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INNATE INTERLEUKIN-17 AND INTERLEUKIN-22 PLAY IMPORTANT ROLES IN VIRAL HEPATITIS

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INNATE INTERLEUKIN-17 AND INTERLEUKIN-22 PLAY IMPORTANT ROLES IN VIRAL HEPATITIS

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DISSERTATION

Presented to the Faculty of the Graduate School of

The University of Texas Medical Branch

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas Medical Branch

April 2014

To my family, for unconditional support and giving me this life full of happiness and inspiration.

谨献给我的家人，让我的生活充满幸福与动力。

ACKNOWLEDGEMENTS

First of all, I would like to express my deepest gratitude to my mentor, Dr. Jiaren Sun, for his excellent guidance, caring, patience, and providing me with a great atmosphere for doing research. When I first came to his lab, my knowledge and skills about immunology were limited. He showed tremendous patience in allowing me to learn the basic immunology and obtain the experimental skills. His scientific guidance and encouragement have been an important source of strength that I can rely on to confront and overcome the difficulties during my graduate study. Without Dr. Sun's support and guidance, I could not complete my graduate study and stand here to defend my dissertation.

I also want to express my gratitude to Dr. Lynn Soong for her tremendous contribution to my graduate study. She helped me with my basic immunology class, proposal writing, paper presentation, and manuscript writing. Her utter enthusiasm for immunology is infectious and has helped to inspire me to become an immunologist. I am also very grateful to Dr. Yingzi Cong for his tireless support of my dissertation research. Whenever needed, Dr. Cong has always provided expert assistance and shared his ideas. I would like to thank Dr. Teh-sheng Chan for giving me valuable scientific career suggestions.

I thank all the members of my dissertation committee, Dr. Jiaren Sun, Dr. Bin Gao, Dr. Yingzi Cong, Dr. Gustavo Valbuena, and Dr. Iryna Pinchuk, for their guidance, advice, and criticism. Their investment of time and effort towards my graduate training are very much appreciated.

I want to thank present and past members of Dr. Sun's laboratory who have provided valuable assistance during my graduate training. They are Dr. Lifei Hou, Dr. Yuejin Liang, Dr. Mayura Desai, Dr. Jiabin Yan, Ms. Yixiao Sun, Dr. Yiqun Xiong, Dr. Shalini Gupta, Dr. Renán A. Aguilar-Valenzuela, and Mr. David Vu. I would especially like to thank Dr. Lifei Hou, who is always willing to help and give his best suggestions and ideas. I would like to thank Dr. Yuejin Liang for helping me with my projects and valuable discussions. I would like thank Dr. Mayura Desai for providing great suggestions.

Many people from other laboratories at UTMB or other institutions have also been tremendously helpful to my education. I acknowledge Dr. Lijun Xin, Dr. Anthony Cao, Dr. Houpu Liu, Dr. Suxia Yao, Dr. Hui Wang and Mr. Eric Carlson for stimulating discussions and for collaborative efforts in my studies. I thank Dr. Yoichiro Iwakura (Tokyo University of Science) and Dr. Chen Dong (The University of Texas MD Anderson Cancer Center) for providing the IL-17A$^{-/-}$ and IL-17F$^{-/-}$ mice, respectively. I thank Amgen and Dr. Tian Wang (UTMB) for providing the IL-17R$^{-/-}$ and $\gamma\delta^{-/-}$ breeding mouse pairs, respectively. I thank Drs. Maria Salvato (University of Maryland) and Robert Tesh (UTMB) for providing LCMV and anti-LCMV antibody, respectively. I thank Dr. Robin Stephens (UTMB) for kindly providing the Rag2$^{-/-}$ mice.

I thank Dr. David Niesel, Dr. Rolf König and Ms. Aneth Zertuche for their excellent work in administering the Microbiology and Immunology program that has been home for me for the last five and half years. I must also thank Ms. Martha Lewis, Ms. Marcela Rubio, Ms. Claudia Salinas, Ms. Analaura Ramos, and Ms. Cecily Garcia for

their excellent administrative and secretarial work. I thank Ms. Mardelle Susman for her excellent editorial assistance in my preparation of proposals, manuscripts and this dissertation during these years. I should thank Dr. Dorian Coppenhaver, Ms. Jessica Linton and Laura Teed for their outstanding work in helping graduate student and administering Graduate School. I also want to extend my gratitude to the McLaughlin Fellowship Fund and Graduate School of Biomedical Sciences for their generous financial support of my dissertation research.

On a more personal level, I want to thank many friends who I have met during my study at UTMB. When I first came to the United States, Dr. Tian Luo and Dr. Daisi Chen helped me tremendously during the initial setting period and made my adjustment to a completely unfamiliar environment a smooth one. I am very grateful to Dr. Xiangsheng Yang, Dr. Xi Lin, Dr. Xiang Lin, and Ms. Yi Wang, my old friends in Houston, for helping me to get re-settled during Hurricane Ike. I would like to thank my friend, Dr. Yingwei Wang, a former visiting graduate student in Dr. Soong's lab, who helped and encouraged me when I confronted problems and difficulties.

Finally, I would like to thank my family, my parents and sisters, who have given me this life full of happiness, wonder, and inspiration. They are always unconditional in supporting me and encouraging me with their best wishes. I would like to thank my wife Xianxiu Wan. She was always there encouraging me and stood by me through the good and bad times.

INNATE INTERLEUKIN-17 AND INTERLEUKIN-22 PLAY IMPORTANT ROLES IN VIRAL HEPATITIS

Publication No. _____

Zuliang Jie M.S.

The University of Texas Medical Branch
at Galveston, 2014

Supervisor: Dr. Jiaren Sun

ABSTRACT

Intrahepatic cell-derived IL-17 and IL-22 are important for T cell priming and hepatoprotection in viral hepatitis, respectively; however, the source and regulation of these cytokines in the liver microenvironment are not well defined. Here, we present evidence for a significant expansion of IL-17- and IL-22-producing cells in mouse liver during viral infection. We found that a subset of IL-17$^+$ and IL-22$^+$ cells expressed no myeloid/lymphoid lineage markers. Instead, they expressed high levels of stem-like markers, IL-7Rα and RORγt, consistent with the newly described group 3 innate lymphoid cells (ILC3s). They contributed significantly to the establishment of the early cytokine milieu in virus-infected mouse livers. Functional studies with mice deficient of IL-17 receptor, IL-17A, and IL-17F further revealed that IL-17 signaling was critical for priming T cell responses in viral hepatitis. Further studies showed that IL-17A repressed IL-17F secretion *in vivo* and *in vitro*; IL-17F$^+$ intrahepatic cells expanded more vigorously in IL-17A knockout animals, permitting efficient antigen-presentation and T cell function. However, IL-17F neither inhibited IL-17A *in vitro* nor regulated its secretion *in vivo*. Interestingly, retinoic acid, secreted by hepatic stellate cells (HSCs), increased in the liver after viral infection. Moreover, hepatic stellate cell-derived retinoic acid promoted IL-22 production by ILC3s in the liver and protected the liver against acute viral hepatitis. Notably, hepatic IL-7 was important for ILC3-derived IL-22 production. The blockade of IL-7Rα *in vivo* significantly decreased IL-22 levels and exacerbated liver inflammation. Collectively, this study has demonstrated the importance of a unique intrahepatic subpopulation ILC3 and its cytokine production during the initial stages of viral infection in the liver. Moreover, the crosstalk among hepatic stellate cells and innate immune cells may play a critical role in modulating immune responses in viral hepatitis.

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LIST OF ABREVIATIONS

Ad	Adenovirus
ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
APC	Antigen-presenting cell
CIA	Collagen-induced arthritis
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
EAE	Experimental autoimmune encephalomyelitis
HAV	Hepatitis A virus
HBeAg	Hepatitis B e antigen
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HDV	Hepatitis D virus
HEV	Hepatitis E virus
HSC	Hepatic stellate cell
IFN	Interferon
IFN-γ	Interferon gamma
IHL	Intrahepatic lymphocyte
IL	Interleukin
ILC	Innate lymphoid cell
ILC1	Group 1 innate lymphoid cell
ILC2	Group 2 innate lymphoid cell
ILC3	Group 3 innate lymphoid cell
i.p.	Intraperitoneal

i.v.	Intravenous
LCMV	Lymphocytic chroriomeningitis virus
LSEC	Liver sinusoidal endothelial cell
mAb	Monoclonal antibody
mDC	Myeloid dendritic cell
MHC-I	Major histocompatibility complex class I
MHC-II	Major histocompatibility complex class II
NCR	Natural cytotoxic receptor
NK	Natural killer
NKT	Natural killer T cell
PD-1	Program death-1
pDC	Plasmacytoid dendritic cell
RA	Retinoic acid
TGF-β	Transforming growth factor beta
Th	T helper
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
Treg	T regulatory cell

CHAPTER 1 INTRODUCTION TO LIVER IMMUNITY

1. 1 Hepatitis viruses

Many different viruses, such as hepatitis A, B, C, D, and E viruses, can cause acute or chronic viral hepatitis, and most of these viral infections can cause acute viral hepatitis. Some of the viruses (namely hepatitis B, C and D viruses) can lead to a chronic stage, which can develop into cirrhosis, fibrosis, and even liver cancer. In addition to the hepatitis viruses A-E, many other viruses can also induce viral hepatitis, e.g., cytomegalovirus, Epstein-Barr virus, herpesvirus, parvovirus, lymphocytic choriomeningitis virus, and adenovirus.

Acute viral hepatitis usually is first noticed due to general ill health, appetite loss, mild fever, fatigue, vomiting, nausea, body aches, itchy skin, and dark urine. Infected individuals develop jaundice at a later stage. In some cases, the illness lasts for several weeks and those infected have gradually recovered. However, a few individuals may develop fulminant hepatic failure, which can cause the patients' death. Chronic infection with HBV or HCV can develop to cirrhosis and even to hepatocellular carcinoma after decades of infection. Herein, I will briefly discuss the hepatitis viruses A through E.

Hepatitis A virus (HAV) belongs to the *Picornavirus* family and is transmitted by the fecal-oral route usually due to ingestion of contaminated food, e.g., raw sea food, but also from personal contact or drinking of contaminated water (1). HAV causes an

acute hepatitis and does not lead to a chronic stage (2). The patient's immune system develops antibodies against virus and prevents future infection. Currently, a vaccine is available that will protect against HAV infection for 10 years.

Hepatitis B virus (HBV) has a circular genome of partially double-stranded DNA and belongs to the *Hepadnaviridae* family (3). Currently, more than 350 million individuals are persistently infected by HBV worldwide. It replicates through the reverse transcription of an RNA intermediate (4). Due to the lack of proofreading of reverse transcriptase, high mutation frequency occurs during the HBV replication process. The most common mutations are found in the precore and core promoter regions that can down-regulate the expression of hepatitis B e antigen (HBeAg) (5). Mutations in the virus surface protein have also resulted in the failure of immune prophylaxis in infants receiving HBV vaccine. In addition, mutations in HBV polymerase can cause resistance to nucleoside and nucleotide analogs, and such mutations are the major barrier to drug treatment.

In most cases, HBV infection can be resolved within an immune-competent adulthood. Adaptive immune responses, especially antigen-specific $CD4^+$ and $CD8^+$ T cells, have been shown to be competent in clearing HBV from hepatocytes. Individuals that generate vigorous, broad-epitope T-cell responses can clear the virus and those persons have recovered spontaneously (6). However, patients that have weak, transient or narrow-epitope T-cell responses are likely to develop chronic hepatitis (7).

In addition, the immune system of infants is generally not fully developed. Thus, vertical transmission from HBV-infected mothers to their infants can cause chronic HBV infection in children. In these cases, HBeAg plays an immune tolerogen role in these cases (8, 9). After secretion, HBeAg circulates into the blood and induces immune tolerance to viral antigens. Moreover, as we discussed above, the development of viral mutants is associated with the escape from humoral and cellular immune responses induced by vaccination.

Hepatitis C virus (HCV) is a single-stranded RNA virus and belongs to the *Flaviviridae* family (10). HCV can be spread through blood contact and can also cross the placenta. HCV infection usually develops into chronic hepatitis, cirrhosis, and hepatocellular carcinoma (10, 11). To date, HCV infection is one of the most common causes of cirrhosis, chronic liver failure, and hepatocellular carcinoma in the US. HCV infects humans and chimpanzees. Currently, no small animal models are available.

The magnitude, diversity and quality of adaptive immune responses play an essential role in resolving HCV infection. Vigorous polyclonal $CD4^+$ and $CD8^+$ T cell responses associated with viral clearance are found in patients with self-limited infection (12). However, a weak and narrowly focused T cell response usually leads to viral persistence and chronic infection (12). During chronic HCV infection, the intrahepatic $CD8^+$ T cells usually develop an exhausted phenotype with low levels of CD127 and high levels of program death-1 (PD-1) expression (13). HCV replication is a

cytoplasm-based, RNA-dependent cycle which lacks proofreading and can generate numerous mutants (14). The virus can still become persistent despite a vigorous $CD4^+$ and $CD8^+$ T cell response by progressive mutational escape. In addition, HCV infection also has direct cellular effects in infected hepatocytes, such as alterations in gene expression, signaling pathways, lipid metabolism, and oxidative stress. To date, there is no vaccine available for the HCV infection (15). Hepatitis C patients can be treated by a combination of interferon and the antiviral drug ribavirin (16).

Hepatitis D virus (HDV) is an RNA virus and belongs to the *Deltaviridae* family. HDV infection requires HBV surface antigen proteins for its assembly (17). Therefore, HDV epidemiology is closely associated with HBV infection. Chronic hepatitis B patients, who are co-infected with HDV, may progress from liver damage to an established cirrhosis and even to fulminant hepatitis (18). Several studies suggested that HDV infection in chronically infected patients significantly increased the risk of developing hepatocellular carcinoma (18, 19).

Hepatitis E virus (HEV) is a positive-sense, single-stranded RNA icosahedral virus, belonging to the *Picornaviridae* family (20). HEV infection can cause acute hepatitis, but not chronic disease. The clinical symptoms are similar to those of hepatitis A. HEV can be transmitted through the fecal-oral route. Interestingly, it replicates in the cytoplasm of hepatocytes and is shed in the feces. Hepatitis E is a self-limiting disease that can be resolved by immune-competent individuals. However, HEV may

occasionally cause fulminant hepatic failure, especially in immune-compromised individuals (21).

1.2 Liver microenvironment and cell types

The liver is the largest solid organ in the body, for which the blood supply comes from two distinctive areas: 80% from the gut (rich in nutrition and food antigens) and 20% from the hepatic artery (rich in oxygen) (22). More than 70% of the total cells in the liver are hepatocytes. The remaining cells are comprised of non-parenchymal cells, including liver sinusoid endothelial cells (LSEC), Kupffer cells, biliary cells, stellate cells and intrahepatic lymphocytes (23). Present evidence demonstrates that the liver is an important part of the body's immune response and is, therefore, considered an immunologic organ (24). Numerous studies have shown that the liver plays an important role in innate and adaptive immune responses against pathogens (22, 24). Here, I will briefly discuss the different cell types in the liver microenvironment.

Hepatocytes, which constitute more than 70% of the total cell population in the liver, are essential for the metabolism and detoxification of antigens and toxins that enter the liver. In addition, hepatocytes can secrete pattern recognition receptors and complement components that play a key role in innate immunity (22). The liver is also an important source of acute phase proteins, which play an essential role in innate immunity and could alleviate liver injury. More importantly, after TLR induction or viral infection, hepatocytes can produce IL-7 and regulate innate $\gamma\delta$ T cells, NKT cells, and

adaptive CD4 and CD8 T cell responses (25). In chronic viral hepatitis, such as chronic HBV- or HCV-infected liver, hepatocytes were found to express high levels of MHC II and co-stimulatory molecules (including CD40, CD80, CD86), suggesting to us that hepatocytes also have an antigen-presenting function (26, 27).

Large populations of resident macrophages located in the liver are the Kupffer cells. They are generated from monocytes in the blood and mainly reside in the hepatic sinusoids. Kupffer cells can interact directly with hepatocytes when they pass through the space of Disse (28). Like other macrophages, Kupffer cells can phagocytose apoptotic cells and microorganisms in the liver (29, 30). Additionally, Kupffer cells have antigen-presenting functions, leading to T cell proliferation and cytokine productions (31). In contrast, Kupffer cells have been shown to be involved in tolerance. They can suppress T cell activation through nitric oxide synthesis (32). Moreover, Kupffer cells can induce alloreactive $CD4^+$ T cell apoptosis by expressing Fas ligand (FasL) in a liver transplant model (33). In addition, Kupffer cells are indispensable in systemic immune tolerance caused by alloantigenic leukocyte injection (34).

The liver contains a large number of resident lymphocytes, such as $CD4^+$, $CD8^+$ T cells, natural killer (NK) and natural killer T (NKT) cells. Most of the $CD8^+$ and $CD4^+$ T cells in the liver have an activated phenotype (35). For example, the intrahepatic $CD4^+$ T cells of mice present a $CD45RB^{low}$ phenotype, and they secrete both IFN-γ

and IL-4 (36). In addition, a subset of liver T cells was reported to have expressed the CD45 isoform B220, which is associated with apoptosis in the liver (37).

The natural killer (NK) cells are present at a high frequency among resident liver lymphocytes (38). They have been known to increase in number during infection of the liver. Type I IFNs have induced chemokine CCL3 expression in cytomegalovirus infection of mice, which is responsible for NK-cell accumulation (39). During experimental liver injury induced by concanavalin A (Con A) injection and adenovirus infection, NK cells expanded rapidly and were found to have a crucial role in T-cell recruitment (40, 41). The NK cells produce IFN-γ that promotes secretion of the chemokine CXCL9 by hepatocytes or LSECs, and this is responsible for T cell accumulation (42). On the contrary, in LCMV infection of mice, activated NK cells can eliminate $CD4^+$ T cells cytolytically leading to $CD8^+$ T cell exhaustion (43). In a murine liver fibrosis model, NK cells can kill the activated stellate cells and ameliorate liver fibrosis (44, 45).

Natural killer T (NKT) cells are at a low frequency in the peripheral lymphoid organ, but they are rich in the liver. The prototype NKT cells express NK1.1, T-cell receptor TCR$\alpha\beta$, and the MHC class-I-like molecule CD1d. Their TCR receptoire usually has limited diversity. The role of NKT cells in the liver is controversial. First, NKT cells were shown to secrete IL-4, which was considered to be anti-inflammatory lymphocytes (46). On the other hand, mice deficient in the CD1d molecule presented

impaired defenses against bacterial infection, such as *Mycobacterium tuberculosis* and *Borrelia burgdorferi* (47, 48). These findings have suggested to us that NKT cells play an important role in protecting against pathogenic infection. In addition, activation of NKT cells *in vivo* has been shown to inhibit melanoma metastases, induce effective immunity to the liver stages of malaria and suppress viral-RNA synthesis in HBV transgenic mice (49-51).

Hepatic stellate cells (HSCs), also known as perisinusoidal cells or Ito cells, are located in the sub-endothelial space of Disse. HSCs store around 75% of vitamin A in the body (52). Once the liver is injured, stellate cells can change into an activated state. Activated HSCs can secrete retinoic acid (RA) through the enzyme-mediated process (45). Moreover, activated HSCs can trans-differentiate into myofibroblasts, producing TGF-β and collagen scar tissue, which can result in liver fibrosis and cirrhosis (53, 54). In addition, emerging evidence indicates that hepatic stellate cells also have antigen-presentation functions (55). Thus, they express MHC class I, MHC class II, and CD1d (56). In addition, HSCs have the potential to respond to innate immune signals via their surface TLR4, CD14, and MD2. They can activate NKT cells and classical T cells. However, their co-expression of the inhibitory molecule program death-ligand 1 (PD-L1) also has been reported to result in T cell inactivation (57). Recently, HSCs have been shown to preferentially generate regulatory T cells by production of retinoic acid, which may contribute to liver tolerance (58).

Liver sinusoidal endothelial cells (LSECs) are perforated by many fenestrations, and they do not form an organized basement membrane. LSECs also have antigen-presenting functions (59). They express the scavenger receptor to take up circulating proteins or antigens. In addition, they express MHC class I, MHC class II and co-stimulatory molecules on the surface, which are similar to the active stimulatory APCs (59). However, they secrete IL-10 when responding to TLR4 antagonism (60). Thus, an *in vitro* study showed that isolated LSECs could induce a T regulatory phenotype of $CD4^+$ T cells, or $CD8^+$ T cell tolerance (61, 62).

Hepatic dendritic cells (DCs) are comprised of a heterogeneous cell population comprised of myeloid and plasmacytoid DCs. Myeloid DCs (mDC) in the liver produce more IL-10 compared to their counterparts in other organs (63). In contrast, hepatic DC can be activated in a pro-inflammatory microenvironment (64). Plasmacytoid DC (pDC) is another subset of DC that is present in the liver (64). Hepatic pDC play an essential role in the primary response against virus infection. They constitutively express toll-like receptor 7 (TLR7) and toll-like receptor 9 (TLR9) and produce large amounts of type I interferon when these receptors are engaged (65). Similar to the mDCs, hepatic pDCs present a tolerogenic cytokine profile when compared to their splenic counterparts, as they produce more IL-10 and less IL-12. It is possible that these tolerogenic features in pDC are partially mediated by their interactions with regulatory T cells (66).

1.3 Immunology and pathogenesis of viral hepatitis

To date, millions of people have been chronically infected by HBV or HCV. In immune-competent adults, HBV infection is a self-limited, transient liver disease; most individuals can clear the virus efficiently. However, in immune-compromised patients or neonates, most HBV infection can develop into persistent hepatitis (67). In contrast to HBV infection, most acute HCV infections (around 70% to 90%) become persistent, and often develop into liver cirrhosis and hepatocellular carcinoma over long periods of time (68).

Most hepatitis viruses, including HBV and HCV, replicate non-cytopathically within primary hepatocytes *in vivo*. Thus, immunologically mediated responses seem to play a key role in determining the pathogenesis and outcome of these diseases (14). It has been suggested that the adaptive immune response, but not innate immunity, mediates liver inflammation in viral hepatitis (69, 70). In addition, emerging evidence indicates that antigen-nonspecific inflammatory cells amplify cytotoxic T lymphocyte (CTL)-induced immunopathology in the liver (71). Vigorous and broad T cell responses can lead to viral clearance and transient acute liver disease (7, 12). On the contrary, inefficient and narrow T cell responses usually result in chronic viral hepatitis (7, 12). Over the decades of infection, continuous low levels of immune-mediated liver damage have led to the development of cirrhosis and hepatocellular carcinoma (72).

The virus-host interaction is a dynamic process: virus attempts to hide its visibility, whereas the host tries to sense and limit virus spread via innate and adaptive immunity. After virus infection, the innate immune system will be activated and attempt to inhibit viral replication by the production of antiviral cytokines (i.e. type I IFN) by infected cells and activation of effector functions of innate cells, including NK, NKT cells and neutrophils (51, 73, 74). Activated NK and NKT cells can be recruited to the infected site rapidly. They can recognize the viral-infected cells and kill them directly or indirectly, by producing pro-inflammatory cytokines and chemokines (51, 74). These pro-inflammatory cytokines and chemokines have anti-viral activities, and more importantly, can recruit inflammatory cells into infected tissue. In murine cytomegalovirus- and adenovirus-infected mice, NK and NKT cells have been suggested to contribute to the pathogenesis of liver inflammation (74). However, in the early phase of HBV or HCV infection, the innate defense mechanisms do not play a significant role in the pathogenesis of liver disorders.

Virus-specific $CD8^+$ cytotoxic T lymphocytes (CTLs) and $CD4^+$ T-helper cells play essential effector and regulatory roles in viral clearance and pathogenesis during viral infection, respectively. They contribute to viral pathogenesis either by killing infected cells directly, or by producing pro-inflammatory cytokines and chemokines to inhibit viral replication (75). Virus-specific $CD8^+$ T cells can lyse the viral-infected hepatocytes in a FasL- and perforin-dependent manner and secrete antiviral cytokines that inhibit virus replication noncytopathically (75). In addition, these

cytokines can also activate liver parenchymal and non-parenchymal cells to produce chemokines that recruit antigen-nonspecific polymorphonuclear and lymphomononuclear cells into the liver, such as neutrophils, monocytes, NK cells, T cells and macrophages (71). These antigen-nonspecific pro-inflammatory cells are outnumbered by virus-specific T cells and exacerbate the liver inflammation initiated by CTLs (71). They can cause liver inflammation through production of cytotoxic and pro-inflammatory mediators, such as perforin, TNF-α, hydrogen peroxide, superoxide species and nitric oxide.

