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THE ROLE OF INVARIANT NATURAL KILLER T (iNKT) CELL ANERGY IN GLYCOLIPID-MEDIATED PROTECTION AGAINST TYPE 1 DIABETES (T1D)

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THE ROLE OF INVARIANT NATURAL KILLER T (iNKT) CELL ANERGY IN GLYCOLIPID-MEDIATED PROTECTION AGAINST TYPE 1 DIABETES (T1D)

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Abstract

Type 1 diabetes (T1D) results from the destruction of pancreatic islet β cells by self-reactive T cells. Treatment of non-obese diabetic (NOD) mice with the potent iNKT cell agonist α-galactosylceramide C26:0 (α-GalCer) or its T_H2-biasing derivative α-galactosylceramide C20:2 (C20:2) confers protection against T1D. After an initial response to α-GalCer, iNKT cells become anergic, exhibiting a significantly blunted response upon subsequent restimulation. Although anergic iNKT cells are more susceptible to apoptosis, they are also responsible for inducing tolerogenic dendritic cells (DCs) upon restimulation, which play an important role in the protection against T1D. My results demonstrate that C20:2 activated iNKT cells enter and recover from anergy more rapidly than α-GalCer, leading to reduced iNKT cell death and the induction of more tolerogenic DCs after a multi-low dose treatment protocol. I propose that these characteristics of C20:2 may render it a more promising drug candidate for the treatment of T1D than α-GalCer.

Keywords

Type 1 diabetes (T1D), non-obese diabetic (NOD) mice, invariant natural killer T (iNKT) cell, α-galactosylceramide C26:0, α-galactosylceramide C20:2, anergy.

Dedication

To my parents, Perry and Keren Tohn,

For their unwavering love, support, and encouragement.

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List of Abbreviations

α	Alpha
α-GalCer	Alpha-galactosylceramide C26:0
Ag	Antigen
APC	Antigen presenting cells
β	Beta
BCR	B cell receptor
BGL	Blood glucose levels
C20:2	Alpha-galactosylceramide C20:2
$CaCl_2$	Calcium chloride
CCR9	Chemokine receptor type 9
CD1	Cluster of differentiation 1
CO_2	Carbon dioxide
CTL	Cytotoxic T lymphocytes
Cy7	Cychrome 7
δ	Delta
DCs	Dendritic cells
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
Foxp3	Forkhead box P3

GAD	Glutamic acid decarboxylase
GM-CSF	Granulocyte/macrophage colony-stimulating factor
h	Hours
H_3PO_4	Phosphoric acid
H_2SO_4	Sulphuric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLA	Human leukocyte antigen
HPLC	High-performance liquid chromatography
IA-2	Insulinoma-associated antigen-2
IDDM	Insulin-dependent diabetes mellitus
IFA	Incomplete Freud's adjuvant
IFN	Interferon
iGb3	Isoglobotrihexosylceramide
IGRP	Islet-specific glucose-6-phosphate catalytic subunit related peptide
IL	Interleukin
iNKT	Invariant natural killer T
Ins	Insulin
i.p.	Intraperitoneal
KCL	Potassium chloride
$KHCO_3$	Potassium bicarbonate
$NaHCO_3$	Sodium bicarbonate
NaN_3	Sodium azide
ng	Nanogram
NH_4Cl	Ammonium chloride
NK	Natural killer

NKT	Natural killer T
nm	Nanometre
NOD	Non-obese diabetic
M	Molar
mAbs	Monoclonal antibodies
mDCs	Myeloid dendritic cells
µg	Microgram
$MgCl_2$	Magnesium chloride
MHC-I	Major histocompatibility complex class I
MHC-II	Major histocompatibility complex class II
Min	Minutes
µL	Microlitre
µm	Micrometre
mM	Millimolar
mRNA	Messenger ribonucleic acid
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PD-1	Programmed death-1
pDCs	Plasmacytoid dendritic cells
PD-L1	Programmed death ligand-1
PD-L2	Programmed death ligand-2
PE	Phycoerythrin
PE-Cy7	Phycoerythrin-cychrome 7
PerCP	Peridinin chlorophyll protein
PerCP-Cy5.5	Peridinin chlorophyll protein-cychrome 5.5

PLN	Draining pancreatic lymph node
PMA	Phorbol 12-myristate 13-acetate
PP	Pancreatic polypeptide-producing
PRRs	Pattern recognition receptors
rpm	Revolutions per minute
SAv-HRP	Streptavidin-horseradish peroxidise
Siglec H	Sialic-acid binding immunoglobulin-like lectin H
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TCR	T cell receptor
T_{eff}	Effector T
TGF	Transforming growth factor
T_H	T helper cell
T_H1	T helper cell class 1
T_H2	T helper cell class 2
T_H17	T helper cell class 17
TLRs	Toll-like receptors
TMB	Tetramethylbenzidine
TNF	Tumour necrosis factor
T_{regs}	Regulatory T cells
ZnT8	Zinc transporter 8

Chapter 1:

Introduction

1.1 The immune system

The mammalian immune system has evolved to provide the host with protection against potentially dangerous pathogens and subsequent infections. This complex system is divided into two main branches: the innate and adaptive immune systems. The basis of an immune response involves the recognition of "danger signals" that may originate from either self/host or non-self/foreign sources. Triggering of an immune response leads to the activation of leukocytes that are capable of either destroying pathogens directly or recruiting help from other immune cells. In addition, the immune system has evolved over time to also consist of specialized regulatory cells that are responsible for controlling the responses of other cell types. Regulatory cells have the ability to either enhance or suppress an ongoing immune response by directly assisting in the destruction of a pathogen or by inhibiting other cells to minimize a potentially harmful inflammatory response [1, 2].

1.1.1 Innate immunity

The coordinated effort of the innate and adaptive immune systems is responsible for the successful clearance of a pathogen [3]. The innate immune system provides rapid, non-specific responses to pathogenic microorganisms and acts as the first line of defense for the host. It is characterized by both physical barriers and specialized immune cells. The epithelial layers of the skin and mucosal surfaces, which line the cavities of the body, act as physical barriers in order to prevent microorganisms from entering internal tissues [4]. In addition, there are physiological barriers that are in place such as temperature, pH, and soluble factors that contribute to limiting the survival, adherence and dissemination of invading microorganisms [4].

Upon successful penetration of the host's physical barriers, pathogens immediately encounter the specialized cells of the innate immune system that reside in the tissues. Upon contact with extracellular microorganisms, mast cells and eosinophils secrete cytotoxic substances into the extracellular matrix to destroy the invading pathogen [4]. Conversely, natural killer (NK) cells are specialized to directly destroy infected cells through the release of apoptosis-inducing molecules such as perforin and granzymes [4]. In addition, NK cells produce copious amounts of interferon (IFN)-γ, a pro-inflammatory cytokine that is crucial in controlling certain infections [4]. Finally, professional phagocytic immune cells such as macrophages, neutrophils, and dendritic cells (DCs) can engulf pathogens and destroy them in phagolysosomes, which are specialized intracellular compartments that possess a highly acidic environment as well as multiple proteolytic enzymes.

Although cells of the innate immune system respond in a non-specific manner, they are still able to distinguish the difference between self and non-self [4]. In general, innate immune cells are able to recognize structural motifs called pathogen associated molecular patterns (PAMPs) that are common to many pathogens through their germ-line encoded pattern recognition receptors (PRRs) [4]. The most well characterized PRRs are the Toll-like receptors (TLRs), each of which are devoted to recognizing distinct microbial components [4]. Upon interaction of a TLR with its appropriate ligand, a signalling cascade is initiated within the immune cell leading to its activation and subsequent secretion of soluble factors [4]. In particular, cytokines and chemokines are released, which are responsible for inducing an inflammatory response within the tissue as well as recruiting neutrophils and other immune cells to the site of infection [4]. Macrophages and DCs, in particular, also upregulate costimulatory molecules on their surface upon TLR- or inflammatory cytokine- induced activation, which are required for the generation of an adaptive immune response [4].

DCs are highly specialized professional antigen presenting cells (APCs) that can secrete anti-viral interferons as well as inflammatory cytokines such as interleukin (IL)-1, IL-12, and tumor necrosis factor (TNF)-α upon activation [5]. In addition, these cells possess antigen (Ag)-capturing capacity combined with a low degradation rate, which enables them to present Ags for long periods of time [6]. DCs also express exceptionally high levels of major histocompatibility complex class II (MHC-II) and costimulatory molecules on their surface, both of which are required to activate an adaptive immune response. What truly makes DCs unique, however, is their migratory ability that enables them to carry Ags captured in the periphery to secondary lymphoid organs and activate immunologically naïve T cells [7-10].

The major subtypes of DCs in both mice and humans are plasmacytoid DCs (pDCs) and myeloid DCs (mDCs). pDCs can be specifically distinguished by their capacity to produce large amounts of type I interferon and are key mediators of antiviral immunity [11]. However, recent evidence demonstrates that a subset of pDCs containing chemokine receptor type 9 (CCR9) possess tolerogenic properties and are able to induce T cells with suppressive activity [12]. pDCs also express lower amounts of MHC-II and costimulatory molecules, and present Ags less efficiently than their myeloid counterparts [13, 14]. As a result, pDCs are not as effective in eliciting a proliferative response from naïve Ag-specific T cells. Therefore, these cells are not believed to play an important role in directly initiating T-cell immune responses [14]. In mice, pDCs can be phenotypically defined by their relatively low expression levels of the integrin CD11c as well as the expression of sialic-acid binding immunoglobulin-like lectin H (Siglec-H) ($CD11c^{low}SiglecH^{+}$) [15].

mDCs are also referred to as conventional DCs, since they seem to participate most directly in Ag presentation and naïve T cell activation. Phenotypically, mDCs are

characterized by their high expression levels of CD11c and their lack of Siglec H in mice ($CD11c^{high}SiglecH^{-}$) [14]. This subset can be further divided into $CD8^{+}$ mDCs and $CD8^{-}$ mDCs, with the majority being $CD8^{-}$. $CD8^{+}$ mDCs are pro-inflammatory, being the major producers of the inflammatory IL-12 cytokine [16-18].

1.1.2 Adaptive immunity

If the innate immune system is unable to successfully clear an invading pathogen on its own, it may elicit help from the cells of the adaptive immune system. In comparison to the non-specific responses of the innate immune system, the adaptive immune system is characterized by Ag specificity and the generation of memory cells that provide long-lasting protection for the host. The effector cells of the adaptive immune system, T and B cells, possess the unique ability to rearrange specialized gene segments (V-D-J gene segments) that allow these cells to develop a sufficiently diverse repertoire of receptors that are capable of recognizing antigenic components of a variety of pathogen motifs [19]. In general, B lymphocytes are responsible for humoral immunity, while T lymphocytes are primarily responsible for cell-mediated immunity; however, these cells also work in concert with other cell subsets to mediate effective adaptive immunity [19].

The primary role of B cells is to recognize Ags with their membrane-bound B cell receptor (BCR) and subsequently produce immunoglobulins, or antibodies, each with a single Ag specificity. There are two different populations of B cells that participate in an immune response. Upon activation, naive B cells will differentiate to plasma cells, which secrete high levels of antibodies [4]. While most plasma cells are short-lived, there is a small fraction of these cells that are long-lived and continually secrete antibodies for extended periods of time, even after Ag clearance [4]. In addition, there is also the

development of long-lived memory B cells, which can rapidly proliferate and differentiate into antibody-secreting plasma cells upon subsequent exposure to the initial Ag [20-22]. Aside from their role as responder cells in the adaptive immune system, B cells also have the ability to interact directly with T cells as an APC. Upon recognition of the BCR with its cognate Ag, the B cell can internalize, degrade, and process the pathogen [4]. Small fragments of the Ag are then loaded intracellularly onto an MHC-II molecule to present to T cells [4]. A defining phenotypic characteristic of human B cells is their surface expression of CD20, which is commonly used as a marker for identification as well as a target for immune therapies [4].

T cells are responsible for generating cell-mediated immune responses. These specialized cells also possess an Ag recognition molecule, termed the T cell receptor (TCR) as well as the co-receptor CD3 [4]. Unlike their B cell counterparts, which can recognize non-processed extracellular Ags present within the environment, T cells are only able to detect Ags that have been processed and subsequently presented by APCs [4]. The TCR is restricted to binding short fragments of degraded proteins that are bound to specialized MHC molecules on the surface of cells. There are two major classes of T cells, each with distinct effector functions, which are distinguished by the expression of specific co-receptors on their cell surface as well as their MHC recognition patterns. MHC class I (MHC-I) molecules are present on virtually all nucleated cells, and present viral and bacterial Ags that are found in the cytosol of these cells to T cells expressing the co-receptor CD8. The function of $CD8^+$ T cells is to lyse infected cells by releasing the cytolytic perforin and apoptosis-inducing granzyme molecules, which is why they are also commonly referred to as cytotoxic T lymphocytes (CTL) [23]. Conversely, pathogens that are located in vesicular compartments of cells are loaded onto MHC-II molecules and can be recognized by $CD4^+$ T cells. This T cell subset is also known as helper T (T_H) cells due to their primary function of activating other immune cells. There

are currently three functional classes of $CD4^+$ T cells: T_H1, T_H2, and the recently identified T_H17 cells [24, 25].

The activation and subsequent differentiation of naïve $CD4^+$ and $CD8^+$ T cells into effector cells requires the engagement of their TCR with cognate Ags presented in the context of an MHC molecule, co-stimulation, and the presence of cytokines [4]. Naïve T cells have a high threshold of peptide-MHC signalling that is needed to activate these cells and therefore require a secondary signal to provide additional stimulation [4]. This signal, termed co-stimulation, is usually delivered upon the engagement of a CD28 molecule, which is expressed on the surface of T cells, with its ligands B7-1 (CD80) or B7-2 (CD86) on Ag presenting DCs [4]. Co-stimulation amplifies the TCR-MHC signal by 100-fold, thus reducing the amount of Ag required for activation [26, 27]. Once activated, naïve T cells undergo clonal expansion and give rise to both short-lived effector cells and long-lived memory cells, both of which have a much lower peptide-MHC threshold for subsequent activation relative to their naïve progenitors. While effector $CD8^+$ T cells elicit a CTL response, cytokines released from APCs or that are present within the environment determine the fate of $CD4^+$ helper T cells [27].

In the presence of IFN-γ and IL-12, $CD4^+$ T cells differentiate into T_H1 cells, which in turn enhance the pro-inflammatory response by secreting large amounts of additional IFN-γ and TNF-α. These cells have been shown to play a significant role in the removal of intracellular pathogens through the activation of macrophages [25, 28-31]. In contrast, $CD4^+$ T cells differentiate into T_H2 cells in the presence of the cytokine IL-4 and are responsible for eliminating extracellular pathogens as well as inducing B cells to elicit an antibody response. T_H2 cells are characteristically anti-inflammatory and secrete cytokines such as IL-4, IL-10, IL-13, and transforming growth factor (TGF)-β [24, 25, 28-31]. T_H2 cells can also activate immune responses such as those that occur in asthma

[32]. Finally, the T_H1/T_H2 paradigm has been recently expanded following the discovery of T_H17 cells, which are induced in the presence of TGF-β and IL-6 [24, 33, 34]. These effector cells secrete the inflammatory cytokine IL-17 and are thought to be involved in regulating anti-microbial defenses at mucosal interfaces, but have also been associated with many human inflammatory conditions [24, 33, 34].

Cells of the adaptive immune system are found in organized sites within the body called lymphoid organs, which can be broadly divided into primary and secondary subclasses [4]. The primary lymphoid organs consist of the bone marrow and the thymus. Both B and T cells originate in the bone marrow, but only B cells undergo maturation there. Conversely, T lymphocyte precursors migrate to the thymus to undergo their differentiation [4]. These lymphocytes then enter the bloodstream and populate the secondary lymphoid organs, which include the spleen and peripheral lymph nodes along with mucosal lymphoid tissues [4, 35]. It is predominantly at these sites that naïve lymphocytes will encounter Ag and elicit an immune response. In general, secondary lymphoid organs are similarly organized and include B and T cells, APCs, stromal cells, and a vascular supply [4, 35]. The lymph nodes are located at the point of convergence of lymphatic vessels and serve to filter extracellular fluid ("lymph") from tissues as well as trap the Ag-bearing DCs that migrate from sites of infection in the lymph [4, 35]. Conversely, the spleen samples Ags present in the blood. Both of these organs are highly compartmentalized and are designed to promote the crucial interactions required to activate the adaptive immune system [4, 35].

1.1.3 Regulatory cells of the immune system

Due to the vast diversity in Ag recognition, long lasting immunological memory, and potent effector activities associated with immune responses, serious damage to the

host may ensue if an aberrant immune response is generated. For these reasons, the immune system has evolved to produce specialized T cell subsets that are capable of controlling the quality, magnitude, and duration of adaptive immune responses. Current evidence suggests that multiple autoimmune diseases are caused by the initiation of uncontrolled immune responses due to defective regulatory T cells. Two types of regulatory T cells subsets in particular have been shown to play key roles in this regulation; the $CD4^+CD25^+$ forkhead box P3 $(Foxp3)^+$ regulatory T cells (T_{regs}) and the invariant natural killer T (iNKT) cells [36]. During strong immune responses, $CD4^+CD25^+Foxp3^+$ T_{regs} play a major role in suppressing the activity of activated cells to maintain homeostatic balance [37]. Conversely, iNKT cells have been shown to be potent immune modulators with the dual ability to enhance and/or dampen an ongoing immune response [38].

1.1.3.1 $CD4^+CD25^+Foxp3^+$ regulatory T cells (T_{regs})

Naturally occurring T_{regs} specifically express the transcription factor Foxp3, which is a master regulator of T_{reg} development and function [37]. These cells account for 10-15% of $CD4^+$ lymphocytes and have been shown to possess highly suppressive capabilities [37, 39-41]. Several mechanisms of T_{reg}-mediated suppression have been proposed, however the exact mechanism has yet to be elucidated [37, 42-45]. There has been evidence to suggest that T_{regs} may act by the secretion of immunosuppressive cytokines such as IL-10, TGF-β, and IL-35, through direct cell contact, as well as by functionally modifying or killing APCs [42, 46]. Importantly, T_{reg} deficiency has been demonstrated to be a cause of various autoimmune diseases, such as type 1 diabetes, in mice as well as in humans [47-51].

1.1.3.2 Invariant natural killer T (iNKT) cells

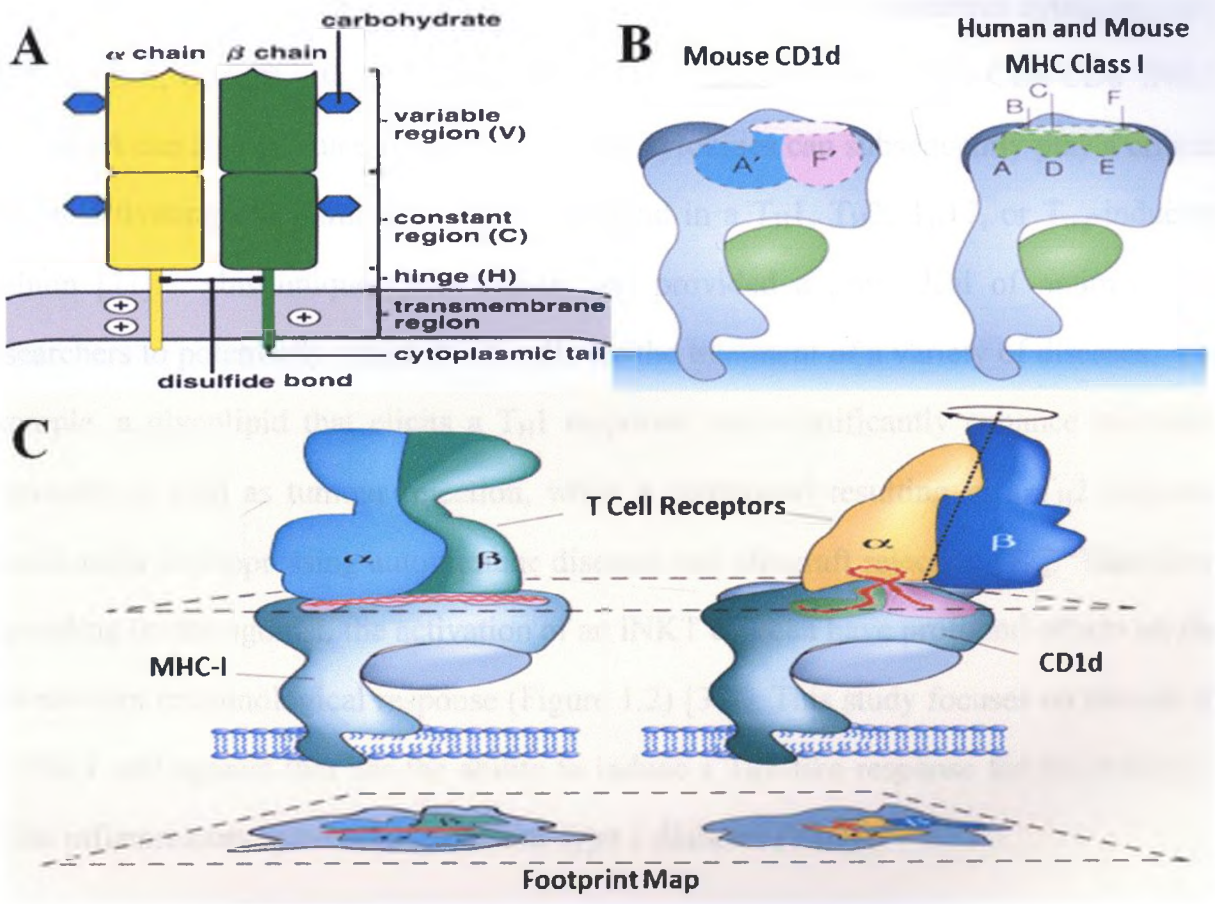
Natural killer T (NKT) cells are a distinct subset of T cells that have the ability to coordinate signals from both the innate and adaptive immune system. These cells have been shown to play an important role in defense against infections with bacteria, fungi, and parasites, in tumour surveillance, as well as in establishing peripheral tolerance [52-54]. NKT cells possess attributes of both T cells as well as NK cells. These cells possess a TCR as well as NK-lineage markers such as NK1.1, NKG2D, CD94, and members of the Ly49 family of NK-cell receptors. NKT cells from certain mouse strains such as BALB/c, CBA, and NOD, however, do not express NK1.1 and therefore are identified by other means. There are currently three separate classes of NKT cells. Type I NKT cells, also called invariant NKT (iNKT) cells, are restricted to recognizing glycolipid Ags presented in the context of the MHC-I-like molecule cluster of differentiation 1 (CD1) d. Murine iNKT cells express a semi-invariant TCR that consists of a Vα14-Jα18 chain paired with either a Vβ8.2, Vβ7, or Vβ2 chain (Vα24-Jα18 and Vβ11 in humans). Type II or "nonclassical" NKT cells are also CD1d restricted, but have a more diverse TCR repertoire. Finally, there is also an NKT-like subclass that consists of multiple different cell types that include CD1d-independent, NK1.1-expressing T cells and multiple others subsets that express semi-invariant TCRs. Since these cells are not dependent on CD1d, it has been recommended that the latter subclass not be called NKT cells to avoid confusion [38, 55]. The most widely studied of these subclasses, and the focus of this thesis, are iNKT cells.

The unique semi-invariant TCR on iNKT cells allows for the recognition of glycolipid Ags that are presented in the context of a CD1 molecule. While there are five isoforms of CD1 molecules in humans (a, b, c, d, and e), mice express only CD1d. Mouse and human CD1d are highly homologous [56-59]. In comparison to the MHC

molecules that present peptide fragments, CD1d is specifically designed to present glycolipids due to its two highly hydrophobic pockets that are present within its binding groove [60]. These pockets, termed the A' and F' channels, are able to accommodate the hydrophobic carbon chains of the lipid moieties, while the polar sugar head of the glycolipid protrudes above the binding groove. This sugar moiety can then be recognized by the iNKT cell TCR [60]. Interestingly, the crystal structure of the TCR-glycolipid-CD1d complex has revealed that relative to the typical TCR-peptide-MHC-I complex, the iNKT TCR is rotated clockwise from the classical position. In this orientation, only the α-chain makes contact with the Ag and also provides the majority of the stabilizing interactions with the CD1d molecule, further demonstrating the importance of the invariant TCR α-chain on iNKT cells (Figure 1.1) [61]. Most murine iNKT cells also express the co-receptor CD4, while the remaining iNKT cells are negative for both CD4 and CD8 [53, 62-64]. Conversely, humans also possess a $CD8^+$ iNKT cell subset [53, 62-64]. In general, iNKT cells are found at the highest frequency in the liver (~10-40%) in mice, however they are also present in the spleen (~1%), thymus (~0.5%), lymph nodes (~0.5%) and peripheral blood (<0.5%) [53, 62-64].

One of the most intriguing attributes of iNKT cells is their ability to potentiate an immune response. These cells have the ability to cross talk with a variety of other cell types, including DCs, macrophages, neutrophils, NK cells, B and T cells, and T_{regs} [54, 65, 66]. Their most defining characteristic, however, is their capacity to rapidly secrete copious amounts of cytokines upon TCR ligation. iNKT cells constitutively express messenger ribonucleic acid (mRNA) encoding IFN-γ and IL-4, which allows these cells to rapidly initiate their effector functions [65]. Interestingly, unlike conventional T cells, iNKT cells are able to synthesize cytokines in response to TCR ligation alone, without costimulation. However, in order to achieve optimal secretion of these cytokines, costimulation is required [67, 68].

Figure 1.1 Recognition of CD1d by an iNKT cell receptor. **(A)** TCRs are composed of α- and β-chains, which are bound to the cell membrane through their transmembrane regions and cytoplasmic tail. Each chain contains a constant region that is highly conserved and a variable region that determines its specificity. iNKT cells in particular possess a semi-invariant TCR consisting of a Vα14-Jα18 chain paired with either a Vβ8.2, Vβ7, or Vβ2 chain (Vα24-Jα18 and Vβ11 in humans). **(B)** The iNKT cell is restricted to recognizing glycolipid Ags that are presented in the context of CD1d, an MHC-I-like molecule. To present glycolipids, the CD1d molecule contains two hydrophobic pockets, the A' and F' channels, into which fit the hydrocarbon chains of the lipid portion of the Ag. This orientation allows for the sugar moiety of the glycolipid to protrude above the binding cleft to be recognized by the TCR (C, right panel). **(C)** In comparison to a TCR-Ag-MHC complex, where both the α- and β-chains make contact with the Ag (red) (left panel), the CD1d-reactive TCR is rotated clockwise from the classical position and pushed laterally so that only its α-chain makes contact with the Ag as well as provide the majority of stabilizing contacts with the CD1d molecule (right panel). A footprint map, or cross-sectional view, of the binding interface between the TCR-Ag-MHC and TCR-glycolipid-CD1d complexes can be seen below. Adapted from Janeway et al. 2005, Moody et al. 2007, and Moody et al. 2005 [4, 69, 70].


A
α chain
β chain
carbohydrate
variable region (V)
constant region (C)
hinge (H)
transmembrane region
cytoplasmic tail
disulfide bond
B
Mouse CD1d
A′
F′
Human and Mouse MHC Class I
C
B
F
A
D
E
C
T Cell Receptors
α
β
MHC-I
CD1d
Footprint Map

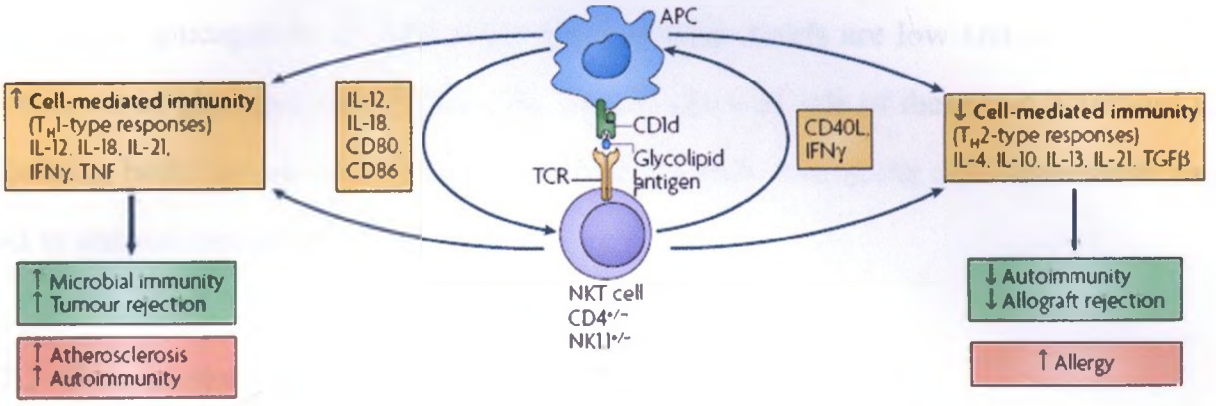
Upon activation, iNKT cells have been shown to secrete numerous cytokines such as IL-2, IL-4, IL-6, IL-10, IL-13, TGF-β, IFN-γ, and TNF-α, while the $CD4^-CD8^-$ iNKT cell subset can also produce IL-17 [71]. These cytokines can subsequently play a critical role in activating other immune cells to respond in a T_H1, T_H2, T_H17, or T_{reg}-inducing fashion [71]. This unique characteristic has provided a great deal of optimism for researchers to potentially target iNKT cells for the treatment of a variety of diseases. For example, a glycolipid that elicits a T_H1 response may significantly enhance microbial immunity as well as tumour rejection, while a compound resulting in a T_H2 response would assist in suppressing autoimmune diseases and allograft rejection [72]. Therefore, depending on the agonist, the activation of an iNKT cell can have profound effects on the downstream immunological response (Figure 1.2) [38]. This study focuses on the use of an iNKT cell agonist that has the ability to induce a T_H2-like response for the treatment of the inflammatory autoimmune disease, type 1 diabetes (T1D).

1.2 The pancreas and T1D

1.2.1 The pancreas

The pancreas is a lobular organ that performs both exocrine and endocrine functions. The exocrine tissue, which composes the vast majority of the pancreas by mass, produces bicarbonate ions and enzymes that aid in digestion within the small intestine [4]. Scattered throughout the exocrine tissue are clusters of endocrine cells called the islets of Langerhans. Each islet consists of a central core of insulin-producing beta (β) cells, composing approximately 75% of the islet, which are surrounded by glucagon-producing alpha (α) cells, delta (δ) cells and pancreatic polypeptide-producing (PP) cells [4]. Together, insulin and glucagon are two antagonizing hormones that are critical in regulating the concentration of blood glucose. In general, when glucose levels

Figure 1.2 iNKT cells mediate a wide range of immune responses. The activation of iNKT cells by a glycolipid Ag presented in the context of a CD1d molecule induces a diverse and robust cytokine response. The resulting immune response can elicit a broad range of downstream effects including enhanced cell-mediated immunity or the induction of tolerance. Depending on the disease of interest, treatment with an iNKT cell agonist that elicits a predominantly T_H1 or T_H2 response can have considerable therapeutic potential. Adapted from Godfrey and Berzins 2007 [72], and Cerundolo et al. 2009 [71].


APC
↑ Cell-mediated immunity
(T_H1-type responses)
IL-12, IL-18, IL-21,
IFNγ, TNF
IL-12,
IL-18,
CD80,
CD86
CD1d
Glycolipid
antigen
TCR
CD40L,
IFNγ
↓ Cell-mediated immunity
(T_H2-type responses)
IL-4, IL-10, IL-13, IL-21, TGFβ
↑ Microbial immunity
↑ Tumour rejection
↑ Atherosclerosis
↑ Autoimmunity
NKT cell
CD4+/−
NK1.1+/−
↓ Autoimmunity
↓ Allograft rejection
↑ Allergy

are high, insulin is released and acts to lower glucose concentration by recruiting cells to take up glucose from the blood as well as inhibit glycogen breakdown in the liver [4]. Conversely, glucagon is secreted when blood glucose levels are low and stimulates the breakdown of glycogen into glucose [4]. Due to the vital role of these two hormones in regulating both fuel use and storage by cells of the body, disrupting this homeostasis can lead to serious consequences.

1.2.2 General characteristics of T1D

T1D is an inflammatory autoimmune disease that is characterized by the destruction of insulin-producing β cells in the islets of the pancreas, resulting in uncontrolled blood glucose levels (BGL) [73]. Conversely, type 2 diabetes (T2D) is characterized by an inability to respond to insulin that is being produced [73]. Diabetes is one of the most prevalent endocrine disorders with approximately 250 million adults having one form of the disease [73]. Approximately 5-15% of diabetic patients have T1D [73].

T1D patients normally control their BGL through daily insulin administration; however, if left untreated, T1D may be deadly [74]. Due to the inability to properly uptake and metabolize glucose, starving cells will instead turn to fatty acid degradation for energy [74]. A by-product of this metabolism is the generation of acidic ketone bodies that accumulate in T1D patients, resulting in decreased blood pH and subsequent development of potentially fatal ketoacidosis [74]. In addition, it is also common for patients to develop other complications such as vascular diseases, myocardial infarction, stroke, renal disease and blindness [74].

1.2.3 Susceptibility to T1D

Both genetic and environmental factors contribute to the susceptibility to T1D. To date, there have been over 20 regions of the genome that are associated with T1D and are categorized as insulin-dependent diabetes mellitus (IDDM) susceptibility loci [75-77]. In both humans and animal models of the disease, the major susceptibility loci are located within the human leukocyte antigen (HLA) (MHC) region of the genome and termed IDDM1. In particular, the presence of HLA-DR3/DR4 and HLA-DQ allomorphs provides the greatest inheritable risk for the development of T1D [75-78]. The promoter region of the insulin gene (IDDM2) has also been identified as a key susceptibility region [79, 80]. The exact mechanism(s) responsible for the increased risk of disease observed with these polymorphisms, however, are still unknown [81, 82]. It is possible that the HLA polymorphisms associated with increased susceptibility to T1D may result in a decreased affinity for certain self-Ags that are found within the islets, resulting in decreased presentation of these Ags in the thymus. This, in turn, may result in a greater number of autoreactive T cells that escape negative selection. A similar scenario may also be responsible for the insulin gene polymorphisms, where decreased insulin synthesis may also reduce the thymic presentation and subsequent tolerance induction in developing T cells [81, 82].

While genetic factors certainly increase the risk of developing T1D, environmental factors are required to initiate the onset of disease [81-83]. This became evident after a study investigating identical monozygotic twins observed that when one of the siblings had T1D, there was only a 50% concordance rate of the other individual developing the disease, indicating that T1D is not solely due to genetic predisposition [83]. While the environmental trigger(s) has yet to be elucidated, there seems to be geographical as well as seasonal variances in the development of T1D. Therefore,

childhood diet [84, 85] and viral infections [86-88] are thought to potentially play a significant role in initiating the disease.

1.2.4 Pathogenesis of T1D

T1D is primarily a T cell mediated disease that can be divided into two distinct stages [89, 90]. The first stage of the disease, insulitis, is defined by the infiltration of the islets of Langerhans by cells of the immune system [89, 90]. This is followed by an inflammatory autoimmune process, which results in β cell death [89, 90]. Overt T1D occurs when the majority of β cells have been destroyed, resulting in the production of inadequate amounts of insulin and a consequent inability to control glucose levels, resulting in hyperglycemia [89, 90]. This usually occurs when approximately 80% of the β cell mass has been depleted [89, 90]. The time frame between islet infiltration and overt diabetes can vary significantly between individuals and range anywhere from a few months to more than 10 years, with some individuals never progressing past insulitis [89, 90].

