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Comprehensive Understanding of Immune Cells in The Pathogenesis of Non−alcoholic Fatty Liver Disease*

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Abstract Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease, defined by several phases, ranging from benign fat accumulation to non-alcoholic steatohepatitis (NASH), which can lead to liver cancer and cirrhosis. Although NAFLD is a disease of disordered metabolism, it also involves several immune cell-mediated inflammatory processes, either promoting and/or suppressing hepatocyte inflammation through the secretion of pro-inflammatory and/or anti-inflammatory factors to influence the NAFLD process. However, the underlying disease mechanism and the role of immune cells in NAFLD are still under investigation, leaving many open-ended questions. In this review, we presented the recent concepts about the interplay of immune cells in the onset and pathogenesis of NAFLD. We also highlighted the specific non-immune cells exhibiting immunological properties of therapeutic significance in NAFLD. We hope that this review will help guide the development of future NAFLD therapeutics.

Key words non-alcoholic fatty liver disease, metabolically associated fatty liver disease (MAFLD), T cells, myeloid cells, mesenchymal stem cells

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Non-alcoholic fatty liver disease (NAFLD) has become one of the most prevalent chronic liver diseases worldwide[1], occurring due to high deposition of fat in the liver in the absence of secondary causes or other liver malignancies. NAFLD includes a wide range of conditions, including simple non-alcoholic fatty liver (NAFL), non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis, and frequent hepatocellular cancer (HCC)[1]. Up to 70% of those with type 2 diabetes melitus (T2DM) have NAFLD, and most obese people who undergo weight-loss surgery have NAFLD. Thus, it is possible to think of NAFLD as the metabolic syndrome's liver component. Considering that NASH is a growing cause of liver transplantation and that obesity, metabolic syndrome, and diabetes have reached pandemic proportions, it is reasonable to predict that the prevalence of NAFLD will rise significantly[1].

The liver is the primary immune organ and the largest metabolic organ. About 10%−20% of the total cells in the liver are immune cells, which include lymphocytes and liver-resident macrophages known as Kupffer cells (KCs) [2-3]. To maintain a distinct immunological tolerance milieu, the immune cells process the blood from the gastrointestinal system, which carries a variety of external antigens and occasionally infections under pathological circumstances[4]. However, the immunological microenvironment also changes when hepatocytes accumulate excessive fat along with changes in metabolic state. According to numerous studies, the

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pathophysiology of NAFLD and NASH was shown to be significantly influenced by immune cells in the liver[1]. Interestingly, some immune cells are recruited to the disease site during the NAFLD onset and secrete cytokines to influence NAFLD development. Recent studies have shown that injecting immune cells or cytokines has reversed the NAFLD development in NAFLD subjects[5-6].

Here, we have first provided an overview of the pathogenesis and clinical characteristics of NAFLD through histological evaluation. After outlining the immune environment of the resting liver, we addressed the impact of different types of immune cells in the progression and pathogenesis of NAFLD in addition to its immune cell-mediated elements, including the role of different immune populations in the evolution from NAFL to NASH. Notably, we also discussed how certain non-immune cells, such as hepatic stellate cells and mesenchymal stem cells, secrete factors that exhibit immunological properties and present therapeutic significance. Lastly, we concluded by providing an overview of the treatment approaches that are now being studied and may be available to treat NAFLD in the future.

1 Pathophysiology of NAFLD

1.1 Spectrum and prevalence of NAFLD

The malignancy of fatty liver in the absence of alcohol consumption was known as NAFLD for 44 years after Ludwig *et al.* [7] observed NASH during histological examinations of liver patients in 1980. NAFLD encompasses various metabolic abnormalities caused by fat accumulation, leading to hepatocellular steatosis, progressive inflammation, ultimately resulting in hepatocyte injury, necrosis, and fibrosis[8]. Recently, a consensus group of experts has proposed that key metabolic dimensions linked with hepatic steatosis may be more described as “metabolic dysfunction-associated fatty liver disease (MAFLD)”[9], without ruling out other etiologies of liver disease such as alcohol consumption or viral hepatitis[10]. However, there is debate in the field about this nomenclature switch, so until the matter is resolved, we will continue to use NAFLD. Notably, there is significant overlap in the definitions of MAFLD and NAFLD; as such, the terms cannot be used synonymously when discussing specific studies[11]. Moreover, NAFLD is being recognized by the American Association for the Study of Liver Diseases (AASLD), the Asian Pacific Association for the Study of the Liver (APASL), British Society of Gastroenterology (BSG), the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) and the European Liver Patient Association (ELPA) for non-alcoholic induced fatty liver pathology [1, 12-13].

Recent reports have shown that the incidence rate of NAFLD is increasing yearly, prevailing higher in males than females. Reports have shown that NAFLD has turned out to be a global disease burden due to the growing trends in sedentary lifestyles and dietary choices in the general population. NAFLD is thought to affect 25% of people, although this number is probably understated because there are no symptoms and few non-invasive diagnostic options (Table 1) [2-3]. According to the survey based on the different diagnostic modalities data (imaging tests, blood tests, liver biopsies, *etc*.) and NAFLD prevalence rate from 2010 to 2019[14], the incidence of NAFLD in Europe and the United States has increased by 36.1%. Similarly, in Asian countries, the number of patients with NAFLD has significantly increased in five years, accounting for 33.9% of the average population, where the prevalence rate of the disease has expanded from middle-aged to young adults [15-16]. Specifically, in China, the geographical distribution of NAFLD patients is alarming. For instance, the prevalence of NAFLD in Shanghai and Beijing has increased 20 times from 2006 (15.0%) to 2016 (31.0%). Based on this analysis, it is proposed that by 2030, the prevalence ratio may increase alarmingly, estimated affected NAFLD individuals more than 300 million in China, 100 million in the United States, and 15−20 million in major European countries[17]. Additionally, recent reports have shown that the global prevalence of NAFLD has risen to 32.4%, which has increased the mortality rate from 0.1% to 0.17%, speculating that NAFLD is the second leading cause of liver transplantation. Unfortunately, the catastrophe of metabolic risk factors has become a major global threat that may shift NAFLD in younger populations in the future[18].

More than 20 phase II and phase III clinical trials are currently being conducted to evaluate the safety and efficacy of potential NASH therapy options because, regrettably, there is currently no proven cure for NASH. Among them, at least 4 candidates are

Table 1 Global prevalence of NAFLD

Global/region of incidence	Prevalence of NAFLD (2019)
Global	~32.4%
North America	24%−48%
United States	~36.1%
South America	~30%
Asia	28%−32.4%
China	29%
Africa	~20%

targeted by immune cells to reduce inflammatory responses in the liver. However, multiple blood-based biomarkers have been identified along with measurements of liver stiffness that assist clinicians in detecting NAFLD patients who are at risk of NASH and liver failure. The identification and management of such patients have become a real challenge for emerging pharmacotherapies to limit NASH onset and its progression toward liver failure. Therefore, more knowledge on how liver immune cells alter during NAFLD and NASH development will help to develop novel treatment approaches for these conditions.

