Immune–metabolic interactions in homeostasis and the progression to NASH

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Title

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Key words

Liver; immunology; NAFLD; NASH; metabolism

Abstract

The incidence of Non-Alcoholic Fatty Liver Disease (NAFLD) has tremendously increased over the past 2 decades. NAFLD ranges from simple steatosis (NAFL) to Non-Alcoholic Steatohepatitis (NASH) and predisposes to fibrosis and hepatocellular carcinoma. The importance of the immune system in hepatic physiology and in the progression of NAFLD is increasingly recognized. At homeostasis, the liver participates in the immune defense against pathogens and in tolerance towards gut-derived microbial compounds. Hepatic immune cells also respond to metabolic stimuli and play a role in NAFLD progression to NASH. We first discuss how metabolic perturbations affect immune cell phenotype and function in NAFL

25 and NASH. Then, we focus on the role of immune cells in liver homeostasis and in the
26 development of NASH.

The liver as an immunological organ

The liver plays a central role in glucose and lipid homeostasis as well as in cholesterol and bile acid synthesis. Hepatocytes account for about 70% of the total liver cell content and execute most hepatic metabolic processes [1]. The remaining liver cells are non-parenchymal cells such as cholangiocytes, liver sinusoidal endothelial cells (LSECs), hepatic stellate cells (HSCs), and resident innate and adaptive immune cells. The main hepatic blood supply (~80%) comes from the gut *via* the portal vein, passes through a network of liver sinusoids, and leaves the parenchyma *via* the central vein. The liver is therefore constantly exposed to metabolites and non-self antigens derived from nutrients and gut microbiota. When pathogens invade the liver, neutrophils are the first responders. They are mobilized from a large bone marrow pool, circulate *via* the blood stream, extravasate into tissue where they immobilize and kill pathogens. Neutrophils act through phagocytosis, production of reactive oxygen species (ROS), release of Neutrophil Extracellular Traps (NET), and secretion of a variety of cytokines. In addition, the antigen-rich portal blood is “probed” by antigen-presenting cells (APCs) and lymphocytes for the presence of non-self compounds as well as for pathogens. In the metabolically healthy liver, this process contributes to the establishment of (antigen-specific) tolerance. This immune tolerant state implies that the liver actively inhibits an overt immune response to harmless food-derived antigens and microbial products. However, to maintain homeostasis, the liver has also to allow efficient responses against infectious agents. APCs, such as dendritic cells (DCs) (represented as plasmacytoid DCs (pDCs) and classical DCs (cDCs)), and Kupffer cells (KCs), the resident hepatic macrophages, are predominantly located in the periportal and pericentral areas. They capture antigens that pass through the liver, process and present them through major histocompatibility complex class-I or II (MHC-I/II) molecules. Immunoglobulin-producing B,

and T lymphocytes are dispersed throughout the parenchyma being present in the portal tracts as well. Various subsets of T cells recognize presented antigens using highly variable T Cell antigen Receptors (TCRs), resulting – in non-inflammatory conditions – in tolerance induction rather than enhancement of T cell responses. In line, in these conditions, antigen presentation to naïve $CD4^+$ T cells induces differentiation of regulatory T cells (Tregs) rather than T helper (Th) 1 (Th1) or 17 (Th17) cells. In addition, $CD8^+$ T cells are highly effective in killing cells infected by intracellular pathogens and damaged cells. Next to conventional $CD4^+$ and $CD8^+$ T cells, non-conventional T cells (natural killer T (NKT) cells) are present in the liver and contribute to immune homeostasis by recognizing specific non-peptide antigens (Figure 1).

In an altered metabolic environment, as occurring in NAFLD, the hepatic immune system can be triggered by 'metabolic overload', leading to an inappropriate immune response. The major risk factor for NAFLD is overnutrition, associated with obesity and type 2 diabetes. Metabolic overload already occurs in early stages of NAFLD characterized by benign steatosis (NAFL) with no evidence of hepatocellular injury [2]. Continuous metabolic overload by high caloric diets generates toxic lipids, such as free cholesterol, free fatty acids, diacylglycerols, and ceramides [3,4]. Nutrient overload causes oxidative and ER stress and hepatocyte apoptosis [5], thereby driving NAFLD progression. The strong association between NAFLD and type 2 diabetes indeed shows that persistent nutrient (glucose) overload affects hepatic cell function. Drastic reduction of caloric intake, such as after bariatric surgery, improves all stages of NAFLD, proving that metabolic overload is a main driver of NAFLD [6]. Metabolic overload first affects hepatic non-immune cells, which, at homeostasis, also exert immune-modulatory functions by participating in antigen presentation contributing to hepatic immune tolerance (Box 1 and 2). Inflammation, the central feature in the progression from

NAFL to NASH is subsequently triggered by metabolic overload and ensuing hepatocyte injury [7]. Pathological NASH develops when fat accumulation in hepatocytes is accompanied by lobular inflammation and hepatocellular ballooning [8,9]. The final stages of NASH are advanced fibrosis/cirrhosis, hepatocellular carcinoma, and liver failure.

In the present review, we will discuss liver immunity at homeostasis and during development and progression of NASH. We will focus on 1) the effects of the altered metabolic environment, predisposing to NASH, on hepatic immune cells, and 2) the role of hepatic immune cells in the development and progression of NASH.

Triggers of the immune response in NAFLD

The altered metabolic environment in NAFLD affects immune cell phenotype and function, hence promoting NASH progression. Indeed, immune cells sense environmental and metabolic cues, such as nutrient and oxygen availability, and microbial metabolites (Box 3), and adapt their metabolic programs accordingly. This part of the review describes the current knowledge about the effects of NAFLD-related alterations in lipid-, glucose-, and cholesterol metabolism on hepatic immune cells.

Lipid metabolism

Hepatic steatosis results from an imbalance between hepatic triglyceride input and output. Hepatic free fatty acid influx rates and *de novo* lipogenesis are increased in NAFLD [10], affecting hepatocytes as well as immune cell function. Accumulation of (toxic) lipids in hepatocytes causes hepatocellular lipotoxicity, which leads to metabolic dysfunction and cellular oxidative and ER stress, and eventually cell death. Injured and dying hepatocytes release apoptotic bodies and extracellular vesicles (EVs) containing RNA, membrane,

cytosolic and nuclear proteins, lipids, and damage-associated molecular patterns (DAMPs) [11], thereby inducing monocyte recruitment and macrophage activation. For example, danger signals, such as histidine-rich glycoprotein (HRG), induce macrophage polarization towards a pro-inflammatory phenotype [12].

Next to hepatocyte-mediated effect of lipids, some studies show a direct effect of lipids on hepatic immune cells. Hepatic macrophages from mice fed a high fat diet display intracellular lipid accumulation which was associated with altered expression of genes involved in lipid synthesis, oxidation, uptake and secretion, and the increased production of inflammatory mediators [13]. Furthermore, *in vitro* assays showed that LPS-mediated activation of these lipid-loaden macrophages promote the recruitment of $CD4^+$ and $CD8^+$ T and B lymphocytes, which is a hallmark of NASH. MRS1, the key macrophage receptor for clearance of circulation lipoproteins, was recently suggested to be critical for the uptake of saturated fatty acids and the subsequent proinflammatory response [14]. These studies do not allow to discriminate between recruited and resident macrophages (or KCs), as the most specific markers for KCs, such as CLEC4F, were not used. However, it is likely that at least some hepatic macrophage subsets can be directly activated by lipid accumulation. More recent studies using single-cell RNAseq have identified distinct macrophage subtypes in livers mice fed a high-fat diet [15,16]. It was suggested that hepatic lipid accumulation promotes the recruitment of a subset of monocyte-derived lipid-associated macrophages (LAMs) – but not resident KCs [15]. Studies on activation of the newly identified macrophage subsets and the potential effects of lipids on their phenotype and function are still awaiting.

Dendritic cells (DCs) are also sensitive to extracellular fatty acids during innate immune activation [17]. High fatty acid concentrations enhance the TLR-mediated innate activation of DCs by inhibiting the activity of hexokinase, the initial enzyme of glycolysis. Consequently,

the glycolytic reprogramming of DCs is impaired and mitochondrial reactive oxygen species (ROS) production is increased. ROS affects the unfolded protein response and induces a specific inflammatory program, characterized by increased IL-23 production [17]. Notably, IL-23 expression is enhanced in livers of mice fed a NASH diet [18]. Finally, a few studies have investigated the effects of specific fatty acids on T cells. Generally, high concentrations of fatty acids are toxic to T cells, and non-toxic concentrations induce proliferation and cytokine production [19–21]. The mechanisms by which fatty acids affect T cell function, especially in NAFLD/NASH, is still unclear and deserves further research.

Glucose metabolism

Hepatic steatosis promotes liver insulin resistance ultimately leading to diabetes, itself a major risk factor for NAFLD. Glucose availability and uptake are critical determinants in directing macrophage differentiation. As DCs, macrophages integrate signals of overnutrition and accordingly reprogram their metabolism [22]. The transcription factor HIF1α plays a key role in the induction of the glycolytic adaptation in inflammatory macrophages. HIF1α expression is regulated both by inflammatory signals, such as pro-inflammatory cytokines, inducing the NF-κB signaling pathway [23], and growth factors, such as GM-CSF, triggering the AKT/mTOR pathway [24,25]. HIF1α, in turn, regulates the expression of glycolytic enzymes and the glucose transporter GLUT1, thereby facilitating rapid glucose uptake. Glucose entry and its metabolization into lactate directly promotes inflammatory macrophage gene expression and activation [26]. Inflammatory macrophages further contribute to hepatic steatosis and inflammation by secretion of cytokines such as TNF-α and IL-1β [27].

Similar as macrophages, T cells are also dependent on the import of extracellular glucose to meet increased metabolic needs during activation. Naïve, resting T cells rely on oxidative phosphorylation to maintain housekeeping functions and cell survival, which is promoted by IL-4 and IL-7 [28]. Upon activation, T cells switch from a quiescent state to proliferative expansion, which requires uptake of essential metabolites to support anabolic growth [29]. T cell activation involves the costimulatory receptor CD28, which induces glucose uptake and glycolysis in T cells to a level that is sufficient for proliferation and function [30]. Glucose availability seems to (partly) control immune cell function since activated immune cells have high metabolic demands. However, more studies are needed to investigate the impact of a nutrient-rich NAFLD environment on immune cell function, not only at the single cell level, but also in cell-cell interactions.

