ROLE OF INVARIANT NATURAL KILLER T CELLS IN TULAREMIA-LIKE DISEASE IN MICE

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To Lieutenant Colonel Robert Hunter Lindsey (1971–2013). You were a great friend and a wonderful role model. You made the world a better place while you were here. I only hope that I can do the same.

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acid ceramidase 1 (ASAH1)

acute lung injury (ALI)

airway hyperreactivity (AHR)

alveolar macrophage (AM)

American Type Culture Collection (ATCC)

antibody (Ab)

antigen presenting cell (APC)

autophagy protein 5 (Atg5)

bone marrow derived dendritic cells (BMDC)

broncheoalveolar lavage fluid (BALF)

C57BL/6J mouse (B6)

cathepsin S (CatS)

cationic lipid–DNA complexes (CLDC)

chronic obstructive pulmonary disease (COPD)

cluster of differentiation (CD)

colony forming units (CFU)

dendritic cell (DC)

diacylglycerol (DAG)

diphtheria toxin (DT)

double negative (DN)

endoplasmic reticulum (ER)

enhanced green fluorescent protein (eGFP)

eosinophil chemotactic factor-L (ECF-L)

experimental autoimmune encephalomyelitis (EAE)

follicular helper NKT cells (NKTfh)

Francisella-containing phagosome (FCP)

germ-free (GF)

glycosphingolipid (GSL)

glycosylated phosphatidylinositol (GPI)

IL-10 producing iNKT (iNKT10)

immunoglobulin (Ig)

induced bronchus-associated lymphoid tissue (iBALT)

inflammatory bowel disease (IBD)

iNKT cell receptor (iNKTCR)

intercellular adhesion molecule 1 (ICAM1)

interferon (IFN)

interleukin (IL)

intraepithelial lymphocytes (IELs)

intranasal (i.n.)

intratracheal (i.t.)

intravenous (i.v.)

invariant chain (Ii)

invariant natural killer T cells (iNKT)

lethal dose, 50% (LD_{50})

live vaccine strain (LVS)

lymph node (LN)

lymphocyte function-associated antigen 1 (LFA-1)

lymphocyte-specific protein tyrosine kinase (Lck)

lysosomal-associated membrane protein (LAMP)

major histocompatibility complex (MHC)

mucosal-associated invariant T cells (MAIT cells)

myeloid differentiation primary response gene 88 (MyD88)

myeloid-derived suppressor cells (MDSCs)

natural killer (NK)

natural killer T cells (NKT)

non-obese diabetic (NOD)

pathogen associated molecular pattern (PAMP)

pattern recognition receptor (PRR)

peripheral blood mononuclear cell (PBMC)

phosphatidylcholine (PC)

phosphatidylinositol mannoside (PIM)

post-inoculation (p.i.)

promyelocytic leukemia zinc finger gene (PLZF)

subcapsular sinus (SCS)

T follicular helper cells (Tfh)

tell cell receptor (TCR)

Th17-like iNKT (iNKT17)

Th1-like iNKT (iNKT1)

Th2-like iNKT (iNKT2)

toll-like receptor (TLR)

tumor necrosis factor (TNF)

α-galactosidase A (α-Gal-A)

α-galactosyl(α1-2)galactosylceramide

(GGC)

α-galactosylceramide (α-GalCer)

β2-microglobulin (β2m)

CHAPTER I

INTRODUCTION

Upon breach of a barrier tissue, microbial invaders encounter a variety of immune cells that are typically considered to belong to either the innate or adaptive arms of the immune system. Cells of the innate arm, such as dendritic cells (DCs), quickly recognize the pathogen through pattern recognition receptors and initiate a local inflammatory response aimed at restricting and reducing the threat. These phagocytic cells take up the pathogen or infected cells and traffic to the local draining lymph nodes where they present processed pathogen-derived antigens to T lymphocytes which in-turn initiate the adaptive phase of the response. Over the next several days, activated T lymphocytes expand, differentiate, and then follow a chemokine trail laid down by innate cells in the infected tissue to the site of active infection where they provide antigen-specific effector functions via their antigen receptors and cytokine arsenals. The binary categorization of immune cells as belonging to either the innate or adaptive arms is challenged by the existence of cells that possess antigen receptors generated by V(D)J recombination, yet respond rapidly to microbial challenge in concert with innate cells. Such “innate-like lymphocytes” include B-1 cells, CD8αα expressing intraepithelial lymphocytes (IELs), γδ T cells, mucosa-associated invariant T cells (MAIT cells), and invariant natural killer T cells (Lanier, 2013; Van Kaer et al., 2015a).

Natural Killer T cells (NKT cells) are a major population of innate-like lymphocytes that were originally defined as cells that co-express αβ T cell receptors (TCR) and surface markers characteristic of natural killer (NK) cells. Unlike conventional T cells which recognize peptide antigens presented by MHC class I or class II molecules, NKT cells are a specialized subset of T cells that recognize glycolipid antigens presented by the MHC class I-like molecule CD1d (Bendelac et al., 1995). Two subtypes of CD1d-restricted NKT cells have been identified based on their expression (or not) of a semi-invariant TCR. The most well-studied NKT cells are called semi-invariant or type I NKT (iNKT) cells. They are semi-invariant in that they express an invariant TCR α-chain (Vα14-Jα18 in mice; Vα24-Jα18 in humans) and one of a limited set of TCR β-chains, which are predominantly Vβ8.2, Vβ7 or Vβ2 in mice and Vβ11 in humans (Bendelac et al., 2007). A second subset of NKT cells known as type II have diverse TCRs and are also presumed to respond to lipid antigens presented by CD1d.

A characteristic feature of iNKT cells is that they populate peripheral organs in a distinctive state of partial activation reminiscent of conventional effector memory T cells, with high surface expression of activation markers such as CD44 and CD69 and low levels of CD62L (Bendelac et al., 2007). iNKT cells develop in the thymus from the same precursor pool as MHC-restricted T cells (Benlagha et al., 2005; Boesteanu et al., 1997; Coles and Raulet, 2000; Gapin et al., 2001; Tilloy et al., 1999). However, unlike conventional T cells, iNKT cells are selected by self-lipids presented on CD1d expressed by double positive thymocytes (Bendelac, 1995). While the presentation of endogenous glycolipids is critical for their thymic

development, continued stimulation by these endogenous ligand/CD1d complexes is not required for iNKT cell survival in the periphery (Bendelac et al., 2007; McNab et al., 2005). CD1d molecules are found on most hematopoietic cells, particularly on antigen presenting cells (APCs) such as DCs and macrophages, with the highest levels being found on marginal zone B cells (Brossay et al., 1997; Dougan et al., 2007; Roark et al., 1998). Hepatocytes, intestinal epithelial cells, granulocytes, keratinocytes, adipocytes, and hematopoietic stem cells also express CD1d molecules (De Santo et al., 2010; Dougan et al., 2007; Huh et al., 2013; Kotsianidis et al., 2006; Mandal et al., 1998).

Antigen recognition and activation of iNKT cells are influenced by the levels of CD1d expressed on the surface of APCs (Skold et al., 2005). Not surprisingly then, a number of pathogens have evolved mechanisms to modulate CD1d expression levels. For example, viruses such as Kaposi sarcoma–associated herpes virus (Sanchez et al., 2005), herpes simplex virus (Yuan et al., 2006) and HIV (Hage et al., 2005) mediate their immune-evasive effects in part by down regulating CD1d expression (Florence et al., 2008; Van Kaer and Joyce, 2006). CD1d on the surface of APCs is enhanced by IFN-γ and either TNF-α or Toll-like receptor (TLR)2 and TLR4 ligands (Brigl et al., 2011; Skold et al., 2005) suggesting that iNKT cells evolved to function at sites of acute inflammation. As demonstrated in various experimental settings, iNKT cells exhibit a functional plasticity that can influence the polarization of adaptive immune responses toward either a Th1 or Th2 profile and may also secrete immunosuppressive cytokines (Fig. 1-1) or directly

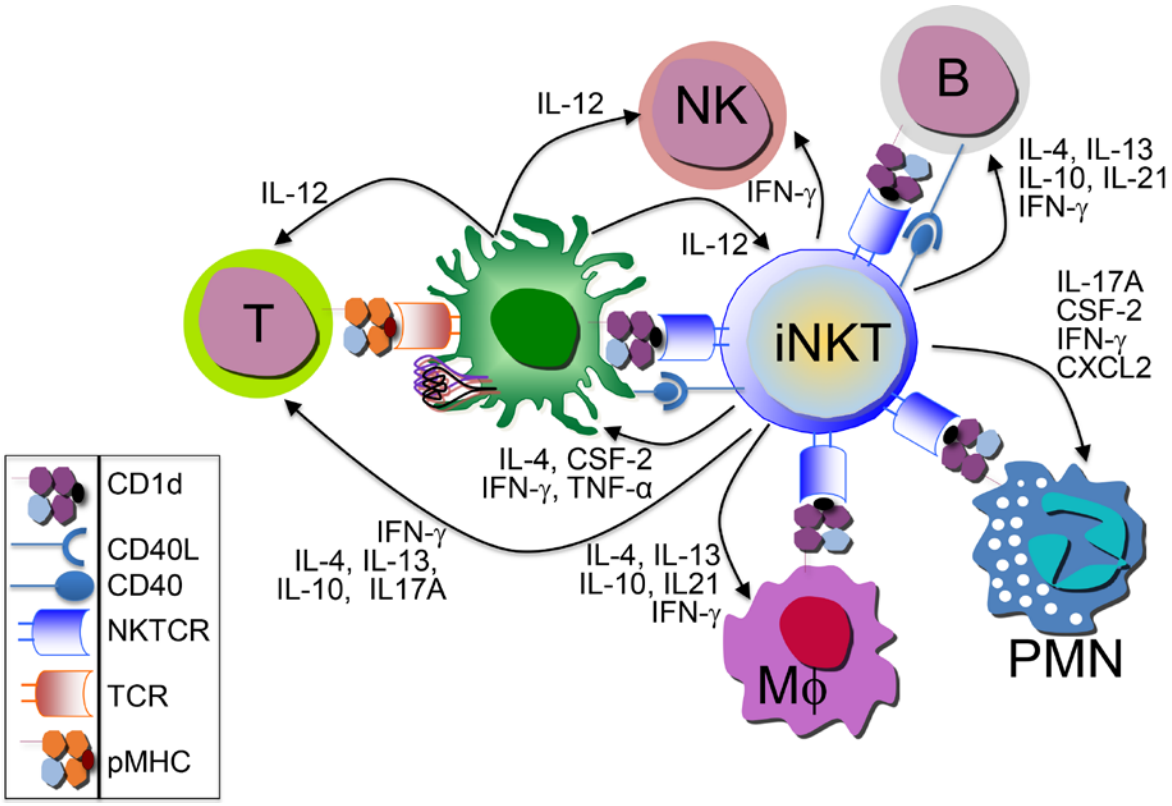


Figure 1-1. The immunological effector functions of iNKT cells.

The interactions between the invariant natural killer (iNKT) cell receptor and its cognate antigen as well as interactions between co-stimulatory molecules CD28 and CD40 and their cognate ligands CD80/86 (B7.1/7.2) and CD40L, respectively, activate iNKT cells. Activated iNKT cells participate in crosstalk between members of the innate and the adaptive immune system by deploying cytokine/chemokine messengers. Upon activation in vivo, iNKT cells rapidly secrete a variety of cytokines/chemokines. These cytokines/chemokines influence the polarization of $CD4^+$ T cells toward T helper (Th)1 or Th2 cells as well as the differentiation of precursor $CD8^+$ T cells to effector lymphocytes, and B cells to antibody-secreting plasma cells. Some of these cytokines/chemokines facilitate the recruitment, activation and differentiation of macrophages and dendritic cells, which result in the production of interleukin (IL)-12 and possibly other factors. IL-12, in turn, stimulates NK cells to secrete IFN-γ. Thus activated iNKT cells have the potential to enhance as well as temper the immune response. Figure adapted from Brennan et al., 2013.

target infected cells or other immune cells via their cytotoxic effector functions (Brennan et al., 2013; Godfrey and Kronenberg, 2004).

Studies of iNKT cell function in infectious disease primarily rely on one of two methods—genetic deletion or *in vivo* antibody (Ab)-mediated depletion—by which to assess their contribution to resistance or pathology. Because no unique iNKT cell-specific marker has been identified, the available Ab-mediated depletion methods deplete both NK and iNKT cells as these two cell types have significant overlap in surface marker expression, making interpretation difficult. Mice made genetically deficient in NKT cells are therefore a better experimental model and were generated previously either by deletion of one of the TCR α-chain gene segment ($J\alpha18^{-/-}$) or by mutation of the restriction element required for thymic NKT cell selection and antigen presentation in peripheral tissues ($CD1d^{-/-}$) (Chen et al., 1997; Cui et al., 1997; Mendiratta et al., 1997; Smiley et al., 1997). While the defect in $J\alpha18^{-/-}$ specifically targets the type I invariant iNKT cells, $CD1d^{-/-}$ mice are deficient in all CD1d-restricted NKT cells, including both type I and the less well-studied type II which have a variable TCR and are therefore unable to be identified using CD1d/α-galactosylceramide (α-GalCer) tetramers (Godfrey et al., 2004). Additionally, iNKT cell activation by endogenous or exogenous lipid antigens can be effectively blocked by the administration of anti-CD1d antibodies (Bleicher et al., 1990; Ilyinskii et al., 2006; Kawano et al., 1997; Szalay et al., 1999).

A recent PubMed search conducted in June 2015 identified more than five thousand studies involving NKT cells with nearly 10 percent of these being published in 2014 alone. Despite this extensive research, new insights into the role

of iNKT cells in health and disease are still being gained and our appreciation of their diverse functions constantly evolving. A brief summary of our current knowledge is presented below.

Immune surveillance by iNKT cells

Central to understanding the role of a leukocyte in disease is charting its trafficking and homing patterns. iNKT cells are perhaps best studied in the mouse spleen and liver where they comprise 1—2% and 20—30% of total lymphocytes, respectively. They are also found at lower frequencies in the skin, lymph nodes, bone marrow, blood, and gastrointestinal tract (Benlagha et al., 2000; Doisne et al., 2009; Matsuda et al., 2000; Thomas et al., 2011). Studies of iNKT cell frequencies in humans are mostly restricted to peripheral blood where their numbers are highly variable (0.001–3%) (Gumperz et al., 2002; Lee et al., 2002; Montoya et al., 2007). Although the numbers of iNKT cells may at first glance seem relatively small, it is important to consider that this small population contains a highly restricted TCR repertoire as compared to conventional T cells. Indeed, the numbers of individual CD4+ and CD8+ T cell clones in the spleen is estimated to be approximately 10 cells (Casrouge et al., 2000; Zarnitsyna et al., 2013). It therefore seems likely that iNKT cells make a significant contribution to the regulation of immune responses at these sites (Barral et al., 2010).

Due to their higher relative frequencies, iNKT cells are most well-studied in the mouse spleen and liver. *In vivo* imaging demonstrated that iNKT cells patrol

randomly throughout the red pulp and marginal zone where they have access to blood entering the spleen. Upon capture of blood-borne antigen by marginal zone macrophages and dendritic cells, iNKT cells are rapidly activated whereupon they arrest and begin to produce cytokines including IFN-γ and IL-4 (Barral et al., 2012).

iNKT cells accumulate and survive in the liver due in part to their high expression level of the chemokine receptor CXCR6 (Geissmann et al., 2005). Here they patrol the sinusoids, where they survey CD1d molecules expressed by hepatocytes, Kupffer cells, hepatic dendritic cells, sinusoid-lining endothelial cells, and hepatic stellate cells (Lee et al., 2010; Winau et al., 2007). Similar to iNKT cell behavior observed in the spleen, hepatic iNKT cells rapidly halt their patrolling upon encounter with blood-borne antigen. These arrested iNKT cells were observed to cluster around Kupffer cells presenting *Borrelia*-derived antigens (the causative agent of Lyme disease) through a mechanism dependent upon iNKT cell-expressed CXCR3 and recognition of its ligand CXCL9 (Lee et al., 2010). Not surprisingly, CXCL9 is among the chemokines elevated in the synovial fluid and skin lesions of human Lyme disease patients (Müllegger et al., 2007; Shin et al., 2010). Interestingly, the same clustering behavior was not observed after α-GalCer (a glycolipid recognized by all Vα14^{+} NKT cells) administration, suggesting that the behavior may be antigen dependent or that it may rely upon other microbial-derived factors.

iNKT cells are differentially distributed within the lymph nodes (LN), with higher frequencies found in mesenteric and pancreatic LNs (similar to the frequencies observed in the spleen), and several fold lower frequencies in

peripheral LNs [popliteal, inguinal, and brachial; (Laloux et al., 2001)]. In the lymph nodes, iNKT cells are found patrolling primarily in the paracortical region along with conventional T cells and, as observed in the spleen, are mainly excluded from the B cell zones (Barral et al., 2010; Barral et al., 2012). They can also be found clustered in the medulla and especially in the interfollicular region in the steady state (Kastenmüller et al., 2012). They are thus part of a group of innate lymphocytes optimally positioned within the LNs to limit systemic pathogen spread. Injection of α-GalCer– or *Sphingomonas*-derived glycosphingolipid (GSL)-1–coated beads results in a further accumulation of iNKT cells in the local draining lymph nodes. These LN iNKT cells rapidly arrest upon antigen administration and are activated by CD169+ macrophages in the subcapsular sinus (SCS), which capture the particulate antigen introduced via the afferent lymphatics (Barral et al., 2010).

Interestingly, iNKT cells in the peripheral lymph nodes seem to be phenotypically distinct from those present in the liver or spleen. Contrary to what has been observed in other organs, LN iNKT cells proliferate poorly after administration of soluble antigen (Harada et al., 2004; Wilson et al., 2003). However, upon arrival of antigen-loaded DCs into the popliteal LN, activated iNKT cells begin to proliferate and produce IL-17 (Doisne et al., 2009). This effect is even more profound when DCs are activated by LPS. Therefore, the identity or activation state of the APC may be important in the functional outcome. Although the significance of these findings is not completely understood, it seems likely that the initial activation of LN iNKT cells is mediated by CD169+ macrophages resident in the SCS that capture and present antigen *in situ*. This initial activation need not be

antigen dependent, as LN-resident iNKT cells were shown to be IL-18R$^+$ (Kastenmüller et al., 2012). IL-18 produced upon inflammasome activation in SCS macrophages, along with a second signal (such as IL-12 or type I IFNs) is sufficient to elicit IFN-γ production from these iNKT cells. Upon arrival of DCs that have encountered antigen in the surrounding tissue, these LN-resident iNKT cells expand dramatically and produce IL-17 under inflammatory conditions. Intriguingly, human iNKT cells have been found to accumulate in chronic inflammatory demyelinating polyneuropathy lesions (Illes et al., 2000) and periodontitis lesions (Yamazaki et al., 2001), suggesting they are recruited to peripheral sites of chronic inflammation (Gumperz et al., 2002).

Perhaps not surprisingly, the behavior of skin-resident iNKT cells is very similar to that observed in the skin-draining peripheral LNs (Doisne et al., 2009). iNKT cells in both compartments have a peculiar phenotype, expressing high levels of CD103, important for leukocyte retention in the epithelial layer (Cepek et al., 1994), CCR6, required for the trafficking of lymphocytes in response to CCL20 produced by epithelial cells (Schutyser et al., 2003), and CD121a, the (IL-1 receptor. These findings are particularly intriguing since E-cadherin, the receptor/ligand for CD103, is not expressed in the LNs or endothelium. Such iNKT cells also do not express CD62L or CCR7, molecules important for homing and retention in the LNs, suggesting that these CD103$^+$CCR6$^+$CD121a$^+$ iNKT cells are recruited and retained in the LN by some as yet undiscovered mechanism, or that they traffic freely through the lymphatics and pause during transit through the LN (Doisne et al., 2009).

Taken together, these studies point toward distinct iNKT cell functions tailored for the particular microenvironment in which they reside and caution against applying lessons learned from studies of iNKT cells found in the liver and spleen to those residing in other tissues. Hence, iNKT cells are one of a group of sentinel cells optimally positioned for early encounter of pathogens inoculated through the skin perhaps by mechanical injury or via tick bite [e.g. *Borrelia burgdorferi* and *Ehrlichia muris* (Kinjo et al., 2006b; Kumar et al., 2000; Lee et al., 2010; Mattner et al., 2005; Tupin et al., 2008)] which would be rapidly disseminated via the blood stream and lymphatics.

Unlike other lymphocytes, iNKT cells in tissues and lymphoid organs do not recirculate (Lynch et al., 2015b; Thomas et al., 2011). Rather, they seem to home to and remain in these locations due in part to their constitutively high levels of lymphocyte function-associated antigen 1 (LFA-1) (Ohteki et al., 1999; Thomas et al., 2011). Studies in parabiotic mice showed that unlike other lymphocytes examined, iNKT cells in lymphoid and nonlymphoid organs failed to recirculate for up to two months after surgery, whereas those in the blood rapidly equilibrated between parabionts (Lynch et al., 2015a; Thomas et al., 2011). This peculiar pattern of homing and retention could be recapitulated in conventional T cells by forced expression of PLZF, the signature transcription factor of iNKT cells (Kovalovsky et al., 2010; Kovalovsky et al., 2008; Savage et al., 2008; Thomas et al., 2011). The authors concluded that PLZF directs a broad innate effector lymphocyte program in iNKT cells where homing and recirculation properties are optimized to rapidly confront bloodborne, lymph-borne, or airborne pathogens in

different tissues. Interestingly, the recently described population of adipose tissue resident iNKT cells does not seem to be dependent on LFA-1 for their retention (Lynch et al., 2015b). The mechanism by which they are maintained in these tissues is still to be revealed but their lack of dependence on LFA-1 is likely linked to their low levels of PLZF expression.

Mucosal surfaces represent large areas where interactions between the host and its surroundings occur and are hence a frontline in the constant battle between the immune system and invading pathogens and environmental insults. These barrier tissues have evolved to provide an initial defense against constant onslaughts from the outside world while at the same time allowing the exchange of gases across the airway epithelium and the absorption of vital nutrients across the epithelial lining of the intestines.

iNKT cells can be found in both the epithelial layer and lamina propria of the large and small intestine (Olszak et al., 2012; Wingender et al., 2012b). Their numbers in the intestinal mucosa are controlled by neonatal colonization of commensal bacteria. Germ-free (GF) mice have increased numbers of iNKT cells in the intestinal mucosa with an immature phenotype marked by hyporesponsiveness to glycolipid antigens (Wingender et al., 2012b). This immature phenotype can be reversed by colonization with iNKT cell-antigen bearing bacteria during early life but not in adult mice (Olszak et al., 2012), demonstrating the importance of the intestinal microbiota in regulating iNKT cell function. Similar increases in the frequency of iNKT cells were observed in the liver and lungs but not spleen or thymus of GF mice on the C57BL/6 background (Olszak et al., 2012).

The increased numbers of iNKT cells in the intestinal mucosa of GF mice were associated with increased levels of the CXCR6 ligand CXCL16. Treatment with a CXCL16-neutralizing antibody reduced the accumulation of iNKT cells in the lungs and colon, but had no effect on numbers in the ileum or thymus. Interestingly, such treatment resulted in an increase in liver iNKT cells, suggesting that these cells were redirected from mucosal sites (Olszak et al., 2012). Through an unknown mechanism, the gut microbiota seems to modulate CXCL16 expression by epithelial cells (Olszak et al., 2012; Zeissig and Blumberg, 2013) thereby controlling the recruitment and function of NKT cells.

Although less well-studied due to their reduced numbers relative to the spleen and liver, iNKT cells have been shown to be enriched among T cells in the lung where they can be found positioned within the lung vasculature (Thomas et al., 2011). These pulmonary iNKT cells are likely also kept in place by LFA1/ICAM1 interactions. Upon exposure to airborne lipid antigens, such as α-GalCer, iNKT cells extravasate into the lung tissue where they can promote inflammation and influence the adaptive immune response (Scanlon et al., 2011). Similar to iNKT cells in the intestinal mucosa, lung iNKT cell populations are also increased in GF mice, suggesting a role for microbial exposure during early life on the establishment of iNKT cell niches within barrier tissues (Olszak et al., 2012). In support of this notion, infection of neonatal mice with influenza virus protected them from later development of allergen-induced airway hyperreactivity (AHR), an effect that was associated with the expansion of $CD8^{-}CD4^{-}$ iNKT cells in the lungs (Chang et al., 2011).

iNKT cells are also thought to play an important role in immune surveillance of the reproductive tract. Intraperitoneal (i.p.) injection of α-GalCer induces abortion in mice in a manner that is dependent on IFN-γ, TNF-α, and perforin (Ito et al., 2000), whereas in nonpregnant mice, α-GalCer treatment protects against genital tract *Chlamydia muridarum* infection (Wang et al., 2012). Interestingly, $CD1d^{-/-}$ mice are no more susceptible to intravaginal *C. muridarum* infection than wild-type mice, but the analysis of leukocyte numbers in infected and uninfected pregnant mice suggested a role for iNKT cells in the control, recruitment, or homeostasis of these populations at the maternal-fetal interface (Habbeddine et al., 2013). Thus, iNKT cells are optimally positioned at ports of microbe entry and antigen exposure.

iNKT cells in disease

Consistent with their immunoregulatory potential, iNKT cells have been implicated in a variety of disease conditions including autoimmunity, infectious diseases, allergic reactions, various cancers, and allograft rejection (Behar and Porcelli, 2007; Berzins et al., 2011; Terabe and Berzofsky, 2008; Tupin et al., 2007; Van Kaer et al., 2013; Van Kaer et al., 2015b; Van Kaer et al., 2015c; Wu and Van Kaer, 2009; Wu and Van Kaer, 2013). They are therefore a potentially important factor in the diagnosis and prevention of human disease. As such, they have been identified as attractive targets for vaccine adjuvants and immunotherapies (Carreno et al., 2014; Cerundolo et al., 2010; Van Kaer et al., 2011b). Conflicting results on the importance of iNKT cells in human disease are common. These differences are likely due to the variety of methods used to identify iNKT cells and the limitations

associated with measuring iNKT cell frequency and function in peripheral blood vs. in the affected tissues (Berzins et al., 2011). Confounding these issues is the inability to discern whether iNKT cell frequencies predispose an individual to a particular disease or are a consequence of the disease itself. Our current knowledge, therefore, is based largely on studies in mice.

The role of murine iNKT cells in infectious diseases has been extensively studied, often with similar conflicting results. Such disparities can likely be attributed to the different methods used to identify NKT cells, differences in mouse strains or models, variations in dose, route of infection, or in the pathogen strain used in similar studies. Such investigations typically conclude either a beneficial function of NKT cells or that these cells are of little consequence. However, in some cases, NKT cells are thought to exacerbate disease. Selected examples of such studies are presented below. A more comprehensive list is provided in Table 1-1.

Beneficial functions of iNKT

Borrelia burgdorferi is a spirochete that causes Lyme-disease, which can lead to disabling symptoms as the bacteria invade the joints, heart, and central nervous system (Biesiada et al., 2012). iNKT cells recognize a diacylglycerol produced by *B. burgdorferi* and rapidly proliferate and produce IFN-γ and IL-4 (Kinjo et al., 2006b). The absence of NKT cells resulted in increased numbers of *B. burgdorferi* in the joints (Lee et al., 2010; Tupin et al., 2008). Interestingly, intravital imaging of these infected joints revealed that iNKT cells in these locations were

Table 1-1. iNKT cells in microbial infection

Organism	Antigen(s)	Role of NKT in infection	Model used	Route of Infection	Ref
Gram-positive					
S. pneumoniae	αGlc DAG	Protective	Jα18$^{-/-}$, CD1d$^{-/-}$	i.n., i.t.	(Brigl et al., 2011; Kawakami et al., 2003; Kinjo et al., 2011)
L. monocytogenes	ND	Protective	CD1d$^{-/-}$	i.v., oral	(Arrunategui-Correa and Kim, 2004)
S. aureus	ND	Not Protective	Jα18$^{-/-}$, CD1d$^{-/-}$	i.v.	(Kwiecinski et al., 2013)
Gram-negative					
P. aeruginosa	ND	Protective Not Protective[#]	CD1d$^{-/-}$ Jα18$^{-/-}$, CD1d$^{-/-}$	i.n. i.t.	(Kinjo et al., 2006a; Nieuwenhuis et al., 2002)
S. typhimurium	ND	Not Protective	CD1d$^{-/-}$	oral	(Berntman et al., 2005; Brigl et al., 2003; Mattner et al., 2005)
S.choleraesuis	ND	Detrimental	Jα18$^{-/-}$	i.p.	(Ishigami et al., 1999)
E. coli (LPS only)	ND	Detrimental	Jα18$^{-/-}$, CD1d$^{-/-}$	i.v.	(Nagarajan and Kronenberg, 2007)
H. pylori	αGlc-acyl-Chol	Protective	Jα18$^{-/-}$	oral	(Ito et al., 2013)
C. trachomatis (muridarum)	GLXA	Detrimental Not Protective	CD1d$^{-/-}$	i.n. intravaginal	(Bilenki et al., 2005) (Habbeddine et al., 2013; Peng et al., 2012)
C. pneumoniae	ND	Protective	Jα18$^{-/-}$, CD1d$^{-/-}$	i.n.	(Joyee et al., 2007)
L. pneumophila	ND	Detrimental	Jα18$^{-/-}$	i.t.	(Hayakawa et al., 2008)
α-Proteobacteria					
S. capsulata	GSL-1 (GlcA-GSL)	Protective (LD) Detrimental (HD)	Jα18$^{-/-}$, CD1d$^{-/-}$	i.v.	(Kinjo et al., 2008; Mattner et al., 2005)
N. aromaticivorans	GSL-1	Induction of PBC	CD1d$^{-/-}$	i.v.	(Mattner et al., 2008)
S. yanoikuyae	GSL-1 (GalA-GSL & GlcA-GSL)	Protective	Jα18$^{-/-}$, CD1d$^{-/-}$	i.v.	(Kinjo et al., 2008; Kinjo et al., 2005)
E. muris	Unknown	Protective	CD1d$^{-/-}$	i.v.	(Mattner et al., 2005)
Spirochetes					
B. burgdorferi	αGal DAG	Protective	CD1d$^{-/-}$	i.d.	(Kinjo et al., 2006b; Kumar et al., 2000; Tupin et al., 2008)
Mycobacteria					
M. tuberculosis	PIM_4	Not Protective Protective[#]	CD1d$^{-/-}$ cell transfer	i.v. aerosol	(Behar et al., 1999; Fischer et al., 2004) (Sada-Ovalle et al., 2008)

Mycobacteria					
M. tuberculosis	PIM_4	Not Protective Protective[#]	$CD1d^{-/-}$ cell transfer	i.v. aerosol	(Behar et al., 1999; Fischer et al., 2004) (Sada-Ovalle et al., 2008)
Parasites					
L. donovani	ND	Protective Not Protective[‡]	$J\alpha18^{-/-}$, $CD1d^{-/-}$ $J\alpha18^{-/-}$, $CD1d^{-/-}$	i.v.	(Amprey et al., 2004; Stanley et al., 2008)
L. major	ND	Protective	$J\alpha18^{-/-}$, depletion	s.c.	(Ishikawa et al., 2000)
T. cruzii	ND	Protective	$J\alpha18^{-/-}$, $CD1d^{-/-}$	i.p.	(Duthie et al., 2005)
T. gondii	ND	Protective Detrimental[#]	$J\alpha18^{-/-}$, $CD1d^{-/-}$ $J\alpha18^{-/-}$, $CD1d^{-/-}$ $V\alpha14^{tg}$	oral	(Smiley et al., 2005) (Ronet et al., 2005)
Viruses					
HSV-1	ND	Protective Not Protective[#]	$J\alpha18^{-/-}$, $CD1d^{-/-}$ $CD1d^{-/-}$	scarification	(Grubor-Bauk et al., 2003) (Cornish et al., 2006)
HSV-2	ND	Protective	$CD1d^{-/-}$	intravaginal	(Ashkar and Rosenthal, 2003)
RSV	ND	Protective	$CD1d^{-/-}$	i.n.	(Johnson et al., 2002)
EMCV-D	ND	Protective	$J\alpha18^{-/-}$, $CD1d^{-/-}$	i.p.	(Exley et al., 2001)
Sendai Virus	ND	Detrimental	$J\alpha18^{-/-}$, $CD1d^{-/-}$	i.n.	(Kim et al., 2008)
Influenza (H1N1 and H3N2)	ND	Protective[#]	$J\alpha18^{-/-}$, $CD1d^{-/-}$	i.n.	(De Santo et al., 2008; Ho et al., 2008; Kok et al., 2012; Paget et al., 2011; Paget et al., 2012)
HBV	ND	Protective	$J\alpha18^{-/-}$, $CD1d^{-/-}$	i.v.	(Zeissig et al., 2012)
LCMV	ND	Non Protective	$CD1d^{-/-}$	i.p.	(Roberts et al., 2004; Spence et al., 2001)
Fungi					
C. neoformans	ND	Not Protective	$J\alpha18^{-/-}$	i.v.	(Kawakami et al., 2001)
A. fumigatus	Asperamide B	Detrimental[‡] (AHR) Protective (early infection)	$CD1d^{-/-}$	i.n. i.t.	(Albacker et al., 2013; Cohen et al., 2011)

Table adapted from (Brigl et al., 2011)

[#]These studies used different bacterial/viral strains

[‡]These studies used mice on different genetic backgrounds

PBC, Primary Biliary Cirrhosis; LD, low dose; HD, high dose; i.n., intranasal; i.p., intraperitoneal; i.t., intratracheal; i.v., intravenous

found surrounding the outside of blood vessels. These joint-resident iNKT cells were shown to interact directly with spirochetes at the blood vessel walls, limiting their dissemination into the surrounding tissue via granzyme-mediated killing (Lee et al., 2014).

Streptococcus pneumoniae is a gram-positive bacterium that asymptomatically resides within the upper airway of humans. However, when *S. pneumoniae* gains access to the lower respiratory tract, it induces an acute inflammatory response resulting in severe disease (Kadioglu et al., 2008). Like *B. burgdorferi*, *S. pneumoniae* produces an α-glycosyldiacyglycerol that activates iNKT cells (Kinjo et al., 2011). Upon recognition of *S. pneumoniae* lipids, pulmonary iNKT cells produce IFN-γ and IL-17 and promote the clearance of bacteria through the recruitment of neutrophils to the infected lung (Kawakami et al., 2003).

Detrimental functions of iNKT

Legionella pneumophila is a gram-negative bacillus that causes acute pneumonia, known as Legionnaires' disease (Diederen, 2008). This pneumonia is often life-threatening and can be nosocomial or community-acquired. iNKT-deficient mice show reduced susceptibility to *L. pneumophila* infection (Hayakawa et al., 2008). This is not due to reduced bacterial burden, but rather to less severe tissue damage in these mice, as indicated by reduced albumin concentrations in bronchoalveolar lavage fluid (BALF). Although the mechanism was not revealed, the decreased susceptibility was not due to an altered Th1/Th2 response. Interestingly, iNKT cell-deficient mice had increased production of IL-1β and IL-18

at early time points owing to increased caspase-1 activity, suggesting an immunosuppressive function for lung iNKT cells.

The obligate intracellular pathogen *Chlamydia trachomatis* is a leading cause of sexually transmitted diseases, blindness, and infertility in humans (Brunham et al., 2000; Schachter, 1978). It is also known to be an important cause of lower respiratory disease in infants (Mishra et al., 2011). As previously discussed, intravaginal infection with the related mouse pathogen *C. muridarum* failed to identify a protective role for iNKT cells during reproductive tract infections (Habbeddine et al., 2013). However, in a mouse model of chlamydial pneumonitis, $CD1d^{-/-}$ mice were found to have reduced susceptibility to intranasal infection with the same pathogen (Bilenki et al., 2005; Joyee et al., 2007). These iNKT-deficient mice showed reduced lung burden and reduced tissue damage which was attributed to a reduced Th2 response as compared to wild-type mice.

These examples serve to highlight the context-specific lessons learned regarding iNKT cell function. They further caution against attempting to apply these lessons to studies of iNKT cells in other disease settings. Hence, our understanding of the intricacies of iNKT cell biology is lacking and further study is required before their immunoregulatory potential can be effectively leveraged.

Effects of α-GalCer treatment on infections

Due to its ability to robustly activate murine $V\alpha14^{+}$ iNKT cells, α-GalCer has been investigated as a means to modulate the immune response to a variety of

infectious diseases. Composite results demonstrate varied efficacy irrespective of whether or not the inciting pathogen produces an iNKT cell ligand. The beneficial role demonstrated for iNKT cells recognizing *S. pneumonia*–derived DAGs is enhanced upon α-GalCer treatment. Mice so treated exhibited enhanced bacterial clearance due to increased recruitment of PMNs into the infected lung (Kawakami et al., 2003; Nakamatsu et al., 2007). This effect was dependent upon IFN-γ produced by activated iNKT cells and transactivated NK cells (Christaki et al., 2015), and resulted in increased production of TNF-α and MIP-2 (Nakamatsu et al., 2007). Similar results were obtained when α-GalCer-treated mice were infected with *C. pneumoniae* (Joyee et al., 2007). Multiple treatments with α-GalCer prior to *P. aeruginosa* infection resulted in increased bacterial clearance by activated alveolar macrophages (Nieuwenhuis et al., 2002). Treated mice exhibited decreased neutrophil influx, increased TNF-α production, and a less severe pneumonia due to early IFN-γ production by activated iNKT cells. However, a later study using a single dose of α-GalCer failed to recapitulate these results (Kinjo et al., 2006b).

α-GalCer-mediated iNKT-cell activation has also been shown to have a beneficial effect on the outcome of systemic experimental infections in mice. αGalCer treatment afforded protection in infection models of murine malaria (Gonzalez-Aseguinolaza et al., 2000), *Cryptococcus neoformans* (Kawakami et al., 2001), hepatitis B virus (Kakimi et al., 2000), and encephalomyocarditis virus (Exley et al., 2001). In each case, the protective effect of α-GalCer was dependent upon the rapid production of IFN-γ by iNKT cells. However, α-GalCer may also elicit the

production of Th2 cytokines, as is the case in mice infected with the intracellular parasite *Toxoplasma gondii* (Ronet et al., 2005). In these mice, αGalCer treatment resulted in reduced IFN-γ but increased IL-4, IL-13, and IL-10 that protected from lethal intestinal inflammation. Interestingly, intranasal infection of mice with *Chlamydia muridarum* following α-GalCer treatment also resulted in a Th2-biased response, which in this instance, proved to be detrimental (Bilenki et al., 2005; Joyee et al., 2007). Hence, the ability of α-GalCer treatment to protect against lethal infectious disease is likely determined by the nature of the pathogen, the character of the protective response at the local site of infection, and the subset(s) of iNKT cells involved.

iNKT cell interactions with other cells

The diverse cytokines and chemokines produced by activated iNKT cells underlie their ability to influence the recruitment and function of a variety of other cell types (Brennan et al., 2013; Van Kaer et al., 2011a). The ability of iNKT cells to interact with CD1d-expressing cells is the basis for these immunoregulatory functions. APCs acquire microbial lipid antigens through phagocytosis of intact microorganisms or by the uptake of lipid binding proteins (van den Elzen et al., 2005). The identity of the APC presenting the lipid antigen as well as the nature of the antigen itself can have a profound influence on the biological outcome of NKT cell activation (Bai et al., 2012).

Dendritic cells

DCs constitutively express CD1d capable of presenting microbial and endogenous lipid antigens to iNKT cells. DCs activate iNKT cells to produce large amounts of IFN-γ and IL-4 (Bai et al., 2012; Bezbradica et al., 2005; Fujii et al., 2002) and have been demonstrated as the major antigen presenting cell responsible for iNKT cell activation *in vivo* (Arora et al., 2014; Bezbradica et al., 2005). Distinct DC subsets differ in their capacity to activate iNKT cells and in the nature of the cytokine responses they elicit (Arora et al., 2014; Bialecki et al., 2011; Macho-Fernandez et al., 2014). This activation is reciprocal in that iNKT-DC interactions induce the rapid maturation of DCs to include up-regulation of costimulatory molecules CD40, CD80, CD86, and surface molecules important in antigen capture and presentation, including DEC205 and MHC class II (Fujii et al., 2003), and the production of IFN-γ, TNF-α, and IL-12 (Carnaud et al., 1999; Fujii et al., 2004; Singh et al., 1999; Vincent et al., 2002).

This bidirectional activation is dependent upon CD40-CD40L interactions with iNKT cells and induces DCs to produce IL-12 (Kitamura et al., 1999), which further amplifies the iNKT cell IFN-γ response. These activated iNKT cells in-turn increase expression of the IL-12 receptor, further amplifying the effect. Mature DCs are potent primers of Th1 CD4+ and CD8+ effector T cells. These DCs recognize microbial antigens through PRRs and increase the production of stimulatory self-lipids and cytokines (primarily IL-12) that amplify iNKT cell activation (Brennan et al., 2013; Brennan et al., 2011; Paget et al., 2007; Salio et al., 2007). This DC-iNKT cell interaction leads to NK cell transactivation (Carnaud et al., 1999), enhanced

responses to protein antigens by B cells (Singh et al., 1999), $CD4^+$ and $CD8^+$ T cells (Fujii et al., 2003; Hermans et al., 2003), and the licensing of dendritic cells for cross-presentation (Semmling et al., 2010). It has recently begun to come clear that iNKT cells may also be involved in controlling the number and phenotype of DCs after bacterial infection or tumor induction (Shekhar et al., 2015; Shimizu et al., 2013). It is through these bidirectional interactions that iNKT cells and DCs cooperate to amplify and direct both innate and adaptive immune responses and are thus the basis for efforts aimed at developing iNKT cell-targeted adjuvants and immunotherapies (Carreno et al., 2014; Singh et al., 2014; Van Kaer et al., 2011b; Wu et al., 2009; Yu and Porcelli, 2005).

Macrophages

Macrophages may be important in iNKT cell activation in a tissue-specific manner. Kupffer cells were shown to be the predominant APC presenting *B. burgdorferi* antigens to iNKT cells in the liver (Lee et al., 2010). In the spleen, marginal zone macrophages capture and present soluble antigen to iNKT cells resulting in rapid activation (Barral et al., 2012). In the lymph nodes, it is the $CD169^+$ macrophages located in the subcapsular sinus that capture and present these antigens to iNKT cells located in the paracortex (Barral et al., 2010).

IFN-γ produced by activated iNKT cells can activate macrophages to phagocytose and kill intracellular bacteria such is the case with *P. aeruginosa* and *M. tuberculosis* (Nieuwenhuis et al., 2002; Sada-Ovalle et al., 2008). iNKT cells have also been shown to relieve the suppressive effect of myeloid-derived

suppressor cells (MDSCs) after influenza virus infection, thereby promoting a more effective virus-specific response (De Santo et al., 2008). In lean adipose tissue, NKT cells are thought to promote the polarization of macrophages to an M2 phenotype, which limits inflammation (Ji et al., 2012; Lynch et al., 2015b; Schipper et al., 2012). However, in the lung, this same type of interaction may contribute to chronic lung disease following viral infection (Kim et al., 2008).

B cells

As mentioned previously, marginal zone B cells have the highest expression levels of CD1d, suggesting a role in activation of iNKT cells (Bosma et al., 2012; Roark et al., 1998). However, the contribution of B cells to iNKT cell activation during infection is not well understood (Bezbradica et al., 2005; Bialecki et al., 2009; Mattner et al., 2008), but they have been suggested to play a role in iNKT cell homeostasis (Bosma et al., 2012). Similar to conventional T cells, iNKT cells can regulate, enhance, and sustain the humoral immune response (Chaudhry and Karadimitris, 2014) through their ability to produce an array of cytokines including IL-4, IL-6, IL-13, and IL-21 (Coquet et al., 2008). Co-administration of protein antigens and α-GalCer results in increased antibody titers in wild-type but not $J\alpha18^{-/-}$ or $CD1d^{-/-}$ mice (Chaudhry and Karadimitris, 2014). In this study, the adjuvant effect of α-GalCer was comparable to other conventionally used adjuvants and could be observed independent of conventional CD4+ T cells. Such an adjuvant effect has been observed with both T-dependent and T-independent antigens (Lang et al., 2006).

