



Immune cell-mediated features of non-alcoholic steatohepatitis

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Abstract | Non-alcoholic fatty liver disease (NAFLD) includes a range of hepatic manifestations, starting with liver steatosis and potentially evolving towards non-alcoholic steatohepatitis (NASH), cirrhosis or even hepatocellular carcinoma. NAFLD is a major health burden, and its incidence is increasing worldwide. Although it is primarily a disease of disturbed metabolism, NAFLD involves several immune cell-mediated inflammatory processes, particularly when reaching the stage of NASH, at which point inflammation becomes integral to the progression of the disease. The hepatic immune cell landscape is diverse at steady state and it further evolves during NASH with direct consequences for disease severity. In this Review, we discuss current concepts related to the role of immune cells in the onset and progression of NASH. A better understanding of the mechanisms by which immune cells contribute to NASH pathogenesis should aid the design of innovative drugs to target NASH, for which current therapeutic options are limited.

Insulin resistance

A state in which cells are not able to respond fully to insulin or induce signalling pathways downstream of the insulin receptor.

Steatosis

Describes the abnormal accumulation of lipids in a tissue.

The prevalence of metabolic diseases, as exemplified by obesity, has increased at an exponential rate over the past few decades. Importantly, in addition to having its own comorbidities, obesity broadly impacts on the health of a patient and increases their susceptibility to other conditions. For example, patients with obesity are more prone to cancer¹ and their susceptibility to infectious diseases is increased, as recently observed for coronavirus disease 2019 (COVID-19)². Long-recognized comorbidities of obesity include insulin resistance and type 2 diabetes, hypertension, dyslipidaemia, cardiovascular disease and fatty liver disease³. Remarkably, non-alcoholic fatty liver disease (NAFLD) is also a major independent risk factor for the development of cardiovascular diseases, including atherosclerosis^{4,5}. There is growing evidence of a tight association between obesity and NAFLD⁶, and considerable research attention is now being directed to this association owing to the increasing prevalence of NAFLD and its ability to evolve towards liver dysfunction and failure. The burden of NAFLD is expected to increase and current projections estimate that, by 2030, more than 300 million individuals will develop NAFLD in China, more than 100 million in the United States and 15–20 million in the major European countries⁷. Current economic costs associated with NAFLD and its comorbidities are extremely high^{8–10}. Thus, strong efforts are underway to develop innovative therapeutics to combat the disease, for which there are currently no approved therapies¹¹.

NAFLD is a heterogeneous condition, the clinical manifestations of which only become problematic at the stage of non-alcoholic steatohepatitis (NASH), when

liver injury, inflammation and fibrosis are superimposed on the initial steatosis¹². Inflammatory processes are involved in the onset and progression of NASH¹² and they also favour its evolution towards more debilitating conditions such as cirrhosis and hepatocellular carcinoma (HCC)¹². Here, we first summarize the clinical features of NAFLD and NASH and their pathophysiology. After describing the immune landscape of the resting liver, we focus on the immune cell-mediated and inflammatory aspects of NASH, in particular discussing the role of diverse immune cell populations in NASH and its transition to HCC. However, we do not cover in any detail the inflammatory processes in NASH involving non-immune cells. Finally, we conclude by summarizing the therapies that are currently under development and possible future therapeutic options to combat NASH.

The spectrum of NAFLD

The occurrence of a fatty liver in the absence of significant alcohol consumption was classified as NAFLD 40 years ago¹³. A consensus group of experts recently proposed that the term ‘metabolic dysfunction-associated fatty liver disease (MAFLD)’ may be used to more accurately describe the key metabolic dimension associated with hepatic steatosis, without excluding other aetiologies of liver disease such as alcohol consumption or viral hepatitis^{6,14}. However, this change in nomenclature is a matter of debate in the field¹⁵; thus, we will continue to use NAFLD until the issue is resolved. It should also be noted that the definition of MAFLD does not entirely overlap with that of NAFLD and therefore the terms are not fully interchangeable when discussing individual

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Metabolic syndrome

A group of five conditions (increased blood pressure, high blood sugar level, excess body fat around the waist, and abnormal cholesterol or triglyceride levels) that can occur together and increase the risk of heart disease, stroke and type 2 diabetes.

Endoplasmic reticulum stress

A state of cellular stress that occurs when protein folding in the ER is impaired, leading to the accumulation of unfolded and/or misfolded proteins.

studies. NAFLD is considered the hepatic manifestation of metabolic syndrome and is highly associated with metabolic comorbidities, including obesity, type 2 diabetes, hyperlipidaemia and hypertension¹⁶. Metabolic syndrome is the most common cause of chronic liver disease and has been estimated to be present in about 25% of the adult population worldwide¹⁶. NAFLD is a progressive disease whereby steatosis (the excessive accumulation of lipids in hepatocytes) constitutes the first disease stage, which can eventually evolve to the more complex stage of NASH. NASH is characterized by the presence of hepatic steatosis (affecting more than 5% of hepatocytes), hepatocellular damage (with distinctive hepatocyte ballooning), signs of inflammation and varying degrees of fibrosis¹². The disease presents with an important heterogeneity in its clinical manifestations and evolution⁶, such that patients with NASH and advanced fibrosis (10–20% of patients with NAFLD)^{17,18} may further progress to cirrhosis, HCC and end-stage liver disease, potentially requiring liver transplantation¹⁹. Thus, NASH is a key step in the clinical progression of NAFLD.

Histological scoring systems remain the 'gold standard' to diagnose NAFLD and to identify patients with NASH who are at greater risk of progression to cirrhosis and HCC. Nevertheless, the disease scoring and staging systems are semiquantitative, subject to sampling error and evaluate only major changes in hepatic tissue remodelling. In this context, emerging techniques that analyse liver biopsies with high-resolution multiphoton microscopy, 3D digital reconstructions and computational simulations are now offering new possibilities for the quantitative characterization of disease progression²⁰. In addition, multiple blood-based biomarkers have been developed that, combined with measurements of liver stiffness, help to guide clinicians in identifying patients with NAFLD who are at risk of progression to NASH^{21–24}. The identification of such patients is a key challenge for disease management but also to improve patient selection and the design of clinical trials for emerging pharmacotherapies. Limiting NASH onset and blocking its transition towards liver failure and HCC are major health challenges worldwide.

Pathophysiology of NASH

NASH is a complex multifactorial disease and its exact aetiology is not completely understood. Overall, multiple triggers are likely to contribute to inducing the hallmarks of NASH, namely steatosis, liver injury and inflammation, which together lead to the onset of fibrosis and sometimes HCC (FIG. 1). Steatosis is, in most cases, an early event in NAFLD but it does not necessarily transition to NASH. Additional stresses are needed to induce NASH onset, such as lipotoxicity, oxidative stress and inflammation, which combine to induce cellular stress pathways leading to hepatocyte death, inflammation and fibrosis development.

Liver steatosis, which is usually characterized by increased liver triglyceride storage, can arise from circulating free fatty acids released upon adipose tissue lipolysis but also from hepatic fatty acid synthesis through de novo lipogenesis²⁵. A major substrate for de novo lipogenesis is fructose²⁶, which is sometimes used in

combination with fat in dietary mouse models of NASH (BOX 1). A recent study showed that fructose intake is sufficient to induce NASH and its evolution towards HCC in the NASH-susceptible *MUP-uPA* mouse model²⁷. In addition to sustaining de novo lipogenesis, fructose also increases gut permeability, which can lead to hepatic inflammation; together, de novo lipogenesis and increased gut permeability precipitate NASH onset and the NASH–HCC transition²⁷. High-fat diet (HFD) feeding also favours NASH and HCC development in *MUP-uPA* mice by inducing endoplasmic reticulum stress and liver injury, in part through production of the pro-inflammatory cytokine tumour necrosis factor (TNF)²⁸. Thus, regardless of the substrate (carbohydrate or fat), the induction of hepatic steatosis favours NASH onset in the *MUP-uPA* mouse model. Importantly, recent studies highlight that diet-induced NASH is accelerated in the presence of dietary cholesterol^{29,30}, favouring the transition to HCC^{31,32}. In HFD-fed *MUP-uPA* mice, endoplasmic reticulum stress-induced caspase 2 activation leads to the activation of sterol regulatory element-binding proteins (SREBPs), which results in cholesterol and triglyceride synthesis and accumulation in the liver³³. Caspase 2 expression is similarly increased in patients with NASH, and genetic or pharmacological inhibition of caspase 2 limits NASH development in HFD-fed *MUP-uPA* mice³³. Overall, the data indicate that hepatic accumulation of triglycerides and cholesterol creates a permissive environment for the development of NASH.

