A distinct subset of hyporesponsive intrahepatic NK cells is partially regulated by IL-10: implications for liver tolerance.

Matthew Gordon Lassen
Walla Walla, Washington

BS Microbiology, Brigham Young University, Aug 2002
MS Molecular Biology, Brigham Young University, Dec 2004

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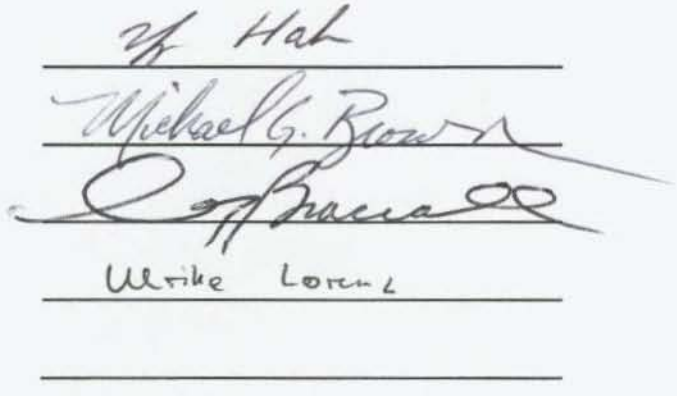

Abstract

The tolerogenic nature of the liver allows daily exposure to gut-derived foreign antigen without causing inflammation, but may facilitate the establishment of persistent infection in the liver. Natural killer (NK) cells make up a large proportion of the lymphocyte population in the liver, and are critical to both the innate and adaptive immune responses. The presence of immature NK cells in the liver has been reported, but the factor(s) involved in shaping the liver NK cell population remains unclear. We hypothesized that the immature phenotype observed on liver NK cells is due to liver specific regulation, leading to a lower functional capacity by liver NK cells.

Compared to splenic NK cells, liver NK cells display a decreased response to IL-12/IL-18 stimulation. Importantly, the liver contains a significant population of NK cells lacking expression of MHC class I-binding Ly49 receptors, whose expression correlates with greater IFN-γ production. A large percentage of intrahepatic $Ly49^-$ NK cells express high levels of the inhibitory receptor NKG2A, are poor producers of IFN-γ, and are the major source of IL-10 among liver NK cells. Splenic NK cells that migrate to the liver following adoptive transfer display phenotypic and functional changes, suggesting that the liver environment can modify NK receptor expression and functional responsiveness.

Importantly, IL-10 is present at high levels within the liver, and administration of IL-10 receptor blocking antibody resulted in a decreased percentage of intrahepatic $NKG2A^+Ly49^-$ NK cells. These data suggest that the liver environment regulates NK cell receptor expression, and that IL-10 is an important regulatory factor in dampening

the responsiveness of liver NK cells in part by maintaining a greater percentage of the hyporesponsive NKG2A^{+}Ly49^{-} NK cells in the liver.

Finally, we demonstrate through NK depletion studies that while CD80/CD86 expression on liver DCs is not affected by NK cells, the absolute number of the more mature liver DC population (CD45^{+}B220^{-}CD11c^{+}MHCII^{Hi} cells) increases following NK cell depletion. These data have important implications for not only liver tolerance but also for chronic liver disease such as chronic hepatitis C virus infection.

Dedication

This thesis is dedicated to my wife, Erika Jeanne Lassen,
and to my parents, Dwight Donald and Jeanette Mary Lassen

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There are many people who have played a part in the completion of the research presented in this dissertation. As I look back over the last several years, I can identify many individuals along the way that contributed to the path that led me to where I am today. Without the guidance and encouragement of these people, I'm sure that I would still be searching for the direction I should take my life. And now that I am looking forward to the next stage of my career, I find that the same thing is happening again. I am convinced that specific individuals and circumstances are placed in our path to guide us down the road where we can make the biggest difference. The key is to be open and humble enough to recognize and follow the signs in the road. I'm grateful to all those who have helped me along the way.

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Table of Contents

Abstract ..... **i**

Dedication ..... **iii**

Acknowledgements ..... **iv**

Table of Contents ..... **viii**

List of Figures ..... **x**

Abbreviations ..... **xii**

Chapter 1: Introduction **1**
NK cell development 1
NK cell activation 3
NK cell receptors 5
NK cell subsets 8
NK cells in defense against viral infection 10
NK cells and the hepatic microenvironment 12

Chapter 2: Statement of rationale and purpose **18**

Chapter 3: Materials and Methods **21**
Mice 21
Cell preparation 21
Flow cytometry and intracellular staining 22
Generation of bone marrow-derived dendritic cells 23
Co-culture assays 23
Adoptive transfer experiments 24
ELISAs 24
IL-10 receptor blockade experiments 25
NK depletion experiments 25
Viral infection experiments 25
Statistical analysis 26

Chapter 4: A distinct subset of hyporesponsive liver NK cells is regulated by IL-10 ..... **27**
Liver natural killer cell receptor expression profile 27
The liver contains a significant subset of NKG2A^{+} NK cells that lack Ly49 expression. 35
Liver NKG2A^{+}Ly49^{-} NK cells have higher cytotoxic potential and decreased capacity to produce IFN-γ in response to cytokine stimulation. 39
Liver environment suppresses functional capacity of NK cells. 56

IL-10 is responsible in part for the immature phenotype of the liver NK cell population....61
NKG2A^{+}Ly49^{-} display full functional competence following viral infection....64

Chapter 5: Impact of liver NK cells on liver DC function 76
NKG2A^{+}Ly49^{-} liver NK cells contain high amount of granzyme B and express elevated levels of CD69. 76
In vivo depletion of NK cells increases total number of mature liver DCs....79
NK cocultures with bone marrow-derived DCs....83
Impact of NK cell depletion on liver DCs 90

Chapter 6: Conclusions and Future Directions....98

Literature Cited....108

List of Figures

FIGURE 1. NK cell gating strategy. 28

FIGURE 2. NK cell receptor expression profile indicates enhanced NKG2A expression and fewer Ly49 expressing NK cells in the liver compared to spleen and blood NK cells. 30

FIGURE 3. Analysis of NK cell maturation markers indicates the liver contains an immature population of NK cells. 33

FIGURE 4. Analysis of NK cell subsets based on CD27 and CD11b expression. 36

FIGURE 5. Analysis of NK cell subsets based on NKG2A and Ly49 expression..... 40

FIGURE 6. Liver NK cells are less responsive to cytokine stimulation. 42

FIGURE 7. Ly49 expressing NK cells demonstrate a higher functional capacity.... 45

FIGURE 8. NKG2A+Ly49- NK cells are hyporesponsive to 5 hour cytokine stimulation...... 47

FIGURE 9. NK cell functional response following 24 hour cytokine stimulation.... 50

FIGURE 10. IFN-γ production by purified NK cells.. 52

FIGURE 11. IL-10 production by liver and spleen NK cells. 54

FIGURE 12. Splenic NK cells that migrate to the liver display greater immature phenotype...... 57

FIGURE 13. Splenic NK cells produce less IFN-γ following migration to the liver. 59

FIGURE 14. NK cells preferentially migrate back to organ of isolation...... 62

FIGURE 15. Liver contains high levels of IL-10. 65

FIGURE 16. IL-10R blockade shifts liver NK cell subsets to greater proportion of Ly49 expressing NK cells...... 67

FIGURE 17. IL-10R blockade does not significantly alter IFN-γ response by liver NK cells. 69

FIGURE 18. Liver NK cell subset composition shifts to greater proportion of Ly49 expressing NK cells following viral infection. 72

FIGURE 19. NKG2A+Ly49- NK cells are display full functional capacity following viral infection. 74

FIGURE 20. Intracellular granzyme B levels in NK cell subsets. 77

FIGURE 21. CD69 expression on NK cell subsets. 80

FIGURE 22. Analysis of bone marrow-derived dendritic cells. 84

FIGURE 23. Analysis of DC maturation following coculture with liver or spleen NK cells. 86

FIGURE 24. NK cell coculture does not modify costimulatory molecule expression of BMDCs. 88

FIGURE 25. Antibody-mediated NK cell depletion using anti-asialo GM-1. 91

FIGURE 26. Liver DC analysis following NK cell depletion. 93

FIGURE 27. Costimulatory molecule expression on liver DCs following NK depletion. 96

Abbreviations

APC	Antigen presenting cell
BMDC	Bone marrow-derived dendritic cell
CMV	Cytomegalovirus
DC	Dendritic cell
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
GM-1	Ganglio-n-tetraosylceramide
GmB	Granzyme B
GM-CSF	Granulocyte macrophage colony-stimulating factor
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HLA	Human leukocyte antigen
ICAM	Inter-cellular adhesion molecule
IFN	Interferon
IL	Interleukin
IL-10R	Interleukin-10 receptor
IP	Intraperitoneal
ITAM	Immunoreceptor tyrosine-based activating motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
IV	Intravenous
KC	Kupffer cell
KIR	Killer cell immunoglobulin-like receptor

KLRG	Killer-cell lectin-like receptor subfamily G
LPS	Lipopolysaccharide
LSEC	Liver sinusoidal endothelial cell
MCMV	Murine cytomegalovirus
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
NF-κB	Nuclear factor κB
NK	Natural killer cell
NKP	Natural killer cell precursor
PBS	Phosphate buffered saline
PD	Programmed death
PolyI:C	Polyinosinic:polycytidylic acid
PRR	Pathogen recognition receptor
SHP	Src homology 2 domain-containing phosphatase
TGF	Transforming growth factor
T_H	T-helper cell
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
VCAM	Vascular cell adhesion molecule
WT	Wildtype

Chapter 1: Introduction

NK cell development

Natural killer (NK) cells have been defined as lymphocytes that develop from bone marrow-derived NK cell precursors (NKPs) in an IL-15-dependent fashion (Di Santo 2006; Farag and Caligiuri 2006). NKPs are defined as precursor cells that possess the ability to differentiate solely into NK cells (Di Santo 2006). While there remains much to learn about NK cell development and maturation, several studies have provided a framework by which an outline of NK cell development stages have been proposed. The earliest committed NKP population in the mouse bone marrow has been described as $CD49b^{-}CD161^{-}CD122^{+}$ (Rosmaraki et al. 2001). NKPs develop into immature NK cells, which express CD161 (NK1.1), maintaining CD122 expression (Kim et al. 2002; Vosshenrich, Samson-Villeger and Di Santo 2005). Both immature and mature NK cells, as well as additional cell types including NKT cells and γδT cells, express NK1.1 (Di Santo 2006). Recently, the cytotoxicity receptor NKp46 has been shown to be expressed only on NK cells, and thus may represent a more specific pan-NK cell marker (Walzer et al. 2007). Immature NK cell development follows a stepwise acquisition of phenotypic markers, including early acquisition of CD94/NKG2 receptors, followed by Ly49 receptors and CD49b (Dx5). CD11b and CD43 also increase with maturation (Dorfman and Raulet 1998; Kim et al. 2002; Sivakumar et al. 1999; Williams et al. 2000).

Although all stages of NK cell development can be identified in the bone marrow, some studies suggest that the bone marrow may not represent the only site of NK cell development. Indeed, a recent study identified a population of thymic-derived NK cells

characterized by the expression of CD127, and dependent on the expression of the transcription factor GATA-3 (Vosshenrich et al. 2006). Furthermore, interleukin-7 (IL-7), while non-essential for NK cell development in the bone marrow, was shown to be critical for thymic-derived NK cell development. Whether thymic-derived NK cells develop from bone marrow-derived NKPs or from a distinct precursor cell remains to be determined. In addition, a distinct population of $NKp46^{+}CD3^{-}$ cells that were IL-15 independent was identified in the gut (Luci et al. 2009). Increasing evidence suggests that peripheral sites of NK cell development do exist, as immature NK cells have been identified in peripheral organs including the spleen and liver (Di Santo 2006). It remains unclear, however, at what stage developing NK cells may migrate from the bone marrow to peripheral organs, and what factor(s) may be regulating their trafficking. Importantly, the functional significance of distinct sites of NK cell development has yet to be demonstrated.

Immature NK cells that develop into mature NK cells have recently been characterized as either educated (also termed "licensed") or uneducated (Huntington, Vosshenrich and Di Santo 2007). An educated NK cell is defined as possessing full functional capacity; that is, an NK cell that responds to stimulation or activation receptor triggering. NK cell education appears to be regulated by signaling through MHC class I receptors, as NK cells that lack the expression of self-specific MHC class I receptors are functionally hyporesponsive (Fernandez et al. 2005; Kim et al. 2005). Indeed, NK cells isolated from MHC class I deficient mice display a lower functional capacity compared to NK cells from wild-type mice. Furthermore, the ITIM (located on the cytoplasmic tail of MHC class I inhibitory receptors) was shown to be critical for full functional capacity

of NK cells (Kim et al. 2005). These data initially were counterintuitive and appeared to be in conflict with the current understanding of NK cell tolerance, as the engagement of self-specific MHC class I receptors is known to be critical in maintaining NK cell tolerance in the face of activating receptor signaling (discussed in greater depth below).

Two models have been proposed to explain these observations, and have been termed the stimulatory receptor model and the disarming model (Raulet and Vance 2006; Yokoyama and Kim 2006). Both models are founded on the idea that NK cell education, or licensing, is aimed at ensuring that a mature NK cell is equipped with a functional inhibitory receptor that acts to control activating receptor signaling. A balance of intracellular signaling initiated through inhibitory and activating receptor engagement regulates NK cell activation, discussed in more detail below. The stimulatory receptor, or arming, model suggests that direct signaling through a MHC class I receptor during NK cell development supplies a signal that conveys functional capacity on the NK cell. The disarming model, similar to the concept of T cell exhaustion, suggests that, in the absence of inhibitory signaling via the MHC class I receptors, continual signaling through activating receptors renders the NK cell hyporesponsive. To date, the data is insufficient to determine which model is correct.

NK cell activation

The reports of the critical role for NK cell education through MHC class I receptors underscores the importance of the balance of anti- and pro-activation signals, mediated through the expression of inhibitory and activating receptors, respectively, that regulate NK cell activation. NK cell self-tolerance is achieved through the interaction of inhibitory receptors with MHC class I and class I-like molecules. Signaling through

inhibitory receptors leads to the phosphorylation of immunoreceptor tyrosine-based inhibition motifs (ITIMs) located on the cytoplasmic tail of inhibitory receptors (Lanier 2005). ITIMs are not unique to NK cell inhibitory receptors, but may be found in other receptors involved in inhibitory functions in numerous cell types (Ravetch and Lanier 2000). ITIM phosphorylation results in the recruitment of the protein tyrosine phosphatase Src homology 2 (SH2) domain-containing phosphatase-1 (SHP-1), leading to the suppression of activating signals received through activating receptor engagement (Binstadt et al. 1996). The cytoplasmic tails of NK cell activating receptors associate with the immunoreceptor tyrosine-based activating motifs (ITAM)-containing adaptor proteins DAP10 or DAP12, which results in signaling through the phosphatidylinositol 3-kinase pathway or Syk and ZAP70 tyrosine kinase pathways, respectively (Lanier 2009). Thus, NK cells are regulated via a balance of potentially continuous sensing of their environment through various activating and inhibitory receptors, with activation occuring when activating signals outweigh inhibitory signals, either through decreased inhibitory signaling or increased activating signaling.

NK cell effector functions include direct cell killing of infected or transformed cells, and secretion of a variety of cytokines, both pro- and anti-inflammatory (Hamerman, Ogasawara and Lanier 2005). The functional capacity of NK cells increases with increased maturation (Vosshenrich, Samson-Villeger and Di Santo 2005). In addition to activating receptor cross-linking, NK cell activation can also be induced by various cytokines, including type I interferons, IL-2, IL-12, IL-15, and IL-18 (Hamerman, Ogasawara and Lanier 2005). Distinct functions have been attributed to different cytokines, with some having synergistic effects. For example, following murine

cytomegalovirus (MCMV) infection of dendritic cells (DCs), type I interferons were required to induce NK cytotoxic activity, while deficiencies in IL-12 and IL-18 had no affect (Andoniou et al. 2005). In contrast, IL-12 and IL-18 are potent inducers of IFN-γ production. In addition, IL-15 has been shown to be important in NK cell "priming" following viral and bacterial infection through transpresentation of membrane-bound IL-15 on the surface of DCs (Lucas et al. 2007).

NK cells can mediate cell lysis via the production of perforin and granzymes, whose exocytosis is regulated by the interaction of multiple activating receptors (Bryceson et al. 2006). NK cells have also been reported to directly kill cells through TNF-related apoptosis-inducing ligand (TRAIL) expression (Takeda et al. 2001). Cytokine production by NK cells can be regulated through both inhibitory and activating receptors. Activating receptor engagement classically leads to production of IFN-γ, which plays a critical role in shaping the subsequent adaptive immune response, as well as tumor necrosis factor alpha (TNF-α) (Lanier 2005; Martin-Fontecha et al. 2004). Anti-inflammatory cytokine production, such as interleukin-10 (IL-10) and transforming growth factor-beta (TGF-β), can be mediated through activating as well as inhibitory receptors. In particular, NKG2A engagement can lead to IL-10 and TGF-β production (Jinushi et al. 2007).

NK cell receptors

As discussed above, NK cell function in mice and humans is regulated by the expression of numerous NK cell inhibitory and activating receptors. In mice, several NK cell receptors have been identified in the Ly49 receptor family with extensive polymorphism (Brown and Scalzo 2008). Ly49 receptors are type II transmembrane

glycoproteins that are expressed mainly on NK cells. Ly49 receptors are expressed as homodimers, with most NK cells expressing 1-4 individual Ly49 receptors. There are 14 identified mouse Ly49 receptors, with most being inhibitory receptors that recognize MHC class I molecules (Yokoyama and Plougastel 2003). Many of the ligands for the activating Ly49 receptors remain unknown, but include MHC class I and MHC class I-like molecules. Signaling through activating Ly49 receptors occurs via the DAP12 adaptor protein (Bakker et al. 2000; Smith et al. 1998). While structurally different from Ly49 receptors, a family of functional homologues of the Ly49 receptor molecules is expressed on human NK cells, referred to as the killer cell immunoglobulin-like receptor (KIR) family. Similar to Ly49 receptors, the KIR family includes both activating and inhibitory receptors (Lanier 2005). Inhibitory KIR consist of a long cytoplasmic tail containing an ITIM, and interact with MHC class I molecules. Activating KIR have short cytoplasmic tails and require the association of DAP12 for delivering a signal (Lanier et al. 1998a). The presence of activating KIR has been reported on effector and memory T cells, and may contribute to autoimmunity under certain conditions (Namekawa et al. 2000; Snyder et al. 2003).

The NKG2 group of receptors are c-type lectin-like receptors that are present in both humans and mice (Gunturi, Berg and Forman 2004). The NKG2 receptors include NKG2A, C, and E, (with NKG2F also expressed in humans) and are expressed at the surface as heterodimers with CD94. NKG2A is inhibitory, with an ITIM present in the cytoplasmic tail, while NKG2C and E receptor engagement results in an activating signal via the adaptor protein DAP12 (Lanier et al. 1998b; Le Drean et al. 1998). The ligand for NKG2A, C, and E is the nonclassical MHC class I molecule HLA-E in humans and Qa-

1b in mice. Expression of HLA-E and Qa-1b is dependent on peptides derived from classical MHC class I molecules and thus acts as an overall measurement of MHC class I expression (Vance, Jamieson and Raulet 1999; Vance et al. 1998). While Ly49 and KIR receptors are thought to be stably maintained once expressed, NKG2 receptor expression is more susceptible to modulation. In particular, IL-10 and TGF-β enhance expression of NKG2A (Jinushi et al. 2007). In addition, NKG2A can be induced on T cells following TCR engagement, and may have a role in limiting immunopathology by increasing the signaling threshold (McMahon et al. 2002; Moser et al. 2002).

Although initially described as being part of the NKG2 group of receptors, NKG2D has very little homology to the other NKG2 receptors. NKG2D does not bind CD94, but is expressed as a homodimer on both mouse and human NK cells (Wu et al. 1999). In mice, two isoforms of NKG2D are present, characterized by either a long or short cytoplasmic tail (Lanier 2005). The long-tailed isoform associates with the adaptor protein DAP10, while the short-tailed isoform can associate with DAP10 or DAP12. Expression of only the long-tailed isoform of NKG2D has been reported in humans. NKG2D is an activating receptor that can also be expressed by $CD8^{+}$T cells, and recognizes ligands induced by cellular stress on virally infected or transformed cells (Bauer et al. 1999). While most NK cells constitutively express NKG2D, the cytokine environment can modulate the level of expression, with IL-15 increasing expression, and TGF-β downregulating expression of NKG2D (Castriconi et al. 2003; Roberts et al. 2001; Sutherland et al. 2002).

Thus, inhibitory receptors include several of the Ly49 receptor family molecules (Ly49A, C, G2, I) in mice, the killer-cell immunoglobulin receptor (KIR) family (KLRL

receptors) in humans, and NKG2A (both mouse and human), which binds the MHC class I-like receptor Qa-1b in mice (HLA-E in humans). Activating receptors include the Ly49 receptor family molecules (Ly49D, H) in mice, the KIR family (KLRS receptors) in humans, and NKG2C/E and NKG2D (in both mouse and humans). Ligands for NK activating receptors include stress-inducible and virally encoded proteins.

NK cell subsets

NK cells were initially defined as lymphocytes that could respond non-specifically to transformed or virally infected target cells without prior sensitization. Over the last several years, scientists have gained an appreciation for the broader role that NK cells play in the overall immune response. As a part of the innate immune response, NK cells represent a first line of defense against viral infection, rapidly responding to and killing infected cells, controlling virus spread during the early response to infection (Hamerman, Ogasawara and Lanier 2005). Through production of a wide range of cytokines, NK cells contribute to the enhancement of the immune response by triggering further activation of additional innate cells and cytokine/chemokine production. NK cells also contribute to the shaping of the adaptive immune response and optimal T cell activation through direct cell-to-cell interactions with DCs, as well as cytokine production that can act directly on either the DC or T cell (Andrews et al. 2003; Martin-Fontecha et al. 2004). NK cells may also help shape the adaptive immune response through selective killing of DCs and/or T cells (Lu et al. 2007; Moretta 2002). Indeed, our understanding of the variety of roles that NK cells may play during an immune response continues to deepen, and has led to an appreciation for the existence of NK cell subsets.

In humans, differential functional capacity has been attributed to distinct NK cell subsets, based on the expression of CD16 and CD56. $CD16^{+}CD56^{Dim}$ NK cells are highly cytotoxic, and make up the bulk of the NK cell population in human peripheral blood (>90%), while $CD16^{-}CD56^{Bright}$ NK cells are poorly cytotoxic and produce higher amounts and a broader array of cytokines (IFN-γ, TNF-α, GM-CSF, IL-10) (Jacobs et al. 2001; Lanier et al. 1986). $CD16^{-}CD56^{Bright}$ NK cells may give rise to $CD16^{+}CD56^{Dim}$ NK cells, as suggested by shorter telomere length in $CD16^{+}CD56^{Dim}$ NK cells, and a rise in the percentage of peripheral blood $CD16^{-}CD56^{Bright}$ NK cells after bone marrow transplantation (Jacobs et al. 1992; Ouyang et al. 2007). A greater proportion of $CD16^{-}CD56^{Bright}$ NK cells are also found in lymph nodes, and may play an important role in driving a T_H1 response (Fehniger et al. 2003; Martin-Fontecha et al. 2004).

In mice, such well-defined NK cell subsets have not been established. Recently, surface expression of CD27 and CD11b has been used to define NK cell subsets on the basis of maturation and function (Hayakawa and Smyth 2006). Adoptive transfer experiments indicated a linear progression of maturation from $CD11b^{-}CD27^{+}$ to $CD11b^{+}CD27^{+}$ to $CD11b^{+}CD27^{-}$. With regards to function, $CD11b^{-}CD27^{+}$ NK cells produce higher amounts of cytokines and show decreased cytotoxicity compared to $CD11b^{+}CD27^{+}$ NK cells. Both of these subsets display a greater functional capacity than the more differentiated $CD11b^{+}CD27^{-}$ NK cells. $CD11b^{+}CD27^{-}$ NK cells also express higher levels of killer-cell lectin-like receptor subfamily G, member 1 (KLRG1), which is an inhibitory receptor that is induced following activation and proliferation (Robbins et al. 2002). In addition, $CD11b^{+}CD27^{-}$ NK cells are predominant in the blood, while $CD11b^{-}CD27^{+}$ NK cells are found at a greater proportion in lymph nodes. Whether the

use of these markers will prove as useful as CD56 and CD16 in humans remains to be determined.

Identifying phenotypically and functionally distinct NK cell subsets may prove increasingly important as our ability to distinguish subtle factors that influence the outcome of infection and cancer therapy continues to improve. In addition, the role that differential sites of maturation may play in the development of such subsets will be of equal importance in our understanding of NK cell biology and their impact on the immune response.

NK cells in defense against viral infection

NK cells play a central role in the innate immune response to intracellular pathogens and in shaping the adaptive response through their ability to directly lyse virally infected cells, secrete both pro-and anti-inflammatory cytokines, and interact with and influence the maturation of DCs. Perhaps the greatest evidence of the important role that NK cells play in the defense against viral infection is the number of mechanisms that viruses have evolved to avoid detection by NK cells. The following are a few examples of viral immunomodulatory mechanisms influencing NK cells; a more complete review of the subject has been published recently (Lodoen and Lanier 2005).

NK cells have been studied extensively in cytomegalovirus (CMV) infection, and multiple virally encoded proteins have been shown to influence the NK cell response. In murine CMV (MCMV), viral proteins gp40 and m155 downregulate the NKG2D stress-induced ligands Rae-1 and H60, respectively (Krmpotic et al. 2002; Lodoen et al. 2004). Gp40 also downregulates MHC class I expression (Ziegler et al. 1997). Interestingly, MCMV protein m157 acts as a ligand for two NK cell receptors, one activating and one

inhibitory (Arase et al. 2002; Smith et al. 2002). In MCMV-resistant C57BL/6 mice, m157 interacts with the activating Ly49H receptor, and has been shown to be critical to control of early viral infection. In contrast, NK cells from MCMV-susceptible 129/J mice lack Ly49H but interact with m157 through the inhibitory receptor Ly49I, which may result in not only the absence of direct viral recognition by NK cells but also induction of inhibitory signaling (Arase et al. 2002).

Human CMV (HCMV) also encode multiple proteins that potentially impact the NK cell response. While several proteins (US2, US3, US6, and US11) are involved in down-regulating MHC class I expression, potentially increasing susceptibility to NK cell targeting, a peptide derived from HCMV-encoded UL40 binds within the peptide groove of HLA-E and stabilizes its expression (Ulbrecht et al. 2000; Wang et al. 2002). In addition, HCMV-encoded UL16 prevents the surface expression of NKG2D ligands ULBP-1, ULBP-2, and MICB (Dunn et al. 2003; Rolle et al. 2003).

Human immunodeficiency virus (HIV)- encoded Nef protein selectively downregulates HLA-A and HLA-B, while leaving HLA-C and HLA-E expression intact (Cohen et al. 1999). As a monitor for overall MHC class I expression, HLA-E expression may play an especially important role in avoiding NK cell recognition. Indeed, a hepatitis C virus (HCV) core-derived peptide has been shown to bind HLA-E and stabilize its expression. In addition, plate-bound HCV E2 protein binds CD81 on NK cells, decreasing their activation (Crotta et al. 2002; Tseng and Klimpel 2002). It is important to note, however, that a recent report using intact HCV virions showed no impact on NK cell function, bringing into question the relevance of the prior studies (Yoon et al. 2009).

Overall, viruses appear to have evolved numerous mechanisms aimed at avoiding detection by NK cells, highlighting their important role in viral immunity.

NK cells and the hepatic microenvironment

The liver is a unique organ that is exposed daily to foreign antigen derived from food and commensal flora that traffic from the gut (Adams, Eksteen and Curbishley 2008; Crispe 2003). Indeed, an estimated seventy five percent of the liver's blood supply flows through the portal vein, which drains the gut. The removal of potential immunogens such as the gram-negative bacterial cell wall component lipopolysaccharide (LPS) flowing from the gut is a critical function of the liver, and requires a level of immune tolerance. The mechanisms underlying liver tolerance to such gut-derived antigen remain incompletely defined, but may include a role for regulatory T cells, elimination of activated T cells, and the production of immunosuppressive cytokines such as IL-10 (Bowen, McCaughan and Bertolino 2005; Crispe 2003; Goddard et al. 2004). The evidence to date suggests that the central players in maintaining liver tolerance are the antigen presenting cells (APCs). The potential APC populations in the liver include Kupffer cells and dendritic cells, as well as the liver sinusoidal endothelial cell (LSEC) and hepatocytes (Adams, Eksteen and Curbishley 2008; Crispe et al. 2006).

Hepatocytes constitute approximately 70% of total liver cells (Racanelli and Rehermann 2006). Hepatocytes may influence the immune response through multiple mechanisms. In response to IL-6, hepatocytes are major producers of acute phase proteins, which are transiently increased in the blood during acute inflammation. In addition, hepatocytes secrete complement factors and soluble pathogen recognition

receptors (PRRs) (Gao, Jeong and Tian 2008). Hepatocytes can also produce a number of cytokines, including IL-10 and TGF-β. Antigen presentation to both CD8 and CD4 T cells is thought to lead to apoptosis, probably by a death-by-neglect mechanism due to the lack of sufficient co-stimulatory molecule expression (Bertolino, Trescol-Biemont and Rabourdin-Combe 1998; Bertolino et al. 1999). Furthermore, direct hepatocyte interaction with NK cells has been shown to decrease the ability of NK cells to drive the maturation of DCs, leading to suboptimal T cell activation (Jinushi et al. 2007).

LSECs can present antigen to both CD4 and CD8 T cells, and constitutively express ICAM-1 and VCAM-1, which are upregulated on endothelial cells by pro-inflammatory cytokines and facilitate integrin-mediated adhesion of activated $CD8^+$ T cells (Knolle et al. 1999; Lohse et al. 1996). While LSECs have been shown to express the costimulatory molecules CD40, CD80, and CD86, they also constitutively express the PD-1 ligand, B7-H1 (Iwai et al. 2003). PD-1 expression is induced upon activation on $CD8^+$ T cells, and may be important for clearance of activated $CD8^+$ T cells. In addition, fenestrations in LSECs allow contact of T cells with hepatocytes and stellate cells (Crispe 2003).