Thus, B cell help can occur through either cognate or noncognate interactions. Noncognate help involves iNKT cell-licensing of APCs to promote the activation of T follicular helper cells (Tfh). These Tfh cells in turn provide help to B cells to promote germinal center formation, class-switch recombination, somatic hypermutation, and the generation of long-lived plasma and memory cells (Tonti et al., 2009). This is believed to be the predominant mechanism of iNKT cell help to B cells when soluble protein antigens are co-administered with soluble α-GalCer.

Cognate interactions between iNKT cells and B cells were subsequently demonstrated via more advanced immunization techniques using haptenated α-GalCer (King et al., 2012; Leadbetter et al., 2008) or α-GalCer and protein antigen linked to bead particles (Barral et al., 2008). Using these more complex antigen formulations, several groups discovered that NKT cells can be induced to express Bcl-6 and become follicular helper cells (NKTfh) upon activation (Chang et al., 2012; King et al., 2012). B cell help from this NKTfh population contributes to rapid immunoglobulin production and some affinity maturation, but does not result in the generation of long-lived memory B cells, suggesting a role for iNKT cells in enhancing the early IgM and IgG responses during acute infection.

NK Cells

After *in vivo* administration of α-GalCer, NK cells are seen to rapidly produce IFN-γ. This NK cell activation depends on CD1d but does not result from NK-iNKT cell interactions (Carnaud et al., 1999), but rather through transactivation dependent upon iNKT cell-derived IFN-γ. This IFN-γ, along with IL-12 produced by iNKT cell-

matured APCs, in-turn activates NK cells. It is this transactivation of NK cells that is thought to be largely responsible for the anti-tumor effect of α-GalCer (Fujii et al., 2013). This NK-iNKT cell axis has also been proposed to be partly responsible for the Th1 vs Th2 biasing effects of certain α-GalCer derivatives, perhaps through the modulation of costimulatory and inhibitory molecules on the surface of APCs (Arora et al., 2014).

Neutrophils

As neutrophils are among the first cells recruited to sites of infection or injury, it is perhaps not surprising that iNKT cells are thought to play a major role in their recruitment. In models of lung infection with *P. aeruginosa* and *S. pneumoniae*, iNKT cells are required for the early recruitment of neutrophils to the site of infection (Kawakami et al., 2003; Nakamatsu et al., 2007; Nieuwenhuis et al., 2002). Although initially proposed to be due to macrophage inflammatory protein 2 (MIP-2) production by activated iNKT cells, this neutrophil recruitment was later thought to be dependent on IFN-γ (Nakamatsu et al., 2007). iNKT cell-derived IL-17 was shown to be important in recruiting neutrophils following influenza virus infection (Michel et al., 2007).

Aside from recruitment, iNKT cells have been shown to modulate neutrophil function. The acute phase protein serum amyloid A (SAA) induces the development of IL-10 producing immunosuppressive neutrophils. CD1d-dependent interactions between neutrophils and iNKT cells can reverse this phenotype promoting neutrophils to produce IL-12 instead (De Santo et al., 2010). Such effects are

reciprocal, in that neutrophils have also been shown to suppress the iNKT cell response in both mice and humans (Wingender et al., 2012a).

The broad distribution of CD1d and the ability of iNKT cells to interact with these CD1d-expressing cells are central to their ability to exert their effector functions. The diverse outcomes of these interactions underscore the complexities inherent in their multiple mechanisms of activation.

iNKT subsets: a division of labor

iNKT cells produce a variety of cytokines upon activation (Coquet et al., 2008; Gumperz et al., 2002). The secretion of Th1 and Th2 cytokines by iNKT cells has long been thought to underlie their immunoregulatory properties, but the mechanism(s) by which these diverse outcomes could be regulated under different disease conditions has remained elusive. One explanation that emerged was the existence of distinct subsets within the CD1d-restricted iNKT cell population (Table 1-2). The relatively recent appreciation that iNKT cells are a heterogenous population suggests that some of the conflicting data obtained in infectious disease models or through administration of synthetic lipid antigens, may be due to the differential activation of discrete iNKT cell subsets. To further complicate the matter, the currently defined subsets are represented in different proportions in various mouse strains (Hammond et al., 2001; Lee et al., 2013; Watarai et al., 2012).

iNKT cells in mice and humans are represented by both $CD4^+$ and $CD4^-$ subsets. In humans, the expression of CD4 has been shown as a predictor for the potential to produce Th2 cytokines, while both subsets exhibit a Th1-type response (Gumperz et al., 2002; Lee et al., 2002; Montoya et al., 2007). Additionally, in humans there exists a $CD8^+$ subset of iNKT cells that produce more IFN-γ and are more cytotoxic than $CD8^-$ iNKT (Motsinger et al., 2002; O'Reilly et al., 2011; van der Vliet et al., 2002). These $CD8^+$ iNKT cells do not seem to be present in mice. The expression of CD4 on mouse iNKT cells also does not seem to be as predictive of their function as is observed with human iNKT cells (Coquet et al., 2008).

Murine $CD8^-CD4^+$ and $CD8^-CD4^-$ (DN) iNKT cells, have traditionally been further subdivided based on their expression of NK1.1 (CD161) (Benlagha et al., 2000; Matsuda et al., 2000; Smyth et al., 2000). However, this marker is not expressed in all mouse strains and does not seem to reliably predict functional distinctions between subsets. As an alternative, recent studies have suggested the use of transcription factors and/or IL-17RB expression to reliably identify iNKT cell subsets (Constantinides and Bendelac, 2013; Lee et al., 2013; Watarai et al., 2012). As such, a proposed classification system for iNKT cells has emerged based on their functional analogy to subsets of MHC-restricted $CD4^+$ T cells (see Table 1-2).

Table 1-2. iNKT cell subsets

	iNKT1	iNKT2	iNKT10	iNKT17
Surface markers	$CD4^{+/-}$, IL-12Rβ, CD122, NKR^{+}, CXCR3, CXCR6,	$CD4^{+}$, NKR^{-}, IL-17RB, CD27, CCR4, CXCR6	$CD4^{+}$, $NK1.1^{-/low}$, CD49d, NRP1, SLAMF6, FR4, KLRG1, PD1	$CD4^{-}$, IL-23R, NKR^{-}, IL-17RB, IL-7Rα^{high}, CCR4, CCR6, CXCR6, CD121a, CD103, CD27, IL-18R
Transcription factors	$PLZF^{low}$, $GATA3^{low/-}$, T-bet	$PLZF^{high}$, GATA3, E4BP4 (IL-25-induced), IRF4	$PLZF^{low/-}$, E4BP4, T-bet, GATA3	$PLZF^{int}$, GATA3, RORγt, IRF4
Cytokines produced	IFN-γ, (IL-4), TNF, granzyme, perforin	IL-4, IL-9, IL-10, IL-13, GM-CSF	IL-10, IL-2	IL-17A, IL-21, IL-22
Proposed functions	Anti-tumor immunity	IL-25 induced AHR	Immune tolerance, resolution of inflammation	Virus/ozone-induced AHR, tissue repair, limiting systemic spread of pathogens
Anatomical location(s)	liver, spleen	lungs, intestine, lymph nodes	adipose tissue	lymph nodes, skin, lungs
References	(Constantinides and Bendelac, 2013; Coquet et al., 2008; Crowe et al., 2005; Lee et al., 2013; Skold and Cardell, 2000; Terashima et al., 2008)	(Constantinides and Bendelac, 2013; Lee et al., 2013; Terashima et al., 2008; Watarai et al., 2012)	(Lynch et al., 2015b; Sag et al., 2014)	(Constantinides and Bendelac, 2013; Coquet et al., 2008; Doisne et al., 2009; Doisne et al., 2011; Kastenmüller et al., 2012; Lee et al., 2013; Michel et al., 2007; Rachitskaya et al., 2008; Watarai et al., 2012)

Adapted from (Brennan et al., 2013; Constantinides and Bendelac, 2013)
NKR, NK lineage receptors including NK1.1, Ly49, NKG2D, CD94, DX5

iNKT1

iNKT1 comprise the majority of iNKT cells in mouse spleen and liver. They can be either $CD4^+$ or $CD4^-$ and the majority express NK1.1. iNKT1, as their name implies, produce predominantly Th1 cytokines, although they are also thought to make IL-4 in response to a strong stimulus. They lack expression of IL-17RB, but unlike other subsets, express CD122, the shared IL-2/IL-15 receptor β-chain, and depend on IL-15 for their development (Castillo et al., 2010; Gordy et al., 2011; Matsuda et al., 2002; Watarai et al., 2012). They can be identified by their expression of T-bet and low levels of GATA-3 (Lee et al., 2013). iNKT1 cells are believed to be those that are primarily responsible for the anti-tumor response elicited by α-GalCer administration (Crowe et al., 2005) and are likely also important in intracellular infections. Because iNKT1 cells are the predominant $NK1.1^+$ iNKT cells in B6 and related strains, it is likely that this subset was overrepresented in early studies that identified iNKT cells only as $CD3^+NK1.1^+$ cells.

iNKT2

iNKT2 are marked by expression of CD4 and IL-17RB and produce predominantly Th2 cytokines upon activation. Unlike iNKT1, they are not dependent on IL-15 for their development (due to a lack of CD122 expression), and can be activated by IL-25 in a manner that is believed to be responsible for iNKT cell-mediated AHR (Kim et al., 2009; Matangkasombut et al., 2009; Terashima et al., 2008; Watarai et al., 2012). This effect is mediated in part by iNKT2 production of IL-13 and IL-4 along with the Th2 chemokines CCL17, CCL22, C10/CCL6, and

eosinophil chemotactic factor-L (ECF-L) and the resulting recruitment of macrophages, eosinophils, neutrophils, and lymphocytes into the lungs (Terashima et al., 2008), resulting in increased tissue damage.

Hogquist and colleagues (Moran et al., 2011) developed a novel reporter mouse, in which the Nur77 promotor/enhancer cassette drives the expression of eGFP transgene upon signaling via the antigen receptor but not by cytokine or inflammatory signals. Using this model, they recently demonstrated that iNKT2 cells are a distinct lineage that can be identified both in the thymus and in the periphery. This subset is marked by constitutive expression of IL-4 and a stronger TCR signal in response to self- or foreign-antigen (Lee et al., 2013). Interestingly, iNKT2 cells were shown to have increased usage of Vβ2 and Vβ7 in their TCRs, a phenotype that may explain their greater perceived recognition of endogenous self-lipids (Schumann et al., 2003; Wei et al., 2006).

iNKT10

More recently, Wingender and colleagues (Sag et al., 2014) described a population of IL-10-producing iNKT cells that could be induced by pre-exposure to α-GalCer *in vivo* (Birkholz et al., 2015; Sag et al., 2014). Interestingly, these "iNKT10" cells could also be found at low frequency in unchallenged mice and in human PBMCs. Unlike what has previously been reported for the larger population of iNKT cells (Wilson et al., 2003), these iNKT10 cells did not downregulate the TCR/CD3 complex following α-GalCer stimulation and expressed several proteins typically associated with regulatory T cells, leading the authors to speculate that

these iNKT10 cells might be important for the maintenance of tolerance in immune-privileged sites. iNKT10 cells resident in adipose tissue are thought to be important for regulating the function of T_{regs} and inducing the polarization of anti-inflammatory M2 macrophages (Lynch et al., 2015a). These iNKT10 cells were recently shown to produce IL-2 and IL-10 but not IFN-γ. Interestingly, unlike other subsets of iNKT, their development and effector functions do not seem to be dependent on PLZF expression (Lynch et al., 2015b).

iNKT17

IL-17A producing NKT cells are contained within the CD4-IL-17RB^{+} population and are enriched in barrier tissues such as the lungs, skin, and peripheral lymph nodes, with lower populations in the spleen and liver (Doisne et al., 2009; Michel et al., 2007). Like iNKT2, they do not express CD122 and therefore develop independently of IL-15, but instead require IL-7 for their survival (Watarai et al., 2012; Webster et al., 2014). iNKT17 can be distinguished from iNKT2 by their constitutive RORγt expression.

iNKT17 have been shown to rapidly produce IL-17A in response to *S. pneumoniae* and *K. pneumoniae* and to induce airway neutrophilia in response to synthetic glycolipid or LPS challenge (Kinjo et al., 2011; Michel et al., 2007; Price et al., 2012). Their production of IL-22 in response to influenza virus infection seems to be important in the maintenance and regeneration of damaged epithelial tissue (Paget et al., 2012), but they may also contribute to ozone-induced AHR (Pichavant

et al., 2008), and the development of experimental autoimmune encephalomyelitis (EAE) in mice.

CD1d trafficking and iNKT cell activation

Unlike antigenic peptides from intracellular or extracellular sources that are generally segregated into MHC class I or MHC class II pathways, respectively, CD1d molecules present lipids derived from both intra- and extracellular sources (Chen et al., 2007; McEwen-Smith et al., 2015). The composite findings of several elegant studies have revealed that the intracellular trafficking of CD1d molecules determines the nature of the bound ligands that are available for surveillance by iNKT cells (Florence et al., 2008). Accordingly, the site of glycolipid loading has been shown to determine the functional outcome of iNKT cell activation (Im et al., 2009).

CD1d is a heterodimer consisting of a heavy chain that is noncovalently associated with the light chain β2-microglobulin and is thus considered MHC class I-like. The extracellular domains of the heavy chain (α1, α2, and α3) are anchored by the transmembrane region, which ends in a short cytoplasmic tail. Akin to MHC class I, CD1d molecules are assembled in the endoplasmic reticulum (ER) where their folding is assisted by the chaperones calnexin and calreticulin and the thiol oxidoreductase ERp57 (Hüttinger et al., 1999; Kang and Cresswell, 2002a; Sugita et al., 1997). In the ER, microsomal triglyceride transfer protein (MTP) assists in loading the hydrophobic groove of CD1d molecules with endogenous phospholipids (Brozovic et al., 2004; De Silva et al., 2002). Once loaded, these newly formed

CD1d molecules traffic the secretory pathway where they are expressed on the cell surface (Fig. 1-2).

Surface-expressed CD1d molecules are recycled through the endosomal vesicular system by virtue of a conserved motif in the cytoplasmic tail. Intracellular trafficking is tightly controlled by binding of adapter proteins 2 and 3 (AP-2 and AP-3) to these tyrosine-containing motifs. It is via this recycling and transit through various endosomal compartments that CD1d molecules acquire not only the endogenous self-lipids to which NKT cells are known to be autoreactive, but also microbial-derived exogenous antigens which become available for presentation to iNKT cells at the cell surface. Alternatively, CD1d has also been shown to associate with the MHC class II chaperone invariant chain (Ii) which targets CD1d directly to lysosomes where it can be loaded without the necessity of recycling from the plasma membrane (Jayawardena-Wolf et al., 2001; Kang and Cresswell, 2002b; Sille et al., 2011a; Sille et al., 2011b). Interestingly, $Ii^{-/-}$ mice develop normal numbers of iNKT cells but show reduced reactivity to α-GalCer or mycobacterial lipids (Sada-Ovalle et al., 2008; Sille et al., 2011b).

The exchange of lipids into the hydrophobic groove of CD1d is facilitated by numerous lysosomal lipid transfer proteins including saposins A, B, C, and D, G_{M2} activator protein (Zhou et al., 2004a), Niemann-Pick type C2 (Schrantz et al., 2007), and catepsin L (Honey et al., 2002). The specific requirements for antigen loading onto CD1d as well as the cellular location at which loading occurs are dependent on the nature of the lipid and likely on the identity and maturation state of the activating APC (Chen et al., 2007; Honey et al., 2002; Zhou et al., 2004a). These findings

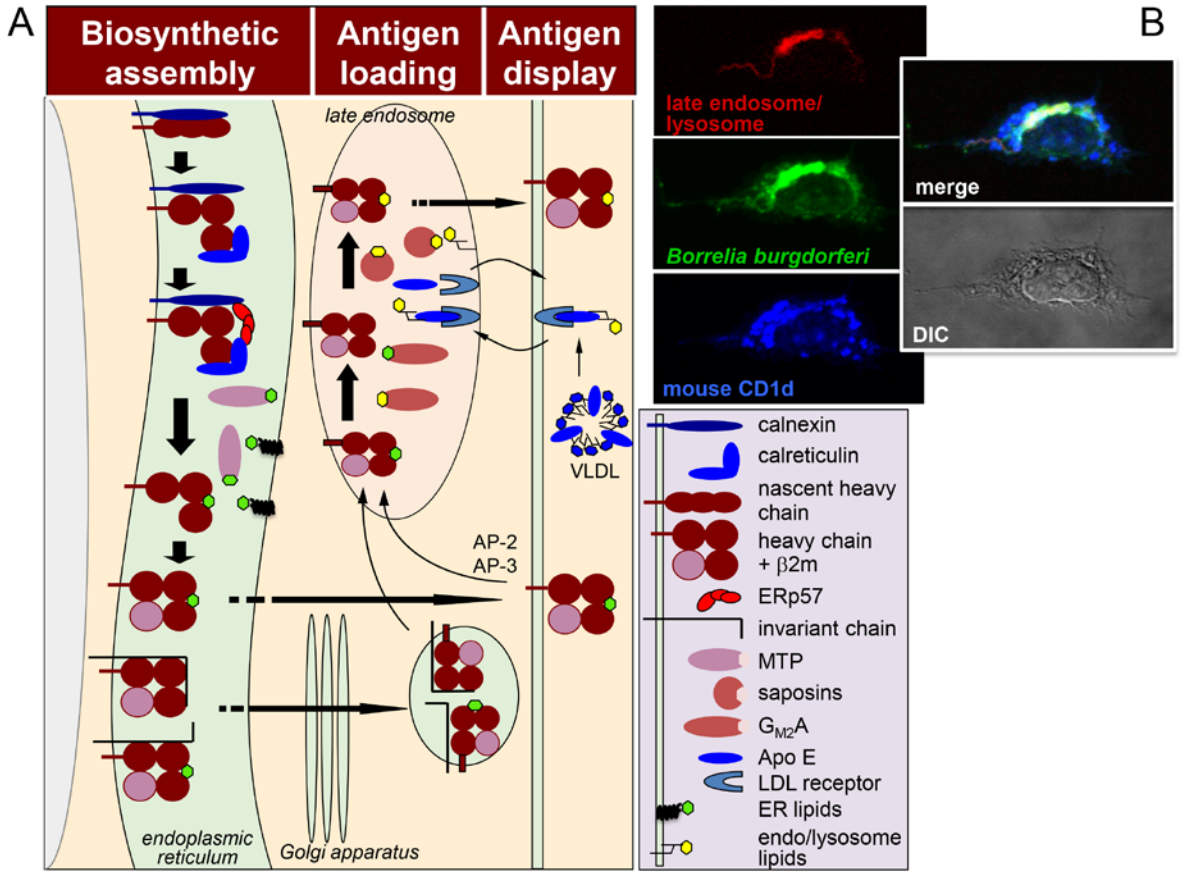


Figure 1-2. Topologic biochemistry and the assembly of CD1d with iNKT cell antigen. (A) CD1d assembly with lipids begins within the rough endoplasmic reticulum (ER) with the assistance of several chaperones. Partially folded α-chain–β2m complex is then thought to bind ER-resident lipids with the assistance of lipid transfer proteins (LTP) such as microsomal triglyceride transfer protein (MTP), a protein that facilitates the assembly of apolipoprotein B. Upon complete assembly, the CD1d–lipid complexes egress from the ER and negotiate the secretory pathway to the plasma membrane. By virtue of a late endosome/lysosome targeting motif within the cytoplasmic tail of CD1d, it recycles through the MHC class II enriched compartment (MIIC). In the MIIC (late endosomes/lysosomes), CD1d exchanges its ER-loaded lipids for antigenic glycolipids that activate iNKT cells *in vivo*. The extraction of bound lipids from CD1d and the loading of antigenic glycolipids are facilitated by lysosomal LTPs such as Saposins (Sap), G_{M2} activator ($G_{M2}A$), and Niemann-Pick C-2 (not shown), which are essential for the enzymatic catabolism of glycolipids. (B) Infection of Mϕ and DC delivers microbes to the CD1d-containing lysosomes. Differential interference contrast (DIC) picture of Mϕ observed under a light microscope showing the gross cellular outline; the prominent structure within this cell is its nucleus. Cellular organelles and their contents are observed by confocal fluorescence microscopy: red, lysosome; green, *B. burgdorferi*; blue, CD1d. Where *B. burgdorferi* colocalizes with lysosomes and CD1d appears white in the merged picture.

further reveal the complex requirements for CD1d loading with structurally diverse lipids (Table 1-3).

Endogenous NKT cell antigens

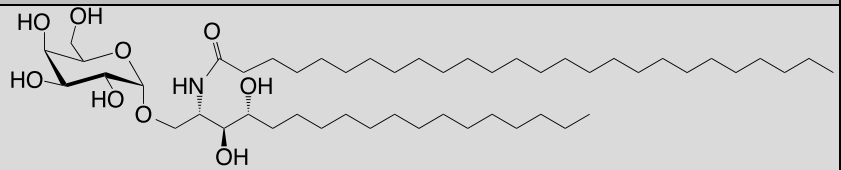
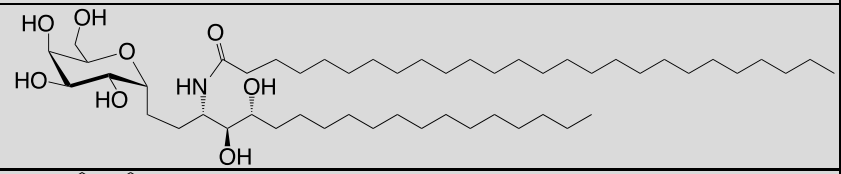
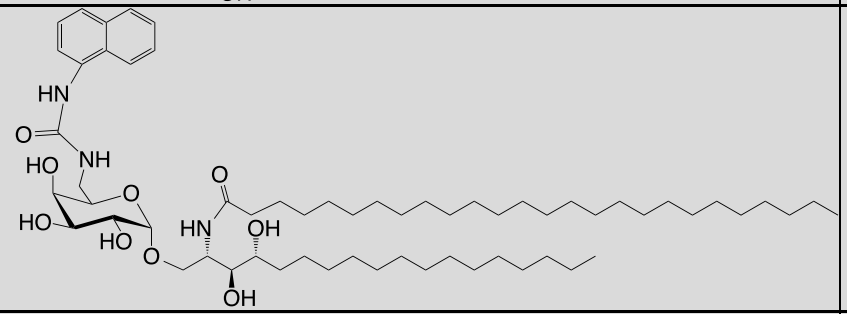
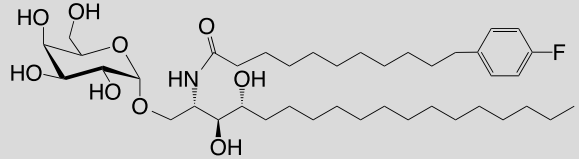
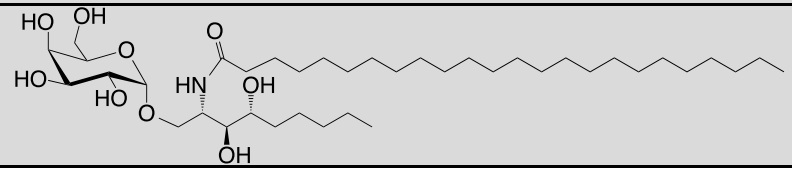
A characteristic feature of iNKT cells is their autoreactivity in that they respond to self-antigens expressed by host cells. Freshly isolated thymic iNKT cells or hybridomas expressing the Vα14 iNKT cell receptor can be activated by a variety of CD1d-expressing cells without the addition of exogenous antigens (Bendelac et al., 1995; Brossay et al., 1998b). As predicted from studies of CD1d trafficking described above, the nature of endogenous lipids bound to CD1d appears to be dictated by the cellular localization of CD1d and the predominant lipid species encountered in these compartments.

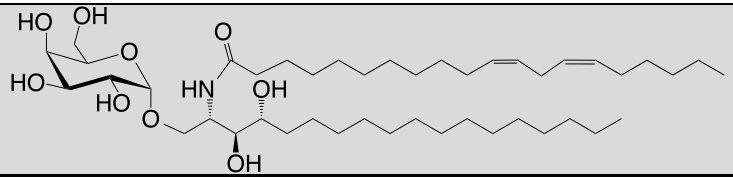
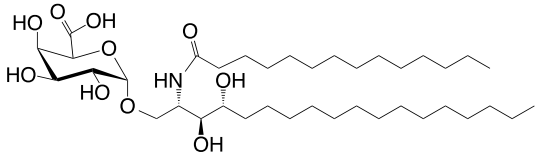
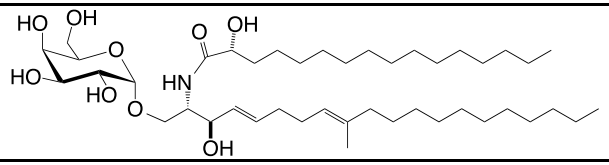
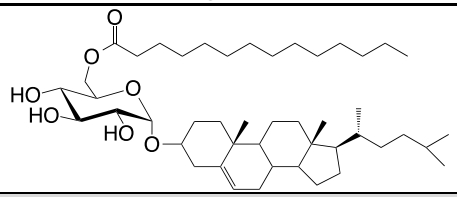
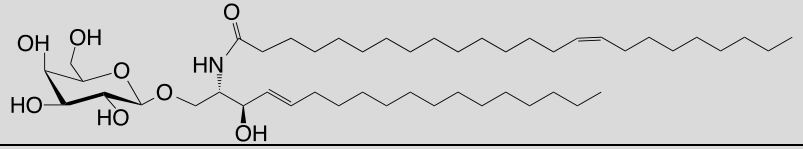
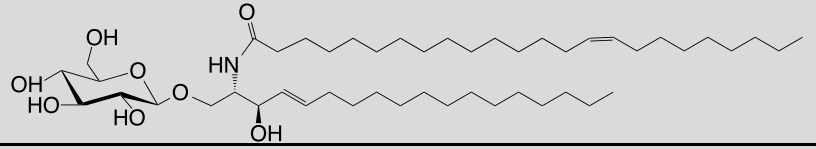
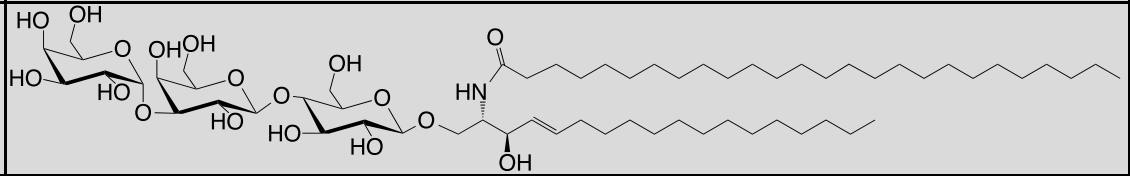
Early studies identified glycosylated phosphatidylinositol (GPI) and unmodified PI as major ligands of murine CD1d (De Silva et al., 2002; Joyce et al., 1998). Using CD1d constructs designed to be either retained in the ER, secreted from the cell upon arrival at the plasma membrane, or proteolytically cleaved following recycling through endosomal compartments, Yuan et al. found each of these constructs to be loaded with discrete populations of lipids (Yuan et al., 2009). CD1d retained in the ER was found to be loaded primarily with phosphatidylcholine (PC), the most abundant phospholipid in the cell, whereas the secreted form that had negotiated the secretory pathway was found to be loaded only with sphingomyelin, a sphingolipid not found in the ER. This suggests that either PC is exchanged in the Golgi apparatus by some unknown mechanism, or that CD1d

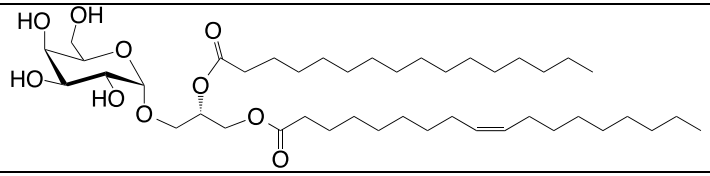
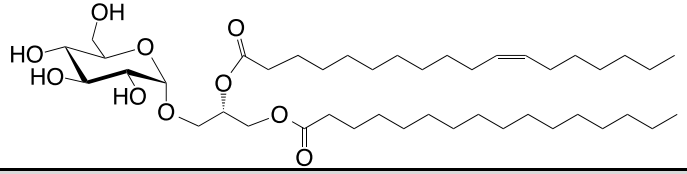
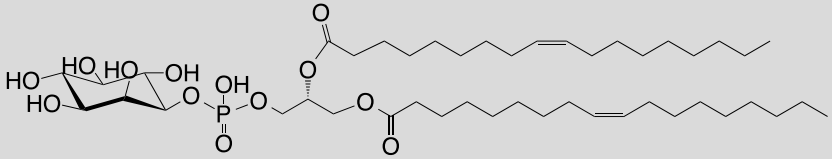
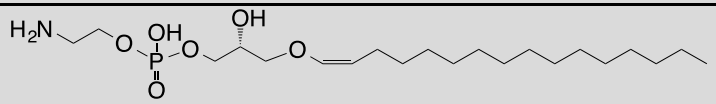
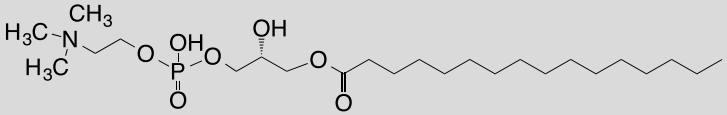
molecules leave the ER in a lipid-free form, whereupon they acquire these lipids in other subcellular compartments. The protease cleavable CD1d was found to be loaded with both PC and sphingomyelin in addition to lysophospholipids acquired in the lysosome. Purified or synthetic preparations of several common cellular phospholipids loaded onto plate-bound CD1d molecules were shown to stimulate iNKT cell hybridomas *in vitro* and this activation was enhanced by loading CD1d under low pH conditions (Gumperz et al., 2000). Later studies identified a wide variety of glycerophospholipids and sphingolipids bound to human CD1d (Cox et al., 2009).

Disparate results from similar experiments are likely due to the sensitivities of the methods employed, the various cell lines used, and the activation state of the CD1d-expressing cells, all of which might influence the menu of cellular lipids available for loading. They should therefore perhaps be viewed as complementary rather than contradictory. Taken together, these findings support the paradigm in which CD1d molecules, in the absence of infection or stress, survey the intracellular environment and accumulate the most abundantly available lipids and that these CD1d-bound lipids are exchanged with an endo/lysosomal lipid(s) during recycling through the endocytic pathway.

Table 1-3. Structure and properties of selected synthetic, microbial, and self iNKT cell agonists

Lipid (class[1])	Chain Length[2]	Structure	Agonist[3]	Ref
αGalCer (GSL)	C18-phyto C26		Very strong; robust IFN-γ, IL-4 and other cytokines; synthetic	(Kawano et al., 1997)
α-*C*-GalCer (GSL)	C18-phyto C26		Weak (mo[4])-to-none (hu); IFN-γ; synthetic	(Schmieg et al., 2003)
NU-αGalCer (GSL)	C18-phyto C26		Very strong; IFN-γ; synthetic	(Aspeslagh et al., 2011)
FPh-αGalCer (GSL)	C18-phyto C10-fluro-phenyl		Very strong; IFN-γ; synthetic	(Li et al., 2010)
OCH (GSL)	C9-phyto C24		Weak (mo)-to-none (hu); IL-4 (low-to-no IFN-γ); synthetic	(Miyamoto et al., 2001)

C20-diene (GSL)	C18-phyto C20:2		Strong; IL-4 (low-to-no IFN-γ); synthetic	(Yu et al., 2005)
αGalU Cer (GSL)	C18-phyto C14		Weak; *Sphingomonas* spp.	(Kinjo et al., 2005; Mattner et al., 2005; Sriram et al., 2005)
Asp B (GSL)	C20:2 (9-Mt) C16 (2-OH)		Weak; *Aspergillus fumigatus*	(Albacker et al., 2013)
αGlc-acyl-Chol	C14		Strong; binds a small NKT cell subset (mo); *Helicobacter pylori*	(Chang et al., 2011)
βGalCer (GSL)	C18 C24:1		Weak; self	(Ortaldo et al., 2004; Parekh et al., 2004)
βGlcCer (GSL)	C18 C24:1		Strong; self	(Brennan et al., 2011)
iGb3 (GSL)	C18-C24		Weak (mo)-to-none (hu); self	(Zhou et al., 2004b)

αGal DAG (GGL)	*sn1*-C18:1 *sn2*-C16		Weak (mo)-to-none (hu); *Borrelia burgdorferi*	(Kinjo et al., 2006b)
αGlc DAG (GGL)	*sn1*-C18:1 *sn2*-C16		Weak; *Streptococcus pneumoniae*	(Kinjo et al., 2011)
PtdIno (GPL)	*sn1*-C18:1 *sn2*-C18:1		Weak (mo)-to-no (hu); self	(Gumperz et al., 2000; Mallevaey et al., 2011)
Plasmalogen (GPL)	*sn1*-C16 vinyl-ether *sn2*-lyso		Positive selection (mo); self	(Facciotti et al., 2012)
Lyso-PtfCho (GPL)	*sn1*-C16 *sn2*-lyso		Weak (hu)-to-none (mo); GM-CSF (no IL-4, IFN-γ); self	(Fox et al., 2009)

[1] GSL, glycosphingolipid; GGL, glycoglycerolipid; glycerophospholipid; [2] sphingosine/phytosphingosine chain length indicated first and *N*-acyl chain length second; [3] agonist strength based on ref (Joyce et al., 2011); [4] mo, mouse; hu, human

Mechanisms of iNKT cell activation

At least two pathways for iNKT cell activation during microbial infection have been described [Fig. 1-3; (Brennan et al., 2013)]. The microbial antigen driven pathway involves TCR-mediated recognition of a microbial lipid presented by CD1d. Cell wall-derived GSLs from *Sphingomonas* spp. (Kinjo et al., 2005; Mattner et al., 2005; Sriram et al., 2005) and diacylglycerols from *Borrelia burgdorferi* (Kinjo et al., 2006b) and *Streptococcus pneumoniae* (Kinjo et al., 2011) were shown to be presented by CD1d and to directly activate iNKT cells in a TCR-dependent manner. Alternatively, iNKT cells can be activated in the absence of a microbial lipid through an innate cytokine/self-lipid driven pathway in which APCs activated in response to Pathogen Associated Molecular Patterns (PAMPs) present weakly-stimulating self-lipids along with a co-stimulating signal provided by IL-12 (Brigl et al., 2003; Mattner et al., 2005; Nagarajan and Kronenberg, 2007). In a modification of this second pathway, when cytokine signals are strong enough, as may be the case with *Escherichia coli* LPS or during viral infections, TCR-mediated recognition of lipid ligands may be mostly dispensable (Juno et al., 2012; Nagarajan and Kronenberg, 2007; Tyznik et al., 2008; Wesley et al., 2008). The cytokine/self-lipid pathway was recently proposed to predominate during most microbial infections, in that IFN-γ production by iNKT cells was dependent on innate signals eliciting IL-12 production from APCs, whether or not a microbial ligand was present (Brigl et al., 2011). Interestingly, IL-12 was not required for iNKT cell activation in response to synthetic lipids despite its requirement for a response to the bacterium from which the lipid was derived. These results led to the conclusion that iNKT cells are uniquely

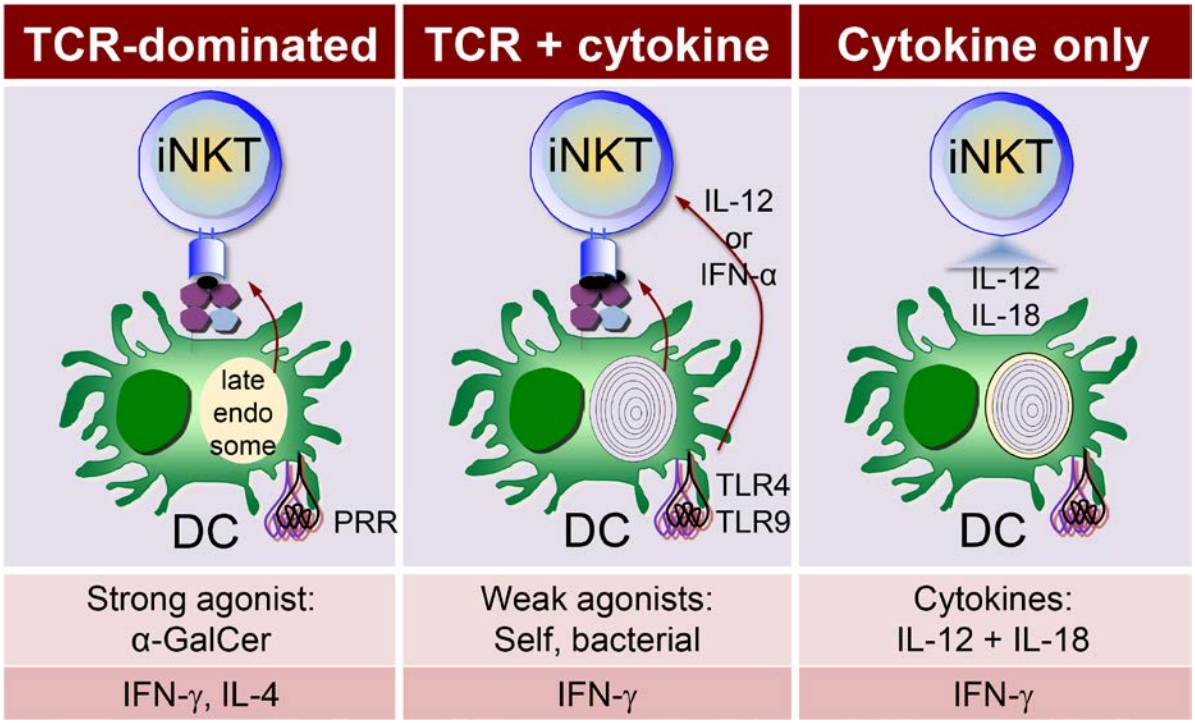


Figure 1-3. Three distinct strategies activate iNKT cells.

Potent iNKT cell agonists—such as α-GalCer—directly activate iNKT cells without the need for a second signal in a TCR-signaling dominated fashion **(left panel)**. Alternatively, microbes containing TLR ligands such as LPS activate iNKT cells by inducing IL-12 production by DCs, which amplifies weak responses elicited upon the recognition of CD1d bound with self-glycolipids by the iNKTCR. Several endogenous lipid agonists have been identified and characterised (see Table 1-3). Some microbes such as *Sphingomonas capsulata*, which are α-Proteobacteria, synthesize α-anomeric glycolipids for their cell walls. These glycolipids, when presented by CD1d, weakly activate iNKT cells directly. In the presence of a second signal—generally a pro-inflammatory cytokine such as IL-12—strongly activate iNKT cells **(middle panel)**. Intriguingly, iNKT cells can also be activated solely by cytokines—mainly IL-12— in a TCR-independent manner **(right panel)**.

equipped to respond to low levels of self- or microbial-antigens under conditions that induce IL-12. However, this IL-12 mediated amplification may not be required in the case of more potent or abundant exogenous lipid antigens.

The identity of the self-lipid that might be responsible for iNKT cell activation under such conditions has remained elusive, although several candidates have been proposed (Brennan et al., 2011; Facciotti et al., 2012; Zhou et al., 2004b). Multiple studies pointed toward a β-linked glycolipid as a probable self-lipid, but a more recent report found that these results were likely the result of commercial lipid stocks being contaminated with α-anomers (Kain et al., 2014). Strikingly, through a series of elegant biochemical and immunological assays, this study identified the endogenous iNKT cell antigen as α-GalCer (and likely also α-GluCer), a lipid whose synthesis was previously thought to be impossible in mammalian cells (Lairson et al., 2008). Furthermore, this study suggested that rather than a lipid whose synthesis is up regulated upon APC activation, iNKT cell activation may be rather controlled by degradative enzymes that limit the availability of these lipids under steady-state conditions (Darmoise et al., 2010; Van Kaer and Joyce, 2010). One might therefore further conclude that pathogens that modulate the expression or function of these enzymes would influence the activation and function of iNKT cells as discussed above.

Synthetic iNKT cell ligands

Much of our understanding on iNKT cell biology is derived from studies using the synthetic form (KRN7000) of α-GalCer. α-GalCer is an α-anomeric glycolipid

originally isolated from the Okinawan marine sponge *Agelas mauritianus*. Its antimetastatic effects were shown to be dependent on its presentation by CD1d and the specific activation of iNKT cells (Brossay et al., 1998a; Burdin et al., 1998; Kawano et al., 1997; Spada et al., 1998). Numerous studies have demonstrated that α-GalCer is a potent activator of both mouse and human iNKT cells, eliciting the production of both Th1 and Th2 cytokines. It therefore emerged as a powerful tool by which to probe the development and function of iNKT cells.

α-GalCer has a C18 sphingosine base and a fully saturated C26 *N*-linked fatty acyl chain. Considerable effort has been made to identify structural variants of α-GalCer that might alter the patterns of cytokine production. The first advance toward this goal came with the synthesis of OCH, an α-GalCer analog where the sphingoid base was truncated to C9:0 and the acyl chain slightly shortened to C24:0 (Miyamoto et al., 2001). Treatment of mice with OCH resulted in a reduced IFN-γ response whereas IL-4 production remained unchanged. This was interpreted as a Th2 bias and was subsequently shown to suppress the development of Th1-mediated EAE in mice. Later studies found that shortening the fatty-acid chain resulted in decreased potency of α-GalCer variants, but the introduction of double bonds could recover the stimulatory activity lost to such truncations (Arora et al., 2014; Yu et al., 2005).

Interestingly, the apparent Th2-skewed cytokine production elicited by these α-GalCer variants is believed to be due to differential transactivation of B and NK cells rather than via direct modulation of iNKT cell-derived cytokines (Arora et al., 2014; Yu et al., 2005). Administration of α-GalCer C20:2 resulted in tempered iNKT

cell expansion and more short-lived activation of B and NK cells as compared to α-GalCer C26 (KRN7000). These disparate responses are believed to be due in part to differential requirements for loading of α-GalCer and its variants onto CD1d, since the Th2-biasing lipids are less dependent on endosomal loading as compared to KRN7000 (Bai et al., 2009; Im et al., 2009; Yu et al., 2005).

Outstanding Questions

Despite almost two decades of research, the question remains as to how iNKT cells in various anatomical locations can so diversely influence disease pathology in response to microbial or environmental insults. The outcome of iNKT cell activation is undoubtedly influenced by the local inflammatory milieu, the types of APCs involved, the nature of the activating lipid(s), and the subset(s) of NKT cells involved in the response. Deciphering the relative contribution of each of these factors in a context-specific manner represents a significant hurdle to our further understanding of iNKT cell function. These efforts are perhaps further stymied by our reliance on synthetic ligands that elicit supraphysiological responses and our inability to translate lessons learned from their study into the context of a natural infection. To further our understanding, we must therefore discover new natural antigens and develop better tools for identification, depletion, and activation of individual iNKT cell subsets before the goal of targeting iNKT cells for therapeutic intervention can be realized.

Toward this goal, I sought a mouse model where the function of iNKT cells could be studied in a physiologically relevant disease setting. I was particularly interested in pulmonary iNKT cells since they have been less well-studied than iNKT cells in other organs. In the lungs, iNKT cells promote inflammation in models of airway hyperreactivity (AHR), acute lung injury (ALI), and chronic obstructive pulmonary disease [COPD;(Paget and Trottein, 2013)]. iNKT cells may also contribute to the inflammatory cascade accompanying sepsis, which is often a complication accompanying bacterial infections of the lung (Leung and Harris, 2011; Munford, 2006). In general, pulmonary iNKT cells are thought to play a protective role in microbial infections, but in some cases, may also exacerbate disease (Paget and Trottein, 2013). However, the mechanisms by which pulmonary iNKT cells contribute to disease pathology remain poorly defined.