The onset of the autoimmune reaction leading to T1D begins with an initial insult to the β cells in the pancreas, resulting in Ag shedding [87, 88, 91]. This may be due to normal β cell turnover in the pancreas or potentially β cell death resulting from an inflammatory response towards a viral infection [87-91]. These self-Ags are in turn engulfed by DCs, which migrate to the draining pancreatic lymph nodes (PLNs) and present these Ags to naïve $CD4^+$ T cells [87-91]. To date, numerous β cell Ags have been shown to be targets of $CD4^+$ T cells in a diabetic patient such as such as insulin, GAD (glutamic acid decarboxylase), IA-2 (insulinoma-associated antigen-2), IGRP (Islet-specific glucose-6-phosphate catalytic subunit related peptide) [92, 93] and ZnT8 (Zinc transporter 8) [94-97]. Due to immune dysregulation [98], there is an increase in the

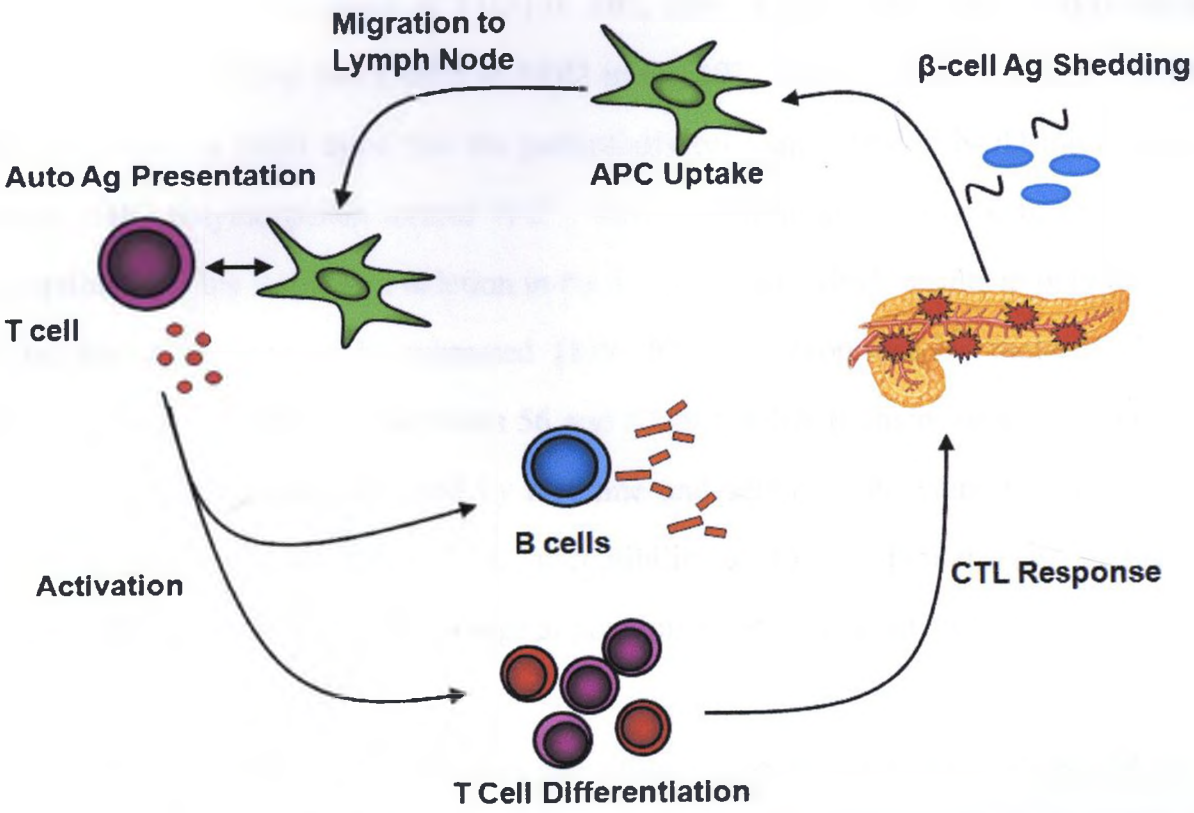
frequency of autoreactive T cells in the PLN, which interact with the presenting DC and become activated. The subsequent activation of these T cells allows them to migrate through tissues and eventually home to the islets where they re-encounter their cognate Ag and begin an inflammatory immune response [87-91]. In addition, the initial activation of these cells also results in cytokine secretion and the transactivation of both B cells and $CD8^+$ T cells to elicit an auto-antibody and CTL response, respectively, leading to further inflammation and β cell death (Figure 1.3). As the β cells are continually destroyed, more Ags are shed and the cycle continues until overt diabetes results [90]. It is important to note that while auto-antibodies directed against insulin have been detected and are currently used as an early diagnostic tool to identify individuals at risk of developing diabetes, there has not been any evidence that these antibodies play a pathological role in the disease [99].

1.2.5 Non-obese diabetic (NOD) mouse model of T1D

The non-obese diabetic (NOD) mouse was initially derived by Makino *et al.* in the late 1970s and is the most widely used animal model for the study of T1D [100]. These mice are valuable research tools due to their propensity to spontaneously develop T1D in a very similar fashion to the human disease. In particular, these mice experience similar stages of disease such as insulitis and hyperglycemia, as well as parallel pathogenic mechanisms such as defective peripheral immune regulation and the presence of auto-Ags and auto-antibodies [101].

The onset of T1D in NOD mice relies on both environmental and genetic factors in a similar fashion to the human disease. The incidence of the disease is greater in mice that are housed in specific-pathogen free environments than those residing in conventional accommodations [102]. Viral infections, diet, and stress have also been

Figure 1.3 Pathogenesis of T1D. Natural β cell turnover or an environmental trigger leads to β cell apoptosis and subsequent auto-Ag shedding. These Ags are taken up by an APC, which migrates to the draining pancreatic lymph node (PLN), where it presents this Ag to autoreactive T cells. Upon activation, these cells release cytokines and assist in the induction of auto-antibody formation and a strong CTL response, which leads to β cell death and further Ag shedding. This cycle continues until overt T1D results.


Migration to
Lymph Node
β-cell Ag Shedding
Auto Ag Presentation
APC Uptake
T cell
B cells
Activation
CTL Response
T Cell Differentiation

shown to modify the incidence of T1D [91, 103, 104]. Genetically, MHC is also one of the major susceptibility loci (*Idd1*) in NOD mice [102]. There are two features of the MHC-II region in NOD mice that are particularly relevant. Firstly, NOD mice have a unique MHC polymorphism termed H-2^{g7}, which is the highest contributor to diabetes susceptibility. This is due to a deletion in the Eα promoter, which results in only the I-A, but not the I-E molecule to be expressed [105-107]. The expressed I-A molecule, I-A^{g7}, also possesses a mutation at positions 56 and 57 of the I-A β chain, where the proline-aspartic acid residues are replaced by histidine and serine. Interestingly, some of the HLA-DQ polymorphisms that induce susceptibility to T1D in humans also contain a mutation that removes a negative charge at position 57 of the β chain [105-107].

Although NOD mouse colonies can differ in the age of onset and incidence of T1D due to environmental factors, leukocyte infiltration and subsequent insulitis is usually detected at 4-6 weeks of age in both males and females [102, 104]. This is followed by β cell death and the spontaneous development of overt T1D can normally be detected by 12-30 weeks of age [102, 104]. Interestingly, in contrast to the humans, NOD mice exhibit a large gender difference in the development of T1D with a much greater frequency of females (70-90%) developing the disease compared to males (10-30%) [102, 104, 106]. It has been demonstrated that female NOD mice exhibit a greater age-dependent reduction in regulatory T cell subsets compared to males [98, 102, 104, 106]. Furthermore, the female sex hormone, estrogen, has been shown to greatly enhances inflammatory T_H1 responses in NOD mice compared to testosterone, which may account for the observed gender discrepancy [108]. For these reasons, in many studies, including this thesis, only female NOD mice are used in experiments.

1.2.6 Treatments for T1D

The most widely used treatment strategy for T1D is to administer insulin subcutaneously to mimic β cell function [73]. While insulin treatment controls blood glucose levels in the patient and increases their life expectancy, if used properly, it is not a cure for T1D [73]. These patients still exhibit chronic autoimmune complications and must continually administer insulin in order to properly regulate BGL [73]. There is a dire need to develop improved treatment methods that may eventually provide long lasting protection from T1D.

Due in large part to the availability of the NOD mouse, a number of therapies that have been successful in this model are now being tested in clinical trials for the use in humans. Therapeutic strategies that are currently being investigated focus on the physical replenishment of β cells and the prevention of the autoimmune response. Transplantation of healthy islets, first described in the Edmonton Protocol, has only shown moderate success in alleviating insulin dependence in humans [109-111]. The main obstacle in this therapy is the relatively rapid destruction of these islets due to the host-versus-graft immune response that ensues after successful transplantation. Graft tolerance only occurs after intense immune suppression that can have serious side effects for the host. In addition, even if graft tolerance is achieved, this therapy would still fail to inhibit the autoimmune recognition of β cell Ags occurring in these patients [110, 111]. Therefore, there are major obstacles hindering the use of β cell replacement therapy, which would most likely have to be employed in combination with different treatments aimed at inhibiting the autoimmune anti-β cell response.

Treatment with monoclonal antibodies (mAbs) directed against T and B cells have thus far been the most well-studied immunomodulatory therapy to date. Administration

of an anti-CD3 mAb results in the inhibition of both $CD4^+$ and $CD8^+$ T cells by preventing Ag recognition in naïve T cells and inducing hyporesponsiveness or death in activated cells [112, 113]. A short, five-day treatment at the time of T1D onset with an anti-CD3 mAb was able to reverse the disease, induce long-term remission, and prevent recurrent immune responses in NOD mice [114, 115]. This promising result prompted the use of a humanized form of this mAb in clinical trials. While patients did exhibit some significant improvements, a large number of patients showed a decline in insulin production after 2 years [116-118]. Anti-CD20 mAbs, which specifically target the total B cell population, have also shown some promising results in NOD mice [119] and have proven safe and efficacious in a recent clinical trial [120, 121]. The main disadvantage of mAb therapies directed against whole cell populations, however, is the risk of total immune suppression and a heightened susceptibility to infections. Clearly, there is a need for more specialized therapy for the treatment of T1D that would ideally elicit protection against the disease without the induction of wide-scale immune suppression.

1.3 Regulatory T cells and T1D

Numerous studies suggest that functional and numerical deficiencies in naturally occurring T_{regs} exist in the NOD mouse, which may play a role in the pathogenesis of T1D [47, 49-51]. Other studies further concluded that these deficiencies are due to an age dependent decline in regulatory cell function over time, which would explain the rather consistent age of disease onset in NOD mice [47, 51, 122, 123]. In addition, various transgenic NOD mouse models such as the TCRα-deficient, Rag1-deficient, or Foxp3-deficient mice, which are all devoid of T_{regs}, display no delay between the onset of insulitis and the development of overt T1D [39]. Furthermore, adoptive transfer of T_{regs} into NOD/Scid mice, which lack T and B lymphocytes due to defective BCR and TCR gene rearrangement, resulted in protection from T1D [49].

Studies in humans have thus far yielded conflicting results. While a number of studies have confirmed functional and numerical deficiencies in humans [48, 124, 125], others have observed no changes in this subset at all [126]. These discrepancies may have occurred for a variety of reasons. Firstly, there is a large variation in the frequency of naturally occurring T_{regs} in the human population. In addition, these studies only used peripheral blood samples from patients, which may not adequately represent what is occurring within the total cell population as functional deficiencies may occur in a site-specific fashion. Therefore, it is possible that on a person-to-person basis, humans exhibit a similar T_{reg} deficiency pattern as mice while diabetes progresses. Other studies [40, 127] have shown that T_{reg} numbers are not diminished in human patients with T1D. Rather, effector T (T_{eff}) cells in such patients may be resistant to the activity of T_{regs} [40, 127]. Current studies are focussing on expanding these suppressive cells for the treatment of various autoimmune diseases, including T1D [41].

Deficiencies in iNKT cells can also have devastating effects in the host and can lead to immune dysregulation and the induction of autoimmune diseases, including T1D [128-130]. Numerous studies have concluded that female NOD mice exhibit numerical and functional defects in NKT cells [98, 128-131]. Although this deficiency has also been reported in human diabetic patients [48, 132], this finding has remained controversial [133]. The two most important genetic loci that control NKT cell numbers in NOD mice are *Nkt1* on chromosome 1and *Nkt2* on chromosome 2, which appears to contribute to diabetes susceptibility [134]. Furthermore, a recently identified third locus has been identified on chromosome 18 (linked to *D19mit149*), which can confer an absolute deficiency in NKT cells [134, 135]. In addition, iNKT cells in NOD mice produce less IL-4, an anti-inflammatory cytokine, after stimulation compared to iNKT cells from non-autoimmune strains such as C57BL/6 or BALB/c [129, 136-138]. Furthermore, NOD CD1d$^{-/-}$ mice, which are completely devoid of iNKT cells, display an

increased incidence of T1D [139]. Conversely, over-expression of iNKT cells using a NOD.Vα14 (Jα18) transgenic mouse model has been shown to protect against T1D [140]. Collectively, this evidence suggests that a deficiency of iNKT cells may be a contributing factor in the development of T1D in NOD mice. Many studies now focus on the activation of iNKT cells to take advantage of their potent cytokine and regulatory responses.

1.4 Glycolipid agonists for the treatment of T1D

1.4.1 Naturally occurring glycolipids

It is widely believed that iNKT cells undergo positive selection in the thymus in a similar fashion to conventional T cells [38]. However, the naturally occurring, endogenous iNKT cell Ag(s) that signals the differentiation of this subset remains elusive. Initial studies suggested that isoglobotrihexosylceramide (iGb3), a degradation product of the enzyme β-hexosaminidase B, may be responsible for this selection due to its ability to stimulate both mouse and human iNKT cells *in vitro* when presented in the context of a CD1d molecule [141-143]. However, recent studies using high-performance liquid chromatography (HPLC), a very sensitive technique used to identify and quantify compounds, failed to detect iGb3 in the thymus of both mice and humans [144]. Similarly, a transgenic approach has also been conducted to test whether iGb3 was physiologically relevant by creating mice that are deficient in iGb3 synthase, and therefore iGb3 [145]. It was observed that these mice possessed normal iNKT cell frequency, development, and function [145]. Identifying the naturally occurring self lipid responsible for iNKT cell differentiation will greatly impact our understanding of iNKT cell development as well as potentially lead to a new wave of therapeutic approaches aimed at altering iNKT cell frequency and function.

While endogenous glycolipid agonists remain ambiguous, several exogenous ligands have been identified that can activate iNKT cells. Consistent with their role as innate-like regulatory cells, iNKT cells are able to respond to a wide range of bacterial Ags in both an indirect and direct fashion. For example, bacteria such as *Salmonella* have the capacity to activate DCs through their TLRs, which in turn can activate iNKT cells through an indirect mechanism [146]. It has been hypothesized that engagement of TLRs on DCs induces the production of cytokines such as IL-12, IL-18, and type I interferons as well as increases the presentation of endogenous Ags such as iGb3 [38, 143, 146, 147]. While iNKT cells can respond weakly to iGb3 as mentioned above, the additional inflammatory cytokines may act to amplify the low affinity interactions between the iNKT cell TCR and the endogenous ligand, thus leading to iNKT cell activation and subsequent secretion of IFN-γ [38, 143, 146, 147]. Conversely, iNKT cells may also be directly activated by microbial glycolipids. In particular, glycosylceramides from the cell wall component of the Gram-negative bacteria *Novosphingobium* and *Borrelia burgdorferi* have both been found to induce iNKT cell activation [143, 148-150]. These results demonstrate the broad scope of potential iNKT cell agonists as well as provide a foundation for the investigation of synthetic glycolipids that share similar properties to known endogenous and exogenous Ags for therapeutic purposes.

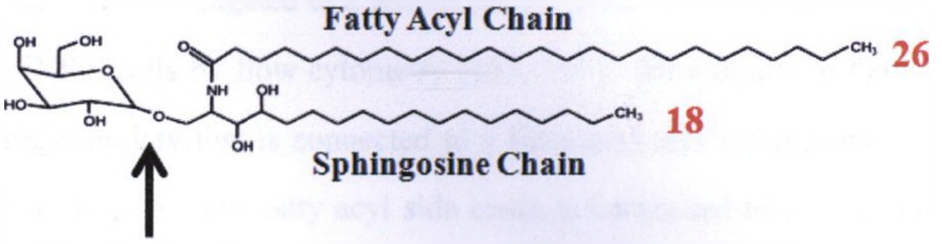
1.4.2 Immunomodulatory effects of α-galactosylceramide C26:0 (α-GalCer)

1.4.2.1 General characteristics of α-GalCer

The glycosphingolipid α-galactosylceramide C26:0 (α-GalCer) was originally discovered by the Kirin Brewery Company in the marine sponge *Agelas mauritianus* and has since become to most widely studied iNKT cell agonist. All iNKT cells from mice, humans, macaques, and rats react with α-GalCer when presented in the context of CD1d in a highly conserved manner [151, 152]. In fact, due to this characteristic, the use of α-

Figure 1.4 Chemical structure of α-GalCer. The compound α-GalCer is a glycolipid agonist that potently activates iNKT cells. It consists of a galactose sugar head that is connected to two hydrophobic fatty acid side chains, termed the fatty acyl and sphingosine chains, by an α-anomeric linkage. The fatty acyl side chain is composed of 26 carbons in length (red), while the sphingosine chain consists of 18 carbons. Adapted from Wu et al. 2009 [38]

α-GalCer

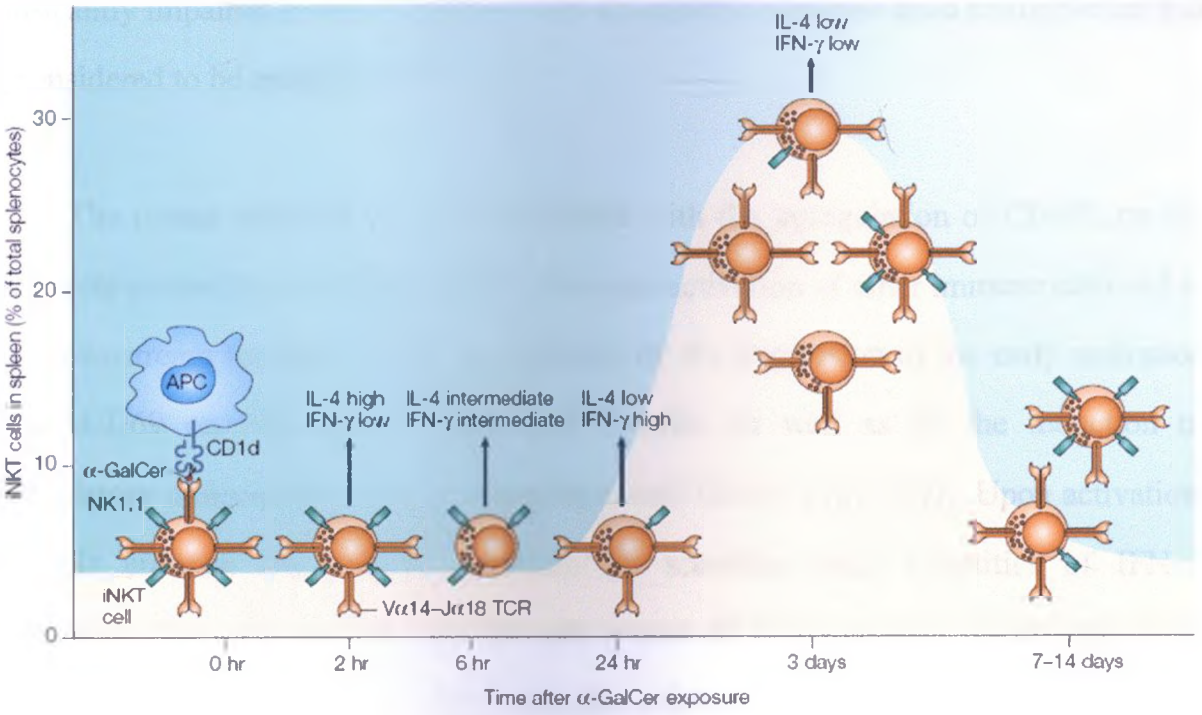

Fatty Acyl Chain
26
18
Sphingosine Chain
α-Anomeric Linkage

GalCer-loaded CD1d tetramers conjugated to a fluorochrome is the most commonly used method of identifying iNKT cells by flow cytometry [153, 154]. Structurally, α-GalCer contains a galactose sugar moiety that is connected to a fatty acyl and sphingosine side chains by an α-anomeric linkage. The fatty acyl side chain is composed of a 26 carbon chain, while the sphingosine chain contains 18 carbons (Figure 1.4). Due to the α-anomeric linkage in α-GalCer, mammalian cells are unable to synthesize this compound; however, it is still a very attractive therapeutic tool due to its potent activation of iNKT cells and subsequent immune responses.

1.4.2.2 iNKT cell responses to α-GalCer

Within 2 hours (h) after injection of α-GalCer into mice, iNKT cells become activated, secrete large amounts of cytokines and upregulate co-receptors such as CD40L [66]. Copious amounts of both T_H1 (IFN-γ, TNF-α) and T_H2 (IL-4, IL-10, IL-13) cytokines as well as IL-2 have been detected following α-GalCer administration [66]. Although multiple cytokines are produced in a short period of time, the immediate response (2-6 h) has been shown to be dominated by IL-4, followed by a shift towards IFN-γ secretion that peaks between 6-24 h after exposure [66]. Interestingly, within 2 h after activation, iNKT cells begin to internalize their TCR for approximately 24 h, therefore rendering these cells undetectable by flow cytometry [66]. As a consequence, it is common to refer to the apparent "decrease" in the iNKT cell frequency during this time as an indication of iNKT cell activation when performing flow cytometry. After 24 h, TCR expression returns to its steady state and the iNKT cells begin to rapidly divide [66]. This leads to a significant clonal expansion in both the spleen and PLNs that peaks 72 h after injection [66]. A contraction phase then ensues, which returns the iNKT cell frequency back to homeostatic levels through apoptosis by one week (Figure 1.5) [66, 155, 156]. Interestingly, after this activation cycle, the surviving iNKT cells are

Figure 1.5 The *in vivo* response of iNKT cells to stimulation with α-GalCer. Within 2 h after administration of α-GalCer, large amounts of IL-4 can be detected in the serum of mice. Between 6-24 h, iNKT cells downregulate their TCR and can no longer be detected by flow cytometry. Also, during this time, there is a shift in the cytokine secretion profile of the iNKT cell to a predominant IFN-γ response. TCR expression levels return to its steady state on iNKT cells after approximately 24 h. This is followed by a rapid expansion phase that peaks at 3 days before returning to homeostatic levels by approximately 7 days. Adapted from Van Kaer, 2005 [66]


iNKT cells in spleen (% of total splenocytes)
30
20
10
0
APC
CD1d
α-GalCer
NK1.1
iNKT cell
IL-4 high
IFN-γ low
IL-4 intermediate
IFN-γ intermediate
IL-4 low
IFN-γ high
IL-4 low
IFN-γ low
Vα14–Jα18 TCR
0 hr
2 hr
6 hr
24 hr
3 days
7–14 days
Time after α-GalCer exposure

significantly impaired in their ability to elicit an immune response upon restimulation and are considered to be anergic [157].

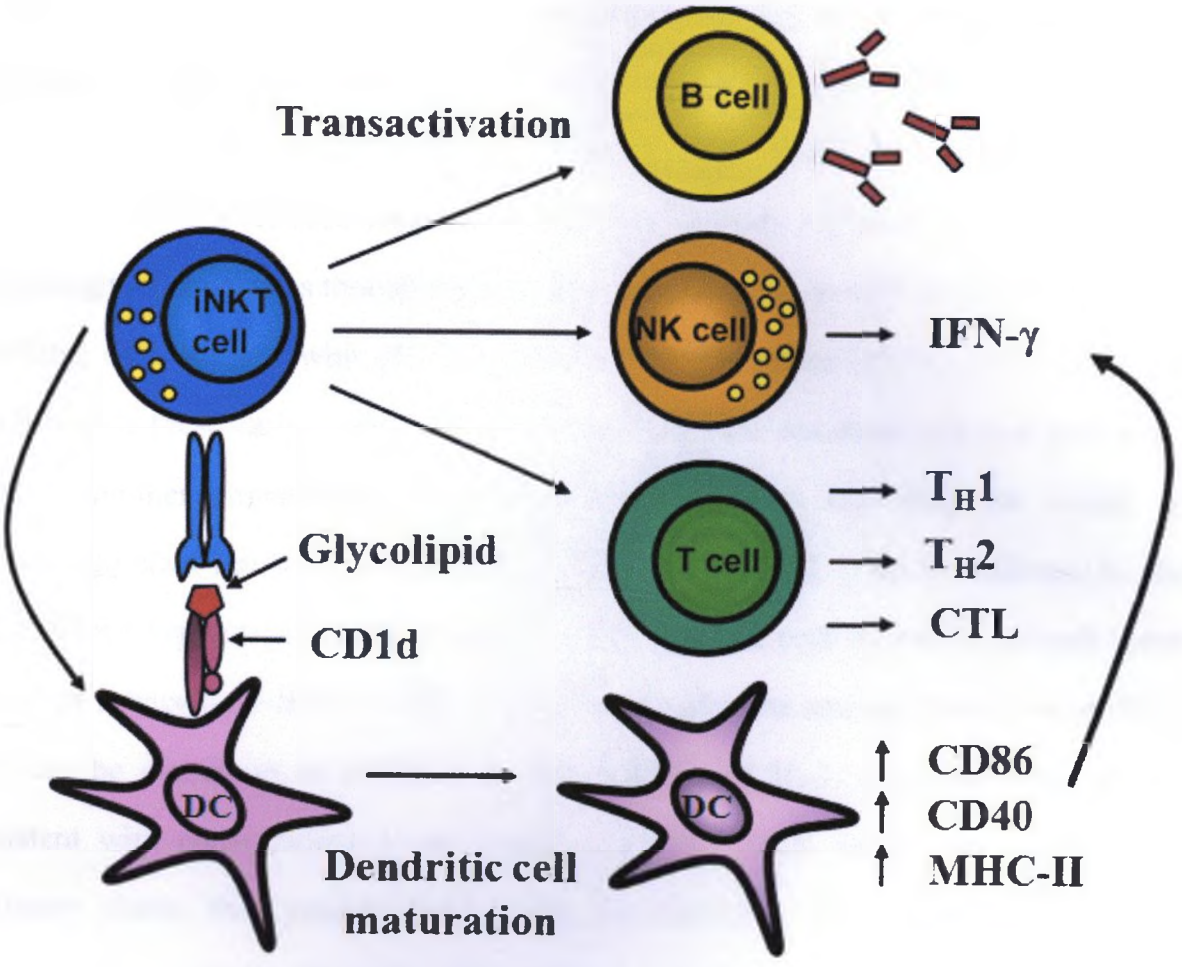
The potent cytokine response combined with the upregulation of CD40L on the iNKT cells elicited by α-GalCer results in the transactivation of other immune cells and a large downstream response. This is evidenced by the expression of the early activation marker CD69 on NK cells, B cells and T cells, as well as by the induction of costimulatory molecules on DCs, macrophages and B cells [157-159]. Upon activation, NK cells enhance the immune response by secreting large quantities of IFN-γ. Interestingly, NK cells are the predominant source of IFN-γ *in vivo* in response to α-GalCer [158, 160]. In addition, iNKT cells also enhance the immune responses mediated by B cells, which have been shown to secrete antibodies, and by T cells by eliciting a T_H1, T_H2 or CTL response (Figure 1.6).

1.4.2.3 α-GalCer induces long-term iNKT cell anergy

In contrast to normal immunological memory generated by T and B cells, iNKT cells exhibit a significantly blunted recall response following the initial administration of α-GalCer, various bacterial pathogens, or sulfatide, a ligand for type II NKT cells [157, 161-165]. Therefore, it appears as though the development of this hyporesponsive state, termed anergy, might be a common outcome of iNKT cell activation. It has been hypothesized that the induction of iNKT cell anergy may be an intrinsic regulatory mechanism that is designed to avoid chronic cytokine production that can lead to an uncontrolled inflammatory response and subsequent damage to the host [38].

After the initial iNKT cell response to a single dose of α-GalCer, these cells remain anergic for up to six weeks [157]. In this hyporesponsive state, iNKT cells

Figure 1.6. Activation of iNKT cells with α-GalCer. Upon activation of iNKT cells with α-GalCer, the subsequent release of cytokines has the ability to transactivate various lymphocyte subsets, further enhancing the immune response. Adapted from Wu et al. 2009 [38]


Transactivation
B cell
iNKT cell
NK cell
IFN-γ
Glycolipid
T cell
T_H1
T_H2
CTL
CD1d
DC
DC
CD86
CD40
MHC-II
Dendritic cell maturation

continue to produce low levels of IL-4 in culture, but fail to proliferate and produce significantly lower levels of IFN-γ and IL-2 upon restimulation [157]. Similar results were observed using intracellular cytokine staining [157]. Therefore, multiple doses of α-GalCer results in a reduced response with a bias towards secretion of T_H2 cytokines. Interestingly, it appears as though the reduced response is due to defects in proximal TCR signalling as treatment with phorbol 12-myristate 13-acetate (PMA) and ionomycin, which bypass these early steps, was able to overcome the observed cytokine deficiency [157]. Another characteristic of anergic iNKT cells is that they are unable to transactivate other immune cells following restimulation [157]. This is evidenced by the lack of CD69 expression on DCs, B cells, T cells, and NK cells as well as a much lower amount of intracellular IFN-γ in NK cells [157]. Finally, the anergic phenotype of iNKT cells can be overcome in culture with the addition of IL-2, a characteristic that is consistent with conventional T cell anergy [157]. Thus, anergy may represent a regulatory event that causes iNKT cells to transform from cells that promote inflammatory responses to cells that suppress inflammation in a ligand-dependent manner.

Although the exact mechanisms of the induction of iNKT cell anergy are not yet fully understood, recent studies have identified an interaction that is essential for the initiation of anergy. Upon activation with α-GalCer, iNKT cells upregulate the inhibitory co-receptor programmed death-1 (PD-1), a member of the CD28 family of co-stimulatory molecules [166, 167]. Interaction of PD-1 with its ligands, programmed death ligand 1 (PD-L1) or PD-L2, on APCs delivers an inhibitory signal, which has been implicated in the induction and maintenance of iNKT cell anergy [167-169]. Inhibiting this interaction using blocking antibodies prevented the induction of iNKT cell anergy in response to α-GalCer, but not to sulfatide or bacterial agonists, indicating the possibility of multiple mechanisms to induce anergy [166, 167]. Furthermore, PD-1 knockout mice have also

been shown to be resistant to α-GalCer-induced iNKT cell anergy. These mice, however, were highly susceptible to developing autoimmunity, which is consistent with the role of PD-1 in regulating conventional T cell tolerance [167].

1.4.2.4 α-GalCer-mediated protection against T1D

Due to its ability to elicit anti-inflammatory responses from iNKT cells upon repeated administration, α-GalCer has been tested in multiple models of disease. Previous studies, including those from our lab, have shown that α-GalCer treatment can prevent NOD mice from developing T1D, even after the onset of insulitis [129, 130, 137]. Interestingly, protection from T1D can only be achieved through multiple doses of α-GalCer, as one or two injections did not delay disease onset [129, 130, 137]. Our lab has optimized this protective procedure, termed multi-low dose protocol, to include intraperitoneal (i.p.) injections of 4 μg of α-GalCer every other day for 3 weeks [129].

Multiple doses of α-GalCer have been shown to result in the accumulation of iNKT cells in the PLN as well as a T_H2-like environment that is characterized by elevated IL-4 and IL-10 production and reduced IFN-γ secretion by iNKT cells [129]. Previous work in our lab has further identified IL-4, but not IL-10, as a key mediator in the protection against T1D [170]. In addition, the T_H2 cytokines elicited by repeated α-GalCer injections such as IL-4, IL-13 and granulocyte/macrophage colony-stimulating factor (GM-CSF) have been observed to promote the differentiation of tolerogenic DCs, which are characterized by their high IL-10 and low IL-12 production, while suppressing inflammatory DCs. It is now believed that these tolerogenic DCs migrate to the PLN and suppress the autoreactive T cells that are responsible for β cell death, while promoting the generation of T_H2 and T_{reg} cell responses to also aid in immune suppression, thus leading

to the protection against T1D [128-130, 137, 171-174]. In fact, our lab has determined that α-GalCer mediated protection against T1D is dependent on T_{reg} activity [36].

The induction of anergy plays an important role in the immunoregulatory effects of iNKT cells and subsequent protection from T1D in NOD mice. Aside from resulting in a significantly reduced and T_H2-biased response upon α-GalCer restimulation, a recent study also demonstrated the role of iNKT cell anergy in tolerogenic DC induction [173]. In the approximately 7 day timeframe between the initial activation of iNKT cells and the induction of anergy following α-GalCer administration, these cells induce the maturation of DCs with immunogenic and inflammatory properties [173]. Conversely, restimulation of already anergic iNKT cells with α-GalCer leads to the induction of non-inflammatory DCs with suppressive capabilities [173]. While this study only investigated the total $CD11c^+$ DC population, it highlighted the importance of iNKT cell anergy in α-GalCer mediated protection from T1D and explained why only multiple doses of glycolipid are therapeutically effective. Previous reports are also consistent with this finding and have identified $CD8^-$ mDCs and pDCs as being tolerogenic subsets that accumulate in the PLNs after α-GalCer treatment, while the inflammatory $CD8^+$ mDCs were reduced in these tissues [128, 171, 173].

While α-GalCer has certainly proven to be a very promising therapeutic target for a variety of diseases, due to the potent cytokine storm and subsequent immune response elicited, administration of this glycolipid can lead to significant pathology and other negative consequences. Treatment of various mouse strains with α-GalCer induces a wide range of negative side effects. For example, transient hepatitis and spontaneous abortion have been observed in mice following α-GalCer injections due to the substantial number of iNKT cells present in the liver and uterus respectively [38, 159, 175, 176]. In addition, intranasal administration of α-GalCer sensitizes mice to developing airway

hypersensitivity [177]. Finally, it has been reported that anergic iNKT cells are more susceptible to activation induced cell death [66]. Due to the long-lasting anergy elicited by α-GalCer, chronic administration of this potent agonist has led to significantly reduced iNKT cell frequencies and therefore has raised concerns about its long term use [66, 178]. In terms of human administration, α-GalCer has thus far proven to be safe in clinical trials, however, only short-term side effects were measured [179-182]. In addition, these studies were primarily focused on cancer therapies and therefore only a few doses of α-GalCer or α-GalCer-pulsed DCs were used to limit iNKT cell anergy and take advantage of the strong T_H1 primary response that is elicited [179-182]. Thus, the long-term effects of such treatment as well as the response to multiple doses of α-GalCer in humans have yet to be determined. Regardless, α-GalCer has shown to be a capable therapeutic agent for a broad range of diseases. It is therefore not surprising that numerous investigators have synthesized derivatives of α-GalCer in an attempt to skew the elicited immune response in a favourable manner for their disease of interest.