Cholesterol metabolism

Elevated cholesterol levels in hepatocytes are associated with NAFLD risk and severity [4,31]. Cholesterol is synthesized *de novo* from acetyl-CoA or imported *via* lipoprotein uptake. Macrophages are important for cholesterol efflux and reverse cholesterol transport from peripheral tissues to the liver *via* high-density lipoprotein (HDL) particles. LXRα is a critical regulator of cholesterol and lipid metabolism in KCs, and links metabolism and inflammation by reducing the production of pro-inflammatory mediators upon stimulation [32,33]. Moreover, cholesterol is a precursor for bile acid synthesis, and (microbial) metabolites of bile acids were recently shown to control the adaptive immune response in the gut [34–36] (Box 3). However, studies about the effect of specific bile acids on hepatic immunity are lacking. Plasma bile acid levels and profiles are reported to be altered in NAFL and NASH [37–40], and a large inter-individual variation in human bile acid pool size and composition

exists [39]. It will be important to further investigate the signaling functions of bile acids and their metabolites in the control of immune cell phenotypes and functions, including inter-individual differences in adaptive immunity.

Several liver immune cell types are involved in NAFLD onset and progression

NASH is characterized by hepatic inflammatory infiltrates and lobular inflammation. Immune cell profiling and transcriptomic analysis identified a hepatic immune- and inflammation-related gene signature in NASH patients, which reversibly associates with NAFL to NASH transition and is characterized by pathways related to antigen presentation and $CD8^+$ T cell activation [18]. Yet a wide range of hepatic immune cell populations have been associated with NAFL progression to NASH, including macrophages, DCs, neutrophils, natural killer cells, (non-conventional) T cells and B cells.

Macrophages are activated in the early phase of liver injury

KCs, the resident macrophages in the liver, are the most abundant hepatic immune cells accounting for ~35% of the non-parenchymal cells. These cells originate from yolk sac-derived erythromyeloid progenitors in the fetal liver. KCs are enriched in mid regions in human and in periportal regions in mouse livers [41,42] and are located along LSECs in the sinusoids, with a significant part of the KC inside the space of Disse to ensure close contact with hepatocytes, HSCs and LSECs [43]. In the metabolically healthy liver, KCs clear common gut-derived antigens, including bacterial antigens and fungal pathogens, from blood by phagocytosing, processing, and presenting antigens to maintain immune homeostasis [44–47]. Both resident (KC) and infiltrating monocyte-derived macrophages play important roles in the development of hepatic steatosis and NASH. Analysis of liver biopsies of patients with

194 different stages of NAFLD show (portal) macrophage infiltration even at the stage of
195 steatosis, before inflammation or fibrosis develop [48–50]. Macrophages are mainly
196 observed around damaged hepatocytes, where they form hepatic crown-like structures
197 (hCLS) [15,51–53]. In hCLS, activated macrophages aggregate and surround hepatocytes
198 containing large lipid droplets and cholesterol crystals. The number of hCLS positively
199 correlates with liver fibrosis [51], suggesting a pathophysiological role in NASH progression.
200 Moreover, several studies in HFD-fed mice show that (selective) depletion of macrophages
201 by gadolinium chloride ($GdCl_3$) [27,54] or clodronate [55] improves hepatic steatosis,
202 hepatocyte insulin resistance, and inflammation. However, as the employed depletion
203 methods target all types of macrophages, firm conclusions about the exact role of KCs in
204 NASH development cannot be drawn from these studies.
205 Upon liver damage macrophages encompass several heterogeneous populations [56].
206 Resident KCs represent the largest macrophage population in the liver. When activated, KCs
207 express high levels of inflammatory markers and are the major source of liver-derived MCP-1
208 (also known as CCL2), which plays an important role in the progression of simple steatosis to
209 NASH) in HFD-fed mice [57]. The second most abundant population are monocyte-derived
210 infiltrating macrophages which express high levels of LY6C and C-C motif chemokine
211 receptor 2 (CCR2), and acquire macrophage characteristics over time [57]. Bonnardel *et al*.
212 showed, in a KC-specific depletion model, that activated HSCs and LSECs induce the
213 expression of monocyte-attracting chemokines and adhesion molecules [43]. Within a short
214 time-frame – 24 h after KC depletion – monocytes colonize the liver and acquire LXRα and
215 ID3 expression, which are an important part of the KC-specific identity program [58].
216 Expression of LXRα, *via* the DLL4-Notch pathway, and ID3 are respectively induced upon
217 interaction with LSECs and hepatocytes, underlining the contribution of different hepatic cell

types in shaping the KC phenotype. Another study, using a similar KC-specific depletion model, confirmed this rapid differentiation of recruited monocytes and showed that tissue environment signals, such as DLL4, TGF-β and desmosterol, induce differentiation by regulating the expression of KC identity transcription factors [59]. In NAFLD, monocyte-derived macrophages are recruited to the liver by the CCL2/CCR2 axis where they promote NASH development. Indeed, monocyte-derived infiltrating macrophages with a pro-inflammatory phenotype are abundant in HFD-fed mice, in *ob/ob* mice [60] and in humans with NAFLD [61]. Pharmacological inhibition of CCR2 decreased monocyte recruitment in the liver and ameliorated NASH in mice [62–64]. Three recent studies aimed to further identify resident and infiltrating macrophages in mice fed either Western-type (high fat and cholesterol) [65], long term NASH-inducing (high fat, high cholesterol, high fructose) [16], or methionine and choline-deficient (MCD) [66] diets (Box 4). Collectively, these studies showed that chronic steatohepatitis leads to permanent changes in the KC pool, with potential long-lasting impact on liver homeostasis and function, especially on the liver response to lipid overload.

The use of different mouse models and protocols to induce steatosis and NASH (*e.g.* with or without additional fructose, choline-deficiency, study duration) with different phenotypic stages of hepatic NAFLD [67] complicates comparison of the identified populations. These studies also underscore the need for a consensus on the macrophage nomenclature. However, the heterogenous macrophage populations clearly play diverse roles in NASH. Functionally characterizing each subset will be of importance to develop more selective therapeutic (immune-based) strategies.

The various subsets of dendritic cells (DCs) are differentially affected in NASH

NASH is associated with the accumulation of intrahepatic DCs and alterations in the proportions of DC subsets. DCs are the major APCs in the liver and in the metabolically healthy liver they control the induction of antigen-specific adaptive immune responses as well as the induction of central and peripheral tolerance to auto-antigens [68,69]. Hepatic DCs (~1% of the non-parenchymal cells) are represented by three populations: plasmacytoid DCs (pDCs), classical type 1 DCs (cDC1s, expressing XCR1 and CD103), priming the $CD8^+$ T cell response, and classical type 2 DCs (cDC2s, expressing CD11b and Sirpα), priming the $CD4^+$ T cell response [70–72]. At homeostasis, hepatic DCs are mostly immature and tolerogenic [73]. However, upon liver injury, activated DCs migrate to the periportal and pericentral areas and draining lymph nodes, secrete pro-inflammatory cytokines, such as IL-6, IL-12, and TNF-α, and express co-stimulatory molecules CD40, CD80 and CD86 [74,75]. The cDC1 ($XCR1^+$)/cDC2 ($CD172a^+$) ratio is decreased in livers of High Fat, High Cholesterol, High Sucrose diet fed mice, mainly due to a proportional (% of $CD45^+$ cells) increase in cDC2 and a slight reduction in cDC1 cells [18]. Moreover, cDC2 positively associate with NASH, while cDC1 negatively correlate with NASH. Surprisingly, the increase in cDC2 cells is paralleled by an increase in hepatic $CD8^+$ T cells, suggesting that changes in the cDC1/cDC2 ratio might fine tune $CD8^+$ T cell activation, likely through complex mechanisms. Indeed, T cell activation by cDC1 cells is classically MHC-I restricted. However, cDC1 cells also regulate the anti-tumor function of $CD8^+$ T cells through MHC-II-dependent regulation of $CD4^+$ helper T cells [76]. Another study reported elevated numbers of both cDC1 and cDC2 cells in mice fed Western, choline-deficient high fat, or MCD diets, while only the abundance of cDC2 cells was increased in high fat diet-fed or *db/db* and *ob/ob* obese mice [77]. The use of different diets and treatment durations to induce NASH-like pathologies, with variable relatedness to the

human pathology [67,78], likely account for the observed differences in cDC1 and cDC2 abundance and ratio.

Immune cell profiling of blood and liver biopsies from NAFLD patients also revealed a NASH-dependent elevation of $CD141^+$ cells, corresponding to murine cDC1 cells [77]. Furthermore, an enrichment in (activated) migratory $CCR7^+$ DC (mDC) upon NASH induction was observed. In line, increased interactions between cDCs with NASH-associated $CD8^+$ T cells were observed in liver-draining lymph nodes of MCD-diet fed mice. A recent study, using *Batf3*$^{-/-}$ mice, which genetically lack cDC1 cells, identified murine $CD103^+$ cDC1 as a protective DC subtype during NASH development [79]. Adoptive transfer of these cells in *Batf3*$^{-/-}$ mice attenuated inflammation and reduced high sucrose diet-induced metabolic disturbances. Finally, specific cDC1 deletion, using XCR1-Cre^{DTA} transgenic mice, protected mice for MCD-induced NASH [77]. The authors suggested that increased cDC1 numbers cause inflammation-related liver damage (NASH), in part *via* their interaction with T cells, while non-inflammatory accumulation of hepatic lipids (simple steatosis) is associated with increased numbers of cDC2 cells. However, lack of cDC2-specific depletion tools prevented so far the functional assessment of their role in NAFL and/or NASH development.

Infiltrating neutrophils facilitate NASH development

Bone-marrow-derived neutrophils mainly circulate *via* the blood stream for immune surveillance and are activated and attracted by inflammation or infection-activated endothelial cells [80]. Neutrophils infiltrate, as first responders, into tissues where they immobilize and kill pathogens acting through the production of reactive oxygen species (ROS), secretion of a variety of cytokines [81], such as IFNγ, TNF-α, IL-4, and IL-10, and the formation of neutrophil extracellular traps (NETs) [82,83]. NETs are formed when

neutrophils actively release their nuclear DNA content, while still maintaining membrane integrity, chemotaxic responsiveness and phagocytosis [84].