Francisella tularensis is a facultative intracellular bacterium that causes the zoonotic disease tularemia. Inhalation of as few as 10 bacteria results in 30-60% mortality if left untreated (Ellis et al., 2002). Due to its high infectivity and low infectious dose, *F. tularensis* is a potential agent of bioterrorism (Dennis et al., 2001) and is classified as a category A agent by the U.S. Centers for Disease Control. Several countries researched, weaponized, and stockpiled *F. tularensis* from the 1940s through the 1990s (Dennis et al., 2001). Much of this research was focused on developing more virulent antibiotic resistant strains of the bacterium, which might someday reemerge. Due to its high infectivity and potential for use as a bioweapon, the NIAID has a long-term research goal of characterizing the innate and adaptive immune responses that occur after initial exposure to *F. tularensis*

(National Institute of Allergy and Infectious Diseases (U.S.), 2002). This goal still remains despite the recent move toward a more broad-spectrum biodefense strategy. This is because the lessons outlined below as learned from the study of *F. tularensis* will likely have broader implications in furthering our understanding of severely acute bacterial infection and pathogenesis.

Survivors of tularemia develop life-long sterilizing immunity, yet efforts to develop a safe and effective vaccine have proven difficult. This is largely due to the fact that infections with different subspecies or strains through disparate routes of inoculation result in widely varied immune responses in animal models (Cowley and Elkins, 2011). Hence, identification of suitable targets for drug or vaccine design has proven elusive. In the murine model of pulmonary tularemia, there is an initial lag in the innate immune response. The delayed response allows the bacterium to replicate relatively unimpeded, spreading to peripheral organs within a matter of days. The resulting bacterial load contributes to systemic inflammation, a major contributor to the pathology and mortality associated with this disease (Mares et al., 2008; Metzger et al., 2007; Sharma et al., 2009; Steiner et al., 2014). IFN-γ is critically important in the host response to *Francisella tularensis* (Anthony et al., 1989; Conlan et al., 2002; Duckett et al., 2005; Elkins et al., 1993; Elkins et al., 1996; Leiby et al., 1992), and multiple cell types have been shown to produce IFN-γ during the early stages of infection (Bokhari et al., 2008; Cowley et al., 2010; De Pascalis et al., 2008; Duckett et al., 2005; Lopez et al., 2004), but the potential role of pulmonary iNKT cells has never been thoroughly investigated. Because all of the cell types susceptible to *F. tularensis* infection have been shown to interact with

iNKT cells, and because iNKT cells are a proven source of early IFN-γ in a variety of other infections, I reasoned that they might contribute to the early response to *F. tularensis* in the lungs. Thus, the central goal of my research was to define the involvement of iNKT cells in the host response to respiratory *F. tularensis* infection. To accomplish this goal, two outstanding questions were identified:

1) How do iNKT cells contribute to the immune response to respiratory infection by *Francisella tularensis*?
2) What is the chemical nature of the *Francisella tularensis*-derived antigen(s) that activates iNKT cells?

How do iNKT cells contribute to the immune response to respiratory infection by *Francisella tularensis*?

Preliminary data from our laboratory indicated that iNKT cells were rapidly activated by parenteral infection with *F. tularensis* subspecies (spp.) *novicida.* This is a pathogen that causes severe disease in mice, but is nonpathogenic in immunocompetent humans (Kingry and Petersen, 2014). Despite a high degree of genetic similarity between *F. tularensis* spp. *novicida* and the more clinically relevant spp. *tularensis* (Type A) and *holarctica* (Type B), *novicida* is not believed to infect animals in nature. It has, however, been extensively used as a surrogate to study the human disease in mice and other small mammals due to its less stringent regulatory requirements. Nevertheless, studies of iNKT cell function using spp. *novicida* are complicated by the fact that unlike Type A and B strains, spp. *novicida* causes a rapid inflammatory cytokine storm due in part to differences in its surface

structure, mechanisms of cellular entry, and the nature and kinetics of the cytokines it elicits. I therefore chose to use an attenuated Type B live vaccine strain (LVS) which still causes severe disease in mice, but perhaps more closely mimics the delayed inflammatory response observed in human tularemia. The results of these studies are detailed in Chapter II.

What is the chemical nature of the *Francisella tularensis*-derived antigen(s) that activates iNKT cells?

As discussed earlier in this chapter, iNKT cells can be activated by at least three mechanisms and the nature of the activation is believed to determine the functional outcome. The best-described means of antigen-independent activation of INKT cells is via TLR4-mediated maturation of APCs in response to LPS. Since the LPS of *F. tularensis* LVS weakly, if at all stimulates APCs through TLR4 (Cole et al., 2006; Cole et al., 2007; Katz et al., 2006), it is likely that any iNKT cell activation in response to LVS would be due to CD1d-mediated recognition of either self- or LVS-derived lipids. Because the entry of *Francisella* into host cells requires access to the endosomal pathway where CD1d-bound lipids are thought to be exchanged, it further seems likely that any *Francisella*-derived lipid capable of being loaded onto CD1d would become available for presentation and subsequent activation of iNKT. The identification of a novel iNKT cell agonist would be of great interest to the field. Preliminary findings related to the identification of *Francisella*-derived lipids are provided in Chapter III.

CHAPTER II

LUNG iNKT CELL ACTIVATION EXACERBATES TULAREMIA-LIKE DISEASE IN MICE

Introduction

The respiratory mucosa is a major site for pathogen entry and hence, requires constant immune surveillance. Like other mucosal surfaces, the lungs are populated by a variety of innate cells and innate-like lymphocytes. One such cell type, iNKT cells, are enriched within the lung vasculature where they are optimally positioned for early antigen encounter (Scanlon et al., 2011). These pulmonary iNKT cells exert diverse functions dependent upon experimental settings (Paget and Trottein, 2013).

To probe the function of lung iNKT cells, I chose a respiratory *Francisella tularensis* infection model as this infection causes lethal pulmonary tularemia. *F. tularensis* is a gram-negative facultative intracellular bacterium, which infects multiple cell types including macrophages, dendritic cells, hepatocytes, neutrophils, and epithelial cells (Hall et al., 2008; Law et al., 2011). The resulting disease targets multiple organs and manifests itself in several forms of differing severity depending on the inciting bacterial strain, dose, and route of infection. Of these, the respiratory route is the most deadly, and the most likely route of infection by weaponized *F. tularensis* (Bossi et al., 2006). After inhalation, patients typically show signs of systemic illness, which may be accompanied by immediate signs of

respiratory disease and can result in death in 30-60% of cases if left untreated (Dennis et al., 2001; Ellis et al., 2002; Foley and Nieto, 2010; Sjostedt, 2007). Although the exact cause of death is unclear, it is likely due to an overwhelming systemic inflammatory response (Steiner et al., 2014). Mice inoculated intranasally (i.n.) with *F. tularensis* fail to mount an effective immune response for the first 48–72h. After this initial immune latency, the response to *F. tularensis* is characterized by a robust local and systemic "cytokine storm" reminiscent of sepsis (Cowley, 2009; D'Elia et al., 2013). Little is known about the role of NKT cells in pulmonary tularemia—in either humans or mice, due in part to the difficulties in distinguishing them from NK cells, which protect mice from tularemia-like disease–but a beneficial role has been implied (Crane et al., 2013). Each of the cell types known to be susceptible to *F. tularensis* infection has been shown to activate NKT cells (Brennan et al., 2013). Therefore, I reasoned that NKT cells might be activated very early after infection and could function in shaping the quality of both the innate and adaptive response.

The results emerging from testing the aformentioned hypothesis revealed that indeed respiratory infection with *F. tularensis* activated iNKT cells which produced interferon (IFN)-γ and propagated a sepsis-like proinflammatory response that led to a lethal phenotype in wild type mice. This proinflammatory response was much tempered in CD1d-deficient mice that lacked NKT cells. Strikingly, however, the mutant mice had increased lymphocytic infiltration in the lungs that organized into structures resembling induced bronchus-associated lymphoid tissue (iBALT) at the peak of infection. Hence, NKT cell activation by *F. tularensis* infection hampers

iBALT formation, which in conjunction with an NKT cell-dependent proinflammatory response, exacerbates severe pulmonary tularemia-like disease in mice.

Results

iNKT cell deficient mice are less susceptible to i.n. LVS infection

IFN-γ is critically required for murine resistance to primary *F. tularensis* LVS infection. The early production of IFN-γ after pulmonary LVS infection is primarily attributed to NK cells and double negative T cells (Cowley and Elkins, 2011). INKT cells are also a source of early IFN-γ and hence, I reasoned that they might contribute to resistance to intranasal (i.n.) LVS infection. I therefore investigated whether iNKT cell deficiency would alter disease outcome. Toward this end, I utilized two different models of NKT cell-deficency: mice generated either by deletion of one of the TCR α-chain gene segments ($J\alpha18^{-/-}$) or by mutation of the restriction element required for thymic NKT cell selection and antigen presentation in peripheral tissues ($CD1d^{-/-}$) (Chen et al., 1997; Cui et al., 1997; Mendiratta et al., 1997; Smiley et al., 1997).

To determine how NKT cell deficiency affects the outcome of pulmonary *F. tularensis* infection, I first determined the LD_{50} to i.n. inoculation of the live vaccine strain (LVS) in C57BL/6 (B6) mice using an established method (Conlan et al., 2011; Reed, 1938). During preliminary experiments, I found that infected mice lost up to 30% of their initial weight beginning d4 p.i.. Although weight loss was observed at all doses tested (500, 2,000, 8,000 and 30,000 CFU), the degree of

disease severity was dependent on the initial inoculum dose. It was only at a dose of 8,000 CFU where I consistently observed moribund mice. At lower doses the majority of mice recovered quickly with few outward signs of disease beyond slightly ruffled fur, which was not consistent between animals. Even at the higher dose, those WT mice that did not succumb by day 12 quickly began to regain weight and appeared otherwise healthy (Fig. 2-1). Other clinical manifestations included ruffled fur, hunched posture, labored breathing, and reduced mobility. Because I found that weight loss alone was not always an accurate predictor of disease severity, a clinical score based on physical appearance was also included in my endpoint criteria (see Materials and Methods). The resulting LD_{50} for i.n. LVS infection in B6 mice was ~6,000–8,000 CFU (data not shown), which was consistent with that previously published by others (Griffin et al., 2015; Kurtz et al., 2013; Malik et al., 2007).

To examine the contribution of NKT cells in resistance to primary pneumonic tularemia, I first assessed disease outcome in $J\alpha18^{-/-}$ mice compared to their wild-type counterparts. Hence, B6 and $J\alpha18^{-/-}$ mice were inoculated i.n. with ~8,000 CFU LVS and monitored for weight loss and signs of morbidity as described in Materials and Methods. When compared to B6, $J\alpha18^{-/-}$ mice showed a significant increase in susceptibility to i.n. LVS infection (Fig. 2-2A), initially suggesting a protective role for iNKT cells. Although $J\alpha18^{-/-}$ mice have historically been used as a model for type I NKT cell deficiency, a recent study demonstrated that they also have a profound defect in conventional αβ T cells, with the loss of an estimated 60% of total TCRα repertoire diversity (Bedel et al., 2012). This report suggests that

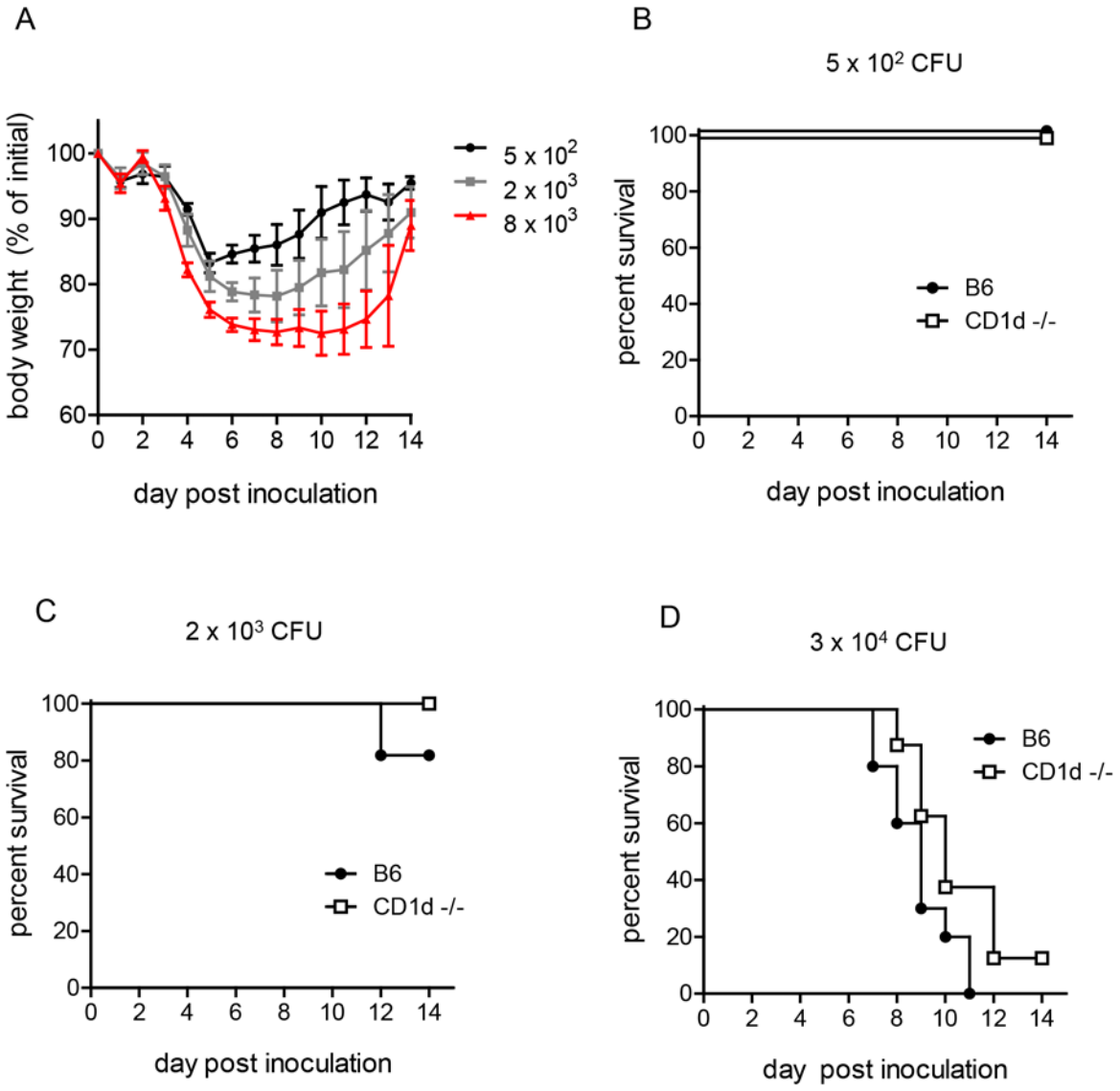


Figure 2-1. Response to intranasal LVS infection is dose-dependent.

(A) Groups of B6 mice were inoculated intranasally with increasing doses of LVS as indicated and monitored daily for weight loss and signs of morbidity. Data are representative of two similar experiments with 5 mice/group. Plotted are mean ± SD. **(B-D)** Groups of B6 or CD1d$^{-/-}$ mice were inoculated intranasally with 5 x 10^2 (B), 2 x 10^3 (C), or 3 x 10^4 (D) CFU LVS and monitored daily for signs of morbidity. Mice were humanely euthanized when weight loss exceeded 30% or when showing obvious signs of distress. Data are representative of two or three experiments with 8-10 mice/group.

the increased susceptibility observed in these mice might be due to this more global T cell deficiency rather than the loss of iNKT cells, since conventional T cells were previously shown to mediate protective immunity to LVS (Cowley and Elkins, 2011; Griffin et al., 2015).

I therefore ascertained the outcome of pulmonary LVS infection in CD1d$^{-/-}$ mice, which have a normal complement of αβ T cells but lack NKT cells. By d4 post-inoculation (p.i.), both B6 and CD1d$^{-/-}$ mice began to show signs of disease, including weight loss and ruffled fur. By d5–6, B6 mice continued to lose more weight and showed more severe outward signs of disease as indicated by clinical score (Figs. 2-2B and C). In striking contrast, by d7, nearly all NKT deficient CD1d$^{-/-}$ mice began to recover and a significantly lower percentage of CD1d$^{-/-}$ mice succumbed to i.n. LVS infection (Fig. 2-2D). By d14, fewer than 50% B6 and almost all CD1d$^{-/-}$ mice regained weight and showed no outward signs of disease, surviving the infection (data not shown). These data suggest that NKT cells serve a deleterious role in pneumonic tularemia. I therefore reasoned that increased iNKT cell number might further exacerbate disease. Thus, Vα14^{tg} mice, which have increased numbers of iNKT cells (Vahl et al., 2013), were infected intranasally and found to have increased susceptibility as predicted (Fig. 2-3). Taken together, these data strongly support a detrimental function of NKT cells in pneumonic tularemia and show that CD1d$^{-/-}$ mice are a better model than Jα18$^{-/-}$ mice to study the role of iNKT cells in this disease. Hence, CD1d$^{-/-}$ mice were used for all subsequent experiments.

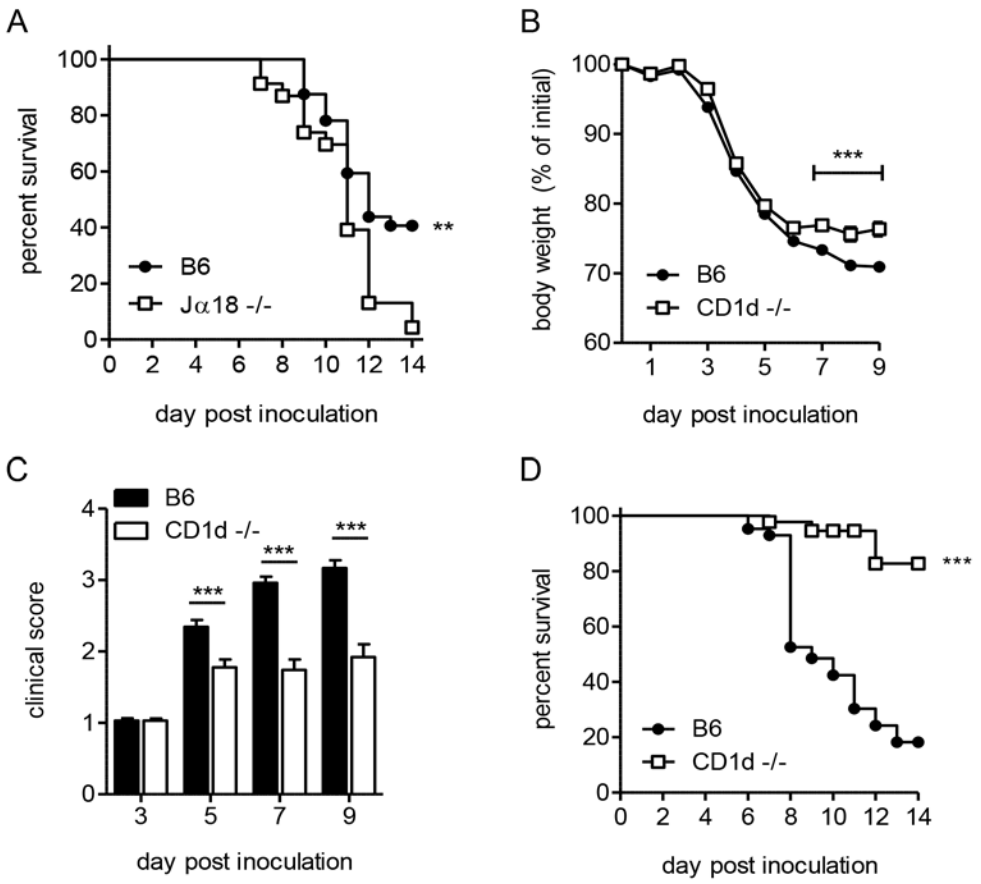


Figure 2-2. CD1d$^{-/-}$ mice are less susceptible to i.n. LVS infection.

(A) Survival of B6 or Jα18$^{-/-}$ mice after i.n. LVS infection. Survival of Jα18$^{-/-}$ and WT mice after intranasal LVS infection (8000 cfu). Curves were compared using Log-rank (Mantel-Cox) Test. Data are from one of three similar experiments with at least 10 mice/group. **(B)** B6 or CD1d$^{-/-}$ mice were infected i.n. with LVS and monitored daily for weight loss. The mean (± SD) weight of 50 animals/group in several independent experiments following intranasal infection (Accumulation of five independent experiments with 10 mice/group in each experiment) is presented. **(C)** Clinical score at various time points p.i. as determined in Materials and Methods. Data are cumulative from four similar experiments (mean + SD) with 5—10 mice/group/time point. Data were analyzed as indicated in Materials and Methods. **(D)** Survival of B6 or CD1d$^{-/-}$ mice after i.n. LVS infection. Cumulative survival curves from 4 experiments with 40 mice/group. Curves were compared using Log-rank (Mantel-Cox) Test. $^{**}p<0.01$, $^{***}p<0.001$.

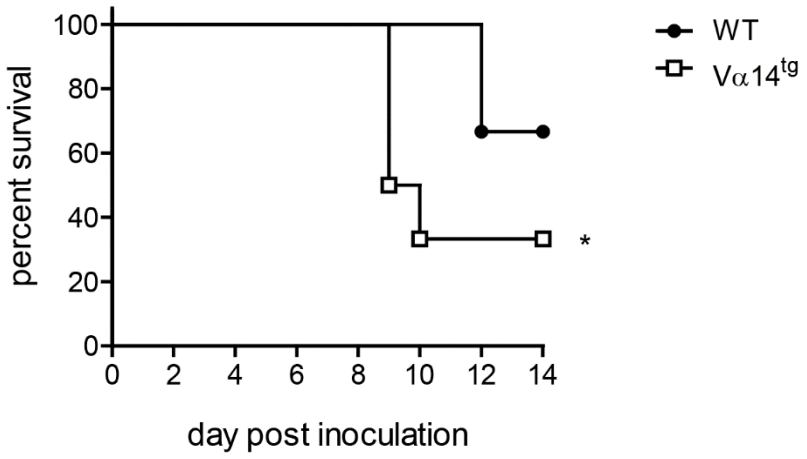


Figure 2-3. Increased iNKT cell numbers exacerbates disease.

Groups of WT or Vα14^{tg} mice were infected intranasally with 8000 CFU LVS and monitored daily for weight loss and signs of morbidity (n=6-9 mice/group). Results are representative from one of three similar experiments.

Intranasal LVS infection recruits NKT cells to the lung interstitium

NKT cells are overrepresented among T cells in the healthy lung compared to their frequency in other organs. These tissue-resident NKT cells do not recirculate but rapidly extravasate into interstitial spaces upon recognition of lipid antigens such as the potent CD1d-restricted agonist α-galactosylceramide (α-GalCer) (Scanlon et al., 2011; Thomas et al., 2011). Whether NKT cells behave similarly in response to bacterial infection, where lipid ligands are presumably of lower affinity (Brennan et al., 2013), is unknown. To visualize the location of NKT cells in naïve lungs, I injected B6 mice with anti-CD45 antibody (αCD45 Ab) i.v., allowed it to circulate through blood for several minutes, and tracked the anatomic localization of lung mononuclear cells by flow cytometry after labeling lung leukocytes with Abs against lineage-specific markers. This quick procedure allows circulating αCD45 Ab to label all intravascular cells ($\alpha CD45^{POS}$), while cells that are in the tissue interstitial space would be $\alpha CD45^{NEG}$. I confirmed that the largest percentage of lymphocytes (T, B, and NK cells) was in the $\alpha CD45^{POS}$ intravascular population as reported previously (Anderson et al., 2014). Interestingly, I found that a proportionately higher frequency of pulmonary NKT cells were present in the interstitium ($\alpha CD45^{NEG}$) when compared to other lymphocytes (Figs. 2-4A and 2-5A). Such localization suggests that pulmonary NKT cells occupy a frontline niche that allows them to rapidly sense and respond to inhaled antigens.

To test the hypothesis that pulmonary NKT cells rapidly engage in host defense at frontline niches, I inoculated B6 mice i.n. with ~LD_{50} of LVS. On d3 p.i., I

injected αCD45 Ab and, after several minutes, lungs were analyzed by flow cytometry for NKT cell localization. I found a significant increase in the frequency of $\alpha CD45^{NEG}$ interstitial NKT cells that was equivalent to the frequency observed after i.n. α-GalCer treatment (Figs. 2-4B and 2-5B). Furthermore, beginning d3 after i.n. LVS inoculation, I found a significant decrease in the frequency of NKT cells in the blood when compared to uninfected controls (Figs. 2-4C and 2-5C Fig.). This reduction in circulating NKT cells was coincident with their increased numbers in the lungs (Figs. 2-4C and D), more specifically and significantly in the lung interstitium ($\alpha CD45^{NEG}$ lung NKT). Thus, i.n. LVS infection rapidly recruits NKT cells from the vasculature into the infected lung interstitium. This finding is consistent with a previous report showing NKT cell extravasation into the lung interstitium after respiratory α-GalCer administration (Scanlon et al., 2011).

Modestly lower bacterial burden correlates with better clinical score in $CD1d^{-/-}$ mice upon pulmonary LVS infection

Two factors can affect overall fitness after infection: pathogen burden and the level of organ pathology. To determine whether the decreased susceptibility of $CD1d^{-/-}$ mice to LVS was due to differences in bacterial burden, I measured LVS burden in lung homogenates at various time points post i.n. LVS inoculation. Lung burden was similar in both groups, with a modest but statistically significant difference (two-fold) seen only at d7 and d9 in infected $CD1d^{-/-}$ mice (Fig. 2-6A), which was concomitant with the observed differences in clinical score and weight loss (Figs. 2-2B and C).

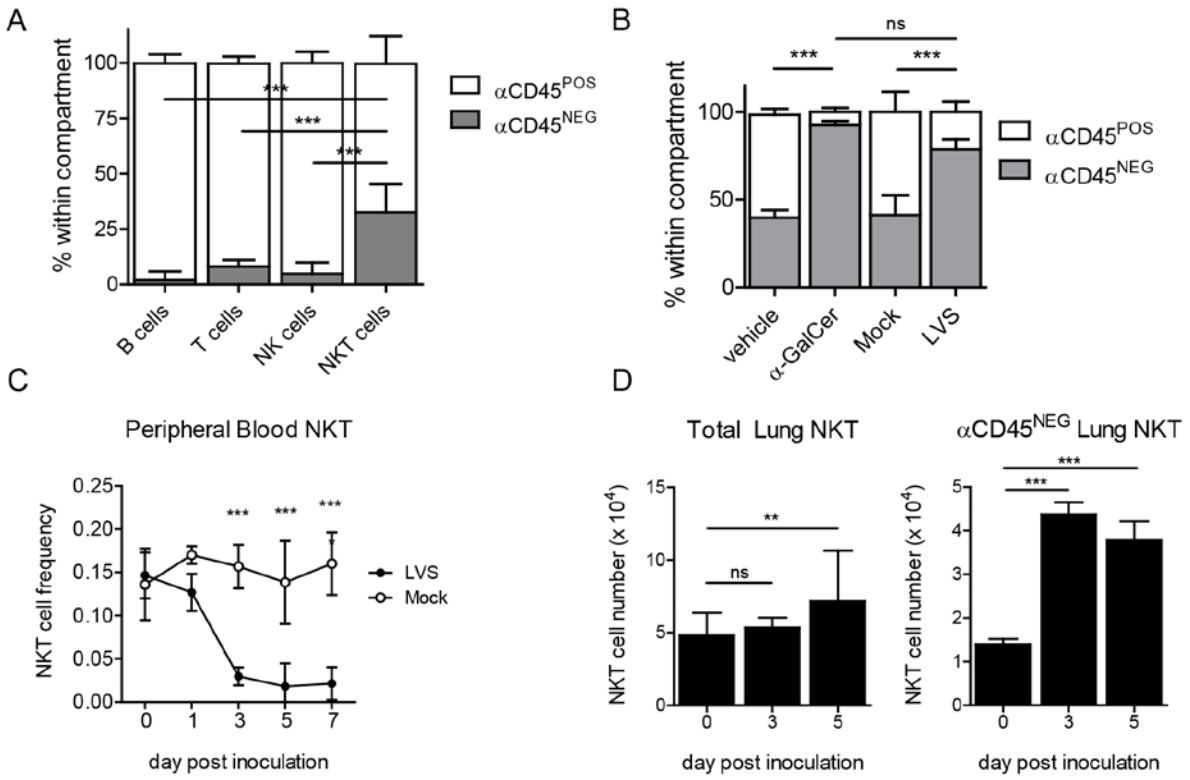


Figure 2-4. iNKT cells are recruited to the lung interstitium in response to LVS infection.

(A) Intravascular αCD45 Ab staining to determine relative distribution of the indicated lymphocytes in naïve lungs. Data combined from four similar experiments. Bars are mean + SD (*n*=13 mice). Interstitial NKT cell frequency was compared to all other lymphocyte populations as described in Materials and Methods. **(B)** Frequency of lung NKT cells among $B220^-$ cells after i.n. αGC treatment or LVS inoculation. Mean + SD from one of two similar experiments (*n*=3-4 mice/group). Frequency of interstitial NKT cells was compared by one-way ANOVA with Tukey's posttest. **(C)** Frequency of NKT cells in peripheral blood of LVS-infected or mock-infected mice. Mean ± SD from one of two similar experiments (*n*=3-4 mice/group). Comparison was made by two-way ANOVA with Bonferroni posttest. **(D)** NKT cell number in total lung or lung interstitium after i.n. LVS infection. Mean + SD combined from two similar experiments (*n*=7 mice/ time point). Day 3 and day 5 post inoculation were compared to naïve mice as described in Materials and Methods. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

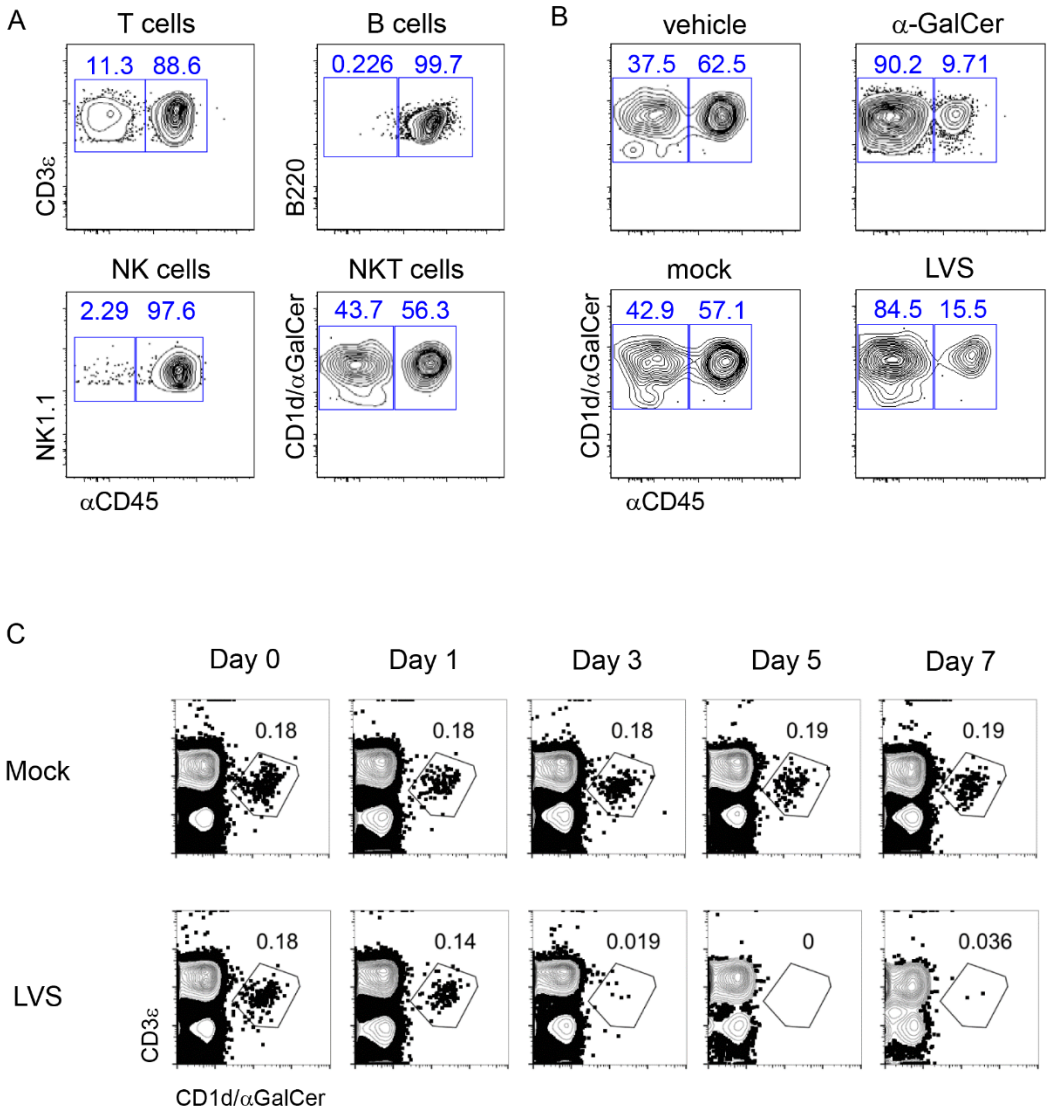


Figure 2-5. iNKT cells are pre-positioned in the lung and recruited into the interstitium after i.n. LVS inoculation.

(A) Representative plots of lung lymphocyte localization in naïve B6 mice. Cells were identified as described in Materials and Methods. Intravascular αCD45 staining was used to discriminate intravascular ($\alpha CD45^{POS}$) and interstitial ($\alpha CD45^{NEG}$) cells. Numbers are percent of each cell type within the respective gate. **(B)** Representative intravascular staining of NKT cell localization d3 after intranasal administration of 2 μg α-GalCer (top) or ~8,000 CFU LVS (bottom). Numbers are percent of $CD3\varepsilon^{+}$CD1d-α-GalCer $tetramer^{+}$ cells. **(C)** Representative NKT staining of blood from mock- or LVS-infected mice at various time points p.i. Numbers in plots are percent of $B220^{-}$ lymphocytes.

Since the observed differences in illness began to appear at d7 p.i., I focused on this time point for subsequent analyses. Differing degrees of lung pathology can result in different animal fitness despite similar bacterial burden (Jamieson et al., 2013; Malik et al., 2007; Slight et al., 2013). To determine whether the difference in morbidity was due to differential lung damage, I measured blood oxygen saturation (SpO_2), which was suggested as an accurate measure of lung pathology (Verhoeven et al., 2009). While both groups of mice showed decreased SpO_2 levels, there were no significant differences observed between groups (Fig. 2-6B). To more directly assess tissue damage, H&E-stained lungs were evaluated at d7 and d9 p.i. As predicted by pulse oximetry data, the findings in the lungs of B6 and $CD1d^{-/-}$ mice were similar. Lungs from both groups of mice displayed focally extensive interstitial pneumonia with perivascular cuffs of mononuclear cells. Inflammatory infiltrate consisting of macrophages and neutrophils filled the alveoli, which also contained necrotic debris and edema with focal areas of necrosis on the alveolar walls (Fig. 2-7).

After i.n. infection, *F. tularensis* rapidly disseminates to the periphery (Ojeda et al., 2008). The kinetics and extent of dissemination are suggested as determinants of disease severity (Bar-Haim et al., 2008; Forestal et al., 2007; Kingry et al., 2011; Sharma et al., 2009). Hence, I measured burden in blood, liver, and spleen. LVS was only transiently detectable in the blood, where levels peaked at d3 p.i., but there were no differences in bacteremia between groups (Fig. 2-8A).

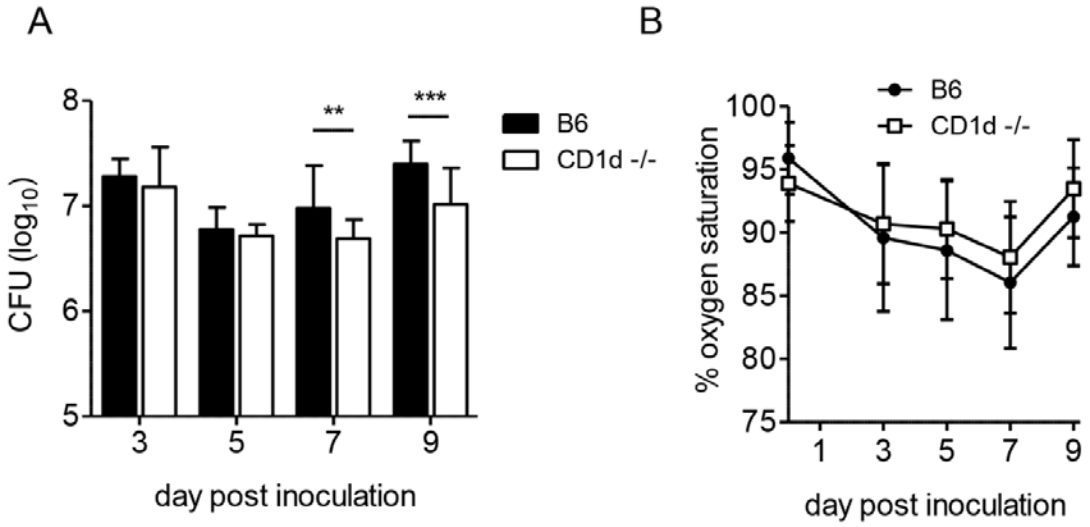


Figure 2-6. CD1d$^{-/-}$ mice have modestly reduced lung LVS burden but no differences in lung pathology.

(A) Lung burden was determined at various times p.i. as described in Materials and Methods. Data are combined from at least 3 separate experiments. Bars are mean + SD of 10–15 mice/group/time point. **(B)** Oxygen saturation was measured as described in Materials and Methods. Data are cumulative of 3 separate experiments with 5–10 mice/group (mean ± SD). Data were analyzed as indicated in Materials and Methods. $^{**}p<0.01$, $^{***}p<0.001$.

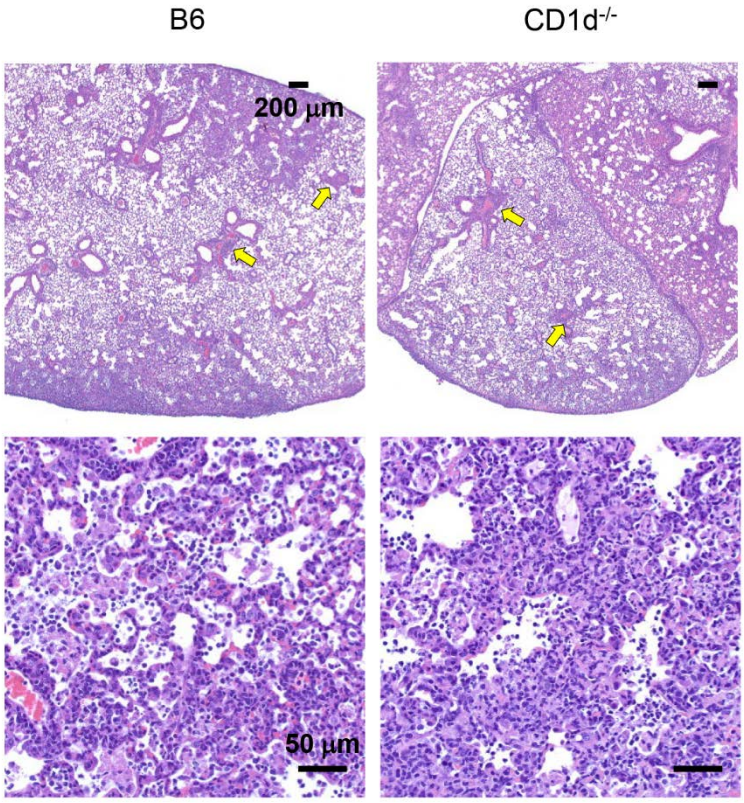


Figure 2-7. Decreased susceptibility in CD1d-/- mice is not due to differential lung damage

H&E stained formalin fixed paraffin embedded (FFPE) lung sections d9 p.i. 40X (top) or 400X (bottom) magnification with scale bars as indicated. Data are representative of three mice per group. Arrows indicate aggregations of lymphocytes.

Liver burden was similar in both groups, but CD1d$^{-/-}$ mice had significantly lower splenic burden d3–7 p.i. (Fig. 2-8A). Further analysis showed that only lung burden—but not liver or spleen—correlated with weight loss at d7 p.i. (Fig. 2-9). Consistent with these findings, I found no difference in susceptibility of CD1d$^{-/-}$ mice to intradermal inoculation with 10^7 CFU of LVS when compared to B6 mice in the same experiment (data not shown), suggesting that the influence of NKT cell activation was manifested primarily in the lungs. Concordant with these findings, histopathological analysis failed to identify any striking differences in either liver or spleen pathology after intranasal inoculation (Fig. 2-8B). The extent of hepatic granuloma formation did not differ between groups (Fig. 2-8B). Contrary to previous reports in BALB/c mice (Chiavolini et al., 2008; Furuya et al., 2013), splenic architecture was mostly intact with some evidence of apoptosis and extramedullary hematopoiesis that did not seem to differ between groups (Fig. 2-8B). In summary, the above data indicate that the milder disease observed in NKT cell-deficient mice cannot be explained by differential pulmonary, hepatic, or splenic histopathology, but is more likely due either to modest differences in bacterial burden in the lungs and peripheral organs, or some other mechanism.