1.4.3 Derivatives of α-GalCer for the treatment of disease

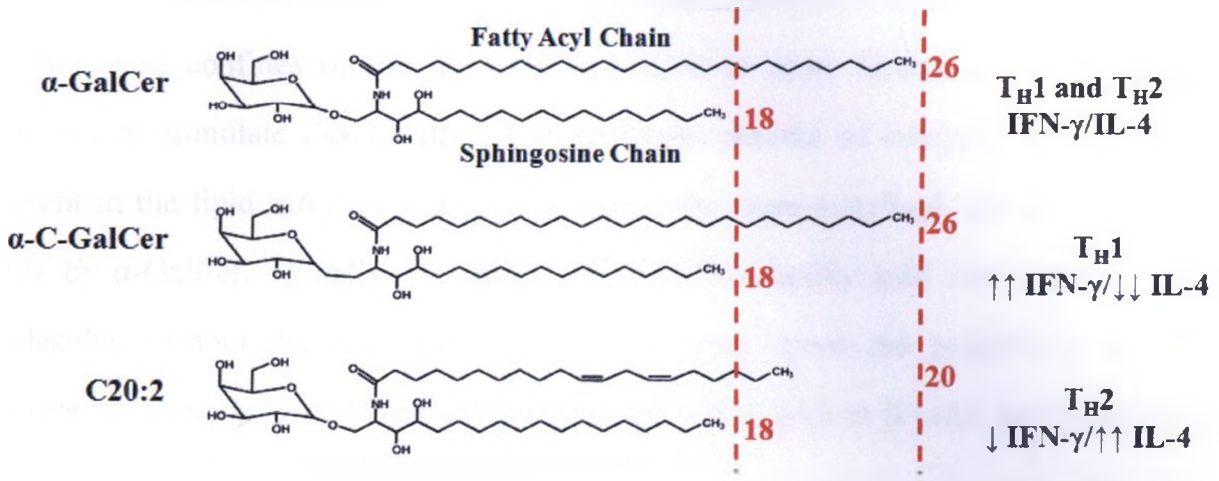
Given the immense diversity of human diseases, it is important to design drugs that efficiently trigger an appropriate immune response without leading to over-activation and potentially harmful side effects. Due to the unique ability of iNKT cells to modulate immune responses after treatment with α-GalCer, many studies have now focussed on designing various glycolipid derivatives in order to bias the subsequent immune response in either a T_H1 or T_H2 fashion. Minor structural changes to α-GalCer have been shown to lead to relatively large differences in iNKT cell responses. For example, the replacement of the glycosidic oxygen molecule with a CH_2 group results in a compound known as α-C-GalCer, which induces a significantly greater T_H1 response compared to α-GalCer with increased levels of IFN-γ and decreased amounts of IL-4 [183]. As such, this compound

is ideally suited for cancer therapies as well as to combat infections such as malaria, which both require strong inflammatory responses [183]. Conversely, T_H2 responses have been shown to be beneficial for treating inflammatory autoimmune diseases such as T1D [38].

A very promising glycolipid that elicits a T_H2 response is α-galactosylceramide C20:2 (C20:2), which has the ability to stimulate both murine and human iNKT cells [184]. In comparison to α-GalCer, the N-acyl variant C20:2 contains a fatty acyl side chain that is truncated from C26 to C20 with the introduction of two sites of unsaturation. These alterations result in a reduced IFN-γ and increased of IL-4 response (Figure 1.7) [184]. Interestingly, while both α-GalCer and C20:2 possess a similar binding affinity for CD1d and can activate iNKT cells with an equal potency, C20:2 administration results in an overall reduced immune response [184, 185]. Further investigation by our lab revealed that the decreased response observed in NOD mice was due to a reduced capacity to sustain iNKT cell activation, which in turn results in a less sustained transactivation of T, B, and NK cells (Ly et. al, 2009, in press, Appendix A). Therefore, the shortened stimulation of NK cells may account for the reduced IFN-γ response observed after C20:2 treatment.

An additional, but not mutually exclusive, explanation for the reduced and less sustained iNKT cell activation involves the differential requirements of α-GalCer and C20:2 loading onto CD1d. Under normal circumstances, α-GalCer requires internalization into an acidic pH environment as well as the assistance of various lipid transport proteins such as saposins to efficiently load onto CD1d, which occurs in the late endosome or lysosome [186, 187]. In comparison, C20:2 has the ability to load directly onto CD1d molecules that are present on the surface of APCs without the need for internalization [188, 189]. As such, the majority of C20:2-CD1d complexes are

Figure 1.7 Structure of α-GalCer and synthetic analogs. Molecular structures of α-GalCer and the synthetic analogs α-C-GalCer and C20:2. Relative to α-GalCer, which elicits a robust T_H1 (IFN-γ) and T_H2 (IL-4) response, replacing the glycosidic oxygen molecule with a CH_2 group results in α-C-GalCer that induces a significantly greater T_H1 response. Conversely, α-GalCer C20:2 (C20:2) contains a fatty acyl side chain that is reduced from 26 to 20 carbons as well as two double bonds and elicits a predominantly T_H2 response. Numbers indicate the length of each carbon chain, while the arrows represent the changes in cytokine secretion relative to α-GalCer. Adapted from Wu et al. 2009 [38]


Fatty Acyl Chain
α-GalCer
26
18
Sphingosine Chain
T_H1 and T_H2
IFN-γ/IL-4
α-C-GalCer
26
18
T_H1
↑↑ IFN-γ/↓↓ IL-4
C20:2
20
18
T_H2
↓ IFN-γ/↑↑ IL-4

presented to iNKT cells outside of lipid rafts [188, 189]. It is therefore possible that both C20:2 and α-GalCer may dissociate from the CD1d molecule at equal rates, however due to the dense confines of the lipid raft, α-GalCer is more likely to re-associate and continue to stimulate iNKT cells. The additional amount of costimulatory molecules present in the lipid raft may also play a role in the more sustained activation of iNKT cells by α-GalCer. Finally, the ability of C20:2 to rapidly load onto surface CD1d molecules without the need for internalization also opens the possibility of C20:2 preferentially being loaded onto non-professional APCs, such as B cells, and thus altering the iNKT cell response [184, 189].

Previous work conducted by Dr. Porcelli's lab, as well as our lab, has demonstrated that a multi-low dose administration of C20:2 significantly delayed and reduced the incidence of T1D in NOD mice with increased efficacy compared to α-GalCer (Ly et. al, 2009, in press, Appendix A) [185]. This protection was also observed to be less dependent on the activity of T_{regs} compared to α-GalCer as the inactivation of these regulatory cells using an anti-CD25 mAb abrogated protection elicited by α-GalCer, but not C20:2 (Ly et. al, 2009, in press, Appendix A). Presumably, the increased IL-4 response combined with the decreased IFN-γ secretion and bystander activation elicited by C20:2 does not require the same level of regulation from T_{regs} to control the immune response. While these are very favourable characteristics of C20:2 for the treatment of T1D, more in depth analyses aimed at investigating the mechanism(s) of protection are still required before such a treatment can be used clinically.

1.5 Rationale, hypothesis, and objectives

As described above, the N-acyl variant C20:2 is a very appealing iNKT cell agonist for the treatment of T1D, however more information is needed regarding the

mechanisms of C20:2 mediated protection before such a drug can be safely administered to humans. The reduced immune response elicited by this glycolipid (Ly et. al, 2009, in press, Appendix A) [184, 185], may be very beneficial in reducing potential long term side effects that may result from a stronger agonist such as α-GalCer. The objective of this study is to further characterize the iNKT cell responses to C20:2 relative to α-GalCer. In particular, this study focuses on the kinetic analysis of iNKT cell activation beginning at the initial cytokine responses and iNKT cell expansion and concluding with the induction of iNKT cell anergy and subsequent modulation of DCs following administration of C20:2. I hypothesize that due to the faster loading of C20:2 onto surface CD1d molecules combined with the less sustained and T_H2-like iNKT cell response, C20:2 administration will result in a more rapid onset and recovery from iNKT cell anergy as well as lead to the induction of DCs with tolerogenic capabilities. The results of this study will further assist in characterizing the molecular mechanisms involved in iNKT cell mediated protection against T1D and provide novel insight into structure/function analysis of α-GalCer derivatives. While C20:2 has yet to be tested clinically, based on the favourable characteristics displayed in previous studies (Ly et al. 2009, in press, Appendix A)[185] as well as my own, I feel that it is a prime candidate for future therapeutic use in humans.

Chapter 2:

Materials and Methods

2.1 Mice

NOD mice were bred in the animal care facility in the Robarts Research Institute at the University of Western Ontario (London, ON). The incidence of T1D in female NOD mice within our colony is 25-30% at 15 weeks of age and >75% by 25 weeks. All experimental mice were female and were maintained in a specific pathogen-free facility in the Animal Care and Veterinary Services at the University of Western Ontario according to institutional guidelines. All experiments were performed in accordance with the Canadian Council for Animal Care guidelines.

2.2 Glycolipids

Synthetic α-GalCer (KRN7000) and its appropriate vehicle control were obtained from Kirin Pharmaceutical Research Laboratories (Gunma, Japan). The lyophilized compound was solubilized in sterile water to a final concentration of 20 microgram (μg)/millilitre (mL) for injections, and was then diluted to a concentration of 100 nanogram (ng)/mL for *in vitro* stimulation of iNKT cells. Synthetic C20:2 was kindly synthesized by Dr. Gurydal Besra (University of Birmingham, Birmingham, UK) and provided by Dr. Steven Porcelli (Albert Einstein College of Medicine, Bronx, NY). The compound was received in a lyophilized form and was dissolved in 1x phosphate buffered saline (PBS) (GIBCO® Invitrogen, Burlington, ON) containing 0.02% Tween 20 and 0.1% dimethyl sulfoxide (DMSO) to a final concentration of 20 μg/mL for injections. C20:2 was further diluted before addition to *in vitro* cultures, resulting in a final concentration of 100 ng/mL of glycolipid in a solution of 0.0001% Tween 20 and 0.005% DMSO. The solution was heat sonicated for 5 minutes (min) and vortexed after each addition.

2.3 Preparation of splenocyte and lymphocyte suspensions

Mice were sacrificed by carbon dioxide (CO_2) asphyxiation. Spleens and PLNs were isolated, maintained at 4°C in ice cold PBS + 2% fetal bovine serum (FBS), and then homogenized by pressing the tissue through a sterile 40 μm nylon filter with a plunger. Cell suspensions were centrifuged at 1500 revolutions per minute (rpm) for 6 minutes at 4°C. Cell pellets were then washed with PBS + 2% FBS, re-centrifuged, resuspended in ACK lysis buffer (sterile water supplemented with 0.15 molar (M) ammonium chloride (NH_4Cl), 10 millimolar (mM) potassium bicarbonate ($KHCO_3$), and 0.1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.2-7.4, approximately 3 mL/spleen, 1 mL/PLN) for 5 min at room temperature to deplete erythrocytes and then washed again in PBS + 2% FBS.

2.4 *In vivo* treatment with glycolipid and serum collection

For single dose experiments, female NOD mice (4-6 week-old) received an intraperitoneal (i.p.) injection of 200 μL of α-GalCer (4 μg), C20:2 (4 μg), or vehicle (control). When a multi-low dose protocol was used, female NOD mice (4-6 week-old) were injected i.p. with α-GalCer (4 μg/dose), C20:2 (4 μg/dose), or vehicle (control) every other day for 3 weeks. Where indicated, blood was extracted at various time points after injections from the inferior vena cava, and then placed in a Microtainer tube (BD Biosciences, Mississauga, ON) to separate the serum after coagulation and incubated overnight at 4°C. Serum was isolated and analyzed by enzyme-linked immunosorbent assay (ELISA) for the presence of IFN-γ and IL-4.

2.5 *In vitro* cultures, ELISAs, and cell proliferation assays

Splenocytes isolated from various treatment groups of mice were suspended in RPMI 1640 tissue culture medium supplemented with 10% heat-inactivated FBS, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 units/mL penicillin, 0.1 mg/mL streptomycin, and 0.05 mM 2-mercaptoethanol (all purchased from Invitrogen). All *in vitro* cultures were stimulated with 100 ng/mL of glycolipid for 72 h at 37°C.

For cytokine analysis, splenocytes were suspended in culture media (5 x 10^6 /mL) and placed in a 24-well tissue culture plate (VWR, Mississauga, ON) in the presence of α-GalCer, C20:2, or vehicle (100 ng/mL). After 72 h, supernatants were collected and analysed by ELISA using paired antibody kits for IL-2, IFN-γ, and IL-4 (BD Biosciences). Briefly, 96-well ELISA plates (VWR) were coated with 100 µL per well of Capture Antibody (1:250 dilution) in Coating Buffer [0.1 M sodium bicarbonate ($NaHCO_3$), pH 9.5], sealed and incubated overnight at 4° C. After washing (PBS + 0.05% Tween-20), plates were blocked with 200 µL/well of assay diluent (1x PBS + 10% FBS) and incubated at room temperature for 1 h. Plates were then washed, loaded with 100 µL of either standard, sample, or control into appropriate wells, sealed, incubated for 2 h at room temperature and washed again. Working Detector reagent (100 µL) [Detection Antibody + Streptavidin-horseradish peroxidase (SAv-HRP), both at a 1:250 dilution in assay diluents] was added to each well, the plates were sealed and incubated for 1 h at room temperature, washed and 100 µL of Substrate Solution (tetramethylbenzidine (TMB) and hydrogen peroxide) was added to each well and incubated for 30 minutes at room temperature in the dark. Finally, 50 µL of Stop Solution [1 M phosphoric acid (H_3PO_4) or 2 M sulphuric acid (H_2SO_4)] was added to

each well and plates were read at a dual wavelength of 450/570 nanometre (nm) using a Benchmark Microplate Reader (Bio-Rad Laboratories, Hercules, CA).

Cell proliferation assays involved culturing 5 x 10^5 splenocytes in 200 µL of culture medium in a 96-well plate (VWR) in the presence of 100 ng/mL of glycolipid or vehicle for 72 h. Cells were pulsed with ^{3}H-thymidine for the final 18 h of culture, harvested, and read on a 1450 Microbeta counter (PerkinElmer, Woodbridge, ON). The relative amount of cell proliferation was measured by determining the amount of ^{3}H-thymidine incorporation.

2.6 Flow cytometry

Leukocytes (20 x 10^6 cells/mL) suspended in fluorescence-activated cell sorting (FACS) staining buffer [1x PBS, 0.1% sodium azide (NaN_3), 1% FBS] were incubated for 15 min on ice with 0.5 µg of Fc block (purified anti-CD16/CD32 antibody, eBiosciences, San Diego, CA) per 10^6 cells. For each stain, 1 x 10^6 cells were incubated for 25 min at 4°C in the dark with antibodies (1:200 dilution in FACS staining buffer) conjugated to various fluorochromes, washed with 2 mL of FACS staining buffer, centrifuged (6 min, 1500 rpm) at 4°C, and fixed with 300 µL of paraformaldehyde solution (1x PBS + 2% paraformaldehyde, pH 7.2) (Polysciences, Niles, IL). Flow cytometry was performed using a FACSCalibur or FACSCanto II and CellQuest Pro or FACSDiva software (BD Biosciences), respectively. Analyses were conducted using FlowJo software (Treestar Inc., Ashland, OR). Non-viable cells were excluded by electronic gating.

For a complete list of fluorescent antibodies used for flow cytometry and of the cell surface expression molecules used for cell subset characterization, refer to Table 2.1. and Table 2.2., respectively. Refer to Appendix A for sample gating strategies.

2.7 Intracellular cytokine staining

Leukocytes were isolated as described above (section 2.3). Prior to the addition of ACK lysis buffer, cells were resuspended in culture media at a concentration of 10^7 cells/mL. Intracellular cytokine staining was then performed using a BD Cytofix/Cytoperm buffer set (BD Biosciences) in accordance with manufacturer's instructions. Cells were cultured in a 24-well plate (VWR) for 3 h in the presence of the protein transport inhibitor, Golgi Stop (1:1000 dilution) (BD Biosciences). Splenocytes were collected, treated with ACK lysis buffer (as described in section 2.3), washed, resuspended in FACS staining buffer and stained as described (section 2.6). Prior to fixation with paraformaldehyde, cells were resuspended in 200 µL of Cytofix/Cytoperm buffer (BD Biosciences) for 20 min at 4°C to fix and permeablize the cell membranes. Samples were then washed with Perm/wash buffer (BD Biosciences) and resuspended in 50 µL of Perm/Wash buffer containing fluorescently labelled antibodies (anti-mouse IL-2, IFN-γ, IL-4, IL-12, or IL-10, 1:25 dilution) for 40 min at 4°C (See **Table 2.1** for complete antibody list). Finally, the cells were washed twice with Perm/wash buffer and once with FACS staining buffer to re-seal the cell membrane before being fixed with 2% paraformaldehyde.

2.8 Magnetic bead cell isolations

A $CD4^+$ T cell negative isolation kit (MACS, Miltenyi Biotec, Auburn, CA) was used according to the manufacturer's protocol to isolate $CD4^+$ T cells. Splenocytes were isolated and processed (as in section 2.3) and resuspended in MACS buffer (1x PBS

Table 2.1: Antibodies, tetramer reagents, and isotype controls used for flow cytometry

Antibody Target	Fluorochrome	Clone	Company
CD3 epsilon	PerCp-Cy5.5	145-2C11	eBiosciences
CD3 epsilon	Allophycocyanin	145-2C11	eBiosciences
CD4	FITC	GK1.5	eBiosciences
CD4	Allophycocyanin -Cy7	GK1.5	Biolegend
CD4	PerCp-Cy5.5	RM4-5	eBiosciences
CD4	PE-Cy7	RM 4-5	eBiosciences
CD8 alpha	FITC	53-6.7	eBiosciences
CD8 alpha	PE	53-6.7	eBiosciences
CD8 alpha	PerCp-Cy5.5	53-6.7	eBiosciences
CD8 alpha	Allophycocyanin	53-6.7	eBiosciences
CD8 alpha	AlexaFluor-700	53-6.7	eBiosciences
CD11c	Allophycocyanin	N418	eBiosciences
CD11c	Allophycocyanin -Cy7	N418	Biolegend
CD11c	FITC	N418	eBiosciences
CD16/32 (Fc block)	Purified	93	eBiosciences
CD274 (PD-L1)	PE	MIH5	eBiosciences
CD279 (PD-1)	PE	J43	eBiosciences
IFN-γ	PE	XMG1.2	eBiosciences
IL-2	PE	JES6-5H4	eBiosciences
IL-4	PE	11B11	eBiosciences
IL-10	PE	JES5-16E3	eBiosciences
IL-10	PerCp-Cy5.5	JES5-16E3	eBiosciences
IL-12	PE	C17.8	eBiosciences
iNKT Cells	Allophycocyanin	PBS57-loaded CD1d tetramer	NIH

iNKT Cells (control)	Allophycocyanin	PBS57-unloaded CD1d tetramer	NIH
Isotype control (IgG)	Allophycocyanin	eBio299Arm	eBiosciences
Isotype control (IgG)	PerCp-Cy5.5	1115PA	eBiosciences
Isotype control (IgG)	PE	eBio299Arm	eBiosciences
Isotype control (IgG2a)	PE	eBio299Arm	eBiosciences
Isotype control (IgG2b)	PE	eB149/10H5	eBiosciences
Isotype control (IgG2b)	PerCp-Cy5.5	eB149/10H5	eBiosciences
Siglec H	FITC	eBio440c	eBiosciences
TCR_{β}	FITC	H57-597	BD Pharmingen

Cy7, Cychrome 7; FITC, Fluorescein isothiocyanate; PE, Phycoerythrin; PE-Cy7, Phycoerythrin-cychrome 7; PerCP, Peridinin chlorophyll protein; PerCP-Cy5.5, Peridinin chlorophyll protein-cychrome 5.5

Table 2.2: Surface antigen phenotype of cell subpopulations

Cell Type	Surface antigen expression
iNKT cell	TCR_{β}^{+} PBS-57-loaded CD1d tetramer^{+}
pDC	Siglec H^{+} $CD11c^{low}$
$CD8^{+}$ mDC	Siglec H^{-} $CD11c^{high}$ $CD8^{+}$
$CD8^{-}$ mDC	Siglec H^{-} $CD11c^{high}$ $CD8^{-}$

supplemented with 0.5% FBS and 2 mM EDTA, degassed, pH 7.2) at a concentration of $2.5x10^8$ cells/mL. Cells were incubated (10 min, 4°C) in the presence of a Biotin-Antibody Cocktail (10 µL/10^7 cells) (MACS, Miltenyi Biotec) consisting of antibodies directed against non-$CD4^+$ cells. Anti-biotin microbeads (20 µL/10^7 cells) were then added to the samples and incubated for an additional 15 min at 4°C. After washing with MACS buffer, the cells were loaded onto a MACS magnetic separation column (Miltenyi Biotec) to isolate the $CD4^+$ T cells, yielding a 95% pure population.

Total DCs were isolated using a CD11c positive isolation kit (MACS, Miltenyi Biotec). Spleens were first cultured with 2 mg/mL of Collagenase D solution [1x PBS supplemented with 2 mg/mL Collagenase D, 10 mM HEPES, 150 mM NaCl, 5 mM potassium chloride (KCL), 1 mM magnesium chloride ($MgCl_2$), and 1.8 mM calcium chloride ($CaCl_2$), pH 7.4] for 30 min at 37°C. Leukocytes were isolated and treated as described (section 2.3) and resuspended in MACS buffer at a concentration of $2.5x10^8$ cells/mL. Anti-mouse $CD11c^+$ Microbeads were added to each sample (100 µL/10^8 cells) and incubated for 15 min at 4°C. After washing, the cells were passed through a MACS magnetic separation column (Miltenyi Biotec) to isolate $CD11c^+$ cells.

2.9 DC functional analysis

For DC cultures, female NOD mice (4-6 week-old) were treated i.p. with glycolipid according to our multi-low dose protocol (see section 2.4). One week after the final dose, spleen and PLN suspensions were pooled and $CD11c^+$ cells were magnetically isolated (see section 2.8) from each treatment group. Concurrently, $CD4^+$ peptide primed T cells were isolated (see section 2.8) from a separate group of female NOD mice (4-6 week-old) that were injected (i.p.) 10 days previously with either vehicle control (PBS) or 100 µg of a B9-23 insulin peptide (Ins B9-23, kindly provided by Dr. B. Singh,

University of Western Ontario, London, ON) emulsified in incomplete Freud's adjuvant (IFA) (Sigma Aldrich, Oakville, ON). Peptide primed-$CD4^+$ T cells were cultured with $CD11c^+$ DCs at a 20:1 ratio and restimulated with 100 μg/mL of Ins B9-23 peptide for 72 h for ELISAs ($2x10^6$ $CD4^+$ T cells + $1x10^5$ DC) and proliferation ($2x10^5$ $CD4^+$ T cells + $1x10^4$ DC) assays. Culture supernatants were collected and analyzed for IL-2, IFN-γ, IL-4 detection using ELISAs (eBiosciences), and cell proliferation was determined by ^3H-thymidine incorporation (1 μCi/well, PerkinElmer) following an 18 h pulse, as described in section 2.5.

2.10 Statistical analysis

Differences were determined by the Student's 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.

Chapter 3:

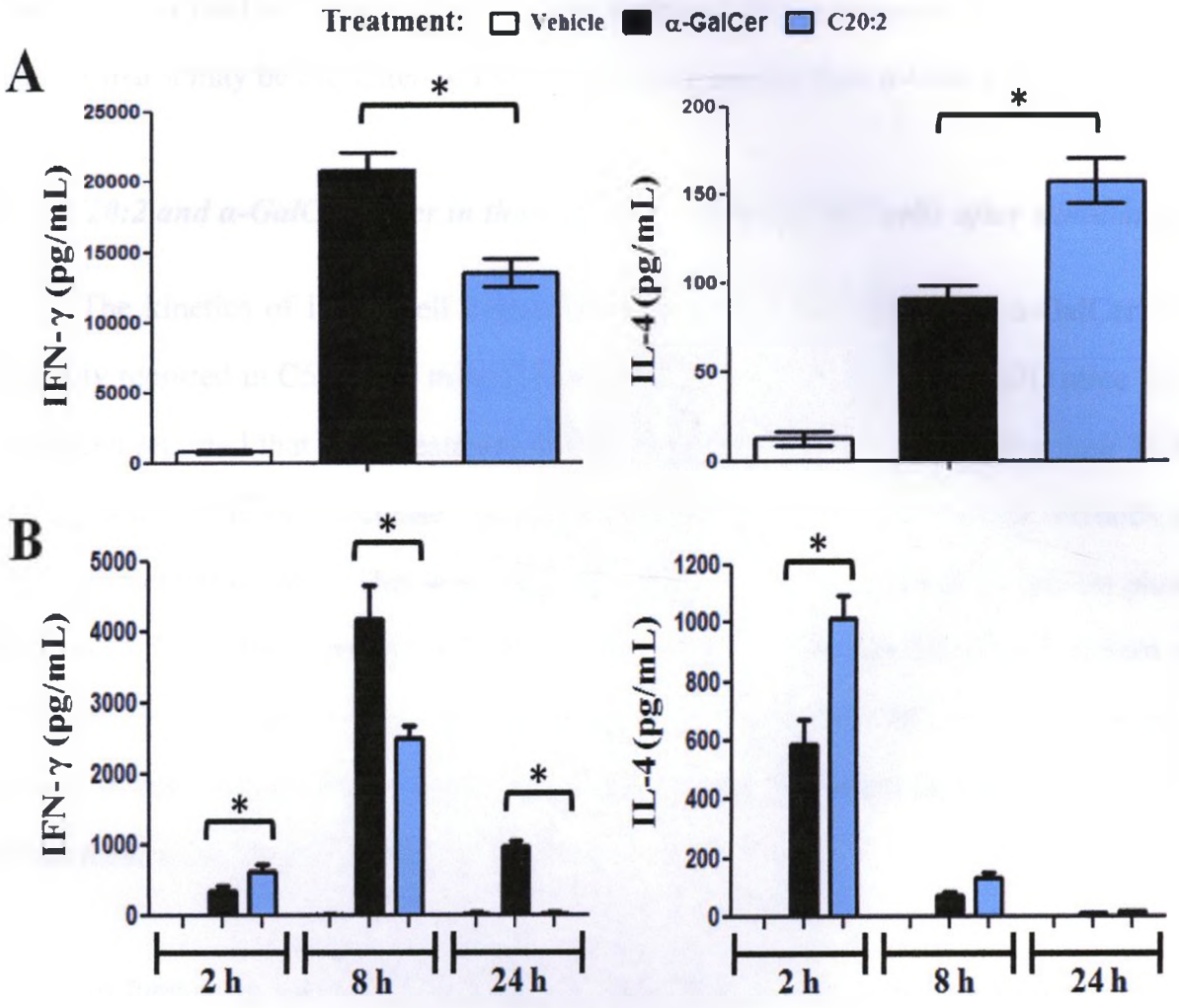
Results

3.1 iNKT cells differ in their primary response to α-GalCer and C20:2

3.1.1 C20:2 activated iNKT cells induce a less sustained and T_H2-biased cytokine response

Relative to α-GalCer, C20:2 contains a shortened fatty acyl side chain from C26 to C20 with the introduction of two unsaturated bonds at carbons 11 and 14 (Figure 1.7). To investigate the response elicited by C20:2 in NOD mice, total splenocytes from naïve female mice (4-6 week-old) were stimulated with 100 ng/mL of either glycolipid *in vitro* for 72 h. Secreted cytokines were analyzed by ELISA and, consistent with previous literature [184, 185, 189], C20:2 treatment was found to stimulate the production of a more T_H2 type response compared to α-GalCer. This was evident from the secretion of significantly less IFN-γ, a pro-inflammatory, T_H1 cytokine, as well as a significant increase in the non-inflammatory, T_H2 cytokine, IL-4 (Figure 3.1 A). This finding was further confirmed *in vivo.* Blood serum was isolated from female NOD mice 2 h, 8 h, and 24 h following the injection of 4 μg of glycolipid. C20:2 treatment led to an early IFN-γ response that was significantly greater (approximately 2-fold) than α-GalCer at the 2 h time point (Figure 3.1 B). By the time of peak secretion at 8 h, however, mice treated with α-GalCer demonstrated a very robust IFN-γ response that was significantly greater than that seen with C20:2. Interestingly, C20:2 treatment led to a less sustained IFN-γ response in the NOD mice that was barely discernible after 24 h compared to α-GalCer, which maintained a strong response throughout the time course. In contrast to IFN-γ, both glycolipids elicited a strong IL-4 response, peaking at 2 h post injection with a small residual response being observed at 8 h. During this peak time of secretion, a significantly greater IL-4 response was observed upon C20:2 treatment relative to α-GalCer. Taken together, these results demonstrate that C20:2 is a T_H2 biasing glycolipid in NOD mice, leading to decreased production of IFN-γ and increased amounts of IL-4

Figure 3.1 Administration of C20:2 elicits a T_H2 type response compared to α-GalCer. (A) Splenocytes from naïve female NOD mice (4-6 week-old) were cultured and stimulated with α-GalCer, C20:2 or vehicle (100 ng/mL) for 72 h. Culture supernatants were analyzed for IFN-γ and IL-4 by ELISA. **(B)** NOD mice (4-6 week-old) were given a single dose (4 μg, i.p.) of glycolipid or vehicle. Serum was collected at 2 h, 8 h, or 24 h after injection and ELISAs were performed to measure levels of IFN-γ and IL-4. Data are representative of at least three independent experiments yielding similar results and are expressed as mean ± SD. N = 5 mice/treatment group/time point. *, significant ($P<0.05$) difference between compared groups.


Treatment: Vehicle α-GalCer C20:2
A
IFN-γ (pg/mL)
25000
20000
15000
10000
5000
0
*
IL-4 (pg/mL)
200
150
100
50
0
*
B
IFN-γ (pg/mL)
5000
4000
3000
2000
1000
0
*
*
*
2 h
8 h
24 h
IL-4 (pg/mL)
1200
1000
800
600
400
200
0
*
2 h
8 h
24 h

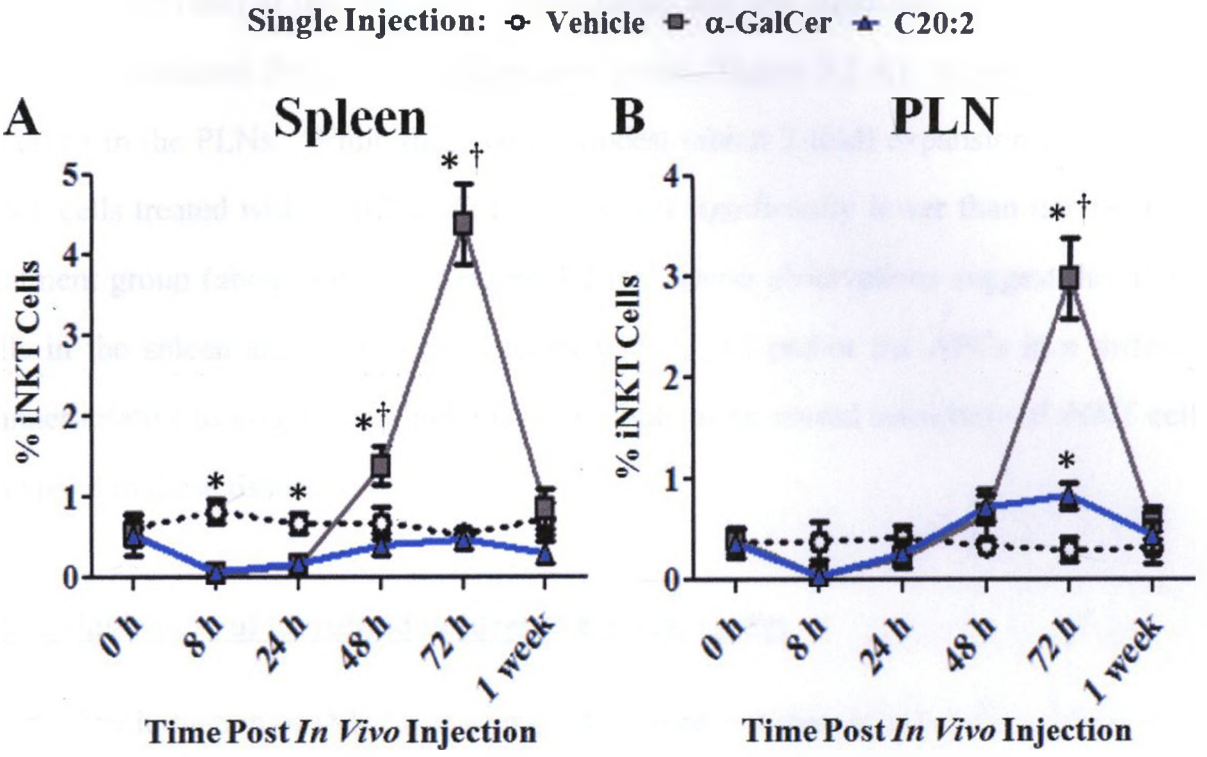
compared to α-GalCer. The earlier and less sustained IFN-γ response elicited by C20:2 suggests that it may be presented to iNKT cells more rapidly than α-GalCer.

3.1.2 C20:2 and α-GalCer differ in their ability to expand iNKT cells after stimulation

The kinetics of iNKT cell expansion following administration of α-GalCer was originally reported in C57BL/6J mice [156, 157] as well as by our lab in NOD mice [36]. It has been reported that after treatment with α-GalCer, iNKT cells internalize their TCR, leading to an apparent "decrease" in cell frequency, based on the current methods of iNKT cell detection [66]. This was subsequently followed by a robust expansion phase that peaked 72 h after exposure to the glycolipid [66]. Although the overall pattern of expansion was similar in NOD mice, the peak frequency of iNKT cells was greatly reduced in these mice, possibly due to the functional and numerical deficiencies of iNKT cells in these mice [36, 98, 128-130].

To further characterize the properties of C20:2, it was important to determine if the kinetics of iNKT cell activation is similar to that of α-GalCer. To investigate iNKT cell expansion, young female NOD mice were injected with a single dose (4 μg, i.p.) of either glycolipid or vehicle. Spleens and PLNs were isolated at various times after treatment (0 h, 8 h, 24 h, 48 h, 72 h, or 1 week), stained with anti-TCRβ-FITC and PBS-57-loaded CD1d tetramer conjugated to allophycocyanin and the frequency of $TCR\beta^{+}tetramer^{+}$ iNKT cells was determined by flow cytometry. In the spleen, both α-GalCer and C20:2 treatments resulted in a significant decrease in iNKT cell frequency at the 8 h and 24 h time points, indicating that activation had occurred (Figure 3.2 A). Interestingly, while iNKT cells treated with α-GalCer exhibited a significant expansion phase that began at 48 h and peaked at 72 h post injection, this was not the case for iNKT cells from mice treated with C20:2. In fact, the frequency of C20:2 treated iNKT cells

Figure 3.2 Kinetics of iNKT cell expansion following administration of α-GalCer or C20:2. Female NOD mice (4-6 week-old) were administered a single dose (4 μg, i.p.) of glycolipid or vehicle and rested for various times (8 h, 24 h, 48 h, 72 h, or 1 week). Spleens **(A)** and PLNs **(B)** were isolated, stained with anti-TCRβ-FITC and α-GalCer-loaded CD1d tetramer and the frequency of $TCR\beta^{+}tetramer^{+}$ iNKT cells was determined by flow cytometry. Data are representative of at least three independent experiments yielding similar results and are expressed as mean ± SD. N = 3 mice/treatment group/time point. *, significant ($P<0.05$) difference between treatment and vehicle values. †, significant ($P<0.05$) difference between α-GalCer and C20:2 treatment values.


