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_____ _____

Dana Christine Tedesco Date

T cell immunity in liver disease

By
Dana Christine Tedesco
Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences
Immunology and Molecular Pathogenesis

_____
Arash Grakoui, Ph.D. Advisor

_____
Brian Evavold, Ph.D. Committee Member

_____
Mandy Ford, Ph.D. Committee Member

_____
Sam Speck, Ph.D. Committee Member

_____
Mehul Suthar, Ph.D. Committee Member

Accepted:

_____
Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____
Date

T cell immunity in liver disease

By

Dana Christine Tedesco

B.S., Muhlenberg College, 2011

Advisor: Arash Grakoui, Ph.D.

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Abstract

T cell immunity in liver disease

By Dana Christine Tedesco

The liver critically participates in a multitude of metabolic functions required for human life. Due to shared blood flow with the gastrointestinal (GI) tract, the liver is perpetually bombarded with antigen rich intestinal blood, oxidative stress, and metabolites, yet maintains order amidst this antigenic chaos. Fibrosis, reversible liver scarring, is one of several mechanisms contributing to preservation of the liver microenvironment. Fibrosis is thought to shunt insults from healthy tissue such that the liver may function unscathed. Once the insult is resolved, fibrosis reverses as healthy tissue replaces scar tissue.

Central to the process fibrosis are activated hepatic stellate cells (HSCs), which upon activation initiate the liver wound healing process. Activated HSC-derived *all-trans* retinoic acid (ATRA) induces Foxp3 expression on CD4+ T cells. These "regulatory" T cells (Tregs) protect the liver from immune mediated injury by CD8+ T cells and other effectors. While beneficial for preservation of the liver microenvironment, these Tregs are implicated in suppressing antiviral responses and permitting chronic hepatotropic infections such as hepatitis C (HCV). Despite dampened CD8+ T cell immunity, chronic liver disease can manifest systemically as antibody mediated disorders due to aberrant B cell functions. These opposing yet co-existent features suggest that fibrosis-driven alterations of intrahepatic CD4+ T cell-help yield extrahepatic consequences. Surprisingly, we found that a subpopulation of fibrosis-elicited Tregs expressing CD40L and therefore represents one mechanism driving the extrahepatic sequelae of chronic liver disease.

Given the peripheral consequences of liver-specific events, it is entirely plausible that peripheral events can influence the liver. Persistent liver injury results in destruction of the liver architecture; this is associated with digestive perturbations such as defective shuttling of bile. Blockages in bile flow, cholestasis, has been linked to intestinal abnormalities, translocation of microbes to the periphery and imbalances in commensal flora. In the clinic, transient improvement in liver functional tests have been reported following antibiotic treatment of patients suffering from choleostatic liver disorders. Although these strategies are not curative, the outcomes suggest that events in GI tract have the potential to augment intrahepatic processes. Here, we found that microbial translocation from the GI tract to the liver augments disease pathology through interaction with γδ-T cells, a non-conventional lymphocyte population selectively enriched and expanded in the liver.

The studies carried out in this dissertation propose that the events in the liver have extrahepatic consequences. Additionally, this work begins to address the effects of extrahepatic events on intrahepatic processes. Overall, deviations associated with chronic liver disease emphasize the liver's remarkable capacity to maintain a balance between tolerance and inflammatory responses while simultaneously inundated with panoply of antigenic stimuli.

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Consequences of intrahepatic environmental cues on T cell functions: a comprehensive review of the literature

Introduction: Meet the liver

Anatomic location can have crucial influence on T cell subset differentiation and functions (1, 2), a principle that is having increasing momentum in the field of T cell biology. The liver microenvironment is no exception. The liver is a vital site of non-redundant metabolic functions required for sustaining life. As such, it is equipped to manage a certain degree of inflammation such that normal metabolic functions may continue uninterrupted. This unique property reputes the liver as a "tolerogenic site" due to constant encounters with a wide array of self-antigens, food antigens, gut commensals, oxidative stress, and metabolites without any appreciable immunologic consequences or tissue damage(3-13). While there are several mechanisms that may govern the tolerogenic nature of the liver, the consequences of persistent insult highlight how deviations from steady state can have dramatic immunologic consequences. The liver is not the only organ affected during chronic liver diseases. Manifestations of disease can include: antibody mediated disorders(14), increased susceptibility to bacterial infections(15-17), insufficient antiviral responses (18-22)and intestinal disorders (23-25). These immunologic consequences suggest that perturbations in the liver microenvironment during chronic inflammation as well as intrahepatic architecture can have deleterious results.

Various etiologic agents can cause liver injury. Some of the most common are: chronic viral infections (hepatitis C virus, HCV; hepatitis B virus, HBV), non-alcoholic fatty liver diseases (NAFLD), alcoholic liver diseases (ALD), genetic insufficiencies, and acute drug toxicities. Despite different mechanisms of eliciting liver damage, nearly all liver

insults converge at hepatic stellate cells (HSCs) activation, which initiates fibrosis (26). When provoked, HSCs become activated and secrete collagen, extracellular matrix proteins, profibrotic cytokines and chemokines, and retinoic acid to begin the tissue repair process. Once the insult is resolved, this process reverses as scar tissue is replaced by healthy tissue (27). This process, fibrosis—reversible liver scarring—initiates the liver wound healing process and functions to siphon the insult away from healthy tissue (26, 27) Ineffective resolution of hepatic insult can progress cirrhosis, irreversible liver scarring, hepatocellular carcinoma (HCC) and/or end-stage liver disease (ESLD). At this point, the only therapeutic intervention is a liver transplant.

While the proverbial "point of no return" remains unclear, alterations to the hepatic architecture because of persistent liver injury have immunologic consequences. Healthy liver tissue is comprised of a series of "hepatic lobules" (28), the primary functional and structural units of the liver (29). Lobules consist of a single cell layer of hepatocytes arranged in a hexagonal shape surrounding a central vein(29). At the vertices are portal triads— a bile duct, and vascular outlets derived from both the hepatic artery and portal vein (29). The arrangement of hepatic lobules in this manner creates the one-cell thick layers of liver tissue, or cell plates. Hepatocyte cell plates are separated from the blood stream by a fenestrated layer of non-parenchymal cell—primarily Liver Sinusoidal Endothelial Cells (LSECs) (30, 31). This porous endothelial layer forms a complex capillary system, termed Liver Sinusoid, that permits exchange of vascular constituents between the sinusoids and the parenchyma(32). Between the endothelium and hepatocyte cell plate lays the Space of Disse. It contains hepatic stellate cells (HSCs) that are critical

for the initiation of liver tissue repair and contain the body's vitamin A stores. Associated with the sinusoidal face of the LSEC monolayer are Kupffer Cells (KCs), liver-resident macrophages, that are constantly bathed in sinusoidal blood flow (28)**.** Additionally, the fenestrated endothelium is the only physical and filtering barrier between the sinusoidal blood and hepatocytes, as LSECs do not have a basement membrane(28, 33, 34). Herein lies a unique property of the liver structure—there is no discrete means of compartmentalizing blood flow like other peripheral lymphoid organs. Thus the architecture of the liver permits circulating cell populations, such as lymphocytes, to interact with tissue resident populations (30, 33).

The liver has a dual blood supply; approximately 70% is derived from the gastro-intestinal tract via the portal vein, while the remainder is derived from the hepatic artery (13, 32)**.** Portal blood contains high concentrations of antigens derived from metabolic processes, as well as gut bacterial components (13). Portal blood is therefore enriched in endotoxin. At steady state, blood originating from the portal tract flows through the sinusoids to lobular central vein (28). As a consequence, antigen rich blood from the portal vein bathes the cellular constituents of the liver until it reaches the hepatic venule and enters systemic circulation (28). Despite constant antigenic exposure, there are no net immune responses to these innocuous antigens. This is due to the complex collaboration of liver antigen-presenting cells, lymphoid cells and non-parenchymal cell interactions and other liver-specific adaptations at steady state.

The liver is comprised of distinct tissue-resident lymphoid and non-lymphoid cells that do not occur in the peripheral counterparts (8, 35). In both humans and mice, the liver lymphoid compartment is enriched in non-conventional subsets including natural killer (NK) cells, and TCRγδ+ T cells (4, 8). Unique liver-resident dendritic cells, Kupffer Cells (KCs), Liver sinusoidal endothelial cells (LSECs) facilitate tolerance at steady state maintaining an immature phenotype, and secretion of IL-10 following encounter of antigen (6, 12, 36, 37). The interaction of intrahepatic APCs and T cells has been implicated as a mechanism of maintaining peripheral tolerance to food antigens, allografts, and self-antigens (13, 38-40). Conversely, the poor immunogenicity of the liver microenvironment is associated with permission of chronic hepatotropic infections and weak anti-tumor responses (6, 8, 11). Due to the composition of the liver lymphoid and non-lymphoid compartments, and the multitude of metabolic and immunologic functions it participates in, the liver is a distinct microenvironment. As such, liver-specific environmental cues can dramatically influence downstream effector functions of T cells.

Dysregulation of these very processes aimed at preserving the hepatic microenvironment can have deleterious consequences in the context of hepatotropic pathogens (6, 41). Fibrosis is the response to inflammatory insult and has three main evolutionary goals: contain the damage, limit immune-mediated injury, and repair the tissue (42). Following injury, activated hepatic stellate cells (HSCs) secrete *all-trans* retinoic acid (ATRA) and other pro-fibrotic mediators such as IL-10, TGFβ (42) to initiate the tissue repair process. HSC-derived ATRA has been shown to induce and stabilize Foxp3 expression in CD4+ T

cells (5, 43). Accumulation of intrahepatic CD4+ Foxp3+ T cells, or “Tregs”, has been implicated in protecting the liver from immune mediated damage (44-46) at the expense of dampening intrahepatic CD8+ T cell responses (5, 9, 47-49). Thus, if injury was caused by a viral infection then CD8+ T cells are primed in an environment rich in IL-10 and TGFβ, cytokines that are not only pro-fibrotic (42) but also tolerogenic and hepatoprotective (35, 50). Thus, liver-specific responses to inflammatory insult may actually increase susceptibility to chronic hepatotropic infections in comparison to conventional priming in peripheral lymphoid organs.

The microenvironment in which a T cell resides can have crucial impact on its differentiation and effector functions. Integration of environmental cues by T cells fundamentally influences outcomes of a given adaptive immune response (51). Environmental perturbations can critically influence elicitation of downstream effector functions. Here, we will review the influence of liver-specific populations and environmental signals on T cell responses during steady state and the effect of deviations in the microenvironment during liver disease on T cell differentiation and effector functions. A more clear understanding of the consequence of anatomic partitioning of a T cell in the liver versus the periphery will better inform vaccination strategies, management of chronic inflammatory diseases, and autoimmune conditions.

I. Liver-specific signals differentially instruct CD4+ T cell responses

Integration of environmental cues by CD4+ T cells critically influences the outcomes of a given adaptive immune response (51). Hepatic CD4+ T cells receive differential cues those of their peripheral counterparts as a result of interaction with liver-resident antigen presenting cells (APC) and other non-parenchymal cells within the liver. Because the liver shares an estimated 70% of blood supply with the gut via portal venous blood, the microenvironment is continually bathed in microbial products, metabolites and food antigens (13). The liver has been implicated as a key anatomic site of maintaining peripheral tolerance via direct and indirect modulation of T cell responses(11, 52). Intrahepatic CD4+ T cell help may be a determinant in responses to innocuous antigens, such as food, versus allergy. Early studies have demonstrated that the liver participates in tolerance to food antigens (38, 53-55). These experiments have found that diverting portal venous blood flow could ameliorate oral tolerance of ingested ovalbumin in naïve mice (55). This suggests that the context in which antigen is presented in the periphery versus intrahepatic presentation can differentially influence the quality of the immune response. Thus, mechanisms of eliciting oral tolerance may be liver-intrinsic.

Interestingly, clinical reports indicate that liver allograft donor-derived food allergies are can be transferred to recipients (56, 57). Clinical management of these new-onset post-transplantation allergies employs T cell based immunosuppressives (57). Later studies in rodent models suggest that inducible regulatory T cells, iTregs, play a role in the outcomes responses to innocuous food antigens (39, 40). iTreg generation is consequence of liver APC-presentation to CD4+ T cells and is regarded as a mechanism of dampening

intrahepatic inflammation (5, 9, 12, 43, 45). Combined, these studies highlight a putative role of intrahepatic presentation of food antigens to CD4+ T cells as a tolerance mechanism that has yet to be explored.

Like food antigens, intrahepatic recognition of commensal microbial products does not elicit an appreciable immune response in healthy tissues (13). This is attributed, in part, to the suboptimal stimulatory capacity of liver-resident dendritic cells (DCs), presumably from low expression of TLR4 in comparison to splenic counterparts (58). Studies in rodent systems indicate that liver derived DCs cultured with CD4+ T cells promote Th2 differentiation following LPS stimulation(58). The response of splenic DCs, on the other hand, resulted in Th1 skewing (58, 59). Following microbial antigen recognition, intrahepatic DCs bias CD4+ T cells toward Th2 responses(58, 59). Th2 responses are associated with hepatic healing and maintenance of the environment milieu under non-inflammatory conditions (4, 6, 60-62)**.** Other intrahepatic APC populations such as Kupffer cells (KCs) and liver sinusoidal endothelial cells (LSECs) have been implicated in eliciting CD4+ T cell response that would favor maintaining the liver microenvironment. Thus, intrahepatic APCs priming CD4+ T cell responses at steady state suggest that the net result does not favor immunogenicity but rather preservation of the liver microenvironment.

CD4+ T cell priming by liver-resident APCs

KC PRIMING. KCs have been implicated in dampening CD4+ T cell responses to cognate antigen. At steady state, KC stimulation of CD4+ T cells also results in a decreased activation state in comparison to splenic DCs (63). Interestingly, co-cultures of CD4+ T cells initially activated by splenic DCs could be suppressed by addition of KCs via prostaglandin secretion (63). This may function as an additional mechanism in which ongoing inflammation is managed within the liver. Mouse models of both chemically-induced and dietary insufficiency-mediated liver injury have demonstrated that KCs in particular lose expression of PD-L1, a tolerogenic mediator that exerts function through interactions with PD-1 to suppress T cell responses (12, 22) and gain expression of immunogenic CD80 (64). As a result, these KCs were able to stimulate immune-mediated liver injury through immunogenic priming of transgenic CD4+ T cell help (64) to CD8+ T cells. This work is one of the first demonstrations of *in vivo* consequences of enhanced immunogenicity acquired by KCs during liver injury.

A hallmark of liver injury is expansion of intrahepatic monocytes and KCs(65) and marked influx of inflammatory monocytes and neutrophils (66). KC expansion is associated with Th2-polarized responses (67) "pro-fibrotic" CD4+ T cells (65). Fibrotic liver KCs influence CD4+ T cell differentiation states through the CCR8/CCL1 pathway (68). Using both the CCl_4 and bile-duct ligation models of liver injury, Heymann and Colleagues (68) found that fibrosis was attenuated in CCR8-/- mice. Their data indicate that deficiencies in the CCR8/CCL1 pathway resulted in reduced intrahepatic Th2/Treg

and skewed more toward “anti-fibrotic” Th1 phenotype (68). Adoptive transfer of CCR8-intact monocytes was sufficient to restore liver pathology in these two models (68). Their data suggests that modulating inflammatory monocytes via chemokine receptor CCR8 can attenuate liver pathology either directly through differentiation or indirectly via Th1-bias. Small molecule inhibitors of CCR8 are under investigation (69). Targeting liver fibrosis through conserved inflammatory pathways has broad therapeutic implications as well as applications to other chronic diseases.

LSEC PRIMING. LSEC are liver resident scavengers that uptake of systemic and/or circulating antigens and present them to intrahepatic lymphocytes(70). In a mouse model, non-fibrotic liver LSECs can present cognate antigen to transgenic-CD4+ T cells are less activated that those primed with cognate antigen by splenic DCs(11, 36, 71). The *in vivo* significance of this finding was found using the ovalbumin-autoimmune hepatitis adoptive transfer model in which liver injury is highly immune mediated (72). Co-transfer of LSEC-primed OT-II Tg-along with OT-I CD8+ T cells (implicated in liver injury), was sufficient to suppress the ongoing immune response and attenuate liver injury (72). Evidence from early work indicates that the effect of naïve CD4+ T cell antigen recognition on LSEC results in IFNγ production, but ineffective at driving full Th1 differentiation (71). A later study concludes the opposite—LSEC are ineffective at CD4+ T cell priming (73). The latter study by Katz et al., calls cellular preparations into question, yet overlooks mouse strain as a factor that could dictate the differential functional outcomes. This caveat could critically influence the outcomes in mouse models of liver injury (74-76)

During liver injury, LSECs acquire immunogenicity that is postulated to be the result of the inflammatory environment they reside (36). Instead of promoting an anergic and/or regulatory CD4+ T cell phenotype, fibrotic liver LSECs gain capacity to stimulate CD4+ T cell proliferation and activation(36). Thus, fibrosis promotes an inflammatory shift in the liver microenvironment that fosters immunogenic alterations in liver-resident APC populations. More study is merited to better understand how the newly acquire stimulatory capacity of liver APCs during

In vivo mouse models have been invaluable for defining immunologic consequences of liver diseases. Although controversial for etiologic studies, intra-peritoneal administration of Carbon Tetrachloride (CCl_4), a hepatotoxin, over a period of time overloads mechanisms for managing oxidative stress and results in marked liver fibrosis (77). Despite systemic administration of CCl_4, peripheral sites such as the spleen are not fazed by the events in the liver (48). Surprisingly, this model system recapitulates features of intrahepatic CD4+ dependent- B cell dysfunction found in human patients with chronic HCV infection (48). In both mouse models and studies of human patients with various etiologic agents of disease hepatic fibrosis results in a liver-specific accumulation of CD4+ Foxp3+ T cells (44). These findings suggest that shared post-injury mechanisms of liver repair exist in both mouse models and human patients of unrelated etiologies. Moreover, these findings validate mouse models as useful tools to study the influence of the fibrotic liver microenvironment on T cell helper functions that are not feasible in human patients

HEPATIC STELLATE CELLS. Fibrotic processes in response to liver injury favor induction of regulatory (Foxp3+) CD4+ T cells. This occurs via hepatic stellate cell-derived *all-trans* retinoic acid, among other pro-fibrotic cytokines, which induces Foxp3 expression on CD4+ T cells (9, 43). In the same study, co-cultures with HSCs- a liver-specific population can elicit Foxp3 expression from splenic CD4+ T cells in responding cognate antigen *in vitro* (43). This work demonstrates that responses by tissue-specific populations can influence CD4+ T cell plasticity and effector functions. Peripheral induction of regulatory T cells, "iTregs" are crucial for maintaining tolerance and preventing disastrous autoimmune episodes (78). During viral infection, induction of Tregs participate in organ protection from immune mediated injury (45). These findings are in line with clinical reports of accumulated CD4+Foxp3+ T cells in the livers of patients suffering from chronic liver disease (3, 9, 19, 22, 43-45). Transfer or induction of CD4+Foxp3+ T cells as a therapeutic to chronic inflammatory diseases has been demonstrated in models of inflammatory bowel diseases (79) Presumably, transfer of regulatory T cells into patients with liver disease may have beneficial outcomes regarding immune mediated injury (80). In light of a putative protective capacity of this population to help control immune-mediated injury; this population is ineffective at controlling the extrahepatic manifestations of liver disease that are consequently largely antibody-mediated autoimmunity (48). Chronic liver diseases such as HCV infection can manifest as systemic Ig-mediated autoimmune symptoms(14, 81, 82). Although auto-reactive B cells exist in healthy individuals, they require proper signaling to generate a response (83). Perturbations in the liver microenvironment may elicit the proper stimulation for these

otherwise dormant B cells through altered CD4+ T cell help. Liver injury results in increased production of complement, c-reactive proteins (CRPs) and acute phase proteins by hepatocytes (84). As such, one plausible source of altered CD4+ T cell help is the generation of iTregs in the presence of HSC-derived ATRA in concert with increased compliment deposition in the injured liver.

Studies of human patients have revealed that stimulation of CD4+ T cells through the complement receptor (CD46) in the presence of MHCII+ APCs, and IL-6 elicit CD4+ Foxp3+ T cells capable of suppressing CD8+ T cell responses(85). In light of suppressive capacity, this population is also capable of stimulating antibody production by B cells(86). Although Treg transfers therapies have shown promise in other disorders such as acute graft-versus host disease and models of inflammatory bowel disorders (79, 87) this may not be the best therapeutic option for liver disease due to the liver-specific alterations of this population during fibrosis (88). In this context, a transfer of Tregs may very well benefit immune-mediated liver injury, while concomitantly stimulating pathogenic antibody production.

II. Hepatic CD8+ T cell responses

Intrahepatic CD8+ T cell priming

Liver APCs have been implicated as a major of maintaining hepatic CD8+ T cell tolerance (6, 36, 70). During steady state—in the absence of any ongoing fibrosis and/or hepatotropic infection—liver APCs contribute to tolerogenic functions of T cells (6). Changes in the hepatic microenvironment can shift the balance from tolerance to immunogenicity. This is apparent as chronic liver diseases can manifest as a loss of systemic tolerance (14, 48, 82, 89). In the absence of fibrosis, TCR-mediated restimulation of LSEC primed CD8+ T cells produce reduced levels of interferon-γ and IL-2 *in vitro* (70). Reactivation LSEC primed T cells of *in vivo* results in increased levels of co-inhibitory receptor PD-1 (70). Although this is beneficial for overall systemic antigen tolerance, the migratory arrest and dampened CD8+ T cell responses is a double-edged sword: tolerance at the expense of sufficient CD8+ T cell immunity.

Mouse models of liver injury have highlighted that LSECs, the population implicated in imprinting distinct functional properties on liver-primed CD8+ T cells, are susceptible to acquiring immunogenic properties as during fibrosis(31, 90-92). During thioacetimide (TAA) mediated liver injury, fibrotic LSECs presenting to CD8+ T cells result in superior IFN-γ and TNF-α production in comparison to non-fibrotic LSEC antigen presentation to CD8+ T cells (36) It is also worth noting that in the context of these experiments, animals were reconstituted with transgenic T cells derived from either the spleen or bone marrow (36, 70). Thus, the promotion of tolerance versus enhanced

immunogenicity can be attributed exclusively to liver-resident populations providing extrinsic signals to CD8+ T cells.

Hepatotropic infections have shed light on the complex role of the liver microenvironment on CD8+ T cell priming and recall responses. Traditional mechanisms of peripheral tolerance, such as antigen experience by immature APCs (93) can lead to clonal deletion of T cells. However, intrahepatic priming of CD8+ T cells by a phenotypically immature APC, such as LSECs, under non-inflammatory conditions results in the generation of a distinct memory population (37). Liver primed memory T cells have differential requirements for reactivation through the TCR in comparison to the same T cells primed by a matured DC. Liver-primed T cells demonstrate a distinct kinetic of effector function with a transient effector period followed by a rapid loss of ability to elicit effector functions following TCR triggering (37). Overall, this may represent a facet of ineffective antiviral responses to hepatotropic infections such as HCV. Due to the nature of the infection and tissue-specific liver danger signals, splenic or other peripheral APC populations may not be appropriately matured. As a result, presentation of antigen-specific CD8+ T cells may result in clonal deletion and/or sub-optimal re-stimulation of liver-primed memory T cells that have trafficked to the periphery. Therefore, this may represent an obstacle in immunity to hepatic infections and an effective vaccination strategy.

Antigen presentation to naïve intrahepatic CD8+ T cells results in rapid induction of effector-like CTL function characterized by improved cytolytic capacity and granzyme B function (94). This has been attributed to the specific effects of rapid IL-6 induction in the hepatic microenvironment upon hepatic APC recognition of MAMPs (95, 96). Properties of liver-resident non-myeloid cells elicited these effects on CD8+ T cells independent of conventional co-stimulatory pathways (94). Hepatic APC presentation to naïve CD8+ T cells results in rapid induction of cytolytic activity(37, 94). In light of this acute response, hepatic APC priming was ineffective to induce cytokine responses in CD8+ T cells in comparison to peripheral APCs (37, 94). Evolutionarily speaking, this acute cytolytic response elicited by intrahepatic APCs may be a means of sequestering liver insult within the liver and limiting inflammation. Peripheral responses on the other hand, may more efficiently elicit cytokine production in order to recruit effector populations. All things considered, this intrahepatic mechanism is favorable for steady state inflammatory insult; it also has the potential to limit any anti-infectious CD8+ T cell immunity in the liver. Due to the transient nature of CD8+ T cell responses, recruitment of phagocytic cells or other lymphocytes to the liver is not achieved, as it would be in the periphery.

The advent of direct-acting antiviral agents to HCV infection has unequivocally improved the quality of life and care for these patients. Despite the successes of these therapies, a protective vaccine for HCV has yet to be developed. A better understanding

of intrahepatic CD8+ T cell responses, priming, and balance of immunity and tolerance will inform novel strategies for vaccination against hepatotropic infections.

Memory Responses activated T cell sink and immune mediated injury.

CD8+ T cells are critical for antiviral immune responses (90, 97-99), however CD8+ T cells contribute to immune mediated injury associated with viral liver diseases (9, 12, 19, 39, 43, 45-47, 100). The liver is enriched in memory CD8+ T cells in comparison to other secondary lymphoid organs (90, 101-103). The liver's role as a memory T cell sink can be appreciated in patients who have undergone splenectomy. While splenectomy predisposes patients to bacterial infections (104), existing memory and innate responses are remarkably intact (105). Intact memory compartments are likely attributed to existing memory CD4+ and CD8+ T cells localizing to the liver (2, 106, 107). Aside from adaptive immunity, the liver also directly participates in innate immunity through production of complement (108, 109) and acute phase proteins (110). While splenectomized patients are more susceptible to bacterial infection (105), it does not equate to the severity of immune deficiencies in patients with mutated or deleted complement experience (111). From an evolutionary standpoint, partitioning these key innate immune components to the liver, but not the spleen may be the result of an earlier adaptation as humans began to encounter pathogens. Although the question of "which came first, viruses or bacteria" remains controversial, from rodents to humans the preferential trafficking of memory T cells to the liver suggests that this property is advantageous enough to be conserved across species.

The liver itself has been implicated as a sink for systemically activated T cells (112) which may serve as a mechanism for controlling CD8+ T cell responses during viral

infections. While factors associated with recruitment of activated CD8+ T cells to the liver are not fully parsed out, cytokine starvation and/or PD-1 expression on activated CD8+ T cells has been implicated the liver's role during the contraction phase (21, 107). Controlling T cell responses to viral infections are critical for effective host immunity and preventing a cytokine storm (113). To this aim, high PD-1 expression on activated antigen-specific T cells during acute infection (18, 114-118) entering the liver from the periphery can interact with PD-L1 expressing hepatocytes and therefore result in apoptotic cell death (119). PD-1/PD-L1 represents only one of many mechanisms implicated in controlling CD8+ T cell functions. CD8+ T cell trafficking is not solely dependent on the PD-1/PD-L1 pathway (119, 120). In a mouse model of autoimmune hepatitis, deficiencies in PD-1 signaling result in marked influx and exacerbation of immune mediated injury by incoming activated CD8+T cells; this was attributed to loss of apoptotic signaling(119). Immune mediated injury by activated CD8+ T cells and other recruited inflammatory populations are critical for induction of liver diseases; hepatoropic virus such as HCV and HBV are not themselves cytopathic (45, 46, 50, 119, 121). Immune-mediated injury is also a pathogenic mechanism of autoimmune liver disorders, chemical induction of liver disease in mouse models (8, 46, 51, 84, 122, 123).

Studies in mouse models of LCMV infection (102, 106) and influenza (103, 124) as well as livers of both healthy patients (101) and HCV-infected patients (21, 107) are enriched in tetramer-positive CD8+ T cells regardless of viral tropism. These studies suggest that there is a conserved mechanism of memory CD8+ T cell trafficking and residence in the liver following successful antiviral responses. Interestingly, transfer of memory flu-

specific transgenic (Tg)- CD8+ T cells into flu-infected mice results in rapid trafficking to the infected lung tissue (125). Tg-CD8+ cell migration was attributed to lung damage in this model (125). Although flu is not a cytopathic virus (126), the rapid cytokine production by memory T cells is critical for modulating lung injury in a dose-dependent manner. Following control of infection, these T cells migrated to the liver despite specific tropism for a virus that does not necessarily infect hepatocytes (125). Tetramer-positive or Tg-CD8+ T cells identified in the livers of influenza-resolved mice exhibited increased activation markers such as CD69, and markers of antigen experience, CD44 (103, 125). Recently, CD44 has been shown to interact with hyaluronin expressed in liver endothelium and hepatocytes (127). This may play a role in preferential localization of antigen-experienced populations to be retained in the liver.

Non-hepatotropic infections such as influenza (flu), Epstein-Barr Virus (EBV) and Cytomegalovirus (CMV) can cause hepatitis (128). In fact, clinical reports indicate that respiratory viruses have a higher incidence of hepatic side effects. In the most severe cases infection can result in fulminant hepatitis and/or liver failure (101, 103, 128-131). Under steady state conditions, the liver may be better equipped to manage the influx of activated CD8+ T cells. However, depending on severity of infection, it is entirely plausible that immune mediated injury may occur when the system is overwhelmed by inflammatory stimuli. Regardless, hepatitis as a result of bystander T cell activation to a non-hepatotropic virus highlights an underappreciated immunomodulatory role of the liver in controlling systemic inflammation.

III. Non-conventional T cell populations

The liver is an anatomic site that is highly enriched in unconventional lymphocytes including γδ-T, NK and NKT cells. γδ-TCR+ comprise 15-25% of intrahepatic T cells (132) while NK/NKT cells account for 5% of total liver lymphocytes and 40% of T cells, respectively (133).The collective actions of these non-conventional lymphocytes critically contribute to early immune responses to pathogens, anti-tumor responses and maintenance of liver homeostasis.

γδ-T cell subsets. γδ-T cells comprise up to 5% of total liver lymphocytes at steady state (134). In fact, this is the first lymphocyte population to colonize both the human (135) and mouse (136) fetal livers. γδ-T cell effector functions a can be dictated by Vγ- chain expression; invariant Vγ-chains— Vγ4 and Vγ6 TCR—TCR-dependent antigen recognition and IL-17 production capabilities (132, 137-143). These invariant γδ-T cells do not have the same thymic selection requirements as conventional αβ-T cells (144). Interestingly, the liver is selectively enriched with invariant γδ-T cell populations in comparison to other peripheral organs (137).

Although IL-17+ γδ-T cells are capable of expanding in response to inflammation (140, 142, 143, 145-147), there is a growing body of work that demonstrates this population is particularly important for TCR-mediated recognition of bacterial pathogens invading host tissues (138, 139, 142, 143, 148, 149). In line with these observations, intrahepatic γδ-T cell IL-17+ production is implicated in protective responses against intestinal bacterial pathogens such as *Listeria* (150). Animals selectively deficient in Vγ4 or total Vδ-/-

experience accelerated disease pathogenesis (147, 150). Vγ6+ T cell responses to bacterial pathogens such as *E.coli* and *Listeria* have been characterized as both protective and pro-inflammatory (151). The outcome of Vγ6/IL-17+ responses to these bugs seems to be dependent on the composition of the inflammatory milieu (151).

Intrahepatic IL-17+ γδ-T cells have been demonstrated to have dual roles in modulating injury. In acute injury settings, such as Con-A induced hepatitis (152) and experimental hepatectomy (141), regeneration but this hepatoprotective population is largely restricted to Vγ4 usage (153). Vγ4- bearing T cells are associated with the Th2-like cytokines (154), and consequently could be considered pro-fibrotic. This subset has been implicated in recruitment of T cells in a model of lung fibrosis (155). However, in chronic models of liver injury, such as high-fat diet (39) and biliary artresia (156-158), γδ-T cell-derived IL-17 is implicated in perpetuating disease pathogenesis; Vγ-chain usage has yet to be elucidated in this context.