Lipid-mediated stresses can lead to liver injury by increasing hepatocyte susceptibility to apoptosis. Indeed, steatosis induced by long-term feeding of mice with a HFD sensitizes hepatocytes to cytokine-induced cell death³⁴, as may occur during NASH when hepatic leukocytes, such as macrophages, locally secrete inflammatory cytokines³⁵. In humans, hepatocyte apoptosis is greater in patients with NASH than in those with simple steatosis and this correlates with increased levels of fibrosis and inflammation³⁶. However, other forms of cell death, such as necroptosis, might also occur during NASH and contribute to its pathogenesis³⁷. In mice, hepatocyte death alone can trigger NASH development. Indeed, deleting *Ikkkg* in hepatocytes (Hep ^{Δ Ikkkg} mice), which impedes the pro-survival effect of nuclear factor- κ B signalling, leads to spontaneous hepatocyte cell death and liver injury in mice fed a standard chow diet³⁸. Hep ^{Δ Ikkkg} mice eventually develop steatosis, NASH and HCC³⁸, which recapitulates the natural course of NAFLD. Of note, increased hepatocyte susceptibility to cell death is also a hallmark of the *MUP-uPA* mouse model. Indeed, increased hepatocyte death is already noticeable in *MUP-uPA* mice compared with wild-type mice fed a standard chow diet and HFD feeding of *MUP-uPA* mice further increases hepatocyte death and precipitates NASH onset²⁸. Also in support of a key role for hepatocyte apoptosis in NASH, deletion of AMP-activated protein kinase (AMPK) in hepatocytes leads to their death and accelerates fibrosis in a diet-induced mouse model of NASH³⁹. Mechanistically, AMPK limits caspase 6 activation and thus caspase 6-mediated hepatocyte death during NASH³⁹. Caspase 6 activation is increased

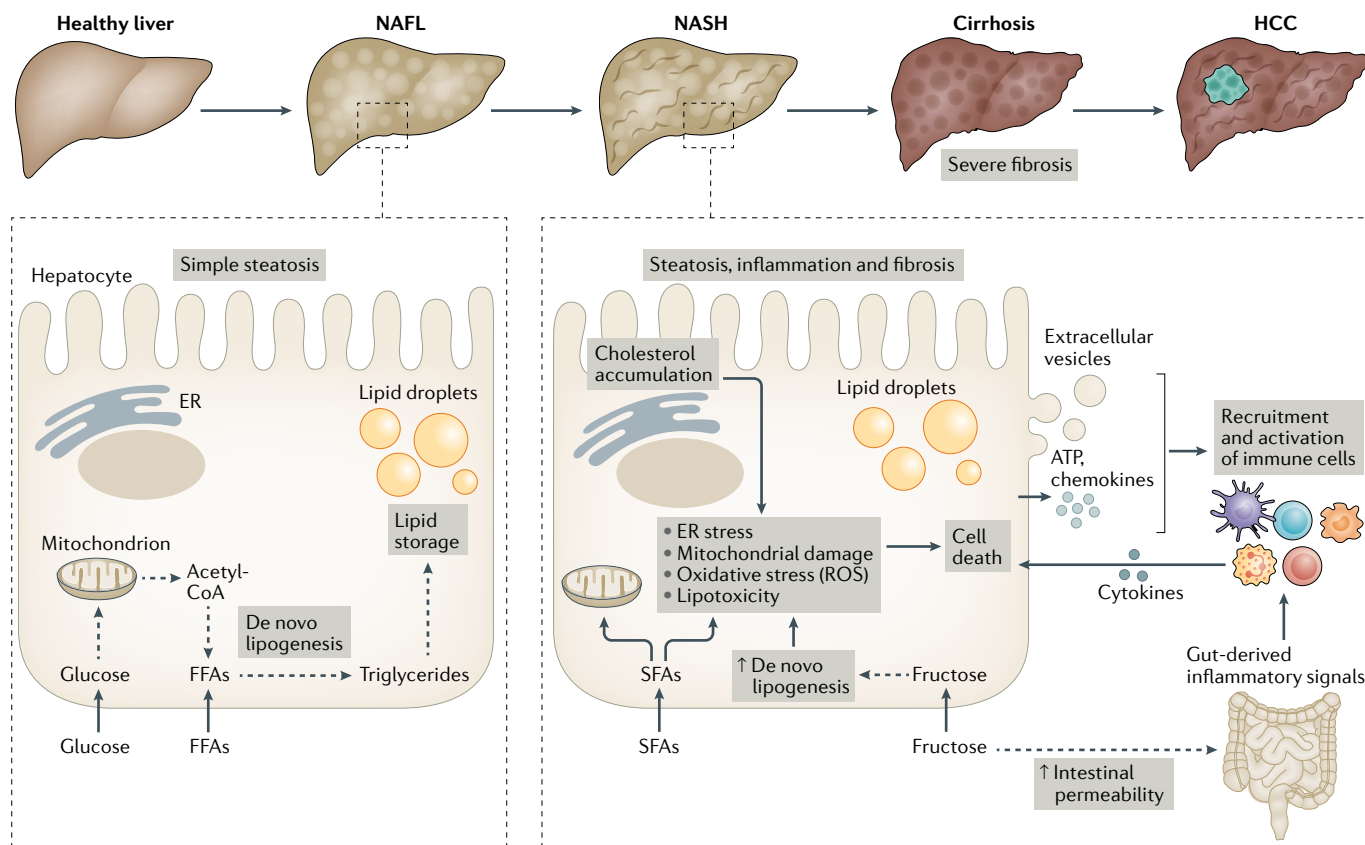


Fig. 1 | Contributing factors in NAFLD progression and NASH pathogenesis. Non-alcoholic fatty liver disease (NAFLD) is a progressive disease that starts with simple steatosis (non-alcoholic fatty liver (NAFL)) and can evolve to a more complex form known as non-alcoholic steatohepatitis (NASH), which is characterized by steatosis, inflammation and fibrosis. NASH can further progress towards cirrhosis and, in some cases, hepatocellular carcinoma (HCC). The NAFL stage is characterized by triglyceride accumulation in hepatocytes through de novo lipogenesis. Lipogenesis is fuelled by the uptake of glucose and free fatty acids (FFAs) and their incorporation into lipid-synthesis pathways. The onset of NASH is associated with multiple cellular stresses in lipid-loaded hepatocytes, including endoplasmic reticulum (ER) stress, mitochondrial damage, oxidative stress

(involving the production of reactive oxygen species (ROS) and/or lipotoxicity. Such cellular stresses can have various causes, including the accumulation of saturated fatty acids (SFAs), increased de novo lipogenesis resulting from increased fructose uptake, or cholesterol accumulation in the ER. In addition to increasing de novo lipogenesis, fructose also increases intestinal permeability, which can lead to the activation of hepatic immune cells by gut-derived inflammatory mediators. This promotes liver inflammation as well as the death of stressed hepatocytes, which also release pro-inflammatory mediators such as ATP, extracellular vesicles and chemokines, further reinforcing the inflammatory process and leading to the development of fibrosis. Exacerbation of this vicious cycle can precipitate the transition towards cirrhosis and eventually the development of HCC.

in various mouse models of NASH and correlates with increased fibrosis in patients with NASH. In mice, caspase 6 inhibition limits the diet-induced hallmarks of NASH³⁹ and broad-spectrum caspase inhibitors also protect against NASH^{40,41}. Importantly, inflammation has a crucial role in hepatocyte death during NASH as limiting TNF-induced signalling reduces hepatocyte death and liver injury, which translates to reduced liver steatosis and fibrosis⁴². Thus, hepatocyte death is a cardinal feature of NASH pathogenesis.

In addition to lipid-mediated stress and hepatocyte death, the liver environment during NASH also leads to oxidative stress in patients and in mouse models^{43,44}. Mice deficient for *Nfe2l2*, which encodes NRF2 (a master regulator of the antioxidant response), are highly susceptible to diet-induced NASH and present with an accelerated transition from simple steatosis to NASH⁴⁵. In addition, oxidative stress is increased in Hep^{ΔIkbkg} mice and antioxidant treatment blunts the progression to NASH in this model³⁸. Increased oxidative stress during

NASH notably induces the oxidation and inactivation of hepatic protein tyrosine phosphatases, including protein tyrosine phosphatase non-receptor type 2 (PTPN2, also known as TCPTP)⁴⁶. *Ptpn2* deletion in hepatocytes leads to uncontrolled signalling through signal transducer and activator of transcription 1 (STAT1) and STAT3 that accelerates the progression of steatosis to NASH and the transition of NASH to HCC, respectively⁴⁶. In addition, it is noteworthy that mitochondrial oxidative stress induced upon hepatic accumulation of free cholesterol increases hepatocyte susceptibility to cytokine-induced cell death⁴⁷. The NASH inflammatory environment, which is mostly controlled by cells of the immune system, has a crucial role here. Indeed, inflammatory mediators, such as TNF, produced by immune cells induce the death of lipid-loaded, stressed hepatocytes that are rendered sensitive to cytokine-mediated cell death⁴⁸. Finally, oxidized phospholipids, which are non-enzymatic products of oxidant-mediated lipid peroxidation, are also produced during NASH and induce mitochondrial

β-oxidation

Catabolic process occurring in the mitochondria by which fatty acids are converted into acetyl-CoA, a major metabolic intermediate.

Mallory–Denk bodies

Cytoplasmic aggregates of damaged cytoskeletal components found in hepatocytes during NASH or alcoholic liver disease.

Portal tracts

Areas of the liver, also known as portal triads, that consist of a bile duct, a small branch of the portal vein and a branch of the hepatic artery.

damage as well as further production of reactive oxygen species (ROS). Antibody-mediated neutralization of oxidized phospholipids decreases oxidative stress together with steatosis, inflammation, fibrosis, hepatocyte death and NASH–HCC progression in a diet-induced mouse model⁴⁹.