Efficient antiviral T cell responses are considered to be primed by viral antigens processed by professional antigen-presenting cells (APCs) in lymphoid organs (76). In contrast, T cell priming within the liver usually causes T cell tolerance and apoptosis (77). HBV or HCV do not effectively infect professional APCs, such as DCs. To generate effective CTL responses, liver-derived DCs have to phagocytose and process apoptotic virus-infected hepatocytes and migrate to the peripheral lymph nodes to prime CTL responses. Primed CTLs expand rapidly, migrate to the infected site, and perform their cytotoxic functions. Individuals who mount vigorous, broad-epitope and polyclonal CTL responses can clear the virus and recover spontaneously. On the contrary, patients with relatively weak, transient, and narrow-epitope T cell responses are more likely to develop chronic hepatitis (7, 12). The underlying mechanisms between these differences are not fully understood yet.

1.4 Animal models for studying viral hepatitis

To date, only nonhuman primates can develop productive HBV or HCV infection that is similar to that in human beings. The chimpanzee is one of the best animal models for studying hepatitis virus infection and related host immune responses. However, several concerns have been raised by using nonhuman primates for research. The cost of maintaining a primate colony is extremely high, and special animal facilities and trained personnel are required. In addition, the National Institutes of Health have recommended that nonhuman primates not be used for biomedical research. Therefore, it is important to develop small animal models for studying HBV or HCV infection.

An important small animal model of HCV infection is the T- and B-cell-deficient mouse (SCID mouse) transplanted with human liver. To produce the chimeric mice, SCID mice are grafted with human hepatocytes (78). However, this model cannot be used to study adaptive immunity, due to their immune deficiency and lack of adaptive T/B cell immunity. They have provided new information about innate responses, viral entry, and HCV neutralization (79).

The transgenic mouse is an important tool for studying HBV or HCV infection. The first transgenic mice containing the complete HBV genome were generated by Guidotti *et al.* in 1995 (80). Work in this model has led to the finding that hepatocytes of HBV transgenic mice can generate high levels of viral particles without any

evidence of cytopathology *in vivo*. HBV particles produced by transgenic mice have been found to be infectious and morphologically similar to human-derived virions (81). By using HBV transgenic mice, scientists have studied the viral replication, entry, pathogenesis, and host antiviral immune responses (82). Several lines of HCV transgenic mice have also been generated (83, 84). The interactions between innate immunity and HCV have been studied by using these models. More recently, it has been reported that use of humanized mice is an advanced approach to study the pathogenesis of HBV/HCV infection (85, 86). These mice have been genetically modified to support engraftment of human hepatocyte progenitor cells and hematopoietic stem cells, leading to liver re-population with human hepatocytes and leukocytes.

1.5 Adenovirus-induced viral hepatitis

Adenovirus (Ad) belongs to the *Adenoviridae* family, which consists of non-enveloped, double-stranded DNA viruses. To date, the recombinant replication-deficient Ad vector is one of the preferred vectors for gene therapy and experimental vaccines (87). As a gene therapy candidate, replication-deficient Ad vectors have several advantages, including the ability to package large quantities of DNA, broad cell tropism, and ease of production in the lab (88). However, there are also several limitations of Ad-based gene therapy. Ad can trigger strong innate and adaptive immune responses, which are induced by viral capsid proteins and adenoviral gene products. After Ad administration, the early innate effector cells composed of

neutrophils, NK cells, and macrophages will infiltrate into the inoculation site. Resident Kupffer cells in the liver take up the administered Ad and act as the first line against viral vectors (89). Ad activates endothelium and mediates leukocytes rolling through P- and E-selectin, which induces the recruitment of neutrophils into the liver within several minutes (90). NK cell activation occurs several hours after the administration of viral vectors, which can secrete a bolus of IFN-γ (41). Pro-inflammatory cytokines and chemokines secreted by these cells contribute to the inflammatory response and recruit additional inflammatory cells. Depletion of Kupffer cells, neutrophils, or NK cells can reduce hepatocyte cell death and liver inflammation caused by Ad infection. Within 24 h, this innate immune response can eliminate about 80% of the adenoviral particles. The clearance of Ad and subsequent elimination of transgenes are major obstacles for Ad gene therapy (91).

The DC is an important antigen-presenting cell in the immune system and can link innate immune response and adaptive immune responses. Ad particles can activate and infect dendritic cells through the viral capsid, which will subsequently induce T cell responses (92, 93). The immune response to adenovirus consists of an innate immune response followed by a T helper 1 (Th1) response, $CD8^+$ cytotoxic T lymphocyte (CTL) response, and the production of neutralization antibody (94). The infiltration of large numbers of $CD4^+$, $CD8^+$ T, and B lymphocytes into the liver occurs around 5 - 7 days post adenovirus infection. It has been suggested that viral-specific $CD8^+$ T cells play a critical role in the virus clearance (95). Simultaneously, they also

contribute to liver inflammation by destruction of infected hepatocytes (96). $CD4^+$ T cells are thought to contribute to antiviral immune responses by secretion of cytokines, thereby providing help for $CD8^+$ T and B cells. The adaptive immune response may be caused by the immunogenicity of adenoviral capsid proteins and transgene protein, which may result in destruction of the infected hepatocytes that express these antigens. Depletion of $CD4^+$ or $CD8^+$ T cells can alleviate liver inflammation and prolong transgene expression (97). Strong Th1 and CTL responses against the viral vector and the transgene induce necroinflammatory hepatitis, and failure to constrain these responses will lead to treatment failure (98).

Liver inflammation caused by Ad infection is characterized by single cell necrosis and megaloblastic changes in hepatocytes. Hepatocyte apoptosis can be observed in the liver after 5 days post infection. The hepatic inflammation in the infected mice is characterized by significant portal and lobular lymphocytic infiltration, which consists of $CD4^+$ T, $CD8^+$ T, monocytes, NK cells, B cells, and granulocytes. Bridging necrosis and many apoptotic bodies can be found in all three adjacent zones. Meanwhile, serum ALT levels reach a peak during the virus infection. When compared to findings at the peak of inflammation, those after two to three weeks exhibit no obvious hepatocyte necrosis or apoptosis, and there is reduced lymphocyte infiltration of lobules.

1.6 IL-17 and IL-17-producing cells in hepatitis

Interleukin-17A (IL-17A), often referred to as IL-17, is produced by the T helper 17 (Th17) cells, which are distinct from the Th1 and Th2 populations (99). Th17 cells mainly produce IL-17A, IL-17F, IL-21 and IL-22, whereas Th1 and Th2 cells preferentially produce IFN-γ and IL-4, respectively (99, 100). To date, five additional structurally related cytokines were identified in the IL-17 family: IL-17B, IL-17C, IL-17D, IL-17E (also known as IL-25), and IL-17F (101). Among the family members, IL-17A and IL-17F share the highest amino acid sequence identity (around 50%), whereas IL-17E is the most divergent when compared to IL-17A (102). The *Il17f* and *Il17a* genes are closely located proximal to one another in the chromosomes in both humans and mice, whereas genes for the other members are located on different chromosomes (102). The IL-17 receptor (IL-17R) family includes five members (IL-17RA to IL-17RE). Functional IL-17 cytokine receptors are comprised of homodimers or heterodimers. For instance, the heterodimer of IL-17RA and IL-17RC serves as a receptor for both IL-17A and IL-17F, whereas the heterodimer of IL-17RA and IL-17RB is a receptor for IL-17E (102).

Initially, IL-17A and IL-17F were shown to be mainly expressed by activated $CD4^+$ T cells, named Th17 cells. Several cytokines are involved in Th17 cell differentiation, survival and effector functions, such as TGF-β, IL-6, IL-1β, IL-23 and IL-21 (103). RORγt is the key transcriptional factor to control the expression of IL-17A/F cytokines (103). Other transcription factors, including AHR, RORα, Batf, and Runx1, also play

important roles in Th17 cell differentiation (102). In addition to Th17 cells, a variety of other cells can also produce IL-17. NKT cells, $\gamma\delta$ T cells, $CD8^+$ T cells, neutrophils, and innate lymphoid cells (ILCs) can produce IL-17 under certain circumstances (104). Moreover, intestinal Paneth cells have been shown to produce IL-17A, whereas IL-17F mRNA is detected in colonic epithelial cells, suggesting to us that IL-17 species produced by non-lymphoid cells may also regulate immune responses (105, 106).

As we discussed above, IL-17A and IL-17F are highly homologous and share the same receptor. They also present similar biological functions. Both IL-17A and IL-17F are suggested to play important roles in host immunity against infection and development of inflammation by inducing pro-inflammatory cytokines, chemokines, and antimicrobial peptides (107, 108). They also recruit neutrophil migration to inflammatory tissue (109, 110). However, IL-17F has a relatively weaker ability to induce pro-inflammatory cytokine expression compared to that of IL-17A.

IL-17A and IL-17F have been indicated to play essential roles in autoimmune diseases (102). It has been demonstrated that IL-23 is important for the development of experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA), indicating involvement of Th17 cells (111, 112). Furthermore, EAE and CIA development are attenuated in IL-17A-deficient mice (106, 113). Interestingly, mice deficient for both IL-17A and IL-17F showed no additional alleviation of these

disorders compared to IL-17A$^{-/-}$ animals, possibly meaning that IL-17F does not have substantial additive or synergistic effects with IL-17A (106). The low activity of IL-17F during autoimmune responses may associate with its weak pro-inflammatory cytokine-inducing ability. On the other hand, both IL-17A and IL-17F were required for immune responses against extracellular bacterial infection (102). IL-17A$^{-/-}$IL-17F$^{-/-}$ mice were more susceptible to spontaneous *S. aureus* infections compared with IL-17F$^{-/-}$ or IL-17A$^{-/-}$ mice, indicating that IL-17A and IL-17F have overlapping roles in these models (114). IL-17A is also required for Th1 cell-type immune responses that protected the host against intracellular pathogens (115). In inflammatory bowel diseases, the roles of IL-17A and IL-17F seem controversial, since both pro-inflammatory or anti-inflammatory functions of IL-17 species have been reported in different experimental systems (116, 117).

Hepatic levels of IL-17 are significantly elevated in various hepatitis models and correlate with the severity of disease (118). IL-17 is mainly produced by Th17 cells in the liver. In addition, other immune cells including $\gamma\delta$ T cells, neutrophils, and NKT cells also contribute to the hepatic IL-17 expression (118). IL-17 is able to target various types of liver cells, such as hepatocytes, Kupffer cells, HSCs, biliary epithelial cells, and LSECs. IL-17 promotes a variety of pro-inflammatory cytokine and chemokine expression through binding of the IL-17R on different types of liver cells, and has a pro-inflammatory role in the pathogenesis of liver disorders.

It is also reported that the number of IL-17^{+} cells correlate with the severity of liver fibrosis in patients with viral hepatitis or alcoholic liver diseases. Furthermore, IL-17-producing cells have been shown to promote liver fibrosis by targeting of Kupffer cells and HSCs in bile duct ligation (BDL)- and CCl4-induced liver fibrosis (119). In Con A-induced T cell hepatitis, the role of IL-17 has been somewhat controversial. Over-expression of IL-17A resulted in massive hepatocyte necrosis, and anti-IL-17A blockage significantly ameliorated the disease (120). In addition, Lafdil *et al.* showed that liver injury was alleviated in Con A-induced hepatitis in IL-17A$^{-/-}$ mice (121). However, Zenewica *et al.* found that IL-17A$^{-/-}$ mice presented liver inflammation similar to that of wild-type mice after Con A injection (122). The discrepancy among these studies is not very clear. Recently, several reports have shown that Th17 cells play an essential role in viral hepatitis. For example, Th17 cells have been reported to be present in both the peripheral blood and liver of chronic hepatitis B and C patients, which indicates that IL-17A and Th17 cells exacerbate liver inflammation in these cases (123, 124). Interestingly, HCV nonstructural protein 4 (NS4) has been shown to induce TGF-β and IL-10 secretion by monocytes, which can suppress the virus-specific Th17 and Th1 cell responses (125). On the one hand, similar to IFN-γ, IL-17A may play a protective role in promoting viral clearance. On the other hand, the potent pro-inflammatory properties may also contribute to liver damage.

1.7 IL-22 and IL-22-producing cells in hepatitis

Interleukin-22 (IL-22) belongs to the IL-10 family, which includes IL-10, IL-19, IL-20, IL-24, IL-26, IL-28, and IL-29 (126). IL-22 is produced by several cell types, such as $CD4^+$ T cells, $CD8^+$ T cells, $\gamma\delta$ T cells, NK cells, NKT cells, neutrophils, and ILCs (127-132). The IL-22 receptor is a heterodimer comprised of IL-10R2 and IL-22R1 subunits (133). IL-10R2 is ubiquitously expressed, whereas IL-22R1 is restricted expressed on intestinal and respiratory epithelial cells, keratinocytes, and hepatocytes (134). It has been shown that IL-22 plays important roles in the host defense against extracellular pathogens at the mucosal surface (135). It maintains epithelial barrier functions and is involved in tissue homeostasis, tissue repair and wound healing (136-139). In contrast, uncontrolled excessive or prolonged IL-22 production can lead to pathology, such as psoriasis (140).

The role of IL-22 in host defense against extracellular pathogens has been studied in different infections of the lungs and intestines. For example, neutralization of IL-22 causes a significant bacterial dissemination in the lungs of animals infected by *K. pneumoniae* (141). IL-22 synergizes with IL-17 to enhance lung repair, as well as production of antimicrobial proteins, and pro-inflammatory chemokines and cytokines. IL-22-deficient mice are more susceptible to the *C. rodentium* infection compared to the wild-type animals (142). In addition, IL-22 also contributes to protection from *Salmonella* infection (143). Taken together, IL-22 protects a host against extracellular pathogens by the following mechanisms: 1) IL-22 maintains the integrity of the

epithelial barrier function during the pathogen invasion. 2) Synergistically with IL-17 or TNF-α, IL-22 induces the secretion of antimicrobial proteins (*i.e.* β-defensin, RegIIIb and RegIIIg) involved in host defense in the skin, the airways, and the intestines. 3) IL-22 promotes the production of inflammatory chemokines, such as CXCL1, CXCL5, and CXCL9 from airway epithelial cells during pathogen infection.

As discussed above, IL-22 induces both innate immunity and wound healing responses in the skin. However, excessive or prolonged expression of IL-22 can result in skin inflammation, such as psoriasis (140), a skin disease exhibiting high IL-22 levels and caused by keratinocyte hyperproliferation, and leukocyte infiltration and activation. In addition, IL-22 can synergize with IL-17 and IFN-γ to exacerbate inflammation by the induction of inflammatory chemokines from keratinocytes (144). In addition, IL-22 has been suggested to have a pathogenic role in rheumatoid arthritis. Increased numbers of Th17 cells producing IL-17 and IL-22 were found in the PBMCs and joints of rheumatoid arthritis patients (145). In a collagen-induced arthritis model, IL-22$^{-/-}$ mice displayed alleviated disease severity, and decreased pro-inflammatory cytokine profiles in the joints compared with those in wild-type animals (146).

More importantly, IL-22 promotes tissue repair and wound healing by enhancing epithelial cell proliferation and survival in various organs, such as the intestines, airways, and the liver (147-149). Many studies indicate that IL-22 plays an important

role in the prevention of hepatocellular damage in various liver injury models (118). Initially, IL-22 was found to be hepatoprotective against liver inflammation triggered by Con A, FasL, and carbon tetrachloride (CCL4) (150, 151). Further, IL-22-deficient mice were found highly susceptible to Con A-induced liver injury, whereas IL-22 transgenic mice with overexpression of IL-22 in the liver were resistant to such injury (122, 152). IL-22 protects against hepatocyte damage and promotes hepatocyte proliferation by activating the STAT3 signaling pathway, by which the expression of anti-apoptotic and mitogenic genes are upregulated (150). In a liver regeneration model, the levels of serum IL-22 protein and hepatic IL-22R1 mRNA expression were significantly increased after partial hepatectomy (153). In addition, IL-22 administration also ameliorated high fat diet- or ethanol-induced liver lipogenesis and hepatic steatosis (147). Recent studies have suggested that IL-22 also promotes liver progenitor cell growth in chronic viral hepatitis patients as well as in experimental mouse models (154).

IL-22 administration has been shown to ameliorate liver fibrosis by targeting hepatic stellate cells in a CCL4-induced liver fibrosis model (148). It is known that the activated HSC is a major source of collagens and TGF-β during hepatic fibrosis. IL-22 over-expression induces the senescence of hepatic stellate cells, decreased liver fibrogenesis and accelerated resolution of fibrosis (148). Moreover, IL-22 also plays a protective role in the development of lung fibrosis (129). Mice chronically infected with *Bacilllus subtilis* develop hypersensitive pneumonitis that progress to lung fibrosis. In

this model, the γδ T cell is the major source of IL-22. Neutralization of IL-22 accelerated lung fibrosis and collagen deposition. In contrast, IL-22 administration significantly alleviated lung fibrosis and decreased $CD4^+$ T cell recruitment to the lungs (129).

IL-22 has also been found to be up-regulated in several infection-related liver injury models; however, the roles of IL-22 in these models have been controversial. IL-22 has been found to play a hepatoprotective role in a primary *Plasmodium chabaudi and Salmonella infection* (143, 155), but IL-22 did not present any protective effects against liver injury infected by *Toxoplasma gondii and Mycobacterium avium* (156, 157). Additionally, IL-22 seems to contribute to the pathogenesis in a HBV transgenic mouse model (158).

1.8 Innate lymphoid cells

Recently, in addition to adaptive lymphoid cells, innate lymphoid cells (ILCs) are a major source of cytokines (159). These ILCs produce several T helper (Th) cell-associated cytokines, whereas they do not express any cell-surface markers related to other immune cell lineages (159). Furthermore, based on their type 1, type 2 and Th17 cell-associated cytokine profiles, these lineage marker-negative ILCs can be categorized into three groups: group 1 ILCs (Th1 cytokines), group 2 ILCs (Th2 cytokines) and group 3 ILCs (Th17 cytokines), respectively.

Group 1 ILCs (ILC1) comprise ILCs such as NK cells that produce type 1 cytokines, notably IFN-γ and TNF-α. In addition to NK cells, other IFN-γ-secreting ILCs have been reported (160).

Group 2 ILCs (ILC2) require IL-7 for their development and produce Th2 cell-associated cytokines in response to stimulation with the cytokines IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) (161-163). Similar to Th2 cells, group 2 ILCs are dependent on the transcription factor GATA3 for their development and function (164). They are involved in tissue repair following acute infection with influenza virus (165). However, group 2 ILCs also mediate pathology in a mouse model of virus-induced allergic asthma (166).

Similar to Th17 cells, group 3 ILCs (ILC3) are capable of producing the cytokines IL-17A and/or IL-22 and depend on RORγt for their development and function. In addition, the development of ILC3s depends on IL-7Rα. Several different subsets of group 3 ILCs have been described. First, the prototypical group 3 ILCs are lymphoid-tissue inducer (LTi) cells, which are important for the secondary lymphoid organ formation during embryogenesis (167). More recently, a second subset of group 3 ILCs expressing the natural cytotoxic receptor (NCR) NKp46, named NCR^+ILC3s, has been found to produce IL-22 but not IL-17A. IL-22-producing ILC3s are crucial for the IL-22-mediated innate immune response against bacterial infection (such as *Citrobacter rodentium*) in the gut (168). However, a third ILC3 subset was

found to mediate pathogenesis in a mouse model of innate colitis (132). This subset produces IFN-γ, IL-17A as well as IL-22, and did not express NKp46.

In the last several years, ILCs have also been found to be involved in innate immunity as well as tissue remodeling (159). However, ILCs in the liver are still not well understood. Recently, it has been shown that group 2 ILCs in the liver can protect against acute viral hepatitis (169), but can also mediate hepatic fibrosis (170). In a con A-induced hepatitis, group 3 ILCs have been shown to play a protective role by their production of IL-22 in the liver (171).

1.9 Objective of this study

Hepatic levels of IL-17 are significantly elevated in viral hepatitis, alcoholic liver disease, autoimmune hepatitis, and hepatocellular carcinoma, and correlate with the severity of disease (118). Among IL-17 family members, IL-17A and IL-17F share the highest sequence homology and similar biological functions. IL-17F is known to be a weaker inducer of pro-inflammatory responses and is produced by a wider range of cell types (106). During viral hepatitis, the cellular source, functions, and gene regulation of IL-17A/F in the liver are not well understood. The hepatoprotective role of IL-22 in liver injury has been well documented; however, little is known about the regulatory pathways controlling IL-22 production in the liver. In addition, the source of the liver-derived IL-22 in viral hepatitis is still not well defined. More recently, ILCs have been found to be involved in innate immunity as well as tissue remodeling in

various models (159). Intrahepatic ILC2s and ILC3s have been shown to protect against acute hepatitis; however, ILC2s also contribute to liver fibrosis (169-171). In viral hepatitis, the role and cytokine profiles of ILCs in the liver are not clearly described.

Therefore, the objective of this dissertation is to identify the cellular source and immune regulation of hepatic IL-17 and IL-22 in viral hepatitis. In addition, we plan to investigate the dynamics and potential roles of intrahepatic ILC3s during the early stages of viral infection in the liver. Collectively, these studies may improve our understanding of innate immunity and its immune regulation in the liver during viral infection.

CHAPTER 2: INTRAHEPATIC INNATE LYMPHOID CELLS SECRETE IL-17A AND IL-17F THAT ARE CRUCIAL FOR T CELL PRIMING IN VIRAL HEPATITIS

2.1 Introduction

Viral hepatitis is one of the most important public health problems globally. Many viruses can cause acute or chronic hepatitis, typically hepatitis A through E, as well as liver infections caused by adenovirus (Ad) and several other viruses. In most cases, patients' immune responses to a virus or a viral strain vary greatly, which leads to a wide range of clinical manifestations and prognosis, from disease resolution to fulminant hepatitis, viral persistence and liver failure. In previous studies, virus-specific $CD8^+$ and $CD4^+$ T cell functions have been found to be critical in viral clearance and disease resolution (95). More recently, IL-17 production has been reported in hepatitis B and C infections (123, 124). In these cases, hepatic levels of IL-17 are significantly elevated in viral hepatitis, alcoholic liver disease, autoimmune hepatitis, and hepatocellular carcinoma, and correlate with the severity of disease (118). In previous work, we found that hepatic IL-17 produced early in Ad infection played a critical role in initiating successful antiviral $CD8^+$ and $CD4^+$ T cell responses (172). To date, the source of the liver-derived IL-17 species are not well understood, and their immune functions remain debatable (120-122, 173, 174).

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[1] *Z. Jie et al., J Immunol 2014 192:3289-300. Used with permission (refer to Appendix on page 97).*

As we mentioned before, IL-17 has been found to act as a potent inducer in T cell-mediated immune responses by activating and recruiting DCs, monocytes and neutrophils in various tissues including the liver (172, 175, 176). It is known to be produced by the Th17 cells and several other cell types (104). While IL-17 is typically associated with destructive tissue damage in autoimmune diseases and bacterial infections (113, 115, 177), more recently, it was found to promote Th1 and CTL responses in anti-tumor immunity (178), inflammatory bowel disease (179), and antiviral immune responses (172). Among IL-17 family members, IL-17A and IL-17F share the highest sequence homology and similar biological functions. Both cytokines bind to the same heterodimeric receptor molecule comprised by the IL-17RA and RC chains (102). Although IL-17F is known to be a weaker inducer of pro-inflammatory responses and is produced by a wider range of cell types (106), its cellular source, functions, and gene regulation in the liver, particularly during the T cell priming phase of viral hepatitis, are not well understood.

Lymphocytic choriomeningitis virus (LCMV) is a prototypical virus used in animal models of acute and persistent hepatitis (182). As we discussed above, adenovirus (Ad) is an important pathogen and one of the preferred vectors for gene and cancer therapy, and experimental vaccines for human immunodeficiency and hepatitis C viruses (180, 181). These viruses target the liver when given intravenously (*i.v.*) and can induce strong innate immune responses, T helper (Th), cytotoxic T lymphocyte (CTL), and B cell responses against viruses (183-186). They are eliminated by innate

immune mechanisms initially (41, 187-189). In subsequent periods, virus elimination and liver pathology are mediated by cytotoxic and helper T cells (94, 186). In previous work, we found that IL-17 was produced in the liver within the first day of Ad infection (172). This brief surge played a critical role in initiating full $CD8^+$ and $CD4^+$ T cell responses. Furthermore, this early IL-17A was produced by $\gamma\delta$ T cells, along with another yet-to-be characterized population in the liver.

