STATE-OF-THE-ART REVIEW

Liver macrophages and inflammation in physiology and physiopathology of non-alcoholic fatty liver disease

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Non-alcoholic fatty liver disease (NAFLD) is the hepatic manifestation of metabolic syndrome, being a common comorbidity of type 2 diabetes and with important links to inflammation and insulin resistance. NAFLD represents a spectrum of liver conditions ranging from steatosis in the form of ectopic lipid storage, to inflammation and fibrosis in nonalcoholic steatohepatitis (NASH). Macrophages that populate the liver play important roles in maintaining liver homeostasis under normal physiology and in promoting inflammation and mediating fibrosis in the progression of NAFLD toward to NASH. Liver macrophages are a heterogenous group of innate immune cells, originating from the yolk sac or from circulating monocytes, that are required to maintain immune tolerance while being exposed portal and pancreatic blood flow rich in nutrients and hormones. Yet, liver macrophages retain a limited capacity to raise the alarm in response to danger signals. We now know that macrophages in the liver play both inflammatory and noninflammatory roles throughout the progression of NAFLD. Macrophage responses are mediated first at the level of cell surface receptors that integrate environmental stimuli, signals are transduced through multiple levels of regulation in the cell, and specific transcriptional

Abbreviations

AP-1, activator protein 1; BMDM, bone marrow-derived macrophages; CCL, chemokine ligand 2; CCR, C-C motif chemokine receptor; CD, cluster of differentiation; CD-HFD, choline-deficient high-fat diet; CETP, cholesteryl ester transfer protein; CLEC4F, C-type lectin domain family 4 member F; CXCL, C-X-C motif ligand; CXCR, C-X-C chemokine receptor; DAMP, damage-associated molecular patterns; DR5, death receptor 5; ER, endoplasmic reticulum; GLUT4, glucose transporter type 4; GM-CSF, granulocyte-macrophage colony-stimulating factor; HCC, hepatocellular carcinoma; HFD, high-fat diet; HIF, hypoxia-inducible factor; HRG, histidine-rich glycoprotein; HSC, hepatic stellate cell; IFN, interferon; IGFBP7, insulin-like growth factor-binding protein 7; IL, interleukin; iNOS, inducible nitric oxide synthase; Insr^{-}/$^{-}$, insulin receptor knockout; IRF, interferon regulatory factor; IκB, inhibitor of kappa-B; JNK, c-Jun N-terminal kinase; KC, Kupffer cell; LDLR, low density lipoprotein receptor; LPS, lipopolysaccharides; LSEC, liver sinusoidal epithelial cells; LXR, liver X receptor; Ly6, lymphocyte antigen; MAPK, mitogen-activated protein kinase; MCD, methionine-choline deficient; MCP-1, monocyte chemoattractant protein 1; M-CSF, macrophage colony-stimulating factor; Mo-, monocyte-derived; MP, macrophage; MUP-uPA, methionine adenosyl transferase 1A knockout mouse with high transient expression of urokinase plasminogen activator in hepatocytes; MyD88, myeloid differentiation primary response 88; NAFLD, non-alcoholic fatty liver disease; NAM, Nash-associated macrophage; NASH, nonalcoholic steatohepatitis; NF-κB, nuclear factor kappa-B; PD-L1, programmed death ligand 1; PI3K, phosphoinositide 3-kinase; PPAR, peroxisome proliferator-activated receptor; PTPROt, protein tyrosine phosphatase receptor type O truncated isoform; RHM, recruited hepatic macrophages; scRNA-seq, single-cell RNA sequencing; SREBP1c, sterol regulatory element-binding protein-1c; STAT, signal transducer and activator of transcription; STING, stimulator of interferon genes; T2D, type 2 diabetes; TGF, transforming growth factor; T$_H$, helper T-cell; TH/TR, thyroid hormone/thyroid hormone receptor; TIM4, T-cell immunoglobulin and mucin domain containing 4; TLR, Toll-like receptor; TNF, tumour necrosis factor; T$_{reg}$, regulatory T cell; TREM2, triggering receptor expressed on myeloid cells; UPR, unfolded protein response; WD, Western diet; XBP1, X-box binding protein 1.

programmes dictate effector functions. These effector functions play paramount roles in determining the course of disease in NAFLD and even more so in the progression towards NASH. The current review covers recent reports in the physiological and pathophysiological roles of liver macrophages in NAFLD. We emphasise the responses of liver macrophages to insulin resistance and the transcriptional machinery that dictates liver macrophage function.

Introduction: Inflammation and metabolic decline in non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) is the most common form of chronic liver disease with an estimated worldwide prevalence of 25% [1,2]. NAFLD is the hepatic manifestation of metabolic syndrome and common comorbidity of type 2 diabetes (T2D), obesity and hypertension. Indeed, around 55% of patients with T2D also have NAFLD [3]. Metabolic and inflammatory disturbances are important parts of the aetiology of NAFLD and of its comorbidities [4,5].

Non-alcoholic fatty liver disease represents a spectrum of conditions ranging from fatty liver, relatively benign steatosis in the form ectopic lipid storage, to nonalcoholic steatohepatitis (NASH) where inflammation and tissue remodelling can impair tissue function and whole-body metabolism. NASH represents the last reversible step of NAFLD, before progression to hepatocellular carcinoma (HCC) [6,7] (Fig. 1).

Over recent decades, considerable progress has been made in understanding the mechanisms of NAFLD development and progression [5]. An important milestone was published by Day and James in 1998 when they put forward their 'two-hit' hypothesis. In this hypothesis, steatosis was considered the first hit and inflammation the second, causing progression through the spectrum of NAFLD towards NASH [8].

Given that T2D and NAFLD are frequent comorbidities, a relationship with insulin sensitivity or secretion was sought in the earliest studies [9]. Initial clinical work found associations between insulin resistance and NAFLD, even in the absence of frank T2D (compromised insulin secretion). Glucose disposal and insulin sensitivity were also found to be progressively impaired going from healthy subjects, to patients with steatotic livers and then in patients with NASH [10,11]. At the cellular level, insulin resistance also contributes to steatosis through two main mechanisms: increased hepatic *de novo* lipogenesis [12] and ectopic lipid storage in response to systemic dyslipidaemia

[13]. Dyslipidaemia arises early in disease course from increased lipolysis in adipose tissue [13].

When hepatocytes reach their lipid storage threshold, lipotoxicity and hepatocellular stress lead to apoptosis [14,15]. Lipid overload and insulin resistance are associated with endoplasmic reticulum (ER) stress and the unfolded protein response (UPR) [15]. Physiologically, X-box binding protein (XBP)-1 mRNA splicing responds to ER stress and promotes cell survival by increasing ER protein folding capacity [16]. However, XBP-1-mediated cell survival fails in NAFLD, resulting in hepatocellular stress, inflammation, further loss of insulin sensitivity and apoptosis [17,18].

The above step is key in initiating inflammation and the transition from benign steatosis to NASH. The initial inflammatory response is largely mediated by tissue-resident macrophages [19]. Upon inflammatory signalling, tissue-resident macrophages recruit other immune cells from circulation, including monocytes that differentiate into macrophages *in situ* and amplify inflammatory signalling [20]. When this cycle is sustained under chronic hepatocellular stress, a macrophage pro-resolution response is also initiated. The resolution of inflammation is beneficial in response to acute inflammation; however, in response to chronic inflammation in the liver the resolution phase is associated with excessive deposition of collagen in extracellular matrix [21]. Fibrosis in later stages of NASH, from excessive collagen deposition, is the result of an exuberant scarring response, which over time significantly remodels the tissue and impedes liver function [21,22].

Macrophages are central to the progression of NAFLD, and their proliferation, differentiation and polarisation are tightly controlled and dependent on extracellular stimuli as well as intracellular signalling cascades [23]. While initially acting as sentinel cells, macrophages are also very important effectors cells that secrete cytokines and chemokines, influencing cells in the microenvironment. This review covers the mechanisms of how liver macrophages undergo activation and contribute to the development and progression of NAFLD. Given the importance of insulin resistance in
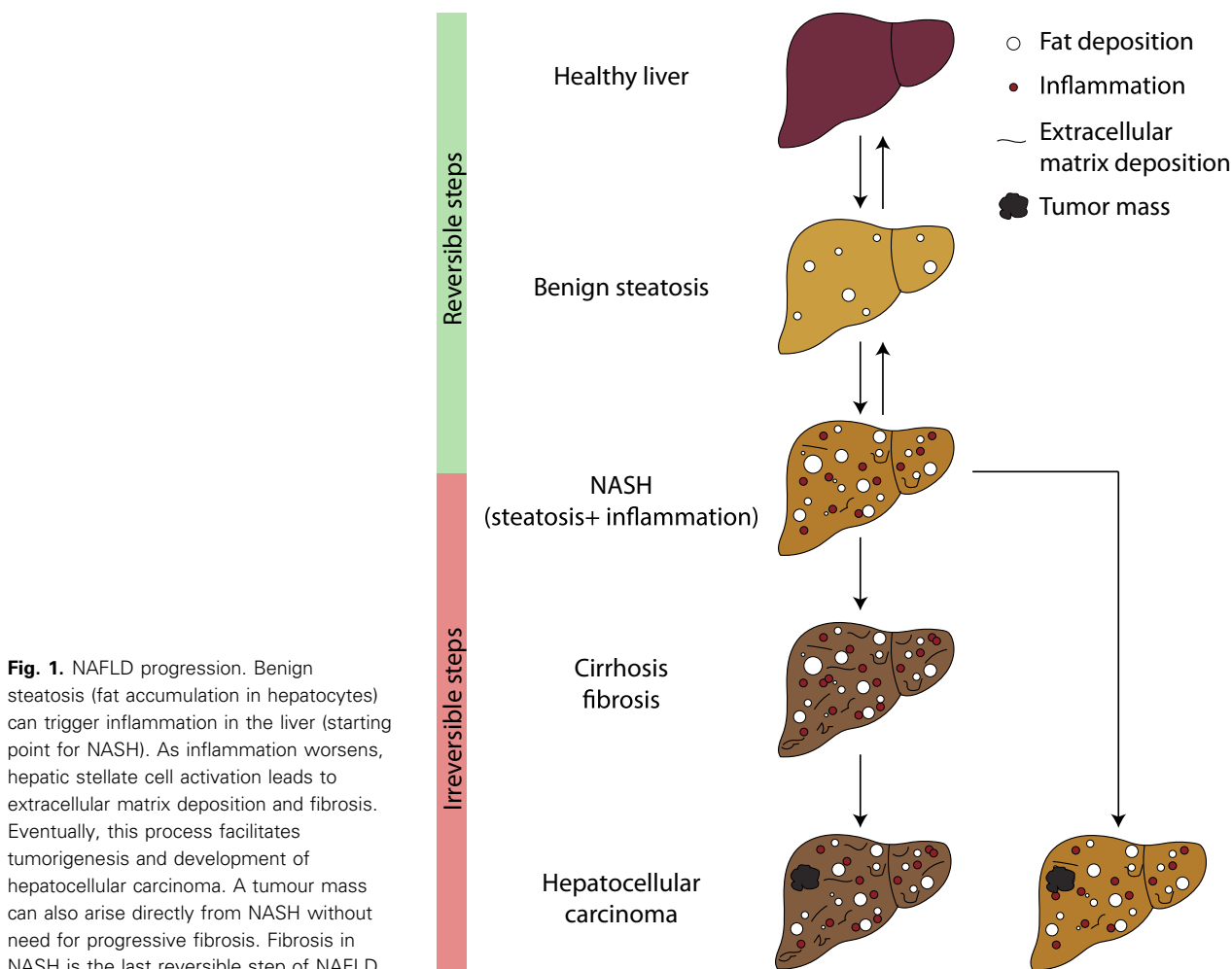
Fig. 1. NAFLD progression. Benign steatosis (fat accumulation in hepatocytes) can trigger inflammation in the liver (starting point for NASH). As inflammation worsens, hepatic stellate cell activation leads to extracellular matrix deposition and fibrosis. Eventually, this process facilitates tumorigenesis and development of hepatocellular carcinoma. A tumour mass can also arise directly from NASH without need for progressive fibrosis. Fibrosis in NASH is the last reversible step of NAFLD.