Increased lymphocyte infiltrates organize into tertiary lymphoid structures in CD1d$^{-/-}$ lungs in response to LVS infection

The differences described thus far become most pronounced at d7 p.i, suggesting that the quality of the adaptive response may be the principal underlying

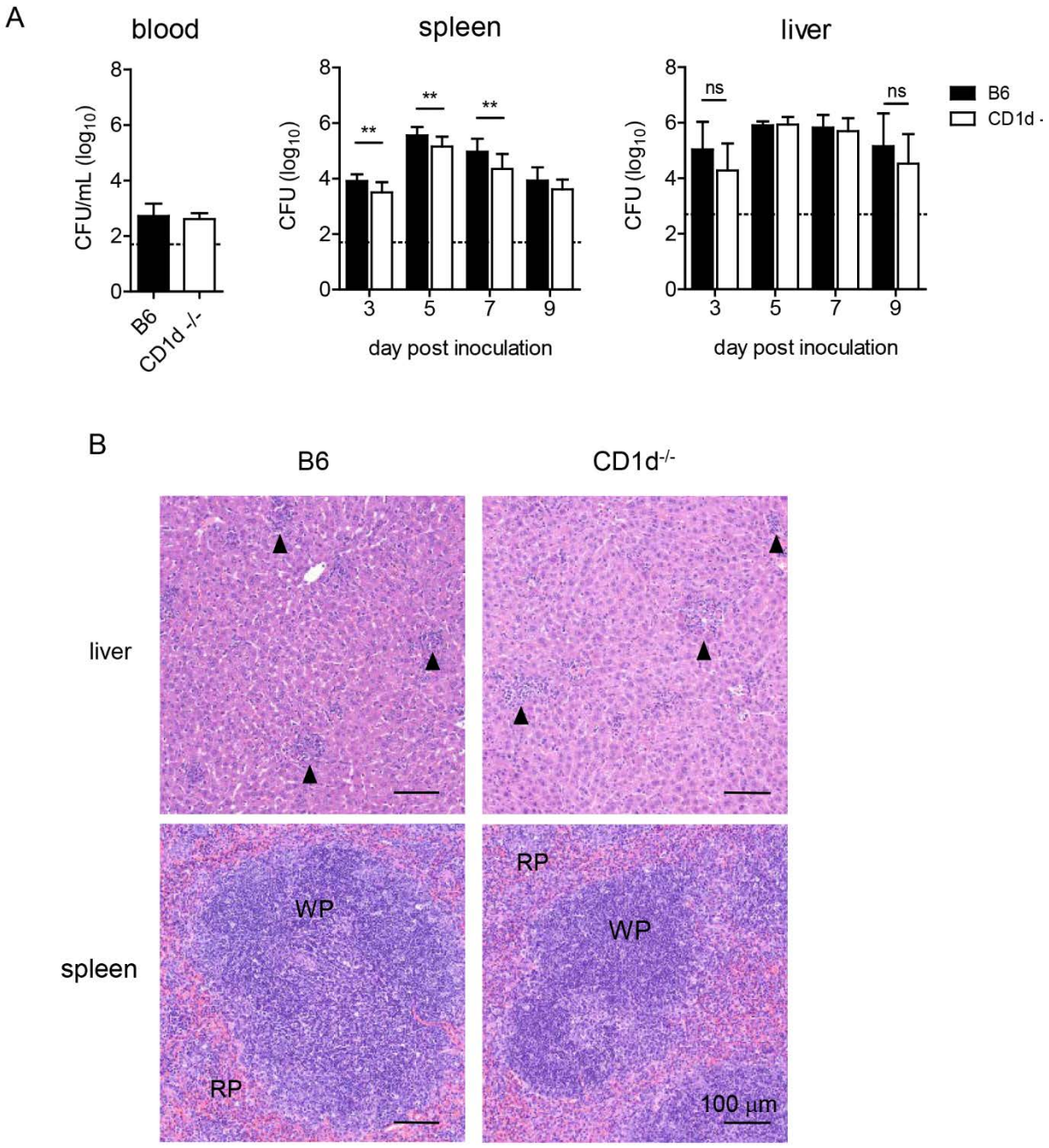
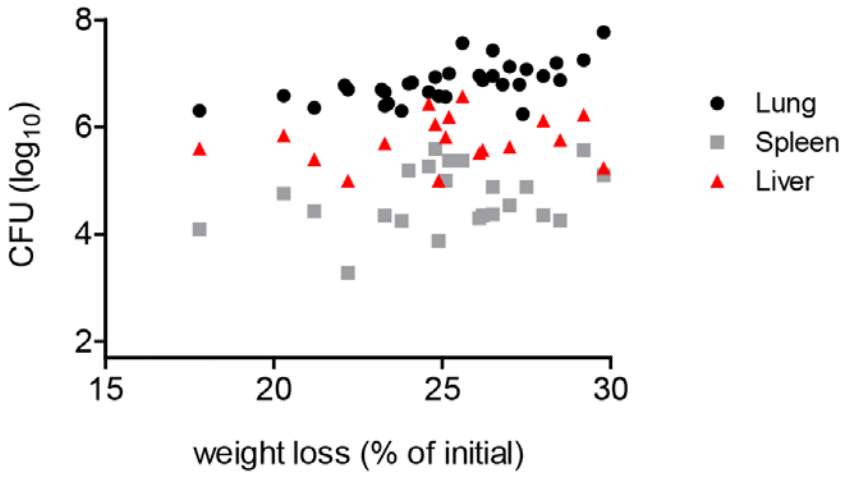


Figure 2-8. CD1d$^{-/-}$ mice have reduced splenic LVS burden but no difference in the liver.

(A) Blood, liver, and spleen burden were determined as in Fig. 4. Data are combined from 3 separate experiments. Bars are mean + SD of 6–10 mice/group/time point. Data were analyzed as indicated in Materials and Methods. $^{**}p<0.01$. **(B)** H&E stained FFPE liver (top) and spleen (bottom) sections d9 p.i., 200X magnification. Data are representative of 3 mice/group. Arrows indicate granulomas.



	Lung	Spleen	Liver
Number of XY Pairs	33	23	18
Spearman r	0.6623	0.2609	0.1394
95% confidence interval	0.4039 to 0.8228	-0.1822 to 0.6159	-0.3634 to 0.5793
P value (two-tailed)	< 0.0001	0.2292	0.5812
P value summary	***	ns	ns

Figure 2-9. Lung burden, but not liver or spleen, are correlated with weight loss after i.n. LVS infection.

LVS burden was determined from homogenized lung, liver, and spleen d7 p.i. Data are cumulative from more than three experiments with *n* values as indicated. Spearman correlation analysis showed that only lung burden was correlated with weight loss at the peak of infection.

cause of the reduced susceptibility observed in CD1d$^{-/-}$ mice. I therefore analyzed lymphocyte numbers in the lungs after LVS infection. Lungs of CD1d$^{-/-}$ mice had consistently higher numbers of both B and T lymphocytes present at d7 p.i. (Fig. 2-10). To directly visualize the localization of these cells—whether the lymphocytic infiltration was in the pulmonary vasculature or had extravasated into the lung tissue—fixed frozen sections of infected lung were stained and examined by confocal microscopy. Consistent with earlier analysis of H&E stained sections, both groups of mice showed extensive perivascular and peribronchiolar infiltration of leukocytes. Significantly however, in addition to an increased cellularity in CD1d$^{-/-}$ mice, there was a striking difference in the degree of organization of the infiltrating immune cells. We found evidence for the formation of tertiary lymphoid structures, iBALT, within the infected lungs of CD1d$^{-/-}$ mice (Fig. 2-11A). These structures were marked by the formation of B cell follicles, which were surrounded by T cell congregates. In contrast, fewer iBALT structures were formed in B6 mice by d7 p.i. (Fig. 2-11B) and those that developed were small and appeared rudimentary in that T and B cells were scattered or at best loosely packed together (Fig. 2-12). Such tertiary lymphoid structures were not observed in uninfected CD1d$^{-/-}$ or B6 mouse lungs (Fig. 2-13) suggesting that iBALTs formed in response to pulmonary LVS infection.

Dendritic cells (DCs) are both necessary and sufficient for the induction of iBALT within the lungs (Foo and Phipps, 2010). Consistent with previous reports, concentrations of CD11c^{+} cells were also observed in and around the B and T cell

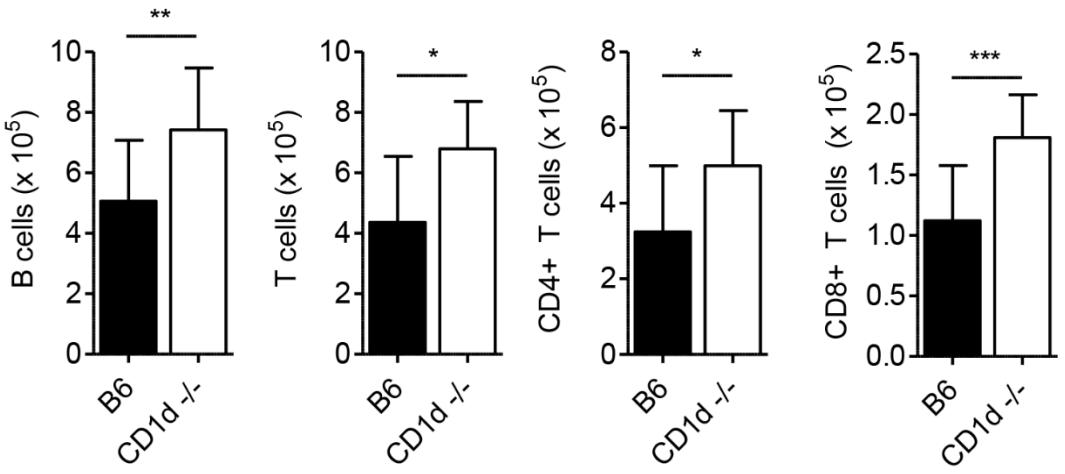


Figure 2-10. Increased lymphocyte numbers in lungs of CD1d$^{-/-}$ mice after i.n. LVS infection.

Groups of B6 or CD1d$^{-/-}$ mice were infected i.n. with LVS. On d7 p.i., lungs were analyzed by flow cytometry for the indicated cell populations. Data are cumulative of 2 experiments with 9–10 mice/group. Bars are mean + SD; compared as indicated in Materials and Methods.

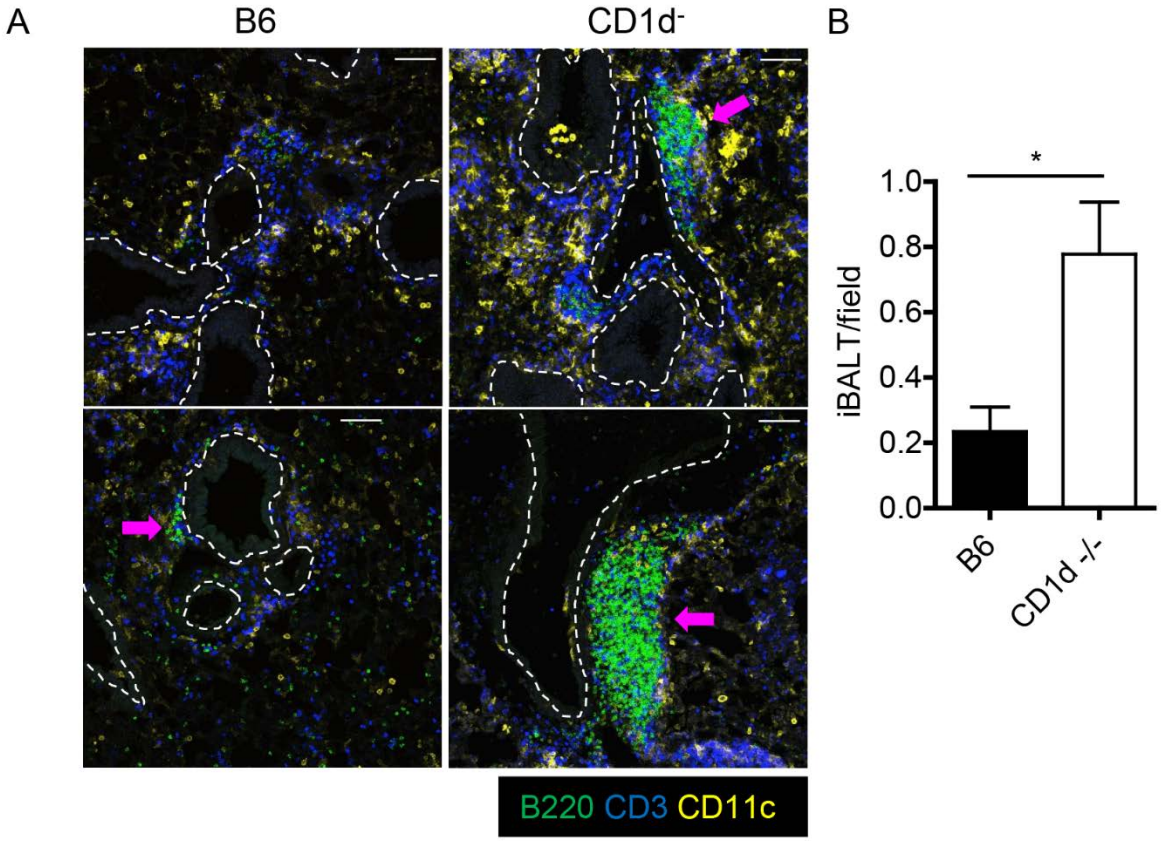


Figure 2-11. Formation of iBALT in CD1d$^{-/-}$ mice after i.n. LVS infection.

(A) Representative images from *F. tularensis* LVS-infected lungs d7 p.i. Images are individual mice from 2 of 3 similar experiments. 20X magnification; bars are 80 μm. Dashed lines indicate large airways as identified by autofluorescence of epithelial cells. Arrows indicate rudimentary iBALT formation in B6 lung and well-developed iBALT in lungs of CD1d$^{-/-}$mice. **(B)** Enumeration of iBALT from d7 infected lung sections. Data are cumulative from three experiments (n=6 mice/group). Bars are mean + SD. Data were compared using Mann Whitney U test. $^{*}p<0.05$

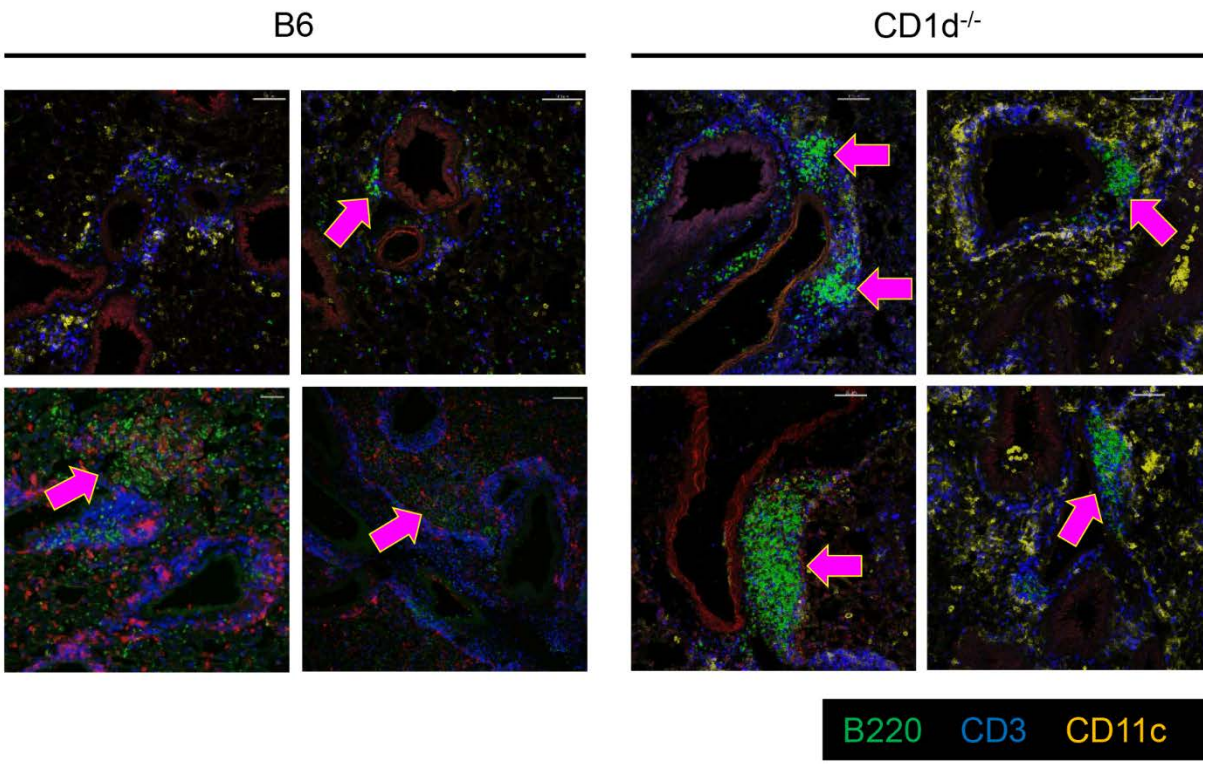


Figure 2-12. Tertiary lymphoid structures are more prominent in lungs of NKT-deficient mice.

Representative sections from B6 (left) and CD1d$^{-/-}$ (right) mice d7 post i.n. inoculation (8,000 CFU LVS). Arrows indicate rudimentary iBALT formation in lungs of B6 mice and well-developed iBALT in CD1d$^{-/-}$ lungs. Each section is from an individual mouse and is representative of three experiments. Bars are 80 μm.

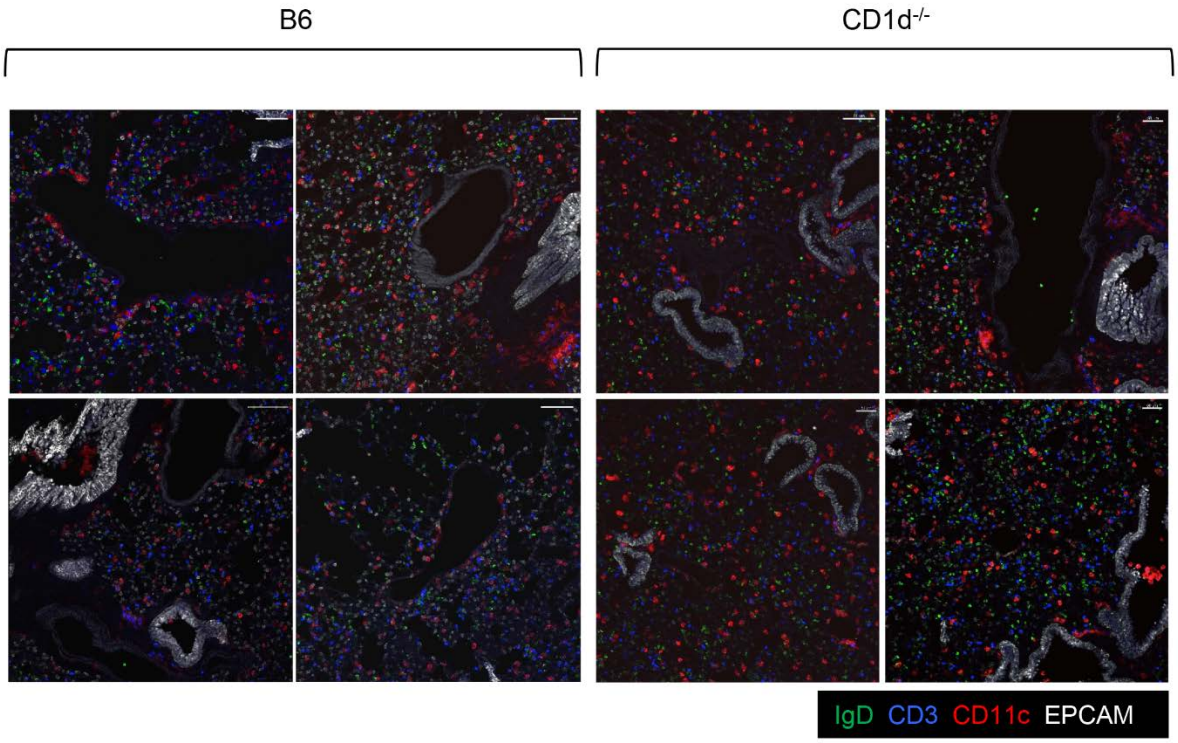


Figure 2-13. iBALT is not observed in the lungs of naïve, uninfected mice.

Representative images of naïve lung sections from B6 (left) or CD1d$^{-/-}$ (right) mice. Sections were stained as indicated.

zones of iBALT formed in CD1d$^{-/-}$ lungs (Fig. 2-11 and 2-12). Further characterization of CD11c^{+} cells within the infected lungs by flow cytometry revealed that all DC subsets identified were increased in the LVS infected lungs of CD1d$^{-/-}$ mice (Fig. 2-14). Considering that DCs have been implicated as a primary vehicle for dissemination of *F. tularensis* from the infected lung (Bar-Haim et al., 2008), this finding may also partially explain the reduced splenic burden in CD1d$^{-/-}$ mice. Hence, iBALT formation in response to LVS infection of the lungs is associated with milder tularemia-like disease in mice and prognosticates recovery.

Decreased neutrophilia in CD1d$^{-/-}$ mice after LVS infection

A previous study showed that increased disease severity in a murine tularemia model was associated with hepatic damage and neutrophilia (Melillo et al., 2013). NKT cells activated by LVS could cause liver damage through direct lysis of infected hepatocytes (Gao et al., 2009; Notas et al., 2009). They might also promote neutrophilia through production of granulocyte colony-stimulating factor (G-CSF), the major neutrophil survival and proliferation factor (von Vietinghoff and Ley, 2008). Thus we monitored these two disease parameters in CD1d$^{-/-}$ mice. Although histological analysis failed to reveal any gross differences in liver pathology (Fig. 2-8B), we measured AST and ALT in the serum of infected animals as a more sensitive indicator of hepatic injury (Fig. 2-15A). CD1d$^{-/-}$ mice had serum ALT levels that were slightly, yet significantly, lower than those measured in B6 mice, which implied slightly more severe liver damage in the latter strain. However,

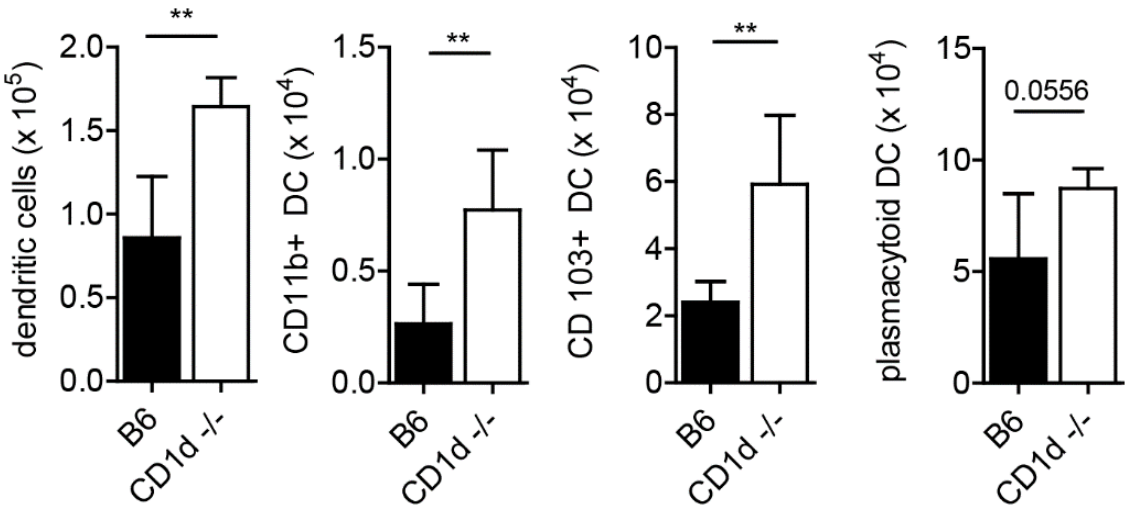


Figure 2-14. CD1d$^{-/-}$ mice have increased numbers of dendritic cells in the lungs. B6 or CD1d$^{-/-}$ mice were inoculated intranasally with 8000 CFU LVS. On d7 post-inoculation, lungs were harvested and assayed for the indicated DC subsets by flow cytometry. DC subsets in infected lungs were identified as described in Materials and Methods. Representative results from 1 of 3 similar experiments (*n*=5 mice/group). Bars are mean + SD; Mann Whitney U test. $^{**}p<0.01$.

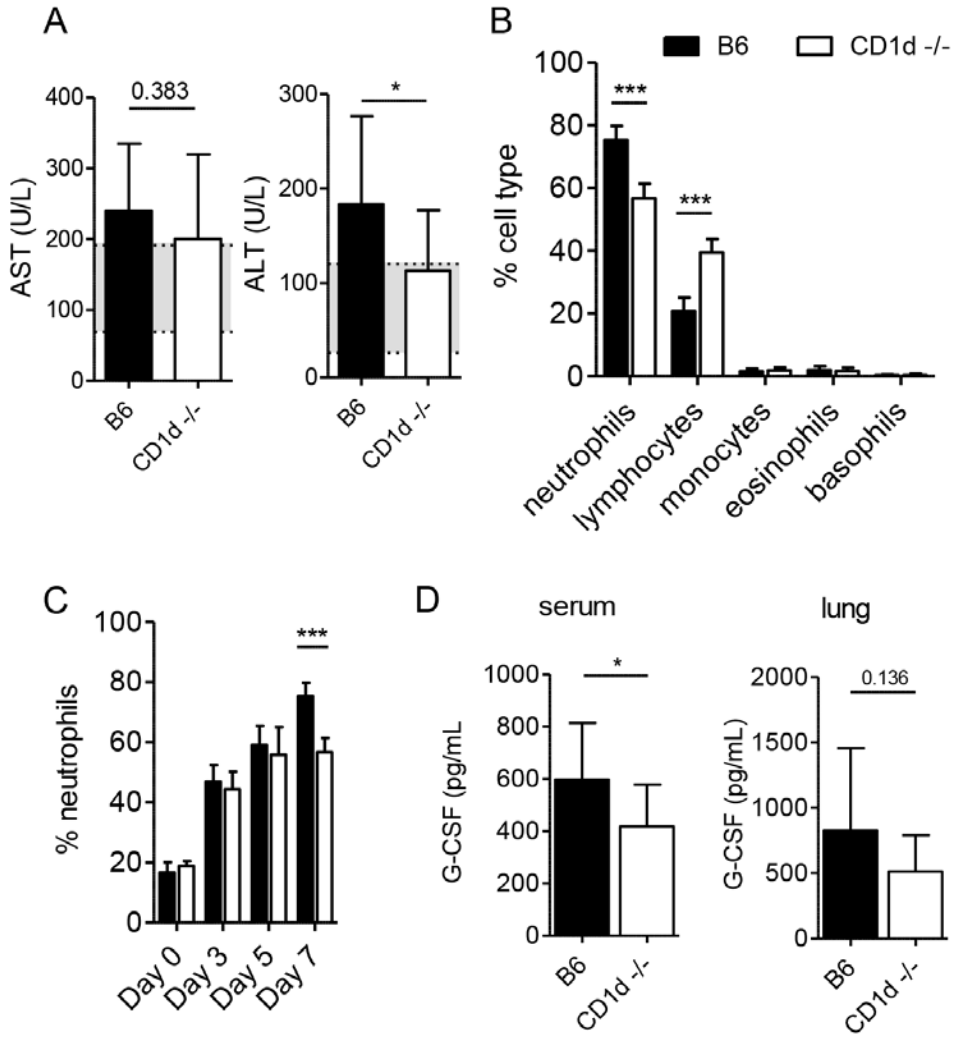


Figure 2-15. CD1d$^{-/-}$ mice have less severe neutrophilia after intranasal LVS infection.

(A) AST and ALT levels were measured in serum of infected mice on d7 p.i. Data are combined from 3 experiments (*n*=10–12). Mean + SD. Shaded regions denote reference range. **(B)** CBC analysis of blood from LVS-infected mice performed on d7. Data are combined from 2 separate experiments (*n*=15 mice/group). Bars are mean + SD. **(C)** Percent neutrophils in blood at the indicated time points p.i. Data are combined from 2 separate experiments. Bars are mean + SD of 5–15 mice/group/time point. **(D)** On d7 serum and lung G-CSF levels were measured by CBA. Data are combined from 2 experiments (*n*=15 mice/group). Bars are mean + SD. Comparisons were made as indicated in Materials and Methods. $^{*}p<0.05$, $^{***}p<0.001$.

when taken together with the absence of widespread necrosis, these mild-to-moderate elevations in circulating hepatic enzymes are not consistent with death from liver failure, particularly considering the similar liver burden in the two groups of mice (Fig. 2-8A). Such slightly elevated ALT levels need not be a result of the death of infected hepatocytes, but might rather be indicative of increased systemic inflammation (Wu et al., 2010).

To monitor the numbers of circulating neutrophils I performed complete blood counts (CBC) at various time points post inoculation. B6 mice did indeed show more pronounced neutrophilia d7 p.i. when compared to CD1d$^{-/-}$ mice (Fig. 2-15B). This difference was not observed in naïve mice and did not appear until d7 p.i. (Fig. 2-15C). I found lower G-CSF levels in the serum but not the lungs of CD1d$^{-/-}$ mice by d7 (Fig. 2-15D), which was consistent with the lower frequency of neutrophils in the blood (Fig. 2-15B). Thus, decreased neutrophilia incited by LVS infection in CD1d$^{-/-}$ mice could be one cause for their better resistance to tularemia.

Tempered inflammatory response in CD1d$^{-/-}$ mice after LVS infection

Although IFN-γ is necessary for resistance to LVS, excessive production of proinflammatory cytokines can be detrimental, particularly after intranasal infection (Cowley, 2009; Slight et al., 2013). Because NKT cells are known to induce IFN-γ production by NK cells (Bezbradica et al., 2005; Carnaud et al., 1999), we therefore asked whether CD1d$^{-/-}$ mice—which have normal numbers of NK cells—might produce less IFN-γ in response to LVS. I found that although the early IFN-γ

response was comparable in both groups of mice (Fig. 2-16), by d7 p.i. CD1d$^{-/-}$ mice had significantly lower levels in both the serum and lung (Figs. 2-16 and 2-17).

Many cytokines produced by NKT cells were suggested to promote sepsis-like inflammatory disease (Kim et al., 2014; Leung and Harris, 2011) and, hence, I measured the levels of those cytokines previously shown to be induced by LVS infection that have also been associated with severe sepsis (Chiavolini et al., 2008; Tisoncik et al., 2012). I found that CD1d$^{-/-}$ mice had consistently lower levels of IFN-γ, IL-6, and TNF-α in both the lungs and serum, with lower levels of MCP-1 and KC in the serum at d7 p.i. (Figs. 2-17A and B) which coincided with modestly reduced burden, reduced weight loss, and less severe outward signs of disease (Fig. 2-2). Hence, NKT deficiency results in a less severe "cytokine storm" in response to i.n. LVS infection.

Reduced susceptibility of CD1d$^{-/-}$ is not dependent on mouse genetic background

Given the observed differences in susceptibility among various mouse strains (Anthony et al., 1988; Chen et al., 2003; Fortier et al., 1991; Lopez et al., 2004), we next ascertained whether CD1d$^{-/-}$ mice in the BALB/c background would exhibit the same phenotype. Consistent with the results in the C57BL6 background, we found that CD1d$^{-/-}$ mice were less susceptible to LVS infection in the BALB/c background as well (Fig 2-18A). As observed with C57BL6 mice, higher doses caused severe disease in both groups, although death was slightly delayed in CD1d-deficient mice.

Mice are highly susceptible to several subspecies of *Francisella* and the extreme virulence of type A (subspecies *tularensis*) or B (subspecies *holarctica*)

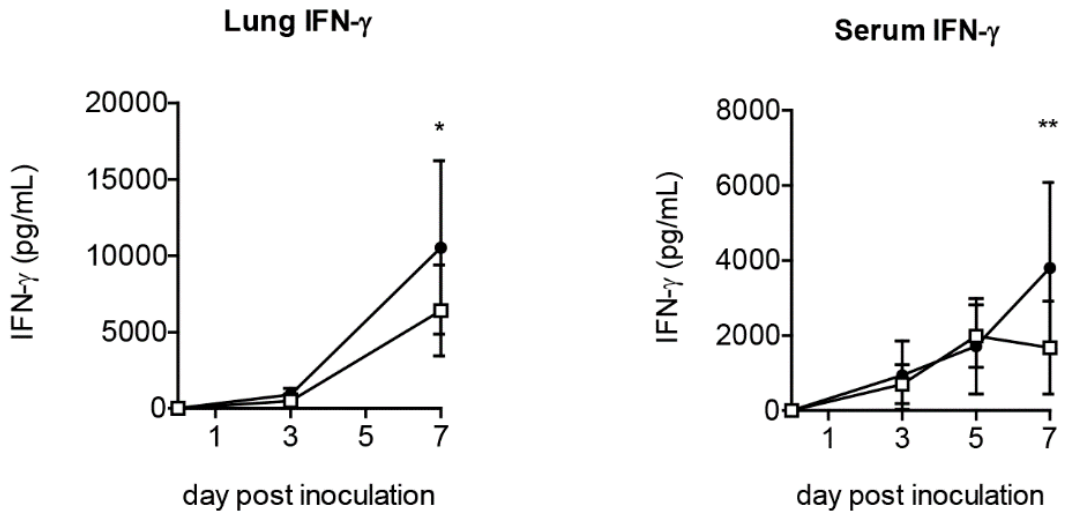


Figure 2-16. CD1d$^{-/-}$ mice have an early IFN-γ response that is comparable to B6 mice.

Lung and serum IFN-γ levels were determined in naïve mice or at various time points p.i. as in Fig 8. Data are combined from 3 independent experiments (*n* = 15 mice/group). Plotted are mean ± SD.

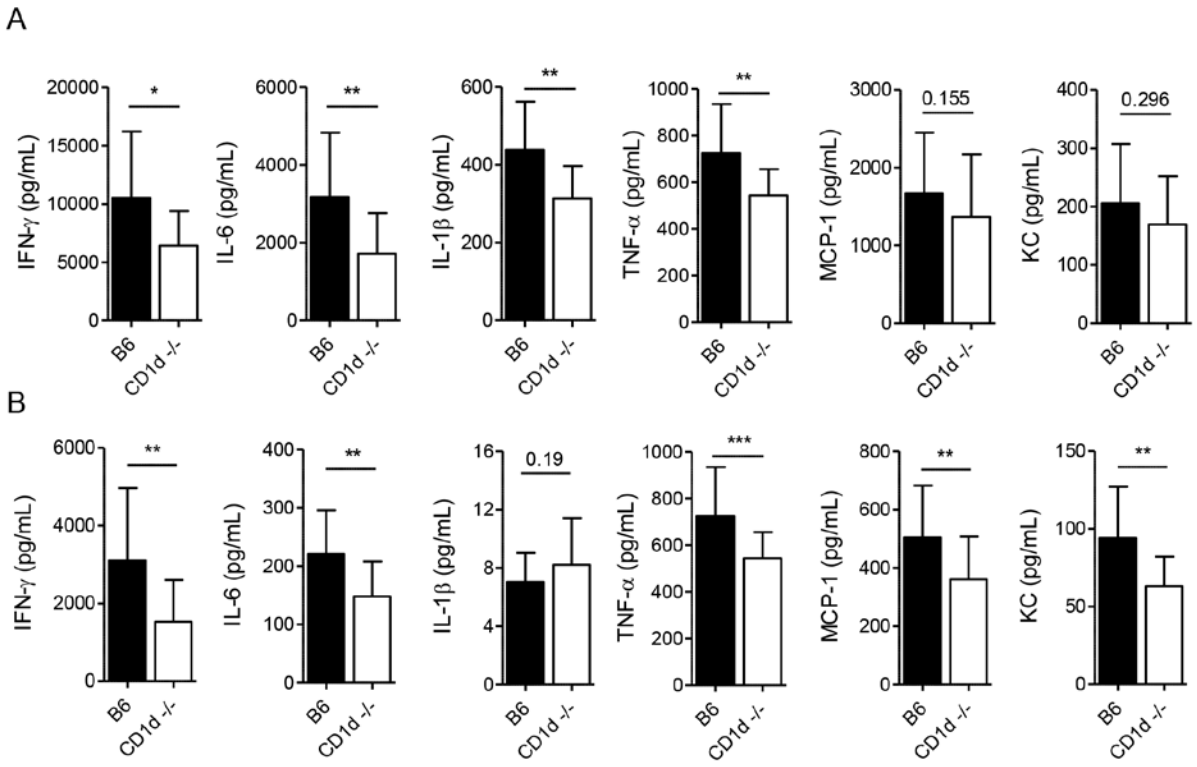


Figure 2-17. CD1d$^{-/-}$ mice exhibit a tempered inflammatory response to LVS.

Cytokine levels were measured at 7d p.i. in lung homogenates **(A)** or serum **(B)** by CBA. Data are combined from at least 3 experiments with 10–20 mice/group. Bars are mean + SD. Comparisons in these experiments were made as indicated in Materials and Methods. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

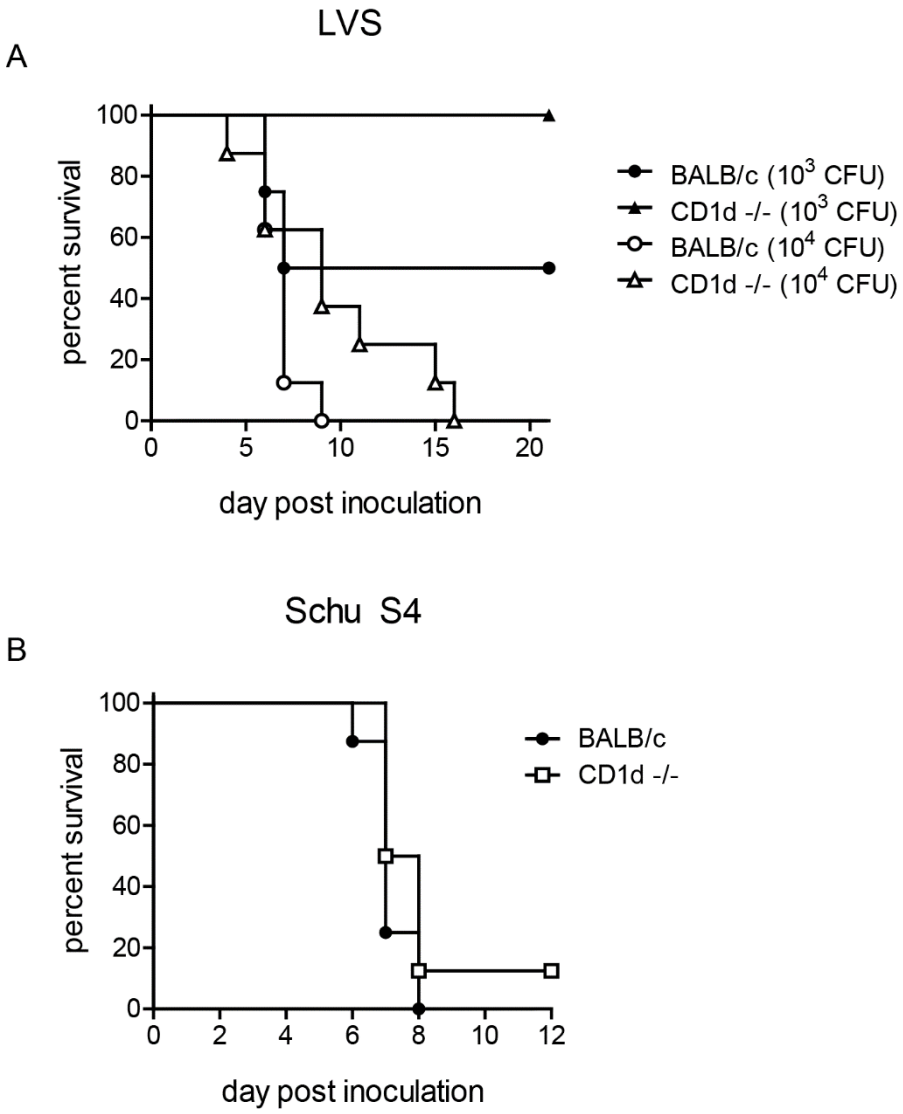


Figure 2-18. Reduced susceptibility of CD1d$^{-/-}$ mice is not dependent on strain background.

(A) Groups of 6-8 week-old BALB/c or CD1d$^{-/-}$ mice were infected intranasally with different doses of LVS as indicated and monitored for survival (*n*=8 mice/group) **(B)** BALB/c or CD1d$^{-/-}$ mice were infected intranasally with 6 CFU Schu S4 and monitored for survival (*n*=8 mice/group)

strains complicates the study of effective immune responses, particularly when administered intranasally (LD_{50}<10) (Cowley and Elkins, 2011). Although LVS has long been used in murine models of experimental tularemia (Anthony and Kongshavn, 1987; Conlan et al., 2011), it is an attenuated type B strain that fails to cause disease in humans. We therefore sought to determine whether $CD1d^{-/-}$ would have a similar resistance to the more virulent type A strain Schu S4. Not surprisingly however, both wild-type and $CD1d^{-/-}$ mice were extremely susceptible to a low-dose (<10 CFU) pulmonary infection with Schu S4 (Fig. 2-18B), similar to the results obtained with higher doses of LVS in both C57BL6 (Fig. 2-1D) and BALB/c (Fig. 2-18A) mice. These data are consistent with previous reports showing reduced efficacy of therapies or mutations that confer resistance to LVS when challenged with Schu S4 (Duckett et al., 2005; Henry et al., 2010; Kirimanjeswara et al., 2007; Malik et al., 2007).

Intranasal α-GalCer treatment elicits a protective inflammatory response

Intranasal administration of α-GalCer recruits NKT cells into the lung interstitium as reported by Bendelac and colleagues ((Scanlon et al., 2011); see also Figs. 2-4B, 2-5B, and 2-19A). Hence, given our data demonstrating a detrimental role for lung NKT cells in *F. tularensis* infection, it is not unreasonable to expect that pretreatment with α-GalCer might exacerbate disease. As such, I treated B6 mice intranasally with α-GalCer or a vehicle control two days prior to intranasal LVS infection. Surprisingly and contrary to what might be expected, α-GalCer-treated mice were completely protected from disease (Fig. 2-19B and C).

In vivo administration of α-GalCer elicits a robust IFN-γ response from both NKT cells (through direct recognition) and NK cells (through transactivation) (Bezbradica et al., 2005; Carnaud et al., 1999; Scanlon et al., 2011). This is significant because studies of lymphocyte trafficking into non-lymphoid tissues — such as the skin, lungs, and vagina— have shown that these tissues are normally not accessible to immune effector cells in the absence of local inflammation ((Shin and Iwasaki, 2013); and our unpublished data). Intranasal treatment with α-GalCer induces such local inflammation that recruits a variety of immune cells into the inflamed lung tissue where they are better positioned to confront an invading pathogen ((Scanlon et al., 2011); and our unpublished data).

Macrophages, which are a primary target for *F. tularensis* infection, become unresponsive to IFN-γ upon pathogen entry into the cytosol, allowing unfettered bacterial replication (Jones et al., 2012). Conversely, uninfected macrophages activated by IFN-γ are able to effectively limit the growth of intracellular *Francisella* (Edwards et al., 2010). It is therefore not surprising that pretreatment with IL-12 or cationic lipid–DNA complexes (CLDC) protects mice from LVS infection through early elicitation of IFN-γ (Duckett et al., 2005; Troyer et al., 2009). Such treatment promotes clearance of *F. tularensis* from the lungs, liver, and spleen after intranasal infection. Similarly, we found that pretreatment with α-GalCer also reduced local and peripheral burden, which is likely due to macrophage activation by IFN-γ similar to that observed after intranasal IL-12 or CLDC treatment (Fig. 2-19D). Therefore, I find it likely that any intervention that results in local or systemic inflammation prior to infection would impart similar resistance to *F. tularensis* infection. However,

these results do leave open the possibility of leveraging the adjuvant effect of α-GalCer in future immunization experiments in an attempt to boost the response to attenuated *F. tularensis* strains.

Discussion

Once infected, the host has two strategies to deal with invasive pathogens: resistance and tolerance. Resistance implies the ability of the host to clear a pathogen or limit its spread whilst tolerance indicates the ability of the host to bear the ensuing pathology (Rivas et al., 2014). Surviving infection from a pathogen such as *F. tularensis* likely requires a balance of both. Previous studies of infection in immunodeficient mice revealed that immunity to *F. tularensis* is mediated by the concerted effort of the innate and adaptive humoral and cellular immune responses. Because the host deploys a wide array of effector mediators in its inflammatory arsenal against the infection a sepsis-like disease ensues (Cowley and Elkins, 2011; Steiner et al., 2014). Hence, fatalities in this infectious disease appear to be caused by an inability to control bacterial growth and dissemination, which precipitates inflammation and causes irreparable collateral tissue damage.

