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THE EXPRESSION AND FUNCTION OF GLYCOSYLPHOSPHATIDYLINOSITOL (GPI)-ANCHORED PROTEINS IN INVARIANT NATURAL KILLER T (/NKT) CELLS

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THE EXPRESSION AND FUNCTION OF GLYCOSYLPHOSPHATIDYLINOSITOL (GPI)-ANCHORED PROTEINS IN INVARIANT NATURAL KILLER T (*i*NKT) CELLS

(Spine title: GPI-anchored proteins in *i*NKT cell activation)

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by

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Abstract

Invariant natural killer T (*i*NKT) cells are a unique lymphocyte subpopulation with potent immunomodulatory properties. They can produce copious amounts of pro- and/or anti-inflammatory cytokines, yet the mechanisms governing the type of immune responses they elicit are not fully understood. Conventional T cell activation can be achieved or augmented by glycosylphosphatidylinositol (GPI)-anchored proteins. Whether this is also true for *i*NKT cells is essentially unexplored. I hypothesized that ligation of GPI-anchored proteins such as Thy-1 and CD55 will enhance *i*NKT cell activation leading to robust cytokine production. Using flow cytometry, mouse *i*NKT cells were found to constitutively express Thy-1. Thy-1 engagement combined with classical TCR stimulation led to *i*NKT cell activation and cytokine production as measured by ELISA and RT-qPCR. Similarly, human *i*NKT cells expressed CD55, the cross-linking of which enhanced TCR-mediated activation. Overall, I have demonstrated that GPI-anchored proteins play a significant role in the magnitude of *i*NKT cell responses.

Keywords

invariant natural killer T (*i*NKT) cells, α-galactosylceramide, GPI-anchored proteins, Thy-1, CD55, cellular activation, costimulation

For my Mom and Dad,

Thank you for your unrelenting support as I embark upon academic journey after journey. Someday soon I will trade in the title of "professional student" for a real job. I promise.

♥

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List of Abbreviations

5'-NT	5'-nucleotidase
α	alpha
α-GalCer	alpha-galactosylceramide
AHR	airway hyperreactivity
ALDH	aldehyde dehydrogenase
AP-1	activator protein-1
APC	allophycocyanin
β	beta
BCR	B cell receptor
BMDC	bone marrow-derived dendritic cell
BSA	bovine serum albumin
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CFSE	carboxyfluorescein diacetate, succinimidyl ester
CO_2	carbon dioxide
CTL	cytotoxic T lymphocyte
CTLA	cytotoxic T lymphocyte antigen
δ	delta
DAF	decay accelerating factor (CD55)
DAG	diacylglycerol
DC	dendritic cell
DEPC	diethyl pyrocarbonate
DN	double negative
ε	epsilon
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-related protein kinase
FACS	fluorescent-activated cell sorting
FcγR	Fc gamma receptor

FCS	fetal calf serum
FITC	fluorescein isothiocyanate
γ	gamma
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GITR	glucocorticoid-induced tumor necrosis factor receptor
GlcN	glucosamine
GM-CSF	granulocyte-macrophage colony-stimulating factor
GPI	glycosylphosphatidylinositol
GTP	guanosine triphosphate
HBSS	Hank's buffered saline solution
HP	hypersensitivity pneumonitis
IFN	interferon
ICOS	inducible T cell costimulator
IL	interleukin
*i*NKT	invariant natural killer T
Ino	inositol
i.p.	intraperitoneally
IP3	inositol triphosphate
ITAM	immunoreceptor tyrosine-based activation motif
JNK	c-jun N-terminal kinase
kDa	kilodalton
$KHCO_3$	potassium bicarbonate
LAT	linker for activation of T cell
mAb	monoclonal antibody
MAC	membrane attack complex
Man	mannose
MAPK	mitogen-activated protein kinase
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MIRF	membrane inhibitor of reactive lysis
μg	microgram

mg	milligram
mL	milliliter
mRNA	messenger ribonucleic acid
NFAT	nuclear factor of activated T cells
NFκB	nuclear factor kappa B
ng	nanogram
NH_4Cl	ammonium chloride
NK	natural killer
NKT	natural killer T
nm	nanometer
P-EthN	phosphoethanolamine
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
pg	picogram
PIG-A	phosphatidylinositol glycan class A
PIP2	phosphatidylinositol bisphosphate
PI-PLC	phosphatidylinositol-phospholipase C
PLC	phospholipase C
PMA	phorbol-12-myristate-13-acetate
PNH	paroxysmal noctural hemoglobinuria
PRR	pattern recognition receptor
PTK	protein tyrosine kinase
qPCR	quantitative polymerase chain reaction
RasGRP	Ras guanyl nucleotide-releasing protein
RNA	ribonucleic acid
RT	reverse transcriptase
SAP	signalling lymphocyte activation molecule-associated protein
SD	standard deviation

SLAM	signalling lymphocyte activation molecule
T_C	T cytotoxic cell
T_H	T helper cell
T_{reg}	T regulatory cell
TCR	T cell receptor
TLR	toll-like receptor
TNF	tumor necrosis factor
*v*NKT	variant natural killer T
VSG	variant surface glycoprotein
XLP	X-linked lymphoproliferative disease
ζ	zeta
ZAP-70	zeta-chain associated protein of 70 kilodalton

CHAPTER 1:

INTRODUCTION

The immune system is a complex network of tissues, cells, and secreted factors whose primary function is to protect the host from infection. Immune responses are mounted when various components of the immune system detect a foreign invader. This triggers the activation of effector cells, which are capable of destroying foreign material or providing help to other immunocytes. To ensure that a controlled response is elicited, several types of regulatory immune cells exist. These regulatory cells can either promote or suppress immune responses in order to minimize bystander host damage while eradicating the foreign material. Examples include $Foxp3^+$ T regulatory (T_{reg}) cells, $CD8^+$ T_{reg} cells, γδ T cells, myeloid-derived suppressor cells, and natural killer T (NKT) cells.

1.1. NKT cells: general introduction

NKT cells are a unique subset of T lymphocytes that possess some characteristics of natural killer (NK) cells. They were first described in the 1980s when several groups independently identified mouse T cells with biased usage of the T cell receptor (TCR) Vβ8 chain (1, 2). A few years later, a subset of Vβ8 chain-biased, double negative ($CD4^-$ $CD8^-$) T cells were identified that expressed the NK cell marker NK1.1 (3). These cells were described as an "NK/T bipotential" subset that was functionally and phenotypically distinct from NK cells. Several years later, these cells were found to express a single invariant TCRα chain, containing the Vα14Jα18 segment, which pairs with either Vβ8, 7, or 2 (4). Since these early findings, the field of NKT immunobiology has exploded and these cells have proven to play important roles in health and disease.

In addition to coexpressing the TCR and NK markers, NKT cells are characterized by their ability to respond to lipid antigens presented by CD1d molecules (5). This is in contrast to conventional T cell activation, where peptide antigens are presented to T cells in the context of major histocompatibility complex (MHC) class I or II (6, 7). CD1d is an MHC class I-like molecule possessing a deep hydrophobic pocket, which makes it ideal for binding water-insoluble lipids (8). Two main subsets of NKT cells exist: invariant NKT (*i*NKT) cells and variant NKT (*v*NKT) cells. This

classification is based on the TCR arrangement of these subsets. *i*NKT cells, also known as classical or type I NKT cells, have an invariant Vα14Jα18 chain paired with a semi-invariant Vβ chain (Vβ8, 7, or 2), while the less commonly studied *v*NKT cells, or type II NKT cells, have a more diverse TCR repertoire [reviewed in (9)]. Both subsets respond to antigens presented in the context of CD1d; however, their antigen specificities do not appear to overlap. *i*NKT cells are often characterized by their responsiveness to the marine sponge-derived glycolipid α-galactosylceramide (α-GalCer), in addition to recognizing microbial glycolipids such as *Sphingomonas* glycosphingolipids and some self antigens (10-12). *v*NKT cell ligands remain relatively unknown, although these cells have been shown to recognize myelin-derived 3'-sulfated galactosylceramide, also known as sulfatide (13). Since most research in the NKT cell field—including this study—relate to the invariant subset, only *i*NKT cells will be discussed from this point forward.

Stimulation of *i*NKT cells with α-GalCer initiates a broad immune response that has widespread effects in the host. Briefly, *i*NKT cells have been shown to aid in bacterial and viral clearance (14, 15), tumor surveillance (16), maintaining self-tolerance (17), and protection against a number of autoimmune diseases such as type 1 diabetes (18). *i*NKT cells have also been implicated in the development of atherosclerosis (19) and airway hyperreactivity (20), and have been shown to promote the development of certain autoimmune diseases (21, 22). Due to these seemingly opposite outcomes, *i*NKT cells have been termed the "double-edged swords" of the immune system (23) **(Figure 1.1.)**. Ultimately, by studying the immunobiology of *i*NKT cells, we can better understand how these cells are able to either promote or suppress immune responses.

1.2. The role of *i*NKT cells in immune responses

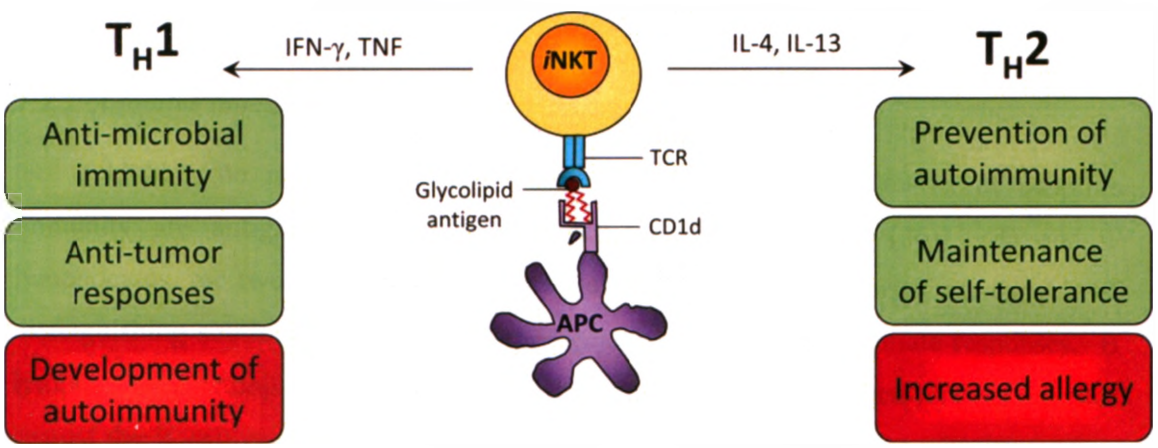
1.2.1. Innate immunity

The innate arm of the immune system consists of several barriers and effector molecules that serve as the first line of defense against invading pathogens. Such barriers include both physical barriers, such as the skin and mucosal surfaces, and physiological

Figure 1.1. The immunomodulatory effects of *i*NKT cell activation. *i*NKT cell recognition of glycolipid antigens, such as α-galactosylceramide, presented by CD1d molecules on antigen presenting cells leads to robust cytokine production that can induce either a T_H1- or T_H2-type response. In most cases, the response is beneficial to the host (shown in green); however, the response is occasionally detrimental (shown in red). Accordingly, *i*NKT cells have been termed the "double-edged swords" of the immune system. APC: antigen-presenting cell; $T_H1/2$: T helper type 1/2; IL: interleukin; IFN: interferon; TNF: tumor necrosis factor.

Adapted from Godfrey and Berzins, 2007 (24)

"Double-Edged Sword"



barriers, which include conditions such as temperature, pH, and various soluble and cell-associated molecules [reviewed in (25)]. Many of the molecules involved in innate immunity, termed pattern recognition receptors (PRRs) have the ability to recognize highly conserved and repetitive microbial motifs known as pathogen-associated molecular patterns (PAMPs). The engagement of PRRs with PAMPs leads to the production of pro-inflammatory cytokines, chemokines, and effector molecules that recruit innate immune cells such as neutrophils and monocytes to the site of damage. Upon arrival, these cells ingest extracellular material and pathogenic organisms by the process of phagocytosis. In addition to neutrophils and monocytes, NK cells also play a role in innate immunity through their rapid activation and ability to recognize and kill targets without prior exposure to antigen. Together, these cell subsets, in addition to numerous others, protect against foreign invaders in a non-specific manner.

1.2.2. Adaptive immunity

Unlike the innate immune system, the distinguishing hallmarks of adaptive immunity are antigenic specificity and memory [reviewed in (26)]. B and T lymphocytes, the two main effector cells of adaptive immune responses, are capable of distinguishing subtle differences among antigens and eliciting appropriate responses. B lymphocytes develop in the bone marrow and express a unique antigen-binding receptor termed the B cell receptor (BCR). When the BCR comes into contact with its cognate antigen, the B cell divides rapidly and differentiates into memory B cells and plasma cells. Long-lived memory B cells persist in the circulation as defenders against future encounters with that unique antigen. Plasma cells, on the other hand, generally have a shorter overall lifespan and secrete copious amounts of antigen-specific antibodies—the major effector molecules of humoral immunity. T lymphocytes, which mature in the thymus, are the other main effector cell of the adaptive immune response. They, too, express a unique antigen-binding receptor (i.e. TCR); however, this receptor only recognizes antigen bound to an MHC molecule (27). Upon encountering antigen, naïve T cells proliferate and differentiate into various effector T cells. The two best characterized subpopulations include T helper (T_H) and T cytotoxic (T_C) cells. T_H cells secrete cytokines that play an important role in activating B cells, T_C cells, macrophages, and

various other immune cells. Depending on the cytokine profile secreted, these T_H cells can be further subdivided into two subsets designated T_H1 and T_H2 (28). The T_H1 subset secretes cytokines that promote cell-mediated functions including inflammation and tissue injury, while the T_H2 subset mediates humoral immunity. Once exposed to cognate antigen and T_H1-derived cytokines, T_C cells differentiate into cytotoxic T lymphocytes (CTLs) that directly kill antigen-expressing cells such as virus-infected cells and tumor cells. The T_H1/T_H2 paradigm has been recently expanded to include a new subset of T_H cells termed T_H17 [reviewed in (29)]. These cells contribute to pathogen clearance by secreting pro-inflammatory cytokines such as interleukin (IL)-17, IL-17F, IL-21, and IL-22. Together, the diverse pool of B and T lymphocytes protect the host from foreign substances by mounting a robust immune response specific for the invading pathogen.

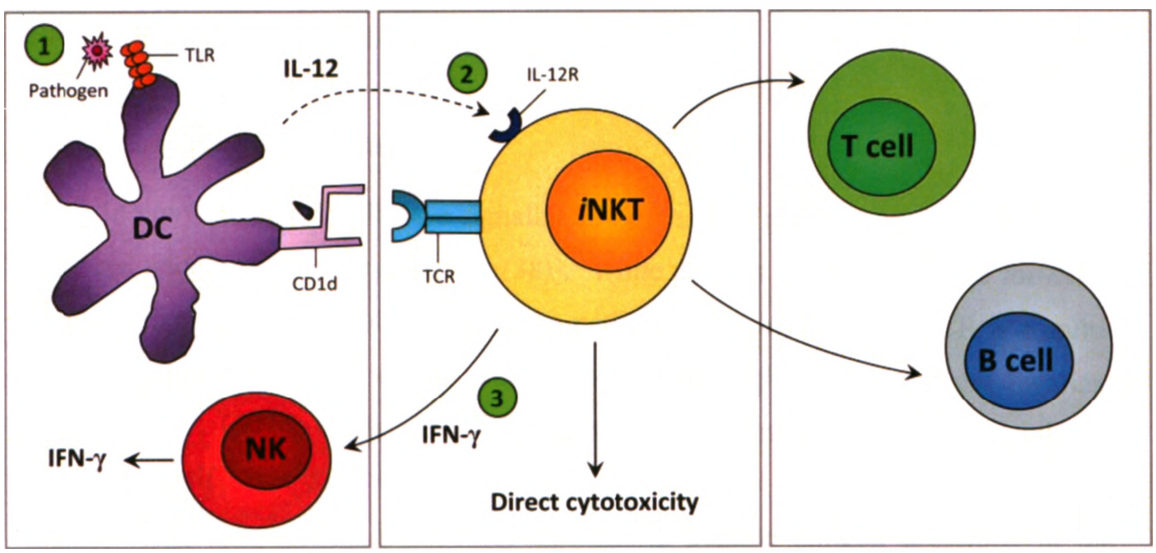
1.2.3. iNKT cells act as a bridge between innate and adaptive immunity

The innate and adaptive arms of the immune system work hand-in-hand to eliminate foreign invaders. *i*NKT cells have been proposed to bridge the two arms of the immune system due to their ability to respond rapidly to antigenic challenge and subsequently transactivate other immune cells [(reviewed in (30)]. In the circulation, *i*NKT cells constitutively express the activation markers CD69 and CD44 (31). They survey the host in a constant state of low-level activation, ready to quickly act when the host is challenged with antigen. When this occurs, bacterial and viral components are often first recognized by PRRs—such as toll-like receptors (TLRs)—on the surface of dendritic cells (DCs). DCs respond by secreting the pro-inflammatory cytokine IL-12, which subsequently enhances *i*NKT cell activation and leads to the rapid release of interferon (IFN)-γ (32). This promotes NK cell, macrophage, and pathogen-specific T_H1 cell activation. In addition, *i*NKT cells support class switching and somatic hypermutation in B cells (33). Together, these effector cells elicit a coordinated immune response that effectively eradicates the invading microbe. By bridging the innate and adaptive arms of the immune system, *i*NKT cells help unify the immune system in order to generate the most effective immune response **(Figure 1.2.)**.

Figure 1.2. ***i*NKT cells act as a functional bridge between innate and adaptive immunity.** *i*NKT cell activation in response to bacterial and viral pathogens is initiated by bacterial and/or viral components binding to PRRs, such as TLRs, on the DC surface (1). This causes DCs to release the pro-inflammatory cytokine IL-12, which binds to IL-12Rs on the *i*NKT cell surface (2). IL-12R binding enhances basal *i*NKT cell activation and leads to the rapid release of IFN-γ (3). IFN-γ promotes the activation of NK cells, T cells, and B cells which together elicit a coordinated immune response that effectively eradicates the invading microbe. TLR: toll-like receptor; DC: dendritic cell; IL-12R: IL-12 receptor.

Adapted from Taniguchi et al., 2003 (30)

innate immunity ↔ ***i*NKT cells** ↔ adaptive immunity



1.3. *i*NKT cell signalling and activation

Upon TCR engagement, an *i*NKT cell becomes activated through a series of signalling events. These events are largely assumed to be similar to those occurring in conventional T cells; however, differences between signalling molecules in T cells and *i*NKT cells have been found (34-36). Upon activation, *i*NKT cells produce copious amounts of cytokines and undergo proliferation. This is followed by eventual contraction of the expanded *i*NKT population in order to maintain a homeostatic size and state. Subsequent encounter with glycolipids often results in a state of long-term *i*NKT cell hyporesponsiveness termed anergy (37). The specific events known to mediate *i*NKT cell activation are described below.

1.3.1. TCR-mediated signalling

In conventional T cells, the signalling cascades that give rise to T cell activation have been widely studied [reviewed in (38)]. While this has not been the case for *i*NKT cells, it is likely that many of the signalling pathways are similar. The TCR is a multi-subunit complex comprised of the antigen-specific TCRαβ heterodimer and invariant subunits CD3γ, CD3δ, CD3ε, and TCRζ that contain immunoreceptor tyrosine-based activation motifs (ITAMs) in their cytoplasmic domains. Signalling cascades are initiated when the TCRαβ heterodimer recognizes peptide antigen presented in the context of self MHC. The earliest documented event following TCR engagement is the phosphorylation of TCR/CD3 ITAMs by the Src family of protein tyrosine kinases (PTKs) Lck and Fyn. These phosphorylated ITAMs serve as docking sites for the SH2 domains of the tyrosine kinase zeta-chain associated protein of 70 kilodalton (ZAP-70), which becomes activated and subsequently phosphorylates a number of adaptor molecules. Linker for activation of T cell (LAT) is a transmembrane adaptor protein that plays an essential role in TCR signalling. Upon phosphorylation by ZAP-70, LAT recruits phospholipase C (PLC)γ1, an enzyme responsible for the degradation of phosphatidylinositol bisphosphate (PIP2) into inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 causes intracellular calcium levels to rise, thereby activating the calcium-binding protein calcineurin. This protein induces translocation of nuclear

factor of activated T cells (NFAT), a transcription factor involved in IL-2 production. Meanwhile, DAG activates several isoforms of protein kinase C (PKC), a family of kinases that play an important role in nuclear factor κ B (NFκB) activation, another transcription factor responsible for IL-2 production. DAG also recruits and activates Ras guanyl nucleotide-releasing protein (RasGRP), which initiates the Ras signalling pathway. Ras, a guanosine triphosphate (GTP)-binding protein, activates a series of serine/threonine and dual-specificity (Ser/Thr and Tyr) kinases eventually leading to the activation of the mitogen-activated protein kinase (MAPK) family members extracellular signal-related protein kinases 1/2 (ERK1/2), c-jun N-terminal kinase (JNK), and p38. These MAPKs directly activate transcription factors such as Jun, Fos, and activator protein-1 (AP-1) that are involved in T cell activation.

1.3.2. Signalling molecules involved in iNKT cell activation

The TCR-mediated signalling pathways involved in *i*NKT cell activation have received little attention to date. On the other hand, the signalling molecules involved in *i*NKT cell ontogeny have been more extensively studied and may also be important for *i*NKT cell function. Differences in signalling pathways between T cells and *i*NKT cells were first seen with Fyn-deficient mice; *i*NKT cell numbers were low but conventional T cell numbers were unaffected (34, 39). Since Fyn interacts with signalling lymphocyte activation molecule (SLAM)-associated protein (SAP)(40), subsequent work investigated *i*NKT cell development in $SAP^{-/-}$ mice and individuals with X-linked lymphoproliferative disease (XLP) who harbour mutations in the gene encoding SAP. Both SAP-deficient mice and XLP patients had reduced numbers of *i*NKT cells but normal levels of conventional T cells (35). This observation suggests that SAP may play a unique role in *i*NKT cell signalling. Among its many functions, SAP has been shown to couple with Rac signalling pathways to influence downstream MAPK activity (41), bind weakly to Lck (42), and cooperate with the TCR to increase activation of NF-κB (43). Unlike conventional T cells, *i*NKT cells are highly dependent on NF-κB for their development (36, 44). Ultimately, many of the signalling molecules that contribute to *i*NKT cell development are also involved in TCR-mediated signalling pathways in conventional T cells. While the role of these molecules in *i*NKT cell activation is currently unknown,

further research in this important area will hopefully shed light on the signalling mechanisms involved in *i*NKT cell activation.

1.3.3. Cytokine production by iNKT cells

When the invariant TCR of an *i*NKT cell recognizes CD1d-bound α-GalCer, the *i*NKT cell responds by secreting large amounts of IL-4 in the first 2-4 hours (45). Such a rapid release of cytokine is made possible by the fact that *i*NKT cells circulate with preformed cytokine mRNA that is waiting to be translated (46). A gradual shift towards IFN-γ production occurs subsequently, with IFN-γ levels peaking at approximately 16-24 hours. In vivo, a substantial amount of IFN-γ is produced by *i*NKT cells themselves; however, most of the detected IFN-γ originates from transactivated NK cells (47). These events are specific to α-GalCer and may vary depending on the glycolipid agonist presented, the costimulatory molecules present on the *i*NKT cell surface, and/or the tissues in which *i*NKT cells reside. In mice, it has been shown that liver *i*NKT cells are better mediators of anti-tumor responses compared to splenic and thymic *i*NKT cells (36, 48). Furthermore, within the liver, double negative (DN) *i*NKT cells are more efficient at tumor cell rejection than the $CD4^+$ subset. These findings are consistent with a previous study demonstrating that human DN *i*NKT cells produce predominantly T_H1-type cytokines while $CD4^+$ *i*NKT cells are skewed towards T_H2-type cytokine production (49).

1.3.4. iNKT cell proliferation, homeostasis, and anergy

It was once believed that mouse *i*NKT cells undergo cell death immediately following activation as they become virtually undetectable by flurochrome-conjugated CD1d tetramers routinely used for *i*NKT cell staining (50). As first demonstrated by Wilson *et al.* (51), it is now widely accepted that *i*NKT cells downregulate their TCR shortly after activation. Reappearance of the TCR occurs 24 hours post-activation and coincides with rapid *i*NKT cell proliferation and expansion. Peak cell numbers are reached on day 3, at which point cytokine secretion stops and *i*NKT cells begin to die off. Homeostatic levels are reached between days 7 and 9 when *i*NKT cell numbers return to normal. Interestingly, restimulation with α-GalCer induces a state of anergy (37).

Anergic *i*NKT cells are hyporesponsive as judged by their inability to proliferate, produce cytokines, and transactivate other immune cells.