the pathogenesis of disease, we also address the role of insulin signalling, and insulin action on liver macrophages.

Insulin signalling and NAFLD

Insulin is an anabolic hormone secreted by pancreatic beta cells and is widely recognised for its role in regulating glucose homeostasis, lipid metabolism and cell growth. The effects of insulin are mediated through the insulin receptor [24,25] and the insulin-like growth factor 1 receptor [26]. When insulin binds to its receptor it activates two major downstream pathways: the phosphoinositide 3-kinase (PI3K) pathway and the mitogen-activated protein kinase (MAPK) pathway [26,27]. The PI3K pathway mediates insulin's metabolic effects including the translocation of glucose transporter (GLUT)-4 in metabolic tissues such as muscle, liver and adipose [26], while the MAPK pathway regulates mitogenesis and growth [27]. Recently,

the insulin receptor has also been shown to directly interact with transcriptional machinery, an additional mechanism for effects in normal physiology and disease [28].

Insulin resistance is the term given to the lack of an appropriate response to physiological levels of insulin, typically determined through systemic metabolic measures such as blood glucose. Insulin resistance is a precursor syndrome to T2D and its comorbidities [29]. In humans, patients presenting with NAFLD are often insulin resistant; however, it is unclear whether insulin resistance is compensatory rather than causal – the challenges to addressing this important question have been recently reviewed [30]. Various murine models of NAFLD have been proposed (detailed below), and in order to reproduce human disease, the model applied will ideally display obesity, insulin resistance and NAFLD concurrently [31]. One of the early mouse models investigating insulin's function, through global targeted disruption of the insulin receptor (Insr$^{-/-}$

mouse), reported liver steatosis and hepatic insulin resistance. The model initially exhibits dramatic metabolic insulin resistance, which is followed by age-dependent morphological and functional changes in the liver [32,33]. This early model suggested that changes in insulin sensitivity are sufficient to initiate NAFLD [32,33].

Insulin and macrophages

While macrophages are less associated with the roles of insulin compared with the majority cells of the metabolic tissues, macrophages do express insulin receptors and downstream intracellular signalling pathways [34,35]. *In vitro* studies investigating the direct effects of insulin on macrophages have shown insulin to have a profound effect on macrophage activation including inflammatory or M1-like polarisation (Table 1) or anti-inflammatory or M2-like polarisation (Table 2). Discrepancies in reports may be due to a lack of consistency in the model of macrophage investigated (different species/tissue type/cell line), concentration or duration of insulin used. The effects of insulin on macrophages are seemingly wide-ranging and thus may reflect macrophage plasticity and ability

Table 1. Evidence supporting pro-inflammatory role of insulin in macrophages. Where insulin resistance is anti-inflammatory.

Model	Summary	Ref
Cell lines ML-1, THP-1, PL-21	Insulin enhances LPS-stimulated IL-1β	[333]
Cell line THP-1	Insulin upregulates TNFα	[334]
Mouse myeloid/ macrophage insulin resistance	Protects against atherosclerosis	[37]
Mouse myeloid/ macrophage insulin resistance	Protects against obesity-induced inflammation	[335]
Human macrophages	Insulin promotes foam cell formation	[336]
Mouse insulin-resistant macrophages	Attenuation of atherosclerosis, promotion of M2-type phenotype when stimulated with pro-inflammatory cytokines	[337]
Mouse macrophages	Insulin and IL-1β synergistically promote inflammation	[338]
Diabetic mouse bone marrow-derived macrophages	Insulin increases TNFα and IL-6 secretion in LPS-stimulated macrophages	[339]
Mouse macrophages	Insulin resistance promotes M2-like phenotype and reduced LPS responses	[340]

Table 2. Evidence supporting anti-inflammatory role of insulin in macrophages. Where insulin resistance is pro-inflammatory.

Model	Summary	Ref
Rat peritoneal macrophages	Insulin enhances phagocytosis capacity and production of H_2O_2	[341]
Obese human mononuclear cells	Insulin inhibits NFκB and stimulates IκB	[342]
Cell line THP-1	Insulin inhibits apoptosis	[343]
Cell line THP-1	Insulin inhibits apoptosis and reduces TNF and IL-1β	[344]
Rat macrophages	Insulin suppresses LPS-induced iNOS and COX-2 expression and NK-κB activation	[345]
Mouse myeloid/ macrophage insulin resistance	Increased macrophage apoptosis and atherosclerotic plaque necrotic core formation	[36]
Mouse insulin-resistant macrophages	Increased macrophage apoptosis	[346]
Cell line THP-1	Insulin pretreatment delays endotoxin mediated macrophage activation	[347]
Mouse insulin-resistant macrophages	Increased LPS IL-1β production	[348]
Mouse insulin-resistant macrophages	Enhanced monocyte adhesion	[349]
Mouse insulin-resistant macrophages	Enhanced vascular wall adhesion and pro-inflammatory mediator adhesion	[350]
Mouse insulin-resistant macrophages	Increased apoptosis	[351]
Cell line RAW264.7 and high fat fed mice +insulin	Reduced foam cell formation, down-regulation of pro-inflammatory cytokines, decreased serum pro-inflammatory mediators and macrophage infiltration	[352]
Mouse insulin-resistant macrophages	Increased atherosclerosis through IFNγ-regulated macrophage network	[353]
Mouse macrophages	Insulin promotes IL-10 expression and attenuates LPS-induced Tnf-α, Il-1β and iNOS expression	[354]
Rat macrophages	Insulin advances infiltration and resolution of macrophages	[355]
Diabetic mouse alveolar and peritoneal macrophages	Insulin reduces TNFα, IL-6 and IL-1β secretion in LPS-stimulated macrophages	[339]
Mouse macrophages	Insulin resistance impairs M2a activation	[356]
Rat macrophages and cell line THP-1	Insulin polarises macrophages to M2 phenotype under high glucose conditions	[357]
Rat macrophages	Insulin restores abnormal macrophage infiltration, promotes efferocytosis and induces M1 to M2 transition	[358]

to respond to the fluctuating nature of blood insulin levels.

Insulin and liver macrophages

Surprisingly, while the impact of macrophages on NAFLD development is appreciated, and the significance of insulin resistance on macrophages in cardiometabolic diseases such as atherosclerosis are recognised, studies investigating the specific role of insulin-resistant macrophages on NAFLD have yet to be reported [36–38]. In obesity-induced insulin resistance in mice, distinct subpopulations of hepatic macrophages, with Kupffer cells (KC), secrete high levels of chemokine ligand (CCL)-2/monocyte chemoattractant protein (MCP)-1. CCL2/MCP-1 acts to recruit 'recruited hepatic macrophages' (RHMs). RHMs in turn enhance the severity of obesity-induced inflammation and hepatic insulin resistance [39]. Recently, Morgantini *et al.* [40] have shown that in obesity-induced insulin resistance in flies, humans and mice; liver macrophages produce noninflammatory factors including insulin-like growth factor-binding protein (IGFBP)-7 that can bind to the insulin receptor, directly regulating liver metabolism independently of inflammation.

Macrophages in liver physiology

There are two major types of hepatic macrophages: monocyte-derived and tissue-resident macrophages. KCs, *bona fide* liver-resident macrophages, are by far the most abundant in the healthy liver. In mice, KCs are identified by their expression of the pan-macrophage marker F4/80, low expression of CD11b, as well as by expression of specific markers such as the C-Type Lectin Domain Family 4 Member F (CLEC4F) or T-Cell Immunoglobulin and Mucin Domain Containing 4 (TIM4) [41–43]. Their development occurs during embryogenesis, from yolk-sac precursors that populate the foetal liver [44–47]. Like other tissue-resident macrophages, KCs are thought to persist in adult mice by self-renewal [48]. From surveillance, to recycling iron and promoting immune tolerance, KCs play important homeostatic roles in normal liver physiology (Fig. 2A).

KCs are located in liver sinusoids, and they continuously survey blood for metabolites and microbial products [41]. Mice lacking KCs show impaired survival following *Listeria monocytogenes* infection, emphasising their importance for the depletion of blood-borne bacteria [49,50]. Similarly, KCs remove damaged or apoptotic cells [51,52].