The mechanism and duration of liver injury may dictate the long-term effects of IL-17 production. Recently, IL-17 has been implicated in hypersensitizing both HSC-cell line LX2 and primary human HSCs to sub-optimal TGFβ (159). While this is advantageous in acute liver wound healing, perhaps prolonged hypersensitivity to profibrotic mediators encourages pathology. Chronic liver diseases are associated with increased intestinal permeability, intestinal dysbiosis and microbial translocation to the liver (160-162). Given the close collaboration of the liver with the GI-tract, it is entirely plausible that IL-17+ γδT cells could potentially expand in response to inappropriate localization of

commensal microbes, however this is speculation at this point. Regardless of the specific role IL-17+ γδ-T cells play in various etiologic agents of disease, imbalances within the intrahepatic γδ-T cell compartment can influence disease progression and outcomes (Tedesco and Thapa, et al. Unpublished). A better understanding of the γδ-T cell compartment during liver diseases will likely yield an attractive therapeutic target for pathogenic processes of liver diseases.

NK and NKT subsets. Approximately 50% of the liver lymphoid compartment is comprised of NK and NKT cells (32). NK/NKT cell subsets have been implicated in anti-tumor responses, antiviral responses, and resolution of fibrosis. NK/NKT subsets coordinate with KCs and other intrahepatic phagocytes to clear pathogenic bacteria from the sinusoids (32). During *Borrelia burgdorferi* infection, KCs uptake bacterial antigens and present on MHC-related molecule, CD1d to NK/NKT in the liver(163). Key to jump-starting this response is stable NK/NKT cell contact with the KC, which relies in KC expression of CXCR3 (163).

Liver-resident NK cells have superior anti-tumor activity in comparison to peripheral blood (164). In comparison to non-specific killing of any non-self target cell, intrahepatic NK cells from healthy donors exert cytotoxic activity against HepG2 hepatoma cell line but leave both donor- and recipient-derived lymphocytes largely unscathed *in vitro* (164). This study demonstrated that cytotoxic activities could be enhanced via IL-2 signaling, leaving cytokine production to a minimum; this was attributed to TNF-superfamily ligand mediated killing. Overall, this and other studies suggest that NK cells function as stealth

anti-tumor immunity. Understanding the alterations in the intrahepatic NK/NKT cell compartment during chronic liver disease creates avenues to understand the link between chronic liver disease, cirrhosis and hepatocellular carcinoma.

IV. Liver transplantation highlights intrinsic management of T cell activation

The overall tolerogenic nature of the liver is exemplified in the context of solid organ transplantation. Early studies of liver transplantation in pigs revealed spontaneous tolerance of the engrafted liver (165). In fact, donor matched liver transplantation in tandem with a rejection prone graft; the kidney for example, can halt rejection episodes (166). As a result, many patients do not require overly aggressive immunosuppressive regimens following orthotopic liver transplantation (167-169). Rejection episodes are largely T cell mediated (5, 170-172) and therefore there have been substantial efforts understanding how intrahepatic T cell immunology can facilitate tolerance to allografts.

One such mechanism proposed by Bertolino and colleagues (173) that used the Des-CD8+ T cell model in which all of the CD8+ T cells are transgenic for self-peptide loaded into H2-K^b MHC class I. By transferring these CD8+ T cells into an intact wild-type C57BL/6 mouse, they found that autoimmunity and hepatitis was largely non-existent even under saturating conditions. This was attributed to CD8+ T cell invasion into hepatocytes, emperipolesis, which were presenting target antigen in the appropriate MHC context. As a result, these T cells became activated and were subsequently destroyed upon entry into the hepatocyte. This putative mechanism of tolerance could be reversed upon inhibition of cytoskeleton motility. Transfer of Des-CD8+ T cells into animals treated with Wortmannin, a pharmacologic inhibitor of myosin light chain kinase, resulted in a dramatic break in tolerance characterized by hepatitis, and other pathologic symptoms. This elegant study suggests that rapid clearance of CD8+ T cells activated

within the liver plays a putative mechanism in which liver allografts can quickly quell any anti-graft responses by activated T cells. This likely contributes to steady state tolerance of the wide variety of food, self and microbial antigens encountered in the liver daily.

Interestingly, emperipolesis has also been observed in livers of patients with HCV and HBV (173) with a markedly increased prevalence in samples obtained from autoimmune hepatitis (AIH) patients (174). However, these disease states are highly associated with loss of tolerance as patients can experience autoimmune sequelae such as immunoglobulin-mediated disorders (14, 175-179). AIH in particular is attributed to immune-mediated damage by ineffective management of activated auto-reactive T cells (180) among other dysfunctions. The clinical pathology associated with AIH despite an increased histologic prevalence of emperipolesis suggests that ongoing episodes of liver injury can alter mechanisms of hepatocyte-mediated tolerance. The relationship between emperipolesis and autoimmune liver disorders has yet to be elucidated.

Transplantation of livers typically result in graft acceptance; however, rejection episodes have provided invaluable evidence of the liver's capability to participate in immune education (181). In a cohort of patients, Dollinger and colleagues found that the activities of graft infiltrating T cells were consistent with naïve T cells undergoing primary activation events (181). It is not clear what determines outcomes of hepatic tolerance, however, there is considerable evidence that liver-resident APCs and non-parenchymal cells participate in graft acceptance (182, 183). Mixed lymphocyte reactions (MLRs) of

LSECs presenting allo-antigens to CD8+ T cells demonstrate an underappreciated role of LSECs tolerizing anti-graft immunity even in complete MHC mismatch (184-186). HSCs undergoing activation as a result to inflammatory events (187) promote increased regulatory T cells via *all-trans* retinoic acid; this population can suppress immune-mediated injury to the graft (43, 188). Another study by Morita and colleagues (189) suggested that regardless of which mechanisms are at play, tolerance of hepatic allografts requires IFNγ, a cytokine commonly associated with rejection. Altogether, these studies implicate liver-intrinsic mechanisms contribute to the unusually high rate of graft acceptance. Transplant exemplifies an under-appreciated role of the liver in tolerance and immunity.

V. Concluding remarks and therapeutic implications

T cell fate and effector functions are dictated by integration of environmental cues and co-stimulatory properties of APCs. The inflammatory and autoimmune manifestations (3, 12, 190-192) of liver diseases suggest that the steady state microenvironment critically contribute systemic tolerance; perturbations can have deleterious consequences. The hepatic architecture permits relatively diffuse blood flow due to the fenestrated endothelial layer (28, 33, 34). Perhaps the transient nature of lymphocyte interactions within the intrahepatic milieu contributes to management of immune responses. Patients with chronic liver diseases can experience increased portal blood pressure due to destruction of the hepatic architecture which can limit blood flow(28, 33, 34). As a result, interaction of infiltrating and resident populations can be prolonged and contribute to exacerbation of inflammation or expand antigen-specific cell populations that would not become activated under steady state conditions (193-195).

The context in which a T cell encounters antigen has critical influence on the ensuing effector functions (1, 2). As the liver participates in a multitude of biologic functions, it is not farfetched to consider the possibility that there is a two-way street—hepatic activities influence peripheral immunity, and vice versa. Thus, the shared blood flow from the GI tract may suggest that the microbiome can influence the outcomes of liver and other chronic inflammatory disorders (84, 160, 161, 196-199). Like many chronic inflammatory disorders, liver diseases are also associated with loss of intestinal integrity and resultant microbial translocation (15, 24, 160, 161, 198, 200). Unconventional T cells in other contexts have the capability to mount receptor-dependent responses to microbes

(138, 139, 200-203). The liver is rich in these unconventional T cell populations, yet the role of responses to inappropriately localized microbial products during liver and other disorders has yet to be elucidated.

Alterations in the liver microenvironment during fibrosis can facilitate activation of typically hypo-responsive liver-resident APCs; resulting in enhanced T cell priming (36, 64). Considering detrimental inflammatory outcomes, activation of intrahepatic APCs seems to reinvigorate or access populations of T cells that have been partitioned to the liver. Harnessing intrahepatic APC activation has incredible therapeutic potential for vaccination strategies against chronic hepatotropic infections and cancers that have evaded the immune system. While this may be a long way off, modulation of the liver microenvironment has the potential to tap into CD4+ T cell help that would have otherwise been anatomically sequestered and non-responsive. A clear understanding of intrahepatic factors and outcomes of CD4+ T cell mediated adaptive immune responses could elicit novel therapeutics for liver diseases as well as autoimmune syndromes and cancers. One may consider strategies to manage the inflammatory responses within the liver during the initiation of fibrosis. Targeting signaling mechanisms with small molecule inhibitors of chemokines, or modulating innate populations perpetuating injury are attractive targets. Alternatively, one could target inflammation associated with liver disease with novel Treg transfer therapeutic strategies (80). In principle, transfer of Tregs can limit immune-mediated injury to the inflamed liver. However, it is critical to consider that the composition of the fibrotic liver microenvironment is permissive to pathogenic fate decisions (204-208). As a result, Treg transfer therapies for liver diseases may

exacerbate the extrahepatic sequelae rather than curtail it. More investigation is necessary to determine the outcome of this therapeutic option.

In summary, the liver is unlike any other anatomic location; it is responsible for metabolic functions, protein production, detoxification, regulation, and a site of immunologic education. While the liver has evolved to manage a certain degree of deviation from steady state, surpassing this "point of no return" has detrimental effects on immunologic tolerance as well as general health. This principle is highlighted in the studies presented in this dissertation. In the first study, we identified a fibrosis-elicited population of intrahepatic CD4+Foxp3+CD40L+ T cells with the capability to suppress CD8+ T cell responses and simultaneously promote extrahepatic Ig-mediated manifestations of liver disease. The second study connects choleostatic liver disease and hepatic microbial translocation which promotes pathology through expansion of IL-17+ γδ T cells. Overall, these findings propose a role for the liver as a rheostat for immunologic harmony.

Chapter 2

CD4+ Foxp3+ T cells promote aberrant IgG production and maintain CD8+ T cell suppression during chronic liver disease

Dana Tedesco[1], Manoj Thapa[1], Sanjeev Gumber[2], Elizabeth J. Elrod[1], Khalidur Rahman[3], Chris C. Ibegbu[1], Joseph F. Magliocca[4], Andrew B. Adams[4], Frank Anania[3], and Arash Grakoui*[1,5]

[1]Emory Vaccine Center, Division of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA.
[2]Division of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, GA.
[3]Division of Digestive Diseases, Emory University School of Medicine, Atlanta, GA.
[4]Department of Surgery, Emory University School of Medicine, Atlanta, GA.
[5]Division of Infectious Disease, Emory University School of Medicine, Atlanta, GA.
[1]Emory Vaccine Center, Division of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA.
[2]Division of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, GA.
[3]Division of Digestive Diseases, Emory University School of Medicine, Atlanta, GA.
[4]Department of Surgery, Emory University School of Medicine, Atlanta, GA.
[5]Division of Infectious Disease, Emory University School of Medicine, Atlanta, GA.

*Corresponding Author:
Arash Grakoui, Division of Infectious diseases, Emory Vaccine Center, Division of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA 30322
Telephone: (404) 727-5850; Fax: (404) 727-7768; Email: arash.grakoui@emory.edu

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Please refer to Appendix 2 for relevant documentation.

I. Abstract

Persistent hepatotropic viral infections are a common etiologic agent of chronic liver disease. Unresolved infection can be attributed to non-functional intrahepatic CD8+ T cell responses. In light of dampened CD8+ T cell responses, liver disease often manifests systemically as Ig-related syndromes due to aberrant B cell functions. These two opposing yet co-existing phenomena implicate the potential of altered CD4+ T cell help. Elevated CD4+ Foxp3+ T cells were evident in both human liver disease and a mouse model of chemically induced liver injury despite marked activation and spontaneous IgG production by intrahepatic B cells. While this population suppressed CD8+ T cell responses, aberrant B cell activities were maintained due to expression of CD40L on a subset of CD4+ Foxp3+ T cells. *In vivo* blockade of CD40L attenuated B cell abnormalities in a mouse model of liver injury. A phenotypically similar population of CD4+ Foxp3+ CD40L+ T cells was found in diseased livers explanted from patients with chronic hepatitis C infection. This population was absent in non-diseased liver tissues and peripheral blood. ***Conclusion:*** Our data indicate that liver disease elicits alterations in the intrahepatic CD4+ T cell compartment that suppress T cell immunity while concomitantly promoting aberrant IgG-mediated manifestations.

II. Introduction

Hepatic fibrosis can be caused by a wide range of inflammatory insults with chronic viral infections (hepatitis C virus, HCV), alcoholic liver disease (ALD), and non-alcoholic fatty liver diseases (NAFLD) among the most common etiologies (209). Key mediators of fibrotic processes are myeofibroblast-like hepatic stellate cells (HSCs) (210). When provoked, HSCs secrete collagen and extracellular matrix proteins to initiate the tissue wound healing process (209, 211). Once the insult is resolved, fibrosis reverses as healthy tissue eventually replaces scar tissue (209). Persistent or ineffective resolution of fibrosis can lead to irreversible liver injuries such as cirrhosis, hepatocellular carcinoma (HCC) and end-stage liver disease (ESLD) (211). At this stage, the only therapeutic intervention is liver transplantation.

Fibrotic processes can elicit alterations in effector immune responses such as CD4+ T cell and B cell responses (212, 213). Activated HSCs secrete *all-trans* retinoic acid (RA), which promotes and stabilizes CD4+ T cell expression of Foxp3 (213-216). Fibrosis-elicited CD4+Foxp3+ T cells that arise in response to liver insult have been attributed to organ protection from immune-mediated injury in mice (217) and in human patients (213, 214, 218). While this is beneficial for the liver, this population has been implicated in aiding establishment of chronic hepatotropic infections, such as HCV, in human patients by suppressing CD8+ T cell responses (22, 214, 219). Aside from the effects on CD4+ T cell functions, HSC-derived RA can augment B cell survival, plasmablast differentiation and IgG production (212). Aberrant B cell function during liver fibrosis has been linked to systemic manifestations such as hyperglobulinemia, elevated titers of autoimmune

anti-nuclear antibody (ANA), and mixed cryoglobulinemia (MC) (reviewed in (220)). The dual effects of fibrotic processes on local suppression of CD8+ T cell responses by accumulation of CD4+Foxp3+T cells with concomitant dysfunctional intrahepatic B cells suggests a potential interplay between fibrosis, CD4+ T cell helper functions and B cells. Here, we investigated the effects of hepatic fibrosis on the CD4+ T cell compartment and its consequence on the IgG-mediated sequelae of liver disease. Using chemically induced liver injury in mice we found that fibrotic animals demonstrated a CD4+ T cell-dependent increase in serum IgG levels. Despite constitutive intrahepatic B cell production of IgG, there was a liver-specific accumulation of "regulatory" CD4+Foxp3+ T cells during liver injury. Fibrosis-elicited CD4+Foxp3
+ T cells effectively suppressed CD8+ T cell responses to cognate antigen while concomitantly permitting B cell activation *in vitro.* Phenotypic analysis demonstrated that a subset of the fibrosis-elicited CD4+Foxp3+ T cell population expressed CD40L and failed to suppress B cell functions *in vitro*. In accordance to our findings in the mouse model, a parallel population and co-incident B cell abnormalities were found in livers explanted from HCV-mediated cirrhotic patients. Taken together, our data suggests that fibrosis drives accumulation of intrahepatic CD4+Foxp3+ T cells that regulate CD8+ T cell responses and promote extrahepatic symptoms of disease.

III. Materials and Methods

Animals. Six to eight week-old C57BL6/J males, C57BL6/J-Tg (TcraTcrb)1100Mjb/J (OT-I) and Foxp3^{GFP} mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in the specific pathogen-free Emory University Vivarium in compliance with the Institutional Animal Care and Use Committee (IACUC) and NIH guidelines for the care and use of laboratory animals.

Human subjects. A total of 22 patients undergoing orthotopic liver transplantation (n=16), or non-HCV, non-ALD, non-NAFLD related liver re-sectioning ("non-fibrotic control tissue", n=6) at Emory Transplant Center of Emory University Hospital were enrolled in the study in accordance with the Emory University Institutional Review Board (IRB) approval (IRB# 00006248). Patient characteristics with clinical information and relevant biological variables are summarized in **Supplementary Tables 2-2 and 2-3**. Written informed consent was obtained from each patient and IRB #00006248 conforms to the guidelines of the 1975 Declaration of Helsinki (revised 2013).

Carbon tetrachloride (CCl_4)-treatment. Mice were injected with CCl_4 (0.5 μg/g of body weight, Sigma) intra-peritoneally (I.P.) mixed with olive oil in 1:10 ratio. Control animals were given olive oil alone. Treatments were administered every 3 days for a total of 12 treatments; animals were sacrificed per IACUC guidelines within one day following final treatment. For CD4 depletion studies, 0.5 mg CD4 depleting antibody (clone GK1.5) was administered I.P. on day -1 and 0 and every 14 days thereafter during CCl_4-treatment. Where applicable, 250 μg anti-CD154 (CD40L, clone MR1) was administered I.P. on

day -1 and 0, or 2 or 3 weeks into CCl_4-treatment regimen. Antibody was given every three days thereafter.

Isolation of liver cell populations. Animals were sacrificed as per IACUC guidelines, and livers were perfused with 1X PBS (Lonza, Switzerland) and harvested. Tissues were processed by enzymatic digestion with 2mg/mL type IV collagenase (Worthington, NY) in serum-free media DMEM-F12 (Lonza). Following incubation, tissue digests were strained through sterile cheesecloth to remove particulate matter, and enzymatic reaction was quenched with complete media. Liver lymphocytes were isolated by Percoll or Ficoll-paque (GE, Sweden) gradients. CD4+ T cells were isolated by negative selection (STEM CELL). Where applicable, further positive selection of CD4+CD25+ T cells was performed using anti-CD25 PE (BD Biosciences) and anti-PE Microbeads (Miltenyi Biotec, CA). B cells were isolated using CD19-positive selection kit for both mouse and human studies (Miltenyi). Purity and composition was assessed on a BD LSRII Custom order system. Human liver interstitial mononuclear cell (LIMC) were isolated as described (221).

Analysis of HSCs activation. HSCs were enriched as described previously (213). Cell suspensions were blocked in FACS Buffer (0.5% FCS, 0.01% Sodium azide, 1X PBS) supplemented with 10% normal mouse serum (Sigma) and anti-CD16/32 (2.4G2) then stained for CD45.2 (104; Tonbo Biosciences, CA). Dead cells were excluded using standard staining procedures for live/dead with Ghost Dye 780 (Tonbo) and fixed with BD Cytofix/Cytoperm (BD Biosciences) according to manufacturer's instructions. To

determine activation, intracellular α-SMA-PE (R&D systems) was stained at room temperature. HSCs were acquired on a FACSAria II equipped with a UV laser (355nm) (BD Biosciences) and were defined as UV auto-fluorescent positive and CD45-negative populations as indicated in **Supplemental Figure S1C.** Auto-fluorescence and debris were excluded using a 405nm laser (450/50BP).

Histology and Immunohistochemistry. Following perfusion, liver sections were immediately fixed in 10% formalin for 24 hours, and transferred to 70% ethanol and paraffin embedded. Standard Sirius Red and H&E staining were performed in the Pathology Core of Emory Vaccine Center. Fibrosis was quantified as described (222), histopathological findings are summarized in **Supplementary Table 2-1.**

Alanine amino-transferase assay. Serum was sampled immediately following animal sacrifice. Levels of alanine amino-transferase (ALT) were detected by standard procedures by the Yerkes Molecular Pathology Core of the Emory Vaccine Center.

Flow cytometry analysis. For mouse studies, cells were blocked as described. The following fluorochrome-conjugated antibodies were purchased from Tonbo Biosciences; anti-CD3 (17A2), CD4 (RM4-5), CD8 (53-6.7), CD25 (PC.61), B220 (RA3-6B2), CD44 (IM7), CD45.2 (104), CD19 (1D3). Anti-CD69 (H1.2F3) was purchased from eBioscience. Anti-ICOS (C398.4A), FasL (MFL3), IgD (11-26c.2a), CD11b (M1/70) and PD-1 (29F-1A12) were purchased from Biolegend. CD95 (Jo2) was purchased from BD biosciences. Where applicable, cells were pre-incubated with biotinylated anti-mouse

CXCR5 (clone 2G8, BD Biosciences) for 1 hour at room temperature, or FasL for 30 minutes at room temperature then followed by either streptavidin-APC or -PE (Invitrogen) and surface markers. Dead cells were excluded with Ghost Dye-780 (Tonbo). Cells were fixed and permeabilized per manufacturer's instructions (Transcription Factor Staining Set; Tonbo) then intracellular stained for Foxp3 (3G3; Tonbo), CD40L (MR1; BD Biosciences), and/or Ki-67 (B56; BD Biosciences). For studies using Annexin-V/ Propidium Iodide (PI), staining was performed per manufacturer's recommendation (BD) and acquired immediately. For human studies, thawed cryopreserved cell suspensions blocked in FACS Buffer containing anti-human CD32 (Miltenyi) and 10% human serum type AB (Sigma). Cells were subsequently stained with relevant antibodies as described: anti-CD3 (OKT3), CD4 (OKT4), CD25 (BC96) (Tonbo). Anti-FoxP3 (259D), CXCR5 (J252D4), ICOS (C398.4A) and PD-1 (EH12.2H7) were purchased from Biolegend. Anti-CD40L (CD154, TRAP-1) was purchased from BD biosciences. All samples were acquired within 24 hours on a BD LSRII Custom Order system. In all FACS experiments, auto-fluorescence and debris were excluded using a 407nm laser (525/40BP 475LP); gates were determined using appropriate isotype and/or fluorescence minus one control. FACS data were analyzed with FlowJo version 9.6.4 (Tree Star, Inc.).

ELISPOT. Milipore multiscreen-IP plates (Milipore, MA) were prepared per manufacturer's instruction and coated with appropriate antibodies in PBS: 2ug/mL Goat Anti-Mouse Ig (Southern Biotech), or 4-8ug/mL anti-human IgG (Rockland Biosciences, NY). The following day, plates were washed and blocked with complete medium. Bulk cell suspensions were prepared, diluted appropriately and incubated in complete IMDM

(10% FCS, 1.0% Pen/Strep) for 18 hours at 37^0C and 5% CO_2. Standard ELISPOT procedures were followed using biotinylated anti-mouse IgG (Sigma) or anti-Human IgG (mABtech) followed by streptavidin-ALP (mABTech). Assay was developed with NBT/BCIP Substrate (ThermoScientific) and analyzed using CTL Immunospot 5.0 software (Cellular Tech Ltd). Data were calculated as antibody-secreting (ASC) spots/10^6 lymphocytes.

Serum Ig ELISA. Nunc Maxi-sorp plates (Thermo-scientific, USA) were coated with 1ug/mL anti-mouse Ig (Southern Biotech) overnight at 4°C. Goat-anti mouse IgG-HRP was used for detection of IgG (Southern Biotech, USA). BD OPT-EIA kit was used for detection (BD Biosciences, USA). Standard curves were generated using known concentrations of mouse IgG.

Co-culture assays. CD19+ B cells ($2.5x10^5$) isolated from naïve C57BL6/J spleen were co-cultured in duplicate with indicated purified CD4+ T cells ($1.25x10^5$) pooled from either control liver, CCl_4-treated liver, control spleen, or B cells alone in the presence of complete IMDM alone or supplemented with 2.0ug/mL LPS (Sigma) for 5 days at 37°C and 5% CO_2. Following incubation, ELISPOT assay was performed as described earlier. FACS for B cell activation was performed (Live B220+CD44+Ki-67+). Where applicable, CD4+Foxp3^{GFP} T cells were used to sort indicated subsets, and cultured with B cells at a 2:1 ratio (B cells: CD4+Foxp3+) as described previously.

CD8+ T cell suppression assay. Splenic CD8+ T cells were purified (Miltenyi) from transgenic mice specific for ovalbumin residues 257-264 (SIINFEKL) (OT-I) and labeled with 5uM CFSE (Biolegend). The CD8-depleted fraction was irradiated (3000rad) and pulsed for one hour with 3ug/mL SIINFEKL at 37°C. Purified CD4+CD25+ T cells derived from indicated organs were cultured in 1:1 ratio with CD8+ T cells ($1x10^5$). Unloaded or SIINFEKL-loaded stimulator cells were added to cultures in a 1:5 ratio (stimulators: CD8). Cultures were incubated for five days at 37°C and 5.0% CO_2. IFNγ in the supernatant was detected via ELISA (BD Biosciences). Cells were stained appropriately for FACS and acquired immediately on a BD LSRII custom order system. Analysis was performed on live, non-autofluorescent, CD8+ T cells expressing the indicated markers.

HEp-2 ANA assay. HEp-2 substrate slides (Antibodies, Inc.) were used for the detection of ANA titers in mouse serum as per manufacturer's recommendations. For mouse studies, secondary anti-IgG FITC (Jackson Immunotech), and for human studies, anti-IgG FITC (Antibodies, Inc.) was used. Bead enriched CD19+ human lymphocytes were seeded at $5x10^4$ per well in the presence of complete IMDM alone or supplemented with 1ug/mL R848 (Invivogen) and 20U/mL rhIL-2 (Roche) and cultured for 5 days at 37^oC with 5% CO_2. Supernatants were harvested for detection of ANA-positive IgG and cells were analyzed by ELISPOT.

Statistical analysis. Statistical analyses were performed using Prism5 software (GraphPad) using a two-tailed Mann-Whitney *U* test, one-way ANOVA or two-tailed

Spearman correlation. Statistical significance was considered *$p<0.05$, **$p<0.005$, ***$p<0.0005$.

IV. Results

Aberrant IgG-production during hepatic fibrosis requires CD4+ T cells

In this study, our aim was to determine the role of CD4+ T cells in aberrant B cell IgG production during liver disease. We found that mice undergoing CCl_4-treatment exhibited characteristic hepatic parenchyma with periportal bridging fibrosis as measured by Sirius red and H&E staining (**Fig. 2-1A)**. Animals demonstrated an elevated serum ALT level consistent with liver injury (**Fig. 2-1B**). CCl_4-treated animals demonstrated a three-fold increase in circulating serum IgG in comparison to oil-treated control animals (**Fig. 2-1C**). Antibody-mediated (clone GK1.5) CD4+ T cell depletion during liver injury markedly reduced serum IgG levels, despite comparable collagen deposition as measured by Sirius red staining, serum ALT and severity of fibrosis lesions (average score 3) (**Fig. 2-1A-C, Supplementary Fig. S2-1A, B & Supplementary Table 2-1)**. Consistent with this finding, FACS analysis of enriched HSC expression of α-smooth muscle actin (α-SMA), an activation marker, was indistinguishable in CD4-intact versus CD4-depleted fibrotic animals (**Fig. S2-1C, D**). Collectively, these data indicate that CCl_4-elicited fibrosis does not require CD4+ T cells; this is in agreement with a previous report (223). Despite comparable indicators of hepatic injury, CD4+ T cell depletion substantially reduced the spontaneous IgG production in the livers of fibrotic animals as detected by direct *ex vivo* ELISPOT analysis of intrahepatic B cells (**Fig. 2-1D**). In contrast, B cells from CD4-intact fibrotic livers constitutively produced IgG in the absence of any stimulation (**Fig. 2-1D)**. This phenomenon was not apparent in splenic B cells (**Fig. S2-2A, B)**. Importantly, serum from CCl_4-treated fibrotic mice demonstrated elevated ANA IgG titers (titers 200), which was not detected in control animals and CD4-depleted fibrotic

animals (titers <50) (**Fig. 2-1E**). Combined together, our data suggest that CD4+ T cells are required for aberrant intrahepatic IgG-production during liver fibrosis.

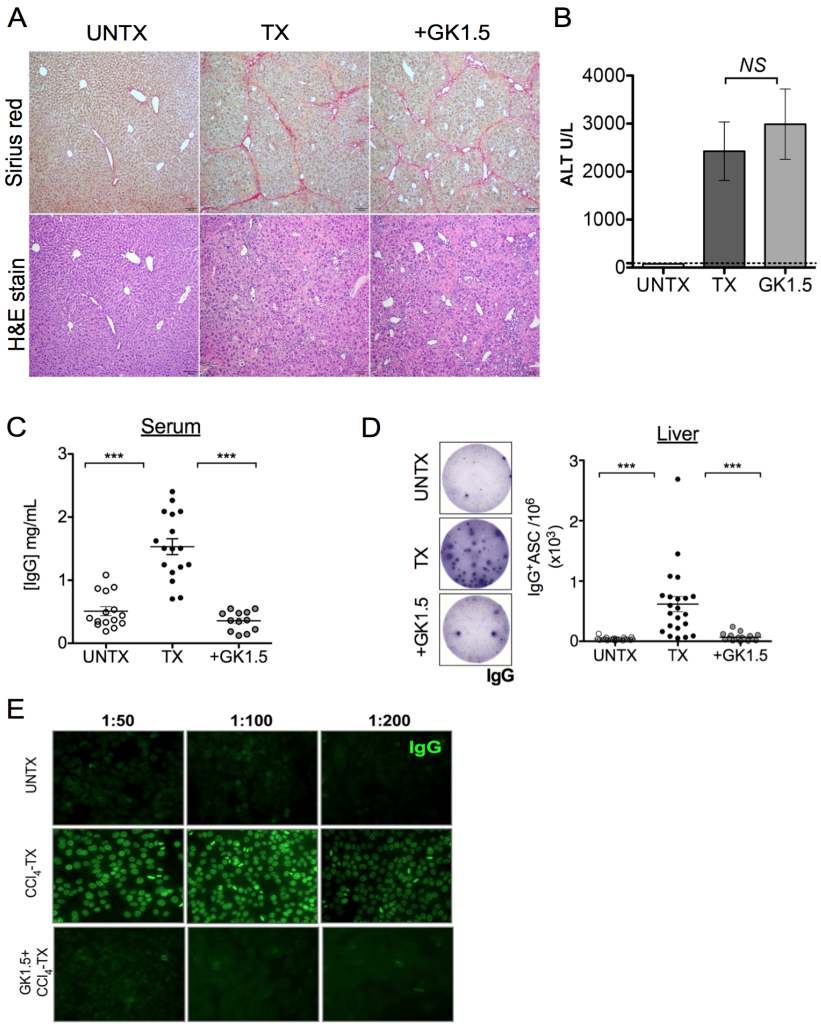

FIGURE 2-1. Aberrant IgG-production during hepatic fibrosis requires CD4+ T cells. C57BL6/J mice were treated with CCl_4 three times per week for a total of 12 treatments to induce liver fibrosis. (**A**) Following treatment, liver tissues were harvested from oil-treated control (UNTX), CCl_4-treated (TX), and CCl_4 plus GK1.5 monoclonal antibody-treated (+GK1.5) mice and processed for histological analyses by Sirius red and H&E staining (magnification 100x). Data are representative of 2 independent experiments (n=5 mice per group). Serum samples were collected from these mice and analyzed for (**B**) ALT levels and (**C**) IgG titers by using a limiting dilution standard IgG ELISA. Graph plot shown is representative of 3 independent experiments (n=3-5 per group). (**D**) Bulk liver lymphocytes isolated from UNTX, TX and +GK1.5 mice were cultured in complete IMDM medium for 18-20 hrs in presence of 5.0% CO_2 at 37˚C, and analyzed for IgG production by ex *vivo* IgG ELISPOT method. (**E**) Serum samples (dilution as indicated) were analyzed for ANA-IgG titers by using a standard HEp-2 substrate staining with anti-mouse IgG FITC. Data shown is representative of 3 independent experiments (n=3-5 mice per group). ***p<0.0005, two-tailed Mann-Whitney *U* test.