1.3.5. Transactivation of other immune cells

Activation of *i*NKT cells with α-GalCer has downstream effects on almost all hematopoietic cells, including DCs, NK cells, and B and T lymphocytes. DC maturation is enhanced following *i*NKT cell activation as measured by heightened IL-12 production and increased expression of MHC class II molecules and B7 and CD40 costimulatory molecules (52). NK cells proliferate, secrete IFN-γ, and exhibit increased cytotoxic activity (47, 53). B cells are activated, demonstrate more sustained memory B cell responses, and antibody production is enhanced (54, 55). As described above, the cytokine profile of $CD4^+$ T cells is influenced by *i*NKT cell activation. Furthermore, *i*NKT cells have been shown to be potent helper cells for cytotoxic T lymphocyte priming (56). The ability of *i*NKT cells to induce such broad immune responses reinforces the importance of understanding the precise events involved in their activation.

1.4. The role of costimulation in *i*NKT cell activation

In conventional T cell activation, two signals are required for a productive response. Interactions between the TCR and a peptide-bound MHC molecule constitute signal one, whereas signal two is transmitted via costimulatory molecules (57). Costimulation is a critical requirement for naïve T cell activation; if signal two is absent, T cells may die or become anergic (58). *i*NKT cells, on the other hand, do not require costimulation for their activation. Mice lacking the costimulatory molecules CD40L or CD28 have impaired IFN-γ and IL-4 secretion in response to α-GalCer stimulation (59); however, further experiments utilizing intracellular staining showed that all *i*NKT cells produce both IFN-γ and IL-4 after α-GalCer administration in $CD40^{-/-}$ and $CD28^{-/-}$ mice (46). This suggests that costimulation is required for optimal cytokine secretion but not for cytokine production. In support of this notion, several molecules including CD28, CD40L, inducible T cell costimulator (ICOS), 4-1BB, glucocorticoid-induced tumor

necrosis factor (TNF) receptor (GITR), and OX40 have been shown to play an important role in α-GalCer-mediated *i*NKT cell activation and are described below.

1.4.1. CD28 and CD40L

In addition to their role in cytokine secretion, the CD28 and CD40L pathways appear to differentially regulate T_H1- and T_H2-type functions of *i*NKT cells. CD28 is required for both IFN-γ and IL-4 secretion since $CD28^{-/-}$ mice showed impaired levels of both cytokines after α-GalCer administration (59). CD40L, on the other hand, only seems to be required for IFN-γ secretion as $CD40^{-/-}$ mice had no change in serum IL-4 levels three hours after α-GalCer administration compared to wildtype mice. Additionally, it has been shown that both of these costimulatory pathways are required for the normal proliferative expansion of *i*NKT cells in vivo (60). $CD40L^{-/-}$ and cytotoxic T lymphocyte antigen (CTLA)-4 immunoglobin (Ig) transgenic mice displayed reduced overall numbers and frequency of *i*NKT cells in both the liver and spleen after α-GalCer injection compared with wildtype mice.

1.4.2. ICOS

ICOS, a member of the CD28 superfamily of costimulatory receptors, has been shown to costimulate *i*NKT cell activation independent of CD28 activity (61). Blockade of ICOS decreases α-GalCer-induced IFN-γ and IL-4 levels both in vitro and in vivo. Like CD28, ICOS is thought to promote both T_H1 and T_H2-type responses equally. ICOS-deficient mice reportedly have partially inhibited *i*NKT cell cytotoxic activity and have reduced T_H1-type responses, as measured by their decreased ability to prevent tumor metastasis, compared with their wildtype counterparts. The T_H2 response in ICOS-deficient mice also appears to be defective (62). In wildtype mice, *i*NKT cells normally promote airway hyperreactivity (AHR); however, $ICOS^{-/-}$ *i*NKT cells were unable to induce AHR. In addition to its effect on cytokine production, ICOS was also shown to be required for $CD4^+$ *i*NKT cell homeostasis. $CD4^+$ *i*NKT cell numbers were significantly reduced in the spleen and liver of both $ICOS^{-/-}$ and $ICOSL^{-/-}$ mice.

1.4.3. 4-1BB

The first indication that 4-1BB, a TNF receptor superfamily member, was involved in *i*NKT cell responses came in 2004 when 4-1BB-deficient mice were found to have impaired *i*NKT cell development (63). Since then, it has been demonstrated that 4-1BB functions as a costimulatory molecule on the surface of *i*NKT cells. Engagement of 4-1BB with an agonistic monoclonal antibody (mAb) enhanced α-GalCer-induced IFN-γ and IL-4 production (64). Likewise, 4-1BB$^{-/-}$ mice had lower serum cytokine levels after α-GalCer administration compared with wildtype mice. In a disease model, 4-1BB engagement exacerbated the induction of *i*NKT cell-mediated AHR in an IL-4Rα- but not IFN-γ-mediated pathway, suggesting that 4-1BB costimulation on *i*NKT cells promotes a T_H2-type response.

1.4.4. GITR

GITR is another member of the TNF receptor superfamily that has been shown to play a role in *i*NKT cell costimulation. GITR engagement using an agonistic mAb, in the presence of TCR signals, resulted in enhanced IL-2, IFN-γ, and IL-4 production, expression of activation markers such as CD25 and CD69, and proliferation (65). These findings were translated to the hypersensitivity pneumonitis (HP) disease model, in which *i*NKT cells function to attenuate HP progression. *i*NKT cells were preincubated with an agonistic anti-GITR Ab and then adoptively transferred into CD1d$^{-/-}$ mice prior to HP induction. The inflammatory responses in mice that received GITR-engaged *i*NKT cells were much less severe than those in mice receiving *i*NKT cells preincubated with control IgG. Cytokine analysis demonstrated that anti-GITR-pretreated *i*NKT cells produced less IFN-γ and more IL-4 than control antibody-pretreated *i*NKT cells in the HP model. Overall, these findings suggest that GITR costimulates *i*NKT cell activation and promotes a T_H2-type response.

1.4.5. OX40

OX40—like CD40L, 4-1BB, and GITR—is a member of the TNF receptor superfamily. Much like the other molecules, OX40 has been shown to play a

costimulatory role in α-GalCer-induced *i*NKT cell activation. OX40L blockade on DCs inhibited their ability to support *i*NKT cell activation, as judged by reduced IFN-γ production (66). Furthermore, in a study using $OX40^{-/-}$ mice, *i*NKT cells produced significantly lower levels of IFN-γ than wildtype *i*NKT cells did after α-GalCer stimulation (67). The role of OX40 in *i*NKT cell activation still needs further investigation as IL-4 levels were not measured and a disease model was not employed in either study.

All of the aforementioned studies have demonstrated that costimulation, while not absolutely essential for *i*NKT cell activation, is an important component of optimal *i*NKT cell responses. Costimulation is involved in *i*NKT cell proliferation, activation, homeostasis, and development. It can differentially regulate T_H1- and T_H2-type responses which, in turn, influence disease outcome. The costimulatory molecules described above might merely be a small snapshot of dozens of other unidentified costimulatory molecules on the *i*NKT cell surface. Ongoing research in the *i*NKT cell field will hopefully reveal some of these molecules and may provide new tools for modulating *i*NKT cell responses. For instance, the role of glycosylphosphatidylinositol (GPI)-anchored proteins as potential costimulators of *i*NKT cells has remained unexplored. This topic forms the basis of my thesis research.

1.5. GPI-anchored proteins

There are a number of proteins that are uniquely linked to the cell membrane through a GPI anchor. The first piece of evidence alluding to the existence of lipid-anchored proteins came from Slein and Logan, who discovered that treatment of cells with the bacterial enzyme phosphatidylinositol-phospholipase C (PI-PLC) led to the selective loss of alkaline phosphatase from the cell surface (68). Ferguson and colleagues were the first to fully elucidate the structure of the GPI anchor using variant surface glycoprotein (VSG) present on the surface of the parasitic protozoan *Trypanosoma brucei* (69). They found that GPI-anchored proteins are linked at their carboxyterminus to a trimannosyl-glucosamine core; the glucosamine core is attached to phosphatidylinositol,

which anchors the structure to the outer leaflet of the plasma membrane through a phosphodiester linkage. In the presence of PI-PLC, the phosphodiester bond is cleaved and the protein is released from the cell surface (**Figure 1.3.**). Endogenous PLC has been found in mammalian tissues, suggesting that it may play a role in downregulating GPI-anchored protein levels on the cell surface (70).

1.5.1. The purpose of the GPI anchor

The GPI anchor is thought to direct proteins to the extracellular surface since conventional intracellular proteins that have been artificially attached to a GPI anchor will be expressed extracellularly (71, 72). More specifically, it has been suggested that the GPI anchor targets proteins to distinct, detergent-insoluble regions of the cell membrane termed lipid rafts (73). These lipid raft microdomains are enriched in cholesterol, glycolipids, and sphingolipids and form associations with the acylated tails of tyrosine kinases and GPI-anchored proteins. The fluid-like architecture within these regions is ideal for highly motile signalling molecules; consequently, lipid rafts have been termed "organizational centers" for signalling (74).

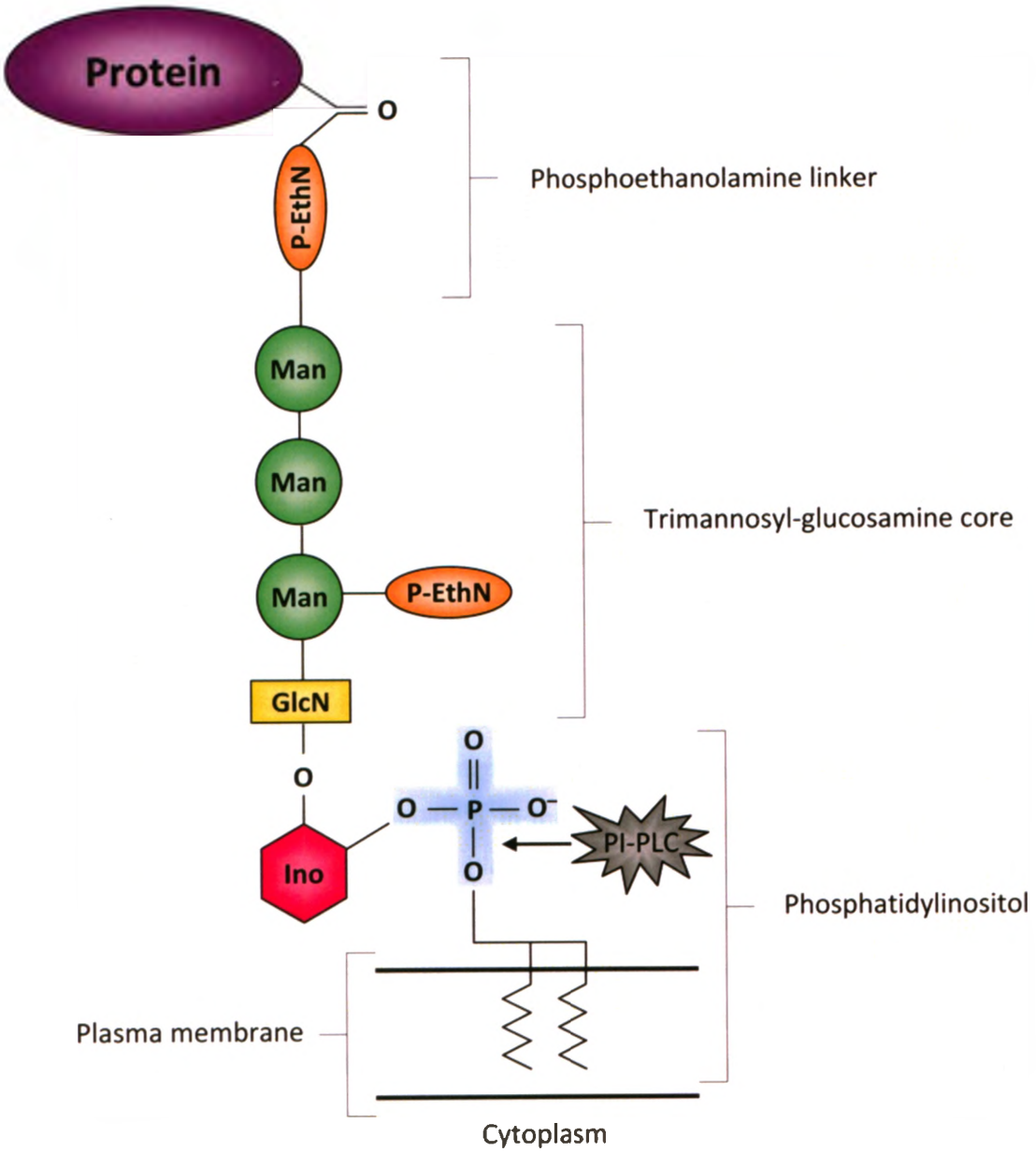
1.5.2. Functions of GPI-anchored proteins

GPI-anchored proteins serve a wide variety of functions on the cell surface. These include, but are not limited to, roles as adhesion molecules, enzymes, antigens, and mediators of cell signalling [reviewed in (75)]. In particular, their ability to modulate cell signalling is an interesting concept since GPI-anchored proteins are exclusively linked to the outer leaflet of the lipid membrane bilayer and lack a transmembrane domain.

Several studies have shown that Ab-mediated cross-linking of numerous GPI-anchored proteins [e.g. 5'-NT (76), Thy-1 (77), Ly-6 (78), DAF (79)] has a downstream effect on signalling events; namely tyrosine, serine, and threonine residue phosphorylation, intracellular calcium mobilization, and transcription factor activation. Interestingly, it is believed that a component of the GPI anchor itself is responsible for signalling since cells expressing a transmembrane form of the GPI-anchored protein decay accelerating factor (DAF), also known as CD55, were not activated when cross-

Figure 1.3. The structure of the GPI anchor. GPI-anchored proteins are linked at their carboxyterminus to a trimannosyl-glucosamine core. The core is attached to phosphatidylinositol, which anchors the structure to the outer leaflet of the plasma membrane through a phosphodiester linkage (shown in blue). In the presence of PI-PLC, the phosphodiester bond is cleaved and the protein is released from the cell surface. P-EthN: phosphoethanolamine; Man: mannose; GlcN: glucosamine; Ino: inositol; PI-PLC: phosphatidylinositol-phospholipase C.

Adapted from Medof et al., 1996 (80) and Paulick and Bertozzi, 2008 (75)


Protein
O
P-EthN
Phosphoethanolamine linker
Man
Man
Man
P-EthN
Trimannosyl-glucosamine core
GlcN
O
Ino
O
O — P — O⁻
O
PI-PLC
Phosphatidylinositol
Plasma membrane
Cytoplasm

linked (81). How an outer leaflet protein interacts with the inner leaflet remains somewhat of a mystery; however, a number of theories to explain signalling through GPI-anchored proteins have been proposed. GPI-anchored proteins have been shown to associate with PTKs, key regulators of cell activation and signal transduction (82). It has been suggested that lipid raft microdomains coalesce when GPI-anchored proteins are cross-linked, which facilitates interactions between PTKs and allows them to reach the cell's required threshold for activation (74, 83). Others propose that GPI-anchored proteins physically interact with transmembrane proteins such as CD45 (84), forming a bridge between the GPI-anchored protein and the inner leaflet of the plasma membrane. Ultimately, these signalling events have been most commonly observed in T lymphocytes, suggesting that signalling induced by GPI-anchored proteins may be mediated by the TCR. For Thy-1- and Ly-6-mediated activation, the presence of the TCR is required for activation to occur (85). Furthermore, T lymphocyte clones that are deficient in GPI anchor biosynthesis have a reduced capacity to signal through their TCR (86). This interdependence between the TCR and GPI-anchored proteins further supports the notion that GPI-anchored proteins play an important role in cell signalling.

1.5.3. Defects in GPI anchor biosynthesis

GPI anchor biosynthesis is mediated by the X-linked phosphatidylinositol glycan class A (*PIG-A*) gene. Somatic mutations in this gene leads to the partial or complete lack of GPI-anchored proteins on all blood cells, giving rise to a disorder called Paroxysmal Noctural Hemoglobinuria (PNH) (87). This condition is characterized by hemolysis, thrombosis, and anemia due to the increased susceptibility of red blood cells to components of the complement cascade (88). Specifically, DAF and Membrane Inhibitor of Reactive Lysis (MIRF or CD59 or protectin) are GPI-anchored proteins that protect red blood cells from complement-mediated lysis in normal individuals (89, 90). Although red blood cell lysis is the dominant manifestation of PNH, these individuals also exhibit underlying immune cell functional abnormalities. For example, T cells from PNH patients have severe defects in TCR-dependent proliferation and IFN-γ production (91).

Most of the work done to better understand the role of GPI-anchored proteins in cell signalling has utilized T lymphocytes. Whether and how GPI-anchored proteins mediate *i*NKT cell signalling have not been previously explored. To investigate this notion, my research has focused on the GPI-anchored proteins Thy-1 (CD90) and CD55 (DAF) on *i*NKT cells.

1.6. Thy-1

1.6.1. The history of Thy-1

Thy-1 is a small, heavily glycosylated GPI-anchored protein (92). It was first identified in the 1960s as a marker to distinguish between lymphocytes of thymic and non-thymic origin (93). Since then, investigations on Thy-1 have led to a number of important discoveries in a wide range of fields including—but not limited to—immunology, neuroscience, and biochemistry. Anti-sera specific for Thy-1 were the first reagents used to distinguish between B cells and T cells (94). It was later observed that Thy-1-specific anti-sera caused spleen and lymph node proliferation, suggesting a role for Thy-1 in T cell activation (95). This was later confirmed by Kroczek *et al.*, who identified an anti-Thy-1 mAb (clone G7) that was capable of activating T cells (96). Structural studies on Thy-1 established the foundation of the immunoglobulin superfamily (97) and led to the first biochemical characterization of a vertebrate GPI anchor (98). Currently, Thy-1 is used as a marker for lipid rafts in murine cells due to its abundance within these specialized areas. Despite five decades of fruitful research on Thy-1, its exact biological function remains somewhat of a mystery.

1.6.2. Characteristics of Thy-1

Homologues of Thy-1 have been identified in both invertebrate and vertebrate species including squid (99), frogs (100), chickens (101), rodents (93, 102), dogs (103), and humans (104). In mice, there are two allelic variants of the Thy-1 gene that code for either Thy-1.1 or Thy-1.2 (105). The latter is expressed in most mouse strains whereas

the former is limited to AKR/J and PL strains. Thy-1.1 and Thy-1.2 differ only in amino acid 89, which are arginine and glutamine in Thy-1.1 and Thy-1.2, respectively (97).

1.6.3. Thy-1 tissue distribution and expression

Thy-1 is expressed on a diverse assortment of cell types including thymocytes (93), lymphocytes (106), neuronal cells (93), fibroblasts (107), and stem cells (108). In all species studied to date, Thy-1 is present on neuronal cells and fibroblasts. In contrast to this conserved expression, Thy-1 expression in the immune system varies largely between species. In mice, Thy-1 is the most heavily expressed surface protein on thymocytes, covering 10-20% of the total cell surface [reviewed in (109)]. As thymocytes mature into T cells, Thy-1 levels decrease [reviewed in (110)]. Mature mouse T cells express Thy-1 at lower levels (~200,000 copies per cell) than thymocytes (~1 million copies per cell). In rats, Thy-1 is found on both early and mature thymocytes; however, this expression is lost on mature T cells. Finally, in humans, Thy-1 expression is strictly confined to a small population of early cortical thymocytes (111).

1.6.4. Functions of Thy-1

The reported functions of Thy-1 are diverse and sometimes contradictory depending on where it is expressed. On neurons and endothelial cells, Thy-1 has adhesion properties and mediates cell-cell interactions [reviewed in (112)]. Neuronal Thy-1 has dual functions, acting as both a receptor on neurons and as a ligand for β3 integrins on astrocytes (113). It is reported to act by either inhibiting (114) or promoting (115) neurite outgrowth. When acting as a ligand, Thy-1 induces focal adhesion formation and stress fiber formation in astrocytes (113). Endothelial cell Thy-1 binds to β2 integrins on the surface of leukocytes, thereby facilitating leukocyte migration and extravasion through the endothelium (116, 117). Thy-1 has also been shown to have adhesive properties in the immune system. Thymocyte adhesion to the thymic epithelium is mediated by Thy-1 interacting with an unknown ligand, suggesting that Thy-1 may play a role in early T cell maturation (118). While the role of Thy-1 as an adhesion molecule is important, most studies on Thy-1 in the immune system have focused on its ability to modulate T cell activation and proliferation.

Thy-1 function in the context of T cells has been primarily studied using an array of anti-Thy-1 mAbs. As a result, differences in antibody specificities and experimental systems have identified conflicting functions for Thy-1. Thy-1 cross-linking stimulates proliferation and IL-2 production by T cells independent from TCR-mediated signalling (77). Interestingly, an intact and functional TCR/CD3 complex must be present in order for T cells to be activated through Thy-1 (85). When combined with conventional TCR stimulation, Thy-1 cross-linking enhances TCR signalling as measured by tyrosine phosphorylation, intracellular calcium influx, and IL-2 production (119). Furthermore, peripheral T cells from Thy-1-deficient mice show reduced calcium influx and tyrosine kinase activity following TCR stimulation (120). Conversely, Thy-1 has also been shown to negatively regulate T cell responses. Thymocytes of Thy-1-deficient mice showed hyperresponsiveness to TCR triggering in the form of increased phosphorylation of TCR subunits, calcium influx, and cell proliferation (121). These conflicting findings reflect the controversy that exists with regard to the function of Thy-1 in T cell biology.

1.6.5. Outstanding questions

Despite the abundance of research on Thy-1 over the past five decades, numerous outstanding and important questions remain. Firstly, the ligand for T cell-associated Thy-1 remains unknown. As mentioned previously, Thy-1 binds to β2 and β3 integrins on neurons and leukocytes such as polymorphonuclear cells, monocytes, and DCs; however, little is known for Thy-1 on T cells. Identification of the putative T cell-associated Thy-1 ligand, if it exists, would reduce our reliance on mAbs to study Thy-1 and would lead to a better understanding of Thy-1's specific biological function. Secondly, precisely how signalling through Thy-1 occurs remains a mystery. As with GPI-anchored proteins in general, many hypotheses have been proposed to explain how Thy-1 transmits an intracellular signal. Potential mechanisms include an association of Thy-1 with the TCR/CD3 complex (85) or other transmembrane proteins such as CD45 (84), the release of bioactive second messengers upon Thy-1 cross-linking (122), and enhanced protein tyrosine kinase phosphorylation due to lipid raft aggregation upon Thy-1 cross-linking (83). Others have simply suggested that the abundance of Thy-1 on the cell surface may have indirect effects on TCR signalling [reviewed in (109)]. Regardless, no clear

consensus has been reached with regards to the mechanism explaining how Thy-1 transmits an intracellular signal. These two outstanding questions combined with the conflicting evidence on Thy-1 function stress the importance of further research to better understand the immunobiology of this ever enigmatic molecule.

1.7. CD55 (Decay Accelerating Factor)

1.7.1. Characteristics of CD55

CD55 is a 70 kilodalton (kDa) protein that is tethered to the cell surface by a GPI anchor. It was first identified in 1969 as a regulator of complement activation (123, 124). Since then, a number of additional functions for this molecule have been revealed. CD55 is present on the surface of erythrocytes, leukocytes, epithelial and endothelial cells in all species studied to date (125). On peripheral blood cells, the primary function of CD55 is to accelerate the decay of both the C3 and C5 convertases of the complement cascade (126). This prevents C3b deposition and the downstream formation of the membrane attack complex (MAC), ultimately protecting these cells from unnecessary complement-mediated lysis. CD55 is also an adhesion receptor for many viral and bacterial pathogens such as enteroviruses and *Escherichia coli* (127, 128). Furthermore, CD55 has been identified as a ligand for the epidermal growth factor receptor CD97 (129), which is expressed on all cells of hematopoietic origin, including macrophages, dendritic cells, and activated leukocytes (130). CD97 is thought to play a role in leukocyte adhesion and migration since blocking this molecule with mAbs impairs granulocyte migration in experimental colitis and pneumonia (131).

1.7.2. The role of CD55 in signal transduction

Like Thy-1 and other GPI-anchored proteins, CD55 has been shown to be involved in signal transduction. In human T cells, treatment with rabbit antiserum to CD55 combined with phorbol ester led to enhanced T cell proliferation (79). Additionally, CD55 was found to associate with the tyrosine kinases Lck and Fyn upon concurrent stimulation with mAbs to CD55 and phorbol esters (81). Moving away from

non-specific mitogenic stimulation, Capasso *et al.* demonstrated that coengagement of CD55 and CD3 resulted in enhanced proliferation and activation of human $CD4^+$ T cells (132). In contrast to these findings, a role for CD55 in suppressing T cell function has been demonstrated using CD55-deficient mice (133, 134). In these studies, it was shown that the T cell hyperresponsiveness seen in CD55-deficient mice was related to increased complement activation, as responses returned to normal in $CD55^{-/-}C3^{-/-}$ or $CD55^{-/-}$Factor $D^{-/-}$ double-knockout mice. Overall, these findings suggest that CD55 has a role in enhancing T cell activation.

