Université de Montréal

Type 3 cytokine responses during Non-Alcoholic Fatty Liver Disease (NAFLD)

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Résumé

Au cours des deux dernières décennies, la stéatose hépatique non alcoolique (NAFLD) a été une maladie épidémique croissante, non seulement dans les pays occidentaux mais également dans le monde entier en raison de l'augmentation continue des modes de vie sédentaires, de l'obésité, et de la résistance à l'insuline. La prévalence mondiale de la NALFD est actuellement estimée à 25% dans la population générale adulte. NALFD est composé d'un éventail d'affections hépatiques s'étendant du foie gras non-alcoolique (NAFL), stéatohépatite non-alcoolique (NASH), fibrose avancée et cirrhose qui peut progresser au carcinome hépatocellulaire (HCC). L'inflammation induite par NASH peut moduler l'activation des cellules stellaires hépatiques (CSH) et donc influencer la progression de la fibrose hépatique. Le rôle de l'inflammation de type 3, qui est caractérisée par la production des cytokines IL-17A et IL-22, dans la fibrose de type NAFLD demeure incompris.

Dans cette thèse, nous avons évalué le rôle d'IL-22 et d'IL-17A dans la fibrose liée à la NAFLD. Des biopsies cliniques de foie NAFLD humain et un modèle murin *in vivo* de NAFLD ont été utilisés et des expériences *in vitro* ont été effectuées. Nous avons démontré que l'expression hépatique d'IL-22 est plus élevée chez les femmes et chez les femelles avec NAFLD versus les hommes et les mâles. Nous avons identifié les neutrophiles et les cellules T, y compris les cellules T Th17, Th22 et γδ, en tant que principaux producteurs d'IL-22 chez les sujets féminins et les souris atteintes de NAFLD. De plus, nous avons démontré que l'absence de la signalisation endogène du récepteur IL-22 (modèle IL-22RA1 knockout) chez les souris femelles avec NAFLD, aggravait les lésions hépatiques, l'inflammation et la fibrose, comparé aux mâles. Cet effet hépatoprotecteur dépend des mécanismes anti-apoptotiques médiés par la signalisation du récepteur IL-22 qui favorisent la survie des hépatocytes et réduisent au minimum les dommages au foie. Nous avons également montré que l'expression hépatique d'IL-22BP est régulé à la hausse chez les souris femelles avec NAFLD comparé aux mâles. Dans ces femelles, le ratio d'ARN messager hépatique de l'IL-22 envers celui de l'IL-22BP est corrélé positivement avec les gènes en aval de cible d'IL-22 (gènes anti-apoptotiques et antioxydants). Par ailleurs, nous avons prouvé que les neutrophiles intrahépatiques produisent l'IL-17A *in situ* dans notre modèle NAFLD et ceci correspondait fortement avec la progression de la fibrose de foie et les dommages hépatiques. Nous avons fourni des preuves préliminaires que l'IL-17A peut induire des pièges extracellulaires de neutrophiles (NET) *in vitro*, et la signature de NETs est impliquée dans la progression de la fibrose hépatique dans notre NAFLD.

Pris ensemble, Ces résultats démontrent qu'identifié un nouveau rôle de l'inflammation de type 3 dans la fibrose liée au NAFLD, où l'action de l'IL-22 est dépendante du sexe et possède des

fonctions hépatoprotectrices contre la fibrose du foie chez les femelles, alors que l'IL-17A agit en tant que cytokine profibrogénique et favorise la fibrose de foie.

Mots-clés : NAFLD, NASH, Fibrose, inflammation, IL-22, IL-17A, et neutrophiles

Abstract

Non-alcoholic fatty liver disease (NAFLD) is a growing epidemic, not only in western countries but also worldwide due to the continuous rise in sedentary lifestyles, obesity, and insulin resistance over the last two decades. The global prevalence of NALFD is currently estimated to be 25% in the general adult population. NAFLD is comprised of a spectrum of liver disease ranging from non-alcoholic fatty liver (NAFL), non-alcoholic steatohepatitis (NASH), advanced fibrosis, and finally cirrhosis that can progress to hepatocellular carcinoma (HCC). NASH-induced inflammation can modulate hepatic stellate cells (HSCs) activation and hence influence hepatic fibrosis progression. The role of type 3 inflammation, which is characterized by the production of the cytokines IL-17A and IL-22, in NAFLD-related fibrosis remain not clear.

In this thesis, we evaluated the role of IL-22 and IL-17A in NAFLD-related fibrosis using clinical liver biopsies from a NAFLD human cohort, an *in vivo* NAFLD mouse model and *in vitro* experiments. We report that hepatic IL-22 expression had sexually dimorphic differences in both humans and mice with NAFLD where it was elevated in females versus males. We identified intrahepatic neutrophils in female subjects with NAFLD as well as T cells, including Th17, Th22, γδ T cells, in female mice with NAFLD as major producers of IL-22. In addition, we demonstrated that lack of endogenous IL-22 receptor signaling (IL-22RA1 knockout model), exacerbated liver injury, inflammation, and fibrosis in female but not male mice with NAFLD. This hepatoprotective effect was dependent on IL-22 receptor signaling-induced anti-apoptotic signals that promote hepatocyte survival and minimize liver damage. We also demonstrated that hepatic IL-22BP expression was upregulated in female mice with NAFLD compared to males, and the hepatic IL-22/IL-22BP mRNA ratio positively correlated with IL-22 downstream target genes (anti-apoptotic and antioxidant genes) in those females. Moreover, we showed that intrahepatic neutrophils produce IL-17A *in situ* in our NAFLD model and this was strongly correlated with progression of liver fibrosis and liver injury. We provided preliminary evidence that IL-17A can induce neutrophil extracellular traps (NETs) *in vitro*, and that NETs are implicated in liver fibrosis progression in our NAFLD model.

Taken together, we identified a novel role for type 3 inflammation in NAFLD-related fibrosis, where IL-22 act in sex-dependent manner and provided hepatoprotective functions against liver fibrosis in females, while IL-17A act as profibrogenic cytokine and promotes liver fibrosis through enhancing NETs.

Keywords: NAFLD, NASH, Fibrosis, inflammation, IL-22, IL-17A, and neutrophils

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Mohamed Abdelnabi

Chapter 1:

Literature Review

I. Introduction

Liver fibrosis is a characteristic hallmark of most types of chronic liver diseases caused by various aetiologies, including viruses, parasites, toxins, alcohol, metabolic dysregulation, or autoimmunity. Liver fibrosis is mainly depicted by activation and proliferation of hepatic stellate cells (HSCs), resulting in pathological accumulation of extracellular matrix (ECM) proteins, including collagen, leading to fibrous scar formation and progressive loss of hepatic architecture and function[1]. Non-alcoholic fatty liver disease (NAFLD) has become the most prevalent chronic liver disease due to the incidence rise of obesity, insulin resistance (IR) and type 2 diabetes mellitus (T2DM)[2]. The liver-related mortality increases exponentially with the escalation in fibrosis stage in patients with NAFLD[3] with therapeutic interventions for clinical management of NAFLD-related fibrosis being limited. NAFLD-related inflammation can enhance activation of HSCs thus accelerating fibrosis progression and development of liver cirrhosis, which represent a high risk for developing hepatocellular carcinoma (HCC)[4]. Indeed, the cross-talk between inflammation, hepatocytes, and HSCs is a critical process in the progression of NAFLD-related fibrosis. Thus, investigating the inflammation in response to the chronic injury by NAFLD is essential to understand pathological mechanisms of liver fibrosis as well as develop appropriate therapies. IL-17 and IL-22 cytokines have been implicated in different contexts of chronic liver inflammation, where IL-17 is associated with disease progression, while IL-22 mediate opposite effects depending on the context[5]. However, little is known about the role of these cytokines in the pathogenesis of NAFLD-related fibrosis. In this section, we will discuss the liver anatomy, its metabolic and immunological functions, the regulatory pathways of HSCs activation during liver fibrosis, NAFLD pathogenesis, and role of IL-17 in acute and chronic liver injuries, including NAFLD.

1. Liver anatomy and functions

1.1. Liver macro and micro anatomy

The liver is the largest vital organ in the human body, accounting for 2-3% of the average body weight. The color of the liver is dark reddish-brown, and it weighs around 3 pounds in adults. The functionality of the liver is very complex, and it involves numerous metabolic and physiological processes which are essential for maintaining normal homeostasis as well as supporting functions of other organs. Anatomically, the liver is located in the right upper quadrant of the abdominal cavity, above the intestine and below the hemidiaphragm, which separates the liver from the lung. The liver consists of two main lobes (right and left), where both further divide into 8 segments (Figure 1), containing thousands of small lobes, known as lobules. The hepatic lobule is considered the structural and functional unit of the liver. With a hexagonal structure, this lobule consists of hepatocytes that arrange radially in plates with the central vein located in the center and the portal tracts (hepatic portal artery, portal vein and small bile ducts) located at the top of the lobule (Figure 2) [6]. The composition of the liver encloses many types of cells, which can be simply classified into parenchymal and non-parenchymal cells. Parenchymal cells are mainly hepatocytes representing most of liver cells (78-80%), while non-parenchymal cells (NPCs) represent the remaining 14–17%. The non-parenchymal cells include various types of cells such as liver sinusoidal endothelial cells (LSECs), biliary epithelial cells, HSCs, and immune cells such as Kupffer cells (KCs) (Please see Figure 4 for the liver immunology).

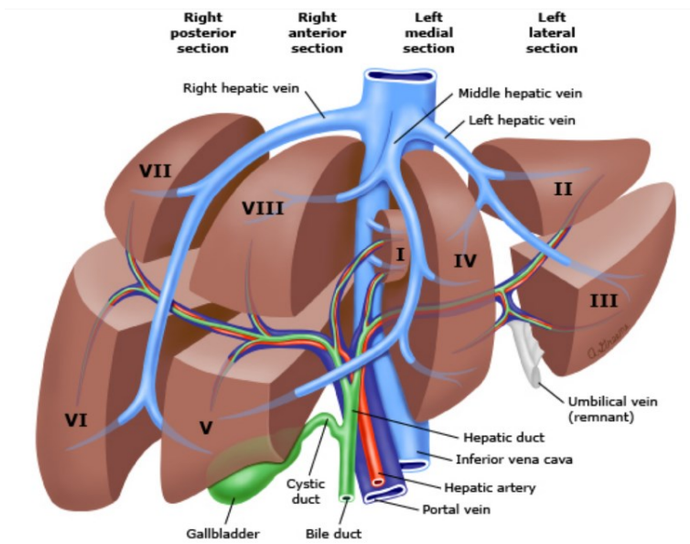

Figure 1. The macro-anatomy of liver

The liver has 4 sections; right and left lateral (anterior) and right and left medial (posterior). These segments composed of 8 independent sections according to Couinaud's system. Segments I to IV from the left lobe, and segments V to VIII form the right lobe. This Figure is adapted from [7] (Reproduced with permission)

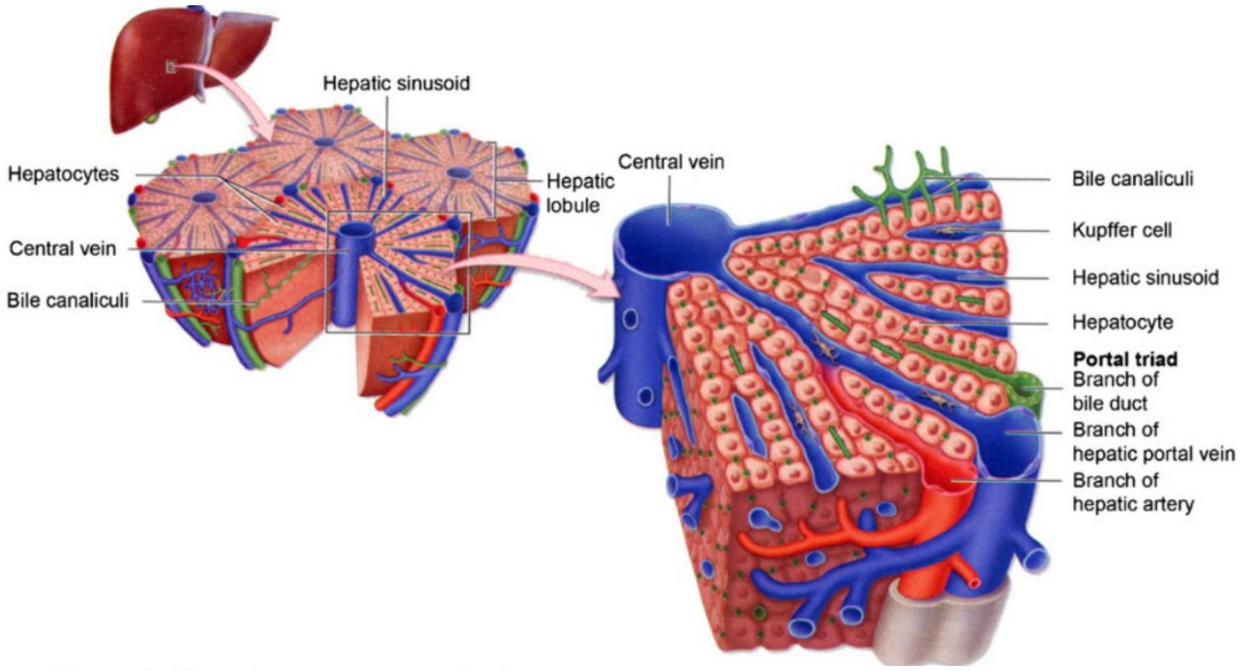

Figure 2. The microanatomy of the liver
Schematic diagram representing the hexagonal microanatomy of the liver composed of six portal triads and a central vein located in the center forming the hepatic functional unit known as lobule. Each portal triad consists of three branches: bile duct, portal vein and hepatic artery. Blood flows from the triads to the central vein through the hepatic sinusoidal space. This Figure is taken from [8]. (Reproduced with permission)

There is a thin barrier in between the hepatocytes, consisting of a network of LSECs. These LSECs are involved in different physiological functions, including nutrient transfer to hepatocytes, pathogen recognition, antigen presentation, and induction of immune tolerance. Indeed, LSECs act as a physical barrier between hepatocytes and blood, where they ensure delivery of nutrient and oxygen-rich blood to the lobule via portal vessels. Importantly, LSECs can undergo fenestration (small holes), allowing the transfer of nutrients and other solutes between sinusoidal blood and the space of Disse, which is a gap or space between hepatocytes and LSECs (Figure 3). This space facilitates transfer of nutrients by slowing down blood flow to enhance metabolite and toxin uptake by hepatocytes and promote the interplay of lymphocytes with antigen presenting cells (APCs), leading to immune tolerance. In addition, HSCs are located in the space of Disse, and in homeostasis, while they are in quiescent state, they contribute to the storage of vitamin A storage that is metabolised by the liver (Figure 3). HSCs are also implicated in modulating innate and adaptive immune responses. On the other hand, the hepatic sinusoids are the house for liver resident KCs, making KCs in close contact with sinusoidal endothelium and hepatocytes (Figure 3). KCs play an essential role in recognizing and clearing pathogens as well as regulating immune tolerance[9-14]. The liver organ is enriched with vasculature, including portal vein, central vein, and hepatic arteries, whereby each minute, the liver receives up to 30% of the total blood volume. Importantly, a dual blood supply reaches the liver, originating from the hepatic portal vein, which accounts for 75-80% of this blood supply, while hepatic arteries provide the

remaining 20-25% of blood supply (oxygenated blood). The hepatic portal vein receives antigen-rich blood derived from the stomach and other peripheral organs such as the spleen and containing high levels of antigens from the diet and microbial toxins from the gut microbiome such as lipopolysaccharide endotoxin (LPS). Thus, the immune system of the liver has evolved to mediate immune surveillance and can be rapidly activated to prevent dissemination of foodborne or bloodborne pathogens and maintain homeostasis. The blood flow in the liver is drained into central veins, which are branches of the hepatic veins and leaves the liver through the inferior vena cava[6, 15, 16].

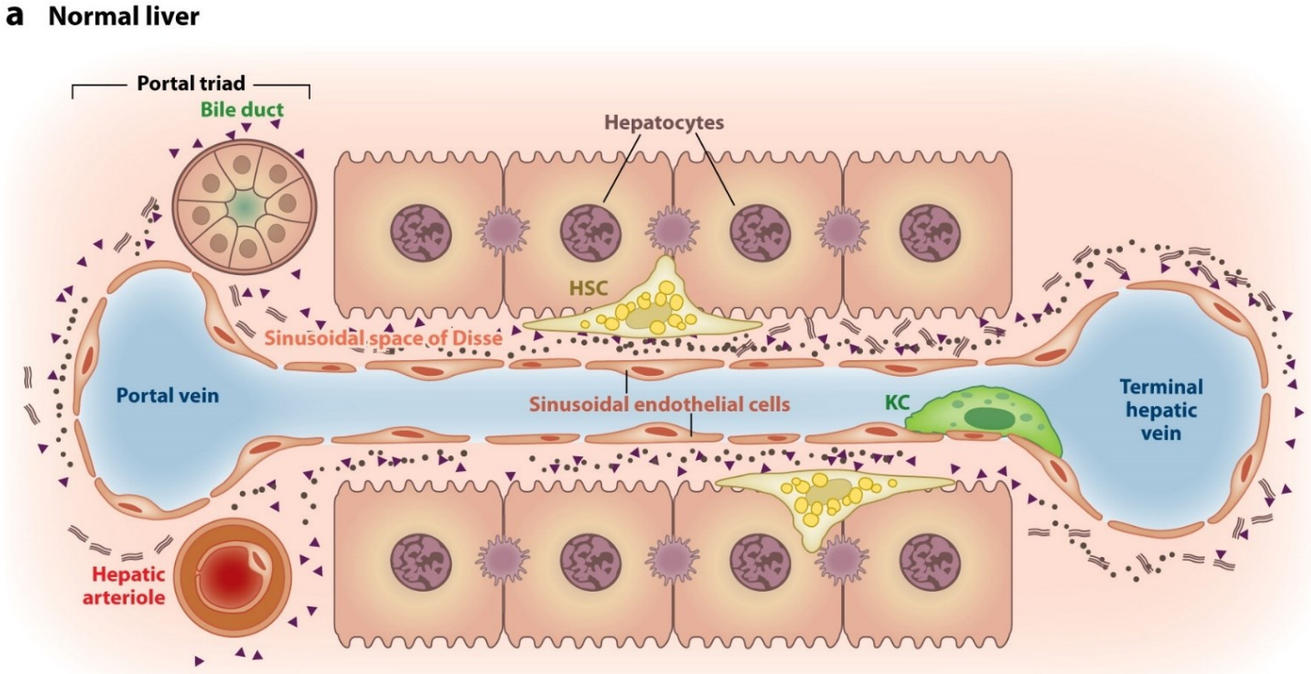

Figure 3. The parenchymal and nonparenchymal cells of the normal liver
Schematic diagram representing normal liver parenchyma containing hepatocytes (epithelial cells) organized as polarized cell layer with microvilli on their surface. Nonparenchymal cells involve fenestrated endothelial cells, HSCs, and KCs. The space of Disse is the pericellular space between endothelial cells and hepatocytes that allows nutrient and metabolic exchange between bloodstream and hepatocytes. HSCs are in quiescent state and located in the space of Disse. KCs are located in hepatic sinusoids and are immobile interacting with LSECs. This Figure is taken from [1](Reproduced with permission)

1.2. Metabolic functions of liver

The liver is a crucial metabolic organ which participates in regulation of body energy metabolism. It acts on different metabolic pathways that connect various organs such as skeletal muscles and adipose tissues (AT). Generally, the gastrointestinal tract (GI) digests the food into simple nutrients, including fatty acids, amino acids, and glucose, followed by their absorption into circulation where they are delivered to the liver through the portal vein. The liver executes different metabolic functions ranging from converting glucose to glycogen, esterification of fatty acids into triacylglycerol (TAG), and metabolism of amino acids to produce energy or synthesize proteins[17]. A brief overview of these biological processes is discussed below.

1.2.1. Glucose metabolism

In the fed state, the blood glucose enters the hepatocytes through a transporter, named GLUT2. A phosphorylation process initiated by glucokinase enzyme, converting glucose into glucose 6-phosphate (G6P), which is metabolized and stored as glycogen through glycogen synthase. In parallel, pancreatic β cells secrete insulin which upregulates glycogen synthase activity in an AKT-dependent manner through inactivation of glycogen synthase kinase 3 (GSK3), leading to increasing glycogen storage[17, 18]. On the other hand, in the fasting state, the stored glycogen is degraded and hydrolyzed by glycogen phosphorylase to produce glucose (glycogenolysis). In parallel, the secretion of insulin is downregulated, while secretion of glucagon hormone from pancreatic α cells is upregulated, which in turn lead to further activation of glycogen phosphorylase by inducing protein kinase A (PKA)[19, 20].

Given that glycogen storage is depleted during long periods of fasting, the liver generates glucose (gluconeogenesis) from different sources, including lactate, pyruvate, glycerol, and amino acids, received by the liver from extrahepatic organs via circulation, or generated within hepatocytes themselves. These substrates, more importantly lactate, undergo different metabolic and biological processes in the liver to generate and release glucose into the circulation. The enzyme phosphoenolpyruvate carboxylase (PEPCK-C) is crucial for gluconeogenesis in the liver. The metabolic imbalances between glycogenesis and glycogenolysis can lead to dysregulation in the metabolism of carbohydrate and lipids, leading to hepatic steatosis. Mice with genetic deletion of PEPCK-C in hepatocytes, failed to undergo gluconeogenesis and develop hepatic steatosis with lipotoxicity-induced liver injury[17, 21, 22].

1.2.2. Lipid or fatty acid metabolism

Fatty acids released into the circulation, whether from adipose tissue or absorbed from digested food in the GI, are received by the liver and enrolled in several metabolic pathways. Once the glucose or carbohydrate is abundant, the liver becomes not only dependent on metabolizing glucose to glycogen, but also converts glucose into fatty acids, a process known as de novo lipogenesis (DNL) or fatty acid synthesis[17]. The insulin can regulate and activate DNL process by inducing mTORC1-activated AKT pathway, which modulate different downstream transcription factors, including Sterol regulatory element binding protein SREBP-1, a master regulator of fatty acid and TAG synthesis in the liver[23]. During DNL, the liver initially converts glucose to pyruvate through glycolysis, which is then metabolized in mitochondria to produce fatty acids. It is worth mentioning that lipogenesis is highly regulated by multiple transcription modulators that control activation of many lipogenic and glycolytic genes, for example, carbohydrate-response-element

binding protein (ChREBP), Liver X receptors (LXRs), farnesoid X receptor (FXR), and peroxisome proliferator-activated receptor γ (PPARγ). The functions of these regulators are reviewed elsewhere[17].

In the fed state, the small intestine digests the dietary fat into fatty acids and releases them into circulation as esterified products, named chylomicrons. These chylomicrons are then delivered to the liver, where they undergo lipolysis and produce nonesterified fatty acids (NEFA). CD36 and fatty acid transport protein 2,4 and 5 (FATP) are the key transporters that allow NEFA to enter the hepatocytes. A deficiency in these carriers was associated with reducing lipid accumulation in the liver of high fat diet (HFD) model, highlighting the important function of these proteins in transporting NEFA into hepatocytes[17, 24, 25]. In hepatocytes, fatty acids, whether from digested food or DNL, are either esterified with glycerol 3-phosphate to produce TAG or cholesterol to generate cholesterol esters. These esterified products are either stored as lipid droplets within hepatocytes or exit into the blood as very low-density lipoprotein (VLDL) and delivered to AT or other organs for metabolism. Besides, fatty acids can be used by the liver to support phospholipid structure, which is a vital component for cell membrane, bile particle, and VLDL[17]. During fasting, fatty acids are exposed to the mitochondrial β oxidation in hepatocytes to generate energy for the liver as well as produce ketone bodies (e.g, hydroxybutyrate and acetone), which act as a metabolic fuel for extrahepatic organs such as muscle. PPARα and its ligand Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) are the key axis involved in the β oxidation of fatty acids, which occurs in mitochondria and peroxisomes[26]. Interestingly, the depletion of PGC-1α in mice fed with obesogenic diet promoted development of hepatic steatosis and oxidative stress[27].

Altogether, the liver controls multiple cellular pathways to regulate glucose and lipid metabolism and maintain homeostasis in the human body. The dysregulation of these vital pathways, irrespective of aetiologies, results in hepatic and systemic metabolic alterations that eventually causes tissue damage.

1.2.3. Bile acid metabolism

The catabolism of cholesterol generates end products, known as bile acids or bile salts. The liver is implicated in this process and can synthesize 0.2 to 0.6 gm per day of bile acids, which interact with phospholipids and cholesterol to form mixed micelles and get stored in the gall bladder. The liver secretes bile acids into the bile, which is reabsorbed by the intestine and finally returns to the liver. Indeed, the bile is very essential for digestion and absorption of several nutrients in the intestine. Also, the bile regulates secretion of lipids, xenobiotics and toxic metabolites[28].

The conversion of cholesterol to bile acids involves a cascade of biosynthetic pathways, including hydroxylation, isomerization, oxidation, cleavage and conjugation. These pathways require 17 distinct enzymes distributed in the cytosol, endoplasmic reticulum, mitochondria, and peroxisomes in order to produce bile acids[28]. During the postprandial period, new cholesterol synthesis is induced in the liver, which subsequently activates the rate-limiting enzyme for bile acid synthesis, known as cholesterol 7α-hydroxylase (CYP7A1), leading to a reduced hepatic cholesterol level and maintaining cholesterol homeostasis[29]. In addition to this, bile acids can mimic insulin action in inducing glycogenesis by activating AKT phosphorylation and consequently suppressing GSK3β activity[30]. Moreover, bile acids can protect hepatocytes against apoptosis by promoting activation of phosphoinositol 3-kinase (PI3K) or AKT [28, 31, 32]. Accordingly, these metabolic protective effects of bile acids are essential for protecting liver against injury from toxic metabolites due to cholesterol or triglycerides accumulation.

Bile acids can also play a role in modulating hepatic inflammation. Hydrophilic bile acids are anti-inflammatory, and they can inhibit the inflammation in the liver by activating FXR signalling and hence suppressing the NF-κB-induced proinflammatory cytokines tumor necrosis factor alpha (TNF-α) and IL-1β[28, 33]. Conversely, hydrophobic bile acids have proinflammatory properties and can stimulate TNF-α and IL-1β expression from Kupffer cells thus enhancing liver injury[34]. Moreover, hydrophobic bile acids upregulate apoptosis in hepatocytes and worsen inflammation[35], while hydrophilic bile acids can protect liver against apoptosis by promoting activation of intracellular cAMP, MAPK and PI3K, which may promote inflammation[28, 31, 36].

Overall, bile acids are essential in regulating different metabolic and immunological functions in the human body. Mutations-induced genetic defects in the bile metabolism, including bile transporters and enzymes responsible for biosynthesis of bile acids, may result in abnormal architecture of the liver and cause severe hepatic disorders such as cholestasis and cholangiocarcinoma.

1.3. Liver immunology

As mentioned above, parenchymal cells represent 78-80%, while NPCs represent 20–30% of liver cells. These NPCs involve various immune populations including APCs, and innate and adaptive immune cells (Figure 4).

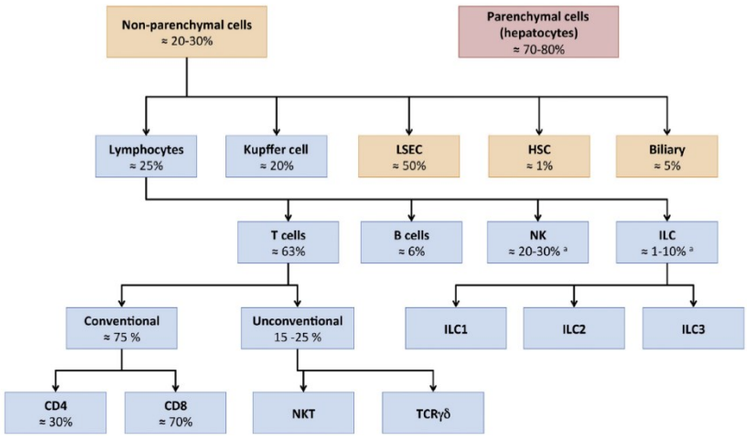

Figure 4. The cellular composition of normal liver
Hepatocytes or parenchymal cells (red box) constitute most of liver cells (70-80%), while nonparenchymal cells (NPCs) represent 20-30% of total liver cells. Liver-resident cells (dark yellow) including LSEC, HSCs and biliary cells represent up to 55% of NPCs, while the immune cells (blue) represent around 45% of NPCs; T cells (conventional and unconventional T cells), B cells, natural killer (NK) cells, natural killer T cells and innate lymphoid cells (ILCs). The Percentages in each box represent the percent of the parent population. Figure is taken from[37] (Reproduced with permission)

KCs

KCs represent 20% of NPCs, constituting the largest population of resident macrophages in the human body[38-40]. KCs remarkably reside in liver sinusoids, mainly around portal tracts, where they interact with HSCs, LSECs, and hepatocytes to acquire important signals for their self-maintenance[38, 41]. Generally, bone marrow-derived monocytes (immature precursors) infiltrate the liver through circulation, where they develop into mature KCs via the action of different growth factors (e.g, macrophage colony stimulating factor)[40, 42]. In addition, KCs have the ability to self-maintain during adulthood by self-renewal mechanisms independent of blood monocytes[43, 44]. KCs are considered, along with neutrophils, the first line of defense against foodborne or bacterial endotoxins passing from the GI to the liver via portal circulation. Also, these cells have a phagocytic activity, contributing to clear dead particles or dying erythrocytes in hepatic parenchyma[45-48]. Other functions of KCs have been reported at the molecular level and are involved in maintaining liver homeostasis. These cells highly express major histocompatibility complex class I (MHC-I), and class II (MHC-II) as well as CD80/CD86 (costimulatory molecules), which allow antigen presentation and the activation of naïve T lymphocytes[49]. Additionally, KCs express high levels of tumor growth factor (TGF-β) and IL-10, which regulate and maintain immune tolerance in the liver [46, 50, 51]. Moreover, in response to blood-borne antigens or endogenous danger signal, both KCs and recruited monocytes-derived macrophages ($Ly6C^{+}CCR2^{+}$) can polarize toward M1 (proinflammatory) or M2 (anti-inflammatory) depending on

different regulatory mechanisms. For instance, Th1 response (IFN-γ) promote M1 polarization, while Th2 response (IL-13 and IL-4) promotes M2 polarization. The phenotypic characteristics of M1 macrophages include high production of oxygen and nitrogen radicals, high expression of proinflammatory cytokines (TNF-α), and strong antimicrobial activity, while M2 macrophages exert immunoregulatory functions, involve in tissue remodeling, and control parasite infections. Both M1 and M2 macrophages play essential role during the inflammation and tissue repair phases of acute liver injury, respectively [52-55].

Natural killer (NK) cells

NK cells are enriched in the liver, representing 20-30% of total lymphocytes, while in peripheral blood, they represent 5-15% of lymphocytes[56, 57]. Hepatic NK cells mediate different functions, ranging from killing infected or precancerous cells, modulating pro- versus anti-inflammatory response balance during liver damage and repair, and suppressing liver fibrosis via inducing apoptosis of HSCs[58, 59].

Natural Killer T (NKT) cells

NK-T cells are innate-like T lymphocytes that combine expression of both conventional T cell (CD3 marker) and NK markers (CD161 in human or NK1.1 in mice)[60]. NKT cells are divided into two major subsets: invariant NKT cells (iNKT) and non-invariant NKT cells. iNKT are generated in the thymus and they express evolutionary semi-invariant Vα24 and V11β T cell receptor (TCR), while non-invariant NKT cells express αβ or γδ TCR repertoire[61]. iNKT cells recognize the lipid antigens (e.g., glycosphingolipid α-galactosylceramide (α-GalCer) or its synthetic analogs) via MHC-class I like molecules, known as CD1d[61-63]. Conversely, non-invariant NKT recognize non-α-GalCer antigens (e.g, sulfatide) presented by the CD1d molecule[61, 64]. The distribution of NKT cells in the liver is very low in humans (10-15% of liver lymphocytes) compared to mice (20-30% of liver lymphocytes)[56, 65]. This may highlight the importance of NKT cells in murine liver immunity compared to humans. Physiologically, NKT cells produce various cytokines upon activation (e.g., IL-17, IL-13, IL-4, IFN-γ), which is dependent on endogenous environment, type of lipid antigen and antigen presenting cell[66-69]. This may influence the inflammation in context of chronic liver disease.

Dendritic cells (DCs)

DCs include both myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) in the healthy liver[51]. mDCs have immature phenotype due to their low expression of the costimulatory molecules CD40,

CD80, and CD86, which mediate tolerance, but not activation of T cells, under normal physiological conditions[51, 70, 71]. This tolerogenic phenotype of mDCs is maintained by the interaction between LSECs and mDCs. In the presence of pathogen-associated molecular pattern (PAMPs)-induced hepatocyte injury, DCs sense, uptake and present antigens through pattern recognition receptors (PRRs) and MHC-I/II. Consequently, hepatic DCs migrate to peripheral lymph nodes, where they acquire mature phenotype and prime T cell responses. On the other hand, hepatic pDCs produce large amounts of type I and type III IFN in response to hepatotropic viral infections[51, 70, 72, 73]. Broadly, under basal conditions, liver DCs are considered less efficient at driving T cell activation than DCs from other tissues, which promotes tolerogenic environment[71]. This is due to the immature phenotype of DCs in the liver, the inhibitory cytokine environment (IL-10 and low IL-12) and DCs-induced Treg development by expressing IL-10, which further promote immunosuppressive environment[70]. In contrast, under specific conditions such as presence of TLR9 ligands, liver DCs, specifically pDCs, are able to induce robust T cell activation by upregulate their expression of costimulatory molecules and enhance their ability to present antigens[70, 74].

Mucosal-Associated Invariant T Cells (MAIT)

MAIT cells are innate immune cells and do not belong to adaptive T cells[75]. In healthy adults, the MAIT cells are enriched (20-50% of intrahepatic T cells) in the liver, mainly localized in the portal tracts, compared to peripheral blood and gut mucosa (around 10% of T cells)[75-77]. MAIT cells strongly express CD161 as well as the conserved semi-invariant TCR (Vα7.2Jα33) in humans[76, 78]. Also, these cells recognise pathogen-related metabolite antigens, in particular vitamin B, which are presented by MHC-class I molecule, known as MR1[78-80]. In addition, MAIT cells can be activated independently of their TCR by responding to pro-inflammatory cytokines such as IL-12 and IL-18[78-80]. Antigen- or cytokine-induced activation of MAIT cells results in secretion of various proinflammatory cytokines, including TNF-α, IL-17A and IFN-γ[76, 81-83], as well as cytotoxic molecules, including perforin and granzyme B[76, 84, 85]. This may highlight the importance of these cells in maintaining immune tolerance in the liver against invading pathogens.

Innate Lymphoid Cells (ILCs)

ILCs are derived from the common lymphoid progenitors (CLPs), though they are innate immune cells, and they do not express antigen specific receptors[86]. The abundance of ILCs is relatively low compared to other lymphocytes in human and mice tissues, though they are a heterogenous population[87]. Since ILCs have similar functional diversity to $CD4^+$ T helper cells, they are

considered the innate counterparts of these T cells. According to the cytokine profile of ILCs, they are divided into 3 subsets: ILC1, ILC2, and ILC3, with Th1-, Th2-, and Th17-like profiles, respectively[88]. ILC1 is the most enriched population among other ILCs in the liver, and they are characterized by the expression of the transcription factor Tbet and IFN-γ[89]. ILC1 have a unique gene signature and they lack perforin-induced cytotoxicity, which distinguish ILC1 from NK cells[89-91]. But ILC1 can mediate cytotoxicity by alternative pathways such as TNF-induced apoptosis[90].

ILC3s produce the cytokine IL-22, which plays an essential role in protecting tissue barriers against invading pathogens[92]. ILC3s are classified into two major sub-populations: $CCR6^+$ lymphoid-tissue inducer (LTi) ILC3s and the $CCR6^-$ ILC3s. Both subpopulations express the retinoic acid receptor-related orphan receptor (RORγt) which is essential for their development and function. The $CCR6^-$ ILC3s includes two subsets, depending on surface expression of the natural cytotoxicity receptors (NCRs): NKp46 in mice and/or NKp44 in human. However, LTi lack NCR expression[93-95]. Accumulating evidence has shown that both LTi and NCR^- ILC3s can express MHC II and hence modulate $CD4^+$ T cell response against intestinal commensal bacteria through antigen-peptide presentation, though this specific response of ILC3s remains unclear in the liver[96-98]. Overall, the function of ILCs during homeostasis and inflammatory conditions in the liver is not well characterized and remains to be elucidated.

Adaptive T Lymphocytes and B Lymphocytes

The liver is enriched in resident T lymphocytes (63% of liver lymphocytes), particularly $CD8^+$ T cells, and the hepatic ratio of $CD8^+$ to $CD4^+$ T cells is higher than that in peripheral blood[56, 57]. The hepatic T lymphocytes pool posses αβ TCR as well as γδ TCR[56, 57, 99]. Like the GI, the liver is enriched with a subset of $CD4^+$ T cells, named Th17, which contributes to mucosal immunity and to the maintenance of homeostasis by producing proinflammatory cytokines such as IL-17 and IL-22[100, 101]. These cytokines mediate diverse physiological functions at the tissue barriers such as inducing expression of antimicrobial peptides in order to protect the mucosa against invading pathogens [101]. Also, IL-22 has hepatoprotective effects and promotes hepatocyte proliferation and survival during acute injury[102]. IL-22 can also be pathogenic especially in the context of high inflammation[103].

The liver has low abundance of B-cells (6%) compared to the peripheral blood. Broadly, the mouse liver B cells are composed of two subsets: the major one is mature bone marrow derived B2 subset ($CD19^+B220^+IgM^+CD23^+CD43^-$) and the less frequent B1 subset ($CD19^+B220^+IgM^+CD23^-CD43^+$)[104, 105]. Although, under physiological conditions, the two

subsets share a common objective, which is to mediate immune response against pathogens or ingested antigens in the liver, their functions are not overlapping[106]. For instance, during antigen stimulation, the B2 subset proliferates and undertakes antibody isotype class switching through T helper-dependent manner, resulting in the production of highly specific IgA, IgG and IgE. On the other hand, B1 subset can mature and proliferate, independent of T helper cells, to plasma cells producing IgM antibodies, which can encounter endogenous antigens such as oxidized phospholipids or proteins from lipid peroxidation[107]. Overall, the underlying mechanisms of B cells in maintaining liver tolerance and homeostasis remain unclear[56].

1.3.1. Liver tolerance

The healthy liver is described as a tolerogenic organ, which is based on the concept that allogenic liver was remarkably tolerated in pigs after transplantation, while allografts of other organs such as lung or kidney were rejected[108] (Figure 5). Also, the liver seems to induce systemic tolerance for other co-transplanted organs, indicating an important tolerogenic potential of the liver[109]. Multiple mechanisms are implicated in the liver to mediate immune tolerance. First, antigen presentation by liver resident APCs, including LSECs, DCs, HSCs and hepatocytes, primes naïve $CD4^+$ or $CD8^+$ T lymphocytes circulating the liver[14, 50, 110-112]. Although, hepatocytes can mechanistically present antigens to T lymphocytes through fenestrations in the endothelium, they lack vital signals for priming T lymphocytes such as costimulatory molecules (signal 2) and IL-12 cytokine (signal 3)[51]. Additionally, in comparison to mature DCs-induced T cell activation in lymph node, hepatic resident DCs have an immature phenotype resulting in a suboptimal activation of T lymphocytes[111, 113, 114]. During inflammation, the expression of inhibitory death-ligand 1 (PD-L1) and galectin-9 by APCs are augmented and inhibit effector functions of T cells through interaction with molecules programmed cell death-1 (PD-1) and T cell immunoglobulin domain and mucin domain-3 (Tim-3), respectively[51, 115-119].

Second, the cytokine milieu and the presence of certain metabolic derivatives can regulate liver tolerance as well. For example, KCs and LSECs can limit T cell responses by secreting IL-10 and TGF-β, which reduce costimulatory molecules expression on APCs and suppress $CD4^+$ T cell activation[46, 50]. Also, KCs and DCs limit proliferation of T lymphocytes by indirectly producing indoleamine-pyrrole 2,3-dioxygenase (IDO) enzyme during tryptophan metabolism, which induces generation of an immunosuppressive compound, known as kynurenine[120, 121]. In addition, pDCs can produce IL-10 and promote immunosuppressive actions of regulatory T cells (Tregs), which further express anti-inflammatory IL-10 and augment expression of inhibitory ligands on APCs[14, 112, 117, 122, 123]. Overall, the diversity of immune populations together with non-

hematopoietic cells play a central role in liver tolerance by limiting local inflammation in response to several inflammatory signals, leading to maintain homeostasis.

On the other hand, during infection, different pathogenic stimuli modulate the immune balance in the liver from tolerance to activation[56]. Liver resident DCs are activated and mature upon release of infection-induced danger signals (damage-associated molecular patterns (DAMPs) or PAMPs) and inflammatory signals (IL-6, IL-1β, apoptotic bodies, and oxidative stress)[56, 124]. Subsequently, mature DCs can either modulate T effector functions by presenting antigens locally in the liver or they capture antigens and migrate to peripheral lymph nodes, where they prime antigen-specific T cell responses[125]. These primed T cells in the lymph nodes migrate back to the liver to induce anti-microbial defensive functions and clear the infection[56, 113]. Of note, it has been shown that unique lymphoid structures, known as intrahepatic myeloid cell aggregate for T cell population expansion" (iMATES), were increased *in vivo* after activation of TLR-9 on monocytes. The formation of iMATEs is dependent on TNF-α signalling. Also, iMATEs upregulated costimulatory molecules, which enhanced expansion of effector T cells that rapidly cleared the infection in the liver[126]. However, the presence of such structures in human liver is unknown and yet to be determined.

Upon systemic pathogen clearance, the liver is responsible for eliminating the residual effector T cells from the circulation. In the absence of inflammation, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) are extensively expressed by LSECs, which facilitate trapping of activated T cells[127, 128]. Importantly, this finding was shown *in vivo* after adoptive transfer of a mixture containing activated and resting T cells to the same recipient mice[128]. The proliferation potential of the retained $CD8^+$ T cells may slightly expand prior to their elimination by apoptosis[127]. In addition to this, the absence of survival signals (cytokine or costimulatory molecules) favors elimination of trapped effector T cells in the liver. Moreover, after pathogen clearance, there is an increase of hepatic expression of PD-L1 and galectin-9 which bind to their corresponding receptors (PD1 and Tim3) on activated T cells, leading to their death via apoptosis[115, 127-130].

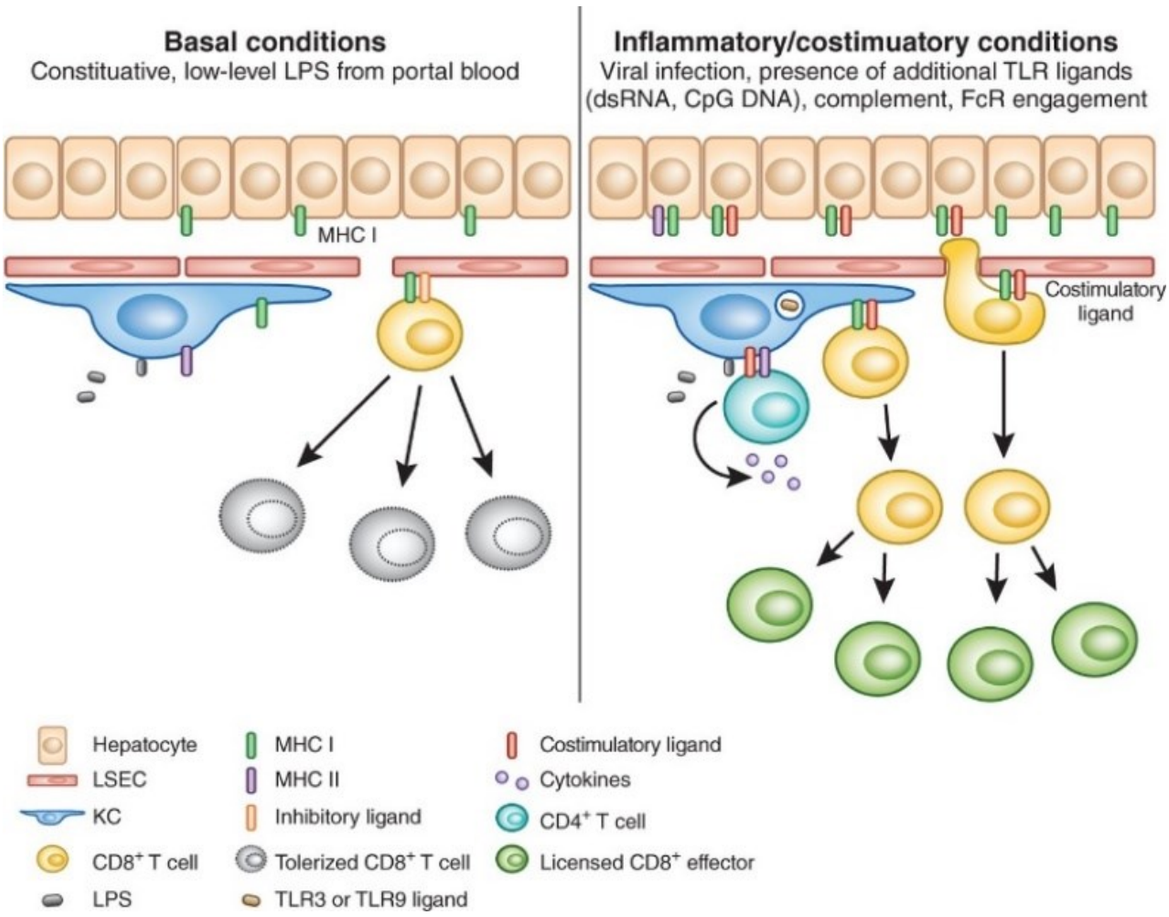

Figure 5. The regulation of T cell activation by liver resident cells during basal conditions and inflammation
In the left panel, the LPS, from portal circulation, is at low level in the liver, which mediates tolerogenic environment; low expression of MHC by APCs and LSECs, expression of inhibitory ligands (e.g., PD-L1) by LESCs, and absence of MHC expression by hepatocytes. These conditions do not allow effector functions of $CD8^+$ T cells and retain their tolerogenic function. In the right panel, during inflammation or infection, the presence of danger signals including TLR ligands, mediates activation of KCs and LSECs which upregulate their expression of MHCs, enhances expression of MHC by hepatocytes, supresses expression of inhibitory molecules and upregulates costimulatory molecules expression. These conditions can overcome the tolerogenic environment and results in priming $CD4^+$ together with $CD8^+$ T cells mediating their effector functions. Figure is taken from [70] (Reproduced with permission)

2. Acute liver Injury

Since the liver is the major site of the infiltrating blood from GI, it is constantly exposed to different types of innocuous antigens from either diet or gut microbiome or microbial toxins. Thus, the immune system of the liver has evolved, including innate and adaptive immune cells, to provide immune tolerance against these harmless antigens to maintain homeostasis[1, 5]. On the other hand, these immune cells play a central role in mediating hepatic tissue protection against acute injury due to invading pathogens or other insults. Indeed, tissue repair is vital for survival of all living organisms[131]. However, the same immune responses involved in tissue repair, when dysregulated or overactive, are implicated in driving pathogenesis and fibrosis progression of

chronic liver diseases (CLD) as well as liver cancer, known as HCC [5]. Here, we discuss the immunological perspectives on the pathogenesis of acute and chronic liver injuries.

There are several types of insults that can induce acute liver injury, including drugs, toxins, bacterial infections, alcohol, and acute resolving viral infections such as hepatitis A. The initial homeostatic response to such insult is the recruitment of platelets and other clotting factors to the injury site to close the wound by forming a transient clot[5, 132]. A self-resolving wound healing response then takes place to clear the insult and restore the normal liver architecture. This healing response consists of 3 distinct but overlapping phases: inflammation, proliferation/repair, and tissue remodeling (Figure 6) [5, 131]. The inflammation phase is an early response, characterized by infiltration of different innate immune cells such as monocytes, neutrophils and macrophages that secrete multiple inflammatory cytokines and chemokines important for clearing the insult or an invading pathogen[5, 54, 133]. Following the insult clearance, the inflammation starts to subside, and the proliferation/repair phase is initiated, where tissue regeneration and repair takes place[5, 131]. Tissue repair involves the formation of ECM as well as new blood vessels (angiogenesis), which is an important process for restoring the basic architecture of liver[5, 131]. Of note, ECM is an intricate network that consists of non-cellular macromolecules forming the liver “scaffolding”, including collagens (types I, III, IV), elastin, laminin, fibronectin, proteoglycans and hyaluronic acid[134]. Finally, in the tissue remodeling phase, the residual inflammatory cells leave the liver or get eliminated by apoptosis, and the temporary ECM is remodeled back to its normal composition[5].

The inflammation phase

Following acute injury, cell death in the liver is induced by necrosis, necroptosis, pyroptosis and apoptosis, which propagate different DAMPS (e.g., adenosine or histones) or PAMPs (e.g., LPS) and chemo-attractants that are recognized by various innate immune cells. KCs and LSECs are also activated during early stage of the inflammatory response further amplifying recruitment of innate immune cells to liver[5, 46, 135]. During the early hours post injury (6-24hr), platelets, neutrophils, and intraperitoneal macrophages expressing “GATA binding factor 6” (GATA-6) are the first immune cells to be recruited at the injury site[132, 133, 136]. A single study, using IF and intravital imaging, revealed that peritoneal macrophages (F4/80^{hi} GATA-6^{+}), which did not colocalize with either KCs or monocytes, rapidly infiltrate liver parenchyma through mesothelial layer as early as 1 hr post-sterile inflammation-induced-acute liver injury. Apparently, neutrophils seem to play an important role in normal wound healing response, as they are involved in clearing the debris or microbes via phagocytosis. Also, neutrophils produce inflammatory cytokines (IL-6 or TNF-α) and antimicrobial peptides against opportunistic infections[5, 132, 133, 137, 138]. These effects

of neutrophils have been demonstrated in acute liver injury models, where blocking neutrophil recruitment resulted in poor tissue repair[137, 139]. The platelets do not only form the temporary clot to close the wound but also promote recruitment of innate immune cells such as neutrophils via expression of different inflammatory mediators[138, 140]. Interestingly, platelets accumulate around the injury site in the liver, specifically in the blood vessels, forming structures-like vessels that facilitate recruitment of neutrophils to the injured area[133, 138].

Monocytes infiltrate the injury site in the liver, likely 24-48hr after arrival of neutrophils[138]. There are two main subsets of monocytes that have been identified in mouse models with acute liver injury: inflammatory ($CCR2^{hi}$, CX_3CR1^{lo}) and anti-inflammatory (CX_3CR1^{hi}, $CCR2^{lo}$) subsets [54, 137]. The inflammatory monocytes infiltrate the liver through the circulation, and they are involved in clearing the debris, secretion of proinflammatory cytokines (TNF-α) as well as digesting injured tissue by secreting proteases[54, 137, 141].

The proliferation/repair phase

In this phase, the inflammatory monocytes can be reprogrammed in situ and acquire an anti-inflammatory phenotype (CX_3CR1^{hi}, $CCR2^{lo}$), which is induced by IL-4 - and IL-10- producing NKT cell[54]. In addition, active HSCs starts to form a temporary matrix and enhance hepatocyte survival via integrin signalling and promote hepatocyte regeneration[5, 142]. Importantly, the anti-inflammatory monocytes express TGF-β, vascular endothelium growth factor (VEGF), and IL-10 to induce tissue regeneration and ECM formation, support revascularization, and promote an anti-inflammatory environment leading to resolution, respectively[54, 137, 143]. In addition, neutrophils as well as GATA-6^+ macrophages contribute to supporting the formation of temporary ECM and revascularization of damaged tissue[136, 137].

The tissue remodelling phase

Once the parenchyma cells begin to repopulate, tissue remodeling is initiated, and several metalloproteinases (MMPs) are secreted to degrade the ECM[5]. In parallel, the active HSCs are then either killed or acquire an inactive phenotype (Figure 7)[144-146]. Interestingly, in this phase, NK cells and IL-10 induce apoptosis of active HSCs to limit the excessive deposition of ECM, which is a critical process to prevent hepatic fibrosis development[59].

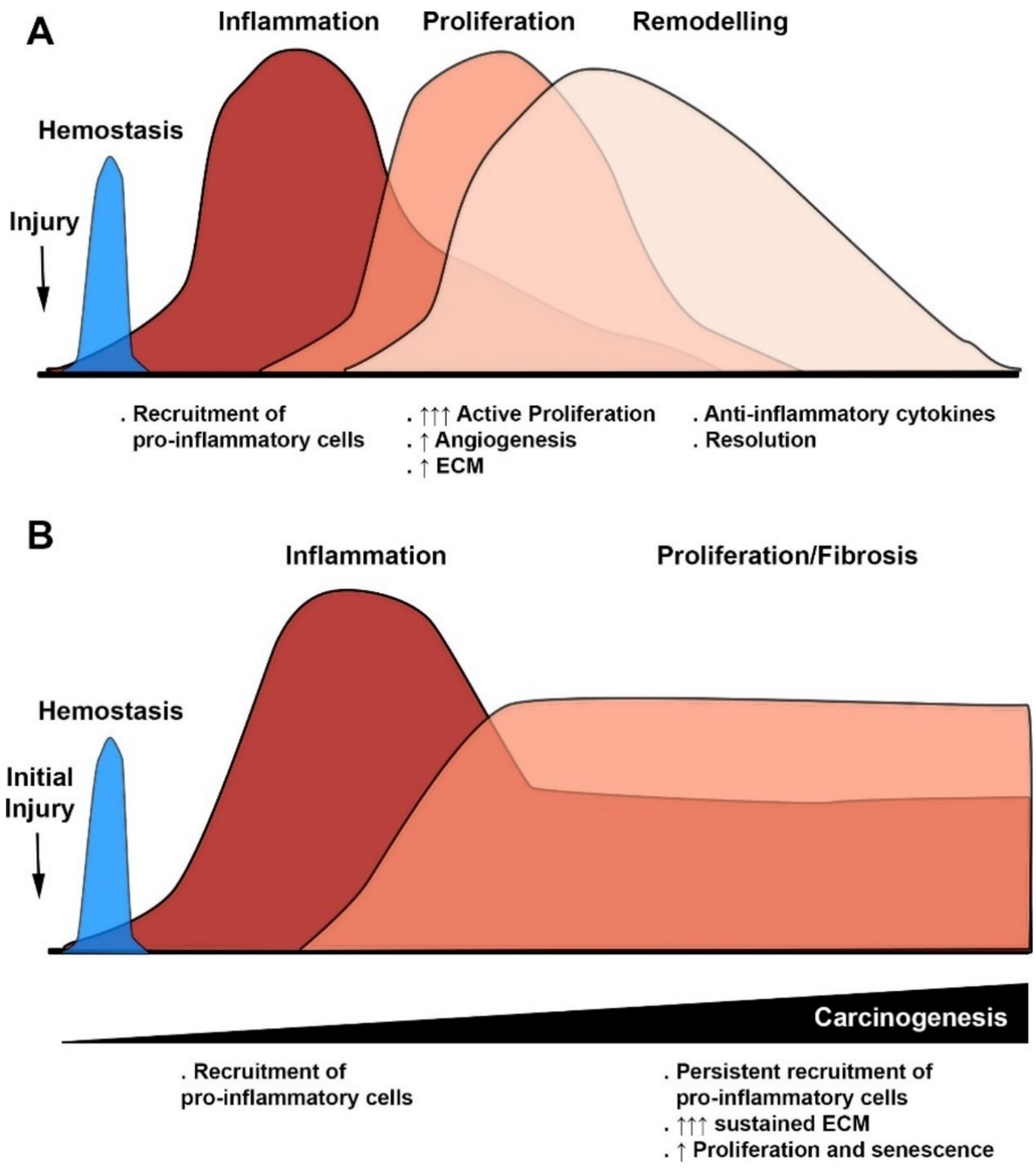

Figure 6. Physiological vs pathological wound healing in response to acute or chronic tissue injury

(**A**) In acute injury, the wound healing response is quickly initiated upon sensing DAMPS or PAMPS. This response is essential to contain the wound and involves 3 phases: inflammation, proliferation/repair, and remodeling. Neutrophils, monocytes and other immune cells are rapidly trafficked to the injury site during the inflammation phase to remove the causative agent of injury, whether pathogen or toxin, as well as eliminating necrotic lesions. In the repair phase, a temporary ECM or scar is formed upon HSCs activation and transdifferentiating into myofibroblasts to support regeneration of new parenchymal tissue as well as revascularization. The pro-resolving macrophages are implicated in this process and can produce several anti-inflammatory and growth factor mediators. Finally, in tissue remodeling phase, the temporary scare is degraded, and HSCs are either deactivated or killed through apoptosis, allowing the tissue to remodeled back to its homeostatic configuration. (**B**) In chronic injury, the persistent exposure to the insult results in sustaining the inflammatory response and promoting fibrosis progression, which increases risk of developing malignant tumors on the long-term. The Figure is taken from [5] (Reproduced with permission)

3. Chronic liver injury and liver fibrosis

Following acute liver injury, the formation of ECM is temporary and reversible upon clearance of the insult, representing a regulated balance between ECM synthesis and degradation. However, the persistence of the injury-causing insult induces chronic inflammation and sustained activation of the tissue repair response, leading to the pathological accumulation of ECM and progressive replacement of liver parenchyma by scar tissue (Figure 6). Thus, the imbalance between deposition and degradation of ECM is fundamental for stimulating hepatic fibrogenesis in response to chronic injury, irrespective of the underlying etiology. Importantly, liver fibrosis progression is a critical condition because it may lead to cirrhosis, a late stage of liver fibrosis with liver function impairment, which is associated with high mortality and poor prognosis[1, 5]. Here, we discuss the molecular and cellular mechanisms that regulate HSCs activation and persistence of liver fibrosis progression in chronic liver injury.

