Peripheral immune cells in NAFLD patients: A spyhole to disease progression

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Summary

Nonalcoholic fatty liver disease (NAFLD) is a worldwide leading cause of chronic liver disease, but we still lack ideal non-invasive tools for diagnosis and evaluation of nonalcoholic steatohepatitis (NASH) and related liver fibrosis in NAFLD population. Systemic immune dysregulations such as metabolic inflammation are believed to play central role in the development of NAFLD, signifying the hope of utilizing quantitative and phenotypic changes in peripheral immune cells among NAFLD patients as a diagnostic tool of NASH and fibrosis. In this review, we summarize the known changes in peripheral immune cells from NAFLD/NASH patients and their potential relationship with NAFLD and NASH progression. Potential challenges and possible solutions for further clinical translation are also discussed.

EBioMedicine 2022;75: 103768
Published online xxx
https://doi.org/10.1016/j.ebiom.2021.103768

Keywords: Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; Leukocyte; Non-invasive diagnosis; Flow cytometry

Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease in the world nowadays, jeopardizing one-fourth of the world's population.[1] In the early disease stage (i.e. nonalcoholic fatty liver, NAFL), it is merely manifested as over-deposit of fat in hepatocytes in the form of steatosis. However, some NAFL patients may develop hepatic injury with hepatocyte ballooning and inflammation, i.e. entering the stage of nonalcoholic steatohepatitis (NASH). NASH patients can further progress to varied degree of fibrosis, or even terminal liver diseases including cirrhosis and hepatocellular carcinoma (HCC).[2] The incidence of NAFLD is related to significant increase in overall mortality and liver-related mortality; but more importantly, increase in overall mortality is in positive correlation with worsening NAFLD histology[3,4], signifying the urgent need for effective recognition of NAFLD histology in order to predicte prognosis and guide therapy. In the era of NAFLD evaluation, liver biopsy, the golden standard for NASH identification, is invasive and consequently hard to be widely applicated, while the non-invasive techniques that have been put into clinical use (such as ultrasound and elastography) only render ideal identification of steatosis and fibrosis but not of NASH.[4–7] Considering NASH as the key transitioning stage of NAFLD progression, new approaches to improve NASH identification should be developed. Although novel non-invasive methods that mainly based on combinative panel of serum molecules and/or imaging modalities, such as NIS4 and FibroScan-AST (FAST) score[8,9], have been tentatively proposed for accurate identification and surveillance of NASH, till now there is still no widely acknowledged non-invasive method[10] (Figure 1).

Harboring numerous innate and adaptive immune cells in the specialized capillary system even in homeostatic state, liver has long been regarded as a central immunological organ and vigorously participates in the maintenance of immune homeostasis.[11] Correspondingly, when immune homeostasis is disrupted under various liver diseases including NAFLD, both innate and adaptive immunity would also participate in the onset and progression of various liver diseases.[12,13] In fact, according to a recent report, even if a short-term high-fat diet administration in mice failed to induce histologically evident hepatic inflammation, proinflammatory gene expression in liver resident immune cells (e.g.

Abbreviations: NAFLD, Nonalcoholic fatty liver disease; NASH, Nonalcoholic steatohepatitis; NAS, NAFLD activity score; FCM, Flow cytometry; PBMC, Peripheral blood mononuclear cell; NK, Natural killer; DC, Dendritic cell

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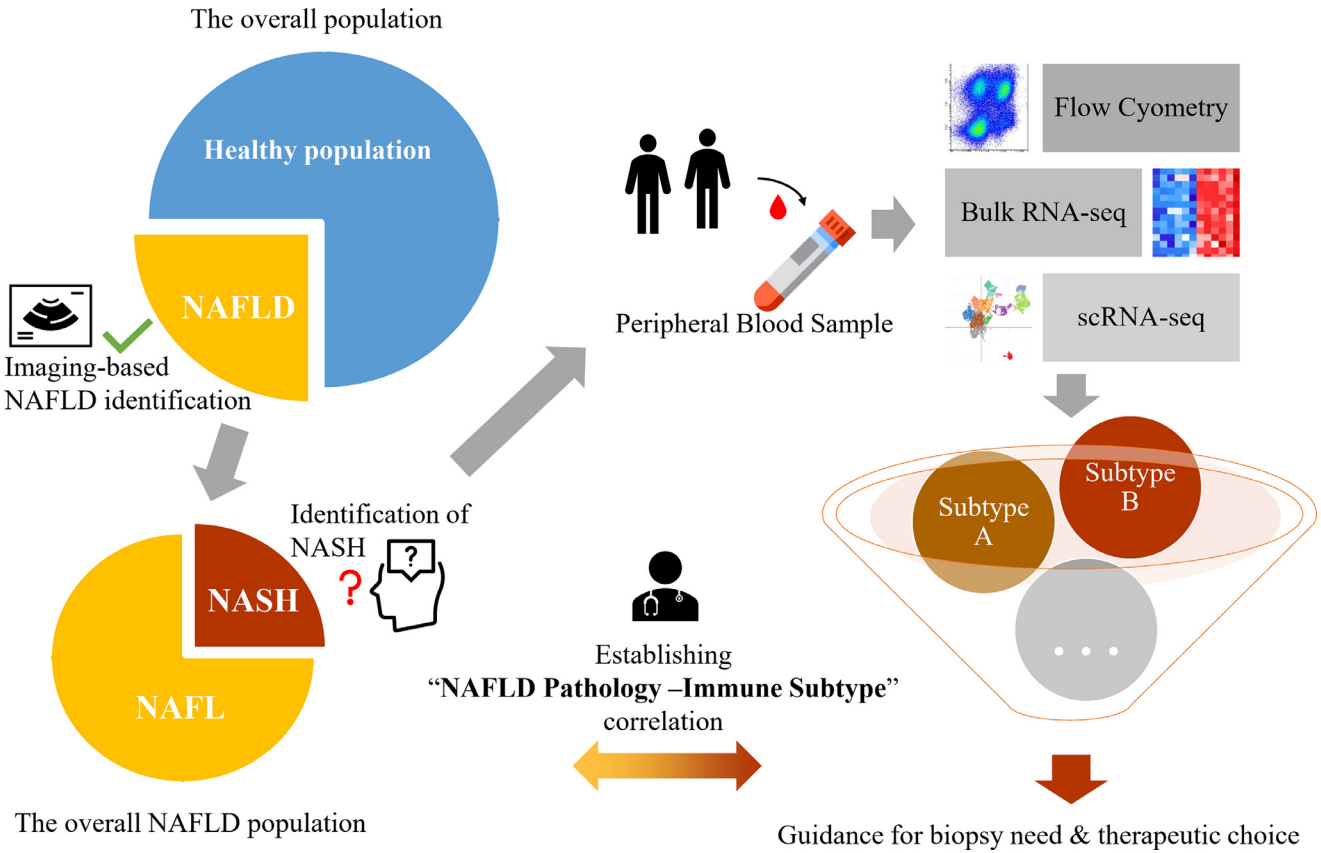

Figure 1. Current dilemma in NAFLD evaluation and a proposed workflow based on characterization of patient peripheral immune cells.

