2017

Molecular Mechanisms and Functional Role of Hepatic Invariant Natural Killer T cell Recruitment Following Sterile Injury in the Liver

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Molecular Mechanisms and Functional Role of Hepatic Invariant Natural Killer T cell Recruitment Following Sterile Injury in the Liver

by

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A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE
DEGREE OF DOCTOR OF PHILOSOPHY

GRADUATE PROGRAM IN IMMUNOLOGY

CALGARY, ALBERTA

APRIL, 2017

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Abstract

After traumatic injury, the body must return to homeostasis as quickly as possible through initial destruction of injured cells and clearance of debris (inflammation) followed by a critical switch towards vascular and tissue reconstruction (non-inflammatory restitution). While many different immune cells are known to infiltrate a site of injury for specific effector functions, it is reasonable to hypothesize that some cells function as directors of inflammation whereupon sensing the degree of injury, these cells orchestrate the local immune response towards a restitution phase by affecting the local cytokine milieu. This progression could be defective in non-resolving sterile injuries where a failure to transition to repair or a persistence of inflammation leads to chronic inflammation.

Invariant Natural Killer T (iNKT) cells are innate lymphocytes that prominently regulate inflammation due to their rapid release of pivotal cytokines during pathological states, which can then differentially impact the downstream immune response and disease outcome. In this thesis, using intravital microscopy, we observed that patrolling iNKT cells in the liver were initially selectively repelled from a site of hepatic injury but were subsequently strategically arrested via self-antigens and cytokines, circumscribing the injured site at exactly the location where monocytes co-localized and hepatocytes proliferated. iNKT cell activation signals were temporally- and spatially-regulated as self-antigen presentation via CD1d occurred first before being closely followed by cytokine signaling. Additionally, both activation signals were located adjacent to the injury and not further away.

Activation of iNKT cells through these two mechanisms resulted in the production of IL-4 but not IFN-γ which promoted increased hepatocyte proliferation, monocyte transition (from classical to tissue repair monocytes) and improved healing. Disruption of any of these
mechanisms led to a delay of wound healing. We show that self-antigens, beyond the known role in iNKT cell development, were fundamental for monocyte transition, appropriate collagen deposition and hepatocyte proliferation. Hepatic iNKT cells were instrumental in directing and modulating the transformation from inflammation to tissue restitution after sterile injury in the liver for essential timely wound repair.
Preface

Some of the material in Chapter 1, the literature review, has been published in an academic, peer reviewed journal and book chapter. Full citations and written permissions for re-use have been obtained for each publication in accordance with thesis guidelines. Furthermore, Chapter 1 to 6 of this thesis has been re-formatted from a manuscript in progress and contributions for this manuscript will be outlined below. Another manuscript in progress is presented in its entirety in Appendix A. Unless explicitly stated, all experiments described in this thesis were performed by Pei Xiong Liew.


A segment of this review article is presented in Chapter 1. Liew PX. authored the manuscript, Kubes P. provided overall supervision and contributed to preparation of manuscript.


A portion of this book chapter is included in Chapter 1. Liew PX. authored the manuscript, Kubes P. provided overall supervision and contributed to the preparation of the chapter.

3) **Liew, PX.**, Lee WY and P. Kubes. *Initial repulsion followed by self-antigen retention are critical for iNKT cells to orchestrate a switch from inflammation to resolution of sterile injury*. Work from this manuscript in preparation is presented in Chapter 1 – 6. All experiments were designed and conducted by myself with the following exception:
Woo-Yong Lee generated CD1d\textsuperscript{ff}VECad-Cre.Cxcr6\textsuperscript{GFP/+} bone marrow chimera mice. Woo-Yong Lee also confirmed the knockdown of CD1d expression using flow cytometry on CD1d\textsuperscript{ff}VECad-Cre mice following tamixofen treatment.

All data analysis and figures were completed by myself with the following exception: Woo-Yong Lee provided the flow cytometry image of knockdown of CD1d expression on CD1d\textsuperscript{ff}VECad-Cre.Cxcr6\textsuperscript{GFP/+} bone marrow chimera mice.

I wrote the original manuscript and implemented changes to the manuscript under the supervision of Dr. Paul Kubes.

4) Liew, PX., Kim JH, Lee WY, Kubes P. Antibody-dependent fragmentation is a newly identified mechanism of cell killing \textit{in vivo}.

Data from this manuscript is presented in its entirety in Appendix A of this thesis. All experiments were designed and conducted by myself with the following exceptions:
Jung Hwan Kim helped harvest leukocytes for lung samples for flow cytometric analysis.
Woo-yong Lee helped with harvesting of hepatic iNKT cells for cell culturing in cell media.

All data analysis and figures were completed by myself. I wrote the original manuscript and made changes under the supervision of Dr. Paul Kubes.
Acknowledgements

Completing a doctorate is not an easy task and there are a number of inspiring people who played significant roles in my education and deserved to be thanked here. I would like to thank Dr. Paul Kubes for his excellent mentorship, training, commitment and the many opportunities he has given to me during my time at his laboratory. I would also like to thank my committee members: Dr. Christopher Mody and Dr. Bertus Eksteen, both who have been instrumental in providing guidance during my PhD and for their time and dedication. My progress and success through my PhD not have been possible without the supervision and unrelenting support that Dr. Kubes’ and my committee members have provided. In addition, I would also like to thank the previous and current program directors of the Immunology research program (Dr. Daniel Muruve and Dr. Donna-Marie McCafferty) for their help in navigating the requirements of the Immunology Graduate Program.

Research is a team effort and I am grateful to the support and generosity in the Kubes’ laboratory. First, I would like to extend my sincere thanks to all past and present colleagues in the Kubes’ lab. I would like to offer special thanks to Dr. Woo-yong Lee for being an outstanding teacher and consistently being there when I needed hands-on help. I would also like to thank Dr. Braedon McDonald, Dr. Ela Kolaczkowska, Dr. Justin Deniset for their advice in writing and the helpful discussions we had. A special thanks to Derrice Knight, Lori Zbytnuik, Marlene Manson and Trecia Nussbaumer for their help or expertise with animals. Also, thanks to Dr. Bjorn Petri, Dr. Bas Surewaard, Dr. Juha Korhonen, Dr. Connie Wong and Dr. Craig Jenne for their help with various experimental techniques. Also, I would like to thank the internal and external examiners (Dr. Donna Senger and Dr. Thierry Mallevaey) for their time and
consideration of my thesis. In addition, I want to thank the Live Cell Imaging and Flow Cytometry core facilities for their help and technical support.

Last but not least, I would like to thank my family for their lifetime support and encouragement for my decision to start on this journey. It has been difficult leaving everything behind in my home country and starting anew in a foreign land. Without them, I wouldn’t have been who and what I am today. To my father, for his invaluable advice and help throughout life and my mother who has always supported me no matter what I did. Many thanks to my grandmother who never had expectations of me but gave everything. Also to my uncle and aunt who have always done much the same. Finally, to Jhoanna, for her support and love.
Dedication

To my family, for their unconditional support and trust, over this long important journey of my life. Especially my grandmother, who has been waiting patiently for the day I would graduate.
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<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
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<tbody>
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<td>αGalCer</td>
<td>Alpha galactosylceramide</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine aminotransferrase</td>
</tr>
<tr>
<td>APAP</td>
<td>Acetaminophen</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>CCl₄</td>
<td>Carbon tetrachloride</td>
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<tr>
<td>CLL</td>
<td>Clodronate liposome</td>
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<td>Con-A</td>
<td>Concanavalin A</td>
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<tr>
<td>CCR2</td>
<td>CC-chemokine receptor 2</td>
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<td>CXCR3</td>
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<tr>
<td>CX₃CR1</td>
<td>CX₃-chemokine receptor 1</td>
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<tr>
<td>DAMPs</td>
<td>Damage associated molecular patterns</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>DDC</td>
<td>3,5-diethoxycarbonyl-1,4-dihydrocollidine</td>
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<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>FWB</td>
<td>Flow wash buffer</td>
</tr>
<tr>
<td>Gr-1</td>
<td>Granulocyte differentiation antigen 1</td>
</tr>
<tr>
<td>iNKT</td>
<td>Invariant Natural Killer T cell</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
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<tr>
<td>LDL</td>
<td>Liver dendritic cell</td>
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<td>Liver sinusoidal endothelial cell</td>
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<td>ICAM-1</td>
<td>Intercellular adhesion molecule 1</td>
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<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
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<tr>
<td>KC</td>
<td>Kupffer cell</td>
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<tr>
<td>MIG</td>
<td>Monokine induced by gamma interferon</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>MIP2</td>
<td>Macrophage inflammatory protein 2</td>
</tr>
<tr>
<td>MLKL</td>
<td>Mixed-lineage kinase domain-like</td>
</tr>
<tr>
<td>NAPQI</td>
<td>N-acetyl-p-benzoquinoneimine</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer cell</td>
</tr>
<tr>
<td>NRP-1a</td>
<td>Neuropilin-1a</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
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<td>Platelet endothelial cell adhesion molecule 1</td>
</tr>
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<td>Propidium Iodide</td>
</tr>
<tr>
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<td>Promyelocytic leukaemia zinc finger protein</td>
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<td>PRR</td>
<td>Pattern recognition receptor</td>
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<tr>
<td>PSGL-1</td>
<td>P-selectin glycoprotein ligand-1</td>
</tr>
<tr>
<td>RIP</td>
<td>Receptor interacting protein</td>
</tr>
<tr>
<td>SHS</td>
<td>Second harmonic signal</td>
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<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor α</td>
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<tr>
<td>TGF-β</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>UT</td>
<td>Untreated</td>
</tr>
<tr>
<td>VECad</td>
<td>Vascular endothelial cadherin</td>
</tr>
<tr>
<td>WT</td>
<td>Wildtype</td>
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Epigraph

“A person who has never made a mistake has never tried anything new.” – Albert Einstein
Chapter One: Introduction and Literature Review

1.1 Introduction

The sterile inflammation response occurs in the absence of infection and underlies medical afflictions such as mechanical trauma, chemical and environmental insults and ischemia-reperfusion injuries in all organs including the liver, heart (myocardial infarction) and brain (stroke) (Rock et al., 2010; Shen et al., 2013). Dysregulation of sterile inflammation can exacerbate and prolong damage to healthy tissue and fundamentally contribute to chronic disease pathology (Kono and Rock, 2008; Lukens et al., 2012). Although much research has been done on sterile injury induced by toxins, clinical interventions or life-style (diet), significantly less knowledge exists in regards to molecular mechanisms and cell types that underlie the early inflammatory phase as well as the progression into a resolution phase (healing) that has been optimized by millions of years of evolution for a timely return to homeostasis. Understanding the molecular mechanisms that underlie a healthy inflammatory response that then seamlessly progresses into repair will advance the design of novel effective therapeutics that treat chronic inflammatory diseases of the Western nations that lack the ability to fully resolve.

1.2 Sterile inflammation in the liver

Sterile (noninfectious) inflammation due to liver injury, including the effects of drug intake and dietary compositions, is a leading cause of liver disease in developed countries (Brempelis and Crispe, 2016; Kubes and Mehal, 2012). In the Western world, the major cause of acute liver failure is drug-induced liver injury, specifically, acetaminophen (APAP) overdose which accounts for close to 50% of acute liver failure cases (Lee, 2012). Other causes of acute liver failure also include toxin-induced liver injury, ischemia-reperfusion and idiosyncratic drug reactions (Cannistra et al., 2016; Lopez and Hendrickson, 2014; Plevris et al., 1998). Acute liver
failure has a high mortality rate if untreated and liver transplants are typically required to improve patient survival (Simpson et al., 2009; Stravitz and Kramer, 2009). Yet, outcomes for patients after liver transplantation are not normal in quality or quantity as transplantation itself comes with complications leading to loss of life-years and metabolic or cardiovascular complications (Neuberger, 2016). Understanding the inflammatory response that occurs after hepatic sterile injury may lead to novel methods to treat liver diseases which could lessen or negate the need for liver transplantation, the final option for patients. In the next few sections, how cell death in the liver leads to an inflammatory response together with the mechanisms and signals which regulate innate immune cell recruitment to sites of hepatic sterile injury will be reviewed.

1.2.1 Liver Architecture and Vascular system

The liver is the largest organ in the body and constitutes about 1/50th of the adult body weight. Although the liver is essential for maintaining metabolic homeostasis and detoxification, it is also designed to optimize immune surveillance. The liver is organized into functional hexagonal lobules consisting of one-cell-thick ‘liver plates’ made up of 15-25 hepatocytes (Ishibashi et al., 2009). The hepatic capillary or sinusoid is formed between two cell plates where blood flows from the portal triad through sinusoids to the central vein (Figure 1-1). Located in these sinusoids are the largest population of macrophages and highest densities of natural killer T (NKT) and natural killer (NK) cells. In combination with other immune cells, this network of sentinel and effector immune cells maximises the ability of the hepatic immune system to respond to microorganisms or non-infectious damage and cell death.
**Figure 1-1. Schematic of a hepatic lobule.**

Hepatocytes, which consists of one-cell-thick rows, are organized hexagonally around a central venule in a hepatic lobule. On the perimeter of each lobule exists the portal triad which is made up of the hepatic artery, portal vein, and bile duct. Mixed (arterial and venous) blood flows from the portal triad to the central vein through sinusoids that are lined with fenestrated endothelial cells which are in turn flanked by hepatocytes. Each lobule is made up of six portal triads and one central vein. LSECs, liver sinusoidal endothelial cells. Reproduced with permission from (Liew and Kubes, 2016).
The liver has a dual blood supply from the hepatic artery (arterial blood) and portal vein (venous blood). The hepatic artery contributes approximately 20-35% of the blood supply while the remainder is delivered by the portal blood (Crispe, 2011; Tygstrup et al., 1962). Approximately 30% of the total volume of blood in the body passes through the liver each minute (Sheth and Bankey, 2001). The design of the hepatic vascular structure slows down the flow so that blood percolates through the honeycomb of sinusoids in the liver allowing contact between molecules in the blood stream and the immune system to be maximised. In fact, blood pressure has been measured to drop 50-fold between arterial blood and sinusoids and about 5-fold between the portal system and sinusoids (Oda et al., 2003).

In the liver, portal blood received from the intestine via the splanchnic circulation is enriched with nutrients and pathogen derived molecules such as lipopolysaccharide (LPS) (Lumsden et al., 1988). The ability of the liver to immunologically filter blood is profound. A 100-fold decrease in LPS concentration can be seen across the liver (Lumsden et al., 1988). As low amounts of LPS (approximately 2 ng/kg) in circulation can cause severe physiological effects in humans and higher amounts can lead to death in humans (Nemzek et al., 2008; Opal et al., 1999), it is imperative that these molecules are efficiently removed by the liver before entering the peripheral circulation. Although larger particles such as intact pathogens have recently been shown to predominantly travel to draining lymph nodes, translocation of whole and intact pathogens from the gut into portal blood can occur during instances of overt damage to the gut (Berg, 1995; Son et al., 2010). In cases of liver failure, loss of these functions as well as other factors (including metabolic stress or insufficient synthesis) leads to host fatality (Helling, 2006; van den Broek et al., 2008).
1.2.2 How cell death in the liver leads to a sterile inflammatory response

The defining characteristic of sterile liver injury is the death of hepatocytes. For example, in APAP-induced toxicity, while initial damage is observed in the endothelial cells, it is followed by extensive hepatocyte cell death at the centrilobular zones of the liver which is the main mechanism of injury (Hinson et al., 2010). A similar mechanism was seen in ischemia-reperfusion injury following liver transplants (Huet et al., 2004; van Golen et al., 2012). Historically, there has been debate whether the mode of hepatocyte cell death was due to apoptosis or necrosis but growing evidence points to necrosis as the dominant pathway of cell death (Hinson et al., 2010; Wang, 2014). During necrosis, indicators of tissue injury known as DAMPs (damage associated molecular patterns) are liberated from dead and dying cells. These DAMPs bind to specific receptors on immune cells which results in their activation and initiation of robust inflammation (Chen and Nunez, 2010; Kono and Rock, 2008). In contrast, death by apoptosis usually fails to elicit an inflammatory response. The list of known DAMPs continues to grow as do the receptors on immune cells which recognize these DAMPs (Table 1-1).

Necrosis has classically been viewed as a nonspecific, passive (unprogrammed) event of cellular lysis due to burn injury or trauma. Characterized by cellular swelling and rupture of the plasma membrane, necrosis results in a general release of all cellular contents into the extracellular environment which leads to significant inflammation (Figure 1-2). Recent studies however, have revealed that necrosis can develop from tightly coordinated programmed cell death pathways known as pyroptosis and necroptosis. Pyroptosis is an inflammasome-dependent process and is initiated by caspase-1 (Fernandes-Alnemri et al., 2009). Initiation of this pathway results in lytic cell death together with the production of inflammatory cytokines interleukin (IL)-1β and IL-18 (Figure 1-2). Cell death through necroptosis is activated by the death receptor
Table 1. Damage-associated molecular patterns (DAMPs) and their receptors.
The above table is a list of currently known intracellular and extracellular DAMPs and the receptors to which they were demonstrated bind to drive cellular activation.
Figure 1-2. Different mechanisms of cell death that regulate inflammatory responses

Illustration depicting a summary of cell death processes by apoptosis, necrosis, pyroptosis and necroptosis. During apoptotic cell death, cellular contents are retained and keep-out signals are released which serves to prevent neutrophil recruitment. In comparison, cell death by necrosis, pyroptosis and necroptosis results in release of DAMPs and induction of sterile inflammation.
(tumor necrosis factor superfamily) or Toll-like receptors (Chan et al., 2015) which activate the receptor interacting protein (RIP) kinases (Figure 1-2). RIP kinases help initiate and execute the necrotic phenotype by recruiting MLKL (mixed-lineage kinase domain-like) proteins which after oligomerizing, insert into the plasma membrane resulting in permeabilization (Sun et al., 2012; Zhao et al., 2012). During this activation, caspase-8 which promotes apoptosis, is inhibited (Linkermann and Green, 2014). Markers of necroptosis have been observed in liver injury models including APAP-induced liver damage (Ramachandran et al., 2013; Roychowdhury et al., 2013). Regardless of the initiating pathway, the end result of necrosis accomplishes the important task of alerting the immune system that cells are disintegrating. As a result, an inflammatory response is rapidly initiated and mobilized to clear and/or contain the damage. As a whole, sterile inflammation functions to protect the host by limiting the injury.

In contrast to necrosis, cells undergoing apoptosis do not trigger an inflammatory response. Apoptotic cells undergo cellular shrinkage and fragmentation but retain membrane integrity and do not release intracellular contents. Apoptotic cell blebs are phagocytosed by local immune cells and enable normal cell turnover and organ development in a dormant manner (Rock and Kono, 2008). Further, anti-inflammatory signals are actively produced by cells undergoing apoptosis to prevent the development of an inflammatory response. For example, ‘find-me’ signals such as nucleotides (adenosine tri-phosphate) or chemokines (fractalkine) are released by apoptotic cells to attract local mononuclear phagocytes but not circulating leukocytes to clear apoptotic bodies (Ravichandran, 2010). Another signal that is produced is the ‘keep-out’ signal which apoptotic cells release to curb neutrophil activation and recruitment (Bournazou et al., 2009). Finally, when phagocytes engulf apoptotic bodies, they release anti-inflammatory mediators to suppress inflammation. For instance, macrophages produce the anti-inflammatory
cytokines, IL-10 and transforming growth factor (TGF)-β with lipid mediators (resolvins and protectins) that suppress the induction of a sterile inflammatory response (Freire-de-Lima et al., 2006; Serhan, 2014).

1.2.3 Homing of innate immune cells to sites of hepatic sterile injury

Following sterile injury, how do innate immune cells migrate to these sites of inflammation? Models of sterile injury in the liver have now uncovered several of the molecular and cellular mechanisms by which immune cells find their way to damaged tissues as well as their functional role in mediating tissue repair and return to homeostasis.

1.2.3.1 Neutrophils

Neutrophils, effector cells of the innate immune system, are polymorphonuclear leukocytes that are among the first leukocytes recruited to an inflammatory site. During sterile injury in the liver, neutrophils begin to infiltrate the site of injury within hours in response to the release of DAMPs (McDonald et al., 2010). During migration, neutrophils were observed to chemotax towards the injury site via the sinusoids instead of proceeding through the interstitium (i.e. through the path of least resistance). Further, neutrophils exhibited swarming behavior within the injury which was dependent on leukotriene B4 produced by the first neutrophils to arrive (Lammermann et al., 2013).

Neutrophil guidance towards the site of injury was precise and accurately controlled. The chemokine, CXCL2 (macrophage inflammatory protein 2 (MIP2)), guided neutrophils towards the foci of damage but this chemokine gradient ended at the edge of the injury (McDonald et al., 2010). Necrotic cells inside the injury were then observed to release chemoattractants to guide neutrophils directly into the injury (McDonald et al., 2010). These necrotactic chemoattractant
signals were known as end-target chemoattractants and employed hierarchical signaling pathways to exert dominance over intermediate-target chemoattractants (e.g. CXCL2) (Foxman et al., 1997; Heit et al., 2002). As a result, polarization of chemotactic machinery and adhesion molecules on the surface of neutrophils occurred, allowing them to migrate specifically to dead cells while ignoring competing signals (Heit et al., 2008; Volmering et al., 2016). Neutrophils were required for normal physiological repair after acute injury. Preventing neutrophil recruitment to these sites of injury resulted in poor repair, characterized by improper clearance of debris, production of extracellular matrix and poor revascularization (Christoffersson et al., 2010; Slaba et al., 2015).

1.2.3.2 Platelets

Platelets are cells that are important in a variety of biological processes including hemostasis and immunity (Herter et al., 2014). Even though they lack a nucleus, platelets are synthetically and metabolically active. Through this active machinery, platelets are able to produce an assortment of cytokines and chemokines which attract neutrophils to sites of inflammation (Grommes et al., 2012; Rossaint and Zarbock, 2015). In addition to the production of inflammatory molecules, platelets are able to affect recruitment of neutrophils in other ways. In several models of sterile inflammation, neutrophil infiltration was discovered to be dependent on the formation of platelet-neutrophil aggregates through the adhesion molecule PSGL-1 (Andonegui et al., 2003; Carvalho-Tavares et al., 2000; Zarbock et al., 2006). Further, platelets were observed to be able to orient neutrophils inside the vasculature by inducing a redistribution of adhesion molecules and chemokine receptors on the surface of neutrophils (Sreeramkumar et al., 2014). This was important to promote directional migration of neutrophils because reduced
neutrophil infiltration was observed when this interaction was blocked. Finally, in a model of focal sterile injury in the liver, platelets rapidly accumulated inside blood vessels surrounding the injury (Slaba et al., 2015). They paved the way for neutrophil migration inside into the injury. If platelet adhesion to endothelium or platelet-neutrophil interaction was blocked, the migration of neutrophils into the injury site was disrupted and resulted in impaired healing of the wound. As a whole, platelets have critically important roles in the immune response to sterile injury.

1.2.3.3 Monocytes

Monocytes are myeloid cells derived from the bone marrow and circulate in the blood stream during basal conditions while also maintaining a splenic reservoir. During inflammation, monocytes are mobilized from the blood and recruited to the site of injury where they differentiate into monocyte-derived macrophages or dendritic cells (DCs) (Brempelis and Crispe, 2016). Generally, it is agreed that there are two subsets of monocytes in mice, the classical inflammatory monocyte and the nonclassical tissue repair monocytes (Geissmann et al., 2003; Palframan et al., 2001; Sunderkotter et al., 2004). These two subtypes are defined by specific combinations of cell surface molecules of Ly6C, CCR2 and CX3CR1 in mice. The classical monocytes express high levels of CCR2 and Ly6C but low levels of CX3CR1 (surface markers in humans: CD14+CD16−) while the nonclassical monocyte expresses high levels of CX3CR1 but low levels of CCR2 and Ly6C (in humans: CD14loCD16+). A third subset of intermediate monocytes has been described in humans with surface markers CD14+CD16+ (Ziegler-Heitbrock and Hofer, 2013).

Functionally, classical Ly6ChiCCR2+CX3CR1− monocytes were observed to be inflammatory cells. They express high levels of the chemokine receptor CCR2 which allows
them to home to sites of acute inflammation where they engulf debris and release inflammatory cytokines and chemokines (Geissmann et al., 2010; Nahrendorf et al., 2010). Further, these cells also release proteases which digest injured tissue to expedite debris clearance. Classical monocytes may also differentiate into tissue resident macrophages or DCs (David et al., 2016). In contrast, the other major subset of monocytes (Ly6C\textsuperscript{lo}CCR2\textsuperscript{-}CX3CR1\textsuperscript{+}) has been described to be anti-inflammatory. These cells can produce anti-inflammatory mediators that promote resolution (such as IL-10 or TGF-β) which switches inflammation towards repair (Geissmann et al., 2010; Nahrendorf et al., 2010; Nahrendorf et al., 2007). In addition, Ly6C\textsuperscript{lo}CCR2\textsuperscript{-}CX3CR1\textsuperscript{+} monocytes have been observed to exhibit a patrolling behavior in tissues (Auffray et al., 2007). In the heart, lung and peritoneum, classical and tissue repair monocytes were described to be recruited independently and in successive waves following injury (Auffray et al., 2007; Landsman et al., 2007; Nahrendorf et al., 2007). However, this recruitment does not exclude the possibility that Ly6C\textsuperscript{hi} monocytes differentiate into Ly6C\textsuperscript{lo} in the blood stream or in the bone marrow before recruitment. In contrast, during sterile injury in the liver, recruited inflammatory monocytes were observed to switch to repair monocytes in situ (Dal-Secco et al., 2015).

In the liver, only a single wave of monocyte recruitment has been observed following liver injury. A current hypothesis in the field is that classical Ly6C\textsuperscript{hi}CCR2\textsuperscript{+}CX3CR1\textsuperscript{-} monocytes are recruited to a site of sterile injury where they may contribute to acute injury but as these cells differentiate into repair Ly6C\textsuperscript{lo}CCR2\textsuperscript{-}CX3CR1\textsuperscript{+} monocytes, these cells now functionally drive tissue repair and resolution (Brempelis and Crispe, 2016). For example, in a carbon tetrachloride (CCl\textsubscript{4})-induced liver injury model, recruited Ly6C\textsuperscript{hi} monocytes during injury became Ly6C\textsuperscript{lo} monocytes which dominated in the later stages of the injury and promoted resolution (Ramachandran et al., 2012). If Ly6C\textsuperscript{lo} monocytes were eliminated, fibrosis occurred. A similar
observation was seen in an APAP-induced liver injury model (Zigmond et al., 2014). Intravital imaging using a focal sterile injury model, CCR2⁺CX3CR1⁻ monocytes were observed to encircle the site of sterile injury at 24 hours and transition into CCR2⁻CX3CR1⁺ monocytes at 48 hours and later (Dal-Secco et al., 2015). This transition was reliant on anti-inflammatory cytokines produced locally at the site of injury and was necessary for the clearance of necrotic cells.

1.2.3.4 Macrophages

The dedicated hepatic macrophage is known as the Kupffer cell (KC). These macrophages are numerous, constituting approximately 80-90% of all tissue macrophages and about 15% of the total liver cell population (Bilzer et al., 2006; Bouwens et al., 1986). Recent evidence suggests that KCs are not homogenous and subpopulations of KCs express different markers that reflect differences in phagocytic ability and cytokine production (Kinoshita et al., 2010). In stark contrast to monocytic and macrophage cell populations in other tissues that actively crawl through the tissue in search of pathogens, KCs are stationary, adherent to liver sinusoidal endothelial cells and located directly in the vasculature (see section 1.7). While numerous populations of immune cells patrol the liver vasculature, KCs are not mobile and extend long pseudopods into multiple sinusoids where they remain with the intent of catching foreign particles. Recent improvements in fluorescent imaging have distinguished these cells from other cell types using markers such as F4/80 (Gordon et al., 2011; Lee et al., 2010).

