Regulation Of Natural Killer T Cell Subset Development And Function By Slam Family Receptors

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REGULATION OF NATURAL KILLER T CELL SUBSET DEVELOPMENT AND FUNCTION BY SLAM FAMILY RECEPTORS

A Dissertation Presented

by

Victoria Lynn DeVault

to

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy Specializing in Cellular, Molecular, and Biomedical Sciences

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ABSTRACT

Semi-invariant natural killer T (iNKT) cells are critical components of the host immune response in peripheral tissues such as the lung, liver, and gut, and they play important roles in cancer, bacterial infections, autoimmunity, wound repair, and atherosclerosis. Tissue-resident iNKT cells exert their effects early in the developing immune response by rapidly producing a wide variety of cytokines and chemokines, and it was recently discovered that different tissues possess iNKT cell subsets that preferentially produce IFN-γ (NKT1), IL-4 (NKT2), or IL-17 (NKT17). Despite their critical role in the immune response, the mechanisms that regulate iNKT cell function in the periphery remain unclear. Signaling lymphocyte activation marker (SLAM) proteins are cell surface-expressed molecular switches that are expressed on all hematopoietic cells. The nine SLAM family receptors serve a variety of functions including promotion of cell-cell adhesion, regulation of cytokine production, co-stimulation, and inhibition. Importantly, SLAM family receptors are critical for the development of iNKT cells. Yet, numerous efforts to ascribe discrete roles of SLAM family receptors in iNKT cell function has proven difficult.

We conducted a comprehensive analysis of SLAM family receptor co-expression on iNKT cell subsets in the lung, spleen, liver, and thymus and identified co-expression profiles that varied in a tissue and strain-dependent manner. Interestingly, we found that SLAM family receptor expression profiles varied among different iNKT cell subsets. In particular, we noted a close association of SLAMf6 expression with the NKT2 and NKT17 subsets in both the periphery and in the thymus. Further investigation using SLAMf6-deficient mice revealed a critical role for SLAMf6 in NKT2 and NKT17 subset development, and in iNKT IL-4 and IL-17 cytokine production in the periphery. This investigation also revealed that the SLAMf6<sup>high</sup> NKT2 and NKT17 subsets exhibited significantly higher proliferative capacity than the NKT1 subset and the NKT2 and NKT17 proliferation was dependent, in part, on SLAMf6 expression.

Since Slam family genes are highly polymorphic, we next investigated whether these polymorphisms regulated iNKT function. We employed a B6.129 congenic mouse exhibiting impaired NK cell function, in which a 6.6 Mbp 129/SvJ locus encompassing Slam genes was introgressed onto the C57BL/6 background. To test the hypothesis that Slam gene polymorphisms regulate iNKT cell function, we refined this genetic interval by generating B6.129 subcongenic lines and assessing iNKT cell function. Unexpectedly, we found that while Slam gene polymorphisms in this model do regulate iNKT cell function, the dominant regulator was in a 0.14 Mbp interval centromeric to the Slam genes. Further experimentation revealed that impaired iNKT cell development and function was associated with changes in the expression of Fcgr3 (Fc gamma receptor III) on iNKT cells, suggesting it as a novel candidate gene regulating iNKT cell function.

Taken together, these data reveal for the first time a specific role for SLAMf6 on NKT2 and NKT17 subset development and function. In addition, these data identify Fcgr3 as a novel candidate gene that regulates iNKT cell subset development and cytokine production. Cumulatively, these data reveal the presence of discrete regulatory mechanisms at work in different iNKT subsets, a finding that has broad implications for our understanding of iNKT-cell mediated immunity.
CITATIONS

Material from this dissertation has been published in the following form:

DEDICATION

To Grandpa Arnold M. Bartz
Science is in our genes.

“A ship in the harbor is safe, but that is not what ships are built for.”
- John A. Shedd
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The pursuit of scientific discovery takes teamwork, collaboration, and the three P’s: patience, persistence, and perseverance. I have been incredibly lucky to have worked with amazing scientists who trained me in ways far greater than I ever expected. First and foremost, a thank-you to my mentor and advisor, Dr. Jon Boyson. Thank-you for the three hour scientific discussions, 3pm coffee breaks, every open door, or time when I just needed a little kick. Thank-you for the thinking stick, the clicking pen, every open or closed circle, color change, and edit. You have molded me into a Scientist, and I am truly amazed at how your passion for science challenges and motivates me. They say that PhD students are often trained in the likeness of their advisors, and I would be honored to be compared as such. To the Boyson lab, without your support I would not be where I am. Oliver, you have shown me every tip and trick to be the most efficient as possible, and I am indebted to you saving me from many late nights. Shawn and Somen, you make every day fun, and you now have free reign on the office, use it wisely. To Linda and Murisa whose work went into this dissertation, thank-you for allowing me to teach you. To my committee, Jason, Matt, and Alan, thank-you for the advice, challenges, and support. Challenge accepted.

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CHAPTER 1: COMPREHENSIVE LITERATURE REVIEW

1.1 Introduction to Innate-Like Cells

In introductory Immunology classes, we are taught that there are two arms of the immune system: the quick responding innate immune system that is triggered within minutes to hours after infection followed by the more specific adaptive immune system (Murphy et al., 2012). There are common cells that fit into each of these arms with dendritic cells, macrophages, neutrophils, and natural killer cells on the innate side, and T and B cells comprising adaptive immunity (Murphy et al., 2012) (Figure 1-1). Recently, the definitions of innate and adaptive have become less static, with the discovery of new subsets of cells comprised of innate lymphoid cells (ILCs) and innate like T cells that bridge the gap between these two classic arms (Gao and Williams, 2015; Mjosberg and Spits, 2012). ILCs and innate like T cells are unique in that they can orchestrate a symphony of immune cell activation allowing the host to combat many different types of infection, be it bacterial, viral, or parasitic.
Innate-lymphoid cells are comprised of three different types: ILC1, ILC2, and ILC3 (Cella et al., 2009; Kiessling et al., 1976; Luci et al., 2009). These subsets are similar to those described for T helper subsets, where ILC1 cells make type 1 cytokines including the pro-inflammatory IFNγ (Fuchs et al., 2013). ILC2 have a type 2 phenotype producing IL-4 and IL-13 (Monticelli et al., 2011; Neill et al., 2010), and ILC3 cells produce IL-17 (Luci et al., 2009). While these cytokines in T helper subsets have been
associated with fighting intracellular bacteria, helminth, and extracellular and fungal infections respectively (Murphy et al., 2012). ILCs are different in that these early waves of cytokine production activate antigen presenting cells, leading to activation of the adaptive immune system (Eberl et al., 2015). ILC’s however, are fully innate cells, and do not have adaptive qualities in that they fail to recognize specific antigens (Colonna, 2018). Innate-like T cells, however, function to bridge that gap.

Innate lymphoid cells and innate-like T cells reside in peripheral tissues where they participate in site specific immune responses (Dusseaux et al., 2011; Gasteiger et al., 2015; Hayday and Tigelaar, 2003; Lee et al., 2015). Innate-like T cells are comprised of natural killer T cells (NKT), gamma-delta T cells (γδT), and the newly described mucosal associated invariant T (MAIT) cells. These cells patrol mucosal barriers such as the gut and lung, providing surveillance and direction for other cell types. In most cases, these innate-like T cells are the first cells to recognize an infection at mucosal sites (Chien et al., 2014; Kurioka et al., 2016; Van Kaer and Joyce, 2005). Gamma-delta T cells were first described in 1986 (Bank et al., 1986; Brenner et al., 1987; Saito et al., 1984), followed by NKT cells in 1987 (Budd et al., 1987; Fowlkes et al., 1987), and MAIT cells in 2003 (Treiner et al., 2003). While the first innate-like T cells have been recognized for over 30yrs, little is known about their precise roles in different types of infection and host immunity. Even less is known about what regulates these cells, and how these determinants modify the cellular responses of these cells. For example, the ligands for gamma-delta T cells are yet to be discovered and we still do not understand how NKT cells are protective in certain bacterial infections, but pathogenic in autoimmune
disorders (Brutkiewicz, 2006). The field has only just discovered that MAIT cells are highly abundant in the human as compared to the mouse model organism (Garner et al., 2018).

One of the most unique innate-like T cells are invariant natural killer T (NKT) cells. First described as having low levels of CD3 and a unique conservation of Vβ7 and Vβ8 chains in the T cell receptor (TCR) (Budd et al., 1987), researchers could not agree on what these unique cells were for 8 years until the NK1.1 T cell, and later termed natural killer T cell, in 1995 was defined (Makino et al., 1995). It was shown that these cells were indeed a separate cell from the previously known innate natural killer cells and adaptive CD4+/CD8+ conventional T cells. Indeed, NKT cells have markers and killing capabilities like that of natural killer cells (Yankelevich et al., 1989), but also have the adaptive quality of MHC-class 1 like restriction (Adachi et al., 1995). Understanding some of the factors that regulate iNKT cell biology was a major goal of this dissertation.

1.2 Introduction to Natural Killer T cells

Natural killer T cells are restricted to the non-classical MHC molecule CD1d and exhibit distinct properties of adaptive T cells and fast acting innate natural killer cells (Adachi et al., 1995; Makino et al., 1995). There are two types of NKT cells, type 1 which use a semi-invariant TCR α chain, and type 2 NKT cells which are less abundant in number in the mouse and use a more varied TCR α chain (Budd et al., 1987; Rhost et al., 2012). Type 1 NKT cells, also known as invariant natural killer T cells (iNKT), use the Vα14/Jα18, Vβ2/Vβ8/Vβ7 semi-invariant TCR (Koseki et al., 1989; Koseki et al.,

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Throughout this thesis, I will only discuss properties of iNKT cells.

iNKT cells are considered the first line of defense upon responding to infection in the peripheral organs such as the spleen, liver, lung, and gut (Crosby and Kronenberg, 2018; Van Kaer and Joyce, 2005). The percentages of iNKT cells circulating in the blood in a mouse is 0.1%, while the spleen, liver, lung, and gut can yield between 0.5-2%, 10-30%, 3-5%, and 10-15% respectively as a percentage of CD45+ cells (Lee et al., 2015; Rymarchyk et al., 2008). In the human, iNKT cells in circulation are variable, in some reports measuring 0.1% to 1% of the leukocytes (Bienemann et al., 2011). iNKT cells become effectors within minutes to hours after activation as evidenced by identification of cytokines such as IFN\(\gamma\), IL-4, and TNF\(\alpha\), in the serum 60min after in vivo challenge (Bendelac and Schwartz, 1991; Christmas et al., 1987; Nishimura et al., 1997). Due to this rapid response, NKT cells may play the role of “generals of the immune system” directing and recruiting other immune cells to carry out their respective roles in combating infection (Carnaud et al., 1999; Hermans et al., 2003; Kitamura et al., 1999). The rapid production of cytokines is in part due to the fact that iNKT cells have pre-formed mRNA transcripts for IFN\(\gamma\), IL-4, and IL-23R and ROR\(\gamma\)t, the latter two of which mediate IL-17 production (Rachitskaya et al., 2008; Stetson et al., 2003). The IFN\(\gamma\) produced by iNKT cells indirectly activates NK cells (Smyth et al., 2002), which aid in killing cells that the body recognizes as foreign (non-self). NKT cells also activate dendritic cells through CD40L binding to CD40 on the APC (Fujii et al., 2004). Activated DCs are then responsible for MHC presentation and activation of conventional
adaptive CD4 and CD8 T cells (Kitamura et al., 1999). NKT cells also help B cells to produce antibodies via the production of IL-4 and increased contact time for the formation of germinal centers (Kitamura et al., 2000) (Chang et al., 2011). Additionally, NKT cell production of IL-17 is important for the activation and recruitment of neutrophils (Li et al., 2007). Thus, cytokine production by NKT cells are important for the activation of many other cells of the immune system.

In addition to cytokine production, NKT cells are prolific cytotoxic cells (Nicol et al., 2000). NKT cells make copious amounts of granzyme b and perforin (Nguyen et al., 2008), and can kill any cells that present a glycolipid on CD1d to the iNKT cell. This is especially important in cancer models where iNKT cells have been shown to be important for preventing tumor growth. In a cytotoxicity killing assay, ligation of NKG2D resulted in increased killing from iNKT cells by degranulation of perforin (Kuylenstierna et al., 2011). Activation of iNKT cells within the tumor microenvironment can also trigger NK cells to produce IFNγ, leading to increased NK cell cytotoxicity and reduced tumor burden (Hayakawa et al., 2002). Thus, iNKT cells serve many roles in the immune system, be it cytokine production to recruit other cells, or direct or indirect cytotoxicity.

Importantly, NKT cells develop by fundamentally different mechanisms than CD4 and CD8 conventional T cells (Bendelac, 1995; Capone et al., 2001). Like conventional T cells, iNKT cells develop and undergo both positive and negative selection in the thymus. Selection of iNKT cells occurs via other double positive thymocytes that express CD1d, not the thymic epithelium as with conventional T cells
(Egawa et al., 2005). Early iNKT cell precursors mature into double positive (CD4^{pos}CD8^{pos}) cells, and move through several developmental stages, finally becoming either CD4^{pos} or CD4^{neg} (Coles and Raulet, 2000). The stages of iNKT cell development can be identified using the iNKT cell markers CD44 and NK1.1 (Benlagha et al., 2002; Benlagha et al., 2005). Stage 0 iNKT cells are CD24^{hi}CD44^{neg}NK1.1^{neg}. Subsequently, iNKT cells progress through Stage 1 CD24^{low}CD44^{neg}NK1.1^{neg}, Stage 2 CD24^{low}CD44^{high}NK1.1^{low}, and mature into Stage 3 CD24^{low}CD44^{high}NK1.1^{high} iNKT cells (Coquet et al., 2008; Gadue and Stein, 2002; Pellicci et al., 2002). Some iNKT cells stay in the thymus as long-term residents (Berzins et al., 2006), while others emigrate from the thymus into the periphery, where they gain expression of NK1.1 (McNab et al., 2005). Recent work has shown diversity and heterogeneity in the subsets of iNKT cells that remain in the thymus and those that migrate to peripheral tissue, but the signals that regulate these movements are incompletely understood.

1.3 Activation of NKT cells

Activation of iNKT cells can be achieved by direct CD1d/TCR interactions. Using the semi-invariant TCR, iNKT cells recognize lipids and glycolipids that are bound to CD1d (Kawano et al., 1997). There are numerous glycolipids that can bind to CD1d, each having different length side chains or different head groups (Birkholz et al., 2015; Tsuji, 2006; Tupin and Kronenberg, 2006). The change in composition or position of the head groups of said glycolipids alters the avidity of the iNKT cell TCR for the CD1d molecule (Parekh et al., 2004; Pellicci et al., 2011). For example, alpha-
galactosylceramide, an activator of NKT cells, can bind to the iNKT TCR with a strong affinity, while β-galactosylceramide has a reduced affinity for the TCR leading to reduced activation and cytokine production of iNKT cells (Morshed et al., 2009). Changes in glycolipid configuration and binding to CD1d can therefore have dramatic effects on iNKT cell responses.

Glycolipids that preferentially activate iNKT cells are derived from self, commensal bacteria such as *Bacteroides fragilis* (Wieland Brown et al., 2013), or pathogenic bacteria such as *Streptococcus pneumonia* and *Borrelia burgdorferi* (Godfrey and Rossjohn, 2011). One of the most potent glycolipid agonists for iNKT cells is alpha-galactosylceramide (αGalCer). Originally derived from a sea sponge, and now shown to be expressed on *Bacteroides* species, the alpha-galactose head group sits above the CD1d molecule directly interacting with the iNKT TCR (Morita et al., 1995). The hydrophobic side chains are buried deep in the CD1d pockets (Koch et al., 2005). The length of the side chain on the glycolipid can affect how the sugar moiety interacts with the TCR (Girardi and Zajonc, 2012), changing the avidity of the CD1d/TCR interaction. This change in the avidity has direct consequences on the magnitude of the iNKT cell cytokine response, wherein some αGalCer-like molecules induce more IL-4 production while others attenuate cytokine production (Parekh et al., 2004). Interestingly, the CD1d/TCR interaction is highly conserved in that αGalCer loaded mouse CD1d can activate human iNKT cells and vice versa (Brossay et al., 1998). This observation suggests the presence of strong evolutionary pressure to maintain the recognition of αGalCer or αGalCer-like compounds by iNKT cells.
The second way that iNKT cells can be activated is indirectly from dendritic cells (DCs) stimulated with TLR ligands such as LPS, self-antigens presented on CD1d, or by IL-12 and IL-18 produced by activated DCs (Brigl et al., 2003). This activation is especially important for the iNKT cell response to bacteria that do not have glycolipids that are directly presented via CD1d. The endogenous pathway allows for iNKT cells to respond to bacterial invasion from pathogens such as *Staphylococcus aureus* and *Salmonella typhimurium* (Kinjo et al., 2011). IL-12 mediated activation of iNKT cells has also been shown in response to mouse cytoomegalovirus (MCMV), indicating a role for iNKT cells in viral infection as well (Wesley et al., 2008) (Tyznik et al., 2014). Therefore, iNKT cells can be activated in numerous ways to aid in the control of infection.

1.4 NKT cell TCR Agonists

iNKT cells can be activated by numerous glycolipid antigens. The most well-known and characterized is αGalCer (Morita et al., 1995). αGalCer, also called KRN7000, was first derived from an Okinawan sea sponge called *Agelas mauritianus*, and was selected initially for use against solid tumors in a B16 melanoma model (Morita et al., 1995). Initial reports stated that KRN7000 activated dendritic cells (Natori et al., 1997). It was not until several years later that Kawano et al. reported that KRN7000 specifically activated mouse splenic iNKT cells in a CD1d dependent manner (Kawano et al., 1997). Around the same time, human iNKT cells were also shown to be able to be
stimulated with KRN7000 (Spada et al., 1998). These observations show that αGalCer is a potent CD1d ligand.

Since the initial description of αGalCer as a CD1d ligand, there have been numerous reports describing CD1d ligands that activate iNKT cells. Several groups reported simultaneously the discovery of CD1d ligands from *Sphingomonas paucimobilis* (Mattner et al., 2005; Sriram et al., 2005; Wu et al., 2005). These cell wall ligands were structurally similar to αGalCer in that these glycosphingolipids (GSL) contain alpha-oriented sugars attached to the lipid backbone (Sriram et al., 2005). Other agonists include the galactosyl diacylglycerol BbGL-II from *Borellia burgdorferi* (Tupin et al., 2008). Stimulation of iNKT cells with this antigen improved joint inflammation and reduced spirochete load in BALB/c mice (Tupin et al., 2008). The initial endogenous CD1d glycolipid discovered was isoglobotrihexosylceramide (iGb3), which has been shown to activate both mouse and human iNKT cells (Mattner et al., 2005; Sanderson et al., 2013; Zhou et al., 2004). Other iNKT cell agonists include glycosylceramides and lysophospholipids (lysophosphatidylcholine), both of which have been reported as endogenous human iNKT self-ligands (Fox et al., 2009; Kain et al., 2015).

While treatment with αGalCer induces iNKT cells to produce both IFNγ and IL-4, the synthesized iNKT cell agonist OCH has been shown to elicit a Th2 bias, priming iNKT cells to make IL-4 (Oki et al., 2004). OCH is a molecule like αGalCer except with a shorter sphingosine moiety (Velmourougane et al., 2009). In an EAE model, OCH was shown to protect C57BL/6 mice stimulated with OCH and sensitized with MOG peptide from disease whereas αGalCer did not (Miyamoto et al., 2001). In contrast to OCH,
modification of a sphinganine backbone instead of sphingosine, and the addition of a hydrocinnamoyl ester to the galactose sugar (Chennamadhavuni et al., 2018) resulted in a compound (AH10-7) capable of inducing higher amounts of IFNγ from both mouse and human iNKT cells. This agonist aided in protection from B16 melanoma metastasis in both mouse and humanized CD1d mouse models (Chennamadhavuni et al., 2018).

Although iNKT cells are resistant to polarization in the traditional sense (Matsuda et al., 2003), modification of CD1d agonists can elicit differential cytokine responses from both mouse and human iNKT cells. This is an active area of investigation as new compounds are being synthesized to specifically activate iNKT cell responses to combat solid tumors (Hung et al., 2017).

1.5 NKT cell Cytokines and Immunity

Cytokine production is one of the major mechanisms through which iNKT cells mediate their action (Figure 1-2). Like T helper cell subsets, NKT cell IFNγ is important for preventing B16 melanoma tumor burden (Smyth et al., 2002), and is being investigated for treatments for other solid tumors (Ma et al., 2018; Tagawa et al., 2013). Interferon-gamma from iNKT cells is also critical for mediating protection in several disparate models of infectious disease (Behar et al., 1999; Gonzalez-Aseguinolaza et al., 2002; Li et al., 2007; Nakamatsu et al., 2007). For example, IFNγ from iNKT cells was shown to protect BALB/c mice from Pseudomonas by increasing the engulfment of bacteria from alveolar macrophages (Nieuwenhuis et al., 2002).
Interleukin-4 from iNKT cells is also critical in many different disease models, especially in the regulation of airway diseases such as allergy, asthma, and transplantation (Akbari O, 2003; Chuang et al., 2018; Meyer EH, 2006). In a model of organ transplantation tolerance, iNKT cell IL-4 induced T-reg production of IL-10, (Hongo et al., 2012) aiding in organ tolerance. Additionally, the iNKT IL-4 production was associated with activation of myeloid derived suppressor cells (MDSCs) which was important for host acceptance of heart and bone marrow transplants (Hongo et al., 2014).

