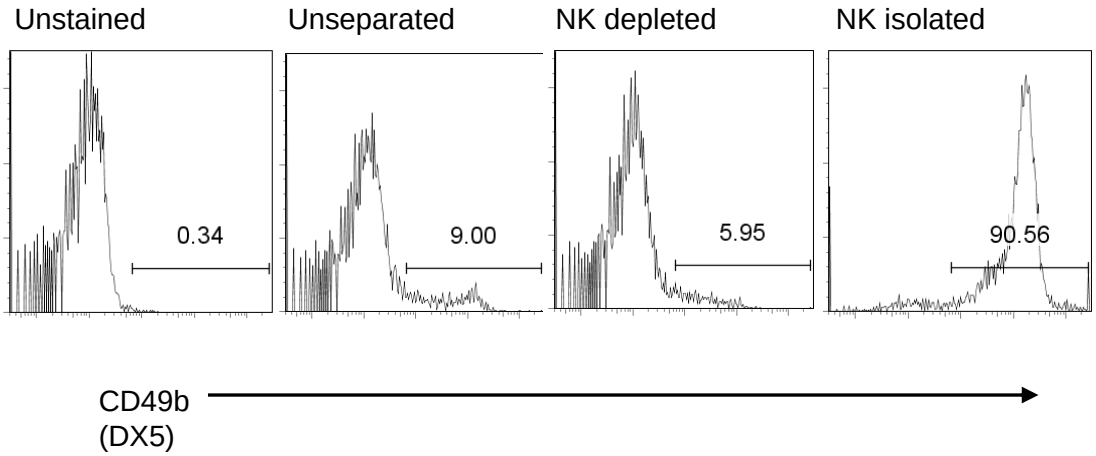
Figure 2-1 **NK cells are responsible for the production of stimulatory cytokines from *in vitro Caulobacter crescentus*-stimulated splenocytes**



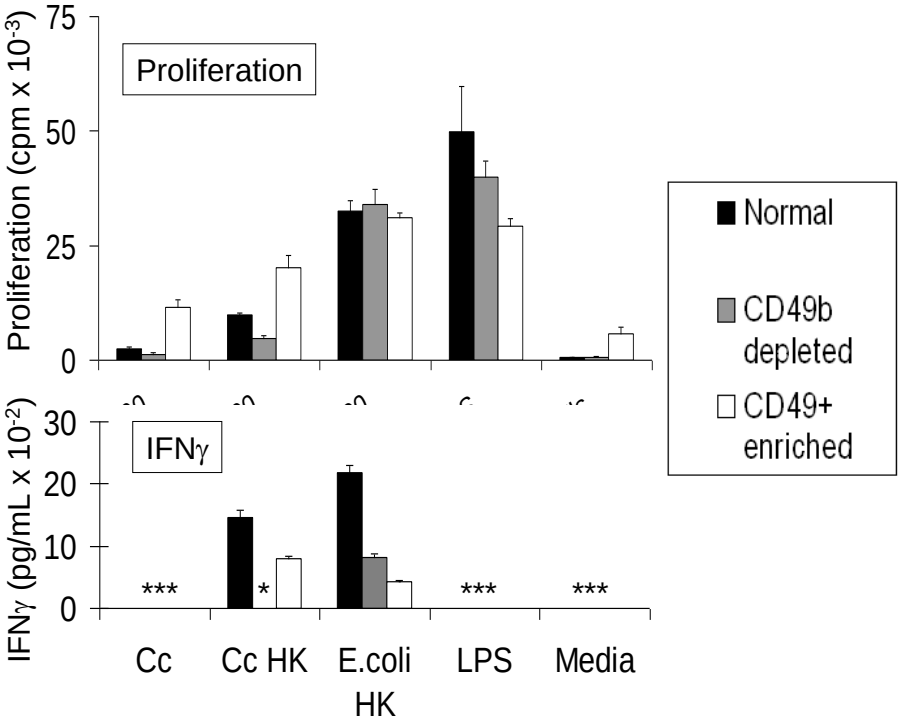
B)


1000ng/mL
Proliferation (cpm)
12000
8000
4000
0
0.98
3.9
15.6
62.5
250
Cc
Cc HK
αGalCer
640
3200
16000
80000
400000
2E+06
Dose (CFU/well)

C)


Unstained
Unseparated
NK depleted
NK isolated
0.34
9.00
5.95
90.56
CD49b
(DX5)

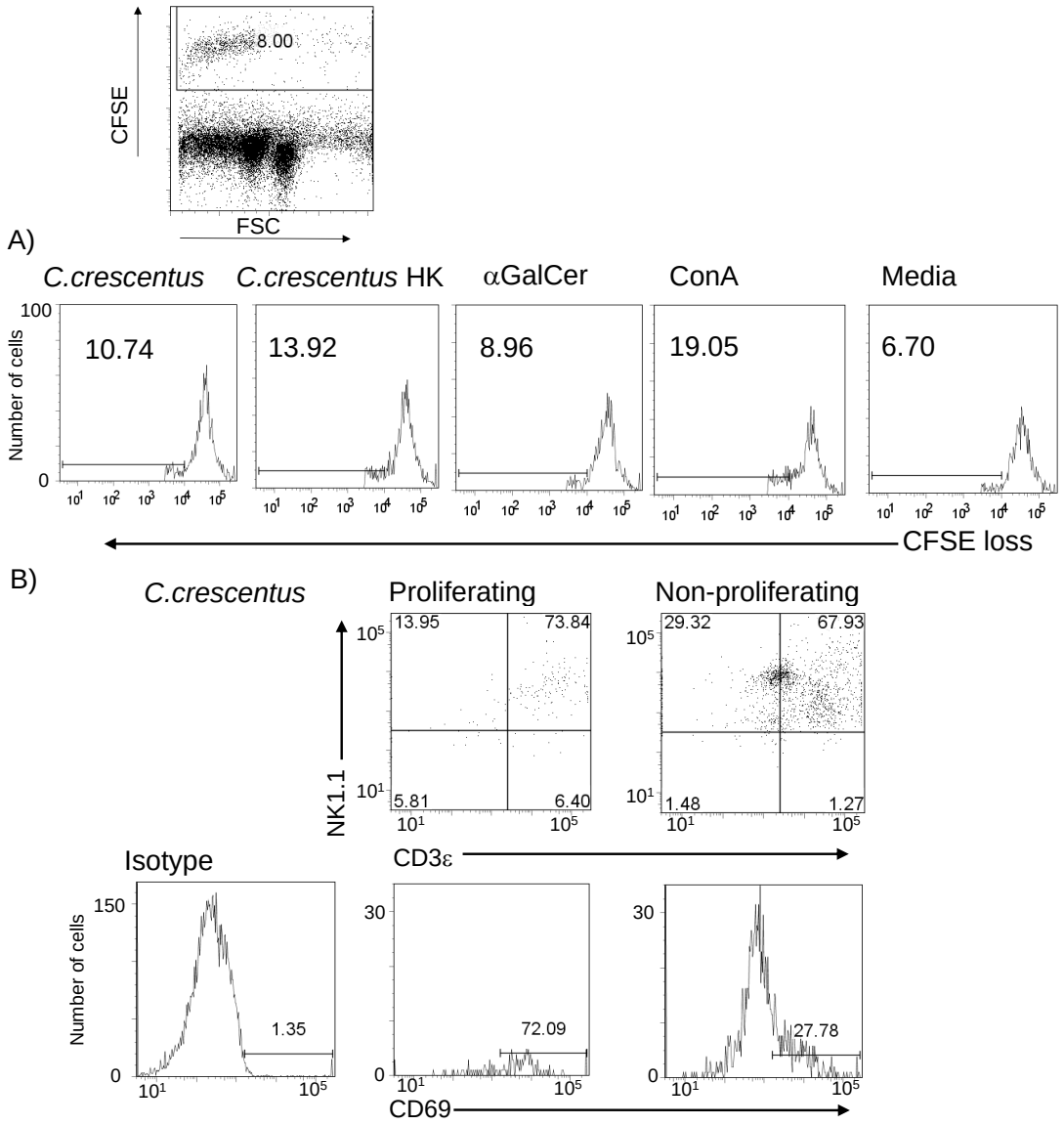
D)



*none detected

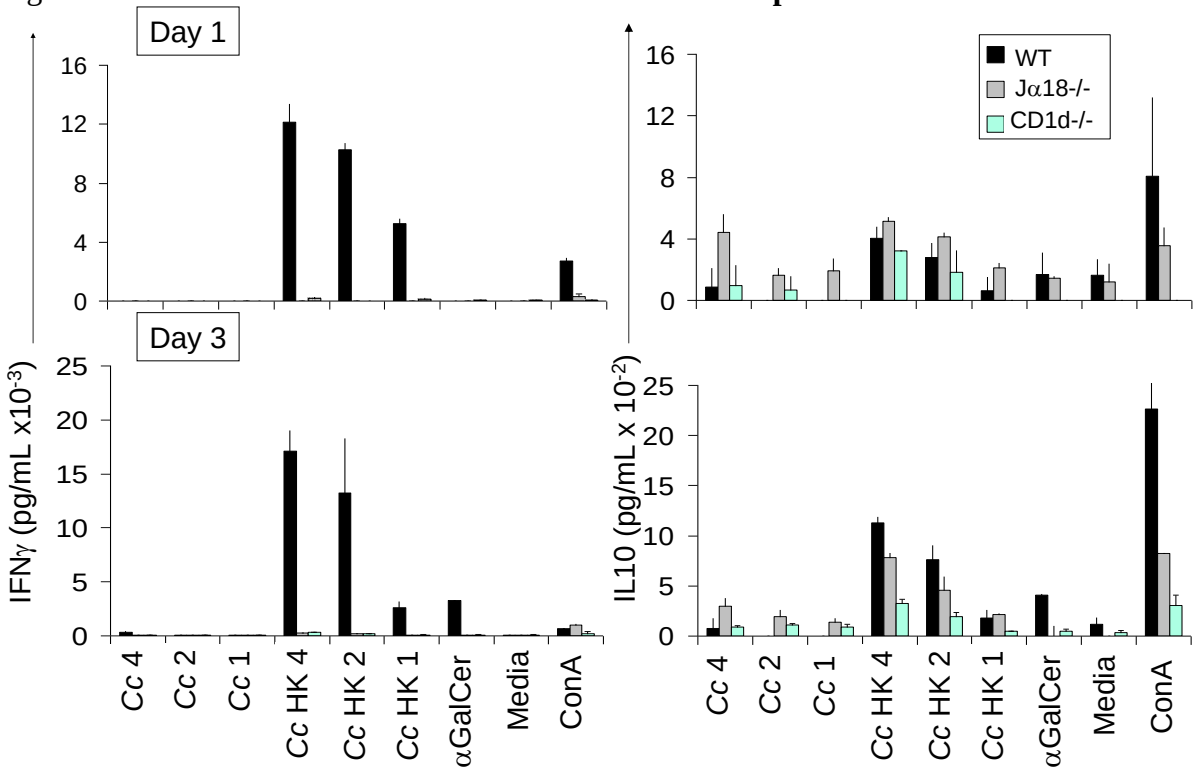
(A) Splenocytes were plated $5x10^5$ cells/well on 96-well plates, along with *Cc* (200,000CFU), heat-killed *Cc* (200,000CFU), heat-killed *E. coli* (200,000CFU) or LPS 1μg/mL and incubated for 3 days until supernatant was collected and cytokines were determined using commercially available cytokine ELISA kits. *E. coli* and LPS were plated as positive controls. Proliferation was measured using a standard ^{3}H-methyl-tritium uptake assay. *Cc* heat-killed for 30 minutes in an 80^{o}C water bath stimulated high levels of proliferation, IFN-γ and TNF-α. Error bars represent the standard error of the mean, BDL indicates the potential presence of cytokine was below the detection limit of the ELISA kit and standard curve. Significance between the stimulation groups and media was determined at $p<0.05$. (B) The proliferation was determined to be dose-dependent. (C) Splenocytes were depleted of CD49b$^+$ NK cells by magnetically-labelled depleting antibody (DX5) and the efficiency of separation was determined by flow cytometry. (D) NK cell depleted splenocytes showed a marked reduction in proliferation and IFN-γ ($p<0.05$) from the heat-killed *Cc*. All data is representative of at least 2 separate repeat experiments.

Figure 2-2 **Proliferating cells are NKT cells**



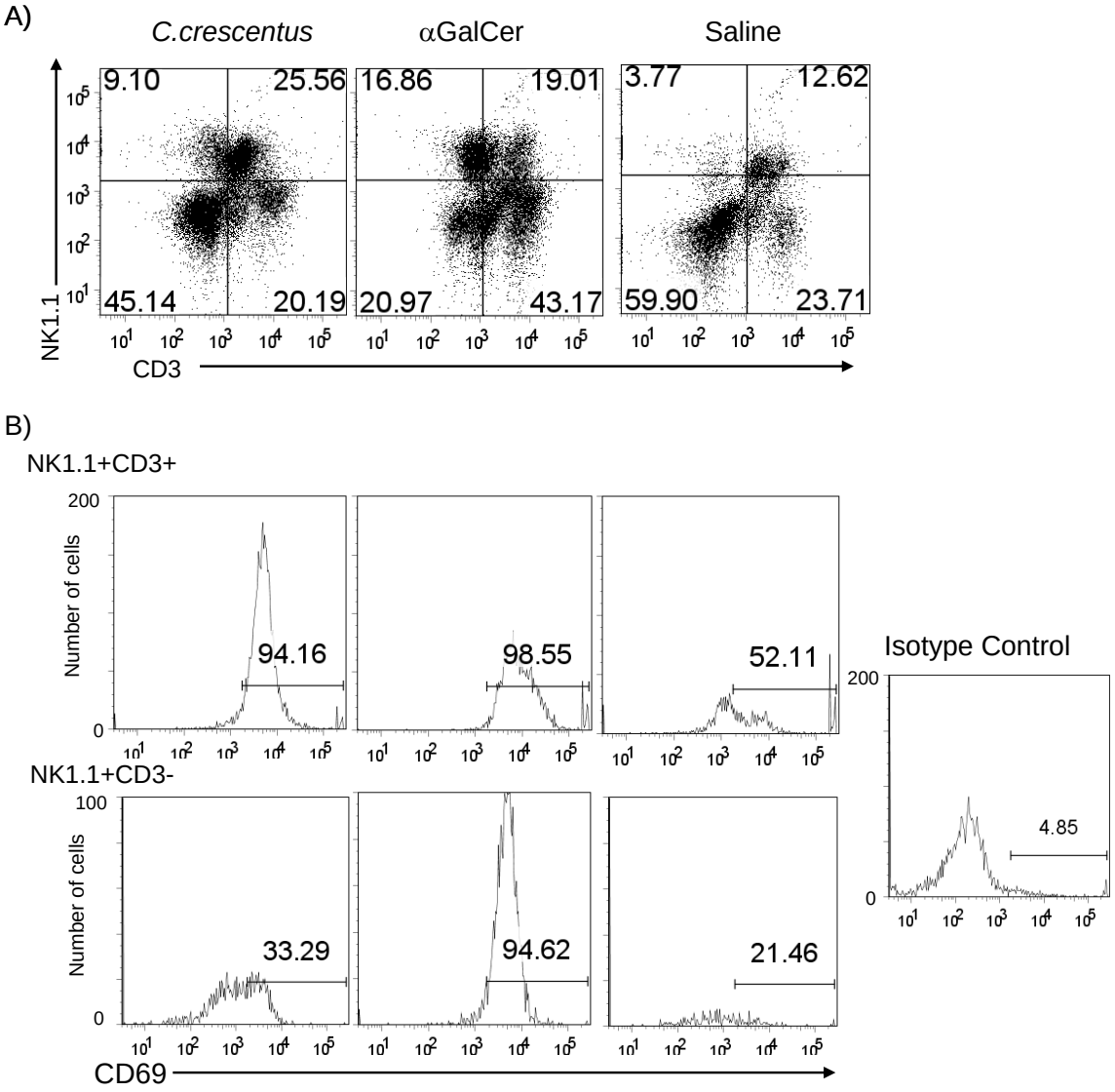
CFSE labelled CD49b$^+$ cells were allowed to proliferate for 3 days in the presence of 2x10^5 CFU of live and heat-killed *Cc*, 1μg of αGalCer or ConA. (A) CFSE+ cells were gated and then plotted onto a histogram to determine the % CFSE loss. CFSE loss indicates proliferation. *Cc*-stimulated cells showed a larger percentage of CFSE loss compared to PBS-treated cells. (B, top) Proliferating and non-proliferating cells from *Cc* stimulated splenocytes were further analyzed to determine if they were NK1.1$^+$ or both NK1.1$^+$ and CD3e$^+$. (B bottom) The CD69 activation marker was also determined on both proliferating and non-proliferating cells. Numbers indicate the percentage of the gated population from the parent population. Additional data regarding the NK1.1 and CD3ε distribution for the other groups is found in appendix 6.1. All data is representative data from 3 similar experiments.

Figure 2-3 **NKT cell knockout animals have a limited response to *Caulobacter crescentus***



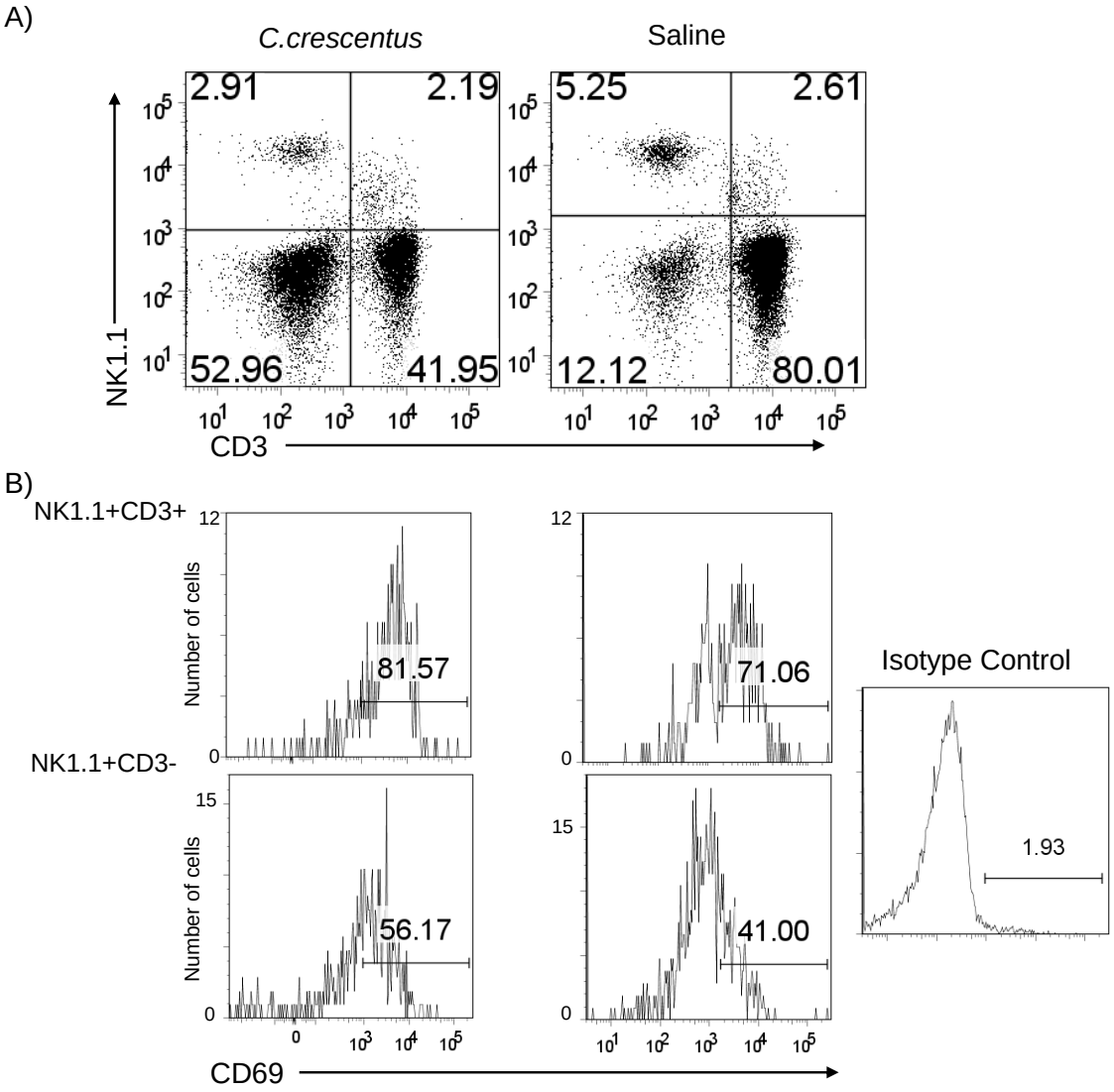
CD1d-/- and Jα18-/- animal spleens were processed and plated onto 96-well plates with *Cc* ($4x10^5$, $2x10^5$, $1x10^5$ CFU), heat-killed *Cc* ($4x10^5$, $2x10^5$, $1x10^5$ CFU) and αGalCer or ConA at 1μg/mL. Heat-killed *Cc* was prepared in 80°C water bath for 30 minutes. Supernatant was collected and analyzed using standard cytokine ELISA kits and sample ODs were compared to a standard curve. Heat-killed *Cc*-induced IFN-γ production did not differ between the two NKT knockout animals; however, Jα18-/- splenocytes had higher production of IL-10 compared to CD1d-/- cultures. Overall, NKT knockout animals had a lower response to heat-killed *Cc* compared to WT animals. This is representative data from two similar experiments.

Figure 2-4 ***Caulobacter crescentus*** **immunization activates NKT cells after 24 hours**



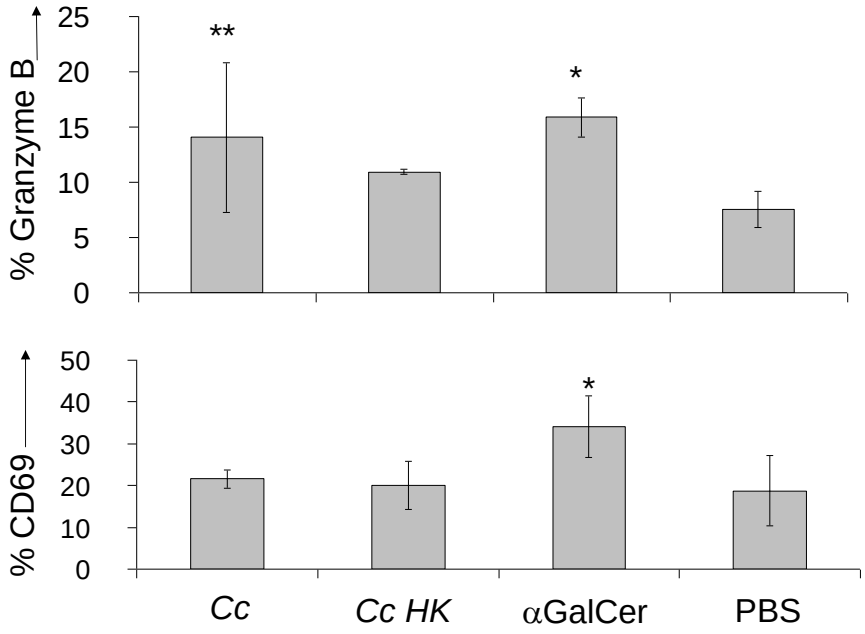
Mice injected intraperitoneally with $20x10^6$ CFU of bacteria or 2μg of αGalCer were euthanized 18hrs after and an IP wash was performed to collect the PE cells. Cells were stained for NK1.1, CD3ε and CD69 and analyzed on flow cytometry. (A) *Cc*-immunized mice showed higher levels of NKT cells in the resident population compared to saline control. (B) Both $NK1.1^+$ and $NK1.1^+$ $CD3^+$ populations from the *Cc*-immunized mice show increased CD69 expression compared to saline. Numbers represent the percent of the total parent population analyzed. This data is representative of two separate, similar assays.