Fibrosis-elicited CD4+Foxp3+ T cells do not suppress B cell activity

Fibrotic processes such as RA production by HSCs have been shown to promote CD4+ T cell expression of Foxp3 and enhance regulatory function (213, 216, 217). We found a liver-specific accumulation of CD4+Foxp3+ T cells following CCl_4-treatment with an increased frequency (15.05 ± 3.8%) in comparison to oil-treated controls (3.15 ± 1.3%). This was not observed in the spleen (**Fig. 2-2A**). Surprisingly, despite increased frequency of "regulatory" phenotype CD4+ T cells, spontaneous IgG production was still evident by fibrotic liver B cells (**Fig. 2-1**). Phenotypic analysis of these intrahepatic B cells reveals a heightened activation state, with an increased frequency of the IgD-CD95+ (Fas) population co-expressing activation markers CD44 and Ki-67 (**Fig. S2-3A).** In line with this observation and in accordance with our previous study, fibrotic liver B cells demonstrated enhanced survival by *ex vivo* Annexin-V/PI analysis **(Fig. S2-3B)** (212). Thus, B cell activation co-exists with a marked increase of CD4+Foxp3+ T cells during hepatic fibrosis.

To investigate this dichotomy, enriched CD4+CD25+ T cells (>80% expressed Foxp3, **Fig. S2-4A**) from the livers and spleens of oil- or CCl_4-treated mice were co-cultured with CD19-enriched splenic B cells in the presence of LPS stimulation (1:2 CD4: B cell ratio) (**Fig. S2-4A**). B cells in co-culture with splenic and control liver-derived CD4+CD25+ T cells markedly reduced this IgG production (260 ± 55.0 IgG+ and 250 ± 155.2 IgG+ ASC/10^6, respectively) (**Fig. 2-2B**). However, CD4+CD25+ T cells derived from fibrotic livers did not inhibit IgG production by B cells (1100 ± 45.1 IgG+ASC/10^6). Likewise, co-culture with fibrotic liver-derived CD4+CD25+ T cells did not suppress the B cell activation (CD44 and Ki-67 expression on Live, B220+ cells)

compared to splenic and control liver CD4+CD25+ T cell co-cultures (**Fig. 2-2C**). The same result was observed in experiments were performed with "untouched" B cells (data not shown). We also investigated the effect of fibrosis on the CD25-negative fraction of CD4+ T cells. Under the same co-culture conditions, all CD4+CD25-negative T cells had a comparable effect on B cell activation (**Fig. S2-4, B-D)**. Thus, these findings suggest that fibrotic liver disease promotes altered CD4+Foxp3+ T: B cell interaction.

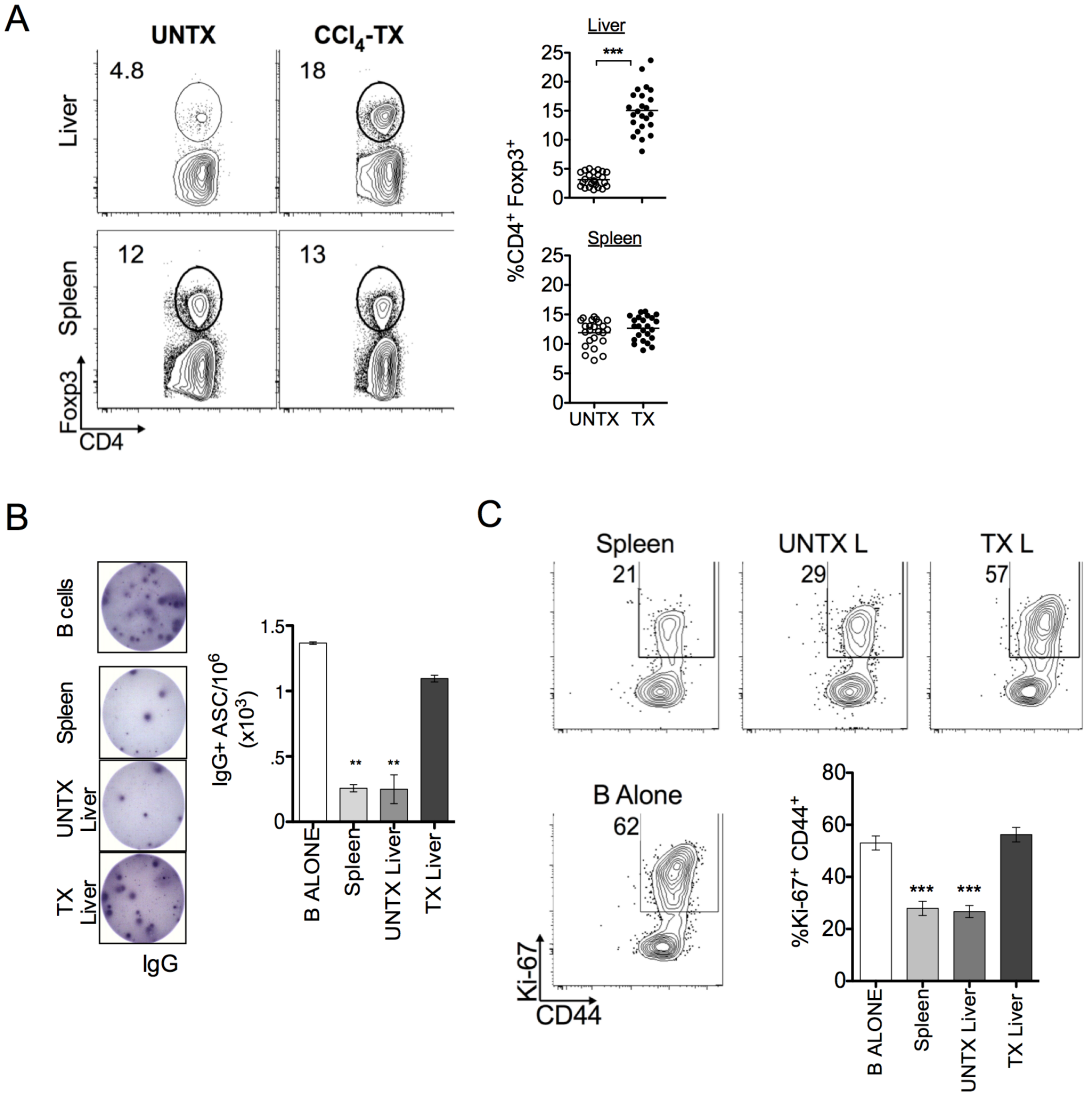

FIGURE 2-2. Fibrosis-elicited CD4+Foxp3+ T cells do not suppress B cell activity.
(**A**) Control (UNTX) and CCl_4-treated (TX) mice were harvested and processed for phenotypic analysis of CD4+Foxp3+ T cells by FACS. FACS plots are representative of 3 independent experiments (n=3-5 mice per group). Frequencies represent the Live, Non-autofluorescent, CD3+ CD4+Foxp3+ T cell gate. (**B**) ELISPOT analysis of B cell co-culture alone or in a 2:1 ratio with CD25-enriched CD4+ T cells in the presence of LPS stimulation for 5 days. The number of IgG producing cells per million ($1x10^6$) was calculated as described, each well was plated in duplicate and manually counted under a microscope. Representative of 5 independent experiments, isolated from pooled lymphocytes (n=3-5 mice per group) is shown. (**C**) Frequency of B220+ Ki-67+ CD44+ B cells co-cultured as indicated in the presence of LPS stimulation for 5 days. Statistics were calculated relative to culture of B cells alone using a two-tailed Mann-Whitney *U* Test (***p<0.0005, **p<0.005, *p<0.05). Error bars represent SEM.

Fibrosis-elicited CD4+Foxp3+ T cells suppress CD8+ T cell responses

Chronic liver disease presents a unique dichotomy of Ig-mediated extrahepatic symptoms and heightened B cell activation (220, 224, 225) that co-exist with intrahepatic immunosuppresive milieu characterized by increased frequencies of CD4+Foxp3+ T cells (22, 213-215, 217). Our own findings are consistent with these B cell-mediated sequelae of disease **(Fig. 2-2)**. Therefore, we aimed to interrogate the suppressive activity of the fibrosis-induced CD4+Foxp3+ T cells on CD8+ T cell activation by cognate antigen in the mouse model. To test this hypothesis, we performed parallel co-culture experiments with CD4+CD25+ T cells (>80% Foxp3+) isolated from spleen, control and fibrotic livers and cultured with CFSE-labeled OT-I CD8+ T cells in the presence of OVA (257-264)-pulsed stimulator cells. After five days of culture, CD4+Foxp3+ T cells comparably reduced CD8+ T cell proliferation, irrespective of their origin (**Fig. 2-3A).** In line with this observation, CD8+ T cells cultured with CD4+Foxp3+ T cells expressed similar levels of activation markers, CD25 and CD44 (**Fig. 2-3A)**. Furthermore, analysis of culture supernatants indicated that all isolated CD4+Foxp3+ T cells significantly suppressed IFNγ production by responding OT-I CD8+ T cells **(Fig. 2-3B)**. These data demonstrate that fibrosis-elicited CD4+Foxp3+ T cells regulate CD8+ T cell responses to the same extent as non-fibrotic liver and their splenic counterparts. Thus, our observations suggest that CD4+Foxp3+ T cells induced by liver injury selectively regulate CD8+ T cell responses while simultaneously permitting B cell activation and IgG production.

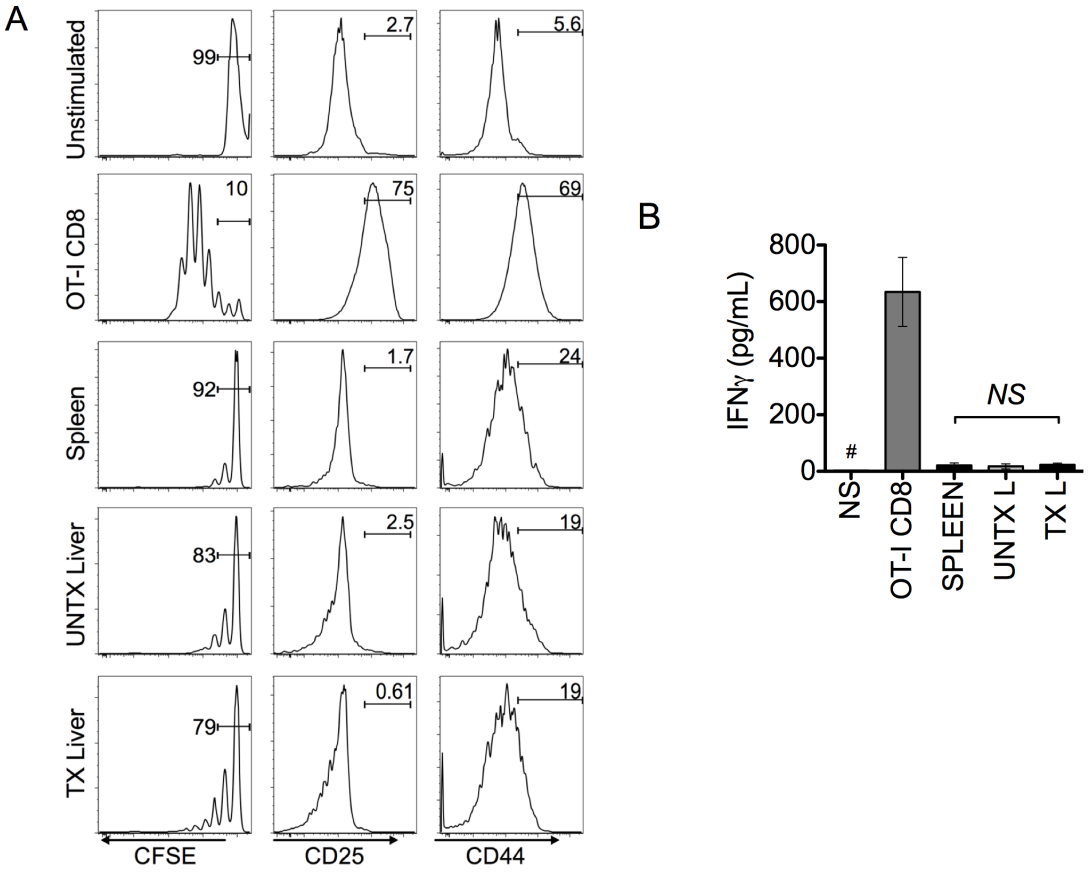

FIGURE 2-3. Fibrotic liver CD4+Foxp3+ T cells suppress CD8+ T cell response.
(**A)** CFSE labeled OT-I CD8+ T cells were cultured in the presence of irradiated CD8-depleted splenocytes pulsed with 3ug/mL ovalbumin peptide (SIINFEKL) 257-264 or unloaded (5:1 ratio, CD8: stimulators). CD4+CD25+ T cells (>80% Foxp3+) were added from indicated sources in a 1:1 ratio with CD8+ T cells. After 5 days of culture, proliferation was assed by CFSE dilution on live, non-auto-fluorescent CD8+ T cells, (**left**). Activation was assessed by CD25 and CD44 expression (**middle and right**). (**B)** ELISA was performed on supernatants to quantify IFNγ production from indicated wells (# denotes IFNγ concentration below 15pg/mL in supernatant). Data are representative of 3 independent experiments with pooled CD4+CD25+ T cells from indicated organs (n=3-5 animals per group). Statistics were calculated using one-way ANOVA of duplicate wells from at least 3 independent experiments (*$p<0.05$); error bars reflect SEM.

Fibrotic liver CD4+Foxp3+ T cells up-regulate CD40L, CD69 and PD-1 expression

Co-existence of B cell activation and abundance of CD4+Foxp3+ T cells with regulatory capacity on CD8+ T cells during fibrotic liver disease may be indicative of altered CD4+ T cell help to B cells. As such, we investigated the effects of fibrosis on the CD4+ T cell compartment by FACS analysis to identify co-expression of Foxp3 with markers that may potentiate B cell activation. We found augmented expression of PD-1, an activation marker implicated immune-modulatory functions(226, 227)); CD69, an activation marker (228); and CD40L (CD154), a molecule critical for IgG-class switching (229), on CD4+Foxp3+ T cells from fibrotic livers (**Fig. 2-4A,** frequencies represent Foxp3+ population). This phenotype was not observed in oil-treated control livers (**Fig. 2-4A**) or spleens (**Fig. S2-5A, B**). Although fibrotic liver CD4+ Foxp3- T cells increased CD69 and PD-1 expression, there was no overt consequence of this activation state with respect to B cell function (**Fig. S2-4, B-D)**. Therefore, liver-specific accumulation of CD4+Foxp3+ T cells during fibrosis represents a heterogeneous population with elevated expression of CD40L, CD69 and PD-1.

With respect to Ig-related syndromes in human liver disease patients, ectopic lymphoid structures have been identified in livers from patients with chronic HCV (230-232). As such, we performed FACS analysis for the presence of T follicular helper (Tfh) or regulatory (Tfr) CD4+ T cells in fibrotic mouse livers using canonical follicular helper markers, CXCR5 and ICOS (230, 233). Although an accumulation of CD4+CXCR5+ICOS+ T cells was observed in both Foxp3+ and Foxp3 negative compartments of CD4+ T cells in fibrotic livers, the frequency of CD4+CXCR5+ICOS+

T cells was significantly increased within Foxp3+ compartment (**Fig. 2-4B**). However, there was no apparent consequence of the two-fold increase of CD4+ Foxp3-negative Tfh-phenotype with respect to B cell activation *in vitro* (**Fig. S2-4, B-D**).

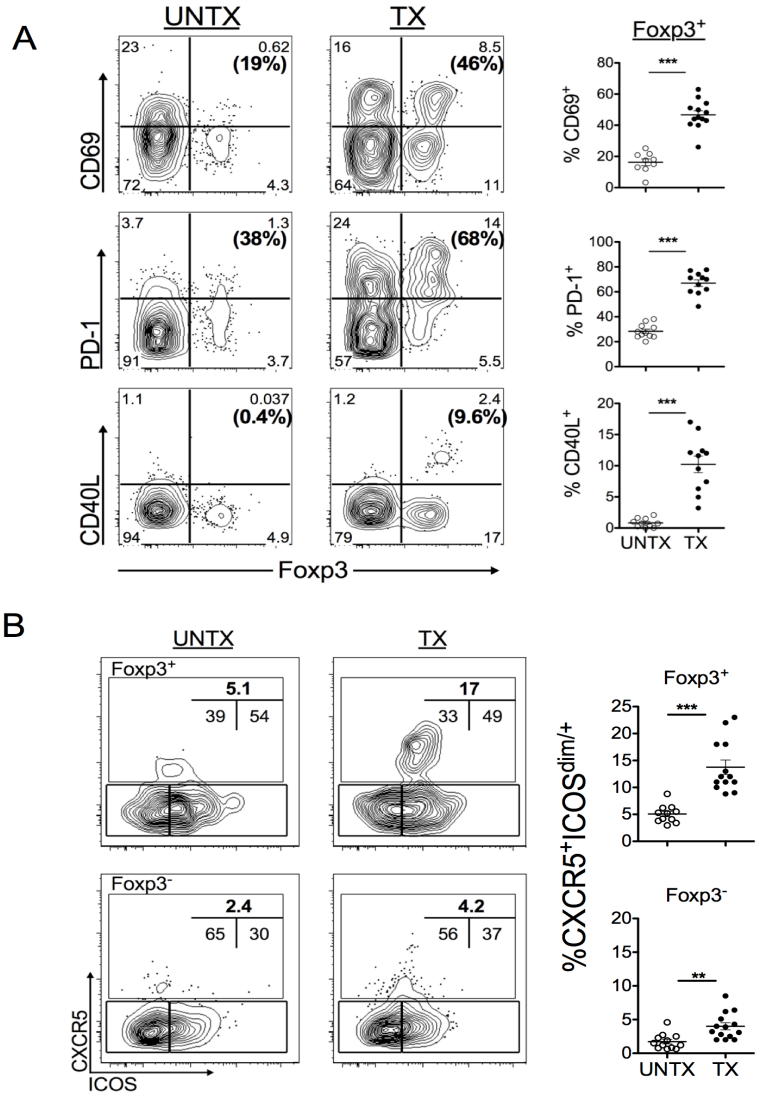

FIGURE 2-4. Fibrotic liver CD4+Foxp3+ T cells up-regulate CD69, PD-1 and CD40L expression. (**A**) FACS analysis of phenotypic changes in the total live, non-autofluorescent hepatic CD4+ T cell compartment following course of CCl_4-treatment. Frequencies (bold) reflect the proportion of CD4+Foxp3+ T cells expressing the specified marker. Gates were defined based on appropriate fluorescent minus one controls. FACS plots are representative of at least 3 independent experiments (n=3-5 mice per group). (**B**) FACS analysis of CD4+Foxp3+ T cells from control liver (UNTX) and fibrotic livers (TX) based on follicular helper markers CXCR5 and ICOS expression is shown. Cells were gated on live, non-autofluorescent CD4+ T cells and indicated Foxp3 expression. FACS plot shown is representative of 3 independent experiments (n=3-5 mice per group). Statistics were calculated using a two-tailed Mann-Whitney *U* Test (***p<0.0005, **p<0.005, *p<0.05).

CD40L expression is localized to CD4+Foxp3+ CXCR5- ICOS- T cells

To further determine the significance of CXCR5 and ICOS expressions on B cell IgG production, we performed co-cultures with FACS-purified CXCR5+ICOSdim/+ (canonical Tfr), CXCR5-ICOS+, and CXCR5-ICOS-CD4+Foxp3+ T cells from transgenic mice that express green fluorescent protein (GFP)-Foxp3 (**Fig. 2-5A**). CD4+Foxp3+ cells expressing CXCR5+ICOS+ and CXCR5-ICOS+ isolated from spleen, oil and fibrotic livers comparably suppressed B cell IgG production in response to LPS (**Fig. 2-5B-C**). Although the CXCR5-ICOS- population derived from spleen and oil-treated livers inhibited IgG production, fibrotic liver CXCR5-ICOS- population permitted LPS-mediated IgG production (**Fig. 2-5D**). Parallel FACS analysis further indicated that CD40L expression predominantly localizes to this population of CD4+Foxp3+ T cells obtained fibrotic livers that are CXCR5-ICOS- (**Fig. 2-5E**). Thus, our observations indicate that fibrosis drives accumulation of CD4+Foxp3+ T cells with varying B cell regulatory functions dictated by CD40L expression.

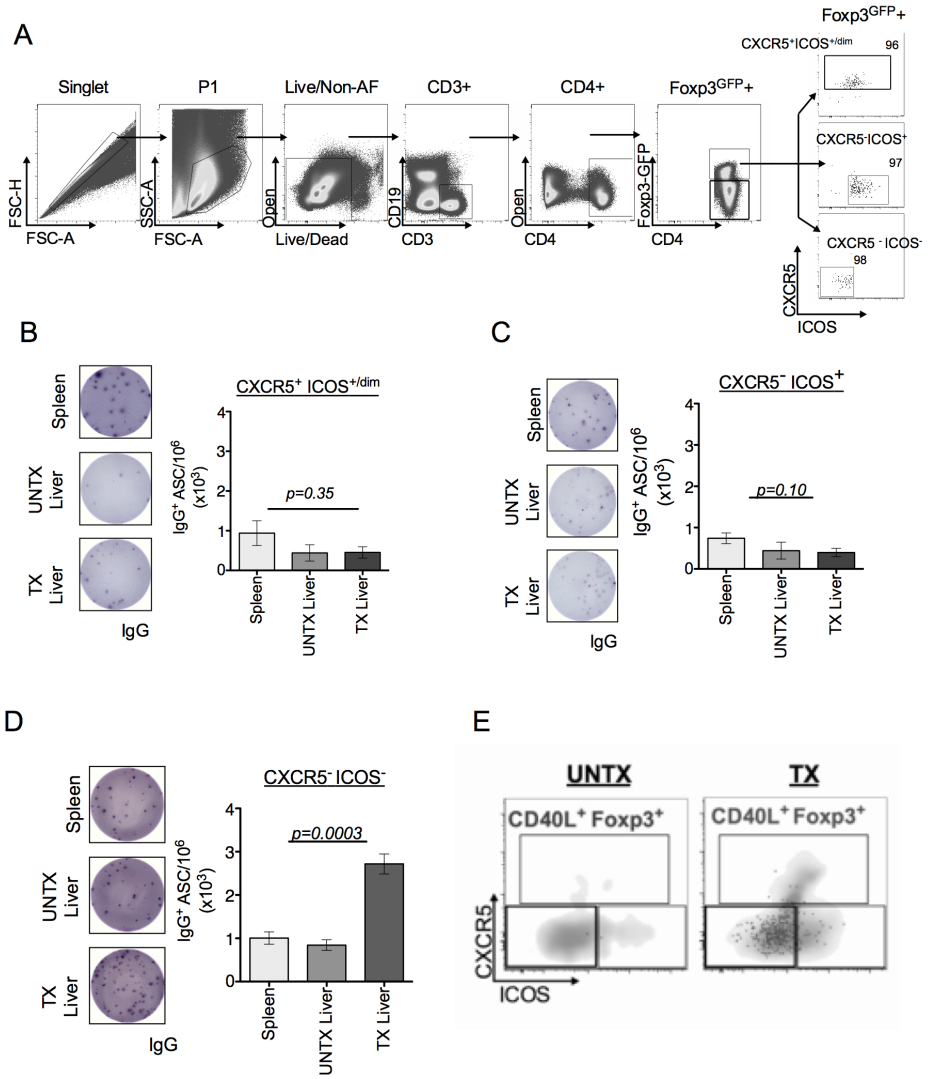

FIGURE 2-5. CD40L is primarily expressed on CXCR5 and ICOS double negative CD4+Foxp3+ T cells. (**A**) Transgenic mice that express GFP-Foxp3 were treated with CCl_4 to induce hepatic fibrosis. CD4+ (GFP-Foxp3+) T cells were sorted based on expression of CXCR5 and ICOS into three populations: CXCR5+ ICOSdim/+ (**B**), CXCR5- ICOS+ (**C**), or CXCR5- ICOS- (**D**) by FACS. Post-sort purity of splenic, untreated liver, and fibrotic liver populations was >95% (**A, far right,** representative of indicated CD4+Foxp3^{GFP}+ subsets). Sort strategy represented is from fibrotic liver lymphocytes. Sorted cells CXCR5+ ICOSdim/+ (**B**), CXCR5-ICOS+ (**C**), or CXCR5-ICOS- (**D**) CD4+Foxp3+T cells from indicated organs were co-cultured with CD19-enriched B cells in ratio of 1:2 (CD4: B cells) for 5 days in the presence of 2ug/mL LPS stimulation and subsequently analyzed for IgG production by ELISPOT. (**E**) FACS plots showing CD40L expression in CD4+Foxp3+ CXCR5- ICOS- T cell compartment in fibrotic (TX) livers in comparison to absence of this population in non-fibrotic (UNTX) livers. Gray density plot represents the total CD4+Foxp3+T cell compartment, black overlay dots represent the CD40L+ population of CD4+Foxp3+ T cells. All data are representative of 3 independent experiments with sorted cell populations pooled from 3-5 animals per group. Statistical significance was determined by one-way ANOVA of duplicate wells of three independent experiments (data represented in **B-D,** ***$p<0.0003$) Error bars represent SEM.

In vivo CD40L blockade during liver injury attenuates IgG aberrations

Interestingly, a previous report identified CD40L expression in total RNA isolated from livers of HCV patients suffering from aberrant Ig-related syndromes (225). Our own findings pinpoint CD40L expression exclusively on CD4+Foxp3+ T cells during liver disease (**Fig. 2-4A**). CD40L-mediated CD4+ T cell help promotes B cell activation and facilitates fine-tuning of immune responses (228, 229) and may implicate this signaling pathway to extrahepatic symptoms of liver disease. To test this hypothesis, we performed antibody-mediated CD40L (clone MR1) blockade prior to ("Day-1"), or during ("Week 2", "Week 3") the course of CCl_4-treatment (**Fig. 2-6A**). Blockade of CD40L signaling before and during CCl_4 treatment regimen attenuated IgG production by intrahepatic B cells (**Fig. 2-6B**). This corresponded to a significant reduction in total serum IgG (**Fig. 2-6C**). Pre-treatment of animals with anti-CD40L resulted in ANA-IgG that was indistinguishable from control animals (titers <50)(**Fig. 2-6D).** Interestingly, later introduction of anti-CD40L treatment (Week 2 and Week 3 groups) attenuated ANA titers (titers 100-200) in comparison to CCl_4-treated animals (titers >400) (**Fig.** 2-**6D).** This may be the result of *in vivo* stability of IgG, which has a reported half-life of approximately 21 days (234). Thus, CD40L blockade may be sufficient to halt production at the level of intrahepatic B cell IgG (**Fig. 2-6B**); ANA-IgG produced before blockade may persist in circulation (**Fig. 2-6D**). These data implicate CD40L expression, which is primarily localized to CD4+Foxp3+ T cells, is critical for aberrant IgG-production during liver disease.

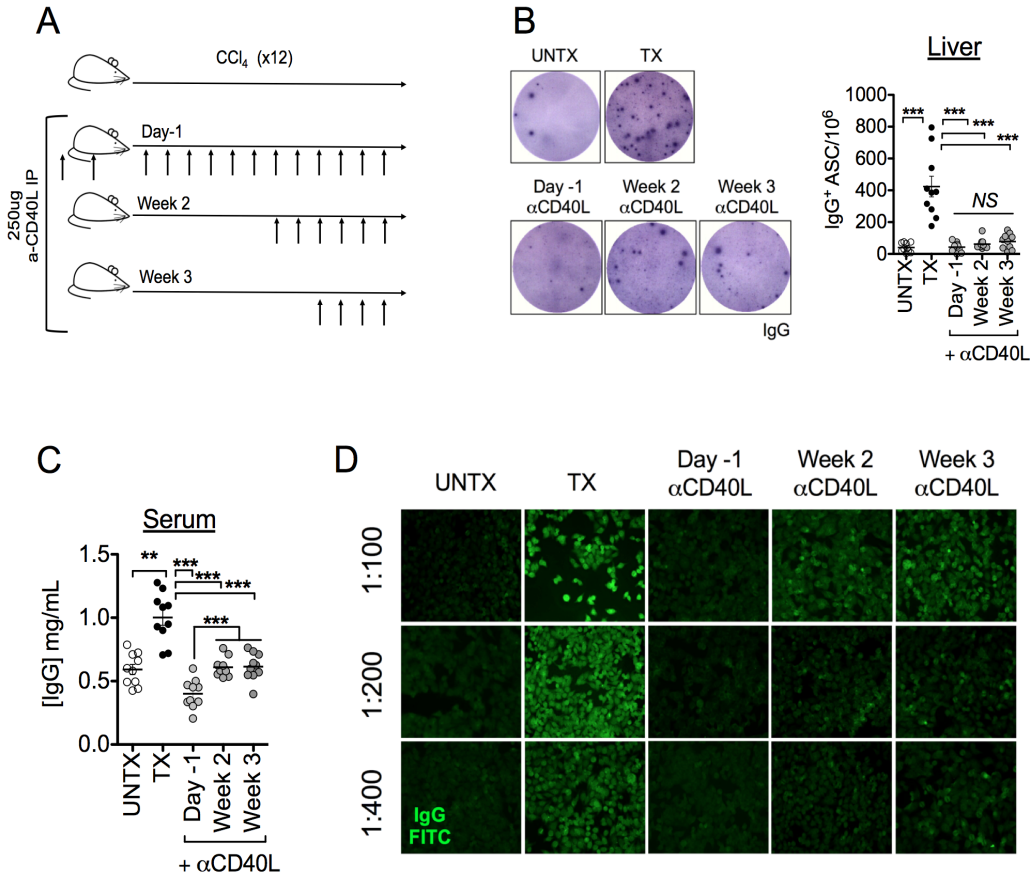

FIGURE 2-6. *In vivo* blockade of CD40L attenuates Ig-mediated manifestations of liver fibrosis. (**A**) Animals were treated I.P. with 250 μg hamster anti-Mouse CD40L one day prior to and upon initiation of CCl_4-treatment ("Day-1"), or begun 2 weeks ("Week 2") or 3 weeks ("Week 3") into CCl_4 regimen and every three days thereafter; antibody administrations are indicated by arrows (**B**) *Ex vivo* ELISPOT of intrahepatic B cell production of IgG in untreated (UNTX), CCl_4 treated (TX), and α-CD40L-treated groups cultured overnight in media alone. (**C**) Serum IgG and (**D**) ANA IgG titers are attenuated when CD40L is blocked during fibrosis. Data are representative of 2 independent experiments (n=5 animals per group). Statistical significance was determined by a two-tailed Mann Whitney *U* test (***p<0.0005, ** p<0.005).

Accumulation of CD4+Foxp3+CD40L+ T cells in explanted HCV patient livers

Next, phenotypic analysis was performed on CD4+Foxp3+ T cells from explanted livers of HCV-infected cirrhotic patients based on canonical follicular helper markers, CXCR5, ICOS, and CD40L expression. Clinical characteristics of explanted cirrhotic livers derived from HCV infected patients and "non-fibrotic control" tissues obtained from donors undergoing surgical re-sectioning for non-HCV, non-NAFLD, non-ALD related medical treatment are summarized in **Supplementary Tables 2-2 and 2-3.** In accordance with our findings in the mouse model, there was a liver-specific increase in CD4+Foxp3+CD25+T cells from HCV explants (n=16, 12.81±5.20%) compared to non-fibrotic controls (n=6, 3.22±1.04%) (**Fig. 2-7A**). This finding was consistent with previous reports of increased frequency of CD4+Foxp3+ T cells in livers of patients infected with HCV (22, 214, 215). Alterations in frequency were not noticed in peripheral blood derived from control or HCV subjects (control subjects n=6, 5.00±1.61%; HCV donors n=6, 6.08±1.24%, $p>0.05$) despite a marked increase observed in HCV patient-matched livers at the same time of transplantation (**Fig. 2-7A**). Furthermore, we found increased PD-1 (55.75±16.5%, n=9) and CD40L (14.86±7.08%, n=9) expression in intrahepatic CD4+Foxp3+ T cells from chronic HCV patients compared to non-fibrotic controls (34.30±9.08%, $p<0.02$, n=6, and 0.50±1.00%, $p<0.005$, n=4, respectively) (**Fig. 2-7B**). Similar to the mouse CCl_4-model of fibrosis, CD40L expression on CD4+Foxp3+ T cells was also predominantly restricted to the subset that lacked canonical follicular helper markers CXCR5 and ICOS (**Fig. 2-7C**).

