Review

JOURNAL OF HEPATOLOGY

Immune mechanisms linking metabolic injury to inflammation and fibrosis in fatty liver disease – novel insights into cellular communication circuits

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Keywords: NAFLD; NASH; Single-cell sequencing; spatial transcriptomics; Kupffer cells; immune-mediated liver disease; macrophages; cancer immunotherapy; HCC; exhausted T cells; PPAR agonists; FXR agonists; scRNA-seq; MAFLD.

Received 16 April 2022; received in revised form 2 June 2022; accepted 6 June 2022; available online 22 June 2022

Summary

Non-alcoholic fatty liver disease (NAFLD) is the most prevalent chronic liver disease and is emerging as the leading cause of cirrhosis, liver transplantation and hepatocellular carcinoma (HCC). NAFLD is a metabolic disease that is considered the hepatic manifestation of the metabolic syndrome; however, during the evolution of NAFLD from steatosis to non-alcoholic steatohepatitis (NASH), to more advanced stages of NASH with liver fibrosis, the immune system plays an integral role. Triggers for inflammation are rooted in hepatic (lipid overload, lipotoxicity, oxidative stress) and extrahepatic (gut-liver axis, adipose tissue, skeletal muscle) systems, resulting in unique immune-mediated pathomechanisms in NAFLD. In recent years, the implementation of single-cell RNA-sequencing and high dimensional multi-omics (proteogenomics, lipidomics) and spatial transcriptomics have tremendously advanced our understanding of the complex heterogeneity of various liver immune cell subsets in health and disease. In NAFLD, several emerging inflammatory mechanisms have been uncovered, including profound macrophage heterogeneity, auto-aggressive T cells, the role of unconventional T cells and platelet-immune cell interactions, potentially yielding novel therapeutics. In this review, we will highlight the recent discoveries related to inflammation in NAFLD, discuss the role of immune cell subsets during the different stages of the disease (including disease regression) and integrate the multiple systems driving inflammation. We propose a refined concept by which the immune system contributes to all stages of NAFLD and discuss open scientific questions arising from this paradigm shift that need to be unravelled in the coming years. Finally, we discuss novel therapeutic approaches to target the multiple triggers of inflammation, including combination therapy via nuclear receptors (FXR agonists, PPAR agonists).

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https://doi.org/10.1016/j.jhep.2022.06.012

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most prevalent chronic liver disease, affecting roughly 25% of the global population.[1,2] NAFLD has a strong association with obesity, type 2 diabetes, and hypertension, and is therefore considered the hepatic manifestation of the metabolic syndrome.[2] In parallel with the rising global prevalence of obesity and type 2 diabetes, the burden of NAFLD is projected to increase rapidly over the coming decade.[3,4] NAFLD is emerging as a leading cause of liver transplantation.[5] NAFLD encompasses a spectrum of liver pathologies ranging from steatosis, to the inflammatory form non-alcoholic steatohepatitis (NASH), which is characterised by lobular inflammation and hepatocyte ballooning (with or without fibrosis), to more advanced stages including cirrhosis and hepatocellular carcinoma (HCC).[6] NAFLD is the fastest growing cause of HCC in Europe and the USA. [7] HCC is the third leading cause of cancer-related death and can occur even in the absence of cirrhosis in patients with NAFLD.[8–10] NAFLD is considered a multisystem metabolic disease[11]; morbidity and mortality in patients are reciprocally influenced by comorbidities and risk factors. The prevalence of NAFLD among individuals with type 2 diabetes is 55% and conversely, patients with NAFLD are 2 to 5 times more likely to develop type 2 diabetes.[12,13] Cardiovascular disease is the leading cause of death in patients with NAFLD (40–45% of deaths), and vice versa, NAFLD is an independent risk factor for cardiovascular disease.[14] The strong impact of metabolic status on fatty liver disease has even prompted an intense debate about whether NAFLD should be "re-defined" as "metabolic dysfunction-associated fatty liver disease" (MAFLD).[15] The involvement of multiple organs in this clinical syndrome indicates common bi-directional pathomechanisms. Indeed, the liver is involved in the development of metabolic dysregulation and may exacerbate insulin resistance, adipose tissue dysfunction and gut dysbiosis, leading to a vicious circle whereby extrahepatic factors further trigger liver damage, resulting in metabolic and

inflammatory insults.[12] On the other hand, storage of fat in adipose tissue or liver may protect from systemic cardio- and neurovascular disease. This subpopulation of patients is referred to as healthy obese individuals.[16] Furthermore, a substantial proportion of patients with morbid obesity show normal liver histology, underlining the complex pathophysiology of these conditions.[17,18] Specific pathways for differential disease manifestations in the context of metabolic risk relate, for instance, to the *TM6SF2* and *PNPLA3* risk alleles for NAFLD, which in turn can protect against vascular morbidity.[19,20]

The clinical management of NAFLD is currently restricted to lifestyle interventions and difficult-to-sustain weight loss, although numerous novel therapies are being evaluated with some promising trial results.[21] The complex and multifactorial pathogenesis of NAFLD make it a challenging disease, and an improved understanding of disease mechanisms will form the basis for the development of better therapeutics. The strongest predictors of liver-related mortality in NAFLD are the presence and stage of liver fibrosis.[22–26] In addition, inflammation is a key driver of disease progression and fibrosis development,[27] evidenced by a strong correlation between the presence of NASH and stage 4 fibrosis.[24] Inflammatory mechanisms are involved along the entire spectrum of NAFLD but particularly at more advanced disease stages, including cirrhosis and during the transition to HCC.[28–30] NAFLD in patients is characterised by profound clinical and histological heterogeneity (rapid *vs.* slow progression, lean *vs.* obese NASH, or NASH-associated HCC with and without cirrhosis). Underlying this heterogeneous clinical presentation, inflammation in NAFLD rarely progresses in a linear fashion but fluctuates between flares and resolution. This might explain why the more dynamic parameter 'inflammation' is a less strong prognostic feature than the more static parameter 'fibrosis' on liver histology, when captured at a single timepoint.[1]

Research in recent years has identified many cellular and molecular targets, however, translation of these findings into disease-modifying treatments has proven challenging. Liver disease research has been propelled forward by the recent technological advances in single-cell multi-omics.[31] With respect to immune mechanisms in NAFLD, employing these novel tools has enabled the identification of unrecognised cell subsets and has increased our awareness of immune cell heterogeneity. Herein, we summarise the current knowledge of the major liver immune cells and their role in NAFLD and review novel concepts of immune-mediated mechanisms in NASH and NASH-to-HCC transition. We propose a conceptual framework that integrates hepatic inflammatory pathways with extrahepatic triggers of inflammation and we discuss unanswered scientific questions.

Inflammation in NAFLD

Liver inflammation is a hallmark of progressive NAFLD in patients; numerous rodent studies have mechanistically demonstrated involvement of immune cells in advancing NASH and fibrosis (Table 1).[32] Recent technological advances including single-cell multi-omics, spatial transcriptomics, multiparametic flow and mass cytometry, as well as multiplex immunofluorescence and intravital microscopy, have provided new insights into how the immune cell composition is reshaped during steatohepatitis both in murine models and patients with various degrees of NAFLD.[31,33–36] We now recognise that NAFLD-associated inflammation mirrors its systemic character, as multiple organ systems fuel liver inflammation (Fig. 1).[1,28,37] The conceptual framework of the pathogenesis of NAFLD proposes that overwhelming metabolic energy substrates, carbohydrates and free fatty acids lead to toxic lipid accumulation in the liver.[27] Hepatic steatosis develops because of increased liver triglyceride storage and hepatic *de novo* lipogenesis resulting in lipotoxicity. NASH onset is linked to oxidative stress, reactive oxygen species, mitochondrial dysfunction and endoplasmic reticulum stress resulting in hepatocellular metabolic dysfunction and injury.[38] Hepatocellular stress induces different modes of cell death including apoptosis, necrosis and necroptosis, and ultimately the release of damage-associated molecular patterns (Fig. 1).[32,39] In addition, hepatocyte senescence, *i.e.* a specific form of cell cycle arrest, can also lead to the release of inflammatory signals, termed the senescence-associated secretory phenotype.[40] Detection of tissue perturbance by immune sentinels and the ensuing immune response induce further damage of stressed hepatocytes, resulting in a vicious cycle and the full picture of necroinflammation. This framework was initially coined the 'two-hit hypothesis'.[41] However, overnutrition and insulin resistance can directly influence immune cell activation and composition in different organ systems.

Inflammation in NAFLD is regulated by multiple intrahepatic and extrahepatic factors (Fig. 1).[29] As a multisystem disease, extrahepatic factors include organ crosstalk with inflammatory signals being derived from the gut, adipose tissue, skeletal muscle and bone marrow (Fig. 1).[42–46] Particularly the intestinal microbiome has garnered much attention in recent years, as NAFLD, NASH and the metabolic syndrome show a strong association with intestinal dysbiosis.[47,48] More recently, alterations of the intestinal virome and mycobiome were linked to NAFLD.[49,50]

As an intrahepatic factor, the biliary compartment is increasingly recognised as a potential driver of inflammation in NAFLD (Fig. 1).[51] Patients with progressive NAFLD frequently exhibit biliary epithelial cell proliferation and a so-called

Key point

NAFLD is the most prevalent chronic liver disease globally and a major cause of liver cirrhosis, liver transplantation and hepatocellular carcinoma.

Table 1. Summary of the major immune cell subsets involved in the pathogenesis of NAFLD derived from patient data and murine models.

Population	Findings in human NAFLD	Mechanisms in rodent NAFLD
Monocytes/ Macrophages	Accumulation of CCR2+ pro-inflammatory macrophages CD9+TREM-2+ scar/lipid-associated macrophages (function?) Presence of Timd4+/- KC subsets	Pro-inflammatory role in aggravating NASH, hepatocyte damage (CCR2+) Replenishment of emKCs with more pro-inflammatory moKCs Early KC activation via DAMPs and PAMPs Distinct KC2 subset increases in steatosis, metabolic gene profile
Neutrophils	Accumulation of neutrophils in liver biopsies Markers of NETs increased in patients Myeloperoxidase increased in human NASH Neutrophil chemoattractants increased in NASH livers	Promote NASH via the release of effector molecules (proteases, elastase, myeloperoxidase, ROS) Blocking NETs beneficial in mice Myeloperoxidase deficient mice or neutrophil depletion protected from NASH
DCs	Increased XCR1+ cDC1s correlate with NASH severity cDC2s correlate with lobular inflammation and hepatocyte ballooning	Increased XCR1+ cDC1s in mice, specific ablation alleviates NASH
T cells	CXCR6+PD-1+CD8+ T cells increased in NASH Auto-aggressive killing of hepatocytes IFN-producing CD4+ T cells increased in patients with NASH Th17 cells are increased in patients Treg cells are decreased in patients	CD8+ T cells promote NASH by aggravating injury IFN-producing CD4+ T cells, promote NASH in mice Th17 cells promote NASH in mice PD-1+CD8+ T cells promote NASH-HCC transition Loss of CD4+ T cells promotes HCC
NKT cells	Increased in human NASH and cirrhosis	CXCR6+ iNKT cells promote steatohepatitis
MAIT cells	Increased in liver, decreased in blood in human NASH Accumulation in fibrotic niche of patients with cirrhosis	Depletion of MAIT cells aggravates NASH suggesting a protective effect
B cells	BAFF was elevated in serum of patients with NAFLD B cells increased in human NAFLD with activated profile Increased anti-OSE titres in patients with NASH	B-cell depletion ameliorated NASH IgA-producing B cells drive NASH Blocking BAFF in mice ameliorated steatohepatitis TNF-α and IL-6 producing B cells promote NASH
γδ T cells	No alterations in γδ T-cell numbers in patients with NASH	γδ T cells increased in murine NASH *Tcrd-/-* mice are protected from experimental NASH
Platelets	Platelet aggregates in human NASH, signs of platelet activation	Anti-platelet therapy improved experimental NASH Platelets recruited immune cells via CD44 and GPIba

Anti-OSE, anti-oxidative stress-derived epitopes; BAFF, B cell activating factor; CCR2, C-C chemokine receptor type 2; cDC, conventional dendritic cells; CXCR6, C-X-C chemokine receptor type 6; DAMPs, damage-associated molecular patterns; DCs, dendritic cells; emKC, embryonic KC; IFN, interferon; KC, Kupffer cells; MAIT, mucosal-associated invariant T; moKC, monocyte-derived KC; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NETs, neutrophil extracellular traps; NKT cells, natural killer T cells; OSE, oxidation-specific epitopes; PAMPs, pathogen-associated molecular patterns; PD-1, Programmed cell death protein 1; ROS, reactive oxygen species; Th, T helper; TNF, tumor necrosis factor; TREM-2, Triggering receptor expressed on myeloid cells 2.