Figure 2-5 ***Caulobacter crescentus*** **immunization activates splenic NKT cells after 3 days**

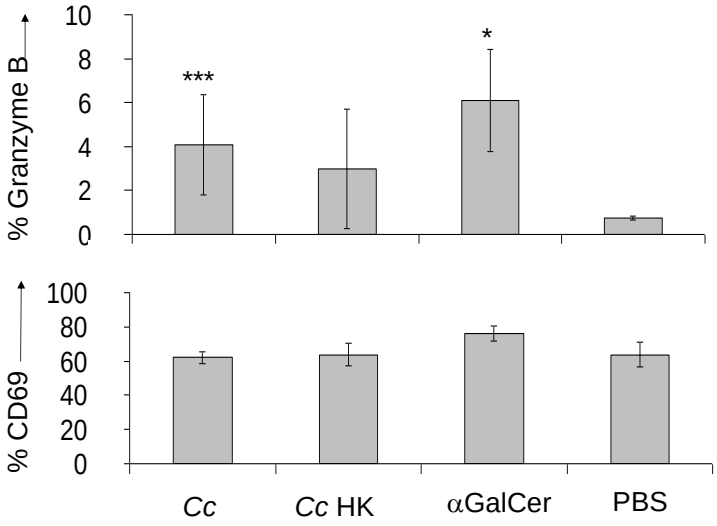


C)

NK1.1+CD3-



NK1.1+CD3+

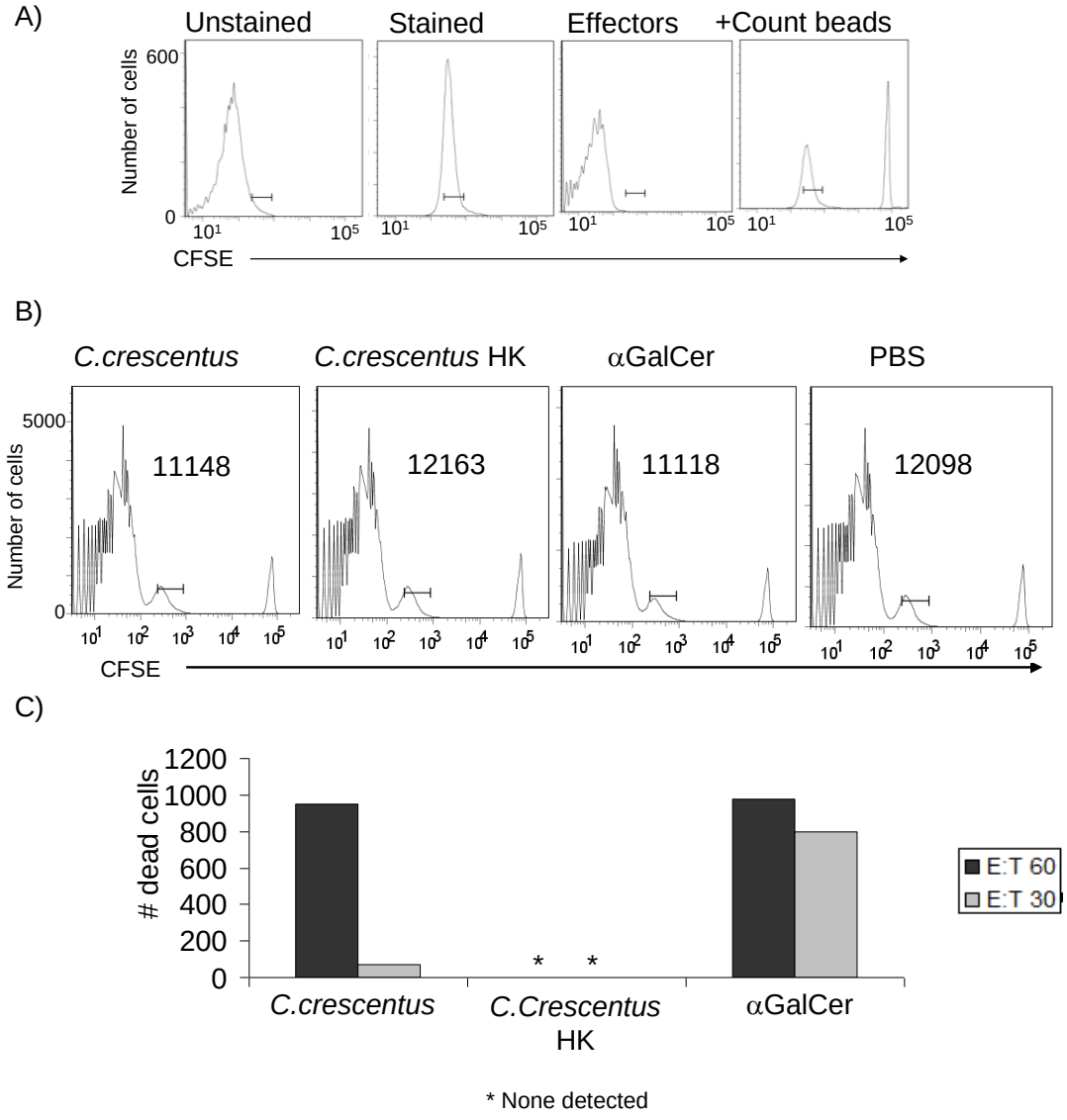


*p<0.05
**p=0.06
***p<0.09

Mice immunized intraperitoneally with $20x10^6$ CFU of live or heat-killed bacteria were euthanized after 3 days. Spleens were collected and processed to yield a single cell suspension. Splenocytes were stained for NK1.1, CD3ε, CD69 and

granzyme B, followed by analysis via flow cytometry. (A) 3 days post-immunization, mouse splenocytes showed little difference in their NKT cell ratios comparing *Cc* immunization to saline. (B) CD69 expression is increased after *Cc* immunization in both NK and NKT cell populations. Numbers represent the gated percentage obtained from the parent population. Data is representative of 3 separate assays. (C) Granzyme B expression in splenocytes from mice immunized 3 times at 5 day apart (N=3) *Cc* immunized mice had an increase in granzyme B increased compared to PBS immunizations. 2μg of αGalCer was administered as a known activator of NKT cells.

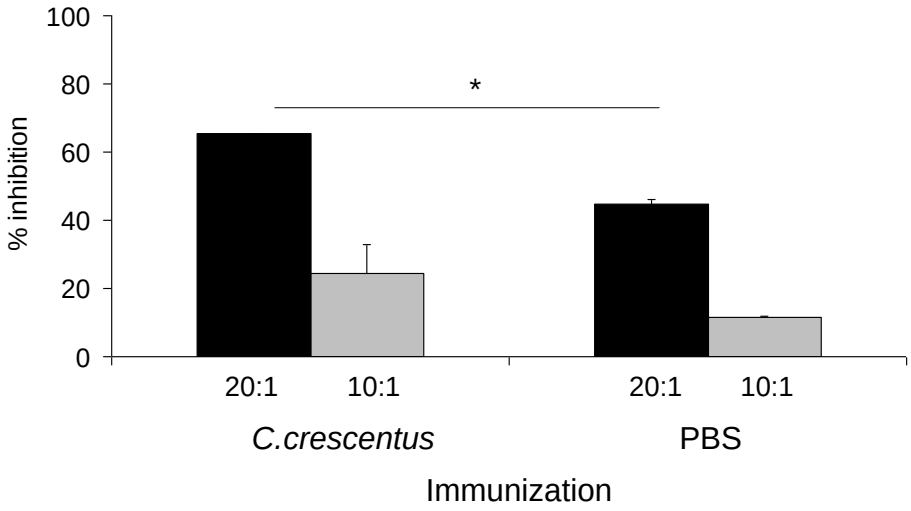
Figure 2-6 ***Caulobacter crescentus*** **immunizations can stimulate peritoneum exudate cells to kill the EL4 tumour cell line**



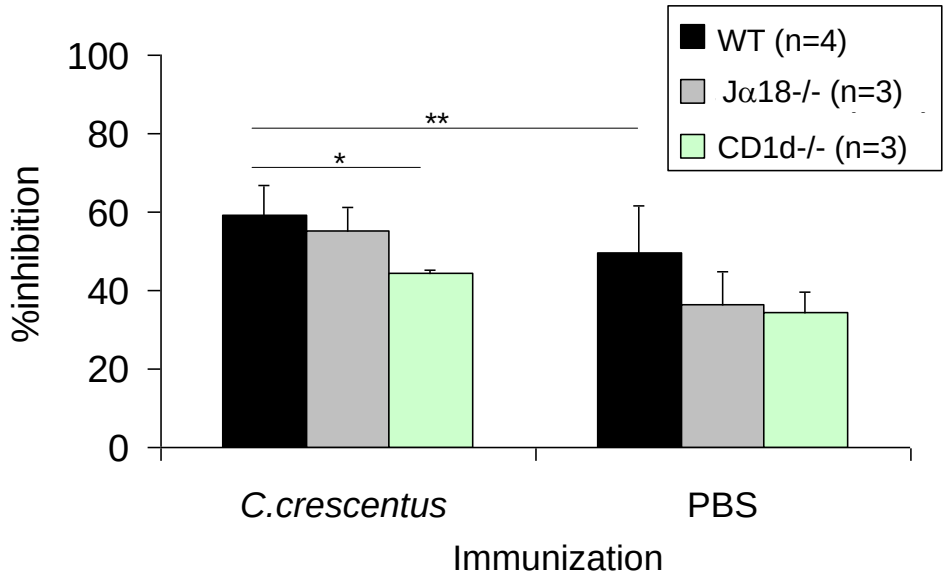
Intraperitoneal injections of $20x10^6$ *Cc* were administered 18 hrs prior to IP wash to collect PE cells. Cells were collected and plated with 10,000 CFSE-stained EL4 cells for 24hrs. (A) They were analyzed using flow cytometry by controlling the number of events taken up in the tube with CountBright beads. Setting of the CFSE+ EL4 cell gate is displayed. (B) Different injection groups were cultured with the CFSE-stained EL4 cells. Exact counts of CFSE+ EL4 cells are displayed. Killing was calculated by subtracting the number of EL4 cells in the media control by the number of EL4 cells in the treated groups. (C) The difference calculated represents the number of dead cells in the culture. This is representative data from 4 similar *ex vivo* killing assays.

Figure 2-7 ***Caulobacter crescentus*** **is capable of stimulating peritoneum exudate cells to inhibit the growth of EL4 cells** ***ex vivo***

A)



B)



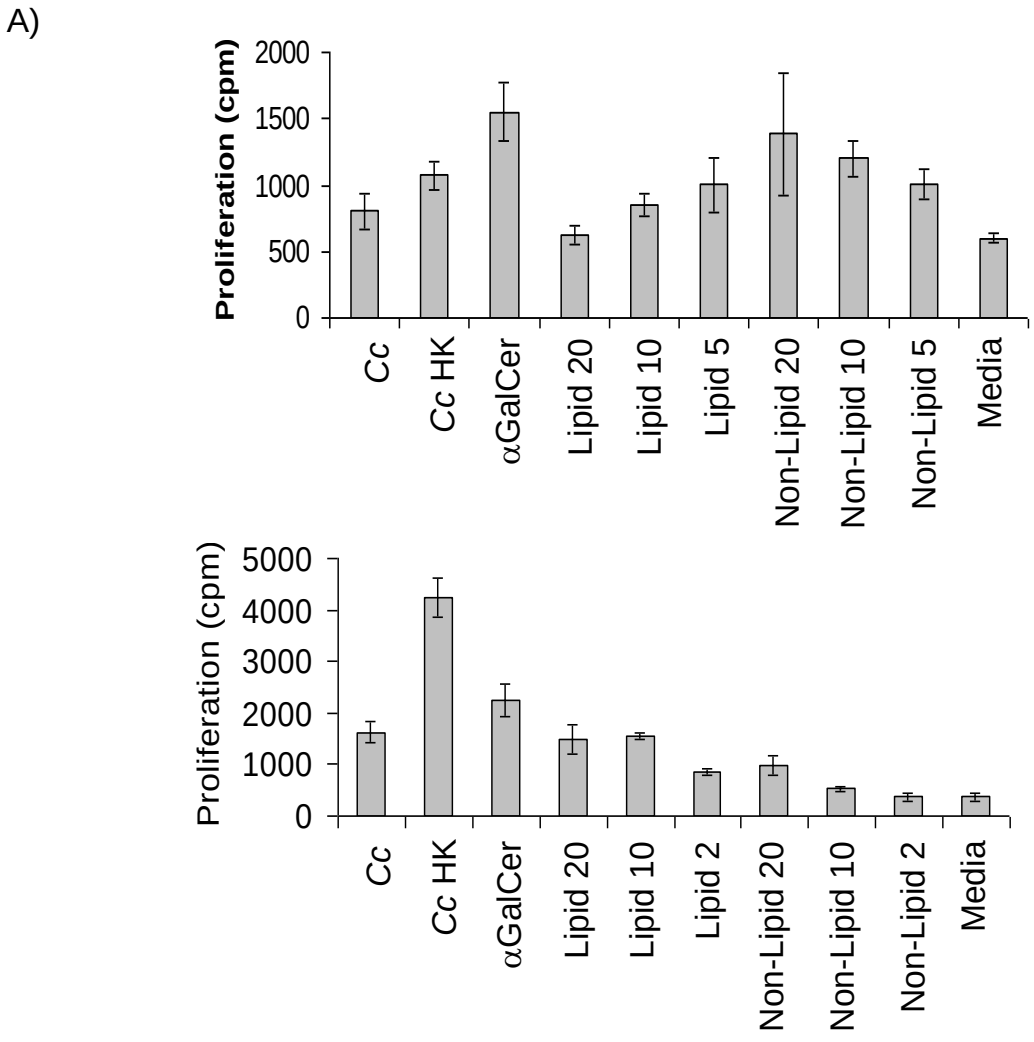
*p<0.05

**p=0.1

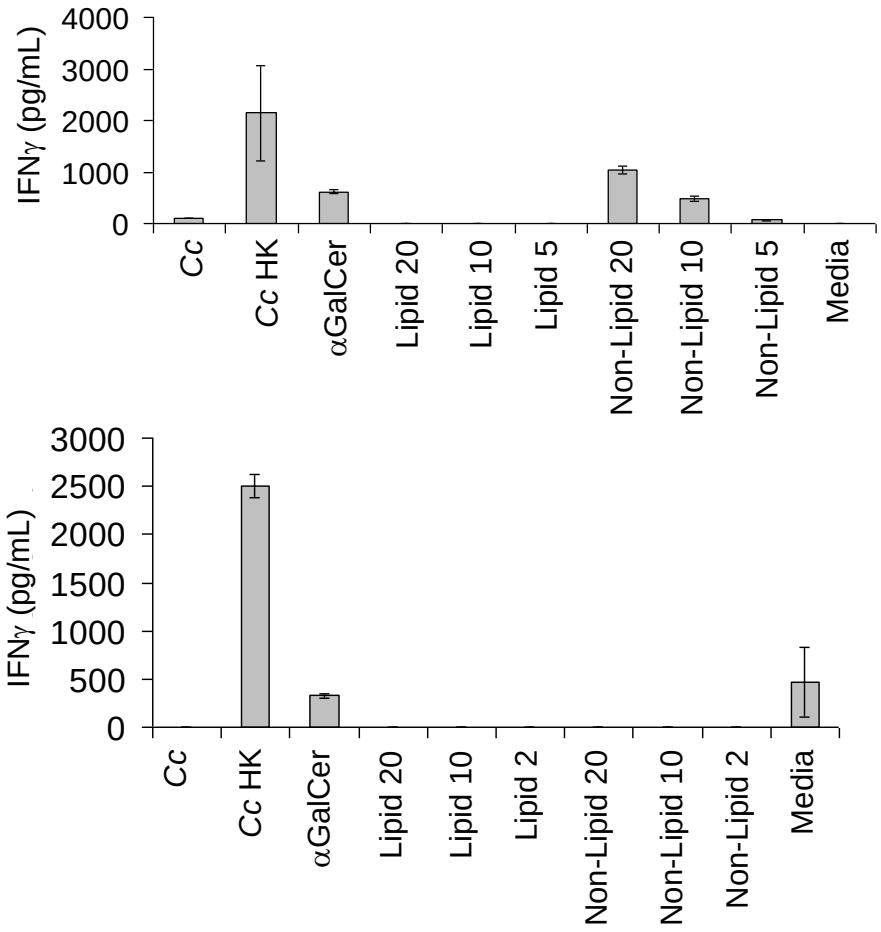
Intraperitoneal injections of *Cc* were administered 18 hrs prior to IP wash in order to collect PE cells. Cells were collected and plated in 96-well plates along with EL4 tumour cells at different effector-to-target ratios. ^{3}H-methyl-thymidine was added to the cultures and left to incubate for 18hrs. Percent inhibition was calculated by comparing the proliferation from sample wells plated in duplicate to EL4 cell line-only wells. (A) The results from one of three representative experiments is shown. Error bars show the standard deviation between the data

points. (B) The cumulative results from 3 separate experiments and the total number of mice used in each group to determine the statistics. Statistics show an independent T-test between groups.

Figure 2-8 **Lipids or non-lipids could be responsible for stimulating NKT cell responses**



B)



Lipids and non-lipid fractions were isolated from *Cc* by the Dyer method of lipid extraction. Fractions were plated *in vitro* onto splenocytes from C57BL/6 mice for 3 days. Supernatant was collected and the culture was pulsed with ^{3}H-methyl-thymidine to determine cellular proliferation over 16-18hrs. (A) The proliferation measured over two separate experiments with two separate preparations of lipid and non-lipid fractions. Proliferation was determined by taking the average cpm from triplicate plated wells. (B) The IFN-γ from the two different experiments was measured using a standard IFN-γ ELISA kit. Two separate experiments are shown chosen from seven similar experiments.

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CHAPTER-3

Caulobacter crescentus can stimulate the activation of dendritic cells: implications for establishing adaptive immunity

A version of this chapter is being prepared and will be submitted for peer reviewed publication.

3.1 Introduction

Cancer is a leading killer of people in the world. It is estimated that over 21,000 people die every day from cancer and cancer related incidents (1). Recent advances in treatment, including the inclusion of immunotherapy options for the treatment of cancer, have been met with great optimism, but limited success. The majority of new treatments being clinically tested or studied rely on the concept of developing a strong adaptive immune response to certain tumour-associated antigens. Treatments such as sipuleucel-T developed by Dendreon are among the first FDA-approved cancer treatments designed to trigger an antigen-specific T cell response to prostate antigen using granulocyte–macrophage colony-stimulating factor as an adjuvant (2).

A recent theory developed to explain the relationship between the immune system and cancer could initiate a closer examination of proposed antigen-specific treatment strategies. The concept of tumour immuno-editing proposed that changes observed in tumours over the course of disease could be explained with Darwinian concepts of evolution (3). It cites an example where transplanted tumours from Rag2-/- mice, deficient in T cells, were far more immunogenic compared to similar tumours derived in wild type mice (3, 4). The rationale for this observation was that the presence of an adaptive immune response added a selection pressure to the tumour and subsequently produced a less immunogenic tumour. Overall, this theory would indicate that if an antigen specific therapy were applied to the tumour and the tumour survived complete eradication, the

resulting tumour would become less immunogenic and present altered tumour-associated antigens.

To overcome this limitation, new therapies should focus on generating strong innate responses targeting cancer in order to target several different tumour survival conditions compared to single antigen targeting in adaptive immunotherapy strategies. One successful, historical example of an immune stimulating cancer therapy was the administration of Coley's Toxins to inoperable sarcoma patients during the early 1900s (5, 6). The concoction consisted of heat killed *Streptococcus pyogenes* and *Serratia marcescens*. The treatment was successful in controlling the growth of sarcomas in 50% of patients, while 20% of cases resulted in the eradication of the tumour (5). Unfortunately the administration of Coley's Toxins never became widespread, as it was associated with severe side effects. The practice was discontinued in 1963. Studies into the ability of Coley's toxins to promote sarcoma clearance have persisted and were initially focused on the TNF-α production from T cells (7, 8). It was later suggested by Tsung et al. that IL-12 played a defining role in shaping the outcome to Coley's toxin treatment (6).

Dendritic cells (DCs) are known producers of IL-12 (9). IL-12 is a heterodimeric protein long understood as a key cytokine for promoting the development of Th1 cellular-mediated responses and IFN-γ production from lymphocytes (9). Recently, it has been reported that IL-12, aside from activating adaptive cell-mediated immunity, could directly stimulate the activation of

tumour-associated macrophages and other myeloid-derived cells to limit tumour growth in mouse models as well (10, 11).

In this study, we evaluate the ability of the non-pathogenic, freshwater bacteria *Caulobacter crescentus (Cc)* to stimulate innate immunity with the aim of controlling tumour growth. We report the ability of this bacteria to stimulate the maturation of DCs. Maturation was determined by measuring cell surface expression of co-stimulatory markers such as CD86, CD40, CD54 and IL-12p70 production. We also show evidence that NK cells can enhance the maturation of DCs activated through *Cc* stimulation.

3.2 Materials and Methods

3.2.1 Animals

Wild-type (WT) C57BL/6 (B6) male and female mice were purchased from Charles River, USA. They were housed by Health Sciences Laboratory Animal Services at the University of Alberta in their conventional environment facility. Jα18-/- on B6 background mice were received as a generous gift from Dr. Kronenberg (La Jolla, California). CD1d-/- B6 background breeding pairs were purchased from Jackson Laboratories (Bar Harbor, USA) and bred in our animal facility (12).

3.2.2 Dendritic cell differentiation

Hind limb bones (tibia and femur) were isolated shortly after euthanization of C57BL/6 male mice. The bones were flushed with PBS using a 27G needle and 5mL syringe. Bone marrow was strained through a 70μm nylon mesh filter (Fisher Scientific; USA) and mechanical pressure was applied using the plunger from a 5mL syringe. Bone marrow cells were washed and red-blood cells (RBC) lysed using filtered double distilled H_2O (ddH_2O) for 10 seconds. Cells were then counted and seeded onto a 24-well tissue culture plate at $1x10^6$ cells/well in RPMI media supplemented with 5% FBS and 800U/mL of GM-CSF. Plates were incubated and the media was changed every 2-3 days according to the pH indicator of the media. After 7-9 days of incubation, the loosely attached cells were collected, washed in PBS and replated in fresh media with $2x10^6$ CFU of *Caulobacter crescentus* in 24-well tissue culture plates. Supernatant was collected 18-20 hrs after incubation and frozen for ELISA. Whole cells were stained for CD11c (clone N418), CD80 (clone 16-10A1), CD86 (clone GL-1), CD54 (clone YN1/1.7.4) and CD40 (clone 3/23) with fluorescently labelled antibodies (BioLegend; San Diego, USA). Cells were fixed in 2% paraformaldehyde and run on a FACSCanto (BD Bioscience; San Jose, USA) flow cytometer and later analyzed using FlowJo software (Treestar; Ashland, USA).

3.2.3 Bacterial growth and maintenance

Caulobacter crescentus (strain CB15) transfected with a plasmid containing a chloramphenicol resistant gene was kindly supplied by Dr. John Smit

(University of British Columbia) and grown on solid PYE agar media plates containing chloramphenicol as a selection antibiotic. An individual colony was harvested from the plate and transferred to liquid PYE media supplemented with chloramphenicol. Bacteria were grown at room temperature and the concentration of the bacteria was determined using a spectrophotometer set to measure the absorbance at the wavelength of 600nm. The following formula was used to determine bacterial colony forming units per mL (A_{600}=1.000($3x10^9$)CFU/mL). Bacterial cultures were then refrigerated until use and diluted as required.

3.2.4 Isolating NK cells

C57BL/6 mouse spleens were collected quickly after euthanization. Spleens were obtained and ground between two sterile frosted glass slides. Single-cell suspensions of splenocytes were washed with PBS followed by RBC lysis performed with ddH_2O for 10 seconds. The panNK magnetic cell separation kit was used to positively select for CD49b expression (clone DX5) on NK cells (Stemcell Technologies; Vancouver, Canada), before being prepared for plating. NK cells were obtained from the magnetically labelled fraction and washed before being plated with BMDCs.

3.2.5 BM-DC NK/NKT cell co-culture

NK/NKT cells were isolated from splenocytes isolated from C57BL/6 mice utilizing the panNK cell EasySep kit (StemCell Technologies; Vancouver,

Canada). NK/NKT cells were co-cultured with BMDCs in 24-well plates overnight in 10% FBS containing RPMI media supplemented with penicillin-streptomycin. In the 24-well experiments, $1x10^6$ BMDCs were seeded with or without $1x10^5$ $CD49^+$ NK cells. Collected supernatant was frozen until cytokine ELISAs could be performed. In the transwell plate experiments, a 24-well plate with pore sizes of 0.4μm was used (Corning; Corning, USA). These plates do not allow cellular contact between the two different cell populations but do allow the transfer of soluble products.

3.2.6 Immunizations

Caulobacter crescentus immunizations were prepared by spinning down *Cc* and resuspending into PBS to remove bacterial growth medium. Heat-killed *Cc* was prepared by incubating the tube for 30 minutes at 80^oC. Single immunizations with our test doses were given intraperitoneally to mice. Mice were left for 8 days prior to being tested for the development of antigen-specific recall responses.

Determining antigen specific recall response, a transgenic strain of *Cc* was used. This strain of CB15 *Cc* was transfected with a plasmid that encodes for a peptide sequence consisting of a 4 times repeating sequence of 'PGSTAPPAHGVTSAPDTRP' from the human MUC1 glycopeptide backbone. This peptide was engineered for expression on the *Cc* protein S-layer. Complete Freunds Adjuvant (CFA) was used to promote peptide antigenicity.

