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REVIEW

T cells: Friends and foes in NASH pathogenesis and hepatocarcinogenesis

Pierluigi Ramadori | **Shing Kam** | **Mathias Heikenwalder**

Division of Chronic Inflammation and Cancer, German Center for Cancer Research (DKFZ), Heidelberg, Germany

Correspondence
Pierluigi Ramadori and Mathias Heikenwälder, Division of Chronic Inflammation and Cancer, German Center for Cancer Research (DKFZ), Im Neuenheimer Feld 242, 69120 Heidelberg, Germany.
Email: p.ramadori@dkfz-heidelberg.de and m.heikenwaelder@dkfz-heidelberg.de

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Abstract

In association with the pandemic spreading of obesity and metabolic syndrome, the prevalence of NAFLD-related HCC is increasing almost exponentially. In recent years, many of the underlining multifactorial causes of NAFLD have been identified, and the cellular mechanisms sustaining disease development have been dissected up to the single-cell level. However, there is still an urgent need to provide clinicians with more therapeutic targets, with particular attention on NAFLD-induced HCC, where immune checkpoint inhibitors do not work as efficiently. Whereas much effort has been invested in elucidating the role of innate immune response in the hepatic NAFLD microenvironment, only in the past decade have novel critical roles been unraveled for T cells in driving chronic inflammation toward HCC. The metabolic and immune microenvironment interact to recreate a tumor-promoting and immune-suppressive terrain, responsible for resistance to anticancer therapy. In this article, we will review the specific functions of several T-cell populations involved in NAFLD and NAFLD-driven HCC. We will illustrate the cellular crosstalk with other immune cells, regulatory networks or stimulatory effects of these interactions, and role of the metabolic microenvironment in influencing immune cell functionality. Finally, we will present the pros and cons of the current therapeutic strategies against NAFLD-related HCC and delineate possible novel approaches for the future.

INTRODUCTION

The recent progress achieved in the prevention and management of viral hepatitis (VH) contributed to contain the rising number of HCCs worldwide. However, HBV and HCV infections still represent the major cause of liver cancer worldwide.[1] On the other hand, in tight association with the global spreading of obesity- and metabolic-related diseases, the prevalence of NAFLD-related HCCs is increasing exponentially.[2] Indeed,

Abbreviations: ASH, alcohol-associated SH; CCL2, C-C motif chemokine ligand 2; CD-HFD, choline-deficient high-fat diet; CXCR6, C-X-C motif chemokine receptor 6; HCG, hepatocarcinogenesis; HFD, high-fat diet; IFNγ, interferon-gamma; KCs, Kupffer cells; Ly6C, lymphocyte antigen 6 complex, locus C; MHC, major histocompatibility complex; NAFLD, non-alcoholic fatty liver disease; NETs, neutrophil extracellular traps; OSEs, oxidative stress–derived epitopes; OXPHOS, oxidative phosphorylation; PAMPs, pathogen-associated molecular patterns; PD1, programmed cell death 1; PD-L1, programmed death ligand 1; RORγt, retinoic-acid–related orphan receptor gamma t; ROS, reactive oxygen species; SH, steatohepatitis; TAAs, tumor-associated antigens; TCR, T-cell receptor; Th, T helper; TLR, Toll-like receptor; Treg, regulatory T; VH, viral hepatitis; WAT, white adipose tissue; WD, Western diet.

NAFLD counts for the most common chronic liver disease, frequently diagnosed in quite advanced progression stages. In fact, NAFLD, and its more modern and comprehensive terminology, metabolic-associated fatty liver disease, describes a wide spectrum of liver alterations ranging from simple steatosis (SS) to steatohepatitis (SH; i.e., NASH) and fibrosis culminating in HCC, with an annual incidence in the USA and Europe ranging from 0.5% to 2.6% in patients diagnosed with cirrhosis.[3]

Intensive research has been performed to understand the mechanisms triggering the transition from SS, defined as abnormal accumulation of triglycerides, to SH, an advanced condition characterized by hepatocyte cell death, lobular inflammation, and initiation of a profibrogenic program. A consistent body of scientific evidence indicates that beyond obvious metabolic alterations leading to energetic imbalance and oxidative as well as endoplasmatic reticulum stress, a wide variety of factors (e.g., sex, lifestyle, genetics and epigenetics, and environmental toxicants) participate and influence progression of the disease.[4] Whereas lipotoxicity seems to be a key event in triggering, on the one hand, uncontrolled reactive oxygen species (ROS) production and, on the other hand, an impaired unfolded protein response, many other intrinsic and extrinsic factors synergistically determine the oscillation between cell death and compensative proliferation.

With the advent of new state-of-the-art technologies, novel light has been shed on this disease. This was achieved by the characterization of a wide number of different immune cell populations orchestrating a complex network of biological events in a well-defined spatiotemporal manner, determining the reversibility and resolution of the process or its irreversible progression to HCC.[5] Moreover, the notion that NAFLD represents a “hepatocentric” condition was finally overcome by decoding bidirectional metabolic and immune lanes of communication with other organs, like gut and adipose tissue, sustaining and maintaining the hepatic microenvironment.

Whereas it is classically believed that the innate immune response takes the stage in the early phases of the disease, recent findings indicate that the adaptive immune response could be responsible not only for the progression of NASH, but also for promoting hepatocyte transformation toward a malignant phenotype (e.g., through metabolic reprogramming of hepatocytes). Nevertheless, the precise origins and cellular temporal dynamics through which lymphocytes intervene in the process of chronic inflammation triggered by metabolic alterations are not yet completely understood. In this review, we provide a brief and concise update about novel findings on the role of T cells in NASH and NASH-derived HCC, illustrating their crosstalk with other immune cells, their interaction with this metabolic microenvironment, and the aspects of their biology that can be exploited to improve the efficacy and safety of immunotherapy in NASH and HCC.

