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- Webb, T.J., Li, J., Sun, W., **Subrahmanyam, P.B.**, Page, C., Frieman, M., Chen, Q. and Kimball, A.S. (2013) *Therapeutic potential of NKT cell adjuvant-based therapies for the treatment of B cell lymphoma*. International Congress of Immunology (ICI), Milan, Italy
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2. Li, J., Sun, W., **Subrahmanyam, P.B.**, Page, C., Younger, K., Tiper, I.V., Frieman, M., Kimball, A.S. and Webb, T.J. (2014) NKT cell responses to B cell lymphoma. *Medical Sciences*, 2(2): 82-97
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Abstract

Title of Dissertation: The Role of Natural Killer T cells in B Cell Lymphoma

Priyanka Balasubrahmanyam, Doctor of Philosophy, 2014

Dissertation Directed by: Tonya Webb, Asst. Professor, Department of Microbiology and Immunology

Natural killer T (NKT) cells are a unique subset of T cells that recognize glycolipid antigens in the context of CD1d, a non-classical MHC class I-like molecule. NKT cells mount strong anti-tumor responses and are a major focus in developing effective cancer immunotherapy. However, little is known about the regulation of CD1d-mediated antigen presentation to NKT cells, particularly in the context of B cell lymphoma. Pro-survival factors of the Bcl-2 family, such as Bcl-xL are often upregulated in B cell lymphomas, and are associated with changes in the endocytic pathway, which is paramount for CD1d-mediated antigen presentation. We hypothesized that Bcl-xL can regulate this process, and found that over-expression or induction of Bcl-xL led to increased CD1d-mediated antigen presentation to NKT cells. Conversely, pharmacological inhibition or shRNA-mediated knockdown of Bcl-xL led to decreased antigen presentation. Surface CD1d expression was unchanged by the modulation of Bcl-xL, but its knockdown resulted in reduced CD1d trafficking to LAMP1⁺ compartments. Furthermore, Rab7, a late endosomal marker was upregulated following Bcl-xL knockdown, and CD1d molecules accumulated in the late endosomes. These results demonstrate that Bcl-xL modulates CD1d-mediated antigen presentation to NKT cells by altering the intracellular trafficking of CD1d. Thus, we have identified a potential tumor recognition mechanism that can

impact current therapies targeting the Bcl-2 family, as well as emerging NKT cell based cancer immunotherapeutic strategies. We further studied the role of NKT cells in mantle cell lymphoma, a particularly aggressive form of non-Hodgkin's lymphoma, *in vivo*, using an IL-14 α and c-Myc double-transgenic mouse model. We found that treatment with a single dose of the NKT cell agonist α -Galactosylceramide, increased survival and caused amelioration of disease. *Ex vivo* restimulation of splenocytes with α -GalCer showed increased IFN- γ responses, providing some insight into the mechanism underlying the enhanced anti-tumor response following α -GalCer administration. These studies indicate that NKT cells play an important role in mediating an effective immune response to lymphoma, warranting further investigation of the CD1d/NKT system. This small but powerful lymphocyte population bears high potential for translation into the next generation of cancer therapy.

The Role of Natural Killer T Cells in B Cell Lymphoma

by
Priyanka Balasubrahmanyam

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, Baltimore in partial fulfillment
of the requirements for the degree of
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List of Abbreviations

α -GalCer- α -Galactosylceramide
ANOVA- analysis of variance
AP- adaptor protein
APAF-1- apoptosis protease-activating factor 1
APC- antigen presenting cell
Bcl-2- B cell CLL/lymphoma 2
BH- Bcl-2 homology
BM- bone marrow
BV- blastoid variant
Ca⁺⁺- Calcium ions
CD- cluster of differentiation
CDK- cyclin-dependent kinase
CLL- chronic lymphocytic leukemia
CTL- cytotoxic T lymphocyte
DC- dendritic cell
DTG- IL-14 α and c-Myc double transgenic
EEA- early endosome antigen
ELISA- enzyme-linked immunosorbent assay
ER- endoplasmic reticulum
ERGIC1- ER golgi intermediate compartment 1
FBS- fetal bovine serum

FoxP3- forkhead box P3

GPI- glycosylphosphatidylinositol

H&E- hematoxylin and eosin

HBsAg- HBV surface antigen

HBV- hepatitis B virus

HDAC- histone deacetylase

HIV- human immunodeficiency virus

HSV- herpes simplex virus

IACUC- institutional animal care and use committee

IBC- institutional biosafety committee

IFN- interferon

Ig- immunoglobulin

iGb3- isoglobotrihexosylceramide

IL- interleukin

ILC- innate lymphoid cell

IP3R- inositol 1,4,5-trisphosphate receptor

LAMP- lysosome-associated membrane protein

LC3- microtubule-associated protein light chain 3

LN- lymph node

MAIT- mucosal-associated invariant T cell

MAPK- mitogen-activated protein kinase

MCL- mantle cell lymphoma

MCMV- mouse cytomegalovirus

MDSC- myeloid-derived suppressor cell

MHC- major histocompatibility complex

MIIC- MHC class II compartment

mTOR- mammalian target of Rapamycin

MTTP- microsomal triglyceride transfer protein

MZB- marginal zone B cell

NHL- non-Hodgkin's lymphoma

NIH- national institutes of health

NK- natural killer

NKT-natural killer T

NLR- NOD-like receptor

OVA- ovalbumin

PAMP- pathogen-associated molecular pattern

PBS- phosphate-buffered saline

PCR- polymerase chain reaction

PI3K- phosphoinositide-3-kinase

PKC- protein kinase C

PLZF- promyelocytic leukemia zinc finger

PM- plasma membrane

PRR- pattern recognition receptor

PTEN- phosphatase and tensin homolog

q-PCR- quantitative PCR

RLR- Rig-I-like receptor

SAP- SLAM-associated protein

SEER- survey epidemiology and end results program

sh- short hairpin

SLAM- signaling lymphocytic activation molecule

SLE- systemic lupus erythematosus

TCR- T cell receptor

Th- T helper

TLR- toll-like receptor

TNF- tumor necrosis factor

TRAIL- TNF-related apoptosis-inducing ligand

Treg- regulatory T cell

VAMP- vesicle-associated membrane protein

VEGF- vascular endothelial growth factor

VV- vaccinia virus

WEHI- Walter and Eliza Hall Institute of Medical Research

Chapter 1: Introduction

1.1 Cancer: a leading cause of death worldwide

The Survey Epidemiology and End Results (SEER) Program (www.seer.cancer.gov) estimates that in 2014, there will be 1,665,540 new cases of cancer and an estimated 585,720 people will succumb to this disease in the United States alone (1) (Table 1.1). Although, the incidence of cancer has been falling at an average 0.7% per year, it is estimated that about 40.8% of all men and women will be diagnosed with some kind of cancer in their lifetime. On average, the number of deaths has dropped by 1.5% per year, and currently 176.4 deaths are estimated per 100,000 men and women. Cancers of different types have varying incidence rates and survival. Breast cancer, melanoma, cancer of the lung and bronchus and prostate cancer are among the most common types of cancer. Cancers arising from hematopoietic cells and lymphoid cells, known as hematologic malignancies, are also high in incidence. Some subtypes like Hodgkin's lymphoma are reducing in incidence with an estimated 9,190 cases in 2014, out of which 1,180 deaths are estimated (Table 1.1). However, the incidence of other hematologic malignancies like leukemia and myeloma is higher, at 52,380 and 24,050 estimated cases respectively (Table 1.1). Furthermore, non-Hodgkin lymphoma, which is also a type of hematologic malignancy has a very high incidence rate with 70,800 estimated new cases and 18,990 estimated deaths in 2014 (Table 1.1). This group of hematologic malignancies will be discussed further in Section 1.2.

Table 1.1: Estimated number of cases and deaths in 2014 for various types of cancer
 Data obtained from the SEER Program (www.seer.cancer.gov) (1).

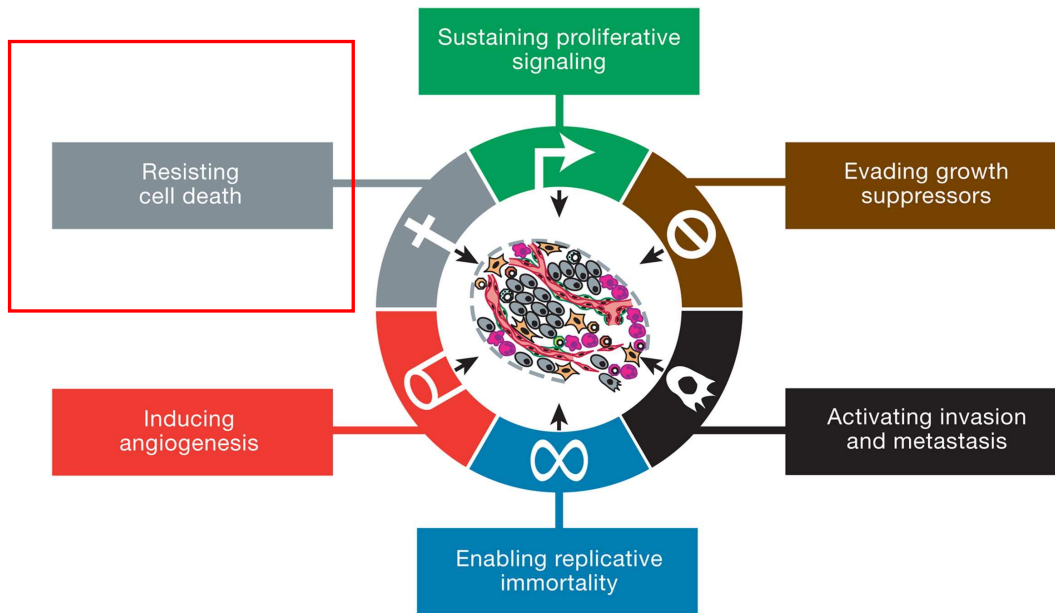
Type of cancer	No. of Cases estimated in 2014	Deaths estimated in 2014
All sites	1,665,540	585,720
Breast cancer	232,670	40,000
Cancer of the colon and rectum	136,830	50,310
Cancer of the lung and bronchus	224,210	159,260
Prostate cancer	233,000	29,480
Melanoma	76,100	9,710
Hematologic malignancies		
Non-Hodgkin lymphoma	70,800	18,990
Leukemia	52,380	24,090
Myeloma	24,050	11,090
Hodgkin lymphoma	9,190	1,180

1.2 Lymphomas

Lymphoma is a hematologic malignancy of the immune system characterized by the accumulation of abnormally high numbers of lymphocytes. About 95% of all lymphomas arise from B cells and are characterized by splenomegaly and lymphadenopathy (2). Lymphomas are divided into two general groups: Hodgkin's disease and non-Hodgkin's lymphoma (NHL). Hodgkin's disease is characterized by the presence of histologically identifiable Hodgkin and Reed-Sternberg cells (3). These Hodgkin and Reed-Sternberg cells are large, multinucleated cells with peculiar morphology, and their presence facilitates the diagnosis of this type of lymphoma (4). Hodgkin's disease is relatively easier to treat and has a favorable outcome with standard radiation and chemotherapy. However, NHL is known to be much harder to treat with poor response to standard therapy and a high relapse rate. Unfortunately, the incidence of NHL has been steadily rising at an average 0.5% from 2002-2011 (1). In fact, NHL is reported to be the 6th and 5th most commonly diagnosed cancer in males and females respectively, in the United States (5). NHL is a heterogeneous group of lymphomas consisting of numerous subtypes that are quite distinct from each other. Diseases like follicular lymphoma, diffuse large B cell lymphoma, Burkitt lymphoma and mantle cell lymphoma are all types of NHL. A major problem in this field is the rise in incidence of more aggressive variants such as blastoid variant mantle cell lymphoma and their occurrence in younger patients (6). Considering the poor outcome of patients with these diseases, and the rising incidence rate, there is an urgent need to develop new therapeutic strategies to help these patients.

1.3 The hallmarks of cancer

The transformation of a cell is not a rare event, yet few of these progress and cause cancer. Under normal circumstances, tumor cells must employ multiple strategies to overcome anti-tumor mechanisms to establish a cancerous mass (Fig. 1.1) (7). For example, one such hallmark of cancer cells is the acquisition of sustained proliferative signaling. This is often achieved by altered signaling pathways, like the B-Raf mutation, which leads to constitutive activation of the MAPK pathway and provides persistent proliferative signals (8). On the other hand, the disruption of negative feedback loops can also lead to sustained proliferation. An important example is the loss of phosphatase and tensin homolog (PTEN). PTEN is a phosphatase that inactivates the phosphoinositide-3-kinase (PI3K) pathway and is often mutated or epigenetically silenced, to inactivate the negative feedback loop and facilitate uncontrolled activation of the PI3K pathway (9, 10). Tumor cells also acquire changes that help them evade growth suppressors like negative cell cycle regulators (e.g. p53), so that these malignant cells can maintain abnormal cell growth and division rates. Some other mechanisms employed by tumor cells are the induction of angiogenesis by deregulated vascular endothelial growth factor (VEGF) signaling, achieving replicative immortality by abnormalities in telomerase activity and evasion of apoptosis by deregulation of the B cell CLL/lymphoma 2 (Bcl-2) family (11, 12). This Bcl-2 family of proteins will be the focus of Section 1.4.



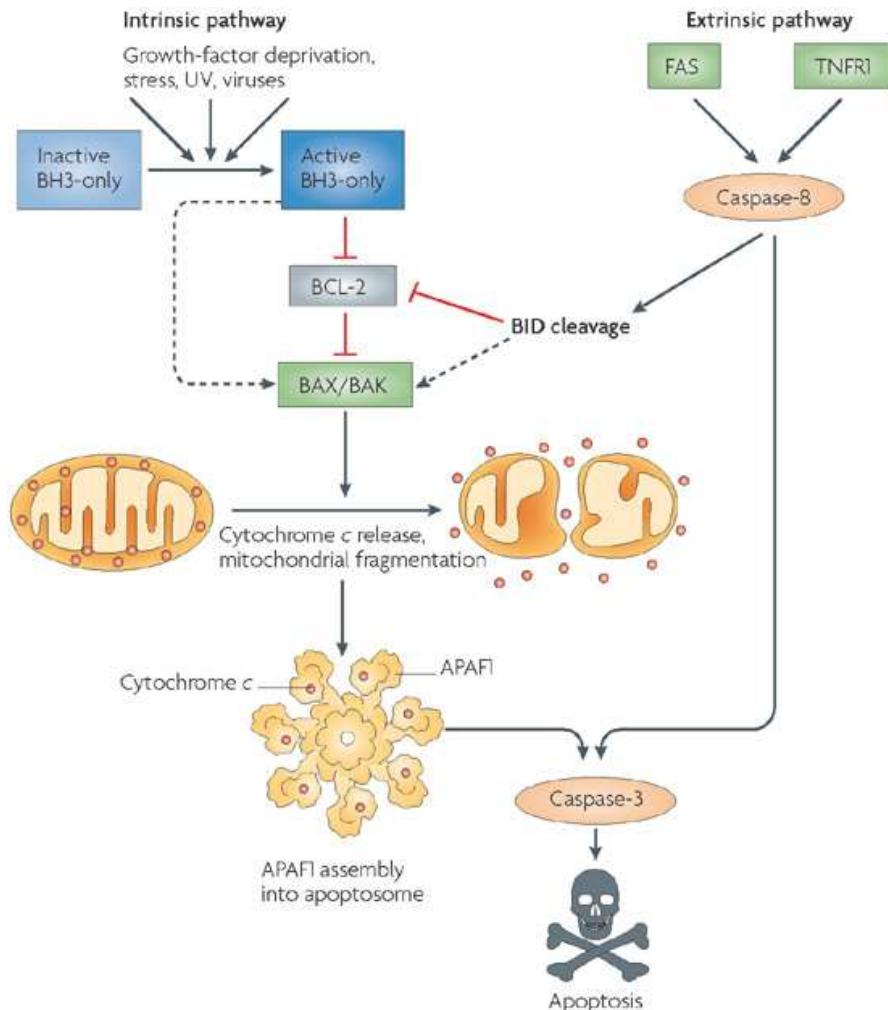
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 Hanahan and Weinberg *Cell* 144 (5), 646-674 (March 2011)

Figure 1.1: The hallmarks of cancer

Malignant cells use multiple mechanisms to grow, survive, proliferate and generate a tumor mass. In general, cells possess mechanisms to limit the growth of neoplastically transformed cells. The classic hallmarks of cancer are six different mechanisms described by Hanahan and Weinberg, by which cells evade these anti-tumor responses and successfully cause disease.

1.4 The Bcl-2-family of proteins

Tumor cells often deregulate apoptotic pathways to evade cell death and enhance their survival. Apoptosis or programmed cell death is a process by which cells receive internal or external signals and initiate signaling pathways that culminate in 'suicide' or cell death. Apoptosis can be initiated by the extrinsic or intrinsic pathway (Fig. 1.2). The extrinsic pathway is initiated by external signals such as Fas ligand or TRAIL that bind receptors on the cell surface (13). The downstream signaling that follows the activation of these receptors, results in the activation of caspase-8 and a subsequent caspase cascade. The intrinsic or mitochondrial pathway of apoptosis is regulated by the Bcl-2 family of proteins (14). Pro-apoptotic members of the Bcl-2 family, Bak and Bax are present on the mitochondrial outer membrane. Upon binding of apoptosis activator proteins like Bid and Bim, these proteins homo-oligomerize and promote the permeabilization of the mitochondrial outer membrane. This allows the escape of cytochrome c from the mitochondria and the recruitment of a scaffolding protein known as apoptosis protease-activating factor 1 (APAF-1) (15). APAF-1 further results in the activation of caspase-9 and eventually causes cell death. The extrinsic and intrinsic pathways are not entirely exclusive, and crosstalk is promoted by Bid which is cleaved by caspase-8 and goes on to activate the intrinsic pathway and amplify the caspase cascade (Fig. 1.2) (16). The Bcl-2 family consists of both pro-apoptotic as well as anti-apoptotic proteins. The founding member Bcl-2, was originally identified from the t(14;18) translocation seen in follicular lymphoma patients, and contains conserved Bcl-2 homology (BH) domains (17, 18).



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Figure 1.2: The extrinsic and intrinsic pathways of apoptosis

Apoptosis or programmed cell death can occur by the extrinsic pathway, which involves activation of Fas or other death receptors. Once activated, downstream signaling pathways lead to the activation of caspase-8 and initiation of the caspase cascade. Intrinsic cell death is regulated by the Bcl-2 family members and involves the release of cytochrome c from the mitochondria. This leads to the activation of caspase-9 and initiates the caspase cascade, resulting in cell death. The extrinsic pathway can also feed into the intrinsic pathway by the cleavage and activation of Bid to amplify the apoptotic response.

Bcl-2 over-expression leads to the development of lymphoma in mice, and also accelerates lymphomas driven by other oncogenes like *myc* (19, 20). Furthermore, the forced expression of Bcl-2 in B cells under the E μ promoter leads to autoimmunity (21). These mice develop autoantibodies to nuclear antigens and glomerulonephritis, similar to human systemic lupus erythematosus (SLE). On the other hand, Bcl-2 knockout mice get fatal polycystic kidney disease due to apoptosis in renal epithelial progenitors. Mature lymphocytes and melanocyte progenitor cells are also lost in these mice (22).

Bcl-x, another member of the Bcl-2 family first described in 1993, was found to be a negative regulator of apoptosis, and its predicted structure was similar to Bcl-2. Bcl-x has two splice variants- Bcl-xL and Bcl-xS. Bcl-xL was found to function similar to Bcl-2 and increase survival, while Bcl-xS countered the ability of Bcl-2 to provide protection from cell death (23). Bcl-xL knockout mice are embryonic lethal at day 13 due to neuronal defects, underscoring its important role in development (24). These Bcl-xL knockout mice show extensive neuronal cell death in the brain and spinal cord. In fact, by embryonic day 12.5 there was extensive neuronal degeneration throughout the maturing brain. The absence of Bcl-xL also impairs hematopoiesis and causes death of immature lymphocytes. Since Bcl-xL knockout mice do not survive long enough to study the maturation of lymphocytes, Bcl-xL knockout embryonic stem cells were injected into RAG2 knockout blastocysts to obtain chimeric mice (24). These studies revealed extensive TUNEL staining in immature lymphocytes. However, the presence of mature T and B cells in the lymph nodes and spleen indicates that the lack of Bcl-xL does not

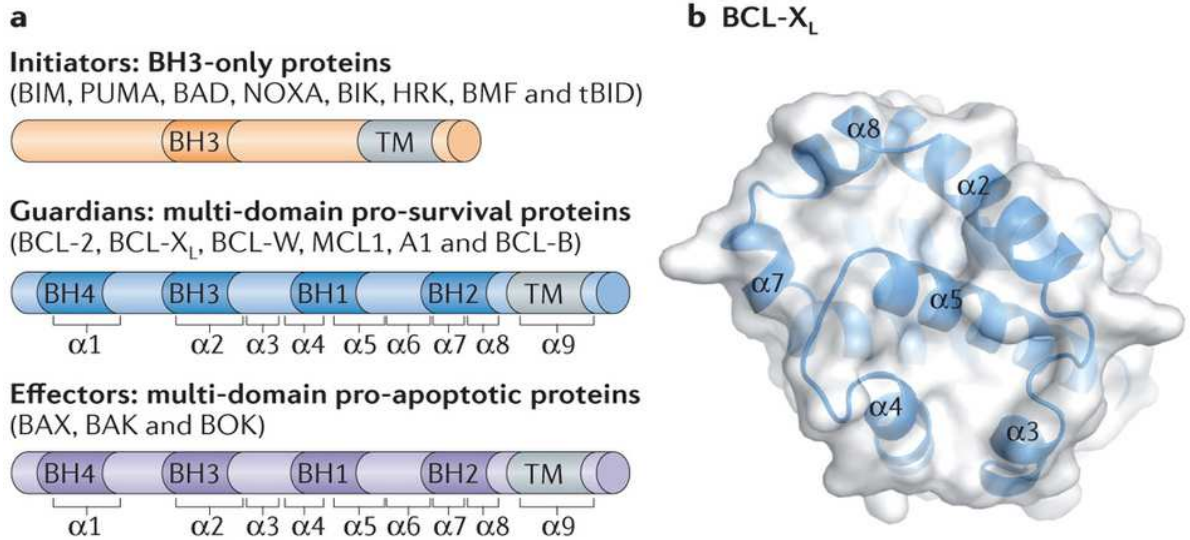
affect lymphocyte maturation, although the numbers of mature cells is reduced. The knockout of Bcl-xL on a single allele leads to reduced spermatogenesis resulting in reduced fertility in males (25). The defects in the development of hematopoietic cells and male germ cells can be rescued by deleting Bim. Another anti-apoptotic Bcl-2 family protein, Mcl-1 also plays a role in development. In fact, the constitutive knockout of Mcl-1 prevents implantation *in utero* (26). Conditional knockout of Mcl-1 has revealed a role for this protein in the survival of a diverse set of cell types including lymphocytes and hematopoietic stem cells (27, 28).

The Bcl-2 Homology (BH)1-4 containing pro and anti-apoptotic proteins of the Bcl-2 family have many structural similarities. They adopt a globular structure, consisting of an amphipathic helical bundle surrounding a central hydrophobic core helix (Fig. 1.3) (29). The BH1, BH2 and BH3 domains form a hydrophobic groove which facilitates binding to other Bcl-2 family members. In fact, the most accepted model for the binding of Bak and Bax is a BH-3-in-groove model (30). According to this model, the BH3 domain of one Bak or Bax molecule inserts into the hydrophobic groove of another, leading to the formation of a symmetric dimer. The dimers are then thought to join by a second interface for the formation of oligomers, although the precise mechanism of this interaction remains to be elucidated. Bcl-xL and Bcl-2 proteins also have a transmembrane domain near their C-terminus. This transmembrane region is required for localization to the mitochondrial outer membrane, as C-terminal transmembrane region deletion mutants of Bcl-xL show diffuse expression in the cell, and there is altered

mitochondrial morphology (31). Interestingly, the anti-apoptotic function of Bcl-xL is retained in this deletion mutant. Bcl-2 and Bcl-xL also have a flexible 'loop' region formed by the BH3 and BH4 domains. Deletion of this loop region does not decrease anti-apoptotic function and may even enhance it (32, 33). Finally, the function of the N-terminal region of Bcl-xL has also been studied and the deletion of the last 76 amino acids near the N-terminus not only leads to the loss of its anti-apoptotic function, but also makes it pro-apoptotic (34, 35).

As mentioned above, the Bcl-2 family pro-survival members play a major role in malignancies. Consequently, there is great interest in the development of drugs to target these proteins. These efforts have resulted in the development of small molecule BH3 mimetics like ABT-263, ABT-199 and WEHI-539 which bind to Bcl-2/Bcl-xL and inhibit their function (36-38). ABT-263 targets both Bcl-xL and Bcl-2 and inhibits their function, leading to the induction of apoptosis in tumor cells. It is currently in early clinical trials for the treatment of CLL (39). However, a side-effect of this drug is thrombocytopenia due to Bcl-xL inhibition (40). ABT-199 is more selective for Bcl-2 and spares platelets, making it more favorable as a chemotherapeutic agent. Recently, Lessene *et al.* reported the design of WEHI-539 which selectively targets Bcl-xL and can be an important tool to study the differences in the roles of these closely related Bcl-2 family members (38).

Many apoptosis-independent roles of Bcl-2 family members are also emerging. The Bcl-2 family members are now known to be involved in mitochondrial dynamics, especially in the processes of mitochondrial fission and fusion (41). They are also capable of boosting mitochondrial energetics, as Bcl-xL influences mitochondrial membrane potential (42). Furthermore, Bcl-xL can also affect mitochondrial bioenergetics by interacting with and sensitizing the inositol 1,4,5-trisphosphate receptor (IP₃R) which plays an important role in the release of Ca⁺⁺ from ER stores (43). The Bcl-2 protein family is also important in the process of autophagy, or engulfment of cellular organelles and contents (discussed further in Chapter 5). Bcl-2 binds Beclin-1, an important player in the autophagic pathway and antagonizes its function (44). Furthermore, this interaction occurs in the ER, indicating that Bcl-2 family members have functions independent of the mitochondria and can play important roles at other sites within the cell.



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Figure 1.3: Structure of Bcl-2 family proteins

(A) The domain structure of the Bcl-2 family members shows BH1-4 domains. Proapoptotic members like Bak and Bax and anti-apoptotic members like Bcl-2 and Bcl-xL have BH1-4 domains and a transmembrane region (TM). BH3 only proteins like Bid and Bim are important for modulating the function of pro-apoptotic and anti-apoptotic members by binding to them through the BH3 domain. (B) The structure of Bcl-xL is shown as a representative of the anti-apoptotic Bcl-2 family proteins. These proteins generally adopt a globular structure with a bundle of amphipathic α -helices surrounding a central hydrophobic core helix.

1.5 The immune system and its ability to eliminate cancers

Another major hallmark of cancerous cells is their ability to avoid immune destruction. This is essential for the tumor because the immune system possesses the ability to eliminate malignancies. This is a multi-layered process and involves early recognition events by mediators of innate immunity, followed by the development of a strong and highly specific adaptive immune response. This means that following neoplastic transformation, cells can be recognized by components of the immune system, which not only mount strong cytotoxic responses, but also release cytokines and other soluble factors that orchestrate the development of a robust and multi-pronged anti-tumor immune response. This role of the immune system in detecting and destroying malignant cells is termed immunosurveillance (45). It allows the components of the immune system to recognize, target and specifically eliminate malignant cells, permitting the host to survive tumor-free. However, tumor cells have developed mechanisms to evade immunosurveillance and cause cancer. This occurs in three phases: the first phase is elimination, where most transformed cells will be recognized and eliminated (46). A transformed cell that escapes these mechanisms has the potential to develop into a tumor. The next stage is equilibrium, which occurs if elimination is not successfully attained. During this phase, tumor cells undergo mutations and other changes that support survival under the selection pressure imposed by the immune system. This process is called immunoediting and results in the development of evasion mechanisms by the tumor cells. The last and final step is escape, where the tumor cells are able to successfully evade the immune system, and continue to grow and proliferate in an uncontrolled fashion. At this

stage the tumor is established, the anti-tumor immune response is either insufficient or ineffective, and a previous healthy individual develops cancer.

Another proposed model to understand the immune response to cancer is the ‘danger’ model. This model, in contrast to the immunosurveillance model proposes that the default response of the immune system to cancer is tolerogenic (47). The rationale behind this is that the immune system constantly encounters harmless infections, dying cells and non-pathogenic microbes. Therefore, cells of the immune system are activated only in the presence of true ‘danger’ signals. Such signals allow the immune system to identify a potentially harmful state of infection or neoplastic transformation and present antigens in the context of secondary costimulatory signals by professional APCs. This would allow the cells to be activated and mount effective responses, but would also ensure that the recognition of harmless antigens does not cause immunopathology. It follows from this that generating an effective anti-tumor response would require overcoming this default ‘off’ signal of the immune system to mount a response against malignant cells (48). It also implies that the mere recognition of tumor antigens is not sufficient to trigger an immune response, and must be accompanied by ‘danger’ signals that would preferentially lead to activation over tolerance. Thus, there is an urgent need to use a multi-pronged approach to make tumors more visible to immune cells and also to do this in an activating and non-tolerogenic environment, to generate an effective anti-tumor immune response.

1.6 Innate and adaptive immunity

Components of the immune system that are involved in early recognition that trigger rapid and only partially specific responses to detected threats are considered parts of innate immunity (49). Physical barriers like epithelial surfaces, cilia and mucus, chemical barriers like low pH in the stomach and biological mechanisms like anti-microbial peptides form the frontline of host defense. Pattern recognition receptors (PRR) like the toll-like receptors (TLR), NOD-like receptors (NLR), RNA-sensing Rig-I-like receptors (RLR) and DNA sensors also constitute an important part of the innate immune response. These components are involved in the recognition of pathogen-associated molecular patterns (PAMP) and initiate early type I interferon responses and mediate transcriptional regulation of important genes. The innate immune system also has cellular components such as granulocytes- neutrophils, eosinophils, basophils and mast cells, and phagocytes like macrophages and dendritic cells (DC). The PRRs and innate cells are not only important in the mounting of early immune responses, but also play a pivotal role in shaping the adaptive immune response. For example, the activation of TLR4 by lipopolysaccharide on DCs results in their maturation, making them more efficient antigen presenting cells for the activation of T cells (50).