Third, IL-17 producing iNKT cells are important for inhibiting growth of ocular Staphylococcus aureus in C57BL/6 mice (St Leger et al., 2018). Another study showed that lung iNKT cells produced IL-17 and IFNγ after activation by respiratory DC’s. This cytokine production subsequently lead to the recruitment of dendritic cells and neutrophils to the lung after Streptococcus pneumonia infection, aiding in increased clearance of the bacteria as compared to dendritic cell depleted controls (Ivanov et al., 2012).
iNKT cells in the germinal center, also termed NKT follicular helper (NKTfh), produce IL-21. IL-21 is known to have many effects on immune cells including activation of macrophages, NK cells, conventional T cells and iNKT cells (Skak et al., 2008). The IL-21 produced by human iNKT cells is crucial for B cell production of IgG
and IgA (Wu et al., 2015). IL-21 from iNKT cells also provides a feedback loop inducing further activation of iNKT cells, NK cells, T cells, and B cells (Coquet et al., 2007). These regulatory mechanisms were especially important for controlling *Mycobacterium tuberculosis* infection (Paidipally et al., 2018). Patients undergoing recombinant IL-21 therapy for the treatment of malignant melanoma showed increases in the frequency of iNKT cells during therapy (Coquet et al., 2013). Taken together, IL-21 from iNKT cells results in activation of other immune cells while further activating iNKT cells.

The regulatory cytokine IL-10 is produced by iNKT cells in disease specific contexts as well. In a model of cardiac ischaemia-reperfusion, αGalCer treated C57BL/6 mice showed reduced blood pressure and increased prevalence of heart remodeling due to an infiltration of IL-10 producing iNKT cells to the heart (Wang et al., 2018). IL-10 producing iNKT cells are also important in regulation of adipose tissue (Sag et al., 2014). iNKT cells are abundant in fat tissue, and over 25% of the iNKT cells in the adipose tissue produce IL-10 (Exley et al., 2014). Interestingly, Jα18−/− and CD1d−/− mice, which lack iNKT cells, gained more weight and had increased serum glucose as compared to wild-type controls. These data implicate IL-10 producing adipose tissue iNKT cells as being critically important for the regulation of metabolic disease (Lynch et al., 2012).

Therefore, the cytokines produced by iNKT cells are important in regulating the immune response to numerous different disease models.
1.6 Compartmentalizing NKT cells: iNKT cell subsets

Recently, new subsets of iNKT cells have been described that possess preformed transcripts of IFNγ (NKT1), IL-4 (NKT2), IL-17 (NKT17), IL-10 (NKTreg), and IL-21 (NKTfh) (Chang et al., 2011; Lee et al., 2013; Lee et al., 2016; Sag et al., 2014). These are functionally similar to their conventional T helper cousins in that they express primarily IFNγ, IL-4, IL-17, IL-10, and IL-4 and IL-21 respectively. Each of these subsets express specific transcription factors that enable their identification. All iNKT cells express the master transcription factor promyelocytic leukaemia zinc finger (PLZF) to various degrees as well as specific transcription factors that regulate cytokine production (Savage et al., 2008). NKT1 cells are PLZF\textsuperscript{low}Tbet\textsuperscript{high}, NKT2 cells PLZF\textsuperscript{high}GATA3\textsuperscript{high}, and NKT17 cells are PLZF\textsuperscript{int}RORγt\textsuperscript{high} (Lee et al., 2013). NKTfh cells express Bcl-6 and markers on their surface similar to conventional Tfh cells including the chemokine CXCR5 and the inhibitory markers PD-1 and ICOS (Chang et al., 2011). NKT10 cells are still an enigma in the field (Sag et al., 2014). These cells do not express PLZF, but E4 promoter-binding protein 4 (E4BP4) which has been implicated in IL-10 and IL-13 production in CD4 T cells (Motomura et al., 2011). Some reports suggest that iNKT10 cells are indeed a distinct subset, while others suggest that earlier subsets may differentiate into iNKT10 cells after stimulation and proliferation (Cameron and Godfrey, 2018). Recently, an additional subset of iNKT2 cells that do not produce IL-4 but express Tbet in addition to GATA3 has been identified (Engel et al., 2016). It has been suggested that this is a precursor population to the IL-4 producing iNKT2 cells (Crosby and Kronenberg, 2018).
iNKT cell subsets are contributors to iNKT cell mediated tissue immunity. Each organ appears to have a specific iNKT cell milieu with great diversity in the percentages of subsets (Lee et al., 2015). For example, NKT1 cells are highly represented in the liver and gut, which receive microbial products from portal circulation. NKT2 cells are enriched in the lung and mesenteric lymph node, and NKT17 cells are highly enriched in the lung and peripheral lymph nodes. Not only do iNKT cell subset frequencies vary among different tissues, but the anatomical location of NKT cell subsets vary within
tissues (Lee et al., 2015). This location specificity is presumably to position iNKT cells at strategic locations for cross-talk with other immune cells and for specific activation requirements. In the spleen, NKT1 cells are thought to reside in the red pulp, while NKT2 cells are located within the T cell zone (Lee et al., 2015). In the lung, NKT17 cells are located within the interstitial tissue, whereas NKT1 and NKT2 cells are primarily in the vasculature (Scanlon et al., 2011; Thanabalasuriar et al., 2016). NKT10 cells are primarily found in the gut while (Sag et al., 2014) liver NKT1 cells patrol the liver sinusoids (Lee et al., 2010). The anatomical locations of iNKT cells appear to have consequences for differences in iNKT cell activation. For example, NKT1 cells in the spleen and liver were activated after intravenous administration of αGalCer whereas only NKT2 and NKT17 cells in the mediastinal lymph node were activated after oral gavage (Lee et al., 2015). These data suggest that location within the tissue and route of activation are all important for the cytokine milieu produced by iNKT cells.

The mechanisms underlying NKT cell subset development in the thymus remain unclear. There are two models to explain how iNKT cell subset development fits into classical models of iNKT cell development. Classic NKT cell staging, discussed in section 1.2, postulates that iNKT cells follow a linear stage model progressing from Stage 0 (CD44\textsuperscript{neg}NK1.1\textsuperscript{neg}), to Stage 3 (CD44\textsuperscript{pos}NK1.1\textsuperscript{pos}) passing through each stage sequentially (Benlagha et al., 2005). Upon the identification of iNKT subsets however, it was found that these developmental stages were characterized by specific NKT cell subsets (Lee et al., 2013). NKT1 cells are the most mature Stage 3 cells, Stage 2 are NKT2, and Stage 1 are both NKT2 and NKT17 cells. The second model suggests that
iNKT cells do not follow a sequential model of development and instead suggests that each iNKT cell subset may have a separate precursor cell (Chen et al., 2017; Lee et al., 2013). Evidence for a precursor population comes from thymic transcriptome analyses that show non-traditional iNKT2 cells can express Tbet, indicating a distinct cell type other than those able to express IL-4 (Engel et al., 2016; Georgiev et al., 2016). To date, the mechanisms that regulate each of the iNKT cell subsets and how they are developmentally controlled in both the thymus and periphery remain undetermined.

While definitive iNKT cell subsets based on transcription factors in the human have not been identified, there are subsets of human iNKT cells that have propensities for different cytokines. The human subsets consist of CD4+CD8neg, CD4negCD8+, DN and DP (O'Reilly et al., 2011). After in vitro stimulation, human iNKT cell subsets produce similar levels of IFNγ and TNFα, but CD4+ iNKT cells specifically make higher levels of IL-4 (Montoya et al., 2007). The CD8+ and DN human iNKT cells are also thought to be more cytotoxic (Baker et al., 2001). The CD4+ human iNKT cells have been shown to provide an important role in antibody production by inducing B cell proliferation (Zeng et al., 2013). Therefore, iNKT cell subsets in both mouse and human contribute to tissue specific immune responses.
1.7 Genetic regulation of NKT cells

Invariant natural killer T cells exhibit immense strain specific diversity in number and function in mice (Chen et al., 2012; Rymarchyk et al., 2008). A comparison of iNKT cell numbers in common inbred strains of mice revealed a 50% difference in the percentage of iNKT cells in the liver (Rymarchyk et al., 2008). C57BL/6 mice, the most commonly used inbred mouse strain, has an above average number of iNKT cells, whereas BALB/c and C3H/HeJ mice harbor half as many (Hammond et al., 2001). Additionally, wild-derived inbred strains of mice such as Cast/EiJ and PWD/PhJ have barely detectable numbers of iNKT cells (Chen et al., 2012). Invariant NKT cell subsets also show variation in number and localization among different inbred strains of mice (Lee et al., 2015). C57BL/6 mice have the most NKT1 cells in all organs while BALB/c mice are enriched in NKT2, and NOD in NKT17. These analyses indicate that the host genetic background is important for the development of iNKT cells.

Strain dependent differences in NKT cell number are associated with strain dependent variation in pathogen clearance. For example, infection with *Borrelia burgdorferi* manifests as joint inflammation in CD1d<sup>−/−</sup> animals on a BALB/c background (Tupin et al., 2008) but myocarditis on a C57BL/6 background (Olson et al., 2009). Data from the Boyson lab show that the magnitude of lung iNKT cell cytokine responses in BALB/c mice was higher than the responses of C57BL/6 after stimulation with both αGalCer and anti-CD3 (Benoit et al., 2015). These data indicate that strain dependent variation affects how iNKT cells respond to infection.
Numerous groups have been working to identify genetic elements that regulate iNKT cell number and function. Early work showed that iNKT cell number was drastically diminished in the autoimmune prone non-obese diabetic (NOD) mouse model. It was also shown that the number of iNKT cells in type 1 diabetic and SLE patients were also diminished (Beristain-Covarrubias et al., 2015; Cho et al., 2011). Upon further investigation to the cause of reduced iNKT cell number in the NOD mouse, the Nkt1 locus located on chromosome 1 was identified (Rocha-Campos et al., 2006). Numerous congenic mice on the NOD and C57BL/6 background were generated, identifying smaller regions of NOD alleles that could control NKT cell number and function (Esteban et al., 2003; Jordan et al., 2004; Jordan et al., 2011; Jordan et al., 2007a). Importantly, a congenic line in which the NOD Nkt1 locus was replaced with C57BL/6 alleles showed no role in the disease susceptibility of type-1 diabetes, indicating that the susceptibility to autoimmune disease in the NOD mouse may be due to several different genetic loci (Rocha-Campos et al., 2006).

Numerous other studies have shown other genetic loci that control iNKT cell number (Wesley et al., 2007; Zhang et al., 2003). One of the most interesting and studied genetic regions, Sle1b on chromosome 1, was identified as a susceptibility locus for SLE in New Zealand White (NZW) mice (Morel et al., 2001). This locus was associated with increased auto-antibody production as well as germinal center formation. When the Sle1b locus was introgressed onto the B6 background, only the mice harboring the NZW alleles at the Sle1b locus (B6.Sle1b) exhibited signs of increased autoimmunity indicating that a genetic determinant within this region provided susceptibility to lupus (Sobel et al.,
This locus contained several hundred immunologically relevant genes, including
the signaling lymphocyte activation markers (Slam genes) which had been associated
with lupus susceptibility in GWAS studies (Cunninghame Graham et al., 2008;
Wandstrat et al., 2004). Similar decreases in iNKT cell numbers were shown when
129X1/SvJ alleles were introgressed onto the C57BL/6 background from chr1: 171.24-
176.09Mbp (Ensemble build m38.p6). B6.129 congenic mice exhibited reduced numbers
of iNKT cells in the liver as well as reduced iNKT cell cytokine production upon
activation (Aktan et al., 2010), indicating that a gene or genes controlling iNKT cell
number was within this region on chromosome 1.

The same region was subsequently investigated for control of iNKT cell subset
numbers. A 6.0Mbp locus on chr1:171-177Mbp derived from NZB mice was
introgressed onto the C57BL/6 background. Importantly, this locus overlapped
extensively with the previous Nkt1, Sle1b, and B6.129 congenic loci and contained the
Slam genes. This locus also appeared to control iNKT cell subset development. B6.NZB
congenic mice exhibited increased numbers of NKT1 and NKT2 cells as compared to
C57BL/6 controls (Baglaenko et al., 2017). While each of the genetic loci reported used
different strains of mice, the data clearly demonstrated that a gene or multiple genes on
chromosome 1 from 171-177Mbp affected iNKT cell development.

These large genetic loci however, contained over 80 immunologically relevant
genes, including the Slam genes, which were suspected candidates in controlling iNKT
cell number and function (Aktan et al., 2010; Wandstrat et al., 2004). The Boyson lab
therefore followed up on these studies and generated numerous congenic mice with
varied 129X1/SvJ congenic intervals and assessed iNKT cell responses. Identification of the candidate gene or genes regulating iNKT cell number and function was a major goal of this dissertation in Chapter 4.

1.8 SLAM receptors: fine-tuning the immune system

Signaling lymphocyte activation markers (SLAMs) are cell surface receptors expressed on all hematopoietic cells (Wu and Veillette, 2016) (Figure 1-4). These receptors are part of the CD2 family of cell surface receptors sharing similar Ig domain folds on their cell surface. The SLAM family receptors, except for SLAMf2 which binds SLAMf4, bind homotypically to self-receptors on opposite cells. Crosslinking of SLAM family receptors results in signaling leading to numerous functions on immune cells such as cell-cell adhesion, activation, cytokine production, cell proliferation, cell death, and cytotoxicity (Cannons et al., 2011). Interestingly, the SLAM family receptors are heterogeneously expressed on different cell types. These patterns of expression could possibly aid in lending specificity of signaling to cell interactions, be it co-stimulation leading to cytokine production from T cells (Howie et al., 2002), adhesion of T cells to B cells or antigen presenting cells (Zhao et al., 2012), antibody production from B cells (Cannons et al., 2006), or cytotoxicity from NK cells (Dong et al., 2012; Meinke and Watzl, 2013). SLAMf1 has been shown to function as a costimulatory receptor on T cells, SLAMf6 signaling aids in B cell help for proliferation and antibody production, while SLAMf4 mediates both inhibitory and activating signals for NK cell cytotoxicity (Chlewicki et al., 2008; Howie et al., 2002; Radomir et al., 2017).
Deletion of SLAM family receptor signaling by mutating the SLAM adaptor protein (SAP) gene has detrimental effects on multiple immune cell functions (Cannons et al., 2006; Nichols et al., 2005a; Nichols et al., 2005b; Rivat et al., 2013). SAP is located on the X-chromosome, and is the mutated gene in X-linked lymphoproliferative disorder (XLP) (Coffey et al., 1998; Nichols et al., 1998; Sayos et al., 1998). Young boys
who acquire this deletion are unable to control Epstein Bar Virus and succumb to the virus at a young age. These patients have increased white blood cells due to defects in activation induced cell death. Conventional CD8 T cells and NK cells fail to kill virally infected cells. These patients also have defects in antibody production and formation of B cell blasts (Purtilo et al., 1975). XLP patients fail to develop iNKT cells, indicating that SLAM/SAP signaling is required for the development of this subset of αβ T cells (Pasquier et al., 2005). Therefore, SLAM family receptor signaling is necessary for the proper functioning and development of immune cells.

SLAM family receptors 1-7, except for SLAMf2, contain immuno-tyrosine switch motifs (ITSM) on their cytoplasmic tails (Shlapatska et al., 2001) (Figure 1-4). ITSMs are different from other immune regulatory motifs such as the immune-tyrosine activation motif (ITAM) and immune-tyrosine inhibitory motif (ITIM). Whereas the activation and inhibitory motifs can only bind adaptor molecules that result in activation or inhibition respectively, the ITSM regulatory tails allow for activating or inhibitory signaling based on the repertoire of adaptor molecules expressed in the cell. The SLAM adaptor molecule, slam adaptor protein (SAP), can bind to the immuno-tyrosine switch motifs, which leads primarily to activation phenotypes (Shlapatska et al., 2001). If SAP is not bound, the inhibitory phosphatase, src-homology region 2 domain containing
phosphatase 1 (SHP-1), can also bind (Kageyama et al., 2012) (Figure 1-5). Which adaptors are bound to the SLAM family receptors in different cells remains controversial. Initial reports observed that SAP was constitutively bound to SLAM family receptors on T cells, while SHP-1 could displace SAP (Dong et al., 2009; Sayos et al., 1998; Tangye et al., 2000), resulting in a decreased activation signal or possibly even an inhibitory signal. An alternative model states that SHP-1 may be constitutively bound and SAP outcompetes SHP-1 for binding to the same ITSM, thereby decreasing the inhibitory
signal (Wilson et al., 2014). Other reports speculate, but have not unequivocally shown, that SAP can be bound to one ITSM and SHP-1 to a second on the same SLAM family receptor (Kageyama et al., 2012). Therefore, SLAM family receptor signaling on different immune cells can have both activating and inhibitory phenotypes.

One of the most striking examples of activation and inhibitory signaling phenotypes is shown by analysis of SLAMf4 expression on NK cell lines (Chlewicki et al., 2008). In vitro, high expression of SLAMf4 resulted in inhibition of cytokine production while a lower level of SLAMf4 correlated with an activation phenotype and increased cytokine production of NK cells. The inhibitory and activation levels were dependent not only on SLAMf4 expression, but on ligand crosslinking (i.e. with SLAMf2), as well as the expression level of SAP. Interestingly, lower levels of SAP were associated with inhibition while higher levels showed activation, indicating that the ratio of adaptor molecule binding to ITSMs on the SLAM family receptor contribute to how the receptor mediates function (Chlewicki et al., 2008).

Furthermore, analysis of SLAMf4 and SLAMf6 in SAP−/− mice show that these receptors can have both inhibitory and activating functions in vivo. In the presence of SAP, SLAMf4 and SLAMf6 were required for activation signals to mediate adhesion of T cells to target cells at the immunological synapse (Zhao et al., 2012). In SAP deficient mice, SHP-1 was recruited to the ITSM, and cell-cell contact time was diminished resulting in decreased cell killing and activation of B cells. Thus, SLAMf4 and SLAMf6 can have an activating function in the presence of SAP, but inhibitory functions without SAP (Zhao et al., 2012).
SLAM family receptor signaling is critical for the development of certain lineages of innate-like T cells. There are SLAM/SAP dependent γδ T cells, innate-like CD8+ T cells, and iNKT cells (De Calisto et al., 2014; Griewank et al., 2007; Kreslavsky et al., 2009). SAP deficient mice lack a specific subset of “NKT-like” gamma-delta T cells that express a characteristic Vγ1.1Vδ6.3 TCR (Kreslavsky et al., 2009). The field still has yet to determine which SLAM family receptors in particular are required for development of this cell type. SLAMf5 deficient mice have an increase in CD8+ innate T cells as compared to wild-type mice, implicating SLAMf5 as a regulator of innate CD8+ T cell development (De Calisto et al., 2014). SLAMf1, SLAMf6, and recently SLAMf3 have all been implicated in the development of iNKT cells, which will be discussed more in the next section (Griewank et al., 2007; Sintes et al., 2013). MAIT cells do not appear to be dependent on SLAM/SAP for development (Garner et al., 2018). To date, SLAM family receptors have not been shown as crucial for the development of conventional T cells, indicating that innate like T cells are more dependent on SLAM family receptor signaling than conventional T cells.

1.8.1. SLAM family receptor 6 (SLAMf6, Ly108, NTB-A)

SLAM family member receptor 6 (SLAMf6) is thought to have diverse effector functions on multiple immune cells. This receptor has been shown to have costimulatory functions on naive CD4 T cells and NK cells, and inhibitory functions on re-stimulated conventional T cells as well as iNKT cells. Initial investigations into the role of SLAMf6 on conventional CD4 T cells showed that SLAMf6 deficient T cells produced less IL-4.
after *Leishmania mexicana* challenge (Howie et al., 2005), as well as reduced phagocytosis of *Salmonella typhimurium* by neutrophils, showing that SLAMf6 could control functions of both innate and adaptive immune responses.

Next, SLAMf6, also termed Ly108, and “NK, T and B cell antigen (NTB-A)” in the human, was identified as a potential susceptibility gene for autoimmunity, especially in systemic lupus erythematosus (SLE) (Wandstrat et al., 2004). The first identification of SLAMf6 as having a role in autoimmunity was due to an increase in the expression of SLAMf6 on autoimmune prone (B6.*Sle1b*) mouse T cells as compared to C57BL/6 mice (Wandstrat et al., 2004). Numerous reports showed that SLAMf6 enhanced signaling in CD4 T cells (Chatterjee et al., 2011), regulated autoantibody production (Keszei et al., 2011), and was important with SAP for B cell help and germinal center formation (Wong et al., 2015). SLAMf6 and SLAMf3 expression were increased on CD4 T cells of SLE patients, indicating that SLAMf6 functioned, in part, to aid in promoting SLE aberrant T cell signaling (Chatterjee et al., 2012).

Recently, SLAMf6 has been shown to have roles as a negative regulator in humoral immunity. Deletion of SLAMf6, SLAMf5, and SLAMf1 resulted in higher antibody responses, T follicular helper cell formation, and germinal center formation. Additionally crosslinking SLAMf6 with an anti-SLAMf6 antibody reduced Tfh formation, and antibody responses, indicating that SLAMf6 also acts as a negative regulator in modulating antibody responses (Wang et al., 2015).

SLAMf6 is also thought to play an active role in binding bacteria such as *Citrobacter rodentium*, *E coli*, and *Salmonella* (van Driel et al., 2015). Engagement of
SLAMf6 with the OmpF and OmpC proteins on *E. coli* allowed SLAMf6 to transmit signaling to activate neutrophils and phagocytic cells. SLAMf6<sup>−/−</sup>Rag<sup>−/−</sup> mice were protected from *C. rodentium* mediated colitis due to decreased inflammatory cell localization to the gut decreasing the pathology from inflammation in this organ (van Driel et al., 2015).

How is it that SLAMf6 could have a myriad of roles in both innate and adaptive immune responses? The answer, in part, is due to the biology of SLAM family receptors themselves. Since SLAM family receptors have ITSM motifs and can bind multiple signaling molecules, as discussed earlier, SLAM receptors can change signaling dependent on expression level and the strength of the signal received. Indeed, SLAMf6 has been shown to act as “immune modulatory switch” providing activation of T cells in the presence of SAP and inhibition of NKT cell development in SAP deficient mice (Kageyama et al., 2012). Chapters 2 and 3 explore how SLAMf6 can have multiple functions on the same immune cell type.