1.2 Pathogenesis of NAFLD

NAFLD is classified into two types: NAFL and NASH. NAFL is associated with fatty degeneration of the liver with negligible hepatocyte necrosis. NAFL is characterized by fat accumulation in the liver. About three perspectives describing excessive fat accumulation in the liver are currently thought to exist, including (1) increased lipolysis of visceral adipose tissue, (2) activation of *de novo* fat in the liver, and (3) high calorie or fat content in the diet[19].

NASH is manifested by steatosis, liver inflammation, and hepatocyte ballooning, with a high proportion of hepatic stellate cells in the activated state compared to NAFL, which is more likely to progress to liver fibrosis. During NASH, lipid accumulation in hepatocytes, oxidative stress, and inflammation work together to induce hepatocyte death, leading to liver damage, inflammation, and tissue fibrosis. During this time, immune cells surrounding the liver regulate the liver microenvironment, further regulating the onset and severity of NASH[20]. The progression of inflammation and fibrosis in NASH forms the basis for cirrhosis and HCC[8]. Statistics showed that approximately 10% of patients with NAFLD will develop cirrhosis and HCC ten years after diagnosis[21-22]. Although NAFLD progresses slowly in the majority of patients, it remains a significant challenge due to the large number of patients with NAFLD.

To better describe the degree of fibrosis in the liver of NAFLD patients due to post-inflammatory repair of hepatocytes, the panel gave a score for the degree of liver fibrosis in NAFLD patients as −1.675+0.037×age (years)+0.094×body mass index (kg/m²)+0.99×aspartate aminotransferase to alanine aminotransferase ratio −0.013×platelet count ($\times10^9$/L) −0.66×albumin concentration (g/dl) +1.13×impaired fasting glucose or diabetes (yes=1, no=0)[1]. A score greater than −1.455 indicates that a patient with NAFLD is at high risk of developing advanced liver fibrosis in the late stages of the disease, and a score greater than 0.675 suggests that the patient already has advanced liver fibrosis. The scoring system has a high accuracy in people over 35 years of age, and the accuracy of the assessment increases progressively between the ages of 35 and 65. There is no comprehensive and established theoretical system to explain the pathogenesis of NAFLD, but several factors have been observed as triggers of NAFLD.

Studies have shown that poor diet (high in oils and fats), specific occupations, and other metabolic disorders (Figure 1), such as chronic smoking and alcohol consumption, inadequate physical activity, obesity, excessive waist circumference, hyperlipidemia, hypopituitarism, T2DM and other factors (such as genetic factors, Figure 1) may contribute to NAFLD, like patients with low-density lipoprotein cholesterol (LDL-C), triglycerides (TG) and total cholesterol (TC). Accumulating evidence has shown that these all are associated in some way with NAFLD[23-24]. However, the presence of fatty liver has been observed in some patients with liver damage, for instance, alcohol-related liver disease, viral hepatitis, and autoimmune hepatitis[22]. A diet enriched in sugar and fat is one of the triggers of NAFLD. However, several studies have reported that NAFLD can also occur as a result of metabolic syndrome. Global epidemiological statistics show that more than half of people with T2DM and about 80% of obese people have NAFLD[25-26], but the prevalence of NAFLD is also being observed in healthy people, referred to as non-obese NAFLD[27].

Inflammation is a critical driver in the progression and development of fibrosis in NAFLD,

and it is involved in the entire progression of NAFLD, including the transition from NASH to cirrhosis with HCC [28]. The crucial role of inflammation in the NAFLD progression suggests that the liver environment is enriched with immune cells, regulating the onset and severity of NASH. Immune cells promote inflammation directly by secreting inflammatory cytokines such as tumor necrosis factor and interleukin-1 beta (IL-1β) or indirectly by activating neighboring immune and non-immune cells. Hence, based on the potential role of immune cells in the NAFLD progression, this review focuses on how the different types of immune cells and the inflammation interplay during NAFLD pathogenesis.

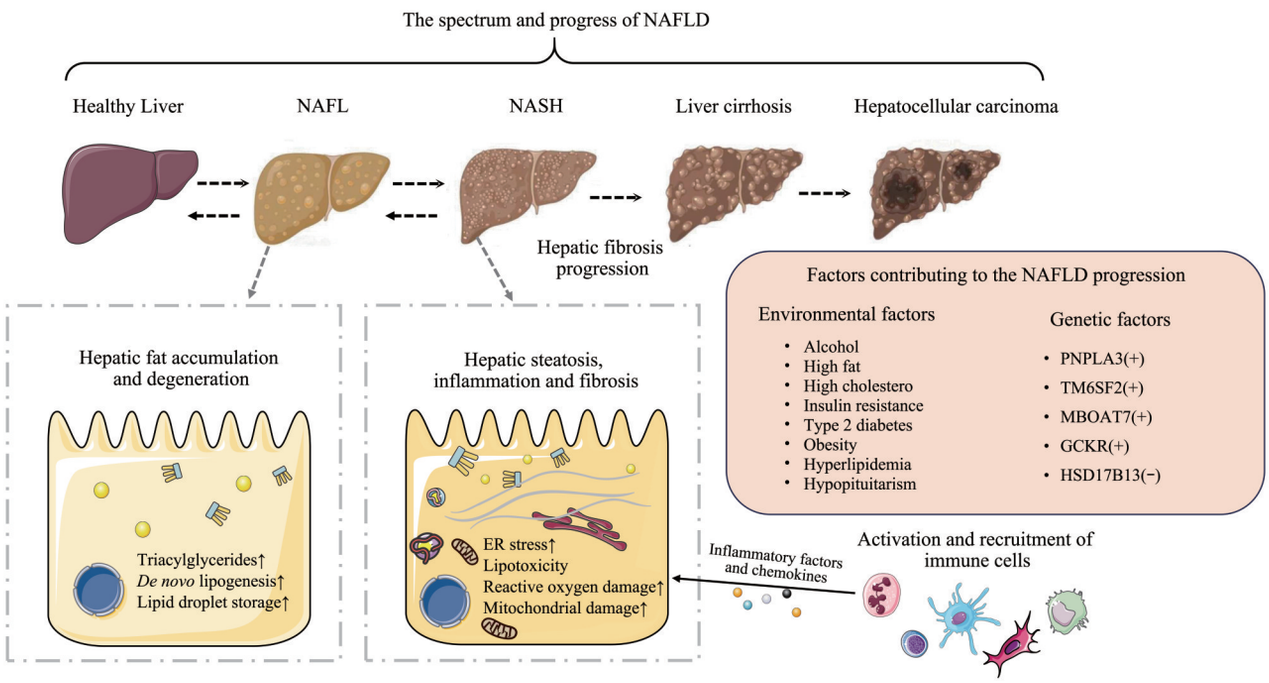

Fig. 1 The spectrum, factors and progress of NAFLD

NAFLD is a progressive disease that can progress from NAFL to a more complex form called NASH. NAFL is characterized by fat accumulation and liver degeneration, but without hepatocellular necrosis. NASH is characterized by steatosis, inflammation and fibrosis and can progress to cirrhosis and, in about 10% of patients, to HCC. Inflammatory mechanisms drive the development of NAFLD, while immune cells in the liver (*e. g.* macrophages) begin to repair the inflammation, and the ductal response is an important marker of liver repair after NAFLD. Both natural environmental and genetic factors can influence the course of NAFLD, such as obesity, alcohol abuse, insulin resistance, type 2 diabetes, hyperlipidemia, hypopituitarism and other comorbidities. Some genes, such as *PNPLA3*, *TM6SF2*, *GCKR* and other genes related to lipid metabolism, are involved in influencing the development and progression of NAFLD.