Infiltration of neutrophils, mainly around steatotic hepatocytes and in the portal tract [50], is associated with the early stage of NAFL/NASH in patients and animal models [80,85–88]. Neutrophils contribute to NAFLD progression and the exacerbation of the inflammatory state by the production of neutrophil-derived myeloperoxidase (MPO), which accelerates ROS production and induces DNA damage [52,89]. NET formation (NETosis) also attracts monocyte-derived macrophages and subsequently induces steatosis-to-NASH progression [87]. In NASH patients, circulating neutrophils suppress the proliferation and activation of $CD4^+$ and $CD8^+$ T cells [90]. Over time, this may induce an inadequate immune-surveillance of liver damage, making patients more prone to the progression of liver damage. Although neutrophils are generally believed to promote NASH, they can also play a beneficial role. Indeed, neutrophils highly express microRNA-223, which upon transfer to hepatocytes elicits anti-inflammatory and anti-fibrotic responses [91]. However, antibody-mediated neutrophil depletion improved hepatic inflammation and fibrosis, and ameliorated HSC activation in high fat, high cholesterol-induced NASH in mice, indicating a detrimental role of neutrophils in NASH. Subsequent chow diet feeding, to study the role of neutrophils during (spontaneous) NASH resolution, showed that neutrophil depletion exacerbated fibrosis, HSC activation, and myeloid cell infiltration during resolution, demonstrating that neutrophils also play a pro-resolutive role in liver injury and fibrosis [92].

The role of conventional $CD4^+$ Th1 and Th17 lymphocytes in the contribution to NASH development deserves further study

312 The main hepatic T cell populations in a healthy liver are conventional $CD4^+$ and $CD8^+$ αβ T
313 cells. Upon activation by APCs [93], they play a critical role in the hepatic immune response.
314 Although the contribution of $CD8^+$ T cells to NAFL and NASH development has been recently
315 extensively studied, data about the precise role of $CD4^+$ T cells are limited.
316 $CD4^+$ T helper 1 (Th1) and T helper 17 (Th17) cells are increased in peripheral blood of NAFL
317 [94] and NASH [18,95–98] patients. Th1 cells are important drivers of inflammation and
318 contribute to NAFLD development by secreting IFNγ [99]. Hepatic infiltration of and IL-17A
319 production by Th17 cells increase steatosis, liver injury and fibrosis [98], while Th17
320 depletion in mice protects against NASH development [100]. The role of $CD4^+$ regulatory T
321 cells (Tregs) in NASH development still remains controversial. Tregs are reported to be
322 reduced in high-fat diet-fed mice [101], while choline-deficient high-fat and high-fat high-
323 carbohydrate diets increase intrahepatic Tregs in mice [102,103]. Mechanistically, circulating
324 IL-10^+ Tregs positively correlate with NASH features. However, as IL-10 is considered to be
325 anti-inflammatory, this likely reflects a counter-adaptive immunoregulatory response
326 [18,104]. A better understanding of the contribution of Tregs to NASH development will
327 provide more insight in the hepatic immune regulation upon a metabolic challenge. Finally,
328 currently no studies mechanistically addressed the role of $CD4^+$ T cells in NASH. Recently it
329 was shown that Th2 cells negatively correlate with NASH activity [18], but the role of Th2
330 cells in NASH remains also to be investigated.
331
332 *$CD8^+$ cytotoxic T lymphocytes: the ultimate effectors in NASH?*
333 Livers of high fat (and high cholesterol) and MCD diet fed mice are characterized by a hepatic
334 infiltration of T cells, which is also observed in livers and peripheral blood of NAFL and NASH
335 patients [18,50,105–108]. $CD8^+$ cytotoxic T cells play a key role in NASH progression, as

administration of neutralizing anti-CD8 antibodies decreased hepatic steatosis and inflammation [106,107]. Our previous work in NAFLD patients showed a strong correlation between circulating and hepatic cytotoxic (perforin and IFNγ-expressing) $CD8^+$ T cells and hepatic markers of inflammation in NASH [18]. Moreover, the cytotoxic potential of $CD8^+$ T cells in NASH was increased as illustrated by the increased expression of perforin, a pore-forming protein, and the cytotoxic proteins granzyme A, a tryptase, and granzyme B, a serine protease, which are key for the effector function of cytotoxic T cells. Regression of NASH-specific features, such as lobular inflammation and ballooning, correlated with a decrease in cytotoxic $CD8^+$ T cells [18]. In line, Dudek *et al.* reported hepatic accumulation of tissue resident ($CXCR6^+$) $CD8^+$ T cells in patients with NASH and in choline-deficient high-fat diet-fed mice, which contribute to liver damage [109]. These short-lived tissue-resident $CXCR6^+$ $CD8^+$ T cells co-express programmed cell death protein 1 (PD1), which is associated with exhaustion. Furthermore, they express genes associated with apoptosis and with effector function, such as granzyme B, Tox, TNF-α, and IFNγ. In the healthy liver, CXCR6 expression in $CD8^+$ T cells is inhibited by FOXO1. However, in NASH patients and mouse models of NASH, serum levels of the FOXO1-inhibitor IL-15 are increased, resulting in a higher expression of CXCR6 on T cells. In an environment with specific stimuli, such as TNF-α or acetate – a short-chain fatty acid which is elevated in NASH livers -, $CXCR6^+$ $CD8^+$ T cells acquire the potential of auto-aggression [109]. $CXCR6^+$ $CD8^+$ T cells were shown to contribute to liver damage by non-specific, MHC-I independent, killing of hepatocytes. This was triggered by ATP released from dying hepatocytes which, *via* the purinergic receptor P2X7, enhances calcium influx in $CXCR6^+$ $CD8^+$ T cells initiating auto-aggression. Tissue damage due to auto-aggression by $CD8^+$ T cells, and the upregulation of PD1 in these cells, can promote the development of liver cancer upon chronic liver damage [110–112]. Although the activation of auto-aggressive

360 $CXCR6^+$ $CD8^+$ T cells is antigen-independent, the strong association between elevated hepatic
361 $CD8^+$ T and cDC1 cells, as discussed above, also points toward a likely role of antigen
362 presentation in $CD8^+$ T cell activation in NASH [18,77] (Figure 4). Future studies will be
363 needed to further elucidate the respective contribution of antigen (in)dependent stimulated
364 $CD8^+$ T cells in the progression of NAFL to NASH.
365 Interestingly, the metabolic environment affects both $CD8^+$ T cell phenotype and function in
366 NASH development. Hepatic $CD8^+$ T cells in Western diet-fed Low Density Lipoprotein
367 Receptor-deficient mice, which display an obese/hyperlipidemic NASH phenotype, express
368 IL-10, TNF-α, and IL-6, and induce hepatic inflammation, macrophage accumulation and HSC
369 activation [113]. Although IL-10 has anti-inflammatory properties, it was hypothesized that
370 IL-10 secretion from $CD8^+$ T cells promotes $CD8^+$ T cell survival and TIMP-1 expression in
371 macrophages, hence stimulating NASH progression. Whereas $CD8^+$ T cells are the primary
372 regulators of HSC activation selectively in an obese/hyperlipidemic environment, $CD8^+$ T cells
373 play a minimal role in NASH development in non-obese animals [113]. Indeed, hepatic $CD8^+$
374 T cells in a lean NASH model, i.e. MCD diet-fed mice, exhibited a different phenotype and
375 expressed low levels of IL-10, IFNγ, and granzyme B. Moreover, these $CD8^+$ T cells did not
376 impact on NASH-associated inflammation nor HSC activation. These findings, indicating that
377 metabolic conditions influence $CD8^+$ T cell phenotype and function, deserve further
378 investigation in additional, more relevant (non-)obese NASH models.
379
380 *The role of non-conventional T lymphocytes in NASH is understudied*
381 The healthy liver contains, next to innate lymphoid cells (Box 5), a substantial number of
382 non-conventional T cells: natural killer T (NKT) cells, mucosal-associated invariant T (MAIT)
383 cells and γδ T cells. NKT cells are the most abundant hepatic non-conventional T cells in mice

[114]. Two types of NKT cells are found: type I NKT cells expressing a semi-invariant TCR (invariant (i)NKT cells), and type II NKT cells, expressing a diverse TCR repertoire [115]. NKT cells recognize lipid antigens presented by CD1d and can rapidly respond by secreting multiple cytokines (IFNγ, IL-4, IL-10 and IL-17A) [116]. CD1d is an MHC-I-like molecule that is reactive to self-derived or microbial lipid antigens, including glycolipids and phospholipids [114]. As CD1d is expressed by antigen presenting cells, such as DCs and KCs [117], NKT cells form a bridge between the innate and adaptive immune systems. NKT cell numbers change during the onset of NAFL and progression to NASH. A reduction in hepatic NKT cell numbers was reported in mouse models on short-term MCD, choline-deficient L-amino acid-deficient (CDAA), or high-fat diets [118,119]. Decreases in hepatic NKT cell numbers can be due to steatosis-induced KC-derived IL-12 and Tim-3/Gal-9 signaling, which promotes NKT cell apoptosis. By contrast, NKT cell numbers increase in advanced NASH. Mice fed a high fat, high cholesterol diet for 16 weeks showed increased NKT cells in liver [106]. NKT cell depletion protected against NASH progression, as these mice exhibited lower NAFLD activity scores (NAS) and AST/ALT levels, less hepatic infiltrating macrophages and decreased expression of genes related to fibrosis [106]. Similarly, a study combining choline-deficiency with a high fat diet for 6 months showed a significant increase in hepatic NKT cells [107]. A possible explanation for the increase in NKT cell numbers is the activation of the Hedgehog pathway in NASH. This results in higher levels of the NKT cell chemokine CXCL16, the adhesion molecule VCAM-1, and IL-15, all promoting NKT viability [120–122]. Several NKT-operated mechanisms can contribute to NASH progression. Activated intrahepatic NKT cells enhance hepatocyte lipid uptake *via* secretion of LIGHT, thus promoting steatosis [107]. Moreover, NKT cells produce IL-17 and IL-22 at the beginning of the steatosis phase, becoming IFNγ/IL-4/IL-13- secreting cells at a later stage of the disease [123]. The differential

activation and cytokine secretion by NKT cells results in an evolving immune response during disease progression. Moreover, the synergistic action of NKT and $CD8^+$ T cells contributes to inflammation and fibrosis [106,123].