At the cellular level, accumulation of the hepatic scar is characterized by persistent activation and transformation of mesenchymal cells, primarily HSCs, into contractile myofibroblasts, leading to ECM deposition[1]. It is worth mentioning that the origin of profibrogenic myofibroblasts is an intense area of debate because myofibroblasts can be derived from other cells rather than HSCs in the liver such as portal fibroblasts, bone marrow-derived fibroblasts (BM-MF), biliary epithelial cells (BECs) and transformation epithelial mesenchymal cells (EMT)[147-150]. Nevertheless, accumulating evidence suggests that HSCs are the primary lineage for myofibroblasts and major driver of liver fibrosis. This has been demonstrated in mice expressing type I collagen reporter (Col1a1- GFP) coupled with the HSC reporter (LraTred tomato), where 82-96% of myofibroblasts were HSCs in origin, irrespective of the chronic insult[151]. Under normal conditions, HSCs are in the resting or quiescent state (qHSCs) and they serve as a reservoir of vitamin A droplets[152]. However, upon liver injury, these cells become activated and lose their stored vitamin A, allowing active HSCs to efficiently migrate to the injury site and deposit ECM[1, 153]. Also, transformation of HSCs into myofibroblasts is associated with distinct phenotypic changes in terms of proliferation, contraction, and release of profibrogenic and proinflammatory cytokines. For example, they produce α-smooth muscle actin (α-SMA) as well as desmin and vimentin. The activation of HSCs consists of two main stages: initiation and perpetuation. Following liver injury, HSCs are initially provoked in response to primarily paracrine stimuli from damaged hepatocytes (apoptotic bodies or oxidative stress) and other neighbouring cells such as KCs-derived TGF-β [1, 154]. Upon persistence of initiation stimuli, active HSCs enter the perpetuation stage, which include both paracrine and autocrine signals maintaining ongoing HSCs activation, leading to scar tissue formation. At this stage, HSCs acquire distinct functional phenotype including proliferation,

contractility, matrix degradation, retinoid loss, chemotaxis, modulation of inflammatory milieu and fibrogenesis (Figure 7) [1] .

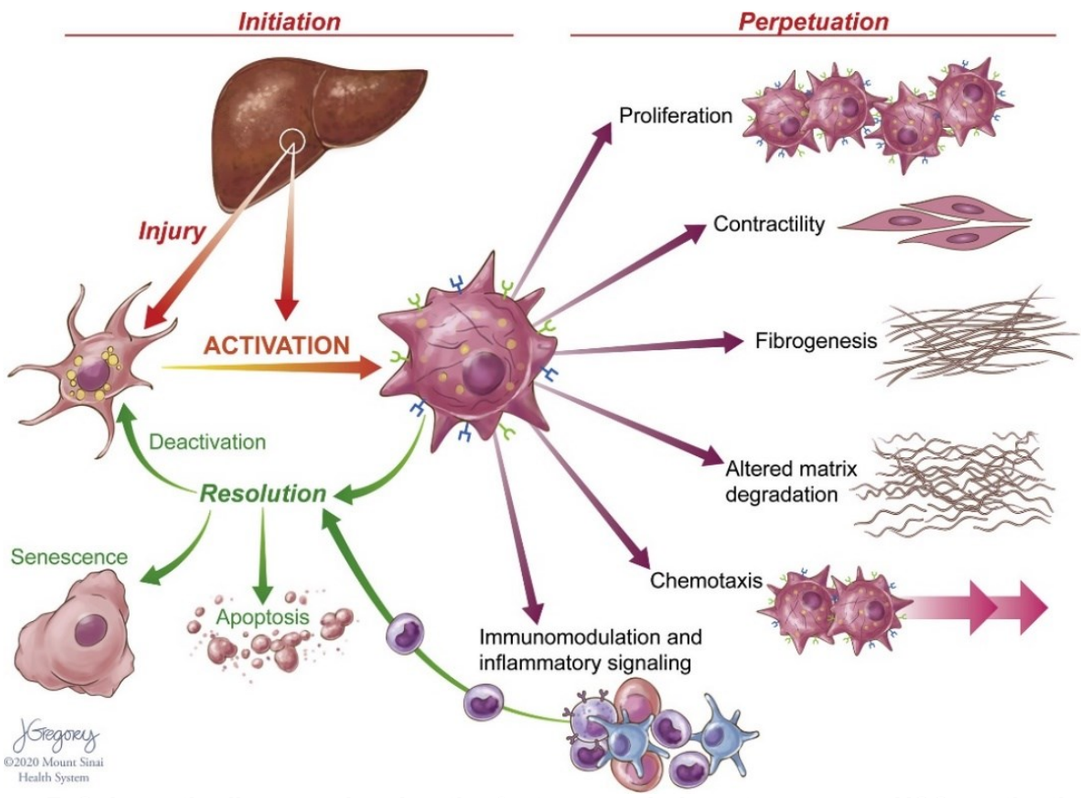

Figure 7. Schematic diagram showing the features and phenotypes upon HSCs activation and resolution
Following liver injury, HSCs initiate transdifferentiating from quiescent state to the active phenotype, which render them responsive to various cytokine and signals that allow HSCs to fully transdifferentiate to myofibroblasts. This is followed by the perpetuation phase, in which myofibroblasts acquire phenotypic changes including proliferation, contractility, altered matrix degradation, retinoid loss, chemotaxis, modulation of inflammatory milieu and fibrogenesis. Upon removal of injurious agent and hence tissue resolve, the HSCs are subjective to 3 fates: apoptosis, deactivation to quiescent state, and/or senescence. This figure is taken from [155] (Reproduced with permission)

3.1. Cytokine regulation of HSCs activation

Since hepatic fibrosis is often preceded by chronic inflammation, several cytokines play a central role in modulating HSCs activity and hence affect liver fibrosis progression. For example, TGF-β, platelet derived growth factor (PDGF), VEGF, connective-tissue growth factor (CTGF) are the most essential profibrogenic cytokines implicated in HSCs activation and collagen production (Figure 8) [156].

TGF-β is secreted as biologically inactive (latent), forming a complex with a latency-associated peptide (LAP), by different immune cells and liver resident cells, including monocytes and KCs[1, 157]. Following liver injury, this latent complex is converted to an active form and bind to its TGF-β type II receptor (TβRII) on HSCs to initiate TGF-β1 signalling. Subsequently, the downstream signalling allows the phosphorylation of the transcription factor Suppressor of Mothers against Decapentaplegic homolog 2 and 3 (SMAD2/3), which promotes the activation of HSCs and the

transcription of collagen type I and type II[158-160]. In addition, TGF- β can indirectly modulate the fibrogenic activity of HSCs through induction of different members of the MAPK family, including c-Jun N-terminal kinase (JNK), p38 and extracellular signal-regulated kinases (ERK)[161, 162]. Moreover, activated HSCs expressing Toll-like receptor 4 (TLR4) in response to LPS suppresses expression of TGF-β inhibitor receptor, known as BMP and activin membrane bound inhibitor (BAMBI), leading to promotion of TGF-β signalling and fibrosis progression[163]. On the other hand, TGF-β-induced SMAD7 results in a negative feedback loop to its own signalling through inhibiting SMAD2 phosphorylation[158]. Also, SMAD7 induces the "SMAD specific E3 ubiquitin protein ligase 2" SMURF2, facilitating degradation of TGF-β receptor by ubiquitination[164, 165]. Although these negative regulatory loops seem to be important in acute liver injury to prevent detrimental effects of TGF-β, Smad 2-mediated fibrosis is found to be constitutively activated in chronic liver injury[158].

PDGF is a potent mitogen that is vital for the growth and proliferation of many liver cells, including HSCs[1]. There are different isoforms of PDGF (A, B, C, and D), which are increased during HSCs activation and associated with liver fibrosis progression[166-168]. PDGFG-B is likely the most important isoform in liver fibrosis, as it directly modulates proliferation and migration of HSCs and myofibroblasts[167, 169]. This isoform is expressed by platelets, macrophages, circulating monocytes, and HSCs[168]. PDGF signalling is mediated through binding to receptor tyrosine kinases (RTK) PDGFR-α and PDGFR-β on HSCs, activating phosphorylation of PI3K/Akt pathway, which enhance proliferation and migration of HSCs[170, 171]. Like TGF-β, PDGF targets MAPK pathways in HSCs as well, which further enhances the proliferation and the fibrogenic activity of HSCs[172].

VEGF plays an important role in resolution of acute liver injury as it supports the angiogenesis and revascularization of hepatic tissue[5, 173, 174]. Neutrophils, HSCs, and LSECs are major producers of VEGF[133, 173, 175]. Specifically, HSCs increases expression of VEGF as well as VEGF receptors (VEGFR-1 and VEGFR-2) upon activation[175-177]. Several studies demonstrated that VEGF induced the proliferation, migration and collagen production of HSCs in chronic liver injury, leading to fibrosis progression[174, 178-180]. Likewise, CTGF shows similar modulatory effects on HSCs, which enhances liver fibrosis[181]. In addition, CTGF synergise the profibrogenic actions of TGF-β by augmenting TGFβ binding to TβRII[182, 183]. Hepatocytes, HSCs, myofibroblasts and BECs express CTGF[183]. CTGF can be negatively regulated by miR-24 which is dependent on the Twist Family BHLH Transcription Factor 1 (TWIST1) pathway, important in regulating fibrosis progression. Notably, dysregulation of TWIST1 or miR-24 were associated with exacerbation of liver fibrosis[184].

Other cytokines such as IL-20, IL-15, IL-17, and IL-22 have been reported to modulate fibrogenesis[156]. IL-20 is generally a proinflammatory cytokine and it induces activation of qHSC by upregulating TGF-β[185]. In contrast, IL-15 is an anti-fibrotic cytokine, which promotes survival of NK cells during liver fibrosis, leading to decreased HSCs activation and collagen production[186]. IL-22 and IL-17 are produced mainly by Th17 cells and play a central role in liver fibrosis of different CLD.

3.2. Hedgehog and other pathways regulating HSCs activation

The interaction between HSCs and ECM can regulate HSCs activation through multiple pathways involved in adhesion, differentiation, migration, and proliferation of myofibroblasts[156]. For example, HSCs interact with ECM components such as collagen I and III through collagen transmembrane receptors, known as integrin (α and β subunits)[187]. Integrin β1 (αvβ1) is expressed by HSCs in the liver, while αvβ3 and αvβ6 integrins are expressed in the lung and the kidneys. These receptors are implicated in fibrosis in different organs. The genetic deficiency of Integrin αv in myofibroblasts, in PDGFRβ Cre–loxP mice, attenuated Carbon tetrachloride (CCl_4)-induced liver fibrosis as well as kidney and lung fibrosis[187]. Mechanistically, integrins interacts with LAP in the liver and releases active TGF-β, leading to enhanced fibrogenesis. Also, myofibroblast can be regulated by integrin β1 through serine/threonine-protein kinase (PAK1) and yes-associated protein 1 (YAP1), which are essential pathways for activation of HSCs[188, 189].

Moreover, Hedgehog (Hh) is an essential regulatory pathway involved in tissue regeneration and liver fibrosis. Hh is a very complex system enclosing many ligands, receptors, and transcription factors[156]. In homeostasis, Hh ligands are not expressed in the liver allowing Patched homolog (PTC) to suppress activation of smoothened homolog (SMO) pathway[190]. Following liver injury, the Hh are expressed in the liver and suppress PTC, leading to activation of SMO. Subsequently, SMO triggers activation of the nuclear transcription factor GLI2, which induces GLI1 and its target protein, osteopontin. This protein is essential for the migration, differentiation, proliferation, and survival of HSCs[156, 191, 192]. Genetic depletion of osteopontin in CCl_4 model suppressed accumulation of myofibroblasts and reduced liver fibrosis[193]. Interestingly, the profibrogenic Hh pathway is upregulated in NAFLD, as a result of caspase2-induced apoptosis of hepatocytes in response to lipotoxicity. Depletion of caspase 2 in NAFLD model, prevented hepatic apoptosis and attenuated fibrosis progression[194].

3.3. Metabolic regulation of HSCs activation

Indeed, the process starting from HSCs activation till generation of ECM and their migration to the injury site requires energy to support these responses. In order to meet these energy

demands, there are certain metabolic pathways acquired by HSCs during their activation and differentiation into myofibroblasts[155]. Here, we focus on the major metabolic pathways regulating HSCs activation, including autophagy, retinoid loss, oxidative stress, lipid metabolism, and hyperinsulinemia (Figure 8).

Autophagy is a natural cellular response inducing digestion of macromolecules or organelles through a lysosome-dependent mechanism to generate energy[195]. Autophagy drives the loss of lipid droplets during HSCs activation by regulating cleavage of retinyl esters into free fatty acids located in the HSC cytoplasmic droplets, and thus fueling activation of these cells[196]. The autophagy-related protein 7 (ATG7) modulates liver fibrosis in the CCl_4 model, where its specific deficiency in HSCs was shown to block autophagosome assembly inhibiting HSCs activation and leading to reduced scar accumulation. Interestingly, these HSCs acquire an active phenotype upon their culture in the presence of exogenous fatty acids[196]. Also, endoplasmic reticulum (ER) stress is common in CLD, and can act as an upstream regulator of autophagy-mediated HSCs activation[1]. ER stress induces inositol-requiring enzyme 1α (IRE1α), which enhances HSCs activation and collagen deposition through p38-MAPK-induced autophagy[197].

In fact, quiescent HSC is the major storage site of retinoids or vitamin A (50-95%) in the human body, the loss of which is a remarkable feature of activation of this cell type following liver injury[152, 156]. However, the involvement of retinoids in activating HSCs and inducing fibrosis is still not clear. One study showed that the deficiency of LRAT, which is an enzyme converting retinol to retinyl esters, did not affect liver fibrosis progression between LRAT-depleted mice and their wild type (WT) littermates. On the contrary, LRAT deficiency reduced development of hepatic tumors, highlighting that retinoid does not activate HSCs, though it prevents HCC development[198]. Nevertheless, HSCs can metabolically oxidize retinol to retinaldehyde through alcohol dehydrogenases 3 (ADH3), which promotes HSCs activation and hence fibrosis progression. This has been shown in liver fibrosis models such as CCl_4, where genetic deletion of ADH3 increased apoptosis of HSCs and enhanced anti-fibrotic activity of NK-cells[199].

CLDs are characterized by excessive release of reactive oxygen species (ROS) from hepatocyte death, leading to HSCs activation[1, 200], mainly via a NADPH oxidase (NOX)-dependent mechanism. Indeed, NOX produces ROS in response to various stimuli[155]. The redox imbalance, due to excessive ROS generation, induces activation of latent TGF-β upregulating NOX1,2,4, and 5 and resulting in a ‘’feeding-forward’’ cycle of HSC activation and collagen deposition[155, 201-203]. Importantly, genetic depletion or pharmacological inhibition of hepatic NOX enzymes (NOX1 or

NOX4) blunts ROS generation and reduces liver fibrosis in CCl_4- or bile-duct ligation (BDL)-induced liver fibrosis models[204, 205].

Activation of HSC can be regulated by lipid metabolism. Adipocytokines, including adiponectin and leptin, can directly act on HSCs and modulate liver fibrosis in CLDs such as NAFLD[156]. Adiponectin decreases HSCs activation by suppressing the PDGF fibrogenic response. The lack of adiponectin expression accelerated liver fibrosis progression in the CCl_4 model, highlighting the anti-fibrotic role of adiponectin[206]. On the contrary, leptin promoted liver fibrosis in diet-induced NAFLD through upregulating TGF-β, COL1A1 and αSMA expression in HSCs, indicating a profibrogenic role of leptin[207]. Also, HSCs can express leptin resulting in a vicious cycle of self-activation[208, 209]. Apart from adipocytokines, accumulation of free cholesterol is associated with liver fibrosis in patients and mice with NAFLD[210, 211]. At the cellular level, free cholesterol sensitizes HSCs to TGF-β-induced activation by upregulating TLR4, which inhibits expression of BAMBI receptor[212]. In the same line of evidence, cholesterol acyltransferase (ACAT) is an enzyme that regulates free cholesterol by catalyzing its conversion to cholesterol ester and hence indirectly limiting HSCs activation. Deficiency of ACAT increases free cholesterol and exacerbates liver fibrosis progression in mice challenged with CCl_4 or BDL[211].

Systemic IR or hyperinsulinemia is characterized by insulin signaling impairment and a dysregulation of glucose metabolism. IR is a major component of the metabolic syndrome (MS), which is considered a risk factor for cardiovascular disease[213]. Also, MS is a common condition in certain CLDs such as NAFLD [213]. HSCs cultured in either insulin- or glucose-rich media are activated, lose their lipid droplets, and increase CTGF and collagen I production, a process highly likely mediated by SMAD-3 transcription factor[214-216]. However, these effects of IR or even hyperglycemia on HSCs is limited to *in vitro* studies. In addition, such effects have not yet been investigated in the context of NAFLD for instance[155].

In summary, all these regulatory pathways of HSCs activation represent possibilities for developing new therapeutic approaches or anti-fibrotic therapies to limit fibrosis progression in the context of chronic liver diseases (CLD).

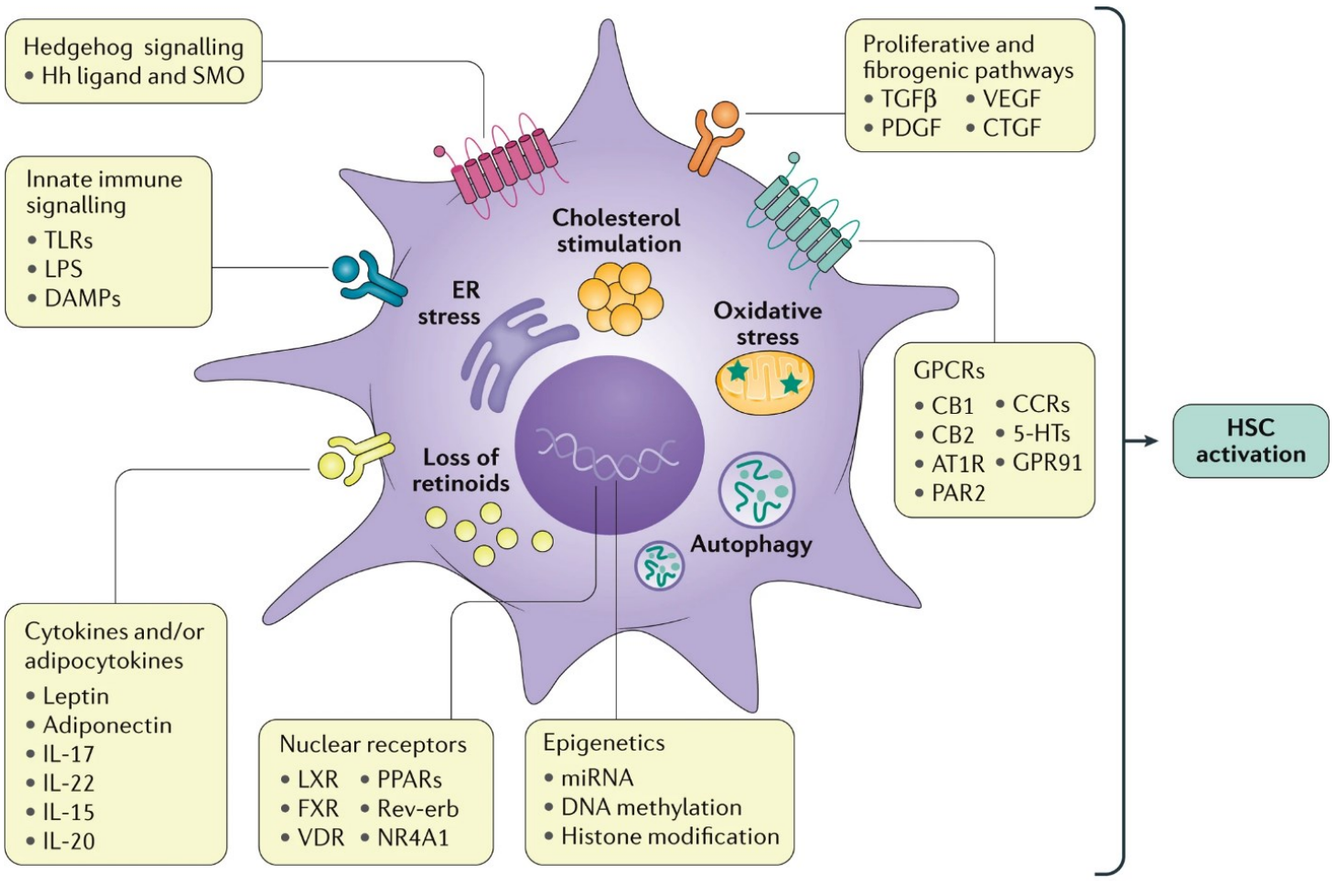

Figure 8. Schematic diagram representing the various regulatory and cytokine pathways of HSCs activation

These pathways include fibrogenic growth factors (TGF-β, VEGF, PDGF, CTGF), hedgehog pathway, cytokines, adipokines, retinoic acid, oxidative stress, autophagy, and cholesterol crystals. The Figure is taken from [156] (Reproduced with permission)

4. Non-alcoholic fatty liver disease (NAFLD)

4.1. NAFLD prevalence and natural history

NAFLD is a growing epidemic disease, not only in the western countries but also worldwide due to the continuous rise in sedentary lifestyles, obesity, and IR over the last two decades. The global prevalence of NALFD is currently estimated to be 25% in the general adult population with the highest rates in the Middle East (32%) and South America (31%), while it is 24% in North America (Figure 9) [2, 217]. In Canada, the number of NAFLD cases is estimated to increase by 20% between 2019 and 2030[218]. NAFLD is also present in lean or non-obese subjects, and its prevalence rate in the US is 18.8% [219]. According to the American association for the study of liver diseases (AASLD), NAFLD diagnosis is based on 3 main categories. First, the presence of hepatic steatosis (HS), either by imaging or histology. Second, alcohol consumption is not excessive (≤ 20gm/day for women and ≤30gm for men). Third, the exclusion of any secondary causes of hepatic fat accumulation such as use of a steatogenic medication over a long term, or history of any monogenic hereditary disorders such as lipodystrophy[220]. NAFLD is an umbrella term that embraces a spectrum of liver diseases ranging from simple HS (NAFL) to fatty liver with inflammation or non-alcoholic steatohepatitis (NASH), to advanced fibrosis and cirrhosis that can progress to HCC [4, 220] (Figure 10). In NAFL, HS represents > 5% of the liver weight without histological evidence of inflammation or hepatocyte ballooning. Conversely, NASH is characterized by > 5% steatosis of the liver weight accompanied with lobular inflammation and hepatocyte ballooning. The fibrosis progression can occur in up to one third of NASH patients, which could further translate to liver cirrhosis or HCC [220-222]. Histologically, the liver fibrosis in NASH has perisinusoidal/pericellular (chicken wire) pattern, which can be evaluated by Picrosirius red or Masson trichrome stains to confirm collagen deposition. During NASH progression, the fibrosis may progress to involve periportal/portal regions or advance to bridging fibrosis or liver cirrhosis[223].

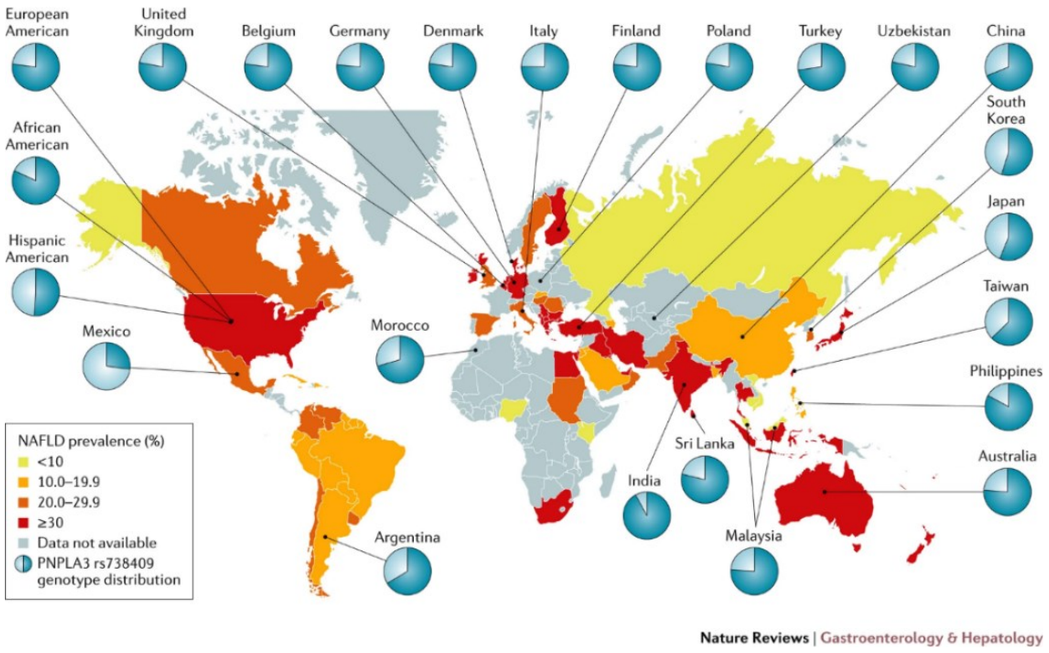

Figure 9. The estimated global prevalence of NAFLD in adult population
South America and the Middle East have the highest prevalence of NAFLD (>30%), followed by Asia (27%), North America and Europe (<25%), while Africa has the lowest prevalence of NAFLD (14%). The Figure is taken from [2] (Reproduced with permission)

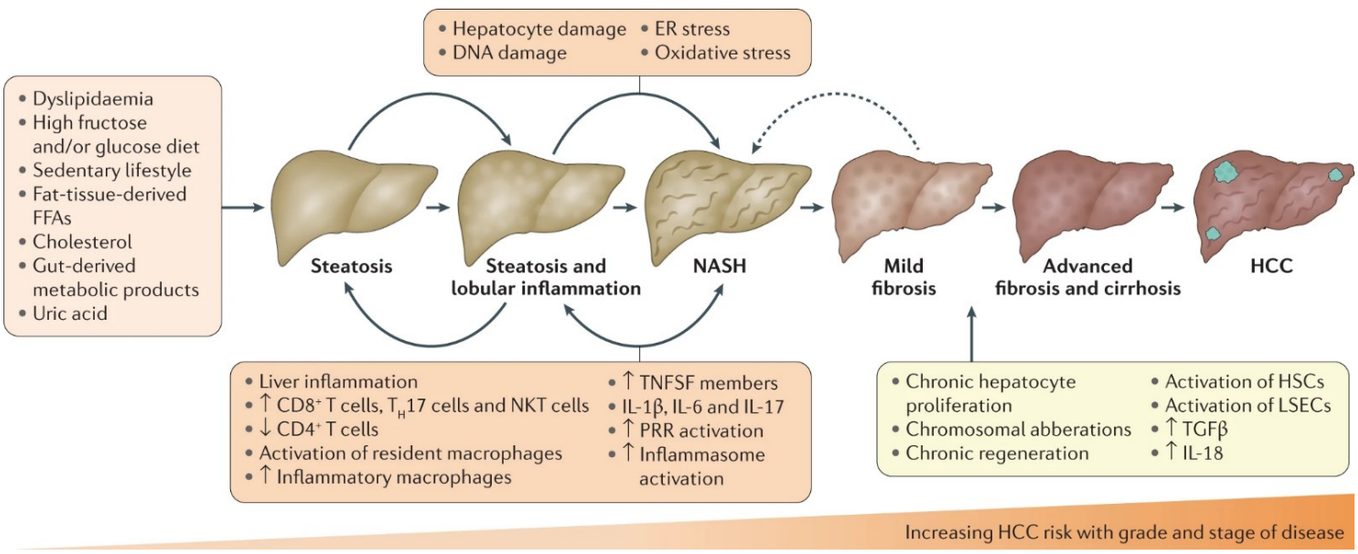

Figure 10. The natural history of NAFLD
Metabolic syndrome and obesity are risk factors for developing NAFLD, which is initiated by fat accumulation in the liver causing hepatic steatosis (NAFL). Histologically, hepatic steatosis is characterized by macrovesicular steatosis, bloated hepatocytes and the nucleus of hepatocytes is displaced. As the disease progress, the excess fat accumulation causes stress and injury to hepatocytes (ballooning) leading to their death, which results in inflammation (NASH) and development of hepatic fibrosis to replace dead hepatocytes. Upon further disease progression, the hepatocytes continue to die, and the scar tissue accumulates over years resulting in liver stiffness and its functional impairment causing liver cirrhosis, which is a high risk of HCC development. The figure is taken from[224] (Reproduced with permission)

4.2. Diagnostic approaches of NAFLD

Most NAFLD patients are asymptomatic and are incidentally diagnosed during heath routine check up or abdominal imaging. They can progress to compensated cirrhosis while being asymptomatic[220, 225]. There are different approaches, invasive and none-invasive ones, for

NAFLD diagnosis. Non-invasive methods such as abdominal imaging and elevated serum aminotransferases can be used for diagnosing hepatic steatosis. Abdominal imaging methods include liver ultrasound, echo-MRI, H-magnetic resonance spectroscopy (H-MRS) and MRI-based proton-density fat fraction PDFF, and they have different degrees of sensitivity and specificity in detecting liver fats in comparison to liver biopsy. However, these imaging techniques are limited due to lack of assessing NASH-related inflammation or degree of liver fibrosis fibrosis[225-229]. Thus, the liver biopsy, an invasive approach, remains the gold standard method for diagnosing and assessing the severity of NASH[225]. Yet, the potential complications and cost of liver biopsy have encouraged the development of other non-invasive methods in the clinical setting. For instance, non-invasive approaches such as NAFLD fibrosis score (NFS), aspartate aminotransferase to platelet ratio index (ARPI) and Fibrosis-4 (FIB-4) Index scores can be used for predicting fibrosis severity. In general, these scoring tests are based on estimations, and they involve different clinical and biochemical measurements including age, gender, serum ALT/AST ratio, serum albumin, serum creatinine, impaired glucose tolerance and platelet count. Nonetheless, these fibrosis scores can predict advanced fibrosis (stage 3-4) but lack sensitivity and specificity to predict moderate fibrosis[220, 225, 230-232].

4.3. Risk factors of NAFLD

NAFLD has many metabolic and non-metabolic risk factors. MS is considered the strongest metabolic risk factor for NAFLD development. Likewise, NAFLD disease could also augment MS comorbidities, highlighting a bidirectional association between NAFLD and MS features[233]. Indeed, NAFLD prevalence is strongly associated with T2DM, hypertension, and visceral obesity in many epidemiological studies [234-237]. In addition, T2DM and hypertension, but not visceral obesity, are significantly associated with NAFLD-related fibrosis and mortality[238-240]. However, it is noteworthy that visceral obesity is associated with IR and lipolysis, and it could release several pro-inflammatory cytokines (e.g., TNF-α) that could promote NAFLD-related fibrosis on the long term[234]. Other non-metabolic risk factors, including genetic polymorphisms, sex, and age have been studied to understand the heterogeneity and complexity of NAFLD. A single nucleotide polymorphism in PNPLA3 gene (rs738409) is strongly associated with NAFLD. PNPLA3 gene encodes for a lipase-protein, which normally regulates lipolysis of hepatic triglycerides, but this rs738409 variant renders lipase inactive causing fat accumulation in the liver. Several variants have been identified for PNPLA3 in NAFLD, but variant M1148 is the most strongly associated with NASH-related inflammation and fibrosis [241-243]. Sex is another important risk factor for NAFLD in either the general population or individuals with MS. NAFLD prevalence is generally more common in males compared to females. However, this sex-based difference is likely inconclusive

and could be cofounded by age. For example, incidence and prevalence of NAFLD was higher in men compared to women, especially at premenopausal age (≤ 50-60 years), while NAFLD prevalent trends became common among postmenopausal women. Interestingly, after the fifth decade, the prevalence of NAFLD is similar among both men and women, or likely at a higher extent in women[217, 244-246]. Thus, while sex could be an independent predictor for NASH-related fibrosis, this remains an area of debate as age, MS, and hepatic inflammation could cofound this result [219, 246-248]. Altogether, female sex could be shielding against NAFLD development and progression at premenopausal age, highlighting the protective effects of estrogen against NAFLD which declines after menopause. Nevertheless, the underlying mechanism beyond this protection remain unstudied in the context of NAFLD.

4.4. Sexual dimorphism in NAFLD

Sexual dimorphism in the immune system is considered akey topic in medicine because it can influence the responses to infectious diseases, development and progression of many inflammatory diseases, transplant rejection, and the response to medications[249]. Although, there is growing evidence in the literature elucidating immunological differences between sexes, immunology has the lowest rank among other ten biological topics for reporting sex differences of human or animals in the published papers[250]. However, currently, many research institutions, funding agencies, and journals have launched policies to promote reporting and analyzing sex in biomedical science to enhance reproducibility.

The phenotypic differences in some components of the immune system between sexes have been reported. For example, females have higher $CD4^+$: $CD8^+$ T cell ratio, B cell numbers, antibody production, peritoneal macrophages, and better phagocytic activity of neutrophils and macrophages than males[251-254]. In contrast, males have higher NK cell number and TLR4-expressing neutrophils and macrophages than females[253, 255, 256]. These findings could generally highlight that adult females have stronger innate and adaptive immune responses than males, which results in faster clearance of infections and better tolerated response to vaccine in females compared to males. However, these strong immune responses in females can be susceptible to dysregulation, contributing to high risk of autoimmunity and chronic inflammation[249, 254]. Thus, the mechanisms regulating this sexual dimorphism in immune response can be mediated by various factors including reproductive hormones, genetics, epigenetics, and environmental factors. For the purpose of this thesis project, we focus on the hormonal effect. The other factors are reviewed elsewhere[249, 254].

Androgens (e.g., testosterone) are the male sex hormones and they are expressed at low levelsin females, while estrogens (e.g., Estradiol) are the female sex hormones and they are produced at low levels in males. These sex hormones are mainly produced by the testes, the ovaries, the adrenal cortex, and the placenta. Generally, androgens and estrogens can act on Androgen receptors (AR) and Estrogen receptors (ER) expressed by many innate and adaptive immune cells, leading to heterogenous immune responses[254, 257, 258]. For example, estradiol supplementation in the mouse model of experimental autoimmune encephalomyelitis (EAE) significantly reduced IL-17 production from circulating Th17, while estrogen deficiency in ovariectomized female mice raised Th17 number and IL-17 production, suggesting an anti-inflammatory role of estrogen in EAE[259, 260]. In contrast, the use of high doses of estradiol showed proinflammatory effects in various species because it enhanced Th2 responses[261]. In addition, androgen deficiency in men is associated with an increase in antibody titers, $CD4^+$: $CD8^+$ T cell ratio, and high levels of proinflammatory cytokines (e,g., IL-1β and TNF-α), suggesting an immunosuppressive function of androgen[262-264].

The liver of both males and females is a major target for steroid sex hormones as it expresses AR and ER, which are attributed to sex differences in biological functions of the liver during health and disease[258]. Interestingly, sexual dimorphism has been reported in ASH and autoimmune liver disease, where the liver of females had severe injury compared to males[265]. In contrast, viral hepatitis- and NASH-induced HCC are more common among males than females[266-268]. Accordingly, several studies investigated either the sex-based difference in liver tissue or the interaction between sexual dimorphism in immune system and the liver in the body. For instance, in rodent NAFLD models, androgen deficiency, due to orchidectomy, induced ER stress in hepatocytes and promoted proinflammatory (NF-kB) signaling. This phenotype was reversed upon administration of testosterone, suggesting an anti-inflammatory role of androgen[269]. In addition, low levels of systemic testosterone was associated with metabolic syndrome, insulin resistance and hepatic steatosis in NAFLD patients, possibly by upregulating lipogenic genes (e.g., FAS)[270, 271]. In contrast, excessive androgen production in females, due to polycystic ovary syndrome, induced hepatic steatosis, overweight and metabolic syndrome, likely by upregulating proinflammatory hepatic MAP2K4[21, 272, 273]. On the other hand, estrogen deficiency, due to ovariectomy, in naïve female mice, results in severe proinflammatory environment in the liver characterized by upregulation of TNF-α, IL-1β, IL-6, and CCR2, compared to controls[274]. Moreover, similar effects were observed in ovariectomized female mice with NAFLD and were associated with enhanced infiltration of proinflammatory hepatic macrophages[275, 276]. Furthermore, estradiol treatment inhibited IL-6 production in KCs in male injected with DEN-

induced HCC[277]. Additionally, another evidence postulated that estradiol could act directly on activated on HSCs and induce their apoptosis by suppressing the activation of the MAPK pathways such as ERK and p38, and hence reduce liver fibrosis progression[278]. This was associated with protection against CCl4 and Thioacetamide-induced liver fibrosis in female mice [279, 280]. Overall, these findings suggest that estradiol could have therapeutic benefit against hepatic inflammation and liver fibrosis in the context of NAFLD.

In summary, sex as biological variable can affect the innate and adaptive immune responses resulting in sex-specific outcomes in many pathological diseases, including NAFLD. Also, as mentioned earlier, females at the premenopausal age seem to be more protected against NAFLD progression than men at the same age, though females develop similar profile of NAFLD progression as men or likely at higher extent after the menopause. The current preclinical evidence suggests a role of sex hormones in modulating metabolic inflammation of NAFLD in both males and females. However, the mechanisms beyond this modulation are still not clear. Also, studying the connection between sexual dimorphism in immunity and liver is more complicated than studying tissue or immune differences between sexes. Nevertheless, additional combined studies are needed because investigating this interaction has crucial effect on advancing our knowledge about sex difference on specific immune cells in context of NAFLD and hence identifying novel therapeutic targets for personalized medicine.

4.5. Complications of NAFLD

The progression of NAFLD-related fibrosis is heterogenous among patients with different rates of severity and different clinical manifestations as well. This could be due to the dynamic and complex pathogenesis of the NAFLD disease[220]. It has been reported that 20-50% of NASH patients may develop liver cirrhosis within 10 years, while estimated annual incidence of NASH-related HCC among those patients with liver cirrhosis is between 0.5% and 2.5%[281-283]. The liver-related mortality increases exponentially with the increase in fibrosis stage in patients with NAFLD[3]. Moreover, and due to lack of effective treatments, NASH has become the second leading cause of HCC-related liver transplantation in the US, increasing from 8.3% in 2002 to exceed 15% by 2017[220, 284]. In Canada, it has been estimated that NASH-related cirrhosis and HCC would increase by 95% between 2019 and 2030. On the other hand, NAFLD also increases the risk of cardiovascular (CVD) complications, which is the most common cause of death in patients with MS [233]. For instance, a Swedish study reported that out of 71 NASH patients, 15.5% and 2.8% died from CVD and liver-related causes, respectively, over almost 14 year-follow up[285], suggesting that NAFLD patients have a higher risk of dying from CVD events compared to liver-

related ones. Also, emerging evidence suggests that CVD risk associated with NASH is independent of other metabolic comorbidities, which possibly could be due to atherogenic dyslipidemia, vascular endothelial dysfunction, and systemic and hepatic inflammation associated with NASH. However, the potential magnitude of these risks has not yet examined [233, 234]. Taken together, these data highlight the burden of NAFLD progression and the urgent need for healthy lifestyle and developing appropriate therapeutic strategies.

4.6. *In vitro* NAFLD models

There are several tissue engineering models that have been designed to generate 3D cultures mimicking architecture of intact liver. These cultures have been genetically modified to develop NAFLD-like phenotype[233]. For example, Organovo bioprinting culture is composed of HepG2 cells, HSCs and umbilical vein cells along with fatty acid accumulation to develop fatty liver[286]. Other 3D cultures such as Hemoshear (coculture system including primary hepatocytes, HSCs, macrophages and fatty acids) and human liver slices have been primarily developed to test the therapeutic potential of drugs in NASH-like systems[287, 288]. However, the application of all these cultures is limited due to either missing the replication of the multicellular architecture of the liver or lacking heterogeneity of human NASH[233].

4.7. NAFLD mouse models

Investigating NALFD progression at the cellular and molecular level in humans is a huge challenge due to the difficulty in accessing liver tissue as well as the complexity of the disease itself including many diverse pathological drivers. Therefore, the utilization of mouse models could be useful to understand the pathogenesis of human NAFLD and hence identify novel therapeutic targets. Over the last two decades, the development of preclinical NAFLD models has substantially increased and advanced our knowledge of disease pathogenesis, with a wide variety of dietary, genetic, and chemically-induced NAFLD. However, none of these current models could be considered as the ideal or the optimal ‘’humanized’’ NASH model that represents the phenotype of human NASH. In fact, these models lack recapitulation of the full spectrum of human NASH, including genetics, metabolic burden, slow progression of liver cirrhosis and HCC, epigenetics, extrahepatic pathological drivers, and heterogeneity of human NASH. Nevertheless, dietary or genetic models provided insights about metabolic burden of NAFLD and development of early onset-low grade inflammation in the liver. In addition, other dietary- or chemically-induced NAFLD models were useful for understanding the possible fibrosis mechanisms of NASH as well as testing new interventional therapies against NASH-related fibrosis[233, 289]. Accordingly, such diversity in preclinical NAFLD models is likely to be influenced by the ultimate research question

or generally the purpose of the study. Here, we summarize examples of available preclinical NAFLD models in (**Table 1, 2, and 3**)

Table 1. Diet-induced NAFLD mouse models

Diet-induced NAFLD mouse models							
Diet	**Composition**	**Duration**	**Metabolic abnormalities**	**Liver Phenotype**	**Privilege(s)**	**Pitfall(s)**	**Reference(s)**
High fat diet (HFD)	• >60% fat • low sugar or cholesterol ingredients	Up to 50 weeks	• Obesity • Adiposity dyslipidemia • IR	• Liver steatosis • Increased transaminases at WK36 of HFD intake • Mild signs of NASH and fibrosis	• Metabolic abnormalities	• NASH is minimal • Phenotype is strain dependent	290
Methionine and choline deficient diet (MCD)	• 0% choline and methionine, • >40% sucrose • 10% fats	3-10 weeks	None	• Dramatic liver injury with robust NASH histological features • Development of severe fibrosis	• Short duration • Low cost compared to other models • Severe fibrosis development in short term • Suitable for testing anti-fibrotic drugs	• Not appropriate for studying NASH because it lacks metabolic abnormalities associated with NASH • Severe weight loss	291, 292
Choline-deficient L-amino acid-defined diet (CDAA)	• 0% choline (L-amino acid) • 70% carbohydrates • 14% fats	8-36 weeks	• Increased plasma lipid profile	• NASH histological features • Mild-moderate fibrosis	• Simple • Lacks weight loss • NASH histology and carcinogenesis	• No IR • No weight gain • Unlike rats, it takes >20 weeks to develop liver fibrosis in mice	293, 294
High fat high cholesterol diet (HF-HC)	• 40% fat • 1.25% cholesterol • 0.5% cholate	12-24 weeks	• Obesity • Increased cholesterol-enriched lipid profile in the plasma	• Aggressive NASH features and fibrosis	• Simple and reliable design • Obesity and weight gain	• Lacks systemic IR • Fibrosis heterogeneity among various mice strains	292, 295

			• Hepatic IR				
High-fat/high-cholesterol/ high-fructose diet (AMLN)	• 40% fat, including 18% trans-fats • 20-22% fructose, • 2% cholesterol and	15-30 weeks	• Obesity • Increased circulating TGs level • IR	• A profound NASH feature, including inflammation and hepatocyte ballooning • Fibrosis grade 2 and 3	• Reproducible • It mimics human NASH at histological and metabolic levels • It mimics western diet • progressive NASH-related fibrosis	• Long duration • Heterogeneity in fibrosis development • Commercially not available due to ban of trans-fat by FDA	[296]
Diet induced animal model of non-alcoholic fatty liver disease (DIAMOND)	• 42% fat • 0.1% cholesterol • high fructose/glucose content in drinking water	16-52 weeks	• Obesity • IR • adiposity • dyslipidemia	• NASH with hepatocyte ballooning • Advanced fibrosis grade 3 and early grade 4) is detected at 36 weeks, while HCC observed along with advanced fibrosis at 52 weeks	• Metabolic abnormalities with adipose inflammation • Molecular transcriptomic signature that mimics human NASH and NASH-related HCC	• Limited to specific strain (inbred strain of a C57Bl6/J and S129S1/svlmJ mice • Reproducibility is unknown • >35 weeks to induce advanced fibrosis • The transcriptomic signature of cholesterol synthesis is not similar to human NASH	[297]

Table 2. Genetic mouse models for NAFLD

Genetic mouse models for NAFLD						
Model	**Description**	**Metabolic abnormalities**	**Liver Phenotype**	**Privilege(s)**	**Pitfall(s)**	**Reference(s)**
ob/ob **(Leptin deficiency model)**	Loss of leptin hormone increases the appetite (hyperphagia) and alters fat distribution from adipose tissue to liver. An additional stimulus such as AMLN or MCD, is needed to induce NASH in this model	• Obesity • IR Adiposity only with AMLN or HFD	• Hepatic steatosis • increase plasma ALT • Marked NASH features, including ballooning degeneration upon AMLN challenge Heterogenous liver fibrosis progression (grade 2-3) after feeding AMLN diet	• Commercially available • Metabolic abnormalities • NASH features	• Absence of leptin mutation in human NASH • Metabolic burden is strain dependent • The model itself has abnormalities with hypothalamic-pituitary-adrenal (HPA axis) and thyroid axis	298 299
db/db **(Leptin resistance model)**	A deficiency of leptin receptor induces resistance to leptin signaling. An additional stimulus such as AMLN or MCD is needed to induce NASH in this model	Same metabolic abnormalities as ob/ob	• Only hepatic steatosis with HFD, but without NASH features • Marked NASH and fibrosis upon feeding MCD	• Commercially available • NASH features, including fibrosis but only with MCD	• Same as ob/ob	298 299
Alr $^{-/-}$	A deficiency of augmenter of liver regeneration (Alr), which is multifunctional protein that regulates liver regeneration through regulating NK cell activity and Kupffer cell activation	None	• Development of hepatic necrosis, inflammation, and bridging fibrosis at 8 weeks after birth • Development of HCC one-year after birth	• No need for dietary stimulus • Useful for studying NASH-related HCC	• Reversible liver steatosis • Not commercially available • Lack of metabolic abnormalities • Absence of this mutation in human NASH	300

Table 3. Chemically induced-NAFLD mouse models

Chemically induced-NAFLD mouse models						
Model	**Description**	**Metabolic abnormalities**	**Liver Phenotype**	**Privilege(s)**	**Pitfall(s)**	**Reference(s)**
STAM	• Injection of low dose of streptozotocin-induced cytotoxicity to pancreatic cells to neonate mice An additional stimulus such as HFD or AMLN is needed to induce NASH	Diabetes and IR	• Liver steatosis, and NASH with hepatocyte ballooning Hepatic fibrosis progression and HCC development	• Commercially available • IR • Useful for investigating NAFLD progression to HCC Induction of advanced fibrosis in short term	• Lacks obesity and weight gain • Not simple and requires technical training	301
CCl_4+ WD	• Injections of low dose (0.32ug/g) of CCl_4 once/week along with feeding western diet (WD) for 12-24 weeks.	• In comparison to controls (WD/oil), this model lacks obesity, IR, and glucose intolerance	• Profound NASH features, including steatosis and ballooning • Development of bridging fibrosis as well as cirrhotic liver • HCC development in 100% of mice	• It may mimic human NASH at transcriptomic levels • Useful for studying development of NASH-related HCC •	• Lack metabolic abnormalities • Lack pattern of perisinusoidal fibrosis observed in human NASH • It does not resemble human NASH etiology	297

4.8. Emerging therapeutic targets for NAFLD treatment

There are yet no approved FDA therapies to treat NASH or NASH-related fibrosis, but many interventional studies are on going[220, 233]. In addition, the pathogenesis of NASH is complex, and involves a crosstalk between different organs, including liver, gut, and adipose tissue. This complexity proposes a heterogeneity of mechanisms driving NASH progression, which represents a challenge for developing therapeutic strategies. Accordingly, the therapeutic management of NASH is primarily focused on lifestyle modifications, as recommended by the AASLD and the European association for the study of the Liver (EASL)[220, 230]. Importantly, weight loss has been remarkably associated with amelioration of all NASH features, including fibrosis, suggesting an effective non-medical therapy[302]. However, the investigation of long-term protective effects of weight loss on NASH severity remains unknown. In addition, the advanced fibrosis in human NASH is a critical driver of mortality and tumor development, which urgently demands pharmacological therapies to minimize disease progression[3, 233, 303].

Since NAFLD is a multifactorial metabolic disorder with different pathological drivers in the liver, including glucose and lipid imbalance, oxidative stress, hepatocyte death, inflammation, and fibrosis[233], it is not surprising that many clinical trials are testing new drugs, whether monotherapy or combination therapy, against these intrahepatic targets. Broadly, the therapeutic strategies involve targets associated with metabolism to reduce hepatic steatosis and improve metabolic burden, and with apoptosis, inflammation and fibrosis to prevent NASH progression. Since many therapeutic drugs are developed and currently studied in NASH clinical trials, it is beyond the scope of this review to discuss all of them. So, we listed some examples in (**Table 4**) below to summarize the preliminary results of the clinical trials.

Metabolic therapies can indirectly inhibit enzyme-induced DNL (Fatty acid synthetase (FAS), acetyl-coA carboxylase (ACC), diacylglycerol acyltransferase (DGAT), and steroyl-coA desaturase (SCD)). Also, metabolic therapies can directly act as synthetic agonists or antagonists

for nuclear receptors (FXR or PPAR agonists or LXR antagonists) to enhance β-oxidation or inhibit DNL, respectively[233]. Of note, obeticholic acid (FXR agonist) and elafibrinor (PPARα/δ agonist) have shown protective effects against not only hepatic steatosis, but also inflammation and fibrosis in NASH patients (**Table 4**)[304, 305].

Other therapies have been developed to target apoptosis, inflammation, and fibrosis. For example, Emricasan, inhibitor of caspase 2-induced apoptosis, failed to show protective effects against NASH severity in three clinical trials[306, 307]. In addition, Selonsertib, inhibitor of apoptosis signal-regulating kinase 1 (ASK1), which is a potent mediator of inflammation-induced apoptosis through activating JNK and NF-kB pathways[308]. Selonsertib attenuated hepatic steatosis and NASH severity, including liver fibrosis, in a short-term phase II trial[309]. Nevertheless, these protective effects have not been confirmed in two phase III clinical trails, even with longer duration of treatment[310]. Antifibrotic drugs include Cenicriviroc, Belapectin, and Simtuzumab. Cenicriviroc is a CCR2/5 antagonist, which inhibits activation and migration of KCs, monocytes, and HSCs[233, 311]. Belapectin suppresses galectin 3 action, which blocks myofibroblasts differentiation and collagen production[312]. Simtuzumab is a monoclonal antibody against lysyl oxidase-2 (LOXL2), an important enzyme for crosslinking of collagen[233]. Cenicriviroc showed protection against liver fibrosis and NASH severity in phase II clinical trial, though these findings did not replicate in phase III studies[313, 314]. Belapectin and Simtuzumab have not shown beneficial effects against NASH-related fibrosis in phase II clinical trials[315, 316]. Overall, numerous drugs have been developed and they are currently being investigated in clinical trials, some show promising results, while others fail to exhibit protective effects.

Table 4. List of ongoing clinical trials of therapeutic drugs for NASH treatment

Drug	Clinical trial	Phase	Mode of action	Clinical improvement					Reference(s)
				IR	Steatosis	Apoptosis	NASH	Fibrosis	
Obeticholic acid	REGENERATE	III	FXR ligand		√		√	√	305
Elafibranor	RESOLVE-IT	III	PPARα/δ ligand	√			√		304
Cilofexor+ firsocostat	ATLAS	II	FXR agonist + ACC inhibitor	√	√		√	√	317
25-hydroxycholesterol 3-sulfate	ACTRN12615000267550	I	LXR inhibitor		√		√	√	Clinicaltrials.gov ACTRN12615001355561
Emricasan	ENCORE-NF ENCORE-PH ENCORE-LF	II	Caspase inhibitor	No difference as compared to placebo					306, 307
Selonsertib	Phase 2	II	ASK1 inhibitor		√		√	√	309
	STELLAR 3, STELLAR 4	III		No difference as compared to placebo					310
Cenicriviroc	CENTAUR	II	CCR2/5 antagonist				√	√	313
	AURORA	III		No difference as compared to placebo					314
Belapectin	NASH-CX	II	Galactin-3 inhibitor	No difference as compared to placebo					315
Simtuzumab	GS-US-321-0105, GS-US-321-0106	II	LOXL-2	No difference as compared to placebo					316

4.9. Pathogenesis of NAFLD

There has been a tremendous research advancement in understanding NAFLD pathogenesis over the last decade. A theory named ‘’ two-hit theory’’ is initially proposed to explain the pathogenesis of NAFLD. In short, the accumulation of fats in liver (NAFL) is the ‘’ first hit ‘’ which alone, is not sufficient to drive NASH, and a ‘’second hit’’ (oxidative stress) is required for mediating liver injury and NASH development. However, this theory is currently outdated and is not generally accepted for two main reasons. First, accumulating evidence have shown that NASH development is not solely dependent on presence of NAFL, and could be driven by multiple molecular pathways. Second, there is heterogeneity in the pathogenic drivers among NASH patients. Therefore, NAFLD pathogenesis is currently complex and multifactorial with several hepatic and extrahepatic alterations involved. IR, dysfunctional adipose tissue, lipotoxicity, different immune cells activation are all key pathogenic drivers that may interact with certain modifiers such as gut dysbiosis and genetic susceptibility in a multiphasic manner to mediate NASH development and progression (Figure 11) [4, 233, 318]. The major pathogenic drivers of NAFLD are discussed below.

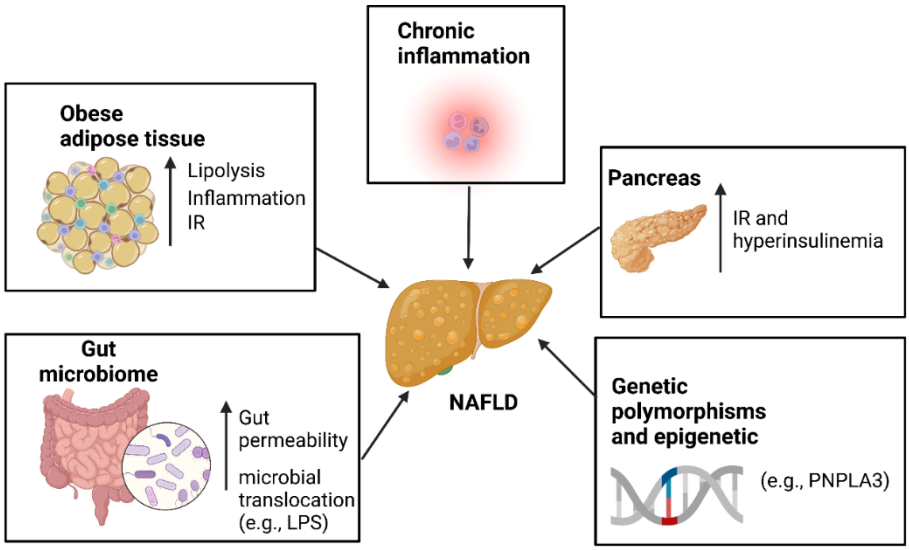

Figure 11. Schematic diagram showing the pathogenic drivers that contribute to the fat accumulation in the liver and to NAFLD progression
Chronic inflammation, IR, gut permeability, lipolysis, genetic polymorphisms and epigenetic are most common pathogenic drivers of NAFLD (Created by BioRender.com)

4.9.1. IR

IR contributes to hepatic steatosis and positively associates with NAFLD pathogenesis[319, 320]. In adipose tissues, IR leads to dysregulated lipolysis, infiltration of inflammatory macrophages, secretion of inflammatory cytokines (e.g., IL-6 and TNF-α) and decreased expression of adipokines (e.g., adiponectin). All these effects reinforce the systemic IR contributing to promote free fatty acids (FFAs) pool in the liver, enhancing lipotoxicity and hepatocyte injury[4, 321-323].