### 1.9 Slam gene polymorphisms

There are 9 SLAM family receptors within the Slam gene family, each of which are termed SLAMf1-SLAMf9 (Cannons et al., 2011). Slams 1-7 are located within a 0.410Mbp region on chromosome 1. Slams 8 and 9 are located another 0.6Mbp telomeric to the main Slam locus located at Chr1:171.55-171.96 (Ensembl Gene Browser, Build 93 GRCm38.p6). These genes and gene arrangement are conserved in both the mouse and
human (Engel et al., 2003), indicating an evolutionary need to constrain these genes (Limaye et al., 2008).

The Slam genes are found in 3 distinct haplotypes in inbred mouse strains, with C57BL/6 harboring one haplotype, while NZW/B, BALB/c, and 129X1/SvJ harbor a second. The third haplotype is only found in wild-derived inbred strains of mice such as CAST/EiJ and PWD. Each of these haplotypes show variability in SLAM family receptor gene expression, copy number, or isoform expression. For example, Slamf4 has 4 pseudogenes duplicated in mice that carry haplotype 2 alleles while C57BL/6 mice, which harbor haplotype 1 alleles, express an inhibitory isoform of the SLAMf6 protein.

Slam polymorphisms have been shown to affect iNKT number and function (Aktan et al., 2010; Esteban et al., 2003; Jordan et al., 2004; Jordan et al., 2007b). Previous reports had indicated immense strain dependent variability in the number of iNKT cells in different inbred strains of mice, raising questions about which genes controlled the development of iNKT cells in these strains. The regions first identified as iNKT cell control genes, Nkt1 and Sle1b, both contained Slam gene polymorphisms in addition to many other immunologically relevant genes (Jordan et al., 2007a; Morel et al., 2001). A major goal of the Boyson lab has been to refine these regions to identify the gene or genes that control iNKT cell number and function.

Slam polymorphisms are also important for infection models. Boyson and Huber showed that mice harboring the autoimmune prone Slam haplotype 2 promoted NKT cell activation after coxsackie-B3 infection resulting in liver pathology. Slam haplotype 1 animals, or C57BL/6, showed more gamma-delta T cell responses, leading to induction
of myocarditis (Huber et al., 2013). In addition, BALB/c mice, which harbor Slam haplotype 2 alleles were more dependent on iNKT cells for the clearance of Pseudomonas from the lung than C57BL/6 mice (Benoit et al., 2015). These infection models describe how Slam polymorphisms may control iNKT cell responses to infection.

1.10 SLAM receptor regulation of NKT cells

iNKT cells are dependent on SLAM and SAP signaling in the thymus for development. Using elegant chimeric models, Griewank et al. showed that SLAMf1 and SLAMf6 with the adaptor molecule SAP are required on double positive thymocytes for iNKT cell selection (Griewank et al., 2007). Subsequent reports have now shown that SLAMf3 is a negative regulator of iNKT cell development (Sintes et al., 2013). Analysis of numerous SLAM family receptor single knockouts, as well as multiple SLAM family receptor knockouts have shown skewed ratios of NKT1, NKT2, and NKT17 subsets as compared to wild-type parental controls, indicating that multiple SLAM family receptor signals play a role in iNKT cell subset development (Baglaenko et al., 2017; Graham et al., 2006; Huang et al., 2016).

The role of SLAM family receptor signaling on iNKT cell subset development is exemplified by the fact that SAP appears to contribute to IL-4 and NKT2 development (Michel et al., 2016). SAP deficient mice crossed with transgenic Vα14 mice, which allow for iNKT cell production even though these mice lack SAP, showed an increase in the level of iNKT17 cells along with a decrease in NKT2 cells (Michel et al., 2016). While these results are indicative of a role for SLAM/SAP signaling in iNKT cell subset
development, the transgenic mouse model used may have confounded results. Initial reports show that this mouse has an increase in NK1.1 NKT cells compared to parental controls, indicating that the Vα14 transgenic has developmental differences in iNKT cell subsets (Bendelac et al., 1996). Additional investigation into the role of SLAM/SAP signaling on the development of iNKT cell subsets is ongoing by us and others.

Several studies have shown that SLAMf6 specifically is a regulator of iNKT cell development. SLAMf6 is required for the induction of the iNKT cell master transcription factor promyelocytic leukaemia zinc finger (PLZF). Treatment of pre-selected thymocytes with anti-SLAMf6 and anti-CD3 induced higher expression of PLZF than treatment with anti-CD3 alone (Dutta et al., 2013). Another report showed that SLAMf6 was a negative regulator of NKT cell development. SLAMf6 deficient mice show a 50% reduction in NKT cells, and SAP deficient mice lack all NKT cells completely (Kageyama et al., 2012; Nichols et al., 2005a). Interestingly, a partial restoration of NKT cells was observed in SLAMf6−/−SAP−/− mice, suggesting that SLAMf6 acts as negative regulator in SAP−/− animals (Kageyama et al., 2012). Additionally, crosslinking of SLAMf6 on APCs and iNKT cells are critical for the cytokine production of iNKT cells in the mouse and human, indicating that SLAMf6 can also act as an activation receptor upon homotypic binding (Baglaenko et al., 2017). SLAMf6 has been shown to be involved in the induction of PLZF, yet the deletion of SLAMf6 by itself is not sufficient to completely block iNKT cell development, indicating that other SLAM family receptors also aid in iNKT cell development. These studies did not address function of SLAMf6 on
iNKT cell subsets. These observations raise questions about the mechanisms of SLAMf6 function on peripheral iNKT cell subsets, which are discussed in Chapters 2 and 3.

Multiple SLAM family receptors can provide both positive and negative signaling in iNKT cell development. As noted above, deletion of SLAMf6 decreases the number of iNKT cells in the thymus by 50% (Dutta and Schwartzberg, 2012). A SLAMf1, SLAMf5, SLAMf6 triple knockout, however, decreases NKT development by over 80% (Huang et al., 2016). This report indicates that SLAMf1 and SLAMf5 may provide positive signaling for NKT cell development while SLAMf6 provides an inhibitory signal. Additional reports observed that total SLAM family receptor knockouts had a less severe decrease in iNKT cells as compared to SAP−/− mice, indicating that all SLAM family receptors can negatively regulate iNKT cell development when SAP is absent (Chen et al., 2017). Interestingly, SAP deficiency induced after iNKT cell development resulted in a decrease in peripheral iNKT cell mediated cytotoxicity, but not cytokine production, indicating that the major role for SAP on iNKT cells could possibly be only in development (Das et al., 2013). These phenotypes highlight the importance of SLAM/SAP signaling in iNKT cell development.

1.11 The role of SLAM family receptors on iNKT cell proliferation

iNKT cells are thought to have an activated phenotype immediately upon emigration from the thymus (Kumar et al., 2017). This primed state not only leads to quick secretion of cytokines, but also rapid proliferation, and as a result, rapid cell death. The balance of this proliferation and cell death has been the focus of numerous studies.
since iNKT cells were discovered, but these studies have been controversial (Eberl and MacDonald, 1998) (Crowe et al., 2003; Subleski et al., 2011). One of the first reports on this topic suggested that liver iNKT cells rapidly undergo apoptosis after activation with \textit{in vivo} anti-CD3\(\varepsilon\) (Eberl and MacDonald, 1998). Numerous reports further showed that this cell death was due to Fas/FasL interactions since FasL is highly expressed on iNKT cells (Leite-de-Moraes et al., 2000). Subsequently, it was shown that splenic NKT cells down regulate their TCR in response to a strong stimulus, making these cells impossible to detect using CD1d/\(\alpha\)GalCer loaded tetramer (Crowe et al., 2003). Recently, both models were found to occur in that NKT cells in the liver undergo activation induced cell death but iNKT cells in the spleen do not. Surprisingly, iNKT cells from both the spleen and liver downregulated their TCR, but cells in the lymph node did not downregulate their TCR or undergo cell death (Subleski et al., 2011). These data point to specific subsets of iNKT cells being more susceptible to cell death than others. Indeed, recent data has shown than NKT2 cells are much more proliferative than NKT1 cells (Cameron and Godfrey, 2018). Further transcriptomic reports indicate that iNKT cell subsets have different propensities and thresholds for activation and cell death (Engel et al., 2016; Georgiev et al., 2016), the implications of which are only now being addressed.

Little is currently known about the role of SLAM family receptors in homeostatic mechanisms of peripheral iNKT cells. Although SLAMf1 and SLAMf6 are required for iNKT cell development (Griewank et al., 2007), it remains to be determined whether SLAMf6-SLAMf6 interactions mediate proliferation or cell death in the periphery. Yet, analysis of reactivation induced cell death shows that SLAMf6 may be
involved in regulating iNKT cell homeostasis. Signaling of SLAMf6 via the adaptor molecule SHP-1 in XLP patients, who lack SAP, caused CD8 T cells to over proliferate and prevented them from undergoing cell death (Snow et al., 2009). When SLAMf6 was knocked-down, however, the resistance to cell death was reversed, and the majority of CD8 T cells underwent apoptosis (Snow et al., 2009). Additionally, SLAMf6 and SLAMf4 were shown to regulate CD8 T cell mediated cytotoxicity against B cells, which is an important function for fighting EBV infection (Zhao et al., 2012).

In the past several years, the unique property of SLAMf6 to change the signaling thresholds for cell death has been exploited in new immuno-therapeutics for leukemia and metastatic melanoma (Eisenberg et al., 2018; Korver et al., 2007). SLAMf6 has been shown to be highly expressed on B cells, with even greater expression on leukemic B cells. Monoclonal antibodies that target SLAMf6 result in enhanced cell death against the leukemic B cells, preventing the cancer from progressing (Korver et al., 2007). In addition, soluble SLAMf6 has been shown to improve CD8 T cell cytotoxicity and activation against melanoma tumors in a mouse model (Eisenberg et al., 2018). These data suggest that SLAMf6 can modulate the cytotoxic and cell death thresholds of lymphocytes, which have important implications in the treatment of cancer.
1.12 Gaps in knowledge of NKT cell regulation

SLAM family receptors have been regulated as modulators of lymphocyte function since 1993 (Garni-Wagner et al., 1993). Although there have been many studies of SLAM family receptors on T cells and NK cells leading to identification of SLAM family receptors mediating cell-cell contact at the immunological synapse, co-stimulation of T and B cells, and inhibition of cytokine production, little is known about how SLAM family receptors function on iNKT cells in the peripheral tissues. Most studies of SLAM family receptors on iNKT cells are centered upon how these receptors mediate signals during development, leading to broad knowledge of how iNKT cell development is fundamentally different from that of conventional T cells. Other reports have investigated cytokine production after thymic iNKT cell stimulation in vitro, but not on iNKT cells from the spleen, lung and liver. Therefore, there is a gap in knowledge of how SLAM family receptors are expressed and if these patterns mediate functionality of peripheral iNKT cell subsets.

The recent discovery of iNKT cell subsets raises new questions about how the SLAM family receptors are involved in development and function of these subsets. We know that SLAMf1 and SLAMf6 with SAP are required for the development of all iNKT cells (Griewank et al., 2007), but how these two proteins affect subset development has not been addressed. Since C57BL/6 mice have mostly NKT1 cells (70% or higher in the spleen) (Lee et al., 2015) (Boyson lab observations), differences in iNKT cell phenotypes have not been discovered until NKT1 cells were separated from NKT2 and NKT17. Additionally, a report of SLAMf6-deficiency on subsets of iNKT cells has not been
performed. Therefore, a major goal of this dissertation was to assess SLAM family receptor patterns of expression and how these patterns controlled NKT cell subset development and peripheral function. A second goal was to analyze specific co-expression of SLAM family receptors on subsets of iNKT cells in both the C57BL/6 and SLAMf6−/− mice, identifying specific roles for SLAMf6 on iNKT cell subsets.

Finally, there have been numerous studies implicating Slam gene polymorphisms in the control of total iNKT cell number and function (Aktan et al., 2010; Baglaenko et al., 2017). Whether these loci control all iNKT cell numbers or just certain subsets still needs to be determined. Most of the susceptibility studies were performed on mice with large congenic loci, containing numerous immunologically relevant genes. There has not been a definitive study showing that the Slam genes were the regulators of iNKT cell number and function. How to refine one of these loci and whether Slam genes may be involved in the regulation of iNKT cell number and function is discussed in Chapter 4.

Therefore, the major goals of this dissertation were first, to broadly analyze SLAM family receptor expression on peripheral iNKT cells in the lung, liver, and spleen and assess the functional implications of these patterns of expression. Second, to assess the role of SLAMf6 signaling on the newly described iNKT cell subsets, and whether this receptor can lead to multiple phenotypes across the iNKT cell lineage. Third, to address whether Slam genes control iNKT cell number and function. These data, overall, provide novel understanding of the mechanisms by which iNKT cells are modulated in secondary organs, and provide context for how these cells contribute to host immunity.
1.13 Literature Review References


inflammation after infection with Borrelia burgdorferi. Proc Natl Acad Sci U S A 105, 19863-19868.


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CHAPTER 2: DELETION OF SLAMf6 REVEALS AN NKT-SUBSET SPECIFIC DEFECT IN IL-17 PRODUCTION

Running Title: SLAMf6 is crucial for NKT17 IL-17 production

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2.1. Abstract

Invariant natural killer T cells (iNKT) are crucial for mediating host immunity in a tissue specific manner. When activated, these cells produce a myriad of cytokines including IFNγ, IL-4, IL-10, and IL-17. Each of these cytokines is primarily produced by specific iNKT cell subsets termed NKT1, NKT2, NKTreg, and NKT17, respectively. The cell surface-expressed family of signaling lymphocyte activation markers (SLAM) have been shown to play a role both in the development and function of iNKT cells, but a role on specific iNKT cell subsets has not been shown. Here, we analyze SLAM family receptor co-expression on iNKT cell subsets revealing tissue and strain dependent differences in SLAM family receptor expression. These observations led us to hypothesize that SLAM family receptors could fine-tune development and function of iNKT cell subsets. Indeed, SLAMf6 was highly expressed on NKT2 and NKT17 subsets. Deficiency of SLAMf6 resulted in decreased NKT2 and NKT17 cell numbers as well as IL-4 and IL-17 production from sorted NKT2/NKT17 cells. Taken together, these data show that SLAM receptor expression patterns define iNKT cell subsets, and that SLAMf6 has a functional role in NKT2 and NKT17 subset specific development. Additionally, this is the first report that SLAMf6 deficiency has a specific effect on NKT17 IL-17 production and NKT2 IL-4 production, showing that SLAM family receptor expression also mediates function of these iNKT cell subsets.
2.2. Introduction

Invariant natural killer T cells are innate-like T cells that play a crucial role in the host immune response (Behar and Porcelli, 2007; Godfrey and Rossjohn, 2011; Gumperz and Brenner, 2001). These invariant cells recognize glycolipids bound by the MHC class-1 like molecule CD1d (Kawano et al., 1997; Spada et al., 1998). When activated by the prototypical agonist alpha-galactosylceramide (αGalCer), iNKT cells make a host of inflammatory mediators such as IFN-γ, IL-4, TNFα, IL-17, and the anti-inflammatory cytokine IL-10 (Coquet et al., 2008; Hameg et al., 2000; Yoshimoto and Paul, 1994).

iNKT cell cytokines play critical roles in immune function and underlie the critical role of iNKT cells in many disease models (Crosby and Kronenberg, 2018). For example, IFN-γ produced by iNKT cells is critical for secondary activation of NK cells and subsequent killing of metastatic tumors in a B16 melanoma model (Smyth et al., 2002), and activation of macrophages by IFNγ-producing lung iNKT cells is crucial for decreasing Pseudomonas bacterial burden in the lung (Nieuwenhuis et al., 2002). Similarly, IL-17 from lung iNKT cells is associated with neutrophil recruitment clearance of Streptococcus pneumoniae from the lung (Ivanov et al., 2012). Finally, iNKT-derived IL-21 has been implicated in aiding clearance of Mycobacterium tuberculosis (Wu et al., 2015), and iNKT-derived IL-10 has been shown to protect mice from metabolic disease (Lynch et al., 2012).

Recently, several subsets of iNKT cells have been identified that preferentially transcribe IFN-γ (NKT1), IL-4 (NKT2), and IL-17 (NKT17) cytokines, much like the T
helper subsets of conventional T cells (Coquet et al., 2008; Hameg et al., 2000; Lee et al., 2013). Invariant NKT cells that produce IL-21 (NKTfh) (Chang et al., 2011) and IL-10 (NKTreg) (Sag et al., 2014) have also been described. The iNKT cell subsets reside preferentially in distinct peripheral tissues, with NKT1 cells homing to the liver, NKT2 in the spleen, NKT17 in the lung and mesenteric lymph node, and NKTreg cells preferentially homing to the gut and adipose tissue (Lee et al., 2015; Lynch et al., 2015). Invariant NKT cell subsets are also highly mouse strain dependent in that C57BL/6 mice possess more NKT1 cells than do BALB/c, while BALB/c possess increased numbers of NKT2 cells. NKT17 cells are most prevalent in NOD mice (Lee et al., 2015). These newly defined subsets could explain the heterogeneity in iNKT cell function and how these cells can regulate the immune response in a tissue-dependent manner. However, the mechanisms by which iNKT cell subsets develop and how they can regulate the immune response are largely unknown.

The signaling lymphocyte activation markers (SLAMs) and the associated signaling adapter protein SAP play critical roles in NKT cell development (Chen et al., 2017; Griewank et al., 2007; Nichols et al., 2005a; Sintes et al., 2013). The nine SLAM family receptors are widely expressed on all hematopoietic cells where they play critical roles in signaling and in cell adhesion (Cannons et al., 2011). Specifically, homophilic interactions among the SLAMf1 and SLAMf6 receptors expressed on thymocytes have been demonstrated to be critical in iNKT cell development (Griewank et al., 2007; Kageyama et al., 2012), while SLAMf3 has been demonstrated to be a negative regulator of NKT cell development (Sintes et al., 2013).
Importantly, SLAM family receptors have been implicated in the modulation of cytokine production in multiple leukocyte subsets. SLAMf6 was demonstrated to be critical in CD4+ T cell IL-4 production (Howie et al., 2005). SLAMf3 and SLAMf6 engagement has been linked to IL-17A production in human lupus patients (Chatterjee et al., 2012) and SLAMf1 leads to increased TNFα and IL-1β after mycobacteria infection (Aversa et al., 1997; Jordan et al., 2007b; Song et al., 2015). However, the extent to which SLAM receptors modulate iNKT cell cytokine production is far less clear. While deletion of multiple SLAM receptors does have a significant impact on iNKT cell cytokine production (Chen et al., 2017; Huang et al., 2016), the pleiotropic nature of the Slam gene family has made it difficult to assign specific functional roles for individual SLAM family receptors to iNKT cells.

Here, we explored the levels of SLAMf1, SLAMf4, and SLAMf6 co-expression on αβ T cells and report the presence of a distinct profile of SLAM family receptor expression on iNKT cells. These SLAM family receptor expression profiles were organ specific and strain-dependent. Importantly, when we extended our analysis to the subsets of iNKT cells, we found that the SLAM family receptor co-expression of SLAMf1, SLAMf4, and SLAMf6 allowed us to delineate specific iNKT cells subsets in the spleen, lung, and liver. We found that SLAMf6high NKT cells were enriched in NKT2 and NKT17 cells, and subsequently demonstrate that iNKT cells from SLAMf6−/− mice exhibit specific defects in IL-4 and IL-17 production that is associated with decreased numbers of NKT2 and NKT17 cells in the thymus.
2.3. Results

2.3.1 Distinct SLAM family receptor expression profiles on conventional αβ T and iNKT cells

SLAM family receptors, together with the intracellular signaling adaptor molecule SAP, are required for the development of iNKT cells (Chen et al., 2017; Chung et al., 2005; Griewank et al., 2007; Kageyama et al., 2012; Nichols et al., 2005a; Sintes et al., 2013). The function of SLAM family receptors on iNKT cells in the periphery, however, is still mostly unclear. Since previous reports suggest that distinct SLAM family receptors exhibit variable expression among NKT cells (Aktan et al., 2010), we assessed the co-expression of three SLAM family receptors, SLAMf1, SLAMf4, and SLAMf6 on iNKT cells and conventional αβ T cells among different organs in two different strains of mice (Figure 1). This analysis revealed the presence of a unique pattern of SLAM family receptor co-expression that distinguished iNKT cells from conventional αβ T cells, and this pattern varied among different tissues (Figure 1). For example, we found that B6 spleen iNKT cells were primarily SLAMf1<sup>neg</sup>SLAMf4<sup>neg</sup>SLAMf6<sup>pos</sup> or SLAMf1<sup>int</sup>SLAMf4<sup>neg</sup>SLAMf6<sup>pos</sup>. In contrast, B6 lung iNKT cells were SLAMf1<sup>neg</sup>SLAMf4<sup>neg</sup>SLAMf6<sup>neg</sup>, SLAMf1<sup>neg</sup>SLAMf4<sup>neg</sup>SLAMf6<sup>pos</sup>, or SLAMf1<sup>pos</sup>SLAMf4<sup>neg</sup>SLAMf6<sup>pos</sup>. Conventional αβ T cells in the B6 spleen and lung, in contrast, were primarily SLAMf1<sup>neg</sup>SLAMf4<sup>neg</sup>SLAMf6<sup>pos</sup> (Figure 1). We also noted that while both iNKT cells and conventional αβ T cells expressed SLAMf6, a distinct population of SLAMf6<sup>high</sup> iNKT cells could be observed in both the spleen and lung. Since SLAM family receptor genes are polymorphic among inbred strains of mice, we
also analyzed the patterns of SLAM co-expression on conventional T cells and iNKT cells in the spleen, liver and lung in BALB/c mice (Figure 1). This analysis revealed significant differences in SLAM family receptor co-expression in both iNKT cells and αβ T cells (Fig. 1). Notably, while B6 lung iNKT cells were \( \text{SLAMf1}^{\text{neg}}\text{SLAMf4}^{\text{neg}}\text{SLAMf6}^{\text{neg}} \), \( \text{SLAMf1}^{\text{neg}}\text{SLAMf4}^{\text{neg}}\text{SLAMf6}^{\text{pos}} \), or \( \text{SLAMf1}^{\text{pos}}\text{SLAMf4}^{\text{neg}}\text{SLAMf6}^{\text{pos}} \), we found that the majority of BALB/c lung iNKT cells were \( \text{SLAMf1}^{\text{neg}}\text{SLAMf4}^{\text{neg}}\text{SLAMf6}^{\text{pos}} \) (Figure 1). Similarly, we noted an increased frequency of \( \text{SLAMf1}^{\text{neg}}\text{SLAMf4}^{\text{neg}}\text{SLAMf6}^{\text{neg}} \) conventional αβ T cells in all organs (Figure 1). Taken together, these data indicated the presence of a unique pattern of SLAM family receptor co-expression that distinguished iNKT cells from their conventional T cell counterparts and that these SLAM receptor expression profiles varied significantly among different peripheral tissues and among different genetic backgrounds.