INTERACTION OF INNATE IMMUNE AND ADAPTIVE IMMUNE CELLS IN NAFLD

Kupffer cells (KCs), the hepatic resident macrophages, are one of the first line of defense of the liver. In NAFLD, KCs can get activated by lipids and their cellular metabolites and produce ROS and proinflammatory cytokines (e.g., IL-1β and TNF) that activate other immune cells or contribute to hepatocyte cell death and regeneration. In experimental models of NASH, KCs were shown to be particularly vulnerable to lipotoxicty and display a limited autonomous turnover being replaced by circulating lymphocyte antigen 6 complex, locus C $(Ly6C)^+$ monocytes, mostly recruited by the chemokine, C-C motif chemokine ligand 2 (CCL2).[6] Although antagonizing the CCL2/C-C motif chemokine receptor 2 (CCR2) axis was shown to improve NASH,[7] CCR2 deficiency in mice undergoing NASH models did not display reduced incidence of HCC.[8] KCs also express Toll-like receptor (TLR) members that act as recognizing molecules for damage-associated molecular pattern molecules (DAMPs) released by injured hepatocytes or pathogen-associated molecular patterns (PAMPs; e.g., lipopolysaccharide) released in the circulation by the altered intestinal barrier during NASH.[9] In the context of NASH and HCC, KCs actively participate in the activation and recruitment of $CD8^+$ T cells in the liver, although the cellular mechanisms of interaction are still unclear (Figure 1). An indirect mechanism involving a physical crosstalk with activated platelets with KCs has been proposed recently to induce production of KC-derived cytokines and chemokines responsible for natural killer T (NKT) and $CD8^+$ T-cell hepatic infiltration with consequent progression of NASH.[10] In other pathological conditions, KCs seem to induce a more tolerogenic differentiation of $CD8^+$ T cells in a major histocompatibility complex (MHC)-I-dependent manner upon IL-2 stimulation.[11] Whereas the role of KCs and monocytes in NASH have been widely investigated in particular for their fibrogenic role through TGF-β-dependent signaling with HSCs, their interaction with T- and B-lymphocytes in promoting tumor progression seems to be etiology dependent and, possibly, associated with their polarization.[12]

For instance, an elegant recent study showed that in HBV-related HCC, M2-polarized tumor-associated macrophages can promote an immunosuppressive microenvironment characterized by increased expression levels of PDCD1 (programmed cell death 1; PD1) and T-cell immunoreceptor with Ig and ITIM domains (TIGIT) on $CD8^+$ T cells and natural killer cells.[13] Similar conclusions were also reported in an experimental model

