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# Immune–metabolic interactions in homeostasis and the progression to NASH

Joanne A Hoogerland, Bart Staels, David Dombrowicz

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1 Title

2 Immune-metabolic interactions in homeostasis and the progression to NASH

3

4 Authors

5 Joanne A. Hoogerland<sup>1</sup>, Bart Staels<sup>1\*</sup>, David Dombrowicz<sup>1\*</sup>

6

7 Affiliations

8 <sup>1</sup>Univ. Lille, Inserm, CHU Lille, Institut Pasteur de Lille, U1011-EGID, F-59000 Lille, France

9 \*Co-senior authors

10

11 Correspondence: david.dombrowicz@univ-lille.fr

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14 Liver; immunology; NAFLD; NASH; metabolism

15

16 Abstract

17 The incidence of Non-Alcoholic Fatty Liver Disease (NAFLD) has tremendously increased over

18 the past 2 decades. NAFLD ranges from simple steatosis (NAFL) to Non-Alcoholic

19 Steatohepatitis (NASH) and predisposes to fibrosis and hepatocellular carcinoma. The

20 importance of the immune system in hepatic physiology and in the progression of NAFLD is

21 increasingly recognized. At homeostasis, the liver participates in the immune defense against

22 pathogens and in tolerance towards gut-derived microbial compounds. Hepatic immune cells

23 also respond to metabolic stimuli and play a role in NAFLD progression to NASH. We first

24 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.

## 27 **The liver as an immunological organ**

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

51 and T lymphocytes are dispersed throughout the parenchyma being present in the portal  
52 tracts as well. Various subsets of T cells recognize presented antigens using highly variable T  
53 Cell antigen Receptors (TCRs), resulting – in non-inflammatory conditions – in tolerance  
54 induction rather than enhancement of T cell responses. In line, in these conditions, antigen  
55 presentation to naïve CD4<sup>+</sup> T cells induces differentiation of regulatory T cells (Tregs) rather  
56 than T helper (Th) 1 (Th1) or 17 (Th17) cells. In addition, CD8<sup>+</sup> T cells are highly effective in  
57 killing cells infected by intracellular pathogens and damaged cells. Next to conventional CD4<sup>+</sup>  
58 and CD8<sup>+</sup> T cells, non-conventional T cells (natural killer T (NKT) cells) are present in the liver  
59 and contribute to immune homeostasis by recognizing specific non-peptide antigens (Figure  
60 1).

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

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

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

83

#### 84 **Triggers of the immune response in NAFLD**

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

91

#### 92 *Lipid metabolism*

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

99 cytosolic and nuclear proteins, lipids, and damage-associated molecular patterns (DAMPs)  
100 [11], thereby inducing monocyte recruitment and macrophage activation. For example,  
101 danger signals, such as histidine-rich glycoprotein (HRG), induce macrophage polarization  
102 towards a pro-inflammatory phenotype [12].

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

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

123 the glycolytic reprogramming of DCs is impaired and mitochondrial reactive oxygen species  
124 (ROS) production is increased. ROS affects the unfolded protein response and induces a  
125 specific inflammatory program, characterized by increased IL-23 production [17]. Notably, IL-  
126 23 expression is enhanced in livers of mice fed a NASH diet [18]. Finally, a few studies have  
127 investigated the effects of specific fatty acids on T cells. Generally, high concentrations of  
128 fatty acids are toxic to T cells, and non-toxic concentrations induce proliferation and  
129 cytokine production [19–21]. The mechanisms by which fatty acids affect T cell function,  
130 especially in NAFLD/NASH, is still unclear and deserves further research.

131

### 132 *Glucose metabolism*

133 Hepatic steatosis promotes liver insulin resistance ultimately leading to diabetes, itself a  
134 major risk factor for NAFLD. Glucose availability and uptake are critical determinants in  
135 directing macrophage differentiation. As DCs, macrophages integrate signals of overnutrition  
136 and accordingly reprogram their metabolism [22]. The transcription factor HIF1 $\alpha$  plays a key  
137 role in the induction of the glycolytic adaptation in inflammatory macrophages. HIF1 $\alpha$   
138 expression is regulated both by inflammatory signals, such as pro-inflammatory cytokines,  
139 inducing the NF- $\kappa$ B signaling pathway [23], and growth factors, such as GM-CSF, triggering  
140 the AKT/mTOR pathway [24,25]. HIF1 $\alpha$ , in turn, regulates the expression of glycolytic  
141 enzymes and the glucose transporter GLUT1, thereby facilitating rapid glucose uptake.  
142 Glucose entry and its metabolization into lactate directly promotes inflammatory  
143 macrophage gene expression and activation [26]. Inflammatory macrophages further  
144 contribute to hepatic steatosis and inflammation by secretion of cytokines such as TNF- $\alpha$   
145 and IL-1 $\beta$  [27].



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

157

#### 158 *Cholesterol metabolism*

159 Elevated cholesterol levels in hepatocytes are associated with NAFLD risk and severity [4,31].  
160 Cholesterol is synthesized *de novo* from acetyl-CoA or imported *via* lipoprotein uptake.  
161 Macrophages are important for cholesterol efflux and reverse cholesterol transport from  
162 peripheral tissues to the liver *via* high-density lipoprotein (HDL) particles. LXR $\alpha$  is a critical  
163 regulator of cholesterol and lipid metabolism in KCs, and links metabolism and inflammation  
164 by reducing the production of pro-inflammatory mediators upon stimulation [32,33].  
165 Moreover, cholesterol is a precursor for bile acid synthesis, and (microbial) metabolites of  
166 bile acids were recently shown to control the adaptive immune response in the gut [34–36]  
167 (Box 3). However, studies about the effect of specific bile acids on hepatic immunity are  
168 lacking. Plasma bile acid levels and profiles are reported to be altered in NAFL and NASH  
169 [37–40], and a large inter-individual variation in human bile acid pool size and composition

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

173

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

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

182

#### 183 *Macrophages are activated in the early phase of liver injury*

184 KCs, the resident macrophages in the liver, are the most abundant hepatic immune cells  
185 accounting for ~35% of the non-parenchymal cells. These cells originate from yolk sac-  
186 derived erythromyeloid progenitors in the fetal liver. KCs are enriched in mid regions in  
187 human and in periportal regions in mouse livers [41,42] and are located along LSECs in the  
188 sinusoids, with a significant part of the KC inside the space of Disse to ensure close contact  
189 with hepatocytes, HSCs and LSECs [43]. In the metabolically healthy liver, KCs clear common  
190 gut-derived antigens, including bacterial antigens and fungal pathogens, from blood by  
191 phagocytosing, processing, and presenting antigens to maintain immune homeostasis [44–  
192 47]. Both resident (KC) and infiltrating monocyte-derived macrophages play important roles  
193 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<sub>3</sub>) [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 LXR $\alpha$  and  
215 ID3 expression, which are an important part of the KC-specific identity program [58].  
216 Expression of LXR $\alpha$ , *via* the DLL4-Notch pathway, and ID3 are respectively induced upon  
217 interaction with LSECs and hepatocytes, underlining the contribution of different hepatic cell