1.8. Rationale and objectives

As described above, little is known concerning the role of GPI-anchored proteins such as Thy-1 and CD55 on T cells. On the T cell surface, GPI-anchored proteins have been shown to possess costimulatory properties capable of enhancing T cell activation. To date, however, the role of these molecules on *i*NKT cells has not been investigated. GPI-anchored proteins may represent a new subset of immunomodulatory molecules on the *i*NKT cell surface. The ability of *i*NKT cells to either promote or suppress immune responses and the relatively unknown mechanisms behind these differential responses necessitate further research in this area.

In order to better understand the factors influencing *i*NKT cell activation, the main objective of this study was to investigate the expression and function of GPI-anchored proteins in *i*NKT cells. The specific aims were: (i) to determine whether the GPI-anchored protein Thy-1 is expressed on mouse *i*NKT cells; (ii) to investigate whether classical TCR-mediated *i*NKT cell activation is enhanced by Thy-1 cross-linking; and (iii) to translate my findings from mouse to human in order to better understand the role of GPI-anchored proteins in human *i*NKT cell activation. I hypothesized that Thy-1, a prototype GPI-anchored protein, is present on mouse *i*NKT cells and that cross-linking of Thy-1 and other GPI-anchored proteins will enhance classical TCR-mediated *i*NKT cell activation resulting in robust cytokine production.

CHAPTER 2:

MATERIALS AND METHODS

2.1. Cell lines and culture medium

DN32.D3 NKT cells ($V\alpha14^+$, $CD4^-CD8^-$ mouse *i*NKT hybridoma) (4) were obtained from Dr. Albert Bendelac (Howard Hughes Medical Institute, Chicago, IL). N38-2C12 NKT cells ($V\alpha14^+$, $CD4^+CD8^-$ mouse *i*NKT hybridoma) (135) were a gift from Dr. Kyoko Hayakawa (Fox Chase Cancer Center, Philadelphia, PA). Human mesenchymal stem cells (aldehyde dehydrogenase high, $ALDH^{hi}$) (136) were generously provided by Dr. David Hess (Robarts Research Institute, The University of Western Ontario, London, ON). All mouse cells were maintained in RPMI-1640 medium (GIBCO® Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal calf serum (FCS) (HyClone, Logan, UT), 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, 0.1 mM MEM non-essential amino acids (all from GIBCO® Invitrogen), 1 mM sodium pyruvate and 50 uM β-mercaptoethanol (both from Sigma-Aldrich Canada Ltd., Oakville, ON). Human cells were cultured in RPMI-1640 containing 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine (hereafter referred to as complete medium).

2.2. Mice

C57BL/6 mice were purchased from Charles River Canada (Senneville, PQ) and maintained under specific pathogen-free conditions in the Animal Care and Veterinary Services unit of The University of Western Ontario. Adult (6-12 week old) female mice were used in all experiments and were cared for in accordance with the guidelines established by the Canadian Council on Animal Care.

2.3. Antibodies and reagents

Monoclonal antibodies to Thy-1 (activating clone G7 and blocking clone 30-H12) were purchased from eBioscience (San Diego, CA). Functional grade isotype controls Rat IgG2c and Mouse IgG1 were from MBL International (Woburn, MA) and eBioscience, respectively. Rat IgG2c was used as a control for anti-Thy-1 mAb clone G7

and Mouse IgG1 was used as a control for anti-CD55 mAb. Monoclonal antibodies against human CD55 (functional grade and PE-conjugated) were purchased from the International Blood Group Reference Laboratory (Bristol, UK). For a list of all fluorescent antibodies used in this study, please see **Tables 2.1., 2.2., and 2.3.**.

Synthetic α-galactosylceramide (α-GalCer) and its vehicle (polysorbate-20) were originally obtained from Kirin Brewery (Tokyo, Japan). Allophycocyanin (APC)-conjugated PBS-57-loaded and -unloaded CD1d tetramers for staining mouse and human *i*NKT cells were generously provided by the National Institutes of Health Tetramer Core Facility (Atlanta, GA). Percoll and Ficoll-Paque density gradients were from GE Healthcare (Chalfont St. Giles, UK). Recombinant mouse IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) were purchased from PeproTech Inc. (Rocky Hill, NJ). Carboxyfluorescein diacetate, succinimidyl ester (CFSE) was purchased from Molecular Probes® Invitrogen.

2.4. Flow cytometric analysis

Rat hybridoma cells (clone 2.4G2) producing a mAb against mouse Fcγ receptor (FcγR) II/III ("Fc block") were obtained from Dr. Jonathan Yewdell (National Institute of Allergy and Infectious Diseases, Bethesda, MD). For mouse cell staining, cells were preincubated with Fc block for 20 minutes before incubation with fluorescent antibodies for 30 minutes on ice. Staining was performed in phosphate-buffered saline (PBS) (GIBCO® Invitrogen) containing 2% FCS and 0.1% sodium azide (Fisher Scientific Company, Ottawa, ON), hereafter referred to as FACS staining buffer. After staining, cells were washed twice prior to fixation in 1% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA). Flow cytometry was performed using a FACSCalibur or FACSCanto II and CellQuest or FACSDiva software (BD Biosciences, San Jose, CA). Analysis was carried out using FlowJo (Treestar, Inc., Ashland, OR).

Table 2.1.: Anti-mouse antibodies used in this research

Target	Fluorochome	Clone	Origin	Company
CD1d	PE	1B1	Rat	BD Pharmingen
CD11c	FITC	N418	Armenian Hamster	eBioscience
CD16/CD32	R-PE	2.4G2	Rat	BD Pharmingen
CD16/CD32	functional grade	93	Rat	eBioscience
CD25	PE	PC61.5	Rat	eBioscience
CD69	PE	H1.2F3	Armenian Hamster	BD Pharmingen
CD80	FITC	16-10A1	Armenian Hamster	eBioscience
CD86	APC	GL1	Rat	eBioscience
CD90	FITC	30-H12	Rat	eBioscience
CD90	PE	30-H12	Rat	eBioscience
CD90	functional grade	G7	Rat	eBioscience
CD90	functional grade	30-H12	Rat	eBioscience
NK1.1	PE	PK136	Mouse	BD Pharmingen
TCRβ	FITC	H57-597	Armenian Hamster	eBioscience

Table 2.2.: Anti-human antibodies used in this research

Target	Fluorochome	Clone	Origin	Company
CD3	FITC	OKT3	Mouse	eBioscience
CD3	APC-eFluor780	UCHT1	Mouse	eBioscience
CD55	PE	BRIC 216	Mouse	U of Bristol
CD55	functional grade	BRIC 216	Mouse	U of Bristol
CD69	PE	FN50	Mouse	eBioscience
CD90	PE	5E10	Mouse	eBioscience

Table 2.3.: Isotype control immunoglobulins used in this research

Isotype	Fluorochrome	Origin	Company
IgG1	FITC	Rat	eBioscience
IgG1	PE	Rat	eBioscience
IgG1	PE	Mouse	eBioscience
IgG1	functional grade	Mouse	eBioscience
IgG2a	APC	Rat	eBioscience
IgG2b	PE	Rat	eBioscience
IgG2c	functional grade	Rat	MBL

2.5. Splenic and hepatic lymphoid mononuclear cell preparation

Mice were sacrificed by carbon dioxide (CO_2) asphyxiation. For isolation of splenic mononuclear cells, spleens were removed and processed in ice-cold PBS (pH 7.2-7.4) using a glass tissue homogenizer. Cell suspensions were depleted of erythrocytes by incubation with ACK lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM EDTA) for 5 minutes at room temperature with occasional mixing. For isolation of hepatic mononuclear cells, the liver was perfused by injecting 10 mL of PBS into the hepatic portal vein using a 10 mL syringe and a 27-gauge needle (Becton Dickinson, Franklin Lakes, NJ). The excised liver was cut into small pieces and pressed through wire mesh with a glass plunger (Sigma-Aldrich Canada Ltd.). The liver homogenate was resuspended in a 33.75% Percoll solution (GE Healthcare) and centrifuged at 700 x g for 12 minutes at 25°C. The pelleted cells were washed and cell suspensions were depleted of erythrocytes as described above.

2.6. Magnetic bead *i*NKT cell isolation

For splenic *i*NKT cell isolation, nylon wool fiber columns (Polysciences Inc. Warrington, PA) were used to deplete cell suspensions of B cells and adherent cells (137). The columns were pre-incubated at 37°C with 20 mL of pre-warmed cRPMI. After 1 hour, 1-2 x 10^8 splenocytes were added to each column and the medium was drained to allow the cells to completely enter the packed wool. Columns were incubated for 1 hour at 37°C. Next, non-adherent cells were collected by opening the stopcock and slowly allowing the cell suspension to exit the column into a 50 mL Falcon tube (Becton Dickinson). The collected cells were spun down and resuspended in cRPMI.

Hepatic lymphoid mononuclear cells and T cell-enriched splenocytes were each resuspended in PBS + 2% FCS + 1 mM EDTA (EasySep buffer) at a concentration of 1-2 x 10^8 cells/mL. Following the manufacturer's instructions, *i*NKT cells were isolated using the EasySep APC Selection Kit (StemCell Technologies, Vancouver, BC). The APC-conjugated antibody used was APC-conjugated PBS-57-loaded mouse CD1d

tetramer at a 1:40 dilution of 25 μL per 1 mL of cell suspension. To improve purity, an additional round of magnetic separation was performed for a total of six 5 minute separations. The purity of isolated *i*NKT cells from both the spleen and the liver was >95% as judged by flow cytometry.

2.7. Hepatic *i*NKT cell sorting

To prevent non-specific antibody binding, liver mononuclear cells were incubated with 20 μL Fc block per million cells for 20 minutes on ice. Cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-TCRβ mAb (clone H57-597) (1:200 dilution in FACS staining buffer) from eBioscience and phycoerythrin (PE)-conjugated anti-NK1.1 mAb (clone PK136) (1:50 dilution in FACS staining buffer) from BD Biosciences for 30 minutes on ice. To prevent clumping, cells were washed and briefly incubated with PBS + 0.1% bovine serum albumin (BSA) + 10 mM EDTA (both from Sigma-Alderich Canada Ltd.) for 10 minutes. Cells were washed again, filtered through a 40-μm strainer (Becton Dickinson), and resuspended at 30-40 x 10^6 cells/mL in Hank's Buffered Saline Solution (HBSS, pH 7.2-7.4) (GIBCO® Invitrogen) with 0.1% BSA. $TCR\beta^+NK1.1^+$ cells were sorted using a FACSVantage cell sorter and FACSDiva software (both BD Biosciences). The purity of sorted *i*NKT cells was >99%.

2.8. Generation of bone marrow-derived dendritic cells (BMDCs)

The femurs and tibias of adult mice were excised and placed in cold PBS. All soft tissue was removed and the bones were sterilized in 70% ethanol for one minute. To obtain bone marrow cells, the ends of the bone were cut and the interior was flushed with cold PBS using a 10 mL syringe and a 27-gauge needle. Bone marrow cells were filtered through a 70-μm strainer (Becton Dickinson) and red blood cells were removed using ACK lysis buffer as described above. Cells were resuspended at 4 x 10^6 cells/mL in cRPMI containing 4 ng/mL GM-CSF and 1000 U/mL IL-4 (both from PeproTech Inc., Rocky Hill, NJ) and 20 x 10^6 cells were seeded into each well of a 6-well polystyrene plate (Becton Dickinson). On days 2 and 4, floating cells were removed and fresh cRPMI

containing GM-CSF and IL-4 was added to the adherent cells. On day 6 when dendritic cells detach, all floating cells were harvested. Surface staining was performed on an aliquot of cells in order to confirm the expression of various DC markers. BMDCs were stained with FITC-conjugated anti-CD11c mAb (1:100 dilution in FACS staining buffer), PE-conjugated anti-CD1d mAb (1:100 dilution in FACS staining buffer), FITC-conjugated anti-CD80 mAb (1:100 dilution in FACS staining buffer), and APC-conjugated anti-CD86 mAb (1:150 dilution in FACS staining buffer) or their respective isotype-matched controls. After this step, unstained cells were used in cell culture as accessory cells.

2.9. In vitro *i*NKT cell activation

Between 20,000 - 100,000 *i*NKT cells in a total volume of 200 μL were seeded in 96-well polystyrene microplates (Becton Dickinson). Flat-bottomed plates were used for activating hybridomas (DN32.D3 and N38-2C12), while U-bottom plates were used for freshly isolated hepatic *i*NKT cells. When activating either the N38-2C12 hybridoma or sorted *i*NKT cells, BMDCs were added to the wells at a ratio of 5 *i*NKT: 1 DC. Cells were stimulated with either α-GalCer, anti-Thy-1 (G7), or a combination of the two. In some experiments, cells were first treated with increasing concentrations of the anti-Thy-1 blocking mAb (clone 30-H12) for 20 minutes prior to the addition of G7. In other experiments, FcγRs II/III were blocked with anti-CD16/32 (5 μg/mL) for 20 minutes prior to the addition of G7. For the *i*NKT hybridomas, culture supernatants were harvested after 24 hours in order to analyze cytokine levels by enzyme-linked immunosorbent assay (ELISA). For sorted *i*NKT cells, culture supernatants were harvested after 48 hours. All cultures were incubated at 37°C and 5% CO_2 for the indicated time periods.

2.10. In vivo *i*NKT cell activation

Mice were injected intraperitoneally (i.p.) with 2 μg of α-GalCer or vehicle. After 72 hours, mice were sacrificed and splenic and hepatic mononuclear cells were isolated

as described above. Cells were stained with FITC-conjugated anti-TCRβ mAb and APC-conjugated PBS-57-loaded mouse CD1d tetramer (both at a 1:100 dilution in FACS staining buffer) and *i*NKT cells ($TCR\beta^+CD1d$ tetramer$^+$) were analyzed using flow cytometry.

2.11. Analysis of cytokine production

The cytokine content of culture supernatants was quantified by ELISA using the Ready-Set-Go kits for mouse IL-2, IL-4, and IFN-γ (eBioscience). Plates were read at a dual wavelength of 450/570 nm using a Benchmark Microplate Reader (Bio-Rad Laboratories, Hercules, CA).

2.12. Reverse transcriptase quantitative PCR (RT-qPCR)

DN32.D3 cells were seeded into 24-well plates (Becton Dickinson) at 1-2 x 10^6 cells/well in a total volume of 1 mL. Cells were stimulated with α-GalCer (100 ng/mL), the indicated doses of anti-Thy-1 mAb G7, or a combination of the two as described above. After 24 hours, the cells were harvested and total RNA was extracted using TRIzol (Invitrogen). RNA concentration and purity were measured using a NanoDrop 1000 (Thermo Scientific, Waltham, MA). One μg of RNA was reverse transcribed into complementary DNA (cDNA) using oligo$(dT)_{18}$ primers within the Advantage RT-for-PCR Kit (Clontech Laboratories Inc., Mountain View, CA). For qPCR, the resulting cDNA was diluted 1:100 in diethyl pyrocarbonate (DEPC)-treated water (Invitrogen) for GAPDH amplification, 1:5 for IL-2 and IL-4 amplification, and remained undiluted for IFN-γ amplification. Briefly, 2.5 μL of cDNA was added to 10 μL QuantiTect SYBR Green (Qiagen Inc., Mississauga, ON), 2.4 μL of 1.25 μM forward and reverse primers, and 5.1 μL DEPC-treated water for a total volume of 20 μL/tube. Primers used for cDNA amplification can be found in **Table 2.4**. The amplification protocol for all genes of interest was as follows: the *Taq* DNA polymerase was activated for 15 minutes at 95°C followed by 40 cycles of denaturation for 15 seconds at 95°C and annealing for 30 seconds at 60°C. A melt analysis was always performed post-cDNA amplification in

Table 2.4.: Primers used for quantitative polymerase chain reaction (qPCR)

Gene of Interest	Primers
GAPDH	F: 5'- CGT CCC GTA GAC AAA ATG GT -3' R: 5'- TTG ATG GCA ACA ATC TCC AC -3'
IL-2	F: 5'- AAC TCC CCA GGA TGC TCA C -3' R: 5'- CGC AGA GGT CCA AGT TCA TC -3'
IL-4	F: 5'- TGA ACG AGG TCA CAG GAG AA -3' R: 5'- CGA GCT CAC TCT CTG TGG TG -3'
IFN-γ	F: 5'- ACA GCA AGG CGA AAA AGG AT -3' R: 5'- TGA GCT CAT TGA ATG CTT GG -3'

order to ensure that only one PCR product was being amplified. Data was acquired on a Rotor-Gene 3000 (Corbett Life Science, Concord, AUS) and analyzed using the $\Delta\Delta C_t$ method (138). This method was applicable since all primers had 100% efficiency as judged by linear regression analysis of a standard cDNA dilution series. mRNA levels were expressed relative to untreated cells, which were assigned an arbitrary expression ratio of 1.

2.13. Human peripheral blood mononuclear cell (PBMC) isolation

All human work was performed in accordance with a protocol approved by the University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects. Peripheral blood was collected from healthy volunteers (male and female, ranging in age from 23 to 44 years old) into heparinized vacutainer tubes (Becton Dickinson) and subsequently diluted with an equal volume of PBS. The diluted blood was overlaid onto a Ficoll-Paque gradient (GE Healthcare) and spun at 800 x g for 30 minutes. Mononuclear cells at the interface were isolated and washed twice with PBS and spun at 1400 rpm and once at 1000 rpm to remove platelets. PBMCs were resuspended in complete medium.

2.14. Thy-1 expression on human *i*NKT cells

Human PBMCs were incubated with APC-conjugated PBS-57-loaded human CD1d tetramer at room temperature (1:100 dilution in FACS staining buffer). After 30 minutes, excess tetramer was removed by washing once in FACS staining buffer. Cells were further stained with either APC-eFluor780-conjugated anti-CD3 mAb (1:20 dilution in FACS staining buffer) or FITC-conjugated anti-CD3 mAb (1:5 dilution in FACS staining buffer) and either PE-conjugated anti-Thy-1 mAb (1:5 dilution in FACS staining buffer) or an isotype control for 30 minutes on ice. Following this step, cells were washed twice with PBS and flow cytometric analysis was immediately performed using a FACSCanto II with FACSDiva software. In some studies, human mesenchymal stem cells were used as a positive control for Thy-1 expression. Cells were harvested and

stained with PE-conjugated anti-Thy-1 mAb or an isotype-matched control as described above.

2.15. CFSE proliferation assay

Human PBMCs were incubated with 5 μM CFSE (Molecular Probes® Invitrogen) for 15 minutes at 37°C. Cells were washed and further incubated in complete medium for 30 minutes. CFSE-stained cells were seeded at 3 x 10^6 cells per well in a 24-well plate. Some wells had been coated overnight with 10 μg/mL anti-CD55 (International Blood Group Reference Laboratory, Bristol, UK) diluted in PBS. Prior to seeding, the plate was extensively washed with PBS. In addition to plate-bound anti-CD55, an optimal dose of α-GalCer (100 ng/mL) was added to some cultures. On day 6, cells were harvested and *i*NKT cells were identified by staining with APC-conjugated PBS-57-loaded human CD1d tetramer (1:100 dilution in FACS staining buffer). Using a FACSCanto II with FACSDiva software, CD1d tetramer$^+$ *i*NKT cells were gated upon and CFSE dilution was analyzed.

2.16. Statistical analysis

For cytokine data, the results are graphed as mean ± standard deviation (SD) in triplicate wells of independent experiments. Differences were determined by Student's two-tailed *t* test using GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA). Differences were considered statistically significant at *P*-values less than 0.05 where *, ** and *** denote $P<0.05$, $P<0.01$, and $P<0.001$, respectively. Data are shown as representative of at least three independent experiments unless otherwise stated.

CHAPTER 3:

RESULTS

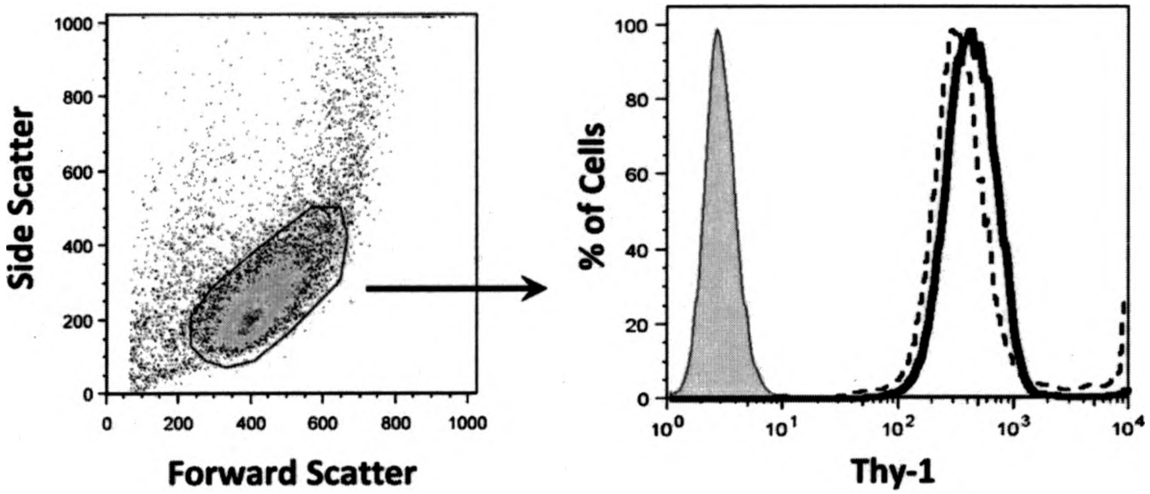
3.1. Thy-1 is highly expressed on both resting and activated mouse *i*NKT cells

Thy-1 (CD90) has been shown to be expressed on mouse T cells (106); however, its presence on *i*NKT cells has yet to be reported. In order to investigate this, DN32.D3 mouse *i*NKT hybridoma cells were left untreated or activated with 100 ng/mL of the *i*NKT cell agonist α-GalCer for 24 hours and were subsequently stained for Thy-1. It was observed that Thy-1 is highly expressed on both resting and activated DN32.D3 cells **(Figure 3.1.)**. This finding was further confirmed in primary mouse cells. Mice were injected i.p. with either α-GalCer or vehicle. Three days later, when *i*NKT cell expansion is known to reach its peak (45), mice were sacrificed. Splenocytes and liver lymphoid mononuclear cells were isolated and Thy-1 expression was analyzed by flow cytometry. Thy-1 was found to be highly expressed on both resting and activated primary *i*NKT cells ($TCR\beta^+$CD1d tetramer$^+$) **(Figure 3.2.)**. As expected, conventional T cells ($TCR\beta^+$CD1d tetramer$^-$) were highly positive for Thy-1, whereas non-T cells ($TCR\beta^-$) did not exhibit any marked expression of Thy-1 (data not shown). Interestingly, the surface expression of Thy-1 on a per cell basis was slightly decreased upon activation in both the hybridoma and the primary *i*NKT cells. Nevertheless, these experiments clearly show abundant expression of Thy-1 on mouse *i*NKT cells regardless of their activation status.