Key point

The pathogenesis of NALFD is multifactorial and comprises metabolic dysregulation, progressive inflammation and fibrogenesis.

"ductular reaction" that correlates with portal inflammation, NASH activity and fibrosis.[52] Ductular reaction refers to histological biliary cell proliferation or hyperplasia and is rooted either in proliferation of cholangiocytes, hepatic progenitor cells or transdifferentiation of hepatocytes to cholangiocytes, which is still a topic of debate.[51] Furthermore, NASH patients with cholestasis had more advanced histological disease compared to age- and sex-matched controls without cholestasis.[53] NASH cirrhosis was characterised by reactive biliary cells that expressed the chemoattractant C-C motif chemokine ligand (CCL)2, suggesting a role in immune cell recruitment.[54] In acute biliary injury in mice and cholestatic liver diseases in humans, high CCL2 expression by biliary epithelial cells was confirmed and, mechanistically, cholangiocytes mediated monocyte recruitment via CCL2 and the integrin αvβ6; vice versa, monocytes induced biliary cell proliferation and regeneration.[55] This suggests an intriguing cellular crosstalk between cholangiocytes and immune cells, potentially driving inflammation in NAFLD (Fig. 1).

Given that the immune system is fundamental in maintaining liver homeostasis, its role during liver inflammation in the different stages of NAFLD is conflicting and might vary during disease progression. During early stages of NAFLD, inflammatory mechanisms clearly drive disease progression,[29] at the same time the liver attempts to resolve inflammation and initiate repair. Ductular reaction is a sign of ongoing liver repair in NAFLD, and the importance of liver macrophages (for example) in this response has been demonstrated.[56] Furthermore, regression of disease, *e.g.* clearance of cell debris or resolution of fibrosis, is actively controlled by inflammatory mechanisms.[29] While inflammatory cytokines and mediators are often increased in NASH – suggesting an overall increase in inflammation –patients with NAFLD may develop HCC, pointing to a loss of sufficient anti-tumour immune surveillance and to imbalanced inflammation.

For most of the major immune cell subsets, we now have data derived from human studies and rodent models that report on cell frequencies in the blood and/or liver, as well as mechanistic studies investigating their role in steatohepatitis (Table 1). However, it is noteworthy that whether an altered immune cell subset is cause or consequence in NASH is difficult to disentangle, especially considering that many different rodent models are used, and cell/organ-specific loss-of-function experiments are scarce. Nonetheless, it is worth detailing the proposed roles that different inflammatory cells play in the pathogenesis of NAFLD. Moreover, it should be noted that immune cell-driven NASH is a multistage process, which interconnects different immune cells, analogous to the metastatic cascade.

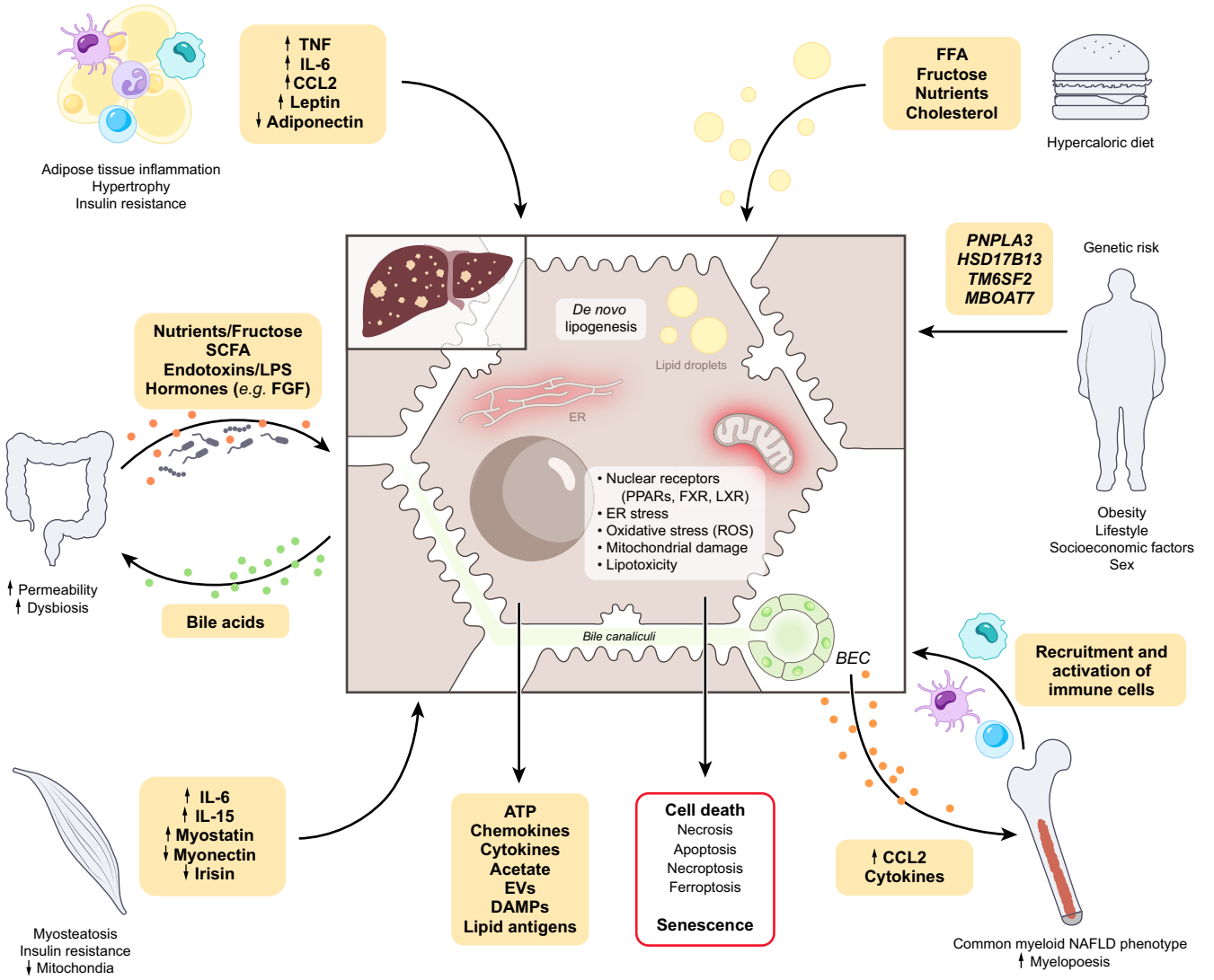

Fig. 1. Triggers of inflammation in NAFLD. Intra- and extrahepatic factors trigger inflammation in NAFLD. Hypercaloric diet, obesity, lifestyle, and genetic risk predispose individuals to NAFLD. Overload of hepatocytes with FFAs and increased *de novo* lipogenesis lead to lipid accumulation in hepatocytes. Fat overload in the liver induces lipotoxicity resulting in ER stress, oxidative stress, ROS production and mitochondrial damage. Stressed hepatocytes release pro-inflammatory mediators and DAMPs resulting in robust immune cell activation and infiltration, further damaging hepatocytes. Different forms of cell death occur as well as hepatocyte senescence, triggering a more pronounced immune response. Cholangiocytes might also release inflammatory mediators. Liver inflammation is also propagated by multiple extrahepatic systems including the adipose tissue, gut, skeletal muscle and bone marrow. BECs, biliary epithelial cells; DAMPs, damage-associated molecular patterns; ER, endoplasmic reticulum; EVs, extracellular vesicles; FGF, fibroblast growth factor; FFAs, free fatty acids; LPS, lipopolysaccharide; NAFLD, non-alcoholic fatty liver disease; SCFAs, short-chain fatty acids; TNF, tumour necrosis factor.

Thus, to develop immune-targeted therapies at the various stages of pathology, it will be important to identify the gaps in our current knowledge and to assemble the 'big picture' of inflammation in NAFLD.

Monocyte and macrophage heterogeneity in NASH

Most organs are equipped with a population of tissue-resident macrophages with unique functions that are essential in maintaining homeostasis.[57] The liver-resident macrophages, Kupffer cells (KCs), are the most abundant population of tissue-resident macrophages in the human body.[58–60] KCs reside in liver sinusoids and are critical sentinels in the liver.[60]

In humans, KCs are less well-characterised than in the mouse system and no bona fide KC marker has been identified. A recently published spatial proteogenomic cell atlas of healthy livers found that human monocytes/macrophages form a single continuum without the clear separation of a distinct population identifiable as KCs.[36] In healthy human livers, using single-cell RNA-sequencing, hepatic macrophages clustered as CD68+MARCO+ and CD68+MARCO- subsets.[61,62] The CD68+MARCO+ subset was deemed KCs and expressed genes

predominantly involved in immune tolerance, whereas the CD68+MARCO- macrophages resembled pro-inflammatory macrophages in mice with higher expression of pro-inflammatory genes. In addition, 2 distinct human KC populations, which differed based on expression of *Timd4* (T cell immunoglobulin and mucin domain-containing 4), were identified using single-cell sequencing.[63] The ontogeny of different human liver macrophages is unknown. Analogous to the mouse system, studies using single-cell technologies have identified resident KCs (CD68+MARCO+Timd4+) and monocyte-derived macrophages (CD68+MARCO-Timd4-) with a more pro-inflammatory gene signature.[36,61] Supporting this view, long-lived donor KCs were identified after HLA-mismatched liver transplantation.[64]

In the healthy murine liver, KCs are characterised based on markers such as F4/80, C-type lectin domain family 4 member F (CLEC4F) and Tim4.[65] KCs are yolk sac-derived and maintained locally by self-renewal; in the mouse system, there is little contribution of bone marrow-derived macrophages to the pool of resident KCs.[66] In the context of liver inflammation and NASH, the pool of liver macrophages is expanded by the recruitment of monocytes that give rise to a phenotypically distinct population of monocyte-derived macrophages.[67] In humans, classical ($CD14^{high}CD16^{neg}$), intermediate ($CD14^{high}CD16^{low}$) and non-classical monocytes ($CD14^{low}CD16^{high}$) are differentiated.[68] In the mouse system, 2 subsets of monocytes have been described, pro-inflammatory $CCR2^{high}Ly6C^{high}$ monocytes and patrolling $CX3CR1^{+}Ly6C^{low}$ monocytes.[69]

In human NAFLD, macrophages are considered key players and an increase in periportal macrophages was an early histological hallmark.[70] C-C motif chemokine receptor (CCR)2+ inflammatory macrophages accumulated in the periportal area in patients with NASH and correlated with disease severity and fibrosis.[71] In mouse models of NASH, monocytes are rapidly recruited to the liver and differentiate into monocyte-derived macrophages (Fig. 2). CCR2+ monocytes are important drivers of liver injury and their therapeutic inhibition reduces the severity of NASH.[59,71,72]

KCs are thought to instigate steatohepatitis as early responders by releasing pro-inflammatory mediators such as tumour necrosis factor (TNF)-α and CCL2.[73] Chemical depletion of KCs during onset of experimental NASH attenuated inflammation and reduced the severity of liver damage, emphasising the importance of KCs in NASH initiation.[74] In addition, free fatty acid-induced release of mitochondrial DNA triggered NLRP3 (NOD-, LLR- and pyrin domain-containing protein 3) inflammasome activation in KCs, resulting in pro-inflammatory interleukin (IL)-1β secretion and progression of experimental NASH.[75] Damage-associated molecular patterns released from dying hepatocytes and pathogen-associated molecular patterns such as lipopolysaccharide (LPS) have been shown to play an important role in NASH pathogenesis.[27,29] LPS activated myeloid cells and thus promoted NASH via toll-like receptor 4 (TLR-4) signalling in a methionine- and choline-deficient (MCD) diet mouse model.[76] A mechanistic link between intestinal dysbiosis and NAFLD is gut barrier dysfunction, which leads to increased translocation of microbial products from the 'leaky gut'. Indeed, patients with NASH have increased endotoxin levels,[77] and NASH severity has been shown to correlate with dysbiosis and a shift in the metabolic function of gut microbiota.[47] Lamina propria macrophages expressing the fractalkine receptor CX3CR1 are critical in maintaining intestinal barrier integrity[78]; gut barrier loss and more severe steatohepatitis were observed in *Cx3cr1*-deficient mice.[79] Furthermore, intestinal dysbiosis disrupts the levels of microbial metabolites, which are critical in maintaining intestinal epithelial integrity and immune homeostasis. In mice fed a high-fat diet, microbiota-derived tryptophan metabolites were depleted in the serum and liver; a lack of these metabolites resulted in increased inflammatory cytokine production by hepatic macrophages and hepatocytes.[80]