2 Hepatic immune cells in NAFLD

The liver, a metabolic and detoxifying organ, is also an essential immune organ[29], enriched in immune cells, primarily located outside the endothelial cells and in the central vein[4, 30]. At different stages of NAFLD, immune cells play different roles[29]. During the early stages of NAFLD, inflammatory mechanisms drive disease progression, while immune cells in the liver (*e. g.*, macrophages) begin to repair the inflammation, and the ductal reaction is an essential marker of liver repair after NAFLD[5-6].

The most common immune cells are natural killer (NK) cells, γδT cells, $CD4^+$ and $CD8^+$ αβT cells, monocytes, B cells, invariant natural killer (iNK) cells, mucosa-associated invariant T cells and dendritic cells (DCs). Of these, long-lived resident cells such as KCs, $CD8^+$ tissue-resident memory T cells (T_{RM}), and intrinsic lymphocytes of type 1 (ILC1) play an essential role in liver disease[20]. During the progressive transformation of NAFL to

NASH, these immune cells, accompanied by steatosis, induce hepatocyte inflammation, leading to hepatocellular damage, ballooning, and necrosis. In the inflammatory process, the accumulation of pro-inflammatory/anti-inflammatory immune cells (*e. g*., monocytes/macrophages, T-lymphocytes, neutrophils) and increased infiltration of the liver are observed along with the activation and *in situ* expansion of liver resident cells (KCs and hepatic stellate cells)[31]. Pro-inflammatory immune cells such as M1 macrophages and CD8 lymphocytes secrete pro-inflammatory cytokines such as tumor necrosis factor (TNF) and interleukin-6 (IL-6), causing insulin resistance and metabolic dysregulation in the organism.

Studies have shown that as NAFLD progresses, the levels of IL-6 in the serum and plasma increase, and a positive correlation is observed among leukocyte, monocyte, and IL-6 levels [32-33]. Secretion of pro-inflammatory factors and chemokines such as IL-1β and chemokine (CC-motif) ligand 2 (CCL2) by resident immune cells such as KCs and DCs further exacerbates inflammatory cell infiltration through oxidative stress, leading to a vicious cycle of apoptosis, releasing apoptotic signals to exacerbate inflammation in the liver environment[34-37]. However, the interplay of the immune cells in NAFLD remained a mystery. This is presumably due to the scarcity of clinical data and insufficient *in vitro* and animal models that make it challenging to replicate the human state fully and precisely. Additionally, the concomitant activation of immune cell subsets and the resulting shift in NAFLD make it challenging to predict whether resulting changes are causes or consequences. Moreover, as the immune cell-driven non-alcoholic fatty liver disease (NAFLD) is a multi-stage process, a comprehensive blueprint of the inflammatory mechanisms involved in NAFLD is necessitated to develop immune-targeted therapies that may offer the best possible outcomes at each stage of the disease. Therefore, to understand the role of immune cells and their underlying mechanism in NAFLD, herein we provide an overview of the different immune cells involved in NAFLD after examining available *in vitro* models, animal models, and clinical data as shown in Figure 2, which may pave the way for developing future immune targeted therapies.

2.1 T cells

T cells are a type of white blood cell called lymphocytes. Several reports have shown that infiltration of lymphocytes, typically focal lymphocyte aggregates of T cells with B cells, is often observed in NAFLD patients[38]. T cell subsets are abundant in the liver, including innate T cells, conventional $CD8^+$ T cells, $CD4^+$ cell subsets, helper T cell 1 (TH1), TH17, *etc*. Additionally, several unconventional T cells are distributed in significantly different amounts in mammals and rodents[4].

Among innate T cell populations, γδT cells are most abundant in the liver as a heterogeneous group of lymphocytes in the hepatic immune system and are unconventional T cells[39]. γδT cells develop and survive in a microbial-dependent manner[40]. Recently, Diedrich *et al*.[41] reported no changes in the γδT cells *in vivo* studies based on human NASH. However, in 2017, Li *et al*.[42] showed an increase in γδT cells in the liver of mice during NAFLD, accompanied by impaired development of γδT cells observed in *Cd1d*-deficient mice, demonstrating that γδT cells in mice promote liver injury during NAFLD. However, Hammerich *et al.*[43] showed that chemokine receptor CCR6-dependent accumulation of γδT cells in the damaged liver inhibited liver inflammation and fibrosis, possibly because γδT cells attenuated liver inflammation by promoting apoptosis of activated hepatic stellate cells as shown in Figure 3.

$CD4^+$ helper T cells are a general term for a group of cells, including TH1, TH2, TH17, and regulatory T cells (Tregs), playing essential roles in maintaining immune tolerance in the liver. However, their dysregulation is a hallmark of the development of chronic liver disease[44]. Usually, regulatory T lymphocytes maintain autoantigen tolerance and suppress excessive inflammatory responses[31]. Rau *et al*.[45] found reduced Tregs expression in peripheral blood and liver in obese individuals compared to non-obese individuals, demonstrating that the transition from NAFL to NASH is accompanied by reduced Tregs expression, where Tregs induces apoptosis in NASH patients[38, 46]. The low number of Tregs during high-fat diet (HFD) -induced steatosis in mice, triggering liver injury by activating the tumor necrosis factor pathway and exacerbating inflammation. On the contrary, restoring Tregs in HFD-fed mice has reversed inflammation[47-48]. Nonetheless, there may be conflicting evidence about Treg involvement in NAFLD in rodent studies. Tregs' interaction with neutrophils was reported to increase the risk of cancer