KCs also have important roles in iron and cholesterol metabolism. They are able to detect and phagocytose damaged erythrocytes and erythrocyte-derived vesicles containing haemoglobin [53,54]. KCs also influence iron reabsorption by regulating hepatocyte hepcidin expression [55]. With regard to cholesterol, all macrophages metabolise lipids, as required by their canonical function of phagocytosing cellular debris and processing lipid-rich elements such as membranes. However, relative to other tissue-resident macrophages, the KC transcriptome is enriched with genes that uptake, process and export cholesterol to extracellular high-density lipoprotein acceptors [42]. Indeed, KCs highly express cholesteryl ester transfer protein (CETP) amongst other genes in lipid processing, which are controlled by well-known transcription factors that regulate cellular lipids (e.g. PPARs, LXR) [42,56]. Physiologically, KCs may require this high lipid processing capacity to cope with dynamic cholesterol synthesis in the liver or to cope with exposure to systemic lipids packaged into lipoproteins in the liver. While KCs are clear drivers of inflammation in NAFLD, [57] their activation spectrum remains to be defined (in the context of M1-/M2-like polarisation), similarly questions remain unanswered with regards to their capacity to accumulate lipids, such as adipose or vascular foams cells, and with regard to their persistence in later stages of NAFLD [58–61].

Immunologically, KCs promote immune tolerance by diverse mechanisms, and their capacity to present antigens and activate T cells is very limited [62]. In mice, as well as in humans, KCs secrete anti-inflammatory cytokines such as interleukin (IL)-10 [63,64]. They also express co-inhibitory molecule Programmed Death Ligand (PD-L)-1, a potent inhibitor of T-cell activity [64]. They can also induce regulatory T-cell (T_{Regs}) differentiation through secretion of prostaglandins [62,64]. Monocyte-derived macrophages (Mo-MPs) or RHMs can also populate the liver and differentiate from (C-C motif chemokine receptor (CCR)-2$^+$ C-X-C 3 chemokine receptor (CX3CR)-1$^+$ lymphocyte antigen (Ly)-6C$^+$ monocytes. Mo-MPs account for a minority of macrophages in the healthy liver [65] but can be rapidly recruited upon liver injury [66] and can persist in chronic diseases such as NAFLD. Like KCs, they are important for erythrocyte clearance and iron recycling during homeostasis [67]. More recently, a third type of macrophage was reported in the liver capsule and these capsular macrophages are derived from bone marrow and play a role in peritoneal-derived pathogen clearance [68].
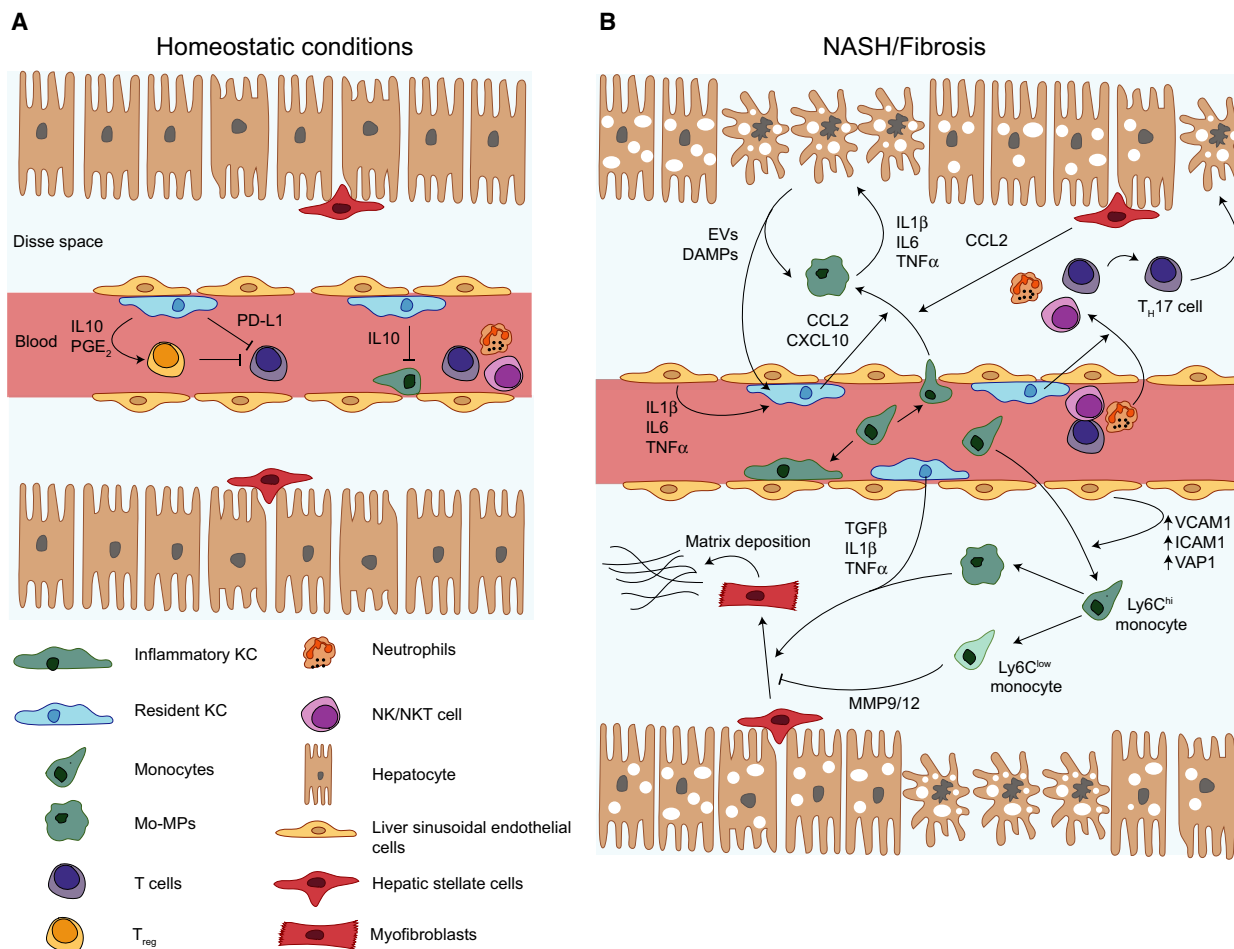
Fig. 2. Cellular crosstalk during NASH. (A) In homeostatic conditions, Kupffer cells (KCs) inhibit monocyte and macrophage recruitment through interleukin 10 (IL-10) secretion. They also promote immune tolerance from T cells by inducing regulatory T cells (T_{reg}) and expressing programmed death ligand 1 (PD-L1). (B) During NASH, apoptotic hepatocytes release danger-associated molecular patterns (DAMPs) that activate KCs. Activated KCs secrete chemokines recruiting monocytes to the liver. Monocytes differentiate into macrophages in situ and produce pro-inflammatory cytokines which drives hepatocyte death and reinforces their pro-inflammatory phenotype. Liver macrophages are also fuelling inflammation by promoting recruitment of other immune cells and T_H17 polarisation. Additionally, KCs and recruited $Ly6C^{hi}$ monocytes can trigger hepatic stellate cells (HSCs) activation through cytokine signalling. HSCs differentiation into myofibroblasts leads to production of extracellular matrix and fibrosis. On the contrary, $Ly6C^{low}$ monocytes are able to inhibit this process. EV, extracellular vesicle; ICAM1, intercellular adhesion molecule 1; MMP, metalloproteinase; Mo-MPs, monocyte-derived macrophages; NK, natural killer; NKT cells, natural killer T cells; PGE_2, prostaglandin E_2; TGFβ, transforming growth factor β; TNF-α, tumour necrosis factor α; VCAM1, vascular cell adhesion molecule 1; VAP1, vascular adhesion protein 1.

Modelling NAFLD physiopathology

To study NAFLD physiopathology and allow the isolation of different cell fractions, including macrophages, murine models are indispensable. Modelling NAFLD in mice comes with its challenges and opportunities. Resistance of mice to spontaneously develop NAFLD upon high-fat feeding had initially led scientists to develop various models that recapitulate isolated events in the disease. In this light, a high-fat diet (HFD) can recapitulate simple steatosis and insulin

resistance, while carbon tetrachloride (CCl_4) induces inflammation and fibrosis without steatosis and a methionine-and-choline–deficient (MCD) diet results in fibrosis, inflammation and steatosis without insulin resistance [31,69,70]. Similarly, surgical ligation of the bile-duct induces cholestatic injury, inflammation and fibrosis in mice, without insulin resistance [71]. Genetic models such as the widely adopted *ob/ob* or *db/db* mice can recapitulate obesity, insulin resistance and to a slight degree liver inflammation, but do not progress beyond steatosis [69]. These above models may be

considered extreme and can only be interpreted as models due to their lacking holistic systemic representation of NAFLD and its comorbidities.

More holistic models exist today that recapitulate a larger part of the NAFLD spectrum, such as the choline-deficient HFD (CD-HFD), high fructose-HFD (HF-HFD) or genetic-based models including the sterol regulatory element-binding protein-1c (SREBP1c) transgenic mouse, methionine adenosyl transferase 1A knockout mouse with high transient expression of urokinase plasminogen activator in hepatocytes (MUP-uPA Tg) and the DIAMOND model [69]. These models, and others, have been recently reviewed in-depth by Febbraio *et al.* [69]. Briefly, through different mechanisms, these models have been shown to recapitulate obesity, insulin resistance, steatosis, inflammation, ER stress and fibrosis in NASH, including a transition towards HCC [69]. Of these models, CD-HFD is gaining popularity, where the lack of choline prevents cholesterol export from hepatocytes, resulting in lipotoxicity and progression of NAFLD. Mice on CD-HFD develop obesity, insulin resistance, glucose intolerance and NAFLD. However, whether the status of other tissues is modified by the lack of choline has not been investigated. The MUP-uPA model recapitulates obesity, insulin resistance and glucose intolerance on HFD, where the key mechanism of hepatocyte ER stress (due to uPA overexpression) leads to mice consistently developing NASH, and up to 85% spontaneously progressing to HCC [69,72].