During the early stages of murine NASH, resident KCs partake in lipid storage, which ultimately renders them incapable of self-renewal, triggering their cell death (Fig. 2).[34,81–84] A series of elegant studies recently demonstrated that in murine NASH, embryonic KCs are gradually lost and subsequently replaced by monocyte-derived KCs (Fig. 2).[82–84] Monocyte-derived KCs lacked *Timd4* expression, which is reminiscent of monocyte-derived KCs that repopulate the liver after genetic ablation of embryonic KCs in healthy mice.[65,85] Embryonic and monocyte-derived KCs shared a similar gene expression profile; however, monocyte-derived KCs were more pro-inflammatory, had limited triglyceride storage and promoted liver injury.[84] Similarly, in healthy and steatotic human livers, a population of *Timd4*- KCs was observed and deemed to be monocyte-derived KCs.[36] The precise mechanism of KC death is unknown. The altered environment during steatosis might impair embryonic KC self-replication, with cells unable to survive because of a lipotoxic gene signature.[37] An alternative suggestion was that the resident KCs lost their identity.[83]

In mice, KCs might be a more heterogeneous population than previously thought. Using single-cell transcriptomics and proteomics, 2 distinct subsets of embryonic KCs were identified based on CD206 and endothelial cell adhesion molecule (ESAM) expression. The authors termed these cells KC1 ($CD206^{lo}ESAM^{-}$) and KC2 ($CD206^{+}ESAM^{+}$) (Fig. 2).[86] KC2s expressed genes involved in metabolic processes, such as lipid metabolism. Mice fed a high-fat diet (HFD) showed a relative increase of KC2s in steatosis. KC2s induced oxidative stress via scavenger receptor CD36 and their depletion

Key point

Inflammation in NAFLD is regulated by intrahepatic factors (lipid accumulation, lipotoxicity, oxidative stress, cell death mechanisms, activation of cholangiocytes and hepatic stellate cells) and extrahepatic factors (gut-liver axis, adipose tissue inflammation, skeletal muscle, bone marrow precursors).

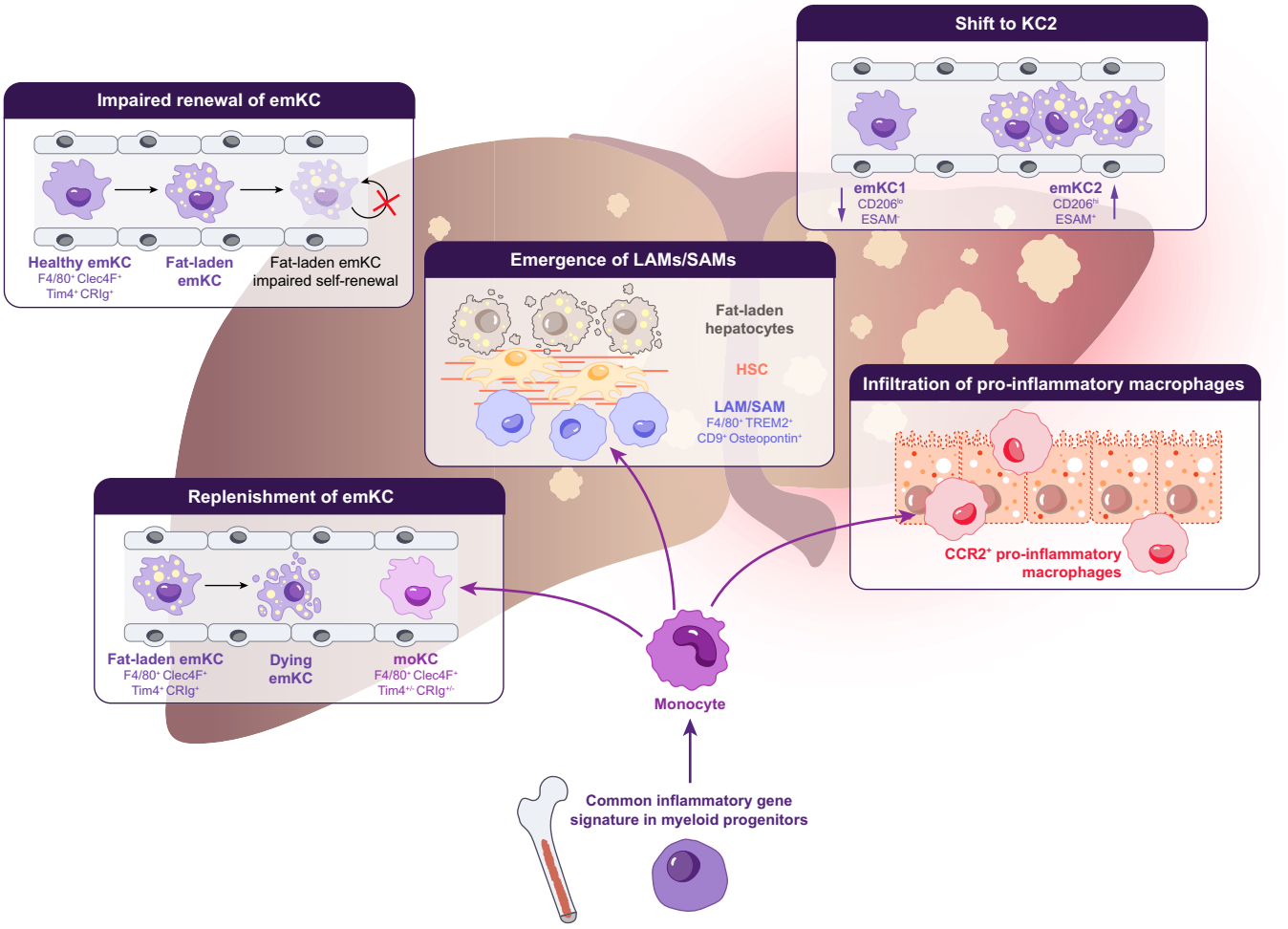

Fig. 2. Macrophage heterogeneity in NAFLD. The figure shows the different fates and cell subsets of monocytes and macrophages during different stages of NAFLD. Embryonic KCs store lipids, subsequently lose the ability to self-replicate and undergo cell death. Monocyte-derived KCs replenish the niche. At the same time, a distinct population of LAMs emerges in proximity to steatosis or as SAMs in the fibrotic niche. CCR2-expressing pro-inflammatory macrophages infiltrate the liver and propagate injury. Among resident KCs, steatosis favours the population of KC2s. Already on the level of bone marrow precursors, NAFLD is characterised by a unique inflammatory gene expression. emKCs, embryonic Kupffer cells; HSCs, hepatic stellate cells; LAMs, lipid-associated macrophages; moKCs, monocyte-derived Kupffer cells; NAFLD, non-alcoholic fatty liver disease; SAMs, scar-associated macrophages.

prevented steatosis and reversed obesity in mice.[86] Another scavenger receptor, macrophage scavenger receptor 1 (*Msr1*), expressed by liver macrophages was also identified as a critical molecule in the pathogenesis of NAFLD.[87] Mice lacking *Msr1* had milder steatohepatitis and Msr1-mediated uptake of saturated fatty acids induced a pro-inflammatory response in macrophages. In patients with NAFLD, *Msr1* transcript levels were correlated with disease activity.[87] These novel observations indicate that among the resident KCs in homeostasis, functionally distinct subsets exist; during obesity and lipid excess, KC2s might promote steatosis and steatohepatitis. On the spectrum of tolerance and immunogenicity, KC2s might therefore be less tolerogenic, and with respect to NAFLD, the KC2 subset was involved in metabolic disturbance. A potential caveat in these studies is the sole liver focus: blocking scavenger receptors or depleting the KC2 subset might decrease liver steatosis but could lead to lipid deposition elsewhere. While for many years it was known that KCs could alter their phenotype (tolerogenic or inflammatory) depending on the host's need, the emergence of KC subsets shows there might be tremendous heterogeneity.

In fibrotic human livers, a distinct macrophage subset was identified based on expression of *CD9* and triggering receptor expressed on myeloid cells 2 (*TREM-2*).[63] Localised in the fibrotic niche, these cells were termed 'scar-associated macrophages' (SAMs).[63] *In silico* trajectory analysis suggested that SAMs were monocyte-derived cells. Their location in the fibrotic niche led to the hypothesis that SAMs might promote liver fibrosis. Indeed, TREM-2+CD9+ SAMs expressed multiple pro-fibrotic genes and were shown to activate hepatic stellate cells *in vitro*.[63] The aforementioned proteogenomic

liver cell atlas also identified a distinct macrophage subset in healthy human livers, characterised by *CD9* and *TREM-2* expression, referred to as 'lipid-associated macrophages' (LAMs).[36] Furthermore, LAMs were found across different species, including human, murine and macaque livers. In healthy human livers, LAMs were found periportally and in close proximity to bile ducts, while in steatotic livers, these cells accumulated pericentrally in areas of steatosis and increased numerically.[36]

In murine steatohepatitis, infiltrating monocytes have at least 2 distinct fates: as monocyte-derived KCs, replenishing lost embryonic KCs, or as LAMs (Fig. 2).[82–84,88] The factors controlling which fate an infiltrating monocyte will undergo are unknown, but it is plausible that the liver microenvironment provides the respective signals.[89] LAMs in murine NASH express genes involved in antigen presentation, extracellular matrix remodelling, endocytosis, and lysosomal degradation.[90] LAMs express a high level of the chemokine osteopontin,[82] which was previously found to be upregulated in human and murine NASH.[91–93] A frequent histological feature of NASH is the formation of hepatic crown-like structures (hCLS), essentially a giant macrophage formation around large lipid droplets.[94] LAMs have been implicated in the formation of these hCLS[81,95]; loss of LAMs prevented the formation of hCLS and was associated with increased fibrosis in a high-fat high-sucrose model of NASH.[81]

The biological function of LAMs is incompletely understood. Analogous to SAMs, their presence in the fibrotic area and expression of osteopontin suggested a disease-promoting role.[82] In contrast, a recent study identified TREM-2-expressing liver macrophages that regulated energy supply and mitochondrial function.[96] TREM-2-deficient mice exhibited accelerated NASH development, and increased body weight and triglyceride levels.[96] LAMs seem to be a conserved population across tissues, as a population of TREM-2-expressing macrophages has been found in adipose tissue of humans and mice with obesity.[97] Depletion of TREM-2 macrophages resulted in severe metabolic dysfunction, suggesting a regulatory function of LAMs in adipose tissue. The precise role of LAMs in NAFLD is obscure and future investigations might uncover more heterogeneity with respect to recruited liver macrophages. Furthermore, based on expression of CD9 and TREM-2, it is likely that LAMs and SAMs refer to the same subset of recruited macrophages. Metabolic disturbances during obesity and steatohepatitis already affected myeloid cell precursors in bone marrow by imprinting a distinct NAFLD-associated inflammatory phenotype on the transcriptional level (Fig. 2).[98] Overall, altered metabolism induces common gene signature changes in the myeloid compartment of bone marrow, adipose and hepatic tissue, reciprocally nurturing inflammation in NAFLD.

Key point

Single-cell RNA-sequencing (scRNA-seq) technologies enable the characterisation of the cellular heterogeneity of immune cells in NAFLD-associated inflammation.