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

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

240

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

242 NASH is associated with the accumulation of intrahepatic DCs and alterations in the  
243 proportions of DC subsets. DCs are the major APCs in the liver and in the metabolically  
244 healthy liver they control the induction of antigen-specific adaptive immune responses as  
245 well as the induction of central and peripheral tolerance to auto-antigens [68,69]. Hepatic  
246 DCs (~1% of the non-parenchymal cells) are represented by three populations: plasmacytoid  
247 DCs (pDCs), classical type 1 DCs (cDC1s, expressing XCR1 and CD103), priming the CD8<sup>+</sup> T cell  
248 response, and classical type 2 DCs (cDC2s, expressing CD11b and Sirpα), priming the CD4<sup>+</sup> T  
249 cell response [70–72]. At homeostasis, hepatic DCs are mostly immature and tolerogenic  
250 [73]. However, upon liver injury, activated DCs migrate to the periportal and pericentral  
251 areas and draining lymph nodes, secrete pro-inflammatory cytokines, such as IL-6, IL-12, and  
252 TNF-α, and express co-stimulatory molecules CD40, CD80 and CD86 [74,75]. The cDC1  
253 (XCR1<sup>+</sup>)/cDC2 (CD172a<sup>+</sup>) ratio is decreased in livers of High Fat, High Cholesterol, High  
254 Sucrose diet fed mice, mainly due to a proportional (% of CD45<sup>+</sup> cells) increase in cDC2 and a  
255 slight reduction in cDC1 cells [18]. Moreover, cDC2 positively associate with NASH, while  
256 cDC1 negatively correlate with NASH. Surprisingly, the increase in cDC2 cells is paralleled by  
257 an increase in hepatic CD8<sup>+</sup> T cells, suggesting that changes in the cDC1/cDC2 ratio might  
258 fine tune CD8<sup>+</sup> T cell activation, likely through complex mechanisms. Indeed, T cell activation  
259 by cDC1 cells is classically MHC-I restricted. However, cDC1 cells also regulate the anti-tumor  
260 function of CD8<sup>+</sup> T cells through MHC-II-dependent regulation of CD4<sup>+</sup> helper T cells [76].  
261 Another study reported elevated numbers of both cDC1 and cDC2 cells in mice fed Western,  
262 choline-deficient high fat, or MCD diets, while only the abundance of cDC2 cells was  
263 increased in high fat diet-fed or *db/db* and *ob/ob* obese mice [77]. The use of different diets  
264 and treatment durations to induce NASH-like pathologies, with variable relatedness to the

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

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

281

### 282 *Infiltrating neutrophils facilitate NASH development*

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

289 neutrophils actively release their nuclear DNA content, while still maintaining membrane  
290 integrity, chemotactic responsiveness and phagocytosis [84].  
291 Infiltration of neutrophils, mainly around steatotic hepatocytes and in the portal tract [50], is  
292 associated with the early stage of NAFL/NASH in patients and animal models [80,85–88].  
293 Neutrophils contribute to NAFLD progression and the exacerbation of the inflammatory  
294 state by the production of neutrophil-derived myeloperoxidase (MPO), which accelerates  
295 ROS production and induces DNA damage [52,89]. NET formation (NETosis) also attracts  
296 monocyte-derived macrophages and subsequently induces steatosis-to-NASH progression  
297 [87]. In NASH patients, circulating neutrophils suppress the proliferation and activation of  
298 CD4<sup>+</sup> and CD8<sup>+</sup> T cells [90]. Over time, this may induce an inadequate immune-surveillance of  
299 liver damage, making patients more prone to the progression of liver damage. Although  
300 neutrophils are generally believed to promote NASH, they can also play a beneficial role.  
301 Indeed, neutrophils highly express microRNA-223, which upon transfer to hepatocytes elicits  
302 anti-inflammatory and anti-fibrotic responses [91]. However, antibody-mediated neutrophil  
303 depletion improved hepatic inflammation and fibrosis, and ameliorated HSC activation in  
304 high fat, high cholesterol-induced NASH in mice, indicating a detrimental role of neutrophils  
305 in NASH. Subsequent chow diet feeding, to study the role of neutrophils during  
306 (spontaneous) NASH resolution, showed that neutrophil depletion exacerbated fibrosis, HSC  
307 activation, and myeloid cell infiltration during resolution, demonstrating that neutrophils  
308 also play a pro-resolutive role in liver injury and fibrosis [92].

309

310 *The role of conventional CD4<sup>+</sup> Th1 and Th17 lymphocytes in the contribution to NASH*  
311 *development deserves further study*

312 The main hepatic T cell populations in a healthy liver are conventional CD4<sup>+</sup> and CD8<sup>+</sup> 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<sup>+</sup> T cells to NAFL and NASH development has been recently  
315 extensively studied, data about the precise role of CD4<sup>+</sup> T cells are limited.  
316 CD4<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> cytotoxic T cells play a key role in NASH progression, as



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

360 CXCR6<sup>+</sup> CD8<sup>+</sup> T cells is antigen-independent, the strong association between elevated hepatic  
361 CD8<sup>+</sup> T and cDC1 cells, as discussed above, also points toward a likely role of antigen  
362 presentation in CD8<sup>+</sup> 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<sup>+</sup> T cells in the progression of NAFL to NASH.

365 Interestingly, the metabolic environment affects both CD8<sup>+</sup> T cell phenotype and function in  
366 NASH development. Hepatic CD8<sup>+</sup> 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- $\alpha$ , 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<sup>+</sup> T cells promotes CD8<sup>+</sup> T cell survival and TIMP-1 expression in  
371 macrophages, hence stimulating NASH progression. Whereas CD8<sup>+</sup> T cells are the primary  
372 regulators of HSC activation selectively in an obese/hyperlipidemic environment, CD8<sup>+</sup> T cells  
373 play a minimal role in NASH development in non-obese animals [113]. Indeed, hepatic CD8<sup>+</sup>  
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, IFN $\gamma$ , and granzyme B. Moreover, these CD8<sup>+</sup> T cells did not  
376 impact on NASH-associated inflammation nor HSC activation. These findings, indicating that  
377 metabolic conditions influence CD8<sup>+</sup> 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  $\gamma\delta$  T cells. NKT cells are the most abundant hepatic non-conventional T cells in mice

384 [114]. Two types of NKT cells are found: type I NKT cells expressing a semi-invariant TCR  
385 (invariant (i)NKT cells), and type II NKT cells, expressing a diverse TCR repertoire [115]. NKT  
386 cells recognize lipid antigens presented by CD1d and can rapidly respond by secreting  
387 multiple cytokines (IFN $\gamma$ , IL-4, IL-10 and IL-17A) [116]. CD1d is an MHC-I-like molecule that is  
388 reactive to self-derived or microbial lipid antigens, including glycolipids and phospholipids  
389 [114]. As CD1d is expressed by antigen presenting cells, such as DCs and KCs [117], NKT cells  
390 form a bridge between the innate and adaptive immune systems. NKT cell numbers change  
391 during the onset of NAFL and progression to NASH. A reduction in hepatic NKT cell numbers  
392 was reported in mouse models on short-term MCD, choline-deficient L-amino acid-deficient  
393 (CDAA), or high-fat diets [118,119]. Decreases in hepatic NKT cell numbers can be due to  
394 steatosis-induced KC-derived IL-12 and Tim-3/Gal-9 signaling, which promotes NKT cell  
395 apoptosis. By contrast, NKT cell numbers increase in advanced NASH. Mice fed a high fat,  
396 high cholesterol diet for 16 weeks showed increased NKT cells in liver [106]. NKT cell  
397 depletion protected against NASH progression, as these mice exhibited lower NAFLD activity  
398 scores (NAS) and AST/ALT levels, less hepatic infiltrating macrophages and decreased  
399 expression of genes related to fibrosis [106]. Similarly, a study combining choline-deficiency  
400 with a high fat diet for 6 months showed a significant increase in hepatic NKT cells [107]. A  
401 possible explanation for the increase in NKT cell numbers is the activation of the Hedgehog  
402 pathway in NASH. This results in higher levels of the NKT cell chemokine CXCL16, the  
403 adhesion molecule VCAM-1, and IL-15, all promoting NKT viability [120–122]. Several NKT-  
404 operated mechanisms can contribute to NASH progression. Activated intrahepatic NKT cells  
405 enhance hepatocyte lipid uptake *via* secretion of LIGHT, thus promoting steatosis [107].  
406 Moreover, NKT cells produce IL-17 and IL-22 at the beginning of the steatosis phase,  
407 becoming IFN $\gamma$ /IL-4/IL-13- secreting cells at a later stage of the disease [123]. The differential