Accordingly, if diagnosed early, antibiotic treatment limits bacterial burden and tempers inflammation thereby controlling morbidity and mortality underlying tularemia. Herein we report that NKT cells respond to intranasal infection by migrating to the infected lung to produce IFN-γ, which promotes clearance and killing of the pathogen. As a trade-off however, the early activation of NKT cells

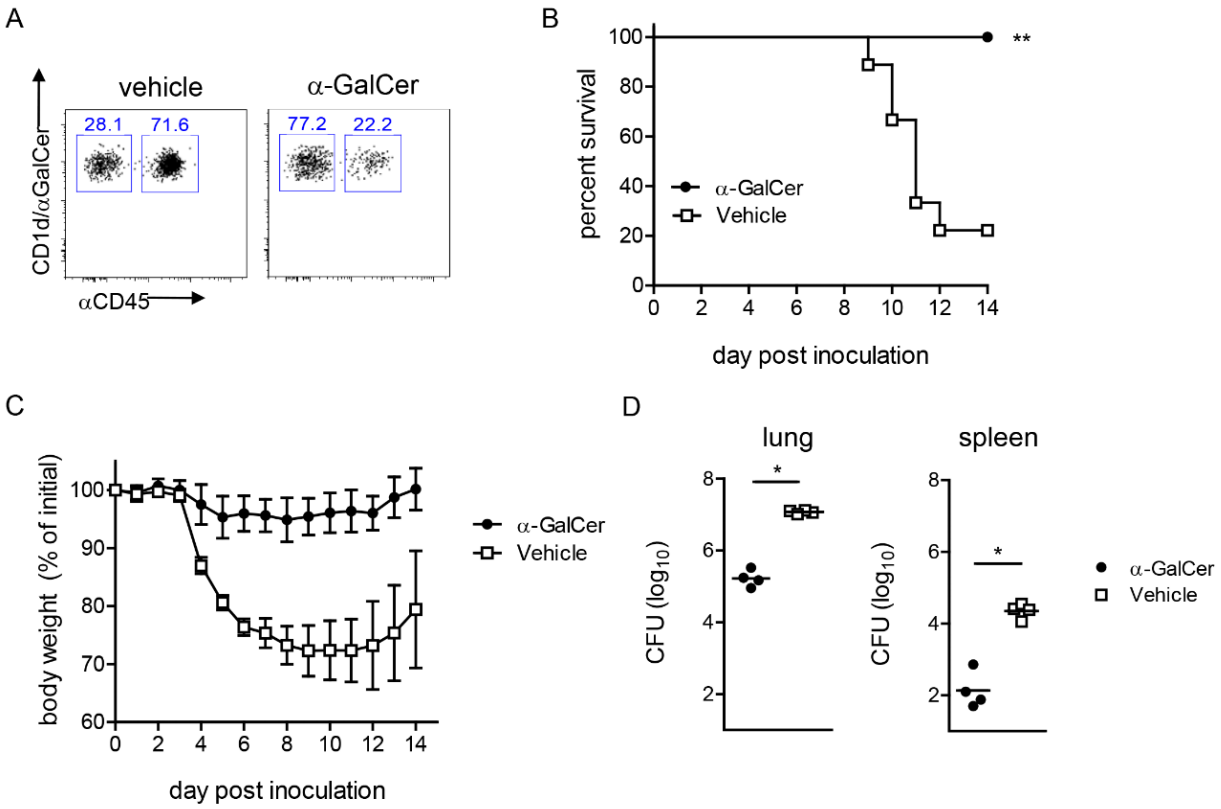


Figure 2-19. α-GalCer treatment protects mice from pulmonary tularemia.

(A) Representative staining of lung NKT cell localization in α-GalCer- and vehicle-treated mice 24 hours post-treatment and prior to LVS infection. Numbers are frequency of NKT within each compartment. **(B)** Groups of B6 mice were treated intranasally with 2 μg α-GalCer or vehicle. After 48 hours, mice were infected intranasally with 8000 CFU LVS and monitored daily for weight loss and signs of morbidity (n = 9 mice/group). Results are representative from one of two similar experiments. **(C)** Weight loss of α-GalCer- or vehicle-treated mice following intranasal LVS infection. Mean ± SD. **(D)** Lung and splenic LVS burden in α-GalCer or vehicle-treated mice day 3 post inoculation. Each symbol represents an individual mouse. Means were compared by Mann-Whitney U test.

causes excessive systemic inflammation, increases neutrophil mobilization, and delays infiltration of lymphocytes into the lungs and formation of protective tertiary lymphoid structures. Hence, CD1d-deficient animals have a tempered inflammatory response and perform clinically better in response to intranasal LVS infection. This advantage, however, can be overcome with higher doses of bacteria or infection with more virulent subspecies. The discordant results obtained using Jα18$^{-/-}$ and CD1d$^{-/-}$ mouse models likely reflect a more global T cell deficiency in Jα18$^{-/-}$ mice and underscore the difficulties in deciphering the role of NKT cells using currently available methods (Tupin et al., 2007). In an effort to address these issues, Chandra and colleagues recently developed a new strain of Jα18-deficient mice which lack iNKT cells while maintaining an otherwise complete TCRα repertoire (Chandra et al., 2015). Such refined models will become important in future studies of iNKT cell function in health and disease.

Akin to CD1d-deficient animals, *F. tularensis* infection of metalloproteinase-9 null as well as IL-17 and IL-10 double-deficient mice showed low disease incidence despite similar bacterial burden (Malik et al., 2007; Slight et al., 2013). In conjunction with a tempered inflammatory response, these immunodeficient mice were able to limit the damage caused by the pathogen or the host immune system and were therefore less susceptible to i.n. *F. tularensis* infection. These findings and our data reported herein, thus enforce the notion that the host response to *F. tularensis* is a balance between resistance to pathogen and tolerance to pathology.

LVS infection of endothelial cells induces the transmigration of neutrophils into infected tissues (Forestal et al., 2003; Moreland et al., 2009). Rather than

contributing to host defense, neutrophils may instead increase disease severity. *F. tularensis*-infected neutrophils assume an extended proinflammatory phenotype, which is thought to contribute to increased tissue destruction (Allen, 2013). Because *F. tularensis* is able to infect and replicate within neutrophils, these cells likely serve as a vehicle for dissemination from the primary site of infection, which might contribute to the reduced peripheral burden observed in CD1d$^{-/-}$ mice (Hall et al., 2008; Mocsai, 2013). Direct interaction with NKT cells has been shown to influence the function of neutrophils (De Santo et al., 2010) and to promote hematopoiesis in both humans and mice (Kotsianidis et al., 2006). NKT cell-neutrophil interactions were also recently shown to exacerbate polymicrobial sepsis (Kim et al., 2014). Although I found no differences in differential cell counts from peripheral blood of naive wild-type and CD1d$^{-/-}$ mice, CD1d$^{-/-}$ mice displayed less severe neutrophilia upon LVS infection. I also found that slightly decreased hepatotoxicity, reduced peripheral burden, and less severe inflammation in NKT cell-deficient CD1d$^{-/-}$ mice corresponded with less severe neutrophilia.

Our studies revealed that acute LVS infection of CD1d$^{-/-}$ mice resulted in increased lymphocytic infiltration in the lung interstitium when compared to B6 mice. The cellular infiltration organized into iBALT at the peak of infection. Although typically associated with chronic inflammatory conditions, iBALT has been shown to form in the lung in response to viral infection (Foo and Phipps, 2010). However, the formation of such structures in response to acute bacterial infection is less well-studied. In the context of pulmonary *F. tularensis* infection, lymphoid aggregates have been described in wild-type mice that survived *F. tularensis* infection after

>50d p.i. whilst bone fide iBALTs were described only in LPS/rPorB vaccinated mice challenged with a high dose of LVS (Chiavolini et al., 2008; Chiavolini et al., 2010). The post-vaccination development of these structures in the lungs was associated with better survival upon i.n. challenge with LVS or the more virulent *F. tularensis* spp. *tularensis* (Chiavolini et al., 2010; Wayne Conlan et al., 2005). Data from these studies suggest that the T and B cells found in iBALT limit the spread of the bacteria from the lung thereby reducing immune-mediated damage to peripheral organs. Contrary to the results of these studies with wild-type animals, our data demonstrate the development of iBALT in the lungs of B6 mice is hampered perhaps by the presence and activation of NKT cells as more robust iBALT formation is observed in $CD1d^{-/-}$ mice during the peak of a primary LVS infection. Such early iBALT formation is suggestive of a more vigorous and effective adaptive immune response resulting in reduced systemic inflammation and therefore less severe disease. These findings suggest a suppressive/regulatory role for pulmonary NKT cells. The regulatory function of NKT cells is just beginning to be elucidated and much of what we know derives from studies utilizing model antigens (Sag et al., 2014). Thus, our findings provide a model in which to study the potentially detrimental functions of iNKT cells in a natural infection.

In the context of intranasal LVS infection, the inability to form iBALT proves to be detrimental, but there may be cases where this outcome is desirable. For example, iBALT formation has been associated with chronic inflammatory conditions such as asthma (Elliot et al., 2004). Therefore, the discovery of an NKT cell-activating ligand(s) derived from *F. tularensis* that can prevent iBALT formation

might be of therapeutic benefit in such instances. Similarly, a better understanding of the regulatory function of NKT cells may lead to advances toward the goals of rational vaccine design.

CHAPTER III

MECHANISMS OF iNKT CELL ACTIVATION AND FUNCTION IN PULMONARY *F. TULARENSIS* INFECTION

Introduction

Common features of most known iNKT cell antigens include a hydrophobic lipid tail that gets buried deeply into the hydrophobic antigen binding pockets of CD1d, and a sugar head that projects above the plain of CD1d and interacts with the invariant iNKT cell TCR α-chain (Borg et al., 2007). The more diverse TCR β-chain makes contact with the CD1d α2 domain, allowing iNKT cells to distinguish distinct structural features in the lipid tails of glycolipid antigens (Florence et al., 2009). In general, the lipid tails consist of a phytosphingosine base and an acyl chain. The length of the phytosphingosine base is believed to control the affinity of the iNKT cell TCR for the CD1d/glycolipid complex (McCarthy et al., 2007), whereas the length of the acyl chain likely affects solubility of the lipid and affinity of binding to CD1d (Im et al., 2009; Sullivan et al., 2010; Wun et al., 2011; Yu et al., 2005).

As discussed in Chapters I and II, iNKT cells possess the potential to rapidly secrete copious amounts of both pro- and anti-inflammatory cytokines upon activation. The character of the ensuing immune responses modulated by this activation are determined by the types of cytokines they produce. The cytokine profile of activated iNKT cells is defined by the structure of the glycolipid antigen, the binding and presentation kinetics and the cellular site of loading onto CD1d, and

the cell membrane location of their presentation to the iNKTCR (Im et al., 2009). The outcome of this activation is additionally influenced by the affinity of the iNKTCR for a given CD1d-glycolipid complex, the costimulatory, coinhibitory, and cytokine signals received, and the subset(s) of iNKT cells receiving these signals (Brennan et al., 2013; Singh et al., 2014; van den Heuvel et al., 2011; Van Kaer et al., 2015a). To begin to gain insights into how *F. tularensis* activates iNKT cells, I performed preliminary experiments directed toward elucidating which subset(s) of iNKT cells were activated in response to intranasal LVS infection and to define the chemical nature of the *Francisella* antigens recognized by the iNKT cells. The outcomes of these investigations are described below.

Results

Multiple iNKT cell subsets are activated by F. tularensis

To begin to determine which subset(s) of iNKT cells were activated in response to intranasal LVS infection, I performed intracellular cytokine staining (ICCS) of freshly isolated lung iNKT cells on day 3 and 5 post-inoculation (Fig. 3-1). Flow cytometric analysis of total lung iNKT cells demonstrated robust activation as indicated by increased CD69 expression (Fig. 3-1A). The predominant cytokine detected in activated iNKT cells was IFN-γ (Fig. 3-1A and B). I was unable to reliably detect intracellular IL-4 in iNKT cells despite several attempts, but a modest amount of IL-17A, granzyme B, and TNF-α were reproducibly detected in several experiments, thus suggesting that iNKT1 and iNKT17 were the predominant

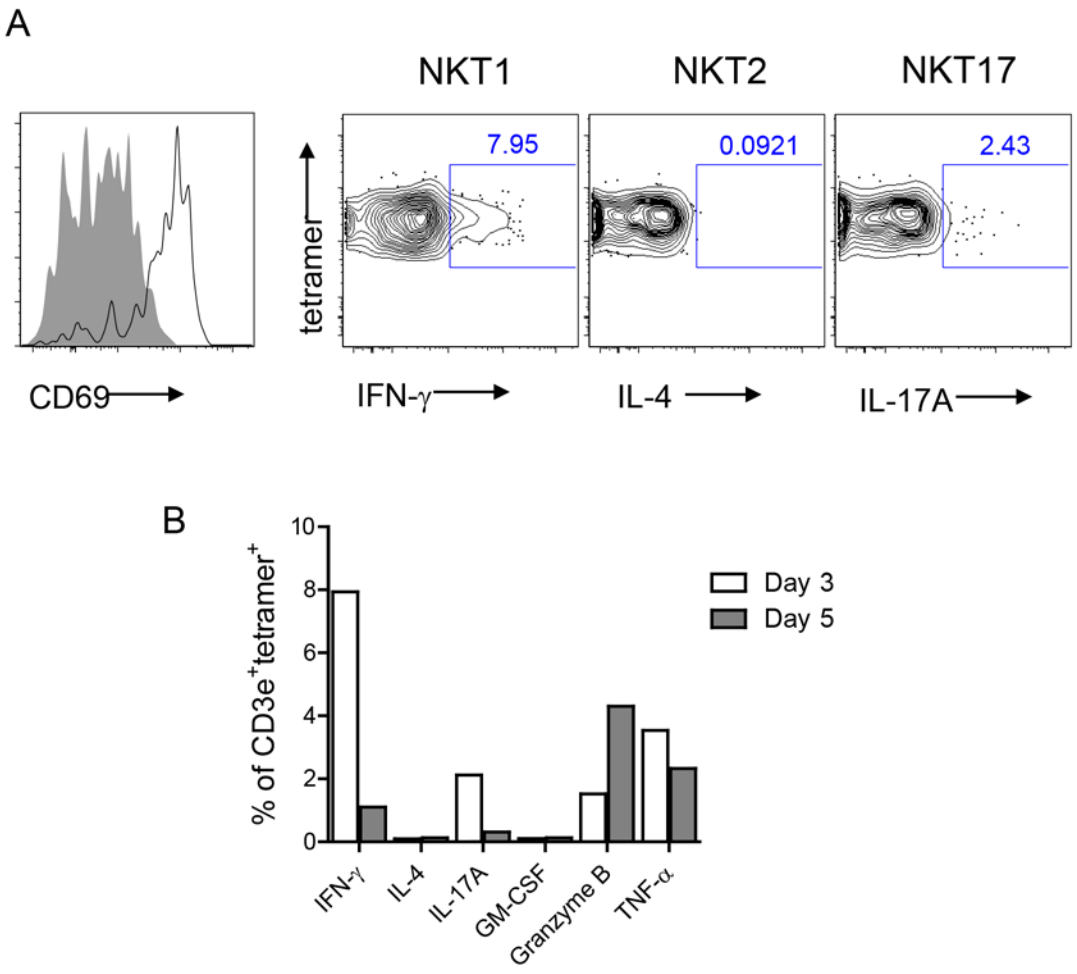


Figure 3-1. Cytokine production by lung iNKT cells.

(A) Lung samples from d3 post-inoculation were pooled and analyzed by ICCS for NKT cell cytokine production. Shown are representative staining from one of three similar experiments. Numbers in plots are % cytokine$^+$ iNKT cells. **(B)** % cytokine$^+$ NKT cells d3 and d5 post-inoculation. Bars represent pooled samples (n = 3 mice). Results are representative from three experiments.

subsets responding to pulmonary LVS infection. These data further support a pro-inflammatory function of iNKT cells in this disease, as suggested by the tempered inflammatory response in iNKT cell-deficient mice described in Chapter II (see Fig. 2-17).

iNKT cell activation by LVS requires contact with infected cells

Aside from activation in response to microbial-derived lipid antigens, iNKT cells can also respond to microbes that do not produce such lipids. APCs responding to microbial products such as TLR ligands produce inflammatory cytokines (including IL-12) which amplify the response of iNKT cells to endogenous lipids presented by CD1d. In many microbial infections, this has been proposed as the predominant means of activation (Brigl et al., 2011). Additionally, iNKT cells can be activated in the absence of TCR stimulation, in a manner that is dependent upon APC-derived IL-12 and IL-18 (Leite-De-Moraes et al., 1999).

In order to determine the mechanism by which iNKT cells are activated in response to LVS, I first used an *in vitro* approach to investigate the possibility of cytokine-driven activation (TCR-independent). Bone marrow-derived macrophages (BMM) were infected with LVS as previously described (Elkins et al., 2001) and co-cultured with B6 splenocytes or cultured in a transwell system where splenocytes were separated from the infected cells by a membrane that is impermeable to bacteria but permissive to the movement of macromolecules (Fig. 3-2). A hallmark of iNKT cell activation is the rapid down-regulation of the TCR resulting in the loss of CD1d/α-GalCer tetramer$^+$ cells (Crowe et al., 2003; Harada et al., 2004; Wilson et

al., 2003). Consistent with these results, I found a complete loss of CD1d/α-GalCer tetramer^{+} cells after 24 hours of co-culture with α-GalCer-loaded macrophages (Fig. 3-2A). Similar results were obtained upon co-culture with LVS-infected macrophages. Importantly, neither LVS nor α-GalCer induced TCR down-regulation by iNKT cells not in contact with loaded or infected macrophages. Furthermore, only those iNKT cells in contact with the infected BMM increased CD69 expression (Fig 3-2B).

These data suggest that the iNKT cell response to LVS infection requires cell-to-cell contact with either the infected cell or perhaps with a phagocytic cell that has internalized an infected cell or cell debris (Shimizu et al., 2007; Wu et al., 2003), and is therefore not due to cytokine-driven (TCR-independent) activation. Importantly, a previous report found that iNKT cells activated *in vivo* after *Salmonella* infection (a bacterium that does not produce any known iNKT cell ligands) did not result in TCR down-regulation, suggesting that this is a phenomenon observed only in response to exogenous lipid antigens presented by CD1d molecules (Wilson et al., 2003). These data therefore support our hypothesis that iNKT cells recognize a lipid present in the cell wall of *F. tularensis*. The less-pronounced TCR down-regulation in response to LVS as compared to α-GalCer suggests that not all iNKT cells are activated by LVS or that the activating lipid(s) provides a weaker stimulus or is present in lower concentration in infected cells.

A putative LVS-derived antigen activates lung-resident iNKT cells through the T cell receptor

As demonstrated in Chapter II (Figs. 2-4 and 2-5), iNKT cells accumulate in the lung interstitium following intranasal LVS infection. To determine whether these accumulating iNKT cells were activated by an LVS-derived lipid, I employed Nur77^{gfp} reporter mice. As recently reported by Holzapfel et al. (Holzapfel et al., 2014), only administration of α-GalCer (or one of its derivatives) or infection with microbes known to produce iNKT cell-activating lipids (*S. pneumoniae* and *S. paucimobilis*) resulted in the induction of increased GFP expression by iNKT cells in Nur77^{gfp} mice. Treatment with TLR agonists or infection with a bacterium thought to increase the expression of a self-lipid (*S. typhimurium*) activated iNKT cells (based on increased CD69 expression and IFN-γ production), but these activated cells did not increase GFP expression. Neither did infection with MCMV, which activates iNKT cells via pro-inflammatory cytokines alone and independent of CD1d-restricted antigen presentation (Tyznik et al., 2008; Wesley et al., 2008).

On d3 post i.n. LVS inoculation, I found that both interstitial and intravascular iNKT cells increased CD69 expression (Fig. 3-3A). Strikingly, however, only the interstitial population showed increased GFP fluorescence (Fig. 3-3A). This result indicated that local LVS antigen presentation occurred within the interstitium to activate LVS-recruited iNKT cells *in situ*. To determine the consequence of interstitial iNKT cell activation, intracellular cytokine staining was once again conducted directly *ex vivo* without any restimulation. I found that the GFP^{HI}

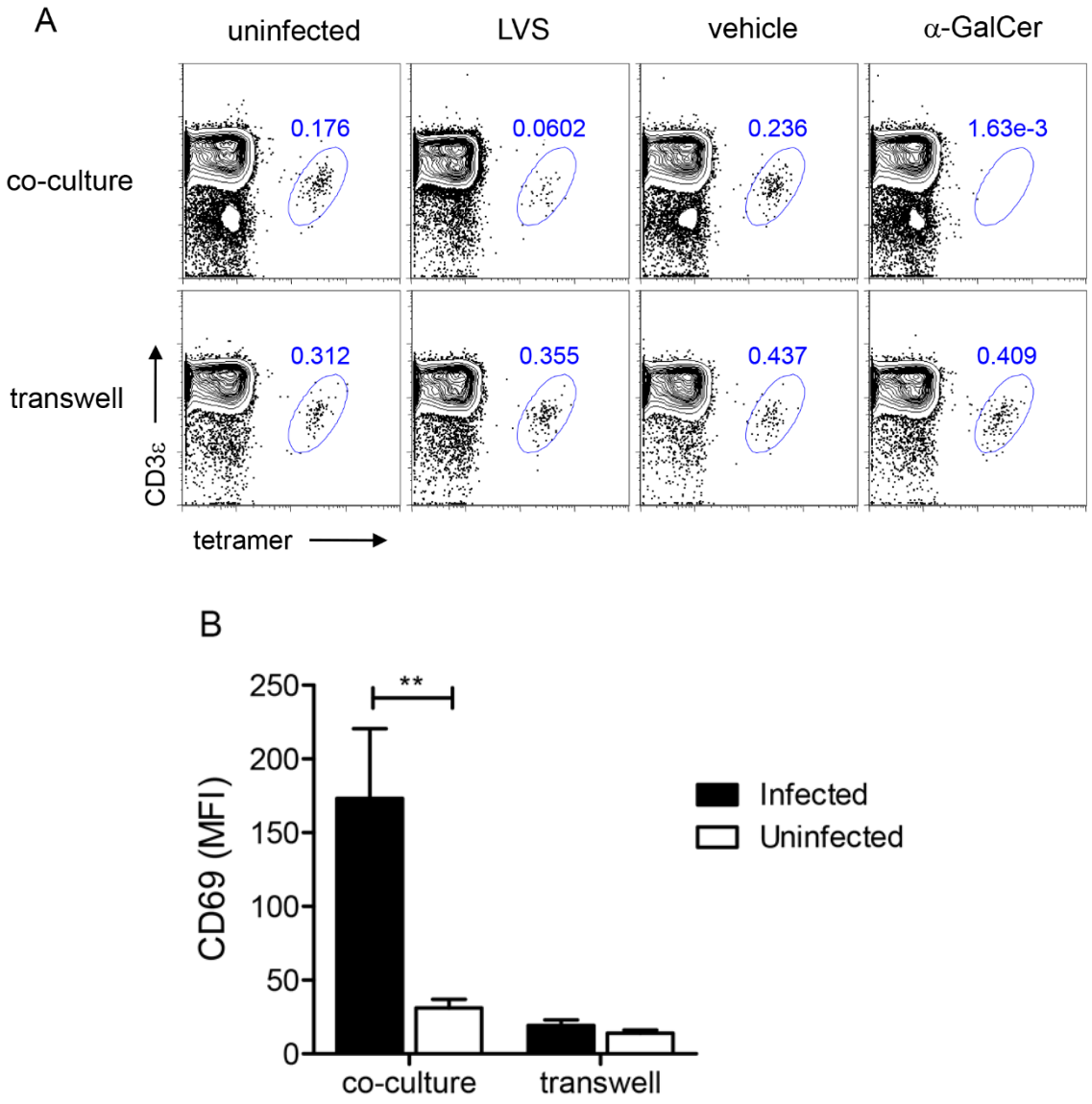


Figure 3-2. iNKT cell activation by LVS requires contact with infected cells.

(A) 2 x 10^5 B6 BM-derived macrophages were infected with LVS for 2 hours or pulsed overnight with 500 ng/mL a-GalCer and then washed with PBS. 1.5 x 10^6 B6 splenocytes were added to the same well (co-culture) or separated by a 0.4 μm membrane. After 24 hours, cells were stained for CD69 expression. Numbers in plots are percent $CD3\varepsilon^+tetramer^+$ cells from among $B220^-$ splenocytes. **(B)** Bars are median fluorescence of CD69 from within iNKT gate (mean of triplicate wells ± SD). Comparisons made by two-tailed t test. $^{**}p<0.01$. Results are representative from one of two similar experiments.

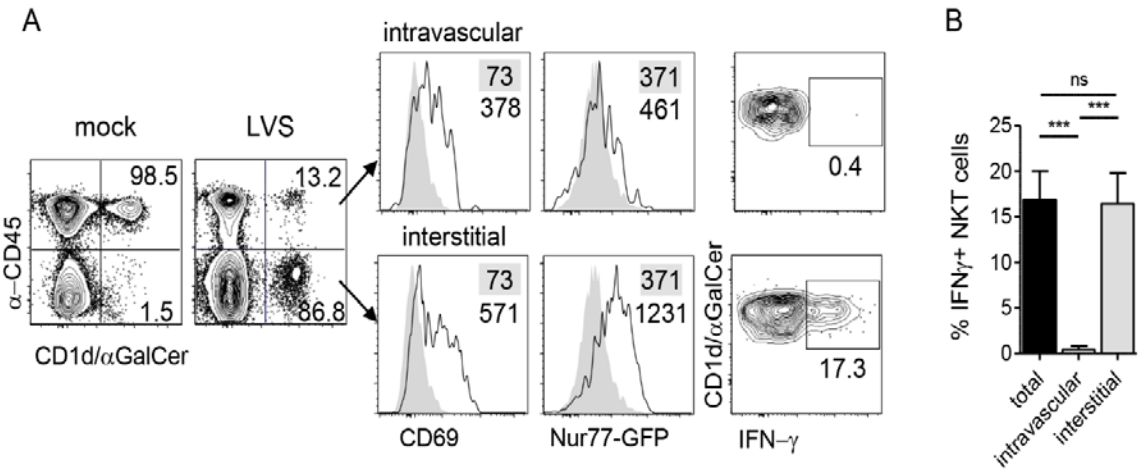


Figure 3-3. *i*NKT cell activation by LVS is TCR-dependent.

(A) Representative plots of NKT cell localization, activation, and IFN-γ production after i.n. LVS infection of Nur77^{gfp} mice. Numbers in contour plots are % iNKT cells. Numbers in histograms are median fluorescence of infected mice (open histograms) and mock-infected controls (shaded histograms). Data are representative of three similar experiments (n=10). **(B)** Percent IFN-γ^{+} ^{i}NKT cells in each compartment after LVS infection. Bars are mean+SD. Data are representative from one of three similar experiments (n=3 mice). Comparison was made by one-way ANOVA with Tukey's posttest. $^{***}p<0.001$.

interstitial iNKT cells but not GFP^{LO} intravascular cells produced IFN-γ (Figs. 3-3A and B), demonstrating that this cytokine production was a result of LVS infection *in vivo*. Taken together with the results presented in Figure 2-4, the above data suggest that upon i.n. *F. tularensis* LVS infection, iNKT cells migrate from the periphery and accumulate within the infected lung where they are activated through their TCR to produce IFN-γ.

Diverse cytokine production by pulmonary iNKT cells in response to LVS infection

To better define the cytokine profile of pulmonary iNKT cells activated after LVS infection, I bred $Nur77^{gfp}$ mice with $Vα14^{tg}$ mice to increase the frequency of iNKT cells in the lungs. The resulting $Nur77^{gfp}$;$Vα14^{tg}$ mice were infected i.n. with LVS and ICCS of lung iNKT cells performed on d3 p.i. In agreement with previous results (Fig. 3-1), GFP expression was mostly restricted to $αCD45^{NEG}$ cells located in the lung interstitium. iNKT cells were found to produce predominantly Th1 cytokines including IFN-γ and TNF-α (Figure 3-4A and B). However, in contrast to earlier results with wild-type B6 mice, the increased iNKT cell population in $Nur77^{gfp}$;$Vα14^{tg}$ mice allowed the detection of a small percentage of iNKT cells producing IL-4. Because IL-4 is thought to be produced primarily in response to microbial but not self-antigens, these data lend further support to the idea that iNKT cells are being activated by an LVS-derived lipid. Interestingly, this experiment demonstrated that unlike IFN-$γ^+$ iNKT cells, those cells that were producing TNF-α seemed to be located predominantly in the lung vasculature. A similar trend was observed with IL-4^+ NKT cells as well (Fig 3-4). Further experiments are required to

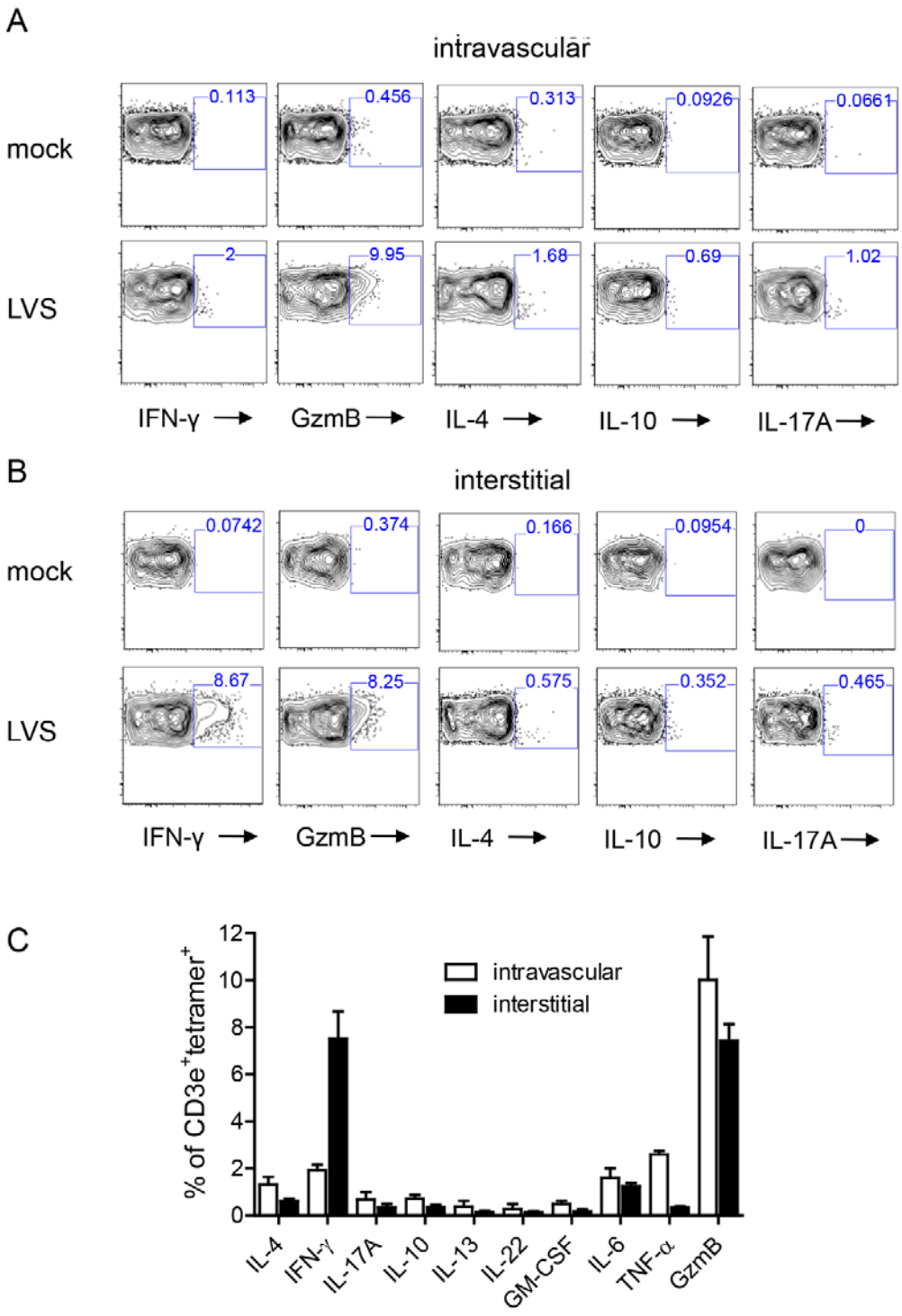


Figure 3-4. Lung iNKT cells produce multiple cytokines in response to LVS. Representative ICCS plots of intravascular **(A)** and interstitial **(B)** lung iNKT cells d3 post-inoculation. **(C)** Mean+SD of individual mice (n = 3). Results are derived from a single experiment.

investigate the significance of these findings, but these results highlight the differential outcome of iNKT cell activation even within the microenvironment of a single tissue.

Studies of iNKT cell effector functions primarily report on their rapid production of Th1 or Th2 cytokines, while less attention has been focused on their cytotoxic potential. Several studies have focused on the ability of iNKT cells to augment the cytotoxic function of NK cells upon *in vivo* treatment with α-GalCer or IL-12. Fewer studies have investigated the direct cytotoxic function of iNKT cells. In one such study, Wingender and colleagues (Wingender et al., 2010) found that iNKT cells from the thymus, spleen, and liver exhibit equivalent cytotoxicity against α-GalCer–loaded A20 B lymphoma cells. This cytotoxicity was correlated with the potency of the activating lipid and the level of CD1d expression on the target cells, and was mediated by Fas/Fas-L interactions. iNKT cells activated by antigen-loaded B cells rapidly upregulated Fas-L which corresponded with an increase in Fas expression by the APCs.

Less well-studied is the *in vivo* cytotoxicity of iNKT cells in response to microbial infection. Given the demonstrated early activation of pulmonary iNKT cells (Figs. 3-1 and 3-3) and the potential for apoptotic cells to precipitate an inflammatory response (Tang et al., 2008), I next wondered whether cytotoxic iNKT cells in the lungs might contribute to the robust inflammation observed after intranasal LVS infection (see Fig. 2-17). I was unable to detect Fas-L expression by activated iNKT cells in the lungs, but surprisingly, pulmonary iNKT cells produced the cytolytic mediator granzyme B regardless of their localization within the infected

lung (Fig 3-4A–C). This granzyme B production was in striking contrast to IFN-γ because it did not seem to depend on TCR signaling, since the majority of GFP^{HI} cells were in the interstitial compartment (Fig. 3-3). In a study by van Gisbergen and colleagues (van Gisbergen et al., 2012), iNKT cells were shown to produce granzyme B in response to innate stimuli but not upon recognition of α-GalCer. These results led the authors to conclude that the effector differentiation of iNKT cells results in the development of either cytokine-producing cells or cytolytic cells depending on the nature of the activating stimuli.

To extend these findings to *F. tularensis* infection, I analyzed interstitial iNKT cells d3 post LVS inoculation. As seen in Figure 3-5, those iNKT cells that were IFN-γ positive also had higher GFP fluorescence compared to the granzyme B^{+} iNKT cells, suggesting that differential signaling was likely at least partially responsible for the diverse cytokine production in response to LVS. These data further highlight the complexities of analyzing iNKT cell function in the context of a bacterial infection where these cells likely respond to multiple stimuli depending on the local inflammatory environment and the specific subset of iNKT cells responding. Further experimentation is required to dissect these issues in our model of pulmonary tularemia, but the suggestion that the cytotoxic function of iNKT cells may play a role is intriguing. Murine iNKT cells were recently shown to augment atherosclerosis through granzyme-mediated cytotoxicity (Li et al., 2015), and in human patients suffering from allergic asthma, iNKT cells were shown to contribute to lung inflammation by granzyme-mediated killing of autologous

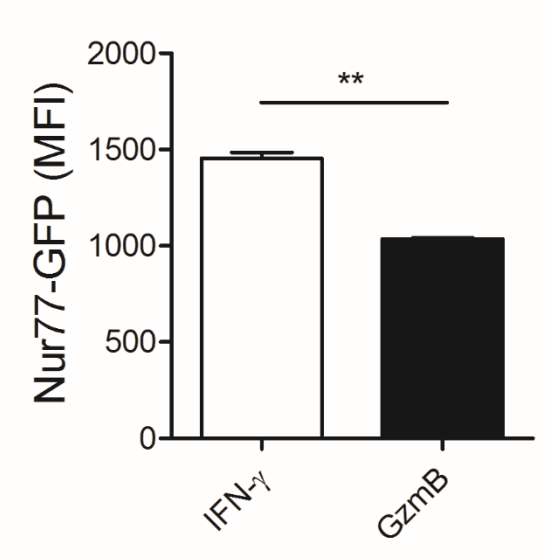


Figure 3-5. Increased TCR signaling in IFN-γ⁺ iNKT cells.

On d3 post-LVS-inoculation, interstitial lung NKT cells were identified by intravascular CD45 staining as in Fig 3-3. NKT cells positive for IFN-γ or granzyme B were assayed for GFP fluorescence intensity. Shown are mean+SD for 3 mice. Means were compared by Welch's t test.

regulatory T cells (T_{reg}) (Nguyen et al., 2008). Whether either of these mechanisms are applicable to the iNKT cell response to LVS is unknown.

Activation of NKT cells by LVS-derived lipid(s)

In an attempt to further elucidate the mechanism(s) by which iNKT cells are activated in response to LVS, we established a collaboration with Dr. Paul Savage who is an expert in lipid chemistry. Crude lipid extracts from *F. tularensis* were prepared as previously described (Albacker et al., 2013). Four lipid fractions were generated and tested for their ability to activate iNKT cells *in vitro*. Total splenocytes contain both APCs and iNKT cells and have therefore been used as a model for the study of iNKT cell activation by exogenous lipids (Burdin et al., 1998). Hence, in an initial experiment, B6 splenocytes were incubated for four days with each lipid fraction. α-GalCer and the reportedly Th1-biasing derivative 7DW8-5 (Li et al., 2010) were used as positive controls. Additionally, I included the *Sphingomonas*-derived GSL-1 (Kinjo et al., 2005; Mattner et al., 2005; Sriram et al., 2005) which was provided by Dr. Savage. DMSO alone served as a negative control.

Consistent with previous data (Coquet et al., 2008), culture of splenocytes with α-GalCer elicited robust production of Th1, Th2, and Th17 cytokines (Fig. 3-6). Interestingly, I did not observe the Th1-biased response previously reported for 7DW8-5. On the contrary, this derivative actually elicited less IFN-γ and more IL-4 as compared to α-GalCer in this assay. As expected, the response to GSL-1 was

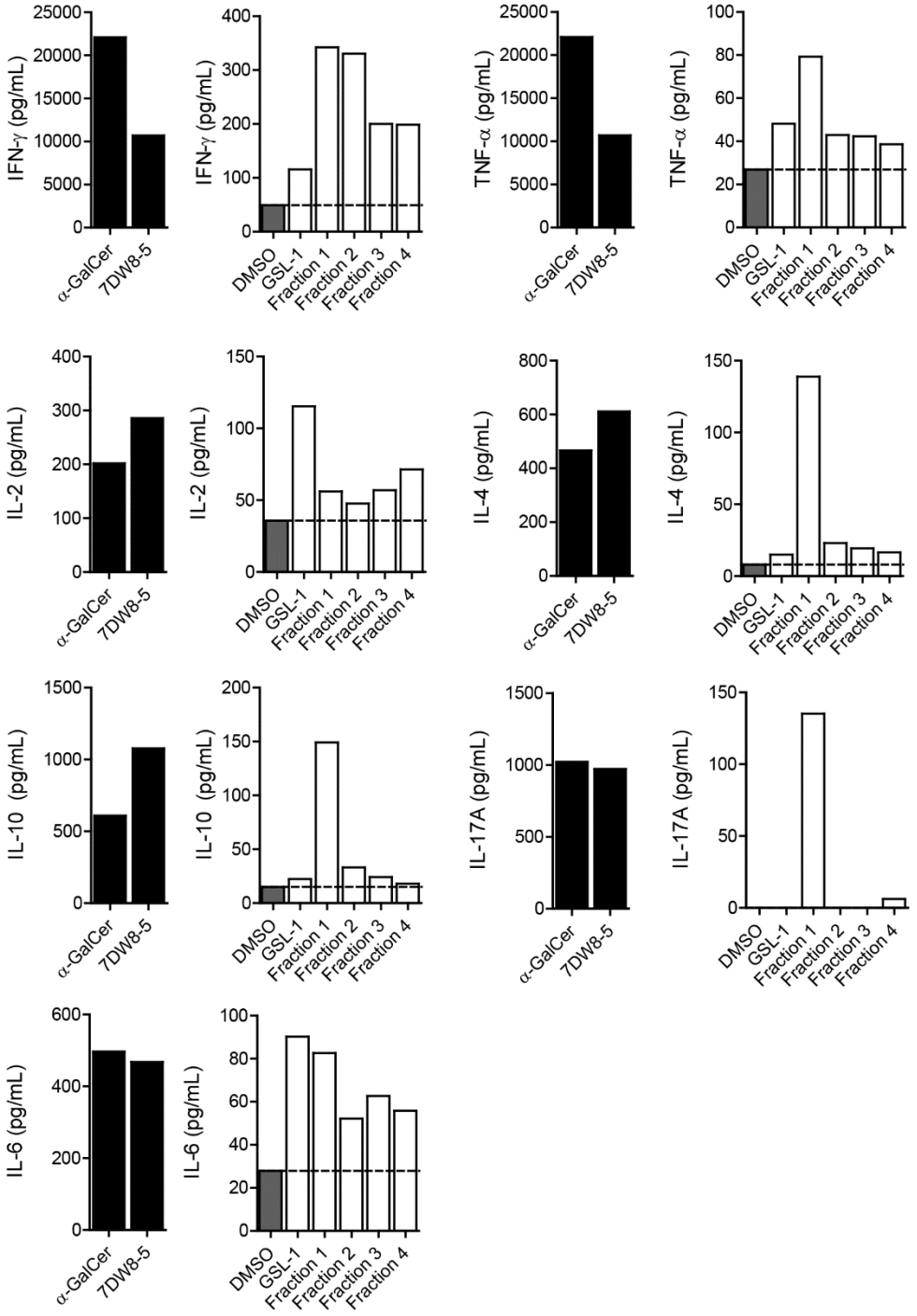


Figure 3-6. Diverse cytokine production in response to LVS lipid fractions *in vitro*.

5 x 10^5 B6 splenocytes were incubated for four days in the presence of 100 ng/mL α-GalCer, 7DW8-5, or GSL-1, or 1 µg/mL LVS lipid fractions as indicated. DMSO was used as a negative control. Supernatants were assayed by CBA for the indicated cytokines.

much less robust than that observed with α-GalCer [Fig. 3-6; (Kinjo et al., 2005)], but importantly, all three synthetic lipids stimulated IL-4 production as expected with antigen-driven iNKT cell activation [Fig. 3-6; (Brigl and Brenner, 2010; Brigl et al., 2011)]. Most importantly, in accordance with my data obtained from infection of Nur77^{gfp} mice (Fig. 3-3), culture with LVS-derived lipid fractions resulted in the production of all cytokines tested, with the most significant levels seen in response to fraction 1 (Fig 3-6). This response was greater than that observed with GSL-1 with respect to IFN-γ, TNF-α, IL-4, IL-10, and IL-17A, suggesting that the major species responsible for iNKT cell activation resides within fraction 1. These data further support our hypothesis that iNKT cell activation in response to *F. tularensis* infection is mediated by iNKT cell recognition of a bacterial lipid. However, these data still do not preclude the possibility that iNKT cells are activated by self-lipids whose expression levels may be altered by some component of the LVS lipid extracts.

In an attempt to further reveal the identity of the activating lipid(s), I made use of an antibody invented by Porcelli and colleagues (Yu et al., 2007). The L363 antibody binds to CD1d only in complex with α-GalCer or several of its derivatives, albeit with lower affinity. It does not recognize CD1d-bound diacylglycerols from *B. burgdorferi* or *S. pneumonia*, and only weakly binds complexes of CD1d and the *Sphingomonas*-derived GalA-GSL (Yu et al., 2012). Importantly, L363 was shown to block the recognition of endogenous self-lipids by autoreactive iNKT cell hybridomas and iNKT cell lines (Kain et al., 2014; Yu et al., 2007). I therefore reasoned that if the activity observed in response to LVS lipid fractions was due to

the increased expression of self-lipids, rather than the presence of an LVS-derived lipid present in the fractions themselves, this activity should be blocked by the addition of L363 to the culture. Thus, Vα14^{tg} splenocytes were cultured with LVS lipid fractions in the presence of L363 (Fig. 3-7). As seen previously with B6 splenocytes, fraction 1 again elicited the most robust activity with all cytokines tested.

Contrary to the results obtained by Yu et al. (Yu et al., 2007), L363 was modestly effective at inhibiting the response to α-GalCer, where levels of IFN-γ, IL-4, TNF-α, and IL-6 were reduced approximately two-fold. Interestingly, the IL-10 response to α-GalCer appeared to be unaffected by antibody treatment. Similar to the results observed with α-GalCer, the response to LVS lipid fractions was reduced, but not completely abrogated by the addition of L363. Not surprisingly, the levels of most cytokines measured in this assay were increased over those observed with B6 splenocytes (Fig. 3-6) owing to the increased numbers of iNKT cells in Vα14^{tg} mice, demonstrating that the response was due either directly or indirectly to iNKT cell activation. The modest reduction in activity observed by L363 treatment suggests that either the antibody is recognizing the agonistic CD1d/bacterial- and/or self-lipid complexes with low affinity, or that iNKT cell activation is mediated by both exogenous lipids, which are not efficiently blocked by L363, and endogenous lipids, which are.