In this work, we found novel innate lymphoid cells (ILCs) as a major source of hepatic IL-17A and IL-17F production in addition to $\gamma\delta$ T cells. In the last several years, ILCs have been found to be involved in innate immunity as well as tissue remodeling (159). In the liver, ILCs have been shown to protect against acute hepatitis (169, 171), but can also mediate hepatic fibrosis (170). In addition, we found that IL-17 signaling was important for adaptive T cell responses in viral hepatitis. IL-17F engagement was crucial for effector T cell functions and antiviral responses. Moreover, IL-17A could negatively regulate IL-17F production. Collectively, this study unveiled a previously unknown source and crosstalk between IL-17A and F in the liver, and may provide potential therapeutic approaches to target ILCs and IL-17 species in viral hepatitis.

2.2 Material and methods

Animals

Female C57BL/6 (B6) and Rag2$^{-/-}$ mice were purchased from the Jackson Laboratory. Mice deficient in IL-17RA (IL-17R$^{-/-}$ mice) were provided by Amgen Inc. Mice deficient in gamma delta T cells ($\gamma\delta^{-/-}$) were kindly provided by Dr. Tian Wang (University of Texas Medical Branch, Galveston, TX). IL-17A$^{-/-}$ and IL-17F$^{-/-}$ mice were reported previously (113, 190). Naïve IL-17R$^{-/-}$, IL-17A$^{-/-}$ and IL-17F$^{-/-}$ mice displayed normal liver function as wild-type mice. All mice were maintained and bred under specific pathogen-free conditions in the animal facility at the University of Texas Medical Branch. Eight- to twelve-wk-old mice were used for all the experiments. All experiments were reviewed and approved by the Institutional Animal Care and Use Committees of the University of Texas Medical Branch. To induce hepatitis, mice were *i.v.* injected with 3×10^9 pfu replication-deficient recombinant Ad carrying the LacZ gene (AdLacZ, purchased from Vector Development Laboratory of Baylor College of Medicine) as described previously (184). Mice were *i.v.* injected with 2×10^6 pfu lymphocytic choriomeningitis virus (LCMV) Clone 13 (a kind gift from Dr. Maria Salvato at the University of Maryland) (191). Titration of LCMV was performed on Vero cell monolayers plated on 24-well plates, followed by the viral quantification of immunological focus assay (192). The antibody of LCMV was kindly provided by Dr. Robert Tesh from the University of Texas Medical Branch.

Antibodies and reagents

Recombinant mouse IL-17A, mouse IL-17F, mouse IL-6 and human TGF-β were purchased from Peprotech. All fluorochrome-labeled monoclonal antibodies (Abs) and their corresponding isotype controls were anti-mouse Abs and purchased from eBioscience (San Diego, CA), BD Pharmingen (San Diego, CA) and Biolegend (San Diego, CA). The following were purchased from eBioscience: PE-conjugated anti-IL-17A (eBio17B7), APC-conjugated anti-IL-17A (eBio17B7), FITC-conjugated anti-IFN-γ (XMG1.2), APC-conjugated anti-IFN-γ (XMG1.2), APC-conjugated anti-TCRγδ (eBioGL3), PE-Cy7-conjugated anti-CD3 (17A2), Pacific blue-conjugated anti-CD4 (GK1.5), PerCp-Cy5.5-conjugated anti-CD8 (53-6.7), APC-conjugated anti-NK1.1 (PK136), Alexa Fluor 488-conjugated anti-CD107a (1D4B), Alexa Fluor 488-conjugated anti-CD107b (ABL-93), FITC conjugated anti-B220 (RA3-6B2), FITC conjugated anti-CD11b (M1/70), FITC conjugated anti-CD11c (N418), FITC conjugated anti-Gr-1 (RB6-8C5), FITC conjugated anti-Ter-119 (TER-119), FITC conjugated anti-NK1.1 (PK136), FITC conjugated anti-CD90.2 (30-H12), APC conjugated anti-RORγt (B2D), and Biotin conjugated anti-c-kit (2B8). APC-conjugated anti-IL-17F (O79-289) and Purified anti-CD16/32 (2.4G2) were purchased from BD Pharmingen. PerCp-Cy5.5 conjugated anti-NKp46 (29A1.4), APC-Cy7 conjugated anti-CD3 (17A2), APC-Cy7 conjugated anti-Sca-1 (D7), PerCp-Cy5.5 conjugated anti-ICOS (C398.4A) were purchased from Biolegend.

H&E and histological scores

Liver specimens were fixed in 10% buffered formalin. Paraffin-embedded sections were stained with H&E for histological evaluation by using a modified Knodell scoring system (193). Briefly, normal liver architecture without remarkable injury or cellular infiltration was scored as 0. A score of 1 represented limited infiltration of inflammatory cells in the portal triad without significant involvement in the lobular and pericentral regions. In addition to these pathological changes, a score of 2 reflected a moderate involvement in the portal areas, accompanied by isolated apoptosis and necrosis in the lobular and pericentral areas. A score of 3 involved extensive lymphocyte infiltration in the portal area with widespread apoptosis and bridging necrosis throughout the liver.

Isolation of intrahepatic lymphocytes

Intrahepatic lymphocytes were isolated according to our previous method with slight modifications (184). Briefly, liver tissue was pressed and collected in complete RPMI-1640. After washing (300 × *g*, 10 min), cell suspensions were re-suspended in complete RPMI-1640 containing collagenase IV (0.05%, Roche Applied Science, Indianapolis, IN) at 37°C for 30 min. After digestion, cell suspensions were passed through 70-μm nylon cell strainers to yield single-cell suspensions. Intrahepatic mononuclear cells were purified by centrifugation (400 × *g*) at room temperature for 30 min over a 30/70% discontinuous Percoll gradient (Sigma). The cells were collected from the interphase, thoroughly washed, and re-suspended in complete

RPMI 1640 containing 10% FBS (Hyclone, Logan, UT). The total numbers of intrahepatic lymphocytes per liver were counted. The relative percentages of $CD4^+$, $CD8^+$ and γδ T cells were measured by flow cytometry, and the absolute numbers of these lymphocyte subpopulations per liver were calculated according to their percentages and the total intrahepatic lymphocyte numbers in each liver.

Intracellular staining

Intracellular staining was performed according to our previous methods (172). Briefly, cells were incubated for 4 h with PMA (50 ng/ml) and ionomycin (750 ng/ml). For the simultaneous detection of surface CD107a/b (LAMP-1/2) and intracellular cytokines, cells were stimulated by plate-coated anti-CD3 mAb (145-2C11, 10 μg/ml, eBioscience) for 4 h, in the presence of GolgiStop (BD Bioscience). After incubation, cells were collected and blocked with FcγR blocker (CD16/32) and stained for specific surface molecules. After surface staining, cells were fixed, permeabilized and stained for intracellular cytokines by using a fixation/permeabilization kit (eBioscience).

Flow cytometry analysis

Murine lymphocytes were blocked with anti-mCD16/CD32 (eBioscience) and stained with fluorochrome-labeled antibodies, and then processed on an LSRII FACSFortessa (Becton Dickinson, San Jose, CA) and analyzed by using FlowJo software (TreeStar, Ashland, OR). All fluorochrome-labeled mAbs and their corresponding isotype controls were purchased from BD Pharmingen (San Diego, CA)

and eBioscience (San Diego, CA).

Polarization of IL-17-producing splenocytes

To polarize IL-17-producing cells, total splenocytes were cultured on the pre-coated anti-CD3 Ab (5 μg/ml) with 10 ng/ml TGF-β, 20 ng/ml IL-6, 10 μg/ml anti-IFN-γ, and 10 μg/ml anti-IL-4. After 4 days of culture, cells were rested for 1 day and then stimulated with PMA (50 ng/ml) and ionomycin (750 ng/ml). Cells were stained with fluorescence-labeled surface Abs, including anti-m-CD3, anti-m-CD4, anti-m-CD8, and anti-m-TCRγδ. After surface staining, cells were fixed, permeabilized and counterstained with fluorescence-labeled Abs for IL-17A and IL-17F.

Real-time PCR

Frozen liver tissues were used to extract genomic DNA and total RNA. DNA was extracted with a DNeasy blood and tissue kit (Qiagen), and total RNA was extracted with an RNeasy Mini kit (Qiagen) and digested with DNase I (Ambion). The concentrations of DNA and RNA were measured by using a spectrophotometer (Eppendorf). For relative quantitation of the cytokine and chemokine mRNA levels, cDNA was prepared from 1 μg of RNA by using an iScript^{TM} Reverse Transcription Kit (Bio-Rad), and 4 μl of the cDNA was amplified in a 25-μl reaction mixture containing 12.5 μl of iQ SYBR Green Supermix (Bio-Rad) and 0.9 μM each of gene-specific forward and reverse primers. The PCR assays were denatured for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. The PCR was performed with

the CFX96 Touch real-time PCR detection system (Bio-Rad). Relative quantitation of mRNA expression was calculated as the fold increase in expression by using the $2^{-\Delta\Delta Ct}$ method. Meanwhile, melting curve analysis was used to check the specificity of the amplification reaction. The sequences of the forward and reverse gene-specific primers used are listed in Table 1.

ELISA assays

Liver proteins were extracted from frozen liver tissues by homogenization on ice in the RIPA Buffer (Cell Signaling) with a protease inhibitor cocktail (Sigma). After centrifugation at 20,000 × *g* for 15 min, the supernatant was collected and protein concentration was measured with a protein assay kit (Bio-Rad). Equal amounts of the liver proteins (100 μg) were loaded for ELISA assays. The levels of IL-17A and IL-17F in the liver proteins were measured by using the ELISA kits (eBioscience) according to the manufacturer's instructions. Detection limits were 4 pg/ml for IL-17A, and 15 pg/ml for IL-17F, respectively.

Statistical analysis

The difference between the two different groups was determined by using Student's *t* test. One-way ANOVA was used for multiple group comparisons (GraphPad Software v4.0). P values < 0.05 were considered significant*, and < 0.01 as highly significant**.

2.3 Results

Early IL-17A/F produced by classical and non-classical intrahepatic cells

Early IL-17A production by intrahepatic γδ T cells is known to be important for adaptive immune responses in Ad-induced hepatitis (172). IL-17F, the closest homolog to IL-17A among members of the IL-17 cytokine family, has partially concordant expression with and shares the same receptor with IL-17A (102). However, the precise role of IL-17F in viral hepatitis is still not well understood. To define the dynamics of IL-17A and IL-17F production in the course of Ad infection, we *i.v.* injected B6 mice with 3×10^9 pfu of AdLacZ. The animals were sacrificed at 0, 12, 24 h, and on days 3 and 6 post-infection. ELISA analysis of liver lysates revealed a significant accumulation of IL-17A and IL-17F during the first 24 h post infection (**Fig. 1A**). Meanwhile, we isolated the intrahepatic lymphocytes (IHLs) and analyzed their intracellular levels of IL-17A and IL-17F by flow cytometry. We found that the IL-17A$^+$ IL-17F$^-$ cells expanded from 1.0% at 0 h to 2.0% at 24 h post-infection (**Fig. 1B**). The absolute cell number of IL-17A$^+$ IL-17F$^-$ cells expanded from 2.2×10^4 cells at 0 h to 8.9×10^4 cells at 24 h post-infection (**Fig. 1C**). In addition, the IL-17A$^+$ IL-17F$^+$ cells expanded robustly, from 0.2% at 0 h to 0.5% at 24 h post-infection (**Fig. 1B**). The absolute cell number of IL-17A$^+$ IL-17F$^+$ cells expanded from 0.4×10^4 cells at 0 h to 2.0×10^4 cells at 24 h post-infection (**Fig. 1C**). IL-17A- or IL-17F-producing cells did not expand in the spleen (data not shown), which suggested to us that this surge of IL-17A$^+$ or IL-17F$^+$ cells was liver-specific. In addition, the increases of IL-17$^+$ cells in the liver were accompanied by a surge of IL-17 in the serum as we reported

previously (172).

Both innate and acquired T cells were reported to produce IL-17A and IL-17F (104). While most IL-17$^+$ T cells in the liver were indeed $\gamma\delta$ T cells at 24 h post-infection (**Fig. 1D**), small populations of IL-17$^+$ $\gamma\delta^-$ intrahepatic lymphocytes were heterogeneous. We therefore characterized the remaining IL-17 producers among IHLs (**Fig. 1D**). To our surprise, in the CD3$^-$ population, the IL-17A-producers did not express lineage markers, such as CD8, CD11b, CD11c, NK1.1, B220, Gr-1, and Ter-119. Further study showed that similar cells from a lineage-negative population in the liver produced IL-17F (**Fig. 2**). Collectively, these data suggested that early surges of IL-17A/F were produced by both classical $\gamma\delta$ T cells, as well as non-classical innate lymphoid cells.

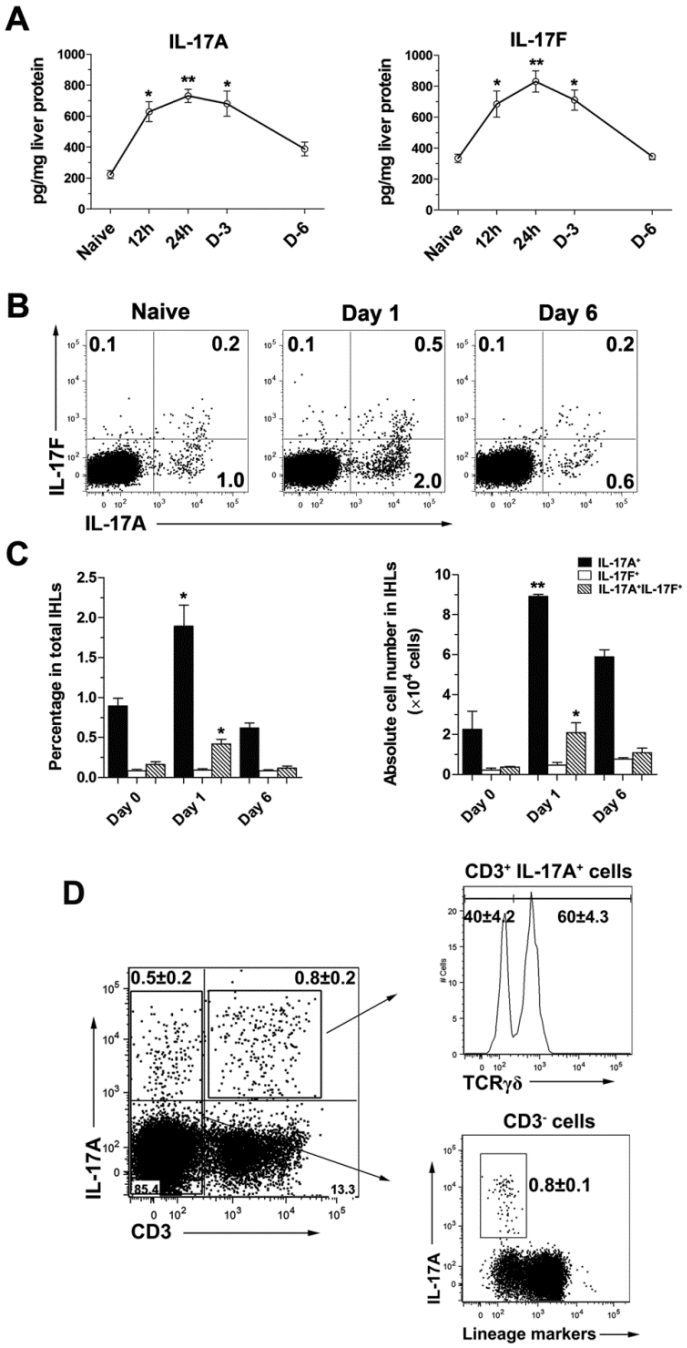

Figure 1 Early IL-17A/F produced by classical and non-classical intrahepatic cells. C57BL/6 mice were injected *i.v.* with 3×10^9 pfu of AdLacZ and sacrificed at the indicated time points. The liver tissues were collected, and IHLs were isolated after perfusion. **(A)** Liver proteins were extracted and liver IL-17A and IL-17F levels were detected by an ELISA. **(B)** IHLs were isolated and stimulated with PMA and ionomycin for 4 h in the presence of GolgiStop. The cells were collected and examined by flow cytometry for intracellular IL-17A and IL-17F. **(C)** *Left panel*: Cumulative statistical results of the percentages of IL-17A$^+$IL-17F$^-$, IL-17A$^-$IL-17F$^+$ and IL-17A$^+$IL-17F$^+$ cells in the liver, respectively. *Right panel*: Cumulative statistical results of absolute cell number of IL-17A$^+$IL-17F$^-$, IL-17A$^-$IL-17F$^+$ and IL-17A$^+$IL-17F$^+$ cells in the liver, respectively. (**D**) Flow cytometric plots of CD3$^+$, CD3$^-$, TCR$\gamma\delta^+$, TCR$\gamma\delta^-$ and lineage-negative cells (lineage markers: CD8, CD11b, CD11c, NK1.1, B220, Gr-1, and Ter-119) producing IL-17A. Values were shown as means ± SEM. Data were compared to the naïve mice and a two-tailed *t* test was used for statistical analysis, **p* < 0.05, ***p* < 0.01.

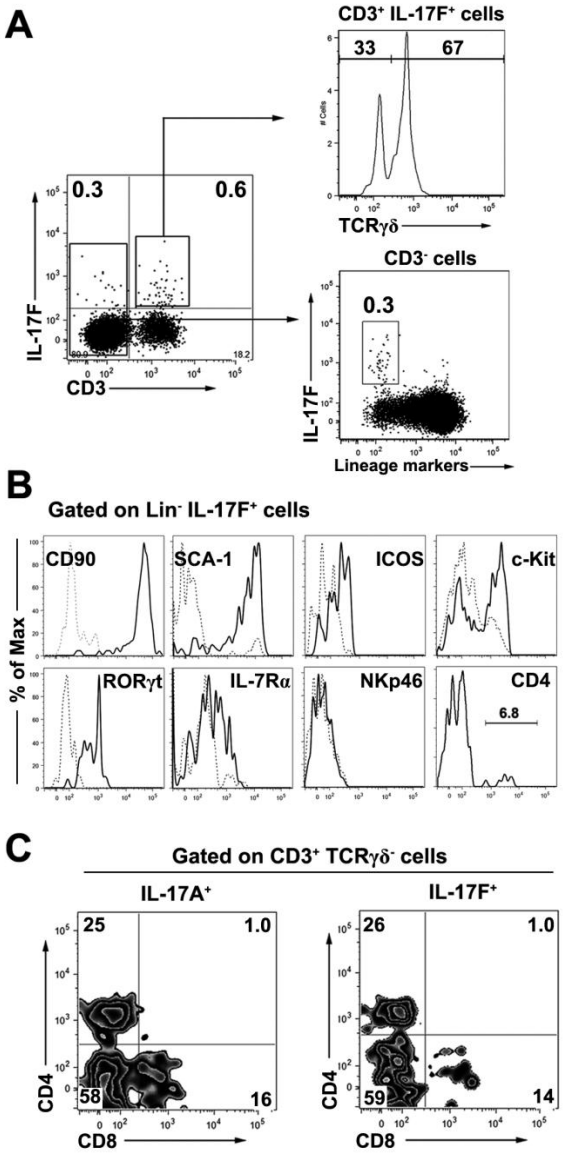

Figure 2 γδ T cells and group 3 innate lymphoid cells were important sources of IL-17F. C57BL/6 mice were injected *i.v.* with 3×10^9 pfu of AdLacZ and sacrificed at day 1 after infection. The IHL were isolated after liver perfusion. IHL were stimulated with PMA and ionomycin for 4 h in the presence of GolgiStop. The cells were collected and examined using flow cytometry for intracellular IL-17F. **(A)** Flow cytometric plot of $CD3^+$, $CD3^-$, $TCR\gamma\delta^+$, $TCR\gamma\delta^-$ and lineage-negative (Lin^-) cells producing IL-17F. **(B)** The Lin^- $IL\text{-}17F^+$ cells in the wild-type group were gated for the further detection of intracellular and surface markers (CD90, Sca-1, ICOS, c-Kit, RORγt, IL-7Rα, NKp46 and CD4). The dotted lines represent the isotype control, and the solid lines indicated Ab staining. **(C)** IHLs were gated on $CD3^+$ $TCR\gamma\delta^-$ cells. $IL\text{-}17A^+$ and $IL\text{-}17F^+$ cells were further analyzed their CD4 and CD8 expression, respectively. (Abbreviation: Lin, lineage)

Group 3 innate lymphoid cells (ILC3s) were important source of IL-17 in the liver

To further characterize these lineage-negative cells that produced IL-17A and IL-17F, we analyzed their surface markers. Interestingly, these cells expressed high levels of CD90, Sca-1, ICOS, c-Kit, RORγt, and IL-7Rα, but low levels of NKp46 and CD4 (**Fig. 3A**). The phenotypical and functional characteristics of these cells were consistent with ILC3s, originally described in the gastrointestinal tract (194). Based on the surface markers of these innate cells in the liver, we further concluded that most of these ILC3s belonged to the $NKp46^-$ ILC3 population. In addition, they expanded robustly and peaked within the first 24 h post Ad-infection and waned in the next few days (**Fig. 3B**). The remaining $IL\text{-}17A^+$ cells were comprised of double-negative, $CD4^+$ and $CD8^+$ T cells (**Fig. 2C**). Th17 cells contributed only small amounts of IL-17 in this model (**Fig. 2C**). Interestingly, similar populations of IHLs produced IL-17F following Ad infection (**Fig. 2 A-C**). To examine whether intrahepatic ILC3s are present in a persistent viral infection model, we *i.v.* injected B6 mice with 2×10^6 pfu of LCMV Clone 13. Similar to our previous findings in Ad model, nearly all $CD3^-$ $IL\text{-}17^+$ cells did not express lineage markers, including CD8, CD11b, CD11c, NK1.1, B220, Gr-1, and Ter-119 (**Fig. 3C**). These results indicated that, in addition to Ad infection, ILC3 secreting IL-17 was also present in the liver after LCMV infection.

To further confirm that ILC3s in the liver can produce the IL-17 species, we examined the IL-17A/F levels in the mice deficient of T and B lymphocytes (195). IHLs from

uninfected Rag2$^{-/-}$ and wild-type mice were analyzed by flow cytometry. In the wild-type animals, there were about 0.8% IL-17A^{+} cells, 0.2% IL-17F^{+} cells and 0.2% IL-17A^{+} IL-17F^{+} cells (**Fig. 3D**). In Rag2$^{-/-}$ mice, however, there were more than 2-fold and 10-fold increases of IL-17A^{+} and IL-17A^{+} IL-17F^{+} cells, respectively, among the total IHLs. These results were confirmed in the lineage-negative IHLs as well (**Fig. 3D**).

Given that ILC3s and γδ T cells were important sources of early IL-17A/F production in the liver, we investigated the role of ILC3-derived IL-17A/F in the outcome of Ad-induced hepatitis using γδ$^{-/-}$ mice. Surprisingly, IL-17A production in γδ$^{-/-}$ mice was comparable to that in the wild-type animals at day 1 post infection (**Fig. 4A**). The γδ^{-} IL-17A^{+} cells in γδ$^{-/-}$ mice increased more than 3-fold compared to those in controls (**Fig. 4A**). Further study revealed that these γδ^{-} IL-17A^{+} cells were composed by ILC3s and double-negative T cells (data not shown). At day 6 post infection, we found Ad-infected γδ$^{-/-}$ mice displayed comparable intrahepatic lymphocytes infiltration and Th1/CTL functions (**Fig. 4B and 4C**). In addition, γδ$^{-/-}$ mice presented similar liver inflammation and pathological scores compared to the control animals (**Fig. 4B**). Taken together, these results indicated that ILC3 in the liver can secret the IL-17 species and, hence, is an important source of IL-17 in the liver.