Due to their distinct location which directly exposes them to the contents of blood and coupled with the expression of a large variety of TLRs, antibody and complement receptors, KCs function as an important sensor for altered tissue integrity or invading microorganisms (Bilzer et
al., 2006; Lee et al., 2010; Wong et al., 2013). For example, the absence of these KC receptors in an infected host results in unrestrained bacteremia and subsequently death (Gorgani et al., 2008) while intravital microscopy reveals efficient bacterial capture by KCs in seconds (Lee et al., 2010; Wong et al., 2013). In contrast, bacteremia can persist for prolonged periods in the absence of KCs (Lee et al., 2010; Zeng et al., 2016). Due to their ability to phagocytose debris of host cells and produce cytokines, KCs are important regulators of collateral host damage and resolution of tissue injury (Taub, 2004). During injury and inflammation, numerous receptors trigger the activation of KCs, leading to the production of chemokines and cytokines which alerts the immune system to the presence of damage (Crispe, 2009; Kolios et al., 2006; Kowalewska et al., 2011). KCs have been demonstrated to bind and phagocytose activated and apoptotic neutrophils thereby limiting local inflammation (Shi et al., 1996; Shi et al., 2001). If this phagocytosis is inhibited, activated neutrophils accumulate in the lung and spleen and cause distal organ injury (Shi et al., 2001). Activated platelets are also cleared by KCs reducing the propensity for a thrombotic event (Grozovsky et al., 2010). As such, KCs are an important immune sentinel cell in the liver.

Nevertheless, since KCs are sessile, it was difficult to envision how KCs migrate, and contribute, to sites of sterile injury. For macrophages to appear at a site of injury, especially during a sudden loss of immune cells during trauma or toxins, a process known as ‘emergency repopulation of macrophages’ has been proposed (David et al., 2016). During this emergency repopulation, monocytes recruited to injury sites subsequently differentiate into resident macrophages. This would be in contrast to replenishment of KCs under basal conditions which is generally composed of in situ proliferation of long-lived descendents of yolk sac progenitor cells that arrived during development (Gomez Perdiguero et al., 2015). To investigate the emergency
repopulation of macrophages, a group depleted phagocytic cells in the liver using clodronate liposomes (David et al., 2016). Using a combination of mass cytometry, gene expression analyses and intravital imaging, it was observed that monocytes were recruited into the liver where they differentiated into either liver macrophages or DCs. However, newly replenished KCs exhibited defective bacterial catching up to two months suggesting that a period of time is required to restore the normal function of these cells. During inflammatory conditions, monocytes have also been observed to adopt macrophage phenotypes and produce different effector cytokines (Crane et al., 2014; Mossanen et al., 2016).

The recruitment of monocytes from vasculature where they subsequently transition into macrophages in response to injury is a common theory. Recently, however, a population of macrophages distinct from monocytes and KCs have been described to be actively recruited from the peritoneal cavity to sterile injury in the liver (Wang and Kubes, 2016). These peritoneal macrophages were GATA6+ and traveled via a transcapsular route from the peritoneal cavity into the liver due to release of purinergic danger signals from dead hepatocytes. GATA6+ macrophages were observed to acquire a repair phenotype within 4 hours and to dismantle nuclei from dead cells by engulfing small fragments similar to trogocytosis. Recruitment of these cells was essential for timely cleanup of cellular debris for wound healing and in a carbon tetrachloride model, lack of these cells resulted in increased mortality.

1.3 Natural Killer T (NKT) cells

1.3.1 A primer to NKT cells and identification of Type I NKT cells/invariant NKT (iNKT) cells

The name ‘NKT cell’ was first conceived about 25 years ago and was used to broadly define a subset of murine T lymphocytes that shared functional and phenotypic characteristics
with the natural killer cell, including the NK1.1 (NKR-P1 or CD161c) surface marker (Bendelac et al., 1997; Godfrey et al., 2004). Although the term NKT cell is now accepted and applied to these cells in both mice and humans, this definition is inaccurate and possibly misleading as NKT cells in certain mouse strains do not express NK1.1 due to the allelic divergence of NK1.1 genes (Brennan et al., 2013; Carlyle et al., 2006). To further complicate this classification, some conventional T cells have been described to spontaneously express NK1.1 after activation (Slifka et al., 2000).

Around the time when NKT cells were identified, a novel process of presenting lipid antigens was discovered (Beckman et al., 1994; Porcelli et al., 1992). This antigen presentation process occurred through the MHC class I-like molecule designated as CD1 (cluster of differentiation 1) that includes CD1a - CD1e (Brigl and Brenner, 2004; Cohen et al., 2009). All of these CD1 molecules present lipids instead of peptides as antigens. While humans express all five CD1 genes, mice express only CD1d. In mammals, CD1d is highly conserved (Barral and Brenner, 2007). Further studies in mice subsequently demonstrated that CD1d molecules presented lipids to invariant T cell receptor (TCR)-bearing cells which also expressed NK1.1 (Bendelac, 1995; Bendelac et al., 1995; Exley et al., 1997). This finding led to the realization that NKT cells were reactive to CD1d and that the invariant TCR α-chain and CD1d were essential for the development of NKT cells. These unique phenotypic characteristics are now used to define NKT cells. An excellent review highlights the detailed timeline of discoveries that contributed to the identification of NKT cells (Godfrey et al., 2004).

The discovery of the compound α-galactosylceramide (αGalCer) in 1997 contributed greatly to the understanding of NKT cells (Kawano et al., 1997). The potent and specific lipid antigen, isolated from a marine sponge sample (likely from an infecting proteobacterium), was
the first identified antigen for a specific population of NKT cells termed Type I NKT cells or invariant NKT (iNKT) cells. Through the use of CD1d tetramers loaded with αGalCer, iNKT cells in mice were discovered to express the invariant Vα14-Jα18 TCR α-chain paired with a β-chain biased towards Vβ2, Vβ3 and Vβ8 (Benlagha et al., 2000; Matsuda et al., 2000). More than 80% of NKT cells were found to express these invariant chains. A similar TCR limited repertoire was found in human iNKT cells which expressed Vα24-Jα18 paired with the Vβ11 chain (Dellabona et al., 1994). Due to large structural and functional similarities between the TCRs expressed by human and mouse iNKT cells, αGalCer can bind to and activate iNKT cells from both species (Brossay et al., 1998). In fact, this property has been taken advantage of by researchers to develop multimeric molecules with loaded synthetic αGalCer to identify iNKT cells ex vivo (Liu et al., 2006). These synthetic loaded tetramers are used in conjunction with anti-CD3 or anti-TCRβ antibodies to identify and enumerate iNKT cells in multi-parameter flow cytometry. In addition to αGalCer, a considerable number of exogenous ligands derived from microorganisms have been identified to activate iNKT cells (Rossjohn et al., 2012). Further, self-derived endogenous lipids as well as the cytokines IL-12 and IL-18 have also been described to activate iNKT cells (Lawson, 2012; Reilly et al., 2010). As iNKT cells can be activated by a range of exogenous and endogenous antigens and diverse inflammatory stimuli (Figure 1-3), they were found to be more important than initially realized in a variety of diseases (Berzins and Ritchie, 2014; Berzins et al., 2011). The research conducted in this thesis will be focused on the role of iNKT cells in sterile injury.
Figure 1-3. Major pathways of iNKT cell activation during inflammation.

(A) A significant number of microorganisms were found to express exogenous antigens that are recognizable by iNKT cells. Here, antigen-presenting cells (APCs) engulf invading microbes during infection and directly present these exogenous antigens via CD1d molecules to the T cell receptor (TCR) on iNKT cells. (B) Many tissues are innervated by the nervous system. During injury to the central nervous system (CNS), signals are transmitted via neurones and result in the release of neurotransmitters such as noradrenaline. These neurotransmitters bind to adrenergic receptors on iNKT cells resulting in the activation. (C, D) During infection and non-infectious injury, pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) are released. These PAMPs and DAMPs bind to pattern-recognition receptors (PRRs) on APCs and result in the production of inflammatory cytokines such as IL-12 and/or...
presentation of self-antigens on CD1d to the TCR of iNKT cells. The synergy of cytokine or self-antigen presentation contributing to iNKT cell activation depends on the type of injury or microbe involved during infection. Reproduced with permission from (Liew and Kubes, 2015).
1.3.1.1 Endogenous lipid ligands

The hunt for endogenous lipid ligands for iNKT cells has been convoluted and remains to be fully resolved. Isoglobotrihexoslyceraminde (iGb3), an α-linked glycolipid, was the first proposed natural iNKT cell ligand (Zhou et al., 2004). This was concluded largely on the observation that iNKT cell development was impaired in Hexb−/− mice (Hexb gene product is responsible for the conversion of iGB4 into iGB3). However, subsequent studies revealed normal levels of iNKT cells in thymus, spleen and liver in iGb3-synthase-deficient mice (Christiansen et al., 2008; Li et al., 2009; Porubsky et al., 2007; Speak et al., 2007) and it was suggested that the defect of iNKT cell in Hexb−/− mice was, in fact, due to altered lysosomal function (Gadola et al., 2006). Also, an ensuing report has demonstrated that humans do not express iGB3 synthase and cannot produce iGB3 (Christiansen et al., 2008). In view of the fact that mammalian cells usually produce β-linked instead of α-linked glycolipids, efforts in this field turned to the possibility that β-linked glycolipids regulate iNKT cell activation and autoreactivity (Godfrey et al., 2011).

In 2011, through a screen of naturally occurring glycosphingolipids, β-glucopyranosylceramide (β-GlcCer) was identified to be a potent activator of murine and human iNKT cells (Brennan et al., 2011). However, this raised a question how the iNKT cell TCR might interact with β linkages as the sugar headgroup projects outwards from the CD1d antigen-binding cleft in contrast to α linkage where the sugar lies flat against CD1d (Pellicci et al., 2011). Recently, new evidence has emerged to indicate that the purified β-GlcCer might have been contaminated by miniscule amounts of α-linked glycolipids that were below the detection levels of analytical instruments (Brennan et al., 2014). Instead, small quantities of α-glycosylceramindes could be constitutively produced in mammalian cells as a result of catabolic enzymes and these were the main endogenous iNKT cell ligands (Kain et al., 2014). It was also
found that multiple α-glycolipids that could activate iNKT cells exist in mammalian sera (calf, goat, rabbit, etc) at low abundance, which was consistent with the physiological function of iNKT cells. The enzymatic activities that are involved in generation of these α-glycolipids as well as how they are regulated under basal or pathological conditions remain to be clarified.

There have been reports of other antigens (such as phospholipids or peptides) that can activate iNKT cells but their functional relevance remains unknown. For example, phospholipids such as lyso-phosphatidylcholine were proposed as self-antigens that could activate iNKT cells. However, stimulatory activity of these antigens was weak and only activated a subset of iNKT cells (Fox et al., 2009; Gumperz et al., 2000). Peptides as antigens for CD1d-restricted T cells have been entertained since the discovery of iNKT cells. Although early studies indicated that CD1d can present peptides (Castano et al., 1995), the difficulties in envisioning the molecular basis of peptide recognition by CD1d and the bias of lipid-reactivity by iNKT cells resulted in this mechanism being set aside. However, two recent reports have renewed interest in the prospect that iNKT cells could react with peptides. First, CD4+Vα14+ CD1d-restricted T cells were reported to recognize a collagen-derived peptide (Liu et al., 2011). Recognition of this peptide resulted in iNKT cell activation and production of a variety of cytokines which suppressed inflammation. Another study examined how peptide antigens might fit into the CD1d groove (Girardi et al., 2016). The authors determined that peptides would adopt a conformation in the CD1d groove which orients residues towards the bottom of the groove, thus allowing its presentation to TCRs. As a whole, all of these observations are important in understanding how antigens affect the effector function and immunological properties of iNKT cells. Defining these antigens is a critical step towards fundamentally comprehending the biology of iNKT cells.
1.3.1.2 Cytokine-driven signals

As researchers initially observed that iNKT cells had a biased TCR repertoire and restricted antigen recognition due to presentation by nonpolymorphic CD1 molecules, investigators asked if iNKT cells could be activated independently from TCR recognition. By treating iNKT cells with IL-12 and IL-18 in vitro and in vivo, it was observed that iNKT cells harvested from the thymus or spleen would become activated and produce interferon-γ (IFN-γ) (Leite-De-Moraes et al., 1999). Subsequently, it was discovered that iNKT cells express the receptors for, and can be activated by, IL-12 (Brigl et al., 2003; Kitamura et al., 1999), and IL-18 (Nagarajan and Kronenberg, 2007). In later studies that involved bacterial and viral infections, iNKT cells could be activated by production of these cytokines in vivo, even in the absence of TCR-CD1d engagement (Brigl et al., 2003; Wesley et al., 2008). To address a possibly synergy between TCR engagement and cytokine signals, a recent report showed that weak iNKT cell TCR engagement resulted in histone modifications at effector cytokine loci which enhanced the responsiveness of iNKT cells (Wang et al., 2012). These data suggest that the activation of iNKT cells is a combination of TCR and cytokine signals, where the modulation and strength of each signal contributes to iNKT cell activation.

Recently, iNKT cells were increasingly observed to respond to many diverse microbial infections and a number of these bacterial pathogens were not known to express antigens that iNKT cells could recognize (Brennan et al., 2013; Crosby and Kronenberg, 2016). This suggested that cytokine-mediated activation of iNKT cells might be the dominant mechanism during infection. To investigate this further, a large panel of different types of bacteria (with or without known iNKT cell antigens) were screened for their ability to contribute to iNKT cell activation (Brigl et al., 2011). In this study, incubation of iNKT cells in vitro with various
bacteria together with MyD88- or IL-12-deficient DCs (which still had CD1d expression) resulted in significantly reduced IFN-γ secretion which suggested that iNKT cell activation might be predominantly cytokine-driven. This phenotype was observed with all bacterial treatment regardless if they expressed antigen that iNKT cell could recognize or not. To determine if cytokine-driven activation of iNKT cells could occur in vivo, the authors injected bacteria (such as S. pneumonia) that are known to express iNKT cell antigens (Kinjo et al., 2005). These pathogens trigger innate receptors (TLRs, NOD1/2) within APCs (such as DCs and macrophages) that consequentially induce antigen presentation and/or release of proinflammatory cytokines (Mattner et al., 2005; Selvanantham et al., 2013; Tomlinson et al., 2014). Similar to in vitro studies, a decrease in IFN-γ secretion and CD69 expression in iNKT cells was observed in IL-12-deficient mice as compared to wildtype following bacterial injection (Brigl et al., 2011). These examples suggest that cytokine-driven signaling might be the dominant form of iNKT cell activation (at least during infection) and would explain how these cells are activated efficiently and rapidly to a variety of bacterial pathogens. Whether these mechanisms (i.e. cytokine-driven iNKT cell activation) might apply in the context of sterile inflammation or chronic inflammatory diseases remain to be seen.

1.3.1.3 Type II Natural Killer T cells

Apart from the iNKT cells (also known as type I NKT cells) introduced above, another set of innate-like CD1d-restricted NKT cells known as type II NKT cells have been described (Behar et al., 1999; Cardell et al., 1995; Park et al., 1998). Type II NKT cells are important for the fact that these cells, like iNKT cells, possess significant immunoregulatory properties when activated. However, type II NKT cells exhibit several key distinguishing features which will be
briefly reviewed here. First, type II NKT cells recognize lipid antigens but express diverse TCR α- and β-chains (Arrenberg et al., 2010; Park et al., 2001) and do not recognize αGalCer (Blomqvist et al., 2009; Jahng et al., 2004) which distinctly separates them from iNKT cells. Instead, some subsets of type II NKT cells are activated following administration of sulfatide or lysophosphatidycholine (Dasgupta and Kumar, 2016). Type II NKT cells also do not possess the ability to be activated by cytokine signals. Next, when type II NKT cells are activated, these cells seem to mostly exhibit immunosuppressive qualities (for example, inhibition in the pro-inflammatory functions of iNKT cells and inhibition of T helper type (Th) 1/Th17 response) (Bandyopadhyay et al., 2016). As a result, type II NKT cells confer protection against several autoimmune and inflammation-induced diseases. However, type II NKT cells are difficult to track experimentally as this group of NKT cells cannot be identified through αGalCer-loaded CD1d tetramers and sulfatide/CD1d tetramers that identify type II NKTs are highly unstable and hard to work with (Bandyopadhyay et al., 2016). Therefore, although type II NKTs form a unique niche in the immunoregulatory cellular repertoire, they are comparatively less characterized and understood (as compared to iNKT cells). Significant progress in identifying and characterizing type II NKT cells is required to further understand their biology in health and disease.

1.3.2 Activation of invariant Natural Killer T cells leading to cytokine production and downstream immunoregulatory effects

Although iNKT cells develop in the thymus, they are generally categorized as innate lymphocytes because iNKT cells exist in a poised effector state when they mature. Accordingly, mature iNKT cells are able to rapidly release large quantities of pro-inflammatory Th1 (for example, IFN-γ) or Th2 (IL-4 and IL-10) cytokines within hours of activation (Bendelac et al.,
2007; Kronenberg, 2005). In mice, resting iNKT cells contain preformed mRNA for both IFN-γ and IL-4 to allow swift cytokine production (Stetson et al., 2003). These cytokines are able to transactivate other immune cells including neutrophils, NK cells, dendritic cells and macrophages during an immune response (Brennan et al., 2013; Van Kaer et al., 2013). Because iNKT cells are able to rapidly release substantial quantities of cytokines that can polarize the immune response, they are hypothesized to be important orchestrators of immunity. For example, activation of iNKT cells during infection results in the secretion of pro-inflammatory cytokines which stimulates the developing immune response to fight off microbial invaders (Brigl et al., 2003; Kinjo et al., 2005; Mattner et al., 2005). A similar protective effect of iNKT cells is also observed during cancer (Ambrosino et al., 2008; Metelitsa, 2011). On the other hand, iNKT cells can strengthen immuno-suppressive pathways during autoimmunity or ischemia-reperfusion injuries such as stroke (Balato et al., 2009; Van Kaer, 2004). In a mouse stroke model, there is increased sympathetic drive which induces iNKT cells to make more IL-10 and less IFN-γ (Wong et al., 2011). This leads to overall immuno-suppression but places individuals at a greater risk to infections. iNKT cells are therefore pivotal in shaping immune responses during diverse pathological states. An ongoing challenge is to unravel the factors that determine if iNKT cells facilitate or suppress an immune response.

Thus far, iNKT cells have been described to produce an array of cytokines such as IL-4, IL-10, IL-17, IFN-γ, tumor necrosis factor-α and transforming growth factor-β (Brennan et al., 2013; Coquet et al., 2007; Sakuishi et al., 2007). How does a single population of cells produce such a large variety of cytokines? The type and quantity of cytokine produced is influenced by several non-mutually exclusive factors. Firstly, the quality of TCR signal (i.e. antigen signal strength and CD1d-binding kinetics) affects the cytokine profile. For example, use of different
αGalCer analogs have been described to result in different ratios of IFN-γ/IL-4 produced (Im et al., 2009; Sullivan et al., 2010; Yu et al., 2005). A similar phenomenon should occur with endogenous antigens compared to relevant foreign antigens. Secondly, targeting of antigen to different antigen-presenting cells will alter the pattern of cytokines made by iNKT cells (Fujii et al., 2002; Lee et al., 2015). Finally, functionally different subsets of iNKT cells have been described based on location and surface phenotype which may promote different outcomes when iNKT cells are activated (see next section) (Brennan et al., 2013; Buechel et al., 2015).

1.3.3 Subsets of invariant Natural Killer T cells

Initially, the development of NKT cells have been proposed to follow a sequential lineage model where developing intermediates produce mostly Th2-type cytokines and mature iNKT cells predominantly produce Th1-type cytokines. Developing iNKT cells were observed to express PLZF which was necessary in establishing the iNKT cell lineage and their maturation (Benlagha et al., 2002; Scanlon et al., 2011). However, the discovery of IL-17 producing iNKT cells (and other subsets) was difficult to fit into this established theory (Michel et al., 2007). To address this, a new concept that divides iNKT cells into iNKT1/2/17 sub-types based on secretion of key cytokines IFN-γ, IL-4 and IL-17 was proposed and is quickly gaining acceptance (Scanlon et al., 2011).

Following this iNKT1/2/17 paradigm, expression of key transcription factors that regulate development of these sub-types was identified using thymic iNKT cells (Lee et al., 2013). iNKT cells that were iNKT1 are defined as PLZF^{lo}T-bet^{−}RORγt^{−} (IFN-γ^{−}), iNKT2 cells are PLZF^{hi}T-bet^{−}RORγt^{−} (IL-4^{−}) and iNKT17 cells are PLZF^{inter}T-bet^{−}RORγt^{+} (IL-17^{+}). The authors also demonstrated that iNKT2 cells produced IL-4 in the steady state and that IL-4-
producing iNKT2 cells do not differentiate into iNKT1 cells (Lee et al., 2013). To further define the transcriptional profiles of these functional subpopulations of iNKT cells, two groups undertook RNA-seq transcriptome analysis of thymic iNKT cell subsets (Engel et al., 2016; Georgiev et al., 2016). Their results broadly support the iNKT1/2/17 concept as they observed subset-specific gene expression and chemokine- and cytokine-encoding genes that were enriched for specific subsets. Nevertheless, both studies also identified significant heterogeneity within each subset as they observed that IL-4 was not only present in iNKT2 but also iNKT1 and iNKT17 cells (Engel et al., 2016; Georgiev et al., 2016). Further, iNKT2 cells displayed the greatest heterogeneity with multiple differentiation states including precursor immature iNKT cells. It is unknown how these immature cells might respond when activated with antigen. These caveats would need to be addressed to better understand the divergent effects observed after iNKT cell activation.

As the above studies were performed using thymic iNKT cells, a study sought to determine the steady-state localization of iNKT cell subsets in the periphery using an *ex vivo* CD1d-tetramer based immunofluorescence technique (Lee et al., 2015). iNKT cell subsets were not symmetrically distributed throughout most tissues. For example, splenic iNKT1 cells were mainly localized to the red pulp of the spleen while iNKT2 cells were localized in the T cells zone. Further, interstrain variation of iNKT cell subsets were observed where C57Bl/6 mice had consistently higher frequency of iNKT1 cells compared to BALB/c mice which generally had more iNKT2 cells across all organs. Interestingly, iNKT1 cells were particularly enriched in the liver (~90%) and this was observed in both C57Bl/6 and BALB/c mice (Lee et al., 2015).
1.3.4 Homing and organ distribution

The distribution of iNKT cells in the periphery has been studied. Mature iNKT cells are distributed widely and have been found in the bone marrow, joints, gastrointestinal tract, skin, lymph nodes, blood, lung, spleen and liver (Brennan et al., 2013; Kronenberg, 2005). Of these, the liver has the highest number of iNKT cells where they represent up to 30% of all lymphocytes followed by the spleen; approximately $1 \times 10^6$ cells can generally be found at these sites. A significant amount of iNKT cells have also been found in adipose tissue although a majority of these cells do not express NK1.1 (in C56Bl/6 mice) and produce mainly IL-4 not IFN-γ (Lynch, 2014). Using congenic parabiotic mouse pairs to follow the in vivo circulation of iNKT cells revealed that iNKT cells are almost an exclusively tissue-resident population (Scanlon et al., 2011). In contrast to B cells, conventional T cells and NK cells which recirculated through all tissues, iNKT cells in liver, lung, spleen and bone marrow all shown little chimerism between parabiotic pairs. As these pairs were followed up to 60 days, iNKT cells in tissues either have a very long dwell time or have insignificant recirculation through tissues. These findings do fit with the concept that iNKT cell phenotype and subsets are location dependent (Lee et al., 2015). So far, the mechanisms that regulate the homing and retention of iNKT cells in various tissues have not been uncovered, although several studies have demonstrated that iNKT cells express a range of chemokine receptors that differ across iNKT cell subsets (Engel et al., 2016; Johnston et al., 2003). These chemokine receptors as well as specific adhesion molecules might regulate their distribution and localization and will be further explored in the next section below.
1.4 Imaging Natural Killer T cells

There are a multitude of publications that describe the activation and cytokine production profiles of iNKT cells in mice and humans. However, their dynamic behavior has only been brought to light recently. The capacity to visualize and observe iNKT cell behavior relies considerably on the labeling method. To date, no lineage-specific fluorescent antibody has been able to label iNKT cells. Isolating iNKT cells and staining them ex vivo with a fluorescent dye (for example, carboxyfluorescein diacetate succinimidyl ester) for adoptive transfer provides a manner to observe their behavior in an organ (Barral et al., 2010). However, this opens the possibility that cellular behavior may be altered by the potential artifact of cell isolation. So far, the best avenue is the use of genetically engineered knock-in mice where fluorescent proteins are inserted into a lineage-specific gene locus (Abe and Fujimori, 2013). Both mouse and human iNKT cells express high levels of the CXCR6 chemokine receptor which has been demonstrated to mediate the survival of iNKT cells in the liver (Germanov et al., 2008; Johnston et al., 2003; Kim et al., 2002). To image iNKT cells in a live animal, a mouse containing enhanced green fluorescent protein (GFP) inserted into the Cxcr6 gene (Cxcr6Gfp+) was generated (Geissmann et al., 2005). iNKT cells have been found to account for 60-80% of all GFPbright cells in the liver. For the first time, the dynamic behavior of iNKT cells in different tissues and organs could be observed.

1.4.1 Liver

Using intravital microscopy, hepatic iNKT cells were seen to crawl along the luminal side of liver sinusoidal endothelial cells without directional bias with an average speed of 10 μm/min (Figure 1-4) (Geissmann et al., 2005; Lee et al., 2010). This distinct behavior is unlike
**Figure 1-4. Intravital imaging of iNKT cells in the liver with Cxcr6Gfp/+ mice.**

A still snapshot of a time-lapse video recorded during imaging in a live animal is presented; iNKT cells (bright green) found in the liver are intravascular and crawl on the luminal side of liver sinusoidal endothelial cells (blue) under basal conditions. When these iNKT cells are activated, they significantly slow their crawling phenotype or completely arrest in the sinusoids. 20X objective, scale bar 50 μm. Adapted from (Liew and Kubes, 2015) with permission.
leukocyte behavior observed in post-capillary venules where leukocytes roll along continuous endothelium (Petri et al., 2008; Phillipson and Kubes, 2011). Detailed analysis of iNKT cell behavior in the liver demonstrated that iNKT cell crawling was random and independent of blood flow (Geissmann et al., 2005).

Resident iNKT cells are enriched in the liver, comprising up to 30% of all lymphocytes as compared to the thymus, lung, colon, bone marrow, spleen, lymph nodes and blood (Bendelac et al., 2007). The cause for the higher frequency of resident iNKT cells in the liver is not completely clear; however, the adhesion molecule leukocyte function-associated antigen-1 (LFA-1, CD11a) has been suggested to be important in retaining iNKT cells in the liver (Thomas et al., 2011). A tandem blockade of LFA-1 and its corresponding ligand, intercellular adhesion molecule 1 (ICAM-1) created a substantial rise in iNKT cells in blood and a reciprocal decrease in their number in the liver. Furthermore, LFA-1-deficient mice have notably reduced numbers of iNKT cells in the liver (Emoto et al., 1999). Although we observed that the crawling phenotype of iNKT cells in liver sinusoids was not affected by LFA-1 and ICAM-1 antibodies, they did detach in collecting venules after treatment with blocking antibodies (Wong and Kubes, 2013). Taken together, these data indicate that LFA-1 and ICAM-1 were perhaps necessary for interactions in larger vessels but not for crawling in sinusoids. However, for iNKT cells that are crawling in liver sinusoids, expression of the chemokine receptor Cxcr6 appears to confer a survival signal as knockout of this gene resulted in impaired survival of hepatic iNKT cells while addition of exogenous CXCL16 caused increased survival of iNKT cells (Geissmann et al., 2005).