3.2.7 Antigen specific recall response

Mice were euthanized 8 days post-immunization and spleens were removed and processed as previously indicated. Splenocytes were then passed through a nylon wool column to enrich the culture of T cells. Naive mouse spleens were also harvested and irradiated at 25Gy in order to generate the antigen presenting cells (APC) of the culture. T cell-enriched culture and APCs were plated in equal numbers ($2.5x10^5$ cells/group) along with recall antigens. Cultures were incubated for 3 days in a 5% CO_2, 37^oC incubator. Culture wells were pulsed with 30µl of 17µCi/mL of ^{3}H-methyl-thymidine overnight, the plates were harvested onto nitrocellulose filter paper and analyzed using liquid scintillation fluid and a Wallac Liquid Scintillation (Perkin Elmer; Waltham, USA) counter to determine the counts per minute (cpm).

3.2.8 Cytokine ELISAs

Cytokine ELISAs were performed as per kit instructions. ELISA kits for IL-10, IL-12p70 and IFN-γ were purchased from both Invitrogen BioSource (Carlsbad, USA) and eBioscience (San Diego, USA). Supernatants were diluted with assay diluent to ensure the level of absorbance would land on the standard curve and plated in duplicate. Absorbance was measured at 450nm. Absorbance values were compared to an assay specific standard curve to determine cytokine concentration. The average cytokine concentrations from duplicate samples were determined along with the standard deviation.

3.2.9 Statistics

Statistics were performed on statistical software SPSS v.13 (IBM; New York, USA). One-way ANOVA was performed between test groups and significance is indicated at $p<0.05$ on post hoc LSD analysis.

3.3 Results

3.3.1 *Caulobacter crescentus* is capable of maturing BMDCs

To determine if *Caulobacter crescentus* could activate DCs, we plated live and heat-killed *Cc* with bone marrow-derived DCs (BMDCs). Here we show that both *Cc* and heat-killed *Cc* have the ability to mature $CD11c^+$ BMDCs by stimulating them to upregulate their costimulatory markers, including CD86, CD54 and CD40 compared to BMDCs in media alone (Figure 3-1). CD86 is important because it is involved in the antigen-specific activation of T cells, whereas CD54 is important in helping to form the immunological synapse where T cells and DCs would interact. CD40 plays a role in both NKT cell and T cell activation as well.

3.3.2 NK/NKT cells enhance the maturation of BMDCs

Along with co-stimulatory marker expression, *Cc* and heat-killed *Cc* were capable of stimulating the production of IL-12p70, a cytokine known for polarizing T cell responses towards a Th1 phenotype (Figure 3-2A). The purified BMDCs were also capable of stimulating IFN-γ production from a co-culture with

$CD49b^+$ NK/NKT cells (Figure 3-2B). NKT cells have been shown previously to to help amplify a BMDC response to infection (13). Interestingly, we detected an enhanced IL-12p70 response when the BMDCs were co-cultured with the isolated NK/NKT cells (Figure 3-2A). To determine if this IL-12p70 enhancement required NK/NKT cell contact, we used a 24-well 0.4μm transwell plate where we cultured the NK/NKT and BMDCs in the same well, but in isolated compartments where only soluble factors were capable of traversing the membrane. We showed that separating the two cell populations abrogates any IL-12p70 enhanced production from the BMDCs (Figure 3-2C). Measuring NK/NKT co-cultured BMDC cell surface markers, we also showed an enhancement in their level of CD40 expression in the presence of NKT cells (Figure 3-3). CD86 appears to be slightly enhanced, while CD54 does not appear to increase in the presence of NK/NKT cells. The enhancement in CD40 expression was also shown to be contact-dependent.

3.3.3 *Caulobacter crescentus* activates NKT cells through an alternative pathway

NKT cells can be activated to secrete IFN-γ either directly through the CD1d-T cell receptor interaction or through indirect methods, such as through IL-12p70 signaling. To determine if the BMDCs are utilizing CD1d to activate NKT cells, we prepared BMDCs from both WT and CD1d-/- mice. After development, these BMDC populations were replated with $CD49b^+$ NK cells that were magnetically isolated from WT mouse spleens and left them to incubate

overnight. We detected equal levels of production of IFN-γ from NKT cells stimulated with both WT BMDCs and CD1d-/- BMDCs (Figure 3-4, top) indicating CD1d independence in *Cc*-stimulated NKT responses. αGalCer, which is known to stimulate NKT cells only through CD1d, displayed its dependence for the WT BMDCs for the production of IFN-γ. The minor production of IFN-γ in the αGalCer treated CD1d-/- BMDC WT $CD49b^+$ NK/NKT cells may be attributed to the presence of some WT antigen presenting cells making it through the magnetic cell separation procedure. Interestingly, this assay also displayed a role for CD1d presentation to lower the amount of IL-10 produced in the $CD49b^+$ NK cell and BMDC co-culture stimulated with live or heat-killed *Cc*, LPS or αGalCer (Figure 3-4, bottom).

3.3.4 *Caulobacter crescentus* immunization stimulates strong adaptive T cell responses to itself

To determine if *Cc* immunization could generate T cell proliferation to an antigen of interest, transgenic *Cc* with a MUC1 peptide inserted into its surrounding S-layer was generated. Mice were immunized with $50x10^6$ CFU via IP injection and left for 8 days. A higher dose of *Cc* was used to ensure antigen delivery. Spleens were then collected and nylon wool-purified cells were plated with irradiated APCs and recall antigens. Proliferation was measured on day 3. We observed that MUC1-transgenic *Cc* was able to stimulate a recall proliferation response to itself; however, when non-transgenic *Cc* was added as the recalled antigen, the proliferation measured was equal (Figure 3-5). This would indicate

that the T cell response was likely directed to a *Cc* antigen and not the transgenic MUC1 insert.

3.4 Discussion

We have shown that *Caulobacter crescentus*, a non-pathogenic bacterium, has the ability to activate key innate immune cells that have a downstream responsibility in developing adaptive immunity. Both DCs and NKT cells are noted for their abilities to modulate the adaptive immune response (14). Understanding how they can influence the development of T and B cells can have a huge impact on controlling disease progression.

Cc has the ability to stimulate both DCs and NKT cells. The maturation of BMDCs is detected in their increased expression of the co-stimulatory markers CD40, CD86 and CD54, followed by the secretion of IL-12p70. The increased expression of co-stimulatory markers could lead to an enhanced ability of DCs to activate antigen-specific T cells. The presence of IL-12 and CD40 suggests that the DCs have the ability to direct a strong cytotoxic element from the immune response (15, 16). The IL-12 cytokine has also been shown to stimulate IFN-γ production in NK and NKT cells (17). This response would support the observation of NK cell activation due to IL-12 being important in stimulating Th1 cells to eliminate murine tumours (15). IL-12 is also being investigated clinically as a treatment for multiple myeloma (18); however, it was found that IL-12 given exogenously could have serious side effects. This could support the development

of treatments that activate endogenously generated IL-12 rather than the exogenous administration of IL-12 into patients.

A novel finding in regards to NKT cell activation was the observation that *Cc*-activated NKT cells do not require CD1d lipid presentation to stimulate IFN-γ; however, contact between NKT and BMDCs was required. The contact dependency we believe could be due to CD40-CD40L interactions which can stimulate the release of IL-12 from DCs, followed by IFN-γ from NKT cells (16, 17, 19, 20). The increase in expression of CD40 on BMDCs in co-culture with NKT cells is supported by previous evidence that showed the ability of NKT cells to upregulate CD40 on DCs via a contact-dependent mechanism (21). The presence of IFN-γ from NKT cells is important, as it has been previously shown to be a potent inducer of anti-tumour (22, 23) and anti-viral effects (24). Interestingly, the production of IL-10 was controlled by the presence of CD1d on the BMDCs cultured with WT $CD49b^+$ NK/NKT cells, which lead us to suggest that the CD1d interaction, although not required to stimulate IFN-γ from NKT cells, is required to lower the secretion of the suppressive cytokine IL-10, most likely from DCs. This observation suggests that the CD1d-TCR pathway and another unknown pathway are important in regulating the environment of developing adaptive immunity by controlling the ratio of pro-inflammatory IFN-γ with the inhibitory IL-10 in regards to *Cc* stimulation.

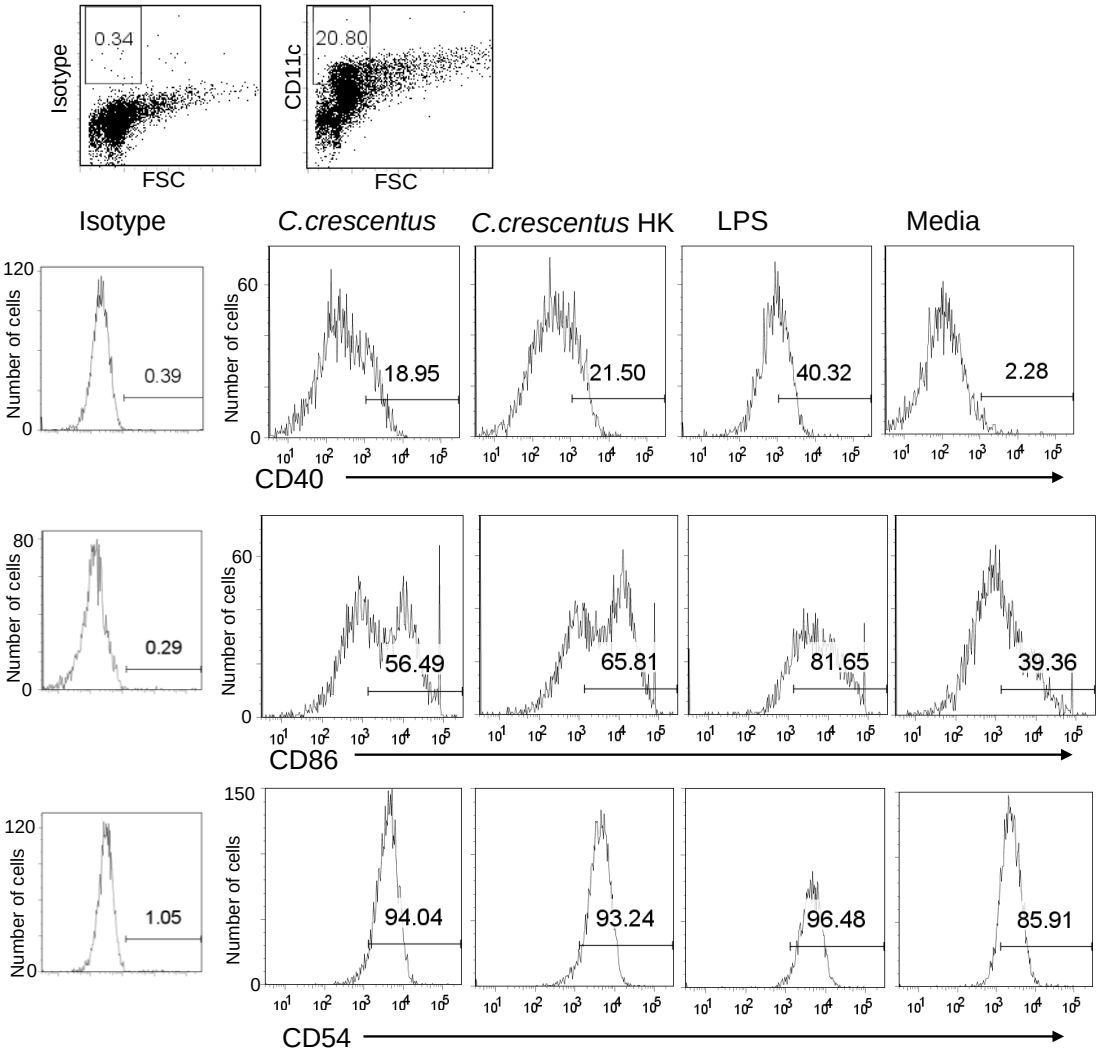
Taken together, the NKT cell population is capable of enhancing the maturation of DCs. Increase in IL-12p70 production by DCs has previously been shown to support the development of $CD8^+$ cytotoxic T cells and cytolytic NK

cells (25). The ability of this non-pathogenic bacterium being able to generate higher amounts of IL-12 could play a role in its development as a potential anti-tumour agent. We also showed evidence that *Cc*, despite being non-pathogenic, can initiate an adaptive immune response against itself through measuring T cell proliferation. The enhanced level of CD40 on the surface of DCs cultured with NKT cells could also support the initiation of a stronger cytotoxic element of the immune system, potentially capable of treating tumours and viral infections (26). Future studies will look into the relationship between *Cc*-activated DCs and antigen-specific T cell stimulation to determine if there is an enhanced cellular immune response. The role of *Cc*-activated DCs in developing tumour immunity also warrants further investigation.

Our data is the first to show that *Caulobacter crescentus* can be used to stimulate both DCs and NKT cells. This has implications in the development of a Th1-based antigen specific immune response. The production of IFN-γ from NKT cells and IL-12 from DCs can also lead to the development of a strong anti-tumour effect. These findings should progress the research concerning potential applications for *Caulobacter crescentus* as an immunotherapy to cancer by stimulating DC maturation.

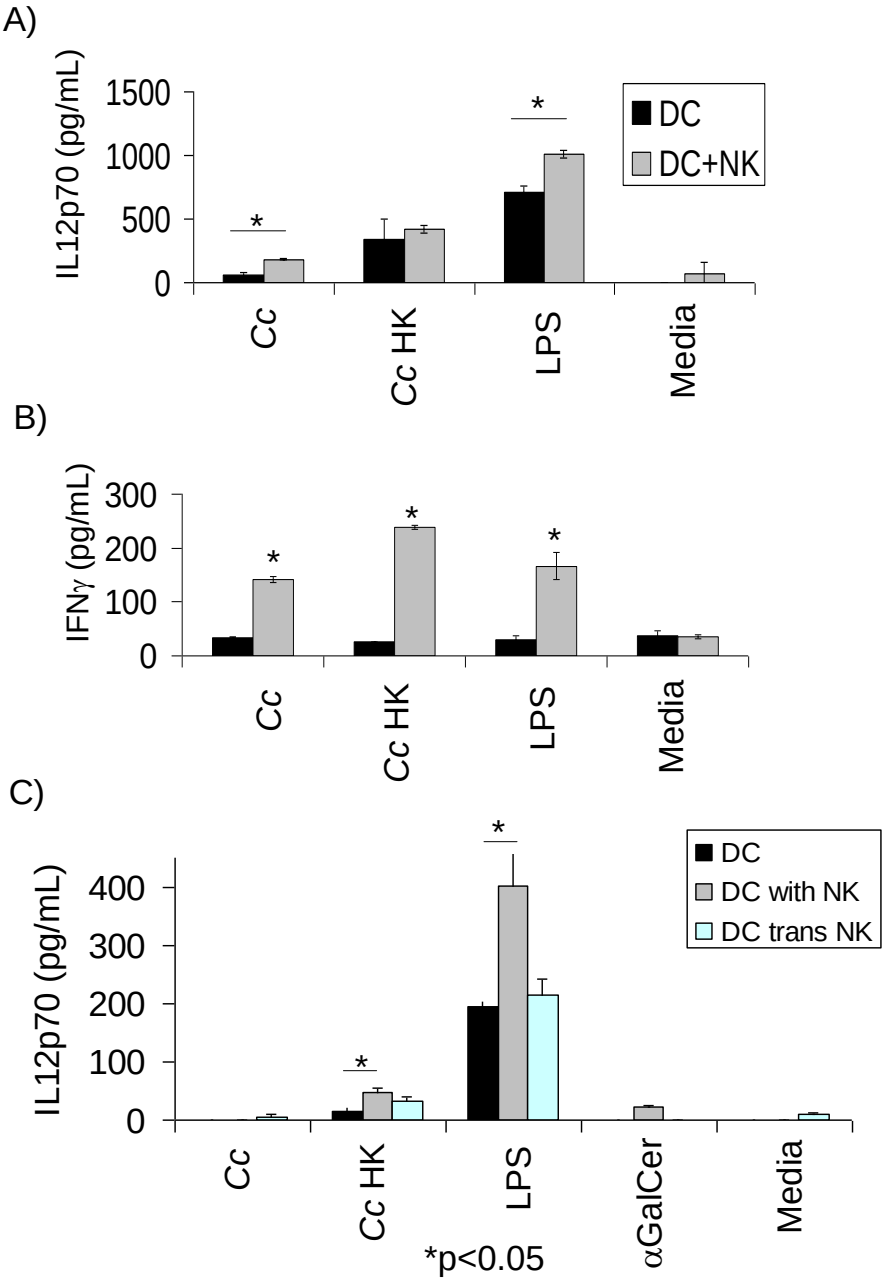
3.5 Figures

Figure 3-1 ***Caulobacter crescentus* can mature BMDCs**



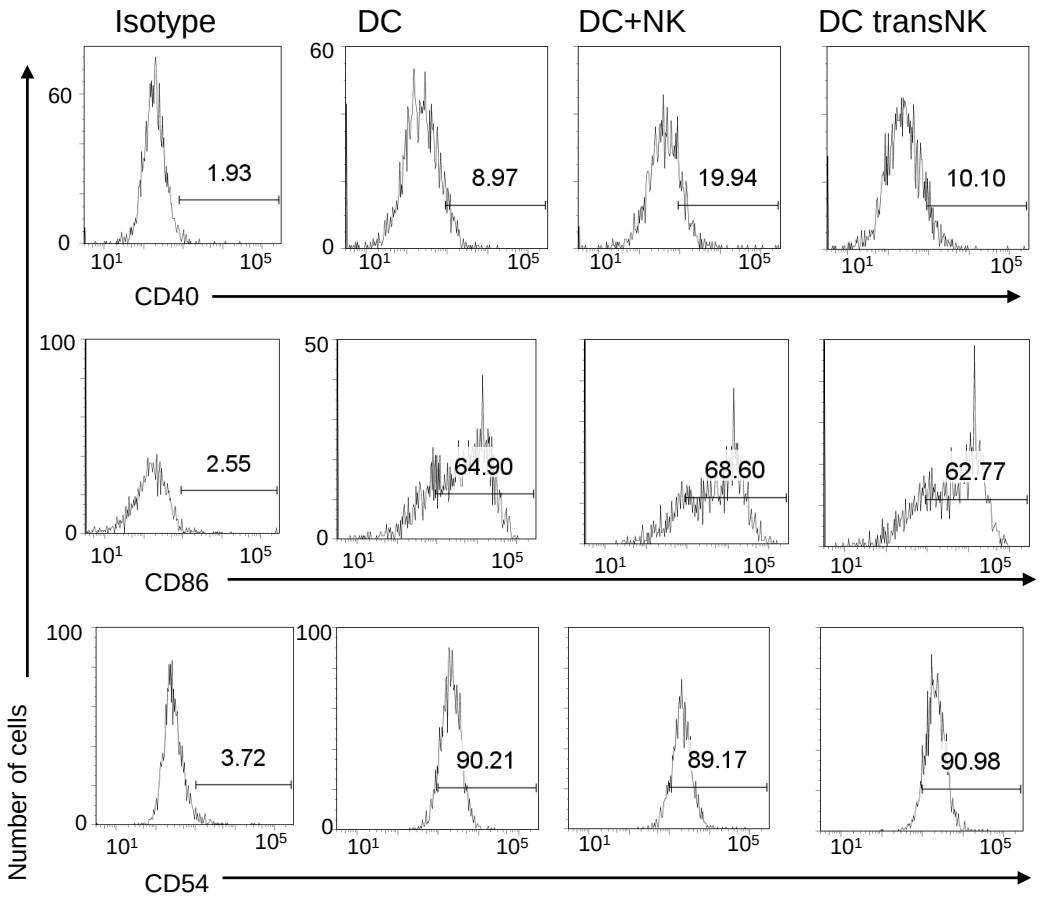
Bone marrow-derived dendritic cells were matured overnight with $2x10^6$ CFU *Cc*, heat-killed *Cc* or 1μg LPS. They were collected, then stained for CD11c, CD40, CD86 and CD54. $CD11c^+$ cells were gated on and their cell surface expression of CD40, CD86 and CD54. Gates were established by utilizing isotype control antibodies specific to each fluorochrome. Numbers shown are the percent positive cells from the parent population. This data is representative data from 5 separate, similar experiments.

Figure 3-2 **BMDCs show a distinct interaction with CD49b$^+$ NK cells when stimulated with *Caulobacter crescentus***



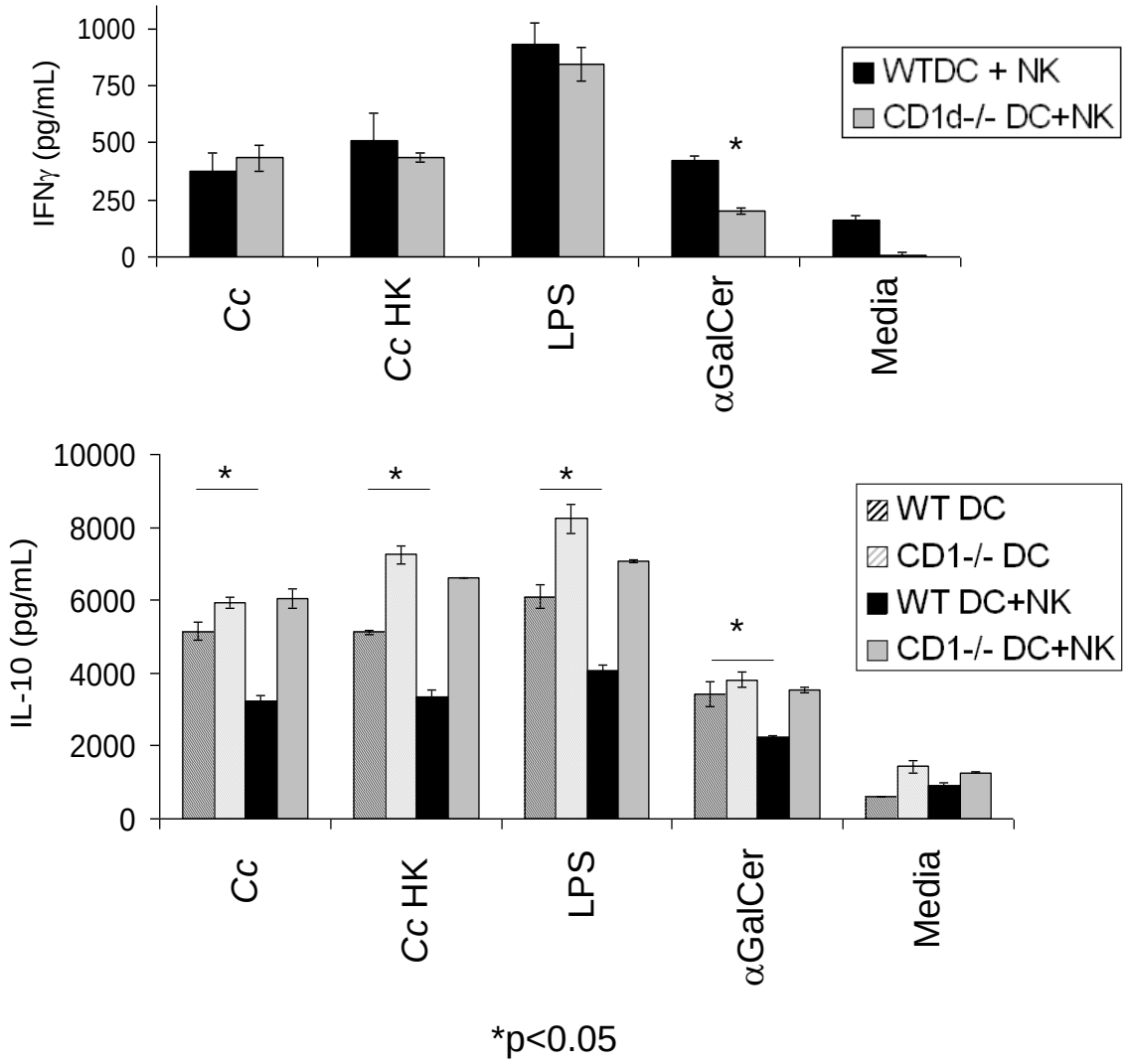
Supernatant was collected from a co-culture of NK cells plated with BMDCs and was tested for (A) IL-12p70 and (B) IFN-γ production after stimulation with $2x10^6$ CFU *Cc*, heat-killed *Cc* or 1μg of LPS. (C) The NK-BMDC co-cultures were also performed in 24-well transwell plates with pore sizes of 0.4μm to determine if NK cell contact with BMDCs was required for the increase in BMDC IL-12p70. The transwell experiment data is representative of two separate experiments.