408 activation and cytokine secretion by NKT cells results in an evolving immune response during  
409 disease progression. Moreover, the synergistic action of NKT and CD8<sup>+</sup> T cells contributes to  
410 inflammation and fibrosis [106,123].

411 MAIT cells are a population of innate-like T lymphocytes that recognize a limited array of  
412 microbial-derived antigens and vitamin B metabolites presented by MHC-I-related molecule  
413 1 (MR1) [124]. In the metabolically healthy liver, they are important for antimicrobial  
414 immunity against bacteria and yeast, and exert cellular cytotoxicity against target cells by  
415 producing pro-inflammatory cytokines such as IFN $\gamma$  and TNF- $\alpha$  [125]. MAIT cells accelerate  
416 inflammation and fibrosis in models of CCL<sub>4</sub>- or bile duct ligation-induced liver injury and  
417 accumulate in livers of NASH patients [126]. By contrast, MAIT cells were found to be  
418 protective against liver inflammation in MCD diet-induced NASH models [127]. Beyond the  
419 fact that very different experimental models were studied, it is difficult to speculate about  
420 possible mechanisms accounting for these opposing results.

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

426

#### 427 *B lymphocytes and antibody production promotes NASH development*

428 B cells are the largest population of hepatic lymphocytes (~20% of all CD45<sup>+</sup> cells in mice)  
429 [130] and are important for both humoral immunity, by producing antibodies, and cellular  
430 immunity, by functioning as APCs. They are located close to the bile ducts and are reported  
431 to function as APCs for MAIT cells by presenting gut-derived bacterial antigens *via* MR-1

432 [131,132]. Most hepatic B cells are mature follicular B cells, which survey for antigens. In the  
433 human liver, immature, “classical” B2, “non-conventional” B1, and antibody-producing  
434 plasma cells have also been detected, although their precise function at steady state is  
435 unclear [133,134].

436 Growing evidence suggests an essential role for B cells in NAFL and NASH development. B  
437 cells accumulate in the portal tract of livers of patients with NASH [50,135], and serum levels  
438 of IgA [136] and B cell-activating factor (BAFF), promoting B cell differentiation and survival,  
439 are increased in NASH patients [137]. Accumulated B cells in livers of HFD- or NASH-diet fed  
440 mice are pro-inflammatory and secrete cytokines such as IL-6 and TNF- $\alpha$  [137–139]. The  
441 (innate) TLR-associated Myeloid Differentiation primary response protein 88 (MyD88)  
442 pathway is required for B cell activation and maturation in NASH [139] and for their pro-  
443 fibrotic role, as shown in a mouse model with CCl<sub>4</sub>-induced liver fibrosis [140]. Moreover,  
444 microbiota-derived metabolites draining into the liver as a result of increased gut  
445 permeability activate intrahepatic B cells during NASH [139].

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

454

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

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

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

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

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



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

535

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983

984 Text boxes

985 **Box 1 – Hepatocytes exert an immune function at homeostasis**

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

997

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

999 *Liver sinusoidal endothelial cells* (LSECs) are, contrary to hepatocytes, in direct contact with  
1000 the blood. Their numerous fenestrae and absence of basal membrane allow efficient  
1001 filtration from the sinusoidal lumen to the space of Disse [150]. LSECs play an important role  
1002 in the clearance of circulating microorganisms [151,152] and molecules [153], as well as in  
1003 mediating immune tolerance [154] under physiological conditions. Antigens that are taken  
1004 up and processed by LSECs can be presented *via* MHC-II to CD4<sup>+</sup> T cells, and *via* MHC-I to  
1005 naïve CD8<sup>+</sup> T cells. Priming of naïve CD4<sup>+</sup> T cells mainly induces the development of  
1006 CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs [155], which is an important mechanism for maintaining hepatic  
1007 tolerance. Antigen presentation by LSECs to CD8<sup>+</sup> T cells results – at low antigen

1008 concentrations – in the downregulation of IFN $\gamma$  and IL-2 cytokine production by CD8<sup>+</sup> T cells,  
1009 leading to antigen-specific T cell tolerance [156,157].

1010 *Hepatic stellate cells* (HSCs) constitute 8-10% of total liver cells. HSCs are located at the  
1011 space of Disse, between the hepatocytes and the LSECs. In a healthy liver, HSCs are  
1012 quiescent, serve as the primary storage site for vitamin A, and are essential in the regulation  
1013 of retinoic acid homeostasis. Moreover, they are the main collagen- and extracellular matrix  
1014 producing cells in liver fibrosis. Recent studies have also identified a role of HSCs in hepatic  
1015 immunology. Antigen uptake and presentation by HSCs in mice is low [158], although they  
1016 express MHC-II, costimulatory molecules such as CD40 and CD80 [159,160], and they  
1017 produce various growth factors, cytokines [161] and chemokines [162]. A recent study using  
1018 single-cell RNA sequencing identified two HSC subpopulations in human liver with one of  
1019 them displaying a gene signature that is reminiscent of antigen-presenting cells [163].  
1020 Although it has been suggested that HSCs can present antigens to T cells [159,164], this  
1021 function is still controversial. However, HSCs do contribute to hepatic immune tolerance *via*  
1022 indirect priming of DC-activated Tregs [165,166]. Treg induction and Th17 inhibition is  
1023 mediated through the release of retinoic acid [167] during priming of T cells by DCs [165]. At  
1024 homeostasis HSCs thus regulate hepatic immune reactivity mainly indirectly by modulating  
1025 the outcome of T cell activation by other APCs.