NKT cells are abundant in the liver, and have been shown to play a central role in the response to tumor and pathogen in the liver (Exley and Koziel 2004; Toura et al. 1999). NKT cells can play a regulatory role through the production of a broad range of pro- and anti-inflammatory cytokines (Vincent, Gumperz and Brenner 2003). In addition, NKT cells, similar to cytotoxic T lymphocytes, mediate direct killing in a perforin dependent manner.

Kupffer cells are critical in endotoxin removal from the blood, and may play an important role in maintaining liver tolerance (Racanelli and Rehermann 2006). Although they can be found throughout the liver, Kupffer cells are found at a higher concentration around the periportal regions of the liver. A recent study demonstrated that LPS treatment of Kupffer cells resulted in high production of the anti-inflammatory cytokine IL-10, compared to polyI:C treatment, while the pro-inflammatory cytokine IL-12 was produced at similar levels in response to both TLR ligands (Tu et al. 2008). This study also suggested an important role for IL-10 in maintaining liver tolerance, and further demonstrated that IL-10 production by Kupffer cells led to decreased NK cell activation.

Several studies have indicated that the liver DC population displays an immature phenotype and are poor activators of T cells. Human liver DCs have been shown to secrete more IL-10, as compared to skin DCs, and were found to be less capable in stimulating T cell proliferation, leading to IL-10-producing T cells (Goddard et al. 2004). In contrast, skin DC activation of T cells resulted in greater proliferation and more IFN-γ production. Interestingly, DCs isolated from human hepatic lymph nodes have also been characterized by high IL-10 production and low allogeneic stimulatory capacity, compared to human inguinal lymph node DCs (Kwekkeboom et al. 2005). Murine liver DCs have also been described as less capable of stimulating T cells in comparison to spleen DCs (Pillarisetty et al. 2004). This report suggested that the differences may be due in part to differences in subset composition. Indeed, the liver reportedly contains a large proportion of plasmacytoid DCs, as well as a $B220^{-}CD11c^{hi}$ and $B220^{-}CD11c^{low}$ DC subsets, expressing high and low levels of costimulatory molecules, respectively (Jomantaite et al. 2004).

Multiple reports have investigated the role of NK cells during viral infection. The most comprehensive data has come from studies of MCMV infection. As noted earlier, MCMV employs multiple mechanisms aimed at avoiding NK cell recognition. A series of studies on the importance of NK cells for early control of MCMV infection in the liver provides a framework for NK cell function during viral infection of the liver. Viral infection of hepatocytes results in production of type I interferons, which induces production of macrophage inflammatory protein-1α (MIP-1α) (Salazar-Mather, Lewis and Biron 2002). MIP-1α is responsible for accumulation of NK cells to the liver, where IFN-γ production by NK cells leads to the expression of MIG and IP-10 (Salazar-Mather, Hamilton and Biron 2000; Salazar-Mather, Orange and Biron 1998). MIG and IP-10 in turn are involved in the recruitment of activated T cells to the liver. Thus, during viral infection, NK cells play a critical role in viral immunity in the liver. In addition to viral infection, NK cells also play an important role in tumor rejection in the liver, and the regenerative response to liver injury (Jinushi et al. 2005; Radaeva et al. 2006; Zwirner et al. 2007).

In contrast, studies relating to the phenotypic and functional capacity of the NK cell population in the naïve liver are more limited. Early reports of a liver-specific NK cell population identified in the rat liver were termed Pit cells, due to the observation of a large number of intracellular granules, which correlated with a high level of cytotoxic activity (Luo et al. 2000; Nakatani et al. 2004). Pit cells express CD69 and were described as an activated NK cell population (Luo et al. 2001). However, data concerning cytokine production and receptor expression on Pit cells is limited.

Other studies have noted the presence of an immature NK cell population within the liver. TRAIL has been reported as a marker of immature NK cells in the fetal and adult mouse liver (Takeda et al. 2005). In addition, fewer Ly49 expressing NK cells, as well as NK cells lacking Dx5 and CD11b expression, within the liver have also been noted (Daniels et al. 2001; Kim et al. 2002). Whether the immature NK cells observed in the liver represent a population that migrated from the bone marrow, the thymus, or, alternatively, developed intrahepatically from a distinct liver precursor cell, has not been determined. In addition, the factor(s) regulating the liver NK cell population are undefined.

Certain pathogens may exploit the tolerogenic environment of the liver in attempts to avoid immune clearance and establish persistent infection. Indeed, the liver is the primary site of persistence for the chronic viral hepatitis agents, hepatitis B (HBV) and hepatitis c (HCV) viruses (Guidotti and Chisari 2006). HCV is particularly intriguing in that an estimated 75-85% of acutely infected individuals are unable to clear the virus and progress to chronic HCV infection. A number of factors have been suggested in the ability of HCV to establish persistent infection, including the development of escape mutants due to the rapid degree of genetic mutation, the multiple reported immunomodulatory affects of the HCV core protein, and the binding of HCV E2 protein to CD81 on NK cells, suppressing their activation (Crotta et al. 2002; Cruise et al. 2005; Eisen-Vandervelde, Yao and Hahn 2004; Large, Kittlesen and Hahn 1999; Lukens et al. 2008; Waggoner, Hall and Hahn 2007).

Although there are conflicting reports, several studies have noted alterations in the NK cell population from chronic HCV patients, including frequency, phenotype and

function (Corado et al. 1997; De Maria et al. 2007; Jinushi et al. 2004; Meier et al. 2005). Expression of the inhibitory receptor NKG2A is reportedly increased on peripheral blood NK cells isolated from chronic HCV patients (Jinushi et al. 2004). In addition, NK cells from chronic HCV patients are less capable in activating DCs, compared to control NK cells. Recently, it was suggested that NK cells may play a central role in the ability of HCV to establish persistent liver infection, and that the NK-DC interaction may be at the core of the immunomodulatory effects targeted by HCV (Golden-Mason and Rosen 2006). Given the central role that DCs play in driving a strong and competent antiviral immune response, and the ability of NK cells to influence the maturation of DCs, disregulating the NK-DC interaction in favor of a weakened T cell response would be an effective means of establishing a persistent infection. It is plausible that HCV and other persistent liver pathogens may take advantage of tolerance mechanisms already in place within the naïve liver, thus emphasizing the importance of investigating such mechanisms that contribute to the daily tolerant liver environment.

Chapter 2: Statement of rationale and purpose

The liver represents a unique immunological environment due to daily exposure to gut-derived foreign antigen, providing a critical barrier to potential inflammation-inducing material. The immune tolerance of the liver has been noted for many years in the high rate of successful liver transplants observed in several species. Indeed, data from the U.S. Scientific Registry of Transplant Recipients indicates that the long-term graft survival (>3 years) for liver transplants between 2005 and 2007 was greater than 70%. This immunological privilege observed in the liver has received a tremendous amount of attention in the scientific community and has been the subject of numerous studies (Adams, Eksteen and Curbishley 2008; Crispe 2009; Knechtle and Kwun 2009). Perhaps even more intriguing is the ability of the liver to rapidly and effectively switch from an environment where T cell engagement typically results in immune tolerance and/or cell death to a strong pro-inflammatory site upon infection, leading to pathogen clearance. However, despite the ability of the liver to successfully clear many pathogens (e.g. MCMV, LCMV), some pathogens, including hepatitis B and hepatitis C (HCV) viruses, may exploit liver tolerance mechanisms in attempts to avoid immune clearance and establish persistent infection. A deeper understanding of the regulatory mechanisms responsible for the initial state of immune tolerance may shed light on potential factors targeted by persistent pathogens.

NK cells represent a large proportion of the lymphocyte population in the liver and may be key players in maintaining liver tolerance through their interactions with a variety of cell types and ability to secrete both pro- and anti-inflammatory cytokines.

Despite recent progress in our knowledge of the diverse functions delivered by NK cells and their vital impact on our defense against disease, the NK cell population in the liver remains relatively unexplored. As noted in chapter one, the liver contains several cell types that appear to contribute to the tolerant liver environment, including LSECs, Kupffer cells (KCs), DCs, and hepatocytes. These studies have enhanced our understanding of liver immunobiology and tolerance. We sought to further increase our knowledge of liver immunobiology by identifying factors regulating liver NK cells that contribute to a tolerant liver environment.

In a recent study, NK cell coculture with hepatocytes has been shown to alter the ability of DCs to prime $CD4^+$ T cells following NK-DC coculture, resulting in a regulatory T cell phenotype and function (Jinushi et al. 2007). Importantly, DC induction of the T cell regulatory phenotype was dependent on NKG2A engagement of NK cells during coculture with hepatocytes.

Interestingly, NKG2A expression is reportedly increased on NK cells from chronic HCV patients, suggesting a role for NKG2A in persistent viral infection in the liver (Jinushi et al. 2004). In addition, these NK cells isolated from chronic HCV patients were impaired in their ability to activate DCs, leading to suboptimal T cell activation, compared to NK cells from normal healthy donors. However, blockade of NKG2A restored the ability of NK cells from chronic HCV patients to activate DCs. Interestingly, NKG2A blockade in these experiments also correlated with lower IL-10 production by the NK cells.

The immunosuppressive role of IL-10 has been well established. With regards to IL-10 function in the liver, several studies have noted higher IL-10 production from liver

DC preparations, compared to other peripheral DCs, including DCs from the spleen and skin (Bosma et al. 2006; Goddard et al. 2004). Additionally, LPS treatment of KCs leads to increased IL-10 production compared to polyinosinic:polycytidylic acid (polyI:C) treatment, and that increased IL-10 production by LPS treatment dampens the ability of the KC to activate NK cells (Tu et al. 2008). In addition, NK-hepatic cell interactions via NKG2A-Qa-1b engagement lead to suboptimal T cell activation by DCs through increased IL-10 and decreased IFN-γ production (Jinushi et al. 2007).

In an effort to better understand the liver NK cell tolerogenic mechanisms potentially exploited by HCV and other persistent liver pathogens, we sought to characterize the receptor expression, subset composition, and functional capacity of the liver NK cell population. Our early observations of increased NKG2A expression in the liver as well as decreased IFN-γ production compared to spleen NK cells led to the hypothesis that the liver-specific factors dampen the NK cell response through increased NKG2A expression. We demonstrate in chapter four that the liver contains a distinct subset of $NKG2A^{+}Ly49^{-}$ NK cells that are functionally hyporesponsive. We further demonstrate that IL-10 contributes to the regulation of the liver NK cell population. In chapter five we investigate the influence of NK cells on the liver DC population through NK depletion studies and NK-DC coculture experiments, demonstrating that the number of mature DCs increases in the liver following NK cell depletion. Further elucidating the mechanisms regulating liver NK cells will increase our understanding of NK cell biology and the role of NK cells in the tolerogenic liver environment, as well as provide valuable insight into the design of novel therapeutic intervention strategies for chronic liver disease.

Chapter 3: Materials and Methods

Mice

C57BL/6 female mice, 6-10 weeks old, were obtained from Taconic. Macrophage inflammatory protein 1-α deficient (MIP1-$\alpha^{-/-}$) and Ly5.1 congenic female mice (both on a C57BL/6 background), 6-10 weeks old, were obtained from The Jackson Laboratory. Mice were housed in a pathogen-free facility at the University of Virginia, Charlottesville. All mice were handled according to protocols approved by the University of Virginia Institutional Animal Care and Use Committee.

Cell preparation

For isolation of intrahepatic leukocytes, the liver was perfused with PBS via the portal vein, followed by PBS plus 0.05% collagenase (Sigma-Aldrich) and then washed with IMDM (Invitrogen) supplemented with 10% newborn calf serum (wash buffer). The liver sections were passed through a wire screen and further digested with PBS plus 0.05% collagenase at 37°C for 20 minutes. Hepatocytes were removed by centrifuging at 40 x g for 4 minutes at 4°C. Mononuclear cells were purified on a 21% nycodenz gradient after centrifugation at 1100 x g for 20 minutes without braking. Splenocytes were prepared by mechanical disruption and isolation over a Ficoll (Atlanta Biologicals, Lawrenceville, GA) gradient centrifugation at 1250 x g for 20 minutes without braking. For preparation of purified NK cells, liver leukocytes and splenocytes were isolated as above, surfaced stained for CD3 and NK1.1, and sorted for $CD3^{-}NK1.1^{+}$ cells using a FACSVantage SE Turbo Sorter (Becton Dickinson) with purities > 98%. For lung cell preparations, lung tissue was finely minced and digested in 183 U/ml Type II collagenase (Worthington,

Lakewood, NJ) in IMDM at 37°C for 30-40 minutes. Lung tissues were then passed through a wire screen. For blood leukocyte preparations, mice were anesthetized with nebutal prior to tissue harvest and blood was collected via a heart stick. Red blood cells were removed by incubation in ACK lysis buffer for 2-3 minutes at room temperature.

Flow cytometry and intracellular staining

Cells were stained with antibodies against B7-H1, B220, CD3, CD11b, CD11c, CD27, CD43, CD45, CD69, CD80, CD86, CD117, CD127, Dx5, Granzyme B, IFN-γ, IL-10R, Ly5.1, Ly49C/I/F/H, Ly49G2, Ly49D, Ly49I, Ly49A, Ly49C/I, MHC class II, NK1.1, NKG2A, and NKG2D (all obtained from eBioscience or BD Biosciences). Anti-Ly49H was kindly provided by Michael Brown (University of Virginia, Charlottesville, VA). For measurements of intracellular levels of granzyme B, NK cells were stained directly ex vivo using Cytofix/Cytoperm (BD Biosciences, San Jose, CA) according to manufacturers instructions. Positive staining was determined by using appropriate isotype controls. For intracellular cytokine staining, NK cells were stimulated with recombinant mouse IL-12 (100 ng/mL) and IL-18 (100 ng/mL) (both from R&D Systems, Minneapolis, MN) in the presence of monensin (BD Biosciences) at a concentration of 1 x 10^6 cells/mL in IMDM containing 10% FBS at 37°C for 5 hours. Cells were stained for intracellular IFN-γ or IL-10 using Cytofix/Cytoperm according to manufacturers instructions. Positive staining was determined using unstimulated cells. All samples were run on a FACSCanto (Becton Dickinson) and analyzed using FlowJo software.

Generation of bone marrow-derived dendritic cells

Femurs were collected from C57BL/6 female mice. After removing the muscle, the femur was washed once in 75% EtOH for 2 minutes, and twice in RPMI for 2 minutes each. Bone marrow was isolated by cutting the ends of the femur and flushing the bone marrow into RPMI using a 23 gauge needle. Bone marrow was then transferred to a 50 mL tube using a 19 gauge needle. Bone marrow cells were pelleted at 1500 rpm for 6 minutes, and resuspended in pre-warmed DC media (RPMI containing 10% FBS and 20 ng/mL recombinant mouse GM-CSF) at approximately 10^7 cells/mL. Bone marrow cells were added to Petri dishes containing 10 mL pre-warmed DC media in a dropwise fashion to the center of each dish and incubated at 37°C and 5% CO_2. On day 3, 10 mL pre-warmed DC media was added to each plate. On day 6, the plates were removed from incubator, allowed to sit for 5-10 minutes, and then 10 mL media was carefully removed from each dish. 10 mL fresh prewarmed DC media was added to each dish. Non-adherant cells on day 6 were used in co-culture assays.

Co-culture assays

Bone marrow-derived dendritic cells (BMDCs) were generated as described above and used on day 6 for coculture assays. Prior to addition of NK cells, BMDCs were incubated with or without lipopolysaccharide (LPS) (1 μg/mL) or polyinosine-polycytidylic acid (polyI:C) (10 μg/mL) for 5-6 hours at 37°C. LPS and polyI:C was washed out prior to addition of NK cells. Purified $CD3^-NK\ 1.1^+$ cells were obtained from liver and spleen leukocyte preparations (described above) on a FACSVantage SE Turbo Sorter (Becton Dickinson). Purity was >98%. NK cells were cultured with BMDCs at a

1:2.5 ratio for 24 hours at 37°C, followed by surface staining for FACS analysis as described above.

Adoptive transfer experiments

Liver and spleen leukocytes were obtained from Ly5.1 congenic mice as described above. NK cells were enriched on an autoMACS Separator (Miltenyi Biotec) using a NK Cell Isolation Kit (Miltenyi Biotec) according to the manufacturers instructions. Approximately 1 x 10^5 $Ly5.1^+$ cells were transferred intravenously into wildtype (Ly5.2) C57BL/6 mice. For experiments involving the transfer of $Ly49^{Hi}$ and $Ly49^{Lo/Neg}$ NK cell subsets, splenocytes were isolated from wildtype $Ly5.2^+$ C57BL/6 mice. $Ly49^{Hi}$ and $Ly49^{Lo/Neg}$ NK cell subsets were sorted on a FACSVantage SE Turbo Sorter (Becton Dickinson), and transferred into naïve Ly5.1 congenic hosts. Transferred cells were detected by flow cytometry in livers and spleens 48 hours post-transfer. All samples were run on a FACSCanto (Becton Dickinson) and analyzed using FlowJo software.

ELISAs

Whole liver and spleen homogenates were obtained from naïve C57BL/6 female mice using a glass pestle. Cells and debris were pelleted at 1500 rpm for 6 minutes. Supernatants were carefully transferred to clean tubes and stored at -80°C. IL-10 levels were measured using a BD OptEIA mouse IL-10 ELISA kit (BD Biosciences, San Jose, CA) according to the manufacturers instructions. Due to significant background staining of liver homogenates, positive IL-10 levels were determined by subtracting background signal from wells containing everything except capture antibody.

IL-10 receptor blockade experiments

For blocking experiments, 250 μg anti-IL-10R (clone 1B1.3a) or rat IgG1 was injected intraperitoneally every two days for six days. Liver and spleen leukocytes were isolated as described above, two days following the last injection. Surface expression of NK cell receptors and intracellular staining for IFN-γ was performed as described above.

NK depletion experiments

For depletion of NK cells, mice were administered intraperitoneally with 250 μg of anti-asialo GM1 (α-GM1) (Wako Chemicals). Mice received up to three injections every 24 hours. Control mice received three injections of 250 μl of PBS intraperitoneally every 24 hours. Livers were harvested from NK-depleted mice at days 1, 2, and 3 of α-GM1 treatment. Each group consisted of three mice.

Viral infection experiments

For analyzing the phenotypic and functional impact of viral infection on liver NK cell subsets, wildtype and macrophage inflammatory protein 1-α deficient (MIP1-$\alpha^{-/-}$) C57BL/6 mice were injected intraperitoneally with 5 x 10^4 pfu of murine cytomegalovirus (MCMV) (Kindly provided by the laboratory of Michael Brown). After 48 hours, total leukocytes were isolated from infected livers, as well as uninfected control mice. Surface expression of NKG2A and Ly49 receptors, as well as intracellular IFN-γ levels following cytokine restimulation, was analyzed on gated NK cells as described in the flow cytometry and intracellular staining section of this chapter.

Statistical analysis

Student's *t* tests were used to evaluate the significance of the differences. A value of $p<0.05$ was regarded as statistically significant.

Chapter 4: A distinct subset of hyporesponsive liver NK cells is regulated by IL-10

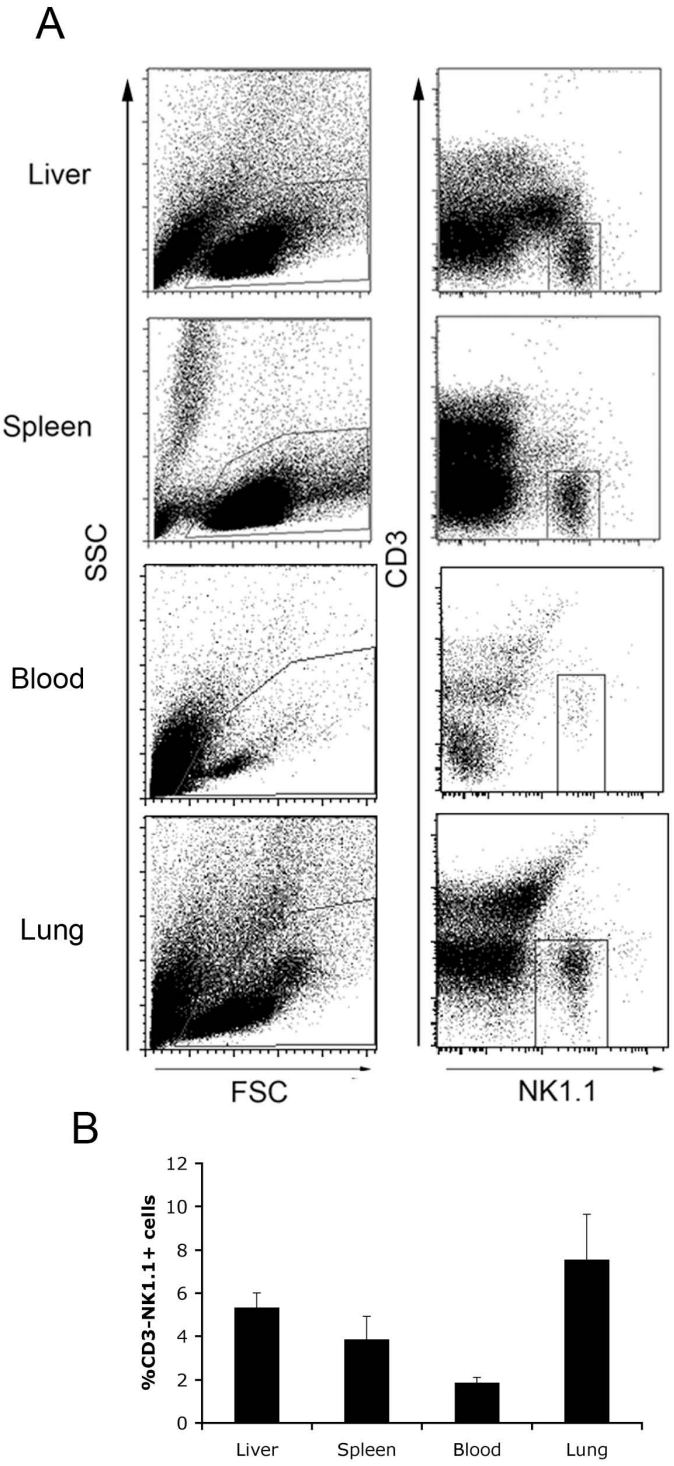
Liver natural killer cell receptor expression profile

NK cells display cell surface inhibitory receptors for self-MHC class I molecules, including Ly49 and NKG2A. Inhibitory receptors are involved in NK tolerance and recognition of transformed or infected target cells. Due to their prominent presence in the liver, as well as their ability to secrete both pro- and anti-inflammatory cytokines and influence the maturational state of DCs, NK cells may play a central role in the tolerogenic nature of the liver. To examine their cell surface expression profile, we stained liver NK cells for various NK cell receptors. Fig. 1 is representative of the gating strategy used to define NK cells throughout this study, and confirms the higher NK cell frequency compared to the spleen, lung, and blood that has been reported in published data.

As compared to splenic NK cells and in accordance with previous work, fewer liver NK cells express the Ly49 receptors tested, either inhibitory or activating (Fig. 2A). In addition, liver NK cells express higher levels of the inhibitory receptor NKG2A, while expressing lower levels of the activating receptor NKG2D. Similar to the spleen, NK cells isolated from the blood also express higher levels of Ly49 receptors and lower NKG2A than liver NK cells, suggesting that the liver environment may be influencing the receptor expression on NK cells (Fig. 2B & C). Alternatively, the distinct receptor expression profile displayed by the liver NK cell population could be the result of a difference in NK cell development or maturation site.

FIGURE 1. NK cell gating strategy. Liver, spleen, and lung tissues were harvested from 6-10 week old naïve female C57BL/6 mice. Blood was collected at the time of harvest via a heart stick. Total leukocytes were isolated and stained for CD3 and NK1.1 expression. (A) NK cells were defined throughout these studies as $CD3^-$ $NK1.1^+$ cells. (B) Quantification of the percentage of NK cells among total leukocyte population isolated from liver, spleen, lung, and blood. Data is representative of at least 3 independent experiments using at least 3 mice per group.

Fig. 1


A
Liver
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SSC
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CD3
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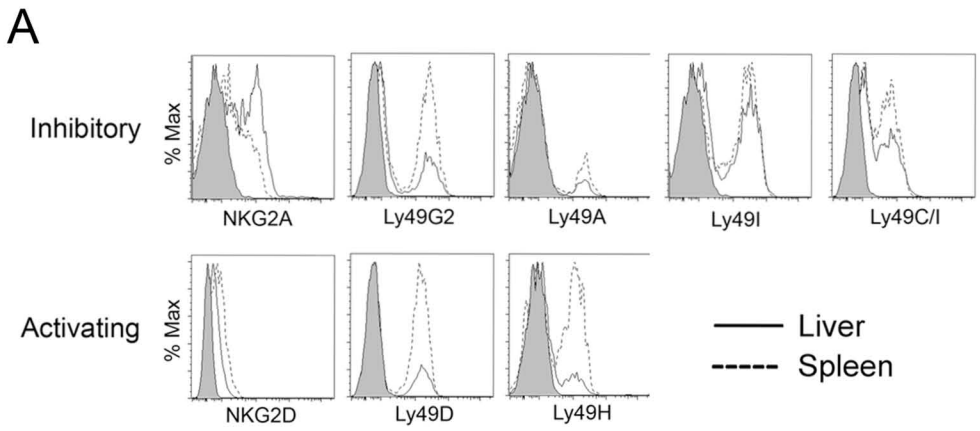
8
10
12
Liver
Spleen
Blood
Lung

FIGURE 2. NK cell receptor expression profile indicates enhanced NKG2A expression and fewer Ly49 expressing NK cells in the liver compared to spleen and blood NK cells. Liver and spleen tissue was harvested from 6-10 week old naïve female C57BL/6 mice. Blood was collected at the time of harvest via a heart stick. Total leukocytes were isolated and stained for various NK cell receptors. (A) Expression of various NK cell receptors on gated liver (solid line) and spleen (dashed line) NK cells. Shaded histograms represent isotype control staining. (B) Combined results from a single experiment demonstrating the expression of various NK cell receptors on NK cells isolated from the liver, spleen, and blood. (C) Combined results from a single experiment of mean fluorescent intensity of NKG2A staining on NK cells from the liver, spleen, and blood. Data is representative of at least 3 independent experiments using 3 mice per group.

Fig. 2


A
Inhibitory
% Max
NKG2A
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Activating
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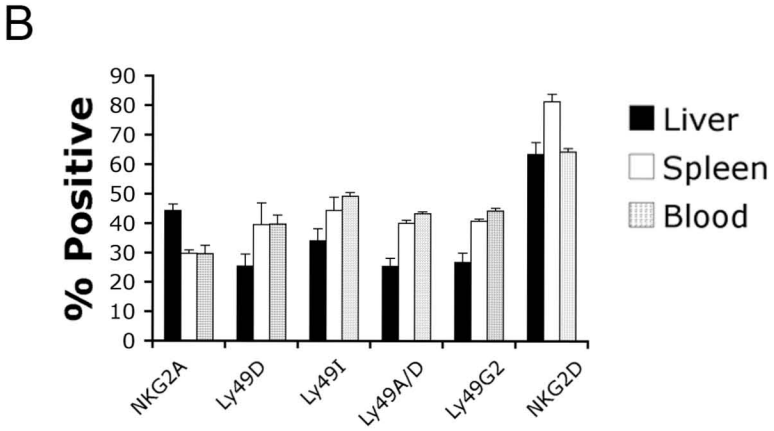
Liver
Spleen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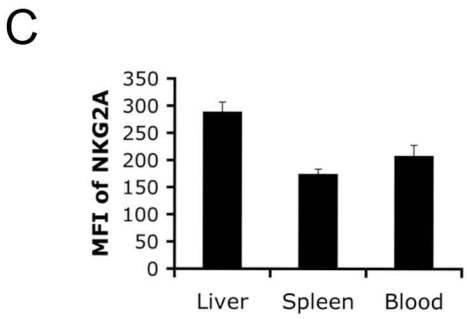
Liver
Spleen
Blood


C
MFI of NKG2A
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Liver
Spleen
Blood

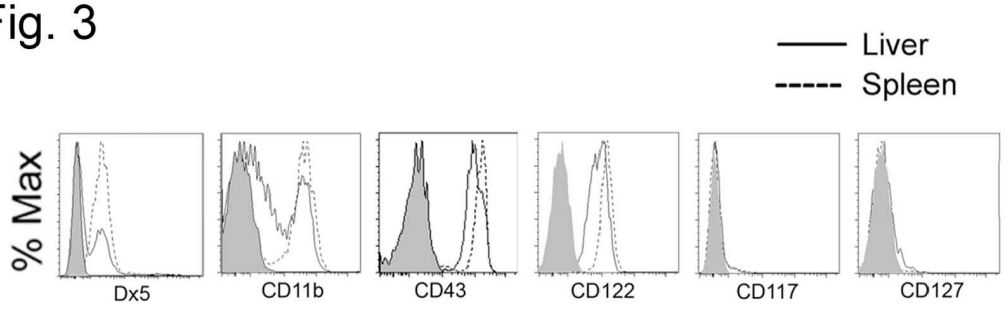
NK cell precursors (NKPs) may migrate from the bone marrow and undergo further development within the liver, resulting in the distinct NK cell receptor expression of liver NK cells. CD43 is a maturation marker present on mature NK cells, and should be absent on NKPs. Analysis of CD43 expression showed that all NK cells from the liver and spleen stain positive for CD43, suggesting that NKPs are not present in the liver (Fig. 3). However, most of the receptor expression during NK cell development has been characterized in the bone marrow. Very little is known about the nature of NKPs outside of the bone marrow, and what factors may influence their receptor expression after leaving the bone marrow.

A unique development pathway for NK cells has been described in the thymus. Thymic-derived NK cells are characterized by the expression of CD127 and are dependent on the expression of the transcription factor GATA-3 (Vosshenrich et al. 2006). In addition, fewer thymic NK cells express Ly49 receptors. Interestingly, a recent study reported that homing to the liver during *Listeria monocytogenes* infection was impaired in GATA-3 deficient mice (Samson et al. 2003). To analyze the potential role for thymic-derived NK cells in populating the liver, we stained for the expression of CD127. In accordance with previous reports, neither NK cells from the liver or spleen stained positive for CD127 expression (Fig. 3), suggesting that thymic-derived NK cells do not have a major impact on the liver NK cell population.