The adaptive arm of the immune system is characterized by a vast diversity of antigen receptors, high specificity of the response and rapid recall responses that are often more potent and effective than the initial response (51). Classically, B cells and T cells are major players in adaptive immunity. After initial recognition of a new antigen, there is

rapid clonal expansion of these lymphocytes, and a highly specific response is generated. Typically, B cells enter germinal center reactions and develop into high-affinity antibody producing plasma cells. T cells, on the other hand differentiate into different subsets and can mediate direct cell killing, cytokine production and also provide help for B cells to produce high-affinity antibodies (51). The development of an effective adaptive response takes longer than innate immune responses, but has the major advantage of immunological memory generation. If the same antigen is encountered again, there will be rapid recall responses, which can quickly and more effectively battle recurring threats.

1.7 Innate-like lymphocytes

Innate-like immunity is a relatively recently coined term used to describe components of the immune system that bear features of both innate and adaptive immunity (Fig. 1.4) (52). Innate like B cells such as marginal zone B (MZB) cells recognize recurring patterns on pathogens and rapidly produce natural IgM antibodies (53). B1 cells, also a part of the innate-like lymphocyte group, bear receptors that have low affinity for a diverse set of antigens and bacterial polysaccharides. Such innate-like responses lack the high specificity afforded by the clonal selection of specific antigen receptors from a large diverse pool, but nevertheless are often sufficient to protect the host from invading pathogens. NK cells rapidly respond to pathogens, especially viruses and mediate cytotoxicity in infected cells and boost interferon responses (54). Another emerging type of innate-like lymphocytes is the innate lymphoid cell (ILC) group (55). These ILCs do not express recombined antigen receptors like conventional B and T cells (56).

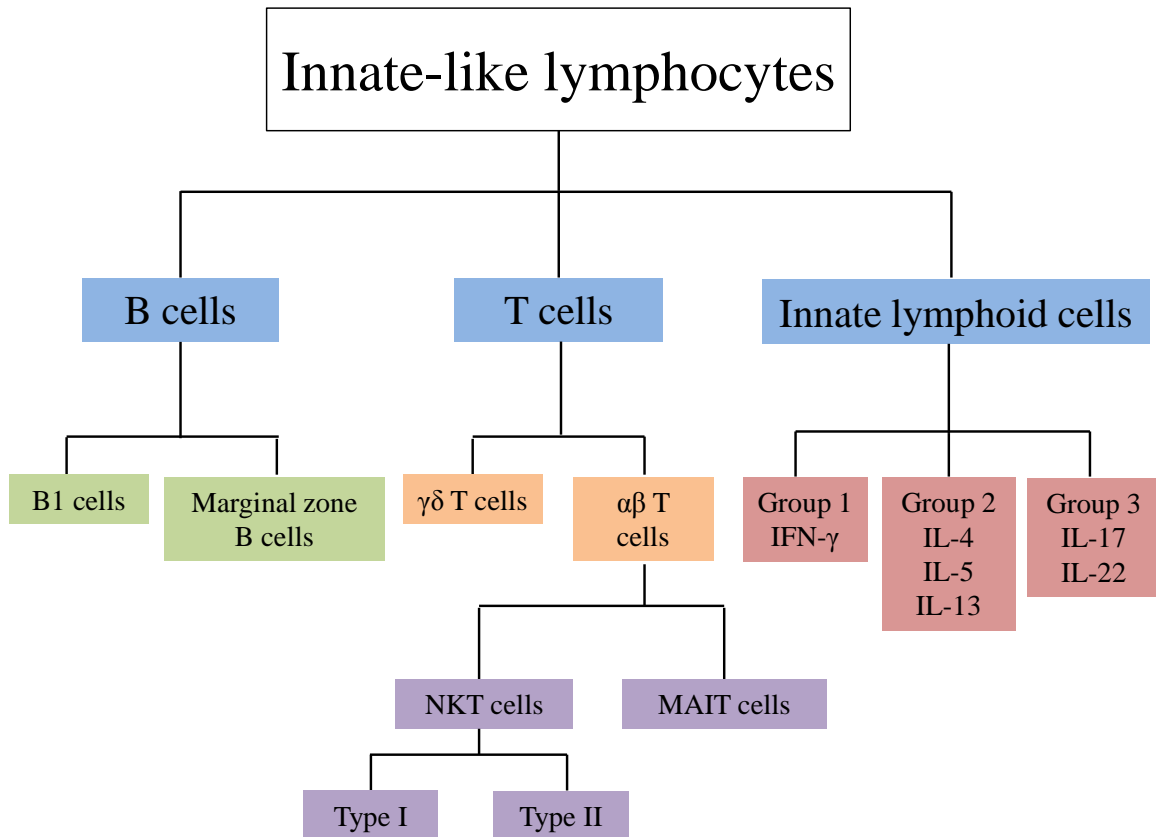


Figure 1.4: Types of innate-like lymphocytes

Innate-like lymphocytes are cells of the immune system that bear features of both innate and adaptive immunity. B1 and marginal zone B cells, have B cell receptors that recognize conserved patterns on pathogens and mount IgM responses. $\gamma\delta$ T, NKT and MAIT cells express T cell receptors with limited diversity and play important roles in health and disease. Finally, innate lymphoid cells, or ILCs do not express any known antigen receptor, but can secrete a variety of cytokines and fulfill important immune functions.

ILCs are thought to be activated by cytokines and express transcription factors that facilitate cytokine production. Recent studies have shown that ILCs can be of different types- ILC1, ILC2, ILC3 and lymphoid tissue inducer (LTi) cells (57). These ILCs can play an important role in the context of infectious diseases as well as in allergy and autoimmunity (58). $\gamma\delta$ T cells are also part of the innate-like system and mediate 'lymphoid stress-surveillance'. These cells are able to respond quickly to stress signals and play important roles in immunity and immunopathology (59). Another subset of innate T cells is the mucosal-associated invariant T (MAIT) cells. MAITs are MR1-restricted and are important in microbial infections at mucosal sites (60). Recent studies have also shown that MR1 can bind and present riboflavin and folic acid metabolites to MAIT cells (61). Although the biological significance of the recognition of vitamin B metabolites remains unknown, it is a valuable tool that will allow us to clearly identify, isolate and activate MAIT cells and better understand their function. Finally, a T cell subset bearing features of NK cells known as natural killer T (NKT) cells also fall in the innate-like category. NKT cells are well known for their role in cancer, infections as well as other conditions, and will be the focus of this study.

1.8 NKT cells

Natural killer T (NKT) cells were originally described in 1987 by Fowlkes et al., as murine thymocytes expressing a restricted TCR repertoire (62). These cells were also found to express NK1.1 similar to Natural Killer (NK) cells and were subsequently named NKT cells (63). The majority of NKT cells express an invariant T cell receptor (TCR) α chain rearrangement- V α 14J α 18 in mice, and V α 24J α 18 in humans, associated with V β chains of limited diversity. These cells are referred to as canonical or invariant or type I NKT (*i*NKT) cells and will be the primary focus of this study (64, 65).

In a screen for anti-tumor agents, a glycosphingolipid α -Galactosylceramide (α -GalCer, KRN7000) was found to have potent anti-tumor activity (66, 67). The anti-tumor property of α -GalCer was attributed to the specific activation of type I NKT cells, which recognize this glycolipid antigen in the context of CD1d, a non-classical, non-polymorphic, Major Histocompatibility Complex (MHC) class I-like molecule (68, 69). In contrast to this, type II NKT cells are CD1d-restricted, but express diverse antigen receptors and do not recognize α -GalCer. Besides the prototypical NKT cell agonist, α -GalCer, other microbial glycolipids that bind CD1d molecules and activate NKT cells have been identified and this will be discussed in more detail in Section 1.9 (70-73). The NKT cell TCR is also known to bind self-glycolipid antigens such as isoglobotrihexosylceramide (iGb3) and glycosylphosphatidylinositol (GPI) presented on CD1d (74, 75). Several groups have studied the structural aspects of glycolipid antigen loading on CD1d molecules using crystal structures of such complexes. These studies

have proposed a model of ‘induced fit’, whereby the CD1d molecule, as well as the NKT cell TCR play an active role in determining the ultimate structural conformation of the bound antigen (76, 77).

Similar to conventional T cells, NKT cells develop in the thymus. However, these cells are selected on double positive cortical thymocytes following the presentation of CD1d in complex with an unknown selecting ligand (78). The development of NKT cells involves activation of the signaling lymphocytic activation molecule (SLAM) family receptors (79). These SLAM family receptors further engage SLAM-associated protein (SAP)-related adaptors, and facilitate the activation of FynT, a Src family kinase encoded by the *fyn* gene, which is necessary for the development of NKT cells (80-82). Following their development, NKT cells acquire a memory phenotype, and can rapidly mount strong effector functions. These cells activate the transcription of IFN- γ and IL-4 genes during development. When these cells migrate to the periphery, their cytokine gene loci are modified by histone acetylation, and the cells are poised with transcripts for rapid cytokine production (83). The transcription factor promyelocytic leukemia zinc finger (PLZF) is important for the initiation of this innate-like effector program, and the homing of NKT cells to the liver (84). NKT cells lacking PLZF retain a naïve phenotype and traffic primarily to the lymph nodes. Conversely, the forced expression of PLZF results in an activated or ‘memory’ phenotype in conventional CD4⁺ T cells (84). NKT cells are activated by glycolipid antigens presented on CD1d molecules, in a complex antigen presentation pathway which will be discussed in Chapter 3. However, NKT cells can also

be activated in a CD1d-independent fashion (85). The CD1d-independent activation reported by Tyznik et al. involved the activation of TLR9 by viral components and the production of IL-12 by DCs. Once activated, NKT cells can mediate direct cytotoxicity, as well as produce large amount of cytokines. The cytotoxic functions of NKT cells can be mediated by multiple mechanisms including perforin and granzymes, similar to NK cells, Fas/FasL interactions as well as TRAIL/TRAIL-receptor engagement. Not much is known about the cytotoxic functions of NKT cells. A recent study by Wingender and group has reported that the *in vivo* cytotoxicity of NKT cells depends largely on CD1d expression by target cells, and the binding affinity of the activating antigen to CD1d (86). Furthermore, this report concluded that unlike NK cells that use perforin/granzymes for cytotoxicity, the engagement of Fas on target cells by FasL expressed on NKT cells was important for their anti-tumor cytolytic activity.

NKT cells are inherently capable of producing Th1/Th2/Th17 cytokines (87-90). The mechanism for the cytokine bias still remains elusive although several groups have proposed possible mechanisms (91). Previously, it was believed that the cytokine response from NKT cells depends mainly on the interaction between the glycolipid antigen and TCR, or the CD1d binding properties of the antigen. This was supported by the observation that ligands like OCH which are known to bind less stably as compared to α -GalCer, elicited a Th2 response (92). On the other hand, compounds like α -C-GalCer which has better binding capabilities, stimulated a stronger Th1 response (93). However, with the development of newer compounds such as α -GalCer acC8 and α -GalCer

acC20:2, the differences in antigen-TCR interaction have proved insufficient to explain the Th1/Th2 bias of different compounds (94). Bai et al. have reported that distinct APCs drive different immune responses (95). This was done using a CD1d conditional knockout mouse model focusing on three major types of APCs, namely DCs, macrophages and B cells. This study showed that Th1-biased ligands are more likely to be presented by DCs, as compared to other APCs. On the contrary, Th2-biased ligands were more likely to be presented on APCs other than DCs. However, a recent study by Arora *et al.* has proposed that CD8 α ⁺ DCs are the most efficient APCs for antigen presentation to NKT cells. Furthermore, this study proposed that the expression of costimulatory/coinhibitory molecules on CD8 α ⁺ DCs determines the cytokine bias following antigen presentation (96). The increased expression of CD70, Rae-1 and CD86 correlated with the presentation of Th1 biased antigens, whereas the expression of PD-L1 and PD-L2 was higher during the presentation of Th2-biased antigens. This study underscores the importance of APCs in eliciting appropriate NKT cell responses, although further investigation will be necessary to confirm the mechanism that drives the Th1/Th2 bias of NKT cell responses.

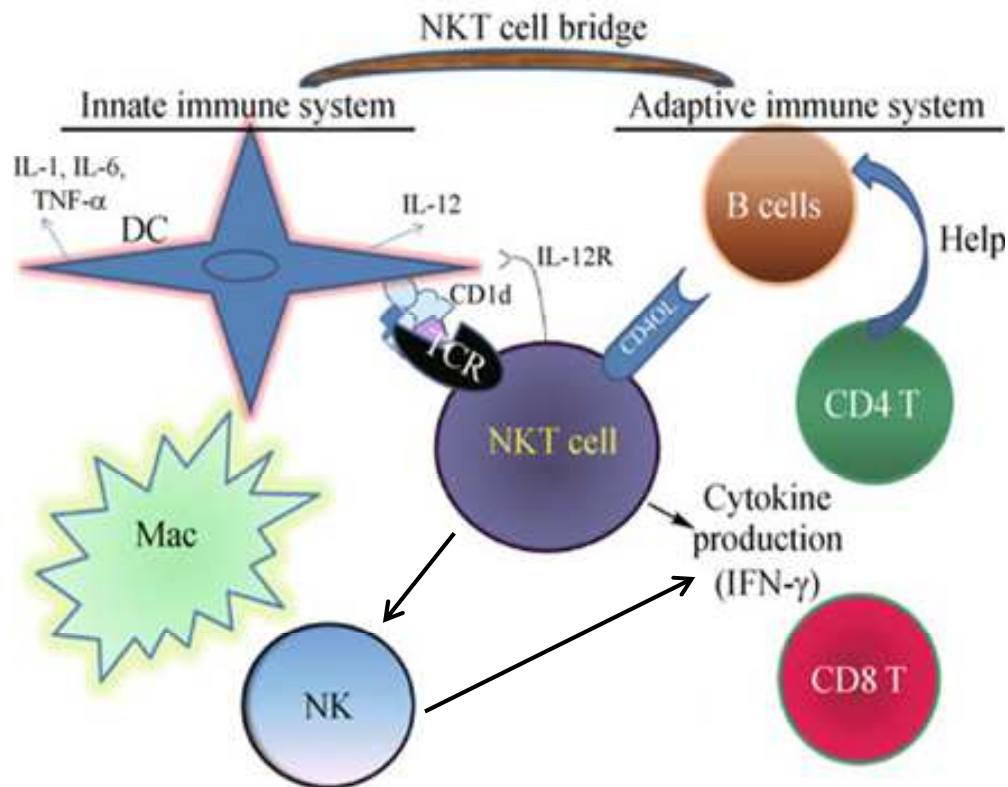
NKT cells help to bridge the innate and adaptive immune systems, especially due to their rapid cytokine production. We know that the anti-tumor immune response is typically characterized by Th1 cytokines such as IFN- γ (97). This leads to the rapid recruitment of CD8⁺ cytotoxic T lymphocytes, which can mount an anti-tumor cytolytic response (98, 99). This IFN- γ response is fortified by a positive feedback loop involving NK cells (100). When DCs present glycolipid antigen to activate NKT cells, they are also activated reciprocally due to CD40L expression on the NKT cell which engages CD40 on the DC (Fig.1.5). This stimulation leads to the secretion of IL-12 by the DC. This IL-12 further activates NK cells which produce more IFN- γ , amplifying the Th1 response. Thus, the primary Th1 response from the NKT cells can be boosted and/or sustained due to DC IL-12 production and NK cell activation (101).

NKT cells can also mount Th2-biased responses. In an OVA-driven mouse model of allergic asthma, J α 18 knockout mice that lack NKT cells were found to have reduced airway hyperreactivity, eosinophilic infiltration and Th2-driven IgE levels, as compared to their NKT cell sufficient counterparts (102). Furthermore, in the absence of classical CD4⁺ T cells, the activation of NKT cells by α -GalCer administration was sufficient to induce airway hyperreactivity by driving IL-4 and IL-13 production (103). Another study by Wingender and colleagues has showed that house dust extract contains antigens that can activate NKT cells and drive airway inflammation in mice (104). The addition of house dust extract had an adjuvant effect to OVA immunization and there was a synergistic effect between NKT cell and classical CD4⁺ T cell function. Akbari and group

have demonstrated that methacholine-induced allergic asthma was significantly reduced in mice lacking NKT cells, further underscoring their role in driving Th2 responses in this context (105). In non-obese diabetic mice, a Th1-driven autoimmunity model, activation of NKT cells by α -GalCer led to reduced disease due to the suppression of IFN- γ and retention of IL-4 production (106). A 2012 study by Lynch *et al.* has revealed that NKT cells are enriched in adipose tissue, but are lost upon the development of obesity (107). This group identified an IL-4 and IL-10-dependent regulatory role for NKT cells in obesity and concluded that NKT cell function is protective in this context. Also, using a mouse peritonitis model, it has been shown that IL-4-producing NKT cells are important in resolving sterile inflammation (108).

In general, NKT cells play a diverse set of biological roles; however different NKT cell subsets vary in their effector functions. NKT cells can express the canonical transcription factors T-bet, GATA-3 or ROR γ t, known as NKT1, NKT2 or NKT17 respectively (109). NKT cells from different organs also show distinct functional properties. Crowe and colleagues have reported that CD4⁻ NKT cells from the liver can reject tumors, in contrast to those from the thymus and spleen (110). NKT cells in mice can be CD4⁻CD8⁻ or CD4⁺, while in humans they can be CD4⁺, CD8⁺ or double negative. These differences have made it difficult to elucidate the precise roles of different NKT cell subsets (111).

Due to their rapid activation and ability to activate other cell types, NKT cells are of great interest in many conditions such as bacterial, viral and parasitic infections, as well as in cancer (112, 113). Many research groups have synthesized analogs of α -GalCer which can be used to activate NKT cells, paving the way for the translational application of NKT cells-based therapy (92, 93, 114-116). Numerous efforts have been directed towards the use of α -GalCer and its analogues as adjuvants in vaccines against malaria, Human Immunodeficiency Virus (HIV) and influenza. In the field of tumor immunology, attempts at using α -GalCer directly as an anti-tumor agent have had limited success, but there is now interest in combination therapy whereby the activation of *i*NKT cells via α -GalCer can be used as an adjuvant with a tumor-specific antigen (117).



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 Subrahmanyam and Webb *Frontiers in Biology* 7(5), 436-444 (October 2012)

Figure 1.5: NKT cells boost IFN- γ responses by activating other cell types

NKT cells are activated by glycolipid antigens presented by CD1d, and rapidly produce large amounts of IFN- γ . The CD40-mediated reciprocal stimulation of DCs by NKT cells results in the production of IL-12 by DCs. This IL-12 from DCs and IFN- γ from NKT cells activates NK cells which are also capable of producing large amounts of IFN- γ . This IFN- γ from NKT and NK cells facilitates the activation of CD8⁺ T cells, which can mount tumor-specific cytotoxic responses. Thus, NKT cells can bridge the innate and adaptive immune systems to boost anti-tumor immunity.

1.9 NKT cell functions

Bacterial infections

α -GalCer, the classical NKT cell activating ligand, has an α -anomeric link between the sugar and the ceramide. This is in contrast to most glycosphingolipids found in nature, making it unlikely that α -GalCer is the natural NKT cell activating ligand. Hence there was an urgent search for other activating glycolipids which would implicate a role for NKT cells in anti-bacterial immunity. The first evidence of such a role emerged in 2005, when glycosphingolipids from *Sphingomonas*, Gram-negative bacteria were shown to activate NKT cells (71). These bacteria do not contain lipopolysaccharide, eliminating the possibility of any involvement of the TLR4, and supporting the idea of direct bacterial recognition by the NKT cell TCR. It was also found that NKT cells were activated *in vivo*, following *Sphingomonas* infection. Mice lacking NKT cells had poor clearance of the bacteria from the liver as compared to their NKT cell sufficient counterparts. Following this study, Kinjo and colleagues reported that diacylglycerols from *Borrelia burgdorferi*, Gram-negative bacteria and the causative agent of Lyme disease, could also activate NKT cells (70). A number of different diacylglycerols were shown to stimulate rapid proliferation of NKT cells and strong cytokine production *in vivo*. The responses could also be correlated to the length and saturation of the acyl chains in these lipids, indicating that the responses were directly dependent upon TCR-mediated recognition. Together, these studies provided the first evidence that NKT cells directly recognize microbial pathogens through their TCR and are important in bacterial clearance. Thereafter, it was found that the spectrum of NKT cell recognition was

broader, and included Gram-positive bacteria (118). *S. Pneumoniae*, the causative agent of pneumonia and group B *Streptococcus*, which causes neonatal sepsis and meningitis, both contain diacylglycerol containing glycolipids which bind CD1d and are presented to NKT cells. The activated NKT cells respond by cytokine release and are required for bacterial clearance. Sada-Ovalle and colleagues have studied the role of NKT cells in *Mycobacterium tuberculosis* (Mtb) infection (119). This group reported that Mtb infected macrophages induced strong IFN- γ response from splenocytes. This response was CD1d-dependent and was attributed to NKT cells. They further adoptively transferred NKT cells into mice infected with Mtb and reported a decreased bacterial burden in the lungs. It was concluded that macrophages present Mtb-associated antigens to NKT cells, which produce IFN- γ and eventually lead to killing of these intracellular bacteria. To further extend the observation that NKT cells suppress Mtb replication, mice were treated with the NKT cell activating ligand, α -GalCer- alone or in combination with isoniazid (120). It was found that NKT cell stimulation by treatment of Mtb-infected mice with α -GalCer led to decreased bacterial burden and increased survival. This effect was enhanced when α -C-GalCer, a C-glycoside analog of α -GalCer was used. This was attributed to the Th1 bias of α -C-GalCer as compared to α -GalCer, because IFN- γ was reported to play the major role in anti-mycobacterial responses mounted by NKT cells. α -GalCer was also found to have an additive protective effect when combined with isoniazid. Such macrophage-mediated activation of NKT cells has also been reported in the context of *Listeria monocytogenes* infection (121). Emoto *et al.* reported increased IFN- γ and nitric oxide (NO) after intraperitoneal administration of α -GalCer in mice infected with *L.*

monocytogenes. Infected macrophages thereafter showed increased respiratory burst, which enabled killing of the bacteria. This study led to the conclusion that administration of α -GalCer causes increased bacterial killing in the phagosomes of infected macrophages and also stimulates IFN- γ responses by NKT cells, leading to amelioration of listeriosis. It was later reported that accelerated recruitment of Gr1⁺ cells and $\gamma\delta$ T cells into the liver also contributed to the anti-bacterial effect of α -GalCer (122).

The role of NKT cells in various Chlamydial infections has also been studied. Wang *et al.* reported that pretreatment of mice with α -GalCer followed by genital tract infection with *Chlamydia muridarum* led to reduced bacterial burden and increased IL-12 and IFN- γ in the lymph nodes and genital tract (123). NKT cell stimulation also enhanced IFN- γ production by NK cells and T cells, and further boosted the Th1 response to this pathogen. A similar Th1-biasing role for NKT cells and involving DCs has been established in a murine infection model of *Chlamydia pneumoniae* (124). Besides aiding Chlamydial clearance in mice, NKT cells have also been reported to have a protective role in *Chlamydia trachomatis*-induced arthritis (125). CD1d^{-/-} infected mice showed poor local bacterial clearance in the joint, and more severe arthritis as compared to wild type controls. This was due to lower IFN- γ production and higher regulatory cytokines such as IL-4 and IL-10. All the above mentioned mouse models were based on C57/BL6 mice and have established a protective role for NKT cells in Chlamydial infections. However, Bilenki and colleagues reported that CD1d^{-/-} mice on a BALB/c background had improved outcome after infection with *Chlamydia trachomatis* and reduced IL-4 and

IgE production (126). Furthermore, treatment of infected mice with α -GalCer enhanced bacterial growth *in vivo* and this could be partially rescued by neutralization of IL-4, suggesting that NKT cells exacerbated disease in this setting by skewing toward a Th2 response. Whether this disparity is a result of differences in mouse strains, which can significantly influence Th1/Th2 balances, is an issue which remains to be addressed. Using a different approach to the modulation of NKT cells *in vivo*, Devera and group studied the effect of NKT cell activation of vaccine-induced, anti-bacterial immunity to *Bacillus anthracis* (127). It was found that the administration of α -GalCer at the time of immunization led to increased neutralizing antibody titers, as well as longer duration of protection after immunization with the protective antigen (PA)-based vaccine against *Bacillus anthracis*. Thus, NKT cells have been shown to play an important role in the immune response to bacterial pathogens. There is significant interest in the modulation of these responses through the use of NKT cell activating ligands alone, as adjuvants for vaccines, or in combination with other treatments.

NKT cells in viral infections

NKT cells are known to have strong anti-viral activity including direct cytotoxicity. Some viruses such as Herpes Simplex Virus (HSV) and Vaccinia virus (VV) are known to alter CD1d-mediated antigen processing and presentation to NKT cells (128, 129). Using a model of infection with mouse cytomegalovirus (MCMV), Reilly *et al.* showed that the activation of NKT cells by administration of α -GalCer led to reduced viral titers, and increased CD8⁺ memory T cells. It was thereby concluded that the activation of NKT cells boosted protective immunity to MCMV in mice. Several reports have shown that HIV infection is associated with decreased numbers of V α 24 β 11 NKT cells in human patients (130-132). The reduction of NKT cells is higher than conventional CD4⁺ T cells. NKT cells have also been found to be functionally impaired, with elevated PD-1 expression during HIV-1 infection (133). Many efforts have been directed towards improving vaccines against HIV by including α -GalCer to activate NKT cells. Prominent among these, is the work published by Courtney *et al.* showing that an HIV vaccine based on peptides from the HIV Envelope protein gp120, along with α -GalCer as an adjuvant could elicit potent immune responses after mucosal delivery in mice (134). Huang and group reported that co-administration of α -GalCer with a DNA vaccine (pADVAX) encoding the Env and Gag proteins of HIV-1 led to improved responses in mice (135). The use of α -GalCer to activate NKT cells led to improved humoral responses, as well as epitope-specific IFN- γ responses. Moreover, the inclusion of α -GalCer also had a dose-sparing effect on the DNA vaccine and has also been used as an adjuvant with polylactic acid based nano-particle vaccines (136).

The activation of NKT cells has also been shown to be important in influenza infection. When compared to wild type controls, CD1d^{-/-} mice which lack NKT cells are known to have a lower survival rate after influenza virus infection (137). The antiviral effect of NKT cells is attributed to the production of IFN- γ , which is important for NK cell activation and also for CD8⁺ T cell responses. IL-22 is also thought to be involved in the protective effect of NKT cells activation toward influenza A infection (138). A major rationale for utilizing NKT cell activation to boost the effectiveness of an influenza vaccine was shown by Ko *et al.* (139). This group reported an important role for NKT cell responses in vaccines using an ovalbumin (OVA) model. They found that the addition of α -GalCer while immunizing mice against OVA improved the immune response upon subsequent challenge with the OVA peptide. Similar to the effect of α -GalCer in HIV vaccines, they found that α -GalCer as an adjuvant increased the therapeutic efficacy of the vaccine at a lower dose. Moreover, the humoral, cytokine and T cell responses were enhanced and more effective. The inclusion of NKT cell activating ligand α -GalCer in an influenza vaccine, A/PR/8/34 (PR8) led to increased IgG and IgA titers. Similarly, formalin-inactivated PR8 influenza virus with α -GalCer resulted in lower viral titers in infected mice and higher PR8 specific antibody titers (140). Thus, NKT cells are known to play an important role in anti-viral immunity, a fact which is being exploited by using NKT cell-activating antigens to improve the immunogenicity of vaccines against viral infections.

NKT cells in anti-tumor immunity

The original discovery of the potent anti-tumor function of NKT cells stemmed from the observation that α -GalCer injected into mice led to a striking reduction in B16 melanoma tumor burden (66, 141). In clinical trials, the treatment of patients with α -GalCer showed that it is well-tolerated and there was no dose-limiting toxicity (117). 5 out of 24 patients showed increased serum TNF- α and GM-CSF. However, no clinical responses were detected, and 7 out of 24 patients experienced stable disease for a median 123 days. The limited effectiveness of α -GalCer is probably because systemic injection of α -GalCer can cause anergy in NKT cells, or because cancer patients have reduced numbers of NKT cells (97, 142-145). This has led to a growing interest in restoring NKT cell numbers, and also using α -GalCer loaded on APCs, or as an adjuvant, to avoid the induction of anergy caused by soluble α -GalCer. Among many efforts made in this direction is the use of a DC based vaccine against hepatocellular carcinoma (99). More specifically, this vaccine was used to target Hepatitis B Virus (HBV)-associated hepatocellular carcinomas which express the HBV surface antigen (HBsAg). This antigen was loaded on to DCs, and α -GalCer was added as an adjuvant. This strategy caused rapid remission from disease in mice. The anti-tumor effect was attributed mainly to cytotoxic CD8⁺ T cell responses. However, IFN- γ and IL-12 are also involved in the anti-tumor response. The adjuvanted vaccine was found to be significantly better than the vaccine alone in terms of mean survival, as well as tumor load. Despite the general belief that NKT cell function can protect against cancer, Bjordahl *et al.* have reported that type I NKT cells led to increased tumor growth in B cell lymphoma by suppressing CD8⁺ T cell responses, which perhaps

points to the importance of context-dependent NKT cell activation and effector function (146). In this study the authors initially discovered that mice lacking T cells had improved disease outcome, however the depletion of CD4⁺ or CD8⁺ T cells had no effect. The lack of type I NKT cells but not type II NKT cells was able to recapitulate these observations. These results indicate that in this system, type I NKT cells were immunosuppressive while type II NKT cells mediated anti-tumor immunity.