Previous studies have demonstrated that T cells arrest their movement when they encounter cognate antigen (Dustin et al., 1997; Munoz et al., 2014). iNKT cells in the liver
exhibit a similar behavior; when αGalCer was injected intravenously, crawling GFP$^{\text{bright}}$ iNKT cells became stationary within an hour (Geissmann et al., 2005). Arrest of hepatic iNKT cells correlated with TCR activation. Further, synergistic effects between the inflammatory cytokines IL-12 and IL-18 applied exogenously also resulted in the arrest and activation of hepatic iNKT cells (Velazquez et al., 2008).

The arrest phenotype and function of hepatic iNKT cells was investigated in the context of ischemia-reperfusion in the brain (stroke). In a mouse stroke model, norepinephrine release by the sympathetic nervous system during stroke caused rapid arrest of iNKT cells in the liver which also associated with iNKT cell activation and CD69 expression (Wong et al., 2011). Hepatic iNKT cells have been previously shown to express adrenergic receptors to receive neural signals (Minagawa et al., 2000). Blockade of CD1d had no effect on the arrest of iNKT cells in this scenario which suggests that classical antigen presentation through CD1d did not play a major role in arrest and activation.

*B. burgdorferi* is a spirochete pathogen that continues to spread in North America (Shapiro, 2014). This pathogen induces Lyme-disease and delayed or inadequate treatment typically leads to disabling symptoms as the bacteria invade the joints, heart and central nervous system (Biesiada et al., 2012). The liver functions as an important organ that is positioned to intercept disseminating pathogens in the blood (Crispe, 2009). This interception is mediated by liver-resident intravascular macrophages (Kupffer cells) which ensnare pathogens from the bloodstream. Visualizing iNKT cell activity in the liver showed dramatically altered iNKT cell behavior after *B. burgdorferi* infection (Lee et al., 2010). Instead of crawling through the liver sinusoids, iNKT cells formed clusters and arrested next to Kupffer cells that had captured *B. burgdorferi*. This clustering occurred as early as four hours after exposure and could be inhibited
by blocking the CXCR3 receptor. Anti-CD1d antibody blocked the firm adhesion of iNKT cells to Kupffer cells as well as the activation of iNKT cells which suggests that Kupffer cells were responsible for presenting antigens to activate iNKT cells. Intravital imaging during this process revealed that Kupffer cells phagocytose *B. burgdorferi* from blood for antigen presentation to iNKT cells which then produce IFN-γ and other inflammatory cytokines after activation. This activated the local hepatic innate immunity system to prevent bacterial dissemination. Therefore, activation of iNKT cells via various mechanisms including CD1d, certain cytokines or even neurotransmission all induce cell arrest within liver sinusoids.

### 1.4.2 Behavior of iNKT cells in other organs (joints, lymph nodes, spleen and lung) differs from those in the liver

Despite the fact that the frequency of iNKT cells in other organs than the liver is low, various studies have highlighted the importance of iNKT cells in these organs in response to blood-borne pathogens (Barral et al., 2012; Lee et al., 2014; Shekhar et al., 2014). The *Cxcr6Gfp/+* mouse has been employed to study the spatial organization, behavior and functional roles of iNKT cells in several organs including the joints, lymph node, spleen and lung. In distinct contrast to the liver, intravital imaging of iNKT cells in joints revealed dramatic localization of these cells around the joint blood vessels but not inside the vessels (Figure 1-5A) (Lee et al., 2014). Joint iNKT cells also exhibited different behavior under basal conditions as they were stationary and non-motile. After *B. burgdorferi* infection, joint iNKT cells switch phenotypes and crawled along vessel walls towards the pathogen, perhaps due to complement activation. *B. burgdorferi* that interacted with iNKT cells subsequently died which suggests that joint iNKT cells limit the dissemination of this pathogen within the joint. Indeed, absence of iNKT cells led to a large number of motile spirochetes outside the vasculature in the joint cavity of mice.
Behavior of iNKT cells in other tissues is significantly different from the liver. In the joint, (A) iNKT cells (bright green) are extravascular and line the capillaries (blue). Joint iNKT cells do not crawl and are stationary next to the joint capillaries. During pathogen invasion in the joint, as in the case of *B. burgdorferi*, iNKT cells begin to crawl along vessel walls. Dissemination of bacteria is limited by joint iNKT cells. Arrows: iNKT cells lining the vasculature. 10X objective, scale bar 110 μm. (B) Still snapshot demonstrating the distribution of iNKT cells (bright green) in different regions of a lymph node. Vasculature of the lymph node is labelled with PECAM-1 (blue). The medullar and interfollicular regions are outlined in yellow while the paracortex is outlined in white. 10X objective, scale bar 210 μm. (C) Snapshot of iNKT cells (bright green) located in the red pulp of spleen show that the cells are located outside the vasculature (blue). Arrows: extravascular iNKT cells. 20X objective, scale bar 50 μm. Adapted from (Liew and Kubes, 2015)
In the lymph node, iNKT cells are located in the medulla and the interfollicular region but mainly absent in the deep paracortex where naïve T cells reside (Figure 1-5B) (Kastenmuller et al., 2012). These iNKT cells are highly motile in the lymph node and actively communicate with resident subcapsular sinus macrophages. During systemic infection, resident macrophages produce IL-18 and complementary cytokines which elicit an innate IFN-γ response from lymph node iNKT cells. On the other hand, iNKT cells were found to be widely distributed throughout the spleen, including B and T cell follicles in the periarteriolar lymphoid sheath, the marginal zone (MZ) as well as the red pulp (Barral et al., 2012; King et al., 2013; Thomas et al., 2011). Dissimilar to the liver, iNKT cells were observed to be crawling outside the vasculature in the spleen (Figure 1-5C) (Barral et al., 2012; Wong and Kubes, 2013). Interestingly, the localization of iNKT cells changes in the spleen during infection or in the presence of cognate lipid antigens. Under these conditions, iNKT cells slow down or arrest and are confined to the MZ where antigen-rich MZ macrophages and dendritic cells reside (Barral et al., 2012; King et al., 2013).

Although there is evidence demonstrating that iNKT cells are important in allergy and airway inflammation, information on the anatomical distribution of iNKT cells in lung, their mechanism of activation and role in lung diseases remain poorly understood (DeKruyff et al., 2014; Shekhar et al., 2014). Recent studies have attempted to address these questions. Two-photon fluorescence microscopy was employed to examine the localization of iNKT cells in a harvested lobe of a murine lung (Scanlon et al., 2011). They revealed that pulmonary iNKT cells mainly resided in the lung microvasculature. Upon exposure to aerosolized lipid antigen, iNKT cells mobilized and extravasated into lung tissue. Thomas et al. (2011) showed that there was an approximate 10- to 20-fold enrichment of iNKT cells in blood drawn from the right or left ventricles of the heart as compared to peripheral blood suggesting that iNKT cells resided in the
blood vessels within the lung. Another study also suggests that some pulmonary iNKT cells can be long-lived (Shimizu et al., 2014). Using parabiotic mice, Bendelac and colleagues (2011) demonstrated that pulmonary iNKT cells do not recirculate between parabiotic mice pairs even after thirty days. To address the lack of information describing the behavior and migrational dynamics of pulmonary iNKT cells, lung intravital imaging was performed (Thanabalasuriar et al., 2016). The majority of pulmonary iNKT cells were intravascular and patrolled the microvasculature with a small population that was extravascular. During inflammation, neutrophils extravasated and produced CCL17 which then paved the way for iNKT cells to leave the vasculature and enter the pulmonary interstitium where they were activated by monocyte-derived DCs (Thanabalasuriar et al., 2016).

1.5 Mouse models for studying host responses in the absence of iNKT cells

Although tools such as glycolipid antigens and tetramers or Cxcr6\textsuperscript{GFP/+} mice exist for the detection of iNKT cells without prior enhancement, they did not provide a means for examining how the host is affected by the absence of iNKT cells during infection or injury. To address this question, two iNKT cell deficient mice were developed; these are the CD1d\textsuperscript{-/-} mice and Jα281KO (Jα18\textsuperscript{-/-} or Traj18\textsuperscript{-/-}) mice. CD1d\textsuperscript{-/-} mice lack Cd1d1 and Cd1d2 loci and as a result, cannot positively select iNKT cells during thymus differentiation (Chen et al., 1997; Smiley et al., 1997). However, a significant caveat of CD1d\textsuperscript{-/-} mice is that in addition to lacking iNKT cells, these CD1d deficient mice also lack type II NKT cells (Crosby and Kronenberg, 2016). As type II NKT cells possess appreciable immunoregulatory properties when activated, studies that employ CD1d\textsuperscript{-/-} mice need to consider the contribution of type II NKT cells.
In contrast, the Jα18\(^{-}\) mice only lack iNKT cells as these mice do not possess the Traj18 gene segment and cannot form the invariant TCR chain that is needed for iNKT cell development (Cui et al., 1997). It must be noted that the original strain of this mouse also had decreased rearrangement of all J\(\alpha\) segments upstream of Traj18 (Bedel et al., 2012). This phenotype was presumably due to incorporation of the neomycin resistance gene cassette selection marker which was not removed and resulted in its transcription from the opposite orientation from \(\alpha\) chain genes. As a result, the product interfered with rearrangement of upstream J\(\alpha\) segments. Although a newer J\(\alpha\)18\(^{-}\) mouse was developed that addressed this problem by removing the neomycin resistance gene, this new strain has yet to be widely tested in different mouse models of disease (Chandra et al., 2015). Despite these limitations, both of these mouse models (CD1d\(^{-}\) and J\(\alpha\)18\(^{-}\)) have been extremely useful in evaluating the functional roles of iNKT cells in the immune response as well as various diseases.

Besides the limitations of iNKT cell deficient mice discussed above, breeding of iNKT cell-deficient mice can be lengthy especially if breeding of iNKT cell deficient mice onto various genetic backgrounds is required. Further, iNKT cell deficient mice lack iNKT cells from birth. To address these issues, a new antibody (NKT14) in development has been described to deplete iNKT cells in the spleen and lung (Scheuplein et al., 2015). We have also discovered and described the mechanism of action of an antibody that is capable of depleting iNKT cells in the liver and lung (see Appendix A). The use of these antibodies could be used as a tool to validate genetic knockout models or to deplete iNKT cells at a time of choice in mouse models prior to disease induction.
1.6 iNKT cells as directors by regulating the inflammatory response after tissue injury

The inflammatory response is critical for host defense against invading pathogens. Known as sterile inflammation, inflammation also occurs when self tissue is damaged in the absence of infection (Chen and Nunez, 2010). Akin to inflammation induced by microbes, sterile inflammation also results in recruitment of neutrophils, monocytes and macrophages and the release of chemokines and pro-inflammatory cytokines such as IL-1 (Kono and Rock, 2008). Sterile inflammation has been identified to underlie many medical afflictions such as burn injuries or ischemia-reperfusion injury in the heart, liver and brain (Rock et al., 2010). Following the initial trauma, the outcome of these afflictions are immunosuppression and susceptibility of the host to subsequent infection. Some medical examples of these complications include patients with acute myocardial infarction, stroke or major burn injuries. Systemically inhibiting inflammation in these conditions can lead to adverse infectious complications. With the ability to react to self or invasive pathogens, iNKT cells are the linchpins which can determine a favorable or detrimental outcome during inflammation in these conditions.

Able to respond to ‘self’ lipid antigens, iNKT cells are able to regulate inflammation during tissue injury (Gapin, 2010; Van Kaer et al., 2013). Several endogenous lipids have been proposed to activate iNKT cells although identification of the primary endogenous lipid antigen is a subject of intense research (Godfrey et al., 2011; Ilan, 2009; Kain et al., 2014). Nevertheless, during tissue injury and cell death, endogenous antigens can serve as danger signals to activate iNKT cells in the absence of exogenous ligands. The functional role of iNKT cells have been investigated in mouse models of burn injury. In a cauterization-induced corneal inflammation model, iNKT cell-deficient mice had increased neutrophil accumulation and higher levels of pro-inflammatory cytokines in the cauterized eye (Oshima et al., 2008). In addition, lack of iNKT
cells led to greater corneal edema and opacity. In this model, iNKT cells played an important role in curbing inflammation and maintained corneal clarity. A similar immunoregulatory effect was observed in a dorsal burn injury model where iNKT cells were found to mediate T cell proliferation after injury by producing IL-4 (Faunce et al., 2003). Production of IL-4 by iNKT cells suppressed antigen-specific T cell delayed-type hypersensitivity after dorsal burn injury.

During stroke, intravital imaging revealed that norepinephrine release rapidly arrested and activated iNKT cells in the liver (Wong et al., 2011). Interestingly, this increased sympathetic drive induced activated iNKT cells to produce increased levels of anti-inflammatory cytokines such as IL-10 which led to post-stroke immunosuppression. This effect likely protects the brain from inflammatory damage (Chamorro et al., 2007) but also leaves the patient open to infection which is a major cause of post-stroke death (Westendorp et al., 2011). In contrast, activating iNKT cells with the potent agonist αGalCer reduced bacterial infection after stroke (Wong et al., 2011). Collectively, these findings suggest that iNKT cell activation was not the determining factor that mediated immunosuppression after stroke but rather the adrenergic activation and modulation of iNKT cells resulted in a shift from pro-inflammatory to anti-inflammatory cytokine production. This also raises the possibility of therapeutically targeting iNKT cells in the liver to quench detrimental neuro-immunosuppression as long as it does not enhance inflammation in the brain.

1.7 Liver microenvironment and invariant Natural Killer T cells

The liver is an important immunological organ permeated not only with immune cells but also with nonimmune cells (for example, liver sinusoidal endothelial cells) that have unique immunological roles and contribute to intrahepatic immunity (Figure 1-6). Many of these cells
Figure 1-6. Distribution of resident hepatic cells which interact with iNKT cells during inflammation.

Illustration of a cross-sectioned liver sinusoid with the positions of resident hepatic immune cells depicted (center). Still snapshots of intravital videos are used to highlight the location of the illustrated cells: (A) Kupffer cells (magenta) stained with F4/80 marker are located inside the sinusoidal lumen and are adjacent to PECAM-stained liver sinusoidal endothelial cells (LSECs) (blue), (B) Patrolling iNKT cells (bright green) are also observed to be inside the sinusoidal lumen marked by PECAM-stained LSECs (blue), with columns of hepatocytes (dull green) alongside the perimeter of sinusoids, (C) In contrast, hepatic stellate cells (HSCs) (bright green)
from a CX3CR1-GFP mouse are observed outside the lumen with dendrite-like extensions that wrap around PECAM-stained (blue) LSECs. Reproduced with permission from (Liew and Kubes, 2016).
can communicate with liver-resident iNKT cells and are similarly affected by activated iNKT cells due to production of cytokines, chemokines and expression of surface molecules. As a result, the cross talk between iNKT cells and CD1d-expressing cognate partners lie at the core of the ability of iNKT cells to orchestrate immune responses. With this in mind, the interactions between iNKT cells and hepatic cells are important for understanding the mechanisms of how iNKT cells affect the outcome of inflammation.

1.7.1 Nonimmune cells with innate immune functions

The cell types mentioned below have unique immunological roles attributed to them in addition to their physiological role in the liver. This section examines how non-immune cells in the liver interact with hepatic iNKT cells and how they might be influenced by this interaction in turn.

1.7.1.1 Hepatocytes

The primary liver cell is the hepatocyte, a polarized epithelial cell that makes up ~65% of all liver cells (Racanelli and Rehermann, 2006; Treyer and Musch, 2013). The principal role of the hepatocyte is metabolism, detoxification and protein production (Ishibashi et al., 2009). Besides these physiological roles, studies have provided strong evidence that hepatocytes can interact in an immunological capacity with T cells. Hepatocytes express MHC I and can be induced to express MHC II under inflammatory conditions (Chiu et al., 1997; Wahl et al., 2008; Warren et al., 2006). Using electron microscopy, hepatocytes and naïve T cells have been shown to physically interact by projecting membrane extensions through the fenestrae of LSECs. These interactions are ICAM-1-dependent and MHC-dependent (Warren et al., 2006). Hepatocytes have also been demonstrated to present antigen to naïve T cells both in vitro and in vivo in mice
where MHC is only expressed on hepatocytes (Balam et al., 2012; Bertolino et al., 2002; Qian et al., 2001). Hepatocytes can also induce an innate immune response. They express pattern recognition receptors which when activated by recognition of pathogen-associated ligands results in production of inflammatory cytokines (Seki and Brenner, 2008; Zhang et al., 2009). The hepatocyte is also responsible for the production of most acute-phase proteins and complement components (Bode et al., 2012) such as C-reactive protein and complement 3 (C3) protein which can bind directly to the microbial surface to eliminate microorganisms and/or serve as an alarm to alert the immune system (Pepys and Hirschfield, 2003; Sabina et al., 2011; Zipfel and Skerka, 2009).

Hepatocytes have also been described to express CD1d and can present antigen to iNKT cells resulting in their activation (Anantha et al., 2014; Geissmann et al., 2005; Mandal et al., 1998; Yang et al., 2007). Exogenous activation of hepatic iNKT cells in vivo appear to be detrimental to hepatocytes. For example, as a result of concanavalin A (Con-A) administration which activates hepatic iNKT cells, iNKT cells rapidly upregulated FasL expression which contributed to Fas ligand-mediated lysis of hepatocytes (Takeda et al., 2000; Toyabe et al., 1997). Further, Con-A activation of hepatic iNKT cells results in IFN-γ secretion which also enhances liver injury (Beldi et al., 2008; Kusters et al., 1996). In a specific model of iNKT cell-mediated liver injury, αGalCer activation of iNKT cells has also been described to induce liver damage (αGalCer-induced liver injury) (Biburger and Tiegs, 2005). In this system, αGalCer was also found to result in up-regulation of FasL on iNKT cells and production of IFN-γ and TNF-α. In contrast to Con-A liver injury, TNF-α but not IFN-γ was found to be required for hepatotoxicity (Biburger and Tiegs, 2005). Interestingly, if αGalCer-pulsed hepatocytes were isolated from the liver and co-cultured with hepatic iNKT cells, these iNKT cells were observed
to produce exclusively IL-4 but not IFN-γ (Anantha et al., 2014). Even when IL-12 was added, IL-4 release by hepatic iNKT cells remained high. Therefore, the type of cytokines released by iNKT cells not only depends on type of antigen or iNKT cell subset but also potentially on the type of antigen presenting cell.

In contrast, hepatic iNKT cells were observed to play a mostly beneficial role during partial hepatectomy (DeAngelis et al., 2012; Minagawa et al., 2000). The number of iNKT cells in the liver was found to increase following partial hepatectomy and they subsequently produced IL-4 where IL-4 production was found to be critical for regrowth of the liver (DeAngelis et al., 2012). Accumulation of iNKT cells were dependent on sympathetic nerve and/or complement activation but not chemokines as the former mechanisms contributed to increase in iNKT cell number (DeAngelis et al., 2012; Minagawa et al., 2000). Modulation of liver regeneration by iNKT cells using αGalCer was attempted. Activation of iNKT cells with αGalCer 36 hours after partial hepatectomy resulted in acceleration of liver regeneration but if mice were pre-treated with αGalCer before surgery, iNKT cells instead produced IFN-γ and inhibited liver regeneration (Nakashima et al., 2006; Yin et al., 2014). As hepatocyte mitosis begins 32 hours after partial hepatectomy and is most prominent 40 - 44 hour after the surgery (Inui et al., 2002; Krupczak-Hollis et al., 2003; Noguchi et al., 1991), inappropriate interference in iNKT cell behavior and/or activity before liver injury must be approached cautiously.

1.7.1.2 Hepatic stellate cells (Ito cells)

Hepatic stellate cells (HSCs) or Ito cells are pericytes of the liver located in the perisinusoidal space of Disse. They comprise approximately 5-8% of all liver cells (Geerts, 2001). These stellate cells have angular rounded cell bodies and extend long cytoplasmic
processes which course along the surface of LSECs and encircle sinusoids at regular intervals (Wake, 2006) (Figure 1-6). Under basal conditions, HSCs play a major role in vitamin A (retinol) homeostasis, extracellular matrix regulation and local control of sinusoidal blood flow (Wake, 2006; Winau et al., 2008). During chronic liver damage or fibrosis, HSCs have been described to become major producers of collagen, structural glycoproteins and proteoglycans by transdifferentiation from a quiescent cell to a myofibroblast (Mederacke et al., 2013; Xu et al., 2014).

Some evidence suggests that HSCs can have an immunological role. HSCs express pattern recognition receptors TLR2 and TLR4, allowing them to recognize and respond to cell wall components of Gram-positive and Gram-negative bacteria (Brun et al., 2005; Paik et al., 2006; Paik et al., 2003). Reports have also demonstrated that HSCs express antigen-presentation molecules MHC I and II as well as CD1d, an MHC-like molecule that presents glycolipid antigens to NKT cells (Bomble et al., 2010; Vinas et al., 2003; Winau et al., 2007). Furthermore, they produce costimulatory molecules CD80 and CD40 which can robustly activate T cells (Vinas et al., 2003; Yu et al., 2004) and fluorescence microscopy has shown close proximity between HSCs and lymphocytes (Muhanna et al., 2007). Although no studies have shown capture and phagocytosis of bacteria by HSCs, these stellate cells do express Fc receptors and complement receptors on their surface presumably for uptake of opsonised molecules (Fimmel et al., 1996; Schieferdecker et al., 1997; Shen et al., 2005). Upon activation, HSCs can secrete cytokines and chemokines to activate or recruit immune cells. For example, HSCs can release monocyte chemoattractant protein 1 (MCP-1/CCL2) which recruits monocytes to sites of inflammation (Marra et al., 1993). HSCs can also secrete IL-6 and TGF-β which affects inflammation and fibrosis in the liver (Thirunavukkarasu et al., 2006).
Ito cells have been investigated in the context of antigen presenting cells to iNKT cells (Winau et al., 2007). Using in vitro T cell proliferation assays, Ito cells pulsed with αGalCer were co-cultured with thymus, spleen and liver iNKT cells. iNKT cells from all three organs proliferated vigorously and this proliferation was abrogated if anti-CD1d was added one hour before co-culture. To examine if Ito cells could also function as APCs in vivo, Ito cells were isolated from both wildtype and CD1d−/− mice, pulsed with αGalCer and re-introduced back into CD1d−/− mice via adoptive transfer. As CD1d−/− mice also did not have iNKT cells, iNKT cells were also transferred in this experiment. Since CD1d−/− mice do not have CD1d molecules, any antigen presentation could only be attributed to transferred Ito cells. In this adoptive transfer scenario, iNKT cells were found to proliferate with transfer of wildtype αGalCer-pulsed Ito cells but not CD1d−/− αGalCer-pulsed Ito cells. However, in this setting, transferred Ito cells are now located in the vasculature instead of the space of Disse. As a result, these findings do require some caution as the physiologic role of intravascular Ito cells has to be considered.

Moreover, it is important to note that many of these studies employed the use of cultured HSCs enriched through density separation to 90-95% purity (Vinas et al., 2003; Yu et al., 2004). As such, potent APCs such as liver dendritic or Kupffer cells may be present in culture. Indeed, during intravital imaging in the response of iNKT cells to systemic B. burgdorferi infection, Ito cells did not seem to present antigen to iNKT cells (Lee et al., 2010). Few bacterial spirochetes were observed in close proximity to Ito cells while the majority was in fact ingested by Kupffer cells. Since Ito cells were located outside the vasculature, they may instead act as a secondary defense against B. burgdorferi cells that avoided ingestion by Kupffer cells. Another important fact of note is that HSCs may alter their phenotype when cultured, increasing their surface expression of antigen presentation molecules which may not accurately reflect the intact liver.
system (Friedman, 2008; Friedman et al., 1989; Mannaerts et al., 2013). A recent study employing fresh 98% ultrapure HSCs populations demonstrated that these cells failed to activate lymphocytes and these HSCs lacked expression of key molecules such as MHC II, CD80/86 and only expressed a low level of CD1d (Ichikawa et al., 2011). As these molecules were only up-regulated after treatment with an inflammatory cytokine, this study suggests that HSCs may not appear as APCs under resting conditions but instead may play a regulatory role during disease conditions.

1.7.1.3 Liver sinusoidal endothelial cells (LSECs)

LSECs are endothelial cells that provide a continuous lining of the liver sinusoids and differ from other endothelial cells both morphologically and functionally (Figure 1-6). LSECs are small cells (~6.5 μm in diameter) that are stretched to a thin layer surrounding the inner surface of hepatic sinusoids (DeLeve, 2013). As LSECs do not contain a regular organized basement membrane, a gap filled with tissue fluid known as the space of Disse is formed between LSECs and hepatocytes (Ishibashi et al., 2009). In addition, LSECs also contain fenestrae which are clustered into sieve plates. These plates are approximately 150nm in diameter, thereby regulating the type of macromolecules which enter the space of Disse (Braet and Wisse, 2002; Wisse et al., 1985). These fenestrae act as a filter or sieve and studies in vivo have shown that macromolecules as small as 12nm can be excluded suggesting some dynamic regulation of the size of fenestrae (Kempka and Kolb-Bachofen, 1988).

LSECs are an integral part of the hepatic innate immune system. They comprise of approximately 50% of all nonparenchymal cells in the liver and are twice as abundant as liver-resident macrophages (Racanelli and Rehermann, 2006). LSECs are equipped with a large
variety of pattern recognition receptors (TLR3, 4, 7 and 9), MHC I and II, costimulatory molecules (CD40, CD80 and CD86) and adhesion molecules (ICAM) required for interaction with lymphocytes (Crispe, 2009; Knolle and Limmer, 2003). In addition, LSECs have also been shown to express CD1d (Geissmann et al., 2005). This array of receptors and molecules provide LSECs with surface characteristics typically observed in professional antigen presenting cells (APCs). Indeed, numerous studies have demonstrated that LSECs can present antigen (Crispe, 2011). Further, these cells express a number of receptors which allow them to internalize circulating proteins and immune complexes, including large macromolecular complexes up to 1 μm in diameter (Knolle and Limmer, 2003; Knolle et al., 1998; Sorensen et al., 2012; Steffan et al., 1986). LSECs are therefore competent to present a wide variety of blood- or gut-derived antigens to T lymphocytes and may form an important fraction of immune surveillance cells in the liver.

iNKT cells have been shown to be important in maintaining the endocytic activity of LSECs (Shishida et al., 2008). The authors employed the use of irradiated allogeneic cells injected via the portal vein to prevent rejection of heart allografts. LSECs endocytose these irradiated cells and exert immunosuppressive effects. In contrast to CD1d−/− mice which rejected heart allografts within 2 months, heart allografts were observed to survive indefinitely in wildtype BALB/c mice. This effect was not due to lack of CD1d molecules as Jα18−/− mice exhibited a similar phenotype. However, the mechanism by which iNKT cells regulate this effect remains unknown. As iNKT cells crawl along the endothelial cells in liver sinusoids, it is likely that they communicate with each other. Nevertheless, the interactions between LSECs and hepatic iNKT cells as well as the functional consequences of these interactions on the heart remain to be clearly defined.
1.7.2 Resident Immune cells

The liver itself also contains large populations of both resident and transient immune cells that are important to immunity in the liver. In the next section, we will review the role and function of these immune cells in the context of iNKT cell interactions.

1.7.2.1 Kupffer cells (liver-resident macrophages)

KCs have been reviewed under section 1.2.3.4 of this thesis. Here, I will discuss the interactions between KCs and iNKT cells. KCs are very effective APCs. They express both MHC I and II as well as CD1d (Crispe, 2011; Lee et al., 2010). However, the ability of KCs to activate lymphocytes depends on the existing microenvironment in the liver. KCs can have dual roles as immunosuppressors or immunoactivators. Under basal resting conditions, KCs are primarily immunosuppressive and inhibit T cell activation. Under continual exposure to low levels of LPS from the gut, KCs express PD-L1 and produce IL-10 which prevents activation of the adaptive immune response (Crispe, 2009; You et al., 2008). During some infections, by contrast, exposure to pathogen-associated molecules that activate TLR3 or 9 or inflammatory cytokines, KCs have the capacity to become APCs. They express high amounts of MHC I, II, CD40, CD80 and CD4+/CD8+ T cells form clusters around KCs (Burgio et al., 1998; Huang et al., 2013).

