The Role of Antigen Receptor Signaling in Activation and Development of invariant Natural Killer T Cells

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Dedication

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Abstract

CD1d-reactive invariant Natural Killer T cells (iNKT) are a T cell subset that have characteristics of both innate immune cells and adaptive immune cells. As a result, they are considered a bridge between the innate and the adaptive immune response. iNKT cells can rapidly secrete a variety of cytokines after activation, and therefore have an important immunomodulatory role during the immune response in many different diseases, such as cancer, asthma, autoimmune disease, and infection. During infections, there are three models of iNKT cell activation: activation requiring microbial antigen, cytokine driven activation, and activation requiring self-antigen. However, how iNKT cells become activated during some infections remains controversial, as activation requiring self-antigen has only been indirectly shown. Therefore, I addressed this controversy using an antigen receptor signal strength reporter mouse, in which Nur77^{gfp} reports stimulation of the T cell receptor (TCR). Although the sensitivity of this reporter mouse was initially observed in conventional T cells, I showed that Nur77^{gfp} is also a sensitive readout for TCR stimulation in iNKT cells. Therefore, several bacterial and viral infections were examined for the role of antigen-dependent versus antigen-independent activation. These experiments showed that Nur77^{gfp} was upregulated during infections with a microbial antigen, and was not upregulated during an infection previously considered to activate iNKT cells by the cytokine driven model. Unexpectedly, the infectious contexts reported to require self-antigen for iNKT cell

activation did not upregulate Nur77^{gfp}, indicating that the main driver of iNKT cell activation is cytokine, and thus activation is antigen-independent. Additionally, as a result of the immunomodulatory capabilities of iNKT cells, iNKT cells have great potential for use in new therapeutics. However, for these therapies to be effective, an exogenous source of iNKT cells is needed. Therefore, I adapted the OP9-DL1 culture system to derive iNKT cells from hematopoietic progenitor cells *in vitro*. I showed that the frequency of cells expressing the iNKT TCR needed to be increased in order to detect iNKT cells using this system. Furthermore, I showed that the addition of IL-15 can enhance the percentage of cells expressing the NKT1 subset transcription factor Tbet. Therefore, this iNKT cell adapted OP9-DL1 culture system can be used as a new method to examine factors that influence iNKT cell development and to identify factors that can skew iNKT cell subset differentiation. Ultimately, these experiments aided in advancing the understanding of iNKT cell activation and development, as well as how to harness the power of iNKT cells to artificially orchestrate an immune response for therapeutic use.

Table of Contents

List of Figures

Chapter 1: Introduction

Innate and Adaptive Immune System

The immune system has evolved to protect the host from a variety of foreign invaders, such as bacteria, fungi, viruses, and parasites. As a result of the selective pressure imposed by these pathogens, all multicellular organisms have an immune system (1). The main function of the immune system is to efficiently recognize and eliminate invading pathogens, but this must be done without causing damage to the host (2). Since there is enormous diversity among pathogens, the immune system has developed two different mechanisms for pathogen recognition: general recognition of molecular patterns shared by a large group of pathogens, or recognition adapted to the specific invading pathogen (1). Immune cells that recognize pathogens by this first mechanism are considered to be part of the innate branch of the immune system, and immune cells that recognize pathogens by this second mechanism are considered to be part of the adaptive branch of the immune system. These two branches of the immune system have different characteristics, which will be described below.

The innate branch of the immune system is the first line of defense against invading pathogens and is found in all multicellular organisms (1). It is found in both plants and animals, and is therefore considered evolutionarily older than the adaptive branch of the immune system (2). The innate immune response is characterized by a fast (within hours), non-specific response to the pathogen, and is mostly carried out by leukocytes from the myeloid lineage of hematopoietic cells, such as macrophages, neutrophils, and dendritic cells. However, Natural Killer cells (NK), which are derived

from the lymphoid lineage of hematopoietic cells, are also considered to be part of the innate immune system.

Innate immune cells can be characterized, in part, by their expression of germ-line encoded receptors called pattern recognition receptors (PRRs), which recognize distinct pathogen associated molecular patterns (PAMPs) (1). PAMPs are repeating molecular structures found only in microorganisms, but not in host cells, thus distinguishing the pathogen from self. Additionally, a PAMP is usually shared by large group of pathogens and is essential for the survival or pathogenicity of the microorganism, and therefore cannot be changed (1). For example, a PRR can recognize the general structure of lipopolysaccharide (LPS), which is a structure shared by all Gram-negative bacteria, but not found in multicellular organisms (1). PRRs can be expressed on the cell surface or in intracellular compartments of innate immune cells, or can alternatively be secreted into the bloodstream and other tissue fluids (1). PRR pathogen recognition can lead to opsonization of the invading pathogen, activation of complement cascades, phagocytosis of the invading pathogen, induction of apopotosis, or activation of signaling pathways, such as proinflammatory signaling pathways (2).

The best characterized class of PRR is a family of receptors known as Toll-like receptors (TLRs) (3). Most mammals have between 10 to 15 different TLRs (3), which recognize different PAMPs. For example, TLR4, a cell surface TLR, recognizes the PAMP mentioned above, LPS, in association with CD14 and MD-2, and TLR9, an endosomal TLR, recognizes the unmethylated dinucleotide CpG repeats found in

bacterial DNA (2). Although different TLRs recognize different PAMPs, all TLRs signal through a conserved signaling cascade, which leads to induction of the transcription factor NFκB (2). NFκB induction leads to the transcription of a variety of genes important for initiating and guiding a subsequent adaptive immune response, such as genes encoding cytokines, chemokines, and co-stimulatory molecules (2). Therefore, the main role of the innate immune system is to rapidly clear pathogens based on general characteristics of the invading pathogen; however, it is also important for initiating the adaptive immune response, which is needed to clear the pathogen when the innate immune response is overwhelmed.

In contrast to the innate branch of the immune system, the adaptive branch of the immune response is characterized by a slower (days as opposed to hours), very specific response to the invading pathogen. Importantly, this specificity to the invading pathogen is maintained by the host to protect against future re-infection, which is referred to as immunological memory and is unique to adaptive immunity (2). Unlike innate immunity, the adaptive immunity is only found in vertebrates, indicating it is evolutionarily younger (1, 2).

The adaptive immune system is mainly composed of leukocytes from the lymphoid lineage, such as B cells and T cells. B cells and T cells arise from a common hematopoietic stem cell in the bone marrow; however, B cell development occurs in bone marrow and T cell development occurs in the thymus. Unlike innate immune cells, whose function is largely dictated by germ-line encoded receptors, B cells and T cells

have highly specialized antigen receptors whose rearrangement allows for very specific recognition of the invading pathogen (1). These receptors are called the B cell receptor (BCR) for B cells or the T cell receptor (TCR) for T cells. For this thesis, I will focus on the TCR. A TCR is comprised of a heterodimeric pairing of two TCR chains. Most commonly, this consists of the TCR β-chain paired with the TCR α-chain ($\alpha\beta^+$ TCR). As mentioned, TCRs have much greater specificity for a particular pathogen than PRRs. During development in the thymus, T cells acquire their antigen receptor specificity through a process of gene rearrangement called V(D)J recombination, which ultimately leads to the generation of more than 10^{15} different receptor specificities (4). This creates a diverse pool of TCRs to ensure that the host will be protected from any potential invading pathogen.

More specifically, a T cell's TCR recognizes a pathogen-derived peptide in the context of a major histocompatibility complex molecule (pMHC). A specialized group of cells, called antigen presenting cells (APCs), process pathogens into peptides for MHC loading and then present the pMHC on their cell surface. However, the number of T cells expressing a TCR that recognizes a specific pMHC in the host is very small. Therefore, to mount an effective immune response, this rare T cell must proliferate to increase the number of T cells expressing this antigen specific TCR. This proliferation event is called clonal expansion, and occurs after the T cell meets its cognate pMHC.

Importantly, clonal expansion is tightly controlled. Since a diverse pool of TCRs is created by V(D)J recombination, it is inevitable that some of these TCRs will be

specific for self-antigen (self). Although many of these T cells expressing self-reactive TCRs are deleted during thymic development by a process called negative selection, some of these T cells "escape" this control point. Therefore, if clonal expansion occurred only through TCR stimulation, these "escaped" T cells would proliferate and attack the host, causing autoimmunity. To prevent this, T cell clonal expansion and subsequent activation requires additional "cues," which are provided by APCs (5). APCs are cells, such as macrophages and dendritic cells, capable of engulfing pathogens (phagocytosis), as well as express a PRR like a TLR. Therefore, in the context of an infection, an APC that engulfs and subsequently presents a pMHC from an invading pathogen will also be stimulated through its PRR to express costimulatory molecules such as CD80/CD86, and secrete cytokines such as IL-12 and type I Interferon, which serve as the additional "cues" required for clonal expansion and proper T cell activation (5). Therefore, the APC presenting a stimulating pMHC will also provide the required costimulation and cytokine signals only in the context of an infection (1-3).

Although innate immunity and adaptive immunity have different roles during the immune response, they must cooperate together to provide the greatest protection for the host. As exemplified by clonal expansion, cells involved in the innate immune response initiate the adaptive immune response. Another example of this cross-talk between innate immunity and adaptive immunity occurs with CD1d-reactive invariant Natural Killer T cells (iNKT). iNKT cells uniquely have characteristics of both innate and adaptive immune cells, and as a result, are considered a bridge between the innate

immune response and the adaptive immune response (6). However, many questions still remain about how iNKT cells contribute to the immune response. For example, the extent to which antigen recognition by the iNKT TCR contributes to the activation of iNKT cells during various infections remains to be fully elucidated, which is the subject of the second chapter of this thesis.

Discovery of "NK T Cells"

The isolation of a new T cell subset was reported in 1987 when three groups published studies identifying a subset of αβ-TCR^+ T cells that expressed intermediate levels of TCR, had a higher frequency of Vβ8 expression than conventional T cells, and lacked expression of the co-receptor molecules CD4 and CD8 (7-9). A few years later, two groups identified a subset of αβ-TCR^+ T cells that also expressed an intermediate level of TCR and a bias towards Vβ8 expression, but additionally expressed the NK cell marker NK1.1 (10, 11). Upon the further investigation of these subsets, it was shown that the $CD4^-$ $CD8^-$ double negative (DN) αβ-TCR^+ T cells could produce important immunoregulatory cytokines such as IFNγ and IL-4 (12), and then a year later, this same ability to produce immunoregulatory cytokines was reported in the subset of $NK1.1^+$ αβ-TCR^+ T cells (13, 14); however, at the time, it was not clear that these reports were describing the same subset (15). Further characterization of the $NK1.1^+$ αβ-TCR^+ cells revealed a developmental requirement for β_2-microglobulin (16, 17), which led to the discovery that these cells are reactive to the MHC class-I-like molecule, CD1d (18). The

majority of these cells were shown to have a very restricted TCR repertoire, which in mice, was composed of the α-chain variable region 14 (Vα14) and the α-chain joining region 18 (Jα18) paired with a restricted subset of TCR β-chains (Vβ8.2, Vβ7 and Vβ2) (19). Additionally, this CD1d-reactive TCR repertoire was conserved in humans and composed of the homologous Vα24-Jα18 paired with Vβ11 (19, 20). Later, in 1995, the term "NK T cells" was used to broadly distinguish this subset of $NK1.1^+$ $\alpha\beta\text{-}TCR^+$ T cells from conventional T cells and NK cells (21).

After the discovery that "NK T cells" are CD1d reactive, it was hypothesized that the antigen recognized by the "NK T" TCR was a lipid antigen (18, 22). However, the first "NK T cell" lipid ligand was not conclusively identified until 1997 (23). During a screen for natural anticancer compounds, the lipid αgalactosylceramide (αGalCer), which is derived from the marine sponge *Agelas mauritianus*, was shown to prolong the survival of mice with B16 melanoma and enhance NK cell activation (24, 25). These effects were later found to be a result of "NK T cell" activation, and thus identified αGalCer as a strong "NK T" TCR agonist that induces "NK T cell" proliferation and rapid production of IFNγ and IL-4 (26). After this initial discovery, further studies found that αGalCer could be used in a CD1d tetramer to track and isolate "NK T" cells due to αGalCer's high affinity for the canonical "NK T" TCR (27, 28). This was an important discovery because NK1.1, which was previously being used for "NK T cell" isolation, is not a reliable marker of "NK T cells": not all mouse strains express NK1.1, and even in mouse

strains that do express NK1.1, not all "NK T cells" express NK1.1. Therefore, "NK T cells" included CD1d-reactive NK1.1$^-$ cells.

All together, these studies expanded the definition of "NK T cells" to include cells that express an $\alpha\beta^+$ TCR and are reactive to lipid antigen presented by CD1d, but do not necessarily express NK1.1 (15, 27, 28). However, this definition revealed two subsets within "NK T cells": type I "NK T cells" which express the canonical Vα14-Jα18 TCR, and type II "NK T cells," which do not express the canonical TCR (non-Vα14) (15, 23). Less is known about type II "NK T cells" which are difficult to isolate for study compared to type I "NK T cells," as they cannot be isolated using the CD1d/αGalCer tetramer (15). Therefore, this thesis will focus on type I "NK T cells," which will now be referred to as CD1d-reactive invariant Natural Killer T cells (iNKT).

iNKT Cell Development and General Characteristics

iNKT cells develop in the thymus, an organ located above the heart. In the thymus, the frequency of iNKT cells is only about 0.5% (23). Initially, iNKT cells follow the same developmental pathway as conventional T cells. Both arise from a common hematopoietic stem cell in the bone marrow, which migrates to the thymus for further development. In the thymus, these progenitor cells go through four CD8$^-$ CD4$^-$ Double Negative (DN) stages before reaching the CD8$^+$ CD4$^+$ Double Positive (DP) stage. However, at the DP stage, the iNKT cell lineage branches away from the conventional T cell lineage. This occurs when rare DP thymocytes expressing the iNKT

TCR are positively selected by a self-lipid presented by CD1d on other DP thymocytes (29, 30). Additionally, iNKT cells undergo negative selection, in which cells that express TCRs with too great of an affinity for the CD1d presented self-lipid, will die (31, 32). The identity of these self-lipids remains controversial, but may include isoglobotrihexosylceramide (iGb3) and/or β-glucosylceramide (βGlcCer) (6, 33).

As mentioned above, iNKT cells are selected by self-lipids presented in the context of CD1d (18, 22). CD1d is a non-polymorphic MHC class I-like molecule that is part of the CD1 family and is highly conserved among mammals (34). It is believed that the CD1 family evolved to present lipids to T cells (35). In addition to being expressed on DP thymocytes, CD1d is expressed mainly on dendritic cells, macrophages, and B cells (18, 23, 29, 36), but has also been shown to be expressed on epithelial cells (37). Furthermore, the cellular trafficking of CD1d has been shown to be important for the presentation of selecting self-lipid (23). After biosynthesis, CD1d rapidly reaches the plasma membrane; however, it is subsequently internalized and recycled between the plasma membrane and the endosomal/lysosomal compartments (38-41). This recycling was found to be dependent on a tyrosine motif in the cytoplasmic tail of CD1d, which, when mutated, greatly impaired iNKT cell selection (40). This suggests that the self-lipid is endosome- and/or lysosome-derived, and CD1d must cycle through these compartments for self-lipid loading and subsequent presentation for iNKT cell selection (42).

In addition to the recognition of stimulating lipid/CD1d presented by other DP thymocytes, iNKT cell selection also requires homophilic interactions between signaling lymphocytic-activation molecule (SLAM) family members (29, 42-46). More specifically, Slamf1 and Slamf6 engagement leads to the downstream recruitment of SLAM-associated protein (SAP) (44, 46). SAP then interacts with the Src Kinase Fyn, which initiates NFκB signaling through PKCθ and promotes iNKT cell survival (42, 47, 48).

After the initial selection of iNKT cells, TCR signals lead to the increased expression of early growth response 2 (Egr2) (49), which directly induces the expression of the main transcriptional regulator of iNKT cell development, PLZF (promyelocytic leukemia zinc finger, encoded by *Zbtb16*) (50-52). PLZF has been shown to direct the innate-like effector differentiation of iNKT cells during thymic development, and is later important for full functionality and proper tissue homing (50, 51, 53, 54). After selection to the iNKT cell lineage, iNKT cell development is classified into four stages. In the first stage, stage 0, these earliest post-selection cells are $CD24^{hi}$ $CD44^{lo}$ $NK1.1^{-}$ (29). In the next stage, stage 1, these cells become $CD24^{lo}$ $CD44^{lo}$ $NK1.1^{-}$ before progressing to stage 2, in which they become $CD24^{lo}$ $CD44^{hi}$ $NK1.1^{-}$ (55). The most "mature" iNKT cells are found in stage 3, in which they become $CD24^{lo}$ $CD44^{hi}$ $NKl.1^{+}$ (55). However, with the recent discoveries of iNKT cell subsets (56), these stage divisions need to be revised.

After PLZF expression is initiated, iNKT cells undergo further differentiation into three main subsets, NKT1, NKT2, and NKT17 (57-59). These three subsets are

distinguished by their transcription factor profiles: NKT1 cells express *Tbx21* (Tbet), NKT2 cells express *Gata3* (GATA3), and NKT17 cells express *Rorc* (RORγt) (56). A recent report separated these three subsets into the three populations based on their expression of PLZF, RORγt, and Tbet: NKT1 cells are $PLZF^{lo}$ $Tbet^{+}$, NKT2 cells are $PLZF^{hi}$ $Tbet^{-}$ $ROR\gamma t^{-}$, and NKT17 cells are $PLZF^{int}$ $ROR\gamma t^{+}$ (57). This subset differentiation is important as it dictates the main cytokine produced by the cell after stimulation, and therefore influences the subsequent immune response. NKT1 cells rapidly produce IFNγ, NKT2 cells produce IL-4, and NKT17 cells produce IL-17 (57).

The mechanisms that drive iNKT cell differentiation into these subsets remains largely unknown (56). One speculation is that the level of PLZF expression may be playing a role in determining each subset (56). Alternatively, cytokines may (also) be influencing subset development. For example, IL-15 may be involved in NKT1 subset differentiation. Previous reports of iNKT cells in IL-15 deficient mice showed reduced thymic iNKT cell numbers (60, 61). Additionally, IL-15 induced the expression of survival factors in thymic iNKT cells, and was shown to be involved in Tbet regulation during iNKT cell development (60). Therefore, IL-15 may potentially play a role in driving NKT1 subset differentiation.

Once selected, iNKT cells in mice become either $CD4^{+}$ $CD8^{-}$ ($CD4^{+}$) or $CD4^{-}$ $CD8^{-}$ (DN). Interestingly, forced CD8 expression in iNKT cells resulted in their deletion in mice (16); however, humans have $CD8^{+}$ iNKT cells (62). Additionally, iNKT cells can express receptors typically associated with NK cells, such as NK1.1 (18), Ly49 (63),

CD161 (64), and NKG2D (65). The regulation and role of these receptors in iNKT cell activation is not well defined, but some studies indicate that they are involved in iNKT cell responses to inflammation that are independent of antigenic lipid presentation (63, 66, 67).

After selection, iNKT cells emigrate to peripheral tissues. In the blood and peripheral lymph nodes, iNKT cells make up about 0.5% of the T cell population (23). In the spleen, mesenteric, and pancreatic lymph nodes, iNKT cells make up about 2.5% of T cells (23). In contrast, in the liver, iNKT cells make up to 30% of the T cell population (23). However, the specific locations of iNKT cells within these organs has been difficult to elucidate due to the technical hurdles associated with using the CD1d/αGalCer tetramer in immunohistochemistry to accurately identify them.

Another distinguishing characteristic of iNKT cells is their activated/memory phenotype during homeostasis (68). iNKT cell express higher levels of the activation markers CD44 and CD69 compared to naïve conventional T cells (68), and are poised for rapid cytokine secretion upon stimulation (69). This rapid cytokine secretion is especially prominent after stimulation with the known iNKT TCR agonist, αGalCer (26). Upon stimulation, iNKT cells can produce a variety of cytokines such as TNFα, IL-2, IL-3, IL-5, IL-6, IL-9, IL-17, IL-21, IL-13, IL-10, TGF-β, and GM-CSF (48, 59, 65), but are most well-known for their ability to secrete IFNγ and IL-4 (26). This cytokine secretion activates other immune cells such as NK cells, T cells, and B cells, which is called transactivation, and therefore influences the subsequent immune response (70).

Importantly, the main technique used to isolate iNKT cells is by CD1d/αGalCer tetramer. However, iNKT cell activation results in TCR internalization (71) and/or activation induced cell death (27, 72). Therefore, the tetramer binding (CD1d/αGalCer tet^+) population that identifies cells as iNKT cells essentially disappears (27), making it difficult to definitively address questions about iNKT cell acute activation.