Thus, lipid loading of hepatocytes, oxidative stress and inflammation function together during NASH to induce hepatocyte death, which leads to liver injury, further inflammation and tissue fibrosis (FIG. 1). The important role of inflammation in these processes suggests that the immune cell-rich hepatic environment can modulate NASH onset and severity. Here, we focus on how immune cell-mediated inflammation impacts NASH pathogenesis, notwithstanding that non-immune cell types, such as hepatocytes and endothelial cells, can also shape hepatic inflammation through different means. For example, hepatocytes can release pro-inflammatory

mediators, such as ATP⁵⁰ or extracellular vesicles^{51,52}, and endothelial cells also produce inflammatory triggers and chemokines such as TNF, IL-6 and CCL2 (REF.⁵³).

Liver immune landscape at steady state

Besides being an essential metabolic and detoxifying organ, the liver is also an important immunological organ that contains a large number of innate and adaptive immune cells⁵⁴. The highly vascularized nature of the liver, together with reduced blood flow in its fenestrated capillary-like vessels known as sinusoids, constitutes a unique environment to maximize the exposure of immune cells to blood-borne and gut-derived pathogens^{55,56}. Indeed, approximately one-third of the body's whole blood volume passes through the liver every minute. Most of that blood flows from the portal vein and drains the gastrointestinal tract, with the remainder (20–25%) originating from the hepatic artery⁵⁷. Most lymphoid and myeloid cell lineages can be found in the liver, residing mainly in the sinusoids but also in the intravascular and subcapsular compartments^{54,58} (FIG. 2). Here, we provide an overview of the key hepatic immune cell populations.

Innate-like T cell populations, including γδ T cells but mostly natural killer T (NKT) cells, are particularly enriched in mouse livers. Notably, invariant NKT (iNKT) cells, which express a CD1d-restricted semi-invariant αβ T cell receptor and recognize lipid-based antigens, represent up to 50% of intrahepatic T cells compared with much lower levels in other mouse tissues⁵⁹. iNKT cells expressing the chemokine receptor CXCR6 patrol the hepatic sinusoids in mice (FIG. 2) under the influence of CXCL16 expressed by liver sinusoidal endothelial cells⁶⁰. It should be noted, however, that iNKT cells are less frequent in human livers (<1% of T cells)⁶¹. By contrast, mucosal-associated invariant T (MAIT) cells, which recognize bacteria-derived vitamin B metabolites through MHC-related 1 (MR1), are rare in mice⁶² but are enriched in the human liver, where they represent 15–45% of the total number of T cells^{63,64}. The liver is also populated with several innate lymphoid cell (ILC) subsets that do not express antigen-specific receptors, including natural killer (NK) cells, ILC1s, ILC2s and ILC3s. In humans, hepatic NK cells predominate over the other ILC subsets^{65,66}. In mice, the main ILC subsets found in the liver are CD49a⁺CD49b⁺ NK cells and tissue-resident CD49a⁺CD49b⁻ ILC1s^{67–69}. As the models used to study ILCs are not specific (for example, models used to deplete NK cells also target ILC1s), the roles of particular subsets in liver physiology and pathology are unclear.

Immune surveillance of the liver also involves conventional CD4⁺ and CD8⁺ αβ T cells, with CD4⁺ T cells present at reduced numbers compared with CD8⁺ T cells⁵⁸. A portion of the CD8⁺ T cells are long-lived resident-memory T cells that are found in the liver parenchyma as well as patrolling the hepatic sinusoids^{70,71} (FIG. 2), where they confer a first-line response to reinfection⁷⁰. Naive B cells and antibody-producing plasma cells are also present in the liver⁷². These plasma cells are mainly found in portal tracts and originate from B cells that are activated in gut-associated lymphoid tissue to produce IgA reactive to commensal bacteria and oral antigens⁷³ (FIG. 2).

Box 1 | Mouse models of fatty liver disease

Multiple mouse models have been generated to study non-alcoholic fatty liver disease (NAFLD) and the transition of non-alcoholic steatohepatitis (NASH) to hepatocellular carcinoma (HCC). The strong association of NAFLD with overnutrition, obesity and insulin resistance has led to the development of various diet-induced models, using dietary formulas enriched in lipids. Moreover, widely used models of obesity, insulin resistance and atherosclerosis, such as *ob/ob* mice and *Apoe*^{-/-} mice, develop features of NAFLD but do not usually transition to NASH and are therefore not discussed in this Review. Mice fed a high-fat diet (HFD) typically develop fatty liver but have limited inflammation and no appreciable fibrosis, which are key components of NASH¹⁶⁹. To circumvent this limitation, the HFD has been supplemented with cholesterol, saturated fatty acids such as palmitate, or fructose and/or sucrose to further promote hepatic insulin resistance, inflammation and mild-to-moderate fibrosis^{49,50,81,151,170}. Occasional evidence of HCC development has also been reported with such diets^{50,81,151}. These supplemented diets, and in particular those rich in fat, cholesterol and fructose and/or sucrose, are often referred to as a 'Western diet' although its composition has not been standardized and varies across laboratories. A recent systematic analysis of the literature on rodent models of NAFLD suggests that high-fat, high-fructose diets most closely recapitulate the overall characteristic features of human NAFLD¹⁷¹. Along these lines, inbred isogenic C57BL/6J×129S1/SvImJ mice (termed DIAMOND mice) fed a cholesterol-enriched HFD with ad libitum consumption of water with a high fructose and glucose content develop NASH with progressive stages of fibrosis, and a major proportion of these mice develop HCC in the long term (>30 weeks of diet)¹⁷². Owing to its mixed genetic background, this model would be more suitable for the preclinical development of therapeutic targets than are knockout or conditional knockout strains, which are more suitable for mechanistic studies. *MUP-uPA* mice, which express the urokinase plasminogen activator (uPA) under control of the hepatocyte-specific *MUP* promoter, are another specific model of NASH. When fed a HFD²⁸ or high-fructose diet²⁷, these mice undergo endoplasmic reticulum stress in hepatocytes and develop obesity, insulin resistance, liver fibrosis and liver inflammation. The appearance of liver tumours is also observed in these mice after 8–10 months of diet. Diets deficient in methionine and/or choline, which are indispensable for hepatic mitochondrial β-oxidation of fatty acids and the synthesis of very low-density lipoprotein, respectively, have been widely used in preclinical NASH research^{151,173}. In particular, the methionine-deficient and choline-deficient diet, enriched in fat and sucrose, favours the rapid onset of hepatic macrovesicular steatosis, lobular inflammation and perisinusoidal fibrosis, which recapitulates the ultrastructural changes observed in human NASH¹⁷⁴. A disadvantage of this diet resides in the hypercatabolism that it induces, leading to weight loss and the absence of insulin resistance. Thus, a choline-deficient diet supplemented with high levels of fat (60% of calories from fat; known as CD-HFD) has been developed to replace the methionine-deficient and choline-deficient diet. Long-term CD-HFD feeding increases fat mass and impairs glucose homeostasis, as well as induces severe steatosis, ballooned hepatocytes, immune cell infiltration, satellitosis, the formation of Mallory–Denk bodies and glycogenated nuclei, all of which are features of human NASH¹¹⁵. In addition, CD-HFD-fed mice have a 25% incidence rate of liver tumours after 12 months of diet¹¹⁵.