Single Injection: Vehicle α-GalCer C20:2
A
Spleen
% iNKT Cells
0
1
2
3
4
5
0 h
8 h
24 h
48 h
72 h
1 week
Time Post *In Vivo* Injection
B
PLN
% iNKT Cells
0
1
2
3
4
0 h
8 h
24 h
48 h
72 h
1 week
Time Post *In Vivo* Injection

never exceeded that of the vehicle treatment group and was significantly less than that of α-GalCer stimulated iNKT cells at these time points (Figure 3.2 A). A similar trend was observed in the PLNs. While there was a modest (about 2-fold) expansion exhibited by iNKT cells treated with C20:2 at 72 h, it was still significantly lower than the α-GalCer treatment group (about 3-4 fold) (Figure 3.2 B). These observations suggest that iNKT cells in the spleen and PLNs may interact with C20:2 and/or the APCs in a different manner relative to α-GalCer, which may contribute to an altered sensitivity of iNKT cells to expand in these tissues.

3.2 A single dose of glycolipid induces iNKT cell anergy

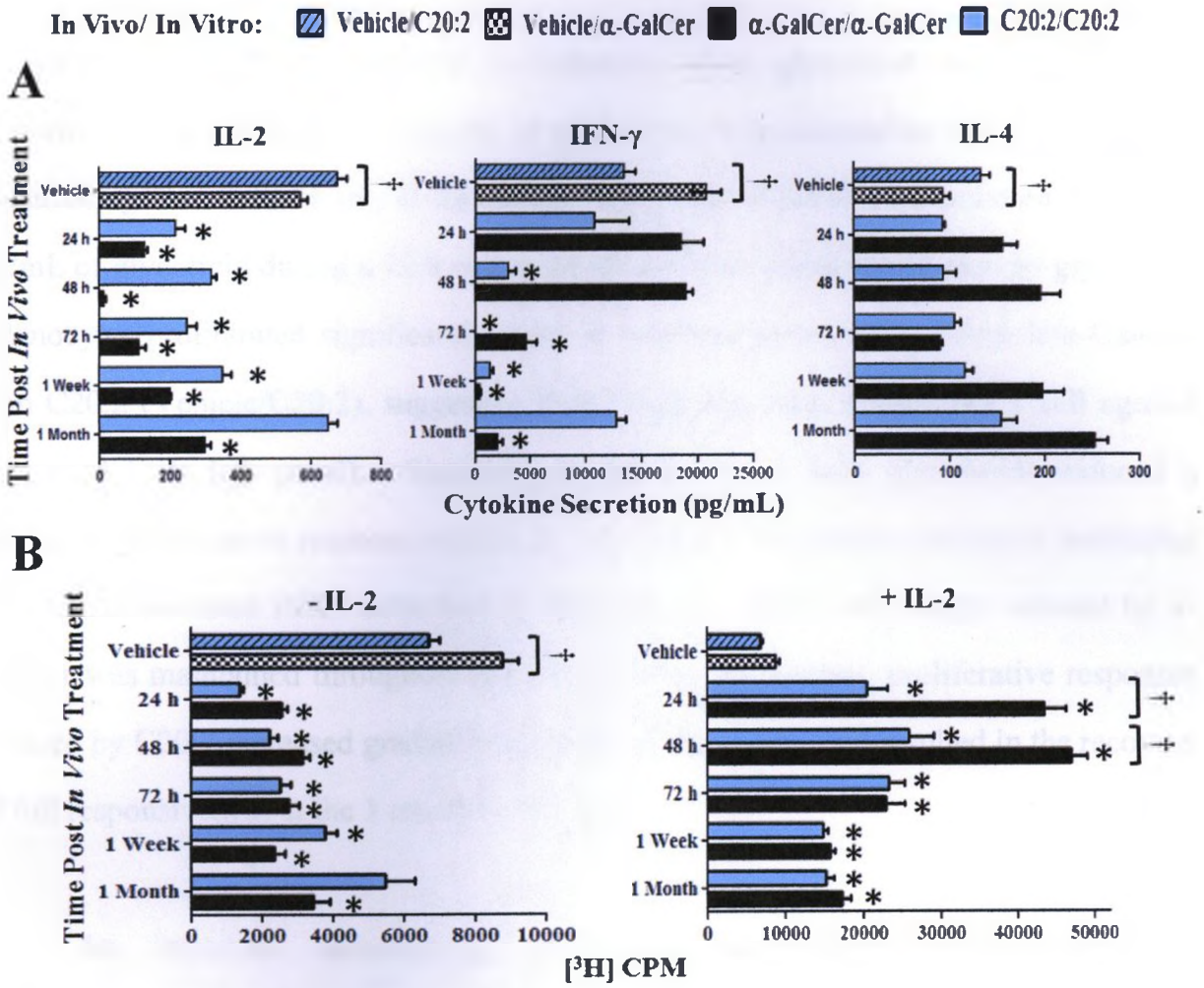
The induction of iNKT cell anergy after administration of α-GalCer has been well characterized. A single dose of this potent agonist renders iNKT cells hyporesponsive to subsequent stimulation, and this state of anergy may last for longer than 1 month [157]. These anergic iNKT cells have a reduced capacity to produce IL-2 and IFN-γ upon α-GalCer restimulation; however, they maintain their ability to stimulate an IL-4 response in a total splenocyte culture [157]. A key inhibitory signalling molecule, PD-1, has recently been identified to be essential for the induction of iNKT cell anergy upon interaction with its ligand PD-L1 [166, 167]. In addition, a previous report demonstrated that restimulation of anergic iNKT cells leads to the induction of tolerogenic DCs, while the stimulation of naïve iNKT cells induces immunogenic DCs upon initial activation [173]. Thus, it appears that iNKT cell anergy plays a role in the response to glycolipid treatment and the alteration of DC function. For these reasons, it was of interest to investigate the kinetics of iNKT cell anergy in response to C20:2 relative to α-GalCer.

3.2.1 Kinetic analysis of total splenocyte responses after glycolipid restimulation in vitro

Initially, a kinetic analysis of the induction of iNKT cell anergy *in vitro* was conducted. Female NOD mice were administered a single dose (4 μg, i.p.) of either glycolipid or vehicle and rested for various times (24 h, 48 h, 72 h, 1 week, or 1 month). Splenic leukocytes were then cultured and restimulated with 100 ng/mL of glycolipid for 72 h, and cytokine secretion was analysed by ELISA (Figure 3.3 A). Consistent with previous results, initial exposure to C20:2 (Vehicle/C20:2 treatment group) elicited a greater T_H2 response, characterized by reduced IFN-γ and increased IL-4 compared to α-GalCer (Vehicle/α-GalCer). Interestingly, C20:2 also induced a significantly greater IL-2 response relative to α-GalCer. These cytokine levels observed after primary stimulation were then used as a baseline to measure iNKT cell hyporesponsiveness. Both glycolipids induced iNKT cell anergy after treatment with a single dose as demonstrated by a significant decrease in the levels of IL-2 and IFN-γ in total splenocyte cultures analyzed after restimulation *in vitro*. In comparison to α-GalCer, however, C20:2 treatment elicited the induction of and recovery from anergy more rapidly. This was evidenced by the faster reduction of IFN-γ levels by C20:2 at the 48 h time point as well as the full recovery of IFN-γ and IL-2 responses at the 1 month time point. Cultures containing iNKT cells from mice that received an α-GalCer injection 1 month prior to restimulation still exhibited significantly blunted IFN-γ and IL-2 responses, indicating that these cells were still anergic. Consistent with a previous report on α-GalCer [157], C20:2 elicited an IL-4 response throughout the time course within the culture.

In addition to cytokine responses, the ability to proliferate after ligand restimulation is also a widely used measure of anergy. Thus, a comparative analysis of

Figure 3.3 Kinetic analysis of anergy induction after treatment with a single dose of glycolipid and restimulation *in vitro*. Female NOD mice (4-6 week-old) were injected with a single dose (4 μg, i.p.) of glycolipid or vehicle. Splenocytes were then isolated at various times (24 h, 48 h, 72 h, 1 week, 1 month) and restimulated *in vitro* with 100 ng/mL of glycolipid for 72 h. **(A)** Supernatants were then analyzed for IL-2, IFN-γ and IL-4 by ELISA. **(B)** Proliferative capacity of the original cultures ± 5ng/mL of IL-2 were assayed by ^{3}H-thymidine incorporation. Data are representative of four independent experiments yielding similar results and are expressed as mean ± SD. N = 3 mice/treatment group/time point. *, significant ($P<0.05$) reduction between primary and recall responses (Vehicle/α-GalCer vs. α-GalCer/α-GalCer, Vehicle/C20:2 vs. C20:2/C20:2). †, significant ($P<0.05$) difference between primary responses to α-GalCer and C20:2 treatments (Vehicle/α-GalCer vs. Vehicle/C20:2).


In Vivo/ In Vitro:
Vehicle/C20:2
Vehicle/α-GalCer
α-GalCer/α-GalCer
C20:2/C20:2
A
IL-2
IFN-γ
IL-4
Time Post In Vivo Treatment
Vehicle
24 h
48 h
72 h
1 Week
1 Month
0
200
400
600
800
5000
10000
15000
20000
25000
100
300
Cytokine Secretion (pg/mL)
B
- IL-2
+ IL-2
2000
4000
6000
8000
10000
30000
40000
50000
[3H] CPM

α-GalCer versus C20:2 induced proliferation upon glycolipid restimulation was performed. The proliferative capacity of splenocytes was assessed as in Figure 3.3 A by quantitating the amount of ^{3}H-thymidine incorporation after restimulation with 100 ng/mL of glycolipid during a 72 h culture *in vitro*. After initial stimulation by glycolipid, splenocytes proliferated significantly more in response to α-GalCer (Vehicle/α-GalCer) than C20:2 (Vehicle/C20:2), suggesting that C20:2 may be a weaker iNKT cell agonist (Figure 3.3 B, left panel). Similarly, restimulation of both glycolipids induced a decreased proliferative response relative to their respective primary responses, indicating that the restimulated iNKT cells had become anergic. iNKT cell anergy induced by α-GalCer was maintained throughout the time course. In contrast, proliferative responses induced by C20:2 increased gradually during the time course, and resulted in the recovery of full responsiveness at the 1 month time point.

An important characteristic of the anergic state is that iNKT cell hyporesponsiveness can be reversed by the addition of IL-2 [190]. This possibility was tested by the addition of 5 ng/mL of IL-2 to the initial cultures. All recall responses obtained for both glycolipids were at least 2-fold greater than their respective primary responses upon addition of IL-2 for the duration of the time course (Figure 3.3 B, right panel). These findings indicate that the reduced recall responses seen *in vitro* are a result of iNKT cell anergy and not activation induced cell death.

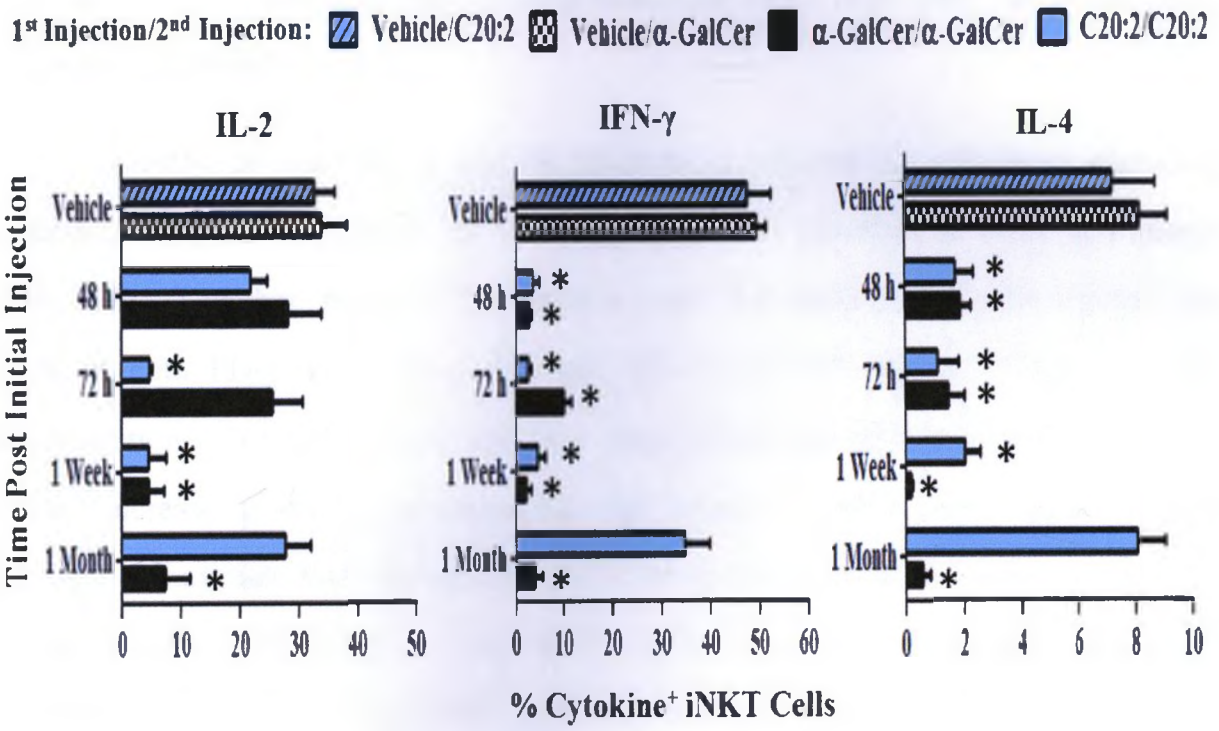
It is important to note that while iNKT cell anergy is evaluated only indirectly in total splenocyte cultures, this method of assessment is still valid and informative. Due to the low frequency of iNKT cells in the spleen, the majority of the cytokine and proliferative responses measured *in vitro* following glycolipid stimulation could be dependent on the transactivation of other immune cells, rather than the iNKT cells

themselves. However, anergic iNKT cells are impaired in their ability to transactivate other leukocytes upon restimulation [157]. Therefore, the observed reduction in the cytokine and proliferative responses of total splenocytes can be directly attributed to iNKT cell anergy. Taken together, these *in vitro* recall responses demonstrate that iNKT cells treated with C20:2 appear to induce and recover from anergy more rapidly than those treated with α-GalCer.

3.2.2 Kinetic analysis of iNKT cell responses after glycolipid restimulation in vivo

To further determine whether iNKT cells become anergic *in vivo,* the levels of intracellular cytokine accumulation in iNKT cells were monitored after glycolipid restimulation. Female NOD mice were administered a single dose (4 μg, i.p.) of either glycolipid or vehicle, rested for various times (48 h, 72 h, 1 week, or 1 month), and then re-injected with an additional dose (4 μg, i.p.) of glycolipid. Splenocytes were harvested 2 h after the second injection, and iNKT cells were stained for their intracellular levels of IL-2, IFN-γ, and IL-4. Consistent with the *in vitro* responses, iNKT cells from mice injected with C20:2 induce and recover from anergy more rapidly than iNKT cells from mice injected with α-GalCer (Figure 3.4). This was particularly evident for levels of intracellular IL-2 detected at the 72 h time point, and for all three cytokines measured at the 1 month time point. Interestingly, iNKT cells *in vivo* exhibited a blunted IL-4 response upon restimulation. This is in contrast to that seen *in vitro* in total splenocyte cultures, which maintain consistent levels of secreted IL-4 upon restimulation (Figure 3.3 A). It is important to note that while a more rapid reduction in the intracellular levels of IFN-γ and IL-4 may occur at the 24 h time point, due to iNKT cell internalization of their TCR following activation, the intracellular levels of these cytokines at this time were below the limit of detection.

Figure 3.4 Kinetic analysis of anergy induction after treatment with a single dose followed by restimulation *in vivo*. Female NOD mice (4-6 week-old) were injected with a single dose (4 μg, i.p.) of glycolipid or vehicle and rested for various times (48 h, 72 h, 1 week, 1 month). At each time point, mice were re-injected with an additional dose (4 μg, i.p.) of glycolipid. Splenocytes were harvested 2 h after the second injection, cultured *in vitro* for 3 h in the presence of a protein transport inhibitor, Golgi Stop, in order to inhibit cytokine secretion. $TCR\beta^{+}tetramer^{+}$ iNKT cells were then stained for intracellular levels of IL-2, IFN-γ, and IL-4. Data are representative of three independent experiments yielding similar results and are expressed as mean ± SD. N = 3 mice/treatment group/time point. *, significant ($P<0.05$) reduction between primary and recall responses (Vehicle/α-GalCer vs. α-GalCer/α-GalCer, Vehicle/C20:2 vs. C20:2/C20:2).

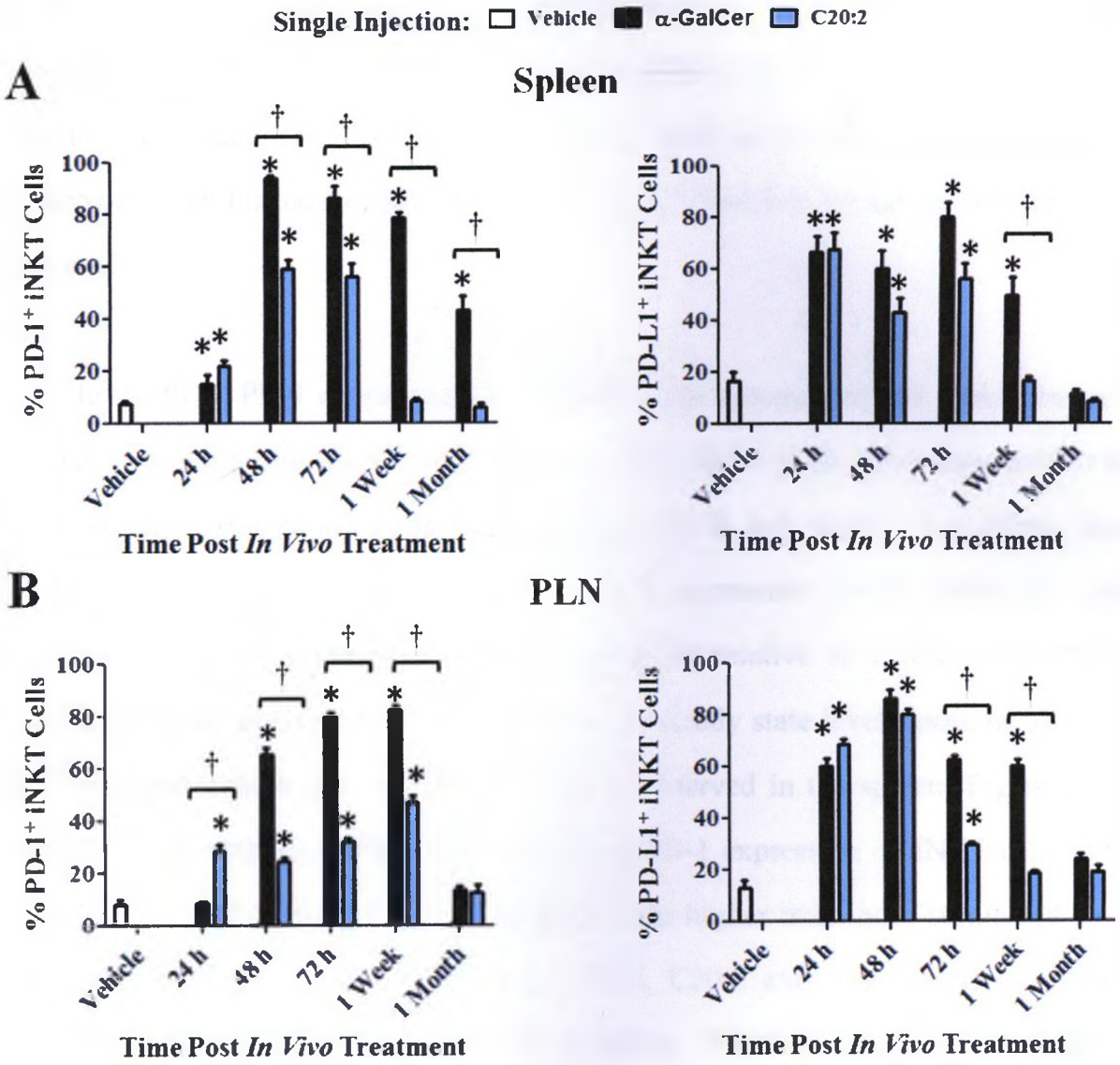

1st Injection/2nd Injection: Vehicle/C20:2 Vehicle/α-GalCer α-GalCer/α-GalCer C20:2/C20:2
IL-2
IFN-γ
IL-4
Time Post Initial Injection
Vehicle
48 h
72 h
1 Week
1 Month
0 10 20 30 40 50
0 10 20 30 40 50 60
0 2 4 6 8 10
% Cytokine+ iNKT Cells

3.2.3 Kinetics of PD-1 and PD-L1 upregulation on iNKT cells after treatment with a single dose of glycolipid

Recently, it was shown that the interaction between the inhibitory signalling molecule, PD-1, and its ligand PD-L1 is crucial for the induction of iNKT cell anergy [166, 167]. Together, these studies demonstrated that naïve iNKT cells express low levels of both PD-1 and PD-L1, but not PD-L2, on their surface. After a single administration of α-GalCer, there is a large, long-lasting upregulation of PD-1 as well as a small increase in PD-L1 expression on the surface of iNKT cells. These studies, however, did not quantitatively measure the level of upregulation and were conducted in C57BL/6 mice. Furthermore, only iNKT cells from the spleen and liver were investigated at the exclusion of iNKT cells in the PLNs, an important immunological site of T cell activation and regulation during the development of T1D.

It was therefore of interest to examine the effects of exposure to α-GalCer and C20:2 on the surface expression levels of PD-1 and PD-L1 on iNKT cells from the PLNs of NOD mice. Female NOD mice were administered a single dose (4 μg, i.p.) of glycolipid or vehicle and rested for various times (24 h, 48 h, 72 h, 1 week, or 1 month). Levels of surface expression of PD-1 and PD-L1 on iNKT cells from the spleen (Figure 3.5 A) and PLNs (Figure 3.5 B) were analyzed by flow cytometry. Consistent with previous results, vehicle treated mice expressed low levels of surface PD-1 and PD-L1, but not PD-L2 (not shown), on iNKT cells from the spleen and PLN. Administration of either α-GalCer or C20:2 upregulated PD-1 expression on splenic iNKT cells beginning at 24 h and peaking between 48-72 h post exposure (Figure 3.5 A, left panel). However, treatment with α-GalCer led to a significantly greater amount of PD-1^+ iNKT cells during this peak expression time compared to C20:2, indicating that it is a stronger iNKT cell agonist. In addition, PD-1 expression levels were still

Figure 3.5 Kinetic analysis of PD-1/PD-L1 upregulation on iNKT cells following treatment with a single dose of glycolipid. Female NOD mice (4-6 week-old) were injected with a single dose (4 μg, i.p.) of glycolipid or vehicle and rested for various times (24 h, 48 h, 72 h, 1 week, and 1 month). Lymphocytes from the spleen **(A)** and PLN **(B)** were isolated, and PD-1 and PD-L1 surface expression levels on $TCR\beta^+tetramer^+$ iNKT cells were analyzed by flow cytometry. Data are expressed as mean $\pm$ SD. N = 5 mice/treatment group/time point. *, significant ($P<0.05$) difference between the vehicle and glycolipid treatment groups. †, significant ($P<0.05$) difference between α-GalCer and C20:2 treatment values.


Single Injection: Vehicle α-GalCer C20:2
A
Spleen
% PD-1+ iNKT Cells
% PD-L1+ iNKT Cells
Vehicle
24 h
48 h
72 h
1 Week
1 Month
Time Post *In Vivo* Treatment
B
PLN
% PD-1+ iNKT Cells
% PD-L1+ iNKT Cells
Time Post *In Vivo* Treatment

significantly increased 1 month after α-GalCer treatment. In contrast, C20:2 treatment elicited a reduced and less sustained upregulation of PD-1 on iNKT cells, as noted by the return to steady state PD-1 expression levels at 1 week post injection. A similar trend was also observed for the surface expression levels of PD-L1 in the spleen (Figure 3.5 A, right panel).

In the PLN, PD-1 expression on iNKT cells was upregulated and peaked between 72 h and 1 week after glycolipid exposure, which revealed a slight delay compared to the kinetics of expression found in the spleen (Figure 3.5 B, left panel). Regardless, there was still a significantly greater increase in PD-1 expression levels during the peak upregulation obtained upon treatment with α-GalCer relative to C20:2. The PD-L1 surface expression levels on iNKT cells returned to steady state levels more rapidly after C20:2 treatment, which was similar to what was observed in the spleen (Figure 3.5 B, right panel). Interestingly, after 24 h, the level of PD-1 expression on iNKT cells in the spleen and PLN following C20:2 administration was higher than those stimulated by α-GalCer (Figure 3.5 A and B, left panels). Thus, C20:2 may lead to the more rapid activation of iNKT cells in comparison to α-GalCer. Furthermore, the faster return of both PD-1 and PD-L1 expression to vehicle levels following C20:2 treatment may play a role in the more rapid recovery of iNKT cells from a state of anergy.

3.3 Immunological effects of multi-low dose administration of glycolipid

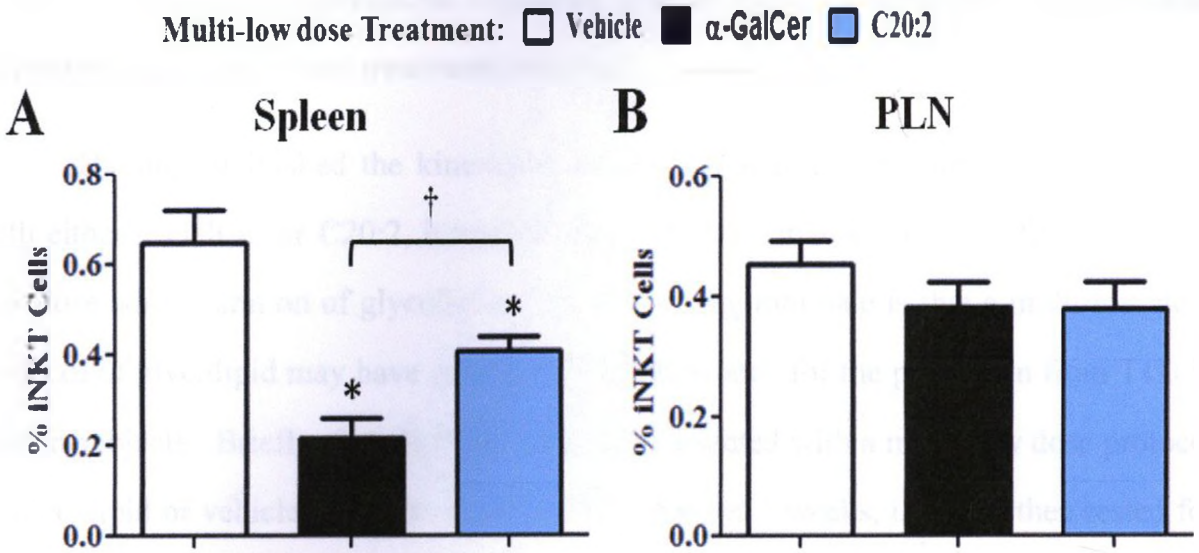
While it is informative to understand the immunological responses elicited by both glycolipids after a single administration, both our lab [129] and other labs [128, 130, 137] have reported that treatment of NOD mice with a single dose of glycolipid does not lead to protection against T1D. Rather, optimal protection from T1D is achieved by treatment with a multi-low dose protocol of either α-GalCer or C20:2 (Ly et. al, 2009, in

press, Appendix A) [128-130, 137, 185]. Therefore, it was of interest to determine the effects of a glycolipid treatment protocol that protects from T1D on target iNKT cells.

3.3.1 Multi-low dose administration of α-GalCer or C20:2 alters the frequency of iNKT cells in the spleen

Although a multi-low dose protocol of α-GalCer treatment protects NOD mice from T1D, the iNKT cell frequency is significantly reduced after such a protocol, presumably due to activation induced cell death [66, 178]. If the therapeutic benefit of such a protocol were to be tested clinically in humans at risk for T1D, it is essential that this treatment does not compromise the patient's immune system. Since iNKT cells mediate many types of immune responses [98], long term depletion of this subset may have detrimental effects and result in a greater susceptibility to infection. Thus, the effect of a multi-low dose treatment with either α-GalCer or C20:2 on the frequency of iNKT cells was investigated. Female NOD mice were treated according to a multi-low dose protocol with α-GalCer, C20:2 or vehicle (4 μg/dose every other day for 3 weeks, i.p.) and were then rested for 1 month. Splenocytes (Figure 3.6 A) and PLN lymphocytes (Figure 3.6 B) were isolated and the frequencies of splenic and PLN iNKT cells were determined using flow cytometry. Administration of either glycolipid markedly reduced the iNKT cell frequency in the spleen, but not PLNs, at 1 month post-treatment (Figure 3.6 A, B). While the iNKT cell frequency in the spleen of mice treated with C20:2 was still reduced compared to vehicle levels, it was significantly higher than that observed in mice that received an α-GalCer treatment regimen (Figure 3.6 A). Thus, chronic administration of C20:2 does not appear to deplete iNKT cells to the same extent as the more potent α-GalCer.

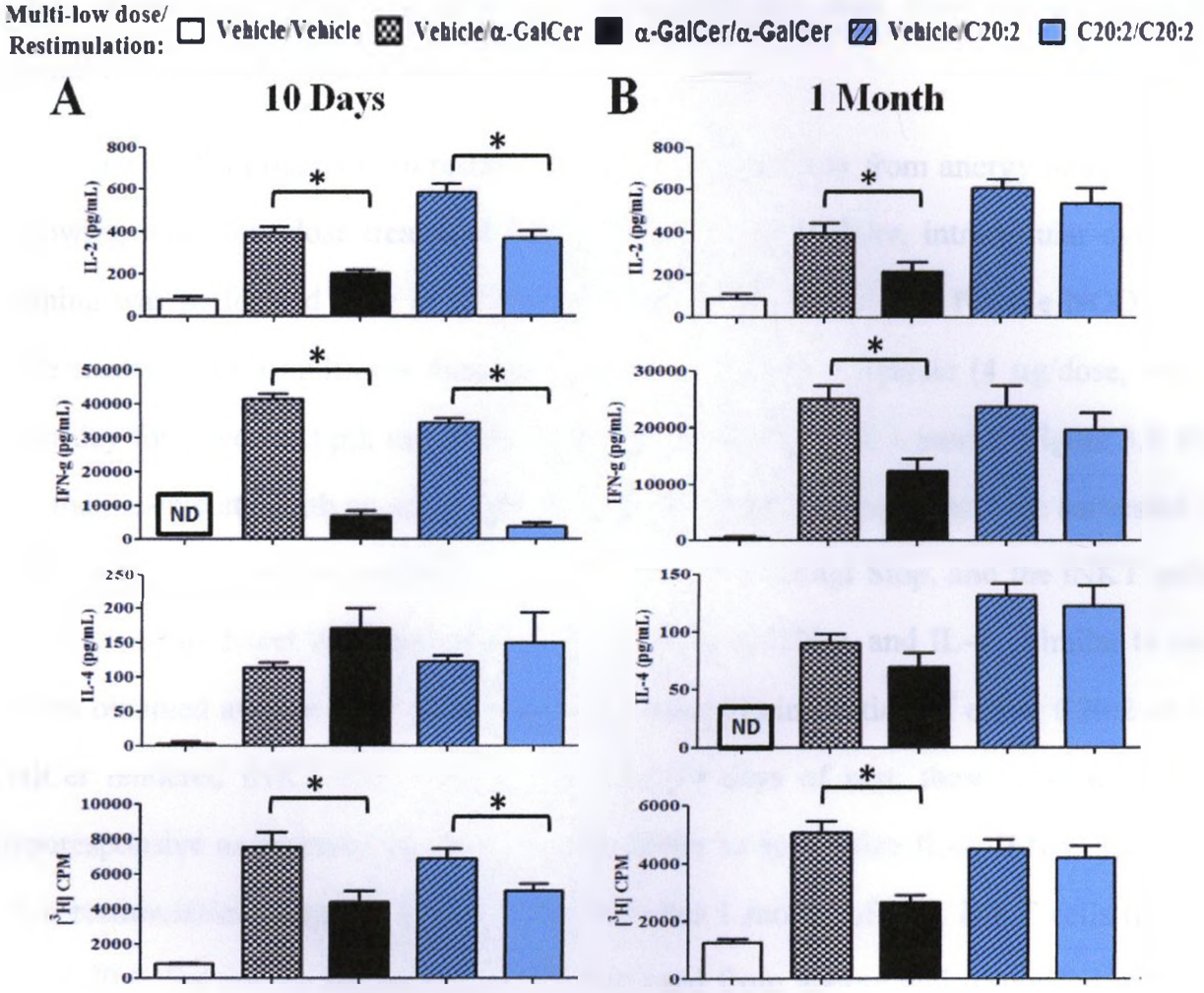
Figure 3.6 Multi-low dose treatment with α-GalCer or C20:2 alters the iNKT cell frequencies in the spleen. Female NOD mice (4-6 week-old) were treated with a multi-low dose protocol of α-GalCer, C20:2 or vehicle (4 μg/dose every other day for three weeks, i.p.) and rested for 1 month. Leukocytes from the spleen **(A)** and PLN **(B)** were isolated and the frequencies of $TCR\beta^{+}tetramer^{+}$ iNKT cells were analyzed by flow cytometry. Data are representative of two independent experiments yielding similar results and are expressed as mean ± SD. N = 5 mice/treatment group/time point. *, significant ($P<0.05$) difference between the vehicle and glycolipid treatment groups. †, significant ($P<0.05$) difference between α-GalCer and C20:2 treatment values.