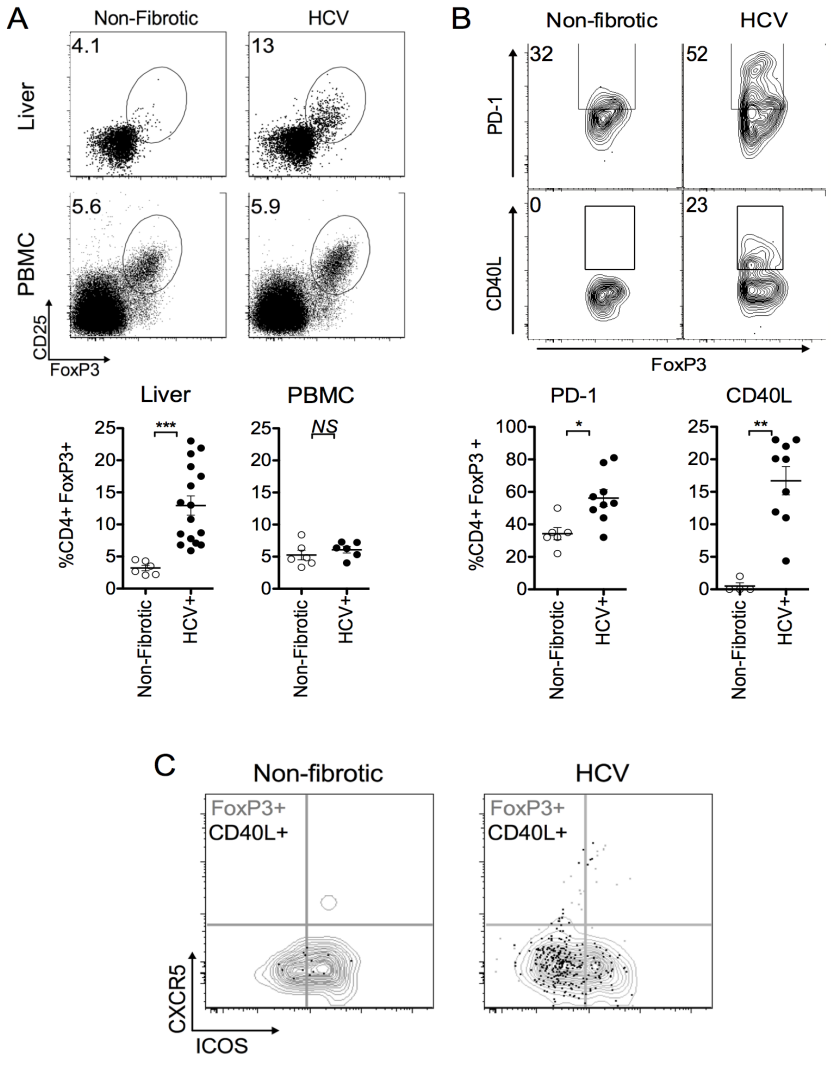

FIGURE 2-7. Phenotypic analysis of CD4+Foxp3+ T cells in explanted livers from HCV patients. (**A**) FACS analysis of cryopreserved livers explanted from cirrhotic HCV human patients (n=16) or patients undergoing non-HCV, non-NASH, non-ALD related surgical re-sectioning ("non-fibrotic control" n=6) (top) and PBMC (control donors, n=6, HCV, matched to livers at time of transplantation n=6), (bottom) based on expression of FoxP3 and CD25. Frequencies were determined by gating on live, non-autofluorescent CD4+ T cells co-expressing CD25 and FoxP3. (**B**) FACS analysis of cryopreserved HCV explanted (n=9) or non-fibrotic control liver (n=4-6) based on CD4+Foxp3+ T cell gated expression of PD-1 and CD40L. (**C**) CD4+Foxp3+ CD40L+ T cells from livers explanted from HCV patients predominantly localize to the CXCR5- ICOS- population. Gray contour plot represents the total CD4+Foxp3+T cell compartment, while black dot overlay represents the CD4+Foxp3+ CD40L+ T cell population. Data are representative of n=9 HCV patients, and n=4 "non-fibrotic control" patients. All statistical analyses (unless otherwise indicated) were calculated by a two-tailed Mann-Whitney *U* test (***p<0.0005, **p<0.005, *p<0.05).

Experiments were performed by Dana Tedesco on samples obtained from the surgical team at the Emory Transplant Center

Aberrant IgG production correlates with frequency of CD4+Foxp3+ T cells in explanted HCV patient livers

Next, we determined whether liver-specific accumulation of CD4+Foxp3+T cells modulates IgG production in chronic HCV patients. In agreement with the murine model, intrahepatic B cells from livers explanted from HCV-infected individuals demonstrated constitutive IgG production in the absence of any stimulation **(Fig. 2-8A)**. This was not observed from peripheral blood or non-fibrotic control intrahepatic B cells (**Fig. 2-8A).** Interestingly, supernatants from cultured B cells isolated from HCV patient livers were positive for ANA-IgG titers independent of stimulation with R848 and IL-2 (**Fig. 2-8B**). R848 and IL-2 stimulation was chosen as human intrahepatic B cells are hypofunctional to LPS, due to very low expression of TLR4 (235). Intrahepatic B cell spontaneous IgG production in HCV patient livers significantly correlated with the frequency of CD4+Foxp3+ T cells (**Fig. 2-8C**). Surprisingly, both parameters were independent of patient viremia (**Fig. S2-6)**. Taken together, characteristics of CD4+Foxp3+ T cells and B cell IgG-mediated aberrations in explanted cirrhotic livers from HCV human patients parallel those in the murine CCl_4-model. Thus, fibrotic liver disease elicits a population of CD4+Foxp3+CD40L+ T cells that promote extrahepatic manifestations of liver disease via aberrant intrahepatic IgG production.

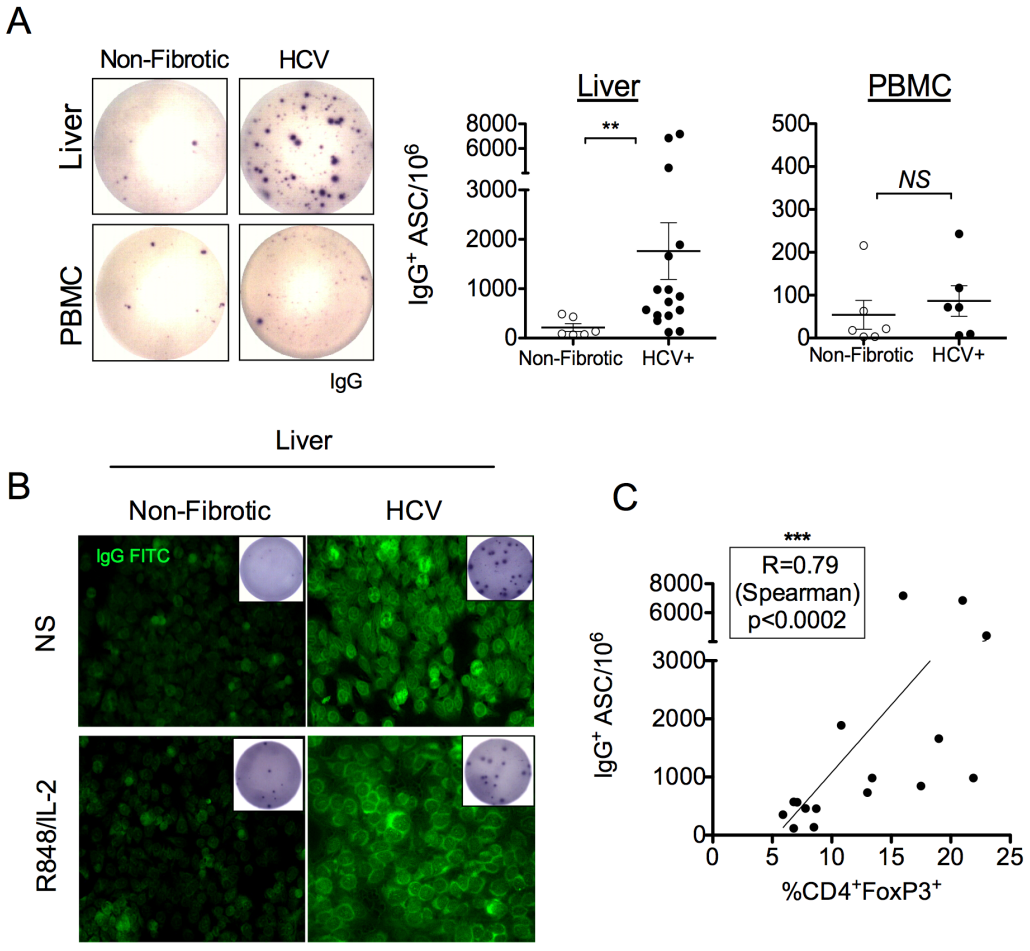

FIGURE 2-8. Aberrant IgG production correlates with frequency of CD4+Foxp3+ T cells in explanted HCV patient livers. (**A**) Spontaneous IgG ELISPOT of cryopreserved non-fibrotic control (n=6) and HCV (n=16) liver lymphocytes (top), or peripheral blood (control subjects, n=6, patient matched HCV, n=6, bottom) lymphocytes, cultured in complete IMDM for 16-18h in 5.0% CO_2 and 37˚C without any stimulation. (**B**) Supernatants of cultured purified intrahepatic B cells from HCV patients have detectable ANA-IgG. Representative images of culture supernatant incubated with HEp-2 ANA substrate and counterstained with anti-IgG FITC. IgG ELISPOT of bead enriched CD19+ B cells ($5.0x10^4$/ well) from non-fibrotic control tissue (n=3) or HCV (n=6) livers after 5 days of culture in IMDM alone or with R848 (1ug/mL) and IL-2 (20U/mL) stimulation was performed to determine the presence of ASC (inset). (**C**) The frequency of intrahepatic CD4+Foxp3+ T cells correlates with spontaneous IgG production in explanted HCV livers (n=16) (Spearman R=0.79, ***p<0.0002, two-tailed).

Experiments were performed by Dana Tedesco on samples obtained from the surgical team at the Emory Transplant Center

V. Discussion

Inflammatory conditions and environmental factors can highly influence CD4+Foxp3+ T cell plasticity and result in various pathology-associated sub-populations; classified as "reprogrammed" Tregs, or "Ex-Tregs" (236). Treg "reprogramming" has been implicated as providing early co-stimulatory signals that promote anti-tumor CTL immunity during vaccination and tumor challenges (228). Induction of this population is reliant on the presence of interleukin-6 (IL-6), and MHCII+ antigen presenting cells (228), both of which are understood to be abundant in the fibrotic liver microenvironment (213, 216). Alternatively, elegant fate mapping and epigenetic studies have indicated that spatial localization of CD4+Foxp3+ T cells can influence *Foxp3* stability and regulatory functions (205); in this context this population is termed "ex-Treg", as this inflammatory population arises from peripheral CD4+Foxp3+ T cells which transiently up-regulate Foxp3. Foxp3 down-regulation or loss of expression has been associated with inflammatory functions (237, 238).

Furthermore, other correlates of liver fibrosis such as complement proteins (239) have also been implicated in CD4+ T cell differentiation into a unique population of CD4+Foxp3+ T cells (207, 208). Stimulation of human PBMC through the complement receptor can result in a unique "Treg" population that expresses CD40L amongst other co-stimulatory markers that can permit dendritic cell maturation, suppress effector T cell responses, and interact with B cells to promote Ig-production (207, 208). Combined together, these findings delineate key factors surrounding CD4+Foxp3+ T cell-mediated regulatory versus pathogenic functions during specific disease or inflammatory states. To

our knowledge, none of these populations have been investigated during liver disease, thus there is a limited understanding of the relationship between the fibrotic liver microenvironment and effect on origin(s) and functional plasticity of CD4+Foxp3+ T cells. Therefore, we cannot conclusively categorize these CD4+Foxp3+ T cells accumulated in the fibrotic livers despite some of the shared traits associated with various disease-related pathogenic Tregs.

In light of the protective role CD4+Foxp3+ T cells play in the maintenance of the hepatic microenvironment and limiting immune-mediated injury to the liver (217, 218), we have identified a key contribution of the CD40L+ sub-population to extrahepatic manifestations of aberrant B cell functions (**Fig. 2-5)**. This finding is consistent with immunologic characteristics of chronic HCV infection in the clinic: ineffective anti-viral CD8+ T cell responses (22, 214, 218, 219, 240) that co-exist with B cell abnormalities (220, 224, 225, 230, 231).

Differential mechanisms of regulation by CD4+Foxp3+ T cells may shed light on this dichotomy. Phenotypically, the bulk CD4+Foxp3+ T cell population demonstrates high levels of PD-1 expression **(Fig. 2-4)**. PD-1 signaling is implicated in suppression of CD8+ T cell functions and exhaustion; both of which contribute to chronic viral infections (241). In contrast, PD-1 expression on CD4+ T cells in B cell follicles has been attributed to modulating the quality and quantity of long-lived plasma cell pool (226, 227). With regard to B cell modulatory markers, our own data draws particular attention to CD40L signaling that arises primarily in fibrotic liver derived intrahepatic

CD4+Foxp3+ T cells (**Fig. 2-4).** Due to the role in Ig-class switching (229), but insignificant influence on CD8+ T cell responses to viral infections (242), CD40L expression may account for contradictory functions of total enriched CD4+CD25+ T cells that suppress CD8+ T cells (**Fig. 2-3)** yet promote B cell activation when sourced from fibrotic livers **(Fig. 2-2)**. In line with this hypothesis, *in vitro* isolation of the CD40L-expressing subset (**Fig. 2-5)** and *in vivo* blockade of CD40L in the mouse model of liver injury (**Fig. 2-6)** suggest that this population critically contributes to extrahepatic IgG aberrations**.**

Although therapeutic potential of anti-CD40L treatment has been realized for treatment of autoimmune conditions (243), its introduction into clinical practice had significant adverse events due to Fc-recognition of the antibody(244) (245). Recent development of Fc-deficient CD40L blockade antibody has achieved the same therapeutic benefits as earlier biologics without complications of its predecessors in pre-clinical trials (246). Our data suggest that such strategies may benefit management of Ig-related sequelae of chronic liver disease (**Fig.2-6)**.

Localization of IgG production and CD40L expressing CD4+Foxp3+ T cells to the liver, but not the periphery has important implications when considering novel "Treg" transfer therapies to promote liver repair (247). The microenvironment in which the "Treg" enters may have critical influence on what types of functions it can promote. This is certainly a consideration in the case of the liver microenvironment during fibrosis or other ongoing inflammatory events. Thus, liver fibrosis highlights a scenario in which accumulation of

regulatory-phenotype T cells may propagate adverse autoantibody mediated pathology, rather than promote tolerance.

The current work demonstrates that chemically induced liver injury in mice can recapitulate both CD4+Foxp3+ CD40L+ T cell phenotype and constitutive intrahepatic IgG production found in explanted livers from cirrhotic patients infected with HCV. Together, these observations imply that shared mechanisms of post-insult hepatic tissue repair govern liver CD4+ T cell phenotype and function. As a result, fibrosis-induced alterations to the intrahepatic CD4+ T cell compartment are not only critical for IgG-anomalies, but also ineffective CD8+ T cell responses associated with chronic liver disease. Decoupling extrahepatic manifestations from the etiologic agent opens up novel avenues for potential therapeutic management of chronic liver disease.

Acknowledgements

We are grateful to the donors and their families of the Emory Transplant Center for invaluable consented participation in this study. We thank the Emory Transplant center team: surgical and nursing staff, coordinators, Shine Thomas for their assistance and cooperation. We thank Victoria M. Velazquez, J. Brett Mendel, Young-Jin Seo and Aryn Price for critical reading of the manuscript and fruitful scientific discussions. We thank Kiran Gill and Barbara Cervasi of the Emory Vaccine Center Flow Cytometry and Pathology Cores for technical assistance throughout the study.

VI. Supplemental Data

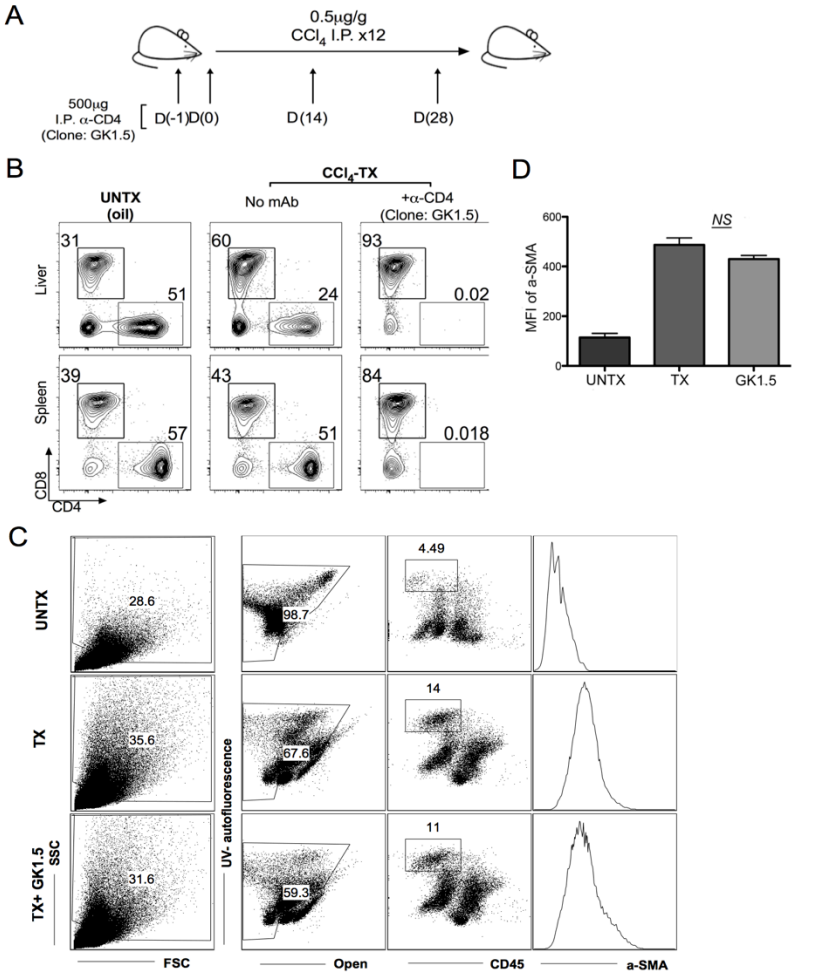

FIGURE S2-1. Antibody mediated depletion of CD4+ T cells in C57BL6/J treated with CCl_4. (**A**) Antibody mediated CD4+ T cell depletion: days -1, and day 0 mice were administered 0.5mg of anti-CD4 (Clone GK1.5) I.P. and CCl_4 treatment beginning day 0, every three days- 12 doses per animal. On days 14 and 28, animals were administered anti-CD4 I.P. (**B**) Representative FACS plots of antibody mediated CD4 depletion in liver (top) and spleen (bottom) at time of animal sacrifice, blood was monitored throughout the study as indicated in the methods section (data not shown). Data are representative of at least 3 independent experiments with 3-5 animals per indicated group. Gated on live non-autofluorescent CD3+ (T cell compartment). (**C**) Mouse livers were processed for liver non-parenchymal cells (NPCs) isolation as described in Materials and Methods. After density gradient enrichment, NPCs were stained with anti-CD45 and analyzed on a FACSAria Cell Sorter II (BD Bioscience) equipped with a UV laser. HSCs were identified as Live, UV autofluorescence-positive (UVAF+), CD45 negative population. For the detection of HSC-associated activation marker, purified liver cells were stained intracellularly with α-SMA. The expression of α-SMA was analyzed on gated HSC population using FlowJo software (Treestar, Ashland, OR). Data shown is representative of 3 independent experiments (n=3-5 mice per group). ***$p<0.0005$, two-tailed Mann-Whitney *U* test. Where applicable, error bars represent SEM.

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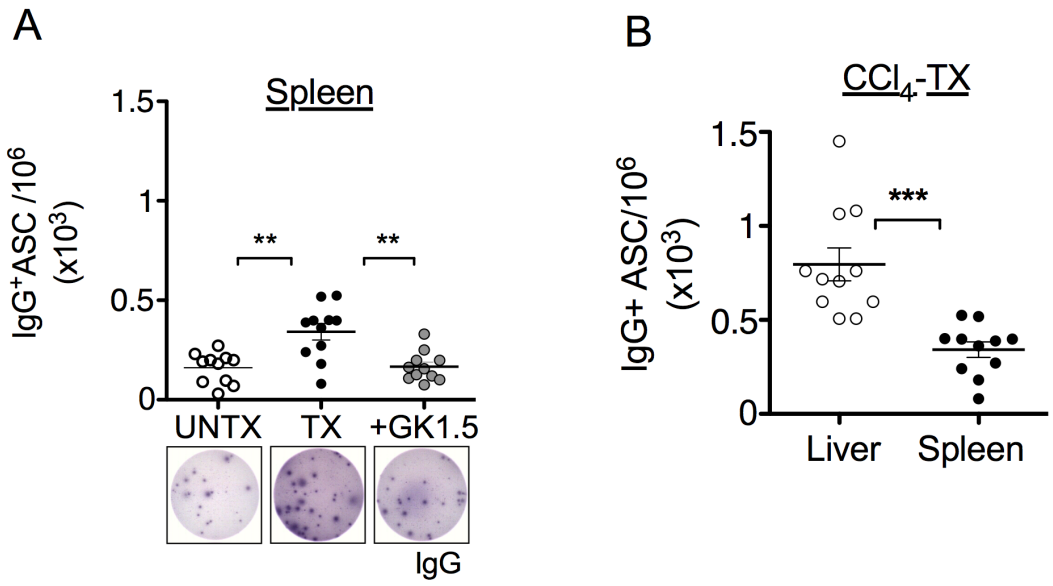

FIGURE S2-2. CCl_4 treatment primarily alters the hepatic B cell compartment.
(**A**) Spontaneous IgG production from splenic lymphocytes; ELISPOT was performed as described. (**B**) Comparison of fibrotic liver (795±291 ASC/10^6) versus animal-matched splenic (342±137 ASC/10^6) frequencies of spontaneous IgG production. All data are representative of three independent experiments, n=3-5 animals per group. Statistical significance was determined by two-tailed Mann-Whitney *U* Test, ***p<0.005.

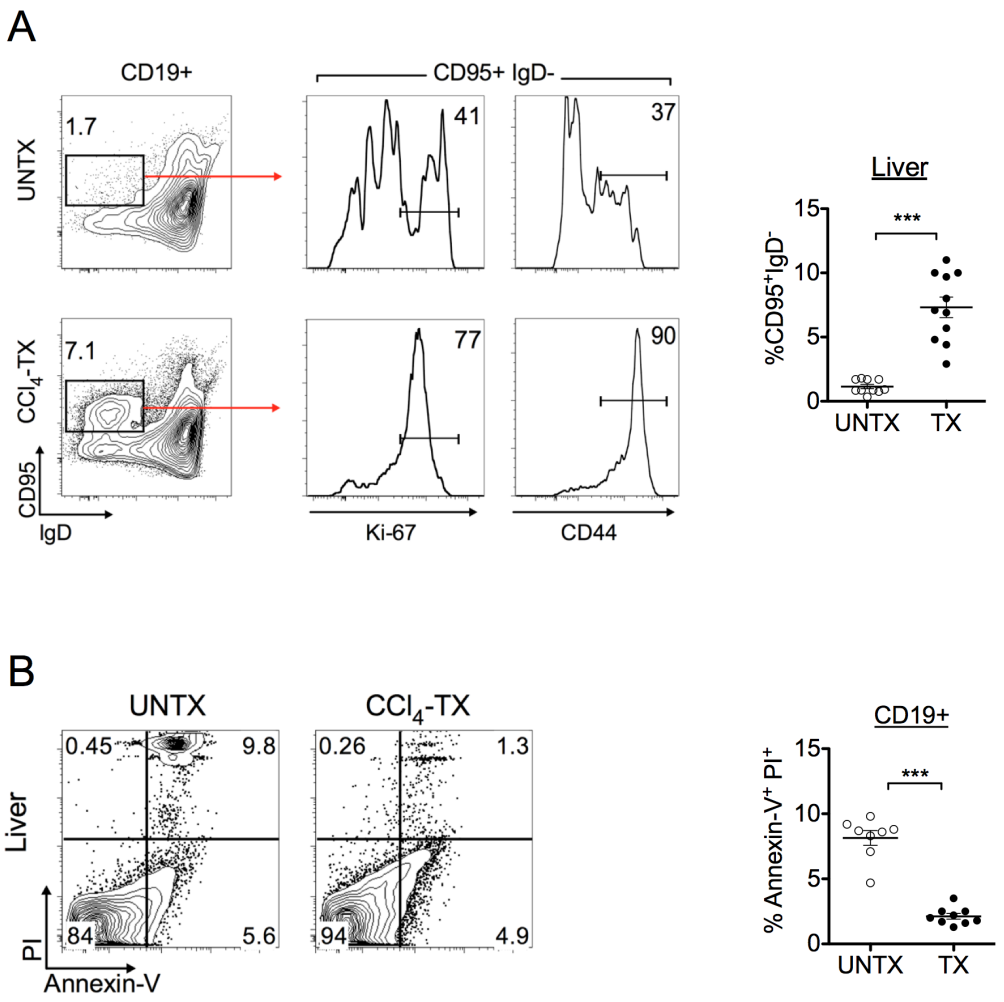

FIGURE S2-3. Increased B cell activation and survival during liver fibrosis. (A) Phenotypic analysis of live, non-autofluorescent, CD19+ B cells from non-fibrotic (UNTX) and fibrotic (TX) livers; left. Right, analysis of Ki-67 and CD44 expression directly *ex vivo* within the CD95+ IgD- population. Fibrotic liver B cells have an increased frequency of CD95+IgD- B cells in comparison to non-fibrotic controls, far right (***$p<0.0005$, two-tailed Mann-Whitney *U* Test). Each dot represents one mouse; data shown are from 3 independent experiments, n=3-5 animals per group. **(B)** Direct *ex vivo* analysis of apoptosis via positive staining for Annexin-V and Propidium Iodide (PI) on hepatic CD19+ B cells. Gates were determined from single stained positive and negative controls. FACS plots and graphs are representative of 2 independent experiments, n =3-5 per group (***$p<0.0005$, two-tailed Mann-Whitney *U* Test).

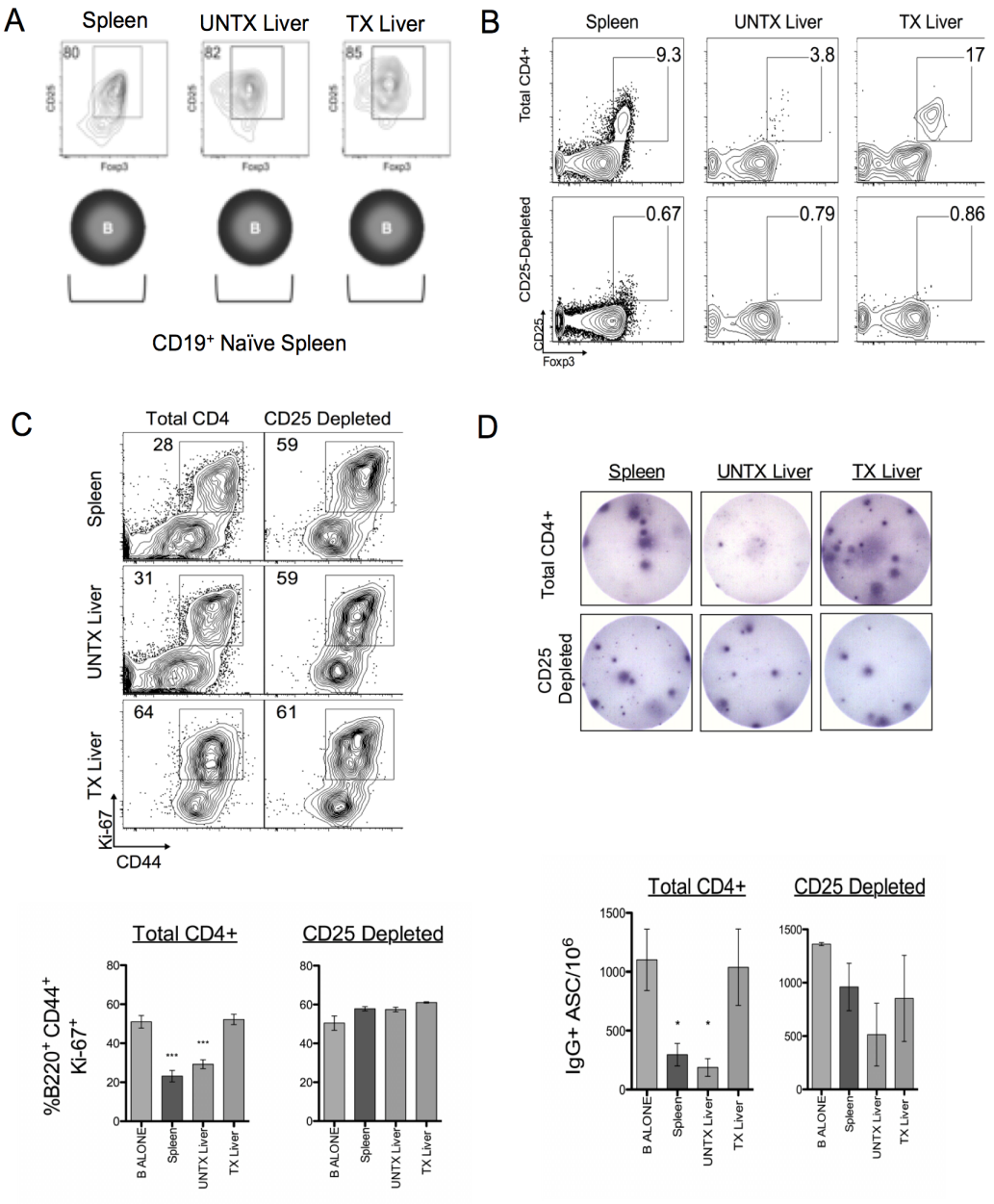

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FIGURE S2-4. CD25 negative CD4+ T cell interaction with B cells is not influenced by fibrosis. (**A**) Foxp3 expression on bead-enriched CD4+CD25+ T cells, representative of indicated organs from 5 independent experiments (pooled cells from n=3-5 per group). (**B**) Total CD4+ T cell compartment expression of CD25 and Foxp3 prior to depletion of CD25+ fraction (top), and Foxp3 expression in CD25 negative fraction of CD4+ T cell compartment (bottom) prior to co-culture with B cells. Data representative of 5 independent experiments of pooled organs from n=3-5 animals per group (**C**) Ki-67 and CD44 co-expression on live, B220+ B cells following 5 days of stimulation with 2 μg/mL LPS and indicated CD4+ T cells. Total CD4+ T cells from spleen and untreated liver reduce expression of activation markers, while fibrotic liver permits activation (left, top). CD25-depleted fraction permits B cell activation to the same extent irrespective of origin. Statistical significance is relative to B cell activation with LPS in the absence of CD4+ T cells (***$p<0.0005$, two-tailed Mann-Whitney *U* Test). (**D**) IgG production from B cells cultured with total CD4+ T cell compartment (top) and CD25-depleted fractions (bottom). All data are representative of at least 5 independent experiments with indicated cell populations pooled from n=3-5 animals per group. Statistical significance is relative to B cell activation with LPS in the absence of CD4+ T cells (**$p<0.005$, *$p<0.05$, two-tailed Mann-Whitney *U* Test). Where applicable, error bars represent SEM.