3.2. Thy-1 cross-linking leads to mouse *i*NKT cell activation

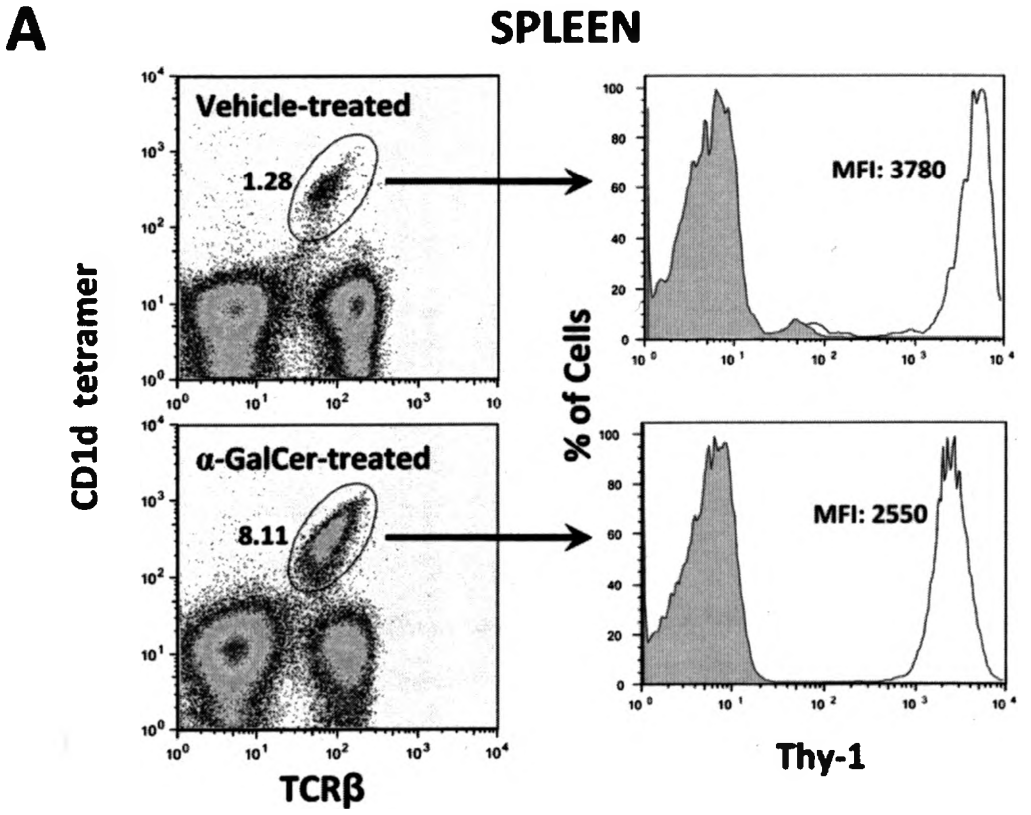
Thy-1 cross-linking has been shown to lead to conventional T cell activation independent of classical TCR-mediated activation as judged by proliferation and IL-2 production (77), suggesting a role for Thy-1 in costimulation of T cell responses. Since little is known regarding the events and costimulatory molecules involved in *i*NKT cell activation, the effects of Thy-1 cross-linking in *i*NKT cells were explored using a soluble anti-Thy-1 mAb (clone G7) that can cross-link Thy-1 on the cell surface (96). DN32.D3 cells were incubated for 24 hours with increasing doses of G7 and IL-2 production was measured as an indicator of activation. Thy-1 cross-linking by G7 induced IL-2 production in a dose dependent manner **(Figure 3.3.)**. DN32.D3 cells that were incubated with 40 μg/mL of a rat IgG2c mAb (isotype control for G7) did not produce

Figure 3.1. Thy-1 is expressed on mouse *i*NKT hybridoma cells. DN32.D3 mouse *i*NKT hybridoma cells were left untreated or were activated with α-GalCer (100 ng/mL) for 24 hours. Cells were harvested and stained with PE-conjugated anti-Thy-1.2 mAb (open histogram) or isotype control (filled histogram). The expression of Thy-1 was analyzed by flow cytometry and the mean fluorescence intensity (MFI) was calculated. Data are representative of 3 experiments yielding similar results.



	MFI
Isotype	2.95
Untreated	425
α-GalCer-treated	409

Figure 3.2. Thy-1 is expressed on mouse splenic and hepatic *i*NKT cells. C57BL/6 mice were injected intraperitoneally with 2 μg of α-GalCer or vehicle. Splenocytes (***A***) and hepatic lymphoid mononuclear cells (***B***) were prepared 72 hours post-injection. Cells were stained with FITC-conjugated anti-TCRβ mAb, APC-conjugated PBS-57-loaded mouse CD1d tetramer, and either PE-conjugated anti-Thy-1.2 mAb (open histogram) or isotype control (filled histogram). $TCR\beta^{+}$CD1d tetramer^{+} *i*NKT cells were gated on and Thy-1 expression was analyzed. Data are representative of 3 experiments yielding similar results. MFI: mean fluorescence intensity.


A
SPLEEN
Vehicle-treated
1.28
α-GalCer-treated
8.11
CD1d tetramer
TCRβ
% of Cells
MFI: 3780
MFI: 2550
Thy-1

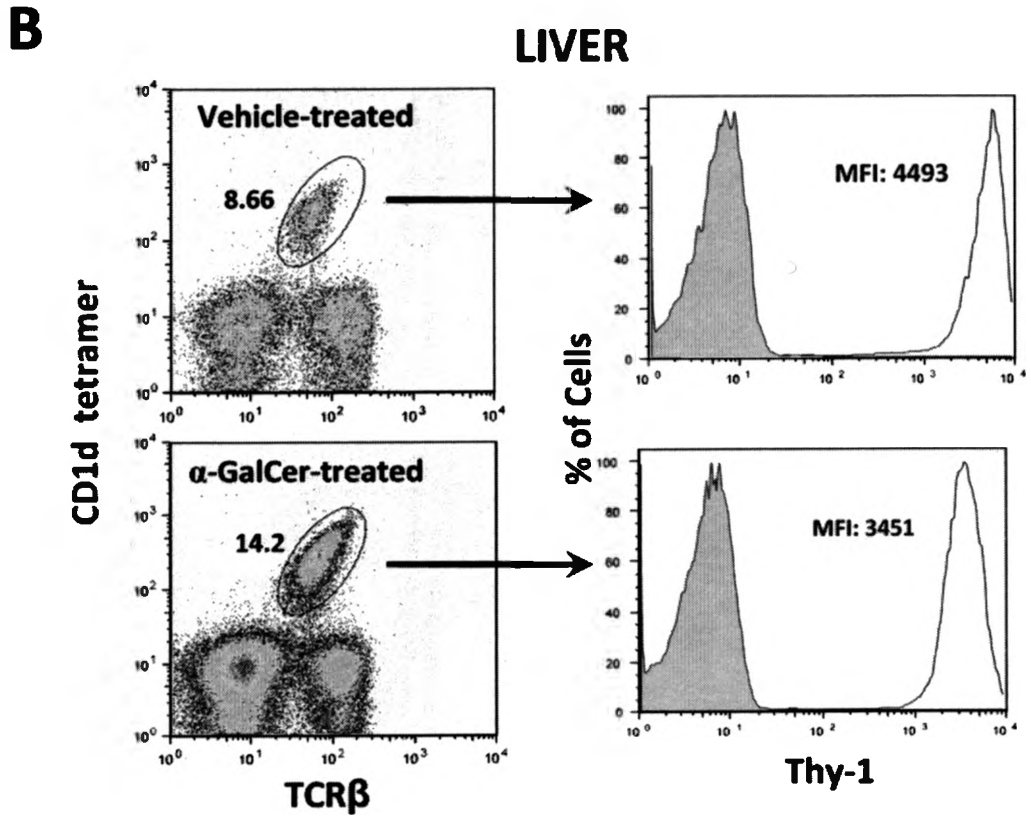
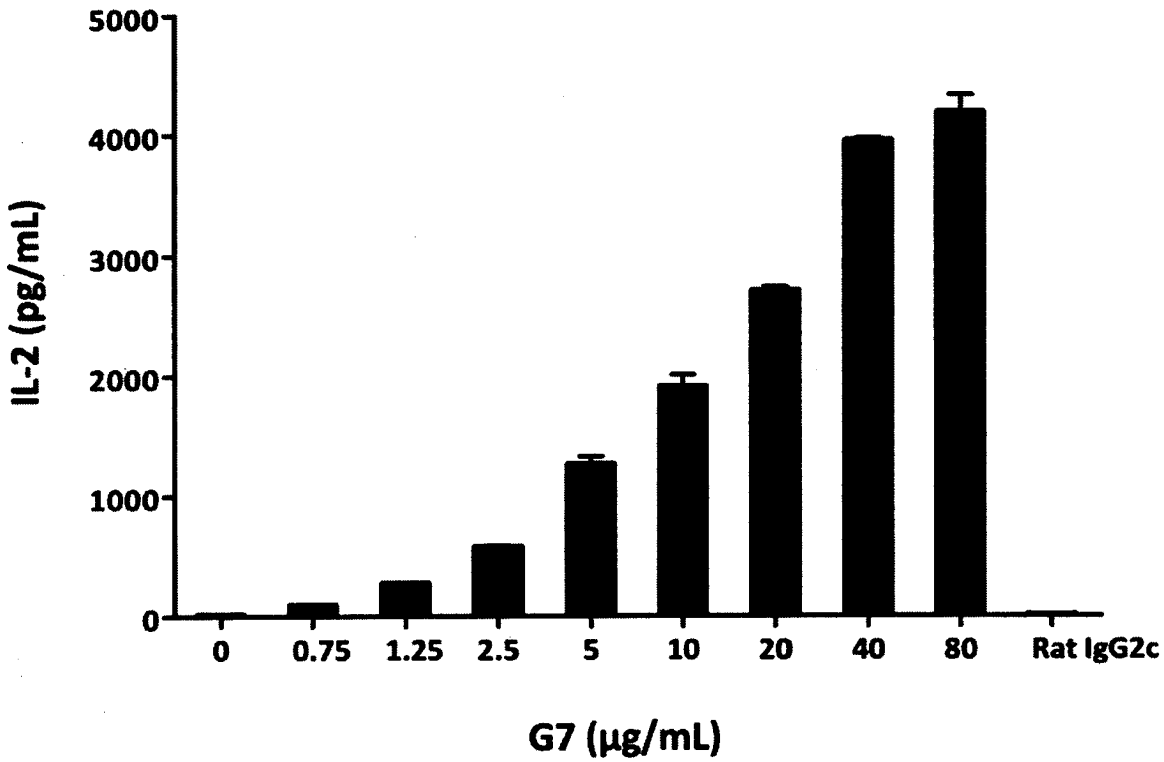

B
LIVER
Vehicle-treated
8.66
α-GalCer-treated
14.2
CD1d tetramer
TCRβ
% of Cells
MFI: 4493
MFI: 3451
Thy-1

Figure 3.3. Thy-1 cross-linking alone leads to mouse *i*NKT cell activation. DN32.D3 cells were seeded at 20,000 cells per well in a 96-well plate. Cells were incubated with various doses of soluble anti-Thy-1 mAb (clone G7) or an isotype control (rat IgG2c). After 24 hours, culture supernatants were harvested and IL-2 levels were measured by ELISA. Error bars represent standard deviation of triplicate wells within each experimental group. Data are representative of 3 experiments yielding similar results.


5000
4000
3000
2000
1000
0
IL-2 (pg/mL)
0
0.75
1.25
2.5
5
10
20
40
80
Rat IgG2c
G7 (µg/mL)

any IL-2. For subsequent experiments utilizing G7, a concentration of 5 μg/mL was chosen as the low dose since it induced IL-2 at concentrations slightly lower than the half maximal levels. For the high dose, 40 μg/mL G7 was selected as this was where the IL-2 response began to plateau.

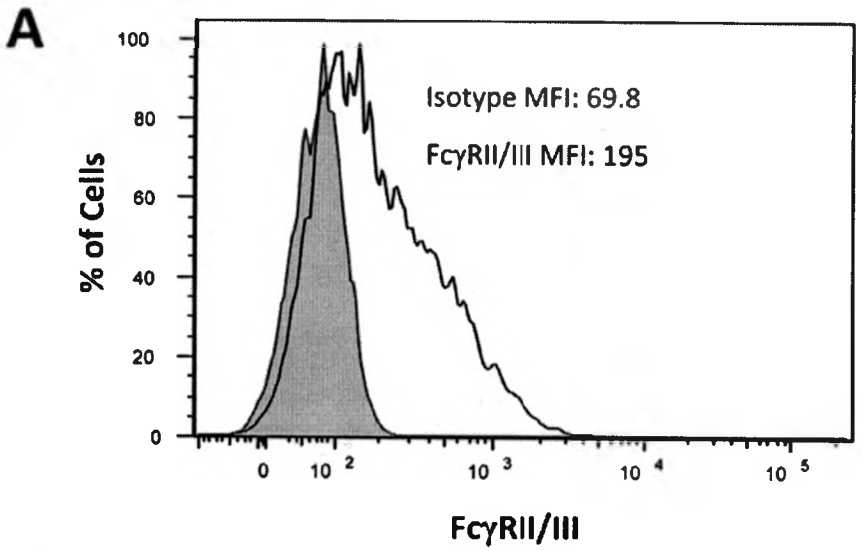
*i*NKT cells have been reported to express FcγRIII (139), a receptor capable of binding the Fc portion of antibodies of IgG isotype. FcγRII/III expression on DN32.D3 cells was examined and it was found that FcγRs were present on these cells **(Figure 3.4A)**. To ensure that *i*NKT cell activation by G7 was a consequence of Thy-1 triggering and not G7 binding to FcγRs, DN32.D3 cells were pre-incubated with 5 μg/mL anti-FcγRII/III mAb for 20 minutes. Following this, increasing doses of soluble G7 were added to the cultures. Blockade of FcγRs failed to decrease Thy-1-mediated IL-2 production **(Figure 3.4B)**, clearly indicating that *i*NKT cell activation was not mediated by non-specific binding of G7 to *i*NKT cells through FcγRs.

3.3. Thy-1 cross-linking enhances α-GalCer-mediated mouse *i*NKT cell activation

3.3.1. iNKT hybridoma cells

Thy-1 is reportedly capable of both activating (119) and suppressing (121) conventional T cell responses depending on the experimental conditions used. Thy-1 is unique among classic costimulatory molecules in that it can act as signal 1 (e.g. in the absence of TCR-mediated signalling) in addition to acting as signal 2 (140). Similar to conventional T cell responses, I have demonstrated that Thy-1 cross-linking leads to *i*NKT cell activation. More importantly, the influence of Thy-1 on TCR-mediated *i*NKT cell activation is unknown. In order to investigate this, the optimal concentration of α-GalCer that would yield the greatest amount of IL-2 production by DN32.D3 cells was first determined. Stimulation with 100 ng/mL of α-GalCer induced the greatest response, which is consistent with what has been widely published in the literature **(Figure 3.5A)**. For measuring activation, the optimal time point was determined to be between 24 and 48 hours post-stimulation, which is also consistent with the literature **(Figure 3.5B)**. The 24 hour time point was chosen for all subsequent experiments utilizing DN32.D3 cells.

Figure 3.4. FcγRs are present on mouse *i*NKT cells but do not contribute to Thy-1-mediated *i*NKT cell activation. ***A)*** DN32.D3 cells were stained with PE-conjugated anti-FcγRII/III mAb (open histogram) or isotype control (filled histogram). FcγRII/III expression on DN32.D3 cells was analyzed by flow cytometry. ***B)*** FcγRs of DN32.D3 cells were blocked by treatment with an anti-FcγRII/III mAb (5 μg/mL). After 20 minutes, indicated doses of soluble G7 were added into the cultures. Culture supernatants were harvested after 24 hours and IL-2 levels were measured by ELISA. Error bars represent standard deviation of triplicate wells within each experimental group. Data are representative of two independent experiments yielding similar results. MFI: mean fluorescence intensity.


A
100
80
60
40
20
0
% of Cells
Isotype MFI: 69.8
FcγRII/III MFI: 195
0
10 2
10 3
10 4
10 5
FcγRII/III

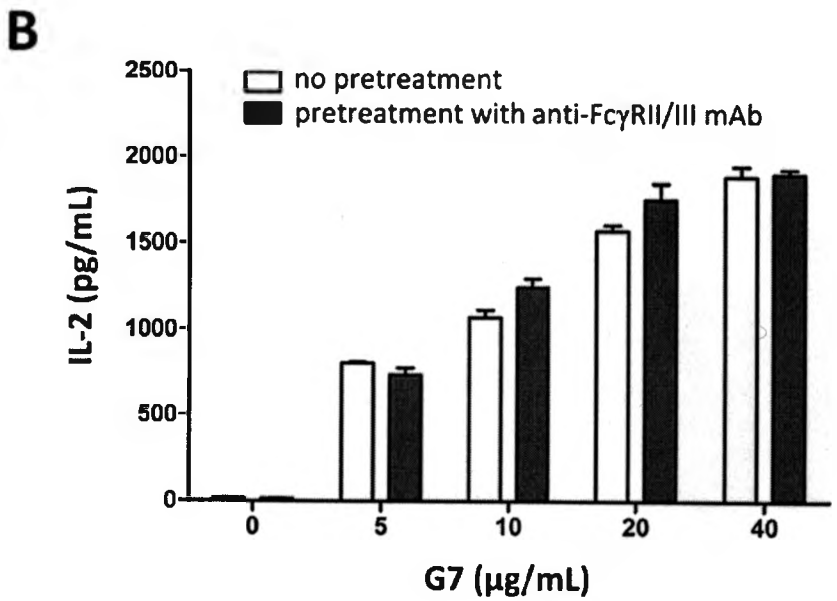
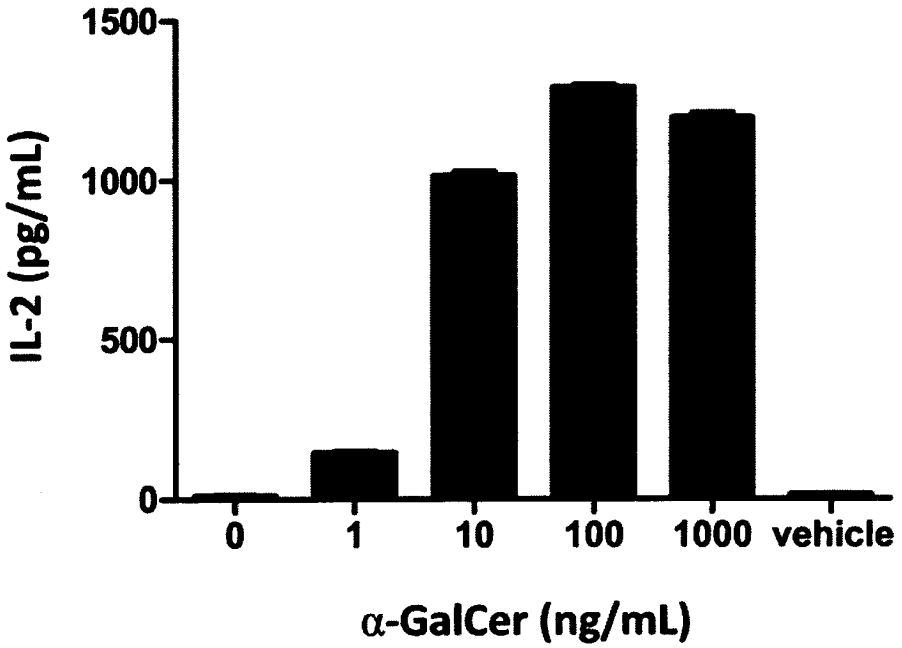
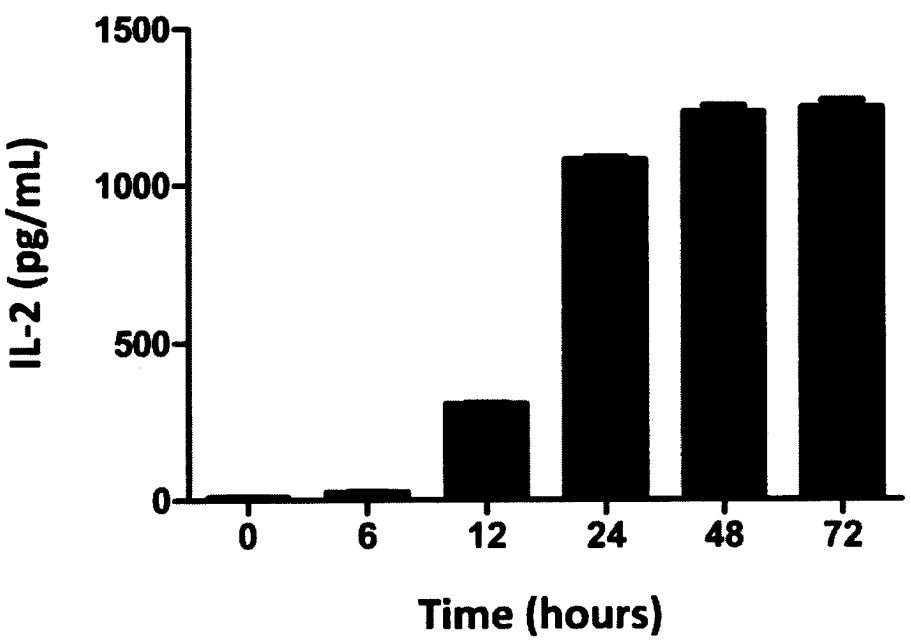

B
2500
2000
1500
1000
500
0
IL-2 (pg/mL)
no pretreatment
pretreatment with anti-FcγRII/III mAb
0
5
10
20
40
G7 (µg/mL)

Figure 3.5. Kinetics of α-GalCer-mediated mouse *i*NKT cell activation. *A)* DN32.D3 cells were incubated with various doses of α-GalCer or 1000 ng/mL of vehicle for 24 hours. Culture supernatants were harvested and IL-2 levels were measured by ELISA. ***B)*** DN32.D3 cells were incubated with 100 ng/mL of α-GalCer. Culture supernatants were harvested at the indicated time points post-stimulation and the IL-2 content of culture supernatants was measured by ELISA. Error bars represent standard deviation of triplicate wells within each experimental group. Data are representative of three independent experiments yielding similar results.

A


1500
1000
500
0
IL-2 (pg/mL)
0
1
10
100
1000
vehicle
α-GalCer (ng/mL)

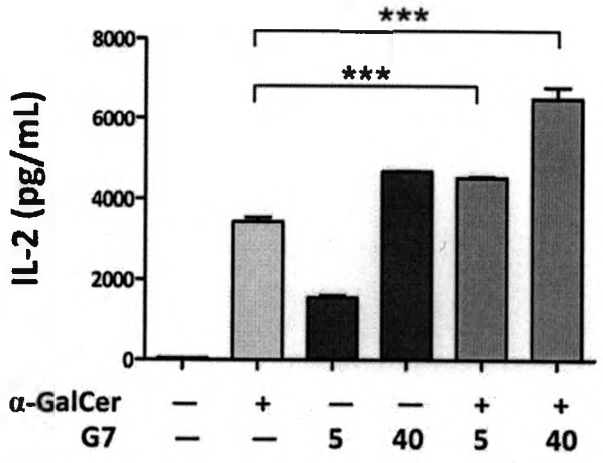
B

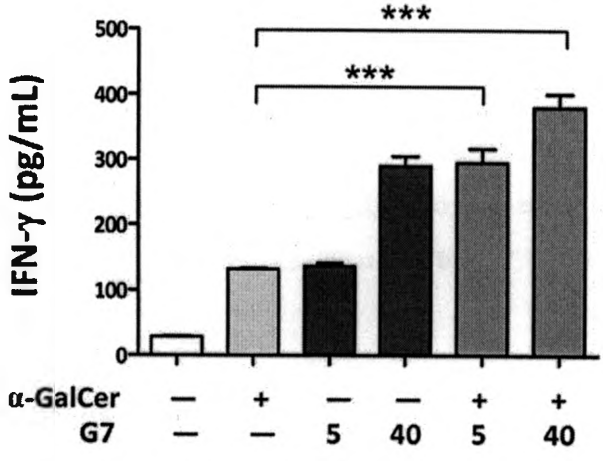

1500
1000
500
0
IL-2 (pg/mL)
0
6
12
24
48
72
Time (hours)

To explore how Thy-1 cross-linking may modulate classical TCR-mediated *i*NKT cell activation, DN32.D3 cells were stimulated with G7 in the presence or absence of α-GalCer. After 24 hours of culture, supernatants were harvested and their cytokine contents were measured. For Thy-1 cross-linking, a low dose (5 μg/mL) and a high dose (40 μg/mL) of G7 were used in order to better characterize Thy-1's ability to activate and/or suppress TCR-mediated *i*NKT cell activation. When DN32.D3 cells were stimulated with α-GalCer and either a low or high dose of G7, IL-2 production was statistically significantly greater than that of either treatment alone **(Figure 3.6., top panel)**. This indicates that Thy-1 cross-linking enhances α-GalCer-mediated *i*NKT cell activation. IFN-γ and IL-4 levels were also examined in order to see whether Thy-1 cross-linking induces the production of prototype *i*NKT cell cytokines. Thy-1 cross-linking alone induced IFN-γ and IL-4 production at both doses; furthermore, it significantly enhanced α-GalCer-mediated IFN-γ and IL-4 production in a similar manner to that seen with IL-2 **(Figure 3.6., middle and bottom panels)**. Given the significance of these findings, it was important to ensure that the results were not exclusive to one *i*NKT hybridoma. In mice, two types of *i*NKT cells exist: $CD4^-CD8^-$ (double negative, or DN) and $CD4^+CD8^-$ ($CD4^+$) *i*NKT cells (141). Since DN32.D3 is a DN *i*NKT hybridoma, these results were corroborated using the $CD4^+$ *i*NKT hybridoma N38-2C12. A similar trend to what was seen with DN32.D3 cells was observed with N38-2C12 cells for all cytokines analyzed **(Figure 3.7.)**. This suggests that enhanced TCR-mediated cytokine production after Thy-1 cross-linking was not influenced by CD4 expression and that our findings can be generalized to both DN and $CD4^+$ *i*NKT cells.