Choice of model and understanding the mechanisms by which NAFLD and NASH develop are critical to correct interpretation of results. In the case of macrophage responses, most models recapitulate the inflammatory hit, to varying degrees, and thus most are applicable. However, results must always be interpreted within the constraints and contexts of the given model, especially in the case of toxic models of fibrosis (CCl$_4$) or in models of global knockout or knockin (e.g. *ob/ob*, *db/db* or SREBP1c Tg).

Macrophages in NAFLD physiopathology

Macrophages are drivers of NAFLD, and human studies show positive correlations between macrophage numbers in the liver and NAFLD severity [73,74]. In mouse models, early depletion of KCs prevents progression of the disease, as well as insulin resistance [75–77]. In macrophage-depleted mice, IL-1β production was decreased while levels of the protective factor peroxisome proliferator-activated protein (PPAR)-α

was increased in hepatocytes [78,79]. Additionally, preventing monocyte entry into the liver through CCR2 blockade improves NASH [74,80] and it is now widely accepted that monocyte recruitment and *in situ* differentiation into macrophages fuels NAFLD progression. In NASH, monocytes replace a fraction of the KC pool by differentiating into monocyte-derived KCs (Mo-KCs) [42,43] which express KC markers, but are functionally different. Mo-KCs express more inflammatory genes potentially contributing to disease progression [43].

Macrophages are at the heart of intense cellular crosstalk in NAFLD, interacting with many liver cell types (Fig. 2B). Macrophages recognise hepatocyte-derived Danger-Associated Molecular Patterns (DAMPs), they secrete cytokines that may alter hepatocyte physiology and promote NAFLD progression [79]. Macrophage-derived cytokines also target hepatic stellate cells (HSCs). Tumour necrosis factor (TNF)-α, IL-1β and transforming growth factor (TGF)-β can all induce HSC activation [81,82]. In turn, HSCs up-regulate several ligands able to attract macrophages and regulate their activity (like CCL2) in NASH [83]. Liver sinusoidal endothelial cells (LSECs) drive anti-inflammatory polarisation of macrophages and down-regulate cytokine and chemokine secretion through nitric oxide production [84]. However, LSECs can also promote monocyte infiltration and contribute to liver inflammation [85,86]. Finally, macrophages can interact with other immune cells. Cytokine secretion by activated T cells can then reinforce macrophage pro-inflammatory phenotype in a feed-forward loop [87]. Additionally, chemokine secretion by activated macrophages leads to recruitment of several immune cell types in the liver [88].

Macrophage subtypes in the liver

The optimisation of single-cell RNA sequencing (scRNA-seq) in recent years has allowed the more precise identification of macrophage subpopulations. Several macrophage subpopulations have been defined in NAFLD. One study identified KCs and three different populations of Mo-MPs. While it is surprising to see the presence of such Mo-MPs already under a normal diet, these cells were enriched in mice fed a western diet (WD). They express less calprotectin, a marker of inflammation, in WD-fed mice, suggesting that these subsets may be protective [65]. Another study in amylin diet-induced NASH showed both KCs and Mo-MPs displayed a pro-inflammatory phenotype compared with controls. Two KC subsets were identified and segregated based on triggering receptor expressed

on myeloid cells (TREM)-2 expression. TREM2-low KCs were predominant in mice fed a normal diet. TREM2-high KCs were almost exclusively found in NASH and were therefore called 'NASH-associated macrophages' (NAMs) [83]. These two reports independently identified different functionally and phenotypically diverse subsets of macrophages in NAFLD. Interestingly however, subsequent reanalysis demonstrated that similar macrophage subsets were found in sequencing data from both studies. This highlights the divergent views in the field with regard to the identity of resident macrophages [89]. Specifically addressing fibrosis, two populations of Mo-MPs have been described based on Ly6C expression. Ly6C^{hi} cells are pro-fibrogenic and express cytokines that are able to activate HSCs such as IL-1β and TGFβ whereas Ly6C^{low} cells derived from Ly6^{hi} monocytes promote resolution of fibrosis and express matrix-degrading factors such as metalloproteinases [90].

Macrophage polarisation in NAFLD

Depending on microenvironmental cues, macrophages display different functional phenotypes. Typically, macrophages have been divided into classically activated M1-like or alternatively activated M2-like macrophages. Macrophages adopt a M1 phenotype in response to Toll-like receptor (TLR) stimulation for example with lipopolysaccharide (LPS), and in response to type 1 cytokines such as interferon (IFN)-γ. These macrophages produce pro-inflammatory cytokines such as IL-1β or TNF-α and are potent antigen presenting cells that are able to induce T_H1/T_H17 cell responses. As a consequence, they are involved in inflammatory responses and are responsible for pathogen killing. On the contrary, M2 macrophages secrete anti-inflammatory cytokines such as IL-10 or TGF-β and elicit T_H2/T_{Reg} responses. They respond to extracellular pathogens, are more tolerogenic and are associated with resolution of inflammation and tissue repair [91].

In a number of tissues, including the liver, M1 macrophages are generally characterised by the expression of CD11c, while CD206 is the common marker of M2 macrophages [92]. With increasing mechanistic studies and novel technologies, this framework has now developed to offer a more comprehensive representation of macrophage heterogeneity and functional diversity. For example, functionally diverse M2 macrophage subtypes have now been identified: M2a, M2b, M2c and M2d [93]. The emergence of transcriptomics and scRNA-seq technologies has further refined that description by emphasising the diversity of tissue macrophages and the highly spectral nature of macrophage polarisation [94,95].

Macrophage polarisation is an important parameter influencing NAFLD progression. Reports with regard to polarisation of liver macrophages in metabolic diseases have been conflicting. While high-fat diet (HFD) has been predominantly associated with M1 polarisation of liver macrophages [58,96], other groups have recently demonstrated that liver macrophages regulate systemic metabolism upon HFD through predominantly noninflammatory signalling [40]. Impaired M2 polarisation was associated with impaired hepatic lipid metabolism and steatosis [97]. In addition, M2 macrophages were shown to induce M1 macrophage apoptosis [96]. Overall, studies suggest that pro-inflammatory macrophages are mostly detrimental in NAFLD while anti-inflammatory macrophages are protective, this remains highly dependent on the stage of NAFLD progression. Indeed, studies have pointed out detrimental roles for M2 macrophages, for example during long-term HFD [60,98]. Polarisation state has different effects depending on disease stage, for example M2-like macrophages may mitigate inflammation in the early stages of NAFLD but promote matrix deposition and fibrosis at later stages.

# Molecular mechanisms of macrophage polarisation in NAFLD

Molecular drivers of macrophage inflammatory status

Macrophage polarisation, and more generally the role of macrophages in liver inflammation during NAFLD, is controlled by multiple pathways and transcription factors. The activity of these transcription factors is triggered by molecular cues coming from the liver but also from other organs of the body. While diverse in nature, in the context of NAFLD, these cues can be divided into two main categories: DAMPs and cytokines, both of which act through cell surface receptors and lead to transcriptional reprogramming in macrophages (Fig. 3).

TLRs and TLR ligands in liver macrophage polarisation

Toll-like receptors are transmembrane proteins expressed by cells of the innate immune system, notably macrophages, and that are activated in response to DAMPs, thirteen TLRs have been identified in mice [99]. Several TLRs play important roles in macrophages during NAFLD progression. TLR4, which
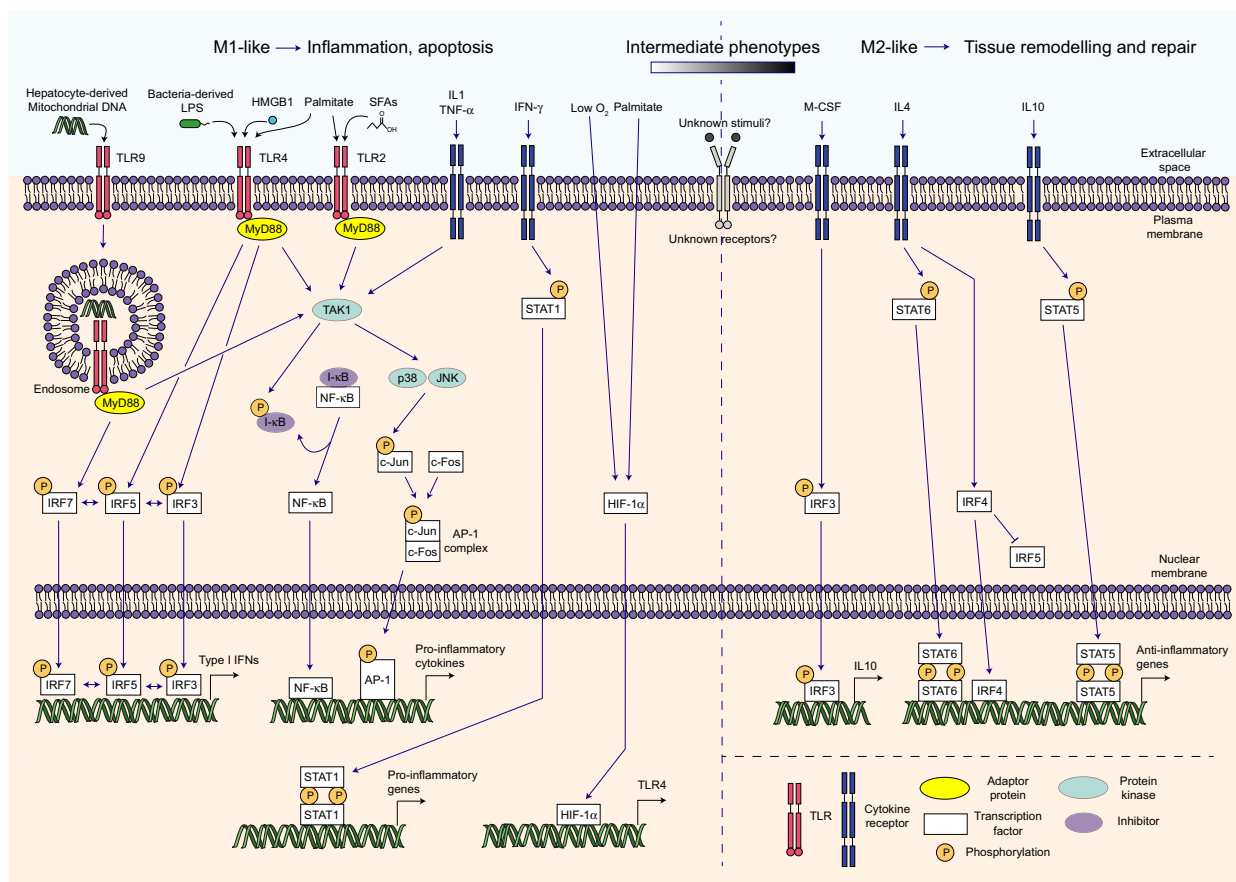
Fig. 3. Transcriptional control of macrophage polarisation. Toll-like receptor (TLR) stimulation by different ligands leads to activation of different intracellular pathways. Mitogen-activated protein kinase kinase kinase 7 (TAK1) activation ultimately leads to phosphorylation of I-κB and c-Jun. Phosphorylation of I-κB enables release of NF-κB and its translocation to the nucleus. c-Jun phosphorylation triggers formation of the activator protein 1 (AP-1) complex through association with c-Fos. NF-κB and AP-1 can then launch transcription of pro-inflammatory genes and cytokines. TLR4 and TLR9 ligation additionally triggers activation of interferon regulatory factor (IRF) 3, 5 and/or 7 and subsequent type I interferon (IFN) production. Cytokine signalling also drives M1-like polarisation, notably through activation of TAK1 and Signal Transducer and Activator of Transcription (STAT) 1. M2-like phenotype is mainly driven by cytokine signalling leading to activation of STAT5/6 and IRF3/4. *In vivo*, a spectrum of intermediate phenotypes exists and it is likely still unknown signalling pathways are involved in this differentiation process. HIF-1α, hypoxia-inducible factor 1α; HMGB1, high-mobility group box 1; JNK, c-Jun N-terminal kinase; M-CSF, macrophage colony-stimulating factor; MyD88, myeloid differentiation primary response 88; SFAs, saturated fatty acids.