Kupffer cells) had been significantly promoted[14], signifying the potential good sensitivity of immune characterization in revealing NASH progression. More importantly, during the pathogenesis of NAFLD, liver is definitively not an isolated organ, but in vigorous crosstalk with other organs (e.g. adipose tissue and gut) through blood circulation in terms of bioactive substances, as well as immune cells.[15–17] It has been recently proposed that multiple organs would enter a state of "metabolic inflammation" during NAFLD pathogenesis, while the liver is one of the susceptible organs yet potentially drives dysfunctions and co-morbidities in other organs.[18] In metabolic diseases including NAFLD and cardiovascular disease, dysregulated metabolism (e.g. defect in cholesterol metabolism) in immune cells can exert direct influence on activation and proliferation of immune cells[19–21], constituting the driving force for metabolic inflammation here. Therefore, a clinical evaluation of NAFLD from the immunological perspective is etiologically plausible.

The concept of metabolic inflammation has been tentatively translated into novel markers for NAFLD, mostly inflammation-related serum proteins and soluble form of immune cell surface proteins.[22–24] However, the formed elements of blood, especially the leukocytes, have long been neglected in the evaluation of NAFLD progression. In this review, we will mainly summarize the changes in frequency and molecular phenotype of peripheral immune cells from NAFLD patients and the potential correlation between these changes and NAFLD progression. The theory of lymphoid lineage development[25,26] and function-oriented immunity types[27] are combined here to categorize the complicated peripheral immune cell subsets (Figure 2). Our following illustration will be based on this categorization.

$CD8^+$ T cells and natural killer (NK) cells

Fundamentals in $CD8^+$ T cells and NK cells

Cytotoxic activity is an important function of the immune system to induce cell death and consequent clearance of target cells, such as infected cells or tumor cells. $CD8^+$ T cells and NK cells are the main cytotoxic effector cells in adaptive immunity and innate immunity respectively. They share similar mechanisms in inducing cell death, including perforin/granzymes system and death ligand expression/release. The cytotoxic activity mainly drives the target cells to "silently" die in the form of non-inflammatory apoptosis, although inflammatory death such as necroptosis and pyroptosis is also potential outcome. In addition to cytotoxic activity, cytotoxic cells can also produce inflammatory cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α).[26,28] NK cells and $CD8^+$ T cells have drastic differences in immune activation and memory formation. $CD8^+$ T cells mainly depend on T cell receptor (TCR)-mediated recognition of specific antigen peptide, and can be divided into naive, effector, central memory and effector memory cell subsets in peripheral blood. In contrast, NK cells rely on the integrated signal from an array of activating and inhibitory receptors to get activation, and are classically categorized as $CD56^{dim}$ and $CD56^{bright}$ subsets that exhibit preferentiality in cytotoxic activity and cytokine secretion respectively, although greater diversity of NK cells *in vivo* is increasingly appreciated according to recent findings.[26,29,30] However, considering the functional similarity of $CD8^+$ T cells and NK cells in cytotoxic activity, these two cell populations should be evaluated together.

Changes in the general NAFLD population

Both peripheral $CD8^+$ cells and NK cells in NAFLD patients are significantly altered. Although the overall peripheral $CD8^+$ cell frequency does not undergo significant change after NAFLD onset [31–33], significant higher percentage of IFN-γ^+ cells among $CD8^+$ T cells were found in NAFLD patients compared with healthy volunteers.[34,35] Besides, there was a $CD8^+$ T cell phenotype skewing from naive ($CD45RA^+$) to activated/memory ($CD45RO^+$) cells and terminal effector ($CCR7^-$ $CD45RA^+$) cells in $CD8^+$ cells of NAFLD patients.[35,36] As for peripheral NK cells in NAFLD patients, these cells seem to enter a state of exhaustion. Multiple researches revealed that the circulating NK cell frequency (especially for the $CD56^{dim}$ subset) diminished in NAFLD patients compared with healthy controls[32,36,37]. Additionally, in $CD56^{dim}$ NK cell subset, activation marker expression including NKp46 and NKp30 were significantly lower in NAFLD patients, while inhibitory (also exhaustive) markers including programmed cell death protein 1 (PD-1) and immunoglobulin-like transcript 2 (ILT2) exhibited significant increase.[32] The basal level of CD107a *in vivo* among $CD56^{dim}$ NK cells was reported to be lower in NAFLD patients[37], but *in vitro* stimulation-induced increase in both IFN-γ production and CD107a expression were found to be impaired.[32]

Apart from the above-mentioned evidence for cell exhaustion, there are two specific changes of peripheral NK cells in NAFLD patients. The first specific change is the drastically elevated $CD38^+$ cell frequency among $CD56^{dim}$ NK cells.[36] CD38 is a multi-functional protein that can display the activity of adenosine diphosphate (ADP)-ribosyl cyclase and cyclic ADP-ribose hydrolase.[38] Although it is found to facilitate the activation and cytotoxic responses of NK cells, its specific role in NK cells remains to be further resolved. The second specific change would be the presence of a new $CD56^{dim}$ NK cell subset characterized by Siglec-7^{dim}Siglec-9^- in only NAFLD patients but not healthy people.[32] Siglec-7, as well as Siglec-9, belongs to the sialic acid-binding