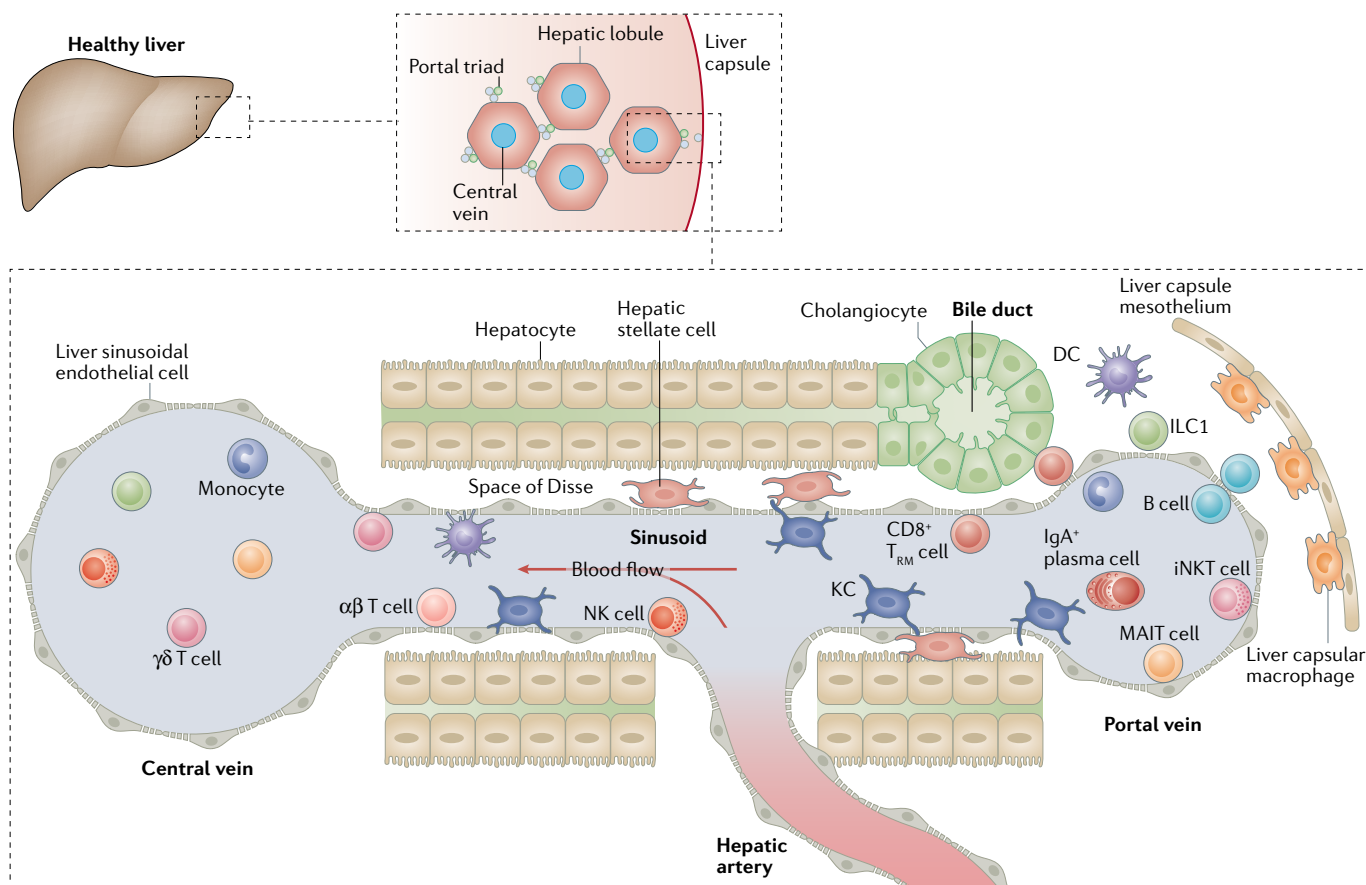


Fig. 2 | Immune landscape of the healthy liver. The liver is composed of lobules that are repeated hexagonal anatomical units. These lobules consist of hepatocytes organized around a central vein that is connected to the hepatic artery and the portal vein via a sinusoidal network. The bile produced by hepatocytes is released into the gastrointestinal tract via bile ducts composed of cholangiocytes. The hepatic artery, portal vein and bile duct together form the portal triads found between hepatic lobules. The blood flow along the portal to central axis (from portal vein and hepatic artery to central vein) creates gradients of oxygen and nutrients, resulting in a spatial division of labour among hepatocytes, a phenomenon known as metabolic zonation. A large spectrum of immune cells is found in the liver. Some circulate or temporarily patrol the hepatic sinusoids or the liver parenchyma, such as natural killer (NK) cells, $\gamma\delta$ T cells,

CD4⁺ and CD8⁺ $\alpha\beta$ T cells, monocytes, B cells, invariant NKT (iNKT) cells, mucosal-associated invariant T (MAIT) cells and dendritic cells (DCs). Long-lived resident cells, such as Kupffer cells (KCs), CD8⁺ tissue-resident memory T (T_{RM}) cells and type 1 innate lymphoid cells (ILC1s) are also found. Liver lobules also have a spatially polarized immune system, known as immune zonation, with KCs, iNKT cells, CD8⁺ T_{RM} cells and IgA⁺ plasma cells being particularly enriched in the periportal regions. KCs, which specifically reside in the sinusoids in close contact with liver sinusoidal endothelial cells, are the most abundant immune cell population in the liver. They also establish connections with hepatic stellate cells and hepatocytes in the space of Disse. Another resident macrophage population occupies the liver capsule and these cells are known as liver capsular macrophages.

Gut-associated lymphoid tissue

Intestinal lymphoid structures, including Peyer's patches and isolated lymphoid follicles, that are particularly rich in antibody-producing plasma cells.

Hepatic stellate cells

(HSCs). Mesenchymal liver cells found in the area between sinusoids and hepatocytes, known as the space of Disse.

With regards to the myeloid compartment, time-of-flight mass cytometry analysis has identified several populations of dendritic cells (DCs), including conventional and plasmacytoid subsets, in mouse livers⁷⁴. Confocal imaging using CD11c and CX3CR1 markers has suggested that DCs are also distributed under the Glisson capsule, the mesothelium that covers the liver⁷⁴. However, these CX3CR1⁺ cells, which have a characteristic dendritic morphology, may in fact correspond to the recently described population of CX3CR1⁺CD11c⁺MHC-II^{hi}F4/80^{low} liver capsular macrophages⁷⁵ (FIG. 2). Liver capsular macrophages derive from blood monocytes and provide immune protection against peritoneal infections⁷⁵. However, the vast majority of liver macrophages are represented by Kupffer cells (KCs), which develop during embryogenesis and are maintained in the healthy adult liver by self-renewal independently from blood monocytes^{76–78}.

KCs exclusively reside in the liver sinusoids (FIG. 2), where they interact closely with liver sinusoidal endothelial cells, hepatic stellate cells (HSCs) and hepatocytes to acquire their tissue-imprinted signature as well as the signals that enable self-maintenance⁷⁹. In addition, chemokines produced by liver sinusoidal endothelial cells determine the asymmetric distribution of KCs within the sinusoids, with an enriched periportal localization⁸⁰.

In summary, the liver is an immune cell-rich environment that, in addition to its role in maintaining homeostasis, undoubtedly has a key role in the pathology of several liver diseases, including NASH.

Immune modulation of NASH pathogenesis

Recent advances in single-cell transcriptomics have allowed for a better appreciation of how the immune cell repertoire is reshaped during NASH in mice^{81,82} but

also in the human cirrhotic liver⁸³. For example, major changes in the myeloid compartment were observed in both mice and humans, with a marked influx of monocytes and monocyte-derived cells^{81–83}. Such changes in the hepatic immune cell landscape are likely to contribute to the uncontrolled inflammatory environment that fuels liver injury and leads to NASH progression. A complex crosstalk between diverse immune cell populations and hepatocytes, HSCs and liver sinusoidal endothelial cells certainly operates during the disease. However, such complexity of interactions is only starting to be uncovered and much of our knowledge so far relates to studies of individual immune cell populations in NASH pathogenesis (FIG. 3), as discussed below. Novel tools, such as single-cell RNA sequencing or single-nucleus RNA sequencing combined with interactome analysis, should allow for a better understanding of the communication axis ongoing during NASH in the near future (BOX 2).

B cells and plasma cells. B cells produce immunoglobulins⁸⁴, present antigens⁸⁵ and secrete cytokines upon Toll-like receptor-mediated activation by pathogen-associated molecular patterns⁸⁶, thereby influencing immune-mediated inflammatory responses in diverse ways. B cells accumulate in the livers of patients with NASH who have high levels of lobular inflammation and fibrosis⁸⁷, which suggests that they might alter the course of the disease. B cells are pro-inflammatory in mouse models of NASH and this involves both B cell receptor-mediated adaptive immune mechanisms and MYD88-dependent innate immune mechanisms⁸⁸. In addition, increased gut permeability and microbiota-derived inflammatory mediators probably contribute to the increased activation of intrahepatic B cells during NASH⁸⁸. Finally, B cell-deficient mice have reduced NASH severity owing to decreased inflammation and fibrosis⁸⁸.

B cells are heterogeneous and can be subdivided into two major lineages. B2 cells are activated in secondary lymphoid organs and receive help from CD4⁺ T helper (T_H) cells to generate antigen-specific, high-affinity antibodies⁸⁴. B1 cells secrete ‘natural’ antibodies (germline-encoded immunoglobulins present in the absence of exogenous antigen stimulation) as part of an innate-like immune response⁸⁹. B2 cell depletion is associated with reduced NASH-associated hepatic fibrosis in mice⁸⁷, whereas the specific role of B1 cells in NASH has not yet been studied. Further highlighting an important role for B2 cells in NASH pathogenesis, serum levels of B cell-activating factor (BAFF), a cytokine that controls the differentiation and survival of B2 cells but not of B1 cells⁹⁰, are increased in patients with NASH and further increased in those with fibrosis⁹¹. In mice, BAFF neutralization limited liver injury during NASH⁸⁷.

Upon activation, B2 cells differentiate into antibody-producing B cells or long-lived antibody-producing plasma cells. The liver is particularly rich in IgA-producing plasma cells at the steady state⁷³ and the number of these cells is further increased during NASH in both mice and humans⁹². In addition, increased serum levels of IgA are associated with advanced hepatic

fibrosis in patients with NASH⁹³. HFD-fed *MUP-uPA* mice that lack IgA develop milder NASH with decreased liver injury and fibrosis⁹². However, although several lines of evidence implicate B cells and IgA in NASH pathogenesis (FIG. 3), the underlying mechanisms warrant further investigation. In addition, the antigen specificity of the B cells that are generated in patients with NASH and involved in disease progression remains to be elucidated.

Dendritic cells. Upon local activation, DCs can migrate to tissue-draining lymph nodes where they interact with naive T cells⁹⁴. In addition to their role in inducing an adaptive immune response in this manner, DCs can also participate in local inflammation through their ability to respond to a wide range of pathogen-associated molecular patterns that engage Toll-like receptors and other pattern recognition receptors⁹⁴. Both subsets of conventional DCs (CD103⁺ cDC1s and CD11b⁺ cDC2s) are present in the liver⁹⁴ and accumulate during NASH in mice^{95,96} but their impact on NASH pathogenesis remains elusive. In humans, the number of liver cDC1s was increased in patients with NASH and increased cDC1 numbers were associated with increased disease hallmarks of NASH⁹⁶. NASH induction in *Batf3*-deficient mice that lack cDC1s led to increased liver triglyceride levels but a similar extent of liver injury⁹⁷. However, such whole-body deletion of *Batf3* could have influenced NASH independently from cDC1 loss. Using a more-specific model of cDC1 depletion, cDC1s were shown to promote liver injury in mice but the mechanisms remain to be clearly defined⁹⁶. By contrast, the role of cDC2s in NASH has not been addressed so far. Overall, further investigation will be needed to better appreciate the role of cDCs in NASH pathogenesis as well as the underlying mechanisms.