Figure 3-3 **BMDC with NK cell co-culture leads to enhanced BMDC activation**



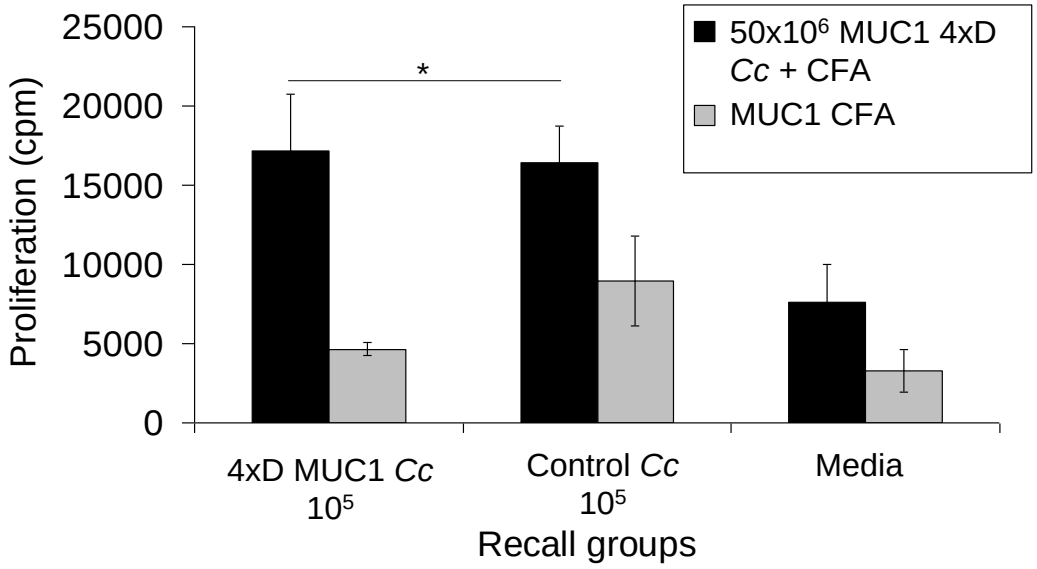
Standard or contact-separated (transwell plate) co-cultures of BMDCs with $CD49b^+$ (DX5) NK cells were stimulated with $2x10^6$ CFU of live *Cc* over 1 day. The cells were harvested, then stained to determine cellular expression of CD40, CD86, and CD54. Numbers are percent positive cells from the parent population of total $CD11c^+$ cells.

Figure 3-4 **CD1d is not required to stimulate IFN-γ from NKT cells in response to *Caulobacter crescentus* stimulation but is required to lower IL-10 production**



Supernatant was collected from a co-culture of wild type NK cells (CD49b+) plated with BMDCs that were matured from wild type or CD1d-/- bone marrow with $2x10^6$ CFU *Cc*, heat-killed *Cc* or 1μg of LPS or αGalCer. No difference was measured between the two different BMDCs when IFN-γ was measured using ELISA however IL-10 ELISA shows CD1d-/- BMDC failed to lower the production of IL-10 from the co-culture. This data is representative from one of two similar experiments.

Figure 3-5 ***Caulobacter crescentus* immunization generates a stronger *Caulobacter crescentus*-specific recall response than MUC1 peptide**



*no statistical difference

Intraperitoneal immunizations with $50x10^6$ CFU of 4xD MUC1 inserted S-layer *Caulobacter crescentus* constructs with complete Freund's adjuvant (CFA) or 100μg of MUC1 peptide suspended in CFA. A proliferation recall response 8 days post-immunization with the transgenic *Cc* and non-MUC1 *Cc*, as well as 50μg/mL MUC1 peptide, was performed. Proliferation was measured by ^{3}H-methyl-thymidine uptake after the 3rd day. Counts per minute (cpm) indicate the level of proliferation. There was no difference detected when using transgenic *Cc* compared to non-MUC1 *Cc*.

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CHAPTER-4

Divergent roles of iNKT cells in tumour immunity

Sections of this chapter are included in papers being prepared and intended for peer reviewed publication

4.1 Introduction

Cancer is estimated to cause the deaths of over 21,000 people each day worldwide (1). That translates to over 7.6 million deaths from cancer a year. Despite constant advances in both treatment and detection technology, cancer remains a leading cause of death. Immunotherapy as a treatment for cancer has provided hope for increasing cancer survival rates but, so far, its impact has been limited. Identifying the differential immune responses generated towards cancer between patients may lead to the development of better targeted treatments for the disease.

Natural killer T cells (NKT) have been identified as a positive prognostic indicator for colorectal cancer patients (2). Patients identified with a high number of tumour-infiltrating NKT cells, through immunohistochemical stains, had a significantly better survival rate following surgical treatment compared to patients who had a low level. Conversely, it was shown that low levels of circulating NKT cell in head and neck squamous cell carcinoma patients were related to poor 3 year survival rates (3).

NKT cells are classically identified as T lymphocytes that express NK phenotypic markers, such as NK1.1 (4). Unlike traditional T cells, NKT cells are positively selected in the thymus by binding to the lipid-presenting MHC class I like molecule CD1d (5). Divergent routes of activation are another differentiating feature of NKT cells. They can be activated, as with T cells, through interaction of their T cell receptor (TCR), though with a foreign lipid presented by the antigen presentation molecule CD1d. Additionally, they can be

activated through recognition of an endogenous lipid associated with CD1d and inflammatory cytokines (6). Their innate activation in response to immune insult is believed to play a role in shaping the antigen-specific adaptive immune response by modulating antigen presenting cells, and by enhancing innate immune responses (7). To promote antigen-specific adaptive immunity, NKT cells have been shown to influence the activity of dendritic cells (DCs) by promoting a shift towards Th1 immunity through the secretion of IL-12p70 (8). Myeloid-DC derived IL-12p70 is believed to be an important cytokine in stimulating effective tumour immunity. Exogenous IL-12p70 delivery to the tumour has been shown to reverse the tolerogenic conditions found within the tumour micro-environment (9, 10). Activated NKT cells have also been suggested to play a role in the quick recruitment of other immune effector cells to combat any infection or cancer (11).

To study NKT cell involvement in disease, various mouse models have been generated that lack different subsets of NKT cells. Invariant NKT (iNKT) cells, or class 1 NKTs, have been identified from their restricted TCR repertoire expressing Vα14-Jα18 TCR chains in mice (4). Mice generated to lack the Jα18 gene segment specifically lack type 1 NKT cells. Type II NKT cells have a less restricted T cell repertoire that interacts with CD1d. Therefore, CD1d knockout animals would lack all CD1d-restricted NKT cells (12).

With our evolving understanding of NKT cells and their relationship with cancer, it is believed that activating NKT cells could be a suitable treatment option for various cancers. αGalactosylceramide (αGalCer) is a glycolipid

product that has been isolated and identified from the sea sponge *Agelas mauritianus* (13). It binds specifically to CD1d and is a known activator of both type 1 and type 2 NKT cells. Type 1 NKT cell activation by αGalCer has been shown to control tumour growth in mice (13, 14). Progression to human clinical trials has, however, returned mixed results when treated solid tumour patients had a decrease in circulating NKT cells after treatment with unbound αGalCer (13). αGalCer-loaded antigen presenting cells have been shown to expand the number of NKT cells in metastatic cancer patients that had failed initial treatment (15). Definitive survival benefits in the human population have yet to be determined. The search for other methods to specifically activate NKT cells has led researchers to discover that non-pathogenic bacteria are common activators of NKT cells (16).

We propose that the use of *Caulobacter crescentus* (*Cc*), non-pathogenic, freshwater bacteria, could activate NKT cells to control the growth of tumours in mice. In this report, we show evidence that *Cc* treatment can slow the initial growth of a subcutaneous EL4 tumour in wild type mice. Interestingly, we found that growth of these subcutaneous tumours was inhibited in untreated Jα18-/- mice. Further investigation found significant differences in the expression level of CD40 and the production of IL-12p70 by Jα18-/- derived DCs. From these findings we further propose that class 1 NKT cells must play an immuno-modulatory role by preventing other CD1d restricted NKT cells from clearing the subcutaneous EL4 tumours and therefore, supporting its overall growth and development.

4.2 Material and Methods

4.2.1 Animals

Wild-type (WT) C57BL/6 male and female mice were purchased from Charles River, USA. They were housed by Health Sciences Laboratory Animal Services at the University of Alberta in their conventional environment facility. Jα18-/- on a B6 background mice were received as a generous gift from Dr. Kronenberg (La Jolla, California). CD1d-/- B6 background breeding pairs were purchased from Jackson Laboratories (Bar Harbor, USA) and bred in our animal facility (12).

4.2.2 Bacterial growth and maintenance

Caulobacter crescentus (strain CB15) transfected with a plasmid containing a chloramphenicol resistance gene, was kindly supplied by Dr. John Smit (University of British Columbia) and grown on solid PYE agar media plates containing chloramphenicol as a selection antibiotic. An individual colony was harvested from the plate and transferred to liquid PYE media supplemented with chloramphenicol. Bacteria were grown at room temperature and the concentration of the bacteria was determined using a spectrophotometer set to measure the absorbance at the wavelength of 600nm. The following formula was used to determine bacterial colony forming units per mL

(A_{600}=1.000($3x10^9$)CFU/mL). Bacterial cultures were then refrigerated until use and diluted as required.

4.2.3 Cell lines

EL4 mouse (C57BL/6) lymphoma cell line was obtained from Biomira Inc. in 2002. It originally came from the American Type Culture Collection (ATCC; Manassas, USA). B16 mouse melanoma cell line (B6 background) was received as a gift from Dr. Maya Shmulevitz (University of Alberta). Both cell lines were maintained in 5%FBS RPMI media supplemented with 2mM L-glutamine, penicillin/streptomycin and 2-mercaptoethanol. Cell lines were confirmed by their observed morphology.

4.2.4 Subcutaneous tumour assays

Mice were immunized intraperitoneally with $20x10^6$ CFU of *Cc* on days -3 and 1. On day 0, $2.5x10^5$ EL4 or B16 tumour cell lines suspended in PBS were injected subcutaneously into the right rear flank of the mice. Tumour measurements were taken daily until the tumours began impeding behaviour, which was around 20 days post-implantation. Tumours were measured using digital callipers measuring the width and length of the tumour at 90^o angles. Area was then calculated by the two measurements taken.

4.2.5 Dendritic cell differentiation

Hind limb bones (tibia and femur) were isolated shortly after euthanization of C57BL/6 male mice. The bones were flushed with PBS using a 27G needle and 5mL syringe. Bone marrow was strained through a 70μm nylon mesh filter (Fisher Scientific; USA) and mechanical pressure was applied using the plunger from a 5mL syringe. Bone marrow cells were washed and red-blood cells (RBC) lysed using filtered double distilled H_2O (ddH_2O) for 10 seconds. Cells were then counted and seeded onto a 24-well tissue culture plate at $1x10^6$ cells/well in RPMI media supplemented with 5% FBS and 800U/mL of GM-CSF. Plates were incubated and the media was changed every 2-3 days according to the pH indicator of the media. After 7-9 days of incubation, the loosely attached cells were collected, washed in PBS and used in co-culture with $CD49^+$ NK cells.

4.2.6 BM-DC NK/NKT cell co-culture

$CD49b^+$ NK/NKT cells were isolated from C57BL/6 isolated splenocytes utilizing the panNK cell EasySep kit (StemCell Technologies; Vancouver, Canada). The kit separates cells based on the NK cell marker CD49b. $1x10^5$ NK/NKT cells were then co-cultured with $1x10^6$ BMDCs in RPMI media containing 10% FBS with penicillin-streptomycin in 24-well plates overnight. 500μl of supernatant was collected and frozen until cytokine ELISAs could be performed.

4.2.7 Cytokine ELISAs

Cytokine ELISAs were performed as per kit instructions. ELISA kits for IL-10, IL-12p70 and IFNγ were purchased from both Invitrogen BioSource (Carlsbad, USA) and eBioscience (San Diego, USA). Supernatants were diluted with assay diluent and plated in duplicate. The absorbance measured at 450nm. Absorbance values were compared to an assay specific standard curve to determine cytokine concentration. The average cytokine concentrations from duplicate samples were determined along with the standard deviation.

4.2.8 Flow Cytometry

NK cells were stained using antibodies purchased from eBioscience (San Diego, USA) for NK1.1 (PK136) and CD3e (145-2C11). Tumour cells and BMDCs were stained with antibody to CD1d (1b1). BMDCs were stained with antibodies to CD11c (clone N418), CD80 (clone 16-10A1), CD86 (clone GL-1), CD54 (clone YN1/1.7.4), CD40 (clone 3/23) and MHC class II I-A (M5/114.15.2) (BioLegend; San Diego, USA). Cells were fixed in 2% paraformaldehyde and run on a FACSCanto (BD Bioscience; San Jose, USA) flow cytometer and later analyzed using FlowJo software (Treestar; Ashland, USA).

4.2.9 Statistics

Statistics were performed on statistical software SPSS v.13 (IBM; New York, USA). One-way and two-way ANOVAs were performed and significance is indicated at $p<0.05$ on post hoc LSD analysis.

4.3 Results

4.3.1 Slower growth of EL4 syngeneic tumours

To determine if *Cc* could control the growth of a syngeneic, subcutaneous tumour mass, we immunized WT C57BL/6 mice with $20x10^6$ CFU of live or heat-killed *Cc* through an intraperitoneal injection 3 days prior to the implantation of an EL4 lymphoma cell line tumour. They received a second dose of *Cc* 1 day after tumour implantation. We tracked the growth of the tumour mass over the course of 20 days. As a control, we performed similar experiments using Jα18-/- and CD1d-/- mice which lack type 1 or all CD1d dependent NKT cells, respectively. The CD1d-/- B16 syngeneic melanoma cell line was also used in a similar assay with WT mice. Using a two-way ANOVA LSD post hoc test we found that the measured tumour area in WT mice treated with *Cc* was significantly smaller on days 13 and 16 when compared to PBS treated mice (Figure 4-1A). In general, the growth of the EL4 tumour in WT mice treated with *Cc* was slower until day 16. A one-way ANOVA found that the *Cc* group overall had significantly smaller tumours than the PBS treated mice over the course of the 20 days. This slower initial EL4 growth was not detected from *Cc* immunized CD1d-/- mice (Figure 4-1B). This indicates the requirement of NKT cells in the initial control of tumour growth. Additionally, the growth of B16 tumour cells, which lacks CD1d expression (Figure 4-2), was not controlled by *Cc* immunizations in WT mice (Figure 4-1C). In both the WT EL4 and B16 tumour models, αGalCer (KRN7000) was able to significantly slow the growth of

tumours over the same span of time as was expected. Interestingly, the Jα18-/- mice largely failed to develop EL4 tumours except for those mice which were not immunized with *Cc* (Figure 4-1D).

4.3.2 Jα18-/- BMDC display enhanced stimulation by *Caulobacter crescentus*

The surprising results observed from the Jα18-/- EL4 tumour mouse model led us to further examine the biology of these mice. We developed BMDCs from WT, CD1d-/- and Jα18-/- mice and plated them with *Cc* to determine their IL-12p70 production. Astonishingly, we found that the Jα18-/- BMDCs produced significantly more IL-12p70 and lower levels of the inhibiting cytokine, IL-10 (Figure 4-3). There was not much difference in the production of IL-12p70 and IL-10 between WT and CD1d-/- BMDCs.

Measuring cell surface markers to determine BMDC maturation and activation, we found that Jα18-/- BMDCs expressed higher levels of CD40, CD86 and CD80 after stimulation with *Cc* compared to WT or CD1d-/- cells (Figure 4-4). Data showing BMDC stimulation with heat-killed *Cc*, LPS and media only is available in appendix 6.2.

CD1d expression was increased after stimulation of BMDCs with heat-killed *Cc* and LPS. It was observed that CD1d expression levels were not different between Jα18-/- and WT BMDCs (Figure 4-5).

4.3.3 Jα18-/- bone marrow cultures contain less NK1.1$^+$ cells

To determine if the difference in BMDC activation could be due to the cells present in the bone marrow differentiation cultures we produced BMDCs from WT, CD1d-/- and Jα18-/- bone marrow. Following their differentiation, we stimulated them with *Cc*, LPS or PBS. After 24 hrs, we stained for the presence of $NK1.1^+$ and $NK1.1^+CD3\varepsilon^+$ dual-positive cells. We found that Jα18-/- cultures generally had fewer $NK1.1^+$ cells present compared to the WT and CD1d-/- BMDC cell cultures (Figure 4-6). This phenomenon was especially evident following maturation with LPS.

4.3.4 Jα18-/- BMDC are stronger stimulators of NK cell activation

Realizing the increased activation of Jα18-/- BMDCs compared to their WT and CD1d-/- counterparts we wanted to determine whether their ability to stimulate $CD49b^+$ NK/NKT cells, obtained from WT spleens, was maintained. This would give us information regarding the activation potential of Jα18-/- BMDCs compared to WT BMDCs. $CD49b^+$ WT spleen cells were positively selected using a magnetic separation kit. They were then co-cultured with BMDCs that were differentiated for 10 days. Heat-killed *Cc* and LPS-stimulated Jα18-/- BMDCs were significantly better than WT BMDCs in their ability to stimulate the $CD49^+$ NK/NKT cells to produce IFN-γ (Figure 4-7).

4.3.5 Jα18-/- mice control the growth of EL4 and B16 tumours

To determine if the unexpected ability of Jα18-/- mice to control subcutaneous EL4 tumours was limited to that specific cell line, we designed a

similar experiment to compare both EL4 and B16 tumour growth in Jα18-/- mice. Immunologically naïve mice were subcutaneously injected with either EL4 or B16 cell lines into the rear right flank. Tumour development was monitored and measured daily. We found that both EL4 and B16 tumours grew significantly slower in Jα18-/- mice compared to WT mice (Figure 4-8).

4.4 Discussion

The infiltration of NKT cells has been suggested as a positive indicator of clinical outcome to cancer (2, 17, 18). Promoting the stimulation of NKT cells is therefore, believed to promote tumour immunity. This has led to the development of a pharmaceutical mode of activation targeting NKT cells. This form of NKT cell activation utilizes αGalCer as the NKT cell TCR agonist. Vigorous pre-clinical trials with mice and current human clinical trials have yet to yield definitive results (19, 20). However, the prospect of NKT cell activation to treat tumours has led us to investigate other avenues of NKT cell activation.

Our data shows an early benefit of *Cc* immunization against CD1d bearing tumours in mice. The control of tumour growth was limited to only *Cc*-treated WT mice and was not observed in CD1d-/- mice, suggesting the involvement of NKT cells. We believe a stronger benefit may be detected if a different immunizing schedule was employed that allowed for the maximum activation of NKT cells over a broad range of time. It would be interesting to determine whether ongoing treatment with *Cc* would have a beneficial effect on tumour mass. Adoptive transfer of WT activated NKT cells into CD1d-/- animals bearing

tumours would also definitively prove whether NKT cells are responsible for the delay in tumour growth. These results are supported by previous studies utilizing αGalCer to successfully treat tumour-bearing mice (13).

Interestingly, our tumour studies yielded some unexpected results. Jα18-/- mice, which lack iNKT cells, displayed a significant ability to prevent the development of $CD1d^+$ and CD1d- tumours from the EL4 and B16 tumour cell lines, respectively. This was in contrast to the finding that iNKT cells help to mediate anti-tumour responses in mice (17, 21, 22)(17). This discrepancy could be due to our use of the Jα18-/- mouse strain to determine iNKT cell involvement in tumour immunity. The unexpected tumour growth curves could be explained due to the difference in Jα18-/- BMDC responses that we measured. Higher levels of IL-12p70 from these BMDCs could be responsible for initiating other forms of cellular immunity, such as NK cells or cytotoxic T cells (23). The development of a strong antigen-specific cytotoxic T cell element is also strongly suggested by our data due to the increased expression of CD40 on the Jα18-/- BMDCs (24), and the preserved ability of the Jα18-/- BMDCs to stimulate strong IFNγ responses from the WT NK cells.

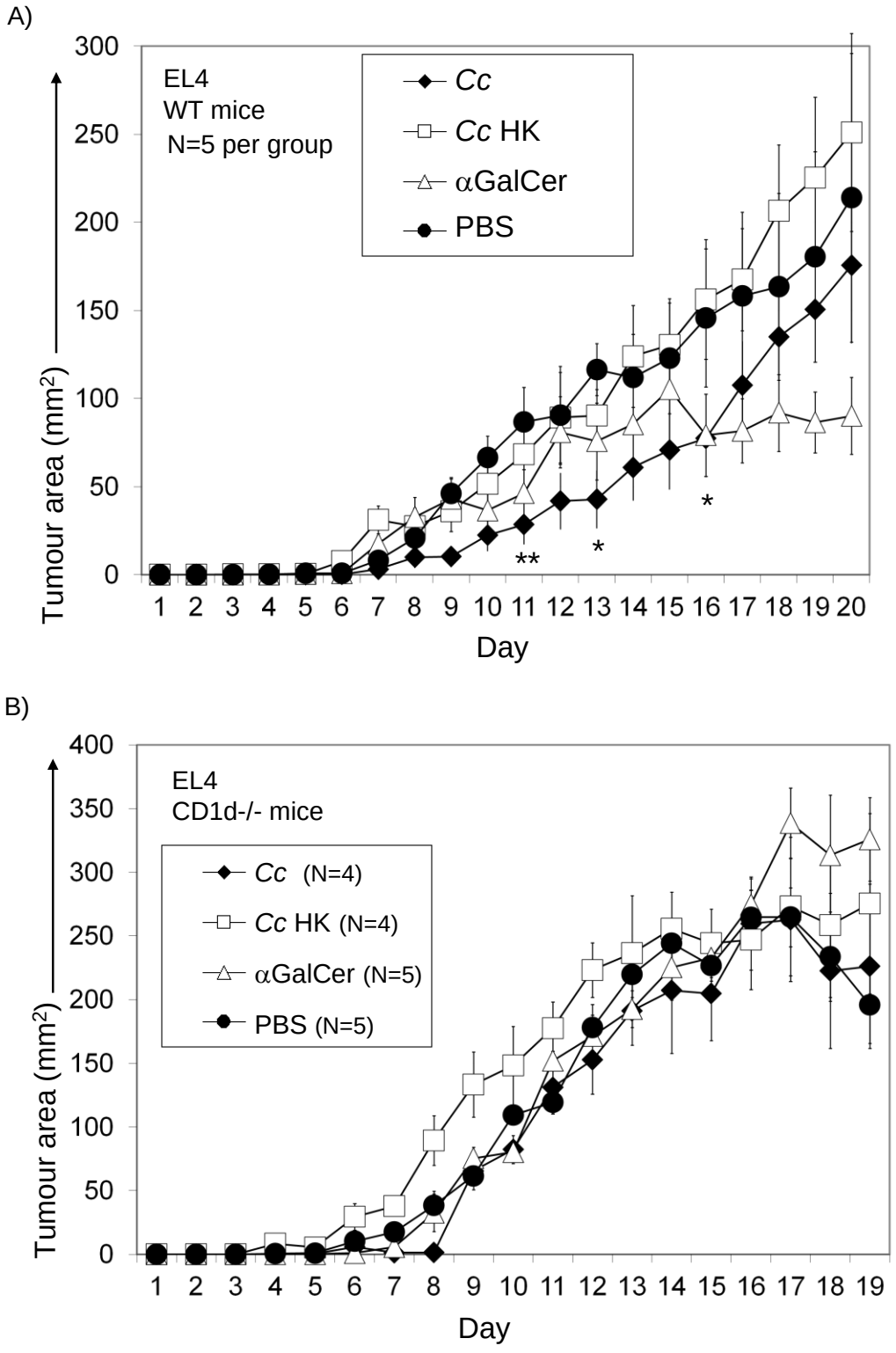
The lack of $J\alpha18^+$ iNKT cells leading to the development of more Th1-stimulating BMDCs is a novel observation. This is a unique observation regarding the requirement of iNKT/NK cells in the balanced development of DCs in the bone marrow, although their presence in the bone marrow has been detected previously (25-27). Current evidence supports the ability of both NK and NKT cells to promote the differentiation of peripheral monocytes into DCs (28, 29).

We believe we have detected, for the first time, the ability for iNKT/NK cells in the bone marrow to influence DC development.