While a good measure of activation, the cytokine content of culture supernatants does not necessarily reflect new cytokine production. Some cytokines may be bound to their corresponding receptors making them undetectable or underdetectable by ELISA (142). The expression of these receptors may also be differentially altered by α-GalCer and/or G7 treatment. Furthermore, post-transcriptional and post-translational regulation may influence the amount of cytokine secreted into culture supernatant. This is influenced by the rates of transcription, mRNA turnover, translation, and protein

Figure 3.6. Thy-1 cross-linking enhances α-GalCer-mediated cytokine secretion by mouse DN32.D3 *i*NKT hybridoma cells. DN32.D3 cells were incubated with either α-GalCer alone (100 ng/mL), a low (5 μg/mL) or high (40 μg/mL) dose of G7, or a combination α-GalCer and G7. After 24 hours, culture supernatants were harvested and IL-2, IFN-γ, and IL-4 levels were measured by ELISA. Error bars represent standard deviation of triplicate wells within each experimental group. Statistical significance is denoted by asterisks, where ** and *** represent $P<0.01$ and $P<0.001$, respectively. Data are representative of three independent experiments yielding similar results.


***
***
8000
6000
4000
2000
0
IL-2 (pg/mL)
α-GalCer — + — — + +
G7 — — 5 40 5 40


***
***
500
400
300
200
100
0
IFN-γ (pg/mL)
α-GalCer — + — — + +
G7 — — 5 40 5 40

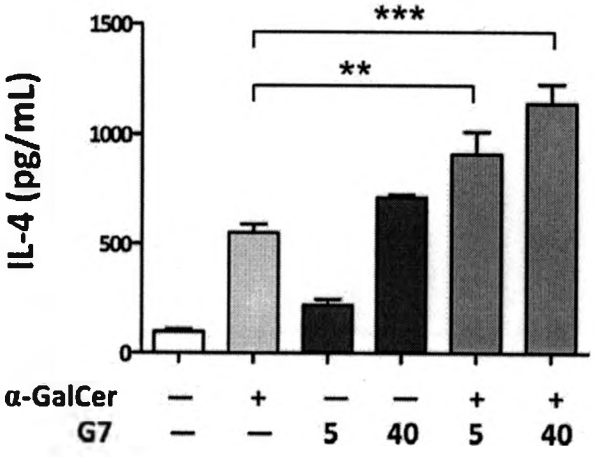
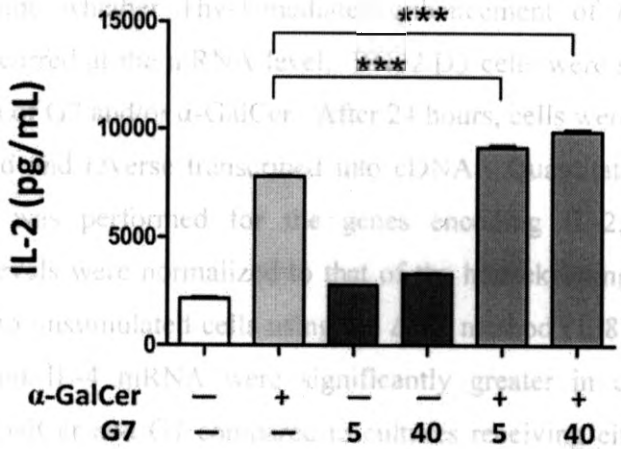
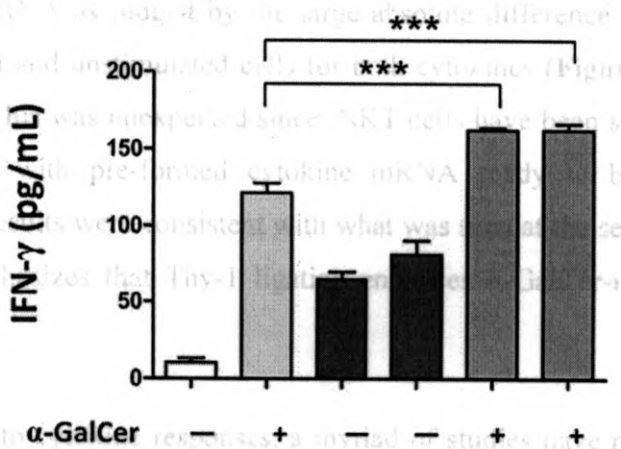
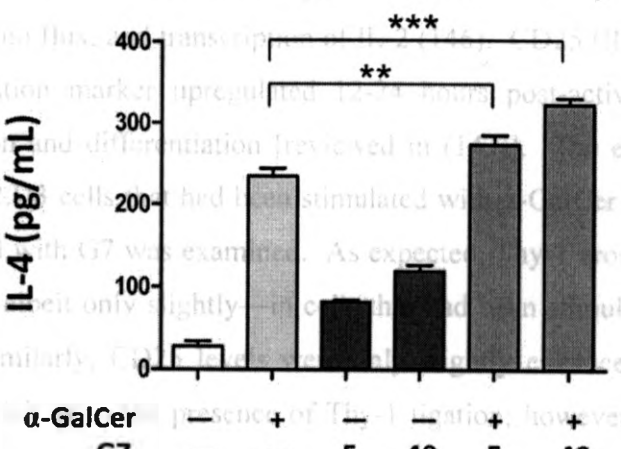

***
**
1500
1000
500
0
IL-4 (pg/mL)
α-GalCer — + — — + +
G7 — — 5 40 5 40

Figure 3.7. Thy-1 cross-linking enhances α-GalCer-mediated cytokine secretion by mouse N38-2C12 *i*NKT hybridoma cells. N38-2C12 cells were incubated with either α-GalCer alone (100 ng/mL), a low (5 μg/mL) or high (40 μg/mL) dose of G7, or a combination of α-GalCer and G7. After 24 hours, culture supernatants were harvested and IL-2, IFN-γ, and IL-4 levels were measured by ELISA. Error bars represent standard deviation of triplicate wells within each experimental group. Statistical significance is denoted by asterisks, where ** and *** represent $P<0.01$ and $P<0.001$, respectively.


IL-2 (pg/mL)
15000
10000
5000
0
***
***
α-GalCer — + — — + +
G7 — — 5 40 5 40

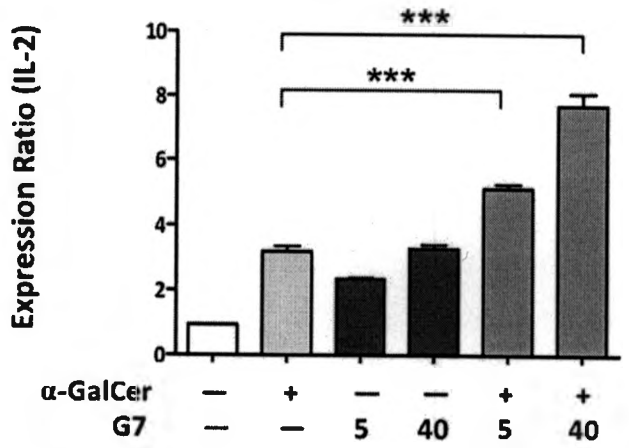

IFN-γ (pg/mL)
200
150
100
50
0
***
***
α-GalCer — + — — + +
G7 — — 5 40 5 40

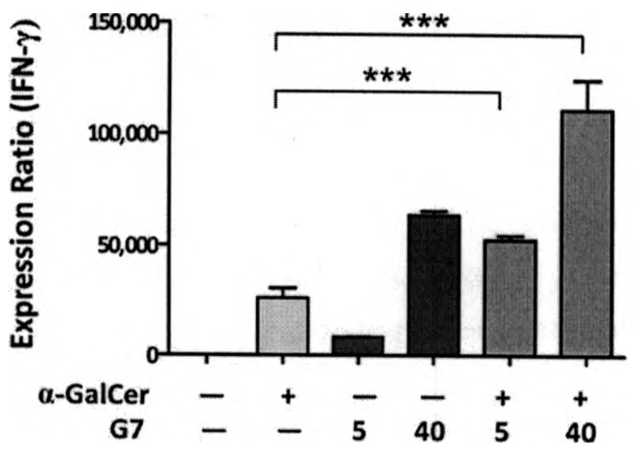

IL-4 (pg/mL)
400
300
200
100
0
***
**
α-GalCer — + — — + +
G7 — — 5 40 5 40

degradation (142). For these reasons, reverse transcriptase (RT) quantitative PCR was utilized to determine whether Thy-1-mediated enhancement of *i*NKT cell cytokine production also occurred at the mRNA level. DN32.D3 cells were stimulated in culture with a combination of G7 and/or α-GalCer. After 24 hours, cells were harvested and total RNA was extracted and reverse transcribed into cDNA. Quantitative—also known as "real-time"—PCR was performed for the genes encoding IL-2, IFN-γ, and IL-4. Cytokine mRNA levels were normalized to that of the housekeeping gene GAPDH and expressed relative to unstimulated cells using the $\Delta\Delta C_t$ method (138). Expression ratios of IL-2, IFN-γ, and IL-4 mRNA were significantly greater in cultures receiving a combination of α-GalCer and G7 compared to cultures receiving either treatment alone **(Figure 3.8.)**. Interestingly, unstimulated DN32.D3 cells had relatively low levels of IFN-γ and IL-4 mRNA as judged by the large absolute difference in expression ratios between stimulated and unstimulated cells for both cytokines **(Figure 3.8., middle and bottom panels)**. This was unexpected since *i*NKT cells have been shown to circulate in an activated state with pre-formed cytokine mRNA ready to be translated (143). Regardless, these results were consistent with what was seen at the secreted protein level, which further emphasizes that Thy-1 ligation enhances α-GalCer-mediated *i*NKT cell activation.

In addition to cytokine responses, a myriad of studies have measured CD69 and CD25 expression on the *i*NKT cell surface as indicators of activation (139, 144, 145). The early activation marker CD69 is upregulated 4 hours after activation and initiates PTK activity, calcium flux, and transcription of IL-2 (146). CD25 (IL-2 receptor α-chain) is a middle activation marker upregulated 12-24 hours post-activation that initiates cellular proliferation and differentiation [reviewed in (147)]. The expression of CD69 and CD25 in DN32.D3 cells that had been stimulated with α-GalCer alone, G7 alone, or α-GalCer combined with G7 was examined. As expected, Thy-1 cross-linking enhanced CD69 expression—albeit only slightly—in cells that had been stimulated with α-GalCer **(Figure 3.9A)**. Similarly, CD25 levels were only slightly enhanced when cells were stimulated with α-GalCer in the presence of Thy-1 ligation; however, CD25 expression remained low regardless of the stimulus **(Figure 3.9B)**. While not conclusive evidence,

Figure 3.8. Cytokine mRNA levels are enhanced when Thy-1 is cross-linked in the presence of α-GalCer. DN32.D3 cells were incubated with either α-GalCer alone (100 ng/mL), a low (5 μg/mL) or high (40 μg/mL) dose of G7, or a combination of α-GalCer and G7. After 24 hours, cells were harvested and total RNA was extracted. RNA was reverse transcribed into cDNA, which was then amplified by qPCR using primers specific for IL-2, IFN-γ, and IL-4. Cytokine mRNA levels were normalized to GAPDH and the normalized values were graphed as expression ratios. Expression ratios were relative to untreated cells, which were assigned a value of 1. Error bars represent standard deviation of triplicate qPCR reactions within each experimental group. Statistical significance is denoted by asterisks, where *** represents $P<0.001$. Data are representative of three independent experiments yielding similar results.


Expression Ratio (IL-2)
10
8
6
4
2
0
***
***
α-GalCer — + — — + +
G7 — — 5 40 5 40


Expression Ratio (IFN-γ)
150,000
100,000
50,000
0
***
***
α-GalCer — + — — + +
G7 — — 5 40 5 40

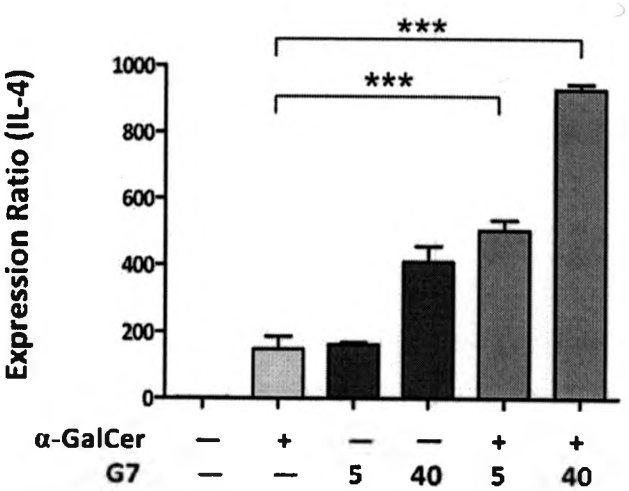
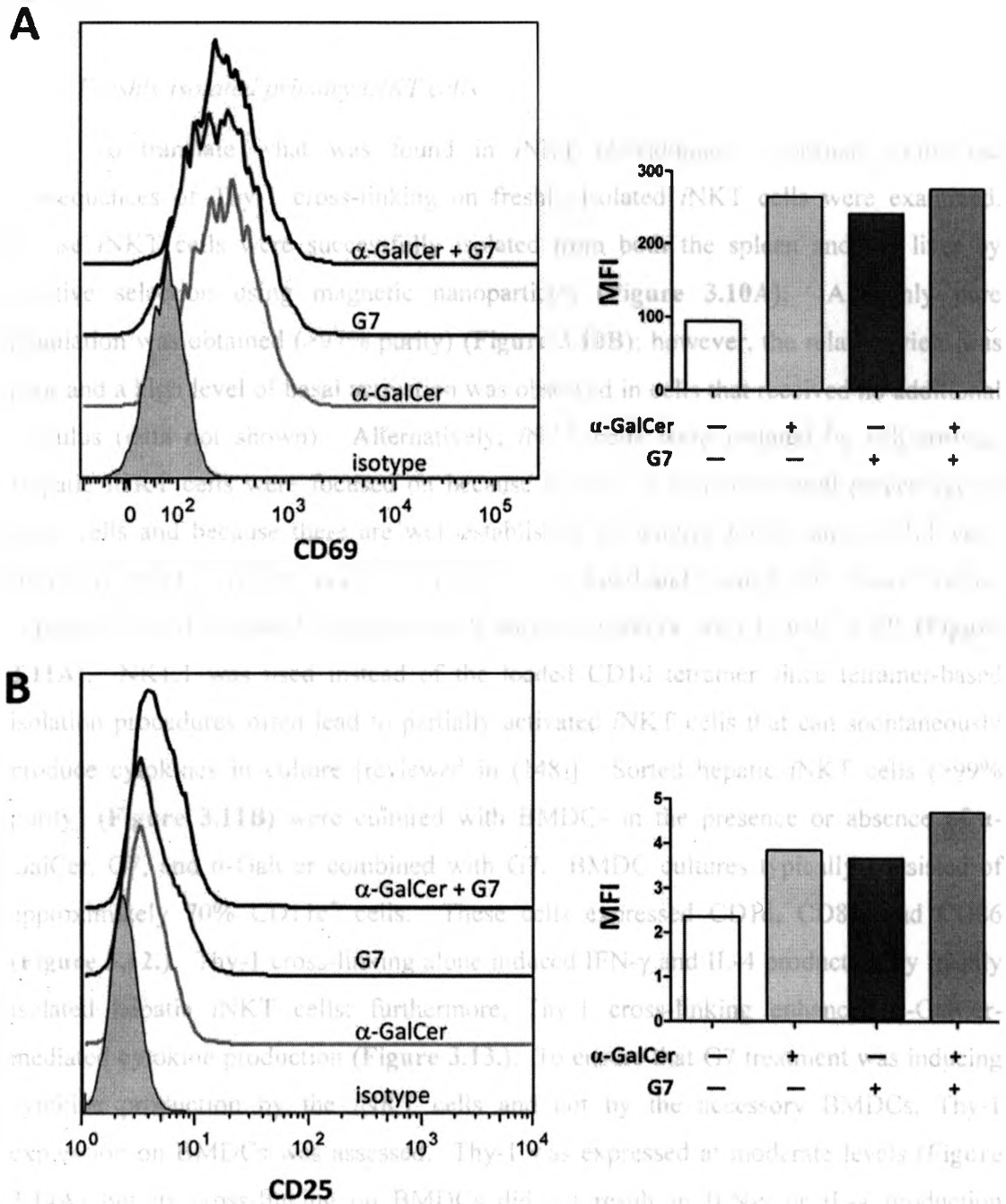

Expression Ratio (IL-4)
1000
800
600
400
200
0
***
***
α-GalCer — + — — + +
G7 — — 5 40 5 40

Figure 3.9. Expression of activation markers CD69 and CD25 is enhanced when Thy-1 is cross-linked in the presence of α-GalCer. DN32.D3 cells were incubated with either α-GalCer alone (100 ng/mL), a low (5 μg/mL) dose of G7, or a combination of α-GalCer and G7. After 24 hours, cells were harvested and separately stained with PE-conjugated anti-CD69 mAb (open histogram) or isotype control (filled histogram) (***A***) and PE-conjugated anti-CD25 mAb (open histogram) or isotype control (filled histogram) (***B***). CD69 and CD25 expression on DN32.D3 cells was analyzed by flow cytometry. Mean fluorescence intensity (MFI) values for each treatment group were graphed. Data are representative of two independent experiments yielding similar results.

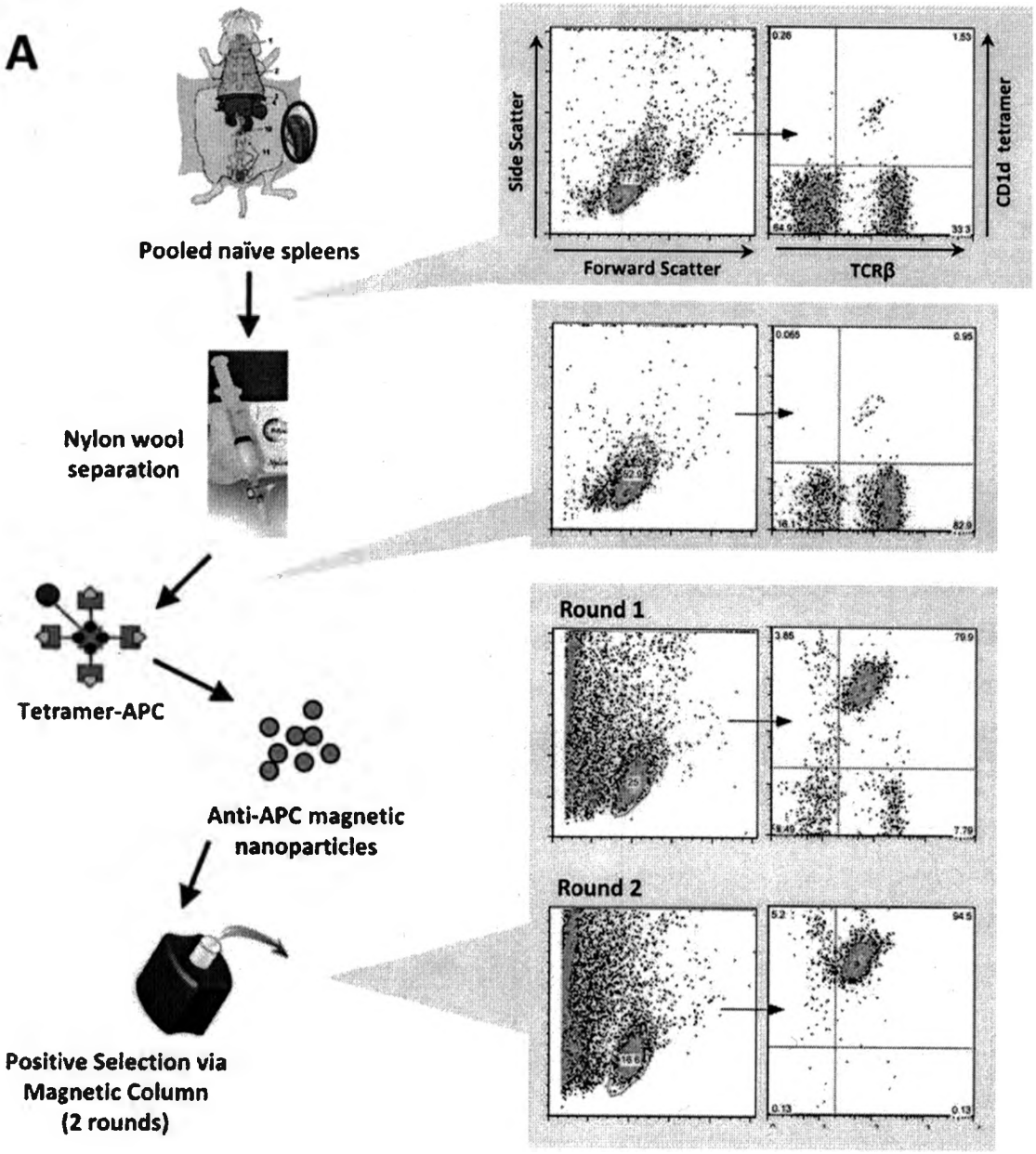

A
α-GalCer + G7
G7
α-GalCer
isotype
0
10^2
10^3
10^4
10^5
CD69
MFI
300
200
100
0
α-GalCer — + — +
G7 — — + +
B
α-GalCer + G7
G7
α-GalCer
isotype
10^0
10^1
10^2
10^3
10^4
CD25
MFI
5
4
3
2
1
0
α-GalCer — + — +
G7 — — + +

these results agree with what was seen at the level of cytokine production and further support the notion that Thy-1 cross-linking enhances α-GalCer-mediated *i*NKT cell activation.

3.3.2. Freshly isolated primary iNKT cells

To translate what was found in *i*NKT hybridomas to primary cells, the consequences of Thy-1 cross-linking on freshly isolated *i*NKT cells were examined. Mouse *i*NKT cells were successfully isolated from both the spleen and the liver by positive selection using magnetic nanoparticles **(Figure 3.10A)**. A highly pure population was obtained (>97% purity) **(Figure 3.10B)**; however, the relative yield was poor and a high level of basal activation was observed in cells that received no additional stimulus (data not shown). Alternatively, *i*NKT cells were isolated by cell sorting. Hepatic *i*NKT cells were focused on because the liver has a substantial percentage of these cells and because there are well-established procedures for isolating *i*NKT cells from this organ. Liver mononuclear cells were isolated and stained with fluorochrome-conjugated mAbs against the *i*NKT cell surface markers NK1.1 and TCRβ **(Figure 3.11A)**. NK1.1 was used instead of the loaded CD1d tetramer since tetramer-based isolation procedures often lead to partially activated *i*NKT cells that can spontaneously produce cytokines in culture [reviewed in (148)]. Sorted hepatic *i*NKT cells (>99% purity) **(Figure 3.11B)** were cultured with BMDCs in the presence or absence of α-GalCer, G7, and α-GalCer combined with G7. BMDC cultures typically consisted of approximately 70% $CD11c^+$ cells. These cells expressed CD1d, CD80, and CD86 **(Figure 3.12.)**. Thy-1 cross-linking alone induced IFN-γ and IL-4 production by freshly isolated hepatic *i*NKT cells; furthermore, Thy-1 cross-linking enhanced α-GalCer-mediated cytokine production **(Figure 3.13.)**. To ensure that G7 treatment was inducing cytokine production by the *i*NKT cells and not by the accessory BMDCs, Thy-1 expression on BMDCs was assessed. Thy-1 was expressed at moderate levels **(Figure 3.14A)** but its cross-linking on BMDCs did not result in IFN-γ or IL-4 production **(Figure 3.14B)**. Overall, what was found using freshly isolated primary hepatic *i*NKT cells was consistent with what was observed using *i*NKT cell hybridomas and provides

Figure 3.10. Isolation of mouse splenic and hepatic *i*NKT cells using magnetic nanoparticles. ***A)*** Six naïve C57BL/6 mice were sacrificed. The spleens and livers were removed and pooled, and both splenocyte and hepatic lymphoid mononuclear cell suspensions were prepared. Nylon wool fiber columns were used to deplete splenocyte suspensions of B cells and adherent cells. Following this step, both splenocytes and hepatic mononuclear cells were stained with APC-conjugated PBS-57-loaded mouse CD1d tetramer and subsequently incubated with anti-APC magnetic nanoparticles. Cells were placed in a magnetic column and CD1d tetramer-bound *i*NKT cells were positively selected over 2 rounds of washes. After each step, an aliquot of cell suspension was removed and stained with FITC-conjugated anti-TCRβ mAb and APC-conjugated PBS-57-loaded mouse CD1d tetramer. Samples were analyzed by flow cytometry and $TCR\beta^{+}$CD1d $tetramer^{+}$ *i*NKT cells were gated on. ***B)*** After two rounds of washes in the magnetic column, splenic (left panel) and hepatic (right panel) cells were stained with FITC-conjugated anti-TCRβ mAb and APC-conjugated PBS-57-loaded mouse CD1d tetramer in order to assess purity. Data are representative of two independent experiments yielding similar results.