As mentioned previously, iNKT cells have characteristics that are similar to both the innate immune response and the adaptive immune response. For example, iNKT cells can very rapidly (within hours) produce cytokines upon stimulation, which is important for guiding the subsequent immune response. This rapid response and ability to influence the adaptive immune response is reminiscent of innate immunity. However, similarly to T cells in the adaptive immune response, iNKT cells undergo V(D)J recombination (19, 42, 73) and can be activated through their TCRs upon stimulation. Therefore, iNKT cells are a unique innate-adaptive hybrid that plays an important immunomodulatory role (70). However, as compared to conventional T cells, the invariant iNKT TCR limits the capacity of iNKT cells to respond to a diverse repertoire of antigens. Interestingly, despite this restricted capacity for antigen recognition, iNKT cells are implicated in wide range of pathogenic infections (70), leading to the question of how iNKT cells are activated during such a variety of infectious contexts.

iNKT Cell Activation During Infections

Currently, there are three main models of iNKT cell activation during infection: 1) activation requiring microbial antigen; 2) activation solely driven by cytokines; or 3) activation requiring self-antigen (74).

For the microbial antigen driven model of iNKT cell activation, previous reports have shown exogenous glycolipid antigens produced by infectious agents can activate iNKT cells through their TCR, and this activation is CD1d-dependent (75-79). For example, iNKT cells are activated by glycosylated diacylglycerol antigens from *Streptococcus pneumoniae* (77, 78) or from *Borrelia burgdorferi* (80), by glycosphingolipids (GSLs) with an α-linked glucuronic or galacturonic acid from *Sphingomonas paucimobilis* (75, 76), or by phosphatidylinositol mannoside from mycobacterial membrane (81). Additionally, asperamide B, a GSL derived from the fungus *Aspergillus fumigatus*, activated iNKT cells through a CD1d-dependent mechanism (82). During these microbial infections, APCs present microbial-derived antigenic lipid, which when recognized by the iNKT TCR, causes iNKT cell activation. Once activated, iNKT cells secrete cytokines, leading to the transactivation of other immune cells and contributing to pathogen clearance (70).

iNKT cells are also able to rapidly produce cytokines during a variety of infections where the pathogen is not known to produce exogenous antigenic lipid. Similarly to some situations with γδ T cells and CD4 and CD8 memory T cells, which can become activated independently of antigen receptor stimulation and is sometimes

referred to as non-cognate activation (83-87), iNKT cell activation in antigen independent contexts are thought to be solely driven by cytokines secreted by APCs (74). For example, this cytokine driven model has been proposed to explain iNKT cell activation during viral infections such as mouse cytomegalovirus (MCMV) (88). After MCMV infection, iNKT cells demonstrated an activated phenotype and produced IFNγ, which resulted from APC secretion of IL-12 and IL-18 (88). There was little or no effect on IFNγ production when CD1d was blocked or deleted, indicating that IL-12 and IL-18 were the main drivers of iNKT cell activation during this infection (88).

However, this cytokine driven model is not thought to occur during all infections that lack exogenous antigenic lipid. Instead, an alternative model of iNKT cell activation dependent on TCR stimulation is proposed to occur (70). More specifically, it is proposed that iNKT cell activation is induced, at least in part, as a result of stimulation of TLRs on APCs, which not only causes the secretion of cytokines such as IL-12 or type I IFN, that participate in the iNKT cell activation process, but which also causes increased synthesis and presentation of an antigenic endogenous lipid by APCs (89-91). This process is sometimes referred to as indirect activation because rather than providing the antigen, the bacteria are inducing the synthesis of both a stimulatory antigen and cytokines by host APCs (74). Two GSLs, iGb3 and βGlcCer, are reported self-lipids that are potential candidates for the antigenic endogenous lipid in this model (6, 33). However, this identification remains speculative, as the full spectrum of antigenic self-lipids has yet to be identified.

Notably, this indirect method of iNKT TCR stimulation is controversial. The main support for this model stems from *Salmonella typhimurium* (*S. typhimurium*) infection data. *S. typhimurium* is a Gram-negative LPS-positive pathogen not reported to contain an iNKT TCR stimulating microbial lipid antigen, but activates iNKT cells to produce IFNγ during infection. More specifically, Brigl et al. reported that after *S. typhimurium* infection, the percentage of iNKT cells producing IFNγ was decreased in infected animals given CD1d blocking antibody, indicating that iNKT cell activation was at least partially dependent on CD1d (92). Further support for this model was reported by Darmoise et al. In this report, it was found that TLR stimulation led to the inhibition of the enzyme α-galactosidase A (α-Gal A) (89). α-Gal A was proposed to convert a stimulatory lipid to a non-stimulatory lipid, and thus the inhibition of this enzyme resulted in the increased accumulation, and therefore presentation, of a stimulatory lipid for iNKT cell activation (89). When α-Gal A deficient bone marrow derived dendritic cells (BMDC) were injected into wild type mice, iNKT cells expressed higher levels of the activation marker CD69 and exhibited a decreased population of CD1d/αGalCer tet^+ cells, which was used as an indication of CD1d-dependent stimulation of iNKT cells *in vivo* (89). Therefore, APC TLR stimulation leads to the presentation of an antigenic endogenous lipid that activates iNKT cells (89).

Further understanding of the indirect activation model, whereby iNKT cells are activated by antigenic endogenous lipid, is important as it may also be occurring in noninfectious diseases such as type I diabetes and asthma (93-95). Greater mechanistic

understanding of how iNKT cells are activated in the absence of exogenous antigenic lipid might also elucidate novel ways to control iNKT cells during infections. However, one challenge to this greater understanding is that it remains difficult to distinguish TCR-mediated from non-TCR mediated iNKT cell activation. Current studies use CD69 upregulation, cytokine production, and reduced recovery of iNKT cells using tetramers, which is presumed to be secondary to TCR internalization, as markers of iNKT cell activation. However, all of these features can also be a consequence of non-TCR mediated activation. Therefore, a more sensitive method is needed to study the role of antigen receptor stimulation in iNKT cell activation during infections *in vivo*. Recently, a novel Nur77^{gfp} BAC transgenic mouse model was developed, which upregulated GFP in T cells in response to antigen receptor stimulation but not inflammatory signals (43). Thus, the aim of Chapter 2 of this thesis was to determine the role of TCR stimulation during antigen-dependent versus -independent iNKT cell activation using the Nur77^{gfp} transgenic mouse.

Nur77^{gfp} Transgenic Mouse

Nur77, also known as *Nr41a*, is an orphan steroid hormone nuclear receptor and an immediate early gene upregulated by TCR stimulation in thymocytes and T cells (96). It is regulated by nuclear factor of activated T cells (NFAT), and can be involved in multiple parts of the TCR signaling cascades (97-99). Although the function of Nur77 has not been fully elucidated, it is thought to play a role in thymocyte apoptosis (100-

102). Interestingly, in a microarray comparing thymocytes undergoing either positive selection or negative selection, *Nr41a* showed a two-fold increase in expression in thymocytes undergoing positive selection in comparison to a ten-fold increase in expression in thymocytes undergoing negative selection (103). This differential expression indicated that a Nur77 reporter mouse may be useful for examining TCR signal strength.

Therefore, a Nur77^{gfp} transgenic (Tg) mouse was developed in which GFP was inserted into the Nur77 locus in a bacterial artificial chromosome (43). Using Kb/OVA-specific OT-I TCR transgenic mice and altered peptide ligands with known affinity for the OT-I TCR, the GFP expression levels in T cells from OT-I Nur77^{gfp} Tg mice were shown to reflect the TCR stimulatory strength of the altered peptide ligand (43). Therefore, GFP expression was a sensitive readout for TCR affinity, as even the lowest affinity peptide variant or a self-peptide was shown to induce GFP above background levels. Importantly, GFP expression was specific for TCR stimulation: stimulation by cytokine alone had no effect on GFP expression in T cells (43). Therefore, the Nur77^{gfp} Tg mouse was shown to be a sensitive reporter of TCR stimulation in T cells (43).

In addition to examining T cells, iNKT cells were examined in the initial characterization of the Nur77^{gfp} Tg mouse. Although GFP expression in iNKT cells in the periphery was very low, GFP expression was upregulated after stimulation with the known strong agonist, αGalCer (43). Additionally, iNKT cell GFP levels were examined during development. As expected, the earliest post-selection iNKT cell exhibited the

highest level of GFP (43), which comports with the idea that iNKT cells are selected by a self-lipid that stimulates the iNKT TCR as described above. Together, these data indicated that the Nur77^{gfp} Tg mouse may be a useful tool to examine antigen-dependent versus antigen-independent models of activation in iNKT cells.

iNKT Cells in Noninfectious Diseases

In addition to contributing to the immune response during infections, iNKT cells have been shown to be important in a variety of disease contexts, such as in asthma, autoimmune disease, and cancer (93). For example, iNKT cell deficient mice failed to develop allergen-induced airway hyperreactivity (AHR), indicating iNKT cells are required for pathogenesis (104). Furthermore, IL-4 and IL-13 produced by iNKT cells were shown to be essential for allergen-induced AHR development (105). However, in the ozone-induced AHR model of asthma, the production of IL-17 by iNKT cells was required for pathogenesis (95, 106). Interestingly, the production of IFNγ after the systemic administration of the strong iNKT cell agonist αGalCer inhibited asthma pathogenesis in an OVA-induced AHR model (107, 108).

In addition to asthma, iNKT cells have been implicated in multiple autoimmune diseases, such as type I diabetes, experimental autoimmune encephalomyelitis and multiple sclerosis (EAE/MS), and systemic lupus erythematosus (SLE) (109). For type I diabetes, studies were performed in NOD mice, which spontaneously develop disease. Interestingly, the iNKT cell population in these mice is numerically and functionally

decreased (110, 111). Furthermore, in CD1d-deficient NOD mice, the onset of disease was accelerated and the incidence of disease was increased (112, 113). However, young NOD mice that received an adoptive transfer of lymphocytes enriched for iNKT cells failed to develop diabetes (111). This suppressive influence of iNKT cells was associated with IL-4 production, implicating that a shift from a TH1 cytokine bias (i.e., IFNγ) toward a TH2 cytokine bias (i.e., IL-4) conferred protection against disease (94, 114). Interestingly, it was also found that NOD mice treated with αGalCer exhibited delayed disease onset and reduced incidence of disease (115). Even when αGalCer treatment was initiated after the onset of invasive insulitis, disease severity was reduced (113). In contrast, the role of iNKT cells in SLE and EAE/MS is less defined. Interestingly, in both diseases, iNKT cells may have a neutral or protective function before disease onset, but after disease onset, may play a pathogenic role (109).

In cancer, iNKT cells are involved the anti-tumor response (116). In one study, iNKT cell deficient mice indicated that iNKT cells are required for protection against spontaneous tumors initiated by MCA, a chemical carcinogen (117, 118). In a B16 melanoma model, iNKT cells were shown to be required for IL-12 mediated tumor rejection (119). Although it is currently unknown how iNKT cells are activated in tumor environments (116), other studies have shown that activation of iNKT cells using αGalCer will further boost the antitumor response. For example, IFNγ produced by αGalCer activated iNKT cells was protective against B16 melanoma metastases (120).

Additionally, activated iNKT cells directly contributed to antitumor cytotoxicity in a perforin-dependent manner (121).

Therefore, clinical trials using αGalCer to activate iNKT cells were initiated (122, 123). Despite being well-tolerated, αGalCer treatment did not effectively boost the anti-tumor response (122, 123). This may be due to observations that cancer patients have a decreased number of iNKT cells compared to the number of iNKT cells in healthy volunteers (124, 125). Furthermore, the iNKT cells from these patients exhibited decreased proliferation and cytokine production after *ex vivo* stimulation (126), indicating the remaining iNKT cells have reduced functional capacities. The reason for the decrease in iNKT cell number and function is not known, but could be a result of autoantigen presented by tumors causing activation induced cell death and/or anergy, or suppression of iNKT cells by Tregs (127, 128). Thus, for αGalCer treatment, or any iNKT cell based therapy, to be effective, the number of functional iNKT cells needs to be supplemented. One way this can be achieved is by an exogenous source of iNKT cells. Therefore, the derivation of iNKT cells from hematopoietic progenitor cells *in vitro* was the aim of Chapter 3 of this thesis.

OP9-DL1 Culture System

Previous work showed that the bone marrow stromal cell line, OP9, can support the differentiation of hematopoietic progenitor cells (HPCs) into multiple lineages, such as B cells, but it did not support T cell development (129, 130). This inability to support

T cell development was hypothesized to be, at least partially, due to the absence of Notch signaling in this system (131). Notch signaling is essential for determining whether lymphocyte progenitors adopt a T cell fate over a B cell fate (131, 132), and is also required throughout T cell development, although the specific details of these requirements are still incompletely understood. Therefore, when OP9 cells were retrovirally transduced to express the Notch ligand Delta-like-1 (DL1), these OP9-DL1 cells supported T cell development from HPCs (131). Additional studies using OP9-DL1 cells showed that T cell development could also be induced in embryonic stem cells (ESCs) (133), progenitor cells from human umbilical cords (134), and human bone marrow-derived progenitor cells (134).

During normal T cell development, lymphoid progenitor cells leave the bone marrow and migrate to the thymus. After entering the thymus, these progenitors are stimulated through the receptor Notch1, which signals T cell lineage commitment and causes these cells to undergo cell proliferation for about a week. Because these cells do not express the co-receptors CD8 or CD4, they are called DN thymocytes, and progress through four DN stages before becoming DP thymocytes. The four DN stages are identified by the differential expressions of the markers CD44 and CD25, in which DN stage 1 cells are $CD44^{+}$ $CD25^{-}$, DN stage 2 cells are $CD44^{+}$ $CD25^{+}$, DN stage 3 cells are $CD44^{lo}$ $CD25^{+}$, and DN stage 4 cells are $CD44^{-}$ $CD25^{-}$ (135). During the DN stages, the TCR β-chain undergoes rearrangement and is subsequently paired with a surrogate pre-T receptor α-chain, which leads to the arrest of β-chain rearrangement and the expression

of CD8 and CD4, thus entering the DP stage. At the DP stage, α-chain rearrangement occurs for the production of the $\alpha\beta^+$ TCR. Importantly, after a productive $\alpha\beta^+$ TCR is found, it is tested for the ability to recognize self-pMHC presented by thymic epithelial cells. TCRs that recognize self-pMHC continue to mature, which is called positive selection, and ensures that a productive TCR that is MHC restricted was assembled (136). TCRs that fail to recognize self-pMHC die. These thymocytes are also tested for the strength of self-pMHC TCR recognition. If the affinity of this interaction is too high, the cells will undergo apoptosis, which is called negative selection and ensures that self-reactive TCRs do not enter the periphery (136). At the last stages of development, these cells express high levels of TCR and cease to express one of the co-receptor molecules to become either $CD4^+$ single positive (SP) or $CD8^+$ SP. Only cells with TCRs that survive both positive and negative selection will fully mature and emigrate to the periphery.

During T cell development supported by OP-DL1 cells, the HPCs undergo the same stages of initial T cell development (131). At the DN stages, they express the same CD44 and CD25 markers and rearrangement of the TCR-β chain as DN thymocytes before progressing to the DP stage (131). However, in *in vitro* cultures, the thymic epithelial cells required for positive selection and negative selection are absent. Therefore, positive selection and negative selection cannot occur, and mature T cells should not develop. However, CD8SP T cells were found in these cultures, and were considered "functional" based upon their ability to produce IFNγ after stimulation with

plate bound CD3 and CD28 antibodies (131, 133). Despite this, the classification of these cells as "functional" CD8 T cells as well as their utility remains controversial.

Although iNKT cells go through the same initial developmental pathway as T cells, they do not go through the same thymic epithelial cell-dependent positive and negative selection. Instead, iNKT cells go through different positive and negative selection processes at the DP stage, which are dependent on DP thymocytes (31, 137). Since iNKT cell development is dependent on DP thymocytes, and the OP9-DL1 culture system faithfully induces T cell development up to the DP stage, the OP9-DL1 culture system could potentially be used to derive a source of supplemental iNKT cells for iNKT cell based therapies.

Three previous studies have examined this possibility (47, 138, 139). More specifically, nuclear transfer from mature iNKT cells (with a Vα14-Jα18 rearranged TCR) were used to create ESC lines, which were then cultured with OP9-DL1 cells and gave rise to iNKT cells (139). The same group also showed splenic iNKT cells could be reprogrammed into induced pluripotent stem cells (iPSCs), which gave rise to iNKT cells when cultured with OP9-DL1 (138). Additionally, a different group showed that iNKT cells could be derived from HPCs, but the transient nature of the iNKT cell population and difficulty with reproducing this data, makes this finding questionable (47).

Although these previous reports showed successful derivation of iNKT cells using the OP9-DL1 culture system, they did not examine transcription factors to identify which subsets were being produced. This distinction is important because the transcription

factors (Tbet, RORγt, and GATA3) that distinguish the iNKT subsets also dictate the cytokine produced by iNKT cells upon stimulation, which affects the subsequent immune response (57). This is especially important for the use of these iNKT cells in iNKT cell based therapies. For example, IFNγ is essential for the antitumor response (116), and therefore NKT1 cells would be the most beneficial subset for this therapeutic use. Hence, Chapter 3 of this thesis also sought to define the culture conditions that result in the differentiation of the NKT1, NKT2, and NKT17 subsets.

Chapter 2: Antigen-dependent Versus -independent Activation of iNKT Cells During Infection

CD1d-reactive invariant natural killer T cells (iNKT) play a vital role in determining the kinetics, extent, and characteristics of an immune response. Insight into their activation by infectious agents is therefore crucial for the general understanding of how iNKT cells contribute to immune responses. Previous reports suggest that iNKT cell activation during infection can be: 1) solely driven by cytokines from innate immune cells; 2) require microbial antigen; or 3) require self-antigen. In this study, we examined the role of antigen receptor stimulation in iNKT cells during several bacterial and viral infections *in vivo*. To test for antigen receptor signaling, Nur77^{gfp} BAC transgenic mice, which upregulate GFP in response to antigen receptor stimulation but not inflammatory signals, were analyzed. iNKT cells from reporter mice infected with mouse cytomegalovirus (MCMV) produced IFNγ, but did not upregulate GFP, consistent with their reported CD1d-independent activation, but IL-12 dependent mode of activation. However *Streptococcus pneumoniae* and *Sphingomonas paucimobilis*, two bacteria known to produce lipid antigens for iNKT cells, induced GFP expression and cytokine production. In contrast, although *Salmonella typhimurium* (*S. typhimurium*) was proposed to induce the presentation of antigenic self-lipid, iNKT cells produced IFNγ, but did not upregulate GFP after infection *in vivo*. Even in CD1d-deficient hosts, iNKT cells were still able to produce IFNγ after *S. typhimurium* infection. Furthermore, while it has been proposed that antigenic endogenous lipid presentation is a result of Toll-like receptor (TLR) stimulation of antigen presenting cells, injection of different TLR agonists led to iNKT cell IFNγ production but not increased GFP expression. These data

indicate that robust iNKT cell responses to bacteria as well as viruses can be obtained in the absence of antigenic iNKT T cell receptor stimulation.

Introduction

CD1d-reactive invariant Natural Killer T cells (iNKT cells) are a distinct lineage of αβ T lymphocytes with an invariant T cell antigen receptor (TCR) composed in mice of the α-chain variable region 14 (Vα14) and the α-chain joining region 18 (Jα18) paired with a restricted subset of TCR-β chains (19). As a result of this TCR expression, iNKT cells are able to recognize several types of glycolipid antigens in the context of CD1d, a non-classical major histocompatibility class I (MHC-I)-like antigen presenting molecule. Upon glycolipid presentation, and most famously with the strong agonist αgalactosylceramide (αGalCer), iNKT cells are able to rapidly produce cytokines such as IFNγ and IL-4 (26). This rapid cytokine production contributes to the recruitment and activation of other cell types during an immune response, which has been shown to influence a variety of diseases, including cancer (119), autoimmunity (140, 141), and pathogenic infection (127). Therefore, insight into their activation is crucial for the general understanding of how iNKT cells contribute to immune responses.

One area that remains to be fully elucidated is the extent to which antigen recognition by the iNKT TCR contributes to the activation of iNKT cells during various infections. Previous reports have shown that iNKT cells can be activated through the TCR by certain infectious agents that produce glycolipid antigens (75-79). For example, iNKT cells are activated by glycosylated diacylglycerol antigens from *Streptococcus pneumoniae* (*S. pneumoniae*) in a CD1d-dependent manner (78). Furthermore, iNKT deficient mice infected with *S. pneumoniae* have significantly reduced survival compared

to infected wild type mice (77). In addition, *Sphingomonas paucimobilis* (*S. paucimobilis*), which is a Gram-negative bacteria that does not express LPS, produces glycosphingolipids (GSLs) with an α-linked glucuronic or galacturonic acid that are able to activate iNKT cells (75, 76, 142). More specifically, it was shown that these GSLs activate iNKT cell proliferation and cytokine secretion, which was dependent on CD1d (79).