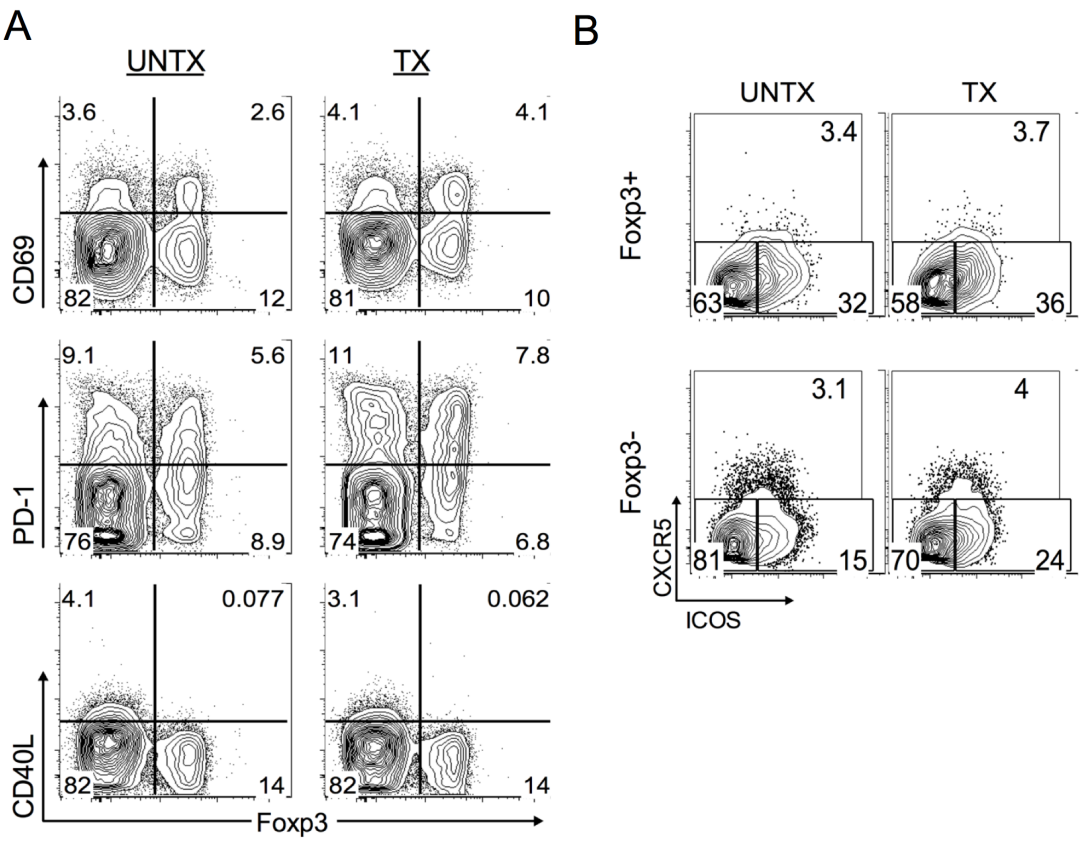


FIGURE S2-5. Phenotypic analysis of splenic CD4+Foxp3+ cells. (**A**) Representative FACS plots of splenic CD4+ T cell phenotype showing CD69, PD-1 and CD40L expression. Gated on live, non-autofluorescent, CD4+ T cells. (**B**) Total CD4+ T cell compartment expression of CXCR5 and ICOS; Foxp3+ population represented on top panel, Foxp3- population represented on bottom panel. All data are representative of at least 3 independent experiments (n=3-5 animals per group).

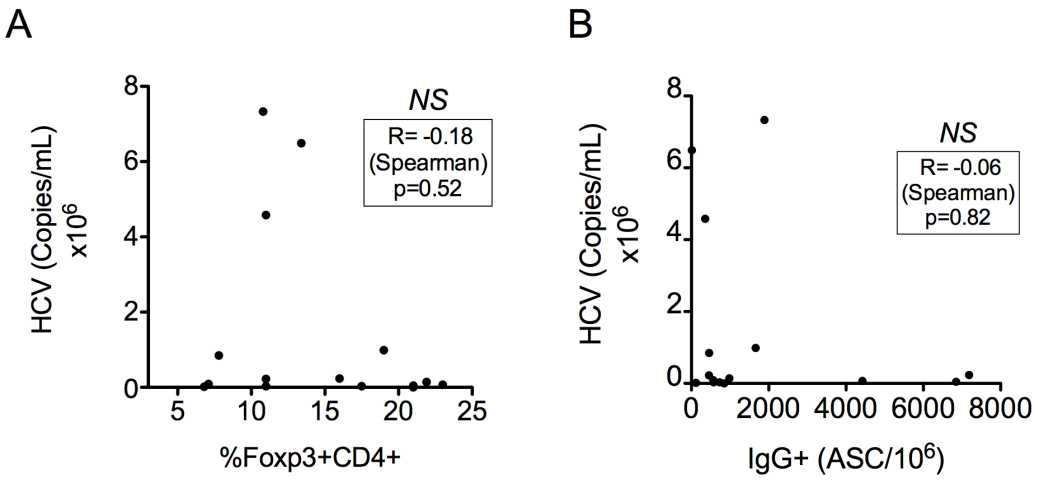


FIGURE S2-6. Intrahepatic accumulation of CD4+Foxp3+ T cells and incidence of spontaneous IgG production are independent of HCV viral load. (**A)** Intrahepatic frequency of CD4+Foxp3+ T cells does not correlate with patient viremia (n=15), R= -0.18 (Spearman), p=0.52 (two tailed, *NS*). (**B)** Spontaneous IgG production by intrahepatic B cells does not correlate with patient viremia (n=15), R= -0.06 (Spearman), p=0.82 (two tailed, *NS*).

Fibrosis Lesion Scoring (adapted from Ishak, et al. J Hepatology 1995; 22:696-9)

Ishak Stage, Categorical description	Score
No fibrosis	0
Expansion of some portal areas with or without short fibrous septa	1
Expansion of most portal areas with or without short fibrous septa	2
Expansion of most portal areas with occasional portal to portal bridging	3
Expansion of portal areas with marked bridging (portal-portal and/or portal-central)	4
Marked bridging with occasional nodules	5
Cirrhosis, probable or definitive	6

Histopathological Evaluation and Fibrosis Lesion Quantification

Animal ID	Slide ID	H&E	Sirius red-Fibrosis score
Group 1	1 C	NSF*	0
	2 C	NSF	0
	3 C	NSF	0
	4 C	NSF	0
	5 C	NSF	0
Group 2	1 Tx	Multifocal moderate areas of hepatocellular necrosis, hydropic degeneration, periportal fibrosis with occasional portal to portal bridging and occasional mononuclear to neutrophilic infiltrates	3
	2 Tx	Multifocal moderate areas of hepatocellular necrosis, hydropic degeneration, periportal fibrosis with occasional portal to portal bridging and occasional mononuclear to neutrophilic infiltrates	3
	3 Tx	Multifocal extensive areas of hepatocellular necrosis, hydropic degeneration, periportal fibrosis with occasional portal to portal bridging and occasional mononuclear to neutrophilic infiltrates	3
	4 Tx	Multifocal moderate areas of hepatocellular necrosis, hydropic degeneration, periportal fibrosis with occasional portal to portal bridging and occasional mononuclear to neutrophilic infiltrates	3
	5 Tx	Multifocal moderate areas of hepatocellular necrosis, hydropic degeneration, periportal fibrosis with occasional portal to portal bridging and occasional mononuclear to neutrophilic infiltrates	3
Group 3	1 GK+Tx	Multifocal extensive areas of hepatocellular necrosis, hydropic degeneration, periportal fibrosis with occasional portal to portal bridging and occasional mononuclear to neutrophilic infiltrates	3
	2 GK+Tx	Multifocal extensive areas of hepatocellular necrosis, hydropic degeneration, periportal fibrosis with occasional portal to portal bridging and occasional mononuclear to neutrophilic infiltrates	3
	3 GK+Tx	Multifocal moderate areas of hepatocellular necrosis, hydropic degeneration, periportal fibrosis with occasional portal to portal bridging and occasional mononuclear to neutrophilic infiltrates	3
	4 GK+Tx	Multifocal moderate areas of hepatocellular necrosis, hydropic degeneration, periportal fibrosis with occasional portal to portal bridging and occasional mononuclear to neutrophilic infiltrates	3
	5 GK+Tx	Multifocal moderate areas of hepatocellular necrosis, hydropic degeneration, periportal fibrosis with occasional portal to portal bridging and occasional mononuclear to neutrophilic infiltrates	3

* NSF: no significant findings; C: oil-treated control, Tx: CCl4-treated, and GK+Tx: CCl4+ GK1.5 antibody treated.

Table S2-1. Fibrosis Lesion Scoring analysis Rubric and Findings. Liver biopsies were analyzed via the Ishak criteria by a qualified pathologist. Table represents one experiment with n=5 individuals per group. These findings are consistent across three independent experiments.

Donor ID	Age	Sex	Cirrhosis	Dx	HCC	HCV RNA (Copies/mL)	ALT
ET101	48	M	YES	HCV	YES	80000	42
ET104	53	M	YES	HCV	YES	7330000	137
ET108	60	M	YES	HCV	YES	233000	45
ET180	63	M	YES	HCV	YES	68700	42
ET196	70	M	YES	HCV	NO	31500	29
ET239	57	M	YES	HCV	YES	3400	27
ET245	47	M	YES	HCV	YES	46900	37
ET261	66	M	YES	HCV	YES	4580000	93
ET262	61	F	YES	HCV	NO	847000	71
ET264	53	F	YES	HCV	NO	225000	30
ET303	67	M	YES	HCV	YES	16300	99
ET308	60	M	YES	HCV	YES	989000	38
ET316	66	F	YES	HCV	YES	44600	26
ET330	50	M	YES	HCV	YES	30400	28
ET334	49	M	YES	HCV	YES	1420000	64
ET371	59	M	YES	HCV	YES	Undet*	30

Table S2-2. Clinical characteristics HCV patients in this study. Explanted cirrhotic liver tissues from HCV infected patients undergoing orthotopic liver transplantation were obtained following informed written consent. Abbreviations: Dx= diagnosis; Undet= undetectable plasma viral load; HCV= hepatitis C virus; HCC= hepatocellular carcinoma; ALT= alanine aminotransferase. Asterisk (*) indicates that donor was treated for HCV infection with PEG-IFNa/Ribavirin course prior to liver transplant.

Donor ID	Age	Sex	Cirrhosis	HCV	HCC	Dx	ALT
ET195	42	M	NO	NEGATIVE	NO	Resection/ Cholangiocarcinoma	35
ET203	42	F	NO	NEGATIVE	NO	Resection/ Hemangioendothelioma	30
ET222	40	M	NO	NEGATIVE	NO	Resection/ Cholangiocarcinoma	45
ET321	24	M	NO	NEGATIVE	NO	Resection/ Negative	32
ET322	Unknown	Unknown	NO	NEGATIVE	NO	Donor liver, resectioned to accommodate pediatric recipient	Unknown
ET331	47	M	NO	NEGATIVE	NO	Resection/ Negative	38

Supplementary Table 3. Clinical characteristics non-fibrotic control subjects in this study. Liver tissues were obtained following informed written consent from patients undergoing surgical re-sectioning for non-HCV, non-NAFLD, non-ALD related medical treatments or re-sectioned donor liver to accommodate pediatric recipient. Livers were considered non-fibrotic as medical staff did not diagnose cirrhosis in these specimens. Abbreviations: Dx= diagnosis; HCV= hepatitis C virus; HCC= hepatocellular carcinoma; ALT= alanine aminotransferase.

Chapter 3

Gut Dysbiosis Propels Pathogenic IL-17A+ Vγ6Jγ1 γδ T-Cells During Cholestatic Liver Disease

Dana Tedesco[1#], **Manoj Thapa**[1#], Chui-Yoke Chin[2,5], Yong Ge[3], Minghao Gong[3], Sanjeev Gumber[4], Patrick Speck[1],Elizabeth J. Elrod[1], Eileen M. Burd[2,5,6], Pablo Pereira[7], Mansour Mohamadzadeh[8,9], David S. Weiss [1,2,5] and Arash Grakoui*[1,2]

[#] **indicates authors contributed equally to this work**

[1]Emory Vaccine Center, Division of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA.
[2] Division of Infectious Diseases, Department of Medicine, Emory University School of Medicine, Atlanta, Georgia 30329, USA
[3]Department of Infectious Disease and Pathology, College of Veterinary Medicine; University of Florida ; Gainesville , FL USA.
[4]Division of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, GA, USA
[5]Emory Antibiotic Resistance Center, Atlanta, GA
[6]Department of Pathology and Laboratory Medicine, Emory University, Atlanta, GA
[7]Department of Immunology, Pasteur Institute, 75015 Paris, France.
[8]Department of Infectious Diseases and Pathology ; University of Florida ; Gainesville , FL USA.
[9]Division of Hepatology, Gastroenterology, and Nutrition; University of Florida ; Gainesville , FL USA.

*Corresponding Author:
Arash Grakoui, Division of Infectious diseases, Emory Vaccine Center, Division of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA 30322
Telephone: (404) 727-5850; Fax: (404) 727-7768; Email: arash.grakoui@emory.edu

I. Abstract

Deficiencies in the MDR3 locus, which encodes a biliary transport protein, are associated with a spectrum of chronic choleostatic liver diseases in human patients. The only therapeutic option is transplantation. Exacerbation of liver disease has been linked to elevated Interleukin (IL)-17A, yet factors governing this IL-17A increase are poorly understood. Using the *Mdr2*$^{-/-}$ mouse model, a genetic ortholog of MDR3, we investigated the role of IL-17A in the pathogenesis of chronic liver disease. At 25 weeks old, *Mdr2*$^{-/-}$ animals spontaneously develop fibrosis due to ineffective transport of bile salts. Fibrotic *Mdr2*$^{-/-}$ mice exhibited elevated serum IL-17A as a consequence of increased production by intrahepatic γδ-T cells. Using a novel *in vivo* antibody labeling technique, we attributed this increase of IL-17 to expansion of invariant Vγ6- γδ T cells, the population that this antibody preferentially targets. Due to the ability of Vγ6- γδ T cell to mount TCR-dependent responses to microbial products, we examined the possibility of microbial translocation to the fibrotic livers. Interestingly, liver homogenates of *Mdr2*$^{-/-}$ mice were positive for gut-associated flora with a striking enrichment in *Lactobacillus*. In accordance with this result, *Mdr2*$^{-/-}$ animals marked intestinal permeability that correlated with of IL-17+ γδ-T cells. Intra-peritoneal administration of fibrotic *Mdr2*$^{-/-}$ liver-derived *Lactobacillus* resulted in increased levels of serum IL-17A and acute hepatic inflammation. This was attenuated when animals were pre-treated with γδ-TCR blocking antibody. Finally, γδ-TCR blocking antibody-treatment of *Mdr2*$^{-/-}$ mice resulted in decreased serum IL-17A that corresponded with significant attenuation of hepatic fibrosis ***Conclusions:*** Together, these data indicate fibrosis is exacerbated by Vγ6- γδ T cell responses to translocated microbes in the liver. This axis may represent a novel target for strategies to manage chronic liver disease.

II. Introduction

Defects in the *ABCB4* locus, which encodes a biliary transport protein MDR3 in humans and Mdr2 in mice are associated with a spectrum of chronic choleostatic liver diseases (248). Patients that lack a functional MDR3 protein cannot transport phospholipids (249, 250), resulting into inability to emulsify toxic bile salts, and participate in downstream metabolic processes (250). Consequently, this cholestasis- reduction in bile flow causes liver injury via build-up of these toxic bile salts (248, 249, 251, 252). Choleostatic liver injury can progress into fibrosis, primary sclerosing cholangitis (PSC), end-stage cirrhosis, and hepatocellular carcinoma (248, 252). In particular, PSC is a heterogenous chronic liver disease, that can lead to end-stage cirrhosis in children and adults worldwide (253-255), and remain one of the leading indications for liver transplantation (256, 257). PSC is a complex liver disease with etiologies that involves genetic, environmental, immunological, and other potential factors i.e. gut dysbiosis (255). An association between PSC and ulcerative colitis in an estimated 75% of Western PSC patients implicates an etiological role for gut dysbiosis in this process (25). An over-presentation of gut commensals *Lactobaciilus, Enterococcus,* and *Fusobacterium* has been recently reported in PSC patients (23). It is most likely that an increased intestinal permeability and alterations in the intrahepatic as well as extrahepatic biliary portals, and biliary epithelial cells (i.e. cholangiocytes) during cholestasis may promote microbial translocation to liver. The liver is an anatomic site that is highly enriched in unconventional T cells including γδ T cells (134), which are capable of modulating liver injuries through IL-17 production. There is mounting evidence that demonstrate IL-17+ γδ T cells expand in response to inflammation (140, 142), particularly important for

TCR-mediated recognition of bacterial pathogens invading host tissues. In acute injury setting, such as Con-A induced hepatitis (152) and experimental hepatectomy regeneration (141), this hepatoprotective population is largely restricted to Vγ4 usage (153). However, in chronic models of liver injury, such as high-fat diet (258) and biliary atresia (157), γδ T cells-derived IL-17 is implicated in perpetuating disease pathogenesis; Vγ-chain usage has yet to be elucidated in this context. Interestingly, IL-17 has also been demonstrated to hypersensitize hepatic stellate cells (HSCs), a sentinel cell types in hepatic fibrosis, to TGF-β; addition of IL-17 to HSC cultures *in vitro* permits a robust response to sub-optimal concentrations (156). While this is advantageous in acute liver wound healing, perhaps prolonged hypersensitivity to profibrotic mediators encourages pathology during chronic liver disease. Therefore, we hypothesize that IL-17+ γδ T cells could potentially expand in respond to inappropriately localized commensal bacteria during liver disease. However, the contributions of these mechanisms in the pathogenic progression of choleostatic liver disease remain largely unknown.

Here we used the multidrug resistance gene 2 knockout (*Mdr2*$^{-/-}$) mouse model, the genetic homolog of human MDR3, to examine the contribution of IL-17+ γδ T cells in choleostatic liver disease. Our findings reveal a novel mechanism for gut dysbiosis that drives a selective expansion of an invariant population of IL-17+ Vγ6Jγ1 γδ T cells in the livers of *Mdr2*$^{-/-}$ animals during disease progression. Expansion of IL-17+ γδ T cells correlates with an enrichment of gut commensal bacteria *Lactobacillus sp.* in *Mdr2*$^{-/-}$ livers. IL-17 response is specific to translocated microbe and correlates with serum IL-17A levels. Most importantly, antibody-mediated blockade of γδTCR attenuated liver fibrosis via targeting Vγ chains of IL-17 producing γδ T cells populations. Our data

implicate γδ T cells as a promising tool for novel immunotherapies against choleostatic liver diseases.

III. Materials and Methods

Animal Experiments

Mdr2$^{-/+}$ (FVB.129P2-Abcb4^{tm1Bor}/J) heterozygotes and FVB/NJ (001800) wild-type (WT) controls obtained from the Jackson Laboratory (ME, USA) were housed in specific pathogen-free environment as per Emory University, NIH and IACUC guidelines. *Mdr2*$^{-/-}$ homozygotes, *Mdr2*$^{-/+}$ heterozygotes and wild-type littermate controls were generated in house by backcrossing littermates until desired true breeding lines were established and confirmed by PCR. Where applicable, anti-Mouse IL-17A (clone: BioXcell, NH USA) administered as 250ug in sterile PBS I.P. every three days or 500ug α-TCR γ / δ (clone: UC7-13D5, in house purified) intravenously (I.V.) every 7-10 days starting at 8 weeks of age; all antibody treatment was continued until time of sacrifice (25 weeks of age). Weights were monitored once per week during the study, and mice were periodically submandibular bled to monitor antibody efficacy.

Histology and Immunohistochemistry

Upon animal sacrifice, liver sections were immediately fixed in 10% formalin overnight and paraffin embedded. Standard Sirius Red and Hematoxylin & Eosin (H&E) staining was performed by the Pathology Core, Yerkes National Primate Research Center (Yerkes), Emory University. Quantification of liver fibrosis was scored by a senior pathologist at Yerkes based on the criteria outlined in (222).

Isolation of Liver Cells and Flow Cytometry

Animals were sacrificed as per IACUC guidelines, and livers were perfused with 1X PBS (Lonza, Switzerland) and harvested. Tissues were processed by enzymatic digestion with 2mg/mL type IV collagenase (Worthington, NY) dissolved in serum-free media HBSS (Lonza). Suspensions were strained through cheesecloth, quenched with complete media, and washed. Liver lymphocytes were isolated by Percoll (GE, Sweden) gradient where applicable. Red blood cells were lysed and resuspended in complete RPMI 1640 (Corning, USA) supplemented with 10% FCS (USA) and 1% Pen/Strep (Lonza, USA). In some experiments, isolates were stimulated with 50ng/mL Phorbol 12-Myristate 13-Acetate (PMA; LC Labs, USA) and 1ug/mL Ionomycin (Enzo Life sciences, USA) for 4 hours in the presence of both Golgi Stop and Golgi Plug (BD Biosciences) per manufacturer's recommendation. Cells suspensions were blocked in FACS Buffer (PBS with 2.5% FCS and 0.1% NaN_3) supplemented with 10% normal mouse serum (Sigma), and anti-mouse CD16/32 (2.4G2), prior to surface staining. The following fluorochrome conjugated antibodies were used in this study: anti-CD3 (17A2), CD8 (53-6.7), CD4 (RM4-5), CD11c (N418), GR1 (RB6-8C5), I/A-I/E (M5/114.15.2), TCR β (H57-597), CD45.1 (A20), IFN γ (XMG1.2) (Tonbo Biosciences, USA). Anti- Ly6C (HK1.4), anti-CD11b (M1/70), TCR γ δ (GL3), goat anti-hamster IgG (Poly4055), anti-TCRV γ 1.1+1.2 (4B2.9), TCRV γ 2 (UC3-10A6), TCRV γ 3 (536), and anti-IL-17A (TC11-18H10.1) were purchased from Biolegend (USA). Anti-mouse TCRV γ 4 (49.2) and TCRV γ 7 (F2.64) were the kind gift of Dr. Pablo Pereira-Esteva (Instuit-Pasteur, France). Dead cells were excluded via staining with GhostDye-780 (Tonbo) using standard procedures. Suspensions were fixed in either 1% paraformaldehyde or fixed and

permeabilized using BD Cytofix/Cytoperm per manufacturer's suggestion (BD) and appropriately intracellular stained where applicable. Events were acquired within 24h on a BD LSRII Custom order system, and were analyzed using FlowJo 9.6.4 (TreeStar, Oregon, USA). Auto-fluorescence and debris were excluded using a 407nm laser (525/50BP 475LP).

Quantitative Polymerase Chain Reaction

At the time of animal sacrifice, a small liver biopsy from mouse was directly kept in 1 mL Trizol (Zymo Research) in an eppendorf tube and kept at -80^{0}C until analysis. Liver tissues were homogenized and processed to isolate mRNA, and to reverse-transcription PCR to generate cDNA using high-capacity cDNA reverse transcription kit (Applied Biosystem). 15 ng of cDNA was used for the real-time qPCR using SYBR Green PCR master mix (Applied Biosystem). PCR reactions were as follows; initial 95^{0}C for 10 min followed by 40 cycles of 95^{0}C for 15 sec, 60^{0}C for 60 sec and 72^{0}C for 30 sec and run using 7900HT Fast Real-Time PCR system (Applied Biosystem). Primer sequences used for liver fibrosis gene markers were; GAPDH- Forward: GTGCCAGCCTCGTCCC, Reverse: ACTGTGCCGTTGAATTTGCC; Collagen1a- Forward: GGAGAGTACTGGATCGACCCTAAC, Reverse: ACACAGGTCTGACCTGTCTCCAT; alpha-smooth muscle actin (α-SMA)- Forward: CGGGAGAAAATGACCCAGATT, Reverse: AGGGACAGCACAGCCTGAATAG; TNF-α- Forward: TCTCATTCCTGCTTGTGGC, Reverse: CACTTGGTGGTTTGCTACG; Claudin1- Forward: AGGTCTGGCGACATTAGTGG,

Reverse: CGTGGTGTTGGGTAAGAGGT. Gene expressions were normalized to housekeeping genes GAPDH and calculated using the $2^{-\Delta\Delta Ct}$ method.

Determination of Serum IL-17A Concentration

Serum was obtained from animals at indicated time point via submandibular bleed, and/or at conclusion of the experiment via cardiac puncture in a serum separator microtube (BD) and processed per manufacturer's instruction. IL-17A levels were assessed using Biolegend mouse IL-17A ELISA MAX Deluxe kit per manufacturer's instruction.

Isolation and Functional Analysis of γδ T Cells

Liver or splenic lymphocytes were isolated from 8-10-week-old *Mdr2*$^{-/-}$ mice as described. Bulk T cells were isolated using negative selection (STEMCELL), and seeded in 96 well plates at [$2x10^5$/well] in complete RPMI media supplemented with 50 units/mL of recombinant human IL-2 (Roche). Cells were incubated overnight either in media alone, in the presence of 50ng/mL PMA plus 1ug/mL ionomycin, LPS (2ug/mL, Sigma), Pam3CSK4 (1ug/mL, Invivogen) or with 10^6/mL of indicated heat killed (95^0C for 1h) microbes isolated from 25-week-old *Mdr2*$^{-/-}$ livers in the presence of Golgi Plug and Golgi Stop (BD) at the manufacturer's recommended concentration. Following stimulation, cells were washed, blocked and stained with the appropriate combinations of antibodies as described. Following staining procedures, events were acquired on a BD LSRII Custom order system, and were analyzed using FlowJo 9.6.4 (TreeStar, Oregon,

USA). Auto-fluorescence and debris were excluded using a 407nm laser (525/50BP 475LP).

Culture and Detection of Commensal Microbes in Liver Homogenates

Chui-Yoke, Weiss lab

Livers were homogenized in 1mL of 1X PBS and plated in limiting dilutions on blood agar plates. After culture in 37 degree-incubator for up to 48 hours, colonies were quantified. Select colonies were identified by clinical MALDI-TOF analysis.

Deep Sequencing of Mouse Fecal Pellets and Analysis

Mouse fecal pellets were collected from 8-weeks-old *Mdr2*$^{-/-}$ homozygotes and FVB/N WT controls, generated as described above in Animal Experiments section, using sterile technique. Fecal pellets were collected again from same corresponding mice 25-weeks-old *Mdr2*$^{-/-}$ homozygotes and FVB/N mice. Fecal pellets were kept at -80^{0}C until analysis. Yong and Minghao, Mansour Lab, University of Florida for Deep sequencing.

Statistical Analysis

All statistical data was obtained using a two-tailed Mann Whitney U test, and where appropriate a two-way ANOVA analysis of variance, using standard methods and Graph Pad Prism 4 software (GraphPad).

IV. Results

Intrahepatic γδ T Cells Are a Predominant Source of IL-17A during Choleostatic Hepatic Fibrogenesis

IL-17A has been demonstrated to drive pathogenesis in multiple liver injuries (259, 260). In this study, our aim was to identify the mechanistic contributions of IL-17A in pathogenesis of cholestatic liver disease in *Mdr2*$^{-/-}$ mice (252). In agreement with other characterizations of this model (249, 252), *Mdr2*$^{-/-}$ mice exhibited characteristic collagen deposition around hepatic bile duct, moderate to severe periportal to bridging fibrosis with marked influx of inflammatory infiltrate at 25 weeks of age (**Figure 3-1A).** Compared to age-matched WT control FVB/N, *Mdr2*$^{-/-}$ mice demonstrated elevated levels of serum IL-17A (**Figure 3-1B**). Intriguingly, administration of IL-17A neutralizing antibody to *Mdr2*$^{-/-}$ animals at 8 weeks of age until 25 weeks was sufficient to reduce hepatic fibrosis score (**Supplementary Table 3-1**)**.** Consistent with reduced collagen deposition, and expressions of collagen-1a and alpha-smooth muscle actin (α-SMA) in liver, we found a marked reduction in infiltrating inflammatory populations such as monocytes (CD11b^{+}Ly6c^{hi}) and neutrophils (CD11b^{+}Gr1^{+}) in animals receiving IL-17A-neutralizing treatment (**Supplementary Figure 3-1**). These data implicated a critical role for IL-17A in pathogenesis of cholestatic liver fibrosis in *Mdr2*$^{-/-}$ mice.

Next, to determine the source of IL-17A in *Mdr2*$^{-/-}$ mice, we performed intra-cellular cytokine staining (ICS) analysis on PMA/Ionomycin stimulated intrahepatic lymphocytes isolated from 25-week-old *Mdr2*$^{-/-}$ mice. *Mdr2*$^{-/-}$ animals demonstrated a marked increase in frequency of IL-17+ T cells (CD3+) in comparison to non-fibrotic controls (**Figure 3-**

1C and D). There were no appreciable changes in splenic frequency of T cell derived IL-17A despite significant changes in the liver (**Figure 3-1E and F).** To our surprise, however, it was not the CD4+ Th17 population, but primarily the γδ TCR+ population that produced the bulk of the intrahepatic IL-17A (**Figure 3-1G and H).** A closer examination of intrahepatic CD4+ T cell and γδ T cell IL-17A production demonstrated a subtle increase in the frequency of CD4+IL-17A+ (**Figure 3-1G)**, while a dramatic increase in the frequency of IL-17A+ γδ T cells in *Mdr2*$^{-/-}$ mice (**Figure 3-1H)**. Thus, elevated serum IL-17A in fibrotic *Mdr2*$^{-/-}$ mice can be attributed to intrahepatic γδ T cell compartment.