A
Pooled naïve spleens
Nylon wool separation
Tetramer-APC
Anti-APC magnetic nanoparticles
Positive Selection via Magnetic Column (2 rounds)
Side Scatter
Forward Scatter
TCRβ
CD1d tetramer
Round 1
Round 2

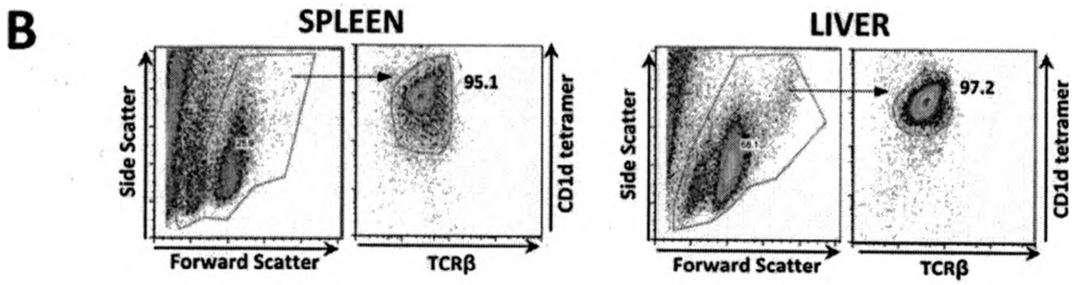
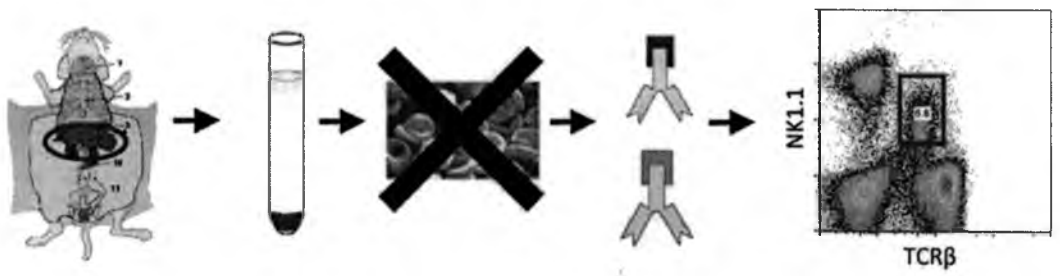

B
SPLEEN
LIVER
Side Scatter
Forward Scatter
TCRβ
CD1d tetramer
95.1
97.2

Figure 3.11. Isolation of mouse hepatic *i*NKT cells using cell sorting. ***A)*** Up to ten naïve C57BL/6 mice were sacrificed. The livers were removed, homogenized with a glass tissue homogenizer, and resuspended in a 33% Percoll density gradient. After centrifugation, the pellets—which consisted of mononuclear cells—were pooled and treated with ACK lysis buffer to remove red blood cells. Cells were stained with FITC-conjugated anti-TCRβ mAb and PE-conjugated anti-NK1.1 mAb prior to cell sorting. ***B)*** $TCR\beta^{+}NK1.1^{+}$ hepatic *i*NKT cells were isolated using a FACSVantage cell sorter with >99% purity.

A



Pooled naïve livers → Percoll density gradient → ACK lysis buffer → Fluorescent staining → FACS sorting

B

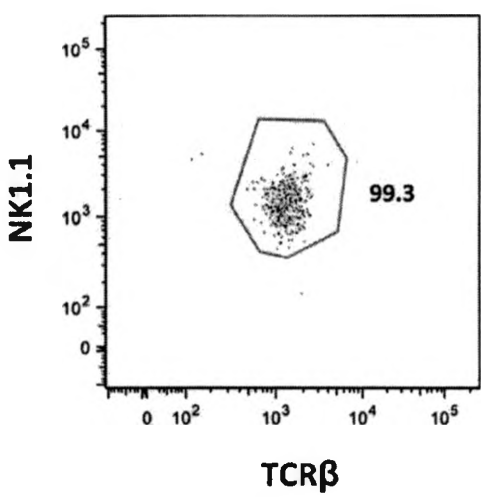


Figure 3.12. Characterization of mouse BMDCs used as accessory cells for sorted hepatic *i*NKT cells. Bone marrow cells were isolated from the tibias and femurs of naïve C57BL/6 mice. Cells were seeded at 20 x 10^6 cells/well in a 6-well plate and were given IL-4 (1000 U/mL) and GM-CSF (4 ng/mL) on days 0, 2, and 4. On day 6 when dendritic cells detach, the floating cells were harvested and stained with fluorescent antibodies against CD11c, CD1d, CD80, and CD80 (open histograms) or isotype-matched fluorescent antibodies (filled histograms). The expression of BMDC surface markers was analyzed by flow cytometry. Data are representative of two independent experiments yielding similar results.

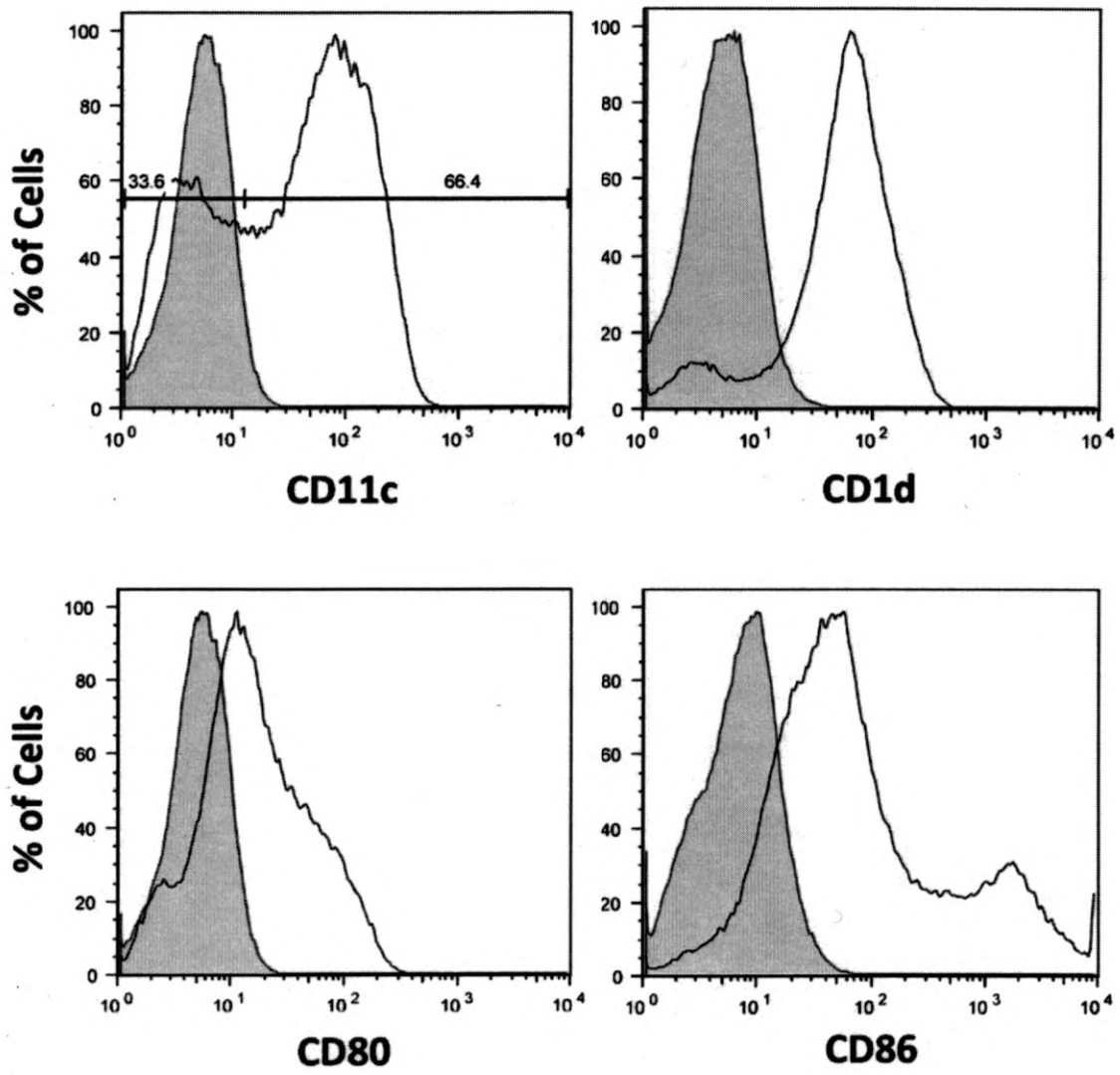
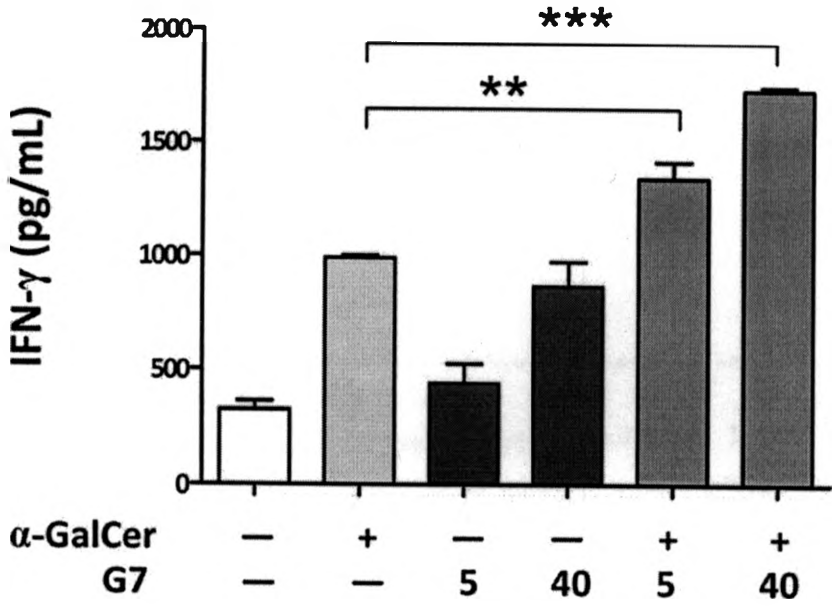

% of Cells
33.6
66.4
CD11c
CD1d
% of Cells
CD80
CD86

Figure 3.13. Thy-1 cross-linking enhances α-GalCer-mediated cytokine secretion by mouse hepatic *i*NKT cells. Isolated $TCR\beta^{+}NK1.1^{+}$ hepatic *i*NKT cells were seeded at 100,000 cells/well in a 96-well plate. BMDCs were added to each well at a ratio of 5 *i*NKT cells: 1 BMDC. Cells were incubated with either α-GalCer alone (100 ng/mL), a low (5 μg/mL) or high (40 μg/mL) dose of G7, or a combination of α-GalCer and G7. After 48 hours, culture supernatants were harvested and IFN-γ and IL-4 levels were measured by ELISA. Error bars represent standard deviation of triplicate wells within each experimental group. Statistical significance is denoted by asterisks, where ** and *** represent $P<0.01$ and $P<0.001$, respectively. Data are representative of three independent experiments yielding similar results.


***
**
2000
1500
1000
500
0
IFN-γ (pg/mL)
α-GalCer — + — — + +
G7 — — 5 40 5 40

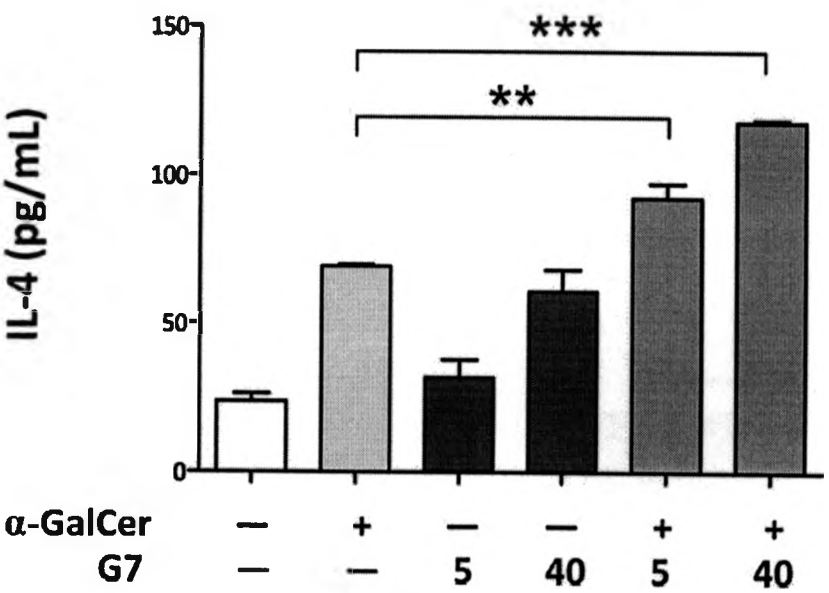
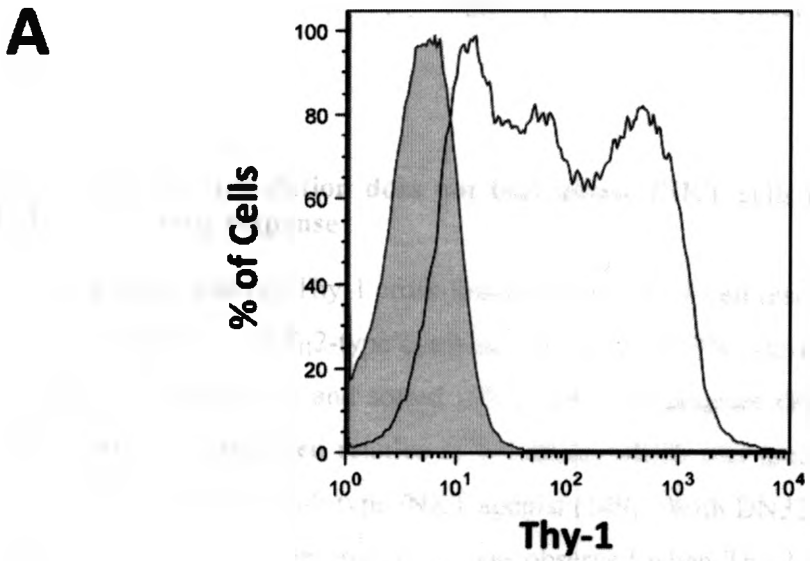
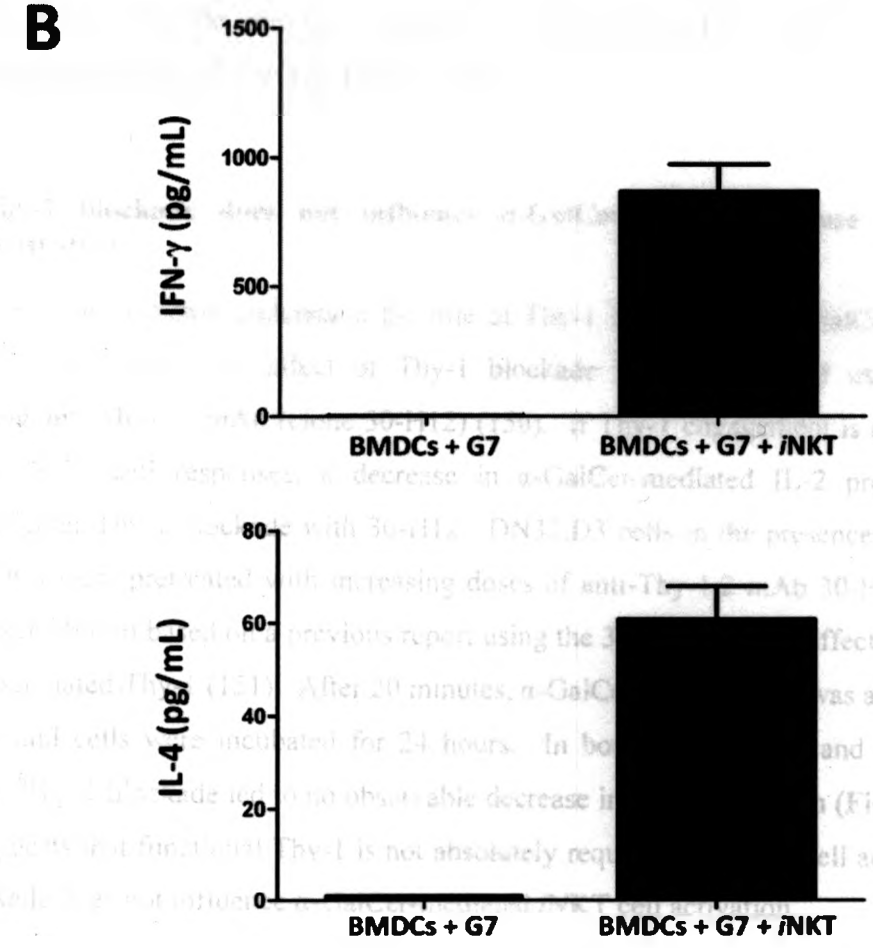

***
**
150
100
50
0
IL-4 (pg/mL)
α-GalCer — + — — + +
G7 — — 5 40 5 40

Figure 3.14. Thy-1 cross-linking on mouse BMDCs does not lead to cytokine production. ***A)*** BMDCs were cultured as previously described. On day 6, cells were harvested and were stained with PE-conjugated anti-Thy-1.2 mAb (open histogram) or isotype control (filled histogram). Thy-1 expression on BMDCs was analyzed by flow cytometry. ***B)*** Day 6 BMDCs were seeded at 20,000 cells/well in a 96-well plate. The G7 mAb (40 μg/mL) was added into the culture. To some wells, 100,000 sorted hepatic *i*NKT cells were added. After 48 hours, culture supernatants were harvested and IFN-γ and IL-4 levels were measured by ELISA. Error bars represent standard deviation of triplicate wells within each experimental group. Data are representative of two independent experiments yielding similar results.


A
% of Cells
100
80
60
40
20
0
10^0
10^1
10^2
10^3
10^4
Thy-1


B
IFN-γ (pg/mL)
1500
1000
500
0
BMDCs + G7
BMDCs + G7 + iNKT
IL-4 (pg/mL)
80
60
40
20
0
BMDCs + G7
BMDCs + G7 + iNKT

further support to the notion that Thy-1 triggering can enhance classical TCR-mediated activation of *i*NKT cells.

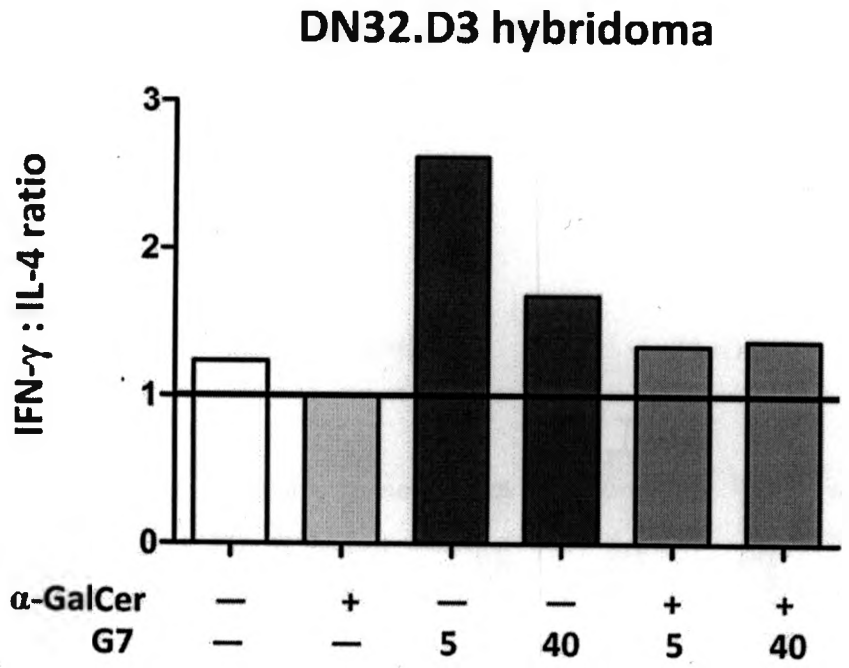
3.4. Thy-1-mediated stimulation does not bias mouse *i*NKT cells towards either a T_H1- or T_H2-type response

To determine whether Thy-1 cross-linking skews *i*NKT cell responses towards the production of either T_H1- or T_H2-type cytokines, the ratio of IFN-γ-to-IL-4 production in both the DN32.D3 hybridoma and sorted *i*NKT cells was graphed **(Figure 3.15.)**. All treatment groups were analyzed relative to α-GalCer, which was assigned a ratio of 1 since it is considered to be a T_H0-type *i*NKT agonist (149). With DN32.D3 cells, a slight bias towards T_H1-type cytokine production was observed when Thy-1 was cross-linked. A similar trend was seen with the $CD4^+$ N38-2C12 cells (data not shown). This was not the case with the more physiological sorted hepatic *i*NKT cells. Since no sound conclusions can be made from this data, it can be inferred that Thy-1 does not bias *i*NKT cells towards either a T_H1- or T_H2-type response.

3.5. Thy-1 blockade does not influence α-GalCer-mediated mouse *i*NKT cell activation

In order to better understand the role of Thy-1 in enhancing α-GalCer-mediated *i*NKT cell activation, the effect of Thy-1 blockade was investigated using a non-activating anti-Thy-1.2 mAb (clone 30-H12) (150). If Thy-1 engagement is required for optimal *i*NKT cell responses, a decrease in α-GalCer-mediated IL-2 production is expected after Thy-1 blockade with 30-H12. DN32.D3 cells in the presence or absence of BMDCs were pretreated with increasing doses of anti-Thy-1.2 mAb 30-H12. These doses were chosen based on a previous report using the 30-H12 mAb to effectively block T cell-associated Thy-1 (151). After 20 minutes, α-GalCer (100 ng/mL) was added to the cultures and cells were incubated for 24 hours. In both the presence and absence of BMDCs, Thy-1 blockade led to no observable decrease in IL-2 production **(Figure 3.16.)**. This suggests that functional Thy-1 is not absolutely required for *i*NKT cell activation as its blockade does not influence α-GalCer-mediated *i*NKT cell activation.

Figure 3.15. Thy-1 cross-linking does not bias mouse *i*NKT cells towards either a T_H1- or T_H2-type response. The ratios of IFN-γ production to IL-4 production for both the DN32.D3 *i*NKT hybridoma (top panel) and sorted hepatic *i*NKT cells (bottom panel) were graphed. Ratios were calculated relative to α-GalCer-treated cells, which were assigned a ratio of 1.


DN32.D3 hybridoma
IFN-γ : IL-4 ratio
3
2
1
0
α-GalCer — + — — + +
G7 — — 5 40 5 40

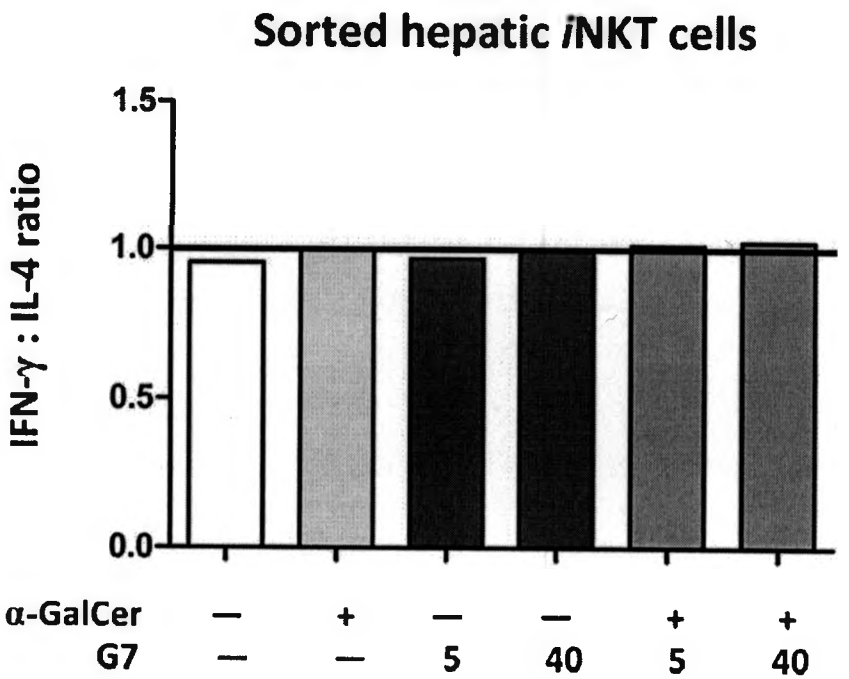
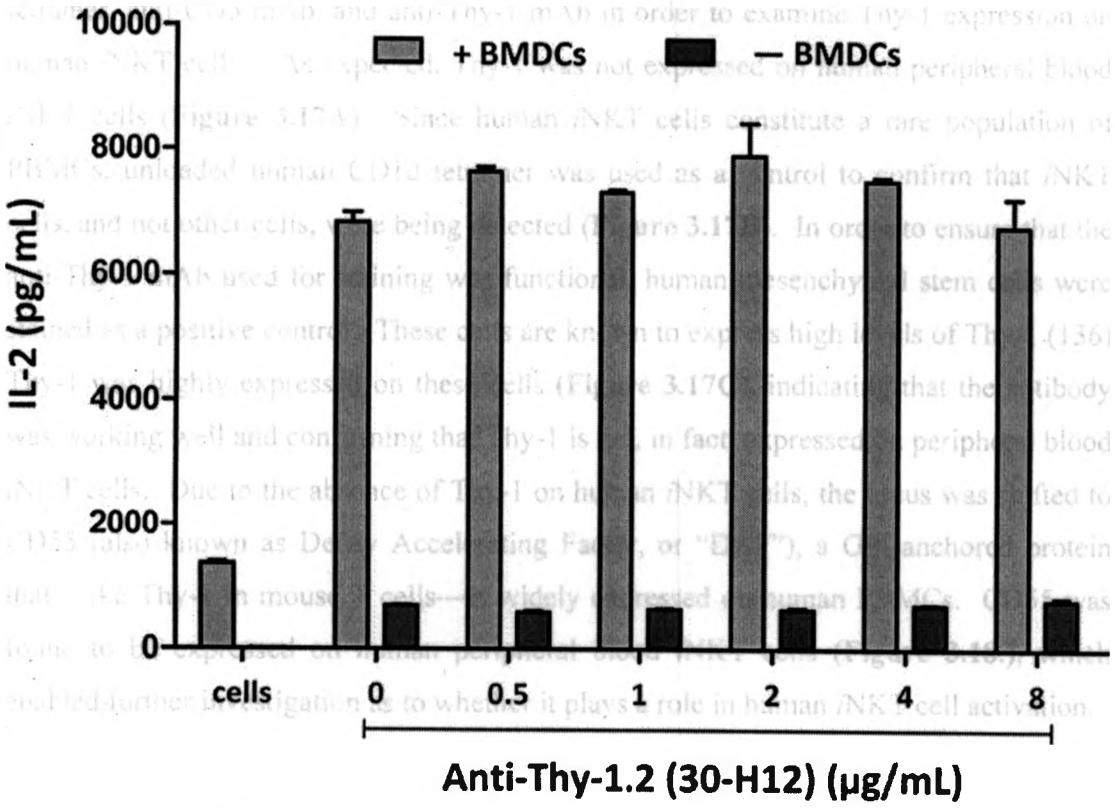

Sorted hepatic iNKT cells
IFN-γ : IL-4 ratio
1.5
1.0
0.5
0.0
α-GalCer — + — — + +
G7 — — 5 40 5 40

Figure 3.16. Thy-1 blockade does not influence α-GalCer-mediated mouse *i*NKT cell activation. DN32.D3 cells were seeded at 20,000 cells/well in a 96-well plate. BMDCs were added to some of the wells at a ratio of 5 *i*NKT cells: 1 BMDC. Blockade of Thy-1 was achieved by incubating cells with increasing doses of a blocking anti-Thy-1.2 mAb (clone 30-H12). After 20 minutes, α-GalCer (100 ng/mL) was added into each well. Culture supernatants were harvested after 24 hours and IL-2 levels were measured by ELISA. Error bars represent standard deviation of triplicate wells within each experimental group. Data are representative of one independent experiment.