In regards to the latter possibility, it has been proposed that iNKT cell responses to bacterial infection are predominantly driven by the synergistic effect of APC-derived inflammatory cytokines and agonistic self-lipids (Brigl et al., 2011; Kain

et al., 2014). I therefore sought to determine what role, if any, innate activation of APCs might play in the activation of iNKT cells by *Francisella*. In contrast to the lipopolysaccharide (LPS) of most bacterial species, *F. tularensis* LPS is a poor TLR4 agonist and also fails to activate cells through TLR2 (Ancuta et al., 1996; Kieffer et al., 2003), yet the macrophage proinflammatory response to live LVS bacteria is overwhelmingly TLR2-dependent (Cole et al., 2006; Cole et al., 2007; Katz et al., 2006). Infection of macrophages with LVS was found to induce the production of several inflammatory cytokines including IFN-γ, TNF-α, and IL-12 (Cole et al., 2007). Interestingly, this effect was dependent upon *de novo* protein synthesis by live bacteria, as heat-killed or formalin-fixed bacteria did not elicit such a response. In similar studies, $TLR4^{-/-}$ dendritic cells were found to have an equivalent response compared to wild-type DCs, whereas this inflammatory response was completely abrogated in TLR2-deficient cells (Katz et al., 2006; Periasamy et al., 2011).

Importantly, purified LPS of *F. tularensis* LVS does not stimulate the production of TNF-α, IFN-γ, IL-12, IL-10, or nitric oxide by murine macrophages (Ancuta et al., 1996; Kieffer et al., 2003). It is therefore unlikely that *Francisella* LPS, which might be present in the fractions, was responsible for iNKT cell activation. However, several TLR2 ligands have been identified in LVS (Forestal et al., 2008; Parra et al., 2010; Thakran et al., 2008). Because our LVS lipid fractions could potentially be contaminated with one of these lipoproteins, I tested $Vα14^{tg}$ splenocytes with a synthetic TLR2 agonist. Such treatment resulted in robust IL-6, TNF-α, and IL-10 production, but contrary to that observed with α-GalCer or LVS

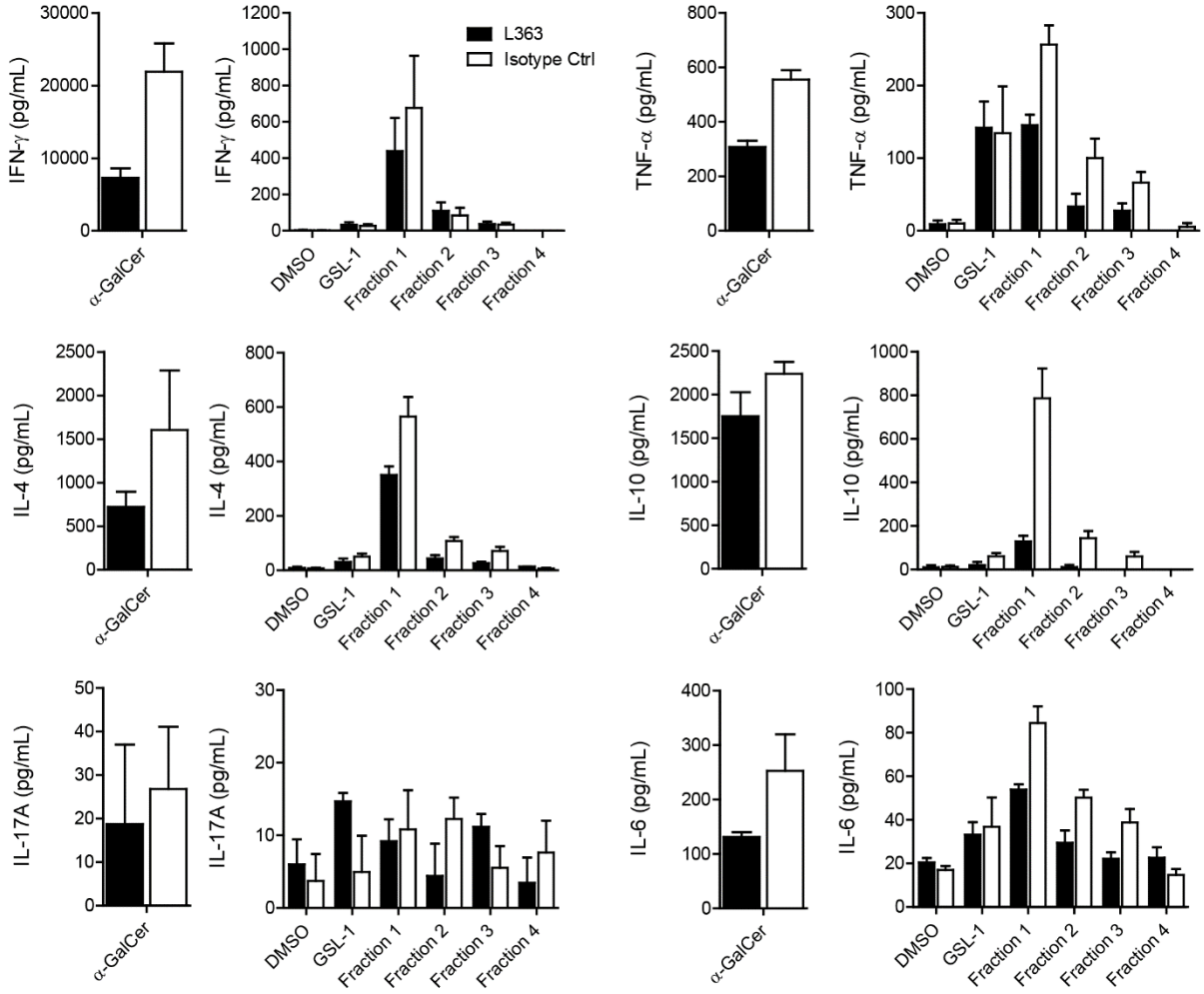


Figure 3-7. L363 antibody does not block iNKT cell activation by LVS lipids.

5 x 10^5 Vα14^{tg} splenocytes were pre-incubated with 40 µg/mL L363 (filled bars) or an isotype control antibody (open bars) and cultured for four days in the presence of 10 ng/mL α-GalCer, 1 µg/mL GSL-1, or 10 µg/mL LVS lipid fractions. Supernatants were assayed for the indicated cytokines by CBA. Bars are mean±SEM of three individual mice.

lipids, no IFN-γ or IL-4 was detected (Figs. 3-7 and 3-8). These results, taken together with the aforementioned studies, make it unlikely that the observed iNKT cell activation by LVS is driven by endogenous lipids.

In the context of a bacterial infection, iNKT cells can respond to both antigen-dependent and antigen-independent activation by recognizing either self- or microbial lipids (or both). Because splenic iNKT cells are capable of responding to multiple stimuli, I next sought to test the activity of the LVS lipid fractions by using iNKT cell hybridomas (Gui et al., 2001). I selected two such hybridomas which previous experience in our lab had shown do not recognize self-lipids presented by thymocytes. The mouse immature DC cell line JAWSII was used as APCs based on their high level of CD1d expression and ability to present α-GalCer and its derivatives [Fig. 3-9A and B; see also (Im et al., 2009)].

As indicated by incubation with JAWSII cells treated with DMSO, these hybridomas produced only modest amounts of IL-2 in response to endogenous lipids (Fig. 3-9C). Treatment with highly purified *E. coli* LPS also failed to induce a response. Conversely, robust IL-2 production was elicited by APCs loaded with α-GalCer or LVS lipids. As seen above with splenocyte cultures (Figs. 3-6 and 3-7), the highest activity was found in fraction 1. The CD1d blocking antibody 1B1 completely abrogated the response to LVS lipids, further confirming that this activation was due to the direct recognition of a lipid antigen contained within the fraction. The inability of 1B1 to block recognition of α-GalCer has been noted previously, and may be hybridoma-dependent (Roark et al., 1998; Yu et al., 2007).

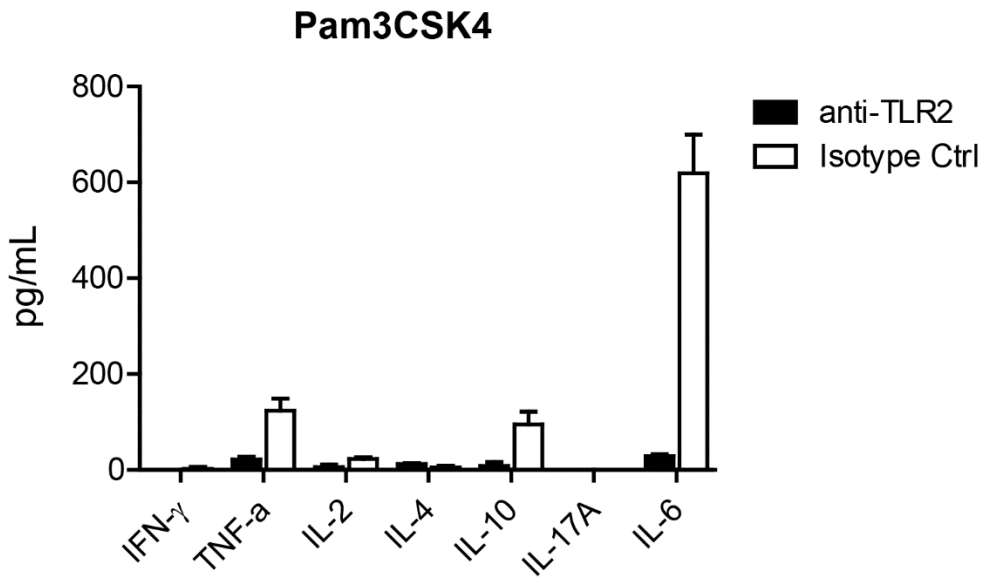


Figure 3-8. iNKT response is not mediated by TLR2 recognition of bacterial lipoproteins.

5 x 10^5 Vα14^{tg} splenocytes were stimulated with 100 ng/mL of the synthetic TLR2 ligand Pam3CSK4 in the presence of 20 µg/mL of TLR2 blocking antibody (filled bars) or an isotype control (open bars). Cytokine levels in the supernatant were measured by CBA after four days of culture. Bars are mean+SEM of three individual mice.

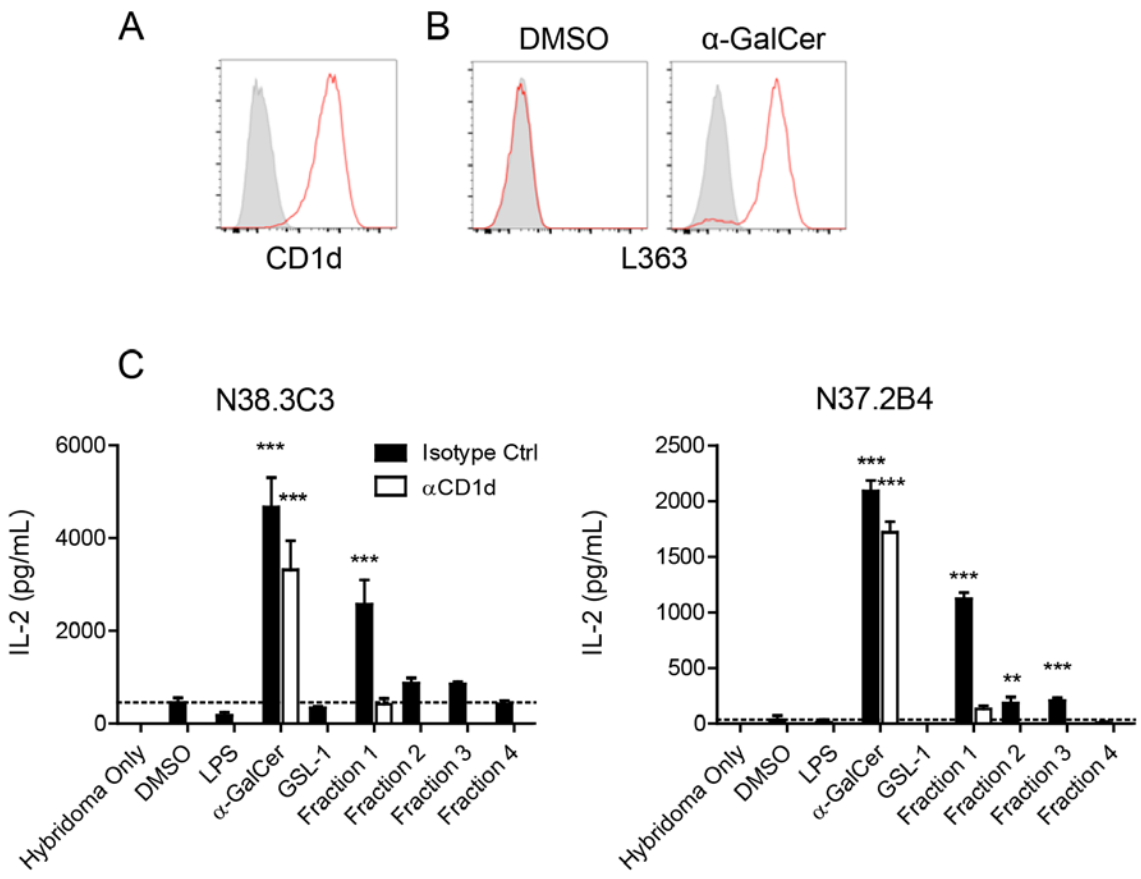


Figure 3-9. Recognition of LVS lipids by iNKT cell hybridomas is CD1d-dependent. **(A)** CD1d expression on JAWSII cells. Shaded histogram is isotype control. Red histogram is surface staining with 1B1 antibody. **(B)** JAWSII cells efficiently present α-GalCer as indicated by staining with L363 antibody on cells pulsed for 48 hours with DMSO (left) or α-GalCer (right). Histograms represented as in (A). **(C)** 5 x 10^4 JAWSII cells were pulsed overnight with 100 ng/mL α-GalCer, 500 ng/mL GSL-1, 100 ng/mL LPS, or 5 ug/mL LVS fractions. APCs were incubated briefly with 40 ug/mL 1B1 or an isotype control before adding 5 x 10^4 of the indicated hybridomas. After 20 hours, IL-2 concentrations in the supernatant were measured by ELISA. Bars are mean ± SD of triplicate wells. Results were compared by One-way ANOVA with Dunnett's Multiple Comparison Test. Significance was determined compared to DMSO control for each hybridoma.

In an attempt to gain further insight into the nature of the LVS-derived iNKT cell agonist lipid, I repeated these iNKT cell hybridoma stimulation assays in the presence or absence of the L363 antibody. Contrary to what was observed with splenocyte cultures (Fig. 3-7), the response of these hybridomas to α-GalCer or LVS lipids was completely abrogated by preincubation with L363 (Fig. 3-10). These results are likely due to the exquisite sensitivity of the monoclonal hybridoma population and the enhanced antigen presentation exhibited by JAWSII cells (Fig. 3-9), and further suggest that iNKT cell activation by *F. tularensis* is due to recognition of a previously unidentified bacterial glycosphingolipid (Yu et al., 2012).

Discussion

All mammalian species investigated thus far express at least one CD1 protein, suggested an important function for these molecules that is conserved across all mammals (Kasmar et al., 2009). Humans express both group 1 (CD1a, CD1b, and CD1c) and group 2 (CD1d) isoforms, whereas mice express only group 2 (Calabi et al., 1989; Hughes, 1991). CD1a, CD1c, and human CD1d are recycled from the plasma membrane to early endosomes, whereas CD1b and mouse CD1d show further trafficking into the endo/lysosomes (Cohen et al., 2009). It is through this recycling that cellular glycerophospholipids and sphingolipids loaded in the secretory pathway are exchanged for other self-lipids or microbial lipids found in these compartments (Dowds et al., 2014). This endo/lysosomal lipid exchange is facilitated by the low pH of these compartments and a variety of lipid processing and transfer proteins. The differing requirements for these factors on the loading of

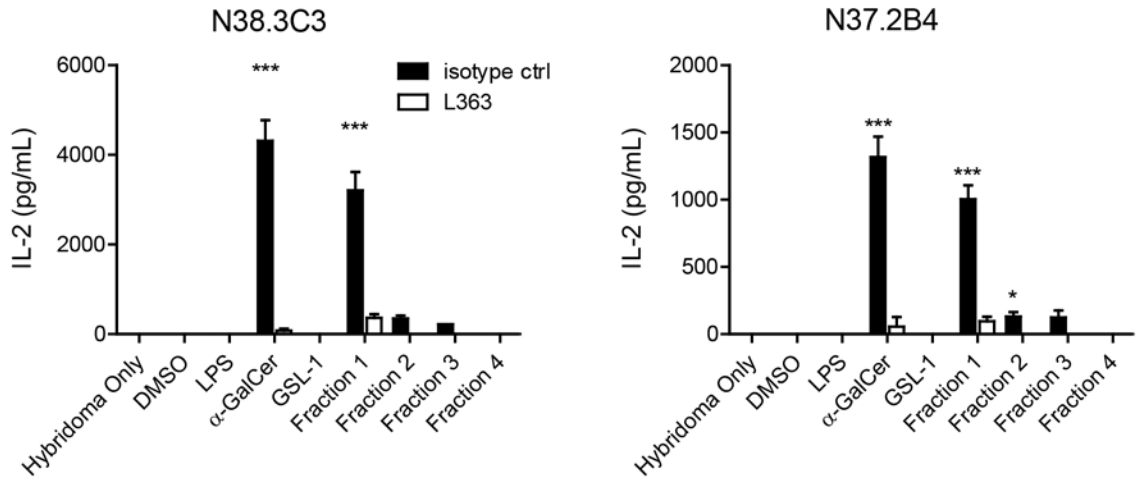


Figure 3-10. Recognition of LVS-derived lipid is antagonized by L363

5 x 10^4 JAWSII cells were pulsed overnight with 10 ng/mL a-GalCer, 10 ug/mL GSL-1, 100 ng/mL LPS, or 10 ug/mL LVS fractions. APCs were incubated briefly with 40 ug/mL L363 (anti-CD1d/α-GalCer) or an isotype control before adding 5 x 10^4 of the indicated hybridomas. After 20 hours, IL-2 concentrations in the supernatant were measured by ELISA. Bars are mean + SD of triplicate wells. Results were compared by One-way ANOVA with Dunnett's Multiple Comparison Test. Comparisons were made relative to DMSO control for each hybridoma.

purified α-GalCer variants is thought to be partly responsible for the apparent cytokine bias of various iNKT cell ligands (Im et al., 2009).

APC-free assays utilizing plate-bound CD1d to stimulate iNKT cell hybridomas have become a “gold standard” for conclusively proving the identity of an iNKT cell ligand. However, due to the sometimes complex cellular requirements for antigen processing and CD1d loading, such assays have proven difficult. I have thus far been unsuccessful in stimulating hybridomas using this method. Although α-GalCer (and to a lesser extent GSL-1) loads easily onto CD1d at neutral pH, the LVS fractions have shown no activity under these conditions. These results suggest either that the LVS-derived lipid(s) have a lower affinity for CD1d and therefore have more stringent loading requirements, or that the LVS lipid extracts contain a precursor form of the antigen that requires further processing by the endo/lysosomal machinery (Dowds et al., 2014).

All of the microbially-derived iNKT cell antigens identified to-date were isolated from extracellular pathogens (Table 1-3). The identification of an iNKT cell agonist lipid from the intracellular pathogen *F. tularensis* therefore represents a potentially novel mechanism of iNKT cell activation. The preliminary data described herein suggest that iNKT cells are robustly activated by a *Francisella*-derived GSL which is likely loaded onto CD1d in an intracellular compartment. The identification and characterization of this lipid promises to provide further insight into the mechanisms of iNKT cell activation during a natural infection. Given the demonstrated role for iNKT cells in promoting the sepsis-like condition associated with our model of experimental tularemia, the development of *F. tularensis* strains

deficient in the pathways required for synthesis of these lipids holds great promise in the development of improved vaccines.

CHAPTER IV

DISCUSSION AND FUTURE DIRECTIONS

In the studies described in Chapters II and III, I found that iNKT cells are activated by intranasal LVS infection and recruited to the lung interstitium during the innate phase of the immune response. These iNKT cells are likely activated by multiple mechanisms, but the production of the predominant cytokine detected, IFN-γ, is dependent on TCR-mediated recognition of a *Francisella*-derived lipid antigen. The pre-activation of iNKT cells via intranasal administration of α-GalCer elicits a local inflammatory response that protects mice from lethal infection, but in the absence of such intervention, the function of these lung-resident NKT cells is detrimental, resulting in an exacerbated systemic inflammatory response that characterizes the murine model of tularemia (Steiner et al., 2014). Accordingly, my experiments with α-GalCer treatment demonstrate that induction of inflammation prior to inoculation is protective against LVS infection (Fig. 2-19).

Interestingly, we found that iNKT cell-deficient mice had decreased susceptibility to a lethal intranasal dose of LVS that was distinguished by increased infiltration of lymphocytes into the infected lungs. These infiltrating cells organized into structures resembling iBALT which were indicative of a timelier and better orchestrated adaptive response that seemed to prognosticate recovery. The formation of such tertiary lymphoid structures was of particular interest because their formation had been previously undescribed in the context of a primary *Francisella* infection, and this represents the first report to suggest a role for iNKT

cells in inhibiting their formation. While these results are phenomenological in nature, they do indeed provide great potential for the future study of iNKT cells in the context of an acute lung infection and promise to provide new insight into their potentially deleterious functions.

Our data demonstrate that NKT cell-deficiency is a gain-of-function mutation that imparts increased resistance to intranasal LVS infection. This is relatively unique with regards to studies of resistance to pulmonary *F. tularensis* infections, with only two exceptions of which we are aware (Henry et al., 2010; Malik et al., 2007). However, rather than a single soluble factor, as was the case in these two examples, our data reveal a single cell type whose function(s) increase susceptibility. Because NKT cells have been demonstrated to produce a variety of soluble factors and exert multiple functions (Brennan et al., 2013; Coquet et al., 2008), elucidating the mechanism involved is a formidable task. There is a paucity of tools available by which to interrogate NKT cell function(s), particularly in the context of a complex disease condition such as tularemia. To investigate each possibility would require a herculean effort involving multiple cell transfers into recipients that are likely already immunocompromised, or neutralizing antibodies against factors that may also be produced by other cell types and that likely have already been shown to increase susceptibility. Any results obtained from such studies might therefore be misleading or inconclusive.

For example, one of the first cell types infected after intranasal LVS inoculation is alveolar macrophages (AM; (Hall et al., 2008). In a recent attempt to analyze the role of AMs in pulmonary tularemia, these cells were specifically

depleted by administration of diphtheria toxin (DT) to human DT receptor transgenic mice (Roberts et al., 2015). Mice depleted of AM were highly susceptible to intranasal LVS challenge, suggesting a protective role for these cells. Upon further analysis, however, the authors found that DT treatment induced systemic inflammation and altered both the cellular composition and cytokine milieu of the lungs, rendering conclusions about the function of AM invalid. Similar results (including increased neutrophilia and monocytosis) after specific cell depletion have been noted by other groups (Loubaki et al., 2013; Tittel et al., 2012).

Although the mechanism by which iNKT cells exert a detrimental/suppressive influence remains elusive, it is intriguing to postulate that this apparent suppressive effect might be mediated by the recently described subset of regulatory iNKT cells termed iNKT10 (Birkholz et al., 2015; Lynch et al., 2015b; Sag et al., 2014). Although these cells have thus far only been described in adipose tissue or after strong antigenic stimulation, their function in the lungs represents a potential explanation for the phenotype observed in $CD1d^{-/-}$ mice after intranasal LVS infection. Moreover, although the precise mechanisms driving the development of iBALT during acute infections have yet to be elucidated, it was recently shown that T_{regs} can inhibit its formation (Foo et al., 2015). Depletion of T_{regs} resulted in increased production of IL-17 which was previously shown to promote the formation of iBALT (Rangel-Moreno et al., 2011). Accordingly, my preliminary experiments using a sublethal dose of LVS have shown increased IL-17 transcript in $CD1d^{-/-}$ lungs d2 p.i. (Fig. 4-1). Aside from producing the immunoregulatory cytokine IL-10, iNKT10 cells are thought to regulate the function of T_{regs} in adipose tissue, in-part

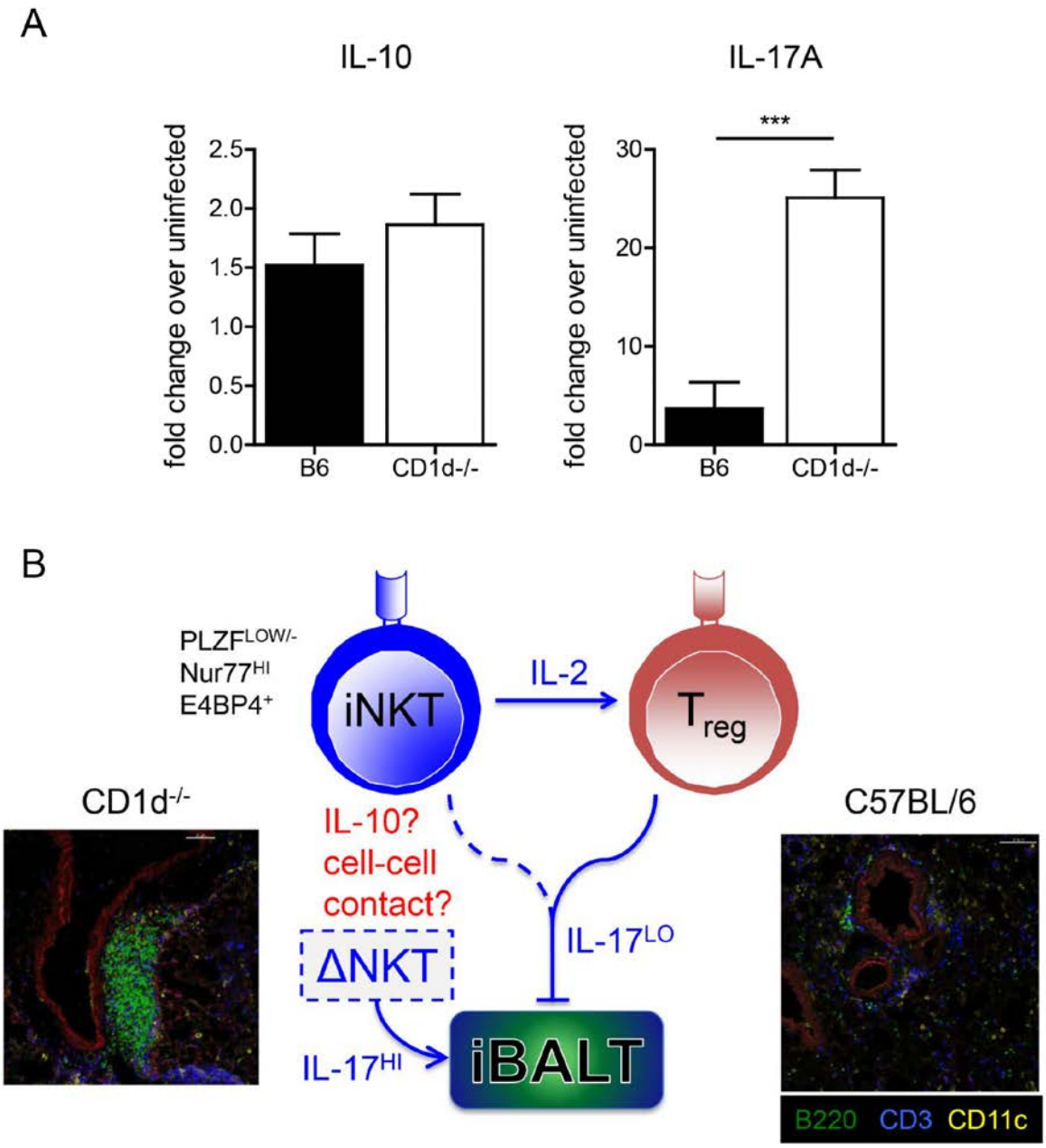


Figure 4-1. iNKT cells inhibit iBALT formation during acute *Francisella* infection

(A) Relative transcript levels of IL-10 and IL-17A in mouse lung d2 p.i.. Bares are mean+SD of four mice/group relative to uninfected controls. Results were compared by Mann-Whitney U test. $^{***}p<0.005$. **(B)** Proposed model of iNKT10 regulation of iBALT formation.

due to their production of IL-2 (Lynch et al., 2015b). They were also shown to directly induce the polarization of anti-inflammatory M2 macrophages. Whether they serve a similar function in the lungs would be of great interest. Future efforts will therefore be aimed at identifying the specific iNKT cell subsets activated and recruited to the lungs by intranasal LVS infection. These studies will require more sophisticated cell staining protocols utilizing a combination of transcription factor and cell surface markers to identify and track each subset (Table 1-2). One strategy might be to use mass cytometry to identify and measure the response of each subset in a single reaction. Because no reliable method exists to deplete individual subsets, or even iNKT cells as a whole, new methods will need to be developed to study their function both *in vitro* and *in vivo*.

Toward this end, I recently developed a testable model. I rederived a previously described mouse strain in which Nur77 is expressed under the control of the Lck proximal promoter (Calnan et al., 1995). Expression of this transgene results in increased negative selection and a complete loss of iNKT cells from the liver, spleen, and thymus (Gordy, 2012). Introgression of the Vα14 transgene restored iNKT cells to wild-type numbers. Nonetheless, I found that these cells were unresponsive to α-GalCer as assessed by IFN-γ and IL-4 production. Subsequent analysis revealed an immature phenotype marked by reduced expression of PLZF. Further studies are required, but the $Nur77^{high}PLZF^{low/-}$ population that predominates in $Nur77^{tg}$;$Vα14^{tg}$ mice resembles the phenotype of adipose tissue-resident iNKT10 cells recently described by Lynch and colleagues (Lynch et al., 2015b). Whether they produce IL-2 and IL-10 remains to be determined, but this

new mouse strain holds potential to be an optimal model by which to study this particular iNKT cell subset *in vivo*.

In an attempt to identify the mechanism by which iNKT cells are activated in response to *Francisella* infection, we utilized a recently described mouse model (Moran et al., 2011) to demonstrate this activation was largely due to TCR recognition of an antigenic lipid. Subsequent experiments using APCs loaded with LVS lipid fractions to stimulate iNKT cell hybridomas support the position that the antigenic lipid is of bacterial origin. Similar experiments with splenocyte cultures revealed diverse cytokine production in response to these lipid fractions alone, further supporting a role for iNKT cells in this response. It is worth noting that previous studies of iNKT cell activation (to include mine) have potentially oversimplified the issue by considering iNKT cells as a relatively homogenous population. More recent data has begun to reveal that distinct subsets exist that might recognize antigens with different affinities or respond to distinct cytokine signals based on differential receptor expression. Based on the presumed tissue localization and distinct phenotype of these iNKT subsets, they should be evaluated independently in order to analyze their function. Thus, further studies designed to identify and characterize the *Francisella*-derived antigen will need to consider not only how it is trafficked and presented by APCs, but which iNKT subsets recognize it and how they respond. These types of questions are likely not best addressed using the prototypical antigen α-GalCer, since it seems to robustly activate most iNKT cells, regardless of the subset to which they belong (Coquet et al., 2008).

Upon contact with macrophages, *F. tularensis* binds to one of several surface receptors and becomes engulfed via asymmetrical membrane protrusions known as pseudopod loops (Clemens et al., 2005). Upon entry into the cell, *Francisella* traffics to early phagosomes known as *Francisella*-containing phagosomes (FCPs). The phagosome is a compartment where the host employs multiple antimicrobial defenses including phagosomal acidification, reactive oxygen species, and antimicrobial peptides. Consequently, *Francisella* has evolved numerous mechanisms to subvert these defenses, the last step of which is escape from the phagosome to reach the cytosol where it can replicate (Jones et al., 2012). Importantly, it has been shown that these FCPs do not contain the endo/lysosomal markers CD63, LAMP-1, or LAMP-2, which are markers of phagosomal maturation (Clemens et al., 2005, 2009), suggesting that the initial entry of live *Francisella* into the cell may not traffic the bacteria far enough along the endocytic pathway for bacterial lipids to be available for loading onto CD1d (Im et al., 2009). This leads to several additional outstanding questions:

1) How are these *Francisella* lipids delivered to the lysosome where the CD1d loading machinery resides?
2) Is the iNKT cell antigen contained within the cell wall of *Francisella*, or does it require further processing by APCs?
3) What additional stimuli are required for iNKT cell activation in response to *Francisella* lipids?
4) Is/are the agonist lipid(s) present in the LVS fractions also produced by other *F. tularensis* subspecies (*tularensis* and *novicida*)?

CD1d surface expression by APCs can be regulated by multiple signals including IFN-γ, type I IFN, and bacterial infection (Raghuraman et al., 2006; Skold et al., 2005). Infection of macrophages with *M. tuberculosis* was shown to increase surface expression of CD1d in infected cells (Sille et al., 2011b). Interestingly, the same degree of CD1d upregulation is not observed in $Ii^{-/-}$ or cathepsin S (CatS)-deficient cells. In this system, Ii expression by the infected macrophages was critical for iNKT cell activation and control of intracellular *M. tuberculosis* growth (Sada-Ovalle et al., 2008). This defect was due to defective antigen loading and/or trafficking of CD1d in the absence of Ii. Consistent with this, α-galactosyl(α1-2)galactosylceramide (GGC) is a lipid that requires internalization and proteolytic removal of the terminal sugars in endo/lysosomal compartments for CD1d-mediated recognition by iNKT cells (Prigozy et al., 2001). The processing of GGC is mediated by the lysosomal enzyme α-galactosidase A (α-Gal-A). Akin to defective presentation of mycobacterial antigens, $Ii^{-/-}$ macrophages are also defective in the processing and presentation of this α-GalCer precursor. This is perhaps not surprising since Ii has also been shown to be important for both endosomal transport and in the presentation of peptide antigens to conventional T cells (Boes et al., 2005; Gregers et al., 2003; Nordeng et al., 2002).

The above data further highlight the importance of an intact cellular lipid loading and processing machinery for the activation of iNKT cells, and suggest that the early escape of *Francisella* from phagosomes may serve to limit the availability of bacterial lipids for processing and loading onto CD1d. However, following replication in the cytosol, *Francisella* enters autophagosomes which subsequently

fuse with lysosomes (Checroun et al., 2006; Cremer et al., 2009). Although the significance of this is not well understood, it has been suggested that this process is initiated by the bacterium and that it may be a strategy evolved to acquire nutrients from the infected cell (Steele et al., 2013), or as a route of escape (Jones et al., 2012). Regardless, it is likely that this fusion of the *Francisella*-containing autophagosome with lysosomes may be required for CD1d loading to occur. Although autophagy has not been shown to be required for the presentation of endogenous or exogenous antigens to iNKT cells (Salio et al., 2014), the autophagosomes induced by *Francisella* are part of a noncanonical Atg5-independent pathway (Steele et al., 2013), and therefore potentially represent a previously unappreciated mechanism of CD1d-dependent antigen presentation.

Although my data support the idea that iNKT cells recognize an LVS-derived lipid, this point is difficult to prove conclusively with the currently available reagents. However, previous studies of CD1d trafficking and antigen presentation provide somewhat of a roadmap to point the way forward. The greatest challenge to these types of studies lies in distinguishing activation due to endogenous versus exogenous lipids (Brigl et al., 2011). α-Gal-A was shown to be involved in the negative regulation of self-lipids after TLR-mediated activation of APCs (Darmoise et al., 2010; Van Kaer and Joyce, 2010). In this study, the authors found that APCs deficient for this enzyme spontaneously activated iNKT cells and hybridomas via increased presentation of endogenous lipids. This was a critical study supporting the idea that the inherent autoreactivity of iNKT cells is controlled by catabolic enzymes (including α-Gal-A) which, under steady-state conditions, degrade self-

lipids that would otherwise be available for presentation to iNKT cells. Upon TLR4 stimulation, α-Gal-A activity was significantly reduced, and these activated APCs were able to in-turn activate iNKT cells without the addition of exogenous lipids. More recently, this finding was extended in a report that surprisingly identified the endogenous iNKT cell antigens as α-glycosylceramides (Kain et al., 2014). These data extended the findings of Darmoise et al. to suggest a two-step mechanism whereby endogenous α-glycosylceramides are degraded by the sequential activity of acid ceramidase 1 (ASAH1), which removes the acyl chain, followed by α-Gal-A mediated cleavage, which then removes the sugar.

To further complicate the issue, iNKT cell activation by these endogenous α-glycosylceramides are blocked by the addition of the L363 antibody, which I used in my study as well. This therefore makes it difficult to distinguish between an exogenous lipid that may be recognized by this antibody (Yu et al., 2012), and endogenous α-GlcCer or α-GalCer that might be presented after TLR ligation. The L363 antibody binds CD1d/α-GalCer complexes in a manner very similar to the iNKT cell TCR (Yu et al., 2012) by binding to both CD1d and the α-linked sugar. Modeling studies performed by Kain et al. (Kain et al., 2014) suggest that L363 should bind an α-linked galactose better than glucose, which was supported by inhibition assays using thymocytes believed to present α-GluCer compared to dendritic cells shown to present α-GalCer.

It should be noted that the experiments by Kain et al. (Kain et al., 2014) relied exclusively on the autoreactive iNKT cell hybridoma DN32.D3 which was robustly activated by RBL cells transfected with mCD1d, by freshly isolated

thymocytes, and by LPS-treated DC3.2 cells (Shen et al., 1997). This activation was completely abrogated by the addition of L363. Similar results were obtained by culturing iNKT cell lines with BMDCs. Contrary to these findings, I found that neither DN32.D3 nor the other Vα14 hybridomas tested (Figs. 3-9B and 3-10) were activated by JAWSII cells, whether or not these APCs were previously activated by LPS, suggesting that JAWSII cells either express a different complement of endogenous glycolipid antigens, or that higher concentrations of LPS must be used to stimulate these APCs. However, when pulsed with LVS lipid fractions, JAWSII cells were able to robustly activate DN32.D3 hybridomas, with resulting IL-2 concentrations greater than five-fold higher than those observed with hybridomas N38.3C3 or N37.2B4, suggesting that the DN32.D3 hybridoma is hyper-responsive to CD1d/self-lipid complexes yet still does not recognize an endogenous lipid presented by JAWSII.

The inability of JAWSII cells to activate iNKT cell hybridomas in the absence of exogenous antigen is intriguing considering their constitutively high CD1d expression (Fig. 3-9A). However, these cells were previously used in hybridoma stimulation assays to detect activation by α-GalCer analogs (Im et al., 2009), which is consistent with my findings that these cells do not readily activate iNKT hybridomas via endogenous lipids. However, future experiments should include the RBL-CD1d cells used by Kain and colleagues (Kain et al., 2014) to ensure that these results are not specific to the APC being used.

Taking these factors into consideration, in future studies it will be important to determine whether infection with *Francisella* LVS affects the activities of the

degradative enzymes that regulate the presentation of endogenous lipids. This can be accomplished by using the α-Gal-A-specific fluorogenic substrate 4-methylunbelliferyl-α-D-galactopyranoside as described (Darmoise et al., 2010), because a reduction in α-Gal-A activity would be indicative of increased presentation of endogenous ligands. Additionally, generation of MyD88-deficient JAWSII and/or RBL-CD1d cells can be used to further preclude the possibility that the activation observed in response to LVS lipids is due to increased presentation of endogenous lipids.

Efforts are currently underway to further purify the LVS lipid fractions in order to identify the iNKT cell ligand. It should be noted that lipids with a low affinity for CD1d may be difficult to load in a cell-free system or on surface-expressed CD1d, as the low pH and lipid loading machinery of the lysosome may be required. As stated in Chapter III, in initial attempts to load LVS lipid fractions onto CD1d, I was unable to demonstrate the activation of iNKT cell hybridomas. However, there was no way to ensure that the lipid(s) were actually binding to CD1d, since the positive control (α-GalCer) is readily loaded onto CD1d at neutral pH (Benlagha et al., 2000; Matsuda et al., 2000). The efficient loading of CD1 molecules with lipids such as α-GalCer involves lysosomal cofactors that function to enhance the transfer of lipids from micelles or membranes into the antigen binding groove of CD1d. The cofactors described thus far are resident intracellular lipid binding and transfer proteins such as saposins, GM2 activator proteins, and Niemann-Pick type C1 and C2 proteins (Sagiv et al., 2007; Schrantz et al., 2007; Yuan et al., 2007; Zhou et al., 2004a). Most of these cofactors have detergent-like activities that facilitate solubilization of

lipids and their exchange between hydrophobic binding sites. In a recent report, Kain et al. (Kain et al., 2014) were able to circumvent this problem by performing CD1d loading at low pH in the presence of recombinant saposin B. Similarly, Im et al. (Im et al., 2009) found that the addition of a detergent facilitated the loading of GSLs containing longer fatty acid chains onto CD1d. Depending on the structure of the activating lipid(s) contained within the LVS fractions, such strategies may need to be employed to perform APC-free hybridoma assays or tetramer staining which would prove conclusively that iNKT cells are responding to a *Francisella*-derived lipid. However, if the putative iNKT cell antigen contained within the fractions requires further processing before being loaded onto CD1d, these assays would be unsuccessful.

Finally, to address the question as to whether or not this lipid(s) is/are also synthesized by the more virulent type A *Francisella* spp., lipids will need to be purified from *F. tularensis* spp. *tularensis* as well. Toward this end, we have recently established a collaboration with a well-known researcher who has published extensively on the pathogenesis of *Francisella*, to include the highly virulent human strain Schu S4. This collaborator has agreed to provide Schu S4 for lipid extraction according to our protocols. If indeed the lipid is identified in both *Francisella tularensis* subspecies, it will be important to demonstrate activation of iNKT cells derived from human PBMCs (Webb et al., 2009)

CHAPTER V

MATERIALS AND METHODS

Ethics Statement

All procedures on mice were performed by approval from the IACUC at Vanderbilt University School of Medicine and at Albany Medical College. Anesthesia was performed using 1—5% isoflurane or 100 mg/kg and 10 mg/kg ketamine and xylazine, respectively. Euthanasia was performed by CO_2 overdose followed by cervical dislocation.

Mice

C57BL/6J mice and Vα14^{tg} mice (C57BL/6-Tg(Cd4-TcraDN32D3)1Aben/J) were purchased from the Jackson Laboratory (Bar Harbor). CD1d$^{-/-}$ mice were a gift from L Van Kaer (Vanderbilt University School of Medicine). Jα18$^{-/-}$ mice were generously provided by M Taniguchi (RIKEN Institute, Yokohama, Japan). PD-1$^{-/-}$ and PD-L1$^{-/-}$ mice were a kind gift from Dr. John Williams (Vanderbilt University School of Medicine). Mice were bred and maintained in the School of Medicine vivarium and provided with food and water ad libitum. LVS infection was performed in an ABSL-2 facility. Vanderbilt's IACUC approved the experiments described here.

Bacterial infection

F. tularensis LVS was provided by S. Khader (Washington University, St. Louis, MO). Preparation of working stocks and CFU determination from infected tissue were performed as described (Conlan et al., 2011). LD_{50} was determined by the method of Reed and Muench (Reed, 1938). Male, 8–12-week old mice were anesthetized by i.p. administration of a ketamine/xylazine mixture and ~8–10x10^3 CFU LVS were administered i.n. in 50μL sterile PBS. Mice were monitored daily for weight loss and signs of morbidity. Criteria for clinical score were developed based upon observation of mice from at least three separate experiments: 1) no outward signs of illness; 2) consistently ruffled fur; 3) hunched back and altered gait; 4) reduced mobility/reaction to stimulus, labored breathing, lethargy. Mice were humanely euthanized when weight loss exceeded 30 percent.

