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Defining cellular and molecular mediators of liver fibrosis

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Abstract

Liver fibrosis, or liver scarring, is a consequence of dysregulated wound healing which leads to excessive extracellular matrix (ECM) accumulation. Fibrosis is the hallmark of chronic liver disease (CLD), and its progression can eventually result in cirrhosis and the development of hepatocellular carcinoma (HCC). CLD is associated with chronic inflammation which is orchestrated by a network of proinflammatory cytokines/chemokines, inflammatory infiltrates, and tissue resident cells. Recent evidence suggests that fibrosis is a reversible process that can be promoted through attenuation of the initiating insult or dissolution of the accumulated ECM itself. Currently, there are no effective anti-fibrotic treatments available. Thus, there remains a clear need for an increased understanding of the factors which promote fibrosis to allow for the strategic development of novel therapeutics to limit the impact of fibrotic disease.

There are various pre-clinical models available to study the pro-inflammatory cellular and molecular mediators that contribute to liver fibrosis. Long-term oral administration of the hepatotoxin thioacetamide (TAA) provides a model in which fibrosis is induced in two phases. An early phase of fibrosis occurs following 1-6-week TAA administration and is characterized by inflammation associated with pericentral monocyte accumulation, HSC activation, and collagen deposition. Following 6-12-week TAA administration, disease is associated with exponentially increased collagen deposition and an expanding ductular reaction (DR) in association with an increase in DR/macrophage interactions.

The Interleukin-17 (IL-17) cytokine family has established roles in multiple diseases which are characterized by fibrosis. Of all IL-17 family cytokines IL-17A, IL-17E, and IL-17F are most well studied. Previous studies have identified a role for IL-17A in the development of liver fibrosis in both preclinical models and human disease. In this regard, IL-17A can both directly activate hepatic stellate cells (HSCs) to promote collagen production, and indirectly promote their activation through evoking proinflammatory cytokine expression by multiple cell types. Although elevated IL-17F expression has been found to be associated with liver fibrosis, whether this

cytokine represents a mechanistic mediator remains to be elucidated. The role of IL-17E during liver fibrosis is controversial with reports of both pathogenic and regulatory functions. Hence, We investigated the relative, temporal and mechanistic contribution of the IL-17A, IL-17F, and IL-17E cytokines to the initiation and perpetuation of liver fibrosis using the TAA model**.** In In vivo experiments using mice deficient in these cytokines or their receptors we investigated the contribution of each signaling pathway to the two phases of TAA-induced fibrosis.

In Chapter 3, we identified a critical role of IL-17RA, the receptor subunit required for IL-17A, IL-17F and IL-17E signaling, in both the early and late phases of TAA-induced liver fibrosis. In contrast, no pathogenic role for IL-17F was observed. We demonstrate that either IL-17A or IL-17E signaling was sufficient to induce the early inflammatory phase (1-6 weeks), however, both cytokines were required for the development of a mature ductular reaction and progression to cirrhosis. Thus, we demonstrate the redundant nature of these cytokines in the inflammatory phase of disease, but their synergistic roles in the later progressive phase (6-12 weeks) of fibrosis. Importantly, these data highlight the potential of IL-17RA blockade, rather than IL-17A or IL-17E individually, as an optimal therapeutic strategy for the treatment of liver fibrosis.

In mechanistic studies, we demonstrated that IL-17A production was increased following 1-week TAA and identified $CCR2^{+}\gamma\delta$ T cells as the primary IL-17A producing liver leukocyte population. We observed IL-17A signaling was found to facilitate the expression IL-6 and TNF in the liver, both of which are established mediators of fibrosis. Furthermore, we demonstrate that both IL-17A and IL-17E function to promote monocyte recruitment in the early phase. Although IL-17A deficiency did not alter the absolute number of infiltrating monocytes, IL-17A signalling was required for the sequestration of monocytes around the central vein (CV). In contrast, in the absence of IL-17E, monocyte infiltration into the liver was markedly attenuated. We demonstrate that after 1 week of TAA treatment, IL-17E signalling, specifically within the non-haematopoietic compartment, significantly increased liver CCL2 levels. Furthermore, we demonstrate that, CCL2 produced from macrophages and eosinophils, but not monocytes was IL-17E dependant. Moreover, the administration of IL-17E to naïve mice induced elevated circulating

and liver CCL2 levels, promoted monocytic infiltration into the liver which was associated with HSC activation and sinusoidal fibrosis. In addition to monocytes, we confirmed CCL2 also promoted the infiltration of $CCR2^{+}IL17A^{+}TNF^{+}\gamma\delta$ T cells into the liver. Thus, IL-17E through CCL2 production promotes the recruitment of pathogenic monocytes and IL-17+γδ T cell infiltration into the liver, whereas, IL-17A signaling contributes to the sequestration of these cells at the central vein, the site of injury initiation in the TAA model. These studies highlight distinct but synergistic roles for IL-17A and IL-17E in driving the inflammatory responses which underpin fibrogenesis.

B cells play a pathogenic role in autoimmune diseases through multiple mechanisms. The mechanisms include secretion of autoantibodies, secretion of inflammatory cytokines and the promotion T cell responses through antigen presentation. Although the contribution of B cells in liver fibrosis is poorly studied, the limited literature suggests innate B cell activation and antibody production as pathogenic mechanisms in liver fibrosis. However, to date, a pathological role for B cells in the TAA liver fibrosis model is undescribed. Here we demonstrated a critical role for B cells in this model. Mechanistically we demonstrated increased B cell activation (CD44 and CD86) associated with elevated TNF production in livers from TAA treated mice when compared to that of naïve mice. Moreover, serum IgG levels were increased in response to TAA treatment, the pathogenicity of which was confirmed using B cell deficient ($\mu MT^{-/-}$) mice. IgG deposition in the livers of TAA treated mice was noted and shown to be localized to the scar regions within the liver parenchyma where macrophages sequester. IgG ligation of FcR on macrophages has previously been shown to elicit pathogenic responses. Using FcγR deficient mice we demonstrate, that while IgG production in response to TAA remained unchanged, the development of fibrosis was significantly attenuated. Thus, B cells contribute to TAA-induced liver fibrosis through B cell activation and cytokine production, IgG deposition and binding to FcγR.

Rho-associated kinase 2 (ROCK2) is a serine–threonine kinase that plays a central role in the control of intracellular signaling cascades involved in the regulation of cytoskeletal reorganization and the secretion of proinflammatory cytokines. ROCK2 has an established role in regulating IL-17A and IL-21 production and macrophage

plasticity. Additionally, through the promotion of T follicular helper cell (TFh) differentiation and IL-21 production, ROCK2 drives germinal center B cell development and antibody production. Increased ROCK2 activity is associated with autoimmunity, and targeted ROCK2 inhibition was recently shown to attenuate pathology in the chronic GVHD, a complication of allogeneic stem cell transplantation which presents with autoimmune like symptoms. To date, the role of ROCK2 in liver fibrosis remains largely unexplored, although increased ROCK2 activity has been shown to correlate with disease severity in the bile duct ligation (BDL) model of liver fibrosis. Chapter 5 outlines our findings from our investigations of the mechanistic contribution of ROCK2 in prevention and treatment of TAA-induced liver fibrosis. We report that KD025, a selective ROCK2 inhibitor, both attenuates fibrogenesis and promotes fibrolysis in the TAA liver fibrosis model. Mechanistically, KD025 inhibited signal transducer and activator of transcription 3 (STAT3) and RAR-related orphan receptor gamma (RORγt) signaling pathways in the liver which in turn attenuated IL-17 production. Furthermore, in addition to pSTAT3, KDO25 inhibited B-cell lymphoma 6 (BCL6) expression, both of which are required for TFh differentiation and function. In line with this, splenic B cell germinal center formation was diminished, resulting in significantly reduced IgG production and deposition in the liver. Additionally, KD025 administration significantly decreased liver macrophage TNF production and altered macrophage distribution. As both IL-17A signalling and IgG FcγR ligation are know to drive inflammatory responses in macrophages, the attenuation of these pathways represents indirect mechanisms by which KD025 inhibits pathogenic macrophage programming. However, in in vitro studies we demonstrated KD025 significantly diminishes STAT3 phosphorylation in bone marrow derived macrophages (BMDM) in response to IL-17A demonstrating additional direct effects on macrophage differentiation. Additionally, the cofilin pathway, another ROCK2 target, was also perturbed by KD025 administration in vivo. Cofilin is a actin-binding protein which regulates cytoskeletal reorganization required for cytokine production, adhesion and migration. Again, using BMDM in vitro we demonstrated KD025 markedly diminished pCofilin levels, which was associated with reduced TNF production and altered MMP profiles. Taken together, we demonstrate the efficacy of ROCK2 inhibition in attenuating the development of TAA-induced liver fibrosis and promoting its regression through direct and indirect effects on macrophage function.

Overall, the data presented in this thesis identifies the synergistic contribution of IL-17A and IL-17E cytokines to liver fibrosis by promoting myeloid recruitment and pathogenic differentiation. Moreover, we identify a critical role for B cells in this model, where aberrant B cell activation results in pathogenic IgG production, tissue deposition, and FcγR ligation. Finally, we identified ROCK2 as a potential therapeutic target to inhibit pathogenic responses induced by different cellular (monocytes/macrophages and B cells) and molecular (STAT3, BCL6, IL-17A, Cofilin) mediators

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Contributions by others to the thesis

Kelli MacDonald and Andrew Clouston made significant contribution to this work, including assisting with experimental design and interpretation of results.

Conception and design of project Associate Prof Kelli MacDonald conceived of the studies, designed individual experiments and helped interpret data. Prof Andrew Clouston contributed to the design of experiments and helped interpret data.

Tissue processing and sectioning Routine histology techniques including tissue processing, paraffin-embedding, sectioning and staining (Sirius red and H&E staining) were performed by Ailsa Davis from the Envio specialist pathology.

Histology scoring Liver histology was graded by specialist pathologists Professor Andrew Clouston and Dr Greg Miller

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Assistance with experiments All experiments were performed with the help of Dr. Michelle Melino

Critical revision of the thesis Associate Prof Kelli MacDonald and Prof Andrew Clouston critically reviewed the thesis

Statement of parts of the thesis submitted to qualify for the award of another degree.

No works submitted towards another degree have been included in this thesis.

Research Involving Human or Animal Subjects

No human subjects were involved in this research. Animal research ethics approval was granted by the QIMR Berghofer Medical Research Institute animal ethics committee (A1503-603M P2096).

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Keywords

Liver fibrosis, thioacetamide, IL-17 signaling, macrophages, B cells, immunoglobulins, ROCK2, STAT3, BCL6, KDO25, regression.

AUSTRALIAN AND NEW ZEALAND STANDARD RESEARCH CLASSIFICATIONS (ANZSRC)

ANZSRC code: 110705 Humoural Immunology and Immunochemistry, 70%
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Fields of Research (FoR) Classification

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List of tables and figures

List of Abbreviations

A list of abbreviations used in this thesis is provided below.

Abbreviations	Definition
BDL	Bile duct ligation
CCL_4	Carbon tetrachloride
CLD	Chronic liver disease
ECM	Extracellular matrix
G-CSF	Granulocyte colony-stimulating factor
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HPC	Hepatic progenitor cells
HSC	Hepatic stellate cells
MAIT	Mucosal associated invariant T cells
MCP1	Monocyte chemoattractant protein 1
MMP	Matrix metalloproteinases
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
PCR	Polymerase chain reaction
TAA	Thioacetamide
TGF-β	Transforming growth factor β
TIMP	Tissue inhibitors of metalloproteinases
MCD	Methyl choline deficient
ConA	Concanavalin A
DMN	Dimethylnitrosamine
DEN	Diethylnitrosamine
ROS	Reactive oxygen species
HMGB1	High mobility group box 1
DAMPs	Damage associated molecular patterns
ATP	Adenosine triphosphate

PDGFRβ	Platelet-derived growth factor receptor beta
L-RAT	Lecithin retinol acyltransferase
GFAP	Glial fibrillary acidic protein
Asma	Alpha smooth muscle actin
PDGF	Platelet-derived growth factor
VEGF	Vascular endothelial growth factor
CTGF	Connective tissue growth factor
LPS	Lipopolysaccharide
TLR	Toll-like receptors
LSEC	Liver sinusoidal endothelial cells
DR	Ductular reaction
CSF-1	Colony-stimulating factor-1
TNF	Tumor necrosis factor alpha
IL	Interleukin
Tfh	T Follicular helper cells
Treg	T regulatory cells
lFNγ	Interferon-gamma
ROR	RAR-related orphan receptor
STAT3	Signal transducer and activator of transcription 3
nTreg	Natural T regulatory cells
iTerg	Induced T regulatory cells
FOXP3	Forkhead box P3
NK	Natural killer cells
MAIT	Mucosal-associated invariant T
TRAIL	TNF-related apoptosis-inducing ligand
NKT	Natural Killer T cells
ILC	Innate lymphoid cells
Cnk	Conventional natural killer cells
LTi	Lymphoid tissue inducer
Bcl6	B-cell lymphoma 6 protein
BAFF	B-cell activating factor
GVHD	Graft versus host disease
RA	Rheumatoid arthritis
SLE	Systematic lupus erythematosus

ICAM	Intercellular Adhesion Molecule 1
CD	Cluster of differentiation
Th	T helper
Tc	T conventional
PI3K	Phosphatidylinositol-3-kinases

Chapter 1: Review of the literature

1.1 CHRONIC LIVER DISEASE (CLD)

Chronic liver disease (CLD) represents a significant global health burden. CLD occurs in response to injury induced by multiple insults such as alcohol exposure, obesity, diabetes, viral infection, autoimmune, chronic inflammatory conditions and drug toxicity. Fibrosis is the hallmark of CLD, and its progression eventually results in cirrhosis and can lead to hepatocellular carcinoma (HCC) **(Figure. 1-1)** [1]. Though HCC is the sixth most common neoplasm worldwide, poor prognosis makes it the principal cause of cancer-related mortality, responsible for 6 million deaths

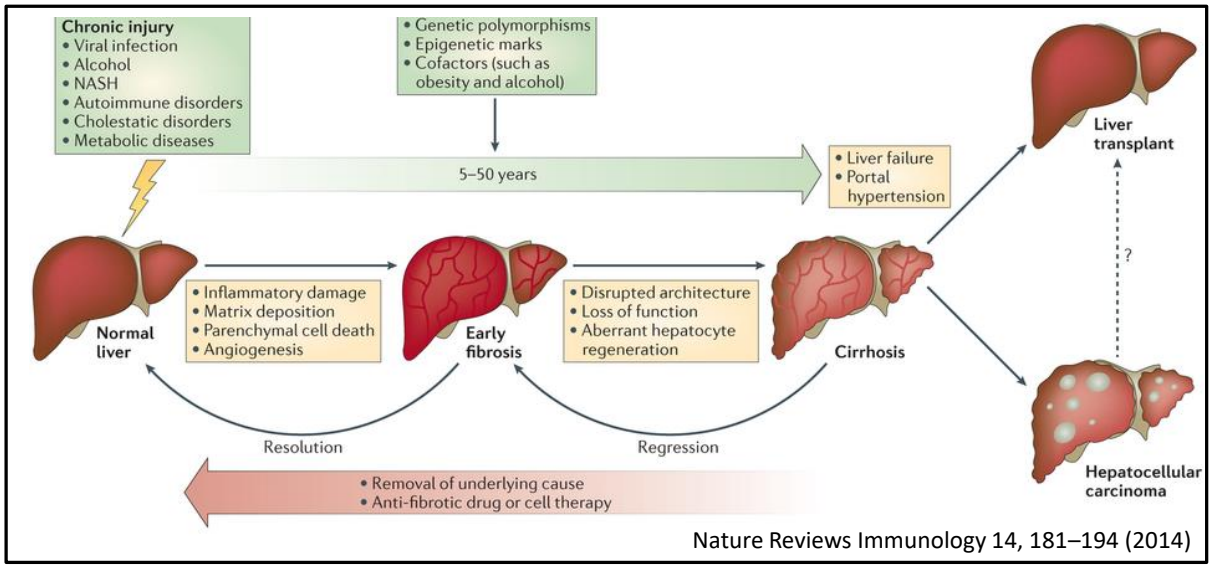


Figure1-1 Phases of liver injury during chronic liver disease: Chronic inflammation leading to fibrosis, cirrhosis and eventually hepatocellular carcinoma (HCC). Nature reviews immunology 14, 181-194 (2014)

annually [2-4]. In the developed world, CLD is a growing clinical burden due in part to an escalating obesity epidemic [2]. Importantly, it is now clear that fibrosis is a reversible process that can be promoted through attenuation of the initiating insult or fibrosis itself; however, there are currently no effective anti-fibrotic treatments available. Thus, there remains a clear need for an increased understanding of the factors which promote fibrosis to allow for the strategic development of novel therapeutics to limit the impact of the fibrotic disease.

1.2 PATHOGENIES OF LIVER FIBROSIS

Extracellular matrix and scar formation: In response to injury, the liver exhibits a unique capacity for repair whereby cellular components are regenerated, leaving no lasting evidence of damage. However, in the setting of chronic inflammation, this repair process becomes pathogenic due to dysregulation of the wound healing response, leading to significant alterations in the extracellular matrix (ECM). Multiple cellular and molecular mediators orchestrate the initiation and progression of liver fibrosis. In response to injury, inflammation rapidly ensues and is characterized by leukocyte infiltration into the liver. The interplay between the inflammatory infiltrates, and tissue-resident cell populations (e.g., epithelial cells, fibroblast, and macrophages) invokes fibrogenesis. Fibrosis is characterized by changes in the amount and composition of collagens and other ECM components (e.g., laminin and fibronectin). In steady state, the ECM is a dynamic structure that undergoes constant remodeling through the actions of matrix metalloproteinases (MMP) and their specific inhibitors, tissue inhibitors of metalloproteinases (TIMP). In the setting of fibrosis, the expression of MMP's and TIMPS is dysregulated which, together with increased collagen production, favors the accumulation of ECM. ECM accumulation, or scarring, results in the disruption of tissue architecture and function and eventually organ failure. In all tissues, myofibroblasts present in the portal tracts and in the sinusoids represent the primary ECM producing cells. In the liver following injury, hepatic stellate cells (HSC) located in the sinusoids are driven to proliferate and differentiate into myofibroblasts which can produce copious amounts of collagen [5]. Therefore, identifying the mechanisms that promote HSC activation and matrix production should identify points of intervention to allow the development of new preventive and therapeutic approaches to treat liver fibrosis.

1.3 ANIMAL MODELS OF LIVER FIBROSIS

Multiple pre-clinical murine models are available to study liver diseases including diet, surgery, genetic, infection, and chemical based models. Diet-based models include methionine-deficient, choline-deficient (MCD) and high fat defined diets promote CLD resembling non-alcoholic steatohepatitis (NASH) and non-alcoholic fatty liver disease (NAFLD) [6, 7]. Non-alcoholic steatohepatitis (NASH) is part of the spectrum of non-alcoholic fatty liver disease (NAFLD), currently the most

common form of the chronic liver disease in the United States and in many parts of the world. However, each of these models have associated limitations. Even though these diets provoke marked inflammation, only inconspicuous and mild-stage fibrosis has been reported with these diet modifications [8] and surgical complications such as bile leakage in the bile duct ligation (BDL) model can confound interpretation and thus limit utility [9] [10]. Genetically modified animals are also used to gain understanding regarding the involvement of specific proteins and signalling pathways during liver fibrosis and thus identify potential new drug targets [11]. However, genetic models hardly develop liver fibrosis due to the genetic perturbation alone, as such require a second stimulus for disease induction [12]. Hence, the process of fibrosis development in genetically modified animals specifies the communication between the environment and the genotype to manifest the disease, as is the case for NASH. The *Schistosoma mansoni* infection model is commonly used to study immune-mediated liver fibrosis stimulated by the parasitic eggs, but utility is limited by the complexity of delivery of parasite eggs that causes infection [13, 14]. Multiple models of hepatotoxin induced liver fibrosis have been developed. The administration of concanavalin A (ConA) [15, 16] causes inflammation and centrilobular fibrosis resembling autoimmune hepatitis. However, the consistence of induction and progression of fibrosis in the ConA model is variable and is influenced by multiple factors including compound preparation. In all animal models the genetic background of the mice, gender, age and the microbiota which can differ vastly between institutions [17]. The administration of ethanol, carbon tetrachloride (CCl_4), thioacetamide (TAA), dimethylnitrosamine (DMN) or diethylnitrosamine (DEN) are also used to induce liver fibrosis. The CCl_4 and TAA liver fibrosis models are most widely utilized and generally well characterized. These models are often selected based on their manifestation of features which recapitulate the pattern of disease in liver fibrosis patients including inflammation, followed by fibrosis and progression to HCC. Additionally, these models also exhibit evidence of fibrotic regression [18]. An unfortunate limitation of CCl_4 treatment is early mortality [19]. Notably, fibrosis induced by TAA differs depending upon the route of administration. Thus, intraperitoneal administration of TAA leads to rapid induction of liver fibrosis whereas oral delivery allows the temporal examination of progressive fibrosis and gradual development of HCC. Long-term oral administration of TAA induces CLD characterized by centrilobular fibrosis progressing to fibrous septation and cirrhosis [20].

1.4 MEDIATORS OF LIVER FIBROSIS

1.4.1 RESIDENT LIVER POPULATION IN FIBROSIS

The liver consists of approximately 80% parenchymal hepatocytes and 20% non-parenchymal cells, including stellate cells, sinusoidal endothelial cells, macrophages (Kupffer cells), biliary epithelial cells and recruited peripheral immune cells. In response to insult, whether it be chemical, infectious or mechanical, the initiation and perpetuation of fibrosis is a consequence of complex interactions between the various lineages of liver-resident and infiltrating immune populations [21]. Studies over the last two decades, predominantly using preclinical models, have greatly increased our understanding of the distinct contributions of these hepatic population to fibrogenesis.

Hepatocytes: Hepatocytes account for over 80% of the liver volume. One of the hepatocyte's primary functions is in the metabolism of xenobiotics (foreign substances such as TAA, alcohol metabolites, hepatitis viruses) circulating in the blood. Xenobiotics target hepatocytes to induce the production of reactive oxygen species (ROS) that cause hepatocyte death [22] [23]. Apoptotic hepatocytes release damage-associated molecular patterns (DAMPs) including nucleic acids, high mobility group box-1 (HMGB1)[24], adenosine triphosphate, (ATP), which act as endogenous stimulators of immune responses following tissue damage. They also work as danger signals in recruiting immune cells (neutrophils, monocytes) to initiate inflammatory responses [25-27]. Furthermore, hepatic stellate cells (HSCs) phagocytose apoptotic hepatocytes for their survival, suggesting the mechanism for fibrosis propagation [28, 29]. It has also been reported that ROS from damaged hepatocytes has a definite role in HSC's migration [30, 31]. Above findings suggest an active role for the hepatocyte in the progression of liver fibrosis.

Hepatic Stellate Cells (HSCs): As the predominant ECM producing liver population, HSCs play a crucial role in the pathogenesis of liver fibrosis [31-33]. HSCs are located between hepatocytes and the endothelial cells in the space of Disse [34]. HSCs are distinguished from other resident liver cells by their expression on the of cell-surface protein platelet-derived growth factor receptor-β (PDGFRβ), the enzyme lecithin retinol acyltransferase (LRAT) [35], and the cytoskeletal proteins

desmin and glial fibrillary acidic protein (GFAP) [36]. The activation of HSCs and their differentiation into extracellular matrix-producing myofibroblasts represents a critical step on the path to fibrosis. Expression of alpha-smooth muscle actin (αSMA) characterizes activated HSCs. Multiple factors trigger the activation, proliferation, and survival of HSCs **(Figure 1-2)** [37].

Transforming growth factor-β (TGFβ) [38-40], platelet-derived growth factor (PDGF) [41, 42], vascular endothelial growth factor (VEGF) [43], and connective tissue growth factor (CTGF) contribute to extracellular matrix production, differentiation, migration, adhesion, and survival of HSCs [44-46]. Also, phagocytosis of apoptotic hepatocytes increases the production of ROS in HSCs [47] which in turn promotes expression of fibrosis-associated genes leading to the production of type 1 collagens, CCL2 and TIMP-1 from HSCs [48, 49]. HSCs express a wide range of toll-like receptors (TLRs) and cytokine receptors (IL-15, IL-17A, IL-20, and TNF), which makes them highly responsive during immune responses. Notably, as a result of gut leakage in response to injury, lipopolysaccharide (LPS) derived from gut bacteria activates HSC through TLR4 signaling [50]. TLR4 can also signal DAMPs (e.g., HMGB1) released from damaged hepatocytes to enhance inflammatory responses [51]. These findings highlight multiple molecular mediators that contribute to HSC function, differentiation, and survival.

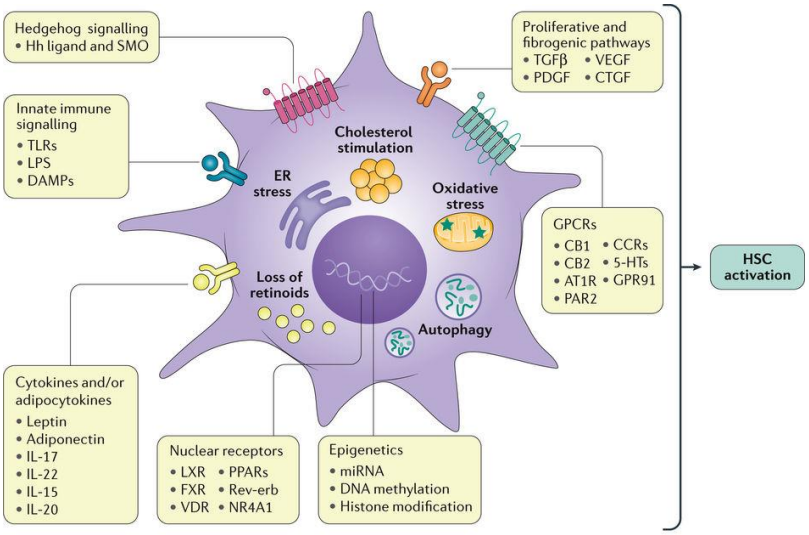


Figure 1-2 Molecules involved in hepatic stellate cells (HSC's) activation: *Nature Reviews Gastroenterology & Hepatology* **14**,397–411 (2017)

Liver endothelial cells (LSECs): LSECs have a highly permeable phenotype allowing the passage of cells and having constant interaction with other liver cells [52]. They have an excellent interface between blood cells, hepatocytes, and HSCs. In normal liver in their differentiated form, LSECs are considered gatekeepers of fibrosis as they maintain HSC quiescence [53]. Differentiation of LSEC is maintained by VEGF [54]. After liver injury, capillarization (loss of LSEC differentiation) attenuates the capacity of LSECs to inhibit the activation of HSCs [54]. These findings suggest that maintenance of LSEC's differentiation can be used as a therapeutic option.

LSECs can promote either liver fibrosis or regeneration depending on the context of injury. During acute liver injury, LSECs encourage regeneration, whereas in chronic liver injury they are implicated in the initiation and progression of disease [55, 56]. Angiocrine signals from LSECs promote regeneration immediately after the injury, but after chronic injury expression of the stromal cell-derived factor 1 receptors, CXCR4 and CXCR7 promote fibrosis [57]. The former drives the fibrogenic pathway which stimulates HSCs whereas the latter promotes pro-regenerative pathways [57].

The ductular reaction (DR): In most chronic liver diseases progressive portal and septal fibrosis are closely associated with a ductular reaction (DR) [58-60]. During DR strings of cholangiocytes will form as canals of Hering and small bile ductules, in a niche of inflammatory and stromal cells near portal tract (PT) [60-63]. Hepatic progenitor cells (HPC) will give rise to these reactive ductules. When mature hepatocyte proliferation is impaired these small bipotential ductules are capable to proliferate, migrate, and differentiate into either hepatocytes or cholangiocytes [58]. During injury, the DR expands dramatically, and its intensity is closely correlated to the extent of fibrosis. HPCs are closely associated with the fibrogenic activity, and they exist in a niche comprised of myofibroblasts, macrophages and other leukocytes and ECM components. Cellular and matrix components present in the DR niche contributed to HPC activation and fate. HPCs, in turn, influence myofibroblasts and inflammatory cells. However, further studies are required to understand the signals that control the induction and progression of the DR [62, 64, 65].

1.4.2 IMMUNOBIOLOGY OF LIVER FIBROSIS

It is increasingly clear that that the immune system plays a central role in the regulation of fibrosis. Residing in or patrolling the liver at steady state are distinct subsets of immune cells (myeloid and lymphoid cells), and many studies have implicated a role for these cells in the pathogenesis of fibrosis in this organ.

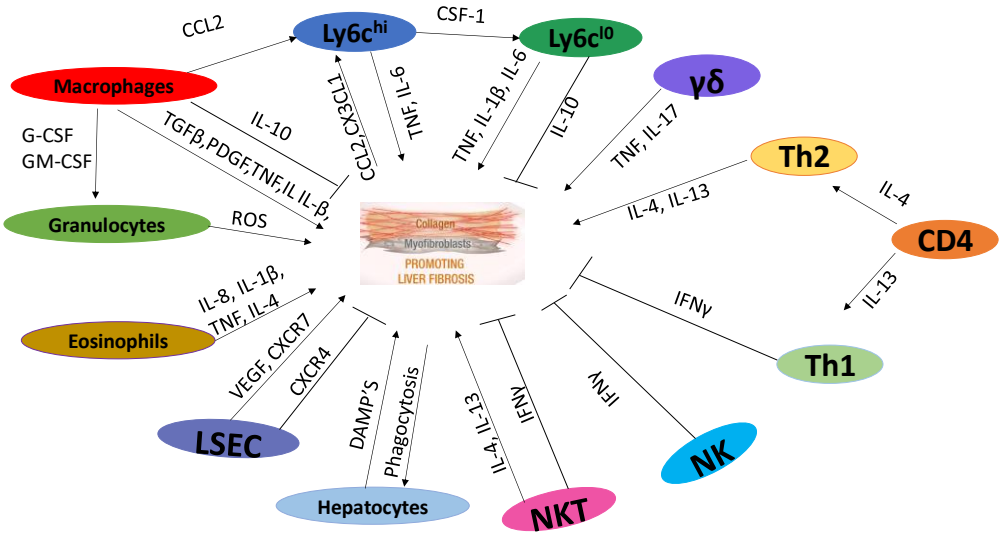


Figure 1-3Interplay between hepatic stellate cells and immune cells: Multiple immune cells produce many factors that modulate property of HSC and vice-versa. Details about the interaction of HSC with individual immune cell subpopulations and their role for liver fibrosis are given section **1.4.2.**

1.4.2.1. Myeloid populations

Monocytes and Macrophages: Monocytes are established critical mediators of fibrosis although the mechanism by which these cells promote fibrosis remain undefined. Monocytes are circulating precursors of macrophages which comprise two major subsets, Ly6c^{hi} and Ly6c^{lo} which can be additionally differentiated by the differential expression of the chemokine receptor CCR2 [66]. Thus, Ly6c^{hi} monocytes express CCR2 and are commonly referred to as inflammatory monocytes. These monocytes are rapidly recruited into inflamed tissues where they secrete inflammatory cytokines [67]. Ly6c^{lo} monocytes lack expression of CCR2 but express high levels of the chemokine receptor CX3CR1 and are commonly referred to as patrolling monocytes or tissue-resident macrophage precursors. Whether these cells are recruited to sites of tissue inflammation from the circulation or differentiate from the infiltrating CCR2+ inflammatory monocytes is unclear.

However, recent literature supports the notion these cells are derived from CCR2+ monocytes at sites of injury [68]. In both steady state and during inflammation $Ly6C^{lo}$ monocytes differentiate into tissue resident macrophages under the influence of colony stimulating factor- 1 (CSF-1) [69]. During inflammation, these $Ly6c^{lo}$ monocytes can also enter tissue and differentiate into macrophages [70]. In parasite-induced liver infection, CCR2 dependent $Ly6c^{hi}$ monocytes are observed in liver and blood [70]. Recent studies using preclinical rat models of acute liver injury suggested that monocytes infiltrate into the liver in a CCR2 and CCL2 dependent manner [68, 71-75]. Also, CCl_4 treated $CCR2^{-/-}$ mice demonstrated reduced monocyte infiltration [76]. However, during liver fibrosis we showed that fibrosis was CCL2 dependent but independent of CCR2, suggesting a controversial role of CCL2 signaling [20].

The liver contains a large population of tissue-resident macrophages (Kupffer cells) which originated from yolk sac precursors with the capacity of self-renewal throughout adult life [77]. Following inflammation, these macrophages are expanded through the recruitment and differentiation of circulating monocytes ($Ly6c^{hi}$). Recently, the expression of the T-cell Immunoglobulin and Mucin (TIM)-4 receptor by KC, was shown to distinguish these liver resident macrophages from infiltrating bone marrow-derived macrophages. Thus, in response to injury, monocytes and monocyte-derived tissue-resident macrophages precursors all contribute to fibrogenesis [78]. Indeed, the depletion of macrophages, either genetically or pharmacologically, has been shown to attenuate myofibroblast activation and fibrosis in multiple murine models of CLD [79]. In the fibrotic liver, macrophages localize near activated myofibroblasts in areas of tissue scarring [80]. Through their production of TGFβ and PDGF macrophages promote HSC activation [81]. Importantly, however, due to their marked functional plasticity, macrophages also play a key role in fibrotic regression. Thus, in the CCl_4 model of liver fibrosis, macrophage depletion during the recovery phase after CCl_4 withdrawal led to the persistence of injury [75, 82]. A deeper understanding of the contribution of these myeloid populations is important because phenotypically and functionally distinct monocyte/macrophage populations may play opposing roles in liver fibrosis and repair.

Neutrophils: Neutrophils are early responders in injury-induced inflammatory responses. During infection, they serve to clear pathogens through phagocytosis, production of ROS and proinflammatory cytokines [83]. Neutrophils are reported as pathogenic mediators of liver fibrosis. Therefore, neutrophil depletion in mice administered with acetaminophen demonstrated reduced liver fibrosis [84]. Mechanistically, neutrophils that are recruited during liver injury will become activated and exacerbate inflammation through their cytotoxic activity and ROS production during ischemia-reperfusion injury and alcoholic hepatitis preclinical models [85, 86]. During injury, activation of resident macrophages and dendritic cells promoted neutrophil (and monocyte) recruitment through their release of G-CSF, CXCL8 (IL-8), CXCL1, 2, 3, CCL2, 3, 4 [86-88]. Additionally, IL-17 produced by γδ T cells and $CD4^+$ T helper cells (Th17) also promoted neutrophil recruitment through the induction of the neutrophil chemokine IL-8 from fibroblasts in renal failure diseases [89]. Once in the liver, neutrophils adhere to parenchymal cells via ICAM-1/Mac-1 interactions which trigger the formation of ROS and release of proteases and hydrogen peroxide through degranulation [90]. Whether neutrophils contribute in all settings of liver fibrosis is unclear as, in the bile duct ligation model, neutrophils have shown a limited contribution [91].

Eosinophils: Activated eosinophils play an essential role in the pathogenesis of various liver diseases including primary cholestatic diseases [92], hepatic allograft rejection [93], hepatitis C and drug-induced liver injury [94]. Eosinophils are recruited into the liver via CCL11, CCL22, IL-2, IL-3, IL-5, and GM-CSF produced by other immune cells [95]. BALB/c mice that are deficient in eosinophils exhibited reduced injury during halothane-induced liver injury [96]. When exposed to necrotic liver cells, eosinophils become activated by upregulating the expression of CD107a [97]. Eosinophil activation following necrotic liver exposure induced the release of IL-1β and IL-18 mediated by caspase-1[97]. Additionally, activated eosinophils also produce TNF, IL-4, and IL-5 in *Schistosoma mansoni* infected patients [98]. IL-4 produced from eosinophils promoted hepatocyte proliferation in regenerating liver [99], suggesting a role in liver regeneration. There are only limited number of studies which investigated the contribution of these cells in liver fibrosis. Thus further studies are required.

1.4.2.2. Lymphoid populations

$CD4^+$ T cells upon activation can differentiate into multiple effector lineages depending on the cytokine milieu. Thus, $CD4^+$ effector cells can be divided into Th1, Th2, Th17, Th9, follicular helper T cells (Tfh), or regulatory T cells (Treg) based on their cytokine expression and function. It is noteworthy that $CD4^+$ T cells also contributes of liver fibrosis. It has been reported that IL-4, IL-5, and IL-13 induced by Th2 cells linked to the development of *Schistosomiasis* infection-induced liver fibrosis [100]. Conversely, cytokines such as IL-12 and interferon γ (IFNγ) which initiates the downstream signaling cascade to develop Th1 cells have demonstrated anti-fibrotic effect in *Schistosomiasis* infection-induced liver fibrosis when compared to Th2 induced cytokines [100]. Also, the imbalance in the production of cytokines by Th1 and Th2 cells lead to liver fibrosis and is demonstrated both in-vitro and in-vivo BALB/c mice treated with CCL_4 or TAA [101]. Splenectomy in these mice partly inhibited the progression of fibrosis by restoring Th1/Th2 balance [102]. The contribution of other $CD4^+$ T cells in liver fibrosis is discussed below.

$CD8^+$ T cells play a major role in the pathogenesis of liver fibrosis induced following hepatitis C virus (HCV) and hepatitis B virus (HBV) infection [103]. However, results indicated that in the presence of HCV-specific IFNγ-secreting $CD8^+$ T cells patients demonstrated less fibrosis suggesting the anti-fibrotic property of $CD8^+$ cells [104]. Conversely, pro-fibrotic property if $CD8^+$ is reported in another study where patients with HCV showed increased expression of RAR-related orphan receptor gamma (RORγt) and IL-17 secretion from Tc17 cells in association with increased disease pathogenesis [105, 106]. $CD8^+$ cell-mediated liver fibrosis is also observed in chemically (CCl_4 and TAA) induced liver fibrosis models [107, 108]. These findings suggest both pro and anti-fibrotic properties of $CD8^+$ cells.