The use of autologous tumor specific antigens to target lymphomas can generate highly specific and effective responses. However, a major limitation to their use is their poor immunogenicity. This was overcome by the use of α -GalCer-loaded A20 lymphoma cells in a mouse model (147). The vaccine was found to elicit effective immune responses to lymphoma in mice in a CD4⁺ T cell dependent manner. Together these studies also shed light on the fact that different immune responses (CD4⁺ and CD8⁺ T cells) are elicited following administration of vaccines with α -GalCer and the specific response elicited may depend upon the type of vaccine, as well as the tumor type. Besides the use of autologous tumor cells and DCs as APCs, attempts have also been directed toward identifying other APCs (148). For example, it has been shown that primary B cells transduced with adenovirus encoding truncated Her-2/neu tumor-specific antigen along with α -GalCer were effective at raising specific antibody responses (149). CD4⁺ T cells, CD8⁺ T cells and NK cells were found to be important for the anti-tumor response elicited by the vaccine. The problem of NKT cell anergy due to antigen presentation by B cells did not pose a problem in this model and could, in fact, be further evidence of

context-dependent activation of NKT cells and subsequent fine tuning of innate and adaptive immune responses. Another study also used the Her-2/neu expressing adenovirus transduction strategy, but the APCs used in this case were Myeloid Derived Suppressor Cells (MDSC) (150). When these cells expressing tumor antigen were loaded with α -GalCer, they did not suppress CD8⁺ T cell responses or induce Forkhead Box P3 (FoxP3) expressing regulatory T (Treg) cells. In fact, the vaccine was found to enhance tumor-specific CTL responses, and the MDSCs underwent phenotypic changes and acted as potent immunogenic APCs (150). In 2010, Kim *et al.* used a combination strategy for an anti-tumor vaccine (151). The regimen consisted of a priming dose of DNA vaccine, followed by DCs transduced to express Human Papilloma Virus Type 16- E7 antigen along with α -GalCer as an adjuvant. However, the subsequent boosters consisted only of DCs loaded with E7 antigen. They reported effective anti-tumor responses with the use of this DNA vaccine boosted with antigen-loaded DCs along with α -GalCer. A recent study used a combination of lenalidomide with α -GalCer-loaded DCs for the treatment of myeloma and was well-tolerated in the tested cohort (152). These studies established a useful role for NKT cells and have set the stage for future translational efforts.

Chapter 2: Materials and methods

2.1 Cell lines, antigens and other reagents

L-CD1d, L-CD1d-DR4 and LMTK-CD1d, are mouse fibroblast cell lines transfected with *mCD1d1* or *mCD1d* and *HLA-DR4* (kindly provided by Dr. Randy Brutkiewicz, Indiana University School of Medicine) and were cultured in DMEM with 10% FBS and 2 mM L-glutamine. Additionally, selection agents G418 sulfate (500 µg/ml) from Mediatech Inc. (Manassas, VA), Zeocin (200 µg/ml) and Puromycin (5 µg/ml) from Santa Cruz Biotechnology (Santa Cruz, CA) were added as required. Unless otherwise specified, all materials for cell culture were purchased from Life Technologies (Carlsbad, CA). WEHI-231 and WEHI-231/Bcl-xL cells were kindly provided by Dr. Gregory Carey (University of Maryland, Baltimore) and cultured in RPMI 1640 with 10% FBS, 100 mM sodium pyruvate, 10 mM non-essential amino acid solution, 1x vitamin solution and 50 µM 2-mercaptoethanol. The mouse type I NKT cell hybridomas DN32.D3 and N38-3C3, as well as the type II NKT cell hybridomas N37-1A12 have been described and were cultured in IMDM supplemented with 5% FBS and 2 mM L-glutamine (69, 153). 17.9 CD4⁺ T cell hybridomas were kindly provided by Dr. Janice Blum (Indiana University School of Medicine) and cultured in RPMI supplemented with 10% FBS, 50 µM 2-mercaptoethanol and 2 mM L-glutamine. All cell lines were maintained in cell culture grade flasks from ThermoScientific (Waltham, MA) at 37°C in a 5% CO₂ environment. Cells were viewed under an Olympus CK2 light microscope from Olympus Corporation (Tokyo, Japan) using a 10x objective. Images were captured using an

Olympus Pen E-PL1 digital camera from Olympus Corporation (Tokyo, Japan). α -Galactosylceramide (α -GalCer) purchased from Enzo Life Sciences (New York City, NY) was used at a final concentration of 100 ng/ml *in vitro* and 2 μ g/mouse *in vivo*. Human Serum Albumin (HSA) was purchased from Sigma-Aldrich (St. Louis, MO) and used at 10 μ M. ABT-263 and ABT-199 (10 or 20 μ M) used for the inhibition of Bcl-xL and/or Bcl-2 were a kind gift from Dr. Gregory Carey (University of Maryland, Baltimore).

2.2 Over-expression and knockdown of Bcl-xL

The use of all recombinant DNA and viral vectors was undertaken only with prior approval from the Institutional Biosafety Committee (IBC) at the University of Maryland Baltimore. All procedures were performed in accordance with the guidelines established by the IBC. LMTK-CD1d cells were transfected with vector alone or vector encoding Bcl-xL, kindly provided by Dr. Mark Williams (University of Maryland, Baltimore). Transfection was carried out using Neofectin one-step transfection reagent from NeoBioLab (Cambridge, MA) as per the manufacturer's directions. Stable transfectants were then cultured in dual selection medium containing G418 sulfate (500 μ g/ml) and Zeocin (200 μ g/ml) and passaged every three days. There were no evident morphological, growth or survival defects in Bcl-xL over-expressing cells as compared to the controls. Over-expression of Bcl-xL was confirmed by Western blotting. Bcl-xL knockdown was attained using lentiviral vectors purchased from Santa Cruz Biotechnology (Santa Cruz, CA). These replication incompetent lentiviral particles

contained a pool of 3-4 short hairpin sequences targeted to the *Bcl2l1* gene that encodes Bcl-xL, or the *Bcl-2* gene and polybrene based transduction was carried out as per the manufacturer's instructions. Stable transductants were cultured in dual selection medium containing G418 sulfate (500 µg/ml) and puromycin (5 µg/ml) with no evident morphological, growth or survival defects, and were passaged every three days. Bcl-xL knockdown was confirmed by Western blotting.

2.3 Cell viability and proliferation assays

LMTK-CD1d cells transfected with Bcl-xL and L-CD1d cells in which Bcl-xL or Bcl-2 has been knocked down and the respective controls were added to 96-well flat bottom microtiter plates (5×10^4 cells/well in 100 µl complete medium). WST-1 was purchased from Roche Applied Science (Penzberg, Germany) and 10 µl was added per well. Cells were incubated for the indicated time periods and absorbance was measured using a standard plate reader. The difference between the absorbance at 450 nm and 690 nm was measured as per the manufacturer's instructions. To test cell viability of L-CD1d-DR4 cells following treatment with ABT-263 or ABT-199, we used the apoptosis detection kit from BioLegend (San Diego, CA). Cells were stained with Annexin-V-FITC and propidium iodide as per the manufacturer's instructions and analyzed by flow cytometry. Cells that were AnnexinV⁺PI⁺ were considered apoptotic while all others were considered viable. AnnexinV or PI single-positive cells were minimal.

2.4 Mice

Female C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used at approximately 6 weeks of age. IL-14 α transgenic mice and c-Myc transgenic mice were kindly provided by Dr. Julian L. Ambrus, Jr. (State University of New York (SUNY) at Buffalo School of Medicine and Biomedical Sciences). IL-14 α and c-Myc double transgenic (DTG) mice have been described before and were obtained by crossing IL-14 α transgenic mice and c-Myc transgenic mice (154). All animals were housed in specific pathogen-free conditions at the University of Maryland Baltimore. All procedures were performed with approval from the University of Maryland Baltimore, Institutional Animal Care and Use Committee (IACUC). For treatment with α -GalCer, each DTG mouse was given 2 μ g of α -GalCer or vehicle alone, injected i.v. using a 29G needle and 1 ml syringe from Med-Vet International (Mettawa, IL). For this treatment, α -GalCer in DMSO at 1 μ g/ μ l was diluted in PBS and each mouse was injected with 100 μ l. Mice were treated at 8 weeks of age and euthanized at approximately 14 weeks of age. During this time, mice were closely monitored for changes in appearance, behavior or responsiveness. After euthanasia, the mice were dissected and thymus, spleen, lymph nodes and bone marrow were obtained. The liver was first perfused by injecting PBS through the hepatic portal vein and then removed. Single cell suspensions of these organs were prepared and used for analysis by flow cytometry, *ex vivo* cocultures, *ex vivo* restimulation or Western blotting. The liver was processed to obtain mononuclear cells using Percoll gradient centrifugation as described below.

2.5 Histological Analyses

Spleen sections from wild type or DTG mice treated with vehicle alone or α -GalCer was fixed in 4% paraformaldehyde in PBS overnight at room temperature. Following fixation, the tissue was washed and transferred to the Histology Core Services at the University of Maryland Baltimore for paraffin embedding and sectioning. Five micrometer sections were prepared and stained with hematoxylin and eosin (H&E). These H&E stained sections were visualized under a light microscope and imaged as described above.

2.6 Ex vivo anti-CD40 stimulation of B cells

After euthanasia and removal of spleens from C57BL/6 mice, a single cell suspension of splenocytes was prepared using a 70 μ m nylon mesh from Fisher Scientific (Waltham, MA) (Fig. 2.1). B cells were isolated using a mouse B cell enrichment kit from Stem Cell Technologies (Vancouver, Canada) as per the manufacturer's instructions. Purity was determined by flow cytometry after staining with FITC-conjugated antibody to B220 (clone RA3-6B2) from BioLegend (San Diego, CA) (Fig. 2.1A). Enriched splenic B cells were suspended in RPMI 1640 with 10% FBS, and other supplements as described above. Primary splenic B cells were treated with 10 μ g/ml of anti-CD40 antibody (clone 1C10) from eBioscience (San Diego, CA). For primary NKT cells, the liver was processed and liver mononuclear cells were obtained as previously described (155). Briefly, the liver was perfused with PBS before removal. Once harvested, the liver was cut into small pieces, and passed through a 70 μ m nylon mesh to prepare a single cell suspension. This suspension was washed twice in NKT buffer (PBS with 2% FBS and

0.02% sodium azide). The cells were then overlaid with a layer of 37.5% Percoll and centrifuged at 700g for 12 minutes at room temperature. The hepatocytes in the upper layer after centrifugation were removed. The pellet containing lymphocytes and erythrocytes was washed in NKT buffer and erythrocytes were lysed by hypotonic shock using ACK lysis buffer from Quality Biologicals Inc. (Gaithersburg, MD). The remaining mononuclear cells were washed twice with NKT buffer. NKT cell percentage was determined by flow cytometry after staining with a FITC-conjugated antibody to the mouse TCR β chain (clone H57-597) from BioLegend (San Diego, CA) and APC-conjugated CD1d tetramers loaded with α -GalCer, kindly provided by the NIH Tetramer Core Facility at Emory University (Atlanta, GA) (Fig. 2.1B). Treated B cells and liver mononuclear cells were cocultured to measure antigen presentation to NKT cells.

2.7 Ex vivo restimulation of NKT cells

Following treatment with α -GalCer or vehicle alone, DTG mice were euthanized and their spleens were processed to obtain a single cell suspension of splenocytes using a 70 μ m nylon mesh strainer. These splenocytes were cultured in 96-well microtiter plates in complete medium, (5×10^5 cells/well) with or without α -GalCer (100 ng/ml). After 48 hours, IFN- γ and IL-4 in the supernatant were measured using the mouse IFN- γ and mouse IL-4 ELISA MAXTM kits from BioLegend (San Diego, CA).

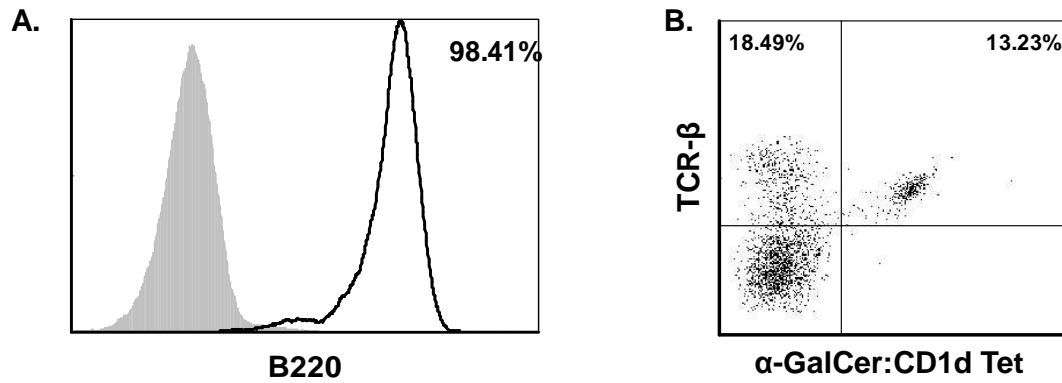


Figure 2.1 Purity of primary mouse splenic B cells, and NKT cells in the liver mononuclear cell fraction

(A) B cells were isolated from mouse splenocytes and surface B220 expression was determined by flow cytometry. (B) Liver mononuclear cells were isolated and the NKT cell population was determined by flow cytometry for TCR- β expression and binding of α -GalCer loaded CD1d tetramers.

2.8 NKT cell hybridoma assay

WEHI-231, L-CD1d or LMTK-CD1d cells (5×10^5 per well) were co-cultured with NKT cell hybridomas (5×10^4 per well) for 20 hours at 37°C in IMDM supplemented with 5% FBS and L-glutamine. Primary mouse B cells were treated with anti-CD40, washed with complete medium and cocultured (10^6 per well) with liver mononuclear cells (2×10^5 per well) for 24 hours at 37°C in complete medium. For Bcl-xL/Bcl-2 inhibition studies, L-CD1d-DR4 cells were treated with ABT-263 or ABT-199 for 4 hours, washed twice with PBS and once with complete medium, and cocultured with NKT cells. For the peptide antigen presentation assay, L-CD1d-DR4 cells were loaded with human serum albumin (HSA) overnight, treated with ABT-263 or ABT-199 for 4 hours, washed extensively and cocultured with 17.9 T cells hybridomas. Following the coculture, supernatants were harvested and IL-2, IFN- γ or IL-4 levels were measured by ELISA. Mouse IL-2 ELISA kit from BD Biosciences (San Jose, CA) and mouse IFN γ and IL-4 ELISA kits from BioLegend (San Diego, CA) were used. Data were graphed using Prism 5.02 from GraphPad (La Jolla, CA).

2.9 CD1d blockade

LMTK-CD1d cells transfected with empty vector alone or Bcl-xL at 5×10^5 cells/well in complete medium were incubated with $10 \mu\text{g/ml}$ of purified anti-CD1d antibodies (clone 1B1) from BioLegend (San Diego, CA) at 37°C for 2 hours before the addition of NKT cell hybridomas.

2.10 Flow cytometry

Cells were stained in staining buffer (PBS containing 0.5% BSA and 2 mM EDTA) for 30 min at 4°C with fluorophore-conjugated antibodies, in accordance with the manufacturer's directions. Primary mouse B cells were treated with mouse Fc block (anti-CD16/32 antibody) from BD Biosciences (San Jose, California) prior to the addition of fluorophore-conjugated antibodies, as per the manufacturer's directions. After staining, cells were washed three times with staining buffer and analyzed by flow cytometry. Data were collected on an LSR II from BD Biosciences (San Jose, CA) and analyzed using FCS Express Version 3 from De Novo Software (Los Angeles, CA).

2.11 Western blotting

Cells were lysed using lysis buffer containing NaCl (150 mM), Tris-Cl (50 mM), EDTA (8.5 mM), sodium azide (0.02%), NP-40 (0.1%) and complete mini protease inhibitor cocktail tablet from Roche Applied Science (Indianapolis, IN) as directed by the manufacturer, prepared in water. Proteins were resolved by electrophoresis on a 4-12% gradient polyacrylamide gel and transferred to a PVDF membrane using the iBlot transfer system. All gels, equipment, buffers and other materials were from Life Technologies (Carlsbad, CA) and were used as per the manufacturer's instructions. Membranes were probed with rabbit anti-mouse Bcl-xL antibody (clone 54H6) from Cell Signaling Technology (Beverly, MA) or rabbit anti-mouse Rab7 (polyclonal) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). GAPDH levels were detected on the same blot as the test protein using rabbit anti-mouse GAPDH antibody (clone 14C10) from Cell

Signaling Technology (Beverly, MA). Dylight-800 conjugated anti-rabbit secondary antibody was purchased from ThermoScientific (Waltham, MA). Membranes were scanned using the Odyssey Imaging System from Li-COR Biosciences (Lincoln, Nebraska).

2.12 Confocal microscopy

Cells were added to a Lab Tek II Chamber slide from Thermo Scientific (Waltham, MA), allowed to adhere overnight and fixed in 1% paraformaldehyde in PBS for 15 minutes at room temperature. Non-specific binding sites were blocked by incubating cells in PermWash buffer from BD Bioscience (San Jose, CA) with 5% rat serum for 1 hour at 4°C. The cells were then incubated with antibodies to CD1d (clone 1H6), kindly provided by Dr. Randy Brutkiewicz (Indiana University School of Medicine, Indianapolis, IN), rat anti-mouse LAMP1 (clone 1D4B) from eBioscience (San Diego, CA), rabbit anti-mouse EEA1 (clone C45B10) from Cell Signaling Technology (Beverly, MA) or rabbit anti-mouse Rab7 (polyclonal) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) at 4°C overnight. After washing, AlexaFluor-546 conjugated anti-mouse IgG, APC conjugated anti-rabbit IgG and AlexaFluor 647 conjugated anti-rat IgG from Life Technologies (Carlsbad, CA) were added and incubated for 1 hour at room temperature in the dark. Slides were then mounted using Vectashield mounting medium containing DAPI from Vector Laboratories (Burlingame, CA). Images were acquired using a 40x lens on the LSM 510, a laser scanning microscope from Carl Zeiss Microscopy (Oberkochen, Germany) using the Zen 2009 software and processed using the Zeiss LSM Image

Browser Version 4.2.0.121. Colocalization was quantified using the same software from 5 different images, and Pearson's correlation for colocalization was represented as percent of control. Additionally, Rab7 staining was quantified by pixel number from 5 different images and represented as percent of control.

2.13 qPCR arrays

The mouse dendritic cell and antigen presenting cell, and mouse autophagy RT² Profiler PCR Arrays and all other reagents were purchased from Qiagen (Venlo, Limburg). mRNA was extracted from samples using the RNeasy Plus Mini kit. cDNA was synthesized using the RT² First Strand kit as per the manufacturer's instructions. This cDNA was loaded on to the PCR array plates as per the manufacturer's directions, and run on an ABI 7900 qPCR machine from Life Technologies (Carlsbad, CA). The qPCR program was set up as per the manufacturer's instructions. All arrays were repeated at least twice and data were analyzed using Qiagen's online RT² Profiler PCR array data analysis software.

2.14 Statistical analyses

All experiments were repeated at least three times. Two-tailed student's t test, one-way analysis of variance (ANOVA) or two-way ANOVA were used as appropriate. Specific experimental groups were compared with controls using the Bonferroni post test. p value less than 0.05 was considered significant. All analyses were performed using Prism 5.02 by GraphPad (La Jolla, CA). *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$.

Chapter 3: The role of Bcl-xL in CD1d-mediated antigen presentation to NKT cells

3.1 Introduction

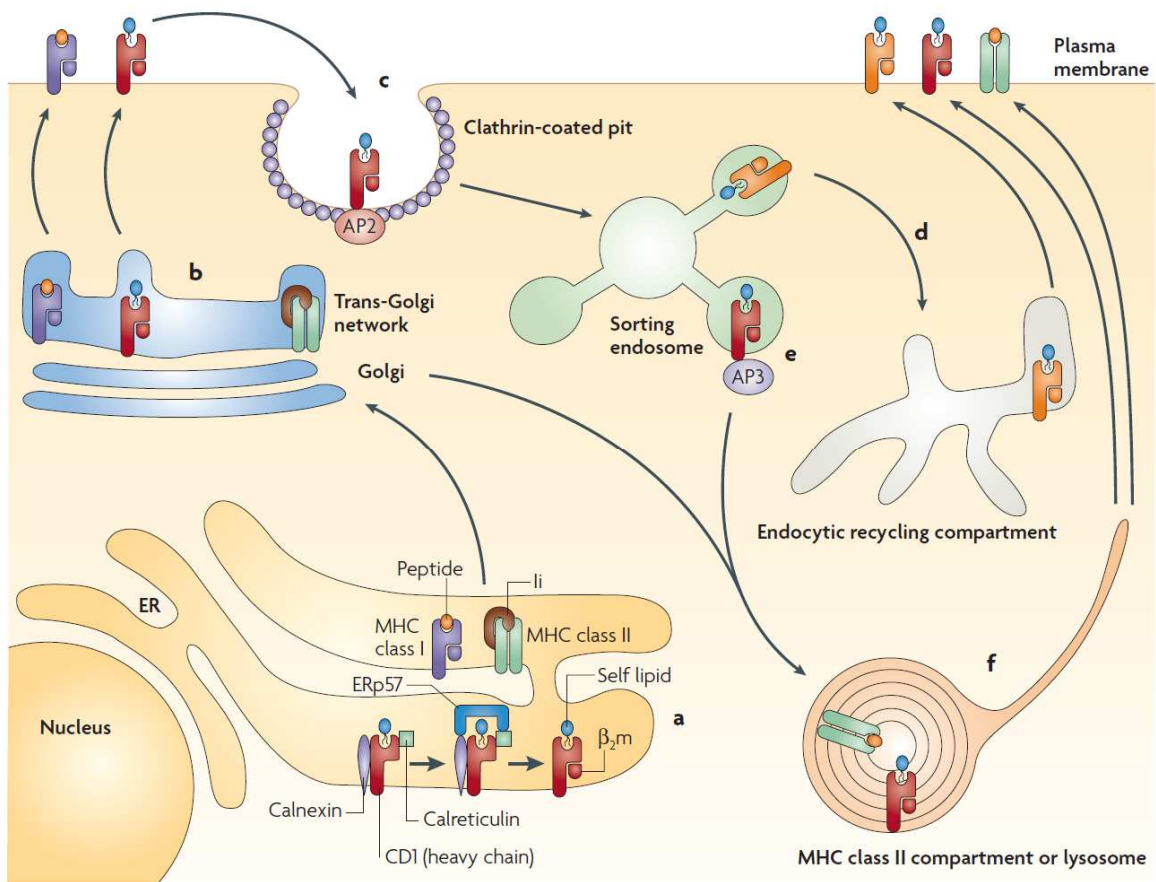
NKT cells are activated by glycolipid antigens in the context of CD1d molecules. One of the most striking and well-established functions of NKT cells is their anti-tumor effect, mediated directly by cytotoxicity, as well as indirectly by cytokine production which leads to the recruitment and activation of other cell types such as NK cells, CD8⁺ T cells and CD4⁺ T cells (156-158). Previous studies in our laboratory have demonstrated that NKT cells can specifically recognize B cells from mantle cell lymphoma (MCL) patients (159). However, the precise mechanisms that underlie the recognition of tumors by NKT cells remain poorly understood. Such mechanisms can be identified through detailed studies on antigen presentation to NKT cells, and its regulation by cell-intrinsic factors.

In contrast to the MHC restriction of classical T cells, NKT cells are CD1d-restricted (65, 68). Mice possess *CD1d1* and *CD1d2* genes, however, *CD1d2* does not encode a functional product, and antigen presentation to NKT cells is dependent upon CD1d1 molecules (referred to as CD1d) (160, 161). The CD1d molecule is structurally similar to MHC class I with a three domain α chain that associates with β 2-microglobulin (β 2m), but unlike the classical MHC class I molecule, CD1d has a hydrophobic antigen binding groove (162, 163). Also, in contrast to the ubiquitous expression of MHC class I, CD1d is mainly expressed on dendritic cells, macrophages, B cells and T cells (164). The process

of CD1d-mediated antigen presentation is complex and begins with the synthesis of the CD1d α chain in the ER (Fig. 3.1) (165). Here chaperones like calnexin, calreticulin and Erp57 ensure that it is properly folded (166). The antigen binding groove of CD1d is occupied by a self-lipid antigen thought to be loaded by the microsomal triglyceride transfer protein (MTTP) (75, 167). After association with β_2m , the CD1d molecule follows the secretory pathway from the ER to the Golgi and reaches the plasma membrane (PM). In order to present an activating endogenous antigen to NKT cells, CD1d molecules recycle from the PM to endocytic compartments due to the presence of a tyrosine based targeting motif (Yxx ϕ where Y is tyrosine, x is any amino acid and ϕ is a hydrophobic amino acid) (168, 169). This is analogous to the invariant chain (Ii) for MHC class II molecules. In fact Ii, which mediates trafficking from the golgi to the lysosomes can associate with CD1d. However, the Yxx ϕ motif is necessary for the proper recycling of CD1d molecules and their trafficking to the endocytic compartments (170). These recycled CD1d molecules can present antigen to type I NKT cells, but type II NKT cells do not require this recycling. Following internalization from the PM, adaptor proteins AP2 and AP3 direct CD1d molecules to the lysosomal compartment, also known as MIIC, where MHC class II molecules are normally loaded with peptide antigens (171, 172). Once in the endocytic recycling compartment, the stabilizing self lipid is exchanged for other activating lipid antigens with the help of saposins (173). These loaded CD1d molecules are then re-expressed on the PM and can be recognized by canonical V α 14J α 18 NKT cells. The localization of CD1d to cholesterol-rich lipid rafts is important for efficient antigen presentation, especially in the presence of low concentrations of

antigens and the disruption of these lipid rafts leads to reduced antigen presentation (174, 175). The complex multi-step process of CD1d-mediated antigen processing and presentation has several potential levels of control, yet very few endogenous regulatory factors have been identified. Prominent among these, are the mitogen-activated protein kinases (MAPK), PKC δ and Rho kinases (176-178). In this study we sought to identify a target that regulates CD1d-mediated antigen presentation, and is relevant to tumor growth and survival.

Anti-apoptotic Bcl-2 family members are known to be expressed at high levels in lymphomas and other malignancies, as discussed in Chapter 1, Section 1.4. Bcl-2 and Bcl-xL are two of the most well-studied anti-apoptotic factors from this family. Bcl-xL, in particular, is a potent anti-apoptotic factor and exerts its function by heterodimerizing with pro-apoptotic Bcl-2 family members (or activators of apoptosis) and preventing the permeabilization of the mitochondrial outer membrane and the escape of cytochrome c. Bcl-xL can also mediate its pro-survival function by causing the retro-translocation of the pro-apoptotic factor Bax, from the mitochondria to the cytosol (179). In addition, Bcl-xL prevents the formation of ceramide channels, which are also involved in mitochondrial membrane permeabilization (32). Although Bcl-xL was traditionally considered a mitochondrial apoptosis-regulator, recent evidence shows that this protein is also present in other compartments such as the ER and can mediate apoptosis-independent functions, as described in Chapter 1. Furthermore, Bcl-xL can affect the intracellular endosomal compartments, which are critical for antigen processing and presentation (180).



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 Barral and Brenner *Nature Reviews Immunology* 7(12), 929-941 (December 2007)

Figure 3.1: CD1d-mediated antigen processing and presentation

CD1d molecules synthesized in the ER, travel to the cell surface via the secretory pathway. They are then internalized and traffic through the endocytic compartments, ultimately reaching the lysosomal compartment, also known as the MIIC. The stabilizing self-glycolipid can then be exchanged for an activating antigen and the recycled CD1d molecules are re-expressed on the cell surface.

In a study by Vento *et al.*, Bcl-xL has been reported to interact with various trafficking related proteins such as calnexin, Rab7, ER golgi intermediate compartment 1 (ERGIC1) and vesicle associated membrane protein 3 (VAMP3), underscoring the multi-faceted effects of Bcl-xL (181). Due to its strong upregulation in lymphomas and its diverse roles in apoptosis, ceramide metabolism and regulation of the endocytic pathway, we hypothesized that Bcl-xL plays a role in the regulation of CD1d-mediated antigen presentation to NKT cells. To examine such a role for Bcl-xL, we conducted a series of overexpression, induction, and knockdown studies. In addition, we examined the effect of pharmacological agents known to disrupt Bcl-xL function and assessed intracellular trafficking to endocytic compartments. Interestingly, we found that the mechanism of regulation is independent of surface CD1d expression, costimulatory molecules and CD1d recycling. Following Bcl-xL knock down, alterations in intracellular trafficking of CD1d were observed. In addition, the expression of Rab7, a late endosomal protein was increased and there was expansion of the late endosomal compartment. CD1d molecules were reduced in the early endosomes and lysosomes, and increased in the late endosomal compartment. These data show that in the absence of Bcl-xL, the expanded late endosomal compartment acts as a CD1d depot and alters antigen presentation, ultimately resulting in decreased NKT cell activation.

3.2 Results

Over-expression of Bcl-xL in antigen presenting cells leads to increased NKT cell responses

As described in Chapter 1, Bcl-xL is a pro-survival member of the Bcl-2 family of proteins and is highly expressed in cancers, especially lymphomas. Bcl-xL also plays important apoptosis-independent roles. Bcl-xL can interact with various trafficking proteins and can also regulate components of the endocytic pathway (180, 181). We therefore hypothesized that Bcl-xL affects the ability of an antigen presenting cell to present glycolipid antigens to NKT cells, independent of its regulation of apoptosis. To determine if the overexpression of Bcl-xL has an effect on CD1d mediated antigen presentation, we utilized WEHI-231, a classical and well-characterized murine B cell lymphoma cell line (182), and compared it to a WEHI-231-derived cell line stably transfected with murine Bcl-xL. Over-expression of Bcl-xL protein was first confirmed by Western blotting (Fig. 3.2 A). Next, the WEHI-231 controls and Bcl-xL transfectants were cocultured with two canonical $V\alpha 14J\alpha 18$ NKT cell hybridomas- DN32.D3 and N38-3C3. These NKT cells produce the T cell growth factor IL-2 upon activation, which is used as the read-out in this system. Following the coculture with WEHI-231 control and WEHI-231/Bcl-xL, we determined the levels of IL-2 in the supernatant by ELISA (Fig. 3.2 B and C). In the absence of exogenously added antigens, the WEHI-231 control, as well as Bcl-xL transfected cell lines, failed to activate NKT cells. These data suggest that the endogenous antigen presented by CD1d molecules in these cells does not activate NKT cells. However, when the potent NKT cell agonist, α -GalCer, was added to the co-

culture, Bcl-xL transfected cells were able to stimulate NKT cells, unlike the untransfected controls. These data demonstrate that CD1d molecules expressed on the cell surface of WEHI/Bcl-xL cells are functional, but these cells do not present an activating endogenous ligand. Furthermore, the presentation of an exogenous antigen is enhanced following the over-expression of Bcl-xL (Fig. 3.2 B and C). To characterize the potential role of Bcl-xL in CD1d-mediated antigen presentation we used LMTK cells as an *in vitro* antigen presenting cell system. LMTK cells, transfected with CD1d (LMTK-CD1d), are known to present an endogenous activating antigen to NKT cells (183). Furthermore, LMTK-CD1d cells can be cocultured with a panel of mouse NKT cell hybridomas, which produce high levels of IL-2 following activation, providing a straightforward system to study CD1d-mediated antigen presentation to NKT cells with minimal confounding factors. In order to directly test if Bcl-xL plays a role in antigen presentation to NKT cells, LMTK-CD1d cells were transfected with an empty control plasmid or a plasmid carrying the *Bcl2l1* gene, which encodes Bcl-xL, resulting in the over-expression of Bcl-xL. There were no evident differences in cell morphology, growth or proliferation (Fig. 3.3 A and B). Bcl-xL over-expression was confirmed by Western blotting (Fig. 3.4 A). Transfection with Bcl-xL did not dramatically alter surface CD1d expression as analyzed by flow cytometry (Fig. 3.4 B). These LMTK-CD1d cells transfected with Bcl-xL or the controls were cocultured with DN32.D3 and N38-3C3, which are canonical type I NKT cells and also N37-1A12, which is a type II NKT cell hybridoma (69, 153). We found that the over-expression of Bcl-xL in LMTK-CD1d cells elicited significantly higher response from all three hybridomas tested (Fig.3.4 C-E).