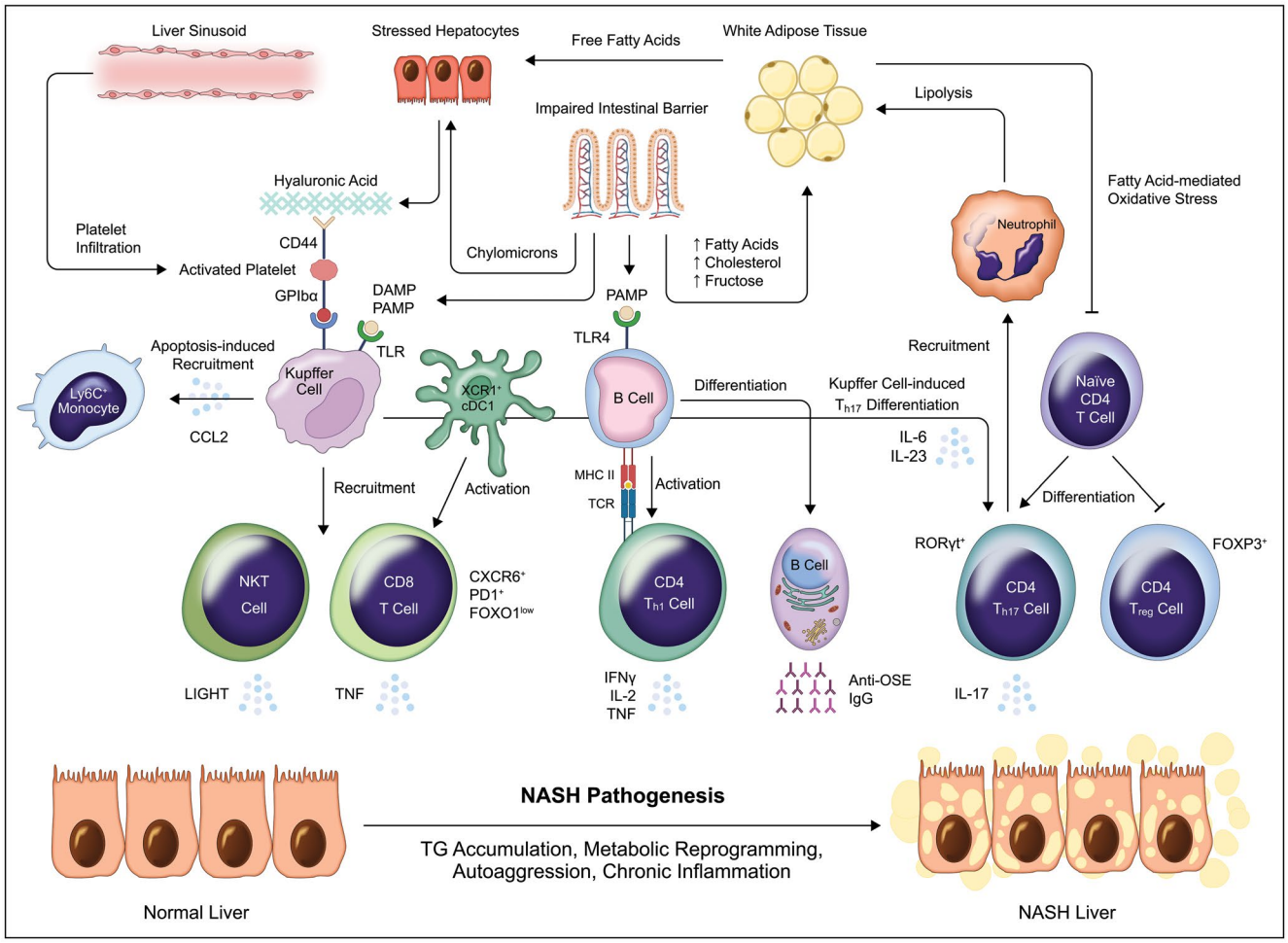

FIGURE 1 Crosstalk between innate and adaptive immune cell populations involved in the pathogenesis of NASH. Hepatic macrophages (KCs) are activated by lipids absorbed from the diet, from lipolysis of the WAT or by interaction with platelets attached to the hepatic extracellular matrix (hyaluronan). Activated KCs release ROS and cytokines that recruit infiltrating monocytes and trigger NKT and aggressive $CD8^+PD1^+$ effector T cells to produce proinflammatory cytokines. KCs can also induce Th17 differentiation by producing IL-23 or IL-6. In parallel, B cells are activated through TLR4 by PAMPs released by the intestine and can differentiate into plasma cells or induce interferon (INF)γ production in $CD4^+$ T cells through interaction with MHC-II. In NASH, lipotoxicity can also reduce intrahepatic $CD4^+$ T cells frequency altering the ratio Th17/Tregs. Neutrophils participate to the inflammatory response upon activation from Th17 that synthesize IL-17 and accumulate in the WAT where they promote lipolysis. cDC1, conventional type 1 dendritic cells; FOXP3, forkhead box P3; GPIbα, glycoprotein Ibα; TG, triglyceride XCR1, XC chemokine receptor 1

of NASH-associated HCC, where M2 polarization of macrophages was shown to promote hepatocarcinogenesis (HCG) through the generation of an immunosuppressive microenvironment (e.g., VEGF release).[14] In this scenario, a comprehensive analysis of cellular crosstalk in the immunomicroenvironment would deserve more attention. Furthermore, it was reported that macrophages/monocytes participate in senescence surveillance upon interaction with $CD4^+$ T cells, therefore exerting antitumor functions in a mouse model of impaired immune surveillance driven by oncogene overexpression with development of HCC.[15] If hepatocyte senescence were an event that might precipitate NASH-related liver alterations, this interesting aspect would deserve further investigations in the future.[16]

Another interesting myeloid population that has been recently shown to contribute to the pathogenesis of NASH is dendritic cells (DCs). A very recent publication identified $XCR1^+$cDC1 as a critical player in the early stages of the disease not only invading the liver parenchyma, but also interacting with $CD8^+$ T cells and activating their effector program in the liver draining lymph nodes[17] (Figure 1). However, another study reported a protective role for cDC1 cells in two experimental nutritional models of NASH; therefore, their role in fibrogenesis and in the late development of HCC is still an object of debate. In fact, under specific metabolic conditions characterized by alterations of lipid and glucose metabolism, the lipid load of hepatic DCs was shown to be a determinant of their immunogenic versus tolerogenic status in the liver.[18] Interesting recent work, based on a transcriptional network analysis performed on NASH patients before and after lifestyle interventions, identified a NASH

transcriptomic profile characterized by changes in genes involved in immune inflammatory processes, antigen presentation, and cytotoxic cells.[19] In particular, the researchers identified specific changes of circulating immune populations revealing an inverse correlation between blood cDC1 and NASH activity whereas cDC2 and $CD8^+$ T cells where strongly associated with hallmarks of NASH, such as lobular inflammation, hepatocyte ballooning, and antigen presentation markers. The same approach was applied for the identification of an immune-mediated cancer field through analysis of gene expression profiles in the overall onset of HCC, which characterized three different features related to an effector T-cell activation, an immune-suppressive and interferon-gamma (IFNγ) signaling proinflammatory profile.[20] Of note, the immunosuppressive phenotype was shown to be an independent predictor of cancer development in humans. In another study, the same researchers later identified a NASH-HCC–specific mutational profile associated with a higher prevalence of an immunosuppressive cancer field.[21]

Neutrophils are also a class of myeloid cells that was reported to infiltrate the liver in several stages of NASH and play a critical role in the transition from fatty liver to SH.[22] In the early phases of the disease, neutrophil depletion by administration of anti-Ly6G antibody and pharmacological inhibition of neutrophil elastase was shown to reduce liver damage and inflammation in murine experimental models of NASH.[23] Recently, neutrophils caught the interest of researchers for their capability to generate neutrophil extracellular traps (NETs) that are detectable also in the later stages of the disease.[24] Indeed, NET formation seems to promote an immune-suppressive environment through programmed death ligand 1 (PD-L1) signaling responsible for T-cell exhaustion in murine models of NASH-induced HCC[25] (Figure 1). Thus, further studies on neutrophils will surely reveal more interesting roles in NASH and NASH-HCC pathogenesis.