Conventional CD4⁺ and CD8⁺ T cells. Conventional CD4⁺ T_H cells adopt a range of polarized cell fates determined by their interactions with specific DC subsets and the cytokine environment^{94,98}. T_H1, T_H2 and T_H17 cell fates are characterized by the expression of interferon- γ (IFN γ), IL-4 and/or IL-13, and IL-17, respectively⁹⁸. The roles of several of these cytokines and their signalling pathways have been studied in the context of NASH. However, as cell types other than CD4⁺ T cells also produce these cytokines, it is difficult to specifically assign the phenotype reported to changes in T_H cell populations and targeted approaches are required to further our understanding.

Mice deficient in IFN γ , the prototypical T_H1 cell cytokine, were markedly protected against liver injury and hepatic fibrosis in a mouse model of NASH⁹⁹. In these animals, decreased fibrosis is associated with a markedly decreased expression of osteopontin, which is a known inducer of liver fibrogenesis¹⁰⁰, but the underlying mechanisms remain largely unexplored. Cellular sources of IFN γ other than T_H1 cells, in particular CD8⁺ T cells, might have contributed to the phenotype. The IFN γ -inducible chemokine CXCL10 is also involved in NASH pathogenesis. CXCL10 induces chemotaxis of CXCR3-expressing cells, including T cells. Circulating

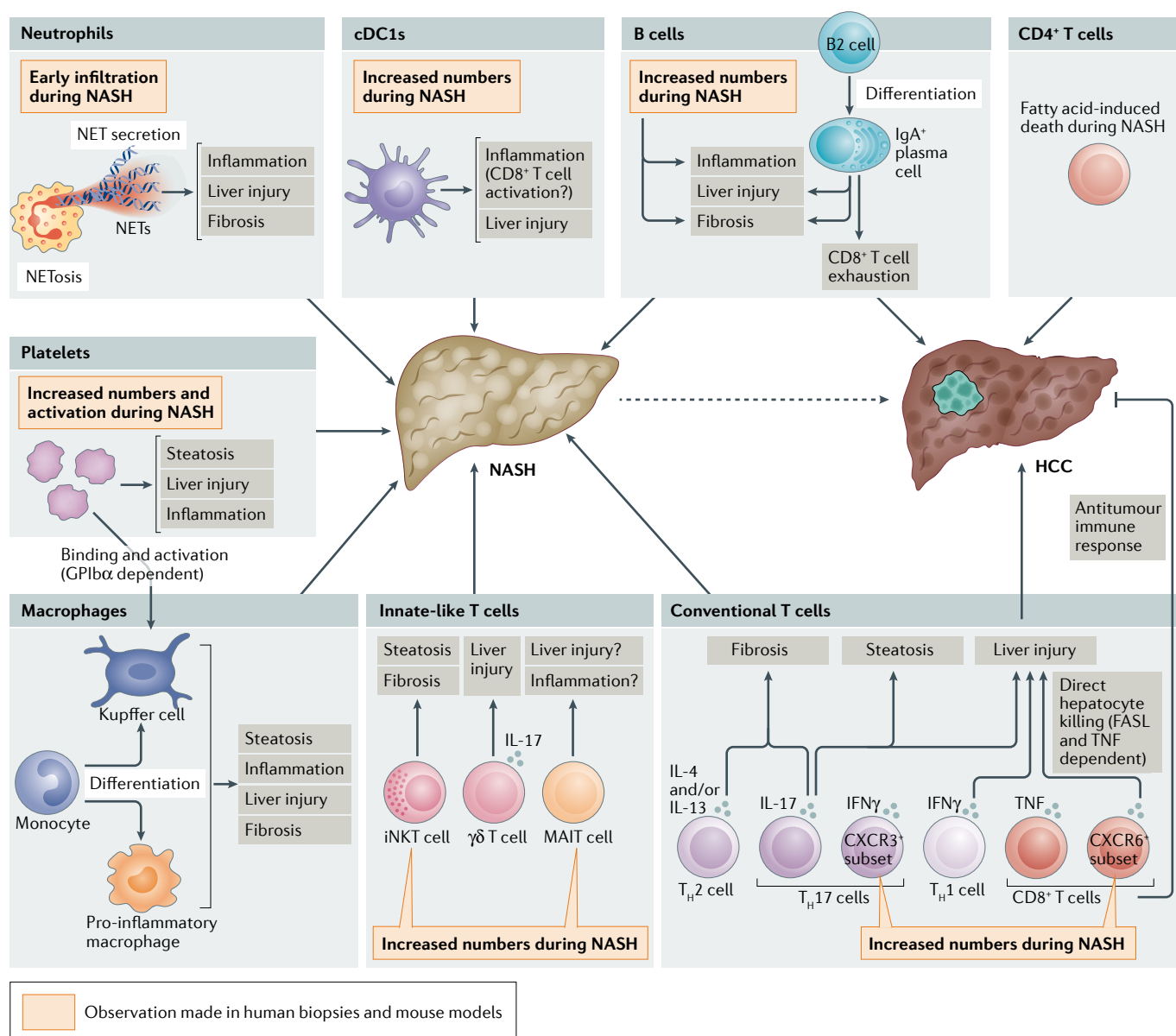


Fig. 3 | Immune modulation of NASH pathogenesis. The hepatic immune cell repertoire is reshaped during non-alcoholic steatohepatitis (NASH) and participates in the uncontrolled inflammatory environment that promotes liver injury (hepatocyte death) and liver fibrosis, further aggravating the disease. These immune cells comprise innate-like T cells, such as invariant natural killer T (iNKT) cells, mucosal-associated invariant T (MAIT) cells and $\gamma\delta$ T cells, but also conventional $CD8^+$ T cells and $CD4^+$ T cell subsets, including interferon- γ (IFN γ)-producing T helper 1 (T_H1) cells, IL-4-producing and/or IL-13-producing T_H2 cells, and IL-17-producing T_H17 cells. Neutrophil accumulation is an early event in NASH that promotes inflammation and liver injury, in particular through the secretion of neutrophil extracellular traps (NETs) or their release upon neutrophil cell death (NETosis). Dendritic cells (DCs) and, in particular, type 1 conventional DCs (cDC1s), increase in number and promote liver inflammation (potentially by activating $CD8^+$ T cells) as well as hepatic injury during NASH. Monocytes are also rapidly recruited to the liver, where they can differentiate into pro-inflammatory macrophages or give rise to monocyte-derived Kupffer cells. Platelets are increased in number and activated during NASH; they promote liver steatosis, inflammation and injury, implicating mechanisms that involve direct binding to and activation of

Kupffer cells in a glycoprotein GPIIb/IIIa-dependent manner. B cells and, in particular, IgA^+ plasma cells also contribute to NASH by promoting liver inflammation, injury and fibrosis. Furthermore, IgA^+ plasma cells can promote the progression to hepatocellular carcinoma (HCC) through their immunosuppressive effects by inducing $CD8^+$ T cell exhaustion. In addition, loss of $CD4^+$ T cells through fatty acid-mediated cytotoxic effects limits their antitumour potential and favours NASH progression to HCC. Finally, $CD8^+$ T cells and, notably, the autoaggressive $CXCR6^+$ subset that is induced by the hepatic microenvironment of steatosis, promote liver damage and the NASH–HCC transition through the secretion of pro-inflammatory cytokines and direct hepatocyte killing in a FASL-dependent and tumour necrosis factor (TNF)-dependent manner. $CD8^+$ T cells can also limit tumour burden through their capacity to mount an antitumour immune response. The contribution of immune cells to the development of NAFLD has been mainly explored in mouse models. Nevertheless, some observations (as indicated in the figure) have been corroborated both in human biopsies and in mouse models of NASH. Our current knowledge is not sufficient to propose an integrated view of NASH pathogenesis involving the crosstalk between immune cells or their temporal involvement and, in many cases, detailed molecular mechanisms are lacking.