Located in the same vascular space as iNKT cells, KCs are extremely efficient at presenting glycolipids to iNKT cells that patrol in the liver sinusoids. Following B. burgdorferi infection, KCs phagocytose Borrelia spirochetes and release CXCL9 to attract iNKT cells to form clusters surrounding KCs (Lee et al., 2010). After cluster formation, KCs then present glycolipids to iNKT cells which activate them to produce IFN-γ and thereby promoting a strong
inflammatory response. Production of inflammatory cytokines by iNKT cells can act in a bi-directional manner as seen in the lung and enhance macrophage phagocytosis and bacterial clearance (Nieuwenhuis et al., 2002; Sada-Ovalle et al., 2008). During hepatosteatosis (fatty liver disease), severe loss of iNKT cells in the liver (but not the spleen) was observed (Kremer et al., 2010). This reduction of iNKT cells was dependent on KCs and IL-12 production as depletion of KCs using clodronate liposomes or use of IL-12 deficient mice restored hepatic iNKT cells in hepatosteatotic mice.

1.7.2.2 Natural Killer Cells

Natural killer cells are large granular innate lymphocytes which exhibit potent cytotoxic activity and cytokine production. Approximately one-third of lymphocytes in the liver are NK cells (Doherty and O'Farrelly, 2000; Gao et al., 2009; Racanelli and Rehermann, 2006). With more than a threefold increase of NK cells in liver compared to blood, hepatic NK cells are clearly resident in the liver (Sojka et al., 2014). NK cells contain a variety of specialized receptors that respond to damaged cells without prior sensitization and can also be activated by a variety of cytokines such as IL-12 and IL-18 (Middleton et al., 2002; Notas et al., 2009). Upon detection and activation, NK cells are able to release an assortment of cytotoxic granules containing granzyme and perforin in a cell-directed fashion (Shi et al., 2011; Vivier et al., 2012). Further, NK cells also can produce cytokines such as IFN-γ and TNF-α which can direct the inflammatory response (Crouse et al., 2015). Due to these properties, NK cells are able to respond rapidly to unexpected changes in the host. NK cells are therefore important local sentinel and effector cells during both infection and sterile inflammation in the liver.
The activation of iNKT cells and their subsequent production of significant amounts of IFN-γ has led to a concept known as NK cell transactivation (Brennan et al., 2013). NK cell transactivation is the secondary activation of NK cells leading to production of IFN-γ and follows the primary activation of another cell type. Using αGalCer to activate iNKT cells, rapid induction of CD69 and IFN-γ was observed in NK cells (Carnaud et al., 1999). This process was dependent on iNKT cells as no NK cell activation was observed if αGalCer was injected into iNKT cell deficient mice. In the spleen, dendritic cell-iNKT cell interaction following αGalCer treatment was the main mechanism for NK cell transactivation (Bezbradica et al., 2005).

1.7.2.3 Liver dendritic cells

Dendritic cells (DCs) are professional antigen presenting cells whose main function is to process antigen and present them to T lymphocytes. Generally, DCs exist in two different states (immature and mature) depending on environmental conditions and each DC state exhibits phenotypic and functional differences (Benencia et al., 2012). Immature DCs possess an antigen-capturing, environment sensing phenotype that is assisted by the large plethora of surface and vesicular pattern recognition receptors (PRRs) they carry (such as TLRs and NOD-like receptors) (Dudek et al., 2013; Worbs et al., 2017). In this immature state, DCs are highly phagocytic and can ingest a variety of antigens such as microbes or necrotic tissue laden with self-antigens through receptors such as CD14 or Fc receptors (Wallet et al., 2005). Ingestion through these pathways leads to antigen processing mechanisms and presentation of antigens through MHC class I or II to T lymphocytes. During this immature stage, DCs form short-term contacts with T cells and do not result in their activation (Hugues et al., 2004). However, the phenotype of DCs changes during pathological conditions. For example, inflammatory stimuli
together with recognition of DAMPs through PRRs trigger DC activation and maturation (Dudek et al., 2013; Wallet et al., 2005). During maturation, DCs upregulate ligands such as CD80, CD86 and MHC class II and increase their interaction and contact time with T cells (Dudek et al., 2013). Furthermore, mature DCs produce various cytokines to enhance the immune responses at the site of injury (Dudek et al., 2013; Wallet et al., 2005). The simultaneous manifestation of appropriate antigen-MHC complexes, immunostimulatory co-factors, cytokines and increased T cell interaction in combination contributes to optimal activation of T cells.

Using both in situ staining and freshly isolated liver dendritic cells (LDCs), studies have unveiled that LDCs exhibit an ‘immature’ or ‘Ag-processing’ phenotype – expressing low levels of MHC II, CD40, CD80 and CD86 (Inaba et al., 1994; Morelli et al., 2000; O'Connell et al., 2000; Woo et al., 1994) but significant levels of CD1d (Anantha et al., 2014; Tatsumi et al., 2008). Furthermore, in contrast to Kupffer cells, LDCs are sparsely distributed throughout the liver (Bosma et al., 2006; Steptoe et al., 2000) and in vivo imaging reveals that LDCs are located outside the vasculature and some appear to be in the sub-capsular space (David et al., 2016). Although possessing an immature phenotype, αGalCer-pulsed LDCs isolated from the liver were able to present this antigen to, and activate hepatic iNKT cells resulting in production of both IFN-γ and IL-4 (Anantha et al., 2014). In another study, injection of αGalCer was able to decrease the tumor load in the liver; this was dependent on the level of CD1d expression on the dendritic cells in the liver (Tatsumi et al., 2008). Nevertheless, considering the localization of LDCs, it is difficult to envision how these cells might interact with hepatic iNKT cells which are crawling in the vasculature. Use of high-resolution intravital microscopy is required to resolve this issue as well as interrogate the inter-compartment communication of these cell types.
1.7.3 Recruitment of immune cells and interaction with iNKT cells in the liver

1.7.3.1 Neutrophils

Since both neutrophils and iNKT cells are among the earliest responders during inflammation, they might share functional duties. Therefore, it is not surprising that in certain situations these two cell types interact and/or modulate each other. In the liver, iNKT cells have been described to modulate the neutrophilic response in some diseases. For example, chronic-plus-binge ethanol feeding increased the number of iNKT cells in the liver and also induced their activation (Mathews et al., 2016). Activation of hepatic iNKT cells in this model resulted in the upregulation of pro-inflammatory cytokines and chemokines (MIP-1, MIP-2 and IL-6) which recruited neutrophils into the liver causing liver injury. iNKT cell deficient mice or blockade of CD1d with anti-CD1d antibody provided protection and decreased injury. In a different model of liver involving bile duct ligation (cholestatic liver injury), iNKT cells were observed to reduce inflammation by suppressing neutrophil recruitment (Wintermeyer et al., 2009). Using iNKT cell deficient mice, increased liver injury was concomitant with increases in neutrophil accumulation. If these iNKT cell deficient mice were injected with iNKT cells before bile duct ligation, rescue of this phenotype was noted (i.e. less neutrophil recruitment, less liver injury).

Although neutrophil numbers are very consistent under basal conditions, infections and inflammation can rapidly elevate circulating neutrophil counts. To investigate if neutrophils might be able modulate iNKT cells, a study was conducted to determine if neutrophil concentration influenced iNKT cell function (Wingender et al., 2012). Using mice that spontaneously develop elevated neutrophil counts (CD18-deficient mice), iNKT cells were observed to be significantly decreased in the liver. In addition, iNKT cells in these mice displayed an impaired cytokine response upon αGalCer stimulation. This was due to a
downregulation of iNKT cell baseline T-bet and GATA3 expression and was cell extrinsic and reversible. The authors also demonstrated that a similar impairment in iNKT cell function occurred during inflammatory conditions in vivo during peritonitis. However, a defect in neutrophil function in these mice (i.e. inability to extravasate into tissues) could lead to subclinical infections and cause functional effects on iNKT cells. As a whole, the various models of disease described above indicate significant cross-talk between iNKT cells and neutrophils.

1.7.3.2 Monocytes

Under section 1.2.3.3 of this thesis, I have already reviewed the response of monocytes to hepatic sterile injury in detail. At this time, reports on interactions between iNKT cells and monocytes in the liver are scarce. To understand the interactions between monocytes and iNKT cells, I will review the interactions between these cell types in other organs. In the lung, iNKT cells have been demonstrated to reduce inflammatory Ly6C<sup>hi</sup> monocytes during infection and decrease lung injury (Kok et al., 2012). Using human iNKT and peripheral blood monocytes, iNKT cells were able to also direct the differentiation of classical monocytes into DCs (Hegde et al., 2007). These iNKT cell-instructed APCs were anti-inflammatory as they were able to dampen neutrophil infiltration and swelling in vivo (Hegde et al., 2009). Collectively, these studies demonstrate it is likely that hepatic iNKT cells, as immune orchestrators, could regulate the recruitment of and/or transition of classical monocytes to tissue repair monocytes.

1.8 A global view: bringing the pieces back together

In the literature review sections above, the various aspects of iNKT biology, activation, interactions with other cells and resultant effector function have been deconstructed (Figure 1-7). However, during inflammation, many of these iNKT cell aspects are intimately interconnected.
Figure 1-7. Interactions of hepatic iNKT cells with various cells in the liver.
Illustration of the cell types in the liver that has been demonstrated to present antigen via CD1d and/or release cytokines to activate hepatic iNKT cells. Following activation, hepatic iNKT cells can release T\textsubscript{h}1 (such as IFN-\(\gamma\)) or T\textsubscript{h}2 (such as IL-4) cytokines depending on the type of antigen presented, antigen-presenting cell and iNKT cell subset. HPC: hepatocyte, EC: endothelial cell, m\textsubscript{\phi}: macrophage, DC: dendritic cell, NK: natural killer cell, iNKT: invariant natural killer T cell, blue solid arrow: cytokines activating iNKT cells, red dashed arrows: cytokines produced by iNKT cells after activation.
To bring together the different facets of iNKT cell biology discussed above, various models of liver diseases will be used as examples to demonstrate how hepatic iNKT cells determine the outcomes of liver injury. Murine models have been employed to study the functional role of iNKT cells after sterile liver injury but current models have so far focused only on the pathophysiological contribution of iNKT cells and their immediate effects (for example, recruitment and activation of iNKT cells during sterile inflammation enhances or protects from liver injury). Two commonly used murine liver injury models, APAP-induced (drug-induced) liver injury and carbon-tetrachloride liver injury, will be used as examples here.

Drug-induced liver injury is a critical problem for the clinic and a substantial challenge for drug discovery and development (Jaeschke et al., 2011). Progress in understanding drug-induced liver injury has been successful in part by the use of predictable hepatotoxins, in particular APAP-induced liver injury (Jaeschke et al., 2014). The intracellular mechanisms of toxicity for APAP have been well-described and are mediated initially by the reactive metabolite N-acetyl-p-benzoquinoneimine (NAPQI) (Jaeschke et al., 2012; Jaeschke et al., 2014). NAPQI binds to mitochondrial proteins which increases mitochondrial oxidant stress, formation of mitochondrial membrane permeability transition pore and causes collapse of membrane potential and cessation of ATP synthesis. A downstream effect of mitochondrial dysfunction is DNA fragmentation and cell necrosis (Jaeschke et al., 2014). Release of DAMPs from necrotic cells induces a robust inflammatory response (see section 1.2.2).

The functional role of iNKT (and NK) cells in APAP-induced liver injury was initially examined by depleting both cell types with anti-NK1.1 antibodies (Liu et al., 2004). Depletion of iNKT and NK cells led to decreased liver damage, lower neutrophil infiltration and a corresponding decrease in expression of inflammatory cytokines and chemokines such as IFN-γ.
and keratinocyte-derived chemokine. Production of IFN-γ was important for liver damage as loss of IFN-γ reduced liver injury (Liu et al., 2004). However, it was discovered later that these phenotypes were observed due to the use of dimethyl sulfoxide (DMSO) as a solvent for acetaminophen (Masson et al., 2008). DMSO itself was found to strongly activate iNKT cells even in the absence of acetaminophen. To address these issues, another group examined the role of iNKT cells specifically by using iNKT cell deficient mice (CD1d<sup>-/-</sup> and Jα18<sup>-/-</sup> mice) (Martin-Murphy et al., 2013). Both strains of iNKT cell deficient mice exhibited increased liver damage in a sub-lethal dose of acetaminophen and when a lethal dose was provided, 75% of iNKT cell deficient mice did not survive in comparison to 100% survival of wildtype mice. However, the immunological contribution of iNKT cells in this study was not determined.

At the other end of the spectrum where iterative toxic damage leads to chronic inflammation and fibrosis, the carbon-tetrachloride injury model has been used to study liver fibrosis (Iredale, 2007). Using a single dose of carbon-tetrachloride (acute injury), iNKT cells were found to be protective as iNKT cell deficient mice (Jα18<sup>-/-</sup>) were more susceptible to liver injury with increased serum alanine aminotransferase (ALT) levels, dying hepatocytes, neutrophil and monocyte infiltration as well as higher levels of pro-inflammatory cytokines (Park et al., 2009). Interestingly, if iNKT cells were exogenously activated with αGalCer, increased liver injury was observed. Multiple doses of carbon-tetrachloride (chronic injury) resulted in a loss of iNKT cell protective effect due to a depletion of iNKT cells. In summary, iNKT cells, together with the cytokines they produce, have emerged as critical regulators of the immune response following sterile injury.
1.9 Statement of Hypothesis and Objectives

As described above, there is evidence that iNKT cells are pivotal in shaping disease outcomes leading to varied pathologies in multiple sterile inflammation models in the liver. However, their functional role in healthy injury and repair in the liver has not been studied systemically nor has the molecular mechanisms which regulate the recruitment and activation of hepatic iNKT cells to sites of sterile injury been defined. **We hypothesize that iNKT cells function as immune orchestrators that modulate the local immune response towards restitution and repair after appraising the inflammatory status of the microenvironment following sterile injury in the liver.** A central objective of this thesis is to identify and characterize the molecular mechanism(s) of hepatic iNKT cell accumulation in the liver microvasculature and clarify its functional role during hepatic sterile inflammatory responses. To achieve this objective, the following aims are planned:

**Aim 1:** To characterize the behavioral phenotype and activation status of hepatic iNKT cells after sterile injury.

**Aim 2:** To elucidate the molecular mechanism(s) that mediate accumulation of hepatic iNKT cells as well as the molecular mechanism(s) which regulate their activation.

**Aim 3:** To determine the functional role of hepatic iNKT cells after sterile inflammation in the liver.
Chapter Two: Methods and Materials

2.1 Animals

All protocols used in this manuscript were approved by the University of Calgary Animal Care Committee (protocol #AC16-0148) and in accordance with guidelines established by the Canadian Council on the Use of Laboratory Animals. All mice were maintained in a specific pathogen-free, double-barrier unit at the Faculty of Medicine, University of Calgary.

2.1.1 Mice

BALB/c, CD1d<sup>+</sup>, B6.CD1d<sup>+</sup> and C57BL/6 mice were purchased from Jackson Laboratories. BALB/c background Cxcr6<sup>GFP/GFP</sup> knock-in and C57BL/6 background Cx3cr1<sup>GFP/GFP</sup> (knock-in) mice were a gift from Dan R. Littman (New York University School of Medicine, New York). Generation of Ccr2<sup>RFP/+</sup> (knock-in) mice have been previously described (Saederup et al., 2010). Cx3cr1<sup>GFP/+</sup>Ccr2<sup>RFP/+</sup> were generated by crossing Cx3cr1<sup>GFP/GFP</sup> with Ccr2<sup>RFP/RFP</sup> mice. Floxed CD1d (C57BL/6-Cd1d1tm1.1Aben/J) and VECadherin-Cre (Cdh5(PAC)-CreERT2) mice were a gift from Albert Bendelac (University of Chicago, Chicago). Endothelial cell-CD1d knockout mice (CD1d<sup>fl/fl</sup>VECad-Cre) were generated by breeding floxed CD1d and VECadherin-Cre mice together.

2.1.2 Generation of chimeric mice (CD1d<sup>fl/fl</sup>VECad-Cre.Cxcr6<sup>GFP/+</sup>)

Bone marrow chimeras were generated through a standard procedure used previously in our laboratory (Andonegui et al., 2003). Bone marrow was isolated from donor Cxcr6<sup>GFP/+</sup> donor mice euthanized by cervical spine dislocation. Endothelial cell-CD1d knockout recipient mice were irradiated with 2 doses of 5 Gy (Gammacell 40 <sup>137</sup>Cs y-radiation source) with a 3 hour interval between doses. Approximately 8 x 10<sup>6</sup> of isolated donor bone marrow cells were
injected intravenously (i.v.) via the tail vein into irradiated recipient mice. Transplant mice received 0.2% neomycin dissolved in water and were kept in germ-free isolator cages for at least 8 weeks to allow for full hematological reconstitution before being used for further treatments. This protocol was previously demonstrated to remove existing bone marrow cells and replace ~99% of cells from donor marrow.

2.1.3 Conditional knockdown of CD1d on endothelial cells

CD1d on endothelial cells were knocked down in CD1d<sup>+/+</sup>VECad-Cre.<i>Cxcr6<sup>GFP/+</sup></i> (Figure 2-1A) through the following protocol. Tamoxifen was dissolved in sterile corn oil overnight at 37°C and injected consecutively for 5 days intraperitoneally (i.p.) into CD1d<sup>+/+</sup>VECad-Cre mice at a dosage of 100 mg/kg mice body weight. Mice were further kept in germ-free cages for 6 days to allow conditional knock down to occur before using the mice for experiments. Knock down of CD1d on endothelial cells were confirmed through flow cytometry (Figure 2-1B).

2.2 Antibodies and reagents

Fluorescein isothiocyanate (FITC)-conjugated anti-CD45r (clone RA3-6B2), eFluor® 660-conjugated anti-CD3 (clone 17A2) and Peridinin chlorophyll (PerCP)-conjugated anti-CD45 (clone 2D1), anti-CD1d (clone 1B1), unconjugated and Alexa Fluor 647-conjugated anti-Gr1 (clone RB6-8C5), anti-IL-12 (clone C17.8), isotype control rat IgG2a, isotype control rat IgG2b, brefeldin A solution (1000X) was purchased from eBioscience (San Diego, CA). Alexa Fluor 647-conjugated anti-CD49b (HMa2) was purchased from Biolegend (San Diego, CA). Alexa Fluor 750-conjugated anti-F4/80 (clone BM8) was obtained from AbLab (University of British Columbia, Vancouver, BC, Canada). Anti-IL-4 (clone 11B11), isotype control rat IgG1 and anti-Gr1 (clone RB6-8C5) was purchased from Bio X Cell (West Lebanon, NH).
Figure 2-1. Knock-down of CD1d expression on endothelial cells in CD1d<sup>f/f</sup>/VECad-Cre mice with tamoxifen treatment

(A) Illustration depicting the generation of mice progeny that do not express CD1d on endothelial cells after treatment with tamoxifen. VECadherin-Cre mice (Mouse A) and floxed CD1d mice (Mouse B) were bred together to generate progenies that expressed Cre recombinase enzyme in endothelial cells after tamoxifen treatment. Cre recombinase expression in endothelial cells would excise CD1d located between loxP sites resulting in removal of CD1d expression.

(B) Flow cytometric analysis of CD1d expression on endothelial cells (CD45<sup>−</sup>CD144<sup>+</sup>) harvested
from the liver of CD1d$^{\text{f/f}}$VECad-Cre$^{-}$ control mice and CD1d$^{\text{f/f}}$VECad-Cre$^{+}$ mouse 6 days after tamoxifen treatment.
Anti-CD16/CD32 (Fc block, clone 2.4G2) and fixation/permeabilization solution kit (BD Cytofix/Cytoperm™) for intracellular staining were purchased from BD (Franklin Lakes, NJ). Polyclonal rabbit anti-Neuropilin 1, anti-Plexin-C1, anti-Plexin-D1 and FITC-conjugated donkey anti-rabbit antibody were purchased from Santa Cruz Biotechnology (Dallas, Texas). Collagenase type IV was purchased from Worthington Biomedical (Lakewood, NJ). Rabbit polyclonal anti-thrombocyte serum was purchased from Cedarlane (Burlington, NC). A small sample of anti-NRP1a was obtained from Genentech (South San Francisco, CA) under a material transfer agreement. PBS57-loaded phycoerythrin (PE)-conjugated mouse CD1d tetramer was obtained from NIH (National Institutes of Health). Rat anti-BrdU was purchased from Abd Serotec (Raleigh, NC) and Alexa Fluor 647 (AF647) conjugated anti-rat IgG was purchased from Cell Signaling (Danvers, MA). Anti-IL-18r (clone 112624) was purchased from R&D systems (Minneapolis, MN). Propidium iodide (PI) and tamoxifen was purchased from Sigma-Aldrich (St. Louis, MO). Polyclonal anti-CXCR3 was a kind gift from Robert M. Strieter (University of Virginia). To visualize the liver vasculature, anti-PECAM-1 (platelet-endothelial cell adhesion molecule 1) (clone 390) was conjugated to Alexa Fluor 647 using a protein labelling kit according to manufacturer’s instructions (Invitrogen, Eugene, OR).

2.3 Experimental protocols and treatments

Kupffer cell depletion was performed by injecting 200 μl (1 mg/ml) of clodronate liposomes via tail vein 36 hours prior to induction of focal necrotic injury. Neutrophils (and a small percentage of monocytes) were depleted by i.p. injection of 200 μg of purified anti-Gr1 24 hours before injury induction. Platelets were depleted by i.v. injection of 100 μl of anti-thrombocyte serum 4 hours before injury induction. CD1d was blocked by administering 200 μg
anti-CD1d antibodies or 200 μg IgG2b isotype control by i.v. 30 minutes before injury induction. For IL-12/IL-18 neutralization experiments, mice were injected i.v. with 200 μg anti-IL12 and 50 μg anti-IL-18r or 250 μg of isotype control IgG2a antibodies 30 minutes before injury induction. For IL-4 neutralization experiments, mice were injected i.v. with 500 μg anti-IL-4 or 500 μg of IgG1 isotype control antibodies 30 minutes before injury induction.

2.4 Focal necrotic injury induction

The focal necrotic injury was performed as previously described. For spinning disk intravital microscopy, a single 0.02 ± 0.001 mm³ focal injury was generated on the surface of the liver to a depth of ~80 μm using the tip of a heated 30-guage needle mounted to an electrocautery pen. For immediate imaging on the microscope, necrotic cells were visualized by perfusing 50 μl of 2 μM propidium iodide solution directly onto the injury. For experiments that required imaging at later time points, the incision was closed with sutures and animals were allowed to recover for imaging at later time points (8 hours - 7 days) after injury. Sham experiments were prepared similarly but without injury induction and imaged identically to injured mice. For liver biopsy analysis via flow cytometry, a cluster of 3 - 4 focal injuries separated by ~1 mm were made on the liver. To obtain enough biopsies for flow cytometric analysis, several clusters of injuries, 0.5 cm apart, were made on the liver.

2.5 Intravital microscopy of murine liver

2.5.1 Preparation of mouse liver for intravital microscopy

Preparation of the murine liver for intravital microscopy was performed as previously described (Lee et al., 2010). Mice were anesthetized by i.p. injection of 200 mg/kg ketamine (Bayer Animal Health) and 10 mg/kg xylazine (Bimeda-MTC). The jugular vein of an
anesthetized mouse was cannulated to allow intravenous delivery of antibodies and additional
anesthetic if required. To maintain body temperature, mice were placed on a heating plate (CU-
201, Live Cell Instruments) at 37°C containing a glass cover slip to image hepatic tissue. A
midline and lateral incision along the costal margin to the midaxillary line was performed to
expose the liver. The mouse was placed in a right lateral position on the heating plate and the
ligaments connecting the liver to the diaphragm were transected to allow externalization of the
liver onto a glass coverslip. Blood flow was maintained intact. To prevent dehydration, exposed
abdominal tissues were covered with saline-soaked gauze. A saline soaked KimWipe®
disposable wiper was gently placed over the liver to restrict movement of the tissue on the
coverslip and to prevent tissue dehydration.

2.5.2 Spinning-disk Confocal Intravital Microscopy (SDIVM)

_Cxcr6^{GFP/+}_ knock-in mice were used for the visualization of hepatic iNKT cells. Intravital
microscopy was performed with an Olympus IX81 inverted microscope (Olympus, Center
Valley, PA), equipped with an Olympus focus drive and motorized stage (Applied Scientific
Instrumentation, Eugene, OR). Images were acquired with 4×/0.16 UPLANAPO and 10×/0.40
UPLANAPO objective lenses. The microscope was linked with a confocal light path (WaveFx;
Quorum Technologies, Guelph, ON, Canada) based on a modified Yokogawa CSU-10 head
(Yokogawa Electric Corporation, Tokyo, Japan). Visualization of iNKT cells and other immune
cells in the liver vasculature was acquired with four laser-excitation wavelengths in rapid
succession (491 nm, 561 nm, 642 nm and 730 nm; Cobolt, Vortran and Omicron) and captured
with appropriate band-pass filters (Semrock and Chroma). Typical exposure times for excitation
wavelengths were 0.2 - 0.5 s. A 512 × 512 back-thinned electron-multiplying charge-coupled
device camera (C9100-13, Hamamatsu, Bridgewater, NJ) was used for fluorescence detection. Z stacks of xy planes (0.5 μm intervals) were recorded with the inverted spinning-disk confocal microscope using either ASI focus drive (Applied Scientific Instrumentation) or Olympus focus drive (Olympus). Volocity software (Perkin Elmer, Waltham, MA) was used for 3D rendering, acquisition and analysis of images and cell tracking.

2.6 Visualizing collagen using second-harmonic signal in multiphoton microscopy

Nonlinear second harmonic signal (SHS) generation in animal tissue was completed using multiphoton microscopy using the crystalline and noncentrosymmetric biological properties of tissue collagen (Cox and Kable, 2006). Intact or injured livers were excised, and kept in cold PBS, and imaged with a FV1000MPE Olympus multiphoton microscope equipped with a Chameleon Ultra Laser Ti:Sapphire laser (Coherent). Multiphoton fluorescence and SHS was employed to visualized hepatic tissue using the 890 nm excitation. The resultant signal was detected with epi/backscattered geometry using appropriate bandpass filters (GFP, 495-540 nm; SHS, 420-460 nm) and nondescanned detectors. Z stacks of the hepatic tissue were recorded at 0.5 μm intervals with a 20x/0.95 N.A. water objective (Olympus). Images from the multiphoton microscopy were exported as OIF files and analyzed in Volocity software.

2.7 Flow cytometry analysis (whole liver and liver biopsies with sterile injury)

Liver-derived lymphocytes were isolated from BALB/c mice using a method previously described with minor modifications (Lee et al., 2010). Briefly, blood was withdrawn by cardiac puncture followed by perfusing the liver with 10 ml saline through the heart. For flow cytometry of cells obtained from the whole liver, livers were finely minced in a digestive medium containing 0.05% collagenase type IV and 0.002% DNase I in HBSS. Liver concentrates were
placed at 37°C for 30 min with gentle agitation. Following that, the concentrate was transferred to ice before generating single cell suspensions by mechanical disruption through a 40 μm nylon mesh. Suspensions were washed with ice-cold PBS (pH 7.4) and centrifuged at 300 × g for 10 min. All suspensions were then resuspended in cold flow wash buffer (FWB; PBS, 2% fetal calf serum, 0.5 mM EDTA). Liver mononuclear cells (MNCs) were also additionally purified through a 37%/70% (vol/vol) Percoll gradient. Purified MNCs were Fc blocked with anti-CD16/CD32 monoclonal antibodies before staining for iNKT cells (CD3+, CD1d-tetramer+) in FWB for 30 mins at 4°C. For semaphorin receptor staining, primary and secondary antibodies were used. Primary antibodies derived from rabbits were used to stain for semaphorin receptors in the same antibody cocktail described above before washing the samples. A secondary fluorophore-conjugated antibody against rabbit was used for sample staining after primary staining. For control samples, no primary antibodies were added. Samples were washed after secondary antibody staining and resuspended in PBS for flow cytometry.