MAIT cells are a population of innate-like T lymphocytes that recognize a limited array of microbial-derived antigens and vitamin B metabolites presented by MHCI-I-related molecule 1 (MR1) [124]. In the metabolically healthy liver, they are important for antimicrobial immunity against bacteria and yeast, and exert cellular cytotoxicity against target cells by producing pro-inflammatory cytokines such as IFNγ and TNF-α [125]. MAIT cells accelerate inflammation and fibrosis in models of CCL_4- or bile duct ligation-induced liver injury and accumulate in livers of NASH patients [126]. By contrast, MAIT cells were found to be protective against liver inflammation in MCD diet-induced NASH models [127]. Beyond the fact that very different experimental models were studied, it is difficult to speculate about possible mechanisms accounting for these opposing results.

γδ T cells express a TCR γ-chain and a δ-chain and are a heterogenous population consisting of multiple subsets. They function as the first line of immune defense against infections and cancer *via* cytotoxic effects and production of IFNγ and IL-17 [128,129]. The liver contains γδ T cell precursors, which primarily develop into $IFN\gamma^+$ γδ T cells, but their role in immune homeostasis and NAFL/NASH progression still needs to be determined [129].

B lymphocytes and antibody production promotes NASH development

B cells are the largest population of hepatic lymphocytes (~20% of all $CD45^+$ cells in mice) [130] and are important for both humoral immunity, by producing antibodies, and cellular immunity, by functioning as APCs. They are located close to the bile ducts and are reported to function as APCs for MAIT cells by presenting gut-derived bacterial antigens *via* MR-1

[131,132]. Most hepatic B cells are mature follicular B cells, which survey for antigens. In the human liver, immature, “classical” B2, “non-conventional” B1, and antibody-producing plasma cells have also been detected, although their precise function at steady state is unclear [133,134].

Growing evidence suggests an essential role for B cells in NAFL and NASH development. B cells accumulate in the portal tract of livers of patients with NASH [50,135], and serum levels of IgA [136] and B cell-activating factor (BAFF), promoting B cell differentiation and survival, are increased in NASH patients [137]. Accumulated B cells in livers of HFD- or NASH-diet fed mice are pro-inflammatory and secrete cytokines such as IL-6 and TNF-α [137–139]. The (innate) TLR-associated Myeloid Differentiation primary response protein 88 (MyD88) pathway is required for B cell activation and maturation in NASH [139] and for their pro-fibrotic role, as shown in a mouse model with CCl_4-induced liver fibrosis [140]. Moreover, microbiota-derived metabolites draining into the liver as a result of increased gut permeability activate intrahepatic B cells during NASH [139].

B2 cells mature towards IgG producing plasma cells, that promote the presentation of oxidative stress-derived antigens to $CD4^+$ T cells and support NASH progression [135]. IgA producing plasma cells also contribute to NASH progression, as mice lacking IgA developed less liver injury and fibrosis upon a HFD [141]. Moreover, B cell-harboring, but antibody-deficient mice (*IgMi* mice) fed a high fat diet were protected from the development of hepatic steatosis and inflammation [142], suggesting a role for ‘pathogenic’ antibodies. Altogether, while hepatic B cell infiltration in NASH is established, the exact activation mechanisms and effector functions are yet to be determined.

Concluding remarks; an integrated and perspective view of immune dysregulation in NASH

NAFL and its progression towards NASH are closely associated with environmental factors, such as overnutrition, and with dysregulation of energy metabolism, such as insulin resistance and obesity. Therefore, dietary and lifestyle interventions are the primary strategy to reduce the prevalence of this most common liver disease. Weight loss improves hepatic steatosis, and there is evidence that diets low in saturated and high in monounsaturated fats and dietary fiber (Mediterranean diet) are beneficial in NAFLD [10,143]. Lowering hepatic steatosis also decreases the risk for progression towards NASH. The fact that bariatric surgery and subsequent great and sustained weight loss can reverse pathological liver features in all stages of NAFLD, including fibrosis [6], proves that energy balance control is beneficial in NAFLD. Still, pharmacological therapies are eagerly awaited and several clinical trials are running to evaluate the effectiveness of different pharmacological targets on the reversal of NASH and/or fibrosis, assessing histological steatosis, hepatocyte ballooning, lobular inflammation and fibrosis stage.

Inflammation is considered a key element of disease progression. The presence of a wide diversity of immune cells in NAFLD livers and their emerging roles in the progression to NASH makes the hepatic immune compartment an attractive target for therapeutic approaches. However, the complexity of different immune cell subsets in the sequential NAFL/NASH stages, and the knowledge gap about cell-cell interactions modifying disease progression, complicates the development of new therapies. An integration of the current knowledge will highlight unanswered questions on the role of immune cells in NASH, which need to be investigated in the future to develop specific immune-modulatory therapeutics for NAFLD (Figure 5, Outstanding questions).

Accumulation of hepatic fat (steatosis), as occurs in energy substrate overload conditions such as diabetes, induces hepatocyte (mitochondrial, oxidative and ER) stress, which

promotes the progression of NAFL towards NASH. Due to the altered metabolic environment, these damaged, necrotic or apoptotic hepatocytes initially induce the recruitment of monocytes, *via* the CCL2/CCR2 axis or other, yet unknown, metabolic and tissue environmental signals. The recruited monocyte-derived macrophages replace resident KCs, whose abundance decreases due to impaired self-renewal. Phenotypically diverse macrophage populations are present in NASH, but how dysregulation of the hepatic macrophage compartment impacts NASH development is yet unknown. Importantly, macrophages are the most abundant hepatic immune cells, but the impact of their altered phenotype on the immune response in NASH still remains to be investigated. It is tempting to speculate that alterations in macrophage subtypes affect antigen presentation, and/or DC and T cell function and phenotype, which are modified in NASH. DC abundance increases in NASH livers, whereas the cDC1/cDC2 ratio decreases. The exact mechanism behind the altered cDC1/cDC2 ratio, as well as its effect on $CD8^+$ T cell activation and the function of cDC2 in NASH are still unknown. However, cDC1 cells interact with $CD8^+$ T cells in NASH, enhancing their inflammatory signature. Moreover, antigen presentation by DCs contributes to the increase in (proinflammatory) Th1 and Th17 $CD4^+$ T cells. As DCs are important hepatic antigen presenting cells, the impact of the altered cDC1/cDC2 ratio in NASH development awaits further investigation. Alterations in $CD4^+$ T cell abundance may subsequently affect the activation B cells, whose abundance is increased in NASH livers. In turn, activated B cells secrete proinflammatory cytokines, such as TNF-α and IL-6, as well as IgA and IgG, and recruit T cells. The exact role of B cells in NASH development, the potential differential role of various B cell subsets, and the possible antigen specificity in NASH remain to be investigated. In a NASH environment, $CD8^+$ T cells acquire the ability to kill hepatocytes, and to activate HSC, hence likely enhancing fibrogenesis. Moreover, their

504 synergistic interaction with NKT cells also promotes hepatic inflammation and fibrosis. Of
505 note, NKT cell abundance is increased in NASH, probably due to IL-15 and CXCL16 secreted
506 by macrophages. LIGHT secretion by NKT cells further enhances hepatocyte lipid
507 accumulation, thus further promoting hepatic steatosis and contributing to NAFLD
508 progression. The cellular interaction between NKT cells and hepatocytes shows integrated
509 communication between parenchymal cells and the immune compartment. As described,
510 major hepatic non-immune cell populations do play an important role in hepatic immunity.
511 This role is fairly well documented at metabolic homeostasis, but information in a
512 metabolically altered context is largely lacking. Further in depth understanding of the
513 important, complex roles of both hepatic immune cells and parenchymal cells in NAFLD
514 progression is necessary for a better understanding of the pathophysiology of this disease
515 and the subsequent development of effective therapeutic strategies.
516
517 *Targeting cDC-T cell interactions: an immune based therapeutic perspective in NASH.*
518 Major recent publications [18,77,109,112] have highlighted the key association of cDCs and
519 $CD8^+$ T cells upon progression of NAFL to NASH. While aberrant activation of $CD8^+$ T cells and
520 development of an auto-aggressive exhausted phenotype appeared independent of antigen
521 presentation, recent data from mice fed a MCD diet to induce NASH show that (mature or
522 migratory) cDC1 and $CD8^+$ T cells physically interact in liver-draining lymph nodes [77]. Since
523 physical interactions are a prerequisite for antigen presentation, it is likely that
524 (conventional) antigen presentation to $CD8^+$ T cells takes place and plays a yet to be
525 established role in NASH development. In line, cDC1 depletion significantly decreased NASH
526 development, and immune cell infiltration, suggesting a functional role for these cells in
527 NASH pathology. Finally, indoleamine 2,3-dioxygenase 1 (IDO1) expressing cDC1 cells

correlated with a prognostic liver signature of NAFLD progression. Inhibition of IDO1, the rate-limiting enzyme that converts L-tryptophan to L-kynurenine, in an *in vitro* system decreased the expression of several of the prognostic NAFLD liver signature genes [144]. While the molecular identification of (a) NASH-specific antigen(s) is awaiting, these data strongly suggest that blocking cDC-$CD8^+$ T cell interactions and thus antigen presentation might represent a promising therapeutic strategy in NASH. Moreover, identification of NASH-specific antigens could open new options for antigen-specific based therapies.

Acknowledgements

This work was supported in part by grants from ANR (EGID ANR-10-LABX-0046, ANR-16-RHUS-0006 PreciNASH and ANR-18-CE15-0024-03 (NASHILCCD8)) (to BS & DD). BS is a recipient of an Advanced European Research Council (ERC) grant (number 694717).