Multi-low dose Treatment: Vehicle α-GalCer C20:2
A
Spleen
% iNKT Cells
0.8
0.6
0.4
0.2
0.0
†
*
*
B
PLN
% iNKT Cells
0.6
0.4
0.2
0.0

3.3.2 Analysis of total splenocyte responses after in vitro restimulation with glycolipid following a multi-low dose treatment protocol

Having established the kinetics of anergy induction for a single dose treatment with either α-GalCer or C20:2, it was of interest to also investigate the effects of multi-low dose administration of glycolipid. The underlying rationale is that a multi-low dose protocol of glycolipid may have greater clinical relevance for the protection from T1D in human subjects. Briefly, female NOD mice were injected with a multi-low dose protocol of glycolipid or vehicle (4 μg/dose, every other day for 3 weeks, i.p.) and then rested for 10 days (Figure 3.7 A) or 1 month (Figure 3.7 B). Splenocytes were then restimulated *in vitro* with 100 ng/mL of glycolipid for 72 h, and were then assayed for their proliferative capacity and cytokine (IL-2, IFN-γ, and IL-4) secretion responses. iNKT cells remained anergic 10 days after a multi-low dose treatment of either glycolipid (Figure 3.7 A), as revealed by the diminished IL-2 and IFN-γ responses to *in vitro* restimulation relative to the primary immune response (Vehicle/α-GalCer or Vehicle/ C20:2). Similarly, the splenocyte proliferative response was also reduced. Interestingly, at the 1 month time point, iNKT cells from mice that received a multi-low dose administration of C20:2 were fully recovered from anergy, however this was not observed for iNKT cells from mice treated with α-GalCer. The IL-2, IFN-γ, and proliferative responses from the C20:2 treatment group all returned to levels that were equal to that of the primary response (Vehicle/C20:2), while those treated with α-GalCer were still significantly reduced. Consistent with the *in vitro* results obtained following the administration of a single dose of glycolipid, secreted levels of IL-4 were consistent throughout the time course for both multi-low dose glycolipid treatments after restimulation. These results indicate that iNKT cells recover from anergy by 1 month after treatment with either a single dose or multi-low dose of C20:2, and this time of recovery is considerably more rapid than that observed for α-GalCer.

Figure 3.7 ***In vitro*** **responses of total splenocytes recover more rapidly from a multi-low dose protocol of C20:2 compared to α-GalCer.** Female NOD mice (4-6 week-old) were administered a multi-low dose protocol (4 μg/dose every other day for 3 weeks, i.p.) of glycolipid or vehicle. Splenocytes were then isolated 10 days **(A)** or 1 month **(B)** after the *in vivo* treatment and restimulated *in vitro* with 100 ng/mL of glycolipid for 72 h. The proliferative capacity and levels of cytokine secretion by these splenic iNKT cells were assayed by ^{3}H-thymidine incorporation and ELISAs, respectively. Data are expressed as mean ± SD. N = 5 mice/treatment group/time point. *, significant ($P<0.05$) difference between indicated treatment groups.


Multi-low dose/
Restimulation:
Vehicle/Vehicle
Vehicle/α-GalCer
α-GalCer/α-GalCer
Vehicle/C20:2
C20:2/C20:2
A
10 Days
B
1 Month
IL-2 (pg/mL)
IFN-g (pg/mL)
IL-4 (pg/mL)
[3H] CPM
ND
ND
*

3.3.3 Analysis of iNKT cell responses after multi-low dose treatment followed by glycolipid restimulation in vivo

To confirm our *in vitro* results that iNKT cells recover from anergy more rapidly following multi-low dose treatment with C20:2 versus α-GalCer, intracellular cytokine staining was performed after *in vivo* restimulation with glycolipid. Female NOD mice were treated with a multi-low dose protocol of glycolipid or vehicle (4 μg/dose, every other day for 3 weeks, i.p.), rested for 10 days (Figure 3.8 A) or 1 month (Figure 3.8 B), and then re-injected with an additional 4 μg of glycolipid. Splenocytes were harvested 2 h after the final injection, cultured *in vitro* for 3 h with Golgi Stop, and the iNKT cells were stained to detect their intracellular levels of IL-2, IFN-γ, and IL-4. Similar to our results obtained after *in vitro* restimulation, chronic administration of either C20:2 or α-GalCer rendered iNKT cells anergic and after 10 days of rest, these cells were still hyporesponsive as assessed by their reduced ability to synthesize IL-2, IFN-γ, and IL-4 upon restimulation (Figure 3.8 A). However, after 1 month of rest, iNKT cells treated with C20:2, but not α-GalCer, were fully recovered from anergy and responded with an equal intensity to iNKT cells experiencing a primary immune response (Vehicle/C20:2) (Figure 3.8 B).

3.3.4 Analysis of PD-1 and PD-L1 surface expression on iNKT cells following a multi-low dose treatment with glycolipid

Due to the importance of PD-1 and PD-L1 interaction for the induction of iNKT cell anergy, the levels of PD-1 and PD-L1 surface expression were analyzed following a multi-low dose treatment with α-GalCer or C20:2. Female NOD mice were treated with a multi-low dose protocol of glycolipid or vehicle (4 μg/dose, every other day for 3 weeks, i.p.) and rested for 10 days (Figure 3.9 A and B) or 1 month (Figure 3.9 C and D). Spleen- and PLN- derived iNKT cells were analyzed by flow cytometry for their surface

Figure 3.8 iNKT cells recover from anergy more rapidly following multi-low dose treatment with C20:2 than α-GalCer. Female NOD mice (4-6 week-old) were treated with a multi-low dose protocol of α-GalCer, C20:2 or vehicle (4 μg/dose every other day for three weeks, i.p.) and rested for either 10 days **(A)** or 1 month **(B)**. Mice were then reinjected with an additional dose (4 μg, i.p.) of glycolipid. Splenocytes were harvested 2 h after the final injection, cultured *in vitro* for 3 h in the presence of a protein transport inhibitor, Golgi Stop, and $TCR\beta^{+}$tetramer^{+} iNKT cells were stained for intracellular levels of IL-2, IFN-γ, and IL-4. Data are representative of two independent experiments yielding similar results and are expressed as mean ± SD. N = 5 mice/treatment group/time point. *, significant ($P<0.05$) reduction between primary and recall responses (Vehicle multi-low dose/α-GalCer vs. α-GalCer multi-low dose/α-GalCer, Vehicle multi-low dose/C20:2 vs. C20:2 multi-low dose/C20:2).

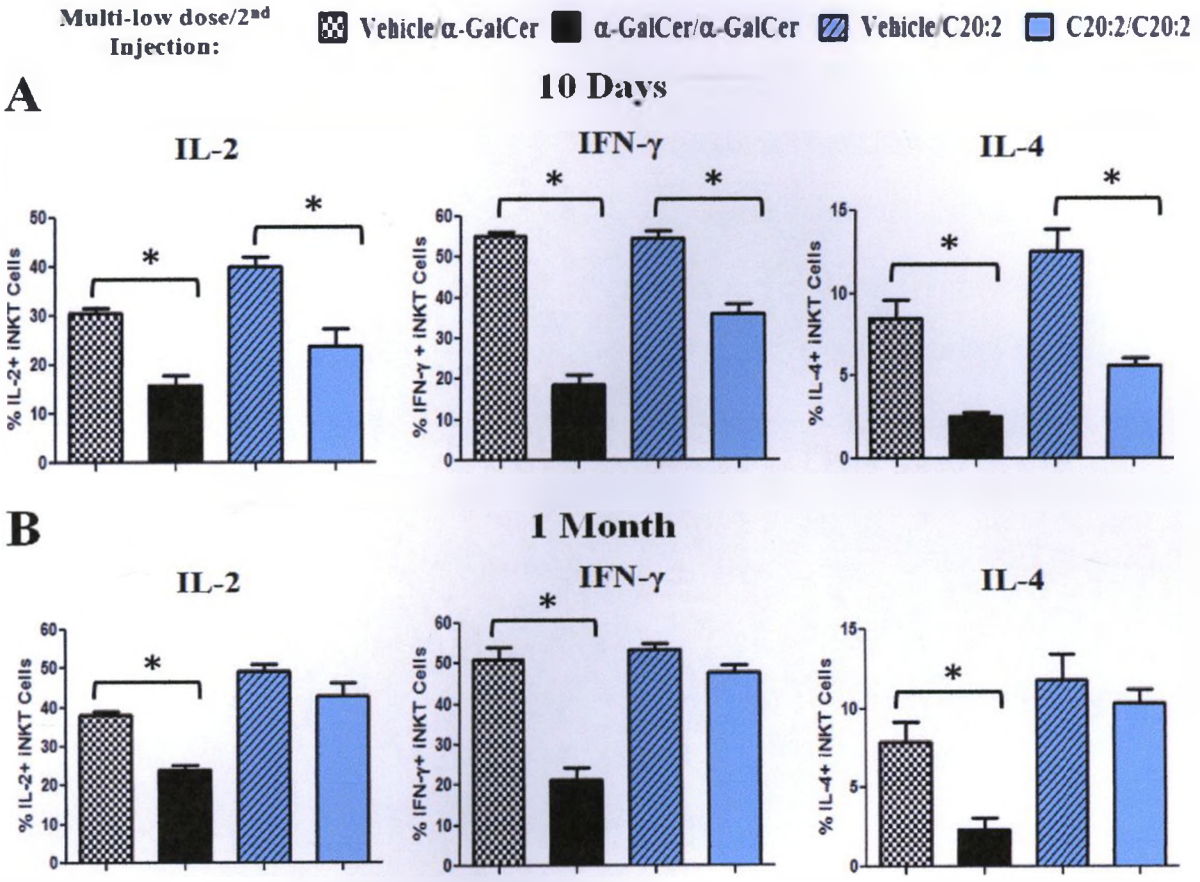
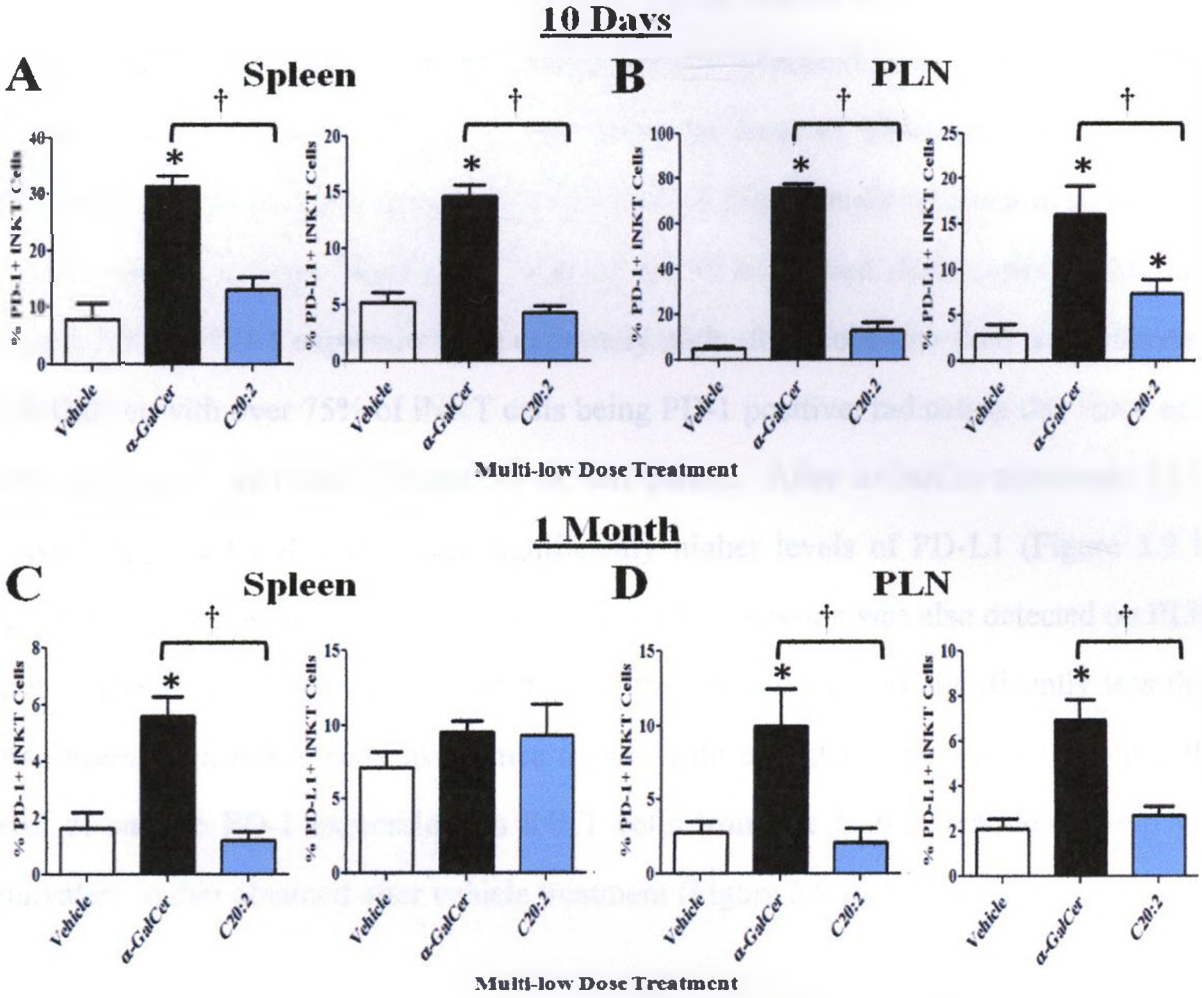

Multi-low dose/2nd Injection:
Vehicle/α-GalCer
α-GalCer/α-GalCer
Vehicle/C20:2
C20:2/C20:2
A
10 Days
IL-2
IFN-γ
IL-4
% IL-2+ iNKT Cells
% IFN-γ + iNKT Cells
% IL-4+ iNKT Cells
B
1 Month
IL-2
IFN-γ
IL-4
% IL-2+ iNKT Cells
% IFN-γ+ iNKT Cells
% IL-4+ iNKT Cells

Figure 3.9 iNKT cells maintain high levels of PD-1 and PD-L1 surface expression for a longer duration following a multi-low dose treatment with α-GalCer than C20:2. Female NOD mice (4-6 week-old) were treated according to a multi-low dose protocol of α-GalCer, C20:2 or vehicle (4 μg/dose every other day for three weeks, i.p.) and rested for either 10 days **(A, B)** or 1 month **(C, D)**. Lymphocytes from the spleen **(A, C)** and PLN **(B, D)** were isolated and the levels of PD-1 and PD-L1 surface expression on $TCR\beta^{+}tetramer^{+}$ iNKT cells were analyzed by flow cytometry. Data are representative of two independent experiments yielding similar results and are expressed as mean ± SD. N = 5 mice/treatment group/time point. *, significant ($P<0.05$) difference between the vehicle and glycolipid treatment groups. †, significant ($P<0.05$) difference between α-GalCer and C20:2 treatment values.


10 Days
A
Spleen
B
PLN
% PD-1+ iNKT Cells
% PD-L1+ iNKT Cells
Vehicle
α-GalCer
C20:2
Multi-low Dose Treatment
1 Month
C
Spleen
D
PLN
Multi-low Dose Treatment

expression of PD-1 and PD-L1. After 10 days of rest, splenic iNKT cells from α-GalCer, but not C20:2, treated mice expressed a significantly increased level of PD-1 and PD-L1 on their surface (Figure 3.9 A). At this time, the level of PD-1 and PD-L1 surface expression on splenic iNKT cells from C20:2 treated mice already returned to steady state (vehicle) levels. Similar data were obtained for PLN-derived iNKT cells at this time (Figure 3.9 B). PD-1 expression was extremely high after multi-low dose administration of α-GalCer with over 75% of iNKT cells being PD-1 positive, indicating that these cells were still highly activated (Figure 3.9 B, left panel). After α-GalCer treatment, PLN-derived iNKT cells also exhibited significantly higher levels of PD-L1 (Figure 3.9 B, right panel). Although a similar increase in PD-L1 expression was also detected on PLN-derived iNKT cells from C20:2 treated mice, this expression was significantly less than that observed on iNKT cells from mice treated with α-GalCer. However, note that the level of surface PD-1 expression on iNKT cells from the C20:2 treatment group was equivalent to that obtained after vehicle treatment (Figure 3.9 B, left panel).

Between 10 days and 1 month post-treatment with α-GalCer, the levels of PD-1 expression on iNKT cells were decreased appreciably in the spleen and PLN (Figure 3.9, C and D), as reflected by reductions in the percentage of PD-1^+ iNKT cells from approximately 32% to 6% and 80% to 10% in the spleen and PLN, respectively. However, the frequency of PD-1^+ iNKT cells observed after α-GalCer treatment was still significantly greater than that observed after treatment with C20:2 or vehicle (Figure 3.9, C and D, left panels). In contrast, at 1 month post-treatment, iNKT cells in the spleen from mice that received multi-dose α-GalCer expressed levels of surface PD-L1 comparable to that of iNKT cells from mice treated with multi-dose C20:2 or vehicle (Figure 3.9, C, right panel). Nonetheless, this was not the case for iNKT cells in the PLN as α-GalCer treated cells still displayed elevated expression levels of PD-L1 relative

to both C20:2 and vehicle values (Figure 3.9, D, right panel). Finally, the elevated PD-L1 expression levels noted in the PLN after C20:2 administration returned to vehicle values by 1 month post-treatment (Figure 3.9, B and D, right panels). Thus, the results obtained for PD-1 and PD-L1 surface expression on iNKT cells after multi-low dose glycolipid treatment closely parallel those observed after single dose glycolipid treatment. Importantly, the shorter duration of PD-1 and PD-L1 upregulation on iNKT cells following C20:2 treatment correlates directly with the faster recovery of iNKT cells from anergy under these conditions of stimulation.

3.4 Activation of iNKT cells with glycolipid alters the frequency and function of DCs

DCs are one of the predominant types of APCs that can activate iNKT cells. Numerous studies have shown that in response to α-GalCer or other glycolipids, iNKT cells rapidly secrete a large amount of cytokines and upregulate CD40L expression on their surface, which in turn leads to the activation of DCs [55, 63, 98, 155, 191]. Depending on the iNKT cell agonist, different DC subsets may be preferentially activated or recruited to various immunological sites, which may enhance or suppress many downstream immunological events [98, 128, 172, 173]. Since α-GalCer is a very potent agonist, the strong response elicited by α-GalCer stimulated iNKT cells can result in DC lysis. Therefore, it is important to investigate the effects of α-GalCer and C20:2 on DC activation and function.

3.4.1 Multi-low dose administration of glycolipid decreases mDC frequency

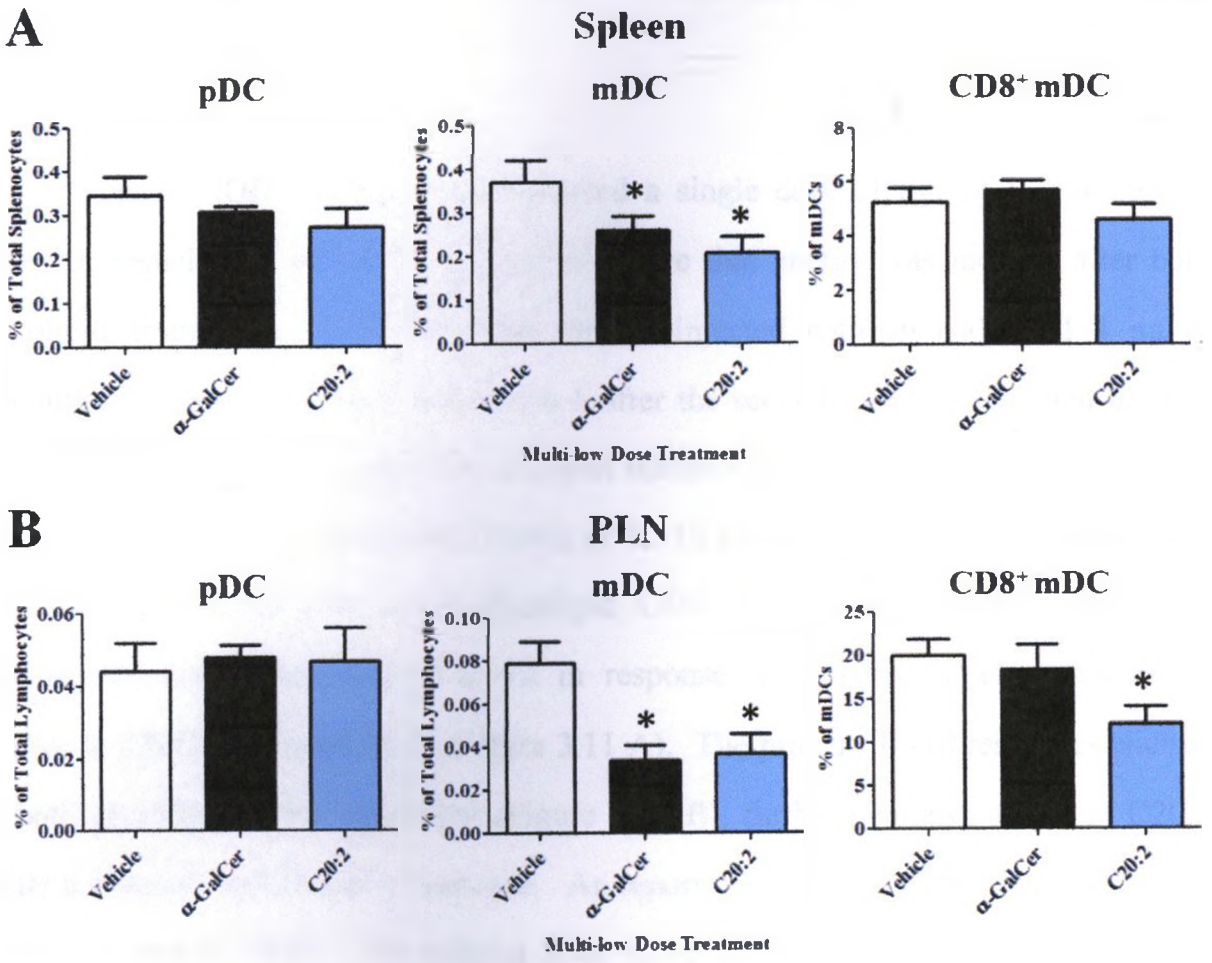
To identify the effect of a protective multi-low dose glycolipid treatment protocol on DCs, the frequencies of various DC subsets were analyzed following this treatment. Female NOD mice were administered a multi-low dose treatment with α-GalCer, C20:2 or vehicle (4 μg/dose every other day for three weeks, i.p.) and rested for 1 month.

Spleen (Figure 3.10 A) and PLN (Figure 3.10 B) lymphocytes were isolated, stained for pDCs (Siglec H^+ $CD11c^{low}$) and mDCs (Siglec H^- $CD11c^{high}$), and analyzed by flow cytometry. The mDC subpopulation was further separated into the $CD8^+$ and $CD8^-$ subsets. Chronic administration of both α-GalCer and C20:2 resulted in a significant decrease in the frequency of total mDCs in the spleen and PLN (Figure 3.10, A and B, middle panels). In addition, C20:2 treatment also significantly reduced the frequency of $CD8^+$ mDCs, thereby altering the $CD8^+$:$CD8^-$ mDC ratio in the PLN, but not the spleen (Figure 3.10, A and B, right panels). Neither glycolipid treatment altered the pDC frequency in the spleen or PLN (Figure 3.10, A and B, left panels). These results demonstrate that both glycolipids preferentially alter the frequency of mDCs, suggesting that this DC subset may be responsible for presenting the majority of glycolipid to iNKT cells. In addition, it is known that $CD8^+$ mDC also mediate pro-inflammatory responses. Therefore, it may be beneficial that both glycolipids reduce the total mDC frequency in the spleen and PLNs, since the consequence would be an overall reduction of the $CD8^+$ mDC subset. Interestingly, treatment with C20:2, but not α-GalCer, also decreases the frequency of $CD8^+$ mDCs within the mDC population, thereby further reducing the frequency of total $CD8^+$ mDCs in the PLNs.

3.4.2 Restimulation with glycolipid alters the phenotypic response of $CD8^+$ mDCs

Murine $CD8^+$ mDCs express significantly higher amounts of CD1d than other DC subsets and are also a major source of the inflammatory cytokine IL-12 *in vivo* [16-18]. Upon activation, mDCs can profoundly influence downstream inflammatory responses. It was recently reported that upon glycolipid restimulation, anergic iNKT cells can induce the function of tolerogenic DCs, which are phenotypically characterized by their decreased IL-12 and increased IL-10 secretion responses [173]. Since our previous results indicated that multi-low dose administration of α-GalCer or C20:2 preferentially

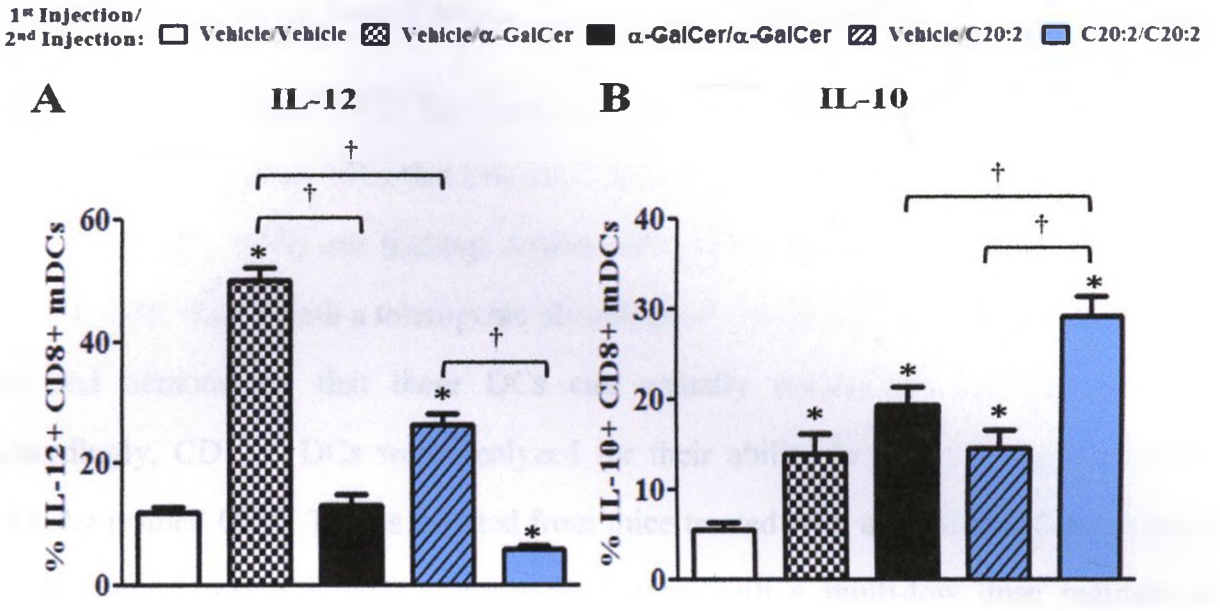
Figure 3.10 Multi-low dose treatment with α-GalCer or C20:2 alters DC frequencies. Female NOD mice (4-6 week-old) were treated with a multi-low dose protocol of α-GalCer, C20:2 or vehicle (4 μg/dose every other day for three weeks, i.p.). One month after the final injection, leukocytes from the spleen **(A)** and PLN **(B)** were collected. The frequencies of pDCs (Siglec H^+ $CD11c^{low}$) and mDCs (Siglec H^- $CD11c^{high}$), which were further separated into $CD11c^{high}$ $CD8^+$ and $CD11c^{high}$ $CD8^-$ subsets, were analyzed by flow cytometry. Data are representative of two independent experiments yielding similar results and are expressed as mean ± SD. N = 5 mice/treatment group/time point. *, significant ($P<0.05$) difference between the vehicle and glycolipid treatment groups.


A
Spleen
pDC
mDC
CD8+ mDC
% of Total Splenocytes
% of mDCs
Vehicle
α-GalCer
C20:2
Multi-low Dose Treatment
B
PLN
pDC
mDC
CD8+ mDC
% of Total Lymphocytes
% of mDCs
Vehicle
α-GalCer
C20:2
Multi-low Dose Treatment

reduced the frequency of mDCs, the primary and recall responses of $CD8^+$ mDCs to both glycolipids was next investigated.

Female NOD mice were administered a single dose (4 μg, i.p.) of α-GalCer, C20:2 or vehicle and rested for 1 week to ensure that anergy was induced after both glycolipid treatments. The mice were then re-injected with an additional 4 μg of glycolipid. Splenocytes were harvested 6 h after the second injection, cultured *in vitro* for 3 h in the presence of the protein transport inhibitor, Golgi Stop, and $CD11c^{high}CD8^+$ mDCs were stained for intracellular levels of IL-12 (Figure 3.11 A) and IL-10 (Figure 3.11 B). Upon initial exposure to glycolipid, $CD8^+$ mDCs responded by synthesizing significantly greater amounts of IL-12 in response to α-GalCer (Vehicle/α-GalCer) relative to C20:2 (Vehicle/C20:2) (Figure 3.11 A). The primary IL-10 responses elicited by both glycolipids were equivalent (Figure 3.11 B), further demonstrating that C20:2 elicits a reduced inflammatory response. As reported [173], the $CD8^+$ mDC responses elicited by anergic iNKT cells differed from those elicited by non-anergic iNKT cells after a recall response (α-GalCer/α-GalCer, C20:2/C20:2). Restimulation of anergic iNKT cells with α-GalCer significantly blunted the IL-12 response to the level observed for vehicle treatment (Figure 3.11 A). In addition, there was a slight increase in intracellular IL-10 levels, however this increase was not significant ($p>0.05$). In contrast, restimulation of anergic iNKT cells with C20:2 induced a more pronounced shift in the $CD8^+$ mDC response. While there was a considerable decrease in intracellular IL-12 synthesis to a level that was significantly lower than the vehicle level, there was also a significant (about two-fold) increase in IL-10 production (Figure 3.11, A and B). Thus, multiple doses of C20:2 induce $CD8^+$ mDCs that possess a greater anti-inflammatory and possibly "tolerogenic" phenotype than those elicited by α-GalCer.

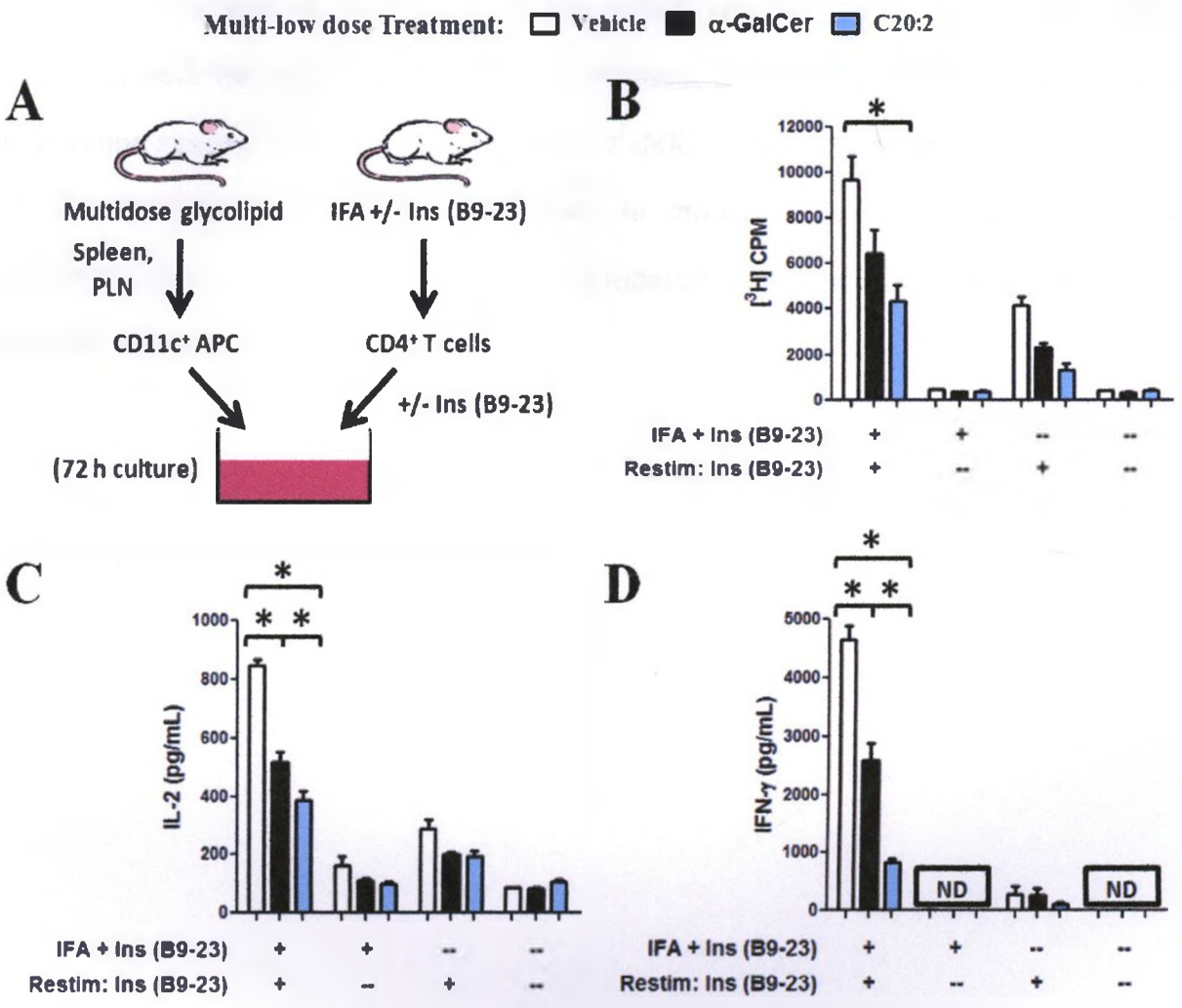
Figure 3.11 Primary and recall responses of CD11c^{high}CD8^{+} mDCs to α-GalCer and C20:2. Female NOD mice (4-6 week-old) were treated with a single dose (4 μg, i.p.) of α-GalCer, C20:2 or vehicle and rested for 1 week to induce iNKT cell anergy. Mice were then re-injected with an additional dose (4 μg, i.p.) of glycolipid. Splenocytes were harvested 6 h after the final injection, cultured *in vitro* for 3 h in the presence of a protein transport inhibitor, Golgi Stop, and CD11c^{high}CD8^{+} mDCs were stained for intracellular levels of IL-12 **(A)** and IL-10 **(B)**. Data are representative of two independent experiments yielding similar results. N = 5 mice/treatment group. *, significant ($P<0.05$) difference between the Vehicle/Vehicle and glycolipid treatment groups. †, significant ($P<0.05$) difference between indicated values.


