REVIEW

Tumor Microenvironment in Hepatocellular Carcinoma: Key Players for Immunotherapy

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Abstract: Hepatocellular carcinoma (HCC) remains a serious medical therapeutic challenge as conventional curative avenues such as surgery and chemotherapy only benefit for few patients with limited tumor burden. Immunotherapy achieves clinical progress in the treatment of this prevalent malignant disease by virtue of the development of tumor immunology; however, most patients have experienced minimal or no clinical benefit in terms of overall survival. The complexity and diversity of tumor microenvironment (TME) built by immune and stromal cell subsets has been considered to be responsible for the insufficiency of immunotherapy. The advance of bioanalytical technology boosts the exploration of the composition and differentiation of these infiltrated cells, which reflect the immune state of the TME and impact the efficacy of the antitumor immune response. Targeting these cells to remodel the TME is one of the important immunotherapeutic approaches to improve HCC treatment. In this review, we focused on the role of these non-cancerous cells in the tumor progression, and elaborated their function on cancer immunotherapy when manipulating them as potential targets.

Keywords: immune cells, stromal cells, tumor microenvironment, hepatocellular carcinoma, immunotherapy

Introduction

Hepatocellular carcinoma (HCC) is a serious prevalent malignancy, and therapeutic options rely on tumor burden and the severity of concurrent liver disease. The early-stage loco-regional tumors are primarily treated with the modalities such as surgery, radiofrequency ablation (RFA), and transarterial embolization (TAE), but high post-operative recurrence usually caused the dreadful prognosis.[1–3] Multikinase inhibitors, representatives as sorafenib, lenvatinib, and regorafenib, have been approved as systemic chemotherapeutic agents in the treatment of advanced-stage unresectable tumors.[4,5] However, higher rate of severe drug-related adverse events results in the interruption of the treatment, thus injuring the efficacy and long-term outcomes of patients.[6] In addition, the intolerance of patients with compromised liver failure to the toxicities also impedes the procurement of maximal benefit from these drugs.[7,8] Some improved strategies like metronomic capecitabine administration at low dose without break, and dose modification of regorafenib based on monitoring real-time outcome have been assessed in clinical trials, and the effect is striking but still needs to be confirmed.[9,10] To improve therapeutic efficacy and life quality of patients, novel approaches different from the above-mentioned mechanisms are urgently required.

Recent advances in studying the connection between chronic inflammation and oncogenesis, and the phenomenon of immune infiltrate in neoplastic tissue indicates that immunotherapy is an appealing strategy to reduce

HCC proliferation, invasion and metastasis.[11] The discovery of T cell-suppressive immune checkpoints including CTLA-4, PD-1, and its ligand PD-L1 illuminates the prospect of cancer immunotherapy. Many antibody-based immune checkpoint inhibitors (ICIs) including ipilimumab (anti-CTLA-4), nivolumab (anti-PD-1), pembrolizumab (anti-PD-1), durvalumab (anti-PD-L1), atezolizumab (anti-PD-L1), etc. have been developed for the treatment of HCC patients, and the effects of these agents were being evaluated in clinical practice.[12–14] Despite these ICIs exhibiting the efficacy in the preliminary use, accumulating evidences in multiple clinical trials indicated the disappointing results for them used as a single-agent, with few obvious benefits for most patients with advance HCC.[15–20] Conversely, the combinations of one ICI with another, or with other anticancer agent with different activity have shown more striking effect. The advantage of the combinations on the efficacy over the monotherapy has been reported in the partial interim outcomes in several ongoing clinical trials.[21] For instance, in the Phase III IMbrave150 trial, the combination of the PD-L1 inhibitor atezolizumab plus the antiangiogenic agent bevacizumab exhibited statistically significant and clinical meaningful benefits over the monotherapy of sorafenib in terms of clinical parameters including objective response rate (ORR), progression-free survival (PFS), and overall survival (OS).[22–24] Final results in most clinical trials are still awaited. Although ICIs seem to play a crucial role as part of combinatorial strategies in the treatment of HCC, the lack of validated biomarkers of response and inconsistent outcomes in various clinical trials restrict patients with advanced HCC to procure maximal benefit from immunotherapy. A greater understanding of the role of potential biomarkers in the diversity of the immune context in tumor lesions is significant on the efficacy of immunotherapy.

Tumor microenvironment (TME) is an intricate ecosystem composed of cancer cells and non-cancerous cells, which plays a fundamental and indispensable role in HCC progression and greatly impacts immunotherapeutic outcomes.[25] In addition to cancer cells, various immune cells and stromal cells, as well as their released molecules, are abundantly filled within the TME, and govern the immune state of the TME in affecting immune response to cancer.[26] The TMEs that are broadly surrounded by immune cells but lack cytotoxic lymphocytes (CTLs) in the tumor core are termed as infiltrated-excluded (I-E) TMEs, which serve as the hallmark of poorly immunogenic or "cold" tumors resistant to immunotherapy.[27] Contrarily, infiltrated-inflamed (I-I) TMEs are characterized by high infiltration of CTLs, and tumors within I-I TMEs are regarded as immunologically "hot" tumors, which are sensitive to immunotherapy with relatively good prognosis and survival rate.[28] This classification emphasizes the importance of immune status for cancer immunotherapy, and thereby a comprehensive exploration of the immune contents within the TME could be favorable to optimize the immunotherapeutic strategies for potential target cells.