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Text boxes

Box 1 – Hepatocytes exert an immune function at homeostasis

While most antigens in the liver are taken up and processed by non-parenchymal APCs, parenchymal cells also possess antigen presentation capacities. Hepatocytes are lined by LSECs, which provide an hepatic barrier to protect the hepatocytes against portal-delivered gut-derived pathogens. However, hepatocytes are accessible for naïve $CD8^+$ T lymphocytes *via* T cell cytoplasmic extensions penetrating the endothelial fenestrations that perforate the LSECs [145,146]. This is of importance for immune surveillance and, for example, for the detection of Hepatitis B Virus-infected hepatocytes by virus-specific $CD8^+$ T cells [146]. Hepatocytes constitutively express MHC-I molecules allowing them to cross-present antigens and to prime naïve $CD8^+$ T cells directly [147], or *via* LSEC-mediated cross-presentation [148]. In a healthy liver, hepatocyte activation of $CD8^+$ T cells generally leads to an abortive immune response and early T cell apoptosis [149], maintaining liver tolerance.

Box 2 – LSEC, cholangiocytes, and HSC act as antigen presenting cells at homeostasis

Liver sinusoidal endothelial cells (LSECs) are, contrary to hepatocytes, in direct contact with the blood. Their numerous fenestrae and absence of basal membrane allow efficient filtration from the sinusoidal lumen to the space of Disse [150]. LSECs play an important role in the clearance of circulating microorganisms [151,152] and molecules [153], as well as in mediating immune tolerance [154] under physiological conditions. Antigens that are taken up and processed by LSECs can be presented *via* MHC-II to $CD4^+$ T cells, and *via* MHC-I to naïve $CD8^+$ T cells. Priming of naïve $CD4^+$ T cells mainly induces the development of $CD4^+CD25^+Foxp3^+$ Tregs [155], which is an important mechanism for maintaining hepatic tolerance. Antigen presentation by LSECs to $CD8^+$ T cells results – at low antigen

concentrations – in the downregulation of IFNγ and IL-2 cytokine production by $CD8^+$ T cells, leading to antigen-specific T cell tolerance [156,157].

Hepatic stellate cells (HSCs) constitute 8-10% of total liver cells. HSCs are located at the space of Disse, between the hepatocytes and the LSECs. In a healthy liver, HSCs are quiescent, serve as the primary storage site for vitamin A, and are essential in the regulation of retinoic acid homeostasis. Moreover, they are the main collagen- and extracellular matrix producing cells in liver fibrosis. Recent studies have also identified a role of HSCs in hepatic immunology. Antigen uptake and presentation by HSCs in mice is low [158], although they express MHC-II, costimulatory molecules such as CD40 and CD80 [159,160], and they produce various growth factors, cytokines [161] and chemokines [162]. A recent study using single-cell RNA sequencing identified two HSC subpopulations in human liver with one of them displaying a gene signature that is reminiscent of antigen-presenting cells [163]. Although it has been suggested that HSCs can present antigens to T cells [159,164], this function is still controversial. However, HSCs do contribute to hepatic immune tolerance *via* indirect priming of DC-activated Tregs [165,166]. Treg induction and Th17 inhibition is mediated through the release of retinoic acid [167] during priming of T cells by DCs [165]. At homeostasis HSCs thus regulate hepatic immune reactivity mainly indirectly by modulating the outcome of T cell activation by other APCs.

Cholangiocytes, or biliary epithelial cells (BECs), line the bile duct and are constantly exposed to bile content. Apart from their function in the regulation of bile flow, composition, and pH, they also play a role in antigen presentation. On the apical side, BECs are exposed to bile and (lipid) antigens present in bile. On the basolateral side, BECs are in close contact with hepatic immune cells. Disorders associated with immune damage of the bile ducts, such as primary biliary cholangitis (PBC) [168,169], are associated with increased basolateral MHC-II

1032 expression by BECs, although *in vitro* assays failed to demonstrate $CD4^+$ T cell-activation
1033 [170]. BECs also constitutively express MHC-I molecules [171] and are capable of presenting
1034 antigens *via* MR1 [131] and CD1d [172] to unconventional T cells such as MAIT and NKT cells.
1035 NKT cells react to lipid antigens presented by CD1d expressed on the basolateral side, and *in*
1036 *vitro* studies have shown that murine and human cholangiocyte cell lines are able to activate
1037 NKT cells upon exposure to lipids and endogenous antigens [172]. Interestingly, CD1d
1038 expression was reported to be a characteristic of healthy BECs, being downregulated in PBC
1039 and alcoholic cirrhosis. This suggests that CD1d-dependent antigen presentation and the
1040 potential ensuing activation of NKT cells is important to maintain immune tolerance in a
1041 healthy liver. However, more studies are needed to fully investigate the role of BECs in
1042 hepatic immune homeostasis.
1043
1044 **Box 3 – Microbial metabolites trigger the immune response**
1045 The gut microbiota is increasingly recognized to contribute to NAFLD development and
1046 progression towards fibrosis [173,174]. Diet-induced microbial dysbiosis damages the gut-
1047 vasculature barrier function [175]. Bacterial translocation into the blood and subsequently in
1048 the liver leads to hepatic inflammation and NASH development [175,176]. Moreover,
1049 microbial-derived compounds, such as short-chain fatty acids, branched-chain amino acids,
1050 and secondary bile acids, affect the pathogenesis of NAFLD [177]. Interestingly, three recent
1051 studies show that (microbial) metabolites of bile acids, which are produced from hepatic
1052 cholesterol, control the adaptive immune response in the gut [34–36] (Figure 2). A bile acid
1053 metabolite library screen for T cell modulatory effects identified two derivatives of
1054 lithocholic acid (LCA) which affect Th17 and Treg differentiation [35]. 3-oxoLCA, on the one
1055 hand, inhibits Th17 differentiation by physically interacting with the transcription factor

retinoic acid receptor-related orphan receptor γt (RORγt). RORγt is a key transcription factor required for induction of IL-17 transcription [178]. 3-oxoLCA inhibits its transcriptional activity and thus Th17 differentiation. Conversely, isoalloLCA promotes Treg differentiation by enhancing mitochondrial ROS production and increasing histone acetylation at the FoxP3 promoter region. Overall, the LCA metabolites 3-oxoLCA and isoalloLCA exert anti-inflammatory actions by directly affecting $CD4^+$ T cell differentiation [179]. Similarly, Song et al. reported that bile acids induce (immunosuppressive) RORγt-expressing Tregs residing in the lamina propria of the colon [36]. The authors speculate that the most abundant intestinal primary bile acid species, such as cholic acid and chenodeoxycholic acid, act *via* the vitamin D receptor (VDR) to maintain a healthy colonic $ROR\gamma t^+$ Treg pool (Figure 2). Recently, the secondary bile acid isoDCA was found to indirectly induce peripheral Tregs in the colon by inhibiting FXR activity in DCs [34]. Collectively, these studies identify an immune suppressive role for bile acid metabolites in the control of the adaptive immune response in the colon. Although these studies are not performed in a NASH context and do not directly relate to the hepatic immune system, they underline the important immune regulatory function of bile acids (and metabolites). NASH is associated with changes in bile acid metabolism and pool composition and altered plasma bile acid levels and profiles [37]. However, so far studies about the effect of altered bile acid profiles or specific bile acid species on hepatic immunity and NASH development are lacking.

Box 4 - Macrophages in NASH: beyond the M1/M2 paradigm

As for macrophages in other tissues, in the past, KCs were subdivided into M1 (classically activated, immunogenic) and M2 (alternatively activated, tolerogenic) cells. However, it is increasingly recognized that KCs exhibit a high plasticity, are highly dynamic and can

differentiate into a wide range of polarization or activation states. Depending on the context, KCs exhibit specific metabolic signatures [180] and M1 and M2 KCs only represent the extreme phenotypes of a wide spectrum.

Upon Western diet feeding, 4 abundant macrophage populations were identified in the liver, including resident KCs and 3 subsets of recruited macrophages: monocyte-derived KCs, macrophages becoming monocyte-derived KCs (termed pre-moKCs), and hepatic lipid-associated macrophages (LAMs). In agreement with other studies [15], resident KCs were gradually lost during NAFLD progression and replaced by monocyte-derived KCs. Interestingly, monocyte-derived KCs resembled resident KCs, while the transcriptome of hepatic LAMs largely differed from that of resident KCs. LAMs expressed lower levels of immune activation-associated genes, such as Il18, Fpr2, Tlr4 and CD38, and had the distinct ability to metabolize lipids. LAMs were only identified in later stages of NAFLD and express Spp1 (next to Trem2, Cd9, Cd63, and Gpnmb), encoding the chemokine osteopontin. Osteopontin was found to be a good biomarker of NASH [181] and is thought to contribute to fibrosis [182]. Moreover, Daemen et al. showed that LAMs preferentially localize to HSC expansion regions, where they aggregate into hepatic crown-like structures (hCLS) [15], suggesting a role for hepatic LAMs in NASH development and liver fibrosis. Another recent study identified low numbers of LAMs already in periportal regions of healthy livers, and showed accumulation of more mature LAMs in pericentral regions in steatotic mouse livers [42]. The exact function of LAMs in the NASH liver is as yet unclear.

A more detailed sc-RNAseq analysis based on transcriptional differences identified 5 macrophage clusters in mice fed control or NASH diets [16]: healthy KCs, NASH KCs, recruited macrophages, LY6C^{hi} recruited macrophages and LY6C^{lo} recruited macrophages (Figure 3). NASH KCs and recruited macrophages were predominantly found in NASH livers,

while healthy livers consisted almost entirely of healthy KCs. Both LY6C$^{hi\&lo}$ recruited macrophages were also predominantly found in NASH livers and differed in gene expression and zonation from recruited macrophages. While recruited macrophages were mainly located in the liver sinusoid, in contact with the LSECs, LY6C$^{hi\&lo}$ recruited macrophages reside proximal to the large portal and central vein vessels in contact with vascular endothelium. These observations underline that disease-promoting metabolic and tissue environmental signals can instruct macrophages to acquire distinct gene expression programs. Mechanistically, MCD diet-induced NASH was associated with impaired KC self-renewal and disease progression with increased recruitment of monocyte-derived KCs [66]. Moreover, death of NASH KCs, possibly induced by increased ER stress, modifies the KC niche and enables repopulation by monocyte-derived KCs. Monocyte-derived KCs are enriched in genes involved in cellular stress and activation, limited hepatic triglyceride storage and increased inflammation.