However, iNKT cells are able to rapidly produce cytokines during a variety of infections where the pathogen is not known to produce an exogenous stimulating lipid (79, 92), raising the question of how iNKT cells are activated to rapidly produce cytokines. It was previously proposed that during these types of infections, iNKT cell activation occurs partly in response to the presentation of an endogenous lipid capable of stimulating the iNKT TCR (79, 89, 92). During these types of infections, iNKT cell responses are induced, at least in part, as a result of stimulation of Toll-like receptors (TLRs) on antigen presenting cells (APCs), which not only causes the secretion of cytokines, such as IL-12 or type I IFN, that participate in the iNKT cell activation process, but which also causes increased synthesis and presentation by the APCs of an antigenic endogenous lipid (89-91). This process is sometimes referred to as indirect activation, because rather than providing the antigen, the bacteria are inducing the synthesis of both a stimulatory antigen and cytokines by host APCs. Although the full spectrum of endogenous stimulatory lipids remains to be identified, and their relative importance remains to be conclusively assessed, two GSLs, isoglobotrihexosylceramide

(iGb3) and β-D-glucopyranosylceramide (βGlcCer) are known to be self-lipids (6, 33, 89). This indirect method of iNKT TCR stimulation is proposed to occur during *Salmonella typhimurium* (*S. typhimurium*) infection, which is a Gram-negative lipopolysaccharide (LPS)-positive pathogen not reported to contain an iNKT TCR stimulating microbial lipid antigen (79, 92). Brigl et al. reported that after *S. typhimurium* infection, the percentage of iNKT cells producing IFNγ was decreased in infected animals given CD1d blocking antibody, indicating that iNKT cell activation is at least partially dependent on CD1d (92).

Yet it is clear that in some situations T lymphocytes, such as γδ T cells and CD4 and CD8 memory T cells, become activated independently of antigen receptor stimulation, which is sometimes referred to as non-cognate activation (83-87). Therefore, it is possible that certain microorganisms are able to activate iNKT cells independently of their TCR (88, 143). This antigen-independent model has been proposed to explain iNKT cell activation during infection with mouse cytomegalovirus (MCMV), which is not reported to contain an iNKT TCR stimulatory lipid (88). Upon infection with MCMV, iNKT cells demonstrated an activated phenotype and produced IFNγ. This activation resulted from APC secretion of IL-12 and IL-18, and there was little or no effect on IFNγ production when CD1d was blocked or deleted (88, 144).

Further understanding of the indirect activation mode, whereby iNKT cells are activated by antigenic endogenous lipids, is important as it may be occurring in diseases such as type I diabetes and asthma (93-95). Greater mechanistic understanding might

also provide a profound way to control iNKT cells during infections. However, one challenge to greater understanding is that it remains difficult to distinguish TCR-mediated from non-TCR mediated activation. The activation parameters typically used in the study of iNKT cells are CD69 upregulation, cytokine production, and reduced recovery of iNKT cells using tetramers presumed to be secondary to TCR internalization. All of these features, however, can also be a consequence of non-TCR mediated activation. In this study, we examined the role of antigen receptor stimulation in iNKT cell activation during infections *in vivo* using Nur77^{gfp} BAC transgenic mice, which upregulate GFP in response to antigen receptor but not inflammatory signals (43). Such mice were infected and examined for cytokine production and GFP expression as indicators of iNKT cell activation and iNKT TCR stimulation, respectively.

Results

iNKT Cells from the Nur77^{gfp} Reporter Mouse Upregulate GFP in Response to Antigenic Lipids

This study sought to demonstrate iNKT cell TCR stimulation by antigen during various infections. However, markers of activation used previously, including CD69 upregulation, TCR downregulation, and cytokine production, can all be induced by stimuli independent of antigen receptor engagement. Previous work showed that Nur77^{gfp} BAC transgenic mice, which encode GFP in the locus of an immediately early gene downstream of the TCR—Nur77 (*Nr4a1*), expressed GFP in T lymphocytes only after antigen receptor stimulation, and not after inflammatory signals, cytokines, or stimuli that promote cell survival or trafficking (43). It was also shown that iNKT cells in this reporter mouse exhibited a very low level of GFP in the periphery at steady state, suggesting that iNKT cells are not continuously receiving stimulation through their TCR (43). However, when injected with the potent TCR agonist αGalCer, iNKT cells greatly increased expression of GFP (43). Therefore, we used the Nur77^{gfp} mouse to examine iNKT TCR activation *in vivo*.

Using OT-I TCR transgenic CD8 T lymphocytes, the Nur77^{gfp} reporter was shown to respond to very weak TCR stimuli, such as those that support positive selection or are incapable of inducing the proliferation of mature T cells (43). Therefore we sought to test the sensitivity of GFP upregulation after iNKT cell TCR stimulation. Most antigenic lipids have lower affinities for the iNKT TCR compared to αGalCer (145, 146),

and we tested OCH, β-D-glucopyranosylceramide (βGlcCer), and isoglobotrihexosylceramide (iGb3), in addition to αGalCer. αGalCer is a glycosphingolipid derived from a marine sponge, and has been shown to strongly activate iNKT cells to produce both IL-4 and IFNγ upon stimulation (26). αGalCer has also been reported to have the strongest affinity for the iNKT TCR (145). OCH is an analog of αGalCer, and is reported to also strongly activate iNKT cells, albeit with a slightly weaker affinity for the iNKT TCR than αGalCer (145, 147). iGb3, and more recently βGlcCer, were identified as putative endogenous self-lipids capable of activating iNKT cells through the TCR (6, 33). iGb3 is a weak activator of iNKT cells with a much lower affinity for the iNKT TCR than αGalCer, in the low μM range compared to as low as 11nM for complexes of αGalCer bound to CD1d (148, 149). βGlcCer, when loaded into CD1d tetramers, is at least capable of binding to a proportion of iNKT cells, suggesting that it is a stronger ligand than iGb3, although an affinity measurement per se has not been reported (6).

We tested the ability of these lipids to activate the iNKT TCR by pulsing bone marrow derived dendritic cells (BMDC), and then intravenously injecting them into $Nur77^{gfp}$ mice. After 18 hours, iNKT cells were isolated and examined directly *ex vivo* by flow cytometry for GFP upregulation. All four lipids induced an upregulation of GFP as compared to iNKT cells from a control mouse injected with unpulsed BMDC (Figure 2-1). αGalCer consistently induced the strongest increase, although OCH induced only slightly less. Of the putative antigenic self-lipids, βGlcCer induced a higher upregulation

of GFP than iGb3. CD69 was also upregulated, showing the same order of expression (αGalCer > OCH > βGlc > iGb3) (Figure 2-1), although its use as a marker of iNKT TCR stimulation during infections is limited by the fact that it can also be upregulated by inflammatory stimuli (43, 150). These results suggest that in the Nur77^{gfp} mouse, GFP expression is a sensitive tool for assessing iNKT TCR stimulation.

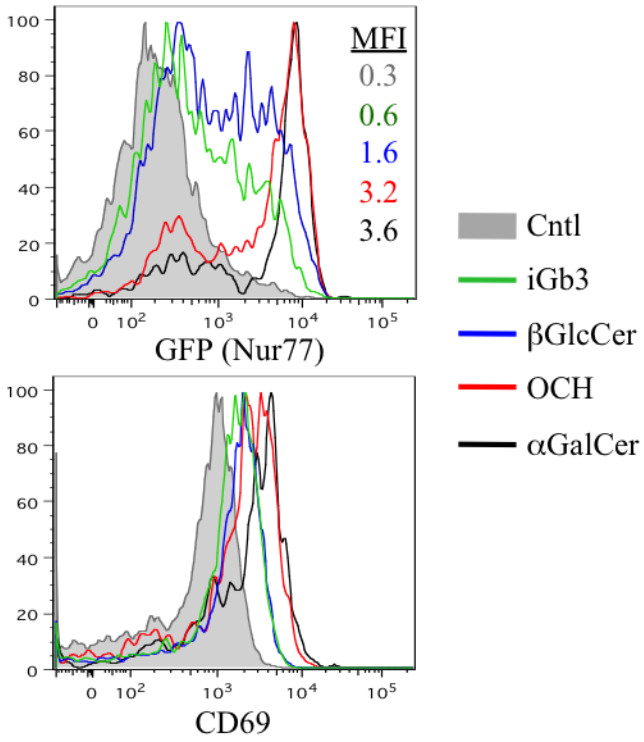

Figure 2-1. iNKT cells upregulated GFP in response to various antigenic lipids, including self-lipids. Expression of GFP (top) and CD69 (bottom) by splenic iNKT cells from B6 Nur77^{gfp} mice 18 h after injection with bone marrow-derived unpulsed DCs (cntrl) or BMDCs pulsed with αGalCer, OCH, or the self-lipids βGlcCer or iGb3 as described in Materials and Methods. Histograms are representative of eight independent experiments. Average relative mean fluorescence intensity (MFI) of GFP (normalized to that in CD4 T cells in each experiment) is indicated.

MCMV Infection Does Not Stimulate iNKT Cells Through Their TCR

MCMV does not contain a stimulatory lipid that activates the iNKT TCR; however, MCMV has been shown to activate iNKT cells during infection *in vivo*, as demonstrated by increased CD25 and CD69 expression, and by IFNγ production detected by intracellular cytokine staining as early as 24 hours after infection (88). This activation is thought to be predominantly a result of IL-12 secretion and IL-18 and/or type I IFN secretion from APCs that were activated through TLR9 (144, 151). Importantly, there was little to no effect on iNKT cell IFNγ production in experiments where CD1d was blocked and/or deleted (88). Since CD1d-mediated antigen is not considered a key-player in the MCMV-induced activation of iNKT cells, we tested this putatively cytokine-mediated model of iNKT cell activation *in vivo* using the Nur77^{gfp} mouse. Similar to previous reports, iNKT cells from Nur77^{gfp} mice infected with MCMV showed a statistically significant increase in the percentage of hepatic iNKT cells that produced IFNγ 36 hours post infection (Figure 2-2B). Interestingly, the level of GFP expression remained the same at all time points examined, suggesting that iNKT cells did not receive TCR stimulation during MCMV infection (Figure 2-2A), and confirming that other inflammatory stimuli produced during infections do not cause GFP upregulation (43).

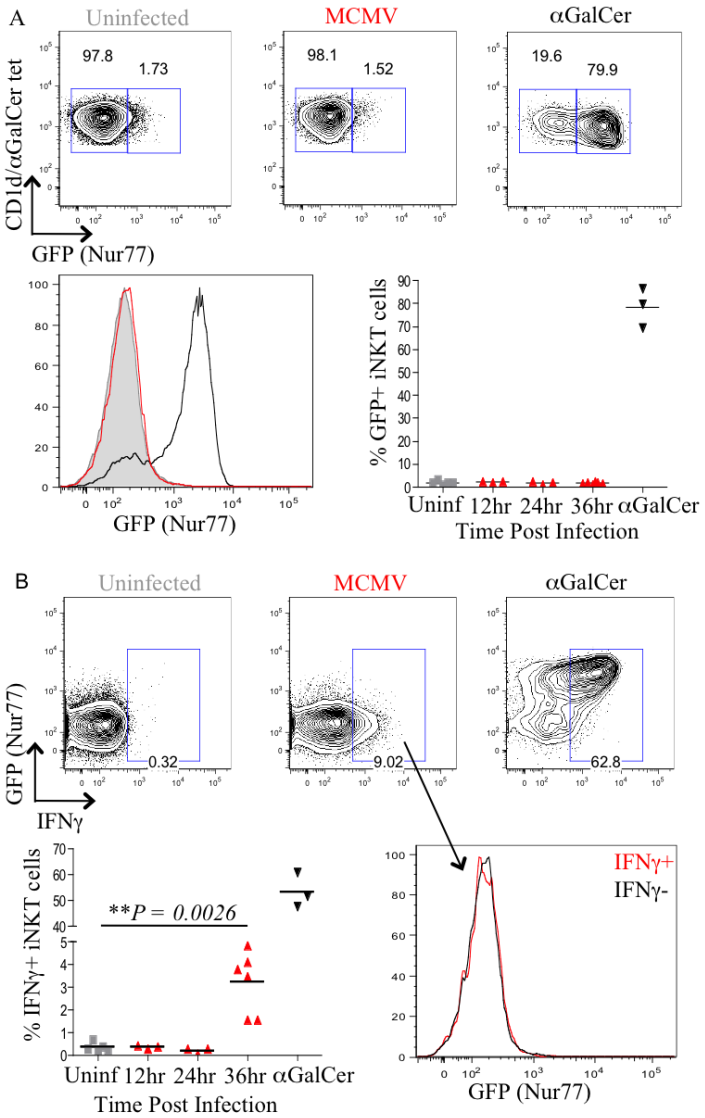

Figure 2-2. MCMV infection induced iNKT cell cytokine production, but not GFP expression. Nur77^{gfp} B6 mice were infected with MCMV *i.p.*, and tet^{+} liver iNKT cells were analyzed by flow cytometry 36 h later for GFP expression (A) and IFNγ production (B). Results show uninfected (left), MCMV infected (middle) and αGalCer treated animals 3 h after injection (right). Data are representative of two independent experiments, with at least three animals per time point. The histogram in (B) shows GFP expression of the IFNγ^{-} iNKT cell population versus the IFNγ^{+} iNKT cell population from an animal 36 h post infection. In graphs, each dot represents one mouse (uninfected n = 6; 12 h post infection n = 3; 24 h post infection n = 3; 36 h post infection n = 6; 3 h post αGalCer injection n = 3). ***P* = 0.0026, percentage uninfected IFNγ^{+} iNKT cells versus percentage 36 h post infection IFNγ^{+} iNKT cells (unpaired two-tailed *t*-test).

S. pneumoniae and *S. paucimobilis* Stimulate iNKT Cells Through Their TCR

We next infected Nur77^{gfp} mice with microbes that are known to produce lipid antigens that activate iNKT cells (75, 78). *S. pneumoniae* is an extracellular Gram-positive pathogen, which produces glycosylated diacylglycerol antigens (77, 78). These antigens are highly abundant in the bacteria, they can stimulate the iNKT TCR, and iNKT cells were shown be important for clearance and host survival (78). *S. paucimobilis* is a Gram-negative bacteria that does not produce LPS, but does produce a GSL with an α-linked glucuronic acid, which also has been shown to directly stimulate the iNKT TCR when presented by CD1d (75, 76). *S. paucimobilis* also produces another GSL with a tetrasaccharide head group, that is either weakly antigenic (152) or not antigenic (153).

We systemically infected Nur77^{gfp} mice with *S. pneumoniae* by intravenous injection, harvested iNKT cells 6 or 20 hours later, and analyzed cytokine by intracellular staining directly *ex vivo* without re-stimulation (Figure 2-3A). As expected, iNKT cells produced IFNγ (Figure 2-3B) and upregulated CD69 (not shown). Consistent with a TCR-dependent activation mode, the expression of GFP increased in iNKT cells as well (Figure 2-3A). This increase was statistically significant compared to the uninfected control mice, but was well below that seen after injection of αGalCer, indicating that although lipids are presented during microbial infections that are able to activate iNKT cells through their TCR, they are weaker agonists than αGalCer. This result is consistent with previous reports that lipid antigens from *S. pneumoniae* presented by CD1d have an affinity for the iNKT TCR in the low μM range, compared to the low nM range for

αGalCer complexes with CD1d (78, 154). Notably, the GFP level was higher in the subset of iNKT cells producing IFNγ, suggesting that a TCR signal activated cytokine production.

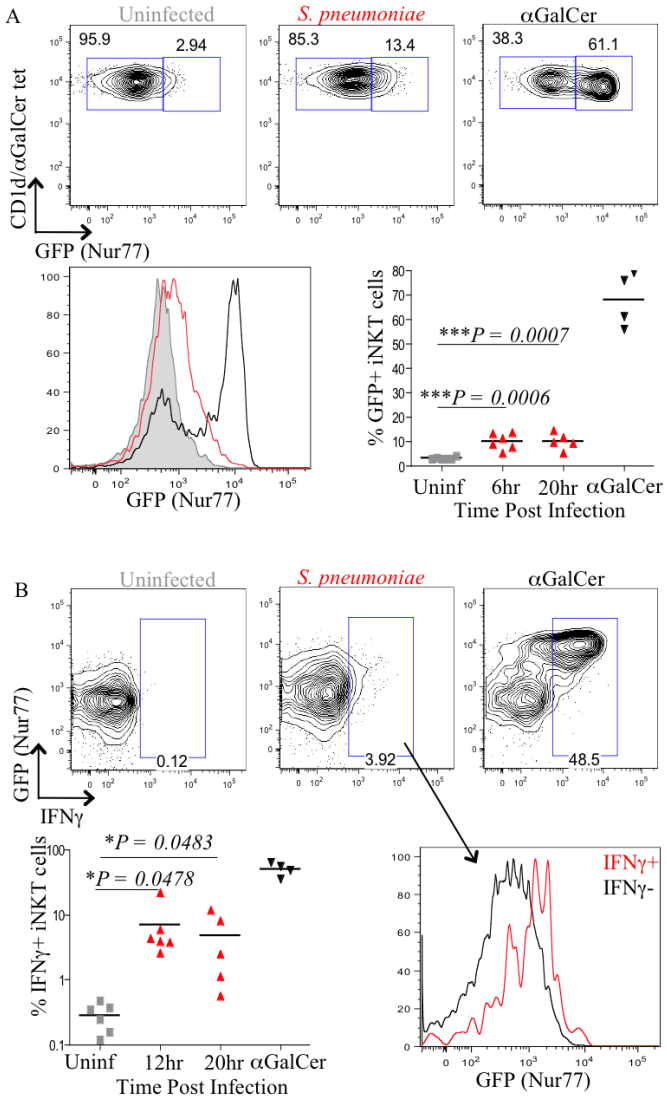

Figure 2-3. *S. pneumoniae* infection induced cytokine production and GFP expression. Nur77^{gfp} B6 mice were infected with *S. pneumoniae i.v.*, and tet^{+} iNKT cells were analyzed by flow cytometry for GFP expression (A) and IFNγ production (B). Results shown are from uninfected mice (left), mice 6 h post *S. pneumoniae* infection (middle) and αGalCer treated animals 2.5 h after injection (right). The histogram in (B) shows GFP expression in the IFNγ^{-} iNKT cell population versus the IFNγ^{+} iNKT cell population from 20 h post infection. Data show representative dot plots of two independent experiments with six animals per time point. For the percentage of GFP^{+} iNKT cells graph (A), ***P = 0.0006, uninfected versus 6 h post infection; and ***P = 0.0007, uninfected versus 20 h post infection. For the percentage of IFN-γ^{+} iNKT cells graph (B), *P = 0.0478, uninfected versus 6 h post infection; *P = 0.0483, uninfected versus 20 h post infection (all using unpaired two-tailed *t*-test).

Similarly, iNKT cells upregulated GFP expression 18 hours after infection with *S. paucimobilis* (Figure 2-4A). This increase was statistically significant compared to the uninfected controls, but again was well below that seen after injection of αGalCer (Figure 2-4A), which is consistent with previous reports (75). For these experiments we used Nur77^{gfp}/KN2 mice that have a human CD2 (hCD2) gene engineered into one allele of the IL-4 locus, because it was shown that *S. paucimobilis* GSLs also induce IL-4 production by iNKT cells (75). *S. paucimobilis* infection resulted in the production of IL-4 by iNKT cells, as judged by increased hCD2 staining, which interestingly was primarily produced by cells that had upregulated GFP (Figure 2-4B), again supporting the idea that a TCR signal activates cytokine production in iNKT cells. Overall, these data indicate that *S.pneumoniae* and *S. paucimobilis* infections, in which the bacteria have glycolipid antigens capable of stimulating the iNKT TCR, activate iNKT cells through TCR stimulation.