From these data, it is evident that Bcl-xL can affect NKT cell activation, since the over-expression of Bcl-xL leads to increased NKT cell responses. Furthermore, we used CD1d blocking antibodies and found that IL-2 production was completely lost following CD1d blockade in the control transfected as well as Bcl-xL transfected LMTK-CD1d cells (Fig. 3.5 A-C). This indicates that regulation of Bcl-xL is specific to CD1d-dependent antigen presentation to NKT cells.

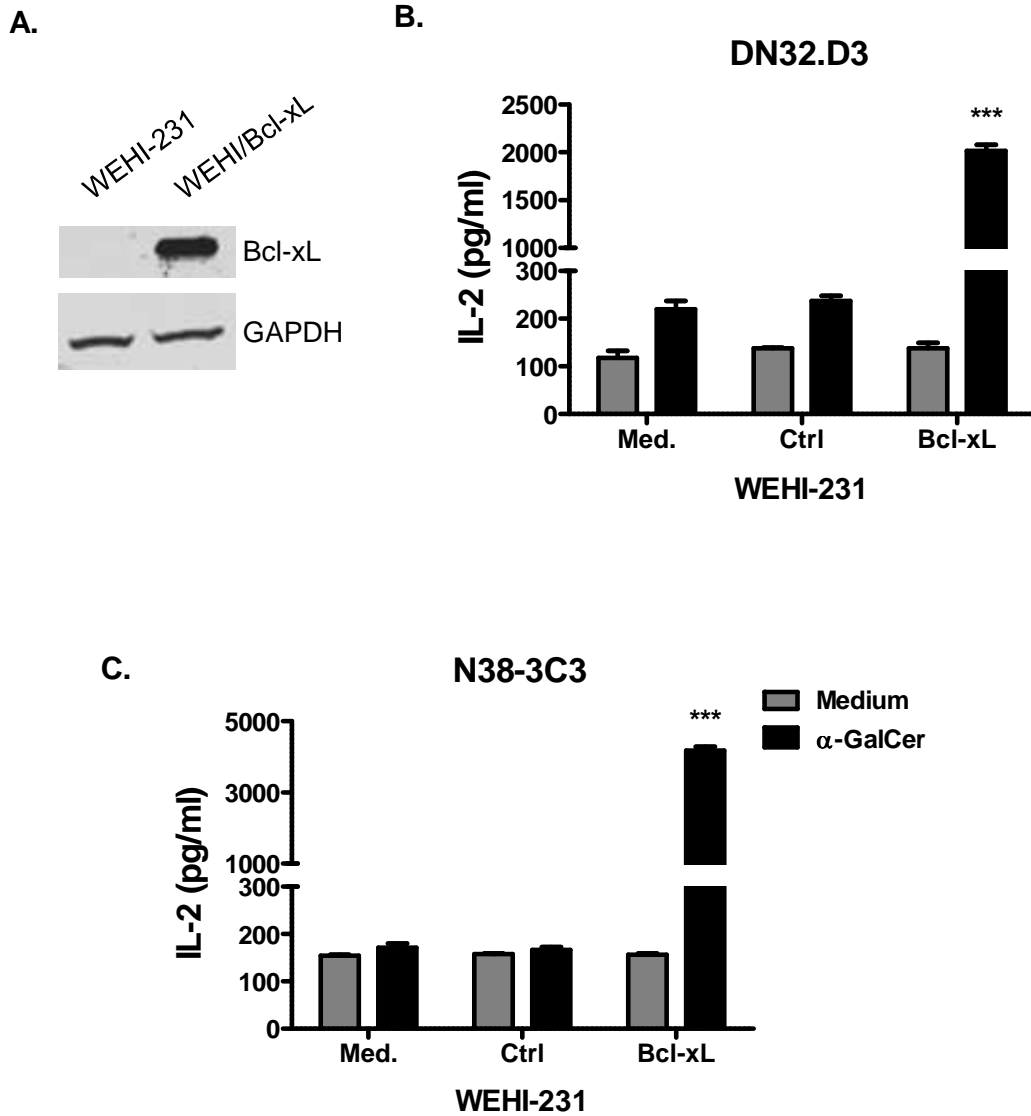


Figure 3.2: Over-expression of Bcl-xL in WEHI-231 cells leads to increased antigen presentation to NKT cells

(A) Western blot showing Bcl-xL protein levels (upper panel) in WEHI-231 control or Bcl-xL over-expressing cells. GAPDH (lower panel) is the loading control. (B-C) Medium alone, WEHI-231 control or Bcl-xL over-expressing cells were cocultured with (B) DN32.D3 or (C) N38-3C3 NKT cell hybridomas in the presence or absence of α -GalCer. IL-2 was measured in the supernatant by ELISA.

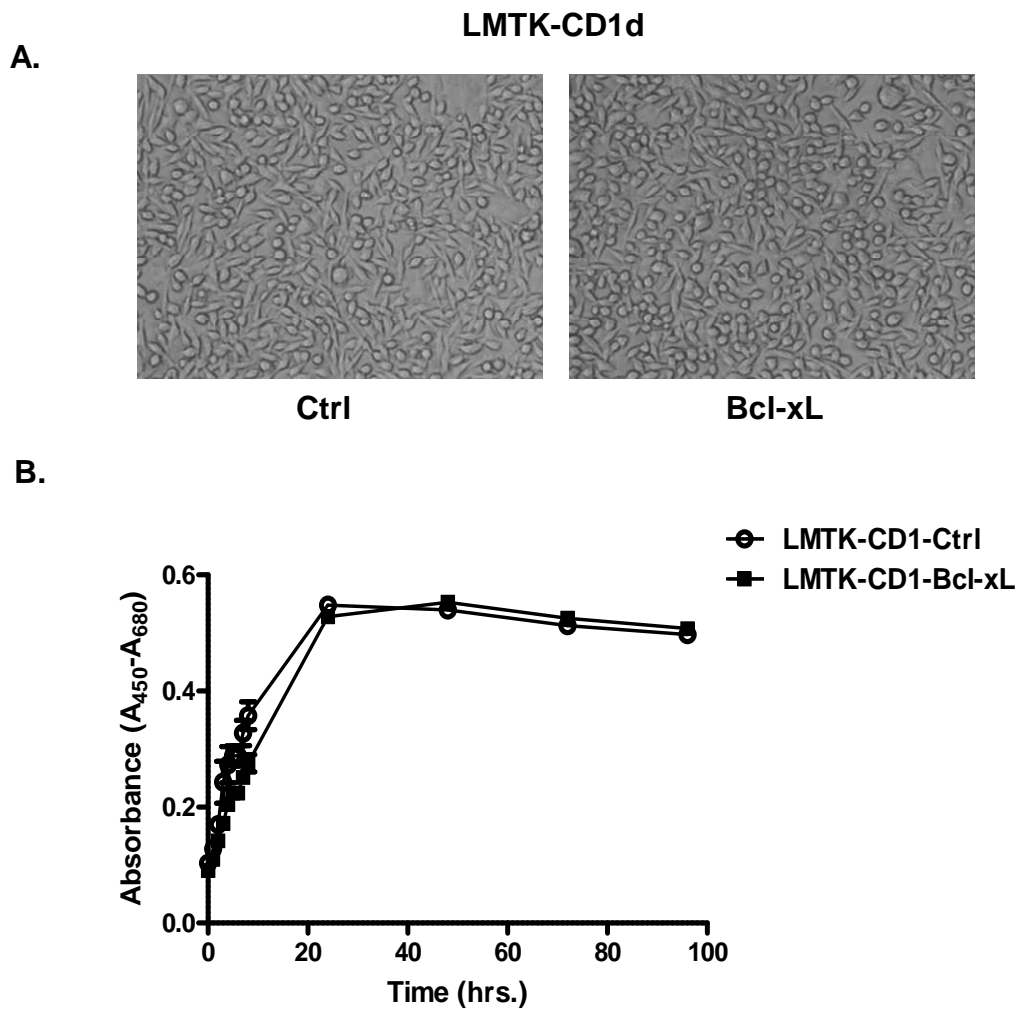


Figure 3.3: Over-expression of Bcl-xL does not alter cell size, morphology, viability or proliferation

(A) LMTK-CD1d cells transfected with empty vector alone or vector encoding Bcl-xL were cultured in cell culture grade flasks in complete medium, and imaged under a light microscope. (B) Control or Bcl-xL over-expressing LMTK-CD1d cells were incubated with WST-1 cell viability and proliferation assay reagent for the indicated time periods. The difference between absorbance at 450 nm and 690 nm is represented.

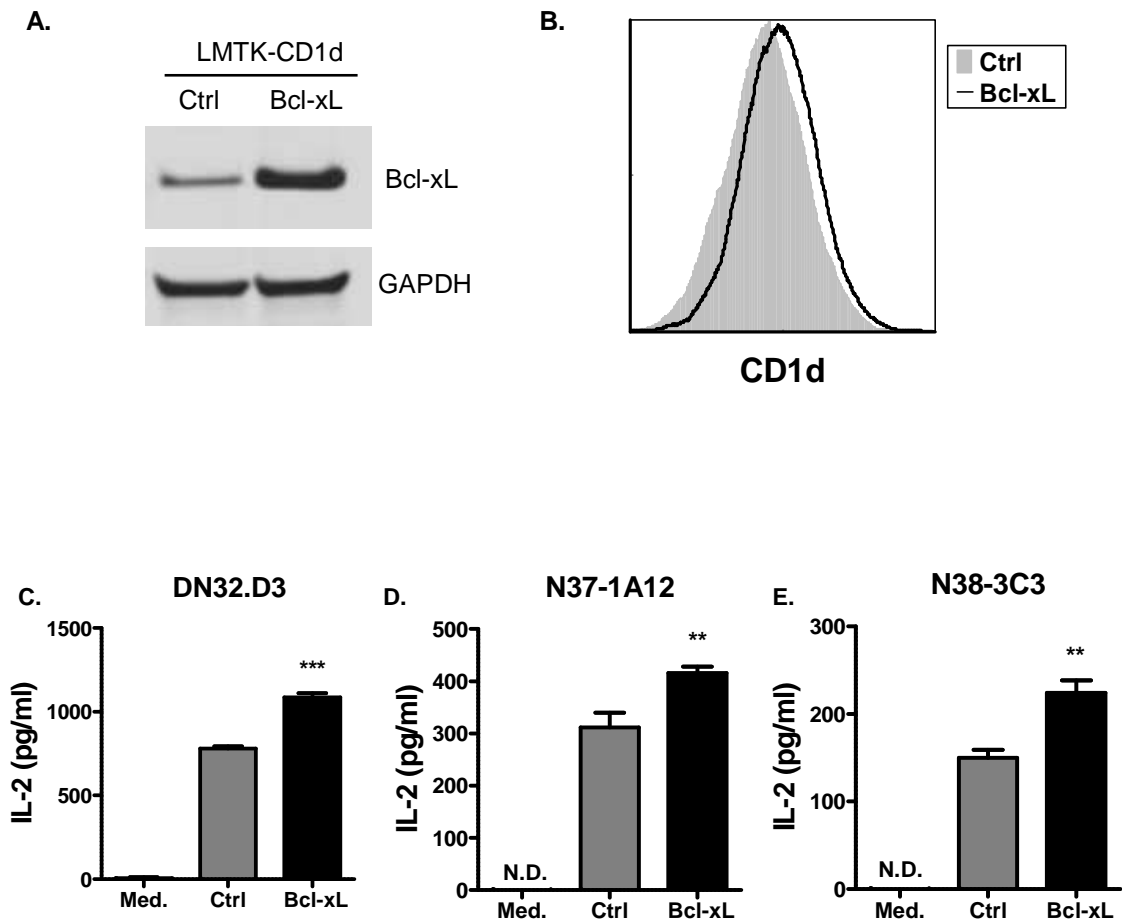


Figure 3.4: Over-expression of Bcl-xL in LMTK-CD1d cells leads to increased CD1d-mediated antigen presentation to NKT cells

(A) Western blot showing Bcl-xL protein levels (upper panel) in LMTK-CD1d cells transfected with empty vector (ctrl) or Bcl-xL. GAPDH (lower panel) is the loading control. (B) Flow cytometry shows surface CD1d expression on LMTK-CD1d cells transfected with Bcl-xL (open histogram) or vector alone (shaded histogram). (C-E) LMTK-CD1d cells transfected with empty vector or vector encoding Bcl-xL were cocultured with (C) DN32.D3, (D) N37-1A12 or (E) N38-3C3 NKT cell hybridomas. IL-2 in the supernatant was measured by ELISA.

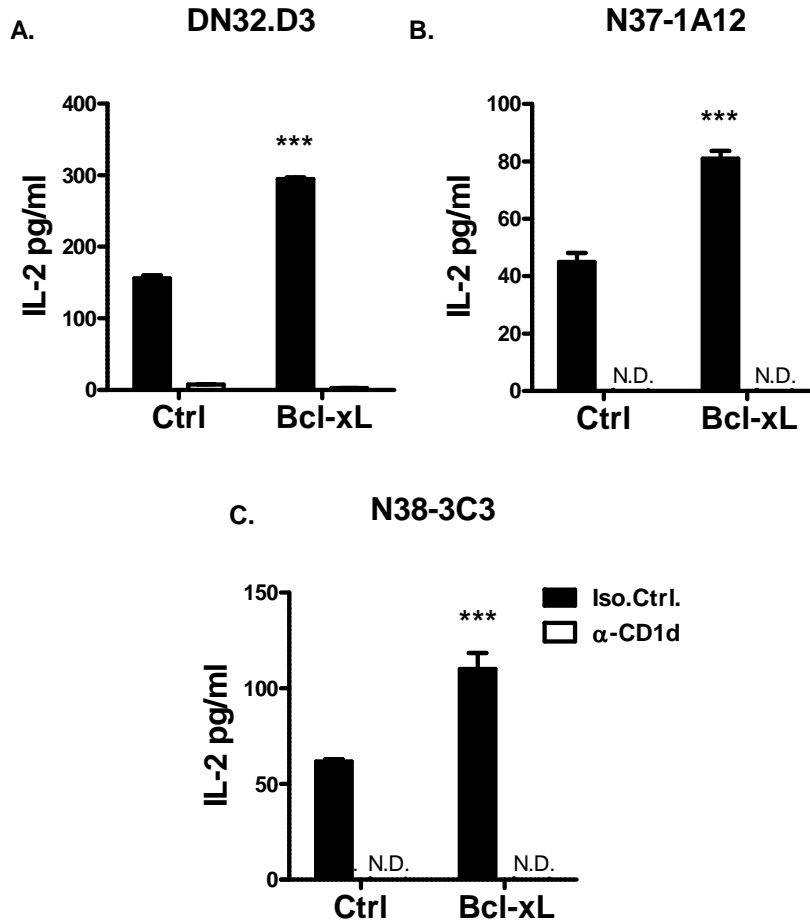


Figure 3.5: Bcl-xL-mediated regulation of NKT cell responses is CD1d-dependent
 (A-C) LMTK-CD1d control and Bcl-xL over-expressing cells were treated with anti-CD1d blocking antibodies prior to the addition of (A) DN32.D3, (B) N37-1A12 or (C) N38-3C3 NKT cell hybridomas. IL-2 in the supernatant was measured by ELISA.

Induction of Bcl-xL by CD40 stimulation in B cells leads to increased NKT cell activation

We found that the over-expression of Bcl-xL in antigen presenting cells leads to enhanced antigen presentation to NKT cells. Next, we wanted to induce Bcl-xL using a biological stimulus and determine if antigen presentation to NKT cells was affected. One of the most well-known aspects of B cell stimulation with anti-CD40 antibody is the upregulation of Bcl-xL, and has been reported extensively in WEHI-231 cells, primary mouse B cells and also in human B cells (184-187). We treated WEHI-231 cells with anti-CD40 antibody (clone 1C10) and confirmed the induction of Bcl-xL protein by Western blotting (Fig. 3.6 A). We found that treatment with anti-CD40 antibody did not alter surface CD1d expression as determined by flow cytometry (Fig. 3.6 B). When cocultured with NKT cell hybridomas in the presence of α -GalCer, WEHI-231 cells treated with anti-CD40 antibody elicited higher NKT cell activation compared to cells treated with isotype control antibody (Fig. 3.6 C and D). This shows that the induction of Bcl-xL in a B cell lymphoma cell line leads to increased NKT cell responses. These results are consistent with the data obtained from over-expression of Bcl-xL (Fig.3.2 and Fig. 3.4), and show that increased Bcl-xL levels lead to a corresponding increase in CD1d-mediated antigen presentation to NKT cells.

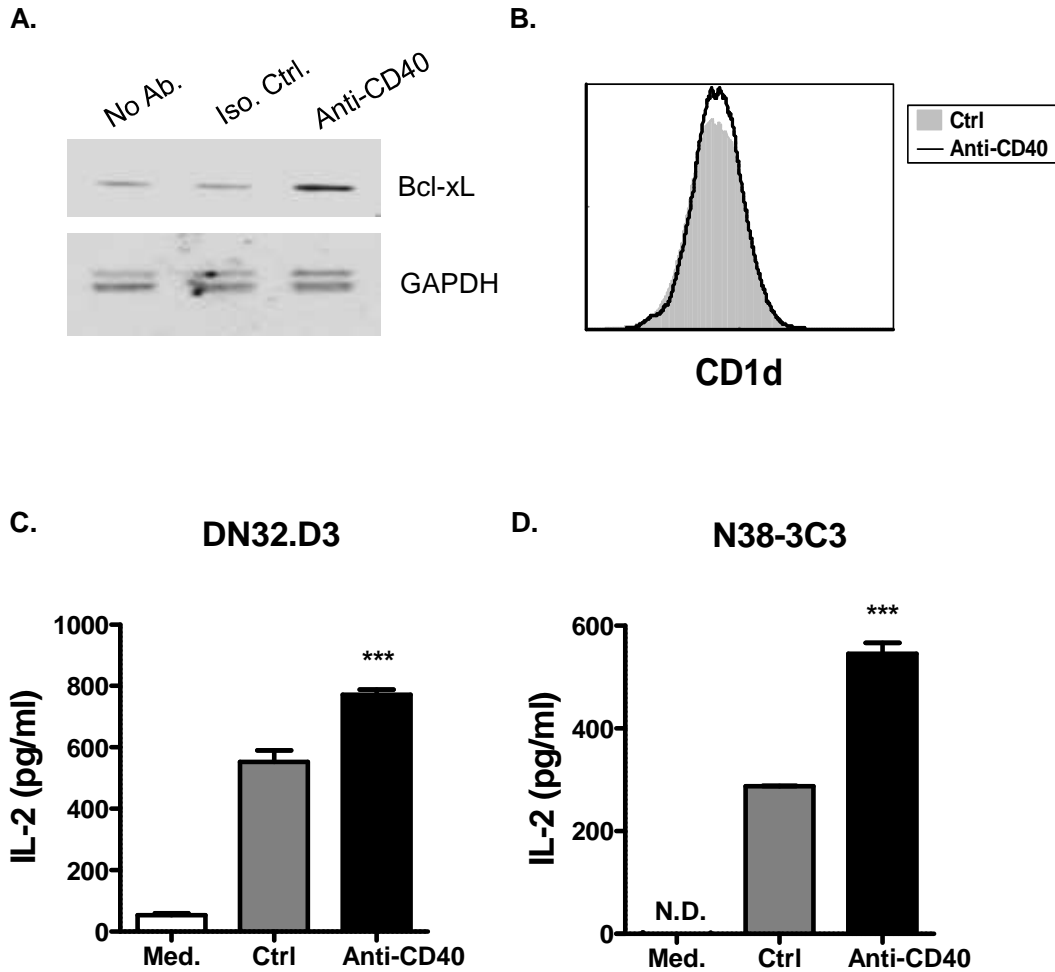


Figure 3.6 Treatment of WEHI-231 with anti-CD40 antibody leads to increased CD1d-mediated antigen presentation to NKT cells

WEHI-231 cells were treated with anti-CD40 antibody or an isotype control antibody for 24 hours. (A) Western blotting shows Bcl-xL protein levels (upper panel) after treatment. GAPDH (lower panel) is the loading control. (B) Flow cytometry showing surface CD1d expression following treatment with isotype control antibody (shaded histogram) or anti-CD40 antibody (black histogram). (C-D) Medium alone or WEHI-231 cells treated with isotype control antibody or anti-CD40 antibody were cocultured with (C) DN32.D3 or (D) N38-3C3 NKT cell hybridomas in the presence of α -GalCer. IL-2 in the supernatant was measured by ELISA.

Bcl-xL-mediated regulation of antigen presentation in primary B cells

To eliminate the possibility of cell line specific effects and validate the results in primary mouse cells, we treated freshly isolated splenic B cells with anti-CD40 and cocultured them with liver mononuclear cells as a source of primary NKT cells (Fig. 3.7). Approximately 40% of T cells in the liver are NKT cells, as compared to the thymus and spleen (1-2%). Also, liver NKT cells are the only subset known to mediate anti-tumor effector functions (188). To study antigen presentation using primary cells, we isolated B cells from murine splenocytes and ensured purity by B220 expression by flow cytometry (Fig. 2.1 A). We obtained the liver mononuclear cells and used antibodies to the TCR β chain and α -GalCer loaded CD1d tetramers to determine NKT cell percentage (Fig. 2.1 B). For the induction of Bcl-xL in B cells, we treated purified primary splenic B cells with anti-CD40 antibody for 24 hours. Western blotting was used to ensure the upregulation of Bcl-xL protein (Fig. 3.8 A). Following treatment, cells were washed and cocultured with liver mononuclear cells in the presence or absence of α -GalCer. NKT cell activation was assessed by measuring IFN- γ or IL-4 in the supernatant by ELISA (Fig. 3.8 B and C). There was no detectable cytokine production in the absence of α -GalCer demonstrating that the observed responses were NKT cell specific. In the presence of α -GalCer, anti-CD40 treated B cells led to increased IFN- γ production by NKT cells as compared to their untreated or isotype control-treated counterparts (Fig. 3.8 B). However, IL-4 production was unchanged after treatment with anti-CD40 antibody (Fig. 3.8 C). All cytokine levels were detectable only in the presence of NKT cell antigens. This indicates that the responses observed are NKT cell driven, in spite of the use of total liver

mononuclear cells in the coculture. In addition, we analyzed the cells by flow cytometry following treatment and found no changes in the surface expression of MHC class I, CD1d, CD80, or CD86 (Fig.3.9 A-D). Together, these data show that Bcl-xL plays a role in CD1d-mediated antigen processing and presentation to NKT cells in an *ex vivo* system using primary mouse B cells and NKT cells.

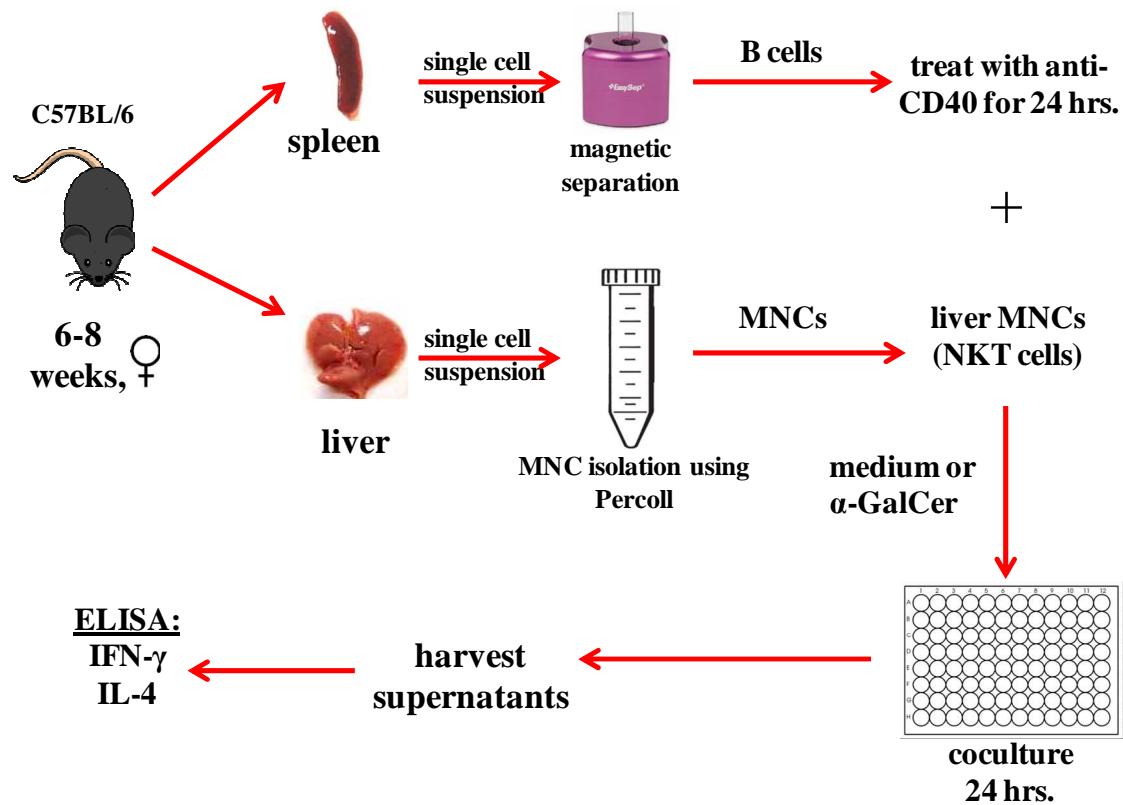


Figure 3.7: Bcl-xL induction in primary mouse B cells

Spleens and livers from C57BL/6 mice were obtained. B cells were isolated from splenocytes using a mouse B cell enrichment kit and treated with anti-CD40 antibody (10 μ g/ml) or an isotype control for 24 hours. Liver mononuclear cells were separated using a Percoll gradient and used as a source of primary NKT cells. Anti-CD40 treated B cells were cocultured with liver mononuclear cells with or without α -GalCer. After 24 hours, IFN- γ and IL-4 were measured in the supernatant by ELISA.

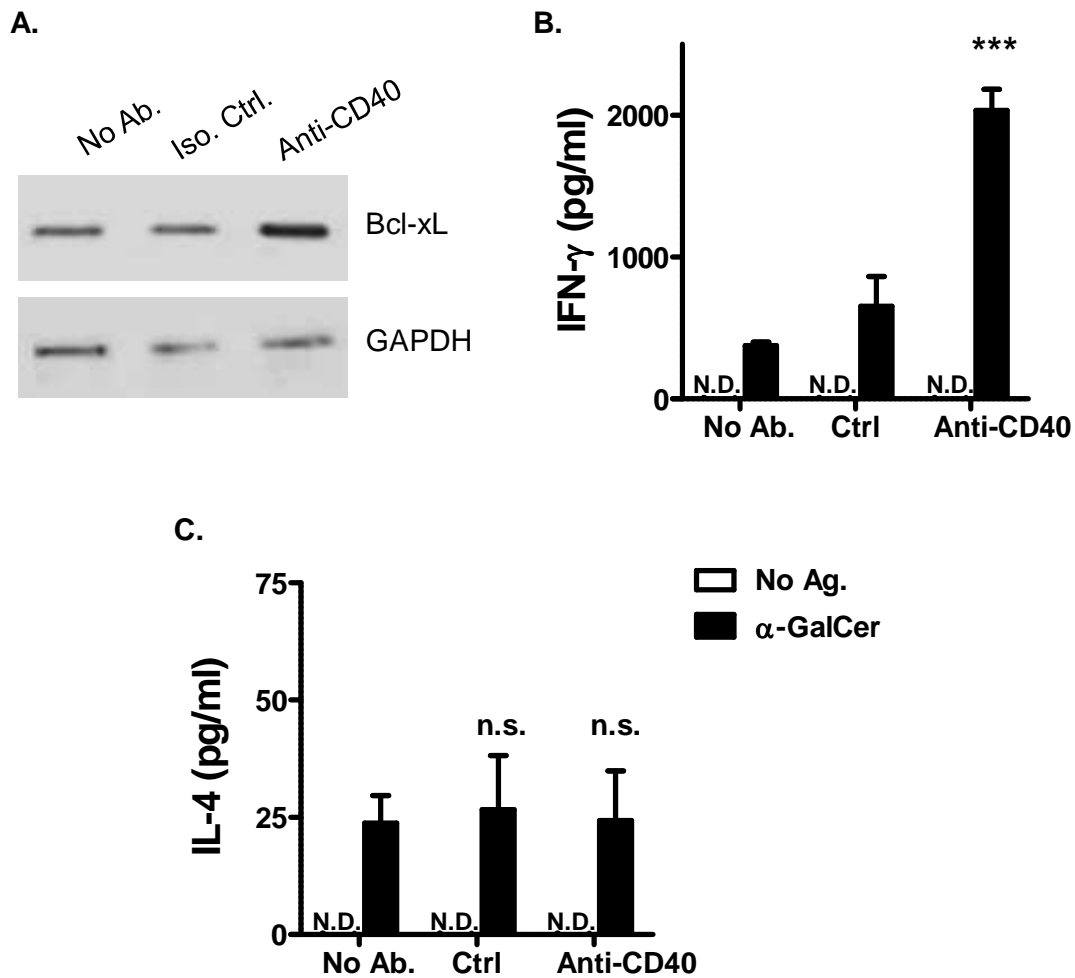


Figure 3.8: Treatment of primary mouse B cells with anti-CD40 antibody leads to increased antigen presentation to primary mouse liver NKT cells

(A) Western blot showing Bcl-xL induction (upper panel) following treatment of primary mouse B cells with anti-CD40 antibody for 24 hours. GAPDH (lower panel) is the loading control. (B-C) Control or Anti-CD40 treated primary B cells were cocultured with liver mononuclear cells in the presence or absence of α -GalCer. (B) IFN- γ or (C) IL-4 was measured in the supernatant by ELISA.