4.9.2. Hepatic Lipotoxicity

NAFLD is characterized by massive influx of FFA into the liver, which are mainly derived from three major sources: dysregulation of lipolysis of triglycerides in adipose tissues as a result of IR of adipocytes, de novo lipogenesis, and dietary fats. In homeostasis, FFA in the liver is mainly metabolized by either esterification to form triglycerides or oxidized via mitochondrial β- oxidation to yield energy. However, in NAFLD, these fates of FFAs are dysregulated, whether due to either an excess supply of FFAs or a defect in their disposal. Consequently, the capacity of the liver to handle this metabolic burden of FFAs is overwhelmed, resulting in lipotoxic metabolites, such as ceramides or diacylglycerols , that induce oxidative stress and ER stress, leading to apoptosis-induced hepatocyte death (Figure 12) [4, 324-327]. Apoptotic hepatocytes are one of the hallmarks of NAFLD progression, which subsequently result in chronic inflammation, fibrosis progression, and HCC[4, 325]. FFA-induced oxidative or ER stress promote liver injury through different mechanisms. Oxidative stress is generally triggered by an imbalance between ROS generation and antioxidant defences. In homeostasis, FFA undergo mitochondrial β-oxidation to yield energy, which is an adaptive mechanism to metabolise FFA. But, in NAFLD, due to surplus of FFA pool in the liver, there is an overproduction of intracellular ROS by mitochondria and peroxisome. Consequently, this oxidative burden induces impairment of antioxidant proteins such as glutathione peroxidase or superoxide dismutase, which eventually lead to mitochondrial dysfunction as well as trigger lipotoxicity-induced hepatic apoptosis[4, 328-331]. For instance, ceramides promote ROS production

within mitochondria, and at the same time, supress β-oxidation[332, 333]. In addition, diacylglycerols or ceramide can provoke activation of NF-kB and inflammasome, which subsequently induce expression of multiple pro-inflammatory cytokines (IL-6, IL-1β, TNF-α), leading to NASH progression[334-336]. Besides, these cytokines can promote recruitment of inflammatory macrophages or neutrophils that release NADPH oxidase-induced ROS, resulting in further promotion of NASH progression[4, 330]. Lipotoxic metabolite can also activate ER stress, which is an adaptive mechanism activated by aggregates of unfolded proteins to prevent accumulation of these proteins and hence halt initiation of apoptosis. Treatment of hepatocytes with lipotoxic metabolites such as palmitate *in vitro* induces calcium depletion in ER, ROS overproduction, and apoptosis[337-339]. In addition, prolonged ER stress causes impairment in many lipoprotein secretions, resulting in accelerating accumulation of lipid droplets in hepatocytes, leading to cell death[340]. ER stress can also induce NF-kB and JNK, which further enhances NASH progression[341, 342]. Finally, ROS overproduction, whether due to mitochondrial dysfunction or ER stress, can either directly activate HSCs and promote fibrosis progression of NASH or indirectly by promoting inflammatory response of NASH[4, 197, 343]. Altogether, NAFLD is associated with chronic impairment in FFA metabolism which causes alterations in oxidant/antioxidant balance. This oxidative stress contributes to NAFLD pathogenesis by inducing generation of lipotoxic metabolites, chronic ER stress, mitochondrial dysfunction, and hepatocellular injury, which in turn promote progression of chronic inflammation (NASH) and liver fibrosis.

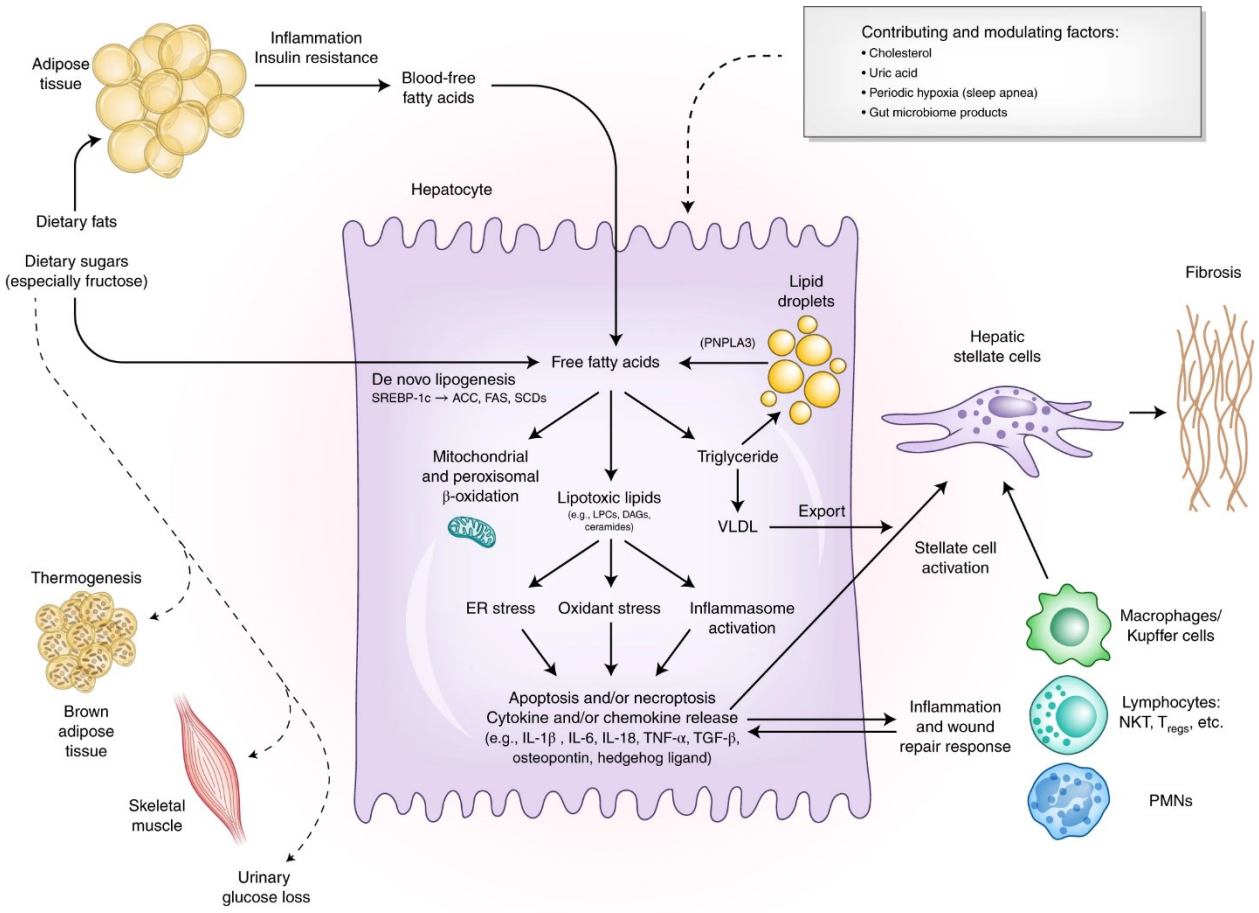

Figure 12. Lipotoxicity is a major driver of NASH pathogenesis
Free fatty acids (FFA), whether originating from DNL of sugars (fructose) or delivered from adipose tissue by lipolysis, massively accumulate in the liver. These FFA are subjected to two main fates in the liver: re-esterification to form triglycerides or undergo mitochondrial β-oxidation. In normal conditions, triglycerides can be exported to other tissues as VLDL through blood to provide metabolic energy, while FFA are metabolized by mitochondrial β-oxidation to yield energy. However, in NAFLD, the triglycerides accumulate in the liver forming lipid droplets leading to hepatic steatosis. Besides, these lipid droplets can themselves act as a source of FFA and undergo lipolysis which further enrich FFA pool in the liver. Also, mitochondrial β-oxidation is disrupted causing a defect in FFA disposal. Consequently, the capacity of liver to handle this metabolic burden of FFAs is overwhelming, resulting in lipotoxic metabolites that induce oxidative stress, ER stress, and hepatocyte death. These effects promote the hepatocyte injury, NASH-related inflammation, HSCs activation and fibrosis progression. The figure is taken from [233] (Reproduced with permission)

4.9.3. Microbiome

The microbiome represents an ecosystem, involving bacteria, fungi, viruses, and other microorganisms living in the human gut. This microbiota actively regulates different physiological functions, including digestion, and absorption of dietary components into portal or systemic circulation, synthesize certain vitamins such as vitamin K, and interact with immune cells at epithelial barriers to protect the host against invading pathogens or foodborne antigens. In a healthy subject, the composition of microbiota involves large abundance of beneficial compared to pathogenic microorganisms (low abundance), representing normal interactions to prevent any potential harm to the host. However, the host genetics and various environmental factors (e.g., diet or physical activity) can cause perturbations in the composition and functions of the gut commensal communities, resulting in gut or intestinal dysbiosis[344-346]. Over the past decade, accumulating evidence has suggested the attribution of gut dysbiosis in NASH pathogenesis. This could be due to microbiome evolution in the human gut, which may be influenced by the western diet rich in carbohydrates and sugar, sedentary lifestyle, obesity, and the large use of prescribed antibiotics in humans as well as farm animals[233, 344]. Several studies demonstrated that gut dysbiosis was implicated in NASH development and progression in both mice and humans[347-351]. Some possible mechanisms have been proposed that link gut dysbiosis and NASH. Most importantly, that dysfunction of the gut barrier could enhance translocation of microbes and microbial-derivates to portal circulation and hence promote steatohepatitis. The presence of high levels of circulating endotoxin in NASH patients as well as NASH mouse models supports this mechanism[352-354]. Indeed, endotoxin provoke activation of several inflammatory innate cells through myeloid differentiation factor 2 (MD2) or TLR4, which in turn promote release of pro-inflammatory cytokines (IL-6 and TNF-α) promoting NASH-related inflammation and fibrosis. The deficiency of these receptors in NASH model, ameliorated the inflammation and liver injury[353]. However, the correlations between circulating endotoxin level and histological NAFLD severity

features are still controversial[355, 356]. Besides, it remains uncertain whether endotoxin disrupts the gut barrier leading to dysbiosis, or whether it is a consequence of diet-induced intestinal dysbiosis[330]. The gut microbiota could also mediate inflammation of the intestinal mucosa, which was characterized by upregulation of the proinflammatory cytokines IL-6 and TNF-α, in parallel with a reduction in the frequencies of $CD8^+$ and $CD4^+$ T cells, including the Th17 subtype and its cytokines IL-17 and IL-22, the latter mediating protective mucosal immunity. Consequently, this resulted in impairment in tight junctions of mucosa, leading to gut dysbiosis and promoting NASH. However, these effects seem to be attributed to the initial phase of NASH development, not to its progression[357, 358]. Overall, there is some progress in linking the microbiome to NASH pathogenesis, though further studies are needed to delineate the underlying mechanisms and to better understand the nature of this association, whether it is direct or indirect through the diet or host genetics or metabolic cofounders.

4.9.4. Inflammation

As lipotoxicity becomes overloaded, stressed hepatocytes release various intracellular DAMPS such as ATP or uric acids that trigger inflammatory responses, including innate and adaptive immunity, to initiate tissue repair mechanisms. Also, PAMPS, which result from presence of bacterial products in the liver due to gut dysbiosis, can mediate NASH-related liver injury. The long-term persistence of this inflammation predisposes to chronic injury and consequently promotes fibrosis progression (Figure 13). We discuss below the various immune cells and their inflammatory role in NASH progression[330].

Innate immunity is the key contributor to hepatic inflammation in NASH. Innate immune cells, including Kupffer cells, express PRR such as TLRs or nucleotide oligomerization domain-like receptor (NLRs) that can recognize both DAMPS and PAMPS[330]. TLRs activate the production of inflammatory (TNF-α and IL-6) and profibrogenic cytokines (TGF-β) that promote inflammation and amplify tissue damage. In NASH, TLR2, TLR4 and TLR9 are overexpressed and are

positively associated with disease progression. Moreover, the genetic depletion of these TLRs substantially attenuates the inflammatory response and decreases disease progression in various NASH models[330, 353, 359, 360]. Activation of NLRs induces inflammasome complex assembly, which results in cell apoptosis through caspase-1 activation and production of pro-inflammatory cytokines, including IL-1β and IL-18. NLRP3 inflammasome is markedly increased in both murine and human NASH, which is positively associated with both hepatocyte apoptosis and pyroptosis. In addition, genetic ablation of this receptor in CDAA-induced NASH model decreased both the inflammation and liver fibrosis progression[233, 334, 361-363].

Macrophages/Kupffer cells (KCs)

An increase in macrophages/Kupffer cells was positively associated with NASH severity in humans and mice with NAFLD[364-366]. Histologically, these cells have been observed as aggregates surrounding steatotic hepatocytes (crown-like structure) in both human and murine NASH[364]. KCs are activated and expanded at the early stage of NASH development by expressing multiple proinflammatory cytokines (IL-6, TNF-α and IL-1β) and chemokines (CCL2, CCL3, CCL5 and CXCL-10) that trigger activation of apoptotic signals and induce recruitment of bone marrow-derived monocytes through the CCL2/CCR2 axis. These monocytes can replace resident KCs and thus promote NASH progression[330, 366-368]. In addition to this, KCs can enhance hepatic fat accumulation, which further promote NASH-related liver injury[369-371]. For instance, in a HFD-induced NASH model, depletion of KCs in early development of NASH, attenuated fat accumulation, liver injury and monocyte recruitment[370]. Moreover, cholesterol-crystal lipoproteins can be stored in the lysosomes of KCs, resulting in NLRP3 inflammasome activation that further promotes hepatocyte death and hence NASH progression[371]. Due to the versatile ability of macrophages, they can be categorized into two major subsets depending on the context of the local environment: proinflammatory M1 type and the anti-inflammatory (patrolling) M2 type[372]. Generally, the M1 macrophages are abundant in human and mice with NASH and are activated

by TLR-signaling pathways, resulting in induction of expression of pro-inflammatory cytokines and chemokines as well as profibrogenic cytokines (TGF-β) that not only enhance hepatocyte death, but also activate HSCs into pro-fibrotic myofibroblasts. At the same time, activated HSCs can express proinflammatory mediators such as CCL2 and macrophage colony-stimulating factors, resulting in further infiltration of inflammatory macrophages and the maintenance of HSCs activation and survival[365, 366, 372-374]. On the other hand, the M2 macrophages induces resolution of inflammation and tissue repair through expression of multiple cytokines (e.g., IL-10 and IL-13). A single study proposed that hepatic M2 macrophages induce apoptosis of their M1 counterparts in the HFD-induced NAFLD model, resulting in resolution of inflammation[375]. Nonetheless, the exact functions of M2 type macrophages remain unknown in both human and murine NASH and demands further investigation.

On the other hand, with the emergence of single-cell RNA sequencing technology, accumulating evidence disputes this dichotomous classification of macrophages and emphasise macrophage heterogeneity and functional diversity. For instance, four different subsets of M2 macrophages have been identified: M2a, M2b, M2c and M2d, based on activating stimulus[376]. All these subsets exhibit anti-inflammatory activities, except M2b subset express both proinflammatory (IL-6 and TNF-α) and anti-inflammatory cytokines (IL-10)[376]. In addition, two subsets of KCs have been identified in livers of NASH patients as well as various NASH models, and they were classified based on triggering receptor expressed on myeloid cells (TREM)-2 expression. Interestingly, TREM2-low KCs were more enriched in the healthy livers, while TREM2-high KCs were exclusively predominant in NASH livers, and hence called NASH-associated macrophages' (NAMs)[377, 378]. This TREM2$^+$ macrophages were mainly localized to sites of fibrosis and hepatocellular injury in NASH livers[379]. Notably, an *in vivo* NASH model with TREM2-deficient macrophages developed defect in lipid handling and exacerbated NASH-related fibrosis, suggesting a protective function of this TREM2$^+$ macrophages[379, 380]. However, the role of TREM2

in NASH pathogenesis is yet nonconclusive and it is still under investigation. In addition, monocyte-derived macrophages (MoMFs) are another subset of macrophages, and they originate from circulating monocytes. This subset represents a minority in healthy liver, but rapidly recruited during liver injury[54, 381]. In the context of liver fibrosis, MoMFs are categorized into two populations depending on their surface expression of Ly6C. Ly6C^{hi} MoMFs originate from Ly6C^{hi} monocytes, and they express profibrogenic and proinflammatory cytokines (TGFβ and IL-1β) that promote liver fibrosis, while Ly6C^{low} MoMFs are derived from Ly6C^{low} monocytes, and they express metalloproteinases that degrade ECM and hence promote resolution of liver fibrosis[382]. Importantly, Krenkel et al identified three subsets of intrahepatic MoMFs in WD-induced NASH model by using single-cell RNA sequencing technique. These subsets expressed less inflammatory marker calprotectin (S100a8 and S100a9) in WD-fed mice compared to those fed with normal diet, highlighting that these subsets may play a protective role in NAFLD[381]. Overall, macrophages are crucial players in NASH pathogenesis, and precise characterization of their subtypes and diverse functions is essential for identifying novel therapeutic target for NAFLD treatment.

Neutrophils

Infiltration of neutrophils into the fatty liver is one of hallmarks of NASH. A substantial increase of peripheral neutrophils to lymphocyte ratio (NLR) has been detected in patients with advanced NASH-related fibrosis compared to those with mild fibrosis[383]. Also, this was associated with hepatocyte ballooning, hepatic steatosis, and lobular inflammation and fibrosis[383, 384]. This may highlight a critical role of neutrophils in promoting NASH progression. Currently, multiple mechanisms have been revealed to understand how neutrophils contribute to NASH pathogenesis and accelerate disease progression.

Like KCs, neutrophils can be activated by DAMPS or PAMPS signals, resulting in release of inflammatory mediators, including myeloperoxidase (MPO), elastase, and ROS which promote

hepatocyte injury. In general, neutrophils express large amounts of MPO, a pro-oxidant enzyme, which interacts with hydrogen peroxide (H_2O_2) and catalyzes the generation of hypochlorous acid/hypochlorite ($HOCl/OCl^-$). Consequently, these oxidants (ROS) promote lipotoxicity in the context of NASH[385]. In addition to this, MPO can modulate the inflammatory microenvironment by promoting macrophage cytotoxicity to release proinflammatory cytokines (e.g, TNF-α)[386] as well as augmenting neutrophil activation via CD11b/CD18 integrin-dependent mechanism[387]. Moreover, MPO-induced oxidative stress can modulate liver fibrosis progression, not only by promoting hepatocyte injury but also by directly activating HSCs [388-390]. Mechanistically, MPO induces hepatocyte death by driving mitochondrial permeability transition through activation of SAPK/JNK[390]. In parallel, MPO induces activation of HSCs in an MAPK- and/or PI3K-AKT-dependent manner as well as it activates TGF-β, which promotes liver fibrosis progression[390, 391]. There was high enrichment of MPO^+ cells in the liver of NASH compared to NAFL patients and this was associated with accumulation of HOCl-related proteins as well as upregulation of chemokines (e.g, CXCL-1) and neutrophil infiltration[385]. MPO deficiency, whether by pharmacological inhibition or genetic deletion, attenuated hepatocyte death, reduced inflammation, and ameliorated fibrosis progression in various diet -induced NASH models[390-392].

Generally, during inflammation, neutrophils express neutrophil elastase (NE), a serine protease enzyme, via degranulation. The secretory NE is often bound to alpha-1-antitrypsin (AAT), which inhibits its activity and hence the NE/ATT ratio determines NE activity. Of note, this ratio was high in the serum and the livers of NASH patients and was associated with NASH severity and NASH-related fibrosis, respectively[393, 394]. In addition, mice with NE deficiency ($Elane^{-/-}$) were protected against WD-induced NASH by reducing hepatic ceramides and inflammatory genes expression (e.g, IL-6)[395]. Apart from NE, neutrophils express other inflammatory mediators such as Lipocalin 2 (LCN2) that is involved in different biological systems, including innate immunity, cell metabolism and cell death[396, 397]. In NASH models, LCN2 level was increased and promoted

neutrophil recruitment to the liver and enhanced the secretion of inflammatory cytokines (IL-1β, TNF-α and CCL2), most likely by upregulating CXCR2-activated MAPK ERK1/2 and by enhancing the crosstalk between neutrophils and macrophages that worsened the inflammation[398, 399].

In homoeostasis, neutrophils can release the neutrophil extracellular trap (NET) structures, including nucleic acids, histones, and antimicrobial peptides, into extracellular space to entrap pathogens or any endogenous danger stimuli, leading to the control of infection and providing host defense[400]. In general, neutrophils are initially activated by their innate immune receptors and then triggered by downstream mediators including mainly mitochondria or NADPH-induced ROS, leading to activation of MPO, NE, and protein-arginine deiminase type 4 (PAD4) to induce chromatin decondensation[401, 402]. PAD4 citrullinates histones, while MPO and NE further promote chromatin decondensation, leading to disruption of the nuclear membrane and the release of chromatin into the cytosol in combination with granular and cytosolic proteins[402]. Indeed, tight regulation of the NET formation is highly critical to maintain homeostasis and prevent excessive tissue damage. However, dysregulated NETs have been evident in infection and sterile diseases, contributing to the disease pathology. Multiple mechanisms have been proposed, which are reviewed in detail elsewhere[400, 403]. Briefly, dysregulated NETs can induce different detrimental functions in chronic diseases such as modulating macrophages to produce inflammatory IL-6 and IL-1β cytokines in atherosclerosis[404], stimulating IFN responses in autoimmunity[405], promoting tumor growth and metastasis[406], and inducing thrombosis by mediating vaso-occlusion[407].

Investigating the role of NETs in NASH pathology and NASH-HCC has become a rapidly growing area. The markers of NETs formation, including MPO-DNA complexes or citrullinated histone H3 were increased in the circulation and the livers of NASH patients and correlated with NAFLD severity [408-411]. Similarly, NET formations were detected in different NASH models, including STAM and MCD-HFD models[408, 412]. Interestingly, treatment of the STAM mice with DNase I or PAD4 deletion ameliorated NASH and abrogated HCC development, but it did not affect liver

steatosis. DNase I administration to MCD-HFD-fed mice ameliorated liver injury, inflammation, and liver fibrosis[412]. The mechanisms underlying the NETs-inducing NASH progression is still unexplored. However, a recent study suggested that NETs promoted Treg differentiation by inducing metabolic reprogramming of naïve $CD4^+$ T cells, specifically OXPHOS, and thus promoting immunosuppressive microenvironment and HCC development in STAM-induced NASH model [411]. This may suggest an essential role of NETs in promoting the cross talk of innate and adaptive immunity in the context of NASH-HCC. Nevertheless, the role of NETs in modulating liver fibrosis progression in NASH remains to be elucidated.

DCs

The role of DCs in human NASH is yet to be established, while in murine NASH, it is complex and controversial. Depletion of DCs in murine NASH livers has worsened or ameliorated the inflammation and liver fibrosis progression. This discrepancy may highlight the lack of robust experimental methods that can appropriately assess the heterogeneity of DCs and delineate the role of DCs in NASH[372, 413, 414].

NK cells

The role of NK cells in human and murine NASH remains controversial. For example, enrichment of activated hepatic NK cells (high NKG2D expression) was positively associated with advanced fibrosis in NASH patients [415]. In contrast, other studies reported negative correlation between fibrosis and the intrahepatic frequency of NK cells in NAFLD patients. In addition, NK cells had less cytotoxicity, though increased IFN-γ production, in murine NASH models. The depletion of NK cells aggravated liver fibrosis progression in these models, likely by increasing the infiltration of MoMFs. NK cells also have antifibrotic function by inducing apoptosis of activated HSCs[59, 416-418]. Thus, further research is needed to dissect the role of NK cells in NAFLD/NASH progression.

NKT cells

NKT cells are depleted at early stage of NAFLD disease, specifically in murine NAFLD models. Interestingly, this loss of NKT cells seem to be dependent on increased apoptosis due to the activation of TIM 3/ /Gal-9 signaling and/or the enhanced IL-12 signaling from Kupffer cells[419, 420]. Importantly, the protective effects of NKT cells were evident in NAFLD models through reducing liver steatosis and metabolic burden[421, 422]. In contrast, NKT cells producing IL-17 and IFN-γ were enriched in the livers of NASH patients with advanced fibrosis, suggesting that NKT could be deficient at early stage of NAFLD, but enriched later in advanced NASH possibly to promote inflammation and fibrosis[191, 423]. In line with this, NKT cells played a fibrogenic role in different NASH models by inducing the osteopontin- and sonic-hedgehog pathways, which in turn activates HSCs and promote liver fibrosis[191, 424]. However, other studies reported antifibrotic role of iNKT cells in NASH [425]. Overall, the role of NKT in NASH progression remain not clear and further research is needed to delineate its role.

γδ T cells

Unlike the classical αβ T cells, γδ T cells express γδ TCR and they lack MHC-dependent peptide presentation. γδ T cells represent a component of innate immune cells as they rapidly respond to invading pathogens by secretion of IL-17 and IFN-γ[426]. Studies that investigated γδ T cells in the context of NAFLD are only limited to animal models, though their number is very rare. There was an expansion of IL-17A$^+$γδ T cells in livers of HFD-induced NASH model. The inflammation burden of NASH and liver injury as well as IR were reduced in γδ TCR-deficient tcrd$^{-/-}$ mice. Interestingly, this amelioration of NASH was dependent on the secretion of IL-17A by γδ T cells[427]. In contrast, another evidence proposed other IL-17-independent mechanisms such as modulating the inflammatory phenotype of CD4$^+$ T cell through upregulating the expression of the CD1d-dependent vascular endothelial growth factor[428]. IL-17A$^+$ γδ T cells may modulate fibrosis progression in NASH, however the underlying mechanisms remain to be determined.

T and B cells:

Accumulating evidence points towards a role for adaptive immunity as an additional factor in promoting NASH-related inflammation[106, 429]. T and B lymphocytes have been detected forming focal aggregates in almost 60% of liver biopsies of NASH patients and this was positively associated with lobular inflammation and fibrosis grade [430]. Moreover, similar observations were detected in murine NASH models, where T and B lymphocytes exacerbate lobular inflammation and liver injury[106, 423, 431]. In NASH, the lymphocytes recruitment to the liver is possibly dependent on stimulation of hepatocyte STAT1, resulting in enhanced expression of lymphocyte chemokine CXCL-9. Conversely, depletion of STAT1 stimulation in hepatocytes, reduced the recruitment of $CD4^+$ and $CD8^+$ T cells and decreased fibrosis progression[432]. In addition, overexpression of vascular adhesion protein 1 (VAP1) by endothelial cells in NASH has also been associated with promoting recruitment of $CD4^+$ T cells to the liver[431].

B cells

Emerging evidence suggests an expansion of intrahepatic B2 cells, in parallel with upregulation of B cell-activating factor (BAFF), in human and murine NASH. BAFF is an essential cytokine for survival and maturation of B cells, which was correlated with inflammation and fibrosis in NASH patients. The selective depletion of B2 cells or neutralization of BAFF in a NASH model inhibited maturation of B2 cells, prevented activation of Th1 response and ameliorated NASH-related fibrosis [430, 433]. In addition, the circulating IgGs against oxidative stress-derived epitopes (OSE) such as malondialdehyde–acetaldehyde adducts (MAA) were increased in adults and pediatrics with NASH, which was positively correlated with the severity of lobular inflammation, and independently predicted liver fibrosis [430, 434, 435]. Similarly, in rodent NASH models, an increase of circulating anti-OSE IgG was associated with upregulation of pro-inflammatory cytokines, including IL-6 and TNF-α, which exacerbated NASH progression[106, 436, 437]. B cells could be profibrogenic in NASH by inducing proinflammatory cytokines, which in turn activate macrophages and HSCs, leading to promotion of liver fibrosis. At the same time, activated HSCs

could support intrahepatic B cell maturation and survival via secreting retinoic acid and CXCL-12, with CXCR4 target in B-cells, resulting in vicious cycle of liver fibrosis progression[377, 436-438]. However, these findings were limited to *in vivo* studies, and their clinical relevance is yet to be determined.

CD8$^+$ T cells (Tc)

An enrichment of intrahepatic cytotoxic Tc cells was observed in human and murine NAFLD, where their recruitment was dependent on IFN-α responses[106, 423, 439]. Tc cells promoted metabolic abnormalities, including IR and glucose intolerance, in a HFD-induced NAFLD model[439]. In addition to this, genetic abrogation of Tc and NKT cells in NASH model resulted in mild hepatic steatosis and inflammation[423, 440]. In this sense, Wolf et al [423] postulated that Tc alone were likely insufficient to promote liver damage in WT mice and the concomitated presence of NKT cells was crucial for developing such injury[423]. Despite this, selective neutralization of CD8 in NASH model reduced inflammation and activation of HSCs, indicating an active role of Tc functionality in pathogenesis of NASH[440]. Taken together, the role of Tc in NASH progression is not well understood, and further studies are needed to characterize kinetics and functionality of Tc.

T helper (CD4$^+$ T) cells

Generally, T helper cells (Th) or CD4$^+$ T cells have different subsets that can mediate different immune responses (inflammatory and/or anti-inflammatory). These subsets have a large degree of plasticity, where their functional classification may change based on the appropriate stimulating signal. The Th subsets were originally classified according to the type of cytokines they produce such as Th1-mediated IFNγ response and Th2-mediated IL-4 response[441]. However, other Th subsets, including regulatory T CD4$^+$ (Treg) and Th17, were later discovered, and the classification was expanded to include the Th1/Th2/Th17/Treg paradigm[442, 443]. Th1 cells are characterized by expression of the transcription factor Tbet and the pro-inflammatory Interferon-

γ (IFN-γ) cytokine. The differentiation of these cells is dependent on IL-12 and IFN-γ signalling via activation of signal transducer and activator of transcription 1 (STAT1) and STAT4. The Th1 response is important for controlling infections against intracellular pathogens, though its dysregulation is associated with tissue necrosis and exacerbation of inflammation in autoimmune diseases.

Th2 cells are characterized by expression of the transcription factor GATA3 and cytokines such as IL-4, IL-13, IL-5. The differentiation of these cells is dependent on IL-2 and IL-4 signalling via activation of STAT6. The Th2 response is protective against extracellular pathogens such as helminths and venoms and mediates tissue repair. Also, Th2-mediated inflammation is implicated in asthma, allergic reactions, and fibrosis[426, 444].

Th17 cells are characterized by the expression of transcription factor RORγt, and they produce IL-17A-F, IL-22 and IL-21. Polarization of Th17 is mainly regulated by IL-6, IL-23, and TGF-β, IL-21 through activation of signal transducers and activators of transcription 3 (STAT3). Th17 cytokines mediate protective immunity against bacteria and fungi at epithelial barriers as well as mediate tissue repair[426, 444, 445]. Dysregulated Th17 responses have been associated with various inflammatory disorders, including colitis, rheumatoid arthritis, T2DM, and autoimmunity[446, 447].

Treg cells are characterized by the expression of the transcription factor FOXP3 and production of IL-10 and TGF-β cytokines. The differentiation of Tregs is mainly driven by TGF-β in the absence of IL-6 and is further induced by IL-2 and retinoic acid via activation of STAT5. Generally, Tregs control immune tolerance through inhibiting auto reactivity to self-antigens and preventing overactivation of effector T cells, which prevent tissue damage during infections. But, dysregulated Treg responses have been implicated in multiple inflammatory diseases including cancer and autoimmunity[426]. These Th subsets are shown to infiltrate the liver in murine and human NASH[426]. In this review, we will discuss Th17 and their cytokines (IL-17 and IL-22) in

details and their implication in liver disease pathogenesis, including NASH. Also, we provide an overview of Th17/Treg imbalance during NASH progression.

In summary, chronic inflammation, involving both innate and adaptive immunity, is a major pathogenic driver of NASH progression to cirrhosis and HCC. The current evidence suggests that innate immune cells, specifically neutrophils and KCs/macrophages, are the main driving forces during NASH development and progression. Also, the adaptive immunity has been implicated in NASH progression and could sustain the inflammatory response to liver fibrosis and HCC. Understanding the role of these immune cells and their underlying mechanisms in NASH is critical for the development of novel therapeutic targets.

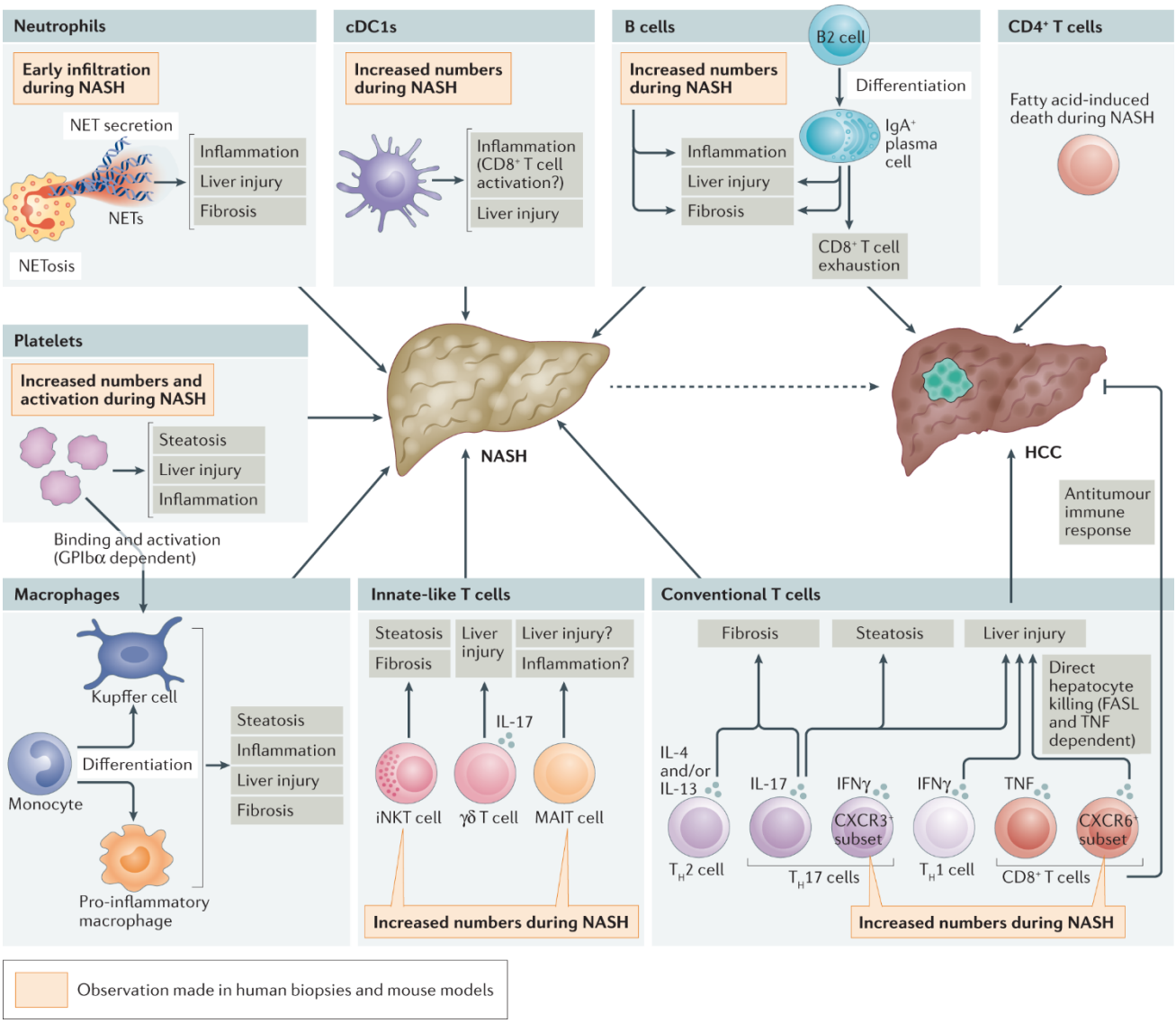

Figure 13. Schematic diagram showing the immune landscape of NASH that contributes to hepatocyte injury, fibrosis progression, and HCC development

The inflammatory immune cells include different innate and adaptive immune cells, all of which have been reported in human and mouse models of NASH. Neutrophils, monocytes, and macrophages are rapidly recruited to the liver and promote NASH-related inflammation and liver injury. DCs enhance the activation of $CD8^+$ T cells, which contribute to liver injury and inflammation through TNF-α-dependent mechanism. Th17, iNKT, MAIT and γδ T cells produce IL-17 cytokine which promote liver inflammation in NASH and fibrosis progression. Also, B2 cells contribute to the liver injury and NASH-related inflammation. Platelets are activated during NASH and promote liver steatosis and liver injury. This figure is taken from [448] (Reproduced with permission)

5. IL-17

The IL-17 family includes six cytokines IL-17A-F, of which IL-17A is the most widely investigated. There is 55% homology in the amino acid sequence between IL-17A and IL-17F, thus both cytokines share cellular sources, receptors, and target tissues, and induce similar pro-inflammatory functions[5, 449, 450]. We will focus on IL-17A, herein after referred to as IL-17.

IL-17 is produced by different adaptive and innate immune cells including, Th17, $CD8^+$ T cells (Tc17), γδ T cells, ILC3s, neutrophils and mast cells[5, 450-452]. RORγt transcription factor is the master regulator of IL-17 expression, while RORα can also regulate IL-17 expression but to a lesser extent[445, 453]. As mentioned earlier, naïve $CD4^+$ T cells can differentiate into Th17-producing cells in response to antigenic stimulation and presence of IL-6 and TGF-β. During the early phase of Th17 differentiation, these cells upregulate expression of RORγt, CCR6 and the IL-23 receptor. IL-23 signalling is very essential in stabilizing the Th17 pathogenic phenotype characterized by the production of both IL-17 and IFNγ[445, 454, 455]. STAT3, interferon regulatory factor 4 (IRF4), aryl hydrocarbon receptor (AhR) and basic leucine zipper ATF-Like transcriptional factor (BATF) are examples of positive regulators of Th17. Tbet and FOXP3 are negative regulators of Th17 as they induce Th1 and Tregs differentiation, respectively[5]. TGF-β can regulate differentiation of both Th17 and Tregs from naïve $CD4^+$ T cells, however this depends on the context of the stimuli, the level of TGF-β, and the presence of other cytokines such as IL-6. Tregs can produce IL-10, which inhibits proliferation and differentiation of Th17 cells and thus blocks effector functions of Th17[5, 426].

IL-17 interacts with the IL-17 receptor (IL-17R) to mediate its signalling. IL-17R, a heterodimer receptor consisting of the IL-17RA and IL-17RC subunits, is expressed by epithelial cells, endothelial cells, fibroblasts as well as macrophages[5, 456-458]. The interaction of IL-17 with IL-17R, propagates activation of multiple downstream signalling pathways, including NF-κB and CCAAT enhancer-binding proteins (C/EBP), and MAPKs/p38/JNK. A non-canonical pathway is also

activated by IL-17, which stabilizes mRNA transcripts of unstable target cytokines or chemokines (Figure 14) [459, 460]. Consequently, these pathways regulate the expression of neutrophil chemo-attractants (e.g., CXCL-1), proinflammatory cytokines and antimicrobial peptides such as defensins, angiogenic factors, and MMPs[461]. These effector functions of IL-17 are essential for protecting mucosal surfaces against extracellular bacteria and fungi in different organs, including the liver[5, 444]. Interestingly, IL-17 itself is not considered as potent inducer of inflammation, though it synergizes with other cytokines such as TNF-α or IL-22 in the tissue microenvironment promoting recruitment of neutrophils and Th17 cells, and hence induces a potent pro-inflammatory response. These effects were implicated in both host protection and disease states[462]. As mentioned above, dysregulated Th17 responses have been associated with various inflammatory disorders, including colitis, rheumatoid arthritis, T2DM, and autoimmunity[446, 447].

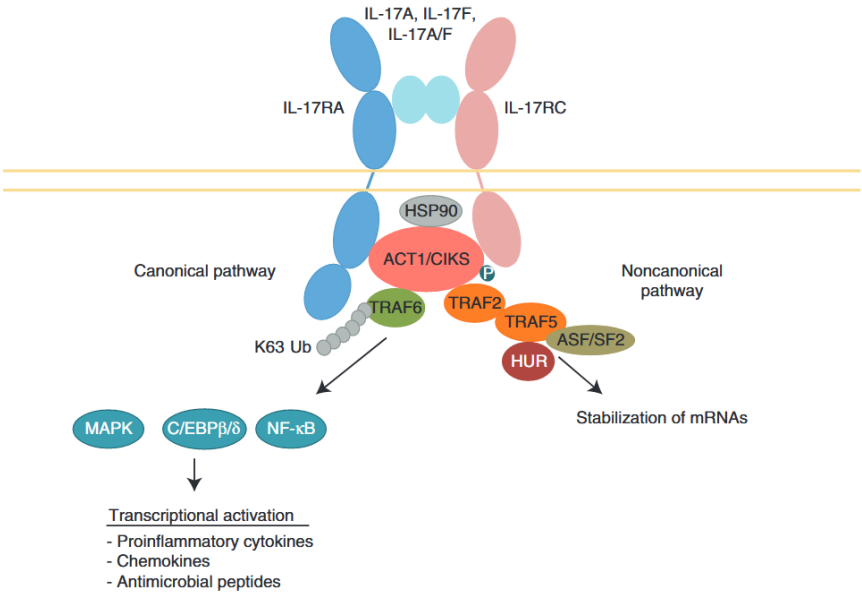

Figure 14. IL-17/IL-17R signaling pathways
Upon binding of IL-17A or IL-17F to IL-17 receptor, this interaction trigger IL-17 downstream signaling through activation of Act1-induced K63-linked ubiquitylation of TRAF6, which results in activation of C/EBPb, MAPK, and NF-kB pathways. This induces the upregulation of antimicrobial peptides and several proinflammatory cytokines and chemokines. During this process, a non-canonical pathway is activated by IL-17, and it depends on the phosphorylation of Act1, which triggers sequestration of messenger RNA (mRNA)-destabilizing factor ASF/SF2 and mRNA-stabilizing factor HuR through recruitment of TRAF2 and TRAF5. These canonical and non-canonical pathways drive the proinflammatory effects of IL-17A and IL-17F. This figure is taken from [460] (Reproduced with permission)

5.1. Role of IL-17 in acute liver injury

Many studies investigated the role of IL-17 in several acute hepatitis models, such as Con-A- and LPS/GaIN-induced hepatitis, where the level of IL-17A increased in the liver and was associated with liver injury[463-467]. Elevated IL-17 levels were also observed in the serum of patients with acute hepatic injury and correlating with severity of injury as well as poor prognosis[468, 469].

In the inflammation phase of acute injury, IL-17 promotes the secretion of several pro-inflammatory chemokines in hepatocytes and fibroblasts (Figure 15), including macrophage inflammatory protein 1-alpha (MIP-1α/CCL3), IFN-γ-inducible protein 10 (IP-10/CXCL10), growth related oncogene-alpha (GRO-α/CXCL1), and IL-8 (CXCL8), which enhances the recruitment of monocytes and neutrophils to the injury site[464, 470]. In addition, IL-17 acts synergistically with TNF-α and results in upregulation of IL-8 further attracting neutrophils [471]. IL-17 also synergizes with IL-1β and IL-6 to promote activation and recruitment of liver resident and pro-inflammatory macrophages, a process worsening the inflammation even though important for elimination of the damaging agent [5, 464, 472].

Other effects of IL-17 have been reported during tissue repair and remodelling phases of acute injury. In the tissue repair phase, hepatocyte proliferation may be targeted by IL-17 signaling, though this effect seems to be indirect as it is dependent on IL-6 signaling which promotes STAT3-induced proliferation[473, 474]. In support of these findings, IL-17A^{+} γδT cells were recruited to the liver of the partial hepatectomy mouse model and induced IL-6 production from macrophages and DCs, which in its turn inhibited IFN-γ^{+} NK-cells and promoted hepatocyte proliferation and regeneration[475, 476]. In addition to its effect on hepatocyte proliferation, IL-17 targeted murine LPCs and promoted their proliferation and expansion *in vitro* and *in vivo*[477]. Also, IL-17 enhanced IL-27 expression in macrophages which favored LPCs differentiation into hepatocytic phenotype[477].

However, further investigation is needed to assess direct or indirect effects of IL-17 on hepatocytes and LPCs proliferation during acute hepatitis.

On another note, angiogenic pathways and LESC proliferation to form new vessels and promote vasculature repair is of critical importance during wound healing (Figure 15)[5]. IL-17 has been reported as a potent enhancer of VEGF production from epithelial cells and fibroblasts to induce angiogenesis in different tumors including HCC[478, 479]. Moreover, stimulation of LESC with TNF-α and IFN-γ facilitated the adhesion of Th17 and Tc17 cells to LSECs by expressing CXCR3 in acute Con-A-induced hepatitis model[480]. These data suggest that IL-17 may play a role in modulating angiogenesis during tissue repair in the liver, though the current evidence remains insufficient.

As previously mentioned, HSCs are fully activated by TGF-β and/or PDGF during the tissue repair phase and they secrete fibrillar collagen. IL-17 can indirectly modulate HSCs and induce fibrogenesis by activating myeloid cells, including KCs, neutrophils, and monocytes, which further drive IL-17 production along with other cytokines such as IL-6 in an inflammatory loop (Figure 15) [464, 470, 472, 473]. Also, Th17 increased α-SMA expression by mouse HSCs, while Treg reduced it [481]. Moreover, other studies showed that IL-17 can cooperate with TGF-β in modulating HSCs and induction of fibrosis. For instance, *in vitro* studies showed that IL-17 sensitized HSCs response to suboptimal doses of TGF-β by upregulating the TGF-β-RII on HSCs in a JNK-induced SMAD2/3-dependent mechansim[482]. Following temporary scar formation, the tissue remodelling phase is initiated to degrade the temporary scar by upregulating expression of MMPs and downregulating expression of tissue inhibitor of metalloproteinases (TIMPs)[5]. Although, IL-17 regulates several MMPs such as MMP1/2/9 in different inflammatory contexts including cancer, its role in ECM remodeling during acute hepatitis remains undefined[483-485].

Given the pro-inflammatory role of IL-17 as mentioned above, the genetic deletion or pharmacological neutralization of IL-17 ameliorated liver injury in most models of acute

hepatitis[463-466, 486]. However, protective effects of IL-17 have been reported in certain models of the disease. For example, in α-galactosylceramide (αGalCer)-induced hepatitis model, IL-17 neutralization aggravated liver damage and inflammation by promoting recruitment of inflammatory monocytes and neutrophils. But this effect of IL-17 could be related to the model itself because αGalCer induces activation of hepatic $CD4^+$ iNKT to produce IL-17, leading to hepatitis with mild-moderate intensity[487]. Likewise, depletion of IL-$17A^+$ γδ T cells, in hepatitis B virus surface antigen transgenic (HBs-Tg) mice treated with ConA, exacerbated inflammation by promoting IFN-γ^+ $CD4^+$ T cells found to be associated with potent liver necrosis[488]. Data of these two studies suggest protective effects of IL-17 in these models via antagonizing the development of type 1 inflammation. Intriguingly, similar effects were observed in acute T cell-mediated colitis model, where IL-17 regulated differentiation of pathogenic Th1 cells[489]. Overall, the role of IL-17 in acute liver injury is complex, acting either as proinflammatory cytokine or as regulator of other types of inflammation.

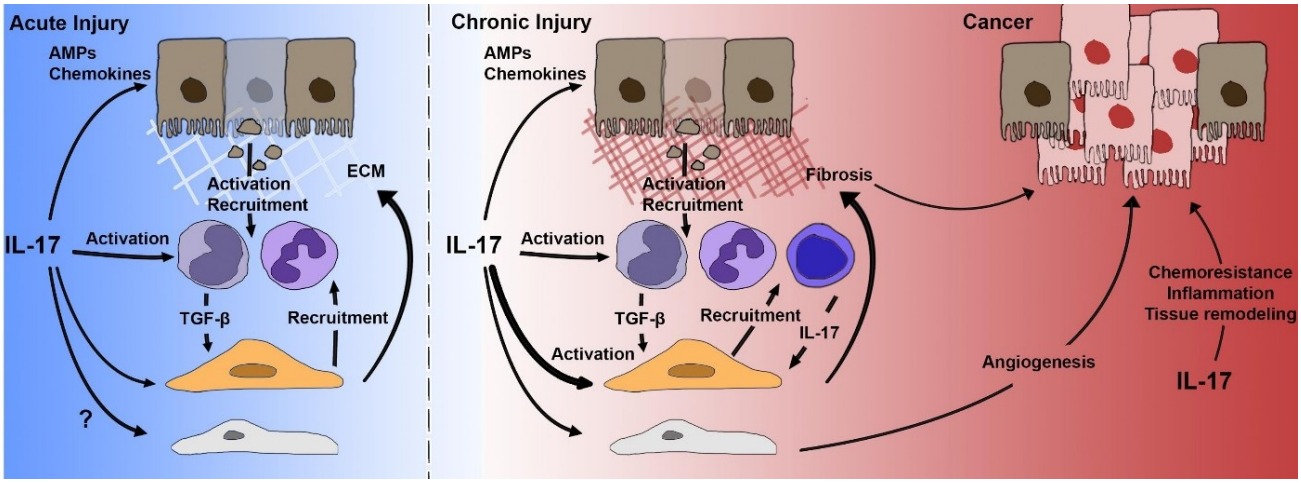

Figure 15. Role of IL-17 in acute and chronic liver injury
During acute injury, IL-17 promotes expression of chemokines that enhance recruitment of inflammatory neutrophils and monocytes to the injury site in order to eliminate the insult. Also, IL-17 induces the expression of antimicrobial peptides (AMPs) to protect epithelial barriers against invading pathogens. In addition, during wound healing, IL-17 may act on LSEC to promote angiogenesis and revascularization. Moreover, IL-17 modulates HSCs activation and enhances generation of ECM that supports tissue repair. On the other hand, in chronic liver injury, the IL-17/IL-17R axis is dysregulated resulting in persistent inflammatory environment as well as HSCs activation, leading to promotion of hepatic injury, ECM deposition, and fibrosis progression. Also,

these inflammatory effects of IL-17 have aggravated tumor progression in HCC. This Figure is taken from [5] (Reproduced with permission)

5.2. Role of IL-17 in chronic liver injury

Many studies have investigated the role of IL-17 in different CLDs, which are characterized by chronic inflammation and fibrosis development that likely progress to liver cirrhosis and HCC. Hepatic Th17/IL-17 axis is commonly upregulated in both human and murine models of CLD and positively associates with disease progression[5]. Stimuli that drive this upregulation of the Th17/IL-17 axis in CLD remain not clear, though some possible mechanisms have been proposed. For example, it is known that damaged hepatocytes express chemokine gradients such as CXCL9, CXCL10, and CCL20, which bind to their receptors CXCR3 and CCR6 on Th17, promoting recruitment of Th17 to the liver and their localization within the fibrotic area[480, 490-492]. Although, such stimulus may be common among all CLDs irrespective of their etiology, other stimuli could drive Th17 and may vary depending on CLD etiology. For example, in HBV, the level of Th17 could be dependent on HBV antigen and serum HMGB1 that activate APCs to produce proinflammatory cytokines (IL-1, IL-6, IL-21, and IL-23) that drive Th17 polarization[493-495]. Also, the release of thymic stromal lymphopoietin by HCV-infected hepatocytes promotes the production of cytokines by APCs and favors Th17 differentiation[496]. In addition, the metabolic burden associated with NAFLD may affect the cellular metabolism of the Th17/IL-17 axis and their differentiation. For example, hypoxia inducible factor 1α (HIF-1α) was shown to promote glycolysis and enhance IL-17 production by T cells *in vitro* and *in vivo*, while its deficiency or blocking glycolysis shifted the T helper cell polarization toward Tregs[497]. Accordingly, the persistent immune imbalance of the Th17/IL-17 axis in context of CLD could indirectly mediate pro-inflammatory effects by driving other inflammatory or profibrogenic signals that promote liver injury and dysfunctional of tissue repair (Figure 15) [5]. For purpose of this review, we briefly discuss these effects of the Th17/IL-17 axis in CLD with more focus on NASH.

There is extensive evidence for involvement of the Th17/IL-17 axis in chronic viral hepatitis (CVH). Peripheral and hepatic Th17 were increased in chronic hepatitis B and C (CHB/CHC) patients and positively correlated with liver injury, inflammation, and recruitment of neutrophils[100, 493, 495, 498-501]. Besides this increase of Th17 in CVH, the Treg count was decreased, leading to a Th17/Treg imbalance which was found to be associated with hepatic injury[502-505] and progression to liver cirrhosis and HCC [504, 506, 507] or to be inversely correlated with mortality[506-508]. Interestingly, CHB patients treated with antiviral therapies such as entecavir or telbivudine had significant reduction in their Th17 response in the liver and blood and this was associated with a reduction in HBV viral load and normalization of serum ALT[509, 510]. Similarly, CHC patients treated with pegylated IFN-α and ribavirin therapy, exhibited decreased IL-17, IL-6 and IFN-α levels in circulation[511]. In addition to viral hepatitis, the Th17/Treg imbalance and enrichment of hepatic IL-17^{+} cells were common in other CLD including ALD, NASH, autoimmune hepatitis (AIH) and positively linked with exacerbation of inflammation and liver injury[470, 512-516]. *In vitro* studies demonstrated that IL-17 mediated recruitment of myeloid cell-driven inflammation, including macrophages and monocytes, enhanced the production of proinflammatory cytokines-induced hepatic injury such as IL-6, IL-1β, and TNF-α[498, 499]. Also, in the CCl_4-induced liver fibrosis model, the lack of IL-17 signaling (IL-17RA$^{-/-}$) ameliorated the inflammation by attenuating neutrophil infiltration and secretion of inflammatory cytokines and by reducing hepatocyte death as well as fibrosis[517]. In line with this, similar effects were observed in liver of AIH model upon IL-17 neutralization[518]. Overall, the enrichment of the Th17/IL-17 axis in CLD induces proinflammatory environment via recruitment and activation of other inflammatory cells that promote liver injury.

5.3. Fibrogenic role of IL-17 in CLD

Beyond its proinflammatory role in CLD and increasing the severity of the disease, IL-17 can modulate liver fibrosis progression as well. There was a positive association between the level of intrahepatic IL-17^{+} cells as well as circulating Th17 and fibrosis grade in chronic HCV or HBV[100,

[498, 499, 519], ALD[470] and NASH[513, 520-522]. We also showed that IL-17-producing cells, including Th17, neutrophils and mast cells, were increased in patients' liver biopsies with advanced fibrosis irrespective of etiology, and mainly localized in the scar area[523]. Although these data suggest a profibrogenic function of IL-17 in CLD, the underlying mechanisms remain controversial.

Given that HSCs express IL-17RA and IL-17RC, stimulating HSCs with IL-17 *in vitro* directly activated HSCs, enhanced their proliferation, and increased their collagen and α-SMA expression in a STAT3- and ERK1/2/p38-dependent manner[472, 517]. On the other hand, other studies reported that the profibrogenic role of IL-17 can be mediated by indirect effects. For instance, we demonstrated that IL-17 sensitized HSCs response to suboptimal doses of TGF-β *in vitro* by upregulating the TGF-β-RII on HSCs and this response was dependent on JNK-induced SMAD2/3 signalling[482]. Moreover, IL-17 activated HSCs to express IL-8 and CXCL-1, which promoted neutrophil recruitment and fibrosis development[470, 524]. Interestingly, blocking IL-17 signaling in HSCs cocultured with IL-17$^+$ MAIT cells reduced expression of IL-8 and CCL2 and consequently attenuated liver fibrosis[525]. In addition, IL-17 upregulated the release of proinflammatory IL-6 and TNF-α as well as profibrogenic TGF-β cytokines by monocytes and KCs via NF-κB- and STAT3-dependent pathways, leading to HSCs activation and increased collagen production[472, 526, 527]. At the same time, HSCs or KCs can further modulate the Th17/IL-17axis during progression of liver fibrosis. KCs, isolated from a hepatitis model, favored differentiation of naïve CD4$^+$ T cells into Th17 *in vitro* via IL-6-dependent mechanism, leading to Th17/Treg imbalance[528, 529]. Additionally, activated HSCs promoted IL-17 production from T cells via TLR3-dependent manner, leading to further Th17/Treg imbalance and exacerbating hepatic fibrogenesis[14, 530]. The profibrogenic activity of IL-17 was further evident in experimental models of liver disease such as CCl_4 or BDL, with liver fibrosis attenuated in IL-17RA$^{-/-}$ mice and associated with reduced expression of profibrogenic genes, including *Col1a1,* actin alpha 2 (*Acta2), Mmp3, Timp1* and *Tgfb*[472]. Intriguingly, the lack of IL-17 signalling in immune cells

including KCs or HSCs inhibited liver fibrosis, though this effect was not observed in hepatocytes or LESCs, highlighting the importance of the fibrogenic function of IL-17 not only in liver resident cells and but also in non-resident ones[472]. In support of these findings, the profibrogenic activity of IL-17 was observed in other liver fibrosis models independent of the cellular source of IL-17 [517, 523, 527, 531-534].

In spite of the well-established evidence of pathogenic function of IL-17 in CLD, few reports showed contradicting findings. The frequency of Th17 in the blood of CHC patients was not elevated compared to acute HCV patients who cleared the infection, and the serum level of IL-17 was independent of liver fibrosis grade in these CHC patients[100]. Similarly, the plasma level of IL-17 was markedly reduced in CHC patients with advanced liver cirrhosis, suggesting regulatory pathways that control IL-17 expression to minimize the fibrosis progression[535]. Another report showed that the proinflammatory function of HSCs, when co-cultured with MAIT cells, was not affected by IL-17 neutralization, but was rather solely dependent on TNF-α secreted by the latter cells[536]. Although these data are unanticipated, the evidence remains insufficient and demands further investigation. In summary, the Th17/IL-17 axis mediates detrimental inflammatory and fibrogenic effects in CLD, leading to disease progression and low survival rate.