1st Injection/
2nd Injection:
Vehicle/Vehicle
Vehicle/α-GalCer
α-GalCer/α-GalCer
Vehicle/C20:2
C20:2/C20:2
A
IL-12
% IL-12+ CD8+ mDCs
0
20
40
60
B
IL-10
% IL-10+ CD8+ mDCs
0
10
20
30
40

3.4.3 Multi-low dose treatment with glycolipid alters DC function

Protection from T1D by treatment with α-GalCer results in part from the induction of tolerogenic DCs that migrate to the PLN and suppress autoreactive T cells [137, 171, 173]. While our findings demonstrated that repeated administration of C20:2 induced $CD8^+$ mDCs with a tolerogenic phenotype, it was important to functionally prove this and demonstrate that these DCs can actually transfer Ag-specific tolerance. Accordingly, $CD11c^+$ DCs were analyzed for their ability to induce a recall response from Ag-primed $CD4^+$ T cells isolated from mice treated with a multi-low dose protocol of glycolipid. Female NOD mice were treated with a multi-low dose regimen of glycolipid or vehicle (4 μg/dose every other day for three weeks, i.p.) and rested for one week. $CD11c^+$ DCs were then isolated and pooled from the spleen and PLNs of these mice. Concurrently, another group of female NOD mice received a single injection (100 μg, i.p.) of the Ins B9-23 peptide or vehicle (1x PBS) emulsified in IFA to generate an immune response towards the peptide. After 10 days of rest, $CD4^+$ T cells were isolated and pooled from the spleens of these mice. $CD11c^+$ cells from mice treated with multi-low dose glycolipid were then cultured with peptide primed $CD4^+$ T cells and restimulated *in vitro* with 100 μg /mL of Ins B9-23 peptide for 72 h (Figure 3.12 A). $CD11c^+$ DCs from the vehicle treated NOD mice elicited a robust activation of primed $CD4^+$ T cells, as evidenced by the induction of cell proliferation as well as IL-2 and IFN-γ secretion in the presence of recall Ag (Figure 3.12, B-D). IL-4 secretion was not detected in any of the cultures (data not shown). In contrast, *in vivo* treatment of NOD mice with C20:2 was more effective than that of α-GalCer in decreasing the stimulatory capacity of $CD11c^+$ DCs. While α-GalCer treated DCs elicited a significantly reduced IL-2 and IFN-γ response from the $CD4^+$ T cells compared to vehicle, the reduced proliferation observed was not significant ($p>0.05$). However, multi-low dose treatment with C20:2 altered DC function in a manner that significantly reduced the $CD4^+$ T cell

Figure 3.12 Multi-low dose stimulation of iNKT cells with C20:2 alters the function of DCs. Female NOD mice (4-5 week-old) were treated with a multi-low dose protocol of α-GalCer, C20:2 or vehicle (4 μg/dose, every other day for 3 weeks). One week after the final dose, spleen and PLN suspensions were pooled and $CD11c^+$ DCs were isolated from each group. Concurrently, Ins B9-23 peptide or vehicle (PBS) emulsified in IFA was administered (100 μg, i.p.) to a separate group of female NOD mice to prime their $CD4^+$ T cells. Ins B9-23 peptide primed-$CD4^+$ T cells were isolated and cultured with $CD11c^+$ DCs in the presence of Ins B9-23 (100 μg/mL) for 72 h **(A)**. A T cell proliferation assay **(B)** as well as IL-2 **(C)** and IFN-γ **(D)** ELISA assays were performed. Data are representative of two independent experiments yielding similar results. *, significant ($P<0.05$) difference between compared groups. ND, not detected.


Multi-low dose Treatment: Vehicle α-GalCer C20:2
A
Multidose glycolipid
IFA +/- Ins (B9-23)
Spleen, PLN
CD11c+ APC
CD4+ T cells
+/- Ins (B9-23)
(72 h culture)
B
[3H] CPM
12000
10000
8000
6000
4000
2000
0
IFA + Ins (B9-23) + + -- --
Restim: Ins (B9-23) + -- + --
C
IL-2 (pg/mL)
1000
800
600
400
200
0
IFA + Ins (B9-23) + + -- --
Restim: Ins (B9-23) + -- + --
D
IFN-γ (pg/mL)
5000
4000
3000
2000
1000
0
ND
ND
IFA + Ins (B9-23) + + -- --
Restim: Ins (B9-23) + -- + --

proliferative response as well as IL-2 and IFN-γ secretion compared to the results obtained in both the vehicle and α-GalCer treatment groups (Figure 3.12, B-D). These observations suggest that chronic activation of iNKT cells by synthetic glycolipids can alter DC function, and that C20:2 appears to induce DCs that possess a reduced stimulatory (more tolerogenic) capacity compared to those DCs stimulated upon treatment with α-GalCer.

Chapter 4:

Discussion

4.1 Summary of results

T1D is an inflammatory autoimmune disease that is characterized by the T cell mediated destruction of insulin-producing pancreatic β cells. It is widely believed that T1D arises, at least in part, due to immune dysregulation [98, 102]. In particular, two main regulatory cell populations, T_{regs} and iNKT cells, have been identified as being numerically and functionally deficient in NOD mice [47-51, 98, 128-130]. Similar results have also been observed in human diabetic patients, although there are still conflicting results in the literature [47-51, 98, 128-130]. These inconsistencies, however, may be due to the large variability that exists within the human population as well as the use of only peripheral blood samples for analysis [47-51, 98, 128-130]. Regardless, numerous studies have focussed on activating and expanding these subsets for therapeutic purposes.

The dual ability to both activate and suppress immune responses make iNKT cells a very promising target for potential drug therapies. The potent iNKT cell agonist, α-GalCer can protect NOD mice against T1D when administered in a multi-low dose protocol [128-130, 137]. While α-GalCer has provided invaluable insight into the mechanisms of iNKT cell activation, due to the very strong cytokine responses and long-lasting transactivation of other immune cells that are elicited, it may not be a suitable drug candidate for the treatment of T1D. The α-GalCer derivative C20:2 is also capable of mediating protection against T1D, and there is evidence to suggest that it does so with higher efficacy than α-GalCer [185]. The overall reduced and predominantly T_H2 response elicited by C20:2 allows this compound to protect against T1D without the requirement for T_{reg} activity, which is not the case for α-GalCer (Ly et. al, 2009, in press, Appendix A).

This study attempted to further characterize the activation of iNKT cells in response to C20:2 administration as well as provide additional insight into the role of anergy induction in the protection against T1D in NOD mice. Consistent with previous studies, in response to a single dose of C20:2, iNKT cells elicited a greater T_H2 response with increased IL-4 and decreased IFN-γ secretion relative to α-GalCer. Interestingly, C20:2-activated iNKT cells did not experience the robust expansion phase that has been reported for α-GalCer treated cells [157]. In addition, C20:2 administration resulted in the faster induction of and recovery from iNKT cell anergy. This property may be very beneficial for future therapeutic use, due to the potential for the faster induction of tolerogenic DCs and a reduction in activation induced cell death. After a multi-low dose treatment with C20:2, the activated iNKT cells exhibited an increased survival rate and also induced DCs that possessed a significantly reduced stimulatory capacity relative to those treated with α-GalCer. These findings suggest that C20:2 may be a more beneficial drug for the treatment of T1D than α-GalCer and warrants further investigation as a candidate for clinical trials in humans at risk for T1D.

4.2 The structure and presentation of glycolipid agonists significantly impacts iNKT cell responses

In comparison to α-GalCer, C20:2 contains a fatty acyl side chain shortened from C26 to C20 as well as two double bonds at carbons 11 and 14 (Figure 1.7). Treatment with C20:2 resulted in a greater T_H2 response with increased levels of IL-4 and decreased IFN-γ during their peak secretion times both *in vitro* and *in vivo,* thus confirming previous results (Figure 3.1) [184]. Interestingly, the IFN-γ response elicited *in vivo* by C20:2 administration was more rapid, but less sustained than α-GalCer as indicated by the 2 h and 24 h time points, respectively (Figure 3.1). In addition, the iNKT cell expansion profile was also significantly altered between α-GalCer and C20:2. Whereas

the injection of α-GalCer elicited a robust iNKT cell expansion in both the spleen and PLNs that peaked after 72 h, C20:2 activated iNKT cells did not expand at all in the spleen and only exhibited a minor (about 2-fold) expansion in the PLN (Figure 3.2).

Two main reasons may explain these results. Firstly, the altered chemical structure of C20:2 may lead to an altered pathway of presentation of this glycolipid by the CD1d molecule and therefore elicit a different iNKT cell response. The A' channel in CD1d, which contains the fatty acyl side chain of glycolipids during presentation, can accommodate chains consisting of a maximum of 26 carbons in length [61]. It has been proposed that while α-GalCer completely fills this space, a compound containing a reduced fatty acyl chain length, such as C20:2, may result in the partial collapse of the unfilled portion of the A' channel, resulting in surface exposed structural changes and potentially an altered response [192].

A second, but not mutually exclusive explanation may revolve around the requirements of both glycolipids to load onto CD1d molecules. Loading of α-GalCer onto CD1d requires many cofactors such as saposins, GM2 activator proteins, and Niemann-Pick type C1 and C2 proteins, which are known to have detergent-like activities that facilitate the solubilization of lipids and their exchange between hydrophobic binding sites [59, 187, 193-195]. As such, α-GalCer must be internalized before it can load onto CD1d in the late endosome or lysosome [59, 187, 193-195]. Conversely, C20:2 has the ability to load directly onto CD1d molecules present on the surface of APCs without the need for internalization [189]. Further analysis revealed that C20:2 can be detected in complex with CD1d as quickly as 30 min after its addition to culture, while the majority of α-GalCer:CD1d complexes were not detected until 18 h [189]. The earlier loading onto CD1d may result in a more rapid activation of iNKT cells and therefore be

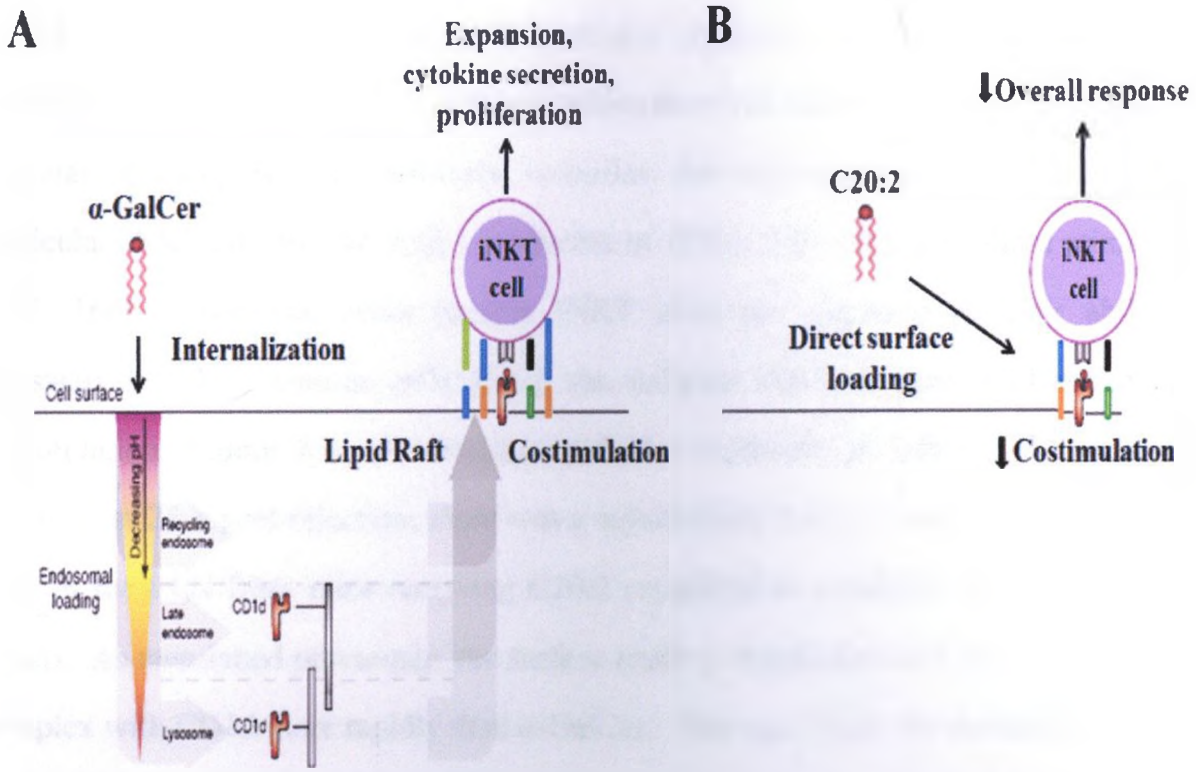
responsible for the increased IFN-γ response observed in the serum at 2 h after C20:2 administration (Figure 3.1 B, left panel) (Ly et. al, 2009, in press, Appendix A).

As a consequence of the differential CD1d loading requirements of α-GalCer and C20:2, each glycolipid is presented to iNKT cells in a different manner. Due to the internalization of α-GalCer, upon loading onto a CD1d molecule, the glycolipid:CD1d complex is shuttled to the surface and presented to iNKT cells in the context of a lipid raft [59, 196, 197]. Importantly, lipid rafts contain an abundance of co-stimulatory molecules and have been shown to be able to deliver iNKT cell activation signals at suboptimal concentrations of Ag [197]. Due to the direct loading of C20:2 onto surface CD1d molecules, the majority of C20:2 molecules are presented to iNKT cells outside of lipid rafts (Figure 4.1) [188, 189]. This critical difference may play a large role in explaining the observed differences in iNKT cell expansion between α-GalCer and C20:2 as well as the prolonged IFN-γ response by α-GalCer stimulated iNKT cells. Proper costimulation has been shown to be required for iNKT cell expansion *in vivo* [198]. In addition, in the presence of costimulation blockade (anti-CD86), iNKT cells are polarized towards a T_H2 cytokine response [199]. The fact that C20:2 is presented outside of lipid rafts, where there is a reduced density of costimulatory molecules, may be responsible for the reduced iNKT cell expansion as well as the greater T_H2 response. Taken together, these results indicate that minor structural changes to glycolipid agonists can lead to large scale differences in iNKT cell responses.

4.3 C20:2 administration leads to a more rapidly induced and less sustained state of anergy in iNKT cells compared to α-GalCer administration

After treatment of NOD mice with a single dose of glycolipid, both α-GalCer and C20:2 induced iNKT cell anergy *in vitro* and *in vivo* (Figures 3.3 and 3.4). However,

Figure 4.1 Differential CD1d loading requirements of α-GalCer and C20:2. (A) The glycolipid α-GalCer requires internalization and the assistance of intracellular lipid transfer proteins to load onto CD1d in the late endosome or lysosome. The α-GalCer:CD1d complex is then shuttled to the surface and presented to iNKT cells in the context of a lipid raft, which is abundant in costimulatory molecules. **(B)** Conversely, C20:2 has the ability to load directly onto CD1d molecules present on the surface of APCs. As a result, the majority of C20:2:CD1d complexes are located outside of lipid rafts. The reduced amount of costimulation present outside of lipid rafts may account for the overall reduced immune response observed after C20:2 administration, compared to α-GalCer. Adapted from Dascher and Brenner, 2003. [59]


A
Expansion,
cytokine secretion,
proliferation
α-GalCer
iNKT
cell
Internalization
Cell surface
Lipid Raft
Costimulation
Decreasing pH
Recycling
endosome
Endosomal
loading
Late
endosome
CD1d
Lysosome
CD1d
B
Overall response
C20:2
iNKT
cell
Direct surface
loading
Costimulation

C20:2 administration resulted in the faster induction of iNKT cell anergy as shown by the more rapid reduction in IFN-γ observed at 48 h *in vitro* (Figure 3.3) and intracellular IL-2 at 72 h (Figure 3.4). While reduced intracellular cytokine staining is a direct measure of iNKT cell anergy, the decreased IFN-γ response observed following *in vitro* restimulation of total splenocytes also indirectly identifies the hyporesponsive iNKT cells. In particular, NK cells are the major producers of IFN-γ following glycolipid stimulation [158, 160]. However, since anergic iNKT cells are impaired in their ability to transactivate other immune cells [157], the reduced IFN-γ responses observed upon restimulation (Figure 3.3 A) correlates with the induction of iNKT cell anergy. In addition, at 24 h post-injection, there was a significantly greater amount of PD-1^+ iNKT cells in the PLN from mice receiving C20:2 compared to α-GalCer (Figure 3.5 B, left panel). As mentioned previously, the surface loading capabilities of C20:2 allow for it to complex with CD1d more rapidly than α-GalCer. This may allow for the faster activation of iNKT cells and therefore lead to the more rapid upregulation of PD-1 and the subsequent induction of anergy.

Since it has been well established that only multiple doses of glycolipid are able to protect NOD mice from T1D [128-130, 137], iNKT cell anergy appears to play an important role in this protection. Initial studies determined that multiple α-GalCer administrations provided favourable benefits in part due to the T_H2 polarization of the immune response that was elicited as well as the induction of tolerogenic DCs in the PLNs [128, 171, 172]. Subsequent analysis revealed that restimulation of anergic iNKT cells resulted in a significantly blunted IFN-γ response while maintaining low levels of IL-4 secretion, thus resulting in T_H2 skewing [157]. Furthermore, these anergic iNKT cells were shown to promote the differentiation of DCs with tolerogenic capabilities upon restimulation [173]. In contrast, in the interval between the initial activation and the induction of anergy, iNKT cells promote immunogenic DCs [173]. Therefore, it appears

that restimulation of anergic iNKT cells may be responsible for the protective effects observed upon treatment of T1D with a suitable glycolipid.

Throughout the experiments conducted, a common theme emerged where C20:2 administration resulted in a faster recovery of iNKT cell anergy relative to α-GalCer both *in vitro* and *in vivo*. This phenomenon occurred regardless of the treatment protocol as both single (Figures 3.3 and 3.4) and multiple (Figures 3.7 and 3.8) doses of glycolipid resulted in full iNKT cell responsiveness to C20:2 restimulation 1 month after the initial treatment. As mentioned previously, it appears as though iNKT cell anergy is a regulatory mechanism that has evolved to prevent the chronic activation of these potent immune cells and thus prevent potential damage to the host [38]. Therefore, it seems reasonable that due to the less sustained activation of iNKT cells elicited by C20:2 relative to α-GalCer, these cells would be regulated by anergy for a shorter duration of time (Ly et. al, 2009, in press, Appendix A).

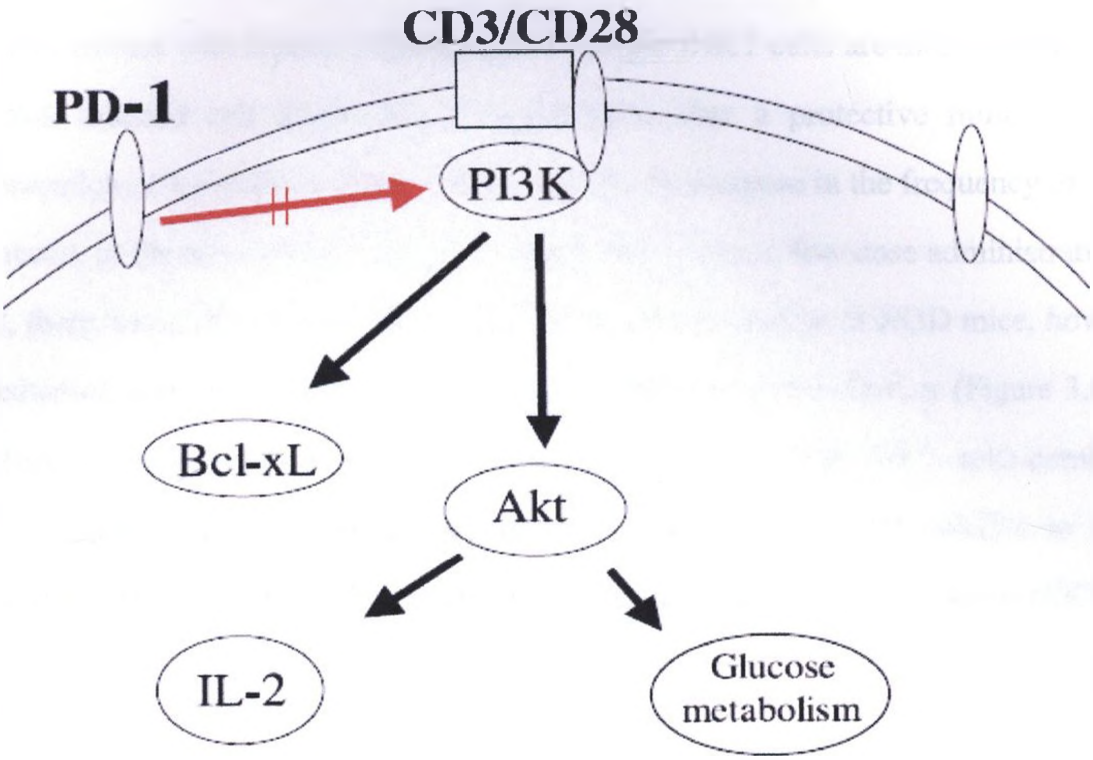
Correlated with the faster recovery from anergy is the more rapid return of PD-1 and PD-L1 levels on iNKT cells in the spleen and PLN to vehicle levels after treatment with both single (Figure 3.5) and multiple (Figure 3.9) doses of C20:2. Under normal conditions, PD-1 expression establishes a threshold of activation that must be overcome, usually through costimulation, before an immune response is initiated [166, 200]. However, during robust activation of conventional T cells, this receptor is upregulated and changes from a "gate keeper" of activation to a veto signal [200]. As such, PD-1 plays a critical role in maintaining tolerance and shutting down excessive immune responses [168, 200-202]. In order to inhibit TCR mediated activation, upon interaction with PD-L1, PD-1 specifically inhibits phosphatidylinositol-3-kinase (PI3K), an important molecule in the early TCR signalling cascade [203, 204]. Under normal conditions, TCR signalling results in increased glucose metabolism and the production of

IL-2 [200, 205] (Figure 4.2). Therefore, blocking PI3K activation would efficiently prevent T cell activation because it would deny the cell the resources required for division, effector function, and differentiation [200]. Interestingly, it has been observed in conventional T cells that IL-2 can override the inhibitory effects of PD-1 ligation [206].

Recent evidence has identified a dual role for the PD-L1 molecule. In addition to binding PD-1 and delivering an inhibitory signal on the PD-1-expressing cell, PD-L1 also has the ability to associate with the costimulatory molecule, B7-1, present on APCs [168, 207]. Functional studies indicated that the B7-1:PD-L1 interaction can inhibit proliferation and cytokine production by the PD-L1-expressing T cell [168, 207]. Thus, it is not surprising that PD-1 and PD-L1 signalling pathways have been shown to be key mediators in the induction of iNKT cell anergy since it is characterized by deficient IL-2 production and proliferation as well as an overall reduced effector response that can be overcome by the addition of exogenous IL-2 (Figure 3.3 B, right panel) [157, 166, 167]. It follows that the less sustained activation of iNKT cells by C20:2 would result in a reduced and less sustained PD-1 and PD-L1 upregulation compared to α-GalCer and thus result in a faster recovery from iNKT cell anergy (Figures 3.5 and 3.9).

The inhibition of iNKT cell activation and the subsequent induction of anergy elicited by PD-1 signalling may also play a role in iNKT cell survival. Upon TCR ligation in conventional T cells, PI3K plays a role in assisting the initiation of the signalling cascade responsible for T cell activation [208-210]. In addition, upon costimulation with CD28, PI3K also leads to the upregulation of Bcl-xL, a survival protein that allows activated T cells to become resistant to apoptosis (Figure 4.2) [208-210]. Interestingly, unlike other inhibitory signalling molecules, such as CTLA-4, PD-1 signalling does not lead to the upregulation of Bcl-xL expression on T cells, suggesting

Figure 4.2 PD-1 signalling pathway. Upon interaction with its ligand PD-L1, PD-1 signalling leads to the inhibition of phosphatidylinositol-3-kinase (PI3K), which effectively abrogates TCR-mediated T cell activation. Adapted from Riley 2009 [200]


CD3/CD28
PD-1
PI3K
Bcl-xL
Akt
IL-2
Glucose
metabolism

that ligation of PD-1 may render T cells more susceptible to apoptosis [200, 204, 211]. This is consistent with reports suggesting that anergic iNKT cells are more susceptible to activation induced cell death [66]. Accordingly, after a protective multi-low dose administration of α-GalCer to NOD mice, a significant decrease in the frequency of iNKT cells occurs in the spleen [66, 178]. Conversely, after a multi-low dose administration of C20:2, there was a decrease in iNKT cell frequency in the spleen of NOD mice, however this reduction was less than that observed after treatment with α-GalCer (Figure 3.6 A). Therefore, the more rapid downregulation of PD-1 and PD-L1 on iNKT cells combined with the faster recovery from anergy in mice treated with C20:2 relative to those receiving α-GalCer appears to be beneficial as it results in a reduced amount of iNKT cell death.

4.4. Multi-dose treatment with C20:2 alters the frequency, phenotype, and function of DCs

It appears as though a major reason why iNKT cell agonists are able to protect NOD mice from T1D is due to the capacity of iNKT cells to modulate DCs. Studies have revealed that after a multi-low dose treatment of NOD mice with α-GalCer, DCs that possess tolerogenic capabilities are recruited to the PLN. Presumably, these tolerogenic DCs inhibit autoreactive T cells and therefore protect against T1D by preventing further islet β cell destruction [128, 171, 172]. It was therefore of interest to investigate the effects of C20:2 on the modulation of DC subsets.

Interestingly, a multi-low dose treatment with C20:2 appears to modify DCs in a more favourable manner than α-GalCer due to the frequency, phenotypic, and functional alterations that are elicited. After a multi-low dose treatment, both α-GalCer and C20:2 significantly reduced the frequency of the total mDC population in both the spleen and

PLN, while the pDC frequency remained unaltered (Figure 3.10 A and B, middle panels). It has been reported that pDCs play a protective role in NOD mice [171], while the $CD8^+$ subset of mDCs have a pro-inflammatory role due to their ability to produce large amounts of IL-12, which in turn can elicit an IFN-γ response from NK cells [16-18, 68]. Therefore, the preferential reduction of the total mDC population by both glycolipids may assist in the protection from T1D in NOD mice by reducing the number of inflammatory $CD8^+$ mDCs present in the secondary lymphoid organs. Importantly, C20:2, but not α-GalCer, also reduced the frequency of $CD8^+$ mDCs in the total mDC population in the PLN, which may provide additional therapeutic benefits (Figure 3.10 B, right panel).

Since multi-low dose treatment with C20:2 was found to reduce the frequency of $CD8^+$ mDCs to a greater degree than that obtained with α-GalCer (Figure 3.10), it was of interest to investigate whether treatment with C20:2 also alters the phenotype of $CD8^+$ mDCs. A single dose of C20:2 was shown to elicit a significantly reduced intracellular IL-12 response in $CD8^+$ mDCs, while maintaining equivalent levels of IL-10 compared to α-GalCer (Vehicle/α-GalCer vs. Vehicle/C20:2) (Figure 3.11). This may provide an additional explanation as to why C20:2 treated iNKT cells induce a greater T_H2 response since a reduction in IL-12 may elicit a lower IFN-γ secretion from NK cells. Upon restimulation of anergic iNKT cells, a second administration of both α-GalCer and C20:2 resulted in a significantly reduced IL-12 $CD8^+$ mDC response, which is characteristic of tolerogenic DCs. Interestingly, restimulation with C20:2, but not α-GalCer, also induced an increased IL-10 response (Vehicle/α-GalCer vs. α-GalCer/α-GalCer, Vehicle/C20:2 vs. C20:2/C20:2) (Figure 3.11). The additional synthesis of IL-10 in the $CD8^+$ mDCs of NOD mice treated with C20:2 suggests that these cells may possess a greater suppressive capacity than those DCs from α-GalCer treated mice. This notion was further supported following the functional analysis of the total DC population from mice that received a protective multi-low dose treatment with glycolipid. Consistent with the phenotypic

characteristics of the $CD8^+$ mDCs, total DCs from mice treated with C20:2 were more effective at suppressing the recall response of peptide-primed $CD4^+$ T cells than those from α-GalCer treated mice, thus illustrating their greater tolerogenic capabilities (Figure 3.12). The induction of DCs with a greater ability to suppress T cell responses is a very important and promising characteristic of C20:2 for the prospective therapeutic treatment of human T1D patients.

4.5 Clinical translation

Overall, this study 1) highlights the potential of glycolipid agonist modulation of iNKT cell activity for the treatment of T1D and 2) demonstrates the effects that chemical modifications to iNKT cell agonists have on the elicited immune response. For the treatment of inflammatory autoimmune diseases such as T1D, C20:2 appears to provide several beneficial immune responses that may translate clinically into a more effective and safer therapeutic alternative to α-GalCer. The less sustained and T_H2-skewed primary immune response, combined with the faster induction of iNKT cell anergy after a single C20:2 injection, may reduce the initial side effects of glycolipid administration compared to α-GalCer. In particular, the lower IFN-γ response elicited by C20:2 administration may limit the negative contribution that α-GalCer treatment has on the ongoing inflammatory response in T1D patients. In addition, by reducing the time frame between the initial activation and the ensuing induction of iNKT cell anergy from 1 week to 72 h compared to α-GalCer, as shown from the intracellular IL-2 production (Figure 3.4, left panel), C20:2 administration may limit the number and function of immunogenic DCs induced upon restimulation [173]. This would further reduce the potential side effects elicited by the inflammatory DCs induced after the initial administration of the drug. In addition, treatment with multiple doses of C20:2 may also diminish the ongoing autoimmune response in T1D patients more rapidly than α-GalCer due to the faster

induction of tolerogenic DCs, thus avoiding unnecessary destruction of additional islet β cells [173].

It is also noteworthy that the faster recovery from iNKT cell anergy observed after C20:2 administration, limited the amount of iNKT cell death that ensued after a multi-low dose treatment protocol. Due to the expanding role of iNKT cells as a key regulator of immune responses [64, 71, 98], it is vital that therapeutic drugs do not render the host immunocompromised. Finally, an intriguing characteristic of C20:2 is its ability to recruit tolerogenic DCs to secondary lymphoid organs that possess greater suppressive capabilities than α-GalCer treated DCs, which may contribute to the more efficient protection from T1D. Taken together, these characteristics of C20:2 make it a prime candidate for future therapeutic use in clinical trials of T1D. Nonetheless, further experimentation on the specific mechanism(s) of C20:2 mediated protection from T1D may be required before such human treatments begin.

4.6. Future directions

While this study complements previous work conducted in the field regarding iNKT cell responses to glycolipid agonists, further work is required to elucidate the complex immune processes that are responsible for mediating protection against T1D. While a T_H2 response is beneficial for treating inflammatory autoimmune diseases, further structure/function analysis is needed to determine what specific alterations to the chemical structure of glycolipids are required to induce such a response. As mentioned previously, the altered requirements for glycolipid loading onto CD1d may be responsible for a T_H2 response; however it is unknown why minor alterations to the fatty acyl side chain would allow C20:2 to load onto CD1d without the need for internalization and

assistance from intracellular lipid transfer proteins. Answering these questions will have a profound effect on our understanding of iNKT cell biology.

As described above, iNKT cell anergy appears to play a major role in protection against T1D in NOD mice, in part due to the subsequent modulation of DCs. Future studies should be aimed at functionally proving the essential role of iNKT cell anergy in protection as well as further analyzing the role of specific DC subsets in disease prevention. In particular, it may be beneficial to take advantage of the recent evidence suggesting that PD-1 signalling is required for iNKT cell anergy. By using blocking antibodies directed against PD-1 and PD-L1, it would be feasible to inhibit the induction of iNKT cell anergy and determine whether or not this prevents the protection against T1D in NOD mice after a multi-low dose treatment of glycolipid. In addition, while C20:2 treatment results in a faster induction of iNKT cell anergy, and theoretically the more rapid recruitment of tolerogenic DCs to PLNs based on previous reports [173], it is important to functionally test this hypothesis by performing a kinetic analysis of the suppressive abilities of DCs following in vivo restimulation of anergic iNKT cells.

Finally, this study demonstrated that restimulation of anergic iNKT cells with C20:2 altered the response of $CD8^+$ mDCs in a manner that mimics a tolerogenic phenotype. However, due to practical limitations, only the total DC population was used in functionally analyzing the suppressive capacity of these cells after a multi-low dose treatment. It is therefore possible that C20:2 may convert $CD8^+$ mDCs from inflammatory mediators to suppressive APCs directly or, conversely, reduce their immunogenic capacity while recruiting other tolerogenic DC subsets, such as $CD8^-$ mDCs or pDCs, to the spleen and PLN instead. A functional analysis of the tolerogenic capabilities of specific DC subsets after a multi-low dose administration of glycolipid

will further enhance our understanding of the mechanisms involved in the protection against T1D.

Chapter 5:

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Appendices

Appendix A: An α-galactosylceramide C20:2 N-acyl variant enhances anti-inflammatory and regulatory T cell-independent responses that prevent type 1 diabetes.

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An α-galactosylceramide C20:2 N-acyl variant enhances anti-inflammatory and regulatory T cell-independent responses that prevent type 1 diabetes

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Summary

Protection from type 1 diabetes (T1D), a T helper type 1 (Th1)-mediated disease, is achievable in non-obese diabetic (NOD) mice by treatment with α-galactosylceramide (α-GalCer) glycolipids that stimulate CD1d-restricted invariant natural killer T (iNK T) cells. While we have reported previously that the C20:2 N-acyl variant of α-GalCer elicits a Th2-biased cytokine response and protects NOD mice from T1D more effectively than a form of α-GalCer that induces mixed Th1 and Th2 responses, it remained to determine whether this protection is accompanied by heightened anti-inflammatory responses. We show that treatment of NOD mice with C20:2 diminished the activation of 'inflammatory' interleukin (IL)-12 producing $CD11c^{high}CD8^{+}$ myeloid dendritic cells (mDCs) and augmented the function of 'tolerogenic' DCs more effectively than treatment with the prototypical iNKT cell activator KRN7000 (α-GalCer C26:0) that induces Th1- and Th2-type responses. These findings correlate with a reduced capacity of C20:2 to sustain the early transactivation of T, B and NK cells. They may also explain our observation that C20:2 activated iNK T cells depend less than KRN7000 activated iNK T cells upon regulation by regulatory T cells for cytokine secretion and protection from T1D. The enhanced anti-inflammatory properties of C20:2 relative to KRN7000 suggest that C20:2 should be evaluated further as a drug to induce iNK T cell-mediated protection from T1D in humans.

Keywords: dendritic cells, diabetes, immunotherapy, NKT cells, regulatory T cells

Introduction

Invariant natural killer T (iNK T) cells comprise a subset of innate lymphocytes that express a semi-invariant T cell receptor (TCR) consisting of Vα14 paired preferentially to Vβ8·2, Vβ7 or Vβ2 in mouse and Vα24 paired to Vβ11 in humans. The defining characteristics of iNK T cells are their ability to recognize a lipid or glycolipid antigen presented on an antigen-presenting cell (APC) by a major histocompatibility complex (MHC) class I-like molecule CD1d [1–3]. All type 1 'classical' iNK T cells recognize and react to the prototypic synthetic glycolipid KRN7000, which is a form of α-galactosyl-ceramide (α-GalCer) C26:0 based on structurally similar immunostimulatory compounds derived originally from a marine sponge [4]. A hallmark of iNK T cells is their ability to produce rapidly and secrete large amounts of both T helper type 1 (Th1) [interferon (IFN)-γ] and Th2 [interleukin (IL)-4] cytokines within hours after activation with KRN7000 [2,3]. Although α-GalCer is most probably not a naturally occurring iNK T cell ligand in mammals, it has been used experimentally to explore the immunomodulatory properties of iNK T cells in many preclinical models of autoimmune disease.