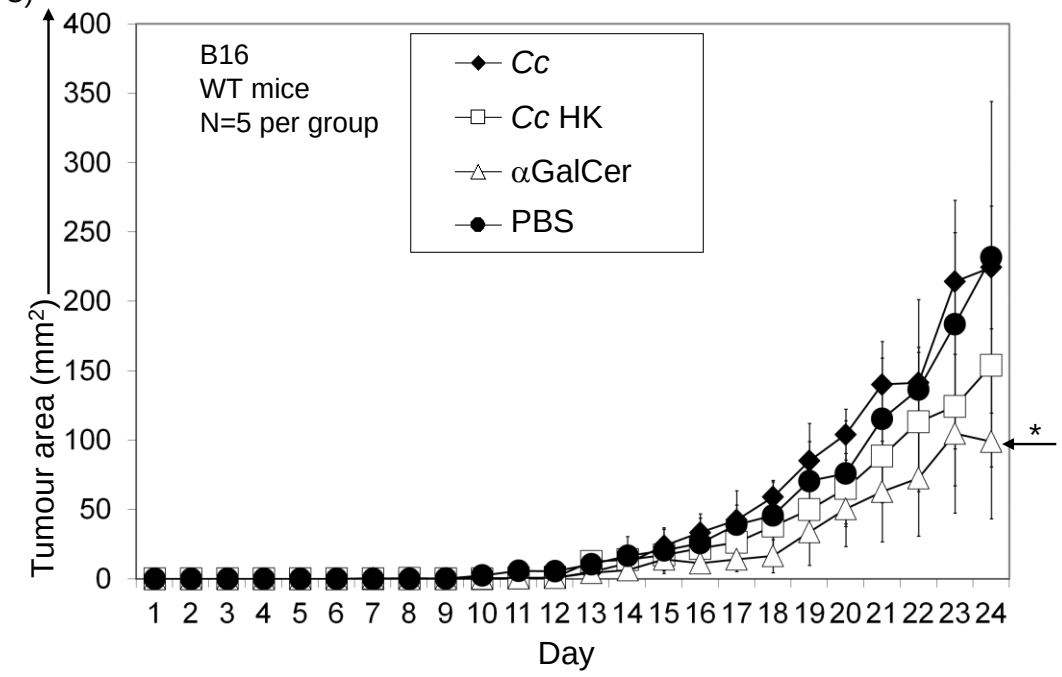
Taken together, we believe we have uncovered evidence to further support the role of DCs in stimulating immune responses directed toward tumour immunity. These responses could be capable of establishing a sufficient adaptive immune response which can control the tumour cells at an early stage. Despite iNKT cells being identified as helping to enhance cellular responses to immunity, we believe that we have uncovered another role for iNKT/NK cells to regulate the pro-inflammatory response through DC development in the bone marrow. Future research should further determine the roles NKT cells and DCs play in establishing tumour immunity, as well as the role of iNKT/NK cells in bone marrow DC development.

4.5 Figures

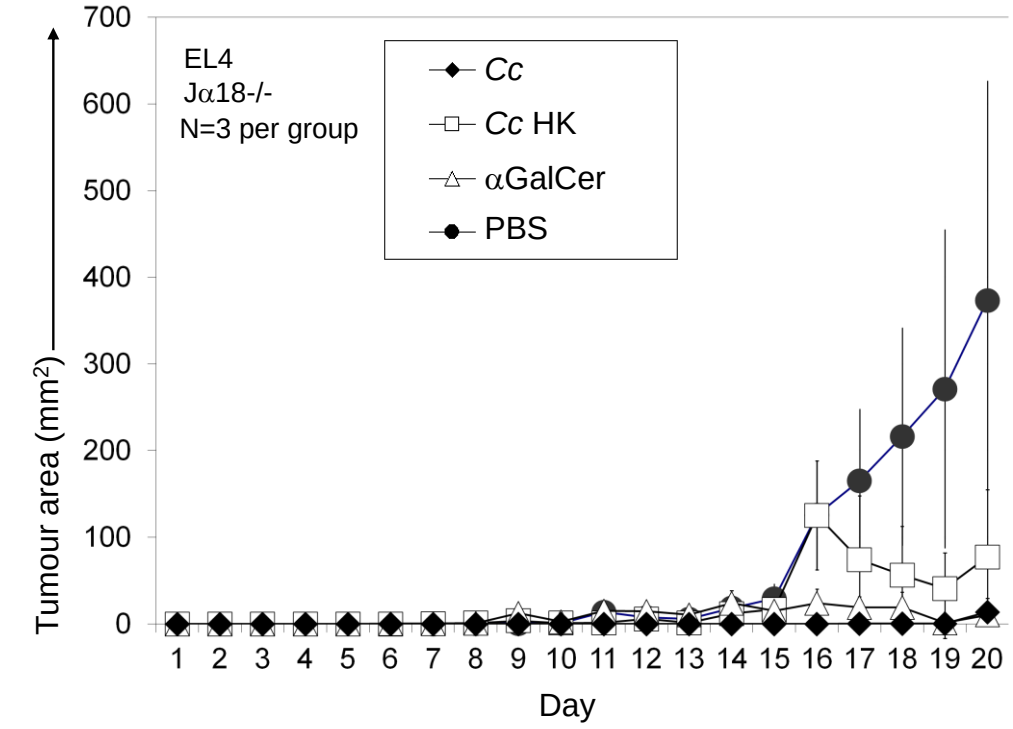
Figure 4-1 **Subcutaneous EL4 tumour grows slower in *C.crescentus* immunized mice**



C)

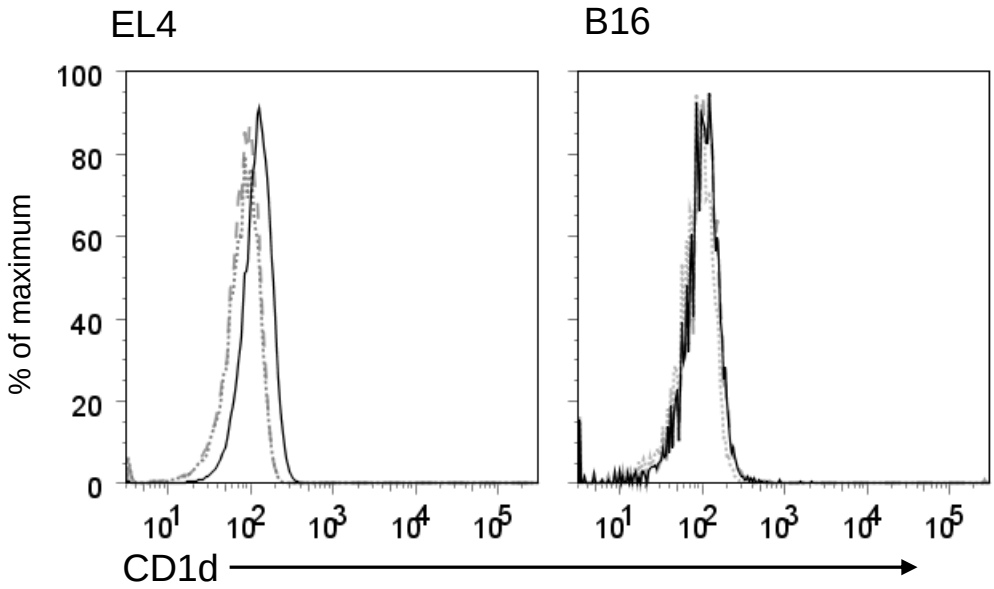

B16
WT mice
N=5 per group
Cc
Cc HK
αGalCer
PBS
Tumour area (mm2)
Day
*

D)


EL4
Jα18-/-
N=3 per group
Cc
Cc HK
αGalCer
PBS
Tumour area (mm2)
Day

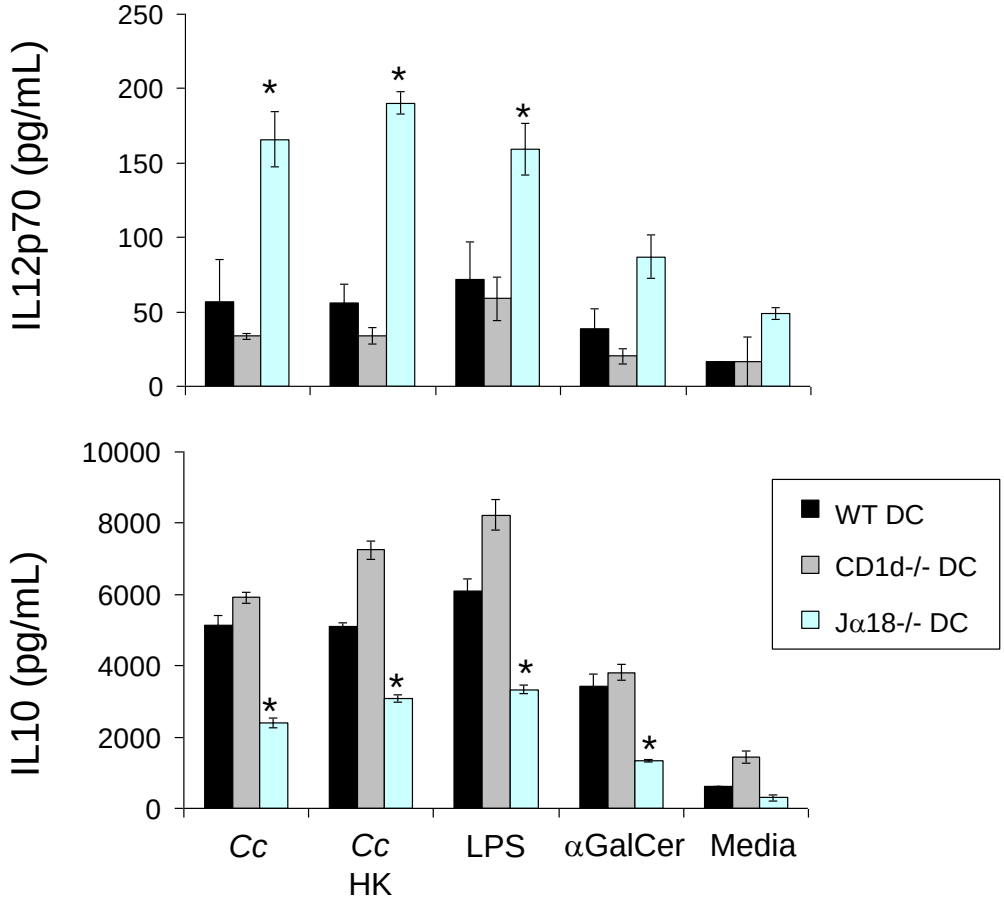
$20x10^6$ CFU of *Cc* or 2μg of αGalCer were injected intraperitoneally into C57BL/6 on days -3 and 1. $2.5x10^5$ EL4 or B16 cells were injected subcutaneously into the right flank of the mice on day 0. Tumour growth was measured daily until mice showed signs of discomfort and the experiment was stopped and mice euthanized. (A) WT mice treated with *Cc* displayed a significantly slower growth curve in their EL4 tumour based on the SPSS Univariate Analysis of Variance (Two-way ANOVA) for the overall growth curves $p<0.05$. *On days 13 and 16, there was a significant difference between *Cc* and PBS ($p<0.05$). ** Day 11 there was a significant trend ($p=0.07$). Days 10-17 there is a trend of difference between *Cc* and PBS treatment ($p<0.2$). (B) Using CD1d-/- animals, we found no difference in the growth of the mean tumour surface area between *Cc* and PBS-treated animals. (C) When B16 cells, which lack CD1d expression, there was no difference in the growth curves between *Cc* and PBS-treated mice, only αGalCer-treated mice had a significant difference from PBS with a Univariate Analysis of Variance on the overall growth curve (* $p<0.05$). (D) EL4 tumours failed to grow in Jα18-/- mice except those treated with PBS. Error bars show the standard error of the mean for each data point from the mice included in the study.

Figure 4-2 **CD1d is present on EL4 tumour cell line and not B16**



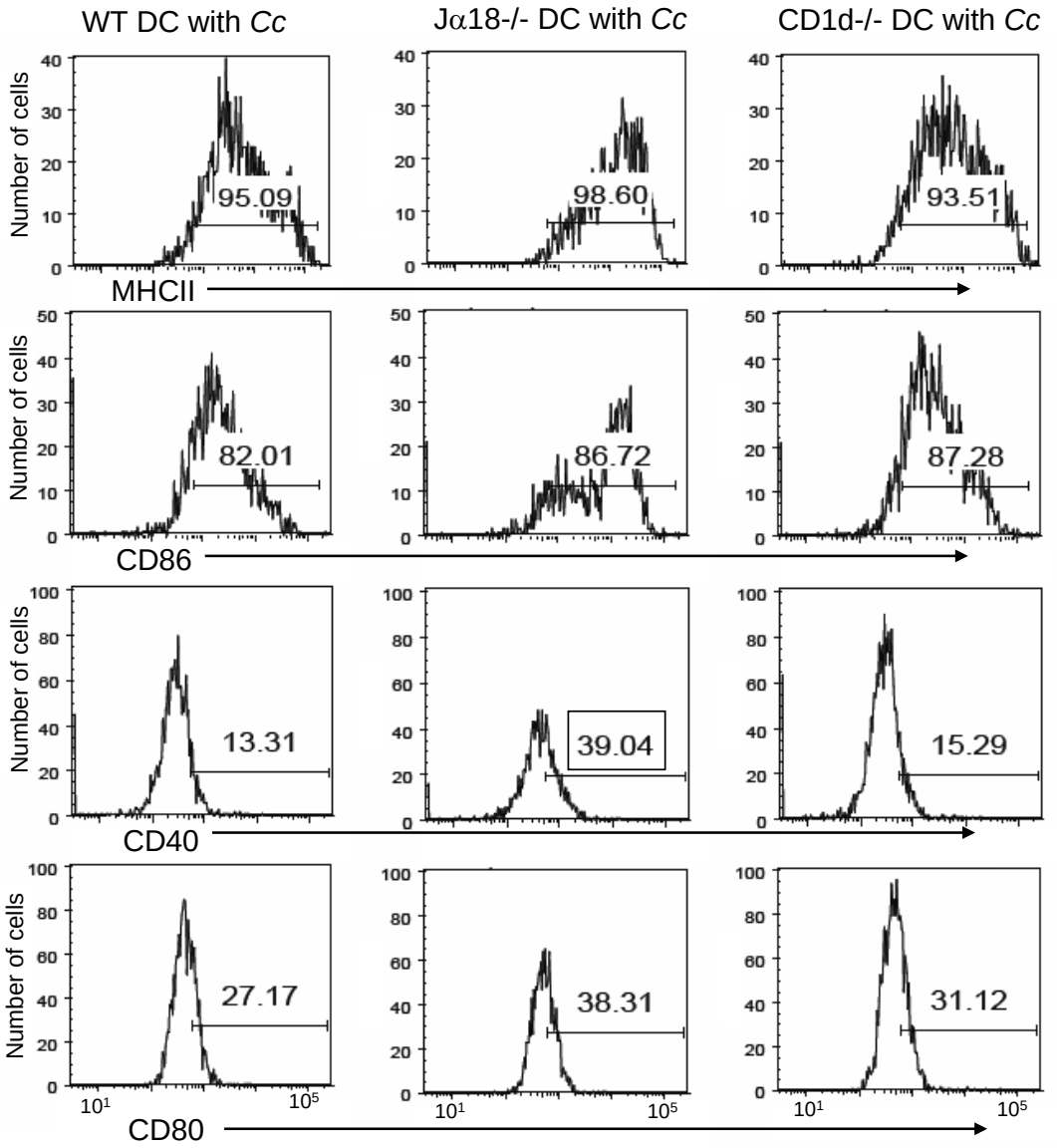
Flow cytometry staining for CD1d was performed and found that EL4 was $CD1d^+$ while B16 was CD1d-/-. Dotted line represents the isotype control. Solid line is αCD1d antibody (1b1).

Figure 4-3 **Jα18-/- BMDCs produce more IL-12p70 and less IL-10 compared to wild type BMDCs**



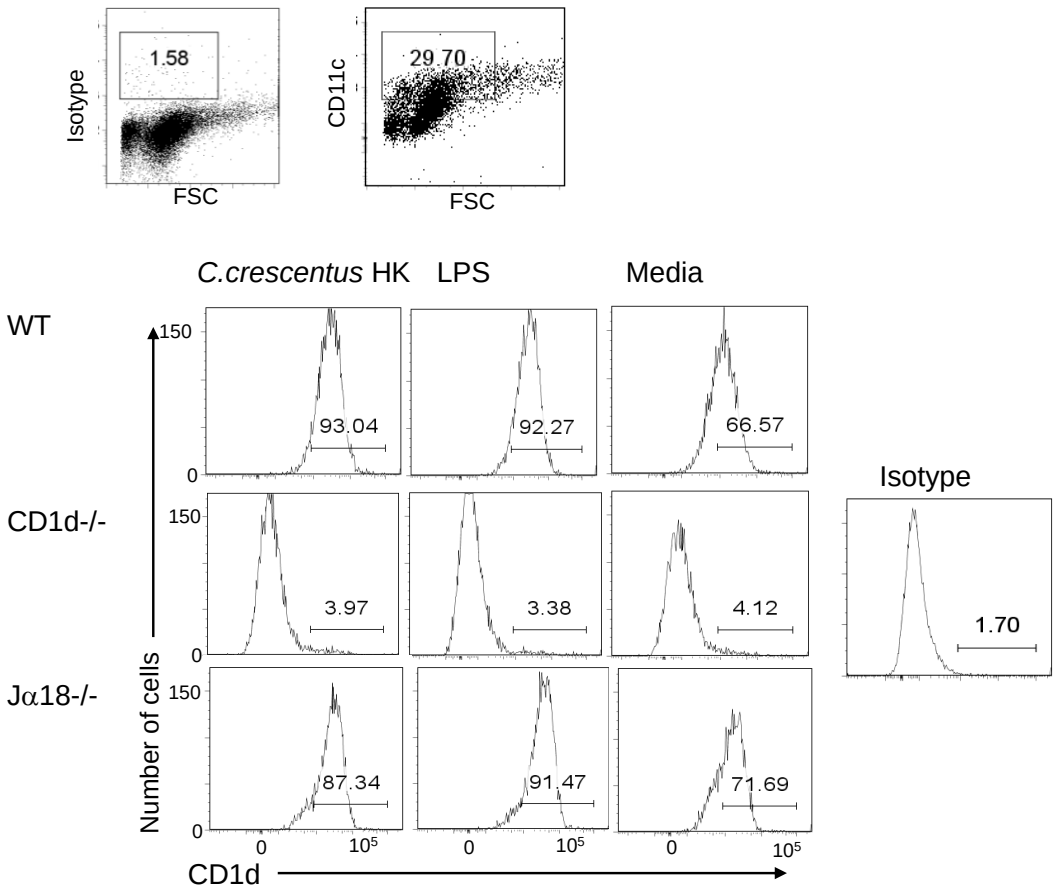
BMDCs were derived from wild type, CD1d-/- and Jα18-/- mice. They were cultured overnight with $2x10^6$ CFU *Cc*, heat-killed *Cc*, 1μg LPS or 1μg αGalCer. Supernatant was collected after 24 hrs and tested for IL-12p70 and IL-10. Jα18-/- BMDCs produced significantly higher levels of IL-12p70 and lower levels of IL-10. Error bars show the standard error of the mean. Data is representative from one of three similar experiments.

Figure 4-4 **Jα18-/- BMDCs have higher levels of co-stimulatory marker expression compared to wild type BMDCs**



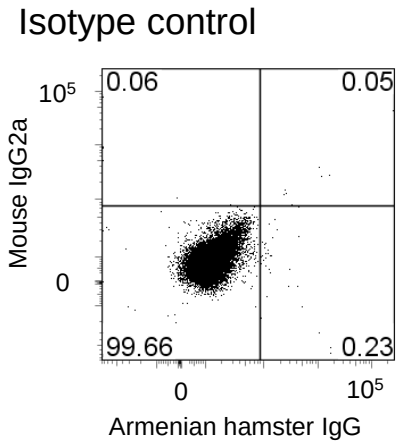
BMDCs were derived from wild type, CD1d-/- and Jα18-/- mice. They were cultured overnight with $2x10^6$ CFU of live *Cc*. They were then stained for CD11c, MHCII, CD86, CD40 and CD80. $CD11c^+$ cells were then gated and the percentage of cells positive for the expression of MHCII, CD86, CD40 and CD80 were determined. This data is a representative of two separate, similar experiments. Expanded data showing BMDC stimulation with heat-killed *Cc*, LPS and media is found in appendix 6.2.

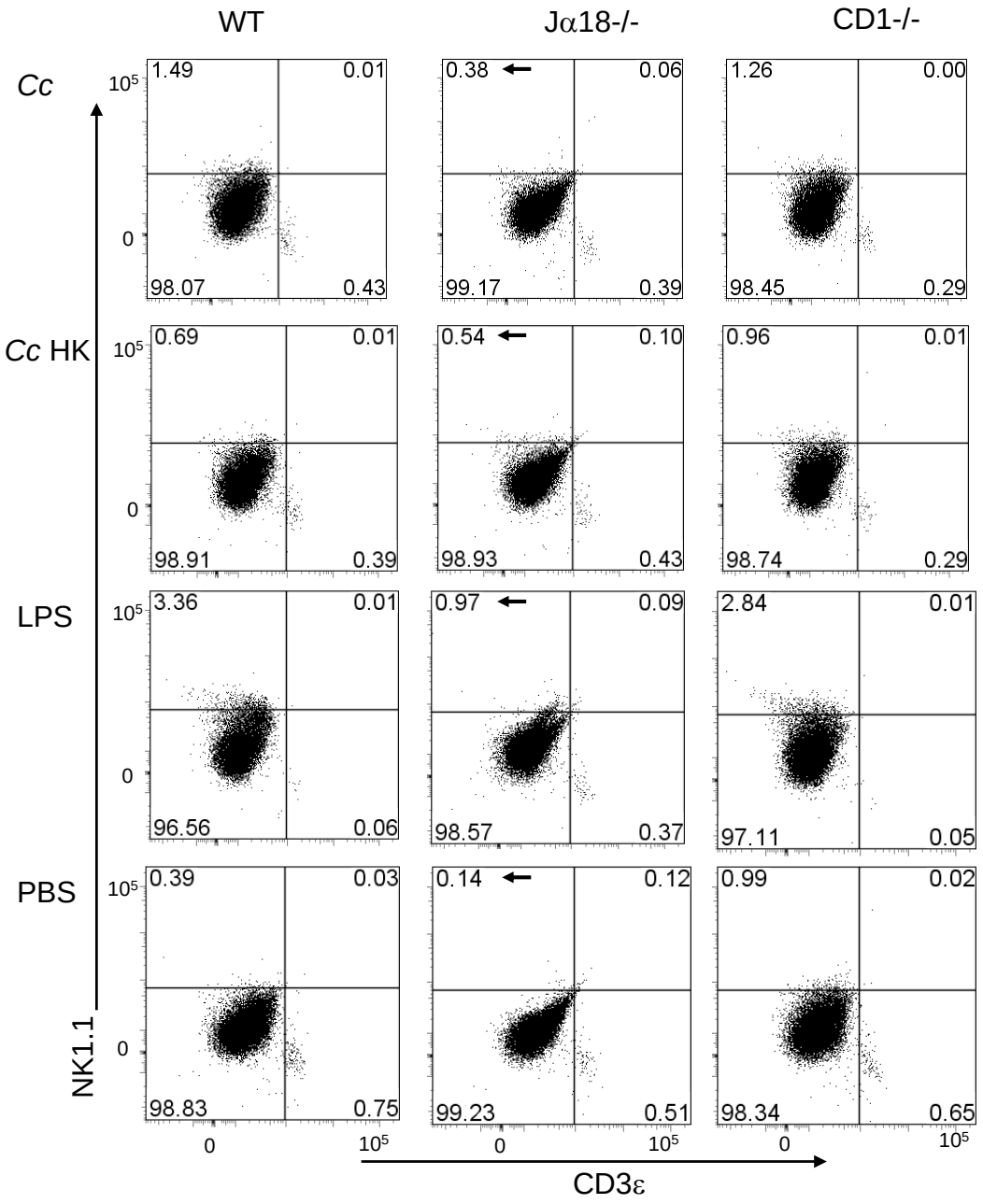
Figure 4-5 **Jα18-/- BMDCs have CD1d expression**



BMDCs were derived from wild type, CD1d-/- and Jα18-/- mice and stimulated overnight with $2x10^6$ CFU heat killed *Cc*, 1μg LPS or media only. CD1d expression was measured on $CD11c^+$ gated cells. This data is from one experiment to confirm lack of CD1d expression on CD1d-/- mouse cells.

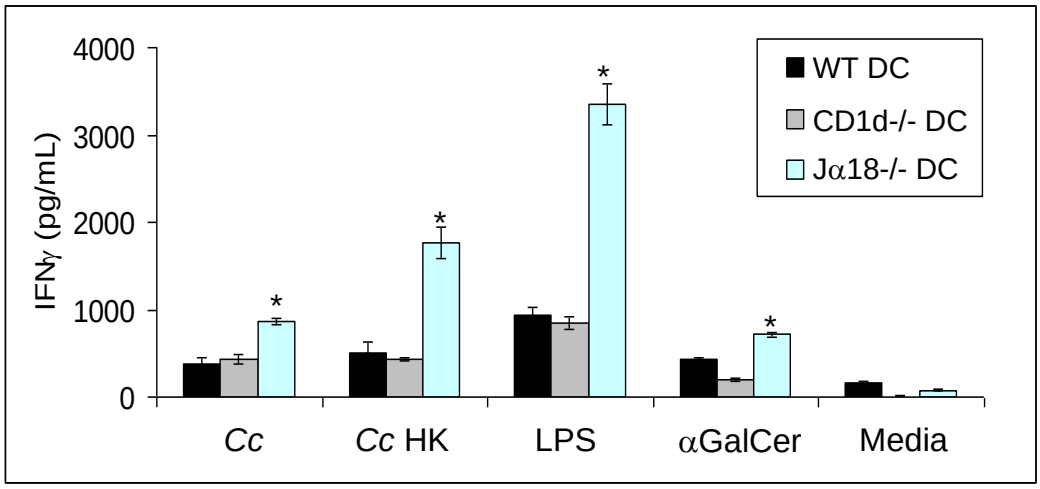
Figure 4-6 **Jα18-/- BMDC cultures contain lower levels of NK1.1$^+$ cells**





Bone marrow obtained from wild type, CD1d-/- and Jα18-/- mice were differentiated into BMDCs using 800U/mL of GM-CSF for 8-10 days. After differentiation, the cultures were matured overnight with $2x10^6$ CFU *Cc*, heat-killed *Cc* or 1μg LPS. Cultures were harvested and stained for CD3ε and NK1.1. Data depicted is representative of two similar experiments.