5.4. Role of IL-17 in NAFLD

Due to the complexity of NAFLD and the involvement of different compartments in driving its pathogenesis, the role of the Th17/IL-17 axis has been largely investigated not only in the liver but also in the AT and intestine. Obesity and T2DM have been linked to varying degrees of elevated Th17, in AT and peripheral blood of both human and murine models[537]. Multiple possible mechanisms have been revealed in this matter. Earlier reports showed that Th17 expansion in AT was mediated by the presence of proinflammatory macrophages (M1)-induced IL-6, IL-1β and IL-23 in obese and diabetic patients[538, 539]. More recent studies demonstrated that specific subsets of DCs ($CD11c^{high}$ $F4/80^{low}$ $CX3CR1^{+}$) and ($CD11c^{+}$ $CD1c^{+}$) promoted expression of

proinflammatory IL-6 and IL-1β, leading to the diversion of naïve T cells toward a Th17 profile in obese mice and morbid obese patients, respectively[540]. Another evidence proposed a cross talk of $CD14^+$ monocytes and adipose-derived stem cells (ASCs) that favored the differentiation of infiltrating T cells into pathogenic Th17 (IL-17/ IFNγ) via promoting IL-1β in obese AT[541]. Also, the IL-17 activity may be influenced by the cellular metabolism that is also responsible for Th17 differentiation and proliferation. Importantly, metabolic nutrients may regulate the interplay between Th17 and Treg, based on their distinct energy requirements. For example, glycolysis pathway is critically essential for inducing Th17 differentiation and proliferation, while Tregs differentiation is induced by oxidative phosphorylation (OXPHOS) of fatty acid substrates[542]. Given that the onset of inflammation in obese AT is generally preceded by hypoxia, HIF-1α levels are consequently increased promoting glycolysis and enhancing IL-17 production by T helper cells, while HIF-1α deficiency or blocking glycolysis shifts the T helper cell polarization toward Tregs[497, 543]. In addition, obesity and nutrient overload have been associated with upregulation of AAC1, which is a crucial enzyme for *de novo* FAS, favoring Th17 differentiation and impairing Tregs[537, 544]. Taken together, these data may clearly imply the importance of cytokine milieu in promoting Th17/Treg imbalance in obesity or T2DM, though these cytokines do not seem to be the sole players and other cellular metabolism pathways of T helper cells may drive this imbalance as well.

The metabolic effects of the Th17/IL-17 axis have been reported in obesity and NAFLD. Several studies demonstrated that either IL-17 neutralization or genetic deletion of IL-17 ($IL\text{-}17^{-/-}$) or its signalling ($IL\text{-}17RA^{-/-}$) increased weight gain and fat mass in diet-induced obesity and/or NAFLD models[521, 522, 545]. Mechanistically, IL-17 activation downregulated the expression of proadipogenic transcription factors (TFs) such as C/EBP-α, and PPARγ, which consequently suppressed the adipogenesis[546-548]. However, IL-17 enhanced IR and inhibited glucose uptake in AT and liver [521, 522, 545, 549, 550]. Accordingly, this inhibitory effect of IL-17 on adipogenesis could appear paradoxical

and may speculate a beneficial attempt from immune system to control obesity or metabolic disorder associated with NAFLD. Nevertheless, NAFLD is associated with chronic low-grade inflammation, which could overcome this beneficial effect of IL-17 through determinantal mechanisms (e.g., IL-6 or IL-1β production by macrophages in AT) that favors inflammation and metabolic syndrome[551]. Interestingly, accumulating evidence showed that Th17 promoted IL-6 and IL-1β secretion not only from macrophages and monocytes, but also adipocytes, which sustained AT inflammation by reinforcing a positive feedback loop[522, 547, 549, 552]. On the other hand, the role of IL-17-mediated hepatic steatosis development in NAFLD remains uncertain. The deficiency of IL-17 activity was associated with increased hepatic steatosis in different NAFLD models, where IL-17 seem to affect hepatic stearoyl-CoA desaturase-1 (SCD-1) expression, but not PPARα or SREBP expression, which are regulators of lipogenesis and lipid oxidation[515, 521, 527, 553]. On the other hand, IL-17 stimulation was also shown to exacerbate fat accumulation in hepatocytes induced by FFA or through upregulating hepatic PPARγ expression[520, 522]. In line with this, IL-17$^+$ cells positively correlated with hepatic steatosis in NAFLD patients[522]. Additional work is warranted to assess the role of IL-17 on hepatic steatosis in context of NAFLD.

Unlike hepatic steatosis, the role of the Th17/IL-17 axis in promoting NASH-related inflammation and liver injury is likely indisputable. The Th17 or IL-17$^+$ cells as well as Th17-related genes, including RORγt, IL-23, IL-21 are enriched in livers of NASH patients compared to healthy subjects. Recently, a unique subset of Th17, known as inflammatory hepatic CXCR3$^+$ IL-17$^+$IFN-γ$^+$ TNF-α$^+$ Th17 (ihTh17) has been identified in the livers of NAFLD patients and correlated with disease severity. This ihTh17 subset is characterized by augmented inflammatory and metabolic gene expression compared to conventional hepatic CXCR3$^-$Th17 (chTh17) cells. Moreover, an increased Th17/Treg ratio in the liver and the peripheral blood has been positively associated with transition from NAFL to NASH in obese patients. This may highlight the involvement of Th17 not only during the progression of the disease but also at the initiation phase of NASH-related

inflammation. Interestingly, one year after bariatric surgery, the Th17/Treg imbalance is inversely changed and its level become similar to heathy subjects, in parallel with NASH amelioration. Similarly, a prospective study reported a decrease in the peripheral and hepatic venous IL-10/IL-17 ratio, which markedly represented an inflammatory state in morbidly obese NASH patients compared to those without NASH[554]. Additionally, genome wide association (GWAS) studies identified single nucleotide polymorphism (SNPs) in the critical pathways of the IL-17 axis such as STAT4, RORγt, and IL-17RA, which were associated with human NAFLD severity and hepatobiliary disease[555, 556]. Accordingly, the number of studies investigating the Th17/IL-17 axis in the pathogenesis of human NASH and NASH-related fibrosis remains limited, most likely due to difficulty and limitations to obtain liver biopsies. Thus, different diet-induced NASH models have been utilized to investigate how the Th17/IL-17 axis modulate liver injury and the progression of liver fibrosis in NASH.

As in humans, an enhanced IL-17 axis as well as Th17/Treg imbalance have been associated with detrimental effects promoting NASH pathogenesis in mice[515, 520-522, 527, 557-562]. The effects of both the Th17/IL-17 axis and Tregs in the pathogenies of NASH are discussed below.

Blocking IL-17 activity, whether pharmacologically (anti-IL-17) or genetically (IL-17$^{-/-}$ or IL17RA$^{-/-}$), ameliorated NASH and was associated with a decrease in hepatocyte injury and in the infiltration of pro-inflammatory immune cells (e.g., granulocytes), chemokines and cytokines in different NASH models[515, 520-522, 527, 553, 558]. The specific mechanisms underlying activation of Th17 and the detrimental effects of IL-17 in NASH are yet to be explored, but certain possible mechanisms have been proposed. Some studies reported hepatic upregulation of CXCL-10 by the Th17/IL-17 axis in an NF-κB/p65-dependent manner, which enhanced recruitment of inflammatory macrophages and T cells to the liver of MCD/or HFD-induced NASH models [527, 553]. In line with this, fatty liver microenvironment promoted CXCR3-CXCL9/10 axis which favored hepatic accumulation of ihTh17 cells in HFD-induced NASH model. Importantly, adoptive transfer

of ihTh17 cells exacerbated NASH progression by augmenting macrophage infiltration and promoting hepatocyte ballooning[561]. However, whether IL-17 directly activate macrophages or promote their recruitment has not been examined in these studies[527, 553, 561]. In addition, Harley et al[521] showed that the Th17/IL-17 axis induced hepatic NADPH oxidase-dependent ROS production in HFD-induced NASH model, most likely by promoting recruitment of neutrophils via CXCL-1, aggravating hepatocyte injury.

Other reports suggested that the Th17/IL-17 axis exacerbated NASH severity by acting synergistically with FFA. For example, *in vitro* treatment of hepatocytes with IL-17 promoted lipotoxic effects of palmitic acid in a JNK-dependent manner[515]. In agreement with this, simulation of HepG2 cells and primary mouse hepatocytes with IL-17 and FFA (palmitic and oleic acids) induced pro-inflammatory IL-6 production, promoted liver steatosis and decreased insulin sensitivity[520]. Apart from the lipotoxicity, IL-17 increased hepatic expression of unconventional prefoldin RPB5 interactor (UPI) in HFD-induced NASH model, promoting hepatic DNA damage and enhancing agranulocytosis and the infiltration of T cells, including Th17, to the liver[522]. Subsequently, this high level of IL-17 may result in a vicious cycle to promote hepatic UPI expression and IL-17 production, leading to further NASH exacerbation.

The Th17/IL-17 axis can play a profibrogenic role in NASH not only by exacerbating inflammation burden and hepatocellular injury, but also by modulating HSCs activation. As previously mentioned, IL-17 itself can directly or indirectly regulate HSCs status and consequently increase collagen production. The development of liver fibrosis is ameliorated in MCD-fed IL-17$^{-/-}$ mice[515], whereas administration of rIL-17 to HFD-induced NASH model exacerbated hepatic fibrosis[522]. Nevertheless, no difference in liver fibrosis was observed in a similar MCD-fed IL-17$^{-/-}$ model[553]. Although, these findings may suggest profibrogenic effects of IL-17 in NASH, further studies are needed to delineate modulation of HSCs by IL-17 in the context of NASH.

6. Role of Tregs in NAFLD

The level of Tregs is relatively increased in the livers of NAFLD patients[411, 563, 564]. Nevertheless, studies investigating hepatic Tregs in human NAFLD are very few and their evaluation techniques of Tregs levels are weak. Of note, a single study showed that the number of resting Tregs was less in the liver and peripheral blood of NASH patients compared to those with NAFL, concurrently with enrichment in Th17 signature. However, the level of activated hepatic Treg was similar between NAFL and NASH patients[513]. Intriguingly, the ratio of Th-17/resting Tregs was able to distinguish NASH versus NAFL patients and was associated with the hepatocyte death marker, known as cytokeratin18[513].

On the other hand, unlike in humans, most studies reported a decrease in the level of hepatic Tregs in different NALFD mouse models[557, 559, 565, 566], though some studies showed no change [432, 515] or even an increased number of these cells[411, 567]. Indeed, mechanisms responsible for these observations remain poorly understood. Of note, Ma et al postulated that hepatic Tregs expressed less B-cell Lymphoma 2 (BCL2) compared to Th17 in HFD-fed induced NASH model, rendering Tregs highly susceptible to oxidative stress-induced apoptosis and consequently favoring a Th17/Treg imbalance[559]. Another evidence suggested a dysfunction of immunosuppressive hepatic DCs or their interference with Treg differentiation through IL-33 signaling[568]. Similarly, to hepatic Tregs, the majority of *in vivo* NAFLD studies showed a decreased number of Tregs in visceral adipose tissue (VAT) and IR. Interestingly, this was likely mediated by PPARγ and TGF-β pathways in VAT. Although, a similar finding was observed in obese and diabetic patients, some studies reported a paradoxical increase of Tregs in VAT of obese subjects with no data available as to their NAFLD diagnosis.

The role of Tregs in NASH pathogenesis remains controversial due to the discrepancy in the data obtained from different NASH models. The decrease of Tregs levels, in HFD-induced NASH models combined with genetic deficiency of CD80/CD86, was associated with aggravation of inflammation in AT and liver[566]. Moreover, expanding Tregs in the leptin-deficient NAFLD model, through treatment with anti-CD3 antibodies and β-glucosylceramide, reduced hepatic steatosis and transaminases levels, but did not affect the inflammation[569]. In line with this, neutralizing CD25 worsened NAFLD related-liver injury[558], while adaptive transfer of Tregs ameliorated NASH *in vivo*[559]. These data may suggest a role for Tregs in mitigating NASH progression. However, detrimental effects of Tregs have also been reported in NASH. For example, adaptive transfer of Tregs or anti-CD3 therapies exacerbated liver injury and hepatic steatosis without affecting steatohepatitis in two different HFD induced-NAFLD models[567, 570]. Interestingly, a recent study supporting the detrimental function of Tregs in a murine NASH-HCC model (CD-HFD/diethylnitrosamine injection), showed that depleting Tregs was capable of inhibiting HCC development and preventing NASH progression[411]. Taken together, these counterintuitive findings could be due to the different NASH models utilized in these studies. In addition, the role of Tregs may possibly differ at various stages of NASH progression (early vs late phases). Also, some data point towards an antifibrotic activity of Tregs due to the secretion of the antifibrotic cytokine IL-10[571], however the role of Tregs in modulating hepatic fibrosis in the context of NASH is largely unexplored. Therefore, further studies are needed to deeply dissect the role of Tregs during NASH.

7. IL-22

IL-22 was discovered in 2000, and it was originally known as IL-10-related T cell-derived inducible factor (IL-TIF) as IL-22 is a novel member of the IL-10 cytokine family, which includes other cytokines such as IL-19, IL-20, IL-24 and IL-26[92, 572]. The human gene of IL-22 is located on chromosome 12q15, encoding a protein of 179 amino acids in length. This protein shares almost 80% homology between mice and human[573]. IL-22 interacts with its receptor (IL-22R) to mediate its signaling[92]. IL22R is a heterodimeric receptor, composed of two subunits IL-22RA1 and IL-10RB2 (Figure 16)[573, 574]. Intriguingly, the IL-22RA1 subunit is mainly expressed by epithelial cells as well as fibroblasts and liver stem/progenitors (LPCs). Thus, unlike other cytokines, the effect(s) of IL-22 is mainly restricted to epithelial cells[575].

The IL-22-IL-22R interaction starts by IL-22 binding to the IL-22RA1 subunit, resulting in conformational changes in the ligand, which enable its binding to the IL-10RB2 subunit and subsequently propagating downstream signalling[576]. IL-22 primarily signals through STAT3, though its activation of STAT1 and STAT5 has also been reported[577]. Following formation of the IL-22-IL-22R complex, phosphorylation of Janus kinase 1(JAK1) and tyrosine kinase 2 (TYK2) is undertaken. Subsequently, JAK induces phosphorylation of IL-22R-asccoiated STAT3 molecules, leading to translocation of STAT3 into the nucleus and regulating expression of target genes such as *Bcl2* [92, 577]. The downstream target genes of IL-22 encode different proteins important for innate immune defense against invading pathogens, including anti-microbial peptides (AMPs), acute phase proteins, antiapoptotic molecules, proinflammatory chemokines and mediators[5, 92]. Apart from the JAK-STAT3 signalling pathway, activation of MAPK, PI3K and AKT–induced mTOR pathways have been also detected in response to IL-22 stimulation (Figure 16)[577, 578].

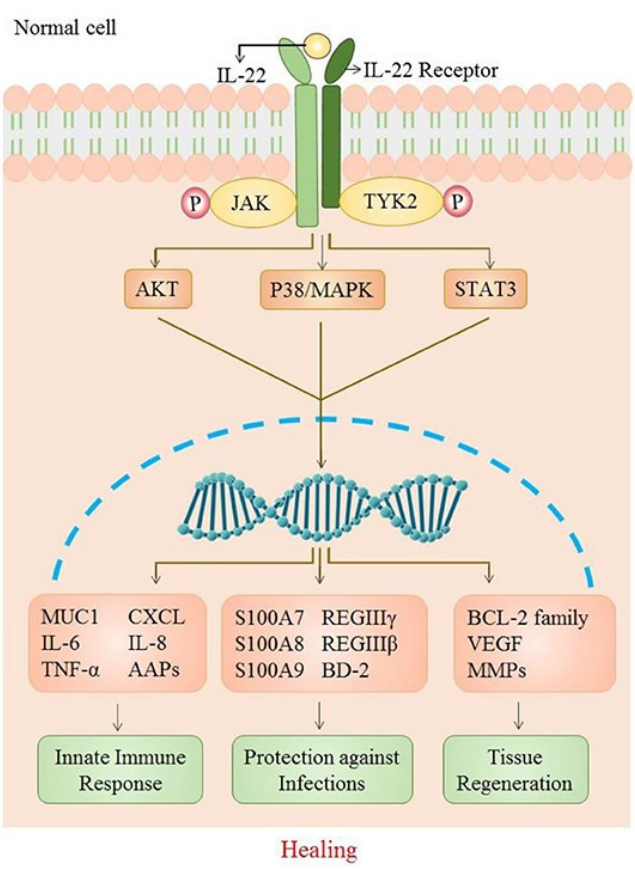

Figure 16. IL-22/IL-22R1 signaling pathway
When IL-22 binds to its receptor, the interaction activates different signaling pathways, including STAT3, MAPK, PI3K and AKT, promoting upregulation of many downstream target genes as indicated. These genes encode different proteins that are essential for enhancing innate immune response against invading pathogens and tissue regeneration. This figure is taken from [579] (Reproduced with permission)

IL-22 is mainly produced by adaptive and innate immune cells, including Th17, Th22, $CD8^+$ T cells (Tc22), γδ-T, NKT, ILC3s, neutrophils and macrophages (Figure 17) [92]. Unlike Th17, Th22 can produce IL-22 and lacks secretion of IL-17 or IFN-γ[580]. IL-22 is expressed in different organs, including the liver, GI, skin, lungs, and brain[92]. The anatomical location and the inflammatory environment are key determinants of major IL-22 producing cells. For example, in a naïve IL-22 reporter mice, ILC3s and $CD4^+$ T cells were the major source of IL-22 in the gut and mesenteric lymph nodes (mLN), respectively, while γδT cells were the main source in the skin and the lungs.

However, in the liver of this reporter mice, IL-22-producing cells were undetected [581]. On the other hand, $CD4^+$ T cells were the predominant source of IL-22 in bleomycin-induced lung inflammation[582]. However, in the ischemia reperfusion liver injury model, $ROR\gamma t^+$ $NKp46^+$ ILC3s were the cells to mainly produce IL-22. These findings may highlight the importance of regulatory pathways that control IL-22 production from these immune cells.

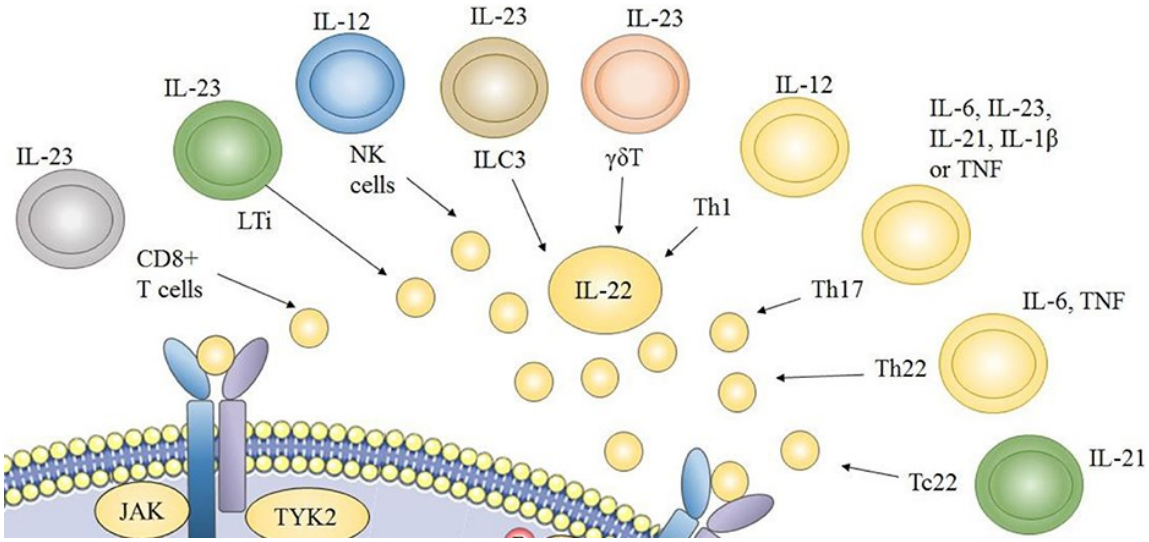

Figure 17. Schematic diagram showing the innate and adaptive IL-22-producing cells
This figure is adapted from [579] (Reproduced with permission)

7.1. Positive Regulation of IL-22 production

7.1.1. Cytokines

IL-23 is the primary inducer of IL-22 by Th17, γδT, ILC3s, and NKT cells in different experimental models (Figure 17), most likely due to the surface expression of IL-23 receptor (IL-23R) on these cells[103, 583-586]. Likewise, IL-1β induces IL-22 production from Th17, ILC3s and NKT cells[587, 588]. The main source of IL-23 *in vivo* is DCs [589], while IL-1β is expressed by neutrophils, macrophages, T cells as well as epithelial cells[92].

7.1.2. Transcription factors

AhR, RORγt, STAT3, and Notch are essential transcriptional factors that regulate IL-22 expression. AhR either directly regulates expression of the IL-22 gene or acts as a regulator of Th17 and ILC3s development[590, 591]. In homeostasis, the inactive form of AhR resides in the cytoplasm as a complex with heat shock protein 90 (Hsp90)[92]. There are various natural Ahr ligands, either from diet or microbiome, such as cyclic AMP or physical shear stress that can activate AhR[592]. Upon activation, the Ahr complex translocate to the nucleus, where Ahr regulate target genes, including IL-22[92]. Triggering AhR and/or STAT3 pathways mediates the IL-6- or TNF-α-induced secretion of IL-22 from Th22 cells[580], while IL-6 and IL-23 drive IL-22 production by Th17 by inducing expression of RORγt[454].

On the other hand, Notch signalling regulates IL-22 expression through several mechanisms. Notch signalling induces differentiation and development of IL-22-producing cells of lymphoid origin[593]. Also, Notch signalling upregulates Hes1, a Notch target gene, that promotes IL-22-induced STAT3 in human epithelial cells. This suggests that Notch signalling not only regulates IL-22 expression but also affects IL-22 targets[594]. Other studies have shown that the Notch signalling can directly influence expression of RORγt, Ahr and STAT3 promoting ILC3s and Th17 development[595-597].

7.2. Negative regulation of IL-22

7.2.1. Cytokines

TGF-β, especially at high concentrations, inhibits Th17 differentiation and reduces IL-22 production, most likely due to downregulating of IL-23R and promoting Treg differentiation[598]. Alternatively, TGF-β can directly inhibit IL-22 production in a dose-dependent manner, likely by inducing transcriptional factor c-Maf which prevents IL-22 transcription. However, this effect is overcome by IL-23 stimulation, leading to enhanced IL-22 production, suggesting that TGF-β-

mediated IL-22 inhibition is reversible [103, 598, 599]. Apart from TGFβ, HIF-1α may influence putative hypoxic response elements in IL-22 promoter and hence block IL-22 expression[600]. Other cytokines, including IL-25, IL-27, and IL-38 that were shown to suppress IL-22 production, though underlying mechanisms remain not clear[92].

7.2.2. IL-22 binding protein (IL-22BP)

IL-22 is the only member of the IL-10 family that has a soluble binding protein, known as IL-22BP or IL-22RA2, to fine-tune IL-22 activity. IL-22BP is a soluble decoy receptor that specifically blocks IL-22 interaction with IL-22RA1 (Figure 18)[601]. This has been shown in several *in vitro* and *in vivo* studies[523, 601-604]. The affinity of IL-22BP for IL-22 binding is higher (1000-fold) than that of IL-22RA1[604, 605], suggesting a tight regulation of IL-22 activity, which is important in the context of homeostasis or disease. In humans, three isoforms of IL-22BP have been reported, while one isoform was reported in mice[606, 607]. IL-22BP is expressed in different epithelial tissues, including the liver and the GI, as well as the lymphatic organs [92]. DCs, eosinophils and $CD4^+$ T cells are the main producers of IL-22BP[603, 608, 609]. Despite the potency of IL-22BP neutralizing activity, the level of IL-22BP expression does not often mirror the level of IL-22 expression. For instance, in models of colitis or LPS administration, the level of IL-22BP decreases when IL-22 level significantly increases[610]. However, the persistence of high IL-22 levels at later periods, can increase IL-22BP substantially and hence inhibit IL-22 activity, suggesting that IL-22BP plays its regulatory role after the early effects of IL-22 have been established[610, 611]. In addition, this process is essential to tightly regulate IL-22 signalling and allow tissue regeneration. Notably, the absence of IL-22BP *in vivo* is associated with dysregulated IL-22 signalling and abnormal wound healing response, leading to tumor development[611].

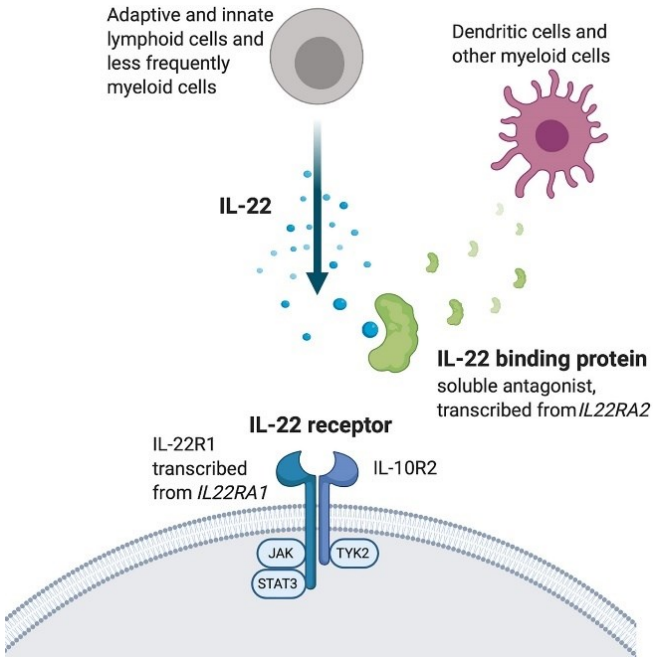

Figure 18. IL-22 binding protein (IL-22BP) acting as an antagonist of IL-22 cytokine and inhibiting IL-22/IL22R1 signaling
Dendritic cells and other myeloid cells produce IL-22BP. This figure is taken from [612] (Reproduced with permission)

7.3. Physiological effects of IL-22

In general, IL-22/IL-22RA1 has been implicated in regulating host defense against invading pathogens, tissue homeostasis and inflammation. Here, and in order to understand the role of IL-22 in acute and chronic liver injury, we first discuss the key physiological effects of IL-22 on epithelial cells, fibroblasts, and stem cells.

Broadly, IL-22 acts on epithelial cells to enhance innate defense mechanisms against invading pathogens, promoting proliferation and tissue regeneration, supressing cellular differentiation, and inducing production of certain chemokines and acute mediators (Figure 14) [613]. It has been shown that IL-22 induces AMPs such as β-defensin 2, S100A7/8/9, regenerating islet derived protein family (REG3β and REG3γ), mucus-associated proteins (MUC1 and MUC3), and LCN2 in various tissues including keratinocytes, the liver, gut, and lungs[578, 586, 614-618]. The anti-microbial

function of IL-22 induced limited replication and the dissemination of *Klebsiella pneumoniae* and in the liver and the lungs [614, 617].

In addition, IL-22 induced-STAT3 promotes proliferation of epithelial cells in several organs, including the liver. Mechanistically, IL-22 inhibits cell apoptosis through inducing the anti-apoptotic molecules (BCL-2, B-cell lymphoma-extra large (BCL-XL), myeloid cell leukaemia sequence 1 (MCL1)) in hepatocytes, which enhance cell survival and protect the tissue against hepatitis[92, 102, 619, 620]. In line with this, similar observations were reported in dextran-induced colitis and hypersensitivity pneumonitis models, where IL-22 deficiency worsened the inflammation-induced tissue injury in the colon [615]and exacerbated fibrosis progression in the lungs[621].

Despite that IL-22RA1 is restricted to epithelial cells, IL-22 can still modulate the recruitment of immune cells. For instance, IL-22 induces neutrophil attraction in the skin via CXCL1/2/5/8 chemokines, while it inhibits Th17 or Th2 recruitment via CCL17 and CCL22 in the lung and skin, respectively[613, 614, 622]. In the liver, IL-22 increases acute-phase proteins such as CXCL-1, serum amyloid A (SAA), haptoglobin, α1-antichymotrypsin and LPS binding protein[573, 610, 623, 624].

Moreover, IL-22 acts on fibroblasts and stem cells in different organs. In the liver, the modulation of HSCs by IL-22 remains controversial as IL-22 may inhibit or activate HSCs[625-627]. However, in colonic myofibroblasts, IL-22 promotes expression of several inflammatory mediators such as CXCL1/6/8, IL-6 and NF-κB, leading to enhanced tissue remodelling response[628]. The proliferative effects of IL-22 on fibroblasts in different organs, including liver, during acute injury are yet to be determined[613]. On the contrary, IL-22 has been shown to promote proliferation of LPCs and intestinal stem cells (ISCs) in a STAT3-dependent mechanism, promoting tissue regeneration response in both acute and chronic injuries [629-631]. Nevertheless, these effects of IL-22 on stem cells remain an area of debate, especially in the intestine, because other reports showed inhibition of ISCs proliferation by IL-22 via activating Wnt and Notch pathways. Therefore, further studies are needed to reconcile these contradicting findings[632, 633].

According to what is mentioned above, there is extensive evidence highlighting that IL-22 is a tissue protective cytokine important for shielding epithelial barriers and promoting tissue regeneration. However, these protective functions of IL-22 have been challenged by several studies showing that IL-22 could play a pathogenic/proinflammatory role[5]. This could be due to the induction of IL-22 to several proinflammatory chemokines and cytokines in epithelial cells or fibroblasts, promoting local or systemic inflammation. For example, in psoriasis-like inflammation models, IL-22-producing Th17 induced dermal inflammation by enhancing proinflammatory neutrophil recruitment in a STAT3-dependent manner [103, 634]. In addition, the inflammatory milieu and the co-expression of other cytokines could drive the IL-22-mediated pathogenic actions. For instance, in the absence of IL-17, IL-22 ameliorated bleomycin-induced lung injury and promoted tissue repair by inducing anti-apoptotic signals. However, in presence of IL-17, IL-22 exacerbated the airway inflammation via promoting neutrophil-driven inflammation[582]. Taken together, these findings may indicate a paradox because of tissue protective and pro-inflammatory effects of IL-22. Alternatively, this could suggest opposite effects of IL-22, which is generally context-dependent and likely dictated by multiple factors including the tissue involved, pathological environment, presence of other cytokines, endogenous versus exogenous IL-22 levels, IL-22BP, and the time of exposure[5, 472, 523, 601, 613, 635-638]. These opposite effects of IL-22 have been reported in acute or chronic liver injury, which are discussed below.

7.4. Role of IL-22 in acute liver injury

As previously mentioned, following acute insult, the healing response consists of 3 phases: inflammation, proliferation/repair, and tissue remodeling[5, 131]. During the inflammation phase, IL-22 plays a hepatoprotective role by inducing expression of glutathione and anti-apoptotic molecules (BCL2, BCL-XL) in hepatocytes, inhibiting hepatocyte death and reducing oxidative stress[102, 619, 620, 639-641] (Figure 17). Recently, another evidence proposed that IL-22 activated autophagy and inhibited apoptosis in hepatocytes in the LPS-induced acute liver injury model,

likely by upregulating ATG4 and ATG7[642]. These protective effects of IL-22 have been evident in different *in vivo* models, including concanavalin A (ConA)- induced T cell hepatitis[102, 619, 641], acute alcoholic hepatitis (ASH) [639], CCl_4 or acetaminophen (APAP)-induced acute hepatitis[620, 643, 644], ischemia reperfusion-induced acute hepatitis[636, 645, 646], and bacteria, parasite and viral-induced acute hepatitis[640, 642, 647, 648].

Moreover, IL-22 contributes to the proliferation/regeneration phase by promoting proliferation of both hepatocytes and liver LPCs through STAT3-induced cyclin D and c-myc expression (Figure 19) [102, 619, 631, 641]. These regenerative effects of IL-22 were directly implicated in the proliferation of hepatocytes in mice undergoing partial hepatectomy and highly contributing to their accelerated recovery [475, 641, 649-651]. Interestingly, similar effects of IL-22 were observed in patients who underwent major liver resections[651].

The role of IL-22 in the tissue remodeling phase of acute liver injury remains not clear. Nevertheless, IL-22 upregulates MMP-1 expression in keratinocytes in a p38- and STAT3-dependent manner, leading to degradation of ECM[578]. As discussed earlier, in this phase, active HSCs are either subjected to apoptosis or revert to their quiescent state[5]. The effect of IL-22 on HSCs is still in dispute as IL-22 may inhibit [625] or activate [626] [523] or even promote senescence of HSCs[635] (Figure 19). In line with the debatable effect of IL-22, an unrecognized role of the gut liver axis in modulating IL-22 immune response during the late phase of acute liver injury has been reported. Nakamoto et al demonstrated that IL-22-producing ILC3s were activated via Lactobacillus species in the gut during the recovery phase of ConA-induced liver injury model. Interestingly, this was associated with restoring damaged intestinal barriers and promoting recruitment of cDCs to the liver, which in turn produced IL-10 and TGF-β that ameliorated liver inflammation upon rechallenge with ConA[652]. However, the precise mechanism beyond such emergence of tolerogenic cDCs to the liver via IL-22 remains undefined. Taken together, IL-22

may play a crucial role in inducing mediators that enhance tissue remodelling post acute injury and re-stablishing liver homeostasis, though the exact mechanisms remain to be elucidated.

In spite the well-established evidence of hepatoprotective role of IL-22 in acute liver injury, several studies have challenged this effect and reported a pathogenic/pro-inflammatory role of IL-22. For example, in the HBV-transgenic T cell adoptive transfer model, neutralizing IL-22 significantly reduced recruitment of pro-inflammatory immune cells and ameliorated acute liver injury[624]. Similarly, using a rat allogeneic liver transplantation model, IL-22 was hepatoprotective during the ischemia-reperfusion injury stage of liver transplantation (day one), however it was detrimental during the acute rejection stage (day 7). This detrimental effect of IL-22 was mediated by upregulating the expression of pro-inflammatory cytokines and chemokines and promoting the Th17/Tregs imbalance, leading to tissue injury exacerbation[636]. In line with this, a proinflammatory role of IL-22 has also been evident in ischemia-reperfusion and APAP-induced liver injury models using IL-22BP-deficient mice (IL-22BP$^{-/-}$). The lack of IL-22BP in these models resulted in uncontrolled regulation of IL-22 signalling and increased death of hepatocytes as well as an upregulated expression of their CXCL-10 promoting infiltration of inflammatory monocytes to liver tissue[602]. In summary, opposite effects of IL-22 have been reported in acute liver injury, which may depend on the specific context of inflammation, time of exposure, the activated signaling pathways in target cells (pro-survival/regeneration vs. pro-inflammatory) and regulation by IL-22BP.

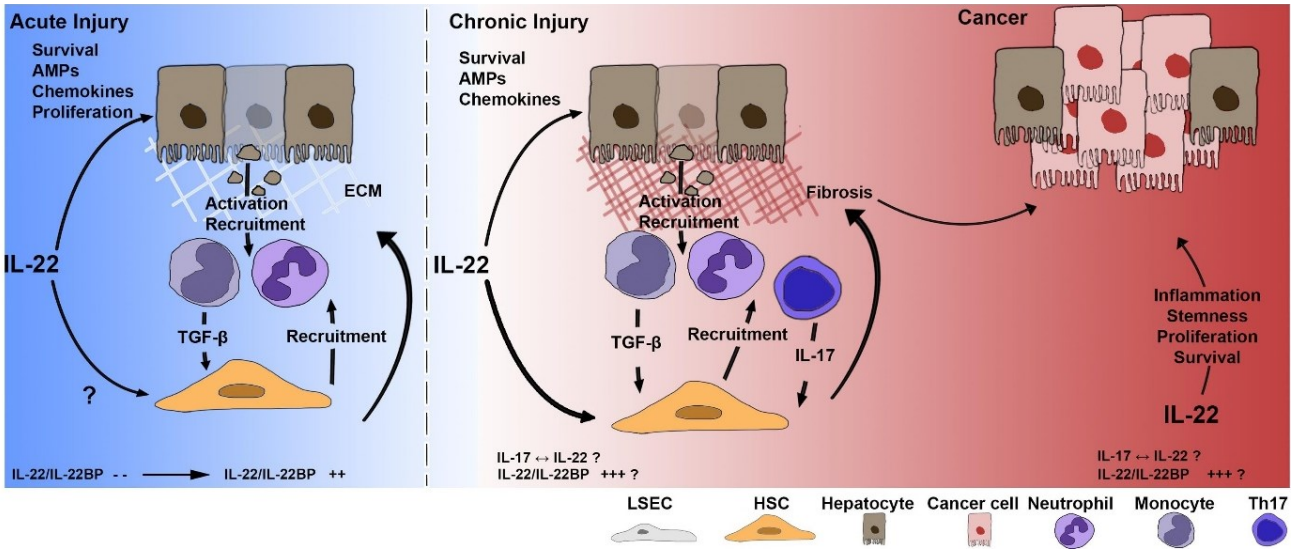

Figure 19. Role of IL-22 in acute and chronic liver injury

IL-22 mediates protective effects against acute liver injury by promoting prosurvival and proliferative signals of hepatocytes through inducing expression of mitogenic and antiapoptotic proteins. Also, IL-22 enhance expression of antimicrobial peptides (AMPs) and acute phase proteins to promote innate defense mechanisms against invading pathogens. The effect of IL-22 on HSC during acute liver injury remain not clear as IL-22 may inhibit or activate HSCs activation. On the other hand, in chronic liver injury, the IL-22 signaling is dysregulated and hepatoprotective effects of IL-22 are likely counterbalanced by proinflammatory effects of IL-22 through inducing recruitment of proinflammatory Th17, which promotes liver fibrosis and carcinogenesis. However, the role of IL-22 in chronic liver injury is context-dependent as opposite effects have been reported in several studies. IL-22BP regulates IL-22 biological activity during acute injury to limit detrimental effects of IL-22 and promote tissue repair. Conversely, the IL-22/IL-22BP ratio in chronic liver injury is likely dysregulated and may enhance proinflammatory effects of IL-22 signaling. Nevertheless, the role of IL-22BP is largely unknown in different pathologies of chronic liver injury, and further studies are needed to dissect its role in the context of CLD. This Figure is taken from [5] (Reproduced with permission).

7.5. Role of IL-22 in chronic liver injury

The IL-22/IL-22RA1 axis is generally dysregulated during chronic inflammation and this has been associated with the pathogenesis of several autoimmune diseases or carcinogenesis, which could be either due to the proinflammatory and proliferative potentials of IL-22 itself or to the presence of other proinflammatory cytokines (e.g., IL-17) that may drive the detrimental effect of IL-22[92]. However, like acute liver injury, opposite effects of IL-22 have been also observed in chronic liver

injury, especially viral hepatitis (Figure 19). The role of IL-22 has been investigated in CLDs including viral hepatitis, alcoholic steatohepatitis (ASH), NASH and liver fibrosis.

7.5.1. Viral hepatitis

IL-22 expression is upregulated in livers of patients with chronic HBV or HCV, where accumulation of Th17 cells in these livers was considered as the major source of IL-22[490, 624, 626, 641, 653]. This high level of IL-22 inhibited neither HBV nor HCV replication in hepatocytes[624, 654]. However, it may suggest an essential role of IL-22 in liver disease progression. Indeed, hepatoprotective effects of IL-22 have been observed in CHB and CHC. IL-22 promoted proliferation of LPCs and induced hepatocyte survival in mice and patients with CHB infection[631]. In line with this, intrahepatic IL-22 expression was inversely correlated with liver fibrosis and inflammation in CHB patients[655]. In HCV, the intrahepatic enrichment of IL-22$^+$ cells and IL-22$^+$IFNγ$^+$ CD4$^+$ T cells was not associated with fibrosis stage[653]. In addition, Sertorio et al observed an increase in genetic variants of the IL-22BP in CHC patients that was associated with severe fibrosis, suggesting a hepatoprotective role of IL-22, possibly by mediating tissue repair and regenerative mechanisms[656]. On the other hand, detrimental effects of IL-22 have been equally reported in chronic HBV and HCV infection. The increase of IL-22 levels in the liver of CHB and CHC patients was positively associated with the inflammation and severity of liver fibrosis [490, 523, 626, 641]. This detrimental effect was possibly dependent on promoting recruitment of proinflammatory Th17 into the liver and inducing activation and proliferation of HSCs [490, 626].

In summary, these inconsistent findings in both CHB and CHC infection may reflect a difference in the patient cohorts studied or the context-dependent role of IL-22. Thus, further investigation is necessary to clarify role of IL-22 in chronic HBV and HCV.

7.5.1. ASH

Unlike viral hepatitis, IL-22 seems to play a hepatoprotective role in ASH. An increase in circulating IL-22-producing $CD4^+$ T cells, including Th17 cells, was observed in ASH patients, which was associated with a better short-term prognosis[657]. In line with this, *in vitro and in vivo* work supported a protective function of IL-22 against ethanol-induced chronic liver injury, which was dependent on STAT3-induced metallothionein 2 (MT2) and LCN2, leading to decreased hepatic apoptosis[658, 659]. Another study reported a decrease in IL-22 production by the gut ILC3s in an ethanol-induced chronic liver injury model, leading to impaired REG3G and promoting gut dysbiosis. Interestingly, treating this model with engineered bacteria producing IL-22 ameliorated gut dysbiosis and reduced liver inflammation, highlighting the importance of IL-22 protective effects against gut dysbiosis during chronic ASH[660]. Despite this, low levels of serum IL-22BP was positively correlated with poor survival in ASH patients with advanced fibrosis, suggesting a pathogenic effect of IL-22[661]. Nevertheless, hepatic IL-22 transcripts were not detected in these patients, adding uncertainty to the role of IL-22 in the liver. Overall, accumulating evidence points toward hepatoprotective effects of IL-22 against chronic ASH, though more studies are demanded to determine role of the IL-22/IL-22BP axis in ASH.

7.5.2. NAFLD

Up to date, most studies reported protective effects of IL-22 in NAFLD. Of note, the protective effects of IL-22 can be generally classified into two main categories: anti-metabolic syndrome and anti-liver injury. Many diet-induced NAFLD models have extensively used to investigate beneficial metabolic effects of IL-22[515, 662-666]. It has been shown that IL-22 decreased body weight, ameliorated glucose intolerance, improved insulin sensitivity, and reduced adiposity and hepatic TGs level in these models[662-666]. Moreover, this was associated with suppression in expression of lipogenic genes such as FAS and elongation of long chain fatty acids member 6 (ELOVL6), which was mediated by JAK1/STAT3 signaling pathways. However, such beneficial effects were

observed in the presence of high levels of exogenous IL-22 (e.g., IL-22Fc) [662-666]. On the other hand, few studies investigated the role of endogenous IL-22 against metabolic abnormalities in HFD-induced NAFLD models, which does not seem to play a significant role, most likely due to very low levels of IL-22 in the circulation and the livers of HFD-fed mice compared to controls[663, 664, 667].

Other beneficial effects of IL-22 against liver injury have been reported in different NASH models[515, 668, 669]. IL-22Fc ameliorated neutrophil-induced oxidative stress via STAT-3-mediated induction of MT1 and MT2 antioxidant proteins and subsequently reduced NASH-related inflammation and fibrosis [668]. In addition, Zai et al [665] demonstrated that liver-target delivery of IL-22 gene in HFD-induced NASH model, where IL-22 activated STAT3-induced Bcl-2 and Nrf2-induced Sod1 pathways, promoted hepatocyte survival and proliferation. However, these effects were observed with high levels of exogenously administered IL-22 (e.g., IL-22Fc)[668]. Studies investigating the role of endogenous IL-22 are limited. Rolla et al[515] demonstrated that endogenous IL-22 produced by Th22 cells antagonised development of inflammation and fibrosis progression in MCD-induced NASH model, but only in the absence of IL-17 (IL-17$^{-/-}$ mice). However, this model lacks metabolic abnormalities associated with NAFLD and does not completely recapitulate human NASH. Moreover, IL-22 producing ILC3s were markedly elevated in a HFD-induced NASH model [669]. The genetic deficiency of ILC3s (RORγt $^{gfp/gfp}$ mice) was associated with higher hepatic steatosis levels, infiltration of the inflammatory macrophages (M1) and worsening of fibrosis progression compared to controls (RORγt $^{gfp/wt}$ mice)[669]. However, the control mice developed NAFL, but not NASH[669].

Overall, there is accumulating evidence pointing toward several beneficial effects of IL-22 in *in vivo* NAFLD models, which is not only protective against metabolic syndrome associated with NAFLD but also against progression of liver fibrosis. Nevertheless, all these studies investigating IL-22 in NASH-related liver injury have certain limitations as mentioned above, rendering the

evidence of a beneficial role of IL-22 in NASH inconclusive and further investigation is warranted. Finally, these studies were limited to male mice and thus data regarding the sex-based immunological difference between males and females in the context of NAFLD remain elusive.

7.5.3. IL-22Fc is a therapeutic potential for treating NASH

Given the potential hepatoprotective effects of IL-22 against various types of liver injury, some clinical studies utilized a recombinant fusion protein of the human IL-22 dimer (IL-22Fc) to evaluate therapeutic application of IL-22 in humans. IL-22Fc had a well tolerability and safety in two phase 1 clinical trials[670, 671]. Although IL-22Fc administration increased the serum level of IL-22 up to 2000 ng/ml (1000 times > IL-22 level in patients with liver cirrhosis) in healthy subjects, the IL-22Fc induced acute phase proteins and the side effects were minimal. These data may suggest a pharmacological benefit of IL-22Fc by suppressing the pathological effects of elevated endogenous IL-22. In addition, in a phase 2a open-label study for moderate to severe ASH patients, IL-22Fc decreased inflammatory markers along with amelioration in the clinical scores such as end-stage liver disease (MELD) score[672]. Moreover, emerging evidence has identified an unrecognized function of IL-22Fc as a mitochondrial protector against liver injury[673, 674]. Interestingly, IL-22 restored metabolic programming (oxidative phosphorylation and glycolysis) and induced autophagy of damaged mitochondria caused by either HFD or acetaminophen-induced liver injury, respectively[675, 676]. This protective effect of IL-22 was mediated by either STAT-3- induced mTOR and AKT activation or STAT-3-activating AMPK pathway, resulting in reduced ROS production and maintaining mitochondrial integrity[675, 676]. Overall, these promising results possibly predict IL-22 therapy to effectively ameliorate NASH progression with minimal side effects, though clinical studies are still lacking.

7.5.4. Liver fibrosis

IL-22 was reported to inhibit activation of HSCs or induce their senescence in a STAT3-dependent manner, leading to decreased HSCs proliferation and hence reduced liver fibrosis. This anti-fibrotic role of IL-22 was observed in several *in vivo* studies using the chronic CCl_4 model[472, 625, 635, 677]. In contrast, other studies reported pro-fibrogenic functions of IL-22. Fabre et al showed that IL-22 promoted hepatic fibrosis by enhancing TGF-β signalling in primary human HSCs via a p38/MAPK–dependent manner. This finding was validated in the chronic CCl_4 model, where IL-22RA1 KO mice had less hepatic fibrosis compared to their WT littermates[523]. In line with this, *in vitro* stimulation of HSCs with IL-22 promoted the proliferation and reduced senescence of HSCs[626]. In addition, systemic IL-22 levels were enriched in a large cohort of patients with liver cirrhosis irrespective of etiology, which was associated with poor prognosis[678]. However, it remains uncertain whether circulating IL-22 levels matched IL-22 levels in these patients' liver.

Altogether, it is too early to conclude on the role of IL-22 in fibrosis progression in CLDs because the increased IL-22 in advanced fibrosis could be explained either as playing an active pathogenic role or could be attempting to repair the exacerbated liver injury that goes along with fibrosis progression. Even though there are important differences between HSC cell lines and the *in vivo* models studied so far, the opposite effects of IL-22 observed may indicate the context-dependent effect of IL-22. Also, the role of IL-22/IL-22BP is largely unexplored in liver fibrosis of CLD. Further studies are warranted, along with better animal models, to identify the pathogenic drivers that shift the balance from IL-22-mediated protection to pathogenesis.

Chapter 2:

Hypothesis and Objectives

Rationale and Hypothesis

Over the past decades, several lines of evidence have demonstrated the involvement of chronic inflammation in the progression of liver fibrosis in different chronic liver diseases, including NASH. Sex differences in NAFLD pathogenesis are reported but the underlying mechanisms remain understudied. Also, sexual dimorphism in immune responses as well as liver tissue have been observed in different chronic liver diseases including NAFLD. Although type 3 inflammation, particularly Th17 cytokines (IL-22 and IL-17A), has been reported in many chronic inflammatory disorders and autoimmunity, the role of these cytokines remains unclear in the context of NAFLD. IL-22 is a pleiotropic cytokine that can be both protective and/or pathogenic during liver injury and inflammation. IL-22 was shown to be hepatoprotective in NAFLD-related liver injury by inducing antioxidant proteins such as MT2 and SOD1 in hepatocytes and hence ameliorates NASH and fibrosis progression. Also, IL-22 mediated protective effects against the metabolic alterations associated with NAFLD, including weight gain, IR, and adiposity. Studies in this matter relied primarily on exogenous administration of IL-22 without examining the role of endogenous IL-22 as well as the cellular sources of IL-22 in NAFLD-related fibrosis. In addition, these studies were limited to male mice and the data regarding the sex-based difference for hepatic IL-22 expression remain unknown. On the other hand, IL-17A was shown to be pathogenic and to mediate the progression of liver fibrosis in NAFLD by inducing recruitment of inflammatory neutrophils and/or activating HSCs that promote hepatic injury and progression of liver fibrosis. Despite the importance of such effect for IL-17A, it has been only reported in Th17 cells and the role of other cellular sources such as neutrophils has not yet been investigated in this context.

We hypothesized that endogenous IL-22 plays an important role in NAFLD-related fibrosis, this role maybe mediated through IL-22 anti-inflammatory and anti-apoptotic function(s) that may promote hepatocyte survival and delay NAFLD related-fibrosis, while IL-17A may have more pro-

inflammatory effects by mediating recruitment and activation of inflammatory immune cells thus leading to enhanced fibrosis in this setting.

Objectives

In order to verify our hypothesis, we designed the following objectives:

1- To evaluate the IL-22 signature in human NAFLD, using clinical liver biopsies and explore whether this signature correlates with the stage of liver fibrosis. In parallel, to validate and characterize this role in mouse models of NAFLD using IL-22RA knock out (IL-22ra1$^{-/-}$) mice. Furthermore, to evaluate the role of sex in IL-22 response during NAFLD in humans and mice

2- To investigate the kinetics and functional mechanisms of IL-17A-producing cells during progression of NAFLD-related fibrosis *in vivo* using HFD-fed WT mice and to establish a correlation with the degree of liver fibrosis. Specifically, to understand the role of IL-17A induced neutrophil recruitment and IL-17A-induced NETs during liver fibrosis.

Chapter 3:

Manuscript 1

Title: Sex-Dependent Hepatoprotective Role of IL-22 Receptor Signaling in Non-Alcoholic Fatty Liver Disease-Related Fibrosis

(Cellular and Molecular Gastroenterology and Hepatology, PMID: 35970323[679])

Sex-Dependent Hepatoprotective Role of IL-22 Receptor Signaling in Non-Alcoholic Fatty Liver Disease-Related Fibrosis

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Authors' contributions:
MNA: Study concept and design; acquisition of data; analysis and interpretation of data; drafting of the manuscript; MFM, NB: Acquisition of data and recruitment of study subjects; GS: Pathological evaluation of mouse liver tissue; VQ-H: Pathological evaluation of human liver tissue, SM: Analysis of publicly available microarray datasets, NJ, JD, ST: Technical support for animal experiments; MB: Coordinated and supervised recruitment of human subjects and provided valuable input on all aspects of the study; JLE: designed experiments and provided valuable input on metabolic aspects of the study; NHS: Study concept and design, obtained funding, supervised the overall study and co-wrote the manuscript. All authors had the opportunity to review the manuscript and provide additional intellectual input.

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Study sponsors had no role in the study design in the collection, analysis, and interpretation of data.

Abstract

Background & Aims: Non-alcoholic fatty liver disease (NAFLD) is a major health problem with complex pathogenesis. Although sex difference in NAFLD pathogenesis has been reported, the mechanisms underlying such difference remain understudied. IL-22 is a pleiotropic cytokine with both protective and/or pathogenic effects during liver injury. IL-22 was shown to be hepatoprotective in NAFLD-related liver injury. However, these studies relied primarily on exogenous administration of IL-22 and did not examine the sex-dependent effect of IL-22. Here, we sought to characterize the role of endogenous IL-22 receptor signaling during NAFLD-induced liver injury in males and females.

Methods: We used immunofluorescence (IF), flow cytometry, histopathological assessment and gene expression analysis to examine IL-22 production and characterize the intrahepatic immune landscape in human subjects with NAFLD (n=20; 11 males and 9 females) and in an *in vivo* western high fat diet-induced NAFLD model in IL-22RA knock out (IL-22ra1$^{-/-}$) mice and their wild type (WT) littermates.

Results: Examination of publicly available datasets from two cohorts with NAFLD demonstrated increased hepatic IL-22 gene expression in females as compared to males. Furthermore, our IF analysis of liver sections from NAFLD subjects (n=20) demonstrated increased infiltration of IL-22 producing cells in females. Similarly, IL-22 producing cells were increased in WT female mice with NAFLD and the hepatic IL-22/IL-22BP mRNA ratio correlated with expression of anti-apoptosis genes. The lack of endogenous IL-22 receptor signaling (IL-22ra1$^{-/-}$) led to exacerbated liver damage, inflammation, apoptosis and liver fibrosis in female but not male mice with NAFLD.

Conclusion: Our data suggest a sex-dependent hepatoprotective antiapoptotic effect of IL-22 receptor signaling during NAFLD-related liver injury in females

Introduction

Non-alcoholic fatty liver disease (NAFLD) has become the most prevalent chronic liver disease due to the rise in obesity, insulin resistance (IR) and type 2 diabetes mellitus (T2DM)[2, 680]. Although, the prevalence of NAFLD seems to be higher in males than females, this sex difference is likely inconclusive and could be cofounded by age[217, 244]. Sex differences in NAFLD pathogenesis are reported but the underlying mechanisms remain understudied[244, 681, 682]. NAFLD spans a wide spectrum of liver disease ranging from hepatic steatosis (HS), non-alcoholic steatohepatitis (NASH), advanced fibrosis, cirrhosis and ultimately hepatocellular carcinoma (HCC)[4, 220]. NASH-induced inflammation can enhance activation of hepatic stellate cells (HSC) thus accelerating fibrosis progression[4, 390]. Importantly, liver-related mortality increases exponentially with the increase in fibrosis stage in patients with NAFLD[3]. Currently, there are no approved therapies for NASH, but many interventional studies are ongoing[4, 220].

IL-22 is a pleiotropic cytokine with both inflammatory and protective effects during injury and repair in various tissues including the liver[92]. IL-22 is a member of the IL-10 cytokine family and is produced by multiple immune cells including Th17, Th22, Tc22 and γδ T cells, NK cells, innate lymphoid cells 3 (ILC3s), macrophages and neutrophils[92, 583, 683, 684]. The IL-22 receptor (IL22R) is composed of two subunits IL-22RA1 and IL-10RB2[92, 607]. The IL-10RB2 subunit is constitutively expressed throughout the body but expression of the IL-22RA1 subunit is mainly limited to epithelial cells, and some fibroblasts[92, 575, 628]. Thus, although IL-22 is produced by many immune cells, its effect(s) is mainly restricted to epithelial cells[92, 575].

IL-22 was reported to be hepatoprotective in various models of liver injury[102, 619, 643, 658, 668]. This effect is mediated by enhancing STAT-3 downstream signaling, including induction of the anti-apoptotic proteins (B-cell lymphoma 2 (BCL2) and B-cell lymphoma-extra large (BCL-xL)), mitogenic proteins (c-MYC and cyclin D1), and antioxidant proteins (metallothionein 2, MT2), leading to prevention of hepatocyte death and enhancing hepatocyte proliferation[102, 619, 643, 658, 668].

IL-22 also induces intrinsic antimicrobial activity in the liver through increased expression of antimicrobial peptides such as lipocalin 2 and serum amyloid A 2 (SAA2)[617]. Furthermore, IL-22 promotes liver regeneration, and was reported to induce senescence of activated HSCs and thus reduce liver fibrosis progression[472, 635, 651]. Finally, emerging evidence has identified IL-22 as mitochondrial protector against liver injury[674]. A recombinant fusion protein of human IL-22 dimer (IL-22Fc) was safe in phase 1 clinical trials[670, 671] and decreased inflammatory markers along with amelioration in model for end-stage liver disease (MELD) scores in a phase 2 trial in alcoholic steatohepatitis (ASH)[672].

IL-22 may also be proinflammatory during HBV infection and in HBV transgenic mouse models[490, 624]. We and others have demonstrated that IL-22 producing cells were significantly enriched in liver tissue samples with advanced fibrosis, in particular in patients with viral hepatitis, suggesting a profibrogenic role of IL-22 during chronic liver injury[523, 626]. Furthermore, we validated this finding *in vivo* in the carbon tetrachloride (CCl_4)- and thioacetamide (TAA)-induced chronic liver injury models, where lack of endogenous IL-22 signaling in IL-22RA1 knockout ($IL22ra1^{-/-}$) mice led to reduced hepatic fibrosis[523]. In contrast, other studies reported that exogenous IL-22 administration in the chronic CCl_4 model was hepatoprotective and resulted in fibrosis resolution[472, 635]. The profibrogenic role of IL-22 was also documented in other organs such as the pancreas[685]. These different observations highlight the dual nature of IL-22 that is likely dictated by multiple factors including the tissue involved, pathological environment, endogenous versus exogenous IL-22 level, and the time of exposure[472, 490, 523, 601, 635].