Metabolic disturbance attracts neutrophils to the liver in NAFLD

Neutrophils are critical first responders of the innate immune system; however, in chronic inflammatory diseases, their ability to liberate toxic molecules including proteases, oxidants, cytokines and neutrophil extracellular traps (NETs) might contribute to tissue damage.[99–101] The role of neutrophils in NASH is incompletely understood. In human NASH, neutrophilic infiltration is frequently observed in the liver.[70,102] Furthermore, patients with NASH had higher hepatic expression of neutrophil chemoattractants such as C-X-C motif chemokine ligand (CXCL)1, IL-8 and E-selectin compared to patients with steatosis.[103] Neutrophil elastase plasma concentrations also correlated with increased severity of NASH.[104]

A study using the MCD diet and high-fat high-cholesterol diet models of NASH provided evidence of the early involvement of neutrophils, as their depletion alleviated NASH.[105] Mice deficient in myeloperoxidase or neutrophil elastase had reduced liver damage in the MCD and Western diet models.[106,107] NETosis is a more recently discovered killing mechanism of neutrophils,[108,109] with increasing evidence implicating NETs in NASH (Fig. 3). Markers of NET formation were elevated in patients with NASH and correlated with NASH severity.[110] A recent study found NETs during early stages of experimental NASH in the MCD/high-fat diet model, while concurrent DNase treatment alleviated NASH severity.[111] Interestingly, in a set of *in vitro* experiments, sphingosine 1 phosphatase receptor 2 redirected neutrophils towards production of NETs. NETs have frequently been reported in patients with obesity, type 2 diabetes and insulin resistance.[99,109,112,113] Moreover, hyperglycaemia seems to predispose neutrophils to produce NETs, which might explain poor healing of diabetic wounds.[114] However, NETosis in metabolic dysregulation, as it pertains to NASH, might merely be a bystander mechanism without causal effect. In mice fed a HFD, blocking NET formation did not dampen adipose tissue inflammation or liver steatosis.[115] Neutrophil depletion in a murine model of toxic liver injury had no effect on fibrosis development in mice.[116] As neutrophils are thought to be short-lived cells, it is still unclear if the abundant population of neutrophils in NASH livers is constantly replenished by fresh bone marrow-derived neutrophils, which is more likely – or if these cells can be sustained for days or even weeks in steatohepatitis. Neutrophils are found in abundance during different stages of NAFLD and a disease-promoting role was suggested in various rodent studies, especially at the onset of NASH.[117] However, their mere presence does not signify a causal role in human NASH. Lastly, to date, neutrophils are

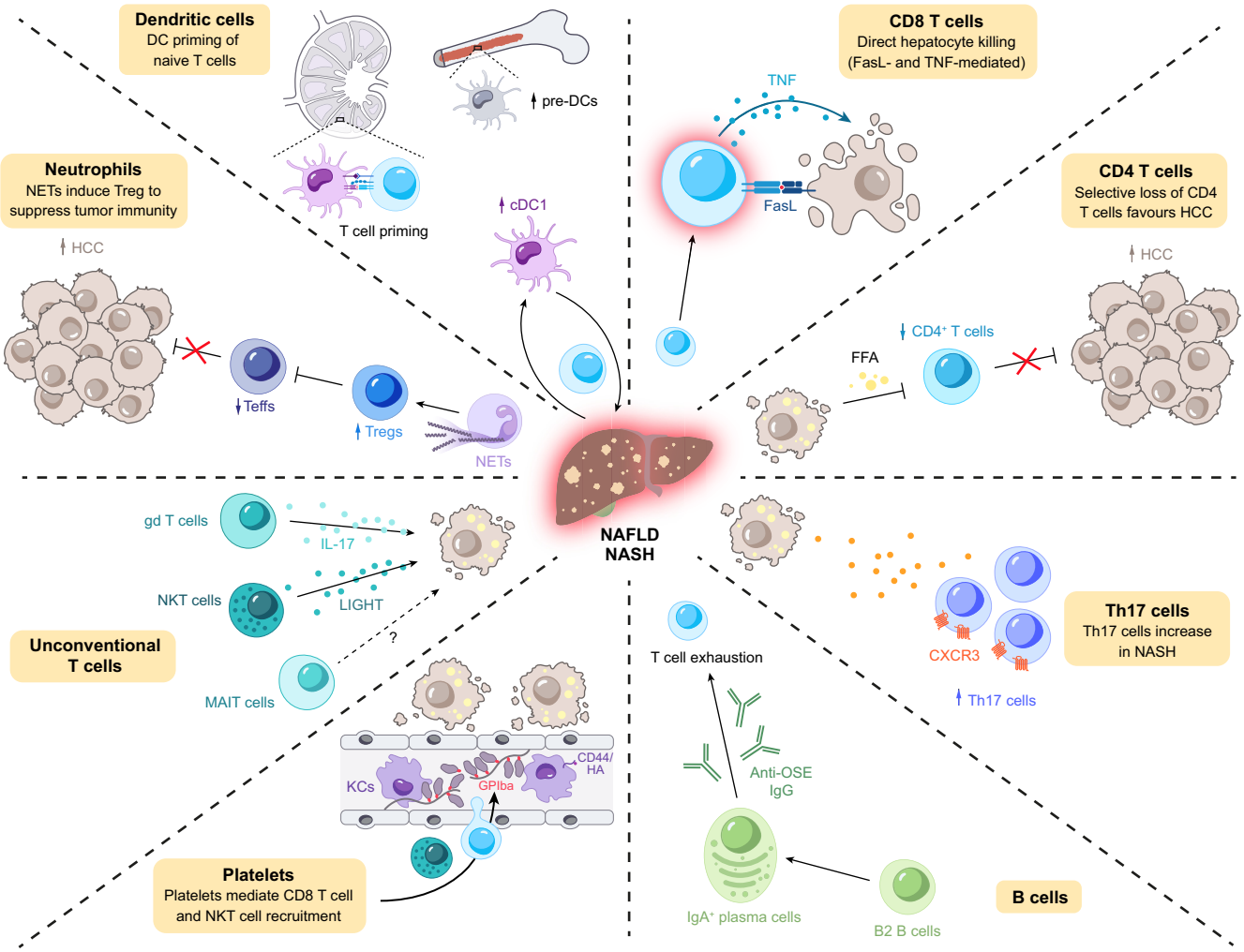

Fig. 3. Recent advances in understanding cellular immune-mediated mechanisms in NAFLD. The figure depicts the reshaping of immune cells during NAFLD, based on recent advances mainly from mouse models. Neutrophils accumulate early during NASH and release NETs that induce Tregs to suppress tumour immunity. DCs, particularly cDC1s increase and corelate with NASH severity. DCs might induce naïve CD8+ T cells in hepatic lymph nodes. Pre-DC precursors are elevated in bone marrow and blood. CD8+ T cells are critical effector cells as CXCR6+CD8+ auto-aggressive T cells directly kill hepatocytes. Platelets form aggregates on Kupffer cells via CD44 and hyaluronan and guide immune cells. B cells, particularly B2 cells contribute to NASH by converting to IgA+ plasma cells and producing anti-OSE antibodies. IgA+ plasma cells promote HCC by suppressing CD8+ T cells. CD4+ T cells are selectively depleted in NASH resulting in hepatocarcinogenesis. Th17 cells drive NASH pathology as pro-inflammatory cells. Unconventional T cells play a role in NASH, but their precise contributions need further evaluation. Anti-OSE, anti-oxidative stress epitopes; cDC, conventional dendritic cells; DCs, dendritic cells; FFAs, free fatty acids; HA, hyaluronan; HCC, hepatocellular carcinoma; KCs, Kupffer cells; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NETs, neutrophil extracellular traps; NKT, natural killer T; OSE, oxidation-specific epitopes; Teffs, effector T cells; Th17, T helper 17; Tregs, regulatory T cells.

considered a fairly homogenous population despite their known pro- and anti-inflammatory functions, and single-cell studies of neutrophils in liver disease are lacking.[117]

Dendritic cells are a critical link between multiple disease-driving systems

Dendritic cells (DCs) connect innate and adaptive immunity by integrating information about tissue environment. As the key antigen-presenting cells of the immune system, DCs can migrate to lymphoid organs to present antigens to T cells.[118] Within the unique hepatic microenvironment, many DC–T-cell interactions occur directly in the liver; however, hepatic DCs are less efficient at stimulating T-cell activation than DCs in other tissues, favouring tolerance.[60] DCs are a heterogeneous population; 2 subsets of conventional (classical) DCs (cDC1s and cDC2s) and plasmacytoid DCs have been distinguished and are identified by a combination of markers, which differ between mice and humans.[119,120] Hepatic DCs preferentially localise to the periportal region[36] and accumulate in patients with NASH.[121,122] A growing number of

studies suggest a disease-promoting role for DCs in NASH based on single-cell sequencing. An abundance of cDCs and specifically chemokine X-C receptor 1 (XCR1)-expressing cDC1s were found in patients with NASH and the frequency of these cells correlated with NASH severity.[123] Hepatic cDC1s also increased in mice fed different steatogenic diets.[123] In addition, NASH induced an increase of cDC progenitors in the bone marrow and blood. Specific depletion of cDC1s attenuated experimental steatohepatitis in mice. Paired sequencing from liver-draining lymph nodes revealed strong interactions of cDC1s with naïve T cells, promoting inflammatory programming of naïve T cells.[123] In addition, cDC2s were also elevated in human and murine steatosis.[123] In contrast, a study investigating the role of cDC1s using *Batf3*-deficient mice, which lack cDC1s, and the MCD diet reported a regulatory role for cDC1s, as *Batf3*-deficient mice progressed rapidly towards steatohepatitis and had an increased influx of inflammatory cells.[124] Fibrosis progression was unaffected, but expression of genes involved in lipid metabolism was altered, and adoptive transfer of CD103+ cDC1s reversed the phenotype.[124] One caveat is that the global deletion of *Batf3* might have other effects on liver inflammation. An emerging concept is the influence of the metabolic microenvironment on function and polarisation of DCs.[125] DCs with higher lipid content showed a more pro-inflammatory phenotype in patients with NASH compared to DCs containing lower levels of lipids.[126] A study analysing the transcriptional network and immune profiles in patients with NASH identified changes in genes regulating inflammatory processes, antigen presentation and cytotoxic cells.[121] Particularly, changes in cDC1s, cDC2s and CD8+ T cells were identified, and cDC2s and CD8+ T cells correlated with lobular inflammation and hepatocyte ballooning. Despite these efforts, the precise role of DCs in NAFLD is still ambiguous, reflecting the different subsets studied and the lack of specific markers, which complicate the interpretation of depletion experiments.[28] However, recent patient-derived data showing an increase of cDCs suggest a disease-promoting role in the inflammatory cascade.

Inflammation in NASH is driven by "auto-aggressive" effector T cells, T helper cells and activated B cells

Lymphocytic infiltration is frequently observed in liver biopsies of patients with NASH, often as focal lymphocytic aggregates consisting of T and B cells, resembling ectopic lymphoid structures.[127] CD8+ T cells are critical effector cells of the adaptive immune system, which are important for killing cancerous or infected cells in an MHC I-restricted antigen-specific fashion.[128] Patients with NASH had a striking hepatic infiltration of CD8+ T cells.[129,130] In another study, increased CD8+ T cells in liver and blood were reported and circulating CD8+ T cells showed a marked activation profile with increased expression of perforin, interferon (IFN)-γ and TNF-α.[121]

In *Rag1*$^{-/-}$ mice, which lack mature B cells, T cells and natural killer (NK) T cells, dietary NASH induced by choline-deficient high-fat diet (CDHFD) feeding is milder with reduced steatosis, parenchymal injury and inflammation.[131] CD8+ T cells and NKT cells promoted liver damage in concert,[129,131] and depletion of CD8+ T cells or NKT cell deficiency led to milder steatohepatitis. Intrahepatic CD8+ T cells were identified as regulators of hepatic gluconeogenesis.[130] In early stages of steatosis, CD8+ T cells did not meaningfully contribute to liver injury but mediated metabolic dysregulation and insulin resistance. CD8-deficient mice had improved metabolic parameters and adoptive transfer of CD8+ T cells isolated from HFD-fed livers induced worsening of glucose metabolism.[130] CD8+ T cells further exhibited the ability to directly activate hepatic stellate cells *in vivo* and *in vitro* in mice with NASH.[132] This study noted a difference regarding the role of CD8+ T cells between models of obese and lean NASH. In mice fed a CDHFD, a lean NASH model, intrahepatic CD8+ T cells were elevated, however, long-term depletion had no effect on liver injury or hepatic stellate cell activation, contrasting with the results from obese NASH mice and indicating that metabolic conditions influence T-cell phenotype.[132] Using single-cell sequencing to characterise the intrahepatic T-cell repertoire in patients with NASH and in mice fed different NASH diets, a recent study identified a conserved and largely expanded population of CD8+ T cells with markers of tissue residency (CXCR6), exhaustion (programmed cell death 1 [PD-1]) and effector function (granzyme B).[133] In a set of mechanistic experiments, the authors identified increased IL-15 signalling in the steatotic liver, which induced downregulation of the transcription factor FOXO1 (forkhead box O1) and upregulation of CXCR6. Metabolic stimuli including acetate and extracellular ATP from dying hepatocytes promoted these cells to release pro-inflammatory cytokines. Furthermore, CXCR6+ CD8+ T cells induced the direct killing of hepatocytes via Fas-FasL interactions (Fig. 3).[133] This killing mechanism was referred to as "auto-aggression" as it was independent of MHC-class-I and thus fundamentally different from the antigen-specific immunity provided by protective CD8+ T cells. Auto-aggressive T cells underline the unavoidable reciprocal impact of metabolism and immunity in NASH as these T cells are rendered 'hyperactivated' by metabolic stimuli. It is unclear if there is an evolutionary benefit of having "auto-aggressive" T cells, which might be overturned in

NASH. It is intriguing to speculate that the auto-aggressive CD8+ T cells found in NASH are primed by aberrant XCR1-expressing DCs, leading to a feed forward loop of liver inflammation. The current body of literature identifies CD8+ T cells as important effector cells in NAFLD: these cells promote metabolic dysregulation and insulin resistance early during steatosis, while "auto-aggressive" T cells directly cause hepatocyte death at later stages.