Experiments with Schu S4 were conducted with approval of the Albany Medical College IACUC (protocols 15-04001 and 15-04002). Six-to-eight week old BALB/c and C.129-$CD1d^{-/-}$ mice were inoculated i.n. with the indicated dose of Schu S4 as described above for LVS.

Cytokine measurements

Cytokines were measured in serum or lung homogenates using Cytokine Bead Array assay (BD Biosciences). Right lobes of lung were homogenized using a Tissue Tearor (Biospec Products, Inc.) in 1 mL sterile PBS containing a cocktail of protease inhibitors (Roche). Homogenates were centrifuged and supernatants frozen at -80°C until analyzed.

Pulse Oximetry

SpO_2 was measured using the PhysioSuite with MouseSTAT Pulse Oximeter (Kent Scientific Corp.). Mice were anesthetized using 1.5% isoflurane for induction and maintenance. Sensor was attached to the right hind foot according to the manufacturer's instructions. Measurements were recorded at 5-second intervals for at least 4 minutes and averaged.

Tissue processing

Spleen, liver, and lungs were processed as previously described (Bezbradica et al., 2006; Scanlon et al., 2011). Left lung lobes were used for flow cytometry and microscopy and right lobes for CFU determination.

Flow Cytometry

NKT cells were analyzed as described (Bezbradica et al., 2006). All data were acquired on an LSR II (BD Biosciences) and analyzed with FlowJo software (FlowJo, LLC). Cell populations were identified as follows: B cells ($CD19^+$); T cells ($CD3\varepsilon^+$); NK cells ($CD3\varepsilon^-NK1.1^+$); NKT cells ($CD3\varepsilon^+$CD1d/αGC tetramer$^+$); DCs ($CD45^+CD64^-CD24^+MHCII^+CD11c^+$). DC subsets were identified as described (Misharin et al., 2013). CD1d monomers were provided by the NIH Tetramer Core Facility. Cell counts were determined using AccuCheck counting beads (Invitrogen). A list of antibodies used is provided in Table 5-1.

Immunofluorescence and Confocal microscopy

To analyze the formation of tertiary lymphoid follicles in the lungs following LVS infection, tissues were fixed in PLP buffer (2% paraformaldehyde, 0.05 M phosphate buffer containing 0.1 M L-lysine, and 2 mg/ml $NaIO_4$) overnight. Tissues were then dehydrated in 30% sucrose and subsequently embedded in OCT media. Twenty micron frozen sections were preincubated with Fc-block (anti-mouse CD16/32 Ab; Biolegend) diluted in PBS containing 2% goat serum and fetal bovine serum (FBS). After incubation for 1 hour at room temperature, sections were washed with PBS and stained with the following antibodies diluted in PBS containing 2% goat serum and FBS for 1 hour at room temperature: anti-B220-Alexa-488 (Biolegend), anti-CD3-allophycocyanin, anti-CD11c-phycoerythrin (Biolegend) and Fc-block. Sections were washed with PBS and mounted using Immu-Mount (Thermo Fisher Scientific). Images were acquired using a Zeiss 780 confocal microscope (Carl Zeiss). The imaging data were processed and analyzed using the Imaris software (Bitplane; Oxford Instruments).

Statistical Analysis

Statistical analyses of representative data were performed using GraphPad Prism version 5.02 for Windows, GraphPad Software, San Diego California USA (www.graphpad.com). Where indicated in the figure legends, data were aggregated across several (from two to four) replicate experiments. To address the clustered nature of the final datasets, we used a linear regression analysis with cluster robust standard error estimation [an extension of Huber-White Sandwich Estimator (Freedman, 2006; Huber, 1967; White, 1980)], a method that accounts for intraclass

correlation when determining statistical significance of regression coefficients. In all reported regression analyses individual data points were entered as the dependent variable, and replicate experiments were identified as the clustering variable. Experimental group type (KO and WT) was entered as the independent variable and was dummy coded for the analyses with "KO" group as the reference category. In such case, the intercept reflects the mean of the reference group (=KO), and the slope reflects the difference between the KO and the WT groups. We have reported herein the significance of coefficients for slopes (differences between group means). Clinical score data was analyzed using Generalized Estimating Equation (GEE) model based on Poisson regression. In the model, clinical score was predicted by group type and day of assessment (dummy coded), and their interaction. We report comparisons between groups at each time of assessment, which were obtained by changing the reference category between days of assessment. All analyses were carried out using R Project for Statistical Computing (http://www.r-project.org/).

Isolation of LVS Lipids

Bacteria was plated on modified MH-agar plates (Conlan et al., 2011) to obtain a lawn, and incubated at 37°C for 48 hours. Each plate was flushed with 10 mL of PBS and glass beads (bacteria easily came off the agar). Bacterial suspension was transferred to 50 mL conical tubes followed by centrifugation for 30 min at 4000 rpm (+4°C). Pellet was carefully resuspended in 45 mL of PBS to reduce contaminants from growth medium, followed by centrifugation. Obtained pellet (~3 g of wet weight, ~1 ml of PBS left to reduce losses of the material) was resuspended in 25

mL of 100% ethanol and stored at -80°C. Lipid fractions were prepared as previously described (Albacker et al., 2013).

Transwell experiments

Bone marrow derived macrophages (BMM) were generated as previously described (Manzanero, 2012; Weischenfeldt and Porse, 2008). Briefly, 2 x 10^5 bone marrow cells were seeded into each bottom chamber of a 24-well HTS transwell plate (Costar). Macrophages were differentiated in DMEM supplemented with 10% L929 supernatant. Media was replaced on days 3 and 6 of culture. On day 7, BMM were infected with LVS at an MOI of 50 as described (Elkins et al., 2001). 1.5 x 10^6 B6 splenocytes were added to each well (co-culture) or to the upper chamber separated by a 0.4 μm membrane (transwell) and cultured for 24 hours. Cells were harvested and analyzed as described.

NKT cell hybridoma assays

NKT cell hybridomas were grown in Opti-MEM I media (Gibco 11058-021) supplemented with 4% FBS, 100 U/mL pen/strep, and 50 μM 2-ME and maintained at 5% CO_2, 37ºC. Prior to using for experiments, cells were sorted based on CD3ε expression. For *in vitro* stimulation, 5 x 10^4 hybridomas were added to 5 x 10^4 lipid-loaded JAWSII cells and incubated for 18-20 hours. Supernatants were harvested and tested for IL-2 by ELISA.

IL-2 ELISA

NUNC Maxisorp plates were coated overnight at 4ºC with 2 μg/mL IL-2 capture antibody (BD 554424) in 0.1 M carbonate coating buffer (100 μL/well). Plates

were washed x4 with wash buffer (PBS + 0.1% TWEEN-20) and blocked with 300 μL/well blocking buffer consisting of 0.1% PBST + 1% BSA (Sigma A2934) for 1 hr at room temperature. Plates were washed x4 and incubated with 100 μL sample (diluted in blocking buffer) for 2 hr at room temperature. Plates were washed x5 and incubated with 1 μg/mL (100 μL volume/well) detection antibody (BD 554426) for 1 hr at room temperature. Plates were washed x5 and incubated with 100 μL/well Avidin-Peroxidase at a 1:400 dilution = 2.5 μg/mL (Sigma A3151) for 30 min at room temperature. Plates were washed x5 and incubated with 100 μL/well room temperature 1-Step Ultra TMB-ELISA substrate (Thermo 34029) for 10 min at room temperature. Reaction was stopped by addition of 50μL 2N H_2SO_4. Absorbance was read on a BioTek Synergy HTX Plate Reader.

Quantitative PCR

Day 2 p.i., right lobes of lungs were harvested into 1 mL RNAlater (Qiagen) and kept at 4ºC overnight. Lung tissue was homogenized in 1 mL TRIzol (Life Technologies)/mg tissue using a Tissue Tearor (Biospec Products, Inc.) according to the manufacturer's instructions. RNA was isolated using an RNeasy Mini Kit (Qiagen) and reverse transcribed using the iScript cDNA Synthesis Kit (BioRad). qPCR reaction was performed on a BioRad IQ5 thermal cycler using SsoFast EvaGreen Supermix and analyzed using the included software. Results were normalized based on the geometric mean Ct values of β-actin and GAPDH for each sample. A list of primers used is provided in Table 5-2.

Cell culture

The murine immature DC cell line JAWSII was purchased from ATCC. JAWSII cells were maintained in αMEM medium (Gibco 12571-063) supplemented with 20% fetal calf serum, 1 mM sodium pyruvate, and 5 ng/mL rmGM-CSF (Tonbo Bioscience). Adherent cells were harvested using Accutase (Innovative Cell Technologies). Splenocyte cultures were maintained in RPMI supplemented with 10% fetal calf serum, 100 U/mL pen/strep, 50 μg/mL gentamicin sulfate, and 50 μM 2-ME.

Table 5-1. List of antibodies used

Antigen	Clone	Fluor	Supplier
B220	RA3-6B2	FITC, PerCP, Pacific Blue	BD Pharmingen
CD11b	M1/70	APC, PerCP-Cy5.5, violetFluor450	BD Pharmingen, Tonbo Bioscience
CD11c	HL3	FITC, APC, PE	BD Pharmingen
CD11c	N418	violetFluor450	Tonbo Bioscience
CD120a (TNFR1)	55R-286	PE	BioLegend
CD120b (TNFR2)	TR75-89	PE	BioLegend
CD134 (OX-40)	OX-86	PE	BioLegend
CD178 (PD-L1)	MFL3	PE	BioLegend
CD19	1D3	violetFluor450, APC-Cy7, FITC	Tonbo Bioscience, BioLegend
CD1d	1B1	Purified, PE	BD Pharmingen
CD1d/α-GalCer	L363	Purified, PE	BioLegend
CD24	M1/69	FITC, PE-Cy7	BD Pharmingen
CD282 (TLR2)	T2.5	Purified	BioLegend
CD357 (GITR)	DTA-1	PE	BioLegend
CD3ε	145-2C11	PerCP-Cy5.5, APC, BV421	BD Pharmingen, BioLegend
CD4	RM4-5	PE, PE-Cy7, FITC	BD Pharmingen
CD4 (depletion)	GK1.5	Purified	BioXCell
CD44	IM7	APC-Cy7	BD Pharmingen
CD69	H1.2F3	PerCP-Cy5.5, PE-Cy7, APC-Cy7	BD Pharmingen
CD80	16-10A1	PE	BD Pharmingen
CD86	GL1	PE	BD Pharmingen
CD86	GL1	PE	BD Pharmingen
CD8α	53-6.7	APC, APC-Cy7	eBioscience

CD8α (depletion)	53-6.72	Purified	BioXCell
CD95 (FAS)	15A7	PE	eBioscience
Egr2	erongr2	PE	eBioscience
F4/80	BM8	APC, BV421	eBioscience, BioLegend
Foxp3	FJK-16S	PE	eBioscience
Gata-3	TWAJ	PE	eBioscience
GM-CSF	MP1-22E9	PE	BioLegend
Gr1	RB6-8C5	APC, APC-Cy7	BD Pharmingen
IFN-γ	XMG1.2	PE, PE-Cy7	BD Pharmingen, PE-Cy7
IL-10	JES5-16E3	PE-Cy7	BioLegend
IL-13	eBio13A	PE	eBioscience
IL-17A	TC11-18H10.1	PE, PE-Cy7	BD Pharmingen, BioLegend
IL-22	Poly5164	PE	BioLegend
IL-4	11B11	PE, PE-Cy7	BD Pharmingen, BioLegend
IL-6	MP5-20F3	PE	BD Pharmingen
Ly49A	A1	FITC	BD Pharmingen
Ly49C/I	5E6	FITC	BD Pharmingen
Ly49D	4E5	FITC	BD Pharmingen
Ly49G2	4D11	FITC	BD Pharmingen
NK1.1	PK136	PE, PE-Cy7, PerCP-Cy5.5	BD Pharmingen
NKG2A/C/E	CX5	FITC	BD Pharmingen
Nur77	12.14	PE	eBioscience
PD-L1 (blocking)	10F.9G2	Purified	BioXCell
PLZF	Mags.21F7	PE	eBioscience
RORγ	AFKJS-9	PE	eBioscience
T-bet	eBio4B10	PE	eBioscience
TCRβ	H57-597	FITC	BD Pharmingen
TNF-α	MP6-XT22	PE	BD Pharmingen

Table 5-2. List of Primers Used in qPCR

Target	Fwd Primer	Rev Primer
IL-10	ATAACTGCACCCACTTCCCA	GGGCATCACTTCTACCAGGT
IL-17A	ACTCTCCACCGCAATGAAGA	CTCTCAGGCTCCCTCTTCAG
B-actin	CCTCTATGCCAACACAGTGC	CCTGCTTGCTGATCCACATC
GAPDH	CTGCGACTTCAACAGCAACT	GAGTTGGGATAGGGCCTCTC

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APPENDIX

LIST OF PUBLICATIONS

1. **Hill, TM**, Bezbradica, JS, Van Kaer, L, and JOYCE, S. A21653: CD1d-restricted natural killer T cells. Encyclopedia of Life Sciences, eLS: A21653, in press 18 September, 2015.

2. **Hill, T.M.**, Gilchuk, P., Cicek, B.B., Osina, M.A., Boyd, K.L., Durrant, D.M., Metzger, D.W., Khanna, K.M., and Joyce, S. (**2015**). Border Patrol Gone Awry: Lung NKT Cell Activation by Francisella tularensis Exacerbates Tularemia-Like Disease. PLoS Pathog *11*, e1004975.

3. Gilchuk, P., **Hill, T.M.**, Wilson, J.T., and Joyce, S. (**2015**). Discovering protective CD8 T cell epitopes-no single immunologic property predicts it! Curr Opin Immunol *34*, 43-51.

4. Gilchuk, P., Spencer, C.T., Conant, S.B., **Hill, T.**, Gray, J.J., Niu, X., Zheng, M., Erickson, J.J., Boyd, K.L., McAfee, K.J., et al. (**2013**). Discovering naturally processed antigenic determinants that confer protective T cell immunity. J Clin Invest 123, 1976-1987.

5. Dragovic, S.M., **Hill, T.**, Christianson, G.J., Kim, S., Elliott, T., Scott, D., Roopenian, D.C., Van Kaer, L., and Joyce, S. (**2011**). Proteasomes, TAP, and endoplasmic reticulum-associated aminopeptidase associated with antigen processing control CD4+ Th cell responses by regulating indirect presentation of MHC class II-restricted cytoplasmic antigens. J Immunol *186*, 6683-6692.

6. Yan, J., Parekh, V.V., Mendez-Fernandez, Y., Olivares-Villagomez, D., Dragovic, S., **Hill, T.**, Roopenian, D.C., Joyce, S., and Van Kaer, L. (**2006**). In vivo role of ER-associated peptidase activity in tailoring peptides for presentation by MHC class Ia and class Ib molecules. J Exp Med *203*, 647-659.

7. Bezbradica, J.S., Gordy, L.E., Stanic, A.K., Dragovic, S., **Hill, T.**, Hawiger, J., Unutmaz, D., Van Kaer, L., and Joyce, S. (**2006**). Granulocyte-macrophage colony-stimulating factor regulates effector differentiation of invariant natural killer T cells during thymic ontogeny. Immunity *25*, 487-497.

8. Bezbradica, J.S., **Hill, T.**, Stanic, A.K., Van Kaer, L., and Joyce, S. (**2005**). Commitment toward the natural T (iNKT) cell lineage occurs at the CD4+8+ stage of thymic ontogeny. Proc Natl Acad Sci U S A *102*, 5114-5119.

PLOS | PATHOGENS

RESEARCH ARTICLE

Border Patrol Gone Awry: Lung NKT Cell Activation by *Francisella tularensis* Exacerbates Tularemia-Like Disease

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Abstract

The respiratory mucosa is a major site for pathogen invasion and, hence, a site requiring constant immune surveillance. The type I, semi-invariant natural killer T (NKT) cells are enriched within the lung vasculature. Despite optimal positioning, the role of NKT cells in respiratory infectious diseases remains poorly understood. Hence, we assessed their function in a murine model of pulmonary tularemia—because tularemia is a sepsis-like proinflammatory disease and NKT cells are known to control the cellular and humoral responses underlying sepsis. Here we show for the first time that respiratory infection with *Francisella tularensis* live vaccine strain resulted in rapid accumulation of NKT cells within the lung interstitium. Activated NKT cells produced interferon-γ and promoted both local and systemic proinflammatory responses. Consistent with these results, NKT cell-deficient mice showed reduced inflammatory cytokine and chemokine response yet they survived the infection better than their wild type counterparts. Strikingly, NKT cell-deficient mice had increased lymphocytic infiltration in the lungs that organized into tertiary lymphoid structures resembling induced bronchus-associated lymphoid tissue (iBALT) at the peak of infection. Thus, NKT cell activation by *F. tularensis* infection hampers iBALT formation and promotes a systemic proinflammatory response, which exacerbates severe pulmonary tularemia-like disease in mice.

Author Summary

NKT cells are innate-like lymphocytes with a demonstrated role in a wide range of diseases. Often cited for their ability to rapidly produce a variety of cytokines upon activation, they

have long been appreciated for their ability to "jump-start" the immune system and to shape the quality of both the innate and adaptive response. This understanding of their function has been deduced from *in vitro* experiments or through the *in vivo* administration of highly potent, chemically synthesized lipid ligands, which may not necessarily reflect a physiologically relevant response as observed in a natural infection. Using a mouse model of pulmonary tularemia, we report that intranasal infection with the live vaccine strain of *F. tularensis* rapidly activates NKT cells and promotes systemic inflammation, increased tissue damage, and a dysregulated immune response resulting in increased morbidity and mortality in infected mice. Our data highlight the detrimental effects of NKT cell activation and identify a potential new target for therapies against pulmonary tularemia.

Introduction

The respiratory mucosa is a major site for pathogen entry and hence, requires constant immune surveillance. Like other mucosal surfaces, the lungs are populated by a variety of innate cells and innate-like lymphocytes. One such cell type, the type I, semi-invariant natural killer T (NKT) cells, are enriched within the lung vasculature where they are optimally positioned for early antigen encounter [1]. These pulmonary NKT cells exert diverse functions dependent upon experimental settings [2].

NKT cells express an invariant TCR α-chain (Vα14-Jα18 in mice and Vα24-Jα18 in humans) and one of a restricted set of TCR β-chains and, hence, called semi-invariant. Their name also reflects their hybrid nature, in that they co-express markers of both NK cells and conventional T cells. Their innate-like character is reflected in their ability to rapidly respond to stimulation by producing a wide variety of cytokines [3]. Several subsets of NKT cells have been identified, each of which may have distinct functional consequences in disease conditions where NKT cells are known to play a role [3–7]. NKT cell functions are controlled by microbial or self-glycolipids presented by CD1d molecules or by pro-inflammatory cytokines produced by activated antigen presenting cells (APCs). The quality and magnitude of the NKT cell response is determined by the mode of activation and the chemical nature of the activating lipid (s) [4]. Activated NKT cells can stimulate APCs, natural killer cells, and other leukocytes through the expression of cytokines and costimulatory molecules, thus functioning at the interface between innate and adaptive immunity [4]. Consequently, NKT cells control microbial and tumour immunity as well as autoimmune diseases [8–10].

In the lungs, NKT cells promote inflammation in models of airway hyperreactivity (AHR), acute lung injury (ALI), and chronic obstructive pulmonary disease (COPD) [2]. NKT cells may also contribute to the inflammatory cascade accompanying sepsis, which is often a complication of bacterial infections of the lung [11,12]. In general, pulmonary NKT cells are thought to play a protective role in microbial infections, but in some cases, may also exacerbate disease [2]. However, the mechanisms by which pulmonary NKT cells contribute to disease pathology remain poorly defined. The disparate results encountered in the literature are likely due to the differential function of individual NKT cell subsets, the various means by which NKT cells are activated in different disease settings, and the use of different NKT-deficient mouse models [13,14].

To probe the function of lung NKT cells, we chose a respiratory *Francisella tularensis* infection model as this infection causes lethal pulmonary tularemia. *F. tularensis* is a gram-negative facultative intracellular bacterium, which infects multiple cell types including macrophages, dendritic cells, hepatocytes, neutrophils, and epithelial cells [15,16]. The resulting disease

targets multiple organs and manifests itself in several forms of differing severity depending on the inciting bacterial strain, dose, and route of infection. Of these, the respiratory route is the most deadly, and the most likely route of infection by weaponized *F. tularensis* [17]. After inhalation, patients typically show signs of systemic illness, which may be accompanied by immediate signs of respiratory disease and can result in death in 30–60% of cases if left untreated [18–21]. Although the exact cause of death is unclear, it is likely due to an overwhelming systemic inflammatory response [22]. Mice inoculated intranasally (i.n.) with *F. tularensis* fail to mount an effective immune response for the first 48–72h. After this initial immune latency, the response to *F. tularensis* is characterized by a robust local and systemic "cytokine storm" reminiscent of sepsis [23,24]. Little is known about the role of NKT cells in pulmonary tularemia—in either humans or mice, due in part to the difficulties in distinguishing them from NK cells, which protect mice from tularemia-like disease-but a beneficial role has been implied [25]. Each of the cell types known to be susceptible to *F. tularensis* infection has been shown to activate NKT cells [4]. Therefore, we reasoned that NKT cells might be activated very early after infection and could function in shaping the quality of both the innate and adaptive response.

The results emerging from testing the afore hypothesis revealed that indeed respiratory infection with *F. tularensis* activated iNKT cells which produced interferon (IFN)-γ and propagated a sepsis-like proinflammatory response that led to a lethal phenotype in wild type mice. This proinflammatory response was much tempered in CD1d-deficient mice that lacked NKT cells. Strikingly, however, the mutant mice had increased lymphocytic infiltration in the lungs that organized into structures resembling induced bronchus-associated lymphoid tissue (iBALT) at the peak of infection. Hence, NKT cell activation by *F tularensis* infection hampers iBALT formation, which in conjunction with an NKT cell-dependent proinflammatory response, exacerbates severe pulmonary tularemia-like disease in mice.

Results

NKT deficient CD1d$^{-/-}$ mice are less susceptible to i.n. LVS infection

IFN-γ is critically required for murine resistance to primary *F. tularensis* LVS infection. The early production of IFN-γ after pulmonary LVS infection is primarily attributed to NK cells and double negative T cells [26]. NKT cells are also a source of early IFN-γ and hence, we reasoned that they might contribute to resistance to i.n. LVS infection. We therefore investigated whether NKT cell deficiency would alter disease outcome. Studies of immune function in infectious disease rely on one of two methods—genetic deletion or *in vivo* depletion—by which to assess their contribution to resistance or pathology. Because no unique NKT cell-specific marker has been identified, the available Ab-mediated depletion methods deplete both NK and NKT cells as these two cell types have significant overlap in surface marker expression, making interpretation difficult. Mice made genetically deficient in NKT cells are therefore a better experimental model and were generated previously either by deletion of one of the TCR α-chain gene segments (Jα18$^{-/-}$) or by mutation of the restriction element required for thymic NKT cell selection and antigen presentation in peripheral tissues (CD1d$^{-/-}$) [27–30].

To determine how NKT cell deficiency affects the outcome of pulmonary *F. tularensis* infection, we first determined the LD_{50} to i.n. inoculation of the live vaccine strain (LVS) in C57BL/6 (B6) mice using an established method [31,32]. During our preliminary experiments, we found that infected mice lost up to 30% of their initial weight beginning d4 p.i.. Although weight loss was observed at all doses tested (500, 2,000, 8,000 and 30,000 cfu), the degree of disease severity was dependent on the initial inoculum dose. It was only at a dose of 8,000 CFU where we consistently observed moribund mice. At lower doses the majority of mice recovered quickly with few outward signs of disease beyond slightly ruffled fur, which was not consistent

between animals. Even at the higher dose, those WT mice that did not succumb by day 12 quickly began to regain weight and appeared otherwise healthy (S1 Fig). Other clinical manifestations included ruffled fur, hunched posture, labored breathing, and reduced mobility. Because we found that weight loss alone was not always an accurate predictor of disease severity, a clinical score based on physical appearance was also included in our endpoint criteria (see Materials and Methods). The resulting LD_{50} for i.n. LVS infection in B6 mice was ~6,000–8,000 cfu (S1 Fig), which was consistent with that previously published by others [33–35].

To examine the contribution of NKT cells in resistance to primary pneumonic tularemia, we first tested Jα18$^{-/-}$ mice. Hence, B6 and Jα18$^{-/-}$ mice were inoculated i.n. with ~8,000 cfu LVS and monitored for weight loss and signs of morbidity as described in Materials and Methods. When compared to B6, Jα18$^{-/-}$ mice showed a significant increase in susceptibility to i.n. LVS infection (Fig 1A), initially suggesting a protective role for iNKT cells. Although Jα18$^{-/-}$

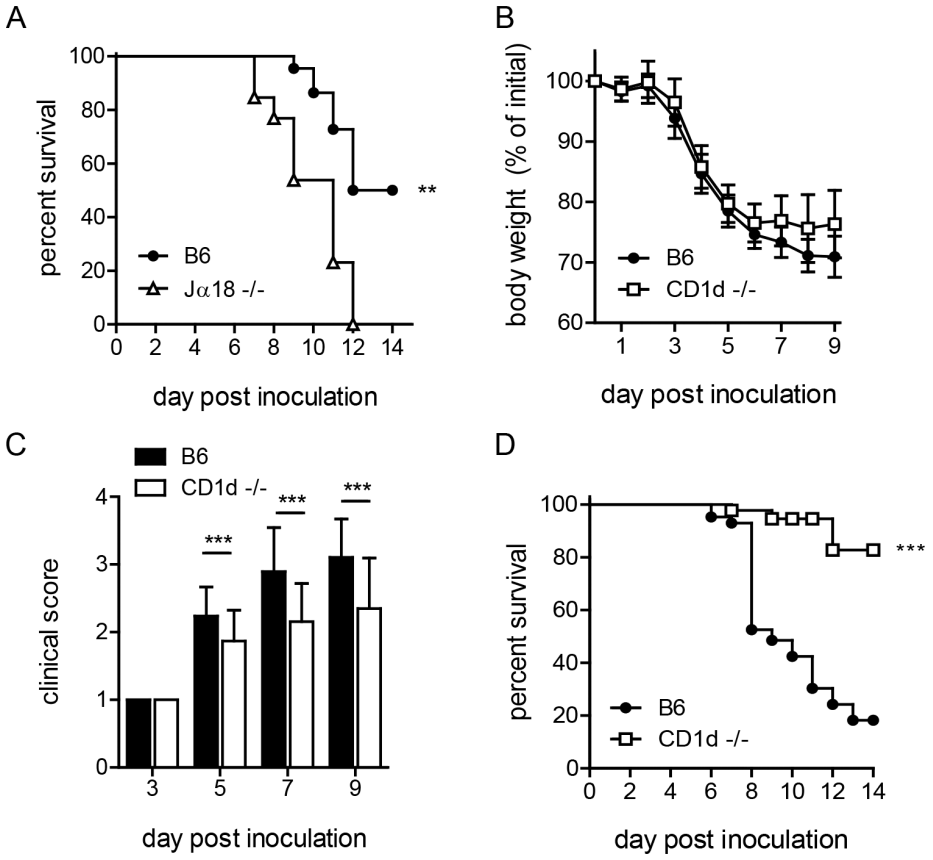

Fig 1. CD1d$^{-/-}$ mice are less susceptible to i.n. LVS infection. (A) Survival of B6 or Jα18$^{-/-}$ mice after i.n. LVS infection. Survival of Jα18$^{-/-}$ and WT mice after intranasal LVS infection (8000 cfu). Curves were compared using Log-rank (Mantel-Cox) Test. Data are from one of three similar experiments with at least 10 mice/group. **(B)** B6 or CD1d$^{-/-}$ mice were infected i.n. with LVS and monitored daily for weight loss. The mean (±SD) weight of 50 animals/group in several independent experiments following intranasal infection (Accumulation of five independent experiments with 10 mice/group in each experiment) is presented. **(C)** Clinical score at various time points p.i. as determined in Materials and Methods. Data are cumulative from four similar experiments (mean+SD) with 5–10 mice/group/time point. Data were analyzed as indicated in Materials and Methods. **(D)** Survival of B6 or CD1d$^{-/-}$ mice after i.n. LVS infection. Cumulative survival curves from 4 experiments with 40 mice/group. Curves were compared using Log-rank (Mantel-Cox) Test. $^{**}p<0.01$, $^{***}p<0.001$.

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mice have historically been used as a model for type I NKT cell deficiency, a recent study demonstrated that they also have a profound defect in conventional αβ T cells, with the loss of an estimated 60% of total TCRα repertoire diversity [36]. This report suggests that the increased susceptibility observed in these mice might be due to this more global T cell deficiency rather than the loss of iNKT cells, since conventional T cells were previously shown to mediate protective immunity to LVS [26,34].

We therefore ascertained the outcome of pulmonary LVS infection in $CD1d^{-/-}$ mice, which have a normal complement of αβ T cells but lack NKT cells. By d4 post-inoculation (p.i.), both B6 and $CD1d^{-/-}$ mice began to show signs of disease, including weight loss and ruffled fur. By d5-6, B6 mice continued to lose more weight and showed more severe outward signs of disease as indicated by clinical score (Fig 1B and 1C). In striking contrast, by d7, nearly all NKT deficient $CD1d^{-/-}$ mice began to recover and a significantly lower percentage of $CD1d^{-/-}$ mice succumbed to i.n. LVS infection (Fig 1D). By d14, fewer than 50% B6 and almost all $CD1d^{-/-}$ mice regained weight and showed no outward signs of disease, surviving the infection (Fig 1D). These data suggest that NKT cells serve a deleterious role in pneumonic tularemia. We therefore reasoned that increased iNKT cell number might further exacerbate disease. Thus, $V\alpha14^{tg}$ mice, which have increased numbers of iNKT cells [37], were infected intranasally and found to have increased susceptibility as predicted (S2 Fig). Taken together, these data strongly support a detrimental function of NKT cells in pneumonic tularemia and show that $CD1d^{-/-}$ mice are better model than $J\alpha18^{-/-}$ mice to study the role of NKT cells in disease. Hence, $CD1d^{-/-}$ mice were used for all subsequent experiments.

Intranasal LVS infection recruits NKT cells to the lung interstitium

NKT cells are overrepresented among T cells in the healthy lung compared to their frequency in other organs. These tissue-resident NKT cells do not recirculate but rapidly extravasate into interstitial spaces upon recognition of lipid antigens such as the potent CD1d-restricted agonist α-galactosylceramide (αGC) [1,38]. Whether NKT cells behave similarly in response to bacterial infection, where lipid ligands are presumably of lower affinity [4], is unknown. To visualize the location of NKT cells in naïve lungs, we injected B6 mice with anti-CD45 antibody (αCD45 Ab) i.v., allowed it to circulate through blood for several minutes, and tracked the anatomic localization of lung mononuclear cells by flow cytometry after labeling lung leukocytes with Abs against lineage-specific markers. This quick procedure allows circulating αCD45 Ab to label all intravascular cells ($\alpha CD45^{POS}$), while cells that are in the tissue interstitial space would be $\alpha CD45^{NEG}$. We confirmed that the largest percentage of lymphocytes (T, B, and NK cells) was in the $\alpha CD45^{POS}$ intravascular population as reported previously [39]. Interestingly, we found that a proportionately higher frequency of pulmonary NKT cells were present in the interstitium ($\alpha CD45^{NEG}$) when compared to other lymphocytes (Fig 2A and S3 Fig). Such localization suggests that pulmonary NKT cells occupy a frontline niche that allows them to rapidly sense and respond to inhaled antigens.

To test the hypothesis that pulmonary NKT cells rapidly engage in host defense at frontline niches, we inoculated B6 mice i.n. with ~LD_{50} of LVS. On d3 p.i., we injected αCD45 Ab and, after several minutes, lungs were analyzed by flow cytometry for NKT cell localization. We found a significant increase in the frequency of $\alpha CD45^{NEG}$ interstitial NKT cells that was equivalent to the frequency observed after i.n. αGC treatment (Fig 2B and S3 Fig). Furthermore, beginning d3 after i.n. LVS inoculation, we found a significant decrease in the frequency of NKT cells in the blood when compared to uninfected controls (Fig 2C and S3 Fig). This reduction in circulating NKT cells was coincident with their increased numbers in the lungs (Fig 2C and 2D), more specifically and significantly in the lung interstitium ($\alpha CD45^{NEG}$ lung

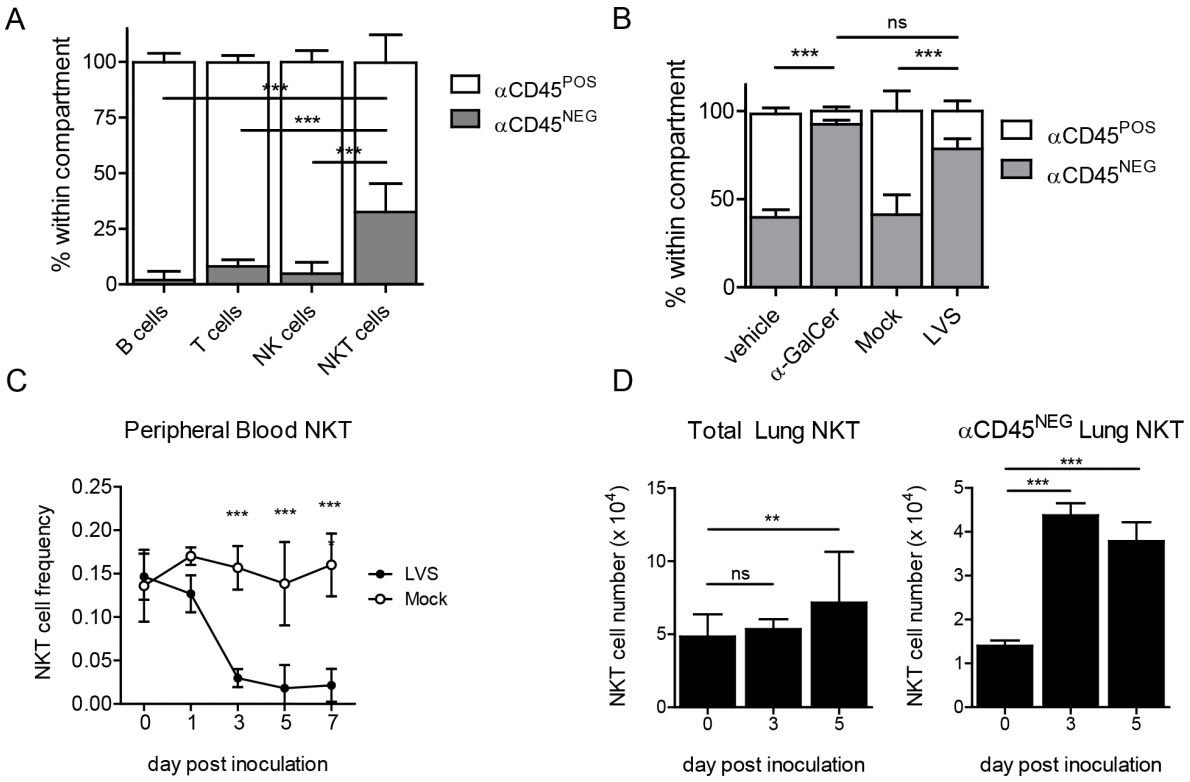

Fig 2. NKT cells are recruited to the lung interstitium in response to LVS infection. (A) Intravascular αCD45 Ab staining to determine relative distribution of the indicated lymphocytes in naïve lungs. Data combined from four similar experiments. Bars are mean+SD (n = 13 mice). Interstitial NKT cell frequency was compared to all other lymphocyte populations as described in Materials and Methods. **(B)** Frequency of lung NKT cells among B220$^-$ cells after i.n. αGC treatment or LVS inoculation. Mean+SD from one of two similar experiments (n = 3–4 mice/group). Frequency of interstitial NKT cells was compared by one-way ANOVA with Tukey's posttest. **(C)** Frequency of NKT cells in peripheral blood of LVS-infected or mock-infected mice. Mean±SD from one of two similar experiments (n = 3–4 mice/group). Comparison was made by two-way ANOVA with Bonferroni posttest. **(D)** NKT cell number in total lung or lung interstitium after i.n. LVS infection. Mean+SD combined from two similar experiments (n = 7 mice/ time point). Day 3 and day 5 post inoculation were compared to naïve mice as described in Materials and Methods. *p<0.05, **p<0.01, ***p<0.001.

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NKT). Thus, i.n. LVS infection rapidly recruits NKT cells from the vasculature into the infected lung interstitium. This finding is consistent with a previous report showing NKT cell extravasation into the lung interstitium after respiratory αGC administration [1].

A putative LVS-derived antigen activates lung-resident NKT cells through the T cell receptor

NKT cells can be activated by recognition of CD1d-bound microbial lipids or self-lipids presented by activated APCs, or by cytokines which can activate these cells independent of TCR stimulation [4]. The mechanism by which NKT cells are activated can influence their function. To determine whether the accumulating NKT cells were activated by LVS infection, we monitored expression of the activation marker CD69 which reports on NKT activation in general, and we employed Nur77^{gfp} reporter mice, which increase GFP expression only upon NKT cell activation via CD1d-restricted microbial glycolipids but not self-lipids [40]. On d3 post i.n. LVS inoculation, we found that both interstitial and intravascular NKT cells increased CD69 expression (Fig 3A). Strikingly, however, only the interstitial population showed increased GFP

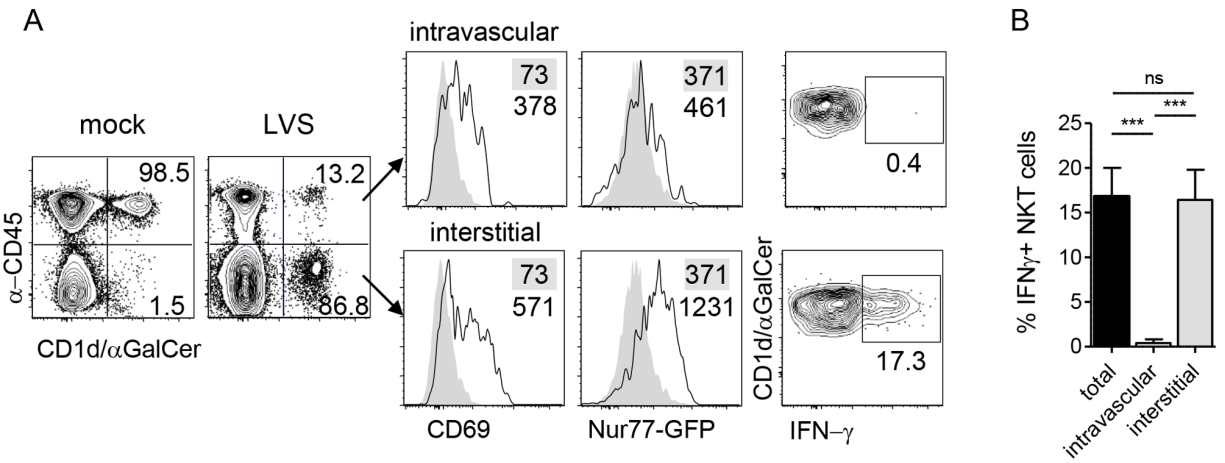

Fig 3. NKT cell activation by LVS is TCR-dependent. (A) Representative plots of NKT cell localization, activation, and IFN-γ production after i.n. LVS infection of Nur77^{gfp} mice. Numbers in contour plots are % NKT cells. Numbers in histograms are median fluorescence of infected mice (open histograms) and mock-infected controls (shaded histograms). Data are representative of three similar experiments (n = 10). **(B)** Percent IFN-γ+ NKT cells in each compartment after LVS infection. Bars are mean+SD. Data are representative from one of three similar experiments (n = 3 mice). Comparison was made by one-way ANOVA with Tukey's posttest. ***p<0.001.

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fluorescence (Fig 3A). This result indicated that local LVS antigen presentation occurred within the interstitium to activate LVS-recruited NKT cells *in situ*.

To determine the consequence of interstitial NKT cell activation, intracellular cytokine staining was conducted directly *ex vivo* without any restimulation. We found that the GFP^{hi} interstitial NKT cells but not GFP^{lo} intravascular cells produced IFN-γ (Fig 3A and 3B), demonstrating that this cytokine production was a result of LVS infection *in vivo*. Taken together, these data suggest that upon i.n. *F. tularensis* LVS infection, NKT cells migrate from the periphery and accumulate within the infected lung where they are activated through their TCR to produce IFN-γ.

Modestly lower bacterial burden correlates with better clinical score in CD1d$^{-/-}$ mice upon pulmonary LVS infection

Two factors can affect overall fitness after infection: pathogen burden and the level of organ pathology. To determine whether the decreased susceptibility of CD1d$^{-/-}$ mice to LVS was due to differences in bacterial burden, we measured LVS burden in lung homogenates at various time points post i.n. LVS inoculation. Lung burden was similar in both groups, with a modest but statistically significant difference (two-fold) seen only at d7 and d9 in infected CD1d$^{-/-}$ mice (Fig 4A), which was concomitant with the observed differences in clinical score and weight loss (Fig 1B and 1C).

Since the observed differences in illness began to appear at d7 p.i., we focused on this time point for subsequent analyses. Differing degrees of lung pathology can result in different animal fitness despite similar bacterial burden [35,41,42]. To determine whether the difference in morbidity was due to differential lung damage, we measured blood oxygen saturation (SpO_2), which was suggested as an accurate measure of lung pathology [43]. While both groups of mice showed decreased SpO_2 levels, there were no significant differences observed between groups (Fig 4B). To more directly assess tissue damage, H&E-stained lungs were evaluated at d7 and d9 p.i. As predicted by pulse oximetry data, the findings in the lungs of B6 and CD1d$^{-/-}$ mice were similar. Lungs from both groups of mice displayed focally extensive interstitial

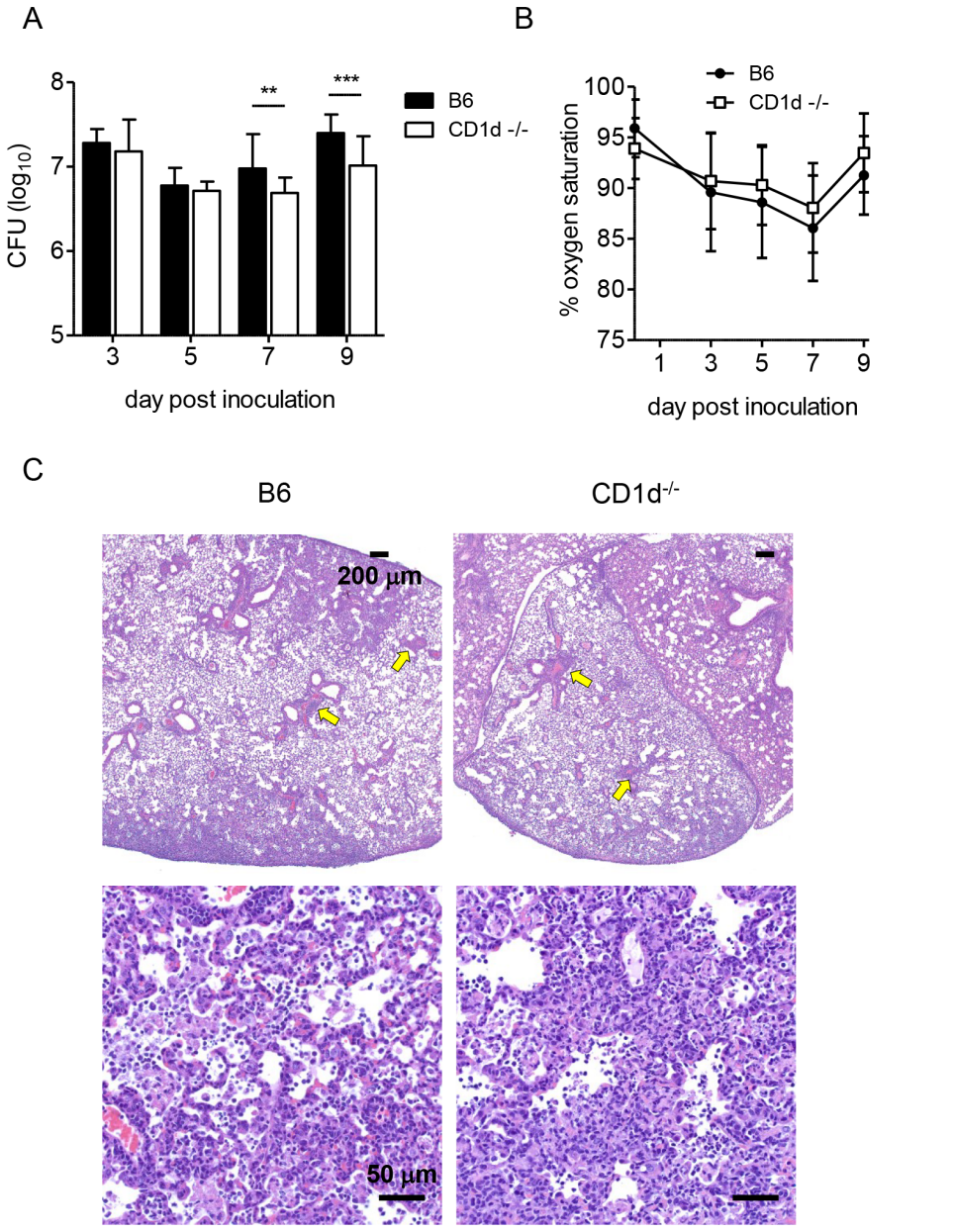

Fig 4. CD1d$^{-/-}$ mice have modestly reduced lung LVS burden but no differences in lung pathology. (A) Lung burden was determined at various times p.i. as described in Materials and Methods. Data are combined from at least 3 separate experiments. Bars are mean+SD of 10–15 mice/group/time point. **(B)** Oxygen saturation was measured as described in Materials and Methods. Data are cumulative of 3 separate experiments with 5–10 mice/group (mean±SD). **(C)** H&E stained formalin fixed paraffin embedded (FFPE) lung sections d9 p.i. 40X (top) or 400X (bottom) magnification with scale bars as indicated. Data are representative of three mice per group. Arrows indicate aggregations of lymphocytes. Data were analyzed as indicated in Materials and Methods. $^{**}p<0.01$, $^{***}p<0.001$.