IL-22 activity is regulated by the IL-22 binding protein (IL-22BP or IL-22RA2), which is a soluble decoy IL-22 receptor that acts as an endogenous high affinity inhibitor of IL-22 [92, 607]. Indeed, a proinflammatory role of IL-22 has been observed in ischemia-reperfusion and acetaminophen-induced liver injury models using IL-22BP deficient mice ($IL\text{-}22BP^{-/-}$)[602]. The lack

of IL-22BP in these models resulted in uncontrolled regulation of IL-22 signaling and exacerbation of hepatocyte death, which was associated with increased CXCL-10 expression in hepatocytes and promoted infiltration of inflammatory monocytes to the liver[602]. In contrast, another study in subjects chronically infected with Schistosoma japonicum, reported that IL-22 transcripts were increased, while IL-22BP transcripts were reduced. This was associated with decreased hepatic fibrosis, suggesting a protective role of IL-22 in these subjects[656].

Most studies report hepatoprotective and beneficial metabolic effects of IL-22 in NAFLD. IL-22 decreased body weight, ameliorated glucose intolerance, improved insulin sensitivity, and reduced adiposity and hepatic triglycerides (TGs) level in various NAFLD models[662-665]. IL-22Fc ameliorated neutrophil-induced oxidative stress via STAT-3-mediated induction of MT1 and MT2 antioxidant proteins and subsequently reduced NASH-related inflammation and fibrosis[668]. However, these effects were observed with high levels of exogenously administered IL-22 (e.g., IL-22Fc)[668]. Studies investigating the role of endogenous IL-22 are limited. Rolla et al[515] demonstrated that endogenous IL-22 produced by Th22 cells antagonised development of inflammation and fibrosis progression in methionine-choline-deficient-diet (MCD)-induced NASH model, but only in the absence of IL-17 (IL-17$^{-/-}$ mice). However, this model lacks metabolic abnormalities associated with NAFLD and does not completely recapitulate human NASH. Finally, these studies were limited to male mice and thus data regarding the sex-based immunological difference between males and females in the context of NAFLD remain elusive.

Here, we investigated the role of endogenous IL-22 receptor signaling in NAFLD-related liver injury in both males and females using a combination of human samples and an *in vivo* mouse model using IL22ra1$^{-/-}$ mice and their wild type (WT) littermates. We report that hepatic IL-22 expression had sexually dimorphic differences in both humans and mice with NAFLD where it was elevated in females versus males. This was associated with an increase in hepatic IL-22BP

expression in female mice with NAFLD compared to males. In addition, the hepatic IL-22/IL-22BP mRNA ratio positively correlated with IL-22 downstream target genes (anti-apoptotic and antioxidant genes) in those females. Lack of endogenous IL-22 receptor signaling in female mice with NAFLD, but not males, exacerbated liver injury, inflammation, and fibrosis.

Results

Increased intrahepatic IL-22 producing cells in female subjects with NAFLD as compared to males

To evaluate the endogenous role of IL-22 in human subjects with NAFLD, we queried two publicly available microarray datasets (GSE106737 and GSE151158) and compared the hepatic IL-22 expression between females and males with NAFLD[686, 687]. We detected a marked upregulation of *Il22* mRNA expression in livers of females compared to males (**Figure 1*A* and *B***). Next, we conducted IF staining to quantify IL-22 producing cells *in situ* in liver biopsy samples from a third cohort of NALFD patients (n=20) recruited at our institution (**Table 1 and Table 2**). There was no difference in neither the NAS nor the fibrosis scores between female and male subjects (**Table 1**), indicating that both groups had comparable NAFLD severity profiles. In line with IL-22 transcriptomic data, IL-22 producing cells were significantly elevated (p=0.0002) in the livers of females compared to males (**Figure 1*C* and *E***). To identify IL-22 producing cells in female subjects with NAFLD, we performed multiplex IF staining of IL-22 and either the T cell marker ($CD3^+$) or the neutrophil marker ($CD66b^+$). Surprisingly, $CD3^+$ T cells identified *in situ* by IF did not colocalize with IL-22 (**Figure 1*F***), while the majority of IL-22 producing cells co-expressed $CD66b^+$, suggesting that they may be neutrophils (**Figure 1*G***). Nevertheless, the lack of detection of IL-22-producing T cells by IF in those females is inconclusive because we could not further characterize IL-22-producing cells by other techniques such as flow cytometry as we did not have access to fresh liver biopsies from these subjects. Overall, these results suggest a sexual dimorphic expression of IL-22 in the context of NAFLD.

Increased intrahepatic IL-22 producing cells in HFD-fed female mice as compared to males

Next, we sought to validate our observation of sexual dimorphic expression of IL-22 in an *in vivo* model of NAFLD. WT C57BL/6N mice, including males and females, were placed on either a HFD that simulates western diet, or a control diet for 30 weeks as described in Materials and Methods. To assess the hepatic infiltration of IL-22-producing cells, we quantified IL-22 cytokine/protein expression *in situ* using IF, and gene expression using qPCR. Interestingly, endogenous IL-22 expression was significantly increased at both the protein and mRNA levels in livers of WT female mice as compared to males ($p<0.001$ and $p<0.0001$, respectively) after 30 weeks on HFD **(Figure 2*A-D*)**. Since IL-23 is established as a key inducer of IL-22 production by different innate and adaptive immune cells[103, 583], we examined hepatic *Il23* gene expression. Similar to IL-22, we observed substantial upregulation of *Il23* expression in the livers of HFD-fed WT female mice compared to their male littermates (**Figure 2*E***). To identify the cellular sources of IL-22 in HFD-fed WT female mice, we performed multiplex IF staining of IL-22 and either the T cell marker ($CD3^+$) or the neutrophil marker ($Ly6G^+$). As observed in our human study, $CD3^+$ T cells identified *in situ* by IF did not colocalize with IL-22 (**Figure 2*F***), but neutrophils ($Ly6G^+$ cells) were one of the IL-22 cellular sources, though they were not the major source (**Figure 2*G***). To better characterize IL-22-producing T cells in our NAFLD model, we extracted the intrahepatic lymphocytes from HFD-fed WT female or male mice and examined their capacity to produce IL-22 by flow cytometry following stimulation with PMA/ionomycin. We observed Th17 ($IL\text{-}22^+$ $IL\text{-}17A^+$ $CD4^+$), Th22 ($IL\text{-}22^+$ $IL\text{-}17A^-$ $CD4^+$), and γδ-T cells ($IL\text{-}22^+$ $CD3^+$ $TCR\gamma\delta^+$) as the major IL-22-producing cells, and to a lesser extent, ILC3s ($CD3^-NKp46^+$) (**Figure 3*A* and *D***). Also, the IL-22 production by these cells was significantly higher in HFD-fed WT female mice compared to the controls ($p<0.05$) (**Figure 3*C-E***). Moreover, the HFD-fed WT female mice exhibited increased IL-22 production by these lymphocyte subsets as compared to the same cells in male mice (**Figure**

3*A* and *B*). This further validates the high level of hepatic IL-22 observed in female mice by IF and qPCR (**Figure 2*A, C* and *D***). Notably, we did not observe a difference in the hepatic frequency of IL-17A-producing Th17 (IL-17A^{+} IL-22^{-} CD4^{+}) between HFD-fed female mice as compared to controls (mean= 1.1 vs 0.80, p=0.5714) (**Figure 3*D* and *E***), which may suggest a low grade of NASH-related inflammation. However, the frequency of IL-22 producing Th17 (IL-22^{+} IL-17A^{+} CD4^{+}) was significantly different between these two groups (p<0.05) (**Figure 3*D* and *E*)**. Altogether, these data suggest that IL-22 expression is upregulated in livers of HFD-fed female mice compared to males with heterogenous cellular source(s) including T cells.

HFD-fed IL-22ra1$^{-/-}$ female and/or male mice developed weight gain and other metabolic alterations

Several studies have reported a protective role of IL-22 against obesity, adiposity, glucose intolerance and IR in different HFD-induced NAFLD models[662-665]. So, we first explored whether endogenous IL-22 receptor signaling protects against metabolic abnormalities associated with NAFLD in our model. IL22ra1$^{-/-}$ female mice started to exhibit significantly higher weight gain after 9 weeks (mean difference= 1.843, p= 0.025) on HFD and up to the termination point (30 weeks) (mean difference= 2.160, p= 0.004) compared to WT (**Figure 4*A***). This was associated with worsened glucose intolerance and insulin resistance at 30 weeks (**Figure 4*C* and *E***). In males, significantly higher weight gain was also observed in IL22ra1$^{-/-}$ male mice compared to WT, starting from 12 weeks (mean difference= 2.567, p= 0.019) and up to 30 weeks (mean difference= 2.956, p= 0.006) (**Figure 4*B***). Although, this was associated with increased insulin resistance at 30 weeks, there was no difference in glucose intolerance (**Figure 4*D* and *F***). Moreover, HFD-fed IL22ra1$^{-/-}$ female mice had markedly higher fat mass, liver index and hepatic steatosis as compared to WT (**Figure 5*A, E* and *G***), with no difference in their lean mass (**Figure 5*C***). Like

females, HFD-fed IL22ra1$^{-/-}$ male mice developed higher adiposity and lean mass compared to WT at 30 weeks, though the difference in fat mass did not reach statistical significance (**Figure 5*B* and *D***). Also, no difference was detected between HFD-fed IL22ra1$^{-/-}$ male mice and WT in terms of liver index and hepatic steatosis (**Figure 5*F* and *H***). Taken together, these data suggest that lack of endogenous IL-22 receptor signaling likely promotes metabolic abnormalities, especially weight gain and insulin resistance, associated with NAFLD in female and male mice.

Loss of IL-22 receptor signaling exacerbates liver injury and NASH-related inflammation in HFD-fed female mice

The hepatic inflammatory response is a key driver of human and murine NASH progression as it promotes advancement of hepatic fibrogenesis, which can eventually lead to cirrhosis[4]. Given that the role of endogenous IL-22 in NASH-related inflammation remains unknown, we asked whether the increase in endogenous IL-22 expression in the fatty livers of WT female mice protected against NASH-related liver injury and inflammation. Interestingly, HFD-fed IL22ra1$^{-/-}$ female mice developed more pronounced liver injury compared to WT, as demonstrated by serum ALT and histological assessment of total NAS score (**Figure 6*A-C***). However, there was no significant difference in the individual pathological categories that make up the NAS score (steatosis, lobular inflammation, and hepatocyte ballooning) between these two groups (**Figure 6*C***). One of the characteristic hallmarks of NASH-associated inflammation is the hepatic infiltration of macrophages and neutrophils[4, 364, 366, 385, 668]. An increase in macrophages/Kupffer cells is positively associated with NASH severity in humans and mice with NAFLD[364, 365]. Activation of macrophages/Kupffer cells promotes release of several pro-inflammatory cytokines (e.g.IL-6) and pro-fibrogenic cytokine (e.g. tumor growth factor β (TGF-β) that exacerbate the inflammatory response in NASH and enhance fibrosis progression by inducing HSC activation[4, 365, 366].

Furthermore, neutrophil infiltration in NASH is associated with increased expression of the myeloperoxidase (MPO) enzyme which promotes fibrosis progression via activation of HSC and aggravation of hepatocyte death[385, 390, 391, 668]. Therefore, using IF, we examined the hepatic infiltration of macrophages ($F4/80^+$) and neutrophils (MPO^+) in our NAFLD model. Hepatic F4/80 and MPO positive areas were significantly higher in HFD-fed IL22ra1$^{-/-}$ female mice compared to WT (**Figure 6*A, D* and *E***). In contrast, we did not observe major differences in liver injury (ALT levels and NAS scores) (**Figure 7*A*-*C***) and inflammation ($F4/80^+$ and MPO^+ areas) between male HFD-fed IL22ra1$^{-/-}$ mice versus WT (**Figure 7*A, D* and *E***). Then, we sought to determine the makeup of other immune cells in the livers and spleen of HFD-fed IL22ra1$^{-/-}$ and WT female or male mice by flow cytometry (**Figure 8 and Figure 9**). There was a large increase in the numbers of all $CD3^+$ T cell subsets, $CD19^+$ B-cells, and NK-T cells ($CD3^+$ $NK1.1^+$), but not NK-cells ($CD3^-$ $CD19^-NK1.1^+$), in the livers and spleens of HFD-fed IL22ra1$^{-/-}$ female mice, but not males, compared to WT (**Figure 8*B-E***). Intriguingly, this was in parallel with an increase in the number of macrophages ($CD11b^+$ $Ly6C^-$ $Ly6G^-$ and $F4/80^+$), monocytes ($CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$) and neutrophils ($CD11b^+$ $Ly6C^{int+}$ $Ly6G^{hi+}$) in the livers and spleens of HFD-fed IL22ra1$^{-/-}$ female mice, but not males, compared to WT (**Figure 9*B-E***). On the other hand, there was no change in the number of dendritic cells (DCs; $CD11b^+$ $Ly6C^{low}$ $Ly6G^-$ $F4/80^-$ $CD11c^+$) in any of the four groups of mice studied (**Figure 9*B-E***). Furthermore, the hepatic mRNA expression of pro-inflammatory cytokines (*Il6*, tumor necrosis factor α (*Tnf-α*), *Il1β*) and inflammatory (C-X-C motif) and (C–C motif) chemokine ligands (*Cxcl-1*, *Cxcl-10*, *Ccl2* and *Ccl3*) were remarkably higher in HFD-fed IL22ra1$^{-/-}$ female mice than WT (**Figure 10*A, C* and *E***). Nonetheless, both HFD-fed IL22ra1$^{-/-}$ male and WT had generally comparable profiles of hepatic pro-inflammatory genes expression except *Ccl2* and *Ccl3* (**Figure 10*B, D* and *F***). Collectively, these findings suggest that lack of

endogenous IL-22 receptor signaling exacerbates NASH-related liver injury and inflammation in HFD-fed female mice, but not males.

Lack of IL-22 receptor signaling promotes progression of NASH-related fibrosis in HFD-fed female, but not the male mice

Hepatic fibrosis is initiated by activation of HSCs to transdifferentiate into myofibroblasts characterized by marked upregulation of type I collagen (COL1A1), alpha-smooth muscle actin (α-SMA, actin alpha 2 ACTA2) and desmin[1, 145]. Thus, based on our findings (**Figures 6-10**), we sought to determine whether lack of endogenous IL-22 receptor signaling in female or male mice will modulate progression of NASH-related fibrosis. As expected, HFD-fed IL22ra1$^{-/-}$ female mice developed advanced NASH-related fibrosis (chicken wire-like perisinusoidal fibrosis) compared to WTs, as illustrated by collagen type I deposition, α-SMA and desmin positive areas measured by PSR staining and IF, respectively (**Figure 11*A, B, D* and *E***). Also, the fibrosis grade was blindly evaluated by an expert pathologist and was consistent with PSR positive area data (**Figure 11*C***). In male mice, there was no difference in liver fibrosis markers and grade between HFD-fed IL22ra1$^{-/-}$ and WT mice (**Figure 12 *A-E***). Next, we examined the mRNA expression levels of the profibrogenic genes *Col1a1*, *Tgfβ*, *Acta2* and *Loxl2* in all groups studied. Consistent with the histological data, these genes were highly upregulated in the livers of HFD-fed IL22ra1$^{-/-}$ female mice, but not males, compared to WT (**Figure 11*F-J* and Figure 12*F-J***). Overall, the endogenous IL-22 receptor signaling likely delays NASH-related fibrosis progression in HFD-fed female mice, while in males, IL-22 receptor signaling seems to play an insignificant role during progression of NASH-related fibrosis.

Endogenous IL-22 receptor signaling protects against HFD-induced liver apoptosis in female, but not the male, mice

Cell death, including apoptosis, is one of the fundamental triggers of NASH progression and has been positively correlated with development of NASH and fibrosis progression[4, 325, 688]. Additionally, IL-22 induces different anti-apoptotic signals in various experimental models of liver injury, leading to protection of hepatocytes from apoptosis and enhancement of their survival[102, 619, 668]. Thus, to better understand the mechanism(s) underlying the severe liver injury observed in HFD-fed IL22ra1$^{-/-}$ female mice, we examined whether hepatocyte cell death is involved. First, we measured the hepatic expression of IL-22 downstream target genes *Bcl2*, *Bcl-xL*, Superoxide dismutase 1 (*Sod1*) and *Mt2*. *Bcl2* and *Bcl-xL* are anti-apoptotic genes, while *Sod1* and *Mt2* are antioxidant genes[102, 619, 665, 668]. Interestingly, the mRNA expression levels of these genes were substantially reduced in HFD-fed IL22ra1$^{-/-}$ female mice, but not males, compared to WT (**Figure 13*A* and *B***). Notably, the hepatic expression of these genes was upregulated in HFD-fed WT female mice compared to those in the chow control group (**Figure 13*A***). Based on these data, we asked whether the IL-22 cytokine, detected in livers of those females, was biologically active. Therefore, we measured the hepatic expression of its regulator *Il22BP*, and it was significantly upregulated in livers of HFD-fed female mice as compared to males, suggesting tight regulation of IL-22 activity by IL-22BP (**Figure 13*C***). Nevertheless, the *Il22*/*IL22BP* mRNA ratio, which should reflect biologically active IL-22, significantly correlated with expression of downstream target genes of IL-22 such as *Bcl2*, *Mt2* and *Sod1* (**Figure 13*D***), suggesting that this regulation process did not limit the overall IL-22 activity.

Next, by using the TUNEL assay, we explored liver cell death in our NAFLD model. Consistent with serum ALT data, the livers of HFD-fed IL22ra1$^{-/-}$ female but not male mice displayed increased cell death as compared WT (**Figure *14A* and *B***). This was reflected by a significant increase in the number of apoptotic bodies observed in HFD-fed IL22ra1$^{-/-}$ females as

compared to WT (**Figure 14*C***). In contrast, we did not detect a difference in the number of apoptotic bodies in the livers of HFD-fed IL22ra1$^{-/-}$ male mice as compared to WT (**Figure 14*D***). Altogether, these results highlight that the lack of IL-22 receptor signaling augments HFD-induced liver cell apoptosis and consequently accelerates the liver injury in female, but not male, mice with NAFLD.

Discussion

In this study we report sexual dimorphism in hepatic IL-22 expression in humans with NAFLD, where females expressed higher levels of IL-22 gene and protein compared to males. We further validated these findings in HFD-fed mice with NAFLD. The lack of IL-22 receptor signaling in female, but not male, mice exacerbated liver injury, apoptosis, inflammation, and consequently liver fibrosis. These results suggest a sex-dependent, hepatoprotective role of IL-22 in NAFLD.

We demonstrated that IL-22 was significantly increased in the livers of female subjects and female mice with NAFLD as compared to males. In line with this, other *in vivo* studies report relatively low serum and hepatic IL-22 levels in HFD-fed male mice compared to controls[663, 664]. Interestingly, a recent report showed comparable plasma IL-22 profile between male subjects with NAFLD and healthy controls[661]. These observations may suggest a regulation of IL-22 expression by the female sex hormone estrogen. Indeed, estrogen is known to modulate inflammatory responses in NAFLD, but the underlying mechanisms remain undefined[689]. For example, significant infiltration of macrophages along with an increase in inflammatory mediators (e.g., TNF-α) and liver fibrosis progression were reported in livers of ovariectomized female mice with NAFLD compared to controls[276]. Furthermore, several observations suggest cross-regulation between estrogen and IL-22. Women with polycystic ovary syndrome, characterized by dysregulated female sex hormones, exhibit significantly lower serum IL-22 than healthy controls[690]. In addition, testosterone or dihydrotestosterone reduced IL-22 production by female murine splenocytes following stimulation by either lipopolysaccharide or αCD3/CD28[691]. Moreover, in the imiquimod-induced psoriasis model, administration of estrogen agonists significantly modulated Th-derived IL-22 thus aggravating psoriasis symptoms[692]. Further *in vivo* studies are warranted to investigate whether endogenous estrogen regulates hepatic IL-22 expression in the context of NAFLD.

We demonstrated that endogenous IL-22 was produced by heterogenous cellular populations including Th17, Th22, γδ-T cells, ILC3s and neutrophils in the livers of HFD-fed

female mice, where IL-22 producing T cells were major producers. Also, we showed that HFD-fed IL-22ra1$^{-/-}$ female mice had relatively higher frequencies of Th17 (IL-17A^{+}IL-22^{-}CD4^{+}) compared to WT, suggesting an exacerbation of NASH-related inflammation in the absence of IL-22 receptor signaling, in parallel with worsening progression of liver fibrosis. In agreement with this, Rolla et al[515] reported a hepatoprotective effect of Th22 and amelioration of NASH-related fibrosis but only in the absence of IL-17 (IL-17$^{-/-}$ male mice). Thus, our results and those of Rolla et al[515] support opposite roles of IL-22 (protective) and IL-17A (pathogenic) during NASH. However, in comparison to Rolla et al[515], we observed multiple cellular sources of IL-22 in the livers of HFD-fed females, not only Th22, which could reflect sex differences and/or utilization of different NASH models (HFD vs MCD).

We observed beneficial metabolic effects of IL-22 receptor signalling against weight gain and IR in HFD-fed WT female or male mice. Wang et al[664] observed similar metabolic alterations during diet‑induced obesity in IL-22R1 KO, but not in IL-22KO mice. These data suggest that other IL-22RA1 ligands such as IL-20 and IL-24 may partially mediate theses metabolic disorders in IL-22ra1 deficient mice upon feeding on a HFD[92, 664]. Although ALT levels often correlate with body weight and/or fat mass[693], we did not observe a significant difference in ALT levels between HFD-fed IL-22ra1$^{-/-}$ and WT male mice. This may be due to the significant increase in the lean mass in HFD-fed IL-22ra1$^{-/-}$ mice as compared to WT while the fat mass was comparable between the two groups. In addition, we showed that HFD-fed IL-22ra1$^{-/-}$ female mice exhibited exacerbated hepatic apoptosis and fibrosis progression compared to WT, which seems to be driven by loss of IL-22-induced antiapoptotic (*Bcl2*) and antioxidant signals (*Sod1* and *Mt2*). Also, we observed an upregulation of IL-22/IL-22BP mRNA ratio, reflecting biologically active IL-22, in the livers of HFD-fed WT females, which significantly correlated with expression of IL-22 target genes (*Bcl2*, *Mt2* and *Sod1*). These data suggest that IL-22 can still play a protective role in this

NAFLD model, even when it is tightly regulated. In line with this, IL-22Fc alleviated oxidative stress-induced hepatocyte death via STAT-3-activating Mt1 and Mt2 in HFD^{+Cxcl1}-induced NASH model, resulting in amelioration of NASH-related fibrosis[668]. In addition, our observations support the findings of Zai et al[665] using liver-targeted delivery of the IL-22 gene in a NASH mouse model, where IL-22 activated STAT3-induced BCL2 and Nrf2-induced SOD1 pathways, resulting in increased hepatocyte survival and proliferation[665]. Furthermore, our data support the *in vitro* work from Hamaguchi et al[669], who showed that IL-22 inhibited palmitate-induced apoptosis of primary hepatocytes. On the other hand, unlike females, we did not observe significant differences in liver apoptosis or fibrosis between HFD-fed IL-22ra1$^{-/-}$ male mice and WT, likely due to low levels of endogenous IL-22 in the livers of HFD-fed male mice. Altogether, our study supports a hepatoprotective function of endogenous IL-22 receptor signaling against liver injury in female mice with NAFLD, while the endogenous IL-22 receptor signaling appears to play no role against liver injury in male mice with NAFLD.

We and others have previously demonstrated that IL-22 has a pathogenic profibrogenic function in humans and in the CCl_4 and TAA models of chronic toxic liver injury[523, 626]. This effect is mediated through enhancement of TGF-β signaling in HSCs in a p38 mitogen-activated protein kinase-dependent manner[523]. The different results obtained here, demonstrating a hepatoprotective effect of IL-22 in a physiological HFD-induced NAFLD model, may reflect the relatively mild to moderate inflammation and heterogenous fibrosis induced in this NAFLD model, in contrast to a toxin-induced model. This is supported by the low TGF-β mRNA expression in the livers of HFD-fed WT females and could represent a context dependent function(s) of IL-22.

There are a few limitations to this study. First, we used a total body IL-22ra1$^{-/-}$ mouse model. Since IL-22RA1 receptor has several ligands such as IL-22, IL-20 and IL-24, our results may be mildly influenced by lack of signaling from other IL-22RA1 ligands. Future investigation using an IL-22$^{-/-}$ model may clarify this issue. Second, the IL-22/IL-22RA1 axis is crucial for

maintaining gut homeostasis which may have been altered in our model[92, 616]. Microbial translocation and microbial-derived products, due to gut dysbiosis, have worsened NASH-related inflammation in humans and mice[349, 694]. Therefore, we cannot exclude a potential influence of gut dysbiosis in the promotion of NASH in this model. Further investigation evaluating the microbiome is needed.

In summary, we provide novel evidence of sexual dimorphism in IL-22 expression in both humans and mice with NAFLD. Our data extend previous observations by demonstrating a hepatoprotective function of IL-22 in the context of NAFLD, in which IL-22 receptor signaling acts in a sex-specific manner and mitigates liver injury, apoptosis, NASH-related inflammation and fibrosis in female mice. These findings should be considered in clinical trials testing IL-22 based therapeutic approaches in treatment of female versus male subjects with NAFLD[670].

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Methods

Patients

NAFLD patients (n=20; females=9 and males=11) were enrolled through the hepatology clinic at the Centre hospitalier de l'Université de Montréal (CHUM), Montreal, Canada. The study was approved from institutional ethics committee (protocol SL09.228) and all the participants signed the informed consent. The main inclusion criteria of the cohort included the history of alcohol consumption, absence of other chronic liver hepatitis (e.g., viral, autoimmune, alcoholic hepatitis) and the NAS score evaluation of liver biopsies. The NAFLD diagnosis was confirmed by an independent pathologist. The demographics and clinical characteristics of the study subjects are summarized in (**Table 1**) and detailed in (**Table 2**). The NAS score and fibrosis grade of participant's liver biopsies were blindly evaluated by an independent pathologist according to the NASH Clinical Research Network (NASH CRN) criteria.

Microarray datasets

Publicly available microarray datasets (GSE151158 and GSE106737) were obtained from two published studies, including two different cohorts of NAFLD patients[686, 687]. The number of NAFLD patients in GSE151158 and GSE106737 datasets are (9-22/group) and (15-24/group), respectively. The diagnoses of NAFLD in both studies were based on liver biopsy. Gene expression data along with gene name annotations were downloaded from the Gene Expression Omnibus (GEO) repository. The normalized values of IL-22 mRNA data were expressed as Read counts and RMA values in GSE151158 and GSE106737, respectively. We stratified the NAFLD patients into females and males in each dataset and then assigned the IL-22 mRNA normalized value to its corresponding NAFLD patient.

Mice

IL22ra1$^{-/-}$ mice and their WT littermates, of C57BL/6N background, were originally rederived as previously described[523]. Heterozygote couples (IL-22ra1$^{+/-}$ x IL-22ra1$^{+/-}$) were used for breeding to generate IL-22ra1$^{-/-}$ mice and WT littermates. Six- to eight-week-old IL-22ra1$^{-/-}$ and their WT littermates, including male and female mice, were fed High-fat diet (HFD, 40% Kcal fat, Cat #D17010102I, Research Diets, US) or Chow diet (CD, 6.2% Kcal fat, Teklad global 18% protein rodent diet, ENVIGO) for 30 weeks. All the mice were terminally euthanized at 30 weeks by using pentobarbital (400 mg/kg) and 2% xylocaine. After dissection, liver, spleen, and blood samples were harvested. All the animal experimental procedures were approved by CRCHUM animal ethics committee, Comité Institutionel de Protection des Animaux (CIPA) (protocol IP18035NSs).

Histology

Human liver biopsy samples were fixed in formalin and processed at the Pathology Laboratory of the CHUM. For the mice, liver specimens were fixed in Tissue Fix (Chaptec, Cat.no.T-50, Montreal, QC) overnight at 4°C, and finally embedded in paraffin for sectioning (BZ-Histo Services Inc., Montreal, QC). The 5-µm thick formalin fixed paraffin embedded (FFPE) sections were deparaffinized and rehydrated, then stained with picrosirius red (PSR) stain (Sigma-Aldrich, Cat.no.365548-5G) with Fast green (Sigma-Aldrich, Cat.no. F7252) or H&E. The H&E stain was performed by BZ-Histo Services Inc. The TUNEL staining was performed by using TUNEL Assay Kit -HRP-DAB Kit (Abcam, ab206386, USA) according to the manufacture's instructions.

Immunofluorescence (IF) staining and Image analysis

The IF technique and image analysis were performed as previously described[695]. Briefly, FFPE liver sections (human or mice) were deparaffinized and rehydrated. For the antigen retrieval step, the sections were immersed in Sodium citrate solution (pH 6) for 10 minutes (with exception to mF4/80 antibody, the incubation was 20 minutes) in an electric high-pressure cooker (Salton).

Then the sections were incubated in 0.1M glycine for 15 minutes at 25°C to reduce autofluorescence, followed by blocking with (10% human serum, 1% BSA, 0.1% Tween 20 and 0.3% Triton-X 100 in PBS solution) for 30 minutes. The sections were then incubated with primary antibodies (**Table 3**) overnight in (1% BSA, 0.1% Tween 20 and 0.3% Triton-X 100 in PBS solution) at 4°C. After this, the sections were washed in PBS-Tween and then incubated with the appropriate secondary antibodies (**Table 3**) in blocking buffer for 1 hour at 25°C. The sections were mounted in Slow fade Gold mounting media with DAPI (Cat.no. S36938, Thermo Fisher Scientific, Fremont, CA, US). Finally, the images were acquired at the CRCHUM molecular pathology platform using Whole slide scanner Olympus BX61VS. For quantification of IL-22 producing cells, FIJI software (Version 1.52a, U. S. National Institutes of Health, US) was used. For all other image analyses, Visiopharm software (Broomfield, CO) was used, including tissue detection (tissue vs non-tissue), identification and automatic calculation of area of interest. Threshold settings based on pixel value was used for generating the calculation of area of interest.

Body composition analysis (fat/lean mass)

At 30 weeks, body composition of experimental mice was measured by using an EchoMRI-100 Body Composition Analyzer (version 2008.01.18) at the rodents-cardiovascular core facility of CRCHUM.

Intraperitoneal glucose tolerance test (IPGTT) and insulin assay

At 30 weeks, experimental mice were food-deprived for 5 h with ad libitum access to water. A bolus of glucose (1.5 g/kg) was administered via intraperitoneal (IP) injection and glycaemia was measured from blood sampled at the tail vein using an Accu-check Performa glucometer at T0 (before injection), 15, 30, 45, 60, 90 and 120 minutes. Tail vein blood samples were collected via a capillary for insulin assays at 0, 15 and 30 min.

Measurement of liver triglycerides

Hepatic triglyceride (TG) content was evaluated by saponification technique using the Bio-protocole223[696]. Briefly, liver samples (100-300 mg) were digested in an ethanolic KOH solution overnight at 55°C. After neutralization with MgCl2, a triacylglycerol GPO kit (Cat.no. F6428, Sigma-Aldrich) was used to measure the glycerol content of the samples. Calculations were performed to estimate TG levels that are presented as a ratio of total liver protein.

Measurement of Serum ALT

Blood samples were drawn using cardiac puncture pf the mice and the serum Alanine Aminotransferase (ALT) levels were measured at the OPTILAB of the CHUM.

Fibrosis and Apoptosis quantification

The fibrotic area (PSR positive area) was determined on the total liver section area and PSR positive area was calculated by applying a threshold method in the green channel using FIJI (version 1.52a, U. S. National Institutes of Health, USA) image analysis software. For apoptosis, the number of apoptotic bodies per field were counted in the total liver section area using FIJI software (version 1.52a, U. S. National Institutes of Health, USA) and the average of apoptotic bodies/field were calculated.

Mouse intrahepatic leukocytes (IHLs) isolation

IHLs were isolated from mice livers using a Percoll gradient (Cat.no.P1644, Sigma-Aldrich) in isotonic solution. Briefly, mice livers were cut into small pieces and digested in collagenase D (0.025IU/mL, Cat.no.110088866001, Roche, Laval, QC) and benzonase (10 IU/mL, Cat.no. 70664-10KUN, EMD Millipore, Germany) at 37°C with rotation for 25 minutes. Then, liver tissues were passed through a 70 µm cell strainer (Cat.no. 22363548, Fisher) followed by centrifugation. The cell pellet was then resuspended in Percoll 40% in 1% HBSS in sterile water (vol/vol) and layered over Percoll 80% in 1% HBSS in sterile water (vol/vol), followed by centrifugation without brakes for 25 minutes. Next, IHLs were washed, and red blood cells (RBCs) were lysed using

ACK lysing buffer (Cat.no.A10492-01, Thermo Fisher Scientific). IHL were directly stained for surface markers or stimulated with PMA/Ionomycin (50ng/mL (Cat.no.P1585) and 1 µg/mL (Cat.no. I-0634), Sigma-Aldrich, respectively) in presence of brefeldin A (5 µg/mL, Cat.no. B6542, Sigma-Aldrich) and monensin (5 µg/mL, Cat.no.M5273, Sigma-Aldrich) for 5 h prior to intracellular staining (ICS).

Mouse Splenocytes isolation

Mouse spleens were digested by direct passing through a 40 µm cell strainer (Cat.no.22363547, Fisher). Then, the cell suspension was centrifuged and resuspended in ACK lysing buffer to lyse RBCs. Splenocytes were then resuspended in RPMI media and filtered. The cells were counted using hemocytometer and then directly stained for flow cytometry analysis.

Flow cytometry

IHL and splenocytes were stained as previously described[523]. Briefly, freshly isolated IHLs and splenocytes were washed with flow cytometry buffer (1% heat inactivated fetal bovine serum (FBS) and 0.01% azide in PBS), followed by incubation with primary antibodies (**Table 4**) at 4°C for 30 min. Next, the cells were washed with flow cytometry buffer and fixed with 1% paraformaldehyde (PFA) in PBS and filtered using polystyrene tubes with cell strainer (Corning science, Mexico). For intracellular cytokine staining (ICS), surface staining was performed first and then the cells were fixed and permeabilized using FOXP3 fixation buffer (Cat.no.005523-00, eBioscience). Then, cells were washed twice with washing buffer (eBioscience) and incubated with antibodies for intracellular antigens at 4°C for 30 min. For detection of live cells, Aqua Live/Dead Fixable Dead Cell Stain kit was used (Cat.no. L34966, Life Technologies, Burlington, ON). Data were acquired using a multicolor BD LSRII flow cytometer (BD Bioscience) equipped with FACS DIVA software version 8 and analyzed using FlowJo software, version 10 (BD Bioscience).

Quantitative real-time PCR (RT-qPCR)

Total RNA was isolated and purified from mouse livers using RNeasy Mini kit plus (Cat.no. 74134, QIAGEN, Germany) according to the manufacture's instructions. Then, 1µg of RNA was reverse transcribed to cDNAs using Transcriptor Universal cDNA Master (Cat.no. 05893151001, Roche, Germany) kit. cDNAs were diluted 1:10 with ultra-pure water and the relative expression of mRNA level was measured by using RT-qPCR with Light Cycler 480 SYBR Green I system (Cat.no. 04707494001, Roche). r28S was used as standard house keeping gene and the $2^{-\Delta\Delta Ct}$ method was applied to calculate the mRNA level. The List of primer sequences used for RT-qPCR are listed in (**Table 5**). Primers for *Tgfβ*, *Col1a1*, *Acta2* and *Loxl2* were purchased from QIAGEN.

Statistical Analysis

GraphPad Prism 7 (La Jolla, CA) and Sigma plot 14 (Version: 1.0.23) were used. Mann-Whitney test was applied to determine differences between two groups. Two-way ANOVA followed by Holm-Sidak's post-hoc test was used to determine difference between groups for glucose intolerance and IR data of mice. Correlations were tested using Spearman's rank correlation and Two-way repeated measures ANOVA followed by Holm-Sidak's post-hoc test was used for determining longitudinal difference in weight gain of mice.

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Figures and figure Legends

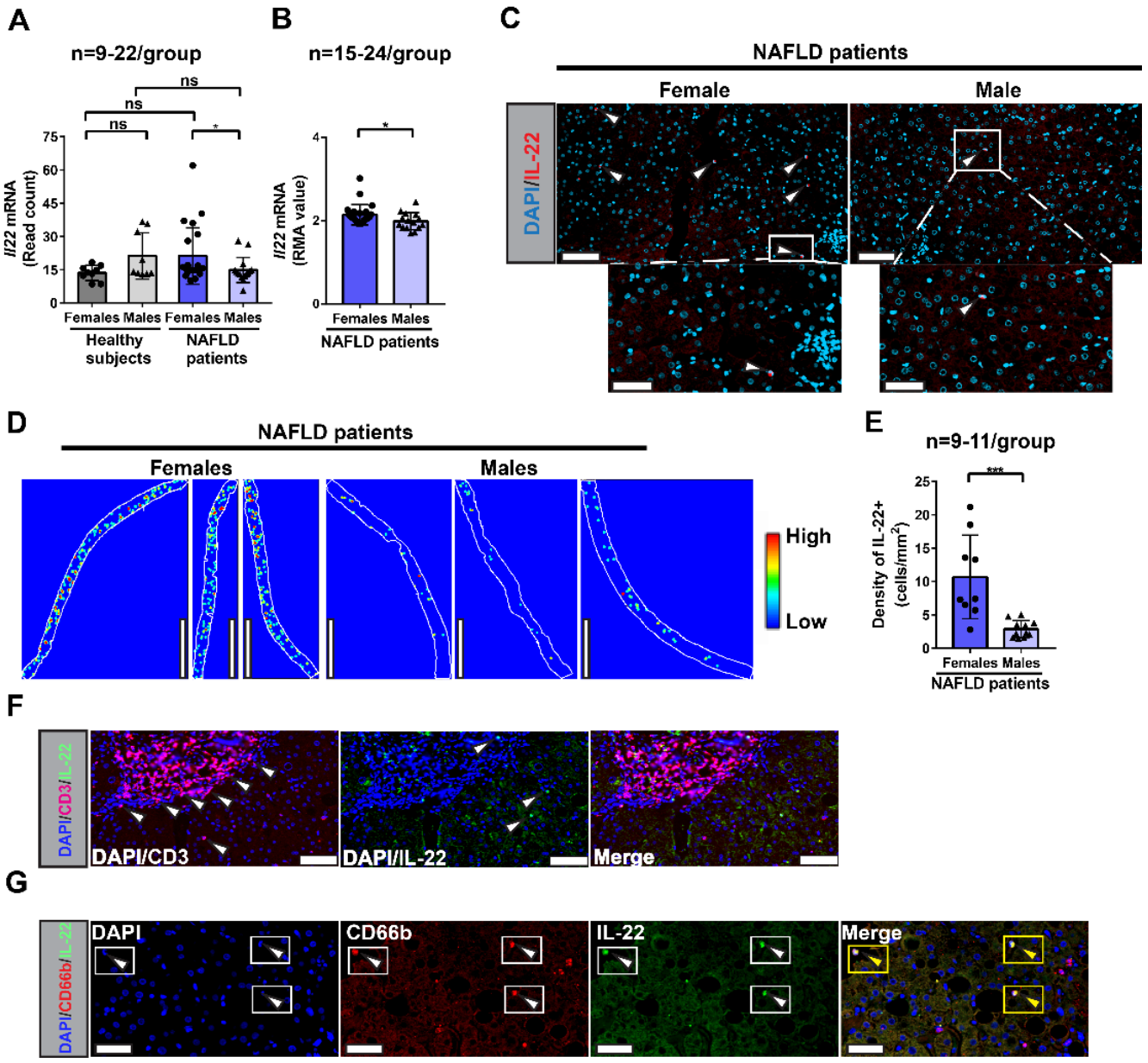

Figure 1. Elevated levels of IL-22$^+$ cells in livers of female versus male patients with NAFLD.

(*A-B*) IL-22 mRNA expression from publicly available microarray datasets (GSE151158 and GSE106737) of two different cohorts of NAFLD patients. *Il22* mRNA levels were normalized and expressed as Read counts and Robust Multiple-array Average (RMA) values in GSE151158 and GSE106737, respectively. **(*C*)** Representative IF images of liver sections stained with anti-IL-22 (red). Lower panels are magnified insets. White arrows indicate IL-22$^+$ cells. Scale bars: 70 μm and 35 μm for upper and lower panel(s), respectively; 20x magnification. **(*D*)** Representative liver tissue heatmaps of IL-22$^+$ cells (scale, blue=0 (low) to red=3 (high) cells/100 μm diameter). Scale bar 3000 μm. **(*E*)** Total density quantification (counts per mm^2) of hepatic IL-22$^+$ cells in our cohort (n=20, females=9 and males=11) performed by FIJI software. **(*F*)** IL-22$^+$ cells did not coexpress the T cell marker (CD3). Representative IF images of CD3 (red) and IL-22 (green) in liver biopsy (FFPE liver section) from a female patient with NAFLD. In Merge, no colocalization between IL-22$^+$ cell and CD3$^+$. Scale bars, 50 μm; 20x magnification. **(*G*)** IF detection of CD66b (red) and IL-22 (green) in liver biopsy (FFPE liver section) from a female patient with NAFLD. Yellow rectangles and/or arrows in the Merge of **(*F*)** indicate IL-22-producing neutrophils (CD66b$^+$ IL-22$^+$). Scale bar: 35 μm; 20x magnification. Data are expressed as mean±SD for 9-24 patients per group: Mann-Whitney test. *p<0.05. ***p<0.001. ns; not significant. Each dot on the bar graphs represents one male (▲) or female (●) patient.

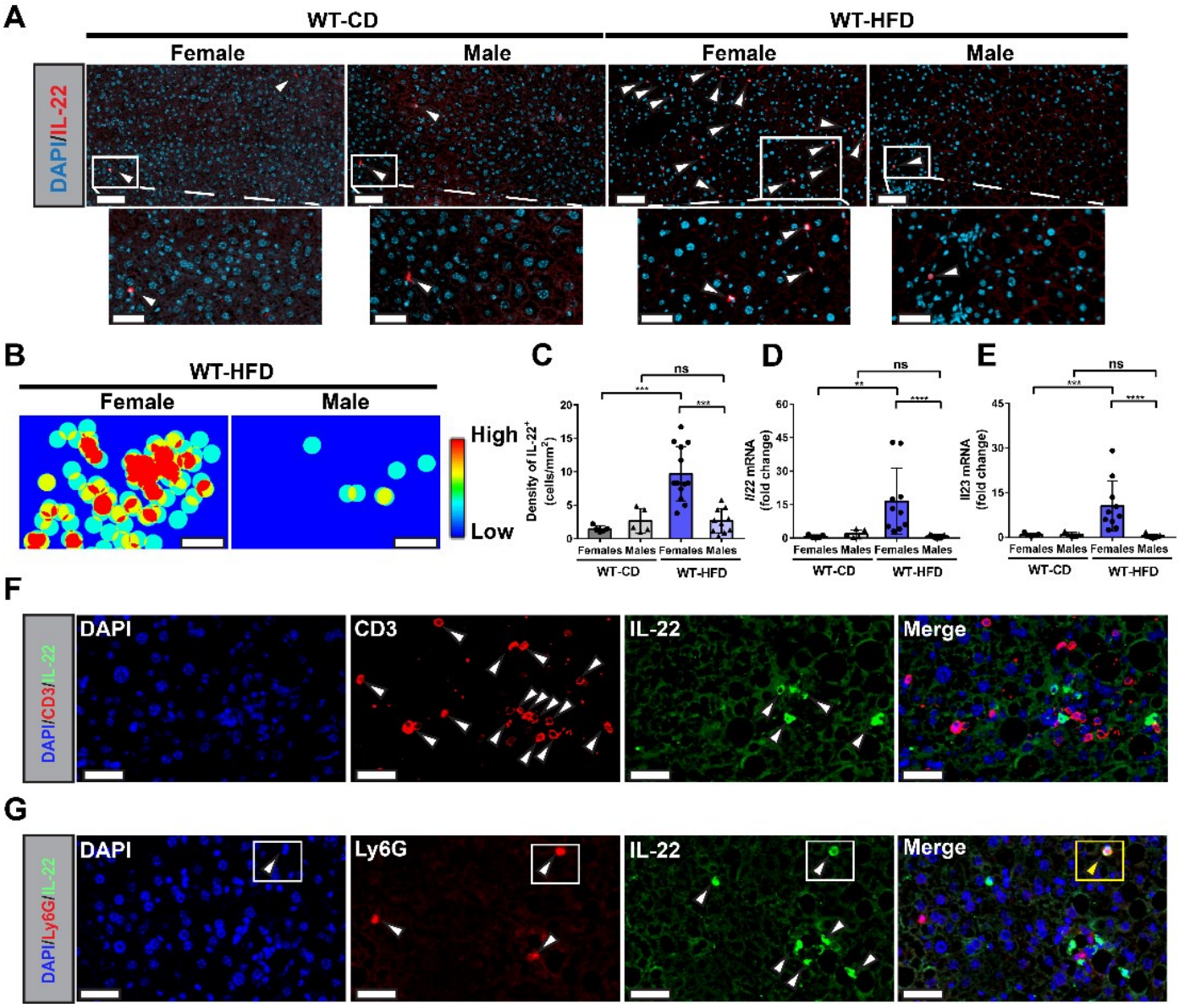
A
WT-CD
WT-HFD
Female
Male
Female
Male
DAPI/IL-22
B
WT-HFD
Female
Male
High
Low
C
Density of IL-22+ (cells/mm2)
ns
***
***
Females Males Females Males
WT-CD
WT-HFD
D
Il22 mRNA (fold change)
ns
**
****
Females Males Females Males
WT-CD
WT-HFD
E
Il23 mRNA (fold change)
ns
***
****
Females Males Females Males
WT-CD
WT-HFD
F
DAPI/CD3/IL-22
DAPI
CD3
IL-22
Merge
G
DAPI/Ly6G/IL-22
DAPI
Ly6G
IL-22
Merge

Figure 2. Elevated density of IL-22$^+$ cells in livers of WT HFD-fed female mice compared to males.

C57BL/6N male (▲) and female (●) mice were fed HFD or CD for 30 weeks as described in materials and methods. **(*A*)** Representative IF images of liver sections stained with anti-IL-22 (red). Lower panels are magnified insets. White arrows indicate IL-22$^+$ cells. Scale bars: 70 μm and 35 μm for upper and lower panel(s), respectively; 20x magnification. **(*B*)** Representative liver tissue heatmaps of IL-22$^+$ cells (scale, blue=0 (low) to red=3 (high) cells/100 μm diameter). Scale bar 300 μm. **(*C*)** Total density quantification (counts per mm2) of hepatic IL-22$^+$ cells performed by FIJI software. **(*D-E*)** Hepatic *Il22* and *Il23* mRNA expression normalized to ribosomal 28s. Data are expressed as fold change. **(*F*)** IL-22$^+$ cells did not coexpress the T cell marker (CD3). Representative IF images of CD3 (red) and IL-22 (green) in in FFPE Liver sections of HFD-fed WT female mice for 30 weeks (WKs). In Merge, no colocalization between IL-22$^+$ cell and CD3$^+$. Scale bars, 35 μm; 20x magnification. **(*G*)** IF detection of IL-22$^+$ (green) and Ly6G$^+$ (red) cells in liver section (FFPE section) of WT female mouse fed HFD for 30 weeks. The rectangle in the middle panel shows the IL-22-producing neutrophils (Ly6G$^+$ IL-22$^+$) in the merge. Scale bar: 35 μm; 20x magnification. Data are expressed as mean±SD (n = 5-12 mice per group, data were pooled from three independent experiments). Mann-Whitney test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

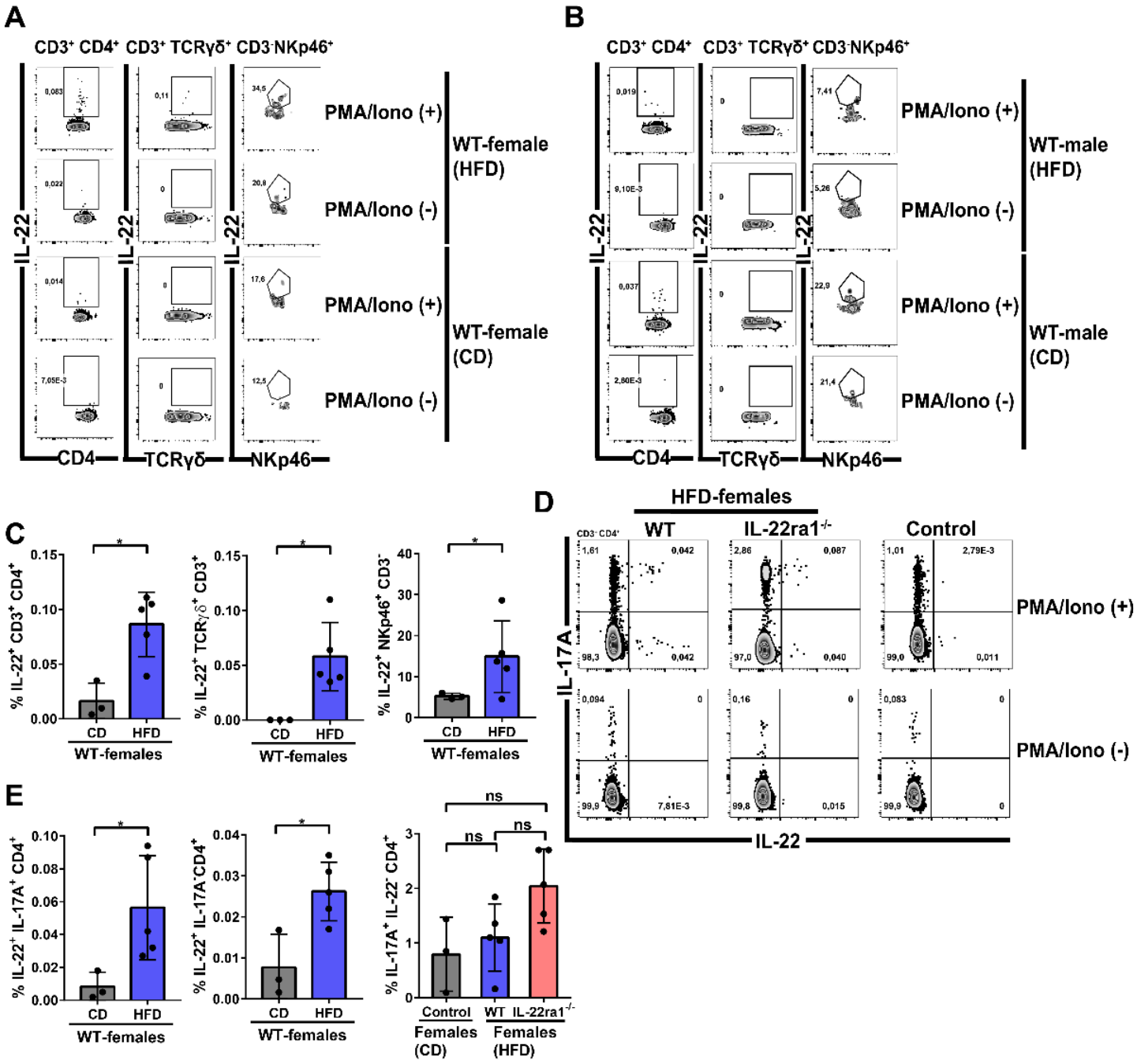
A
CD3+ CD4+
CD3+ TCRγδ+
CD3-NKp46+
IL-22
CD4
TCRγδ
NKp46
PMA/Iono (+)
PMA/Iono (-)
WT-female (HFD)
WT-female (CD)
B
WT-male (HFD)
WT-male (CD)
C
% IL-22+ CD3+ CD4+
% IL-22+ TCRγδ+ CD3+
% IL-22+ NKp46+ CD3-
CD
HFD
WT-females
D
HFD-females
WT
IL-22ra1-/-
Control
IL-17A
IL-22
E
% IL-22+ IL-17A+ CD4+
% IL-22+ IL-17A-CD4+
% IL-17A+ IL-22- CD4+
ns
Control Females (CD)
WT Females (HFD)
IL-22ra1-/- Females (HFD)

Figure 3. Intrahepatic T cells are major producers of IL-22 in HFD-fed WT female mice. Representative flow cytometry plots showing intrahepatic IL-22-producing cells: $CD4^+$ T cells (IL-$22^+CD3^+CD4^+$), γδ-T cells (IL-$22^+CD3^+TCR\gamma\delta^+$) and ILC3s (IL-$22^+CD3^-NKp46^+$) and their frequencies in WT female (***A***) and male mice (***B***). The intrahepatic lymphocytes were extracted from livers of HFD or CD-fed WT female mice at 30 weeks (WKs) and then stimulated with/without PMA/ionomycin (PMA/Iono) for 5 hours. **(*C*)** The frequency quantification of IL-22 producing $CD4^+$ T cells or γδ-T cells or ILC3s in **(*A*)**. **(*D*)** Representative flow cytometry plots showing frequencies of IL-22 and/or IL-17A $CD4^+$ T cells, including Th17 (IL-22^+ IL-$17A^+$ $CD4^+$ and/or IL-$17A^+$ IL-22^- $CD4^+$) and Th22 (IL-22^+ IL-$17A^-$ $CD4^+$), in livers of HFD-fed IL-22ra$1^{-/-}$ female mice and their WT littermates. **(*E*)** The frequency quantification of **(*D*)**. Data are expressed as mean±SD (n = 3-5 mice per group). Mann-Whitney test. *p<0.05. ns; not significant.

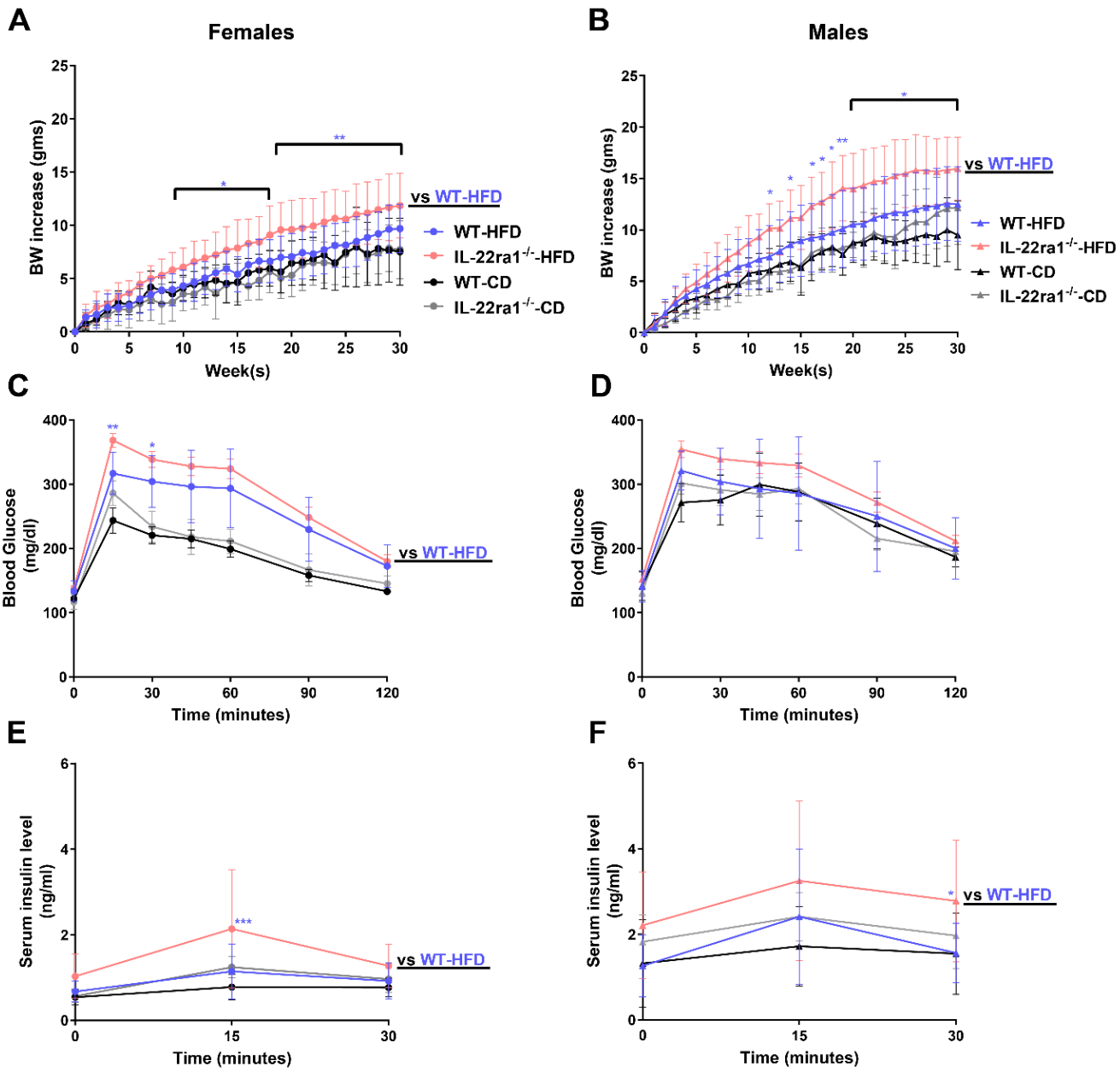
A
Females
BW increase (gms)
Week(s)
vs WT-HFD
WT-HFD
IL-22ra1-/--HFD
WT-CD
IL-22ra1-/--CD
B
Males
BW increase (gms)
Week(s)
vs WT-HFD
WT-HFD
IL-22ra1-/--HFD
WT-CD
IL-22ra1-/--CD
C
Blood Glucose (mg/dl)
Time (minutes)
vs WT-HFD
D
Blood Glucose (mg/dl)
Time (minutes)
E
Serum insulin level (ng/ml)
Time (minutes)
vs WT-HFD
F
Serum insulin level (ng/ml)
Time (minutes)
vs WT-HFD

Figure 4. HFD-fed IL-22ra1$^{-/-}$ female and/or male mice develop significant weight gain and insulin resistance compared to their WT littermates at 30 weeks.