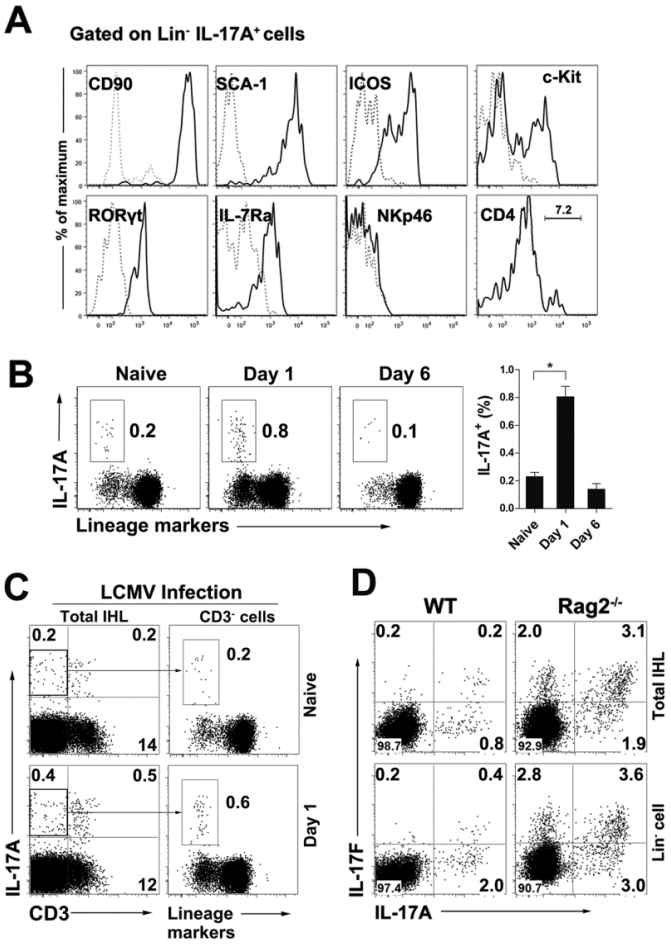

Figure 3 Group 3 innate lymphoid cell was an important source of IL-17A.

(A) Mice were infected as Fig. 1 and sacrificed at day 1 after infection. The IHL were isolated after liver perfusion and stimulated with PMA and ionomycin for 4 h in the presence of GolgiStop. The IHLs were gated on $CD3^-$ population. The cells were further gated on negative populations of lineage (Lin) markers (CD8, CD11b, CD11c, NK1.1, B220, Gr-1, and Ter-119) and analyzed for intracellular IL-17A. The Lin^- $IL\text{-}17A^+$ cells were gated for detection of surface and intracellular markers (CD90, Sca-1, ICOS, c-Kit, RORγt, IL-7Rα, NKp46 and CD4). The dotted lines represent the isotype control and the solid lines indicated Ab staining. **(B)** Mice were infected as Fig. 1 and sacrificed at the indicated time points. IHLs were stimulated and gated on $CD3^-$ population. The cells were gated on negative populations of lineage markers, and intracellular IL-17A was analyzed by flow cytometry. *Right panel*: Cumulative statistical results of flow cytometry data. **(C)** Mice were injected *i.v.* with 2×10^6 pfu of LCMV clone 13 and sacrificed at day 1 after infection. IHLs were isolated and stimulated with PMA and ionomycin. The cells were collected and examined by flow cytometry for intracellular IL-17A. IHLs were further gated on $CD3^-$ population and analyzed the IL-17A and lineage markers. (**D**) The IHL from $Rag2^{-/-}$ and wild-type mice were isolated and stimulated with PMA and ionomycin for 4 h in the presence of GolgiStop. Intracellular IL-17A and IL-17F of total IHL and lineage negative population were analyzed by flow cytometry. (Abbreviation: Lin, lineage)

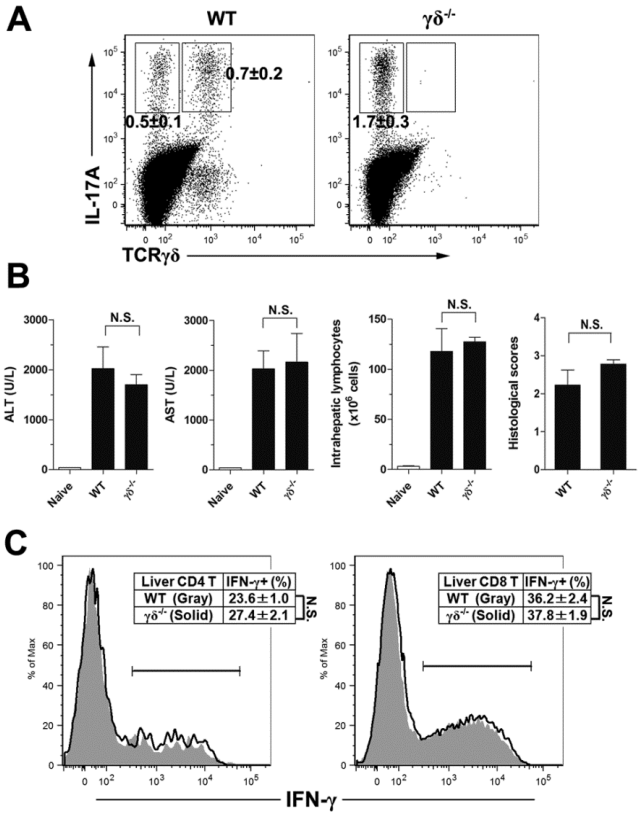

Figure 4 Deficiency of γδ T cells did not affect early IL-17A production and Th1/CTL responses in Ad-induced hepatitis. (A) C57BL/6 and $\gamma\delta^{-/-}$ mice were injected *i.v.* with 3 × 10^9 pfu of AdLacZ and sacrificed at day 1 post infection. IHLs were isolated and stimulated with PMA and ionomycin for 4 h in the presence of GolgiStop. The cells were collected and examined by flow cytometry for intracellular IL-17A. **(B)** Ad-infected C57BL/6 and $\gamma\delta^{-/-}$ mice were sacrificed at day 6 post infection. Serum ALT/AST levels, absolute cell numbers of total IHLs and histological scores of wild-type and $\gamma\delta^{-/-}$ mice are shown. **(C)** Ad-infected C57BL/6 and $\gamma\delta^{-/-}$ mice were sacrificed at day 6 post infection. IHLs were isolated and stimulated with anti-CD3 for 4 h in the presence of GolgiStop. The cells were then stained for surface markers (CD3, CD4 and CD8) and analyzed for intracellular IFN-γ. The experiment was repeated three times independently, and representative graphs are shown (n = 3 - 6 mice per group). Values were shown as means ± SEM. A two-tailed *t* test was used for statistical analysis (N.S. indicates no significance).

IL-17 signaling was critical for adaptive T cell responses and infiltration

Ad infection induced strong $CD4^+$ T cell and $CD8^+$ T cell recruitment into the liver. To determine whether this early surge of IL-17A and IL-17F signals through IL-17 receptor (IL-17R) and mediates T cell responses, we injected the wild-type and IL-$17R^{-/-}$ mice with 3 × 10^9 pfu of AdLacZ. At day 6 post infection, we examined the

accumulation of total intrahepatic lymphocytes, $CD4^+$ T cells and $CD8^+$ T cells in these animals. Compared with the control animals, IL-$17R^{-/-}$ mice developed significantly less lymphocyte infiltration, with reduced numbers of $CD4^+$ and $CD8^+$ T cells (**Fig. 5A**). A lack of IL-17 signaling significantly reduced IFN-γ-producing ability among $CD8^+$ T cells in the liver (**Fig. 5B**). In addition, considerable IFN-γ^+ cells (14 ± 0.5%) in the control mice expressed LAMP-1/2 (CD107a/b), indicative of their ability to de-granulate cytolytic vesicles. In the IL-$17R^{-/-}$ animals, due to the reduced IFN-γ-producing ability of intrahepatic $CD8^+$ T cells, significantly lower percentages (9 ± 2%) and fewer numbers of these cells are IFN-γ^+ CD107a/b^+ ones (**Fig. 5B**). As found with $CD8^+$ T cells, lower percentages and fewer numbers of $CD4^+$ T cells expressed IFN-γ (**Fig. 5C**).The presence or absence of IL-17 signaling did not significantly change the percentages of Treg cells in the liver (**Fig. 5C**). Due to the decrease in the total number of IHLs in IL-$17R^{-/-}$ mice, there were fewer numbers of Treg cells in these animals (**Fig. 5C**).

To determine the effect of IL-17 signaling on disease outcomes, we examined the pathology of Ad-infected IL-$17R^{-/-}$ mice. The control animals developed hepatitis characterized by inflammatory infiltration, hepatocytes with megaloblastic changes, and single-cell necrosis at 6 days post-infection (**Fig. 6A**). Compared to the wild-type mice, the Ad-infected IL-$17R^{-/-}$ mice displayed much milder inflammation and lower pathological scores (**Fig. 6**), as well as lower serum ALT and AST levels (**Fig. 6B**). Taken together, the lack of IL-17 signaling impaired the recruitment and functions of

intrahepatic Th1/CTL responses and ameliorated Ad-induced hepatitis.

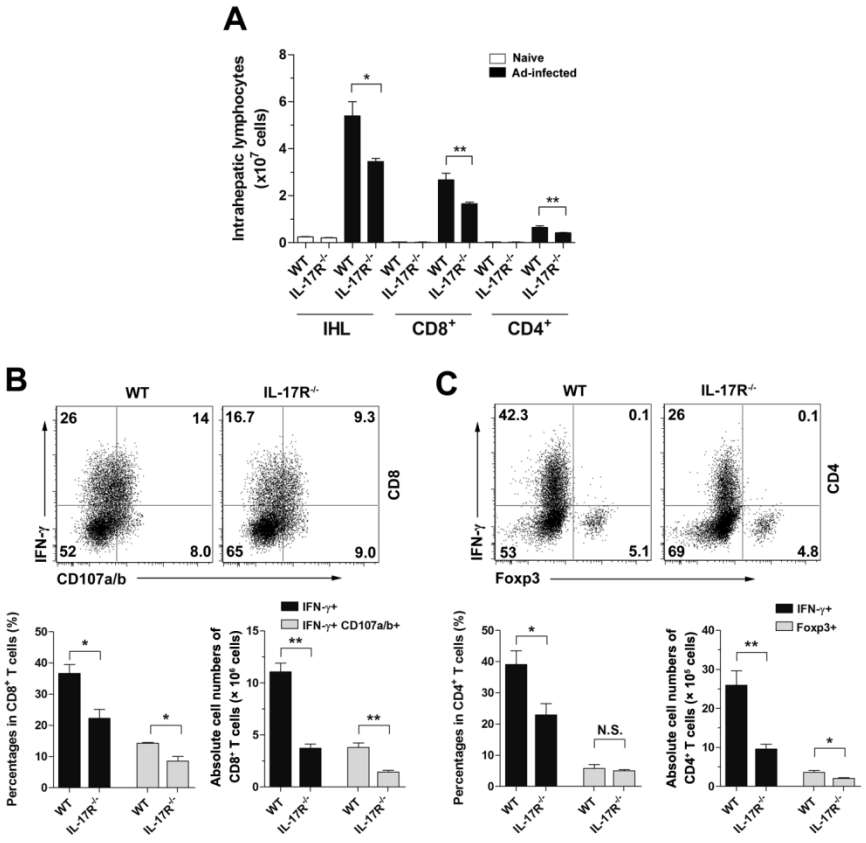

Figure 5 Impaired Th1 and CTL functions in Ad-infected IL-17R$^{-/-}$ mice. C57BL/6 and IL-17R$^{-/-}$ mice were injected *i.v.* with 3 × 10^9 pfu of AdLacZ and sacrificed at day 6 post infection. IHLs were isolated and stimulated with anti-CD3 for 4 h in the presence of GolgiStop. The cells were then stained for surface markers (CD3, CD4 and CD8) and intracellular cytokines and examined by flow cytometry. Shown are representative flow cytometric results. **(A)** Absolute cell numbers of total IHLs and intrahepatic CD4$^+$ and CD8$^+$ T cells of Ad-infected wild-type and IL-17R$^{-/-}$ mice. **(B)** *Upper panel:* Flow cytometric analysis of IFN-γ and CD107a/b levels of intrahepatic CD8$^+$ T cells. *Lower panel:* Cumulative statistical results of the percentages and absolute cell numbers of IFN-γ$^+$ and IFN-γ$^+$ CD107a/b$^+$ cells in the intrahepatic CD8$^+$ T cells. **(C)** *Upper panel:* Flow cytometric analysis of IFN-γ and Foxp3 of intrahepatic CD4$^+$ T cells. *Lower panel:* Cumulative statistical results of the percentages and absolute cell numbers of IFN-γ$^+$ and Foxp3$^+$ cells in the intrahepatic CD4$^+$ T cells. The experiment was repeated two to three times independently, and a representative graph is shown (n = 6 – 8 mice per group). Values were shown as means ± SEM. A two-tailed *t* test was used for group-to-group comparison. Results were expressed with asterisks (*p < 0.05; **p <0.01).

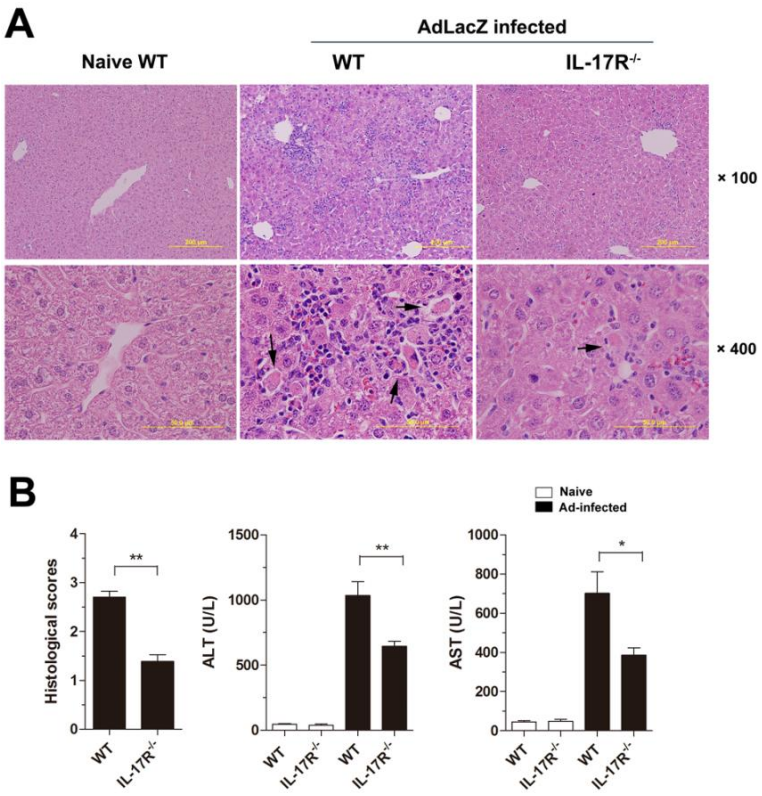

Figure 6 Lack of IL-17RA signaling ameliorated Ad-induced hepatitis. C57BL/6 and IL-17R$^{-/-}$ mice were injected *i.v.* with 3 × 10^9 pfu of AdLacZ and sacrificed at day 6 post infection. The serum was prepared and liver tissues were isolated after perfusion. **(A)** Liver tissues were obtained from uninfected wild-type, Ad infected wild-type and IL-17R$^{-/-}$ mice, and the tissue section were stained with H&E. Shown are representative images. The arrows indicate apoptotic bodies in the liver sections. Original magnification: *upper panels*, × 100; *lower panels*, ×400. **(B)** Cumulative graphical representation of the histological scores, serum ALT and AST levels of wild-type and IL-17R$^{-/-}$ mice. The experiment was repeated two to three times independently, and representative graphs are shown (n = 6 – 8 mice per group). Values were shown as means ± SEM. A two-tailed *t* test was used for group-to-group comparison. Results were indicated with asterisks (*p < 0.05; **p <0.01).

IL-17F engagement was necessary for effective cytokine and chemokine responses in the liver

IL-17A and IL-17F bind to the same heterodimeric receptor comprised of IL-17RA and IL-17RC (102). Having demonstrated that IL-17R$^{-/-}$ mice displayed greatly reduced hepatic inflammation following Ad inoculation, we investigated which ligand was responsible for IL-17R signaling and immune-mediated liver injury. We injected IL-17A$^{-/-}$, IL-17F$^{-/-}$ and control mice with 3 × 10^9 pfu of AdLacZ. Surprisingly, IL-17A$^{-/-}$

mice developed serum ALT and IHL infiltration comparable to those in the wild-type mice (**Fig. 7A and 7B**). However, the IL-17F$^{-/-}$ mice displayed significantly reduced liver injury and lower pathological scores (**Fig. 7A and 7B**). Likewise, they presented lower serum ALT levels and fewer numbers of infiltrating IHLs (**Fig. 7B**). Consistent with the liver inflammation and infiltrated lymphocytes, lower percentages of CD8^{+} T cells in IL-17F$^{-/-}$ mice expressed IFN-γ compared with the findings in the control animals (**Fig. 7C**). However, those in the IL-17A$^{-/-}$ mice produced similar levels of IFN-γ compared to the controls (**Fig. 7C**). Furthermore, liver pro-inflammatory cytokines and chemokines, including IFN-γ, TNF-α, CXCL9 and CXCL10, were significantly decreased in IL-17F$^{-/-}$, but not in IL-17A$^{-/-}$ mice, compared to those in wild-type animals (**Fig. 7D**).

By using quantitative real-time PCR analysis, we found no significant difference in the viral copy numbers among the wild-type, IL-17R$^{-/-}$, IL-17A$^{-/-}$ and IL-17F$^{-/-}$ groups on day 7 post Ad infection ($p > 0.05$; **Fig. 8**). Although there was a steady reduction of the viral genome in all groups, no statistical difference was found among these mice on day 14, as well as on day 21. Following *i.v.* injection of Ad in mice, a majority of the viruses was eliminated quickly by the innate immune mechanisms within 24 h

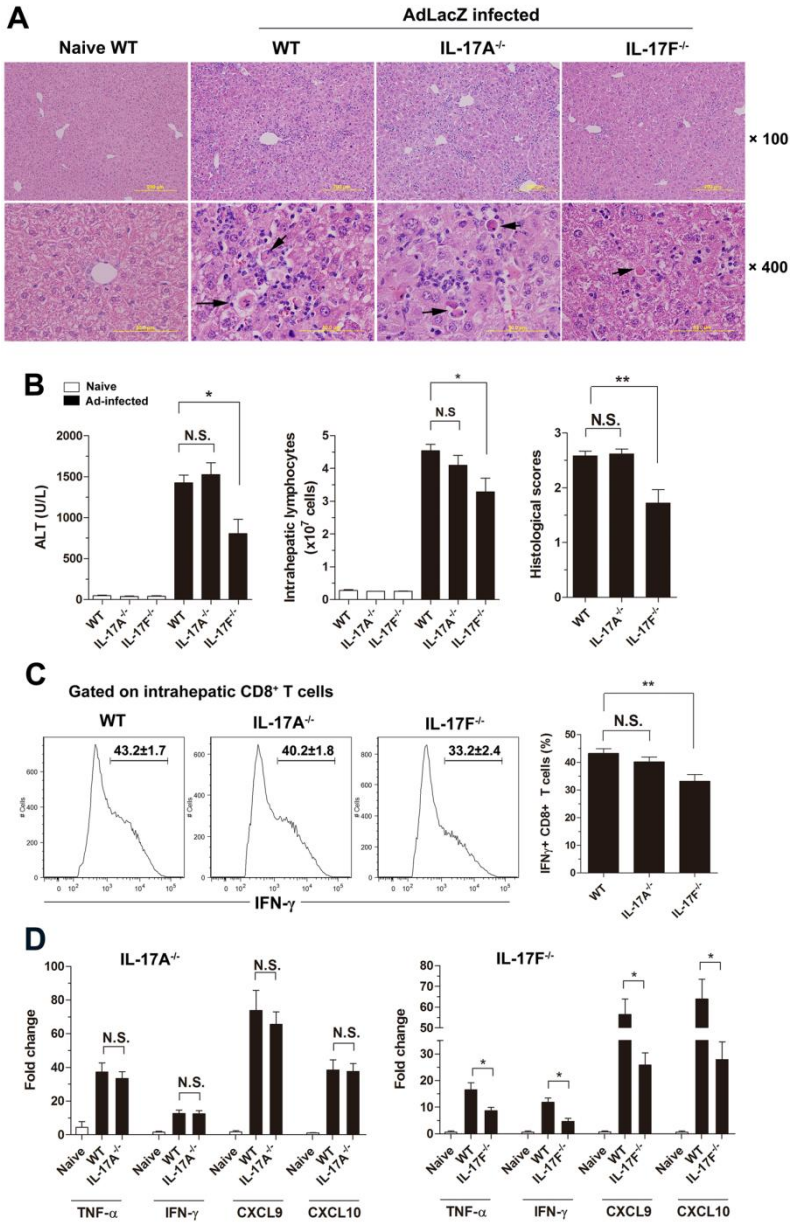

Figure 7 IL-17F deficiency, but not IL-17A deficiency, resulted in impaired CTL functions and alleviated Ad-induced hepatitis. C57BL/6, IL-17A$^{-/-}$ and IL-17F$^{-/-}$ mice were injected *i.v.* with 3 × 10^9 pfu of AdLacZ and sacrificed at day 6 post infection. **(A)** Liver tissues from uninfected and infected mice were collected and stained with H&E. Shown are representative images. **(B)** The serum was prepared, and intrahepatic lymphocytes were isolated after perfusion. Serum ALT levels (*right panel*) and numbers of total intrahepatic lymphocytes (*middle panel*) of wild-type, IL-17A$^{-/-}$ and IL-17F$^{-/-}$ mice were shown. *Right panel*: cumulative graphical representation of the histological scores. **(C)** IHLs were isolated and stimulated with anti-CD3 for 4 h in the presence of GolgiStop. The cells were stained for surface markers (CD3 and CD8) and then analyzed for intracellular IFN-γ. Cells were gated on CD3$^+$ CD8$^+$ cells. **(D)** Liver mRNA levels of IFN-γ, TNF-α, CXCL9 and CXCL10 of wild-type, IL-17A$^{-/-}$ and IL-17F$^{-/-}$ mice were analyzed by qRT-PCR. The experiment was repeated three times independently, and representative graphs are shown (n = 8 – 10 mice per group). (N.S. indicates no significance; **p* < 0.05; ***p* <0.01).

(187). However, overzealous T cell responses may result in increased necroinflammatory hepatitis without accelerating viral elimination *in vivo* (169, 172, 184, 189). These results suggested to us that IL-17A/F signaling affected lymphocyte infiltration and hepatic inflammation, rather than viral clearance in the liver in Ad-induced acute hepatitis. Thus, further investigations in chronic infection models, such as LCMV infection, are needed to define the role of IL-17 in virus clearance.

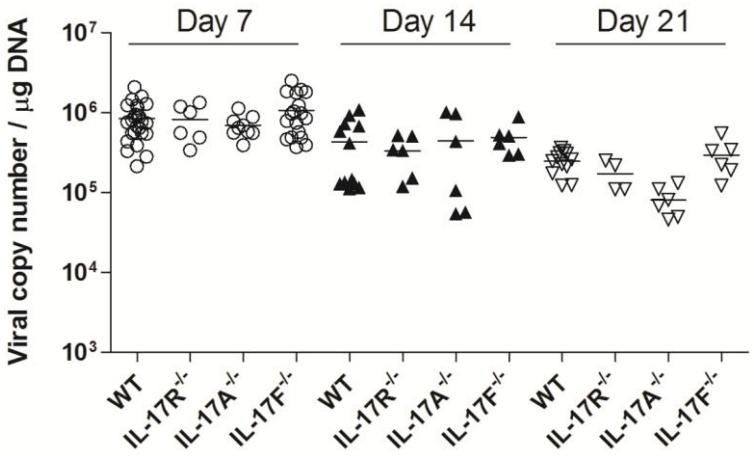

Figure 8 Clearance of intrahepatic AdLacZ from infected wild-type, IL-17R$^{-/-}$, IL-17A$^{-/-}$ and IL-17F$^{-/-}$ mice. The AdLacZ genome in the livers of the infected mice at days 7, 14 and 21 was quantitated by real-time PCR analysis. Each plot represents an individual mouse, and the data were pooled from two to three independent experiments.