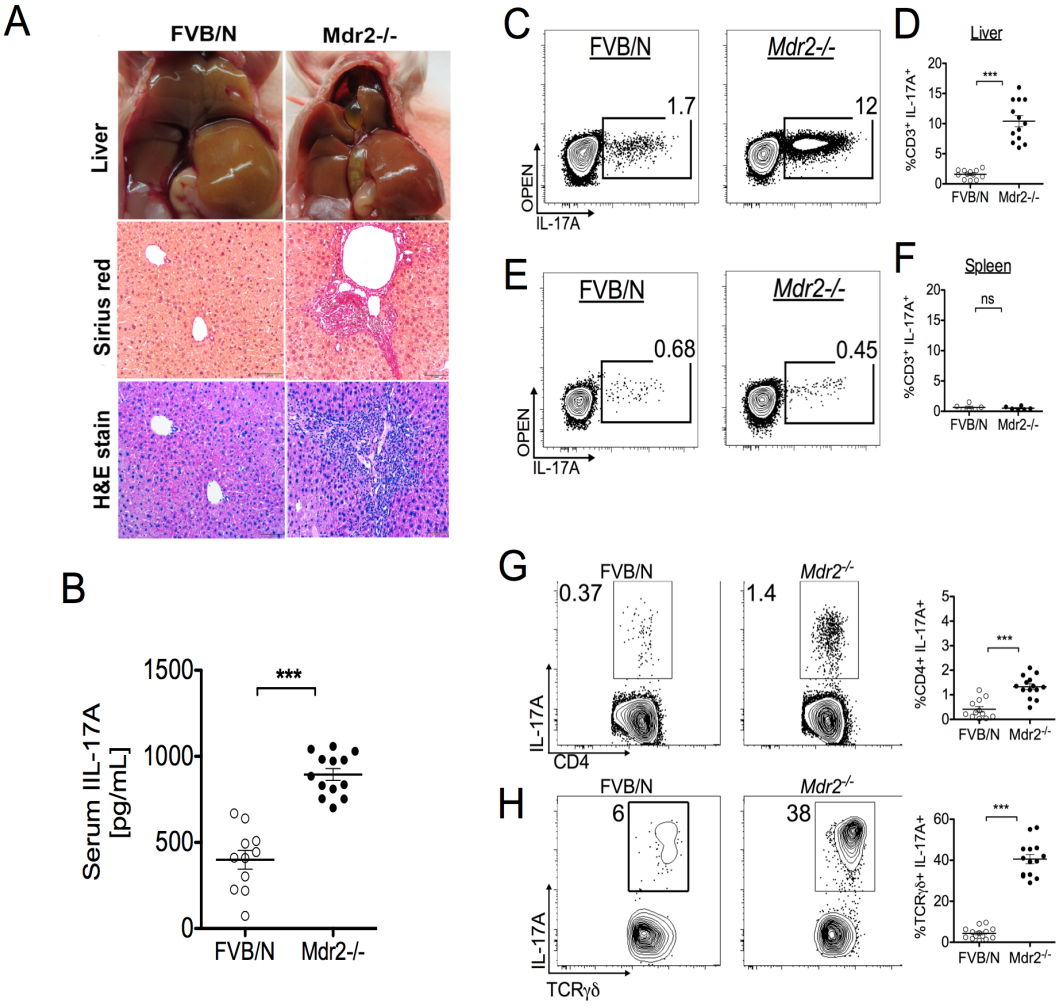


Figure 3-1. Intrahepatic γδ-T cells are a predominant source of IL-17A during chronic liver disease. (A) Histological analysis of 25-wk-old *Mdr2*$^{-/-}$ mice indicate a characteristic collagen deposition around bile duct (Sirius red stain, mag.10X) and immune cells infiltration (H&E stain, mag. 10X) compared to WT counterpart, FVB/N. (**B**) Serum samples were obtained from 25-wk-old *Mdr2*$^{-/-}$ or age-matched FVB/N mice immediately following sacrifice and the levels of IL-17A were detected using a standard ELISA. **(C-F)** Bulk lymphocytes isolated from livers (C, D) and spleens (E, F) were stimulated with PMA and Ionomycin for 4 hrs to determine frequency of Live, Non-autofluorescent CD3+ (T cells) that produce IL-17A from livers (C) and spleens (E) derived from the indicated group. **(G)** Quantitation of frequencies of IL-17A+ CD4+ T cells (G, Top) and γ/δ-T cells (G, bottom) from livers are shown. Representative figures from more than 3 independent experiments are shown. ***P<0.001, Two-tailed Mann Whitney test).

Liver-specific Alterations of the γδ T Cell Compartment During Cholestatic Disease

Anatomic compartmentalization and functions of γδ T cells, such as propensity to make IL-17, are dictated by composition of the TCR (137). To determine Vγ-chain usage in *Mdr2*$^{-/-}$ livers, we performed a FACS analysis using commercially available antibodies directed against Vγ1.1+1.2, Vγ2, Vγ3 in combination with Vγ4 and Vγ7 within TCRβ-CD3+ gate. Due to the limitations of reagent availability, we also took a novel approach of *in vivo* administration of anti-γδTCR (Clone: UC7-13D5) and detection with anti-hamster IgG to account for the remaining unidentified Vγ-chains indicated as "other" in this assay (**Supplementary Figure 3-2)**. This approach revealed dramatic alterations within the composition of the intrahepatic γδ T cell compartment in *Mdr2*$^{-/-}$ animals in comparison to age-matched WT controls (**Figure 3-2A)**. The most dramatic increase in fibrotic livers was the accumulation of *in vivo* anti-γδTCR+ T cells (**Figure 3-2A).** This was liver-specific, as the splenic γδ T cell compartment was not appreciably changed in fibrotic animals (**Figure 3-2B)**. As such, we hypothesized that this population may account for increases in IL-17A+ γδ T cells in livers of *Mdr2*$^{-/-}$ mice. To this aim, we isolated lymphocytes from livers and spleens of both WT and knock out animals and performed a brief PMA/Ionomycin stimulation to interrogate which Vγ-chains comprised the IL-17+ population. IL-17A was primarily derived from *in vivo* labeled γδ T cells with Vγ2 and Vγ4 comprising the remainder (**Figure 3-2C).** Consistent with our earlier observation, splenic IL-17A production was very minor in comparison to the liver; Vγ2, Vγ4 and *in vivo* labeled γδ T cells comprised this population (**Figure 3-2D).** Overall, these data indicate that the fibrotic liver γδ T cell compartment is altered in favor of IL-17A γδ T cell subsets.

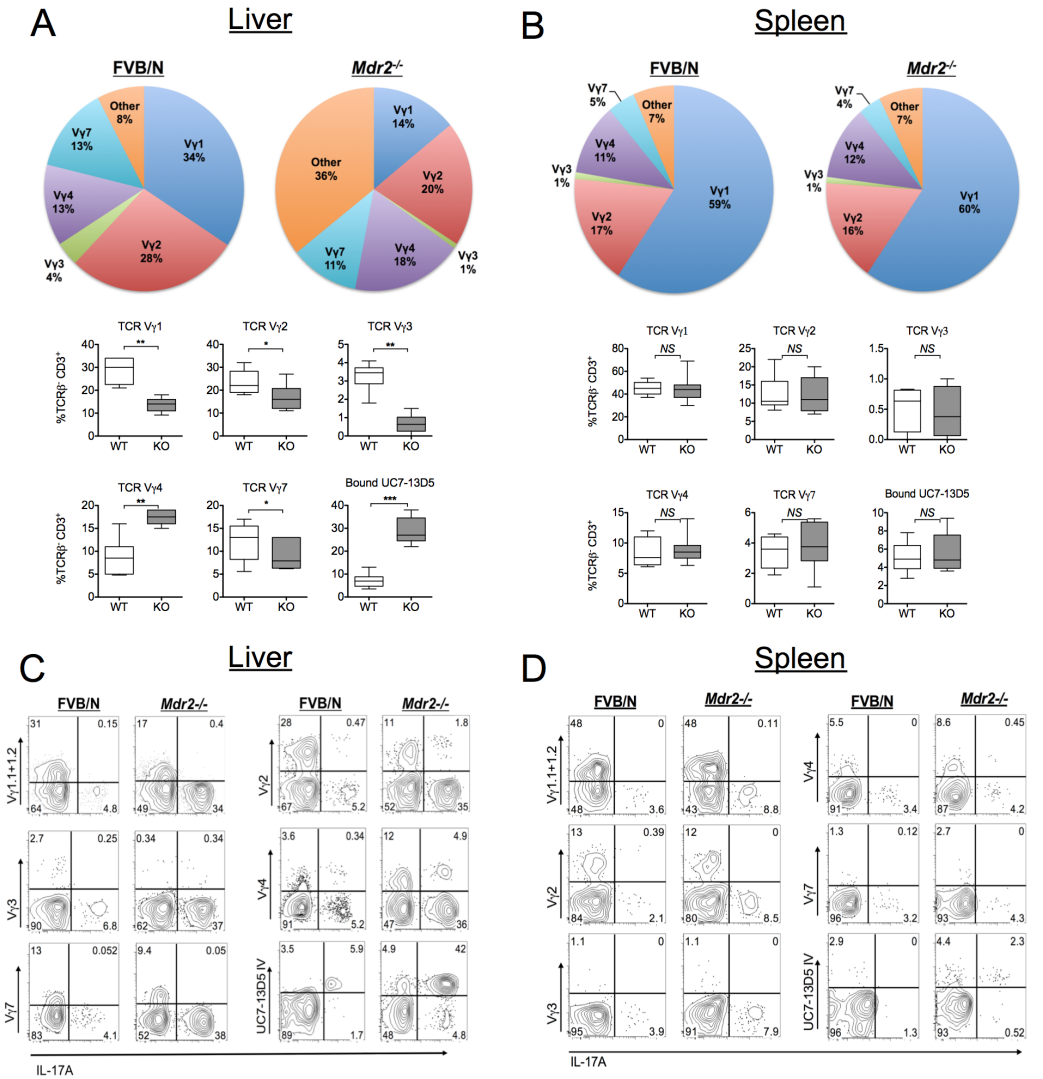


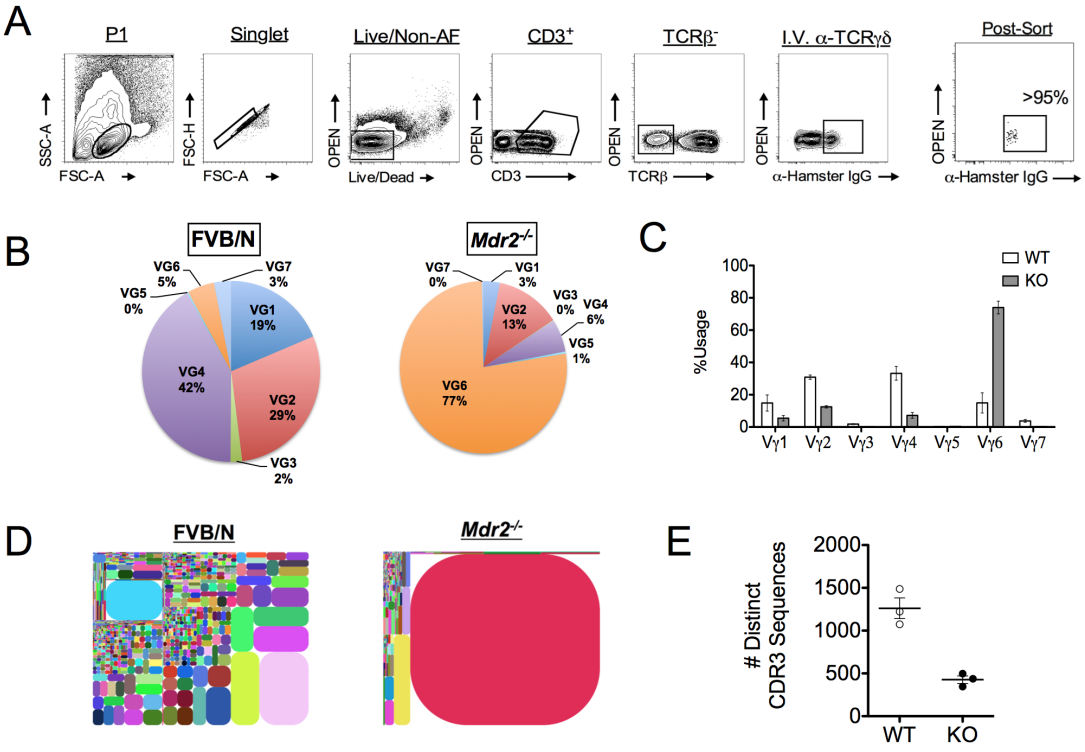
Figure 3-2. Fibrotic liver γδ T cell compartment is altered in favor of IL-17 producing subsets. Age-matched *Mdr2*$^{-/-}$ or FVB/N mice were administered 500ug of anti-γδTCR (Clone UC7-13D5; Hamster IgG) or appropriate Hamster-IgG isotype control intravenously and sacrificed one day following treatment. Livers and spleens were harvested; and bulk lymphocytes were first stained with anti-hamster IgG, followed by TCRVγ1.1-1.2, Vγ2, Vγ3, and for Vγ4, Vγ7 in combination with appropriate surface antibodies. Frequencies were determined based on the percentage of the indicated Vγ-chain within the Live non-autofluorescent TCRβ- CD3+ gate (**A and B**). "Other" refers to *in vivo* bound and *ex vivo* detectable UC7-13D5 (A, top; B, Top). To determine the IL-17A producing capacity of these subsets, lymphocytes were stimulated with 50ng/mL PMA plus 1ug/mL ionomycin in the presence of Golgi Plug and Golgi Stop for 4 hours at 37°C, and subsequently stained for intracellular IL-17A **(C and D)**. Statistical significance was determined by a two-tailed Mann-Whitney Test, *p<0.05, **p<0.005, ***p<0.0001. Error bars reflect the standard error of mean (SEM). All data are representative of at least three independent experiments (n=3-5 age-matched animals per indicated group).

Fibrosis Drives Intrahepatic Expansion of IL-17A+ Invariant V γ 6J γ 1 γδ *T Cells*

To understand the mechanisms of γδ T cell contributions in our model, we first needed to elucidate the actions for *in vivo* administration of this antibody. Therefore, we isolated intrahepatic and splenic lymphocytes from animals treated with anti-γδTCR (UC7-13D5) and control *Mdr2*$^{-/-}$ mice and stained with a fluorochrome-conjugated anti-hamster IgG. Then in a second step, lymphocytes were stained with appropriate surface antibodies plus an alternative monoclonal antibody directed against the γδTCR (clone GL3). In the liver, we could detect hamster IgG solely in mice that were treated with anti-γδTCR (UC7-13D5) **(Supplementary Figure 3-2A and B).** This observation is in accordance with another study which used this approach to determine that *in vivo* treatment with UC7-13D5 made γδT cells "invisible" opposed to depletion (261). In both fibrotic and WT animals, this antibody preferentially targeted Vγ chains of T cells capable of IL-17A production upon PMA/Ionomycin stimulation **(Supplementary Figure 3-2B).**

To further identify Vγ-chains that are targeted by *in vivo* administration of anti-γδTCR, we utilized our labeling approach to FACS-sort the antibody-bound subset in WT versus *Mdr2*$^{-/-}$ livers (**Figure 3-3A)**. Consistent with our previous experiments, we found an accumulation of *in vivo* labeled γδ T cells in knockout mice in comparison to WT controls**.** The purified population was sequenced to identify VγJγ usage as well as determine the CDR3 diversity. In WT mice, *in vivo* labeling targeted predominantly Vγ4, Vγ2 and Vγ1 populations (**Figure 3-3B, left and C)**. *Mdr2*$^{-/-}$ mice, on the other hand, revealed that *in vivo* labeling targeted predominantly Vγ6 bearing γδ T cells (**Figure 3-3B, right and C)**. Examination of the overall CDR3 region diversity indicated that the Vγ6

population is a monoclonal/invariant **(Figure 3-3D).** Consistent with this analysis, in vivo labeled γδ T cells from knockout livers demonstrated a substantial reduction in the number of CDR3 sequences present in the sample **(Figure 3-3E).** Analysis of peptide sequences of the most prevalent CDR3 indicates a massive expansion of Vγ6Jγ1, an invariant population of γδ T cells in the livers of *Mdr2*$^{-/-}$ animals, comprising approximately 76% of the total *in vivo* labeled population **(Figure 3-3F)**. The same analysis of WT livers demonstrates that 2 out of 3 mice demonstrate Vγ6Jγ1 as the most prevalent population, whereas Vγ4Jγ1 was the most prevalent in the remaining control mouse **(Figure 3-3F)**. Altogether, these data indicate that liver fibrosis drives expansion of IL-17A+ invariant Vγ6Jγ1 γδ T cells; this and other IL-17+ populations are largely targeted by *in vivo* administration of anti-γδTCR.



F

Sample ID	CDR3 Peptide Sequence	Vg usage	Jg usage	#positive reads	#total reads	Percentage in sample
1 WT	SYGYSSGFHKV	mTRGV4	mTRGJ1	17936	239445	7.490655474
2 WT	ACWDSSGFHKV	mTRGV6	mTRGJ1	30982	232323	13.33574377
3 WT	ACWDSSGFHKV	mTRGV6	mTRGJ1	62240	241045	25.82090481
1 KO	ACWDSSGFHKV	mTRGV6	mTRGJ1	206246	271147	76.0642751
2 KO	ACWDSSGFHKV	mTRGV6	mTRGJ1	162482	254116	63.94009035
3 KO	ACWDSSGFHKV	mTRGV6	mTRGJ1	204418	266106	76.81826039

Figure 3-3. Fibrosis drives expansion of IL-17A+ invariant Vγ6Jγ1 γδ-T cells. Three FVB/N-wild type and three *Mdr2*$^{-/-}$ animals were administered 500ug of anti-γδTCR (Clone UC7-13D5; Hamster IgG) intravenously and sacrificed one day following treatment. One wild-type and one *Mdr2*$^{-/-}$ animal was administered appropriate Hamster-IgG isotype control in the same fashion for gating control purposes. Lymphocytes were stained as described and sorted based on positivity for bound UC7-13D5 in the live non-autofluorescent CD3+ TCRβ- anti-Hamster IgG+ **(A).** Post-sort purity was >95% in all animals **(B)**, and *in vivo* bound antibody+ populations were sequenced to identify Vγ-chains usage. **(C)** Vγ chain usage of sorted population bound by *in vivo* administration of anti-TCRγδ, and diversity diagram of the sorted populations based prevalence of Vγ-chain using a unique CDR3 **(D).** Total unique CDR3 sequences identified in antibody bound populations of individual mice **(E).** In 2 out of 3 WT animals; invariant Vγ6Jγ1 chain was the most prevalent population identified by *in vivo* administration of anti-TCRγδ **(F, top)**, while this population was the most prevalent in 3 out of 3 *Mdr2-/-*animals **(F, bottom).**

Increased Gut Permeability & Dysbiosis Favor Microbial Translocation and Enrichment in Fibrotic liver

Chronic inflammatory conditions such as liver disease can contribute to the loss of intestinal integrity (262) and result in translocation of gut commensals to the periphery (200, 263). Since Vγ6Jγ1 γδ T cells are largely implicated in TCR-dependent responses to microbial products (140), we sought to determine whether microbial translocation in the liver could influence IL-17A production by γδ T cells. For this purpose, we first extracted fecal DNA from 25-weeks-old WT (n=5) and *Mdr2*$^{-/-}$ (n=5) mice and processed for 16s ribosomal DNA sequencing to determine the relative enrichment of various bacterial families in the intestine. Interestingly, linear discriminant analysis effect size (LEfSe) predictions for bacterial families found in the fecal DNA implicated a marked enrichment (LDA score) of *Lactobacillus, Bacilli, Turicibacter, Unclassified Streptococcus, and Prevotella* in the intestine of *Mdr2*$^{-/-}$ mice (red) compared to WT animals (green) (**Figure 3-4A**). Likewise, cladogram representation indicated the close phylogenetic relationship among bacterial families identified in the *Mdr2*$^{-/-}$ mice (**Figure 3-4B**). Interestingly, LEfSe predictions also implicated the enrichment of *Lactobacillus* and other bacterial families as young as 8 weeks of age, the beginning of disease onset in *Mdr2*$^{-/-}$ mice (249, 252) (**Supplementary Figure 3-4**). This finding correlated with our observation of increased intestinal barrier permeability in 8-weeks-old *Mdr2*$^{-/-}$ animals as detected by oral administration of FITC-dextran assay (**Figure 3-4C**). The intestinal permeability was even more pronounced at 25 weeks of age, when animals experience the peak of liver fibrosis (**Figure 3-4C**). Intriguingly, this profile corresponded to the expansion of IL-17A+ γδ T cells in the livers and the increasing serum levels of IL-17A (**Supplementary Figure 3-3A and B**).

Furthermore, by using a standard bacteriological culture technique, we found that the total colony forming units (CFU) per gram of whole liver homogenates is significantly higher in *Mdr2*$^{-/-}$ livers compared to WT animals (**Figure 3-4D**). Remarkably, *Lactobacillus* was the predominant bacterial species detected only in *Mdr2*$^{-/-}$ but not in WT livers (**Figure 3-4E**). The isolated bacterial colonies were subjected to confirmatory diagnosis by using matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry analysis for bacterial surface proteins (**Supplementary Figure 3-5**). MS profiles of bacterial colonies isolated from the liver homogenates identified *Lactobacillus sp.* only in *Mdr2*$^{-/-}$ livers. To investigate the mechanisms that favor *Lactobacillus* enrichment in *Mdr2*$^{-/-}$ mice liver, we performed *in vitro* bile salt and pH tolerance assays using isolated strains of *Lactobacillus.* A non-specific commensal bacterium *Ralstonia picketti* was chosen as control. Strikingly, *Lactobacillus* exhibited bile salts (1.5%) and pH (2) resistance after 4 hours of treatment, while *Ralstonia picketti* was highly sensitive to both conditions (**Figure 3-4F**). Taken together, these data suggest that cholestatic liver disease selectively enriches intestinal flora in *Mdr2*$^{-/-}$ mice, which corresponds to inappropriately localized and enriched *Lactobacillus* in fibrotic microenvironment.

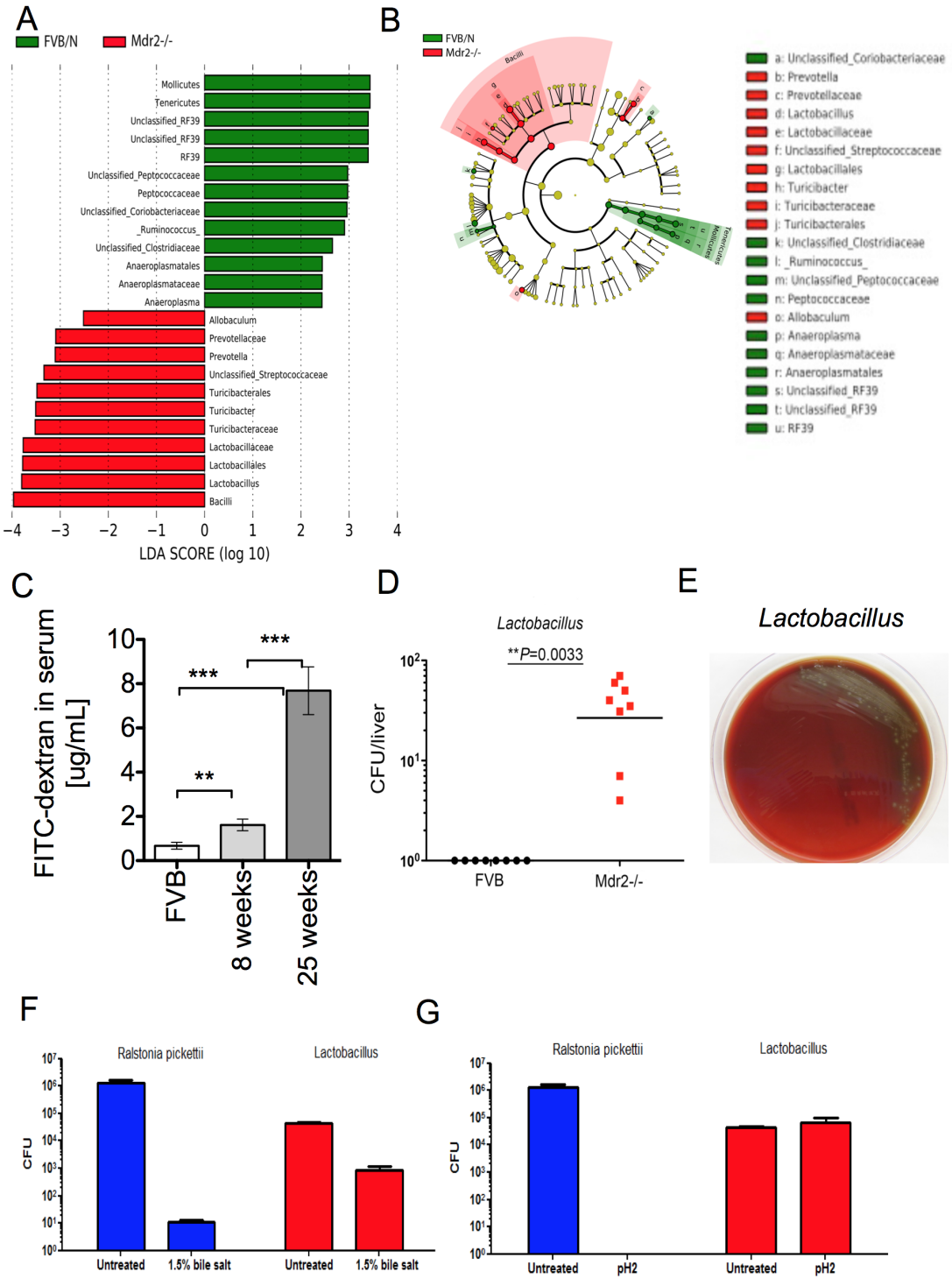


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Figure 3-4. Increased Gut Permeability and Dysbiosis Favor Microbial Translocation and Enrichment in Fibrotic Liver. (**A**). Linear discriminant analysis size effect (LEfSe) predictions for bacterial families found in fecal pellets of 25-wk-old *Mdr2*$^{-/-}$ (red) and FVB/N (green) mice are shown. Linear discriminant analysis (LDA) score represents log changes in bacterial families shown. (**B**). The Cladogram representing the phylogenetic relationship between bacterial families (red = *Mdr2*$^{-/-}$ and green = FVB/N) is shown. (**C**) Gut permeability was determined in FVB/N, 8-wk-old and 25-wk-old *Mdr2*$^{-/-}$ animals by using FITC-Dextran oral feeding assay. (**D**) Using standard bacterial culture methods, whole liver homogenates were plated on blood agar plates and 24 hours following plating, colonies were quantified as CFU/gram of tissue in 25-wk-old *Mdr2*$^{-/-}$ *and* WT controls. (**E**). Bacterial colonies detected on blood agar were subjected to clinical identification as described in the Supplementary sections; and *Lactobacillus;* predominant bacterial species present in *Mdr2*$^{-/-}$ gut, were also detected in *Mdr2*$^{-/-}$ livers but not in WT livers. (**F**). Bile salt and pH tolerance assays show the bile salt and pH tolerance property of *Lactobacillus* indicating the possible mechanism by which *Lactobacillus* are selectively enriched in the *Mdr2*$^{-/-}$ livers. Statistical analysis werewas performed by one way analysis of variance (ANOVA) for C. *$p<0.05$, **$p<0.005$, ***$p<0.0001$. Error bars reflect the standard error of mean (SEM).

Please note author contributions for this figure are as follows:
A and B: Mohmadzadeh lab members (University of Florida). C: Dana Tedesco D: Dana Tedesco obtained the mouse samples, and Chui-Yoke Chin performed bacterial assays. E: Collective efforts of Chui-Yoke Chin and Eileen Burd. F. Collaborative effort between Manoj Thapa and Chui-Yoke Chin.

Intrahepatic γδ T Cells Produce IL-17 in Response to Translocated Gut Microbiota

γδ T cells can respond in a TCR-dependent manner to microbial products (138, 139). As such, we next examined if γδ T cells isolated from *Mdr2*$^{-/-}$ livers could respond to bacteria isolated from *Mdr2*$^{-/-}$ liver homogenates. Indeed, *in vitro* stimulation of isolated bulk T cells from 25-week-old *Mdr2*$^{-/-}$ livers with heat-killed *Lactobacillus* resulted in IL-17A production **(Figure 3-5A)**. To rule out non-specific stimulation by toll like receptors (TLRs) or other innate mechanisms, we also stimulated control wells with Pam3Csk4, a TLR2 agonist, and LPS, a TLR4 agonist. To our surprise, IL-17 responses were not elicited against these stimuli (**Figure 3-5A, right).** We did not observe any appreciable splenic γδ T cell IL-17A in response to these stimuli (**Figure 3-5A, top).** To assess the overall functionality of these γδ T cells, we also examined IFN-γ responses (**Figure 3-5B)**. Intrahepatic γδ T cell responses to heat-killed microbes resulted in weak IFN-γ production in comparison to IL-17A **(Figure 5B)**. Splenic γδ T cells were not appreciably influenced by microbial stimuli (**Figure 5B, bottom)**. Thus, these data indicate that intrahepatic γδ T cells can specifically respond to *Mdr2*$^{-/-}$ liver-derived microbes *in vitro.*

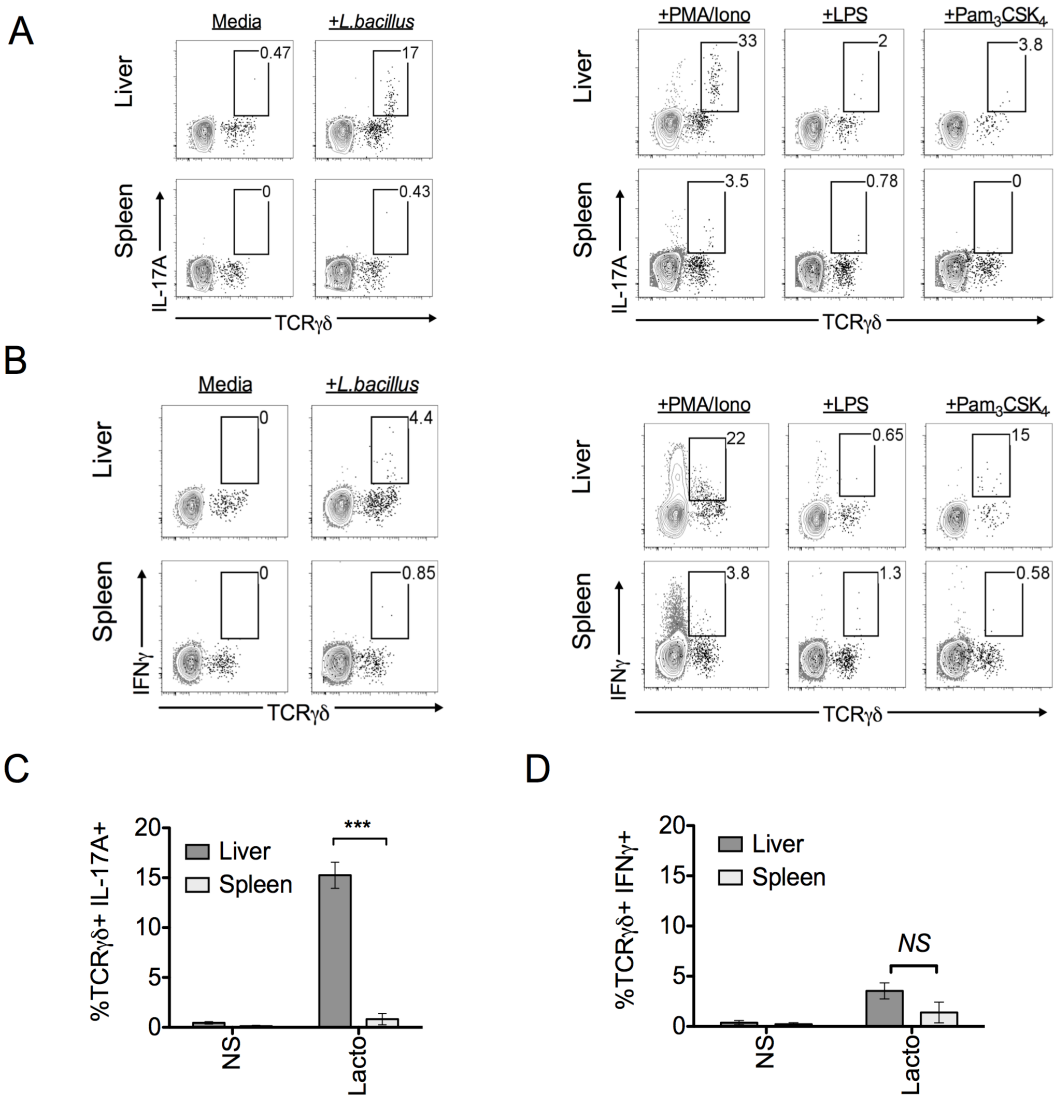


Figure 3-5. Intrahepatic T cells produce IL-17 in response to translocated gut microbiota. Bulk T cells were isolated from Mdr2$^{-/-}$ livers and spleens via negative selection. Liver and spleen T cells were seeded at 2x10^5 cells per well and stimulated for 24 hrs in the presence of media (complete RPMI +20U/mL IL-2), or heat killed *Lactobacillus* (10^6/mL) species isolated from Mdr2$^{-/-}$ livers. T cells were analyzed for IL-17A (**A**) and IFNγ (**B**) responses via intracellular cytokine staining. To rule out non-specific TLR pathways, control wells were stimulated with 2ug/mL LPS, or 1ug/mL Pam_3CSK_4; PMA/Ionomycin stimulation served as a positive control (**A and B; far right**). Frequency of IL-17A (**C**) and IFNγ (**D**) production by TCRβ- TCRγδ+ were compared in the liver versus spleen. Numbers on FACS plots represent the proportion of the TCRβ- TCRγδ+ population producing the indicate cytokine. Gates were determined using appropriate unstimulated and isotype controls. Statistical significance was determined by a two-tailed Mann-Whitney Test, *p<0.05, **p<0.005, ***p<0.0001. Data are representative of atleast 3 independent experiments with n=3-5/group

Administration of Mdr2$^{-/-}$ Liver-derived Lactobacillus Induces IL-17 Responses in WT Mice

Intrahepatic γδ T cells have the capacity to respond to translocated microbiota in fibrotic livers. To determine the implications of these responses on perpetuating hepatic inflammatory processes, we administered 1x10^6 CFU of liver-derived *Lactobacillus* to WT animals (**Figure 3-6A)**. Intra-peritoneal (I.P.) injection was chosen to ensure that these bacteria were introduced into the periphery rather than colonize the intact intestinal tracts. Indeed, I.P. inoculation of FVB/N mice resulted in bacterial colonization of the liver after 18-24 hours as detected by standard bacteriological culture technique **(Supplementary Fig S3-6)**. Introduction of *Mdr2$^{-/-}$* liver-derived *Lactobacillus* resulted in a marked surge of serum IL-17A in WT animals (**Figure 3-6B).** This corresponded with an influx of hepatic inflammatory mediators such as CD11b$^+$Gr-1$^+$ neutrophils (**Figure 3-6C and D)**, which was present in a low frequency in the spleens of these animals (**Figure 3-6C, bottom)**. This is consistent with a low frequency of microbe-responsive γδ T cells in the spleen**.** Our *in vitro* data highlighted a potential role of intrahepatic γδ T cell IL-17 in response to translocated gut microbes (**Figure 3-5)**. We treated FVB/N mice with anti-γδTCR or Hamster IgG isotype control one day prior to infection with *Lactobacillus.* Following bacterial inoculation, we found that blockade of the γδ TCR resulted in a subtle reduction of serum IL-17 levels (**Figure 3-6B).** However, a dramatic effect was found in the livers of these mice, with a marked reduction in hepatic inflammatory infiltration (**Figure 3-6C, right).** Altogether, these data suggest that intrahepatic γδ T cells can mount inflammatory IL-17 responses to inappropriately localized commensal microbiota.