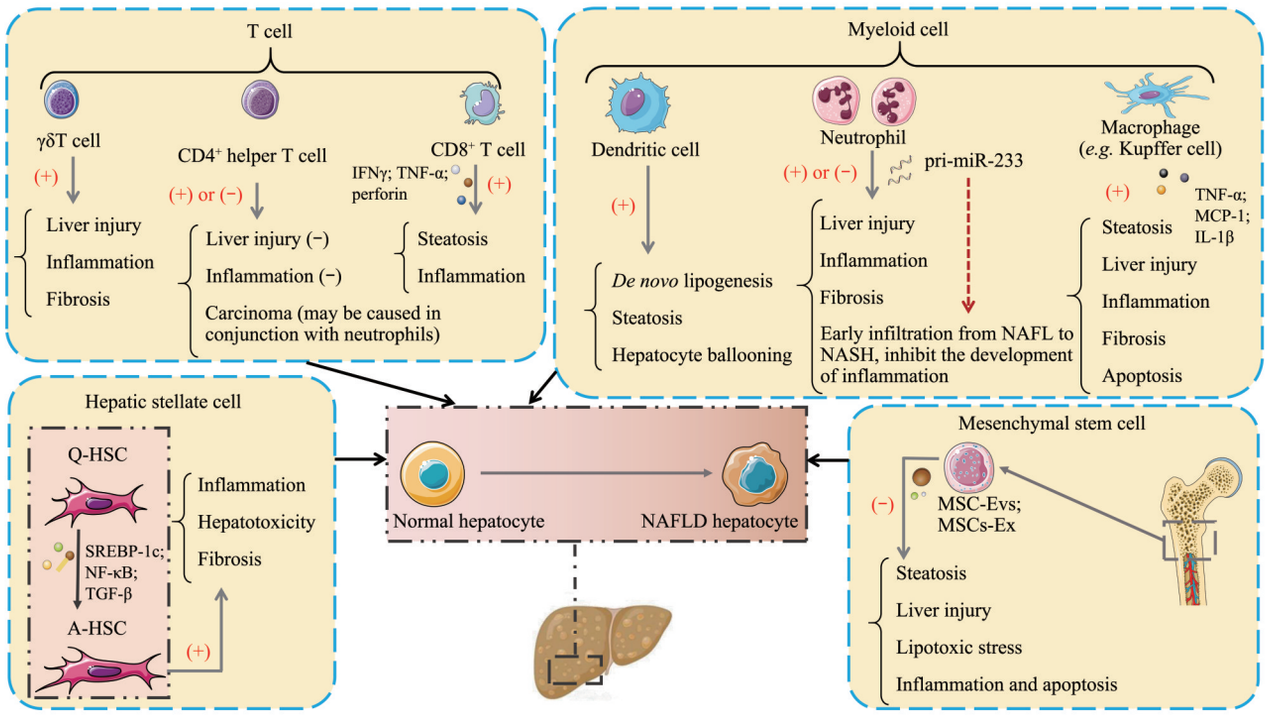

Fig. 2　The role of different types of immune cells on hepatocytes in NAFLD

During NAFLD, immune cells from the body are recruited to the liver and participate in the inflammatory environment of the liver, with important effects on liver damage (hepatocyte death) and liver fibrosis, and play an important role in the progression of NAFLD. These immune cells include T cells such as mucosa-associated invariant T cells (MAIT), invariant natural killer T cells (iNKT) and γδT cells, but also $CD8^+$ T cells and $CD4^+$ T cell subsets. $CD8^+$ T cells play an important role in hepatocyte lipotoxicity and inflammation by secreting interferon-γ (IFNγ), tumor necrosis factor-α (TNF-α) and perforin during NAFLD. Dendritic cells (DCs) increase in number during NASH and promote liver inflammation (possibly by activating $CD8^+$ T cells) and liver injury. Myeloid cells, such as Neutrophils, which aggregation is an early event in NAFLD that promotes or suppresses inflammation and liver injury in the early stages, particularly through the secretion of neutrophil extracellular traps (NETs) or pri-miR-233-containing exosomes. Mesenchymal stem cells are able to attenuate the inflammatory, lipotoxic and apoptotic processes of hepatocytes through their exosomes and extracellular vesicles to alleviate NAFLD. Macrophages are also quickly recruited to the liver, where they can differentiate into pro-inflammatory monocyte-derived KCs and replace umbilical cord-derived KCs as one of the factors causing hepatocyte inflammation. Immune cells act differently in different animals and even in the same animal at different stages of the disease. For example, γδT cells, which can promote inflammation, are also able to reduce liver inflammation by promoting apoptosis of activated hepatic stellate cells.

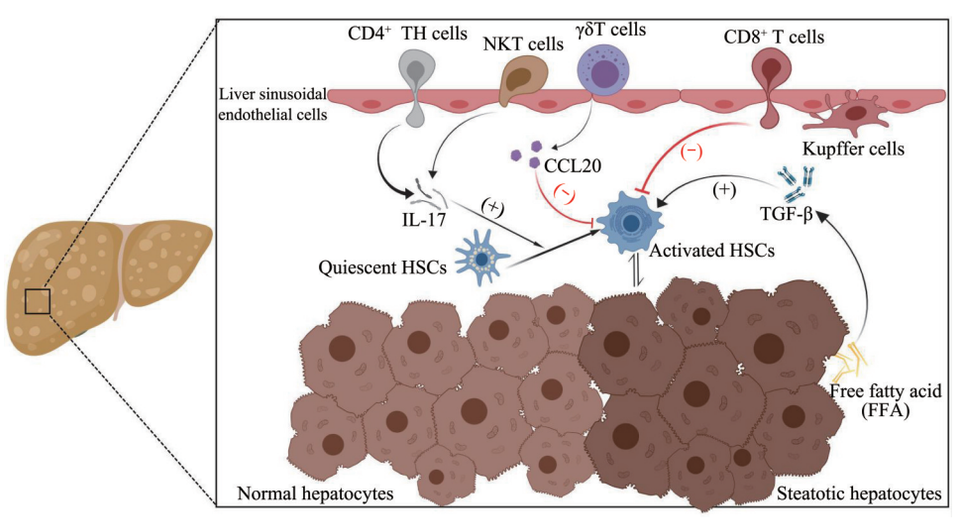

Fig. 3　T cells promote hepatic stellate cell activation and regulate NAFLD progression in hepatocytes

T helper 17 (TH17) cells and NKT cells increase fibrosis and inflammation by producing IL-17, whereas $CD8^+$ T cells and γδT cells limit fibrogenesis by inducing apoptosis in myofibroblasts.

development in a mouse model of NASH based on a choline-deficient, high-fat diet (CDHFD) [49]. Furthermore, transplantation of Treg from the mice to the liver of BALB/c mice fed a high-fat, high-sugar diet elevated the severity of NASH[50].

$CD8^+$ T cells are critical effector cells of the acquired immune system, significantly increased in the liver and blood of patients with NAFLD, and present an activation profile[51-52]. Activated $CD8^+$ T cells mainly produce IFNγ, TNF-α, and cytotoxic molecules (*e. g*., perforin) [53-54]. According to one study, during NAFLD, there is a notable rise in $CXCR6^+CD8^+$ T cells and an increase in $CD8^+$ T cells in both humans and mice[55]. Programmed cell death 1 (PD1), a key marker of T cell failure, is a transmembrane protein expressed primarily on the surface of activated T cells. After PD1 activation, $CXCR6^+CD8^+$ T cell activation accelerated in the NASH process in mice models[56]. On the other hand, knockdown of T cell and natural killer T (NKT) cell expression in a CDHFD mouse model of NASH reduced steatosis, liver parenchymal damage, and inflammatory injury in mice[57]. In the early stages of NAFLD, $CD8^+$ T cells mediated dysregulation of hepatic environmental metabolism and insulin resistance, and they also accelerated inflammation and hepatocyte injury. Additionally, $CD8^+$ T cells and NKT cells synergistically promoted the development of inflammation in NAFLD patients[57]. Similarly, different metabolic conditions have an impact on $CD8^+$ T cell phenotypes. In a mouse NASH model with an HFD construct, $CD8^+$ T cells were found to be able to activate hepatic stellate cells directly; however, in a mouse NASH model with a CDHFD construct, there was an increase in intrahepatic $CD8^+$ T cells without any discernible impact on liver injury or hepatic stellate cell activation[58].