Box 5 – Hepatic innate lymphoid cells in NAFLD: a controversial role

Liver innate lymphoid cells mostly include natural killer (NK) cells and closely-related group 1 lymphoid cells (ILC1s). They both lack antigen-specific receptors and produce the signature cytokine IFNγ [114,183] in response to IL-12, IL-15, or IL-18 secreted by DCs, macrophages and T lymphocytes [184,185]. Mouse hepatic NK, unlike ILC1, require the transcription factor EOMES for their development, while ILC1 express the transcription factor Zpf683. Moreover, the development of hepatic ILC1s from its liver-resident progenitor cells is promoted by IFNγ production by ILC1s themselves [186].

In the metabolically healthy liver, NK cells rapidly recognize and kill viral-infected or tumor-transformed cells in the absence of specific immunization by releasing perforin and

granzymes [185]. Hepatic ILC1s exhibit cytotoxicity mediated through granzyme A [187] and B, perforin, TRAIL, and FasL [188]. By expressing inhibitory receptors, such as PD-1 (programmed cell death-1) and lymphocyte-activation protein-3 (LAG-3), ILC1s regulate the anti-viral activity of hepatic T cells [189], thereby preventing hepatic virus infection.

Conflicting data exist on changes in ILC1 and NK cell numbers and proportions in blood and livers of NAFL/NASH patients [190–192] and in livers of mouse NAFLD models [193–196], probably due to differences in disease stage and the use of different NASH-inducing protocols. On the one hand, NK cells have a detrimental role in NAFL to NASH progression as TRAIL-producing NK cells were shown to promote a pro-inflammatory environment at an early NAFLD stage [197]. On the other hand, NK cells were reported to exert anti-fibrotic effects [193,195] and to actively contribute to limiting NASH-associated tissue damage and fibrosis by killing activated HSCs, the main collagen producing cells in fibrosis [198]. A better characterization of (tissue resident) NK cells and the seemingly controversial role of NK cells as contributors or modulators of NASH progression should be further investigated.

Figure legends

Figure 1. Hepatic immunity in healthy liver.

At homeostasis the liver exhibits an immune tolerant state, while it also allows efficient responses against infectious agents. LSECs prime naïve $CD4^+$ T cells and induce Treg differentiation. KCs are important to maintain immune homeostasis by clearing common gut-derived antigens. $CD8^+$ effector T cells scan and recognize MHC-I/peptide complexes presented by hepatocytes to respond to intracellular pathogens and damaged cells.

BEC; biliary epithelial cells, MHC-I/II; major histocompatibility complex, class I/II, TCR; T cell receptor, HSC; hepatic stellate cell, KC; Kupffer cell, neutro; neutrophils, Treg; regulatory T cell, CD4; $CD4^+$ T cell, CD8; $CD8^+$ T cell, NKT; natural killer T cell, pDC; plasmacytoid dendritic cell, cDC; conventional dendritic cell.

Figure 2. The immune suppressive role of bile acids and their metabolites affect T cell homeostasis in the colonic lamina propria and control the adaptive immune response.

The most abundant primary bile acids (CA and CDCA) maintain a healthy colonic RORγt Treg pool *via* VDR signaling. 3-oxo-LCA, which is formed by the gut microbiota, inhibits Th17 differentiation by physical interaction with RORγt. IsoalloLCA promotes Treg differentiation by enhancing mitoROS and FoxP3 acetylation. isoDCA indirectly induces pTreg generation by inhibiting FXR transcriptional activity in DCs.

CA; cholic acid, CDCA; chenodeoxycholic acid, 3-oxo-LCA; 3-oxo-litocholic acid, isoalloLCA; isoallolitocholic acid, isoDCA; isodeoxycholic acid, mitoROS; mitochondrial reactive oxygen species, VDR; vitamin D receptor, RORγt; retinoid-related orphan receptor γt, FoxP3; forkhead box P3, FXR; farnesoid X receptor, Treg; regulatory T cell, Th17; T helper 17, pTreg; peripheral Treg.

Figure 3. Kupffer cell populations in healthy and NASH livers, according to Seidman *et al.* [16] and Bonnardel *et al.* [43]

KC-H cells are enriched in healthy liver. KC-H loss upon NASH-induced liver injury, most probably due to apoptosis, induces TNFα and IL-1-dependent receptor activation of HSCs and LSECs. VCAM, SEL-E, and CCL2 upregulation results in monocyte recruitment. LSEC DLL4 expression drives monocyte differentiation and supports niche specialization via the DLL4-Notch pathway. KC-hepatocyte interactions induce ID3 expression, LSECs and HSCs induce LXRα expression. 4 macrophage populations are enriched in NASH liver: KC-N, KN-RM (both localized in liver sinusoid), Ly6C^{hi}-RM, and Ly6C^{lo}-RM (both localized around portal and central vein vasculature).

HSC; hepatic stellate cell, TNFα; tumor necrosis factor α, IL-1; interleukin-1, VCAM; vascular cell adhesion molecule, SEL-E; selectin-E, DLL4; delta like canonical notch ligand 4, LXRα; liver X receptor alpha, ID3; inhibitor of differentiation 3, KC-H; healthy Kupffer cells, KC-N; NASH-Kupffer cells, KN-RM; recruited macrophages, Ly6C^{hi}-RM; Ly6C^{hi} recruited macrophages, Ly6C^{lo}-RM; Ly6C^{lo} recruited macrophages.

Figure 4. Cytotoxic CD8^{+} T cells in NASH.

(a) IL-15-driven transcriptional (re)programming and metabolic stimuli promote auto-aggressive CXCR6^{+} CD8+ T cells displaying enhanced expression of PD1 and GZMB. Auto-aggression is MHC-I-independent, as the purinergic receptor P2X7 responds to ATP released from dying hepatocytes. Calcium influx is enhanced to initiate auto-aggression. (b) GZMA and perforin expressing CD8^{+} T cells associate with NASH. The link between the altered cDC1/cDC2 ratio and antigen dependent CD8^{+} T cell activation remains to be investigated.

GZMA; granzyme A, GZMB; granzyme B, PD1; programmed cell death protein 1, CXCR6; C-X-C motif chemokine receptor 6, IL-15; interleukin 15, FOXO1; forkhead box O1, P2X7; purinergic receptor P2X 7, MHC-I; major histocompatibility complex, class I, TCR; T cell receptor, cDC1; conventional dendritic cells 1, cDC2; conventional dendritic cells 2.

Figure 5. Immune dysregulation in NASH liver.

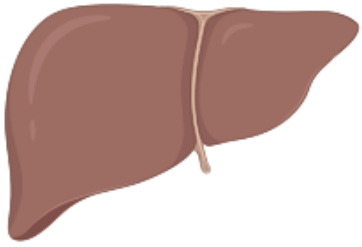
Macrophages are recruited *via* the CCL2/CCR2 axis or by other (environmental) signals and replace resident KCs. DC abundance increases in NASH livers and cDC1 cells enhance the inflammatory signature of $CD8^+$ T cells. The cDC1/cDC2 ratio decreases, but its effect on $CD4^+$ and $CD8^+$ T cell activation and the function of cDC2 in NASH is unknown. B cells secrete proinflammatory cytokines, such as TNFa and IL-6, as well as IgA, although the exact role of B cells in NASH remains to be investigated. $CD8^+$ T cells in a NASH environment acquire the ability to kill hepatocytes, and to induce HSC activation, thereby enhancing the development of fibrosis. The synergistic action of $CD8^+$ T cells with NKT cells might lead to NASH progression. LIGHT secretion by NKT cells enhances hepatocyte lipid accumulation, thus promoting hepatic steatosis, while the secretion of proinflammatory cytokines, such as IFNγ, may recruit and activate other immune cell types, enhancing immune dysregulation and NASH progression. NASH-related metabolites, such as excess glucose and fatty acids and increase in plasma bile acid levels also affects phenotype and function of the hepatic immune compartment.

HSC; hepatic stellate cells, CCL2; C-C motif chemokine ligand 2, CCR2; C-C motif chemokine receptor 2, ATP; adenosinetriphosphate, NKT; natural killer T cells, IFNγ; interferon γ, IL; interleukin, MPO; myeloperoxidase, NETosis; NET (neutrophil extracellular traps) formation, CXCR6; C-X-C motif chemokine receptor 6, cDC1; classical type 1 DCs, cDC2; classical type 2

DCs, DCs; dendritic cells, moKC; monocyte-derived Kupffer cells, Th1; T helper 1, Th17; T helper 17, IgA; immunoglobuline A, TNFα; tumor necrosis factor α, ER stress; endoplasmic reticulum stress, KC; Kupffer cells, FA; fatty acids, chol; cholesterol, BA; bile acids.

Figures are created with BioRender.com

Figure 1



- Immune tolerant state
- Efficient response against infectious agents
- T cell tolerance induction
- Regulatory T cell development