IL-17A negatively regulated IL-17F secretion in the liver

IL-17A$^{-/-}$ mice developed Ad-induced hepatitis similar to that of the wild-type animals (**Fig. 7**). We speculated that IL-17F could compensate for IL-17A deficiency in these animals. To test this hypothesis, we infected the IL-17A$^{-/-}$, IL-17F$^{-/-}$ and control mice with AdLacZ, and compared their IL-17 levels at 24 h post infection. Interestingly, in IL-17A$^{-/-}$ mice, IL-17F^{+} $\gamma\delta$ T cells increased more than 3 fold compared with those in

controls (**Fig. 9A**). In IL-17F$^{-/-}$ mice, however, there was no such an increase among IL-17A^{+} cells (**Fig. 9B**). This IL-17F increase was also observed in the uninfected IL-17A$^{-/-}$ mice (data not shown). Furthermore, IL-17R$^{-/-}$ mice seemed to produce more IL-17A and IL-17F than wild-type animals in both uninfected and infected animals (data not shown). Consistent with an earlier report (110, 196), our results suggest that IL-17A has a strong negative feedback loop, repressing its own production and that of IL-17F through IL-17R.

To test whether IL-17A can inhibit IL-17F production *in vitro*, we isolated the naïve splenocytes and cultured them under the Th17 differentiation conditions in the presence of rIL-17A and rIL-17F, respectively. We found that IL-17A significantly suppressed IL-17F production *in vitro* in CD4^{+}, CD8^{+}, $\gamma\delta^{+}$ T cells, and total splenocytes in a dose-dependent manner (**Fig. 9C**). However, IL-17F did not affect IL-17A production from these cells (**Fig. 9D**).

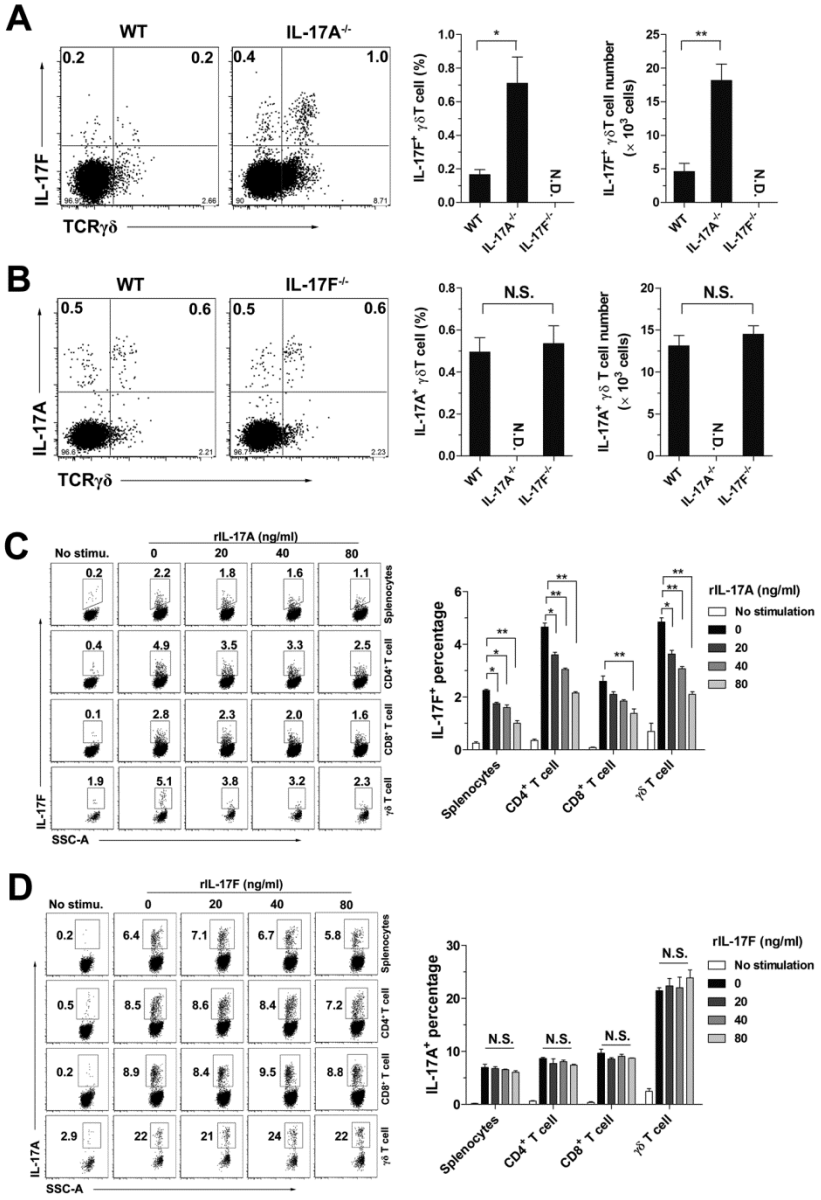

Figure 9 IL-17A negatively regulated IL-17F secretion in the liver *in vivo* and *in vitro*. C57BL/6, IL-17A$^{-/-}$ and IL-17F$^{-/-}$ mice were injected *i.v.* with 3 × 10^9 pfu of AdLacZ and sacrificed at 24 h post infection. IHLs were isolated and stimulated with PMA and Ionomycin for 4 h in the presence of GolgiStop. **(A)** *Left panel*: IL-17F production in γδ$^-$ and γδ$^+$ cells of wild-type and IL-17A$^{-/-}$ mice. *Right panel:* Cumulative statistical results from flow cytometry data. **(B)** *Left panel*: IL-17A production in γδ$^-$ and γδ$^+$ cells of wild-type and IL-17F$^{-/-}$ mice. *Right panel*: Cumulative statistical results of flow cytometry data. **(C)** Total splenocytes were cultured on the pre-coated anti-CD3 Ab with TGF-β, IL-6, anti-IFN-γ, and anti-IL-4. After 4 day culture, cells were rested for 1 day and then stimulated with PMA and ionomycin. Different concentrations of IL-17A were added during the culture process. Intracellular IL-17F levels of total splenocytes, CD4$^+$, CD8$^+$ and γδ T cells were detected by flow cytometry (*left panel*). Cumulative statistical results of flow cytometry data were shown (*right panel*). **(D)** Different concentrations of IL-17F were added during the culture process. Intracellular IL-17A levels of total splenocytes, CD4$^+$, CD8$^+$ and γδ T cells were detected by flow cytometry (*left panel*). Cumulative statistical results of flow cytometry data were shown (*right panel*). Asterisks indicate results (N.S. indicates no significance; **p* < 0.05; ***p* <0.01).

2.4 Discussion

ILCs are essential effectors of innate immunity and have an important role in tissue remodeling (159). They are characterized by the absence of lineage markers as well as lymphoid morphology. Recently, ILCs have been categorized into three groups based on their cytokines and transcriptional factors (159). Group 1 comprises ILCs that produce IFN-γ. Group 2 consists of ILCs that produce type 2 cytokines (including IL-5 and IL-13). Group 3 includes ILC subsets that produce IL-17 and/or IL-22 and depend on the transcriptional factor RORγt for their development and function. ILC3s also have been shown to play important roles in intestinal immunity and homeostasis (165, 197). However, the phenotype and role of ILC3s in the liver is unclear. In this study, we found that several intrahepatic cell populations secreted IL-17A and IL-17F locally shortly after Ad infection (**Fig. 1**). Among these cells, γδ T cells were highest in number, as we reported previously (172). Among $\gamma\delta^-$ cells, we revealed that a previously uncharacterized population constituted a major group of IL-17 producers. These lineage marker-negative, stem cell marker-positive cells belong to group 3 ILCs (**Figs. 1 and 3**). Based on their CD4 and NKp46 expression profiles, we further speculate that most of these ILC3s belonged to the $NKp46^-$ ILC3 population, and a small population belonged to the $CD4^+$ lymphoid-tissue inducer (LTi) cells in the liver (**Fig. 3**). Using $Rag2^{-/-}$ and $\gamma\delta^{-/-}$ mice, we further confirmed that ILC3s are indeed potent IL-17 producers and an integral part of the immune defense system in the liver (**Figs. 3 and 4**). Moreover, in addition to Ad model, we also observed that ILC3s were

present in mouse liver after LCMV infection (**Fig. 3C**). To the best of our knowledge, this is the first report for detailed study of ILC3 subsets in the liver after viral infection.

IL-17A and IL-17F belong to the IL-17 superfamily. They were originally reported to be predominantly produced by activated Th17 along with several other cell types (e.g., $CD8^+$ T cells, $\gamma\delta$ cells, NKT cells, double negative T cells) (104, 106). They were typically linked to destructive tissue damage in autoimmune diseases and bacterial infections (113, 115, 177). More recent evidence has pointed to their involvement in promoting Th1 and CTL responses in anti-tumor immunity (178), inflammatory bowel disease (179), and antiviral immune responses (172). Both IL-17A and F have been found to be required for immune responses against extracellular bacterium such as *S. aureus* infection (106, 115). At present, the immunoregulatory effects of the IL-17 species on the antigen-presentation process, CTL and Th responses in virus-infected liver are not well understood. On the other hand, in the presence of IL-17A, IL-17F was thought to be dispensable for disease progression in EAE and CIA (113, 198). The roles of IL-17A and F seem to be more controversial as either pro-inflammatory or anti-inflammatory mediators in inflammatory bowel disease (116, 117).

IL-17-producing cells are known to play a role in autoimmune and viral hepatitis (123, 199, 200). Several studies showed that Th17 cells could promote the activation of stellate cells and Kupffer cells, which, in turn, may aggravate liver fibrosis and the inflammatory response in chronic hepatitis (119, 201). There is a paucity of data

addressing the possible involvement in DC activation and T cell-priming. In a concanavalin (Con) A-induced hepatitis model, over-expression of IL-17A resulted in massive hepatocyte necrosis, and anti-IL-17A blockage significantly ameliorated the disease (120). In addition, Lafdil *et al.* showed that liver injury was alleviated in Con A-induced hepatitis among IL-17-deficient mice (121). In a separate study, however, IL-17A deficiency did not seem to thwart T cell activation and liver inflammation (122). We speculate that these discrepancies are attributable to the compensatory IL-17F production, as we showed in this report (**Figs. 7 and 9**). Secondly, Con A-induced liver injury is an extremely acute hepatitis model, in which lectin-activated NKT cells play a critical role (202). Also, the dose of Con A injection and the timing of liver injury assessment may also contribute to the discrepancies among these studies. In this study, we report that adaptive T cell responses and associated liver injury were dependent on IL-17 signaling (**Figs. 5 and 6**). Surprisingly, these clinical parameters were not affected by the lack of IL-17A in the gene knockout animals in this study (**Fig. 7**). However, in our previous observation, IL-17A neutralization by monoclonal Ab clearly hampered DC activation and ameliorated liver inflammation upon viral infection (172). Additional experiments revealed that IL-17A$^{-/-}$ mice displayed a greater than 3-fold increase in IL-17F^{+} cells following viral infection (**Fig. 7A**). Interestingly, this compensatory IL-17F increase was also observed in un-infected IL-17A$^{-/-}$ animals (data not shown). Finally, *in vitro* experiments directly confirmed that IL-17A represses IL-17F secretion in $\gamma\delta$, CD4^{+} and CD8^{+} T cells (**Fig. 9C**). These results are consistent with previous reports that IL-17A controls IL-17F production

through an IL-17R-dependent, short-loop inhibition mechanism (110, 196). In the absence of IL-17A, however, IL-17F could compensate and maintain baseline neutrophil counts in mice (110). In a recent commentary, it was proposed that IL-17A and F can cause negative feedback of their own and each other's synthesis via IL-17R (203). However, we did not observe IL-17F-mediated IL-17A inhibition in our studies (**Fig. 9D**). In addition, in IL-17F$^{-/-}$ animals, there was no compensatory IL-17A increase or rescue of T cell functions (**Figs. 9B and 7**). Although IL-17A and IL-17F have some functional redundancy in viral infection, our results unveil a mechanism underlying the seeming discrepancies between IL-17A- and IL-17F-deficient mice and underscore the unique functions of IL-17F in T cell responses to viral infection in the liver.

Recombinant Ad is one of the preferred vectors for gene therapy, cancer therapy, and experimental vaccines (180, 181). However, it can also induce strong Th, CTL and B cell responses against the viral vector and the transgene (183, 184). At day 1 post Ad infection, we observed an elevation of IFN-β, IL-7, IL-23 and TNF-α in addition to IL-17 (172). Although the IL-17-producing cell expansion and IL-17 level increase were relatively brief (**Fig. 1A and B**), the IFN-γ, TNF-α, IL-1β, CXCL9 and CXCL10 levels continued to persist into day 6 post infection {**Fig. 7D** and (172)}. In this report, we found that IL-17 signaling blockage in IL-17RA knockout mice developed less CD4$^+$ and CD8$^+$ T cell infiltration and displayed much milder liver inflammation and ALT and AST elevations (**Figs. 5 and 6**). Our results indicate that blockade of

IL-17/IL-17RA signaling pathway may represent a novel therapeutic intervention to constrain liver injury when using Ad for gene therapy.

In summary, we have defined the early source and function of hepatic IL-17, which are important for DC activation and T cell priming in viral hepatitis. We have provided evidence that besides $\gamma\delta$ T cell, ILC3 seemed as a significant source of IL-17A/F in the liver within the first few hours and days of viral infection using Ad and LCMV models. Furthermore, this surge of IL-17 mediated DC licensing and adaptive immune responses through binding to IL-17R. Also, while we know that IL-17A is a dominant species constituting the cytokine microenvironment and regulating IL-17F production, in its absence, IL-17F^{+} IHLs expanded significantly and compensated for IL-17A deficiency in an IL-17 signaling pathway-dependent fashion. On the other hand, IL-17F deficiency resulted in compromised T cell priming and tissue infiltration. Collectively, this study indicates that innate IL-17A/F signaling is important for adaptive immune responses in viral hepatitis. Our study unveiled a previously unknown source and crosstalk between IL-17A and F and may provide potentially important information aimed at targeting ILCs and IL-17 species in acute and chronic viral hepatitis.

CHAPTER 3: RETIONIC ACID PROMOTES IL-22 PRODUCED BY GROUP 3 INNATE LYMPHOID CELLS AND REGULATES IMMUNE RESPONSES IN VIRAL HEPATITIS

3.1 Introduction

Virus-specific $CD8^+$ and $CD4^+$ T cell functions have been shown to be critical in viral clearance and disease resolution (69, 204). However, T cell responses also played a central role in hepatocellular injury through multiple mechanisms, including cytolytic pathways and the release of various pro-inflammatory cytokines (205). On the contrary, activated T cells also produce anti-apoptotic cytokines, including IL-6 and IL-22, as well as anti-inflammatory cytokines such as IL-10, indicating a potential role in liver repairing and remodeling (150, 206, 207).

As we mentioned above, IL-22 can be produced by various cells, including Th17, Th22, $\gamma\delta$ T, NK, neutrophils, and innate lymphoid cells (ILCs) (127-132). IL-22 exerts its functions by binding to a heterodimeric receptor comprised of IL-10R2 and IL-22R1 (208). It mainly targets epithelial cells and hepatocytes due to restricted expression of IL-22R1 on these cells (134). As a survival factor for hepatocytes, IL-22 plays a critical role in prevention of hepatocellular damage in a variety of liver injury models (147, 148, 150, 152, 209). Through binding to IL-22R1 on hepatocytes and

[2] *Submitted to Journal of Immunology*

intestinal epithelial cells, IL-22 protects against concanavalin A (Con A)-induced hepatitis, inflammatory bowel disease, ulcerative colitis, and lung disease (150, 210-212). Although the hepatoprotective role of IL-22 in liver injury has been well established, its role in virus-induced hepatitis is still debatable (158, 213). Little is known about the regulatory pathways controlling IL-22 production in the liver. Although IL-23R signaling, RORγt and the nuclear factors aryl hydrocarbon receptor (AHR) have been indicated in promoting IL-22 production (127, 144, 159, 214), their regulation and requirement for other factors have not been thoroughly investigated. To date, the source and regulation of the liver-derived IL-22 in viral hepatitis is not well understood.

Hepatic stellate cells (HSCs) store around 75% of the body's supply of vitamin A (retinol) under normal conditions (215). However, in response to liver injury, the quiescent HSCs can be activated and then it secretes retinoic acid (RA), a vitamin A metabolite (45, 52). These early activated HSCs gradually lose their retinol storage through either secretion or dehydrogenation into retinal by alcohol dehydrogenase (ADH) and conversion into RA by retinal aldehyde dehydrogenase (ALDH) (216). It has been shown that HSC-derived RA can promote the induction of $CD4^+$ $Foxp3^+$ T regulatory cells (Treg) and inhibit the differentiation of IL-17-producing $CD4^+$ T cells *in vitro* (58). More recently, RA secreted by intestine dendritic cells (DCs) has been shown to promote IL-22 production by γδ T cells and group 3 innate lymphoid cells (ILCs) in the intestine (217). Currently, the role of HSCs in liver fibrosis has been well

documented (53, 218); however, little is known about the roles of HSC and HSC-derived RA in viral hepatitis.

In this study, we found group 3 ILCs as a major source of IL-22 production in the liver during viral infection. In the last several years, ILCs have been found to be involved in innate immunity as well as tissue remodeling (159). In the liver, ILCs have been shown to protect against acute hepatitis (169, 171), prime T cell responses, but can also mediate liver fibrosis (170). In addition, the HSC-derived RA levels increased in the liver following viral infection. Moreover, RA promoted IL-22 production by ILC3s and protected the liver from inflammation and injury. Notably, hepatic IL-7 was important for IL-22 production by IHLs. The blockade of IL-7Rα *in vivo* significantly decreased IL-22 levels and exacerbated hepatic inflammation. In conclusion, this study has demonstrated the importance of a unique intrahepatic subpopulation ILC3 and its cytokine production during the early stages of viral hepatitis.

3.2 Material and methods

Animals

Female C57BL/6 (B6) and Rag2$^{-/-}$ mice were purchased from the Jackson Laboratory. All mice were maintained and bred under specific pathogen-free conditions in the animal facility at the University of Texas Medical Branch. Eight- to twelve-week-old mice were used for all the experiments. All experiments were reviewed and approved by the Institutional Animal Care and Use Committees of the University of Texas Medical Branch. To induce hepatitis, mice were *i.v.* injected with 3×10^9 pfu replication-deficient recombinant Ad carrying the LacZ gene (AdLacZ, purchased from Vector Development Laboratory of Baylor College of Medicine) as described previously (184)**.** Mice were *i.v.* injected with 2×10^6 pfu lymphocytic choriomeningitis virus (LCMV) Clone 13 (a kind gift from Dr. Maria Salvato at the University of Maryland) (191). Titration of LCMV was performed on Vero cell monolayers plated on 24-well plates, followed by the viral quantification of immunological focus assay (192). The antibody of LCMV was kindly provided by Dr. Robert Tesh from the University of Texas Medical Branch.

Abs and reagents

All fluorochrome-labeled monoclonal antibodies (Abs) and their corresponding isotype controls were anti-mouse Abs and purchased from eBioscience (San Diego, CA), BD Pharmingen (San Diego, CA) and Biolegend (San Diego, CA). The following were purchased from eBioscience: PE-conjugated anti-IL-22 (1H8PWSR),

APC-conjugated anti-IL-17A (eBio17B7), FITC-conjugated anti-IFN-γ (XMG1.2), APC-conjugated anti-IFN-γ (XMG1.2), APC-conjugated anti-TCRγδ (eBioGL3), PE-Cy7-conjugated anti-CD3 (17A2), Pacific blue-conjugated anti-CD4 (GK1.5), PerCp-Cy5.5-conjugated anti-CD8 (53-6.7), APC-conjugated anti-NK1.1 (PK136), FITC conjugated anti-B220 (RA3-6B2), FITC conjugated anti-CD11b (M1/70), FITC conjugated anti-CD11c (N418), FITC conjugated anti-Gr-1 (RB6-8C5), FITC conjugated anti-Ter-119 (TER-119), FITC conjugated anti-NK1.1 (PK136), APC conjugated anti-RORγt (B2D), and Biotin conjugated anti-c-kit (2B8), and eFluor 506 conjugated fixable viability dye. Purified anti-CD16/32 (2.4G2) and anti-IL-7Rα mAb (SB/14) were purchased from BD Pharmingen. PerCp-Cy5.5 conjugated anti-NKp46 (29A1.4), APC-Cy7 conjugated anti-CD3 (17A2), APC-Cy7 conjugated anti-Sca-1 (D7), PerCp-Cy5.5 conjugated anti-ICOS (C398.4A) were purchased from Biolegend. Anti-IL-7 polyclonal Ab was purchased from R&D. Recombinant murine IL-6, IL-1β, IL-23, IL-2 and IL-7 were purchased from Peprotech. The *all-trans* retinoic acid (RA) was purchased from Enzo Life Sciences. STAT3 inhibitor VII and STAT5 inhibitor were purchased from Calbiochem.

In vivo administration of retinoic acid (RA)

To induce hepatitis, mice were *i.v.* injected with 3×10^9 pfu AdLacZ as described previously. Mice were treated *i.p.* with 250 μg all-trans RA (Enzo Life Sciences) everyday post infection. DMSO was administered *i.p.* as a control. Mice were euthanized at day 6 post-infection.

In vivo blocking of IL-7Rα or neutralization of IL-7

To block the effects of IL-7, mice were injected *i.p.* with 100 μg anti-mouse IL-7Rα mAb (clone: SB/14, BD Bioscience) or 100 μg anti-mouse IL-7 polyclonal Ab (R&D) at days -1, 0, 1, 3 and 5 post Ad infection. Mice were euthanized at day 6 post-infection. Normal rat or goat IgG (Sigma-Aldrich) was administered *i.p.* as an isotype control.

Isolation of intrahepatic lymphocytes

Intrahepatic lymphocytes were isolated according to our previous method with slight modifications (184). Briefly, liver tissue was pressed and collected in complete RPMI-1640. After washing (300 × *g*, 10 min), cell suspensions were re-suspended in complete RPMI-1640 containing collagenase IV (0.05%, Roche Applied Science, Indianapolis, IN) at 37°C for 30 min. After digestion, cell suspensions were passed through 70-μm nylon cell strainers to yield single-cell suspensions. Intrahepatic mononuclear cells were purified by centrifugation (400 × *g*) at room temperature for 30 min over a 30/70% discontinuous Percoll gradient (Sigma). The cells were collected from the interphase, thoroughly washed, and re-suspended in complete RPMI 1640 containing 10% FBS (Hyclone, Logan, UT). The total numbers of intrahepatic lymphocytes per liver were counted. The relative percentages of $CD4^+$, $CD8^+$ and γδ T cells were measured by flow cytometry, and the absolute numbers of these lymphocyte subpopulations per liver were calculated according to their percentages and the total intrahepatic lymphocyte numbers in each liver.

Isolation of hepatocytes and hepatic stellate cells

Primary hepatocytes were isolated from B6 mice by an adaptation of a two-step collagenase perfusion technique (219). Briefly, mouse liver was perfused with HBSS (pH 7.4, without calcium and magnesium) containing 1 mM EGTA and 10 mM HEPES for 10 min, followed by HBSS with calcium and magnesium plus collagenase D (Roche Applied Science, Indianapolis, IN) for 10 min at 37^{o}C. The digested liver was then excised, rinsed, and disaggregated in a 150-mm polystyrene Petri dish. Subsequently, the disaggregated material was filtered through a 70-μm cell strainer, and the filtrate was gently centrifuged for 3 min at 50 × *g*. The pellet was re-suspended in 45% Percoll in PBS and centrifuged at 50 × *g* for 10 min. After the enrichment by Percoll isodensity purification, the cells were washed and gently centrifuged, and the pellets were resuspended in the DEME supplemented with 10 mM HEPES, 2 mM L-glutamine, ITS (Sigma) and 10% fetal bovine serum.

Primary hepatic stellate cells were isolated from B6 mice by an adaptation of the two-step protocol (220). The hepatic stellate cells were perisinusoidal localized in the liver. In order to make stellate cells accessible to isolation from the space of Disse, mouse livers were perfused *in situ* with the digestive enzymes Pronase E (Calbiochem) and Collagenase D. Following perfusion, the liver tissue was subjected to additional enzymatic treatment with Pronase E, Collagenase D and DNase *in vitro* for 30 min at 37^{o}C. The digested liver was then excised, rinsed, and disaggregated in

a 150-mm polystyrene Petri dish. Subsequently, the disaggregated material was filtered through a 70-μm cell strainer, and the filtrate was centrifuged at 300 × *g* for 10 min. The pellet was resuspended in 10% OptiPrep^{TM} density gradient medium (Sigma-Aldrich) in PBS and centrifuged at 1,400 × *g* for 20 min. We added 1 ml of RPMI on the top of the density gradient medium. Hepatic stellate cells were collected from the interphase of RPMI and 10% OptiPrep^{TM} density gradient medium. After being thoroughly washed, cells were re-suspended in complete RPMI 1640 containing 10% FBS (Hyclone, Logan, UT).