CD4+ T helper (Th) cells are broadly categorised into Th1, Th2, Th17 and regulatory T cells (Tregs); their balance is critical to maintaining liver immune tolerance, and dysregulation of regulatory and effector T helper cells is a hallmark of chronic liver diseases.[134] The role of CD4+ T cells in NAFLD is less clear and studies using single-cell technologies will likely provide novel insights in the coming years. Numerous studies reported that Th1 and Th17 cells are elevated in patients with NASH.[135] Particularly, IFN-γ-expressing CD4+ T cells were enriched in NASH and mice deficient in IFN-γ fed the MCD diet had milder steatohepatitis and reduced infiltration of inflammatory macrophages.[136,137] In a humanised mouse model fed a high-fat high-calorie diet, human CD4+ T cells accumulated in the liver and their depletion reduced liver inflammation and fibrosis.[138] OX40 is a costimulatory molecule that promotes T-cell division and survival.[139] Mice fed different steatogenic diets had increased OX40 expression in CD4+ T cells and OX40-deficient mice were protected from steatohepatitis via Th1 and Th17 differentiation and suppressed monocyte recruitment.[140] Furthermore, patients with NASH had increased OX40 levels in the serum.

In humans, intrahepatic Th17 cells were increased across the entire spectrum of NAFLD, and higher in NASH compared to steatosis.[137] Bariatric surgery improved steatosis, NASH, and led to a normalisation of Th17 cell frequencies.[137] In mice lacking IL-17, HFD or MCD diet feeding reduced hepatocellular damage and liver inflammation, while steatosis was unchanged.[141–143] Interestingly, a microbiome signature known to induce IL-17 production was sufficient to accelerate liver damage.[142] A subset of inflammatory hepatic CXCR3+Th17 (ihTh17) cells promoted obesity and steatohepatitis in murine models.[144] These ihTh17 cells exhibited enhanced glycolytic activity and production of inflammatory cytokines IFN-γ, TNF-α and IL-17. Therapeutically, glycolysis inhibition, ihTh17 cell-specific ablation of IFN-γ or interference with the CXCR3 axis protected against steatohepatitis.[144] Moreover, feeding mice a high-fat high-fructose diet resulted in hepatic and adipose tissue infiltration of Th17 cells and reduced adipose tissue Tregs.[145] These T-cell disruptions in adipose tissue and liver were sustained even after switching mice to normal chow.

Tregs, important immune regulatory cells, were reduced in patients with NAFLD compared to healthy controls.[137] In murine models, data on the role of Tregs are controversial. In one study, Tregs were reduced in HFD-fed mice, while adoptive Treg transfer before LPS challenge reduced subsequent liver inflammation.[146] Tregs were shown to be more prone to apoptosis in steatohepatitis.[127] Furthermore, increased levels of leptin reduced Treg frequency in obese individuals and mice.[147] In contrast, a recent study reported increased intrahepatic Tregs in mice fed a CDHFD and given a diethylnitrosamine injection.[148] Furthermore, high-fat high-carbohydrate feeding of BALB/c mice resulted in increased intrahepatic Tregs, however, adoptive Treg transfer exacerbated experimental NASH.[149]

Patients with NAFLD exhibit a pronounced imbalance between intrahepatic Th17 cells and Tregs[137]; however, it is mechanistically unclear if this is driven by local immune dysregulation or induced by extrahepatic factors. Interestingly, recent work identified microbial bile acid metabolism as a critical regulator of the Th17/Treg balance in the intestine.[150,151] Furthermore, secondary bile acids were critical in inducing peripheral Tregs in the colon and thereby regulating colonic immune homeostasis.[152] It remains to be investigated if altered bile acid metabolism in NAFLD also skews the T-cell balance locally in the liver or if these effects are regulated distally in the colon via the gut-liver axis.

T-cell alterations were also found in the intestine of patients with NAFLD, with fewer CD4+ and CD8+ T cells in patients, and an increase in inflammatory cytokines and disruption of tight junctions, potentially linking intestinal inflammation to gut barrier dysfunction.[153] Accordingly, treatment with a gut local anti-inflammatory agent dampened bowel inflammation, decreased insulin resistance and improved liver steatosis in mice fed a HFD.[154]

B lymphocytes have important immunological functions, including production of antibodies, antigen presentation and cytokine secretion. The B cell compartment of the liver is incompletely understood, likely owing to the fact that B cells make up only 5% of intrahepatic lymphocytes in humans; however, their numbers in mice are much higher.[155] B cells are a heterogeneous population that can be broadly divided into B1 and B2 cells. B2 cells arise from haematopoietic progenitors in bone marrow and migrate to secondary lymphoid organs, where they are aided by CD4+ T helper cells to generate high-affinity antibodies, before maturing into antibody-producing plasma cells. B1 cells are more 'innate-like' and produce 'natural' antibodies rapidly following antigen encounter, independent of T-cell help.[156] An additional population of B cells with immunosuppressive properties are regulatory B cells.[157] In NASH, B cells are found within the inflammatory infiltrate of patients and are activated during the onset of

Key point

Cell death of resident embryonic Kupffer cells and replenishment by monocyte-derived phagocytes is an emerging concept in experimental steatohepatitis.

steatohepatitis.[158] In patients with NASH, B cells accumulated in the liver, showed an activated profile and correlated with disease severity.[159] In mice, the transcriptional landscape of B cells reflected a broadly activated pro-inflammatory phenotype, including increased levels of pro-inflammatory cytokines, such as TNF-α and IL-6. B-cell depletion ameliorated NASH induced by a high-fat high-carbohydrate diet. Mechanistically, B cell-specific deletion of MyD88 (myeloid differentiation primary response 88) ameliorated NASH, while the B-cell response also involved direct B cell-receptor signalling, indicating both innate and adaptive mechanisms of activation in NASH.[159] Lastly, faecal microbiota transplantation of human NAFLD microbiota into mice led to accumulation and activation of intrahepatic B cells, indicating that the microbiome might partake in activating B cells in NASH.[159]

Key point

Lipid-associated macrophages (LAMs) expressing TREM2 and CD9 accumulate in steatotic livers.

Furthering the role of B2 cells in NASH, serum levels of B cell-activating factor (BAFF), which controls survival of B2 cells,[160] were elevated in patients with NASH and correlated with the degree of fibrosis.[161] In mice, neutralising BAFF reduced steatohepatitis.[158] Another study found IgA-expressing plasma cells, which differentiate from B2 cells after activation, to be increased in livers of patients with NASH (Fig. 3).[162] Interestingly, these cells expressed high levels of IL-10 and promoted hepatocarcinogenesis by suppressing CD8+ T-cell responses. Furthermore, IgA levels were shown to predict the degree of fibrosis in NASH[163] and mice lacking IgA exhibited milder steatohepatitis.[162] B cells in murine NASH co-localised with T-cell clusters, presented epitopes to CD4+ T cells (in turn activating the myeloid compartment via pro-inflammatory mediators), and developed into plasma cells producing anti-OSE (oxidative stress-derived epitopes) IgG.[158] Patients with NASH had elevated anti-OSE antibody titers, which positively correlated with liver inflammation.[164] While the presence of B cells in NASH is established, potential mechanisms of activation and effector function are less well understood.[165] B2 cells accumulated in adipose tissue, contributing to local inflammation via the production of pathogenic IgG antibodies, activation of T cells and local macrophages and signalling via the leukotriene B4 receptor.[166,167] Furthermore, loss of regulatory B cells during obesity resulted in adipose tissue inflammation and insulin resistance.[168] Recent single-cell data suggests B cells could activate hepatic stellate cells, as ligand-receptor interactome analyses of NASH livers identified increased CXCL12 expression by activated stellate cells; a potential target could be CXCR4 expressed by B cells.[90,165] A role for B cells in activating stellate cells and promoting liver fibrosis was previously reported.[169] Hence, in addition to antibody production, B cell-derived cytokine production and stellate cell activation seem to be important in NASH and NASH-associated fibrosis.[165]

Collectively, recent studies relying on single-cell transcriptomic data have clearly established a role for CD8+ T cells, CD4+ T cells and B cells in the immune pathogenesis of NASH.

Innate lymphoid cells as novel participants in NASH pathogenesis

Innate lymphoid cells (ILCs) are lymphocytes that lack the antigen-specific receptors of T and B cells. As tissue-resident cells, ILCs are integrated into tissues and their ability to rapidly respond to stress signals by producing cytokines makes them an integral part of liver immunity.[170] ILCs consists of 5 subsets – NK cells, ILC1s, ILC2s, ILC3s and lymphoid tissue inducer cells[171] – based on developmental and functional trajectories. The tissue residency phenotype of ILCs emphasises that these cells are critical in organ homeostasis and metabolism, besides their important immune function. Emerging evidence links ILCs to metabolic dysregulation in obesity, insulin resistance and NAFLD.[172]

NK cells are critical for host defense and kill target cells by secreting perforins and granzymes.[173] In humans, these cells are further divided into $CD56^{dim}$ cells, which circulate and can directly kill pathogens, or $CD56^{bright}$ cells, which are found in tissues and mainly act via production of cytokines.[174] $CD56^{dim}$ NK cells were decreased in peripheral blood but increased in the liver of patients with NAFLD.[175] Other studies failed to detect differences in the frequency of peripheral and hepatic NK cells between patients with NASH and healthy individuals.[176,177] In keeping with the multisystem character of inflammation in NAFLD, NK cells accumulated in adipose tissue of mice fed a HFD, where they produced pro-inflammatory cytokines and polarised adipose tissue macrophages towards a pro-inflammatory phenotype.[178,179] NK cell depletion improved insulin resistance in mice, identifying NK cells as potential mediators of adipose tissue inflammation.[179] While these studies provide evidence that NK cells promote obesity and insulin resistance, the role of NK cells in NASH is controversial.[176] In a murine study using the MCD diet, NK cells seemed to be protective, by altering the liver macrophage compartment, and depletion of NK cells resulted in accelerated fibrogenesis.[180] Data from human NK cells further support the influence of the microenvironment on NK cell activity, which is severely altered in obesity.[176] In obese children and adults with NAFLD, NK cells exhibited a metabolically stressed phenotype, including increased glycolysis and reactive oxygen species production resulting in loss of cytotoxicity.[181,182] Interestingly, lipid accumulation in NK cells caused immune 'paralysis' by inhibiting mTOR-mediated glycolysis. *In vivo*, the lipotoxic environment impaired NK cell-mediated tumour suppression, potentially providing a link to hepatocarcinogenesis in NASH.[181]