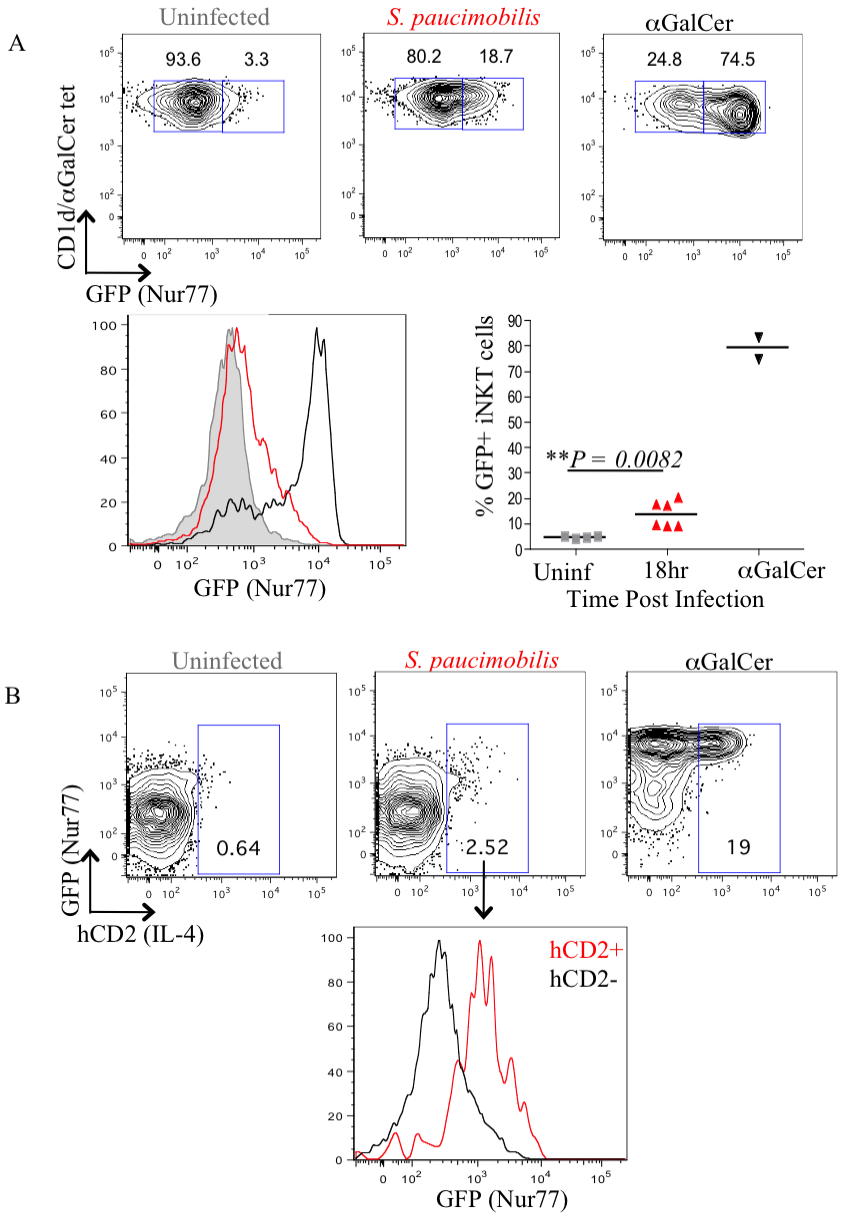

Figure 2-4. *S. paucimobilis* infection induced cytokine production and GFP expression. IL-4 reporter (KN2) Nur77^{gfp} B6 mice were infected with *S. paucimobilis* *i.v.*, and tet^{+} iNKT cells were analyzed by flow cytometry 18 h post infectionfor GFP (A) and human CD2 expression (B). Histograms in (A) show GFP expression for uninfected (left), 18 h infected (middle) and 3 h αGalCer injected (right) animals. The histogram in (B) shows GFP expression on the hCD2^{-} iNKT cell population versus the hCD2^{+} iNKT cell population from an animal 18 h post infection. Data show representative dot plots of two independent experiments with four to six animals per time point. $^{**}P = 0.0082$, uninfected versus 18 h post infection (unpaired two-tailed *t*-test).

S. typhimurium Infection Does Not Stimulate iNKT Cells Through Their TCR

Having analyzed a cytokine-mediated model of activation and two direct activation models, we next analyzed a third proposed model of iNKT cell activation. In this case, we infected mice with a microbe that has not been shown to produce a microbial antigen for iNKT TCR stimulation, but putatively causes APCs to present an antigenic endogenous self-lipid that stimulates the iNKT TCR, namely *S. typhimurium* (79, 92). *S. typhimurium* is a Gram-negative LPS-positive pathogen in which a microbial lipid antigen has not been detected, but which causes the activation of iNKT cells that has been shown to be partially CD1d dependent, but which is also dependent on MyD88 expression by the APCs (79) and on the ability of these APCs to produce IL-12 when activated (92). Unexpectedly, when Nur77^{gfp} mice were infected with *S. typhimurium* for 20 hours, iNKT cells did not upregulate GFP expression (Figure 2-5A). Despite this, there was increased expression of CD69 (data not shown) and an increase in the percentage of iNKT cells producing IFNγ (Figure 2-5B). The iNKT cells producing IFNγ did not have a higher mean expression of GFP (Figure 2-5B), unlike what was observed for cytokine producing iNKT cells in *S. pneumoniae* or *S. paucimobilis* infection (Figure 2-3B and Figure 2-4B). To ensure that we did not miss a relevant time point *in vivo*, we evaluated GFP expression at earlier (4 hours) and later (72 hours) times, but did not observe a GFP increase (Figure 2-5A). However, at all of the tested time points, there was a statistically significant increase in the percentage of iNKT cells producing IFNγ

(Figure 2-5B). These data suggest that iNKT cells were not receiving stimulation through their TCR during *S. typhimurium* infection, despite being activated to produce IFNγ.

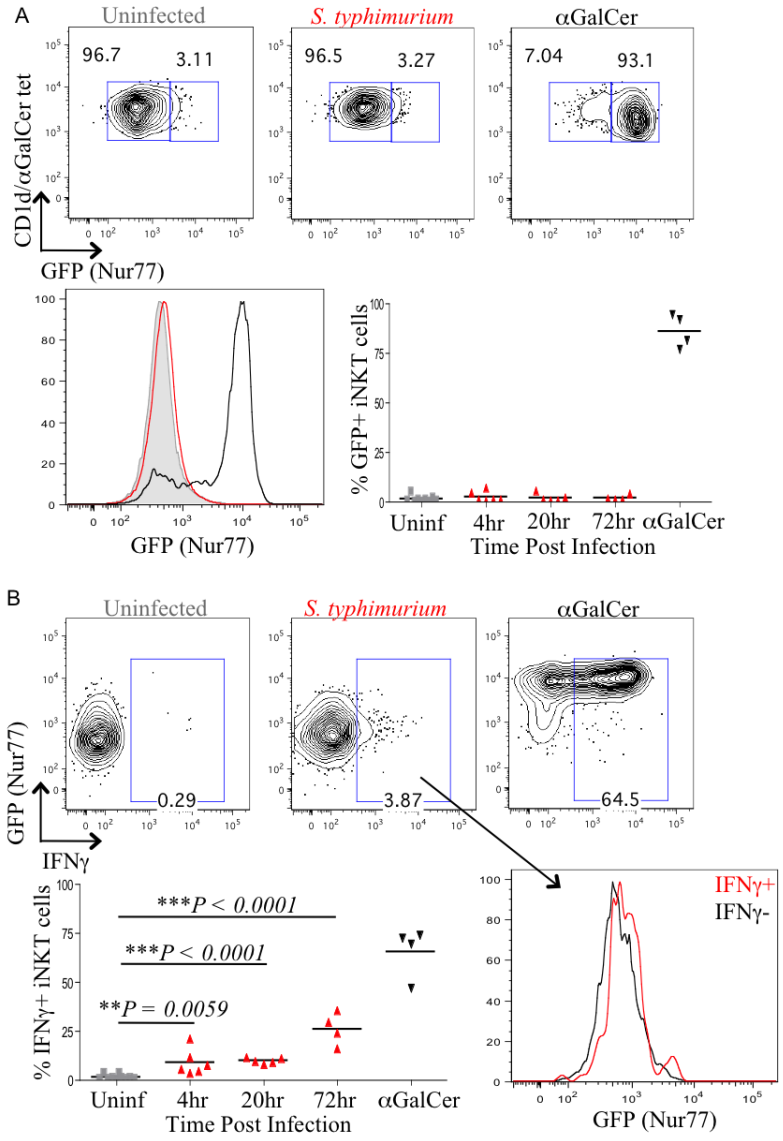

Figure 2-5. *S. typhimurium* infection induced cytokine production, but not GFP expression. Nur77^{gfp} B6 mice were infected with *S. typhimurium i.v.*, and tet^{+} iNKT cells were analyzed by flow cytometry directly *ex vivo* for GFP expression (A) and IFNγ production (B). The histogram shows uninfected mice (left), 20 h *S. typhimurium* infected (middle) and 3 h αGalCer injected (right) animals. The histogram in (B) shows GFP expression of the IFNγ^{-} iNKT cell population versus the IFNγ^{+} iNKT cell population from an animal 20 h post infection. Data show representative dot plots and histogram of four independent experiments with four to six animals per time point. For the percentage of IFNγ^{+} iNKT cells graph (B), $^{**}P = 0.0059$, uninfected versus 4 h post infection; $^{***}P < 0.0001$, uninfected versus 20 h post infection; $^{***}P < 0.0001$, uninfected versus 72 h post infection (all using unpaired two-tailed *t*-test).

Given that this result contradicts previous reports (90, 92), we tested if iNKT cells could produce IFNγ in the absence of CD1d expressed by APCs during *S. typhimurium* infection. This was done by enriching for iNKT cells from the liver and spleen of Vα14 TCR transgenic crossed to $Nur77^{gfp}$ transgenic mice, and by adoptively transferring these cells into either CD1d-deficient or WT mice. Host mice were infected with *S. typhimurium* 24 hours after receiving the iNKT cells, and then the transferred iNKT cells were analyzed 20 hours after infection. The transferred iNKT cells did not upregulate GFP expression regardless of whether they were recovered from CD1d-deficient or WT hosts (Figure 2-6), consistent with the result obtained previously (Figure 2-5). Despite this, iNKT cells from both types of infected hosts produced IFNγ, and the percentage of cytokine producing iNKT cells was not significantly different. These data suggest that iNKT cells were activated independently of CD1d, and thus independently of endogenous self-lipid antigen, during *S. typhimurium* infection.

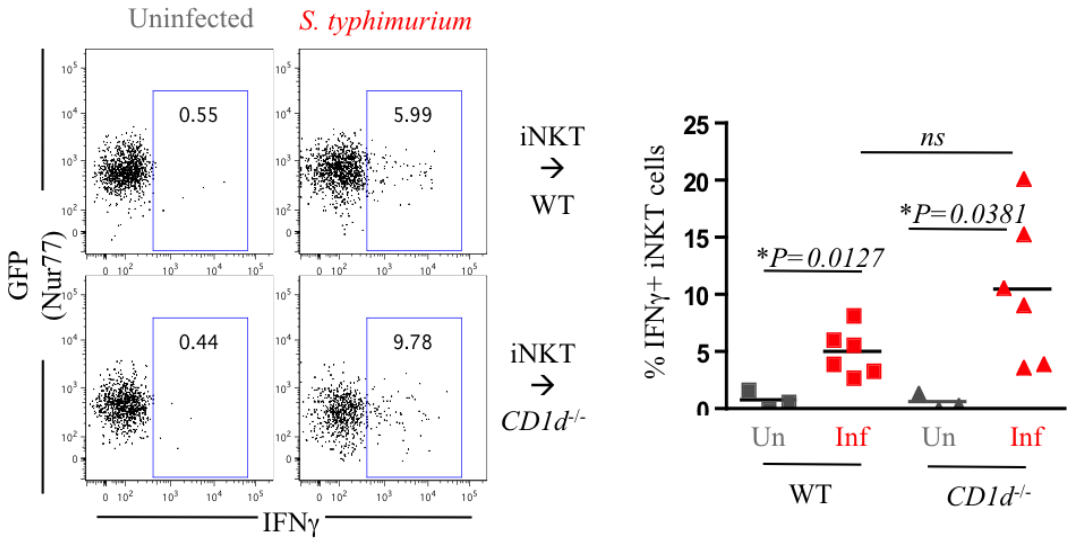

Figure 2-6. iNKT cell cytokine production was CD1d independent during *S. typhimurium* infection. iNKT cells were purified from Nur77^{gfp} Vα14 TCR transgenic B6 mice and transferred into either WT hosts (iNKT → WT) or CD1d$^{-/-}$ hosts (iNKT → CD1d$^{-/-}$). Mice were then infected with *S. typhimurium i.v.*, and the transferred tet^{+} iNKT cells were isolated and analyzed by flow cytometry for intracellular IFNγ 20 h post infection. Dot plots (left) show representative data of iNKT cells isolated from an uninfected WT host (top, left), an uninfected CD1d$^{-/-}$ host (bottom, left), an infected WT host (top, right) or infected CD1d$^{-/-}$ host (bottom, right) from two independent experiments with three to six animals per group. *P = 0.0127, uninfected in WT host versus 20 h post infection in WT host; *P = 0.0381, uninfected in CD1d$^{-/-}$ host versus 20 h post infection in CD1d$^{-/-}$ host (all using unpaired two-tailed t-test).

TLR Stimulation Does Not Cause TCR-dependent Activation of iNKT Cells

The presentation of antigenic endogenous self-lipids by CD1d leading to the stimulation of iNKT cells has been proposed to occur as a result of TLR stimulation of APCs (89-91). Specifically, stimulation of TLR4 or TLR9 was suggested to lead to increased synthesis and/or presentation of antigenic endogenous self-lipids (79, 89-91). In order to test this, we injected Nur77^{gfp} mice with either LPS to stimulate TLR4, or CpG oligodeoxynucleotides (ODNs) to stimulate TLR9, and examined iNKT cell

activation 4, 24, or 36 hours later. There was no increase in GFP expression in iNKT cells at any time point after TLR activation, despite robust production of IFNγ and elevated expression of CD69 at 4 hours for LPS and 36 hours for CpG (Figure 2-7 and data not shown).

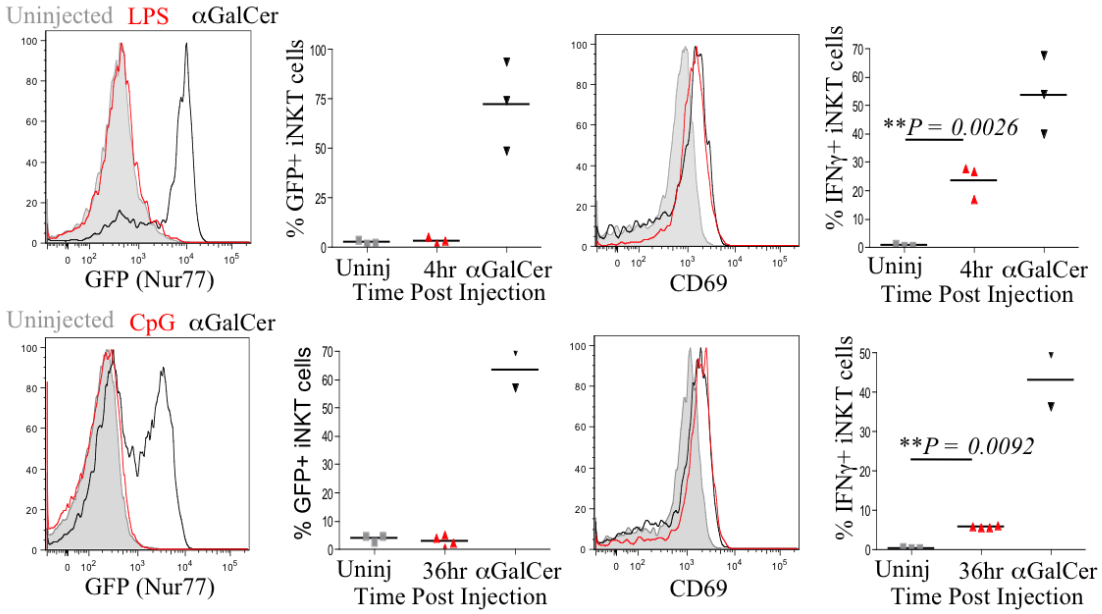

Figure 2-7. iNKT cell cytokine production was CD1d independent during TLR stimulation. Nur77^{gfp} B6 mice were injected with LPS (top panel) or CpG (bottom panel) *i.v.*, and tet^{+} iNKT cells were analyzed by flow cytometry 4 or 36 h later, respectively, for GFP expression, CD69 expression, and IFNγ production. Representative histograms show GFP expression (left) or CD69 expression (middle) for uninjected, LPS or CPG injected, or 2 to 3 h αGalCer injected animals from two independent experiments with 3 animals per group. For the percentage of IFNγ^{+} iNKT cells graph (right, top), ***P* = 0.0026, uninjected versus 4 h post LPS injection. For the percentage of IFNγ^{+} iNKT cells graph (right, bottom), ***P* = 0.0092, uninjected versus 36 h post CpG injection (all using unpaired two-tailed *t*-test).

This contradicts the conclusions from a previous report, in which it was found that TLR stimulation led to the inhibition of the enzyme α-galactosidase A (α-Gal A) (89). α-Gal A was proposed to convert a stimulatory lipid to a non-stimulatory lipid, and thus the inhibition of this enzyme would result in the increased presentation of the stimulatory lipid for iNKT cell activation (89). Therefore, we used the Nur77^{gfp} mice with α-Galactosidase A deficient (α-Gal A$^{-/-}$) mice to test this notion. Initially, we looked at GFP expression in Nur77^{gfp}:α-Gal A$^{-/-}$ mixed bone marrow chimeras to examine iNKT cell activation in the steady state *in vivo*. However, we saw no GFP expression in the recovered Nur77^{gfp} iNKT cells (Figure 2-8). Next, we analyzed a model of acute activation of iNKT cells by examining GFP expression after adoptive transfer of iNKT cells from Nur77^{gfp} Vα14 TCR transgenic mice into α-Gal A$^{-/-}$ or WT hosts. The Nur77^{gfp} Vα14 TCR transgenic iNKT cells did not have increased GFP expression when recovered from α-Gal A$^{-/-}$ hosts (Figure 2-9). Lastly, we performed a direct comparison with the reported data, but analyzed GFP expression as a measure of iNKT TCR stimulation in addition to the decrease in the αGalCer-loaded CD1d-tetramer^{+} TCRβ^{+} population used in the previous report as a measure of activation (89). Similar to the prior study, we transferred α-Gal A$^{-/-}$ BMDC, βGlcCer pulsed α-Gal A$^{-/-}$ BMDC, or WT BMDC into Nur77^{gfp} recipients. iNKT cells from animals injected with α-Gal A$^{-/-}$ BMDC failed to upregulate GFP in comparison to animals injected with WT BMDC, while increased GFP expression was seen in iNKT cells from animals injected with βGlcCer pulsed α-Gal A$^{-/-}$ BMDC (Figure 2-10).

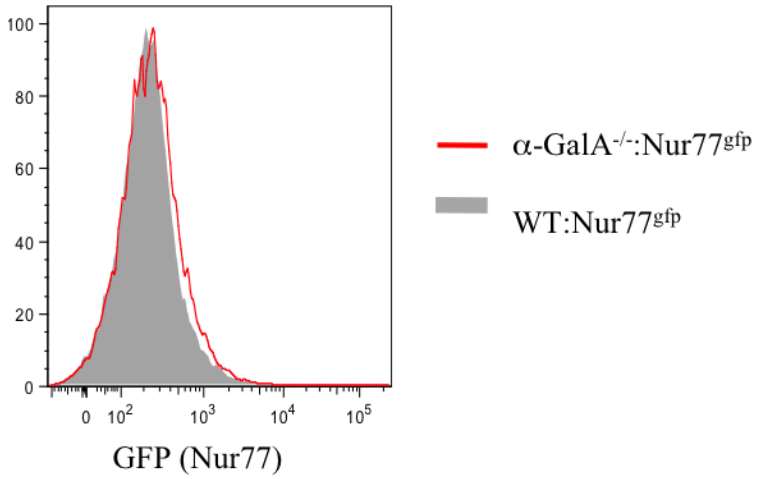

Figure 2-8. iNKT cells were not activated in α-GalA$^{-/-}$:Nur77^{gfp} chimeras. Nur77^{gfp} iNKT cells were isolated from α-GalA$^{-/-}$:Nur77^{gfp} mixed bone marrow chimeras and analyzed by flow cytometry for GFP expression. Histogram shows GFP expression for Nur77^{gfp} iNKT cells from a WT:Nur77^{gfp} mixed bone marrow chimera and Nur77^{gfp} iNKT cells from α-GalA$^{-/-}$:Nur77^{gfp} mixed bone marrow chimera. Data shown is representative of three independent experiments (WT:Nur77^{gfp} = 3; α-GalA$^{-/-}$:Nur77^{gfp} = 10).