The pathogenesis of type 1 diabetes (T1D), an autoimmune disease characterized by the T cell-mediated destruction of insulin-producing pancreatic islet β cells, arises in part from functional deficiencies in regulatory T cell (T_{reg}) populations, including $CD4^{+}CD25^{+}$forkhead box P3 ($Foxp3^{+}$) (T_{reg}) cells and iNK T cells [5,6]. Studies conducted in the NOD mouse model of experimental T1D [7] have shown that many therapies that prevent T1D depend upon their ability to activate T_{regs} and/or iNK T cells [5,6]. Although several investigators reported that activation of iNK T cells with KRN7000 can protect NOD mice from

developing T1D [8–10], the mechanism(s) of iNK T cell-mediated protection against T1D is not well understood. Current evidence suggests that therapeutic effects elicited by activation of iNK T cells elicited upon KRN7000 treatment of NOD mice occur in an IL-4-dependent manner [11] and are associated with a Th2-like environment [8,9] that promotes the recruitment of tolerogenic dendritic cells (DCs) to the draining pancreatic lymph nodes (PLN) [12–14]. We have demonstrated that iNK T-mediated protection from T1D induced by KRN7000 requires the activity of $CD4^+CD25^+$ T cells that are enriched for $CD4^+CD25^+Foxp3^+$ T_{regs} [15]. Young (4–5 weeks old) NOD mice treated *in vivo* first with an anti-CD25 monoclonal antibody (mAb) to inactivate T_{regs} and then with KRN7000 to activate iNK T cells still developed T1D spontaneously. Importantly, we found that KRN7000 induces the surface expression of CD25 by only about one-third of activated iNK T cells in wild-type NOD mice [15], indicating that anti-CD25 treatment does not inactivate the majority of activated iNK T cells. These findings support the notion that T_{regs} may be required to down-regulate the activity of iNK T cells stimulated by synthetic α-GalCer ligands.

Since the discovery of KRN7000, several investigators have generated other synthetic analogues of α-GalCer that polarize iNK T cells towards Th1- or Th2-type cytokine responses [16–18]. In the case of a Th1-mediated autoimmune disease such as T1D, the use of an analogue that enhances IL-4 production significantly while reducing simultaneously the synthesis of a strong proinflammatory cytokine such as IFN-γ represents a desirable therapeutic approach to the prevention of T1D [17,18]. One such analogue, termed α-GalCer C20:2 (referred to as C20:2 hereafter), in which the C26:0 N-acyl group of KRN7000 is replaced by an 11,14 *cis* unsaturated 20 carbon fatty acid, can potently activate iNK T cells and promote Th2-biased responses associated with improved clinical and immunological outcomes in the prevention of T1D in NOD mice when compared to the parent glycolipid α-GalCer [18,19]. Having shown that the activity of T_{regs} is required for iNK T cells activated with KRN7000 to protect NOD mice from T1D [15], it was of interest to determine whether this is also the case for C20:2-induced protection from T1D and, if so, the mechanisms involved. Interestingly, we report in this study that iNK T cells activated by C20:2 depend less on regulation by $CD4^+CD25^+$ T_{reg} cells than do iNK T cells activated by KRN7000 for protection from T1D. This decreased dependency of C20:2-induced protection on T_{reg} function correlated with its reduced capacity to sustain the transactivation of T cells, B cells and NK cells. In addition, treatment of NOD mice with C20:2 diminished the activation of 'inflammatory' IL-12-producing $CD11c^{high}CD8^+$ myeloid dendritic cells (mDCs) and augmented the function of 'tolerogenic' DCs more effectively than treatment with KRN7000. The latter findings may explain why C20:2-activated iNK T cells depend less than KRN7000-activated iNK T cells on regulation by T_{regs} for cytokine secretion and protection from T1D. Our results demonstrate that greater anti-inflammatory activity is associated with protection from T1D induced by C20:2 than that induced by KRN7000 and presumably other forms of a-GalCer that induce mixed Th1- and Th2-type cytokine responses. These data suggest that C20:2 should be evaluated further as a target drug for iNK T cell-mediated protection from T1D in humans.

Materials and methods

Mice

NOD/Del and C57BL/6 mice were bred in a specific pathogen-free barrier facility at the Robarts Research Institute (London, ON, Canada). The incidence of T1D in female NOD mice in our colony is 25–30% at 15 weeks of age and ≥75% by 25 weeks. All experimental mice were female and were maintained in a specific pathogen-free facility in the Animal Care and Veterinary Services at the University of Western Ontario according to institutional guidelines.

Antibody and reagents

KRN7000 (α-GalCer) and its proprietary vehicle control was kindly provided by Kirin Pharmaceutical Research Laboratories (Gunma, Japan), solubilized in water and injected intraperitoneally (i.p.) into mice (4 μg/dose) every other day for 3 weeks, as described previously [15]. C20:2 was synthesized as described previously [18] and dissolved at 200 μg/ml in phosphate-buffered saline (PBS) containing 0·05% Tween 20, and injected i.p. (4 μg/mouse, in 250 μl of vehicle consisting of PBS plus 0·05% Tween 20) or vehicle (PBS plus 0·05% Tween 20). Fluorescein isothiocyanate (FITC)-conjugated anti-TCR-β (H57-597), anti-CD25 (7D4), anti-B220 (RA3-6B2), anti-Pan NK cells (DX5), anti-Siglec H (eBio440c); phycoerythrin (PE)-conjugated anti-CD69 (H1·2F3), anti-I-A^d (AMS-32·1), anti-CD86 (Gl-1), anti-CD40 (MR1), anti-IL-4 (11B11), anti-IFN-γ (XMG1·2), anti-IL-12p40/70 (C15·6); peridinin chlorophyll (PerCP)-conjugated anti-CD8α (53–2·1), anti-CD4 (RM4-5), anti-CD3ε (145-2C11); and APC-conjugated anti-CD11c (N418) mAbs were purchased from eBiosciences or BD Biosciences. [3] Fluorescently labelled tetrameric CD1d molecules loaded with α-GaCer (KRN7000) were prepared as described previously [20]. The anti-CD25 (PC61) mAb used to inactivate $CD4^+CD25^+$ T_{reg} cells *in vivo* was prepared in house from hybridomas (American Type Culture Collection no. TIB 222). RPMI-1640 tissue culture medium was supplemented with 10% heat-inactivated fetal calf serum (FCS), 10 mM HEPES buffer, 1 mM Na pyruvate, 2 mM L-glutamine, 100 units/ml penicillin, 0·1 mg/ml streptomycin and 0·05 mM 2-mercaptoethanol (ME) (all purchased from Invitrogen Life Technologies). [4]

Monitoring of diabetes

Mice were monitored beginning at 12 weeks of age for hyperglycaemia by measurement of blood glucose levels (BGL) twice weekly using an Ascensia ELITE glucometer and strips (Bayer, Toronto, ON, Canada), as described previously [15]. Mice were considered diabetic when two consecutive BGL readings of >11·1 mmol/l were obtained.

In vivo treatment with glycolipid

To monitor the spontaneous development of T1D, NOD mice (4–5 weeks old) were first injected intravenously (i.v.) with 500 μg of either anti-CD25 mAb (PC61) to inactivate $CD25^+$ cells or isotype control immunoglobulin G (IgG), rested for 3 days, treated by i.p. injection with KRN7000 (4 μg/dose), C20:2 (4 μg/dose) or vehicle (control) every other day for 3 weeks and then monitored for the incidence of T1D, as reported previously [15].

Cell purification and flow cytometry

Single-cell lymphocyte suspensions were prepared from the spleen and PLN [15]. Non-viable cells were excluded by electronic gating for all experiments. For intracellular cytokine staining, cell suspensions were cultured without further stimulation at 10^7 cells/ml in culture media containing Golgi Stop (BD Biosciences) for 3–4 h. Intracellular staining for IL-4, IFN-γ and IL-12 was performed using a BD Cytofix/Cytoperm buffer set (BD Biosciences), according to the manufacturer's protocol. Flow cytometry was performed using a fluorescence activated cell sorter (FACS)Calibur instrument (BD Biosciences), and the acquired data were analysed using FlowJo software (Tree Star Inc.).

In vitro cultures and enzyme-linked immunosorbent assay (ELISA)

Splenocytes obtained from various treatment groups of mice as indicated after the last α-GalCer treatment were cultured (5×10^6 cells/ml) for 72 h in the absence or presence (restimulation) of KRN7000, C20:2 or control (100 ng/ml, or as indicated). A standard sandwich ELISA was performed for mouse cytokines using paired antibody kits for IFN-γ, IL-13 (eBiosciences) or IL-4 (BD Biosciences). For signal detection, streptavidin–horseradish peroxidase (HRP) conjugate and development solution from BD OptiEIA Reagent Set A (BD Biosciences) were used. For DC cultures, NOD mice were treated i.p. with glycolipid according to our multi-low-dose protocol (4 μg/dose every other day for 3 weeks), as reported previously [15]. At 1 week after the last dose, spleen and PLN suspensions were pooled and $CD11c^+$ microbeads (Miltenyi Biotec) were used to sort $CD11c^+$ DCs, according to the manufacturer's instructions for each treatment group. Concurrently, $CD4^+$ peptide-primed T cells were isolated ($CD4^+$ T cell-negative selection kit; Miltenyi Biotec) from NOD mice injected (i.p.) 10 days previously with either vehicle control (PBS) or 100 μg of a B9-23 insulin peptide (Ins B9-23, kindly provided by Dr B. Singh, University of Western Ontario, London, ON, Canada) emulsified in incomplete Freund's adjuvant (IFA) (Sigma Aldrich) or dissolved in PBS. Peptide primed-$CD4^+$ T cells were cultured with $CD11c^+$ DC at a 20 : 1 ratio in the presence of the Ins B9-23 peptide for 72 h for the ELISA (2×10^6 T cells + 1×10^5 DC) and proliferation (5×10^5 T + 1×10^4 DC) assays, respectively. Culture supernatants were collected for IL-2, IFN-γ and IL-4 detection using a standard sandwich ELISA (eBiosciences), and cell proliferation was determined by [^{3}H]-thymidine pulse (1 μCi/well; PerkinElmer) for the last 18 h of culture, harvested and read on a 1450 Microbeta counter (PerkinElmer).

Statistics

Statistical significance of cell expansion as well as cytokine production and secretion assays were conducted using two-way analysis of variance (ANOVA) comparisons with Bonferroni post-tests. The incidence of T1D was compared using a Mantel–Cox log rank test. In all experiments, differences were considered significant when P was less than 0·05.

Results

C20:2 activated iN K T cells induce a Th2-biased cytokine response

Relative to KRN7000, the structure of C20:2 consists of a fatty acyl chain reduced from C26 to C20 with unsaturations at carbons 11 and 14 (Fig. 1a). Unique among Th2-biased glycolipid analogues, C20:2 has a relatively similar binding affinity to CD1d as α-GalCer (Kd = 1·83 μM *versus* Kd = 1·29 μM, respectively) [21], and based on staining with glycolipid-loaded CD1d tetramers has been shown to be recognized by the same population of iNK T cells as α-GalCer [18,19]. Accordingly, we analysed initially whether the proliferative capacity and cytokine secretion profile of iNK T cells from NOD mice stimulated *in vitro* by KRN7000 or C20:2 are similar. Indeed, the proliferative responses of NOD splenocytes stimulated by KRN7000 or C20:2 were found to be very similar (Fig. 1b). Supernatants from C20:2 activated splenocytes generally contained a higher concentration of IL-4 and lower concentration of IFN-γ than supernatants from KRN7000-stimulated splenocytes (Fig. 1c), consistent with the reported ability of C20:2 to induce a Th2-biased response [19]. More importantly, the induction of a Th2-biased response by C20:2 was even more pronounced *in vivo*, as NOD mice treated first with isotype control IgG and then with C20:2 (IgG/C20:2) yielded a greater serum IL-4

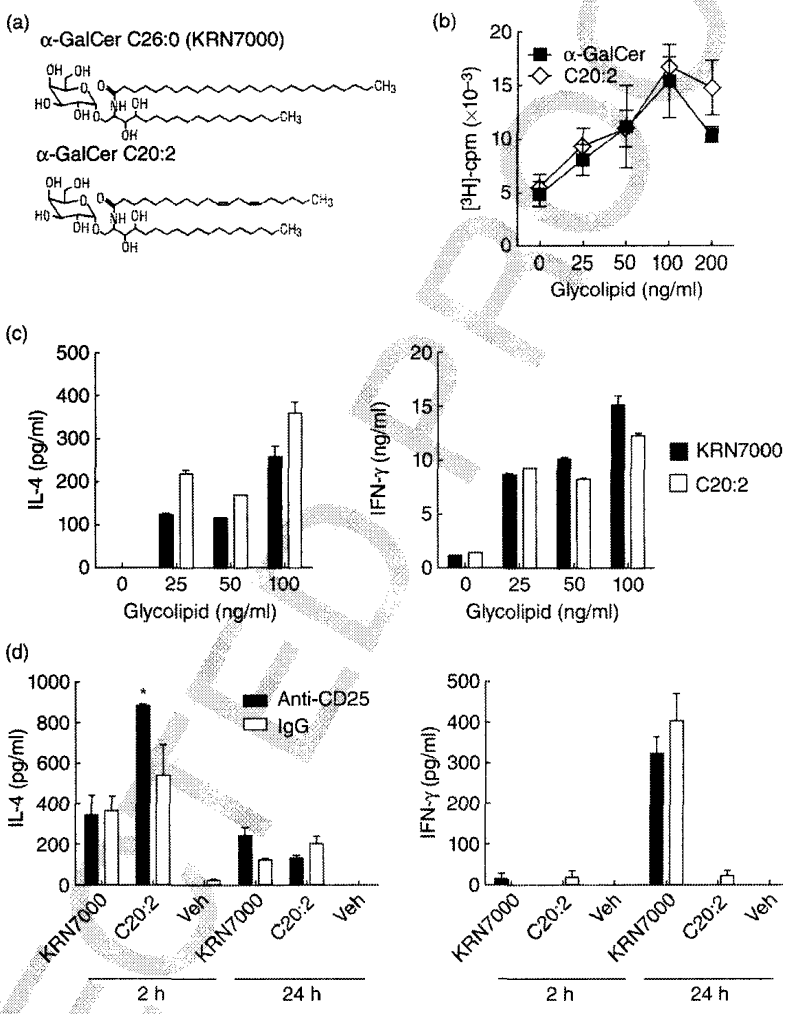


Fig. 1. C20:2-activated invariant natural killer T (iNK T) cells induce a T helper type 2 (Th2)-biased cytokine response. (a) Structures of synthetic glycolipids α-galactosyl-ceramide (α-GalCer) C26:0 (KRN7000) and α-GalCer C20:2. Splenocytes from non-obese diabetic (NOD) mice (5–6 weeks old) were stimulated *in vitro* with the indicated concentrations of glycolipids for 72 h, and were then assayed by [^{3}H]-thymidine incorporation for their proliferative capacity (b) or enzyme-linked immunosorbent assay (ELISA) for their cytokine secretion (c). Data show the means ± standard deviation (s.d.) for six replicates pooled from two independent experiments containing three mice per group. (d) NOD mice (5–6 weeks old) were injected once intravenously with 500 μg of anti-CD25 monoclonal antibody or immunoglobulin G (IgG), rested for 3 days, and then treated intraperitoneally with 4 μg of KRN7000, C20:2 or vehicle. Serum was collected from the mice at the indicated times post-treatment and analysed by ELISA for the concentrations of interleukin-4 and interferon-γ. Data show the means ± s.d. of three to five individual mice per group. *$P < 0{\cdot}01$ compared to IgG/glycolipid treatment.

response than did IgG/KRN7000-treated mice at 2 h post-treatment (Fig. 1d, left panel). Interestingly, the IgG/C20:2 serum IL-4 response was increased further ~twofold at 2 h in anti-CD25/C20:2-treated mice, whereas no differences in IL-4 or IFN-γ responses were detected between anti-CD25 *versus* IgG-treated mice at 24 h. Note that the anti-CD25 mAb used here inactivates rather than depletes T_{regs} in NOD mice [15,22]. Considerably lower IL-4 responses were seen at 24 h post-treatment, and the responses obtained in IgG/KRN7000- and IgG/C20:2-treated mice were not significant. While only a very weak serum IFN-γ response was detected at 2 h in IgG/KRN7000- and IgG/C20:2-treated mice, a markedly higher serum IFN-γ response was observed at 24 h in IgG/KRN7000-treated mice than in IgG/C20:2-treated mice (Fig. 1d, right panel). Thus, C20:2 can induce a Th2-biased cytokine response by iNK T cells in NOD mice, and prior inactivation of T_{regs} by treatment of these mice with an anti-CD25 mAb can augment this Th2-type response further.

C20:2-activated iNK T cells are less dependent than KRN7000-activated iNK T cells on CD4$^+$CD25$^+$ T_{reg} cells for protection against T1D

Given that T_{reg} cells are required to regulate KRN7000-induced iNK T-mediated protection of NOD mice from T1D [15], and having shown that C20:2 elicits a Th2-biased iNK T cytokine response in NOD mice (Fig. 1), we next investigated whether C20:2-activated iNK T cells require the activity of T_{regs} for protection from T1D. Young NOD mice (4–5 weeks old) were administered a single dose of anti-CD25 mAb, rested for 3 days, and treated with a multi-low-dose protocol of glycolipid known to protect NOD mice from T1D [9]. Mice treated with vehicle only developed T1D spontaneously beginning at 12–15 weeks of age in mice receiving anti-CD25 or control IgG, with more than 75% of mice becoming diabetic by 30 weeks of age. Similarly, mice that received anti-CD25 mAb and KRN7000 developed T1D beginning at 12 weeks of age and greater than 75% of mice

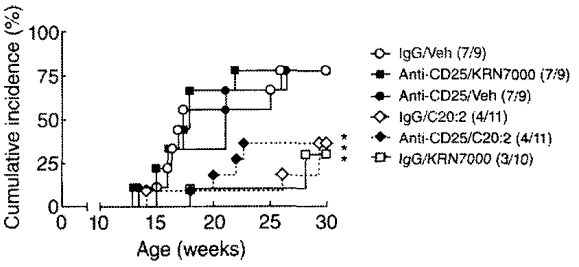


Fig. 2. C20:2-activated invariant natural killer T (iNK T) cells depend less on $CD4^+CD25^+$ regulatory T cell (T_{reg}) activity than KRN7000-activated iNK T cells for protection against type 1 diabetes (T1D). Non-obese diabetic (NOD) mice (4–5 weeks old) were administered anti-CD25 or immunoglobulin G (IgG), rested, and then treated with a multi-low-dose protocol of KRN7000, C20:2 or vehicle (4 μg/dose, every other day for 3 weeks). The incidence of T1D in these mice was monitored to 30 weeks of age. Results show the cumulative incidence of T1D from two independent experiments conducted using four to six mice per group. $^*P < 0{\cdot}04$ compared to vehicle-treated mice.

were diabetic by 30 weeks of age (Fig. 2), which was consistent with our previous report [15]. However, we were surprised to find that mice treated with anti-CD25 mAb and C20:2 were relatively protected from T1D, with only 35% of mice developing T1D by 30 weeks of age (Fig. 2). A similar level of protection was seen in mice treated with control IgG and either C20:2 or KRN7000. These results suggest that C20:2-activated iNK T cells depend less than KRN7000-activated iNK T cells on regulation by T_{regs} for protection against T1D.

KRN7000 and C20:2 differ in their capacity to expand iNK T cells in the spleen

The observation that KRN7000- and C20:2-activated iNK T cells differ in their level of requirement of T_{reg} regulation for protection from T1D raised the possibility that these two glycolipid analogues may vary in their capacity to activate iNK T cells. To test this possibility, we administered KRN7000 or C20:2 to NOD mice *in vivo* and assayed the kinetics of iNK T cell expansion in the spleen and PLN, two sites of localization of activated iNK T cells that mediate protection from T1D. Using α-GalCer/CD1d tetramers to identify iNK T cells, we observed that both KRN7000 and C20:2 induce iNK T cell TCR down-regulation by 24 h after glycolipid administration, which is typical of iNK T cell activation *in vivo* [23,24]. A comparison of the frequency of iNK T cells in the spleen at 72 h and 2 h post-treatment revealed that whereas KRN7000 stimulated the proliferation and expansion (seven- to eightfold) of spleen iNK T cells ($3{\cdot}72 \pm 0{\cdot}7\%$ *versus* $0{\cdot}54 \pm 0{\cdot}1\%$), the frequency of C20:2-activated spleen iNK T cells remained at homeostatic levels ($0{\cdot}45 \pm 0{\cdot}04\%$ *versus* $0{\cdot}37 \pm 0{\cdot}1\%$) (Fig. 3a). A similar comparison of iNK T cell frequencies in the PLN at 72 h and 2 h post-treatment showed that both KRN7000 ($2{\cdot}63 \pm 0{\cdot}4\%$ *versus* $0{\cdot}31 \pm 0{\cdot}1\%$) and C20:2 ($0{\cdot}97 \pm 0{\cdot}1\%$ *versus* $0{\cdot}13 \pm 0{\cdot}1\%$) stimulated the proliferation and expansion (seven- to eightfold) of iNK T cells (Fig. 3b). A similar kinetics of expansion was also observed in NOD mice treated with anti-CD25 mAb prior to glycolipid administration (our unpublished observations). However, at 72 h after treatment, we observed differences in the absolute number of iNK T cells in the spleen between mice treated first with anti-CD25 or IgG and then with KRN7000 (Fig. 3a, right panel). This difference was not observed upon treatment with C20:2. The differences noted in the spleen for KRN7000-activated iNK T cells were not detected in the PLN at 72 h post-treatment (Fig. 3b, right panel). Thus, iNK T cells in the spleen but not PLN appear to be sensitive to a different level of expansion induced by KRN7000 *versus* C20:2. Different subsets of DCs interact with iNK T cells in the spleen and PLN, and may account for this variation in sensitivity to iNK T cell activation and expansion.

KRN7000 and C20:2 differ in their capacity to activate iNK T cells for cytokine secretion. Next, we analysed the ability of activated iNK T cells to secrete cytokines upon glycolipid restimulation. Because the greatest difference in iNK T cell expansion was observed at 72 h post-glycolipid treatment *in vivo*, splenocytes were restimulated at this time-point *in vitro* and their secretion of IFN-γ, IL-4 and IL-13 was analysed by ELISA. In support of a role of T_{regs} in the down-regulation of immune responses, we observed an increase in cytokine secretion by splenocytes from mice treated with anti-CD25 prior to glycolipid or vehicle by comparison to splenocytes from IgG-treated mice (Fig. 4a). A fourfold and fivefold increase in IFN-γ secretion over that of vehicle control was detected in mice treated with anti-CD25/KRN7000 and IgG/KRN7000, respectively. These increases in IFN-γ secretion were not evident in C20:2-treated mice (Fig. 4a) in which the basal levels of IFN-γ secretion detected were similar to those in vehicle control-treated mice. Thus, inactivation by anti-CD25 seems to inhibit the ability of T_{regs} to regulate iNK T cell secretion of IFN-γ after stimulation by KRN7000, but not C20:2.

Th2 cytokine responses also differ between KRN7000- and C20:2-activated iNK T cells. Consistent with the observation that C20:2 induces Th2-biased responses, we found that splenocytes from C20:2-treated mice, irrespective of previous T_{reg} inactivation, produced greater amounts of IL-4 and IL-13 compared to splenocytes from KRN7000-treated mice (Fig. 4a). Interestingly, the greatest difference between C20:2- and KRN7000-induced secretion of Th2 cytokines was noted in mice treated with control IgG. Whereas T_{reg} inactivation resulted in an increase in IFN-γ secretion by splenocytes from KRN7000- and IgG-treated mice, only a marginal increase in C20:2-induced Th2 cytokine secretion was triggered upon T_{reg} inactivation. Thus, although C20:2-activated iNK T cells in the spleen do not expand appreciably at 72 h post-stimulation, these cells

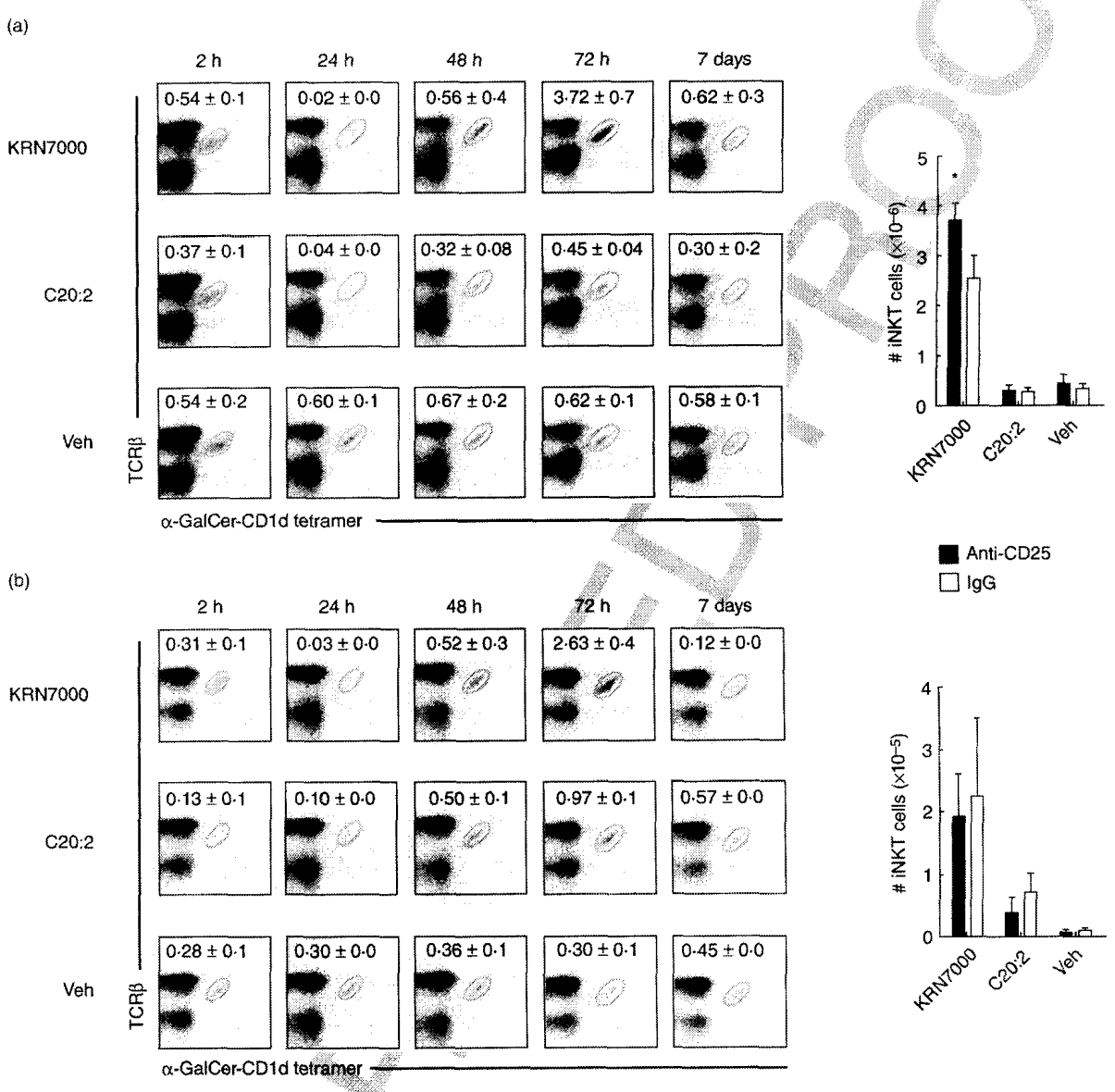


Fig. 3. Kinetics of invariant natural killer T (iNK T) cell expansion activated with KRN7000 or C20:2. Non-obese diabetic (NOD) mice (4–5 weeks old) were administered anti-CD25 or immunoglobulin G (IgG), rested, and then treated with KRN7000, C20:2 or vehicle, as in Fig. 1. Spleen (a) or pancreatic lymph nodes (PLN) (b) lymphocytes were harvested at the indicated time-points post-glycolipid treatment for flow cytometric analysis of their frequency of iNK T cells. Dot plots from a representative experiment are shown with the means ± standard deviation (s.d.) of gated [T cell receptor (TCR)-β$^+$ and α-galactosyl-ceramide C26:0 (α-GalCer)-CD1d tetramer$^+$] iNK T cells from three or five individual mice per group (a and b, left panel). Absolute numbers of iNK T cells observed at 3 days post-glycolipid treatment are shown for each group (a and b, right panel). Data represent the means ± s.d. from three individual mice per group. $^*P < 0{\cdot}001$ compared to IgG for a given glycolipid treatment.

retain their ability to secrete Th2 cytokines. While T_{regs} appear to control the level of Th1 and Th2 cytokine secretion induced by KRN7000-activated iNK T cells, T_{regs} seem to exert less stringent control of cytokine secretion by C20:2-activated iNK T cells.

Glycolipid-induced iNK T cell cytokine bias probably originates from interactions with APCs and the transactivation of other immune cells, rather than biasing iNK T cells themselves, which are resistant to cytokine polarization in C57BL/6 mice [25]. To test whether C20:2 polarizes cytokine expression directly by iNK T cells *in vivo* in NOD mice, we analysed C20:2-activated iNK T cells for their pattern of intracellular cytokine expression. Prior to iNK T TCR down-regulation at 2 h after glycolipid administration

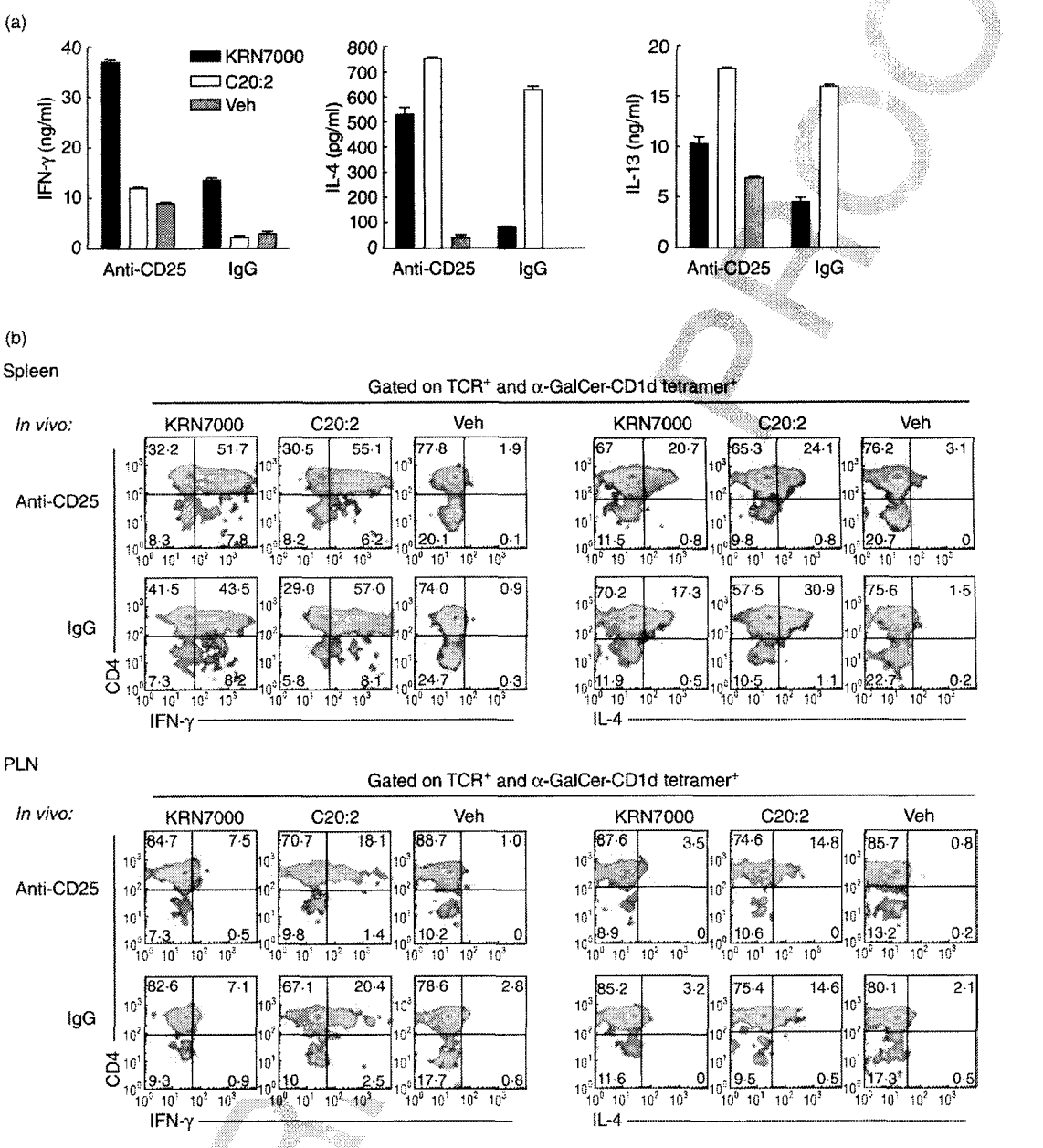


Fig. 4. C20:2 restimulation induces T helper type 2 (Th2) cytokine production by invariant natural killer T (iNK T) cells but does not polarize iNK T cells to a Th2-type response. Non-obese diabetic (NOD) mice (4–5 weeks old) were administered anti-CD25 or immunoglobulin G (IgG), rested, and then treated with KRN7000, C20:2 or vehicle, as in Fig. 1. (a) Splenocytes harvested at 3 days post-glycolipid treatment were restimulated *in vitro* for 72 h with KRN7000 or C20:2 (100 ng/ml), or vehicle, and their levels of cytokine [interferon (IFN)-γ, interleukin (IL)-4, IL-13] secretion were assayed. Data represent the means ± SD of triplicate measurements. Representative data from two independent experiments containing three mice per group are shown. (b) Spleen and PLN lymphocytes harvested from mice at 2 h post-glycolipid treatment were cultured *in vitro* for 3 h in the presence of a protein transport inhibitor and Golgi Stop, and stained for intracellular IL-4 and IFN-γ in gated $CD4^+$ iNK T cells. Representative pseudocolour contour plots from three independent and reproducible experiments are shown. Percentages of total cells in each quadrant are indicated.

in vivo, iNK T cells in the spleen and PLN of NOD mice were stained directly for their intracellular accumulation of IFN-γ and IL-4 without further stimulation *in vitro*. Gated TCR$^+$ and α-GalCer/CD1d tetramer$^+$ iNK T cells from both C20:2- and KRN7000-treated splenocytes expressed intracellular IFN-γ and IL-4, predominantly in CD4$^+$ iNK T cells (Fig. 4b). Small increases were noted in the percentages of CD4$^+$ iNK T cells from mice treated with anti-CD25 *versus* IgG and then either KRN7000 or C20:2, with the greatest difference observed in IFN-γ accumulation between anti-CD25/KRN7000 (51·7%)- and IgG/KRN7000 (43·5%)-treated mice. Of note, activation of iNK T cells in the spleen and PLN from IgG/C20:2-treated mice contained more IL-4- and IFN-γ-producing cells than in the spleen and PLN from IgG/KRN7000-treated mice (Fig. 4b). At this early time-point, we did not observe any bias in cytokine secretion by NOD iNK T cells, as C20:2 and KRN7000 induced an equivalent level of accumulation of intracellular IL-4 and IFN-γ.