Intracellular staining

Intracellular staining was performed according to our previous methods (172). Briefly, cells were incubated for 4 h with PMA (50 ng/ml) and ionomycin (750 ng/ml) in the presence of GolgiStop (BD Bioscience). After incubation, cells were collected, stained with fixable viability dye, blocked with FcγR blocker (CD16/32) and stained for specific surface molecules. After surface staining, cells were fixed, permeabilized and stained for intracellular cytokines by using a fixation/permeabilization kit (eBioscience).

Flow cytometry analysis

Murine lymphocytes were blocked with anti-mouse CD16/CD32 (eBioscience) and stained with fluorochrome-labeled antibodies, and then processed on an LSRII FACSFortessa (Becton Dickinson, San Jose, CA) and analyzed by using FlowJo software (TreeStar, Ashland, OR). All fluorochrome-labeled mAbs and their

corresponding isotype controls were purchased from BD Pharmingen (San Diego, CA) and eBioscience (San Diego, CA).

Aldehyde dehydrogenase activity assay

Cell aldehyde dehydrogenase (ALDH) activity was determined by using the ALDEFLUOR staining kit (Stemcell Technologies, Tukwila, WA) according to the manufacturer's instructions. Briefly, cells were re-suspended at 10^6 cells/ml in ALDEFLUOR assay buffer containing activated ALDEFLUOR substrate with or without the ALDH inhibitor diethylaminobenzaldehyde (DEAB) and incubated at 37°C for 30 min. ALDEFLUOR-reactive cells were detected in the FITC channel by flow cytometry.

Real-time PCR

Frozen liver tissues were used to extract genomic DNA and total RNA. DNA was extracted with a DNeasy blood and tissue kit (Qiagen), and total RNA was extracted with an RNeasy Mini kit (Qiagen) and digested with DNase I (Ambion). The concentrations of DNA and RNA were measured by using a spectrophotometer (Eppendorf). For relative quantitation of the cytokine and chemokine mRNA levels, cDNA was prepared from 1 μg of RNA by using an iScript™ Reverse Transcription Kit (Bio-Rad), and 4 μl of the cDNA was amplified in a 25-μl reaction mixture containing 12.5 μl of iQ SYBR Green Supermix (Bio-Rad) and 0.9 μM each of gene-specific forward and reverse primers. The PCR assays were denatured for 10 min at 95°C,

followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. The PCR was performed with the CFX96 Touch real-time PCR detection system (Bio-Rad). Relative quantitation of mRNA expression was calculated as the fold increase in expression by using the $2^{-\Delta\Delta Ct}$ method. Meanwhile, melting curve analysis was used to check the specificity of the amplification reaction. The sequences of the forward and reverse gene-specific primers used are listed in Table 1.

ELISA assays

Liver proteins were extracted from frozen liver tissues by homogenization on ice in the RIPA Buffer (Cell Signaling) with a protease inhibitor cocktail (Sigma). After centrifugation at 20,000 × *g* for 15 min, the supernatant was collected and protein concentration was measured with a protein assay kit (Bio-Rad). Equal amounts of the liver proteins (100 μg) were loaded for ELISA assays. The levels of IL-22 in the liver proteins were measured by using the ELISA kits (eBioscience) according to the manufacturer's instructions. Detection limits were 5 pg/ml for IL-22.

Statistical analysis

The difference between the two different groups was determined by using Student's t test. One-way ANOVA was used for multiple group comparisons (GraphPad Software v4.0). P values < 0.05 were considered significant*, and < 0.01 as highly significant**.

3.3 RESULTS

Early surges of IL-22 produced by classical and non-classical intrahepatic cells

To define the dynamics of IL-22 production in the liver, we *i.v.* injected B6 mice with 3 × 10^9 pfu of AdLacZ. The animals were sacrificed at 0, 12, 24 h, and on days 3 and 6 post-infection. ELISA analysis of liver lysates revealed a significant accumulation of IL-22 during the first three days post infection (**Fig. 10A**). Meanwhile, we isolated the intrahepatic lymphocytes (IHLs) and analyzed their intracellular levels of IL-22 and IL-17 by flow cytometry. We found that the intrahepatic IL-17^+ cells expanded from 0.5% at 0 h to 1.3% at 24 h post-infection as we reported previously (**Fig. 10B**). More importantly, the IL-22^+ cells expanded robustly, from 0.2% at 0 h to 0.7% at 24 h post-infection (**Fig. 10B**). The absolute cell number of IL-22^+ cells expanded from 0.9 × 10^4 cells at 0 h to 2.6 × 10^4 cells at 24 h post-infection (**Fig. 10B**). In addition, we found that IL-22 can be detected in the liver, but not in the serum (data not shown).

To define which cell types in the liver can produce IL-22, we isolated the intrahepatic lymphocytes (IHLs) at day 1 post-infection and analyzed their intracellular levels of IL-22 by flow cytometry (**Fig. 10C**). We found that most of the IL-22-producing cells were CD11b^+ cells and lineage negative populations (lineage markers: CD8, CD11b, CD11c, NK1.1, B220, Gr-1, and Ter-119). Other cell types, such as CD4 T, CD8 T, $\gamma\delta$ T, NK and CD11c^+ cells, did not produce IL-22. In addition, IL-17-producers were mainly CD3^+ T cells and group 3 innate lymphoid cells (ILC3s). Furthermore, we gated on different cell types and analyzed their intracellular IL-22 and IL-17 (**Fig. 11A**).

Interestingly, we found that $CD4^+$ T cells, $CD8^+$ T cells and γδ T cells can produce IL-17 but not IL-22. NK cells and $CD11c^+$ dendritic cells did not produce IL-17 or IL-22. Interestingly, $Gr\text{-}1^+$ $CD11b^+$ cells can produce IL-22 but not IL-17 (**Fig. 11A**). Notably, we found the lineage negative populations (innate lymphoid cells) can produce both IL-17 and IL-22 (**Fig. 11A**). Collectively, these data suggested to us that early surges of IL-22 were mainly produced by $Gr\text{-}1^+$ $CD11b^+$ cells and innate lymphoid cells in the liver.

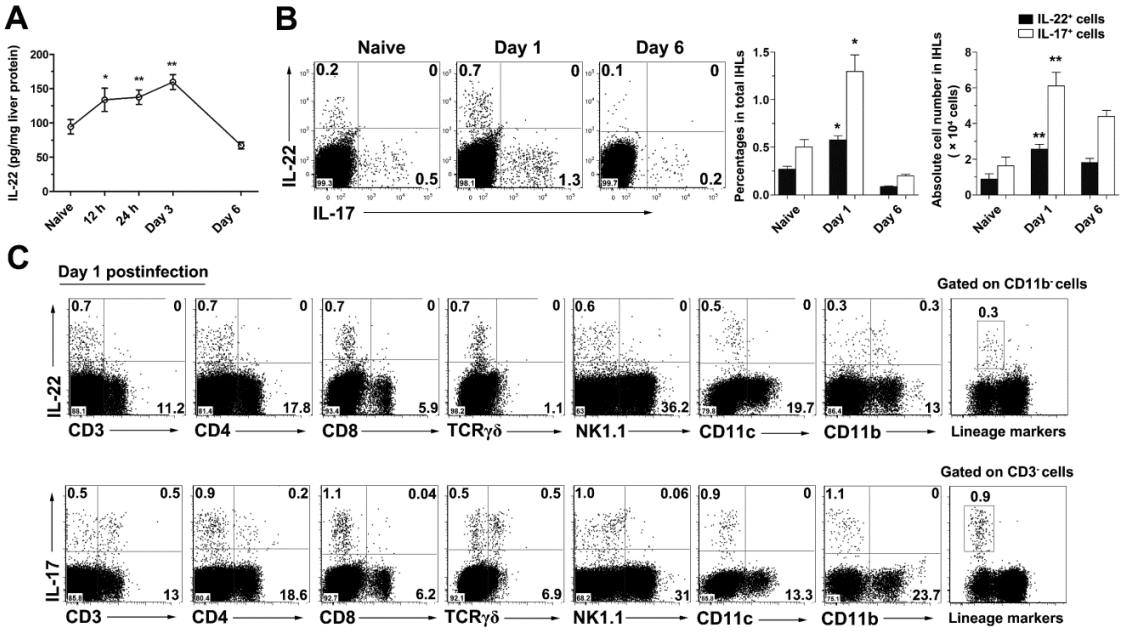

Figure 10 Early surges of IL-22 produced by classical and non-classical intrahepatic cells. Mice were infected as Fig. 1 and sacrificed at the indicated time points. The liver tissues were collected, and IHLs were isolated after perfusion. **(A)** Liver proteins were extracted and liver IL-22 level was detected by an ELISA. **(B)** IHLs were isolated and stimulated with PMA and ionomycin for 4 h in the presence of GolgiStop. The cells were collected and examined by flow cytometry for intracellular IL-22 and IL-17. *Right panel*: Cumulative statistical results of the percentages and absolute cell number of $IL\text{-}22^+$ and $IL\text{-}17^+$ cells in the liver, respectively. (**C**) IHLs from day 1 infected mice were *in vitro* stimulated with PMA and ionomycin for 4 h in the presence of GolgiStop and analyzed by flow cytometry for intracellular IL-22 and IL-17 expression. Expression of IL-22 (*upper panel*) and IL-17 (*lower panel*) was analyzed in $CD3^+$, $CD4^+$, $CD8\alpha^+$, $TCR\gamma\delta^+$, $NK1.1^+$, $CD11c^+$, $CD11b^+$ and lineage negative cells (lineage markers: CD3, CD8, CD11b, CD11c, NK1.1, B220, Gr-1, and Ter-119). Representative *dot plots* are shown. Values were shown as means ± SEM. Data were compared to the naïve mice and a two-tailed *t* test was used for statistical analysis, $*p < 0.05$, $**p < 0.01$.

Group 3 ILCs and Gr-1$^+$ CD11b$^+$ cells were important sources of IL-22 in the liver

To further characterize these lineage-negative cells that produced IL-22, we analyzed their surface markers. Interestingly, these cells expressed high levels of Sca-1, ICOS, RORγt and low levels of IL-7Rα on their surface, but did not express NKp46 (**Fig. 11B**). The phenotypical and functional characteristics of these cells were consistent with ILC3s, previously described in the gastrointestinal tract (159). We further concluded that most of these ILC3s belonged to the NKp46$^-$ ILC3 population. These cells expanded robustly and peaked within the first 24 h post-infection and waned in the next few days (**Fig. 11C**). In addition to these IL-22$^+$ ILC3s, we found Gr-1$^+$CD11b$^+$ cells can also produce IL-22 in the liver post viral infection (**Fig. 11A**). It has been reported that neutrophils in the intestine are capable producing IL-22 and protect against inflammatory bowel disease (131). We speculate that these Gr-1$^+$CD11b$^+$ cells in the liver were either neutrophils or inflammatory monocytes, since these cells express Gr-1 and CD11b on their surface and can be activated rapidly post viral infection.

To further confirm that ILC3s in the liver can produce IL-22, we examined the IL-22 levels in the Rag2$^{-/-}$ mice that are deficient of T and B lymphocytes (195). IHLs from uninfected Rag2$^{-/-}$ and wild-type mice were analyzed by flow cytometry. In the wild-type animals, there were about 0.5% IL-22$^+$ cells and 0.5% IL-17$^+$ cells (**Fig. 11D**). In Rag2$^{-/-}$ mice, however, there were more than 3-fold and 7-fold increases of IL-22$^+$

and IL-17^{+} cells, respectively, among the total IHLs (**Fig. 11D**), suggesting to us that innate immune cells are capable of producing IL-17 and IL-22 in the liver.

To examine whether intrahepatic ILC3s are present in a persistent viral infection model, we *i.v.* injected B6 mice with 2 × 10^{6} pfu of LCMV Clone 13. Similar to our previous findings in Ad model, most IL-22-producing cells in the liver were Gr-1^{+}CD11b^{+} cell and ILC3s after viral infection (**Fig. 11E** and **11F**). In addition, these ILCs expanded robustly and increased within the first 24 h post LCMV-infection (data not shown). These results suggested to us that IL-22-secreting ILCs also existed in the liver and expanded after LCMV infection.

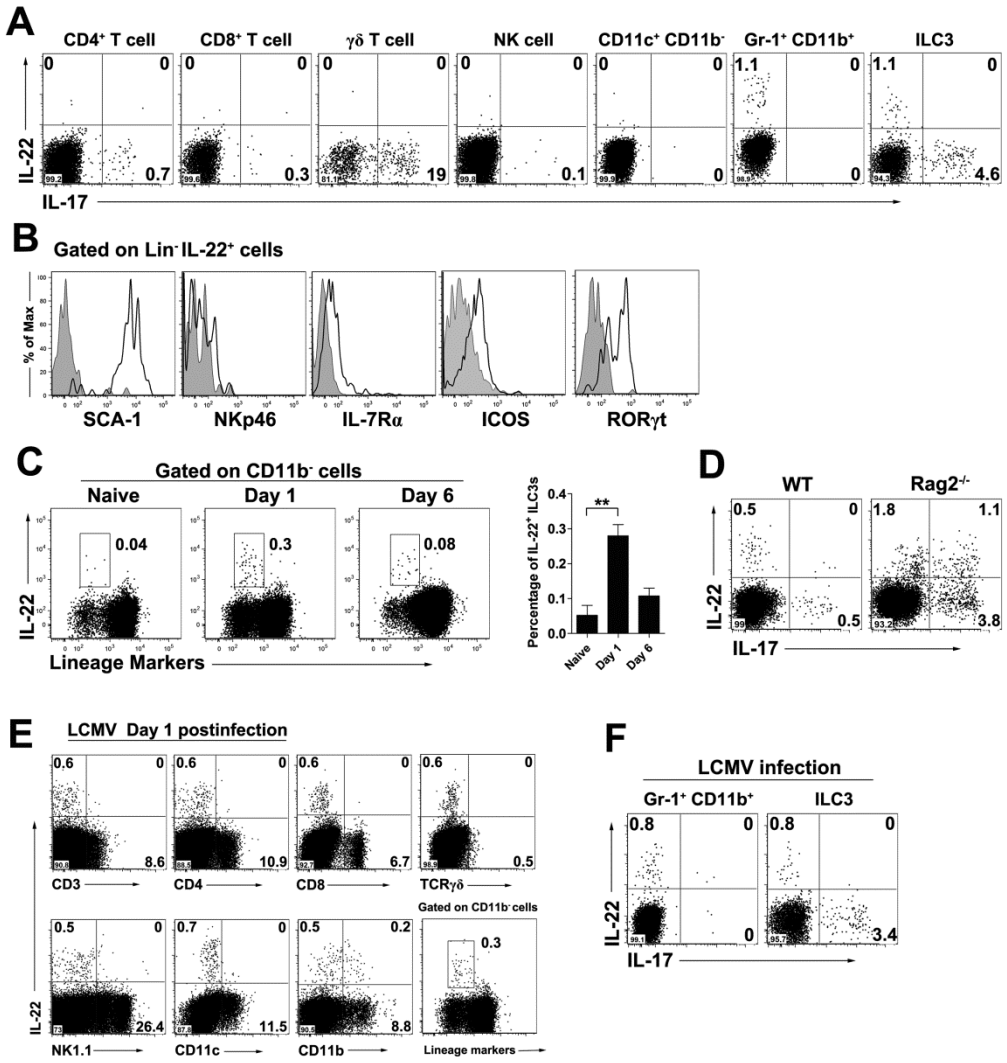

Figure 11 Group 3 ILCs and Gr-1$^+$ CD11b$^+$ cells were important sources of IL-22. (A) Mice were infected as Fig. 1 and sacrificed at day 1 after infection. The IHL were isolated after liver perfusion and stimulated with PMA and ionomycin for 4 h in the presence of GolgiStop. Flow cytometric analysis of IL-22 and IL-17 productions in CD4$^+$ T, CD8$^+$ T, γδ T, NK, CD11c$^+$, Gr-1$^+$ CD11b$^+$ and ILC3s at day 1 post-infection. **(B)** The IHLs were gated on CD11b$^-$ population. The cells were further gated on negative populations of lineage (Lin) markers (CD3, CD8, CD11c, NK1.1, B220, Gr-1, and Ter-119) and analyzed for intracellular IL-22. The Lin$^-$ IL-22$^+$ cells were gated for detection of surface and intracellular markers (Sca-1, NKp46, IL-7Rα, ICOS, and RORγt). Shaded graphs: isotype control, solid lines: Ab staining. **(C)** Mice were infected as Fig. 1 and sacrificed at the indicated time points. IHLs were stimulated and gated on CD11b$^-$ population. The cells were gated on lineage-negative population and intracellular IL-22 was analyzed by flow cytometry. *Right panel*: Cumulative statistical results of flow cytometry data. (**D**) The IHL from Rag2$^{-/-}$ and wild-type mice were isolated and stimulated with PMA and ionomycin for 4 h in the presence of GolgiStop. Intracellular IL-22 and IL-17 of total IHL were analyzed by flow cytometry. **(E and F)** Mice were injected *i.v.* with 2 × 10^6 pfu of LCMV clone 13 and sacrificed at day 1 post-infection. IHLs were isolated and stimulated with PMA and ionomycin for 4 h in the presence of GolgiStop. **(E)** Expression of IL-22 was analyzed in CD3$^+$, CD4$^+$, CD8α$^+$, TCRγδ$^+$, NK1.1$^+$, CD11c$^+$, CD11b$^+$ and lineage-negative cells. **(F)** Flow cytometric analysis of IL-22 and IL-17 productions in Gr-1$^+$ CD11b$^+$ and ILC3s at day 1 post virus infection. (Abbreviation: Lin, lineage)

Retinoic acid alleviated liver injury in viral hepatitis

The vitamin A metabolite retinoic acid (RA) is mainly produced by hepatic stellate cells (HSCs) and hepatocytes in the liver (45, 53). RA has been shown to associate with enhanced IL-22 production by ILCs in intestinal inflammation (217). We hypothesize that RA can promote IL-22 production *in vivo*, leading to hepatoprotection in viral hepatitis. First, we examined whether endogenous RA is produced during development of Ad-induced hepatitis. We examined activities of aldehyde dehydrogenases (ALDH), the enzymes involved in retinol metabolism and RA synthesis. It can cleave a fluorescent synthetic substrate, ALDEFLUOR, which accumulates within the cell after cleavage. Here, we found the total number of ALDEFLUOR$^+$ HSCs significantly increased in the liver of mice infected with Ad (**Fig. 12A**). However, hepatocytes did not demonstrate active ALDH activities (data not shown). These results suggested to us that RA production by HSCs increased during Ad infection and may play a role in limiting liver inflammation.

To investigate the effects of RA during viral hepatitis, we treated mice with all-trans retinoic acid (250 μg/mouse, *i.p.*) or DMSO daily from day 1 to 5 after Ad infection. Ad infection induced strong CD4$^+$ T cell and CD8$^+$ T cell recruitment into the liver. The control animals developed hepatitis characterized by inflammatory lymphocytes infiltration, hepatocytes with megaloblastic changes, and single-cell necrosis at 6 days post-infection. Compared to the control group, the RA-treated mice displayed much milder inflammation and lower serum ALT and AST levels (**Fig. 12B and C**).

Next, we examined the accumulation of total intrahepatic lymphocytes and splenoctyes in these animals. Compared with the control animals, RA-treated mice developed much fewer splenocytes and intrahepatic lymphocyte infiltration (**Fig. 12B**).

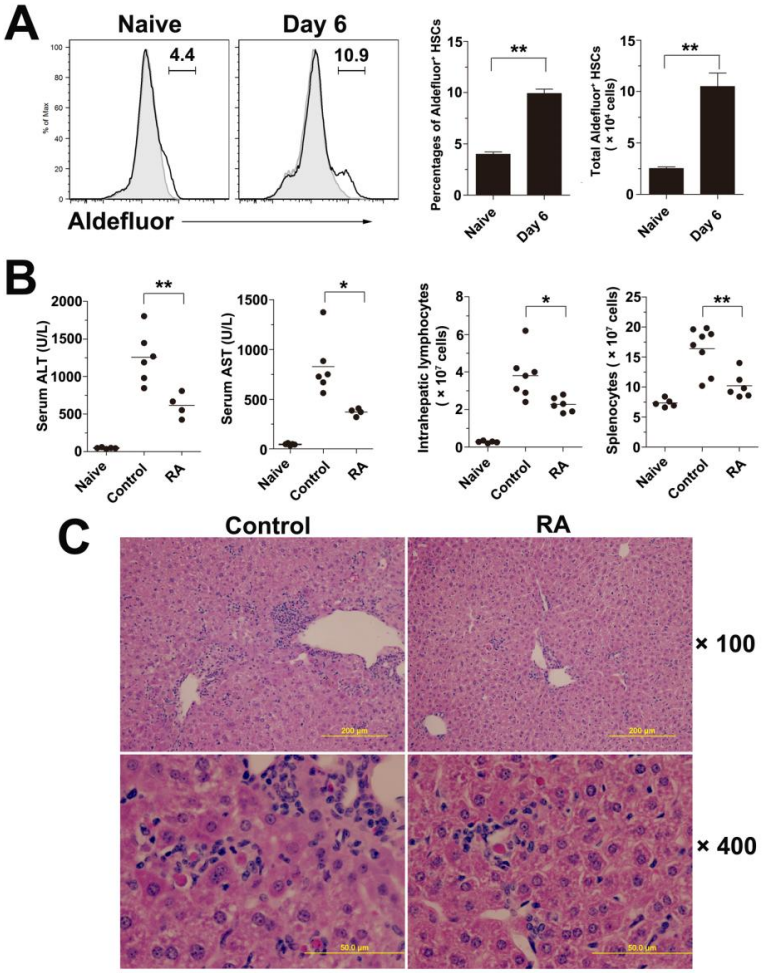

Figure 12 RA alleviated liver injury in Ad-induced viral hepatitis. (A) C57BL/6 mice were injected *i.v.* with 3×10^9 pfu of AdLacZ and sacrificed at day 6 post infection. Hepatic stellate cells were isolated and stained with ALDEFLUOR. The cells were collected and examined by flow cytometry for ALDEFLUOR. The shaded lines represent the ALDEFLUOR-negative control and the solid lines indicated experimental group. *Right panel*: Cumulative statistical results of the percentages and absolute cell number of Aldefluor$^+$ hepatic stellate cells in the liver, respectively. **(B)** B6 mice were injected *i.v.* with 3×10^9 pfu of AdLacZ. Mice were injected *i.p.* with 250 μg retinoic acid (RA) or DMSO (control) daily and sacrificed at day 6 post-infection. *Left panels*: serum ALT and AST levels of control and RA injected group. *Right panels*: total IHLs and splenocytes of control and RA injected group. **(C)** Liver tissues were obtained from Ad-infected control and RA-treated mice. Representative H&E image is shown. Results were indicated with asterisks (*$p < 0.05$; **$p < 0.01$).