2.2 Myeloid cells

Myeloid cells, which consist of macrophages, DCs, monocytes and granulocytes (especially neutrophils), are a major component of the immunosuppressive network[59]. Myeloid cells secrete inflammatory factors and influence the course of NAFLD and influence the course of NAFLD by expressing IL-6 receptor alpha (IL-6Rα) following secreting the pro-inflammatory factor IL-6 under the regulation of hepatocytes, megakaryocytes, and leukocyte subsets[60-62]. During NASH, inflammatory signals cause the liver to recruit macrophages, increasing the hepatic macrophage pool[63]. Miller *et al*. [64] observed that mice lacking *IL-6* had an increased risk of steatosis and liver injury following a high-fat diet, but wild type (WT) mice had an increased risk of liver cancer detection. Thus, it suggests that IL-6Rα is an essential mediator of inflammation linked to obesity; consequently, myeloid cells are critical for the degree of inflammation in NAFLD[65]. It was found that macrophages not only secrete inflammatory factors during the early phases of NASH, but they also inhibit the production of IL-6 throughout some stages of chronic liver disease, thereby delaying the course of liver disease[65-66].

2.2.1 Dendritic cells (DCs)

DCs are antigen-presenting cells that are vital links between several disease-driving systems[67]. Two subpopulations of DCs, $CD103^+$cDC1s (cDC1s) and $CD11b^+$cDC2s (cDC2s), exist in both humans and mice, where cDC2s are closely associated with the appearance of hepatocyte ballooning in hepatic lobular inflammation[29]. DCs capture and process antigens, migrate to lymphoid organs, deliver antigens to T cells, and secrete cytokines to initiate the immune response[68-69]. DCs act as a cellular link between innate and acquired immunity, secreting type I interferon and providing antigen to T cells during viral infection[70-71]. Most DCs in the liver are located in the periportal region and are recruited during a high-fat diet or the onset of NAFLD[72-73]. In the hepatic microenvironment, many DC-T cell interactions occur directly in the liver, although hepatic DCs are less efficient at stimulating T cell activation than DCs in other tissues[72].

However, the precise role of DCs in NAFLD remains unknown, as it is still unclear whether they promote or inhibit the disease. This could be because mice and humans have two distinct types of hepatic DCs, which vary slightly in terms of their adipogenesis and the expression of markers linked to lipid metabolism and adipogenesis, pointing to differences in the results as well as the absence of study-specific markers[28, 67]. Henning *et al*[74]. performed dendritic cell depletion experiments in chimeric mice by transferring bone marrow from CD11c-DTR mice to wild-type mice and found that DCs play a protective role in NAFLD by reducing inflammation. However, experiments by Connolly *et al.* [75] found that DCs promote the process of inflammation and fibrosis in hepatocytes. In addition,

Deczkowska *et al.*[73] found that targeted elimination of cDC1s reduced the extent of steatohepatitis in mice. According to the research of Ibrahim *et al.*[76], the livers of mice and humans have distinct functions for dendritic cell populations with different lipid concentrations in terms of tolerance and immunity. Compared to low lipid DCs, high lipid DCs were likelier to exhibit pro-inflammatory responses.

2.2.2 Neutrophils

One of the early features of NAFLD is the aggregation and infiltration of neutrophils, observed in patients during NASH[77-79]. Disturbances in liver metabolism lead to the migration of neutrophils to sites of inflammation. In chronic inflammation such as NAFLD, neutrophils appear to aggregate and release proteases, neutrophil extracellular traps (NETs), and reactive oxygen species (ROS) to induce hepatocellular damage, inflammation, and fibrosis to influence the NAFLD progression[80-81].

As neutrophils have a limited life span, it remained unknown whether the abundant neutrophil population found in the liver during NASH are transited from bone marrow-derived neutrophils. During NASH, neutrophils play a significant role in promoting or inhibiting inflammation development, whereas several studies supported the protective role of neutrophils in suppressing inflammation processes[82]. According to He *et al.*[83-84], NAFLD is associated with hepatocellular steatosis and inflammation, where extra hepatocellular free fatty acids regulate the APOE/PU-1 signaling pathway by upregulating neutrophil miR-223 expressions. miR-223 forms a feedback loop, which is transported *via* vesicle encapsulation from neutrophils to hepatocytes and reduces the inflammatory, fibrosis-related process in hepatocytes by inhibiting the expression of genes associated with inflammation and fibrosis in hepatocytes. Similarly, a study performed on a mouse model of a high cholesterol, fed with a high-fat diet demonstrated that neutrophils play a crucial role in reducing inflammation in the early stages of NASH through neutrophil proteases[78].

In patients, the amount of NETs secreted by neutrophils during NASH correlates with the severity of NASH[85]. In a mouse model of HFD, Zhao *et al.*[78] found that the presence of NETs *in vivo* early in NASH reduced the severity of NASH upon DNase treatment administration. Some studies have shown that neutrophils play a role in promoting the development of inflammation in the NAFLD process. To support this notion, Gao *et al.*[86-87] showed that neutrophils are also involved in the infiltration of hepatocytes under lipotoxic conditions and promote hepatocyte injury and inflammation through the production of reactive oxygen species.

miR-233 is a key fine-tuner in regulating hyperinflammatory responses and neutrophil activation. It also ameliorates alcohol or drug-induced hepatocyte injury by inhibiting neutrophil infiltration in response to acute neutrophil injury[83, 88]. miR-233 is expressed at higher levels in neutrophils, about ten times higher than in other cells, followed by macrophages, and at deficient levels in hepatocytes, pointing out that neutrophils specifically express miR-233[89-90]. Similarly, a study by He *et al.*[84] showed that immune cells, primarily neutrophils, have upregulated miR-233 levels in hepatocytes during the NAFLD process. However, the underlying mechanism of cell signaling between neutrophils and hepatocytes resulting in the transfer of miR-233 remains unknown. Recent studies have shown that under HFD induction, downstream free fatty acids stimulate neutrophils to express pri-miR-233, which is modified and transferred to hepatocytes *via* vesicles and recognized by LDL receptors to transfer mature miR-233 into hepatocytes. Convincing studies have shown that miR-233 is specifically activated within hepatocytes and exhibits a protective role by inhibiting genes linked with fibrosis and inflammation, reducing hepatic damage inflammation and fibrosis in NAFLD[83-84]. Recently, several studies reported that neutrophil accumulation and infiltration occur only in the early stages of NASH. However, this effect disappears in the later stages of NASH, suggesting that neutrophil elastase and NET could be used as markers to detect the early stages of NAFLD development [78, 91-92].

2.2.3 Kupffer cells

Hepatic macrophages consist mainly of tissue-resident KCs and monocyte-derived macrophages (MoMFs). They are characterized by high phenotypic and functional diversity and plasticity[93-94]. KCs are the most prevalent tissue-resident macrophages in the body and the largest resident immune cell population in the liver, performing unique functions in the liver[95]. Together with DCs and hepatic sinusoidal endothelial cells, they form the reticuloendothelial

system[72].