Generally, the immune cells infiltrating in the TME could be sorted into two subsets based on their contribution to the immune character of the TME. Myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), and regulatory T (Treg) cells, which are responsible for the building of immunosuppressive environment, are termed as suppressive immune cells. In contrast, cytotoxic CD8$^+$ T cells, CD4$^+$ T cells with a pro-inflammatory T helper 1 phenotype, and natural killer (NK) cells, which work together to exert antitumor effects, are ascribed to the sort of stimulatory immune cells. Additionally, stromal cells existing in tumor lesions also contribute to the establishment of HCC immune microenvironment via the regulation of immune cells by releasing inflammatory molecules. In this review, we will discuss the role of these cells in controlling tumor growth by orchestrating the construction of the TME, and the therapeutic strategies via targeting their activity and function, thereby enhancing the antitumor immunotherapeutic efficacy.
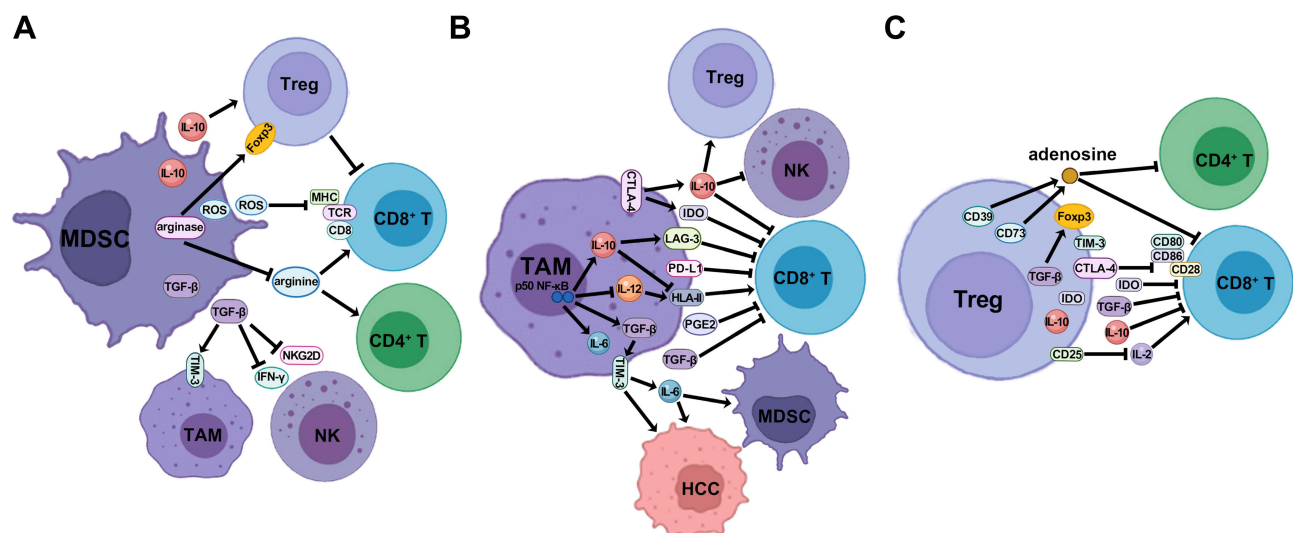
Suppressive Immune Cells

In physiological disease-free state, the liver exhibits intrinsic immune tolerance dominated by suppressive immune cells to inhibit inappropriate inflammatory response when processing multiple antigens derived from the gut and portal vein blood circulation. In the pathological state of chronic inflammation when the complex immune balance is disrupted, the predominance of suppressive immune cells in the microenvironment is responsible for immune escape of cancer cells, facilitating the development of liver tumor.[29] Thus, dampening the function of these cells via manipulating the expression of immunoregulatory molecules from them is an effective way of immunotherapy in HCC.

Myeloid-Derived Suppressor Cells

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous group of immature myeloid cells infiltrating in the TME. MDSCs play an instrumental role in promoting tumor growth, angiogenesis and metastasis in HCC through the subversion of antitumor immunity by releasing immunosuppressive signal molecules. Clinical studies have shown that increased number of MDSCs in tumor tissue and peripheral blood is associated with tumor progression in patients with HCC, and reduction of these cells can effectively inhibit tumor recurrence after resection or chemotherapy.[30] Therefore, MDSCs are able to act as the biomarker to evaluate the therapeutic effect and the potential target in the immunotherapy of HCC.

As one of the suppressive immune cells, MDSCs exhibit the primary function to cause T cell exhaustion, thus favoring immune escape of tumor cells. In a study using spontaneous HCC mouse model, MDSCs were found to trigger the suppression of CD8$^+$ T cells immune response.[31] The in-depth studies indicated that MDSCs are able to increase arginase activity and then deplete extracellular arginine, thus impairing CD4$^+$ and CD8$^+$ T cell proliferation. Meanwhile, the increase of arginase activity promotes the expression of Foxp3 on CD4$^+$ T cells, inducing differentiation and expansion of Treg cells, which blunts the effector T cell function.[32] Therefore, suppression of arginase activity in MDSCs is a promising antitumor immunotherapeutic strategy in HCC.