C20:2-activated iNK T cells elicit reduced bystander cell activation

As IFN-γ was detected in the serum of KRN7000-treated mice only at 24 h post-administration (Fig. 1d), interactions between iNK T cells and other cell types may influence the level of IFN-γ secretion at later times after iNK T cell activation. To compare the ability of C20:2 and KRN7000 to transactivate other immune cells, we assayed the activation of B cells and NK cells in the spleen at 6 h and 24 h post-glycolipid administration. Based on the percentages of B220$^+$CD69$^+$ cells detected, B cell activation was evident in both C20:2- and KRN7000-treated mice. At 6 h post-glycolipid administration, the level of B cell activation was similar between C20:2- and KRN7000-treated mice, irrespective of anti-CD25 treatment (Fig. 5a). Differences between C20:2 and KRN7000 were observed at 24 h post-glycolipid administration, when the percentage of CD69$^+$ B cells activated by C20:2 was reduced about twofold in mice treated with anti-CD25 or IgG. Similar results were obtained for the transactivation of T cells (our unpublished observations). Analyses of NK cell activation profiles revealed that whereas C20:2 and KRN7000 both activate NK cells at an early time-point (6 h), only KRN7000 sustains this activation for a longer time, as the frequency of DX5$^+$CD69$^+$ NK cells was reduced by 1·6-fold and 2·5-fold, respectively, in anti-CD25/C20:2- and IgG/C20:2-treated mice at 24 h (Fig. 5a). Moreover, at 6 h after activation *in vivo* both C20:2 and KRN7000 induced an equivalent accumulation of IFN-γ in NK cells, while at 12 h post-treatment the percentage of NK$^+$IFN-γ$^+$ cells in C20:2-treated mice was reduced about twofold compared to that in KRN7000-treated mice (Fig. 5b). Thus, C20:2 and KRN7000 each induce immune cell transactivation, but only KRN7000-activated iNK T cells sustain this transactivation.

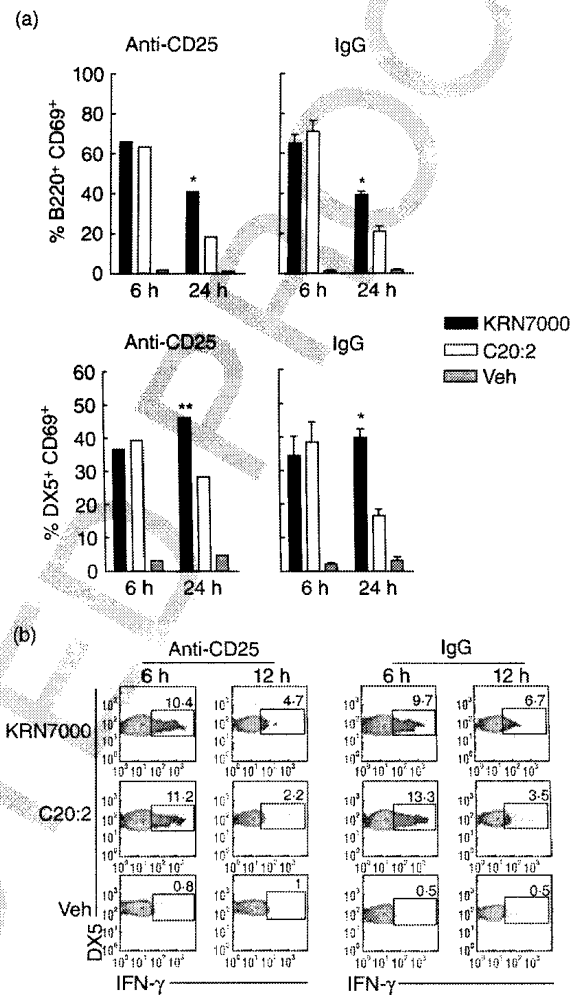


Fig. 5. C20:2-activated invariant natural killer T (iNK T) cells show a reduced ability to sustain the transactivation of B cells and NK cells. Non-obese diabetic (NOD) mice (4–5 weeks old) were administered anti-CD25 or immunoglobulin G (IgG), rested, and then treated with KRN7000, C20:2 or vehicle, as in Fig. 1. (a) Splenocytes were harvested at the indicated time-points post-glycolipid treatment, gated on B cells (B220$^+$CD3$^-$) or NK cells (DX5$^+$CD3$^-$) and the frequency of CD69$^+$ cells was analysed by flow cytometry. Data represent the means ± standard deviation (s.d.) of from three or four individual mice per group. (b) Splenocytes harvested from the mice at the indicated time-points post-glycolipid treatment were cultured *in vitro* for 3 h in the presence of a protein transport inhibitor and Golgi Stop. Gated NK cells (DX5$^+$CD3$^-$) were stained for intracellular interferon (IFN)-γ and representative contour plots from two or three independent and reproducible experiments are shown. The percentage of IFN-γ$^+$ cells in the gated NK cell populations are indicated. $^*P < 0{\cdot}01$ compared between glycolipids for a given anti-CD25 or IgG treatment; $^{**}P < 0{\cdot}05$ compared between glycolipids for a given treatment group.

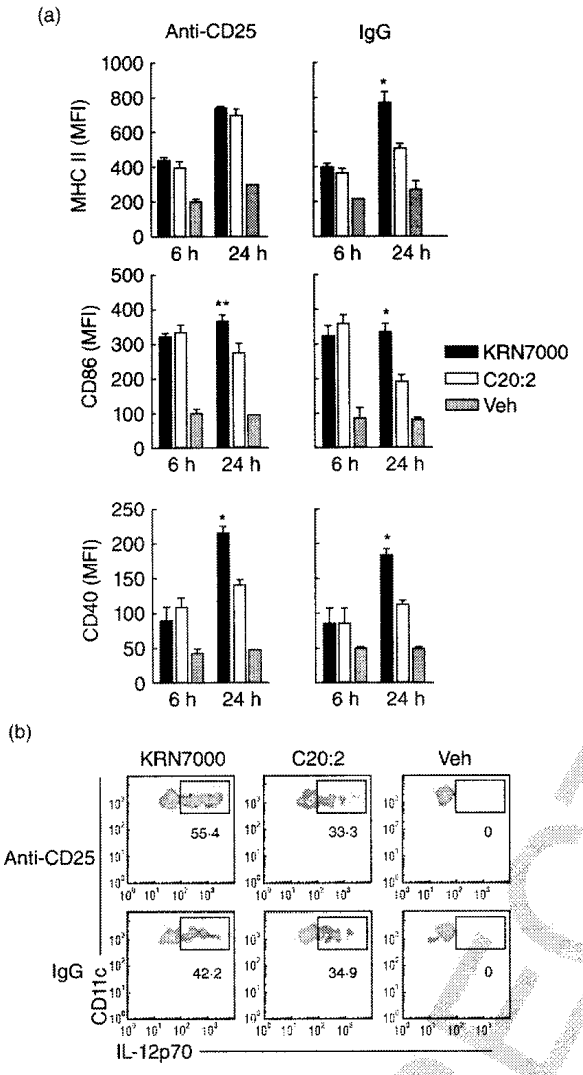


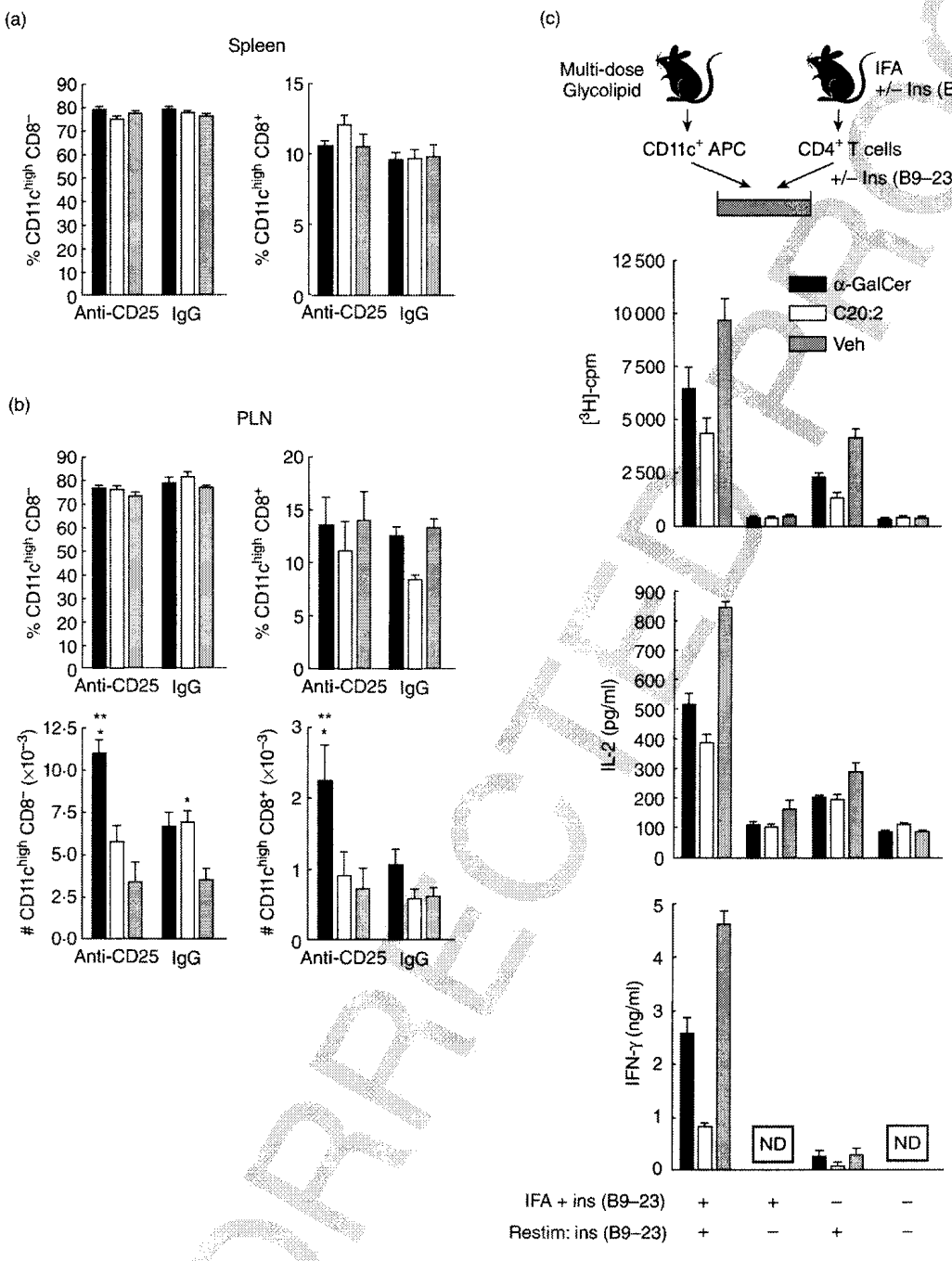
Fig. 6. Both C20:2- and KRN7000-activated invariant natural killer T (iNK T) cells induce dendritic cell (DC) maturation but only KRN7000 sustains this maturation. Non-obese diabetic (NOD) mice (4–5 weeks old) were administered anti-CD25 or immunoglobulin G (IgG), rested, and then treated with KRN7000, C20:2 or vehicle, as in Fig. 1. Splenocytes were harvested at the indicated time-points post-glycolipid treatment. (a) $CD11c^{high}$ mDC were gated and the mean fluorescence intensity (MFI) of the complete FL-2 channel staining for major histocompatibility complex (MHC) class II, CD86 and CD40 were graphed for the 6 h and 24 h time-points after glycolipid treatment. Data represent the means ± standard deviation (s.d.) of four individual mice per group. (b) At 6 h post-treatment with glycolipid, splenocytes were harvested, cultured *in vitro* for 3 h in the presence of a protein transport inhibitor and Golgi Stop and $CD11c^{high}CD8^{+}$ mDCs (Siglec $H^{-}CD11c^{high}$) were then gated and stained for intracellular interleukin-12. Representative contour plots from one of two independent and reproducible experiments are shown. $^{*}P < 0{\cdot}01$ compared between glycolipids for a given treatment group; $^{**}P < 0{\cdot}05$ compared between glycolipids for a given treatment group.

C20:2-activated iNK T cells influence DC function

DCs present glycolipid and translate iNK T activation signals to other immune cells [26], and KRN7000 but not C20:2 sustains B and NK cell transactivation. Accordingly, we investigated whether KRN7000 and C20:2 induce DC maturation and function differentially. $CD11c^{+}$ DCs were assayed by flow cytometry for their surface expression of activation and maturation markers at early and late time-points after iNK T cell activation. At 6 h post-treatment, C20:2- and KRN7000-activated $CD11c^{+}$ mDCs equivalently, as evaluated by the up-regulation of MHC class II, CD86 and CD40 expression, and the levels of mDC activation observed in IgG-treated mice were similar to those in anti-CD25-treated mice (Fig. 6a). At 24 h post-treatment, when KRN7000-activated iNK T cells up-regulate further the surface expression of co-stimulatory molecules on mDCs, a significant reduction in the expression was observed on mDCs in C20:2-treated mice. While mDCs from anti-CD25/C20:2- and anti-CD25/KRN7000-treated mice displayed a similar level of MHC class II expression, CD86 and CD40 expression was decreased on mDCs from anti-CD25/C20:2-treated mice (Fig. 6a). Thus, KRN7000 stimulation of iNK T cells elicits more robust mDC activation than C20:2 stimulation. This difference in mDC activation may account for the greater bystander activation of B, T and NK cells noted for KRN7000 relative to C20:2.

The latter notion is supported further by our finding that the intracellular expression of IL-12p70 at 6 h post-administration in $CD11c^{high}CD8^{+}$ mDCs was greater in anti-CD25/KRN7000-treated than anti-CD25/C20:2-treated mice (Fig. 6b). We detected very little IL-12p70 secretion in $CD11c^{high}CD8^{-}$ mDCs (data not shown). These results are consistent with the increased level of serum IFN-γ observed in anti-CD25/KRN7000-treated mice (Fig. 1d). Thus, the decreased ability of C20:2-stimulated iNK T cells to sustain the transactivation of other immune cells may be due in part to the reduced activation of $CD11c^{high}CD8^{+}$ mDCs.

Previous studies have suggested that iNK T cell-mediated protection against T1D may be due to the recruitment of tolerogenic DCs that suppress rather than prime effector T cells [12–14]. More recent analyses of APCs that mediate the development of T1D demonstrate that plasmacytoid DCs (pDCs) may play a role in reducing inflammation and insulitis [27]. Using Siglec H, a siglec-like molecule that binds specifically precursor pDCs [28] and CD11c as surface markers that distinguish between pDCs and conventional mDCs, we determined if the frequencies of these DC subsets are altered in mice treated with a therapeutic dose of glycolipid. Young NOD mice (4–5 weeks old) were administered a single dose of anti-CD25 mAb or control IgG, rested for 3 days, and then treated with a multi-low-dose protocol



of KRN7000 or C20:2. One week after the last dose, spleen and PLN lymphocytes were harvested for flow cytometric analysis of DC subsets. No differences were detected in the frequency of Siglec H$^+$CD11c^{low} pDCs (data not shown) or CD11c^{high}CD8$^{-/+}$ mDCs in the spleens of mice treated with either glycolipid or control vehicle (Fig. 7a). However, the frequency of CD11c^{high}CD8$^+$ mDCs but not CD11c^{high}CD8$^-$ mDCs was reduced in the PLN of C20:2-treated mice compared to the frequencies observed in KRN7000- and vehicle-treated mice (Fig. 7b). The greatest reduction was observed in mice pretreated with IgG rather than anti-CD25. Note that treatment with KRN7000 or C20:2 each increased the absolute number of CD11c^{high}CD8$^-$ mDCs recruited to the PLN relative to control vehicle treatment. Thus, C20:2 activation of iNK T cells leads to the differential recruitment of CD11c^{high}CD8$^-$ and CD11c^{high}CD8$^+$ mDCs to the PLN of

Fig. 7. Activation of invariant natural killer T (iNK T) cells with C20:2 alters the frequency and function of dendritic cells (DCs). Non-obese diabetic (NOD) mice (4–5 weeks old) were administered anti-CD25 or immunoglobulin G (IgG), rested, and then treated with KRN7000, C20:2 or vehicle, as in Fig. 2. Splenocytes and pancreatic lymph nodes (PLN) were collected at 1 week after the last glycolipid dose. (a) Plasmacytoid DCs (pDCs) (Siglec H^+CD11c^{low}) and myeloid DCs (mDCs) (Siglec H^-CD11c^{high}) were gated as indicated for DC subset analysis, and mDCs were separated further into the $CD11c^{high}CD8^-$ and $CD11c^{high}CD8^+$ subsets. (b) Splenocytes and PLN were gated as in (a), and the frequencies and PLN absolute number of the various DC subsets were analysed. Data shown represent the means ± standard deviation (s.d.) of five mice per group. (c) NOD mice (4–5 weeks old) were treated with a multi-low-dose protocol of KRN7000 or C20:2 (4 μg/dose, every other day for 3 weeks), or vehicle. One week after the last dose, spleen and PLN suspensions were pooled and $CD11c^+$ DCs from each group were sorted. Concurrently, the Ins B9-23 peptide emulsified in incomplete Freund's adjuvant (IFA) or vehicle was administered to a separate group of NOD mice to prime their $CD4^+$ T cells. Fluorescence activated cell sorted (FACS) Ins B9-23 peptide primed-$CD4^+$ T cells were cultured with $CD11c^+$ DCs in the presence of Ins B9-23 for 72 h. T cell proliferation and enzyme-linked immunosorbent assay (ELISA) assays of cytokine concentrations [interleukin (IL)-2, interferon (IFN)-γ, IL-4] in culture supernatants were then performed. $^*P < 0{\cdot}05$ compared to vehicle within a given treatment group; $^{**}P < 0{\cdot}05$ compared to C20:2 within a given treatment group.

treated mice, and T_{reg} inactivation enhances the recruitment of both mDC subsets but only in mice treated with KRN7000.

To determine if glycolipid treatment alters the function of DCs, we assayed the capacity of $CD11c^+$ DCs to stimulate antigen-primed $CD4^+$ T cells. $CD11c^+$ DCs from NOD mice treated with a multi-low-dose protocol of glycolipid were co-cultured with Ins B9-23 peptide primed $CD4^+$ T cells and assayed for their capacity to stimulate a T cell recall response (Fig. 7c). $CD11c^+$ DCs from NOD mice administered control vehicle elicited the robust activation of primed $CD4^+$ T cells, as evidenced by the induction of cell proliferation as well as IL-2 and IFN-γ secretion in the presence of recall antigen (Fig. 7c). IL-4 secretion was not detected in any of the cultures (data not shown). In contrast, *in vivo* treatment of NOD mice with C20:2 was more effective than that of KRN7000 treatment in decreasing the stimulatory capacity of $CD11c^+$ DCs, with the decreases noted for C20:2 being ≥twofold. These observations suggest that chronic activation of iNK T cells by synthetic glycolipids can alter DC antigen-presenting capacity, and that *in vivo* treatment of NOD mice with C20:2 appears to induce DC function with less stimulatory activity than treatment with KRN7000.

Discussion

Immunomodulation of iNK T cells with glycolipids represents a potential therapeutic strategy for protection against autoimmune T1D. Notwithstanding, activation of iNK T cells by KRN7000, the prototypic form of α-GalCer that is used currently for iNK T cell analysis and immunomodulation, can be problematic. This glycolipid induces not only 'protective' Th2 cytokine responses but also strong pro-inflammatory Th1 cytokine responses, depending on the system under study [6]. Moreover, as we reported, T_{reg} inactivation by anti-CD25 treatment augments the ability of KRN7000 to induce IFN-γ responses that exacerbate rather than protect from T1D in NOD mice [15] 0. Thus, the use of modified analogues of α-GalCer that reduce Th1 responses and amplify Th2 responses may be preferable due to their ability to direct iNK T cell functions towards more desirable outcomes. In this study, we analysed iNK T cell activation and function in response to a Th2-biased glycolipid analogue of α-GalCer termed C20:2. We confirmed that C20:2 reduces IFN-γ and enhances IL-4 and IL-13 secretion by iNK T cells in NOD mice and protects them from T1D [19]. Interestingly, however, we found that C20:2-activated iNK T cells depend less on T_{reg} activity than KRN7000-activated iNK T cells for protection. This may result from the reduced capacity of C20:2 to sustain the transactivation of T, B, NK and mature myeloid DCs. Previously, C20:2 was reported to provide improved clinical outcomes against T1D when compared to the α-GalCer-related compound C24:0, which induces a mixed Th1- and Th2-type cytokine response indistinguishable from KRN7000 [19]. Thus, based on these anti-inflammatory functional properties of C20:2 and its capacity to bind to and activate human iNK T cells [19], our findings support further the idea that C20:2 should be evaluated as a candidate target drug to indue iNK T cell-mediated protection from T1D in humans.

The ability of T_{regs} to suppress autoimmunity and protect against autoimmune T1D [6] is currently receiving much attention due to its potential clinical benefit [29,30]. In this regard, it is important to consider that this beneficial effect of T_{regs} in T1D may be attributable in part to their ability to suppress the proinflammatory functions of activated iNK T cells [31,32]. Given that T1D patients may have functional differences in T_{reg} activity [33], and that KRN7000-activated iNK T cells depend on T_{reg} cells for protection against NOD T1D [15], we investigated the effect of T_{regs} on C20:2-activated iNK T cells during the spontaneous development of T1D. In contrast to the high incidence of T1D observed in anti-CD25/KRN7000-treated mice, anti-CD25/C20:2-treated mice were protected from T1D. Based primarily on two sets of findings, we reason that the different outcome noted between the KRN7000- and C20:2-treated mice arises from the differential ability of these glycolipids to activate iNK T cells rather than the ability of anti-CD25 treatment to inhibit iNK T cell activation. First, the effect of anti-CD25 on T_{reg} inactivation exceeded that on iNK T cell activation, as iNK T cells expanded to a similar extent in mice treated with either anti-CD25 or control IgG. At 3 days post-KRN7000

treatment, spleen cellularity was elevated and this was accompanied by an increase in the absolute number of iNK T cells in the spleen of anti-CD25/KRN7000-treated mice. Secondly, glycolipid activation of iNK T cells *in vivo* and restimulation *in vitro* enhanced significantly cytokine secretion by iNK T cells from anti-CD25-treated compared to IgG-treated mice. In particular, KRN7000 experienced iNK T cells secreted much more IFN-γ than C20:2-activated iNK T cells, not only when left unstimulated but also after restimulation with glycolipid. Presumably, this increase in IFN-γ secretion contributed to the development of T1D. In contrast, iNK T cells from C20:2-treated mice retained their ability to secrete Th2 cytokines and were protected from T1D. Taken together, our findings indicate that the inactivation of T_{regs} augments the function of iNK T cells and are consistent with the idea that T_{regs} can down-regulate iNK T cell-mediated immune responses. Thus, anti-CD25 mAb treatment may 'unmask' the potentially harmful side effects of KRN7000-activated iNK T cells, whereas the effect of this treatment on C20:2-treated iNK T cells is minimal. This observation may be relevant clinically to prediabetic patients who may be deficient in the number and/or function of T_{regs} [33]. Thus, an intervention protocol that treats prediabetic subjects with C20:2 and relies less on T_{reg} activity may increase the benefit : risk ratio of these subjects due to its reduced ability to stimulate proinflammatory responses.

Several differences in the activation of iNK T cell-mediated responses by C20:2 and KRN7000 were observed. One of the most striking was the inability of C20:2 to sustain an increase in the level of IFN-γ in the sera of C20:2-treated mice even after T_{reg} inactivation. As iNK T cells are resistant to polarization [25], this response was due probably to the inability of C20:2-activated iNK T cells to sustain the activation of a downstream target cell(s). NK cells are probable candidates, as they are major contributors to IFN-γ synthesis. Depletion of NK cells by treatment with anti-asialo-GM1 antibody can abrogate IFN-γ in serum induced by KRN7000, a mechanism that is dependent upon IL-12 signalling [25]. Our results presented here demonstrate that C20:2 may not sustain the activation of NK cells and B cells as measured by their lower surface expression of CD69. We also found a reduction in the frequency of intracellular IFN-γ-expressing NK cells as late as 24 h after C20:2 treatment. Despite this reduced ability of C20:2 to sustain cellular transactivation, the initial activation of iNK T cells by C20:2 at an earlier time-point (6 h) was equivalent to that seen with KRN7000 and sufficient to induce early bursts of NK cell and B cell activity. No significant differences in CD69 expression were detected on NK cells and B cells from anti-CD25-treated *versus* IgG-treated mice. Therefore, the major effect of T_{reg} inactivation by anti-CD25 appears to be on the modulation of cytokine secretion. Our findings suggest that that the initial burst of activation of iNK T cells stimulated by C20:2 is not deficient, but rather that C20:2-activated iNK T cells fail to sustain their initial level of iNK T cell activation and transactivation that leads to an increase in the serum concentration of IFN-γ.

The capacity of iNK T cells to interact and modulate DC activity by co-stimulation is important for the translation of iNK T cell signals to immune responses. The initial iNK T cell cytokine burst induced by KRN7000 occurs independently of CD40–CD40L and CD80/86–CD28 signalling [34], but the expansion and immunogenicity induced by KRN7000-activated iNK T cells is dependent on these signalling pathways [26,34]. iNK T cells activated from mice deficient in CD40–CD40L signalling do not undergo expansion and cannot induce T cell activation to third-party antigens such as ovalbumin (OVA) [26,34]. As reported for KRN7000 [26], we found that C20:2 can activate and mature $CD11c^{high}$ mDC cells upon administration of glycolipid. Up-regulation of MHC class II, CD86, and CD40 were detected at 6 h post-treatment. However, as observed for NK and B cell transactivation, mDCs from C20:2-treated mice were less capable than mDCs from mKRN7000-treated mice in sustaining the surface expression of co-stimulatory molecules required for iNK T cell expansion. The latter observation may explain why C20:2-treated iNK T cells induced less IL-12 secretion by $CD11c^{high}CD8^{+}$ DCs, which may account for the reduced levels of serum IFN-γ detected in downstream responses. Interestingly, the activation of iNK T cells can induce the migration of $CD11c^{high}$ DCs capable of 'tolerizing' autoreactive T cells in the PLN of NOD mice [12,13]. As the transfer of syngeneic PLN DC into young NOD mice protects against T1D [12,35], iNK T cell recruitment of DC may restore a deficiency in DC function inherent in NOD mice [35,36]. Our observations also indicate that glycolipid treatment modulates the frequency and function of DCs. Furthermore, we did not detect differences in the frequency of pDCs upon iNK T cell activation, although the function of these pDCs was not assayed. While glycolipid administration stimulated the recruitment of $CD11c^{high}CD8^{-}$ mDCs to the PLN of NOD mice, as reported previously [12], the frequency of $CD11c^{high}CD8^{+}$ mDCs recruited to the PLN using our treatment regimen was lower in C20:2-treated than KRN7000-treated mice. Importantly, $CD11c^{high}CD8^{+}$ mDCs were found to be the major producers of IL-12. In addition, iNK T cell-mediated recruitment of DC is probably dependent upon the glycolipid treatment regimen. One weekly dose of C20:2 administered for 7 weeks resulted in an increased recruitment of DC subsets in the PLN of NOD mice at 30 weeks of age [19], whereas the current study indicates that treatment every other day for 3 weeks starting at 4 weeks of age resulted in a reduced frequency of $CD11c^{high}CD8^{+}$ mDC when analysed at 8–9 weeks of age. The difference in treatment regimen and/or age of the mice at time of killing may account for the apparent difference in DC subset proportions observed. None the less, both results suggest that glycolipid treatment modulates the frequency of recruited DC, but how this correlates with reduced T1D requires further experimentation and is beyond the scope of

this study. Interestingly, T_{reg} inactivation resulted in a significant increase in the recruitment of both $CD11c^{high}CD8^{-}$ and $CD11c^{high}CD8^{+}$ mDCs in KRN7000-treated mice, suggesting that DC recruitment alone is insufficient to prevent T1D in the absence of functional T_{regs}. Thus, our observations using the current treatment regimen suggest that *in vivo* treatment of NOD mice with C20:2 diminish the activation of 'inflammatory' IL-12-producing $CD11c^{high}CD8^{+}$ mDCs and augment the function of 'tolerogenic' DCs more effectively than treatment with KRN7000. Moreover, the latter findings may explain why C20:2-activated iNK T cells depend less than KRN7000-activated iNK T cells on regulation by T_{regs} for cytokine secretion and protection from T1D.

Our study demonstrates that T_{regs} play either a major or more minor role in the control of iNK T cell function that varies according to the durability of the activation signal elicited by a given glycolipid. Both the KRN7000 and C20:2 forms of α-GalCer induce a strong early signal in iNK T cells. The signal induced by KRN7000 sustains iNK T cell activation for a sufficiently long time (several days) to induce robust downstream responses, and T_{regs} are required for iNK T cell-mediated protection from T1D. Conversely, the signal induced by C20:2 in iNK T cells is not sustained beyond 6 h, reduced downstream responses are stimulated and iNK T cells are less dependent on the activity of T_{regs} for iNK T cell protection from T1D. These results are consistent with a model in which the extent of T_{reg} suppression required for a given immune response correlates directly with the level of T effector cell activation [37]. Accordingly, iNK T cells activated by KRN7000 promote a more 'proinflammatory' response that appears to increase the requirement for T_{reg} activity to return iNK T cells to a homeostatic level of activation (Fig. S1). Alternatively, activation of iNK T cells by C20:2 yields a more anti-inflammatory response that is less dependent on T_{regs} for the maintenance of iNK T cell homeostasis. INK T cell activation by a Th2-biased ligand such as C20:2 may provide a better outcome and a 'safer' therapeutic alternative due to its decreased propensity to elicit proinflammatory responses. Thus, our study further underscores the importance of selective iNK T cell modulation for the immunotherapy of T1D.

Acknowledgements

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Disclosure

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Model of differential activation of invariant natural killer T (iNK T) cells by KRN7000 and C20:2. The activation of iNK T cells by a glycolipid antigen leads to the rapid release of the immunomodulatory cytokines interleukin (IL)-4 and interferon (IFN)-γ. The T helper type 2 (Th2)-biased glycolipid C20:2 can modulate differentially iNK T cell-induced responses that lead to more efficacious protection against type 1 diabetes (T1D). Whereas KRN7000 activation leads to iNK T cell secretion of Th1 and Th2 cytokines and robust bystander cell activation, C20:2-activated iNK T cells secrete elevated levels of Th2 cytokines and elicit reduced bystander cell activation. Furthermore, KRN7000-activated iNK T cells are dependent on regulation by regulatory T cells (T_{reg}) for protection against T1D, whereas C20:2-activated iNK T cells are less dependent on the regulation by T_{reg} cells. Thus, given the ability of IL-4 to prevent T1D and the possibility that differential T_{reg} activity may exist *in vivo*, C20:2 may be a better candidate drug than KRN7000 for the prevention of T1D.

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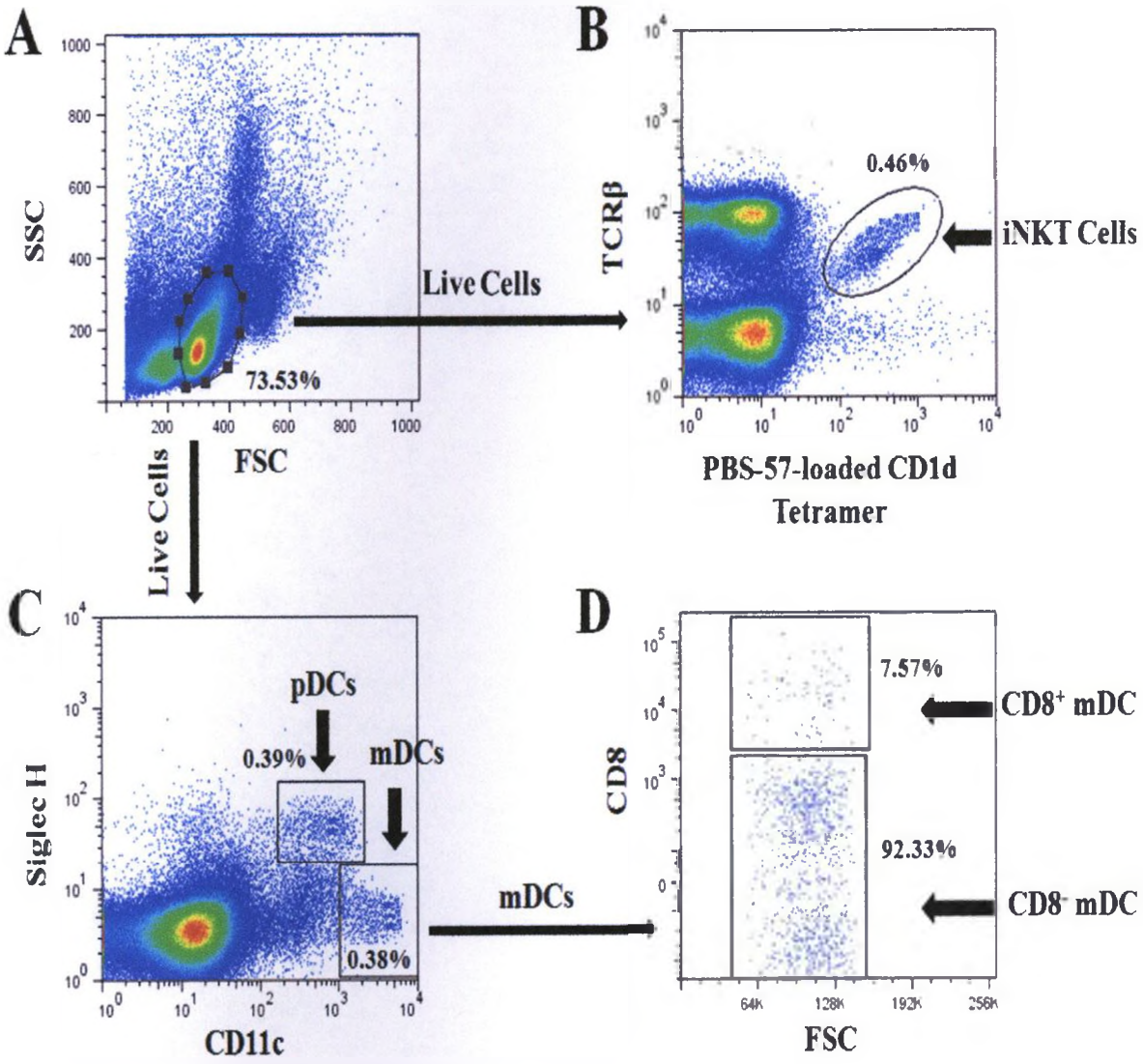
Appendix B: Gating strategies for cell subsets. Upon completion of flow cytometry, data was analyzed using FlowJo software. Only viable cells, based on forward scatter and side scatter values, were used for analysis **(A).** Within the live gate, iNKT cells were characterized by their expression of TCRβ and PBS-57-loaded CD1d tetramer ($TCR\beta^+tetramer^+$) **(B).** Dendritic cell subsets were identified by the surface expression of Siglec H and CD11c. Plasmacytoid DCs (pDCs) and myeloid DCs (mDCs) were characterized as Siglec H^+ $CD11c^{low}$ and Siglec H^- $CD11c^{high}$, respectively **(C).** mDCs were further differentiated based on their expression of CD8 **(D).**

Appendix B: Gating strategies for cell subsets



Appendix C: Ethics approval for research involving animals

02.01.09
*This is the 1st Renewal of this protocol
*A Full Protocol submission will be required in 2012

Dear Dr. **Delovitch**

Your Animal Use Protocol form entitled

T Cell Regulation of Type 1 Diabetes

has had its yearly renewal approved by the Animal Use Subcommittee.

This approval is valid from **02.01.09 to 01.31.10**

The protocol number for this project remains as **2006-025**

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.
   If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.
4. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

c.c. M Pickering, W Lagerwall

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