THE ADAPTIVE IMMUNE RESPONSE IN NAFLD

B cells

The contribution of the adaptive immune response to the pathophysiology of NAFLD and its progression to HCC is a finding that was strongly consolidated in the past decade. In the mouse, B cells represent up to 50% of the lymphocyte population, whereas in humans they account only for 8% of total lymphocytes.[26,27] They have been shown to take part in several pathological conditions, such as VH and alcohol-associated SH (ASH), and actively contribute to fibrosis development triggering HSC activation.[28]

Presence of infiltrating clusters of B- and T-lymphocytes was reported in human hepatic biopsies from NASH-diagnosed patients and in liver of different models of experimental NASH.[29,30] The role of infiltrating B cells in NASH is still poorly explored. B-cell activating factor was reported to be overexpressed in patients with NASH and associated with histology grade.[31] It was recently shown that B-cell infiltration in NASH livers colocalized with T-cell aggregates and positively correlated with oxidative stress–derived epitopes (OSEs) released from damaged hepatocytes.[32] In this work, B cells were shown, on the one hand, to present the epitopes to $CD4^+$ T helper (Th) 1 cells, which, in turn, activated the myeloid compartment through release of proinflammatory cytokines. On the other hand, they were shown to develop into plasma cells producing anti-OSE immunoglobulins (IgG). In a more recent study, intrahepatic B cells were shown to display a proinflammatory character and increased antigen-presentation ability.[33] In their work, the researchers provided evidence for the presence of a TLR4 receptor on B cells and of an innate response nature of this cell population, promoting a proinflammatory/profibogenic phenotype through activation of TLR4/myeloid differentiation primary response 88 intracellular signaling, possibly through gut-derived bacterial products. In a recent article by Shalapour et al., it was shown that liver-resident immunoglobulin A–producing cells might affect the tumor surveillance function in NASH-HCC, suppressing $CD8^+$ T cells by expressing high levels of PD-L1 and by IL-10 production.[34,35] However, further research is still needed to dissect the role and origins of this heterogeneous adaptive immune cell population in the context of NASH and NASH-HCC, which most likely exerts different functions in hepatic disease.

T cells

Conventional T cells, expressing αβ T-cell receptor (TCRαβ), are abundant in the healthy liver and represent more than 50% of the whole lymphocyte population. They recognize peptides bound to the MHC on antigen-presenting cells (APCs) and are mainly classified in $CD8^+$ and $CD4^+$ T cells.[36] $CD8^+$ cells are subdivided in tissue resident memory (TRM) that seem to patrol the liver and display a crucial role in viral infections and cytotoxic T cells that are activated by the interaction of MHC-I on APCs and exert cytotoxic functions by releasing cytokines such as TNFα and granzyme B.[37] $CD4^+$ T cells interact preferentially with MCH-II and have a more regulatory role, mainly maintaining the functionality of other immune cell populations.[38]

The specific $CD4^+$ T-cell subsets are described in detail later.

Cytotoxic $CD8^+$ T cells in NAFLD

In the recent past, the role of $CD4^+$ and $CD8^+$ T cells has been explored with more accuracy in different experimental NASH models, but also in the context of a clinical setting of NASH. In the context of chronic VH (e.g., HBV/HCV), lymphocyte organization in the hepatic parenchyma is typically resembled by ectopic lymphoid structures.[39] In the context of NASH, infiltrating immune cells colonize the liver differently: T and B cells are rather organized in a dispersed manner.[40] In mice, a long-term choline-deficient high-fat diet (CD-HFD) regimen was shown to recapitulate the chronic metabolic dysfunction of NASH and NASH-related HCC in humans, even recapitulating most of the histopathological features (e.g., ballooning, Mallory-Denk bodies) as well as the organization of dispersed inflammatory reactions.[8] This model resulted in diffuse hepatic penetration and activation of $CD8^+$ and NKT cells as observed in the human situation, in concomitant up-regulation of the inflammatory cytokine, LIGHT (LT-related inducible ligand that competes for glycoprotein D binding to herpesvirus entry mediator on T cells), and expression of effector function markers.

Of note, the use of $Rag1^{-/-}$ mice, lacking lymphocyte immune populations, resulted in a reversal of the NASH phenotype, independently of the metabolic alterations observed systemically (e.g., obesity). Similarly, depletion of $CD8^+$ T cells also resulted in the prevention of liver damage and inflammation without alterations of the systemic metabolic phenotype, providing clear evidence for a role of $CD8^+$ T cells and their secreted cytokines in NASH development and progression to HCC. Notably, these findings were reproduced in different nutritional and genetic NASH models associated with obesity, but, strikingly, $CD8^+$ cells seem not to play a central role in models of lean NASH.[41,42] It now appears that cholemia might act as a multiplication factor of lean NASH and NASH-HCC transition.[43] Moreover, obesity was recently shown to trigger signal transducer and activator of transcription 3–dependent induction of HCC, independently of the development of T-cell–driven NASH and fibrosis.[44] Therefore, the role of T cells in promoting HCG in this experimental context is an aspect that requires further investigations.

However, further characterization of the adaptive immune response in NASH recently revealed unexpected biological functions of $CD8^+$ T cells that shed light on the efficacy of current therapeutic strategies for the treatment of NASH-related HCC. In fact, novel findings contributed to the identification of a specific subpopulation of resident $CD8^+PD1^+$ cells responsible for the NASH phenotype and characterized by a sort of "hyperactivation" status characterized by the expression of the chemokine receptor, C-X-C motif chemokine receptor 6 (CXCR6), and by a down-regulation of the transcription factor, forkhead box O1 (FOXO1).[45] Mechanistically, the investigators could show, by an elegant series of *in vitro* experiments, that the acquisition of this autoaggressive character is mainly related to an IL-15-dependent mechanism. Moreover, they demonstrated that the metabolic changes occurring in the NASH microenvironment of mice, fed a CD-HFD and a Western diet (WD) through increased concentrations of acetate released by steatotic hepatocytes, contribute to increased levels of TNF and elevated expression of PD1 by $CXCR6^+CD8^+$ T cells. Furthermore, this specific population can induce hepatocyte apoptosis by Fas/Fas ligand (FasL) interaction, therefore explaining their detrimental role in NASH. These results indicate that the etiology-related specific metabolic and immune microenvironment unavoidably interacts with and influences the evolution of the hepatic disease favoring and sustaining hepatic survival and regeneration (Figure 1). Moreover, another interesting recent study explored the role of resident CD8 T cells in the context of NASH-induced fibrosis, highlighting a key interaction of CD8 T cells with HSCs, resulting in resolution of the fibrogenic process.[46] Of note, the investigators reported an increase of CD8 T cells in liver of mice fed a high-fructose, high-cholesterol (HFHC) dietary model of NASH, which was still persistent once the metabolic phenotype was reversed by switching the nutritional regimens to a normal chow diet after 24 weeks of treatment. Interestingly, deletion of CD8 T cells inhibited the resolution process as observed by persistent liver inflammation and fibrosis. Dissecting the CD8 cell population by single-cell RNA analysis, the investigators characterized a hepatic resident memory T cell (Trm) subpopulation of $CD44^+CD62L^-CD69^+CD8^+$ T cells also expressing resident markers, such as CXCR6, responsible for resolution of the fibrotic process. Accordingly, these resident T cells were shown to interact with HSCs by C-C motif chemokine receptor 5–driven chemotaxis and induce apoptosis in HSC by Fas-FasL contact.