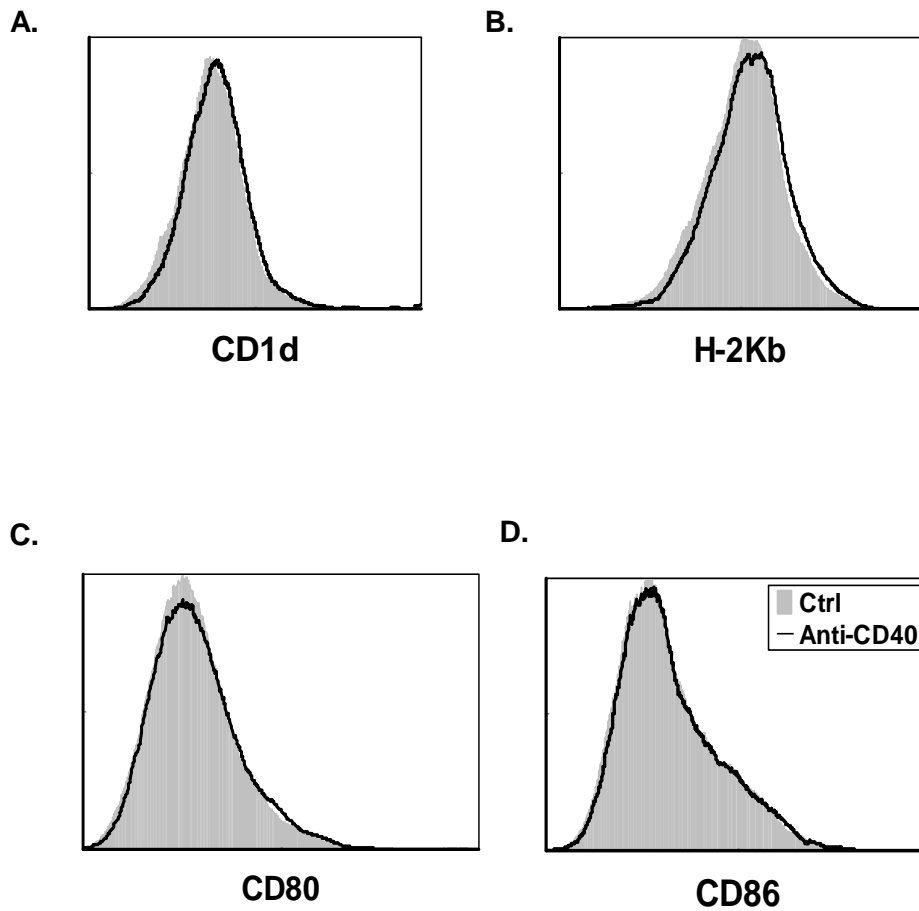


Figure 3.9: Cell surface expression of CD1d, MHC class I and classic costimulatory molecules is unchanged following treatment with anti-CD40 antibody

Primary splenic mouse B cells were treated with anti-CD40 or an isotype control antibody for 24 hours. Cell surface expression of (A) CD1d, (B) MHC class I (H-2K^b), (C) CD80 and (D) CD86 was determined by flow cytometry.

Pharmacological inhibition of Bcl-xL leads to reduced NKT cell responses

In order to study the effect of Bcl-xL inhibition on CD1d-mediated antigen presentation to NKT cells, we used small molecule inhibitors that bind the Bcl-2 Homology 3 (BH3) domain of Bcl-xL. BH3-BH3 interactions between proteins of the Bcl-2 family are critical for their function. We treated L-CD1d cells with two small molecule BH3 mimetics, ABT-263 and ABT-199 (36, 37) which are functional inhibitors. ABT-263 targets both Bcl-xL and Bcl-2, while ABT-199 is Bcl-2 specific. We treated L-CD1d-DR4 cells with ABT-263 or ABT-199 for 4 hours and used Western blotting for Bcl-xL (Fig. 3.10 A). Following the drug treatment, cells were washed extensively and cocultured with NKT cell hybridomas (Fig. 3.10 B). We found that inhibition of Bcl-xL/Bcl-2 using ABT-263 led to decreased CD1d-mediated antigen presentation to NKT cells. In contrast, inhibition of Bcl-2 only by treating with ABT-199 had no effect on antigen presentation to NKT cells (Fig. 3.10 B). These data demonstrate that BH3 domain-based functional inhibition of Bcl-xL but not Bcl-2 leads to reduced CD1d-mediated antigen presentation to NKT cells. Since ABT-263 and ABT-199 can induce apoptosis at higher concentrations, cell viability was determined by Annexin V-PI staining, where Annexin V⁺ PI⁺ cells were considered apoptotic and the remainder of cells were considered viable (single positive cells were minimal). There were no differences in viability between treated and untreated groups, indicating that our selected treatment concentrations were not sufficient to induce cell death and the role of Bcl-xL in CD1d-mediated antigen presentation is independent of apoptosis (Fig. 3.11 A). Also, there were no evident changes in cell surface expression of CD1d following treatment

with ABT-263 or ABT-199, as determined by flow cytometry (Fig. 3.11 B). The antigen presentation pathway of CD1d is similar to classical MHC class II in that it involves the lysosomal or MHC class II compartment (MIIC). CD1d molecules are recycled from the cell surface and end up in the MIIC, while MHC class II molecules are transported to this compartment from the golgi by the Ii (Section 3.1). However, antigen presentation by both CD1d and MHC class II requires trafficking to the lysosomes or MIIC. We sought to determine the effect of Bcl-xL inhibition on peptide antigen presentation to classical CD4⁺ T cells by MHC class II molecules (189). L-CD1d-DR4 cells loaded with human serum albumin (HSA) were treated with ABT-263 or ABT-199, washed extensively and cocultured with the 17.9 T cell hybridoma line (Fig. 3.12 A). Similar to CD1d-mediated antigen presentation, we found that the presentation of HSA was reduced following treatment with ABT-263 but was unaffected by treatment with ABT-199. Surface expression of HLA-DR was determined by flow cytometry and found to be unchanged (Fig. 3.12 B). Thus, the pharmacological inhibition of Bcl-xL leads to decreased CD1d-mediated antigen presentation to NKT cells, as well as MHC class II-mediated antigen presentation to classical CD4⁺ T cells.

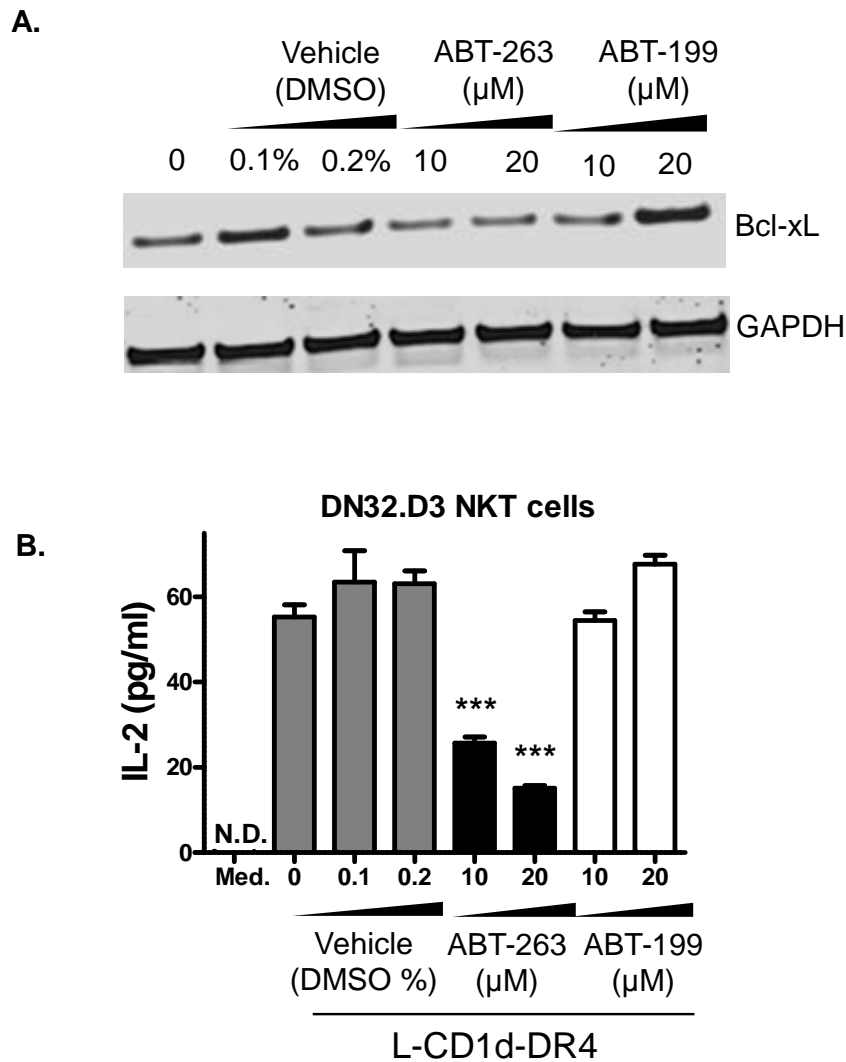


Figure 3.10: Pharmacological inhibition of Bcl-xL and Bcl-2 but not Bcl-2 alone leads to reduced antigen presentation to NKT cells

(A) L-CD1d cells were treated with vehicle alone (DMSO), ABT-263 or ABT-199 for four hours. Western blotting shows Bcl-xL protein levels (upper panel). GAPDH (lower panel) is the loading control. (B) After treatment, cells were washed extensively and cocultured with DN32.D3 NKT cell hybridomas. After 24 hours, IL-2 released in the supernatant was measured by ELISA

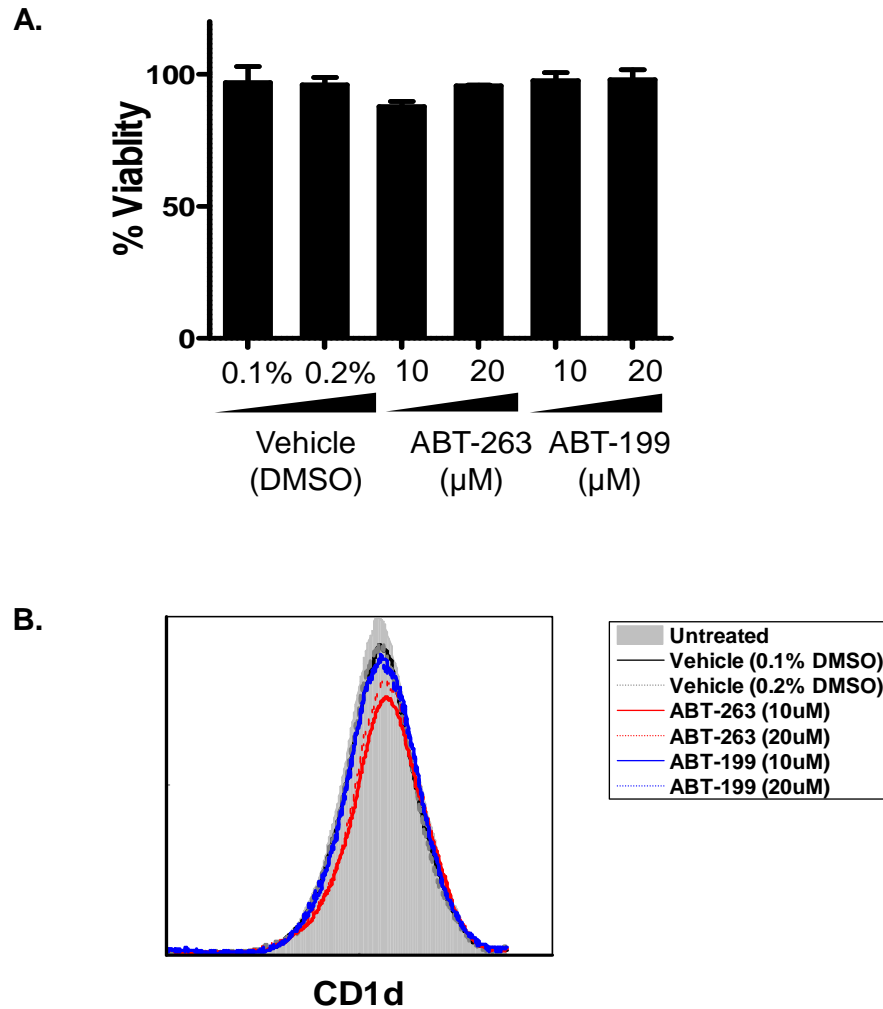


Figure 3.11: Cell viability and surface CD1d expression are unchanged after treatment with Bcl-xL/Bcl-2 inhibitors

(A) L-CD1d cells treated with vehicle alone, ABT-263 or ABT-199 for 4 hours were stained for AnnexinV and PI. AnnexinV⁺PI⁻ cells were considered viable and represented as percent of untreated control. (AnnexinV⁺ or PI⁺ single positive cells were minimal). (B) Following treatment with ABT-263 or ABT-199, surface CD1d expression was determined by flow cytometry.

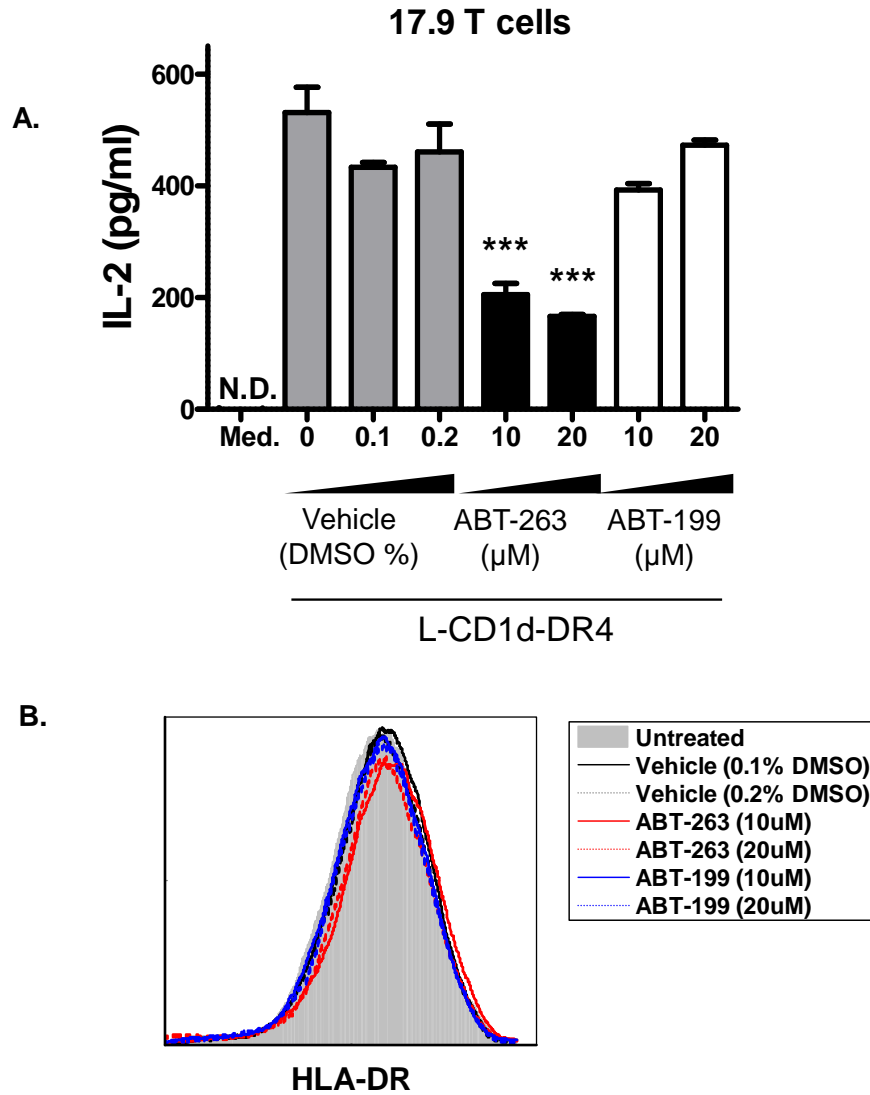


Figure 3.12: Pharmacological inhibition of Bcl-xL and Bcl-2 but not Bcl-2 alone leads to reduced antigen presentation by MHC Class II to classical CD4⁺ T cells

(A) L-CD1d-DR4 cells were treated with vehicle alone (DMSO), ABT-263 or ABT-199 for four hours, washed extensively and cocultured with DN32.D3 NKT cell hybridomas. After 24 hours, IL-2 released in the supernatant was measured by ELISA. (B) After treatment with ABT-263 or ABT-199, surface HLA-DR expression was determined by flow cytometry.

Stable knock-down of Bcl-xL causes inhibition of CD1d-mediated antigen presentation to NKT cells

L-CD1d cells express high levels of Bcl-xL, therefore it is a good system to investigate the effects of Bcl-xL knockdown on CD1d-mediated antigen processing and presentation. We used lentiviral particles carrying a pool of shRNA sequences targeting the *Bcl2l1* or the *Bcl2* gene which encode the Bcl-xL and Bcl-2 protein respectively. After transduction, stable cell lines were generated and maintained in culture. Cells were regularly passaged at the same rate and there were no apparent differences in cell morphology, growth or proliferation following stable knock down (Fig. 3.13 A and B). Bcl-xL or Bcl-2 knock down was confirmed by Western blotting (Fig. 3.14 A). There were no evident changes in surface CD1d expression following transduction and selection of stable integrants, as determined by flow cytometric analyses (Fig. 3.14 B). Following the knockdown of Bcl-xL or Bcl-2, L-CD1d cells were cocultured with NKT cell hybridomas. Cells transduced with scramble control shRNA were used as controls. The knockdown of Bcl-xL or Bcl-2 led to significantly decreased NKT cell activation as indicated by a reduction in IL-2 production by both DN32.D3 and N37-1A12 NKT cell hybridomas (Fig. 3.14 C and D).

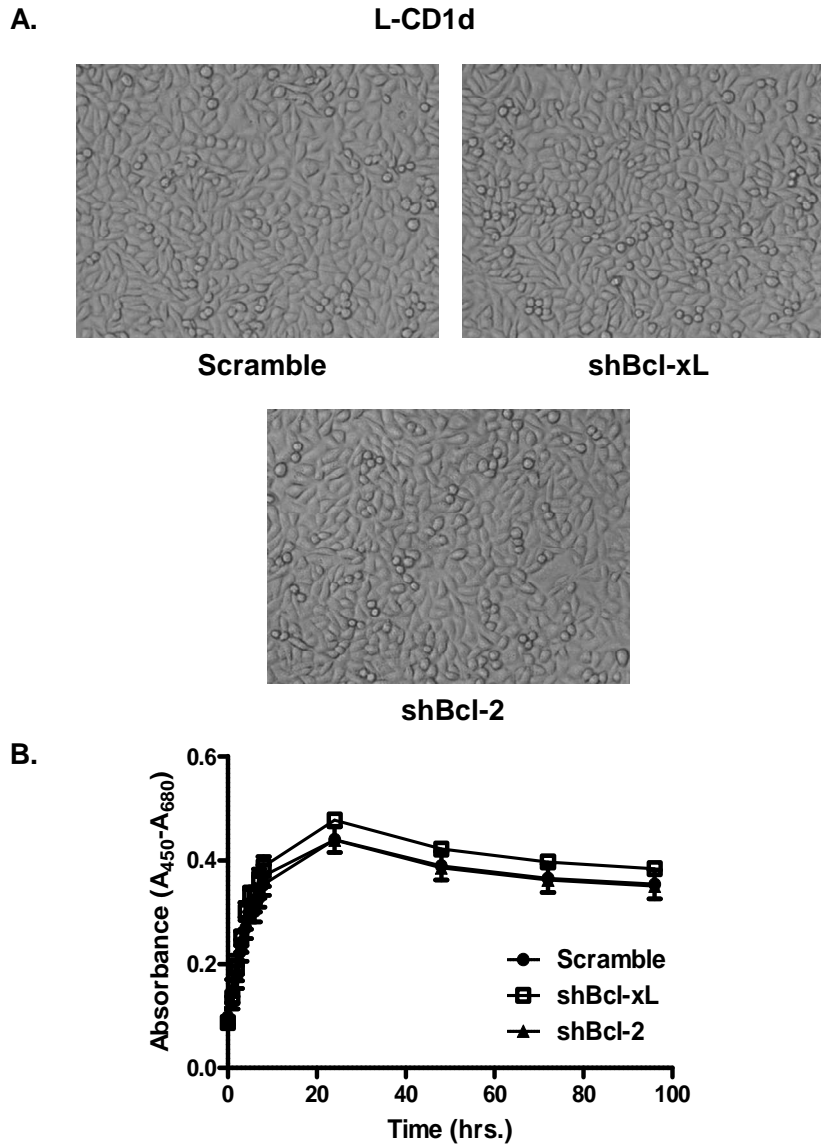


Figure 3.13: shRNA-mediated knockdown of Bcl-xL or Bcl-2 does not alter cell size, morphology, viability or proliferation

(A) L-CD1d cells transduced with lentiviral particles carrying a scramble control or shRNA targeting Bcl-xL or Bcl-2, were cultured cell culture treated flasks in complete medium, and imaged under a light microscope. (B) Control or Bcl-xL over-expressing LMTK-CD1d cells were incubated with WST-1 cell viability and proliferation assay reagent for the indicated time periods. The difference between absorbance at 450 nm and 690 nm is represented.

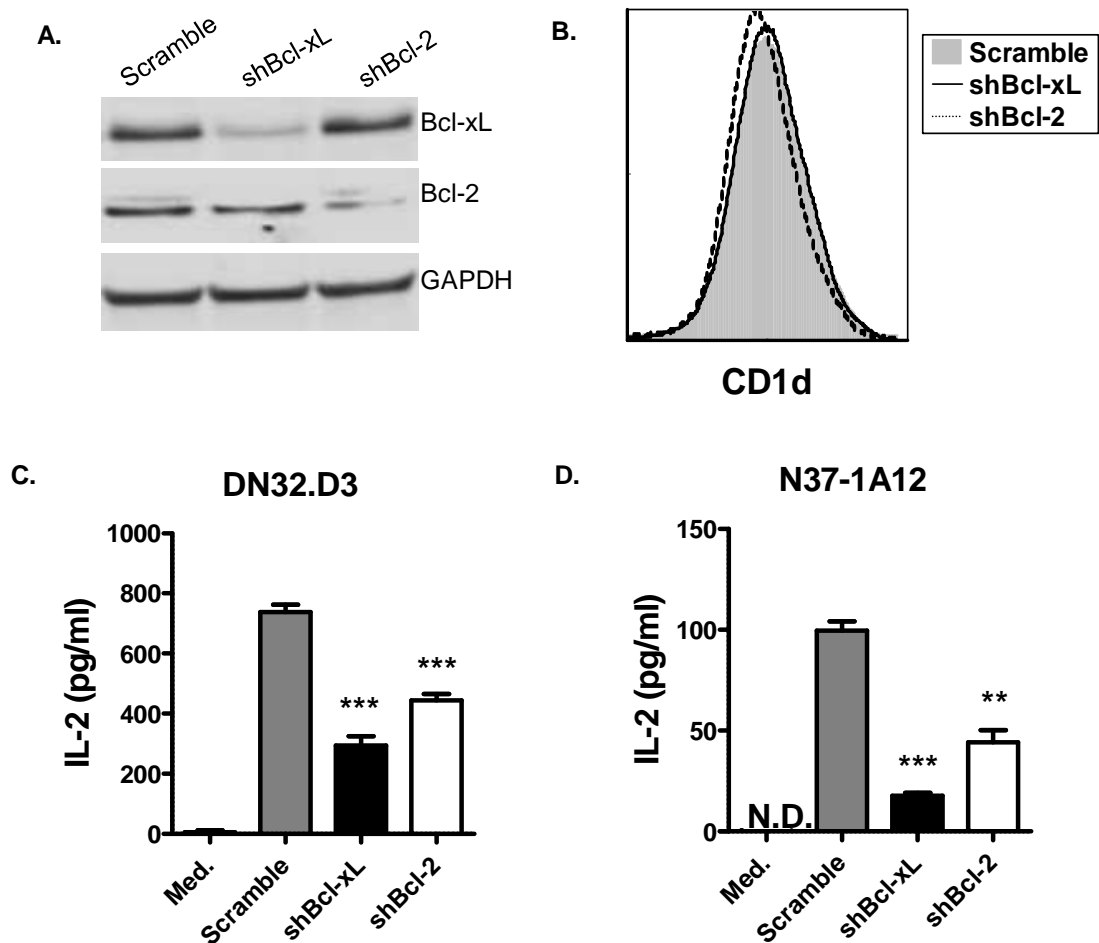


Figure 3.14: shRNA-mediated knockdown of Bcl-xL or Bcl-2 leads to reduced CD1d-mediated antigen presentation to NKT cells

(A) Western blot showing Bcl-xL (upper panel) or Bcl-2 (middle panel) protein in L-CD1d cells transduced with lentiviral particles carrying a scramble control or shRNA targeting Bcl-xL or Bcl-2. GAPDH (lower panel) is the loading control. (B) Cell surface CD1d following the knockdown of Bcl-xL or Bcl-2 in L-CD1d cells was determined by flow cytometry. (C-D) After stable knockdown of Bcl-xL or Bcl-2, L-CD1d cells were cocultured with (C) DN32.D3 or (D) N37-1A12 NKT cell hybridomas and IL-2 in the supernatant was measured by ELISA.

Bcl-xL regulates intracellular CD1d trafficking

Bcl-xL knockdown results in a significant reduction in CD1d-mediated antigen presentation to NKT cells; however, the levels of surface CD1d expression are comparable to the controls. In order to address the possibility of time-dependent changes in CD1d expression, we incubated control or Bcl-xL/Bcl-2 knockdown L-CD1d cells with α -GalCer for 0, 2, 4, 8, 16 and 24 hours. Surface expression of CD1d and CD1d: α -GalCer complexes was determined using antibodies to CD1d alone (clone 1B1) or in complex with α -GalCer (clone L363). We found no striking differences between the scramble transduced cells and cells in which Bcl-xL or Bcl-2 was knocked down, indicating that the observed effects cannot be attributed to subtle temporal changes in CD1d expression (Fig. 3.15 A and B). The over-expression or knockdown of Bcl-xL altered the activation of N37-1A12, a type II NKT cell hybridoma. These NKT cells do not require CD1d recycling for antigen presentation, suggesting that this is not the mechanism by which Bcl-xL mediates its regulation. We therefore hypothesized that Bcl-xL alters the intracellular trafficking of CD1d molecules. To determine the precise mechanism of regulation, we first analyzed the intracellular localization of CD1d in L-CD1d cells after Bcl-xL knockdown. Under normal conditions, CD1d is localized in the lysosomal compartment, as seen by its colocalization with LAMP1 (lysosome associated membrane protein 1, CD107a) (190). We used confocal microscopy to determine the intracellular location of CD1d following Bcl-xL knockdown. As expected, control L-CD1d cells (scramble) showed strong colocalization of CD1d with LAMP1 (Fig. 3.16 A). Interestingly, after Bcl-xL knockdown (shBcl-xL) this colocalization was significantly

reduced (Fig. 3.16 A and B). These data show that following Bcl-xL knockdown, there is a decrease in CD1d molecules in the LAMP1⁺ compartment, and this is at least one mechanism by which Bcl-xL regulates antigen presentation to NKT cells. Thus, Bcl-xL can regulate the intracellular trafficking of CD1d molecules to the LAMP1⁺ lysosomal compartment, also known as the MIIC.