IL-22ra1$^{-/-}$ female (●) or male mice (▲) and their WT littermates were fed HFD or CD for 30 weeks (WKs). **(*A-B*)** Measurements of total body weight gain (grams) over time, (***C-D***) intraperitoneal glucose tolerance test (IPGTT) at 30 weeks, and (***E-F***) measurements of serum insulin at 30 weeks for female (***A***, ***C*** **and** ***E***) and male (***B***, ***D*** **and** ***F***) mice. Data are expressed as mean± SD for 8-22 mice per group/sex (data were pooled from three independent experiments): Two-way repeated measures ANOVA followed by post-hoc test (Holm-Sidak's multiple comparisons test) was used for **(*A* and *B*)**. Regular two-way ANOVA followed by post-hoc test (Holm-Sidak's multiple comparisons test) was used for (***C-D***). *p<0.05, **p<0.01, ***p<0.001. ns; not significant.

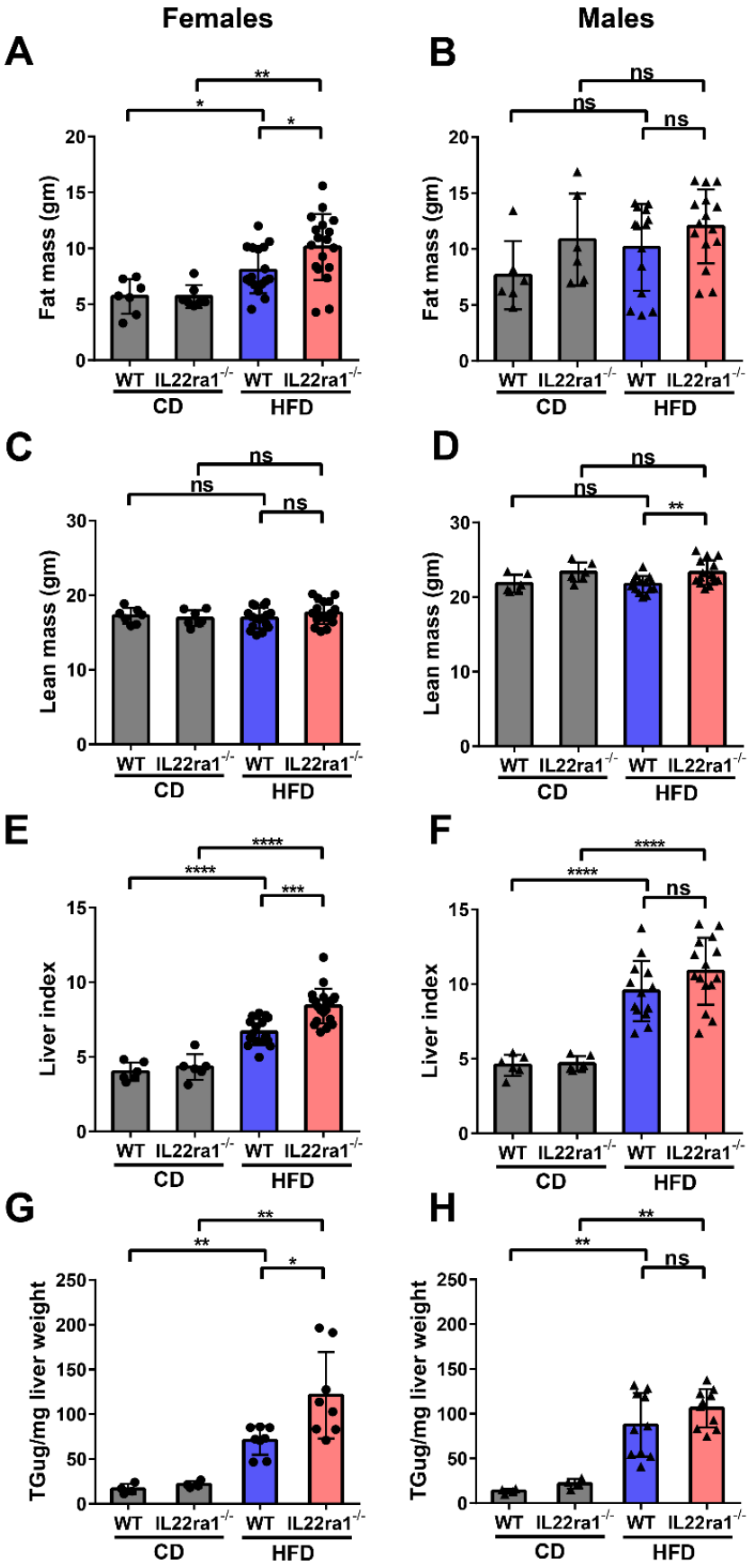
Females
Males
A
B
C
D
E
F
G
H
Fat mass (gm)
Lean mass (gm)
Liver index
TGug/mg liver weight
WT
IL22ra1-/-
CD
HFD
ns
*
**
***
****

Figure 5. HFD-fed female and/or male mice develop adiposity and hepatic steatosis at 30 weeks.

(*A-B*) Measurements of fat mass (g), **(*C-D*)** lean mass (g), **(*E-F*)** liver index (liver/body weight ratio), and **(*G-H*)** liver TG level (TG, ug/mg liver weight) at 30 weeks (WKs) for female (***A*, *C*, *E* and *G***) and male mice (***B*, *D*, *F* and *H***). Data are expressed as mean± SD (n= 4-18 mice per group/sex, data were pooled from three independent experiments). Mann-Whitney test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. ns; not significant.

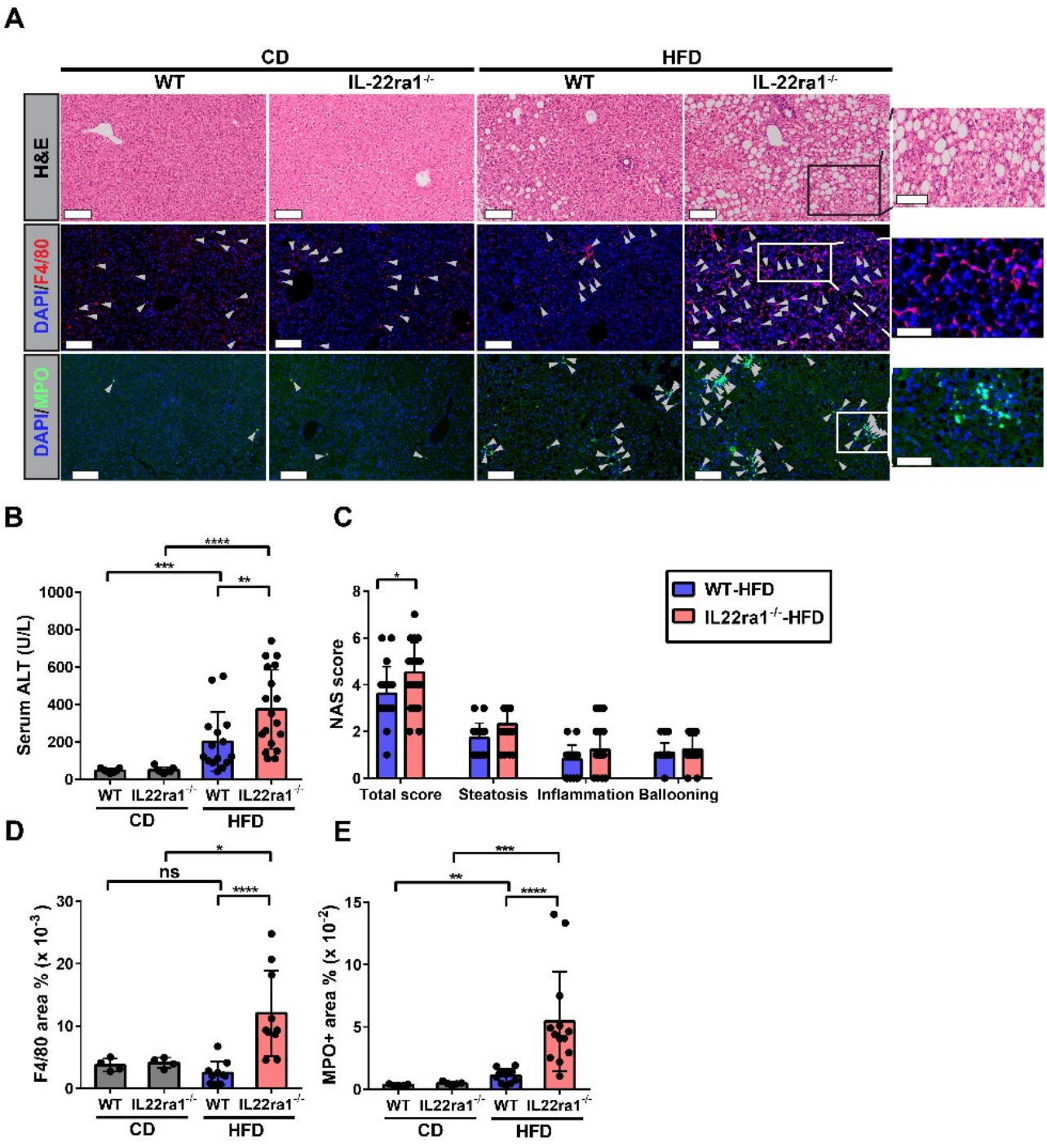
A
CD
HFD
WT
IL-22ra1-/-
WT
IL-22ra1-/-
H&E
DAPI/F4/80
DAPI/MPO
B
Serum ALT (U/L)
1000
800
600
400
200
0
****
***
**
WT
IL22ra1-/-
WT
IL22ra1-/-
CD
HFD
C
NAS score
8
6
4
2
0
*
WT-HFD
IL22ra1-/--HFD
Total score
Steatosis
Inflammation
Ballooning
D
F4/80 area % (x 10-3)
30
20
10
0
*
ns
****
WT
IL22ra1-/-
WT
IL22ra1-/-
CD
HFD
E
MPO+ area % (x 10-2)
15
10
5
0
***
**
****
WT
IL22ra1-/-
WT
IL22ra1-/-
CD
HFD

Figure 6. Lack of IL-22 receptor signaling exacerbates liver injury and degree of inflammation-induced NASH in HFD-fed female mice.

IL22ra1$^{-/-}$ female or male mice and their WT littermates fed on either HFD or CD for 30 weeks (WKs). **(*A*)** Representative microscopic view of liver sections from IL22ra1$^{-/-}$ and WT female mice stained with H&E stain and IF staining of macrophage marker F4/80^{+} (red cells delineated by arrows) and the neutrophil marker, MPO^{+} (green cells delineated by arrows). Scale bars: 100 μm; 20x magnification. The rightmost panels are magnified insets. Scale bars of insets 50 μm for the H&E image and 35 μm for both MPO^{+} and F4/80^{+} IF images. **(*B*)** Measurements of serum ALT. **(*C*)** Blinded pathological evaluation of NAS score (steatosis grade, lobular inflammation and hepatocyte ballooning) by an expert pathologist. **(*D-E*)** Visiopharm quantification of F4/80^{+} **(*D*)** and MPO^{+} **(*E*)** areas in livers of female mice. Data are expressed as mean±SD for 5-22 mice per group (data were pooled from three independent experiments): Mann-Whitney test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. ns; not significant.

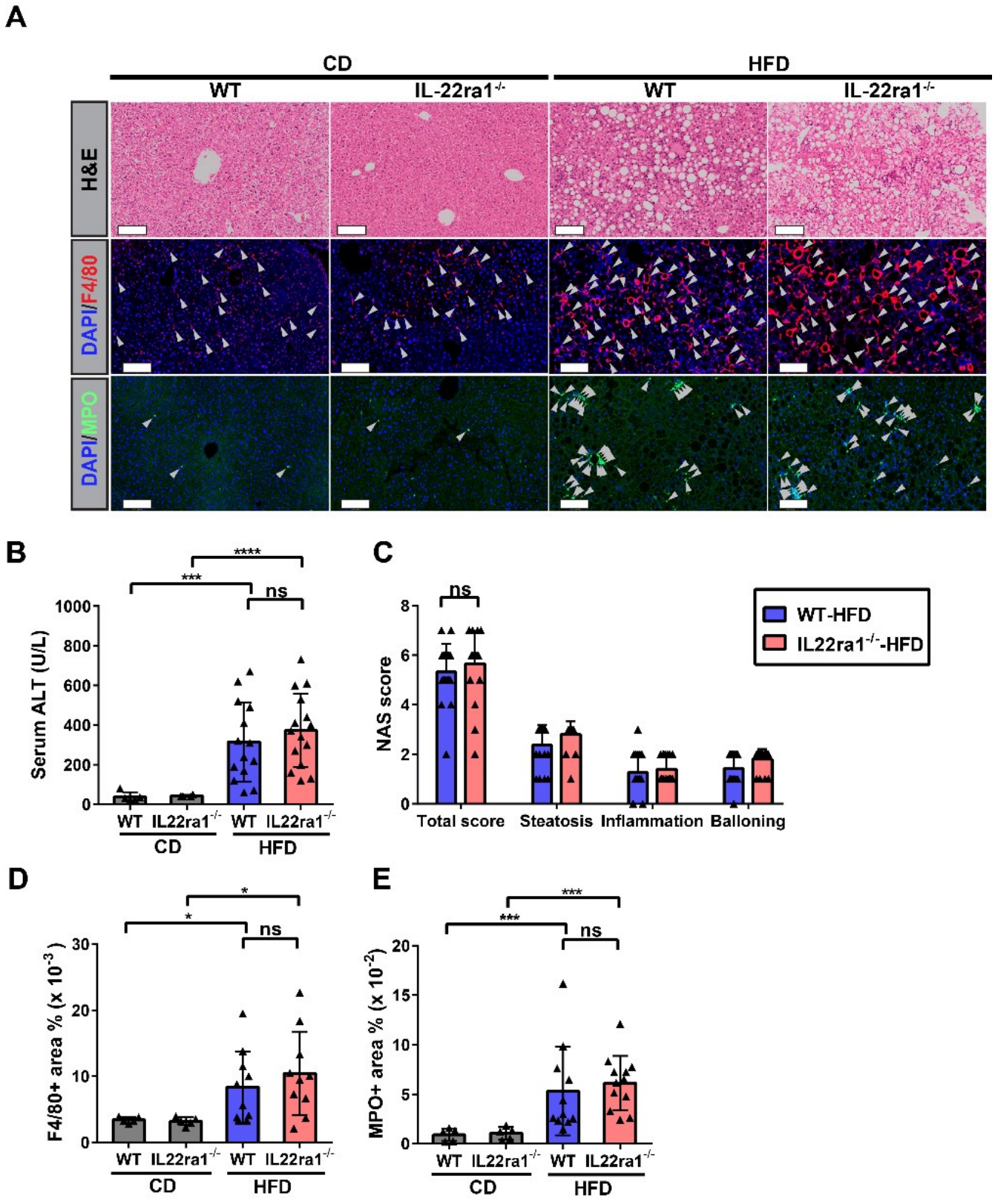
A
CD
HFD
WT
IL-22ra1-/-
WT
IL-22ra1-/-
H&E
DAPI/F4/80
DAPI/MPO
B
Serum ALT (U/L)
1000
800
600
400
200
0
****
***
ns
WT
IL22ra1-/-
WT
IL22ra1-/-
CD
HFD
C
NAS score
8
6
4
2
0
ns
WT-HFD
IL22ra1-/--HFD
Total score
Steatosis
Inflammation
Balloning
D
F4/80+ area % (x 10-3)
30
20
10
0
*
*
ns
WT
IL22ra1-/-
WT
IL22ra1-/-
CD
HFD
E
MPO+ area % (x 10-2)
20
15
10
5
0
***
***
ns
WT
IL22ra1-/-
WT
IL22ra1-/-
CD
HFD

Figure 7. HFD-fed IL-22ra1$^{-/-}$ male mice have comparable profile of inflammation-induced NASH compared to their WT littermates.

(*A*) Representative microscopic view of liver sections of IL-22ra1$^{-/-}$ and WT male mice stained with H&E stain and IF staining of macrophage marker, F4/80^{+} (red cells delineated by arrows) and the neutrophil marker, MPO^{+} (green cells delineated by arrows). Scale bars: 100 μm; 20x magnification. **(*B*)** Measurements of serum ALT. **(*C*)** Blinded pathological evaluation of NAS score (steatosis grade, lobular inflammation and hepatocyte ballooning) by an expert pathologist. **(*D-E*)** Visiopharm quantification of F4/80^{+} **(*D*)** and MPO^{+} **(*E*)** areas in livers of male mice. Data are expressed as mean±SD for 5-20 mice per group (data were pooled from three independent experiments): Mann-Whitney test. *p<0.05, ***p<0.001, ****p<0.0001. ns; not significant.

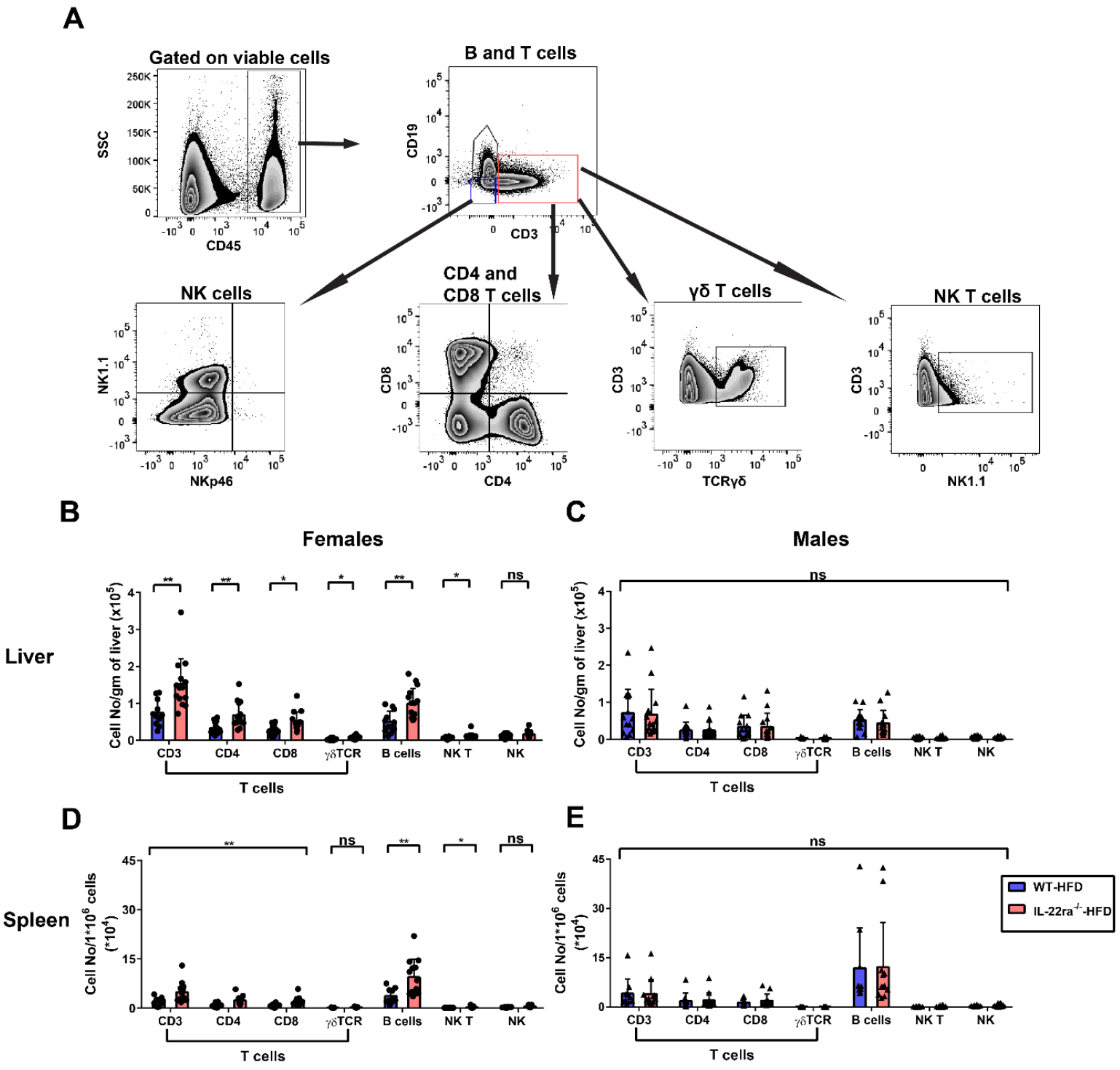
A
Gated on viable cells
SSC
CD45
B and T cells
CD19
CD3
NK cells
NK1.1
NKp46
CD4 and
CD8 T cells
CD8
CD4
γδ T cells
CD3
TCRγδ
NK T cells
CD3
NK1.1
B
Females
C
Males
Liver
Cell No/gm of liver (x10^5)
CD3
CD4
CD8
γδTCR
B cells
NK T
NK
T cells
ns
D
E
Spleen
Cell No/1*10^6 cells (*10^4)
WT-HFD
IL-22ra-/--HFD

Figure 8. HFD-fed IL-22ra1$^{-/-}$ female mice, but not males, develop an increase in the absolute number of adaptive immune cells in their livers and spleen compared to their WT littermates at 30 weeks.

lHLs and splenocytes were extracted from fatty livers and spleen of IL-22ra1$^{-/-}$ and WT female or male mice, respectively and analyzed by flow cytometry. **(*A*)** Representative FACS plot showing an outline for the gating strategy of B cells (CD45^{+} CD19^{+} CD3^{-}), T cells (CD45^{+} CD3^{+} CD19^{-}), NK cells (CD45^{+} CD19^{-} CD3^{-} NK1.1^{+} NKp46^{-}), CD4^{+} T cells (CD3^{+} CD19^{-} CD4^{+}), CD8^{+} T cells (CD3^{+} CD19^{-} CD8^{+}), TCRγδ T cells (CD3^{+} CD19^{-} TCRγδ^{+}) and NK-T cells (CD3^{+} CD19^{-} NK1.1^{+}). The indicated numbers of cell subsets of lHLs **(*B*-*C*)** and splenocytes **(*D*-*E*)** represent cell number/g of liver and splenocyte number/10^{6} cell for female **(*B* and *D*)** and male mice **(*C* and *E*)**, respectively. Data are expressed as mean± SD for 10-13 mice per group/sex (data were pooled from three independent experiments): Mann-Whitney test. *p<0.05, **p<0.01. ns; not significant.

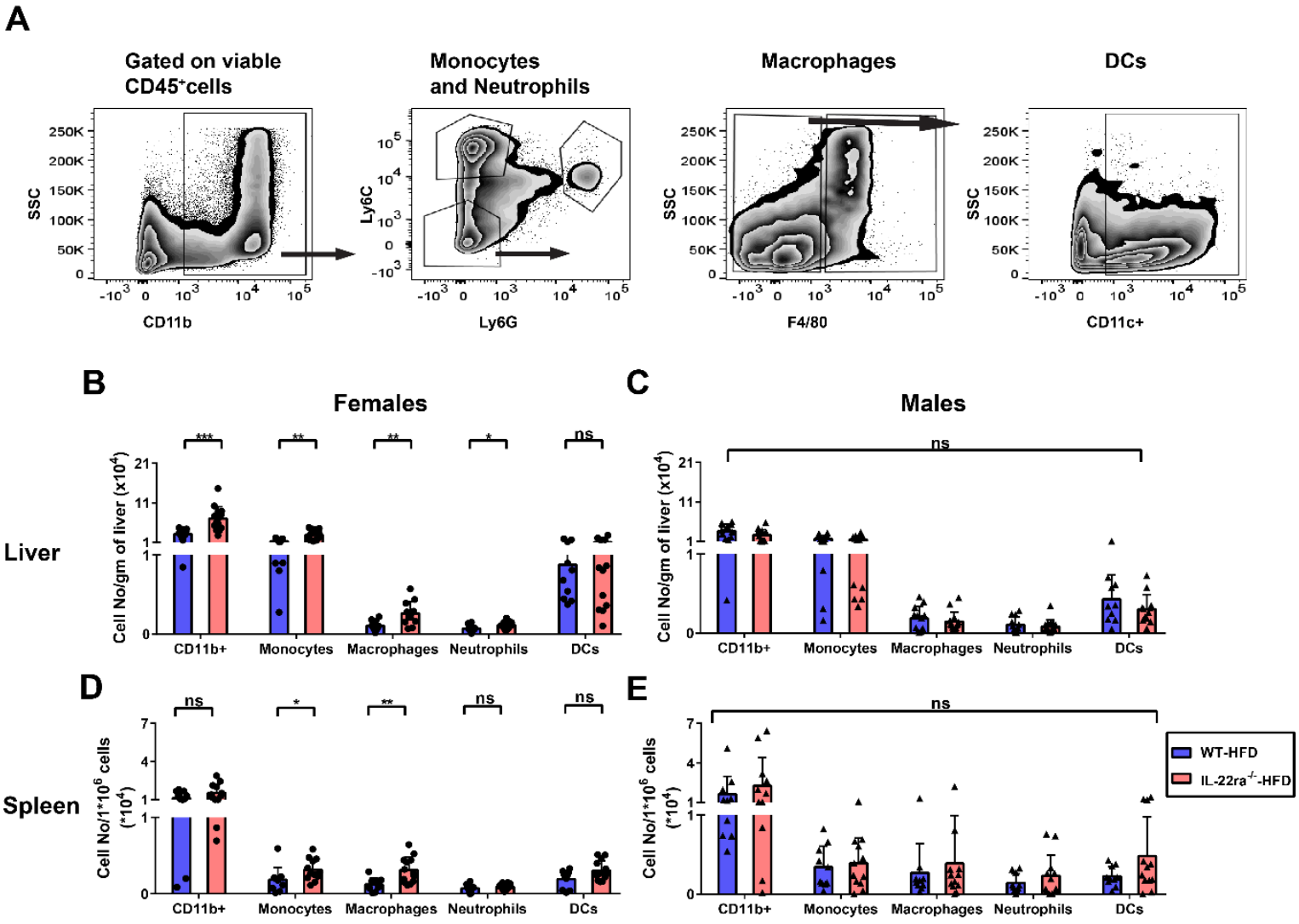
A
Gated on viable CD45+cells
Monocytes and Neutrophils
Macrophages
DCs
SSC
CD11b
Ly6C
Ly6G
F4/80
CD11c+
B
Females
C
Males
Liver
Cell No/gm of liver (x10^4)
CD11b+
Monocytes
Macrophages
Neutrophils
DCs
ns
D
E
Spleen
Cell No/1*10^6 cells (*10^4)
WT-HFD
IL-22ra-/--HFD

Figure 9. HFD-fed IL-22ra1$^{-/-}$ female mice, but not males, develop an increase in the absolute number of innate immune cells in their livers and spleen compared to their WT littermates at 30 weeks.

IHLs and splenocytes were extracted from fatty livers and spleen of IL-22ra1$^{-/-}$ and WT female or male mice, respectively and analyzed by flow cytometry. (***A***) Representative zebra plots showing an outline for the gating strategy of Granulocytes (CD45^{+} CD11b^{+}), Neutrophils (CD11b^{+} Ly6C^{int} Ly6G^{+}), Monocytes (CD11b^{+} Ly6C^{hi} Ly6G^{-}), Macrophages (CD11b^{+} Ly6C^{-} Ly6G^{-} F4/80^{+}) and Dendritic cells (DCs) (CD11b^{+} Ly6C^{-} Ly6G^{-} F4/80^{-} CD11c^{+}). The indicated numbers of cell subsets of IHLs **(*B-C*)** and splenocytes **(*D-E*)** represent cell number/gm of liver and splenocyte number/10^6 cell for female **(*B* and *D*)** and male mice **(*C* and *E*)**, respectively. Data are expressed as mean±SD for 10-13 mice per group/sex (data were pooled from three independent experiments): Mann-Whitney test. *p<0.05, **p<0.01, ***p<0.001. ns; not significant.

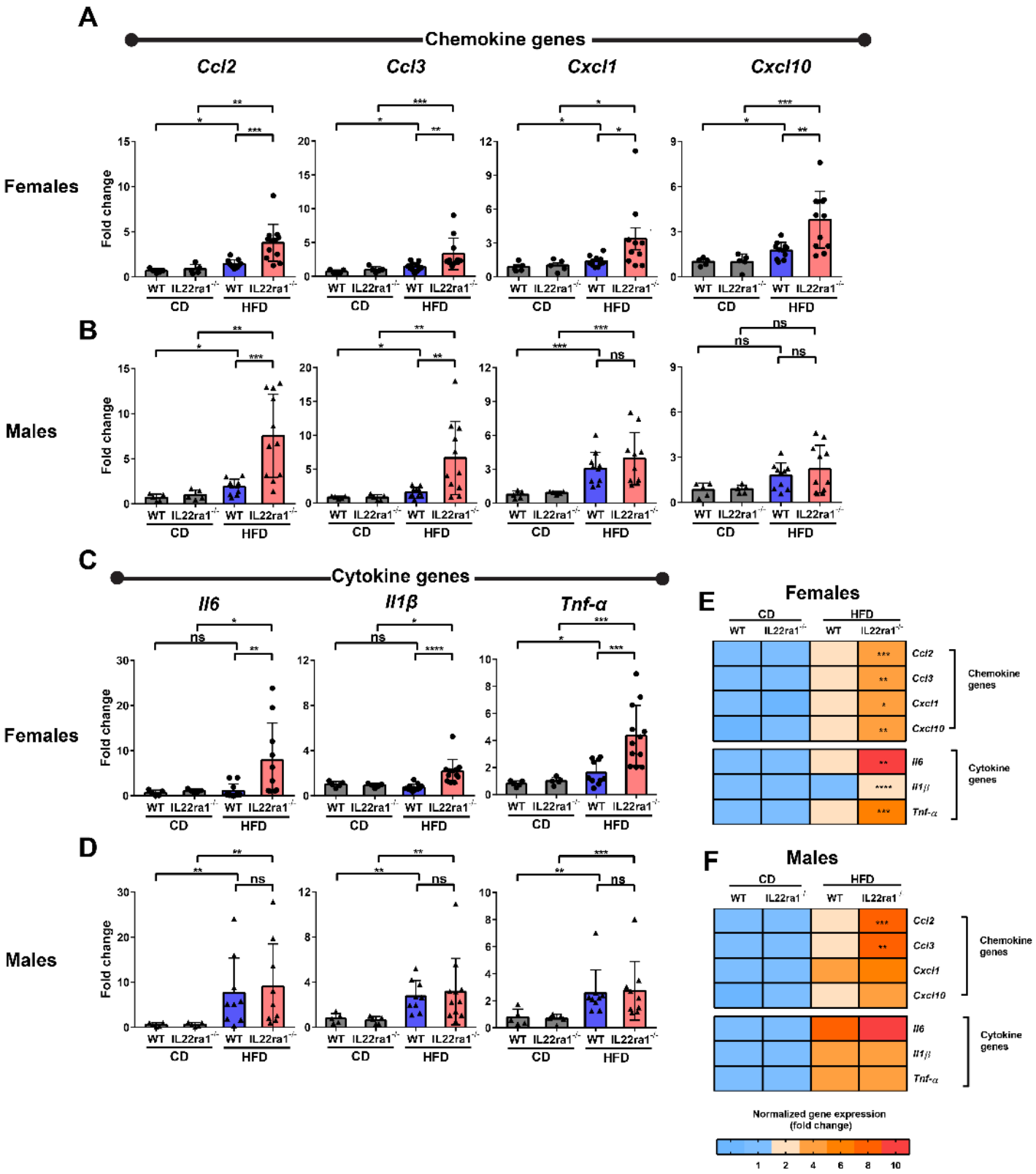
A
Chemokine genes
Ccl2
Ccl3
Cxcl1
Cxcl10
Females
Fold change
WT IL22ra1-/- WT IL22ra1-/-
CD HFD
B
Males
C
Cytokine genes
Il6
Il1β
Tnf-α
D
E
Females
CD HFD
WT IL22ra1-/- WT IL22ra1-/-
Ccl2
Ccl3
Cxcl1
Cxcl10
Chemokine genes
Il6
Il1β
Tnf-α
Cytokine genes
F
Males
Normalized gene expression
(fold change)
1 2 4 6 8 10

Figure 10. Absence of IL-22 receptor signaling results in significant dysregulation of hepatic inflammatory genes in HFD-fed female mice, but not males.

(*A*-*B*) Bar graphs of pro-inflammatory chemokine and/or **(*C*-*D*)** cytokine gene expression (normalized to r28s) as indicated and represented as fold change for female (***A* and *C***) and male mice **(*B* and *D*)**. (***E*-*F***) Heatmaps representing a summary of gene(s) expression in (***A*-*D***) for female **(*E*)** and male **(*F*)**. Asterisk(s) in **(*E* and *F*)** indicate statistical significance between HFD-fed IL22ra1$^{-/-}$ group and their WT littermates. Data are expressed as mean±SD for 5-13 mice per group/sex (data were pooled from three independent experiments): Mann-Whitney test. **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001. ns; not significant.

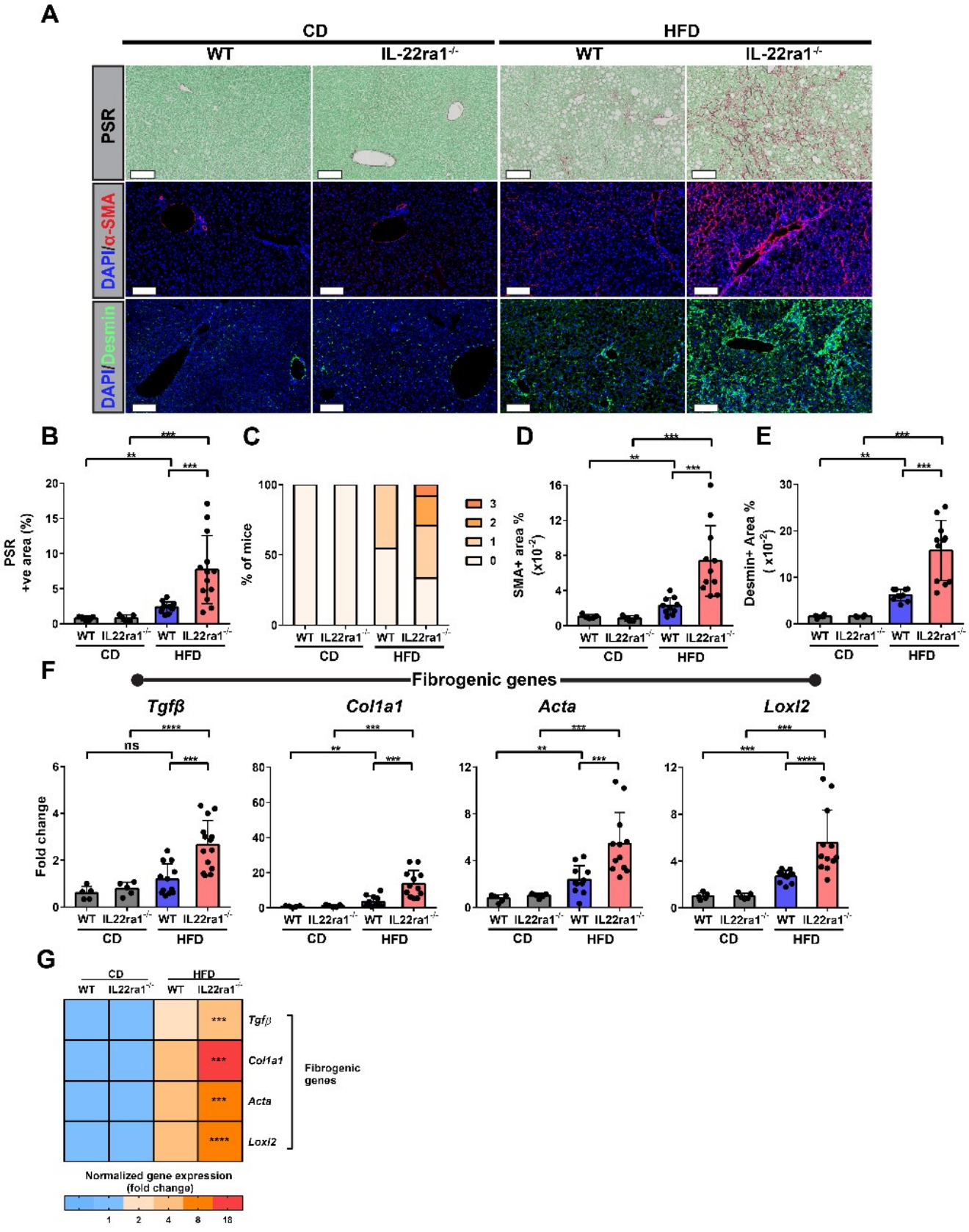
A
CD
HFD
WT
IL-22ra1-/-
WT
IL-22ra1-/-
PSR
DAPI/α-SMA
DAPI/Desmin
B
PSR +ve area (%)
C
% of mice
3
2
1
0
D
SMA+ area % (x10-2)
E
Desmin+ Area % (x10-2)
WT IL22ra1-/- WT IL22ra1-/-
CD
HFD
F
Fibrogenic genes
Tgfβ
Col1a1
Acta
Loxl2
Fold change
ns
G
Tgfβ
Col1a1
Acta
Loxl2
Fibrogenic genes
Normalized gene expression (fold change)
1
2
4
8
16

Figure 11. Loss of IL-22 receptor signaling induces severe NASH-related fibrosis in HFD-fed female mice

IL22ra1$^{-/-}$ female and their WT littermates were fed on either HFD or CD for 30 weeks (WKs). **(*A*)** Representative microscopic and IF images of liver sections of IL22ra1$^{-/-}$ female mice and their WT littermates stained with picrosirius red (collagen shown in red), or α-SMA (Red) or Desmin (green). Scale bars 100 μm; 20x magnification. **(*B*)** FIJI quantification of PSR +ve area in livers of female mice. **(*C*)** Blinded pathological evaluation of liver fibrosis grade of female mice by an expert pathologist. **(*D-E*)** Visiopharm quantification of α-SMA **(*D*)** and Desmin **(*E*)** +ve areas in livers of IL22ra1$^{-/-}$ and WT female mice after HFD or CD treatment for 30 weeks. **(*F-G*)** Bar graphs and heatmap of qPCR data of profibrogenic gene expression (normalized to r28s) as indicated and represented as fold change. Asterisk(s) in **(*G*)** indicate statistical significance between HFD-fed IL22ra1$^{-/-}$ group and HFD-fed WT group. Data are expressed as mean± SD for 5-22 mice per group (data were pooled from three independent experiment): Mann-Whitney test. ***p*<0.01, ****p*<0.001, *****p*<0.0001. ns; not significant.

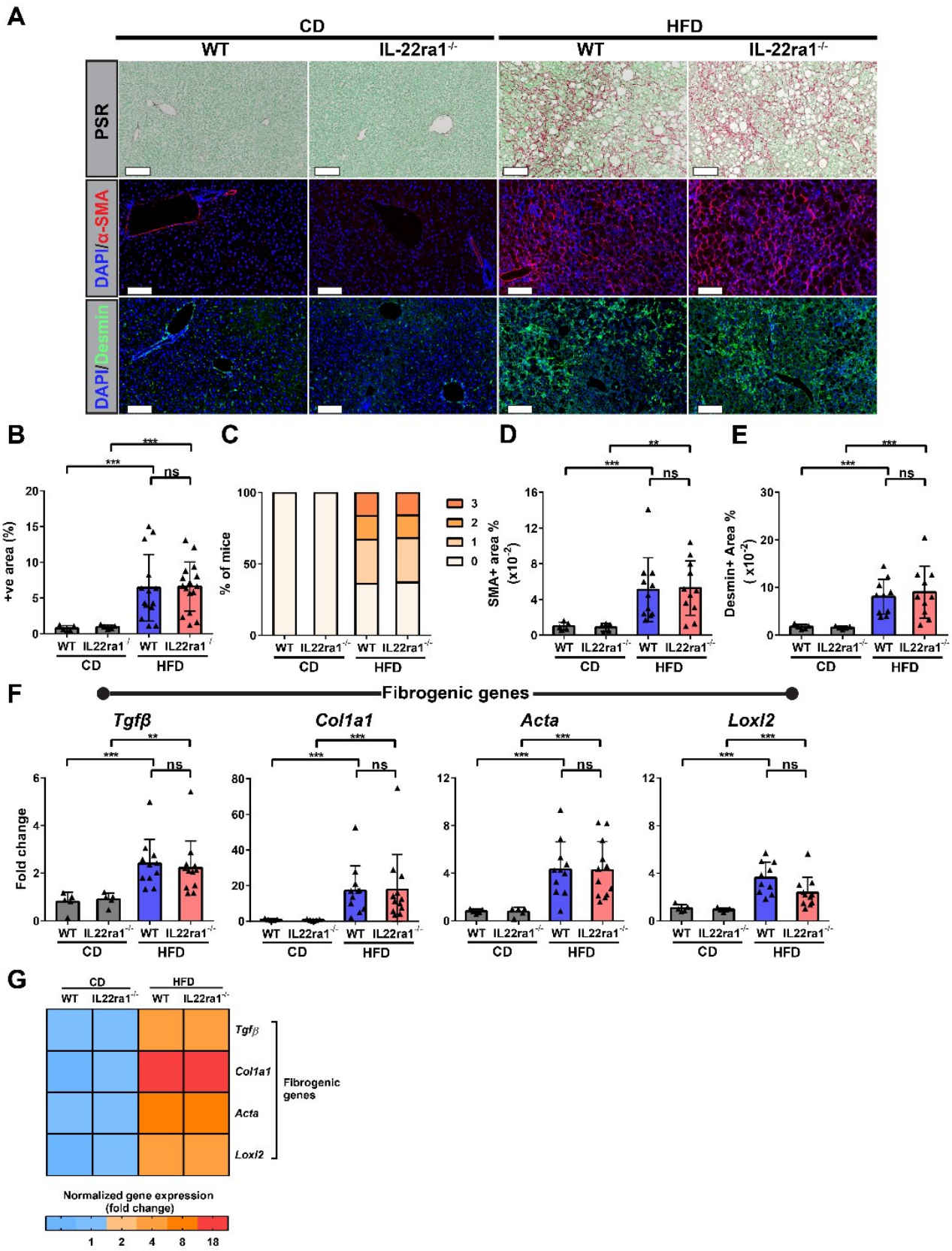
A
CD
HFD
WT
IL-22ra1-/-
WT
IL-22ra1-/-
PSR
DAPI/α-SMA
DAPI/Desmin
B
PSR +ve area (%)
C
% of mice
3
2
1
0
D
SMA+ area % (x10-2)
E
Desmin+ Area % (x10-2)
WT IL22ra1-/- WT IL22ra1-/-
CD
HFD
***
**
ns
F
Fibrogenic genes
Tgfβ
Col1a1
Acta
Loxl2
Fold change
G
Tgfβ
Col1a1
Acta
Loxl2
Fibrogenic genes
Normalized gene expression (fold change)
1
2
4
8
18

Figure 12. HFD-fed IL-22ra1$^{-/-}$ male mice have comparable profiles of NASH-related fibrosis compared to their WT littermates.

(*A*) Representative microscopic and IF images of liver sections of IL22ra1$^{-/-}$ male mice and their WT littermates stained with PSR (collagen in red), or α-SMA (red) or Desmin (green). Scale bars 100 μm; 20x magnification. **(*B*)** FIJI quantification of PSR +ve area in livers of male mice. **(*C*)** Blinded pathological evaluation of liver fibrosis grade of male mice by an expert pathologist. **(*D-E*)** Visiopharm quantification of α-SMA (***D***) and Desmin (***E***) +ve areas in livers of IL22ra1$^{-/-}$ and WT female mice after HFD or CD treatment for 30 weeks. **(*F-G*)** Bar graphs and heatmap of qPCR data of profibrogenic gene expression (normalized to r28s) as indicated and represented as fold change. Asterisk(s) in **(*G*)** indicate statistical significance between HFD-fed IL22ra1$^{-/-}$ group and HFD-fed WT group. Data are expressed as mean± SD for 5-20 mice per group (data were pooled from three independent experiment): Mann-Whitney test. ***p*<0.01, ****p*<0.001, *****p*<0.0001. ns; not significant.

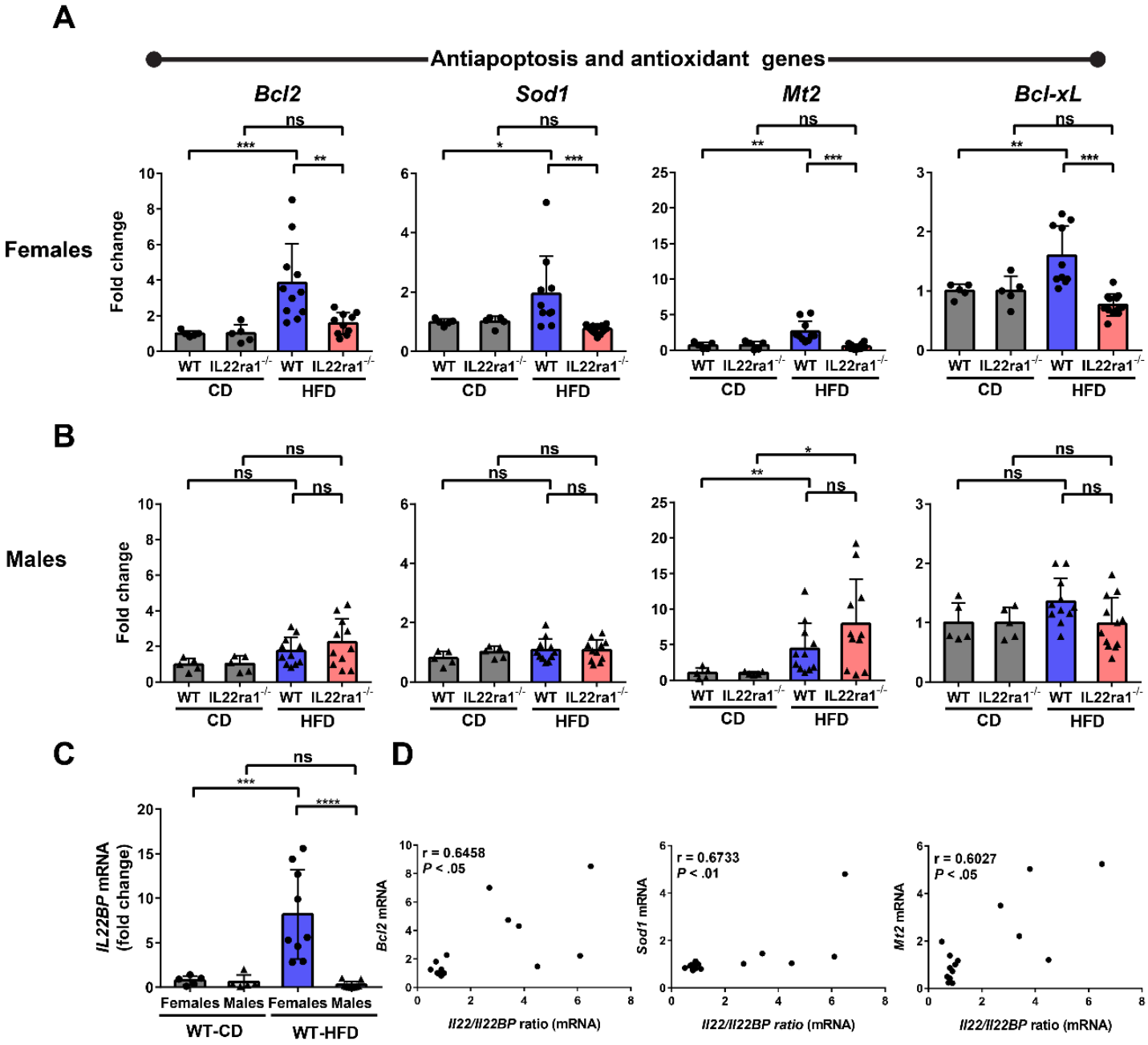
A
Antiapoptosis and antioxidant genes
Bcl2
Sod1
Mt2
Bcl-xL
Females
Fold change
WT IL22ra1-/- WT IL22ra1-/-
CD HFD
B
Males
C
IL22BP mRNA (fold change)
Females Males Females Males
WT-CD WT-HFD
D
Bcl2 mRNA
r = 0.6458
P < .05
Sod1 mRNA
r = 0.6733
P < .01
Mt2 mRNA
r = 0.6027
P < .05
Il22/Il22BP ratio (mRNA)

Figure 13. A positive correlation between hepatic IL-22/IL-22BP ratio and IL-22-induced anti-apoptotic genes in WT female mice.

Female (●) and male (▲) mice were fed HFD or CD for 30 weeks. RNA was extracted from fatty livers of WT female and male mice, converted to cDNA followed by qPCR. **(*A*-*B*)** The expression of anti-apoptotic and antioxidant genes as indicated in livers of IL22ra1$^{-/-}$ female **(*A*)** or male **(*B*)** mice and their WT littermates at 30 weeks. Data are normalized to r28s and represented as fold change. *Il22BP* **(*C*)** mRNA expressions were normalized to r28s and data represented as fold change. **(D)** Spearman correlation graphs between IL-22/IL-22BP ratio (mRNA) in livers of HFD-fed WT female mice and IL-22 downstream target genes: *Bcl2*, *Sod1* and *Mt2* mRNA. Data are expressed as mean± SD for 5-12 mice per group/sex (data were pooled from three independent experiments): Mann-Whitney test. *p<0.05, **p<0.01, ***$p<0.01$, ****$p<0.0001$. ns; not significant.

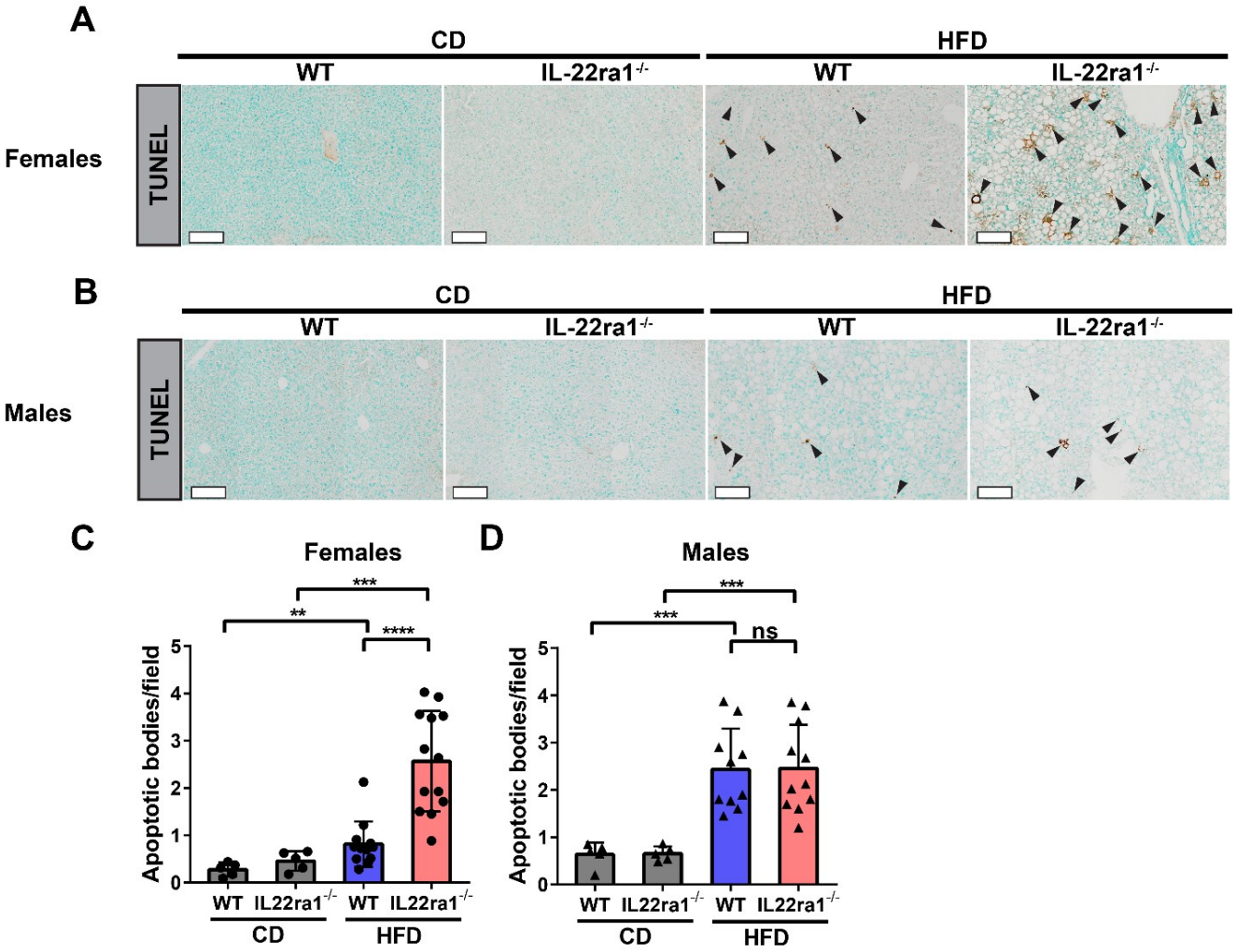

Figure 14. Endogenous IL-22 receptor signaling protects against hepatic apoptosis in HFD-fed WT female mice, but not males.

(***A-B***) Representative microscopic view of liver sections stained with TUNEL stain of IL22ra1$^{-/-}$ and WT female (***A***) or male ***(B)*** mice after HFD treatment for 30 weeks. Scale bars 100 μm. Black arrows indicate apoptosis. (***C-D***) FIJI quantification of apoptotic bodies (count/field) for IL22ra1$^{-/-}$ and WT female (***C***) or (***D***) male mice. Data are expressed as mean±SD for 5-13 mice per group/sex (data were pooled from three independent experiments): Mann-Whitney test. ***p*<0.01, ****p*<0.001, *****p*<0.0001. ns; not significant.