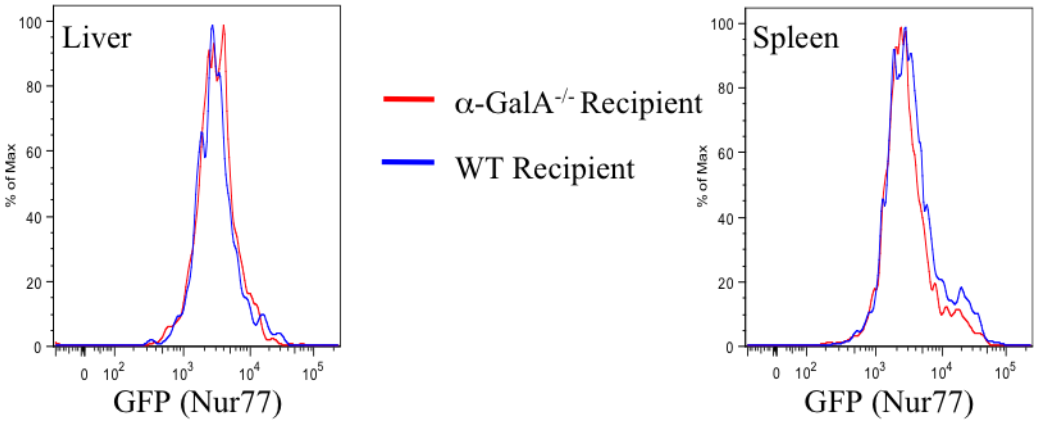

Figure 2-9. iNKT were not activated after transfer into α-Gal A$^{-/-}$ mice. iNKT cells were enriched from congenic (CD45.1) Nur77^{gfp} Vα14 TCR transgenic B6 mice and transferred into either WT or α-GalA$^{-/-}$ recipients. One day later, the transferred cells were recovered with magnetic beads, and tet^{+} cells analyzed by flow cytometry. Histograms show representative data of hepatic or splenic iNKT cells isolated from a WT recipient or α-GalA$^{-/-}$ recipient from one experiment (WT recipient = 3; α-GalA$^{-/-}$ recipient = 3).

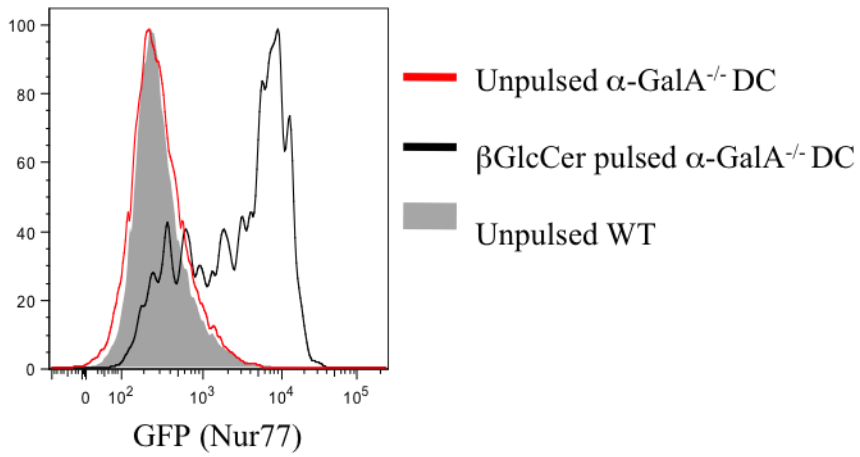

Figure 2-10. α-Gal A$^{-/-}$ BMDCs transferred into Nur77^{gfp} mice did not activate iNKT cells. Unpulsed α-Gal A$^{-/-}$ BMDC, α-Gal A$^{-/-}$ BMDC pulsed with βGlcCer, or unpulsed WT BMDC were transferred into Nur77^{gfp} mice, and tet^{+} iNKT cells were analyzed by flow cytometry 18 h later. Histogram shows representative GFP expression for iNKT cells from mice that received unpulsed WT BMDC, unpulsed α-Gal A$^{-/-}$ BMDC, or α-Gal A$^{-/-}$ BMDC pulsed with βGlcCer from two independent experiments (WT BMDC = 2, unpulsed α-Gal A$^{-/-}$ BMDC = 4, α-Gal A$^{-/-}$ BMDC pulsed with βGlcCer =2).

Activation of iNKT cells can lead to a decreased recovery when using tetramer due to TCR internalization (71) and/or activation induced cell death (27, 72). This reduced recovery has been used as a measure of iNKT cell activation *in vivo*. However, mice from α-Gal A$^{-/-}$ BMDC injected groups exhibited variability in the percentage of αGalCer-loaded CD1d-tetramer^{+} TCRβ^{+} populations recovered (Figure 2-11). Overall, our data are not consistent with the hypothesis that TLR stimulation of APCs leads to the presentation of antigenic endogenous self-lipid for the stimulation of iNKT TCRs.

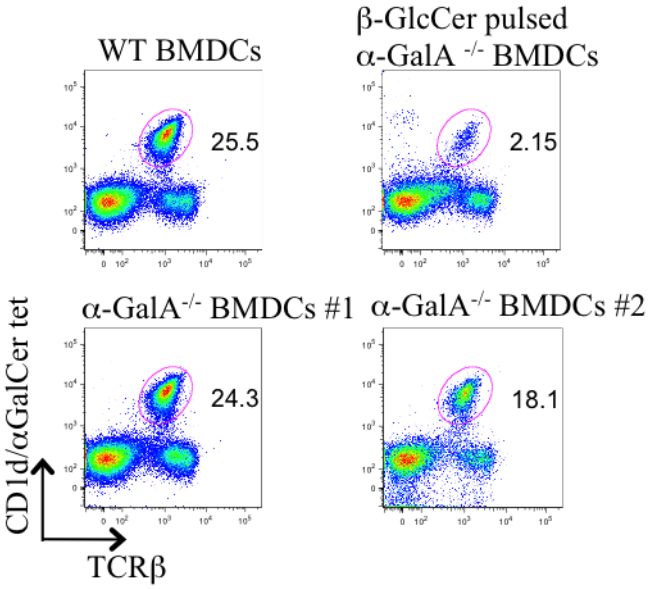

Figure 2-11. Inconsistent TCR internalization in iNKT cells after injection of α-Gal A$^{-/-}$ BMDC. Unpulsed α-Gal A$^{-/-}$ BMDC, α-Gal A$^{-/-}$ BMDC pulsed with βGlcCer, or WT BMDC were transferred into Nur77^{gfp} mice. After 18 h, the tet^{+} iNKT cells were gated and showed an inconsistent reduction in proportion compared to WT (presumed to reflect TCR internalization) when injected with α-Gal A$^{-/-}$ BMDC (compare bottom left to bottom right).

Discussion

The invariant nature of the iNKT TCR α-chain implies that the potential repertoire of lipids capable of stimulating iNKT cells must be restricted, which indicates that iNKT cells are only activated in a limited context of conditions. Yet, diverse structures have been reported for both the microbial and self-lipids recognized by these cells including GSLs, diacylglyerols, phospholipids and cholesterol containing compounds (70). Also, several TCR independent modes of activation of iNKT cells have been reported, including the cytokine-mediated activation described above, but also stimulation mediated by engagement of activating NK receptors such as NKG2D (66) and activation of iNKT cells in the liver by engagement of β-adrenergic receptors (155). Furthermore, it has been shown that iNKT cells are able to rapidly produce cytokines not only in response to bacterial, viral, and fungal infections, but also in response to cancers, various inflammatory conditions, and autoimmune diseases (23). This ability to respond in such diverse contexts raises the question as to the importance of TCR-dependent pathways in those situations in which iNKT cells play a role in the immune response, and also, when the TCR is involved, the relative weight of recognition of self versus foreign glycolipids.

Here we used the $Nur77^{gfp}$ mouse as a sensitive tool to examine the role of iNKT cell TCR stimulation during various infections. We showed that this reporter mouse can detect iNKT cell responses to self glycolipid antigens presented by CD1d that have an affinity for the iNKT cell TCR of approximately 5 μM or weaker, which is nearly 10^3-

fold weaker than the affinity for αGalCer presented by CD1d. Using this system, we showed that iNKT cells are stimulated through their TCR when infected with pathogens known to have antigenic lipids, such as in *S. pneumoniae* infection or *S. paucimobilis* infection.

Using infection with MCMV, we showed that iNKT cells became activated in the absence of TCR stimulation, consistent with other results showing that cytokines from APCs were sufficient to activate a protective response by iNKT cells after this infection (88, 151). Further evidence showing that iNKT cells can undergo TCR-independent stimulation was recently reported. Using a transgenic mouse in which the iNKT TCR could be conditionally ablated, it was shown that TCR-negative iNKT cells could still mount a response to LPS by producing similar amounts of IFNγ as compared to iNKT cells that retained TCR expression (156).

More surprising was our findings that iNKT cell TCR stimulation could not be detected after either *S. typhimurium* infection or TLR stimulation of APCs, as these observations run counter to a currently popular model (70). It could be argued that the reporter mouse is not sufficiently sensitive, and that while we have tested some of the weakest reported TCR agonists, that *S. typhimurium* induces the presentation of a self-antigen that has a very low abundance and/or TCR affinity, and therefore cannot give an activation signal in the Nur77^{gfp} mice. We consider this unlikely, however, because when transferred to CD1d-deficient hosts, we found that the iNKT cell IFNγ response to *S. typhimurium* was not impaired. Regarding the reported TCR-dependence of the iNKT

cell response to *S. typhimurium*, much of the data relied on *in vitro* studies. For example, *in vitro* experiments showing that CD1d$^{-/-}$ BMDC were ineffective at inducing IFNγ production by tetramer sorted iNKT cells in response to *S. typhimurium* are difficult to reconcile with our data. *In vivo* studies used CD1d-blocking antibodies to demonstrate TCR dependent IFNγ production. It is possible that antibodies to CD1d could have had a direct effect on APCs independently of iNKT cells, which led to a reduction in inflammatory cytokine production, and a consequent reduction in IFNγ production by iNKT cells (157).

Similarly, we did not find that TLR activation of APCs induced a detectable TCR signal in iNKT cells, although it was previously reported to increase the synthesis of enzymes responsible for the synthesis of stimulatory self-antigens (6, 91, 92, 158). Although again, other *in vivo* experiments showed that iNKT cells with and without a TCR produced similar levels of IFNγ in response to LPS (156). One possible explanation is that a stronger stimulation of the innate immune system achieved *in vivo* may somehow inhibit TCR-dependent activation of iNKT cells. TLR stimulation was also reported to inhibit an enzyme, α-Gal A, that would degrade a putative lipid self-antigen (89). Regarding the role of α-Gal A, using the Nur77^{gfp} reporter mouse, we did not find evidence that APCs in mice deficient for this enzyme induced a TCR signal in iNKT cells *in vivo*. In the α-Gal A deficient model, the previous data supporting TCR-dependent activation of iNKT cells relied upon loss of the αGalCer-loaded CD1d-tetramer^{+} TCRβ^{+} population and on the increased expression of the surface marker CD69 (89). In our

hands, however, population loss was not a consistent and reliable measure of TCR-mediated iNKT cell activation, and CD69 expression is not strictly TCR dependent, as it is also influenced by inflammatory stimuli.

In the cases of infection with *S. pneumoniae* and *S. paucimobilis*, in which there was a detectable TCR signal in the Nur77^{gfp} reporter mouse, the question remains as to whether that signal was due to the presence of a foreign antigen or the induction of the synthesis of a self-antigen, although these alternatives are not mutually exclusive and both types of antigens could be present (6). The evidence provided here is only correlative with the need for a foreign antigen to induce a TCR-mediated signal in the infections studied; although if self-antigen were dominant during each of the three bacterial infections, it is puzzling that a signal from the reporter mouse could only be obtained using bacteria with a foreign antigen. There is no evidence that the iNKT cells from the *S. typhimurium* infected mice were less activated; in fact, by intracellular cytokine staining they were at least as activated as the populations from *S. pneumoniae* or *S. paucimobilis* infected mice. It has not been possible to resolve the issue of foreign versus self-antigen recognition so far, however, as it has not been feasible to remove either the microbial antigen or the self-antigen by genetic deletion, without causing unacceptable secondary effects. For example, *S. pneumoniae* mutants that cannot synthesize the glycosylated diacylglycerol antigen have proved not to be viable (M. Kronenberg, unpublished). Regarding the self-antigen, problems include the diversity of self-antigens reported, with both GSLs and phospholipids, and secondary effects on the

health of the mice and the functioning of the endosomal system in APCs when basic components of glycolipid synthesis pathways are eliminated (30, 159).

Although *S. typhimurium* infection did not stimulate the iNKT TCR as a result of endogenous lipid antigen presentation in our experiments, we are not proposing that this pathway for iNKT cell activation is unimportant. It is known that iNKT cells do receive TCR stimulation from endogenous lipid during iNKT cell thymic selection (160, 161). For example, in the Nur77^{gfp} mice, intrathymic stage 0 iNKT cell precursors expressed a high level of GFP (43). Furthermore, thymic NKT2 cells from BALB/c mice, which produce IL-4 at steady state, showed an increased expression of GFP compared to NKT1 or NKT17 cells (57). iNKT TCR stimulation may also be important during homeostasis, as a recent report indicated its importance in detection of inhibitory lipids required for the regulation of symbiotic microbe homeostasis (162). Additionally, other types of infections remain to be tested for iNKT cell TCR stimulation as was done in this report. For example, infection with the helminth parasite *Schistosoma mansoni*, in which iNKT cell activation has been shown to be dependent on CD1d after schistosome egg encounter, remains one of a number of possible examples of iNKT cell TCR stimulation mediated by an antigenic endogenous lipid, as a foreign lipid from *S. mansoni* has not been reported (163).

In conclusion, we have demonstrated that the Nur77^{gfp} mouse provides a sensitive tool for directly measuring iNKT cell TCR stimulation and detecting endogenous and foreign lipid agonists even when they vary greatly in potency. Additionally, our data

provided insight into the role of iNKT cell TCR stimulation in iNKT cell activation during infections, differentiating between infections that are dependent on TCR stimulation and infections that are not. We showed that TCR-independent activation applies to bacterial as well as viral infections, and furthermore, that the ability to detect TCR-mediated activation *in vivo* correlated with the presence of foreign antigen. This approach may be useful to identify those situations in which a self-antigen mediated activation of iNKT cells is most relevant, for example, in the context of sterile inflammatory conditions such as ischemia reperfusion injury (164).

Materials and Methods

Mice

Nur77^{gfp} B6 mice were previously described (43). B6 (C57BL/6NCr) and B6.SJL (B6-LY.5/Cr) mice were obtained from the National Cancer Institute. CD1d$^{-/-}$ B6 (B6.129S6-Cd1d1/Cd1d2tm1Spb/J) mice and Vα14 TCR transgenic B6 (C57BL/6-Tg(Cd4-TcraDN32D3)1Alben/J) mice were obtained from The Jackson Laboratory. Fabry mice deficient for the enzyme α-galactosidase A (B6;129-*Gla*^{tm1Kul}/J) were obtained from F. Winau (Harvard Medical School). KN2 mice on the B6 background were obtained from M. Mohrs (Trudeau Institute). KN2 Nur77^{gfp} B6 mice were generated by the crossing of KN2 and Nur77^{gfp} mice. Nur77^{gfp} Vα14 TCR transgenic B6 mice were generated by the crossing of Vα14 TCR transgenic B6 and Nur77^{gfp} B6 mice. All animal experimentation was approved by and performed according to the guidelines from the Institutional Animal Care and Use Committees at the University of Minnesota or at the La Jolla Institute for Allergy & Immunology.

Flow Cytometry, Antibodies, and Intracellular Cytokine Staining

Single-cell suspensions were prepared from spleens, and hepatic mononuclear cells were separated by Percoll gradient centrifugation. All cells were resuspended in staining buffer (PBS with 5% FCS). Antibodies to surface markers were added, and cells were stained on ice for 20 min. Antibodies used were from eBioscience, BD, or BioLegend. For iNKT cell staining, CD1d monomers loaded with biotinylated PBS57 or

unloaded monomers were obtained from the tetramer facility of the US National Institutes of Health. Cells were analyzed on an LSRII (Becton Dickinson) and data was processed with FlowJo software (TreeStar). A dump strategy (CD11b, F4/80, and CD8) was used to eliminate non-specific events.

For intracellular detection of cytokines, cells were stained for surface markers as described above, and then fixed and permeabilized using the Cytofix/CytoPerm buffer (BD Biosciences) according to the manufacturer's protocol. Anti-IFNγ (eBioscience) was added and the cells were stained on ice for 30 min. Cells were washed twice and then resuspended in staining buffer before analysis on an LSRII.

iNKT Cell Purification

For iNKT cell adoptive transfer experiments, a single-cell suspension was prepared from Nur77^{gfp} Vα14 TCR transgenic mice spleens, and from hepatic mononuclear cells separated by Percoll gradient centrifugation. This single-cell suspension was depleted of B cells and CD8 T cells using anti-CD19 and anti-CD8α conjugated to magnetic beads, and MACS columns (Miltenyi Biotec), according to manufacturer's protocol. The percentage of iNKT cells in the enriched fraction was ~30%, and the equivalent of ~3x10^6 iNKT cells was adoptively transferred per mouse.

In Vivo Infections

Mouse cytomegalovirus (MCMV-K181) was obtained from C. Benedict (La Jolla Institute for Allergy & Immunology). Mice were infected intra-peritoneally with $1x10^5$ PFU diluted in 300 ul PBS. *Sphingomonas paucimobilis* was obtained from M. Kronenberg (La Jolla Institute for Allergy & Immunology). *S. paucimobilis* cultured in Tryptic Soy Broth (BD) at 37°C was collected at a mid-log phase and washed with PBS. Mice were inoculated intravenously with ~$1x10^9$ CFU diluted in 200 ul PBS. *Streptococcus pneumoniae* URF918 (clinical isolate, serotype 3) was obtained from M. Kronenberg (La Jolla Institute for Allergy & Immunology). *S. pneumoniae* cultured in Todd-Hewitt broth (BD) at 37°C were collected at a mid-log phase and then washed with PBS. Mice were inoculated intravenously with ~$1x10^7$ CFU diluted in 200 ul PBS. *Salmonella typhimurium* (SL1344) was obtained from S. McSorley (University of California, Davis). *S. typhimurium* cultured in Todd-Hewitt broth (BD) at 37°C was collected at a mid-log phase and then washed with PBS. Mice were inoculated intravenously with ~$1x10^6$ CFU diluted in 200 ul PBS. As a control for all infections, 2 ug of αGalCer diluted in 200 ul of PBS was injected intravenously, and spleen and liver were harvested two to four hours later.

TLR Agonists

Mice were injected intravenously in a final volume of 200 ul with 50 ug of *E. coli* derived LPS diluted in PBS, or with 10 ug of ODN 1826 diluted in endotoxin free water. Spleen and liver were analyzed at the indicated timepoints after injection.

Lipid-pulsed Bone Marrow-derived Dendritic Cells

Bone marrow cells from femurs of mice were cultured for seven days ($5x10^6$ cells/well) at 37°C in six-well cell culture dishes with complete RPMI medium in the presence of recombinant murine GM-CSF (50 ng/ml, PeproTech) and IL-4 (10 ng/ml, PeproTech). On day six, cells were pulsed with either 100 ng–1 ug/ml of αGalCer (KRN7000, Avanti Polar Lipids), 1 ug/ml OCH (Alexis Biochemicals), 1 ug/ml of βGlcCer (C24:1 Glucosyl(β) Ceramide (d18:1/24:1(15Z)), Avanti Polar Lipids), 1ug/ml of iGb3 provided by D. Zhou (MD Anderson) or 1 ul/ml solvent (2:1 methanol:chloroform) for 12–15 hours at 37°C. BMDCs were intravenously injected into $Nur77^{gfp}$ mice ($0.5–1.0x10^6$cells/mouse), and endogenous splenic iNKT cells were analyzed 16 h later.

Mixed-bone Marrow Chimeras

Total bone marrow cells were prepared from the femurs of $Nur77^{gfp}$ B6.SJL ($CD45.1^+CD45.1^+$), B6 ($CD45.2^+CD45.2^+$), or Fabry B6 ($CD45.2^+CD45.2^+$) donor mice, and samples were depleted of mature T cells with anti-Thy1.2 (30-H12; Biolegend) and

complement. Recipient mice ($CD45.2^+$ $CD45.1^+$) were lethally irradiated (1,000 rads) and received $1x10^7$ adult bone marrow cells. Chimeras were analyzed eight weeks after transplantation.

Statistical Analysis

Prism software (GraphPad) was used for statistical analysis. Unpaired two-tailed *t*-tests were used for data analysis.