Developing NK cells have been shown to sequentially express NKG2A, followed by Ly49 (Kim et al. 2002); thus, the predominance of NKG2A expression and the relatively low expression of Ly49 receptors may reflect the presence of a population of immature NK cells in the liver. Indeed, the presence of immature NK cells in the adult

FIGURE 3. Analysis of NK cell maturation markers indicates the liver contains an immature population of NK cells. Expression of NK cell maturation markers on NK cells isolated from the liver (solid line) and spleen (dashed line). Shaded histograms represent isotype control staining. Data is representative of at least 3 independent experiments using 3 mice per group.

Fig. 3



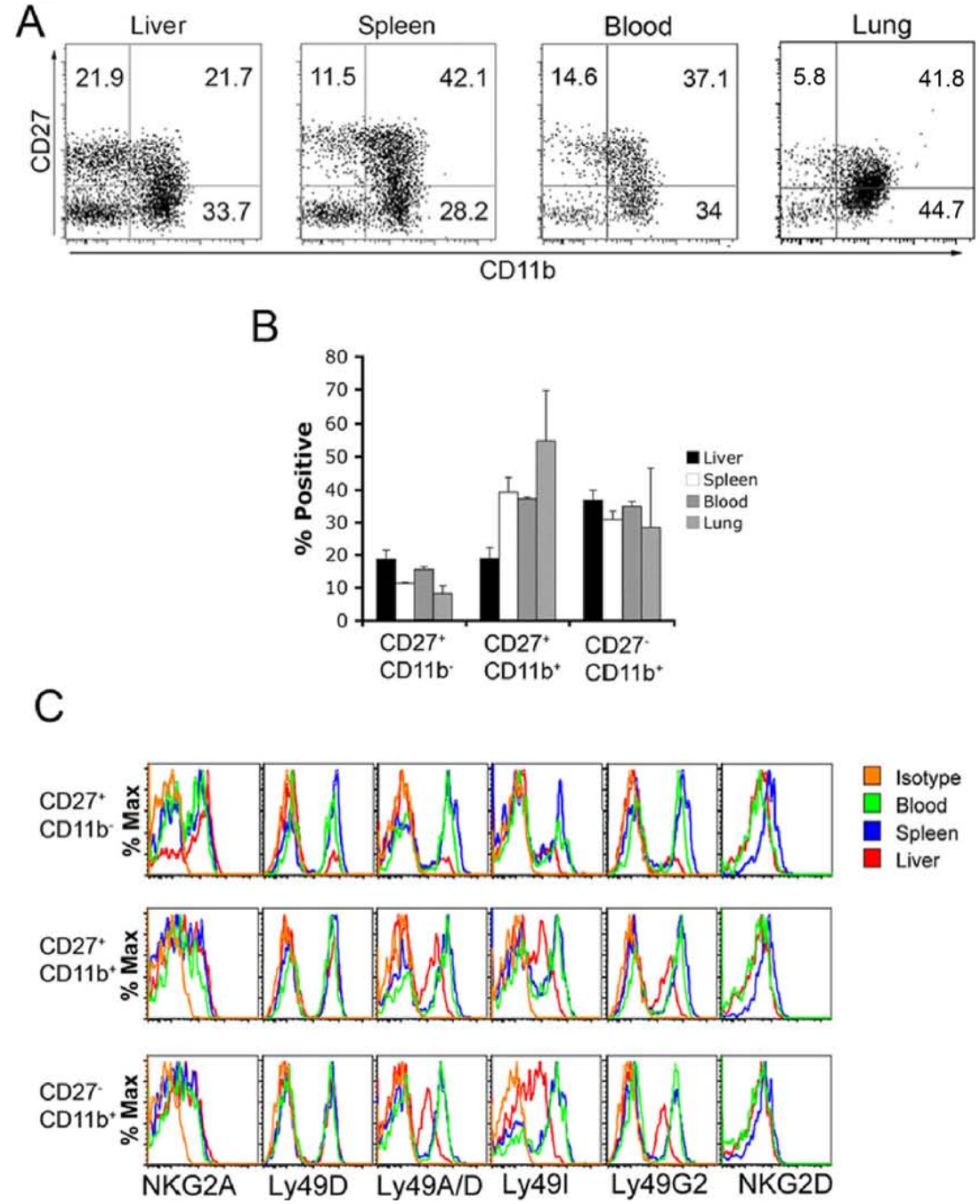
mouse liver has been reported (Kim et al. 2002; Takeda et al. 2005). Our data confirms a significant population of $Dx5^{neg}$ and $CD11b^{low}$ NK cells is found in the liver, but not the spleen (Fig. 3). This data seems to be contradictory to the expression of CD43, expressed on mature NK cells, found on all liver NK cells, suggesting that liver NK cells may represent a distinct NK cell population.

The liver contains a significant subset of $NKG2A^+$ NK cells that lack Ly49 expression.

As discussed earlier, the range of functions associated with NK cells may be explained by the existence of various NK cell subsets displaying distinct functional roles. The markers CD27 and CD11b have been used to separate NK cells based on maturation. A recent report showed that $CD27^+CD11b^-$ NK cells appear first after bone marrow reconstitution, followed by $CD27^+CD11b^+$ and then $CD27^-CD11b^+$ NK cells (Hayakawa and Smyth 2006). Our analysis confirmed that all CD27/CD11b subsets reported previously are present in the liver, spleen, lung, and blood (Fig. 4A). However, in contrast to published data (Hayakawa and Smyth 2006), we found that the liver contains a greater proportion of $CD27^+CD11b^-$ NK cells compared to spleen NK cells, which are almost entirely the more mature $CD27^+CD11b^+$ and $CD27^-CD11b^+$ subsets. The discrepancy between the two findings is unclear. Several studies have noted a greater percentage of immature NK cells in the liver compared to other peripheral organs, suggesting that an increased proportion of the less mature $CD27^+CD11b^-$ NK cell subset in the liver is not surprising. While most available data do not use CD27 and CD11b jointly in identifying NK cells in the liver, our findings are in line with current

FIGURE 4. Analysis of NK cell subsets based on CD27 and CD11b expression. (A) Liver, spleen, blood, and lung NK cell subsets defined according to surface expression of CD27 and CD11b. Plots represent expression on gated NK cells from total leukocyte isolations. (B) Combined results from a single experiment analyzing CD27/CD11b NK cell subsets. (C) Expression of NK cell receptors within CD27/CD11b NK cell subsets. Liver and spleen data is representative of at least 3 independent experiments using 3 mice per group. Blood and lung data is representative of 2 independent experiments using 3 mice per group.

Fig. 4


A
Liver
Spleen
Blood
Lung
21.9
21.7
33.7
11.5
42.1
28.2
14.6
37.1
34
5.8
41.8
44.7
CD27
CD11b
B
% Positive
80
70
60
50
40
30
20
10
0
Liver
Spleen
Blood
Lung
CD27+ CD11b-
CD27+ CD11b+
CD27- CD11b+
C
CD27+ CD11b-
CD27+ CD11b+
CD27- CD11b+
% Max
Isotype
Blood
Spleen
Liver
NKG2A
Ly49D
Ly49A/D
Ly49I
Ly49G2
NKG2D

published data in showing a population of NK cells with a less mature phenotype. Alternatively, the conflicting observations in the two reports could reflect differences in the liver cell preparations.

If the difference in receptor expression observed on the total liver NK cell population is due to a greater proportion of an immature NK cell subset, then the receptor expression profile within a particular NK cell subset should be identical, regardless of the organ where the NK cells were isolated from. When NK cell receptor expression was analyzed within NK subsets based on CD27 and CD11b expression, we found that receptor expression was similar among all NK subsets on NK cells from the spleen and blood. In contrast, liver NK cells display a unique receptor expression, most notably in the $CD27^{+}CD11b^{-}$ NK cell subset (Fig. 4C). While there were some differences observed among the different organs in the more mature $CD27^{+}CD11b^{+}$ and $CD27^{-}CD11b^{+}$ NK cells subsets, the greatest difference was seen in the $CD27^{+}CD11b^{-}$ NK cell subset.

As CD27 and CD11b appear limited in their utility to characterize the distinctive nature of the liver NK cell population, we sought for a novel means of separating the liver NK cells into subsets. NKG2A expression occurs early during NK cell development, followed by the acquisition of Ly49 receptors in a variegated manner, with mature NK cells typically expressing 1-4 Ly49 receptors (Joncker and Raulet 2008). As NKG2A is expressed on NK cells at high levels in the liver, while Ly49 receptor expression is decreased, we analyzed the proportion of liver NK cells expressing NKG2A but lacking expression of Ly49 receptors by staining with a cocktail of Ly49 receptors (C, I, F, H, G2, D). Our results showed that the liver contains a prominent subset of NK cells expressing NKG2A but lacking Ly49 receptor expression, while spleen, blood, and

lung NK cells are almost entirely positive for Ly49 expression (Fig. 5A). We further analyzed the proportion of $NKG2A^{+}Ly49^{-}$ NK cells within NK cell subsets defined by CD27 and CD11b expression (Fig. 5C). Our analysis revealed that increased positive staining for Ly49 receptors correlated with increased maturational state, as defined by CD27 and CD11b staining, in both the liver and the spleen. However, in all CD27/CD11b subsets analyzed, the liver contains a significant proportion of $NKG2A^{+}Ly49^{-}$ NK cells.

Taken together, the receptor expression profile of liver NK cells presented here supports that the liver contains a large population of NK cells that display an earlier maturation stage phenotype than other NK cells present in the periphery. In addition, these data suggest that the liver contains a distinct population of liver NK cells that displays a unique NK cell receptor expression profile.

Liver $NKG2A^{+}Ly49^{-}$ NK cells have decreased capacity to produce IFN-γ in response to cytokine stimulation.

To further define the liver NK cell population, we analyzed the functional attributes of liver NK cells compared to spleen NK cells. IL-12 and IL-18 are important cytokines in driving pro-inflammatory responses to pathogens, as well as potent activators and inducers of cytokine production by NK cells. Following stimulation with IL-12 and IL-18, a smaller proportion of liver NK cells produced IFN-γ as compared to spleen NK cells (Fig. 6A). Although the percentage of liver IFN-γ^{+} NK cells was not statistically different within a single experiment, the overall trend in multiple experiments consistently showed a lower percentage of liver NK cells produce IFN-γ (Fig. 6B). In

FIGURE 5. Analysis of NK cell subsets based on NKG2A and Ly49 expression. (A) Liver, spleen, blood, and lung NK cell subsets according to surface expression of NKG2A and a cocktail of Ly49 receptors (C/I/F/H/G2/D). (B) Combined results from a single experiment showing NK cell subset composition based on $NKG2A^{+}Ly49^{-}$ and $Ly49^{+}$ NK cells within the liver, spleen, blood, and lung. (C) NKG2A/Ly49 expression on $CD27^{+}CD11b^{-}$, $CD27^{+}CD11b^{+}$, and $CD27^{-}CD11b^{+}$ subsets of liver and spleen NK cells. Liver and spleen data is representative of at least 3 independent experiments using 3 mice per group. Blood and lung data is representative of 2 independent experiments using 3 mice per group.

Fig. 5

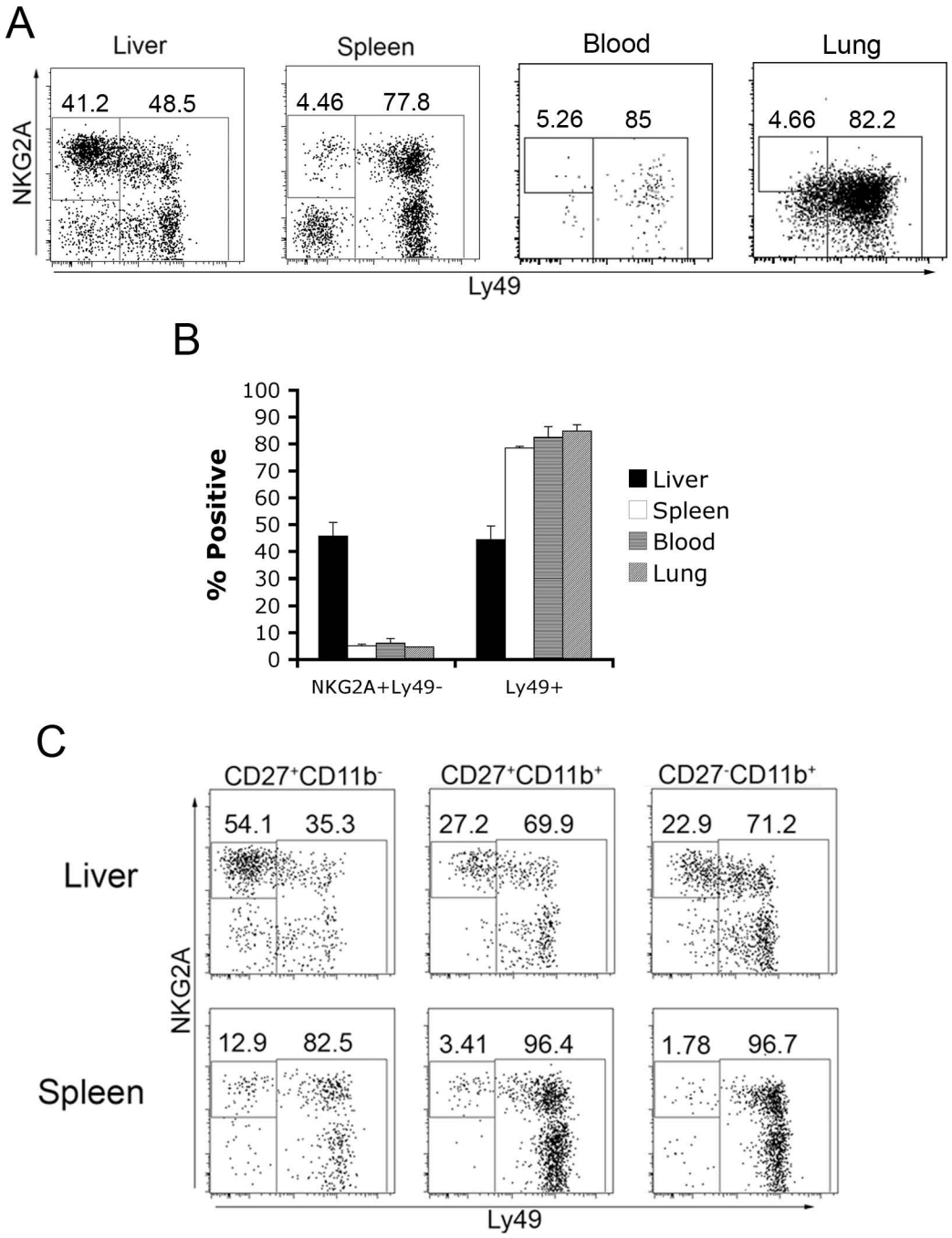
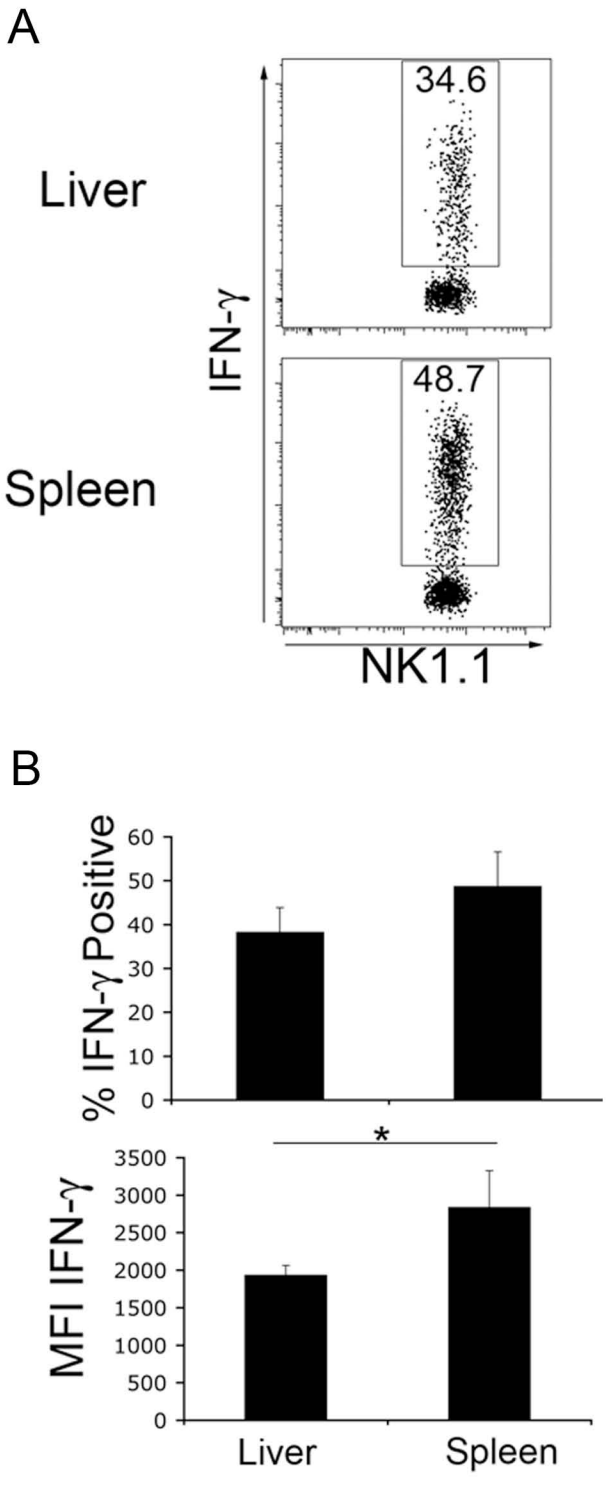

A
Liver
Spleen
Blood
Lung
41.2
48.5
4.46
77.8
5.26
85
4.66
82.2
NKG2A
Ly49
B
% Positive
100
90
80
70
60
50
40
30
20
10
0
Liver
Spleen
Blood
Lung
NKG2A+Ly49-
Ly49+
C
CD27+CD11b-
CD27+CD11b+
CD27-CD11b+
Liver
Spleen
54.1
35.3
27.2
69.9
22.9
71.2
12.9
82.5
3.41
96.4
1.78
96.7
NKG2A
Ly49

FIGURE 6. Liver NK cells are less responsive to cytokine stimulation. Total female C57BL/6 liver or spleen leukocytes were stimulated with IL-12 and IL-18, followed by intracellular staining for IFN-γ. Plots are gated on $CD3^{-}NK1.1^{+}$ cells. (A) IFN-γ production by total liver and spleen NK cells. (B) Combined results from a single experiment showing percentage of IFN-γ positive liver and spleen NK cells as well as mean fluorescent intensity of intracellular IFN-γ staining. Data is representative of 3 independent experiments using 3 mice per group. * = $p<.05$.

Fig. 6


A
Liver
Spleen
34.6
48.7
IFN-γ
NK1.1
B
% IFN-γ Positive
60
50
40
30
20
10
0
*
MFI IFN-γ
3500
3000
2500
2000
1500
1000
500
0
Liver
Spleen

addition, the mean fluorescence intensity of intracellular IFN-γ staining was significantly lower in liver NK cells (Fig. 6B).

Because recent studies have shown that surface expression of inhibitory NK receptors for self-MHC class I ligands coincides with acquisition of effector functions by individual NK cells (Yokoyama and Kim 2006), we considered that the liver NK cell population might be less functionally competent due to the larger proportion of NK cells lacking Ly49 receptor expression. When NK cells were gated according to Ly49 receptor expression, we found that Ly49 receptor expression did indeed correlate with greater IFN-γ production, in both the liver and spleen (Fig. 7). Thus, in accord with previous findings, liver and spleen NK cells expressing Ly49 receptors display functional competence.

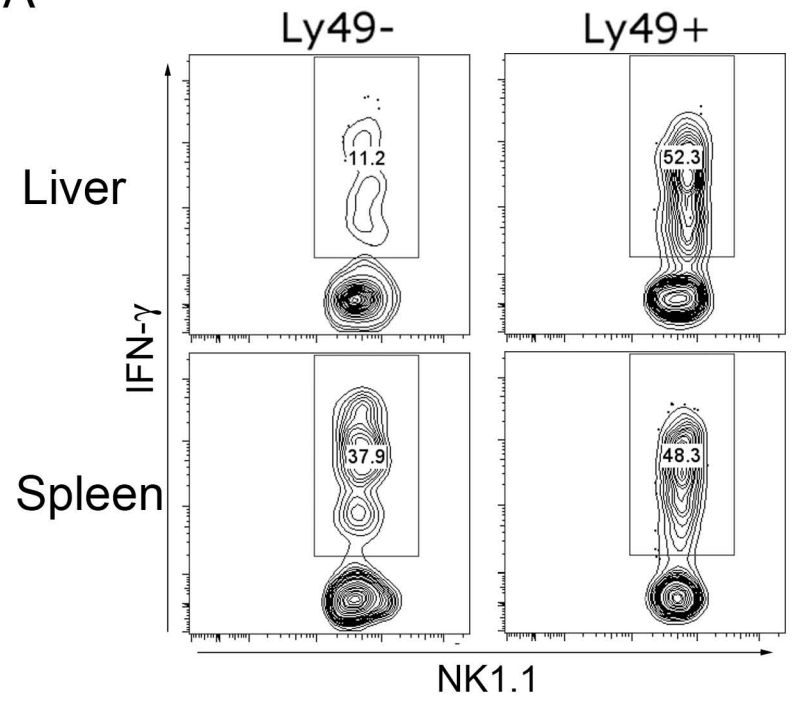
Due to the abundance of NK cells expressing NKG2A in the liver, we next examined the effect of NKG2A expression on the functional capacity of liver and spleen NK cells. As expected, liver and spleen NK cells lacking expression of both NKG2A and Ly49 receptors were hyporesponsive to stimulation, while those NK cells expressing Ly49 receptors readily produced IFN-γ (Fig. 8A & B). Furthermore, NKG2A expression contributed little, if any, additional control to NK cells with Ly49 receptors expressed since $NKG2A^{+}Ly49^{+}$ and $NKG2A^{-}Ly49^{+}$ NK cells in the liver and spleen produced comparable IFN-γ. However, the production of IFN-γ from $NKG2A^{+}Ly49^{-}$ NK cells in the liver was greatly reduced compared to spleen $NKG2A^{+}Ly49^{-}$ NK cells. We further found that the level of NKG2A expression is significantly higher on $NKG2A^{+}Ly49^{-}$ NK cells in the liver, compared to all other NKG2A expressing NK cells in the liver or spleen (Fig. 8C).

FIGURE 7. Ly49 expressing NK cells demonstrate a higher functional capacity.

(A) IFN-γ production in response to IL-12 and IL-18 stimulation by $Ly49^-$ and $Ly49^+$ (C/I/F/H/G2/D) liver and spleen NK cells. (B) Combined results from a single experiment showing intracellular IFN-γ staining by NK cell subsets gated according to Ly49 expression. Data is representative of 3 independent experiments using 3 mice per group. * = $p<.05$, *** = $p<.0005$.

Fig. 7

A


Ly49-
Ly49+
Liver
Spleen
IFN-γ
NK1.1
11.2
52.3
37.9
48.3

B

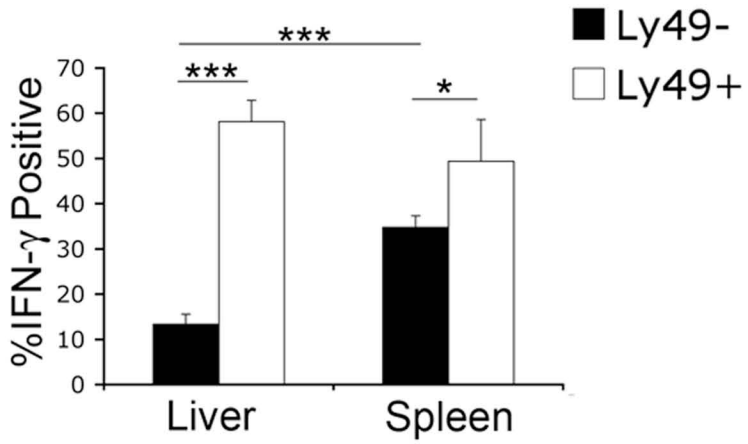
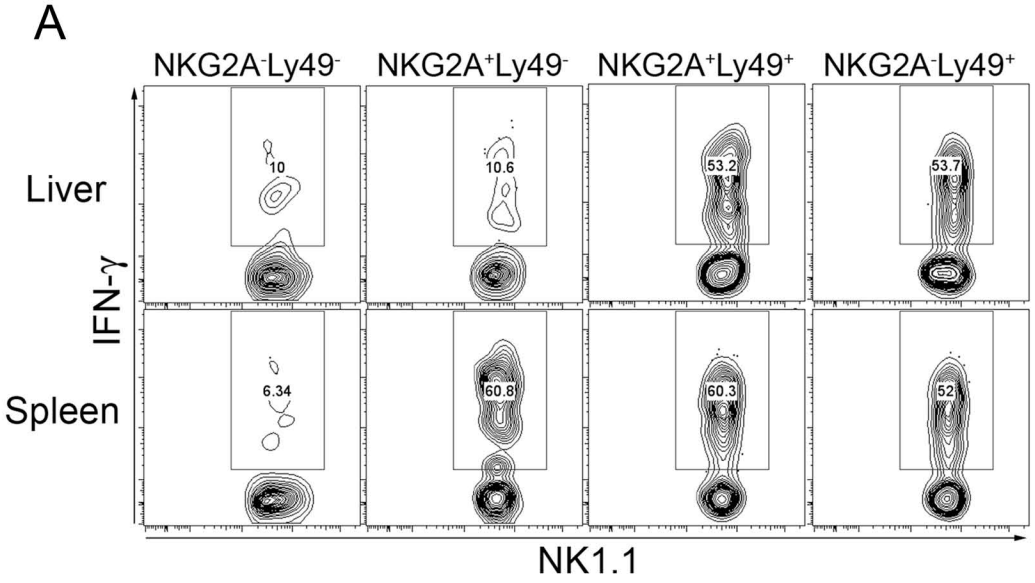
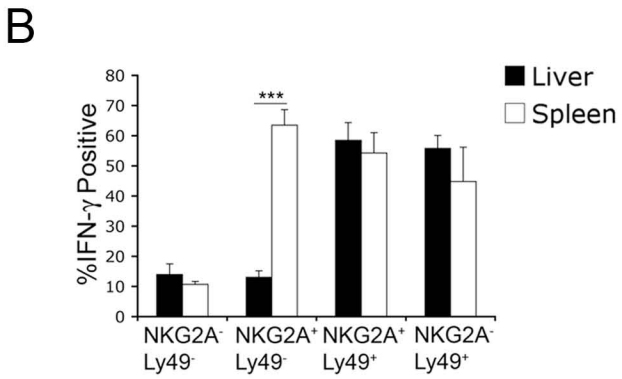
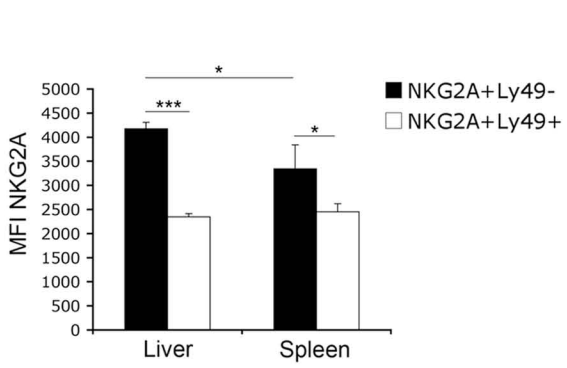

Ly49-
Ly49+
%IFN-γ Positive
0
10
20
30
40
50
60
70
***
***
*
Liver
Spleen

FIGURE 8. NKG2A+Ly49- NK cells are hyporesponsive to 5 hour cytokine stimulation. (A) IFN-γ production by NK cell subsets based on NKG2A and Ly49 expression on liver and spleen NK cells in response to IL-12 and IL-18 stimulation for 5 hours. (B) Combined results from a single experiment showing intracellular IFN-γ staining by NKG2A/Ly49 NK cell subsets. (C) Combined results from a single experiment of mean fluorescent intensity of NKG2A staining on $Ly49^-$ and $Ly49^+$ NK cells. Data is representative of three independent experiments containing 3 mice per experiment. * = $p<.05$, *** = $p<.0005$.

Fig. 8


A
NKG2A-Ly49-
NKG2A+Ly49-
NKG2A+Ly49+
NKG2A-Ly49+
Liver
Spleen
IFN-γ
10
10.6
53.2
53.7
6.34
60.8
60.3
52
NK1.1


B
%IFN-γ Positive
Liver
Spleen
***
NKG2A- Ly49-
NKG2A+ Ly49-
NKG2A+ Ly49+
NKG2A- Ly49+


C
MFI NKG2A
NKG2A+Ly49-
NKG2A+Ly49+
*
***
*
Liver
Spleen

To determine if prolonged cytokine exposure could reverse the hyporesponsive nature of NKG2A$^+$Ly49$^-$ liver NK cells, we stimulated liver and spleen cells with IL-12 and IL-18 for 24 hours. Intracellular staining for IFN-γ expression showed similar results as the 5 hour cytokine stimulations (Fig. 9). Thus, IL-12 and IL-18 stimulation appear to be insufficient for inducing IFN-γ production by NKG2A$^+$Ly49$^-$ liver NK cells.

Several studies have reported tolerogenic activity for various cell types within the liver, including DCs and KCs. Within the stimulation conditions used in Figs. 6-9, it is possible that additional intrahepatic cells may be dampening the ability of the liver NK cells to produce IFN-γ in response to IL-12/IL-18, or conversely, additional splenocytes enhancing the functional response of splenic NK cells. To assess the direct ability of liver NK cells to respond to cytokine stimulation, sorted liver and spleen NK cells were stimulated as above. Unexpectedly, sorted liver NK cells are dramatically less responsive to cytokine stimulation compared to spleen NK cells in all subsets analyzed (Fig. 10). These results suggested that the liver environment is suppressing the functional responsiveness of all NK cell subsets, and that cytokine stimulation alone is insufficient to overcome the suppression. However, cytokine stimulation in the context of additional liver leukocytes restores full functional competence in Ly49$^+$ liver NK cells, but not NKG2A$^+$Ly49$^-$ liver NK cells. These data further suggest that the stimulation requirements for the induction of IFN-γ differ among Ly49$^+$ and NKG2A$^+$Ly49$^-$ liver NK cells.