Indeed, growing evidence highlights the importance of the metabolic crosstalk between the tumor and its microenvironment in supporting HCC growth and reshaping antitumor immunity. Recently, methionine metabolism was shown to generate a liver-cancer–promoting environment by sustaining tumor growth and inducing epigenetic changes in $CD8^+$ T cells leading to an exhaustion phenotype.[47] These findings, other than offering valid prognostic biomarkers predicting immunotherapy efficacy in patients with HCC, also propose therapeutic targets for the development of combinatorial therapies. On a similar note, another novel interesting study reported dramatic evidence on the effects of targeting PD1 in the context of NASH-related HCC.[48] In this specific case, two experimental

nutritional models of NASH-induced HCC (CD-HFD and WD) were used to show a massive accumulation of $CD8^+$ T cells in NASH and NASH-HCC liver tissues with an autoaggressive character as described in the article above. Surprisingly, the administration of anti-PD1 therapy failed to reduce the incidence of HCC in these murine models and, indeed, induced an increment of infiltrating $CD8^+PD1^+$ T cells, expressing high levels of effector and exhaustion markers with a residency signature (Figure 2). From a clinical perspective, these results did not really surprise, given that clinical studies already reported the lack of efficacy of immune therapy in NASH-induced HCC. Moreover, the same researchers could illustrate the results of a meta-analysis indicating a poorer overall survival response in patients diagnosed with nonviral HCC treated with immunotherapy as compared to patients diagnosed with viral HCC. Therefore, beyond the limitations of the human data, this work underlines the importance of a stratification according to the etiology that might influence the functionality of the immune system according to a specific microenvironment. In a similar context, it was found that NASH diminishes the effect of immunotherapy against intrahepatic metastasis (e.g., colon cancer).[49]

Role of $CD4^+$ T cells in NAFLD

In the setting of metabolic alterations, such as NASH and NASH-related HCC, the role of $CD4^+$ T lymphocytes is less understood and, given the wide heterogeneity of this cell population, their functions and origins are still quite puzzling. Using two nutritional models of NASH, methionine-deficient diet (MCD) and choline-deficient, L-amino-defined (CDAA) diet, on top of a murine transgenic model overexpressing the oncogene Myc, it was recently shown that $CD4^+$ T cells are much more vulnerable to an altered metabolic microenvironment as compared to $CD8^+$ cells.[50] Although both nutritional models might not optimally represent a clinical NASH condition characterized by obesity and metabolic