Liver biopsies of thermal injuries were excised with a 2 mm biopsy punch and single cell suspensions were generated from the liver by mechanical disruption through a 40 μm nylon mesh. Samples were Fc blocked with anti-CD16/CD32 monoclonal antibodies before staining with appropriate fluorophore-conjugated antibodies in FWB for 30 mins at 4°C. For intracellular cytokine staining experiments, additional steps were added. Following harvest of liver biopsies, samples were immediately placed into PBS containing 3.0 μg/ml brefeldin A solution to inhibit intracellular protein transport and enhance detection of intracellular cytokines. After washes and staining of cell surface markers (CD45+, CD3+, CD1d-tetramer+), samples were subjected to a fixation and permeabilization step for intracellular staining according to manufacturer’s kit instructions. Samples were then washed with Perm/Wash™ buffer from the kit before re-
suspending in PBS for cytometric analysis. All samples were analyzed on an Attune acoustic focusing cytometer (Applied Biosystems). For iNKT cell analysis, only mononuclear cells (MNCs) were gated in FSC versus SSC flow cytometric plots followed by gating for single cells. Figures were generated with Attune Cytometer software or FlowJo (Tree star).

2.8 Immunofluorescent staining and imaging

2.8.1 Frozen-sections

Frozen OCT-embedded liver sections of BALB/c mice were used for BrdU+ cell staining and identification. 5 μm tissue sections of the hepatic focal necrotic injury were obtained and dried overnight at room temperature in the dark. Tissue sections were fixed with ethanol and paraformaldehyde before antigen retrieval. Antigen retrieval was performed according to manufacturer’s instructions (BD Retrievagen kit). Sections were blocked with 20% goat serum before primary staining with rat anti-BrdU antibodies and secondary staining with anti-rat fluorophore-conjugated antibodies. Anti-fade fluorescence mounting media was added to the section before mounting the slides for imaging and image acquisition with the spinning disk confocal microscope.

2.8.2 Whole-mount

A small section (~ 5 mm) of the liver containing the sterile injury was harvested and incubated overnight at 4°C with a 1:100 dilution of PBS57-loaded PE-conjugated CD1d tetramer in PBS. The next day, the liver section was washed twice with PBS before mounting onto a slide for image acquisition with the spinning disk confocal microscope. A layer of moist Kimwipe® was placed over the liver section to prevent it from drying out as well as to hold it in place during imaging.
2.9 Image and Video Analysis

2.9.1 Analysis in the number of hepatic iNKT cells after sterile injury

The number of hepatic iNKT cells inside or surrounding the injury up to 400 μm away was analysed using ImageJ software. Images were exported using Volocity into TIFF image files and imported into ImageJ. A region-of-interest (ROI) section was drawn around the sterile injury to determine the exact size and area (Figure 2-2). Distances of 100 μm each, up to 400 μm away from the injury, was marked in 4 vertices surrounding the injury. By drawing a circle circumscribing these 4 equidistant vertices, this allowed the specific area in μm² to be calculated. For sham-injury mice, a circle of approximately the same size compared to a sterile injury site was superimposed onto the image (Figure 2-2). This imaginary circle was used to calculate areas up to 400 μm away as described above. GFP+ cells (bright green) were manually counted in each 100 μm radii circle for quantification and analysis.

2.9.2 Analysis of hepatic iNKT cells behavior

Behavior of hepatic iNKT cells under basal conditions or after sterile injury was analysed with Volocity software using time-lapse images acquired with the 10x objective. Manual tracking of crawling or stationary GFP+ cells (bright green) was performed over a 30 minute recording of images. Cells were considered stationary if they did not crawl more than half a cellular body length over this 30 minute period of analysis. For analysis of hepatic iNKT cells infiltrating or U-turning at a site of sterile injury 4 hours post-injury, an imaginary line (similar to the circle described above in section 2.9.1) was drawn around an injury or superimposed onto sham mice. This allowed systematic tracking of hepatic iNKT cells which would either infiltrate/cross or U-turn at the line.
Figure 2-2. Illustration depicting the analysis of hepatic iNKT cells after sterile injury
Representative images of sterile injury and sham operation used for quantifying the number and behavior of hepatic iNKT cells. A circle (red) was drawn either around a thermal injury (sterile injury image) or an imaginary injury (sham operation image). Subsequently, circles of increasing 100 μm radius (yellow) was drawn around the original circle. This allowed the quantification of hepatic iNKT cells and specific area in μm².
2.9.3 Analysis of hepatic iNKT cells from whole-mount immunofluorescence staining

Volocity software was used to analyse hepatic iNKT cells following whole-mount staining. Number of iNKT cells, i.e. dual positive cells GFP+ (green) and PBS57-loaded PE-conjugated CD1d tetramer+ (red) cells were counted using the software. Percentage of iNKT cells was determined by calculating number of yellow dual positive cells divided by total green cells (GFP+ but not PBS57-loaded PE-conjugated CD1d tetramer+).

2.9.4 Analysis of BrdU+ cells from immunofluorescence staining

ImageJ software was employed to quantify the number of BrdU+ cells. Images were exported from Volocity in TIFF files for use in ImageJ. The area of injury was determined as described in section 2.9.2. AF647-BrdU+ cells (in blue) were manually counted and quantified within the area of the injury.

2.9.5 Analysis of second harmonic signal of collagen from multiphoton microscopy

Volocity software was employed to analyze second harmonic signal. Z-stack images obtained from the multiphoton microscopy were compressed into a single extended focus image (focus stacking) which allowed a consistent method to analyze the images. Intensity of the second harmonic signal was quantified using the Volocity software for collagen fibers inside as well as outside the injury. Autofluorescence signals within the injury which were detected in multiple channels were excluded from this quantification through use of region-of-interest exclusions.
2.9.6 Cellular tracking of iNKT cell behavior and crawling with Sema3A-infused filter paper treatment

iNKT cells behavior was analyzed using Volocity software. The edge of the filter paper was used to define an imaginary end line upon which all GFP+ cells within 100 μm of that line were tracked manually. The starting point of all cell tracks were subsequently calculated and adjusted to begin from origin (i.e. x-coordinates: 0, y-coordinates: 0) to allow all tracks to be plotted from identical root source. To obtain the net vector movement of cells, the end coordinates from all the tracked cells were averaged and plotted on a Cartesian coordinate system.

2.9.7 Analysis of monocyte hues

Monocyte hues were measured as previously described (Dal-Secco et al., 2015). In brief, 10× images for each experiment acquired using Volocity software, were exported into TIF images to be used in ImageJ. Only the green and red fluorescence emission channels were kept and merged into a RGB image (where the blue channel was blank). Images were segmented using the Color Threshold Tool in the brightness channel in the hue, saturation and brightness (HSB) color space. Threshold levels for each dataset were identical in the same imaging session. Pixels in the segmented color region were categorized according to the hue (red, orange, yellow or green) before the relative percentage of pixels in each color category was calculated.

2.10 Statistical analysis

All values are expressed in mean ± SEM. Data were compared with either unpaired Student’s t-test or one-way ANOVA with Bonferroni multiple comparisons post hoc test. Statistical significance was accepted at $P < 0.05$. 
Chapter Three: The early phase of the iNKT cell response to hepatic sterile injury

3.1 Kinetics of the iNKT cell response

Platelets, neutrophils, peritoneal macrophages and monocytes are recruited to a site of focal sterile injury in the liver (Dal-Secco et al., 2015; McDonald et al., 2010; Slaba et al., 2015; Wang and Kubes, 2016). To visualize the response of hepatic iNKT cells to focal sterile injury using the same model, mice expressing enhanced green fluorescent protein under the control of the Cxcr6 promoter (Cxcr6Gfp/+) were used. In the liver, approximately 60 - 80% of GFP+ cells are iNKT cells and the remaining GFP+ cells are NK and conventional T cells (Geissmann et al., 2005). Hepatic iNKT cells were evenly distributed throughout the liver and did not form clusters or accumulate in any specific area (Figure 3-1A). They were also observed to reside exclusively in the sinusoids and crawled in a random pattern under basal conditions (Figure 3-1B and C) as previously described (Geissmann et al., 2005; Lee et al., 2010; Wong et al., 2011).

In contrast to neutrophils which infiltrated into the necrotic lesion within 4 hours (McDonald et al., 2010), iNKT cells did not accumulate or infiltrate into the injury by this time point. Instead, iNKT cells were observed to begin accumulating around the lesion at 8 hours and were increasingly retained around the lesion up to 24 hours post-injury (Figure 3-2A and B). Interestingly, this accumulation of iNKT cells around the lesion was similar to what we observed for CCR2RFP/+ monocytes which encircled the lesion within the first 24 hours and then entered the injury at 48 hrs (Dal-Secco et al., 2015). The ring of iNKT cells was also observed to infiltrate into the injured site at 48 hours and they were seen to remain inside the injury up till 7 days post-injury (Figure 3-2A and C). In view of the different stages observed during the iNKT cell response to sterile injury, three different phases of this response were delineated; an
Figure 3-1. Distribution and behavior of iNKT cells under physiological basal conditions

(A) Representative stitched image (combining 70 different fields of view) of a section of the liver of Cxcr6^{GFP/+} mice without injury, n ≥ 3, bright green = iNKT cells. Scale bar is 300 μm.

(B) Representative still image of crawling iNKT cells, white arrows represent live tracks of iNKT cells imaged over 30 minutes, n ≥ 3, bright green = iNKT cells. Scale bar is 100 μm. (C) Cell tracks of all iNKT cells obtained from 2 fields of view in 3 sham injury-operated mice. Cell tracks were adjusted to begin from a common point of origin (x-coordinates: 0, y-coordinates: 0) and then overlaid over each other in the Cartesian coordinate system.
Figure 3-2. Kinetics of iNKT cell response to sterile injury

(A) Representative still images of the iNKT cell response to sterile injury induced by thermal probe over time up to 7 days, bright green = iNKT cells, red = dead cells (propidium iodide), n ≥ 3, scale bar is 100 μm. (B) Quantification in the number of GFP+ cells within 100 μm of the injury over time, n ≥ 3, P** < 0.01, P*** < 0.001 by one-way ANOVA, n.d.: not determined. (C) Quantification in the number of GFP+ cells inside the injury over time, n ≥ 3, P** < 0.01, P*** < 0.001, P**** < 0.0001 by one-way ANOVA, P### < 0.001 by t test.
early repulsive phase, an intermediate retention phase and a late infiltration phase (Figure 3-2B and C). This thesis will address these three phases; the early phase (Chapter 3), the intermediate phase (Chapter 4) and the late phase (Chapter 5) in sequential order.

To confirm that the accumulating CXCR6$^{GFP/+}$ cells surrounding the injury were iNKT cells in our focal sterile injury model, two separate approaches were used. First, immunofluorescence whole mount staining of the injured site from a Cxcr6$^{GFP/+}$ mouse was performed using loaded CD1d-tetramers (Figure 3-3A and B). Concurrently, flow cytometry of harvested 2 mm biopsies of multiple focal injuries was also employed (Figure 3-3C). These approaches confirmed that a significant majority of GFP$^+$ cells surrounding the injury were CD1d-tetramer$^+$ iNKT cells.
Figure 3-3. Accumulating GFP+ cells are mainly iNKT cells

(A) Representative image of fluorescence whole-mount staining of injured site from Cxcr6GFP/+ mouse, n = 3. Scale bar is 100 μm. (B) Quantification in the number of PBS57-loaded CD1d-Tetramer+ GFP+ iNKT cells from whole-mount staining, n = 3, P*** < 0.001 from t test. (C) Flow cytometric analysis of percentage of GFP+ cells that are iNKT cells obtained from a biopsy of the liver injury or from an uninjured lobe of the liver.
3.2 Behavior of iNKT cells during the early phase

The cellular dynamics of the iNKT cell response was next assessed at each of the three phases: early, mid and late. Unexpectedly, iNKT cells approach the injury at 4 hours (the early phase), make a 180° U-turn and were repelled from the immediate area (Figure 3-4A and 3-7B). Under normal conditions, the likelihood of a crawling iNKT cell making U-turns was very rare (Figure 3-1B, C and 3-5B). Imaging under sham injury conditions for 30 minutes revealed numerous cells crossing an imaginary line (see Figure 2-2, section 2.9 for methods) whereas if this line was placed surrounding the injury, no iNKT cell crossed the line to infiltrate into the injury (Figure 3-4A, B and 3-5A). In fact, every one of these cells U-turned for the first 4 hours post injury (Figure 3-5B).

The U-turns were not due to other cells at the injury site. There were a significant number of neutrophils entering the lesion at 4 hours (McDonald et al., 2010). However, use of antibodies to deplete neutrophils (Figure 3-5C) did not reduce the number of iNKT cell U-turns (Figure 3-5B). Platelets were observed to completely line the sinusoids after injury (Slaba et al., 2015). Anti-thrombocyte serum was used to induce platelet depletion (Figure 3-5D) but this treatment also did not result in any change in iNKT cell U-turns (Figure 3-5B). We also investigated if liver-resident macrophages (Kupffer cells, KCs) played any roles in iNKT cell U-turns by depleting them using clodronate liposomes (CLL) (Figure 3-5E). CLL treatment had no impact on iNKT cell U-turns (Figure 3-5B). Tandem depletion of platelets and KCs also did not affect this iNKT cell behaviour (Figure 3-5B). Semaphorins are molecules which mediate a fugetactic response where immune cells are chemically repelled from a site (Takamatsu and Kumanogoh, 2012; Takamatsu et al., 2010). We identified that iNKT cells expressed primarily the receptor
Figure 3-4. Hepatic iNKT cells U-turn and leave from site of sterile injury

Hepatic iNKT cells do not infiltrate into an area of sterile injury during the early phase (4 hours) but U-turn and depart the area. (A) Representative time-lapse image of iNKT cell behavior at injury site 4 hours after injury, bright green = iNKT cells, red = dead cells, white arrows demonstrate iNKT cell tracks crawling towards injury, yellow arrows are iNKT cell tracks after U-turn from injury, scale is 50 μm. (B) Representative image of iNKT cell behavior under sham injury conditions with an imaginary injury(line) superimposed onto the image for analysis, yellow arrows are actual iNKT cell crawling tracks, scale is 50 μm.
Figure 3-5. Mechanisms that affect hepatic iNKT cell U-turns after sterile injury

(A) Quantification in the number of hepatic iNKT cells that infiltrate into a sham imaginary site or site of sterile injury under different conditions, n ≥ 3, P* < 0.05 by t test. (B) Quantification in the number of hepatic iNKT cells that U-turn from a sham imaginary site or sites of sterile injury
under different treatment conditions, n ≥ 3, P## < 0.001, P** < 0.01 by t test. Depletion of various cells and different treatments to examine iNKT cell behavior after sterile injury; (C) Representative in vivo still image of neutrophil response 4 hours after sterile injury in wildtype (−Neut-depl) and neutrophil depleted mice (+Neut-Depl), blue = neutrophils, red = dead cells, n = 3, scale is 100 μm. (D) Representative still intravital image of platelets lining sinusoids 4 hours after sterile injury in wildtype (−platelet-depl) and platelet depleted mice (+platelet-depl), blue = platelets, red = dead cells, n = 3, scale is 100 μm. (E) Representative still image of Kupffer cells present in sinusoids 4 hours after sterile injury in wildtype (−CLL) and clodronate treated (+CLL) mice, magenta = Kupffer cells, red = dead cells, n = 3, scale is 100 μm. (F) Expression of Neuropilin-1 (NRP1a), Plexin-C1 and Plexin-D1 on iNKT cells (CD3+CD1d-tetramer+) determined by flow cytometry.
(NRPIa) for class 3 semaphorins (Sema3A) (Figure 3-5F). Receptors for other classes of semaphorins (Class 7 receptor: Plexin-C1 and Class 4 receptor: Plexin-D1) was detected at low levels on iNKT cells (Figure 3-5F). Antibody blockade of the NRPIa receptor failed to alter the iNKT cell U-turn response (Figure 3-5B).

To examine if anti-NRPIa antibody blockade was operative, we set up an *in vivo* functional assay using a 2 mm filter paper perfused with Sema3A placed onto the surface of the liver with or without anti-NRPIa antibody blockade (Figure 3-6A). All iNKT cells moving towards or away from the paper (within 100 μm) were visualized and tracked (Figure 3-6A, inset, area between dotted or whole yellow line). The net movement of iNKT cells was computed from these representative tracks. Filter paper perfused with only PBS solution alone did not result in any change in iNKT cell crawling behavior as they continued to migrate randomly in all directions as previously observed (Figure 3-6B, first panel). In contrast, iNKT cells were observed to display overall movement away from the filter paper with Sema3A which was abolished with anti-NRPIa treatment (Figure 3-6B, second and third panel).

CXCL9 (MIG, monokine induced by gamma interferon) is a chemokine that attracts iNKT cells (Johnston et al., 2003). During infection, KCs have been demonstrated to release CXCL9 which binds to CXCR3 on iNKT cells (Lee et al., 2010). This chemokine attraction results in iNKT cells forming clusters surrounding KCs and allowing KCs to present antigens to activate iNKT cells. In an effort to override the repulsion mechanism that resulted in iNKT cell U-turns, we perfused CXCL9 over the injured site at 4 hours to provide a chemo-attractive gradient to recruit iNKT cells into the injury. Although we observed an increased number of iNKT cells which approached the injury site, this simply increased the number of U-turns at the injured site (Figure 3-5B). No iNKT cells entered the injury (Figure 3-5A) suggesting a
Figure 3-6. In vivo functional assay to determine behavior of hepatic iNKT cells after Sema3A treatment with and without anti-NRP1a antibody

Stitched image of filter paper placed onto the surface of the liver obtained from 9 different fields of view, bright green = iNKT cells, scale is 500 μm; inset: higher magnified image for iNKT cell behavior tracking after placement of filter paper. (B) Top panels: total cell tracks of all iNKT cells within 100 μm of the paper from 3 mice under different treatment conditions, cell track diagrams are arranged in a way that sources of the treatment (e.g. Sema3A) are to the right (east) of the plot from cell origin; Bottom panels: net vector movement of iNKT cells determined from all iNKT cell tracks, all treatment sources occur from the right (east) of the plot.
formidable physical barrier to iNKT cell entry into the sterile injury site. It is worth mentioning that this behavior was selective to iNKT cells as neutrophils entered the injured sites and never did U-turns.

3.3 iNKT cells U-turn specifically at collapsed sinusoids

To examine if a physical barrier might exist to prevent iNKT cells from infiltrating the site of sterile injury, several approaches were used. By staining the sinusoids with an intravenous injection of PECAM-1 antibody before (red) and after (blue) inducing thermal injury, we could delineate the exact position where the sinusoids stopped being patent (stopped being blue but retained red staining) (Figure 3-7A). A higher magnification power was employed to observe iNKT cell behavior at this boundary and iNKT cells were seen to specifically U-turn at this edge of collapsed sinusoids caused by the injury (Figure 3-7B and C). The width of PECAM-1 stained sinusoids was measured at this higher magnification and there was a significant decrease in the width of PECAM-1 stained sinusoids inside the injury compared to uninjured liver areas (Figure 3-8A). To confirm that the lumen of these collapsed sinusoids were not patent 4 hours after injury, fluorescent albumin was injected and no perfusion inside the injury was observed in contrast to sinusoids outside the injury which were well-perfused (Figure 3-8B); consistent with previously published results (Slaba et al., 2015). In contrast, if fluorescent albumin was injected 3 days after sterile injury, significant leakage of serum albumin into the injury was observed (Figure 3-8B).
Figure 3-7. iNKT cell U-turn behavior occurs specifically at edge of collapse sinusoids

(A) Representative image demonstrating staining of sinusoids and remnants of sinusoids using PECAM-1 antibodies before (red) or after (blue) induction of sterile injury. Scale bar is 50 μm.

(B) Representative still images over time of iNKT cells U-turn specifically at collapsed sinusoids over time, yellow arrow: iNKT cell approaching collapsed sinusoid, red arrow: iNKT cell U-turn and leaving collapsed sinusoid, scale is 25 μm. (C) Quantification of iNKT cells U-turn at regular vs sinusoid remnants, n ≥ 3 mice, P*** < 0.001 by t test.
Figure 3-8. Sinusoids inside the injury are not patent immediately after sterile injury

(A) Width of PECAM-1 stained sinusoids under basal untreated conditions as well as after sterile injury, n = 3 mice, P*** < 0.001 by t test. (B) Representative still images of albumin perfusion (blue) over time at 0, 0.5, 5 and 20 minutes after albumin perfusion; albumin was injected at 4 hours and 3 days post-injury (red), scale bar = 100 μm.
Chapter Four: Intermediate Retention Phase of iNKT response to sterile injury

4.1 Behavior of iNKT cells during the intermediate retention phase

In contrast to the U-turns observed during the initial repulsive phase, a significant change in iNKT cell behavior was observed during the intermediate retention phase which occurred at 8 and 24 hours after sterile injury. At 8 hours, the iNKT cells no longer displayed U-turn behavior. Instead, iNKT cells arrested their crawling behavior in what appeared a strategic position closely abutting the lesion boundary (Figure 4-1A). Approximately 50% of iNKT cells arrested (Figure 4-1B) with the remaining iNKT cells crawling at less than 2.5 µm/min (Figure 4-1D, 8 hr). Continued arrest of iNKT cells was also observed at 24 hours as iNKT cells continued to accumulate around the injured site (Figure 3-2C and 4-1C). The arrest of iNKT cells was not due to surgical stress as iNKT cells in sham operations performed at 8 hours and 24 hours continued crawling at velocities observed at basal conditions (Figure 4-1E). In addition, the arrest of iNKT cells were specific to the boundary of the lesion as iNKT cells that were more than 100 µm away from the injury continued to crawl randomly until they reached the injury site. This suggested that there was an immobilized local arrest molecule.

Arrest of iNKT cells next to the injury correlated with activation and expression of CD69 marker (Figure 4-2A) as have been previously observed (Lee et al., 2010; Wingender et al., 2011; Wong et al., 2011). In contrast to systemic B. burgdorferi infection where Kupffer cells presented bacterial antigens to activate iNKT cells (Lee et al., 2010), activated iNKT cells in the sterile injury model did not produce IFN-γ (Figure 4-2B) but instead released IL-4 (Figure 4-2C). At 48 hours, iNKT cells finally migrated into the injured site (Figure 3-2C) and no further arrest of iNKT cells inside the injury was noted (Figure 4-1D, 48 and 72 hr).
Figure 4-1. iNKT cells arrest abutting the necrotic lesion during the intermediate phase

iNKT cells arrest abutting injured site at 8 and 24 hours. (A) Representative still images demonstrating arrest of iNKT cells (white arrows) over 60 minutes, scale is 25 μm. (B) Quantification of stationary iNKT cells within 100 μm of injured site at 8 hours, n ≥ 3, P** <
0.01 by *t* test. (C) Quantification of stationary iNKT cells within 100 μm of injured site at 24 hours, *n* ≥ 3, P** < 0.01 by *t* test. (D) Velocity profiles of iNKT cells within 100 μm of injury under basal conditions and 4, 8, 24, 48 and 72 hours after induction of sterile injury, *n* ≥ 3 mice. (E) Velocity profiles of iNKT cells in sham surgery mice, 8 and 24 hours after sham surgery, *n* = 3.
Figure 4-2. Activation of hepatic iNKT cells results in production of IL-4

CD69 expression and intracellular cytokine staining of hepatic iNKT cells (CD45⁺CD3⁺CD1d-tetramer⁺) harvested from biopsies of thermal injuries performed in the liver. (A) Representative flow cytometry image of CD69 expression of iNKT cells in uninjured mice and after sterile injury at 24 hours, n = 3 mice. For intracellular staining, harvested biopsies were incubated 5 - 10 mins in Brefeldin A before mechanical disruption and subsequent antibody staining (see methods); (B) Representative flow cytometry image of intracellular IFN-γ staining of iNKT cells in uninjured mice and after sterile injury at 24 hours, n = 2 mice. (C) Representative flow cytometry plot of intracellular IL-4 staining of iNKT cells in uninjured mice and after sterile injury at 24 hours, n = 3 mice.
4.2 Biphasic mechanism of arrest of iNKT cells after sterile injury

4.2.1 Initial iNKT cell arrest is dependent on endogenous antigens presented via CD1d

As iNKT cells displayed a difference in temporal accumulation and cellular dynamics as compared to neutrophils, monocytes and peritoneal macrophages, we hypothesize that iNKT cells have an independent mechanism of accumulation. Previous studies have demonstrated that iNKT cells cease crawling behavior upon presentation of exogenous antigenic ligands via CD1d by antigen presenting cells (APCs) (Geissmann et al., 2005; Lee et al., 2010; Velazquez et al., 2008). iNKT cell arrest also correlated with the activation of iNKT cells (Geissmann et al., 2005; Lee et al., 2010; Velazquez et al., 2008; Wong et al., 2011). In our sterile injury model, blockade of CD1d self-antigen presentation with anti-CD1d antibodies prevented iNKT cell arrest at 8 hours (Figure 4-3A and B). In addition, the accumulation of iNKT cells around the injured site was also completely abrogated after CD1d antibody blockade at 8 hours (Figure 4-3C) while anti-CXCR3 antibody treatment had no effect (Figure 4-3D). This was in contrast to the CXCR3-dependent recruitment of hepatic iNKT cells during B. burgdorferi infection which resulted in the formation of iNKT cell clusters surrounding KCs (Lee et al., 2010). No changes in iNKT cell arrest or accumulation was observed at 8 hours post-injury with isotype control antibodies (Figure 4-3B and C).
Figure 4-3. Initial iNKT cells arrest around sites of sterile injury during intermediate retention phase is due to endogenous antigen presentation via CD1d

(A) Representative still images of crawling iNKT cells after anti-CD1d antibody blockade at 8 hours, scale is 25 μm. (B) Quantification of stationary iNKT cells within 100 μm of injury after anti-CD1d antibody treatment at 8 hours, n ≥ 3 mice, P*** < 0.001 by t test. (C) Number of iNKT cells up to 400 μm of the injury without antibody treatment (black bars), anti-CD1d treatment (white bars) and isotype treatment (grey bars) at 8 hours, n ≥ 3 mice, P† < 0.05, P** <
0.01 by $t$ test. (D) Quantification in the number of iNKT cells within 100 μm of the injury after anti-CXCR3 treatment at 8 hours post-injury, $n \geq 3$ mice.
4.2.2 Subsequent activation of iNKT cells during the intermediate retention phase was dependent on cytokines IL-12 and IL-18

Unexpectedly, no changes in iNKT cell arrest were noted at 24 hours when employing the same anti-CD1d antibody treatment (Figure 4-4A), suggesting different arrest molecule(s) at this time point. Innate cytokine-driven signals have been described to activate iNKT cells after bacterial infection in vitro and in vivo (Brigl et al., 2011). Additionally, other investigators demonstrated that iNKT cells can arrest through synergistic effects by exogenously applying cytokines IL-12 and IL-18 (Velazquez et al., 2008). Blocking IL-12 and IL-18 with antibodies in the sterile injury model prevented arrest of iNKT cells at 24 hours (Figure 4-4B). Cytokine-driven signaling which resulted in iNKT cell arrest and accumulation only occurred after the initial CD1d antigen presentation as blocking IL-12 and IL-18 at 8 hours did not affect iNKT cell arrest or accumulation (Figure 4-4C and D).