Since the NKG2A$^+$Ly49$^-$ NK cell subset in the liver is a poor producer of IFN-γ, we sought to determine if this subset produced other cytokines. NKG2A engagement on NK cells has been reported to result in the production of IL-10, therefore we analyzed

FIGURE 9. NK cell functional response following 24 hour cytokine stimulation. (A) IFN-γ production by total NK cell population from the liver and spleen. (B) IFN-γ production by NK cell subsets based on NKG2A and Ly49 expression on liver and spleen NK cells in response to IL-12 and IL-18 stimulation for 24 hours. (C) Combined results from a single experiment showing intracellular IFN-γ staining by NKG2A/Ly49 NK cell subsets. Data is representative of two independent experiments containing 3 mice per experiment. * = $p<.05$, *** = $p<.0005$.

Fig. 9

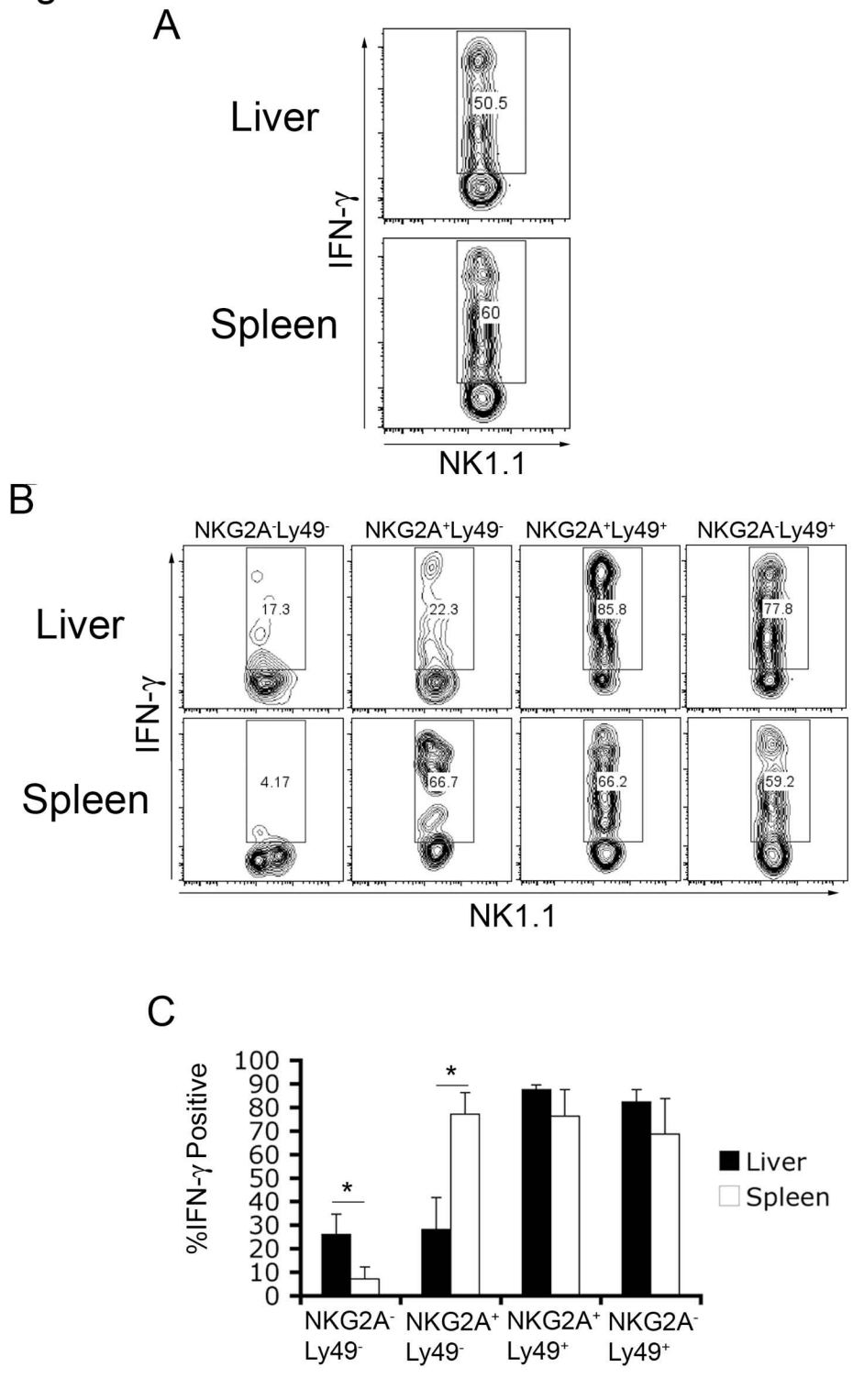

A
Liver
Spleen
IFN-γ
NK1.1
50.5
60
B
NKG2A-Ly49-
NKG2A+Ly49-
NKG2A+Ly49+
NKG2A-Ly49+
Liver
Spleen
IFN-γ
NK1.1
17.3
22.3
85.8
77.8
4.17
66.7
66.2
59.2
C
%IFN-γ Positive
100
90
80
70
60
50
40
30
20
10
0
*
*
NKG2A- Ly49-
NKG2A+ Ly49-
NKG2A+ Ly49+
NKG2A- Ly49+
Liver
Spleen

FIGURE 10. IFN-γ production by purified NK cells. Purified NK cells were obtained from liver and spleen leukocyte preparations from 6-10 week old C57BL/6 mice. (A) IFN-γ production by total purified NK cells in response to 5 hour IL-12 and IL-18 stimulation. (B) IFN-γ production by purified liver and spleen NK cells gated according to NKG2A and Ly49 expression in response to IL-12 and IL-18 stimulation for 5 hours. Data is representative of pooled purified NK cells from two independent experiments containing at least 6 mice per experiment.

Fig. 10

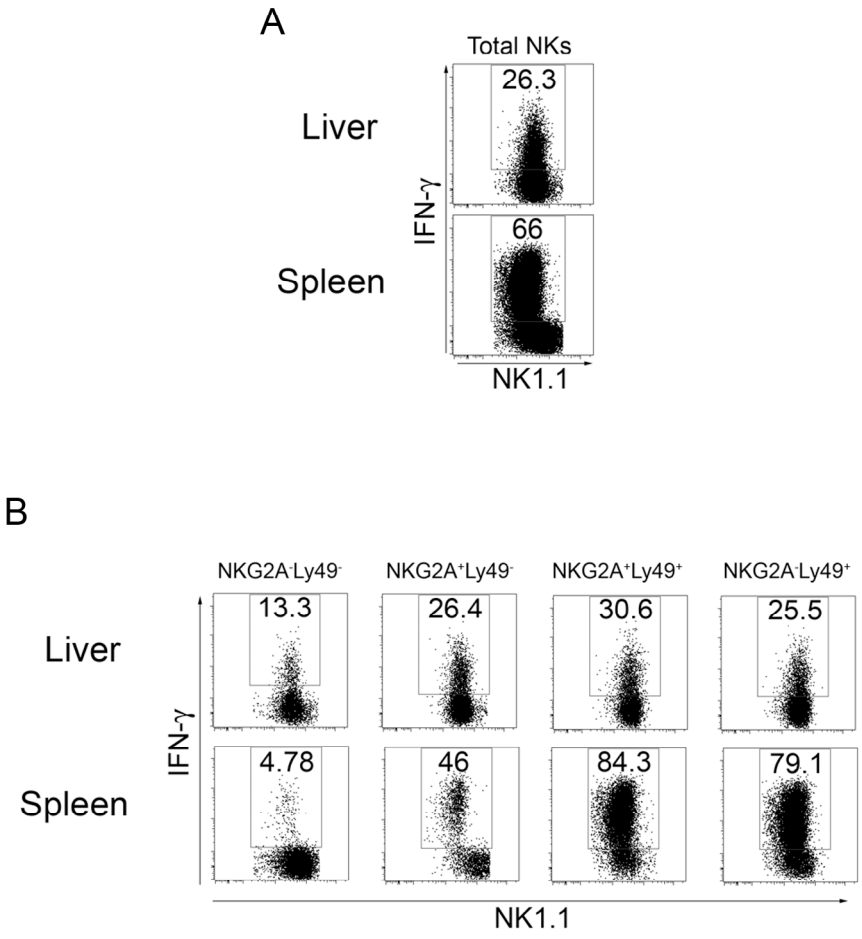
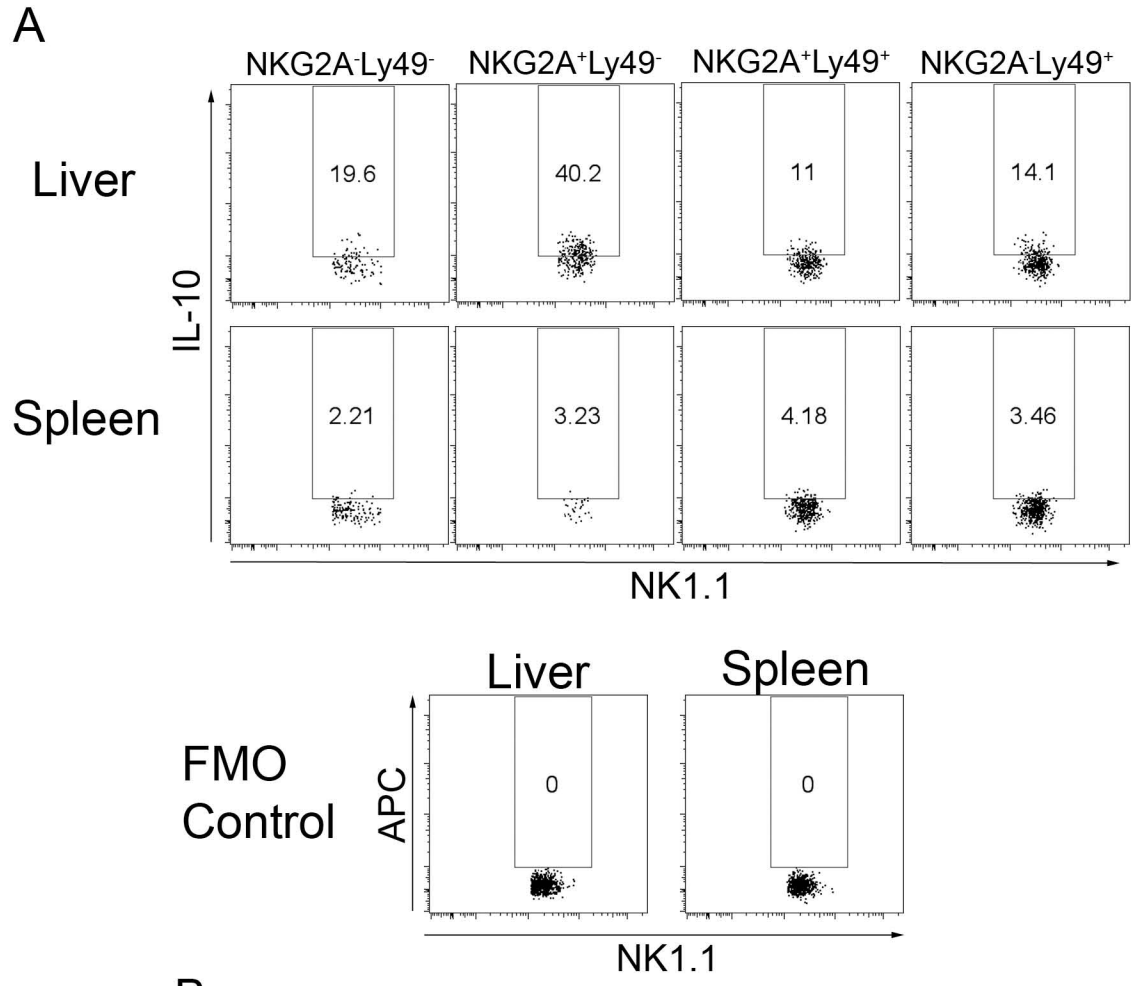

A
Total NKs
Liver
Spleen
26.3
66
IFN-γ
NK1.1
B
NKG2A-Ly49-
NKG2A+Ly49-
NKG2A+Ly49+
NKG2A-Ly49+
Liver
Spleen
13.3
26.4
30.6
25.5
4.78
46
84.3
79.1
IFN-γ
NK1.1

FIGURE 11. IL-10 production by liver and spleen NK cells. Intracellular expression of IL-10 was analyzed following IL-12 and IL-18 stimulation for 5 hours. (A) Intracellular IL-10 levels in NK cell subsets based on NKG2A and Ly49 expression on liver and spleen NK cells. FMO controls represent background staining for the APC channel on $CD3^{-}NK1.1^{+}$ gate. (B) Combined results from a single experiment showing intracellular IL-10 staining by NKG2A/Ly49 NK cell subsets. Data is representative of two independent experiments containing 3 mice per experiment.

Fig. 11


A
NKG2A-Ly49-
NKG2A+Ly49-
NKG2A+Ly49+
NKG2A-Ly49+
Liver
Spleen
19.6
40.2
11
14.1
2.21
3.23
4.18
3.46
IL-10
NK1.1
FMO Control
Liver
Spleen
0
0
APC
NK1.1
B
%IL-10 Positive
45
40
35
30
25
20
15
10
5
0
*
***
*
*
NKG2A- Ly49-
NKG2A+ Ly49-
NKG2A+ Ly49+
NKG2A- Ly49+
Liver
Spleen

their ability to produce IL-10 following IL-12/IL-18 stimulation. Our analysis showed that liver NK cells do stain positive for low levels of IL-10, while most spleen NK cells were negative (Fig. 11). Furthermore, $NKG2A^{+}Ly49^{-}$ NK cells appear to be the major producer of IL-10 among liver NK cells. Importantly, a dramatic difference in IL-10 production is seen between $NKG2A^{+}Ly49^{-}$ NK cells in the liver compared to the spleen.

These data provide further evidence that liver NK cells represent a distinct NK cell population, and suggest that the liver environment may be regulating the phenotype and function of liver NK cells. Additionally, the phenotype and functional capacity of $NKG2A^{+}Ly49^{-}$ NK cells appear to be diverse depending on the peripheral location.

Liver environment suppresses functional capacity of NK cells.

The receptor profile and lower functional responsiveness of the liver NK cell may be due to preferential retention/recruitment of less mature NK cells, or, alternatively, the liver environment may modify NK cell receptor expression and functional responsiveness. To address this question, we performed adoptive transfer experiments utilizing a CD45 (Ly5) mismatch. We examined the NK cell receptor expression on adoptively transferred splenic NK cells that had migrated to the liver or spleen 48 hours post-transfer. Adoptively transferred splenic NK cells ($Ly5.1^{+}$) that migrated to the liver showed a greater proportion of $NKG2A^{+}$ cells that lack Ly49 expression, while splenic NK cells that migrated back to the spleen were almost entirely $Ly49^{+}$ (Fig. 12A). In addition, a greater proportion of splenic NK cells that migrated to the liver display the less mature $CD27^{+}CD11b^{-}$ phenotype (Fig. 12B).

These data indicate that the liver can modify receptor expression on NK cells, potentially influencing their functional competence. Indeed, a lower proportion of

FIGURE 12. Splenic NK cells that migrate to the liver display greater immature phenotype. Splenic NK cells were isolated from 6-10 week old naïve female Ly5.1 congenic C57BL/6 mice. Approximately 6 x 10^5 $Ly5.1^+$ NK cells were adoptively transferred intravenously into C57BL/6 mice ($Ly5.2^+$). 48 hours post-transfer, liver and spleen leukocytes were isolated and stained for CD3, NK1.1, Ly5.1, and various NK cell receptors. Plots represent data from $CD3^-NK1.1^+$ gated cells. (A) Staining for NKG2A and Ly49 receptors on adoptively transferred ($Ly5.1^+$) and endogenous ($Ly5.1^-$) NK cells. (B) Staining for CD27 and CD11b molecules on adoptively transferred ($Ly5.1^+$) and endogenous ($Ly5.1^-$) NK cells. Data is representative of pooled samples of 2-3 mice per group in two independent experiments.

Fig. 12

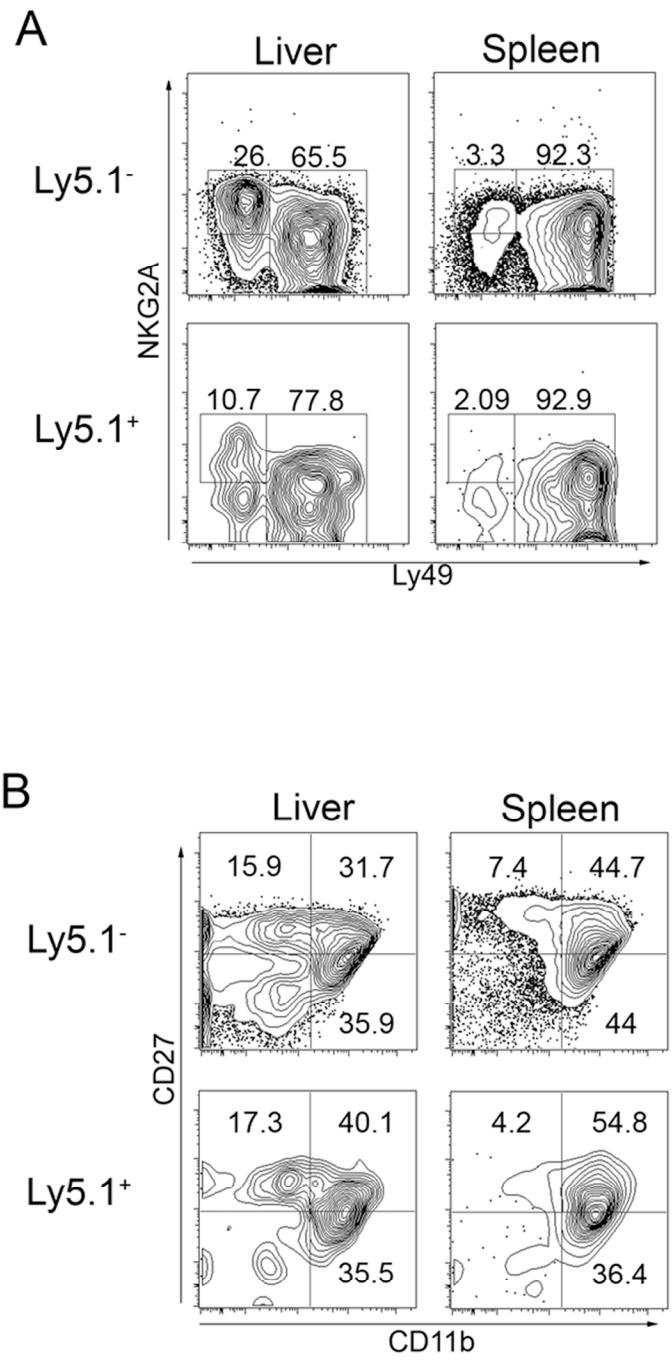
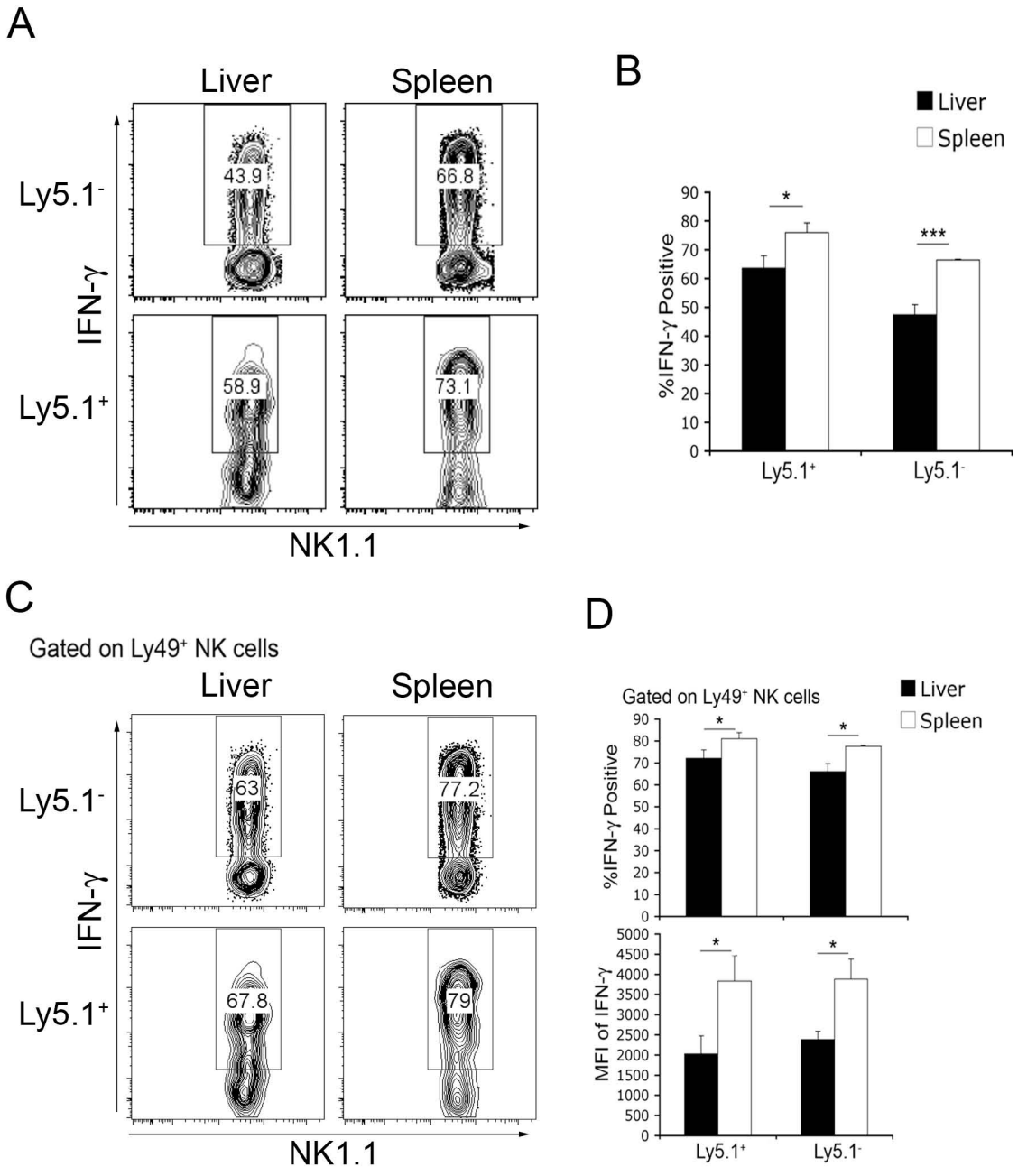

A
Liver
Spleen
Ly5.1-
Ly5.1+
NKG2A
Ly49
26
65.5
3.3
92.3
10.7
77.8
2.09
92.9
B
Liver
Spleen
Ly5.1-
Ly5.1+
CD27
CD11b
15.9
31.7
35.9
7.4
44.7
44
17.3
40.1
35.5
4.2
54.8
36.4

FIGURE 13. Splenic NK cells are impaired following migration to the liver. Splenic NK cells were enriched from $Ly5.1^+$ mice and adoptively transferred intravenously into C57BL/6 ($Ly5.1^-$) mice. $CD3^+NK1.1^-$ gated NK cells from liver and spleen were analyzed 48 hours following transfer. (A) Dot plots are representative of intracellular IFN-γ staining of transferred ($Ly5.1^+$) and endogenous ($Ly5.1^-$) total NK cells. (B) Combined results from a single experiment of IFN-γ production by transferred ($Ly5.1^+$) and endogenous ($Ly5.1^-$) total NK cell populations. (C) Representative dot plots of intracellular IFN-γ staining in gated $Ly49^+$ NK cells. (D) Combined IFN-γ results from transferred ($Ly5.1^+$) and endogenous ($Ly5.1^-$) $Ly49^+$ NK cells. Data is representative of two independent experiments with 2-3 mice per group. * = $p<.05$, *** = $p<.0005$.

Fig. 13


A
Liver
Spleen
Ly5.1⁻
Ly5.1⁺
IFN-γ
43.9
66.8
58.9
73.1
NK1.1
B
Liver
Spleen
%IFN-γ Positive
*
***
Ly5.1⁺
Ly5.1⁻
C
Gated on Ly49⁺ NK cells
Liver
Spleen
Ly5.1⁻
Ly5.1⁺
IFN-γ
63
77.2
67.8
79
NK1.1
D
Gated on Ly49⁺ NK cells
Liver
Spleen
%IFN-γ Positive
MFI of IFN-γ
Ly5.1⁺
Ly5.1⁻

splenic NK cells that migrated to the liver were IFN-γ^+ following IL-12/IL-18 stimulation (Fig. 13A & B). In addition, when IFN-γ production was examined in Ly49 expressing NK cells, both the percent positive as well as the mean fluorescence intensity was lower in those NK cells that migrated to the liver, compared to those that migrated to the spleen (Fig. 13C & D).

To further address the possibility of a preferential retention of immature NK cells within the liver, we sorted splenic NK cells into $Ly49^{Hi}$ and $Ly49^{Lo/Neg}$ subsets and transferred them into separate hosts. Regardless of Ly49 receptor expression, adoptively transferred spleen NK cells showed no preference to migrate to the liver over the spleen (Fig. 14A).

In contrast, when liver NK cells were adoptively transferred, they displayed a preference to migrate back to the liver. Indeed, transferred liver NK cells were virtually undetectable in the spleen (Fig. 14B). Interestingly, the transferred cells that migrate back to the liver were enriched for the $NKG2A^+Ly49^-$ subset of NK cells, suggesting distinct factors regulating the trafficking of specific NK cell subsets (Fig. 14C).

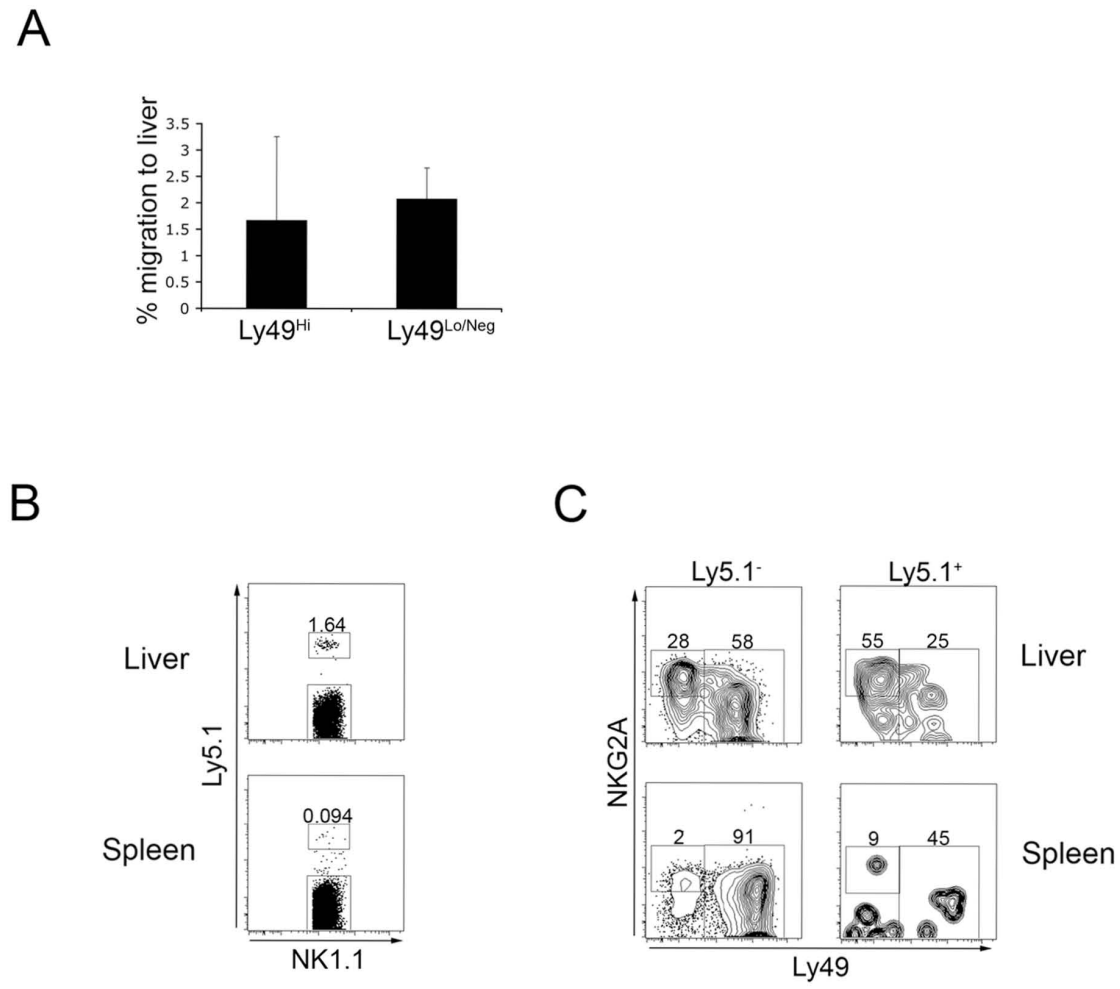
Taken together, these data suggest that the normal liver environment is sufficient to dampen spleen NK cell IFN-γ production and that $NKG2A^+Ly49^-$ NK cells are enriched in the liver following adoptive transfer of liver NK cells.

IL-10 is responsible in part for the immature phenotype of the liver NK cell population.

The immunosuppressive nature of IL-10 has been well documented in many facets of the immune response, and may be critical to the tolerogenic nature of the liver (Couper, Blount and Riley 2008). Indeed, a recent report suggested that high levels of

FIGURE 14. NK cells preferentially migrate back to organ of isolation. Splenic or liver NK cells were isolated from naïve female wildtype (Ly5.2) or Ly5.1 congenic, respectively, C57BL/6 mice, and transferred intravenously into Ly5 mismatched hosts. 48 hours post-transfer, liver and spleen leukocytes were isolated and stained for CD3, NK1.1, Ly5.1, and various NK cell receptors. Plots represent data from $CD3^{-}NK1.1^{+}$ gated cells. For adoptive transfer of $Ly49^{Hi}/Ly49^{Lo}$ NK cells, $Ly5.2^{+}$ splenic NK cells were sorted into $Ly49^{Hi}$ and $Ly49^{Lo/Neg}$ populations, and adoptively transferred separately into Ly5.1 congenic C57BL/6 mice. (A) Percentage of cells that migrated to the liver was calculated by dividing the absolute number of transferred cells that migrated to the liver by the absolute number of transferred cells that migrated to the spleen and liver ((liver absolute #/(spleen absolute # + liver absolute #)) x 100). Data represents combined data from two independent experiments. (B) $Ly5.1^{+}$ liver NK cells were adoptively transferred intravenously and detected in liver and spleen 48 hrs. post-transfer. (C) NKG2A and pan Ly49 staining on transferred ($Ly5.1^{+}$ NKs) and endogenous ($Ly5.1^{-}$) NK cells. Data is B & C is representative of two independent experiments with 2-3 mice per group.