responds to bacterial LPS, is pivotal in KCs activation. Steatosis promotes lipid accumulation in macrophages, which become more sensitive to LPS-mediated TLR4 stimulation, promoting inflammation [100]. This is of particular relevance since microbial dysbiosis and microbial products coming from the gut are increasingly shown to potentiate NAFLD [101]. Other TLRs and their ligands play a part in macrophage polarisation during NAFLD. Dying hepatocytes, for example, release DAMPs that are recognised by TLRs. Histidine-rich glycoprotein (HRG), a protein that is abundantly produced by hepatocytes, induces proinflammatory cytokines in macrophages, even though

the receptor and transduction pathway implicated are yet to be discovered. As a result, HRG-deficient mice were partially protected against steatohepatitis induced by a MCD diet [102]. Hepatocyte-derived mitochondrial DNA and extracellular vesicles also trigger a proinflammatory phenotype through respective ligation of TLR9 and Death receptor 5 (DR5) and therefore also drive NAFLD progression [103,104]. Finally, free fatty acids, especially saturated fatty acids, have been associated with macrophage-driven inflammation. Saturated fatty acids induce IL-1 and inducible nitric oxide synthase (iNOS) expression in macrophages *in vitro* through TLR4-dependent nuclear factor (NF)-κB

activity while unsaturated fatty acids inhibit this process [105]. In another report, palmitate was shown to induce IL-1 expression after TLR2 stimulation [106]. TLR4-palmitate interaction in infiltrating macrophages has also been shown to drive NAFLD, indicating that different macrophage subsets may have specificities in recognising different sources of free fatty acids [107].

Cytokine signalling and liver macrophage polarisation

During NAFLD, activated KCs become pro-inflammatory and secrete cytokines such as IL-1β, IL-6 or TNF-α which promote inflammation and monocyte recruitment to the liver [108]. Several cytokines play key roles in NAFLD. Early TNF-α secretion by KCs drives hepatic steatosis and inflammation [108,109]. Similarly, IL-1β is a potent driver of NAFLD and produced by KCs in the early phases of the disease [108,110]. It has an important role in promoting monocyte recruitment, inflammation and steatosis [79,110]. In addition, both TNF-α and IL-1β, as well as TGFβ, promote liver fibrosis by activating HSCs and promoting their survival [81,82]. IL-6 has a more complex role in the course of NAFLD. It seems to have a protective effect against liver injury and hepatocyte death but may promote NASH progression and fibrosis at high levels [111,112].

Other cytokines favouring M1 polarisation, while less studied, also contribute to NAFLD progression [113]. Interestingly, IFNγ deficiency has been associated with decreased production of pro-inflammatory cytokines, decreased inflammation and fibrosis in a mouse model of NASH [114]. Granulocyte–macrophage colony-stimulating factor (GM-CSF) promotes M1 polarisation and subsequent fibrosis in a model of virus-related fibrosis, suggesting that it could also play a similar role during NAFLD [115]. However, M2-polarising cytokines such as IL-10 also have a role in NAFLD. IL-10 production was reported in livers from mice fed a HFD alongside pro-inflammatory cytokines. Its blockade is associated with increased TNF-α and IL-1β levels and impaired insulin sensitivity [116].

Transcriptional control of liver macrophage polarisation

Transcriptional control downstream of TLR ligation

In response to their respective ligands, TLRs can trigger different intracellular pathways. TLR3 and TLR4 activate NF-κB, Activator protein (AP)-1 and Interferon

Regulatory Factors (IRF)-3 and -5 while TLRs 7, 8 and 9 activate IRF7 instead of IRF3 (Fig. 3) [99].

In quiescent macrophages, NF-κB activity is hindered by inhibitor of κB (IκB). Upon TLR stimulation, phosphorylation of IκB releases NF-κB, and NF-κB then translocates to induce transcription of target genes [117]. NF-κB is a key regulator of M1 polarisation [118,119]. It is responsible for production of pro-inflammatory cytokines such as IL-1, IL-6, IL-12 or TNF-α [118]. During NAFLD, NF-κB seems to have an important role in triggering inflammation. Indeed, one upstream regulator of NF-κB, glucocorticoid-induced leucine zipper (Gilz), has been shown to be down-regulated in macrophages during NAFLD. Its overexpression in macrophages results in decreased pro-inflammatory cytokine secretion and decreased hepatic inflammation [120]. Decreased activity of NF-κB due to loss of Protein tyrosine phosphatase receptor type O truncated isoform (PTPROt) activity in liver macrophages is also associated with decreased inflammation [121].

AP-1 is a complex formed of 2 proteins, c-Jun and c-Fos. After TLR stimulation, AP-1 is activated through c-Jun phosphorylation by MAPK, specifically by p38 and c-Jun N-terminal kinase (JNK). AP-1 and NF-kB are closely linked and regulate similar transcriptional programmes [122]. JNK has been shown to promote M1 polarisation of adipose tissue macrophages, while pro-inflammatory cytokine production in response to palmitate *in vitro* is JNK-dependent [123,124]. Studies in other macrophages populations indicate a strongly pro-inflammatory role for AP-1, yet its role and regulation in liver macrophages remains to be entirely elucidated. A recent study has reported that c-Jun/AP-1 plays different roles in hepatocytes and in nonparenchymal liver cells (NPLCs, a significant proportion of which is macrophages) [125]. Schulien *et al* report that while expression in hepatocytes correlated with transition from steatosis to NASH, c-Jun expression in NPLCs specifically correlated with fibrosis. In hepatocytes, c-Jun promotes survival, preventing the regenerative ductal reaction and fibrosis, whereas in NPLCs c-Jun promotes ductal regeneration and fibrosis through regulating both osteopontin and CD44 expression [125]. Whether this mechanism is mediated wholly or in part by macrophages remains to be demonstrated. A recent study of macrophage-specific p38 deficiency demonstrated that, as canonically described, p38 maintains its pro-inflammatory actions in liver macrophages and this promotes the progression of NASH [126].

IRF3 has a more complex role in macrophage polarisation. BMDM differentiated with Macrophage Colony-Stimulating Factor (M-CSF) display are

predominantly M2-like and activate IRF3 [127]. Its overexpression in microglia blunts the production of IL-1β and TNF-α in response to IL-1β and IFNγ, while boosting IL-10 secretion [128]. However, IRF3 also triggers the expression of pro-inflammatory factors CCL5 [129] and IFN-β, as well as CXCL9 and CXCL10 [130]. Studies showed that the Stimulator of Interferon Genes (STING)-IRF3 cascade is activated in livers of mice fed a HFD [131]. Upon inactivation of STING in myeloid cells, inflammation and steatosis are decreased, suggesting a potential role for this axis in regulating liver macrophage inflammatory status [132]. Likewise, IRFs 5 and 7 has been associated with M1 polarisation in response to LPS [133,134]. Following TLR signalling, IRFs 3, 5 and 7 are responsible for type I IFNs production [99,135]. Type I IFNs are increasingly regarded as important players in NAFLD. In particular, they have been shown to induce T-cell recruitment, secretion of pro-inflammatory cytokines and subsequent insulin resistance [92,136].

Other transcription factors, such as hypoxia inducible factor (HIF)-1α, can be indirectly involved in TLR-mediated macrophage polarisation, HIF-1α is stabilised and activated in response to hypoxia [137,138]. It was shown to induce M1 polarisation *in vitro* and to have an important role in macrophage function, both under normoxic or hypoxic conditions [139,140]. TLR stimulation can also induce HIF-1α activity though transcriptional control by NF-κB [141,142]. Liver macrophages from mice fed a MCD diet have enhanced HIF-1α expression. Mice overexpressing HIF-1α in myeloid cells display increased levels of pro-inflammatory cytokines and increased steatosis compared with controls, both under a chow diet or MCD [143,144]. Moreover, palmitate was shown to induce HIF-1α activity [143,144]. These results suggest a macrophage-specific role for TLR-induced HIF-1α in liver inflammation and pathology in NASH. A role for HIF-1α in liver fibrosis has also been reported even if the precise mechanisms still remain to be elucidated [145]. Additionally, HIF-1α is able to increase TLR4 in response to hypoxia, thereby sensitising macrophages to LPS stimulation [137]. Hypoxia has been reported to happen during NAFLD [146], which suggests that HIF-1α may also increase hepatic inflammation indirectly through macrophage sensitisation to TLR ligands.