Table 1. NAFLD patients' demographics and clinical characteristics

	Female patients (n=9)	Male patients (n=11)
Age (years)	50.11± 14.39	49.09 ± 11.65
Weight (Kg)	88.89 ± 20.92	96.86 ± 23.57
ALT (IU/L)	66.67± 28.18	75.33 ± 60.64
NAS score Criteria		
Hepatic steatosis	1.889 ± 0.928	1.917 ± 0.793
Lobular inflammation	1.444 ± 0.527	1 ± 0.8528
Hepatocyte ballooning	1.444 ± 0.8819	1.083 ± 0.793
Total NAS score	4.889 ± 1.616	4 ± 2.045
Fibrosis score		
NASH CRN criteria	2.778 ± 1.093	1.917 ± 1.165

Data are presented as mean±SD

Table 2. Clinical data and patient characteristics of 20 patients (Females=9 and males= 11) with NAFLD disease selected in the study

Patient	Diagnosis	Sex	Age	Weight (Kg)	ALT (IU/ml)	Fibrosis grade (Metavir score)	H&E (Total NAS Score)	IF IL-22+ cells
Males (N=11)								
LB051	NAFLD	M	62	62	53	F2	6	√
LB054	NASH	M	41	124	249	F3	5	√
LB059	NASH	M	52	80	66	F0	1	√
LB078	NASH	M	57	93.2	195	F2	2	√
LB084	NAFLD	M	33	76	138	F1-2	5	√
LB099	NAFLD	M	66	N/A	12	F1	2	√
LB102	NASH	M	38	101	65	F0	3	√
LB123	NAFLD	M	52	102	35	F3	5	√
LB125	NAFLD	M	43	140	196	F3	6	√
LB130	NAFLD	M	35	81	115	F2	6	√
LB133	NAFLD	M	61	109.4	16	F1	1	√
Females (N=9)								
LB062	NASH	F	59	100.4	58	F4	5	√
LB076	NASH	F	25	100	138	F1	6	√
LB079	NAFLD	F	52	55	23	F4	2	√
LB093	NASH	F	37	N/A	19	F2	6	√
LB097	NASH	F	62	89	204	F3-F4	3	√
LB118	NASH	F	40	120	45	F2	5	√
LB119	NASH	F	73	93	51	F4	6	√
LB128	NASH	F	51	63	94	F2	7	√
LB131	NASH	F	52	90.7	46	F3-F4	4	√

Table 3. List of IF primary and secondary antibodies

Antibodies	Supplier	Catalog number	Concentration
Primary antibodies			
Anti-hIL-22	Cloud-Clone Corp, USA	MAC032Hu22	1:100
Anti-hCD66b	Novus Biotechnologies	G10F5	1:100
Anti-mIL-22	Santa Cruz Biotechnology, CA	SC-14436	1:100
Anti-mLy6G	BioLegend	127602	1:50
Anti-mCD3	Abcam	Ab16669	1:100
Anti-h/mMPO	R&D	AF3667	1:100
Anti-mF4/80	Synaptic System, Germany	397004	1:50
Anti-mSMA-α	Sigma-Aldrich	A2547	1:100
Anti-mDesmin	Thermo Fisher Scientific, USA	PA5-1670	1:100
Secondary antibodies			
Donkey Anti-mouse CF568	Sigma-Aldrich, USA	SAB4600315	1:500
Donkey Anti-mouse A647	Thermo Fisher Scientific	A31571	1:500
Donkey Anti-mouse IgM A488	Jackson ImmunoResearch	715-545-020	1:300
Donkey Anti-goat A568	Thermo Fisher Scientific	A11057	1:500
Chicken Anti-Rat A678	Thermo Fisher Scientific	A21472	1:250
Donkey Anti-Rabbit A678	Thermo Fisher Scientific	A31573	1:500
Donkey Anti-Rabbit A488	Thermo Fisher Scientific	A21206	1:500
Donkey Anti-Guinea pig A678	Jackson ImmunoResearch	706-605-148	1:500

Table 4. List of FC antibodies

FC Antibodies	Supplier	Catalog number
CD45-BV650	BioLegend	103151
CD3-PECF594	BD bioscience	562286
CD4-BUV496	BD bioscience	612952
CD8-A700	BioLegend	100730
TCRγδ-BV421	BD bioscience	744118
CD19-APC-H7	BD bioscience	560143
NK1.1-A488	BioLegend	108718
NKp46-SB600	ebioscience	63-3551-82
CD11b-BV421	ebioscience	63-0112-82
Ly6C-PECF594	BD bioscience	562728
Ly6G-A488	BioLegend	127626
F4/80-BUV395	BD bioscience	565614
CD11c-PE	BD bioscience	565592
IL-17A-APC	eBioscience	17-7177-81
IL-22-PE	eBioscience	12-7227-82

Table 5. List of primer sequences

Gene Name	Forward (5'-3')	Reverse (5'-3')	Reference
Il22	ATGAGTTTTTCCCTTATGGGGAC	GCTGGAAGTTGGACACCTCAA	[667]
Il6	AGGATACCACTCCCAACAGACCT	CAAGTGCATCATCGTTGTTCATAC	[697]
Il1β	GGCAGGCAGTATCACTCATT	GAGGATGGGCTCTTCTTCAAA	[698]
Tnf-α	ACTCCAGGCGGTGCCTATGT	GTGAGGGTCTGGGCCATAGAA	[699]
Il23 p-19	CCCCCTTCTCCGTTCCAA	GGGCAGCTATGGCCAAAAA	[700]
Cxcl1	GGATTCACCTCAAGAACATCCAG	ATCTTTTGGACAATTTTCTGAACC	[701]
Cxcl10	CTTCTGAAAGGTGACCAGCC	GTCGCACCTCCACATAGCTT	[702]
Ccl2	TCTGGACCCATTCCTTCTTGG	TCAGCCAGATGCAGTTAACGC	[668]
Ccl3	GTGGAATCTTCCGGCTGTAG	ACCATGACACTCTGCAACCA	[703]
Bcl2	ATGCCTTTGTGGAACTATATGGC	GGTATGCACCCAGAGTGATGC	[704]
Bcl-xl	GCTGCATTGTTCCCGTAGAG	GTTGGATGGCCACCTATCTG	[636]
Sod1	GAGACCTGGGCAATGTGACT	GTTTACTGCGCAATCCCAAT	[705]
Mt2	GCCTGCAAATGCAAACAATGC	AGCTGCACTTGTCGGAAGC	[668]
28s	CGAGATTCCCACTGTCCCTA	GGGGCCTCCCACTTATTCTA	[482]
Acta2	QT00140119		N/A
Col1a1	QT00162204		
Tgfβ1	QT00145250		
Loxl2	QT00129052		

Chapter 4:

Manuscript 2

Title: Profibrogenic role of IL-17A-induced NET in Non-Alcoholic Fatty Liver Disease (NAFLD)

(In Preparation)

Profibrogenic role of IL-17A-induced NET in Non-Alcoholic Fatty Liver Disease (NAFLD)

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Authors' contributions:
MNA: Study concept and design; acquisition of data; analysis and interpretation of data; drafting of the manuscript; MFN: Acquisition of data, GS: Pathological evaluation of mouse liver tissue; JD: Acquisition of data and technical support for animal experiments; NHS: Study concept and design, obtained funding, supervised the overall study and co-wrote the manuscript. All authors had the opportunity to review the manuscript and provide additional intellectual input.

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Introduction:

Non-alcoholic fatty liver disease (NAFLD) has become the most prevalent chronic liver disease due to the rise in obesity, insulin resistance (IR) and type 2 diabetes (T2DM)[2, 680]. NAFLD embraces a wide spectrum of liver disease ranging from hepatic steatosis (HS), non-alcoholic steatohepatitis (NASH), advanced fibrosis, cirrhosis and ultimately hepatocellular carcinoma (HCC)[4, 220]. NASH-induced inflammation is associated with activation of hepatic stellate cells (HSC) and thus accelerating fibrosis progression[4, 390]. Importantly, liver-related mortality increases exponentially with the increase in fibrosis stage in patients with NAFLD[3]. Currently, there are no approved therapies for NASH, but many interventional studies are ongoing[4, 220].

IL-17 is a cytokine with proinflammatory activity, and it is a member of the IL-17 family that comprises six members: IL-17A-F, of which IL-17A is the most widely investigated. IL-17A is produced by many immune cells, including Th17, $CD8^+$ T cells (Tc17), γδ T cells, innate lymphoid cells 3 (ILC3s), neutrophils and mast cells[5, 451, 523]. IL-17 receptor (IL-17R), a heterodimer receptor consisting of IL-17RA and IL-17RC subunits, is expressed by epithelial cells, endothelial cells, and fibroblasts as well as macrophages [5, 456, 458]. The IL-17/IL-17R axis regulates the expression of proinflammatory cytokines, neutrophil chemo-attractants (e.g., CXCL-1), antimicrobial peptides (AMPs), angiogenic factors, and matrix metalloproteinases (MMPs). These effector functions of IL-17 are essential for protecting mucosal surfaces against extracellular bacteria and fungi in different organs, including the liver, but also contribute to the pathogenesis of autoimmunity, inflammatory diseases, fibrosis and carcinogenesis [5, 446, 523, 706].

There is well-established evidence that IL-17A mediates proinflammatory as well as profibrogenic effects, which are associated with injury severity, inflammation and liver fibrosis in both mice and humans with chronic liver diseases. For instance, an increase of Th17/Treg balance in chronic viral hepatitis (CVH) was associated with hepatic injury[502-505] or progression to liver cirrhosis and HCC [504, 506, 507] and inversely correlated with mortality[506-508]. In addition, we also showed that the

density of intrahepatic IL-17-producing cells, including Th17, neutrophils and mast cells, was increased in patients' liver biopsies with advanced fibrosis irrespective of etiology [523]. Moreover, liver fibrosis, induced by CCl_4 or bile duct ligation (BDL), was attenuated in IL-17RA$^{-/-}$ mice and this was associated with reduced expression of profibrogenic genes, including *Col-α1*, *Acta2*, and *Tgfβ* [472, 517]. A similar observation was reported in other models of liver fibrosis [531, 532, 534].

Mechanistically, these detrimental effects of IL-17 are mediated by promoting recruitment of proinflammatory monocytes and neutrophils leading to fibrosis development. In addition, IL-17 can directly activate HSCs *in vitro*, and increase collagen and α-SMA expression in a STAT3- and ERK1/2/p38-dependent manner[472, 517]. Also, IL-17A can indirectly modulate HSCs and induce fibrogenesis by activating Kupffer cells (KCs), neutrophils, and monocytes, which further drives the production of TGF-β as well as IL-17 in an inflammatory loop [464, 470, 472, 473]. Moreover, *in vitro* work showed that IL-17A sensitized HSCs response to suboptimal doses of TGF-β by upregulating their TGF-β-RII expression, via a JNK-induced SMAD2/3 signalling pathway and hence enhanced fibrogenesis [482].

On the other hand, some studies reported beneficial metabolic effects of IL-17 against weight gain and fat mass in diet-induced obesity and/or NAFLD models[521, 522, 545], where IL-17 activation suppressed the expression of proadipogenic transcription factors such as Peroxisome proliferator- activated receptor gamma (PPARγ). On the contrary, IL-17 was reported to enhance IR and to inhibit glucose uptake in adipose tissue and liver in various NAFLD models[521, 522, 545, 549, 550]. The role of IL-17-induced hepatic steatosis development in NAFLD remains controversial. The lack of IL-17 activity was associated with increased hepatic steatosis in different NAFLD models [521, 527, 553]. In contrast, *in vitro* stimulation of hepatocytes with IL-17 promoted fat accumulation, likely by upregulating hepatic PPARγ expression[520, 522].

Furthermore, other studies reported the involvement of the IL-17 axis in promoting NASH-related inflammation and liver fibrosis. The Th17/Treg ratio, in the liver and peripheral blood of obese patients, has been positively associated with transition from NAFL to NASH[513]. In addition, recently, a unique subset of Th17, known as inflammatory hepatic $CXCR3^{+}$ $IL\text{-}17^{+}$$IFN\text{-}\gamma^{+}$ $TNF\text{-}\alpha^{+}$ Th17 (ihTh17,) has been identified in the liver of NAFLD patients and correlated with disease severity. This ihTh17 subpopulation is characterized by augmented inflammatory and metabolic genes expression compared to conventional hepatic $CXCR3^{-}$Th17 (chTh17) cells[561]. Moreover, blocking IL-17 activity ameliorated NASH-related inflammation and liver fibrosis in different NASH models[515, 520-522, 527]. However, all these studies were mainly limited to Th17 and lacked investigation of other IL-17-producing cells such as neutrophils.

Neutrophils can contribute to NASH pathogenesis through release of inflammatory mediators including myeloperoxidase (MPO), cytokines, and the neutrophil extracellular trap structures (NETs)[707]. Neutrophils producing IL-17 have been reported in autoimmune diseases as well as chronic liver diseases, especially CVH and were associated with liver fibrosis progression[523, 706, 708]. In addition, in normal conditions, neutrophils release NETs, comprising nucleic acids, histones, and antimicrobial peptides (AMPs), into the extracellular space to entrap pathogens, leading to host defense. The markers of NETs formation, including MPO-DNA complexes or citrullinated histone H3 (CitH3) were increased in circulation and livers of human and murine NASH and correlated with NAFLD severity, suggesting a pathogenic function of these NETs [408-412]. Interestingly, IL-17A was reported to induce NET formation, which enhanced liver necrosis in acute ischemia reperfusion model, while anti-IL-17A inhibited NETs and ameliorated liver injury[709]. Moreover, IL-17A expressed in NET structures promoted the fibrotic activity and collagen deposition of differentiated lung fibroblasts[710]. Nevertheless, the role of IL-17-induced NETs and its impact on hepatic fibrosis progression in the context of NASH remains unknown.

Here, we investigated the kinetics of intrahepatic IL-17A-producing cells during progression of NAFLD-related fibrosis using a high fat diet (HFD)-fed mouse model at two time points: 15 or 30 weeks on the diet. Also, we investigated the role of IL-17A-induced NET formation and its role in NAFLD-related fibrosis. We report marked increase in the level of intrahepatic IL-17A-producing cells, including neutrophils and T cells, in mice at 30 weeks on HFD compared to those at 15 weeks. Neutrophils and/or neutrophils producing IL-17A positively correlated with advanced liver fibrosis and liver injury in mice at 30 weeks on HFD. Also, we demonstrated that *in vitro* stimulation of bone marrow neutrophils with IL-17A induced NETs formation, while anti-IL-17A inhibited NETs formation. Our data highly suggest an active role of IL-17A^{+} neutrophils in NAFLD-related fibrosis.

Materials and Methods:

Mice

21- to 23-weeks old vs 36-38 weeks old male WT mice, with C57BL/6N background, were fed High-fat diet (HFD, 40% Kcal fat, Cat #D17010102I, Research Diets, US) or Chow diet (CD, 6.2% Kcal fat, Teklad global 18% protein rodent diet, ENVIGO) for 15 or 30 weeks. All mice were terminally euthanized at 15- or 30-weeks using pentobarbital (400 mg/kg) and 2% xylocaine. After dissection, liver and blood samples were harvested. All animal experimental procedures were approved by CRCHUM animal ethics committee, Comité Institutionel de Protection des Animaux (CIPA) (protocol IP18035NSs).

Body composition analysis (fat/lean mass)

At 15 or 30 weeks of HFD or CD, body composition of experimental mice was measured using an EchoMRI-100 Body Composition Analyzer (version 2008.01.18) at the rodents-cardiovascular core facility of CRCHUM.

Intraperitoneal glucose tolerance test (IPGTT) and insulin assay

After 15 or 30 weeks on HFD or CD, experimental mice were food-deprived for 5 h with ad libitum access to water. A bolus of glucose (1.5 g/kg) was administered via intraperitoneal (IP) injection and glycaemia was measured from blood sampled at the tail vein using an Accu-check Performa glucometer at T0 (before injection), 15, 30, 45, 60, 90 and 120 minutes. Tail vein blood samples were collected via a capillary for insulin assays at 0 min.

Measurement of Serum ALT

Blood samples were drawn using cardiac puncture of the mice and the serum Alanine Aminotransferase (ALT) levels were measured at the OPTILAB of the CHUM.

Fibrosis quantification

The fibrotic area (PSR positive area) was determined on the total liver section area and PSR positive area was calculated by applying a threshold method in the green channel using FIJI (version 1.52a, U. S. National Institutes of Health, USA) image analysis software.

Histology

Liver specimens of mice were fixed in Tissue Fix (Chaptec, Cat.no.T-50, Montreal, QC) overnight at 4°C, and finally embedded in paraffin for sectioning (BZ-Histo Services Inc., Montreal, QC). The 5-µm thick FFPE sections were deparaffinized and rehydrated, then stained with picrosirius red (PSR) stain (Sigma-Aldrich, Cat.no.365548-5G) with Fast green (Sigma-Aldrich, Cat.no. F7252) or H&E. The H&E stain was performed by BZ-Histo Services Inc.

Immunofluorescence (IF) staining and Image analysis

The IF technique and image analysis were performed as previously described[695]. Briefly, FFPE liver sections (human or mice) were deparaffinized and rehydrated. For the antigen retrieval step, sections were immersed in Sodium citrate solution (pH 6) for 10 minutes (with exception to mF4/80 antibody, the incubation was 20 minutes) in an electric high-pressure cooker (Salton). Then sections were incubated in 0.1M glycine for 15 minutes at 25°C to reduce autofluorescence, followed by blocking with (10% human serum, 1% BSA, 0.1% Tween 20 and 0.3% Triton-X 100 in PBS solution) for 30 minutes. Sections were then incubated with primary antibodies (**Table 1**) overnight in (1% BSA, 0.1% Tween 20 and 0.3% Triton-X 100 in PBS solution) at 4°C. Next, sections were washed in PBS-Tween and then incubated with the appropriate secondary antibodies (**Table 1**) in blocking buffer for 1 hour at 25°C, to be mounted in Slow fade Gold mounting media with DAPI (Cat.no. S36938, Thermo Fisher Scientific, Fremont, CA, US). Finally, images were acquired at the CRCHUM molecular pathology platform using Whole slide scanner Olympus BX61VS. For quantification of IL-17A or neutrophils producing IL-17A, Visiopharm software (Broomfield, CO) was used, including tissue detection (tissue vs non-tissue),

identification and automatic calculation of area of interest. Threshold settings based on pixel value was used for generating the density of IL-17A cells or neutrophils producing IL-17A.

Mouse intrahepatic leukocytes (IHLs) isolation

IHLs were isolated from mice livers using a Percoll gradient (Cat.no.P1644, Sigma-Aldrich) in isotonic solution. Briefly, mice livers were cut into small pieces and digested in collagenase D (0.025IU/mL, Cat.no.110088866001, Roche, Laval, QC) and benzonase (10 IU/mL, Cat.no. 70664-10KUN, EMD Millipore, Germany) at 37°C with rotation for 25 minutes. Then, liver tissues were passed through a 70 µm cell strainer (Cat.no. 22363548, Fisher) followed by centrifugation. The cell pellet was then resuspended in Percoll 40% in 1% HBSS in sterile water (vol/vol) and layered over Percoll 80% in 1% HBSS in sterile water (vol/vol), followed by centrifugation without brakes for 25 minutes. Next, IHLs were washed, and red blood cells (RBCs) were lysed using ACK lysing buffer (Cat.no.A10492-01, Thermo Fisher Scientific). IHL were directly stained for surface markers or stimulated with PMA/Ionomycin (50ng/mL (Cat.no.P1585) and 1 µg/mL (Cat.no. I-0634), Sigma-Aldrich, respectively) in presence of brefeldin A (5 µg/mL, Cat.no. B6542, Sigma-Aldrich) and monensin (5 µg/mL, Cat.no.M5273, Sigma-Aldrich) for 5 h prior to intracellular staining (ICS).

Flow cytometry

IHL were stained as previously described[523]. Briefly, freshly isolated IHLs and splenocytes were washed with FACS Buffer (1% heat inactivated fetal bovine serum (FBS) and 0.01% azide in PBS), followed by incubation with primary antibodies (**Table 2**) at 4°C for 30 min. Next, cells were washed with FACS Buffer and fixed with 1% paraformaldehyde (PFA) in PBS and filtered using polystyrene tubes with cell strainer (Corning science, Mexico). For intracellular cytokine staining (ICS), surface staining was performed first and then cells were fixed and permeabilized using FOXP3 fixation buffer (Cat.no.005523-00, eBioscience). Next, cells were washed twice with washing buffer (eBioscience) and incubated with antibodies for intracellular antigens at 4°C for

30 min. For detection of live cells, Aqua Live/Dead Fixable Dead Cell Stain kit was used (Cat.no. L34966, Life Technologies, Burlington, ON). Data were acquired using a multicolor BD LSRII flow cytometer (BD Bioscience) equipped with FACS DIVA software version 8 and analyzed using FlowJo software, version 10 (BD Bioscience).

Western blot

Total liver proteins from HFD- or CD-fed mice were extracted in RIPA buffer (NaCl, Nonidet P-40, Sodium Dodecyl Sulfate (SDS), Na Deoxycholate, Triton-X 100, Tris-HCl buffer [pH 8]) in the presence of protease and phosphatase inhibitors (Roche, Cat.no. 05 892 791 001 and 04 906 837 001). A Pierce rapid Gold BCA Protein Assay (Thermofisher, Cat.no. a53227) was used to quantify total proteins in each sample, and 20ug total proteins were loaded on 15% polyacrylamide gel and transferred onto PVDF membrane (GE Cat.no. RPN303F). Blots were blocked in 5% non fat dry milk (BioShop Cat.no. SKI400), and then incubated with primary antibodies overnight at 4 °C, anti-CitH3 antibody (1:5000, Abcam, Cat.no. ab5103) or anti- β-actin antibody (1:5000, Cloud-Clone Corp, Cat.no. CAB340Hu22), followed by incubation with secondary HRP-conjugated antibody (1:2500, Cell Signaling Technology, Cat.no.7074S) at room temperature for 1 hour. Finally, blots were developed with the ECLTM Prime Western Blotting Detection Reagent (Cytiva, Cat.no. RPN2232) and images were acquired using Bio-Rad instrument (ChemiDocTM MP Imaging System). β-actin was used as a loading control.

***In vitro* NET formation**

A naïve mouse was sacrificed, its Tibia and Femurs harvested, and all attached connective tissues were removed. The metaphysis of Tibia and Femurs was exposed using scissors and the bone marrow (BM) was collected by applying microcentrifugation at 10, 000 g for 15 sec. BM cells were then suspended in ACK lysing buffer to remove RBCs. Then, mouse mature neutrophils were isolated from BM cells using discontinuous histopaque gradient, including histopaque 1119 and histopaque 1017, at ratio 1:1. The isolated purity of neutrophils was confirmed by flow

cytometry (>95%) based on surface staining for neutrophils ($CD45^+$ $CD11b^+$ $Ly6C^{int}$ and $Ly6G^{hi}$). Isolated mature neutrophils (2×10^5 cells) were suspended in RPMI 1640 without phenol red and seeded in a glass cover slide with 8 wells. Then, neutrophils were allowed to adhere on the slide for 30-60 minutes in the incubator (37°C, 5% CO2), followed by stimulation with rIL-17A (80 pg/ml) in presence or absence of anti-IL-17A (0.5 ug/ml) for 4 hours under the same conditions. An isotype (normal Rat IgG) was used in the absence of anti-IL-17A. Stimulation with PMA (20nM) was used as a positive control, while unstimulated neutrophils were used as negative controls. After the stimulation period, supernatants were discarded, and adhering neutrophils were fixed in 4% PFA overnight at 4°C. Next, the PFA was removed, and wells washed with PBS, followed by incubation with blocking buffer (PBS + 0.3% triton +1% BSA + 5% human serum) for 30 min at room temperature. Then, adhering neutrophils were incubated with primary antibodies, including anti-MPO (1:400 dilution) and anti-CitH3 (1:200), for 1 hour in the incubator (37°C, 5% CO2). After several washes with PBS-Tween, neutrophils were incubated with secondary antibodies, including donkey anti-rabbit (1:500 dilution) and donkey anti-goat (1:500 dilution) antibodies, for 1 hour at room temperature. Following multiple washes again with PBS-Tween, the slide was allowed to dry and then mounted with DAPI to stain nuclei or extracellular DNA of NET. Finally, the images were acquired at the CRCHUM molecular pathology platform using Whole slide scanner Olympus BX61VS. For quantification of % NETosis (number of NETs/total number of neutrophils* 100), FIJI software (Version 1.53q, U. S. National Institutes of Health, US) with DANA macros were used as described in [711].

Quantitative real-time PCR (RT-qPCR)

Total RNA was isolated and purified from mouse livers using RNeasy Mini kit plus (Cat.no. 74134, QIAGEN, Germany) according to the manufacture's instructions. Then, 1µg of RNA was reverse transcribed to cDNAs using Transcriptor Universal cDNA Master (Cat.no. 05893151001, Roche, Germany) kit. cDNAs were diluted 1:10 with ultra-pure water and the relative expression of mRNA

level was measured by using RT-qPCR with Light Cycler 480 SYBR Green I system (Cat.no. 04707494001, Roche). r28S was used as standard house keeping gene and the $2^{-\Delta\Delta Ct}$ method was applied to calculate the mRNA level. Primers for *Tgfβ* (QT00145250), *Col1a1* (QT00162204), *Acta2* (QT00140119) and *Loxl2* (QT00129052) genes were purchased from QIAGEN.

Statistical Analysis

GraphPad Prism 7 (La Jolla, CA) and Sigma plot 14 (Version: 1.0.23) were used. Mann-Whitney test was applied to determine differences between two groups. Correlations were tested using Spearman's rank correlation.

Results

HFD-fed mice develop significant metabolic abnormalities and liver injury after 30 weeks on feeding compared to 15 weeks.

To develop an in vivo NAFLD model, WT C57BL/6N male mice were placed on either a HFD that simulates western diet, or a control diet for 15 or 30 weeks as described in Materials and Methods. We first explored the metabolic abnormalities associated with our NAFLD model. HFD-fed mice developed significant increase in weight gain, fat, and lean mass after 30 weeks of feeding compared to 15 weeks (**Supplementary Figure 1A-C**). This was accompanied by an increase in insulin resistance, but not glucose intolerance (**Supplementary Figure 1D-E**). However, after 15 weeks of feeding, there was no difference in weight gain, fat or lean mass, serum insulin, and glucose intolerance between HFD and CD groups (**Supplementary Figure 1A-E**). Mice fed with HFD developed higher liver index, and serum ALT after 30 weeks of feeding as compared to 15 weeks (**Supplementary Figure 1F-G**). Also, both HFD-fed groups had significant higher serum ALT compared to control groups (**Supplementary Figure 1G**). Taken together, these data highlight that long-term feeding of HFD (30 weeks) promoted the development of metabolic alterations and liver injury as compared to short-term feeding (15 weeks).

Neutrophils positively correlated with exacerbation of liver fibrosis in HFD-fed mice after 30 weeks of feeding compared to 15 weeks

Histologically, NASH is characterized by hepatic steatosis, lobular inflammation (steatohepatitis), liver injury (hepatocyte ballooning) and various degrees of fibrosis[220]. The chronic lobular inflammation is a key driver of human and murine NASH progression as promotes advancement of hepatic fibrogenesis, which can eventually lead to cirrhosis[4]. Also, liver fibrosis is initiated by activation of HSCs to transdifferentiate into myofibroblasts characterized by marked upregulation of type I collagen (COL1A1) and alpha-smooth muscle actin (α-SMA, ACTA2) [1, 145]. Given that neutrophil infiltration is one of the characteristic hallmarks of NASH-related inflammation and has

been associated with NASH severity and liver fibrosis progression[385, 390, 391], we examined the hepatic infiltration of neutrophils ($Ly6G^+$) in our NAFLD model, using IF. Interestingly, we observed an increase in the hepatic density of neutrophils in HFD-fed mice after 30 weeks of feeding as compared to 15 weeks (**Figure 1A-B**). This was associated with advancement in NASH pathological severity and NASH-related fibrosis (chicken wire-like perisinusoidal fibrosis), as illustrated by the NAFLD activity score (NAS) and collagen type I deposition, measured by H&E and PSR staining, respectively (**Figure 1A, C-D**). However, the NAS score of HFD-fed mice at 30 weeks as compared to those at 15 weeks did not reach a significant level (mean=5.153 vs 4.076 p=0.0796) (**Figure 1C**). The fibrosis grade was blindly evaluated by an expert pathologist and was consistent with the PSR positive area data above (**Figure 1E**).

Next, we examined the mRNA expression levels of the profibrogenic genes *Col1a1*, *Tgfβ*, *Acta2* and *Loxl2* in all groups studied. Consistent with the histological data, these genes were highly upregulated in livers of HFD-fed mice after 30 of feeding as compared to 15 weeks (**Figure 1F-G**). Finally, we detected a positive correlation between hepatic density of neutrophils and liver fibrosis (PSR +ve area) and liver injury (total NAS score) in mice after 30 weeks on HFD (**Figure 1H-J**). Overall, these data suggest a pathogenic role of neutrophils in progression of NASH-related fibrosis.

Intrahepatic IL-17A$^+$ neutrophils positively correlated with liver fibrosis and liver injury in HFD-fed mice after 30 weeks of feeding

Next, using IF and flow cytometry, we sought to examine the infiltration of intrahepatic IL-17A producing cells in all groups studied. We observed an elevation in the density of intrahepatic IL-17A-producing cells in HFD-fed mice after 30 weeks of feeding compared to those at 15 weeks, as evidenced by IF (**Figure 2A-B**). To identify the cellular sources of IL-17A in our NAFLD model, we performed multiplex IF staining of IL-17A and either the T helper cell marker ($CD4^+$) or the

neutrophil marker (MPO^+). The $CD4^+$ T cells identified *in situ* by IF did not colocalize with IL-17A (**Figure 2C**), and neutrophils (MPO^+ cells) were the major source of IL-17A (**Figure 2D**). Consistent with the neutrophil data in **Figure 1**, the density of neutrophils producing IL-17A (MPO^+ $IL\text{-}17A^+$) positively correlated with liver fibrosis (PSR +ve area) and liver injury (total NAS score) (**Figure 2E-F**). To better characterize IL-17A-producing T cells in our NAFLD model, we extracted the intrahepatic lymphocytes from HFD- or CD-fed mice and examined their capacity to produce IL-17A by flow cytometry following stimulation with PMA/ionomycin. We observed Th17 ($IL\text{-}17A^+$ $CD3^+$ $CD4^+$), γδ T cells ($IL\text{-}17A^+$ $CD3^+$ $TCR\gamma\delta^+$) as major IL-17A producing T cells, and to a lesser extent, Tc17 ($IL\text{-}17A^+$ $CD3^+$ $CD8^+$) (**Figure 2G**). HFD-fed mice exhibited increased IL-17A production by these lymphocyte subsets after 30 weeks of feeding as compared to 15 weeks (**Figure 2G**). Altogether, these data suggest an active role of intrahepatic IL-17A producing cells, primarily neutrophils and T cells, in NAFLD-related fibrosis.

IL-17A induced NET formation *in vitro*

Previous studies reported a profibrogenic role of IL-17 in chronic inflammation-induced fibrosis involving many organs including the liver [517, 523, 712], and demonstrated the presence of IL-17 during NET formation in different contexts of chronic inflammation [710, 713, 714]. Also, based on our data in **Figures 1 and 2**, neutrophils producing IL-17A are likely promoting liver fibrosis progression in our NASH model. Thus, we aimed here at evaluating if IL-17A could be promoting liver fibrosis in NASH through inducing NET formation. For this purpose, we first isolated BM mature neutrophils from naïve mouse, and confirmed their purity using flow cytometry (>95%) based on surface staining for neutrophils ($CD45^+$ $CD11b^+$ $Ly6C^{int}$ and $Ly6G^{hi}$) flow cytometry (**Supplementary Figure 2**). Then, we stimulated these neutrophils with rIL-17A in the presence or absence of anti-IL-17A for 4 hours as described in Materials and Methods. Interestingly, we observed the

coexpression of NET markers, including extracellular DNA, MPO, and CitH3, upon rIL-17A stimulation, while anti-IL-17A inhibited NET formation, as evidenced by IF (**Figure 3A-C**). Also, the expression of CitH3 protein was increased in livers of HFD-fed mice after 30 weeks of feeding as compared to 15 weeks as well as controls (**Figure 3D**). Overall, these preliminary results may suggest a pathogenic role of IL-17A-induced NET in NASH-related fibrosis, though additional work is still required to test this concept.

Discussion:

In this study, we report marked elevation of intrahepatic IL-17A-producing cells, including neutrophils and T cells, in mice with advanced NAFLD-related fibrosis (30 weeks on HFD) compared to those with mild fibrosis (15 weeks on HFD). Neutrophils producing IL-17A were positively correlated with hepatic fibrosis and liver injury after 30 weeks of feeding. These results suggest an active role for IL-17A^{+} neutrophils in NAFLD-related fibrosis.

We showed that the density of intrahepatic IL-17A-producing cells *in situ*, primarily neutrophils, were increased in mice with advanced NAFLD-related fibrosis (30 weeks on HFD) compared to those with mild fibrosis (15 weeks on HFD). This was strongly associated with liver fibrosis and liver injury at 30 weeks. Our data are in agreement with other reports demonstrating the enrichment of IL-17A^{+} neutrophils in livers of patients with advanced fibrosis irrespective of the etiology, with the evidence that IL-17A^{+} neutrophils were mainly localized in the scar area [523, 708]. In addition, we previously showed that an RORγt antagonist reduced fibrosis and collagen deposition *in vivo* in the CCl_4 model of chronic liver injury, and this was associated with reduction in the intrahepatic IL-17A-producing cells, including neutrophils. Moreover, the lack of IL-17 signaling (IL-17RA$^{-/-}$) reduced liver fibrosis and collagen deposition in experimental models of liver fibrosis such as CCl_4 or BDL[472]. The profibrogenic role of neutrophils producing IL-17A was also reported in other organs such as lung [715].

As for neutrophils, we demonstrated that the frequency of IL-17A-producing T cells, including Th17 and γδ T cells were highly elevated in mice with advanced NAFLD-related fibrosis (30 weeks on HFD) compared to those with mild fibrosis (15 weeks on HFD). In spite the fact that HFD-fed mice did not develop systemic metabolic abnormalities associated with NAFLD after 15 weeks of feeding, the frequency of intrahepatic Th17 and IL-17A^{+}γδ T cells were higher than the same lymphocytes in control group. This could be consistent with similar observations from MCD-

induced NASH model that lacks metabolic syndrome associated with NAFLD, and where Th17 cells were increased after the first week of feeding, highlighting the importance of this subset at the initiation phase of NASH[515]. Also, the kinetics of intrahepatic Th17 from our NASH model are in agreement with many studies that reported Th17/Treg imbalance during NASH initiation as well as NASH-related fibrosis progression in experimental NASH models [515, 520, 522, 557, 559]. This was associated with an increase in the hepatic expression of inflammatory cytokines such as IL-6, TNF-α, and TGF-β [515]. A similar observation was reported in human NASH, where the Th17/Treg ratio, in the liver and peripheral blood of obese patients, was positively associated with transition from NAFL to NASH. Interestingly, one year after bariatric surgery, this Th17/Treg imbalance was shown to reverse to its level in heathy subjects, in parallel with NASH amelioration[513]. Moreover, our observation regarding intrahepatic IL-17A-producing γδ T cells in our NAFLD model support findings reported by Li et al [427], who demonstrated an increase of IL-17A^{+}γδ T cells in livers of HFD-induced NASH model. This was associated with elevation in ALT, IR, and neutrophil infiltration, but not liver fibrosis [427]. However, in contrary to the authors' observations [427] reporting no increase of Th17 cells, we showed the involvement of IL-17A^{+}γδ T cells as well as Th17 during the early and late phases of liver fibrosis in our NASH model.

We demonstrated that *in vitro* stimulation of mature bone marrow neutrophils with IL-17A induced NETs formation, while anti-IL-17A inhibited NETs formation. Moreover, we observed an increase in the expression of CitH3 (NET marker) in livers of HFD-fed mice after 30 weeks of feeding compared to 15 weeks, which could suggest a pathogenic role of NETs in modulating liver fibrosis progression in NASH, but not the development of fibrosis. Although the data of IL-17A-induced NETs in the context of NASH-related fibrosis remain unknown, a single study reported that IL-17A induced NET formation and enhanced liver necrosis in acute ischemia reperfusion model, while anti-IL-17A suppressed NETs and reduced liver injury[709]. Also, human neutrophils were shown to release IL-17 during the process of forming NETs, which synergistically

act with DNA/histone of NETs to activate lung fibroblasts, and hence promoted collagen deposition[713]. Moreover, DNase I administration (NET inhibitor) ameliorated liver injury, inflammation, and liver fibrosis in methionine-choline-deficient and high-fat diet (MCD-HF)-induced NASH model[412]. Overall, our results regarding IL-17A-induced NETs and its implication in NASH-related fibrosis are still preliminary and require additional investigation.

There are few limitations to this study. First, we only used an *in vivo* model of NAFLD, and thus further longitudinal studies, involving human patients with NAFLD-related fibrosis, are warranted to validate the kinetics of neutrophils producing IL-17A in this setting. Second, our data showed that neutrophils produced IL-17A using only IF methods. Other techniques such as flow cytometry are needed to confirm this finding.

In conclusion, we demonstrated the kinetics of intrahepatic IL-17A-producing cells during the early and late phases of NASH-related fibrosis, and identified neutrophils and T cells as major producers of IL-17A in this setting. Our data suggest a profibrogenic role of neutrophils producing IL-17A in NASH while providing preliminary evidence of IL-17A-induced NET formation and its probable implication in liver fibrosis progression in NASH. Additional work using cocultures of IL-17A-induced NETs and primary murine HSCs *in vitro* should be performed to test the potential effect of NET formation in liver fibrogenesis.

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Figures and figure Legends

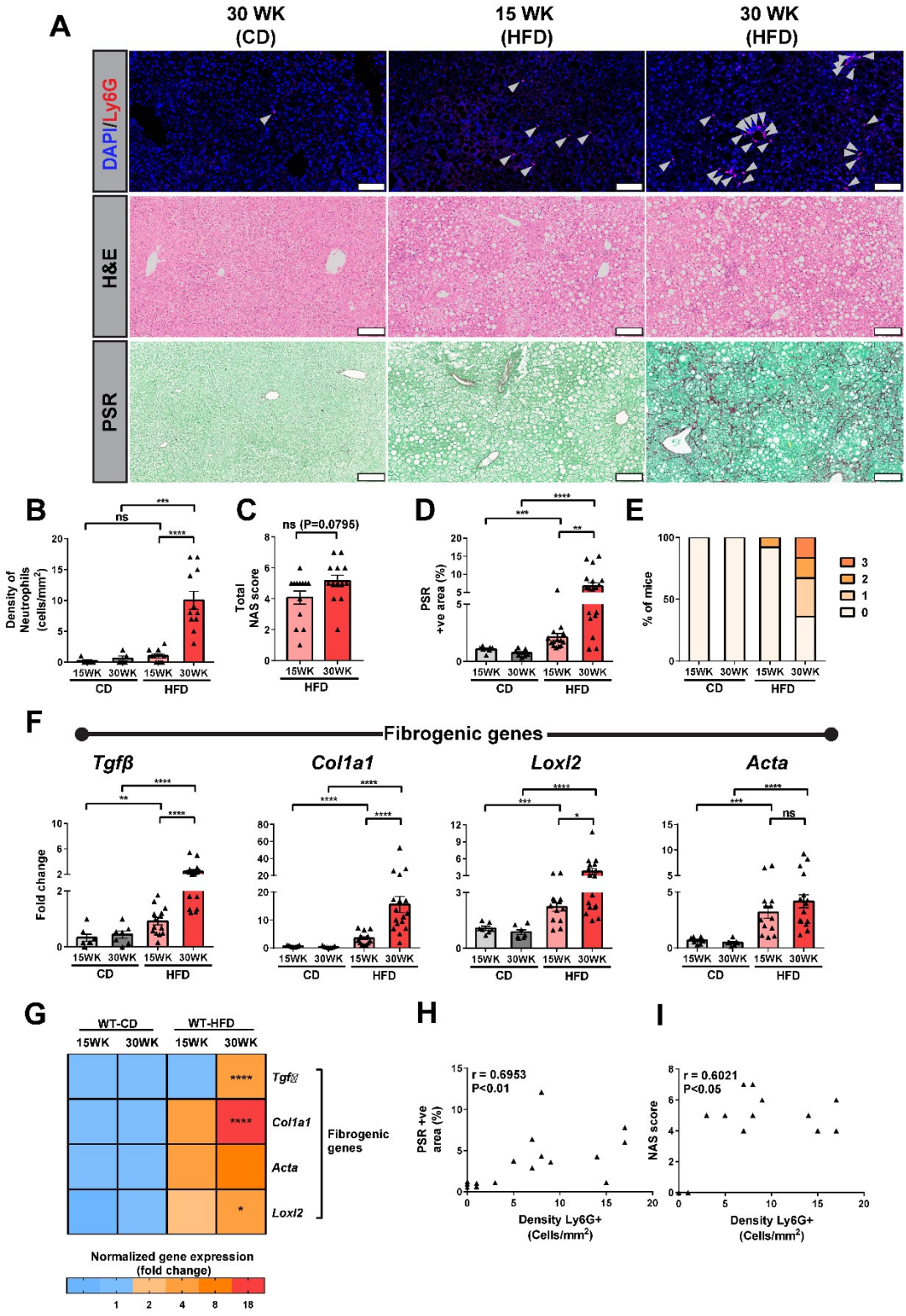


Figure 1. Neutrophils are positively associated with progression of liver fibrosis in HFD-fed mice after 30 weeks of feeding compared to 15 weeks.

WT male mice (▲) were fed HFD or CD for 15 or 30 weeks (WK). **(A)** Representative microscopic view of liver sections from stained CD- or HFD-fed mice with H&E stain, picrosirius red, and IF staining of neutrophil marker, $Ly6G^+$ (red cells delineated by arrowheads). Scale bars: 50 μm; 20x magnification. **(B)** Total density quantification (counts per mm2) of hepatic neutrophils ($Ly6G^+$) cells performed by Visiopharm software. **(C)** Blinded pathological evaluation of NAS score (steatosis grade, lobular inflammation, and hepatocyte ballooning) by an expert pathologist. **(D)** FIJI quantification of PSR +ve area in livers of CD- or HFD-fed mice. **(E)** Blinded pathological evaluation of liver fibrosis grade by an expert pathologist. **(F-G)** Bar graphs and heatmap of qPCR data of profibrogenic gene expression (normalized to r28s) as indicated and represented as fold change. Asterisk(s) in **(F)** indicate statistical significance between HFD-fed mice after 30 WKs of feeding compared to 15 WKs. (**H-I**) Spearman correlation graphs between neutrophils ($Ly6G^+$) and PSR +ve area **(H)** or NAS score **(I)** in mice after 30 WKs. Data are expressed as mean± SEM for 5-18 mice per group (data were pooled from three independent experiment). Statistical analysis was performed using Mann-Whitney test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. ns; not significant.

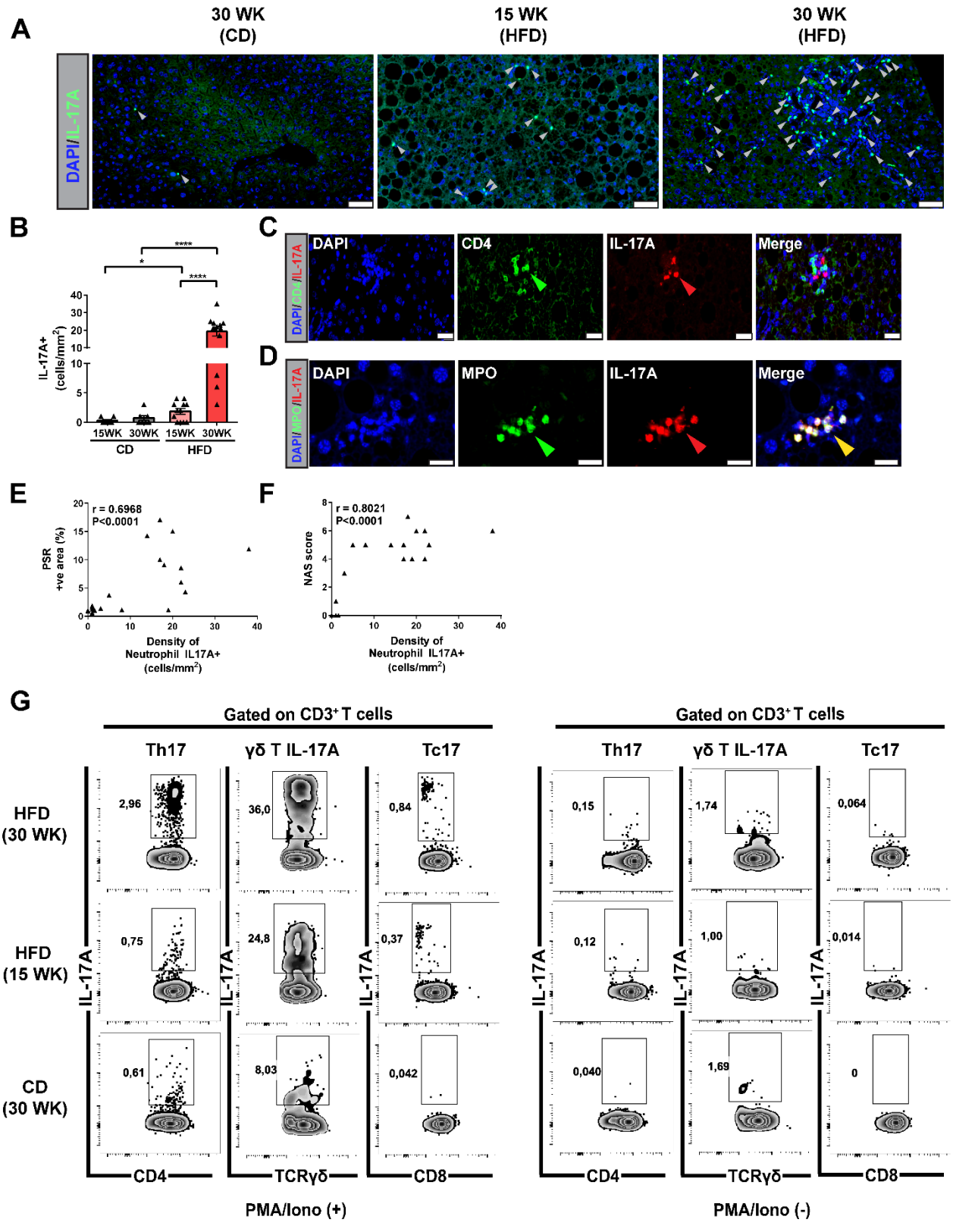
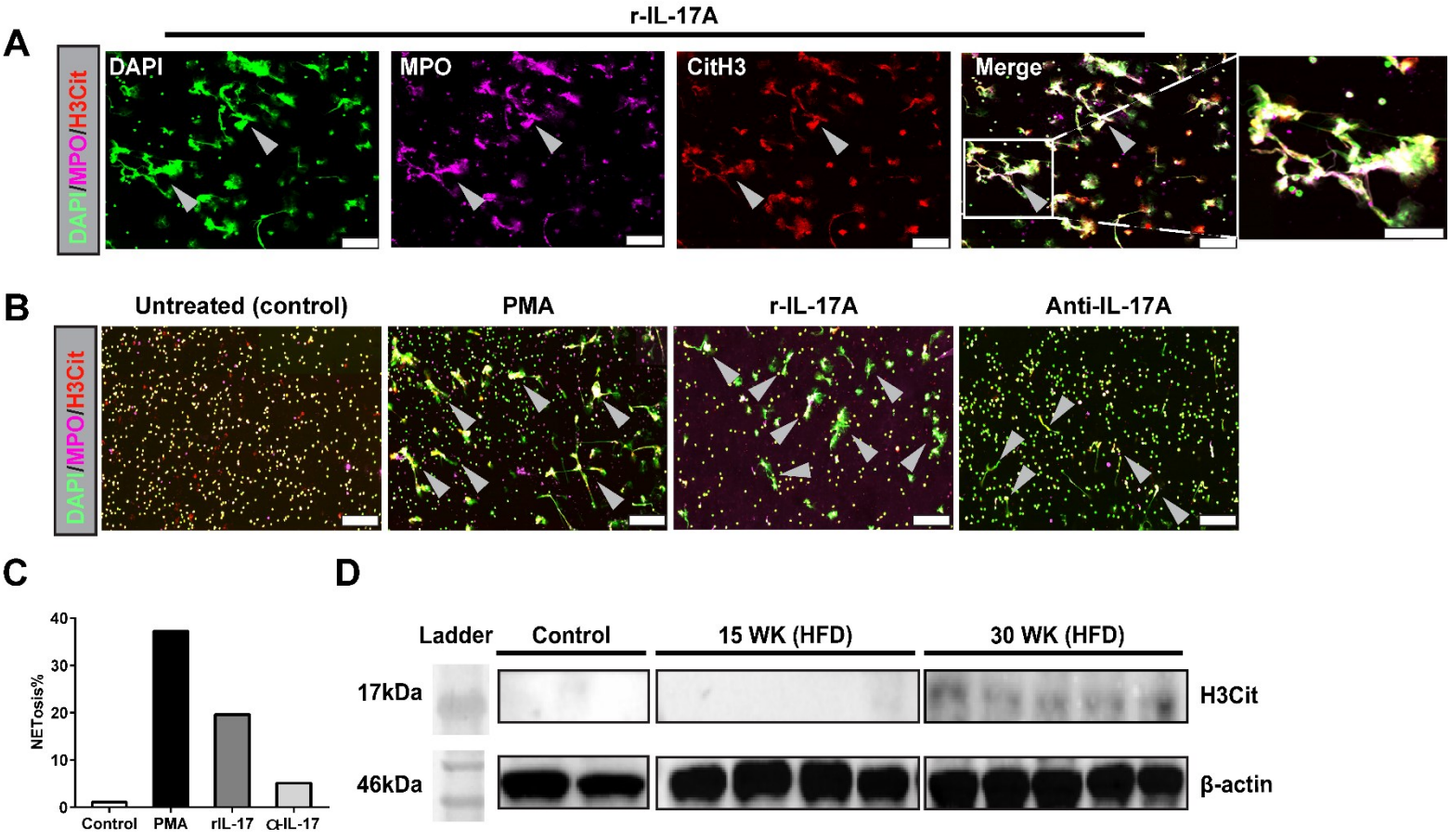

A
30 WK (CD)
15 WK (HFD)
30 WK (HFD)
DAPI/IL-17A
B
IL-17A+ (cells/mm²)
15WK 30WK 15WK 30WK
CD
HFD
C
DAPI/CD4/IL-17A
DAPI
CD4
IL-17A
Merge
D
DAPI/MPO/IL-17A
DAPI
MPO
IL-17A
Merge
E
r = 0.6968
P<0.0001
PSR +ve area (%)
Density of Neutrophil IL17A+ (cells/mm²)
F
r = 0.8021
P<0.0001
NAS score
Density of Neutrophil IL17A+ (cells/mm²)
G
Gated on CD3⁺ T cells
Th17
γδ T IL-17A
Tc17
HFD (30 WK)
HFD (15 WK)
CD (30 WK)
2,96
36,0
0,84
0,75
24,8
0,37
0,61
8,03
0,042
0,15
1,74
0,064
0,12
1,00
0,014
0,040
1,69
0
IL-17A
CD4
TCRγδ
CD8
PMA/Iono (+)
PMA/Iono (-)

Figure 2. Neutrophils producing IL-17A are positively correlated with liver fibrosis in HFD-fed mice after 30 weeks of feeding.

WT male mice (▲) were fed HFD or CD for 15 or 30 weeks (WK). **(A)** Representative IF images of liver sections from CD- or HFD-fed mice stained with anti-IL17A (green). Scale bars: 50 μm; 20x magnification. **(B)** Total density quantification (counts per mm^2) of hepatic IL-17A$^+$ cells performed by Visiopharm software. **(C)** Representative IF images of liver sections from HFD-fed mice after 30 WKs of feeding stained with CD4 (green) and anti-IL-17A (Red). In Merge, no colocalization between IL-17A$^+$ cell and CD4$^+$. Scale bars, 30 μm; 20x magnification. **(D)** Representative IF images of liver sections from HFD-fed mice after 30 WKs of feeding stained with anti-IL-17A (green) and anti-MPO (Red). Yellow arrow in the Merge of **(D)** indicate IL-17A-producing neutrophils (MPO$^+$ IL-17A$^+$). Scale bars, 20 μm; 20x magnification. **(E-F)** Spearman correlation graphs between neutrophils producing IL-17A (MPO$^+$ IL-17A$^+$) and PSR +ve area **(E)** or NAS score **(F)** after 30 WKs of feeding. **(G)** Representative flow cytometry plots showing intrahepatic IL-17A-producing T cells: CD4$^+$ T cells (IL-17A$^+$CD3$^+$CD4$^+$), γδ-T cells (IL-17A$^+$CD3$^+$TCRγδ$^+$) and CD8$^+$ (IL-17A$^+$CD3$^+$CD8$^+$) and their frequencies in HFD- or CD-fed mice after 15 or 30 WKs of feeding. The intrahepatic lymphocytes were extracted from livers of HFD- or CD-fed WT male mice after 15 or 30 WKs of feeding and then stimulated with/without PMA/ionomycin (PMA/Iono) for 5 hours. Data are expressed as mean± SEM for 5-20 mice per group (data were pooled from three independent experiment). Statistical analysis was performed using Mann Whitney test. *p<0.05, ***p<0.001.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α-IL-17
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Ladder
Control
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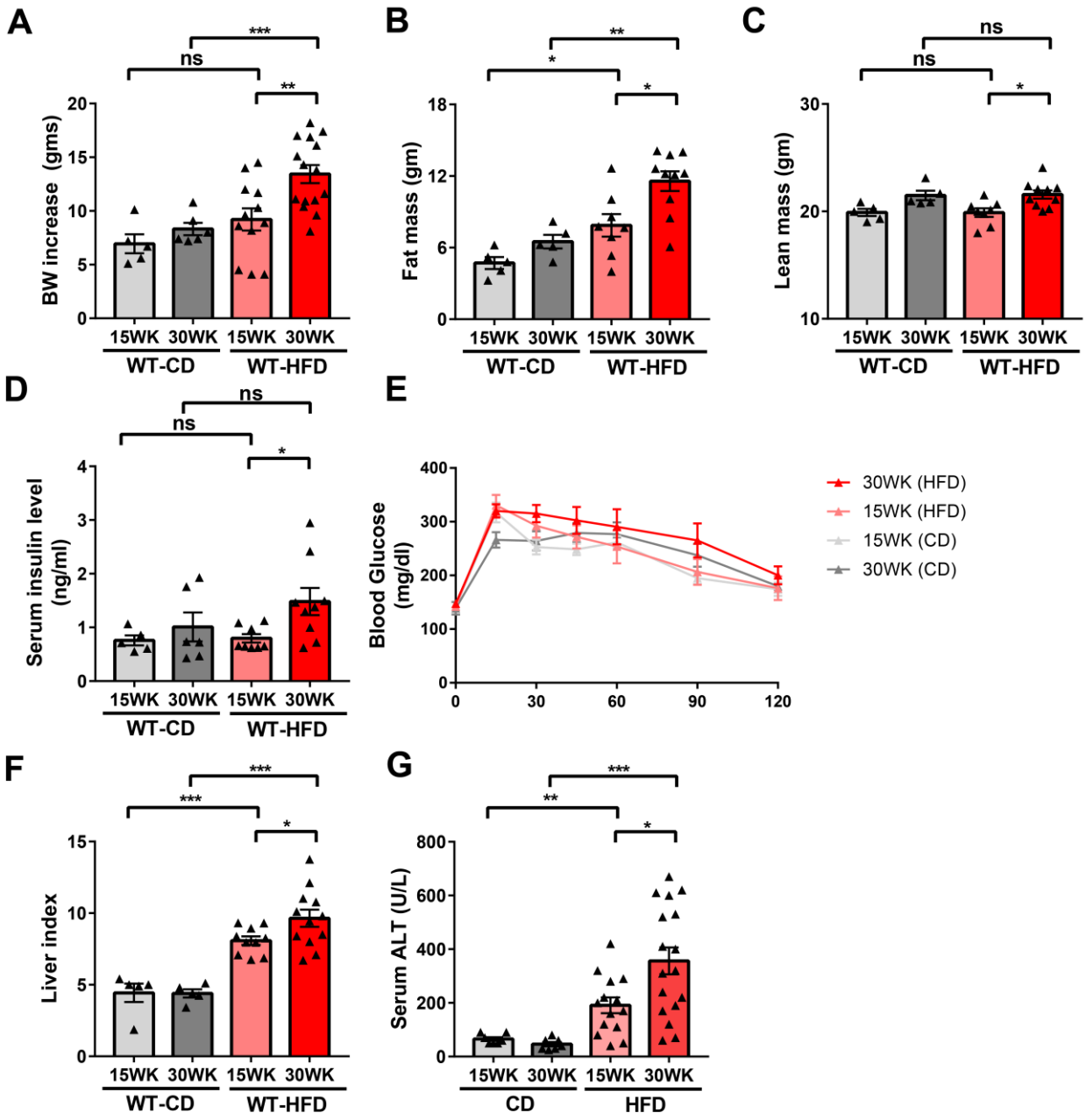
17kDa
H3Cit
46kDa
β-actin

Figure 3. IL-17A induces NET formation *in vitro.*

(A) Representative IF images of isolated bone marrow neutrophils (mature) treated with rIL-17A (50 ng/ml) to induce NETosis. Grey arrows indicate coexpression of NET markers; extracellular DNA (green), myeloperoxidase (MPO, magenta) and citrullinated histone 3 (CitH3, red). Scale bars, 80 μm; 20x magnification. The rightmost panel is magnified inset. Scale bar of inset is 35 μm. **(B)** Representative IF images of isolated bone marrow neutrophils (mature) treated with PMA or rIL-17A (80 pg/ml) in presence or absence of anti-IL-17 (0.5 ug/ml) to induce or block NETosis, respectively. PMA or rIL-17A treatment induced NET formation, while anti-IL-17A inhibited it. Grey arrows indicate NET markers mentioned in **(A)**. **(C)** Quantification of NETosis % (number of NETs/total number of neutrophils* 100) in **(B)** performed by FIJI software, n=1. **(D)** CitH3 protein expression in the livers of HFD- mice after 15 (n=4 mice) or 30 WKs (n=5 mice) of feeding. Control represent CitH3 protein expression from the livers of CD-fed mice for 15 (n=1) or 30 WKs (n=1). The level of CitH3 were increased at HFD-fed group after 30 WKs compared to 15 WKs of feeding. β-actin used as internal control.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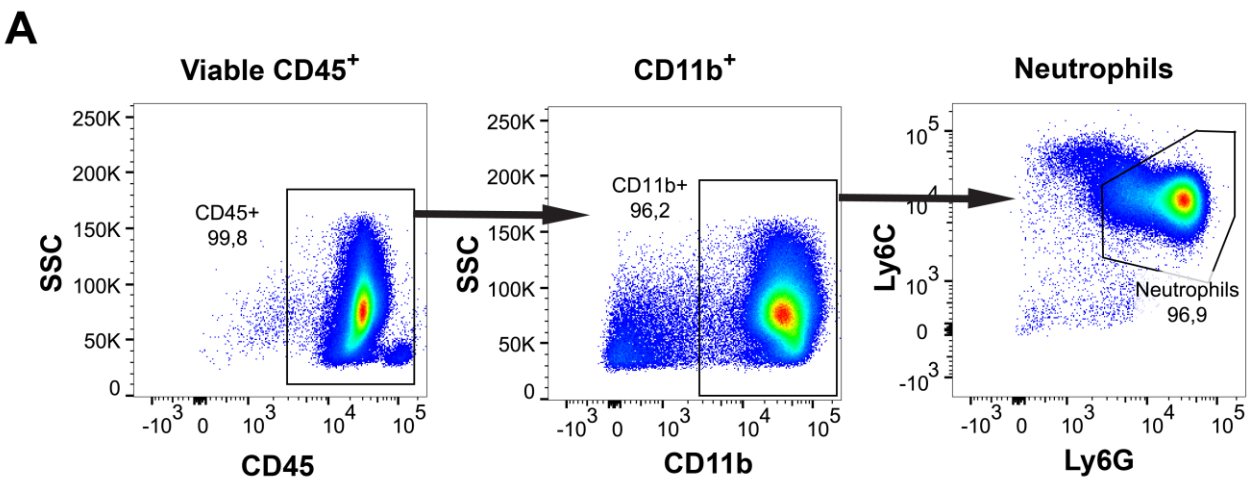
E
Blood Glucose (mg/dl)
30WK (HFD)
15WK (HFD)
15WK (CD)
30WK (CD)
F
Liver index
G
Serum ALT (U/L)
CD HFD

Supplementary Figure 1. HFD-fed mice develop significant metabolic abnormalities and liver injury at 30 compared to 15 weeks.