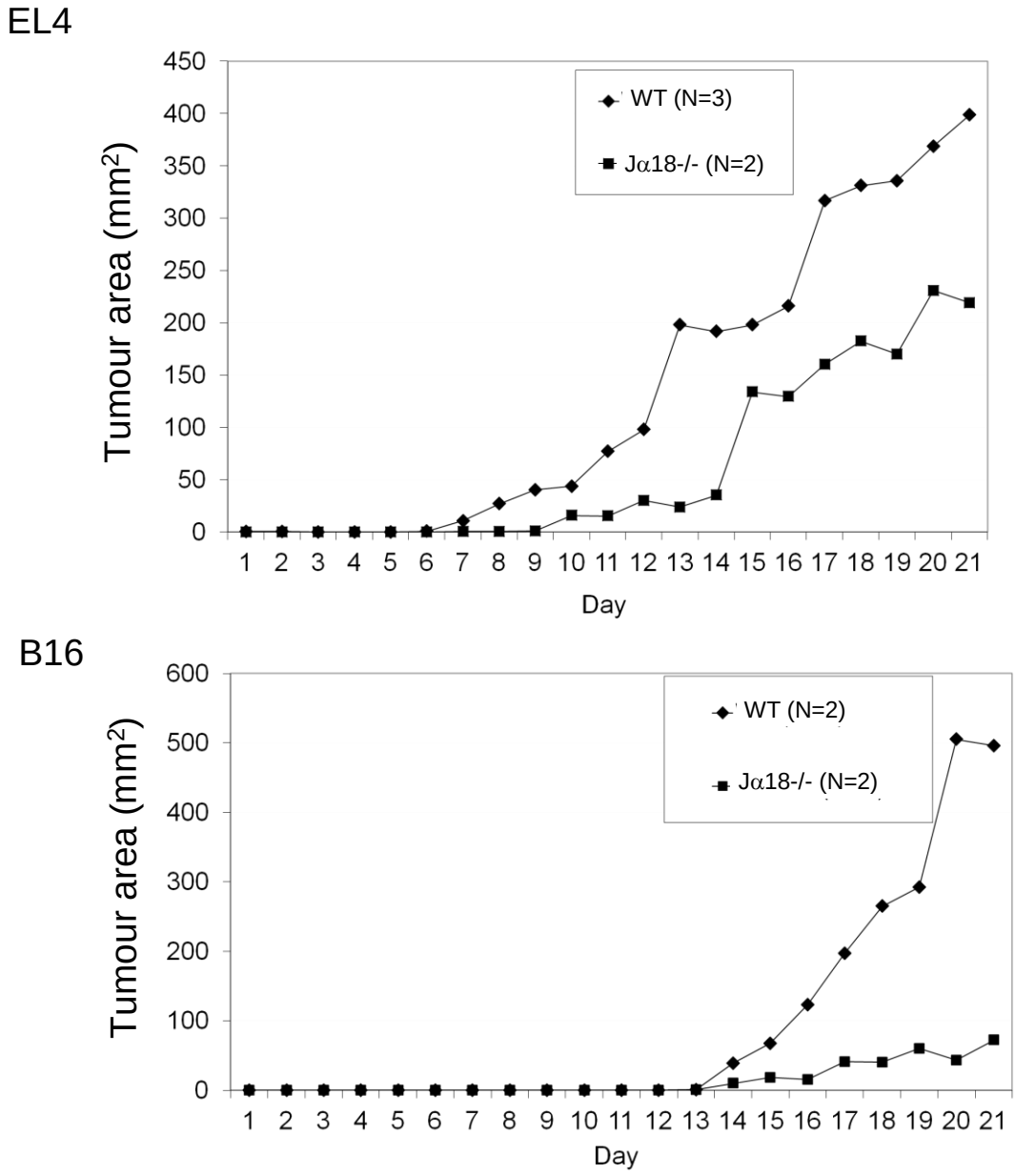
Figure 4-7 **Jα18-/- BMDCs can stimulate wild type NK/NKT cells to produce IFN-γ to a greater degree compared to WT BMDCs**



*p<0.05

BMDCs obtained from wild type, CD1d-/- and Jα18-/- mice were co-cultured with wild type NK cells ($CD49b^+$) for 24 hrs with $2x10^6$ CFU of *Cc*, heat-killed *Cc* or 1μg LPS. Supernatant was collected and tested for IFNγ by ELISA. Jα18-/- BMDCs can significantly stimulate higher IFN-γ from wild type NK cells. This data is representative from one of two similar assays.

Figure 4-8 **Jα18-/- mice develop EL4 and B16 tumours slower than wild type mice**



Wild type and Jα18-/- mice were injected into their right rear flanks with $2.5x10^5$ EL4 or B16 tumour cells. Tumour growth was measured daily until mice showed signs of discomfort and the experiment was stopped and mice euthanized.

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CHAPTER-5

General Discussion

This thesis has focused on the immunological response stimulated by *Caulobacter crescentus* (*Cc*) and the ability of that response to control the growth of $CD1d^+$ tumours. We used a combination of NKT cell knockout mice and magnetically isolated NK cells in our experiments to identify the involvement of NKT cells in *Cc* induced tumour immunity. It is the first report to show the use of non-pathogenic bacteria to stimulate innate immunity in order to initiate tumour immunity. It is our belief that these responses could re-establish equilibrium or initiate immunosurveillance, which leads to the eradication of the tumour (1).

Additionally, in the progress of these studies, we were able to elucidate a potential role that iNKT cells could play in the development of bone marrow derived dendritic cells (BMDCs) and modulating anti-tumour immunity. Although the data was collected under the premise of promoting tumour immunity, it can be additionally applied to a wide range of other scientific fields of study. These findings and concepts will all be discussed in more detail in the following sections.

5.1 Activation of natural killer T cells by *Caulobacter crescentus*

Initially, our studies focused on the activation of natural killer T cells (NKT), as it is currently understood that NKT cells play a role in establishing and controlling tumour immunity (2). We found that the non-pathogenic bacterium, *Caulobacter crescentus*, led to the specific activation of NKT cells. This activation was determined through expression of both CD69 and granzyme B on NKT cells, along with the production of IFN-γ and cellular proliferation. The

activation of NKT cells by non-pathogenic bacteria is supported by research from Kinjo and colleagues using non-pathogenic *Sphingomonas* bacteria (3, 4).

NKT cells are known to be activated by either direct recognition of a foreign lipid presented by CD1d or through recognition of an endogenous lipid through CD1d followed by co-stimulation with CD40 and IL-12 (4). In our initial experiments, we were unable to determine which route led to the activation of *Cc*-induced NKT cells. Plating the lipid and non-lipid fractions isolated from *Cc* resulted in ambiguous observations which did not clearly identify a requirement for CD1d presentation of a foreign lipid product. In our later experiments, utilizing CD1d-/- and wild type (WT) bone marrow derived-dendritic cells (BMDCs), our data hints at a possible CD1d independent mechanism of NKT cell activation by *Cc*. Activation was determined through the production of IFN-γ from co-cultures of CD1d-/- BMDC and WT $CD49b^+$ NK cells obtained through a magnetic NK cell enrichment assay; we can not exclude the possibility of WT antigen presenting cells expressing CD1d contaminating the assay. As expected, αGalCer, a known CD1d dependent activator of NKT cells, had significantly lower production of IFN-γ in co-cultures utilizing CD1d-/- BMDCs. Interestingly, CD1d presentation was required for down-regulating the production of IL-10 from the co-cultures. This finding suggests an alternative pathway to stimulate the production of IFN-γ from NKT cells. Our data would appear to support others who have reported a population of CD1d-independent NKT cells that are matured from mice lacking β2-microglobulin, a protein essential in the structure of CD1d (5). However, we do not believe this population is responsible

for *Cc*-induced NKT cell activation, since CD1d-/- and Jα18-/- splenocytes ultimately did not respond to *Cc in vitro*.

Previous murine studies have shown that NKT cell activation was capable of controlling the growth of cancer (6-8). Both of our *in vivo* tumour studies and *ex vivo* killing of EL4 tumour cells compliment the current understanding regarding the role of NKT cells in tumour immunity. We are the first to show that NKT cell activation by the non-pathogenic *Cc* bacteria can control the growth of a tumour cell line. This data adds to the previous study by Bhatnagar and collegues who showed *Cc* immunizations in mice prevented the growth of Lewis lung carcinoma (9).

The importance of NKT cells in promoting a successful immune response targeting viral and bacterial infections is only recently documented (10, 11). The ability to stimulate NKT cell activation through both CD1d-dependent and independent pathways potentially gives the cell population a certain level of flexibility in being able to respond to different infections. In the case of viruses, such as herpes simplex virus 1 (HSV-1) (12) and human immunodeficiency virus (HIV) (13), it has been shown they can down-regulate the cell surface expression of CD1d in infected cells. This would indicate the potential evolution of a viral escape mechanism to avoid classical detection and activation of NKT cells. Our newly uncovered mode of CD1d-independent NKT cell activation could potentially point to a new branch in the evolutionary arms race between mammalian immune systems and viral/bacterial parasites. The ability of *Cc* to induce this novel type of NKT cell activation could be significant in excising its

ability to be a human pathogen. Further research may reveal a link between CD1d-independent activation and the classification of pathogenic or non-pathogenic micro-organisms.

5.2 Activation of dendritic cells by *Caulobacter crescentus*

Dendritic cells (DCs) can play an important role in developing tumour immunity. Acting as a bridge between innate and adaptive immunity, their level of activation can shape the adaptive immune response towards either a Th1 or Th2 phenotype (14). Th1 responses are characterized by IFN-γ production and the development of cytotoxic effector cells (15). This type of response has been shown to be important in the maintenance of immunosurveillance, as well as establishing tumour-targeting cytotoxic cells (16-18). Our data regarding DC activation confirms that *Cc*, despite being non-pathogenic, could potentially shape a Th1 adaptive immune response.

BMDC activation was determined through measuring the cell surface expression of CD80, CD86, CD40 and CD54, as well as the production of IL-12p70. CD80 and CD86 are both costimulatory molecules which are required for the priming of an antigen-specific T cell response (19). CD40 and IL-12p70 are important in directing the development of a Th1 antigen-specific T cell response (14, 15). CD54 is an adhesion molecule important in the formation of the immunological synapse while lowering the amount of antigen required for developing the T cell response (14).

Culturing BMDCs with *Cc* resulted in the increased expression of all the measured BMDC activation markers. Taken together, *Cc*-activated DCs present a novel therapy that could increase antigen presentation of tumour-associated antigens (TAAs) acquired up by tumour-infiltrating DCs. This would, theoretically, increase the diversity of antigen-specific Th1 cytotoxic responses directed at the tumour. IL-12p70 production by activated DCs could also play a role in mediating a change in the tumour micro-environment (20), further supporting the development of tumour immunity. Together, these DC-mediated effector responses could re-establish the equilibrium between the tumour and immune system while controlling the progression of the disease (1, 21).

The observation that *Cc* could potentiate an immunological shift favouring Th1 responses could additionally provide a source of treatment for diseases, such as allergies and asthma, which display a dominant Th2 immunological response to allergen (22). One of the dominant theories that attempt to explain the etiology of asthma and allergy implicates a hyper-polarization towards Th2 responses in affected patients. Th2 polarization in allergy patients has been commonly attributed to a limited, early life exposure to bacteria (22). *Cc* is naturally found in bodies of fresh water (23). Taking into account our data concerning *Cc* activation of DCs, the prevalence of allergies and asthma in the western population could be simply attributed to sanitation and water treatment conditions prior to water consumption. This may ultimately limit exposure to Th1 polarizing *Cc* in early life and therefore, increase the risk in the future development of allergies and asthsma.

5.3 *Caulobacter crescentus* modulates the interaction between NKT and DCs

DCs and NKT cells have both been described as cell populations that bridge the innate with adaptive immune systems (4, 24). DCs have been classically tasked with presenting antigens to T cells, while NKT cells play a role in shaping the adaptive immune response through their cytokine production (4, 24). Others have previously shown that NKT cell contact with DCs can stimulate the production of IL-12p70 (10, 17). This finding is confirmed by our data which showed that *Cc* stimulation of a co-culture containing DCs and NKT cells led to an enhanced production of IL-12p70, as well as CD40 expression on DCs. It is clear that their cooperation after *Cc* activation would further increase the probability of establishing a strong Th1 immune response which could target cancer.

5.4 Jα18^{+} iNKT cells play a role in supporting tumour growth

A surprising observation during our studies into the relationship between NKT cells and cancer involved the lack of tumour growth in mice devoid of iNKT cells. This result was in stark contrast to the conventional belief that iNKT cells play an important role in tumour immunosurveillance (24, 25). When we subcutaneously implanted EL4 and B16 tumour cells into these mice, there was a significant reduction in tumour growth compared to WT mice.

Examining the biology of Jα18-/- mice, we found that BMDCs generated from these mice had a significantly higher level of CD40 and IL-12p70

expression. These responses suggest a role for their DCs in establishing a robust cellular immunity that could control the exogenously delivered syngeneic tumours. Interestingly, in our early experiments, it was found that Jα18-/- splenoctyes stimulated *in vitro* with *Cc* led to abrogated IFN-γ production, while the production of IL-10 was increased. This data suggests that immunosurveillance in Jα18-/- mice is independent of iNKT cell-produced IFN-γ. DCs could play an important role in establishing tumour immunosurveillance in these mice (26). Recent reports have identified a new subset of killer DCs which can be identified by their expression of NK1.1 and B220. However, in our bone marrow differentiation experiments, we detected a lack of NK1.1 expression in the Jα18-/- mice (27, 28). We believe that, in Jα18-/- mice, the classic DC response is generating Th1 immunity that is capable of controlling the growth of syngeneic tumours. The failure to detect IFN-γ in our studies could possibly be due to the early nature of our experiments designed to detect IFN-γ from innate responding cells such as NKT cells.

Despite seeming advantages in the context of preventing the establishment of tumours, Jα18-/- mice and humans have not been selected for in the evolutionary tree. This theory is supported by a 2010 study where Jα18-/- mice developed higher levels of auto-reactive antibodies when injected with apoptotic bodies in a model of systemic lupus erythematosus (29). This study, although limited to autoantibody development, presented along with our data would suggest Jα18-/- mice may be at a higher risk for autoimmune diseases.

5.5 Future Directions

This thesis has focused on the *Cc*-specific activation of DC and NKT cells. We believe that it sets the foundation towards the study of non-pathogenic bacteria as immune stimulators to potentiate the development of tumour immunity. Future studies should determine the ability of DCs to present tumour antigens and whether *Cc* immunizations can increase the repertoire of T cells targeting tumour-associated antigens in an effort to re-establish tumour-host equilibrium. The role of NKT cells should also be further explored to determine their relationship with DCs. Studying the potential existence of a *Cc* stimulated CD1d-independent pathway leading to the activation of NKT cells will help our overall understanding of NKT cell biology. If this pathway is definitely proven to exist, effort should be taken to relate CD1d-independent NKT cell activation with the ability of bacteria and viruses to be either commensal or pathogenic invaders of the body.

The ability of *Cc* to induce Th1 stimulating DCs need to be further explored in other model systems including allergy and asthma. A link between early life, natural exposure to *Cc*, found in freshwater, and the development of allergies or asthma later in life should be explored. Additionally, the ability of *Cc* to polarize a Th1 response could be useful in the later treatment of patients who suffer asthma or allergies.

Efforts to develop *Cc* into a tumour treatment primarily to promote the stable development of tumour equilibrium should be avoided. Notably, the re-establishment of tumour equilibrium with the immune system may only be

temporary and could lead to further immuno-editing of the tumour resulting in a very low immunogenic tumour. Schreiber and collegues suggest that tumour equilibrium could potentially be maintained for the duration of life in a cancer patient (1), however if the tumour is constantly experiencing immuno-editing pressures, the possibility of another relapse into tumour escape is possible and therefore should be discouraged through physical removal of the tumour mass. Using *Cc* to activate DCs and NKT cells to potentially target a greater number of TAAs could re-establish tumour equilibrium however, we believe this gives a window of opportunity to eventually remove the tumour. Failure to remove the tumour mass may result in the future development of a severely non-immunogenic tumour which may not respond to any subsequent therapeutic approaches. Future studies should examine the long term consequences of a tumour treated with *Cc* and whether these tumours are more or less immunogenic than those that received no treatment.

The differential tumour responses by Jα18-/- and CD1d-/- mice indicate the presence of NKT cell subsets. Studies to define the appropriate NKT cell sub-populations that enhance tumour immunity can lead to improved targeted immunotherapies in the future. Elucidating the role of NKT cells in the development of DCs will allow us to better understand the biology of DC development. This understanding could lead to targeted therapies that knock down specific NKT cell subsets and allow for the development of Th1-polarized DCs. To determine if Jα18-/- mice DCs are primed to trigger a Th1 response, future studies could determine the antigen specific T cell recall response after

tumour exposure, looking at IFN-γ production. Further studies into the human population should determine if those who lack NKT cells, such as patients suffering from Wiskott-Aldrich syndrome (30), are more or less likely to develop a cancer over their lifetimes. In addition, the potential development of autoimmune diseases in the Jα18-/- mouse model needs to be determined. Autoimmune data may help explain why the seemingly beneficial Jα18-/- phenotype has not evolved naturally in mouse and humans.

Finally, studies to define the molecular patterns of *Cc* that stimulate innate immunity may reveal novel activation pathways. This would also allow for the purification of the *Cc* products for use in future studies to avoid any variability of results that can be attributed in using whole bacteria to stimulate immunity. These defined products could also serve as a therapeutic approach to initiate CD1d-independent activation of NKT cells which could be beneficial in fighting viruses that down-regulate CD1d.

In the future, these studies may support the use of non-pathogenic bacteria for use as immune stimulators to initiate successful tumour immunity leading to the clearance of tumours. In addition, the role of *Cc* in promoting Th1 responses may lead to a further understanding of its natural interactions with the human body.

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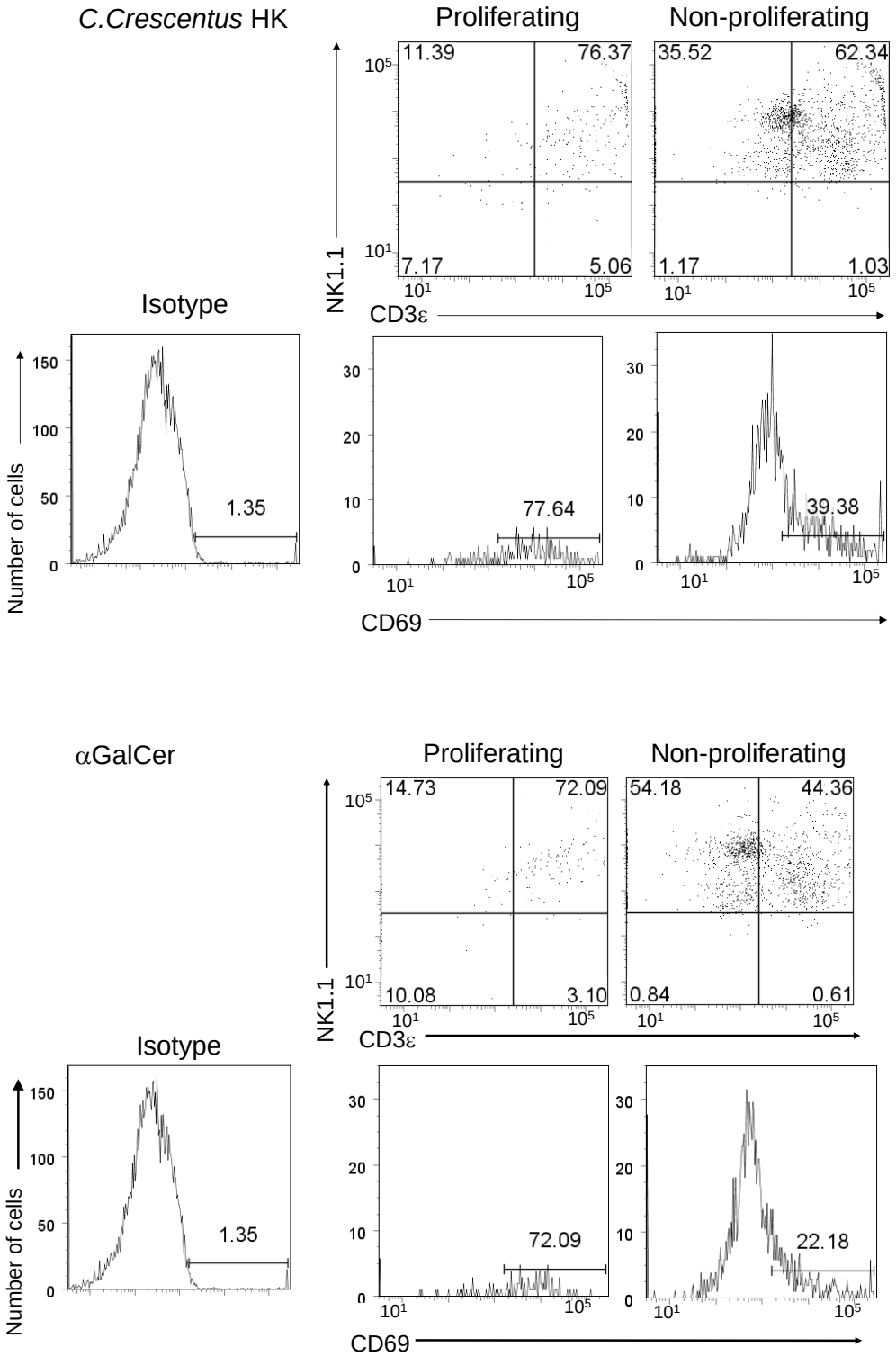
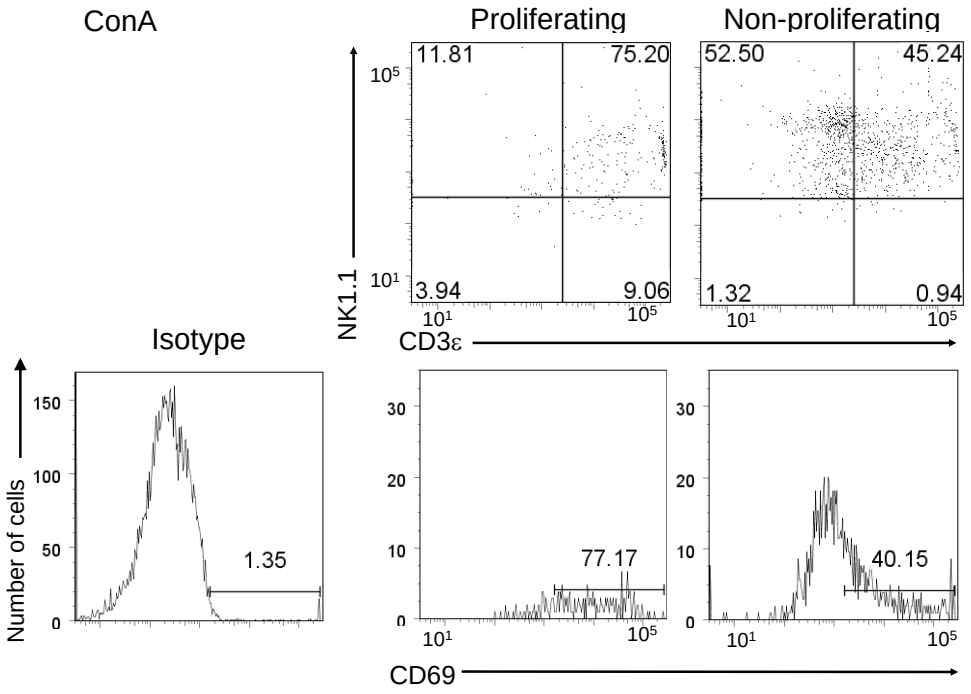
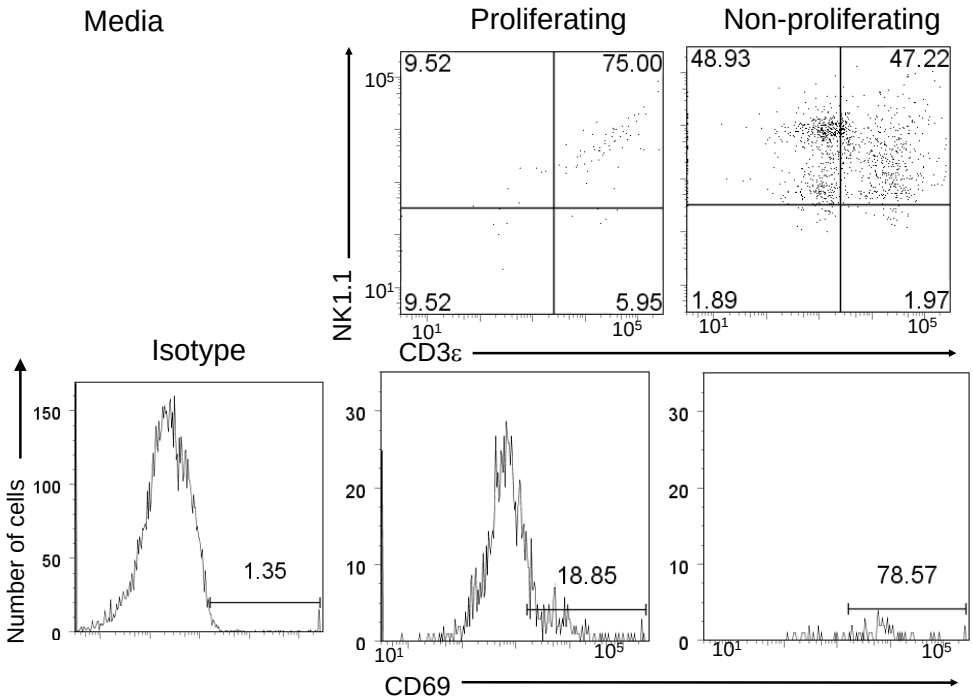
6.0 Appendix