Transcriptional control through cytokine signalling

Cytokines such as IFN-γ, IL-1β, IL-4 or IL-10 can activate different transcription factors to orient macrophage polarisation, among which NF-κB, AP-1 or members of the IRF and Signal Transducer and Activator of Transcription (STAT) families.

Interferon regulatory factors

The IRF family comprises nine members of transcription factors [147]. IRFs 3, 5 and 7 have critical roles in M1 polarisation. GM-CSF-treated macrophages display an M1-like phenotype and highly express IRF5 in particular. Macrophages transfected *in vitro* with a siRNA targeting IRF5 lose their ability to produce pro-inflammatory cytokines like IL-12 in response to LPS [148]. Alzaid *et al.* showed that IRF5 is also metabolically responsive and its expression in macrophages during NAFLD was responsible for M1 polarisation and secretion of pro-inflammatory and pro-apoptotic mediators. This translated into liver inflammation, Fas-dependent hepatocyte death and fibrosis [92,149].

Other members of the IRF family can induce M1 polarisation. IRF1 expression is induced *in vitro* in macrophages treated with IFN-γ. IRF1 can then synergise with NF-κB to trigger IL-12, iNOS and IFN-β expression [150]. IRF8 is activated through the Notch pathway during LPS stimulation and is crucial for transcription of typical M1-related genes in this context [151]. It was shown to collaborate with other transcription factors, such as IRF1 or STAT1, to drive pro-inflammatory genes transcription in response to IFN-γ [152]. On the contrary, IRF4 has a clear role in M2 polarisation in response to IL-4. IRF4-deficient macrophages secrete more cytokines such as TNF-α, IL-6 but also IL-10 [153]. Lysine demethylase 6B (KDM6B) was shown to enhance IRF4 production in IL-4-stimulated macrophages, an event directly promoting M2 polarisation [154]. Additionally, IRF4 can suppress IRF5 activity by competing for binding to the Myeloid differentiation primary response 88 (MyD88), a crucial adaptor protein in TLR signalling [155].

Signal transducers and activators of transcription

The STAT family is composed of seven members [156]. STAT1 is phosphorylated and activated in response to IFN-γ, one of the canonical stimuli of M1 polarisation [157]. STAT1-deficient macrophages lose the induction of IFN-γ-activated genes such as inducible nitric oxide synthase (iNOS) or Class II major histocompatibility complex transactivator (CIITA) [158]. STAT1 also plays a key role in type I IFNs ability to induce M1 macrophages through STAT1:STAT2 dimers and IFN-stimulated gene factor 3 (ISGF3)

[159,160]. Likewise, STAT5 is classically regarded as inducing M1 polarisation in response to GM-CSF [127]. However, broader assessment of GM-CSF–responsive genes has revealed that STAT5 may in fact induce both M1- and M2-related genes, resulting in an intermediate phenotype [161]. STAT3 and STAT6 promote M2-like macrophage polarisation. STAT6-deficient macrophages lose their ability to respond to IL-4 [162] and the ability to induce a number of M2-related genes [160]. IL-6 and IL-10 induce STAT3, and STAT3-deficiency leads to greater accumulation of pro-inflammatory macrophages and susceptibility to inflammatory conditions, namely enterocolitis [163–165].

Nuclear receptors in gene regulation and as therapeutic targets in NAFLD and NASH

The nuclear receptor (NR) superfamily of transcription factors control transcription in response to specific ligands [166]. Agonists for these receptors range from hormones to vitamins, to fatty acids and cholesterol [167] as well as synthetic and pharmaceutical ligands that currently represent about 16% of all approved drugs [168]. NRs play important functions in regulating hepatic lipid and glucose metabolism as well as multiple inflammatory pathways and immune responses. As such, they are prime candidates to modulate NAFLD development [169]. Indeed, many NRs have shown promising potential as targets for anti-NAFLD therapeutics. To date, 48 NRs that share structural and functional characteristics have been described in humans [166]. Of these, 17 have been linked to NAFLD, either using synthetic ligands that target them in experimental models of disease or in models of global, hepatic- or, in few cases, myeloid-specific NR deficiency showing changes in liver steatosis and/or the development of steatohepatitis. For a detailed description of how these receptors function and the roles they play, we refer the reader to a recent comprehensive review [169]. Here, we focus on receptors that are or have been drug targets in clinical trials for NAFLD and NASH.

Thyroid hormone receptor β

Thyroid hormone (TH) receptor β (TRβ) is the TR isoform thought to be responsible for the main beneficial effects of TH on liver [170]. TRβ regulates gene expression by binding to TH response elements (TREs) in regulatory regions within target genes, mostly as heterodimers with the retinoid X receptor or RXR

[171]. Unliganded TR represses basal gene expression by recruiting a corepressor complex [172]. Ligand (TH) binding then leads to the dissociation of corepressors and favours the recruitment of coactivators promoting chromatin accessibility thereby increasing gene transcription [173]. In this manner, TR enhances the expression of genes involved in fatty acid metabolism [174]. TR also inhibits the expression of lipogenic genes promoting steatosis [175].

TH metabolism and TH status have been linked to various aspects of the immune response [176] and recent reports suggest that innate immune cells are important TH targets and that intracellular TH plays essential roles on several innate immune cell types, including monocytes and liver macrophages [176]. Functional studies have shown TH pro-inflammatory actions in macrophages. A shift towards an M1 phenotype alongside an inhibition of M2 polarisation was reported in bone marrow-derived macrophages [177]. Intriguingly, polarisation was associated with changes in $TR\alpha_1 : TR\beta_1$ ratio, suggesting the relative abundance of TR isoforms may be linked to macrophage phenotype [177]. However, this study contrasts with reports that found no effect on macrophage polarisation [178] and it has been speculated that this could be due to differences in the hormone concentrations used [179].

Some TH actions are mediated through signal transduction mechanisms, for instance, through cell surface integrins leading to PI3K activation followed by the iNOS upregulation, nitrite production and bacterial killing [180]. Other studies have concluded that higher levels of bioavailable TH increase macrophage phagocytic capacity [181]. The effects of intracellular TH are partly mediated via TRα [182] and unstimulated macrophages deficient in TRα show low-grade inflammation suggesting a TRα-mediated anti-inflammatory response [182]. TH stimulation leads to KC hyperplasia and enhanced phagocytosis [183]. TH has shown pro-inflammatory actions in KCs involving NFκB activation [184] and acute-phase responses in liver involving increased STAT3 activation [185], in turn increasing hepatic iNOS activity, enhancing production of reactive oxygen species and hepatic oxidative stress [186]. However, another study showed conflicting results in models of endotoxemia [187]. Clearly, more research is needed to establish whether similar mechanisms occur in other inflammatory contexts including NAFLD and to establish better *in vitro* models to replicate not only the inflammatory, but also dyslipidaemic environment in this disease.

Overall, THs have been shown to be beneficial for liver metabolism through: (a) an increase in energy

expenditure via ATP consumption, membrane permeability and effects on mitochondrial biogenesis and activation [188], and (b) lipid metabolism such as cholesterol clearance by LDLR, cholesterol biosynthesis and metabolism through regulation of CYP7A1, a key bile acid synthesis enzyme [189]. In addition, hypothyroidism has been considered a risk factor for NAFLD [190], while TH administration improves lipid profiles in experimental models of NAFLD [191]. Unfortunately, these beneficial effects are accompanied by thyrotoxicosis and harmful effects in the brain [192]. Work on animal models stresses the importance of TRs for the hepatic actions of TH [193]. Using individual TRα1 and TRβ, knockout mice treated with TH and dietary cholesterol showed that CYP7A1 regulation was lost only in TRβ knockout mice and that TH administration was not able to modulate cholesterol levels [194], suggesting a key role for TRβ. Consistently, TRβ mutant mice are unable to bind TH and develop liver steatosis [195].

As TRβ is the predominant isoform in liver, efforts have focussed on the development of TH analogs capable of uncoupling beneficial liver actions (triglyceride and cholesterol lowering) from deleterious effects [196]. TRβ agonists modulate lipid metabolism pathways and reduce hepatic steatosis and inflammation in animal models [197] as well as improve liver function in clinical trials in patients with NAFLD and NASH [191]. Unfortunately, Sobetirome (GC-1) and Eprotirome (KB2115), early examples of TRβ-selective thyromimetics showing encouraging effects against hypercholesterolemia and NASH, in the absence of adverse side effects, were stopped after Phase 1 and Phases 2–3 clinical trials, respectively [170]. Resmetirom (MGL-3196), another liver-directed and TRβ-selective agonist, successfully reduced steatosis and was advanced to Phase 3 trials. Other compounds are being evaluated in Phase 2 trials [198] or have shown promising preclinical effects [199]. These studies suggest that the most recent classes of thyromimetics are promising alternatives to existing NASH therapies.

Peroxisome proliferator-activated receptors

Peroxisome proliferator-activated receptors (PPARs) are a nuclear receptor subfamily with key actions on glucose and lipid metabolism as well as on inflammatory and fibrotic processes. Thus, PPARs are considered interesting NAFLD therapeutic targets for improving liver function and showing beneficial liver, cardiovascular and diabetes-related outcomes [200]. The role of PPARs in the development of NAFLD has been recently reviewed in detail [201].

PPARs were first described as ligand-activated transcription factors that promote peroxisome proliferation [202], and subsequently, they have been shown to be involved in function of other organelles, mainly mitochondria, showing pleiotropic actions [203]. Three PPAR isotypes have been described – α, β/δ and γ, with two subtypes: γ1 and γ2, and with each isotype showing a specific pattern of tissue and cell-type expression [203]. Additionally, substantial species-specific differences, especially for PPARα, exist and must be considered when translating findings from experimental models [204]. Specifically, PPARα activity in human liver is lower compared with rodents with reported differences in PPARα expression, ligand activation and biological responses [205].