Fig. 14


A
% migration to liver
3.5
3
2.5
2
1.5
1
0.5
0
Ly49Hi
Ly49Lo/Neg
B
Liver
Spleen
Ly5.1
NK1.1
1.64
0.094
C
Ly5.1-
Ly5.1+
NKG2A
Ly49
28
58
55
25
2
91
9
45
Liver
Spleen

IL-10 produced by KCs in response to LPS may contribute to liver tolerance by dampening the effect of pro-inflammatory cytokines present in the liver (Tu et al. 2008). Figure 15A shows that liver homogenates contain a high amount of IL-10, compared to spleen homogenates, as measured by ELISA. In addition, IL-10 receptor is expressed on both liver and spleen NK cells (Fig. 15B).

To analyze the role of IL-10 in the regulation of the liver NK cell population, we administered IL-10R blocking antibody every two days over a six day period. Unexpectedly, IL-10R blockade resulted in an increase of $Ly49^+$ NK cells in the liver (Fig. 16A & B). This increase in the proportion of $Ly49^+$ NK cells, and accompanying decrease in the proportion of $NKG2A^+Ly49^-$ NK cells, was specific to the liver compartment. In addition, there was no significant difference in the absolute number of NK cells between treatments (Fig. 16C).

Surprisingly, IL-10R blockade did not significantly increase the production of IFN-γ by liver NK cells in response to cytokine stimulation, in any of the NK cell subsets analyzed (Fig. 17). Although a trend in increased IFN-γ production was observed in NK cells from both the liver and the spleen of αIL-10R treated mice, most notably in Ly49 expressing NK cells, it did not reach statistical significance.

$NKG2A^+Ly49^-$ display full functional competence following viral infection.

Upon viral infection, the liver can switch from a tolerant environment to one that can support a strong pro-inflammatory response that results in viral clearance. To determine if viral infection is sufficient to restore functional competence in $NKG2A^+Ly49^-$ liver NK cells, we analyzed liver NK cells 48 hours following murine cytomegalovirus (MCMV) infection. Since MCMV infection is known to induce a rapid

FIGURE 15. Liver contains high levels of IL-10. (A) Liver and spleen tissue was isolated from naïve 6-10 week old C57BL/6 female mice and weighed prior to homogenization. Tissue was gently homogenized in iscove's media using a glass pestle. Cells and debris were then pelleted by centrifugation and supernatants collected and stored at -80°C. IL-10 levels were measured in triplicate by ELISA. Cytokine levels represent positive signal above background (wells containing no capture antibody). (B) IL-10 receptor expression on total liver (solid line) and spleen (dashed line) NK cells. Data is representative of three independent experiments using three mice. ** = $p<.005$.

Fig. 15

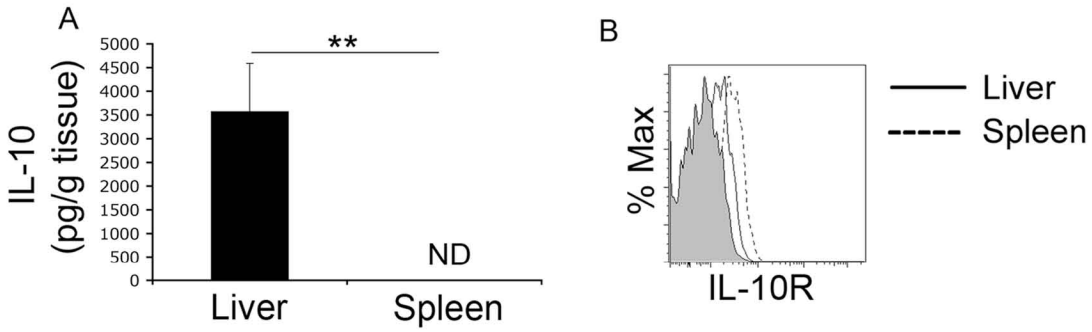

A
IL-10
(pg/g tissue)
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
**
ND
Liver
Spleen
B
% Max
IL-10R
Liver
Spleen

FIGURE 16. IL-10R blockade shifts liver NK cell subsets to greater proportion of Ly49 expressing NK cells. 6-10 week old female C57BL/6 mice were injected intraperitoneally with 250 μg of IL-10 receptor blocking antibody or isotype control every 2 days over a six day period. Total leukocytes from liver and spleen tissue were isolated. (A) Expression of NKG2A and Ly49 receptors on liver and spleen NK cells from control and anti-IL-10R treated mice. (B) Combined results from a single experiment showing $NKG2A^{+}Ly49^{-}$ and $Ly49^{+}$ NK cell subsets. (C) Combined results from a single experiment showing absolute numbers of $Ly49^{+}$ and $NKG2A^{+}Ly49^{-}$ NK cells following IL-10R blockade or control antibody injection. Data is representative of three independent experiments using three mice per group. * = $p<.05$.

Fig. 16

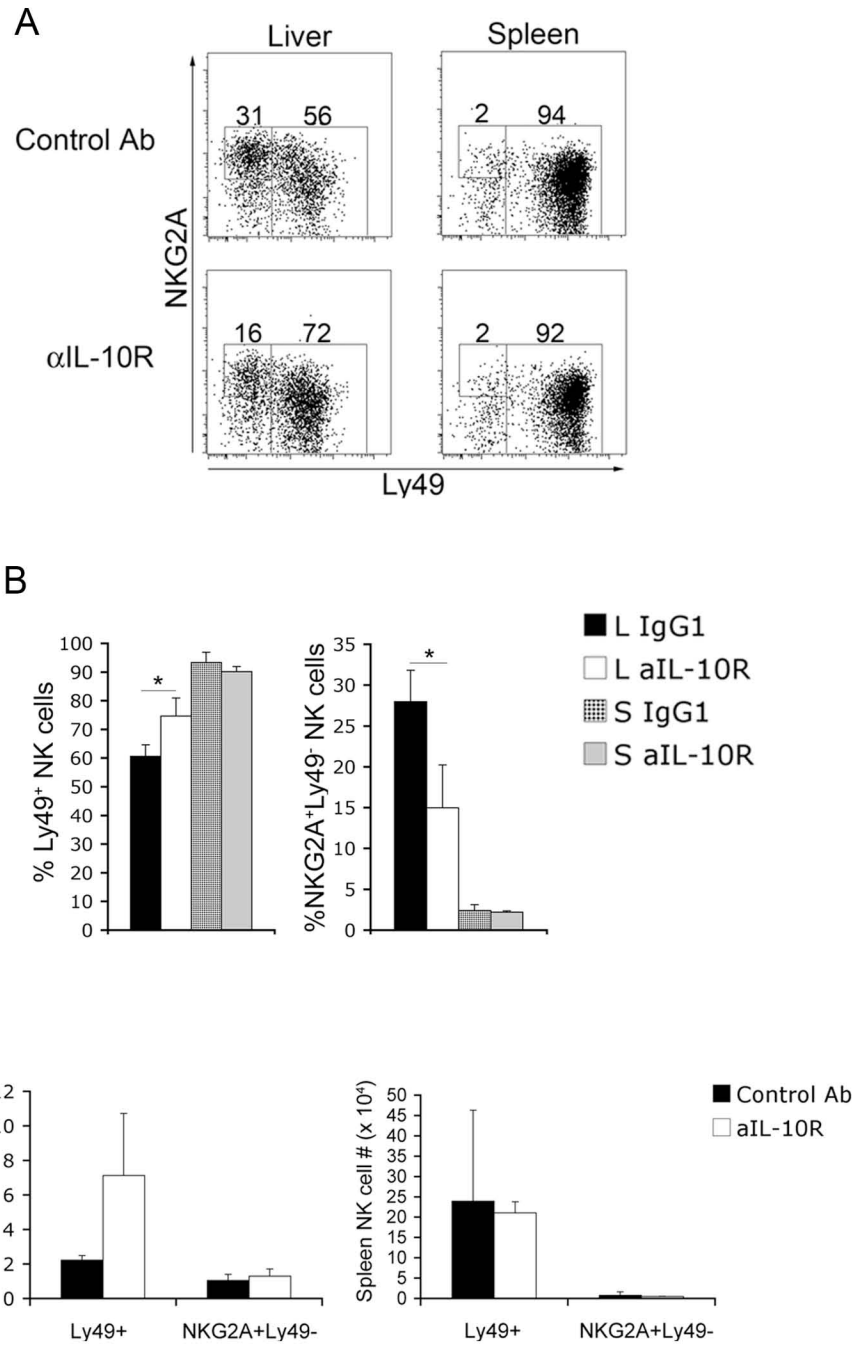
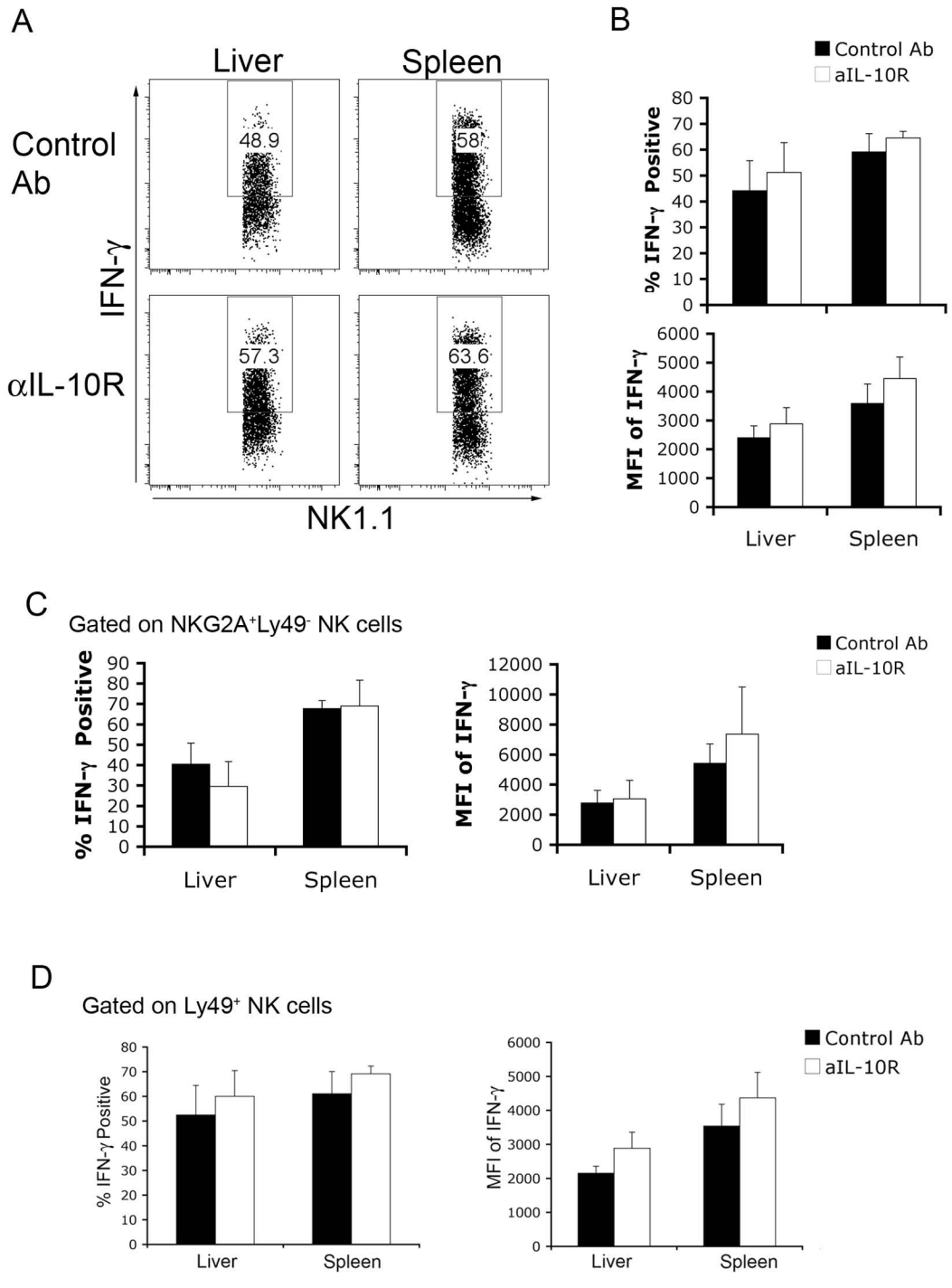

A
Liver
Spleen
Control Ab
αIL-10R
NKG2A
Ly49
31
56
2
94
16
72
2
92
B
% Ly49+ NK cells
%NKG2A+Ly49- NK cells
L IgG1
L aIL-10R
S IgG1
S aIL-10R
*
*
C
Liver NK cell # (x 10^4)
Spleen NK cell # (x 10^4)
Control Ab
aIL-10R
Ly49+
NKG2A+Ly49-
Ly49+
NKG2A+Ly49-

FIGURE 17. IL-10R blockade does not significantly alter IFN-γ response by liver NK cells. (A) IFN-γ production by total liver and spleen NK cells. (B) Combined results from a single experiment showing percentage of IFN-γ positive liver and spleen NK cells as well as mean fluorescent intensity of intracellular IFN-γ staining from control and anti-IL-10R treated mice. (C) Combined results of a single experiment displaying the percentage of IFN-γ^+ NK cells and the mean fluorescence intensity of intracellular IFN-γ staining in liver and spleen NKG2A$^+$Ly49$^-$ NK cells from control and anti-IL-10R treated mice. (D) Combined results of a single experiment displaying the percentage of IFN-γ^+ NK cells and the mean fluorescence intensity of intracellular IFN-γ staining in liver and spleen Ly49$^+$ NK cells from control and anti-IL-10R treated mice. Data is representative of two independent experiments using 3 mice per group.

Fig. 17


A
Liver
Spleen
Control Ab
αIL-10R
IFN-γ
48.9
58
57.3
63.6
NK1.1
B
Control Ab
aIL-10R
% IFN-γ Positive
MFI of IFN-γ
Liver
Spleen
C
Gated on NKG2A+Ly49- NK cells
% IFN-γ Positive
MFI of IFN-γ
Control Ab
aIL-10R
Liver
Spleen
D
Gated on Ly49+ NK cells
% IFN-γ Positive
MFI of IFN-γ
Control Ab
aIL-10R
Liver
Spleen

recruitment of NK cells to the liver, we also analyzed the effect of viral infection on liver NK cells in mice deficient for MIP1-α (MIP1-$\alpha^{-/-}$), which is critical for the recruitment of NK cells to the liver in MCMV infection (Salazar-Mather, Orange and Biron 1998). As shown in Fig. 18, the percentage of NKG2A^{+}Ly49^{-} NK cells in the liver decreases significantly following MCMV infection, in both wildtype (WT) and MIP1-$\alpha^{-/-}$ mice. However, the mechanism responsible for the observed decrease in the percentage of NKG2A^{+}Ly49^{-} NK cells in the liver is unclear.

We further analyzed the functional capacity of liver NK cells 48 hours following MCMV infection in WT and MIP1-$\alpha^{-/-}$ mice. In response to IL-12 and IL-18 stimulation, NKG2A^{+}Ly49^{-} from both WT and MIP1-$\alpha^{-/-}$ MCMV infected mice displayed full functional competence, as assessed by IFN-γ production (Fig. 19A & B). Importantly, this increased functional response correlated with a decrease in NKG2A expression on NKG2A^{+}Ly49^{-} NK cells (Fig. 19C). In addition, a greater percentage of Ly49 expressing NK cells also produce IFN-γ in response to cytokine stimulation following viral infection.

These data suggest that upon viral infection, a significant proportion of NKG2A^{+}Ly49^{-} liver NK cells may acquire Ly49 expression. Furthermore, the NKG2A^{+}Ly49^{-} NK cells still present in the liver following viral infection are fully competent in their ability to produce IFN-γ. Thus, while the normal liver appears to suppress the functional responsiveness of NKG2A^{+}Ly49^{-} NK cells, the virally infected liver can restore functional competence.

FIGURE 18. Liver NK cell subset composition shifts to greater proportion of Ly49 expressing NK cells following viral infection. Wildtype (WT) C57BL/6 or MIP1-$\alpha^{-/-}$ mice on the C57BL/6 background were injected intraperitoneally with 5 x 10^4 pfu of murine cytomegalovirus (MCMV). Liver leukocytes were harvested 48 hours post-injection and stained for NK cell subsets. (A) Plots represent NKG2A and Ly49 (C/I/F/H, G2, & D) staining on gated CD3-NK1.1+ cells from naïve or MCMV-infected WT or MIP1-$\alpha^{-/-}$ mice. (B) Combined results from a single experiment showing the NKG2A/Ly49 subset composition. * = $p<.05$.

Fig. 18

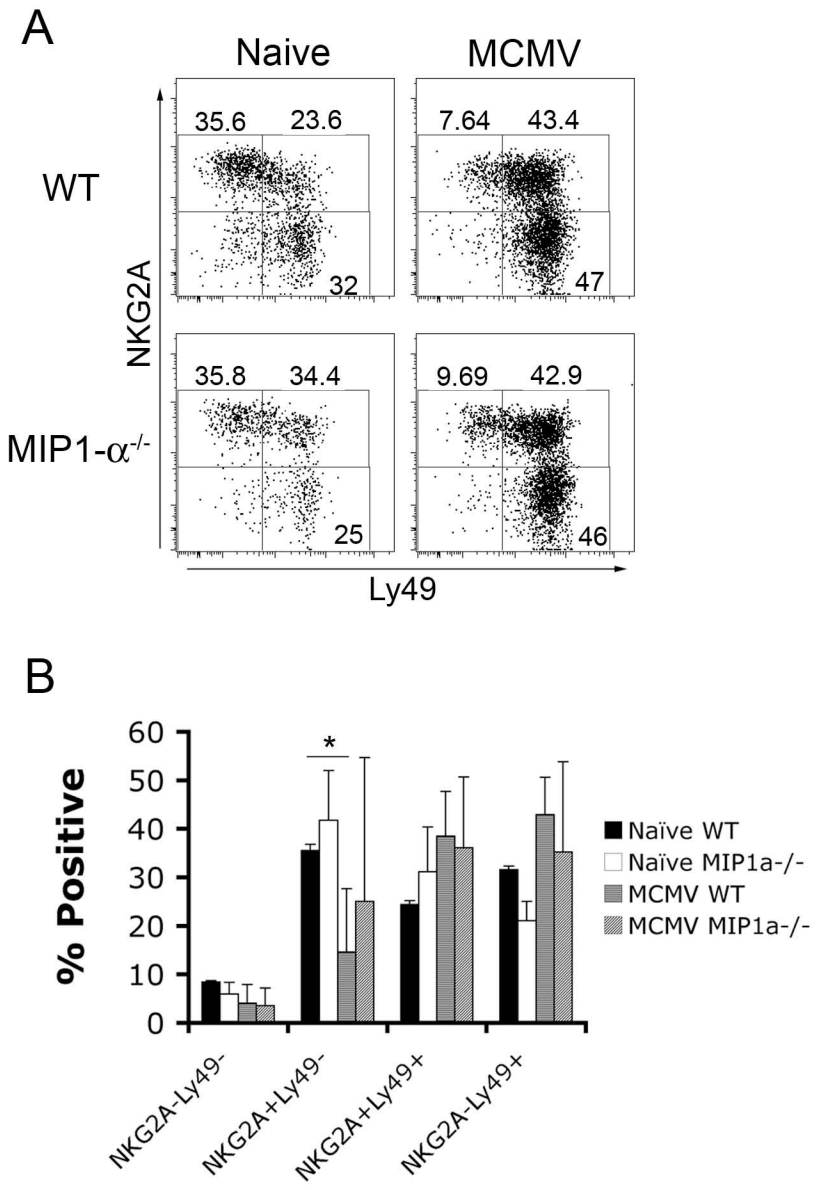
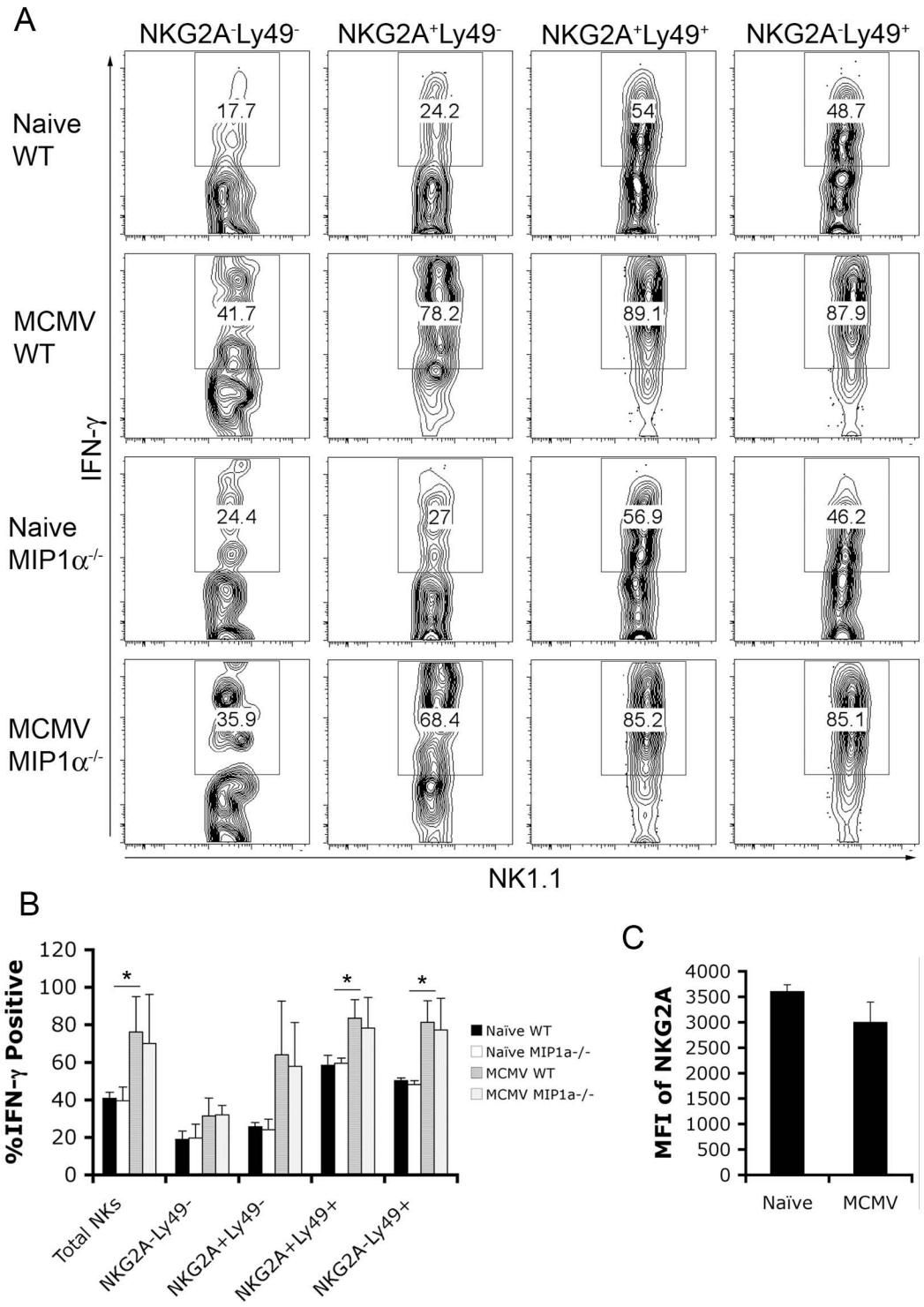

A
Naive
MCMV
WT
MIP1-α-/-
NKG2A
Ly49
35.6
23.6
32
7.64
43.4
47
35.8
34.4
25
9.69
42.9
46
B
% Positive
60
50
40
30
20
10
0
*
Naïve WT
Naïve MIP1a-/-
MCMV WT
MCMV MIP1a-/-
NKG2A-Ly49-
NKG2A+Ly49-
NKG2A+Ly49+
NKG2A-Ly49+

FIGURE 19. NKG2A+Ly49- NK cells are display full functional capacity following viral infection. Wildtype (WT) C57BL/6 or MIP1-$\alpha^{-/-}$ mice on the C57BL/6 background were injected intraperitoneally with 5 x 10^4 pfu of murine cytomegalovirus (MCMV). Liver leukocytes were harvested 48 hours post-injection and stimulated with IL-12 and IL-18 for 5 hours. (A) Representative dot plots of intracellular IFN-γ staining in NK cell subsets from naïve or viral infected WT or MIP1-$\alpha^{-/-}$ mice. (B) Combined results from a single experiment using 3 mice per group. (C) Combined results from a single experiment of mean fluorescent intensity of NKG2A staining on NKG2A$^+$Ly49$^-$ NK cells from naïve or MCMV-infected wildtype mice.

Fig. 19


A
NKG2A-Ly49-
NKG2A+Ly49-
NKG2A+Ly49+
NKG2A-Ly49+
Naive WT
MCMV WT
Naive MIP1α-/-
MCMV MIP1α-/-
17.7
24.2
54
48.7
41.7
78.2
89.1
87.9
24.4
27
56.9
46.2
35.9
68.4
85.2
85.1
IFN-γ
NK1.1
B
%IFN-γ Positive
120
100
80
60
40
20
0
*
*
*
Naïve WT
Naïve MIP1a-/-
MCMV WT
MCMV MIP1a-/-
Total NKs
NKG2A-Ly49-
NKG2A+Ly49-
NKG2A+Ly49+
NKG2A-Ly49+
C
MFI of NKG2A
4000
3500
3000
2500
2000
1500
1000
500
0
Naïve
MCMV

Chapter 5: Impact of liver NK cells on liver DC function

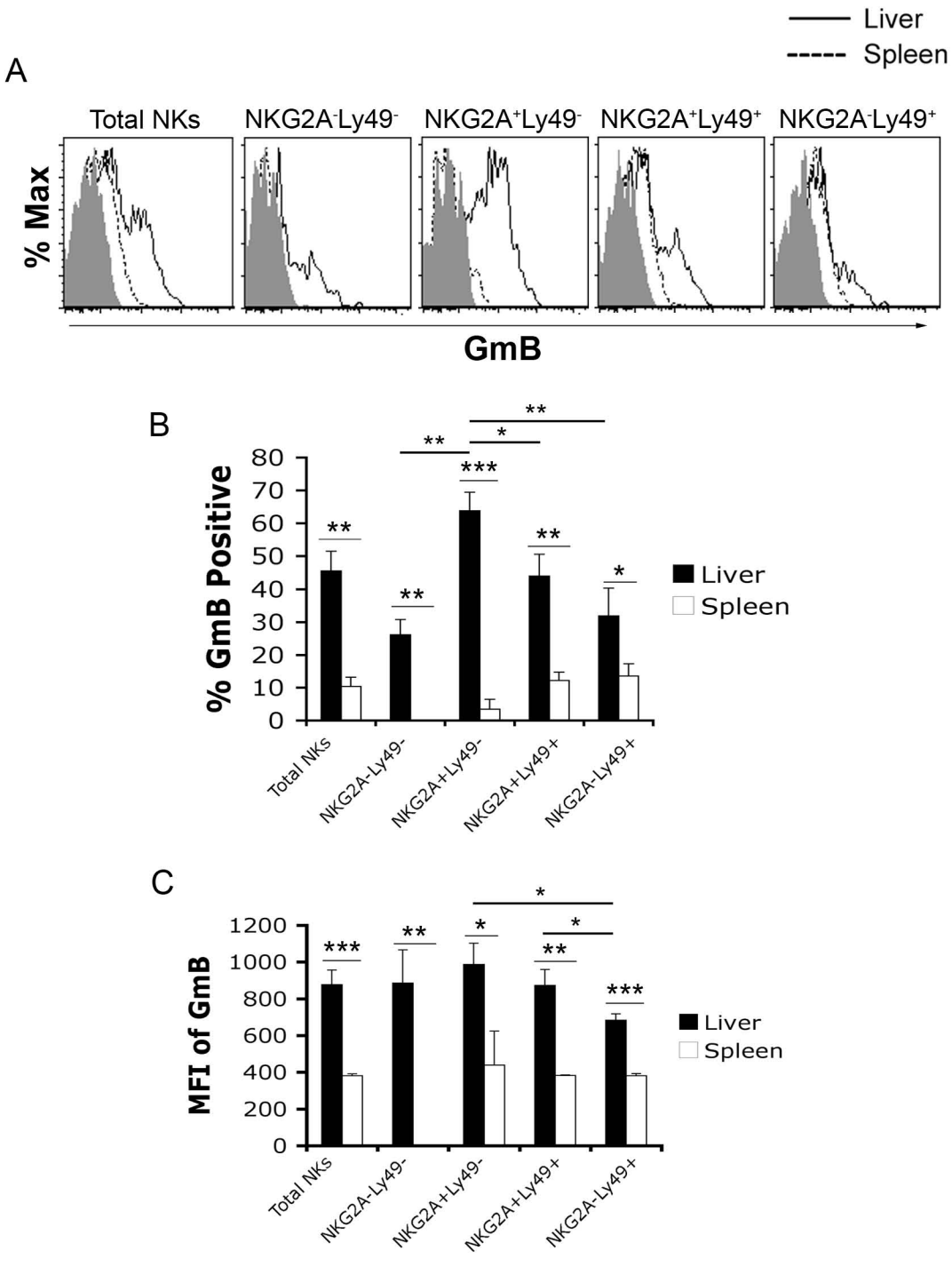
NKG2A$^+$Ly49$^-$ liver NK cells contain high amount of granzyme B and express elevated levels of CD69.