In addition to arginase, MDSCs were found to generate other immunosuppressive molecules to regulate multiple immune cells, which dampen T cell immune response to HCC. A recent in vivo study demonstrated that MDSCs induced the higher production of reactive oxygen species and peroxynitrite, which abrogated the binding of MHC to CD8$^+$ T cells through the nitration of TCR-CD8 complex, leading to the tolerance to antigen-specific CD8$^+$ T cell for favoring tumor escape.[33] Moreover, MDSCs produce TGF-β to stimulate the expression of TIM-3 on macrophage, which promotes the macrophage differentiation to immune-inhibitory phenotype.[34] TGF-β also mediates MDSCs to abrogate hepatic NK cell activity via reducing cytotoxicity, NKG2D expression and IFN-γ production in NK cells.[35] Additionally, MDSCs show the function to expand Treg cells and promote CD4$^+$ T cell differentiation to the T_H2 phenotype through the production of IL-10, which induce tumor cell proliferation, invasion and metastasis.[36] Collectively, since MDSCs serve as an important component in the TME to promote tumorigenesis through the regulation of various immunosuppressive cytokines, it is of great significance in the HCC immunotherapy to target MDSCs via cell number decrease or the related cytokine blockade (Figure 1A).

Figure 1 The mechanism of suppressive immune cells in the TME to promote HCC formation and the potential targets in these cells for HCC immunotherapy. (**A**) MDSCs suppress CD8$^+$ T, CD4$^+$ T, and NK cells, and stimulate TAMs and Treg cells by releasing various immunosuppressive molecules. (**B**) TAMs suppress CD8$^+$ T and NK cells, and stimulate MDSC, Treg and HCC cells by releasing various immunosuppressive molecules and expressing a variety of immune checkpoints on the surface. (**C**) Treg cells suppress CD8$^+$ T and CD4$^+$ T cells by releasing various immunosuppressive molecules and expressing a variety of immune checkpoints on the surface.

Tumor-Associated Macrophages

Macrophages are a group of monocyte-derived immune cells with the plasticity to differentiate into two diverse cell populations with opposite functions on cancer cell proliferation.[37] Classically activated macrophages, or M1 macrophages, possess tumoricidal activity via the release of antitumor inflammatory cytokines. Contrarily, alternatively activated macrophages, or M2 macrophages, promote the tumorigenesis by facilitating cancer cell proliferation. The advantageous phenotype of macrophages in the tumor, or the ratio of M1 versus M2 macrophages governs the status of TME, which exhibits anti-tumorigenic or pro-tumorigenic characters.

M1 macrophages produce pro-inflammatory cytokines, such as IL-12, and have the potential to stimulate effector T cell proliferation and function. They also stimulate the generation of reactive oxygen species (ROS) and nitric oxide synthase (NOS2) that promotes arginine metabolism into nitric oxide (NO) and citrulline, resulting in the microbicidal and tumoricidal effect.[38,39] Extracellular matrix protein SPON2 has been evidenced to be essential for recruiting M1 macrophages via binding to its integrin receptor α4β1. The combination of SPON2 and α4β1 activates RhoA and Rac1 to upregulate the expression of F-actin, leading to the accumulation of M1 macrophage in the tumor lesion to induce the antitumor immune response.[40] Several studies have manifested that M1 macrophages can be induced to phagocytose tumor cells by blocking the interaction between signal regulatory protein alpha (SIRPα) and CD47, and this therapeutic strategy has been assessed in multiple clinical trials in cancer.[41] In addition, chemotherapeutic drug sorafenib has been evidenced to trigger the pro-inflammatory activity of macrophages, reverse the polarization to M2, and enhance IL12 secretion by M1 macrophage.[42] Moreover, the ability of sorafenib to promote M1 activation could be consolidated by the natural CCR2 antagonist, indicating the combination of an immunomodulatory with a multikinase inhibitor could be an effective modality for M1 macrophage activation in HCC immunotherapy.[43]

Tumor-associated macrophages (TAMs) are mainly M2 macrophages that predominate in the HCC microenvironment, which are nurtured to favor tumor initiation, progression and metastasis. According to clinical data, high TAM infiltration in tumors correlates with short survival and poor prognosis in HCC patients.[44] Reprogramming TAM towards tumoricidal M1 phenotype seems to be a promising avenue to tumor regression. Studies have evidenced that the accumulation of p50 NF-κB factors in macrophages is responsible for the TAM-governed immunosuppression in HCC.[45,46] Therefore, decreasing p50 NF-κB factor in TAM to reinvigorate the antitumor M1 activity may be one approach for HCC treatment.

TAMs conduct immunosuppressive functions via the expression of anti-inflammatory cytokines, chemokines and growth factors to mediate the cell–cell mutual interaction. Previous literature has demonstrated that TAMs could produce IL-6 to exacerbate the immunosuppressive setting in favor of HCC progression.[36] The increased expression of IL-6 enhanced the yield of IL-10 in MDSCs, which induced the development of TAMs. This positive feedback loop between TAMs and MDSCs promotes immunosuppressive effect of cancer. TAMs per se produce high level of IL-10 and low level of IL-12, which inhibits the expression of HLA-II molecule in macrophages, blocking antigen presentation to T cell and thus disrupting the stimulation of immune response to HCC. Moreover, IL-10 also stimulates Treg cell expansion and blocks NK cell activation, which is associated with HCC development. Suppression of IL-6 by neutralizing antibodies could abolish the effect of TAM on upregulating pro-tumorigenic signaling cascades, leading to the activation of antitumor T cell response to HCC.