To determine whether the initial CD1d-dependent arrest of iNKT cells was required for the subsequent cytokine retention around the injury at 24 hrs, mice were pretreated with CD1d antibody 30 minutes prior to injury. Using this protocol, fewer iNKT cells were retained around the injured site at 24 hours (Figure 4-4E), suggesting that the initial CD1d-induced arrest was required for the cytokine retention at the later time point. IL-12- and IL-18-dependent arrest was also necessary for continued retention of iNKT cells around the injury at 24 hours as accumulation was attenuated after anti-IL-12 and anti-IL-18 antibody treatment (Figure 4-4F), which was given 30 minutes before induction of injury. Isotype control antibodies did not affect iNKT cell arrest or retention within the 100um around the injury site (Figure 4-4E and 4F).
Figure 4-4. Subsequent iNKT cells arrest around sites of sterile injury during the intermediate retention phase is due to cytokine-driven (IL-12 and IL-18) signaling.

(A) Quantification of stationary iNKT cells within 100 μm of injury after anti-CD1d treatment at 24 hours, n = 3. (B) Quantification of stationary iNKT cells within 100 μm of injury after anti-IL-12 and anti-IL-18 treatment at 24 hours, n ≥ 3 mice, P** < 0.01 by t test. (C) Percentage of stationary GFP+ cells within 100 μm of injury after anti-IL-12 and anti-IL-18 treatment at 8 hours, n = 3 mice. (D) Number of GFP+ cells within 100 μm of injury after anti-IL-12 and anti-
IL-18 treatment at 8 hours. (E) Number of iNKT cells within 100 μm of the injury without antibody treatment (black bars), anti-CD1d treatment (white bars) or isotype treatment (grey bars) at 24 hours, n ≥ 3 mice, P* < 0.05, by t test. (F) Number of iNKT cells within 100 μm of the injury without antibody treatment (black bars), with anti-IL-12 and anti-IL-18 treatment (white bars) or isotype treatment (grey bars) at 24 hours, n ≥ 3 mice, P* < 0.05 by t test. (A-F) All antibodies were given before induction of sterile injury.
4.3 Kupffer cells and endothelium present CD1d-dependent antigens to iNKT cells after sterile injury

Several CD1d-expressing APCs exist in the liver including KCs, liver sinusoidal endothelial cells and hepatic stellate cells (Ito cells) and they have been demonstrated to present exogenous ligands to iNKT cells (Geissmann et al., 2005; Lee et al., 2010). KCs have been reported to be the most effective presenters of CD1d-relevant glycolipids (Lee et al., 2010). We wanted to explore the possibility that KCs could also present self-antigens in this sterile, non-infectious model. Using F4/80\(^+\) staining during intravital imaging, we observed that KCs were evenly distributed throughout the liver all the way to the necrotic lesion at 8 hours (Figure 4-5A). Clodronate liposome (CLL) treatment (Lee et al., 2010) was used to deplete KCs, but no reduction in the arrest of iNKT cells was observed at 8 hours after CLL treatment (Figure 4-5B).

Liver sinusoidal endothelial cells (LSECs) have been described to express high levels of CD1d on their cell surface (Geissmann et al., 2005). To test if LSECs were involved in presenting ligands to arrest iNKT cells via CD1d, LSEC-CD1d knockout mice were generated by breeding mice that carry a floxed allele of the CD1d gene to mice containing an inducible Cre recombinase under the control of VE-cadherin, a vascular endothelial cell promoter (see Figure 2-1A). Induction of Cre with tamoxifen decreased CD1d expression on endothelial cells (Figure 2-1B). However, no significant reduction in iNKT cell arrest was noted at 8 hours after injury in LSEC-CD1d knockout mice (Figure 4-5B). Noteworthy, iNKT cells in LSEC-CD1d knockout mice arrested adjacent (<5 µm) to KCs (Figure 4-5C (red arrows)). When LSEC-CD1d knockout mice were treated with CLL and imaged at 8 hours after injury, a significant decrease in arrested iNKT cells was noted (Figure 4-5B). However, depletion of CD1d on both KCs and endothelium
Figure 4-5. Cells that contribute to iNKT cell arrest and activation after sterile injury.

(A) Representative stitched image of injured site (red) with Kupffer cells (magenta) and sinusoids (blue) at 8 hours, scale is 50 μm. (B) Quantification of stationary iNKT cells within 100 μm of injury without treatment (black bars), clodronate treated mice (white bars), endothelial cells without CD1d (grey bars) and VECadCreCD1d(KO) mice with clodronate treatment (diagonal patterned bars) at 8 hours, n ≥ 3 mice, P** < 0.01 by t test. (C) Representative still image of iNKT cells arrested next to Kupffer cells (red arrows), scale is 100 μm.
was still not sufficient to completely ablate iNKT cells arrest, suggesting other CD1d presenting cells may also play a role in presenting self-antigens to the iNKT cells.
Chapter Five: iNKT cells modulate healing of the hepatic sterile injury site during the late infiltration phase

5.1 Regulated healing of the injured site by iNKT cells

Given that iNKT cells infiltrate into the injured site 3 - 7 days after the initial injury, during which repair of the wound occurs, we hypothesized that these iNKT cells were responsible for regulating the local wound healing process. To understand the functional effects of iNKT cells on repair of the wound, we compared wildtype BALB/c mice and CD1d<sup>−/−</sup> mice. No changes in the size of the injury were observed between BALB/c or CD1d<sup>−/−</sup> mice at 4 hours or 3 days after the injury (see Figure 5-1B). Instead, healing of the injury occurred at 7 days as we observed a reduction in the size of the injury by 70% in BALB/c mice (Figure 5-1A and B). Healing transpired concurrently with a restoration of sinusoids from the outer perimeter of the injury inwards towards the center (Figure 5-1A). In comparison, healing (size of the injury) was significantly delayed in CD1d<sup>−/−</sup> mice at 7 days (Figure 5-1B).

Two major mechanisms that contribute to the reduction in the size of an injury are (1) contraction to pull edges of the wound together (Racine-Samson et al., 1997; Van De Water et al., 2013) and (2) the local proliferation of cells at the site of injury to restore tissue architecture and function (Miyaoka and Miyajima, 2013). We first examined if wound contraction might influence injury size in our model. Contractile action, mediated by myofibroblasts, compacts the wound, influences cell size near the injury and promotes wound closure (Van De Water et al., 2013). However, hepatocyte cell size near the injury or further away (~300μm) (Figure 5-2A, insets) were not significantly different (Figure 5-2B), suggesting that contraction did not play a significant role in reducing the size of the injury in our model. We then considered if there were differences in the number of liver parenchymal cells by enumerating the number of hepatocytes
Figure 5-1. iNKT cells are important for healing of the injury site

(A) Representative still image of wound size (no sinusoid stain inside injury) at 4 hours and 7 days after injury, sinusoids stained with PECAM-1 (red), scale is 100 μm. (B) Quantification of size of injury over time between wildtype BALB/c and CD1d<sup>-/-</sup> mice, n ≥ 3 mice, P* < 0.05, P** < 0.01 by t test.
Figure 5-2. iNKT cells regulate hepatocyte cell density which is important for wound healing

(A) Representative image demonstrating how the analysis of the number of hepatocytes next to the injury at 7 days after injury was performed, scale is 50 μm, inset: size of hepatocytes next to the injury versus away (> 300 μm), scale is 10 μm. (B) Quantification of length of hepatocytes at the injury versus further away (> 300μm), n ≥ 3 mice. (C) Quantification of hepatocytes cell density between wildtype BALB/c and CD1d−/− mice at 7 days after injury, n ≥ 3 mice, P* < 0.05 by t test.
(between yellow and red dotted lines) between BALB/c and CD1d<sup>−/−</sup> mice (Figure 5-2A). A lower density of hepatocytes surrounding the injury in CD1d<sup>−/−</sup> mice compared to BALB/c mice was observed (Figure 5-2C).

Hepatocyte mitosis following hepatic injury is necessary to restore normal liver architecture and function; dividing hepatocytes closest to the area of necrosis replace dead cells (Bajt et al., 2003; Miyaoka and Miyajima, 2013). Bromodeoxyuridine (BrdU) was used to label proliferating cells <i>in vivo</i> prior to harvesting frozen sections of injured hepatic tissue for immunofluorescence staining. At 72 hours, antibody staining for BrdU<sup>+</sup> cells revealed many more proliferating cells near the edge of the injured site where iNKT cells localize as compared to CD1d<sup>−/−</sup> mice which lacked iNKT cells (Figure 5-3A and B). The number of proliferating cells has decreased significantly at 7 days for both BALB/c and CD1d<sup>−/−</sup> mice and no significant difference in BrdU<sup>+</sup> cells were now observed between these two strains of mice at 7 days (Figure 5-3B).
Figure 5-3. Proliferating cells help repair of the wound during the late phase

(A) Representative immunofluorescence staining of BrdU⁺ cells in between wildtype BALB/c and CD1d⁻/- mice, scale is 200 μm (4X) and 40 μm (20X). (B) Quantification of BrdU⁺ cells between wildtype BALB/c and CD1d⁻/- mice at 3 and 7 days after injury, n ≥ 3 mice, P** < 0.01, P### < 0.001 by t test.
5.2 Localized switch from inflammation to restitution at the site of sterile injury

5.2.1 Neutrophil infiltrates were not altered in CD1d<sup>-/-</sup> mice

Preliminary results that were not included in this thesis demonstrated that the kinetics of the initial neutrophil response during sterile injury peak and decrease during the intermediate retention phase of iNKT cells (Wang and Kubes, unpublished observations). These recruited neutrophils that were inside the injury were necessary for dismantling collapsed sinusoids and removing cellular debris leading to appropriate healing of the wound. To examine if iNKT cells might affect the kinetics and magnitude of the neutrophil response and therefore wound healing in the later phase, we chose to evaluate whether infiltration of neutrophils differed in wildtype BALB/c mice versus CD1d<sup>-/-</sup> mice during the intermediate phase. No differences in neutrophil numbers were observed inside the injury or within 100 μm in BALB/c mice or CD1d<sup>-/-</sup> mice at both 4 and 24 hours (Figure 5-4A and B) suggesting that regulation of wound healing by iNKT cells did not occur by perturbing the recruited neutrophil population.

5.2.2 Monocyte switch from inflammatory to reparative phenotype

The accumulation, activation and production of IL-4 by iNKT cells during the retention (2<sup>nd</sup> phase) coincided with the cytokine-driven switch of recruited inflammatory CCR2<sup>hi</sup>CX<sub>3</sub>CR1<sup>low</sup> monocytes to reparative CCR2<sup>low</sup>CX<sub>3</sub>CR1<sup>hi</sup> monocytes in the proximity of the lesion (Dal-Secco et al., 2015). We showed previously that this switch in monocyte phenotype was critical for optimal repair after injury but how this happened was not elucidated. We wanted to investigate if iNKT cells would play a role in modulating the local reprogramming of monocytes after injury and affect debris clearance. We bred the CD1d<sup>-/-</sup> mice to CCR2<sup>RFP/+</sup>CX<sub>3</sub>CR1<sup>GFP/+</sup> mice and compared these with wildtype CCR2<sup>RFP/+</sup>CX<sub>3</sub>CR1<sup>GFP/+</sup> mice after sterile injury. Using the rainbow hue analysis to delineate the spectrum of monocytes
Figure 5-4. Recruitment of neutrophils were not affected in CD1d<sup>-/-</sup> mice after sterile injury

(A) Number of neutrophils inside the injury or within 100 μm of the injury in BALB/c (black bars) mice or CD1d<sup>-/-</sup> (white bars) mice at 4 hours after injury, n = 3. (B) Number of neutrophils inside the injury or within 100 μm of the injury in BALB/c (black bars) mice or CD1d<sup>-/-</sup> (white bars) mice at 24 hours after injury, n = 3.
detected at the lesion, we were able to compare the continuum of monocyte phenotypes (Dal-Secco et al., 2015). We observed that there were substantially more CCR2\textsuperscript{hi}CX\textsuperscript{3}CR1\textsuperscript{low} (red) cells compared to the spectrum of orange and yellow cells in the absence of NKT cells at 24 hours (Figure 5-5A and 5-5B) with a resultant increase in cellular debris (Figure 5-5C).

Normal collagen deposition in the injury area (compared to healthy tissue) was observed in wildtype mice but CD1\textsuperscript{d}\textsuperscript{+} mice had excessive collagen deposition that qualitatively did not appear to be in a honeycomb pattern that was seen in the uninjured tissue (Figure 5-5D). This aberrant excessive deposition of collagen is consistent with fibrosis and/or scar formation (Figure 5-5E).
Figure 5-5. iNKT cells regulate transition of monocytes after sterile injury

(A) Representative image of inflammatory (red-CCR2^{hi}CX3Cr1^{low}) and reparative monocytes (green-CCR2^{low}CX3Cr1^{hi}) in C57B6 and B6.CD1d^{-/-}CCR2^{rfp/wt}CX3Cr1^{gfp/wt} mice 24 h after injury, scale is 100 μm. (B) Analysis of monocytic hues after sterile injury in C57B6 and B6.CD1d^{-/-}CCR2^{rfp/wt}CX3Cr1^{gfp/wt} mice 24 h after injury, n ≥ 3 mice, P* < 0.05, P^1 < 0.05, P^# < 0.01 by t test. (C) Quantification in the amount of dead cells inside the injury between wildtype and CD1d^{-/-} mice 72 h after injury, n = 3, P* < 0.05 by t test. (D) Representative images of 2^{nd} harmonic signal (collagen) from multiphoton imaging between wildtype BALB/c versus CD1d^{-/-} mice 72 hr after injury, top panel; green: autofluorescence signal, magenta: collagen, bottom panel; same image but with removal of green signal, magenta: collagen. (E) Quantification of the intensity of 2^{nd} harmonic signal (collagen) obtained from multiphoton microscopy between wildtype and CD1d^{-/-} mice inside the injury versus outside the injury (intensity ratio), 72 h after injury, n = 3, P* < 0.05 by t test.
5.3 Preventing iNKT cell activation or neutralizing IL-4 results in poorer healing

We have reported above that in an absence of NKT cells, wound healing in the liver was delayed with aberrant collagen deposition after sterile injury. Since CD1d<sup>−/−</sup> mice lack NKT cells congenitally, impaired wound healing could be due to a compensatory developmental response in immunity due to a lack of NKT cells for these mice. To further characterize the functional role of iNKT cells in regulating healing of the injury, we decided to target the activation of iNKT cells by inhibiting activation through systemic administration of anti-CD1d antibodies or anti-IL-12 and anti-IL-18 antibodies. Preventing iNKT cell activation through anti-CD1d or anti-IL-12 and anti-IL-18 or a unison of three antibodies significantly delayed wound healing to a level comparable to CD1d<sup>−/−</sup> mice (Figure 5-6A). Hepatocyte cell density around the injury was also decreased using these interventions (Figure 5-6B).

iNKT cells are important immunoregulatory T cells where the category of cytokine produced (T<sub>H1</sub>-type or T<sub>H2</sub>-type) after iNKT cell activation is important in determining the functional outcome during inflammation. For example, hepatic iNKT cells release IFN-γ (T<sub>H1</sub>-type) which limited the spread of *B. burgdorferi* during systemic infection while these same cells released IL-10 leading to immunosuppression after stroke (T<sub>H2</sub>-type) (Lee et al., 2010; Wong et al., 2011). Beyond a role in modulating a phenotypic switch in monocytes, we hypothesize that iNKT cells, through IL-4 secretion, might contribute directly to repair itself. Indeed, murine hepatocytes have been demonstrated to express the IL-4 receptor and proliferate in response to IL-4 during different disease states *in vivo* (Goh et al., 2013). In our sterile injury model, IL-4 was neutralized with antibodies to determine if the IL-4 produced by iNKT cells affects wound healing.
Figure 5-6. Preventing iNKT cell activation results in poor healing similar to CD1d⁻/⁻ mice

(A) Quantification of injury size after antibody treatments given 30 - 60 mins before injury or in CD1d⁻/⁻ mice; tissues were imaged 7 days post-injury, n ≥ 3 mice, P* < 0.05, P*** < 0.001, P**** < 0.0001 by one-way ANOVA, P# < 0.05 by t test. (B) Quantification of hepatocyte cell density after antibody treatments 30 - 60 mins before injury or in CD1d⁻/⁻ mice; tissues were imaged 7 days post-injury, n ≥ 3 mice, P* < 0.05 by t test.
Wound size was significantly increased and was comparable to CD1d<sup>−/−</sup> mice with anti-IL-4 antibody treatment (Figure 5-7A). In addition, a decrease in hepatocyte cell density surrounding the injury was also observed when IL-4 was neutralized with antibodies (Figure 5-7B).
Figure 5-7. Neutralizing IL-4 significantly impairs healing to level observed in CD1d$^{-/-}$ mice

(A) Quantification of injury size in wildtype BALB/c, CD1d$^{-/-}$ mice or anti-IL-4 treatment given 30 - 60 mins before injury and tissues were imaged 7 days after injury, n = 3 mice, P** < 0.01, P*** < 0.001 by t test. (B) Quantification of hepatocyte cell density in wildtype BALB/c, CD1d$^{-/-}$ mice or anti-IL-4 treatment given 30 - 60 mins before injury and tissues were imaged 7 days after injury, n ≥ 3 mice, P* < 0.05 by t test.
Chapter Six: General Discussion

6.1 General discussion

6.1.1 Approach and review of hypothesis

Hepatic iNKT cells have important roles in hepatic inflammation by determining the outcome in various liver diseases (Bandyopadhyay et al., 2016; Swain, 2010). However, very little was known about the molecular mechanisms to which hepatic iNKT cells employs to fulfil this role. As such, we took an alternative but focused approach that allowed us to clearly elucidate the mechanisms by which iNKT cells regulate healing in situ. We employed a model of focal sterile inflammation in the liver that we have previously established, where many aspects of the early inflammatory response including the recruitment of neutrophils, monocytes, platelets and peritoneal macrophages have been uncovered (Dal-Secco et al., 2015; McDonald et al., 2010; Slaba et al., 2015; Wang and Kubes, 2016). Using this model, we also showed that inflammatory monocytes surround the injury and were educated by the local environment to switch to repair monocytes (Dal-Secco et al., 2015). However, what cells were involved in mediating this switch and the sources of anti-inflammatory cytokines were never clearly defined.

In our approach, we first reasoned that hepatic iNKT cells may be the missing link here and hypothesized that self-antigens released during hepatic sterile injury would be critical in informing (i.e activating) iNKT cells of the status of the local microenvironment. Second, activation of iNKT cells should also be tightly regulated (either spatially or temporally) as uncontrolled release of cytokines into the blood stream is potentially harmful. Third, iNKT cells would function as a chief executive/decision-maker where upon sensing the injury they would determine whether to continue/perpetuate inflammation or produce anti-inflammatory cytokines to initiate healing. Finally, the cytokines released by iNKT cells could either modulate other
downstream innate immune cells to regulate repair or act directly on parenchymal cells for wound healing. Therefore, we hypothesize that hepatic iNKT cells function as immune orchestrators that modulate the local immune response towards restitution and repair upon sampling the local environment after sterile injury in the liver.

6.1.2 Unique sterile injury model

We chose to use a focal sterile injury model induced by thermal heat to study the response of hepatic iNKT cells after injury. This model was chosen for a number of reasons. First, the focal nature of this injury results in a centralized area of tissue damage. This allows the visualization of the entire temporal and localization response of hepatic iNKT cells without the disrupting influence of multiple injury sites. Further, our sterile injury model allows us to compare the behavior of iNKT cells in the periphery versus the injury site. In fact, we have observed iNKT cells that were not in the vicinity of the injury continue crawling randomly. In contrast, in conventional models of liver injury such as drug- or toxin-induced liver injuries where the damage is more diffuse, the hepatic environment as a whole is altered. In these situations, precise dissection of the mechanisms regulating iNKT cell behavior would be difficult. Next, we have previously established this model as a robust self-resolving injury model where we are able to follow the fate of various innate immune cells from an inflammatory stage to the resolution of inflammation and wound healing. Multiple cell types including neutrophils, monocytes and peritoneal macrophages were observed to respond to the site of sterile injury in the liver and contribute functionally to the clearance of cellular debris as well as normal healing of the lesion (Dal-Secco et al., 2015; McDonald et al., 2010; Slaba et al., 2015; Wang and Kubes, 2016). This is in comparison to non-healing models of liver injury where resolution is never
achieved and results in a dysregulation of cells. Here, this focal sterile injury model was successfully employed to study the phenotypic behavior of individual iNKT cells and to assess their functional role in the resolution of inflammation and/or contribution to tissue repair.

6.1.3 Distinctive iNKT cell behavior response to sterile injury

Using spinning-disc confocal intravital microscopy to track hepatic iNKT cells after a focal sterile injury provided critical temporal and spatial information regarding their behavior and functional role (Figure 6-1). First and foremost, iNKT cell recruitment was fundamentally different, from our previous reports of neutrophils, peritoneal macrophages and some inflammatory monocytes which infiltrated the afflicted tissue immediately to initiate removal of dead tissue (Dal-Secco et al., 2015; McDonald et al., 2010; Wang and Kubes, 2016). By contrast, iNKT cells were overtly precluded from entering the injured site (Figure 6-1A). While neutrophils have potent proteases including neutrophil elastase and matrix metalloproteinases to enter the injury site across collapsed vessels, iNKT cells lack these molecules to burrow into the injured tissue. Although we identified numerous chemotactic alarmins for neutrophil infiltration into the injured site, even bathing the injury with the potent iNKT cell chemoattractant CXCL9, did not coax iNKT cells into the injury site. In fact, the iNKT cells still made U-turns and left the injury site, suggesting that perhaps in addition to a barrier, something might be overtly repelling the iNKT cells.

Next, iNKT cells visibly arrested and were retained at the border of the injury for at least 48 hours by antigen presentation via CD1d and subsequently by cytokines where they appeared
(A) Initial repulsion phase

Liver sinusoid

Murine Abdominal Cavity

Liver lobes

Injury

iNKT cells

180° Uturn

iNKT

(B) Intermediate retention phase

Liver sinusoid

CD1d presentation

Sterile injury:
IL-12 and IL-18

Mφ

Injury

iNKT cells arrest

Intermediate to late phase

iNKT cell Infiltration
- Increased cellular proliferation
- Increased hepatocyte density
- Decreased wound injury size
- Clearance of debris

Proinflammatory monocytes to anti-inflammatory monocytes

Activation → IL-4 Production

iNKT cells accumulate around injury
Figure 6-1. Three temporal phases (repulsion, retention and infiltration) of iNKT cell response to sterile injury in the liver

After sterile injury in the liver, hepatic iNKT cells exhibit three different kinetic phases during their response. (A) In the first phase, iNKT cells approach the injured site and subsequently made a 180° U-turn before leaving the area. This was due to a physical barrier at the injury site which prevent the entry of iNKT cells. (B) Next, in the second phase, iNKT cells arrested close to the injury site due to presentation of self-antigens via CD1d and cytokine-driven signals which were mediated by liver sinusoidal endothelial cells and Kupffer cells in the area. This resulted in the accumulation of iNKT cells surrounding the injury and also activation of iNKT cells. iNKT cells produced IL-4 after activation. In the intermediate phase and late infiltration phase, iNKT cells continued to produce IL-4 which had a number of functional effects including (i) proliferation of hepatocytes, (ii) a switch in monocyte phenotype from pro-inflammatory to anti-inflammatory, (iii) improved clearance of cellular debris. These cumulative effects led to a regulated healing of the wound. EC: endothelial cells, mφ: macrophages (Kupffer cells), iNKT cells: invariant natural killer T cells.
to contribute to 1) monocyte conversion from inflammatory to repair phenotype and 2) the proliferation of hepatocytes (Figure 6-1B). LSECs, KCs and other CD1d expressing cells were central players in this arrest of iNKT cells. Only at 48 - 72 hours did the iNKT cells enter the injury site. Blockade of the CXCR3 chemokine receptor did not affect the accumulation of iNKT cells surrounding the injury. This was in contrast to B. burgdorferi infection where iNKT cells localized around Kupffer cells due to chemokines acting on CXCR3 (Lee et al., 2010). Chemokines were also observed to not have any role in recruiting iNKT cells to regenerating livers after partial hepatectomy (DeAngelis et al., 2012).

In our model of sterile injury, the iNKT cells strategically arrested in the midst of a ring of monocytes and proliferating hepatocytes. The lack of iNKT cells resulted in delayed conversion of the monocytes, decreased cellular proliferation with a concurrent increase in collagen deposition and decreased wound repair. It is tempting to suggest that the detainment of infiltration of iNKT cells allows debridement of tissue by other immune cells prior to the switch to critical repair.

6.1.4 Spatial and temporal activation of iNKT cells during sterile injury

6.1.4.1 Endogenous glycolipid ligands

The identity of natural endogenous ligands for iNKT cells remains elusive although recent data has demonstrated that small quantities of α-glycosylceramides could be constitutively produced in mammalian cells as a result of catabolic enzymes and these were proposed to be the main endogenous iNKT cell ligands (Kain et al., 2014). Functionally, self-antigens are important in mediating the positive selection and final maturation of iNKT cells in the thymus (Brennan et al., 2013; Facciotti et al., 2012). Whether self-antigens are important
during activation of peripheral iNKT cells remained unknown. For example, some studies report that CD1d expression is not required for the proliferation or maintenance of peripheral iNKT cells (Matsuda et al 2002, Nat Immunol, McNab et al 2005, JI, Wencker et al 2013 Nat Immunol). Other studies which examined the role of iNKT cells during infection propose that self-antigens play only minor roles during peripheral iNKT cell responses (Brennan et al., 2013). Instead, cytokine-driven signals were dominant in activating iNKT cells (Brigl et al., 2011). Further, although self-antigens can be presented on CD1d, their effect on iNKT cell crawling phenotype and behavior is unknown.

Here, we show that in a healthy repair model, they play a critical role in arresting iNKT cells and strategically localizing them at the site where hepatocytes are proliferating and monocytes switch phenotypes. For the first time, our data reveal that endogenous antigens play a significant role beyond iNKT cell development to direct and localize these cells at sites of injury and that presentation of self-antigens via CD1d was functionally important in vivo to guide healing and repair.

6.1.4.2 Innate cytokine-driven signals

In contrast to the role of endogenous lipid antigens in activating peripheral iNKT cells, activation of iNKT cells by non-TCR signals or cytokine driven activation have been proposed to be critical for the physiological functions of iNKT cells (Brennan et al., 2013). In fact, using diverse bacteria that express or did not express iNKT cell CD1d-recognizable antigen, a recent study revealed that iNKT cell activation critically depended on cytokine signals instead of cognate antigen (Brigl et al., 2011). As a result, the authors propose a model where virtually all infectious agents predominantly activate iNKT cells through cytokines produced by APCs after
TLR-mediated activation. However, in inflammatory diseases which occur independently of exogenous microbial bacterial antigens, the contribution of self-antigens versus cytokine signals is less clear.

From prior in vivo imaging studies, iNKT cells have been shown to arrest in blood vessels either by the presentation of CD1d-dependent bacterial antigen or exogenously applied IL-12 and IL-18 (Lee et al., 2010; Velazquez et al., 2008). In this sterile model of injury and repair, we observed that CD1d antigen presentation was the first activating molecule as blockade of CD1d with antibodies resulted in iNKT cells resuming a crawling phenotype. Blocking IL-12 and IL-18 at this time point had no effect on arrest or retention of iNKT cells around the injury. However, we observed that the CD1d antigen presentation lasted only a few hours being closely followed by cytokine activation 16 hours later as blocking CD1d had no effect at the 24 hour time point. This was in contrast to the neutralization of IL-12 and IL-18 which led to reduced retention of hepatic iNKT cells. Therefore, the sequence of cell activation was initially through CD1d antigen presentation followed by cytokine driven signaling. While one study has shown that IL-12 might induce iNKT cell hyporesponsiveness to bacteria (Choi et al., 2008), another revealed that IL-12 could enhance responsiveness in iNKT cells in response to weak antigen presentation via CD1d through histone modification of relevant genetic loci (Wang et al., 2012). Our in vivo model suggests CD1d may prime cells to respond more potently to cytokines but also that cytokines are necessary for the continued activation and localization of iNKT cells.