Recent studies found ILC2s in visceral adipose tissue of humans and mice, maintained by IL-33 and obesity was characterised by loss of ILC2s.[183] Furthermore, ILC2s contribute to metabolic homeostasis by sustaining eosinophils and polarising macrophages to regulate adiposity and insulin resistance.[183,184] In humans with liver fibrosis, ILC2s correlated with severity of fibrosis.[185] In mice, hepatocyte injury induced IL-33 signalling, resulting in ILC2 activation and propagation of fibrosis via IL-13-dependent stellate cell activation.[185] During development of obesity, IFN-γ-producing ILC1/NK cells accumulate in adipose tissue, skewing the immune milieu towards a more pro-inflammatory phenotype and thereby promoting insulin resistance.[179,186,187] IL-22 deficiency by ILC3s resulted in aggravation of metabolic disorders in obese mice and restoration of IL-22-producing ILC3s improved insulin sensitivity, preserved the intestinal barrier and decreased inflammation in adipose tissue of obese mice.[188] It had previously been shown that IL-22 ameliorated experimental liver fibrosis by inducing stellate cell senescence via upregulation of signal transducer and activator of transcription (STAT)3.[189] Furthermore, the therapeutic potential of IL-22 was recently suggested in a murine study using overexpression of the neutrophil chemoattractant CXCL1 and feeding of HFD as a model system.[190] Although not linked to specific cell types, IL-22 treatment reduced inflammation in mice.[190]

Diverse populations of unconventional T cells emerge in steatohepatitis

Unconventional T cells are a heterogeneous group of lymphocytes of the liver immune system (Fig. 3). The most important subsets of unconventional T cells include mucosal-associated invariant T (MAIT) cells, γδ T cells and NKT cells.[191] Unconventional T cells account for roughly 10% of circulating T cells, however, in the liver they represent the majority of T cells.[191] There are key differences between humans and mice as MAIT cells represent between 15–45% of liver T cells in humans, but are rare in mice, and conversely invariant NKT (iNKT) cells constitute between 30–50% of intrahepatic T cells in mice but less than 1% in healthy humans.[66]

The role of MAIT cells during NASH is still obscure and warrants further investigation. One study noted an increase of intrahepatic MAIT cells in patients and a decrease in peripheral blood.[192] In addition, MAIT cells were increased in the fibrotic septa of patients with cirrhosis due to alcohol-related steatohepatitis and NASH.[193] As functional studies on the role of MAIT cells in NASH are limited, most of our understanding is derived from mouse studies. Depletion of MAIT cells in the MCD diet model in mice aggravated liver injury; their protective effect seemed to be mediated by polarising macrophages towards an anti-inflammatory phenotype. On the contrary, during obesity, MAIT cells promoted adipose tissue inflammation by skewing macrophages towards a pro-inflammatory phenotype, resulting in pronounced insulin resistance, dysbiosis and metabolic dysfunction.[194] Furthermore, MAIT cell inhibition served as a potential treatment in this study. These results indicate different mechanisms of immune regulation of MAIT cells depending on the tissue niche. In mouse models of liver fibrosis induced by bile duct ligation or chronic toxicity, a pro-inflammatory and pro-fibrogenic role for MAIT cells (via induction of hepatic stellate cells) was ascribed[193] and further confirmed in humans by *ex vivo* coculture experiments.[195]

NKT cells can be divided into type I NKT (iNKT) cells and type II NKT cells.[196] The relative abundance of iNKT cells and their ability to recognise lipid-based antigens presented by the MHC class-I-like molecule CD1d make iNKT cells an interesting player in the inflammatory response during steatohepatitis. Indeed, hepatic NKT cell numbers were increased in patients with NASH.[131,197] Similarly, in murine steatohepatitis, NKT cells were enriched and mice deficient in CD1d had milder steatohepatitis.[197] Mechanistically, it was shown that NKT cells mediated steatosis by secretion of the cytokine LIGHT which prompted free fatty acid uptake by hepatocytes (Fig. 3).[131] NKT cells express the chemokine receptor CXCR6 and during experimental steatohepatitis, CXCR6 and its ligand CXCL16 were shown to be critical in the recruitment of NKT cells to the liver.[198] Mechanistically, NKT cells expressed pro-inflammatory cytokines, CXCR6 deficiency reduced liver fibrosis and adoptive transfer of NKT cells aggravated liver fibrosis in steatohepatitis.[198] Given the opposing distribution of NKT cells and MAIT cells between humans and mice, findings about NKT cells from rodent models need to be interpreted with caution, and NKT cells have been suggested as the murine counterpart of MAIT cells in humans from a functional perspective.[199]

γδ T cells express a T-cell receptor (TCR) γ-chain and δ-chain; many aspects of their biology are incompletely understood and the frequency of γδ T cells varies between humans and mice. Furthermore, both humans and mice have CD1d-restricted γδ T cells that recognise lipid antigens.[191] In human NAFLD, the frequency of hepatic γδ T cells was unchanged.[175] In mice fed high-fat high-cholesterol diet, hepatic γδ T cells were increased, and the production of IL-17A was identified as a disease-promoting factor, as specific depletion of IL-17 in γδ T cells alleviated NASH (Fig. 3).[200] Since CD1d-deficient mice also have impaired γδ T cells, the protective effect observed in these mice might be partially attributed to altered γδ T cells.[201] IL-17 producing γδ T cells depend on CCR6 and its ligand CCL20 for recruitment to the liver.[202] Expression of CCR6 and CCL20 was increased in human cirrhosis, and

Key point

Conventional dendritic cells (cDCs) and particularly cDC1s are elevated in NAFLD bridging innate and adaptive immunity.

mice deficient in CCR6 had aggravated liver fibrosis and inflammation in 2 models of chronic liver injury, including the MCD diet.[203] In this study, adoptive transfer of γδ T cells reduced liver inflammation, likely by promoting apoptosis of activated hepatic stellate cells.

Platelets bridge innate and adaptive immune cells in NASH

Platelets are essential in hemostasis and wound healing; however, more recently their role in inflammation and infection has been appreciated.[204] Using intravital microscopy, a crucial contribution to the KC-mediated capture of pathogens was observed.[204] KC–platelet adhesion was mediated via platelet receptor GPIIb, and blocking this interaction led to rapid mortality in mice with bloodstream infections. Platelets play a role in the pathogenesis of obesity, metabolic syndrome and atherosclerosis.[205,206] Platelets were also investigated in NASH and anti-platelet therapy alleviated experimental NASH in rats and mice.[207] During steatosis, platelets accumulate in the liver early on through CD44 expressed on their surface, binding to hyaluronic acid presented on the cell surface of stressed hepatocytes, liver sinusoidal endothelial cells and KCs.[34] Indeed, platelet aggregates on KCs were detected early during steatosis and these platelets mediated the subsequent recruitment of CD8+ T cells and NKT cells in a GPIba-dependent manner by secreting exosomes (Fig. 3).[34] In a clinical trial, anti-platelet therapy improved fatty liver disease in patients, identifying platelet aggregation as a potential target in NASH.[34] Bone marrow adiposity, frequently observed in obesity, directly effects platelets: megakaryocyte maturation was increased in medullar adiposity, leading to increased thrombogenicity and activation of platelets, thus potentially providing a feed forward loop of hepatic platelet aggregation in NASH.[208]

Key point

Auto-aggressive CD8 T cells attack steatotic hepatocytes and are characterised by tissue residency (CXCR6) and exhaustion (PD-1) markers.

Inflammatory mechanisms driving disease regression

Inflammation in NAFLD is rarely a constant process, but instead transitions through phases of aggravated inflammation and periods of resolution in most patients. While the chronic inflammatory phase has been studied in detail, our understanding of the resolution phase during NASH is still limited. For many years, resolution of inflammation was viewed as a passive event; however, it is increasingly apparent that the immune system has developed sophisticated mechanisms to mediate the switch from inflammation to resolution. At the centre is the orchestrated interplay between different immune cells, particularly macrophages and neutrophils (Fig. 4).[209] The precise inflammatory mechanisms driving disease regression in a chronic setting such as NAFLD are not well understood. However, it is conceivable that the presence of multiple triggers of inflammation (Fig. 1) hinders a coordinated resolving cascade, resulting in defective resolution.

A recent study investigated the transcriptional landscape of patients with NAFLD before and after lifestyle intervention.[121] Patients responding to treatment had downregulated transcripts of numerous genes related to pro-inflammatory cytokines, immune cell infiltration, and antigen presentation.[121] Liver macrophages are important cells in the resolution of liver damage and inflammation, both in acute and chronic disease models.[209,210] In liver fibrosis, depleting liver macrophages during the recovery phase prolonged healing with failure of matrix degradation.[211] Subsequently, infiltrating restorative macrophages were identified that promoted resolution by expression of matrix metalloproteinases (MMP-9, MMP-12 and MMP-13), anti-inflammatory mediators (IL-10) and growth factors.[212] Stabilin1 was identified as a critical receptor on monocyte-derived macrophages mediating a restorative phenotype.[213] Macrophages that expressed MerTK were shown to promote the resolution of inflammation in patients and mice with acute liver failure.[214] On the contrary, a gene polymorphism associated with lower hepatic expression of *MerTK* was protective in patients with NAFLD.[215] Furthermore, global or myeloid-specific deletion of *MerTK* in NASH diet-fed mice decreased liver fibrosis, a protective effect mediated by decreased stellate cell activation.[216] Hence, the role of *MerTK* in macrophages could be dependent on the model. In different models of liver fibrosis and isolated human macrophages, uptake of products of oxidative lipid peroxidation resulted in suppressed pro-inflammatory cytokine production and reduced collagen deposition (Fig. 4).[213] It is unclear whether restorative macrophages are recruited specifically during resolution or if these cells are generated locally via phenotype switching of pro-inflammatory macrophages, as seen in acute sterile injury.[217] There is evidence that pro-repair macrophages can promote apoptosis of pro-inflammatory macrophages via IL-10 in mice fed a HFD.[218] In murine NASH, activation of autophagy in liver macrophages favoured their maturation towards a reparative phenotype.[219,220]

Increasing evidence suggests that neutrophils play an important role in tissue repair and breakdown of extracellular matrix in liver fibrosis[221–223]; hence their presence during steatohepatitis might be part of the restorative response. Neutrophil depletion in the restorative phase after MCD diet feeding impaired liver healing and was accompanied by impaired phenotype switching of pro-inflammatory macrophages towards repair macrophages.[221] Neutrophils released extracellular vesicles (EVs) containing microRNA-223 as one specific mechanism to limit steatohepatitis in mice.[221,224,225] Uptake of miRNA-233 by hepatocytes