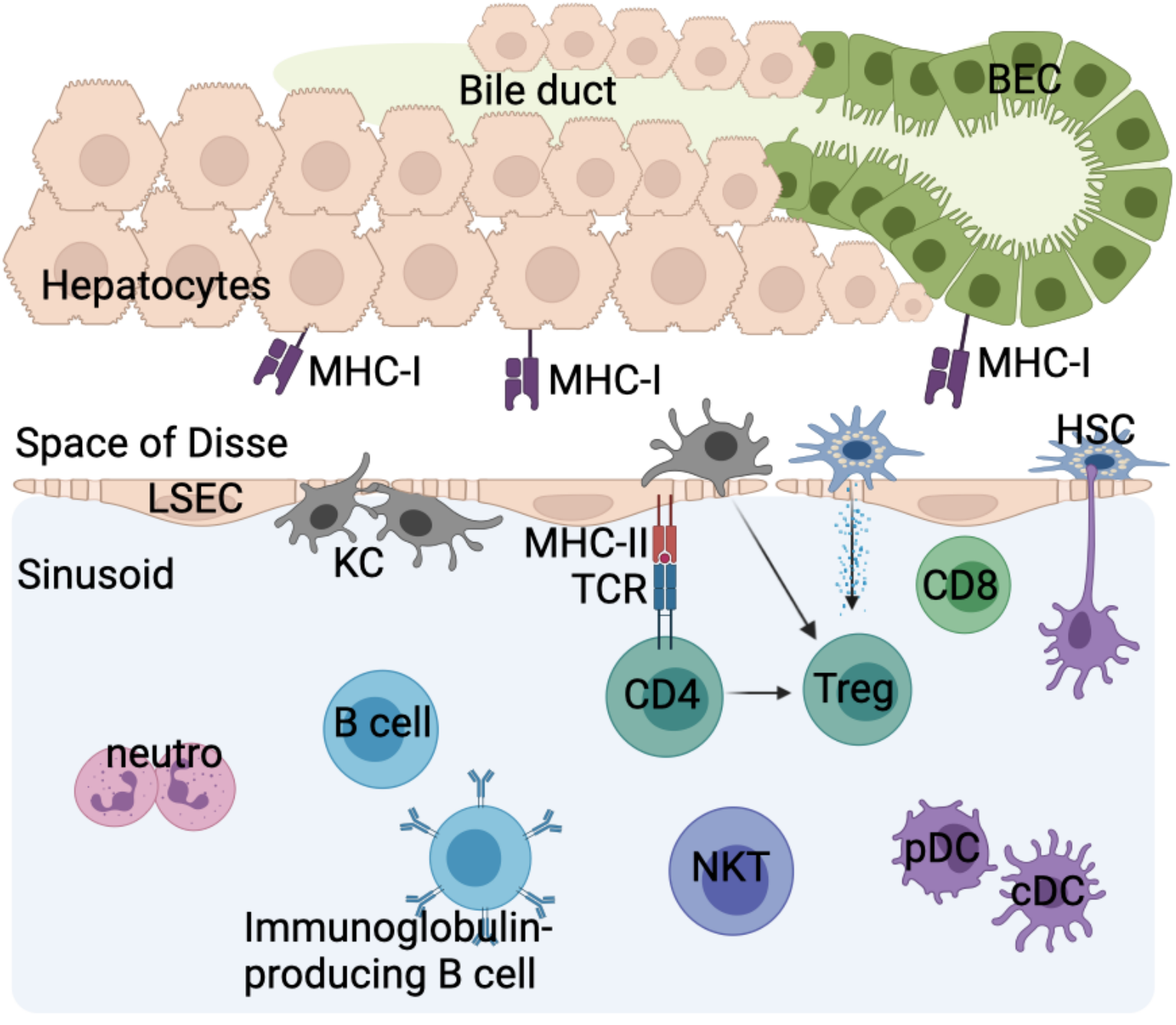


Figure 2

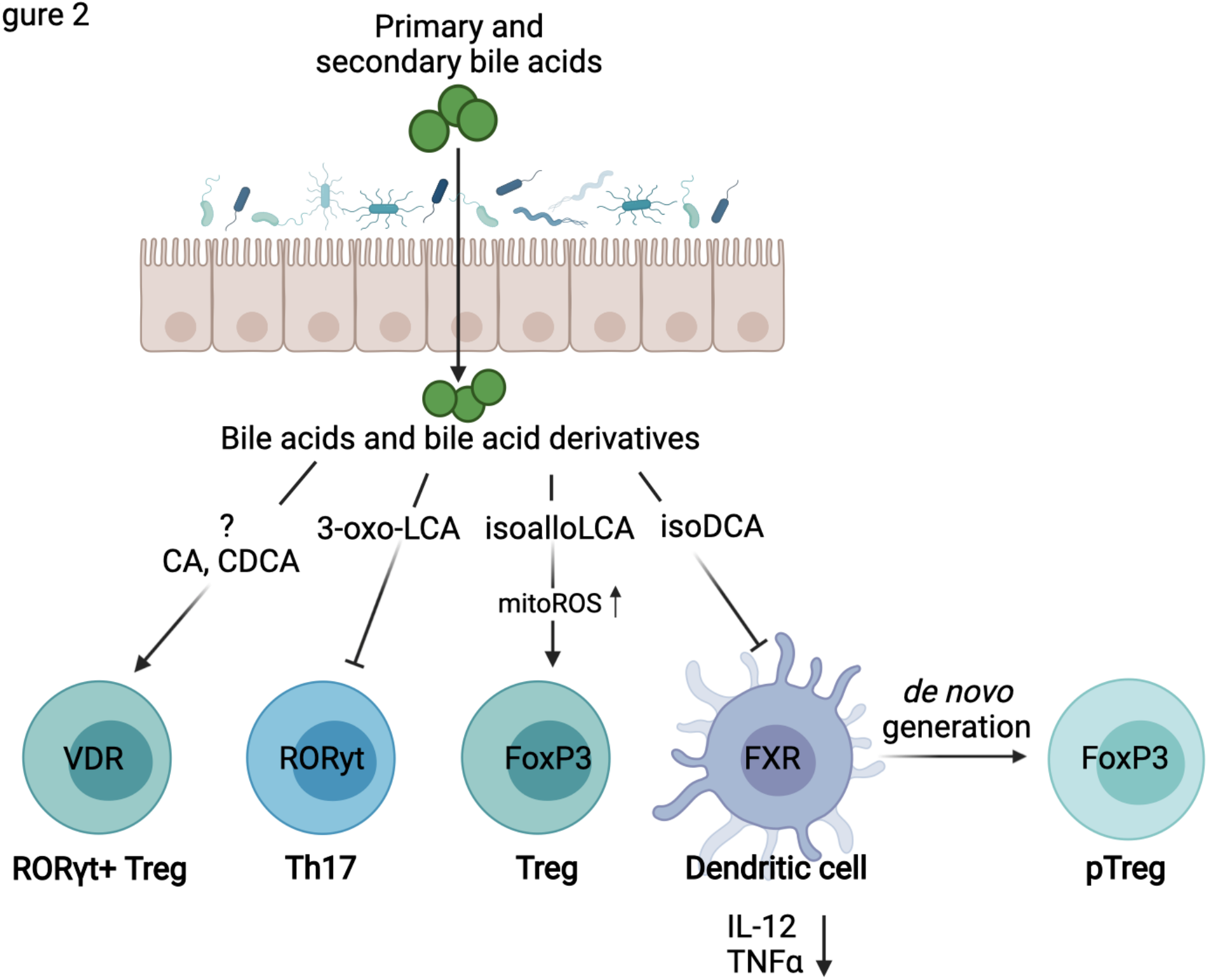
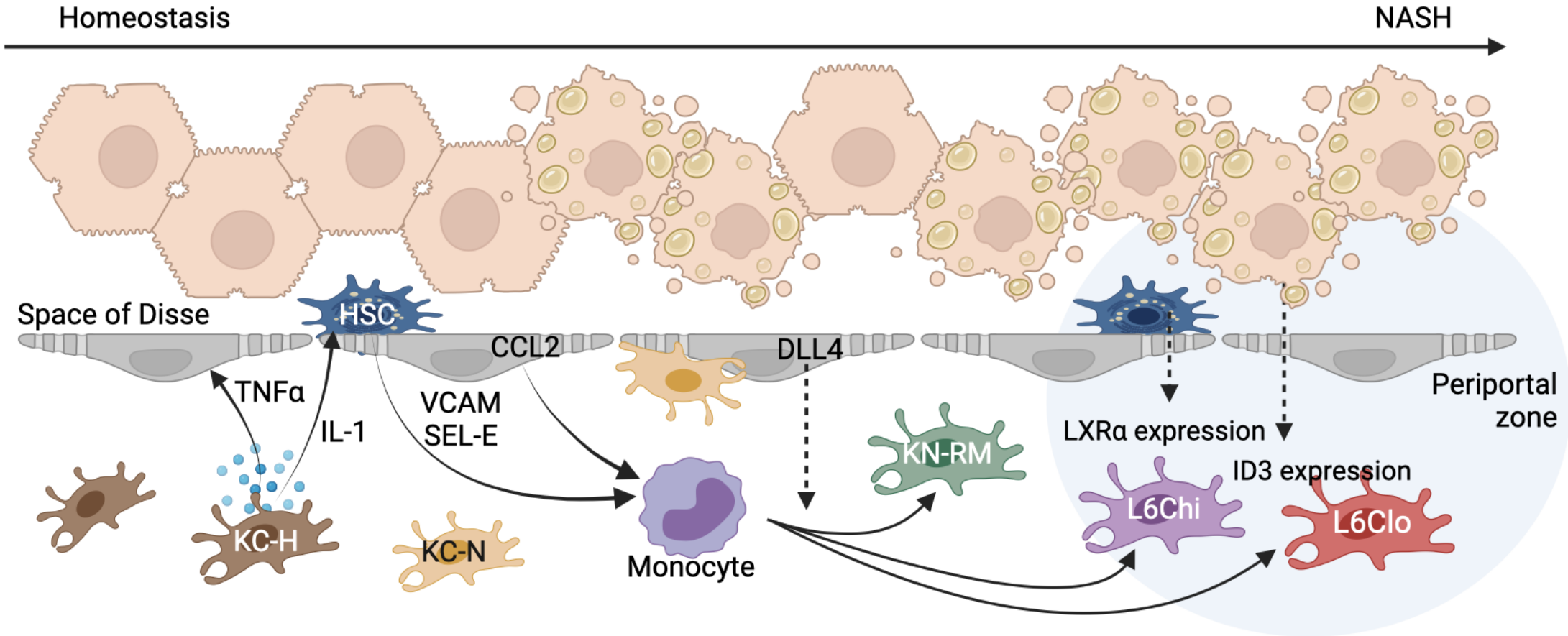

Primary and
secondary bile acids
Bile acids and bile acid derivatives
?
CA, CDCA
3-oxo-LCA
isoalloLCA
isoDCA
mitoROS
VDR
RORγt
FoxP3
FXR
de novo
generation
FoxP3
RORγt+ Treg
Th17
Treg
Dendritic cell
pTreg
IL-12
TNFα

Figure 3



Cell population	KC-H (healthy Kupffer cells)	KC-N (NASH-Kupffer cells)	KN-RM (recruited macrophage occupying the KC-N nice)	Ly6C^{hi}-RM (Ly6C^{hi} recruited macrophage)	Ly6C^{lo}-RM (Ly6C^{lo} recruited macrophage)
Enriched in	Healthy liver	NASH liver	NASH liver	NASH liver	NASH liver
Localization	Liver sinusoid	Liver sinusoid	Liver sinusoid	Around portal and central vein vasculature	Around portal and central vein vasculature
Identity genes	F4/80, Maf, Cd163, Clec4f, Timd4	F4/80, Maf, Clec4f, Cd5l, Fabp7, Ccl24	F4/80, Maf, Clec4f Cd63, Cdh5	Ly6c2, Ccr2, Itgam	Cd209a, Cd7, Itgam