10000
8000
6000
4000
2000
0
IL-2 (pg/mL)
+ BMDCs
— BMDCs
cells
0
0.5
1
2
4
8
Anti-Thy-1.2 (30-H12) (µg/mL)

3.6. Thy-1 is not expressed on human peripheral blood *i*NKT cells

To extend what was found in the mouse model, the effects of GPI-anchored protein cross-linking on human *i*NKT cell responses were explored. It is known that Thy-1 is not expressed on peripheral conventional T cells in humans (111). Nevertheless, the expression of Thy-1 on human *i*NKT cells was examined. Freshly isolated PBMCs were isolated from healthy human donors and stained with PBS-57-loaded human CD1d tetramer, anti-CD3 mAb, and anti-Thy-1 mAb in order to examine Thy-1 expression on human *i*NKT cells. As expected, Thy-1 was not expressed on human peripheral blood *i*NKT cells **(Figure 3.17A)**. Since human *i*NKT cells constitute a rare population of PBMCs, unloaded human CD1d tetramer was used as a control to confirm that *i*NKT cells, and not other cells, were being detected **(Figure 3.17B)**. In order to ensure that the anti-Thy-1 mAb used for staining was functional, human mesenchymal stem cells were stained as a positive control. These cells are known to express high levels of Thy-1 (136). Thy-1 was highly expressed on these cells **(Figure 3.17C)**, indicating that the antibody was working well and confirming that Thy-1 is not, in fact, expressed on peripheral blood *i*NKT cells. Due to the absence of Thy-1 on human *i*NKT cells, the focus was shifted to CD55 (also known as Decay Accelerating Factor, or "DAF"), a GPI-anchored protein that—like Thy-1 in mouse T cells—is widely expressed on human PBMCs. CD55 was found to be expressed on human peripheral blood *i*NKT cells **(Figure 3.18.)**, which enabled further investigation as to whether it plays a role in human *i*NKT cell activation.

3.7. CD55 cross-linking enhances α-GalCer-mediated human *i*NKT cell activation

In human conventional T cells, CD55 ligation has been shown to enhance classical TCR-mediated activation (132). To determine the effects of CD55 cross-linking on human *i*NKT cell activation, we incubated CFSE-stained human PBMCs with plate-bound mAb to CD55 (anti-CD55) alone, α-GalCer alone, or plate-bound anti-CD55 combined with α-GalCer. On day 6, *i*NKT cell proliferation, as judged by CFSE dye dilution, was measured as an indicator of activation. CD55 cross-linking alone had no effect on *i*NKT cell proliferation; however, *i*NKT cell proliferation was enhanced when anti-CD55 was combined with α-GalCer stimulation **(Figure 3.19A)**. The effect of anti-

Figure 3.17. Thy-1 is not expressed on human peripheral blood *i*NKT cells. ***A)*** Peripheral blood was collected from healthy donors and mononuclear cells were isolated using a Ficoll-Paque gradient. Cells were stained with FITC-conjugated anti-CD3 mAb, APC-conjugated PBS-57-loaded human CD1d tetramer, and PE-conjugated anti-Thy-1 mAb (open histogram) or isotype control (filled histogram). $CD3^+$ CD1d $tetramer^+$ *i*NKT cells were gated on and Thy-1 expression was analyzed. Data are representative of two independent experiments yielding similar results. ***B)*** Peripheral blood mononuclear cells were stained with FITC-conjugated anti-CD3 mAb and APC-conjugated unloaded human CD1d tetramer. Cells were analyzed by flow cytometry to confirm the absence of non-specific binding by the unloaded CD1d tetramer. ***C)*** Human mesenchymal stem cells were stained with PE-conjugated anti-Thy-1 mAb (open histogram) or isotype control (filled histogram). Thy-1 expression was analyzed by flow cytometry.

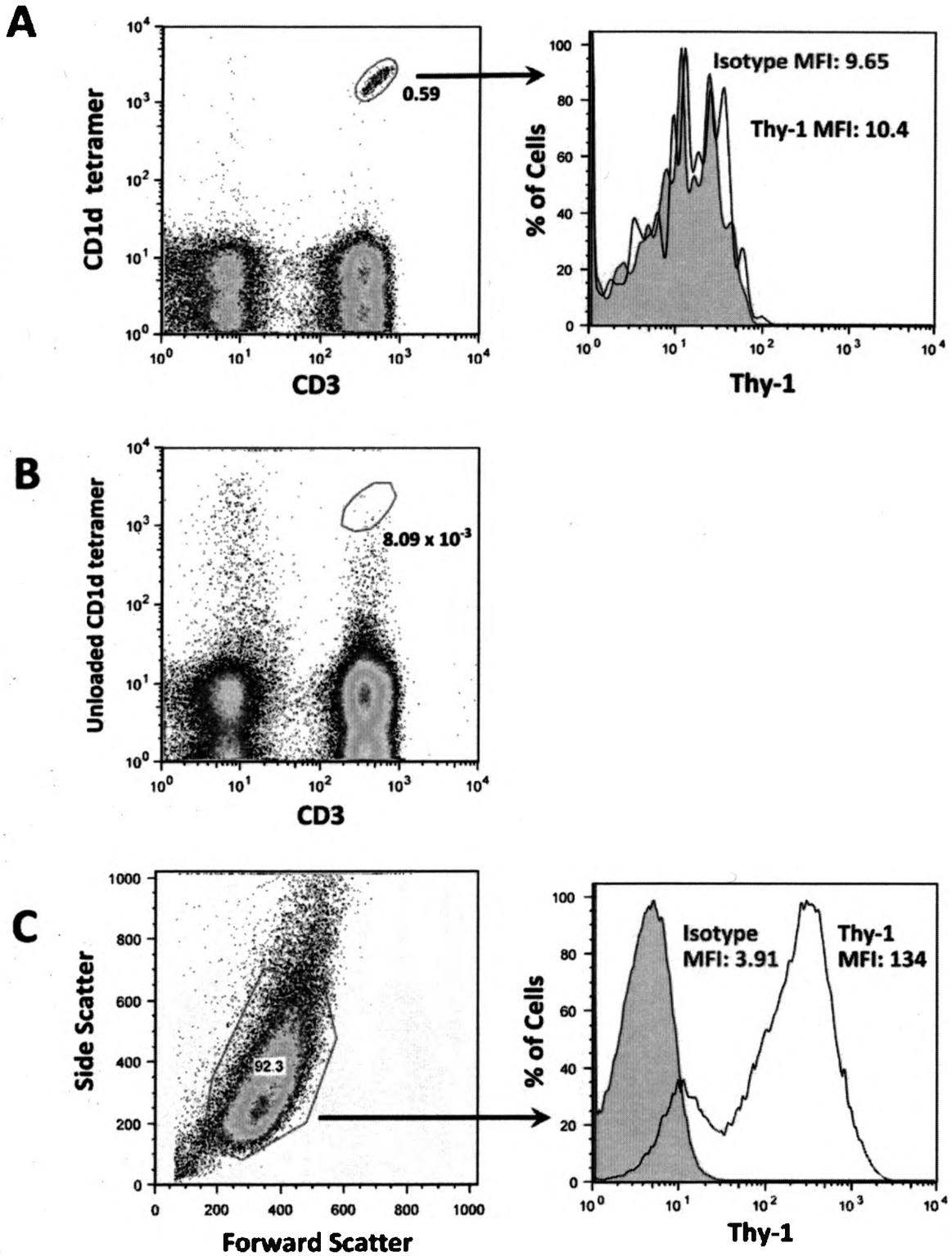
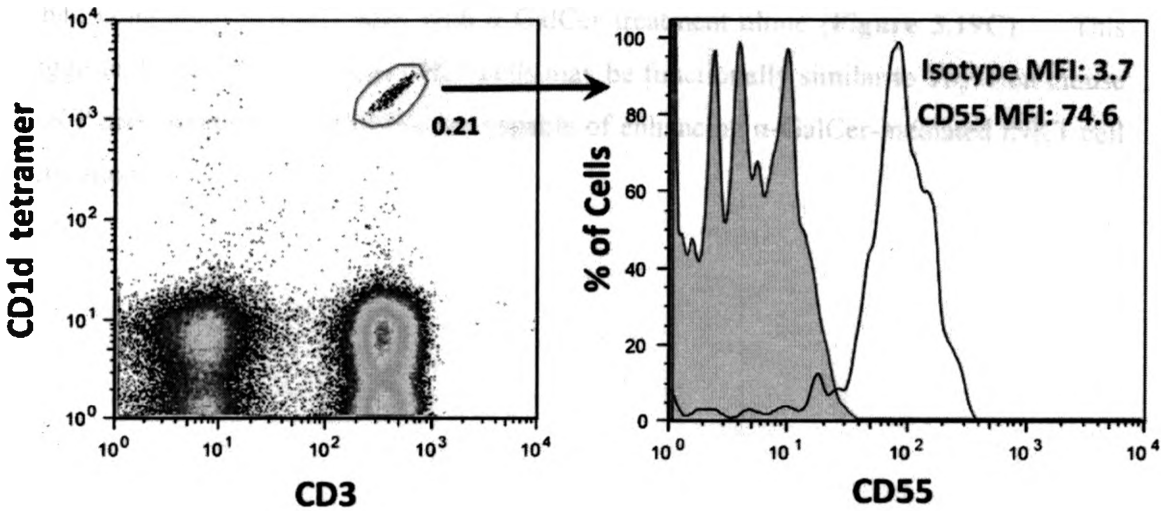
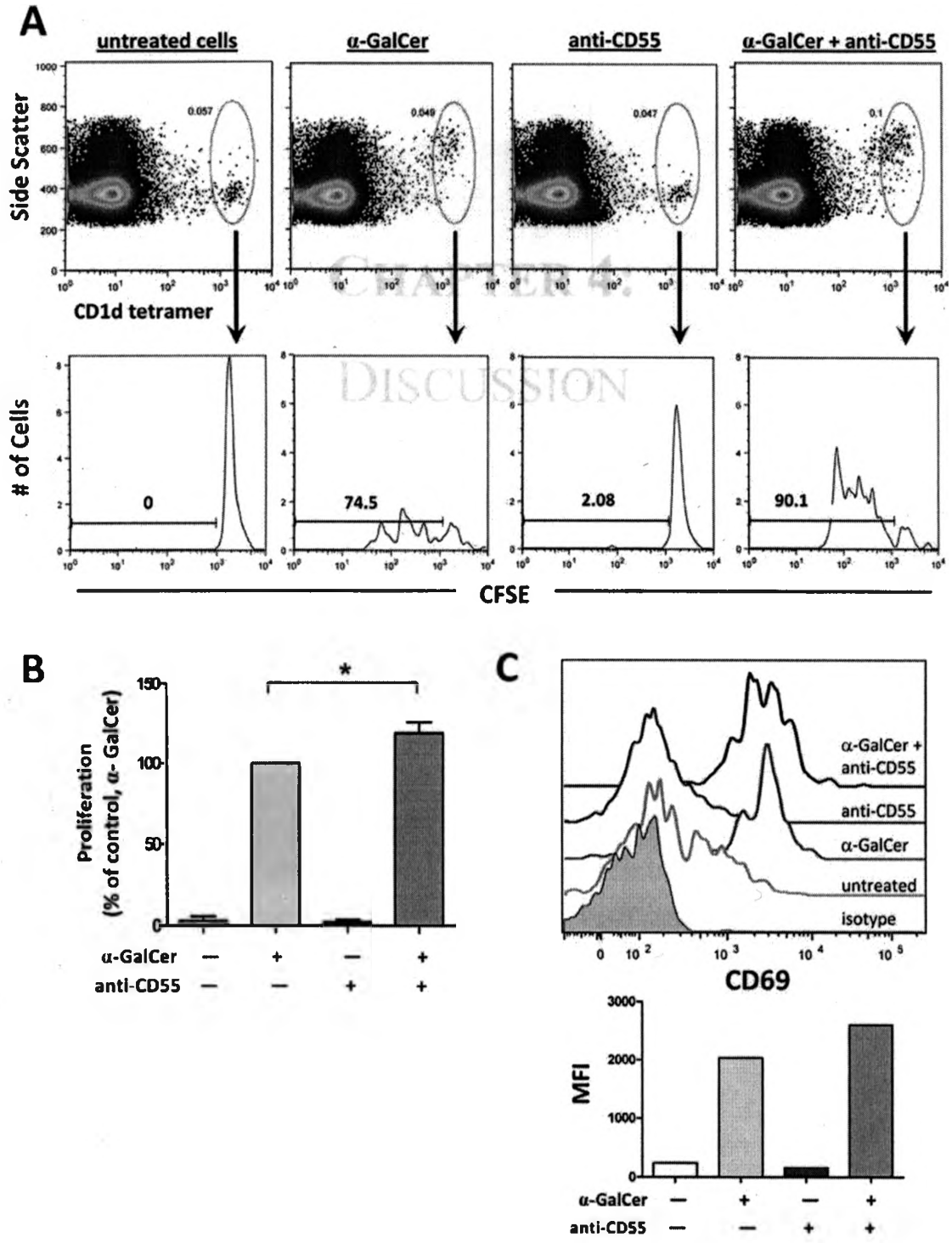

A
CD1d tetramer
CD3
0.59
% of Cells
Thy-1
Isotype MFI: 9.65
Thy-1 MFI: 10.4
B
Unloaded CD1d tetramer
CD3
8.09 x 10-3
C
Side Scatter
Forward Scatter
92.3
% of Cells
Thy-1
Isotype
MFI: 3.91
Thy-1
MFI: 134

Figure 3.18. CD55 is expressed on human peripheral blood *i*NKT cells. Peripheral blood was collected from healthy donors and mononuclear cells were isolated using a Ficoll-Paque gradient. Cells were stained with FITC-conjugated anti-CD3 mAb, APC-conjugated PBS-57-loaded human CD1d tetramer, and PE-conjugated anti-CD55 mAb (open histogram) or isotype control (filled histogram). $CD3^+$ CD1d $tetramer^+$ *i*NKT cells were gated on and CD55 expression was analyzed by flow cytometry.


CD1d tetramer
CD3
0.21
% of Cells
CD55
Isotype MFI: 3.7
CD55 MFI: 74.6

CD55 was specific since cells incubated with both plate-bound mouse IgG1 (isotype control for anti-CD55) and α-GalCer showed no difference in proliferation compared to cells treated with α-GalCer alone (data not shown). For each donor, the proportion of *i*NKT cells undergoing proliferation in each treatment group was expressed relative to the proportion of *i*NKT cells undergoing proliferation following α-GalCer treatment (α-GalCer treatment = 100% proliferation) and the mean percent of control from a total of 4 donors was graphed **(Figure 3.19B)**. The data clearly demonstrated that CD55 cross-linking enhances α-GalCer-mediated human *i*NKT cell activation. In addition to *i*NKT cell proliferation, CD55 cross-linking enhanced α-GalCer-induced CD69 expression when compared to levels seen with α-GalCer treatment alone **(Figure 3.19C)**. This suggests that CD55 on human *i*NKT cells may be functionally similar to Thy-1 on mouse *i*NKT cells since both molecules are capable of enhancing α-GalCer-mediated *i*NKT cell activation.

Figure 3.19. CD55 cross-linking enhances α-GalCer-mediated human *i*NKT cell activation. ***A)*** Human PBMCs were labeled with 5 μM CFSE and seeded at 3 x 10^6 cells/well in a 24-well plate. Some wells contained plate-bound anti-CD55 and some wells received 100 ng/mL α-GalCer. After 6 days of culture, cells were harvested and stained with APC-conjugated PBS-57-loaded human CD1d tetramer. Using flow cytometry, CD1d tetramer$^+$ *i*NKT cells were gated upon and CFSE dilution was analyzed as a measure of *i*NKT cell proliferation. Data are representative of four independent experiments yielding similar results. ***B)*** *i*NKT cell proliferation results were averaged from 4 healthy donors. For each donor, cells treated with α-GalCer served as the positive control and were assigned a value of 100%. The proliferation values resulting from all other treatment groups were expressed as a percentage of the control. Error bars represent standard error of the mean. Statistical significance is denoted by asterisks, where * represents $P<0.05$. ***C)*** Human PBMCs were seeded at 3 x 10^6 cells/well in a 24-well plate. Some wells contained plate-bound anti-CD55 and some wells received 100 ng/mL α-GalCer. After 6 days of culture, cells were harvested and stained with APC-eFluor780-conjugated anti-CD3 mAb, APC-conjugated PBS-57-loaded human CD1d tetramer, and PE-conjugated anti-CD69 mAb (open histogram) or isotype control (filled histogram). CD3$^+$CD1d tetramer$^+$ *i*NKT cells were gated upon and CD69 expression was analyzed as a measure of *i*NKT cell activation. Mean fluorescence intensity (MFI) values for each treatment group were graphed. Data are representative of two independent experiments yielding similar results.


A
untreated cells
α-GalCer
anti-CD55
α-GalCer + anti-CD55
Side Scatter
CD1d tetramer
# of Cells
0
74.5
2.08
90.1
CFSE
B
Proliferation
(% of control, α- GalCer)
α-GalCer
anti-CD55
C
α-GalCer +
anti-CD55
anti-CD55
α-GalCer
untreated
isotype
CD69
MFI

CHAPTER 4:

DISCUSSION

4.1. Research goals

*i*NKT cells play an important role in modulating immune responses. Molecular interactions between the invariant TCR and lipid-bound CD1d molecules are at the heart of *i*NKT cell activation, yet there are a variety of accessory molecules on the *i*NKT cell surface that influence how TCR-mediated signals are transduced. In conventional T cells, accessory signals, including costimulation, are necessary for cytokine production. Engagement of costimulatory molecules such as CD28, CD40L, ICOS, 4-1BB, and OX40 has been shown to enhance signalling through the TCR and thus lead to productive T cell activation [reviewed in (38)]. Without these costimulatory signals, signalling through the TCR alone results in a hyporesponsive state termed anergy or even apoptotic cell death (152).

In contrast, *i*NKT cells are not absolutely dependent on these accessory molecules for their activation. Several studies suggest that costimulation is required for optimal cytokine secretion but not for de novo cytokine production. For example, mice lacking CD28 or CD40L have impaired cytokine secretion in response to α-GalCer stimulation (59), but retain the ability to synthesize cytokine mRNA (46). Furthermore, CD28 and CD40L seem to be required for normal proliferative expansion of *i*NKT cells (60). Other costimulatory molecules on the *i*NKT cell surface have been shown to contribute to optimal *i*NKT cell responses. ICOS blockade decreases α-GalCer-induced IFN-γ and IL-4 production (61). 4-1BB-deficient mice have lower serum cytokine levels after α-GalCer administration compared with wildtype mice (64). GITR engagement, in the presence of TCR signals, results in enhanced IL-2, IFN-γ, and IL-4 production, expression of activation markers such as CD25 and CD69, and proliferation (65). In the absence of OX40, *i*NKT cells produce significantly lower levels of IFN-γ after α-GalCer stimulation (66). From these studies it is clear that research investigating costimulatory molecules on the *i*NKT cell surface has advanced our understanding of *i*NKT cell responses.

In addition to the aforementioned "classical" costimulatory molecules, there are numerous other molecules on *i*NKT cells that may also influence their activation. GPI-

anchored proteins constitute a family of molecules that are abundantly expressed on the lymphocyte cell surface. To date, the role of these molecules on *i*NKT cells has not been investigated; however, GPI-anchored proteins have been shown to play a role in T cell activation. On the T cell surface, the prototypic mouse GPI-anchored protein Thy-1 has been shown to possess costimulatory properties. Thy-1 engagement together with TCR stimulation induces mouse T cell proliferation comparable to that seen following TCR and CD28 coligation (140). In humans, coengagement of CD55 and CD3 results in enhanced proliferation and activation of $CD4^+$ T cells (132).

In this study, I sought to investigate the expression and function of GPI-anchored proteins in *i*NKT cells. Firstly, Thy-1 was found to be expressed on both resting and activated mouse *i*NKT cells. Thy-1 cross-linking alone led to *i*NKT cell activation as measured by IL-2 production. Furthermore, Thy-1 cross-linking in conjunction with TCR stimulation enhanced *i*NKT cell activation compared to cells that had only received TCR stimulation as judged by cytokine production, cytokine secretion, and expression of activation markers. Our findings could be extrapolated from mice to humans. Thy-1 was absent from the human *i*NKT cell surface; however, CD55 was found to be expressed on these cells. Like Thy-1 on mouse *i*NKT cells, CD55 cross-linking enhanced TCR-mediated human *i*NKT cell activation. These findings suggest that GPI-anchored proteins play an important role in enhancing *i*NKT cell activation.

4.2. Thy-1 is highly expressed on both resting and activated mouse *i*NKT cells

To my knowledge, this study is the first to demonstrate Thy-1 expression on *i*NKT cells. Thy-1 was found to be highly expressed on both resting and α-GalCer-activated DN32.D3 *i*NKT hybridoma cells **(Figure 3.1.)** and splenic and hepatic mouse *i*NKT cells **(Figure 3.2.)**. Interestingly, the surface expression of Thy-1 on a per cell basis was slightly decreased upon activation in both the hybridoma and in primary *i*NKT cells. One possible explanation for this finding is that Thy-1 may be internalized upon activation. This result is similar to that observed for the TCR on *i*NKT cells, where the TCR is downregulated shortly after stimulation but reappears by day 3 (51). Whether or

not this is also the case for Thy-1 requires further experimentation. Alternatively, activation may lead to increased PI-PLC activity—the enzyme responsible for cleaving the phosphodiester bond of phosphatidylinositol within the GPI anchor—which would enhance Thy-1 shedding. This theory is plausible considering that products released due to GPI-anchored protein cleavage have been hypothesized to be involved in signal transduction pathways [reviewed in (153)]. Additionally, the cleavage of GPI-anchored proteins may serve as a regulatory mechanism to prevent overactivation of *i*NKT cells. Contrary to our observation that Thy-1 levels decreased following *i*NKT cell activation, in conventional T cell activation GPI-anchored proteins appear to be resistant to bacterial PI-PLC (154). The physiological relevance of Thy-1 downregulation after activation is unknown; however, these experiments clearly show abundant expression of Thy-1 on mouse *i*NKT cells regardless of their activation status. In future studies it would be interesting to investigate whether Thy-1 surface expression increases after the initial downregulation, or if the abundance of Thy-1 on the surface compensates for the downregulation.