Box 2 | Crosstalk between immune and parenchymal cells during NASH

Various immune cell subsets affect different aspects of the pathogenesis of non-alcoholic steatohepatitis (NASH), including liver steatosis, inflammation, injury and fibrosis. Notably, immune cells promote inflammation directly by secreting inflammatory cytokines, such as tumour necrosis factor and IL-1 β , but also indirectly by activating neighbouring immune and non-immune cells. In terms of liver injury, which mostly arises from hepatocyte death, immune cells can crosstalk with hepatocytes to promote their cytokine-induced death. Furthermore, the interaction of immune cells with hepatic stellate cells (HSCs)¹⁷⁵ can promote fibrogenesis. Overall, it remains poorly defined whether immune cells affect NASH pathogenesis directly by signalling to hepatocytes (liver injury) and HSCs (fibrosis) and/or indirectly through a more complex network of interactions. Indeed, although scattered evidence suggests that there is crosstalk between macrophages and HSCs or liver sinusoidal endothelial cells during NASH¹⁷⁶, a clear integrated view is still missing. Methods such as single-cell RNA sequencing or single-nucleus RNA sequencing combined with novel bioinformatic tools that can generate ligand–receptor interaction maps¹⁷⁷ would allow for identification of the most relevant pathways of crosstalk. This has recently been attempted in the context of NASH using single-cell RNA sequencing, highlighting HSC-secreted factors that could potentially impact the functions of neighbouring cells⁸². Of note, single-nucleus RNA sequencing would be particularly interesting to use as it offers the ability to more easily study cells, such as hepatocytes and HSCs, that are difficult to retrieve after classical tissue dissociation methods. This approach would give an integrated view of the intercellular communications operating during NASH and better resolve their complexity. Such an approach has recently been used to study the hepatic cellular communications that enable monocytes to differentiate into Kupffer cells⁷⁹. In addition, perturbation of a specific immune cell population and analysis of its impact on the ligand–receptor interaction map may help to better understand how a single immune cell population can have broad effects on neighbouring cells. This will aid in uncovering the interactions between several cell types and furthering our understanding of how these interactions coordinate NASH-induced liver injury. This knowledge should eventually define the communication networks and crucial nodes that control the immune-mediated inflammatory processes that promote NASH.

levels of CXCL10 are increased in patients with NASH and *Cxcl10* deletion or antibody-mediated neutralization of CXCL10 limits steatosis, liver injury and fibrosis in mice¹⁰¹. *Cxcr3* deficiency also reduced NASH pathogenesis in mice¹⁰². Thus, dampened CXCL10–CXCR3 signalling could partially explain the impact of IFN γ deficiency on NASH.

The role of several T_H2 cell-associated cytokines in NASH has also been addressed. Serum levels of IL-13 are increased in patients with NASH and expression levels of its receptor IL-13RA2 are increased in the liver¹⁰³. IL-13RA2 is expressed by HSCs and cytotoxin-mediated killing of IL-13RA2⁺ cells impedes liver fibrosis in a rodent model of NASH¹⁰³. Production of the type 2 cytokines IL-4, IL-5 and IL-13 is induced by IL-33 and, consistent with the known role of type 2 cytokines in extracellular matrix production¹⁰⁴, IL-33 treatment promotes hepatic fibrosis in mice¹⁰⁵. However, treatment with IL-33 also limits hepatic triglyceride storage and results in a small decrease in liver injury in a mouse model of NASH¹⁰⁵. Overall, therefore, the role of T_H2 cell-mediated immunity in NASH remains unclear.

T_H17 cells can have either homeostatic functions that maintain the intestinal barrier in response to commensals or pathogenic functions that fuel inflammatory diseases when elicited by pathogens¹⁰⁶. Hepatic T_H17 cells¹⁰⁷ and the expression of T_H17 cell-associated genes¹⁰⁸ are increased in patients with NASH. Similarly, T_H17 cells are increased in mouse models of NASH^{109–111}, in particular a subset of pro-inflammatory CXCR3⁺ T_H17 cells

that drives NASH pathogenesis¹¹². In mice deficient for unconventional prefoldin RPB5 interactor (*Uri1*) in hepatocytes (Hep^{*ΔUri1*} mice), NASH is exacerbated owing to DNA damage in hepatocytes and this is associated with the induction of T_H17 cells and increased hepatic IL-17A production¹¹⁰. Neutralizing IL-17A using a monoclonal antibody or suppressing the generation of T_H17 cells with the ROR γ t inhibitor digoxin decreases the hallmarks of NASH in Hep^{*ΔUri1*} mice¹¹⁰. *Il17a* deficiency is equally protective in a different mouse model of NASH¹¹¹ and recombinant IL-17A treatment increases hepatic DNA damage, steatosis, liver injury and fibrosis in wild-type mice fed a NASH-inducing diet¹¹⁰. Mechanistically, impaired IL-17-induced signalling in myeloid cells protects Hep^{*ΔUri1*} mice from NASH, which suggests the existence of a major pathway of crosstalk between IL-17-producing cells, including T_H17 cells, and phagocytes that drives NASH¹¹⁰. Consistent with the pro-fibrotic effects of cytokines produced by T_H1, T_H2 and T_H17 cells reported above, the depletion of all CD4⁺ T cells in a humanized mouse model of NASH blunted hepatic fibrosis¹¹³.

CD8⁺ T cells mainly produce IFN γ , TNF and cytotoxic molecules such as perforins¹¹⁴. Hepatic CD8⁺ T cells are increased in number during NASH in both mice and humans^{92,115}, in particular CD8⁺ T cells expressing CXCR6 (REF.¹¹⁶). Lipid-conditioned CXCR6⁺ CD8⁺ T cells induce hepatocyte killing in a perforin-independent, FasL (CD95L)-dependent manner¹¹⁶. Consequently, in a diet-induced mouse model of NASH, CD8⁺ T cell depletion blunted liver injury¹¹⁵. Perforin 1-deficient mice have exacerbated NASH hallmarks, which was associated with an increased number and activation status of hepatic CD8⁺ T cells¹¹⁷. Perforin deficiency is known to favour CD8⁺ T cell activation in viral infection^{118,119}. This effect is cell-extrinsic and involves the persistence of immunostimulatory DCs in the absence of perforin-mediated killing of antigen-loaded DCs^{120,121}. Finally, CXCR6⁺ CD8⁺ T cells that accumulate during NASH express the exhaustion marker PD1 and PD1 blockade exacerbated CD8⁺ T cell activation resulting in accelerated NASH development in mice¹²². Thus, CD8⁺ T cells are likely to promote hepatic injury during NASH.

Overall, an integrated mechanistic view of how T cell subsets are activated and cooperate to promote hepatic inflammation during NASH is missing. Most of the current literature refers to cytokines produced by T cell subsets rather than the T cells themselves. In addition, although CD8⁺ T cell-mediated killing of hepatocytes seems to occur in an antigen-independent manner during NASH¹¹⁶, it remains unknown whether adaptive, antigen-specific T cell responses are also involved. Further investigations will be needed to fill these gaps in our knowledge as well as to elucidate how these pathways could be targeted therapeutically without impairing immune defences.

Innate-like T cells. iNKT cells are enriched in the mouse liver as compared to other organs and are increased in mouse models of NASH as well as in patients with NASH^{115,123}. Their role in NASH pathogenesis has been

Neutrophil extracellular traps (NETs). Structures released by neutrophils that are primarily composed of a scaffold of chromatin fibres and antimicrobial proteins.

evaluated using *Cd1d*-deficient or *Traj18*-deficient mice¹²⁴, in which iNKT cells do not develop. iNKT cells promote liver fibrosis^{124,125} through their ability to increase liver expression of osteopontin¹²⁴, which is known to stimulate fibrogenesis during NASH in both mice and humans¹⁰⁰. A more recent study shows that iNKT cells also favour liver steatosis and, together with CD8⁺ T cells that induce liver damage, are necessary for NASH development¹¹⁵. iNKT cells are heterogeneous and encompass T-bet⁺ iNKT1 cells, GATA3⁺ iNKT2 cells and ROR γ t⁺ iNKT17 cells, which selectively produce IFN γ , IL-4 and IL-17, respectively¹²⁶. As type 2 cytokines, such as IL-4, are known to support collagen production and extracellular matrix deposition¹⁰⁴, it would be interesting to assess the specific role of iNKT2 cells in NASH-induced fibrosis.

$\gamma\delta$ T cells are another subset of innate-like T cells present in the steady-state liver, where they develop and are maintained in a microbiota-dependent manner¹²⁷. The number of $\gamma\delta$ T cells in the mouse liver increases during NASH and they promote hepatic injury¹²⁷. Importantly, the development of liver $\gamma\delta$ T cells is impaired in *Cd1d*^{-/-} mice and this may contribute to the dampened NASH phenotype that is observed in these mice¹²⁴.

One study reported that MAIT cells increase in number during NASH pathogenesis in both mice and humans and that their deficiency promotes liver inflammation and injury¹²⁸. However, it remains unclear how MAIT cells are protective in the context of diet-induced NASH whereas they are pro-inflammatory and pathogenic during diet-induced obesity¹²⁹. In addition, the impact of MAIT cells on fibrosis was not addressed in this study but a previous work has described a pro-fibrogenic role of MAIT cells in models of acute liver injury¹³⁰. Thus, deciphering the role of MAIT cells in NASH, particularly with regards to hepatic fibrosis, warrants further investigation.

Platelets. In addition to their front-line role in coagulation and haemostasis, platelets are also involved in the regulation of inflammatory processes¹³¹. For example, the interaction of platelets with blood-borne pathogens facilitates KC-mediated bacterial clearance in the liver¹³². In addition, circulating platelets interact with monocytes to favour atherosclerotic plaque development by promoting arterial inflammation and further leukocyte recruitment^{133,134}. Platelets are also involved in the development of NASH (FIG. 3). Indeed, anti-platelet therapy was previously shown to dampen NASH pathogenesis in rats¹³⁵, although the underlying mechanisms remain uncertain. A recent study showed that platelet activation and adhesion (but not aggregation) as well as platelet-derived granules are crucial in the promotion of NASH¹³⁶. Platelets interact with KCs at both early and late stages of NASH and promote liver steatosis, inflammation and injury in mice¹³⁶. In addition, platelets promote the accumulation of inflammatory cells in the liver during NASH in a glycoprotein GPIIb α -dependent manner. In line with these observations, platelets are a promising therapeutic target in NASH¹³⁶.