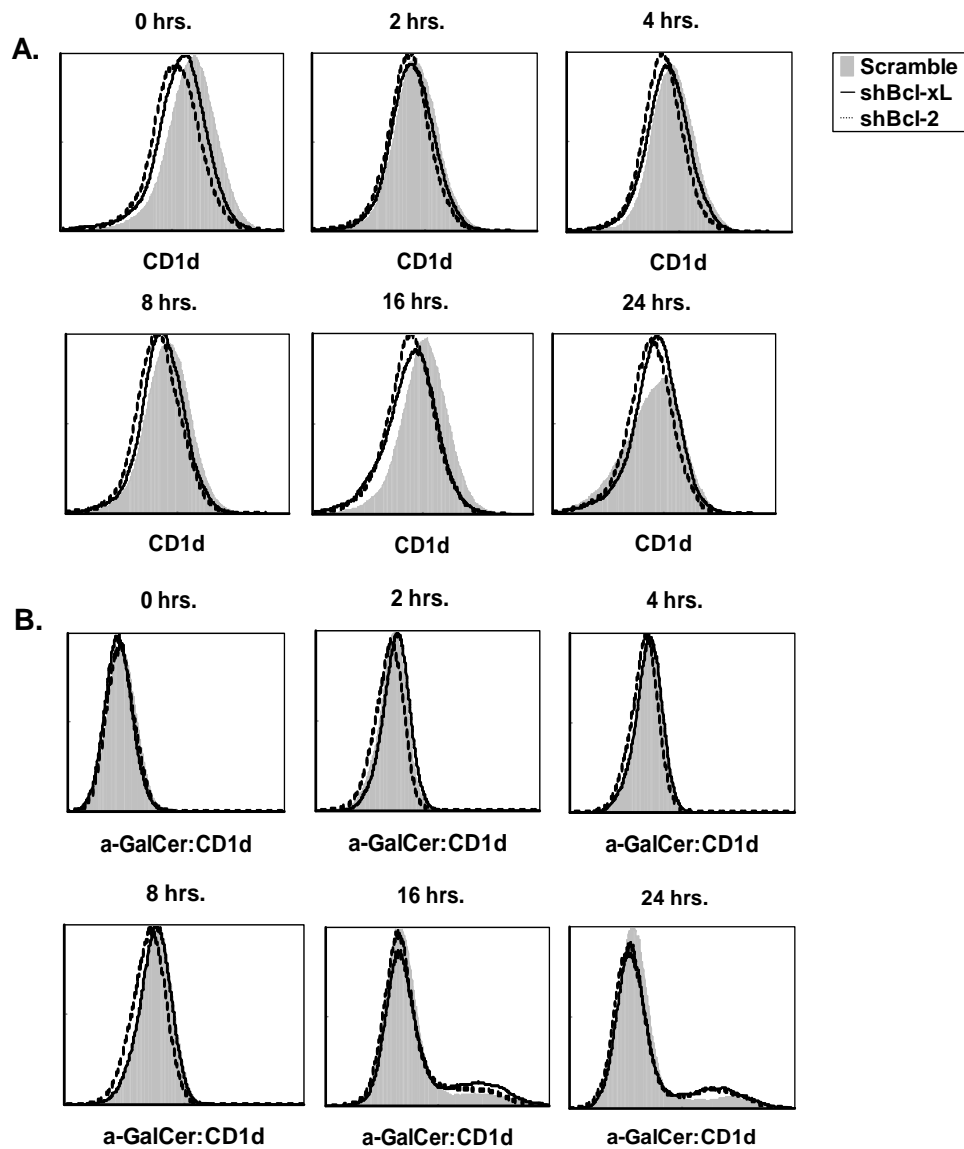


Figure 3.15: Surface expression of CD1d and CD1d:α-GalCer complexes at multiple time points is unchanged after knockdown of Bcl-xL or Bcl-2

L-CD1d cells transduced with lentiviral particles carrying a scramble control or shRNA targeting Bcl-xL or Bcl-2 were incubated with α-GalCer for the indicated time periods. Cell surface expression of (A) CD1d and (B) CD1d:α-GalCer complexes was determined by flow cytometry.

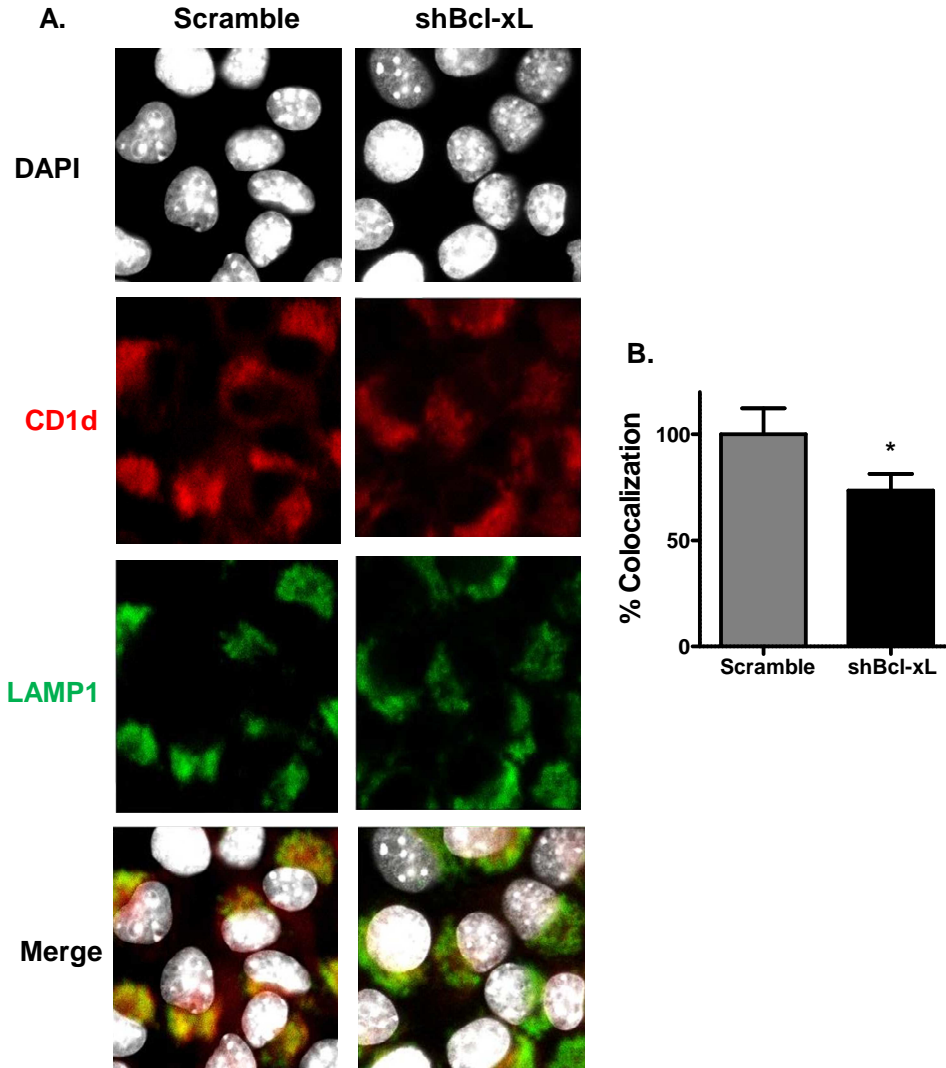


Figure 3.16: Reduced expression of CD1d molecules in the LAMP1⁺ lysosomal compartment following Bcl-xL knockdown

(A) Confocal microscopy was used to determine the intracellular localization of CD1d. L-CD1d cells transduced with a scramble shRNA (left column) or an shRNA sequence targeting Bcl-xL (shBcl-xL, right column) were fixed, permeabilized and stained with antibodies to CD1d (red), and LAMP1 (green), a marker of the lysosomal compartment. DAPI (white) was used to visualize the nucleus. The merge (lowermost panels) show that CD1d is localized in the LAMP1⁺ late endosomal/lysosomal compartment in the control (yellow) while this localization is reduced following Bcl-xL knockdown. (B) Quantification of CD1d and LAMP1 colocalization in scramble control or Bcl-xL knockdown cells.

Bcl-xL causes expansion of the Rab7⁺ late endosomal compartment

We found that Bcl-xL knockdown leads to the loss of CD1d from the LAMP1⁺ compartment (Fig. 3.16 A and B). We further sought to determine an alternative intracellular compartment where CD1d molecules accumulate following Bcl-xL knockdown. We found that similar to the LAMP1⁺ compartment, there was reduced colocalization of CD1d with EEA1, a marker for early endosomes, following Bcl-xL knockdown (Fig. 3.17 A and B). This shows that CD1d molecules are reduced in the LAMP1⁺ lysosomes as well as the EEA1⁺ early endosomes, indicating that CD1d molecules might have accumulated in a different intracellular compartment, impairing antigen presentation after Bcl-xL knockdown.

A previous report has shown that Bcl-xL can bind to Rab7, which is a Ras-related GTP binding protein present in late endosomes (191). Rab7 is critical to endosome biogenesis and the endocytic pathway. We found that there was greater Rab7 staining after Bcl-xL knockdown as compared to the scramble control (Fig. 3.18 A and B). The increased Rab7 staining indicates an expansion of the late endosomal compartment, while the probability of colocalization of CD1d and Rab7 remained unchanged, suggesting that following Bcl-xL knockdown, CD1d molecules may be accumulating in the late endosomes (Fig. 3.18 A, B and C). We also examined Rab7 protein levels by Western blotting and found that following Bcl-xL knockdown, Rab7 protein was increased as compared to the scramble (Fig. 3.18 D). The knockdown of Bcl-2 did not cause any change in Rab7 expression, as seen by Western blotting (Fig. 3.18 D). Thus, knocking down Bcl-xL leads to up-

regulation of Rab7 and expansion of the late endosomal compartment. Furthermore, this expanded endosomal compartment acts as a depot for CD1d molecules, resulting in impaired trafficking to the lysosomal compartment, which is critical for antigen presentation (3.19). The results indicate that under normal conditions, Bcl-xL regulates the endosomes and limits the size of the late endosomal compartment. When Bcl-xL is reduced, this regulation is lost, leading to upregulation of Rab7 and expansion of the late endosomal compartment (Fig. 3.19).

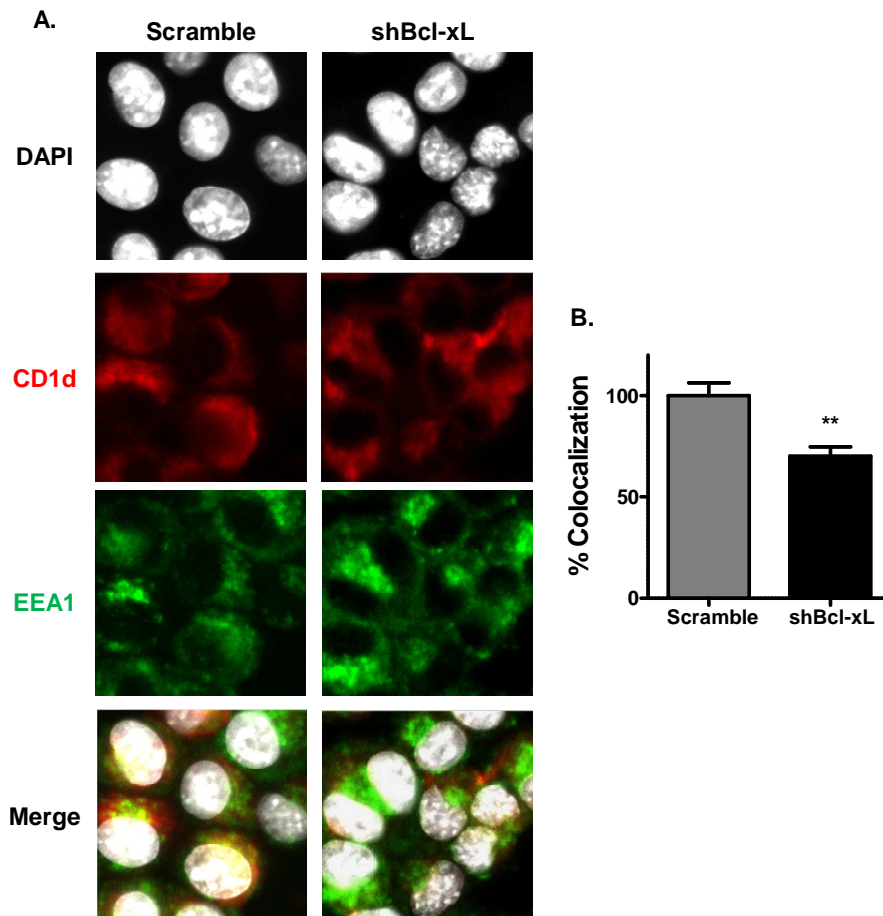


Figure 3.17: Reduced expression of CD1d in early endosomes after Bcl-xL knockdown

(A) Confocal microscopy was used to analyze control or Bcl-xL knockdown L-CD1d cells stained with antibodies against CD1d (red) or EEA1 (green), a marker for early endosomes. The lowermost panels show colocalization of CD1d with EEA1, which is indicative of its presence in the early endosomal compartment. As compared to the control, Bcl-xL knockdown cells show less localization of CD1d with EEA1 (yellow). (B) Quantification of CD1d and EEA1 colocalization in scramble control or Bcl-xL knockdown cells.

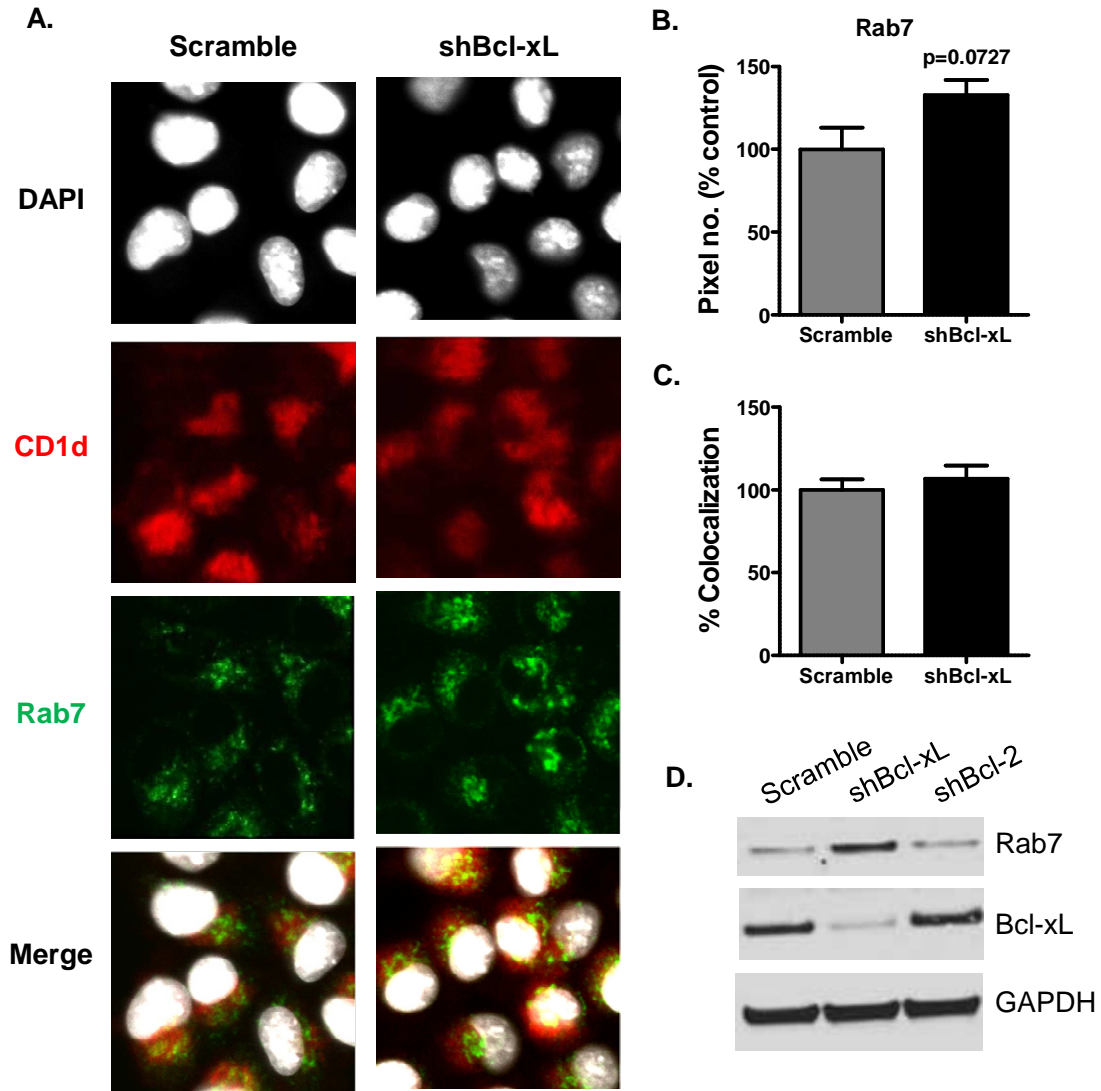


Figure 3.18: Increased Rab7 expression and accumulation of CD1d molecules in the late endosomes following Bcl-xL knockdown

(A) Confocal microscopy was used to analyze control or Bcl-xL knockdown L-CD1d cells stained with antibodies to CD1d (red) and Rab7 (green), a late endosomal marker. Following Bcl-xL knockdown, there is increased staining of Rab7 (green). (B) Quantification of Rab7 staining based on pixel number in scramble control or Bcl-xL knockdown cells. (C) Quantification of colocalization of CD1d and Rab7 in scramble control or Bcl-xL knockdown cells. (D) Western blotting shows that Rab7 protein is upregulated following Bcl-xL but not Bcl-2 knockdown (upper panel) and GAPDH is shown as the loading control (lower panel).

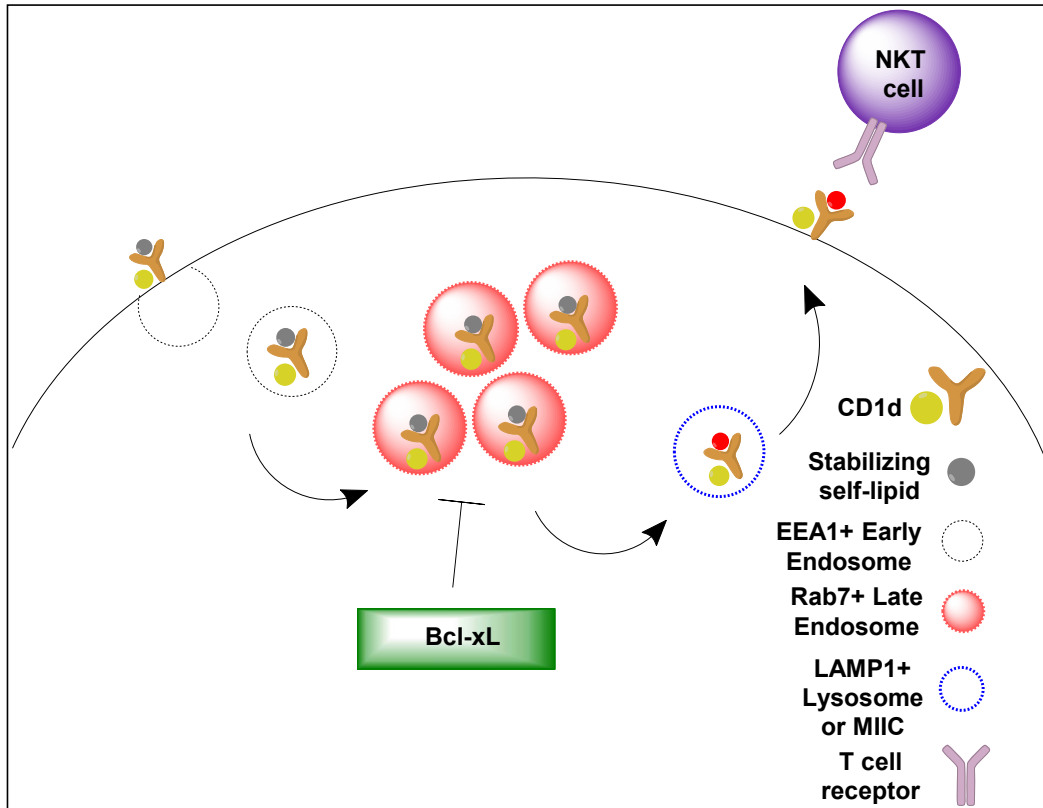


Figure 3.19: Proposed mechanism by which Bcl-xL regulates CD1d-mediated antigen processing and presentation

Under normal conditions, CD1d molecules traffic through the early endosomes, late endosomes and finally the lysosomes, where they are loaded with antigens and re-expressed on the cell surface. In Bcl-xL knockdown cells, there is a decrease in CD1d molecules in the LAMP1⁺ compartment (lysosomes or MIIC). Furthermore, Rab7 is upregulated and there is expansion of the late endosomal compartment. This results in the accumulation of CD1d molecules in the late endosomes. Thus, the data from this study show that Bcl-xL plays a role in the regulation of CD1d-mediated antigen presentation to NKT cells by altering CD1d trafficking through the endocytic pathway.

3.3 Discussion

In this study, we have shown that the over-expression of Bcl-xL in antigen presenting cells leads to increased activation of NKT cells (Fig. 3.2 and 3.4). The induction of Bcl-xL in a B cell lymphoma cell line, as well as in primary mouse B cells by treatment with anti-CD40 antibody, led to increased NKT cell activation (Fig. 3.6 and 3.8). Interestingly, following the induction of Bcl-xL in primary mouse B cells, we saw significantly increased IFN- γ levels, while IL-4 levels were unchanged (Fig. 3.8B and C). We have previously described the important role of IFN- γ in anti-tumor responses in Section 1.9. The results from Fig. 3.8 further emphasize the relevance of our discovery that Bcl-xL is involved in the regulation of NKT cell activation. In the context of lymphoma, the expression of Bcl-xL can potentially drive high NKT cell activation and induce IFN- γ production. Such a Th1-biased response could be highly beneficial in this setting and make NKT cell activation a highly effective means to boost anti-tumor immunity.

In contrast to the overexpression or induction of Bcl-xL, pharmacological inhibition or shRNA mediated knockdown of Bcl-xL led to decreased antigen presentation to NKT cells (Fig. 3.10 and 3.14). Surface CD1d expression was unchanged after Bcl-xL overexpression, induction, inhibition or knockdown. Therefore, we hypothesized that Bcl-xL regulates the intracellular CD1d-mediated antigen processing and presentation pathway. In line with this, we found that following Bcl-xL knockdown, CD1d molecules were reduced in the LAMP1⁺ compartment (Fig. 3.16). There was Rab7 upregulation and CD1d molecules accumulated in the late endosomal compartment (Fig. 3.18). Thus, we

have identified a novel function for Bcl-xL in CD1d-mediated antigen processing and presentation to NKT cells, that involves altered trafficking of CD1d through the endocytic pathway (Fig. 3.19). We found that the pharmacological inhibition of Bcl-xL and Bcl-2 by ABT-263 resulted in decreased antigen presentation to NKT cells, as well as classical CD4⁺ T cells (Fig. 3.10). However, the selective inhibition of Bcl-2 using ABT-199 had no effect. It is important to note that ABT-263 and ABT-199 are BH3 mimetics, and functionally inhibit Bcl-xL and/or Bcl-2 by binding its BH3 domain, and preventing dimerization with other BH3 containing proteins. This type of inhibition is distinct from shRNA mediated knockdown which is a simple loss in Bcl-xL or Bcl-2 protein expression. We have discovered that although knocking down Bcl-xL or Bcl-2 led to reduced antigen presentation, inhibition of Bcl-xL and Bcl-2, but not Bcl-2 alone led to reduced antigen presentation to NKT cells. This suggests that Bcl-xL and Bcl-2 may be able to regulate antigen presentation by different mechanisms. Our studies indicate that Bcl-xL-mediated regulation is dependent upon the function of its BH3 domain and involves increased Rab7, and altered trafficking of CD1d molecules through the endocytic compartments. However, the ability of Bcl-2 to regulate CD1d-mediated antigen presentation was not dependent upon BH3 domain-mediated interactions, and there was no evidence of Rab7 upregulation following Bcl-2 knockdown (Fig. 3.10 and 3.18). These findings highlight an important functional difference between the structurally similar Bcl-xL and Bcl-2 proteins.

Our data demonstrate that the intracellular trafficking of CD1d was altered following Bcl-xL knockdown, and Rab7 expression was increased. A previous study has reported a direct interaction between Bcl-xL and Rab7 (181). However, we do not know if this interaction is sufficient to explain the upregulation of Rab7 following Bcl-xL knockdown. It is possible that Bcl-xL causes expansion of the late endosomal compartment through an indirect mechanism, and the up-regulation of Rab7 is a consequence of this expansion. Further studies may reveal more intermediates through which Bcl-xL mediates its regulation on CD1d-mediated antigen presentation. It is well established that the antigen presentation pathway through the endocytic compartments requires trafficking through the low pH LAMP1⁺ lysosomal compartment or MIIC for proper antigen presentation. The requirement for accessory factors available only in the MIIC, or the presence of inhibitory factors in the late endosomes, can be a potential explanation for the observed results. The involvement of other enhancing or inhibitory factors requires further investigation and can lead to an important advancement in our understanding of the regulation of antigen presentation pathways, ultimately resulting in the development of strategies to modulate antigen presentation not only in cancer but also in any other setting where boosting of the immune response is desired. This issue will be further addressed using a qPCR array-based approach in Chapter 5.

Currently, there is great interest in using NKT cell-based cancer therapy as a novel strategy to improve available treatments (192). Studies in our laboratory have demonstrated that as compared to healthy B cells, lymphoma cells have altered CD1d-mediated antigen presentation to NKT cells (159). The finding that Bcl-xL regulates antigen presentation to NKT cells is not only a potential mechanism of tumor recognition by NKT cells, but can also have direct translational impact by allowing the development of more effective NKT cell based anti-tumor immunotherapy. Furthermore, in this study, we present evidence that Bcl-xL can also regulate peptide antigen presentation by MHC class II molecules to classical CD4⁺ T cells. This could potentially have an even larger impact on anti-tumor immunity and the tumor microenvironment, as CD4⁺ T cells play a critical role in this context. In summary, we have found that Bcl-xL plays a role in the regulation of CD1d-mediated antigen presentation to NKT cells through alterations in CD1d trafficking. Cells in which Bcl-xL expression is reduced have high Rab7 expression and an expanded late endosomal compartment. This indicates that Bcl-xL has more general effects on the endocytic pathway even under non-apoptotic conditions and can change CD1d trafficking and affect antigen presentation to NKT cells. Our study reveals a novel function of Bcl-xL, which is a major target for the development of therapeutics (193), and may help in designing better immunotherapy.

Chapter 4: The role of NKT cells *in vivo* in a mouse model of mantle cell lymphoma

4.1 Introduction

Mantle cell lymphoma (MCL) is an aggressive non-Hodgkin lymphoma that is known to respond poorly to available therapeutics (194, 195). MCL usually appears in patients in their sixth decade of life and has a median survival time of 5-7 years. Patients undergoing primary treatment show some remission but the relapse rate is high, and the disease remains incurable. MCL can occur in its classic or blastoid variant form with the latter being much more aggressive and likely to occur in younger patients. Blastoid variant MCL portends poor prognosis and has a shorter median survival time than classic MCL, reported at 14.3 months (196). Genetically, MCL is characterized by the t(11;14)(q13;q32) translocation which results in the aberrant expression of Cyclin D1 (6). This translocation occurs in the pre-B cell stage during rearrangement of the IgH variable region in the bone marrow. However, full neoplastic state is attained at a later stage of B cell differentiation and results in malignant mature naïve B cells. Following the initial translocation event, there are also secondary genetic events such as mutations in the 3'UTR, which result in a truncated form of Cyclin D1 transcripts. These truncated transcripts are missing microRNA binding sites, leading to increased Cyclin D1 mRNA stability, which further contributes to the overexpression of Cyclin D1 protein (197).

Contrary to the original notion that mature naïve B cells are the only players in MCL, as evidenced by their CD5 and IgM/IgD status, there is also evidence for antigen experienced B cells in this malignancy. Thus, despite the conventional notion that MCL arises from mature pre-germinal center B cells, there is great cellular heterogeneity in the disease. Cyclin D1 is considered to be at the center of MCL pathogenesis due to its ability to regulate the cell cycle and is heavily relied upon for diagnosis (194). However, there is also evidence of patients lacking the upregulation of Cyclin D1 and this is sometimes compensated for by CyclinD2 or 3, although the patients clinically exhibit regular MCL. Once Cyclin D1 expression is upregulated, there is deregulation of the cell cycle. This is because Cyclin D1 binds to Cyclin Dependent Kinases (CDK) 4/6, which results in the activation of the transcriptional factor E2F due to phosphorylation of its inhibitor retinoblastoma 1 (RB1). This leads to the activation of CyclinE/CDK2 and promotes entry into the cell cycle by facilitating transition from the G₁ to the S phase. The over-expression of Cyclin D1 alone was found to be insufficient for the initiation of MCL in nude mice. However, the expression of a mutated form of Cyclin D1 that is constitutively nuclear, was sufficient for transformation (198). The nuclear Cyclin D1 promotes entry of resting lymphocytes into the S phase of the cell cycle. This potentially explains the dominance of mature naïve antigen inexperienced B cells in MCL. Typically, MCL B cells are from primary follicles or from the mantle zone of secondary follicles. However, some patients exhibit evidence of antigen experience and hypermutated IgH variable region and this is correlated with better clinical outcome (199). The molecular characteristics of MCL result in high genomic instability resulting

in the deregulation of other factors involved in cell cycle control, DNA damage response, signal transduction and apoptosis. These secondary genetic abnormalities further accelerate MCL lymphomagenesis and increase its aggressiveness. Important among these are the PI3K/AKT/mTOR, WNT, Hedgehog, NF- κ B and B cell receptor (BCR) signaling pathways and are all targets for MCL therapy.

The deregulation of apoptosis in MCL is also an important aspect and occurs through the Bcl-2 family members. Malignant cells often overexpress Bcl-2, Bcl-xL and Mcl-1 which are all potent anti-apoptotic members of the Bcl-2 family (200-202). Consequently, there is a lot of interest in inducing apoptosis by using agents that target the Bcl-2 family members. One of the more successful strategies has been the use of small molecule inhibitors like ABT-737 and its improved, orally bioavailable form, ABT-263 which is in clinical trials. These drugs functionally inhibit anti-apoptotic members like Bcl-2 and Bcl-xL by mimicking the BH3 domains that are important for protein-protein interactions (Section 1.4) (203, 204). Despite the use of various strategies like targeting secondary pathways, trying to limit the expression of Cyclin D1, proteasome inhibitors, histone deacetylase (HDAC) inhibitors and the apoptotic pathways, there is still a strong need for improvement. In spite of the use of multiple approaches to treatment, MCL remains incurable. The treatment of MCL is further complicated by the high genomic instability, involvement of apoptosis and proliferation pathways and the complicated role of the microenvironment, underscoring the need for alternative therapeutic approaches that are more effective or can be used in conjunction with currently available therapies (194). In

this situation, understanding the immune response to MCL can be very beneficial in the development of innovative strategies that not only target malignant B cells but also help in activating the immune system. A previous study has reported that using α -GalCer loaded autologous tumor cells was successfully used to treat an E μ -Myc lymphoma in mice (205). In this setting, the adjuvant effect of α -GalCer is important in driving the anti-tumor response, since early NKT cell activation helps to rapidly polarize the cytokine milieu to Th1 and favor effective anti-tumor immunity. This adjuvant effect of NKT cell activation by α -GalCer and its analogues is known to be effective in bacterial, viral and parasitic infections, as well as in cancer (Section 1.9). In this study we sought to determine the role of NKT cell activation using exogenous antigen on the progression of MCL-BV in a spontaneous mouse model.