In addition, TAMs are able to regulate the expression of multiple immune checkpoints to sustain the immunosuppressive state in favor of HCC growth and metastasis. It has been reported that TAMs upregulate the expression of PD-L1 and immunosuppressive molecules, such as IL-10, TGF-β, and prostaglandin 2 (PGE2), which suppress cytotoxic T cell immune response.[47] LAG-3 is an immune checkpoint that acts synergistically with PD-L1 to promote cancer evasion from immunity.[48] A study in human HCC samples revealed that the expression of LAG-3 was associated with the TAM-mediated pro-HCC effect. Abrogation of IL-10 could block the pro-tumorigenic effect via the decrease of LAG-3 in TAM, suggesting TAM could regulate LAG-3 via the production of IL-10.[49] Moreover, CTLA-4 expression could induce the production of IL-10 and IDO in TAMs, which inhibit T cell activation and proliferation in a negative feedback way, and promote the conversion of naive CD4$^+$ T cells to Foxp3$^+$ Treg cells in the HCC microenvironment.[50] TGF-β stimulates the transcription and high expression of TIM-3 on TAMs, which promotes the secretion of anti-inflammatory

cytokines and enhances IL-6-induced tumor growth in HCC patients.[34] Furthermore, the TIM-3 expression promotes TAM to generate exosomes that transfer functional CD11b/CD18 proteins to HCC cell, endowing HCC cells with migratory activity.[51] Anti-TIM-3 antibodies have shown antitumor efficacy by suppressing TAM activity and inducing cytotoxic T cell immune activity.[52] Depleting M2-TAMs or transforming M2 to M1 phenotype can effectively reduce the production of pro-tumorigenic cytokines and signaling proteins, which ameliorates immunosuppression and reinvigorates antitumor immune response.[53] Therefore, M2-TAMs that serve as the target used to exploit the feasible immunotherapy for HCC have been documented and could be a new approach (Figure 1B).

Regulatory T Cells

Regulatory T (Treg) cells are an immunosuppressive subpopulation of CD4$^+$ T cells with the expression of inhibitory receptors CD25, CTLA-4, and TIM-3 on the cellular surface, and especially, the typical transcription factor Foxp3. Evidence demonstrates that Foxp3$^+$ Treg cells increase in peripheral blood from HCC patients, and infiltrate into the tumor, which serves as an independent prognostic factor for overall survival.[54] High infiltration of Treg cells in the tumor is usually associated with short survival rate and poor prognosis for HCC treatment. Studies have manifested that CCR6-CCL20 signaling axis plays a key role in recruiting Treg cells in the tumor lesion, and tumor-derived TGF-β signaling promotes the expression of Foxp3 in naive CD4$^+$ T cells, the hallmark of the differentiation to mature Treg cells.[54,55] Multikinase inhibitor sorafenib was found to reduce the frequency of Tregs infiltrating in tumor lesion by suppressing TGF-β signaling.[56] These evidences suggest that the activity of Tregs is regulated by the anti-inflammatory and immunosuppressive cytokines and chemokines in the TME.[57]

Tregs exert immunosuppressive effect and promote tumor proliferation orchestrated by a variety of immunosuppressive signaling molecules. It has been reported that the Treg cell is activated by TCR engagement concurrent with IL-10 and TGF-β signaling.[58] IL-2 functions to inhibit the accumulation of Tregs and promote cytotoxic T cell infiltration, while the expression of CD25 on Treg cell membrane can bind to IL-2 to reduce their content in the extracellular space. CTLA-4 is constitutively expressed on Treg cells, and competitively binds to CD80 and CD86 on the membrane of antigen-presenting cells (APCs), thus inhibiting the activity of the co-stimulatory molecule CD28 on effector T cells.[59] CTLA-4 also stimulates Treg cell activation and differentiation by increasing the immunosuppressive molecules IDO, IL-10 and TGF-β, which robustly enhances immunosuppressive state in HCC.[60,61] CTLA-4 blocking antibodies reinvigorate T cell immune response and alleviate the immunosuppressive HCC microenvironment via antibody-dependent elimination of Treg cells. In addition, Treg cells express high level of CD39 and CD73, which work as ectoenzymes to catalyze the conversion of ATP or ADP to adenosine.[62] Adenosine functions to induce CD4$^+$ and CD8$^+$ T cells exhaustion via the activation of A$_2$AR signaling, while targeting Treg cells via the blockade of A$_2$AR is considered to be a valuable auxiliary approach for HCC immunotherapy.[63] Taken together, suppressing Treg cells favors the goal of HCC immunotherapy via curbing T cell depletion and increasing stimulatory immune cell infiltration, allowing the elimination of tumor cells and the prevention of tumor development (Figure 1C).