### 2.3.2 SLAM family receptor co-expression profiles define NKT cell subsets

The iNKT cell population is comprised of functionally distinct NKT1, NKT2, and NKT17 subsets (Lee et al., 2013). Therefore, we asked whether the SLAM family receptor co-expression profiles varied among the different iNKT cell subsets. iNKT cell subsets are defined according to their expression of the transcription factors PLZF and RORγt (Lee et al., 2013). NKT1 cells express low levels of PLZF and no RORγt. NKT2 cells express high levels of PLZF and no RORγt, and NKT17 express intermediate levels of PLZF and RORγt (Figure 2A). Interestingly, analysis of splenic iNKT subsets revealed distinct differences in the SLAM family receptor expression profile on each subset.
(Figure 2A). NKT1 cells were primarily SLAMf1^{int}SLAMf4^{low}SLAMf6^{int}. All iNKT2 cells were SLAMf1^{low}SLAMf4^{low}SLAMf6^{high} (Figure 2A). NKT17 cells were comprised of two distinct populations, one that is SLAMf1^{high}SLAMf4^{low}SLAMf6^{low}, and a second that is SLAMf1^{low}SLAMf4^{low}SLAMf6^{high} (Figure 2A). These data demonstrated that iNKT cell subsets were each characterized by a distinct SLAM family receptor expression profile.

The frequency of NKT cell subsets varies among different organs (Lee et al., 2015), and it has been reported that NKT1 cells are enriched in the liver, NKT2 cells in the lung, and NKT17 cells in the lung and lymph node (Lee et al., 2015). Therefore, we asked whether SLAM family receptor expression profiles on NKT cells also varied in an organ-specific manner. We analyzed SLAM family receptor expression on the different iNKT cell subsets in the spleen, lung, and liver of C57BL/6 and BALB/c mice (Figure 2B). Interestingly, we found that the lung was enriched in SLAMf1^{high}SLAMf4^{low}SLAMf6^{low} and SLAMf1^{low}SLAMf4^{low}SLAMf6^{high} cells consistent with NKT17 cells (Figure 2B). Consistent with a previous report of tissue-and strain-dependent variation in NKT cell subset frequencies (Lee et al., 2015), we observed a greater frequency of NKT17 cells in the lungs of BALB/c mice and a concomitant increase of SLAMf1^{high}SLAMf4^{low}SLAMf6^{low} iNKT cells (Figure 2B). Similarly, BALB/c spleen and lung had an increased abundance of SLAMf6^{high} NKT2 cells, and livers of both strains were dominated by SLAMf1^{int}SLAMf4^{low}SLAMf6^{int} NKT1 cells (Figure 2B). Taken together, these data suggest that SLAM family receptor expression profiles stably mark functionally distinct NKT cell subsets in distinct peripheral tissues.
2.3.3 SLAM family receptor co-expression profiles on iNKT cell subsets are established during thymic development

NKT1, NKT2, and NKT17 subsets are established during thymic development (Lee et al., 2013; Lee et al., 2015). Therefore, we asked whether the SLAM family receptor expression profiles observed in the periphery were established coincident with NKT cell subset development in the thymus. Analysis of CD4^+CD8^+ double positive (DP) thymocytes, CD4 and CD8 single positive (SP) thymocytes, and NKT cells in the thymus revealed that DP thymocytes were uniformly SLAMf1^{high}SLAMf4^{low}SLAMf6^{high}, consistent with previous reports (Chen et al., 2017; Griewank et al., 2007; Jordan et al., 2007a). Expression of SLAMf6 decreased significantly in CD4 and CD8 SP populations, while SLAMf1 decreased only slightly (Figure 3A). In contrast to the homogeneous SLAM family receptor expression on conventional T cells, thymic iNKT cells exhibited distinct SLAM family receptor expression profiles. We observed a distinct SLAMf1^{low}SLAMf4^{low}SLAMf6^{high} population, as well as a SLAMf1^{low}SLAMf4^{high}SLAMf6^{low} population (Fig. 3A).

We next investigated whether the thymic SLAM family receptor profiles varied in a strain-dependent manner as was observed in the peripheral tissues. Interestingly, we noted an increased percentage of iNKT cells from C57BL/6 mice that were SLAMf1^{neg}SLAMf4^{neg}SLAMf6^{neg} (Figure 3B). Conversely, we observed an enrichment of SLAMf6^{pos} iNKT cells in BALB/c mice, consistent with increased NKT2 and NKT17 cells in this strain (Lee et al., 2015) (Figure 2B and 3E). Strain-dependent variation in
SLAM family receptor expression profiles were not only confined to iNKT cells, as we noted significant differences between C57BL/6 and BALB/c conventional αβ T cells (Figure 3B). Taken together, these data suggested that the SLAM family receptor expression profiles on iNKT cells and conventional T cells observed in the peripheral tissues are established during thymic development and that the distinct expression profiles are established upon divergence of the NKT and conventional αβ T cell lineages.

The classic developmental stages for thymic iNKT cells are defined by the expression of CD44 and NK1.1 (Benlagha et al., 2002; Pellicci et al., 2002): Stage 1 is CD44\textsuperscript{neg}NK1.1\textsuperscript{neg}, Stage 2 is CD44\textsuperscript{high}NK1.1\textsuperscript{neg}, and Stage 3 is CD44\textsuperscript{high}NK1.1\textsuperscript{high} (Figure 3C/D). We confirmed recent reports that developmental stages defined by CD44 and NK1.1 are roughly correlated with the NKT1, NKT2, and NKT17 subsets (Fig. 3C). We next analyzed the SLAM family receptor co-expression on each of the iNKT cell stages. We found that the SLAM family receptor expression patterns mirrored the expression patterns we observed on the iNKT cells after gating with PLZF and RORγt in the periphery (Figure 3D). Stage 1 iNKT cells expressed both SLAMf1 and SLAMf6 which was indicative of both an NKT2 and NKT17 phenotype (Figure 3D). Stage 2 iNKT cells express high SLAMf6, reminiscent of NKT2 cells (Figure 3D). Finally, the majority of Stage 3 iNKT cells are low in both SLAMf1 and SLAMf6, with a subpopulation of cells expressing SLAMf4 (Figure 3D), which is indicative of an NKT1 phenotype.

Finally, we assessed whether the distinct marking of iNKT cell subsets by SLAM family receptor expression profiles was strain-dependent in the thymus as was observed in the periphery. We found that the NKT1 subset in both strains exhibited two distinct
populations, one that was $SLAMf1^{\text{int}}SLAMf4^{\text{low}}SLAMf6^{\text{low}}$ and a second that was $SLAMf1^{\text{low}}SLAMf4^{\text{high}}SLAMf6^{\text{low}}$ (Fig. 3E). In contrast, NKT2 cells were characterized by a $SLAMf1^{\text{low}}SLAMf4^{\text{low}}SLAMf6^{\text{high}}$ phenotype (Figure 3E). NKT17 cells in both strains also showed two populations of cells, $SLAMf1^{\text{high}}SLAMf4^{\text{low}}SLAMf6^{\text{high}}$ and $SLAMf1^{\text{high}}SLAMf4^{\text{low}}SLAMf6^{\text{low}}$. Intriguingly, the SLAM family receptor expression profiles in either C57BL/6 or BALB/c marked the same thymic iNKT cell subsets. Together, these data suggest that the SLAM family receptor expression profiles that mark functionally distinct NKT cell subsets in the periphery are established during thymic development.

### 2.3.4 SLAMf6 deficiency results in a defect in NKT17 cells and IL-17 production

Given our findings that SLAMf6 was preferentially expressed on NKT2 and NKT17 cells, we investigated whether SLAMf6 played a significant role in the development of these specific NKT cell subsets. We first compared the number of iNKT cell subsets between C57BL/6 and SLAMf6−/− mice. We observed a significantly lower number of thymic NKT2 and NKT17, but not NKT1, subsets in SLAMf6−/− mice compared to their C57BL/6 counterparts (Figure 4A). Analysis of the splenic NKT cell subsets revealed a significant decrease in the number of NKT17, but not NKT1 cells (Figure 4B). While there was a decrease in the number of iNKT2 cells in the spleen, this did not reach statistical significance (Figure 4B). Therefore, these data suggested that the NKT cell subsets on which SLAMf6 is preferentially expressed are present at significantly lower numbers in SLAMf6-deficient mice.
Next, we investigated whether SLAMf6-deficient mice exhibited an impaired ability to produce NKT2 and/or NKT17 cytokines. We sorted spleen NKT cells from C57BL/6 and B6.Slamf6−/− mice and cultured them for 72 h on anti-CD3-coated plates. An analysis of NKT cell cytokine production revealed a significant decrease in the production of IL-2 and IL-17 in SLAMf6−/− NKT cells (Figure 4C). We observed no difference between the two mouse strains in IL-4, IL-5, or IL-13 production (Figure 4C). These data indicated that SLAMf6-deficient NKT cells exhibited an impaired ability to produce IL-17, consistent with the lower numbers of NKT17 cells in these mice. To investigate whether NKT17 cells were impaired in their ability to produce IL-17, we compared IL-17 production between B6 and B6.SLAMf6−/− NKT17 cells. To identify NKT17 cells in B6.SLAMf6−/− mice, we used the surrogate markers ICOS (Cameron and Godfrey, 2018; Engel et al., 2016) and NRP-1 (Milpied et al., 2011), which allowed us to identify the NKT2 and NKT17 populations that also express high levels of SLAMf6 (Figure 4D). In our hands, we found that ICOS^hi^NRP-1^hi^ cells contain the NKT2 and NKT17 populations, while the ICOS^lo^NRP-1^lo^ subset contains the NKT1 cells (Figure 4D).

We sorted ICOS^lo^NRP-1^lo^ and ICOS^hi^NRP-1^hi^ iNKT subsets from C57BL/6 and B6.SLAMf6−/− mice and assessed IL-17 production after culturing equal numbers of cells for 72 h on anti-CD3-coated plates (Figure 4E). As expected, there was a low level of detectable IL-17 produced in the ICOS^lo^NRP-1^lo^ population in both the wild-type and B6.SLAMf6−/− mice, consistent with the prevalence of NKT1 cells in this population (Figure 4E). Interestingly, IL-17 production in the ICOS^hi^NRP-1^hi^ population was 2.5
fold lower in the NKT17 cells from B6.SLAMf6−/− mice. More surprising was that the IL-4 production from the ICOS^{high}NRP-1^{high} NKT2 and NKT17 B6.SLAMf6−/− population was also significantly decreased (Figure 4E). Taken together, these data suggested that SLAMf6 plays a critical NKT2/17 subset specific role in regulation of NKT IL-17 and IL-4 production.
2.4. Discussion

SLAM family receptors are widely expressed on all hematopoietic cells and individual leukocyte populations are known to express a distinct, characteristic complement of SLAM family receptors (Cannons et al., 2011). Here, we find that examination of just 3 SLAM family receptors reveals distinct patterns of SLAM family receptor co-expression that not only distinguish iNKT cells from their conventional αβ T cell counterparts, but distinguish functionally distinct subsets of iNKT cells. Furthermore, we find that these SLAM receptor expression profiles stably mark iNKT cell subsets in naïve mice in different tissues and in different strains of mice. Our finding that the development and function of SLAMf6\textsuperscript{high} NKT2 and NKT17 subsets are dependent on SLAMf6 indicates a segregation of SLAM receptor roles in regulating iNKT cell function not previously appreciated. Indeed, these findings are reminiscent of a recent report demonstrating that SLAM receptor expression profiles defined functionally distinct populations of hematopoietic stem cells (Oguro et al., 2013). In addition, recent findings from our laboratory indicate the presence of SLAM receptor expression profiles that distinguish functional γδ subsets in mice (manuscript in preparation). These findings demonstrate that the heterogeneity in SLAM family receptor expression is much greater than previously thought, and suggest that discrete modulation of the array of SLAM family receptors expressed by a cell may be a mechanism used to fine-tune cell function.

Despite the critical role played by the SLAM/SAP signaling pathway in iNKT cell development, elucidation of the specific functions regulated by individual SLAM family receptors has proven difficult. While deletion of multiple SLAM family receptors
results in impaired iNKT cell function (Hu et al., 2016), it has been difficult to identify significant impairments in iNKT cell function in mice deficient in only a single SLAM family receptor. One study using an inducible conditional SAP knockout mouse to delete SAP after iNKT cells had developed, reported that deletion of SAP had no effect on IFN-γ or IL-4 production and found a defect only in iNKT cell cytotoxicity (Das et al., 2013). Our data demonstrating that SLAMf6 specifically regulates NKT2 and NKT17, but not NKT1, is consistent with this finding since most iNKT cell cytotoxicity is mediated by NKT1 cells. In addition, our data indicate that after in vivo αGalCer administration, the vast majority of IL-4 is produced by the predominant NKT1 population and not by the minor NKT2 population (data not shown), providing a possible explanation for why this defect was not previously observed. This would be consistent with a recent report demonstrating that all thymic iNKT subsets, including NKT1 cells, can produce IL-4 after stimulation (Cameron and Godfrey, 2018; Georgiev et al., 2016; Tang et al., 2014).

Using a Vα14 transgenic mouse to force iNKT cell development in the absence of SAP, Michel et al., demonstrated that iNKT cells developing in the absence of SAP exhibited a marked defect in NKT2 cells but an expansion of NKT17 cells (Michel et al., 2016). These data are in partial agreement with our observation that mice deficient in SLAMf6, which signals using the SAP adapter, are also deficient in NKT2 cells. While our data differed in that we observed a reduction in NKT17 cells, this discrepancy could be due to either the different experimental approaches used, or it could point to the involvement of additional SLAM family receptors in the regulation of the NKT17 subset.
Studies using SLAMf1/5/6 triple knockouts demonstrated significantly reduced numbers of iNKT cells versus those observed in SLAMf6–deficient mice (Huang et al., 2016), suggesting that SLAMf1, SLAMf5, and SLAMf6 can each contribute to the positive signaling required for iNKT cell development (Chen et al., 2017). Our data, combined with these previous reports, suggest that SLAMf6 may have the greatest effect on the development of NKT2 and NKT17 cells while SLAMf1 and SLAMf5 may affect NKT1 cells specifically, or provide compensatory signaling in the absence of SLAMf6 on this subset.

Our data suggest that SLAMf6 has a specific effect on iNKT cell IL-17 production. Unlike the case with IL-4, which can be produced by NKT1 and NKT2 cells after stimulation by αGalCer, we found that only NKT17 cells produced IL-17 consistent with a recent report (Cameron and Godfrey, 2018). SLAMf6 has previously been implicated in the production of IL-17 (Tang et al., 2014). In a study comparing the cytokine production of splenic IL-17 producing iNKT cells, SLAMf6\textsuperscript{low}CD4\textsuperscript{neg}NK1.1\textsuperscript{neg} NKT17 cells produced IL-17, whereas SLAMf6\textsuperscript{high}CD4\textsuperscript{pos}NK1.1\textsuperscript{neg} produced IL-21. While the reason for these discrepant findings is not clear, they may result from the use in that study of autoimmune-prone NZB/W mice. In addition, we note that a small percentage of CD4\textsuperscript{neg}SLAMf6\textsuperscript{high} cells, which comprise up to 30-50% of the NKT17 cells in the spleen (Cameron and Godfrey, 2018), were not analyzed (Tang et al., 2014).

*Slam* family genes are highly polymorphic and these polymorphisms are known to have functional consequences. Recently, it was shown that mice that possess *Slam* haplotype 2 as opposed to *Slam* haplotype 1 alleles exhibit a decreased NKT1/NKT2
ratio while the NKT17 subset was unaffected (Baglaenko et al., 2017). There are profound differences in the level of SLAM receptor expression as well as receptor signaling capabilities between the Slam haplotypes (Wandstrat et al., 2004). For example, Slam haplotype 1 contains an isoform for SLAMf6 that is more inhibitory than the isoform present in Slam haplotype 2 expressing animals (Keszei et al., 2011). SLAMf1 and SLAMf6 receptor expression is decreased in Slam haplotype 2 animals, while SLAMf3 expression is increased (Baglaenko et al., 2017). Our data demonstrating that SLAMf6 plays a role in NKT2 development suggests the possibility that the strain-dependent differences in NKT2 subsets result from the differing signaling capabilities of the SLAMf6 isoforms expressed in each of these strains.

Together, our data linking SLAM family receptor expression profiles with functional iNKT cell subsets provides a new conceptual framework that may help elucidate the specific roles of SLAM family receptors on iNKT cells as well as other leukocyte subsets. Specifically, the data demonstrating that SLAMf6 specifically regulates NKT2 and NKT17 development could represent an important link in our understanding the connection between iNKT cell development and iNKT cell functional programming.
2.5. Materials and Methods

2.5.1 Mice and Reagents

Female C57BL/6 and BALB/cJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were bred at the University of Vermont. SLAMf6−/− mice were described previously (Dutta et al., 2013). Mice were bred and housed in the specific pathogen-free barrier facility at the University of Vermont. All procedures involving animals were approved by the University of Vermont Institutional Animal Care and Use Committee.

2.5.2 Flow cytometry

Splenocytes and thymocytes were obtained by gently pressing through a 70 µm nylon mesh. Liver leukocytes were obtained by gently pressing through a 70 µm nylon mesh, followed by washing in PBS, and separation of hepatocytes via a 33.8% Percoll gradient (GE Healthcare, Chicago, IL) as described (Rymarchyk et al., 2008). Lung leukocytes were isolated via mechanical dissociation followed by enzymatic degradation using 200µg/mL DNase (Sigma Aldrich, St. Louis, MO) and 1mg/mL Collagenase Type IV (Life Technologies, ThermoFisher, Waltham, MA) as described (Benoit et al., 2015). RBC’s were lysed using Gey’s solution. Cells were stained at 4°C in PBS + 2% FBS containing 0.2% sodium azide for 30 min., after which they were washed and resuspended in PBS + 1% PFA when appropriate. Surface antibodies used in these studies were anti-CD16/32 (Fc block), CD4, CD45, TCRβ, NK1.1, Nkp46, CD8,
SLAMf1, SLAMf4, SLAMf3, SLAMf2, SLAMf5, SLAMf6, ICOS, and NRP-1 (Biolegend, San Diego, CA). Additional antibodies CD11b, CD19, and CD44 were obtained from BD biosciences. UV Live Dead staining reagent was included in all experiments (Life Technologies, ThermoFisher, Waltham, MA). CD1d tetramer loaded with PBS-57 was provided by the National Institutes of Health (NIH) tetramer facility (Emory University Vaccine Center, Atlanta, GA).

For nuclear transcription factor staining, cells were surface stained as above, after which they were fixed overnight using the Foxp3 transcription factor staining buffer set (EBiosciences, ThermoFisher, Waltham, MA). The next day cells were washed, blocked with rat IgG, and stained with anti-PLZF (9E12) and RORγt (Q31-378) (BD Biosciences). After staining, cells were washed, resuspended, and data were immediately collected on a LSRII flow cytometer (BD Biosciences, Franklin Lake, NJ) and analyzed with FlowJo software (FlowJo LLC).

### 2.5.3 Analysis of SLAM receptor expression patterns

Three-way analysis of SLAM receptor expression was performed with combinatorial gating using FlowJo software (FlowJo LLC). In brief, iNKT cells were first identified using anti-TCRβ and CD1d tetramer. Positive SLAMf1, SLAMf4, and SLAMf6 gates were identified using specific SLAM fluorescence minus-one controls. Combination gates were then applied for the positive gates resulting in multiple combination averages of the permutations of SLAM receptor expression on iNKT cells.
When appropriate, percent expression for multiple mice were averaged and plotted in GraphPad Prism (GraphPad Software, San Diego, CA).

2.5.4 Statistics

All animal experiments were conducted in a non-randomized and non-blinded fashion. Statistical analyses were performed using an unpaired T test or multiple T tests where appropriate. In all cases, tests were considered significant when \( p \leq 0.05 \). All groups were shown to have similar variance in distribution. Sample size estimates were based on previous experience. When data were combined from multiple different experiments, data were first normalized to the C57BL/6 reference strain in each experiment. Normalization was accomplished by dividing each data point in an experiment by the average of the C57BL/6 response in that experiment. All statistical analysis was performed using Graph Pad Prism (GraphPad Software, San Diego, CA).