6.1 Extended figures of Figure 2-2

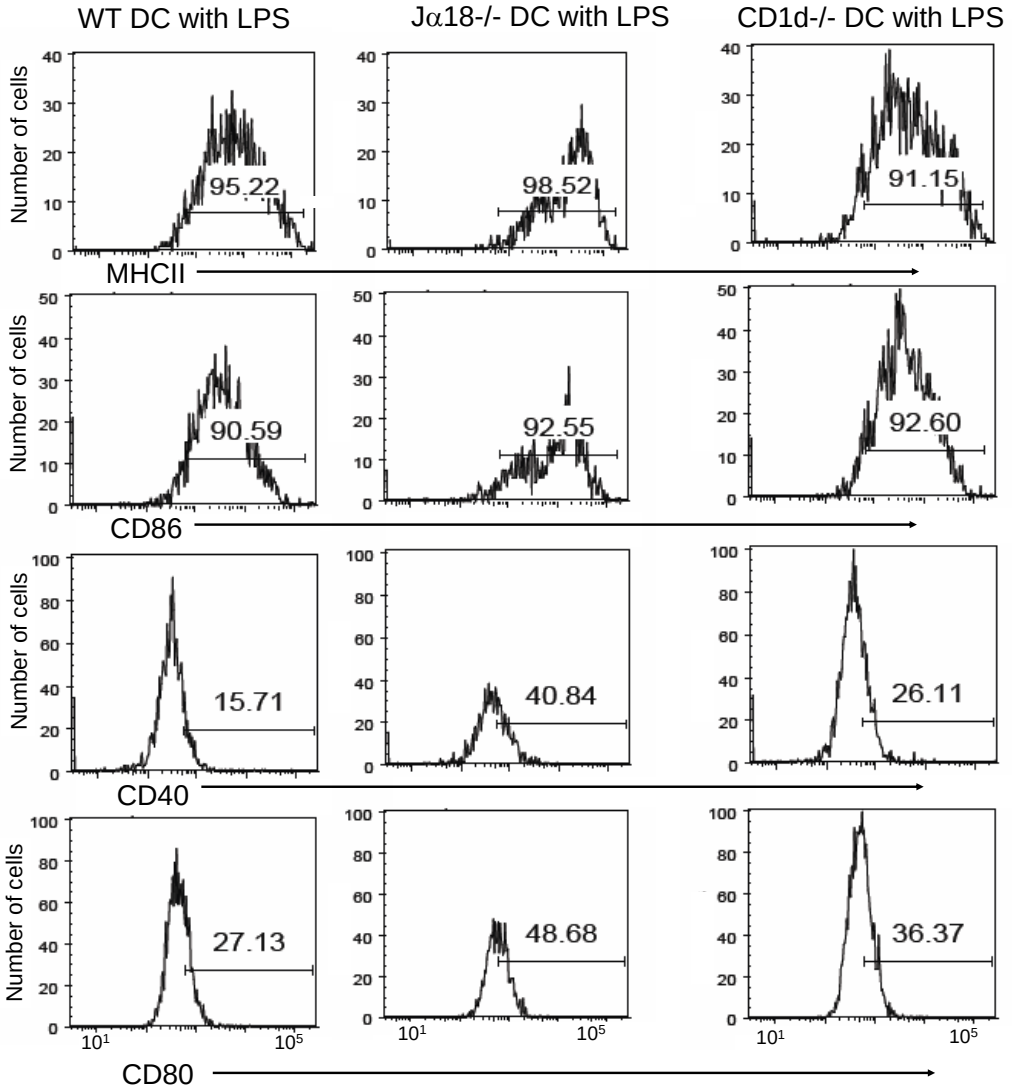



ConA
Proliferating
Non-proliferating
11.81
75.20
52.50
45.24
3.94
9.06
1.32
0.94
NK1.1
CD3ε
Isotype
Number of cells
1.35
77.17
40.15
CD69

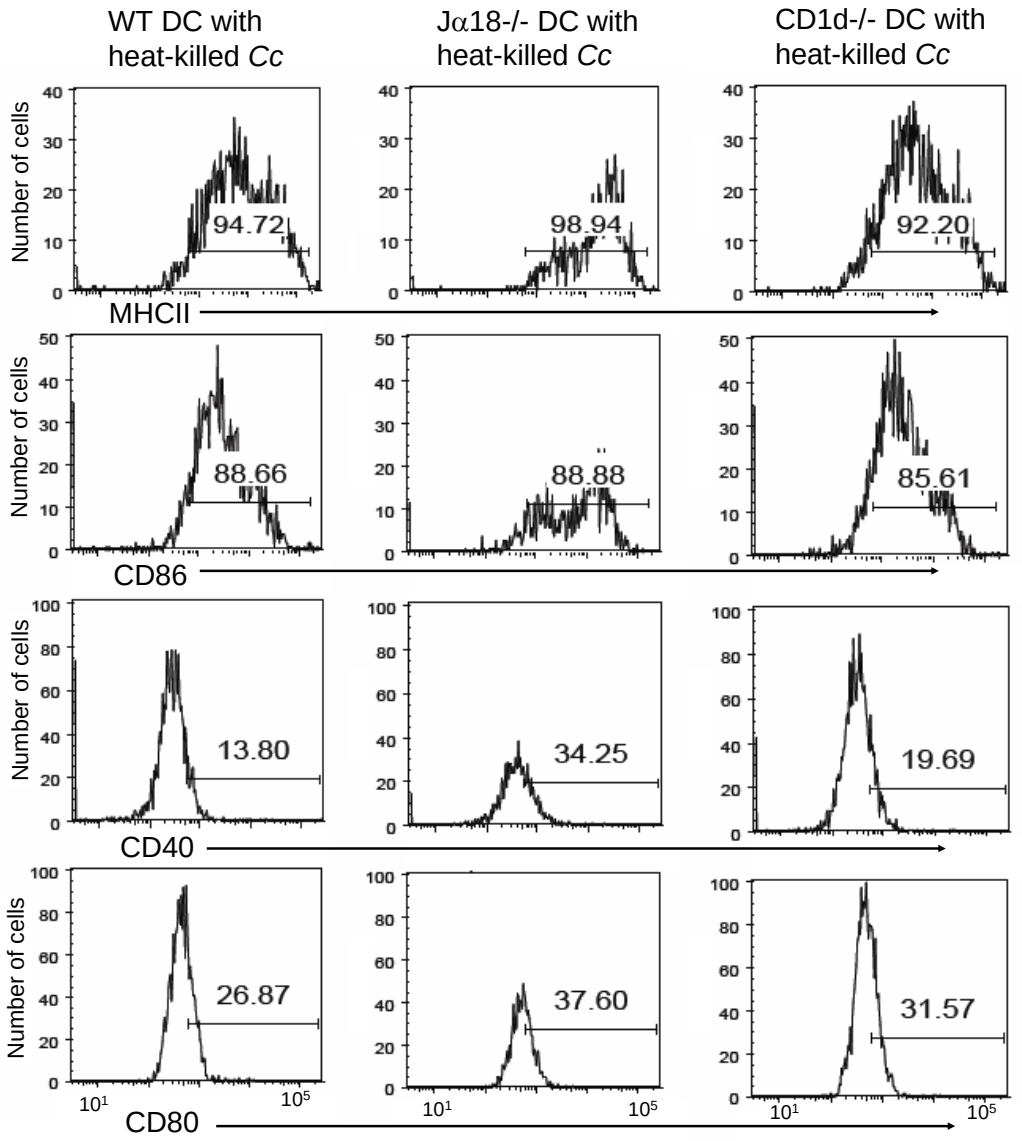

Media
Proliferating
Non-proliferating
9.52
75.00
48.93
47.22
9.52
5.95
1.89
1.97
NK1.1
CD3ε
Isotype
Number of cells
1.35
18.85
78.57
CD69

Flow cytometry data showing the distribution of $NK1.1^+$ and $CD3\varepsilon^+$ cells from the proliferating and non-proliferating cells induced by heat-killed *C. crescentus*, αGalCer, ConA or media. Proliferating cells had higher proportions of dual positive NK1.1 and CD3ε NKT cells. Additionally, CD69 early activation marker expression levels were determined from proliferating and non-proliferating cells. All conditions, except media, stimulated >70% expression levels of CD69 in proliferating cells.

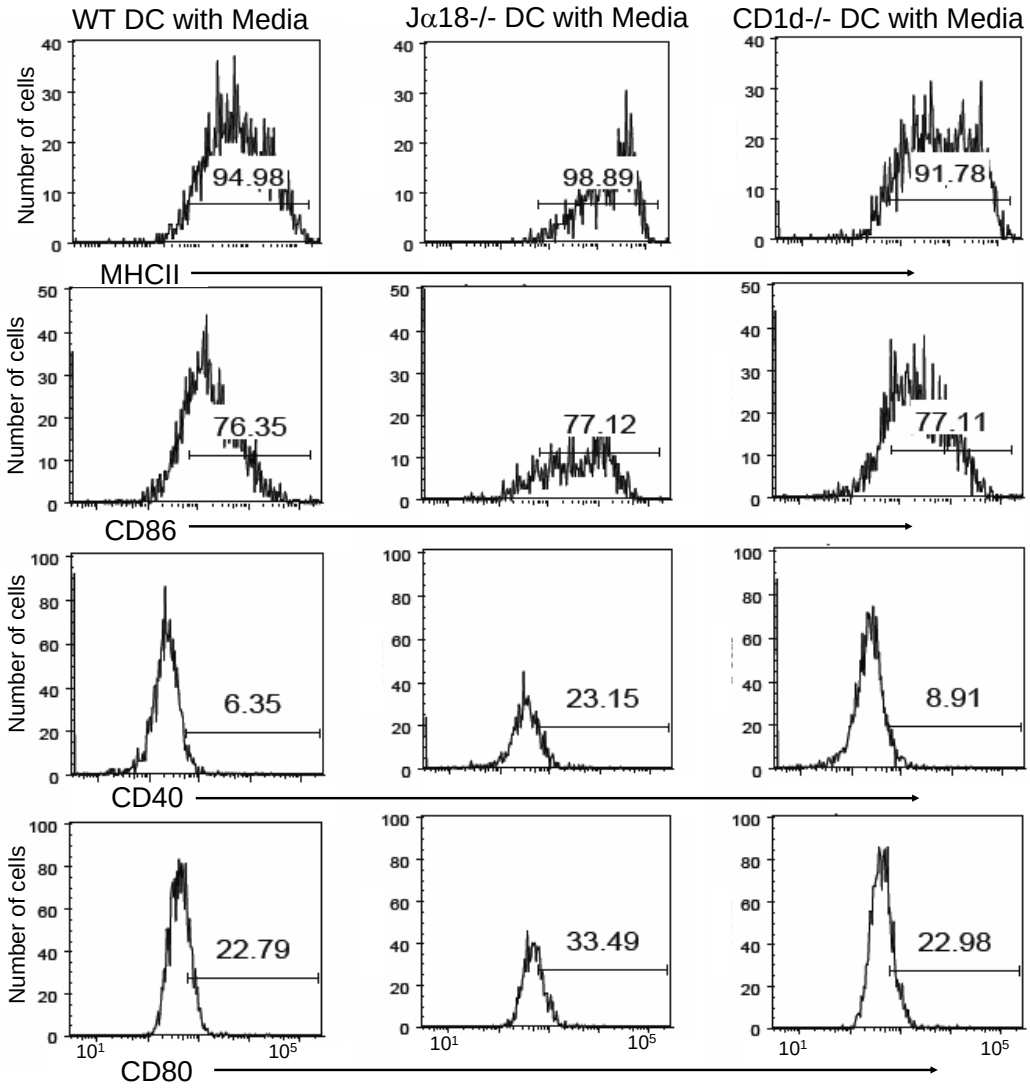
6.2 Extended figures of Figure 4-4



BMDCs were derived from wild type, CD1d-/- and Jα18-/- mice. They were cultured overnight with 1μg of LPS. The percent of gated positive cells was determined from the parent $CD11c^{+}$ population. An increased expression of co-stimulatory markers was detected from BMDCs cultured from Jα18-/- mice.



BMDCs were derived from wild type, CD1d-/- and Jα18-/- mice. They were cultured overnight with $2x10^6$ CFU of heat-killed *Cc*. The percent of gated positive cells was determined from the parent $CD11c^+$ population. An increased expression of co-stimulatory markers was detected from BMDCs cultured from Jα18-/- mice.



BMDCs were derived from wild type, CD1d-/- and Jα18-/- mice. They were cultured overnight with media alone. The percent of gated positive cells was determined from the parent $CD11c^{+}$ population. An increased expression of co-stimulatory markers was detected from BMDCs cultured from Jα18-/- mice.

6.3 Publication of MS paper

Cellular Immunology citation:

Loo, E. W., M. J. Krantz, and B. Agrawal. 2012. High dose antigen treatment with a peptide epitope of myelin basic protein modulates T cells in multiple sclerosis patients. *Cell. Immunol.* 280: 10-15.

High dose antigen treatment with a peptide epitope of myelin basic protein modulates T cells in multiple sclerosis patients

Eric W. Loo
Department of Surgery, Faculty of Medicine and Dentistry, University of Alberta, Edmonton, Canada

Mark J. Krantz
BioMS Medical Corp, Edmonton, Canada

Babita Agrawal
Department of Surgery, Faculty of Medicine and Dentistry, University of Alberta, Edmonton, Canada

1. Introduction

Multiple Sclerosis (MS) is a complicated autoimmune disease affecting the central nervous system of patients often leading to neurological disability. The origin and progression of MS is still not well understood and both genetic and environmental factors appear to play a role in its development.

In a 2005 study it was estimated that over 240 of every 100 000 Canadians are afflicted by MS [1]. This leaves Canada with some of the highest incidents of MS in the world. Progressive forms of MS are especially debilitating because there are no signs of remittance during this course of MS. The patients suffer a

constant increase in their disability over time (MS Society of Canada). MRI scans of the brain and central nervous system (CNS) characterize areas of active neural degradation in 'plaques'. These plaques are sites of active inflammation harbouring infiltrating immune cells, especially T cells [2].

Disease incidence and progression of MS have many competing etiologies, autoimmune responses have been suspected as a major cause of MS. The presence of $CD4^+$ and $CD8^+$ T cells in neuronal plaques and the even distribution of MHC class II positive microglia cells implicates an immune involvement in the disease [3]. Myelin basic protein (MBP) reactive T cells have been found in the blood of both MS patients and healthy volunteers however, it is believed that the inflammatory phenotype of those T cells could be greater in diseased individuals [4]. The importance of MBP reactivity to MS disease is confirmed through mouse models of MS where the administration of MBP with inflammatory adjuvants, such as Freunds complete adjuvant, into mice initiates an experimental autoimmune encephalomyelitis (EAE) that largely resembles MS [5]. MBP immunodominant regions have also been characterized through antigen determination of MS patient B and T cells [6].

Currently the treatments offered to MS patients have focused on establishing an immunosuppressive environment or altering the type of T cell response in their CNS. IFN-β1b was one of the first treatments utilized against MS [7]. IFN-β functions as an immunosuppressive cytokine. Since the etiology of MS is still unknown it seemed prudent to suppress all immune functions to limit neuroinflammation. This belief has led to a number of off label uses of

immunosuppressive drugs; these include cyclophosphamide, azathioprine, methotrexate [8]. Immunosuppressives however, are known for their numerous side effects including inhibition of the routine clearance of common commensal bacteria and in severe cases, hepatotoxicity. Using Glatiramer acetate to alter the T cell response from a Th1 to a Th2 response has also shown limited success by extending the time between MS disease relapses [9]. Side effects and limited success has led the push to develop selective immune therapies against MS which largely target modes of immune activation [10]. These include monoclonal antibodies against B-cells and common immune synapse associated adhesion markers [8]. Cost and limited success remain an issue in wide spread acceptance of monoclonal antibody therapies.

One treatment that has been considered to re-establish a tolerogenic environment within the CNS utilizes the phenomenon of high dose antigen tolerance [11]. The treatment considered uses a peptide consisting of the immune T and B-cell immunodominant region found in HLA DR2 MS patients [6]. A very successful phase 2 clinical trial was conducted that showed a strong clinical benefit in treatment for MS, measured by the Expanded Disability Status Scale (EDSS) [6]. The peptide, located within MBP, consists of the amino acid sequence between 82-98. Previous clinical data assessed few biological factors. It was found that there was no correlation between EDSS scores and MBP specific antibody titres [6].

A link regarding the number of circulating regulatory T cells ($CD3^{+}CD4^{+}CD25^{hi}$) has been disputed among MS researchers for some time. In a

recent review on regulatory T regulatory in MS, they discuss that some investigators report a lower number of circulating Tregs while others do not [12]. It is generally understood that MS patient Tregs appear to have a defect in their ability to suppress an immunological response [13]. Studies performed on mice show the importance that regulatory T cells (Tregs) play in the resolution of an EAE inflammation episode [14]. Their numbers are also seen to be elevated in cancer patients [15] which are shown to be responsible for the tolerogenic environment that exists within tumors and prevents successful immune clearance of tumors.

We, therefore, hypothesized that upon high dose administration of MBP8298 there will be an increase in the number of circulating Tregs or a reversal in their unresponsiveness. Our results demonstrated an increase in the number of Tregs in patients' peripheral blood mononuclear cells 6 wks and 6-months post treatment with high dose of MBP peptide. We also unexpectedly recorded evidence of peripheral T-cell anergy and the ability of MBP8298 treatment to reverse that state in MS patients.

2. Methods

2.1. Patients

10 chronic progressive MS patients selected to receive MBP8298 based on compassionate care admission were selected for this study by Dr. Kenneth Warren (University of Alberta Hospital). Patients were given 500mg of MBP8298 intravenously every 6 months until the end of their prescribed study. Blood was taken before their first treatment with MBP8298. Blood was also drawn during a

6 week followup visit and 6 months after treatment prior to their next MBP8298 administration. The use of patients' blood samples was approved by the Human Research Ethics Board at the University of Alberta.

2.2. Cell culture

PBMCs from whole blood were isolated using Ficoll-paque (R&D Systems, USA). PBMCs were then plated $2x10^5$ cells/well in a standard 96 well tissue culture plate. Cells were treated in triplicate with 1ug/mL of phytohemagglutinin (PHA), poke weed mitogen (PWM) (Sigma, USA) in AIM-V media (Invitrogen, USA). They were incubated at 37^oC 5% CO_2 for 3-4 days before supernatant collection. Supernatant was collected by pooling from the wells that were given the same mitogen and samples were frozen at -70^oC until required. Cells were then immediately pulsed with 17μCi of ^3H-thymidine (Amersham Pharmacia, Canada) overnight and then harvested onto micro-cellulose membranes to determine thymidine uptake in a standard thymidine proliferation assay.

2.3. Cytokine ELISAs

IL-17A, TGF-β and IFN-γ were measured using standard ELISA kits (eBioscience, USA and Biosource, Canada). Culture supernatants were diluted 1/10 so that absorbance would fit on the standard curve produced by the standards run on each plate.

2.4. Flow Cytometry

PBMCs were stained with antibodies Q4120 (anti-CD4) labeled with FITC (Sigma, USA), M-A251 (anti-CD25) labeled with PE (BD Pharmingen, USA) and 236A/E7 (Foxp3) labeled with APC (ebioscience, USA) along with corresponding isotype controls. Cells were stained in 5mL flow cytometry tubes (BD Falcon, USA) where they were blocked before staining with 5% human AB serum in staining buffer. Staining was performed on ice for 30 minutes in the dark. Cells were then washed and fixed in 2% (w/v) paraformaldehyde (Sigma, USA) before permeabilization with 0.3% (w/v) saponin buffer (Sigma, USA). Intracellular staining for Foxp3 was carried out for another 30 minutes in the dark on ice. Cells were fixed and analyzed within 24hrs on a FACSCanto (BD, USA).

2.5. Healthy Controls

Healthy control blood was obtained from normal donors. Nine different donors were analyzed together to generate the healthy control group. $2x10^6$ PBMCs were plated in standard tissue culture treated 24 well plates in 1mL of AIM-V media. Culture supernatant was harvested on day 3 and cytokines were tested. To compare cytokine concentrations with the MS test groups, concentrations were divided by half to make comparable concentrations.

Three donors were tested for their, CD4 and CD25 expression using the same flow cytometry staining protocol used on MS patient samples.

2.6. Statistics

Patient data was pooled and tested using a standard one-way ANOVA followed by a tukey post hoc test to determine individual relationships between groups. SPSS (IBM, USA) statistical software was used to make the calculations.

3. Results

3.1. Increase in the percentage of $CD4^{+}CD25^{hi}$ cells in PBMCs of patients after treatment with MBP8298

Whole PBMCs obtained from patients before treatment, 6-wks and 6-months post-treatment were stained for CD4/25 expression (Figure 1). Overall the $CD25^{+}$ T cells population was increased in the $CD4^{+}$ T cells. These results suggest an overall activation of $CD4^{+}$ T cells after treatment with high dose of MBP8298. However, since CD25 cannot be used as a sole marker for Tregs, because activated T cells also express CD25, we used hi-CD25 expression as a marker for Tregs as has been suggested by Baecher-Allen et al. [16]. We compared the frequency and percentage of circulating $CD4^{+}CD25^{hi}$ cells before and after treatment. We found that the $CD25^{hi}$ population became more abundant in both time periods post treatment compared to before treatment with MBP8298 (Figure 1a). The increase post treatment was significant when data from all 10 patients enrolled in this study were pooled together (Figure 1b). Interestingly, the population of circulating $CD4^{+}CD25^{hi}$ cells in healthy patients did not differ from pre-treatment frequencies in MS patients. We also show the average expression levels of CD25/IL2RA from CD4+ cells rose after treatment, approaching the levels seen in healthy individuals (Figure 1c).

Human Tregs were first characterized as $CD4^{+}CD25hi^{+}$ T cells by several groups in 2001 [17, 18]. Transcription factor Forkhead box P3 (FoxP3) was first described as a master control gene for mouse Treg cell development and function [19]. Subsequently, FoxP3 was shown to be a marker for human Tregs as well [20]. However, in later studies, it was shown that both CD25 and FoxP3 expression could be induced in human naïve $CD4^{+}$ T cells through T cell activation blurring the identification of $FoxP3^{+}$ cells as pure Treg cells in humans. The expression of FoxP3 by both activated and regulatory $CD4^{+}$ T cells in humans could be one component of homeostatic programming initiated by these cells to exert negative feedback during the course of an immune response [21]. To examine whether the increased $CD25^{+}$ cells belong to the regulatory T cell subsets, we measured the level of FoxP3 expressed intracellularly. We found that there was a significant increase in the percentage of cells expressing FoxP3 6 months post treatment compared to pretreatment or 6-weeks post treatment groups (Figure 2). The delay in the upregulation of FoxP3 could be due to the time it took the body to wind down autoimmune responses and re-establish a regulatory environment.