The data presented in chapter 4 demonstrated that the liver NK cell population contains a significant proportion of a distinct NKG2A$^+$Ly49$^-$ NK cell subset with phenotypic and functional features that are unique to the liver, including fewer IFN-γ producing cells when stimulated with IL-12 and IL-18. Although NKG2A$^+$Ly49$^-$ liver NK cells are poor producers of IFN-γ, they may provide other functions in the normal liver. Indeed, our analysis of IL-10 production showed that the NKG2A$^+$Ly49$^-$ NK cell subset is the major source of IL-10 among liver NK cells. We sought to further analyze the functional potential of liver NK cell subsets by examining granzyme B (GmB) levels.

NK cells can mediate direct cell killing through the production of GmB. We analyzed the levels of intracellular GmB in liver and spleen NK cells directly ex vivo. Approximately 10% of spleen NK cells stain positive for intracellular GmB directly ex vivo, compared to approximately 40% of liver NK cells (Fig. 20A & B). When GmB levels were analyzed within NK cell subsets according to NKG2A and Ly49 expression, we found that the NKG2A$^+$Ly49$^-$ NK cell subset in the liver expresses higher levels of GmB than other liver NK cells (Fig. 20B & C). In comparison, GmB expression in spleen NK cells shows a very different pattern. The majority of GmB positive spleen NK cells are Ly49$^+$, while only a fraction of NKG2A$^+$Ly49$^-$ spleen NK cells stain positive for GmB. These data suggest that while the cytokine profile of NKG2A$^+$Ly49$^-$ liver NK

FIGURE 20. Intracellular granzyme B levels in NK cell subsets. Intracellular granzyme B levels were analyzed in NK cell subsets directly ex vivo. (A) Plots represent intracellular granzyme B staining in total NK cells and gated NK cell subsets based on NKG2A and Ly49 expression on liver (solid line) and spleen (dashed line). (B) Combined results from a single experiment showing positive granzyme B staining in liver and spleen NK cell subsets. (C) Combined results from a single experiment showing mean fluorescent intensity (MFI) of intracellular granzyme B staining. Data is representative of two independent experiments containing 3 mice per experiment. * = $p<.05$, ** = $p<.005$, *** = $p<.0005$.

Fig. 20


A
Liver
Spleen
Total NKs
NKG2A-Ly49-
NKG2A+Ly49-
NKG2A+Ly49+
NKG2A-Ly49+
% Max
GmB
B
% GmB Positive
Liver
Spleen
Total NKs
NKG2A-Ly49-
NKG2A+Ly49-
NKG2A+Ly49+
NKG2A-Ly49+
C
MFI of GmB
Liver
Spleen
Total NKs
NKG2A-Ly49-
NKG2A+Ly49-
NKG2A+Ly49+
NKG2A-Ly49+

cells appears to be more immunosuppressive, they may be an important NK cell subset in mediating cytotoxicity within the normal liver.

The greater expression of GmB by liver NK cells may reflect the constant exposure of the liver to gut-derived foreign material. Expression of CD69 on liver NK cells has been reported previously. Our analysis of CD69 expression confirmed its higher expression on liver NK cells compared to spleen NK cells (Fig. 21A). Further analysis of liver NK cell subsets revealed that nearly all $NKG2A^{+}Ly49^{-}$ liver NK cells expressed CD69 at high levels, compared to other liver NK cell subsets, providing further evidence that the $NKG2A^{+}Ly49^{-}$ liver NK cell subset is a distinct NK cell population (Fig. 21B & C).

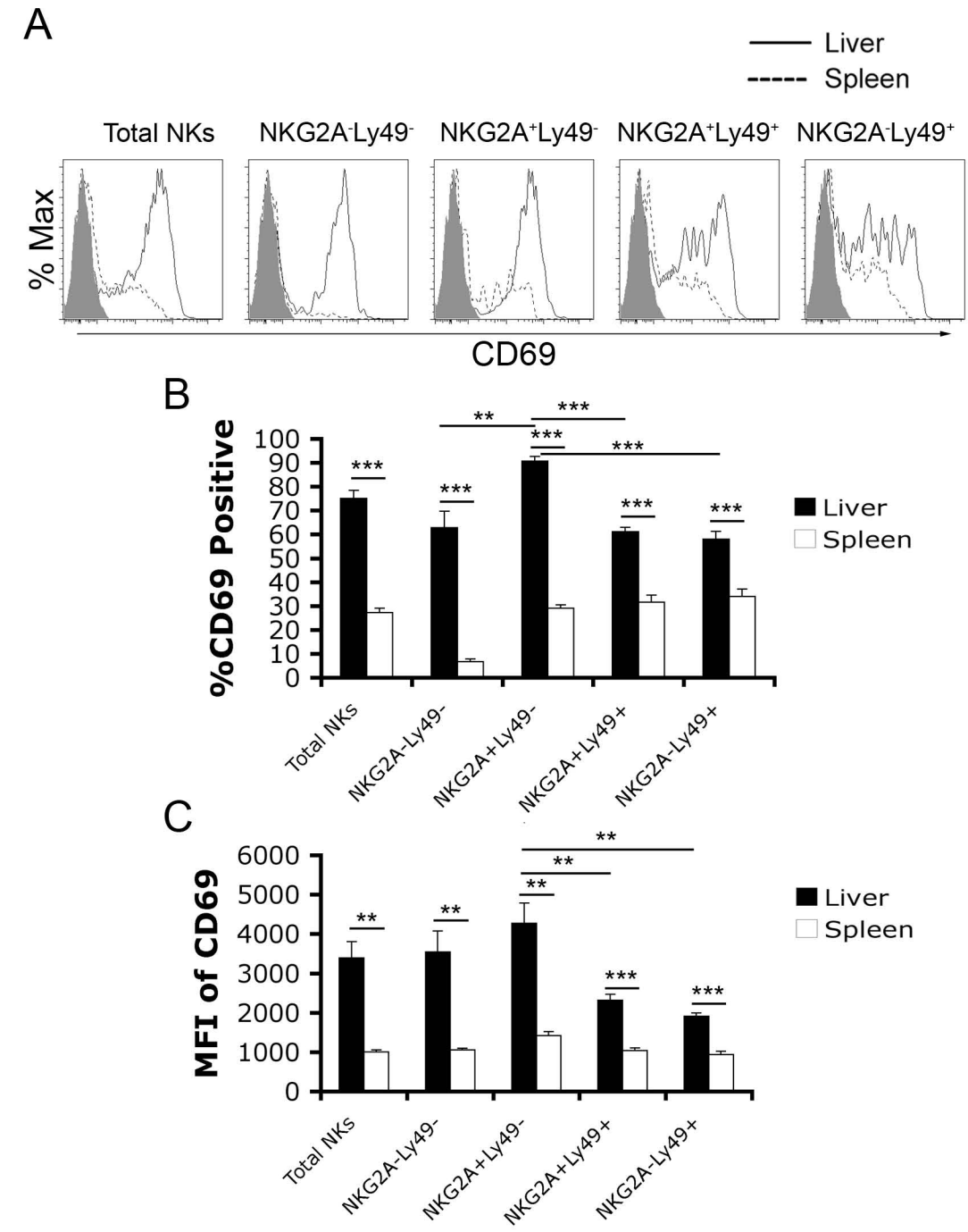
In vivo depletion of NK cells increases total number of mature liver DCs

As the liver is a tolerogenic organ, and NK cells have the ability to influence the maturation and function of a variety of cell types, we hypothesized that the liver NK cell population plays an important role in maintaining the tolerant environment critical to the function of the normal liver. To begin to investigate the functional impact of the liver NK cell population, we investigated the effect of liver NK cells on the maturation of dendritic cells (DCs).

As discussed earlier, multiple studies have indicated that, in comparison to other DC populations such as skin and spleen DCs, the liver DC population is tolerogenic in nature, including poor T cell stimulatory capacity, lower costimulatory molecule expression, greater IL-10 production, and less antigen uptake (Goddard et al. 2004; Kwekkeboom et al. 2005). It has been proposed that these observations may be

FIGURE 21. CD69 expression on NK cell subsets. CD69 expression was analyzed on NK cell subsets from the liver and spleen. (A) Representative histograms showing CD69 expression on total NKs and NKG2A/Ly49 NK cells subsets in the liver (solid line) and spleen (dashed line). Shaded histogram represents isotype control staining. (B) Combined results from a single experiment showing positive CD69 staining in liver and spleen NK cell subsets. (C) Combined results from a single experiment showing mean fluorescent intensity (MFI) of CD69 staining. Data is representative of two independent experiments containing 3 mice per experiment. ** = $p<.005$, *** = $p<.0005$.

Fig. 21


A
Liver
Spleen
Total NKs
NKG2A-Ly49-
NKG2A+Ly49-
NKG2A+Ly49+
NKG2A-Ly49+
% Max
CD69
B
%CD69 Positive
Liver
Spleen
Total NKs
NKG2A-Ly49-
NKG2A+Ly49-
NKG2A+Ly49+
NKG2A-Ly49+
C
MFI of CD69
Liver
Spleen
Total NKs
NKG2A-Ly49-
NKG2A+Ly49-
NKG2A+Ly49+
NKG2A-Ly49+

explained in part due to the subtype composition of the liver DC population (Pillarisetty et al. 2004). The conventional DC population in the liver consists of a major $B220^{-}$ $CD11c^{Low}$ and a minor $B220^{-}CD11c^{Hi}$ DC subset, expressing low and high levels of costimulatory molecules, respectively (Jomantaite et al. 2004). The liver also contains a significant population of plasmacytoid DCs, which are less capable of stimulating T cell activation. The factors regulating the liver DC population subtype composition and costimulatory molecule expression remain unclear. As the interaction between NK cells and DCs is known to play an important role in both the innate and adaptive immune responses, we hypothesized that liver NK cells contribute to the immature profile reported for liver DCs.

The reciprocal activation and maturation observed in NK-DC crosstalk appears to be achieved through both soluble mediators and cell-cell contact. Type I IFN, IL-12, and IL-18 production by DCs, as well as trans-presentation of surface bound IL-15 and upregulation of NKG2D ligands contribute to the ability of DCs to activate NK cells (Hamerman, Ogasawara and Lanier 2005; Mortier et al. 2008). DC-mediated NK cell activation results in enhanced cytotoxicity, IFN-γ production, CD69 expression, and proliferation (Hamerman, Ogasawara and Lanier 2005). On the opposite side, NK-mediated maturation of DCs requires direct cell contact and secretion of IFN-γ and TNF-α, resulting in upregulation of costimulatory molecules and enhanced T cell stimulatory capacity (Gerosa et al. 2002; Piccioli et al. 2002). Importantly, NK cells also have the ability to kill immature DCs, thus further influencing the outcome of the adaptive immune response (Moretta 2002).

NK cocultures with bone marrow-derived DCs

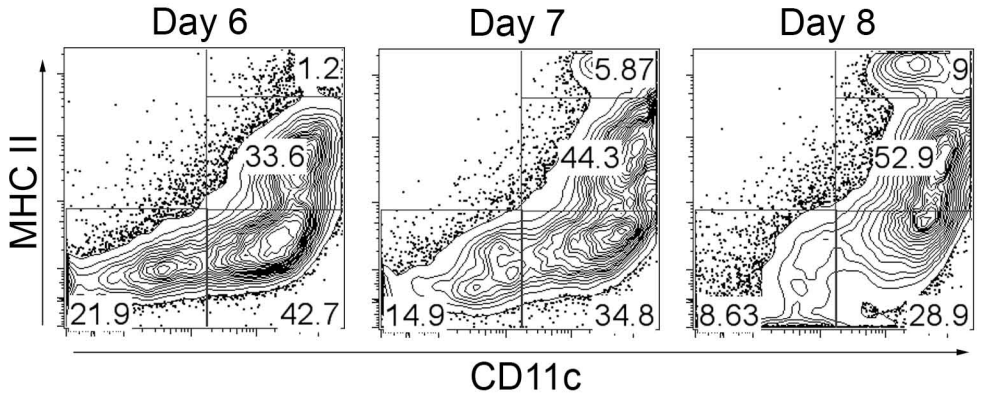
To investigate the ability of the liver NK cell population to drive DC maturation, we performed coculture experiments of bone marrow-derived DCs (BMDCs) and purified liver or spleen NK cells. BMDCs were used at day 6 of BMDC generation, due to the greater proportion of immature BMDCs (Fig. 22A). We observed that the upregulation of CD80 and CD86 on BMDCs correlated with the induction and upregulation of CD11c and MHC class II (Fig. 22B).

Prior to the addition of the NK cells, BMDCs were incubated for 5-6 hours with or without LPS or polyI:C. Purified liver or spleen NK cells were then cocultured with BMDCs for 24 hours, followed by DC subset analysis and CD80/CD86 expression levels. BMDCs upregulated MHC class II in response to both LPS and polyI:C stimulation (Fig. 23). In addition, the presence of either liver or spleen NK cells slightly increased the expression of MHC class II in all conditions. However, no difference was observed between liver and spleen NK cells in their ability to alter MHC class II expression or the proportion of DC subsets according to CD11c and MHC class II expression.
The expression of CD80 and CD86 was also analyzed among the $CD11c^{+}MHCII^{Lo/Int}$ and $CD11c^{+}MHCII^{Hi}$ BMDC subsets. Among the $CD11c^{+}MHCII^{Lo/Int}$ BMDC subset, LPS and polyI:C had little effect on the expression of CD80 and CD86 (Fig. 24A). In addition, the presence of either liver or spleen NK cells failed to modulate costimulatory molecule expression under any condition among the $CD11c^{+}MHCII^{Lo/Int}$ BMDCs. In contrast, $CD11c^{+}MHCII^{Hi}$ BMDCs showed enhanced expression of both CD80 and CD86 by LPS, while polyI:C had little impact (Fig. 24B). Furthermore, in the absence of TLR stimulation, both liver and spleen NK cells increased the expression of CD80 and

FIGURE 22. Analysis of bone marrow-derived dendritic cells. Bone marrow-derived dendritic cells (BMDCs) were generated from C57BL/6 mice. BMDCs were harvested at days 6, 7, and 8 of BMDC generation. (A) BMDC maturation stages according to CD11c and MHC class II expression. (B) Analysis of CD80 and CD86 expression on different BMDC maturation stages.

Fig. 22

A


Day 6
Day 7
Day 8
MHC II
CD11c
1.2
33.6
21.9
42.7
5.87
44.3
14.9
34.8
9
52.9
8.63
28.9

B

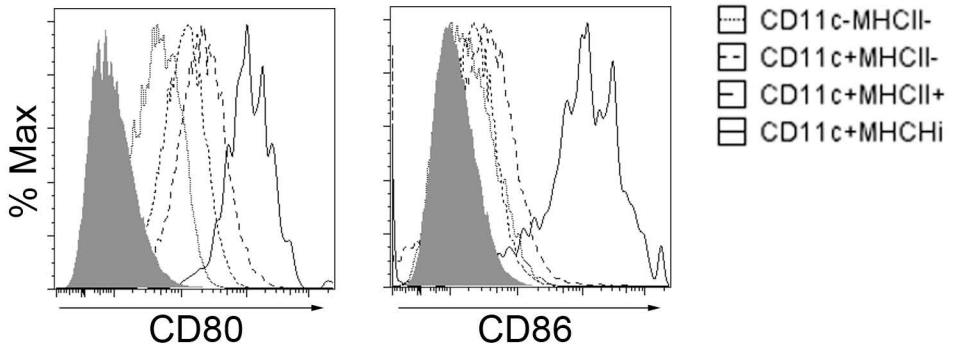

% Max
CD80
CD86
CD11c-MHCII-
CD11c+MHCII-
CD11c+MHCII+
CD11c+MHCHi

FIGURE 23. Analysis of DC maturation following coculture with liver or spleen NK cells. Bone marrow-derived dendritic cells (BMDCs) were harvested at day 6 of BMDC generation and incubated at 37°C for 5-6 hours with or without LPS (1 μg/mL) or polyI:C (10 μg/mL) in RPMI containing 10% FBS. LPS and polyI:C were then washed out, and purified liver or spleen NK cells were added to the BMDCs for 24 hours at 37°C. After 24 hours, BMDCs were analyzed for the expression of CD11c and MHC class II.

Fig. 23

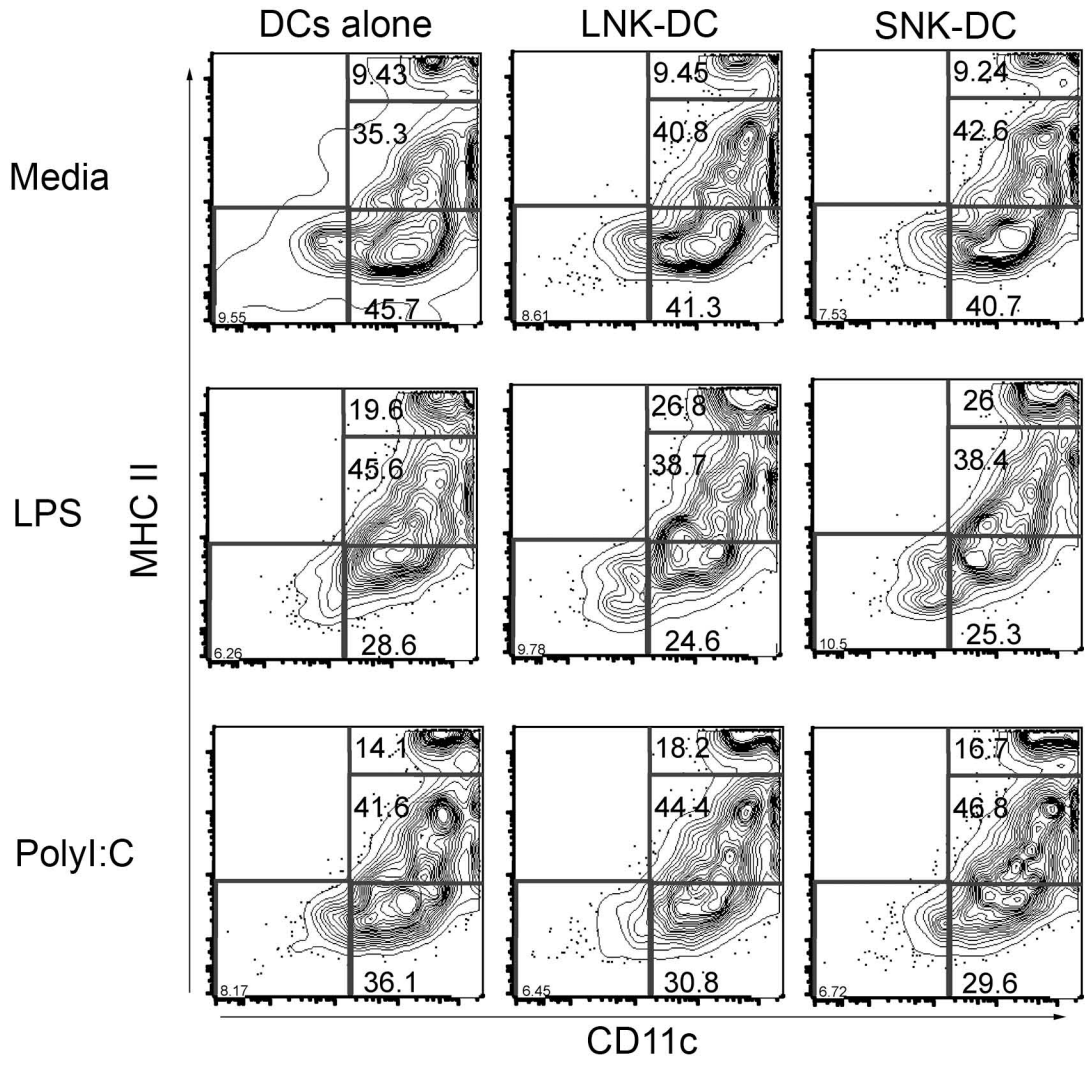
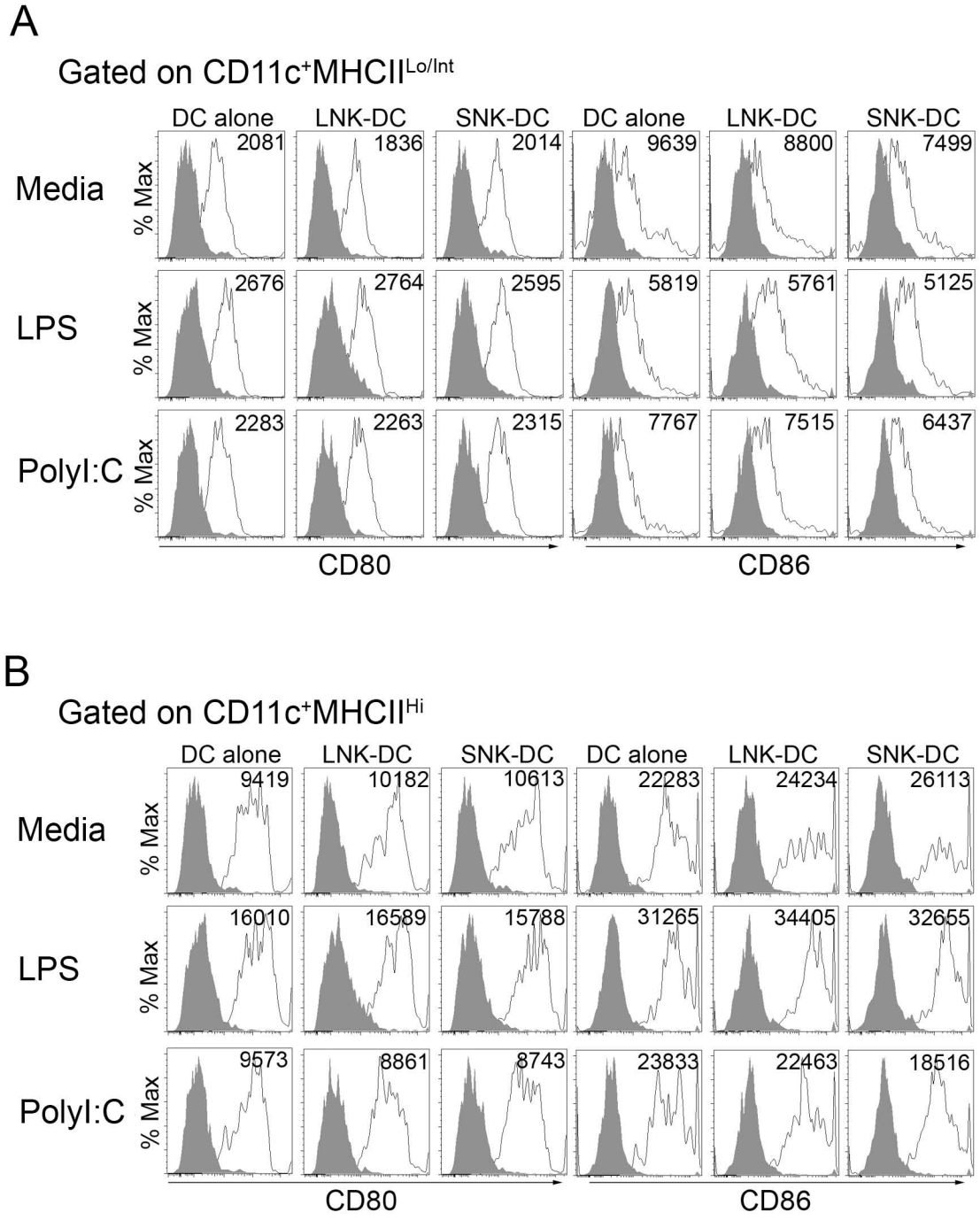

DCs alone
LNK-DC
SNK-DC
Media
LPS
PolyI:C
MHC II
CD11c
9.43
35.3
45.7
9.55
9.45
40.8
41.3
8.61
9.24
42.6
40.7
7.53
19.6
45.6
28.6
6.26
26.8
38.7
24.6
9.78
26
38.4
25.3
10.5
14.1
41.6
36.1
8.17
18.2
44.4
30.8
6.45
16.7
46.8
29.6
6.72

FIGURE 24. NK cell coculture does not modify costimulatory molecule expression of BMDCs. NK cell cocultures with BMDCs were set up as described for figure 21. After 24 hours of coculture, BMDCs were stained for the surface expression of CD80 and CD86. (A) CD80 and CD86 expression on $CD11c^{+}MHCII^{Lo/Int}$ gated BMDCs. Values in plots represent the mean fluorescent intensity of staining on positive cells. Shaded histograms represent isotype control staining. (B) CD80 and CD86 expression on $CD11c^{+}MHCII^{Hi}$ gated BMDCs. Values in plots represent the mean fluorescent intensity of staining on positive cells. Shaded histograms represent isotype control staining.

Fig. 24


A
Gated on CD11c+MHCIILo/Int
DC alone
LNK-DC
SNK-DC
DC alone
LNK-DC
SNK-DC
Media
LPS
PolyI:C
% Max
2081
1836
2014
9639
8800
7499
2676
2764
2595
5819
5761
5125
2283
2263
2315
7767
7515
6437
CD80
CD86
B
Gated on CD11c+MHCIIHi
DC alone
LNK-DC
SNK-DC
DC alone
LNK-DC
SNK-DC
Media
LPS
PolyI:C
% Max
9419
10182
10613
22283
24234
26113
16010
16589
15788
31265
34405
32655
9573
8861
8743
23833
22463
18516
CD80
CD86

CD86 on $CD11c^{+}MHCII^{Hi}$ BMDCs. However, no differences were seen between liver and spleen NK cells.

Impact of NK cell depletion on liver DCs

To investigate the *in vivo* impact of NK cells on the liver DC population, we depleted NK cells via intraperitoneal administration of antibody against asialo-GM1 (αGM-1). Injections were administered every 24 hours for up to three days, and livers were harvested after 1, 2, or 3 days of injections (Fig. 25A). Control mice received PBS injections every 24 hours for 3 days. To determine the effectiveness of NK cell depletion within the liver, we analyzed the $CD3^{-}NK1.1^{+}$ cell population in livers from αGM-1 treated and control mice. FACS analysis showed that following two and three injections of αGM-1, the liver NK cell population was virtually undetectable (Fig. 25B). The percentage of liver NK cells was reduced following one αGM-1 injection, but still readily detectable. In addition, the total number of NK cells in the liver was greatly reduced following one αGM-1 injection, and almost completely depleted following two and three injections (Fig. 25C).

Next we analyzed the liver DC subset composition following NK cell depletion. Figure 26A outlines the gating strategy used in these experiments for defining the liver DC populations. The liver DC subsets were defined as $CD11c^{Lo}MHCII^{+}$ and $CD11c^{+}MHCII^{Hi}$ cells within the $CD45^{+}B220^{-}$ gate. Following NK cell depletion, we noted a dramatic decrease in a population of $CD11c^{Lo}MHCII^{-/Lo}$ cells. As NK cells can express low levels of CD11c and MHC class II (Caminschi et al. 2007; Laouar et al. 2005), we looked at the expression of NK1.1 within this gate. Virtually all of the $CD11c^{Lo}MHCII^{-/Lo}$ cells in a control mouse stained positive for NK1.1 (Fig. 26C). NK

FIGURE 25. Antibody-mediated NK cell depletion using anti-asialo GM-1.

C57BL/6 mice were injected intraperitoneally with 300 μg of anti-asialo GM-1 (anti-GM-1)every 24 hours for up to three days. Control mice were injected intraperitoneally with 300 μL phosphate buffered saline every 24 hours for three days. (A) Injection schedule for administration of anti-GM-1. Each group consisted of three mice and received anti-GM-1 or PBS injections every 24 hours following the initial injection until the day of harvest. (B) Isolated liver leukocytes were combined in each group and stained for the expression of CD3 and NK1.1. (C) Graph showing the total number of NK cells isolated in each treatment group.