6.1.4.3 Multiple cell types contribute to iNKT cell activation

In the liver, both immune cells and non-immune cells (e.g. hepatocytes, liver sinusoidal endothelial cells, Ito cells) have been described to express CD1d molecules suggesting that these
cells might be capable to present antigen to iNKT cells (Geissmann et al., 2005; Winau et al., 2007). In vivo, Kupffer cells have been shown to present antigen to iNKT cells resulting in formation of clusters of iNKT cells as well as their arrest during infection (Lee et al., 2010). When CD1d-deficient mice were reconstituted with CD1d-sufficient Ito cells in the blood, antigen presentation to iNKT cells was also noted (Winau et al., 2007). In this thesis, neither clodronate liposome treatment nor CD1d-endothelial cell knockdown alone achieved statistical significance in iNKT cell arrest but when these two treatments were combined, significant reductions in the arrest of iNKT cells were observed. Having said that, since clodronate liposome treatment also depletes dendritic cells and possibly Ito cells (Lee and Kubes, unpublished data), it seems that multiple APCs exist near the site of sterile injury and all of these contribute to the activation of iNKT cells. These collective mechanisms operating in vivo appear to be possible redundant pathways allowing iNKT cell activation during hepatic sterile injury.

6.1.5 Hepatic iNKT cells as directors of inflammation and healing

Activated iNKT cells can interact with a wide assortment of cells from the innate and adaptive immune system. Together with the iNKT cells’ ability to respond to a variety of inflammatory stimuli resulting in production of modulatory cytokines, iNKT cells are uniquely positioned as early directors of inflammation where they can subsequently mediate its outcome (restitution versus poor healing or continued inflammation). For example, we previously reported that inflammatory CCR2\textsuperscript{hi}CX3CR1\textsuperscript{low} monocytes transitioned, in situ, into reparative CCR2\textsuperscript{low}CX3CR1\textsuperscript{hi} monocytes at the site of injury (Dal-Secco et al., 2015), but the molecular mechanism and cell type that mediated this phenotypic switch remained unknown. Herein, we demonstrate that 1) iNKT cells localize specifically at the site where these monocytes were
reported to switch their phenotype, 2) the iNKT cells produce an abundance of IL-4, which we previously reported was critical for the monocyte transition and 3) in the absence of iNKT cells, there is a delayed transition of monocyte phenotypes. Further, iNKT cells had a direct effect on hepatocyte proliferation as lack of iNKT cells resulted in delayed repair and revascularization of the injury. These observations were consistent with a previous study where production of IL-4 (but not IFN-γ) by iNKT cells after two-thirds partial hepatectomy resulted in improved regeneration of the liver (DeAngelis et al., 2012).

Specific cytokine production correlated with iNKT cell function has been observed in clinically-relevant mouse models of hepatic disease. For example, in partial hepatectomy, iNKT cells were observed to be beneficial for re-growth of the liver due to production of iNKT cell mediated IL-4 (DeAngelis et al., 2012). If iNKT cells were activated with αGalCer, which also causes iNKT cell arrest, iNKT cells instead produce IFN-γ which inhibited liver regeneration (Yin et al., 2014). Similar findings were also observed in CCl₄-induced liver injury (Park et al., 2009) and αGalCer-induced liver injury (Biburger and Tieg, 2005). Nevertheless, the mechanisms by which iNKT cells respond to and affect liver injury remain incompletely understood. It is possible that in these models studied previously, iNKT cells were not appropriately localized to modulate optimal repair and/or were potentiated to produce the wrong cytokines when activated exogenously. This suggests that interfering with hepatic iNKT cell functionality has to be approached with caution and raises clinical implications as αGalCer is currently in trials for treatment in various human diseases. With the identification of a critical role for iNKT cells in healthy repair in our sterile injury model, completely eliminating their contribution in non-healing injury or inappropriately activating iNKT cells is unlikely to benefit the patient.
6.2 Clinical relevance and translation aspects

Our focal sterile injury model in the liver was developed with the intent to dissect the molecular mechanisms of the innate immune response to hepatic non-infectious injury. Nevertheless, electrocautery (i.e. treatment of tissue with a high temperature probe)-based instruments and techniques are commonly used during liver resections to stop bleeding and to remove larger metastasis in hepatocellular carcinoma (Delis and Dervenis, 2008; Morise et al., 2014; Romano et al., 2012). Although efficient at controlling bleeding and removing unwanted/harmful tissues, cauterization results in significant tissue destruction and a potent inflammatory response (Dal-Secco et al., 2015; McDonald et al., 2010; Slaba et al., 2015; Wang and Kubes, 2016). As liver resections are performed to address underlying liver disease, cauterization and the resultant tissue damage may contribute to further liver complications (Guo et al., 2014; Wrighton et al., 2012). In this thesis, following sterile injury in the liver, hepatic iNKT cells are recruited to the injury site where they play an important role in regulating healing of the injury. Instead of prescribing general anti-inflammatory drugs to suppress the immune system following liver surgery (Soleimanpour et al., 2016), immunomodulation by activating hepatic iNKT cells using specific glycolipid ligands that bias hepatic iNKT cells towards a Th2 response might be a better approach to regulate the optimal healing of the liver. In the sections below, future experiments are described to identify knowledge gaps in iNKT cell biology that are needed for the development of iNKT cells as a cell-based therapy product in the clinic.

6.3 Future directions

6.3.1 Invariant Natural Killer T cell variety and subtypes

Sub-divisions of iNKT cells into iNKT1 (producing mainly IFN-γ), iNKT2 (producing IL-4) and iNKT17 (producing IL-17), have been quickly gaining acceptance (Constantinides and
Bendelac, 2013; Lee et al., 2013; Lee et al., 2015). Nevertheless, substantial plasticity exists between these sub-types as both iNKT1 and iNKT17 have been described to produce IL-4 (in addition to the iNKT2 subset) suggesting that the subsets are not a uniform population of cells (Georgiev et al., 2016). Further, the iNKT1/2/17-concept has yet to integrate how recognition of different antigens by iNKT cells result in a production of different polarizing cytokines; for example the αGalCer analogue, OCH, results in mainly IL-4 production in comparison to αGalCer where iNKT cells predominantly produces IFN-γ (Anantha et al., 2014; DeAngelis et al., 2012). In our sterile injury model, iNKT cells were observed to only produce IL-4 but not IFN-γ. To validate the iNKT1/2/17-concept, further research is needed to understand if different sub-types of hepatic iNKT cells are recruited after sterile injury in the liver and how they might affect wound healing. It is not understood whether specific subtype(s) of iNKT cells have been recruited after sterile injury or if a recruited subtype regulates cytokine secretion through up-regulation of key transcription factors (for example, PLZF<sub>lo</sub>T-bet<sup>−</sup>(iNKT1) to PLZF<sub>hi</sub>T-bet<sup>−</sup>(iNKT2)). Further, it is also unknown if the type of self-antigen presented in our sterile injury affects the IL-4 production observed in our model.

### 6.3.2 Identifying endogenous ligands that activate iNKT cells after sterile injury

Although the endogenous antigenic ligands that contribute to iNKT cell activation remains unclear, improvements in biochemical technologies have moved this field several steps closer and have provided several promising candidate antigens (Brennan et al., 2014; Kain et al., 2014). For example, new techniques such as multiple reaction monitoring mass spectrometry allows increased specificity and sensitivity of detection from complex biological samples (Kain et al., 2014). Use of these techniques would be ideal to identify the types of endogenous ligands
that activate iNKT cells following sterile injury. Our focal sterile injury model provides an excellent opportunity here as we have already dissected the kinetics and mechanisms of iNKT cell activation during sterile inflammation. Fundamentally, this approach would provide a platform for understanding iNKT cell function. Subsequently, it would open new ways for understanding and predicting iNKT cell activity in clinical settings as well as an opportunity for immunotherapeutic modulation of iNKT cells by controlling the amount/type of ligand for treatment during infections, autoimmunity or chronic inflammation settings.

6.3.3 Behavior and functional role of hepatic iNKT cells in clinically relevant models of hepatic disease

Our sterile injury model, which was intended to be a basic self-resolving inflammatory model to understand mechanisms in which iNKT cells contribute to wound repair, helped us to directly visualize the sequence of events and interplay between iNKT cells and other immune cells. As an obvious extension to our work, systematically analyzing the behavior, localization and functional role of iNKT cells in liver inflammation models that are more pertinent to the clinic would provide critical information on how these cells might be beneficial or not and if they could be manipulated during clinical settings; for example, in drug-induced liver injuries.

Drug-induced liver injuries are a major health problem that challenges health care professionals. Overdose by acetaminophen itself accounts for nearly 40% of acute liver failures (Holt and Ju, 2006). Using mouse models of acetaminophen overdose, iNKT cells were observed to play a beneficial role after overdose as lack of iNKT cells resulted in increased liver damage as observed by serum ALT levels (Martin-Murphy et al., 2013). However, the kinetics of the iNKT cell response and how iNKT cells function as key participants in this model was not
elucidated. Our own preliminary experiments using a similar acetaminophen overdose model echoes these observations where iNKT cells were protective (see appendix A). Innate immune cells are main sources of anti-inflammatory cytokines which have been shown to play a hepatoprotective role (Holt and Ju, 2006). Perhaps through strategic localization around areas of liver damage and release of anti-inflammatory cytokines and growth factors at sites of tissue injury, iNKT cells regulate resolution and restitution after liver damage. Embarking on studies using these clinical models would be of relevance for human acute liver diseases and would be useful when planning human trials involving treatments that activate iNKT cells.

6.3.4 Behavior and functional role of iNKT cells in chronic inflammation

Acute inflammation occurs immediately after sterile injury and is necessary to initiate the healing process. Yet when the inflammatory process becomes dysregulated or overactive (as in the cases of autoinflammatory diseases or fatty liver disease), the resultant inflammation develops into a chronic pathological form (Murakami and Hirano, 2012). To date, many murine studies of chronic inflammation in the liver have focused on comparing sham-treated animals to animals with chronic liver disease. However, to have a comprehensive understanding of disease pathogenesis, it would be more accurate to compare models of self-resolving physiological sterile inflammation against models of pathological chronic inflammation. The identification of vital differences between these two inflammatory states will reveal new therapeutic opportunities for the treatment of chronic liver diseases, especially in patients with end-stage liver disease where few options exist outside of liver transplantation.

Chronic inflammation in the liver has been characterized primarily through murine models that mimic non-alcoholic fatty liver disease or primary biliary cholangitis where iNKT
cells have been described to play significant roles (Lau et al., 2017; Schrumpf et al., 2017; Van Kaer et al., 2013). One well-established model that is used to study chronic inflammation in the liver is the chronic feeding of 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) which results in the formation of hepatocellular inclusion bodies that are associated with steatohepatitis, metabolic liver diseases and chronic cholestatic liver diseases (Fickert et al., 2007). In a DDC model of chronic inflammation in the liver, neutrophils exhibited an altered behavioral phenotype as compared to the neutrophilic response after acute sterile injury (Lee and Kubes, unpublished observations). It would be possible to employ the same techniques described in my thesis to characterize the behavioral phenotype and functional role of iNKT cells in this chronic liver injury for comparison to our focal sterile injury model.

6.4 Conclusion

Exceptional advances in technology have enabled phenomenal discoveries to be made in innate immunity. The observations presented in this thesis contribute significant novel insight into the biology of iNKT cells and their effector functions in the liver. We identified unique iNKT cells behavioral responses to hepatic focal sterile injury that involves an initial repulsive step followed by arrest and retention at sites of injury. In addition, we have discovered that the first phase was due to a physical barrier while the second phase was dependent on self-antigen presentation followed by cytokine-driven signals. In this way, we have also learnt that the arrest signals were temporally- and spatially-regulated as CD1d presentation occurred first before cytokine signaling and that both signals were located closely abutting the injury but not further away. This resulted in iNKT cell activation and production of important Th2 cytokines (IL-4) which regulated hepatocyte proliferation and a switch from inflammation towards restitution. We
also provided evidence that this response was important in wound healing as lack of iNKT cells, preventing iNKT cell activation or blocking IL-4 resulted in poor healing. We are enthusiastic about these observations because we not only demonstrated a beautiful example of how iNKT cells detect a change in the microenvironment, but we also showed how these cells orchestrate the transition from inflammation into healing. As such, completely eliminating their contribution during injury in clinical settings is not likely to benefit patients. Further, this novel mechanism likely plays significant roles in fibrosis and chronic inflammatory conditions and our study would inform many future studies that examine these pathological events.
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APPENDIX A: ANTIBODY-DEPENDENT FRAGMENTATION IS A NEWLY IDENTIFIED MECHANISM OF CELL KILLING IN VIVO.

A.1. PROLOGUE

During the progress of my PhD, we explored the possibility that we might be able to specifically deplete iNKT cells in the liver through antibody treatments instead of relying on iNKT cell deficient mice. The results of those experiments are presented in this appendix section as a prepared manuscript in progress. The contributions of co-authors in this manuscript have been described in the Preface of my thesis.

A.2. ABSTRACT

The prevailing view is that therapeutic antibodies deplete cells through opsonization and subsequent phagocytosis, complement-dependent lysis or antibody-dependent cell-mediated cytotoxicity. We used high resolution in vivo imaging to identify a new antibody-dependent cell death pathway where Kupffer cells ripped large fragments off crawling antibody-coated iNKT cells. This antibody-dependent fragmentation process resulted in lethality and depletion of crawling iNKT cells in the liver sinusoids and lung capillaries. iNKT cell depletion was FcG-receptor dependent and required iNKT cell crawling. Blood, spleen or joint iNKT cells that did not crawl were not depleted. The antibody required high glycosylation for sufficiently strong binding of the iNKT cells to the Fc Receptors on Kupffer cells. Using an acetaminophen overdose model, this approach functionally depleted hepatic iNKT cells and affected the severity of liver injury. This study reveals a new mechanism of antibody-dependent killing in vivo and raises implications for the design of new antibodies for cancer and auto-reactive immune cells.
A.3. INTRODUCTION

Development of antibodies to eliminate target cells has become a hugely successful experimental and therapeutic approach. Despite their growing widespread use, with many antibodies moving into the clinical arena, the mechanism by which these antibodies function is still very poorly understood. It is however predicted that after the antibodies bind a target cell, they induce one of three forms of cell death; 1) antibody-dependent cell-mediated cytotoxicity (ADCC), 2) complement-dependent cytotoxicity (CDC) and 3) antibody-dependent phagocytosis (Alduaij and Illidge, 2011; Scott et al., 2012). In ADCC-mediated cell death, the binding of cytotoxic cells (for example, NK cells) to antibody-opsonized tumor cells result in the release of vesicular contents such as perforin and granzymes which lyses and kills the tumor cells (Teillaud, 2012). Although these antibodies can also activate complement to induce membrane disruption and cell death, this mechanism is not considered to be the dominant mechanism of killing (Alduaij and Illidge, 2011; Weiner, 2007). Opsonization of cells is also thought to induce phagocytosis, an event commonly seen in vitro.

Recent intravital imaging work has shown that the anti-CD20 antibody (rituximab) which targets B cell lymphomas induces a phagocytic mechanism by Kupffer cells lining the liver sinusoids (Montalvao et al., 2013). When opsonized B cells were injected into the mainstream of blood, they flowed towards intravascular Kupffer cells, were ensnared and phagocytosed resulting in cell death. Indeed, the anti-CD20 antibody has been so effective in B cell depletion that it is now regularly employed as a hematological cancer therapeutic and represents a breakthrough in the treatment of B cell malignancies (Chan and Carter, 2010; Maloney, 2012; Weiner et al., 2010). Antibody directed immunotherapy is becoming an extremely promising strategy to target tumor cells in cancer but can also be used to target inappropriately activated
immune cells in autoimmune disease. In addition to anti-CD20-specific antibodies, CD52 specific alemtuzumab, Her2/neu-specific trastuzumab, EGRF-specific cetiuximab and anti-GD-2 antibodies are all under investigation in clinical trials to target cancer or immune cells (Beck et al., 2010; Havrdova et al., 2015; Lavaud and Andre, 2014; Yazdi et al., 2015). Moreover, many new antibodies are now being used to selectively deplete immune cells in vivo. For example, antibodies have been used to successfully deplete B cells (Montalvao et al., 2013), natural killer cells (Cook and Whitmire, 2013; Monnier and Zabel, 2014; Walzer et al., 2007), dendritic cells (Jung et al., 2002), neutrophils (Carr et al., 2011; Daley et al., 2008) and a variety of T cells including CD4+ T cells (Arora et al., 2006; Gavett et al., 1994), CD8+ T cells (Cano et al., 2000; Jones et al., 2002), γδ T cells (Wu et al., 2007), iNKT cells (Scheuplein et al., 2015) and Tregs (Setiady et al., 2010). It is presumed that all these antibodies functionally deplete target cells with the same mechanism, even though many populations of cancer cells and immune cells might not flow through the mainstream of blood but instead transverse or crawl along the luminal surface of blood vessels. There is absolutely no information how a crawling cell might be eradicated. In addition, the size of target cells could limit the ability of the phagocytosing cell to engulf the target cell. Under these conditions, it is conceivable that a sufficiently large cell cannot be targeted and depleted by antibodies.

Immense effort has been made by biopharmaceutical companies to engineer third generation antibodies that can induce higher effector responses. Critical to all these events are Fc receptors. The constant fragment (Fc) of an antibody is necessary for interactions with immune cells and efficacy of the antibody depends upon Fc modifications which occur during production in the host. For example, absence of Fc glycosylation dramatically reduces binding affinity as well as certain modifications to amino acids in the Fc region (Hogarth and Pietersz, 2012;
Leabman et al., 2013; Liu, 2015). Typically, third generation antibodies have increased Fc-linked glycosylation to improve ADCC although other mechanisms of killing may be enhanced concurrently (Beck et al., 2010; Cang et al., 2012). In fact, there are at least 40 therapeutic antibodies approved for human use and it is estimated that 30% of all new drugs will soon be antibodies but their mechanisms of action remain unclear (Beck et al., 2008; Liu, 2015). For example, the CC chemokine receptor 4 (CCR4)-targeting glyco-engineered antibody, mogamulizumab, has enhanced ADCC with no known explanation, but has recently been approved in Japan for use in patients with relapsed and refractory CCR4-positive adult T cell leukemia/lymphoma (Beck and Reichert, 2012). Similarly, although the complex interacting network between CXCR3 and its ligands or cellular expression have not been fully understood, companies have now patented the use of anti-CXCR3 antibodies in the treatment of cancer and autoimmune diseases (Mackay, 2014; Vela et al., 2015). Regardless of mechanism, antibodies against specific cell surface proteins are a commonly used technique to deplete immune cell populations in vivo. Since most interrogations are done in cell culture systems devoid of blood flow and other important in vivo micro-environmental factors, mechanisms of action of these antibodies remain equivocal.

In this study, we show a novel antibody-dependent cellular killing mechanism that is dependent on the specific antibody as well as the distribution of the target protein and the specific behavior of the target cell within select organs. Using spinning-disk confocal microscopy with 3D reconstruction capabilities revealed that immobilized Kupffer cells via FcγRII and FcγRIII grabbed crawling invariant Natural Killer T (iNKT) cells in the presence of an antibody (CXCR3-173) but rather than inducing phagocytosis or any other form of cell death, they repeatedly ripped off the trailing edge of these crawling cells which ultimately led to
depletion of iNKT cells in the liver. This is strikingly different from phagocytosis and we term this antibody-dependent fragmentation. We also show that this can be an extremely selective and efficient approach by demonstrating that depletion of these iNKT cells by antibody resulted in identical effects as a knockout mouse devoid of iNKT cells.
A.4. RESULTS

A.4.1. iNKT cells in liver and lung are specifically depleted by an anti-CXCR3 antibody 
(CXCR3-173)

CXCR3-173 antibody was injected intravenously into CXCR6-GFP mice, in which 60-
80% of GFP+ cells are iNKT cells in the liver (Geissmann et al., 2005; Lee et al., 2010; Wong et
al., 2011). The distribution of iNKT cells up to 4 days after antibody treatment was visualized
and quantified using intravital microscopy. To obtain a comprehensive perspective on the
exposed liver lobe, 70 different fields of view were captured sequentially and these images were
stitched into a single view (Figure A-S1). In the liver of untreated mice, iNKT cells were
distributed evenly throughout the liver and resided exclusively in the sinusoids, as described
previously (Geissmann et al., 2005; Lee et al., 2010; Wong et al., 2011). Two days and four days
after CXCR3-173 treatment, a 10-fold decrease was noted in the number of GFP+ cells in the
liver (Figures A-1A, A-1B and A-S1).

To confirm that intravenous CXCR3-173 treatment caused a specific depletion of the
iNKT cell population, the liver, lung, spleen and blood lymphocytes were isolated for flow
cytometric analysis. The data confirmed that hepatic iNKT cells (CD45+B220−CD3+CD1d
tetramer+) were depleted after four days. Hepatic iNKT cells decreased significantly in the liver
(Figures A-1C and A-1E). In addition, CXCR3-173 treatment also reduced iNKT cells in the
lung after four days (Figures A-1D and A-1F). There was no change in the iNKT cell population
in the spleen or blood after CXCR3-173 treatment (Figures A-S2A and A-S2B) or other tissues
we examined (such as the joints). To exclude the possibility of non-specific iNKT cell depletion
due to the simple injection of antibodies into blood, experiments were repeated with an isotype
control antibody. iNKT cells in the liver and lung were not depleted after injection of the isotype antibody (Figures A-S2C and A-S2D).

Other cell types including T cells, B cells and NK cells have been described to express the CXCR3 receptor (Groom and Luster, 2011; Lacotte et al., 2009) although these cells do not patrol the sinusoids like iNKT cells. This antibody has been previously described not to deplete naïve and activated CD4+ T cells or CD44+ memory T cells even with up to 1 mg of given antibody (Uppaluri et al., 2008). To further examine if other cells are depleted, the number of T cells, B cells and NK cells was examined at 2 days and 4 days after CXCR3-173 treatment using flow cytometry. No change was observed in whole number of CD3+ T cells, B220+ B cells or NK cells in the liver (Figures A-2A – A-2C) and lung (Figures A-2D – A-2F).

**A.4.2. iNKT cell depletion in liver occurs through fragmentation by Kupffer cells**

A previous study has demonstrated that the liver was a major site for B cell depletion after anti-CD20 treatment where Kupffer cells mediate the arrest and engulfment of B cells circulating in liver sinusoids (Montalvao et al., 2013). To investigate if iNKT cell depletion after CXCR3-173 treatment is mediated by Kupffer cells, intravital imaging of the liver of CXCR6-GFP mice was performed. In the liver, iNKT cells crawled in a random pattern under basal conditions, at times changing directions, as previously described (Lee et al., 2010; Wong et al., 2011). Kupffer cells were sessile macrophages that resided in the lumen of sinusoids. iNKT cells crawled over Kupffer cells without a change in velocity or cell behavior (shown later). Strikingly, within minutes of CXCR3-173 antibody injection, iNKT cells began leaving GFP+-fragments behind as they crawled across Kupffer cells (Figures A-3A and A-3D, inset). As the iNKT cell remained motile during this event, the acquisition of fragments occurred primarily
from the trailing end or tail of the crawling iNKT cell. One or multiple fragments were removed from GFP+ iNKT cells as they traversed the immobilized Kupffer cell with or against blood flow (Figures A-3A and A-3D). Approximately 60% of GFP+ cells had fragments acquired from the trailing end of the cell (Figures A-3E). Fragmentation continued to occur until the cell became so small that it stopped crawling. Isotype control antibody treatment caused no fragmentation (Figures A-3B and A-3C) or alterations in iNKT cell behavior (Figures A-S3A – A-S3C). As early as 24 hours after CXCR3-173 antibody treatment, a very noticeable number of iNKT cells were smaller from flow cytometric analysis. Other forms of cell death including phagocytosis and lysis were never observed.

Numerous forms of cell death including apoptosis, necrosis and autophagy have increased annexin V binding as cellular corpses ultimately lose their plasma membrane integrity (Kemal Duru et al., 2000; Leiter et al., 2005; Tait et al., 2014; Vermes et al., 1995; Wallberg et al., 2016). Following fragmentation, annexin V expression was also detected in hepatic iNKT cells (Figures A-3F). We also performed Z-stack image acquisition and 3D reconstruction after CXCR3-173 antibody treatment. The trailing edge of the crawling iNKT cell appeared to be attached to the Kupffer cell, stretching the iNKT cell, prior to the tail becoming a fragment in the Z-stack 3D image (Figures A-3G and inset). Whole-cell phagocytosis of hepatic iNKT cells was not observed after CXCR3-173 treatment.

The Kupffer cells appear to be the main mechanism mediating the depletion of hepatic iNKT cells. Clodronate liposomes (CLL) injected intravenously removed almost all Kupffer cells at 36 hours after CLL injection. Normal levels of iNKT cells were seen in the liver in mice subjected to tandem CLL and CXCR3-173 treatment as opposed to complete depletion by CXCR3-173 antibody alone (Figures A-4A – A-4C). Kupffer cells clearly accounted for all of
the depletion of GFP⁺-iNKT cells as Kupffer cell-depleted mice given CXCR3-173 antibody and mice receiving nothing had identical iNKT cell numbers (Figures A-4C). This observation was also confirmed using flow cytometry where normal levels of iNKT cells in the liver were noted when Kupffer cells were depleted prior to giving the CXCR3-173 antibody (Figures A-4D).

The role of Fcγ receptors were examined in the fragmentation of iNKT cells in vivo. iNKT cells in the liver were not depleted 4 days after CXCR3-173 antibody treatment in Fcγ receptor-deficient mice (Figures A-4D). FcγRII and FcγRIII were blocked with anti-CD16/anti-CD32 antibodies in CXCR6-GFP mice before the CXCR3-173 antibody was injected. iNKT cells crawled over Kupffer cells with no fragment formation in the sinusoids similar to basal untreated conditions (Figures A-4E).

Distribution of CXCR3 molecules on the surface of GFP⁺-iNKT cells were examined in vivo by injecting small amounts of PE-conjugated CXCR3-173 antibody. The majority of iNKT cells had CXCR3 molecules which were concentrated towards the tail end of the cell (Figures A-4F). This non-uniform distribution was only seen on polarized cells as iNKT cells with a round morphology had homogenous distribution of CXCR3 (Figures A-4G).

**A.4.3. Crawling of iNKT cells is critical for the CXCR3-173 induced fragmentation**

The commonality between iNKT cells in lung and liver is that they crawl within blood vessels whereas this does not occur in spleen (Wong and Kubes, 2013) or joints (Lee et al., 2014). Two approaches were used to stop iNKT cell crawling. First, anti-TCRβ antibody was given. Upon injection of anti-TCRβ antibody, 95% of iNKT cells slowed (< 5μm/min) or arrested (Figures A-5A and A-5B). Under these conditions, essentially no fragmentation could be seen even if the arrested iNKT cell was on top of a Kupffer cell suggesting that the fragments
were being ripped from the crawling iNKT cell. Unexpectedly, when an iNKT cell stopped on top of the Kupffer cell, the Kupffer cell did not phagocytose the iNKT cell suggesting that CXCR3-173 antibody induces fragmentation but not complete opsonization. Next, α-galactosylceramide (αGalCer), a lipid ligand that results in the arrest of approximately 50% of iNKT cells was injected (Geissmann et al., 2005; Velazquez et al., 2008). 40% of iNKT cells remained crawling at significant speeds (> 5μm/min) after αGalCer treatment (Figures A-5C). Tandem treatment of mice with αGalCer and CXCR3-173 decreased the number of cell fragmentation events by half (Figures A-5D). Intriguingly, only crawling iNKT cells were subject to fragmentation whereas arrested iNKT cells did not undergo fragmentation even if they were on top of a Kupffer cell (Figures A-5E). With fewer crawling iNKT cells, fewer of these cells contacted Kupffer cells after tandem αGalCer and CXCR3-173 treatment which accounted for the decrease in fragmentation events (Figures A-5D and A-5F).