Th17 cells and their secreted cytokines have a significant role in fibrosis. Proinflammatory cytokines that are dominantly produced by Th17 cells include IL-17, IL-21, IL-22, and TNF. TGFβ and IL-6 or IL-21 contributes to the differentiation of Th17 cells from naïve T cells [109], and differentiated Th17 cells are stabilized by IL-23 [110]. Transcription factors such as signal transducer and activator of

transcription 3 (STAT3) and RAR-related orphan receptor gamma t (RORγt) are involved in the development of Th17 cells. In the liver, Th17 responses have been found during fibrosis associated with NASH, alcoholic liver disease, cholestatic liver diseases, antimicrobial defense, and BDL [111]. Halofuginone (HF) and KD025 inhibit the expression of STAT3, RORγt and IL-17 production [112, 113]. A more in-depth discussion of Th17 cells can be found in section 1.6.

Regulatory T cells (Treg) are critical in the maintenance of peripheral tolerance and the regulation of immune responses. Broadly, $CD4^+$ Treg can be classified into two subsets: natural Treg (nTreg) which are produced in the thymus and induced Tregs (iTreg) that are generated in the periphery. Both nTreg and iTreg are characterized by their expression of the transcription factor forkhead box P3 (FoxP3), and the majority of Tregs express high levels of CD25. The high-affinity of IL-2R expressing Tregs are implicated as participants in a variety of fibrotic diseases. Foxp3-expressing Treg cells are important producers of immunosuppressive cytokines, such as IL-10 and TGF-β1which control effector cell function [114]. One study has suggested that IL-10-secreting Treg cells protected mice from fibrosis [115]. Also, in cholestasis-induced liver injury, $FoxP3^+$ Treg inhibited Th17 induced liver injury [116]. Tregs were also involved in immune regulation of CCL_4 induced liver fibrosis by suppressing pathogenic property of macrophages, Th1 and natural killer (NK) cells [117, 118]. Hence these findings suggest that Treg cells may inhibit progressive fibrotic disease by negatively regulating the inflammatory responses. Conversely, Treg cells are also an important source of TGF-β1, with at least a few studies reporting that TGF-β–producing Treg induce rather than inhibit fibrosis [119, 120]. However, it remains unclear that why Treg cells have antifibrotic activity in some situations and profibrotic activity in others.

Gamma delta (γδ) T cells have been implicated in various liver diseases and models that are associated with fibrosis including fulminant hepatitis [121], autoimmune hepatitis [122], HBV [123] and HCV [124-126]. They secrete large amounts of pro-inflammatory cytokines, including IFNγ, IL-17, IL-4, IL-5, IL-10, IL-13, TGFβ and GM-CSF [127]. Through this secretion, γδT cells link the innate and adaptive immune systems [128]. IL-17 secretion is of significance, and a pathogenic role of IL-17-producing γδT cells has been implicated in autoimmune liver diseases

[122, 129] and *Schistosoma japonicum* infection [130]. IL-17-producing γδT cells originating in the gut were shown to contribute to fibrosis and inflammation of bile ducts in animal models and patients with chronic biliary disease [131]. Recently it has also been reported that hepatocyte-derived exosomes activated TLR3 in HSC and exacerbated liver fibrosis by inducing IL-17A production in γδT cells [132]. Conversely, γδT cells are shown to be protective through their inhibition of NKT cells in immune-mediated liver disease induced by ConA but this protective function appeared to be independent of IL-17 [121]. However, the anti-fibrotic pathway of IL-17^+ γδT cells expressing CCR6 is reported during chronic liver injury by inhibiting the inflammatory reaction and the activation of collagen-producing HSCs [133]. Further mechanistic studies related to tumour microenvironment revealed that CCR2 and CCL2-deficient mice displayed reduced γδT cell infiltrates [134]. However, the association of CCL2/CCR2 recruited γδT cell in association with liver fibrosis is not clear. Above findings suggest both protective and pathogenic properties of γδT cells. However, it is not clear whether this diverse property of γδT cells during liver diseases is due to the specific receptors they express or due to the redundant cytokine profile of γδT cells. Thus, it is important to define different γδT cell subsets in acute and chronic liver inflammation as well as the cytokines they produce to assess whether these cells might be useful as a therapeutic target for the treatment of liver disease.

Mucosal-associated invariant T cells (MAIT) are non-conventional T cells with both adaptive and innate immune properties [135]. These cells respond to vitamin B metabolites derived from bacteria that are presented on MR1 which MHC is class 1 like molecule [136]**.** MAIT cells are enriched in liver tissue as well as the gastrointestinal tract and are characterized by high-level expression of cell surface CD161 and secretion of IL-17A [137]. These cells are found to be associated with unique anti-bacterial function [138]. MAIT cells express receptors such as CXCR6 and CCR6, that allow them to home to the liver [139, 140]. In addition to MR1, IL-18 and IL-12 cytokines also activate MAIT cells because these innate-like T cells express receptors for IL-18 and IL-12 [141-143]. During HCV infection MAIT cells were activated in an IL-18 dependent manner and increased disease severity [144]. Activated MAIT cells promote HSC activation in patients with the autoimmune liver disease [145]. Conversely, IFN-α mediated MAIT cell activation was associated with therapeutic responses through the induction of IFN-γ [144]. MAIT cells are

predominant IL-17 producers targeting multiple cell types in the liver to produce pro-inflammatory cytokines and chemokines [137, 146][136]. Hence MAIT cells could be important contributors to hepatic inflammation and fibrosis. Further studies are needed to understand the nature and type of ligands produced by MAIT cells that could be involved in liver diseases.

Natural killer (NK) cells are abundant in liver and provide the first line of defense against invading microbes, pathogens and toxic substances [147]. During HCV and CCL_4 induced liver fibrosis, NK cells in the liver can induce apoptosis of activated HSC in a TNF-related ligand (TRAIL), Fas-L and NKG2D dependent manner and also produce antifibrotic mediators such as IFN-γ that protect against fibrosis. [148-150]. In support of these studies mice depleted of NK cells had demonstrated increased liver fibrosis [151]. Thus, these findings suggest an antifibrotic property of NK cells. Conversely, the fibrotic property of IL-17A secreting NK cells was documented in ischemia-reperfusion liver injury (IRI) [152]. Since NK cells are reported to be a mediator of liver fibrosis, further investigations are required to fully appreciate the protective vs. pathogenic roles of NK cells in fibrotic liver injury.

Natural killer T (NKT) cells play a diverse role in liver injury due to the heterogeneity of NKT cells and the variety of cytokines produced. They are classified as CD1d-restricted invariant NKT and non-invariant NKT cells [153]. In liver, antigen presenting cells present antigens to CD1d on NKT cells, leading to the release of Th1 (IFN, TNF), Th2 (IL-4, IL-5, IL-10) or Th17 (IL-17, IL-22) cytokines which in turn activate other innate immune cells and adaptive T cells [154]. Accumulation of NKT cells was observed in NASH through activation of Hedgehog pathway [155]. The profibrotic effect of NKT cells is implicated in HBV transgenic mice [156, 157], TAA-induced liver fibrosis [156], primary biliary cholangitis [158], and NASH [155] through HSC activation and immune modulation [156, 157]. Similar to NK cells, NKT cells are also reported to be antifibrotic by inhibiting HSC activation and IFN-γ production through direct killing [159, 160]. Further studies are required to understand the complex role of NKT cells in liver injury and fibrosis

Innate Lymphoid cells (ILC) are heterogeneous innate immune cells. There are three subsets of ILCs based on cytokines (type 1, type 2, and Th17 cell cytokines) they produce: group 1 ILC (consists of ILC1s and conventional NK (cNK) cells); group 2 ILC (consists of ILC2s); and group 3 ILC (consists of ILC3s and lymphoid tissue inducer (LTi) cells) [161]. ILCs are detected abundantly in the liver, with the dominant subsets of ILC being cNK cells and ILC1s (comprising of nearly 95% of all ILCs) [162]. Although ILC2s and ILC3s in the liver are quite rare (comprising of nearly 5% of all ILCs), the roles of these different ILC subpopulations in liver disease have gained recent interest. Hepatic cNK cells contributed to HBV or Con A-induced liver fibrosis by promoting the antiviral activity of $CD8^+$ T cells [161, 163]. Depletion of ILC2s significantly ameliorated murine immune-mediated hepatitis [164]. Despite the low percentage, hepatic ILC3s are involved in the pathogenesis of murine and human liver diseases *via* secretion of proinflammatory cytokines (IL-22 and IL-17) [165, 166]. Thus, above findings suggest that excessive activation of ILCs can lead to chronic pathologies (e.g., inflammation, liver injury, or fibrosis). Further research is required to understand the protective or pathogenic mechanisms of different ILCs in liver diseases.

B cells represent half of the intrahepatic lymphocyte population [167] that induce hepatic fibrosis through cytokine release and antibody production [167]. Patients with CLD have increased B cells in their livers. In the CCl_4 model, the frequency of activated B cells (CD44 and CD86) were elevated with increased TNF production [168, 169]. This suggests the role of B cell activation and cytokine production during liver fibrosis. B cells that are activated in T-cell dependent manner will promote germinal center (GC) formation to induce antibody or immunoglobulin production [170] [171]. Supporting this, recently increased production of immunoglobulin (IgG) was documented in the livers of CCL_4 treated mice [168, 169]. Also, auto-antibodies from B cells were detected in the sera of patients with alcoholic liver disease, NAFLD, viral hepatitis, drug-induced hepatitis, HCC including autoimmune liver diseases for which these are essential for disease diagnosis and to judge prognosis [172]. This suggests a role for antibodies during liver fibrosis, however association of GC formation during liver fibrosis is not known.

It has already been reported that (chronic graft versus host disease) T follicular helper cells (Tfh) through upregulation of B-cell lymphoma 6 (Bcl6) transcription

factor and IL-21 production will activate B cells to promote B cell differentiation and GC formation [173, 174]. Additionally, under the influence of IL-21, γδ T cells will acquire Tfh cells phenotype to induce GC formation [175]. However, the association of these cellular and molecular mediators (Tfh, Bcl6, IL-21) in promoting GC formation during liver fibrosis is not clear. Given that activated B cells and GC B cells will induce antibody production, the mechanism through which these antibodies contribute to liver fibrosis is not clear. Evidence suggests that, after antibody binding to target antigen, the possible mechanisms include 'complement activation' and 'antibody-mediated cellular cytotoxicity. During complement activation, a number of distant plasma proteins will either directly attack pathogen or antibody bound pathogen to induce inflammatory responses that can help fight infection whereas during antibody-mediated effector function, antibodies will form as immune complexes and signal through FcγR (expressed by immune cells) to trigger both pro and anti-inflammatory immune responses [176]. FcγR consists of both activating and inhibitory receptors [176]. Signaling through activating FcγR may trigger cellular cytotoxicity, cytokine release, and phagocytosis whereas signaling through inhibitory receptors downregulates effector functions [176]. Previously, it has been observed that mice deficient in FcγR demonstrated reduced cartilage damage during rheumatoid arthritis [177] which highlights the role of FcγR in disease pathogenesis. However, the association of FcγR during liver fibrosis is not clear. Even though the role of B cells has been implicated in liver fibrosis, the mechanisms that activate B cells and induce immunoglobulin deposition during hepatic fibrosis need further investigation.

1.5 IL-17 IN LIVER FIBROSIS

Cytokines and their receptors. The IL-17 family of cytokines plays an important role in host defence against microbial organisms and also contributes to the pathology of multiple inflammatory disease settings including autoimmunity and liver fibrosis (discussed in IL-17A section). Functionally, IL-17 family cytokines alter cell differentiation pathways and induce the expression of a variety of pro-inflammatory cytokines, chemokines, and cell adhesion molecules in a wide range of cell types. The IL-17 cytokine family is comprised of 6 established members: IL-17/IL-17A, IL-17B, IL-17C, IL-17D, IL-17E/IL-25, and IL-17F. These cytokines are produced by multiple cell types of both hematopoietic and non-hematopoietic origin

and are secreted as disulfide-linked homo or heterodimers, except IL-17B, which is secreted as a non-covalent homodimer. The biological function of the IL-17 family cytokines is mediated through the IL-17 receptor (IL-17R) family expressed on the surface of target cells. The IL-17R family members include IL-17RA, IL-17RB, IL-17RC, IL-17RD and IL-17RE which are expressed on cells of both hematopoietic (e.g., monocytes/macrophages, granulocytes and eosinophils) and non-hematopoietic (e.g., fibroblasts, hepatocytes, endothelial cells, HSC) origin [178].

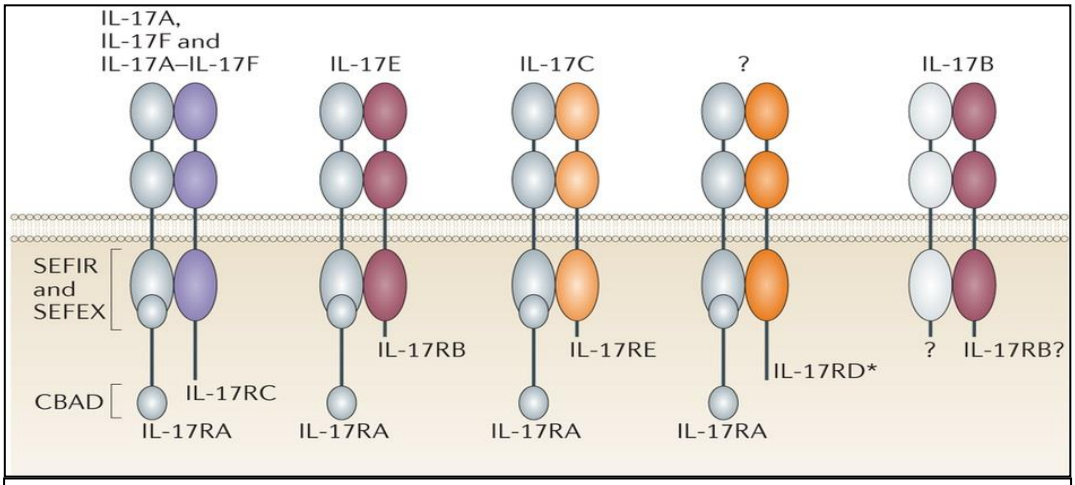


Figure 1-4 Signalling of IL-17 family ligands and its receptors *Nature Reviews Immunology* **volume9**, pages 556–567 (2009)

The IL-17 family cytokine/receptor interactions required for downstream signaling are unique in that IL-17RA utilizes a common recognition strategy to bind to several IL-17 family members and subsequently pairs with a second IL-17R subunit required for signaling **(Figure: 1-3).** Thus, initial IL-17RA receptor binding by IL-17 cytokines alters that cytokines affinity and specificity for a second IL-17 receptor, resulting in heterodimeric receptor complex formation. In this way, IL-17RA acts as a shared receptor with multiple different signaling complexes [179]. IL-17E, also known as IL-25, signals through a heterodimeric receptor comprised of IL-17RA and IL-17RB. It is also established that IL-17C signals through the IL-17RA/IL-17RE receptor complex, but the cognate receptors for IL-17B and IL-17D are less defined. IL-17B signals through a receptor comprised of an IL-17RB subunit complexed with undefined subunit, and the receptor for IL-17D remains to be elucidated [180]. Relatively little is known regarding the physiological roles of IL-17B, IL-17C or IL-17D, however, similar to IL-17A, signaling by these cytokines in target cells is reported to promote proinflammatory responses. The known functions and expression of various IL17 family cytokines are detailed in (**Table 1-1).**

Table 1-1 IL-17 cytokines and receptors expression.

Cytokine	Receptor	Producers	Responders	Function
IL17A	IL17RA	CD4[181],CD8[182], mast cells, macrophages[183], B cells[184]	Eosinophils[185], CD4[186],	G-CSF, IL6, IL18[187], CXCL5[188], GROα, and MIP-1β[185],
	IL17RC	Macrophages[183] , γδT cells, NKT, MAIT cells[189], LTicells and neutrophils[190]	HSC, monocytes[191]	CCL20[192], MMP3, MMP13, collagen
IL17E	IL17RA	Eosinophils[193], Lung epithelial cells	Monocytes[193]	IL13, IL4, IL10, G-CSF[193]
	IL17RB	alveolar macrophages macrophages[194]	Fibroblasts[195], naïve T cells	CCL5,CCL11,CXCL-8[195]
IL17F	IL17RA	CD4, CD8, B cells[184], mastcells,	Eosinophils[185]	G-CSF[196], CCL20, IL-1β, IL-6, IL-8,
	IL17RC	macrophages[183]γδT cells, NKT cells, LTi cells, basophils, activated monocytes, Hepatic stellate cells[197]	Bronchial epithelial cells[198], endothelial cells, fibroblasts,	GROα, and MIP-1β[185], IL-11[198] and IGF[199]
IL17A/F	IL17RA	CD4[181],CD8[182], mast cells, macrophages[183],	Eosinophils[185],	CXCL5[188],CXCL1, IL6[200]
	IL17RC	B cells[184], γδT cells, NKT cells, LTi cells,	Kupffer cells [200], CD4 cells[201]	
IL17B	IL17RB	-	-	TNF[202], IL-6[202]
IL17C	IL17RA	Epithelial cells[203, 204],CD4Tcells, DC, and	-	TNF, IL-6
	IL17RE	Macrophages	CD4	IL-2
IL17D	-	-	Endothelial cells	IL-6, IL-8, and GM-CSF

IL-17A. IL-17A shares 50% structural homology with IL-17F and is formally referred as IL-17 in the literature [205]. IL-17A and IL-17F form IL-17A-IL-17A and IL-17F-IL-17F homodimers or together form IL-17A/IL-17F heterodimeric cytokines and signal through the receptors IL-17RA and IL-17RC. Noticeably, IL-17A and IL-17F exhibit differential binding affinity and specificity to the IL-17RA/RC receptor. The IL-17A homodimer preferentially binds IL-17RA whereas IL-17F preferentially associates with IL-17RC [206]. However, IL-17A/ IL-17F heterodimer interacts equally with either the IL-17RA or IL-17RC receptor chain complexes. Functionally, IL-17A and IL-17F mediate pro-inflammatory responses. However, evidence suggests that these cytokines have overlapping yet distinct roles in host immune and defense mechanisms [207]**.**

T cells, neutrophils, macrophages [208], NK cells [209], γδT cells [210, 211], activated $CD4^+$ and $CD8^+$ T cells (Th17 and Tc17 respectively), lymphoid tissue inducer (LTi) [212] and MAIT cells have all been demonstrated to produce IL-17A. IL-17A has been implicated in several autoimmune [210, 213] and inflammatory diseases [214, 215] including rheumatoid arthritis (RA) [215, 216], psoriasis [217], atherosclerosis [217] and graft versus host diseases (GVHD) [218]. During inflammatory responses, IL-17A-producing T cells increased the production of TNF and IL-12 [219-222] suggesting a role in inducing inflammatory cytokines. Moreover, in experimental autoimmune encephalomyelitis (EAE), IL-6 secreted from fibroblasts in response to IL-17A signaling had amplified inflammation in an autocrine manner [223]. This suggests that IL-17A signaling induces a positive feedback loop for IL-6 signaling to maintain the inflammatory cycle. It has also been reported that IL-17A synergizes with B-cell activating factor (BAFF) to protect B cells from apoptosis, thereby increasing the number of autoantibody-producing cells [224] in the setting of systemic lupus erythematosus (SLE). Sometimes during chronic inflammation such as arthritis, disease continues in the presence of reduced IL-17A producing cells which can occur due to molecular changes induced during early exposure of mesenchymal (synoviocytes, fibroblasts) cells to IL17A is sufficient to maintain cell survival by inducing the anti-apoptotic molecule synovolin [205, 225] [226]. This means that the use of IL-17A inhibitors during the early stage of inflammation is sufficient to induce a long-lasting reduction of arthritis [226]. During acute and chronic inflammation, IL-17A has a modest effect, whereas its effect is greatly increased when combined with other cytokines [227]. IL-17A and

TNF have synergic interactions, where IL-17 increases the expression of TNF receptor to enhance its effect [228]. The combination of inhibitors to both TNF and IL-17 represents a strong therapeutic approach in inflammatory disorders [225]. A role for IL-17A has been reported in monocyte to macrophage differentiation (osteoclasts) through NFkB ligand (RANKL) in rheumatoid arthritis [229, 230]. Apart from pathogenic effects, IL-17A has also been reported to be protective in T cell-mediated intestinal inflammation by suppressing Th1 differentiation [231].

In addition to other inflammatory and autoimmune diseases, IL-17A has also been implicated as an important mediator of liver fibrosis in various pre-clinical models of chronic liver disease. In ConA hepatitis (which is T cell-mediated), mice deficient in IL-17A developed the same degree of injury as wild-type controls, suggesting that IL-17A plays no significant role in this type of liver injury [232]. IL-17 has been linked to bacterial infections in the liver, where it is believed to play a protective role rather than being pathogenic [233]. Conversely, in parasitic infection models, mice infected with the helminth *Schistosoma mansoni* developed fibrosis in an IL-17 dependent manner [234]. In BDL and CCl_4-induced liver injury, IL-17A signaling in inflammatory cells promoted liver fibrosis with increased expression of pro-inflammatory and pro-fibrogenic mediators such as IL-6, TGFβ, MMP-3, TNF, TIMP-1, and αSMA [211, 235]. IL-17A also induced collagen production by activated myofibroblasts through the STAT3 signaling pathway [211]. Additionally, it promoted liver neutrophil infiltration with increased liver enzymes in an ischemia-reperfusion model [236, 237]. Signaling through IL-17RA contributed in peripheral blood monocyte differentiation [238] noticeably. This supports a role for IL-17 in recruitment and differentiation of immune cells. While a lot of work has been done regarding the contribution of IL-17A in different autoimmune and inflammatory diseases, however, the association of IL-17A with other immune cells in liver fibrosis is yet to be investigated.

IL-17F: Amongst IL-17 family members, IL-17F shows the highest homology to IL-17A, and both can exist as heterodimers. Former findings suggest that IL-17A negatively regulates the production of IL-17F [239]. Signaling triggered by IL-17F is 10-30-fold weaker than IL-17A regarding downstream gene activation, whereas the IL-17A /F heterodimer acts at an intermediate level.

IL-17F is produced by γδ and other T cells, activated monocytes [197], B cells, basophils, mast cells and bronchial epithelial cells and is induced in response to IL-33 [240]. T cells treated with TGFβ transiently upregulated the expression of IL-17F but not IL-17A at both gene expression and protein levels [241, 242]. IL-17F is reported to be associated with asthma, chronic obstructive pulmonary disease[243], EAE, colitis and Crohn's disease [244]. Additionally, during early stages of bacterial infections, IL-17F and IL-17A are equally important in controlling the bacterial burden, whereas at later stages IL-17F appears to be more critical [245]. Although IL-17F is pathogenic in various diseases, mice deficient with IL-17F displayed similar onset of EAE as wild-type controls [240]. IL-17F has been reported to induce the production of GCSF, TNF [246], CCL2, CCL5, CCL7 [240], TGFβ and IL-2 from endothelial cells [197], GM-CSF from human bronchial epithelial cells, and IL-6 and CXCL1 from fibroblasts [243, 247, 248], suggesting wide-ranging effects of IL-17F signaling in inducing inflammatory mediators.

IL-17F has also been implicated in liver fibrosis. Clinically patients with HCV-associated HCC have increased levels of IL-17F [249]. This suggests the potential usage of IL-17F as a biological marker of fibrosis progression and HCV-associated HCC. *In-vitro* studies using human hepatocarcinoma cell lines demonstrated the role of IL-17F in reducing the expression of pro-angiogenic factors such (IL-6, IL-8, and VEGF) which led to the inhibition of tumor angiogenesis [250], demonstrating a protective role for IL-17F. Though there is limited research regarding the role of IL-17F in liver fibrosis, further studies are required to understand the mechanistic role of IL-17F during liver fibrosis.

IL-17E/IL-25: IL-17E signals through binding the IL-17RA and -RB heterodimeric receptor complex [251, 252]. Its signaling requires both receptors and the absence of either blocks IL-17E signaling. IL-17E is defined as a Th2 cell-derived cytokine [253]. IL-17E only shares 16-20% amino acid sequence identity with IL-17A [254]. IL-17E is produced by T cells, macrophages [194], basophils, epithelial cells [255], endothelial cells [256] pulmonary eosinophils [193, 257] and mast cells [258]. IL-17E is reported in various diseases models which include DSS-induced colitis [259], asthma [257, 260], colon cancer [261], breast cancer [262], pulmonary fibrosis [263, 264], systematic sclerosis [265], and inflammatory bowel disease [266]. Mechanistically, IL-17E promoted type 2-based immune reactions, with increased

expression of IL-5, IL-4, IL-13, serum IgG, IgE and recruitment of eosinophils into the lung during airway inflammation [253, 254, 267-269] [270, 271]. ILC2s produce these type 2 cytokines IL-4, IL-5, and IL-13 in response to IL-17E stimulation [272]. IL-17E is also described to play a pathogenic role in colonic inflammation with increased production of IL-13 and IL-6 [273]. In-vitro studies showed that IL-17E signaling induced up-regulation of IL-13 and TNF in T84 colonic epithelial cells [259] and IL-8, MIP-1, MCP-1 and IL-6 from both epithelial cells [259] and activated eosinophils [274]. This highlights the role of IL-17E in inducing inflammatory cytokines that promote cellular infiltration. Conversely, the protective of IL-17E is reported during DSS-induced colitis, associated with reduced inflammation and increased survival [275]. Therefore, the above findings indicate both a protective and pathogenic role of IL-17E depending on the disease or model.

IL-17E also contributes to liver fibrosis. IL-17E from macrophages potentially induced collagen production from HSC during *C. sinensis* liver fibrosis [276]. *C.sinensis*-will induce lysophospholipase A (*Cs*LysoPLA) which upregulated the expression of pro-fibrotic genes in a HSC cell line (LX-2) [277]. In addition to *C. sinensis,* hepatocyte-specific Autotaxin (ATX) expression also induced lysophospholipase to activate HSC in CCl_4-induced liver fibrosis [278]. These findings suggest that lysophospholipase from hepatocytes following liver injury and also from C. *sinensis* will induce HSC activation through IL-17E. Conversely, a protective role of IL-17E has been described in mice with fulminant hepatitis [279] and bile duct ligation-induced liver fibrosis which is associated with increased immunosuppressive Gr1+CD11b+ cells and decreased proliferation of T cells [280]. Though IL-17E is well studied in allergic asthma and colonic inflammation, its function in the context liver disease needs further investigation.

IL-17B: IL-17B signals through IL-17RB which couples with an orphan receptor. Expression of IL-17B is organ-specific; it is expressed in human pancreas, small intestine, and stomach [281]. Expression of IL-17B is found in activated T cells [282], neutrophils [283], germinal center B cells [284] or chondrocytes [285]. The protective role of IL-17B has been described in a murine model of collagen-induced arthritis [286]. As discussed earlier, deficiency of IL-17E led to decreased DSS-induced colitis, whereas deficiency of IL-17B showed opposite phenotype by increasing IL-6 and IL-13 production [287]. This suggests that IL-17B inhibits IL-17E

signaling by suppressing its ability to bind to IL-17RB. IL-17B enhanced TNF induced G-CSF and IL-6 secretion from fibroblasts in patients with RA [283]. Recently it has been reported that patients with pulmonary inflammation or SLE have increased IL-17B expression [288, 289]. These studies discovered the role of IL-17B as a proinflammatory cytokine implicated in the pathogenesis of inflammatory and autoimmune diseases. In addition to this, there are several other studies which highlight a strong contribution of IL-17B in tumor development. For instance, IL-17B/IL-17RB promoted breast and pancreatic tumorigenesis by inducing the production of anti-apoptotic factor Bcl2, and other inflammatory mediators such as CCL20, CXCL1, IL-8, and TFF1 to promote cancer cell survival and leukocyte recruitment cell recruitment in distant organs [290] [291]. Blocking of the IL-17RB/IL-17B signaling pathway has reduced tumorigenicity of breast cancer and pancreatic metastasis [290, 291]. There is some evidence regarding the contribution of IL-17B in inflammatory and autoimmune diseases, but further research is needed to understand the biological function of IL-17B in liver fibrosis.

IL-17 C, D: Far less is known about the cellular sources, receptors/target cells and biological functions of the remaining IL-17 family cytokines. IL-17C signals through IL-17RE and IL-17RA [292]. IL-17C is detected in keratinocytes and tracheal epithelial cells [203, 204]. IL-17C expression has been reported in some bacterial infections and autoimmune diseases like psoriasis [293, 294]. Also, elevated levels of IL-17C are observed in mice with autoimmune hepatitis. IL-17D is another cytokine whose function is not well defined. Similar to IL-17B, IL-17D is also expressed in a variety of organs and tissues, including heart, pancreas, and adipose tissue [295]. It can stimulate endothelial cells to induce the expression of IL-6, IL-8, and GM-CSF [295]. Though there is some evidence regarding the contribution of these cytokines under inflammatory conditions, however, the role of these cytokines in liver diseases is not well described.

IL-17 Receptors (IL-17R):

The IL-17 receptors are shown in Figure 1.3.

IL-17RA signal IL-17A, IL-17F, IL-17E and IL-17C with different binding affinities along with other co-receptors IL-17RB, IL-17RC, IL-17RD, and IL-17RE. IL-17RA is expressed on both hematopoietic and non-hematopoietic cells [178]. Blocking IL-

17RA is a therapeutic option in patients with asthma by decreasing airway hyperreactivity and pulmonary inflammation [296]. IL-17RA has also been implicated in liver fibrosis. In *Schistosoma japonicum*-induced liver fibrosis, IL-17RA$^{-/-}$ mice showed reduced injury with a significant reduction in the expression of IL1β, IL-6, TNF, CXCL1, IL-14 and IL-17E after 8 and 15 weeks of infection [297]. Previous findings indicated that blocking of IL-17RA signaling reduced Ly6c^{low} monocytes in blood, spleen and peritoneal cavity [238]. As mentioned above Ly6c^{low} are important in the pathogenesis of liver fibrosis. Further investigations have shown that reduced Ly6c^{low} monocytes are due to the defect in Ly6c^{high} to Ly6c^{low} transition in the absence of IL-17RA signaling [238]. These findings suggest a contribution of IL-17RA signaling in cell homeostasis and promoting hepatic fibrogenesis..

IL-17RB IL-17RB is expressed on NKT cells [257, 260], activated portal myofibroblasts [298], innate lymphoid cells 2 (ILC2) [263], eosinophils [274], and macrophages [299]. IL-7RB signaling in ILC 2 ’s has been reported to induce collagen deposition during lung fibrosis [263]. It also induces the production of CCL20, CXCL1, and IL-8 to promote pancreatic cancer metastasis [300]. These findings suggest the role of IL-17RB signaling in inducing pro-inflammatory and pro-fibrogenic mediators. L-17RB signals both IL-17B and IL-17E with higher binding affinity for IL-17E when compared to IL-17B [254]. It has been reported that IL-17B dampens IL-17E signaling by restricting its binding to IL-17RB and IL-17RA and stopping IL-6 production. The opposing roles of IL-17B and IL-17E (IL-17E is pathogenic, IL-17B is protective) is observed during mucosal inflammation [287]. Though there is some evidence regarding the expression of IL-17RB and its inflammatory responses, however its contribution to liver fibrosis is not addressed.

IL-17RC is essential to signal IL-17A, IL-17F and IL-17A/F. IL-17RC will not pre-associated with IL-17RA on its surface but, it required IL-17A to induce the formation of the IL-17RA-RC heterodimeric receptor complex. It has been reported that mice deficient in IL-17RC have reduced EAE including reduced expression of CXCL1, CXCL2, CCL2, and CCL5 [301]. The role of IL-7RC in liver fibrosis has not yet been studied.

IL-17RD signals IL-17A [302], associating with IL-17RA to form a heterodimeric receptor complex [303] which is involved in neutrophil recruitment. IL-17RD is expressed in both epithelial and endothelial cells. The role of IL-7RD in liver fibrosis has not yet been studied.

IL-17RE signals IL-17C [304], associating with IL-17RA to form a heterodimeric receptor complex. IL-17RE signaling induces antibacterial mediators during bacterial infection [305]. IL-17RE is also implicated in autoimmune hepatitis by inducing the production of IL-2 from hepatic $CD4^+$ T cells to promote the activation of NK cells and liver damage [306] (ref). Thus, protective role during bacterial liver infections and pathogenic role during autoimmune hepatitis suggest the opposing effects of IL-17RE signaling. Further studies are required to validate the pathogenic effects of IL-17RE signaling in liver fibrosis.

1.6 DRUGS TARGETING IL-17 SIGNALLING

Several monoclonal antibodies have been developed to block IL-17 signaling pathway and its contribution during the development of disease pathogenesis. Two antibodies targeting IL-17A have been tested in clinical trials: humanized IL-17A-specific antibodies Ixekizumab (LY2439821) and Secukinumab (also known as AIN457). Other antibodies targeting IL-17A are in clinical development, such as RG4934 and SCH-900117. Antibody targeting IL-17RA, brodalumab (AMG 827), is also in clinical development. Clinical outcome following the usage of these antibodies under different settings is discussed in section **1.7**. In addition to monoclonal antibodies, significant efforts were made to inhibit IL-17A secretion by blocking key transcription factor such as RORγt [307]. This transcription factor was targeted using synthetic ligands such as Rho kinase inhibitors or ROCK2 inhibitors (SR1001) which can block the activity of RORγt [308]. Further details regarding the contribution of ROCK2 during disease settings is discussed in sections **1.8** and **1.9**. Another potential inhibitor of Th17 cell differentiation is to inhibit the signaling pathway of phosphoinositide 3-kinase δ-subunit (PI3Kδ)[309]. Further investigation will be needed to determine whether any of these signaling pathway inhibitors are suitable to inhibit liver fibrogenesis.

1.7 CLINICAL RESULTS FOLLOWING THE INHIBITION OF IL-17 SIGNALLING.

Inhibition of IL-17A is observed for the first time during Phase I clinical trial using IL-17A-specific antibody (secukinumab), in patients with psoriasis [310]. A single injection of secukinumab reduced the skin lesions covering the surface area, as well as the number of IL-17A producing cells in the skin biopsy samples. Secukinumab was also studied in the patients with rheumatoid arthritis and uveitis [310]. Further results of a Phase II study demonstrated the safety and efficacy of secukinumab for the treatment of ankylosing spondylitis [311]. Patients treated with ixekizumaban, another IL-17A blocking antibody during Phase II trial have shown improved clinical parameters in patients with rheumatoid arthritis [312]. Also, administration of IL-17RA blocking antibody (brodalumab) demonstrated improved skin lesions in patients with psoriasis [313]. In contrast, brodalumab and ixekizumab had no effect in improving intestinal inflammation [314]. The efficacy of these IL-17 inhibitors in liver fibrosis is not yet studied in clinical trials, and the combination of positive and negative outcomes in other inflammatory diseases highlights the complexities of IL-17-targeted treatment. Generally, the circulating level of IL-17A in normal patients is low [205], but IL-17A still contributes to diseases in combination with other cytokines. Targeting other IL-17 family cytokines also needs to be considered for further study. At present, drugs targeting IL-17A and IL-17RA are only in clinical trials. However, targeting other IL-17 family cytokines also need to be considered for effective therapeutic options.

1.8 ROLE OF ROCK2 IN LIVER FIBROSIS

Rho-associated coiled-coil forming protein kinase 2 (ROCK2) –a serine-threonine kinase – is an established mediator of fibrosis in multiple disease settings. ROCK2 is activated by RhoA associated GTPase [315]. ROCK2 activation is also induced by pro-fibrogenic biochemical mediators which includes IL-17A [316] and TGFβ [317]. ROCK contains an N-terminally located kinase domain, a coiled-coiled region followed by a Rho-binding domain (RBD). The kinase activity is turned on when Rho-GTP binds to the Rho-binding domain of ROCK. Kinase activity is off when ROCK is intramolecularly folded between the C-terminal cluster of RBD domain and the PH domain to the N-terminal kinase domain [315].

ROCK-mediated effects are elicited by phosphorylation of down-stream targets including myosin light chain, LIM kinase and cofilin, which are associated with the regulation of actin cytoskeleton dynamics and cell contractility.[318] These processes underpin multiple cellular functions including cell migration activation, differentiation, and survival. Non-selective ROCK1/2 inhibition has been demonstrated to ameliorate fibrosis in experimental models of pulmonary,[319] cardiac,[320] renal,[321] dermal[322] and liver fibrosis[323, 324]. Thus, ROCK activation plays a key role in the development of fibrosis in most organs. Although ROCK1 and ROCK2 exhibit a high degree of homology,[325, 326] they are functionally non-redundant. Both isoforms have implicated roles in fibrogenesis, however, the efficacy of isoform specific targeting, and the distinct mechanisms by which the specific ROCK isoforms contribute to the pathophysiology of hepatic fibrosis remain an enigma.

ROCK2 has been implicated to have a role in liver fibrosis associated with BDL. During BDL, mice treated with anti-aldosterone demonstrated decreased fibrosis associated with the decreased ROCK2 expression [327]. However, the function of ROCK2 in liver fibrosis is not defined. In addition to liver fibrosis, role ROCK2 is also implicated in idiopathic pulmonary fibrosis (IPF), myocardial fibrosis, and peritoneal fibrosis in which Rho-associated kinases are associated in cytoskeletal reorganization and remodeling of participating fibrotic cells [328-330]. GVHD [112, 113] and auto-immune disease [331, 332] like SLE are also associated with ROCK2 induced cellular and molecular functions. In the setting of GVHD, which manifests multiple organ fibrosis, ROCK2 is associated in promoting inflammatory cytokines [322]. Evidence strongly suggests the importance of ROCK2 in fibrosis and other disease settings. Further study is needed to clarify the role of ROCK2 in chronic liver injury and fibrosis.