Stimulatory Immune Cells

Contrary to inhibitory immune cells, stimulatory immune cells are considered as the effector cells that are regulated to induce or boost antitumor immune response. The essential functions of stimulatory immune cells on anticancer immunity include the surveillance, detection and destruction of tumor cells. The quantity and type of stimulatory immune cells infiltrating in the TME determine the strength and efficacy of the immune response to cancer. However, due to the inherent immunosuppressive specificity in HCC, the activity of stimulatory immune cells is usually succumbed, resulting in insufficient immune response. Moreover, as the effector cells in the immune system, the activities of stimulatory immune cells are generally influenced by cytokines, chemokines and signal proteins. Therefore, elucidating the function of various types of stimulatory immune cells, and their regulation in the HCC progression is crucial to revitalize the antitumor immune response.
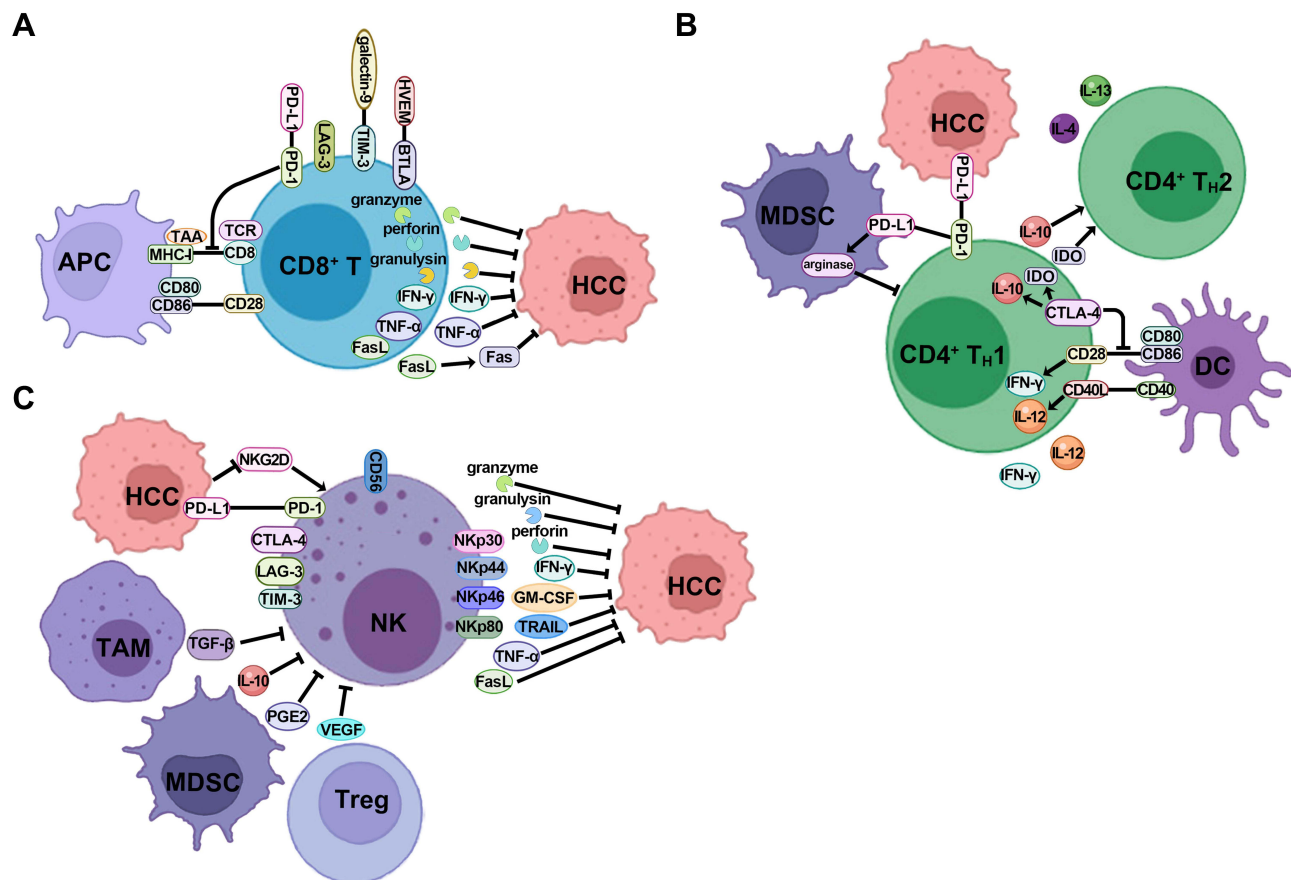
CD8^{+} T Cells

CD8^{+} T lymphocytes are the most powerful effector lineage of stimulatory immune cells in the antitumor immune response. CD8 and T cell receptor (TCR) form a co-stimulatory complex to be engaged with MHC-I and the presented tumor-associated antigen (TAA) peptide on target cells, leading to the activation of CD8^{+} T cells cytotoxicity.[64] The interaction of CD28 on CD8^{+} T cell and CD80 or CD86 highly expressed on APCs, increases antigen-reactive sensitivity of CD8^{+} T cells to enhance cell proliferation and pro-inflammatory cytokine production. Activated CD8^{+} cytotoxic T cells secrete death-inducing granules containing granzymes, perforin and granulysin, which provoke pore formation in the tumor cell membrane and subsequently induce cell death.[65] In addition, cytotoxic CD8^{+} T cells secrete pro-inflammatory cytokines IFN-γ and TNF-α that mediate antitumor immunity through signal transduction. Fas ligand (FasL) is expressed on cytotoxic CD8^{+} T cells, and the ligation with Fas on tumor cells induces the activation of caspase signaling, leading to the apoptosis of tumor cells.[66] Thus, the number of CD8^{+} T cell infiltrates is positively correlated with the improvement of overall survival.