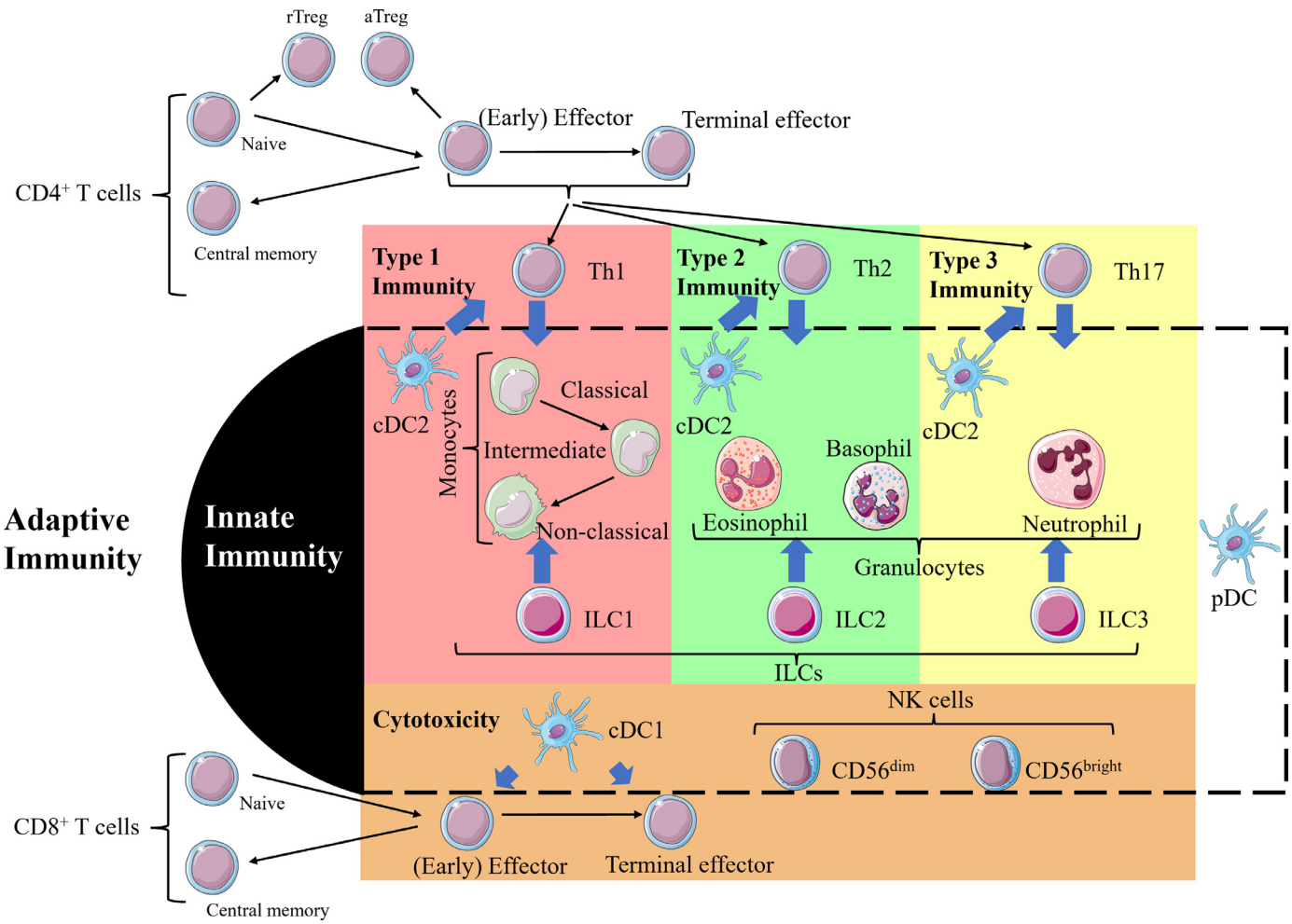

Figure 2. Major peripheral immune cell subsets mentioned in NAFLD-related clinical researches.

According to recognition mode, peripheral blood immune cells can be divided into adaptive immune cells (cells outside the dotted rectangle) and innate immune cells (cells inside the dotted rectangle). Adaptive immune cells mainly include $CD4^+$ T cells, $CD8^+$ T cells and B cells (B cells are not shown in the Figure), while innate immune cells mainly include monocytes, granulocytes, dendritic cells (DCs), natural killer (NK) cells and innate lymphoid cells (ILCs). Both peripheral $CD4^+$ and $CD8^+$ T cells can be divided into naïve, effector (terminal effector cells and early effector cells), and central memory cells. $CD8^+$ effector T cells, as well as NK cells (possessing two $CD56^{dim}$ and $CD56^{bright}$ subsets), mainly undertake cytotoxicity activity. In contrast, $CD4^+$ effector T cells usually undergo polarization into Th1, Th2 and Th17 cells, playing modulatory role in type 1, type 2 and type 3 immunity respectively. Their counterparts in ILCs, i.e. ILC1, IL2 and ILC3, can play similar modulatory role in corresponding immunity module. Additionally, some Naïve $CD4^+$ T cells and effector $CD4^+$ T cells can differentiate into resting Tregs (rTregs) and activated Tregs (aTregs) respectively. The main undertakers of three immunity modules are myeloid cell subsets, i.e. monocytes for type 1 immunity, basophils and eosinophils for type 2 immunity, and neutrophils for type 3 immunity. Among them, monocyte can be further divided into classical, intermediate and non-classical subsets. Finally, DCs can be divided into three major subsets: type 1 conventional DC (cDC1) for $CD8^+$ T cell priming, type 2 conventional DC (cDC2) for $CD4^+$ T cell priming, and plasmacytoid DC (pDC) for viral infection recognition. Black thin arrows denote "differentiation direction", while blue thick arrows denote "functional influence".

immunoglobulin-like lectin (Siglec) family and is regarded as important inhibitory checkpoint for NK cell activation.[39] This Siglec-7^{dim}Siglec-9^{-} NK cell subset was regarded as a highly dysfunctional subset, because it has reduced expression of activation markers (including NKp30, NKp46, IFN-γ and CD107a) and augmented expression of inhibitory markers (including ILT2 and PD-1).[32] This cell subset might have good diagnostic value in NAFLD pathological diagnosis since it could be clearly resolved in a Siglec-7^{dim} CD57^{+} manner by flow cytometry.