2.5.5 Cell isolation and culture for ex vivo cytokine analysis

Splenocytes were enriched for NKT cells using the STEMCELL EasySep CD4+ negative selection kit according to the manufacturer’s instructions. After which, cells were stained with Live/Dead efluor780 (EBiosiences), anti-TCR\( \beta \), CD11b, CD8, Nkp46, ICOS and NRP-1, and the addition of CD1d/PBS-57 loaded-tetramer. NKT cells were subsequently purified by fluorescent activated cells sorting (FACS Aria III). Cell purity for all sorts was between 80% and 95%. After sorting, cells were placed in modified Click’s culture medium (5% FBS, 2mM glutamine, Pen/Strep, 50\( \mu \)M 2-mercaptoethanol,
20µg/mL gentamycin) and incubated on anti-CD3 (5 µg/ml) and anti-CD28 (1 µg/ml)-coated plates at 37 °C and 5% CO₂. 72 h later, supernatants were harvested and analyzed via Milliplex assay or ELISA, according to the manufacturer’s instructions (EMD Millipore, Darmstadt, Germany). The milliplex kits used were the Mouse cytokine/chemokine magnetic bead panel followed by a custom kit containing GM-CSF, IFNγ, IL-2, IL-4, IL-5, IL-10, IL-13, and IL-17.

2.5.6 ELISA

Interleukin-4 and IL-17 ELISAs were performed on cultured supernatants from sorted NKT cell cultures as above. IL-4 capture was plated on a COSTAR high protein binding plate overnight at 4°C. After which, the plate was washed, blocked, and detection, strep-HRP, TMB substrate, and stop solution added after the appropriate incubations. All IL-4 reagents were obtained from Biolegend. TMB substrate was from BD Biosciences. IL-17 ELISA was performed via the R&D mouse IL-17 Duo kit according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN).
2.6. Acknowledgements

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2.7. References


2.8. Figures

C57BL/6

Spleen
Liver
Lung

BALB/c

Spleen
Liver
Lung

NKT

Conv. T

Bar charts showing the percentage of NKT and Conv. T cells in various tissues for C57BL/6 and BALB/c strains.
FIGURE 2-1: Strain-dependent and organ-specific heterogeneity in SLAM family receptor co-expression profiles on iNKT cells.

Representative contour plots of SLAMf1, SLAMf4, and SLAMf6 receptor expression on spleen, liver, and lung conventional αβ T cells and iNKT cells from B6 (upper) and BALB/c mice (lower). iNKT cells and conventional αβ T cells were identified using CD1d-tetramer/PBS57 and TCRβ. Three-way expression profiles of the different SLAM receptors were calculated using combination gating and are presented as a percentage of total iNKT or αβ T cells. Data is representative of 4 separate experiments using B6 mice (n =18 mice), and 2 separate experiments using BALB/c mice (n = 10 mice).
FIGURE 2-2: SLAM family receptor co-expression marks iNKT cell subsets in peripheral tissues.

iNKT cell subsets from the spleen, lung, and liver were identified using CD1d-tetramer/PBS57 and TCRβ followed by staining for transcription factors. A). SLAM family receptor expression profiles on spleen iNKT cell subsets. Top: Gating scheme used for identification of iNKT cell subsets using PLZF and RORγt expression. Bottom: Co-expression of SLAMf1, SLAMf4, and SLAMf6 on iNKT1 (green), iNKT2 (red), and iNKT17 (blue) subsets. B). Strain-dependent and organ-specific heterogeneity in SLAM receptor co-expression profiles on iNKT cell subsets. Representative flow cytometry
comparing SLAMf1, SLAMf4, and SLAMf6 receptor expression in C57BL/6 mice (*top*) and BALB/c mice (*bottom*). Overlays of NKT1 (*grey*), NKT2 (*red*), and NKT17 (*blue*) subsets reveal that SLAM family receptor expression profile marking of NKT cell subsets are relatively stable amidst strain-dependent and organ-specific variability in the frequency of NKT cell subsets. Data is representative of 4 separate experiments using B6 mice (total of 18 mice), and 2 separate experiments using BALB/c mice (total of 10 mice). All mice were age- and sex-matched in each experiment.
FIGURE 2-3: SLAM family receptor expression profiles are established during thymic development.

Thymic iNKT cells and conventional αβ T cells from C57BL/6 or BALB/c mice were identified using TCRβ and CD1d-tetramer/PBS-57 or CD4 and CD8, respectively. A). Representative flow cytometry plots of SLAM family receptor expression profiles on C57BL/6 thymic CD4+ , CD8+ , DP, and iNKT cells. Examination of SLAMf1, SLAMf4, and SLAMf6 co-expression revealed heterogeneous co-expression on iNKT cells but not conventional T cells. B). Cumulative SLAM family receptor co-expression profiles on thymic iNKT and conventional T cells from C57BL/6 and BALB/c mice. C). iNKT cell subset profiles correlate with C57BL/6 iNKT cell developmental stages. Representative flow cytometry of iNKT subsets identified using PLZF and RORγt, followed by analysis of CD44 versus NK1.1 expression. D). Distinct SLAM family receptor expression profiles associated with NKT cell developmental stages. Thymic NKT cells were identified using TCRβ and CD1d-tetramer/PBS57, then stained with CD44 and NK1.1 to identify classical NKT cell developmental stages and SLAMf1, SLAMf4, and SLAMf6 expression was evaluated. Representative flow cytometry of SLAMf1, SLAMf4, and SLAMf6 co-expression on Stage 1 (blue), Stage 2 (red), and Stage 3 (green) iNKT cells. E). Strain-dependent SLAM family receptor expression profiles are established during thymic development. Representative flow cytometry plot overlays of SLAMf1, SLAMf4, and SLAMf6 expression on NKT1 (grey), NKT2 (red), and NKT17 (blue) subsets. Data are representative of 2 separate experiments, n= 9 mice per strain per experiment. Mice were age- and sex- in each experiment.
FIGURE 2-4: SLAMf6-dependent NKT2 and NKT17 development and function.

A). Decrease in thymus iNKT2 and iNKT17 subsets in SLAMf6⁻/⁻ animals. *Top:* Representative flow cytometry plots of thymic iNKT cell subsets defined using PLZF and RORγt staining. Subsets are indicated by colored boxes: NKT1 (green), NKT2 (red), and NKT17 (blue). *Bottom:* Relative number of iNKT cell subsets in B6 and SLAMf6⁻/⁻ animals. Data are the cumulative quantification of 4 separate experiments. Statistical analysis was performed using an unpaired T test. *p ≤ 0.05, **p ≤ 0.01*

B). Decrease in spleen NKT17 cells in SLAMf6⁻/⁻ animals. *Upper:* Representative flow cytometry of iNKT cells and iNKT cell subsets between C57BL/6 and SLAMf6⁻/⁻ animals identified as above. *Lower:* Relative number of total splenic iNKT cells and iNKT cell subsets between B6 and SLAMf6⁻/⁻ animals. Data are the cumulative quantification of 4 separate experiments. Statistical analysis was performed using an unpaired T test. **p ≤ 0.01*

C). NKT-intrinsic reduction in IL-17 production from SLAMf6⁻/⁻ iNKT cells. iNKT cells from C57BL/6 and SLAMf6⁻/⁻ animals were sorted and incubated on anti-CD3-coated plates for 72 h, after which supernatant cytokine levels were assessed using Milliplex. Data represent the cumulative results of 2 separate experiments. Statistical analysis was performed using an unpaired T test. ***p ≤ 0.001, ****p ≤ 0.0001*

D). Confirmation of NKT2-specific surrogate markers ICOS and NRP-1 for use in sorting. *Top:* iNKT cell subsets (shown in colored boxes) were evaluated for ICOS, NRP-1, and SLAMf6 expression. All three proteins marked NKT2 and NKT17 subsets. *Bottom:* Gating
strategy for isolating specific iNKT1 versus iNKT2 and iNKT17 subsets using ICOS and NRP-1. Representative flow cytometry of iNKT cells identified using CD1d-tetramer/PBS57 and TCRβ. Middle: ICOS\textsuperscript{high}NRP1\textsuperscript{high} identify NKT2 and NKT17 cells while ICOS\textsuperscript{low}NRP1\textsuperscript{low} identify NKT1 cells in both C57BL/6 and SLAMf6\textsuperscript{-/} mice. E). Significantly reduced IL-17 and IL-4 production from sorted ICOS\textsuperscript{high}NRP-1\textsuperscript{high} NKT2/NKT17 subsets in Slamf6\textsuperscript{-/} mice. Sorted NKT2/NKT17 and NKT1 subsets were stimulated on anti-CD3-coated plates for 72hrs. IL-4 and IL-17 production from supernatants were determined via ELISA. Data are representative of 2 separate experiments. Statistical analysis was performed using multiple T tests. **p ≤ 0.01 ****p ≤ 0.0001. All mice were 6-9wk old females in each experiment.
CHAPTER 3: IDENTIFICATION OF A SLAMF6-DEPENDENT PROLIFERATIVE SUBSET OF NKT CELLS

Running Title: SLAMf6 is crucial for iNKT cell proliferation

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3.1. Abstract

Semi-invariant natural killer T cells (iNKT) are important mediators of host immunity. This innate-T cell subset is now thought to encompass specific subsets responsible for the production of IFNγ (NKT1), IL-4 (NKT2), and IL-17 (NKT17). While these cell subsets are distinct within the thymus, less is known about the regulation of iNKT cell subsets in the periphery. Regulation of iNKT cells in general is thought to occur via the signaling lymphocyte activation markers (SLAMs), which are required with the SLAM family receptor adaptor protein (SAP) for the development of iNKT cells. A complete analysis of SLAM family receptors on iNKT cell subsets has not been completed. Here, we show that SLAMf6, which can have both activating and inhibitory function on iNKT cells, is responsible for cytokine production and the proliferation of NKT2 and NKT17 subsets after activation. Additionally, we show preferential expansion of an NKT2 cell like population 3 days after activation with the iNKT cell agonist αGalCer. These data reveal a role for SLAMf6 on the regulation of distinct NKT2 and NKT17 subsets in the periphery. Our data also challenge the definitions of iNKT cell subsets, revealing potential changes to the iNKT cell milieu after activation. Taken together, these data improve our understanding of the mechanisms regulating NKT1, NKT2, and NKT17 behavior upon activation in the periphery.
3.2. Introduction

Invariant natural killer T (iNKT) cells are innate-like T cells that play critical roles in the host immune response (Behar and Porcelli, 2007; Godfrey and Rossjohn, 2011; Gumperz and Brenner, 2001). iNKT cells recognize glycolipids bound by the MHC class-I like molecule CD1d (Kawano et al., 1997; Spada et al., 1998). When activated by the prototypical agonist alpha-galactosylceramide (αGalCer), iNKT cells produce a wide variety of cytokines such as IFNγ, IL-4, TNFα, IL-17, and IL-10 (Coquet et al., 2008; Hameg et al., 2000; Yoshimoto and Paul, 1994). During iNKT cell development, three distinct iNKT cell subsets have been described: NKT1, which produces IFN-γ and is characterized by low PLZF and high T-bet expression, NKT2, which produces IL-4, IL-5, and IL-13 and is characterized by high PLZF expression, and NKT17, which produces primarily IL-17 and is characterized by high RORγt expression (Lee et al., 2013; Lee et al., 2015).

NKT cell development is highly dependent on signaling by multiple SLAM family receptors and their associated signal adapter protein SAP (Griewank et al., 2007; Nichols et al., 2005a; Sintes et al., 2013). The best characterized interactions are SLAMf1-SLAMf1 and SLAMf6-SLAMf6 interactions that are thought to occur between developing iNKT cells and neighboring DP thymocytes that express high levels of both of these proteins as well as CD1d (Coles and Raulet, 2000; Gapin et al., 2001; Griewank et al., 2007). Multiple engagements of different SLAM family receptors appear to be necessary for iNKT cell development as mice deficient in either SLAMf1 or SLAMf6 alone are able to mediate selection of iNKT cells, albeit to lower levels than their wild-
type counterparts (Griewank et al., 2007; Huang et al., 2016). Although SLAM family receptor signaling is critical for iNKT cell development, the exact mechanisms through which they act is still unclear. Accumulating data suggest that SLAMf6 induces the expression of PLZF and Egr2, transcription factors critical for iNKT cell development (Baglaenko et al., 2017; Dutta et al., 2013; Tuttle et al., 2018). However, it has also been reported that SLAMf6 possesses inhibitory functions that are modulated by the SAP signaling adapter (Kageyama et al., 2012). Finally, very little is known about the role of SLAMf6 in regulation of the development of different iNKT cell subsets and how this affects iNKT cell function in peripheral tissues.

We have previously shown that SLAMf1, SLAMf4, and SLAMf6 are co-expressed in a heterogeneous manner among different iNKT cell subsets (Chapter 2). We found that SLAMf6 was highly expressed on all NKT2 cells and a significant fraction of NKT17 cells, and that SLAMf6 regulated IL-17 and IL-4 cytokine production from NKT17 and NKT2 subsets, respectively (Chapter 2). We next examined whether SLAMf6 could also affect the proliferation of iNKT cells in a subset specific manner.

Here, we report that the SLAMf6-expressing NKT2 and NKT17 subsets in the peripheral tissues encompass the majority of the proliferating NKT cells both in vivo and in vitro. Ki67 and BrdU+ NKT cells are highly enriched in SLAMf6high NKT2 and NKT17 cells. Using surrogate markers ICOS and NRP-1 for NKT2 and NKT17 (without the use of SLAMf6), we showed that NKT2 and NKT17 cells are also more proliferative than NKT1 cells in the B6.SLAMf6−/− mouse. Interestingly, SLAMf6 deficient NKT2 and NKT17 cells did not proliferate to the same level as C57BL/6 iNKT cells. Finally, we
show that NKT2 cells may preferentially expand after activation with the prototypical glycolipid alpha-galactosylceramide (αGalCer). This manuscript supports a model in which SLAMf6 has multiple roles on iNKT cells subsets, not only in activation of cytokine production, but also in maintenance of iNKT cell subset homeostasis.
3.3. Results

3.3.1 SLAMF6 marks highly proliferative NKT2 and NKT17 cells

To investigate the proliferative capacity of thymic iNKT cells, we assessed the expression of Ki67, a marker of cells in either the S or G2/M phase of the cell cycle, on thymic iNKT cell subsets. A comparison of naïve thymic iNKT cell subsets in the C57BL/6 mouse (Figure 1A) revealed significant differences in Ki67 expression among NKT1, NKT2, and NKT17 subsets. Whereas high levels of Ki67 expression were observed in the NKT2 and NKT17 subsets, little Ki67 expression was observed in the NKT1 subset which comprises the majority of thymic iNKT cells (Figure 1A). Calculation of the frequency of Ki67+ iNKT cells revealed that a significantly higher percentage of NKT2 and NKT17 cells were Ki67+ (Figure 1A), indicating that the thymic NKT2 and NKT17 subsets constitute the highly proliferative iNKT cells in the thymus.

Since we previously observed that the population of SLAMf6\textsuperscript{high} NKT cells in the thymus was composed primarily of NKT2 and NKT17 subsets (Chapter 2), we investigated the association of SLAMf6 expression with Ki67. An analysis of total thymic iNKT cells revealed a clear correlation between high SLAMf6 expression and Ki67 expression (Figure 1B). These data indicated that the vast majority of proliferating Ki67+ NKT cells in the thymus were SLAMf6\textsuperscript{high} NKT2 and NKT17 cells.
3.3.2 SLAMf6 marks highly proliferative peripheral NKT2 and NKT17 cells after activation

We next investigated whether SLAMf6 expression on iNKT cells in peripheral tissues was associated with proliferative capacity. First, we confirmed that SLAMf6\textsuperscript{high} iNKT cells marked the NKT2 (PLZF\textsuperscript{high}ROR\textgreek{t}\textsuperscript{low}) and NKT17 (PLZF\textsuperscript{int}ROR\textgreek{t}\textsuperscript{high}) subsets while SLAMf6\textsuperscript{low} iNKT cells marked the NKT1 (PLZF\textsuperscript{low}ROR\textgreek{t}\textsuperscript{low}) and NKT17 spleen iNKT cells (Figure 2A). To determine whether there was a difference in the proliferative capacity of the SLAMf6\textsuperscript{high} (NKT2/NKT17) cells and SLAMf6\textsuperscript{low} (NKT1/NKT17) iNKT cells, we sorted spleen iNKT cells based on their SLAMf6 expression (Supplementary Figure 1) and assessed their proliferation after anti-CD3 stimulation. FACS-sorted iNKT cell subsets were labelled with VioTracker fluorescent dye and cultured for three days on anti-CD3-coated plates after which we assessed proliferation (Figure 2B). Interestingly, we observed a significantly higher number of live SLAMf6\textsuperscript{high} iNKT cells versus their SLAMf6\textsuperscript{low} counterparts, and we found that SLAMf6\textsuperscript{high} iNKT cells had proliferated significantly more than the SLAMf6\textsuperscript{low} iNKT cells (Figure 2B). The association of SLAMf6 with increased proliferative capacity was not specific to C57BL/6 NKT cells, as we also observed increased proliferation in SLAMf6\textsuperscript{high} iNKT cells from BALB/c mice (Supplementary Figure 2). Taken together, these data indicate that SLAMf6 expression marks NKT2 and NKT17 cells that are homeostatically proliferating \textit{in vivo}, and that SLAMf6 expressing NKT2/NKT17 cells exhibit a significantly higher proliferative capacity than their SLAMf6\textsuperscript{low} NKT1 counterparts after stimulation.
3.3.3 Expansion of SLAMf6<sup>hi</sup> NKT2-like cells after in vivo activation

Next, we evaluated the relationship of SLAMf6 expression on iNKT cells and proliferation in vivo after activation with the more physiological CD1d ligand, αGalCer, which induces rapid proliferation of NKT cells (Kawano et al., 1997) (Supplementary Figure 3). First, we assessed SLAM family receptor expression on iNKT cells 3 and 5 days after αGalCer administration. This analysis revealed a 12.5-fold increase in the expression of SLAMf6 on NKT cells 3 days after activation (Figure 3A). In contrast, we observed a 3.5 and 3.2 fold increase in SLAMf1 and SLAMf3 expression respectively at this time (Figure 3A). Interestingly, while iNKT cell SLAMf6 expression in naïve mice was quite heterogeneous, SLAMf6 expression was uniformly high 3 days after activation and cells retained this level of expression 5 days after activation (Figure 3A), at which time iNKT cell numbers were returning to baseline levels (Supplementary Figure 4). In contrast, the expression of SLAMf1 and SLAMf3 had returned to baseline levels 5 days after activation. These data reveal that after activation and expansion of iNKT cells, the iNKT cell population is dominated by SLAMf6<sup>hi</sup> iNKT cells.

Since we have shown that SLAMf6 is preferentially expressed on naïve NKT2 and NKT17 cells, we next determined whether the SLAMf6<sup>hi</sup> iNKT cells present on day 3 exhibited an NKT2 phenotype. A comparison of splenic iNKT cell subsets between vehicle-treated and αGalCer-treated mice revealed a dramatic increase in a population of PLZf<sup>hi</sup>RORγt<sup>low</sup>SLAMf6<sup>hi</sup> iNKT cells (Figure 3B), which was reminiscent of the phenotype of NKT2 cells. To determine the proliferative capacity of the iNKT cell subsets in vivo, we assessed BrdU incorporation after αGalCer administration. B6 mice
were challenged with αGalCer i.p., BrdU was administered 48 h later, and iNKT BrdU incorporation was assessed 72 h after αGalCer administration. After 72hrs, iNKT cells showed increased proliferation, with 20% of all iNKT cells actively incorporating BrdU at this time point (Figure 3C). Interestingly, the only cells that were actively incorporating BrdU were the PLZF<sup>high</sup> iNKT cells (Figure 3C). Together, these data suggested that a PLZF<sup>high</sup> SLAMf6<sup>high</sup> NKT2-like population of iNKT cells preferentially expanded after activation in vivo. Whether these cells are, in fact, NKT2 cells remains unclear since an analysis of the NKT1-specific transcriptional regulator T-bet revealed that it was also highly expressed in the PLZF<sup>high</sup> SLAMf6<sup>high</sup> “NKT2-like” population of iNKT cells 3 days after activation (data not shown), raising the possibility that altered expression of the diagnostic PLZF, RORγt, and T-bet transcription factors after activation may preclude their use for identification of iNKT cell subsets.

3.3.4 Slamf6-dependent proliferation of NKT2/NKT17 subsets

Since our data indicated that SLAMf6 expression was closely associated with iNKT cell proliferation in vivo and in vitro, we next investigated whether SLAMf6 regulated iNKT cell proliferation by assessing the proliferative capacity of SLAMf6-deficient iNKT cell subsets. Since our previous results suggested that identification of iNKT cell subsets in vivo after activation was difficult, we compared proliferation in vitro using anti-CD3 stimulation. To identify iNKT cell subsets in both C57BL/6 and B6.SLAMf6<sup>−/−</sup> mice, we employed the previously identified surrogate markers ICOS (NKT2) and NRP-1 (NKT2 and NKT17) (See Chapter 2, Figure 4D) that we
demonstrated reproducibly distinguished the NKT1 subset from the NKT2/NKT17 subsets in both B6 and B6.SLAMf6<sup>−/−</sup> mice (Chapter 2).