1.9 ROCK2 ACTIVITY AND ITS DOWNSTREAM TARGETS

Evidence suggests that ROCK2 activity promotes inflammation through various mechanisms **(Figure 1-5).** During GVHD and autoimmune disease like SLE, ROCK2 signaling mediates inflammatory responses through the phosphorylation of downstream transcription factors like STAT3, RORγt, and BCL6 in the targeted cell population [333-336]. Several studies demonstrated that T cells lacking STAT3

expression inhibited the expression of RORγt and downstream IL-17A production in acute and chronic GVHD suggesting the importance of ROCK2 in IL-17 production [112, 113, 337, 338].

ROCK2-phosphorylated STAT3 also induces BCL6 expression in T follicular helper (Tfh) cells to promote B cell activation, differentiation, germinal center (GC) formation and antibody production in secondary lymphoid organs of mice with cGVHD [339, 340]. These findings complement earlier studies suggesting that IL-21 generated from Tfh cells through Bcl6 expression influence B cells to maintain GCs and immunoglobulin deposition [174]. [338, 341-343]. Additionally, IL-21 contributes to the differentiation of IL-17 producing γδT cells [344]. ROCK2 activation is also reported in the polarization of macrophage phenotype from M1 to M2 to induce pathogenic responses which implicate its role in macrophage differentiation [345-347]. Furthermore, in support of this IL-17 which is secreted through ROCK activation also promotes pathogenic differentiation and function of macrophages [348-350]. These findings suggest that ROCK2 induce inflammation through activation, survival, and differentiation. These findings also specified that ROCK2 signaling promotes IL-17 secretion, macrophage differentiation, and IL-21 induced B cell development.

In addition to inflammation ROCK2 activity also contributes to cytoskeletal remodeling. It promotes the phosphorylation of cofilin indirectly through the LIM-Kinase pathway, resulting in actin polymerization, stress fiber formation and cell migration [351, 352]. The activity of cofilin is reversibly regulated through phosphorylation and dephosphorylation, where the phosphorylated form (pcofilin) become inactive [353]. Phosphorylation of cofilin (inactive) is a factor affecting the actin cytoskeleton organization promoting cell adhesion and migration [353]. HSC isolated from steatotic livers displayed increased contractility associated with increased ROCK2 expression and cofilin phosphorylation [354]. Furthermore, cofilin also contributes to the migration of T cells, mesenchymal cells and macrophages [351-355]. These findings suggest that ROCK2 is involved in cell function, differentiation, and migration.

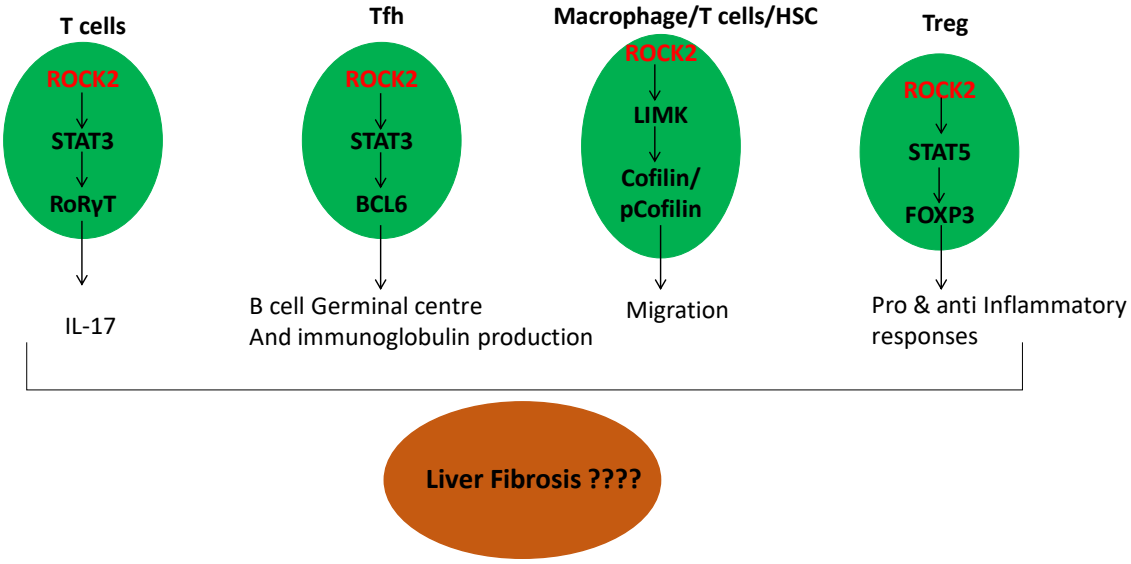


Figure 1-5ROCK2 signalling pathways: ROCK2 activates STAT3/RORγT to induce the expression of IL-17A and promote inflammatory responses. ROCK2-phosphorylated STAT3 also induces BCL6 expression in T follicular helper (Tfh) cells to promote B cell activation, differentiation, germinal center (GC) formation and immunoglobulin production. ROCK2 promotes cell migration through pCofilin induced cytoskeletal remodelling. Conversely, it inhibits the expression of STAT3/Foxp3. However, the contribution of above mentioned ROCK2 signaling pathways in liver fibrosis in unkown.

Though ROCK2 has been implicated in various diseases, surprisingly little is known regarding the contribution of ROCK2 in liver fibrosis. Given the importance of IL-17, monocytes/macrophages and immunoglobulins during liver fibrosis, the above findings highlight the role of ROCK2 activity in the pathogenesis of liver fibrosis. Supporting this, the association of ROCK2 in liver fibrosis has been reported in bile duct ligation (BDL) model [327]. However, the mechanistic contribution of ROCK2 in liver fibrosis is unexplored. KD025 is a potent and selective ROCK2 inhibitor which has been tested in multiple pre-clinical mouse models including GVHD [338, 342] and rheumatoid arthritis [356]) and is in now in clinical trials (rheumatoid arthritis, idiopathic pulmonary fibrosis (IPF) and GVHD) [337]). In multiple studies, ROCK2 suppression of disease was associated with diminished IL-17 and IL-21 production [112, 113]. Hence, further studies are required to demonstrate the efficacy of ROCK2 inhibition in prevention and reversal of liver fibrosis and identify the key mechanisms inducing this effect.

1.10 CONCLUSION

Despite extensive research and advances in the mechanisms responsible for the chronic liver disease, therapies for the effective treatment of liver fibrosis remain limited. However, identification of the cellular and molecular mediators of liver fibrosis highlights some potential therapeutic targets that are suitable for future development.

Considerable efforts have given us a better understanding of the biological functions of IL-17 family cytokines. Though IL-17 cytokines have been implicated in liver fibrosis, many questions remain. It is unclear which cells produce and respond to the various IL-17 family members during liver injury. How the signaling of these cytokines function in the pathogenesis of liver fibrosis still needs investigation. Recognising that monocytes/macrophages are critical mediators of liver fibrosis, the role of different IL-17 family cytokines in regulating monocyte/macrophage recruitment and function during liver fibrogenesis still needs elucidation. Other interesting contributors to liver fibrosis are B cells, and although there is some evidence suggesting a role in liver fibrosis, further studies are required to understand the mechanism of B cell activation and the role of immunoglobulin deposition during liver fibrosis. Furthermore, inhibition of the molecule ROCK2 may help to understand the mechanistic interplay between inflammatory cells, including monocytes/macrophages and B cells, and some of their associated molecular mediators (IL-17, RORγt, STAT3, Bcl6, and pcofilin) in the formation of liver fibrosis.

The experiments described in this thesis increase the understanding of the pathogenesis of chronic liver disease at both cellular and molecular levels and have underlined novel therapeutic approaches to inhibit disease progression. My initial findings highlight the contribution of IL-17RA signaling during chronic liver injury. Our studies also explain the contribution of B cells in liver fibrosis through GC formation and immunoglobulin deposition. Finally, the experiments show the potential of the ROCK2 inhibitor KDO25 in inhibiting the interactions between different cellular and molecular mediators associated with liver fibrosis.

1.11 STUDY OBJECTIVE.

Hypothesis:

Monocytes/macrophages and B cells are critical and distinct contributors to the initiation and perpetuation of liver fibrosis. IL-17 cytokines and ROCK signaling represent mechanistic and targetable pathways for the attenuation of the pro-fibrogenic function in myeloid and B cells.

Aims:

1. To determine the relative contribution of IL-17A, IL-17F, and IL-17E to fibrosis in the TAA hepatotoxin model of CLD (Chapter 3).
2. To determine the mechanistic contribution of B cells to liver fibrosis in response to TAA treatment (Chapter 4).
3. To determine the therapeutic potential of ROCK2 inhibition for the attenuation of TAA-induced fibrosis and delineate mechanistic pathways (Chapter 5).

Chapter 2: Materials and methods

2.1 ANIMALS AND ETHICS

Mouse strains:

All procedures were approved by the QIMR Berghofer Medical Research Institute animal ethics committee (A1503-603M P2096). Female C57Bl/6j (B6. WT, CD45.2, H-2^b), $Ptprc^a$ (B6. Ptprc, CD45.1, H-2^b) mice were purchased from the Animal Resources Centre (W.A., Australia).

Transgenic mouse strains:

IL-17RA$^{-/-}$ (B6) [357], IL-17RB$^{-/-}$ (B6), IL-17A$^{-/-}$ (B6) [358], IL-17F$^{-/-}$ (B6) [240] µmt$^{-/-}$ (B6) [359], and FCγR$^{-/-}$ (B6) mice were bred and housed at QIMR Berghofer Medical Research Institute. IL-17ACre and Rosa26eYFP mice from C57BL/6 background were provided by Dr. B Stockinger (Medical research council NIMR, Mill Hill, UK) and subsequently crossed by our lab at QIMR animal breeding facility to generate IL-17Acre/Rosa26YFP mice for experiments. All mice were housed in sterilized microisolator cages and received acidified autoclaved water (pH 2.5) during experimental housing. Approval for all mouse experiments and procedures obtained from the institutional animal ethics committees of the QIMR Berghofer Medical Research Institute.

IL-17RBKO (IL-17RB$^{-/-}$) chimeras:

Chimeric recipients were generated using WT (B6 or Ptprca) and IL-17RB$^{-/-}$ (B6) mice as both donor and recipient cohorts. Recipients were lethally irradiated (1000 cGy) and transplanted with 5x10^6 bone marrow from donars **(Table 2.1).** Chimeras were left to reconstitute for three months. Reconstitution of donor engraftment was confirmed by analysis of CD45.1 and CD45.2 congenic markers in peripheral blood. Animals were subsequently used for TAA experiments.

2.2 INJURY

Thioacetamide (TAA) administration

Liver fibrosis was induced by treating mice with TAA (Sigma) (supplemented in the drinking water at 300 mg/L) for 1 to 12 weeks. Mice were transferred into clean cages once a week with their water was replaced with fresh TAA water at this time.

KDO25 administration

KDO25 (provided by Bruce Blazar, University of Minnesota) was administered along with TAA. During 1-week TAA treatment KDO25 was administered daily in parallel with TAA. During 8-week TAA treatment KDO25 was administered during last two weeks of TAA treatments. 4 mg of KDO25 in 100 ul of 0.2 % methylcellulose was administered orally into each mouse.

2.3 IMMUNOHISTOCHEMICAL ANALYSIS

Tissue preparation:

For paraffin embedding, the formalin-fixed liver tissues were transferred to 70% ethanol for storage embedding process. Paraffin embedding was done at envoi pathologists, Brisbane. For frozen sections, formalin fixed liver tissues were transferred into 30% sucrose and incubated at 4^{o}C for 24 hrs to avoid crystal formation, and after which the tissues were mounted by placing in optimal cutting temperature (OCT) (Tissue-Tek*) embedding compound and stored at -80^{o}C for long-term storage. Frozen sections were sent to QIMR histology services to cut slides. Slides from frozen sections were warmed to room temperature before staining.

Sirius Red (SR) and hematoxylin and eosin (H&E) staining:

Sirius Red (SR) and hematoxylin and eosin (H&E) staining was performed on deparaffinized of tissue slides using an automated slide staining machine (Leica Autostainer XL).

Pan-keratin (CK-WSS) and myofibroblasts activation (α-SMA) staining:

For CK-WSS (bile duct and ductular epithelium and HPC) or α-SMA (activated stellate cells and myofibroblasts) staining, deparaffinised tissue slides underwent heat-induced antigen retrieval using Tris-EDTA buffer (10mM Tris /1 mM EDTA solution, pH9) in a microwave on full-power for 3 min, then 50% power for 5 min to break methylene bridges that cross-link proteins to expose antigenic sites. After this heat retrieval, tissue slides were allowed to cool and washed twice in TBS. To avoid non-specific background due to endogenous peroxidase present within the tissue, slides were pre-treated with ready to use peroxidase block (DAKO, Glostrup, Denmark) for 10 minutes and washed in TBS before antibody treatment. Slides were incubated for 1 hr with CK-WSS (1:500 dilution in TBS, DAKO) or α-SMA (1:500 dilution in TBS, DAKO) and washed in TBS for 2-3 minutes. Then slides were incubated for 30 minutes with HRP-linked anti-rabbit (Dako EnVision anti-rabbit HRP kit) and anti-mouse (Dako EnVision anti-mouse HRP kit) ready-to-use secondary antibodies for CK-WSS & α-SMA respectively and washed in TBS for 2-3 minutes. Finally, slides were stained with DAB (DAKO) as per manufacturer protocol to visualize the signal from the HRP conjugated secondary antibody.

F4/80 staining:

For F4/80 (monocytes and macrophages) staining, antigen retrieval on deparaffinised tissue slides was performed using enzymatic digestion (Carezyme Trypsin, Biocare Medical, Concord, CA) for 15 min at room temperature (prior to enzymatic digestion trypsin was pre-heated at 30 degrees for 10 minutes) and then washed in TBS for 5 minutes. Tissues were then blocked for 1 hour in 10% fetal calf serum/10% normal goat serum (diluted in TBS) to avoid non-specific binding of antibodies and for 30 min in peroxidase block (1% H_2O_2 in TBS) to avoid non-specific staining. Slides were then incubated for 1 hr with anti-mouse F4/80 antibody (1:400 dilution in TBS, AbDSerotec, Kidlington, UK) followed 5 minutes washing in TBS. Slides were further incubated with secondary antibody Goat anti-rat (1:300 dilution in TBS, Santa Cruz) for 30 min. Finally, slides were incubated with streptavidin HRP (1:300 dilution in TBS, JACKSON Immuno-research) for 30 min and stained using DAB (DAKO).

Peanut agglutinin (PNA) staining:

Peanut agglutinin (PNA, Vector Laboratories) was used to stain germinal center B cells. For PNA staining deparaffinized tissue slides underwent heat-induced antigen retrieval using Tris-EDTA buffer as described above. Tissue slides were allowed to cool and incubated for 10 min with ready-to-use streptavidin/biotin block (Vector Laboratories) and washed. Tissue slides were then incubated with PNA (1:100) for 1hr. Finally, streptavidin HRP (1:300 dilution in TBS, JACKSON Immuno-research) was added for 30 min followed by DAB staining (DAKO).

IL-17A and γδ staining:

Monoclonal antibodies that were used to stain frozen sections are biotinylated γδT cell receptor (Abcam) and anti-IL-17A (Abcam). Before antibody staining, frozen section slides were air dried overnight at room temperature. Slides were rehydrated in TBS for 5 minutes. Endogenous peroxidase activity was blocked by incubating the slides in 1% H_2O_2 (diluted in TBS) for 15 min at room temperature and washed in water for 5 minutes. Non-specific antibody binding is inhibited by incubating the slides in Sniper + 2% BSA (Biocare) for 30 min. Slides were further incubated in biotinylated γδT cell receptor (Abcam) and anti-IL-17A (Abcam) antibodies diluted in Sniper + 2% BSA (1:100) for 60 minutes. Finally, streptavidin HRP (1:300 dilution in TBS, JACKSON Immuno-research) was added for 30 min followed by DAB staining (DAKO).

All slides stained by immunohistochemical methods were imaged with Aperio ScanScope XT Slide Scanner (Aperio, CA) and analyzed with Aperio ImageScope software (Aperio, CA).

Quantitative image analysis

For quantitative image analysis of immunolabelled slides, Aperio ImageScope version 12.1.0.5029 was used. Sirius red (SR) stained collagen fibers were quantified using the algorithm, 'positive pixel count V9' with the following parameters: Hue value=0, Hue width=0.1, colour saturation threshold= 0.2. Total area analyzed per each slide was 5-10 mm^2 not including capsule and portal tracts larger than 150 µm **(Figure 2-1A)**. The area which is SR positive was shown as total positivity (Npositive/Ntotal) in annotations menu.

Similarly, pan-keratin (CK-WSS) stained slides were also quantified using 'positive pixel count V9' with the following parameters: Hue value=0.1, Hue width=0.1, colour saturation threshold= 0.2. Cells which are CK-WSS positive were shown in brown colour **(Figure 2-1B)**. Total area analyzed per each slide is 5-10 mm^2 including portal tracts. The area which is CK-WSS positive was shown as total positivity (NPositive/Ntotal) in annotations menu.

F480 and SMA positive cells were counted manually using Aperio ImageScope. A total of 5 separate areas of 200 µm x 200 µm (away from central vein) were quantified for each slide. The average number of positive cells present in 5 separate areas gives some of total positive cells per slide.

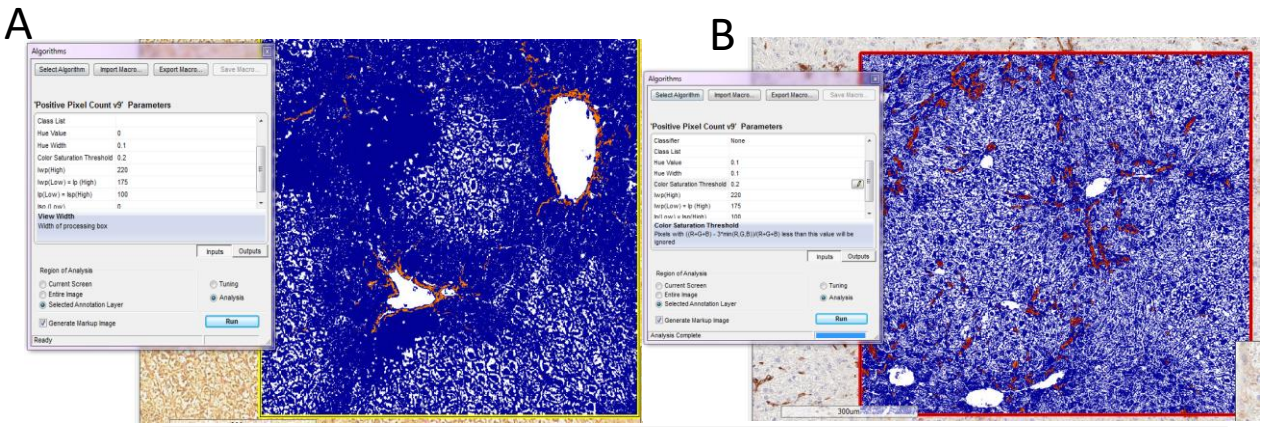


Figure 2-1: Example of quantitative image analysis of (A) Sirius red (SR) and (B)ductular reaction (DR) using aperio image scope.

2.4 CELL ENUMERATION AND FUNCTIONAL STUDIES

Isolation of hepatic leukocytes:

For hepatic leukocyte isolation, livers were perfused with 2 ml of collagenase solution comprising hank's based salt solution (Sigma) containing 1 mg/ml Collagenase Type IV (Sigma) and 0.1 mg/ml DNase I (Sigma). Liver perfusion was performed via the portal vein using a 26G needle. The liver was then removed and placed in a tube containing 7 ml of collagenase solution. A scalpel blade was used to dice the liver into a "slurry" and make a cell suspension. The cell suspension was incubated at 37°C for 40 min, and then pressed through a 70 µM cell strainer (Becton Dickinson). The cells were then washed twice at 500 x g for 5 minutes in cold wash buffer containing PBS and 2% heat-inactivated fetal bovine serum (Gibco

life technologies). The washed cells were resuspended in 12 ml of 33% Percoll (Sigma) in PBS and then centrifuged at 600 x g for 15 min with no brake to separate cells based on their density. The supernatant containing hepatocytes was discarded, and red blood cells were lysed from the resulting nonparenchymal cell pellet by resuspending in Gey's solution **(Appendix A)** and incubating for 2 min. Cells were then washed twice and resuspended in cold wash buffer for flow analysis. Cell yield was quantified using Beckman coulter counter.

Hepatocyte isolation:

Before hepatocyte isolation, mouse was anesthetized by injecting of a mixture of 40mg/gm Ketamine and 2mg/gm Xylazine in 200 µL of saline. The liver was perfused via portal vein with perfusion buffer (PB) **(Appendix A)** for 6 min and then with enzyme buffer (EB) **(Appendix A)** for 6 min at 420 ml/hr using a hepatocyte perfusion pump (Kd Scientific pump) with the perfused blood exiting through the inferior vena cava. After perfusion, the liver was removed carefully and transferred into chilled sterile iscove's modified Dulbecco's medium (IMDM, Sigma) containing 10% heat-inactivated fetal bovine serum (Gibco life technologies). The liver membrane was opened using clean forceps to release digested cells in a single cell suspension. Cells were strained using a 70 µm cell strainer to remove undigested cells and connective tissues. The cells were washed using IMDM **(Appendix A)** at 500 x g for 5 minutes, and hepatocytes will be pelleted down at the bottom. Pellet was resuspended in 10 ml of 50% percoll (Sigma) in 0.15 M NaCl and spun at 1800 x g for 15 min with no brake at 20^{o}C. Intact live hepatocytes will penetrate through percoll layer and were pelleted at the bottom of the tube.

Flow cytometric analysis of surface staining:

Freshly isolated hepatic leukocytes (1 x 10^{6}) were incubated with 30µl of FcBlock 2.4G2 (BD Pharmingen, San Diego, CA) for 10 min at 4 degrees before adding antibody cocktail. Fluorescently-labeled antibodies used to stain the cells are listed in **Table 2.2.** Antibody cocktail was prepared by adding 0.5µl of each antibody in a final volume of 10µl wash buffer **(Appendix A)**. Fc Blocked cells were incubated in 10µl of antibody master mix in the dark for 20 minutes followed by washing with wash buffer for 2 minutes at 2000rpm. Fluorescent data was acquired by flow cytometry using LSR Fortessa (BD Biosciences) and BD FACSDiva (v7.0) software. Acquired live events were analysed using FlowJo software (v9.7 OR USA).

Compensation was accurately set among fluorescence channels specific for each antibody to avoid high degree of emission overlap between the channels. Unstained cells were used to adjust forward scatter (FSC) and side scatter (SSC) to clearly distinguish the live cells from dead. Compensation controls were used for each fluorochrome to adjust voltages. The absolute number of each cell type was calculated by multiplying their frequency by the total number of viable hepatic leukocytes per liver and normalized with liver weights.

Intracellular Staining (ICS) and Flow Cytometry:

Intracellular staining was performed by using BD Cytofix/Cytoperm™ solution kit (BD Bioscience). 5 x 10^6 of hepatic leukocytes were cultured with PMA (50 ng/ml, Sigma) and Ionomycin (500 ng/ml, Sigma) in the culture media (IMDM, Sigma) for 4hr. After 1 hour of PMA/ionomycin stimulation, Brefeldin A (Biolegend) was added to the medium and the cells incubated for a further 3 hours. After stimulation, the cells were surface labeled as described above in the flow cytometry section. The cells were then washed in wash buffer and permeabilized with 250 µl of ready-to-use cytoperm contained in the kit mentioned above for 20 min at room temperature. Permeabilised cells were washed twice using 1ml of 1X permeabilization wash buffer. 1X permeabilization wash buffer was prepared by diluting 20x permeabilization wash buffer in milliQ water to make it 1X. Fcγ receptors were blocked using 2.4G2 before intracellular antibody staining to avoid non-specific staining. All samples were acquired on BD LSR Fortessa (BD Biosciences) using BD FACSDiva (v7.0) compensated as discussed earlier and analyzed with FlowJo software (v9.7 OR USA).

Isolation of monocytes and macrophages with flow cytometry sorting

After hepatic leucocyte isolation, 5-10 x 10^6 cells were surface stained with CD11B-PERCPCY5.5 (Biolegend), F4/80-PE (Biolegend), LY6G-APC-CY7 (Biolegend), SIGLECF-PE.CF594 (Biolegend) antibodies. Antibody master mix was prepared by adding 0.5µl of each antibody in a final volume of 10µl wash buffer. Fcγ receptor block (2.4G2) was added before surface staining to avoid non-specific antibody staining. cells were incubated in 10ul of antibody master mix in the dark for 20 minutes followed by washing with wash buffer for 2 minutes at 2000rpm,

supernatant was discarded, and cells were resuspended in 250 µl of RMPI+FCS **(Appendix A).** Monocytes and macrophages were sorted using Aria II-SSMRC-8.18 (BD Bioscience). Cells that are positively stained for monocytes and macrophages were collected into the flow tubes containing RMPI+FCS. Collection tubes were washed and resuspended for 2 minutes at 2000rpm and cells resuspended in 1ml of trizol reagent for gene expression analysis.

Cytokine administration

For *in vivo* administration, of cytokine, a group of mice were intraperitoneally administered with 0.5 ug of IL-17E (R&D systems) resuspended in 0.1% BSA every day or with control (0.1% BSA in filter-sterilized saline) for up to 4 days. On 5^{th} day liver tissues were taken for IHC and flow cytometry analysis as mentioned above and blood was collected for further analysis.

Blood collection and processing

All mice were terminally eye bleed to collect blood. For serum blood was collected in eppendorf which are allowed to clot at 4 degrees for 3-4 hours. Finally, eppendorf's were spun at 10,000rpm for 10minutes to collect serum.

2.5 GENE EXPRESSION STUDIES

RNA extraction

Ribonucleic acid (RNA) was extracted using Trizol Reagent (Invitrogen, Life Technologies) in the fume hood. RNaseZAP (Thermofisher) was used to clean bench surface and all equipment in order to remove RNA degradation due to RNAse contamination. Liver tissue (0.2-0.4mg) and sorted cell (10×10^5 to 10×10^6 cells) populations were resuspended in 1ml of Trizol and homogenized by repetitive pipetting. 200 µl of chloroform was added to the tubes and vigorously shaken for approximately 15 seconds, followed by incubation at room temperature for 3 min. Samples were then centrifuged at 12,000 x g for 15 min at 4 degrees. The aqueous phase (upper) was carefully transferred to a fresh tube avoiding the white deoxyribonucleic acid (DNA) interphase. 1 µL glycogen (5 µg/µL-Invitrogen) diluted in RNase-free water (Thermofisher) and 500 µl isopropanol was added to the tubes, which were incubated for 30 min at -20^0C or overnight at -70^0C to induce RNA precipitation. Finally, the solution was centrifuged at 4^0C for 15 min at 12,000×g.

The supernatant was discarded and the pellet was resuspended in 1ml of 80% ethanol (diluted in RNase free water-Thermofisher) and centrifuged at 4^0C for 5 min at 7,500×g. Supernatant was discarded and the pellet was air dried and dissolved in 10 µl of RNase-free water (Thermofisher) and stored at −80°C until cDNA preparation. Quantity of RNA was assessed using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

Reverse transcription and cDNA amplification:

In order to remove any DNA contamination from Trizol RNA preparations, a DNAse treatment was performed on each RNA sample (maximum 2 µg of RNA was used in total volume of 7 µL of RNase-free water) by adding containing 1 µl Invitrogen 10X DNAse Buffer and 1 µl Invitrogen DNAse I Enzyme to each sample. The tubes were vortexed and briefly spun down, then incubated for 15 min at room temperature. Following this, 2 µl of 12.5 mM EDTA was added to each sample and the sample incubated in the thermal cycler at 65ºC for 10 min. The samples were then immediately placed on ice for at least 1 min. Next, the reverse transcription (RT) of the RNA was started by adding 1 µl of 5ug/uL promega oligo dT and 1 µl of 10 mM dNTPs to each sample. The samples were incubated in the thermal cycler at 65ºC for 5 min and immediately placed on ice for 1 minute. Then, 7 µl of a master mix containing 4µl of 5X 1st strand buffer, 1µl of 100 mM DTT, 1µl of Promega RNAsin enzyme at 40U/µl and 1µl Superscript III enzyme (50 U, Invitrogen Life Technologies,) was added to each sample. The samples were placed in the thermal cycler (Applied Biosystems) and incubated at 50ºC for 60 minutes, followed by 70ºC for 15 minutes to inactivate the enzyme. The generated cDNA was stored at -20ºC.

Quantitative real-time PCR (RT-qPCR):

Real-time quantitative polymerization chain reaction (RT-qPCR) was performed in microAmp optimal PCR plates (Applied Biosystems) using SYBR Green or TaqMan assays. Forward and reverse primers were resuspended in DNAse/RNAse free water (100nmol/ml). Primer master mix solutions were prepared by pooling forward and reverse primers at a 1/10 dilution in DNAse/RNAse free water; final working primer solution was (10nmol/ml). For SYBR Green, a commercial reagent SYBR Green (Applied Biosystems) mix which contained AmpliTaq Gold® DNA Polymerase (for the DNA amplification) with syber green dye molecules was used. 1 in 10 is a final dilution (diluted in RNase free water) of cDNA per reaction. Reactions were conducted in a real-Time PCR machine (ViiA™ 7 Real-Time PCR

System, Applied Biosystems). The Real-time PCR was performed by cycling the samples under the following conditions: 50°C for 2 min then 95°C for 10 min, then 40 cycles of 95°C for 15s then 60°C (primer annealing step) for 1min, 72°C (Extension step) for 1 min. Product specificity was checked by applying a melting curve from 60°C to 95°C.

At the end of the RT-PCR, the ViiA7 software (QuantStudioTM Real-Time PCR Software) generate the Ct values for each sample which represent the number of cycles required to reach the intensity of fluorescence at the threshold level.

Water was used as negative control in all reactions to verify the absence of DNA contamination in the PCR reaction. In addition, we quantified the transcript of interest in the calibrator which is used as a positive control and as the reference to calculate the relative quantity of the transcript in the samples. Finally, the Hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used as an endogenous control gene to normalize for variations in the starting amount of RNA. All primer pairs were ordered from Integrated DNA technologies **(Table 2-3)**

2.6 PROTEIN ANALYSIS

Liver lysate preparation:

0.02 to 0.06 mg of liver (right lobe) was dissected from the mouse and snap frozen immediately in liquid nitrogen and stored at -80°. For liver lysate preparation, snap frozen liver tissue was ground in 1ml of lysis buffer (T-PER® Tissue Protein Extraction Reagent, Thermofisher) containing protease inhibitor tablets (Sigma). The sample was centrifuged at 10,000 × g for 5 minutes to pellet cell/tissue debris. The supernatant was collected and stored -80°. to continue downstream analysis.

Cytokine Analysis:

Serum and liver cytokine concentrations were determined using the cytometric bead array kit from BD Biosciences Pharmingen and BioLegend'sLEGENDplex™ according to the manufacturer's protocol.

Protein estimation:

Standard (STD) curves were prepared at 0, 50, 75, 100, 150, 200, 300 µg/ml concentrations in MilliQ water. Protein estimation was performed by dissolving samples at 1/100 in MilliQ water to get protein concentration within the standard range. In 96 well plate, add 20 µl diluted STD curve or diluted samples to each well in duplicate. Add 180 µl Bradford solution to each well (2ml Bradford reagent in 8 ml water). Incubate the plate at room temperature for 5 minutes. Read plate for absorbance on plate reader at 595nM.

Western Blots:

Western blotting was performed using SDS-PAGE Electrophoresis System (BIO-RAD). 20 ug of liver lysate was electrophoresed on a 7.5 or 10% Tris gel with Tris running buffer. The separated proteins were blotted on to a PVDF membrane. The membranes were probed with primary antibodies against pSTAT3, pSTAT5, pCofilin, B-Actin and BCL6. HRP conjugated secondary antibodies were then added. Secondary antibodies were detected by using enhanced chemiluminescence.

2.7 STATISTICAL ANALYSIS

Given that most of the data were not normally distributed, we used A non-parametric Man Whitney-U test for comparison of two groups, and a non-parametric Kruskal Wallis (K.W) test for comparison of three or more groups. The data were summarized as median and interquartile range and presented as box and whisker plots. When the data appeared to follow normal distribution, we used a Two-way Anova test to compare the mean difference of two factors simultaneously between three or more groups. Here the data were presented as means and Standard error of the mean (SEM). Two-tailed p-value of <0.05 was considered as statistically significant. All data were analysed using Prism version 7 software (GraphPad).

Table 2-1 Conditions for generating bone marrow chimeras.

Recipient	Donor	Radiation conditioning	Number of donor cells
PTP	PTP	1000cGy	$5X10^6$
PTP	IL-17RB-/-	1000cGy	$5X10^6$
IL-17RB-/-	PTP	1000cGy	$5X10^6$
IL-17RB-/-	IL-17RB-/-	1000cGy	$5X10^6$

Table 2-2 List of antibodies for flow cytometry.

Antibody	Catalogue No.	Clone
CD3-CF594	562286; BD Horizon	145-2C11
CD3e-Pacific Blue	100334; Biolegend	145-2C11
CD3-PE	100308; Biolegend	145-2C11
CD4-Pacific Blue	100428; Biolegend	RM4-5
CD4-PerCP-Cy5.5	550954; BD Pharminogen	RM4-5
CD4-APC	100412; Biolegend	RM4-5
CD8-Pacific Blue	558106; BD Pharminogen	53-6.7
CD8-PE/Cy7	100722; Biolegend	53-6.7
F4/80-PE	123110; Biolegend	BM8
F4/80-PE/Cy7	123114; Biolegend	BM8
IA/IE (MHC II)-FITC	107607; Biolegend	M5/114.15.2
IA/IE (MHC II)-PE/Cy7	107630; Biolegend	M5/114.15.2
IA/IE (MHC II)-Pacific Blue	107620; Biolegend	M5/114.15.2
Ly6G-APC/Cy7	127628; Biolegend	1A8
Mac-1 (CD11b)-PerCP/Cy5.5	101228; Biolegend	M1/70
NK1.1-APC	108710; Biolegend	PK136
SiglecF-AF647	562680; BD Pharminogen	E50-2440
Γδ T- BV421	118120; Biolegend	Gl3
IL-17RA (CD217)-PE	12-7182-82; eBioscience	PAJ-17R
IL-17A-AF700	506914; Biolegend	TC11-18H10
TNF-APC	506308; Biolegend	MP6-XT22
IL-6-PE	504504; Biolegend	MP5-20F3
CCL2/MCP1-AF647	1C479A; R&D Systems	24822
LY6C-Pacific Blue	128014; Biolegend	HK1.4
CD19-FITC	101506; Biolegend	6D5

CCR2-APC	FAB5538A; R&D Systems	475301
CD44-PE	103008; Biolegend	1M7
CD86-AF700	105023; Biolegend	GL-1
IFNγ- PECy7	505826; Biolegend	XMG1.2
IFNγ- Pacific Blue	505818; Biolegend	XMG1.2
IL-17F-PE	12-7471-80; eBioscience	eBio18F10

Table 2-3 List of primers

Primer	Forward	Reverse
IL-17RA	TTCCCAAGCCAGTTGCAGA	CTACGGGCAAGATGCCATTGA
IL-17RB	GGCTGCCTAAACCACGTAATG	CCCGTTGAATGAGAATCGTGT
Collagen 1-A1	CACCCCAGCGAAGAACTCATAC	CCCCTTCTACGTTGTATTCAAACTG
MMP2	TTGCAGGAGACAAGTTCTGGAGATA	CACGACGGCATCCAGGTTAT
MMP9	AGGGGCGTGTCTGGAGATTC	TCCAGGGCACACCAGAGAAC
MMP12	AGCTTTCCAAGTCTGGAGTGATG	GCATGGGCTAGTGTACCACCTT
MMP13	ACAAAGATTATCCCCGCCTCAT	GGCCCATTGAAAAAGTAGATATAG CC
TIMP1	AAGGGCTAAATTCATGGGTTCC	ACAGCCTTGAATCCTTTTAGCATC
CSF-1	CGACATGGCTGGGCTCCC	CGCATGGTCTCATCTATTAT
TNF	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC
MCP-1	GCTGACCCCAAGAAGGAATG	GTGCTTGAGGTGGTTGTGGA
TWEAK	GTACTGTCAGGTGCACTTTGATG	ATCCGAAGGGAAGACCCTGG
HPRT	CCCAGCGTCGTGATTAGCG	GCCACAATGTGATGGCCTCC

Chapter 3: IL-17A and E synergistically contribute to TAA-induced liver fibrosis.

3.1 INTRODUCTION.

Fibrosis is an accumulation of excess extracellular matrix (ECM) due to a perturbed normal wound healing response which results in tissue scarring**.** Prolonged chronic injury may cause dysregulation of normal processes and result in chronic inflammation orchestrated by a network of proinflammatory cytokines/chemokines, inflammatory infiltrates, and tissue resident cells. Events leading to fibrosis consist of (1) injury to the tissue, (2) recruitment of inflammatory cells, (3) release of proinflammatory and fibrogenic cytokines, and finally (4) activation of collagen-producing cells**.** Fibrosis is the outcome in various diseases including chronic respiratory tract infections [360], chronic graft versus host disease (cGVHD) [361], systemic sclerosis [362], psoriasis [363], atherosclerosis [364], and intestinal diseases [365].

Of the proinflammatory cytokine mediators of fibrosis, the interleukin-17 (IL-17) cytokine family has established role in many of the diseases mentioned above including psoriasis [217], GVHD [218], pulmonary fibrosis [243], Crohn's disease [244] as well as liver fibrosis [211, 232, 235, 249, 276, 366]. The IL-17 family is complex consists of multiple cytokine members with multiple receptors (as reviewed earlier in Chapter 1). Of the IL-17 family cytokines, IL-17A, IL-17F, and IL-17E (IL-25) are implicated as mediators of liver fibrosis with distinct mechanisms. IL-17A and IL-17F signal through a common heterodimeric receptor complex IL-17RA/IL-17RC [178]. IL-17E also signals through IL-17RA, coupled with IL-17RB.