Chapter 3: Deriving iNKT Cells from Hematopoetic Progenitor Cells *In Vitro*

CD1d-restricted invariant Natural Killer T cells (iNKT cells) play a vital role in determining the kinetics, extent, and characteristics of an immunological response. iNKT cells influence an immune response by producing large amounts of the cytokines IFNγ, IL-4, and IL-17, from three major subsets: NKT1, NKT2, and NKT17. iNKT cells have been shown to be involved in cancer, autoimmune disease, asthma, and infection. Therefore, there is great potential for the use of iNKT cell based therapeutics to beneficially influence the immune response. In this study, we sought to develop an *in vitro* system to generate iNKT cells, and to define the conditions that result in the iNKT cell subset differentiation into NKT1, NKT2, and NKT17. We used the bone marrow stromal cell line OP9 engineered to ectopically express the Notch ligand, Delta-like-1 (DL1), to induce fetal liver hematopoietic progenitor cells to become iNKT cells. We found that it was necessary to use a source of Vα14 T cell receptor (TCR) transgenic (Tg) fetal liver hematopoietic progenitor cells in order to detect a CD1d/αgalactosylceramide tetramer$^+$ (CD1d/αGalCer tet$^+$) TCRβ$^+$ population. These CD1d/αGalCer tet$^+$ TCRβ$^+$ cells expressed PLZF and Tbet similarly to the iNKT cell subset NKT1; however, there was a temporal component to this expression: CD1d/αGalCer tet$^+$ TCRβ$^+$ cells were initially detected, followed by the detection of PLZF$^+$ CD1d/αGalCer tet$^+$ TCRβ$^+$ cells, and then lastly, by the detection of PLZF^{lo} Tbet$^+$ CD1d/αGalCer tet$^+$ TCRβ$^+$ cells. Interestingly, the addition of IL-15 enhanced the percentage of CD1d/αGalCer tet$^+$ TCRβ$^+$ cells expressing Tbet. The function of these cells was assessed by adoptive transfer and stimulation *in vivo*, where upon these OP9-

DL1 derived CD1d/αGalCer tet^{+} TCRβ^{+} cells rapidly produced IFNγ. Overall, this study showed that the Vα14 TCR Tg fetal liver OP9-DL1 culture system successfully gave rise to CD1d/αGalCer tet^{+} TCRβ^{+} NKT1-like cells that expressed the important iNKT cell transcription factors PLZF and Tbet, as well as rapidly produced IFNγ after stimulation. These findings indicate that this culture system can be used as a powerful tool for the further analysis of iNKT cell development, and potentially for iNKT cell-based therapies.

Introduction

CD1d-restricted Natural Killer T cells (iNKT cells) are a distinct lineage of αβ T lymphocytes with a semi-invariant T cell receptor (TCR) (19). In mice, this iNKT TCR is composed of the TCR α-chain variable region 14 (Vα14) and the α-chain joining region 18 (Jα18) paired with a restricted subset of TCR-β chains (Vβ8.2, 7 or 2) (19). Upon stimulation, iNKT cells are able to rapidly produce cytokines, such as IFNγ, IL-4, and IL-17 (48). This rapid cytokine production contributes to the activation of other cell types during the immune response, which has been shown to influence a variety of diseases, including cancer (119), autoimmunity (140, 141), and pathogenic infection (127).

More specifically in the context of cancer, iNKT cells have been shown to boost the anti-tumor response (116). In a B16 melanoma model, iNKT cells were shown to be required for IL-12 mediated tumor rejection (119). In another study, IFNγ produced by iNKT cells activated by the strong agonist αgalactosylceramide (αGalCer) was shown to be protective against B16 melanoma metastases (120). Additionally, activated iNKT cells were shown to be directly involved in the antitumor response by contributing to antitumor cytotoxicity (121). However, it has also been shown that the frequency of iNKT cells in cancer patients were significantly lower than in healthy volunteers (122, 124, 126), and that this decreased iNKT cell number predicted poor clinical outcomes (125). Furthermore, the iNKT cells from these patients exhibited decreased proliferation and cytokine production after *ex vivo* stimulation (126). Together, these studies indicate

an iNKT cell-based therapeutic could be a powerful new cancer treatment; however, for such a treatment to be effective, a patient will need to have a sufficient number of functional iNKT cells. Therefore, in this study we sought to derive a source of exogenous iNKT cells.

Previous work has shown that the stromal cell line, OP9, can support the differentiation of hematopoietic progenitor cells (HPCs) into multiple lineages, such as B cells (129, 130). However, only when OP9 cells were retrovirally transduced to express the Notch ligand Delta-like-1 (DL1) were these cells able to support T cell development (131). The expression of DL1 allowed for activation of Notch signaling, which is essential for determining whether lymphocyte progenitors adopt a T cell fate over a B cell fate (131). With the ectopic expression of DL1, OP9-DL1 cells co-cultured with HPCs were able to differentiate into the T cell lineage specific Double Negative (DN) stages and then into the $CD4^+$ $CD8^+$ Double Positive (DP) stage similarly to the T cell development that occurs in the thymus (131). However, these cultures do not yield fully mature, functional T cells in the absence of thymic epithelial cells, as they are unable to undergo normal positive selection.

Importantly, iNKT cells follow the same progression through T cell development until the DP stage (29, 42, 137). At the DP stage, rare DP thymocytes expressing the iNKT TCR are positively selected by a self-lipid presented by CD1d expressed on other DP thymocytes (29, 42, 43). Homophilic interactions between SLAM family members expressed on the surface of DP thymocytes are also required to initiate essential signals

for iNKT cell development (42, 44-46). During or immediately after this selection event, one of the main transcriptional regulators of iNKT cell development, PLZF (promyelocytic leukemia zinc finger, encoded by *Zbtb16*), is expressed (50, 51). PLZF has been shown to direct the innate-like effector differentiation of iNKT cells during thymic development, and is later important for appropriate cellular trafficking and functionality (50, 51). After PLZF expression is initiated, iNKT cells undergo further differentiation into three main subsets, NKT1, NKT2 and NKT17 (57). These three subsets are distinguished by their transcription factor profiles, and can be separated into the three populations based on their expression of PLZF, RORγt and Tbet: NKT1 cells are $PLZF^{lo}$ $Tbet^{+}$, NKT2 cells are $PLZF^{hi}$ $Tbet^{-}$ $ROR\gamma t^{-}$, and NKT17 cells are $PLZF^{int}$ $ROR\gamma t^{+}$ (57). This subset differentiation is important as it dictates the main cytokine produced by the cells after stimulation, which influences the subsequent immune response. NKT1 cells produce IFNγ, NKT2 cells produce IL-4, and NKT17 produce IL-17 (56, 57).

Since iNKT cells develop from DP cells, the OP9-DL1 culture system can be used to derive iNKT cells. Three previous studies have examined this possibility using HPCs, iNKT cell derived embryonic stem cell (ESC) lines, or iNKT cell induced pluripotent stem cells (iPSCs) cultured with OP9-DL1 to derive iNKT cells *in vitro* (47, 138, 139). However, the iNKT cells derived from HPCs cultured with OP9-DL1 were very transient, making this finding debatable (47). Additionally, since iNKT cells are a heterogenous

population, determination of what subsets developed is very important for future therapeutic use, which was not done in any of the previous studies.

Here, we showed that using HPCs with a fixed Vα14-Jα18 TCR was necessary to detect a CD1d/αgalactosylceramide tetramer^{+} (CD1d/αGalCer tet^{+}) TCRβ^{+} population. We found that CD1d/αGal tet^{+} TCRβ^{+} population was undetectable using wild type HPCs, which was presumably due to the low frequency of generating the appropriate Vα14/Jα18 recombination event. We then examined the transcription factor profile of the derived CD1d/αGal tet^{+} TCRβ^{+} cells to determine which iNKT subsets were present, and tested these cells for cytokine production after TCR stimulation *in vivo*.

Results

Induction of T Cell Differentiation in Vα14 TCR Tg Fetal Liver Hematopoietic Progenitor Cells Using the OP9-DL1 Culture System

This study sought to demonstrate an *in vitro* approach to derive iNKT cells from hematopoietic progenitor cells (HPCs). Previous work showed that the bone marrow stromal cell line, OP9, can support the differentiation of HPCs into multiple lineages, such as B cells (129, 130), and that when these OP9 cells were retrovirally transduced to express the Notch ligand DL1, this cell line supported T cell development (131). Since iNKT cells follow the same developmental pathway as conventional T cells until the DP stage (29, 42, 137), we used the OP9-DL1 culture system to derive iNKT cells from HPCs.

At the DP stage of T cell development, iNKT cells are selected from the DP pool of thymocytes based upon the interaction between their invariant TCR and the selecting lipid ligand presented by other DP thymocytes (29, 137). However, the frequency of DP thymocytes that express the required TCR is very small in the wild type (WT) animals. Therefore, we decided to use Vα14 TCR Transgenic (Vα14 TCR Tg) animals as the source of HPCs (Vα14 HPCs). Since Vα14 TCR Tg mice express a fixed Vα14-Jα18 TCR, the percentage of DP thymocytes that express the invariant TCR required for iNKT cell selection is greatly increased (165). However, since we are using a TCR transgenic source of HPCs, we first wanted to ensure that this TCR expression was not altering the ability of the Vα14 HPCs to develop normally.

We first tested the ability of Vα14 HPCs to undergo B cell development. To do this, HPCs were isolated from the fetal livers of day 15 Vα14 TCR Tg embryos. HPCs were enriched for $CD24^{lo}$ Lin^{-} cells by CD24 complement depletion and then placed into culture with OP9 cells. Cells were harvested at day 7, 12, and 17, and were analyzed by flow cytometry for B cell development using CD19. By day 12 there was a large population of $CD19^{+}$ cells, which was still present at day 17, indicating that Vα14 HPCs had differentiated into the B cell lineage (Figure 3-1A). These cells were also analyzed for markers of T cell development. During T cell development, T cell progenitors go through four Double Negative (DN) stages before reaching the $CD8^{+}$ $CD4^{+}$ Double Positive (DP) stage. The progression through the DN stages is marked by differential expression of the markers CD25 and CD44, in which DN stage 1 cells are $CD44^{+}$ $CD25^{-}$, DN stage 2 cells are $CD44^{+}$ $CD25^{+}$, DN stage 3 cells are $CD44^{lo}$ $CD25^{+}$, and DN stage 4 cells are $CD44^{-}$ $CD25^{-}$. After DN stage 4, cells will then progress to the DP stage. The cells derived from the Vα14 HPCs did not express the markers for the four DN stages or the DP stage, indicating they did not undergo T cell lineage commitment under these conditions (Figure 3-1A). Therefore, expression of the Vα14-Jα18 fixed TCR did not interfere with the ability of HPCs to undergo B cell development nor did it predispose these HPCs to undergo T cell development in the absence of Notch signaling.

Next, we tested whether the Vα14 HPCs would undergo T cell commitment similarly to HPCs from WT animals. As done in the OP9 cell co-culture, HPCs were isolated from fetal livers of day 15 WT embryos or Vα14 TCR Tg embryos. After

enriching for HPCs, these cells were placed in parallel cultures with OP9-DL1 cells. Cells were then harvested at day 7, 12, 17, 22, 26, 33, and 37, and were again examined by flow cytometry for B cell development using CD19, and for T cell development using CD25, CD44, CD4, and CD8. Cells derived from both WT HPCs and Vα14 HPCs did not express CD19, indicating that B cell development was equally inhibited in both cultures at all time points (Figure 3-1B and data not shown). Additionally, cells derived from both cultures underwent similar T cell lineage commitment. These cells followed a similar progression through the four DN stages as shown by their CD25 and CD44 expression, as well as a similar emergence of a DP population at day 12 that was maintained at all subsequent time points (Figure 3-1B). These results indicate that cells derived from Vα14 HPCs underwent similar Notch-dependent T cell commitment as cells derived from WT HPCs.

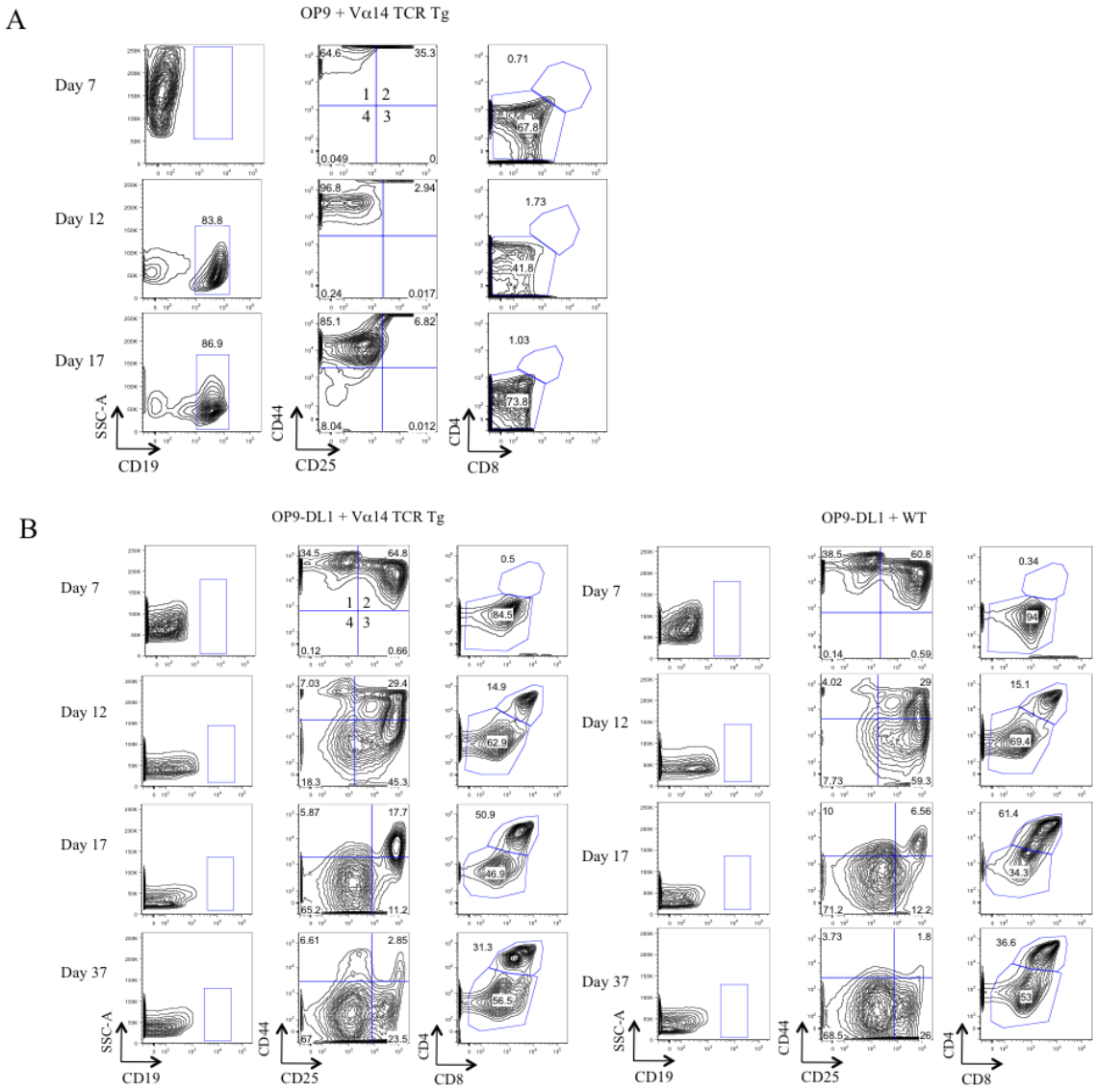

Figure 3-1. Vα14 TCR Tg hematopoietic progenitors underwent Notch-dependent T cell commitment similar to WT hematopoietic progenitor cells. Flow cytometric analysis for the B cell marker (CD19) or T cell development markers (CD25, CD44, CD4, and CD8) from Vα14 HPCs cultured with OP9 cells (A), Vα14 HPCs cultured with OP9-DL1 cells (B, right), or WT HPCs cultured with OP9-DL1 cells (B, left). Cells were harvested at time points indicated. Numbers in quadrants for CD44 and CD25 expression indicate DN stages. Contour plots in (A) are representative of two to four co-cultures at each time point from three independent experiments. Contour plots in (B, right) are representative of two to four co-cultures at each time point from five independent experiments. Contour plots in (B, left) are representative of four co-cultures at each time point.

Vα14 TCR Tg Fetal Liver Hematopoietic Progenitor Cells Give Rise to CD1d/αGalCer Tetramer Binding Cells Using the OP9-DL1 Culture System

iNKT cells follow the same developmental pathway as conventional T cells until the DP stage (29, 137). At the DP stage, DP thymocytes that express the iNKT cell TCR are positively selected by interactions with other DP thymocytes to become iNKT cells (42). As shown above, Vα14 HPCs cultured with OP9-DL1 gave rise to a large population of DP cells (Figure 3-1B). Therefore, we next tested the cultures for the presence of a CD1d/αGalCer tet^+ $TCR\beta^+$ population. The cultures were examined at day 7, 12, 17, 22, 26, 33, and 37 (Figure 3-2 and data not shown). At day 7, no CD1d/αGalCer tet^+ $TCR\beta^+$ population was present. However, by day 12 and at every time point after, there was a stable population of CD1d/αGalCer tet^+ $TCR\beta^+$ cells. The emergence of CD1d/αGalCer tet^+ $TCR\beta^+$ cells coincided with the appearance of DPs in culture (Figure 3-1B), indicating that these cells are most likely arising from the DP population as would be expected based on normal iNKT cell development. Interestingly, in a parallel culture using WT HPCs, no population of CD1d/αGalCer tet^+ $TCR\beta^+$ cells was detected (Figure 3-2B). Thus using Vα14 HPCs was necessary to increase the frequency of DPs expressing the required iNKT TCR in order to obtain a detectable population of CD1d/αGalCer tet^+ $TCR\beta^+$ population.

We additionally performed controls to ensure that the population in the culture was a CD1d/αGalCer tetramer specific population. Both control Vα14 TCR Tg thymocytes and cells harvested from a day 22 Vα14 co-culture were stained with either a

CD1d/αGalCer loaded tetramer or a CD1d/unloaded tetramer (Figure 3-2A). The CD1d/αGalCer loaded tetramer, which will bind to the iNKT TCR, should accurately stain the population of cells expressing the specific TCR expressed by iNKT cells (27). In contrast, the CD1d/unloaded tetramer, which will not be able to bind the iNKT TCR, should not detect any tetramer^{+} population. As expected, the cells from the Vα14 TCR Tg thymus stained with the CD1d/αGalCer tetramer had a robust population of tetramer^{+} cells, which was not seen when stained with the CD1d/αGalCer unloaded tetramer. Similarly, the day 22 Vα14 co-culture's cells stained with the CD1d/αGalCer tetramer had a robust population of tetramer^{+} cells, and the cells stained with the CD1d/αGalCer unloaded tetramer did not. Therefore, the CD1d/αGalCer tetramer is accurately staining cells harvested from the OP9-DL1 co-culture system that express the iNKT TCR, confirming that CD1d/αGalCer tet^{+} cells can be derived from Vα14 HPCs using this culture system.