Monocyte chemoattractant protein-1 (MCP-1) is also known as CCL2. KCs are known to contribute to the early progression of NAFLD by secreting the chemokine TNF-α and MCP-1 in the early stages of NAFLD disease, as confirmed by the study of Tosello-Trampont *et al*.[96-97] which was performed on HFD mouse model of NAFLD. KCs play a vital role in the early stages of NAFL and NASH. During early stages of fat accumulation and degeneration in NAFLD, free fatty acids may affect the expression and release of hepatic mitochondrial DNA and activate the release of inflammatory vesicles NOD-, LLR- and pyrin domain-containing protein 3 (NLRP3) and pro-inflammatory factor IL-1β in KCs, promoting the development of liver inflammation[98-99]. Indeed, studies have shown that the severity of NAFLD is associated with dysbiosis of the gut flora and a shift in the metabolic function of the gut microbiota[100]. In Cx3Cr1-deficient mice, intestinal epithelial integrity is disrupted due to intestinal barrier dysfunction, and inflammatory factors enter KCs and hepatocytes through the intestinal epithelium, exacerbating the development of NAFLD inflammation in mice[101-102].

Recent studies have shown that most of the KCs produced during NAFLD in rodents are derived from monocytes, observed in the hepatic sinusoids after immunostaining[103-105]. Monocyte-derived KCs proliferate and differentiate during NAFLD and progressively replace embryonic-derived KCs. Monocyte-derived KCs lack part of their gene expression profile (*e. g*. *Timd4*−) compared to embryonic-derived KCs, but are more likely to cause inflammation during NASH and may contribute more to liver injury[99, 105-106]. Although KCs derived from embryos are more susceptible to lipotoxic stress and mortality than monocytes, they also facilitate the entry of triglyceride into hepatocytes for lipid droplet storage, which is crucial for the buildup of saturated fatty acids in early NAFLD[20, 29]. Accumulating evidence has shown that activation of monocyte-derived KCs is influenced by macrophage scavenger receptor 1 (*Msr1*), a receptor that is also a key molecule in the pathogenesis of NAFLD[107]. *Msr1*-mediated uptake of saturated fatty acids accelerated the inflammatory response in the liver of NAFLD mice, whereas knockdown of *Msr1* reduced the extent of steatohepatitis in mice[107].

Along with the inflammation-mediated advancement of NAFLD facilitated by KCs, the disease progression is linked to a closely controlled metabolic interaction between hepatocytes and KCs. A study performed by Canbay *et al*.[108-109] showed that in late NAFLD, KCs are activated by phagocytosis of apoptotic vesicles and accelerate the hepatocyte apoptosis. The NASH process is characterized by significant changes in KCs enhancers and gene expression, partial loss of KCs properties, and induction of Trem2 and Cd9 gene expression, leading to cell death[110]. Seidman *et al*.[104] found that NASH-induced changes in KCs enhancers were driven by AP-1 and EGR, which reprogrammed LXR functions required for KCs recognition and survival. These findings reveal mechanisms by which disease-related environmental signals direct resident and recruited macrophages to acquire distinct gene expression programs and functions. These findings demonstrate the mechanisms by which disease-related environmental signals control the differential gene expression programs and corresponding functions of resident and recruited macrophages. Moreover, KCs regulate lipid metabolism in hepatocytes and promote lipid accumulation in hepatocytes by expressing tumor necrosis factor and IL-1β, thereby altering fatty acid oxidation, triglyceride accumulation, and insulin response in hepatocytes. IL-1β is reported to exacerbate steatosis by binding to IL-1 receptor type 1 (IL-1R1) and inhibiting the expression and activity of peroxisome proliferator-activated receptor α (PPARα) in hepatocytes, leading to downregulation of genes involved in fatty acid oxidation[111].

2.2.4 Monocyte-derived macrophages

To promote the coordinated recruitment of immune cells, non-parenchymal cells such as macrophages and liver cells release chemokines to attract immune cells to the site of injury, MoMFs are one such immune cell[112]. They play an important role in the homeostasis and normal physiology of the body by removing metabolic waste and cellular debris, regulating iron homeostasis through erythrocyte phagocytosis and iron recycling, maintaining cholesterol homeostasis, maintaining immune tolerance and promoting antimicrobial defence[113-114].

MoMFs play a critical role in the pathogenesis of NASH due to their context-dependent polarization and significant functional plasticsity. These macrophages, regulating inflammatory, fibrogenic and tissue repair responses[93], include the

lipopolysaccharides (LPS) co-receptor, immunoglobulin receptors, scavenging receptors and pattern recognition receptors, including Toll-like receptors (TLR), such as TLR4 or TLR9, which detect damage-associated molecular patterns (DAMPs) [115]. For example, activation of TLR4 by free fatty acids leads to the release of pro-inflammatory cytokines (*e.g*. TNF, IL-6)[115].

MoMFs can replace KCs and express the phenotypes of lipid-associated macrophages (LAMs) or scar-associated macrophages (SAMs), which express CD9 and osteopontin (OPN) [103-105, 116]. The significance of recruited monocyte-derived macrophages in NASH has recently been highlighted by single-cell RNA sequencing (scRNA-seq). scRNA-seq analysis of human liver and mouse models has shown that LAM (SAM) migration to steatotic regions is facilitated by increased CCL2 expression by activated hepatic stellate cells (A-HSCs), promoting both fibrogenesis and fibrosis resolution[117-118]. A recent study found that macrophages take up various lipids and lipoprotein particles and convert triglycerides to free fatty acids for extracellular removal, which is necessary for the design of future therapeutic interventions to correct lipid overaccumulation and related complications[119-120].

2.3 Some non−immune cells

2.3.1 Hepatic stellate cells

Quiescent hepatic stellate cells (Q-HSCs) are located between the hepatocytes and the endothelial cells of the hepatic sinusoids, which are pericytes in the peri-sinusoidal space and account for 1.4% of the total liver volume, approximately 8% of the total number of hepatocytes[121]. Activation of Q-HSCs is associated with the expression of transcriptional lipogenic factors and steroid regulatory element binding protein 1c (SREBP-1c). Under the influence of inflammatory and chemotactic factors, hepatic stellate cells undergo a transition from a quiescent state to (A-HSCs), which triggers elongated morphologically and termed myofibroblast-like stellate cells[122].

Inflammation triggers the activation of HSCs, which can modulate immune mechanisms through chemokines and cytokines, or transdifferentiate into matrix-forming fibroblasts[123]. HSCs trans-differentiation depends on the inflammatory activity of hepatic immune cells, and KCs promote HSC activation by secreting tumor necrosis factor and reactive oxygen species. Hepatic macrophages can also contribute to the activation of HSCs in a nuclear factor-kappa B (NF-κB) -dependent manner, thereby indirectly influencing hepatic inflammation and fibrosis[124]. Under lipotoxic conditions, the hormone leptin, secreted by adipocytes, drives the transformation and differentiation of hepatic stellate cells[125]. Wang *et al*. [126] showed that drug-induced hepatotoxicity and liver fibrosis were inhibited in rats lacking leptin receptors, and it was hypothesized that leptin could activate HSCs trans-differentiation and maintain the myofibroblast phenotype by stimulating TGF-β expression in KCs and activating the Hedgehog (Hh) signaling pathway. A recent study by Marcos *et al*. [127] found that A-HSCs release fatty acids and carnitine palmitoyltransferase 1A (CPT1A), which is responsible for lipid synthesis, was elevated in HSCs from patients with liver fibrosis and in a mouse model of liver fibrosis. Knocking down of CPT1A expression has inhibited the HSCs activation and attenuated the progression of liver fibrosis. Additionally, $CD8^+$ T cells were also reported to regulate and activate HSCs and induce inflammation in NASH patients[58].