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pneumonia with perivascular cuffs of mononuclear cells. Inflammatory infiltrate consisting of macrophages and neutrophils filled the alveoli, which also contained necrotic debris and edema with focal areas of necrosis on the alveolar walls (Fig 4C).

After i.n. infection, *F. tularensis* rapidly disseminates to the periphery [44]. The kinetics and extent of dissemination are suggested as determinants of disease severity [45–48]. Hence, we measured burden in blood, liver, and spleen. LVS was only transiently detectable in the blood, where levels peaked at d3 p.i., but there were no differences in bacteremia between groups (Fig 5A). Liver burden was similar in both groups, but CD1d$^{-/-}$ mice had significantly lower splenic burden d3–7 p.i. (Fig 5A). Further analysis showed that only lung burden—but not liver or spleen—correlated with weight loss at d7 p.i. (S4 Fig). Consistent with these findings, histopathological analysis failed to identify any striking differences in either liver or spleen pathology after intranasal inoculation (Fig 5B). The extent of hepatic granuloma formation did not differ between groups (Fig 5B). Contrary to previous reports in BALB/c mice [49,50], splenic architecture was mostly intact with some evidence of apoptosis and extramedullary hematopoiesis that did not seem to differ between groups (Fig 5B).

In summary, the above data indicate that the modest differences in bacterial burden in the lungs and peripheral organs, or another mechanism, but not differential pulmonary, hepatic, or splenic histopathology could explain the milder disease observed in NKT cell deficient mice.

Increased lymphocyte infiltrates organize into tertiary lymphoid structures in CD1d$^{-/-}$ lungs in response to LVS infection

The differences described thus far become most pronounced at d7 p.i, suggesting that the quality of the adaptive response may be the principal underlying cause of the reduced susceptibility observed in CD1d$^{-/-}$ mice. We therefore analyzed lymphocyte numbers in the lungs after LVS infection. Lungs of CD1d$^{-/-}$ mice had consistently higher numbers of both B and T lymphocytes present at d7 p.i. (Fig 6A). To directly visualize the localization of these cells—whether the lymphocytic infiltration was in the pulmonary vasculature or had extravasated into the lung tissue—fixed frozen sections of infected lung were stained and examined by confocal microscopy. Consistent with earlier analysis of H&E stained sections, both groups of mice showed extensive perivascular and peribronchiolar infiltration of leukocytes. Significantly however, in addition to an increased cellularity in CD1d$^{-/-}$ mice, there was a striking difference in the degree of organization of the infiltrating immune cells. We found evidence for the formation of tertiary lymphoid structures, iBALT, within the infected lungs of CD1d$^{-/-}$ mice (Fig 6B). These structures were marked by the formation of B cell follicles, which were surrounded by T cell congregates. In contrast, fewer iBALT structures were formed in B6 mice by d7 p.i. (Fig 6C) and those that developed were small and appeared rudimentary in that T and B cells were scattered or at best loosely packed together (S5 Fig). Such tertiary lymphoid structures were not observed in uninfected CD1d$^{-/-}$ or B6 mouse lungs (S6 Fig) suggesting that iBALTs formed in response to pulmonary LVS infection.

Dendritic cells (DCs) are both necessary and sufficient for the induction of iBALT within the lungs [51]. Consistent with previous reports, concentrations of CD11c^{+} cells were also observed in and around the B and T cell zones of iBALT formed in CD1d$^{-/-}$ lungs (Fig 6B). Further characterization of CD11c^{+} cells within the infected lungs by flow cytometry revealed that all DC subsets identified were increased in the LVS infected lungs of CD1d$^{-/-}$ mice (Fig 6D). Considering that DCs have been implicated as a primary vehicle for dissemination of *F. tularensis* from the infected lung [45], this finding may also partially explain the reduced splenic burden in CD1d$^{-/-}$ mice. Hence, iBALT formation in response to LVS infection of the lungs is associated with milder tularemia-like disease in mice and prognosticates recovery.

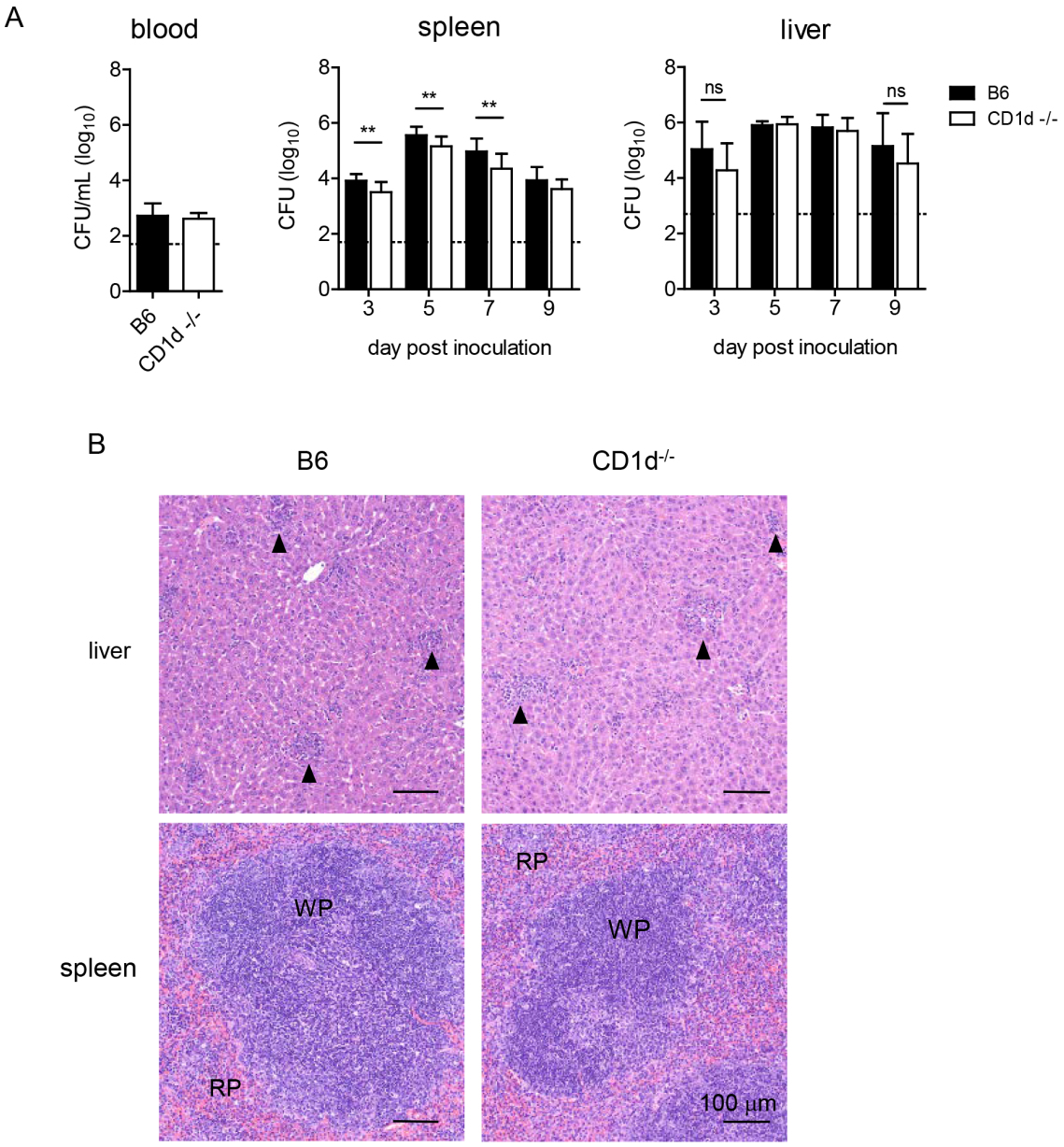

Fig 5. CD1d$^{-/-}$ mice have reduced splenic LVS burden but no difference in the liver. (A) Blood, liver, and spleen burden were determined as in Fig 4. Data are combined from 3 separate experiments. Bars are mean+SD of 6–10 mice/group/time point. Data were analyzed as indicated in Materials and Methods. $^{**}p<0.01$. **(B)** H&E stained FFPE liver (top) and spleen (bottom) sections d9 p.i., 200X magnification. Data are representative of 3 mice/group. Arrows indicate granulomas.

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Decreased neutrophilia in CD1d$^{-/-}$ mice after LVS infection

A previous study showed that increased disease severity in a murine tularemia model was associated with hepatic damage and neutrophilia [52]. NKT cells activated by LVS could cause liver damage through direct lysis of infected hepatocytes [53,54]. They might also promote neutrophilia through production of granulocyte colony-stimulating factor (G-CSF), the major neutrophil survival and proliferation factor [55]. Thus we monitored these two disease parameters in

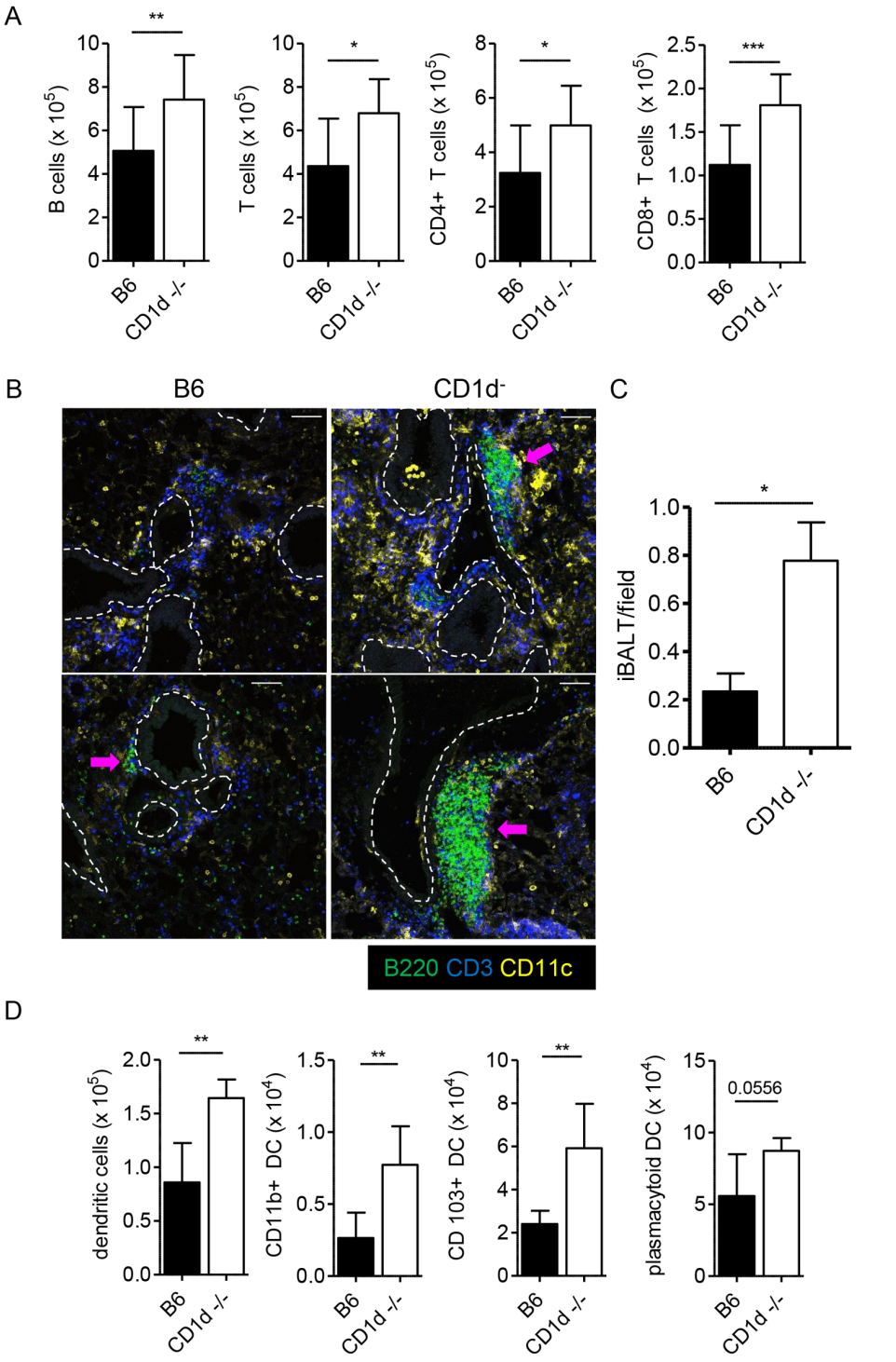

Fig 6. Formation of iBALT in CD1d$^{-/-}$ mice after i.n. LVS infection. (A) Groups of B6 or CD1d$^{-/-}$ mice were infected i.n. with LVS. On d7 p.i., lungs were analyzed by flow cytometry for the indicated cell populations. Data are cumulative of 2 experiments with 9–10 mice/group. Bars are mean+SD; compared as indicated in Materials and Methods. **(B)** Representative images from *F. tularensis* LVS-infected lungs d7 p.i. Images are individual mice from 2 of 3 similar experiments. 20X magnification; bars are 80 μm. Dashed lines indicate large airways as identified by autofluorescence of epithelial cells. Arrows indicate rudimentary iBALT

formation in B6 lung and well-developed iBALT in lungs of CD1d$^{-/-}$mice. **(C)** Enumeration of iBALT from d7 infected lung sections. Data are cumulative from three experiments (n = 6 mice/group). Bars are mean+SD. Data were compared using Mann Whitney U test. **(D)** DC subsets in infected lungs were identified as described in Experimental Procedures. Representative results from 1 of 3 similar experiments (n = 5 mice/group). Bars are mean+SD; Mann Whitney U test. *p<0.05, **p<0.01, ***p<0.001.

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CD1d$^{-/-}$ mice. Although histological analysis failed to reveal any gross differences in liver pathology (Fig 5B), we measured AST and ALT in the serum of infected animals as a more sensitive indicator of hepatic injury (Fig 7A). CD1d$^{-/-}$ mice had serum ALT levels that were slightly, yet significantly, lower than those measured in B6 mice, which implied slightly more severe liver damage in the latter strain. However, when taken together with the absence of widespread necrosis, these mild-to-moderate elevations in circulating hepatic enzymes are not consistent with death from liver failure, particularly considering the similar liver burden in the two groups of mice (Fig 5). Such slightly elevated ALT levels need not be a result of the death of infected hepatocytes, but might rather be indicative of increased systemic inflammation [56].

To monitor the numbers of circulating neutrophils we performed complete blood counts (CBC) at various time points post inoculation. B6 mice did indeed show more pronounced neutrophilia d7 p.i. when compared to CD1d$^{-/-}$ mice (Fig 7B). This difference was not observed in naïve mice and did not appear until d7 p.i. (Fig 7C). We found lower G-CSF levels in the serum but not the lungs of CD1d$^{-/-}$ mice by d7 (Fig 7D), which was consistent with the lower frequency of neutrophils in the blood (Fig 7B). Thus, decreased neutrophilia incited by LVS infection in CD1d$^{-/-}$ mice could be one cause for their increased resistance to tularemia.

Tempered inflammatory response in CD1d$^{-/-}$ mice after LVS infection

Although IFN-γ is necessary for resistance to LVS, excessive production of proinflammatory cytokines can be detrimental, particularly after intranasal infection [24,41]. Because NKT cells are known to induce IFN-γ production by NK cells [57,58], we therefore asked whether CD1d$^{-/-}$ mice—which have normal numbers of NK cells—might produce less IFN-γ in response to LVS. We found that although the early IFN-γ response was comparable in both groups of mice (S7 Fig), by d7 p.i. CD1d$^{-/-}$ mice had significantly lower levels in both the serum and lung (Fig 8A and 8B).

Many cytokines produced by NKT cells were suggested to promote sepsis-like inflammatory disease [11,59] and, hence, we measured the levels of those cytokines previously shown to be induced by LVS infection that have also been associated with severe sepsis [49,60]. We found that CD1d$^{-/-}$ mice had consistently lower levels of IFN-γ, IL-6, and TNF-α in both the lungs and serum, with lower levels of MCP-1 and KC in the serum at d7 p.i. (Fig 8A and 8B) which coincided with modestly reduced burden, reduced weight loss, and less severe outward signs of disease (Fig 1). Hence, NKT deficiency results in a less severe "cytokine storm" in response to i.n. LVS infection.

Given the observed differences in susceptibility among various mouse strains [61–64], we next ascertained whether CD1d$^{-/-}$ mice in the BALB/c background would exhibit the same phenotype. Consistent with the results in C57BL6 background, we found that CD1d$^{-/-}$ mice were less susceptible to LVS infection in the BALB/c background as well (Fig 9A). As observed with C57BL6 mice, higher doses caused severe disease in both groups, although death was slightly delayed in CD1d-deficient mice.

Mice are highly susceptible to several subspecies of *Francisella* and the extreme virulence of type A (subspecies *tularensis*) or B (subspecies *holarctica*) strains complicates the study of effective immune responses, particularly when administered intranasally (LD_{50}<10) [26].

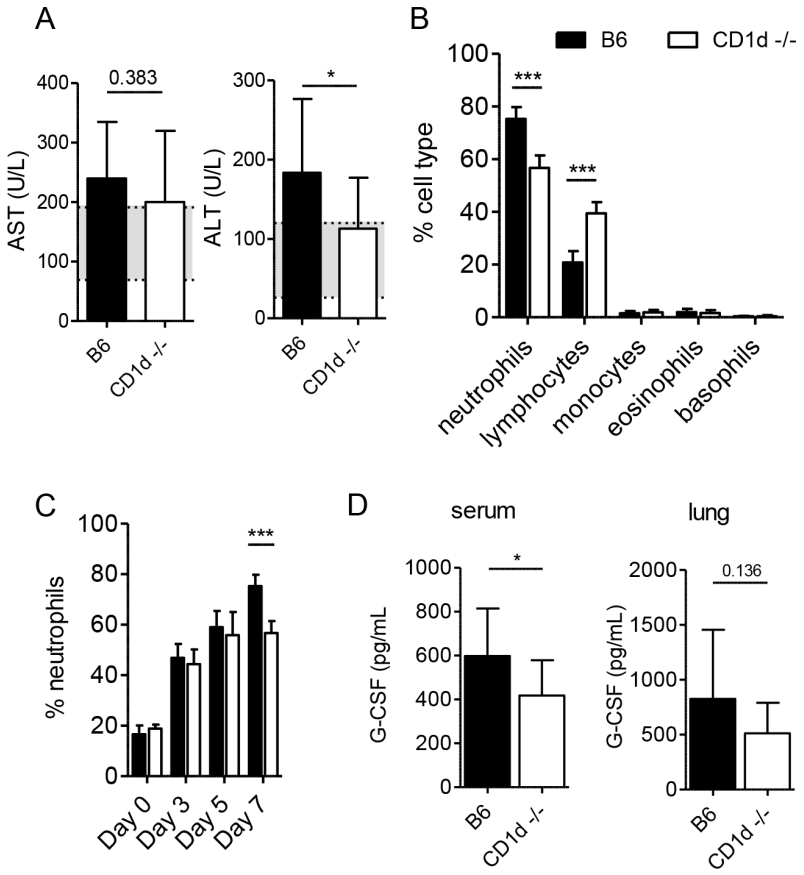

Fig 7. CD1d$^{-/-}$ mice have less severe neutrophilia after intranasal LVS infection. (A) AST and ALT levels were measured in serum of infected mice on d7 p.i. Data are combined from 3 experiments (n = 10–12). Mean+SD. Shaded regions denote reference range. **(B)** CBC analysis of blood from LVS-infected mice performed on d7. Data are combined from 2 separate experiments (n = 15 mice/group). Bars are mean+SD. **(C)** Percent neutrophils in blood at the indicated time points p.i. Data are combined from 2 separate experiments. Bars are mean+SD of 5–15 mice/group/time point. **(D)** On d7 serum and lung G-CSF levels were measured by CBA. Data are combined from 2 experiments (n = 15 mice/group). Bars are mean+SD. Comparisons were made as indicated in Materials and Methods. *p<0.05, ***p<0.001.

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Although LVS has long been used in murine models of experimental tularemia [31,65], it is an attenuated type B strain that fails to cause disease in humans. We therefore sought to determine whether CD1d$^{-/-}$ would have a similar resistance to the more virulent type A strain Schu S4. Not surprisingly however, both wild-type and CD1d$^{-/-}$ mice were extremely susceptible to a low-dose (<10 CFU) pulmonary infection with Schu S4 (Fig 9B), similar to the results obtained with higher doses of LVS in both C57BL6 (S1 Fig) and BALB/c (Fig 9A) mice. These data are consistent with previous reports showing reduced efficacy of therapies or mutations that confer resistance to LVS when challenged with Schu S4 [35,66–68].

Discussion

Once infected, the host has two strategies to deal with invasive pathogens: resistance and tolerance. Resistance implies the ability of the host to clear a pathogen or limit its spread whilst tolerance indicates the ability of the host to bear the ensuing pathology [69]. Surviving infection

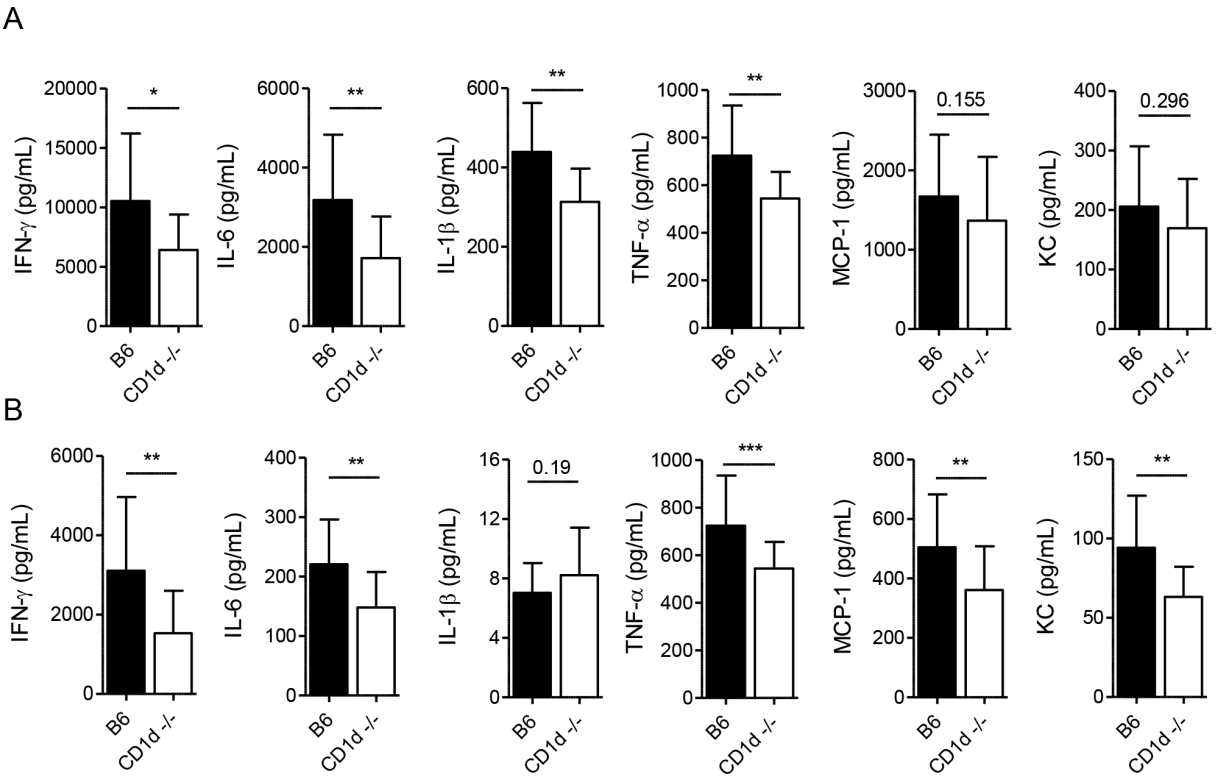

Fig 8. CD1d$^{-/-}$ mice exhibit a tempered inflammatory response to LVS. Cytokine levels were measured at 7d p.i. in lung homogenates **(A)** or serum **(B)** by CBA. Data are combined from at least 3 experiments with 10–20 mice/group. Bars are mean+SD. Comparisons in these experiments were made as indicated in Materials and Methods. *p<0.05, **p<0.01, ***p<0.001.

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from a pathogen such as *F. tularensis* likely requires a balance of both. Previous studies of infection in immunodeficient mice revealed that immunity to *F. tularensis* is mediated by the concerted effort of the innate and adaptive humoral and cellular immune responses. Because the host deploys a wide array of effector mediators in its inflammatory arsenal against the infection a sepsis-like disease ensues [22,26]. Hence, fatalities in this infectious disease appear to be caused by an inability to control bacterial growth and dissemination, which precipitates inflammation and causes irreparable collateral tissue damage. Accordingly, if diagnosed early, antibiotic treatment limits bacterial burden and tempers inflammation thereby controlling morbidity and mortality underlying tularemia. Herein we report that NKT cells respond to intranasal infection by migrating to the infected lung to produce IFN-γ, which promotes clearance and killing of the pathogen. As a trade-off however, the early activation of NKT cells causes excessive systemic inflammation, increases neutrophil mobilization, and delays infiltration of lymphocytes into the lungs and formation of protective tertiary lymphoid structures. Hence, CD1d-deficient animals have a tempered inflammatory response and perform clinically better in response to intranasal LVS infection. This advantage, however, can be overcome with higher doses of bacteria or infection with more virulent subspecies. The discordant results obtained using Jα18$^{-/-}$ and CD1d$^{-/-}$ mouse models likely reflect a more global T cell deficiency in Jα18$^{-/-}$ mice and underscore the difficulties in deciphering the role of NKT cells using currently available methods [14].

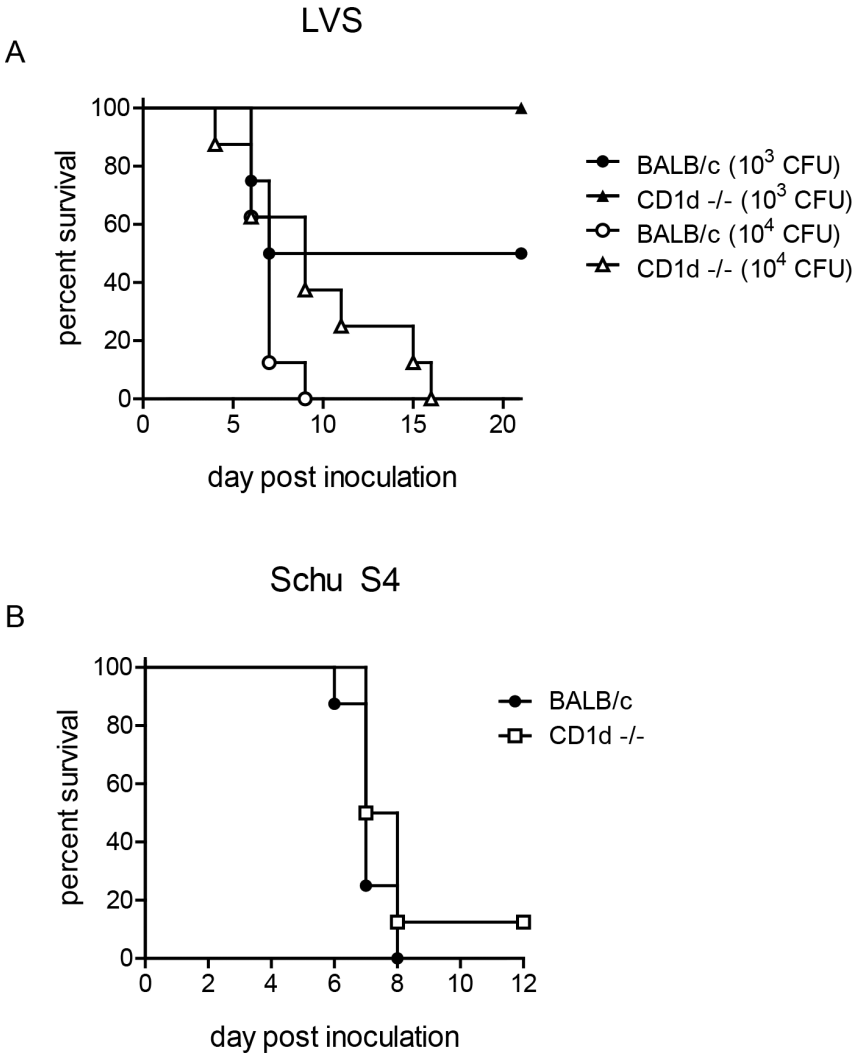

Fig 9. Reduced susceptibility of CD1d$^{-/-}$ mice is not dependent on strain background. (A) Groups of 6–8 week-old BALB/c or CD1d$^{-/-}$ mice were infected intranasally with different doses of LVS as indicated and monitored for survival (n = 8 mice/group) **(B)** BALB/c or CD1d$^{-/-}$ mice were infected intranasally with 6 cfu Schu S4 and monitored for survival (n = 8 mice/group).

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Akin to CD1d-deficient animals, *F. tularensis* infection of metalloproteinase-9 null as well as IL-17 and IL-10 double-deficient mice showed low disease incidence despite similar bacterial burden [35,41]. In conjunction with a tempered inflammatory response, these immunodeficient mice were able to limit the damage caused by the pathogen or the host immune system and were therefore less susceptible to i.n. *F. tularensis* infection. These findings and our data reported herein, thus enforce the notion that the host response to *F. tularensis* is a balance between resistance to pathogen and tolerance to pathology.

LVS infection of endothelial cells induces the transmigration of neutrophils into infected tissues [70,71]. Rather than contributing to host defense, neutrophils may instead increase disease severity. *F. tularensis*-infected neutrophils assume an extended proinflammatory phenotype, which is thought to contribute to increased tissue destruction [72]. Because *F. tularensis* is able

to infect and replicate within neutrophils, these cells likely serve as a vehicle for dissemination from the primary site of infection, which might contribute to the reduced peripheral burden observed in CD1d$^{-/-}$ mice [15,73]. Direct interaction with NKT cells has been shown to influence the function of neutrophils [74] and to promote hematopoiesis in both humans and mice [75]. NKT cell-neutrophil interactions were also recently shown to exacerbate polymicrobial sepsis [59]. Although we found no differences in differential cell counts from peripheral blood of naive wild-type and CD1d$^{-/-}$ mice, CD1d$^{-/-}$ mice displayed less severe neutrophilia upon LVS infection. We also found that slightly decreased hepatotoxicity, reduced peripheral burden, and less severe inflammation in NKT cell-deficient CD1d$^{-/-}$ mice corresponded with less severe neutrophilia.

Our studies revealed that acute LVS infection of CD1d$^{-/-}$ mice resulted in increased lymphocytic infiltration in the lung interstitium when compared to B6 mice. The cellular infiltration organized into iBALT at the peak of infection. Although typically associated with chronic inflammatory conditions, iBALT has been shown to form in the lung in response to viral infection [51]. However, the formation of such structures in response to acute bacterial infection is less well-studied. In the context of pulmonary *F. tularensis* infection, lymphoid aggregates have been described in wild-type mice that survived *F. tularensis* infection after >50d p.i. whilst bone fide iBALTs were described only in LPS/rPorB vaccinated mice challenged with a high dose of LVS [49,76]. The post-vaccination development of these structures in the lungs was associated with better survival upon i.n. challenge with LVS or the more virulent *F. tularensis* subspp. *tularensis* [76,77]. Data from these studies suggest that the T and B cells found in iBALT limit the spread of the bacteria from the lung thereby reducing immune-mediated damage to peripheral organs. Contrary to the results of these studies with wild-type animals, our data demonstrate the development of iBALT in the lungs of B6 mice is hampered perhaps by the presence and activation of NKT cells as more robust iBALT formation is observed in CD1d$^{-/-}$ mice during the peak of a primary LVS infection. Such early iBALT formation is suggestive of a more vigorous and effective adaptive immune response resulting in reduced systemic inflammation and therefore less severe disease. These findings suggest a suppressive/regulatory role for pulmonary NKT cells. The regulatory function of NKT cells is just beginning to be elucidated and much of what we know derives from studies utilizing model antigens [7]. Thus, our findings provide a model in which to study the potentially detrimental functions of iNKT cells in a natural infection.

In the context of intranasal LVS infection, the inability to form iBALT proves to be detrimental, but there may be cases where this outcome is desirable. For example, iBALT formation has been associated with chronic inflammatory conditions such as asthma [78]. Therefore, the discovery of an NKT cell-activating ligand(s) derived from *F. tularensis* that can prevent iBALT formation might be of therapeutic benefit in such instances. Similarly, a better understanding of the regulatory function of NKT cells may lead to advances toward the goals of rational vaccine design.

Materials and Methods

Ethics statement

All procedures on mice were performed by approval from the IACUC at Vanderbilt University School of Medicine and at Albany Medical College. Anesthesia was performed using 1–5% isoflurane or 100 mg/kg and 10 mg/kg ketamine and xylazine, respectively. Euthanasia was performed by CO_2 overdose followed by cervical dislocation.

Mice

C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor). CD1d$^{-/-}$ mice were a gift from L Van Kaer (Vanderbilt University School of Medicine). Jα18$^{-/-}$ mice were generously provided by M Taniguchi (RIKEN Institute, Yokohama, Japan). Mice were bred and maintained in the School of Medicine vivarium and provided with food and water ad libitum. LVS infection was performed in an ABSL-2 facility. Vanderbilt's IACUC approved the experiments described here.

Bacterial infection

F. tularensis LVS was provided by S. Khader (Washington University, St. Louis, MO). Preparation of working stocks and CFU determination from infected tissue were performed as described [31]. LD_{50} was determined by the method of Reed and Muench [32]. Male, 8–12-week old mice were anesthetized by i.p. administration of a ketamine/xylazine mixture and ~8–10x10^3 CFU LVS were administered i.n. in 50μL sterile PBS. Mice were monitored daily for weight loss and signs of morbidity. Criteria for clinical score were developed based upon observation of mice from at least three separate experiments: 1) no outward signs of illness; 2) consistently ruffled fur; 3) hunched back and altered gait; 4) reduced mobility/reaction to stimulus, labored breathing, lethargy. Mice were humanely euthanized when weight loss exceeded 30 percent.

Experiments with Schu S4 were conducted with approval of the Albany Medical College IACUC (protocols 15–04001 and 15–04002). Six-to-eight week old BALB/c and C.129-CD1d$^{-/-}$ mice were inoculated i.n. with the indicated dose of Schu S4 as described above for LVS.

Cytokine measurements

Cytokines were measured in serum or lung homogenates using Cytokine Bead Array assay (BD Biosciences). Right lobes of lung were homogenized using a Tissue Tearor (Biospec Products, Inc.) in 1 mL sterile PBS containing a cocktail of protease inhibitors (Roche). Homogenates were centrifuged and supernatants frozen at -80°C until analyzed.

Pulse Oximetry

SpO_2 was measured using the PhysioSuite with MouseSTAT Pulse Oximeter (Kent Scientific Corp.). Mice were anesthetized using 1.5% isoflurane for induction and maintenance. Sensor was attached to the right hind foot according to the manufacturer's instructions. Measurements were recorded at 5-second intervals for at least 4 minutes and averaged.

Tissue processing

Spleen, liver, and lungs were processed as previously described [1,79]. Left lung lobes were used for flow cytometry and microscopy and right lobes for CFU determination.

Flow cytometry

NKT cells were analyzed as described [79]. All data were acquired on an LSR II (BD Biosciences) and analyzed with FlowJo software (FlowJo, LLC). Cell populations were identified as follows: B cells (CD19$^+$); T cells (CD3ε$^+$); NK cells (CD3ε$^-$NK1.1$^+$); NKT cells (CD3ε$^+$CD1d/αGC tetramer$^+$); DCs (CD45$^+$CD64$^-$CD24$^+$MHCII$^+$CD11c$^+$). DC subsets were identified as described [80]. CD1d monomers were provided by the NIH Tetramer Core Facility. Cell counts were determined using AccuCheck counting beads (Invitrogen).

Immunofluorescence and confocal microscopy

To analyze the formation of tertiary lymphoid follicles in the lungs following LVS infection, tissues were fixed in PLP buffer (2% paraformaldehyde, 0.05 M phosphate buffer containing 0.1 M L-lysine, and 2 mg/ml $NaIO_4$) overnight. Tissues were then dehydrated in 30% sucrose and subsequently embedded in OCT media. Twenty micron frozen sections were preincubated with Fc-block (anti-mouse CD16/32 Ab; Biolegend) diluted in PBS containing 2% goat serum and fetal bovine serum (FBS). After incubation for 1 hour at room temperature, sections were washed with PBS and stained with the following antibodies diluted in PBS containing 2% goat serum and FBS for 1 hour at room temperature: anti-B220-Alexa-488 (Biolegend), anti-CD3-allophycocyanin, anti-CD11c-phycoerythrin (Biolegend) and Fc-block. Sections were washed with PBS and mounted using Immu-Mount (Thermo Fisher Scientific). Images were acquired using a Zeiss 780 confocal microscope (Carl Zeiss). The imaging data were processed and analyzed using the Imaris software (Bitplane; Oxford Instruments).

Statistical analysis

Statistical analyses of representative data were performed using GraphPad Prism version 5.02 for Windows, GraphPad Software, San Diego California USA (www.graphpad.com). Where indicated in the figure legends, data were aggregated across several (from two to four) replicate experiments. To address the clustered nature of the final datasets, we used a linear regression analysis with cluster robust standard error estimation (an extension of Huber-White Sandwich Estimator [81–83]), a method that accounts for intraclass correlation when determining statistical significance of regression coefficients. In all reported regression analyses individual data points were entered as the dependent variable, and replicate experiments were identified as the clustering variable. Experimental group type (KO and WT) was entered as the independent variable and was dummy coded for the analyses with "KO" group as the reference category. In such case, the intercept reflects the mean of the reference group (= KO), and the slope reflects the difference between the KO and the WT groups. We have reported herein the significance of coefficients for slopes (differences between group means). Clinical score data was analyzed using Generalized Estimating Equation (GEE) model based on Poisson regression. In the model, clinical score was predicted by group type and day of assessment (dummy coded), and their interaction. We report comparisons between groups at each time of assessment, which were obtained by changing the reference category between days of assessment. All analyses were carried out using R Project for Statistical Computing (http://www.r-project.org/).

Supporting Information

S1 Fig. (A) Groups of B6 mice were inoculated intranasally with increasing doses of LVS as indicated and monitored daily for weight loss and signs of morbidity. Data are representative of two similar experiments with 5 mice/group. Plotted are mean ± SD. (B-D) Groups of B6 or $CD1d^{-/-}$ mice were inoculated intranasally with 5 x 10^2 (B), 2 x 10^3 (C), or 3 x 10^4 (D) cfu LVS and monitored daily for signs of morbidity. Mice were humanely euthanized when weight loss exceeded 30% or when showing obvious signs of distress. Data are representative of two or three experiments with 8–10 mice/group.
(TIF)

S2 Fig. Increased NKT cell numbers exacerbates disease. Groups of WT or $V\alpha14^{tg}$ mice were infected intranasally with 8000 cfu LVS and monitored daily for weight loss and signs of morbidity (n = 6–9 mice/group). Results are representative from one of three similar experiments.
(TIF)

S3 Fig. NKT cells are pre-positioned in the lung and recruited into the interstitium after i.n. LVS inoculation. (A) Representative plots of lung lymphocyte localization in naïve B6 mice. Cells were identified as described in Materials and Methods. Intravascular αCD45 staining was used to discriminate intravascular ($\alpha CD45^{POS}$) and interstitial ($\alpha CD45^{NEG}$) cells. Numbers are percent of each cell type within the respective gate. **(B)** Representative intravascular staining of NKT cell localization d3 after intranasal administration of 2 μg αGalCer (top) or ~8,000 cfu LVS (bottom). Numbers are percent of $CD3\varepsilon^{+}CD1d/\alpha GalCer$ tetramer^{+} cells. **(C)** Representative NKT staining of blood from mock- or LVS-infected mice at various time points p.i. Numbers in plots are percent of $B220^{-}$ lymphocytes.
(TIF)

S4 Fig. Lung burden, but not liver or spleen, are correlated with weight loss after i.n. LVS infection. LVS burden was determined from homogenized lung, liver, and spleen d7 p.i. Data are cumulative from more than three experiments with *n* values as indicated. Spearman correlation analysis showed that only lung burden was correlated with weight loss at the peak of infection.
(TIF)

S5 Fig. Tertiary lymphoid structures are more prominent in lungs of NKT-deficient mice. Representative sections from B6 (left) and $CD1d^{-/-}$ (right) mice d7 post i.n. inoculation (8,000 cfu LVS).
(TIF)

S6 Fig. iBALT is not observed in the lungs of naïve, uninfected mice. Representative images of naïve lung sections from B6 (left) or $CD1d^{-/-}$ (right) mice.
(TIF)

S7 Fig. $CD1d^{-/-}$ mice have an early IFN-γ response that is comparable to B6 mice. Lung and serum IFN-γ levels were determined in naïve mice or at various time points p.i. as in Fig 8. Data are combined from 3 independent experiments (n = 15 mice/group). Plotted are mean ±SD.
(TIF)

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Author Contributions

Conceived and designed the experiments: TMH PG BBC DMD KLB DWM KMK SJ. Performed the experiments: TMH PG BBC DMD KLB. Analyzed the data: TMH PG BBC MAO DMD KLB DWM KMK SJ. Contributed reagents/materials/analysis tools: TMH PG BBC MAO DMD KLB DWM KMK SJ. Wrote the paper: TMH BBC MAO SJ.

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