Since the activation of effector CD8^{+} T cells is an important pattern to resist tumorigenesis, multiple suppressive factors are impressed to restrict T lymphocyte activity for tumor escape from immunity. In HCC, the immunosuppressive microenvironment induces the expression of various immune checkpoints on effector T lymphocytes, which function as co-inhibitory factors to interrupt the immune response. PD-1 that expresses on activated T lymphocytes induces T cell exhaustion via blocking TCR signaling through the engagement of PD-1 and PD-L1.[67] Moreover, the high expression of PD-1 on effector CD8^{+} T cells in HCC microenvironment is observed, and the number of PD-1^{+}CD8^{+} T cells is related to HCC progression and post-operative recurrence.[68] TIM-3 is another immune checkpoint that is upregulated in CD8^{+} T lymphocytes, causing the exhaustion of effector T cells via binding to the soluble protein galectin-9, whereas disrupting the interaction of TIM-3 and galectin-9 is effective in enhancing antitumor immunity.[69] LAG-3 expresses on activated CD8^{+} T cells and acts synergistically with PD-1 to promote cancer evasion from immunity through the suppression of MHC-II molecules.[48] Blockade of LAG-3 enhances antitumor T cell immune response, and dual inhibition of PD-1 and LAG-3 results in synergistic restoration of T cell immunity.[70] Another co-inhibitory molecule BTLA is overexpressed on activated CD8^{+} T cells in HCC patients, and BTLA^{+}CD8^{+} T cell could be effectively inhibited by binding to its ligand HVEM that is expressed on tumor cells.[71] Evidence shows that HCC patients with high HVEM expression exhibit more advanced disease and poor overall survival, concurrent with reduced lymphocyte infiltration and diminished antitumor mediators in peripheral blood and tumor tissues.[72] Thus, the blockade of immune checkpoints to revitalize the repressed CD8^{+} T cells is a potent strategy for CD8^{+} T cell-based immunotherapy (Figure 2A).

Adoptive cell transfer of engineered CD8^{+} T cells with chimeric antigen receptors (CARs) is an emerging immunotherapeutic approach that specifies and augments CD8^{+} T cell functionality. CAR-T cells are constructed by fusing the determinant of the tumor-specific antigen with an intracellular signaling domain in the autologous CD8^{+} T cells, and the engineered tumor-reactive CAR-T cells are expanded and reinfused back into the patient.[73,74] However, CAR-T cells show limited efficacy in patient with solid tumors including HCC for the difficulty in penetrating the fibrotic extracellular matrix barrier. A study indicated that engineered human CAR-T cells to secrete IL-7 and CCL19 could improve T cell infiltration and enhance tumor suppression efficacy in xenograft HCC mouse model.[75] A Phase I clinical trial in advanced HCC patients showed that the treatment with anti-GPC3-, IL-7- and CCL19-expressing CAR-T cells resulted in complete tumor disappearance 30 days post intratumor injection.[75] Therefore, the increase of cell penetration and TAA-specific sensitivity for CAR-T cells promotes progress in HCC immunotherapy.

CD4^{+} T Helper 1 Cells

CD4^{+} T cells are a heterogeneous subgroup of T lymphocytes that differentiate to diverse phenotypes, such as helper 1(T$_H$1) and helper 2 (T$_H$2), with the conduction of pro- or anti-inflammatory functions, respectively. CD4[+] T$_H$1 cells are stimulatory immune cells mainly working as an adjuvant regulator to enhance CD8[+] T cell antitumor immunity. By recognizing dendritic cells (DCs)-processed antigen and/or being exposed to DCs-derived IFN-I and IL-12, CD4[+] T cells are stimulated to proliferate and differentiate into T$_H$1 phenotype. The co-stimulatory signals acquired from the binding of CD28 on the T cells to CD80 and CD86 on the DCs further induce the activation of CD4[+] T$_H$1 to produce

Figure 2 The mechanism of stimulatory immune cells to inhibit HCC formation and the cross-talk with suppressive immune cells in the TME. (**A**) CD8$^+$ T cells function as an effector, activated by APC through the engagement of co-stimulatory surface molecules, to inhibit HCC cells by releasing cytotoxic molecules and pro-inflammatory cytokines. The activity of CD8$^+$ T cells is dampened by the expression of immune checkpoints on the surface. (**B**) CD4$^+$ T$_H$1 cells are stimulated by DCs via the binding of co-stimulatory molecules, and inhibited by MDSCs and HCC cells through the expression of immune checkpoints, and reprogrammed to CD4$^+$ T$_H$2 phenotype via the induction of anti-inflammatory cytokines. (**C**) NK cells suppress HCC cells via releasing a variety of cytotoxic molecules and pro-inflammatory cytokines, and are abrogated by TAM, MDSC and Treg cells via the secretion of anti-inflammatory cytokines, and by cancer cells via the expression of immune checkpoints.

IFN-γ.[76] The interaction of CD40L expressed on activated CD4$^+$ T cells with CD40 on APC promotes IL-12 generation and T$_H$1 differentiation.[77] This positive feedback effect promotes the accumulation of T$_H$1 cells in the TME. CD4$^+$ T$_H$1 cells manipulate DCs to present antigen peptides to CD8$^+$ T cells, facilitating the development of cytotoxic CD8$^+$ T lymphocytes for efficient antitumor immune response. Therefore, CD4$^+$ T$_H$1 cells involve the immune elimination of tumor cells through the regulation of effector T cells, and increased activity of T$_H$1 cells marked with the expression of cytokines IL-1, IL-2 and IFN-γ is associated with good prognosis in HCC clinical practices.