Since the iNKT cells were crawling after administration of the CXCR3-173 antibody, it seemed unlikely the antibody was causing cell death via complement or ADCC. Nevertheless, Figure A-S4A demonstrated that addition of antibody to iNKT cells in vivo and then harvesting the cells for in vitro cell culture, revealed no direct untoward effects of this antibody to iNKT cells after 3 days. In addition, administration of the antibody to cultures of iNKT cells also caused no increase in apoptosis as determined by annexin V expression (Figures A-S4B and A-S4C).

To examine if other CXCR3 blocking antibodies could deplete iNKT cells, a rabbit polyclonal anti-CXCR3 antibody was injected into mice. Although this polyclonal antibody does not affect iNKT cell crawling under basal conditions, it was previously shown to block the chemotaxis of iNKT cells towards Kupffer cells (producing CXCL9) after Borrelia infection.
(Lee et al., 2014). No significant decrease in iNKT cells were observed 2 days or 4 days after polyclonal anti-CXCR3 treatment suggesting that CXCR3-173 specifically causes fragmentation (Figures A-S5A and A-S5B). All natural antibodies are glycosylated although glycan compositions and structures vary depending on host cells (i.e. mammalian origin). Alterations in glycoforms change binding affinities to Fc receptors (e.g. absence of glycosylation reduces or eliminates binding to Fc receptors) and result in modified immune effector functions (Liu, 2015; Tayi, 2015). To induce fragmentation, a firm interaction between the Fc region of the antibody with the Fc receptor was predicted. To determine whether the glycosylation properties of the CXCR3-173 antibody induced the firm binding, CXCR3-173 antibody was deglycosylated with PNGase F. No fragmentation of iNKT cells was observed when deglycosylated CXCR3-173 was injected in vivo (Figures A-6A and A-6B). Deglycosylation of CXCR3-173 antibody was confirmed by SDS-PAGE analysis where the heavy chain of the deglycosylated antibody migrated faster than the untreated control (Figure A-6C) which is consistent with previous observations (Magnelli et al., 2011; Meuris et al., 2014). To confirm that the deglycosylated CXCR3-173 antibody could still bind to iNKT cells, PE-conjugated CXCR3-173 antibody was subjected to the same deglycosylation treatment before intravital imaging. PE-CXCR3 was observed to localize to the trailing edge of the crawling iNKT cell (Figure A-6D) as previously observed (Figure A-4F). No degradation of the antibody was observed after deglycosylation treatment as visualized by SDS-PAGE.
A.4.4. Validating iNKT cell depletion using CXCR3-173 antibody in liver-specific acetaminophen (APAP) overdose model

To ensure that the depletion of iNKT cells translated to impaired function, we used an APAP model of liver injury. APAP overdose results in severe, fulminant liver injury. Animal models of APAP-induced liver failure closely mirror those in human patients with formation of reactive metabolites, hepatocyte cell death and activation/contribution of immune cells to liver injury (Jaeschke et al., 2011). The role of iNKT cells in APAP overdose have been examined with genetic NKT cell knockout models, which suggests a protective role of iNKT cells (Martin-Murphy et al., 2013). At a dose of 350 mg/kg APAP, only 25% of wild-type mice died whereas all CD1d— mice died (Figure A-7A), which was consistent with previously published data (Martin-Murphy et al., 2013). Similarly, 80% of mice treated with the CXCR3-173 antibodies did not survive. At a lower 300 mg/kg APAP dose, all mice survived till 24 hours with the exception of one CD1d++ mouse (Figure A-7B). This permitted the taking of blood samples. Marked increases in serum alanine aminotransferase (ALT) levels, as a marker of hepatocyte injury, were observed in all three groups of mice (Figure A-7C). However, the CXCR3-173 treated mice had significantly higher ALT levels than the wild-type mice and the CD1d++ mice had significantly higher levels than the CXCR3-173 antibody treated mice at 8 hours and 24 hours. From the ALT data, it was clear that the CD1d++ mice which lacked both type 1 iNKT and type 2 NKT cells, had greater injury than CXCR3-173 antibody treated mice in response to APAP-induced liver injury. As such, B6.Jα18++ mice, which lack only iNKT cells, were also subjected to APAP-induced liver injury and similar levels of serum ALT levels were detected in CXCR3-173 treated C57Bl/6 mice versus B6.Jα18++ mice (Figure A-7D). Further, B6.Jα18++ mice treated with CXCR3-173 antibody also had similar levels of serum ALT levels suggesting
that CXCR3-173 antibody depleted only iNKT cells and not type 2 NKT cells (Figure A-7D). Treatment of wildtype mice with polyclonal anti-CXCR3 antibody did not increase serum ALT levels after acetaminophen treatment (data not shown).
A.5. DISCUSSION

In our study, high resolution imaging in vivo using spinning disk microscopy revealed a pathway of cell death that required an antibody with functionally high binding to Fc, predominantly on Kupffer cells of the liver sinusoids. The latter is important as the process occurred under shear conditions and required motility by the target cell. The sessile Kupffer cell binds to the antibody that opsonized the crawling target cell which caused large fragments to detach from the cell. Fragments were a few microns in diameter but sometimes were as large as the remaining cell. It is becoming clear that immune cells are able to “gnaw” at the plasma membrane of other cells and exchange proteins and/or remove proteins from a cell and internalize them. This phenomenon has been described as immune cell shaving or trogocytosis and can remove up to 80% of a molecule from the surface of a cell (Beum et al., 2011). We do not consider the iNKT cell ripping by Kupffer cells as a form of trogocytic type process due to the size of the particles. Rather, we coin the term antibody-dependent fragmentation to indicate that these were large fragments of the cell that were actively ripped from the target cell. It was not unusual to see a cell crawl across a Kupffer cell and leave behind a large fragment, reverse direction and traverse the Kupffer cell a second time leaving behind a second large fragment and this second fragmentation appeared to deliver a death blow; the iNKT cells stopped crawling and formed structures reminiscent of shistocytes or fragmented red blood cells.

There have been numerous mechanisms by which cell death can be induced including the autonomous cell death pathways: apoptosis and autophagy. Necrosis usually occurs due to destructive perturbations that lead to lysis. These can occur when an immune cell releases toxic factors including oxidants, proteases and pore forming molecules to induce cell death. Of course, the best studied mechanism by which one cell eliminates another cell is through phagocytosis
wherein an immune cell engulfs another cell. Indeed, a recent study described the engulfment of B cells and B cell lymphoma through a two-step process following anti-CD20 antibody administration (Montalvao et al., 2013). Initially, rapid attachment of circulating B cells to Kupffer cells occurred, followed by immediate phagocytosis. In this study, hepatic iNKT cells were not circulating but rather crawling within the sinusoids so there was no need for an initial capture. However, there was also no evidence of phagocytosis after antibody treatment. In contrast, Kupffer cells grabbed crawling iNKT cells via Fcγ receptors and tear fragments off crawling iNKT cells upon CXCR3-173 antibody treatment. This was not dependent on directional shear as iNKT cells crawling within flow would lose a portion of membrane, reverse direction, crawl against flow and lose another piece of their membrane. It was however, dependent upon the crawling by iNKT cells because arrest of iNKT cells, even on top of Kupffer cells, eliminated fragmentation and iNKT cell disappearance. Although two different modes of iNKT cell arrest were used, one cannot exclude the possibility that activation rather than arrest prevents membrane ripping. However, the membrane ripping only occurred in organs where the iNKT cells crawled inside the vasculature and not in organs where iNKT cells were localized extravascularly (e.g. joint (Lee et al., 2014) or spleen (Wong and Kubes, 2013)).

Our data would suggest that we depleted functionally iNKT cells within the liver and lung but not other organs, as their functional role in protecting the liver from toxic molecules like acetaminophen was absent after CXCR3-173 administration. Since iNKT cells can play important central detrimental roles in immune responses to autoimmune disease, allergy and liver fibrosis, this approach could be used to therapeutically intervene in the process (Liew and Kubes, 2015; Van Kaer et al., 2013). Extending this work to immunotherapy, one could now strategically design antibodies by altering glycosylation in Fc regions to induce fragmentation of
tumor cells and patrolling monocytes which are known to benefit tumor growth at the expense of the host. In addition, this antibody depleting approach could be useful as an experimental tool. NKT cell deficient mice have provided great insights into NKT cell biology but have certain limitations. CD1d deficient mice are also deficient in other CD1d restricted cells such as type 2 NKT cells and this subset of NKT cells are now emerging as important players in health and disease (Marrero et al., 2015). Finally, the CXCR3-173 antibody could be useful as an initial screen prior to lengthy breeding protocols of iNKT cell deficient mice onto various genetic mouse backgrounds or used as another tool to validate genetic knockout models.

Collectively, these studies establish fragmentation, as a novel cell death pathway mediated by Kupffer cells leading to antibody-mediated target cell depletion. With the ability to image interactions of immune cells at improved resolution with spinning disk intravital microscopy, we have provided significant insight into this effector mechanism and demonstrate that it is quite distinct from phagocytotic mechanisms previously identified. Furthermore, we have identified a novel use of an antibody that can effectively deplete iNKT cells in the liver and lung. This creates new experimental possibilities that can increase our understanding of the role of iNKT cells in disease and facilitate development of novel therapeutic strategies.
A.6. EXPERIMENTAL PROCEDURES

A.6.1. Mice

BALB/c, Fcγ knockout and C57Bl/6 mice were purchased from The Jackson Laboratory. CXCR6-GFP knock-in mice on the BALB/c background were a gift from Dan R. Littman (New York University School of Medicine, New York). B6.Jα18−/− mice were a gift from Brent Johnston (Dalhousie University, Halifax, Nova Scotia, Canada). All mice were maintained in a specific pathogen-free, double-barrier unit at the Faculty of Medicine, University of Calgary. All protocols used in this manuscript were approved by the University of Calgary Animal Care Committee (protocol #AC16-0148) and in accordance with guidelines established by the Canadian Council for the Use of Laboratory Animals.

A.6.2. Antibodies and treatments

Armenian Hamster IgG functional grade purified anti-CXCR3 (clone CXCR3-173), Armenian Hamster IgG functional grade purified anti-TCRβ (clone H57-597), Armenian Hamster IgG functional grade purified non-binding isotype control (clone eBio299Arm), fluorescein isothiocyanate (FITC)-conjugated anti-CD45r (clone RA3-6B2), eFluor® 660-conjugated anti-CD3 (clone 17A2), Perdinin chlorophyll (PerCP)-conjugated anti-CD45 (clone 2D1), phycoerythrin (PE)-conjugated anti-CXCR3 (clone CXCR3-173) and allophycocyanin (APC)-Annexin V kit was purchased from eBioscience (San Diego, CA). Alexa Fluor 750-conjugated anti-F4/80 (clone BM8) was obtained from AbLab (University of British Columbia, Vancouver, BC, Canada). PBS57-loaded PE-conjugated mouse CD1d tetramer was obtained from NIH (National Institutes of Health). α-GalCer was purchased from Funakoshi co. ltd (Tokyo, Japan). To deplete iNKT cells in liver and lung, 200 μg of CXCR3-173 was intravenously administered via tail vein two or four days prior to flow cytometry analysis or intravital microscopy. To
investigate the mechanism of iNKT cell depletion, different treatments were used. Either 200 μg of CXCR3-173 or anti-TCRβ was injected via the jugular vein before imaging. In similar depletion mechanism experiments, α-GalCer (dissolved in 0.5% Tween 20 and 0.9% NaCl, 5 μg/mouse) with or without CXCR3-173 antibodies were injected via the jugular vein. Annexin V expression of iNKT cells was examined 24 hours after 200 μg of CXCR3-173 was injected via the tail vein. To investigate the role of Fcγ receptors in iNKT cell depletion after CXCR3-173 antibody treatment, 200 μg of anti-CD16/anti-CD32 were injected into CXCR6-GFP mice via the jugular vein 30 minutes before intravital microscopy and allowed to circulate before injection of CXCR3-173 antibodies. To visualize the liver vasculature, anti-PECAM-1 (clone 390) was conjugated to Alexa Fluor 647 using a protein labeling kit according to manufacturer’s instructions (Invitrogen, Eugene, OR) and injected via the jugular vein before intravital imaging. Kupffer cell depletion was performed by injecting 200 μl of clodronate liposomes via tail vein 36 hours prior to CXCR3-173 treatment as previously described (Lee et al., 2010).

A.6.3. Spinning-disk confocal intravital microscopy (SDIVM)

CXCR6-GFP mice were used for the visualization of hepatic iNKT cells (Lee et al., 2010; Wong et al., 2011). Intravital microscopy was performed with an Olympus IX81 inverted microscope (Olympus, Center Valley, PA), equipped with an Olympus focus drive and motorized stage (Applied Scientific Instrumentation, Eugene, OR). Images were acquired with 10×/0.40 UPLANSAPO and 20×/0.45 LUCPLANFLN objective lenses. The microscope was linked with a confocal light path (WaveFx; Quorum Technologies, Guelph, ON, Canada) based on a modified Yokogawa CSU-10 head (Yokogawa Electric Corporation, Tokyo, Japan). Activity of iNKT cells in the liver vasculature was acquired with four laser-excitation wavelengths in rapid succession (491 nm, 561 nm, 642 nm and 730 nm; Cobolt, Vortran and Omicron) and captured
with appropriate band-pass filters (Semrock and Chroma). Typical exposure times for excitation wavelengths were 0.2-0.5 s. A 512×512 back-thinned electron-multiplying charge-coupled device camera (C9100-13, Hamamatsu, Bridgewater, NJ) was used for fluorescence detection. Z stacks of xy planes (0.5 μm intervals) were recorded with the inverted spinning-disc confocal microscope using either ASI focus drive (Applied Scientific Instrumentation) or Olympus focus drive (Olympus). Volocity software (Perkin Elmer, Waltham, MA) was used for 3D rendering, acquisition and analysis of images.

**A.6.4. Preparation of mouse liver for SDIVM**

Preparation of the murine liver for intravital microscopy was performed as previously described (Lee et al., 2010). Briefly, the jugular vein of an anesthetized mouse was cannulated to permit intravenous delivery of antibodies and additional anesthetic, as required. To maintain body temperature, mice were placed on a heating plate (CU-201, Live Cell Instruments) at 37°C. A midline and lateral incision along the costal margin to the midaxillary line was performed to expose the liver. The mouse was placed on a right lateral position on the heating plate and the ligaments connecting the liver to the diaphragm were severed to allow externalization of the liver onto a glass coverslip. To prevent dehydration, exposed abdominal tissues were covered with saline-soaked gauze. A saline soaked KimWipe® disposable wipe was gently placed over the liver to restrict movement of the tissue on the slide and to prevent tissue dehydration.

**A.6.5. Liver, spleen, lung and blood cells isolation and flow cytometry analysis**

Liver-, spleen-, and lung-derived lymphocytes were isolated from BALB/c mice using a method previously described (Lee et al., 2010). Briefly, blood was collected from anesthetized mice by cardiac puncture and red blood cells were lysed with ACK lysis buffer (Lonza, Switzerland). Leukocytes were first washed with cold PBS and resuspended in cold FACS wash buffer (FWB;
PBS, 2% fetal calf serum, 0.5 mM EDTA). Livers and lungs were excised and finely minced in a
digestive medium containing 0.05% collagenase type IV (Worthington Biomedical) and 0.002% 
DNase I in HBSS (for liver) or collagenase type I (Worthington Biomedical) and 0.002% DNase I in PBS (for lung). Concentrates were placed at 37°C for 30 min with gentle agitation (for liver) 
or without (for lung). The spleen was excised and collected in cold PBS. Subsequently, single 
cell suspensions were generated from the liver and lung concentrates and spleen by a mechanism 
of disruption through a 40 μm nylon mesh. Suspensions were washed with ice-cold PBS (pH 7.4) 
and centrifuged at 300 × g for 10 min. Liver mononuclear cells (MNCs) were also additionally 
purified through a 37%/70% (vol/vol) Percoll gradient. All suspensions were then resuspended in 
cold FWB and counted in 0.4% trypan blue using a hemocytometer. All samples were analyzed 
on an Attune acoustic focusing cytometer (Applied Biosystems). For iNKT cell analysis, only 
MNCs were gated in FSC versus SSC flow cytometric plots. The absolute number of MNCs was 
standardized by tissue weight (for liver, lung and spleen) or milliliters for blood.

A.6.6. Cell culture of iNKT cells

For in vitro cell culture experiments, hepatic iNKT cells from CXCR6-GFP mice were harvested 
45 minutes after administration of CXCR3-173 antibody and incubated with RPMI-1640 media 
in 250 ml cell culture flasks for 3 days at 37°C and 5% CO₂. For in vitro CXCR3-173 incubation 
treatments, hepatic iNKT cells from BALB/c mice were harvested and incubated as described 
above for 24 hours. iNKT cells were incubated with CXCR3-173 for 30 minutes before staining 
for annexin V expression. Medium was supplemented with 10% fetal bovine serum, 1% 
penicillin/streptomycin cocktail and 1% Glutamax™. All cell culture media and supplements 
were purchased from Thermo Fisher Scientific (Burlington, ON).

A.6.7. Deglycosylation of antibodies
CXCR3-173 and PE-conjugated CXCR3-173 were deglycosylated under non-denaturing conditions. 200 μg of CXCR3-173 antibody was incubated with 1,875 units of glycerol-free PNGase F (New England Biolabs, Ipswich, MA) according to manufacturer’s protocol for 24 hours at 37°C before in vivo injection. 2 μg of PE-conjugated CXCR3-173 was incubated with 500 units of glycerol-free PNGase F for 24 hours at 37°C before imaging studies. For SDS-PAGE analysis, 5 μg of CXCR3-173 antibody was incubated with or without PNGase F as described above before gel analysis. SDS-PAGE gel was stained with 0.25% coomassie blue (Sigma-Aldrich, Oakville, Ontario, Canada) solution dissolved in a methanol/acetic acid mixture.

A.6.8. Acetaminophen (APAP) overdose treatment

Treatments were performed as described (Martin-Murphy et al., 2013). Mice were allowed food and water ad libitum until the start of experiment. Before treatment, mice were fasted overnight (16 hours). APAP was dissolved in warm saline and administered by intraperitoneal injection (350mg/kg or 300mg/kg doses). Food was restored immediately after APAP treatment. These doses are known to be sublethal in wild-type mice. After various time points, blood was harvested by cardiac puncture and liver tissues collected for histological analysis.

A.6.9. Statistical analysis

All values are expressed in mean ± SEM. Data were compared with either unpaired Student’s t-test, one-way ANOVA or two-way ANOVA with Bonferroni multiple comparisons post hoc test. Statistical significance was accepted at $P < 0.05$. 
A.7. REFERENCES


A.8. FIGURE AND FIGURE LEGENDS

Figure A-1: iNKT cells in the liver and lung are depleted by CXCR3-173 antibody.
(A) Intravital images of CXCR6-GFP liver 0, 2 and 4 days after CXCR3-173 antibody treatment. Bright green: iNKT cells. Scale bar: 50 μm. (B) Enumeration of GFP+ cells from intravital
microscopy after treatment with CXCR3-173 antibody, n = 3 for all mice. *P < 0.01 by one-way ANOVA, error bars: SEM. (C, D) Representative flow cytometric plot demonstrating decrease of iNKT cells in liver (C) and lung (D) after CXCR3-173 antibody treatment over 4 days. (E, F) Absolute number of iNKT cells in the liver (E) and lung (F) after CXCR3-173 treatment at day 4, n ≥ 3 mice, *P < 0.05, **P < 0.01 by t test, error bars: SEM. See also Figure S1.
Figure A-2: Population of T cells, B cells and natural killer (NK) cells remain consistent after CXCR3-173 treatment.

Absolute number of CD3⁺ T cells in (A) liver and (D) lung, n ≥ 3 mice. Absolute number of B220⁺ B cells in (B) liver and (E) lung, n ≥ 3 mice. Absolute number of NK cells in (C) liver and (F) lung, n ≥ 3 mice. All error bars are SEM.
Figure A-3: iNKT cell fragmentation by Kupffer cells.
(A) Intravital images of iNKT cells fragmentation after CXCR3-173 treatment. Bright Green: iNKT cells, Blue: Alexa Fluor 647-labelled PECAM-1, Magenta: Alexa Fluor 750-labelled F4/80+ Kupffer cells. Scale bar: 50 μm. iNKT cell in red inset used for panel (D) demonstrating
fragmentation over time. (B) No fragmentation of iNKT cells occurred with isotype control treatment. Bright Green: iNKT cells, Blue: Alexa Fluor 647-labelled PECAM-1, Magenta: Alexa Fluor 750-labelled F4/80+ Kupffer cells. Scale bar: 50 μm. (C) Quantification of fragmentation after CXCR3-173 or isotype control, **P < 0.01 by t test, error bars are SEM. (D) Time-lapse intravital images from panel (A) demonstrating fragmentation of single iNKT cell; inset: enlarged image of fragment ripped from iNKT cells by Kupffer cells at 6 min post CXCR3-173 antibody treatment. Scale bars: 25 μm. (E) Percentage of GFP+ cells that undergo fragmentation after CXCR3-173, n = 3, **P < 0.001 by t test, error bars are SEM. (F) Annexin V expression in CD45+CD3+CD1d-tetramer+ hepatic iNKT cells with (red) and without (blue) CXCR3-173 treatment. (G) Representative 3D reconstruction image of tail-end (white arrow) of crawling iNKT cell during fragmentation after CXCR3-173 antibody treatment. Green: iNKT cell, Magenta: Alexa Fluor 750-labelled F4/80+ Kupffer cells. Scale bars: 50 μm. See also Figure S2.
Figure A-4: iNKT cell depletion in the liver is dependent on Kupffer cells and mediated by Fcγ receptor.

(A, B) Representative intravital image of CXCR6-GFP mice treated with CXCR3-173 antibody alone (A) or tandem CLL and CXCR3-173 antibodies (B). Bright green: iNKT cell. Scale bar:
200 μm. (C) Enumeration of GFP+ cells from intravital image after treatment with either CXCR3-173 antibody alone or CLL and CXCR3-173 antibodies, n = 3 for all mice, *P < 0.05 by one way ANOVA, error bars are SEM. (D) Percentage of iNKT cells in liver after CXCR3-173 antibody treatment alone or in tandem with CLL treatment. Livers are harvested at 4 days after treatment and percentage of iNKT cells were determined by flow cytometry (CD3+CD1d-tet+ cells), n ≥ 3 mice, *** P < 0.001 by one way ANOVA, error bars are SEM. (E) Intravital image of CXCR6-GFP mice pre-treated with anti-CD16/anti-CD32 antibodies prior to CXCR3-173 antibody treatment. Bright Green: iNKT cells, Blue: Alexa Fluor 647-labelled PECAM-1, Magenta: Alexa Fluor 750-labelled F4/80+ Kupffer cells. Scale bar: 50 μm. (F) Tail-localized distribution of CXCR3 molecules (red) on the surface of crawling iNKT cells (green) in vivo over time. Numbers in top right corner represents minutes of video recording. Scale bars: 10 μm. (G) Homogenous distribution of CXCR3 molecules (red) on surface of non-crawling iNKT cells (green). Scale bars: 10 μm.
Figure A-5: Fragmentation of iNKT cells is dependent on iNKT cell crawling.
(A) Intravital image over time of GFP+ cells after anti-TCRβ antibody treatment. Scale bar: 50 μm. (B) Distribution of velocity of GFP+ cells in untreated mice, anti-TCRβ, CXCR3-173 or αGalCer treated mice. (C) Percentage of iNKT cells >5 μm/min after anti-TCRβ or αGalCer treatment, **P < 0.01 by t test, error bars are SEM. (D) Number of fragmentation events in field of view after antibody treatments, *P < 0.05 by t test CXCR3-173 treatment against tandem αGalCer and CXCR3-173 treatment, ***P < 0.001 by one way ANOVA, error bars are SEM. (E) Number of fragmentation events in crawling or stationary iNKT cells after tandem αGalCer/CXCR3-173 treatment, *P < 0.05 by t test, error bars are SEM. (F) Kupffer cell contact by crawling or arrested iNKT cells with various treatments, n = 3. *P < 0.05 by t test, treatment groups were tested against identical cell behaviors (crawling vs crawling or arrested vs arrested).
Figure A-6: No fragmentation of iNKT cells with deglycosylated CXCR3-173 antibody.

(A) Representative still images from intravital imaging demonstrating no iNKT cell fragmentation after *in vivo* injection of deglycosylated CXCR3-173 antibody. Bright Green: iNKT cells, Blue: Alexa Fluor 647-labelled PECAM-1, Magenta: Alexa Fluor 750-labelled...
F4/80+ Kupffer cells. Scale bar: 50 μm. (B) Enumeration of fragmentation events after antibody treatment, n = 3 for all mice, ***P < 0.001 by one way ANOVA, error bars are SEM. (C) SDS-PAGE analysis of untreated versus deglycosylated CXCR3-173 antibody. HC: heavy chain, LC: light chain. (D) Representative intravital snapshots demonstrating binding of deglycosylated PE-conjugated CXCR3-173 antibody to iNKT cells. Bright Green: iNKT cells, Red: deglycosylated PE-conjugated CXCR3-173 antibody.
Figure A-7: iNKT cells and acetaminophen overdose liver injury.

(A) Survival of BALB/c, CXCR3-173 treated mice or CD1d<sup>-/-</sup> mice after 350 mg/kg acetaminophen (APAP) treatment. (B) Survival of BALB/c, CXCR3-173 treated mice or CD1d<sup>-/-</sup> mice after 300 mg/kg APAP treatment. (C) Serum ALT levels in BALB/c, CXCR3-173 treated mice or CD1d<sup>-/-</sup> mice after 300 mg/kg APAP, *P < 0.05, *P < 0.01, *P < 0.001 by t-test, error bars are SEM. (D) Serum ALT in C57Bl/6 CXCR3-173 treated mice, B6.Jα18<sup>+/+</sup> mice or CXCR3-173 treated B6.Jα18<sup>-/-</sup> mice 8 hours after 300 mg/kg APAP. (D) Serum ALT levels in BALB/c, CXCR3-173 treated mice or polyclonal anti-CXCR3 treated mice at 8 hours after 300 mg/kg APAP.
Figure A-S1: Stitched intravital image of CXCR6-GFP mice

Still image of GFP+ cells under untreated conditions, 2 or 4 days after CXCR3-173 antibody treatment. Scale bars are 400 μm.
Figure A-S2: iNKT cells in blood and spleen not depleted by CXCR3-173 treatment.
(A) Absolute number of iNKT cells in spleen, n ≥ 3 mice. (B) Absolute number of iNKT cells in peripheral blood, n ≥ 3 mice. (C, D) Absolute number of iNKT cells in liver (C) and lung (D) after isotype antibody treatment at day 4. All error bars are SEM.
Figure A-S3: Isotype antibody did not alter iNKT cell behavior.


(B) Percentage of stationary iNKT cells from cell tracking after 30 mins of intravital imaging, n \( \geq 3 \) mice. (C) Velocity frequency distribution of iNKT cells under basal and isotype antibody treated conditions. All error bars are SEM.
Figure A-S4: CXCR3-173 antibody alone is not lethal to iNKT cells.

(A) 3 days culture of iNKT cells in vitro after injection of CXCR3-173 into mice. (B) Annexin V expression of iNKT cells after incubation with CXCR3-173 antibodies in vitro.
Figure A-S5: Polyclonal anti-CXCR3 antibody treatment does not deplete iNKT cells.

(A) Stitched intravital image of the liver of CXCR6-GFP mice 0, 2 and 4 days after polyclonal anti-CXCR3 antibody. Scale bars are 400 µm. (B) Quantification of number of GFP+ cells from stitched intravital image. All error bars are SEM.