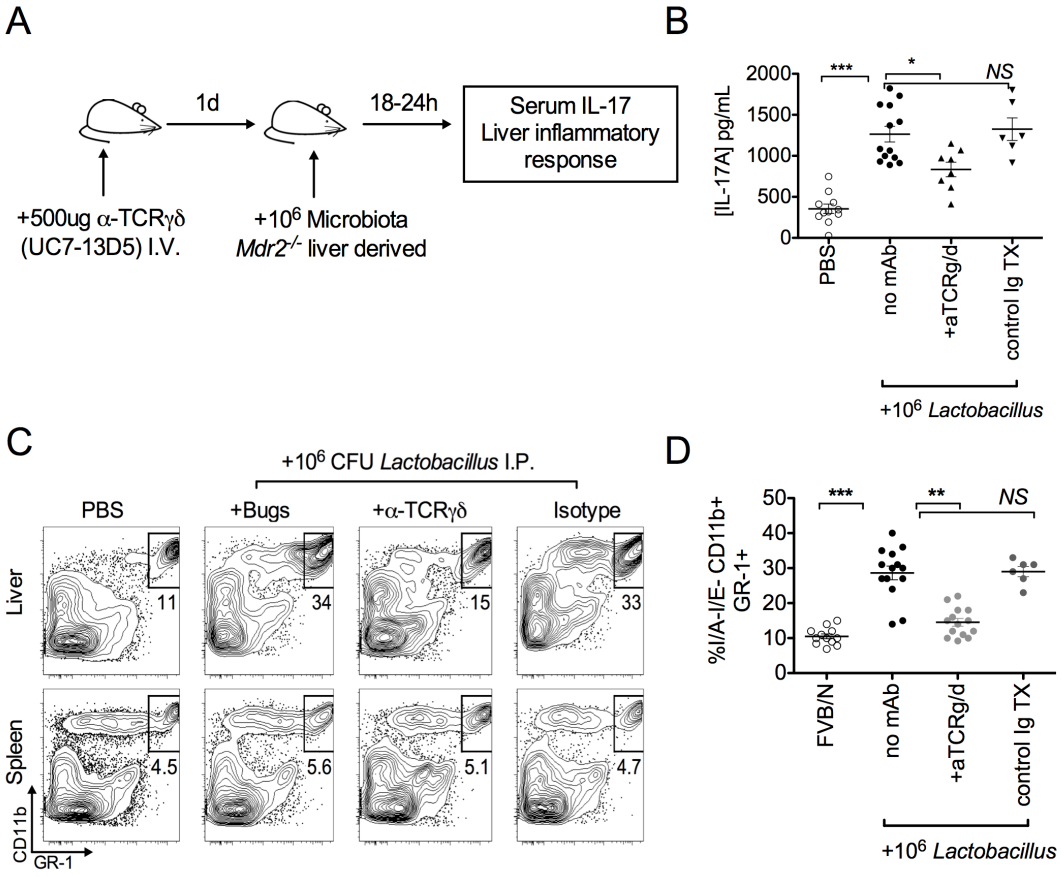


Figure 3-6. Administration of *Lactobacillus* can induce hepatic inflammation and increase serum IL-17 in wild-type FVB/N mice. FVB/N mice were either untreated or administered 500ug anti-TCR, or appropriate isotype control I.V. one day prior to intraperitoneal inoculation with 10^6 live ***Lactobacilli*** isolated from *Mdr2*$^{-/-}$ livers**. (A)**. 18-24 hours following IP infection, animals were sacrificed and serum was immediately harvested for ELISA analysis of serum IL-17A concentration **(B).** Livers and spleens were analyzed for infiltrating neutrophils (Live, Non-autofluorescent (Non-AF), Lineage (CD3/CD19) negative, MHC class II negative, CD11b+, GR-1+ (Ly6G)) as indicators of inflammation **(C, left).** Frequency of hepatic neutrophilic infiltrates are represented **(C, right)**. Data are representative of 3 independent experiments with 3-5 mice per group. Statistical significance was determined by a two-tailed Mann-Whitney Test, *p<0.05, **p<0.005, ***p<0.0001. Error bars reflect the standard error of mean (SEM).

Antibody-mediated Blockade of the γδTCR Attenuates Fibrosis

γδ T cells have been implicated in perpetuating liver injuries (132, 264) through interaction with inflammatory populations and IL-17A signaling. We took an antibody-mediated approach to target the γδTCR and examine whether γδ T cells are required for the pathogenesis of liver fibrosis in our model. Due to limited availability of commercially availability of FVB/N background for specific-cell population knockouts (IMSR Database http://www.findmice.org/index)**,** an antibody-mediated approach by infusing *Mdr2*$^{-/-}$ animals with monoclonal antibody directed against the γδTCR (Clone: UC7-13D5) I.V. every 10 days from 8 weeks of age to 25 weeks of age was adapted (264). Compared to untreated *Mdr2*$^{-/-}$ mice (average score 3), anti-γδTCR treated animals exhibited reduced fibrosis lesion (average score 2) and a substantial reduction in serum IL-17A levels (**Figure 3-7A, and B**), and expressions of fibrosis-associated genes such as Collagen-1a, α-SMA and TNF-α in liver (**Figure 3-7C).** Interestingly, we found that antibody-treated animals demonstrated serum IL-17A levels that were indistinguishable from WT control animals; a two-fold reduction in comparison to levels found in age matched *Mdr2*$^{-/-}$ serum (**Figure 3-7B)**. A trend of reduced serum ALT was found that did not reach statistical significance (**Figure 3-7D)**. In addition, the reduced serum IL-17A concentration observed in antibody-treated *Mdr2*$^{-/-}$ corresponded to a marked decrease in frequency of intrahepatic inflammatory monocyte and neutrophil populations in comparison to untreated *Mdr2*$^{-/-}$ animals (**Figure 3-7E).** Overall, these data indicate that intrahepatic γδT cells responding to inappropriately localized gut microbes promote pathogenesis of cholestatic liver disease via IL-17A production.

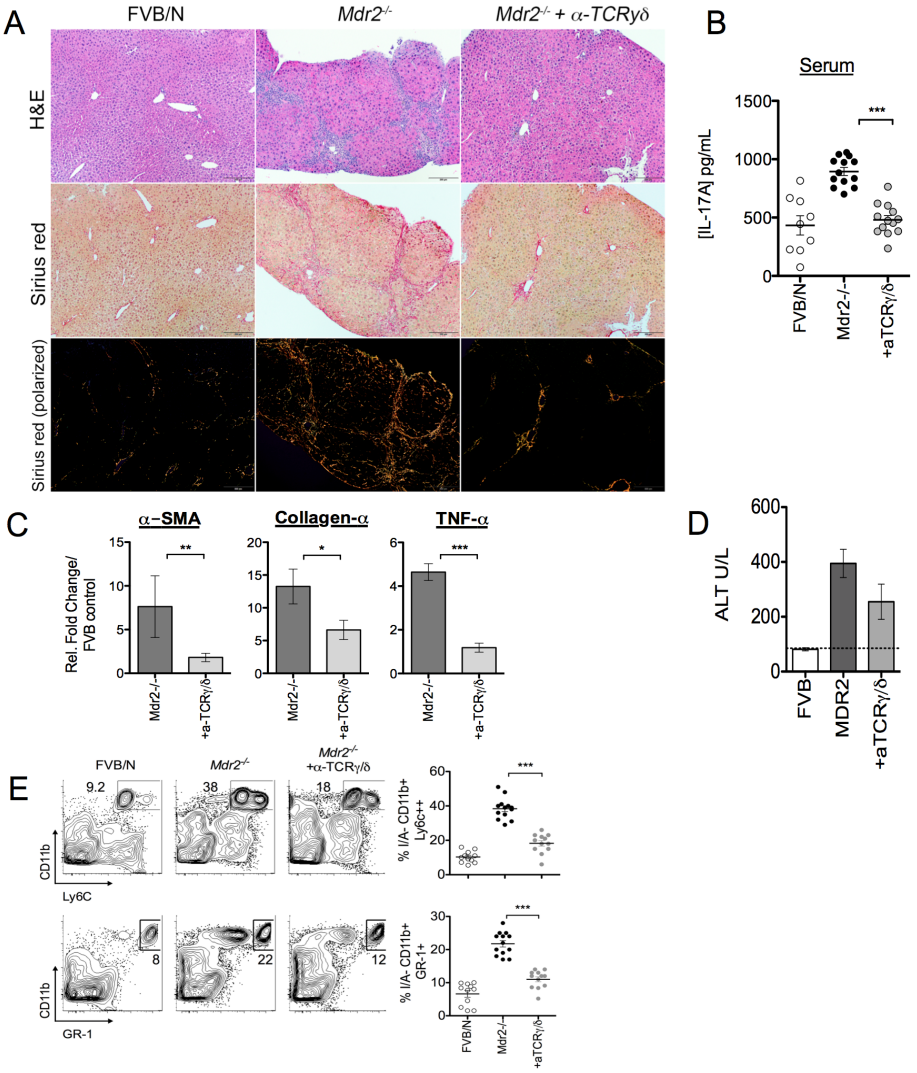


Figure 3-7. Antibody-mediated blockade of the TCR attenuates hepatic fibrosis. *Mdr2*$^{-/-}$ were treated i.v. with 500ug anti-TCR (clone UC7-13D5) every 10 days from 8-wk to 25- wk. (**A**) Histological analysis of representative liver samples for H&E, Sirius red and polarized Sirius red staining (Mag. 10x) are shown (**B**). Fibrosis gene expression in total liver tissues by RT-PCR analysis, (**C**) Serum ALT and (**D**) IL-17A were determined at the end pointend-point (25-wk) from FVB/N, *Mdr2*$^{-/-}$ and antibody-treated *Mdr2*$^{-/-}$ (+anti-TCR). (**E**) ELISA analysis of serum samples following animal sacrifice from the indicated 25-wk-old animals. Analysis of the frequency of infiltrating intrahepatic inflammatory myeloid populations; monocytes (top, gated on Live, Non-autofluorescent (Non-AF), Lineage (CD3/CD19) negative, MHC class II negative, CD11b+, Ly6C+), and neutrophils (bottom, gated on Live, Non-autofluorescent (Non-AF), Lineage (CD3/CD19) negative, MHC class II negative, CD11b+, GR-1+ (Ly6G). Statistical significance was determined by a two-tailed Mann-Whitney Test, *p<0.05, **p<0.005, ***p<0.0001. Data are representative of 3 independent experiments, n=3-5/group. Error bars reflect the standard error of mean (SEM).

Panel A was generated by Sanjeev Gumber, Panel C was generated by Manoj Thapa on Dana Tedesco's samples

V. Discussion

Anatomic sequestration of commensal flora relies on intact barriers to ensure that microbes serve their respective functions in discrete locations. Disruption of intestinal barriers is associated with various etiologies of chronic liver diseases (200, 249, 263, 265-267). Alterations in the liver microenvironment during cholestatic liver disease can promote microbial translocation to liver tissues. Particularly, toxic bile acids (BAs) associated with cholestasis contribute to increased oxidative stress, hepatocyte death, inflammation and suppression of Kupffer cell (KC) phagocytic functions(268). The effects of BAs on KC functions contribute to the both ineffective clearance of fibrotic tissues (269) and permission of intrahepatic microbial accumulation (268). Our data demonstrate that biochemical processes of cholestasis in *Mdr2*$^{-/-}$ mice select intestinal microflora for certain bacterial species such as *Lactobacillus, Bacilli, Turicibacter, Unclassified Streptococcus,* and *Prevotella.* These findings are in agreement with PSC patients, in whom *Lactobacillus* and other related bacterial families have been found selectively enriched regardless of treatment regimens or co-existent intestinal disorders (23). Interestingly, *Lactobacillus* is the predominant bacterial species detected only in *Mdr2*$^{-/-}$ but not in WT livers. Our findings from bile salt and pH tolerance assays further explain the possible mechanisms for selective enrichment of these bugs in *Mdr2*$^{-/-}$ liver microenvironment. Here, we also demonstrate that translocation of *Lactobacillus* triggers the expansion of invariant Vγ6Jγ1 IL-17+ γδ T cells in the liver of *Mdr2*$^{-/-}$ mice. This subset of γδ T cells is the predominant source of IL-17 production; its expansion correlates with serum IL-17 levels during disease progression.

The liver is highly enriched in unconventional lymphocytes; γδ T cells comprise 5% of the total compartment (134). The functions Intrahepatic IL-17+ γδ T cells have dichotomous roles in modulating liver injury depending on the acute vs. chronic settings. Intrahepatic IL-17+ γδ T cells can be protective in acute injury settings such as Con-A induced hepatitis (152) and experimental hepatectomy (141), while this population is implicated in perpetuating disease pathogenesis in chronic models such as high-fat diet (39) and biliary atresia (156, 157). In our model, we observed an attenuation of fibrosis in anti-γδTCR-treated *Mdr2*$^{-/-}$ animals with a substantial reduction in serum IL-17A levels. Regardless of the specific role IL-17+ γδ T cells play in each etiology of liver disease, imbalances within the intrahepatic γδ T cell compartment can influence disease progression and outcome. The same principle is illustrated during cholestatic liver fibrosis in the *Mdr2*$^{-/-}$ model**,** and make the γδ T cell compartment an attractive therapeutic target for liver diseases.

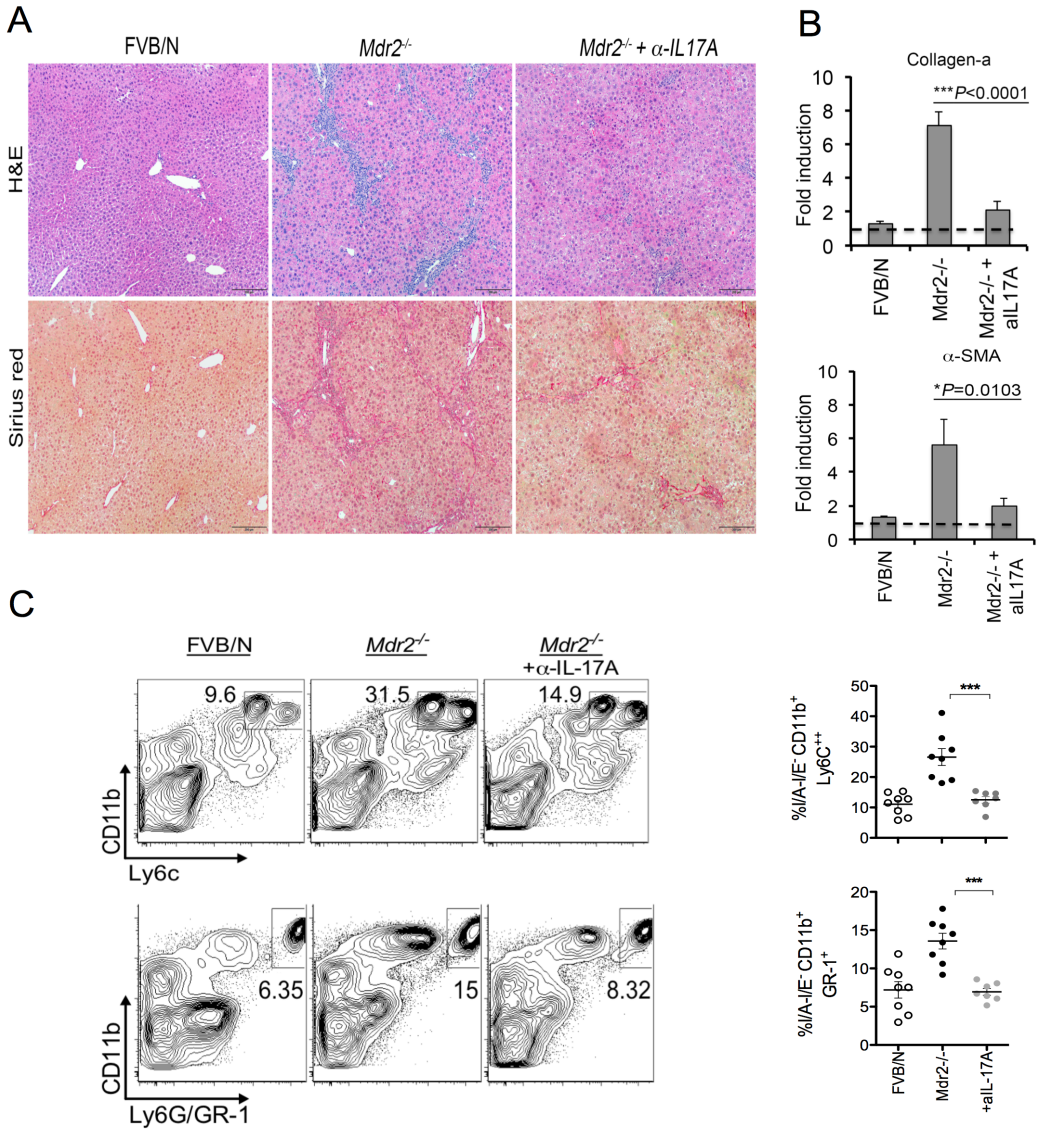
During liver disease, however, IL-17 responses in the liver can perpetuate destruction of biliary networks and hepatic architecture via hyper sensitization of HSCs to profibrotic mediators (140). This highlights the importance of appropriate anatomic distribution of microbes. In our model, an expansion of microbe-responsive γδ T cells corresponded with increased serum IL-17A, intestinal permeability, and disease progression. While these γδ T cells bear the invariant Vγ6 γδTCR and are capable of IL-17 mediated responses to microbes(138, 139, 142), microbial species alone may not be sufficient for increased composition as subset has been shown to expand in response to inflammation (140, 270). Therefore, the appropriateness of intestinal microbial genera that are good for

the gut could be bad for the liver. Furthermore, commensal bacteria play a crucial role in shaping the immune responses in healthy mice (271). Germ-free (GF) mice which lack commensal microflora have a range of immune defects that include ineffective regulatory T cells, and increased Th2-CD4+ T cell differentiation (271). In combination, these features of the GF mouse T cell compartment would favor perpetuation of liver damage (218) and may account for the exacerbation of liver injury reported by Tabiban and colleagues (272). In addition, clinical reports suggest treating PSC patients by Metronidazole therapy, which targets anaerobic microbes such as *Lactobacillus* (273)transiently corrects plasma levels of liver enzymes (23). This strategy was not curative because the majority of study participants still required a liver transplant (274). Additionally, there is evidence that suggests GF *Mdr2*$^{-/-}$ mice exhibit exacerbated symptoms of cholestasis and liver disease (272). These studies suggest that barrier loss alone and microbial translocation does not cause liver disease associated with genetic insufficiencies in the *MDR3/Mdr2* biliary transport protein.

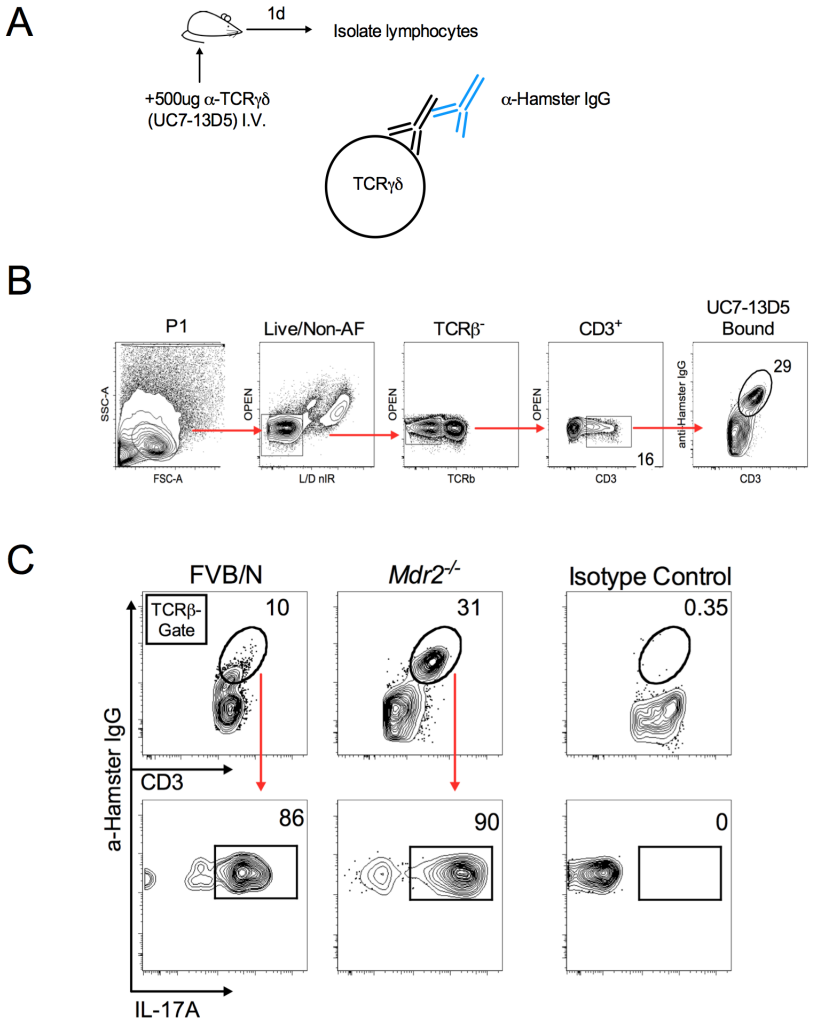
Although IL-17+ γδ T cells are capable of expanding in response to inflammation (140, 142, 143, 145, 146), there is a growing body of work that demonstrates this population is particularly important for TCR-mediated recognition of bacterial pathogens invading host tissues (138, 139, 142, 143). Our data suggest fibrotic livers have an expanded blockade, and therefore may inhibit TCR dependent recognition of translocated microbes that reside in the fibrotic liver. WT mice administered with *Lactobacillus* corroborate this finding, as acute hepatitis is attenuated when anti-γδTCR is administered prior to inoculation. This in conjunction with the therapeutic effect of anti-γδTCR antibody administration in the

Mdr2$^{-/-}$ mice supports this notion. Taken together, our findings hold considerable promise for γδ T cells as the therapeutic target in cholestatic liver diseases.

VI. Supplemental Data



Supplementary Fig. S3-1. Neutralization of IL-17A attenuates hepatic inflammation in *Mdr2*$^{-/-}$ mice. *Mdr2*$^{-/-}$ mice were administered 250ug anti-IL-17A I.P. two times per wk from 8 wks of age until the experimental endpoint, 25 wks of age. **(A).** Histological analysis of representative liver samples for H&E, and Sirius red staining (Mag. 10x) are shown. **(B).** Fibrosis gene expression in total liver tissues by RT-PCR analysis are shown. (**C**). Frequency of infiltrating intrahepatic inflammatory myeloid populations; monocytes (**top**, gated on Live, non-autofluorescent (Non-AF), Lineage (CD3/CD19) negative, MHC class II negative, CD11b+, Ly6C+), and neutrophils (**bottom**, gated on Live, Non-autofluorescent (Non-AF), Lineage (CD3/CD19) negative, MHC class II negative, CD11b+, GR-1+ (Ly6G)), **(C)**. Data are representative of 2 independent experiments and statistical significance was determined by a two-tailed Mann-Whitney Test, *p<0.05, **p<0.005, ***p<0.0001.



Supplementary Fig. S3-2. In vivo administration of anti-γδTCR (clone UC7-13D5) preferentially targets IL-17 producing subsets. Animals were administered 500ug of anti-γδTCR (Clone UC7-13D5; Hamster IgG) or appropriate Hamster-IgG isotype control intravenously and sacrificed one day following treatment (**A**). To detect *in vivo* antibody targets, lymphocytes were isolated from livers and stained first with anti-Hamster IgG conjugated to AlexaFluor 647 (Clone Poly4055) (**A**). Excess antibody was washed off, and lymphocytes were incubated with appropriate surface antibodies against TCRβ and CD3. To identify positive staining, cells were gated on live non-autofluorescent TCRβ-, CD3+ population (**B**). To determine IL-17 production capability of this subset, bulk lymphocytes were stimulated with 50ng/mL PMA plus 1ug/mL ionomycin in the presence of Golgi Plug and Golgi Stop for 4 hours at 37°C. This population was gated first on TCRβ- CD3+ a-Hamster IgG+ and then IL-17A production was assessed (**C**). Data are representative of 3 independent experiments, with 3-5 mice per group. Gates were determined using proper isotype and/or fluorescent minus one controls.

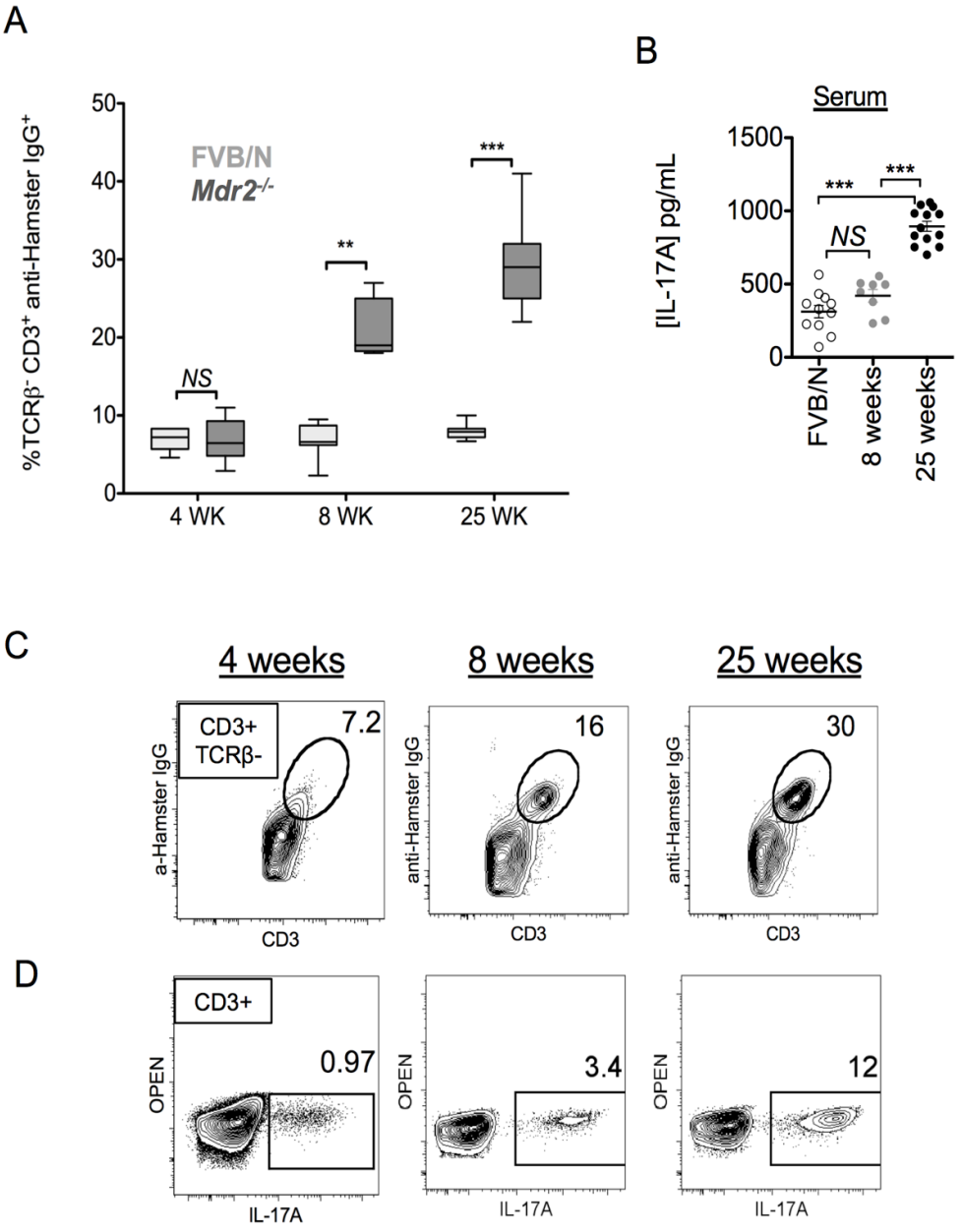


Figure S3-3 Accumulation of intrahepatic in vivo bound anti-TCR + corresponds with increased serum IL-17A levels in *Mdr2*$^{-/-}$ mice.