4.3. Thy-1 cross-linking on its own induces mouse *i*NKT cell activation

Thy-1 cross-linking alone led to robust mouse *i*NKT cell activation, even in the absence of classical TCR stimulation and accessory cells **(Figure 3.3.)**. This finding is consistent with previous studies demonstrating that Thy-1 plays a mitogenic role in T cell activation, perhaps due to the relative abundance of surface Thy-1 and the presence of Thy-1 and the TCR in similar lipid rafts. In T cells, G7 mAb-mediated cross-linking of Thy-1 is capable of inducing proliferation, IL-2 production, and CD25 upregulation (77). More recently, it has been shown that T cell activation in response to Thy-1 cross-linking alone is not optimal compared to Thy-1 cross-linking in the presence of costimulation provided by CD28 ligation (140). This is supported by the finding that Thy-1-mediated activation with mAbs other than G7 requires the presence of mitogenic 4β-phorbol-12-myristate-13-acetate (PMA), an activator of PKCs, for T cell proliferation (96). Even G7 and PMA are known to act synergistically in the context of conventional T cell activation

(155). Overall, in conventional T cells it seems that additional signals are required for appreciable Thy-1-mediated activation.

In this study, Thy-1 cross-linking on the *i*NKT hybridoma DN32.D3 in the absence of accessory cells led to significant IL-2 production comparable to that seen after stimulation with the prototype *i*NKT cell agonist α-GalCer **(Figure 3.6.)**. Furthermore, consistent with what was previously shown in T cells using both FcγR-deficient mice and anti-FcγRII/III mAb (140), FcγRs were not found to be involved in Thy-1-mediated *i*NKT cell activation **(Figure 3.4B)**. This is most probably due to the physicochemical properties of anti-Thy-1 mAb G7, which is a self-aggregating mAb (96). The ability of Thy-1 cross-linking to induce a comparable *i*NKT cell response to that of α-GalCer stimulation could be due to the fact that *i*NKT cells, unlike conventional T cells, exist in a partially activated state (31). Thus, the signals generated by Thy-1 cross-linking in the absence of costimulation may be sufficient to overcome the cell's threshold for activation. Building on this, we investigated how costimulation influences Thy-1-mediated activation in N38-2C12 *i*NKT cells, which require accessory cells (i.e. BMDCs) for their activation. Interestingly, following Thy-1 crosslinking, decreased IL-2 production was seen when BMDCs were present; in their absence, IL-2 production was higher **(Appendix A)**. The presence of Thy-1 on BMDCs **(Figure 3.14A)** may have competed with Thy-1 on *i*NKT cells, thus reducing the interactions between anti-Thy-1 mAb and Thy-1 on the *i*NKT cell surface. There is also the possibility that molecules on the BMDC surface contributed to enhanced activation-induced cell death of *i*NKT cells following Thy-1 ligation. Alternatively, costimulation may inhibit Thy-1-mediated *i*NKT cell activation in certain subsets of cells. This clearly demonstrates the pitfalls of working with cell lines, which do not always mirror physiological conditions as closely as believed. Nevertheless, given the difficult nature of obtaining large quantities of purified *i*NKT cells, cell lines have proven to be useful tools for studying *i*NKT cell responses.

A number of mechanisms have been proposed to explain how mitogenic stimulation through Thy-1 occurs. First, an intact and functional TCR/CD3 complex is

required since Thy-1 cross-linking does not activate TCR/CD3-deficient T cells (85). There is likely a functional association between Thy-1 and some elements of the TCR signalling pathway, as Thy-1- and TCR-mediated signalling events are similar in many respects (155). Furthermore, both Thy-1 and the TCR have been shown to physically associate with CD45 (84). This suggests that a ternary complex might exist such that cross-linking of Thy-1 would be equivalent to TCR engagement. Alternatively, it has been proposed that the abundant expression of Thy-1 on the T cell surface may have an indirect effect on TCR signal transduction [reviewed in (109)]. Others have found that cross-linking of GPI-anchored proteins such as Thy-1, which is enriched within lipid rafts, leads to lipid raft aggregation and clustering of signalling molecules which may, in turn, trigger signalling cascades (74, 83). These proposed mechanisms, many of which have been studied in T cells, would likely also apply to *i*NKT cells. Nevertheless, understanding how, mechanistically, Thy-1 cross-linking induces *i*NKT cell activation is an important question worth investigating.

4.4. Thy-1 cross-linking enhances TCR-mediated mouse *i*NKT cell activation

Thy-1 cross-linking combined with α-GalCer stimulation led to enhanced *i*NKT cell cytokine production **(Figures 3.6., 3.7., 3.8.)** compared with cells that had been stimulated with α-GalCer alone. This suggests that Thy-1 can function as costimulatory molecule on *i*NKT cells that weakly, yet still appreciably, enhances *i*NKT cell responses. Unlike conventional costimulatory molecules, however, Thy-1 is also capable of triggering *i*NKT cell activation in the apparent absence of classical TCR ligation **(Figure 3.3.)**. I use the term "apparent" because *i*NKT cells are known to respond to endogenous lipids that may be constitutively present in the environment (12, 45) and thus a constitutively low level of TCR-mediated activation may exist. These findings are consistent with what has been shown in T cells, where Thy-1 is capable of providing either signal 1 or signal 2 depending on the availability of other signalling molecule ligands (140). In the future, it would be interesting to examine the effect of Thy-1 triggering in conjunction with the engagement of classical costimulatory molecules in *i*NKT cells.

Thy-1 cross-linking on both $CD4^-CD8^-$ (DN) and $CD4^+$ *i*NKT hybridoma cells enhanced the cytokine response to α-GalCer **(Figures 3.6. & 3.7.)**. Interestingly, the response to Thy-1 cross-linking alone was much less potent in the $CD4^+$ *i*NKT cell hybridoma (N38-2C12) compared to the DN *i*NKT cell hybridoma (DN32.D3) and never exceeded the response elicited by α-GalCer. In conventional T cells, $CD4^+$ is a coreceptor that augments cytokine production through the recruitment of Lck and by physically stabilizing TCR:MHC interactions [reviewed in (156)]. In *i*NKT cells, CD4 interacts with CD1d (157) and its engagement with mAb enhances *i*NKT cell activation (158); however, CD4 expression is not necessary for maximal *i*NKT cell activation (159). One possible explanation for our findings is that the presence of CD4 on the *i*NKT cell surface may physically interfere with maximal Thy-1 cross-linking due to its sheer abundance. Alternatively, different hybridomas may respond differently to the same stimuli since they are not always an accurate representation of primary cell function in vivo.

In addition to enhanced cytokine production, Thy-1 cross-linking together with α-GalCer stimulation led to slightly upregulated CD69 and CD25 expression **(Figure 3.9.)**. In the case of CD25, only a slight increase in surface expression was seen even after stimulation solely with α-GalCer. A similar result was seen by Kim *et al.* in hepatic primary *i*NKT cells that had been stimulated with α-GalCer for 24 hours (65), yet the authors did not elaborate on this finding in their article. There exists the remote possibility that CD25 expression on *i*NKT cells, particularly the DN32.D3 *i*NKT cell hybridoma, is inconsistent and perhaps not as good a marker for *i*NKT cell activation as once thought. Further confirmation of *i*NKT cell activation could be achieved using additional indicators of activation status such as CD62L and CD44.

The results seen in *i*NKT hybridoma cells were confirmed in primary hepatic *i*NKT cells that were purified by cell sorting. Only IFN-γ and IL-4 production was used to assess activation **(Figure 3.13.)** due to the limited number of purified *i*NKT cells that could be acquired in any given sort. An appreciable number of cells were recovered from

each sort when the liver was used as the source, yet caution must be taken when applying these findings to all *i*NKT cells. Functional differences between *i*NKT cells of different organs have been reported; for example, liver-derived *i*NKT cells are better mediators of tumor rejection than their thymic and splenic counterparts (48). Examining splenic *i*NKT cell responses to Thy-1 cross-linking in parallel with hepatic *i*NKT cell responses would have strengthened my findings. Unfortunately, this was not feasible since *i*NKT cells constitute such a small population of splenocytes (approximately 1.5%) (160) which limits their purification. The sorting approach utilized the *i*NKT cell markers NK1.1 and TCRβ **(Figure 3.11.)**. Since NK1.1 is also expressed on NK cells (161) and *v*NKT cells (162), it is not a perfect *i*NKT cell marker and it is possible that non-*i*NKT cell contaminants were present in culture. There are reportedly cells that express both NK1.1 and the TCR but are CD1d-independent (163). Furthermore, some CD1d-dependent NKT cells do not express NK1.1 (164), which may suggest that the sorting strategy excluded some *i*NKT cells. However, it is reassuring that very similar *i*NKT cell frequencies were always found when CD1d tetramer and NK1.1 staining were performed in parallel **(Appendix B)**. While CD1d tetramers could have been used instead of NK1.1, this may lead to partially activated *i*NKT cells that can spontaneously produce cytokines in culture [reviewed in (148)]. In addition to purified hepatic *i*NKT cells, BMDCs were added to culture to establish more physiological conditions. It was observed that BMDCs do express Thy-1, but Thy-1 cross-linking did not induce cytokine production by BMDCs **(Figure 3.14.)**. Further experimentation is required to test the possibility that BMDCs may respond differently to anti-Thy-1 mAb in the presence of *i*NKT cells.

In both *i*NKT cell hybridomas and primary hepatic *i*NKT cells, Thy-1 cross-linking enhanced TCR-mediated production of IL-2, IFN-γ, and IL-4. Of greater interest than absolute cytokine production was the ratio of IFN-γ-to-IL-4 production, as this indicates whether Thy-1 skews *i*NKT cells towards either a T_H1- or T_H2-type response. A slight bias towards T_H1 cytokine production was observed when Thy-1 was cross-linked in DN32.D3 cells, but not in primary *i*NKT cells **(Figure 3.15.)**. Other studies have shown that certain costimulatory molecules can skew *i*NKT cell responses. For example, OX40 may promote T_H1-type responses (66), while 4-1BB and GITR promote

T_H2-type responses (64, 65). Most of these studies used disease models (i.e. airway hyperreactivity) in mice to examine T_H skewing but unfortunately this was not a feasible option in the current study. Thy-1, unlike other costimulatory molecules, can trigger potent T cell and *i*NKT cell activation on its own. This suggests that injection of anti-Thy-1 mAb into mice would lead to non-specific activation of immune cells resembling that induced by polyclonal mitogens. This would ultimately lead to a cytokine storm and its associated morbidity and/or mortality.

Although Thy-1 cross-linking enhanced *i*NKT cell activation, its functionality was not required for α-GalCer-induced activation to occur. Blocking Thy-1 with the anti-Thy-1.2 mAb 30-H12 led to no noticeable dampening of TCR-mediated activation **(Figure 3.16.)**. This is the opposite of what has been described in conventional T cells. In T cells, blocking Thy-1 with this mAb inhibits TCR-mediated T cell activation, which suggests that 30-H12 prevents the interaction between Thy-1 and its putative ligand on accessory cells (151). In contrast, the above findings suggest that the putative ligand for *i*NKT cell Thy-1, if it exists, is not present on either BMDCs or *i*NKT cells themselves. Alternatively, the ligand for Thy-1 may be present on either of these cell types but may not be essential for *i*NKT cell responses. This seems plausible given that conventional T cells and *i*NKT cells differ considerably in their requirement for costimulation.

4.5. CD55 on the human peripheral blood *i*NKT cell surface may be functionally similar to mouse Thy-1

Thy-1 was not expressed on human peripheral blood *i*NKT cells **(Figure 3.17.)**; however, this does not exclude the possibility that Thy-1 may be expressed on *i*NKT cells found in other tissues or organs. This notion was not further investigated due to the lack of availability of human biopsy samples. Also, this was beyond the scope of this study. Ultimately, this finding was somewhat expected since Thy-1 is absent from conventional human T cells (111).

Various GPI-anchored proteins are expressed on human lymphocytes, such as CD48 (165), CD52 (166), CD55 (89), and CD59 (167). CD55 was investigated in order

to determine how GPI-anchored proteins modulate human *i*NKT cell responses since it has been relatively well-studied and various CD55-specific reagents are commercially available. Furthermore, it has been shown that CD55 cross-linking on the surface of conventional human T cells enhances TCR-mediated activation (132). Like in T cells, CD55 cross-linking enhanced α-GalCer-induced human *i*NKT cell activation as judged by proliferation and CD69 expression **(Figure 3.19.)**. Interestingly, unlike Thy-1, cross-linking of CD55 on its own led to no significant increase in proliferation. This suggests that there may be species-related functional differences between GPI-anchored proteins (mouse vs. human), or that there may be functional differences between individual GPI-anchored proteins (Thy-1 vs. CD55) beyond those characteristics imparted by the GPI-anchor itself. Alternatively, the abundance of each of these molecules on the cell surface may correlate with their ability to induce activation. On T cells, there may exist as many as 1 million copies of Thy-1 per cell [reviewed in (109)], whereas CD55 is only present at approximately 9,000 molecules per cell (168). Likewise, MFI levels of Thy-1 and CD55 expressed on the *i*NKT cell surface indicate that Thy-1 is much more abundant on mouse *i*NKT cells **(Figure 3.2.)** than CD55 is on human *i*NKT cells **(Figure 3.18.)**.

Ultimately, working with human peripheral blood *i*NKT cells has not proven to be easy since they constitute a rare population. The *i*NKT cell frequency was often lower than 0.1% in the blood of healthy subjects, which made it difficult to identify individuals with a detectable population. Furthermore, as with many human studies [reviewed in (169)], there was large variability between individual donors. Due to the challenging nature of isolating these cells in sufficient quantities for culture, it was not possible to investigate human *i*NKT cell function in the absence of other lymphoid cells. Access to human *i*NKT cell clones will enable further investigations into the role of GPI-anchored proteins expressed by human *i*NKT cells. For example, T_H1- and T_H2-type cytokine production following CD55 cross-linking and α-GalCer stimulation could be quantified as an additional measure of activation.

4.6. Impact of this research on better understanding *i*NKT cell responses

Overall, this study demonstrates for the first time that GPI-anchored proteins may represent a subset of immunomodulatory molecules on the *i*NKT cell surface. A number of costimulatory and inhibitory molecules have been identified to date; however, our basic understanding of *i*NKT cell immunobiology remains far from complete. *i*NKT cells can elicit a broad range of sometimes contradictory immune responses (e.g. *i*NKT cells have been shown to either cause, or protect against, autoimmune diseases) [reviewed in (170)], yet the mechanisms governing these differential responses are not fully understood. The findings presented in this study may be one small piece of a large puzzle that influences how *i*NKT cells are being regulated and/or regulate other cells in health and disease. Notable advances in the field include clinical trials that have successfully used *i*NKT cell agonists as cell-based immunotherapeutics for cancer (171, 172).

α-GalCer, a rather strong agonist for *i*NKT cells, was used in this study to induce *i*NKT cell activation. The role of GPI-anchored proteins in *i*NKT cell responses may vary depending on the stimulus strength. As such, it would be interesting to see if the results from this study differ in the presence of weaker *i*NKT cell agonists that have been shown to function in a T_H1- or T_H2-biased manner (173, 174). Ultimately, I hope that future studies can build upon these findings so that the role of GPI-anchored proteins in *i*NKT cell responses can be better understood.

4.7. Future directions

Numerous outstanding questions remain with respect to the role of GPI-anchored proteins in *i*NKT cell responses. How do GPI-anchored proteins transmit signals in *i*NKT cells? What is the natural ligand for *i*NKT cell-associated Thy-1? Does the absence of GPI-anchored proteins influence human *i*NKT cell responses and/or development?

As described above, a number of mechanisms have been proposed to explain how GPI-anchored proteins transmit intracellular signals. Despite decades of research, no clear consensus has been reached. In conventional T cells, differences between Thy-1- and TCR-mediated signal transduction pathways have been identified (155). These pathways have not been studied in *i*NKT cells and it is possible that differences beyond those observed in conventional T cells may exist. This could provide clues as to *how* Thy-1 transmits signals in *i*NKT cells and *if* and *where* Thy-1-mediated signalling converges with conventional TCR-mediated signalling pathways.

Integrins on neurons, polymorphonuclear cells, monocytes, and dendritic cells have been identified as the natural ligands for Thy-1 on endothelial cells (113, 116, 117). Nonetheless, the ligand(s) for Thy-1 on both conventional T cells and *i*NKT cells remains unknown. This is an area worthy of investigation since it would reduce our reliance on mAbs to study Thy-1 and would lead to a better understanding of the biological function of Thy-1 in T cells.

The GPI anchor has been proposed as a potential self-ligand that could be involved in *i*NKT cell development (175, 176). Consequently, it would be interesting to investigate whether individuals who lack GPI-anchored proteins, for instance patients with PNH, have defects in either *i*NKT cell development or function. To do this, blood samples from PNH patients need to be screened for the presence of *i*NKT cells. If *i*NKT cells are not present, this would suggest that the GPI anchor is a critical component for *i*NKT cell development. If *i*NKT cells are present, their ability to respond to normal stimuli should be tested. Diminished *i*NKT responses to α-GalCer (compared to healthy donors) would strengthen our findings and further support the notion that GPI-anchored proteins contribute to optimal *i*NKT cell responses.

CHAPTER 5:

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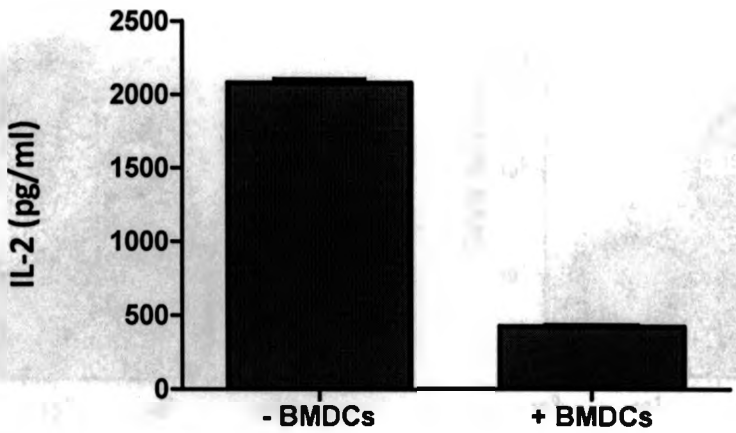
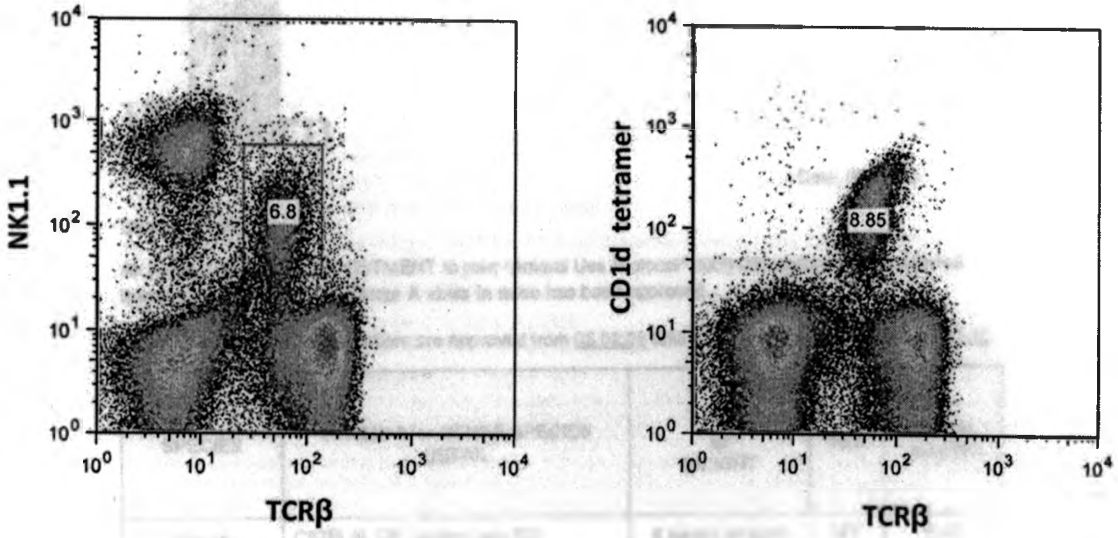
APPENDICES

APPENDIX A



BMDCs dampen the response of N38-2C12 *i*NKT hybridoma cells to Thy-1 cross-linking. *i*NKT cells were seeded at 20,000 cells per well in a 96-well plate and BMDCs were added to some wells at a ratio of 5 *i*NKT : 1 BMDC. Cells were incubated with anti-Thy-1 mAb G7 at a concentration of 5 μg/mL. After 24 hours, culture supernatants were harvested and IL-2 levels were measured by ELISA. Error bars represent standard deviation of triplicate wells.

APPENDIX B



Flow cytometric analysis of *i*NKT cell frequencies comparing CD1d tetramer staining to NK1.1 staining. Naïve mice were sacrificed and their livers were removed. Hepatic lymphoid mononuclear cells were isolated and stained with FITC-conjugated anti-TCRβ mAb and either APC-conjugated PBS-57-loaded mouse CD1d tetramer or PE-conjugated anti-NK1.1 mAb. Double positive cells were gated on and the frequency of *i*NKT cells detected from each staining procedure was compared.

APPENDIX C

Date: 08.26.08

Dear Dr. Haeryfar

An ANIMAL NUMBER ADJUSTMENT to your "Animal Use Protocol" 2006-065 entitled **Cell-mediated immune responses to influenza A virus in mice** has been approved.

These strains and animal numbers are approved from 06.02.08 until the protocol full expiry date 08.31.10.

SPECIES	STRAIN &/or OTHER SPECIES DETAIL	AGE or WEIGHT	SEX	ANIMAL NUMBER
Mouse	C57BL/6, CR, strain code 027	6 weeks or older	M/F	2150
Mouse	BALB/c, CR, strain code 028	6 weeks or older	M/F	930
Mouse	DBA/2,CR, strain code 026	6 weeks or older	M/F	300
Mouse	TdT KO, we already have a breeding colony in Room 6028	6 weeks or older	M/F	250 from 6 pairs NOT being bred simultaneo usly
Mouse	Ikaros L/L mice, Non commercially available	6 weeks or older	M/F	50
Mouse	OT-1,JAX stock number 005432	6 weeks or older	M/F	50
Mouse	CD-1 KO, JAX stock number 006330	6 weeks or older	M/F	135
Mouse	Thy1.1 congenic, JAX stock number 005686	6 weeks or older	M/F	50
Mouse	RIP2 KO, Non commercially available	6 weeks or older	M/F	50
Mouse	Jα18 KO, Non commercially available	6 weeks or older	M/F	834

c.c. Approval Letter - W Lagerwerf

***The* University *of* Western Ontario**
Animal Use Subcommittee / University Council on Animal Care
Health Sciences Centre, • London, Ontario • CANADA – N6A 5C1
PH: 519-661-2111 ext. 86770 • FL 519-661-2028 • www.uwo.ca / animal

APPENDIX D

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Use of Human Subjects - Ethics Approval Notice

Principal Investigator: Dr. M. Haeryfar
Review Number: 15439E **Review Level:** Expedited
Review Date: September 10, 2008
Protocol Title: Regulation of Immune Responses by Natural Killer T (NKT) and Natural Killer (NK) cells
Department and Institution: Microbiology & Immunology, University of Western Ontario
Sponsor: CIHR-CANADIAN INSTITUTE OF HEALTH RESEARCH
Ethics Approval Date: October 30, 2008 **Expiry Date:** October 31, 2013
Documents Reviewed and Approved: UWO Protocol, Letter of Information and Consent.
Documents Received for Information:

This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced study on the approval date noted above. The membership of this REB also complies with the membership requirements for REB's as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the UWO Updated Approval Request Form.

During the course of the research, no deviations from, or changes to, the protocol or consent form may be initiated without prior written approval from the HSREB except when necessary to eliminate immediate hazards to the subject or when the change(s) involve only logistical or administrative aspects of the study (e.g. change of monitor, telephone number). Expedited review of minor change(s) in ongoing studies will be considered. Subjects must receive a copy of the signed information/consent documentation.

Investigators must promptly also report to the HSREB:

a) changes increasing the risk to the participant(s) and/or affecting significantly the conduct of the study;
b) all adverse and unexpected experiences or events that are both serious and unexpected;
c) new information that may adversely affect the safety of the subjects or the conduct of the study.

If these changes/adverse events require a change to the information/consent documentation, and/or recruitment advertisement, the newly revised information/consent documentation, and/or advertisement, must be submitted to this office for approval.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.

Chair of HSREB: Dr. Joseph Gilbert

Ethics Officer to Contact for Further Information

☐ Janice Sutherland	☐ Elizabeth Wambolt	☑ Grace Kelly	☐ Denise Grafton

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cc: ORE File