1026 *Cholangiocytes*, or biliary epithelial cells (BECs), line the bile duct and are constantly exposed  
1027 to bile content. Apart from their function in the regulation of bile flow, composition, and pH,  
1028 they also play a role in antigen presentation. On the apical side, BECs are exposed to bile and  
1029 (lipid) antigens present in bile. On the basolateral side, BECs are in close contact with hepatic  
1030 immune cells. Disorders associated with immune damage of the bile ducts, such as primary  
1031 biliary cholangitis (PBC) [168,169], are associated with increased basolateral MHC-II

1032 expression by BECs, although *in vitro* assays failed to demonstrate CD4<sup>+</sup> 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

1056 retinoic acid receptor-related orphan receptor  $\gamma$ t (ROR $\gamma$ t). ROR $\gamma$ t is a key transcription factor  
1057 required for induction of IL-17 transcription [178]. 3-oxoLCA inhibits its transcriptional  
1058 activity and thus Th17 differentiation. Conversely, isoalloLCA promotes Treg differentiation  
1059 by enhancing mitochondrial ROS production and increasing histone acetylation at the FoxP3  
1060 promoter region. Overall, the LCA metabolites 3-oxoLCA and isoalloLCA exert anti-  
1061 inflammatory actions by directly affecting CD4<sup>+</sup> T cell differentiation [179]. Similarly, Song et  
1062 al. reported that bile acids induce (immunosuppressive) ROR $\gamma$ t-expressing Tregs residing in  
1063 the lamina propria of the colon [36]. The authors speculate that the most abundant  
1064 intestinal primary bile acid species, such as cholic acid and chenodeoxycholic acid, act *via* the  
1065 vitamin D receptor (VDR) to maintain a healthy colonic ROR $\gamma$ t<sup>+</sup> Treg pool (Figure 2). Recently,  
1066 the secondary bile acid isoDCA was found to indirectly induce peripheral Tregs in the colon  
1067 by inhibiting FXR activity in DCs [34]. Collectively, these studies identify an immune  
1068 suppressive role for bile acid metabolites in the control of the adaptive immune response in  
1069 the colon. Although these studies are not performed in a NASH context and do not directly  
1070 relate to the hepatic immune system, they underline the important immune regulatory  
1071 function of bile acids (and metabolites). NASH is associated with changes in bile acid  
1072 metabolism and pool composition and altered plasma bile acid levels and profiles [37].  
1073 However, so far studies about the effect of altered bile acid profiles or specific bile acid  
1074 species on hepatic immunity and NASH development are lacking.

1075

#### 1076 **Box 4 - Macrophages in NASH: beyond the M1/M2 paradigm**

1077 As for macrophages in other tissues, in the past, KCs were subdivided into M1 (classically  
1078 activated, immunogenic) and M2 (alternatively activated, tolerogenic) cells. However, it is  
1079 increasingly recognized that KCs exhibit a high plasticity, are highly dynamic and can



1080 differentiate into a wide range of polarization or activation states. Depending on the  
1081 context, KCs exhibit specific metabolic signatures [180] and M1 and M2 KCs only represent  
1082 the extreme phenotypes of a wide spectrum.

1083 Upon Western diet feeding, 4 abundant macrophage populations were identified in the liver,  
1084 including resident KCs and 3 subsets of recruited macrophages: monocyte-derived KCs,  
1085 macrophages becoming monocyte-derived KCs (termed pre-moKCs), and hepatic lipid-  
1086 associated macrophages (LAMs). In agreement with other studies [15], resident KCs were  
1087 gradually lost during NAFLD progression and replaced by monocyte-derived KCs.  
1088 Interestingly, monocyte-derived KCs resembled resident KCs, while the transcriptome of  
1089 hepatic LAMs largely differed from that of resident KCs. LAMs expressed lower levels of  
1090 immune activation-associated genes, such as Il18, Fpr2, Tlr4 and CD38, and had the distinct  
1091 ability to metabolize lipids. LAMs were only identified in later stages of NAFLD and express  
1092 *Spp1* (next to *Trem2*, *Cd9*, *Cd63*, and *Gpnmb*), encoding the chemokine osteopontin.  
1093 Osteopontin was found to be a good biomarker of NASH [181] and is thought to contribute  
1094 to fibrosis [182]. Moreover, Daemen et al. showed that LAMs preferentially localize to HSC  
1095 expansion regions, where they aggregate into hepatic crown-like structures (hCLS) [15],  
1096 suggesting a role for hepatic LAMs in NASH development and liver fibrosis. Another recent  
1097 study identified low numbers of LAMs already in periportal regions of healthy livers, and  
1098 showed accumulation of more mature LAMs in pericentral regions in steatotic mouse livers  
1099 [42]. The exact function of LAMs in the NASH liver is as yet unclear.

1100 A more detailed sc-RNAseq analysis based on transcriptional differences identified 5  
1101 macrophage clusters in mice fed control or NASH diets [16]: healthy KCs, NASH KCs,  
1102 recruited macrophages, LY6C<sup>hi</sup> recruited macrophages and LY6C<sup>lo</sup> recruited macrophages  
1103 (Figure 3). NASH KCs and recruited macrophages were predominantly found in NASH livers,

1104 while healthy livers consisted almost entirely of healthy KCs. Both LY6C<sup>hi&lo</sup> recruited  
1105 macrophages were also predominantly found in NASH livers and differed in gene expression  
1106 and zonation from recruited macrophages. While recruited macrophages were mainly  
1107 located in the liver sinusoid, in contact with the LSECs, LY6C<sup>hi&lo</sup> recruited macrophages  
1108 reside proximal to the large portal and central vein vessels in contact with vascular  
1109 endothelium. These observations underline that disease-promoting metabolic and tissue  
1110 environmental signals can instruct macrophages to acquire distinct gene expression  
1111 programs. Mechanistically, MCD diet-induced NASH was associated with impaired KC self-  
1112 renewal and disease progression with increased recruitment of monocyte-derived KCs [66].  
1113 Moreover, death of NASH KCs, possibly induced by increased ER stress, modifies the KC  
1114 niche and enables repopulation by monocyte-derived KCs. Monocyte-derived KCs are  
1115 enriched in genes involved in cellular stress and activation, limited hepatic triglyceride  
1116 storage and increased inflammation.

1117

#### 1118 **Box 5 – Hepatic innate lymphoid cells in NAFLD: a controversial role**

1119 Liver innate lymphoid cells mostly include natural killer (NK) cells and closely-related group 1  
1120 lymphoid cells (ILC1s). They both lack antigen-specific receptors and produce the signature  
1121 cytokine IFN $\gamma$  [114,183] in response to IL-12, IL-15, or IL-18 secreted by DCs, macrophages  
1122 and T lymphocytes [184,185]. Mouse hepatic NK, unlike ILC1, require the transcription factor  
1123 EOMES for their development, while ILC1 express the transcription factor Zfp683. Moreover,  
1124 the development of hepatic ILC1s from its liver-resident progenitor cells is promoted by IFN $\gamma$   
1125 production by ILC1s themselves [186].

1126 In the metabolically healthy liver, NK cells rapidly recognize and kill viral-infected or tumor-  
1127 transformed cells in the absence of specific immunization by releasing perforin and

1128 granzymes [185]. Hepatic ILC1s exhibit cytotoxicity mediated through granzyme A [187] and  
1129 B, perforin, TRAIL, and FasL [188]. By expressing inhibitory receptors, such as PD-1  
1130 (programmed cell death-1) and lymphocyte-activation protein-3 (LAG-3), ILC1s regulate the  
1131 anti-viral activity of hepatic T cells [189], thereby preventing hepatic virus infection.