Changes in association with NASH

The vital role of cytotoxic effector cells in NASH progression has been tentatively unveiled in animal models. NK cells and CD8^{+} T cells were found to participate in not only metabolic dysregulations such as insulin resistance and hepatic steatosis[40,41], but also tissue injury and inflammation during NASH progression. NK cells can induce hepatocyte death via cytotoxic activity and promote aberrant macrophage infiltration and/or activation via inflammatory cytokine secretion in both liver and adipose tissue.[40] As for CD8^{+} T cells, deleterious role of CD8^{+} T cells in macrophage recruitment to adipose tissue, as well as NASH-HCC transition, have been reported[41] and been similarly validated by our group's recent work in transgenic pig model of metabolic diseases.[42] Recently, a novel subset of CXCR6^{+} hepatic CD8^{+} T cells with high expression in both granzymes and exhaustion markers was found in NAFLD mice model. Contrary to classical theory of "restricted antigen recognition" for CD8^{+} T cell activation, this CXCR6^{+} hepatic CD8^{+} T cell subset can be directly activated in an "auto-aggression" way, i.e. via dual activation of interleukin-15 (IL-15) and acetate, and lead to pathogenic non-specific killing of hepatocytes, as well as potentially concomitant increase in atherosclerosis risk.[43,44]

Accordingly, in addition to the changes between NAFLD population and healthy people, some of the changes in peripheral NK cell and CD8^{+} T cell are correlated with NAFLD activity score (NAS). Plasma perforin level was elevated in NASH patients compared with healthy controls and in positive correlation with NAFLD histological changes including hepatocyte ballooning, indicating a systemic elevation of cytotoxic activity in NASH progression.[45] Similarly, activated cytotoxic state of CD8^{+} T cells (especially the percentage of perforin^{+} cells among CD8^{+} T cells) was strongly correlated with ballooning score and total NAS score.[46] As for the inflammatory state of CD8^{+} T cells, markers for CD8^{+} T cell inflammatory state (i.e. IFN-γ^{+} or TNF^{+}) were only poorly or not correlated with NAS score.[35,46] Moreover, NASH patients were found to have significantly increased frequency of IFN-γ^{+} blood CD8^{+} cells among non-diabetic NAFLD patients, but its level was comparable between NAFL and NASH subgroups among diabetic NAFLD patients.[46] Therefore, activation markers of blood CD8^{+} cells might be promising markers of NASH progression for non-diabetic NAFLD patients. Additionally, CD8^{+} T cells in NASH patients exhibited higher Toll-Like Receptor 9 (TLR9) expression than their counterparts in NAFL patients.[34] TLR9 is an intracellular nucleic acid-sensing pattern recognition receptor, and its over-activation by elevated circulating level of mitochondrial DNA in NAFLD patients has been deemed as a driver of NAFLD progression.[47] Notably, TLR9 expression of circulating CD8^{+} T cells in healthy volunteers also appeared to be higher than NAFL patients at a comparable level to NASH patients[34], indicating the need of establishing NAFLD diagnosis before future utilization of this marker.

For NK cells, there are debates about their relationship with NAS score. Haas et al. found that the overall peripheral NK cell frequency was in positive correlation with NAS score[46], while no significant correlation between NK cell frequency and NAS score among NAFLD patients was detected in another study[32], indicating the debatable role of NK cell frequency in predicting NASH among NAFLD patients. Likewise, NK group 2 member D (NKG2D) was reported to be the most prominently changed activating receptor after NAFLD onset, yet its change direction is quite debatable. While Diedrich et al. found that the NKG2D expression of peripheral NK cells was significantly lower in the overall NAFLD population[36], another study found significantly elevated NKG2D expression among peripheral NK cells in NASH patients but not in NAFL patients or healthy volunteers.[33]

Changes in association with NAFLD-related fibrosis

For NAFLD-related fibrosis, recent studies about peripheral cytotoxic cells mainly focus on NK cells. In basic studies, NK cells in liver can mediate direct killing of activated fibrogenic hepatic stellate cells (HSCs) through cytotoxic activity, but this ability might be compromised because of persistent metabolic stress during advanced fibrosis stage, i.e. "metabolic paralysis".[40] In clinical studies, alterations in peripheral NK cell frequency and phenotype are signified in NAFLD patients with higher fibrosis degree, possibly indicating a compensatory reaction for hepatic fibrosis. For example, plasma perforin level was in positive correlation with fibrosis stage of NASH patients.[45] Furthermore, NAFLD patients with advanced fibrosis have significant higher percentage of CD56^{dim} NK cells, as well as decreased insulin receptor expression, among total NK cells than either healthy people or NAFLD patients without advanced fibrosis.[37] Likewise, among inhibitory markers, T-cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT) in peripheral CD56^{dim} NK

cells was found to specifically overexpressed among NAFLD patients with advanced fibrosis.[32]

$CD4^+$ T cells

Fundamentals in $CD4^+$ T cells and innate lymphoid cells

Apart from the cytotoxic $CD8^+$ T cell, another major T lymphocyte population in peripheral blood is $CD4^+$ T cell that mainly play a modulatory role in diverse immune responses through cytokine secretion. Although naive $CD4^+$ and $CD8^+$ T cells are similar in TCR-mediated recognition and memory-forming potentiality, $CD4^+$ T cells have distinctively functional plasticity, i.e. the ability to polarize into functionally distinct subsets under different cytokine stimulations and different co-stimulatory signal strength.[48] Consequently, both effector and memory $CD4^+$ T cells could be further subdivided into Th1, Th2 and Th17 subsets[48,49], i.e. the three key mediators for type 1, type 2 and type 3 immunity respectively.[27] Their functional versatility is manifested by different preference in cytokine expression (e.g. IFN-γ for Th1, interleukin-4 (IL-4) or interleukin-13 (IL-13) for Th2, and interleukin-17 (IL-17) or interleukin-22 (IL-22) for Th17) and surface marker expression.[48] In addition to immune activating roles, some $CD4^+$ T cells can also differentiate into regulatory T cells (Tregs) that play inhibitory role in immune responses, and these cells can be further divided into resting Tregs (rTregs) and activated Tregs (aTregs) according to developmental and functional heterogeneity.[50] Changes in these immunity types persist throughout the development of immune-related diseases[27], therefore corresponding characterization of $CD4^+$ T cells may conversely help us learn about the disease state or severity.

Similar with NK cells that mirror $CD8^+$ T cells in terms of cytotoxic activity, there is also an innate immune cell population called "innate lymphoid cells" (ILCs) that mirror $CD4^+$ T cells in terms of immune modulatory activity. The highly heterogeneous ILCs can be divided into 3 subsets (ILC1, ILC2 and ILC3) as functional counterparts of Th1, Th2 and Th17 respectively. Although ILCs are scarce in the peripheral blood of healthy donors[51] and barely explored in NAFLD patients (therefore will not be further discussed in this review), there have been basic studies indicating that these cells can participate in NAFLD pathogenesis [52–54], indicating the potential value of peripheral ILCs in future NAFLD studies.