PPARα

PPARα, encoded by the *NR1C1* gene, binds to several saturated and unsaturated fatty acids, whereas the other isotypes show affinity mostly restricted to polyunsaturated fatty acids [206]. PPARα is predominantly expressed in tissues with high fatty acid oxidation rates such as skeletal muscle, liver – mostly in hepatocytes – heart, kidney and brown adipose tissue [207]. Besides hepatocytes, PPARα is expressed in sinusoidal endothelial cells and in HSCs [208]. In the liver, this nuclear receptor acts as a nutrient sensor and its expression and activity are stimulated by fasting or a fat-rich diet [209]. PPARα functions as a transcription factor mostly as a heterodimer with RXR and, upon ligand binding, activates genes associated with mitochondrial and peroxisomal fatty acid oxidation [210]. PPARα can also repress gene expression, by interfering with the glucocorticoid receptor [211] or by tethering to other transcription factors [212]. Regarding NAFLD development, it is worth noting that a fat-rich diet elevates hepatic PPARα expression in a circadian rhythmic manner and that the lipid-lowering effect of a PPARα agonist is more prominent when PPARα expression peaks [212,213]. Additionally, PPARα dampens NASH fibrotic and inflammatory gene expression through protein–protein interactions with pro-inflammatory transcription factors NF-kB and AP-1 [210,214].

Multiple studies in preclinical models or PPARα-deficient mice show PPARα is a critical regulator of target genes involved in fatty acid metabolism and ketogenesis. Specifically, it regulates fatty acid transport, peroxisomal and mitochondrial β-oxidation and lipolysis, and influences the production of apolipoproteins [215]. This reduces triglyceride-rich lipoproteins

and triglyceride accumulation in the liver, whereas plasma HDL cholesterol is increased [215]. Consistently, preclinical studies show that deficiency in PPARα, either in global or liver-specific-deficient mice, leads to more severe NASH [216,217], which can be improved or prevented by specific PPARα ligands [217–219]. Interestingly, expression of a PPARα mutant that only shows transrepressive activity in mice confers protection against NASH but not steatosis, whereas mice expressing wild-type PPARα are protected from both NASH and steatosis [210] highlighting the importance of this activity in the overall effects of PPARα.

Considering that approximately 50% of PPARα target genes are conserved between mice and humans [220], it is relevant that this experimental evidence agrees with existing clinical findings (see below). Additionally, hepatic PPARα expression inversely correlates with severity in patients with NASH, visceral fat and insulin resistance, and improved liver histology positively correlates with increased PPARα expression [221]. Accordingly, PPARα was considered a promising therapeutic target for NAFLD, though the number of clinical studies evaluating single PPARα ligands is low [201,218]. Drugs of the fibrate class that predominantly act as PPARα ligands such as Clofibrate and Fenofibrate have been used clinically to treat hypertriglyceridemia, without affecting insulin sensitivity or hepatic steatosis [222–225]. Disappointingly, their effect on NASH was not proven [226,227], which could be due in part to the species-specific differences mentioned above. Exploiting the concept of selective PPAR modulators based on differences in receptor and coactivator binding, other fibrate compounds (Gemfibrozil, Pemafibrate) are currently being tested in clinical studies based on their promising clinical profiles [228–230]. In addition, targeting both PPARα and PPARβ/δ with Elafibranor has shown promising anti-NASH properties in a clinical trial [231], reporting improved glycaemic control and lipid profile, reduction in hepatic and muscle insulin resistance and steatohepatitis [232]. Recruitment was recently terminated on the phase III RESOLVE-IT clinical trial (NCT02704403) assessing Elafibranor for NASH resolution [233]. Results at termination of the study have not yet been published; however, interim analyses in May 2020 revealed a near significant ($P = 0.066$) resolution of NASH without worsening fibrosis in patients treated with Elafibranor compared with placebo [234]. Elafibranor was found to be safe and well tolerated, consistent with a previous study in biliary cholangitis that reported improvement in a number of disease markers [234,235].

PPARβ/δ

PPARβ/δ which is encoded by *NR1C2* and expressed in hepatocytes, sinusoidal endothelial cells, HSCs and KCs also has an important role in liver metabolism [236]. This receptor activates glucose utilisation, hepatic lipogenesis and lipoprotein metabolism, as confirmed by transcriptomic analyses in PPARβ/δ-deficient mice [237]. In addition, PPARβ/δ increases the production of monounsaturated fatty acids and protects against lipotoxicity and saturated fatty acid cytotoxicity *in vitro* [238]. It appears PPARα is predominant in the fasting state whereas PPARβ/δ is equally involved in both fasting and fed states [239].

PPARβ/δ also regulates the expression of key genes in innate immunity and inflammation [237,240]. In the absence of ligand, PPARβ/δ has pro-inflammatory effects mainly in atherosclerotic models. Ligand binding exerts anti-inflammatory effects, such as the suppression of pro-inflammatory adhesion molecules on endothelial cells [241,242]. PPARβ/δ ligands promote a more anti-inflammatory phenotype in KCs resulting in improved metabolic and hepatic dysregulation [97]. In addition, PPARβ/δ may have a potential role in wound healing, as its activation in fibroblasts increases α-smooth muscle actin production and myofibroblast differentiation [243,244]. Importantly, synthetic PPARβ/δ ligands mimic the endogenous activation of PPARβ/δ, although different responses have been reported for different ligands [245]. Additionally, the selective PPARβ/δ agonist Seladelpar improves dyslipidaemic lipid profiles in overweight or obesity patients at risk of CVD [246] although these compounds have not been as broadly tested as the fibrate PPARα agonists.

PPARγ

Finally, PPARγ is encoded by *NR1C3* and is expressed in the liver, yet less than adipose tissue where it is a master regulator of multiple metabolic pathways. As other PPARS, PPARγ forms a heterodimer with RXR to control gene expression. In addition, as shown in cistrome studies in peritoneal macrophages, macrophage lineage factors SPI1 (PU.1) and CEBPβ are present with PPARγ in regulatory sites [247,248], enhancing permissive chromatin configurations. In liver, PPARγ is induced by obesity in mice [249] although this is not seen in patients with NASH [221]. Hepatocyte-specific PPARγ deficiency protects mice from steatosis in diet-induced or genetic obesity in mice by reducing expression of genes promoting lipogenesis and lipid transport [250,251]. In contrast, PPARγ agonists, including antidiabetic thiazolidinedione drugs (TZDs), improve

NAFLD partly by reshuffling fatty acids and triglycerides to privilege storage in adipose tissue [252].

PPARγ is also present in macrophages, KCs and HSCs. In liver and other tissues, PPARγ binds to the p65 subunit of the NF-κB complex to dampen NF-κB-driven inflammatory gene expression [253]. A PPARγ sumoylation-dependent pathway was described to mediate some of the anti-inflammatory actions of this receptor [254]. In KCs, PPARγ agonists inhibit pro-inflammatory gene expression leading to lower inflammation and hepatosteatosis [58]. Consistently, inhibition of PPARγ with a specific antagonist promotes the M2c anti-inflammatory phenotype in human monocyte-derived macrophages [255], although despite the concomitant induction of MerTK expression, cells do not show enhanced efferocytosis. In HSCs, PPARγ is predominantly expressed in the quiescent state and lowered in the activated state. Ligand activation in these cells or in experimental models of fibrosis reduces collagen levels, but the mechanism underlying this regulation still need to be refined [256,257]. Finally, PPARγ also improves endothelial cell inflammation and function in patients with diabetes and atherosclerosis [258], controls vascular homeostasis and decreases blood pressure in patients with diabetes, leading to reduced CVD risk [259].

Hepatic PPARγ expression is elevated in patients with NAFLD and NASH [260], and PPARγ agonists are promising therapeutics. The TZD class of PPARγ agonist antidiabetics, including rosiglitazone and pioglitazone. TZDs ameliorate steatosis and inflammation, but have shown only minimal reduction in fibrosis [258,259,261–263]. A PPARα/γ dual agonist Saroglitazar improves cardiovascular risk profiles in diabetics [264,265] and after promising results in animal models of NASH [266] is being tested in a randomised clinical trial [258].

This subfamily of nuclear receptors represents a great example of how simultaneous activation of multiple isotypes could be a more efficacious therapeutic approach by targeting multiple pathways that contribute to the development and progression of NASH. Early studies with Lanifibranor (IVA337), which activates all three PPAR subtypes and acts on multiple NASH-affected pathways [267,268], showed it was effective at preventing and even inducing regression of pre-existing fibrotic lesions in different organs [269,270]. This occurred in the absence of deleterious effects of TZDs while improving insulin sensitivity and lipid profiles in NASH [267]. Remarkably, Lanifibranor actions on inflammation, fibrosis and macrophage accumulation and activation seem stronger than single and dual PPAR agonists in several models

of NASH [271]. Lanifibranor is now part of a phase IIb trial in patients with NASH without cirrhosis. To date, significant reductions in steatohepatitis, regression of fibrosis and improved glycaemic control and lipid profile have been reported [272], suggesting pan-PPAR agonism could have a strong therapeutic potential and be a promising therapeutic strategy for NASH.

Farnesoid X receptor

Farnesoid X receptor (FXR), encoded by *NR1H4* gene, whose expression is attenuated in NASH patients [273], was originally labelled as an orphan receptor and subsequently considered 'adopted' as free or conjugated bile acids were recognised to be endogenous ligands [274–276]. Its impact on the regulation of key aspects of metabolic, inflammatory and fibrotic pathways has been recently covered in detail [277]. FXR is highly expressed in liver [274] and acts, through FXR response elements, mainly as a heterodimer with RXR [278]. The liver receptor homolog-1 (LRH-1) is also present in a substantial number of FXR-binding sites and induces gene transcription mostly in lipid metabolic pathways [279,280]. Other studies have proposed direct transcriptional repression by FXR in the regulation of lipoprotein metabolism and as an important contributor to its anti-inflammatory effects through a motif independent of the canonical one [281–284].