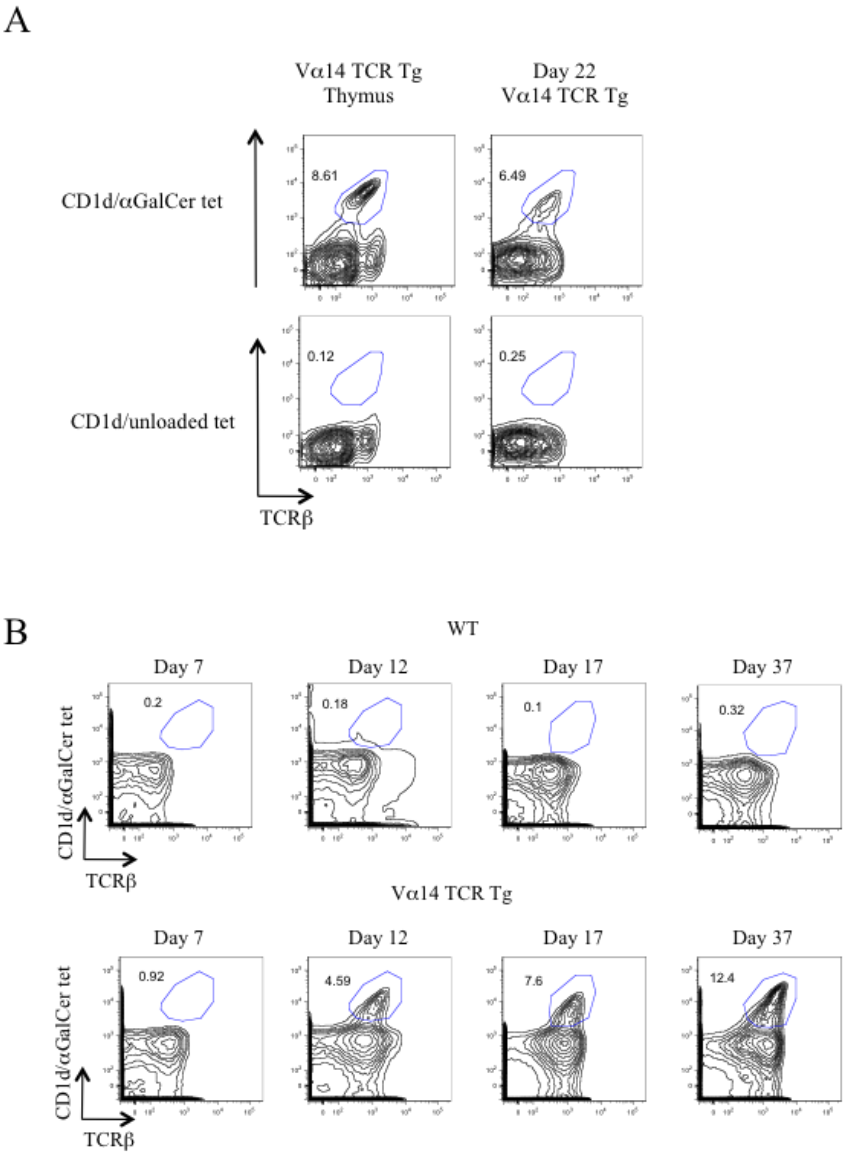

Figure 3-2. A transgenic Vα14-Jα18 TCR was necessary to detect CD1d/αGalCer tetramer binding cells in the OP9-DL1 culture system. Thymocytes from a Vα14 TCR Tg thymus (A, left column) or cells from a day 22 Vα14 co-culture (A, right column) were stained with either CD1d/αGalCer loaded tetramer (A, top row) or CD1d/unloaded tetramer (A, bottom row). WT co-culture cells (B, top row) or Vα14 co-culture cells (B, bottom row) were analyzed by flow cytometry for a CD1d/αGalCer tet$^+$ TCRβ$^+$ population at the indicated time points. Contour plots in (A) are representative of three experiments or four co-cultures. Contour plots in (B) are representative of two to four co-cultures from one (WT) or five (Vα14) independent experiments.

CD1d/αGalCer Tet$^+$ TCRβ$^+$ Cells Express the Transcription Factor PLZF

Although a population of CD1d/αGalCer tet$^+$ TCRβ$^+$ cells are present in the OP9-DL1 culture system by day 12, the ability to bind CD1d/αGalCer tetramer does not alone mean that these cells are iNKT cells. The ability to bind tetramer merely indicates that the cells have the correct TCR that will strongly interact with the tetramer. This is exemplified in Vα14 TCR Tg CD1d$^{-/-}$ mice. Such mice have a detectable population of CD1d/αGalCer tet$^+$ TCRβ$^+$ cells (Figure 3-3A); however, these animals lacked CD1d, which is required for iNKT selection. These CD1d/αGalCer tet$^+$ TCRβ$^+$ cells exhibited a lack of PLZF expression, indicating they were not selected and therefore not iNKT cells (Figure 3-3A) (50, 51). Thus we next examined PLZF expression in the CD1d/αGalCer tet$^+$ TCRβ$^+$ population from co-cultures at day 12, 17, 22, 26, 33, and 37 (Figure 3-3B and data not shown). At day 12, the CD1d/αGalCer tet$^+$ TCRβ$^+$ population did not express PLZF, indicating that these cells had not yet been positively selected by other DP cells to initiate PLZF expression. However, by day 17, there is a small percentage of CD1d/αGalCer tet$^+$ TCRβ$^+$ population that expressed PLZF, which continued to increase at subsequent time points. By day 26, the proportion of PLZF$^+$ CD1d/αGalCer tet$^+$ TCRβ$^+$ cells had become significantly increased compared with day 12 (Figure 3-3C). These results show that PLZF can be induced in the CD1d/αGalCer tet$^+$ TCRβ$^+$ population using the OP9-DL1 culture system, indicating these cells were selected and initiated the iNKT cell developmental program.

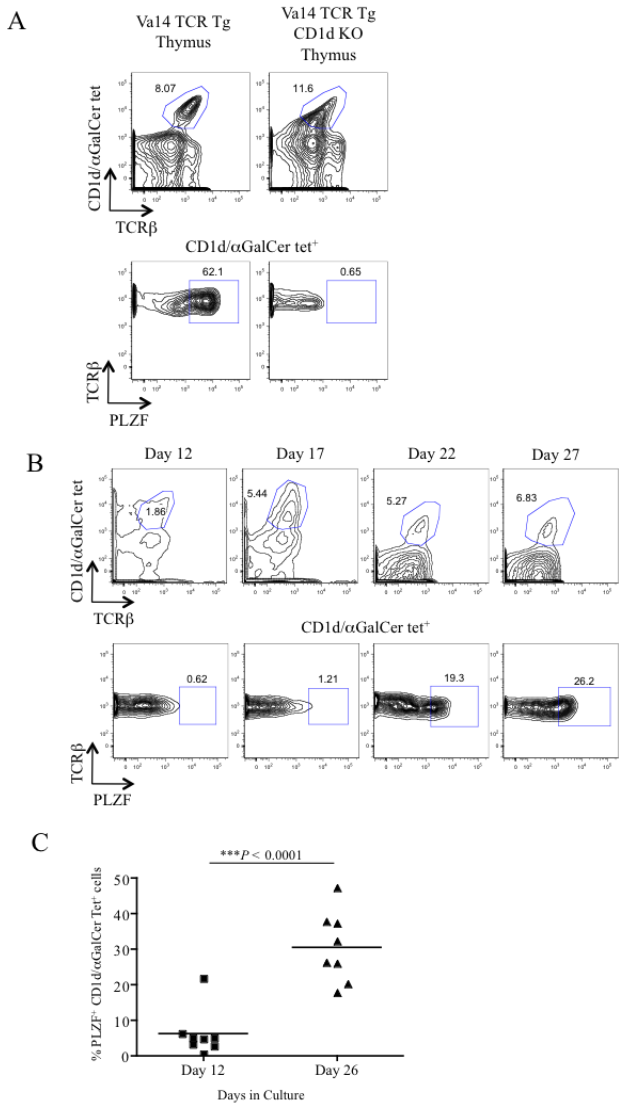

Figure 3-3. PLZF was induced in CD1d/αGalCer tet$^+$ TCRβ$^+$ cells. Thymocytes from a Vα14 TCR Tg thymus or Vα14 TCR Tg CD1d$^{-/-}$ thymus were examined by flow cytometry for CD1d/αGalCer tet$^+$ TCRβ$^+$ cells (A, top). The CD1d/αGalCer tet$^+$ TCRβ$^+$ population were then analyzed for PLZF expression (A, bottom). Cells from Vα14 co-culture were also examined by flow cytometry for CD1d/αGalCer tet$^+$ TCRβ$^+$ cells at the times indicated (B, top). These CD1d/αGalCer tet$^+$ TCRβ$^+$ populations were then analyzed for PLZF expression at the times indicated (B, bottom). PLZF expression of the CD1d/αGalCer tet$^+$ TCRβ$^+$ population was significantly increased in cells analyzed on day 26 versus day 12 of the co-culture. Contour plots are representative of two to four co-cultures from one to three independent experiments. In the graph, each dot indicates one co-culture (day 12 = 8; day 26 = 8). ***$P < 0.001$, percentage of PLZF$^+$ CD1d/αGalCer tet$^+$ TCRβ$^+$ cells from day 12 versus day 26 (unpaired two-tailed *t*-test).

CD1d/αGalCer Tet$^+$ TCRβ$^+$ Cells Express the Transcription Factor Tbet, Which Is Enhanced by the Addition of IL-15

The percentage of PLZF$^+$ CD1d/αGalCer tet$^+$ TCRβ$^+$ cells continued to increase until day 37, where there was an observable drop in the proportion (Figure 3-4A). One possible explanation for this drop is that the CD1d/αGalCer tet$^+$ TCRβ$^+$ cells are differentiating into the NKT1 subset. NKT1 cells are characterized by lower PLZF (PLZF^{lo}) expression accompanied by the expression of the transcription factor Tbet (57). Therefore, we examined Tbet expression in the CD1d/αGalCer tet$^+$ TCRβ$^+$ population. Tbet expression was not increased until after PLZF expression was initiated (Figure 3-4A). Interestingly, the largest increase in the percentage of CD1d/αGalCer tet$^+$ TCRβ$^+$ cells expressing Tbet coincided with the observed late decrease in the percentage of CD1d/αGalCer tet$^+$ TCRβ$^+$ cells expressing PLZF, indicating that the Tbet$^+$ cells were mimicking the PLZF^{lo} Tbet$^+$ transcription factor profile of NKT1 cells.

However, the percentage of Tbet expressing CD1d/αGalCer tet$^+$ TCRβ$^+$ cells was low. Since Tbet expression is important for iNKT cell IFNγ production, we sought to determine if we could increase the percentage of CD1d/αGalCer tet$^+$ TCRβ$^+$ cells expressing Tbet, and thus skew more cells toward the NKT1 lineage. It was previously shown that IL-15 is important for iNKT cell survival and is involved in Tbet regulation in cells expressing the NKT1 cell phenotype (60, 61). Therefore, we added IL-15 to co-cultures at day 7 or day 17. This addition of IL-15 greatly increased the percentage of CD1d/αGalCer tet$^+$ TCRβ$^+$ cells expressing the PLZF^{lo} Tbet$^+$ transcription factor profile

(Figure 3-4B). More specifically, at day 33, co-cultures with IL-15 showed a significant increase in the percentage of CD1d/αGalCer tet^+ $TCR\beta^+$ expressing Tbet as compared to parallel co-cultures without IL-15 (Figure 3-4C). Together, this data showed that some CD1d/αGalCer tet^+ $TCR\beta^+$ cells from the OP9-DL1 culture system expressed Tbet with a similar $PLZF^{lo}$ $Tbet^+$ transcription factor profile as NKT1 cells, and the proportion was enhanced by the addition of IL-15.

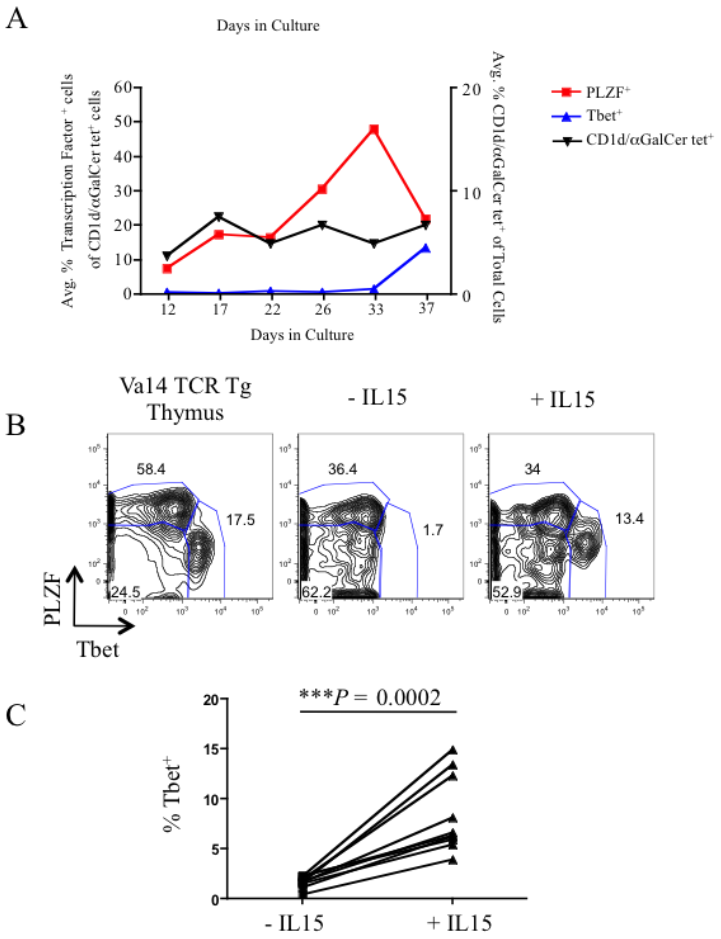

Figure 3-4. IL-15 enhanced the percentage of Tbet$^+$ CD1d/αGalCer tet$^+$ cells in the OP9-DL1 culture system. The average percentage of CD1d/αGalCer tet$^+$ TCRβ$^+$ cells of total cells, PLZF$^+$ cells of CD1d/αGalCer tet$^+$ TCRβ$^+$ cells, or Tbet$^+$ cells of CD1d/αGalCer tet$^+$ TCRβ$^+$ were analyzed from Vα14 co-cultures at the indicated time points (A). CD1d/αGalCer tet$^+$ TCRb$^+$ cells from a Vα14 TCR Tg thymus, day 33 Vα14 co-culture, or day 33 Vα14 co-culture with added IL-15 were analyzed for PLZF and Tbet expression (B). The percentage of CD1d/αGalCer tet$^+$ TCRβ$^+$ Tbet$^+$ cells from day 33 Vα14 co-cultures with added IL-15 was significantly higher than the percentage of Tbet$^+$ CD1d/αGalCer tet$^+$ TCRβ$^+$ cells from day 33 Vα14 cocultures without added IL-15 (C). The graph in (A) shows the average of each positive population of two to four co-cultures from four independent experiments. The contour plots show representative data of eight co-cultures from two independent experiments. For the graph in (C), each dot represents one co-culture from two independent experiments (-IL15 = 8; +IL15 = 8). ***P = 0.0002, percentage of Tbet$^+$ CD1d/αGalCer tet$^+$ TCRβ$^+$ cells without added IL-15 versus percentage of Tbet$^+$ CD1d/αGalCer tet$^+$ TCRβ$^+$ cells with added IL-15 (paired two-tailed *t*-test).

CD1d/αGalCer Tet$^+$ TCRβ$^+$ Cells Produce IFNγ After Stimulation with αGalCer

Since some of the CD1d/αGalCer tet$^+$ TCRβ$^+$ population resembled the PLZF^{lo} Tbet$^+$ transcription factor profile of the NKT1 subset, we next tested if these cells were also functionally similar to NKT1 cells *in vivo* (Figure 3-5A). When stimulated with the strong iNKT cell agonist lipid αGalCer, NKT1 cells rapidly produce IFNγ (57). In order to test this, cells from day 37 of Vα14 OP9-DL1 co-cultures (Figure 3-5B) were injected *i.v.* into congenically different mice. After 12 hours the mice were injected *i.v.* with αGalCer. Three hours later, the spleen and liver were harvested, and the injected cells were recovered based on their congenic marker expression using Magnetic Assisted Cell Sorting (MACS) before analysis by flow cytometry. A population of injected cells was recovered in the both the liver and spleen, indicating that the cells from the OP9-DL1 co-culture were capable of homing to these tissues (Figure 3-5C). A population of these cells was also found in the thymus (data not shown). Only the cells from mice stimulated with αGalCer produced IFNγ (Figure 3-5C). Together, these data indicate that the CD1d/αGalCer tet$^+$ TCRβ$^+$ cells derived from Vα14 HPCs using the OP9-DL1 co-culture system functionally mimic NKT1 cells by producing IFNγ upon stimulation.

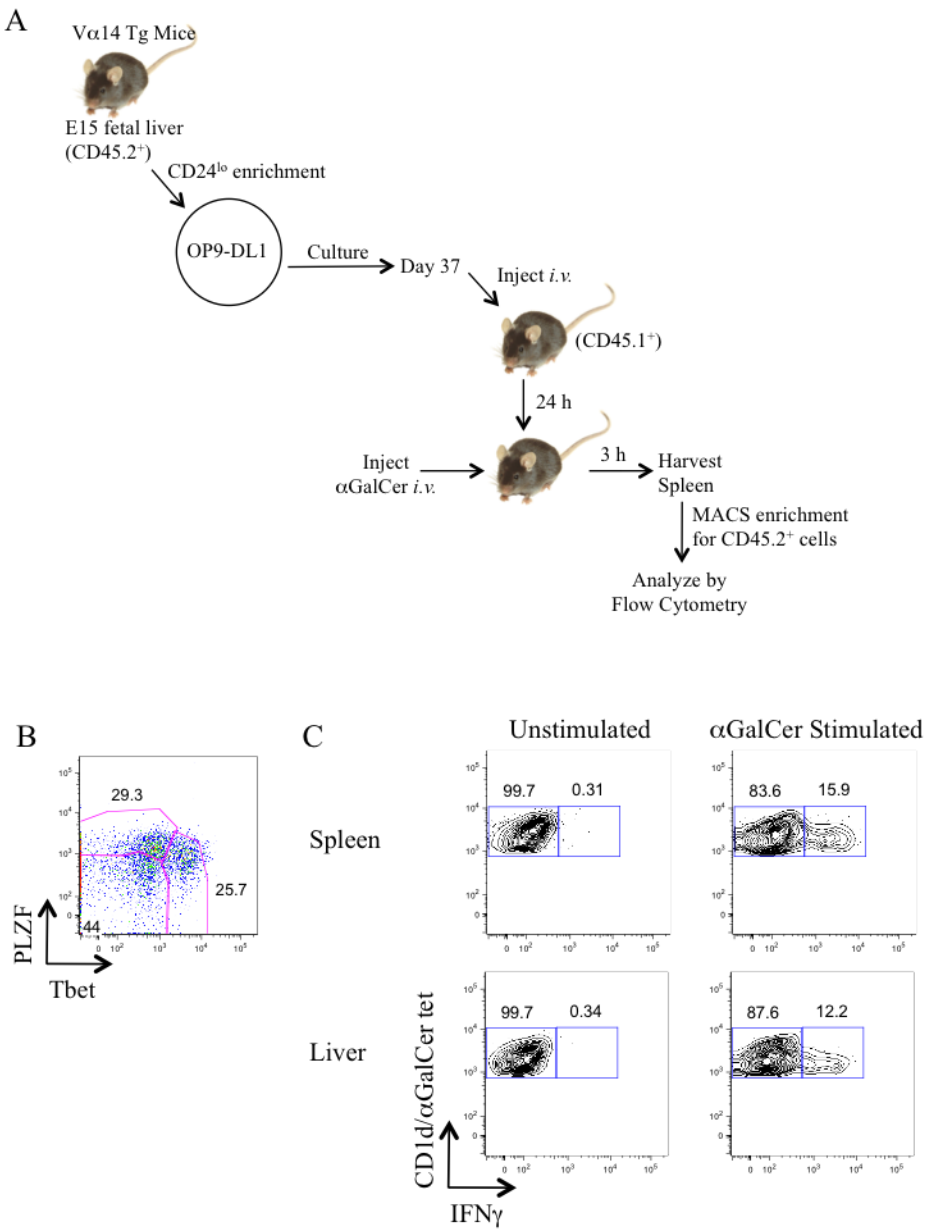

Figure 3-5. CD1d/αGalCer tet$^+$ TCRβ$^+$ cells produced IFNγ upon stimulation with αGalCer *in vivo*. Schematic of Vα14 HPC co-culture with OP9-DL1, transfer to mice, and *in vivo* stimulation with αGalCer (A). The PLZF and Tbet expression of the day 37 cells transferred into mice (B). The percentage of transferred CD1d/αGalCer tet$^+$ TCRβ$^+$ cells expressing IFNγ harvested from the spleen (C, top row) or liver (C, bottom row) from either unstimulated mice (C, left column) or stimulated mice (C, right column). Contour plots are representative of two mice from one experiment.

Discussion

iNKT cells have been shown to influence a variety of diseases, such as cancer, autoimmune disease, and various infectious diseases. This influence is thought to be due to their ability to rapidly produce cyotkines, which can be artificially initiated by the strong agonist, αGalCer (26). For example, in some cancers, it has been shown that IFNγ produced by αGalCer activated iNKT cells aids in the antitumor response by activating other immune cells and by direct tumor cytotoxicity (120, 121). However, clinical trials using αGalCer to boost the antitumor response have not proven particularly effective (122, 123). This may be due to decreased numbers of iNKT cells observed in cancer patients (124, 125). Furthermore, the iNKT cells that remain have decreased cytokine production and proliferation after *ex vivo* stimulation (126). The reason for the decrease in iNKT cell number and function is not known, but could be a result of autoantigen presented by tumors causing activation induced cell death and/or anergy, or suppression of iNKT cells by Tregs (127, 128). Therefore, for any iNKT cell based therapy to be effective, the number of functional iNKT cells needs to be supplemented. One way this can be achieved is by cell therapy with iNKT cells. In this study, we used the OP9-DL1 culture system to derive iNKT cells from HPCs. We showed that HPCs gave rise to CD1d/αGalCer tet^+ $TCR\beta^+$ cells that expressed PLZF, an important transcription factor required for normal iNKT cell development. A portion of these cells also expressed Tbet, an important transcription factor for the development of NKT1 cells (57), and similarly to NKT1 cells, these cells produced IFNγ after stimulation with αGalCer.