2.3.2 Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are non-terminal differentiated adult stem cells possessing the potential for multidirectional differentiation and self-renew. MSCs are derived from bone marrow and are found throughout the body. MSCs have the potential to differentiate into osteoblasts, chondrogenic cells, and adipocytes and express CD105, CD73, and CD90 without expressing CD45, CD34, CD14, or CD11b, CD79a, or CD19[128-129]. Interestingly, recently, MSCs have been derived from bone marrow, the umbilical cord, and adipose tissue and have been utilized in clinical research and various useful therapeutics for organ damage and neurological injury.

Clinical evidence and animal studies confirm that MSCs function therapeutically through extracellular vesicles (EVs) and exosomes rather than through differentiation (MSC-EVs)[130-132]. Recent studies have shown that MSC-EVs are an effective biological tool widely used in various clinical trials to alleviate NAFLD and steatosis[133]. MSC-EVs were found to repair NAFLD by altering the NASH process through steatosis, including metabolic homeostasis,

inflammation, and fibrosis[134].

El-Derany *et al*.[135] found that bone marrow mesenchymal stem cell-derived exosomes inhibited downregulation of Caspase-2 translation in hepatocytes through upregulation of miR-96-5p, which significantly upregulated fatty acid oxidation and activation of mitochondrial autophagy, exerting an inhibitory effect on hepatic steatosis and apoptosis. In another study, miR-233 encapsulated in human mesenchymal stem cell exosomes (hBM-MSCs-Exs) was reported to ameliorate liver fibrosis in NAFLD mice by inhibiting E2F1 and slowing liver fat accumulation[136]. In an investigation on the mechanism of human umbilical cord mesenchymal stem cell-derived small extracellular vesicles (hUC-MSCs-sEVs) for the treatment of type 2 diabetic rats, Yap *et al*.[137] found that hUC-MSCs-sEVs improved insulin resistance in T2DM rats by activating the AMPK pathway and aided hepatic glucose and lipid metabolism. Furthermore, MSCs-sEVs are also reported to regulate macrophage polarization by inhibiting the Kruppel-like factor 6/STAT3 pathway, inhibiting pro-inflammatory macrophages, and promoting anti-inflammatory macrophages to protect against liver fibrosis in mice[138].

Based on prior research on the therapeutic significance of MSCs, we have illustrated in Figure 4 how MSCs can be utilized for NAFLD's therapeutic strategies. Massive liposynthesis and fat accumulation occur in hepatocytes as NAFLD progresses, leading to lipotoxicity, ROS, and mitochondrial dysfunction, impairing hepatocyte structure and function. To regulate liver function and alleviate hepatocyte lipotoxicity and mitochondrial oxidation, MSCs secrete MSC-EVs during the early stage of NAFLD to lessen liver toxicity. For instance, (1) MCSs encapsulated with miR-96-5p enter the liver to inhibit Caspase-2 release, which in turn downregulate fat oxidation and mitochondrial autophagy, suggesting that MSCs can slow the early progression of NAFLD by attenuating hepatocellular steatosis and apoptosis; (2) MSCs-EVs can activate the APMK pathway and promote insulin resistance after entering the liver through blood vessels. MSCs-EVs containing miR-233 may lower fat accumulation and synthesis in the liver and promote hepatic glucose-lipid metabolism, thus signifying the therapeutic potential of MSCs in NAFLD.

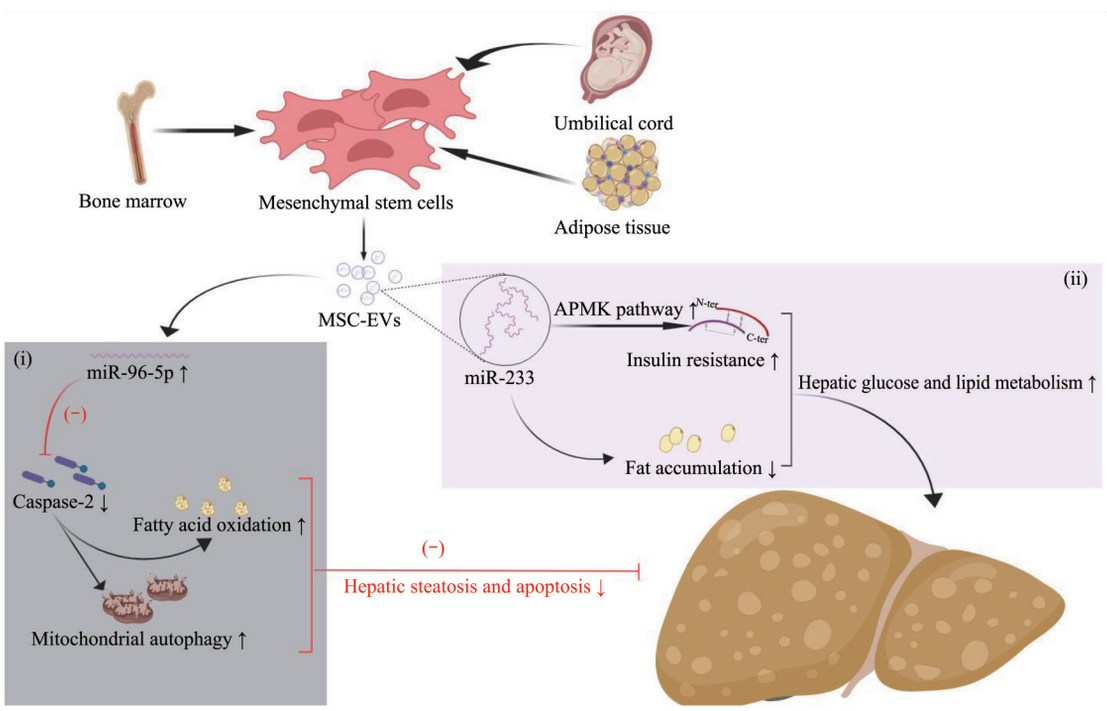

Fig. 4 Mesenchymal stem cells inhibit hepatocellular steatosis and promote hepatocellular lipid metabolism in NAFLD

During NAFLD, MSCs-EVs are secreted by MSCs and transported into the liver to regulate hepatic inflammation through activation of the APMK pathway with decreased Caspase-2 expression. Hepatocytes show an accelerated rate of fatty acid metabolism with a reduction in fat accumulation and a reduction in inflammation and fibrosis.