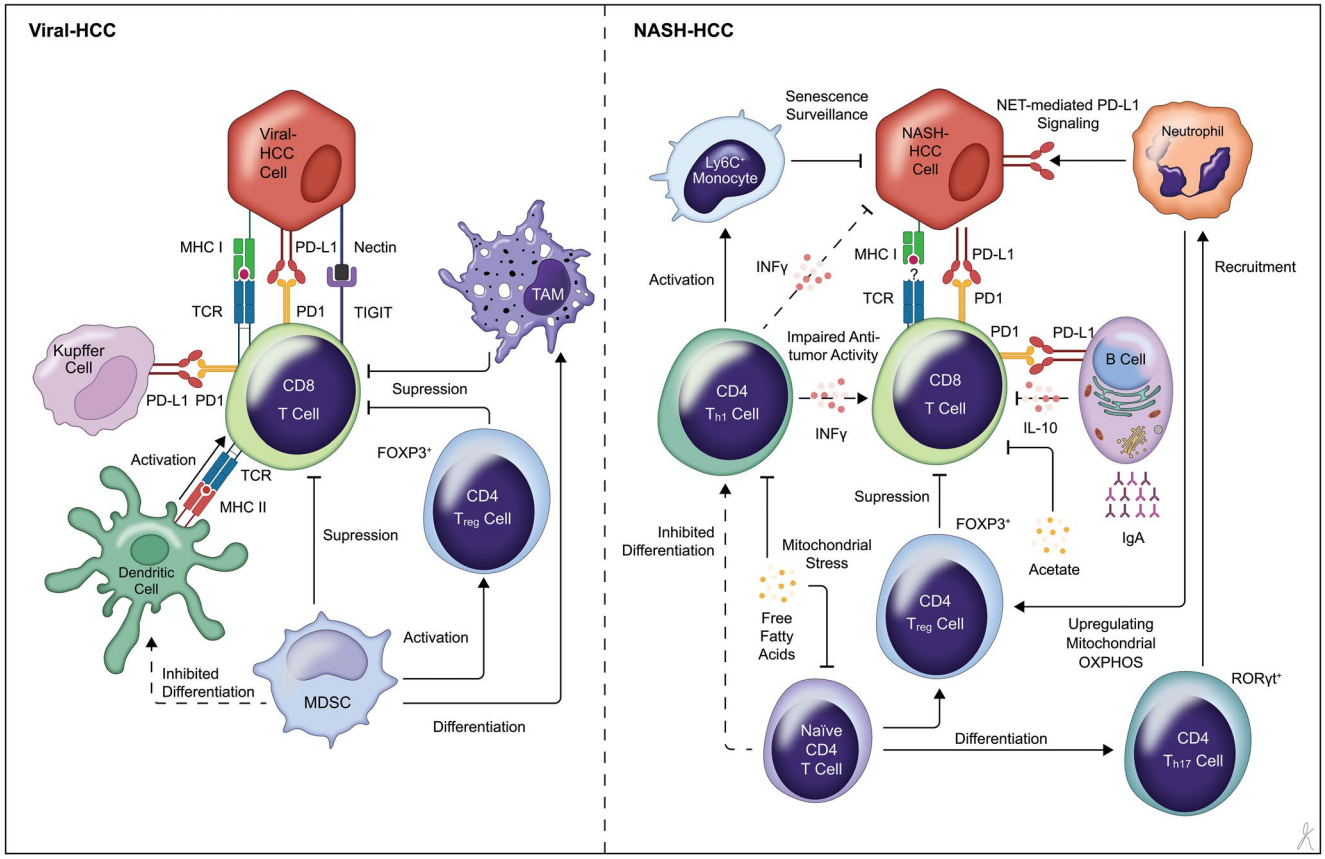

FIGURE 2 Antitumor immune responses in viral and nonviral HCC. In viral HCC, $CD8^+$ T cells interact with tumor cells presenting TAAs by MHC-I interaction. In response, tumor cells dampen $CD8^+$ T-cell activation via exposing PD-L1 and Nectin on their surface. In this frame, DCs also induce $CD8^+$ cell activation by MHC-II/TCR interaction, whereas macrophages and $DC4^+$ Tregs seem to play an immune-suppressive role. In NASH-HCC, autoaggressive $CD8^+$ T cells seem to lose their immune surveillance properties as a consequence of the interaction with the metabolic microenvironment. Reduced Th1 response and increased Tregs inhibition together with enhanced PD-L1 binding from other cell populations contribute to impaired antitumor activity. FOXP3, forkhead box P3; MDSC, myeloid-derived suppressor cells; TAM, tumor-associated macrophages; TIGIT, T cell immunoreceptor with Ig and ITIM domains

syndrome, dysregulation of lipid metabolism and the release of specific lipid metabolites (e.g., linoleic acid) induce a selective loss of resident $CD4^+$ cells as a consequence of their increased capacity to take up lipids leading to a burst of endogenous oxidative stress in relation to their consistent mitochondrial mass. Whereas this selective loss of T-cell subsets seems not to occur in other obesity-related models of NASH-HCC with the same efficacy (e.g., CD-HFD), it is an interesting field of research to be followed up on and might still require further investigation. Indeed, several studies found rather increased numbers of intrahepatic CD4 T cells in murine models of NASH, such as a WD and high-fat diet (HFD),[33,51] not necessarily related to HCG. Therefore, a role for $CD4^+$ T cells should also be better analyzed in the context of a WD- or CD-HFD–induced NASH-HCC to exclude a diet-specific phenotype.[48] (Figure 2). A deeper analysis of T-cell lymphocyte subpopulations revealed an enrichment of retinoic-acid–related orphan receptor gamma t $(ROR\gamma t)^+$ $CD4^+$ T cells producing high amounts of IL-17, at the expenses of a reduced regulatory T (Treg) population. Interestingly, using an experimental transgenic murine model, expressing hepatic unconventional prefoldin retinol binding protein 5 interactor, in combination with high caloric feeding (HFD), the research group of Nabil Djouder showed that the hepatocyte damage induced by genotoxic stress was able to trigger a selective recruitment of Th17 cells releasing a high amount of IL-17.[52] The investigators further demonstrated that IL-17 activates lipolysis of the white adipose tissue (WAT) and actively contributes to the development of hepatic steatosis, NASH, and the NASH-derived HCC phenotype. Accordingly, a systemic increase of circulating IL-17 was reported in patients with NASH, but also NASH and ASH.[53-55] On the basis of the observation that NASH results in loss of $CD4^+$ cells, the same group further analyzed this aspect in NASH-associated metastatic growth using a murine model of intrahepatic and subcutaneous injection of melanoma and colon cell lines in mice undergoing MCD or CDAA diets.[49] Accordingly, SH resulted in a reduction of tumor-infiltrating $CD4^+$ cells and in suppression of their antitumor activity. In line with their previous findings, the investigators showed that antioxidant therapy (*N*-acetylcysteine) was able to reinvigorate the vitality and functionality of $CD4^+$ T-lymphocytes in this specific setting.

In apparent contrast to these findings, recent studies reported an active role for central and effector memory $CD4^+$ cells in promoting and sustaining liver inflammation and fibrosis in an HFD combined with a carbohydrate enriched drinking water murine model of NASH.[56] However, novel evidence seems to support an intestinal and mesenteric lymph-nodal origin of $CD4^+$ T cells migrating to the liver in response to dysbiosis and responsible for this phenotype rather than a local activation of resident populations.[57] Moreover, adoptive transfer of CD4-derived double-negative T cells was recently shown to prevent NASH progression in different experimental models of NASH, resulting in a selective decrease of $CD4^+$ T cells with reduced IL-17 production in the liver and adipose tissue.[58]

Thus, the local and systemic metabolic conditions characterizing the development of liver disease emerged as a major determinant in tuning the functions and orientation of the immune response. These considerations further warrant the importance of understanding the metabolic changes of the immune microenvironment in the preclinical and clinical evaluation of anticancer therapies. Moreover, it should also not be forgotten that NASH in humans is much more heterogeneous than initially thought, dictated by the individual diet, lifestyle, and immune status of each patient—demanding for better stratification of NASH patients—almost on a personalized level.