Increased levels of IL-17A have been reported in patients with liver fibrosis associated with hepatitis B virus (HBV) infection and primary biliary cholangitis (PBC) [368]. During liver fibrosis, IL-17A is produced by γδT, $CD4^+$, and neutrophils [369, 370] [235]. Mechanistically, during murine hepatotoxin (CCl_4) and physical (BDL) injury models direct IL-17A signaling in hepatic stellate cells (HSCs) has been shown to induce liver fibrosis [190, 211, 366]. IL-17A also promotes liver

fibrosis by inducing pro-fibrogenic mediators such as IL-6, TGFβ, MMP, TNF, and TIMP from both resident and infiltrating cell populations [369, 370] [235]. Additionally, IL-17A is also associated with neutrophil infiltration by increasing the expression of CXCL-2 and ICAM-1 during ischemia-reperfusion injury [236, 237]. The role of IL-17A in monocytic infiltration [191] and pathogenic differentiation and function of macrophages [348] is also observed in-vitro and in rheumatoid arthritis (RA). This suggests the importance of IL-17A signaling in cell recruitment, activation, and survival during liver fibrosis. In addition to IL-17A, IL-17F also utilizes the IL-17RA/IL-17RC receptor complex. While IL-17A is most abundantly studied of the IL-17 cytokine family, IL-17F has also been implicated as profibrogenic mediator. Increased serum IL-17F levels are detected in patients with hepatitis C virus (HCV) associated fibrosis [249]. Additionally, $CD4^+$ and $CD8^+$ T cells producing IL-17F are increased during fibrosis in mice fed with a methionine-choline-deficient diet (MCDD) [371]. Studies in psoriasis identified the pathogenic nature of IL-17F through the induction of IL-6, IL-8 and CCL20 [372, 373]. Although a pathogenic role for IL-17F in autoimmune disease has been described, whether it participates similarly in liver fibrosis, is unknown and further studies are required.

IL-17E signals through a heterodimeric complex of IL-17RA and IL-17RB subunits. The pathogenic [276] and protective effects [210] [279] of IL-17E are reported during parasite and BDL induced liver fibrosis respectively. Pathologically, IL-17E can induce liver fibrosis by upregulating the expression of smooth muscle actin protein (SMA) and collagen deposition [276]. The role of IL-17E is well studied in pulmonary diseases. Mechanistically, IL-17E is reported to induce the production of IL-13 and other inflammatory cytokines (IL-8, MIP-1, MCP-1, and IL-6) during in-vitro and in-vivo studies [259, 274, 374]. Though IL-17E is reported during liver fibrosis, the mechanism by which it contributes to liver fibrosis is not clear.

These findings highlight that blocking of IL-17A could be an effective therapy. However, studies related to the role of IL-17F and IL-17E during liver fibrosis are very limited. There is a need to investigate the relative contribution of IL-17A, IL-17E and IL-17F cytokines for the development of optimal therapeutic strategies. Hence, we examined the relative, mechanistic and temporal contribution of the IL-17 family cytokines to the initiation and perpetuation of liver fibrosis in a murine model of hepatoxin induced liver injury. Of all available multiple pre-clinical murine

models, the thioacetamide (TAA)-induced liver fibrosis model resembles all important properties of human liver fibrosis, which includes inflammation, regeneration, fiber formation and regression [18]. We have previously reported that TAA treatment induced two phases of liver fibrosis [20]. During the early phase (following 1 to 6 weeks TAA treatment) fine fibrosis radiating from the central vein is associated with pericentral F4/80 monocyte accumulation. During the late, progressive phase (following 6 to 12 weeks TAA treatment) septal fibrosis is associated with expansion and migration of a ductular reaction (DR). A niche of DR consists of cholangiocytes and hepatic progenitor cells (HPC), macrophages and HSCs. Also, collagen 1A, which is one of the dominant ECM, is also increased following TAA treatment. Hence, in the current study, we examined the impact of IL-17A, IL-17E, and IL-17F cytokines during two phases of TAA-induced liver fibrosis.

3.2 RESULTS

3.2.1 Differential contribution of IL-17 cytokine family members to TAA-induced liver fibrosis

In initial experiments, we examined the temporal development of liver fibrosis in wildtype (WT) and IL-17RA$^{-/-}$ mice in response to oral administration of TAA. As IL-17RA is a critical receptor component for the signaling of IL-17A, IL-17F, and IL-17E, IL-17RA$^{-/-}$ mice are devoid of signaling of all three of these cytokines. Mice were administered with TAA for periods of 1, 6 and 12 weeks, after which livers were harvested to perform sirius red (SR) staining, and aperio image analysis to visualize and quantify collagen deposition. Livers from IL-17RA$^{-/-}$ mice exhibited significantly reduced TAA-induced collagen deposition at all time points as compared to livers from WT mice **(Figure. 3.1A and D).** As IL-17A and IL-17F utilize a common ternary complex of the IL-17RA and IL-17RC receptors for signaling, we next examined the relative contribution of these cytokines to TAA-induced fibrosis using IL-17A$^{-/-}$ and IL-17F$^{-/-}$ mice. Notably, SR staining revealed a significant reduction in fibrosis in livers from IL-17A$^{-/-}$ mice compared to livers from WT mice at both the 1 and 6-week time points. However, by 12 weeks fibrosis had increased and was equivalent to that measured in livers from TAA treated WT mice, indicating that fibrosis was delayed but not prevented. In contrast, IL-17F deficiency did not alter the induction or progression of fibrosis, as demonstrated by equivalent SR staining of livers from WT and IL-17F$^{-/-}$ mice at all time points **(Figure. 3.1B and D).** IL-17E also signals through a heterodimeric complex of IL-17RA and IL-17RB receptors. Thus to examine the contribution of IL-17E cytokine to TAA-induced liver fibrosis, we used IL-17RB$^{-/-}$ mice lacking IL-17E signaling, whereas IL-17A and IL-17F signaling remain intact. SR staining of livers from TAA treated IL-17RB$^{-/-}$ mice demonstrated significantly reduced fibrosis at all time points **(Figure. 3.1Cand D)**. Of note, however, there was a consistent trend, although not significant, to increased collagen deposition in livers from IL-17RB$^{-/-}$ mice compared to those from IL-17RA$^{-/-}$ mice. To further assess the impact of IL-17 signaling deficiencies on fibrosis, in subsequent experiments we quantified mRNA expression of Collagen 1A (*Col1a*), an established predominant collagen species evoked during liver fibrosis [20]. Quantitative real-time PCR (qRT-PCR) identified *Col1a* was dominantly upregulated at the 6-week time point in the livers from TAA treated WT mice

(Figure. 3.1E). Compared to expression levels in WT mice at this time point, *Col1a* levels were reduced in IL-17RA$^{-/-}$, IL-17A$^{-/-}$, and IL-17RB$^{-/-}$ but not IL-17F$^{-/-}$ mice **(Figure. 3.1F).** However, *Col1a* levels were significantly reduced in IL-17RB$^{-/-}$ when compared to IL-17A$^{-/-}$ suggesting the strong role of IL-17E in inducing *Col1a*.

Taken together these findings demonstrate pathogenic roles for IL-17A and IL-17E in TAA-induced liver fibrosis. In contrast, IL-17F signaling does not appear to contribute to pathogenesis in this model. Notably, IL-17RA deficiency resulted in attenuation of fibrosis at 1,6, and 12 weeks, including prevention of both the early inflammatory phase (1-6 weeks) and the later progressive phase (6-12 weeks) of fibrosis which characterizes this model. Importantly, however, the restricted absence of either IL-17A or IL-17E which require IL-17RA for signaling, only attenuated fibrosis at the 1 and 6-week time points. In both the IL-17A$^{-/-}$ and IL-17RB$^{-/-}$ mice, between weeks 6 and12 there was a rapid progression of fibrosis. Thus, during early phase (1-6 weeks) either IL-17A or IL-17E signaling is sufficient to induce fibrosis demonstrating the redundant nature of these cytokines. However, during the late progressive phase (6-12 weeks), IL-17A and IL-17E both signaling together is required to promote fibrosis demonstrating the synergistic contribution of these cytokines. Hence, the blockade of IL-17RA is required for long-term protection.

3.2.2 Signaling of IL-17 family cytokines is required for the development of the ductular reaction (DR).

An expanded DR has previously been reported to be associated with fibrosis following 6 and 12 weeks of TAA treatment [20]. Thus, we examined the temporal changes in DR during TAA-induced liver fibrosis in WT and IL-17RA$^{-/-}$ mice **(Figure. 3.2A and D)**. CK-WSS (DR) staining and aperio image analysis was performed on the livers harvested following 1,6 and 12-week TAA treatment to visualize and quantify DR expansion. In support of our initial findings, DR expansion was only observed at 6 and 12-week whereas at 1-week only single CK-WSS^{+} HPC were evident at the portal tract. When compared to WT livers, DR expansion was substantially altered in the livers from IL-17RA$^{-/-}$ mice demonstrating reduced DR expansion. We next examined the relative contribution of IL-17A and IL-17F (which both commonly signal through IL-17RA) cytokines to DR in the livers of IL-17A$^{-/-}$ and IL-17F$^{-/-}$ mice following 6 and 12-week TAA treatment **(Figure. 3.2B and D)**. Notably, IL-17A$^{-/-}$ mice displayed reduced DR expansion at 6-weeks. However, by

12-weeks expansion of DR had increased and was equivalent to WT mice. In contrast, IL-17F deficiency did not alter DR expansion, as demonstrated by equivalent CK-WSS staining in the livers from WT and IL-17F$^{-/-}$ mice at all time points **(Figure. 3.2B and D).** We next examined the contribution of IL-17E (also signals through IL-17RA along with IL-17RB) signaling to DR in the liver of IL-17RB$^{-/-}$ (lack IL-17E signaling) mice following TAA treatment at similar time points. IL-17RB$^{-/-}$ mice demonstrated significantly reduced DR expansion at 6-weeks, with reduced trend at 12-week time point **(Figure. 3.1C and D)**. However, DR expansion was found to be significantly increased in livers from the IL-17RB$^{-/-}$ mice compared to those from IL-17RA$^{-/-}$ mice, once again suggesting the combined role of both IL-17A and IL-17E during 12-weeks injury.

Taken together these findings demonstrate that IL-17RA deficiency resulted in attenuation of DR expansion at both 6 and12 weeks. However, the restricted absence of either IL-17A or IL-17E which require IL-17RA for signaling only attenuated DR expansion at 6-weeks but not at 12-weeks. Thus, either IL-17A or IL-17E signaling is sufficient to initiate DR. However, at 12-week reduced DR only in IL-17RA$^{-/-}$ suggests that both IL-17A and IL-17E act synergistically to maintain DR expansion. In contrast, IL-17F signaling does not appear to contribute to the DR.

3.2.3 The IL-17 family cytokines are required for the activation of myofibroblasts.

Hepatic stellate cells (HSCs) or myofibroblasts are the predominant ECM producing liver population and play a crucial role in the pathogenesis of liver fibrosis [31-33]. Activated myofibroblasts are characterized by the expression of alpha-smooth muscle actin (αSMA). αSMA^{+} activated myofibroblasts are seen at the pericentral regions following 1-week TAA treatment and after 6 weeks this staining progresses to septa linking the centrilobular regions **(Fig 3.1A)**. Our previous finding demonstrated that IL-17F was not found to be a critical mediator in this (TAA) model. We thus investigated the individual, and combined contribution of IL-17A and IL-17E cytokines in activating myofibroblasts by using livers from 1 and 6-week TAA treated IL-17RA$^{-/-}$, IL-17A$^{-/-}$, IL-17RB$^{-/-}$ and WT mice **(Figure. 3.3A and B**). Livers from 1-week TAA treated IL-17RA$^{-/-}$, IL-17A$^{-/-}$ and IL-17RB$^{-/-}$ mice exhibited significantly reduced SMA^{+} myofibroblasts at pericentral regions when compared to WT mice. However, pericentral SMA^{+} cells were significantly increased in IL-17A$^{-/-}$

when compared to IL-17RA$^{-/-}$ and IL-17RB$^{-/-}$ (commonly inhibits IL-17E signaling), implicating the strong contribution of IL-17E in activating myofibroblasts at the 1-week time point. Additionally, 6-week TAA livers from IL-17RA$^{-/-}$, IL-17A$^{-/-}$, and IL-17RB$^{-/-}$ mice demonstrated reduced SMA^{+} myofibroblasts in comparison to livers from WT mice.

Thus, we demonstrated that IL-17A or IL-17E contributes to myofibroblasts activation at 1-week. Notably, reduced SMA^{+} cells observed in the absence of IL-17RA and IL-17RB when compared to IL-17A suggests the predominant role of IL-17E in promoting myofibroblast activation at 1-week. However, at 6-week both IL-17A and IL-17E equally contribute to myofibroblasts activation. Together, our findings suggest that both IL-17A and IL-17E cytokines contribute to myofibroblast activation at all times points examined, though with differing intensity.

3.2.4 The IL-17 signaling is required for peri-central monocytic accumulation.

To examine the temporal changes of inflammatory infiltrates during TAA-induced liver injury, we initially enumerated multiple myeloid (monocytes-CD11b^{+}F4/80^{+}, eosinophils -Siglec-F, and granulocytes-Ly6G) and lymphoid (T cells-CD3 and B cells-CD19) cell populations in WT mice following 1, 6 and 12-weeks of TAA treatment. Flow cytometric analysis on liver mononuclear cells demonstrated increased myeloid cells following 1-week TAA treatment whereas, lymphoid cells did not demonstrate any significant difference at all time points. This implicates the contribution of myeloid cells during 1-week TAA **(Figure. 3.4A) (Gating strategy in supplementary figure 3.1).** We next sought to determine the individual and combined contribution of IL-17A and IL-17E cytokines in promoting myeloid cell infiltration using WT, IL-17RA$^{-/-}$, IL-17A$^{-/-}$ and IL-17RB$^{-/-}$ mice following 1-week TAA. Notably, IL-17RA$^{-/-}$ and IL-17RB$^{-/-}$ mice demonstrated substantially decreased monocytes, eosinophils, and granulocytes when compared to WT mice. Conversely, monocyte, eosinophil, and granulocyte numbers were equivalent between IL-17A$^{-/-}$ and WT mice **(Figure. 3.4B-D).** Collectively, these data demonstrated reduced myeloid infiltration in the absence of IL-17RA and IL-17RB signaling (which commonly inhibits the signaling of IL-17E cytokine) but not in the absence of IL-17A, highlighting the importance of IL-17E cytokine in myeloid cell infiltration.

Of all myeloid cells, monocytes are reported to be critical mediators of liver fibrosis. We also previously reported an increased number of F4/80^{+} monocytes around the central vein (CV) associated with fine centrilobular fibrosis following 1-week TAA [20], which is the predominant site of tissue injury in this model. Therefore, to examine the contribution of IL-17A and IL-17E cytokines in recruiting F4/80 cells at CV regions, we performed F4/80 IHC staining on the livers of 1-week TAA treated WT, IL-17RA$^{-/-}$, IL-17A$^{-/-}$ and IL-17RB$^{-/-}$ mice **(Figure. 3.4E and F)**. Livers from IL-17RA$^{-/-}$ and IL-17RB$^{-/-}$ mice demonstrated a marked reduction in pericentral F4/80+ monocyte accumulation when compared to livers from WT mice. The pericentral accumulation of F4/80^{+} monocytes was also significantly reduced in IL-17A$^{-/-}$ in which absolute monocyte number was unperturbed. These findings demonstrated reduced F4/80^{+} accumulation observed in the absence of IL-17RA and IL-17RB suggesting that IL-17E signaling promotes the accumulation of F4/80^{+} monocytes around the CV. However, in the absence of IL-17A the degree of monocytic infiltration was unchanged but there was reduced accumulation of F4/80^{+} cells around the CV, which suggests that IL-17A signaling has role in localising monocytes at the CV.

3.2.5 IL-17E/IL-17RB signaling promotes CCL2 production for the recruitment of monocytes.

Having noted the association of monocytes with TAA injury, we next examined the mechanism of monocyte recruitment. It has been reported that monocyte chemoattractant protein (MCP-1/CCL2) is a potent chemokine required for monocyte recruitment [76, 375]. We previously reported increased CCL2 levels following 1-week TAA when compared to naïve animals [20]. Therefore, to examine the contribution of IL-17A and IL-17E cytokines in inducing the production of CCL2 we measured liver CCL2 protein levels in WT, IL-17RA$^{-/-}$, IL-17A$^{-/-}$, and IL-17RB$^{-/-}$ mice following 1-week TAA. CCL2 concentration was similar between IL-17A$^{-/-}$ and WT, but IL-17RA$^{-/-}$ and IL-17RB$^{-/-}$ mice demonstrated significantly reduced levels of CCL2 **(Figure 3.5A)**. As mentioned earlier, IL-17E utilizes both the IL-17RA and IL-17RB receptor components for signaling we thus hypothesized that IL-17E signaling might promote CCL2 production. Based on this hypothesis, recombinant IL-17E was administered intraperitoneally (I.P) to naïve WT mice daily for four days (5µg/day) before harvest. Serum CCL2 levels were markedly increased in IL-17E

administered WT mice **(Figure. 3.5B)**. In tandem with increased CCL2 levels, flow cytometric analysis on collagen digested livers demonstrated increased monocytes as well as increased eosinophils and granulocytes **(Figure. 3.5C)**, thereby suggesting that IL-17E promotes CCL2 production for the recruitment of monocytes and other myeloid cells. Additionally, to examine the cellular source for CCL2 production, we performed intracellular staining (ICS) on cells from collagen digested WT and IL-17RB$^{-/-}$ livers (taken 6 weeks after TAA administration), following 4-hour in-vitro LPS re-stimulation (20ng/ml). Notably, macrophages and eosinophils were demonstrated to be major producers of CCL2 through IL-17RB signaling **(Figure. 3.5D)**. Additionally, IL-17E treated mice exhibited increased liver collagen deposition (SR) and myofibroblast activation (SMA) **(Figure. 3.5E).** Thus, our study demonstrated the contribution of IL-17E cytokine in promoting CCL2 production from macrophages following 1-week TAA.

3.2.6 IL-17E/IL-17RB signaling in non-hematopoietic cells contributes to liver fibrosis.

Further investigations were made to determine whether the protection against TAA-induced liver fibrosis observed in the absence of IL-17E signaling was mediated by cells located in either the hematopoietic or non-hematopoietic compartments. Chimeras were generated by transferring bone marrow cells from WT or IL-17RB$^{-/-}$ mice into lethally irradiated WT and IL-17RB$^{-/-}$ recipients, allowing deficient signalling to be confined to either the hematopoietic or non-hematopoietic cells. After a four-month period to allow haematopoietic reconstitution, liver fibrosis was induced in these mice following 6-weeks TAA treatment. Recipients lacking IL-17RB signaling in the non-hematopoietic compartment or both compartments (WT →IL-17RB$^{-/-;}$ 17RB$^{-/-}$ →17RB$^{-/-}$) developed significantly less fibrosis than those in which the signaling was intact in both compartments or the hematopoietic compartment in isolation (WT→WT; IL-17RB$^{-/-}$ →WT)(**Figure. 3.6A-B**). Reduced fibrosis was also associated with reduced accumulation of SMA^{+} myofibroblasts. To further assess the contribution of IL-17E signaling in hematopoietic or non-hematopoietic compartments subsequent experiments were performed to quantify mRNA expression of IL-17RB in the whole liver, isolated liver mononuclear cells (MNCs), and hepatocytes from naive and 1-week TAA treated WT mice. qRT-PCR identified that IL-17RB was dominantly expressed by the whole liver (consists 80% hepatocytes) and hepatocytes and to a lesser extent by MNCs (**Figure. 3.6C**). Together, our findings suggested that IL-17E/IL-17RB signaling in non-

hematopoietic cells contributes to TAA-induced liver fibrosis. Notably, within the non-hematopoietic compartment, predominant expression of IL-17RB on hepatocytes suggests that IL-17E signaling on these cells may contribute to liver fibrosis.

3.2.7 IL-17A and IL-17E cytokine promote the expression of proinflammatory cytokines.

Various pro-inflammatory cytokines contribute to liver fibrosis. Thus, we examined the contribution of IL-17A and IL-17E cytokines in inducing the expression of pro-inflammatory cytokines by using livers from WT, IL-17RA$^{-/-}$, IL-17A$^{-/-}$, and IL-17RB$^{-/-}$ mice following 1 & 6-week TAA treatment. It has been reported that IL-17A directly or through the induction of IL-6 or TNF from macrophages activates HSCs to induce collagen deposition [211, 376, 377]. Consistent with reduced liver fibrosis, 1-week TAA treated IL-17RA$^{-/-}$, IL-17A$^{-/-}$ and IL-17RB$^{-/-}$ mice displayed reduced *IL-6* and *Tnf* expression when compared to WT **(Figure. 3.7A)**. We also noted that progenitor TNF-related weak inducer of apoptosis (*Tweak*) which is found to be associated with the DR [378] was increased in the livers of WT mice following 6-weeks TAA, but reduced in livers from IL-17RA$^{-/-}$, IL-17A$^{-/-}$ and IL-17RB$^{-/-}$ mice **(Figure. 3.7B)**. These data suggest the role of IL-17A and IL-17E cytokines in promoting the production of *Tnf* and *IL-6* during 1-week TAA; *Tnf* and *Tweak* during 6-week TAA. It has been reported that macrophages are the major producers of pathogenic TNF and IL-6 during liver fibrosis [376]. Hence, reduced *Tnf* and *IL-6* observed in the absence of IL-17E signaling is due to an overall reduction in absolute monocyte number. However, reduced *Tnf* and *IL-6* in livers from IL-17A$^{-/-}$ mice, with preserved monocyte number, is the direct result of ablated IL-17A signaling.

We previously reported that the DR niche during 6-week TAA is associated with macrophage accumulation [20]. Colony stimulating factor (CSF-1) is essential for survival and differentiation of macrophages from monocytes [69] [379]. Therefore to examine whether IL-17A and/or IL-17E influence Csf-1 *expression,* we measured *Csf-1* mRNA levels in the livers from WT, IL-17RA$^{-/-}$, IL-17A$^{-/-}$, and IL-17RB$^{-/-}$ mice following 1-week TAA treatment. Our qRT-PCR results demonstrated increased *Csf-1* expression following TAA treatment and observed a reduced trend in CSF-1R mRNA in livers from IL-17RA$^{-/-}$ and IL-17RB$^{-/-}$ but not in IL-17A$^{-/-}$ mice, once again emphasizing the importance of IL-17E signaling **(Figure. 3.7C)**. Also, when studied

using chimeric mice to restrict IL17RB signalling to either hematopoietic or non-hematopoietic compartments or both, there was a greater reduction in C*sf-1* expression following 6-week TAA treatment when IL-17RB signalling was blocked in the non-hematopoietic compartment compared with hematopoietic cells, but the greatest reduction occurred when all cells were IL17RB$^{-/-}$ **(Figure. 3.7D)**. We have reported previously that CSF-1R blockade with the anti-CSF-1R monoclonal antibody M279 results in reduced DR development with a significant reduction in collagen deposition and progressive fibrosis [20]. Therefore to examine the pathogenic role of CSF-1 in TAA-induced liver fibrosis, WT mice were injected (I.P) with M279 (400ug/ml of M279/day) or control antibody for 3 days along with 1-week TAA. Consistent with previous findings M279 treated mice demonstrated reduced fibrosis (Sirius red) associated with reduced myofibroblast activation (SMA^{+}) and pericentral monocyte accumulation (F4/80^{+}) **(Figure. 3.7E)**. Flow cytometric analysis of collagen digested livers highlighted decreased inflammatory monocytes (Ly6c^{hi}) and macrophage precursors (Ly6c^{lo}) **(Figure. 3.F)**. Together our findings suggest that the signaling of IL-17RB mainly in non-hematopoietic cells promotes macrophage differentiation through *Csf-1* whereas IL-17A signaling induces the production of *Tnf* and *IL-6* through IL-17RA signaling on hematopoietic cells **(supplementary figure 3.3)**.

3.2.8 IL-17A is predominantly expressed by γδ T cells following TAA treatment.

Having demonstrated that IL-17A signaling was associated with TAA-induced liver fibrosis with an important role in inducing proinflammatory cytokines, we aimed to define which cell population(s) were the source of IL-17A and could be responsible for the observed pathogenic effect. Using IL17eYFP fate map reporter mice in which yellow fluorescent protein (YFP) is expressed constitutively once a cell has made IL-17A [380], we digested liver tissue and phenotyped the IL-17A producing cell populations therein. We identified CD3^{+} cells as the predominant IL-17A expressing population within the liver, which doubled in frequency following 1-week TAA treatment **(Figure. 3.8A)**. Further analysis of the CD3^{+} subsets (γδ T, CD4^{+} T, CD8^{+} T, CD4^{neg}CD8^{neg} T, natural killer (NK) and NKT cells) highlighted that γδ T cells were major IL-17A producing cells and found to be increased in number **(Figure. 3.8B)** and frequency **(Figure. 3.8C)** following 1 and 6 week TAA treatment. Knowing that MAIT cells are also one of the major IL-17A producing cells, we looked for IL-17A producing MAIT cells in livers from WT mice. 88% of MAIT cells

were $CD4^{neg}CD8^{neg}$ which are meager fraction when compared to γδ T cells **(Figure. 3.8D)**. As mentioned earlier there was no striking difference in the frequency of IL-17A -producing $CD4^{neg}CD8^{neg}$ cells following 1 and 6 weeks TAA.

3.2.9 Localization of IL-17A producing γδT cells around the central vein.

γδ T cells are differentiated to IFNγ or IL-17A producers during the developmental stages in the thymus [381]. Additionally, IL-17A producing γδ T cells co-express IL-17F [382]. Also, IL-17A synergize with TNF during inflammation [383]. Therefore, we characterized different γδ T cell subsets by performing ICS on naïve, and 1-week TAA treated WT following 4hr PMA (100ng/ml) + ionomycin (50ng/ml) stimulation. Notably, following 1-week TAA the increased differentiation of γδ T cells was restricted to the IL-17A and not the IFNγ producers **(Figure. 3.9A)**. Additionally, the frequency of IL-17A producing γδ T cells was doubled with no change in the proportion of cells co-expressing IL-17A and IL-17F **(Figure. 3.9B)**. However, the frequency of $IL\text{-}17A^{+}$ TNFγδ T cells were increased 2-fold following 1-week of TAA treatment **(Figure. 3.9 C-D)**. Importantly, immunohistochemistry data revealed that in addition to $F4/80^{+}$ monocytes and SMA^{+} myofibroblasts [20], IL-17A-producing γδ T cells were also located around the central vein following 1 week of TAA treatment **(Figure. 3.9E)**.

3.2.10 IL-17E induced CCL2 is important in recruiting IL-17A producing γδT cells.

To determine the contribution of IL-17 signaling in recruiting γδT cells, immunohistochemistry was performed in the livers of WT, $IL\text{-}17RA^{-/-}$, $IL\text{-}17A^{-/-}$ and $IL\text{-}17RB^{-/-}$ mice following 1-week TAA. The recruitment of γδ T cells around central vein were unchanged between the livers of WT and $IL\text{-}17A^{-/-}$ mice. Notably, $IL\text{-}17RA^{-/-}$ and $IL\text{-}17RB^{-/-}$ displayed reduced γδ T cells around the central vein when compared to WT **(Figure. 3.10A)**. As mentioned earlier, IL-17E utilizes both the IL-17RA and IL-17RB receptor components for signaling, hence reduced γδ T cells exhibited in the absence of IL-17RA and IL-17RB receptors demonstrate the importance of IL-17E signaling in promoting γδ T cells recruitment. It has been reported that CCL2 will promote the recruitment of IL-17A+γδ T cells through their expression of CCR2 [384]. In above experiments we demonstrated the role of IL-17E cytokine in CCL2 production **(section 1.2.5).** Therefore, the contribution of CCL2 in the recruitment of γδT cells was investigated in $CCL2^{-/-}$ mice following TAA

treatment. In comparison to WT mice, 1-week TAA treated CCL2$^{-/-}$ mice displayed markedly reduced γδT around the CV **(Figure. 3.10B)**. Additionally, flow cytometric analysis also confirmed the expression of CCR2 in IL-17A^{+} γδ T cells **(Figure. 3.10C)** providing evidence that these cells were recruited to the liver in a CCL2/CCR2 dependent manner. As previously illustrated in **Figure 3.9C**, the frequency of IL-17A^{+} TNF^{-}producing γδ T cells substantially increased following 1-week TAA. Notably, in the absence of IL-17A, γδ T cells still produce TNF in response to injury **(Figure. 3.10D).** TNF is important in activating myofibroblasts to induce collagen deposition [385] . Hence, TNF production observed in the absence of IL-17A singling will still induce myofibroblasts activation, explaining why livers from 1-week TAA IL-17A$^{-/-}$ mice displayed increased SMA^{+} cells when compared to IL-17RA$^{-/-}$ and IL-17RB$^{-/-}$ mice, although they were significantly reduced when compared to WT mice **(Figure 3.3).** Together, our data demonstrate that though TNF is important in myofibroblasts activation its production from γδ T cells in the absence of IL-17A signaling is insufficient to induce maximum myofibroblasts activation, highlighting the importance of synergistic production of IL-17A and TNF from γδ T cells during liver fibrosis.

3.3 DISCUSSION

The IL-17 family of cytokines have been shown to play an important role in chronic fibrosing diseases including chronic liver disease. This study provides new insight into the mechanistic and relative contribution of different IL-17 family cytokines during different phases of fibrosis in the chronic injury model using TAA administration. We specifically demonstrated that IL-17A and IL-17E act synergistically during the pathogenesis of TAA-induced liver fibrosis. Blockade of IL-17A and IL-17E individually demonstrated reduced fibrosis following 1 and 6-week TAA treatment. However, combined blockade of IL-17A and IL-17E (by blocking IL-17RA) was required for long-term protection (12-week TAA).

Monocytes are established as critical mediators of fibrosis although the nature of specific monocyte population(s) and the mechanism by which these cells promote fibrosis remain incompletely defined [386, 387]. Our data illustrate that IL-17 has an important role in the recruitment and differentiation of monocytes with chronic inflammatory injury induced by TAA. In the absence of IL-17RA (IL-17RA$^{-/-}$) signaling there was reduced monocytic infiltration following 1-week TAA treatment associated with reduced myofibroblast activation and collagen deposition. Additionally, the absolute number of eosinophils and granulocytes were also reduced in IL-17RA$^{-/-}$ mice. We previously demonstrated that fibrosis induced following 6 and 12-week TAA treatment was associated with DR expansion and macrophage differentiation [20] and we extended those findings here by showing significantly reduced DR expansion in IL-17RA deficient mice, implicating IL-17 signalling in the development of the DR. Thus, IL-17 family members have a key role in both the early inflammatory phase of injury as well as the later progressive phase characterized by the development of extensive fibrosis with septal formation associated with an expanded DR.

IL-17RA is a common receptor for IL-17A and IL-17F as well as IL-17E (IL-25). Our study highlights the individual and combined mechanistic contribution of each these cytokines in promoting TAA-induced liver fibrosis during both early and late phases. . Following 1-week TAA, mice deficient in IL-17A demonstrated reduced monocyte accumulation around the CV while the absolute number in the liver was unchanged, suggesting a role for IL-17A signaling in sequesteration of monocytes around the

CV (the site of major injury) rather than recruiting them as has been found in other models [388]. Similarly and in contrast to recent findings observed in ischemia-reperfusion (IR) liver injury, neutrophil infiltration also remained unchanged in IL-17A$^{-/-}$ mice following TAA [236, 237] demonstrating that in the TAA model, IL-17A does not contribute to myeloid cell recruitment. Mice deficient in IL-17A also demonstrated delayed fibrosis and development of the DR at 6 weeks, but this was only temporary and by 12 weeks the degree of injury was similar to that seen in WT animals, indicating a redundant role for IL-17A in the progressive phase.

Our studies provide insight into how IL-17A may promote fibrogenesis. Some reports have demonstrated the role of IL-17A in inducing pro-fibrogenic mediators such as IL-6, TGFβ, and TNF, from resident and inflammatory cells [235] and we confirmed IL-17A-dependent expression of the inflammatory cytokines IL-6 and TNF. TNF induced from macrophages in response to IL-17A increases the activation and survival of HSCs [389]. Additionally, IL-17A can also activate HSCs in STAT3 dependent manner [211]. IL-17A and TNF have synergistic interactions, where IL-17 enhances the expression of TNF receptor [228]. TNF improves the effect of IL-17 by increasing the levels of protein expression. Combined suppression of these synergistic cytokines results in enhanced anti-inflammatory effects [225]. We have shown that IL-17A^{+}TNF γδT cells were increased following TAA treatment, but since blockade of IL-17A alone (IL-17A$^{-/-}$ mice) did not prevent fibrosis these appear to represent only part of the stimulatory response during injury and are insufficient to induce strong HSC activation.

During the late phase of injury we previously reported that expansion and migration of the DR is associated with macrophage interaction, with approximately 50% of the liver macrophages in contact with the DR [20]. Although IL-17A has been implicated in the pathogenic differentiation of macrophages suggesting a potential contribution to late phase fibrosis (6-12 weeks) [348-350], we found that IL-17A deficient mice had normal expression of CSF-1, which is essential for macrophage differentiation and survival [379]. IL-17A did have an effect on TWEAK expression, the major growth factor for HPC and the DR [390] (which is produced by macrophages and NK cells), likely the cause of delayed DR development seen in IL-17A$^{-/-}$ mice. Next, we extended our study by examining which cells produced IL-17A and found that γδT cells were the major producers of this cytokine. IL-17A-expressing γδT cells

have been implicated in other diverse liver diseases including viral hepatitis [122, 129], *Schistosoma japonicum* infection [130] and chronic biliary disease [131]. Our finding of increased IL-17A-producing γδT cells in the centrilobular regions of maximal injury further support a role for these cells in the liver injury responses.

While the pathogenic role of IL-17A was confirmed, IL-17F was found to be non-pathogenic during TAA-induced liver fibrosis at all time points.

Our studies indicate that IL-17E appears to have a role in the early and progressive liver injury in this model. IL-17E signals through a heterodimer of IL-17RA (also used for IL-17A and IL-17R signalling) and co-receptor IL-17RB, the latter allowing us to selectively inhibit IL-17E signalling using IL-17RB$^{-/-}$ mice in the experiments described above. Though IL-17E signaling is reported to protective in fulminant liver injury [279], we identified its role as pathogenic during TAA-induced liver fibrosis. IL-17RB$^{-/-}$ mice demonstrated reduced monocytic (F4/80) infiltration and centrilobular accumulation following 1-week TAA treatment associated with reduced activation of HSC and reduced early collagen deposition around the CV. IL-17E had a role in monocytic infiltration. CCL2 has been reported to be an essential chemokine for monocyte recruitment [68, 71-75] and is also important in CCl_4-induced liver fibrosis [76]. However, our earlier studies of TAA-induced liver fibrosis found that CCL2 deficiency attenuated early monocyte infiltration and fibrosis but was not mediated exclusively by CCR2 ligation [20].. The role of IL-17 family cytokines in inducing chemokines during liver fibrosis is not known but some evidence suggests that IL-17E promotes the induction of chemokines (CCL2, MIP-1) that lead to monocyte recruitment [259]. In the current studies, reduced CCL2 levels in IL-17RA$^{-/-}$ and IL-17RB$^{-/-}$ but not IL-17A$^{-/-}$micehighlight the importance of IL-17E in CCL2 expression. We have shown that macrophages, which are major producers of CCL2, demonstrated reduced CCL2 production in the absence of IL-17E signaling. Additionally, our studies identified increased CCL2 and increased monocytic infiltration in recombinant IL-17E administered mice, increased activation of HSC and sinusoidal fibrosis within days of administration. The CCL2/CCR2 axis has also been demonstrated to promote infiltration of IL-17 producing γδT cells in the tumor microenvironment [134, 384]. The role of this CCL2/CCR2 axis in recruiting γδT cell during liver fibrosis has not been clear and we determined that IL-17A-producing γδT cells were recruited in a CCL2/CCR2 dependent manner. Our data

also revealed that IL-17RB signaling, particularly in non-hematopoietic cells predominantly hepatocytes, contributed to liver fibrosis. However, the role of IL-17E dependent IL-17RB signaling in hepatocytes is yet to be known.

CSF-1 signaling, needed for macrophage differentiation and survival [379], appears critical for the progression of liver fibrosis and development of the DR from our earlier studies [20]. Here, we extended our understanding by showing the key role of IL-17RB signaling, in inducing the production of CSF-1 promoting late phase injury at 6-12 weeks. Studies in chimeric animals showed that CSF-1 production was greater from non-hematopoietic cells, than recruited hematopoietic cells. Similar to IL-17RA$^{-/-}$ and IL-17A$^{-/-}$ animals, reduced expression of IL-6 and TNFwas also observed in IL-17RB$^{-/-}$ mice due to reduced inflammatory cells that produce these cytokines. We were unable to determine the cellular sources of IL-17E since the relevant mice were not available, but a similar strategy to that used in IL-17A interogation could be used. IL-17E is typically expressed by a greater range of cells including epithelial cells and granulocytes [255].

Thus, our study demonstrates that IL-17E signaling promotes monocyte and IL-17^{+}γδT recruitment through the induction of CCL2 whereas IL-17A signaling promotes the activation of HSCs through the induction of pro-inflammatory cytokines IL-6 and TNF as well as other mediators such as TWEAK during the early phase. IL-17RB signaling on hepatocytes is associated with production of CSF-1 leading to macrophage differentiation and the development of fibrosis. However, inhibition of both IL-17A and IL-17E by blockade of IL-17RA signaling is required for long-term protection from progressive septal fibrosis.