Similar to CD8$^+$ T cells, CD4$^+$ T$_H$1 cells are inhibited by the expression of multiple immune checkpoints and immunosuppressive cell infiltrates in the TME. CTLA-4 is induced to express on activated CD4$^+$ T cells, and competes to block the engagement of CD28 with CD80 or CD86, preventing the initiation of immune response.[78] CTLA-4 also promotes the secretion of the immunosuppressive molecules IL-10 and IDO to foster differentiation of CD4$^+$ T cells to the T$_H$2 phenotype with the production of IL-4 and IL-13 that potentiate the immune evasion of tumor growth.[61] CTLA-4 blockade is evidenced to reverse the effect on repressing CD4$^+$ T cells activity, increasing the T$_H$1-associated cytokines IL-2 and IFN-γ. The high level of PD-1 expressed on CD4$^+$ T cells binds to PD-L1 on both APCs and tumor cells, resulting in T cell exhaustion and tumor cell viability.[79] The binding of PD-1 and PD-L1 on MDSCs gives rise to the release of arginase, which causes CD4$^+$ T cell repression by depleting arginine.[32] Interruption of the binding of PD-1 to PD-L1 by neutralizing antibody effectively promotes CD4$^+$ T$_H$1 differentiation concomitant with increased

level of pro-inflammatory cytokines. Thus, checkpoint blockade mainly targeting the inhibitory molecules is an effective approach to reverse the repression of CD4$^+$ T cells and enhance antitumor T cell activity (Figure 2B).

Adoptive cell transfer of genetically modified CD4$^+$ T cell is also an immunotherapeutic option for the augmentation of CD4$^+$ T cell function. Early experiments indicated that the transfer of CD4$^+$ T cells greatly enhanced CD8$^+$ T cell antitumor response. Compared with CD8$^+$ T cells, CD4$^+$ T cells are apt to infiltrate and proliferate in tumor tissue, highlighting their importance in the antitumor immunity.[80] Recently, adoptive transfer of tumor-infiltrating lymphocytes (TIL), supplemented with IL-2, has become one of the personalized anticancer immunotherapies, and genetic modification of TIL-derived CD4$^+$ T cells is efficient to control tumor growth. A study indicated that the transfer of autologous TIL-derived CD4$^+$ T cells modified with ERBB2IP mutation into a patient with metastatic cholangiocarcinoma increased the durable suppressive response to tumor relapse.[81] Another clinical case evidenced that the transfer of CD4$^+$ T cells specific for a mutation of BRAF, a tumor-specific antigen, achieved long-term tumor control upon the expansion and persistence of T cell immunity.[82] Thus, these findings reveal the importance of CD4$^+$ T cells as a highly potent and clinically crucial subset for effective T cell-based immunotherapy.

Natural Killer Cells

Natural killer (NK) cells are stimulatory immune cells of the innate immune system, and stand at the forefront of the body's defense systems.[83] The characteristic phenotype of NK cells is the expression of CD56, and the absence of CD3 and TCR.[84] In contrast to T cells, NK cells recognize the tumor-associated surface proteins and the special MHC-I molecules on the malignant cells without requiring antigen sensitization. In cancer immunotherapy, NK cells show the potential benefit with the rapid recognition and elimination of malignant cells with little side reaction. NK cells can eliminate the majority of tumor cells in the initial stage; however, the activity of NK cells generally decreases with cancer advance.[85] Therefore, the infiltration and cytotoxicity of NK cells in the cancer tissue is an important indicator for treatment efficacy and survival of patients.[86]

NK cells recognize and kill tumor cells via the communication of signal receptors with their ligands on the surface of target cells, such as damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs).[87] The upregulation of stimulatory signal receptors through intercellular interaction provokes the antitumor activity of NK cells, which triggers to release toxins including granzymes, granulysin, and perforin, and death receptor ligands, including IFN-γ, TNF-α, TRAIL and FasL.[88] NKp30, NKp44, NKp46 and NKp80 that constitutively express on NK cells are the important stimulatory signal receptors for the activation of NK cell cytotoxicity.[89] The usage of co-stimulatory signals from specific antibodies to bridge NK cells and target cells further improves the efficacy of NK cell immune repulsion to cancer.[90] A study evidenced that a bispecific antibody targeting CD16 on effector cells and CD30 on tumor cells enhanced the connection of both cells, which produced more cytotoxic granules for the effective lysis of tumor cells.