Role of Th cells in NAFLD

Taking into account the different subsets of $CD4^+$ T cells, the situation in NASH and NASH-HCC encounters further levels of complexity in mice and humans. The main effector $CD4^+$ T-cell populations can be grouped as Th1, Th2, and Th17.[59] Th1 cells are typically characterized by the production of specific cytokines IFNγ, IL-2, and TNF. They have been reported to be increase in persons diagnosed with NASH and in obese mice fed high caloric diets.[60,61] However, their role in NASH has been poorly explored, and controversial evidence in experimental murine models exists. Indeed, although $CD4^+$ Th1 were shown to participate in the immunosurveillance of senescent transforming hepatocytes,[15] most of the studies on NASH reported a general decrease of these CD4 T cells in the liver (Figure 2). Moreover, they might be exerting regulatory functions on macrophage polarization also through production of IFNγ, but their functions in the different stages of the disease are still unclear. Similarly, also for Th2 cells, published data are scarce. These cells are mainly characterized by their capability of producing IL-4, IL-5, and IL-13 and expressing the transcription factor, GATA binding protein 3.[62] They are also altered in peripheral blood and in the adipose tissue of obese patients, but their role in the liver has not been studied so far. In mesenteric lymph nodes, the ratio of Th1/Th2 was shown to be altered in murine models of NAFLD.[63] The subset of Th17 is characterized by a cell population of $CD4^+$ T cells expressing the transcription factor, RORγt, and by production of IL-17.[64] The importance of Th17 in NAFLD-associated obesity has been widely investigated in human and murine NAFLD. In humans, the transition from simple fatty liver to SH was shown to be determined by increased hepatic Th17 and expression of IL-17, accompanied by reduced frequency

of Tregs.[65] Accordingly, a recent experimental study showed a constant time-dependent increase of infiltrating Th17 cells in liver and WAT of obese mice fed a high-fat, high-fructose diet.[66] Notably, administration of anti-IL-17A pharmacological therapy resulted in reduced inflammation without improvement of steatosis and liver damage. In this line, inhibition of lipogenesis through pharmacological inhibition of acetyl-CoA carboxylase 1 and 2 was shown to inhibit the differentiation of naïve $CD4^+$ T cells into a proinflammatory Th17 subset,[67] highlighting the importance of lipogenesis in T-cell polarization.[68] A similar study, analyzing Th17 activity in NAFLD in the setting of diet-induced obesity, was recently published.[69] In this case, the researchers showed that the NAFLD microenvironment and the metabolic systemic alterations induce IL-17A production and an increase of circulating and hepatic Th17 $CD4^+$ T cells. Notably, these cells display a proinflammatory phenotype regulated by the expression of C-X-C motif chemokine receptor 3 and induced by a metabolic reprogramming triggering a glycolytic process. In another metabolic setting characterized by ASH, a similar increase of Th17 was repetitively reported, and genetic deletion of RORγt resulted in the inhibition of alcohol-induced gut inflammation and dampened liver damage.[70] A better understanding of the metabolic changes shaping T-cell differentiation and orientation in different liver etiologies will be important for the development of personalized therapies.

Role of tregs in NAFLD

T regulatory cells, identified as $CD4^+FoxP3^+CD25^+$ Tregs, display a pronounced regulatory role and exert more tolerant anti-inflammatory functions through production of IL-10.[71] Given their immunosuppressive nature, Tregs might promote immunosurveillance escape of cancer cells and were reported to clonally expanded in HCC.[72] However, the exact role of Tregs in the development of NASH is unclea,r and their role in this setting remains controversial.[73] Recent findings in HFHC nutritional murine models indicate that Treg frequency increases in the NASH liver, which might counterbalance the $CD8^+$ T-cell effector proportion.[74] However, adoptive transfer of Tregs in mice lacking B- and T-lymphocytes ($Rag1^{-/-}$) failed to rescue the aggravation of inflammation observed upon high caloric feeding. Indeed, Treg transfer seemed to exacerbate the metabolic phenotype. Similarly, a more recent interesting work attempted to shed light on the cellular mechanisms contributing to the increased frequency of Tregs observed in several NASH models.[75] The researchers used several murine models of NASH-induced HCC, such as CD-HFD in combination with diethylnitrosamine injection and the stelic animal model, to monitor a decrease in the frequency of total $CD4^+$ T cells, but an increase in the proportion of Tregs ($CD4^+Foxp3^+$), inducing an immunosuppressive microenvironment in the premalignant stages of disease. A limitation of this study is represented by the lack of data regarding the absolute numbers of intrahepatic CD4 T cells, which would indicate actual changes of this cell population. However, genetic (DEREG mouse model) and pharmacological (anti-CD25) deletion of Tregs resulted in a significant reduction of steatosis, fibrosis, and tumor burden in these NASH models. The researchers further demonstrated that the interaction of naïve $CD4^+$ T cells with neutrophils is responsible for their differentiation into Tregs, and this process occurs through a metabolic reprogramming enhancing mitochondrial oxidative phosphorylation (OXPHOS) triggered by TLR4 pathway activation (Figure 2). Also, in this case, metabolic changes altering the balance of OXPHOS/glycolysis sustain the polarization of T-lymphocytes determining progression of the disease.[75]

T CELLS IN NASH AND NASH-HCC IMMUNOTHERAPY

The novel introduction of immunotherapies in the treatment and management of late-stage liver cancer has certainly brought beneficial effects in the survival and lifestyle of patients. As compared to the classical systemic treatment approach represented by, for example, sorafenib/regorafenib, the introduction of combined therapy, such as anti-PD-L1 (atezolizumab) and anti-VEGFA (bevacizumab), has been shown to increase the overall survival rate of HCC patients in several clinical trials.[76] Nevertheless, recent clinical and experimental studies revealed etiology-specific outcomes that cautioned the clinical community to stratify patients according to the cause of liver disease. In fact, studies performed in the past decades were able to identify tumor-associated antigens (TAAs) efficiently recognized by T cells, indicating an actual T-cell response against HCC.[77,78] However, the effectiveness and safety of this response were recently shown to differ dramatically among patients and, seemingly, according to the causative agents generating a specific tumor microenvironment.