Figure 4

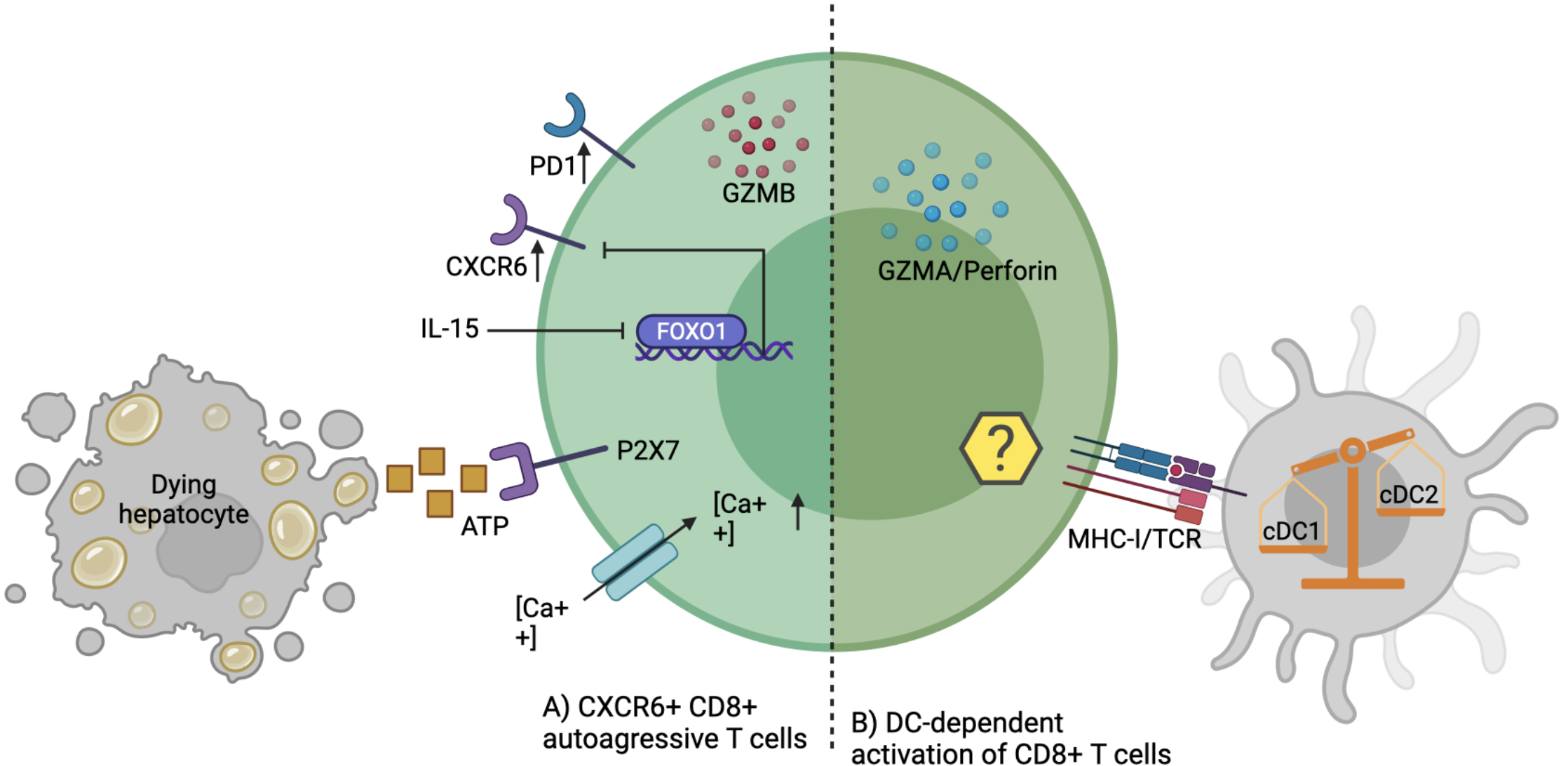
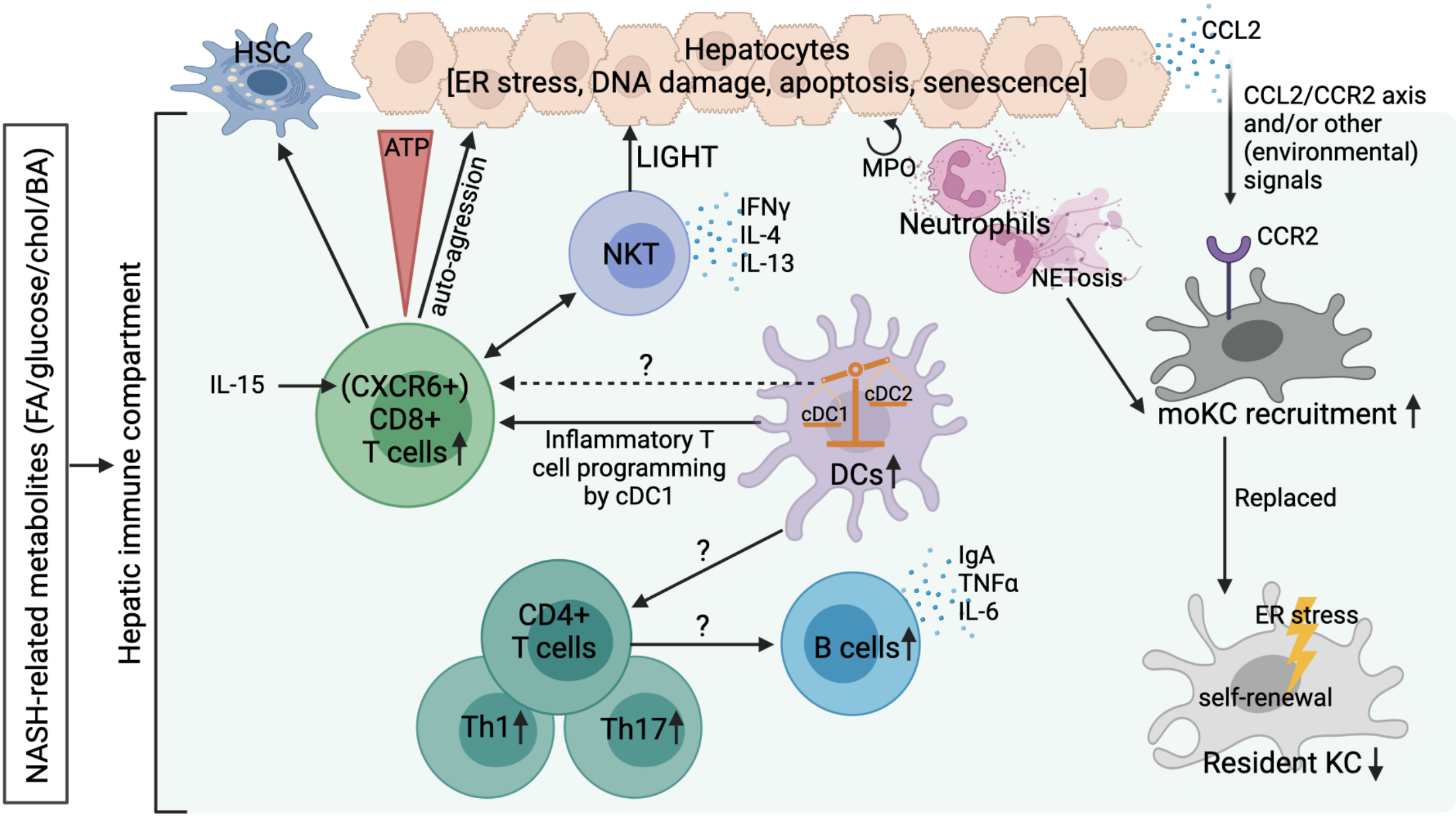

PD1
GZMB
CXCR6
GZMA/Perforin
IL-15
FOXO1
Dying hepatocyte
P2X7
ATP
[Ca++]
[Ca++]
?
MHC-I/TCR
cDC1
cDC2
A) CXCR6+ CD8+ autoagressive T cells
B) DC-dependent activation of CD8+ T cells

Figure 5


NASH-related metabolites (FA/glucose/chol/BA)
Hepatic immune compartment
HSC
Hepatocytes
[ER stress, DNA damage, apoptosis, senescence]
CCL2
CCL2/CCR2 axis and/or other (environmental) signals
ATP
auto-agression
LIGHT
MPO
Neutrophils
NETosis
NKT
IFNγ
IL-4
IL-13
CCR2
IL-15
(CXCR6+) CD8+ T cells
?
cDC1
cDC2
DCs
Inflammatory T cell programming by cDC1
moKC recruitment
Replaced
?
IgA
TNFα
IL-6
CD4+ T cells
?
B cells
Th1
Th17
ER stress
self-renewal
Resident KC

Highlights

- The altered metabolic environment in non-alcoholic fatty liver disease (NAFLD) promotes hepatocyte metabolic dysfunction and cellular stress, affecting the highly diverse hepatic immune compartment.
- Resident macrophages are activated in the early phase of liver injury and disease-promoting metabolic and tissue environmental signals alter their gene expression programs resulting in an identity loss.
- In a NASH environment, cDC and $CD8^+$ T cell populations are altered. CD8 cells with an "exhausted phenotype" are activated and kill hepatocytes, hence promoting tissue damage.
- The phenotype and function of hepatic immune cells is reshaped during the progression of NAFL to non-alcoholic steatohepatitis (NASH), but the role of the cellular interactions in disease progression is only starting to be uncovered.

Outstanding questions

1. How does the dysregulation of the macrophage compartment impact NASH development?
2. Does an altered cDC1/cDC2 ratio rather than absolute numbers play a role in NASH?
3. Is antigen-dependent activation of CD8 T lymphocytes playing a role in NASH?
4. Do various B cell subsets play a differential role in NASH development?