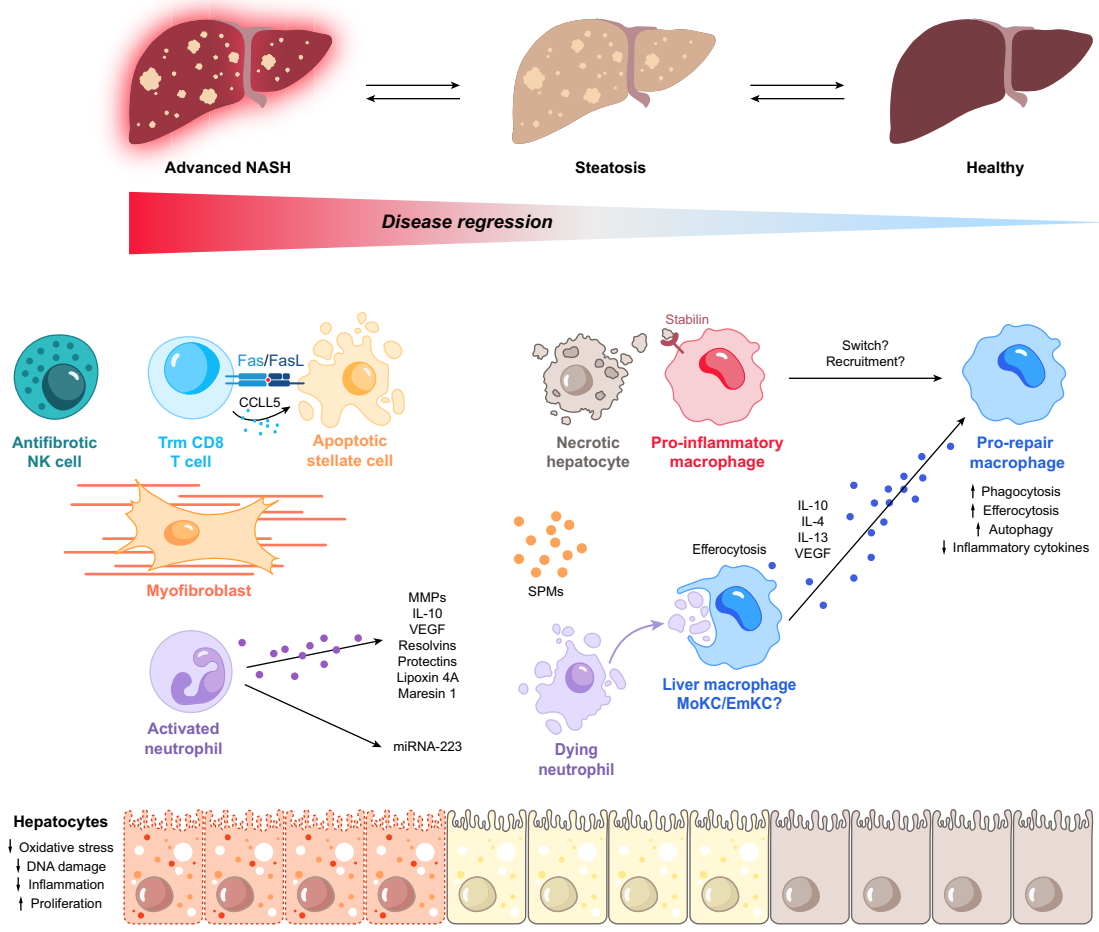

Fig. 4. Mechanisms of disease regression of NAFLD. Resolution of inflammation is an active process involving SPMs derived from omega 3 (n-3) and omega 6 (n-6) PUFAs, including protectins, resolvins, lipoxins and maresins. These mediators are liberated and dampen inflammation, however their disbalance might contribute to ongoing inflammation in NASH. Neutrophils are emerging as important pro-resolving cells, as they release many anti-inflammatory/pro-resolution molecules including IL-10, VEGF, SPMs and microRNA-223. Dying neutrophils are phagocytosed by macrophages (efferocytosis) inducing a pro-repair phenotype switch in macrophages. Restorative macrophages emerge (Ly6C^{lo} in mice), characterised by anti-inflammatory cytokine and growth factor production, degrading extracellular matrix and favouring resolution. Other cells contribute to resolving fibrosis by inducing stellate cell apoptosis including Trm T cells and NK cells. MMPs, metalloproteinases; NASH, non-alcoholic steatohepatitis; NK, natural killer; PUFAs, polyunsaturated fatty acids; SPMs, specialised pro-resolving mediators; Trm T cells, tissue-resident memory T cells.

was dependent on the low-density lipoprotein receptor on hepatocytes and dampened pro-inflammatory and pro-fibrotic gene expression.[225]

In two dietary mouse models of NASH, single-cell transcriptomics revealed an expansion of tissue-resident memory CD8+ T cells (CD69+CD103-CD8+ Trm) cells during the resolution phase of NASH.[226] Depletion of CD8+ T cells during resolution or antagonism of IL-15 signalling prolonged healing. Mechanistically, CD8+ Trm were directly involved in a CCR5-dependent, FasL-Fas-mediated killing of hepatic stellate cells, thereby promoting resolution of liver fibrosis.[226] This mechanism of disease regression might work in concert with NK cells, as NK cells were previously shown to have antifibrotic properties by directly targeting activated stellate cells.[227–229] These studies suggest a conserved communication circuit by which cytotoxic lymphocytes silence pro-fibrotic cells to induce resolution of disease.

On a molecular level, a family of lipid mediators generated during inflammatory responses has garnered attention in recent years, promoting the resolution of inflammation by acting as "stop signals".[230] Specialised pro-resolving mediators (SPMs) include lipoxins, resolvins and protectins, which are derived from the omega-3 essential fatty acids eicosapentaenoic acid and docosahexaenoic acid.[231] SPMs exert protective effects via a wide range of cell types, including dampened neutrophil and monocyte influx, increased phagocytosis of apoptotic neutrophils by macrophages (efferocytosis) and downregulation of inflammatory transcription factors.[100,230,231] The precise role of pro-resolution lipid mediators in NASH is unclear but given that they are derived from eicosapentaenoic acid and docosahexaenoic acid, which

are known to be altered in steatosis,[232] a failure of pro-resolution mechanisms in NASH is assumed.[29] Therapeutic administration of resolvin D1 to HFD-fed mice was associated with reduced liver inflammation.[233] LXA4, a synthetic lipoxin analogue, attenuated steatosis in mice.[234] Maresin 1 was recently shown to promote a pro-repair phenotype of liver macrophages via binding to retinoic acid-related orphan receptor α.[235] By inducing a pro-repair phenotype in infiltrating liver macrophages, administration of maresin 1 was protective in mice with HFD-induced NASH.[235] Current data suggest that a focus on pro-resolution pathways and their mediators could represent a novel therapeutic avenue in NAFLD.[236,237]

The role of inflammation in the transition to NASH-associated HCC

Necroinflammation, hepatocyte cell death, oxidative stress and ongoing liver repair increase the risk of DNA damage and hepatocarcinogenesis.[238] Obesity *per se* is linked to increased cancer risk in many organs,[239] involving mechanisms that alter immune function and endocrine changes. In addition, liver-specific mechanisms during NAFLD, including metabolic and oxidative stress and profound immune dysregulation, contribute to hepatocarcinogenesis.[240] For instance, oxidative stress in hepatocytes activates inflammatory pathways such as STAT1 and STAT3, which are known pro-inflammatory transcription factors and often act in concert. Inflammation leading to NASH was induced by high levels of STAT1, whereas high levels of STAT3 independently promoted HCC development in mice, demonstrating the uncoupling of inflammation and carcinogenesis in the NASH-to-HCC transition.[241] The precise role of the immune system in the context of NASH-associated HCC is controversial, and likely dependent on the status of the underlying NASH, the genetic risk profile, and other factors such as comorbidities, the microbiome, and the environment. Chronic sterile inflammation promotes the progression of NASH towards advanced fibrosis and increases the risk of cancer. The liver activates repair pathways, and thus might provide an environment for malignant hepatocytes to escape immune surveillance. In recent years, the roles of different immune cells in the NASH-to-HCC transition have been unravelled. Long-term feeding of CDHFD leads to NASH-associated HCC in mice, which appears to be driven by CD8+ T cells and NKT cells.[131] Furthermore, platelets promoted NASH and HCC, whereas anti-platelet therapy alleviated NASH and reduced HCC incidence in mice.[34] In a diet-induced NASH-HCC mouse model, CD8+ T cells lacked efficient immunosurveillance and promoted HCC development.[242]

Key point

Platelets are emerging inflammatory players already in early NAFLD that initiate immune cell infiltration.

Interestingly, therapeutic anti-PD-1 administration did not lead to tumour regression, and paradoxically, preventive treatment with anti-PD-1 antibodies expanded PD-1+ CD8+ T cells, aggravated steatohepatitis and increased HCC incidence.[242] The observed exhausted PD-1+CXCR6+ CD8+ T cells potentially promoted HCC by directly killing hepatocytes in an "auto-aggressive" fashion.[133,242] A meta-analysis of clinical trials and 2 retrospective cohort studies evaluating the role of checkpoint inhibitors in HCC revealed that when stratifying patients into viral and non-viral HCC, the benefit of immune checkpoint inhibitors was restricted to patients with viral HCC.[242] In contrast, CD8+ T cells had anti-tumorigenic effects in HFD-fed MUP-uPA mice, protecting them from NASH-induced HCC.[162] Here, CD8+ T cells had little effect on the underlying NASH. Furthermore, anti-PD-L1 therapy reduced CD8+ T cell exhaustion and promoted anti-tumour immunity.[162] In patients with HCC, a larger number of tumour-infiltrating CD8+ T cells was shown to correlate with improved survival.[243] Integrating the findings of these studies, it appears that an abundance of exhausted CD8+ T cells (CXCR6+PD-1+CD8+ T cells) leads to tumorigenesis in mice and could indicate a phenotype switch of CD8+ T cell subsets from cytotoxic (anti-tumour) to exhausted (tumour promoting). Depending on the model, checkpoint inhibition in experimental HCC either promotes or reduces the frequency of these cells. Potentially intrahepatic PD-1+ CD8+ T cells might serve as a prognostic marker for treatment response to checkpoint inhibitors in HCC.

Another mechanism involved in NASH-related HCC was the selective reduction of CD4+ T cells, induced by direct toxicity of free fatty acids on hepatic CD4+ T cells in humans and mice through mitochondrial reactive oxygen species production.[244] CD4+ T-cell loss resulted in inefficient tumour surveillance and accelerated hepatocarcinogenesis in mice.[244] CD4+ T cells were previously shown to be critical in the immune response against pre-malignant senescent hepatocytes, and antibody depletion of CD4+ T cells resulted in increased HCC.[245] Furthermore, in mice fed the MCD diet, tumour immunotherapy using anti-OX40 antibodies was ineffective in melanoma and colon cancer metastasis compared to control mice with normal levels of CD4+ T cells.[246] In contrast, Th17 cells promoted NASH and HCC via IL-17A signalling.[247] In HFD-fed mice with overexpression of transcriptional repressor unconventional prefoldin RPB5 interactor, DNA damage in hepatocytes triggered IL-17A release, which caused neutrophil infiltration in adipose tissue, leading to insulin resistance, steatohepatitis and HCC.[247] In this study, Th17-mediated acceleration of the underlying NASH might have promoted

hepatocarcinogenesis as opposed to the tumour-suppressive role of CD4+ T cells.

Neutrophils were also implicated in NASH-associated HCC development through the release of NETs.[248] In the streptozotocin and HFD model in mice, inhibiting NET formation through DNase treatment or mice deficient in PAD4 (peptidyl arginine deiminase type 4) decreased NASH and HCC.[248] A mechanistic explanation was recently suggested by a study using the CDHFD and diethylnitrosamine mouse model.[148] An increase in NETs and Tregs was detected compared to controls and Treg depletion prevented HCC (Fig. 3). RNA-sequencing revealed an impact of NETs on gene expression profiles of naïve T cells, promoting Treg differentiation in a TLR-4-dependent manner. Finally, blockade of NETs reduced Tregs and subsequent HCC incidence.[148] The precise role of other myeloid cells, such as KCs or LAMs, in the NASH-to-HCC transition has not been fully elucidated. Recent data obtained from single-cell RNA-sequencing of (non-NAFLD-associated) HCC detailing the immune cell landscape of HCC found a diverse spectrum of macrophages, with one subset having a more immunosuppressive gene expression profile (tumour promoting) and another subset having a more immunogenic phenotype (anti-tumour).[249]

Thus, the immune system plays opposing roles in NASH-associated hepatocarcinogenesis. While for many decades, the tumour-promoting role of inflammation has been emphasised – with steatohepatitis being a seeding ground for malignant transformation of proliferative hepatocytes and the failure of tumour-suppressive immune functions – we now understand the anti-tumour activities of immune cells in NASH better. With novel high dimensional sequencing tools, we are learning more about the different subsets of immune cells that are involved, both in promoting NASH and HCC. The consequences for improving systemic therapy in patients with NASH-related HCC, such as response to immune checkpoint inhibition, are prospectively evaluated in future studies.

Potential implications of immune mechanisms in NAFLD for therapeutic interventions

Specific therapeutics for NAFLD are still lacking, despite a growing number of positive early-stage clinical trials.[21] Targeting inflammation appears principally a promising strategy, however, unspecific immunosuppression will likely not be successful, and potentially harm patients.[250] The novel inflammatory pathways identified might lead to more specific immunomodulatory strategies. Preclinical and clinical data for cenicriviroc, a dual CCR2/CCR5 antagonist targeting monocyte and lymphocyte recruitment in NASH, were encouraging[251]; however, a phase III trial was recently terminated due to lack of efficacy in the interim analysis.[21] It is unclear why cenicriviroc was unsuccessful, however, a redundancy of the CCR2-mediated pathway or the aforementioned heterogeneity of myeloid cells – potentially resulting in inhibition of restorative cells – could be an explanation. Alternative strategies to target liver macrophages have been proposed and tested in preclinical and early-stage clinical settings.[30,252] Direct delivery of dexamethasone to liver macrophages via the CD163 receptor[253] reduced pro-inflammatory cytokines, liver inflammation and fibrosis in a dietary NASH model in rats. Galectin 3 is highly expressed by macrophages and is involved in liver fibrogenesis.[254] Inhibiting or deleting galectin 3 in mouse models of NASH improved liver fibrosis.[255,256] The recently published phase IIb trial evaluating belapectin, a galectin 3 inhibitor, did not reveal improved portal hypertension or fibrosis in patients with NASH cirrhosis.[257]

Targeting adaptive immune cells, such as B cells or Th17 cells, holds promise, given that rituximab (anti-CD20) and belimumab (anti-BAFF) targeting B cells or anti-IL-17 monoclonal antibodies have been used for years in patients with autoimmune disorders.[165,258] Another therapeutic avenue follows the discovery of auto-aggressive CD8+ T cells in NASH.[133] Since exhaustion of these cells might be linked to metabolic disturbance, restoring glycolytic pathways and mitochondrial function, as seen in exhausted T cells in chronic viral infection,[259] might reduce their auto-aggressive potential. Anti-platelet therapy could become an effective NASH therapy, preventing the transition from NASH to HCC.[260] However, potential side effects of long-term treatment must not be underestimated. Thus, novel platelet-specific drugs that block platelet-related inflammation without affecting haemostasis are warranted.