Changes in the general NAFLD population

Peripheral $CD4^+$ T cells of NAFLD patients exhibited enhanced activation, as well as shifted polarization towards Th2 cells. It remains debatable whether there is significant difference in the overall frequency of $CD4^+$ cells between NAFLD patients and healthy volunteers.[31,36] However, similar with the changes in $CD8^+$ T cells, peripheral $CD4^+$ T cells of NAFLD patients also showed shrinkage in naive cell compartment and concomitant expansion in activated cell compartments, such as activated/memory ($CD45RO^+$) cells, terminal effector ($CCR7^-$ $CD45RA^+$), $OX40^+$ cells and pre-activated ($CD25^+CD45RA^+$) cells.[31,35,36] Additionally, significantly decreased frequency of either PD-1^+ cells or $CTLA4^+$ cells (inhibition markers) was ubiquitously found among multiple $CD4^+$ cell subsets of NAFLD patients.[31] As for Th1/Th2/Th17 polarization, some studies revealed augmented peripheral Th2 cell frequency among NAFLD patients.[31,36] In contrast, it varies among different studies whether the frequency of peripheral Th1, Th17 and Treg cells are significantly different between NAFLD patients and healthy people.[31,35,36,55]

Changes in association with NASH

$CD4^+$ T cells play important role in the pathogenesis of NAFLD according to basic studies. The cell fate of $CD4^+$ T cells can be influenced by the NAFLD microenvironment. For example, due to great susceptibility of $CD4^+$ T cells for lipotoxity, hepatic microenvironment with excessive fatty acid can lead to aberrant accumulation of reactive oxygen species (ROS) and consequent selective loss of intra-hepatic $CD4^+$ T cell (especially Treg cells).[56] In turn, $CD4^+$ T cells can influence NAFLD pathological progression in the form of altered functional differentiation. The pro-inflammatory Th1 subset can promote insulin resistance and adipose tissue inflammation, while the anti-inflammatory Th2 subset can alleviate insulin resistance and obesity, although the role of these two subsets in hepatic pathological changes remains unexplored.[41] In addition, accumulating evidence showed that the highly pro-inflammatory Th17 subset can promote liver inflammation and injury in NASH.[41,57]

Changes in the overall activation state, as well as polarization direction, of the peripheral $CD4^+$ T cell compartment may signify diagnostic markers for differentiating NASH from NAFL. For the overall activation state, NAFLD patients with higher NAS score (especially hepatocyte ballooning) were characterized by significant increase in effector memory cells and a trend of decrease in naive cell frequency correspondingly in peripheral $CD4^+$ T cells.[46] Therefore, $CD4^+$ T cell activation, characterized by the skewing from naive cells into "effector memory" or "terminal effector" cells, may be commonly applied for prediction of NASH. However, when it comes to peripheral T cell polarization, its correlation with NAS score seems to be quite complicated and debatable. Haas et al. reported that both Th1 (characterized by $CXCR3^+$, TNF-α^+ or IFN-γ^+) and Th17 (characterized by either $CD161^+$ or $CXCR3^-CCR6^+$) cell percentages among $CD4^+$ cells are positively correlated

with NAS score, especially with lobular inflammation.[46] Similarly, peripheral blood mononuclear cell (PBMC) from NASH patients showed significant elevated IFN-γ mRNA expression compared with NAFL patients.[58] As for Th2 polarization, although no significant correlation was found between IL-5 or IL-13 expressing $CD4^+$ T cells and NAS score, Th2 cell frequency was intriguingly in strong negative correlation with lobular inflammation, ballooning and total NAS score.[46] However, in contrast with aforementioned studies, Rau et al. found no significant difference between NAFL patients and NASH patients in terms of blood Th1, Th2, Th17, rTreg or aTreg cell frequency when these subsets were evaluated separately. To cope with the inefficacy of these cell frequencies in distinguishing NASH patients from NAFL patients, they proposed the ratio between different T helper subsets and rTreg, and found Th17/rTreg was specifically elevated in NASH patients but not in NAFL patients or healthy volunteers, while both Th1/rTreg and Th2/rTreg also exhibited significant elevation in NASH patients. Accordingly, both Th17/rTreg and Th2/rTreg dramatically decreased in NAFLD patients after underwent bariatric surgery, indicating a close correlation between these ratios and possible regression of NASH.[55]

In addition to the changed activation state, another adverse event called "T cell exhaustion" seem to be present in peripheral $CD4^+$ T cells during NAFLD progression. T cell exhaustion refers to the impaired effector function of activated T cells under persistent antigen and/or inflammatory signals, accompanied with increased expression of inhibitory receptors.[59] Interleukin-10 (IL-10) and PD-1 are the $CD4^+$ T cell exhaustion markers that have been explored in NAFLD patient blood. Multiple studies indicated that IL-10 seems to be in positive correlation with NASH progression. By evaluating mRNA expression of PBMC from biopsy-proven NAFLD patients, Kado et al. found significantly elevated IL-10 expression in patients with NASH compared with NAFL. In addition, IL-10 expression level was in positive correlation with hepatocyte ballooning and fibrosis.[58] Likewise, the frequency of IL-10-expressing cells among peripheral $CD4^+$ T cells showed the most prominent and significant positive correlation with NAS score. Meanwhile, both Treg cells and Th2 cells showed inverse correlation with NASH activity.[46] Although Th2 cells and Treg cells, according to classical description, are regarded as the major producers of the inhibitory cytokine IL-10 among $CD4^+$ T cells, it has been widely appreciated that all T helper subsets possess the non-redundant ability to produce IL-10, contributing to both desirable self-limitation during inflammation regression and undesirable persistence of pathological chronic inflammation.[60] Therefore, both IL-10 mRNA expression and $IL\text{-}10^+$ cell frequency among $CD4^+$ cells (especially T helper cells) promise to have high prognostic value in predicting NASH progression (especially hepatocyte ballooning).

Additionally, similar with the expression pattern in $CD8^+$ T cells, TLR9 expression level in peripheral $CD4^+$ cells of NAFL patients was lower than both NASH patients and healthy people.[34] Therefore, TLR9 expression level in both $CD4^+$ cells and $CD8^+$ cells may have diagnostic value for differentiating NAFL patients and NASH patients.