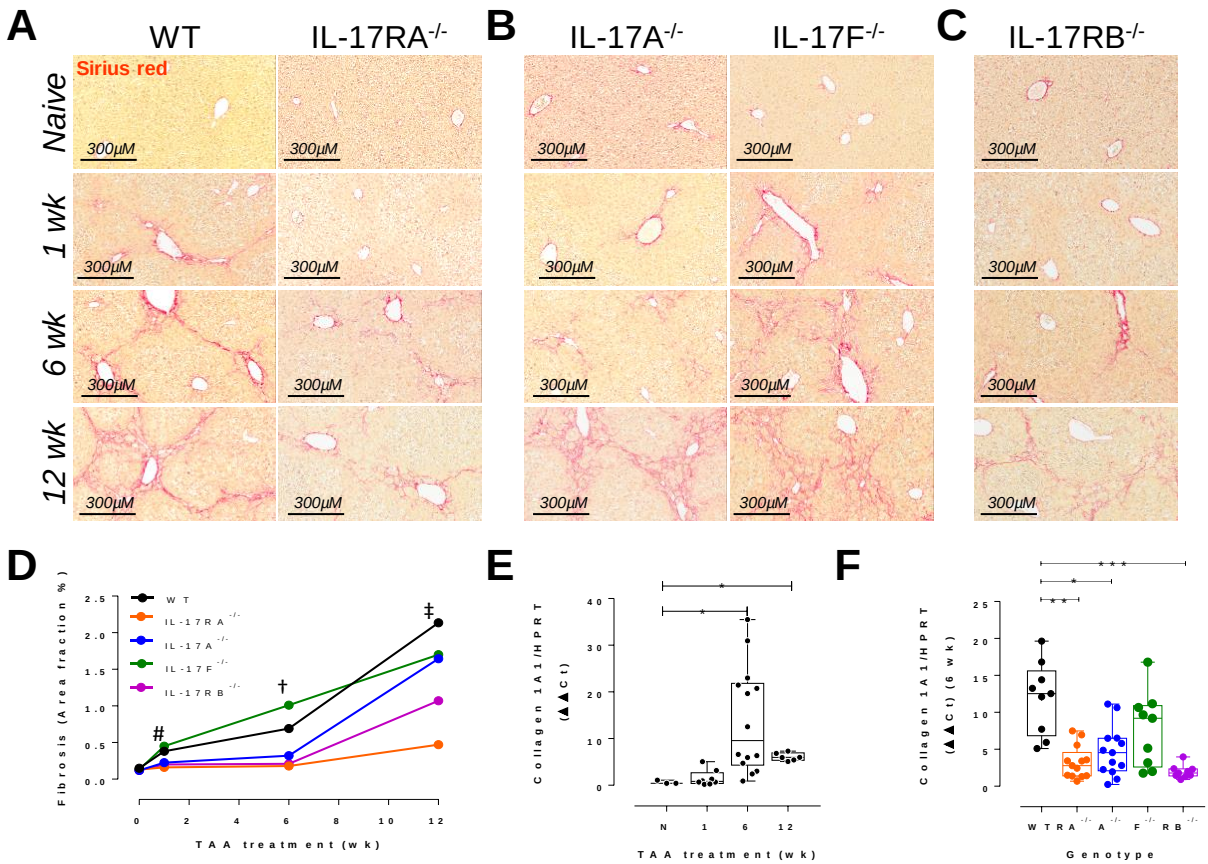


Figure 3-1 Differential contribution of IL-17 cytokine family members to TAA-induced liver fibrosis. (A) Histochemical staining for Sirius red in representative liver sections from naïve, 1, 6 and 12-week TAA-treated C57BL/6 (WT), IL-17RA$^{-/-}$, (B) IL-17A$^{-/-}$, IL-17F$^{-/-}$ and (C) IL-17RB$^{-/-}$ mice. (D) Aperio quantification of Sirius red staining (n= 4-14 animals/group/timepoint). Data are presented as mean ± SEM. * p< 0.05 Mann Whitney-U test. (E) Expression of pro-fibrogenic gene Col1-A1 in whole livers from naive and time course TAA-treated mice (n=3-14 animals/timepoint). Data are presented as median and interquartile range * p< 0.05 (naïve verses 6-week TAA; naïve verses 12-week TAA), Mann Whitney-U test. (F) Expression of pro-fibrogenic gene Col1-A1 in whole livers from 6-week TAA treated WT, IL-17RA$^{-/-}$, IL-17A$^{-/-}$, IL-17F$^{-/-}$ and IL-17RB$^{-/-}$ mice (n=9-14 animals/time point). Data are presented as median and interquartile range *p<0.05 **p<0.01, ***p<0.001. Kruskal Wallis (K.W) test.

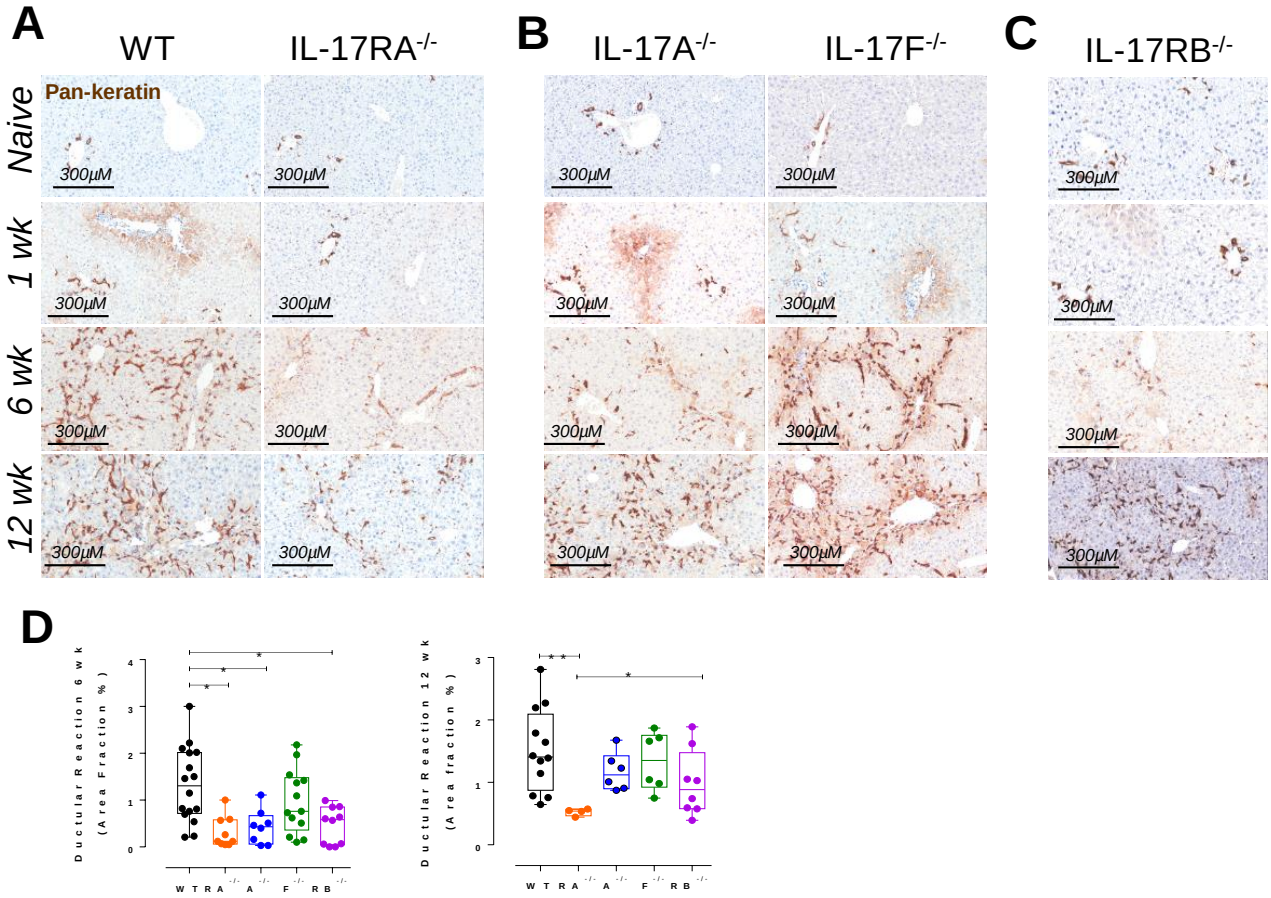


Figure 3-2 Role of IL-17 family cytokines in the development of ductular reaction: A) Immunolabelling for the DR (pan-keratin) in representative liver sections from naïve, 1, 6 and 12-week TAA-treated C57BL/6 (WT), IL-17RA$^{-/-}$, (B) IL-17A$^{-/-}$, IL-17F$^{-/-}$ and (C) IL-17RB$^{-/-}$ mice. Aperio image analysis in pan-keratin stained liver sections of (D) 6 and 12-week TAA treated mice (n=10-16 animals/group/time point). Data are presented as median and interquartile range. * p< 0.05,** p< 0.01, *** p< 0.001, Kruskal Wallis (K.W) test.

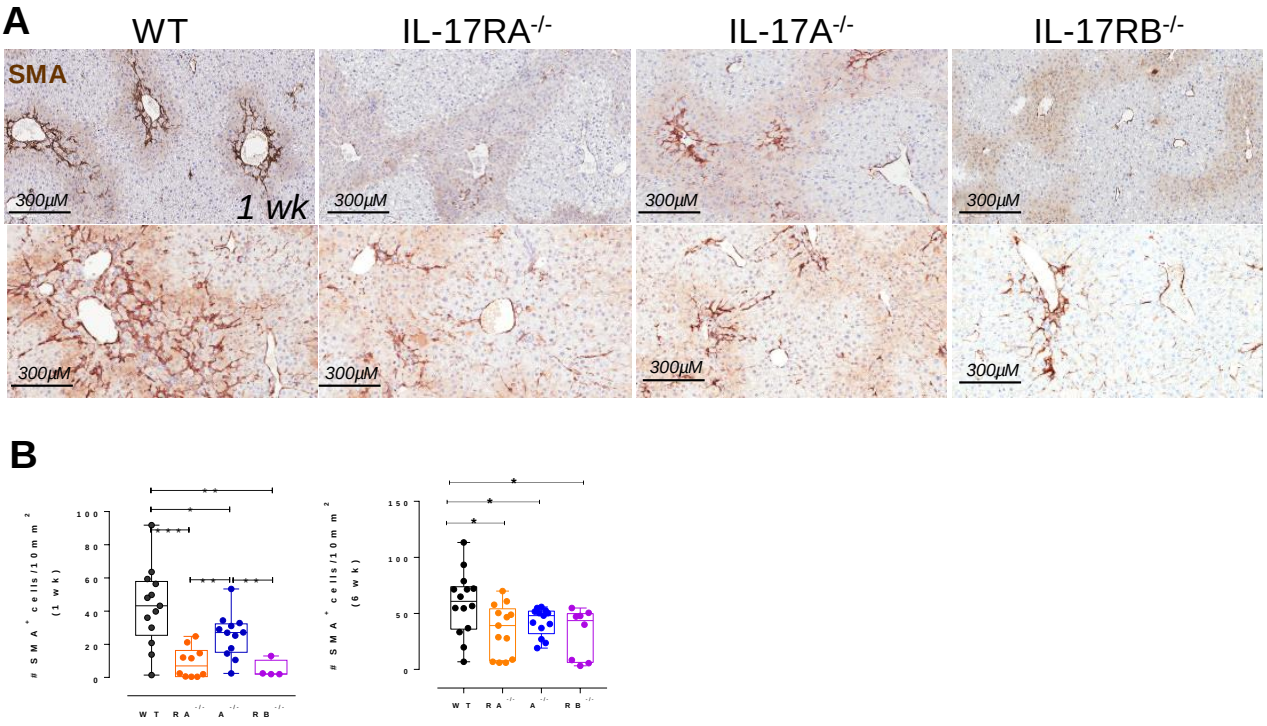


Figure 3-3 Role of IL-17 family cytokines in activating myofibroblasts (A) α-sma immunohistochemistry staining and (B) Quantification in 1 and 6-week TAA treated WT, IL-17RA$^{-/-}$, IL-17A$^{-/-}$, and IL-17RB$^{-/-}$ livers. For 'B' results were shown as average of five 10 X 20 counted areas per section (n=8-14 sections/group). Data are presented as median and interquartile range. * p< 0.05 (1-week SMA: WT verses IL-17A$^{-/-}$) (6-week SMA: WT verses IL-17A$^{-/-}$; WT verses IL-17RA$^{-/-}$; WT verses IL-17RB$^{-/-}$), ** p< 0.01 (1-week SMA: WT verses IL-17RB$^{-/-}$; IL-17A$^{-/-}$ verses IL-17RA$^{-/-}$; IL-17A$^{-/-}$ verses IL-17RB$^{-/-}$;), *** p< 0.001 (1-week SMA: WT verses IL-17RA$^{-/-}$). Mann Whitney-U test.

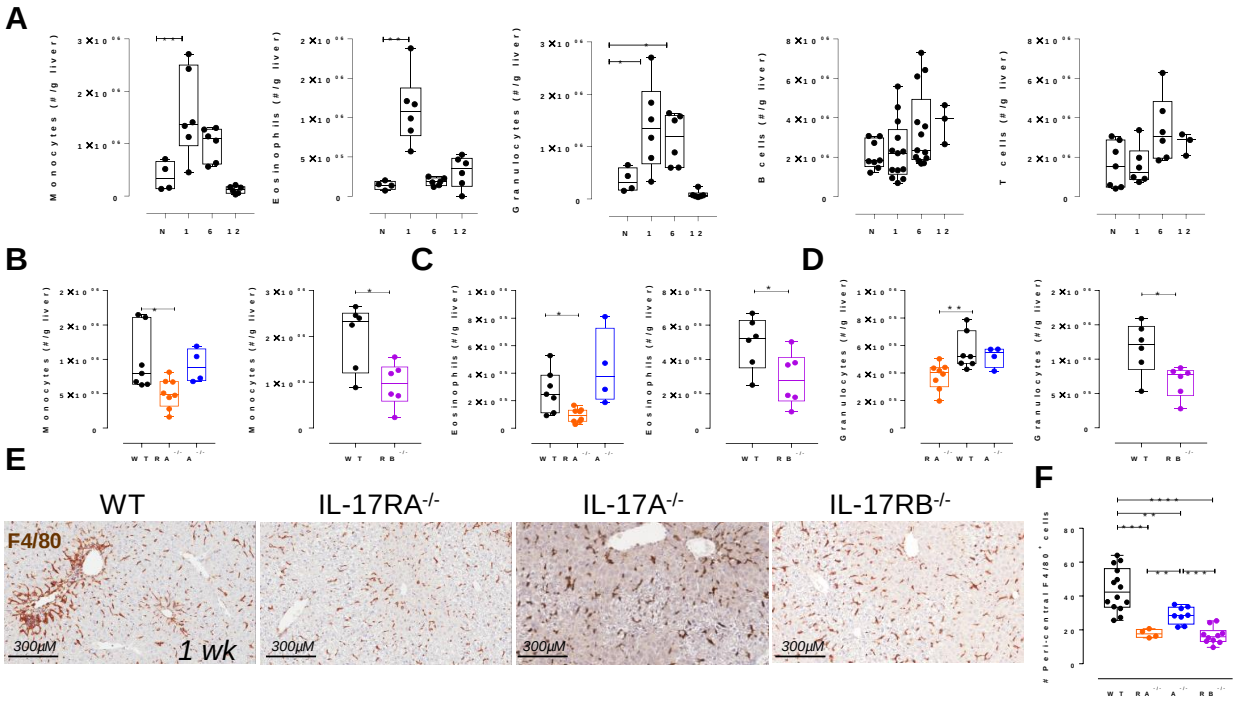


Figure 3-4 IL-17 family cytokines are required for myeloid infiltration, and pericentral monocyte accumulation: (A) An absolute number of monocytes, eosinophils, granulocytes, T, and B cells in the livers of 1,6 and 12-week TAA treated mice (n = 3-12/group). Data are presented as median and interquartile range. * p< 0.05 (Granulocytes: naïve verses 1-week; naïve verses 6-week), ** p< 0.01 (Monocytes: naïve verses 1-week) (Eosinophils: naïve verses 1-week). Mann Whitney-U test. (B-D) An absolute number of monocytes, eosinophils, and granulocytes in the livers of 1-week TAA treated WT, IL-17RA$^{-/-}$, IL-17RB$^{-/-}$ and IL-17A$^{-/-}$ mice (n = 4-8/group). Data are presented as median and interquartile range. * p< 0.05 (1-week Monocytes: WT verses IL-17RA$^{-/-}$; WT verses IL-17RB$^{-/-}$) (1-week Eosinophils: WT verses IL-17RA$^{-/-}$; WT verses IL-17RB$^{-/-}$) (1-week Granulocytes: WT verses IL-17RA$^{-/-}$; WT verses IL-17RB$^{-/-}$), Mann Whitney-U test. (E) F4/80^{+} Immunohistochemistry staining and (F) Quantification in 1-week TAA treated WT, IL-17RA$^{-/-}$, IL-17A$^{-/-}$, and IL-17RB$^{-/-}$ livers. For 'F' results were shown as average of five counted areas per section (n=8-14 sections/group). Data are presented as median and interquartile range. ** p< 0.01 (WT verses IL-17A$^{-/-}$; IL-17RA$^{-/-}$ verses IL-17A$^{-/-}$), *** p< 0.001 (WT verses IL-17RA$^{-/-}$; WT verses IL-17RB$^{-/-;}$ IL-17A$^{-/-}$ verses IL-17RB$^{-/-}$). Mann Whitney-U test.

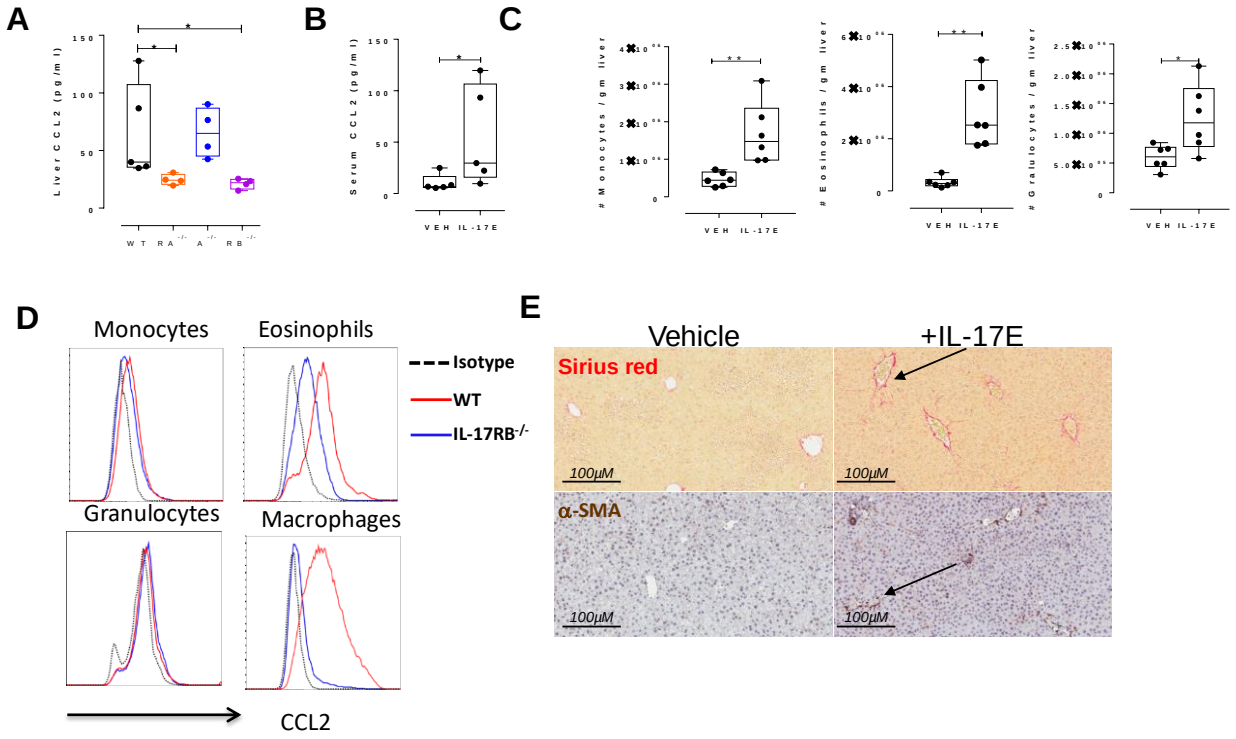


Figure 3-5 IL-17E induces CCL2 production for the recruitment of monocytes: (A) CCL2 levels were measured using cytometric bead array in the livers of WT, IL-17RA$^{-/-}$, IL-17A$^{-/-}$, and IL-17RB$^{-/-}$ following 1-week TAA treatment (n = 4/group). (B) Serum CCL2 levels in naïve mice administered with 5μg/ml IL-17E for 4 days (n = 5/group). (C) An absolute number of monocytes, eosinophils, and granulocytes in the livers of IL-17E treated mice (n = 5/group). (D) Representative histograms are illustrating CCL2 expression, where isolated liver cell fraction from 1-week TAA treated WT and IL-17RB$^{-/-}$ mice were in vitro stimulated with LPS (50ng/ml) for 4 hours and analyzed by flow cytometry for intracellular CCL2 expression. (E) Vehicle and IL-17E treated liver sections were analyzed for immunohistochemistry Sirius red and SMA staining. Data are presented as median and interquartile range. * p< 0.05 (Liver CCL2: WT verses IL-17RA-/-; WT verses IL-17RB-/-)(Serum CCL2: vehicle verses IL-17E)(Granulocytes: vehicle verses IL-17E), ** p < 0.01 (Monocytes: vehicle verses IL-17E; Eosinophils: vehicle verses IL-17E). Mann Whitney-U test.

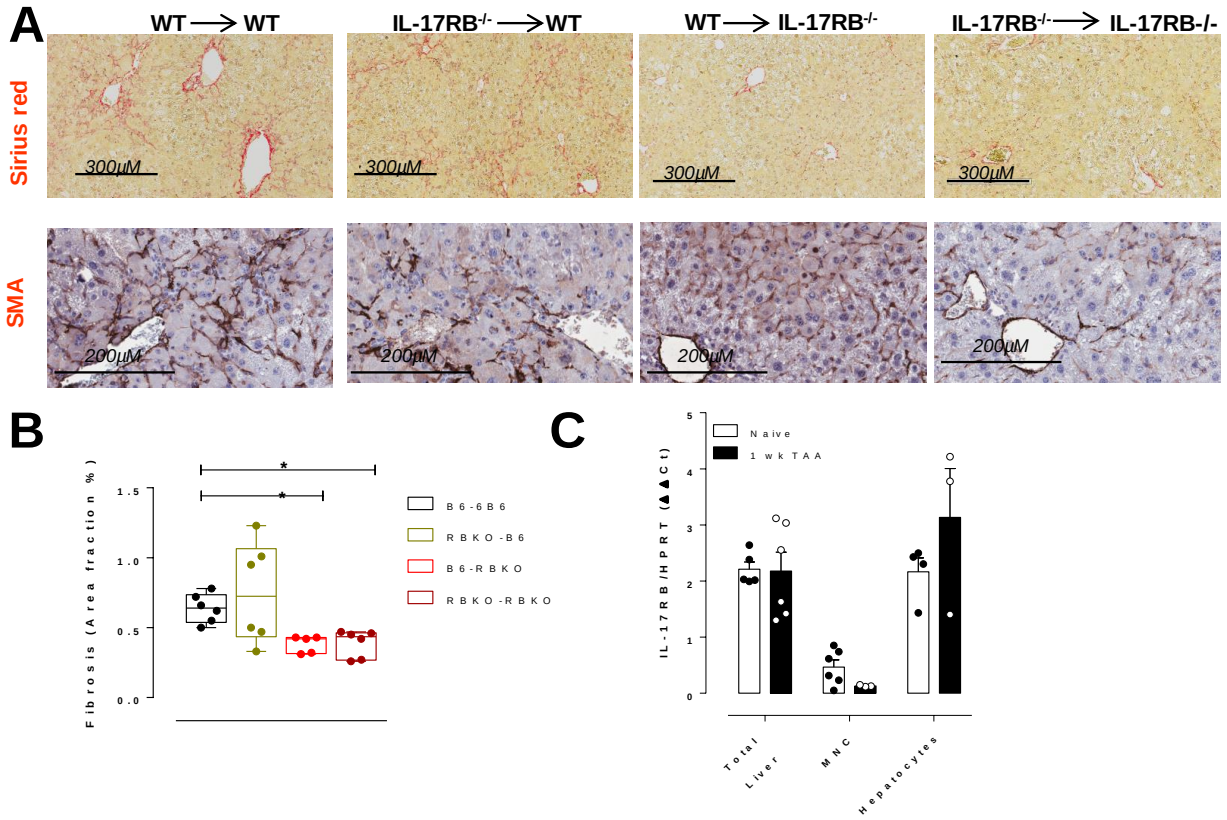


Figure 3-6 IL-17RB signaling in non-hematopoietic cells contributes to TAA-induced liver fibrosis. (A) Development of liver fibrosis in bone marrow chimeric mice was compared in the livers of TAA treated WT→WT, IL-17RB$^{-/-}$ →WT, WT→IL-17RA$^{-/-}$, IL-17RB$^{-/-}$ →IL-17RB$^{-/-}$, and IL-17RA$^{-/-}$ → IL-17RA$^{-/-}$ by Sirius red staining and α-SMA immunohistochemistry. (B) Sirius red aperio image analysis (n=6 animals/time point). Data are presented as median and interquartile range. * p< 0.05, Kruskal Wallis (K.W) test. (C) A graph illustrating the expression of IL-17RB in the whole liver, MNC, and hepatocytes of naïve and 1-week TAA treated mice (n = 3-6/group). Data are presented as means ±SEM * p< 0.05, Two-way ANOVA.

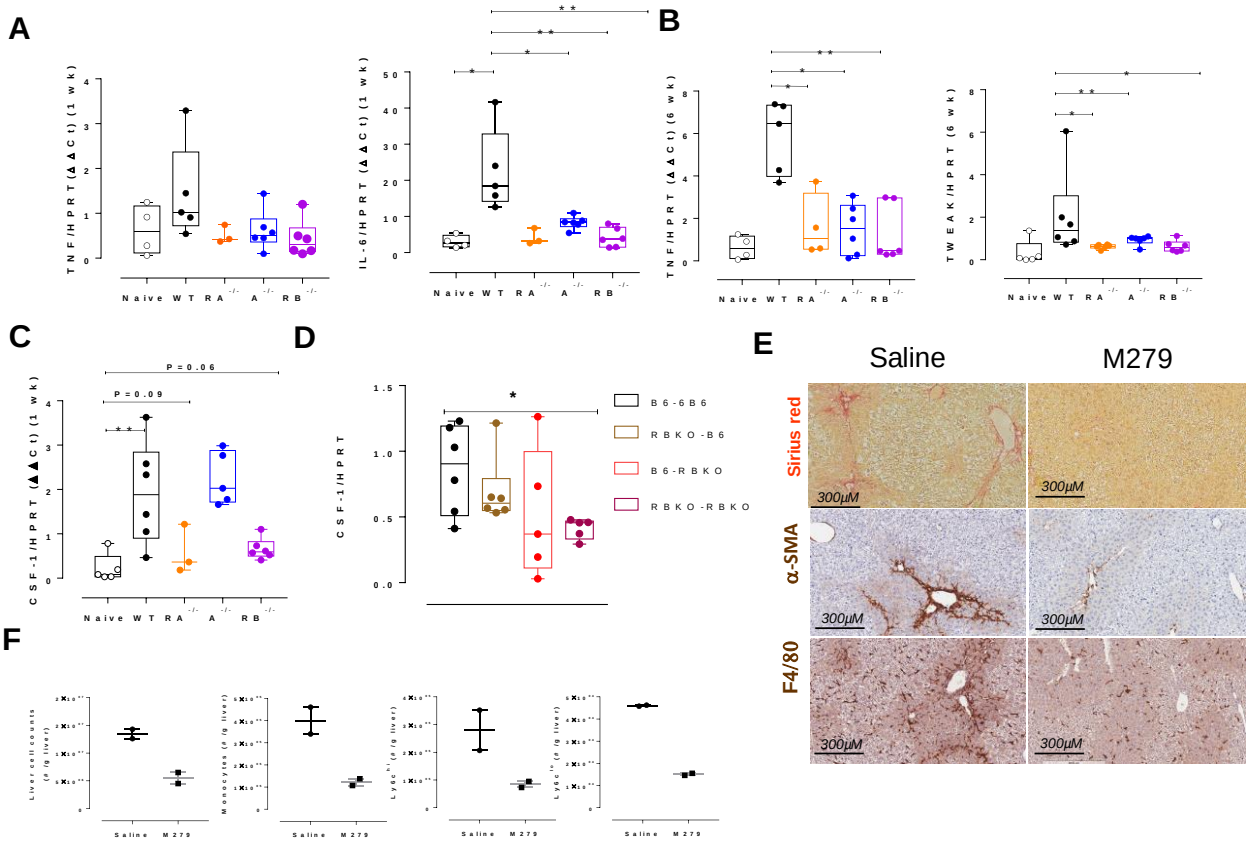


Figure 3-7 IL-17 family cytokines induce the production of proinflammatory cytokines Graphs illustrating the expression of TNF, IL-6 and TWEAK following (A)1-week and (B) 6-week TAA treated in WT, IL-17RA$^{-/-}$, IL-17A$^{-/-}$, and IL-17RB$^{-/-}$ (n = 3-6/group). Data are presented as median and interquartile range * p< 0.05 (1-week IL-16: naïve verses WT; WT verses IL-17RA$^{-/-}$) (6-week TNF: WT verses IL-17A$^{-/-}$; WT verses IL-17RA$^{-/-}$) (6-week TWEAK: WT verses IL-17RA$^{-/-}$; WT verses IL-17RB$^{-/-}$), ** p < 0.01 (1-week IL-16: WT verses IL-17A$^{-/-}$; WT verses IL-17RB$^{-/-}$) (6-week TNF: WT verses IL-17RB$^{-/-}$) (6-week TWEAK: WT verses IL-17A$^{-/-}$). Mann Whitney-U test. Graphs illustrating the expression of CSF-1 in (C) WT, IL-17RA$^{-/-}$, IL-17A$^{-/-}$, and IL-17RB$^{-/-}$ (n 3-6/group) following 1-week TAA treated and in (D) IL-17RB$^{-/-}$ bone marrow chimeras following 6-week TAA (n 3-6/group). Data are presented as median and interquartile range * p< 0.05 (CSF-1 in bone marrow chimeras: B6-B6 verses IL-17RB$^{-/-}$- IL-17RB$^{-/-}$), (1-week CSF-1: naive verses WT), ** p < 0.01 (1-week CSF-1: naive verses WT). Mann Whitney-U test. (E) Representative images showing immunohistochemistry staining of Sirius red, SMA, and F4/80+ in mice treated with saline/M279 ((400μg/ml) along with 1-week TAA. (F) An absolute number of monocytes, Ly6c^{hi} and Ly6c^{lo} in M279 treated mice (n = 2/group). Data are presented as median and interquartile range. Mann Whitney-U test.

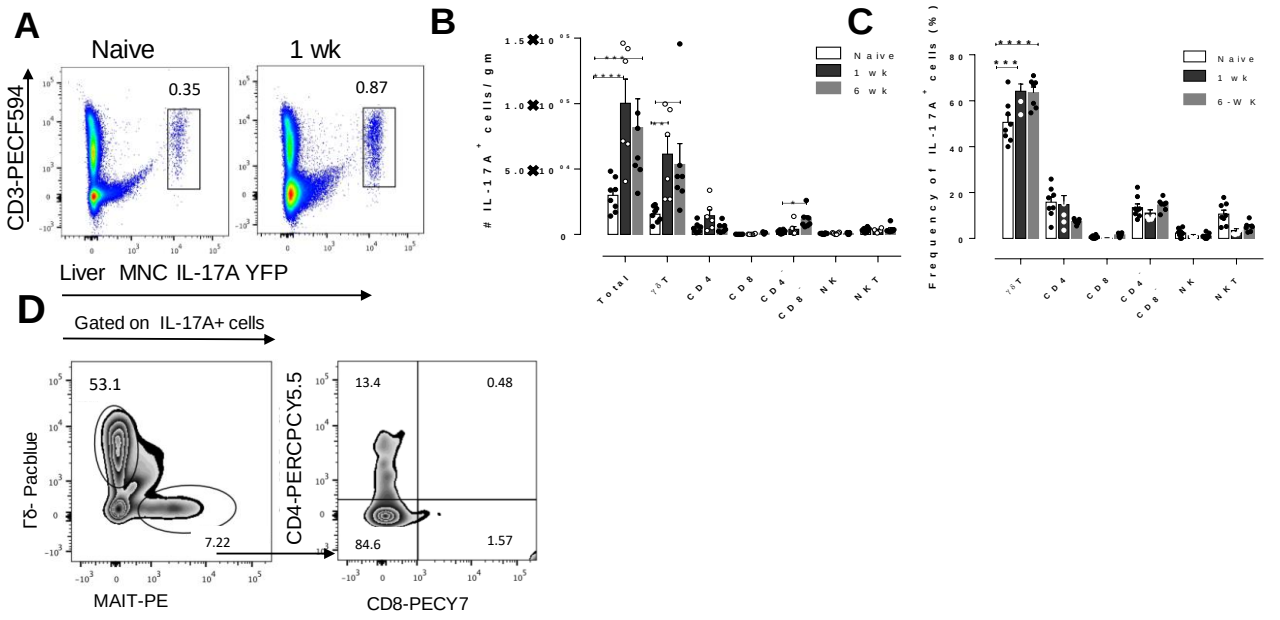


Figure 3-8 IL-17A is predominantly expressed by γδ T cells following TAA treatment. (A) Representative dot plot illustrating the frequency of $CD3^{+}IL\text{-}17AYFP^{+}$ cells in collagenase digested livers from naive, and 1-week TAA treated IL-17 YFP fate reporter mice. (B) Absolute numbers and (C) frequency of IL-17A producing γδ T cells, CD4 T, CD8 T, $CD4^{neg}CD8^{neg}$ T (DN), NK and NK T cells from naïve, 1 and 6-week TAA treated IL-17 YFP fate reporter mice (n = 6-8/group). (D) Representative contour plots are highlighting frequency of IL-17A producing MAIT cells. Data are presented as means ±SEM * p< 0.05 ,** p< 0.01, *** p< 0.001. Two-way ANOVA.

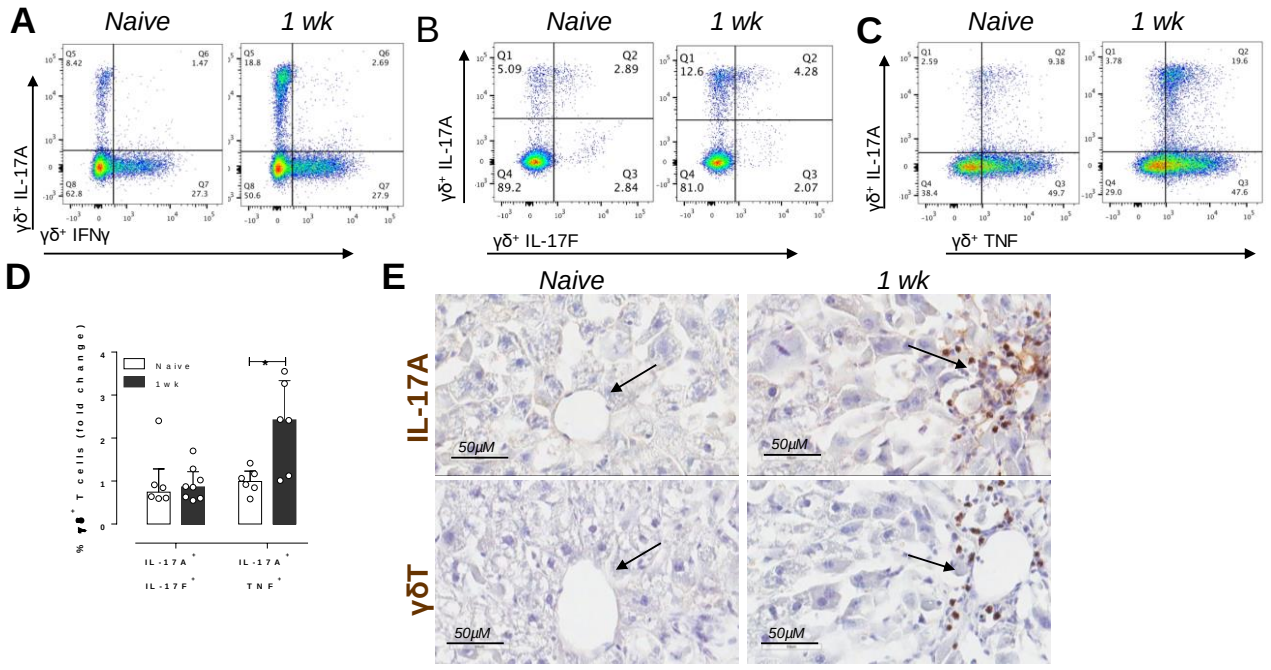


Figure 3-9 Pericentral localization of IL-17A producing γδ T cells following 1-week TAA. Liver cell fraction from naïve and 1-week TAA treated IL-17 YFP fate reporter mice were *in vitro* stimulated with PMA (100ng/ml)+Ionomycin (50ng/ml) for 4 hours, and analyzed by flow cytometry for intracellular (A) IFNγ, (D) IL-17F and (C) TNF expression in IL-17 producing γδ T cells (D) Fold change of $IL17A^{+}IL\text{-}17F^{+}$ and $IL\text{-}17A^{+}TNF^{+}$ producing γδ T cells from naïve and 1-week TAA treated IL-17 YFP fate reporter mice (n = 6-8/group). (H) Immunohistochemistry staining of IL-17A (top panel) and γδ T cells (lower panel) from naïve and 1-week TAA treated livers. Black arrows indicate positive staining. Data are presented as means ±SEM * p< 0.05 Two-way ANOVA.