1132 Conflicting data exist on changes in ILC1 and NK cell numbers and proportions in blood and  
1133 livers of NAFL/NASH patients [190–192] and in livers of mouse NAFLD models [193–196],  
1134 probably due to differences in disease stage and the use of different NASH-inducing  
1135 protocols. On the one hand, NK cells have a detrimental role in NAFL to NASH progression as  
1136 TRAIL-producing NK cells were shown to promote a pro-inflammatory environment at an  
1137 early NAFLD stage [197]. On the other hand, NK cells were reported to exert anti-fibrotic  
1138 effects [193,195] and to actively contribute to limiting NASH-associated tissue damage and  
1139 fibrosis by killing activated HSCs, the main collagen producing cells in fibrosis [198]. A better  
1140 characterization of (tissue resident) NK cells and the seemingly controversial role of NK cells  
1141 as contributors or modulators of NASH progression should be further investigated.

1142 Figure legends

1143 *Figure 1.* Hepatic immunity in healthy liver.

1144 At homeostasis the liver exhibits an immune tolerant state, while it also allows efficient  
1145 responses against infectious agents. LSECs prime naïve CD4<sup>+</sup> T cells and induce Treg  
1146 differentiation. KCs are important to maintain immune homeostasis by clearing common  
1147 gut-derived antigens. CD8<sup>+</sup> effector T cells scan and recognize MHC-I/peptide complexes  
1148 presented by hepatocytes to respond to intracellular pathogens and damaged cells.

1149 BEC; biliary epithelial cells, MHC-I/II; major histocompatibility complex, class I/II, TCR; T cell  
1150 receptor, HSC; hepatic stellate cell, KC; Kupffer cell, neutro; neutrophils, Treg; regulatory T  
1151 cell, CD4; CD4<sup>+</sup> T cell, CD8; CD8<sup>+</sup> T cell, NKT; natural killer T cell, pDC; plasmacytoid dendritic  
1152 cell, cDC; conventional dendritic cell.

1153

1154 *Figure 2.* The immune suppressive role of bile acids and their metabolites affect T cell  
1155 homeostasis in the colonic lamina propria and control the adaptive immune response.

1156 The most abundant primary bile acids (CA and CDCA) maintain a healthy colonic ROR $\gamma$ t Treg  
1157 pool *via* VDR signaling. 3-oxo-LCA, which is formed by the gut microbiota, inhibits Th17  
1158 differentiation by physical interaction with ROR $\gamma$ t. IsoalloLCA promotes Treg differentiation  
1159 by enhancing mitoROS and FoxP3 acetylation. isoDCA indirectly induces pTreg generation by  
1160 inhibiting FXR transcriptional activity in DCs.

1161 CA; cholic acid, CDCA; chenodeoxycholic acid, 3-oxo-LCA; 3-oxo-lithocholic acid, isoalloLCA;  
1162 isoallolithocholic acid, isoDCA; isodeoxycholic acid, mitoROS; mitochondrial reactive oxygen  
1163 species, VDR; vitamin D receptor, ROR $\gamma$ t; retinoid-related orphan receptor  $\gamma$ t, FoxP3;  
1164 forkhead box P3, FXR; farnesoid X receptor, Treg; regulatory T cell, Th17; T helper 17, pTreg;  
1165 peripheral Treg.

1166

1167 *Figure 3.* Kupffer cell populations in healthy and NASH livers, according to Seidman *et al.* [16]

1168 and Bonnardel *et al.* [43]

1169 KC-H cells are enriched in healthy liver. KC-H loss upon NASH-induced liver injury, most

1170 probably due to apoptosis, induces TNF $\alpha$  and IL-1-dependent receptor activation of HSCs

1171 and LSECs. VCAM, SEL-E, and CCL2 upregulation results in monocyte recruitment. LSEC DLL4

1172 expression drives monocyte differentiation and supports niche specialization via the DLL4-

1173 Notch pathway. KC-hepatocyte interactions induce ID3 expression, LSECs and HSCs induce

1174 LXR $\alpha$  expression. 4 macrophage populations are enriched in NASH liver: KC-N, KN-RM (both

1175 localized in liver sinusoid), Ly6C<sup>hi</sup>-RM, and Ly6C<sup>lo</sup>-RM (both localized around portal and

1176 central vein vasculature).

1177 HSC; hepatic stellate cell, TNF $\alpha$ ; tumor necrosis factor  $\alpha$ , IL-1; interleukin-1, VCAM; vascular

1178 cell adhesion molecule, SEL-E; selectin-E, DLL4; delta like canonical notch ligand 4, LXR $\alpha$ ; liver

1179 X receptor alpha, ID3; inhibitor of differentiation 3, KC-H; healthy Kupffer cells, KC-N; NASH-

1180 Kupffer cells, KN-RM; recruited macrophages, Ly6C<sup>hi</sup>-RM; Ly6C<sup>hi</sup> recruited macrophages,

1181 Ly6C<sup>lo</sup>-RM; Ly6C<sup>lo</sup> recruited macrophages.

1182

1183 *Figure 4.* Cytotoxic CD8<sup>+</sup> T cells in NASH.

1184 (a) IL-15-driven transcriptional (re)programming and metabolic stimuli promote auto-

1185 aggressive CXCR6<sup>+</sup> CD8<sup>+</sup> T cells displaying enhanced expression of PD1 and GZMB. Auto-

1186 aggression is MHC-I-independent, as the purinergic receptor P2X7 responds to ATP released

1187 from dying hepatocytes. Calcium influx is enhanced to initiate auto-aggression. (b) GZMA

1188 and perforin expressing CD8<sup>+</sup> T cells associate with NASH. The link between the altered

1189 cDC1/cDC2 ratio and antigen dependent CD8<sup>+</sup> T cell activation remains to be investigated.

1190 GZMA; granzyme A, GZMB; granzyme B, PD1; programmed cell death protein 1, CXCR6; C-X-  
1191 C motif chemokine receptor 6, IL-15; interleukin 15, FOXO1; forkhead box O1, P2X7;  
1192 purinergic receptor P2X 7, MHC-I; major histocompatibility complex, class I, TCR; T cell  
1193 receptor, cDC1; conventional dendritic cells 1, cDC2; conventional dendritic cells 2.

1194

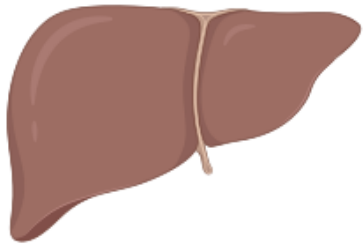
1195 *Figure 5. Immune dysregulation in NASH liver.*

1196 Macrophages are recruited *via* the CCL2/CCR2 axis or by other (environmental) signals and  
1197 replace resident KCs. DC abundance increases in NASH livers and cDC1 cells enhance the  
1198 inflammatory signature of CD8<sup>+</sup> T cells. The cDC1/cDC2 ratio decreases, but its effect on  
1199 CD4<sup>+</sup> and CD8<sup>+</sup> T cell activation and the function of cDC2 in NASH is unknown. B cells secrete  
1200 proinflammatory cytokines, such as TNF $\alpha$  and IL-6, as well as IgA, although the exact role of B  
1201 cells in NASH remains to be investigated. CD8<sup>+</sup> T cells in a NASH environment acquire the  
1202 ability to kill hepatocytes, and to induce HSC activation, thereby enhancing the development  
1203 of fibrosis. The synergistic action of CD8<sup>+</sup> T cells with NKT cells might lead to NASH  
1204 progression. LIGHT secretion by NKT cells enhances hepatocyte lipid accumulation, thus  
1205 promoting hepatic steatosis, while the secretion of proinflammatory cytokines, such as IFN $\gamma$ ,  
1206 may recruit and activate other immune cell types, enhancing immune dysregulation and  
1207 NASH progression. NASH-related metabolites, such as excess glucose and fatty acids and  
1208 increase in plasma bile acid levels also affects phenotype and function of the hepatic  
1209 immune compartment.