Immunotherapy, aimed at fibrosis could be a ground-breaking new treatment in NASH. Engineered chimeric antigen receptor (CAR) T cells have been shown to eliminate activated cardiac fibroblasts, ameliorating cardiac fibrosis in a pioneering study.[261] Recently, CAR T cells designed to combat senescent cells via recognition of urokinase-type plasminogen activator receptor (uPAR), were shown to efficiently reduce fibrosis in different *in vivo* rodent models, including experimental NASH and chronic carbon tetrachloride toxin administration.[262] Specifically, uPAR-expressing hepatic stellate cells and macrophages were removed by CAR T-cell therapy. Another innovative approach is adoptive cellular therapy. First-in-human studies exist, such as autologous macrophage infusion in patients with cirrhosis,[263] or infusion of Tregs in patients with autoimmune hepatitis.[264] However, given the many uncertainties regarding stability, homing properties and specificity, many more studies will be needed before this approach becomes a viable alternative in NAFLD. Alternative strategies addressing the

Key point

NASH-associated HCC is enabled by inflammation-associated pro-tumorigenic mechanisms and insufficient immune surveillance against (pre-)malignant cells.

dysregulated Treg axis in NAFLD involve either administering an oral anti-CD3 antibody,[265] which in preclinical work was shown to induce Tregs,[266] or adding low-dose IL-2 to expand Tregs *in vivo*.[267]

An emerging strategy involves simultaneously targeting the metabolic and inflammatory arms of NAFLD pathogenesis (*i.e.* combination therapy) using drugs with multiple mechanisms of actions, such as peroxisome proliferator-activated receptor (PPAR) agonists, farnesoid X receptor (FXR) agonists or thyroid hormone receptor-β agonists.[268,269] These nuclear receptors (NRs) restore the metabolic disturbance in NAFLD, but also have multiple effects on immune cell functions, overall, favourably modulating the inflammatory environment in preclinical studies of NAFLD.[12,270,271] For instance, the PPAR agonist lanifibranor that targets all PPAR isoforms has direct anti-inflammatory effects on hepatic macrophages via PPARβ/δ and direct anti-fibrotic effects on stellate cells via PPARγ in NAFLD mouse models.[272] Liver X receptor (LXR) is a NR that regulates triglyceride and cholesterol metabolism. In a recent study, pharmacologic increase of the LXR ligand desmosterol resulted in increased LXR signalling and downstream increases in the SPM maresin-1, effectively skewing macrophages towards a pro-resolution phenotype.[273] This might indicate that pharmacologic targeting of LXR or other NRs, to reverse the metabolic disturbance in NASH, could also strengthen pro-resolution pathways in NASH.

Disturbed bile acid homeostasis is considered an additional driver of NAFLD pathogenesis[274] and has thus spawned numerous preclinical and clinical studies investigating therapeutic modulation of bile acid signalling in NASH.[271,275] FXR is a bile acid-activated NR that regulates bile acid, lipid and glucose metabolism and is hence central to metabolic homeostasis.[276] FXR is expressed in multiple extrahepatic tissues including the intestine and adrenal glands. In the liver, FXR signalling acts on various parenchymal and non-parenchymal cells, including hepatocytes, cholangiocytes, and immune cells (macrophages, NK cells and dendritic cells).[277] FXR signalling modulates inflammatory pathways by dampening pro-inflammatory cytokine production, inflammasome activation and upregulating anti-inflammatory mediators.[278] In murine models of NASH, therapeutic administration of FXR agonists skewed liver macrophages towards an anti-inflammatory phenotype, reducing steatosis, liver inflammation and fibrosis.[279,280] Importantly, obeticholic acid, a steroidal FXR agonist, has shown promise in phase II and III clinical trials in patients with NASH,[281] including the ongoing phase III REGENERATE trial, which reported an improvement of fibrosis on interim analysis without worsening of NASH.[282] By potentially restoring bile acid metabolism and dampening inflammation, pharmacologic FXR agonism thus remains an important pillar for future combination therapy in NAFLD.[283]

Challenges and perspectives

Inflammation is critical in progressive NAFLD and modulating the immune response is a promising therapeutic strategy. Observational studies from patients and mechanistic studies derived from rodent models have identified numerous inflammatory pathways that promote or regulate NAFLD (Table 1). At the same time, clinical management to date is restricted to difficult-to-sustain lifestyle interventions and many clinical trials have yielded frustrating results. The reciprocal communication circuits in NAFLD, encompassing metabolic dysregulation, sterile chronic inflammation, and the contribution of multiple immunologically active liver cells, make NAFLD a challenging disease. The complexity is increasing further, as extrahepatic disease modifiers (nutrition, gut microbiota, adipose tissue signals, endocrine dysfunction, or concomitant medication) are being integrated into a dynamic understanding of pathomechanisms in NAFLD (Fig. 1). The past few years have advanced our understanding of the complex immune cell subsets in the healthy liver and in NAFLD. While (pronounced) inflammation may only be observed during NASH, preclinical studies show profound changes of the liver-resident immune system already during obesity and steatosis, indicating that the immune system is involved across the entire spectrum of NAFLD. Furthermore, in recognising the diversity of hepatic tissue-resident immune cells, the traditional black and white distinction between steatosis (no inflammation = benign) and NASH (with inflammation = severe) might be too simplistic in identifying patients at risk. It becomes clear that NASH exists in different flavours, further increasing the heterogeneity of the metabolic and immunologic landscape of the liver as well as its response to therapies.[238,284] Indeed, metabolic dysregulation has profound effects on many immune cells before the detection of classical 'liver inflammation', as graded by conventional H&E-based histology. Along those lines, a call for a renewed framework of immune responses beyond the classical role in infection and injury has recently been proposed.[285] While many fundamental principles have been established in rodents, increasingly state-of-the-art multi-omics has been used to investigate human immunology. Implementing novel tools such as single-nucleus RNA-sequencing (suitable also for fragile cells such as hepatocytes) combined with interactome analysis should improve our understanding of the communication circuits during NASH. Particularly, the cellular crosstalk between hepatocytes, hepatic stellate cells, endothelial cells, and immune cells is still incompletely understood.

While on a per cell basis, alterations for each of these cells have been documented, novel bioinformatic tools such as receptor-ligand interaction maps might shed more light on critical pathways of cross communication.[37]

Numerous rodent models of NAFLD exist, highlighting different features of human NAFLD[286]; however, it remains challenging to compare findings regarding immune cells between studies due to the different models used. NAFLD is also heterogenous on the human side, thus the future challenge will be to stratify patients with specific metabolic/immunologic disease better and detect which mouse models reflect which NAFLD subtype in humans. In fact, many differences between mouse models and human patient samples are apparent.[287] Lastly, we are only beginning to understand, that immune cells can directly contribute to liver cell death, *e.g.* via the "auto-aggressive" actions of CD8+ T cells.[133] A major limitation to the study of immune cell behaviour in steatohepatitis has been the lack of tools such as intravital imaging that allow for the detection of such behaviour due to technical challenges induced by autofluorescence. However, first *in vivo* imaging studies have been published,[34,35] and novel microscopy tools, including spectral detection and tunable detectors can overcome some of the technical challenges, resulting in more robust evidence for immune-mediated mechanisms in NASH.[288,289] Harnessing the potential of novel tools for basic research and improving our understanding of immune cell subsets will come with the challenge of translating these abundant data points into actionable therapeutic targets. An improved understanding of the complex communication circuits between inflammation and metabolism in NAFLD will aid in developing better and more stage-specific treatments. Targeting inflammation and/or metabolism might be most beneficial in early-stage NAFLD, while at later stages antifibrotic or combination therapy is warranted. Stratifying patients based on disease stage, risk profile and modifiers will also enable better designed trials and more personalised treatment options.

Abbreviations

Anti-OSE, anti-oxidative stress-derived epitopes; BAFF, B cell-activating factor; CAR, chimeric antigen receptor; CCL, C-C motif chemokine ligand; CDHFD, choline-deficient high-fat diet; CXCL, C-X-C motif chemokine ligand; CLEC4F, C-type lectin domain family 4 member F; DAMPs, damage-associated molecular patterns; DC, dendritic cell, DHA, docosahexaenoic acid; EVs, extracellular vesicles; EPA, eicosapentaenoic acid; FXR, farnesyl X receptor; HFD, high-fat diet; hCLS, hepatic crown-like structure; IFN, interferon; IL-, interleukin-; iNKT, invariant NKT; KC, Kupffer cell, LAMs, lipid-associated macrophages; LPS, lipopolysaccharide; MAIT cell, mucosal-associated invariant T cell; MCD, methionine-deficient; MerTK, Mer tyrosine kinase; MMP, matrix metalloproteinases; Msr1, macrophage scavenger receptor 1; NETs, neutrophil extracellular traps; NK(T), natural killer (T); NLRP3, NOD-, LLR- and pyrin domain-containing protein 3; PAD4, peptidyl arginine deaminase type IV; PPAR, peroxisome proliferator-activated receptor; ROR, retinoic acid-related orphan receptor; SAMs, scar-associated macrophages; SPMs, specialised pro-resolving mediators; STAT, signal transducer and activator of transcription; TCR, T cell receptor; Th, T helper; Timd4, T-cell immunoglobulin and mucin domain containing 4; TLR-4, toll-like receptor 4; TNF, tumour necrosis factor; Tregs, regulatory T cells; TREM-2, triggering receptor expressed on myeloid cells 2; Trm, tissue resident memory; uPAR, urokinase-type plasminogen activator receptor; URI, unconventional prefoldin RPB5 interactor; XCR1, chemokine X-C receptor 1.

Financial support

The authors received no financial support to write this manuscript.

Conflict of interest

Work in the lab of FT has been supported by research grants from Gilead, Allergan, Bristol-Myers Squibb and Inventiva. The other authors declare no conflict of interest.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

MP and FT wrote the initial draft and designed the figures. RS, JH, PK and MH edited the manuscript, wrote parts of the manuscript, and gave critical input.

Acknowledgements

MP is supported by the BIH clinician scientist program. FT is supported by the German Research Foundation (DFG SFB/TRR 296 and CRC1382, Project-ID 403224013) and the German Ministry of Education and Research (BMBF DEEP-HCC consortium). PK is supported by the NSERC Discover grant (RGPIN/07191-2019), the Heart & Stroke Foundation of Canada, CIHR and Canada Research Chairs Program. MH is supported by an European

Reseach Council, EU (ERC) Consolidator grant (HepatoMetaboPath), SFBTR179 Project-ID 272983813, SFB/TR 209 Project-ID 314905040, SFBTR1335 Project-ID 360372040, the Wilhelm Sander-Stiftung, the Rainer Hoenig Stiftung, a Horizon 2020 grant (Hepcar), Research Foundation Flanders (FWO) under grant 30826052 (EOS Convention MODEL-IDI), Deutsche Krebshilfe projects 70113166 and 70113167, German-Israeli Cooperation in Cancer Research (DKFZ-MOST), and the Helmholtz-Gemeinschaft, Zukunftsthema "Immunology and Inflammation" (ZT-0027). RFS is supported by NIH grants CA190844 and CA228483. JH is supported by the German Ministry of Research and Education (BmBF) through the Liver Systems Medicine (LiSyM) network grant and ERACOSysMed (Dynaflow) grant.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2022.06.012.

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Author names in bold designate shared co-first authorship

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