Changes in association with NAFLD-related fibrosis

$CD4^+$ T cells, especially the Th2 and Th17 subsets, have long been implicated in the pathogenesis of liver fibrosis.[61] However, the association between peripheral $CD4^+$ T cells and NAFLD-related fibrosis has been barely explored in both animal models and NAFLD patients.[41] In contrast with changes in NASH progression, decreased frequency of Th1-polarized $CXCR3^+$ cells, as well as exhausted $PD\text{-}1^+$ cells, was found in NAFLD patients with higher stage of hepatic fibrosis[31], possibly indicating the opposing role of $CD4^+$ T cell-related immunological changes in NASH progression and fibrosis worsening. A noticeable $CD4^+$ T cell subset here is $CD25^+CD45RA^+$ cells, possibly the "terminal effector" subset. Frequency of $CD25^+CD45RA^+$ cells among peripheral $CD4^+$ T cells was not only found to be higher in NAFLD patients with advanced fibrosis, but also maintained at this high level in NAFLD patients with fibrosis progression proven by repeated biopsy. For NAFLD patients without fibrosis progression at repeated biopsy, on the contrary, cell frequency of frequency of $CD25^+CD45RA^+$ cells would witness a significant decrease[31], indicating the potential value of peripheral $CD25^+CD45RA^+$ cell frequency in fibrosis worsening among NAFLD patients.

Monocytes and dendritic cells

Fundamentals in monocytes and dendritic cells

Apart from above-mentioned lymphocytes, rest of the peripheral leukocyte compartment is mainly constituted of myeloid cells including monocytes, dendritic cells (DCs) and granulocytes (neutrophils, eosinophil and basophils). Among them, neutrophils, i.e. the most abundant granulocyte subset in peripheral blood, are the major inflammatory infiltrating cells in human NASH biopsy specimens and have been regarded as a driving force for tissue injury and inflammation in NASH.[62] More importantly, phenotypic and functional changes, including increased expression of CD62L and increased efficacy in suppressing *ex vivo* proliferation and activation of T cell, have been reported in peripheral neutrophils from NASH patients compared with NAFL patients.[63] Neutrophils-related indices (e.g. Neutrophil-to-Lymphocyte Ratio, Lipocalin-2) also exhibit predictive value in NAFLD progression and have been finely reviewed elsewhere.[24,64,65] However, granulocytes do not belong to the commonly employed PBMC

compartment in the aim of study standardization, leading to limited number of granulocyte-related clinical researches in the era of NAFLD. Consequently, we will mainly focus on monocytes and DCs in this section.

Monocytes occupy the major myeloid cell compartment of PBMC. Based on differential expression of CD14 and CD16, monocytes in human peripheral blood could be basically divided into three distinct subsets, i.e. the short-lived classical monocytes ($CD14^{hi}$ $CD16^{-}$), intermediate monocytes ($CD14^{int}$ $CD16^{+}$) and the long-lived non-classical monocytes ($CD14^{lo}$ $CD16^{hi}$). As the major monocyte subset in human peripheral blood, classical monocytes can either infiltrate various tissues and differentiate into monocyte-derived macrophages (i.e. the classically defined "monocyte-to-macrophage" *in vivo*), or stay in the blood and differentiate into intermediate and non-classical subsets sequentially. Notably, with deepened understanding of mononuclear phagocyte system, diverse potential differentiation directions have been acknowledged in addition to the above two directions, including replacing embryonically derived "tissue resident macrophages", and acquiring "DC-like" or "Neutrophil-like" phenotype.[66,67]

Despite similarity in developmental origin (i.e. monocyte-DC progenitors in bone marrow) when compared with monocytes, DCs are manifested by strong antigen presentation capacity, and consequently play a vital role in T cell priming. Based on differential expression of CD123, CD141 and CD1c, DCs are divided into 3 major subsets, i.e. plasmacytoid DC (pDC), type 1 conventional DC (cDC1) and type 2 conventional DC (cDC2). In terms of difference in cellular function, cDC1s mainly facilitate the activation of $CD8^{+}$ T cells and NK cells through their special "cross-presentation" capacity for endogenous antigens, whereas cDC2s mainly participate in the activation of $CD4^{+}$ T cells through the classic presentation of exogenous antigens. In addition, pDCs uniquely participate in anti-viral responses through TLR7/TLR9-mediated recognition of viral nucleic acid.[68,69]

Changes in the general NAFLD population

According to researches based on complete blood count and flow cytometry-based PBMC analysis, frequency of the overall peripheral monocyte population exhibited a slight but statistically significant increase among NAFLD patients compared with healthy volunteers.[70,71] More importantly, multiple studies in terms of the three monocyte subsets unanimously revealed an overt shift towards intermediate and non-classical monocytes in NAFLD patients.[70,72,73] In addition, expression of C-C motif chemokine receptor 2 (CCR2) in peripheral monocytes was ubiquitously compromised in NAFLD patients regardless of different pathological stages, but the expression of C-C motif chemokine receptor 4 (CCR4) was upregulated in NAFLD patients conversely.[72] Toll-Like Receptor 4 (TLR4) expression, as well as *ex vivo* secretion of the classical proinflammatory triad (interleukin-1β (IL-1β), interleukin-6 (IL-6) and TNF-α), was drastically elevated among peripheral monocytes from NAFLD patients, especially in NAFLD patients with obesity.[74]

In contrast with the relatively abundant monocytes among PBMCs (around 10%), the DC subsets are all quite scarce in the peripheral blood, but alterations in peripheral DC phenotype and frequency also exist in NAFLD patients. Although peripheral DCs from NAFLD patients possessed enhanced endocytosis capacity in comparison with healthy people, their allostimulatory capacity after *ex vivo* lipopolysaccharide (LPS) stimulation was drastically impaired[75], indicating an activated yet functionally immature state of peripheral DCs during NAFLD pathogenesis.

Changes in association with NASH

The role of macrophage in NAFLD pathogenesis have been extensively explored in basic studies. In short, hepatic macrophages, abnormally activated by gut-originated endotoxin and lipoapoptotic hepatocytes, can exacerbate liver inflammation and tissue damage in NASH progression; dysregulated adipose tissue macrophages can additionally contribute to insulin resistance.[76,77] For example, our group recently found that exosomal miR-192-5p secreted by lipoapoptotic hepatocytes could lead to aberrant increase of pro-inflammatory cytokine secretion among hepatic macrophages.[78] With recent advances in lineage tracking studies, the inflammatory monocyte-derived macrophages (MoMFs) are now regarded as the major contributor to macrophage number increase and macrophage-related damage during NASH, while the self-renewal liver-resident macrophages (i.e. Kupffer cells) undergo decrease in cell number and can only get partly replenished by monocyte-originated, functionally immature "Kupffer cell-like" macrophages.[79–81] Nevertheless, these findings unanimously signify an essential role for monocyte as the source for NASH-progression related macrophage.