Neutrophils. In contrast to their beneficial roles during infection, neutrophils usually have a detrimental effect on chronic inflammatory disorders through the production of ROS, cytokines, proteases and neutrophil extracellular traps (NETs)^{137,138}. In NASH, hepatic neutrophil infiltration is evident in both mouse models and human biopsies^{139–142}. Neutrophil accumulation is an early event in mouse models of NASH^{141–143}. Neutrophil depletion impairs the early stages of NASH in mice by limiting inflammation and liver injury but such effects are no longer observed as NASH progresses¹⁴¹. Inhibition of the serine protease neutrophil elastase similarly impacts the early phases of NASH¹⁴¹. Neutrophil elastase is released as a constituent of NETs¹⁴⁴, which are found very early in the liver during NASH development in mice¹⁴² and at increased levels in the circulation of patients with NASH¹⁴³. Dismantling NETs using deoxyribonuclease I limits hepatic inflammation, liver injury and fibrosis in mice^{142,143}, which indicates the detrimental effect of these structures on NASH progression. Overall, neutrophils seem to have a role in the early pathogenesis of NASH through NET formation (FIG. 3) but their contribution to later stages of the disease remains unclear.

Macrophages. During NASH, inflammatory cues drive the hepatic recruitment of blood monocytes that locally differentiate into monocyte-derived macrophages, thereby increasing the size of the macrophage pool in the liver¹⁴⁵. Recent studies have shed light on the diversity of hepatic macrophages in mouse models of NASH^{35,81,146}.

A major finding is that self-maintenance of embryonically derived KCs is impaired in NASH as KCs with low cell surface expression levels of TIMD4 are observed in diseased mouse liver^{35,81,146}. Such TIMD4^{low} KCs are reminiscent of the monocyte-derived KCs that are generated after non-physiological depletion of embryonically derived KCs in mice^{79,147,148}, which suggests that monocyte-derived KCs are generated during NASH. Indeed, fate-mapping experiments document monocyte participation in the mouse KC pool during NASH^{35,146} and immunostaining studies have shown that these monocyte-derived KCs localize to hepatic sinusoids, similarly to embryonically derived KCs^{81,146}. The generation of monocyte-derived KCs is a response to the increased death of embryonically derived KCs during NASH³⁵ and is aimed at maintaining KC numbers. Transcriptomic analysis reveals the enrichment of a lipotoxicity gene signature in embryonically derived KCs as well as in monocyte-derived KCs during NASH. Such a cellular stress signature probably explains both why embryonically derived KCs die during NASH and their inability to efficiently self-renew. Although the generation of monocyte-derived KCs maintains the KC pool in the liver, their transcriptomic landscape differs from that of embryonically derived KCs^{35,81,146}. In particular, monocyte-derived KCs do not acquire the full gene expression spectrum associated with accessory functions of embryonically derived KCs (such as erythrophagocytosis) and they have a more pronounced inflammatory profile than their embryonically derived counterparts³⁵. Finally, at a functional level, monocyte-derived KCs and embryonically derived KCs differently impact the liver

response to NASH. Monocyte-derived KCs limit hepatic triglyceride storage but they promote liver injury to a greater extent than embryonically derived KCs³⁵ (FIG. 4). Thus, KC homeostasis is markedly impaired during NASH and has an impact on liver pathology.

In addition to their contribution to the KC pool, monocytes also follow a classical pathway of differentiation during NASH, leading to the generation of monocyte-derived inflammatory macrophages. Of note, the liver environment during NASH has a systemic effect on monocytes as they already show NASH-associated transcriptional alterations in mouse bone marrow¹⁴⁹. In the liver, monocyte-derived macrophages express high levels of *Spp1*, *Itgax*, *Gpnmb*, *Cd9* and *Trem2* (among other markers)^{81,82,146}, which are also found at some levels in monocyte-derived KCs³⁵. In mice, the monocyte-derived macrophages that accumulate in the liver during NASH resemble the lipid-associated macrophages that are present in obese white adipose tissue¹⁵⁰, which suggests that metabolic inflammation induces a common gene signature in monocyte-derived macrophages in different tissues and different metabolic contexts. In terms of their function, monocyte-derived macrophages in mouse liver localize to areas of tissue fibrosis in close proximity to desmin⁺ HSCs⁸¹, which suggests that they may participate in hepatic fibrosis (FIG. 4). Similar observations were made in human cirrhotic liver⁸³. Indeed, a TREM2⁺CD9⁺ monocyte-derived macrophage population with profibrotic properties accumulates during liver fibrosis in humans⁸³.

Overall, as mentioned above, diverse immune cell populations are involved in NASH pathogenesis and the

roles of other immune cell subsets, such as NK cells and ILCs, remain to be clarified. Thus, it is likely that a coordinated response involving crosstalk between immune cells contributes to the hepatic inflammatory environment observed during NASH. However, the contours of such an integrated response typifying NASH pathogenesis have largely been unexplored so far. It is too early to speculate regarding a specific sequence of immune cell activation during NASH, with the exception that neutrophils are known to be recruited early during the disease and have been described to only impact NASH pathogenesis at early stages. Similarly to neutrophils, monocytes are likely to be rapidly recruited to the liver but their contribution to the KC pool might vary in time and magnitude depending on the model. Another important point is that many of the immune cell populations that are known to alter the course of NASH are already present in the steady-state liver and are thus susceptible to being activated locally as soon as NASH triggers are induced. Again, the time and magnitude of such activation might differ between models. Thus, kinetic studies on different mouse models of NASH are needed to better appreciate the dynamic accumulation of immune cells and their activation. Our current knowledge is not sufficient to propose an integrated view of NASH pathogenesis but emerging technologies, such as single-cell RNA sequencing or time-of-flight mass cytometry, are likely to fill this knowledge gap in the near future. Indeed, kinetic, large-scale analysis of the accumulation and activation of immune cell populations over the course of NASH would help to propose a model typifying NASH-driven inflammation.

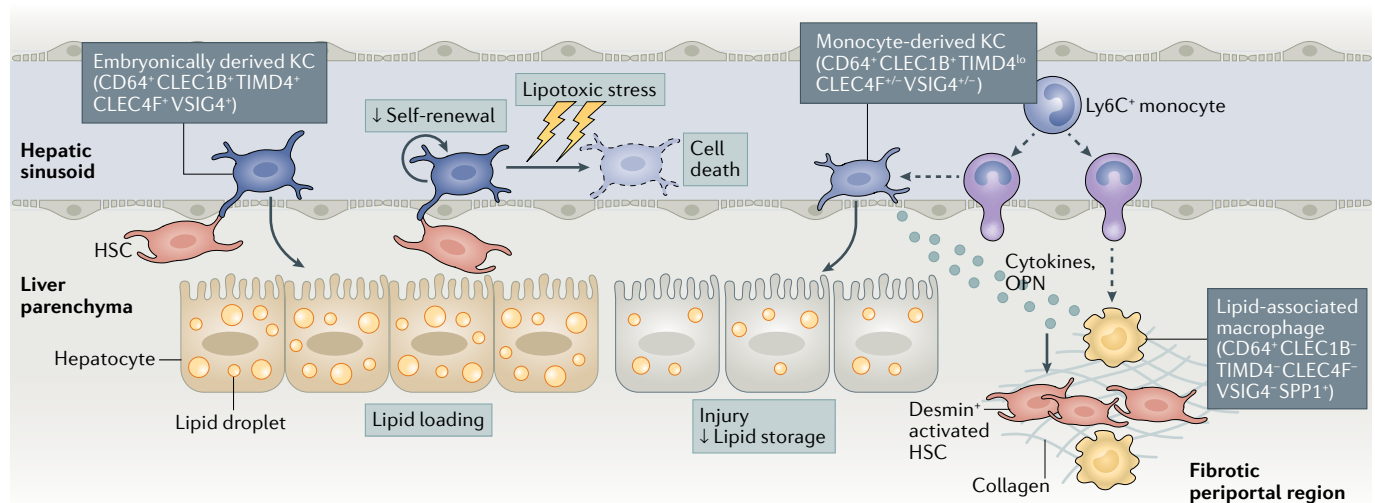


Fig. 4 | The diversity of hepatic macrophages during NASH. During non-alcoholic steatohepatitis (NASH), embryonically derived Kupffer cells (KCs), which are tissue-resident macrophages that inhabit the sinusoids of the healthy liver, are gradually lost. In response to the NASH environment, they lose their capacity to self-renew and show accelerated signs of cell death owing to lipotoxic stress. To maintain the KC pool, circulating Ly6C⁺ monocytes are recruited to the liver sinusoids, where they differentiate into monocyte-derived KCs. These monocyte-derived KCs express the pan-macrophage marker CD64 and have high levels of expression of CLEC1B (the prototypical marker of embryonically derived KCs) but lower levels of expression of other markers of embryonically derived KCs (such as CLEC4F, VSIG4 and TIMD4). Monocyte-derived KCs

are more pro-inflammatory than embryonically derived KCs and they affect the liver response to NASH by limiting liver lipid storage and promoting liver injury. Other macrophages, also derived from circulating monocytes, populate the liver during NASH. They express no typical markers of KCs and are prevalent in fibrotic areas positive for desmin, a marker of activated hepatic stellate cells (HSCs). These monocyte-derived macrophages have been termed lipid-associated macrophages and they resemble the TREM2⁺CD9⁺ scar-associated macrophages that have been described in human fibrotic livers. Lipid-associated macrophages and monocyte-derived KCs express high levels of *SPP1*, the gene encoding osteopontin (OPN), which is a strong inducer of liver fibrogenesis.