Fig. 25

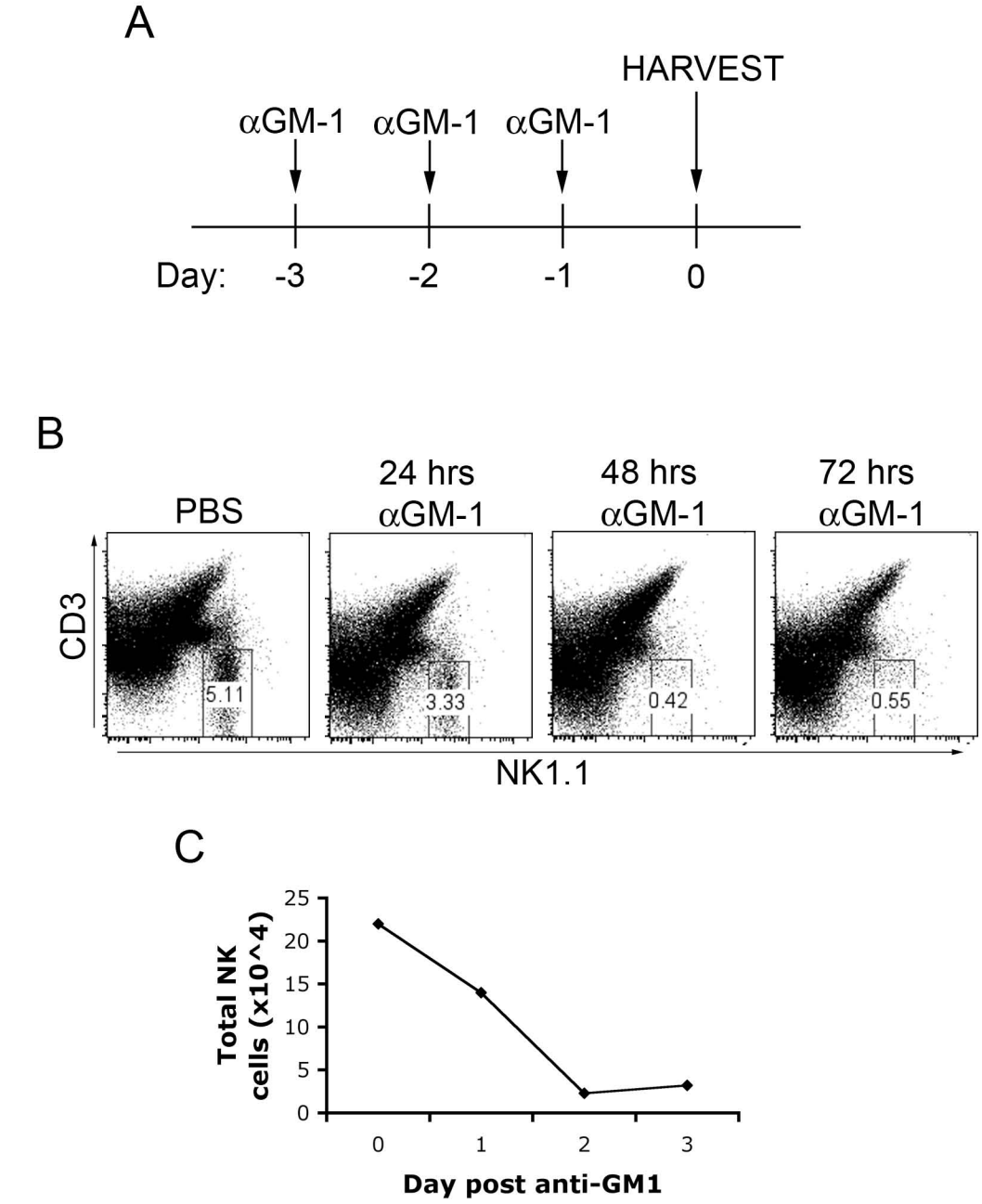
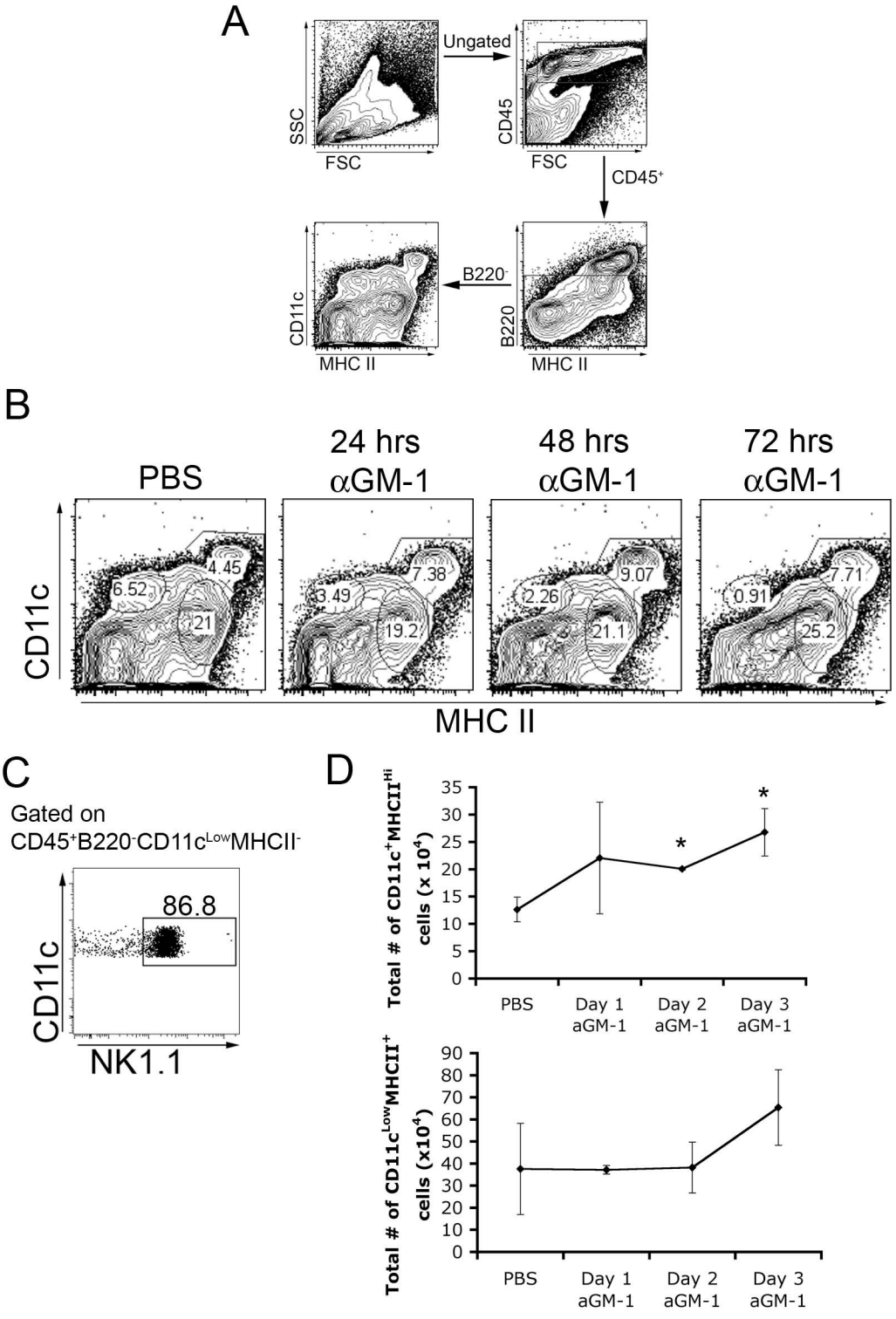

A
HARVEST
αGM-1
αGM-1
αGM-1
Day:
-3
-2
-1
0
B
PBS
24 hrs αGM-1
48 hrs αGM-1
72 hrs αGM-1
CD3
5.11
3.33
0.42
0.55
NK1.1
C
Total NK cells (x10^4)
25
20
15
10
5
0
0
1
2
3
Day post anti-GM1

FIGURE 26. Liver DC analysis following NK cell depletion. Liver leukocytes from NK depleted and control mice were isolated and stained for DC markers. (A) Gating strategy for defining conventional liver DCs. Liver DCs were defined as $CD45^{+}B220^{-}CD11c^{+}MHCII^{Hi}$ cells. (B) Dot plots showing the percentage of conventional liver DCs ($CD11c^{+}MHCII^{Hi}$) in control and NK depleted mice. (C) Dot plot showing NK1.1 expression on $CD11c^{Lo}MHCII^{-/Lo}$ cells from control mice. (D) Absolute numbers of conventional liver DCs from control and NK depleted mice. * = $p<.05$.

Fig. 26


A
Ungated
SSC
FSC
CD45
FSC
CD45+
B220-
CD11c
MHC II
B220
MHC II
B
PBS
24 hrs αGM-1
48 hrs αGM-1
72 hrs αGM-1
CD11c
MHC II
6.52
4.45
21
3.49
7.38
19.2
2.26
9.07
21.1
0.91
7.71
25.2
C
Gated on
CD45+B220-CD11cLowMHCII-
86.8
CD11c
NK1.1
D
Total # of CD11c+MHCIIHi cells (x 10^4)
PBS
Day 1 aGM-1
Day 2 aGM-1
Day 3 aGM-1
Total # of CD11cLowMHCII+ cells (x10^4)
PBS
Day 1 aGM-1
Day 2 aGM-1
Day 3 aGM-1

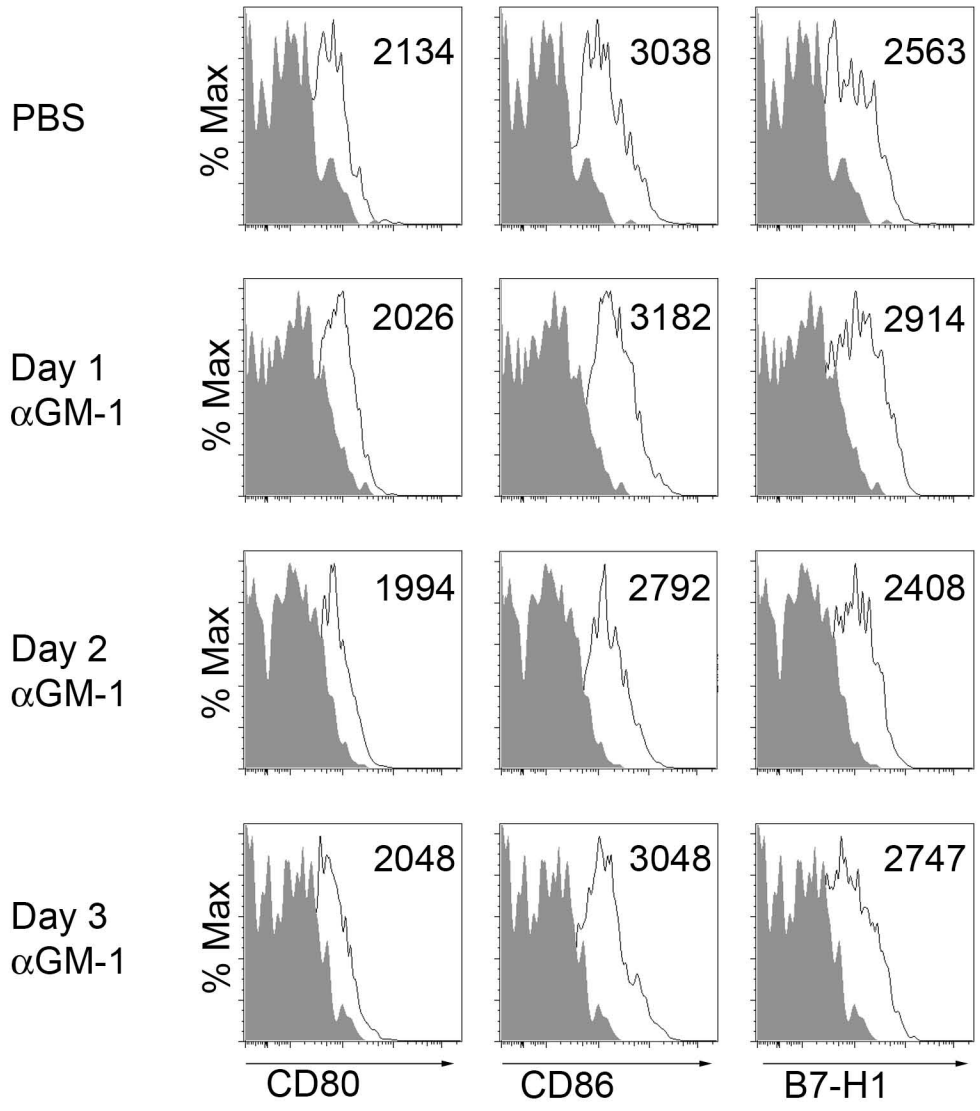
depletion also correlated with an increase in the percentage of the $CD11c^{+}MHCII^{Hi}$ DCs, but had no affect on the percentage of $CD11c^{Lo}MHCII^{+}$ DCs (Fig. 26B). This shift in the proportion of DC subsets was also seen in the total number of DCs, with NK depleted animals showing a significant increase in the number of $CD11c^{+}MHCII^{Hi}$ DCs following two and three injections of αGM-1 (Fig. 26D).

The impact of NK depletion on costimulatory molecules expressed by DCs was also analyzed, by staining for the expression of CD80 and CD86. Figure 27 shows that NK depletion had no impact on the expression of CD80 or CD86 on $CD11c^{+}MHCII^{Hi}$ DCs. Similar results were observed for CD80 and CD86 expression on $CD11c^{Lo}MHCII^{+}$ DCs. In addition, we looked at the impact of NK depletion on the expression of the PD-1 ligand, B7-homologue 1 (B7-H1). The expression of B7-H1 on $CD11c^{+}MHCII^{Hi}$ DCs was not affected by NK depletion (Fig. 27). Similar results were observed for B7-H1 expression on $CD11c^{Lo}MHCII^{+}$ DCs.

These data suggest that liver NK cells have little impact on the expression of the costimulatory molecules CD80 and CD86, as well as the inhibitory molecule B7-H1, on liver DCs. In contrast, NK cells do contribute to the overall subset composition of the liver DC population, as NK depletion results in an increase of the more mature $CD11c^{+}MHCII^{Hi}$ DCs in the liver. Further work is warranted to determine if the observed shift in liver DC subsets and higher number of $CD11c^{+}MHCII^{Hi}$ DCs correlates with an increased T cell stimulatory capacity by the liver DC population.

FIGURE 27. Costimulatory molecule expression on liver DCs following NK depletion. Liver leukocytes from NK depleted and control mice were isolated and stained for costimulatory molecules. Histograms represent staining on $CD45^{+}B220^{-}CD11c^{+}MHCII^{Hi}$ liver cells from control and NK depleted mice. Values in plots represent the mean fluorescent intensity of staining on positive cells. Shaded histograms represent isotype control staining.

Fig. 27


PBS
Day 1 αGM-1
Day 2 αGM-1
Day 3 αGM-1
% Max
2134
3038
2563
2026
3182
2914
1994
2792
2408
2048
3048
2747
CD80
CD86
B7-H1

Chapter 6: Conclusions and Future Directions

The normal liver is exposed to a daily barrage of gut-derived foreign antigen that must be absorbed and dealt with in a manner that does not elicit an inflammatory response. The liver is therefore armed with a variety of immune tolerance mechanisms that may include induction of regulatory T cells and elimination of activated T cells. Recent reports have highlighted cells with antigen presenting capabilities, such as liver sinusoidal epithelial cells (LSECs), dendritic cells (DCs), and kupffer cells (KCs) as playing a central role in maintaining liver tolerance. The findings presented in this thesis suggest that an additional contribution to liver tolerance may include regulation of certain NK cell subsets, rendering them functionally less responsive.

Through these data we demonstrated that the liver NK cell population is less responsive to cytokine-inducing stimulation. Multiple factors may be contributing to lower IFN-γ production by liver NK cells, including a decreased proportion of Ly49 expressing NK cells. Indeed, Ly49 expression on NK cells correlated with greater IFN-γ production in liver and spleen NK cells. A reduced functional response among liver NK cells corresponds with a smaller proportion of NK cells expressing Ly49 receptors. This increased functional response by $Ly49^+$ NK cells most likely reflects a more mature state of development, as the ability to secrete IFN-γ increases with maturation. However, sorted $Ly49^+$ liver NK cells were less responsive to cytokine stimulation than $Ly49^+$ NK cells from the spleen, indicating that there are likely additional factors unique to the liver influencing the NK cell functional response.

NKG2A is a major inhibitory receptor expressed by liver NK cells; its high expression coincided with hyporesponsiveness observed in intrahepatic $NKG2A^{+}Ly49^{-}$ NK cells, suggesting a significant role for NKG2A in regulating liver NK cell effector functions. Importantly, Ly49 expression appears sufficient to overcome this hyporesponsiveness, as there was no statistical difference in IFN-γ production between $NKG2A^{+}Ly49^{+}$ and $NKG2A^{-}Ly49^{+}$ NK cells.

It is interesting to note that a majority of fetal NK cells have been reported to express CD94/NKG2 molecules, while lacking surface expression of Ly49 receptors. Liver $NKG2A^{+}Ly49^{-}$ NK cells, while less responsive to cytokine stimulation than Ly49 expressing NK cells, may have other functions within the liver. Indeed, liver $NKG2A^{+}Ly49^{-}$ NK cells appear to be a distinct NK cell subset with unique phenotypic and functional characteristics. While few $NKG2A^{+}Ly49^{-}$ liver NK cells produced IFN-γ, a large proportion stained positive for low levels of IL-10. In addition, our examination of intracellular granzyme B revealed that liver NK cells contain higher amounts compared to spleen NK cells. However, upon further analysis we found that the $NKG2A^{+}Ly49^{-}$ liver NK cell subset contained higher intracellular amounts of granzyme B than all other liver NK cell subsets. This is most intriguing due to the observation that the weak immunostimulatory function associated with the liver DC population may in part be due to differences in subtype composition (Jomantaite et al. 2004; Pillarisetty et al. 2004). Further analysis is required to determine if the cytolytic potential and/or cytokine production of specific NK cell subsets in the liver may contribute to the shaping of the liver DC population. Interestingly, a recent report showed that the killing of

immature DCs by human NK cells was mediated by a subset of NK cells that express NKG2A but lack killer Ig-like receptors (KIR) expression (Della Chiesa et al. 2003).

In addition to the functional differences observed in $NKG2A^{+}Ly49^{-}$ liver NK cells, a number of phenotypic differences were also very interesting. While lacking Ly49 expression, which is associated with a less mature NK cell developmental stage, all liver NK cells stained positive for CD43, a marker typically expressed on mature NK cells. Analysis of NKG2A/Ly49 NK cell subsets within CD27/CD11b subsets further revealed that the $NKG2A^{+}Ly49^{-}$ NK cell subset is present at significant proportions even in the $CD27^{+}CD11b^{+}$ and $CD27^{-}CD11b^{+}$ liver NK cells, while these same CD11b expressing subsets in the spleen were virtually void of NK cells lacking Ly49 expression. Furthermore, while expression of CD69 is elevated on a majority of liver NK cells, compared to a small fraction of spleen NK cells, a significantly higher percentage of $NKG2A^{+}Ly49^{-}$ liver NK cells, as well as enhanced levels of expression, are positive for CD69 expression compared to all other liver NK cell subsets. The adoptive transfer of liver NK cells also suggested that trafficking of $NKG2A^{+}Ly49^{-}$ liver NK cells may be differentially regulated. These data, together with the enhanced levels of NKG2A observed on $NKG2A^{+}Ly49^{-}$ liver NK cells, strongly suggest that $NKG2A^{+}Ly49^{-}$ NK cells in the liver represent a unique NK cell population with distinct phenotypic and functional characteristics.

The adoptive transfer experiments of spleen NK cells reported here suggest that the liver environment can modify NK cell functional responsiveness and surface marker expression. Although the adoptive transfer experiments we performed are not definitive in showing modification of surface marker expression, the lack of a preferential

recruitment of Ly49^{-} NK cells to the liver, as shown in the separate transfer of Ly49$^{-/Lo}$ and Ly49^{+} spleen NK cells, strengthens the theory that factors specific to the liver environment lead to a modulation of the NK cell receptor expression profile upon migration to the liver.

There are several possibilities that may explain these observations. As discussed briefly in the introduction section of this thesis, the contribution of peripheral maturation sites for NK cells is relatively unknown, although recent reports have given traction to the idea that the bone marrow may not be the only site of NK cell development and maturation. Thymic-derived NK cells are characterized by the expression of CD127 and are dependent on several unique developmental factors distinct from spleen NK cells, including IL-7 and the transcription factor GATA-3 (Vosshenrich et al. 2006). Importantly, thymic-derived NK cells were dependent on hematopoetic precursors. More recently, a distinct population of NKp46^{+}CD3^{-} cells was identified in the gut (Luci et al. 2009). While NKp46 is expressed exclusively on NK cells, this novel gut-derived NKp46^{+}CD3^{-} population was IL-15 independent, suggesting a developmental pathway at least partially independent of the bone marrow. The potential for trafficking of NK cell precursors (NKPs) outside of the bone marrow has been proposed by some researchers (Di Santo 2006), but has yet to be investigated. The liver may represent a site that NKPs or another hematopoetic precursor traffic to undergo further maturation, giving rise to the distinct NKG2A^{+}Ly49^{-} liver NK cell subset described in this thesis. Additional experiments should be performed to assess the contribution of migrating NKPs on the liver NK cell subset composition. Irradiation experiments may prove useful in identifying the NK cells that appear first in the liver following bone marrow

reconstitution. In addition, following genetically labeled NKPs over time after adoptive transfer may provide insight into peripheral sites that could support their development outside of the bone marrow, as well as the fate of such additional maturation sites.

NK cells may also migrate to the liver following development in the bone marrow and undergo liver specific modifications that give rise to the NK cell subset composition present in liver cell preparations. Our adoptive transfer experiments suggest that the ability of the liver environment to modify both surface marker expression and functional capacity contributes at least in part to the distinctiveness of the liver NK cell population. Further investigation is needed to identify additional factors involved in shaping the liver NK cell population. The data presented in this thesis identifies IL-10 as one factor contributing to the NK cell subset composition in the liver.

Given the immunosuppressive functions of IL-10 and its high levels in the liver determined by ELISA, IL-10 may play a critical role in maintaining liver tolerance. IL-10 has been reported to inhibit the expression of MHC class II and the costimulatory molecules B7-1/B7-2 on DCs and macrophages, influencing their ability to optimally activate T cells (de Waal Malefyt et al. 1991; Ding et al. 1993; Willems et al. 1994). IL-10 may also lead to lower NK cell activation through inhibition of IL-12 and IL-18 production, and KC production of IL-10 has been shown to directly dampen NK cell activation and decrease IFN-γ production (D'Andrea et al. 1993; Tu et al. 2008). Our data suggest that IL-10 may also contribute to liver tolerance by decreasing the percentage of NK cells expressing Ly49 receptors within the liver. Indeed, blocking the IL-10R leads to an increase of Ly49 expressing NK cells in the liver. Notably, we also

observed a greater percentage of Ly49 expressing NK cells in the livers of IL-10-deficient mice, as compared to wildtype C57BL/6 mice (data not shown).

The mechanism of IL-10 regulation of NK cells in the liver remains unclear. One possibility is that IL-10 may directly regulate Ly49 expression through the inhibition of NF-kB activation, which has been suggested to play a role in regulating Ly49 receptor expression (Pascal et al. 2007; Yoshidome et al. 1999). Alternatively, IL-10R blockade may modify the chemokine profile of the liver, resulting in different NK cell subsets migrating to the liver (Li et al. 2004). Indeed, while we favor a hyporesponsiveness induced by the liver environment, the results of our adoptive transfer experiments could be explained by a preferential migration of less functionally responsive NK cells. The potential role of differential chemokine production in the IL-10R blockade phenotype reported here is under investigation.

Liver DCs and KCs are likely candidates for regulating the NK cell population. Recent studies discussed above have highlighted receptor expression differences on liver DCs and KCs compared to spleen DCs and peritoneal macrophages, noting lower T cell activation capabilities by liver DCs and KCs. Whether liver DCs and/or KCs contribute to the immature phenotype and lower functional response of liver NK cells reported here is also under investigation.

Importantly, the results of our viral infection experiments suggest that liver NK cells are fully capable of responding in an infectious environment. The factors mediating the perceived functional restoration and maturation of liver NK cells are undefined at this time, but most likely include type I IFNs.

An important question driven by the data presented in this thesis is the functional impact on the liver environment of a NK cell population that is less responsive to cytokine stimulation. We began to address this question in this thesis through *in vitro* NK-DC cocultures and *in vivo* analysis of the liver DC population following NK cell depletion.

NK-DC cocultures failed to reveal any impact on the BMDCs unique to liver NK cells. However, several factors were problematic with this experiment. First, as compared to the BMDC phenotype at day 6 prior to coculture, BMDCs displayed increased maturation after 24 hours of culture in the absence of GM-CSF, TLR ligation, or NK cells. Repeating NK-DC cocultures with BMDCs at an earlier maturation stage, such as day 4, would be worthwhile. Second, while LPS increased the expression of CD80 and CD86 in $CD11c^{+}MHCII^{Hi}$ BMDCs, polyI:C had no affect. Neither LPS nor polyI:C had any measurable affect on CD80 or CD86 expression on $CD11c^{+}MHCII^{Lo/Int}$ BMDCs. Optimizing the dose of polyI:C should be done in future NK-DC cocultures. Third, although both liver and spleen NK cells increased CD80 and CD86 expression on $CD11c^{+}MHCII^{Hi}$ BMDCs, the increases were very modest. Increased NK:DC ratios may reveal greater differences. Caution should be taken, however, as high NK:DC ratios have been shown to result in DC killing. Fourth, a major obstacle in performing these NK-DC cocultures is obtaining sufficient numbers of purified liver NK cells. A typical yield for one C57BL/6 liver was 0.5-1.0 x 10^5 NK cells, severely limiting the size and scope of the experiment. The ultimate direction foreseen with these studies is to establish the specific impact of the $NKG2A^{+}Ly49^{-}$ NK cells on liver function; cell sorting yields may prove to be the greatest challenge in the successful completion of such studies.

NK cell depletion results in an increase in the numbers of $CD11c^{+}MHCII^{Hi}$ DCs in the liver. Expression of CD80 and CD86 was unaffected on either the $CD11c^{+}MHCII^{Hi}$ or $CD11c^{Lo}MHCII^{+}$ liver DCs. The expression of the co-inhibitory receptor B7-H1 was similarly unaffected. The expression of additional costimulatory molecules such as CD83 and CD40L should be done to assess the impact of NK cell depletion. In addition, it would be very interesting to determine differences in the expression of NK cell receptor ligands by liver DCs following NK cell depletion.

Whether the observed shift towards a more mature DC population in the liver is sufficient to modify liver DC function or liver tolerance needs to be addressed in a direct functional assay. DC function could be investigated initially through *in vitro* T cell stimulation assays using TCR transgenic T cells stimulated by antigen pulsed DCs from control or NK depleted mice. In addition to total NK cell depletion, the impact of purified specific NK cell subsets on liver DC function could be analyzed *in vitro*.

The *in vivo* impact on the ability of the liver to clear virus should also be analyzed. In choosing an appropriate viral model, adenovirus may prove to be very useful. Intravenous (i.v.) administration of adenovirus results in almost an exclusive infection of the liver. In addition, studies in our lab have shown that i.v. adenovirus infection results in suboptimal T cell priming in the liver leading to an impaired $CD8^{+}$ T cell response. Assessing the $CD8^{+}$ T cell response to i.v. adenovirus infection following NK cell depletion would provide a readout for liver APC function. The specific role of liver DCs could be further determined through *in vitro* T cell stimulatory assays following adenovirus infection in control and NK depleted mice.

These findings have important significance for not only liver tolerance but for chronic liver disease. Certain pathogens such as HCV may exploit the tolerogenic nature of the liver in evading the immune system and establishing persistent infection. Several reports have indicated an impairment of NK cells associated with chronic HCV infection, leading to a proposed model that targeting NK cells is central to HCV persistence in the liver (Golden-Mason and Rosen 2006). In addition, the outcome of HCV infection may be influenced by certain killer-cell immunoglobulin-like receptors (KIR)/HLA-type combinations, as weaker inhibitory KIR/HLA combinations positively correlated with resolution of infection (Khakoo et al. 2004). Interestingly, our unpublished observations suggest that infection with an HCV core protein expressing adenovirus (Ad-core) results in enhanced levels of NKG2A on liver NK cells, compared to infection with a beta-gal expressing adenovirus (Ad-LacZ). Furthermore, NK cells isolated from Ad-core infected livers show reduced IFN-γ production compared to Ad-LacZ-infected liver NK cells (Lassen et al. unpublished observations). Lower NK production of IFN-γ could result in suboptimal DC maturation, leading to inefficient T cell activation.

These data presented here identify a NK cell subset present in the liver that displays distinct phenotypic and functional characteristics. These data further suggest an important contribution for IL-10 in regulating the functional capacity of the liver NK cell population, in part by decreasing the percentage of the more functionally responsive $Ly49^{+}$ subset of NK cells. Further characterization and elucidation of the mechanism of IL-10 regulation of liver NK cells may increase our understanding of liver tolerance and may lead to the development of novel and improved therapeutic strategies for persistent liver pathogens. In addition, future studies on the functional impact of NKG2A+Ly49-

NK cells on liver tolerance and response to viral infection may help us better understand how the liver maintains a tight balance between tolerance and immunity to pathogens.

Literature Cited

Adams, D. H., B. Eksteen, and S. M. Curbishley. 2008. "Immunology of the gut and liver: a love/hate relationship." *Gut* 57:838-48.

Andoniou, C. E., S. L. van Dommelen, V. Voigt, D. M. Andrews, G. Brizard, C. Asselin-Paturel, T. Delale, K. J. Stacey, G. Trinchieri, and M. A. Degli-Esposti. 2005. "Interaction between conventional dendritic cells and natural killer cells is integral to the activation of effective antiviral immunity." *Nat Immunol* 6:1011-9.

Andrews, D. M., A. A. Scalzo, W. M. Yokoyama, M. J. Smyth, and M. A. Degli-Esposti. 2003. "Functional interactions between dendritic cells and NK cells during viral infection." *Nat Immunol* 4:175-81.

Arase, H., E. S. Mocarski, A. E. Campbell, A. B. Hill, and L. L. Lanier. 2002. "Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors." *Science* 296:1323-6.

Bakker, A. B., R. M. Hoek, A. Cerwenka, B. Blom, L. Lucian, T. McNeil, R. Murray, L. H. Phillips, J. D. Sedgwick, and L. L. Lanier. 2000. "DAP12-deficient mice fail to develop autoimmunity due to impaired antigen priming." *Immunity* 13:345-53.

Bauer, S., V. Groh, J. Wu, A. Steinle, J. H. Phillips, L. L. Lanier, and T. Spies. 1999. "Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA." *Science* 285:727-9.

Bertolino, P., M. C. Trescol-Biemont, and C. Rabourdin-Combe. 1998. "Hepatocytes induce functional activation of naive CD8+ T lymphocytes but fail to promote survival." *Eur J Immunol* 28:221-36.

Bertolino, P., M. C. Trescol-Biemont, J. Thomas, B. Fazekas de St Groth, M. Pihlgren, J. Marvel, and C. Rabourdin-Combe. 1999. "Death by neglect as a deletional mechanism of peripheral tolerance." *Int Immunol* 11:1225-38.

Binstadt, B. A., K. M. Brumbaugh, C. J. Dick, A. M. Scharenberg, B. L. Williams, M. Colonna, L. L. Lanier, J. P. Kinet, R. T. Abraham, and P. J. Leibson. 1996. "Sequential involvement of Lck and SHP-1 with MHC-recognizing receptors on NK cells inhibits FcR-initiated tyrosine kinase activation." *Immunity* 5:629-38.

Bosma, B. M., H. J. Metselaar, S. Mancham, P. P. Boor, J. G. Kusters, G. Kazemier, H. W. Tilanus, E. J. Kuipers, and J. Kwekkeboom. 2006. "Characterization of human liver dendritic cells in liver grafts and perfusates." *Liver Transpl* 12:384-93.

Bowen, D. G., G. W. McCaughan, and P. Bertolino. 2005. "Intrahepatic immunity: a tale of two sites?" *Trends Immunol* 26:512-7.

Brown, M. G., and A. A. Scalzo. 2008. "NK gene complex dynamics and selection for NK cell receptors." *Semin Immunol* 20:361-8.

Bryceson, Y. T., M. E. March, H. G. Ljunggren, and E. O. Long. 2006. "Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion." *Blood* 107:159-66.

Caminschi, I., F. Ahmet, K. Heger, J. Brady, S. L. Nutt, D. Vremec, S. Pietersz, M. H. Lahoud, L. Schofield, D. S. Hansen, M. O'Keeffe, M. J. Smyth, S. Bedoui, G. M. Davey, J. A. Villadangos, W. R. Heath, and K. Shortman. 2007. "Putative IKDCs

are functionally and developmentally similar to natural killer cells, but not to dendritic cells." *J Exp Med* 204:2579-90.