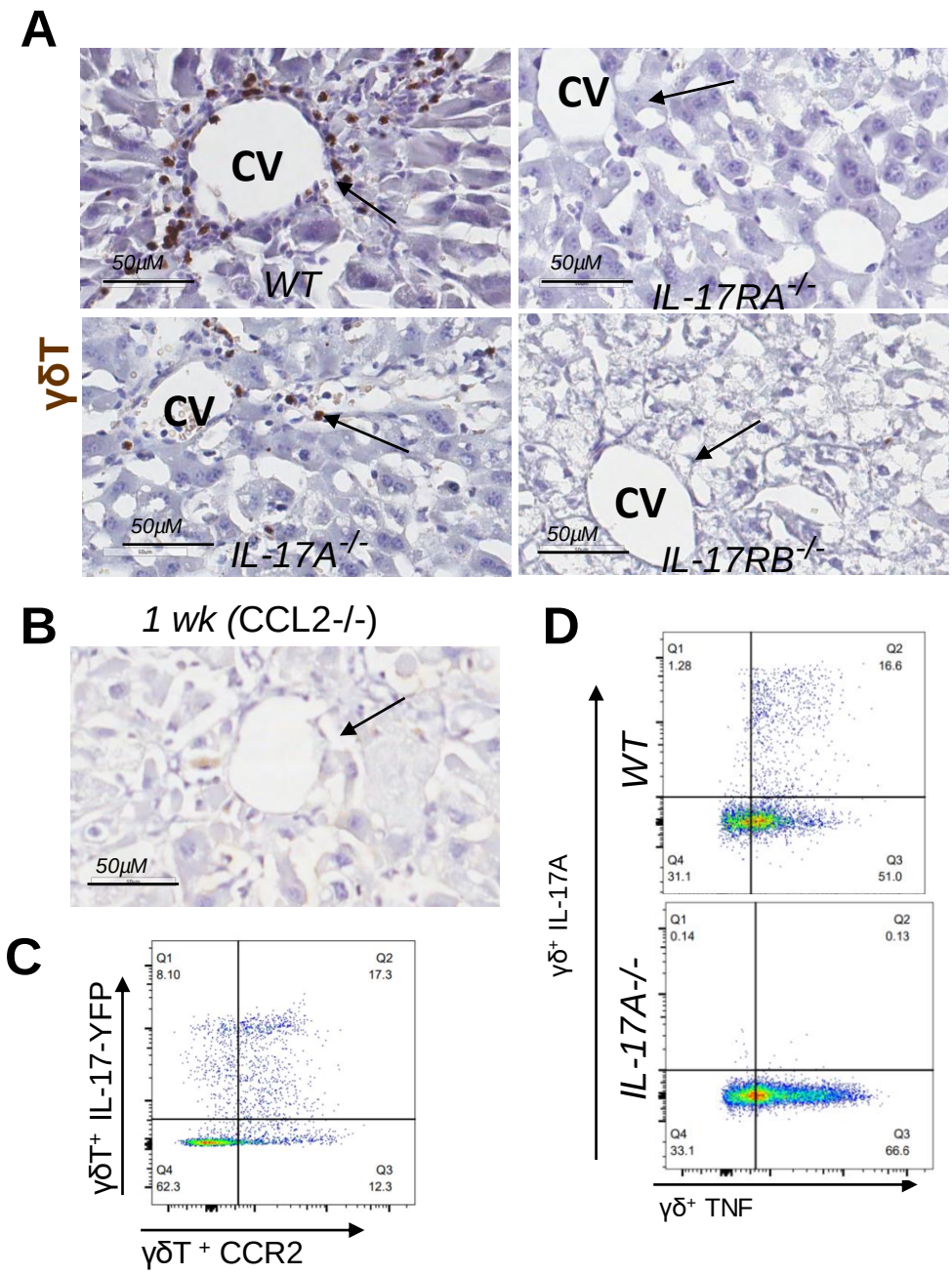
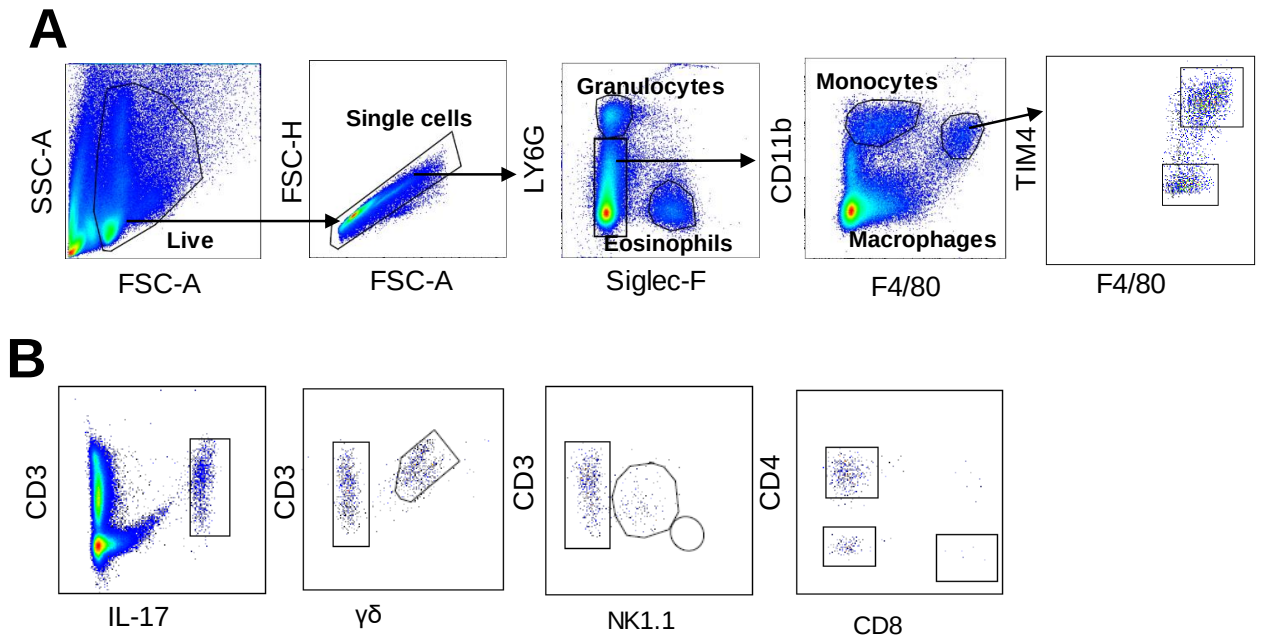
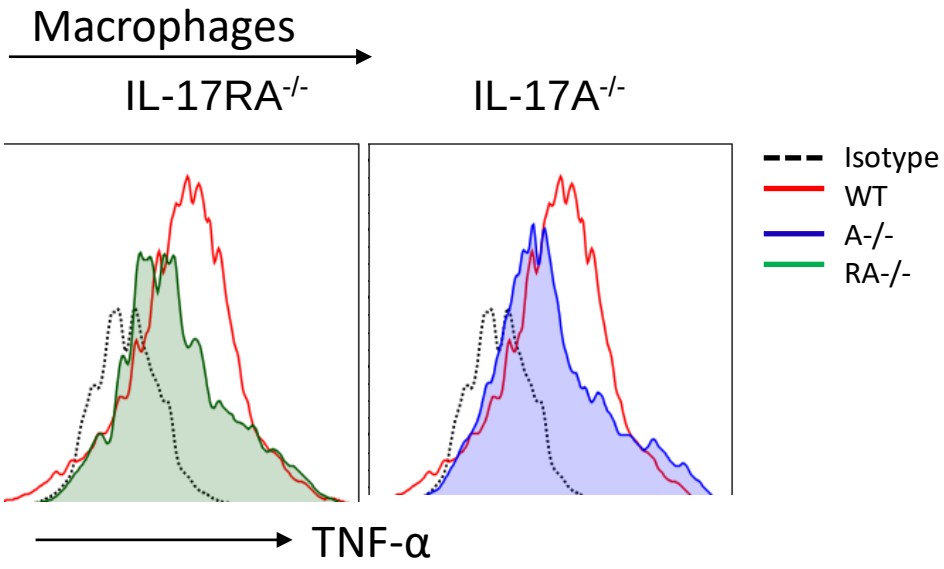


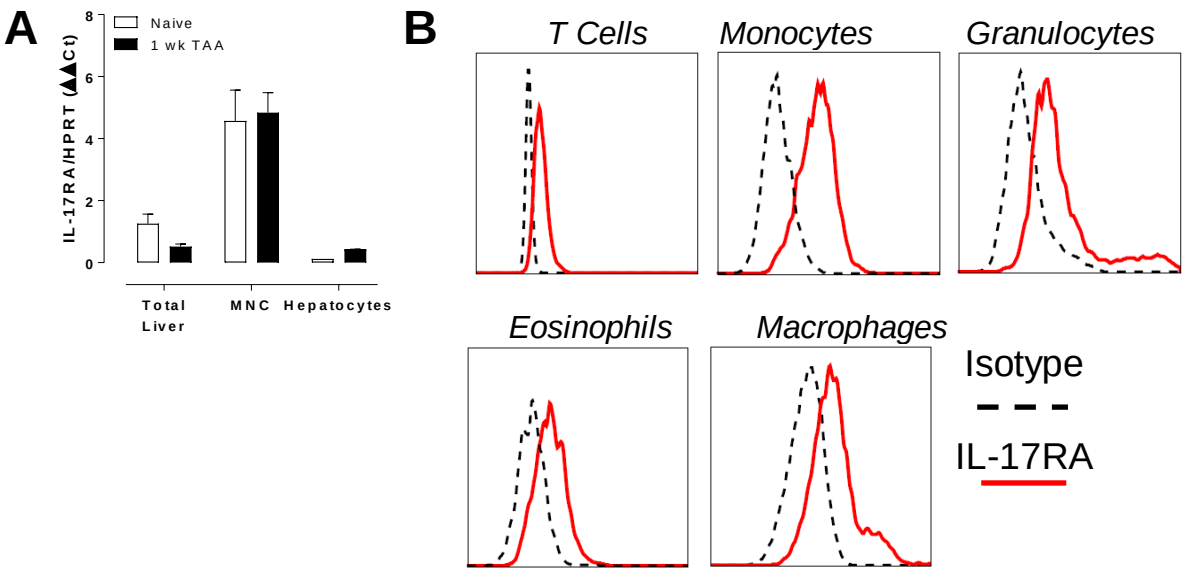
Figure 3-10 CCL2 mediates the infiltration of IL-17 producing γδ T cells. Immunolabelling staining showing (A) co-localization of γδ T cells in livers of WT, IL-17RA$^{-/-}$, IL-17A$^{-/-}$ and IL-17RB$^{-/-}$ and (B) CCL2$^{-/-}$ following 1-week TAA treatment. (C) Representative contour plots illustrating CCR2 expression on γδT^{+} IL-17-YFP cells. (D) Representative counter plots highlighting the expression of TNF in 1-week TAA treated WT, and IL-17A$^{-/-}$



Supplementary figure 3-1 Gating strategy of (A) Myeloid cells and (B) IL-17A producing cells.



Supplementary figure 3-2 production of TNF by macrophages. Representative histograms are illustrating TNF expression, where isolated liver cell fraction from 1-week TAA treated WT, IL-17RA$^{-/-}$ IL-17A$^{-/-}$ mice were *in vitro* stimulated with LPS (50ng/ml) for 4 hours and analyzed by flow cytometry for intracellular TNF expression.



Supplementary figure 3-3 Expression of IL-17RA in hematopoietic cells. (A) A graph illustrating the expression of IL-17RA in the whole liver, MNC, and hepatocytes of naïve and 1-week TAA treated mice (n = 3-6/group). (B) Representative histograms are illustrating IL-17RA expression in isolated liver cell fraction. Data are presented as median and interquartile range * p< 0.05, Mann Whitney-U test

Chapter 4: Binding of B cell immunoglobulins to FcγR is associated with TAA-induced liver fibrosis

4.1 INTRODUCTION

B cells are important contributors to immune responses during both physiological and pathological conditions. B cells contribute to immune responses through multiple functions, including secretion of antibodies and inflammatory cytokines, and shaping T cell responses through antigen presentation [391]. The contribution of B cells is well documented in autoimmune diseases such as multiple sclerosis (MS) [392], rheumatoid arthritis (RA) [393, 394], and systemic lupus erythematosus (SLE) [395, 396], and diseases such as graft versus host disease (GVHD) [397]. Most of these autoimmune diseases are driven by a dysregulated function of immune network involving B cells, T cells, and other immune cells.

Generally, B cells contributes to disease pathogenesis through antibody production. One of the mechanism through which antibodies mediate disease pathogenesis include 'antibody-dependent cell-mediated cytotoxicity (ADCC)' [398, 399]. ADCC is an immune mechanism through which FcγR bearing effector cells (monocytes, macrophages, neutrophils, mast cells, and NK cells) will ligate to antibody coated target cells expressing pathogen-derived antigens on their surface [176, 399]. The ligation of FcγR to antibody will promote phagocytosis, and induce the production of cytokines, chemokines, and other inflammatory mediators central to both protective and pathogenic properties [176]. The pathogenic property of autoantibody production is reported during disease pathogenesis of GVHD [397] [400], and MS [401]. During autoimmune diseases, B cells, under the influence of IL-21 produced by follicular T helper (Tfh) cells, differentiate into germinal center (GC) B cells to promote antibody production [174, 338, 341-343]. Recently, γδT cells are also reported to provide B cell help for antibody production during parasite infection [344, 402] [403]. These findings suggest the importance of T and B cell interaction to induce B cell-dependent antibody production, which in turn promotes FcγR mediated pathogenic function.

Accumulating evidence also suggests that apart from antibody production, activated B cells also contribute to immune responses through cytokine production. Production of tumor necrosis factor (TNF), IL-6 from activated B cells is observed during disease pathogenesis of cardiomyopathy [404] and MS [405].

B cells, which represent half of the intrahepatic lymphocyte population, have also reported in liver fibrosis and [167]. In one study using the hepatotoxin (CCl_4) induced liver fibrosis model, B cells were demonstrated to contribute to fibrosis independently of T cell activation or antibody production [168]. Conversely, another study using the same model demonstrated that liver fibrosis is associated with increased antibody production along with elevated B cell activation markers (CD44 and CD86) and TNF production [169]. Above findings suggest the importance of innate B cell activity by inducing the production of TNF during liver fibrosis. However, the role of IgG production remains controversial. In the current studies, we examined the role of B cells in the TAA model of liver fibrosis, and demonstrate their critical role through aberrant B cell activation followed cytokine production and pathogenic IgG production and its binding to FcR.

4.2 RESULTS

4.2.1 B cells contribute to TAA-induced liver fibrosis.

In this study, we aimed to identify the contribution of B cells to liver fibrosis using TAA-induced liver fibrosis model. In initial studies, mice harboring a targeted deletion of the Igμ heavy chain ($\mu mt^{-/-}$) were used to assess the role of B cells [359]. Wild type (WT) and $\mu mt^{-/-}$ mice were treated with TAA for a period of 6-week after which livers were harvested to perform immunohistochemical sirius red (SR), smooth muscle actin (SMA) and ductular reaction ($CK\text{-}WSS^{+}$) staining to visualize collagen deposition, myofibroblasts activation, and expansion of ductular reaction (DR) respectively. Livers from $\mu mt^{-/-}$ mice displayed significantly reduced TAA-induced collagen deposition associated with reduced activation of myofibroblasts and DR expansion **(Figure 4.1A and B)**. Together our data reveal an important pathogenic role for B cells in TAA-induced liver fibrosis.

4.2.2 The innate activity of B cells contributes to liver fibrosis.

To further investigate the mechanism by which B cells contribute to TAA-induced liver fibrosis we initially enumerated total B cells in the dissociated livers of mice that were treated with TAA for 6-week. Flow cytometry results demonstrated no marked difference in the number of hepatic B cells (CD19+) in 6-week TAA-treated mice in comparison to naïve **(Figure 4.2A)**. However, an increased number of activated B cell, characterized by the expression of CD44 and CD86 was noted in 6-week TAA-treated mice in comparison to naïve **(Figure 4.2A)**. Moreover, B cells isolated from the livers of mice treated with TAA for 6 weeks, produced increased TNF in response to PMA/Ionomycin stimulation when compared to hepatic B cells from uninjured mice **(Figure 4.2B)**. As mentioned earlier TNF is a potent cytokine involved in the activation and survival of hepatic stellate cells [385, 406]. Taken together, our data identify B cell induced TNF production as a potential mechanism of HSC activation and collagen production during TAA induced liver fibrosis.

4.2.3 TAA-induced liver fibrosis is associated with antibody deposition.

It has been reported that immunoglobulins produced following B cell activation and differentiation contributes to pathogenic immune responses [407]. The contribution of antibody production during hepatotoxin induced (CCL_4) liver fibrosis is controversial [167][169]. Therefore, serum immunoglobulin (IgG) levels were measured in naive and 6-week TAA treated WT and $\mu mt^{-/-}$ mice. In comparison to naive, 6-week TAA treated mice demonstrated increased serum IgG levels whereas, these levels were significantly reduced in $\mu mt^{-/-}$ when compared to both naïve and 6-week TAA WT **(Figure 4.3A),** thus confirming the role of B cells in IgG production following TAA treatment. Subsequent experiments were performed to examine the intrahepatic distribution of IgG during liver fibrosis. Immunohistochemical (IHC) IgG staining was performed in the livers from naïve and 6-week TAA-treated mice. Compared to naïve, 6-week TAA treated displayed increased IgG deposition across the scar regions adhering within the septae. **(Figure 4.3B)**. Taken together, our data demonstrate the association between B cells and antibody production following TAA treatment.

4.2.4 Antibody binding to Fc gamma receptor (FcγR) is associated with TAA-induced liver fibrosis.

One of the mechanisms through which antibodies mediate pathogenic immune responses is through FcγR binding [176]. FcγR is expressed by specific effector hematopoietic cells (Monocytes/macrophages, dendritic cells, basophils, mast cells, and neutrophils) to trigger pathogenic immune responses [408]. Therefore, we investigated the pathogenic contribution of FcγR during liver fibrosis. For this, we treated WT and $Fc\gamma R^{-/-}$ (ablated FcγR) mice with TAA for a period of 6-week after which livers were harvested to perform SR and SMA staining. In comparison to naïve, livers from $Fc\gamma R^{-/-}$ mice displayed significantly reduced collagen deposition associated with reduced activation of myofibroblasts **(Figure 4.4 A and B).** Serum IgG levels were also measured in WT and $Fc\gamma R^{-/-}$ mice following 6-week TAA treatment. When compared to WT, $Fc\gamma R^{-/-}$ demonstrated increased serum IgG levels **(Figure 4.4C)**. In the context of increased serum IgG levels, our IHC data displayed reduced IgG distribution in the livers of TAA treated $Fc\gamma R^{-/-}$ mice suggests the binding of IgG to FcγR as a potential mechanism driving liver fibrosis. Together our data demonstrates the association between IgG and FcγR during TAA induced liver fibrosis.

4.2.5 TAA induced liver fibrosis is associated with germinal center (GC) formation.

Its has been reported that B cells under the influence of IL-21 produced by follicular T helper (Tfh) cells, differentiate into germinal center (GC) B cells to promote antibody production in secondary lymphoid organs [174, 338, 341-343]. These findings suggest the role of GC formation in antibody production. Therefore, we next examined GC formation in the spleens of TAA-treated mice using peanut-agglutin (PNA) immunohistochemical staining. When compared to naïve, 6-weeks TAA treated mice displayed increased GC in the spleen which is consistent with serum IgG production **(Figure 4.5A**). Taken together, our data showed an association between splenic GC formation and antibody production during TAA induced liver fibrosis.

4.3 DISCUSSION

B cells have been shown to play an important role in the pathogenesis of liver fibrosis. Our study identified a pathogenic role for B cells in theTAA induced liver fibrosis model and demonstrated GC B cell development and pathogenic antibody production.

Characteristically, B cells are multifunctional as they express antigen-specific receptors, immune modulatory receptors (CD40), and pathogen targeting receptors including FcRs, complement receptors, and TLRs having both beneficial and dangerous signals [409-411]. Although B cells were identified as mediators of TAA induced liver fibrosis, the mechanism by which these cells promote fibrosis remains undefined. Consistent with previous findings, our study illustrated that mice deficient in B cells ($\mu mt^{-/-}$) demonstrated reduced myofibroblast activation followed by reduced collagen deposition following 6-weeks TAA treatment [168, 412]. We previously reported that fibrosis which was, induced following 6-week TAA treatment, was associated with DR expansion and macrophage differentiation [20]. Extending these findings our current study demonstrated that mice deficient in B cells displayed significantly reduced DR expansion. Thus, B cells appear to play a key role during TAA induced liver fibrosis characterized by the development of extensive fibrosis with septal formation associated with an expanded DR.

The mechanism through which B cells contribute to TAA-induced liver fibrosis is not clear. Therefore, to investigate the mechanism, we initially enumerated total B cell numbers in the liver. Our results demonstrated no marked difference in intrahepatic B cell number between naive and TAA treated mice. Interestingly, consistent with previous findings [412], intrahepatic B cell activation (characterized by the expression of CD44 and CD86) was increased in 6-weeks TAA treated mice. The innate function of activated B cells has previously been reported in CCL_4 induced liver fibrosis [412]. Moreover, we demonstrated increased production of TNF, an important cytokine in HSC activation and survival during the later phases of fibrosis [385, 406]. Thus, the data suggests TNF secretion by activated B cells is a potential mechanism contributing to TAA-induced liver fibrosis.

In addition to cytokine production, B cells also contribute to disease pathogenesis through antibody production [401]. T-cell dependent B cells activation is crucial for

antibody production [413]. Mechanistically, Tfh cells promote B cell activation, GC formation and antibody production through the production of IL-21[174, 338, 341-343]. Additionally, antigen-stimulated γδ T cells acquire Tfh phenotype to promote antibody production from B cells by up-regulating chemoattractant markers and IL-21 production [344] [403]. The pathogenic property of antibody production is observed during disease pathogenesis of GVHD [397] [400], and MS [401]. The role of antibody production during liver fibrosis is controversial whereby one study using the hepatotoxin (CCL_4) model demonstrated that liver fibrosis is independent of antibody production [168]. Conversely, a second study using the same model demonstrated increased IgG levels associated with liver fibrosis [169]. Moreover, several pathogenic auto-antibodies are detected in the sera of patients with the alcoholic liver disease, non-alcoholic fatty liver disease (NAFLD), viral hepatitis, drug-induced hepatitis, and HCC as well as autoimmune liver diseases which are essential for diagnosis and prognosis [172, 412, 414, 415]. In addition to the above findings, we showed that TAA induced liver fibrosis was associated with increased splenic germinal centre (GC) B cells and serum IgG levels. IgG deposition in the livers of TAA treated mice was shown to be localized at the scar regions within the liver parenchyma along with macrophages demonstrating the close association between IgG and macrophages. Although increased IgG production was associated with liver fibrosis in our model, the type of antigens that stimulate antibody production is undefined. Nevertheless, previous findings documented the association of collagen-related antigens in the serum of patients with alcohol-induced liver fibrosis [416]. Additionally, it has also been reported that collagen itself is a potential self-antigen in collagen induced arthritis [417, 418]. Therefore, in support of the above findings, our study suggests that collagen may be an antigen during liver fibrosis development in the TAA model.

Although TAA induced liver fibrosis was associated with increased antibody production, the mechanism by which antibodies induce liver fibrosis is unclear. It has been reported that antibodies will induce danger signals through direct antigen attack to clear pathogens or through the formation of immune complexes around the antigen and signal through FcγRs to mediate cellular cytotoxicity [398, 399]. The family of FcγRs regulate antibody-mediated effector functions with both pro and anti-inflammatory responses. FcγR consists of both activating and inhibitory receptors [176]. Signaling through activated FcγR triggers cellular cytotoxicity, cytokine release, and phagocytosis whereas, signaling through inhibitory receptors downregulates effector functions [176]. Four different classes of FcγRs, including FcγRI, FcγRIIB, FcγRIII

and FcγRIV whereby each receptor signals different IgG antibodies with varying binding affinities [419, 420]. The evidence suggests that restoring the function of inhibitory FcγR in human autoimmune diseases may be a novel treatment for autoimmune disease patients. Similarly, inhibiting the dominant role of activating FcγRs prevents the pathogenic effect of autoantibodies. In the liver, FcγRs are predominantly expressed by macrophages with both an activating and inhibitory function [421]. The role of FcγRs during liver fibrosis is not defined. Our study illustrates that mice deficient in FcγR (FcγR $^{-/-}$) demonstrated reduced myofibroblast activation associated with reduced collagen deposition following 6-weeks TAA treatment. Additionally, mice deficient in FcγR also demonstrated increased serum IgG levels. Although, serum IgG levels were increased, reduced IgG distribution in the livers FcγR$^{-/-}$ mice highlighted an association between IgG and FcγR in liver fibrosis. We also observed that IgG was sequestered around the central vein within the scar region along with macrophages. Previous findings reported that FcγR is predominantly expressed by macrophages [421]. However, we still need to investigate the cells that express FcγR in our model. We identified that the innate function of B cells and the binding of IgG to FcγR were associated with liver fibrosis. Taken together our study implies an association between B cells and fibrosis development.

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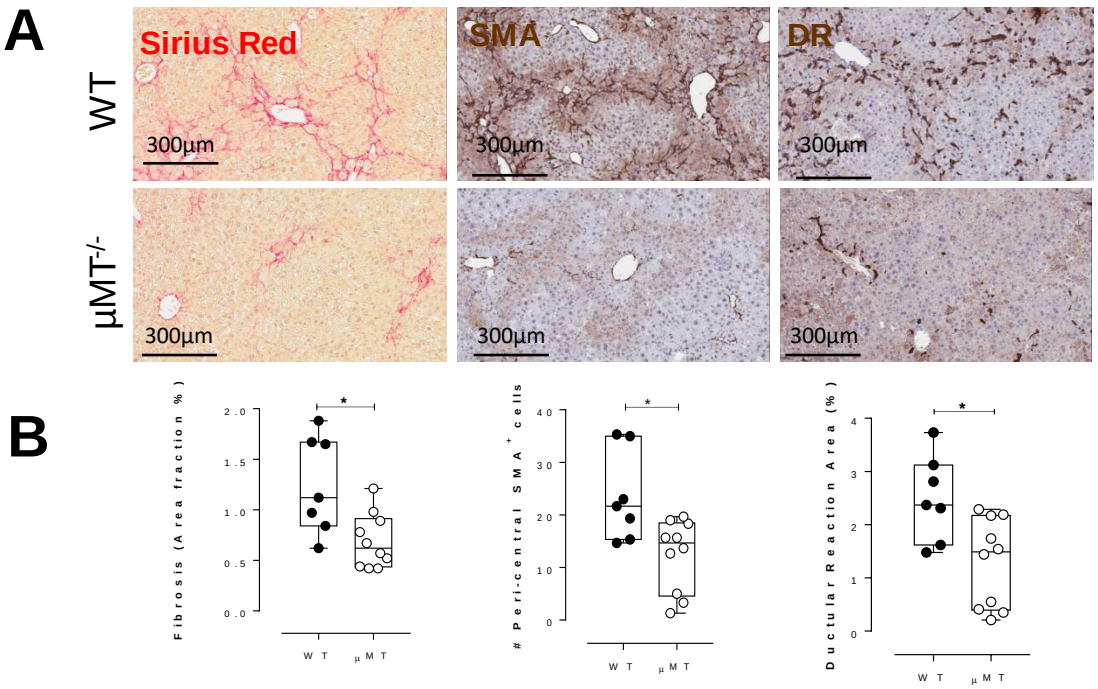


Figure 4-1 Contribution of B cells to TAA-induced liver fibrosis. (A) Histochemical staining for Sirius red (SR), smooth muscle activation (SMA), and ductular reaction (DR) staining in representative liver sections from 6-week TAA-treated C57BL/6 (WT) and μmt$^{-/-}$ mice. (B) Aperio quantification of (SR) Sirius red, smooth muscle activation (SMA), and ductular reaction (DR) staining (n= 7-10 animals/group/time point). Data are presented as median and interquartile range * p< 0.05, Mann Whitney-U test.

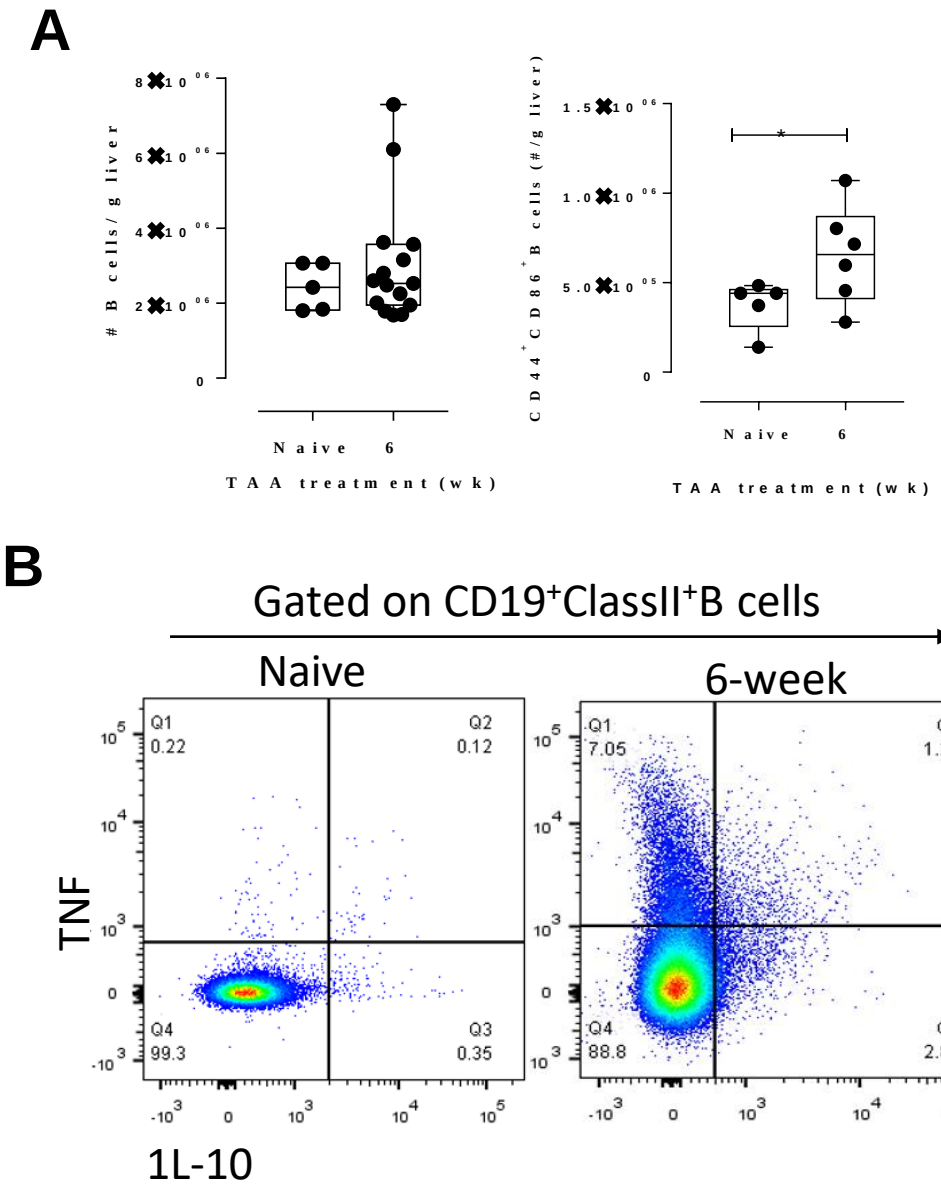


Figure 4-2 B cells contribute to TAA-induced liver fibrosis through innate immune responses. (A) Representative graphs showing the absolute number of total B cells and activated B cells in naïve and 6-week TAA treated mice (n=3 to 9/group/time point) (B) Representative plots illustrating the production of TNF and IL-10 in naïve, and 6-week TAA treated mice. Data are presented as median and interquartile range * p< 0.05, Mann Whitney-U test & T-test.

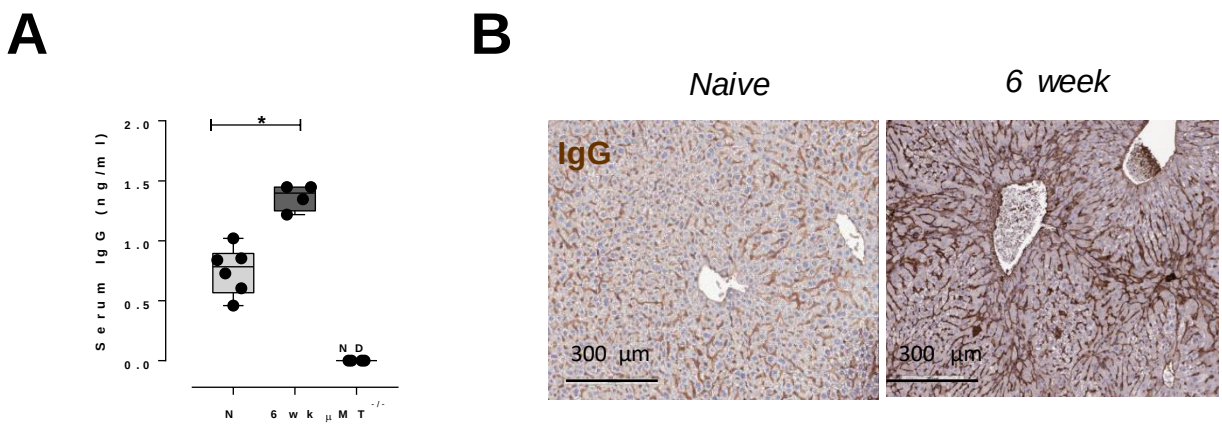


Figure 4-3 Immunoglobulin production and deposition following 6-week TAA. (A) Serum IgG levels in naïve and 6-week TAA WT, µmt$^{-/-}$ mice. (n= 6-12 animals/group/time point) (B) Histochemical staining for IgG distribution in representative liver sections from naïve and 6-week TAA treated mice. Data are presented as median and interquartile range. * p< 0.05 (naïve verses 6-week). Mann Whitney-U test.

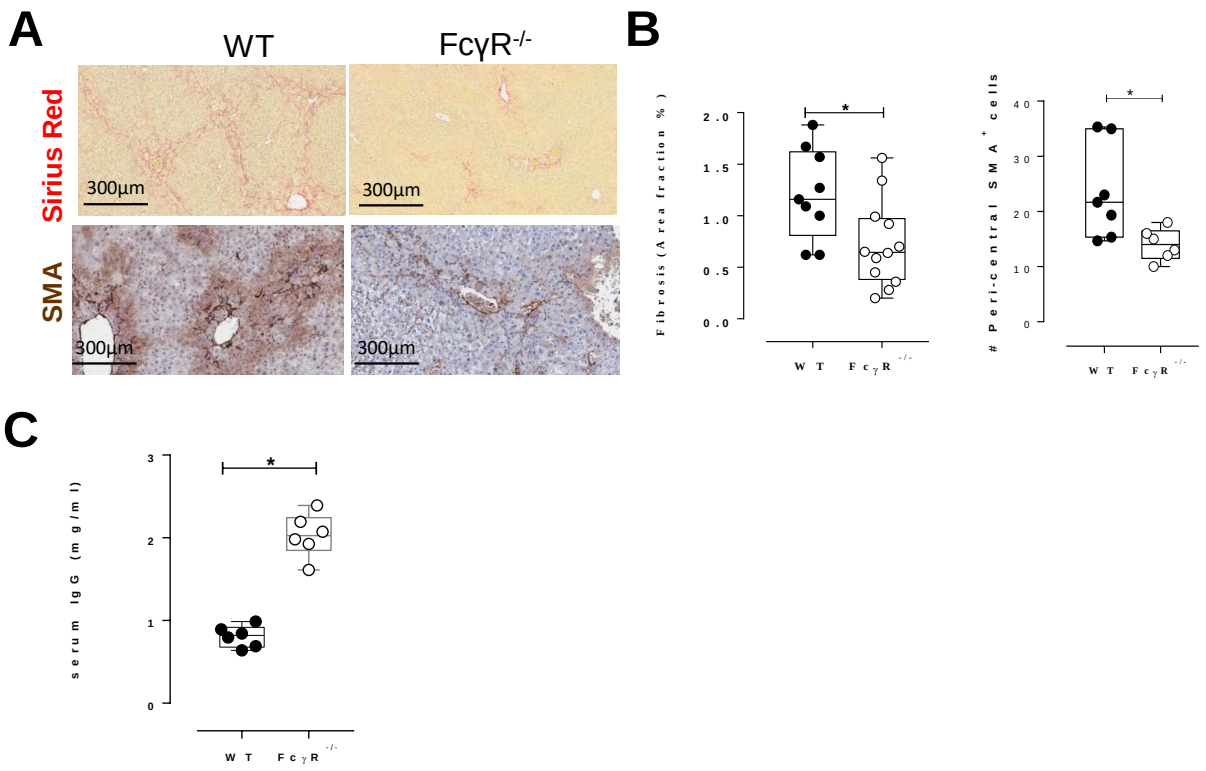


Figure 4-4 Binding of immunoglobulins to FCγR is associated with TAA-induced liver fibrosis. Histochemical staining for (A) Sirius red and SMA in representative liver sections from 6-week TAA-treated C57BL/6 (WT) and FcγR$^{-/-}$ mice. (B) Aperio quantification of SR and SMA staining (n= 6-12 animals/group/time point). (C) Serum IgG levels in 6-week TAA WT and, FcγR$^{-/-}$ mice (n= 6 animals/group/time point). Data are presented as median and interquartile range * p< 0.05, Mann Whitney-U test.