Immune cells in the NASH–HCC transition

NASH is a major risk factor for developing HCC¹², as influenced by the hepatic inflammatory environment involving the immune cell populations described above¹⁵¹. In a mouse model of diet-induced NASH and HCC, CD8⁺ T cells and NKT cells were crucial for the transition to HCC by promoting both liver steatosis and damage¹¹⁵. In this model, the inability to reach the NASH stage upon depletion of CD8⁺ T cells and NKT cells is likely to underlie the protection from HCC development. By contrast, CD8⁺ T cells protect HFD-fed *MUP-uPA* mice lacking IgA from NASH-induced HCC⁹². In these mice, CD8⁺ T cells have a limited effect on promoting NASH and instead resistance to HCC is associated with a decrease in the number of exhausted CD8⁺ T cells. Consequently, PD1 blockade reverses T cell exhaustion in *MUP-uPA* mice fed a HFD, which leads to increased antitumour immunity and reduced tumour incidence⁹². Thus, CD8⁺ T cells have a key antitumour role in the HFD-fed *MUP-uPA* mouse model. In addition, *Cd8a*-deficient mice develop a higher tumour burden in other models of NASH-induced HCC⁹². In this study, CD8⁺ T cells have a limited, albeit significant, effect on NASH severity, which probably explains why their antitumour effects are apparent. In conclusion, the effect of CD8⁺ T cells in promoting NASH onset might, in other models, mask their well-described antitumour potential¹⁵².

CD4⁺ T cells were shown to impede HCC development in a mouse model of NASH-potentiated HCC¹⁵³. In this model, fatty acids induce CD4⁺ T cell death through mitochondrial ROS production, whereas ROS scavenging limits CD4⁺ T cell loss and reduces tumour burden¹⁵³. The effects of CD4⁺ T cells on tumour growth are attributed to their ability to mount a tumour-specific immune response rather than to an effect on NASH progression. However, the opposite effect of CD4⁺ T cells has been described in another model of NASH-promoted HCC. Indeed, IL-17A-producing T_H17 cells drive NASH development and its progression to HCC through IL-17A-induced signalling in myeloid cells¹¹⁰. In this study, the ability of T_H17 cells to accelerate NASH progression, rather than their role in the antitumour immune response, is most likely to explain the faster transition towards HCC. Thus, depending on the NASH–HCC model used, CD4⁺ T cells can modulate NASH-driven HCC through different modes of action. Of note, if NASH progression is affected by an immune cell subset, this will mainly determine the ability of that subset to alter the NASH–HCC transition.

Innate immune cells can also impact NASH-induced HCC. As mentioned above, neutrophils promote the development of NASH through the release of NETs¹⁴³. Limiting NET production decreases NASH-associated inflammation and reduces NASH-induced HCC¹⁴³ probably due to limited NASH development. The roles of other myeloid cells, such as KCs, in NASH-induced HCC have not yet been explored, although there is abundant literature on the role of macrophages in HCC development in general.

Overall, immune cells can limit HCC onset either by controlling NASH development or by blocking the NASH–HCC transition. To better understand the mode

of action of diverse immune cell types, approaches aimed at modulating specific immune cell populations in a temporal manner need to be developed.

Therapeutic prospects

There are currently no approved therapies for NASH in the clinic¹². Approaches targeting the metabolic arm of the disease have been tested. For example, peroxisome proliferator-activated receptor (PPAR) agonists have been previously developed and tested in clinical studies¹¹. Although they are mainly known for their roles in modulating lipid metabolism, PPAR γ and PPAR δ also have immunomodulatory properties, such as anti-inflammatory actions on macrophages^{154,155}, which may contribute to the potential clinical benefit in NASH. More recently, obeticholic acid (OCA), a ligand for farnesyl X receptor (FXR; a nuclear receptor for bile acids) that is already approved for the treatment of primary biliary cholangitis¹⁵⁶, has been repurposed to combat NASH. FXR activation by OCA inhibits bile acid production and promotes their efflux from hepatocytes, thereby limiting their accumulation in the liver¹⁵⁷. OCA improves NASH score and decreases fibrosis in an interim analysis of an ongoing phase III study¹⁵⁸. However, there are concerns that patients receiving OCA might have dysregulated cholesterol metabolism¹⁵⁹, which could increase their risk of cardiovascular disease. Similarly to the PPARs, FXR is also expressed by immune cells and the immunomodulatory effects of OCA cannot be excluded, although firm evidence for such effects is lacking. Finally, the GLP1 receptor agonist semaglutide, which was previously developed to promote weight loss and counteract type 2 diabetes, has shown promising effects on NASH resolution in patients¹⁶⁰ and further studies will determine if it could be a potential therapeutic option for NASH.

As discussed above, hepatocyte death is a crucial factor in NASH initiation and perpetuation and caspase inhibitors have been tested in this regard. However, the pan-caspase inhibitor Emricasan, which was shown to limit hepatocyte apoptosis, inflammation and fibrosis in preclinical models⁴⁰, was unsuccessful in patients with NASH¹⁶¹. This may limit the commercial interest to test drugs targeting apoptosis or, at best, may motivate the use of more specific caspase inhibitors such as those targeting caspase 6, which have shown promising effects in preclinical models³⁹.

Treatments specifically focusing on the immunoinflammatory arm of NASH have also been evaluated, in particular Cenicriviroc (CVC). CVC is a dual CCR2 and CCR5 antagonist that has been shown to limit fibrosis without affecting other aspects of NASH in mice^{162,163}. The chemokine receptors CCR2 and CCR5 are expressed by monocytes as well as T cells and CVC would thus be expected to limit immune cell infiltration of the liver during NASH. Although CVC showed promising results in phase II trials, the phase III study was recently terminated owing to a lack of efficacy¹¹. This is particularly disappointing as CVC was at the frontline of approaches to limit fibrosis in patients with NASH, which is of particular importance as fibrosis can lead to permanent effects on the liver such as cirrhosis¹⁶⁴.

Peroxisome proliferator-activated receptor (PPAR). Part of a group of ligand-controlled transcription factors of the nuclear receptor family that have major regulatory roles in controlling metabolic functions as well as anti-inflammatory properties.

Thus, use of CVC might be combined with the blocking of other chemokine receptors controlling T cell recruitment to more efficiently limit NASH. Indeed, in addition to CCR2 and CCR5, T cell-specific chemokine receptors, such as CXCR3 or CXCR6, the latter being recently identified to mark a subset of NASH-associated autoaggressive CD8⁺ T cells¹¹⁶, could be targeted.

At this stage, alternative therapeutic options to limit hepatic fibrosis are not available, which is motivating the design of innovative strategies. Along these lines, a recent study highlights that an immunotherapeutic approach can be used to limit extracellular matrix deposition in a mouse model of NASH. The approach is based on the development of chimeric antigen receptor (CAR) T cells that can target and destroy myofibroblasts¹⁶⁵, as previously studied in models of cardiac fibrosis¹⁶⁶. In this study, CAR T cells are designed to recognize and destroy urokinase-type plasminogen activator receptor (uPAR)-expressing senescent cells that fuel tissue fibrosis in different organs¹⁶⁵. In the NASH liver, CAR T cells target and eliminate uPAR⁺ HSCs and macrophages, which reduces fibrosis and improves liver function¹⁶⁵. Finally, the use of autologous macrophage infusion has been shown to limit liver fibrosis in mice¹⁶⁷ and a recent safety profile study has shown the feasibility of such an approach in humans¹⁶⁸. This approach might represent an alternative avenue to limit liver fibrosis during NASH.

Conclusions and perspectives

NAFLD is a complex, multifactorial disease with NASH being its first crucial step towards severe liver alterations. Although it develops on a background of altered

metabolism, NASH has a strong immunoinflammatory dimension. A vast network of immune cells is mobilized during NASH and we have described here how they can promote the transition from simple steatosis to NASH as well as the evolution of NASH to cirrhosis and HCC. However, our understanding of the inflammatory cues that drive NASH is fragmented and further investigation is required to better appreciate the role of specific immune cell subsets in the disease. In addition, an integrated view of the interactions between different types of immune cells as well as between immune cells and stromal cells is required to appreciate the complexity of NASH. Mouse models of NASH are continuing to improve and are likely to help determine causality between specific immune cell populations and disease progression. This new information will need to be coupled to the better phenotyping of patients using novel methods such as single-cell or single-nucleus RNA sequencing, which would allow for a better appreciation of the immune landscape that characterizes patients with NASH. In addition, comparing the immune landscape in humans with NASH to that observed in different mouse models could be used to determine the superiority of a specific preclinical model over another. Together, these developments should foster a research effort to develop innovative therapeutic strategies to limit the global burden of NASH. Thus, the upcoming years will be the scene of intensive basic, preclinical and clinical research aimed at filling the urgent need for drugs to combat the NASH epidemic.

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