We sorted ICOS<sup>high</sup>NRP-1<sup>high</sup> (NKT2/NKT17) and ICOS<sup>low</sup>NRP-1<sup>low</sup> (NKT1) splenic iNKT cells, stimulated equal numbers of cells with anti-CD3 and anti-CD28, and assessed the number of viable cells after 72 hours. As expected, we observed a significantly higher number of live ICOS<sup>high</sup>NRP-1<sup>high</sup> NKT2/NKT17 cells compared to their ICOS<sup>low</sup>NRP-1<sup>low</sup> NKT1 counterparts (Figure 4). Interestingly, we observed a striking decrease in the number of the ICOS<sup>high</sup>NRP-1<sup>high</sup> NKT2 cells in B6.SLAMf6<sup>−/−</sup> mice compared to their C57BL/6 counterparts (Figure 4). Taken together, these data reveal a specific role for SLAMf6 in the regulation of SLAMf6<sup>high</sup> NKT2/NKT17 iNKT cell proliferation after activation.
3.4. Discussion

SLAM family receptors and the signaling adapter protein SAP play critical roles in iNKT cell development (Griewank et al., 2007; Kageyama et al., 2012; Nichols et al., 2005a). However, elucidation of the specific roles that individual SLAM family receptors play in iNKT cells has been difficult. Here, we show that SLAMf6 is highly expressed on homeostatically proliferating iNKT cells that we identify as NKT2 and NKT17 subsets. In addition, we demonstrate that SLAMf6 controls activation-induced proliferation of NKT2 and NKT17, but not NKT1, subsets. These data not only reveal a specific role for SLAMf6 in iNKT cell function, but also indicate that functions of individual SLAM family receptors are not evenly distributed among each of the iNKT cell subsets.

Our finding that SLAMf6 controls the proliferation of NKT2 and NKT17 subsets is consistent with recent transcriptomic studies of thymic iNKT cell subsets which found that the NKT2 subset is enriched in both SLAMf6 and cell cycle gene transcripts (Engel et al., 2016; Georgiev et al., 2016). In addition, recent reports demonstrate that thymic NKT2 and NKT17 cells are significantly more proliferative than their NKT1 counterparts (Cameron and Godfrey, 2018) and that all iNKT cell subsets pass through an IL-13-expressing (i.e., NKT2-like) intermediate during thymic development (O'Brien et al., 2016). These studies suggest that the NKT2 and NKT17 subsets may represent a cycling population that is a precursor to the NKT1 subset (Engel et al., 2016; Lee et al., 2015). Together with these findings, our data suggests that SLAMf6 may function at an early stage of thymic development to foster cycling and expansion of the NKT2 and NKT17 subsets. Our data are not consistent, however, with a model in which NKT2 and NKT17...
subsets are precursors to NKT1 cells since the absence of SLAMf6 did not have significant impact on the NKT1 subset.

Consistent with its proliferative phenotype, we observed an increase in an “NKT2-like” population of iNKT cells 3 days after αGalCer administration. These cells expressed high levels of PLZF and SLAMf6, but also expressed high levels of T-bet, making an unambiguous NKT subset identification difficult. The expanded iNKT cells could be NKT1 cells exhibiting increased PLZF expression after activation, but most reports suggest that PLZF is only induced after TCR signaling in the thymus and is not inducible on mature T cells (Savage et al., 2011; Zhang et al., 2015). While T-bet is widely used as a marker of NKT1 cells, a population of PLZF<sup>high</sup>Tbet<sup>high</sup> NKT2 precursors has been described (Engel et al., 2016; Pellicci et al., 2002), which could represent the SLAMf6<sup>high</sup> NKT subset that we observe in the spleen 3 days after activation.

Whether highly proliferative SLAMf6<sup>high</sup> NKT2 cells preferentially expand in the periphery or whether they represent recent thymic emigrants is unclear. NRP-1 which is highly expressed on the SLAMf6<sup>high</sup> NKT2 and NKT17 subsets has been suggested to mark recent thymic emigrants (Milpied et al., 2011), and it was demonstrated that NKT2 cells express more homing markers than do NKT1 cells, and that NKT2 and NKT17 emigrate from the thymus at higher rates than NKT1 (Georgiev et al., 2016). After activation with αGalCer, iNKT cells either downregulate their TCR or undergo activation induced cell death (Subleski et al., 2011; Wilson et al., 2003). Liver iNKT cells, which are comprised of primarily NKT1 cells (Lee et al., 2015), appear to rapidly undergo cell
death within 24 h of αGalCer treatment, which is followed by repopulation of the liver with iNKT cells that are ICOS$^{\text{high}}$TCRβ$^{\text{high}}$NK1.1$^{\text{neg}}$, a phenotype consistent with what we now know are NKT2 cells (Subleski et al., 2011). We report here that these repopulating cells also expressed high levels of SLAMf6, another marker of NKT2 cells. Additional experiments will be needed to determine the relative contributions of the proliferation of peripheral iNKT cells and contributions from the recent thymic emigrant pool in the SLAMf6-dependent proliferation of iNKT cells.

Regulation provided by SLAMf6 is thought to occur at the immunological synapse upon TCR engagement (Tuttle et al., 2018; Zhao et al., 2012). A recent report observed that SLAMf6 crosslinking is required for the upregulation of Egr2, a protein activated downstream of TCR engagement (Tuttle et al., 2018). Once Egr2 expression reached a sustained, high level, PLZf expression was induced, allowing for the differentiation of iNKT cell subsets (Dutta et al., 2013; Tuttle et al., 2018). These data are consistent with previous reports revealing SLAMf6 as an inducer of PLZf on double positive thymocytes (Dutta et al., 2013). Interestingly, we did not see any significant differences in PLZf expression between C57BL/6 and SLAMf6$^{-/-}$ iNKT cell subsets in either the thymus or the spleen (Supplementary Figure 4), suggesting the possibility that other SLAM receptors may be compensating in the absence of SLAMf6.

Although it has been known for some time that both SLAMf1 and SLAMf6 contribute to iNKT cell development (Griewank et al., 2007), the relative contributions of these SLAM receptors has been difficult to define. In this manuscript, we demonstrate a link between SLAMf6 that is highly expressed on NKT2 and NKT17 cells and their
increased proliferative capacity. Our finding that SLAMf6 primarily regulate NKT2 and NKT17 proliferation suggests the possibility that the requirement for both SLAMf1 and SLAMf6 may not simply be due to their additive effects. Rather, each SLAM receptor may play a distinct role in iNKT cell development. These observations may aid in our understanding of how NKT cell subsets develop and proliferate in tissues, which may have important consequences on tissue-specific immunity.
3.5. Materials and Methods

3.5.1 Mice and reagents

C57BL/6 and BALB/cJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME). B6.Slamf6−/− mice have been described previously (Dutta et al., 2013). Mice were bred in the specific pathogen free barrier facility at the University of Vermont. All procedures involving animals were approved by the University of Vermont Institutional Animal Care and Use Committee. αGalCer (Avanti Lipids) was resuspended in PBS + 0.05% Tween20, and administered to mice intraperitoneally (i.p.).

3.5.2 Flow cytometry

Splenocytes and thymocytes were obtained by gently pressing through a 70 μm nylon mesh. Splenic red blood cells were lysed using Gey’s solution. Cells were stained at 4°C in PBS + 2% FBS containing 0.2% sodium azide for 30 min., after which they were washed and resuspended in PBS + 1% PFA when appropriate. Surface antibodies used in these studies were anti-CD16/32 (Fc block), CD4, CD45, TCRβ, NK1.1, Nkp46, CD8, SLAMf1, SLAMf3, SLAMf6, ICOS, and NRP-1 (Biolegend, San Diego, CA). CD11b and CD19 antibodies were obtained from BD Biosciences. UV Live Dead staining reagent was included in surface and transcription factor stains (Life Technologies, ThermoFisher, Waltham, MA), with the exception of stains for cell sorting in which efluor780 Live/Dead stain was used. CD1d tetramer loaded with PBS-57 was provided by the National Institutes of Health (NIH) tetramer facility (Emory University Vaccine Center, Atlanta, GA).
For nuclear transcription factor staining, cells were surface stained as above, after which they were fixed overnight using the Foxp3 transcription factor staining buffer set (EBiosciences, ThermoFisher, Waltham, MA). The next day, cells were washed, blocked with rat IgG, and stained with anti-PLZF (9E12) (Biolegend) and RORγt (Q31-378) (BD Biosciences), and Ki67 (Biolegend). After staining, cells were washed, resuspended, and data were immediately collected on a LSRII flow cytometer (BD Biosciences, Franklin Lake, NJ) and analyzed with FlowJo software (FlowJo LLC).

### 3.5.3 In vitro proliferation of sorted NKT cells

Splenocytes were enriched for NKT cells using the STEMCELL EasySep CD4+ negative selection kit according to the manufacturer’s instructions. After which, cells were stained with Live/Dead efluor780 (EBiosciences), anti-TCRβ, CD11b, CD8, Nkp46, ICOS and NRP-1, and the addition of CD1d/PBS-57 loaded-tetramer. NKT cells were sort-purified using fluorescent activated cell sorting (FACS Aria III). Cell purity for all sorts was between 80% and 95%. After sorting, cells were stained with Viotracker blue for 30min. Subsequently, 40,000 cells/well were placed in modified Click’s culture medium (5% FBS, 2mM glutamine, Pen/Strep, 50µM 2-mercaptoethanol, 20µg/mL gentamycin) and incubated on anti-CD3 (5 µg/ml) coated plates in the presence of soluble CD28 (1 µg/ml) at 37 °C and 5% CO2. 72 h later, supernatants were harvested and analyzed via ELISA (Biolegend and R&D systems).

After culture, supernatants were harvested and media replaced with cold PBS + 2% FBS. Cells were harvested from each well and stained with propidium iodide for
quantification of live/dead. Harvested cells were immediately analyzed on a MACSQuant Vyb flow cytometer. All data was analyzed using FlowJo Software (FlowJo LLC).

### 3.5.4 BrdU incorporation

Bromodeoxyuridine was resuspended in PBS at 10mg/mL. Mice were injected with 1mg 24 hrs prior to the scheduled end-point. A second injection was administered i.p. 4hrs prior to harvest. Cells were surface stained as above, then fixed in 1% PFA overnight. The next day, the BD Biosciences BrdU staining and fixation kit was used according to manufacturer’s instructions to prepare the cells for the transcription factor stain. Antibodies used in these experiments were anti-BrdU, anti-PLZF, anti-RORγt and anti-SLAMf6.

### 3.5.5 Statistics

All animal experiments were conducted in a non-randomized and non-blinded fashion. Statistical analyses were performed using an unpaired T test, multiple T tests, or 2-way ANOVA where appropriate. In all cases, tests were considered significant when p ≤ 0.05. All groups were shown to have similar variance in distribution. Sample size estimates were based on previous experience. When data were combined from multiple different experiments, data were first normalized to the C57BL/6 reference strain in each experiment. Normalization was accomplished by dividing each data point in an experiment by the average of the C57BL/6 response in that experiment. All statistical analyses were performed using Graph Pad Prism (GraphPad Software, San Diego, CA).
3.6. Acknowledgements

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3.7. References


3.8 Figures

**FIGURE 3-1: SLAMf6 expression marks proliferating thymic NKT2 and NKT17 subsets.**

C57BL/6 thymocytes were harvested and iNKT cells identified using CD1d-tetramer/PBS57 and TCRβ. iNKT cell subsets were identified using transcription factors PLZF and RORγt as indicated in the colored boxes A). Thymus NKT2 and NKT17 subsets are highly proliferative in comparison to their NKT1 counterparts. *Upper:* Representative contour plot demonstrating gating strategy to identify NKT cell subsets and median fluorescence intensity of Ki67 on iNKT cell subsets. *Lower:* Quantification of the frequency of Ki67 expression in distinct iNKT subsets. Statistical analysis was performed using multiple t tests followed by the Holm-Sidak multiple comparisons test. ****p ≤ 0.0001 B). Increased SLAMf6 expression on Ki67+ iNKT cells. *Upper:* Representative contour plots of thymus iNKT cells stained with SLAMf6 *Lower:*...
Quantification of the frequency of Ki67+ cells expressing SLAMf6. Data are representative of 2 separate experiments. Statistical analysis was performed using an unpaired t test ****p ≤ 0.0001
FIGURE 3-2: SLAMf6\textsuperscript{high} iNKT cells are more proliferative than SLAMf6\textsuperscript{low} iNKT cells.

C57BL/6 splenic SLAMf6\textsuperscript{high} and SLAMf6\textsuperscript{low} iNKT cells were sorted, plated at equal numbers, and stimulated with anti-CD3 and CD28 for 72hrs. A). Representative flow cytometry gating for sorting iNKT cell subsets. SLAMf6\textsuperscript{high} cells encompass NKT2 and NKT17 cells while SLAMf6\textsuperscript{low} populations are NKT1 and NKT17 cells. B). Representative flow cytometry of live iNKT cells recovered after sorting SLAMf6\textsuperscript{low} and SLAMf6\textsuperscript{high} iNKT cells. Middle: Quantification of live cell counts recovered. Right: Representative flow cytometry histograms of iNKT cells stained with Viotracker and mean fluorescence intensity of either isotype or anti-CD3 stimulated SLAMf6\textsuperscript{high} or
SLAMf6<sup>low</sup> iNKT cells. Data is representative of 3 separate experiments. Statistical analysis was performed using one-way ANOVA followed by Tukey’s multiple comparison test. *** p ≤ 0.001, **** p ≤ 0.0001
FIGURE 3-3: SLAMf6<sup>high</sup>PLZF<sup>high</sup> iNKT cells preferentially expand after in vivo activation.

C57BL/6 mice were treated with either vehicle or αGalCer for 72h. A). All iNKT cells in the spleen after in vivo αGalCer stimulation express high levels of SLAMf6. Representative flow cytometry of SLAMf6, SLAMf1, and SLAMf3 expression of iNKT cells in vehicle treated (heavy dark line), and αGalCer treated (shaded histogram) C57BL/6 mice. Middle: SLAM receptor staining on conventional T cells treated with or without αGalCer. Data is representative of 3 separate experiments. Right: Median fluorescence intensity of SLAMf1, SLAMf3, and SLAMf6 on splenic iNKT cells in naïve (day 0), and αGalCer treated (day 3 and 5) mice. All data is representative of 2 separate experiments at each time point. Statistical analysis was performed using two-way ANOVA followed by Tukey’s multiple comparison test. **** p ≤ 0.0001 B). Expansion of iNKT2-like cells in the spleen after administration of αGalCer. Representative flow cytometry of iNKT cell subsets after gating on SLAMf6<sup>high</sup> and SLAMf6<sup>low</sup> populations before (left) and after αGalCer challenge (right). C). Incorporation of BrdU in iNKT cell subset 72h after αGalCer administration. Upper: Representative flow cytometry of BrdU<sup>+</sup> iNKT cells stained with either isotype control (black) or anti-BrdU (red). Conventional T cell BrdU incorporation is shown for comparison (right). Lower: Representative flow cytometry of PLZF<sup>high</sup> iNKT cells incorporating BrdU. SLAMf6<sup>+</sup> iNKT cell incorporation of BrdU is shown at right.
C57BL/6 naïve splenocytes were harvested, and NKT cells identified using CD1d-tetramer/PBS57 and TCRβ. ICOS^{high}NRP-1^{high} and ICOS^{low}NRP-1^{low} iNKT cells were sorted and plated in equal numbers on anti-CD3 with CD28 for 72hrs. SLAMf6 deficient ICOS^{high}NRP-1^{high} iNKT cells have a defect in cell growth after stimulation. *Left:* Representative flow cytometry of live C57BL/6 (*top*) and B6.SLAMf6^{-/-} (*bottom*) iNKT cells 72hrs after stimulation. *Right:* Live cell number of C57BL/6 versus B6.SLAMf6^{-/-} iNKT cells recovered. Data is representative of 2 separate sorts. Live cell numbers are the combined numbers of two separate experiments normalized to the B6 control. Statistical
analysis was performed using a 2-way ANOVA followed by Sidak multiple comparison test. ***p ≤ 0.001. All C57BL/6 and SLAMf6−/− mice were females aged 7-10wks.
SUPPLEMENTAL FIGURE 3-1: Sorting iNKT cells using SLAMf6 or ICOS and NRP-1 surrogate markers yielded iNKT cells with 78.4-96.6% purity.

A). Representative post-sort analysis sorting iNKT cells using CD1d-tetramer/PBS57 and SLAMf6\(^{\text{high}}\) and SLAMf6\(^{\text{low}}\). B). Representative post-sort analysis sorting iNKT cells using CD1d-tetramer/PBS57 and ICOS\(^{\text{high}}\)NRP-1\(^{\text{high}}\) and ICOS\(^{\text{low}}\)NRP-1\(^{\text{low}}\) in both
C57BL/6 mice (*top*) and B6.SLAM6<sup>+</sup> mice. Data is representative of 2 experiments for each sort. All mice were female and age matched between 7-10wks.

**SUPPLEMENTAL FIGURE 3-2: BALBc SLAMf6<sub>high</sub> splenic iNKT cells are more proliferative than SLAMf6<sub>low</sub> iNKT cells.**

BALBc splenocytes were harvested, sorted after first gating on iNKT cells using CD1d-tetramer/PBS57 and TCRβ, then on levels of SLAMf6. Cells were plated in equal numbers on anti-CD3 with CD28 for 72hrs. *Left:* Representative flow cytometry of live iNKT cells after 72hrs. *Right:* Proliferation of BALBc iNKT cell SLAMf6<sub>high</sub> and SLAMf6<sub>low</sub> iNKT cells as indicated by VioTracker blue proliferation dye. MFI is reported for the anti-CD3 and CD28 stimulated and the isotype control. Data is representative of one experiment. BALBc female mice aged 20wks were used.
C57BL/6 mice were challenged with αGalCer. At each time-point the number of liver iNKT cells were identified using CD1d-tetramer/PBS57 and TCRβ. Data is the cumulative numbers of 3 separate experiments. C57BL/6 female mice aged 10-14wks were used.
SUPPLEMENTAL FIGURE 3-4: C57BL/6 and B6.SLAMf6−/− iNKT cells express similar levels of PLZF.

Naïve thymic (left) and spleen (right) iNKT cells were identified using CD1d-tetramer/PBS57 and TCRβ. The mean fluorescence intensity of PLZF was determined on total iNKT cells and each iNKT cell subset. Data is representative from 1 experiment. Statistical analysis was performed using multiple t test and Holm-Sidak multiple comparisons test. All mice were female aged 14wks.
CHAPTER 4: REGULATION OF IN Variant NK T CELL DEVELOPMENT
AND FUNCTION BY A 0.14 MBP LOCUS ON CHROMOSOME 1: A
POSSIBLE ROLE FOR Fcgr3


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4.1. Abstract

Invariant NKT (iNKT) cells are tissue-resident innate-like T cells critical to the host immune response. We previously identified a 6.6 Mbp region on chromosome 1 as a major regulator of iNKT cell number and function in C57BL/6 and 129X1/SvJ mice. Here, we fine-mapped this locus by assessing the iNKT cell response to alpha-galactosylceramide (αGalCer) in a series of B6.129 congenic lines. This analysis revealed the presence of at least two genetic elements that regulate iNKT cell cytokine production in response to αGalCer. While one of these genetic elements mapped to the B6.129c6 interval containing Slam genes, the dominant regulator in this region mapped to the 0.14 Mbp B6.129c3 interval. In addition, we found that numbers of thymic iNKT cells and DP thymocytes were significantly lower in B6.129c3 mice, indicating that this interval also regulates iNKT cell development. Candidate gene analysis revealed a 5-fold increase in Fcgr3 expression in B6.129c3 iNKT cells, and we observed increased expression of FcγR3 protein on B6.129c3 iNKT cells, NK cells, and neutrophils. These data identify the B6.129c3 interval as a novel locus regulating the response of iNKT cells to glycosphingolipid, revealing a link between this phenotype and a polymorphism that regulates Fcgr3 expression.
4.2. Introduction

Semi-invariant iNKT cells comprise an unusual innate-like T cell subset that plays significant roles in the host immune response to bacterial and viral pathogens (Behar and Porcelli, 2007; Godfrey and Rossjohn, 2011; Gumperz and Brenner, 2001). iNKT cells recognize glycolipids and glycosphingolipids presented by the MHC class I-like molecule CD1d (Kawano et al., 1997; Pellicci et al., 2009; Spada et al., 1998). The prototypical glycosphingolipid agonist alpha-galactosylceramide (αGalCer) is structurally similar to glycosphingolipids from Bacteroides fragilis (Wieland Brown et al., 2013) and is a potent activator of iNKT cells (Borg et al., 2007; Kawano et al., 1997; Kawano et al., 1998; Koch et al., 2005; Takahashi et al., 2000). Upon activation by αGalCer presented by CD1d, iNKT cells rapidly produce large amounts of chemokines and cytokines (Coquet et al., 2008; Gumperz et al., 2002; Yoshimoto and Paul, 1994) and contribute to an orchestrated activation of both innate and adaptive immune cells including dendritic cells, macrophages, and natural killer (NK) cells (Carnaud et al., 1999; Eberl and MacDonald, 2000; Hermans et al., 2003; Kitamura et al., 1999; Sada-Ovalle et al., 2008). The iNKT cell subset, therefore, is uniquely poised to shape the quality and magnitude of the developing host immune response.

Invariant NKT cell number and function varies dramatically among mice of different genetic backgrounds. Wild-derived inbred strains (e.g., PWD/PhJ, Cast/EiJ) have barely detectable numbers of iNKT cells (Borg et al., 2014; Chen et al., 2012), and there is significant strain-dependent variability even among common laboratory inbred strains (Chen et al., 2012; Gombert JM, 1996; Hammond et al., 2001; Lee et al., 2015;
Rymarchyk et al., 2008). Accumulating evidence suggests that the genetic background has a significant influence on the role of iNKT cells in the host immune response. For example, iNKT cells are critical in the clearance of the opportunistic pathogen *Pseudomonas aeruginosa* from the lung in BALB/cJ mice, but are dispensable in C57BL/6J mice (Benoit et al., 2015). Similarly, pathology in iNKT cell-deficient mice infected with *Borrelia burgdorferi* manifests as joint inflammation in BALB/c mice (Tupin et al., 2008) and as myocarditis in C57BL/6J mice (Olson et al., 2009). Therefore, a thorough understanding of the genetic determinants that regulate iNKT cell development and function is necessary to understand the role of iNKT cells in the host immune response.