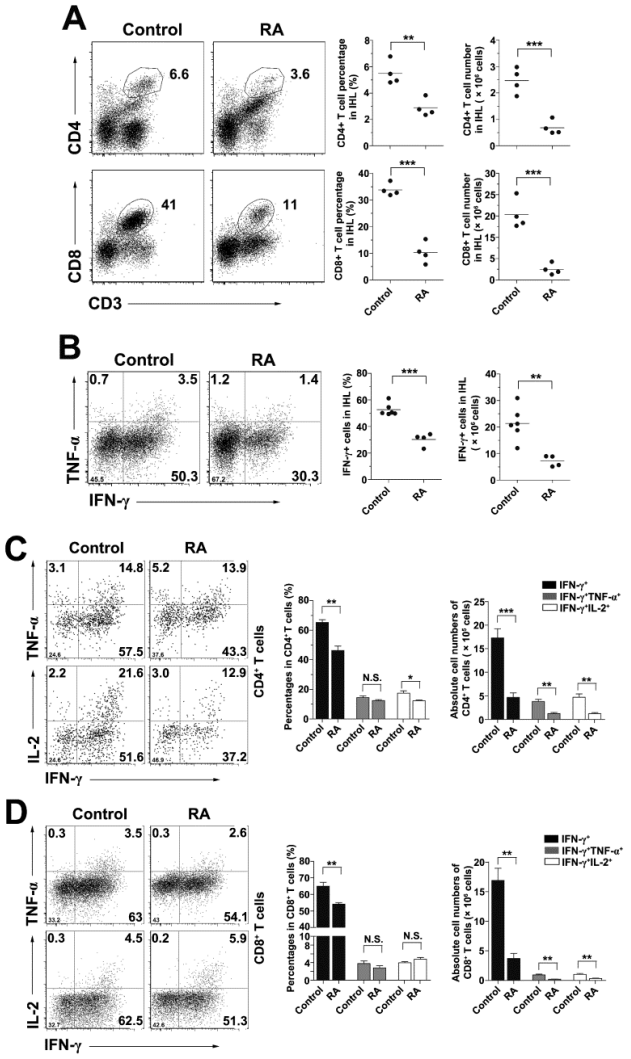

Figure 13 RA treatment halted Th1 and CTL functions in Ad-infected mice. C57BL/6 mice were injected *i.v.* with 3×10^9 pfu of AdLacZ. Meanwhile, mice were injected *i.p.* with 250 μg retinoic acid (RA) or DMSO (control) daily and sacrificed at day 6 post-infection. **(A)** IHLs were stained for surface markers (CD3, CD4 and CD8) and examined by flow cytometry. *Left panel*: flow cytometric plot of intrahepatic $CD4^+$ and $CD8^+$ T cells. Shown are representative flow cytometric results. *Right panel*: Cumulative statistical results of the percentages and absolute cell number of $CD8^+$ and $CD4^+$ T cells in the liver. **(B)** IHLs were isolated and stimulated with PMA and Ionomycin for 4 h in the presence of GolgiStop. The cells were then stained for intracellular cytokines (IFN-γ and TNF-α) and examined by flow cytometry. *Right panel*: Cumulative statistical results of the percentages and absolute cell number of IFN-γ^+ cells in the liver. **(C and D)** IHLs were stimulated with PMA and Ionomycin for 4 h in the presence of GolgiStop. The cells were then stained for intracellular cytokines (IFN-γ, TNF-α and IL-2) and examined by flow cytometry. $CD4^+$ T cell **(C)** and $CD8^+$ T cell **(D)** were gated and its intracellular cytokines (IFN-γ, TNF-α and IL-2) were analyzed, respectively. *Left panel*: flow cytometric plot of IFN-γ, TNF-α and IL-2 among intrahepatic $CD4^+$ or $CD8^+$ T cells. Shown are representative flow cytometric results. *Right panel*: Cumulative statistical results of the percentages and absolute cell number of IFN-γ^+, IFN-γ^+ TNF-α^+, and IFN-γ^+ IL-2^+ $CD4^+$ or $CD8^+$ T cells in the liver, respectively.

Previous studies showed that RA negatively regulates Th1 cell responses (221, 222). Here, we found that RA treatment significantly decreased the percentages of intrahepatic $CD4^+$ and $CD8^+$ T cells (**Fig. 13A**), as well as the pro-inflammatory cytokine productions by IHLs (**Fig. 13B**). Furthermore, RA treatment significantly reduced IFN-γ-producing ability among $CD4^+$ T cells in the liver (**Fig. 13C**). Due to the reduced number of intrahepatic $CD4^+$ T cells, significantly fewer numbers of these cells are IFN-γ^+ TNF-α^+ or IFN-γ^+ IL-2^+ (**Fig. 13C**). As found with $CD4^+$ T cells, lower percentages and fewer numbers of intrahepatic $CD8^+$ T cells expressed IFN-γ (**Fig. 13D**). In the spleen, RA treatment remarkably decreased the percentages of $CXCR3^+$ and $CD44^+$ $CD62L^-$ populations in both $CD4^+$ and $CD8^+$ T cells (**Fig. 14**). These findings demonstrated that treatment with RA protected against liver damage and suggested to us that endogenous RA may play a role in limiting liver inflammation.

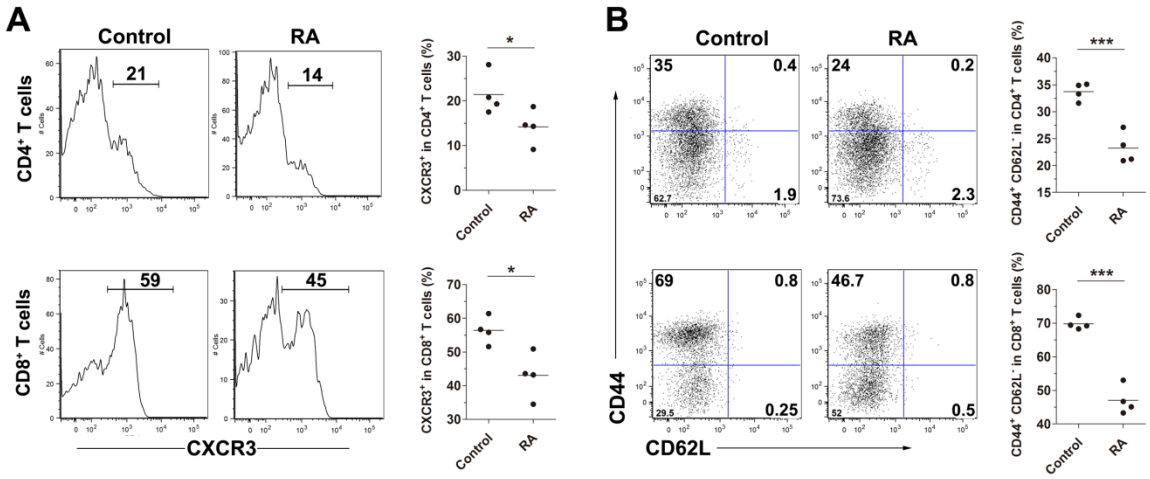

Figure 14 RA treatment suppressed splenic $CD4^+$ and $CD8^+$ T cell activation. C57BL/6 mice were injected *i.v.* with 3 × 10^9 pfu of AdLacZ. Meanwhile, mice were injected *i.p.* with 250 g retinoic acid (RA) or DMSO (control) daily and sacrificed at day 6 post-infection. Splenocytes were stained for surface markers (CD3, CD4 and CD8) and examined by flow cytometry. (**A)** Flow cytometric analysis of CXCR3 of splenic $CD4^+$ and $CD8^+$ T cells. (**B)** Flow cytometric analysis of CD44 and CD62L of splenic $CD4^+$ and $CD8^+$ T cells.

Retinoic acid promoted IL-22 production by ILC3s in the liver

RA has been suggested to associate with increased IL-22 production by innate lymphoid cells and γδ T cells in the intestine (217). To investigate whether RA can enhance hepatic IL-22 production during viral hepatitis, RA-treated and control animals were sacrificed and analyzed for IL-22 levels at day 6 post-infection. Here, we found that RA administration significantly enhanced IL-22 and IL-17 production by IHLs (**Fig. 15A**). Further study showed that RA can promote ILC3 to produce IL-22 in the liver (**Fig. 15B**). We gated on these lineage-negative cells and found that RA treatment can significantly enhance IL-22-producing ability, but not IL-17-producing ability, in ILC3s (**Fig. 15C**).

It has been shown that HSC-derived RA can promote the induction of $CD4^+$ $Foxp3^+$ T regulatory cells *in vitro* (58, 223, 224). To our surprise, we found that RA treatment decreased $Foxp3^+$ T regulatory cells in the liver (**Fig. 15D**). We considered that the decreased numbers of Treg cells may be due to the lower percentages and fewer cell numbers of total $CD3^+$ T lymphocytes after RA treatment. To examine this, we gated on intrahepatic $CD3^+$ T cells and analyzed the $Foxp3^+$ population. Interestingly, we found that RA treatment did not change the percentages of $Foxp3^+$ cells among intrahepatic $CD3^+$ T cells (**Fig. 15D**).

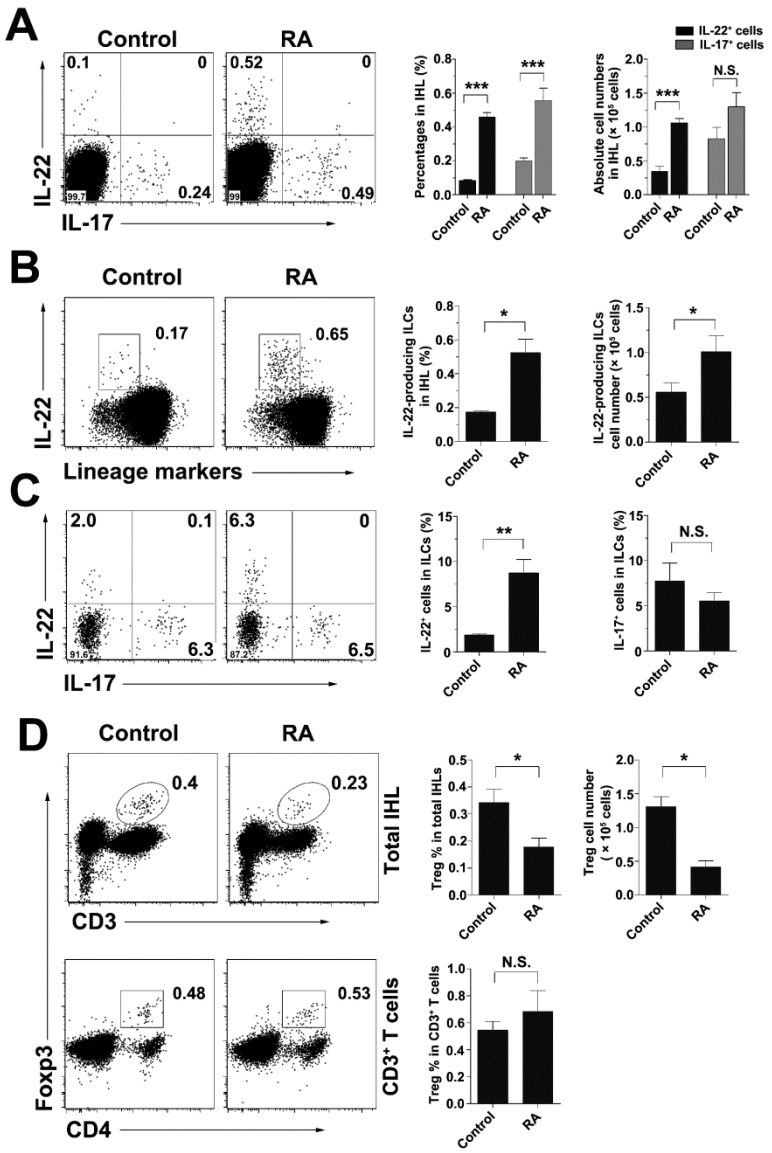

Figure 15 RA promoted IL-22 production by ILC3s in the liver. C57BL/6 mice were injected *i.v.* with 3×10^9 pfu of AdLacZ. Meanwhile, mice were injected *i.p.* with 250 μg retinoic acid (RA) or DMSO (control) daily and sacrificed at day 6 post-infection. **(A)** IHLs were isolated and stimulated with PMA and Ionomycin for 4 h in the presence of GolgiStop. The cells were then stained for intracellular cytokines (IL-22 and IL-17) and examined by flow cytometry. *Right panel*: Cumulative statistical results of the percentages and absolute cell number of IL-22$^+$ and IL-17$^+$ cells in the liver, respectively. **(B)** IHLs were gated on CD11b$^-$ population. IL-22 producing cells were analyzed against lineage (Lin) markers (CD3, CD8, CD11c, NK1.1, B220, Gr-1, and Ter-119) by flow cytometry. *Right panel*: Cumulative statistical results of the percentages and absolute cell number of IL-22$^+$ cells, respectively. **(C)** The cells were further gated on lineage negative populations and analyzed for intracellular IL-22 and IL-17. *Right panel*: Cumulative statistical results of the percentages of IL-22$^+$ and IL-17$^+$ cells among ILC3s, respectively. **(D)** Flow cytometric analysis of Foxp3 of intrahepatic IHLs (*upper panel*) and CD3$^+$ T cells (*lower panel*). *Right panel:* Cumulative statistical results of the percentages and absolute cell numbers of Foxp3$^+$ cells in the intrahepatic IHLs or CD3$^+$ T cells, respectively. Results were expressed with asterisks (*p < 0.05; **p <0.01).

Hepatic IL-7 is important for intrahepatic lymphocytes to produce IL-22

Hepatocytes could produce IL-7, a potent immune-stimulatory cytokine, in response to IFN-I stimulation or TLR4 induction (25, 172). We found that IL-7 production in the liver peaked at 24 h post Ad infection, which was accompanied by an elevation of IL-22 levels in the liver, as well as IL-22R1 expression on hepatocytes (**Figs. 10 and 16A**). To determine the role of hepatic IL-7 in viral hepatitis, Ad infected mice were *i.p.* injected with anti-IL-7Rα or isotype Rat IgG on day -1, 1, 5 post infection. Mice were sacrificed at day 6, and liver inflammation was assessed. Interestingly, liver injury in IL-7Rα blockade group was more severe than the control group (**Fig. 16B**). More necrotic hepatocytes and infiltrated lymphocytes were found in IL-7Rα blockade group (**Fig. 16C**). However, the phenotype and the function of intrahepatic T cells were not affected by IL-7Rα antagonism (data not shown). In addition, IL-7 neutralization also exacerbated liver inflammation in Ad-induced hepatitis (**Fig. 16D**). This finding raised the question: whether IL-7 alleviates liver injury through protecting hepatocyte directly or regulates other cytokines in the liver. We found that hepatocytes do not express IL-7R receptor (data not shown), indicating that IL-7 may protect hepatocytes through regulating other cytokines in viral hepatitis.

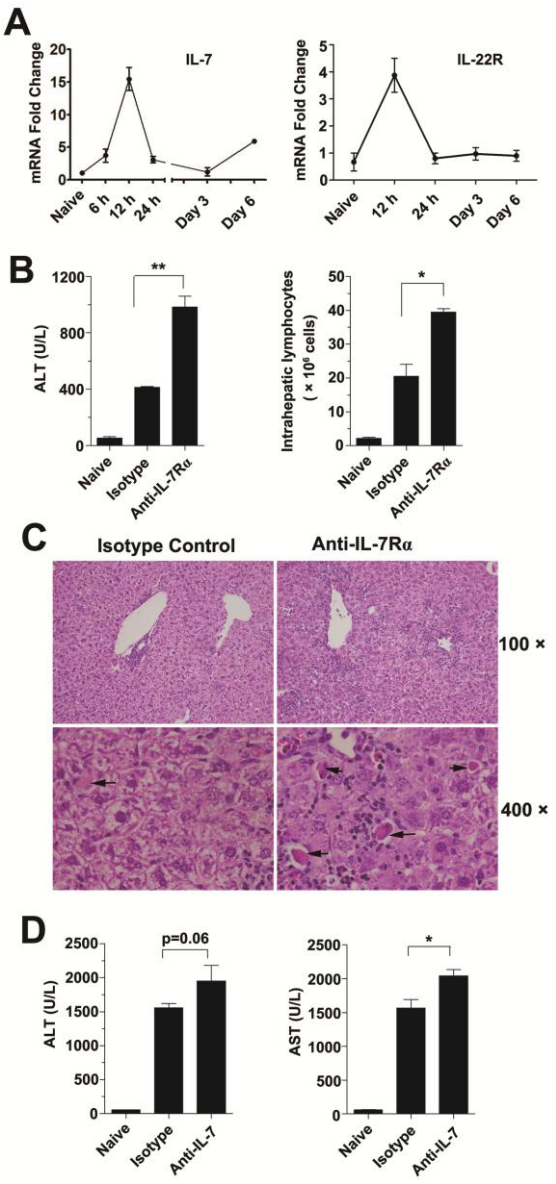

Figure 16 Exacerbated liver injury by IL-7R or IL-7 neutralization in Ad-induced hepatitis. C57BL/6 mice were injected *i.v.* with 3×10^9 pfu of Ad and sacrificed at the indicated time points. After perfusion, liver tissues were isolated, and total RNA was extracted for qRT-PCR analysis of IL-7. Meanwhile, hepatocytes were isolated and total RNA was extracted for qRT-PCR analysis of IL-22R1. Results were expressed as fold change to corresponding mRNA levels in naive mice. **(B)** Age- and sex- matched C57BL/6 mice were *i.v.* injected with 3×10^9 pfu Ad-LacZ. Mice were *i.p*. injected with anti-IL-7Rα or isotype Rat IgG (100 μg per mouse) on day -1, 1, 3, 5 post infection. Mice were sacrificed at day 6 post infection. Shown are serum ALT and intrahepatic lymphocytes in different groups. **(C)** Liver H-E slides of anti-IL-7Rα and Rat IgG injected group. **(D)** B6 mice were *i.v.* injected with 3×10^9 pfu Ad-LacZ. Mice were *i.p*. injected with anti-IL-7 (100 μg per mouse) or isotype Rat IgG on day -1, 1, 3, 5 post infection. Mice were sacrificed at day 6 post infection. Shown are serum ALT and AST in different groups. The experiment was repeated two to three times independently, and a representative graph is shown (n = 4 – 6 mice per group). Values were shown as means ± SEM. A two-tailed *t* test was used for group-to-group comparison. Results were expressed with asterisks (*p<0.05; **p<0.01).

To determine the cytokines that can be affected by IL-7R blockade, we measured the mRNA levels of cytokines and chemokines of liver tissue by qRT-PCR. We found that the pro-inflammatory cytokines and chemokines, including IFN-γ, TNF-α, IL-1β, IL-6, CXCL9 and CXCL10 were significantly up-regulated in the IL-7Rα blockade group (**Fig. 17A**). Notably, IL-22 mRNA levels of intrahepatic lymphocytes were significantly decreased in IL-7Rα blockade group (**Fig. 17A**). In chronic LCMV-induced hepatitis, IL-7 has been shown to promote IL-22 production and protect hepatocytes against apoptosis (225). Our data in Ad-induced acute hepatitis are consistent with those observed in chronic LCMV model (225).

Moreover, IL-7 can promote intrahepatic lymphocytes to produce IL-22 in a dose-dependent manner *in vitro* (**Fig. 17B**). It has been reported that IL-7 can induce IL-17 production in γδ T cells and $CD4^+$ T cells through regulating the phosphorylation of STAT3 or STAT5 pathway (226). To determine which pathway of IL-7 promotes IHL producing IL-22, we cultured IHLs with IL-7 in the presence or absence of the STAT3 or STAT5 antagonist (*STAT3 inhibitor VII or STAT5 inhibitor*), respectively. We found that IL-7 promoted IL-22 production by IHLs depending on both STAT3 and STAT5 signaling (**Fig. 17C**). Collectively, these data indicated that, in viral hepatitis, hepatic IL-7 can promote IHL-derived IL-22 production to protect liver through STAT3 and STAT5 signaling pathways.

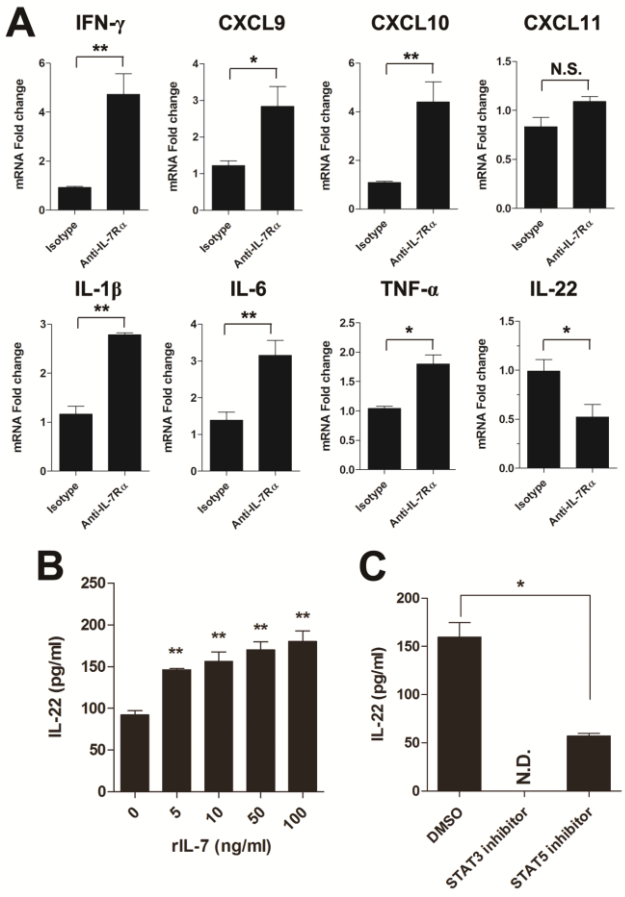

Figure 17 Intrahepatic lymphocytes required IL-7 to produce IL-22. (A) Age- and sex- matched C57BL/6 mice were *i.v.* injected with 3×10^9 pfu Ad-LacZ. Mice were *i.p*. injected with anti-IL-7Rα or isotype Rat IgG (100 μg per mouse) on day -1, 1, 3, 5 post infection. Mice were sacrifice at day 6 post infection. After perfusion, liver tissues were isolated, and total RNA was extracted for qRT-PCR analysis of IFN-γ, CXCL9, CXCL10, CXCL11, IL-1β, IL-6, and TNF-α. IHLs were isolated and mRNA level of IL-22 were analyzed by qRT-PCR. **(B)** IHLs were isolated and cultured with different concentrations of IL-7 for 2 days. Supernatants were collected and analyzed for IL-22 levels. **(C)** IHLs were isolated and cultured with 50μg/ml IL-7 for 2 days. STAT3 or STAT5 inhibitors were added during the culture. Supernatants were collected and analyzed by ELISA. The experiment was repeated two to three times independently, and a representative graph is shown (n =4 – 6 mice per group). Values were shown as means ± SEM. A two-tailed *t* test was used for group-to-group comparison. Results were expressed with asterisks (**p*<0.05; ***p*<0.01).

3.4 Discussion

Innate lymphoid cells (ILCs) represent an important family of innate effector cells which are characterized by the absence of lineage markers as well as lymphoid morphology (159, 227). As we mentioned above, ILCs have been categorized into three groups based on their transcription factors and cytokine profiles (159, 227). Group 1 ILCs comprises natural killer cells and other ILC1 cells that produce IFN-γ. Group 2 ILCs are $GATA3^{+}$ and produce type 2 cytokines (including IL-5 and IL-13). Group 3 includes ILC subsets that produce IL-17 and/or IL-22 and depend on the transcriptional factor RORγt for their development and function. ILC3s also have been shown to play important roles in intestinal immunity and homeostasis (132, 197, 228). However, the phenotype and role of ILC3s in the liver is not well understood. Our previous study showed that intrahepatic ILC3s can secrete IL-17A and IL-17F, which are crucial for adaptive T cell responses in viral hepatitis (**Chapter 2**). In this work, we found that, hepatic ILC3s can also produce a hepatoprotective cytokine IL-22 in addition to IL-17 (**Figs. 10 and 11**). Based on their NKp46 expression profile, we further speculate that most of these ILC3s belonged to the $NKp46^{-}$ ILC3 population in the liver (**Fig. 11**). By using $Rag2^{-/-}$ mice, we confirmed that ILC3s are indeed potent IL-17 and IL-22 producers and an integral part of the immune defense system in the liver (**Fig. 11**). Moreover, in addition to the Ad model, we also observed that ILC3s were present in mouse liver after LCMV clone-13 infection (**Fig. 11C**). To the best of our knowledge, this is the first report of a detailed study of ILC3 secreting IL-22 subsets in the liver following viral infection.

Cellular sources of IL-22 are various and diverse. It has been reported that IL-22 can be produced by different types of cells, including Th17, Th22, γδ T, NK, neutrophils, and ILCs (127-132). In this study, we found that intrahepatic $CD4^+$, $CD8^+$, γδ T cells, NK cells and $CD11c^+$ DCs did not produce IL-22 (**Figs. 10 and 11**). In addition to ILC3s, $Gr\text{-}1^+$ $CD11b^+$ cells can also produce IL-22 in the liver (**Fig. 11**). Until recently, it has been shown that neutrophils, a major source of IL-22 in the intestine, can protect against Dextran Sodium Sulfate (DSS)-induced colitis (131). Together, our data showed that early surges of IL-22 in the liver were mainly produced by $Gr\text{-}1^+$ $CD11b^+$ cells and ILC3s following viral infection.