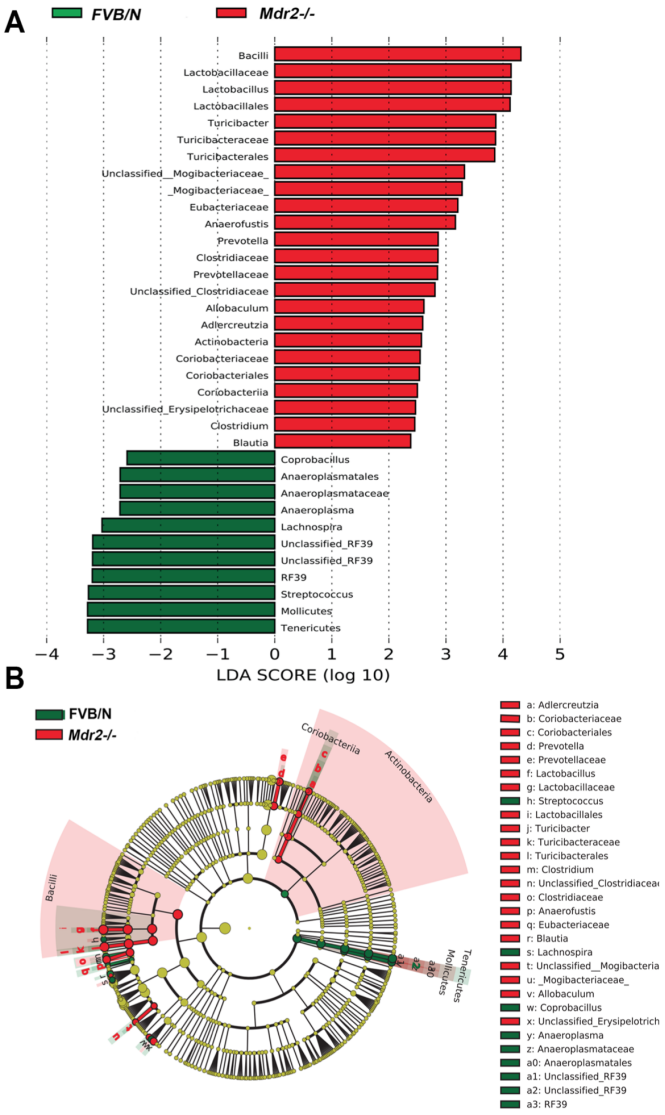


Figure S3-4. Gut microbial composition is altered in 8-wk-old Mdr2$^{-/-}$ mice. (A) Linear discriminant analysis size effect (LEfSe) predictions for bacterial families found in fecal specimens of 8-wk-old *Mdr2*$^{-/-}$ (red) and FVB/N (green) mice are shown. Linear discriminant analysis (LDA) score represents log changes in bacterial families shown. (B) The Cladogram representing the phylogenetic relationship between bacterial families (red = *Mdr2*$^{-/-}$ and green = FVB/N) is shown. *Lactobacillus, Bacilli, Turicibacter, and Prevotella* represent the highly enriched bacterial families in *Mdr2*$^{-/-}$ mice.

These data were obtained as a collaborative effort between Manoj Thapa and the Mohamadzadeh lab

A

Identification of culture isolates (MALDI-TOF MS; bioMerieux, Durham, NC, USA)

Isolates	MALDI-TOF organism identification	Confidence value	Comments
Lactobacillus	*Lactobacillus acidophilus* *Lactobacillus gasseri*	50% 50%	This identification is from the non-clinically validated database and we report as *Lactobacillus sp.*
Ralstonia picketti 1	*Ralstonia picketti*	99.9%	

B

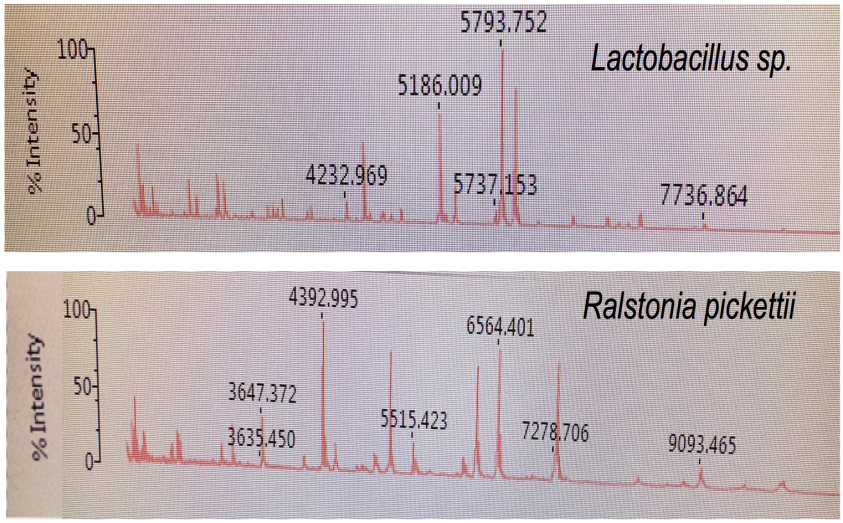


Figure S3-5. Identification of culture isolates. (**A**). The isolates were analyzed using Vitek MS matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS). Identifications were generated using IVD Knowledge Base Version 2.0 with the confidence scores as presented in the Table. (B). Representative Vitek MS profiles of bacterial colonies isolated from the liver homogenates of *Mdr2*$^{-/-}$ and FVB/N mice are shown.

These data were obtained in collaboration with Chui-Yoke Chin and Eileen Burd

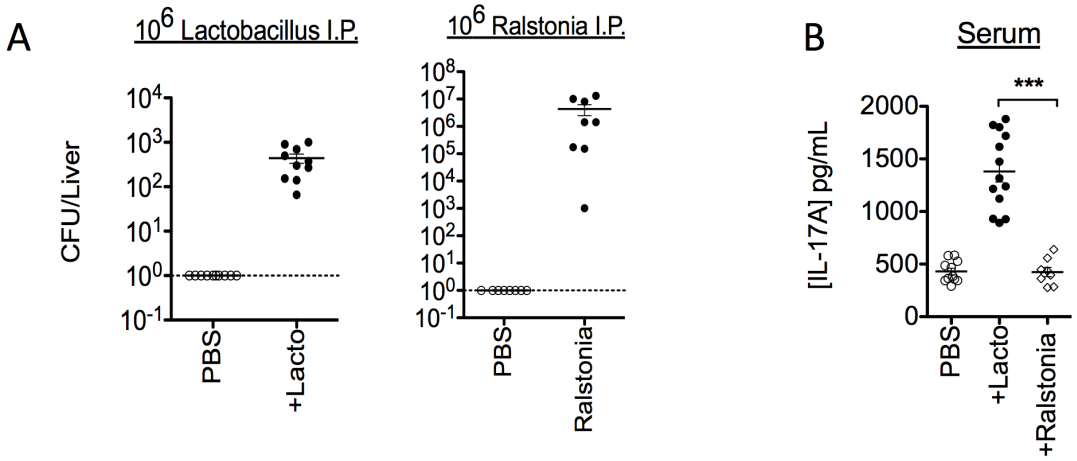


Figure S3-6. Increased IL-17A in serum in FVB/N mice inoculated with *Lactobacillus*. FVB/N mice were inoculated I.P. with 10^6 of either *Mdr2-/-* liver-derived *Lactobacillus* or *Ralstonia* another microbe sometimes found in the same intestinal compartment as *Lactobacillus*. Standard bacterial culture of liver homogenates indicated hepatic monocolonization of the indicated microbe at the time of animal sacrifice **(A)**. Despite hepatic bacteremia, only *Lactobacillus* inoculated animals demonstrated a marked spike in serum IL-17 **(B)**. All data are representative of at least 2 independent experiments, n=3-5/group. Statistical significance was calculated using a two-tailed Mann-Whitney Test ($p<0.0001$***)

Panel A was generated by Chui-Yoke Chin from samples obtained by Dana Tedesco

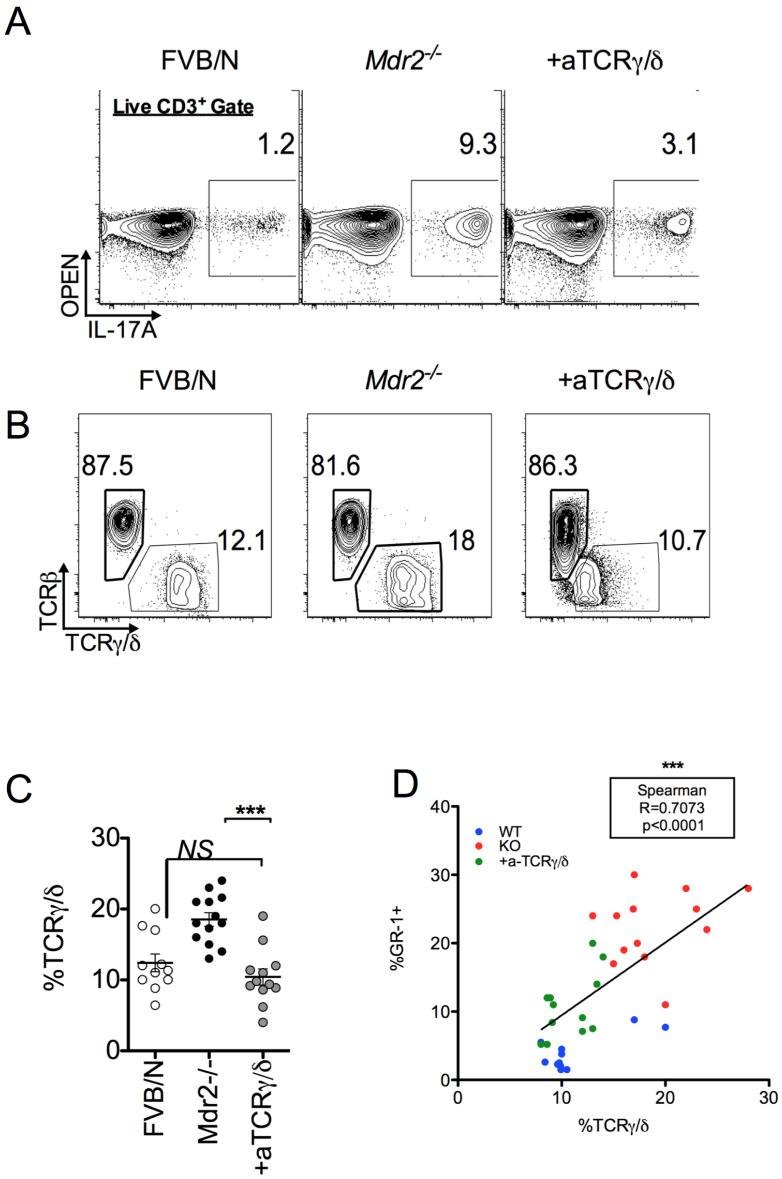


Figure S3-7. Blockade of TCR results in reduced of intrahepatic expansion of IL-17+ population and corresponds to reduced neutrophilic infiltration. Following treatment with anti-TCR, 25-week-old *Mdr2-/-* mice were sacrificed and intrahepatic lymphocytes were isolated. **(A)** Anti-TCR treated animals exhibited reduction in T cell-derived (CD3+) IL-17 following a 4h PMA/Ionomycin stimulation. **(B)** Anti-TCR reduced -T cell expansion in (Gated on Live, Non-AF, CD3+ compartment) indicated by reduced frequency of Live, Non-AF, CD3+, TCR+ **(C;** two-tailed Mann-Whitney Test, p<0.0001***, *NS*= non-significant**).** Intrahepatic neutrophil infiltration (Live, Non-AF, CD3/19-, MHCII-, CD11b+ GR-1+) correlates with intrahepatic frequency of -T cells (**D;** spearman correlation, two-tailed, p<0.0001***).** Data are representative of 3 independent experiments, n=3-5 mice per indicated group.

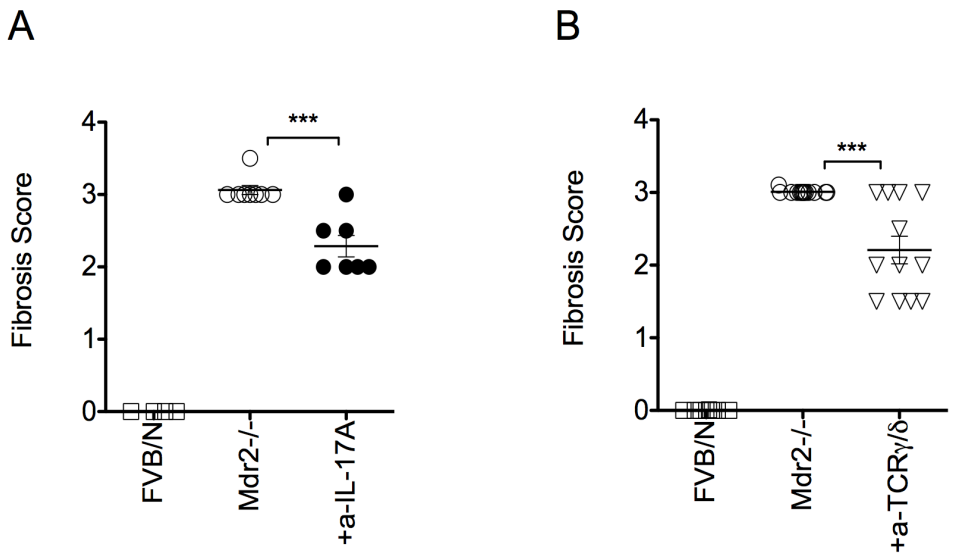


Figure S3-8. Fibrosis scores in control *Mdr2*$^{-/-}$ vs. anti-IL17A treated *Mdr2*$^{-/-}$ mice (25 weeks-old) (A) and *Mdr2*$^{-/-}$ vs. anti-TCR treated *Mdr2*$^{-/-}$ mice (25-weeks-old) (B). Statistical analysis were performed by using two-tailed Mann-Whitney test, *p<0.05, **p<0.005, ***p<0.0001. Error bars reflect the standard error of mean (SEM).

These data were obtained by Dr. Sanjeev Gumber, a senior pathologist, performing the analysis on samples from Dana Tedesco.

Ishak Stage, Categorical description	Score
No fibrosis	0
Expansion of some portal areas with or without short fibrous septa	1
Expansion of most portal areas with or without short fibrous septa	2
Expansion of most portal areas with occasional portal to portal bridging	3
Expansion of portal areas with marked bridging (portal-portal and/or portal-central)	4
Marked bridging with occasional nodules (incomplete cirrhosis)	5
Cirrhosis, probable or definitive	6

Adapted from: Ishak, et al. J Hepatol 1995;22:696-9

Animal ID	H&E	Sirius Red Score
1 FVB/N	NSF	0
2 FVB/N	NSF	0
3 FVB/N	NSF	0
4 FVB/N	NSF	0
5 FVB/N	NSF	0
1 *Mdr2 -/-*	Moderate to marked Periportal fibrosis with ocassional portal to portal bridging and expansion of portal areas with mild to moderate mononuclear infiltrates	3.5
2 *Mdr2-/-*	Moderate Periportal fibrosis with ocassional portal to portal bridging and expansion of portal areas with moderate mononuclear infiltrates	3
3 *Mdr2-/-*	Moderate Periportal fibrosis with ocassional portal to portal bridging and expansion of portal areas with moderate mononuclear infiltrates	3
4 *Mdr2-/-*	Moderate Periportal fibrosis with ocassional portal to portal bridging and expansion of portal areas with moderate mononuclear infiltrates	3
5 *Mdr2-/-*	Moderate Periportal fibrosis with ocassional portal to portal bridging and expansion of portal areas with moderate mononuclear infiltrates	3
1 *Mdr2-/-* + aTCRgd	Expansion of some to most portal areas with or without short fibrous septa intermixed with small numbers of mononuclear infiltrates	1.5
2 *Mdr2-/-* + aTCRgd	Expansion of some to most portal areas with or without short fibrous septa intermixed with small numbers of mononuclear infiltrates	1.5
3 *Mdr2-/-* + aTCRgd	Mild to Moderate Periportal fibrosis with ocassional portal to portal bridging and expansion of portal areas with moderate mononuclear infiltrates	2.5
4 *Mdr2-/-* + aTCRgd	Expansion of most portal areas with or without short fibrous septa intermixed with small numbers of mononuclear infiltrates	2
5 *Mdr2-/-* + aTCRgd	Moderate Periportal fibrosis with ocassional portal to portal bridging and expansion of portal areas with moderate mononuclear infiltrates	3
1 *Mdr2-/-* + a-IL17A	Mild to Moderate Periportal fibrosis with ocassional portal to portal bridging and expansion of portal areas with moderate mononuclear infiltrates	2.5
2 *Mdr2-/-* + a-IL17A	Moderate Periportal fibrosis with ocassional portal to portal bridging and expansion of portal areas with moderate mononuclear infiltrates	3
3 *Mdr2-/-* + a-IL17A	Mild to Moderate Periportal fibrosis with ocassional portal to portal bridging and expansion of portal areas with moderate mononuclear infiltrates	2.5
4 *Mdr2-/-* + a-IL17A	Expansion of most portal areas with or without short fibrous septa intermixed with small numbers of mononuclear infiltrates	2
5 *Mdr2-/-* + a-IL17A	Expansion of most portal areas with or without short fibrous septa intermixed with small numbers of mononuclear infiltrates	2

Table S3-1. Fibrosis scoring criteria and representative findings

Chapter 4

Discussion

The liver critically participates in a multitude of metabolic and immunologic events. As a result, it is constitutively bombarded with a variety of antigens and other inflammatory stimuli. At steady state, however, these antigens do not elicit an appreciable immune response. This is thought to be the result of the liver's tolerogenic nature and intrinsic ability to heal itself upon noxious stimuli. Once the insult is cleared, this fibrosis, liver wound healing, reverses as healthy tissue replaces scar tissue. Persistent insult, regardless of the specific etiologic agent, highlights the immunologic consequences of a perturbed liver microenvironment. The studies presented in this dissertation demonstrate that liver fibrosis has profound influence on intrahepatic T cell functions.

I. When a "Treg" is not really a "Treg": intrahepatic environmental cues can tip the balance between tolerance and immune responses

The liver is not the only organ affected by chronic fibrosis. Extrahepatic symptoms of disease include Ig-mediated autoimmune disorders, as well as skin, intestinal and even neurologic disorders (275); all of which co-exist with an increased instance of CD4+Foxp3+ "regulatory" T cells and ineffective CD8+ T cell responses (48). At steady state, these sequelae are non-existent due to a variety of mechanisms in place to maintain the tolerogenic nature of the liver and a threshold to manage minor insults. Persistent injury-related imbalances in pro- and anti-inflammatory signals can overcome the tolerogenic nature of the liver (276).

Although it remains unclear if the liver is enriched in autoreactive B cell populations, the preferential trafficking of activated and memory T cell populations (112) adds plausibility to this scenario. Our own studies using human patient samples indicate that

intrahepatic B cells are exclusive producers of constitutive IgG; patient matched PBMC do not exhibit this phenomenon (**Fig. 2-8).** In line with this observation, only intrahepatic B cell-derived IgG exhibited detectable ANA-IgG (**Fig 2-8,** and data not shown). Interestingly, preliminary co-cultures of fibrotic mouse-liver CD4+Foxp3+ T cells with splenic B cells promoted IgG production **(Fig 2-2)**, although it was not ANA reactive (Appendix 3 Figure A3-1). An important consideration regarding our current work presented in **Chapter 2** is that we do not know, nor is it entirely parsed out in the field, if these activated intrahepatic B cells are naïve and a product of *de novo* activation or if these B cells were initially hypo responsive and then driven into activation due to loss of tolerance. Additionally, it is unclear what antigen, if any, these B cells are specifically responding to. Both these areas are active avenues of investigation in ours and other labs. From our own studies, the data indicate that intrahepatic environmental cues alter CD4+Foxp3+ T cells to permit aberrant IgG production; this break in B cell-mediated tolerance is dependent on CD4+ T cell help (**Chapter 2)**.

In the context of liver disease, fibrosis-elicited Foxp3-expressing CD4+ T cells both suppress/ "regulate" CD8+ T cell responses while promoting extrahepatic symptoms of disease. As such, Treg transfer therapies that have shown encouraging outcomes in experimental models (5, 39, 40, 51) as well as clinical settings (277) may not be suited to treat liver diseases (80). In a sense, the unique functions and local milieu of the liver could be considered a "privileged site"—there are no other anatomic compartments that remotely function like the liver. Conservation of T cell abnormalities across various etiologies of hepatic diseases suggest that chronic fibrosis, not the cause of disease,

drives alterations in CD4+ T cell help. Thus, if tightly regulated mechanisms of liver wound healing go awry due to prolonged injury, immunologic consequences ensue. The liver specific nature immune dysregulation in both human and mouse models of disease studied here (48) brings forward a key caveat of studying liver disease: events that occur in the liver may not be accurately represented in peripheral tissues. Therefore, it is prudent that investigators err on the side of caution when drawing conclusions about liver disease in studies where only peripheral blood was available. Fortunately, the advent of mouse models available to address various means of hepatic injury has improved our understanding of liver diseases. In an ideal world, both mouse models and human liver samples are available to investigators and better inform model choices.

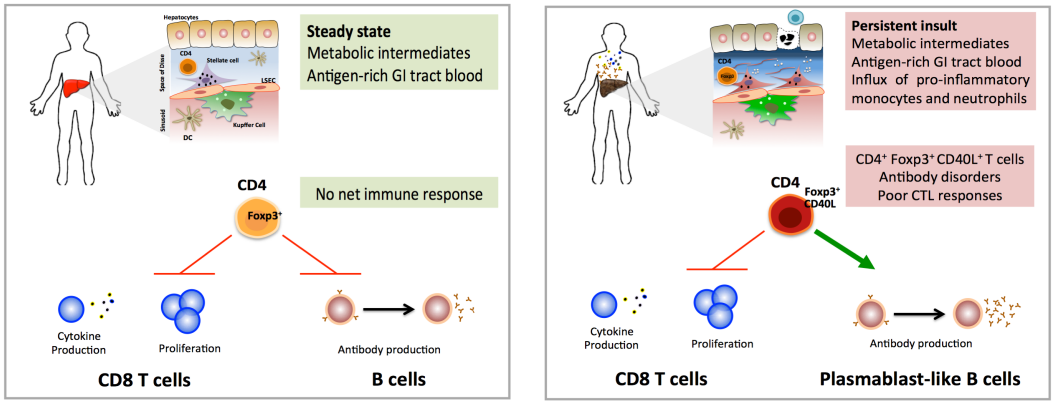


Figure 4-1. The fibrotic liver microenvironment promotes aberrant CD4+ T cell functions. The liver has the unique ability to repair itself upon injury. This serves the purpose of liver wound repair as well as maintenance of metabolic functions without an appreciable immune response. This is due, in part, to the interactions of intrahepatic CD4+ T cells and liver-resident APCs. Steady-state CD4+ Foxp3+ T cells are induced by HSC derived *all-trans* retinoic acid, or by recognition of cognate antigen presented by intrahepatic APCs. The resulting "regulatory" T cells suppress immune mediated injury by CD8+ T cells and B cell functions (**left**). If liver insult is not adequately resolved, the fibrotic liver microenvironment promotes a population of CD4+ Foxp3+ T cells that retain the ability to suppress CD8+ T cells. A sub-population stimulates B cells through CD40L expression. This seems to critically participate to the extra-hepatic antibody mediated disorders and poor CTL responses associated with chronic liver diseases (**right**).

II. Gut-Liver Axis is disrupted during liver diseases; implications on pathologic outcomes

Liver diseases highlight the critical role maintenance of the liver microenvironment plays in peripheral tolerance and immune homeostasis. The liver is constantly bathed in microbial products as a result of sharing portal venous blood flow with the gut (13, 278). Mechanisms implicated in management of these physiologic levels of LPS through KC production of IL-6 and TNFα (4). Although counter-intuitive, the inflammatory effects are counteracted by high levels of IL-10 and IL-4, a profibrotic cytokine, production (96, 121). *In vitro* studies indicate continuous low levels of microbial exposure over time results in a tolerogenic effect characterized by IL-10 responses, which gradually inhibit IL-6 and TNFa production (71, 95). This mechanism is sensitive to high concentrations of endotoxin, such as a bacterial infection, and favors IL-6/TNFa production in order to mount a proper immune response to pathogen(95). During liver fibrosis, imbalances in pro-inflammatory cytokines, such as IL-6 and TNFα affect anatomic locations other than the liver. Inflammatory mediators such as TNF-α can promote disruption of intestinal and many other epithelial barriers (267) as well as promote hepatic fibrosis (279). Disruption of intestinal barriers is associated with various etiologies of chronic liver diseases (200, 249, 263, 265-267). In the absence of any underlying bacterial infection, liver diseases are associated with a marked state of intestinal dysbiosis (13, 200). Together the gastrointestinal side effects link intrahepatic disturbances with compromised intestinal integrity.

Regarding our own studies in the *Mdr2-/-* mouse model, choleostatic liver diseases are the outcome of ineffective bile flow that leads to accumulation of toxic bile acids (BAs). BAs then play a role in oxidative stress, hepatocyte death, inflammation and suppression of Kupffer cell (KC) phagocytic functions (268). The effects of BAs on KC functions contribute to the both ineffective clearance of fibrotic tissues (269) and permission of intrahepatic microbial accumulation (268). In our model, translocated microbes, particularly lactobacillus, perpetuate inflammation via activation of intrahepatic Vγ6-bearing γδ- T cells that respond by producing IL-17A **(Chapter 3).** These findings in the *Mdr2-/-* model suggest that intrahepatic events like reflux of BAs can select for specific commensal micro flora and thereby promote disease-associated dysbiosis (**Fig. 3-4)**.

Microbial translocation associated with chronic inflammatory disorders shows the essential function of appropriate anatomic segregation in immunologic homeostasis. Steady state IL-17 responses in the proper context contribute to barrier maintenance (280). In the intestinal tract, commensal microbes drive IL-17 production from lymphocytes (203, 280, 281). Like hepatic wound healing, intestinal responses require tight regulation-imbalances in IL-17 are associated with pathologic outcomes of bowel disorders, and compromise intestinal integrity. Disturbance of barrier functions highlight the deleterious outcomes of inappropriate IL-17 responses to commensals, such as those that translocated into the liver in the *Mdr2-/-* mouse model (**Figs. 3-5, 3-6, and 3-7).** This is in-line with patients reports of recurrent episodes of bacterial peritonitis during chronic liver diseases(17). In mouse models, IL-17+ γδ T cells contribute to mucosal barrier immunity against bacterial pathogens (281). Despite the beneficial effects of enriched IL-17+ γδ T

cells reported at steady state in other anatomic sites (138, 139, 142, 143), our data suggests that intrahepatic overabundance of this population contributes to the pathology of chronic liver diseases.

Surprisingly, the reported functions of intrahepatic γδ- T cell derived IL-17A are contradictory. On one hand, Intrahepatic IL-17+ γδ T cells can be protective in acute injury settings such as Con-A induced hepatitis (152) and experimental hepatectomy (141). On the other hand, this population can also contribute to disease pathogenesis in chronic models such as high-fat diet (39) and biliary atresia (156-158). Overall, the paradoxical outcomes of these studies suggest that duration and persistence of injury dictate the therapeutic versus pathogenic effect of IL-17A. This warrants closer investigation prior to implementing IL-17A inhibitors in the clinic.

III. The liver does not exist in a vacuum

The systemic consequences of liver diseases, suggest a greater role for the liver in modulating peripheral homeostasis as well as an interconnection of the liver and other anatomic compartments. One compelling argument is that entire body's blood supply passes through the liver around 360 times per day (32)—the liver sequesters antigen rich intestinal blood components from circulation (13). Therefore, it is plausible that the liver can be influenced by inflammation in other peripheral compartments, such as the gastro-intestinal tract and vice versa.

Aside from the association of liver diseases with GI disorders (24), recent work has linked liver inflammation to a variety of psychiatric (282, 283), neurologic (284-286), and even respiratory (287) abnormalities. Some groups suggest that perturbations in the GI tract may be the real driving force behind these disorders (288). However, examination of clinical reports of hepatic encephalopathy (HE), a neurologic disorder occurring in up to 70% of cirrhotic patients (199, 289, 290), suggest otherwise. Acute episodes of HE in cirrhotic patients can be treated with antibiotics such as Rifaximin (290) or Metronadizole (291). Although liver disease is not reversed, transient improvement in liver enzymes and cognition supports a relationship between liver functions and neurologic functions (13, 16, 199, 292). While GI tract disturbances and dysbiosis may contribute to pathologic outcomes of liver disease, these clinical observations propose that events in the liver may dictate the scope of the GI tract's influence on systemic responses. This is an active and exciting avenue and more likely these pathway connections will be elucidated soon as the field expands.

Conversely, non-hepatotropic events such respiratory viral infections carry the risk of acute hepatitis, and in the most severe cases, liver failure (101, 103, 128-131). A variety of mechanisms may account for non-hepatotropic viral infection-related liver failure. For one, the liver facilitates acute phase responses (110). Thus, the magnitude of the acute phase response could potentially facilitate immune-mediated injury(9, 12, 19, 39, 43, 45-47, 100). Alternatively, the liver's role in corralling peripherally activated T cells may invite more damage (293). At this point in time, there seem to be more questions than answers about the effects of marked peripheral inflammation or acute viral infection on liver homeostasis. However, understanding which peripheral signals can surpass the hepatic inflammatory threshold has potential to inform therapeutic strategies for sepsis and other infections associated with acute organ failure.

Overall, the studies presented in this dissertation demonstrate that fibrosis drastically alters the liver microenvironment. Independent of etiology, these environmental cues drive a population of CD4+ Foxp3+CD40L+ T cells that suppress CD8+ T cell responses and simultaneously drive extrahepatic sequelae of disease. If one only considers the liver, fibrosis-elicited CD4+Foxp3+ T cells fulfill their duty to limit CD8+ T cell-mediated immune injury. Systemic sequelae tell a very different story; events in the fibrotic liver affect the periphery. Digestive disturbances such as those observed in the *Mdr2-/-*model of liver injury indicate that metabolic insufficiencies and buildup of toxic BAs not only causes liver damage, but also actively participates in dysbiosis via selection of microbes. Collateral damage to the liver occurs as a result expansion of pathogenic intrahepatic IL-

17+ Vγ6+ T cell responses to translocated microbes. Together, these studies highlight the complex coordination of intra-hepatic and systemic processes that govern pathologic outcomes of liver disease. Therefore, the liver does not exist in a vacuum. Instead, the liver continues to maintain functional capacity while constantly inundated with antigens endemic to its microenvironment. While the liver manages a physiologic degree of inflammatory insult, surpassing this threshold results in deleterious immunologic and metabolic consequences. Therefore, disease-elicited T cell responses highlight an underappreciated yet critical role of the liver in immunologic harmony.

Appendices

Appendix 1.

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Appendix 2.

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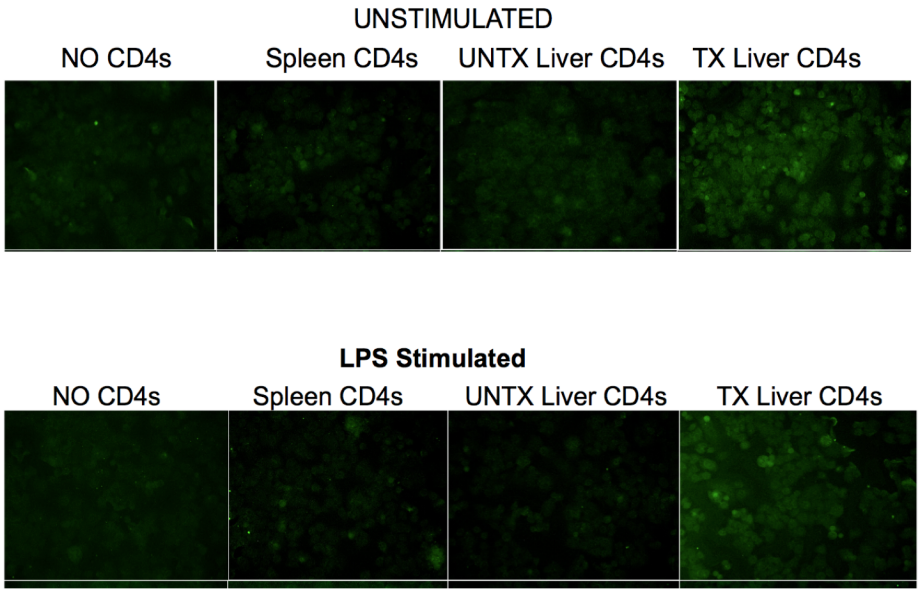

Figure A3-1. ANA-assay of naive splenic B cells co-cultured with the indicated total CD4+ T cell source. Un-diluted Supernatants from $2x10^5$ Naive Splenic B cells co-culturd with $1x10^5$ total CD4+ T cells in the presence or absence of 2ug/mL LPS for five days. IgG production was confirmed in these cultures via ELISPOT **(Supplemental Fig S2-4)**. The IgG detected in these co-cultures were not positive of ANA-titers