Given recent findings about how the metabolic microenvironment could not only affect tumor growth, but also shape the quality and quantity of immune responses, our understanding of the efficacy and selectivity of immunotherapy has improved. In this direction, clinical data reporting IFNγ production by TAA-specific T cells indicated a positive immune response in 22 of 32 patients with HBV-related HCC and 32 of 42 patients with HCV-related HCC, but only 6 of 18 patients with NASH-related HCC displayed a positive immune response.[79] These findings show a poorer immune response in patients with

NASH-related HCC, and a growing body of evidence supports this analysis, indicating a scarce therapeutic efficacy of immunotherapy in the context of metabolic disorders.[80] Whereas the lack of efficacy in this case was shown to be related to a $CD8^+$ T-cell–dependent mechanism of autoaggression and loss of immune surveillance, further research will be required to understand the effects of combined immunotherapy that might dampen $CD8^+$ T-cell reactivity reinvigorating their antitumor capacity. Accordingly, other clinical trials indicate that combined administration of anti-VEGF (bevacizumab) and anti-PD-L1 (atezolizumab) seems to revert the therapeutic effects of immunotherapy alone in nonviral HCCs, prolonging progression free survival.[81] Further preclinical and clinical investigations are required to clarify and confirm the beneficial effects of this combined therapy, and the cellular mechanisms are not yet fully understood. However, anti-VEGF therapy was shown to redirect cytotoxic T cells in the intratumoral environment, possibly by indirect normalization of the endothelium and direct dampening of T-cell exhaustion.[82] Moreover, stratifying metabolic targets in the tumor microenvironment and intensively investigating their effects on antitumor immunity might provide a further level of efficacy for immune therapy in this field. In this direction, considering the interesting amount of clinical studies evaluating the effects of antilipogenic, metabolic, and antifibrotic drugs on NASH patients, the development of combinatorial therapies, including immune checkpoint inhibitors, should be deeply analyzed starting from the level of preclinical NASH models. Notably, a clinical trial has been recently launched on a cohort of 106 cancer patients including also HCC diagnosed persons, where the effects of anti-PD1 (nivolumab or pembrolizumab) in combination with metformin or rosiglitazone (peroxisome proliferator-activated receptor gamma agonist) on best overall response and progression free survival will be evaluated (ClinicalTrials.gov Identifier: NCT04114136).

CONCLUSION AND FUTURE PERSPECTIVES

Recent experimental and clinical evidence indicates that NASH develops as a hepatic metabolic disturbance that drives an uncontrolled chronic inflammatory process directed by autoaggressive T cells involving also other innate and adaptive immune cells. Beyond monocyte/macrophage cell populations—classically involved in this process and retained to be the fuse of the inflammatory burst—also $CD8^+$ and $CD4^+$ T and B cells are emerging as protagonists of a highly more complex sustainable phenomenon. Although T cells seem to be responsible for a given phenotype, the mechanisms of activation, interaction with other cell types, and dynamics of infiltration seem to be different among the population according to genetic, epigenetic, and socioenvironmental characteristics (e.g., including lifestyle, type of diet, and exercise). From preclinical studies, we learned that several genetic and nutritional experimental models of NASH are characterized by reproducible key inflammatory events found in humans that nevertheless develop and progress with different intensity, kinetics, and outcomes. We also observed that diet composition in distinct nutritional models strongly influences not only hepatocyte functionality triggering metabolic reprogramming, but also shapes the hepatic immune microenvironment.[83] In NAFLD, the metabolic microenvironment was shown to influence $CD8^+$ T-cell functionality through a metabolic reprogramming that sustained their adaptability in these conditions and hindered targeted effector activities. In return, it is reasonable to think that the release of inflammatory/metabolic mediators from T cells and other immune cells might be able to modulate directly or indirectly the metabolic/effector functions of hepatocytes. Although this aspect will require deeper analysis, the comprehension of such a bidirectional process would be important not only for the development of combinatorial therapies, but also for the stratification and validation of future therapeutic approaches. In fact, the heterogeneity of HCC and the different response to specific therapeutic regimens might certainly also depend on the individual nature of the hepatic microenvironment—which in NASH/MAFLD also might be manifold—with many different liver disease subtypes. Understanding the mechanisms regulating the interaction between the metabolic and immune microenvironment in liver cancer will be crucial not only to improve the efficacy of current therapeutic strategies, but also to individuate, among HCC patients with NASH etiology, specific conditions (e.g., adiposity, insulin resistance, and hypercholesterolemia) that will lead to personalized treatments supporting and maintaining an optimal functionality of the immune response.

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AUTHOR CONTRIBUTIONS

PR: conception and design, drafting the article, preparation, creation of the published work, specifically writing the initial draft. SK: visualization, preparation and creation of the figures, revising the article for intellectual content. MH: conception and design of the article, final approval of the version to be published, supervision and funding acquisition.

CONFLICT OF INTEREST

Nothing to report.

ORCID

Pierluigi Ramadori https://orcid.org/0000-0001-6581-9648

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