IL-22 has been found to be up-regulated and indicated as a pro-inflammatory cytokine in the pathogenesis in various human diseases and in animal models, including rheumatoid arthritis, psoriasis, and Crohn's disease (140, 145, 229). On the contrary, IL-22 has also been shown to prevent mice from liver injury, inflammatory bowel disease, ulcerative colitis and lung disease (122, 147, 150, 152, 209-212). IL-22 is an essential survival factor for hepatocytes and plays an important role in protecting against hepatocellular damage in various models of liver injury, including T cell-mediated hepatitis, high-fat diet-induced fatty liver, alcoholic liver injury and liver fibrosis (147, 148, 150, 152, 209). In addition to its protective role in hepatitis, IL-22 has also been indicated as a pro-inflammatory cytokine by using an HBV transgenic model (158). It is possible that IL-22 has dual functions in viral hepatitis: increasing

liver inflammation by induction of acute phase proteins and chemokines produced by hepatocyte (134, 158, 213), but also alleviating liver injury by preventing hepatocyte damage and subsequently reducing necrosis-related liver inflammation (150).

It has been shown that the IL-23R signaling pathway, as well as the nuclear factors aryl hydrocarbon receptor (AHR) and RORγt are essential for promoting IL-22 production (127, 144, 159, 214). More recently, RA secreted by intestinal DCs has been shown to promote IL-22 production by γδ T cells and ILC3s in the intestine (217). Interestingly, HSCs store around 75% of retinol in the whole body under healthy conditions (215). However, when the liver is injured, HSCs will be activated and lose their storage of retinol through either secretion or dehydrogenation into retinal by alcohol dehydrogenase (ADH) and conversion into RA by retinal dehydrogenase (ALDH) (45, 52). In the current study, we found that the numbers of $ALDH^+$ HSCs were significantly increased in the liver following viral infection (**Fig. 12A**), indicating an endogenous RA production in the liver. Furthermore, we found that RA administration alleviated liver inflammation by inhibiting adaptive $CD4^+$ and $CD8^+$ T cell activation, recruitment and cytokine productions (**Fig. 13**). More importantly, RA promoted hepatic ILC3s to produce IL-22 (**Fig. 15B**), which was consistent with previous findings in the intestine (217). After gating on ILCs, we found that RA treatment can significantly promote their IL-22-producing ability, without affecting IL-17-producing ability (**Fig. 15C**). It has been indicated that RA could either inhibit or promote IL-17 production by Th17 cells in different disease models, which depends

on the dose and timing of RA-treatment, as well as cytokine microenvironments (223, 230-232).

Unlike the *in vitro* observation that RA strongly enhances Treg cell conversion (58, 223, 224, 233), our data showed that RA treatment did not increase the population of Treg cells *in vivo* (**Fig. 15D**). Interestingly, Mucida *et al.* found that RA treatment did not enhance the differentiation of $Foxp3^+$ Treg cells in mice infected with *Listeria monocytogenes* (230). Xiao *et al.* also showed a minor expansion of Treg cells by RA in an experimental autoimmune encephalomyelitis (EAE), which was due to the strong induction of IL-6, TNF-α and IL-1β during the inflammatory process and inhibited the enhancement of Foxp3 by RA (223). In addition, it has been shown that under strongly inflammatory conditions Treg cells are derived from nTreg cells but not generated *de novo* (234).

The liver is known as an immune tolerant organ. However, hepatocytes can also regulate the homeostasis and function of adaptive Th1 and CTL responses under inflammation conditions (184, 235, 236). Recently, we and others have shown that hepatocytes can produce large amounts of IL-7 in response to viral challenge or TLR4 activation (25, 172). Hepatic IL-7 is also important for $CD4^+$, $CD8^+$ and NKT cell responses in the liver (25, 237). In mice infected with LCMV clone 13, IL-7 administration not only augmented virus-specific T cell responses and hastened virus clearance, but also increased serum IL-22 levels and protected the liver against viral

hepatitis (225). Previous study showed that IL-7 was important for IL-17 production by γδ T and Th17 cells (172, 226, 238). Here, we found that blockade of IL-7 signaling in Ad-induced hepatitis decreased hepatic IL-22 levels and further exacerbated liver injury (**Fig. 16B**). An *in vitro* study confirmed that IL-7 is important for intrahepatic lymphocytes to produce IL-22 (**Fig. 17B**). Moreover, we found that IL-7-induced IL-22 production by IHLs was dependent on both STAT3 and STAT5 signaling pathways (**Fig. 17C**). Together, these data indicated that hepatic IL-7 can promote IL-22 productions by intrahepatic lymphocytes and protect liver in acute viral hepatitis.

In this study, we found ILC3s as a major source of IL-22 production in virus-infected liver. In addition, HSC-derived RA increased and promoted ILC3-derived IL-22, which protected the liver from inflammation-related injuries. Notably, hepatic IL-7 was important for IL-22 production by IHLs. In conclusion, this study has demonstrated the importance of a unique intrahepatic ILC3 and its cytokine production during the initial stages of viral infection in the liver. The crosstalk among the HSCs, hepatocytes and innate immune cells plays a key role in modulating immune responses. Modulation of these communications may be a potential therapeutic approach to treat liver diseases.

CHAPTER 4: CONCLUDING REMARKS

Viral hepatitis is one of the most important public health problems globally. To date, there are five well characterized human hepatitis viruses (from A-E). In most cases, patients' immune responses to different viruses vary greatly, from disease resolution to fulminant hepatitis, viral persistence and even liver failure. Virus-specific $CD8^+$ and $CD4^+$ T cell functions have been suggested to be critical in viral clearance and disease resolution. Recently, IL-17 and IL-22 production has been reported in hepatitis B and C infections. Hepatic levels of IL-17 and IL-22 are also significantly elevated in alcoholic liver disease, autoimmune hepatitis, and hepatocellular carcinoma, and correlate with the severity of disease. Interestingly, IL-17 and IL-22 played distinctive roles during viral hepatitis. To date, the cellular source of liver-derived IL-17 and IL-22 species are not well understood, and their immune functions remain debatable.

In this dissertation, we use Ad- and LCMV-induced hepatitis as viral hepatitis models. They can induce strong innate immune responses, T helper, cytotoxic T lymphocyte, and B cell responses against viruses. Herein, we examined the early phases of innate immune response and its subsequent outcome during viral infection. Intrahepatic innate cells can produce both IL-17 and IL-22 in the initial stage of viral infection, and contribute to distinctive roles in viral hepatitis.

How do our findings contribute to an understanding of hepatitis?

In this work, we found group 3 innate lymphoid cells (ILC3s) as a major source of hepatic IL-17A, IL-17F and IL-22 production in early stages of viral infection. In the liver, ILCs have been shown to protect against acute hepatitis, but can also mediate hepatic fibrosis. Here, we found that IL-17A/F signaling was important for adaptive T cell responses in viral hepatitis. IL-17F engagement was crucial for effector T cell functions and antiviral responses. Moreover, IL-17A could negatively regulate IL-17F production. In addition, hepatic stellate cell (HSC)-derived retinoic acid increased and promoted IL-22 production by ILC3s, which protected liver from inflammatory response-related injuries. Notably, hepatic IL-7 was important for IHL-derived IL-22 production. In conclusion, this study has demonstrated the importance of a unique intrahepatic ILC3 and its cytokine production during the early stages of viral infection in the liver. This study unveiled a previously unknown source and crosstalk between IL-17A and F in the liver, and may provide potential therapeutic approaches to target ILCs and IL-17 species in viral hepatitis. In addition, the crosstalk among the hepatic stellate cell, hepatocytes and innate immune cells plays a key role in regulating immune responses. Modulation of these communications may be a potential therapeutic approach to treat liver diseases.

4.1 Role of innate IL-17A and IL-17F in viral hepatitis

IL-17A and IL-17F belong to the IL-17 superfamily. They were initially reported to be produced by activated Th17 along with several other cell types (*e.g.*, $CD8^+$ T cells, $\gamma\delta$ T cells, NKT cells, neutrophils, and ILCs). They were involved in destructive tissue damage in autoimmune diseases and bacterial infections. More recent evidence has suggested their roles in promoting Th1 and CTL responses in anti-tumor immunity, inflammatory bowel disease, and antiviral immune responses. Both IL-17A and F are required for immune responses against extracellular bacterium such as *S. aureus* infection. However, in the presence of IL-17A, IL-17F was considered to be dispensable for disease progression in autoimmune diseases. The roles of IL-17A and F seem to be controversial as either pro-inflammatory or anti-inflammatory mediators in inflammatory bowel disease.

IL-17-producing cells are known to play a role in different hepatitis. Here, we identified ILC3s as a major source of hepatic IL-17A and IL-17F production in early stage of viral infection (**Figs. 1 and 2, chapter 1)**. Further, we observed that IL-17RA deficiency halted adaptive T cell responses and ameliorated liver inflammation upon viral infection (**Figs. 5 and 6, chapter 1)**. Additional experiments revealed that $IL\text{-}17A^{-/-}$ mice displayed an increase in $IL\text{-}17F^+$ cells following viral infection (**Fig. 9A, chapter 1**). *In vitro* experiments directly confirmed that IL-17A represses IL-17F secretion in $\gamma\delta$, $CD4^+$ and $CD8^+$ T cells (**Fig. 9C, chapter 1**). These results are consistent with previous reports that IL-17A controls IL-17F production through an

IL-17R-dependent, short-loop inhibition mechanism. Herein, we did not observe IL-17F-mediated IL-17A inhibition in this study (**Fig. 9D, chapter 1**). Consistently, in IL-17F$^{-/-}$ animals, there was no compensatory IL-17A increase or rescue of T cell functions (**Fig. 9B, chapter 1**). Although IL-17A and IL-17F have some functional redundancy in viral infection, our results unveil a mechanism underlying the seeming discrepancies between IL-17A- and IL-17F-deficient mice and underscore the unique functions of IL-17F in T cell responses to viral infection in the liver.

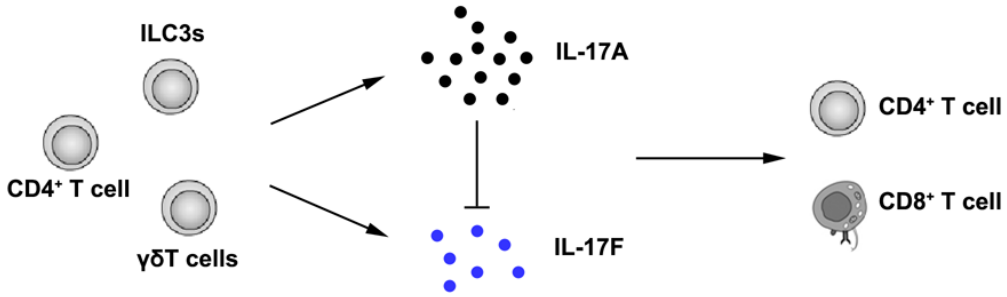

Diagram 1 Intrahepatic innate lymphoid cells and γδ T cells can secrete IL-17A and IL-17F that are crucial for adaptive immune response in acute viral hepatitis. γδ T cells and ILC3 are major sources of IL-17A/F in the liver following viral infection. IL-17 signaling was important for adaptive T cell responses in viral hepatitis. IL-17A could negatively regulate IL-17F production in the liver.

Together, we have defined the early source and function of hepatic IL-17, which are important for DC activation and T cell priming in viral hepatitis. We have provided evidence that γδ T cells and ILC3 are major source of IL-17A/F in the liver of viral infection using Ad and LCMV models. Furthermore, innate IL-17 mediated DC licensing and adaptive immune responses. In addition, we found that IL-17A is a dominant species constituting the cytokine microenvironment and regulates IL-17F production. This study suggests that innate IL-17A/F signaling is crucial for adaptive immune responses in viral hepatitis (**Diagram 1**).

4.2 Regulation and the role of IL-22 in the liver

IL-22 belongs to the IL-10 family. It has been shown that IL-22 can be produced by various types of cells, including Th17, Th22, γδT, NK, neutrophils, and ILCs. IL-22 exerts its functions by binding to a heterodimeric receptor comprised by IL-10R2 and IL-22R1. It mainly targets epithelial cells and hepatocytes due to the restricted expression of IL-22R1 on these cells. As a survival factor for hepatocytes, IL-22 is recognized in many studies as playing a critical role in the prevention of hepatocellular damage in various liver injury models. To date, the source and regulation of the liver-derived IL-22 in viral hepatitis are still not well studied.

In this study, we found that intrahepatic $CD4^+$, $CD8^+$, γδ T cells, NK cells and $CD11c^+$ DCs did not produce IL-22 at day 1 after Ad and LCMV infection (**Fig. 10, chapter 2**). Here we found that, in addition to ILC3s, $Gr\text{-}1^+$ $CD11b^+$ cells can also produce IL-22 in the liver (**Fig. 11, chapter 2**). More recently, it has been shown that neutrophils, a major source of IL-22 in the intestine, can protect against Dextran Sodium Sulfate (DSS)-induced colitis (131).

In this work, we found that $ALDH^+$ HSCs were increased in the liver after Ad infection (**Fig. 12A, chapter 2**), suggesting to us that there was an endogenous RA production in the liver. Furthermore, we found that RA treatment alleviated liver inflammation by inhibiting adaptive $CD4^+$ and $CD8^+$ T cell activation, recruitment and cytokine productions (**Figs. 13 and 14, chapter 2**). More importantly, RA promoted hepatic

ILC3s to produce IL-22 (**Fig. 15B, chapter 2**), which was consistent with previous findings in the intestine (217). Furthermore, we found that RA treatment can significantly promote IL-22-producing ability, but not IL-17-producing ability, in ILC3s (**Fig. 15C, chapter 2**). Unlike the *in vitro* observation that RA strongly enhances Treg cell conversion (58, 223, 224, 233), our results showed that RA administration did not increase the population of Treg cells *in vivo* (**Fig. 15D, chapter 2**).

Here, we found that the blockade of IL-7 signaling in Ad-indcued hepatitis decreased hepatic IL-22 levels and exacerbated liver injury. *In vitro* study confirmed that IL-7 is important for intrahepatic lymphocytes to produce IL-22 (**Fig. 17B, chapter 2**). Moreover, we found that IL-7 promotes IL-22 production by IHLs depending on both STAT3 and STAT5 pathways (**Fig. 17C, chapter 2**). These data suggested to us that hepatic IL-7 is important for IL-22 production by IHLs in acute viral hepatitis.

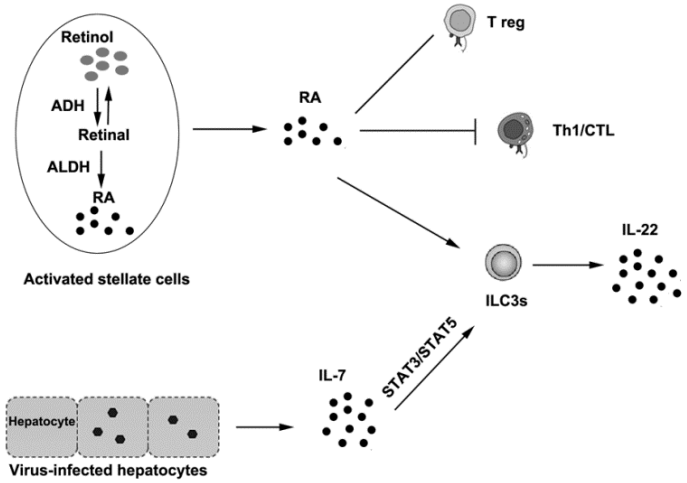

Diagram 2 Model for how hepatic RA signaling and IL-7 modulate immune responses in the liver. Quiescent HSCs store 75% of the body's supply of retinol (vitamin A). During the early stages of activation, retinol can be metabolized into retinal through dehydrogenase (ADH) and subsequently metabolized into RA through ALDH1 and ALDH2. RA treatment halted Th1 and CTL functions in viral infected liver. However, RA promoted IL-22 production by ILC3s in the liver. It seems that RA did not affect Treg population among IHLs. Hepatic IL-7 can promote the IL-22 production by intrahepatic IHLs through STAT3 and STAT5 signaling, which is protective in acute viral hepatitis.

In this part of work, we found group 3 ILCs as a major source of hepatic IL-22 production in viral infection. In addition, RA derived from HSC promoted IL-22 production by ILC3s, and protected liver from inflammatory response-related injuries. Notably, hepatic IL-7 was important for ILC3-derived IL-22 production. This study has demonstrated the importance of a unique intrahepatic ILC3 and its cytokine production during the initial stages of viral infection in the liver (**Diagram 2**).

In summary, in this dissertation work we evaluated the effect of IL-17A/F and IL-22 in the immune response to hepatotropic viruses. These two molecules are secreted by innate cells in the liver after viral infection. The cellular source of IL-17 and IL-22 seems similar; however, they conferred distinctive roles in viral hepatitis through immune-mediated mechanisms. Our findings help us to understand that innate

immune regulations are crucial for adaptive immune responses and liver protection. Future studies using models of chronic infection would help to define the role and mechanisms of these molecules and ILCs in the immune response to viruses. Our current data suggest that ILCs may be an interesting target for cell therapy against viral hepatitis.

Table 1. Primer pairs for qRT-PCR assays in the dissertation

Gene Name	Primer Sequence
GAPDH	Forward 5'-TGGAAAGCTGTGGCGTGAT-3' Reverse 5'-TGCTTCACCACCTTCTTGAT-3'
IFN-γ	Forward 5'-ATGAACGCTACACACTGCATC-3' Reverse 5'-CCATCCTTTTGCCAGTTCCTC-3'
CXCL9	Forward 5'-GGCACGATCCACTACAAATCC-3' Reverse 5'-GGTTTGATCTCCGTTCTTCAGT-3'
CXCL10	Forward 5'-CCAAGTGCTGCCGTCATTTTC-3' Reverse 5'-GGCTCGCAGGGATGATTTCAA-3'
CXCL11	Forward 5'-GGCTTCCTTATGTTCAAACAGGG-3' Reverse 5'-GCCGTTACTCGGGTAAATTACA-3'
TNF-α	Forward 5'-CCCTCACACTCAGATCATCTTCT-3' Reverse 5'-CTTTGAGATCCATGCCGTTG-3'
IL-1β	Forward 5'-GCAACTGTTCCTGAACTCAACT-3' Reverse 5'-ATCTTTTGGGGTCCGTCAACT-3'
IL-22	Forward 5'-GTGGGATCCCTGATGGCTGTCCTGCAG-3' Reverse 5'-AGCGAATTCTCGCTCAGACTGCAAGCAT-3'
IL-22R1	Forward 5'-CTACGTGTGCCGAGTGAAGA-3' Reverse 5'-AAGCGTAGGGGTTGAAAGGT-3'
IL-7	Forward 5'-TTCCTCCACTGATCCTTGTTCT-3' Reverse 5'-AGCAGCTTCCTTTGTATCATCAC-3'
IL-6	Forward 5'-CTGCAAGAGACTTCCATCCAG-3' Reverse 5'-AGTGGTATAGACAGGTCTGTTGG-3'
Hexon	Forward 5'-GAGCCAGCATTAAGTTTGATAGCA-3' Reverse 5'-AGATAGTCGTTAAAGGACTGGTCGTT-3'

APPENDIX

April 3, 2014

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Dear Dr. Jie,

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VITA

Zuliang Jie was born in Guangze, Fujian Province in China in 1984. In 2001, he went to Xiamen University, a beautiful university on a small island, to study biological sciences. In 2005, he graduated and pursued a master's degree at the same University. In the summer of 2008, he was accepted by the Graduate Program in Microbiology and Immunology, University of Texas Medical Branch (UTMB), in a beautiful island city Galveston.

During his graduate education in UTMB, Zuliang Jie has received several awards. He was funded by the James McLaughlin Pre-doctoral Fellowship from 2011 to 2013. He also received the American Society for Virology Student Travel Award at the 31st Annual Meeting in Madison, WI, in 2012. In 2013, Zuliang received the American Association of Immunologist Trainee Abstract Award at the AAI Annual Meeting in Honolulu, HI. In 2013, he received the Robert Shop PhD Endowed Scholarship at UTMB. In 2014, Zuliang was given the American Association of Immunologist Trainee Abstract Award at the AAI Annual Meeting in Pittsburgh, Pennsylvania.

Zuliang Jie married Xianxiu Wan, currently a graduate student at UTMB, on December 2009 in Guangze, Fujian, China.

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EDUCATION

Degree	Institution	Field of Study	Years
B.S.	Xiamen University	Biology	2001-2005
M.S.	Xiamen Univeristy	Biochemistry and Molecular Biology	2005-2008

PUBLICATIONS

Peer-reviewed publications:

1. **Z. Jie**, L. Xu and F. Yang. The C-terminal region of envelope protein VP38 from White Spot Syndrome Virus is indispensable for interaction with VP24. ***Arch. Virol.*** 2008.153: 2103-2106.
2. Y. Xiao, **Z. Jie**, M. Wang, G. Lin and W. Wang. Leaf and stem anatomical responses to periodical waterlogging in simulated tidal floods in mangrove Avicennia marina seedlings. ***Aquat. Bot.*** 2009.91: 231-237.
3. J. Yan[#], **Z. Jie**[#], L. Hou, J. Wanderley, L. Soong, S. Gupta, S. Qiu, T. Chan and J. Sun. Parenchymal expression of CD40 exacerbates adenovirus-induced hepatitis. ***Hepatology*** 2011.53:1455-67. ([#]co-first author)
4. L. Hou[#], **Z. Jie**[#], M. Desai, Y. Liang, L. Soong, T. Wang and J. Sun. Early IL-17 production by intrahepatic T cells is important for adaptive immune responses in viral hepatitis. ***J. Immunol.*** 2013.190:621-9. ([#]co-first author)
5. Y. Liang, **Z. Jie**, L. Hou, R. Aguilar-Valenzuela, D. Vu, L. Soong and J. Sun. IL-33 induces nuocytes and modulates liver injury in viral hepatitis. ***J. Immunol.*** 2013.190:5666-75.
6. **Z. Jie**, Y. Liang, L. Hou, C. Dong, Y. Iwakura, L. Soong, Y. Cong and J. Sun. Intrahepatic innate lymphoid cells secrete IL-17A and IL-17F that are crucial for T cell priming in viral infection. ***J. Immunol.*** 2014.192:3289-300.

Manuscript submitted or in preparation

1. **Z. Jie**, Y. Liang, L. Hou, L. Soong, Y. Cong and J. Sun. Hepatic stellate cell-derived retinoic acid regulates group 3 innate lymphoid cells and modulates viral hepatitis. *In preparation.*
2. L. Hou, **Z. Jie**, Y. Liang, M. Desai, L. Soong and J. Sun. Type 1 interferon-induced IL-7 maintains $CD8^+$ T cell responses and homeostasis by down-regulating PD-1 expression in viral hepatitis. ***Cell. Mol. Immunol.*** *Submitted*.

Abstracts presented at professional meetings

1. **Z. Jie,** J. Yan, L. Hou, L. Soong and J. Sun Parenchymal expression of CD40 exacerbates adenovirus-induced hepatitis. 2011, SCBA-Texas Chapter Meeting (Oral Presentation)
2. **Z. Jie**, L. Hou, Y. Liang, M. Desai and J. Sun IL-7 regulates the expansion of IL-17-producing γδ T cells and antiviral responses in mouse liver. 2012, SCBA-Texas Chapter Meeting (Oral Presentation)
3. **Z. Jie,** L. Hou, Y. Liang, M. Desai, L. Soong, T. Wang and J. Sun IL-7 regulates the expansion of IL-17-producing γδ T cells and antiviral responses in mouse liver. 2012, The 31st Annual Meeting of the American Society for Virology (Poster Presentation)
4. Y. Liang, **Z. Jie**, L. Hou, R. A. Aguilar-Valenzuela and J. Sun IL-33 induces nuocytes and protects liver from excessive injury in viral hepatitis. 2012, The 31st Annual Meeting of the American Society for Virology (Poster Presentation)
5. **Z. Jie**, L. Hou, Y. Liang, M. Desai, L. Soong, T. Wang and J. Sun IL-7 regulates the expansion of IL-17-producing γδ T cells and antiviral responses in mouse liver. 2012, The Emerging Viruses: Disease Models and Strategies for Vaccine Development Symposium (Poster Presentation)
6. **Z. Jie**, L. Hou, Y. Liang and J. Sun Early IL-17 production by intrahepatic T cells is important for adaptive immune responses in viral hepatitis. 2013, IMMUNOLOGY 2013™ AAI Annual Meeting (Oral Presentation)