Numerous reports have described polymorphic genetic loci that regulate iNKT cell number and function (Aktan et al., 2010; Araujo et al., 2000; Borg et al., 2014; Esteban LM, 2003; Fletcher et al., 2008; Jordan et al., 2007b; Wesley et al., 2007; Zhang et al., 2003). We and others have identified a region on chromosome 1 that regulates iNKT cell development and the response to αGalCer (Aktan et al., 2010; Gombert JM, 1996; Jordan et al., 2007b; Rocha-Campos et al., 2006). We previously demonstrated that iNKT cells in 129X1/SvJ mice produced significantly lower amounts of cytokine after αGalCer challenge than did iNKT cells in C57BL/6J mice. Using B6.129 congenic mice, we identified the genetic interval spanning from rs222297065 to D1MIT115 (Chr1: 171.03 - 177.68 Mbp) as a regulator of the response of iNKT cells to *in vivo* αGalCer challenge (Aktan et al., 2010). This ~6.6 Mbp locus is densely populated with numerous immunologically relevant genes, including signaling lymphocyte activation markers
(SLAMs) that modulate iNKT cell development and function (Griewank et al., 2007). Interestingly, this locus overlaps extensively with several autoimmune susceptibility loci (Edberg et al., 2002; Morel et al., 2001; Wandstrat et al., 2004) and there are numerous reports of an association between iNKT cell numbers and autoimmunity (Baxter et al., 1997; Cardell, 2006; Esteban et al., 2003; Gombert JM, 1996).

To refine this interval and identify candidate genes that regulated the responsiveness of iNKT cells to αGalCer, we generated additional B6.129 subcongenic lines with overlapping intervals. Here, we report the mapping of the iNKT cell response to αGalCer to a minimal 0.14 Mbp interval (Chr1: 171.032-171.170) containing 4 genes and 2 microRNAs. In addition, we found that this interval regulates total thymocyte numbers and total iNKT cell number. Finally, we identify Fcgr3 as a possible candidate iNKT cell regulatory gene due to the association of increased iNKT cell FcγR3 expression and impaired response of iNKT cells to αGalCer stimulation observed in B6.129c3 mice.
4.3. Results

4.3.1 Refinement of the 129X1/SvJ interval on chromosome 1

We previously reported that a 6.6 Mbp genetic region on chromosome 1 containing the *Slam* genes regulated iNKT cell function (Aktan et al., 2010). Given previous reports that SLAMf1 and SLAMf6 are required for iNKT cell development and the *Slam* genes have been reported to regulate thymic iNKT cell numbers (Aktan et al., 2010; Jordan et al., 2011), we hypothesized that polymorphisms in one or more of the *Slam* genes are responsible for the differences in the iNKT cell response to αGalCer between C57BL/6 and 129X1/SvJ mice. To test this hypothesis, we generated 4 subcongenic strains: B6.129c2, B6.129c3, B6.129c4, and B6.129c6 with overlapping 129X1/SvJ intervals ranging from 0.14 Mbp to 1.1 Mbp that spanned the centromeric region of the B6.129c1 interval containing *Slam* family genes (Figure 1a). Mapping of the 129 derived-interval boundaries (Figure 1b) revealed that B6.129c2 and B6.129c6 strains possess *Slam* family genes derived from 129X1/SvJ, while B6.129c3 and B6.129c4 strains possess *Slam* family genes derived from C57BL/6J.

4.3.2 Invariant NKT cell cytokine production in response to αGalCer maps to a 0.14Mbp region on chromosome 1

To determine which 129X1/SvJ intervals regulate the iNKT cell response to glycolipid, we assessed serum IFNγ and IL-4 cytokine levels after administration of αGalCer to C57BL/6 and B6.129 congenic strains. This analysis revealed that each of the tested B6.129 congenic strains exhibited significantly reduced levels of serum cytokines
in comparison to the B6 control (Figure 2a). To determine whether this decrease in αGalCer-induced cytokine was due to reduced production by iNKT cells, we evaluated IFNγ and IL-4 production by CD1dtetramer/PBS57-gated cells in response to αGalCer using intracellular staining and flow cytometry (Figure 2b). Consistent with the reduced serum cytokine production in the B6.129 congenic strains in response to αGalCer administration, we observed significantly lower levels of iNKT IFN-γ and IL-4 production in B6.129c2, B6.129c3, and B6.129c4 strains as compared to the B6 parental strain (Figure 2b). These data indicated that the dominant regulator of iNKT cell cytokine production in response to αGalCer mapped to the minimal 0.14 Mbp B6.129c3 interval.

Interestingly, examination of the cytokine production in B6.129c6 mice revealed an intermediate phenotype. iNKT IFN-γ and IL-4 production in B6.129c6 mice were significantly higher in comparison to B6.129c3 mice, while B6.129c6 IFN-γ was significantly lower in comparison to B6 parental mice (Fig. 2b). B6.129c6 IL-4 was also lower than B6 mice, although this reduction did not reach statistical significance (p=0.0880) (Figure 2b). Together, these data indicated the presence of at least two genetic elements in the B6.129c2 region that regulate iNKT cell cytokine production in response to αGalCer: a dominant regulator mapping to the 0.14 Mbp B6.129c3 interval that did not contain Slam genes, and a second subdominant regulator mapping to the B6.129c6 interval containing Slam genes.

Differences in in vivo iNKT cell cytokine production could be the result of an iNKT cell-intrinsic phenomenon, or from differences in antigen processing and presentation. To determine whether the difference in iNKT cell cytokine production was
an iNKT-intrinsic phenomenon, we directly compared cytokine production from purified B6 and B6.129c3 iNKT cells after stimulation. Equal numbers of sorted splenic iNKT cells from B6 WT and B6.129c3 mice were stimulated with anti-CD3 and anti-CD28, after which supernatants were analyzed for cytokine and chemokine production. We observed a significant reduction in cytokines and chemokine secretion from B6.129c3 iNKT cells compared to their B6 counterparts (Figure 3). Taken together, these data indicate that one or more of the genes contained within the B6.129c3 interval regulates iNKT cell cytokine production through an iNKT-intrinsic process.

4.3.3 Reduced numbers of thymic iNKT cells in B6.129c3 mice

Since it has been previously reported that the Nkt1 locus that encompasses the B6.129c3 congenic interval regulates thymic iNKT cell number (Jordan et al., 2007b), we asked whether this phenotype could be mapped to the B6.129c3 interval. A comparison of thymic iNKT cell numbers revealed a significant reduction in B6.129c3 thymic iNKT cells compared to their B6 counterparts (Figure 4a). Further analysis revealed that there was no significant difference in the overall frequency of thymic iNKT cells (data not shown), and that the decrease in thymic iNKT cell number in B6.129c3 mice was associated with a decrease in total thymocyte number (Figure 4a). Specifically, we observed a specific decrease in the number of DP, but not DN, CD4 or CD8 SP, B6.129c3 thymocytes. These data suggest that the B6.129c3 interval indirectly regulates iNKT cell development through its influence on the DP stage of thymocyte development.
Accumulating data suggest that there is significant strain-dependent variation in the distribution of iNKT cell subsets (Lee et al., 2013; Lee et al., 2015). Therefore, we considered the possibility that the iNKT cell-intrinsic differences in cytokine production was due to regulation of the distribution of iNKT cell subsets by the B6.129c3 congenic interval. iNKT1, iNKT2, and iNKT17 subsets were identified according to their expression of PLZF and RORγt (Lee et al., 2013) (Figure 4b, left). Examination of the frequency and number of thymic iNKT cell subsets in C57BL/6 and B6.129c3 mice revealed a decrease in the numbers of iNKT1 and iNKT2 cells, consistent with the decrease in total iNKT cell numbers (Figure 4b). The iNKT17 subset also showed a trend toward lower numbers in the B6.129c3 mouse, albeit not significantly lower. Taken together, these results suggest that the B6.129c3 interval regulates the number of thymic iNKT cells but does not significantly affect the distribution of iNKT cell subsets.

4.3.4 Increased expression of FcγR3 on B6.129c3 iNKT cells

The B6.129c3 congenic interval contains 4 genes and two micro-RNAs (Table 1). Examination of gene expression data using the Immgen database (Heng et al., 2008) suggested that one of the genes, *Mpz* (myelin protein zero) is not expressed in leukocytes, and we confirmed this data using PCR and gene-specific primers (data not shown).

We next investigated whether one or more of the remaining three genes, *Fcgr3* (Fc receptor, IgG, low affinity III), *Cfap126* (cilia and flagella associated protein), or *Sdhc* (succinate dehydrogenase complex c), were differentially expressed in B6.129c3 naive mice compared to C57BL/6 controls. We sorted splenic iNKT cells from C57BL/6
and B6.129c3 mice and used quantitative PCR to assess gene expression. Interestingly, we found that \( \text{Fcgr3} \) gene expression was significantly higher (5.5-fold change) on B6.129c3 iNKT cells compared to their B6 counterparts (Figure 5a). In contrast, no significant differences in \( \text{Cfap126} \) or \( \text{Sdhc} \) expression were observed between the two strains.

We next determined whether the increase in \( \text{Fcgr3} \) gene expression correlated with an increase in protein expression on the surface of B6.129c3 iNKT cells. Similar to a previous report (Kim et al., 2006), we observed a very low, but detectable level of Fc\( \gamma \)R3 expression on naïve iNKT cells from B6.129c3 mice as well as from parental strain 129X1/SvJ mice (Figure 5b). To confirm the increased expression of Fc\( \gamma \)R3 in B6.129c3 mice, we also assessed expression on neutrophils that express significant levels of this protein. This analysis similarly revealed increased Fc\( \gamma \)R3 expression on neutrophils from both B6.129c3 and 129X1/SvJ parental mice (Fig. 5b). We then compared iNKT Fc\( \gamma \)R3 expression between \( \alpha \)-GalCer-treated B6 and B6.129c3 mice to determine if activation altered the relative expression. While this analysis revealed significantly greater Fc\( \gamma \)R3 on iNKT cells and NK cells, the relative expression difference was similar to that seen in naïve mice (Fig. 5c). Together, these data suggest that one or more polymorphisms in the B6.129c3 interval regulates \( \text{Fcgr3} \) gene and cell surface expression, and identifies \( \text{Fcgr3} \) as a candidate gene responsible for regulation of the iNKT cell responsiveness to \( \alpha \)-GalCer.
4.4. Discussion

The ability of iNKT cells to rapidly produce large amounts of cytokines and chemokines in response to pathogens puts them in a unique position to shape the developing host immune response. In this context, natural genetic variants that modulate iNKT cell function could have significant downstream effects on the host immune response to pathogens. We recently demonstrated that two seemingly conflicting reports regarding the role of iNKT cells in the clearance of *Pseudomonas aeruginosa* from the lung (Nieuwenhuis et al., 2002; Stevenson et al., 1995) could be reconciled when genetic background was taken into consideration (Benoit et al., 2015). For reasons that are still unclear, BALB/c iNKT cells make a significant contribution to the host immune response after *P. aeruginosa* infection, but B6 iNKT cells do not. The strain-dependent role of iNKT cells in bacterial clearance was associated with strain-dependent differences in the response of lung iNKT cells to αGalCer. Elucidation of genetic variants that regulate iNKT cell function is necessary to understand their role in the host immune response.

Invariant NKT cell number, function, and tissue-specific homing varies widely among inbred strains of mice (Aktan et al., 2010; Lee et al., 2013; Lee et al., 2015; Rymarchyk et al., 2008) and several polymorphic loci that regulate iNKT cell number have been identified (Aktan et al., 2010; Borg et al., 2014; Esteban et al., 2003; Fletcher et al., 2008; Gombert JM, 1996; Jordan et al., 2004; Jordan et al., 2007b; Rocha-Campos et al., 2006). In an investigation into the role of iNKT cells in type 1 diabetes, it was reported that autoimmune-prone non-obese diabetic (NOD) mice possessed very low numbers of iNKT cells (Baxter et al., 1997; Godfrey et al., 1997; Gombert JM, 1996).
genetic locus, Nkt1, regulating the number of thymic iNKT cells was identified and mapped to a distal region on chromosome 1 (D1MIT15 - D1MIT155). The Nkt1 locus contained several immunologically relevant genes, including CD247, FcyR2b, FcyR3, and the Slam family of receptors including Slamf1 and Slamf6 (Esteban LM, 2003; Rocha-Campos et al., 2006), which are required for iNKT cell development in the thymus (Griewank et al., 2007). A subsequent report implicated Slamf1 in the regulation of thymic iNKT cell numbers as well as iNKT IL-4, but not IFNγ production (Jordan et al., 2007b). Here, we have refined this locus further and have found evidence suggesting the presence of at least two regulators of NKT cell number and function. Our data suggest that Slam genes may not be the major regulators of the iNKT cell response to αGalCer in this region. Although B6.129c6 mice that possess the Slam129 and FcgrB6 alleles exhibited reduced IFN-γ and IL-4 production in comparison to B6 mice, the dominant effect was observed in B6.129c3 and B6.129c4 mice that possess SlamB6 and Fcgr129 alleles. Conversely, the observation that B6.129c6 iNKT cell cytokine production was significantly greater than their B6.129c3 counterparts in both cytokines measured is consistent with the presence of an additional gene or genetic modifier regulating this phenotype. Together, these data suggest the possibility that there are multiple genetic elements in the larger B6.129c2 interval that regulate the function of iNKT cells: a novel genetic element that we identified in the B6.129c3 interval, and one or more genes (most likely Slam genes) or genetic modifiers in the B6.129c6 interval.

We identified Fcgr3 as a candidate gene in regulating the iNKT cell response. FcγR3 (CD16) is an activating receptor that binds IgG and signals through an immune-
tyrosine activation domain (ITAM) and is highly expressed on monocytes, macrophages, neutrophils, and NK cells (Kim et al., 2006; Nimmerjahn and Ravetch, 2007). We found that FcγR3 gene and protein expression was significantly increased on iNKT cells, NK cells, and neutrophils from B6.129c3 and 129X1/SvJ parental mice compared to C57BL/6 controls. The underlying basis for this differential expression is still unclear. A comparison of Fcgr3 promoter sequence (as defined by the Eukaryotic promoter database, (Dreos et al., 2017) between C57BL/6 and 129S1/SvImJ strains revealed no nucleotide differences. However, over 100 upstream variants were identified, suggesting the possibility that a substitution in an enhancer element could affect expression (data not shown).

Here, we confirm previous reports that iNKT cells express low, but detectable levels of FcγR3 (Araujo et al., 2011; Kim et al., 2006; Park-Min et al., 2007). These previous reports demonstrate that engagement of Fc receptors can significantly modulate iNKT cell function. In one study, intravenous Ig (IVIG) treatment was found to exert some of its effect through its action on iNKT cells (Park-Min et al., 2007), and IVIG administration to C57BL/6 mice resulted in diminished iNKT cell activation. Co-administration of αGalCer and IVIG resulted in severely reduced IL-4 and IFNγ production compared to mice treated with αGalCer alone, and this inhibition was dependent on FcγR3 (Araujo et al., 2011). Our finding that increased expression of FcγR3 on B6.129c3 iNKT cells is associated with decreased cytokine production is consistent with these data, although it remains unclear whether there is increased FcγR3 signaling in B6.129c3 iNKT cells.
Given the association of increased \textit{Fcgr3} in B6.129c3 mice and alterations in thymocyte number, it is possible that the NKT cell-intrinsic changes in function stem from differences in development. FcγR3 is expressed on developing thymocytes (Rodewald et al., 1992; Sandor et al., 1994) and \textit{Fcεr1γ}, which encodes the signaling chain of FcγR3 contributes to TCR signaling (Flamand et al., 1996; Shores et al., 1997). Interestingly, \textit{Fcεr1γ} was recently demonstrated to be over-expressed in NKT1 cells, and deletion of \textit{Fcεr1γ} resulted in altered frequencies of NKT1 and NKT2 subsets (Georgiev et al., 2016). Whether the impaired iNKT cell function observed in B6.129c3 mice is associated altered FcγR3 and/or \textit{Fcεr1γ} signaling will require further study.

Although our results suggest a role for \textit{Fcgr3} in the regulation of iNKT cells, we cannot rule out the other genetic elements that lie within the B6.129c3 region. We did not observe differences in gene expression of \textit{Sdhc} or \textit{Cfap126} genes on sorted splenic iNKT cells. Mutations in the \textit{Sdha, b,} and \textit{c} genes have been linked to susceptibility to mitochondrial disorders (Renkema et al., 2015). \textit{Cfap126} has been shown to have a function on pancreatic β cells (Bader et al., 2016), but studies of this gene on iNKT cells have not been performed. Several microRNAs have had reported roles in regulating iNKT cell development, including the Let-7 family of microRNAs (Burocchi et al., 2015; Fedeli et al., 2016; Frias et al., 2017). The microRNA Mir6546 present in the B6.129c3 interval appears to be rare (Castellano and Stebbing, 2013; Meunier et al., 2013) and its function has not been studied in immune cells. Therefore, although this interval contains multiple genes and genetic elements, the most likely iNKT cell regulator within our interval is \textit{Fcgr3}. 

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In summary, we have fine-mapped a locus that regulates the iNKT cell response to the agonist glycolipid αGalCer. Our findings suggest that the previously described Nkt1 locus contains at least two genetic elements that regulate NKT cell number and function, and that the dominant regulator in the region maps to a congenic interval containing Fcgr3, identifying it as a probable candidate gene for these phenotypes. Further characterization of this genetic locus and of the Fc receptor signaling pathway will aid in our understanding of the role of iNKT cells in host immunity.
4.5. Materials and Methods

4.5.1 Mice and Reagents

C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6.129c1 mice were described previously (Wandstrat et al., 2004), and B6.129c2, B6.129c3, B6.129c4 and B6.129c6 strains were generated by backcrossing B6.129c1 mice to C57BL/6J mice, and intercrossing the heterozygous progeny. Offspring in which recombinants were identified were backcrossed B6 to allow for the generation of homozygous congenic lines. The congenic interval boundaries were determined using primer sets designed to amplify across informative insertions/deletions and SNPs (Table 2). PCR products were sequenced to confirm boundaries. All mice were age and sex matched as indicated in the figure legends. All mice were housed and bred in the specific pathogen-free barrier facility at the University of Vermont. The alpha-galactosylceramide (Avanti Polar Lipids, Alabaster, Alabama) was prepared as described previously (Aktan et al., 2010) and administered i.p. at indicated doses in a 100µL volume. All procedures involving animals were approved by the University of Vermont Institutional Animal Care and Use Committee.

4.5.2 Serum cytokine analysis

Mice were bled via cardiac puncture, after which serum was collected and frozen at -20°C until analysis by ELISA according to the manufacturer’s instructions (Biolegend, San Diego, CA, BD Biosciences, Franklin Lakes, NJ).
4.5.3 Flow cytometry

Splenocytes and thymocytes were obtained by gently pressing through a 70 μm nylon mesh. RBCs were lysed using Gey’s solution. Cells were stained at 4°C in PBS + 2% FBS containing 0.2% sodium azide for 30 min., after which they were washed and resuspended in PBS + 1% PFA. Abs used in these experiments were anti-CD4 (RM4-5), CD45 (30-F11), TCRβ (H57-597), NK1.1 (PK136), CD8 (53-6.7) (Biolegend), and CD11b (M1/70), CD16/32 (2.4G2) and CD19 (6D5) (BD Biosciences). UV Live Dead staining reagent was included in all experiments (Life technologies, ThermoFisher, Grand Island, NY). CD1d tetramer loaded with PBS-57 was provided by the National Institutes of Health (NIH) tetramer facility (Emory University Vaccine Center, Atlanta, GA).

For intracellular cytokine staining (ICS), cells were isolated from the spleen as described above and stained with Ab to surface markers. In every ICS experiment, cells were analyzed directly ex vivo without further treatment. After washing in staining buffer, cells were fixed with 4% PFA for 30 min at 4°C. After washing in permeabilization buffer (0.1% saponin, 1% FCS, 0.1% sodium azide in PBS), cells were stained with anti-IL-4 (11B11) and anti-IFNγ (XMG1.2) or isotype-matched control antibodies. All intracellular antibodies were from Biolegend. Data was collected on an LSRII (BD Biosciences) and analyzed using FlowJo software (FlowJo LLC, Ashland, OR).

Purified CD16/32 (Clone 93) (Biolegend) was used in all samples prior to the addition of Abs to block nonspecific Ab binding, except for samples in which FcγR3 expression was being assessed. In those cases, Fc block was not performed, and conjugated anti-CD16/CD32 (2.4G2) (BD Biosciences, San Jose, CA) was added to the
surface stain master mix. As controls for FcγR3 staining and to control for the addition of conjugated 2.4G2 on all Fc receptors, cells were stained with unconjugated 2.4G2 Ab in order to block receptors and subsequently stained with conjugated 2.4G2. As a separate control, cells were stained with unconjugated 2.4G2 Ab and subsequently stained with an isotype matched control (IgG2a, Biolegend).

For nuclear transcription factor staining, cells were surface stained as above, after which they were fixed overnight using the Foxp3 transcription factor staining buffer set (eBiosciences/ThermoFisher, San Diego, CA). The next day cells were washed, blocked with rat IgG, and stained with anti-PLZF (9E12) and RORγt (Q31-378) (BD). After staining, cells were washed, resuspended, and data were immediately collected on a LSRII flow cytometer (BD Bioscience) and analyzed with FlowJo software (FlowJo LLC).