C57BL/6N WT male mice (▲) were fed HFD or CD for 15 or 30 weeks (WK). Measurements of **(A)** total body weight gain (grams, gm), **(B)** fat mass (gm) **(C)** lean mass (gm), **(D)** serum insulin, **(E)** intraperitoneal glucose tolerance test (IPGTT), **(F)** liver index (liver/body weight ratio), **(G)** serum ALT were undertaken at 15 or 30 WKs of feeding. Data are expressed as mean± SEM for 5-18 mice per group (data were pooled from three independent experiments). Statistical analysis was performed using Mann-Whitney test. *p<0.05, **p<0.01, ***p<0.001. ns; not significant.



Supplementary Figure 2. Gating strategy for checking isolated mature neutrophils from murine bone marrow.

Tibia and Femurs were isolated from naïve mice and bone marrow was collected by centrifugation. Neutrophils were then isolated from bone marrow using discontinuous histopaque gradient and then analyzed by flow cytometry. Neutrophils were identified using surface staining of neutrophil markers ($CD45^+$ $CD11b^+$ $Ly6C^{int}$ and $Ly6G^{hi}$), and their purity was confirmed (> 95%).

Table 1. List of IF primary and secondary antibodies

Antibodies	Supplier	Catalog number	Dilution
Primary antibodies			
Anti-IL-17A	Cloud-Clone Corp, USA	PAA063Mu01	1:100
Anti-CD4	Abcam	Ab16669	1:100
Anti-Ly6G	BioLegend	127602	1:50
Anti-MPO	R&D	AF3667	1:100
Anti-CitH3	Abcam	ab5103	1:100
Secondary antibodies			
Donkey Anti-goat A568	Thermo Fisher Scientific	A11057	1:500
Chicken Anti-Rat A678	Thermo Fisher Scientific	A21472	1:250
Donkey Anti-Rabbit A678	Thermo Fisher Scientific	A31573	1:500
Donkey Anti-Rabbit A488	Thermo Fisher Scientific	A21206	1:500
Donkey Anti- Guinea pig A678	Jackson ImmunoResearch	706-605-148	1:500

Table 2. List of FC antibodies

FC Antibodies	Supplier	Catalog number
CD45-BV650	BioLegend	103151
CD3-PECF594	BD bioscience	562286
CD4-BUV496	BD bioscience	612952
CD8-A700	BioLegend	100730
TCRγδ-BV421	BD bioscience	744118
CD11b-BV421	eBioscience	63-3551-82
Ly6C-PECF594	BD bioscience	562728
Ly6G-A488	BioLegend	127626
IL-17A-APC	eBioscience	17-7177-81

Chapter 5:

Discussion and Conclusion

1. General conclusions

Inflammation is the body's immune response to tissue injury, which is essential to eliminate tissue insult or invading pathogen and allow tissue repair[5]. However, chronic or dysregulated inflammation results in different immunopathologies that might progress to severe diseases with poor outcomes. Chronic hepatic inflammation in response to persistent tissue insult is a key driver of liver fibrosis progression and/or carcinogenesis in different chronic liver diseases, including NASH[506, 507, 513, 515]. The chronic inflammatory response is complex and encloses three types of immunity: type 1/2/3 responses or Th1/Th2/Th17, respectively[5, 426]. IL-17A and IL-22 are the two major cytokines of the type 3 immunity and have been reported in different chronic inflammatory disorders, autoimmunity, and cancer[5, 103, 446, 479, 490, 523, 706, 716]. However, the role of these cytokines in the progression of NAFLD-related fibrosis remains not clear.

The objective of this study is to characterize the role of IL-22 and IL-17A *in vivo* in the progression of NAFLD-related fibrosis, to elucidate their cellular sources and to evaluate possible mechanisms by which they contribute to NAFLD-related fibrosis using *in vitro* and *in vivo* models.

Our data demonstrate the contribution of type 3 inflammation in NAFLD-related fibrosis with IL-22 acting as protective and IL-17A as a pathogenic player. We provided novel evidence of sexual dimorphism in IL-22 expression in both humans and mice with NAFLD and reported IL-22 receptor signaling to function in a sex-specific manner mitigating liver injury, NASH-related inflammation and fibrosis in female mice. These outcomes resulted from the anti-apoptotic effect of the IL-22 receptor signaling in female mice with NAFLD. Also, we demonstrated a profibrogenic role of IL17A-producing neutrophils in NAFLD, likely mediated by induction of NET formation. This could indicate a novel mechanism for IL-17A-induced NAFLD-related fibrosis and hence represent a potential therapeutic target for the treatment of liver fibrosis in the context of NAFLD.

We investigated a cohort of 20 patients with varying degrees of NAFLD-related fibrosis, including 11 males and 9 females. This cohort of NAFLD patients allowed us to characterize and quantify IL-22-producing cells in humans with NAFLD. We observed an enrichment of IL-22-producing cells, likely neutrophils, in liver biopsies obtained from female patients with NAFLD compared to males, though such high level of IL-22^{+} cells did not correlate with liver fibrosis. We also validated the IL-22 signature in two publicly available microarray datasets, including two different NAFLD cohorts. In addition, we developed an *in vivo* NAFLD model, and demonstrated the IL-22 signature herein where intrahepatic Th17, Th22 and γδ T cells were the major IL-22-producing cells in female mice with NAFLD. These observations suggest a sexual dimorphic expression of IL-22 in the context of NAFLD (Manuscript 1).

Next, we characterized the role of endogenous IL-22 in NAFLD-related fibrosis in both females and males using *in vivo* mouse model including IL22ra1$^{-/-}$ mice and their WT littermates (Manuscript 1). We demonstrated that the lack of IL-22 receptor signaling (IL22ra1$^{-/-}$) exacerbated liver injury, inflammation, liver fibrosis in female but not male mice (Manuscript 1). Also, we showed that hepatic inflammation in HFD-fed IL-22ra1$^{-/-}$ female mice is characterized by relatively higher frequencies of IL-17A^{+} IL-22^{-} Th17 compared to WT, indicating an exacerbation of NASH-related inflammation in the absence of IL-22 receptor signaling (Manuscript 1, Figure 3). To investigate the possible mechanisms underlying the severe liver injury observed in HFD-fed IL22ra1$^{-/-}$ female mice, we examined whether hepatocyte cell death is involved. We initially have demonstrated that the expression of IL-22 downstream target genes, including antiapoptotic genes (*Bcl2*, *Bcl-xL*) and antioxidant genes (*Sod1* and *Mt2)* were substantially decreased in HFD-fed IL22ra1$^{-/-}$ female but not in male mice compared to WT (Manuscript 1, Figure 13). This was indeed associated with increased cell death in HFD-fed IL22ra1$^{-/-}$ female but not in male mice

compared to WT (Manuscript 1, Figure 14). Finally, we explored for the first time the role of IL-22BP in the context of NAFLD using our *in vivo* NAFLD model. We demonstrated an upregulation in the hepatic expression of IL-22BP in female, but not in male mice with NAFLD, and a significant correlation of the hepatic IL-22/IL-22BP mRNA ratio with the expression of downstream target genes of IL-22 such as *Bcl2*, *Mt2* and *Sod1* in these females (Manuscript 1, Figure 14). This observation could indicate that the regulation process by IL-22BP did not limit the overall activity of IL-22. Taken together, these findings suggest a sex-dependent hepatoprotective antiapoptotic effect of endogenous IL-22 receptor signaling during NAFLD-related liver injury in females.

On the other hand, we also studied the kinetics of intrahepatic IL-17A-producing cells during the progression of NAFLD-related fibrosis using an *in vivo* mouse model (WT male mice) at two time points: 15- and 30-weeks post-diet initiation (HFD or control). We demonstrated an enrichment of intrahepatic IL-17A-producing cells, including neutrophils and T cells, at late phases of liver fibrosis (30 weeks) compared to the early phase (15 weeks) (Manuscript 2, Figure 2). Also, we reported that the number of IL17A-producing neutrophils positively correlated with advanced liver fibrosis and liver injury at 30 weeks (Manuscript 2, Figure 2). To investigate the possible mechanisms by which IL-17A^{+} neutrophils promote the progression of NAFLD-related fibrosis, we investigated IL-17A-induced NET formation and whether these NETs induce HSCs activation and fibrogenesis *in vitro*. We observed an increase of NETosis percentage upon IL-17A stimulation *in vitro*, while anti-IL-17A Ab inhibited such effect. Also, we showed that the expression of CitH3 protein (NET marker) was increased in the livers of HFD-fed mice at 30 as compared to 15 weeks (Manuscript 2, Figure 3). These results may suggest a pathogenic role of IL-17A-induced NETs in NAFLD-related fibrosis. However, additional work with cocultures of IL-17A-induced NETs and primary murine HSCs *in vitro* are still needed to test this concept.

In summary, our data demonstrate the contribution of type 3 inflammation in NAFLD-related fibrosis with IL-22 acting as protective and IL-17A as a pathogenic player. We provided novel evidence of sexual dimorphism in IL-22 expression in both humans and mice with NAFLD and reported that IL-22 receptor signaling functions in a sex-specific manner mitigating liver injury, NASH-related inflammation and fibrosis in female mice. These outcomes resulted from the anti-apoptotic effect of the IL-22 receptor signaling in female mice with NAFLD. Also, we demonstrated a profibrogenic role of IL17A-producing neutrophils in NAFLD, likely mediated by induction of NET formation. This could indicate a novel mechanism for IL-17A-induced NAFLD-related fibrosis and hence represent a potential therapeutic target for the treatment of liver fibrosis in the context of NAFLD.

2. NAFLD cohorts

To do this study, we established a cohort of NAFLD liver biopsies through the collaboration with the CHUM hepatology clinic. This collaboration allowed the accessibility to the clinical data of the patients and their paraffin-fixed liver tissue, but not to fresh fragments of their liver biopsy. The main inclusion criteria of the cohort included the history of alcohol consumption, absence of other chronic liver hepatitis (e.g., viral, autoimmune, alcoholic hepatitis) and the NAS score evaluation of liver biopsies. Exclusion criteria for this study included patients receiving liver transplant or immunosuppressants which influence immune response of IHL, presence of severe autoimmune diseases such as Chron's disease or systemic lupus erythematosus as such conditions severely affect immune responses and thus could alter our results, and cases with suboptimal fragments from liver biopsy which makes it difficult to assess NAFLD pathological features.

Given that liver biopsy is an invasive method and cannot be used for the purpose of research only and that the use of alternative non-invasive tests in order to diagnose NAFLD is still limited [225-229], we established our cohort with NAFLD patients recruited as part of a clinical follow-up and their diagnosis mainly based on the liver biopsy. Moreover, we queried two publicly

available microarray datasets (GSE106737 and GSE151158), including two different NAFLD cohorts with females and males [686, 687]. Our cohort included 20 NAFLD patients, females=9 and males=11. We would have wanted to increase the sample size of our cohort, however, after the COVID-19 outbreak, we faced many challenges in the recruitment process as many elective procedures (including liver biopsies) and in-person visits were cancelled and replaced by telephone visits to minimise risk of COVID-19 transmission. Therefore, we could not increase the sample size of our cohort. Despite these challenges, we were able to establish a relatively homogeneous cohort with a mean age of 50.11 and 49.09 for females and males with NAFLD, respectively. In addition, there was no difference in both NAS and fibrosis scores between female and male patients (NAS score mean= 4.889 vs 4.0, *p*= 0.3392, fibrosis score mean= 2.778 vs 1.917, *p*=0.1440) (Manuscript 1, Table 1), indicating that both female and male patients had comparable profiles of NAFLD severity. Moreover, we queried two publicly available microarray datasets (GSE106737 and GSE151158), including two different NAFLD cohorts with females and males [686, 687]. The number of NAFLD patients in GSE151158 and GSE106737 datasets were (9-22/group) and (15-24/group), respectively. The diagnosis of NAFLD in both studies was based on liver biopsy. We explored the age and the NAS score of NAFLD cohort from GSE151158 dataset and we found that the age and NAFLD severity between female and male patients were comparable (mean= 45.27 vs 48.31, *p*= 0.4683, and mean= 4.143 vs 3.563, *p*=0.1714, respectively, data not shown in Manuscript 1). Overall, these NAFLD cohorts allowed us to compare female vs male groups without the impact of confounding factors such as age and NAFLD severity on our results. Nevertheless, in GSE106737 datasets, the NAS score evaluation for each patient was not publicly available, so we could not stratify NAS scores of female vs male patients.

3. Development of NAFLD model

To study the role of endogenous IL-22 *in vivo* in NAFLD-related fibrosis, we developed a western HFD-induced NAFLD model in IL-22RA knock out (IL-22ra1$^{-/-}$) mice and their WT littermates, with a C57BL/6N background. Our HFD was established in collaboration with Dr. Wynn at the NIH (now at Pfizer)[717], with the capacity of inducing the metabolic syndrome features, NASH pathological features, and hepatic fibrosis lesions, which could mimic human NASH. In fact, Dr. Wynn previously used HFD (Cat # D09100301, Research Diets, US) that contained trans-fat which provoked severe liver injury and hepatic fibrosis progression in mice after a short-term feeding (15 weeks)[717]. Indeed, these privileges make this model more preferable than other diets models such as MCD model because it lacks metabolic abnormalities associated with NAFLD, or other HFD models that associated with minimal NASH phenotype or fibrosis progression. However, the US government banned all rodent diets containing trans fat in 2017. Therefore, we used an alternative to this diet (D17010102I, Research Diets, US) that is trans-fat free but induced evident advanced fibrosis only after long-term feeding (30 Weeks).

4. Endogenous hepatic IL-22 signature in human and mice with NAFLD

4.1. A sexual dimorphism of hepatic IL-22 expression in both human and mice with NAFLD

Studies investigating the role of endogenous IL-22 in NAFLD in both mice and humans are limited. Rolla et al[515] demonstrated that endogenous IL-22 produced by Th22 cells antagonized the development of inflammation and fibrosis progression in MCD-induced NASH model, but only in the absence of IL-17 (IL-17$^{-/-}$ mice). However, this model lacks metabolic abnormalities associated with NAFLD and does not completely recapitulate human NASH. Other *in vivo* studies reported relatively low serum and hepatic IL-22 levels in HFD-induced NALFD model, and this

was not associated with any beneficial effects against metabolic abnormalities associated with NAFLD[663, 664]. All these studies were limited to male mice and thus data regarding the sex-based immunological difference between males and females in the context of NAFLD remain elusive.

Interestingly, our data quantifying IL-22 in liver of patients with NAFLD revealed marked elevation in hepatic IL-22 expression at both the protein and mRNA levels in females as compared to males with NAFLD (Manuscript 1) but with no evident correlation, at least in patients from our cohort, with liver fibrosis or liver injury, suggesting that the increase of IL-22 levels is likely not dependent on NAFLD severity (data not shown). Consistent with our human data, a similar IL-22 signature was detected only in the livers of female mice with NAFLD (Manuscript 1). These results are in line with previous reports demonstrating comparable levels of endogenous IL-22 in both circulation and liver of male humans and mice with NAFLD [661, 663, 664]. Moreover, a recent study demonstrated that hepatic IL-22 expression was enhanced in female compared to male mice post acetaminophen-induced liver injury[691], suggesting sexual dimorphism in hepatic IL-22 expression during acute liver injury. Accordingly, these observations may suggest a regulation of IL-22 expression by the female sex hormone estrogen. Indeed, there is emerging evidence from few reports that may support this notion. Women with polycystic ovary syndrome, characterized by dysregulated female sex hormones, exhibit significantly lower serum IL-22 than healthy controls[690]. In addition, testosterone or dihydrotestosterone reduced IL-22 production by female murine splenocytes following stimulation by either lipopolysaccharide or αCD3/CD28[691]. Moreover, in the imiquimod-induced psoriasis model, administration of estrogen agonists significantly modulated Th-derived IL-22 thus aggravating psoriasis symptoms[692].

In summary, our study compared the hepatic IL-22 expression between females and males in three different human NAFLD cohorts and an *in vivo* NAFLD model and demonstrated that females expressed higher levels of IL-22 gene and protein compared to males. This IL-22 signature brings new perspective to the research of IL-22 in chronic liver diseases, especially

NAFLD, since to date, most studies investigating endogenous IL-22 in NAFLD were limited to male sex and lacked the inclusion of females.

4.2. Intrahepatic neutrophils and T cells are the major IL-22 producing cells in female subjects and mice with NAFLD, respectively

Knowledge of endogenous IL-22 cellular sources involved in NAFLD is very limited, most likely because several studies use the recombinant IL-22 to investigate the functions of IL-22 in *in vivo* NAFLD models. In our study, by performing multiplex IF, we observed neutrophils producing IL-22, but not T cells, in livers of female subjects and mice with NAFLD (Manuscript 1). However, the use of flow cytometry techniques, but only with livers from female mice with NAFLD since we did not have access to fresh liver biopsies from patients, revealed Th17, Th22, γδ-T cells as the major IL-22-producing T cells, and to a lesser extent, ILC3s. We believe that the discordance in flow cytometry and IF results with regards to IL-22 producing T cells could be due to the higher sensitivity of flow cytometry techniques upon stimulation with PMA/Ionomycin compared to IF, which can detect cells actively producing cytokines *in situ*. Rolla et al [515] demonstrated that Th-derived IL22 was the major source of IL-22 in MCD-induced NASH model during the absence of IL-17A (IL-17$^{-/-}$ male mice). This finding was mainly detected by flow cytometry using PMA/ionomycin stimulation to intrahepatic T cells. Nevertheless, in comparison to Rolla et al [515], using the same method, we observed multiple cellular sources of IL-22 in the livers of HFD-fed WT females, not only Th22. In addition, our results could support the finding of Hamaguchi et al, who demonstrated a marked elevation in IL-22 producing ILC3s in livers of HFD-induced NAFLD model (males). However, we only observed this in livers of WT female mice with NAFLD, not males. In addition, Hamaguchi et al only measured the hepatic IL-22 expression by qPCR in mice deficient in ILC3s (RORγt $^{gfp/gfp}$) vs heterozygote controls (RORγt $^{gfp/wt}$)[669]. The findings of these studies could reflect sex difference or utilization of different NAFLD models. Another recent study reported sex difference in hepatic IL-22 expression between female and male mice post

acetaminophen-induced acute liver injury, though the authors did not characterize the IL-22 producing cells in this model[691]. Although the role of neutrophils producing IL-22 in liver disease remains unknown, neutrophils producing IL-22 were shown to upregulate AMPs, such as RegIIIβ and S100A8, in the colonic epithelium resulting in protection against dextran sodium sulfate-induced colitis in mice. Overall, our results showed redundancy in IL-22 cellular sources in the context of NAFLD which is a novel finding that has not previously reported.

5. Role of IL-22 in NAFLD-related fibrosis

The sexual dimorphism in hepatic IL-22 expression in both human and mice with NAFLD provokes an important question about the role of endogenous IL-22, whether protective or pathogenic, in this context. Therefore, we decided to characterize the function of this cytokine in our *in vivo* NAFLD model, including female and male mice.

5.1. The lack of IL-22 receptor signaling is associated with metabolic abnormalities in female and male mice with NAFLD

There is a bidirectional association between NAFLD and MS comorbidities[233]. Many studies have reported beneficial effects of IL-22 against obesity, adiposity, glucose intolerance and IR in different HFD-induced NAFLD models[662-665]. However, these protective effects are limited to exogenous administration of IL-22 to these NAFLD models. In this study, our NAFLD model developed the MS features, and the absence of IL-22 receptor signaling promoted the metabolic abnormalities, especially weight gain and IR, in female and male mice with NAFLD. These results are consistent with Wang et al[664], who observed similar metabolic alterations during diet-induced obesity in IL-22R1 KO, but not in IL-22KO mice. Thus, these observations suggest that other IL-22RA1 ligands such as IL-20 and IL-24 may partially mediate theses metabolic disorders in IL-22ra1 deficient mice upon feeding on HFD[92, 664] (please see limitations and future perspectives).

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5.2. Lack of IL-22 receptor signaling exacerbates liver injury, apoptosis, inflammation, and promotes progression of NASH-related fibrosis in female, but not male, mice.

We showed that the liver of HFD-fed IL22ra1$^{-/-}$ female mice developed advanced NASH-related fibrosis (chicken wire-like perisinusoidal fibrosis) compared to WT, which is associated with an increase in HSCs activation by expression of α-SMA and desmin (Manuscript 1). The difference in collagen deposition was associated with a difference in hepatic apoptosis, inflammation, serum ALT, and NAS score (Manuscript 1). Moreover, this severe phenotype is likely to be driven by loss of IL-22-induced anti-apoptotic (Bcl2) and anti-oxidant signals (Sod1 and Mt2) (Manuscript 1). On the contrary, there was no difference in hepatic apoptosis, inflammation, serum ALT and liver fibrosis profiles between HFD-fed IL22ra1$^{-/-}$ male mice and their WT littermates (Manuscript 1). Our data highly suggest that IL-22 receptor signaling protects hepatocytes from apoptosis, promotes their survival, and hence delays progression of NAFLD-related fibrosis in female, but not male mice (Figure 20). In line with this, Hwang et al [668] demonstrated that IL-22Fc alleviated oxidative stress-induced hepatocyte death via STAT-3-activating Mt1 and Mt2 in HFD^{+Cxcl1}-induced NASH model, resulting in amelioration of NASH-related fibrosis. However, this model is limited by the supraphysiological level of CXCL-1 which markedly exceeds the CXCL-1 level observed in NASH patients. In addition, our data support the findings of Zai et al[665] using liver-targeted delivery of the IL-22 gene in a NASH mouse model, where IL-22 activated STAT3-induced BCL2 and Nrf2-induced SOD1 pathways, resulting in increased hepatocyte survival and proliferation[665]. However, Zai et al[665] did not investigate these protective effects of IL-22 against NASH-related fibrosis.

On the other hand, we and others have previously demonstrated that IL-22 has a profibrogenic function in humans and in the CCl_4 and TAA models of chronic toxic liver injury[523, 626]. This effect

is mediated through enhancement of TGF-β signaling in HSCs in a p38 mitogen-activated protein kinase-dependent manner[523]. The difference with results obtained in the present study, demonstrating a hepatoprotective effect of IL-22 in a physiological HFD-induced NAFLD model, may reflect the relatively mild to moderate inflammation and heterogenous fibrosis induced in this NAFLD model, in contrast to a toxin-induced model. This argument is supported by the low TGF-β mRNA expression in livers of HFD-fed WT females and could represent a context-dependent function(s) of IL-22. Furthermore, our IF analysis demonstrate that IL-22-producing cells were mainly localized in the parenchyma of livers of HFD-fed WT females (data not shown), indicating that IL-22 signaling is possibly targeting hepatocytes and promoting their survival. On the contrary, in the CCl_4 model, IL-22^+ cells were mainly localized in the scar area (non-parenchyma), in close proximity to HSCs, suggesting more direct activation of HSC via promoting TGF-β signaling[523].

Although IL-22 does not directly modulate immune cells, it can influence generation of different inflammatory mediators in the epithelial cells depending on the inflammatory environment. Some studies reported a pathogenic proinflammatory functions of IL-22 upon HBV recognition through upregulating hepatic expression of chemokine CXCL-10 and CCL20, which positively correlated with Th17 recruitment and liver fibrosis progression[490, 624]. In contrast, we did not observe such pathogenic effect of IL-22 in our NAFLD model, and even the hepatic inflammation was more pronounced in livers of HFD-fed IL22ra1$^{-/-}$ female as compared to WT (Manuscript 1, Figure 6 and 10) highly likely suggesting the context-dependent function(s) of IL-22 in steatohepatitis versus viral hepatitis.

In summary, the pathogenic effects of IL-22 receptor signaling, whether profibrogenic or proinflammatory effects, were not evident in our NAFLD model, and our results support a hepatoprotective function of endogenous IL-22 receptor signaling against liver injury in female mice with NAFLD, while the endogenous IL-22 receptor signaling appears to play no role against

liver injury in male mice with NAFLD. This sex-dependent hepatoprotective effect of IL-22 receptor signaling is driven by inducing antiapoptotic and antioxidant signals in the context of NAFLD.

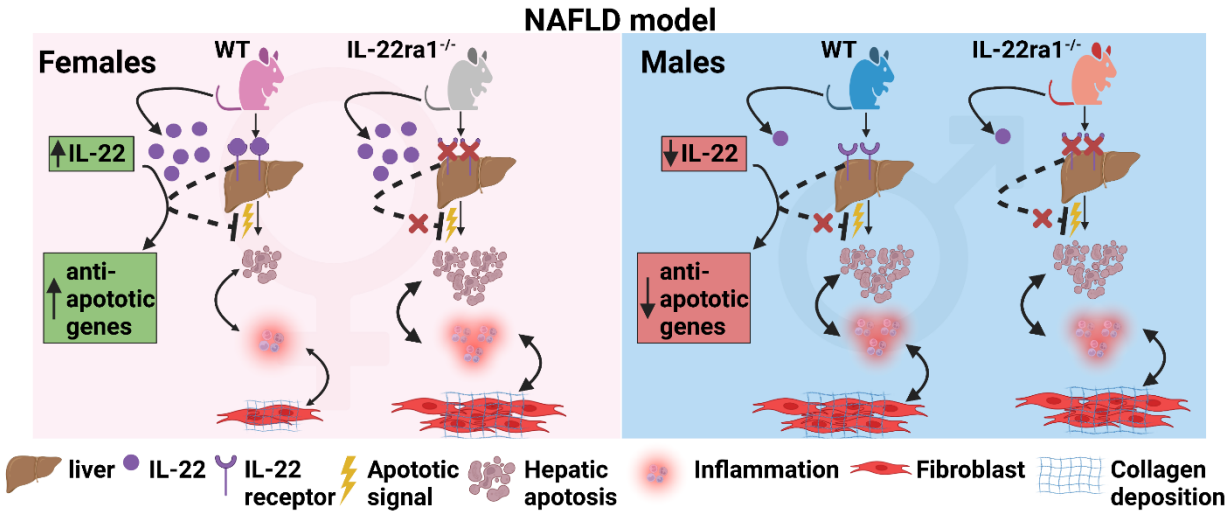

Figure 20. Graphical summary of sex-dependent hepatoprotective effect of IL-22 receptor signaling in NAFLD-related liver injury. This figure is taken from[679] (Reproduced with permission)

5.3. Regulation of IL-22 by IL-22BP

The endogenous levels of IL-22 can be regulated by the IL-22 binding protein (IL-22BP) [601, 602]. However, in NASH, the role of IL-22BP remains largely unknown[601]. In our study, we provided novel evidence that endogenous IL-22BP is highly expressed in the livers of female mice with NAFLD, without limiting the overall IL-22 activity in this context. In addition, these data could be in line with the findings of few reports demonstrating protective effects of IL-22BP in acetaminophen-induced liver injury and/or alcoholic hepatitis[602, 661]. Low plasma IL-22BP was associated with 1 year mortality in patients with alcoholic hepatitis, highlighting the importance of IL-22BP in limiting pathogenic effects of endogenous IL-22 in this context[559]. In line with this, the lack of IL-22BP in acetaminophen-induced acute liver injury model exacerbated hepatic injury due to uncontrolled IL-22 signaling [602]. This dysregulation of IL-22 signaling was associated with

upregulating hepatic CXCL-10 expression and hence promoted recruitment of inflammatory monocytes to the liver leading to exacerbation of liver damage[602]. Thus, the regulation of IL-22BP to IL-22 signaling in our NAFLD model seem to be protective and could succumb IL-22 activity to mediate hepatoprotective effects. However, it is important to mention that we were limited to the measurement of hepatic IL-22BP expression by qPCR in our NAFLD model, and we could not characterize IL-22BP protein expression and its cellular sources by IF due to lack of effective antibodies to be used in tissue sections. Therefore, additional work is needed to validate this observation at the protein level, as well as using IL-22BP$^{-/-}$ mice to dissect role of IL-22BP in the context of NAFLD.

6. Role of IL-17A in NAFLD-related fibrosis

IL-17A is a proinflammatory cytokine that has been implicated in pathogenesis of autoimmunity, inflammatory diseases, fibrosis and carcinogenesis [5, 446, 523, 706]. Also, we and others have demonstrated an increase of IL-17A producing cells in patients' liver biopsies with advanced fibrosis irrespective of etiology [100, 470, 506, 523]. Indeed, many studies reported proinflammatory functions of Th17/IL-17A axis in NAFLD-related fibrosis, though the role of neutrophils producing IL-17A in this context is still not clear. Therefore, we decided to characterize the function of IL-17A^{+} neutrophils in NAFLD-related fibrosis using *in vivo and in vitro* studies.

6.1. Correlations between neutrophils producing IL-17A and liver fibrosis

We demonstrated a remarkable increase of neutrophils and IL-17A-producing cells that include neutrophils and T cells, in livers of mice with advanced NAFLD-related fibrosis (30 weeks) as compared to those with mild fibrosis (15 weeks) (Manuscript 2). These kinetics of Th17 and IL-17A-producing γδ T cells in our NAFLD model are consistent with previous observations in human

and mice with NAFLD [427, 513, 515, 520, 522, 557, 559]. Also, intrahepatic neutrophils and T cells induced IL-17A expression in ASH patients, and this was associated with lobular inflammation[470]. Importantly, we also observed that most of hepatic neutrophils (MPO^+) detected *in situ* at 30 weeks were IL-17A$^+$ and were strongly correlated with advanced liver fibrosis and liver injury (Manuscript 2). These results are in line with previous reports showing the enrichment of IL-17A$^+$ neutrophils in livers of patients with advanced fibrosis irrespective of the etiology, and the localization of these cells to the scar area [523, 708]. However, IL-17A$^+$ neutrophils in the liver of our NAFLD model were not only localized to the scar area, but also presented at the parenchyma and/or surrounding the lipid droplets (data not shown). This observation could support findings of Tang et al [520] showing that IL-17$^+$ cells were detected in close proximity to ballooning hepatocytes and macrovesical lesions in livers of NASH patients. In addition, we previously showed that RORγt antagonist reduced fibrosis and collagen deposition *in vivo* in CCl_4 model of chronic liver injury, and this was associated with reduction in the IL-17A$^+$ neutrophils [523]. Blocking IL-17 activity *in vivo*, whether pharmacologically or genetically, ameliorated NASH progression and was associated with a decrease in hepatocyte injury, oxidative stress, infiltration of pro-inflammatory immune cells (e.g., granulocytes), and liver fibrosis in different NASH models[515, 520-522, 527, 553, 558]. The underlying mechanisms of these detrimental effects of IL-17 in NASH are yet to be explored. Neutrophils releasing NETs has been implicated in various chronic inflammatory disorders, including NASH [707, 718]. However, little is known about the role of IL-17-induced NETs and its impact on hepatic fibrosis progression in NASH. Our data herein demonstrated that NETs formation is increased in livers of HFD-fed mice at 30 compared to 15 weeks, in parallel with the increase of intrahepatic IL-17A producing cells and progression of liver fibrosis (Manuscript 2), highly suggesting a pathogenic role of NETs in progression of NAFLD-related fibrosis. In line with this, administration of NET inhibitor (DNase I) ameliorated liver inflammation and fibrosis in MCD-

HF-induced NASH model [412]. However, the role of IL-17A-induced NET was not investigated in this model. In addition, the mechanisms underlying NETs-induced NASH progression remain unknown. However, a recent study proposed that NETs promote immunosuppressive milieu in STAM-induced NASH-related HCC through inducing Treg differentiation from naïve $CD4^+$ T cells by upregulating their metabolic reprogramming profile[411]. This may suggest an important role of NETs in promoting cross talk of innate and adaptive immunity in context of NASH-HCC. Moreover, Zhang et al [719] demonstrated that IL-17A triggered NETs formation in pancreatic ductal adenocarcinoma model, and pharmacological inhibition of neutrophils or PAD4-dependent NETosis phenocopied IL-17A neutralization, which enhanced cytotoxicity of $CD8^+$ T cells against this tumor. Another report showed that IL-17A mediated NET formation enhancing liver necrosis in acute ischemia reperfusion model, while anti-IL-17A administration suppressed NETs and reduced liver injury[709]. Although the findings of these studies may suggest a detrimental effect of IL-17A-induced NETs on epithelial and immune cells during acute or chronic liver injury, the impact of this effect on HSCs and fibrogenesis in the context of NASH remains unexplored.

In spite all evidence to date, data of the IL-17A-induced NETs are preliminary and further work are still needed (please see limitations and future perspectives).

7. Conclusions, Limitations, and future directions

7.1. General conclusions

The progression of hepatic fibrosis is associated with chronic inflammatory dysregulation in response to persistent tissue insult. This chronic inflammation is characterized by activation of different immune cells and cytokine signatures. Type III inflammation, with IL-17A and IL-22 cytokine signature, have been reported in different contexts of chronic inflammatory disorders and autoimmunity, though their role in NAFLD-related fibrosis remain elusive. The study of hepatic

inflammatory response in humans represents a major challenge due to the difficulty in accessing liver biopsies. Consequently, development of murine NAFLD model is essential to understand the role of these cytokines during the progression of liver fibrosis. The main objectives of this study are to characterize the role of IL-22 and IL-17A *in vivo* in the progression of NAFLD-related fibrosis, to identify their cellular sources, and finally to elucidate the possible mechanisms by which they contribute to progression of NAFLD-related fibrosis *in vitro* and/or *in vivo.* The main conclusion of our study is that IL-22 and IL-17A are common signature in NAFLD-related fibrosis, where IL-22 acts in a sex-dependent manner and provided hepatoprotective functions against fibrosis in females, while IL-17A functions as a profibrogenic cytokine and promotes liver fibrosis. We provided novel evidence of sexual dimorphism in hepatic IL-22 expression in both humans and mice with NAFLD, as demonstrated by IF, microarray, and qPCR data. Also, IL-22 receptor signaling acts in a sex-specific manner and mitigates liver injury, NASH-related inflammation, and fibrosis in female mice, as illustrated by IF, histological, and qPCR data. These effects were dependent on the anti-apoptotic function of IL-22 receptor signaling, which enhances hepatocyte survival in these female mice. Also, we demonstrated a strong correlation between neutrophils producing IL-17A and progression of NAFLD-related fibrosis, which likely to be mediated by inducing NET formation.

7.2. Limitations and future directions

Longitudinal studies investigating role of IL-22 in human NAFLD-related fibrosis

Our study is the first report showing the sexual dimorphism in hepatic IL-22 expression in a NAFLD cohort with varying degrees of liver fibrosis (n=20, females=9 and males=11). However, we were unable to conduct longitudinal study to investigate IL-22 signature during progression of NAFLD-related fibrosis in both female and male patients for two main reasons. First, the COVID-

19 outbreak caused many challenges in the follow-up and the recruitment process because many elective procedures (including liver biopsies) and in-person visits were cancelled and replaced by telephone visits to minimize risk of COVID-19 transmission. Second, we started recruiting these NAFLD patients in 2017, and it is considered normal that these patients did not undergo a new liver biopsy during the follow-up of their clinical visits. Therefore, we were limited to the cross-sectional study. Nevertheless, this limitation can be overcome via accessing a bank of archived NAFLD liver biopsies embedded in paraffin as well as the development of optimized identification panels for IL-22 and its cellular sources using IF, which will allow conducting a longitudinal study in the future.

Age of female patients with NAFLD

It is known that prevalence of NAFLD is higher in male compared to female, especially at premenopausal age (≤ 50-60 years), while NAFLD prevalent trends became more common among post-menopausal women[244]. This may highlight the protective effects of estrogen against NAFLD which declines after menopause. Also, the increase of hepatic IL-22 levels in female versus male patients in our cohort may suggest hormonal regulation of IL-22 expression by estrogen. However, we could not stratify the female patients in our study according to their menopausal vs premenopausal age because of the small sample size of our cohort. Thus, we believe that classifying female patients with NAFLD according to their menopausal age (pre- vs post-menopause) should be highly considered in future longitudinal studies allowing proper assessment of this factor and its impact on hepatic IL-22 expression in the context of NAFLD.

The effect of sex hormones on hepatic IL-22 expression in the context of NAFLD

In line with the argument above, female hormones, especially estrogen, might be the reason for the sexual difference in hepatic IL-22 expression between female and male mice with NAFLD. Indeed, there are few reports in the literature pointing towards modulation of IL-22 expression by

estrogen hormone or estrogen agonists[690, 692]. Additional studies using ovariectomized female mice or castrated male mice are needed to address the mechanistic figure behind this sex difference in IL-22 expression.

Characterization of IL-22-, IL-22BP-, and IL-17A-producing cells in the liver

In our study, by performing multiplex IF, we observed neutrophils producing IL-22, but not T cells, in livers of female subjects and mice with NAFLD (Manuscript 1). However, the use of flow cytometry techniques, but only with livers from female mice with NAFLD since we did not have access to fresh liver biopsies from patients, revealed Th17, Th22, γδ-T cells as the major IL-22-producing T cells, and to a lesser extent, ILC3s. We believe that the discordance in flow cytometry and IF results with regards to IL-22 producing T cells could be due to the higher sensitivity of flow cytometry techniques upon stimulation with PMA/Ionomycin compared to IF, which can detect cells actively producing cytokines in situ.

Our IF analysis undertaken on liver sections from human patients or mice with NAFLD and showing that the majority of intrahepatic IL-22-producing cells were not T cells, was in discordance with our flow cytometry results reporting Th17, Th22, IL-22$^+$ γδ T cells, and ILC3s in livers of HFD-fed mice. Since we unfortunately did not have access to fresh liver biopsies from human patients, we were limited to only using IF methods on human liver sections, having low sensitivity as compared to flow cytometry techniques upon stimulation with PMA/Ionomycin.

Moreover, even though we identified neutrophils as cells producing IL-17A *in situ* using IF, optimization of *ex vivo* stimulation assays for neutrophils and assessing IL-17A production by other techniques such as flow cytometry are needed to confirm this observation. Furthermore, we could not characterize IL-22BP *in situ* by IF due to lack of functional antibodies against IL-22BP. Many studies have shown that IL-22 and IL-17A are produced by NK cell, ILC3s, macrophages and mast cells[5, 92]. Therefore, further studies with better functional antibodies are necessary to

characterize IL-22-, IL-22BP- and IL-17A-producing cells in FFPE specimens. Experiments using reporter mice (IL22-Cre.R26R-eYFP or Il22bp$^{LacZ+/-}$ or Il17a-Cre.Rosa26- eYFP) could also allow detection of these cells *in vivo* in the context of NAFLD.

The use of IL-22RA1 KO (IL-22ra1$^{-/-}$) model

Since IL-22RA1 receptor has several ligands such as IL-22, IL-20 and IL-24, our results may be mildly influenced by lack of signaling from other IL-22RA1 ligands. Therefore, we measured hepatic expression of IL-20 and IL-24 by qPCR. There was high upregulation of IL-24 mRNA in livers of HFD-fed WT female compared to those in control group, while there was no difference in IL-20 mRNA level between these two groups. Yet, the fold increase of IL-22 mRNA was significantly higher compared to both IL-24 and IL-20 in HFD-fed WT female mice (data not shown). In addition, IL-20 and/or IL-24 signalling is not only limited to the IL-22RA1 subunit but also can be mediated via IL-20R1-IL-20R2 complex. Consequently, knocking out IL-22RA1 or pharmacologically blocking IL-22RA1 could only partially block IL-20 and IL-24 signalling[613]. In addition, Chiu et al [185] demonstrated that the administration of neutralizing antibodies against IL-20R1 is sufficient to inhibit liver fibrosis in the CCl_4 model, highlighting that IL-20 signaling is poorly mediated via IL-22RA1 in the liver. Finally, we demonstrated that the hepatic expression of the IL-22 downstream target genes, including *Bcl2, Bcl-xL, Sod1, Mt2*, was substantially reduced in HFD-fed IL22ra1$^{-/-}$ female mice compared to their WT littermates (Manuscript 1). To the best of our knowledge, all these genes are major targets of IL-22/IL-22RA1 downstream signaling, but not IL-20 or IL-24[102, 619, 620, 665, 668]. Taken together, these findings suggest that the effects we observed in IL-22ra1$^{-/-}$ female mice is specific to IL-22 signaling, not IL-20 or IL-24. Nevertheless, future investigation using an IL-22$^{-/-}$ model may validate this phenotype.

The role of the microbiome in our NAFLD model

The IL-22/IL-22RA1 axis is crucial for maintaining gut homeostasis as it maintains integrity of epithelial barriers and promotes production of AMPs against invading pathogens [92, 616]. Also, microbial translocation and microbial-derived products, due to gut dysbiosis, have worsened NASH-related inflammation in humans and mice[349, 694]. Since we used a total body IL-22ra1$^{-/-}$ model, this IL-22/IL-22RA1 axis in the gut of mice may have been altered. Therefore, we cannot exclude a potential influence of gut dysbiosis in the promotion of NASH in our model. Currently, we established a project to investigate the effect of the microbiome composition and alterations on NASH progression in our model. We collected stool samples prior to HFD feeding (baseline) and at 30-WK (endpoint) from IL-22ra1$^{-/-}$ mice and their WT littermates, including females and males. Also, we extracted microbial DNA from these samples and 16S sequencing (MiSeq PE 250bp) was performed. Finally, the bioinformatic analysis is currently ongoing. These data should provide some insights onto the role of the microbiome in our model.

The impact of IL-17A-induced NETs on liver fibrosis *in vitro* and *in vivo*

We demonstrated that IL-17A promoted NET formation *in vitro*, and the signature of NETs was detected in livers of our *in vivo* NAFLD model in parallel with advancement of liver fibrosis (Manuscript 2). We could not evaluate the effect of IL-17A-induced NETs on HSCs activation and fibrogenesis *in vitro.* Therefore, additional work involving cocultures of IL-17A-induced NETs and primary murine HSCs *in vitro* should be performed to test the potential effect of IL-17A-induced NETs on liver fibrogenesis. Moreover, administration of r-IL17A or anti-IL-17A to our NAFLD model is another future direction to test whether IL-17A-induced NETs promote liver fibrosis in the context of NAFLD.

7.3. Significance of the study

Our study provides a first insight into sexually dimorphic differences in hepatic endogenous IL-22 expression in both humans and mice with NAFLD and demonstrates elevated IL-22 in females

versus males. We have shown conceptually that this increase of IL-22 levels is associated with delayed liver fibrosis progression in female mice with NAFLD, likely by inducing antiapoptotic signals that promote hepatocyte survival. Indeed, the hepatoprotective effects of IL-22 has drawn considerable attention for the use of IL-22 as potential therapeutic target for chronic liver diseases, specifically ASH and NASH. Few clinical trials have been conducted to investigate the therapeutic application of IL-22, specifically IL-22Fc, in humans. For instance, IL-22Fc has shown well tolerability and minimal side effects in healthy subjects in two phase 1 clinical trials[670, 671]. Also, IL-22Fc demonstrated anti-inflammatory effects along with amelioration in MELD clinical score in phase 2a open-label study for moderate to severe ASH patients[672]. These promising results could predict IL-22 therapy to be effective against NASH, nevertheless clinical studies are still lacking. Accordingly, the hepatoprotective and sex-dependent IL-22 signature observed in our study should be highly considered in clinical trials when testing IL-22-based therapeutic approaches in treatments of female versus male subjects with NAFLD in order to minimize adverse events and maximize therapeutic benefits. Also, identifying this IL-22 signature opens the door for future studies aiming at characterizing the hormonal (estrogen and/or androgen) regulation mechanisms behind this sex-related difference and hence could represent a major therapeutic target for treating liver fibrosis progression in NAFLD. In addition, we provided novel evidence as to the involvement of endogenous IL-22BP in regulating IL-22 activity in livers of female mice with NAFLD. In fact, studies investigating the role of IL-22BP in NAFLD are largely lacking, hence our results provide useful insights for future work aiming at deeply exploring the role of IL-22BP in this context. Moreover, we provided preliminary evidence of a profibrogenic role of IL-17A induced-NET formation in NAFLD. Such mechanism could represent a novel therapeutic target mainly aiming at inhibiting IL-17A pathway and limiting fibrosis progression in the context of NAFLD. In fact, up to date, two main strategies: direct and indirect options, have been developed to target IL-17/IL-17RA axis in chronic inflammatory diseases in humans, and consequently controlling the harmful manifestations of IL-17 in such context. Direct targeting IL-17A pathway

include monoclonal anti-IL-17A antibodies (secukinumab and ixekizumab) or anti-IL-17RA (brodalumab)[720]. These antibodies have been approved for treatment of chronic inflammatory diseases such as psoriasis, psoriatic arthritis and ankylosing spondylitis[721]. Several other anti-IL-17A antibodies, such as CNTO 6785, CJM112, and BCD085, are currently being tested in clinical trials[721]. The indirect targeting of IL-17A pathway involve blocking generation of Th17 subset and its related cytokines, including IL-17A, IL-17F, and IL-22. For example, anti-IL-6 (tocilizumab or the anti-JAK tofacitinib), anti-IL-1β (anakinra or canakinumab) and anti-IL-23 (tildrakizumab or guselkumab) are currently tested in clinical trials[720, 722, 723]. Also, small molecules targeting or inhibiting RORγt-induced Th17 differentiation have shown restoration of Th17/Treg balance *in vitro* and in experimental autoimmune encephalomyelitis[724-726]. Also, we previously demonstrated that RORγt antagonist (GSK805) inhibited intrahepatic IL-17A$^+$ cells, including neutrophils, along with reduction in liver fibrosis in CCl_4 model[523]. Although all these strategies are considered promising for targeting IL-17A pathway to limit progression of inflammation and liver fibrosis in CLD in humans, the risk of adverse events is a major concern with such approaches due to blocking protective effects of IL-17A against bacteria and fungal infections[727, 728]. Therefore, the evaluation of therapeutic benefit/risk balance of IL-17A inhibition in chronic liver disease is still needed to assess its safety and efficacy and hence determine its validity as therapeutic option for NASH.

APPENDECIES

APPENDIX I: The candidate's contribution to the articles

Author's contribution to the first manuscript "Sex-Dependent Hepatoprotective Role of IL-22 Receptor Signaling in Non-Alcoholic Fatty Liver Disease-Related Fibrosis"

Mohamed N. Abdelnabi (MNA) participated in study concept and design, acquired all the data presented in the figures, performed analysis and interpretation of data, prepared all figures and tables, and wrote the manuscript. Manuel Flores Molina (MFM) and Nathalie Bédard (NB) participated in acquiring the data and recruitment of NAFLD subjects from the CHUM hospital. Geneviève Soucy (GN) did the pathological evaluation of mouse liver tissue, including NAS score and liver fibrosis grade. Vincent Quoc-Huy (VQH) evaluated NAS score and liver fibrosis of the NAFLD cohort. Sabrina Mazouz (SM) participated in the analysis of publicly available microarray datasets. Nathalie Jouvet (NJ), Jessica Dion (JD), and Sarah Tran (ST) provided technical support for animal experiments. Marc Bilodeau (MB) coordinated and supervised recruitment of human subjects and provided valuable input on all aspects of the study. Jennifer L. Estall (JLE) participated in designing the experiments and provided valuable input on metabolic aspects of the study. NHS supervised the whole study including concept and design, obtaining the fund, and co-wrote the manuscript. The data and results presented in this article were all generated during the Ph.D. study of the candidate (MNA). This article is in press at Cellular and Molecular Gastroenterology and Hepatology journal.

Author's contribution to the second manuscript " Profibrogenic role of IL-17A-induced NET in non-Alcoholic Fatty Liver Disease (NAFLD) "

Mohamed N. Abdelnabi (MNA) participated in study concept and design, generated all the data presented in the figures, performed analysis and interpretation of data, prepared all figures and tables, and wrote the manuscript. Manuel Flores Molina (MFM) participated in acquiring the data, Geneviève Soucy (GN) did the pathological evaluation of mouse liver tissue, including NAS score and liver fibrosis grade. Jessica Dion (JD) participated in acquiring the data and provided technical

support for animal experiments. Naglaa H. Shoukry (NHS) NHS supervised the whole study including concept and design, obtaining the fund, and co-wrote the manuscript. The data and results presented in this article were all generated during the Ph.D. study of the candidate (MNA). This article is in under preparation.

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Curriculum Vitae

Mohamed Abdelnabi

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Education

2017/1 (2022/11)	Doctorate, PhD degree, Immunology, Université de Montréal Degree Status: In Progress Thesis Title: Role of intrahepatic immune response, specifically IL-22 and IL-17A cytokines, in nonalcoholic steatohepatitis (NASH)-related fibrosis Supervisors: Shoukry, Naglaa
2014/9 - 2015/10	Master's Thesis, Master of science, Molecular Medicine, University of Sheffield Degree Status: Completed Thesis Title: Modulation of CXCR4 cellular localization in multiple myeloma cells, new insights into cancer therapies Supervisors: Richards, Gareth
2003/9 - 2008/6	Bachelor's Honours, bachelor’s degree, Pharmacy, Misr International University Degree Status: Completed Supervisors: N/A

Recognitions and Awards

2022/5	Frank Bialystok Award (Canadian dollar) Prize / Award I received this award (500$) for the best PhD student poster presentation at the 11th Canadian Symposium on hepatitis C virus (HCV)
2019/5	Graduate Student Poster Award (Canadian dollar) CanHepC network Prize / Award I received this award (400$) for the best PhD student poster presentation at the 8th Canadian Symposium on hepatitis C virus (HCV)
2018/7 - 2022/9	PhD Fellowship - 25,000 (Canadian dollar) Canadian Network on Hepatitis C Prize / Award The CanHepC network guarantees a PhD fellowship with a minimum stipend of $25,000 per year, renewable for a period up to 4 year(s) starting July 1st, 2018, till September 2022

2017/1 - 2018/12	Bourse d'exemption des droits supplémentaires de scolarité (Foreign student fee exemption) - 35,415 (Canadian dollar) University of Montreal I received this scholarship from University of Montreal to waive the international tuition fees of my PhD study
2003/9 - 2008/6	bachelor's degree in pharmacy with first class honor - 40,000 (Egyptian pound) Misr International University

Publications

1. Abdelnabi MN, Molina MF, Soucy G, Trinh QH, Bédard N, Mazouz S, Jouvet N, Dion J, Tran S, Bilodeau M, Estall JL, Shoukry NH. (2022). Sex-Dependent Hepatoprotective Role of IL-22 Receptor Signaling in Non-Alcoholic Fatty Liver Disease-Related Fibrosis. Cellular and Molecular Gastroentrology and Hepatology.
   First Listed Author
   In Press,
   Refereed? Yes, Open Access?: Yes
2. Molina MF, Abdelnabi MN, Mazouz S, Baighman DV, Trinh VQH, Muhammad S, Bedard N, Laverde DO, Hassan GH, Di Polo A, Shoukry NH. (2022). Distinct spatial distribution and roles of Kupffer cells and monocyte-derived macrophages in mouse acute liver injury. Frontiers in Immunology.
   Co-Author
   Accepted,
   Refereed? Yes, Open Access?: Yes
3. Rheault M, Abdelnabi MN, Atif J, Gobran S, Greenwald Z, Fontaine G, Jeong D, Delaunay CL, Kolla G, Marathe G, Makuza JD, Mazouz S, Mortazhejri S, Li J, Liu CH, Palmer M, Castilho AMP, Saeed Y, Sag M, Shengir M, Udheister STP, Wallace HL, D'souza S. (2021). Open Letter: Hepatitis Can't Wait Teachings from COVID-19 to combat Hepatitis B and C viruses. McGill Daily.
   Co-Author
   Published,
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4. Molina MF, Fabre T, Cleret-Buhot A, Soucy G, Meunier L, Abdelnabi MN, Belforte N, Turcotte S, Shoukry NH. (2019). Visualization, Quantification, and Mapping of Immune Cell Populations in the Tumor Microenvironment. JoVE.
   Co-Author
   Published,
   Refereed? Yes, Open Access?: No
5. Molina MF, Abdelnabi MN, Fabre T, Shoukry NH. (2018). Type 3 cytokines in liver fibrosis and liver cancer. Cytokine.
   Co-Author
   Published,
   Refereed? Yes, Open Access?: No

Presentations

1. Abdelnabi MN, Molina MF, Soucy G, Trinh QH, Bédard N, Mazouz S, Dion J, Tran S, Bilodeau M, Shoukry NH. (2022). Sex-Dependent Hepatoprotective Role of Il-22 In NAFLD-Related Fibrosis (Poster). The European Association for the Study of the Liver (EASL), International Liver Congress

2022, London, United Kingdom
Main Audience: Researcher

2. Abdelnabi MN, Molina MF, Soucy G, Trinh QH, Bédard N, Mazouz S, Dion J, Tran S, Bilodeau M, Shoukry NH. (2022). Sex-Dependent Hepatoprotective Role Of IL-22 In NAFLD-Related Fibrosis (Poster). Canadian Liver meeting 2022 (CLM2022), Ottawa, Canada
Main Audience: Researcher

3. Molina MF, Abdelnabi MN, Bédard N, Muhammad S, Cleret-Buhot A, Shoukry NH. (2020). Spatio-Temporal Characterization Of The Immune Landscape During Acute Liver Injury (Poster). American Association for the study of liver Diseases (AASLD), The Liver Meeting Digital Experience, United States Main Audience: Researcher

4. Abdelnabi MN, Molina MF, Fabre T, Bilodeau M, Soucy G, Shoukry NH. (2019). Neutrophils are the major producers of the pro-fibrogenic cytokine IL-17A in non-alcoholic fatty liver (NAFLD) (Poster). Canadian Liver meeting 2019 (CLM2019), Montreal, Canada
Main Audience: Researcher

5. Molina MF, Fabre T, Abdelnabi MN, Bilodeau M, Soucy G, Turcotte S, Shoukry NH. (2018). Neutrophils and mast cells are major producers of IL-17 in hepatocellular carcinoma (Poster). Single Topic Conference 2018-Hepatic Fibrosis: New Concepts and Controversies. American Association for the study of liver Diseases (AASLD), Texas, United States
Main Audience: Researcher

6. Abdelnabi MN, Molina MF, Fabre T, Soucy G, Shoukry NH. (2017). Role of the intrahepatic immune response in non-alcoholic fatty liver disease (NAFLD) (Poster). 5th Day of research students of the Department of Microbiology, Immunology and Infectious Disease. University of Montreal, Montreal, Canada Main Audience: Researcher

7. Molina MF, Fabre T, Abdelnabi MN, Bilodeau M, Soucy G, Turcotte S, Shoukry NH. (2017). Novel Combined Multiplex Immunofluorescence and Histochemistry for Detecting IL17 and IL22 producing cells in Hepatocellular Carcinoma Tissue (Poster). Canadian Microscopy and Cytometry symposium (CMCS), Montreal, Canada
Main Audience: Researcher

User Profile

Researcher Status: Doctoral Student
Research Career Start Date:
2014/09/15

Key Theory / Methodology: Animal model (acute and chronic liver injuries), flow cytometry, cell sorting, immunofluorescence, immunohistochemistery, western blot, real-time qPCR, mammalian cell culture, immunoprecipitation, and cAMP assay

Fields of Application: Biomedical Aspects of Human Health, Pathogenesis and Treatment of Diseases

Disciplines Trained In: Immunology, Molecular Biology, Pharmacy

Areas of Research: Immune Mediators: Cytokines and Chemokines

Research Specialization Keywords: Animal model (mice), Immunology, Liver disease, Liver inflammation and fibrosis, Neutrophils, Nonalcoholic fatty liver disease (NAFLD), T lymphocytes

Research Disciplines: Gastroenterology, Immunology, Pharmacology

Community and Volunteer Activities

2018/7	Volunteer, Canadian Network on Hepatitis C (CanHepC) As being a member of CanHepC, I participated with other CanHepC members in the World Hepatitis Day (July 28th, 2018). Based on the World Hepatitis Alliance campaign (WHO) theme "**Find the Missing Millions**", we chose to focus messaging on people born between 1945 and 1975 (Baby Boomers) as well as foreign born individuals from countries with high rates of HCV infection encouraging them to get tested and informing them about the treatments available. We set up information kiosks in Du Boisé Public Library, Montreal, QC, Canada. We printed Over 1000 flyers in 3 languages (French, English and Arabic) explaining the importance of getting tested with contact information of a clinic at the CHUM that offers walk-in HCV assessment and treatment were distributed with incentives such as pens and cookies
2015/9 - 2015/11	Volunteer, Bloodwise Bloodwise is a non-profit organization, and its main mission is funding a world-class research that can aid in preventing blood Cancer. My main duties were collecting donations and raising people's awarness of blood cancer.