NKT cells are known to be reduced in number and function in cancer patients (97, 142, 159). However, little is known about the cause and effect relationship between the disease and NKT cell number. Furthermore, little is known about the role of NKT cells in an aggressive hematological malignancy like MCL. We therefore studied the role of NKT cells using a spontaneous mouse model of blastoid variant MCL (MCL-BV), which was first described in 2007 (154). This mouse model of MCL-BV is a double transgenic mouse obtained from IL-14 α and c-Myc transgenic mice.

IL-14, also known as taxilin or high molecular weight B cell growth factor (HMW-BCGF) was originally identified from a Burkitt lymphoma cell line (206). This 60 kDa protein is encoded by the plus strand of the *il14* gene. In response to IL-14, B cells showed increased size, expression of activation markers and move from the resting phase to the S phase of the cell cycle (207, 208). IL-14 is a growth factor for germinal center B cells, B1 cells and may also help to expand subsets of memory B cells (209). IL-14 α transgenic mice express IL-14 in the B cell compartment under the E μ promoter. At the age of 14-18 months, 95% of these mice develop CD5⁺ CD19⁺ Cyclin D1-negative lymphomas (210). This lymphomagenesis is frequently preceded by the development of autoimmunity, similar to human Sjogren's syndrome with detectable autoantibodies, sialadenitis and immune complex mediated nephritis. On the other hand E μ -Myc mice also develop lymphoma at 12 months of age (211). We used IL-14 α and c-Myc double transgenic mice (DTG) to understand the role of NKT cells in this disease.

The IL-14 α /c-Myc double transgenic mice (DTG) develop lymphoma within 3-4 months of age (154). The disease is clinically similar to human MCL. DTG mice at this age typically exhibit splenomegaly and lymphadenopathy. Histologically, loss of lymphoid architecture can be visualized by H&E staining of splenic sections as the disease progresses. Immunohistochemically, disease is visible as high Cyclin D1 expression concomitant with the loss of lymphoid architecture in spleen sections.

4.2 Results

Treatment of DTG mice with α -GalCer improves survival

We wanted to test the ability of NKT cells to improve the outcome of MCL in DTG mice. We have recently shown that DTG mice with severe disease show reduced NKT cell responses (159). We therefore wanted to test the role of NKT cells upon activation by an exogenous antigen. Our previously report has shown that NKT cell function in DTG is reduced at around 10-15 weeks of age, when tested *ex vivo* (159). This correlates with the development of disease as seen by splenomegaly and lymphadenopathy. We sought to study the effect of treatment with α -GalCer at a point when these mice have developed MCL, but do not show visible splenomegaly, and their NKT cells are still functional. Therefore, DTG mice at 8 weeks of age were treated with vehicle alone (n=11) or with 2 μ g of α -GalCer (n=10) (Fig. 4.1). The α -GalCer was injected i.v. and animals were monitored closely for symptoms of disease. Littermate controls were used for each experiment. After a single injection, mice were euthanized 6-8 weeks after treatment. Animals that showed disease-related symptoms like increased size due to lymphadenopathy, difficulty breathing, reduced movement and poor responsiveness were euthanized along with the remaining animals in the experiment. For survival curves, mice that exhibited symptoms mentioned above were considered terminal while mice without such symptoms were counted as viable. We found that vehicle treated mice were more likely to show overt symptoms of MCL and had to be euthanized when compared with their α -GalCer treated counterparts. Thus, following the treatment of littermate DTG mice with a single dose of α -GalCer, there was improved survival (Fig. 4.2).

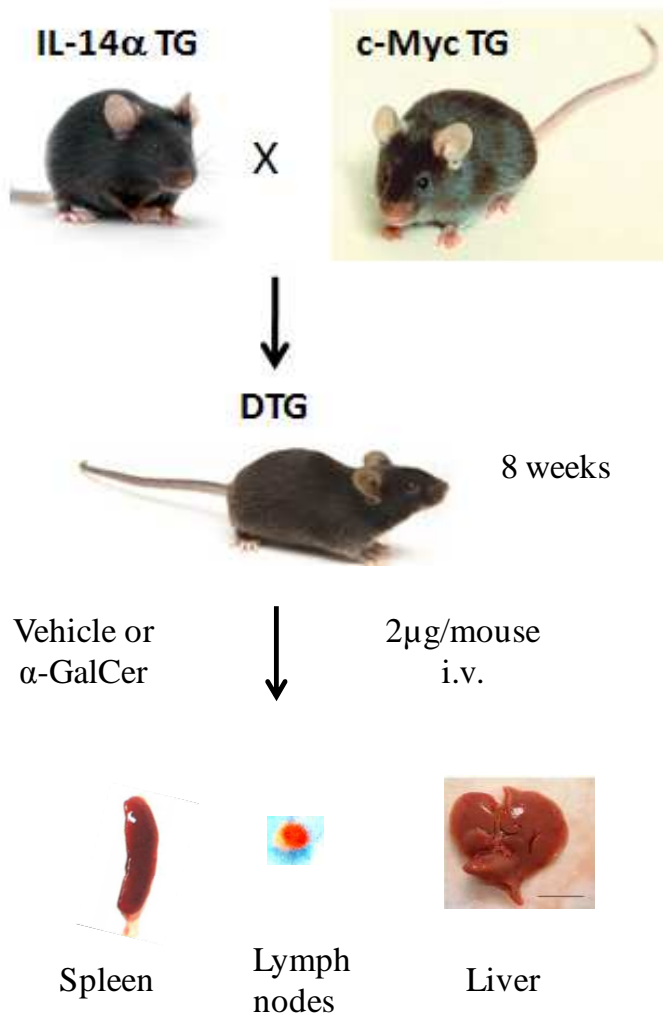


Figure 4.1: Schematic of experimental design for the treatment of DTG mice with the NKT cell agonist α -GalCer

8 week old DTG mice were treated with vehicle (0.2% DMSO in PBS) or 2 μ g/animal of α -GalCer in a single dose. 6-8 weeks after injection, mice were euthanized and their thymus, spleen, liver, lymph nodes and bone marrow were removed and analyzed by flow cytometry. Spleen sections were analyzed by H&E staining and splenocytes were used to test *ex vivo* restimulation of NKT cells.

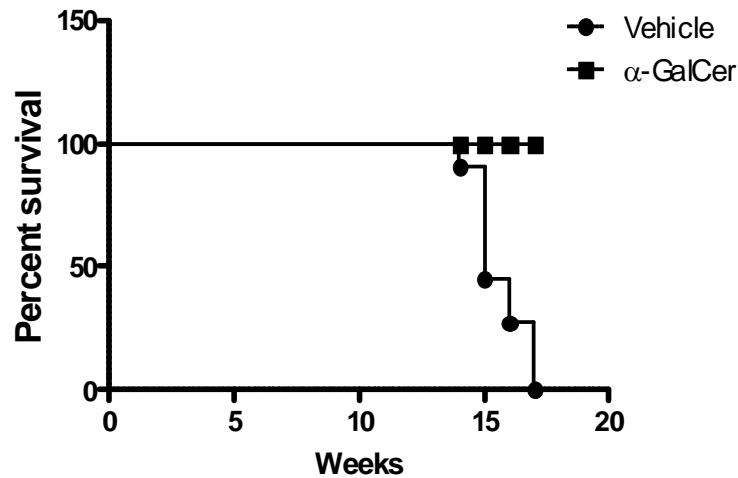


Figure 4.2: Treatment of DTG mice with α -GalCer leads to improved survival

8 week old DTG mice were treated with vehicle (0.2% DMSO in PBS) or 2 μ g/animal of α -GalCer at 8 weeks of age. Mice were observed closely for symptoms like enlarged lymph nodes, difficulty breathing and reduced mobility. Mice that looked normal and showed normal mobility were considered viable while mice one or more of the above symptoms were considered terminal. All experiments were performed using littermates.

α -GalCer treated DTG mice show reduced splenomegaly and lymphadenopathy

DTG mice are known to develop severe splenomegaly and lymphadenopathy at 3-4 months of age (154). We have previously found that NKT cells showed reduced responsiveness to *ex vivo* stimulation when DTG mice have full-blown disease (159). General T cell stimulation using anti-CD3/CD28 *ex vivo*, also resulted in reduced cytokine production during severe MCL (159). In this study, we euthanized mice 6-8 weeks after injection of α -GalCer and examined their spleens and lymph nodes. We found that following treatment with a single dose of α -GalCer, mice had reduced splenomegaly and lymphadenopathy (Fig. 4.3). We found that α -GalCer treated mice had smaller spleens and lymph nodes as compared to their vehicle treated controls. These data show that the activation of NKT cells by injecting α -GalCer leads to reduced progression of MCL. Lymph nodes were smaller in treated mice as compared to the controls, however, the size was not similar to wild type mice (data not shown). We can therefore conclude that MCL was not completely cured after treatment with α -GalCer, but the progression of this disease was stalled following the activation of NKT cells.

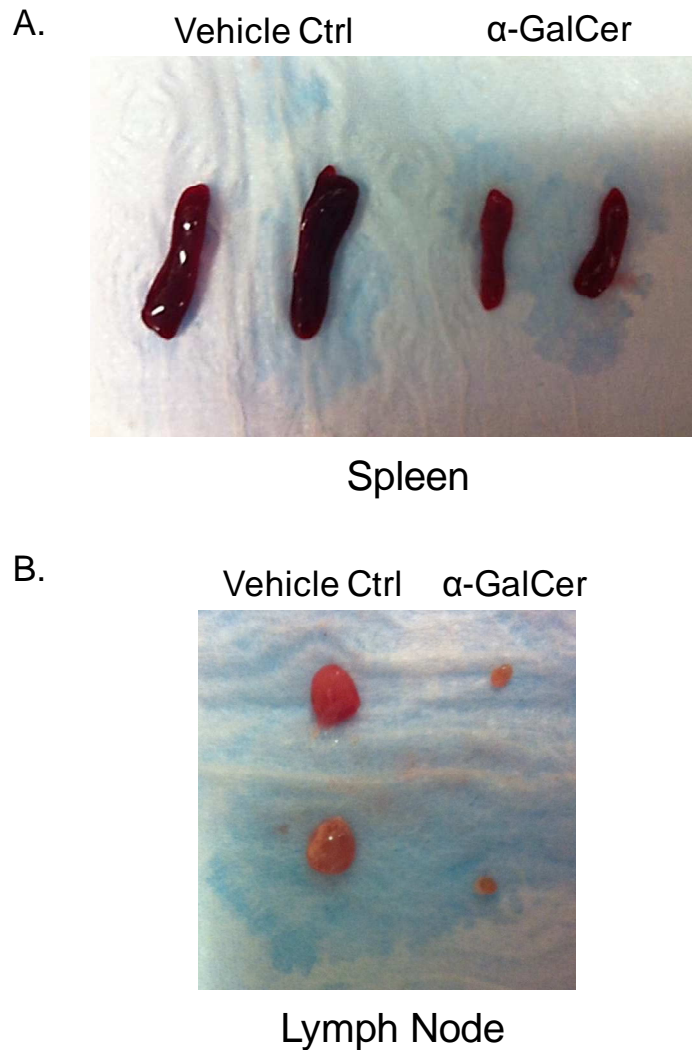


Figure 4.3: Treatment of DTG mice with a single dose of α -GalCer leads to reduced splenomegaly and lymphadenopathy

8 week old DTG mice were treated with vehicle (0.2% DMSO in PBS) or 2 μ g/animal of α -GalCer. After 6 weeks, mice were euthanized and their (A) spleens and (B) lymph nodes were removed and photographed. One representative of a total n=21. 10 mice were treated with α -GalCer and 11 were vehicle controls.

Treatment with α -GalCer leads to reduced MCL pathology in the spleen

The onset of MCL results in major changes in the spleen. As the number and size of B cell increases, B cell follicles can no longer be distinctly seen by H&E staining of spleen sections. In MCL-BV there is also a large number of malignant blastoid B cells that are larger in size and have densely packed chromatin (195). As MCL progresses, the loss of splenic architecture can be clearly visualized and eventually, there is complete loss of well-organized B cell follicles. Following treatment of mice with α -GalCer we used H&E staining of spleen sections to assess MCL-related pathology. We found that mice treated with α -GalCer showed improved splenic architecture as compared to vehicle treated controls (Fig. 4.4). There was a reduced frequency of blastoid B cells in the treated mice as compared to the controls (indicated by arrows in Fig. 4.4). These data further support the conclusion that treatment with an NKT cell agonist stalls the progression of MCL-BV. Previous reports have indicated that injection with α -GalCer can lead to reduced NKT cell percentages in secondary lymphoid organs, and their subsequent recovery. Work in our lab has shown that DTG mice have reduced NKT cell function as the disease progresses (159). We first determined NKT cell percentages in the thymus, spleen, liver, lymph nodes and bone marrow, 6-8 weeks after the injection of α -GalCer. We found that treatment with α -GalCer did not lead to any changes in NKT cell percentage in any of the organs analyzed (Fig. 4.5). Two potential explanations for this are that either our experimental design was successful at eliminating the loss of NKT cells that usually accompanies treatment; or the time period after injection was sufficient for the recovery of NKT cells.

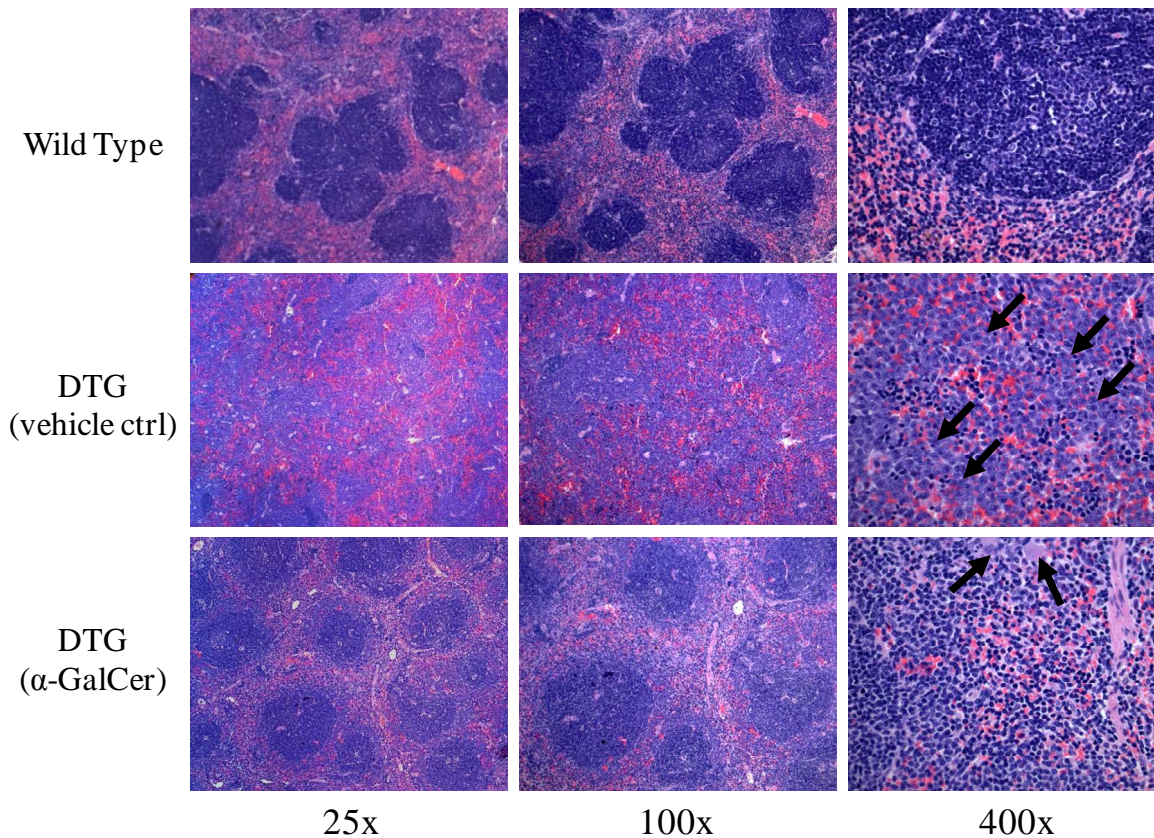


Figure 4.4: Treatment of DTG mice with α -GalCer leads to improved splenic histopathology

8 week old DTG mice were treated with vehicle (0.2% DMSO in PBS) or 2 μ g/animal of α -GalCer. After 6 weeks, mice were euthanized and their spleens were removed. Spleen sections were mounted and H&E staining was used to visualize lymphoid architecture. One representative of a total n=21. 10 mice were treated with α -GalCer and 11 were vehicle controls. All experiments were performed using littermates. Arrows show examples of MCL-BV B cells.

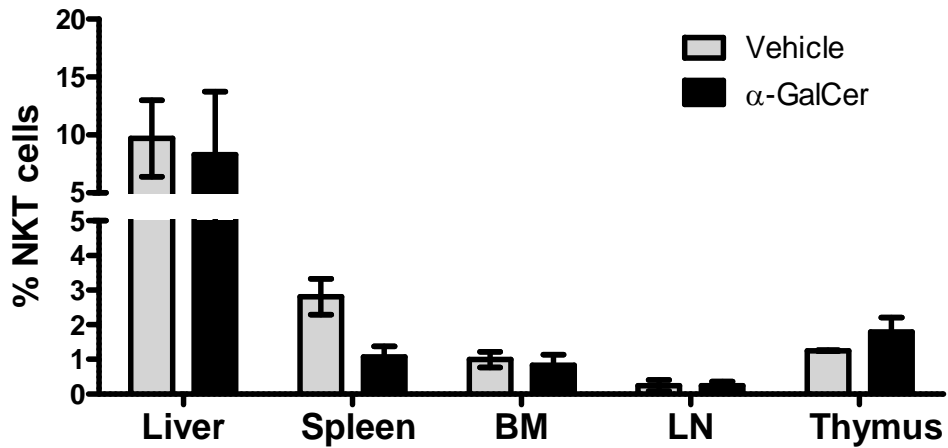


Figure 4.5: NKT cell percentages in various organs after injection of α -GalCer

8 week old DTG mice were treated with vehicle (0.2% DMSO in PBS) or 2 μ g/animal of α -GalCer. After 6 weeks, mice were euthanized and the thymus, spleen, liver, lymph nodes and bone marrow were processed into single cell suspensions. Flow cytometry was used to determine NKT cell percentages as TCR β ⁺ CD1d: α -GalCer tetramer⁺ cells.

NKT cell activation in DTG leads to increased IFN- γ production

Previous studies have shown that the production of IFN- γ by NKT cells can have a major impact on the outcome of anti-tumor responses (212, 213). In fact this strong IFN- γ response is thought to be important for the adjuvant effect of NKT cell activating antigens (Section 1.9). We therefore examined IFN- γ production in splenocytes *ex vivo* to gain insight into the mechanism underlying the improved disease outcome obtained after treatment with α -GalCer. We obtained the splenocytes from α -GalCer treated or control mice and cultured them *ex vivo* for 48 hours with or without α -GalCer. We found that in the absence of *ex vivo* restimulation, there was increased IFN- γ in the supernatants from the cultured splenocytes (Fig. 4.6 A). In vehicle treated mice there was no detectable IFN- γ in the absence of exogenously added NKT cell antigen. However, splenocytes from α -GalCer treated mice showed increased IFN- γ response (Fig. 4.6 A). Upon the addition of α -GalCer to the culture, splenocytes from vehicle treated mice showed some IFN- γ responses. Splenocytes from α -GalCer treated mice showed a much stronger response to antigen by producing larger amounts of IFN- γ (Fig. 4.6 A). No differences were observed in IL-4 production following treatment with α -GalCer in the presence or absence of exogenously added antigen (Fig. 4.6 B). This demonstrates that the injection of α -GalCer favors IFN- γ and not IL-4 responses in this context. These results indicate that high IFN- γ production may be one of the mechanisms by which α -GalCer mediates its anti-tumor effect, although further studies will be needed to fully understand the details and main cell types involved in the overall therapeutic effect.

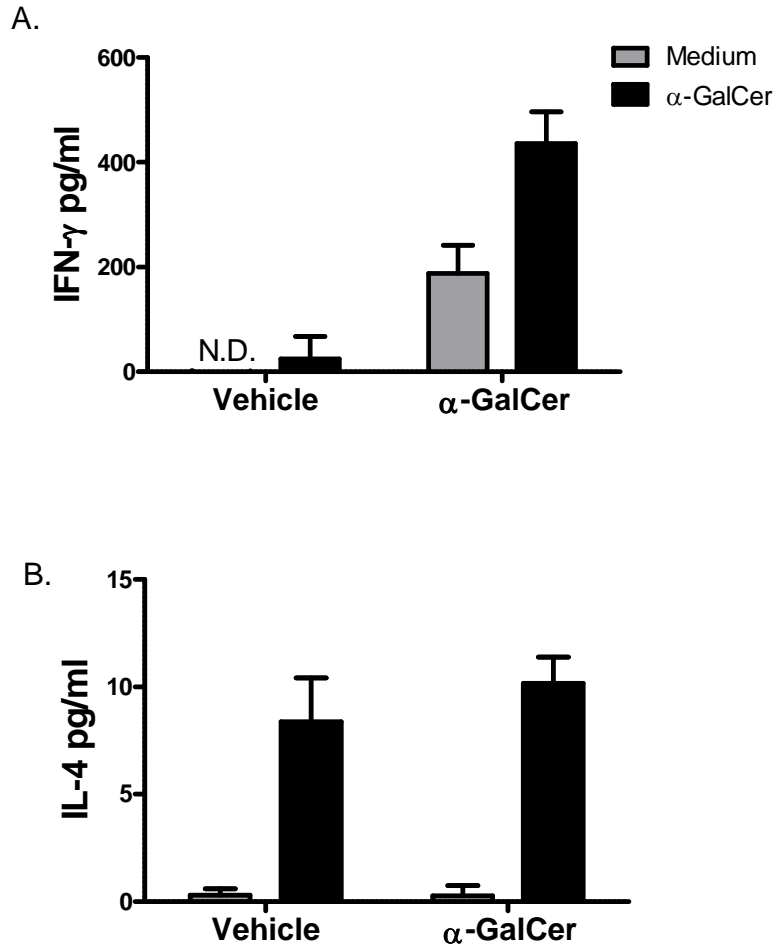


Figure 4.6: Treatment of DTG mice with α -GalCer leads to increased *ex vivo* IFN- γ but not IL-4 responses

8 week old DTG mice were treated with vehicle (0.2% DMSO in PBS) or 2 μ g/animal of α -GalCer. After 6 weeks, mice were euthanized and splenocytes were cultured *ex vivo* with medium alone or with α -GalCer for restimulation. (A) IFN- γ and (B) IL-4 in the supernatant were measured by ELISA.

4.3 Discussion

The IL-14 α /c-Myc DTG mouse model clinically recapitulates human MCL. Patients with t(11;14) translocation can be disease free for a long time before an increase in the number of Cyclin D1 over-expressing B cells. Thus, similar to human MCL, the DTG mice used in this study develop MCL-BV spontaneously from underlying genetic modifications which drive lymphomagenesis. DTG mice develop an extremely aggressive form of MCL, i.e. MCL-BV as seen by gross pathology as well as histopathology. This spontaneously driven mouse model is thus a useful system to study endogenous NKT cell responses during an aggressive hematologic malignancy. However, like any model system, it has some limitations. For example, the IL-14 α transgenic mice are on a C57BL/6 background and c-Myc transgenic mice are on a 129 background, thereby making the DTG mouse a mixed strain. This results in experimental limitations as DTG mice cannot be directly compared to wild type mice. Therefore, throughout this study we have used littermate controls in all experiments. The top row of panels in Fig. 4.4 show H&E staining of wild type spleen sections as a representation of normal splenic architecture and are not intended for direct comparison. However, the H&E staining from untreated and mice treated with α -GalCer are derived from littermates and can be directly interpreted. Another caveat of the DTG mouse model is its spontaneity. Although a good representation of clinical lymphomagenesis, this poses experimental problems due some uncertainty in the timeline of expected disease progression.

In this genetically driven DTG mouse model of MCL, we evaluated the role of NKT cells by treating mice with an NKT cell agonist. Treatment with a single dose of α -GalCer was sufficient to stall disease progression, improve survival and reduce pathology (Fig. 4.2, 4.3 and 4.4). Furthermore, NKT cell percentages were not changed and their function was enhanced by α -GalCer treatment (Fig. 4.5). Splenocytes from α -GalCer treated mice showed increased IFN- γ at baseline as well as after *ex vivo* restimulation, as compared to splenocytes from vehicle treated mice. This indicates that following treatment with α -GalCer, NKT cell function is increased at 6-8 weeks post treatment. Furthermore, there was no change in IL-4 levels, showing that NKT cell activation in the context of this disease favors IFN- γ production, which may further aid in mounting effective anti-tumor responses. It is interesting to speculate that other cell types may also be involved in amplifying this IFN- γ response (214). As described in Section 1.9, NKT cells can produce large amounts of IFN- γ which can subsequently activate NK cells (Fig. 1.5). Activated NK cells can then produce more IFN- γ , forming a feed-forward loop. This highly IFN- γ rich milieu can then help polarize CD8⁺ T cell responses. In fact the activation of CD4⁺ T cells can also aid the IFN- γ production as well as the responses mounted by CD8⁺ T cells. Once activated and in the presence of high IFN- γ , CD8⁺ T cells can mount strong anti-tumor responses and can mediate direct cytotoxicity towards malignant B cells. Thus, a single dose of NKT cell activating antigen can stall the progression of MCL, and other cell types are probably critical in achieving effective anti-tumor immunity. The prediction from this would be that getting rid of any of these cell types would result in a partial reduction in the overall effect observed after NKT cell

activation, although further studies in this experimental model will be required to confirm this. Thus, we hypothesize that NKT cells can ‘jumpstart’ the immune system by providing a favorable cytokine environment for the activation of other cell types which can also mount effective responses. In an aggressive hematological malignancy like MCL-BV, and at a time when disease onset has already begun, the involvement of diverse cell types can play an important role in achieving a favorable disease outcome. Thus, a small lymphocyte population like NKT cells can make a major difference in shaping anti-tumor immunity and result in dramatic improvement in disease by orchestrating an effective immune response.

Chapter 5: qPCR array analyses of Bcl-xL knockdown cells

5.1 Accessory factors in Bcl-xL-mediated regulation of NKT cell activation

The results from chapter 3 have shown that Bcl-xL regulates CD1d-mediated antigen presentation to NKT cells. The over-expression or induction of Bcl-xL led to increased NKT cell activation. On the other hand, pharmacological inhibition or shRNA-mediated knockdown of Bcl-xL resulted in reduced antigen presentation. Furthermore, the knockdown of Bcl-xL resulted in loss of CD1d molecules from the LAMP1⁺ lysosomal compartment. Rab7 was up-regulated and CD1d molecules accumulated in the Rab7⁺ late endosomes. We sought to further identify other factors that may be involved in this Bcl-xL-mediated regulation of antigen presentation to NKT cells. We tested the expression of a large pool of factors that are relevant to antigen processing and presentation using a PCR array-based approach. This PCR array allowed us to test the expression of a diverse set of molecules like cytokines, chemokines, costimulatory molecules and cell adhesion molecules. We compared L-CD1d cells transduced with a scramble shRNA sequence or with shRNA targeting Bcl-xL using this qPCR array, to identify proteins that are important in the Bcl-xL-mediated regulation of antigen presentation (Table 5.1). As changes in chemokines, cytokines and adhesion molecules can significantly impact the regulation of NKT responses by Bcl-xL *in vivo*, this strategy can provide valuable insight into what role this regulation might play in a real-life spatio-temporal setting. Table 5.1 shows the qPCR array results and genes up or downregulated by more than 2 fold have been highlighted in bold typeface. The genes *csf2* and *cd209a* that encode for the

cytokine GM-CSF and CD209 (DC-SIGN) respectively, were both highly upregulated after the knockdown of Bcl-xL. Perhaps in normal cells, Bcl-xL plays a role in suppressing the expression of these genes and the upregulation of Bcl-xL during lymphomagenesis may provide a mechanism of escape for immune recognition. Furthermore, CD209 is a C-type lectin receptor that can recognize carbohydrate moieties on pathogens and helps in internalizing antigens. The internalized antigens are then targeted to the endocytic pathway through the early endosomes, late endosomes and finally to the lysosomes, or MIIC. Thus CD209 helps to make these antigens available for cross-presentation (215, 216). We found that the chemokine CCL2 and one of its receptors CCR2 were also upregulated following Bcl-xL knockdown. This chemokine is important in the recruitment of monocytes and perhaps even other cell types. The CCL2-CCR2 interaction has also been implicated in macrophage polarization (217). The gene *erbB2*, which encodes HER-2 was found to be downregulated following Bcl-xL knockdown. Our q-PCR array data reveal a lot of genes that have important functions in cancer. Further investigation will be needed to determine how the up/down regulation of these genes by Bcl-xL may also play a role in antigen presentation to NKT cells.