4.5.4 Cell isolation and culture for ex vivo cytokine analysis

Splenocytes were enriched for CD4 T cells by depletion of CD8 (53-6.7), CD11b (M1/70), MHCII (M5-114) (BioXcell, West Lebanon, NH) with anti-rat and anti-mouse IgG magnetic beads (Qiagen, Hilden, Germany). After enrichment, cells were stained with anti-TCRβ, CD1dtetramer/PBS57, and DAPI, and iNKT cells were purified by fluorescence activated cell sorting (FACS Aria III). Cell purity for all sorts was between 83% and 97%. After sorting, cells were placed in modified Click’s culture medium (5% FBS, 2mM glutamine, Pen/Strep, 50µM 2-mercaptoethanol, 20µg/mL gentamycin) and incubated on anti-CD3 (5 µg/ml) and anti-CD28 (1 µg/ml)-coated plates at 37°C and 5%
CO₂. 72 h later, supernatants were harvested and analyzed via Milliplex assay, according to the manufacturer’s instructions (EMD Millipore, Darmstadt, Germany). The milliplex kits used were the Mouse cytokine/chemokine magnetic bead panel followed by a custom kit containing GM-CSF, IFNγ, IL-2, IL-4, IL-5, IL-10, IL-13, and IL-17.

Quantitative real-time PCR

Sorted iNKT cells were washed in cold PBS, after which cell pellets were snap frozen and kept at -80°C until RNA was isolated. RNA isolation was performed using the RNeasy Micro kit according to the manufacturer’s instructions (Qiagen, Hilden, Germany). cDNA was synthesized using Superscript III (ThermoFisher, San Diego, CA). Mpz and Pcp4l-1 expression was evaluated by PCR using the gene specific primers: Mpz forward 5’-CGGACAGGGAAATCTATGGTGC-3’, reverse 5’-TGGTAGCGCCAGGTAAAAGAG-3’. Pcp4l-1 forward 5’-ATGAGCGAGCTTAACACCCAAA-3’, reverse 5’-CTGCCAGGGCTTCCCTTTTTC-3’ using cDNA derived from central nervous system tissue (a gift from D. Krementsov, University of Vermont). Expression of Fcgr3, Sdhc, and Cfap126 was evaluated using Assay on Demand (AOD) Taqman probes (Life Technologies, Carlsbad, CA) according to the manufacturer’s instructions. qPCR assay on demand (AOD) was run using the Perfecta qPCR SuperMix (Quanta Biosciences, Beverly MA). Samples were incubated with UNG SuperMIX at 45°C for 5m, then denatured initially at 95°C for 3m, followed by: 95°C denaturation for 15 s, and a combined 60°C annealing and extension step for 45s for 40 cycles. Fold change was calculated by determining 2^ΔΔCT using mouse β-Actin
as the endogenous control. Data was collected on an ABI Prism 7900HT Sequence Detection System.

4.5.5 Statistics

All animal experiments were conducted in a non-randomized and non-blinded fashion. One way-ANOVA, two-way ANOVA, or unpaired T tests were used where appropriate. ANOVA post-hoc analysis comparisons were made using Dunnett’s test, Sidak’s multiple comparisons test, Tukey’s multiple comparisons test, or Holm-Sidak’s multiple comparisons test where appropriate. In all cases, tests were considered significant when \( p \leq 0.05 \). All groups were shown to have similar variance in distribution.

Sample size estimates were based on previous experience. In Figure 2B, 3 mice were excluded from the analysis due to poor αGalCer injections. When data were combined from multiple different experiments, data were first normalized to the C57BL/6 reference strain in each experiment. Normalization was accomplished by dividing each data point in an experiment by the average of the C57BL/6 response in that experiment. All statistical analysis was performed using Graph Pad Prism (GraphPad Software, San Diego, CA).
4.6. Acknowledgements

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4.7. References


NKT cell development and function by a 0.14 Mbp locus on chromosome 1: a possible role for Fcgr3. Genes Immun.


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Association of extensive polymorphisms in the SLAM/CD2 gene cluster with murine lupus. Immunity 21, 769-780.


4.8. Figures

**FIGURE 4-1**: Fine-mapping of the B6.129c1 congenic interval

A). *Left* Schematic of the chromosome 1 congenic intervals used in this study. Black regions denote C57BL/6 derived regions, white denotes 129X1/SvJ derived regions, and the cross-hatched region is either C57BL/6 or 129X1/SvJ derived (undefined). For reference, the *Slam* family genes are denoted in red and the Fc receptor gene family is denoted in green. B). Number of protein-coding genes predicted in each subcongenic interval. *Lower*: Reference SNPs that define boundaries for each congenic line on the centromeric and telomeric ends.
FIGURE 4-2: Regulation of αGalCer-induced iNKT cell cytokine production maps to a 0.14 Mbp region on chromosome 1

A). Decreased serum cytokine in B6.129 congenic mice in response to αGalCer. Blood was collected from B6 or B6.129 congenic mice 2 h after αGalCer administration. Serum cytokine levels were assessed using ELISA. Statistical analysis was performed using 2-way ANOVA followed by Tukey’s multiple comparisons test, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 B). Top: Representative intracellular staining of cytokine-producing iNKT cells in response to αGalCer. Splenocytes were isolated 2 h after αGalCer administration. iNKT cells were identified using CD1d-tetramer/PBS57 and TCRβ. The percentages of iNKT cells expressing cytokines were determined using an isotype control for each mouse. Lower: Decreased iNKT cell cytokine production in B6.129 congenic mice in response to αGalCer. The data represent the relative level of iNKT cell cytokine production. Data are the combined data from 5 separate experiments using female mice 8 -14wks of age and are presented as the mean ± s.d. All mice were age-matched to controls in each experiment. Statistical analysis was performed using 2-way ANOVA followed by Tukey’s multiple comparison test. * = comparison of B6 to B6.129 congenics. # = comparison of B6.129c3 to B6.129 congenics. *,# p ≤ 0.05, **,## p ≤ 0.01, ### p ≤ 0.001, ####p ≤ 0.0001.
FIGURE 4-3: Reduced cytokine production in B6.129c3 is iNKT cell-intrinsic. iNKT cells were sorted from C57BL/6 or B6.129c3 splenocytes.

Cells were stimulated using anti-CD3 and anti-CD28 for 72 h, after which supernatants were analyzed using Milliplex. Data represent the cumulative results from 3 separate experiments using 8 - 11week old female mice. Mice were age-matched in each experiment. Statistical analysis was performed using a 2-way ANOVA followed by the Holm-Sidak multiple comparison test and is reported as the mean normalized concentration ± s.d. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001
FIGURE 4-4: iNKT cell numbers in the thymus are regulated by the B6.129c3 interval.

A). iNKT cell (left), total thymocyte (middle), thymocyte subset (right) numbers were compared between C57BL/6 and B6.129c3 mice. B). No significant difference in the distribution of thymic iNKT subsets between B6 and B6.129c3 mice. iNKT cell subsets were identified based on PLZF and RORγt expression as indicated by colored boxes (left). Cumulative numbers of iNKT cell subsets are shown (right). Data represent the cumulative results of 4 separate experiments using 9 – 13 week old female mice. Mice were age matched in each experiment. Statistical analysis was performed using an unpaired T-test. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ .001
Increased *Fcgr3* gene and protein expression on B6.129c3 iNKT cells. A) Increased *Fcgr3* gene expression in B6.129c3 splenic iNKT cells. Spleen iNKT cells were sorted using CD1d tetramer/PBS-57 for RNA isolation. Gene expression was determined using quantitative PCR. Data is reported as the fold change relative to B6. B) Increased cell surface protein expression of FcγR3 on naive B6.129c3 iNKT and neutrophils. Representative flow cytometry histograms show expression levels of FcγR3 on B6.129c3 iNKT cells (upper) and neutrophils (lower). Shaded dark histograms represent cells
blocked with unconjugated 2.4G2 Ab and subsequently stained with conjugated isotype control. Heavy lined histograms are cells stained with conjugated 2.4G2 Ab. The numbers represent the median fluorescence intensity (MFI). Graphs depict the normalized MFI of 2.4G2 relative to the B6 control. Data represents the mean ± s.d. and is the cumulative data from 2 separate experiments of female mice aged 6-8wks. C) Increased cell surface protein expression of FcγR3 on αGalCer treated B6.129c3 iNKT and NK cells. Shaded dark histograms represent cells blocked with unconjugated 2.4G2 Ab and subsequently stained with conjugated isotype control. Heavy lined histograms are cells stained with conjugated 2.4G2 Ab. The numbers represent the median fluorescence intensity (MFI). Graphs depict normalized MFI of 2.4G2 relative to the B6 control. Data represents the mean ± s.d. and is representative data from 2 independent experiments using 10 or 14 week old female mice. Statistics were performed using a 1-way ANOVA and followed by a post-hoc comparison to B6 controls using Dunnett’s test. **p ≤ .01, ****p ≤ 0.0001
Table 1. Genetic elements within the B6.129c3 interval.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Name</th>
<th>Position</th>
<th>Non-synonymous SNPs</th>
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</thead>
<tbody>
<tr>
<td>Fcgr3</td>
<td>Fc receptor, IgG low affinity III</td>
<td>171.051-171.064</td>
<td>12</td>
</tr>
<tr>
<td>Cfap126</td>
<td>Cilia and flagella associated protein</td>
<td>171.113-171.126</td>
<td>1</td>
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<td>Mpz</td>
<td>Myelin protein zero</td>
<td>171.150-171.161</td>
<td>1</td>
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<tr>
<td>Sdhc</td>
<td>Succinate dehydrogenase complex subunit C</td>
<td>171.127-171.150</td>
<td>2</td>
</tr>
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<td>Mir6546</td>
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<tr>
<td>GM27844</td>
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<td>171.079</td>
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</tr>
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</table>

Gene positions were determined according to Ensembl release 90. Structural variants, missense variants, and splice variants were determined using the Mouse Phenome Database, Sanger 4 and NCBI dbSNP databases. SNP values are those that result in amino acid changes between C57BL/6 and 129S1/SvImJ. The B6.129c3 interval boundaries are on chromosome 1 location 171.032 Mbp to 171.170 Mbp.
CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

Initial identification of an unusual T cell expressing the Vβ8 invariant TCR was performed in 1987 (Budd et al., 1987; Fowlkes et al., 1987). It was not until 8 years later that these cells were defined as a new αβ T cell subset called natural killer T cells (Macdonald, 2007). In the 8 years that it took for iNKT cells to be agreed upon, reports were generated showing how these cells did not fit the definition of a traditional T cell. Immunologists describe new cells by defined functional or phenotypic markers that fit into a defined “box”. Natural killer T cells, so named due to both function and the markers they express, do not fit into a classic box for immune cells, and have been challenging to define. In some instances, they are protective, in others, pathogenic (Crosby and Kronenberg, 2018). After activation they make numerous Th1, Th2, Th17, or Treg cytokines, displaying a diverse repertoire of function. Some iNKT cells are cytotoxic, some undergo activation induced cell death, and others are long lived. It can be safely said that iNKT cells do not fit into any one box, but many, bridging both innate and adaptive immunity and showcasing incredibly versatility to contribute to host immunity (Godfrey et al., 2004) (Figure 5-1).

Diversity within the iNKT cell lineage and among the iNKT cell subsets may be important for the ways in which iNKT cells contribute to host immune responses. Since the SLAM receptors are heterogeneously expressed on iNKT cells (Aktan et al., 2010), we can envision a scenario in which interactions with a SLAM family receptor expressing antigen presenting cell can change the functionality of the iNKT cell subsets.
It has already been shown that SLAMf6 crosslinking on an APC and iNKT cell are required for proper IFNγ and IL-4 production (Baglaenko et al., 2017). There is evidence to suggest that a certain subset of dendritic cells can present antigen that results in a Th1 Th2 bias, but the mechanisms for how this presentation could be specific to a certain iNKT cell subset is currently unknown (Arora et al., 2014). Does SLAM family receptor crosslinking induce the production of cytokines from the APC, which then changes how the iNKT cell responds? Does one subset lead to increased kinetics of immune cell activation or vice versa? Is the repertoire of SLAM family receptors on the APC just as important for effector function as the SLAM family receptor repertoire on the iNKT cell? These questions, given our added knowledge of iNKT cell subsets, and our contribution of how SLAM family receptors modulate iNKT cell subset responses, can now be addressed.

An interesting point of further investigation is whether organ specific iNKT cells can be stimulated with different agonists at the same time. While this is likely a normal occurrence within different tissues with self-glycolipids, it would be interesting to “polarize” iNKT cell subsets into distinct cytokine or cytotoxic inducing cells for different disease treatments. We already know that there are distinct ligands that can induce IFNγ versus IL-4, such as αGalCer and OCH respectively (Sullivan et al., 2010; Velmourougane et al., 2009), but how these agonists induce specific iNKT cell TCRβ chains and subsets leading to cytokine production has only recently been investigated (Cameron, Godfrey 2015). Several of these agonists are being used as adjuvants to induce a more robust immune cell activation, but these treatments have only shown modest
ability to increase iNKT cell responses in humans (Giaccone et al., 2002; Ishikawa et al., 2005). One interesting line of study would be to deliver specific iNKT cell agonists that would activate distinct iNKT cell subsets.

Our data shows that the SLAM family expression patterns on iNKT cell subsets are determined during thymic development. This suggests additional programming and lineages where iNKT cells are “destined” to become NKT1, NKT2, or NKT17. Before the work shared in this dissertation, the role of SLAM receptors in this process had not been studied in detail.

Recent reports identifying determinants of iNKT cell development give us some clues into how iNKT subset cell development could be regulated. Programming of the NKT cell lineage is said to require signaling through Id (inhibitor of DNA binding and differentiation) and E proteins (helix-loop-helix proteins), which serve as transcriptional regulators, often functioning to antagonize the function of the other (Roy and Zhuang, 2018). Downstream of Id proteins is a protein responsible for the induction of the master transcription factor PLZF termed Egr2 (early growth response protein). Surprisingly, SLAMf6 has been shown to induce expression of PLZF via the regulation of Egr2 after ligation of the TCR on iNKT cells (Tuttle et al., 2018). E proteins, which function downstream from the TCR and SLAM family receptor crosslinking, can bind to the PLZF promoter, while Id proteins inhibit this interaction, indicating that these proteins can aid in the regulation of iNKT cell PLZF expression. Intriguingly, Id proteins are required for the development of iNKT cells, but appear to inhibit production of the SAP dependent innate-like Vγ1.1Vδ6.3 subset as well as NKT2 cells (D'Cruz et al., 2014; Verykokakis et
(Michel et al., 2010), indicating that Id proteins are intimately involved in the developmental programs of these subsets.

Additional data suggest that upon deletion of SAP in a Vα14 transgenic model, development of NKT2 cells is decreased while NKT17 cells are increased and NKT1 cells are not affected, indicating separate, but overlapping signaling mechanisms in iNKT cell development (Michel et al., 2016). Taken together, we speculate that these reports suggest that TCR ligation in concert with SLAM family receptor crosslinking potentiates separate signaling cascades involving E and Id protein regulation of iNKT cell subset development. Our findings that SLAMf6 specifically regulates NKT2 and NKT17, but not NKT1, subsets suggests that signaling from distinct SLAM family receptors augments TCR signals in developing iNKT cells that ultimately shape their differentiation fate. The molecular nature of these signaling mechanisms during iNKT cell development currently needs more investigation.

In Chapter 3, we found that NKT2 and NKT17 cells were more proliferative than their NKT1 counterparts. We show that after αGalCer-mediated expansion of iNKT cells, there appears to be an influx of NKT2-like iNKT cells. There are several possibilities for this observation. First, this phenotype could result from the upregulation of PLZF and Tbet after activation in the predominant spleen NKT1 cell population, resulting in their acquisition of an NKT2 phenotype. Alternatively, proliferative NKT2 cells could be preferentially expanding and repopulating the peripheral organs, thereby outnumbering NKT1 cells 3 days after activation. The first model would suggest that the iNKT cell subset definitions based on PLZF, Tbet, GATA3 and RORγt expression are
useful only in resting iNKT cell populations, and suggests a degree of plasticity in the iNKT cell population. Although plasticity among conventional T helper cells is now recognized (Gagliani et al., 2015), the degree of plasticity exhibited by the different NKT cell subsets remains unknown. The second model suggests that the ratio of iNKT cell subsets changes after activation due to the repopulation of the periphery with NKT2 cells, possibly from cells repopulating the thymus. We know that repeated administration of αGalCer over a period of weeks reveals the presence of IL-10-producing NKT cells (NKT10) (Sag et al., 2014). Whether this results from the differentiation of NKT cells or the preferential expansion of a small pre-existing NKT10 subset remains to be determined. Interestingly in Chapter 3, supplemental figure 1, we show that the number of iNKT cells in the spleen returns toward baseline levels 5 days after activation with αGalCer. An analysis of the iNKT cell subsets at this time-point could reveal whether a different subset is present or whether immature NKT2 precursors then convert into NKT1 and NKT2 cells once in the tissue. In the future, adoptive transfers of different iNKT cell subsets marked with congenic markers will be necessary to address these questions in the future. In summary, while NKT1, NKT2, and NKT17 subsets are apparent during development (Lee et al., 2013), these definitions are less apparent after peripheral activation, indicating a degree of plasticity that has yet to be determined.

We show in Chapter 4 that Slam haplotypes play a role in regulating iNKT cell subset development and function (Aktan et al., 2010; DeVault et al., 2018). However, we found the altered iNKT cell function in B6.129 congenic mice was not primarily due to allelic differences among the Slam genes. Rather, we identified Fcgr3 as a potential
candidate in this regulation. Investigation into the exact mechanisms that control \textit{Fcgr3} regulation of iNKT cells are currently ongoing in the lab. Our data in Chapter 4 suggested both \textit{Fcgr3} and SLAM family receptors as regulating the iNKT cell responses. While these genes do not necessarily have to work together and epistatic mechanisms may be at play, there is some speculation that these receptors do work in concert. Interestingly, FcγR3 appears to signal synergistically with the CD2 receptor in activation of NK cells (Liu et al., 2016). SLAM family receptors are said to be a part of the CD2 family of receptors and SLAMf2 can bind hetero-typically to CD2 (van der Merwe et al., 1995). We therefore speculate that SLAM family receptors, such as CD2, could act as co-receptors for activation and signaling through FcγR3. Additionally, this data could suggest that other SLAM family receptors, such as SLAMf4 (which binds to SLAMf2), may have the ability to bind to CD2, allowing for crosslinking and co-receptor functions of CD2 to FcγR3.

The work provided within this dissertation could have broader implications for human innate T cell biology. The data we show here raise questions as to whether similar SLAM family receptor modulation is present on human innate-like T cells and iNKT cells. Data has suggested evidence for human iNKT cell subsets, although they are less defined than those in the mouse (Zeng et al., 2013). To date, a complete analysis of SLAM family receptor expression on human iNKT, γδT, or MAIT cells has not been completed. We have already discussed that there are SAP dependent γδ T cells and iNKT cells (Nichols et al., 2005a; Verykokakis et al., 2010). MAIT cells do not appear to be SAP dependent, but they do express SLAM receptors. Humans happen to have a high
number of MAIT cells that are dependent on B cells and the gut microbiome for
development (Chandra and Kronenberg, 2015). While subsets of MAIT cells have not
been determined, SLAM family receptors could play a role in MAIT cell effector
functions, especially upon engagement with an APC. Similar SLAM family receptor
analyses on γδ T cells and ILC populations are currently being performed in the lab,
suggesting that differing SLAM receptor expression mediating function are not unique to
iNKT cell subsets. Instead, these patterns lend diversity to many different innate-like
cells in the mouse, and potentially, the human.

With the renewed interest in human cancer immunotherapies, iNKT cell subsets
are being thought of as important cells in modulating the immune response toward solid
tumors such as melanoma and neuroblastoma (Wolf et al., 2018). We show in Chapter 3
that NKT2 and NKT17 cells are more proliferative than their NKT1 counterparts,
indicating that some iNKT cell subsets may be better for expansion for immunotherapy.
In addition, the repertoire of cytotoxic iNKT cells to kill tumors, as well as cytokine
producing iNKT cells (if they are not the same), should be taken into consideration. In a
search of clinical trials at the US National Library of Medicine website, there were over
30 NKT cell related clinical trials (as of 09/20/2018). In most of these studies, iNKT cells
were isolated from the patient, expanded in vitro, and then given back to the same patient
to stimulate the immune system, especially NK cells and cytotoxic T cells. Some of the
data from completed trials show that expanded iNKT cells produced only IFNγ and no
IL-4 or IL-10 (Exley et al., 2017), suggesting that only one type of iNKT cell may have
been expanded. Although this difference in cytokine production may have been from the
specific expansion protocol (Exley et al., 2014), it would be beneficial to trials in the future to determine whether subsets of iNKT cells can be expanded independently for differential cytokine production, and to potentially, aid in stimulating different parts of the immune system for treating different types of cancers.

In conclusion, this dissertation reveals specificity of distinct regulatory mechanisms on iNKT cell subsets provided by the SLAM family of signaling receptors as well as a novel candidate gene, *Fcgr3* (Figure 5-1). This work provides context for the diverse and specific functions of iNKT cell mediated host immunity.
Summary

A). The regulatory mechanisms behind how iNKT cells produce a myriad of cytokines, home to particular tissues, and how particular SLAM family receptors specifically function on iNKT cells have not been elucidated B). Upon the identification of iNKT cell subsets, information about the tissue localization and specific cytokine production of these cells began to emerge. In this dissertation, we reveal specific SLAM family receptor expression profiles on distinct iNKT cell subsets. C). SLAMf6, highly expressed on NKT2 and NKT17 cells, specifically affects the cytokine production and cell growth of NKT2 and NKT17 cells, but not that of NKT1 cells. This work identifies SLAM family receptors as crucial regulators of NKT cell subset development and function.

FIGURE 5-1: Diversity SLAM family receptor expression patterns controls the development and function of iNKT cell subsets


Chatterjee, M., Rauen, T., Kis-Toth, K., Kyttaris, V.C., Hedrich, C.M., Terhorst, C., and Tsokos, G.C. (2012). Increased expression of SLAM receptors SLAMF3 and SLAMF6 in
systemic lupus erythematosus T lymphocytes promotes Th17 differentiation. Journal of Immunology 188, 1206-1212.


analysis of the NKT cell control gene Nkt2 implicates the peroxisomal protein Pxmp4. Journal of Immunology 181, 3400-3412.


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