Interestingly, we found that the days when PLZF or Tbet were expressed varied between independent co-cultures. In some co-cultures, PLZF expression was initiated by day 17, whereas in other co-cultures, this expression did not begin until day 22 or day 26. This same variability was seen with Tbet expression, in which there was one co-culture where Tbet expression was initiated by day 26 whereas in other co-cultures, the same level of Tbet expression was not seen until day 33 or day 37. In contrast, in parallel co-cultures within the same experiment, PLZF and Tbet expression initiation occurred on the same days. We hypothesize that this variability between independent cultures was due to differences in the plated cell density, as the plated cell density varied between independent experiments but not within experiments, indicating that interactions between cells is important not only for the initial selection of iNKT cells, but may also be important for later transcription factor initiation.

Importantly, we found that it was necessary to use HPCs with a fixed Vα14-Jα18 TCR to detect the CD1d/αGalCer tet^+ $TCR\beta^+$ population. We hypothesize that this is due to the low frequency of WT DP cells expressing the semi-invariant iNKT TCR. However, this is in opposition to a previous report which showed that HPCs cultured with OP9-DL1 gave rise to a population of iNKT cells, albeit very transiently (47). This iNKT cell population was only seen on day 17 of co-culture, and was not observed before or after this time point. Although it is difficult to reconcile this result with our data, as we saw no CD1d/αGalCer tet^+ $TCR\beta^+$ population in WT cultures at any time point examined (day 7, 12, 17, 22, 26, 33, and 37; Figure 3-2 and data not shown), it is possible

that we missed the small window in which this iNKT cell population appeared. However, others have tried to recapitulate this data, but, to our knowledge, no one has done so successfully (T. Malleveay, personal communication).

Our data showed that NKT1-like cells can be derived from these co-cultures, indicating this system can potentially be used to derive iNKT cells for therapeutic uses where IFNγ is important. However, as mentioned above, we found that it was necessary to use HPCs from Vα14 TCR Tg mice. The need for HPCs that have increased expression of the invariant iNKT TCR is a potential barrier to using this co-culture strategy for therapeutic applications in humans, as the frequency of the invariant iNKT cell TCR expression in humans is similarly as low as in wild type mice. One possible solution to this would be to retrovirally transduce HPCs to express the iNKT TCR. Two recent reports provide two alternative strategies that also addressed this problem. In one study, nuclear transfer from mature iNKT cells (with a Vα14-Jα18 rearranged TCR) were used to create embryonic stem cell (ESC) lines, which were then cultured with OP9-DL1 cells (139). DNA extracted from these ESC lines were positive for the iNKT TCR, and therefore when cultured with OP9-DL1 cells, successfully gave rise to iNKT cells. As there are potential ethical issues surrounding the use of embryos to create these iNKT-ESC lines, the same group showed splenic iNKT cells could be reprogrammed into induced pluripotent stem cells (iPSCs) (138). These iPSCs were then cultured with OP9-DL1 cells, and differentiated into iNKT cells. *In vitro* stimulation with αGalCer showed these derived iNKT cells could produce IFNγ; however, no *in vivo* tests were performed.

Although these two previous reports showed successful derivation of iNKT cells from ESCs or iPSCs, unlike our work, they did not examine transcription factors to identify which subsets were being produced. This distinction is important, as PLZF is required for iNKT cell differentiation, and other transcription factors (Tbet, RORγt, and GATA3) distinguish the iNKT subsets, which dictates the cytokine produced upon stimulation and affects the subsequent immune response (57). Interestingly, Watarai et al. observed that stimulated iPSC-derived iNKT cells from an earlier time point (day 20) were not functional (as compared to day 25) (138). They reasoned that these stimulated iNKT cells were not able to produce cytokine because they were too immature, but provided no further explanation. We hypothesize that this observation was due to lack of PLZF and/or Tbet expression in these cells. Tbet controls expression of IFNγ (166), and thus iNKT cells that express Tbet produce IFNγ (57). Additionally, we observed a kinetic pattern of CD1d/αGalCer tet^+ $TCR\beta^+$ cells, PLZF expression, and then Tbet expression, sequentially. This pattern of expression comports with the lack of IFNγ production seen by the immature iNKT cells in contrast to the later iNKT cells that were able to produce IFNγ. Also, in our *in vivo* functional experiment, not all the injected Vα14 day 37 cells produced IFNγ upon stimulation. Since not all the injected cells expressed Tbet, we hypothesize that the IFNγ producing cells were only the cells that expressed Tbet. This indicates that Tbet expression, and more generally identification by transcription factor expression, is important before the therapeutic use of these derived iNKT cells.

In conclusion, we have demonstrated that HPCs co-cultured with OP9-DL1 cells gave rise to NKT1-like cells. These cells expressed both PLZF and Tbet, and the percentage of cells expressing Tbet was enhanced by the addition of IL-15. Notably, the frequency of iNKT TCR expressing cells needed to be increased above WT levels to detect a CD1d/αGalCer tet^+ $TCR\beta^+$ population. Together, these data indicate that this culture system can be used as a new tool to address many difficult questions about iNKT cell development, such as what factors and/or cytokines are important in the differentiation of iNKT cell subsets. This work also advances previous work toward the achievement of iNKT cell-based therapies that harness the different cytokine-producing abilities of each iNKT cell subset for personalized disease treatment.

Methods and Materials

Mice

B6 (C57BL/6NCr) and B6.SJL (B6-LY.5/Cr) mice were obtained from the National Cancer Institute. CD1d$^{-/-}$ B6 (B6.129S6-Cd1d1/Cd1d2tm1Spb/J) mice and Vα14 TCR Tg B6 (C57BL/6-Tg(Cd4-TcraDN32D3)1Alben/J) mice were obtained from The Jackson Laboratory. Vα14 TCR Tg CD1d$^{-/-}$ B6 mice were generated by the crossing of CD1d$^{-/-}$ B6 and Vα14 TCR Tg B6 mice. Timed-pregnant B6 or Vα14 TCR Tg B6 were used as the source of day 15 embryos for harvesting fetal liver. All animal experimentation was approved by and performed according to the guidelines from the Institutional Animal Care and Use Committees at the University of Minnesota.

Cell Lines

The OP9-GFP and O9-DL1 cell lines were obtained from D. Kaufman (University of Minnesota). OP9-GFP cells and OP9-DL1 cells were cultured as monolayers in OP9 media (αMEM supplemented with 20% FCS, 100 IU/ml penicillin, 100 ug/ml streptomycin, 20 ug/ml gentamicin, and 2.2g/liter of sodium bicarbonate).

OP9 and OP9-DL1 Co-cultures

Hematopoietic progenitor cell co-culture with OP9-GFP cells or OP9-DL1 cells was setup following a previously described protocol (167). Briefly, day 15 embryos were obtained from timed-pregnant females, and fetal livers were extracted. PCR was used to

identify Vα14 TCR Tg genotype of each embryo before pooling fetal livers. Hematopoietic progenitor cells (HPCs) were then isolated by enrichment for $CD24^{lo}/Lin^{-}$ cells by anti-CD24 antibody/complement-mediated depletion (Carlyle 1998). HPCs were then seeded at 6×10^5 cells/plate into 10 cm tissue culture dishes containing a confluent monolayer of OP9-GFP cells or OP9-DL1 cells. All co-cultures were performed in the presence of 1 ng/ml IL-7 (PeproTech) and 5 ng/ml Flt3L (R&D Systems).

At days 7, 12, 17, 22, 26, 33 and 37, non-adherent cells were collected by vigorous pipetting, and filtered through a 70 μm filter. The adherent cells were treated with 0.25% trypsin (Gibco) for 5 min before adding OP9 media and transferring the resulting single-cell suspension to a new 10 cm dish for 45 min. The non-adherent cells were filtered through a 70 μm filter and added to the previously collected non-adherent cells. These cells were then split either 1:2 or 1:4 and replated to a 10 cm dish containing a fresh monolayer of OP9-GFP cells or OP9-DL1 cells and fresh OP9 media with new IL-7 (1 ng/ml, Peprotech) and Flt3L (5 ng/ml, R&D Systems). Alternatively, some cultures also received 10 ng/ml IL-15 (eBioscience) starting at day 7 or day 17.

PCR

Genomic DNA was purified from day 15 embryos. DNA samples were amplified using the following primers to test for Vα14-Jα18 TCR transgene: forward, 5'-TGTAGGCTCAGATTCCAACC-3' and reverse, 5'-CTCCAAAATGCAGCCTCCCTAAG-3'. Products were separated by agarose gel electrophoresis and visualized by ethidium

bromide staining. Only embryos genotyped as positive for Vα14-Jα18 TCR were used as the source of Vα14 HPCs.

Flow Cytometry, Antibodies, Intracellular Cytokine Staining, and Transcription Factor Staining

For co-cultures, single-cell suspensions were prepared from the pooled non-adherent cells as described above. For *in vivo* experiments, single-cell suspensions were prepared from spleens, and hepatic mononuclear cells were separated by Percoll gradient centrifugation. All cells were resuspended in staining buffer (PBS with 5% FCS). Antibodies to surface markers were added, and cells were stained on ice for 20 min. Antibodies used were from eBioscience, BD, or BioLegend. For iNKT cell staining, CD1d monomers loaded with biotinylated PBS57 or unloaded monomers were obtained from the tetramer facility of the US National Institutes of Health. Cells were analyzed on an LSRII (Becton Dickinson) and data was processed with FlowJo software (TreeStar).

For intracellular detection of transcription factors, cells were stained for surface markers as described above, and fixed with a Foxp3 staining buffer set (eBioscience), and incubated with anti-PLZF (BD Pharmigen) and anti-Tbet (eBioscience) for 30 min on ice. Cells were washed once before analysis on an LSRII.

For intracellular detection of cytokines, cells were stained for surface markers as described above, and then fixed and permeabilized using the Cytofix/CytoPerm buffer (BD Biosciences) according to the manufacturer's protocol. Anti-IFNγ (eBioscience) was

added and the cells were stained on ice for 30 min. Cells were washed twice and then resuspended in staining buffer before analysis on an LSRII.

In Vivo αGalCer Stimulation

For *in vivo* αgalactosylceramide (αGalCer) stimulation of co-culture-derived cells, a single-cell suspension of non-adherent cells from the OP9-DL1 co-culture was prepared as described above. These cells were washed twice with PBS before *i.v.* injection into congenically different B6.SJL ($CD45.1^+$) mice. The injected cells were allowed to park for 24 h before *i.v.* injection of 2 μg of αGalCer (KRN7000, Avanti Polar Lipids) diluted in 200 μl PBS. Three hours later liver, spleen, and thymus were harvested.

Co-culture-derived Cell Enrichment

Single-cell suspensions were prepared from spleens and thymi, and hepatic mononuclear cells were separated by Percoll gradient centrifugation. The single-cell suspensions were enriched for co-culture-derived cells using anti-CD45.2 conjugated to magnetic beads, and MACS columns (Miltenyi Biotec), according to manufacturer's protocol.

Statistical Analysis

Prism software (GraphPad) was used for statistical analysis. Unpaired or paired two-tailed *t*-tests were used for data analysis.

Chapter 4: Discussion

Conclusions

The work presented in the second chapter of this thesis showed the Nur77^{gfp} transgenic (Tg) mouse is a useful tool to investigate iNKT TCR stimulation during infection. GFP sensitively reported stimulation by both weak and strong lipid agonists, as well as by both foreign- and self-lipid agonist; however, GFP upregulation does not distinguish TCR stimulation achieved by foreign-lipid versus TCR stimulation achieved by self-lipid. Importantly, GFP was only upregulated in iNKT cells by TCR stimulation and not by inflammatory cytokines.

Although the presented data indicated the indirect model of iNKT cell activation was not occurring during *Salmonella typhimurium* infection or after TLR agonist injection, this activation model could be occurring in other infectious contexts; for example, during parasitic infections, which were not examined in this thesis. Two potentially interesting candidates for study are *Trypanosoma cruzi* (*T. cruzi*) and *Schistosoma mansoni* (*S. mansoni*). Infections with these parasites showed iNKT cell activation was dependent on CD1d (163, 168). Interestingly, iNKT cell activation during *T. cruzi* infection, although also dependent on IL-12, was independent of MyD88, an adaptor protein essential for the TLR activation of NFκB, and therefore iNKT cell activation was independent of TLR stimulation (168). During *S. mansoni* infection, iNKT cell activation in MyD88-deficient or IL-12 deficient mice was similar to WT, indicating that iNKT cell activation was independent of both TLR stimulation and IL-12 (163). Therefore, iNKT cell activation during parasitic infections may be distinct from

the iNKT cell activation examined during bacterial or viral infections in this thesis, and would be interesting to further explore using the Nur77^{gfp} Tg reporter.

Another interesting area that was not explored in this thesis was iNKT cell activation during noninfectious diseases. For example, in both allergen-induced and ozone-induced airway hyperreactivity, it is unknown if iNKT cell activation occurs as a result of TCR stimulation by endogenous lipid expressed as a result of the inflammatory environment, or if the inflammatory environment itself stimulates iNKT cells independently of antigen (95). In cancer, in one study, iNKT cells from cancer patients exhibited decreased proliferation and cytokine production upon stimulation *ex vivo* (126). This anergic-like phenotype indicated that these cells were strongly stimulated (128). However, how iNKT cells are stimulated in cancers is not known. Similarly to the models of iNKT cell activation during infection and asthma, it is hypothesized that in cancer, iNKT cells are activated by a stimulatory lipid from tumors (169) and/or are activated as a result of tumor-mediated inflammation (116). Therefore, Nur77^{gfp} Tg iNKT cells could similarly help to elucidate these important questions about iNKT cell activation during noninfectious diseases.

One question requiring further investigation is to what extent TCR stimulation is required for iNKT cell activation during infections where iNKT TCR stimulation was observed. The presented data showed iNKT cell TCR stimulation is occurring during *Streptococcus pneumoniae* infection and *Sphingomonas paucimobilis* infection; however, the data does not indicate if this TCR stimulation is required for iNKT cell activation.

Instead, it is possible that these infections also induce cytokines by innate cells, such as IL-12 by dendritic cells, which are sufficient for robust iNKT cell activation in the absence of iNKT TCR stimulation (90). However, this question is difficult examine since the iNKT TCR is required for selection, but could potentially be studied using a recently developed mouse model that induces TCR ablation in mature iNKT cells (156).

Another question that remains unanswered is why the presented *in vivo* infection data from CD1d-deficient mice showed CD1d-independent iNKT activation after *Salmonella typhimurium* infection or TLR agonist injection, whereas the *in vitro* data using CD1d-deficient APCs indicated a CD1d-requirement for this activation (90). One possible explanation is that the stronger stimulation of the innate immune system achieved *in vivo* may somehow inhibit TCR dependent activation of iNKT cells. Therefore, TCR dependent activation is only observed in the isolated culture conditions that occur *in vitro*. Thus, it would be very interesting to test if iNKT cells from a Nur77^{gfp} Tg mouse will upregulate GFP after *S. typhimurium* infection or after stimulation with TLR agonists *in vitro*. However, unstimulated iNKT cells unexpectedly exhibited elevated GFP levels in culture (data not shown), which is a problem that must be addressed before this question can be examined.

Interestingly, the activation of iNKT cells during infections seems to be largely independent of TCR stimulation. Additionally, even in contexts where stimulatory lipid is present, cytokine activation seems to be the dominate activation mechanism. Therefore, why do iNKT cells have a TCR? In my opinion, the main role of the iNKT TCR is for

iNKT cell selection and the acquisition of the iNKT activated/memory phenotype, which allows iNKT cells to very rapidly secrete cytokine upon activation.

However, recent reports indicate that the iNKT TCR may additionally be important for determining iNKT cell homeostasis. In one study, the smaller iNKT cell population found in specific pathogen free (SPF) mice conferred protection against experimental iNKT cell-mediated oxazolone-induced colitis as compared to germ free mice (170). This difference in iNKT cell populations between the mice indicated that bacterial sphingolipids may be playing a role in the establishment of iNKT cell populations (170). The same group did a follow up study in which they identified *Bacteroides fragilis* as one of the bacteria responsible for this effect (162). More specifically, they showed it produced a sphingolipid that affected the expansion of the iNKT cell population during development in germ free mice (162). Interestingly, the function of this lipid is inhibitory instead of stimulatory. However, the mechanism of inhibition is unclear; therefore, it would be interesting to look at this phenomenon in $Nur77^{gfp}$ Tg mice to investigate if stimulation of the iNKT TCR is involved similarly to activating lipids.

The work in the third chapter of this thesis presented a new culture system using Vα14 Tg hematopoietic progenitor cells to derive iNKT cells *in vitro*. Importantly, this new culture system induced a robust and stable population of CD1d/αGalCer tet^+ cells, which will allow for further examination of factors that influence iNKT cell development. Additionally, this culture system can be used to investigate what factors

are needed to skew iNKT cell subset development to derive the subset most beneficial for individual disease treatment.

For example, IFNγ production has been shown to essential for the antitumor response (61). Therefore, NKT1 cells, which produce IFNγ, would be the subset most beneficial for therapeutic use (57). In this work, I tested whether IL-15 could skew the cultured cells to develop into the NKT1 subset. Interestingly, the addition of IL-15 resulted in an increase in the percentage of CD1d/αGalCer tet^+ cells expressing Tbet. However, it is unclear whether this increase was due to increased cell survival, or if IL-15 caused more cells to express Tbet. However, because Tbet promotes the upregulation of CD122 (the IL-15 receptor β chain) (61), it seems more likely that the addition of IL-15 is having a survival effect on these cultured cells. Therefore, the factors involved in the initiating Tbet expression and NKT1 subset differentiation remain to be identified. Yet another group of lymphocytes, which like iNKT cells, have characteristics similar to innate immune cells, also have subsets similar to the iNKT cell subsets (171). Therefore, factors involved in the differentiation of these cells, which are called innate lymphoid cells (ILCs), are also potential candidates for involvement in iNKT cell subset differentiation. The ILC subset most similar to NKT1 cells are cytotoxic ILCs. Therefore, based upon cytoxic ILC development, factors that could skew iNKT cell development toward NKT1 cells are IL-12 and/or IL-18 (171). These cytokines could also be tested in combination with IL-15 (171).

In contrast, the therapeutic use of NKT1 cells for treatment of type I diabetes would be harmful. Previous studies about the role of iNKT cells in type I diabetes indicate that aid provided by iNKT cells stems from their ability to shift the TH1 cytokine bias toward a TH2 cytokine bias (94, 114). Therefore, NKT2 cells would be the most beneficial subset for type I diabetes treatment. However, the factors that initiate the development of NKT2 also remain to be identified. The ILC that most resembles the NKT2 are Type 2 ILCs (ILC2). Based upon ILC2 development factors, IL-25 and/or IL-33 are potential candidates for skewing iNKT cell development toward NKT2 cells (171).

Although a therapeutic use for the NKT17 subset is unclear, determining the factors involved in the development of this subset is still important for the general understanding how this subset can influence an immune response. For example, IL-17 produced by NKT17 cells is required for the development of some asthma models (106). Therefore, insight into the development of this subset may provide insight into the pathogenesis of IL-17 dependent asthma. Again, the factors that are involved in NKT17 development remain to be identified. However, the ILC most similar to NKT17 cells are Rorγt^{+} ILCs. The factors that influence the development of these ILCs are IL-1β and IL-23 (171). Therefore either or both of these cytokines are potential candidates for skewing iNKT cell development toward the NKT17 subset.

In conclusion, the work done in this thesis identified the $Nur77^{gfp}$ Tg mouse as a reporter specific for TCR stimulation in iNKT cells. Here, antigen-independent activation was identified as the main mechanism for iNKT cell activation during

Salmonella typhimurium infection, and injection of TLR agonists implicated that TLR-stimulating infections are also activating iNKT cells in an antigen-independent manner. Furthermore, this work indicated that the Nur77^{gfp} Tg mouse can be used to explore the role of the antigen stimulation in additional infections, as well as in disease models where the role of antigen stimulation in iNKT cell activation is unclear. This additional insight into when the different models of iNKT cells activation occur may provide new ways to control iNKT cells during a variety of diseases. This work also identified a new culture system for the *in vitro* derivation of an exogenous source of iNKT cells. It provided a new platform for examining the conditions that influence iNKT cell development and for determining the specific factors that can skew iNKT subset development. The ability to derive a specific iNKT cell subset is essential for iNKT cell therapies, as different iNKT cells subset will have different effects on the same disease. Therefore, this work has identified new tools for further understanding of how iNKT cells develop and are activated, which is crucial for harnessing the immunomodulatory potential of iNKT cells for use in iNKT cell based therapeutics.

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