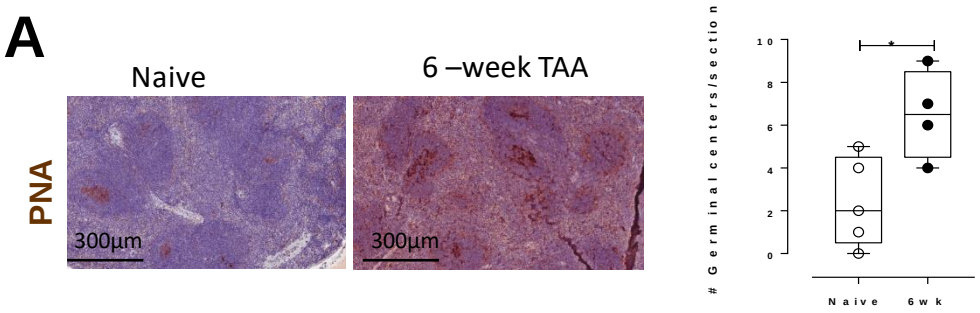
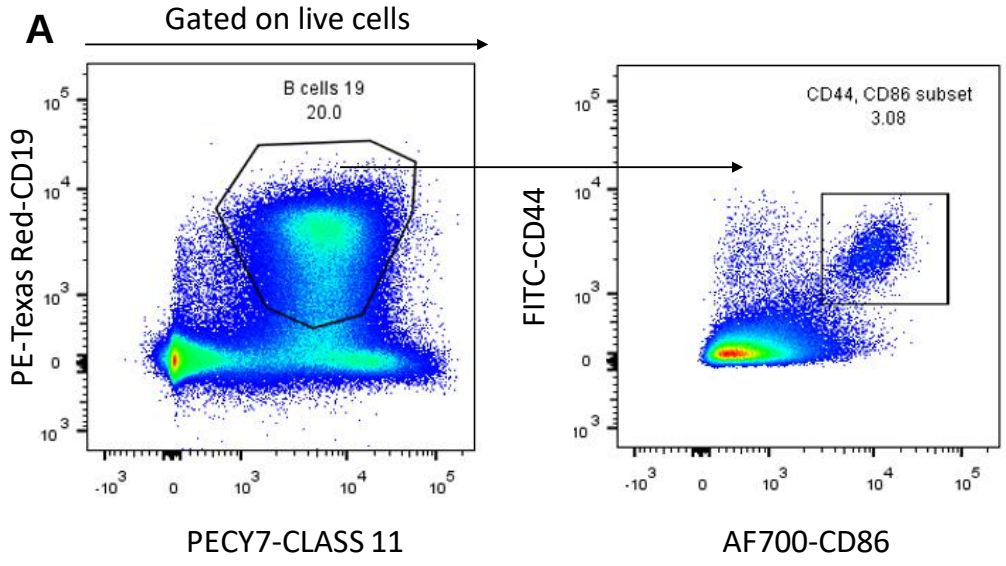


Figure 4-5 TAA induced liver fibrosis is associated with germinal center formation. Histochemical staining and quantification of PNA in mice treated with and without TAA. Data are presented as median and interquartile range * p< 0.05, Mann Whitney-U test.

Supplementary figure:



Supplementary figure 4-1 Gating strategy showing activated B cells

Chapter 5: KDO25 inhibits ROCK2 induced pathogenic effects in monocytes/macrophages and B cells following TAA treatment.

5.1 INTRODUCTION

Rho-associated coiled-coil forming protein kinase 2 (ROCK2) is a serine-threonine kinase and an established mediator of fibrosis in multiple disease settings. ROCK2 is activated by RhoA associated GTPase [315] and is induced by multiple biochemical mediators including established fibrogenic mediators such as IL-17A [316] and TGFβ [317]. In the setting of graft versus host disease (GVHD), rheumatoid arthritis (RA), psoriasis, and systemic lupus erythematosus (SLE) ROCK2 has been shown to promote inflammatory responses [322, 332, 422]. Moreover, in the setting of idiopathic pulmonary fibrosis (IPF), myocardial fibrosis, and, peritoneal fibrosis Rho-associated kinases function in cytoskeletal reorganization and remodeling of participating fibrotic cells [328-330].

ROCK2 promotes inflammatory responses through leukocyte infiltration, activation, differentiation, and survival. Inflammatory responses induced by ROCK2 are mediated through the phosphorylation and expression of its downstream transcription factors such as signal transducer and activator of transcription 3 (STAT3), RAR-related orphan receptor gamma (RORγt), and B-cell lymphoma 6 (BCL6) in the targeted cell population [333-336]. Previously, it has been demonstrated that STAT3 and RORγt expression following ROCK2 activation is required to promote the differentiation of IL-17 producing cells during GVDH and autoimmunity, [112, 113, 337, 338]. Also, BCL6 expression in follicular helper cells (Tfh) following ROCK2 activation is reported to induce IL-21 production. IL-21 from Tfh will, in turn, provide survival signals to germinal center (GC) B cells to promote pathogenic immunoglobulin production [338, 341-343], linking ROCK2 activity to the induction and perpetuation of antibody responses. Additionally, in the presence of IL-21, γδT cells are reported to acquire Tfh phenotype and interact with B cells in IL-21 dependent manner to promote pathogenic antibody production against microbial

pathogens [175, 344]. Importantly, blocking of this signaling pathway has been shown to be effective in attenuating IL-17A and immunoglobulin induced pathogenesis in various diseases that manifest fibrosis.

Monocytes/macrophages are established critical mediators of fibrosis. In response to injury, they contribute to fibrogenesis in part through their activation of myofibroblasts and also important in matrix remodeling through the production of matrix metalloproteases (MMP's) [78, 423] [78, 81]. IL-17A, an established pro-inflammatory cytokine, is reported to directly promote the pathogenic differentiation and function of macrophages [348-350]. In-vitro studies demonstrated that IL-17 induces the production of pro-inflammatory cytokines (TNF, IL-6, and IL-1β) and also acts as a chemokine to recruit monocytes [191, 424]. Importantly, ROCK2 has been shown to promote macrophage differentiation and induce proinflammatory responses in a cell-intrinsic manner both in vitro and in vivo [345-347]. Thus, ROCK2 has a broad role in promoting inflammatory signals by inducing the production of IL-17A and IL-21 cytokines and promoting the pathogenic function of B cells and macrophages.

A role for ROCK2 in cytoskeletal remodeling/reorganization is well established [351, 352]. Cofilin is an actin-binding protein which regulates dynamic cytoskeletal reorganization [353]. Cofilin activity is reversibly regulated through phosphorylation and dephosphorylation where the phosphorylated form, pcofilin, is inactive [353]. ROCK2 induced cofilin phosphorylation contributes to actin cytoskeleton organization [351-354] required for the adhesion and migration of multiple cell types including T cells, myofibroblasts, macrophages and cancer cells [425] [426] [353] [427]. Given the importance of monocyte migration and macrophage adhesion within the scarred liver tissue, the above observations implicate a mechanistic role for macrophage specific cofilin activity in liver fibrosis.

Surprisingly little is known regarding the contribution of ROCK2 in liver fibrosis. Given the importance of IL-17, monocytes/macrophages and immunoglobulins during liver fibrosis, the above findings highlight the role of ROCK2 activity in the pathogenesis of liver fibrosis. In support of this, recent findings suggest the association of ROCK2 in bile duct ligation (BDL) induced liver fibrosis [327]. However, the mechanistic contribution of ROCK2 in liver fibrosis is unexplored. KD025 is a potent and selective ROCK2 inhibitor which has been tested in multiple pre-clinical mouse models including GVHD [338, 342] and rheumatoid arthritis

[356]) and is in now in clinical trials (rheumatoid arthritis, idiopathic pulmonary fibrosis (IPF) and GVHD) [337]). In multiple studies, ROCK2 suppression of disease was associated with diminished IL-17 and IL-21 production [112, 113]. In this study, we demonstrated the efficacy of ROCK2 inhibition in prevention and reversal of liver fibrosis in the TAA model and identified the key mechanisms underpinning this effect.

5.2 RESULTS

5.2.1 ROCK2 signaling blockade diminishes onset of TAA-induced liver fibrosis, SMA$^+$ myofibroblasts and F4/80$^+$ cell infiltration.

In initial experiments, we examined the contribution of ROCK2 signaling to the initiation of TAA-induced liver fibrosis. Mice were co-administered TAA with vehicle (0.4% methylcellulose, daily oral gavage) or KD025 (100 mg/kg/day, daily oral gavage) for a period of 1-week. KD025 treatment significantly reduced TAA-induced collagen deposition as demonstrated by sirius red (SR) staining and aperio image analysis **(Figure 5.1A)**. Additionally, immunolabelling for smooth muscle actin (α-SMA staining) and monocytes/macrophages (F4/80$^+$ staining) demonstrated a significant reduction in the number of activated myofibroblasts and pericentral F4/80$^+$ monocyte sequestration which represents characteristic features of early injury in this model [20] **(Figure 5.1B and C).** Thus, we demonstrate a critical role for ROCK2 signaling in the initiation of TAA-induced liver fibrosis, highlighting the potential of ROCK2 inhibition as a preventive therapeutic strategy.

5.2.2 KDO25 displays therapeutic potential for the treatment of established liver fibrosis.

To establish the therapeutic potential of KD025, mice were treated with TAA for 6-weeks to induce extensive fibrosis and then co-administered TAA with vehicle or KD025 for a further 2 weeks (in total 8-weeks). Livers isolated from 6-week TAA treated mice exhibited extensive collagen deposition and myofibroblast activation as demonstrated by SR and anti-SMA IHC staining **(Figure 5.2 A, B).** Moreover, pan-keratin staining (CK-WSS) identified the development and expansion of a ductular reaction (DR) at the 6-week time point **(Figure 5.2C).** Notably, between the period of 6 and 8 weeks, there was no significant progression in collagen deposition, myofibroblast activation or the DR expansion. However, KD025 administration promoted fibrolysis as highlighted by SR staining demonstrating diminished collagen deposition and significant thinning of fibrotic septa **(Figure 5.2 A).** Furthermore, SMA$^+$ myofibroblast numbers were significantly reduced in livers from KDO25 treated mice compared to those from vehicle-treated mice **(Figure 5.2 B).** Additionally, livers from KD025-treated mice displayed a substantially reduced DR in comparison to vehicle-treated animals, demonstrating the role of ROCK2 in DR maturation **(Figure 5.2 C).** As discussed in Chapter 3, *Collagen1a (col1a)* is the

dominantly expressed collagen species expressed in the liver after 6-week TAA administration. In line with reduced SR staining, RT-qPCR demonstrated a significant reduction *col1a* mRNA production in livers from KDO25 treated mice **(Figure 5.2D)**.

Although poorly studied, there is evidence that TAA-induced liver fibrosis is not readily amenable to spontaneous regression [18]. Hence, to gain greater insight into the anti-fibrotic efficacy of KD025 treatment, we examined the degree of spontaneous fibrotic regression in this model, following TAA withdrawal. Thus, mice were treated with TAA for a 6-week period, after which TAA administration was ceased for a period of 1 or 2 weeks. In line with a previous report of long-term persistence of fibrosis following TAA withdrawal for 12-weeks, our SR staining **(Figure 5.3A)** and aperio image analysis indicated no decrease in collagen deposition after 1-week withdrawal whereas, moreover, an apparent trend toward fibrotic progression was evident following the 2-week withdrawal period **(Figure 5.3B)**. These data support that ROCK2 inhibition by KD025 activily promotes pathways to attenuate collagen deposition, reverse the DR and facilitate ECM turn over to allow fibrotic regression**.**

5.2.3 ROCK2 inhibition reduces pSTAT3 phosphorylation and RORγT expression.

The mechanism through which ROCK2 inhibition attenuated liver fibrosis was examined by investigating the expression of its downstream targets. Given the confirmed role for IL-17 in this model, and that the importance of ROCK2 in IL-17 production through STAT3 and RORγT, we next performed western blot analysis of pSTAT3 and RORγT expression in mice treated with TAA for 1 or 6-weeks co-administered with vehicle or KD025. Western blotting demonstrated a significant reduction in STAT3 phosphorylation associated with the reduced trend in RORγT expression in the livers of 1-week TAA treated mice co-administered with KDO25 when compared to vehicle-treated animals **(Figure 5.4A).** Similarly, administration of KD025 between the period of 6 and 8 weeks markedly reduced in STAT3 phosphorylation and RORγT expression when compared to vehicle-treated **(Figure 5.4B)**. Intrestingly, when compared to 6-weeks TAA treatment, mice co-administered with vehicle (0.4% methylcellulose) demonstrated increased STAT3 phosphorylation. However, it is uncertain weather this increased STAT3 was due to methylcellulose. Having noted a significant decrease in pSTAT3 and RORγT levels

in the livers from mice treated with KDO25 we next examined the impact of KD025 on *IL-17a* expression using qRT-PCR. Livers from mice co-administered with vehicle and TAA between 6 and 8-week exhibited no difference in *IL-17a* expression. However, following KDO25 administration *IL-17a* expression was significantly reduced **(Figure 5.4C)**. Together, our findings demonstrate that protective effect of KDO25 is at least in part due to limiting pro-fibrogenic IL-17 production.

5.2.4 ROCK2 activation is required for BCL6 expression, germinal center formation and immunoglobulin deposition following TAA treatment.

BCL6 is a known target of ROCK2, and previous studies in GVHD have identified that KDO25 inhibits the expression of BCL6 and its downstream IL-21 production, GC B cell differentiation and immunoglobulin production [338, 341-343]. Having demonstrated the pathogenic role of GC B cells and immunoglobulin deposition in the TAA model (discussed in chapter 4), we next performed western blot analysis of BCL6 expression in mice treated with TAA for 6-weeks co-administered with vehicle or KD025 for a further 2-weeks (in total 8-week TAA). Western blot analysis of livers from 8-week TAA+ vehicle-treated mice demonstrated increased BCL6 expression when compared to 6-week TAA treatment whereas, following KD025 administration BCL6 expression was significantly reduced **(Figure 5.5A)**. However, it is uncertain weather this increased BCL6 expression in 8-week TAA+ vehicle-treated mice was due to vehicle (0.4% methylcellulose). As BCL6 promotes GC B cell formation through TFh differentiation and IL-21 production [428], in next experiments, we addressed whether diminished BCL6 levels were contributing to the attenuation of fibrosis via inhibition of GC B cell development and antibody production. In secondary lymphoid organs, GC develops in B cell follicles during T cell-dependent antibody response. GC consists of two zones; light zone and dark zone. T cells interact with B cells in the light zone (T cell zone) which stimulates B cells to enter the follicle to form dark regions (germinal center) [429-431]. Hence, we performed PNA/CD3/B220 immunofluorescence triple staining to identify germinal centers in spleens from 6-week TAA treated mice co-administered with TAA and vehicle or KD025-for the final 2 weeks. Consistent with diminished BCL6 expression confocal imaging showed KDO25 administered mice displayed reduced splenic GC's formation when compared to vehicle-treated animals **(Figure 5.5B)**. In line with

reduced splenic GC, KDO25 treated mice also demonstrated significantly decreased circulating IgG levels **(Figure 5.5C)** and liver IgG deposition **(Figure 5.5D)**. Thus, our data identify that in addition to inhibiting IL-17 production, ROCK2 inhibition attenuates TAA-induced fibrosis through the disruption of GC B cell development and pathogenic antibody production.

5.2.5 KD025 alters macrophage function by inhibiting TNF and MMP production.

In addition to its effects in driving IL-17 differentiation, pSTAT3 has been reported to alter the functionality of cells of multiple lineages including macrophages [432, 433]. One of the mechanisms by which IL-17 drives fibrosis is through the production of proinflammatory mediators from macrophages [369, 370] [235]. Recently, IL-17A induced STAT3 signaling has been reported in macrophages to induce pro-proinflammatory mediators [191, 424]. Additionally, we also demonstrated the role of IL-17A in TNF production from macrophages during TAA-induced liver fibrosis (chapter 3). Moreover, ROCK2 signaling in macrophages has been demonstrated to promote an alternative M2 phenotype, similar to the profile of macrophages which accumulate in fibrotic tissues, suggesting the link between IL-17A, STAT3, and ROCK2 [345-347]. Thus, in next experiments, we addressed whether the protective effects of KD025 are in part mediated through the attenuation of altered macrophage function. Therefore, CSF-1 derived bone marrow- macrophages (BMDMs) were generated in-vitro to investigate if KD025 altered ROCK2 induced STAT3 phosphorylation in macrophages. For this CSF-1 derived BMDMs were cultured with KD025 (10 µM) or IL-17A (10 µM) or KDO25+IL-17A for 20 minutes to examine STAT3 phosphorylation using western blotting. BMDMs cultured with IL-17A demonstrated increased STAT3 phosphorylation which was reduced following KDO25 or IL-17A+KDO25 (**Figure 5.6A**) treatment, demonstrating the role of KDO25 in inhibiting IL-17A mediated STAT3 phosphorylation in macrophages.

Macrophages also induces the production of pro-inflammatory cytokines which include tumor necrosis factor alpha (TNF) [434, 435]. Therefore, using qRT-PCR we initially examined if KDO25 inhibits *Tnf* expression in the whole liver following TAA. Between the period of 6 and 8 weeks of TAA treatment, consistent to unchanged fibrosis *Tnf* expression also remained unchanged. However, treatment with KD025 indicated a remarkable reduction in *Tnf* expression (**Figure 5.6B**). Subsequent experiments were performed on liver mononuclear cells (MNCs) isolated from

digested livers from 6-week TAA treated mice co-administered with KD025 or vehicle in the last 2 weeks and asked whether KD025 was altering TNF production from monocyte and macrophage. **(Figure 5.6C).** In comparison to the vehicle, KD025 treatment substantially reduced TNF production from both monocyte and macrophage populations in response to LPS stimulation. Further, CSF-1 derived BMDMs were cultured with KD025 (10 µM) or vehicle for 6hrs to ask weather KDO25 directly inhibits TNF production from macrophages. qRT-PCR analysis demonstrated reduced T*nf* expression in BMDM cultured in KDO25 when compared to vehicle-treated (**Figure 5.6D**).

Additionally, macrophages are also an important source of MMP's involved in ECM turn over [81, 423]. Here, we demonstrated that MMP's were significantly altered in BMDMs following KDO25 treatment, associated with increased MMP12 and 13 with a trend toward increased MMP2 (**Figure 5.6E**). This suggests that ROCK2 drives a macrophage phenotype that is inefficient in matrix turnover due to inhibition of MMPs. Moreover, the capacity of KD025 to promote fibrotic regression may be explained in part by restoring macrophage MMP production. Together, these data suggest the direct and indirect (through IL-17A) role of ROCK2 in altering macrophage function.

5.2.6 KD025 alters macrophage migration by inhibiting the phosphorylation of cofilin.

Cofilin is known target of ROCK2 implicated in cytoskeletal reorganization during fibrosis [328-330]. We demonstrated the importance of ROCK2 in macrophage functionality. Cofilin has already been shown to control macrophage adherence and motility [436]. Hence, our study is focused on the role of Cofilin in macrophage function in response to ROCK2 activation during liver injury. Initially, western blot analysis of pCofilin levels in whole liver lysates was performed in 6-week TAA mice co-administered with KDO25 or vehicle for further 2-weeks (in total 8-weeks). Cofilin phosphorylation remained unchanged between 6 and 8-week TAA mice. However, following KDO25 administration phosphorylation of Cofilin was significantly reduced (**Figure 5.7A**). Further, CSF-1 derived BMDMs were cultured with KD025 (10 µM) or DMSO control for 20 min (**Figure 5.7B**) to ask weather KDO25 directly inhibits Cofilin phosphorylation in macrophages. BMDMs cultured with KD025 diminished phosphorylation of Cofilin, which confirming active and potentially pathogenic ROCK2 signaling in macrophages. Furthermore, absolute

macrophage numbers were examined in the livers from 8-week TAA treated mice co-administered with vehicle or KD025, to understand if altered macrophage function induced by KDO25 was actually due to reduced macrophage number. Flow cytometry analysis demonstrated macrophage numbers remained comparable between KDO25 and vehicle-treated group (**Figure 5.8C**). However, our F4/80 + IHC staining displayed that in the fibrotic liver macrophages sequester in the scar regions (fibrotic septa) whereas, following KD025 treatment, macrophages were more evenly distributed throughout the tissue (**Figure 5.7D**), demonstrating the role of KDO25 in inhibiting the macrophage adherence around the scar region. Additionally, IHC analysis was performed using an anti-cofilin antibody to examine the distribution of cofilin in the tissues. Livers from mice treated with KDO25 displayed reduced cofilin across scar regions when compared to vehicle treated. Together, our data suggest that during fibrosis, in addition to altering macrophage phenotype, ROCK2 inhibits macrophage migration and promotes their retention in fibrotic septae through its inhibition of cofilin mediated cytoskeleton remodeling.

5.3 DISCUSSION

ROCK2 has been shown to play an important role in inflammation and tissue remodeling in multiple chronic diseases that lead to fibrosis. In this study, we report the role of ROCK2 in promoting liver fibrosis through multiple mechanisms. We specifically demonstrated the role of ROCK2 in promoting proinflammatory cytokine IL-17A expression, pathogenic immunoglobulin production by B cells and driving profibrotic macrophage activity.

ROCK2 inhibition has been reported to attenuate disease pathogenesis of GVHD [437], pulmonary fibrosis [328], psoriasis [438], autoimmune EAE [439], rheumatoid arthritis [440] and systematic lupus erythematosus [332, 439]. Inhibition of ROCK2 in the above mentioned models is effective not only in prevention but also in the treatment of disease [319, 328, 437]. The established mechanisms by which ROCK contribute to disease pathogenesis is through IL-17A induced inflammatory responses [191, 424], B cells induced immunoglobulin deposition [338, 341-343], and pathogenic macrophage function [345-347]. Given the importance of IL-17, monocytes/macrophages and immunoglobulins during liver fibrosis, the above mechanisms highlight the role of ROCK2 activity in the pathogenesis of liver fibrosis. Recently, upregulation of ROCK2 expression is reported during BDL induced liver fibrosis [327], however, the mechanism by which ROCK2 induce liver fibrosis is not clear. We demonstrated that the inhibition of ROCK2 by the small molecule inhibitor KD025 prevented the development of fibrosis associated with reduced pericentral monocyte recruitment and myofibroblasts activation in 1-week TAA treated mice. This highlights the role of KDO25 in inhibiting the recruitment of inflammatory cells to prevent the development of fibrosis. In addition to prevention, ROCK2 inhibition also promoted the regression of established fibrosis. Herein we reported that mice co-administered with KD025 following 6-week TAA treatment promoted regression of liver fibrosis which was demonstrated by diminished collagen deposition and significant thinning of fibrotic septa when compared to vehicle-treated animals. KDO25 treatment also altered DR which is the characteristic feature of 6-week TAA treatment. We previously reported that F4/80^{+} monocytes/macrophages are more organized in a central-central distribution associated with DR niche [20]. However, following KDO25 treatment, F4/80^{+} cells that are residing within scar region were evenly distributed by altering DR. Hence, our study postulated that one of the anti-fibrotic property of ROCK2 inhibition is

through altering macrophage retention and migration to promote regression of fibrosis.

Extracellular matrix (ECM) degradation also promotes fibrosis regression [441]. We demonstrated that during KDO25 mediated fibrotic regression *Collagen1a* (abundantly found in ECM) in the livers was significantly reduced indicating the role of KDO25 in collagen degradation. It has been well described that matrix metalloproteinases (MMPs), play a pivotal role in promoting ECM degradation that is essential for wound healing [442]. Of all available MMPs, the anti-fibrotic property of MMP-2 [443-446] and MMP-13 [447, 448] is reported in hepatotoxin (CCL_4), and physical (BDL) injured liver fibrosis models both by inhibiting collagen production and promoting collagen degradation. MMP-12 is also reported during liver fibrosis with conflicting results where one group demonstrated reduced fibrosis in *Mmp12*$^{-/-}$ animals compared with wild-type controls [449] whereas, another observed no difference in fibrosis between the two genotypes [450]. Macrophages which group with HSCs to induce collagen deposition are also observed to produce MMPs that control ECM turns over [78, 81]. However, the importance of ROCK2 in inducing MMPs from macrophages is not clear. Thus, using *in-vitro* CSF-1 derived BMDM we demonstrated that *Mmp-2*, *Mmp-12*, and *Mmp-13* that are under-expressed were upregulated following KDO25 treatment to encourage ECM degradation and promote fibrotic regression. Though our *in-vitro* findings using BMDM demonstrated the importance of KDO25 in inducing MMPs from macrophages, further studies are required to confirm these observations by using liver macrophage. However, the question remains here is if macrophages are removed from tissue scar following KDO25 treatment, how does MMP production is altering ECM?

Previous studies have shown that KD025 during disease pathogenesis limits IL-17A and immunoglobulin production [319, 328, 437]. In chapter 3 and 4 we explained the pathogenic role of IL-17A and immunoglobulins by effecting monocytes/macrophages in TAA-induced liver fibrosis model (discussed below). Therefore, in addition to altered macrophage migration and ECM production, KDO25 is also important in macrophage-induced inflammatory responses.

STAT3 and RORγt are one of the downstream targets of ROCK2 which promote inflammatory responses [112, 113, 337, 338]. We observed that livers (following 8-week TAA) from KDO25 treated mice demonstrated reduced STAT3

phosphorylation and RORγT expression when compared to vehicle treated. The reduction in pSTAT3 and RORγT attenuates differentiation of IL-17 producing cells leading to reduced IL-17 expression. pSTAT3 has been reported to alter the functionality of macrophages which are reported to be important cellular mediators of liver fibrosis [432, 433]. Recently, IL-17A is reported to directly activate macrophages in STAT3-dependent manner to induce the production of proinflammatory mediators [191, 424]. Moreover, ROCK2 signaling is also stated in macrophages to promote an alternative macrophage phenotype, similar to the profile of macrophages which accumulate in fibrotic tissues [345-347]. All these findings suggest the link between ROCK2, IL-17A, and STAT3 to induce pathogenic macrophage phenotype. Hence, using *in-vitro* CSF-1 generated bone marrow-derived macrophages (BMDM) we demonstrated that KDO25 directly inhibited pSTAT3 in macrophages which was induced in response to IL-17A. We previously reported the role of IL-17A signaling in promoting proinflammatory (TNF) macrophage phenotype during liver fibrosis (Chapter 3). Here, using *in-vitro* and *in-vivo* studies, we demonstrated that TNF production from macrophages was significantly reduced in response to KDO25 when compared to vehicle-treated during 8-week TAA treatment. Together our data demonstrated the direct (through pSTAT3) and indirect (through IL-17) role of ROCK2 in promoting inflammatory macrophage phenotype.

Notably, when compared to 6-weeks TAA treatment, mice co-administered with vehicle (0.4% methylcellulose) demonstrated a mild increase in STAT3 phosphorylation and BCL6 expression. At this stage, it is uncertain whether this was due to methylcellulose and further investigations are required to clarify this issue. Unfortunately, this is a limitation of the study as KDO25 is only soluble in DMSO or methylcellulose. We selected methylcellulose as the vehicle in our *in vivo* experiments as it is non-toxic and can be safely used for oral administration. Importantly, however, all results were compared relative to methylcellulose alone.

Immunoglobulins will also contribute to pathogenic macrophage activation by binding to FCγR [407]. We demonstrated that TAA-induced liver fibrosis is associated with the binding of immunoglobulins to FCγR (chapter 4). B cell lymphoma 6 (BCL6) is also a known target of ROCK2 that contributes to B cell differentiation and immunoglobulin production [338, 341-343]. Mechanistically, BCL6 mediates the interaction between Tfh and B cells to promote B cell germinal

center (GC) formation and immunoglobulin production [429-431]. Although immunoglobulin deposition has been noted in the TAA-induced liver fibrosis (chapter 4), we also demonstrated increased expression of liver BCL6 and splenic GC formation during 8-week TAA treatment. Additionally, during KDO25 induced fibrotic regression we have shown reduced BCL6 expression, GC formation, and immunoglobulin production. Thus, our data demonstrated that KDO25 indirectly inhibited pathogenic macrophage function by inhibiting IL-17A and immunoglobulin induced proinflammatory responses.

ROCK2 also controls macrophage adherence and migration by inducing cytoskeletal re-organization [436]. Cofilin is known target of ROCK2 established in cytoskeletal reorganization [328-330]. We demonstrated that livers (following 8-week TAA) from KDO25 treated mice exhibited reduced pCofilin when compared to vehicle treated. Cofilin has already been shown to control macrophage adherence and motility [436]. Therefore using, using CSF-1 generated BMDM, we demonstrated that KDO25 directly inhibited pCofilin in macrophages, highlighting the importance of ROCK2 induced pCofilin in macrophages during liver fibrosis. Given that KDO25 treatment promoted macrophage migration by altering their retention within the septae implicates the role of pCofilin in macrophage migration. Thus, our data demonstrated that ROCK2 inhibits macrophage migration and promotes their retention in fibrotic septae through its inhibition of cofilin mediated cytoskeleton remodeling.

To obtain considerable understanding into the anti-fibrotic efficacy of KD025 treatment, we examined the degree of spontaneous fibrotic regression following TAA withdrawal. Recent findings suggest that withdrawal of TAA for 8 weeks following 12-week TAA administration (IP) in rats promoted partial spontaneous regression [18]. This suggests that in our model for the fibrosis to promote spontaneous regression it might take a long time to reverse pathogenic inflammatory responses and ECM remodeling mechanisms. However, identifying the targets that cease these responses might significantly promote quick reversal of fibrosis. We identified ROCK2 as a dominant mediator of liver fibrosis. Taken together our data demonstrated a remarkable capacity for ROCK2 inhibition by KD025 to attenuate macrophage-induced pro-inflammatory responses mediated through IL-17A and IgG production. Additionally, ROCK2 inhibition also promoted collagen degradation by upregulating macrophage-induced MMP production to

allow fibrotic regression. These findings highlight that ROCK2 as a therapeutic target that promoted fibrosis regression by inhibiting the interaction between cellular and molecular mediators that contribute to liver fibrosis.

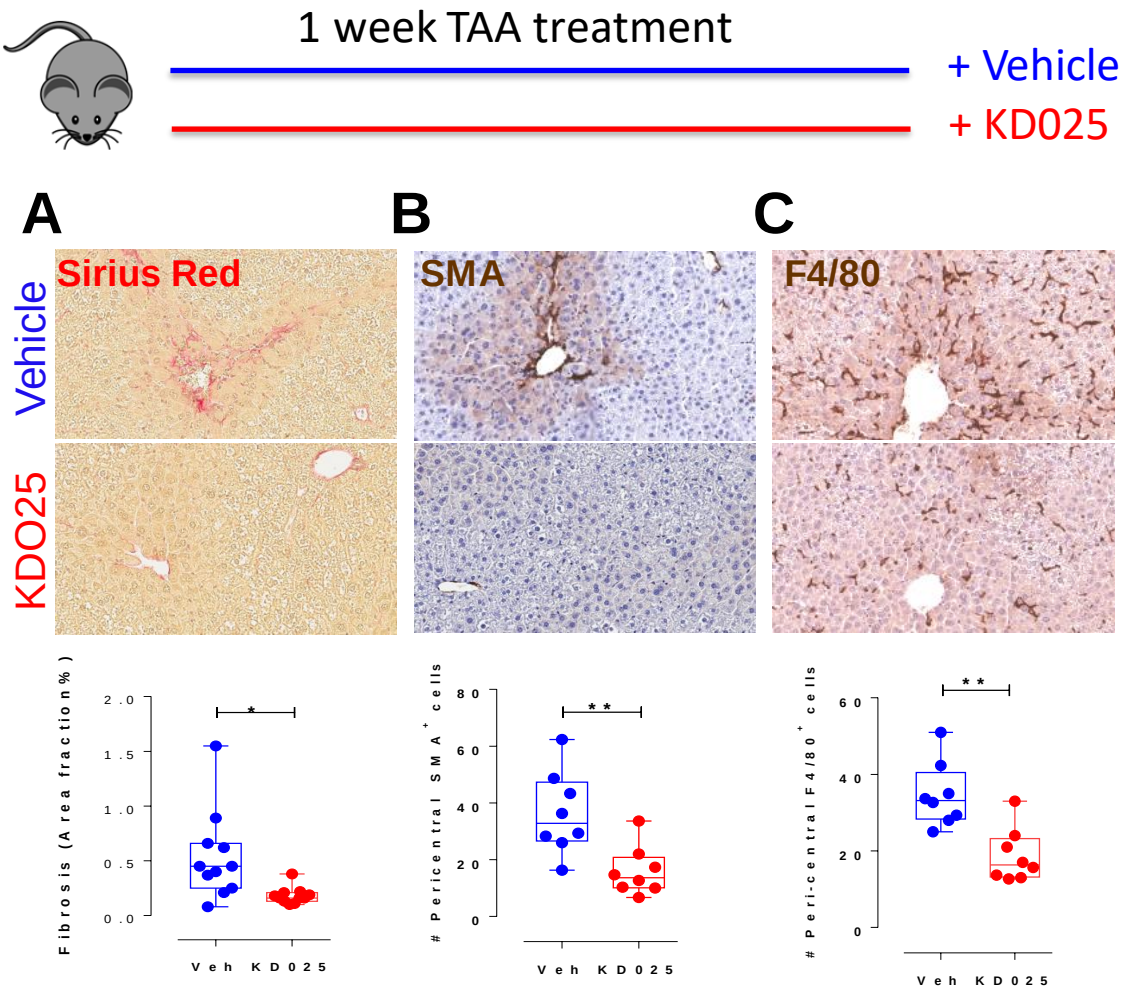


Figure 5-1 KDO25 inhibits TAA-induced liver fibrosis. Histochemical staining and quantification of (A) Sirius red (SR) (B) smooth muscle actin (SMA), and (C) F4/80 in representative liver sections from 1-week TAA-treated C57BL/6 mice co-administered with 0.4% methylcellulose vehicle control or 100mg/kg KDO25. Data are presented as the median and interquartile range (n=11 animals/group/time point). * p< 0.05, ** p< 0.01 Mann Whitney-U test.

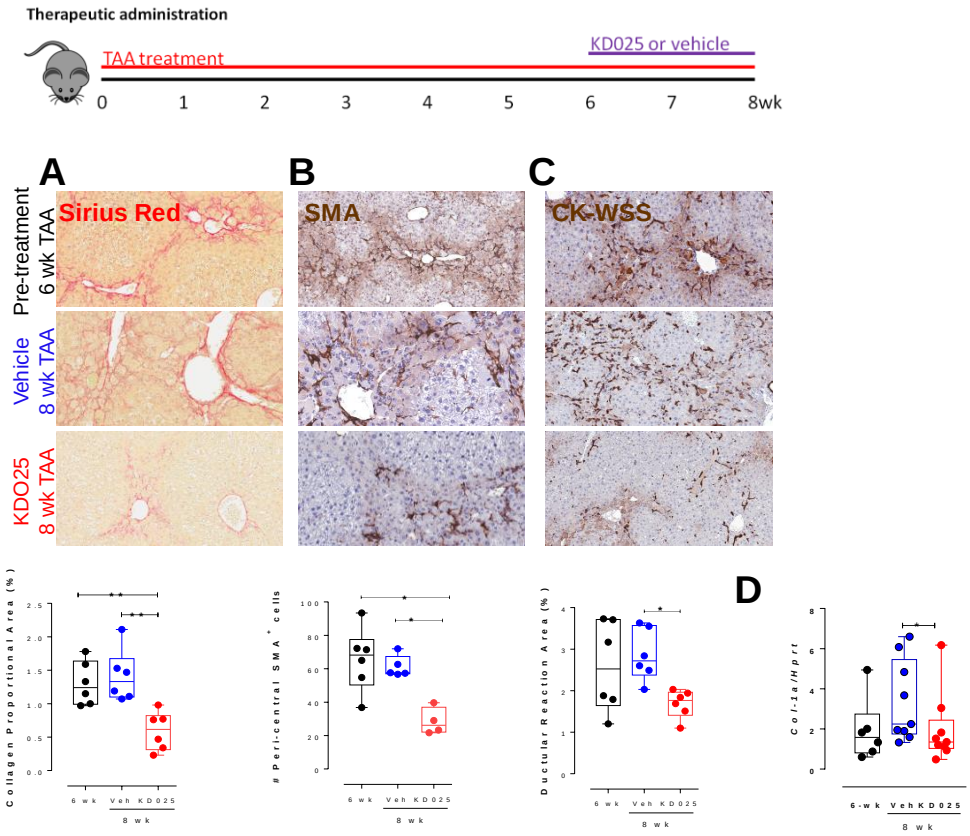


Figure 5-2 KDO25 promotes regression of TAA-induced liver fibrosis. Histochemical staining and aperio quantification of (A) Sirius red (SR), (B) smooth muscle activation (SMA), and (C) Pan-keratin (CK-WSS) staining in representative liver sections from C57BL/6 (WT) co-administered 0.4% methylcellulose vehicle control or 100mg/kg KDO25 after 6-week TAA-treatment. (D) Expression of *col-1a* in whole liver (n= 6 animals/group/time point). Representative images and data are presented as median and interquartile range * p< 0.05 (Pericentral SMA: 6-week TAA verses 8-week vehicle + TAA; 6-week TAA verses 8-Week KDO25 + TAA) (Ductular reaction: 8-week vehicle + TAA verses 8-week KDO25 + TAA) (Col1A: 8-week vehicle + TAA verses 8-week KDO25 + TAA), ** p< 0.01 (Collagen proportion area: 6-week TAA verses 8-week vehicle + TAA; 6-week TAA verses 8-Week KDO25 + TAA). Mann Whitney-U test.