FXR is a well-established regulator of bile acid homeostasis showing tissue-specific roles in the liver and intestine [285]. Upon activation, FXR reduces the levels of its ligands by suppressing bile acid synthesis through CYP7A1 [275], an example of a negative feedback regulatory loop. In addition, FXR is critical in regulating the enterohepatic circulation of bile acids by affecting the expression of several transporters [286–289] and in regulating lipid and glucose homeostasis. FXR activation lowers blood lipid levels as it inhibits fatty acid synthesis [290,291], decreases hepatic secretion of VLDL [292] and increases triglyceride hydrolysis and clearance as well as fatty acid oxidation [293–296]. Conflicting evidence exists regarding FXR actions in glucose homeostasis [277] which could be due to species differences between humans and mice [297,298]. Nevertheless, FXR likely plays an important role as FXR-deficient mice develop steatosis, show elevated circulating FFAs and glucose levels, and are insulin resistant [299]. In addition, FXR activation may improve glucose dysregulation, as either FXR activation or hepatic overexpression significantly lowers blood glucose levels and FFA levels, and improves

insulin sensitivity in both *db/db* diabetic and wild-type mice [300].

FXR is also a homeostatic regulator that suppresses liver inflammation and fibrosis. Pretreatment of HepG2 cells and primary hepatocytes with FXR agonists suppresses NF-κB-mediated inflammation in an FXR-dependent manner [301]. In NASH models, synthetic FXR agonists lower MCP-1 chemokine expression leading to significantly reduced hepatic inflammatory cell infiltration [301,302]. Moreover, FXR-deficient mice display strong hepatic inflammation in response to LPS, concomitant liver necrosis and a significant increase in inflammatory molecules such as iNOS, COX-2 and IFN-γ [301]. A growing body of evidence suggests that bile acids modulate intestinal and liver immune cells [303–305] and the role played by bile acid receptors has been reviewed in detail [303]. Briefly, FXR is expressed by circulating monocytes and both intestinal and liver macrophages [306]. FXR activation in human and rodent macrophages shows effective anti-inflammatory activities, and FXR is required for the TLR9-dependent inhibition of pro-inflammatory responses of intestinal macrophages [307]. Transrepression of inflammatory genes in macrophages by FXR ligands involves complex mechanisms that are both SHP-dependent and SHP-independent [306–308]. In addition, ligand-induced sumoylation of FXR has also been implicated in the regulation of NF-κB and AP1-driven gene expression [309]. Beyond NF-κB-mediated mechanisms, FXR may exert anti-inflammatory actions indirectly, for instance by reducing cholestasis and the levels of toxic bile acid production and accumulation in the liver [277].

Macrophage phenotypic shift has also been described for FXR. Treatment of an obese and diabetic mouse model of NAFLD mice with the semi-synthetic bile acid and FXR agonist obeticholic acid (OCA) improves liver histology and increases expression of M2 markers and the proportion of intrahepatic anti-inflammatory monocytes [310]. In addition, non-specific ligands for FXR also acting on another bile acid receptor [311], reverse liver steatosis and fibrosis along with markers of inflammation, shifting macrophage polarisation towards an M2-like phenotype [311,312]. Whether these modulatory effects of the hepatic immune system add to the metabolic effects of FXR ligands in the clinic requires further investigations. Moreover, FXR activation suppresses the development of hepatic fibrosis both by reducing fibrosis and by inducing antifibrotic gene expression in HSCs. HSC inactivation is also achieved by ligand-activated FXR inducing a transcriptional regulatory cascade involving other nuclear receptors, namely the small heterodimeric partner SHP and PPARγ [256,313,314].

Both steroidal and nonsteroidal FXR agonists have been developed for the treatment of liver diseases. Based on previous favourable results [315], OCA was investigated in the phase IIb Farnesoid X Receptor Ligand Obeticholic Acid in NASH Treatment (FLINT) multicentre trial in patients with noncirrhotic NASH [316]. OCA improved biochemical and histological features of NASH when compared with placebo without the worsening of fibrosis. Unfortunately, no difference was observed on the resolution of NASH and effects on ALP, lipids and blood glucose observed in the placebo group associated with weight loss were absent or even reversed in OCA-treated patients [317]. In addition, unfavourable dyslipidaemia occurred in the OCA treatment group [316]. OCA, FDA approved for biliary cholangitis therapy, was further evaluated in a NASH phase III trial REGENERATE [318], with a disappointing outcome [319]. Nevertheless, FXR remains an attractive target for NAFLD. For instance, safety and efficacy of the nonsteroidal FXR agonist Cilofexor (GS-9674) was evaluated in a phase II study for other liver conditions [320]. Cilofexor improved inflammatory biomarkers alongside significant reductions in serum markers of liver injury [320]. In a phase II trial in NASH noncirrhotic patients, Cilofexor significantly improved hepatic steatosis, liver biochemistry and bile acids without affecting serum lipids [321]. Moreover, Tropifexor significantly reduced oxidative stress, steatosis, inflammation and fibrosis in mouse models of NASH [322]. Time will tell whether this nonsteroidal FXR agonist also proves beneficial in NASH patients.

Closing remarks: Challenges and future perspectives

NAFLD is extremely complex, due to its multiple aetiologies and the large spectrum of liver states that ranges from steatosis to inflammation and fibrosis. The complexity of NAFLD is also present at the cellular and molecular levels, where we now know that macrophages play an important role in both maintaining normal physiology and in the pathophysiology of NAFLD. As reviewed, liver macrophages are central actors of NAFLD progression, they receive and respond to signals from systemic circulation, such as insulin or lipolysis products from adipose tissue, and they are exposed to nutrient-rich blood from portal circulation as well as a multitude of signals from the liver microenvironment. As part of the innate immune system, macrophages must act primarily as sentinels,

keeping the peace then raising the alarm when homeostasis is disturbed. Raising this alarm is a tightly regulated process where a number of molecular actors within the cell integrate afferent signals and coordinate efferent responses. The responses of these very important cells can dictate disease course in NAFLD. While recent decades have accumulated a wealth of knowledge, much is yet to be learned about how therapeutically target these cells in NAFLD.

In insulin dynamics, NAFLD and liver macrophages

Strong mechanistic associations have been drawn between insulin resistance and NAFLD, yet the physiological and physiopathological adaptations of liver macrophages remain to be fully understood. Insulin levels oscillate from low levels when fasting, to higher postprandial levels. Basal levels of insulin in the blood are also different in healthy people compared with people with prediabetes or diabetes (reviewed recently [323]). An added layer of complexity for investigating the physiological and pathophysiological levels of insulin on liver macrophage function include the location and action of the liver. The liver is immediately downstream of the pancreas and clears 40–80% of the insulin [324,325]. The amplitude of insulin's oscillations are thought to be ~ 100-fold higher in the portal vein than in the systemic circulation [326]. Therefore, even in health, liver macrophages are exposed to higher levels of insulin compared with other tissue-resident macrophages which may have consequences, affecting trained immunity in macrophages for example, these effects of insulin have been recently reviewed [327].

Macrophage heterogeneity and challenges in therapeutic targeting

The plasticity of macrophage terminal differentiation is now widely recognised, similarly macrophage polarisation states and effector functions now exist on a sliding scale with the classical and alternative states being at the extremes. The technical advances of recent years, namely single-cell sequencing and high-density cytometric methods, have allowed this appreciation of macrophage heterogeneity. Novel functional classification of macrophage subsets is an area of active research, booming in a number of fields and specially in study of the liver and pathogenesis of NAFLD [94,328]. Despite the currently wide application of these technologies, one technical hurdle that has been a long-standing subject of discussion in the field is the *in vivo* modelling of NAFLD [31]. Given the range of models available that reproduce the different components of NAFLD, macrophage populations are also very likely to vary across these numerous *in vivo* models. Future studies are working towards consistency and specificity with regard to the different models available and in the way that data are reported, with the multiple models being increasingly applied as mechanistic representations of different stages of disease. This trend enables a more thorough understanding of the importance of different macrophage subsets in NAFLD. Accordingly, it is necessary to decipher macrophage heterogeneity across different models of NAFLD and to understand cellular and molecular drivers of this intra- and inter-model heterogeneity. The respective role of embryonic KCs, inflammatory KCs and Mo-MPs, and their different subsets, in liver disease can now be investigated with great precision. Understanding macrophage heterogeneity in kinetic studies will also be of value. Such an approach will allow understanding of how cellular diversity arises, leading to the therapeutic targeting of detrimental subsets at the appropriate time without influencing other potentially beneficial subsets.

Challenges in translatability and NAFLD clinical evaluation

One of the most important milestones of research in NAFLD was the proposal of the two-hit hypothesis in 1998. Since then, clear experimental evidence has implicated insulin resistance, lipotoxicity and inflammation in the pathogenesis of NAFLD. Yet a number of well-known barriers exist in the field with regard to the translatability of certain findings from basic research to clinical practice, which in itself has clear priorities to improve staging and diagnosis of NAFLD.

The main barrier to translatability is in the complex modelling of NAFLD. Today no single murine model recapitulates the whole spectrum of NAFLD, the models applied will allow at best the integration of one or two stages, taking into account one or two factors. For example, a high-fat diet will robustly reproduce insulin resistance; however, the liver is not affected beyond simple steatosis. Diets deficient in certain amino acids (methionine/choline) may induce moderate steatohepatitis and when combined with high fat will also induce obesity and insulin resistance; however, the depletion of amino acids reduces the physiological relevance to human disease. Similarly, surgical (bile-duct ligation) and toxic (CCl_4) models that mimic steatohepatitis and fibrosis are far from physiological. Taking into account the above models and other

genetic models, reviewed by [31,69], the scientific community can still gain a lot of mechanistic insight with regard to discrete components of the disease that can be currently reproduced. While challenging to encapsulate the entire spectrum of NAFLD as well as its comorbidities, it is also of mechanistic value that these models allow the isolated study of the different components of NAFLD. The application of these models must in future be interpreted as such, until a holistic and physiologically relevant model is developed.

Two major clinical barriers that are areas of active work are the noninvasive staging and the detection of NAFLD. Currently, a very widely used and accepted staging method is the SAF score, which histologically grades steatosis, activity and fibrosis in NAFLD. However, establishing a SAF score requires an invasive biopsy and a recognised limit to this method is considerable heterogeneity in the staging of advanced fibrosis [329]. It is for this reason that a future priority in the field is the development of sensitive noninvasive virtual biopsies, notably through the application of imaging techniques (CT, MRI) in conjunction with serological and immunological parameters. A number of large-scale trials are currently tackling this issue (LITMUS, NIMBLE, QUID-NASH) [330–332].

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

RT, MCG, IP-T, GC, NV and FA wrote the manuscript.

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