Castriconi, R., C. Cantoni, M. Della Chiesa, M. Vitale, E. Marcenaro, R. Conte, R. Biassoni, C. Bottino, L. Moretta, and A. Moretta. 2003. "Transforming growth factor beta 1 inhibits expression of NKp30 and NKG2D receptors: consequences for the NK-mediated killing of dendritic cells." *Proc Natl Acad Sci U S A* 100:4120-5.

Cohen, G. B., R. T. Gandhi, D. M. Davis, O. Mandelboim, B. K. Chen, J. L. Strominger, and D. Baltimore. 1999. "The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells." *Immunity* 10:661-71.

Corado, J., F. Toro, H. Rivera, N. E. Bianco, L. Deibis, and J. B. De Sanctis. 1997. "Impairment of natural killer (NK) cytotoxic activity in hepatitis C virus (HCV) infection." *Clin Exp Immunol* 109:451-7.

Couper, K. N., D. G. Blount, and E. M. Riley. 2008. "IL-10: the master regulator of immunity to infection." *J Immunol* 180:5771-7.

Crispe, I. N. 2003. "Hepatic T cells and liver tolerance." *Nat Rev Immunol* 3:51-62.

—. 2009. "The liver as a lymphoid organ." *Annu Rev Immunol* 27:147-63.

Crispe, I. N., M. Giannandrea, I. Klein, B. John, B. Sampson, and S. Wuensch. 2006. "Cellular and molecular mechanisms of liver tolerance." *Immunol Rev* 213:101-18.

Crotta, S., A. Stilla, A. Wack, A. D'Andrea, S. Nuti, U. D'Oro, M. Mosca, F. Filliponi, R. M. Brunetto, F. Bonino, S. Abrignani, and N. M. Valiante. 2002. "Inhibition of natural killer cells through engagement of CD81 by the major hepatitis C virus envelope protein." *J Exp Med* 195:35-41.

Cruise, M. W., H. M. Melief, J. Lukens, C. Soguero, and Y. S. Hahn. 2005. "Increased Fas ligand expression of CD4+ T cells by HCV core induces T cell-dependent hepatic inflammation." *J Leukoc Biol* 78:412-25.

D'Andrea, A., M. Aste-Amezaga, N. M. Valiante, X. Ma, M. Kubin, and G. Trinchieri. 1993. "Interleukin 10 (IL-10) inhibits human lymphocyte interferon gamma-production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells." *J Exp Med* 178:1041-8.

Daniels, K. A., G. Devora, W. C. Lai, C. L. O'Donnell, M. Bennett, and R. M. Welsh. 2001. "Murine cytomegalovirus is regulated by a discrete subset of natural killer cells reactive with monoclonal antibody to Ly49H." *J Exp Med* 194:29-44.

De Maria, A., M. Fogli, S. Mazza, M. Basso, A. Picciotto, P. Costa, S. Congia, M. C. Mingari, and L. Moretta. 2007. "Increased natural cytotoxicity receptor expression and relevant IL-10 production in NK cells from chronically infected viremic HCV patients." *Eur J Immunol* 37:445-55.

de Waal Malefyt, R., J. Haanen, H. Spits, M. G. Roncarolo, A. te Velde, C. Figdor, K. Johnson, R. Kastelein, H. Yssel, and J. E. de Vries. 1991. "Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression." *J Exp Med* 174:915-24.

Della Chiesa, M., M. Vitale, S. Carlomagno, G. Ferlazzo, L. Moretta, and A. Moretta. 2003. "The natural killer cell-mediated killing of autologous dendritic cells is

confined to a cell subset expressing CD94/NKG2A, but lacking inhibitory killer Ig-like receptors." *Eur J Immunol* 33:1657-66.

Di Santo, J. P. 2006. "Natural killer cell developmental pathways: a question of balance." *Annu Rev Immunol* 24:257-86.

Ding, L., P. S. Linsley, L. Y. Huang, R. N. Germain, and E. M. Shevach. 1993. "IL-10 inhibits macrophage costimulatory activity by selectively inhibiting the up-regulation of B7 expression." *J Immunol* 151:1224-34.

Dorfman, J. R., and D. H. Raulet. 1998. "Acquisition of Ly49 receptor expression by developing natural killer cells." *J Exp Med* 187:609-18.

Dunn, C., N. J. Chalupny, C. L. Sutherland, S. Dosch, P. V. Sivakumar, D. C. Johnson, and D. Cosman. 2003. "Human cytomegalovirus glycoprotein UL16 causes intracellular sequestration of NKG2D ligands, protecting against natural killer cell cytotoxicity." *J Exp Med* 197:1427-39.

Eisen-Vandervelde, A. L., Z. Q. Yao, and Y. S. Hahn. 2004. "The molecular basis of HCV-mediated immune dysregulation." *Clin Immunol* 111:16-21.

Exley, M. A., and M. J. Koziel. 2004. "To be or not to be NKT: natural killer T cells in the liver." *Hepatology* 40:1033-40.

Farag, S. S., and M. A. Caligiuri. 2006. "Human natural killer cell development and biology." *Blood Rev* 20:123-37.

Fehniger, T. A., M. A. Cooper, G. J. Nuovo, M. Cella, F. Facchetti, M. Colonna, and M. A. Caligiuri. 2003. "CD56bright natural killer cells are present in human lymph nodes and are activated by T cell-derived IL-2: a potential new link between adaptive and innate immunity." *Blood* 101:3052-7.

Fernandez, N. C., E. Treiner, R. E. Vance, A. M. Jamieson, S. Lemieux, and D. H. Raulet. 2005. "A subset of natural killer cells achieves self-tolerance without expressing inhibitory receptors specific for self-MHC molecules." *Blood* 105:4416-23.

Gao, B., W. I. Jeong, and Z. Tian. 2008. "Liver: An organ with predominant innate immunity." *Hepatology* 47:729-36.

Gerosa, F., B. Baldani-Guerra, C. Nisii, V. Marchesini, G. Carra, and G. Trinchieri. 2002. "Reciprocal activating interaction between natural killer cells and dendritic cells." *J Exp Med* 195:327-33.

Goddard, S., J. Youster, E. Morgan, and D. H. Adams. 2004. "Interleukin-10 secretion differentiates dendritic cells from human liver and skin." *Am J Pathol* 164:511-9.

Golden-Mason, L., and H. R. Rosen. 2006. "Natural killer cells: primary target for hepatitis C virus immune evasion strategies?" *Liver Transpl* 12:363-72.

Guidotti, L. G., and F. V. Chisari. 2006. "Immunobiology and pathogenesis of viral hepatitis." *Annu Rev Pathol* 1:23-61.

Gunturi, A., R. E. Berg, and J. Forman. 2004. "The role of CD94/NKG2 in innate and adaptive immunity." *Immunol Res* 30:29-34.

Hamerman, J. A., K. Ogasawara, and L. L. Lanier. 2005. "NK cells in innate immunity." *Curr Opin Immunol* 17:29-35.

Hayakawa, Y., and M. J. Smyth. 2006. "CD27 dissects mature NK cells into two subsets with distinct responsiveness and migratory capacity." *J Immunol* 176:1517-24.

Huntington, N. D., C. A. Vosshenrich, and J. P. Di Santo. 2007. "Developmental pathways that generate natural-killer-cell diversity in mice and humans." *Nat Rev Immunol* 7:703-14.

Iwai, Y., S. Terawaki, M. Ikegawa, T. Okazaki, and T. Honjo. 2003. "PD-1 inhibits antiviral immunity at the effector phase in the liver." *J Exp Med* 198:39-50.

Jacobs, R., G. Hintzen, A. Kemper, K. Beul, S. Kempf, G. Behrens, K. W. Sykora, and R. E. Schmidt. 2001. "CD56bright cells differ in their KIR repertoire and cytotoxic features from CD56dim NK cells." *Eur J Immunol* 31:3121-7.

Jacobs, R., M. Stoll, G. Stratmann, R. Leo, H. Link, and R. E. Schmidt. 1992. "CD16-CD56+ natural killer cells after bone marrow transplantation." *Blood* 79:3239-44.

Jinushi, M., T. Takehara, T. Tatsumi, N. Hiramatsu, R. Sakamori, S. Yamaguchi, and N. Hayashi. 2005. "Impairment of natural killer cell and dendritic cell functions by the soluble form of MHC class I-related chain A in advanced human hepatocellular carcinomas." *J Hepatol* 43:1013-20.

Jinushi, M., T. Takehara, T. Tatsumi, T. Kanto, T. Miyagi, T. Suzuki, Y. Kanazawa, N. Hiramatsu, and N. Hayashi. 2004. "Negative regulation of NK cell activities by inhibitory receptor CD94/NKG2A leads to altered NK cell-induced modulation of dendritic cell functions in chronic hepatitis C virus infection." *J Immunol* 173:6072-81.

Jinushi, M., T. Takehara, T. Tatsumi, S. Yamaguchi, R. Sakamori, N. Hiramatsu, T. Kanto, K. Ohkawa, and N. Hayashi. 2007. "Natural killer cell and hepatic cell interaction via NKG2A leads to dendritic cell-mediated induction of CD4 CD25 T cells with PD-1-dependent regulatory activities." *Immunology* 120:73-82.

Jomantaite, I., N. Dikopoulos, A. Kroger, F. Leithauser, H. Hauser, R. Schirmbeck, and J. Reimann. 2004. "Hepatic dendritic cell subsets in the mouse." *Eur J Immunol* 34:355-65.

Joncker, N. T., and D. H. Raulet. 2008. "Regulation of NK cell responsiveness to achieve self-tolerance and maximal responses to diseased target cells." *Immunol Rev* 224:85-97.

Khakoo, S. I., C. L. Thio, M. P. Martin, C. R. Brooks, X. Gao, J. Astemborski, J. Cheng, J. J. Goedert, D. Vlahov, M. Hilgartner, S. Cox, A. M. Little, G. J. Alexander, M. E. Cramp, S. J. O'Brien, W. M. Rosenberg, D. L. Thomas, and M. Carrington. 2004. "HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection." *Science* 305:872-4.

Kim, S., K. Iizuka, H. S. Kang, A. Dokun, A. R. French, S. Greco, and W. M. Yokoyama. 2002. "In vivo developmental stages in murine natural killer cell maturation." *Nat Immunol* 3:523-8.

Kim, S., J. Poursine-Laurent, S. M. Truscott, L. Lybarger, Y. J. Song, L. Yang, A. R. French, J. B. Sunwoo, S. Lemieux, T. H. Hansen, and W. M. Yokoyama. 2005. "Licensing of natural killer cells by host major histocompatibility complex class I molecules." *Nature* 436:709-13.

Knechtle, S. J., and J. Kwun. 2009. "Unique aspects of rejection and tolerance in liver transplantation." *Semin Liver Dis* 29:91-101.

Knolle, P. A., E. Schmitt, S. Jin, T. Germann, R. Duchmann, S. Hegenbarth, G. Gerken, and A. W. Lohse. 1999. "Induction of cytokine production in naive CD4(+) T

cells by antigen-presenting murine liver sinusoidal endothelial cells but failure to induce differentiation toward Th1 cells." *Gastroenterology* 116:1428-40.

Krmpotic, A., D. H. Busch, I. Bubic, F. Gebhardt, H. Hengel, M. Hasan, A. A. Scalzo, U. H. Koszinowski, and S. Jonjic. 2002. "MCMV glycoprotein gp40 confers virus resistance to CD8+ T cells and NK cells in vivo." *Nat Immunol* 3:529-35.

Kwekkeboom, J., P. P. Boor, E. Sen, J. G. Kusters, H. A. Drexhage, E. C. de Jong, H. W. Tilanus, J. N. Ijzermans, and H. J. Metselaar. 2005. "Human liver myeloid dendritic cells maturate in vivo into effector DC with a poor allogeneic T-cell stimulatory capacity." *Transplant Proc* 37:15-6.

Lanier, L. L. 2005. "NK cell recognition." *Annu Rev Immunol* 23:225-74.

—. 2009. "DAP10- and DAP12-associated receptors in innate immunity." *Immunol Rev* 227:150-60.

Lanier, L. L., B. C. Corliss, J. Wu, C. Leong, and J. H. Phillips. 1998a. "Immunoreceptor DAP12 bearing a tyrosine-based activation motif is involved in activating NK cells." *Nature* 391:703-7.

Lanier, L. L., B. Corliss, J. Wu, and J. H. Phillips. 1998b. "Association of DAP12 with activating CD94/NKG2C NK cell receptors." *Immunity* 8:693-701.

Lanier, L. L., A. M. Le, C. I. Civin, M. R. Loken, and J. H. Phillips. 1986. "The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes." *J Immunol* 136:4480-6.

Laouar, Y., F. S. Sutterwala, L. Gorelik, and R. A. Flavell. 2005. "Transforming growth factor-beta controls T helper type 1 cell development through regulation of natural killer cell interferon-gamma." *Nat Immunol* 6:600-7.

Large, M. K., D. J. Kittlesen, and Y. S. Hahn. 1999. "Suppression of host immune response by the core protein of hepatitis C virus: possible implications for hepatitis C virus persistence." *J Immunol* 162:931-8.

Le Drean, E., F. Vely, L. Olcese, A. Cambiaggi, S. Guia, G. Krystal, N. Gervois, A. Moretta, F. Jotereau, and E. Vivier. 1998. "Inhibition of antigen-induced T cell response and antibody-induced NK cell cytotoxicity by NKG2A: association of NKG2A with SHP-1 and SHP-2 protein-tyrosine phosphatases." *Eur J Immunol* 28:264-76.

Li, X., D. Klintman, T. Sato, G. Hedlund, R. Schramm, B. Jeppsson, and H. Thorlacius. 2004. "Interleukin-10 mediates the protective effect of Linomide by reducing CXC chemokine production in endotoxin-induced liver injury." *Br J Pharmacol* 143:865-71.

Lodoen, M. B., G. Abenes, S. Umamoto, J. P. Houchins, F. Liu, and L. L. Lanier. 2004. "The cytomegalovirus m155 gene product subverts natural killer cell antiviral protection by disruption of H60-NKG2D interactions." *J Exp Med* 200:1075-81.

Lodoen, M. B., and L. L. Lanier. 2005. "Viral modulation of NK cell immunity." *Nat Rev Microbiol* 3:59-69.

Lohse, A. W., P. A. Knolle, K. Bilo, A. Uhrig, C. Waldmann, M. Ibe, E. Schmitt, G. Gerken, and K. H. Meyer Zum Buschenfelde. 1996. "Antigen-presenting function and B7 expression of murine sinusoidal endothelial cells and Kupffer cells." *Gastroenterology* 110:1175-81.

Lu, L., K. Ikizawa, D. Hu, M. B. Werneck, K. W. Wucherpfennig, and H. Cantor. 2007. "Regulation of activated CD4+ T cells by NK cells via the Qa-1-NKG2A inhibitory pathway." *Immunity* 26:593-604.

Lucas, M., W. Schachterle, K. Oberle, P. Aichele, and A. Diefenbach. 2007. "Dendritic cells prime natural killer cells by trans-presenting interleukin 15." *Immunity* 26:503-17.

Luci, C., A. Reynders, Ivanov, II, C. Cognet, L. Chiche, L. Chasson, J. Hardwigsen, E. Anguiano, J. Banchereau, D. Chaussabel, M. Dalod, D. R. Littman, E. Vivier, and E. Tomasello. 2009. "Influence of the transcription factor RORgammat on the development of NKp46+ cell populations in gut and skin." *Nat Immunol* 10:75-82.

Lukens, J. R., M. W. Cruise, M. G. Lassen, and Y. S. Hahn. 2008. "Blockade of PD-1/B7-H1 interaction restores effector CD8+ T cell responses in a hepatitis C virus core murine model." *J Immunol* 180:4875-84.

Luo, D., K. Vanderkerken, M. C. Chen, D. Vermijlen, K. Asosingh, E. Willems, V. Triantis, D. L. Eizirik, P. J. Kuppen, and E. Wisse. 2001. "Rat hepatic natural killer cells (pit cells) express mRNA and protein similar to in vitro interleukin-2 activated spleen natural killer cells." *Cell Immunol* 210:41-8.

Luo, D. Z., D. Vermijlen, B. Ahishali, V. Triantis, G. Plakoutsi, F. Braet, K. Vanderkerken, and E. Wisse. 2000. "On the cell biology of pit cells, the liver-specific NK cells." *World J Gastroenterol* 6:1-11.

Martin-Fontecha, A., L. L. Thomsen, S. Brett, C. Gerard, M. Lipp, A. Lanzavecchia, and F. Sallusto. 2004. "Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1 priming." *Nat Immunol* 5:1260-5.

McMahon, C. W., A. J. Zajac, A. M. Jamieson, L. Corral, G. E. Hammer, R. Ahmed, and D. H. Raulet. 2002. "Viral and bacterial infections induce expression of multiple NK cell receptors in responding CD8(+) T cells." *J Immunol* 169:1444-52.

Meier, U. C., R. E. Owen, E. Taylor, A. Worth, N. Naoumov, C. Willberg, K. Tang, P. Newton, P. Pellegrino, I. Williams, P. Klenerman, and P. Borrow. 2005. "Shared alterations in NK cell frequency, phenotype, and function in chronic human immunodeficiency virus and hepatitis C virus infections." *J Virol* 79:12365-74.

Moretta, A. 2002. "Natural killer cells and dendritic cells: rendezvous in abused tissues." *Nat Rev Immunol* 2:957-64.

Mortier, E., T. Woo, R. Advincula, S. Gozalo, and A. Ma. 2008. "IL-15Ralpha chaperones IL-15 to stable dendritic cell membrane complexes that activate NK cells via trans presentation." *J Exp Med* 205:1213-25.

Moser, J. M., J. Gibbs, P. E. Jensen, and A. E. Lukacher. 2002. "CD94-NKG2A receptors regulate antiviral CD8(+) T cell responses." *Nat Immunol* 3:189-95.

Nakatani, K., K. Kaneda, S. Seki, and Y. Nakajima. 2004. "Pit cells as liver-associated natural killer cells: morphology and function." *Med Electron Microsc* 37:29-36.

Namekawa, T., M. R. Snyder, J. H. Yen, B. E. Goehring, P. J. Leibson, C. M. Weyand, and J. J. Goronzy. 2000. "Killer cell activating receptors function as costimulatory molecules on CD4+CD28null T cells clonally expanded in rheumatoid arthritis." *J Immunol* 165:1138-45.

Ouyang, Q., G. Baerlocher, I. Vulto, and P. M. Lansdorp. 2007. "Telomere length in human natural killer cell subsets." *Ann N Y Acad Sci* 1106:240-52.

Pascal, V., N. R. Nathan, E. Claudio, U. Siebenlist, and S. K. Anderson. 2007. "NF-kappa B p50/p65 affects the frequency of Ly49 gene expression by NK cells." *J Immunol* 179:1751-9.

Piccioli, D., S. Sbrana, E. Melandri, and N. M. Valiante. 2002. "Contact-dependent stimulation and inhibition of dendritic cells by natural killer cells." *J Exp Med* 195:335-41.

Pillarisetty, V. G., A. B. Shah, G. Miller, J. I. Bleier, and R. P. DeMatteo. 2004. "Liver dendritic cells are less immunogenic than spleen dendritic cells because of differences in subtype composition." *J Immunol* 172:1009-17.

Racanelli, V., and B. Rehermann. 2006. "The liver as an immunological organ." *Hepatology* 43:S54-62.

Radaeva, S., R. Sun, B. Jaruga, V. T. Nguyen, Z. Tian, and B. Gao. 2006. "Natural killer cells ameliorate liver fibrosis by killing activated stellate cells in NKG2D-dependent and tumor necrosis factor-related apoptosis-inducing ligand-dependent manners." *Gastroenterology* 130:435-52.

Raulet, D. H., and R. E. Vance. 2006. "Self-tolerance of natural killer cells." *Nat Rev Immunol* 6:520-31.

Ravetch, J. V., and L. L. Lanier. 2000. "Immune inhibitory receptors." *Science* 290:84-9.

Robbins, S. H., K. B. Nguyen, N. Takahashi, T. Mikayama, C. A. Biron, and L. Brossay. 2002. "Cutting edge: inhibitory functions of the killer cell lectin-like receptor G1 molecule during the activation of mouse NK cells." *J Immunol* 168:2585-9.

Roberts, A. I., L. Lee, E. Schwarz, V. Groh, T. Spies, E. C. Ebert, and B. Jabri. 2001. "NKG2D receptors induced by IL-15 costimulate CD28-negative effector CTL in the tissue microenvironment." *J Immunol* 167:5527-30.

Rolle, A., M. Mousavi-Jazi, M. Eriksson, J. Odeberg, C. Soderberg-Naucler, D. Cosman, K. Karre, and C. Cerboni. 2003. "Effects of human cytomegalovirus infection on ligands for the activating NKG2D receptor of NK cells: up-regulation of UL16-binding protein (ULBP)1 and ULBP2 is counteracted by the viral UL16 protein." *J Immunol* 171:902-8.

Rosmaraki, E. E., I. Douagi, C. Roth, F. Colucci, A. Cumano, and J. P. Di Santo. 2001. "Identification of committed NK cell progenitors in adult murine bone marrow." *Eur J Immunol* 31:1900-9.

Salazar-Mather, T. P., T. A. Hamilton, and C. A. Biron. 2000. "A chemokine-to-cytokine-to-chemokine cascade critical in antiviral defense." *J Clin Invest* 105:985-93.

Salazar-Mather, T. P., C. A. Lewis, and C. A. Biron. 2002. "Type I interferons regulate inflammatory cell trafficking and macrophage inflammatory protein 1alpha delivery to the liver." *J Clin Invest* 110:321-30.

Salazar-Mather, T. P., J. S. Orange, and C. A. Biron. 1998. "Early murine cytomegalovirus (MCMV) infection induces liver natural killer (NK) cell inflammation and protection through macrophage inflammatory protein 1alpha (MIP-1alpha)-dependent pathways." *J Exp Med* 187:1-14.

Samson, S. I., O. Richard, M. Tavian, T. Ranson, C. A. Vosshenrich, F. Colucci, J. Buer, F. Grosveld, I. Godin, and J. P. Di Santo. 2003. "GATA-3 promotes maturation, IFN-gamma production, and liver-specific homing of NK cells." *Immunity* 19:701-11.

Sivakumar, P. V., A. Gunturi, M. Salcedo, J. D. Schatzle, W. C. Lai, Z. Kurepa, L. Pitcher, M. S. Seaman, F. A. Lemonnier, M. Bennett, J. Forman, and V. Kumar. 1999. "Cutting edge: expression of functional CD94/NKG2A inhibitory receptors on fetal NK1.1+Ly-49- cells: a possible mechanism of tolerance during NK cell development." *J Immunol* 162:6976-80.

Smith, H. R., J. W. Heusel, I. K. Mehta, S. Kim, B. G. Dorner, O. V. Naidenko, K. Iizuka, H. Furukawa, D. L. Beckman, J. T. Pingel, A. A. Scalzo, D. H. Fremont, and W. M. Yokoyama. 2002. "Recognition of a virus-encoded ligand by a natural killer cell activation receptor." *Proc Natl Acad Sci U S A* 99:8826-31.

Smith, K. M., J. Wu, A. B. Bakker, J. H. Phillips, and L. L. Lanier. 1998. "Ly-49D and Ly-49H associate with mouse DAP12 and form activating receptors." *J Immunol* 161:7-10.

Snyder, M. R., M. Lucas, E. Vivier, C. M. Weyand, and J. J. Goronzy. 2003. "Selective activation of the c-Jun NH2-terminal protein kinase signaling pathway by stimulatory KIR in the absence of KARAP/DAP12 in CD4+ T cells." *J Exp Med* 197:437-49.

Sutherland, C. L., N. J. Chalupny, K. Schooley, T. VandenBos, M. Kubin, and D. Cosman. 2002. "UL16-binding proteins, novel MHC class I-related proteins, bind to NKG2D and activate multiple signaling pathways in primary NK cells." *J Immunol* 168:671-9.

Takeda, K., E. Cretney, Y. Hayakawa, T. Ota, H. Akiba, K. Ogasawara, H. Yagita, K. Kinoshita, K. Okumura, and M. J. Smyth. 2005. "TRAIL identifies immature natural killer cells in newborn mice and adult mouse liver." *Blood* 105:2082-9.

Takeda, K., Y. Hayakawa, M. J. Smyth, N. Kayagaki, N. Yamaguchi, S. Kakuta, Y. Iwakura, H. Yagita, and K. Okumura. 2001. "Involvement of tumor necrosis factor-related apoptosis-inducing ligand in surveillance of tumor metastasis by liver natural killer cells." *Nat Med* 7:94-100.

Toura, I., T. Kawano, Y. Akutsu, T. Nakayama, T. Ochiai, and M. Taniguchi. 1999. "Cutting edge: inhibition of experimental tumor metastasis by dendritic cells pulsed with alpha-galactosylceramide." *J Immunol* 163:2387-91.

Tseng, C. T., and G. R. Klimpel. 2002. "Binding of the hepatitis C virus envelope protein E2 to CD81 inhibits natural killer cell functions." *J Exp Med* 195:43-9.

Tu, Z., A. Bozorgzadeh, R. H. Pierce, J. Kurtis, I. N. Crispe, and M. S. Orloff. 2008. "TLR-dependent cross talk between human Kupffer cells and NK cells." *J Exp Med* 205:233-44.

Ulbrecht, M., S. Martinozzi, M. Grzeschik, H. Hengel, J. W. Ellwart, M. Pla, and E. H. Weiss. 2000. "Cutting edge: the human cytomegalovirus UL40 gene product contains a ligand for HLA-E and prevents NK cell-mediated lysis." *J Immunol* 164:5019-22.

Vance, R. E., A. M. Jamieson, and D. H. Raulet. 1999. "Recognition of the class Ib molecule Qa-1(b) by putative activating receptors CD94/NKG2C and CD94/NKG2E on mouse natural killer cells." *J Exp Med* 190:1801-12.

Vance, R. E., J. R. Kraft, J. D. Altman, P. E. Jensen, and D. H. Raulet. 1998. "Mouse CD94/NKG2A is a natural killer cell receptor for the nonclassical major histocompatibility complex (MHC) class I molecule Qa-1(b)." *J Exp Med* 188:1841-8.

Vincent, M. S., J. E. Gumperz, and M. B. Brenner. 2003. "Understanding the function of CD1-restricted T cells." *Nat Immunol* 4:517-23.

Vosshenrich, C. A., M. E. Garcia-Ojeda, S. I. Samson-Villeger, V. Pasqualetto, L. Enault, O. Richard-Le Goff, E. Corcuff, D. Guy-Grand, B. Rocha, A. Cumano, L. Rogge, S. Ezine, and J. P. Di Santo. 2006. "A thymic pathway of mouse natural killer cell development characterized by expression of GATA-3 and CD127." *Nat Immunol* 7:1217-24.

Vosshenrich, C. A., S. I. Samson-Villeger, and J. P. Di Santo. 2005. "Distinguishing features of developing natural killer cells." *Curr Opin Immunol* 17:151-8.

Waggoner, S. N., C. H. Hall, and Y. S. Hahn. 2007. "HCV core protein interaction with gC1q receptor inhibits Th1 differentiation of CD4+ T cells via suppression of dendritic cell IL-12 production." *J Leukoc Biol* 82:1407-19.

Walzer, T., M. Blery, J. Chaix, N. Fuseri, L. Chasson, S. H. Robbins, S. Jaeger, P. Andre, L. Gauthier, L. Daniel, K. Chemin, Y. Morel, M. Dalod, J. Imbert, M. Pierres, A. Moretta, F. Romagne, and E. Vivier. 2007. "Identification, activation, and selective in vivo ablation of mouse NK cells via NKp46." *Proc Natl Acad Sci U S A* 104:3384-9.

Wang, E. C., B. McSharry, C. Retiere, P. Tomasec, S. Williams, L. K. Borysiewicz, V. M. Braud, and G. W. Wilkinson. 2002. "UL40-mediated NK evasion during productive infection with human cytomegalovirus." *Proc Natl Acad Sci U S A* 99:7570-5.

Willems, F., A. Marchant, J. P. Delville, C. Gerard, A. Delvaux, T. Velu, M. de Boer, and M. Goldman. 1994. "Interleukin-10 inhibits B7 and intercellular adhesion molecule-1 expression on human monocytes." *Eur J Immunol* 24:1007-9.

Williams, N. S., A. Kubota, M. Bennett, V. Kumar, and F. Takei. 2000. "Clonal analysis of NK cell development from bone marrow progenitors in vitro: orderly acquisition of receptor gene expression." *Eur J Immunol* 30:2074-82.

Wu, J., Y. Song, A. B. Bakker, S. Bauer, T. Spies, L. L. Lanier, and J. H. Phillips. 1999. "An activating immunoreceptor complex formed by NKG2D and DAP10." *Science* 285:730-2.

Yokoyama, W. M., and S. Kim. 2006. "Licensing of natural killer cells by self-major histocompatibility complex class I." *Immunol Rev* 214:143-54.

Yokoyama, W. M., and B. F. Plougastel. 2003. "Immune functions encoded by the natural killer gene complex." *Nat Rev Immunol* 3:304-16.

Yoon, J. C., M. Shiina, G. Ahlenstiel, and B. Rehermann. 2009. "Natural killer cell function is intact after direct exposure to infectious hepatitis C virions." *Hepatology* 49:12-21.

Yoshidome, H., A. Kato, M. J. Edwards, and A. B. Lentsch. 1999. "Interleukin-10 inhibits pulmonary NF-kappaB activation and lung injury induced by hepatic ischemia-reperfusion." *Am J Physiol* 277:L919-23.

Ziegler, H., R. Thale, P. Lucin, W. Muranyi, T. Flohr, H. Hengel, H. Farrell, W. Rawlinson, and U. H. Koszinowski. 1997. "A mouse cytomegalovirus glycoprotein retains MHC class I complexes in the ERGIC/cis-Golgi compartments." *Immunity* 6:57-66.

Zwirner, N. W., M. B. Fuertes, M. V. Girart, C. I. Domaica, and L. E. Rossi. 2007. "Cytokine-driven regulation of NK cell functions in tumor immunity: role of the MICA-NKG2D system." *Cytokine Growth Factor Rev* 18:159-70.