Since the decade, a great deal of research has been done on MSCs, with the primary research directions being as follows. (1) The extraction of high-quality, less toxic MSC-EVs and the availability of cost-effective, large-scale EV production with safe isolation and identification methods[139]. (2) Identifying higher-quality secretions, which could significantly down-regulate pro-inflammatory factors to aid NAFLD therapeutics. For instance, a recent discovery of edible exosome-like nanoparticles (ELNs) has been observed as a promising and alternative strategy for treating NAFLD patients[140-141].

3 Overview of research advances in NAFLD therapeutics

Usually, the intrahepatic environment, enriched in immune cells, is involved in hepatocyte damage and fibrosis in an inflammatory environment to influence the NAFLD process. However, other than immune cells, several non-immune cells in the liver also trigger NAFLD pathology. For instance, hepatocytes also secrete EVs and adenosine triphosphate (ATP); endothelial cells, which produce inflammatory triggers and chemokines such as IL-6[142], TNF- α and CCL2 to promote the inflammatory process in liver[143-144], making commemorable challenges in NAFLD therapeutics and disease management. Therefore, to develop therapeutic approaches intervening in disease progression, it has become crucial to understand the inflammatory cellular mediators and pathways that orchestrate the development and progression of NAFLD and its associated pathology. To date, an increasing number of experimental approaches have been applied *in vivo* mouse models to treat and mitigate NASH. For example, in mice with CCl4- and methionine- and choline-deficient diet (MCD)-induced liver injury, treatment with neutralizing antibodies against CXCL16, a cytokine that promotes the accumulation of NKT in the liver, has reduced hepatic macrophage aggregation and the progression of steatosis[145]. Parallel to this, Zhang *et al*. [146] successfully decreased hepatocyte damage brought on by NAFLD in mice fed MCD by enhancing the degree of steatohepatitis and anti-CXCL10 monoclonal antibody. In addition, some drugs have been found to alleviate the NAFLD process by affecting the expression of immune cell-associated pro-inflammatory factors. Another study performed by Zhou *et al*. [147] showed that an α-7 nicotinic receptor agonist can alleviate liver inflammation in mice by downregulating NF-κB and extracellular signal-regulated kinase (ERK)-dependent signaling pathways.

Nevertheless, several drugs targeting NAFLD are currently in clinical research, but their results remain unimpressive. This might be brought about by the intricacy of the mechanisms behind fibrosis and inflammation as well as the redundant nature of fibrotic and inflammatory signals. The body has developed several overlapping defense mechanisms against fibrogenesis and inflammation to preserve tissue homeostasis. Clinical trials with combinations have not yielded encouraging results despite encouraging preclinical data in animal models[94, 148-149]. Converging shreds of evidence has shown that traditional Chinese medicine (TCM) has advantages in treating NAFLD from a holistic theory and dialectical treatment[150]. TCM treats NAFLD with an emphasis on the holistic theory of liver preservation, which takes many different forms and features into account in terms of mechanisms. These include regulation of the intestinal microbiota, antioxidative stress, lipid metabolism, anti-inflammation, and anti-fibrosis[151]. For example, the flavonoids, triterpenoids, astragalus polysaccharides, and other alkaloids found in *Astragalus* presented therapeutic and preventive benefits by reducing hepatic steatosis, inhibiting inflammation and hepatic fibrosis[152], protecting against liver damage in all stages of NAFLD[153].

4 Conclusions and perspectives

The rising prevalence of NAFLD yearly is becoming a global havoc due to its complexity and multifactorial disease, causing severe liver damage. Although NAFLD develops due to altered metabolism, it also exhibits strong immunoinflammatory dimensions. During NASH, a vast network of immune cells is activated, and in this review, we have explained how these immune cells can facilitate or suppress the progression of NASH from basic steatosis to cirrhosis and HCC. Nevertheless, further research is necessary to fully understand the significance of immune cell subsets in the illness, as our knowledge of the inflammatory

cues that cause NASH is fragmented and remains a significant challenge in developing effective therapeutics for disease management.

Furthermore, understanding the intricacy of NASH necessitates an integrated perspective on the interactions between various immune cell types and between immune cells and hepatocytes, which may aid in uncovering the mystery of molecular inflammatory mechanisms in the liver leading to NAFLD. Additionally, more advancements in mouse models of NASH are anticipated, which will help to establish the causal relationship between particular immune cell populations and the advancement of the illness. Consequently, better phenotyping of patients by cutting-edge techniques like single-cell or single-nucleus RNA sequencing is necessary in conjunction with this new knowledge to better understand the immunological landscape that defines NASH patients.

Similarly, the advent of *in vitro* research has grown with the introduction of multi-cell co-culture and organ-on-a-chip technologies. The spatial structure of biological cells can be replicated *in vitro* using 3D cell co-culture technology, allowing researchers to explore the distinct spatial functions of these cells and open new avenues for developing novel therapeutics and investigating pathological mechanisms in disease models. Future research on the relationship between various organs should investigate information transfer and exchange between cell-cell, cell-organ, and organ-organ. Additionally, co-culturing hepatocytes and immune cells and implementing them on chips may prove feasible and have potential applications. Recent data analysis of structured and unstructured data in organoids using an artificial intelligence layer has demonstrated the synergy between artificial intelligence and organoids[154], opening new avenues for subsequent analysis of the complex structure of multi-organoids and the complex disease mechanism of NAFLD, as well as multi-drug targeting to study the effects of drugs on NAFLD.

Moreover, it is proposed that integrating all types of data from patient clinical samples, advanced *in vitro* cellular models, and preclinical rodent models may aid in exploring new targets for targeted therapy[155], which may assist in the better understanding of the intricate pathways linking metabolism, inflammation, and fibrosis in NAFL and NASH, and improve the development of more effective, stage-specific and personalized treatments. Together, these developments should encourage research into cutting-edge therapeutic modalities to lower the prevalence of NAFLD globally and necessitate high standards of basic, preclinical, and clinical research in the future to fulfill the pressing demand for medications to address the NAFLD pandemic.

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免疫细胞在非酒精性脂肪性肝病发病机制中的作用*

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摘要 非酒精性脂肪性肝病（NAFLD）是最常见的慢性肝病，是从脂肪堆积到变性炎症，并出现非酒精性脂肪性肝炎（NASH），直至引发肝纤维化、肝硬化甚至肝癌的一系列过程。NAFLD目前已经是全球感染人群占比最大的肝病且发病率逐年上升。NAFLD是一种新陈代谢紊乱的疾病，但它也涉及多种免疫细胞介导的炎症过程，通过分泌促炎和/或抗炎因子来促进和/或抑制肝细胞炎症，从而影响NAFLD的进程。然而，NAFLD潜在的疾病机制及免疫细胞在其中的作用仍在研究之中，留下了许多悬而未决的问题。本综述介绍了免疫细胞在NAFLD发病和致病过程中相互作用的最新概念，重点介绍了在NAFLD中表现出具有治疗意义的免疫学特性的特定非免疫细胞，以便更好地了解表现出治疗特性的免疫/非免疫细胞的作用机制，从而在未来设计出治疗NAFLD的创新性和更具特异性的药物。

关键词 非酒精性脂肪性肝病，代谢相关性脂肪性肝病（MAFLD），T细胞，髓样细胞，间充质干细胞
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