Table 5.1: Dendritic cell and antigen presenting cell qPCR array to study changes in gene expression following the knockdown of Bcl-xL in L-CD1d cells

Gene	Fold Difference	Fold Up- or Down- Regulation
	shBcl-xL/Scramble	shBcl-xL/Scramble
Ccl11	0.78	-1.28
Ccl12	1.69	1.69
Ccl17	0.54	-1.87
Ccl19	1.03	1.03
Ccl2	0.75	-1.34
Ccl20	1.21	1.21
Ccl3	3.1	3.1
Ccl4	1.28	1.28
Ccl5	0.64	-1.57
Ccl7	0.88	-1.14
Ccl8	0.98	-1.02
Ccr1	0.79	-1.27
Ccr2	2.18	2.18
Ccr3	1.28	1.28
Ccr5	1.28	1.28
Ccr9	0.96	-1.04
Cd1d1	0.73	-1.38
Cd1d2	1.94	1.94
Cd2	1.79	1.79
Cd209a	6.56	6.56
Cd28	0.78	-1.28
Cd33	0.47	-2.14
Cd36	1.28	1.28
Cd4	0.69	-1.44
Cd40	1.06	1.06
Cd40lg	1.28	1.28
Cd44	0.64	-1.56
Cd74	0.48	-2.1
Cd80	0.81	-1.24
Cd86	1.31	1.31
Cd8a	1.28	1.28

Table 5.1 continued

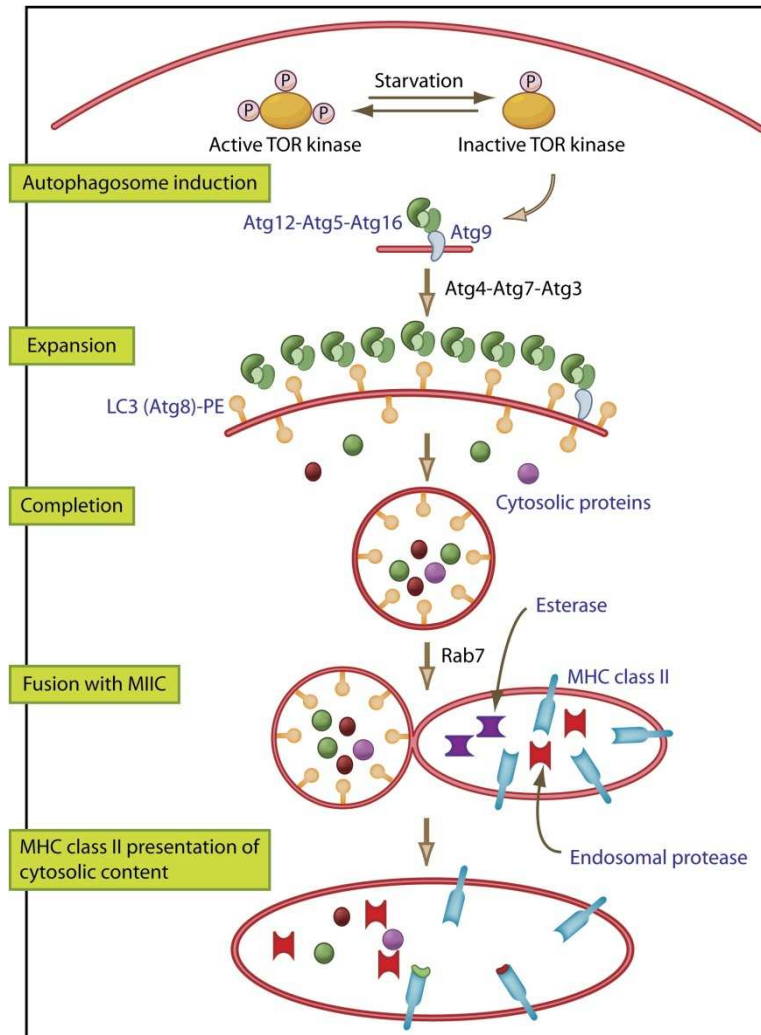
Gene	Fold Difference	Fold Up- or Down- Regulation
	shBcl-xL/Scramble	shBcl-xL/Scramble
Cdc42	0.73	-1.37
Cdkn1a	0.39	-2.56
Clec4b2	1.28	1.28
Csf1r	1.07	1.07
Csf2	7.4	7.4
Cxcl1	0.95	-1.05
Cxcl10	0.7	-1.42
Cxcl12	1.41	1.41
Cxcl2	0.52	-1.92
Cxcr1	1.28	1.28
Cxcr4	1.28	1.28
ErbB2	0.45	-2.24
Fas	1.01	1.01
Fcer1a	0.97	-1.03
Fcer2a	0.71	-1.4
Fcgr1	0.62	-1.6
Fcgrt	0.69	-1.44
Flt3	1.45	1.45
Flt3l	0.87	-1.14
H2-DMa	0.95	-1.05
Icam1	0.67	-1.5
Icam2	0.57	-1.75
Ifng	1.45	1.45
Il10	1.28	1.28
Il12a	0.58	-1.73
Il12b	1.28	1.28
Il16	0.66	-1.5
Il2	1.28	1.28
Il6	0.76	-1.31
Irf7	1.97	1.97
Itgam	0.78	-1.28
Itgb2	0.57	-1.76
Lrp1	0.84	-1.19
Lyn	0.8	-1.25
Mif	0.59	-1.68

Table 5.1 Continued

Gene	Fold Difference	Fold Up- or Down- Regulation
	shBcl-xL/Scramble	shBcl-xL/Scramble
Nfkb1	0.85	-1.18
Ptprc	1.28	1.28
Rac1	0.77	-1.31
Rag1	2.14	2.14
Relb	1.26	1.26
Stat3	0.83	-1.21
Tap2	0.71	-1.4
Tapbp	0.77	-1.3
Tgfb1	0.61	-1.64
Thbs1	0.74	-1.36
Tlr1	0.95	-1.06
Tlr2	1.04	1.04
Tlr7	1.14	1.14
Tlr9	1.61	1.61
Tnf	0.72	-1.4

5.2 A potential role for autophagy in Bcl-xL-mediated regulation of antigen presentation to NKT cells

The word autophagy literally means ‘eating oneself’. During autophagy, cells engulf their own components, as a response to nutritional deprivation or other stress signals. Cellular organelles or parts of the cytoplasm are sequestered in vesicles known as autophagosomes. These autophagosomes then fuse with lysosomes and the sequestered content is targeted for degradation (218). The Atg proteins play a major role in the initiation, formation and expansion of the autophagosomes. Nutrition starvation or other cellular stress signals such as temperature, radiation or changes in osmolarity can trigger the formation of the autophagic membrane (Fig. 5.1). Signaling pathways like mammalian target of Rapamycin (mTOR) and PI3K converge with the autophagic pathway, which is especially important in the autophagic response to metabolic stress. Most autophagic stimuli involve the mTOR pathway, however, hypoxia can also induce autophagy, and this occurs through the autophagic protein Beclin-1. As the Atg proteins assemble, the microtubule-associated protein light chain 3 (LC3) is concomitantly recruited (219). LC3 is lipidated with phosphatidylethanolamine and binds the membrane of the autophagosome to recruit cargo for inclusion in the vesicle. This lipidation of LC3 is one of the most common ways by which autophagy is detected (220).



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Figure 5.1: Major steps and players in the autophagic pathway

Cellular stress signals like nutrient starvation initiate the formation of the autophagosome, usually through mTOR. Atg proteins are recruited and the autophagosomal complex is formed. This membrane expands to form a complete vesicle and LC3 is concomitantly lipidated and recruited. This autophagosome then matures with the aid of Rab7 and fuses with the lysosomes or MIIC. Thus the autophagic pathway converges with the endocytic pathway and can cause alterations in antigen presentation, especially by MHC class II which is loaded with antigens in the acidic MIIC compartment (230).

The Bcl-2 family members are integrally linked with the autophagic process. Under normal conditions, Beclin-1 is bound to Bcl-2 through BH3 domain-mediated interactions. Hypoxia can induce BH3 containing proteins that bind Beclin-1, and free it from Bcl-2, to induce autophagy (44). Thus Bcl-2 binds Beclin-1 and is generally thought to be a negative regulator of autophagy (221). Similarly, Bcl-xL is known to interact with Beclin-1 through its BH3 domain (222). Bcl-2 and Bcl-xL also interact with the autophagic protein Atg5. Truncated Atg5 translocates from the cytosol to the mitochondrial, where it binds Bcl-xL and triggers apoptosis (223). In this way, components of the autophagic system can feed into the apoptotic pathway, and this crosstalk is facilitated by Bcl-2 family proteins. The regulation of Ca^{++} by anti-apoptotic Bcl-2 proteins in the ER also influences autophagy, since Ca^{++} flux is also an inducer of autophagy (224). Bcl-2 family members regulate autophagy through multiple mechanisms, and small molecule BH3 mimetics like ABT-737 also induce autophagy in addition to apoptosis (225). The autophagic and endocytic pathways converge to deliver their cargo for lysosomal degradation. Consequently, many of the Rab family GTPases are important in autophagy (226). Furthermore, Rab7 is important for the maturation of autophagosomes (227). This helps to direct the autophagic cargo along microtubules and deliver them to the lysosomes (228). The convergence of the autophagic and endocytic pathways can also impact antigen presentation (229). Autophagy entails the engulfment of cellular material and can deliver it to the endocytic compartments for presentation on MHC class II (or for cross-presentation on MHC class I) (230). Autophagy plays a role in the normal antigen processing and presentation pathway, as $Atg5^{-/-}$ DCs show impaired

antigen presentation (231). The downregulation of autophagy in DCs can also result in poor DC maturation, as seen in the context HIV infection (232). Autophagy plays a role in T cell development during the selection of CD4⁺ T cells, and is important for the induction of tolerance (233).

A large body of literature describes the role of autophagy in antigen presentation by classical MHC molecules. However, little is known about the effect of autophagy on CD1d-mediated antigen presentation to NKT cells. CD1d-mediated antigen presentation resembles both MHC class I and II, and involves the endocytic pathway. Our data demonstrate that Bcl-xL regulates this process. Given the important role that Bcl-2 family members play in autophagy, it is plausible that alterations in autophagy are involved in this regulation exerted by Bcl-xL. The observation that Rab7 is upregulated following Bcl-xL knockdown, could also be another potential indication of autophagic changes. Further investigation of autophagic markers can help tease this out and improve our understanding of CD1d-mediated antigen presentation. To initiate this work, we performed some preliminary studies using a qPCR array to test the expression of autophagy-related proteins. As in the previous section, we compared L-CD1d cells transduced with scramble shRNA or shRNA targeting Bcl-xL (Table 5.2).

Table 5.2: qPCR array to study changes in the expression of autophagy-related genes following the knockdown of Bcl-xL in L-CD1d cells

Gene	Fold Difference	Fold Up- or Down-Regulation
	shBcl-xL/Scramble	shBcl-xL/Scramble
Akt1	0.97	-1.03
Ambra1	0.94	-1.07
App	0.7	-1.43
Atg10	0.82	-1.21
Atg12	0.84	-1.19
Atg16l1	1.01	1.01
Atg16l2	0.9	-1.11
Atg3	0.85	-1.17
Atg4a	1.09	1.09
Atg4b	0.81	-1.24
Atg4c	0.87	-1.15
Atg4d	1.02	1.02
Atg5	1	-1
Atg7	1.04	1.04
Atg9a	1.05	1.05
Atg9b	1.15	1.15
Bax	0.97	-1.03
Bcl2	1.02	1.02
Bcl2l1	0.35	-2.82
Becn1	0.79	-1.26
Bid	0.66	-1.52
Bnip3	0.88	-1.14
Casp3	1	-1
Casp8	0.97	-1.03
Cdkn1b	1.2	1.2
Cdkn2a	0.8	-1.25
Cln3	0.77	-1.31
Ctsb	0.89	-1.13
Ctsd	0.73	-1.37
Ctss	1.76	1.76
Cxcr4	0.8	-1.25
Dapk1	1.04	1.04

Table 5.2 Continued

Gene	Fold Difference	Fold Up- or Down-Regulation
	shBcl-xL/Scramble	shBcl-xL/Scramble
Dram1	1.06	1.06
Dram2	1.09	1.09
Eif2ak3	0.89	-1.12
Eif4g1	0.97	-1.03
Esr1	0.42	-2.39
Fadd	1.13	1.13
Fas	1.42	1.42
Gaa	0.93	-1.08
Gabarap	0.78	-1.28
Gabarap11	0.87	-1.15
Gabarap12	0.97	-1.03
Hdac1	1.18	1.18
Hdac6	0.91	-1.09
Hgs	0.93	-1.08
Hsp90aa1	0.85	-1.17
Hspa8	1.09	1.09
Htt	0.72	-1.39
Ifng	0.98	-1.02
Igf1	2.09	2.09
Ins2	0.43	-2.31
Irgm1	1.07	1.07
Lamp1	0.97	-1.04
Map11c3a	0.89	-1.12
Map11c3b	0.93	-1.07
Mapk14	0.8	-1.25
Mapk8	0.93	-1.07
Mtor	0.84	-1.19
Nfkb1	0.84	-1.19
Npc1	0.95	-1.06
Pik3c3	0.96	-1.04
Pik3cg	2.79	2.79
Pik3r4	1.06	1.06
Prkaa1	0.88	-1.13
Pten	1.28	1.28

Table 5.2 Continued

Gene	Fold Difference	Fold Up- or Down-Regulation
	shBcl-xL/Scramble	shBcl-xL/Scramble
Rab24	0.94	-1.07
Rb1	1.09	1.09
Rgs19	0.91	-1.1
Rps6kb1	0.91	-1.1
Snca	0.8	-1.25
Sqstm1	0.8	-1.25
Tgfb1	1.03	1.03
Tgm2	1.15	1.15
Tmem74	0.65	-1.53
Tnf	1.05	1.05
Tnfsf10	0.84	-1.19
Trp53	1.02	1.02
Ulk1	1.14	1.14
Ulk2	1.01	1.01
Uvrag	0.92	-1.09

The table above shows that as expected, the expression of Bcl-xL was reduced as compared to the scramble control transduced cells. Furthermore, there is no change in the expression of Bcl-2. This confirms that we were able to selectively knockdown Bcl-xL without any changes in Bcl-2 expression levels. From the results of this q-PCR array, we did not see changes in any of the Atg proteins tested. However, we found that the expression of *esr1* which encodes the estrogen receptor 1 (ER1) and *ins2* which encodes Insulin II was downregulated by more than 2 fold. On the other hand *igf1* which encodes the insulin-like growth factor 1, and *pik3cg* which encodes PI3K- γ were upregulated following Bcl-xL knockdown. Changes in autophagy due to Bcl-xL knockdown in our system will have to be further studied by LC3 lipidation and confocal microscopy. This will allow us to definitively determine if there are any autophagic changes in the cells and aid in elucidating its role in the regulation of antigen presentation.

Chapter 6: Further discussion and future directions

6.1: The role of Bcl-xL in CD1d-mediated antigen presentation to NKT cells

In Chapter 3, we have exhaustively shown that the modulation of Bcl-xL leads to altered NKT cell responses. More specifically, we found that increasing the level of Bcl-xL by overexpression or induction led to a corresponding increase in CD1d-mediated antigen presentation to NKT cells (Fig. 3.2, 3.4, 3.6 and 3.8). We found that inhibition or knockdown of Bcl-xL led to a corresponding decrease in antigen presentation (Fig. 3.10 and 3.12). We have also shown that this regulation is apoptosis-independent and CD1d-dependent (Fig. 3.5 and 3.11). Thus we have identified a novel role for Bcl-xL in the regulation of antigen presentation to NKT cells. From previous studies in the lab, we know that NKT cells can specifically recognize MCL B cells as compared to healthy B cells (159). We therefore hypothesize that the regulation of NKT cell activation by Bcl-xL may constitute a mechanism underlying this recognition. An alternative possibility is that the upregulation of Bcl-xL leads to over-stimulation of NKT cells, causing loss in number and function, which is observed during cancer. Further investigation will be required to determine if this is a major pathway allowing NKT cells to recognize and respond to lymphoma B cells. Interestingly, the induction of Bcl-xL in primary mouse B cells led to increased IFN- γ but not IL-4 production by NKT cells. This may be because of the use of liver NKT cells in our studies, as this subset of NKT cells is known to

mount strong anti-tumor responses and has a higher proportion of IFN- γ -producing NKT1 cells (109). Although the precise mechanism for the skew remains unknown, the observed responses are in line with our proposed hypothesis that NKT cells may recognize lymphoma cells due to high Bcl-xL expression and mount strong IFN- γ responses against them. It will be interesting to see if this Th1 bias is also observed if splenic NKT cells are used instead of liver NKT cells, to determine if the cytokine bias is driven by Bcl-xL-mediated regulation of antigen presentation, or by the inherent properties of the NKT cells used.

Throughout our study of Bcl-xL in antigen presentation to NKT cells, we did not detect appreciable changes in surface CD1d expression. However, there were alterations in the intracellular trafficking of CD1d. We found that the trafficking of CD1d molecules to the LAMP1⁺ lysosomes, or MIIC was reduced following Bcl-xL knockdown (Fig. 3.16). However, the LAMP1⁺ MIIC compartment was intact, as seen by confocal microscopy. We do not know if these CD1d molecules have bypassed the MIIC completely, or if CD1d molecules pass through this compartment rapidly. Our steady-state confocal analyses are insufficient to determine this and further investigation will be required to understand the precise mechanism underlying the altered trafficking of CD1d molecules. We further wanted to understand how this altered intracellular trafficking affects NKT cell activation. The low pH MIIC compartment is a distinct cellular organelle that facilitates antigen presentation by classical MHC class II as well as CD1d. It follows then that if CD1d molecules are reduced in the MIIC, antigen presentation might be impaired

due to a lack of accessory factors like saposins which facilitate lipid antigen processing and presentation by CD1d (234). Alternatively, a new accessory factor present in the MIIC might be the reason underlying the reduced antigen presentation following Bcl-xL knockdown. To further explore this possibility, we conducted qPCR array analyses and found changes in the expression of CD209, a molecule involved in delivering carbohydrate-containing antigens to the endocytic pathway and eventually the MIIC (216). As NKT cells recognize glycolipid antigens, it will be interesting to further study this molecule to determine if this receptor may play a role in delivering glycolipid antigens to the MIIC for loading on to CD1d molecules.

We also found that similar to CD1d-mediated antigen presentation, antigen presentation by MHC class II to classical T cells was also reduced following Bcl-xL knockdown (Fig. 3.12). We have shown that Bcl-xL can regulate the trafficking of CD1d molecules to the MIIC. We would therefore predict that the trafficking of HLA-DR to the MIIC would be similarly reduced. Further investigation will be required to test this hypothesis and ascertain the mechanism by which Bcl-xL affects MHC class II-mediated antigen presentation. Since antigen presentation by both CD1d and MHC class II were reduced after Bcl-xL inhibition, we hypothesize that alterations in trafficking to the MIIC are responsible for the observed effects. We do not know if the reduced trafficking of CD1d and MHC class II molecules to the MIIC leads to a decrease in antigen presentation due to the unavailability of a common accessory factor present in the MIIC, or distinct factors that affect either CD1d or MHC class II.

We have discovered that the knockdown of Bcl-xL can lead to reduced CD1d in the lysosomes (Fig. 3.16). Lysosomes are important low-pH cellular organelles involved in the degradation of cellular components (235, 236). The lysosomes also contain a large number of enzymes that help degrade the nucleic acids, carbohydrates, proteins and lipids. Lysosomes also play an important role in antigen presentation, apoptosis and autophagy (235). Perhaps, similar to CD1d, the trafficking of other molecules to the lysosomes may also be reduced following Bcl-xL knockdown. Alternatively, the observed effect of Bcl-xL might be a ‘side-effect’ of its anti-apoptotic role. The loss of lysosomal integrity is a part of the cellular apoptotic response and Bcl-xL can inhibit this by preventing the cleavage of Rabaptin-5, an effector protein found in the early endosomes (180). This indirectly blocks endosomal fusion and breakdown of the endo-lysosomal network. Alternatively, it will be interesting to study the effects of other lysosome-related pathways on NKT cell activation, as discussed below.

Autophagy is a process that is intimately linked to the endosomes and lysosomes. The autophagic process, described in Section 5.2, intersects the endocytic pathway to target the contents of the autophagosomes to the lysosomes for degradation. Bcl-xL and Bcl-2 are generally considered inhibitors of autophagy. We can therefore deduce that as the knockdown of Bcl-xL leads to decreased antigen presentation, it would concomitantly cause increased autophagy. This increased autophagy may be responsible for the expanded late endosomal compartment observed in (Fig. 3.18). Thus, the possible existence of essential accessory factors described above, may be one or more components

of the autophagic machinery. Our qPCR array analyses have yielded some preliminary data, but further investigation will be needed to fully understand if the autophagic pathway is involved in our observed results (Table 5.2). Further studies using the traditional method of detecting LC3 lipidation, will help shed light on this matter. Specific modulation of the autophagic pathway by deleting or overexpressing autophagy drivers will help dissect their role in CD1d-mediated antigen presentation.

Our studies from Chapter 3 have revealed that Rab7, a late endosomal protein is upregulated after Bcl-xL but not Bcl-2 knockdown (Fig. 3.18). We propose that this leads to accumulation of CD1d molecules in the late endosomes. However, we do not know whether the expansion of the late endosomal compartment is a cause or a consequence of Rab7 upregulation. The study by Vento and colleagues has shown that Bcl-xL directly interacts with Rab7 using tandem affinity purification (181). Although we have conducted some preliminary coimmunoprecipitation studies, further efforts will be needed to obtain conclusive results. These experiments will allow us to determine if the interaction between Bcl-xL and Rab7 seen using affinity chromatography can be reproduced using cell lysates from our system.

6.2 NKT cell responses in a mouse model of severe mantle cell lymphoma

A previous study by Mattarollo *et al.* has shown that NKT cell activation can boost anti-tumor immunity to B cell lymphoma (205). In this study, the authors transplanted tumor cells from E μ -Myc mice, and treated with autologous tumor cells loaded with α -GalCer. This study showed that the activation of NKT cells by α -GalCer resulted in reduced tumor growth and long-term protection against lymphoma. The effect was attributed to NKT, NK and CD8⁺ T cells. IFN- γ and IL-12 as a driver for IFN- γ were the main components of the immune response. The authors concluded that a large systemic level of IFN- γ afforded protection in the treated mice. This work supports the notion that an NKT cell activating antigen used as an adjuvant can lead to an effective IFN- γ -dominated anti-tumor immune response. Data from our study described in Chapter 4 are in agreement with these findings; however our system differs from theirs in two main aspects. Firstly, our DTG mouse model is a spontaneous mouse model for MCL-BV. Secondly, we used the NKT cell agonist α -GalCer alone and not as an adjuvant. Thus, we treated an aggressive hematologic malignancy with a single dose of α -GalCer alone and were able to see improved survival and splenic histology, as well as reduced splenomegaly and lymphadenopathy. We noted high IFN- γ levels but not IL-4 following treatment of mice with α -GalCer. Thus, contrary to Bjordahl *et al.*, but in agreement with Mattarollo *et al.*, we found that NKT cell activation boosts IFN- γ responses and mediates anti-tumor immunity (146, 205). It is also interesting to note that our investigation on Bcl-xL showed an increase in IFN- γ responses, which further supports a role for this protein in eliciting protective NKT cell immune responses in lymphoma.

6.3 Ascertaining the role of NKT cells in vivo in a mouse model of mantle cell lymphoma

The results from chapter 4 showed that the stimulation of NKT cells by injecting α -GalCer led to improved disease outcome in IL-14 α /c-Myc double transgenic (DTG) mice. We found that treatment with a single dose of NKT cell agonist can cause stall disease progression, even in an aggressive spontaneous model of blastoid variant mantle cell lymphoma. These results show that even a small lymphocyte subpopulation can cause drastic effects by potent modulation of the immune response. We found that following treatment with α -GalCer, there were increased IFN- γ responses in splenocytes at baseline, as well as after *ex vivo* restimulation with α -GalCer. This demonstrates that NKT cells play a strong protective role in mantle cell lymphoma, and this effect involves IFN- γ secretion. With these promising results, we plan to directly confirm the role of NKT cells in this disease model by generating CD1d^{-/-} DTG mice (Fig. 5.2). This study is currently in progress, and involves crossing the IL-14 α transgenic mice on to a CD1d^{-/-} background. c-Myc transgenic mice will be crossed on to a CD1d^{-/-} background. Finally, the IL-14 α transgenic CD1d^{-/-} mice will be crossed with c-Myc transgenic CD1d^{-/-} mice to obtain CD1d^{-/-} DTG mice. These DTG mice will completely lack NKT cells, and will be instrumental in definitively determining the role of NKT cells in mantle cell lymphoma. These CD1d^{-/-} DTG mice will be thoroughly immunophenotyped, and their disease progression will be monitored from onset to end stage, to determine mantle cell lymphoma pathogenesis in the absence of NKT cells. We hypothesize that CD1d^{-/-} DTG mice will show more rapid disease progression and succumb to this aggressive

hematological malignancy. Based on our results from α -GalCer injection, we also expect that mice will show lower survival and exacerbated histopathology in the spleen. An assessment of IFN- γ production by T cells and NK cells can also help to test our hypothesis that NKT cells boost an IFN- γ positive feedback loop involving other cell types and fortify anti-tumor responses.

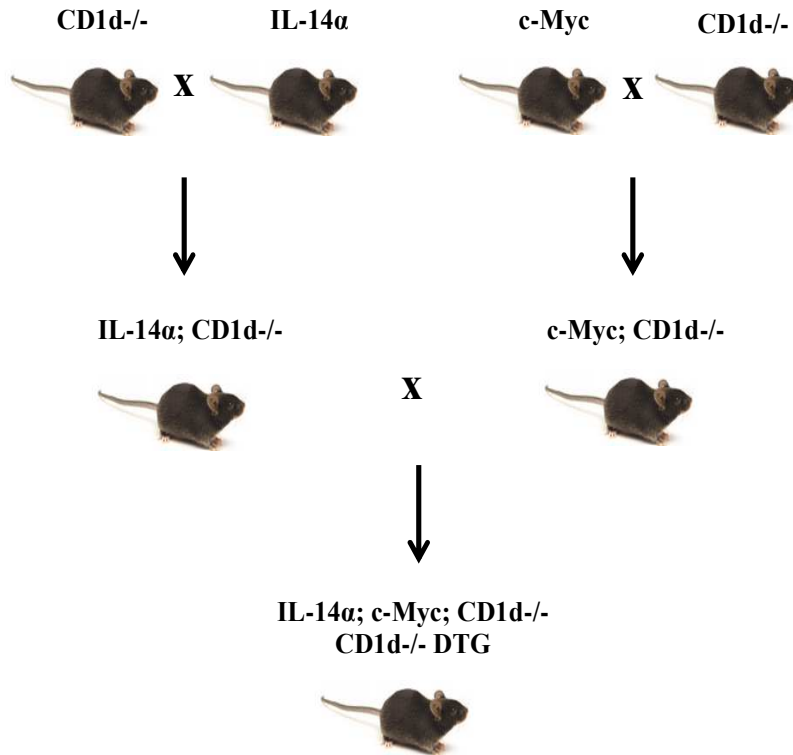


Figure 5.2: Generation of the $CD1d^{-/-}$ DTG mouse, a model for spontaneous mantle cell lymphoma in the absence of NKT cells

$IL-14\alpha$ transgenic mice will be crossed with $CD1d^{-/-}$ mice. $c-Myc$ transgenic mice will also be crossed with $CD1d^{-/-}$ mice. $IL-14\alpha; CD1d^{-/-}$ and $c-Myc; CD1d^{-/-}$ mice will then be crossed to obtain $CD1d^{-/-}$ DTG mice. These DTG mice will have the genetic drivers for MCL, and will also lack NKT cells due to the loss of $CD1d$. This model will help to confirm the role of NKT cells in an aggressive hematological malignancy like MCL.

5.4 Significance and implications

In this study, we first identified a novel role for Bcl-xL in CD1d-mediated antigen presentation to NKT cells. We then found that altered intracellular CD1d trafficking was involved in this regulation. This novel regulatory pathway involving Bcl-xL may allow NKT cells to recognize lymphoma cells, which express high levels of Bcl-xL. Thus, the up-regulation of Bcl-xL as a means to evade apoptotic signals, may actually make lymphoma cells more visible to the immune system. Perhaps, as cells increase the levels of anti-apoptotic proteins like Bcl-xL, their ability to present antigens increases, and they can be readily recognized by NKT cells. NKT cells can then mediate direct cytotoxicity or cytokine responses, and facilitate their elimination. It can be hypothesized that such a mechanism allows the host to detect and destroy malignantly transformed cells and remain disease free. Thus, the appropriate recognition of lymphomas and the activation of NKT cells, may be an important part of anti-tumor immunity in healthy individuals.

However, an alternate possibility is that the high expression of Bcl-xL drives antigen presentation, and subsequently results in excessive NKT cell stimulation. It is already known that NKT cells are reduced in cancer patients (142, 159, 237). We also recently reported that NKT cell function is reduced following the development of lymphoma in DTG and c-Myc transgenic mice (159). It is therefore plausible that the increased expression of Bcl-xL results in the over-stimulation of NKT cells. This can lead to anergy or activation-induced cell death in NKT cells, explaining their loss in number and function during cancer.

Studies using the DTG mouse model of mantle cell lymphoma have shown that treatment with a single dose of NKT cell activating agonist can stall disease progression. This implies that NKT cells play a protective role in this disease. Upcoming studies using CD1d^{-/-} DTG mice will help to confirm the role of NKT cells. This means that NKT cells may provide a new avenue for treatment of an incurable, highly recalcitrant disease like mantle cell lymphoma. In the context of current studies on the translational application of NKT cell agonists, this work will help realize the goal of novel therapy in a disease that greatly needs such advancement (152). In fact, the Webb lab has recently published a method for the rapid assessment of NKT cell function using quantitative PCR. This method can facilitate the screening of patients to identify good candidates for NKT cell-based therapy (142). A strong fundamental understanding of NKT cell biology, and the development of strategies for the effective modulation of NKT cell functions, are together paving the way for the therapeutic use of NKT cells in the near future.

Summary

Our studies on CD1d-mediated antigen processing and presentation have revealed a novel regulatory role for Bcl-xL in NKT cell responses. We found that the over-expression or induction of Bcl-xL led to increased NKT cell responses. On the other hand, pharmacological inhibition or knockdown of Bcl-xL led to reduced NKT cell responses. Importantly, we demonstrate that the inhibition of Bcl-xL using small molecule BH3 mimetics, also resulted in reduced MHC class II-mediated antigen presentation to classical CD4⁺ T cells. We present evidence that this regulation of antigen presentation by Bcl-xL was CD1d-dependent and apoptosis-independent. Furthermore, surface CD1d expression was unchanged following the modulation of CD1d, although the intracellular CD1d localization was altered. The knockdown of Bcl-xL led to loss of CD1d molecules from the LAMP1⁺ compartment. Rab7 was upregulated, and CD1d molecules accumulated in the late endosomes. Together this work demonstrates that Bcl-xL regulates CD1d-mediated antigen presentation to NKT cells by altering CD1d trafficking through the endocytic pathway.

In vivo, using a spontaneous mouse model of blastoid variant mantle cell lymphoma, we have shown that the activation of NKT cells by a single injection of α -GalCer was sufficient to stall disease progression. Treated animals showed reduced splenomegaly and lymphadenopathy compared to their untreated controls. Splenic histopathology was also dramatically improved. Finally, strong IFN- γ responses were observed in splenocytes from mice treated with α -GalCer, indicating that this might be a possible mechanism underlying the improved disease outcome that follows NKT cell activation.

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