Changes in peripheral monocytes may reflect the underlying metabolic and immune dysregulations during NASH progression. The phenotypic shift of monocyte towards intermediate and non-classical subsets have been found in positive correlation with hepatic steatosis level, waist-to-hip ratio, waist circumstance and fasting glucose[46,70,73], possibly signifying their close relationship with the metabolic dysregulations. More importantly, the frequency of both intermediate and non-classical subsets were strongly positively correlated with the NAS score and each individual score (steatosis, lobular inflammation and ballooning), while classical subset frequency showed no correlation with these pathological changes.[46] Arias-Loste et al. found

that peripheral monocyte expression of Toll-Like Receptor 6 (TLR6), was specifically elevated in obese patients with NAFLD but not in either healthy volunteers or obese patients without NAFLD. Moreover, monocytes from NASH patients could produce higher level of IL-6 after TLR6 agonist *ex vivo* administration in comparison with monocytes from NAFL patients[82], signifying a promising diagnostic value of monocyte-expressed TLR6 in identifying NASH among NAFLD patients.

As for DCs, although the role of DCs have not been vigorously explored in NAFLD-related basic studies, acquisition of proinflammatory capacity and enhanced lipid storage are two phenotypic changes found among hepatic dendritic cells in NAFLD microenvironment, indicating the potential important role of DCs in NAFLD pathogenesis.[83] Later researches on peripheral DCs in NAFLD patients mainly concentrate on the frequency changes in DC subsets, but the findings remain controversial. Haas et al. found cDC2 frequency among PBMC was in significantly positive correlation with hepatocyte ballooning, while cDC1 frequency was not correlated with NAS score.[46] In contrast, Deczkowska et al. found cDC1 percentage among total leukocytes in both NAFL and NASH patients was higher than healthy people, but cDC2 percentage was comparable between groups.[84] In addition, pDC, the scarcest peripheral DC subset, exhibited diminished peripheral frequency in NAFLD patients[32,33], but whether this decrease is correlated with progression from NAFL to NASH is yet to be validated.

Changes in association with NAFLD-related fibrosis

During the onset and progression of NAFLD-related fibrosis, MoMFs can play profibrotic role in the way of activating hepatic stellate cells and facilitating myofibroblast survival according to multiple basic studies.[76,77] Apart from the above-mentioned hepatic injury and inflammation, changes in peripheral monocytes among NAFLD patients are also linked with NAFLD-related fibrosis. Peripheral monocyte expression of Siglec-1, a unique Siglec family member that participate in macrophage recruitment instead of inhibitory function, showed a modest elevation specifically in cirrhotic NAFLD patients. In contrast, the phagocytic capacity of peripheral monocytes was specifically hampered in non-cirrhotic NAFLD patients but not in cirrhotic NAFLD patients.[72] In addition, among peripheral monocytes from non-cirrhotic NAFLD patients, RNA expression of inflammatory cytokines (including IL-1β, IL-6 and TNF-α), as well as lipid metabolism-related CD36 and PLIN2, was elevated in NAFLD patients and positively correlated with fibrosis stage.[85] In addition to phenotypic and functional changes in monocyte itself, serum monocyte-originated microparticles and soluble form of cell surface protein in NAFLD patients are also found to reflect NAFLD pathological severity, which have been finely reviewed elsewhere.[24,86]

Potential challenges and possible solutions

From the above-mentioned studies, we can see that cell subset frequencies (summarized in Table 1) and molecular phenotypes (summarized in Table 2) of peripheral immune cells from NAFLD patients really undergo significant change, some of which are correlated to NASH progression and NAFLD-related fibrosis, signifying characterization of peripheral blood immune cells as a promising non-invasive approach for NASH progression. However, we have to admit that it is difficult to directly compare peripheral-leukocyte-based diagnostic algorithm with other established non-invasive diagnostic algorithm (e.g. the aforementioned NIS4 and FAST score) for validation of superiority in peripheral-leukocyte-based algorithm. This is mainly because peripheral immune cell characterization must be performed on freshly acquired and prepared blood samples with specialized expertise, making it hard and expensive to reach a comparable sample size to studies of other non-invasive algorithm studies. In addition, some challenges may loom and require to be handled in regard to proper "pre-stratification" of patients and some technical problems in immune cell characterization.

Pre-stratification of patients

NAFLD patients in real-world clinical settings are often complicated with other diseases that may also cause significant changes to peripheral immune cells. The most vivid example would be the diseases that have incorporated peripheral leukocyte testings into diagnostic evaluation, such as infectious diseases and immunodeficiency diseases.[87–90] In addition, co-morbidity of metabolic diseases (e.g. type 2 diabetes) and drug intake (e.g. metformin) have been proven to significantly alter the molecular phenotype of $CD8^+$ T cells and monocytes among NAFLD patients in the previously mentioned studies.[46,74] Therefore, prior to assessment of immune landscape, NAFLD patients need to be stratified according to co-morbidities and drug-use history. Similarly, metabolic genetic predisposition, such as polymorphism in PNPLA3, may also exert potential impact in both systemic and hepatic immune responses[42,91], therefore should also be tentatively considered.

For further improvement of diagnostic efficacy, healthy people without NAFLD, as well as NAFLD patients with cirrhosis, should be pre-excluded ahead of peripheral leukocytes analysis (Figure 1). This is partly because the present imaging modalities have already had high diagnostic-accuracy and cost-effectiveness in distinguishing NAFLD patients from non-NAFLD population, as well as recognizing the NAFLD patients with established cirrhosis. Another important reason is that, some immunological indices exhibit different changing patterns in NAFLD patients at different disease stages, such as aforementioned changes in NK cell frequency[37], TLR9 level among T cells[34] and Siglec-1 level among

Cell Population	Cell subset	Frequency change		
		NAFLD vs. Healthy people	NASH vs. NAFL	Late vs. early stage of fibrosis
$CD8^+$ T cell				
	Naïve cells	↓		
	Activated and/or memory cells	↑		
	IFN-γ^+	↑	↑*	
	$Perforin^+$		↑	
NK cell		↓	?	
	$CD56^{dim}$	↓		↑
	$Siglec\text{-}7^{dim}Siglec\text{-}9^-$ $CD57^+$	↑		
$CD4^+$ T cell				
	Naïve cells	↓	↓	
	Activated and/or memory cell	↑	↑**	↑***
	PD-1^+ or $CTLA4^+$	↓		↓
	IL-10^+		↑	
	Th1	?	?	↓
	Th2	↑	?	
	Th17	?	?	
	Treg	?	↓	
Monocyte		↑		
	Classical	↓		
	Intermediate	↑	↑	
	Non-classical	↑	↑	
Dendritic cells				
	cDC1		?	
	cDC2		?	
	pDC	↓		

***Table 1*: Changes in cell frequency of different peripheral immune cells in NAFLD patients.**
"↑", upregulation/positive correlation; "↓", downregulation/negative correlation; "?", contradictory between studies; blank, no significant correlation/yet to be studied; *, in non-diabetic patient; **, effector memory subset; ***, terminal effector subset.