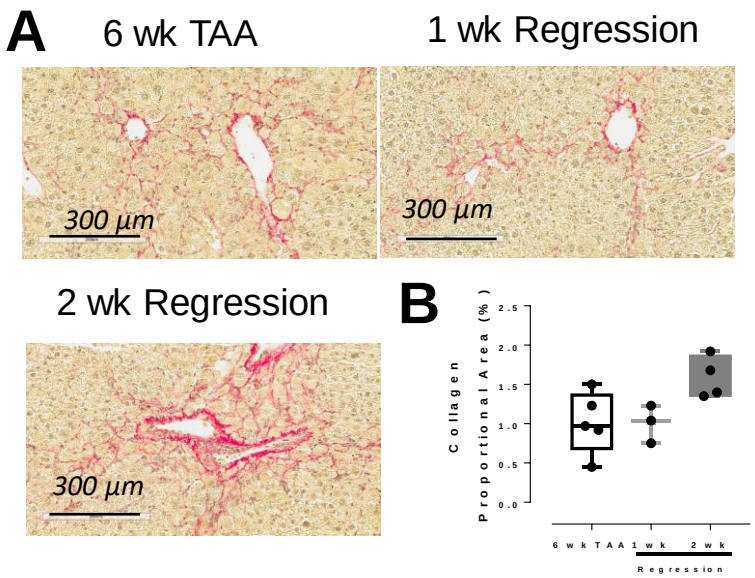


Figure 5-3 TAA withdrawal does not promote fibrotic regression. (A) Histochemical staining and (B) Aperio quantification of sirius red (SR) staining in representative liver sections from C57BL/6 (WT) after 6-week TAA-treatment followed by TAA withdrawal for 1 and 2 weeks to investigate fibrosis regression. (n= 3-6 animals/group/time point). Representative images and data are presented as median and interquartile range One-way ANOVA (K.W).

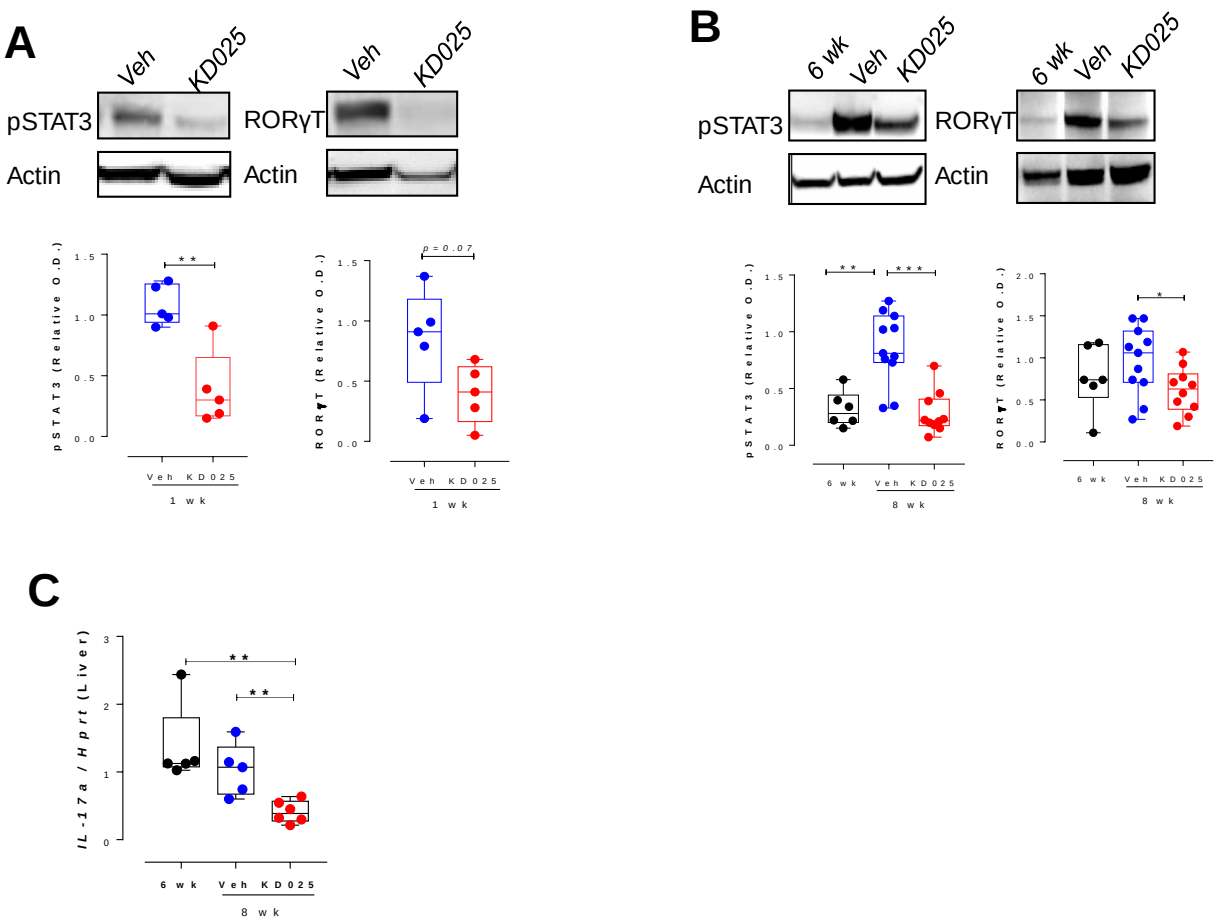


Figure 5-4 KDO25 inhibits STAT3 phosphorylation, RORγt, and IL-17A expression in the liver. To investigate whether KDO25 promotes regression of liver fibrosis through the inhibition of STAT3 phosphorylation, RORγt, and IL-17A expression, we initially treated one group of mice with TAA for 6-weeks to establish fibrosis. Subsequently, we used this 6-week TAA treatment as a common starting point for therapeutic administration experiments where either KDO25 and vehicle were co-administered with TAA for the final two weeks (8wk total). Whole livers were assessed for STAT3 phosphorylation and RORγt expression using western blots and normalized to actin expression following (A) 1-week TAA treatment and (B) 6-week TAA treatment, co-administered with either 0.4% methylcellulose or 100mg/kg KDO25. (n= 5-11animals/group). (C) qRT-PCR is determining the expression of *IL-17a* in whole liver (n= 5-6 animals/group). Data are presented as median with interquartile * p< 0.05, ** p< 0.01, *** p< 0.001 Mann Whitney-U test.

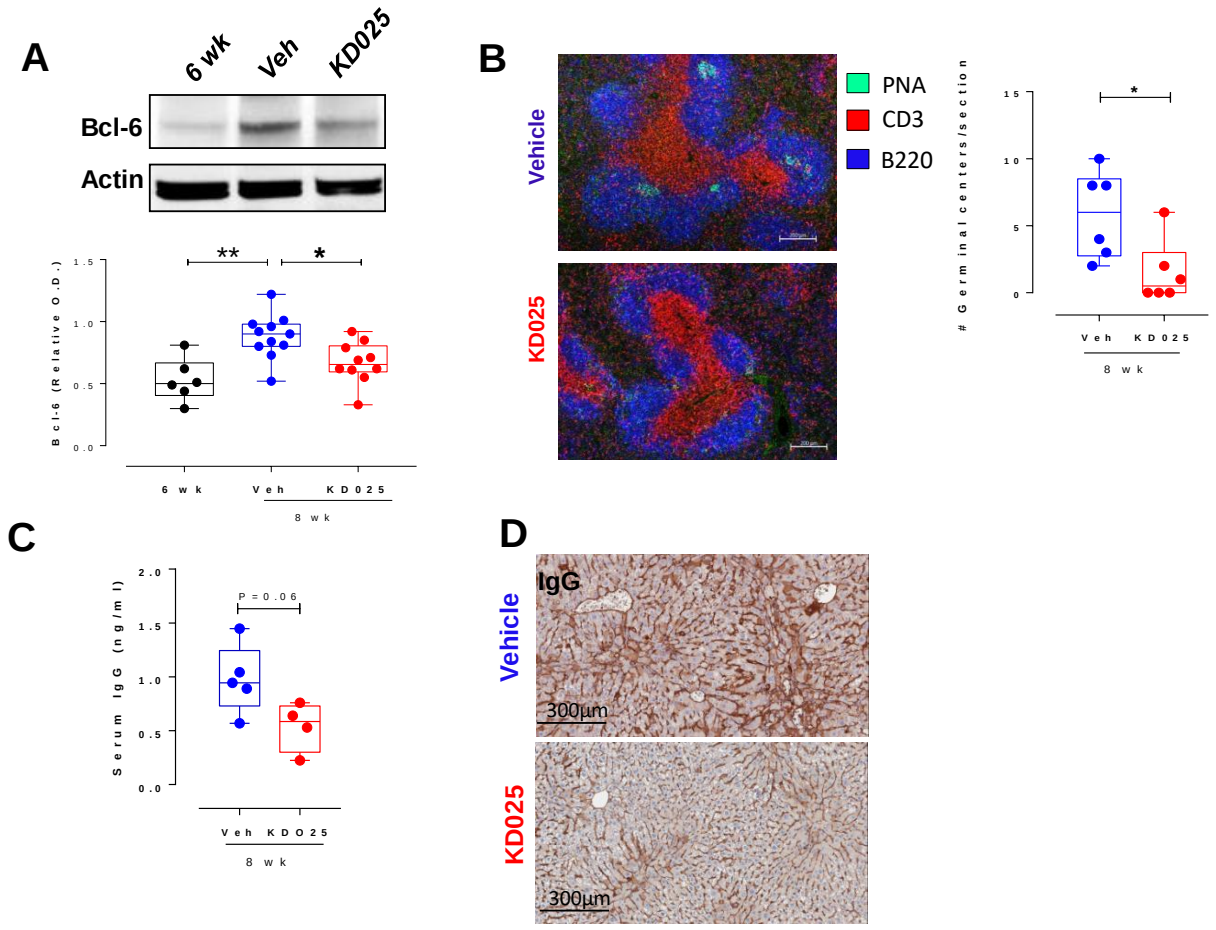


Figure 5-5 KDO25 inhibits BCL6 expression and germinal center formation in the liver. To investigate the impact of KDO25 promotes regression through the inhibition of on Bcl6 expression and GC formation, we initially treated one group of mice with TAA for 6-weeks to establish fibrosis. Subsequently, we used this 6-week TAA treatment as a common starting point for regression experiment where either KDO25 and vehicle were administered for the final two weeks (8wk total). (A) Western blots showing BCL6 expression in whole liver following 6-week TAA treatment, co-administered with 0.4% methylcellulose vehicle control or 100mg/kg KDO25 for further 2-weeks (n= 5-6 animals/group). (B) Confocal image representing anatomic structure of germinal center formation (GC) in spleens using immunofluorescence triple staining stained with anti-CD3 (red), anti-B220 (blue), and carbohydrate-peanut-agglutin (green) (n= 6 animals/group). (C) Serum immunoglobulin (IgG) levels (n= 4-5 animals/group) and (B) Histochemical staining for immunoglobulin deposition in the liver in 6-week TAA treated mice co-administered with 0.4% methylcellulose vehicle control or 100mg/kg. KDO25 Data are presented as median with interquartile * p< 0.05, ** p< 0.01 Mann Whitney-U test.

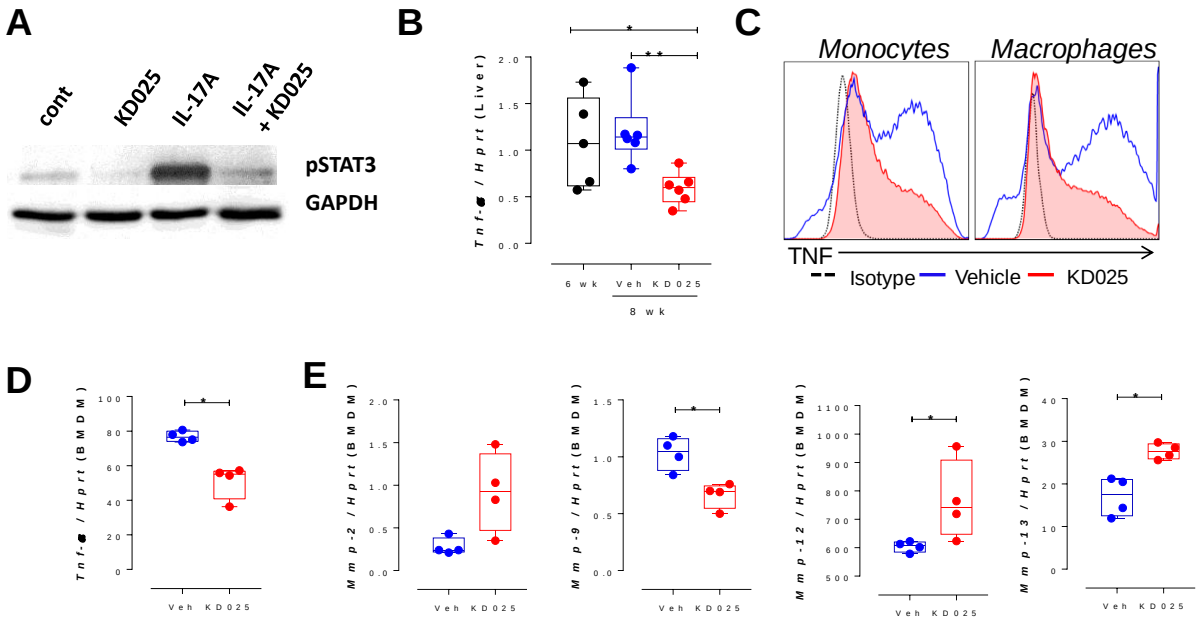


Figure 5-6 KDO25 alters macrophage function. (A) CSF-1 generated bone narrow derived macrophages were used to assess STAT3 phosphorylation by treating them with KD025 (10 µM) or IL-17A (10 µM) or KDO25+IL-17A or nil for 20 minutes before performing western blot analysis. Mice treated with TAA for 6-weeks, co-administered with 0.4% methylcellulose vehicle control or 100mg/kg KDO25 were used to determine (B) *Tnf* expression in whole liver (n-5-6 animals/group) and (C) TNF production from monocytes and macrophages following In-vitro LPS (100ng/ml) stimulation of liver cells. Data are presented as median and interquartile range * p< 0.05 (TNF liver: 6-week TAA verses 8-week KDO25 + TAA), ** p<0.01 (TNF liver: 8-week vehicle + TAA verses 8-week KDO25 + TAA). Mann Whitney-U test. CSF-1 generated bone narrow derived macrophages were treated with KD025 (10 µM) or DMSO control for 6 hr to determine the expression of (D) T*nf* and (E) MMP's (n= 4 animals/group). Data are presented as median and interquartile range * p< 0.05 Mann Whitney-U test.

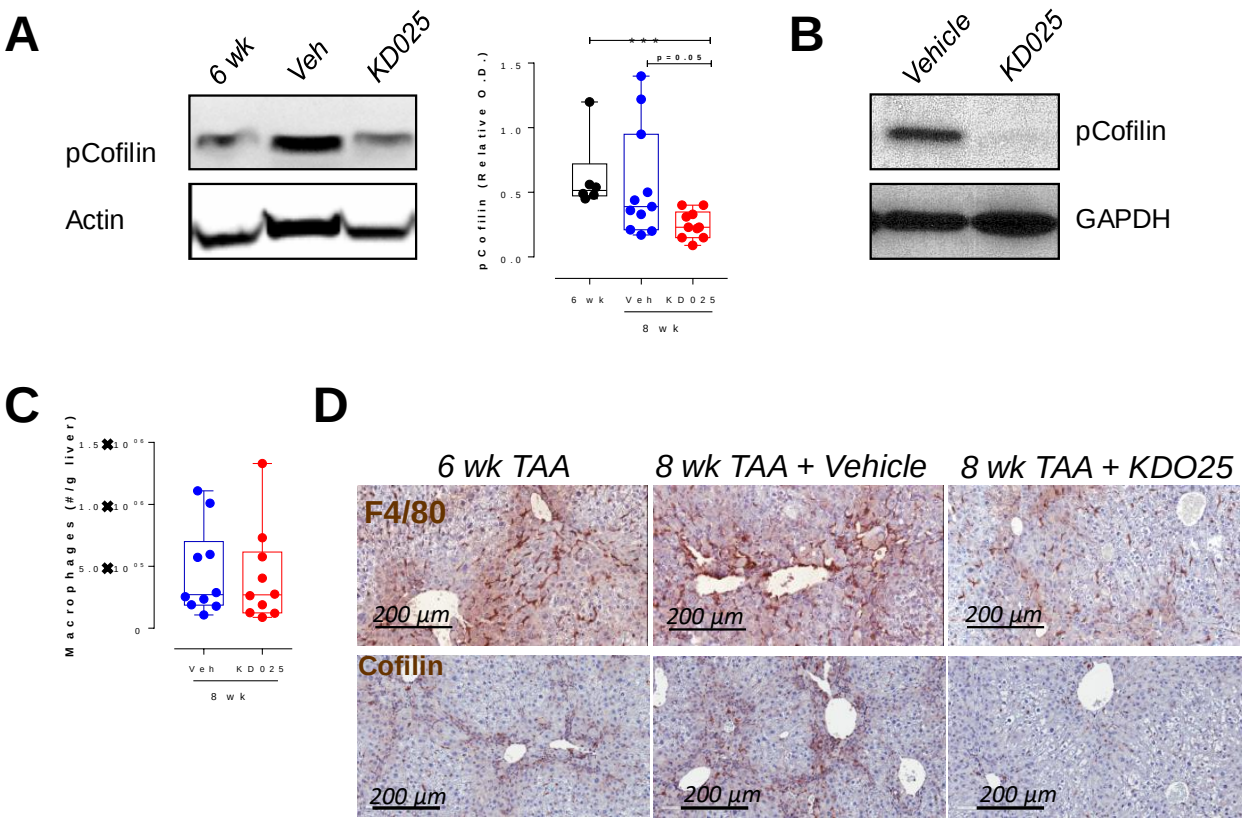


Figure 5-7 KDO25 alters macrophage migration. (A) Western blots determining the Cofilin phosphorylation in whole liver normalised with actin following 6-week TAA treatment, co-administered with 0.4% methylcellulose vehicle control or 100mg/kg KDO25 (n= 5-11animals/group). (B) CSF-1 generated bone narrow derived macrophages were used to assess Cofilin phosphorylation by treating them with KD025 (10 µM), DMSO control for 20 min before performing western blot analysis. (C) An absolute number of macrophages in total liver PBMC's (n =10 animals/group) and (D) Histochemical staining for F4/80 and cofilin in representative liver sections following 6-week TAA treatment, co-administered with 0.4% methylcellulose vehicle control or 100mg/kg KDO25. Data are presented as median and interquartile range *** p< 0.001(pCofilin: 6-week TAA verses 8-week KDO25+TAA) Mann Whitney-U test.

Chapter 6: Summary and Future directions

6.1. Summary:

Liver fibrosis is a consequence of chronic liver disease (CLD). Its progression can result in cirrhosis and the development of hepatocellular carcinoma (HCC). Liver fibrosis results from the interplay between proinflammatory cytokines/chemokines, inflammatory infiltrates, and tissue resident cells. Currently, there are no effective antifibrotics. One of the biggest challenges is to develop antifibrotic agents which not only inhibit the development of fibrosis but are able to reverse already established fibrosis. This thesis aimed to examine the different cellular and molecular mediators that contribute to liver fibrosis and also identify the active therapeutic target to inhibit progression and promote regression of fibrosis.

Fibrosis was induced by treating mice with thioacetamide (TAA) for 1, 6 and 12 weeks. TAA-induced liver fibrosis is an excellent model to investigate the various stages of fibrosis. The TAA model has many advantages as it is cost-effective, easy to administer and does not require surgical intervention. As recently described, the TAA liver injury model induces two phases of fibrosis [20]. After 1-6 week of TAA treatment, early collagen deposition is observed solely at the central vein; however, from 6-12 weeks injury progresses to central-central linkage with thick bands of collagenous septa [20]. During TAA treatment, early fibrosis is characterized by peri-central monocyte infiltration whereas, DR maturation with increased macrophage association characterizes late-stage fibrosis. Taking these parameters into consideration, we investigated different cellular and molecular mediators to the two phases of TAA-induced fibrosis.

In chapter 3 identified the critical requirement of IL-17 family cytokines during two phases of TAA-induced liver fibrosis. We confirmed that in the absence of IL-17A and IL-17E signaling, fibrosis and its related parameters were reduced following 1-6-week TAA, but not at the 12-week time point. However, fibrosis was significantly reduced in the absence of IL-17RA (devoid of both IL-17A and IL-17E signaling) at all time points examined. This study also identifies the mechanistic contributions of IL-17A and IL-17E cytokine during injury 1) Following TAA, IL-17E through CCL2

production promotes monocytic and CCR^{+} IL-17^{+}γδ T cell infiltration whereas, 2) IL-17A produced from γδ T cell contributes to peri-central monocytic sequestration without altering monocytic infiltration. We also demonstrated that IL-17A induces the production of proinflammatory cytokines (TNF and IL-6) which contribute to the pathogenesis of disease in this model. Thus, the inhibition of any of these signaling pathways (IL-7A or IL-17E) is sufficient to inhibit early liver fibrosis whereas, during late phase, both these cytokines synergistically contribute to disease pathogenesis.

In chapter 4 we also examined the role of B cells in liver fibrosis. Two recent publications have identified the pathogenic effects of B cells during liver fibrosis which is mediated by T cell-mediated B cell activation and antibody production [168] [169]. We identified that in the setting of TAA-induced liver fibrosis B cell-deficient mice displayed reduced fibrosis associated with reduced DR expansion. We observed increased intrahepatic B cell activation (CD44 and CD86) in mice treated with TAA for 6 weeks. Functionally, activated B cells are demonstrated to induce pro-inflammatory gene signature producing higher levels of TNF. In addition to pro-inflammatory gene signature activated B cells are also reported to induce the production of antibodies during the pathogenesis of various auto-immune diseases. We confirmed that TAA-induced liver fibrosis was also associated with antibody (IgG) deposition within the scar regions along with macrophages. The mechanism through which antibodies induce pathogenic effects is through FcγR ligation. We observed that although IgG was increased, decreased collagen deposition in the absence of FcγR demonstrates the importance of IgG and FcγR ligation during liver fibrosis. Together, our studies identified that B cells contribute to liver fibrosis through B cell activation, antibody deposition and its binding to FcγR.

Chapter 5 identifies the role of Rho-associated kinase 2 (ROCK2) in prevention and treatment of TAA-induced liver fibrosis. One publication has identified the upregulated expression of ROCK2 in BDL induced liver fibrosis model. ROCK2 has an established role in IL-17A production, B cell development, and pathogenic macrophage function during autoimmune and inflammatory diseases. Given the importance of IL-17A, B cells, and macrophages in TAA model, above findings suggest the role of ROCK2 in our model. Here, we identified that KD025 a selective ROCK2 inhibitor prevented fibrosis by attenuating IL-17 production through the inhibition of signal transducer and activator of transcription 3 (STAT3) and RAR-

related orphan receptor gamma (RORγt) signaling pathways. We also identified that that KDO25 attenuated splenic B cell germinal center formation and liver IgG deposition by inhibiting the expression of B-cell lymphoma 6 (BCL6) transcription factor. KDO25 has directly altered macrophage function by downregulating IL-17A mediated pSTAT3 expression along with reduced TNF production. We also confirmed the role of KDO25 in fibrotic regression. During fibrotic regression, KDO25 will disrupt the association between DR/F480 within the scar region and promotes macrophage migration away from the scar through pCofilin mediated cytoskeletal rearrangements. We also observed that during regression of fibrosis, KDO25 promotes collagen degradation by restoring the production of MMP's (Mmp-2, 12, and, 13.) from macrophages. Together, these data demonstrated the role of ROCK2 in promoting proinflammatory cytokine IL-17A expression, pathogenic immunoglobulin production by B cells and driving profibrotic macrophage activity.

In summary, this thesis presents the mechanistic contributions of IL-17A and IL-17E signaling during liver fibrosis mediated through myeloid infiltration and function. Additionally, we also identified the mechanisms associated with B cell-induced liver fibrosis. Finally, we identified the therapeutic role of KDO25 in by inhibiting the pathogenic responses induced by different cellular (monocytes/macrophages and B cells) and molecular (STAT3, BCL6, IL-17A, Cofilin) mediators.

6.2. Mechanism of TAA induced liver fibrosis:

TAA has been reported to induce oxidative stress to hepatocytes which eventually leads to the production of DAMPS and recruitment of inflammatory mediators (Predominantly monocytes). Collectively, our study identified multiple cellular and molecular mediators that contribute to TAA-induced liver fibrosis **(Figure 6-1).** Monocytes/macrophages are reported as critical mediators of TAA-induced liver fibrosis. Initially, TAA-induced liver fibrosis promotes the recruitment of pericentral CCR2+ monocytes. Eventually, CSF-1 will promotes the differentiation of CCR2+ monocytes into CX3CR1+ monocytes and pathogenic macrophages. Our data identified that during the early phase of TAA-induced liver fibrosis, IL-17E/IL-17RB signaling on macrophages will promote the recruitment of CCR2+monocyte and γδT cells through the production of CCL2. Subsequently, activation of RORγt and STAT3 in γδT cells induced the production of IL-17A in our model. Mechanistically, IL-17A signaling on monocytes facilitate pericentral monocytic sequestration

following IL-17E induced monocytes recruitment. Additionally, IL-17A signaling induced the production of inflammatory cytokines such as: (a) TNF from macrophages in STAT3 dependent manner and (b) IL-6 in the absence of IL-17A signaling for which the mechanism was unidentified. Finally, the redundant nature of both IL-17A and IL-17E during early liver fibrosis was identified whereas during late phase, both IL-17A and IL-7E synergistically contribute to TAA-induced liver fibrosis. Our study also observed that IL-17A signaling induced the production of TWEAK, which has been reported in the contribution of DR maturation and the development of fibrosis. However, further studies are required to elucidate whether there are additional mechanisms where IL-17 family cytokines directly or indirectly contribute to TAA-induced DR maturation. Other cellular mediators which were associated with TAA-induced liver fibrosis are B cells. Contribution of these B cells during TAA-induced liver fibrosis was associated with BCL6 induced B cell activation, germinal center formation, antibody (IgG)production and its binding to FcγR. We also observed that IgG was sequestered around central vein within the scar region along with macrophages. Previous findings reported that FcγR is dominantly expressed by macrophages. However, we still need to investigate the cells that express FcγR in our model. In order to prevent and promote the regression of TAA-induced liver fibrosis, ROCK2 inhibitor (KDO25) was used which inhibited the pathogenic responses induced by different cellular and molecular mediators. KDO25 a) Inhibited STAT3 and RORγt signaling pathways which in turn attenuated IL-17 production in the livers of TAA treated mice, b) inhibited BCL6 expression resulting in reduced splenic B cell germinal center formation and diminished IgG production in the livers c) diminished STAT3 phosphorylation and TNF production in response to IL17A in macrophages d) diminished pCofilin expression from macrophage promoting regression of fibrosis through the inhibition of macrophage migration from fibrotic septae . Thus, these studies identify ROCK2 as a potential therapeutic target to inhibit pathogenic responses induced by different cellular (monocytes/macrophages and B cells) and molecular (STAT3, BCL6, IL-17A, Cofilin) mediators during TAA-induced liver fibrosis

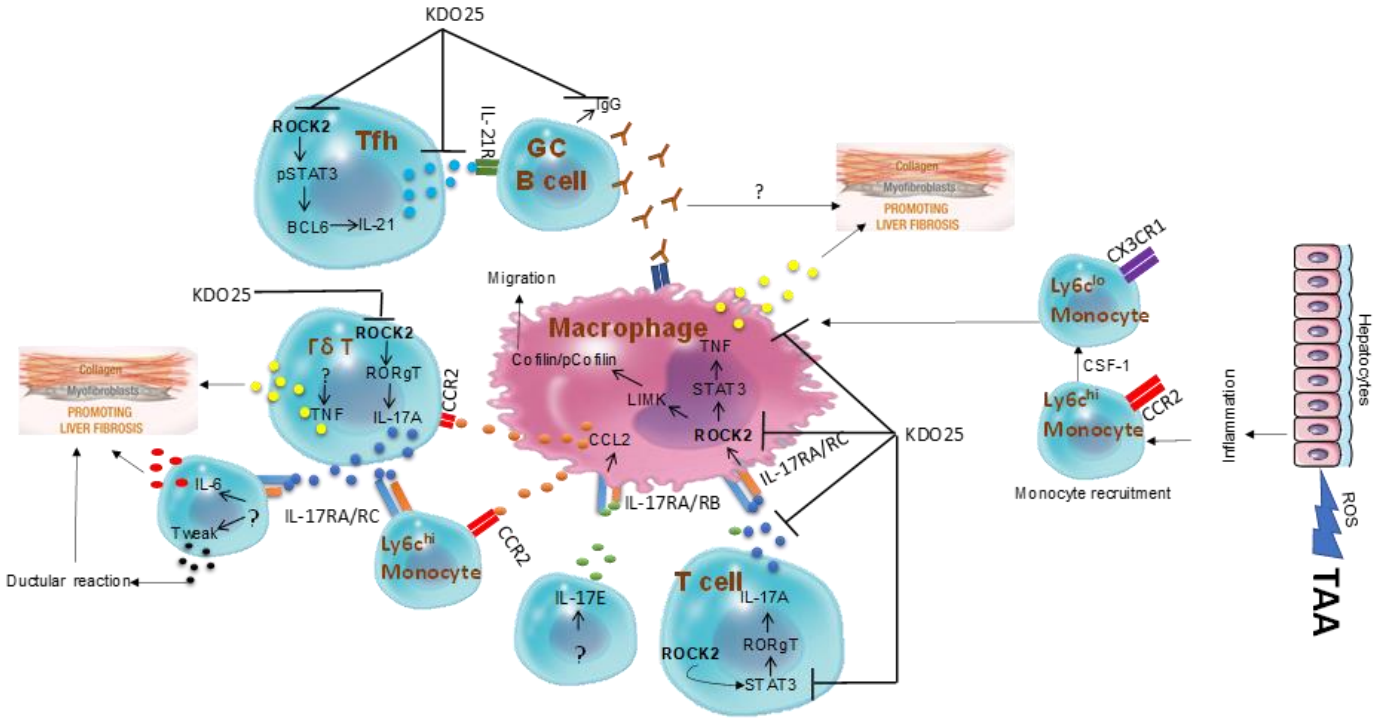


Figure 6-1Mechanism of TAA induced liver fibrosis:

During early phase fibrosis (1-week TAA) L-17E through IL-17RA/IL-17RB signaling induce the production of CCL2 from macrophages. CCL2 production promotes the recruitment of pathogenic CCR2+ monocytes and CCR2+IL-17+γδ T cell infiltration into the liver. IL-17A through IL-17RA signaling was required for the sequestration of monocytes around the central vein (CV) during 1-week TAA treatment. IL-17A signaling also facilitate the expression IL-6 and TNF in the liver, both of which are established mediators of fibrosis. IL-21 production from T follicular helper cell (TFh) promotes germinal center B cell development and antibody (IgG) production during 6-week TAA treatment. Association of IgG and FcR on macrophages were shown to elicit pathogenic responses. ROCK2 has an established role in regulating IL-17A and IL-21 production induce macrophage plasticity. KD025, a selective ROCK2 inhibitor, inhibited signal transducer and activator of transcription 3 (STAT3) and RAR-related orphan receptor gamma (RORγt) signaling pathways which in turn attenuated IL-17 production in the livers of mice following 1 and 8-week TAA treatment. KDO25 also inhibited B-cell lymphoma 6 (BCL6) expression resulting in significantly reduced B cell germinal center formation and diminished IgG production in the livers of TAA treated mice. Additionally, KD025 administration significantly diminishes STAT3 phosphorylation and TNF production in response to IL17A in macrophages. Cofilin pathway, is another ROCK2 target which regulates cytoskeletal reorganization required for cytokine production, adhesion and migration. KD025 markedly diminished pCofilin expression from macrophage. Overall, our findings suggested the synergistic contribution of IL17A and IL-17E cytokines by promoting myeloid recruitment and pathogenic differentiation. Moreover, we identify a critical role for B cells in pathogenic IgG production and its association with FcγR. Finally, we identified ROCK2 as a potential therapeutic target to inhibit pathogenic responses induced by different cellular (monocytes/macrophages and B cells) and molecular (STAT3, BCL6, IL-17A, Cofilin) mediators during TAA induced liver fibrosis.

6.3. Future work:

TAA induced liver fibrosis was associated with immunoglobulin (IgG) production, however, the pathogenic role of IgG elicited during TAA-induced liver fibrosis is undefined. Having demonstrated an increased serum IgG levels in mice with established fibrosis, future studies on adaptive transfer of serum (containing IgG) from these mice to naïve WT mice might lead to understanding the pathogenicity of IgG in liver fibrosis. Binding of IgG to FcγR was associated with liver fibrosis. However, the mechanism of this binding is not found. Our study displayed that IgG was sequestered around central vein along with macrophages. Therefore further studies like macrophage depletion in $Fc\gamma R^{-/-}$ (prior to adaptive serum IgG transfer) and generation of bone marrow chimeras where FcγR is restricted to hematopoietic cells might reveal the binding of IgG to FcγR on macrophages.

We have shown that degradation of ECM (Collagen1A) promotes regression of fibrosis. As per the previous findings MMPs, play a pivotal role in promoting ECM degradation that is essential for wound healing [442]. Our studies demonstrated that macrophages are an important source of MMP's and were shown to be profoundly regulated by KDO25 to encourage ECM degradation and promote fibrolysis. Therefore further studies are required to understand the mechanisms by which KDO25 promotes fibrolysis. Thus, the examination of the effects of ROCK2 inhibition on macrophages in MMP expression and function (zymograpgy) should provide further insight into profibrotic mechanisms of ROCK2. Eventually, this would help to define the permissive role of macrophages in fibrotic regression. Although the importance of KDO25 in macrophages was thoroughly studied in our model, further studies investigating the role of KDO25 in other immune and liver resident cells (HSC and LSEC) using RNA sequencing would help to understand the relative contribution of ROCK2 among different cells.

Our study identified an important role of IL-17E signaling in inducing CCL2 from macrophages which in turn promotes pericentral monocytic infiltration. Having identified that the expression of IL-17RB was largely restricted to hepatocytes future studies will focus on establishing the direct effects of IL-17E signaling in hepatocytes that promote fibrosis. Thus, administration of recombinant IL-17E to chimeric mice where IL-17RB signaling is restricted to non-hematopoietic cells

would lead to understand the profibrotic mechanisms of IL-17E induced through hepatocytes. Along these lines, interrogation of IL-17E elicited hepatocyte cytokine and growth factors (in-vivo and in-vitro) could be used to identify the intermediate(s) factors responsible for the induction of CCL2 from macrophages. Additionally, our lab reported that macrophages were closely associated with DR maturation during 6-week TAA treatment which internally demonstrated the association of IL-17 signaling with DR maturation. Further investigations on the effects of IL-17 signalling on macrophages (in-vitro and in-vivo) in expression and function various profibrotic genes associated with DR would lead to identify the mechanistic role of IL-17 signalling in promoting DR.

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Appendices

Appendix A: List of reagents used.

- Tris-EDTA Buffer (10mM Tris Base/1 mM EDTA solution, pH9)

  Tris- 1.21gm

  EDTA- 0.37gm

  RO water- 1l

- 0.5M EDTA:

  EDTA (disodium ethylenediamine tetraacetate.$2H_20$)- 186.1GM

  Distilled water- 800ml

  NaOH pellets- 18-20gm

  PH- 8.0

- WASH BUFFER:

  1X PBS – 1l

  Heat inactivated FCS (Gibco) – 20ml

  0.5M EDTA- 10ml

- IMDM Complete:

  IMDM (SAFC)- 500ml

  Heat inactivated FCS (Gibco) – 50ml

  Pencillin/Streptamycin (Gibco) - 5ml

  L-Glut 100x (Hyclone 200mM)- 5ml

  Non-essential amino acids 100x (Gibco) – 5ml

  Sodium Pyruvate 100x (100mM)- 5ml

  2 Mercapto ethanol 100x -1ml

- Collegenase digest solution for 10ml:

  Hank's Based Salt Solution (HBSS)- 10ml

Collagenase Type IV (D) (Sigma)- 10mg

1 mg/ml DNase I (Sigma)- 20µl

- 30% Pecoll gradient for 25ml:

  Percoll (GE Healthcare #17-0891-01) - 8.44 ml
  10 x PBS - 0.94 ml
  1 x PBS - 15.63 ml

- GEY'S lysis buffer:

Gey's Stock A	NH_4Cl	35gm
	KCl	1.85gm
	$Na_2HPO_412H_2C$	1.5gm (or Na_2HPO_4 0.63g)
	KH_2PO_4	0.12gm
	Glucose	5.0gm
	Phenol red	50.0mg
	Bring to 1000mls with distilled water	
Gey's Stock B	$MgCl_26H_2O$	0.42gm
	$MgSO_4.7H_2O$	0.14gm
	$CaCl_2$	0.34gm (or $CaCl_2.2H_20$ 0.45g)
	Bring to 100mls with distilled water	
Gey's Stock C	$NaHCO_3$	2.25gm
	Bring to 100mls with distilled water	
	1 X Gey's Working Solution	
	20 parts stock A	
	5 parts stock B	
	5 parts stock C	
	70 parts distilled water	
	Sterile fillter 0.22µm	

- Preperfusion buffer (PB) (10×)

NaCl	80.00 gm
KCl	4.00 gm
$NaH_2PO_4•2H_2O$	0.78 gm
$Na_2HPO_4•12H_2O$	1.51 gm

$NaHCO_3$ 3.50 gm

EGTA 1.90 gm

Glucose 9.00 gm

Phenol red 0.06 gm

Dissolved in 1 L ddH_2O and adjust pH to 7.4.

- Enzyme buffer (EB) (10×)

  NaCl 80.00 gm

  KCl 4.00 gm

  $NaH_2PO_4 \cdot 2H_2O$ 0.78 gm

  $Na_2HPO_4 \cdot 12H_2O$ 1.51 gm

  $NaHCO_3$ 3.50 gm

  HEPES 23.80 gm

  Phenol red 0.06 gm

  Dissolved in 1 L ddH_2O and adjust pH to 7.4.

  [Add 20µl $CaCl_2$(2.5M) to 100 ml of 1x EB buffer first, mixed well, then add 10 mg of collagenase IV before the experiment]

- Liver lysis buffer (1ml per 0.02-0.06mg liver)

  T-PER® Tissue Protein Extraction Reagent 10ml

  Protein inhibitor tablets 1