1210 HSC; hepatic stellate cells, CCL2; C-C motif chemokine ligand 2, CCR2; C-C motif chemokine  
1211 receptor 2, ATP; adenosinetriphosphate, NKT; natural killer T cells, IFN $\gamma$ ; interferon  $\gamma$ , IL;  
1212 interleukin, MPO; myeloperoxidase, NETosis; NET (neutrophil extracellular traps) formation,  
1213 CXCR6; C-X-C motif chemokine receptor 6, cDC1; classical type 1 DCs, cDC2; classical type 2

1214 DCs, DCs; dendritic cells, moKC; monocyte-derived Kupffer cells, Th1; T helper 1, Th17; T  
1215 helper 17, IgA; immunoglobuline A, TNF $\alpha$ ; tumor necrosis factor  $\alpha$ , ER stress; endoplasmic  
1216 reticulum stress, KC; Kupffer cells, FA; fatty acids, chol; cholesterol, BA; bile acids.  
1217  
1218 Figures are created with BioRender.com

# Figure 1



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

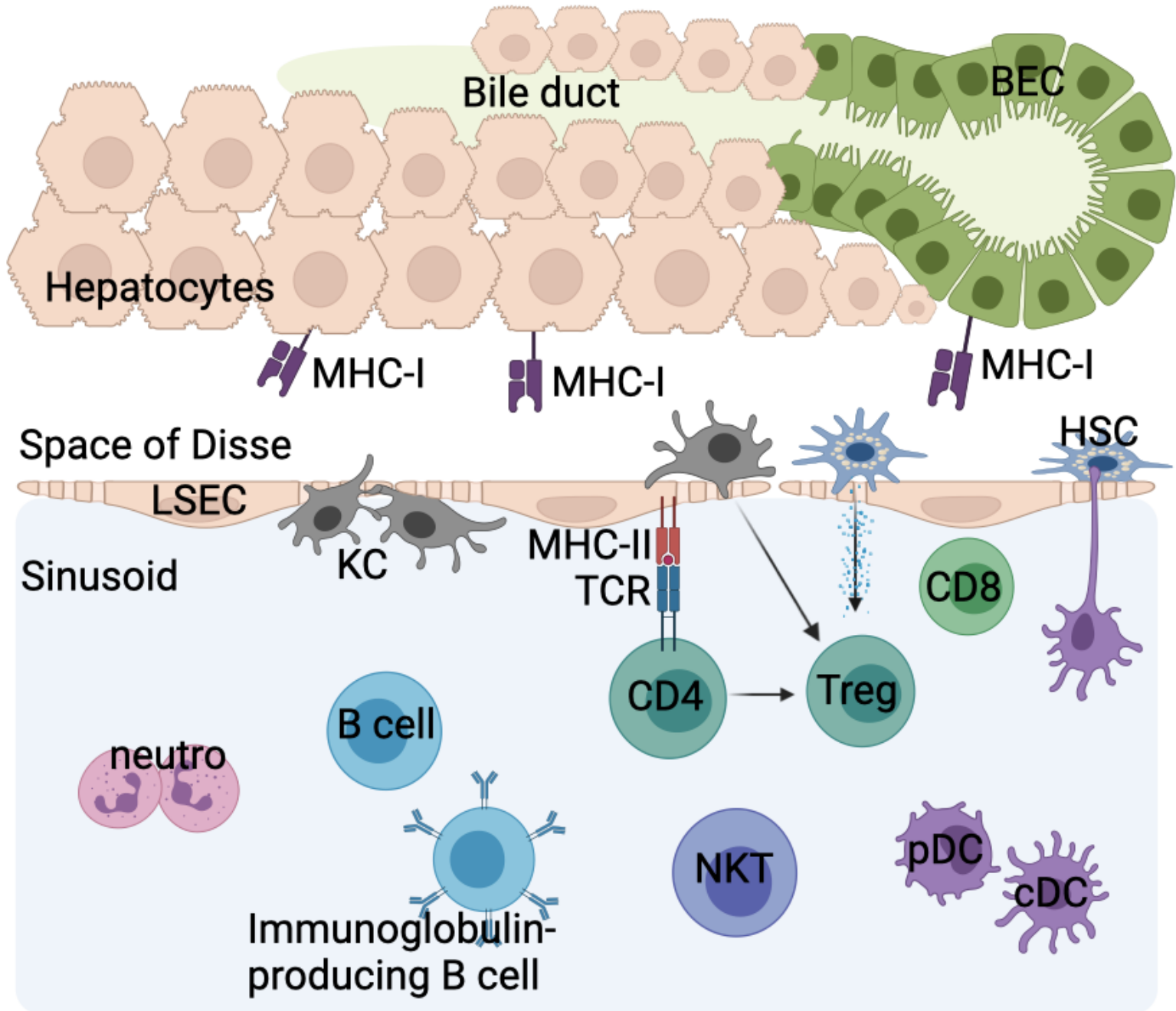




Figure 2

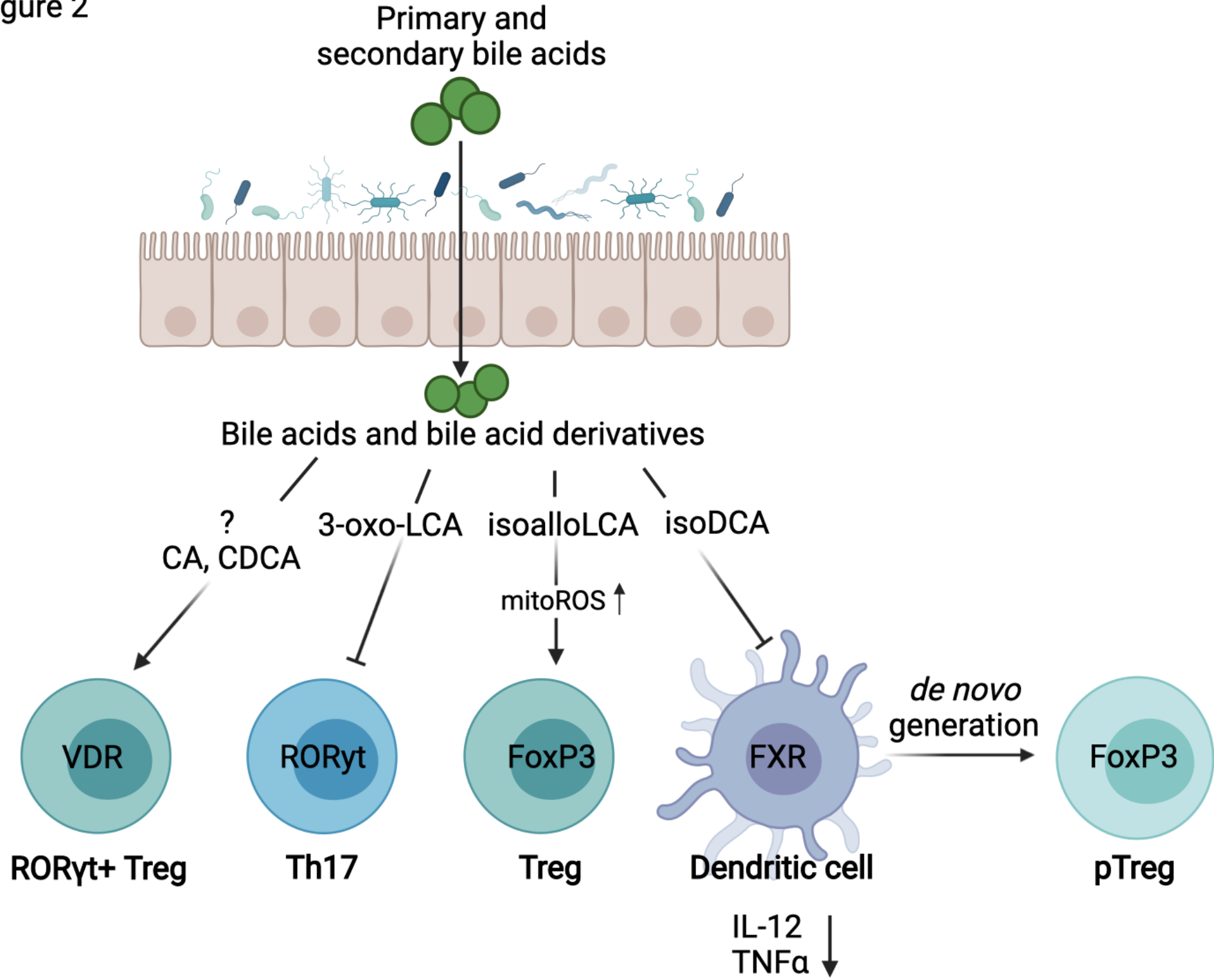
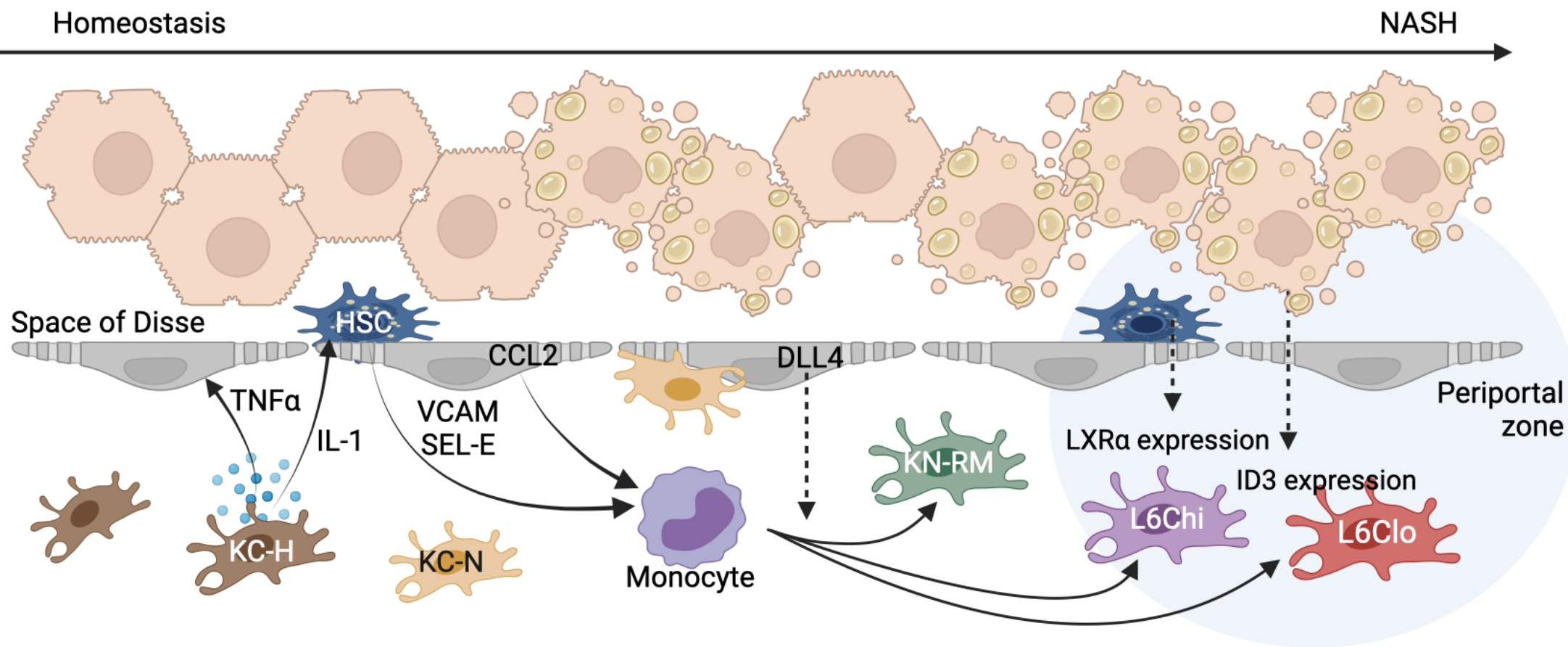


Figure 3








					
<b>Cell population</b>	KC-H (healthy Kupffer cells)	KC-N (NASH-Kupffer cells)	KN-RM (recruited macrophage occupying the KC-N niche)	Ly6C <sup>hi</sup> -RM (Ly6C <sup>hi</sup> recruited macrophage)	Ly6C <sup>lo</sup> -RM (Ly6C <sup>lo</sup> recruited macrophage)
<b>Enriched in</b>	Healthy liver	NASH liver	NASH liver	NASH liver	NASH liver
<b>Localization</b>	Liver sinusoid	Liver sinusoid	Liver sinusoid	Around portal and central vein vasculature	Around portal and central vein vasculature
<b>Identity genes</b>	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