Cell Population	Cell surface marker	Intracellular marker / assayed function
$CD8^+$ T cell		Perforin, granzymes, IFN-γ, TNF, TLR9
NK cell	NKp46, NKp30	Perforin, IFN-γ
	PD-1, ILT2, CD107a	
	CD38	
	Siglec-7, Siglec-9	
	TIGIT	
$CD4^+$ T cell	PD-1, CTLA4, OX40	GATA3, IL-10, IFN-γ, TNF, TLR9
Monocyte	CCR2, CCR4, TLR4, TLR6, Siglec-1, CD36	IL-1β, IL-6 and TNF-α
		phagocytic capacity, PLIN2
Dendritic cells		Endocytosis capacity, allostimulatory capacity

***Table 2*: Markers or functions with altered level among different peripheral immune cells from NAFLD patients.**

monocytes.[72] A possible explanation might be that the potential immunological mechanisms behind hepatic steatosis, inflammation and fibrosis in NAFLD are distinct from each other (e.g. differently dominant role of type 1 and type 2 immunity in hepatic inflammation and fibrosis respectively[92]).

Technical problems

Some obstacles also stem from technical problems in immune cell characterization *per se*. Currently, the most widely used technique for multi-parametric characterization of peripheral immune cells is flow cytometry (FCM). However, many aforementioned markers, such as

chemokine receptors and intracellular cytokines, require laborious processing (e.g. *ex vivo* culture, fixation and permeabilization) for desirable staining and often end up with undesirable results with low signal-to-noise ratio in FCM detection when compared with cell surface markers (especially the well-characterized CD markers), limiting their translational value for clinical evaluation. In addition, granulocytes that are typically discarded in the frequently exploited PBMC isolation, might also possess potential phenotypic changes and consequently deserve tentative exploration in the future.

Another set of technical problems lies with data analysis. Peripheral immune cell frequency and phenotypes are complicatedly subject to multiple factors aside from NAFLD pathogenesis, making stratification of NAFLD patients by an absolute cut-off value of each individual marker theoretically unrealistic. In contrast, improved efficacy of NAFLD progression identification may be achieved via phenotypic differences in multivariate space of the FCM result, as proposed in early researches.[33] Correspondingly, future data analysis of peripheral immune cell from NAFLD patients should incorporate "dimension reduction", thus allowing high efficient discovery of new "integrated marker" (e.g. Th17/Treg ratio[55] and Siglec-7^{dim}Siglec-9^{-} NK cell subpopulation[32]). However, it is undeniable that the multivariate characteristic in immune cell analysis inevitably increases the difficulty in unifying analysis standard across different studies. For example, which parent population should be used when calculating cell frequency of interested cell population? Which markers should be used for defining a certain cell population? Therefore, data analysis standard should be unified before these markers translating into future clinical utilization.

Aside from FCM in the above discussion, some other technologies have also been introduced into this field, such as transcriptome sequencing of isolated PBMC, mass cytometry and single cell RNA sequencing (scRNA-seq)[32,58] (Figure 1). Higher dimensional data can be derived via these technologies, potentially enabling the identifications of more novel markers. However, tremendous cost limits these technologies in the era of research and makes them hard to be put into clinical utility at present.

Concluding remarks and outstanding questions

In this review, we mainly summarize recent findings in peripheral immune changes among NAFLD patients, in order to assess the feasibility of novel NAFLD progression evaluation based on these research advances. Accompanied with NAFLD progression in patients, both peripheral lymphocytes and myeloid cells undergo significant phenotypic changes. According to current literature, the phenotypic changes that have been extensively studied in immunology need to be highlighted here as potential biomarkers: increased activation of cytotoxic cells and $CD4^+$ T cells, increased exhaustion of NK cells and $CD4^+$ T cells, skewed $CD4^+$ T cell polarization, and phenotypic shift of monocyte towards non-classical subsets. In addition, novel cell subsets such as Siglec-7^{dim}Siglec-9^{-} NK cells and novel markers such as the Siglec family, as well as the scarcely explored TLR family members (e.g. TLR9 and TLR6), also deserve further studies. Acting on the contention that systemic metabolic inflammation actively participates in NAFLD progression, we believe that multi-parametric characterization of peripheral blood immune cells through these markers could be a promising non-invasive approach for assessing NAFLD progression. In addition to diagnostic value, changes in these markers may also imply critical yet poorly recognized immune changes in NASH pathogenesis, and may translate into new therapeutic targets for NASH in the future.

The following outstanding questions remain to be solved in future studies. Firstly, what are the markers that have the best diagnostic value in identifying fibrotic NASH and predicting NAFLD progression risk? A standardized marker panel with good diagnostic efficacy should thereafter be built in order to facilitate clinical practice. A corresponding protocol should also be built to minimize disturbance from technical problems. Secondly, current researches are mainly based on cross-sectional observation, but we still lack prospective study of peripheral immune cells for better understanding their relationship with NAFLD progression or regression. Thirdly, what else can significantly distort the peripheral immune changes of NAFLD patients in addition to factors such as co-morbidities? Finally, giving that NAFL progression to NASH and fibrosis may differ in pathophysiology, would there be any integrated immunity modules (possibly resembling or even partially overlapping "type 1, 2, 3 immunity" module) that can be reflected by changes in peripheral blood and conversely facilitate better diagnosis?

Search strategy and selection criteria

Data for this Review were identified by searches of PubMed, Web of Science, and references from relevant articles using the search terms "NAFLD patient", "peripheral leukocytes", "T cell", "NK cell", "monocyte", and "dendritic cell". Only articles published in English between 2008 and 2021 were included, with a preference for those published after 2018.

Declaration of interests

The authors have no conflict of interests related to this publication.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (81873565, 82170593),

Shanghai Leading Talent Plan 2017, Collaborative Innovation Program of Shanghai Municipal Health Commission (2020CXJQ01) and Hospital Funded Clinical Research, Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine (17CSK04). The funders had no role in study design, data collection, data analysis, interpretation, and writing of the paper.

Contributors

Conception and design: (JGF), funding acquisition and supervision: (JGF), collection and assembly of data: (SZL), Manuscript writing: (SZL), revision and final approval of manuscript: (JGF). All authors have read and approved the submitted manuscript.

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