3.2. Treatment with MBP8298 reverses T cell hyporesponsiveness

Circulating PBMCs were plated in the presence of poke weed mitogen (PWM) and phytohemagluttinin (PHA) to determine the underlying status of the adaptive immune system before and after high dose antigen treatment. The proliferation of these cells as well as the cytokines produced suggests that after treatment with

MBP8298 they become more responsive to mitogen stimulation. This was especially true in the cultures treated with PHA compared to PWM. Proliferation by PBMCs is statistically lower in pre-treatment cells compared to either of the post treatment time frames (Figure 3).

IFN-γ, which is a principle effector and regulating cytokine is not present in the cell cultures from cells obtained pre-treatment (Figure 4). However, 6-wks and 6-months post treatment, significant amounts of IFN-γ was found in the culture supernatant of PHA treated PBMCs. The return of IFN-γ after treatment with MBP8298 suggests that the treatment has reversed the hyporesponsiveness that was originally present.

IL-17A, which is a cytokine initially found to be important in autoimmune disorders and only now being studied for its role as an effector cytokine was found to be in lower levels pre-treatment as well (Figure 4). However, substantial amounts of IL-17A were found in the culture supernatants of cells stimulated with PHA in after treatment samples. This further supports our belief that high dose antigen treatment is modulating the T cell responsiveness.

TGF-β, a regulatory cytokine, was interesting in that, in the cells cultured with PHA in the pretreatment samples, relatively high levels of TGF-β was present (Figure 4), whereas the amounts were almost undetectable in cells obtained after treatment.

4. Discussion

It has been recently reported that the phase 3 clinical trial for the use of MBP8298 for use on progressive patients has failed to reach statistical endpoints in EDSS scores when it was compared to placebo. This result was not anticipated after the widely successful phase 2 trial performed earlier. The difference between the trials was an improving EDSS score from the placebo treatment group in phase 3 [22]. Such setbacks are not uncommon in the field of clinical research developing treatments for MS [23]. Despite the clinical setbacks, the biological data from such clinical trials are still crucial in helping us understand biological responses to the treatment administered.

It is becoming widely understood that many autoimmune diseases, such as MS, could have their etiology based on an underlying immune dysfunction. There have been reports that in MS there exists a lack of immune suppression by $CD25^+$ Treg cells and overall responsiveness by peripheral T-cells [13]. Our data clearly supports this observation by displaying a form of peripheral T-cell hyporesponsiveness in our MS patients. There is also new evidence to suggest that an IL2RA/CD25 polymorphism may exist that pre-disposes people to MS [24]. The polymorphisms found in the IL2RA/CD25 gene locus could be responsible for CD25 having less affinity for IL-2, but that remains to be tested.

Our data appears to support the link between IL2RA/CD25 and MS. The increase in CD25 expression after treatment with high dose self antigen would suggest that. This population also appears to be increased in the proportion of $FoxP3^+$ cells which would indicate an increased number of circulating regulatory T cells. Their appearance 6 months after initial treatment may suggest that an

active modulation of peripheral T cells is taking place in the treated patients. An increase in CD25 on Tregs could aid in binding up free IL-2 through cytokine deprivation of active T cells in the central nervous system and circulation [25]. The surge of CD25 could also help push the immune system back into homeostasis or if the lower affinity IL-2 polymorphism did exist then an increase in CD25 on the cell surface could potentially help combat that. It is also proposed that the increase in CD25 could lead to better activation of Tregs through environmentally available IL-2, stimulating their other suppressive activities [26].

The reversal of peripheral T-cell hyporesponsiveness after treatment with high dose antigen lends support to the initial idea that this condition could prevent the body from re-establishing homeostasis [27]. IFN-γ could be a principle cytokine that plays a role in re-establishing homeostasis. It has long been seen as an effector cytokine however, there is a new belief that it may also act as an immune regulator/suppressant in high concentrations [28]. This regulatory property of IFN-γ is already being shown to be required in the treatment of a systemic lupus in a mouse model [29]. If this mode of regulation was lost on the circulating PBMCs before treatment, this would explain any episode of uncontrolled inflammation. The response observed in the healthy patients would further suggest a role for IFN-γ to be a regulator against autoimmune disease. The return of this regulation could prove important in future studies regarding MS. This goes against the observations seen by Kinnman et al who found that the mitogenic reactivity of blood lymphocytes was not different between healthy and MS patients [30]. This could be because the groups they looked at did not involve

MS patients with advanced disease. Any link between the overall increase in CD25 on the cells and this response remains to be tested but there would appear to be a link as the IL-2 receptor increase on the cell surface may lead to the observed increase in IFN-γ that was measured. The induction of suppression is significantly influenced by cytokines. The cytokines IFN-γ and IL-17A have also been linked to many autoimmune disorders including MS [31]. It is believed that IL-17 producing T cells are naturally occurring and are somehow stimulated to recognize auto-antigens. New evidence has challenged the role of IL-17 as an effector cytokine for autoimmune disease. The mice with induced EAE treated with an anti-IL-17 monoclonal antibody had a reduction in symptoms but not a total abrogation of EAE lesions indicating that other cytokines must be present to signal for inflammation [32]. Alternate theory now suggests that IL-17 is a constituent of the normal course of inflammation and its expression occurs early on to strengthen an adaptive immune response compared to other more studied cytokines such as IFN-γ [33]. Our studies in contrast have shown that a reversal in peripheral hyporesponsiveness restored the ability of circulating PBMCs to produce IFN-γ and IL-17A. These cytokines may play an important part in disease progression at the site of infection but in the periphery they may play an equally important role in returning homeostasis to the MS patient. It is curious that with the increase in peripheral regulatory T cells that there is a decrease in TGF-β following PHA stimulation. The source of TGF-β would need to be confirmed through further observations. However, the presence of TGF-β after PHA stimulation from the pre-treatment group supports the idea that it is aiding in

maintaining peripheral T-cell hyporesponsiveness. Its disappearance after treatment would indicate that IFN-γ and IL-17A responding T cells have re-established homeostasis in the periphery, measured through their stimulation with PHA.

In this study we have shown that high dose treatment with a known B cell recognizing auto-epitope of myelin basic protein is capable of reversing the mitogenic hyporesponsiveness of PBMCs. This reversal is also characterized by an increase in the number of $CD4^{+}CD25^{hi}$ cells detected in the circulation of these patients. Whether these cells have the ability to suppress an immune response remains to be seen. This research is the first to show the reversal of immunological hyporesponsiveness in MS patients after a high dose antigen treatment is administered. Our results suggest that the regulation of T cells is an active process and require them to be active and producing certain regulatory/effector cytokines.

If using other high dose antigens can return IFN-γ regulation and increase the effectiveness of regulatory T cells then we have a potential new avenue to explore for treating autoimmune diseases with deregulated IFN-γ. Diseases that lack IFN-γ regulation still need to be defined because of the lack of focus afforded to the field by our past discrimination of IFN-γ, believing it only acted as a stimulating, effector cytokine.

A link regarding the number of circulating regulatory T cells ($CD3^{+}CD4^{+}CD25^{hi}$) has been disputed among MS researchers for some time. Many acknowledge that the circulating frequency of Tregs do not differ between

MS and healthy individuals, however, there does appear to be a defect in the ability of these cells to suppress an immunological response [13]. Our data would support the idea that the defect could be overall T-cell hyporesponsiveness. It is not disputed that Tregs can play a major role in re-establishing and maintaining tolerance in models of autoimmunity and cancer [14, 15]. We now believe that circulating T cells, including Tregs, in patients have simply become anergic and that high dose antigen treatment can reverse their anergy and therefore increase their CD25/IL2R expression and their function as regulatory T cells.

5. Acknowledgements

We would like to thank Jodie Peynado and Yuan Yuan for their technical assistance and for generating the healthy control data sets. Financial support for this work was provided by BioMS Medical Corporations.

6. Figures

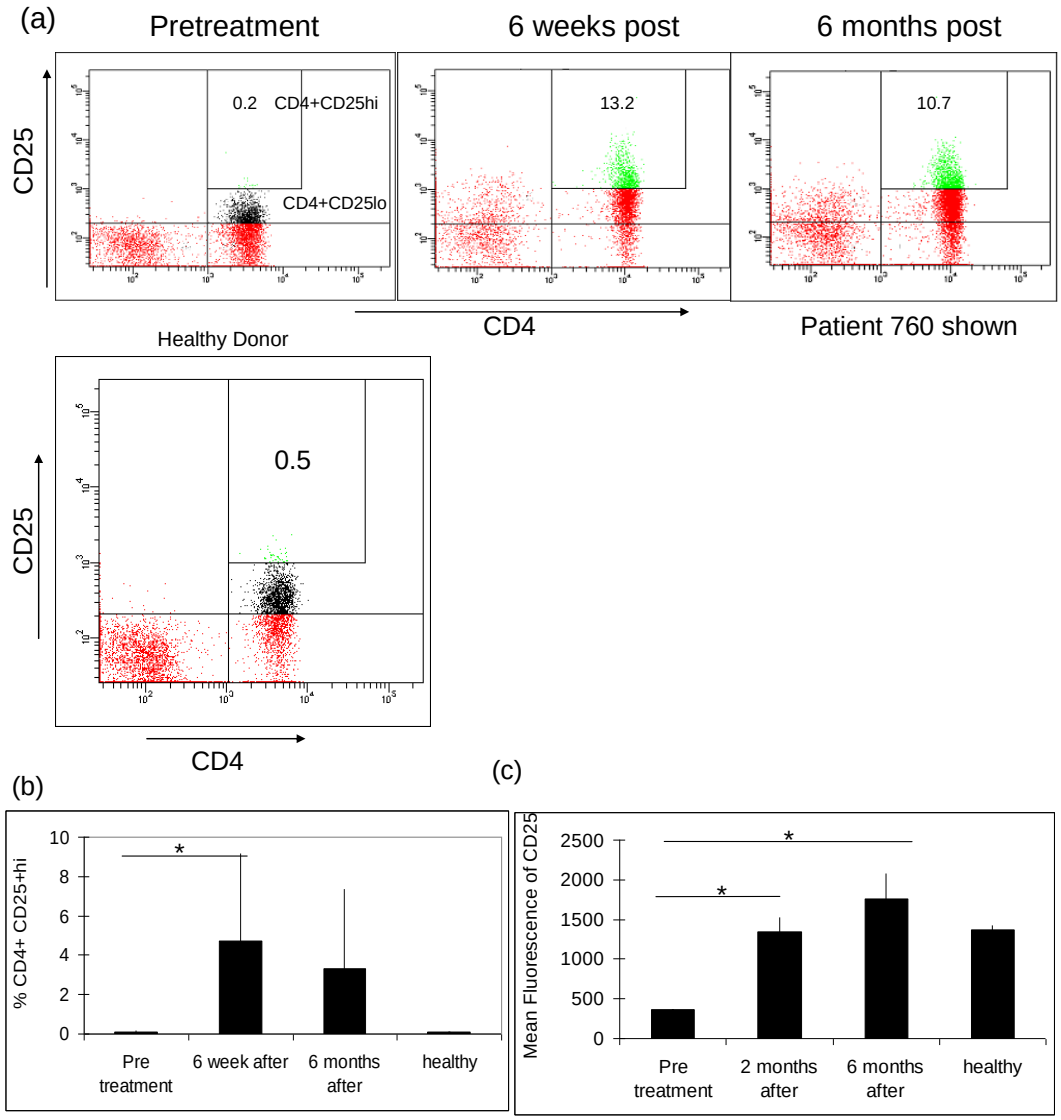


Figure 1) a) Flow cytometric data representative from one patient included in the study. Dot plots shown were taken from pre, 6 weeks post and 6 months post high dose MBP8298 treatment. All plots were originally gated on the FSClo SSClo lymphocyte gate. Cells were stained with anti-CD4 (Q4120) and anti-CD25 (M-A251) b) Taking the CD4+CD25hi cell population averages of all 10 patients included in the study the averages are compared between pre, post and 6 months post treatment. c) The mean fluorescence intensity of CD25 expression on the gated cells averaged from all 10 patients in the study. A significant increase is reported to be $p<0.05$.
Flow cytometric data taken from one healthy control patient gated from the lymphocyte gate. The gated population shows the proportion of CD4+CD25hi

cells measured with antibodies Q4120 and M-A251 respectively. The mean fluorescence of CD25 and the proportion of Foxp3 from this one patient is shown.

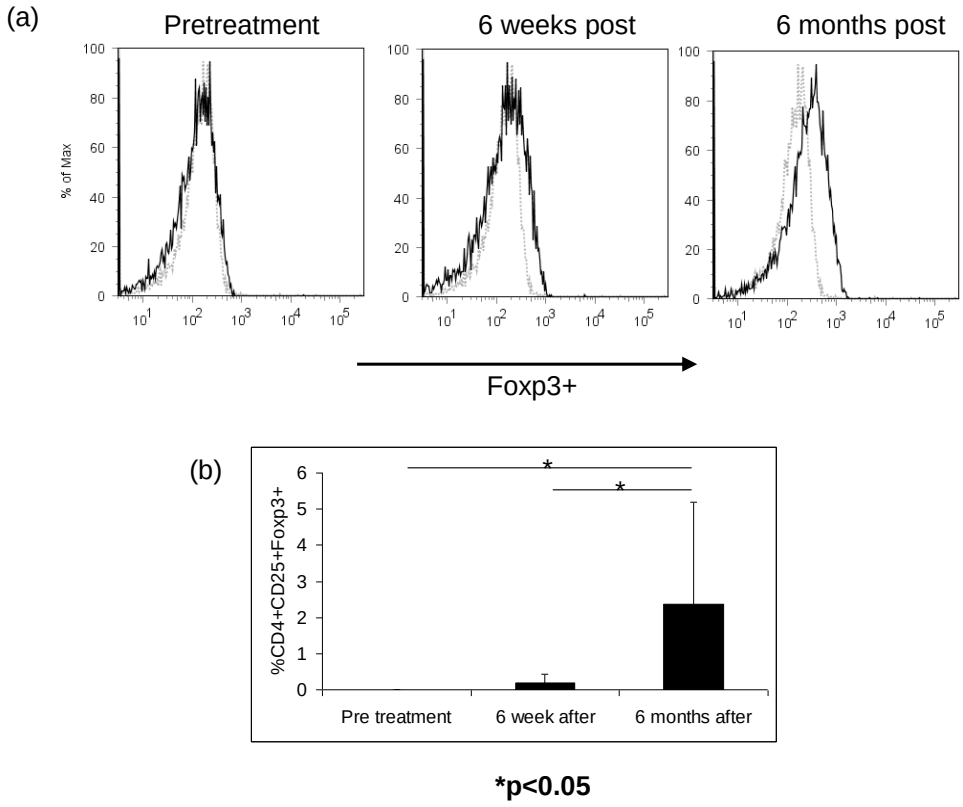
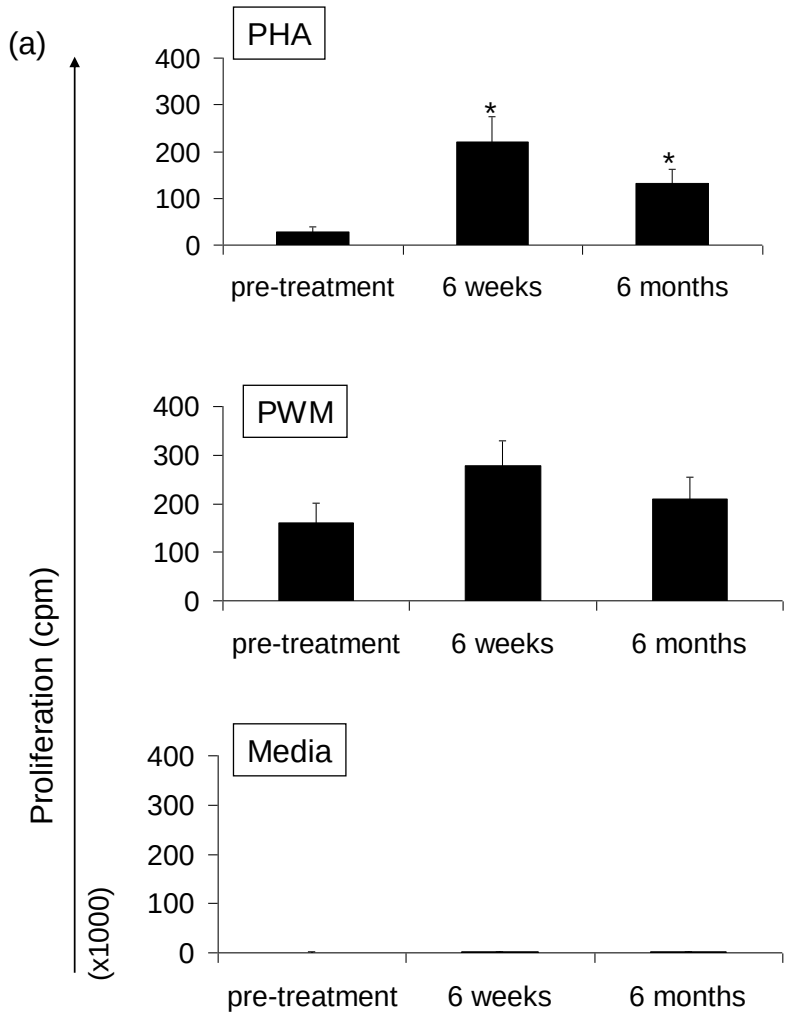


Figure 2) a) Flow cytometric data from one patient is displayed showing the level of intracellular Foxp3 measured by antibody 236A/E7 expression from all CD4+CD25+ cells. Data plots are shown for pre, post and 6 months post treatment with MBP8298. Dotted grey line is the isotype control while the solid black line indicates Foxp3 expression level. b) Grouping all 10 patient proportions of Foxp3 expressing cell populations together. Statistical significance is indicated to be $p<0.05$.


(a)
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*
*
pre-treatment
6 weeks
6 months
PWM
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300
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pre-treatment
6 weeks
6 months
Media
400
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200
100
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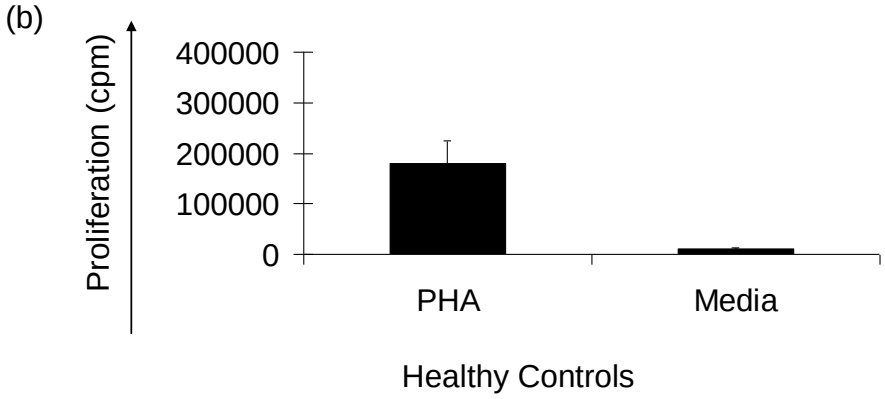
pre-treatment
6 weeks
6 months
Proliferation (cpm)
(x1000)

***p<0.05**


(b)
Proliferation (cpm)
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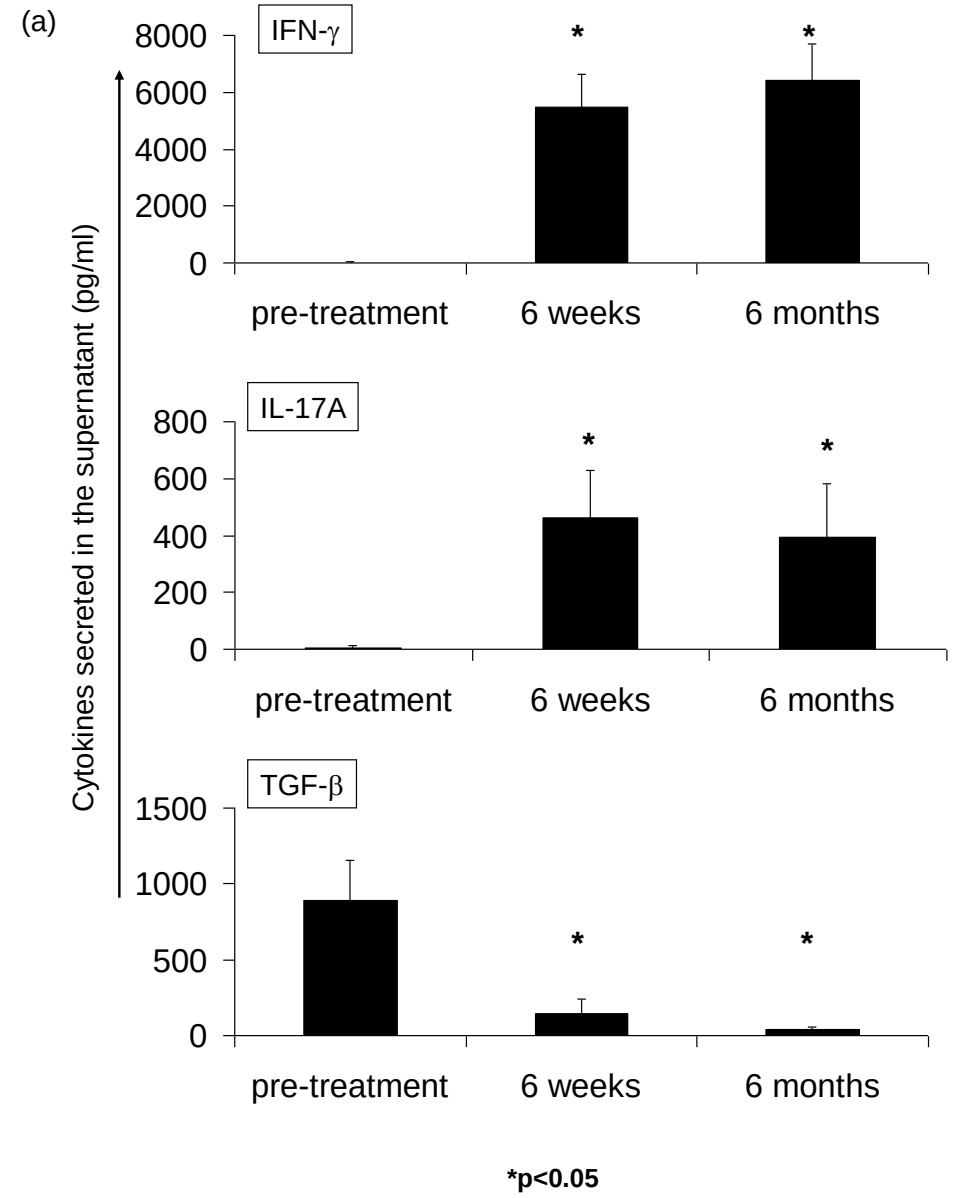
PHA
Media
Healthy Controls

Figure 3) a) The proliferation induced by PHA, PWM and media as measured by an average of all 10 patients in the study combined separated by pre, 6 weeks post and 6 months post high dose MBP8298 treatment. Proliferation was measured through beta counting of 3H-thymidine labelled cells. Significance is measured as $p<0.05$. b) The cumulative healthy control data from 9 pooled donors showing proliferation of PBMCs in the presence of PHA. Proliferation was measured through 3H-thymidine uptake and subsequent beta counting.


(a)
IFN-γ
8000
6000
4000
2000
0
*
*
pre-treatment
6 weeks
6 months
IL-17A
800
600
400
200
0
*
*
pre-treatment
6 weeks
6 months
TGF-β
1500
1000
500
0
*
*
pre-treatment
6 weeks
6 months
Cytokines secreted in the supernatant (pg/ml)
*p<0.05

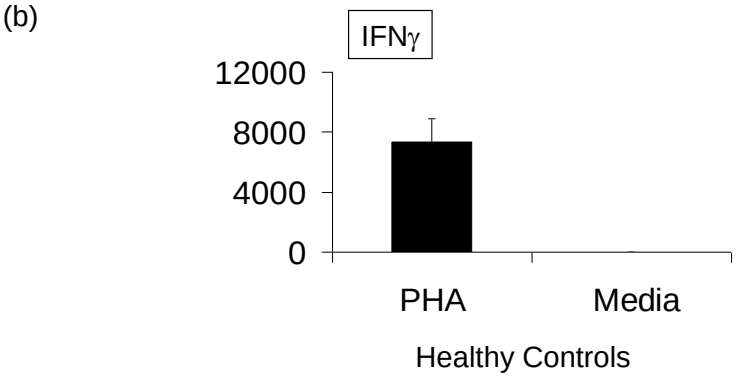

(b)
IFNγ
12000
8000
4000
0
PHA
Media
Healthy Controls

Figure 4) a) ELISA measured IFN-γ (Biosource), TGF-β and IL-17A (eBioscience) released by PHA stimulated PBMCs from patients pre, post and 6 months post treatment with MBP8298. Data has been averaged together and significance is defined as $p<0.05$. b) The cumulative healthy control data from 9 pooled donors showing IFNγ expression of PBMCs in the presence of PHA or media alone. IFNγ was measured through ELISA.

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