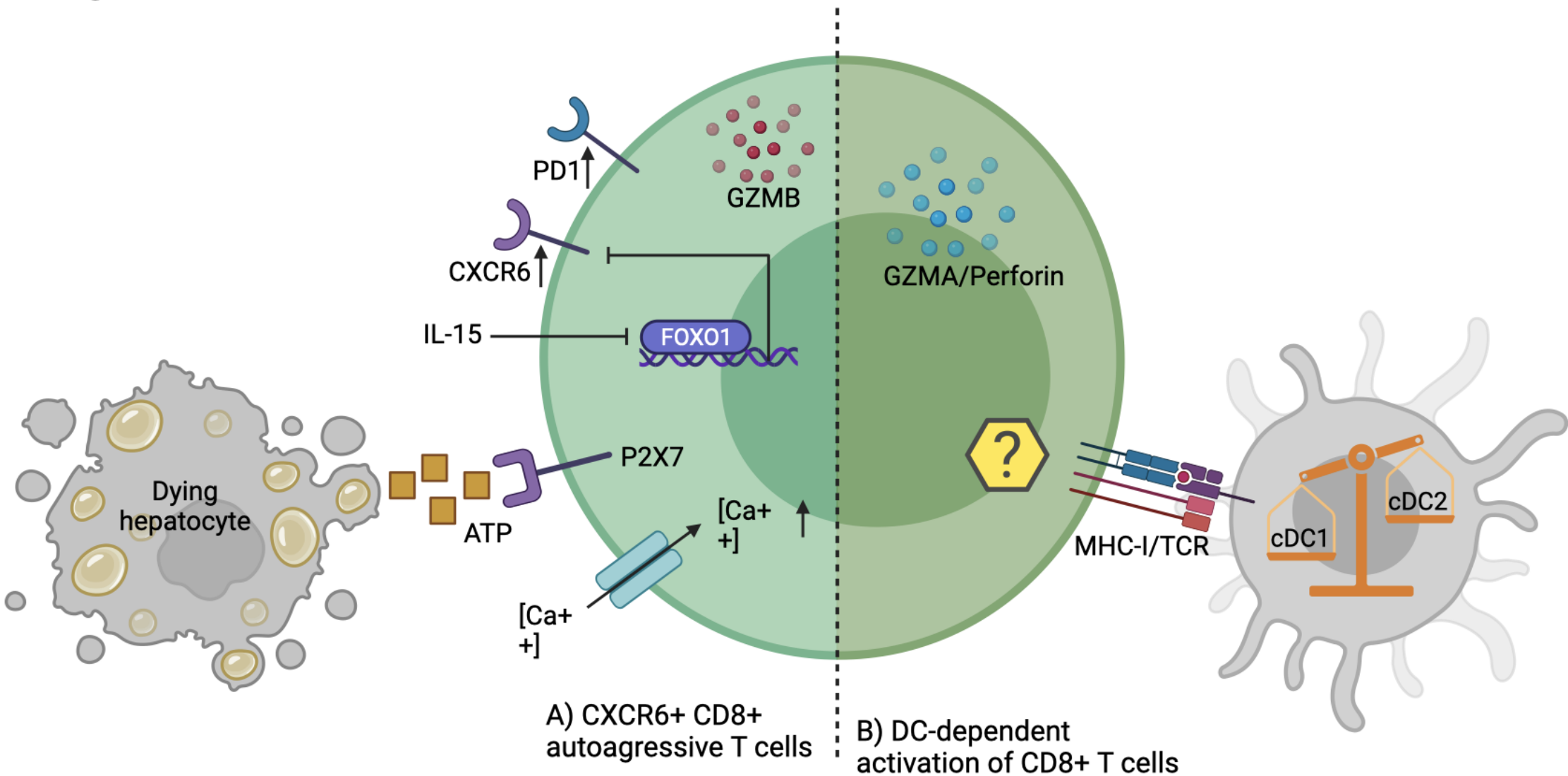
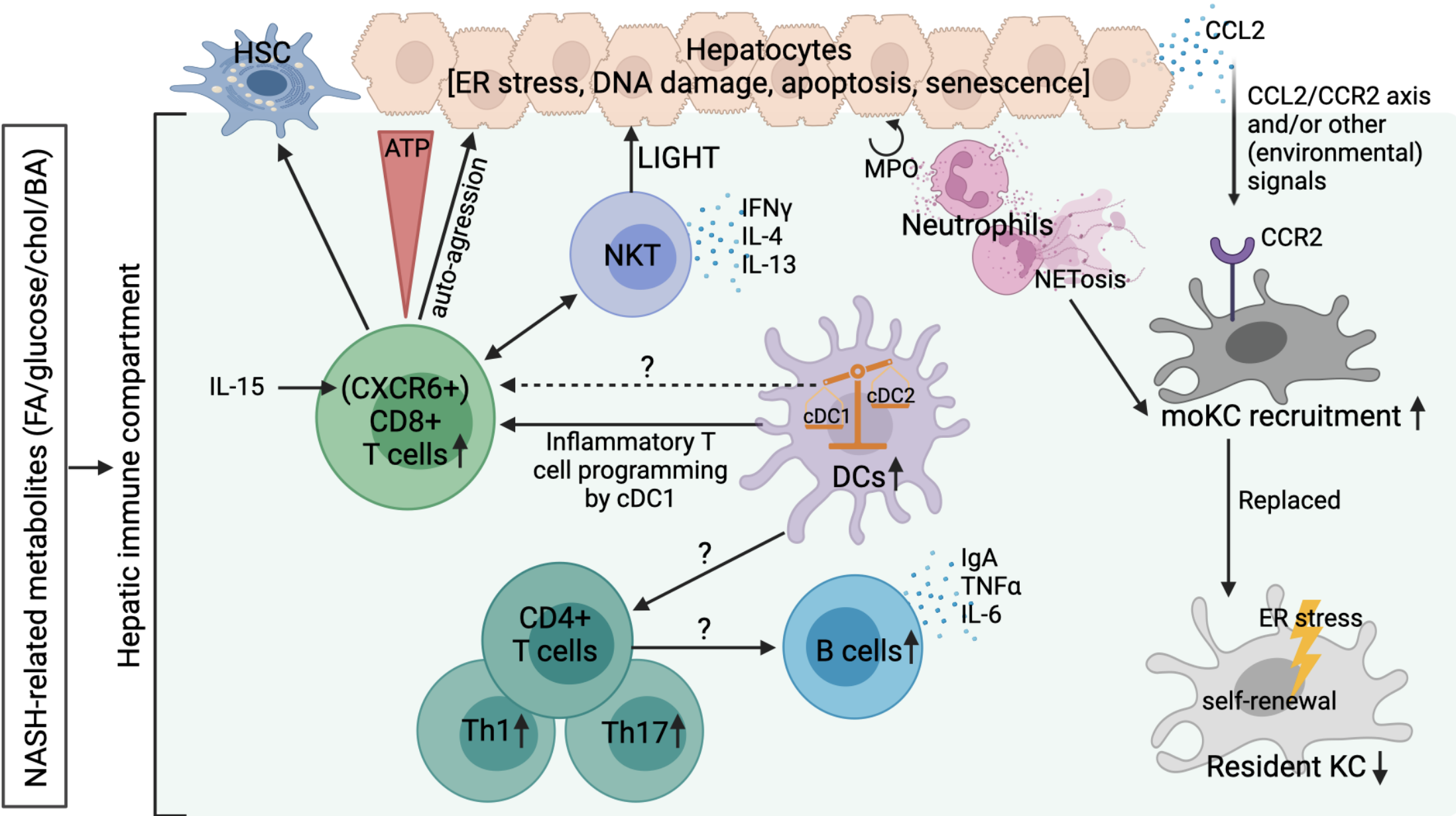


Figure 5



## 1 Highlights

2

3 • The altered metabolic environment in non-alcoholic fatty liver disease (NAFLD) promotes  
4 hepatocyte metabolic dysfunction and cellular stress, affecting the highly diverse hepatic  
5 immune compartment.

6 • Resident macrophages are activated in the early phase of liver injury and disease-  
7 promoting metabolic and tissue environmental signals alter their gene expression  
8 programs resulting in an identity loss.

9 • In a NASH environment, cDC and CD8<sup>+</sup> T cell populations are altered. CD8 cells with an  
10 “exhausted phenotype” are activated and kill hepatocytes, hence promoting tissue  
11 damage.

12 • The phenotype and function of hepatic immune cells is reshaped during the progression  
13 of NAFL to non-alcoholic steatohepatitis (NASH), but the role of the cellular interactions  
14 in disease progression is only starting to be uncovered.

1 Outstanding questions

2

3 1. How does the dysregulation of the macrophage compartment impact NASH

4 development?

5 2. Does an altered cDC1/cDC2 ratio rather than absolute numbers play a role in NASH?

6 3. Is antigen-dependent activation of CD8 T lymphocytes playing a role in NASH?

7 4. Do various B cell subsets play a differential role in NASH development?