Apart from stimulatory signal receptors, the immune inhibitory receptors such as PD-1, CTLA-4, LAG-3 and TIM-3 are expressed on NK cells, and the binding to their ligands generated by tumor cells blocks the NK cell antitumor response. The usage of anti-PD-1 antibody was evidenced to improve the antitumor activity of NK cells, and the data robustly suggested a possible role of NK cells in targeting the PD-1/PD-L1 interactions in immunotherapy.[91] Moreover, tumor cells may produce metalloproteinase to cleave NKG2D ligands from their surfaces and remove them by endocytosis, inhibiting the activation of NK cells.[92] Metalloproteinase inhibitors prevent tumor cells from spoiling NKG2D ligands and potentiate the revitalization of NK cell activity. In addition, MDSCs, Treg cells, TAMs and stromal cells accumulated in HCC microenvironment contribute to the dysfunction of NK cells by secreting various inhibitory cytokines. TGF-β and PGE2 reduce NK cell proliferation and cytotoxicity. Cancer-associated fibroblasts produce the dense, fibrotic ECM to prohibit the penetration and navigation of NK cells within the TME. TAMs stimulate angiogenesis and promote immune suppression via releasing VEGF, TGF-β and IL-10, which attract immunosuppressive MDSCs and stimulate the release of immunosuppressive cytokines. Therefore, relieving the restriction of immune checkpoints and/or suppressive immune cells is a powerful strategy to reinvigorate NK cell antitumor immune response (Figure 2C).

Stromal Cells

Stromal cells are a collection of a variety of non-immune mesenchymal cells including cancer-associated fibroblasts (CAFs), hepatic stellate cells (HSCs), endothelial cells, and dendritic cells (DCs), which are essential components in the building of HCC microenvironment.[93] Besides providing the scaffold structure, stromal cells possess the immunosuppressive, cytoprotective, stromagenic and proangiogenic properties of supporting progressive tumor growth and invasion by opposing immune-mediated cancer rejection through the expression of immune checkpoints and immunosuppressive factors. Evidence indicates that stromal cells generate diverse immunosuppressive cytokines and growth factors including TGF-β, IL-4, IL-10, CSF-1 and VEGF, which contribute to the immunosuppressive specificity of HCC microenvironment.[60] TGF-β and IL-10 work as the instrumental mediators to maintain immunosuppressive setting through the induction of Foxp3$^+$ Treg cells, inhibition of DC immunogenic functions, suppression of T_H1 responses and abolition of NK cell activity.[42] CSF-1 and IL-4 promote macrophage differentiation into M2 phenotype, and TAM infiltration in the TME.[94] VEGF conducts multiple tumor-promoting functions not only by promoting angiogenesis, but also by recruiting MDSCs and Tregs into the TME for the subversion of cytotoxic CD8$^+$ T cell antitumor immune response.[95,96] Accordingly, it has been proposed that the modification of stromal cells might potentially restore successful antitumor immunity.

Cancer-Associated Fibroblast

Cancer-associated fibroblasts (CAFs), a major component of tumor stroma, are typically evolved from the tumor-infiltrating fibroblast with the stem cell-like plasticity.[97] In HCC, CAFs not only construct the scaffold in the tumor milieu, but also promote tumor progression via secreting various cytokines and growth factors.[98] Intercellular communication between tumor cells and fibroblasts provokes the differentiation and maturation of CAF in the TME. A study demonstrated that high level of tumor cell-derived miRNA-21 has been correlated with the activation of CAFs and high vessel density in HCC patients.[99] MiRNA-21 was confirmed to stimulate the activity of CAFs to secrete angiogenetic cytokines such as VEGF, MMP2, MMP9 and TGF-β through the activation of PDK1/ATK signaling, leading to HCC progression. Importantly, the CAF-mediated oncogenic effect could be attenuated by upregulation of PTEN, suggesting PTEN is one of the regulators of CAFs. Therefore, PTEN might serve as the target to suppress CAF in the treatment of HCC.

In addition to being regulated by tumor cells, CAFs could educate tumor cells to sustain pluripotent properties of self-renewal, metastasis and tumorigenicity in HCC. CD24 is identified as a marker of stem cells, and its overexpression on HCC cells is associated with poor prognosis.[100] Hepatocyte growth factor (HGF) and IL-6 secreted by CAFs promote pluripotent characteristics of CD24$^+$ HCC cells via the activation and phosphorylation of STAT3 signaling, thereby promoting tumorigenesis and cancer metastasis.[101]

Increasing studies have also emphasized the major role of CAFs in shaping the immunosuppressive condition for tumor progression by influencing immune cells in the TME. CAFs recruit the circulating MDSCs by secreting CCL2 to recognize and bind to CCL2R, leading to the suppression of immune response.[102] Knockdown of CCL2 by anti-CCL2 antibody remarkably abrogates the migration of MDSC and CAF-mediated HCC progression. In addition, CAFs induce the immune tolerance of HCC cells by recruiting the PD-L1-expressing neutrophils through the secretion of IL-6, while inhibition of STAT3-PD-L1 signaling cascade could attenuate the immunosuppressive effect.[103] CAF-derived IL-6 also prompts the accumulation of regulatory DCs through the activation of STAT3, and educates them to upregulate Treg production by secreting TGF-β in the TME.[104] Besides, CAFs produce cyclooxygenase-2 (COX-2) and IL-8 to provoke TAMs to release TNF and PDGF, which in turn promote CAFs activation.[105] Furthermore, HCC-derived CAFs inhibit the activation of NK cells by releasing immunosuppressive PGE2 and IDO, thus forming the immune tolerance setting for HCC development.[106] These evidences demonstrated that CAFs play an important role in the development of cancer cells, and targeting CAFs may be an effective way in HCC treatment (Figure 3A).

Hepatic Stellate Cells

Hepatic stellate cells (HSCs) are a significant component of stromal cells in HCC microenvironment, and secrete extracellular matrix proteins such as collagen I and III that contribute to fibrosis and HCC development.[107] The
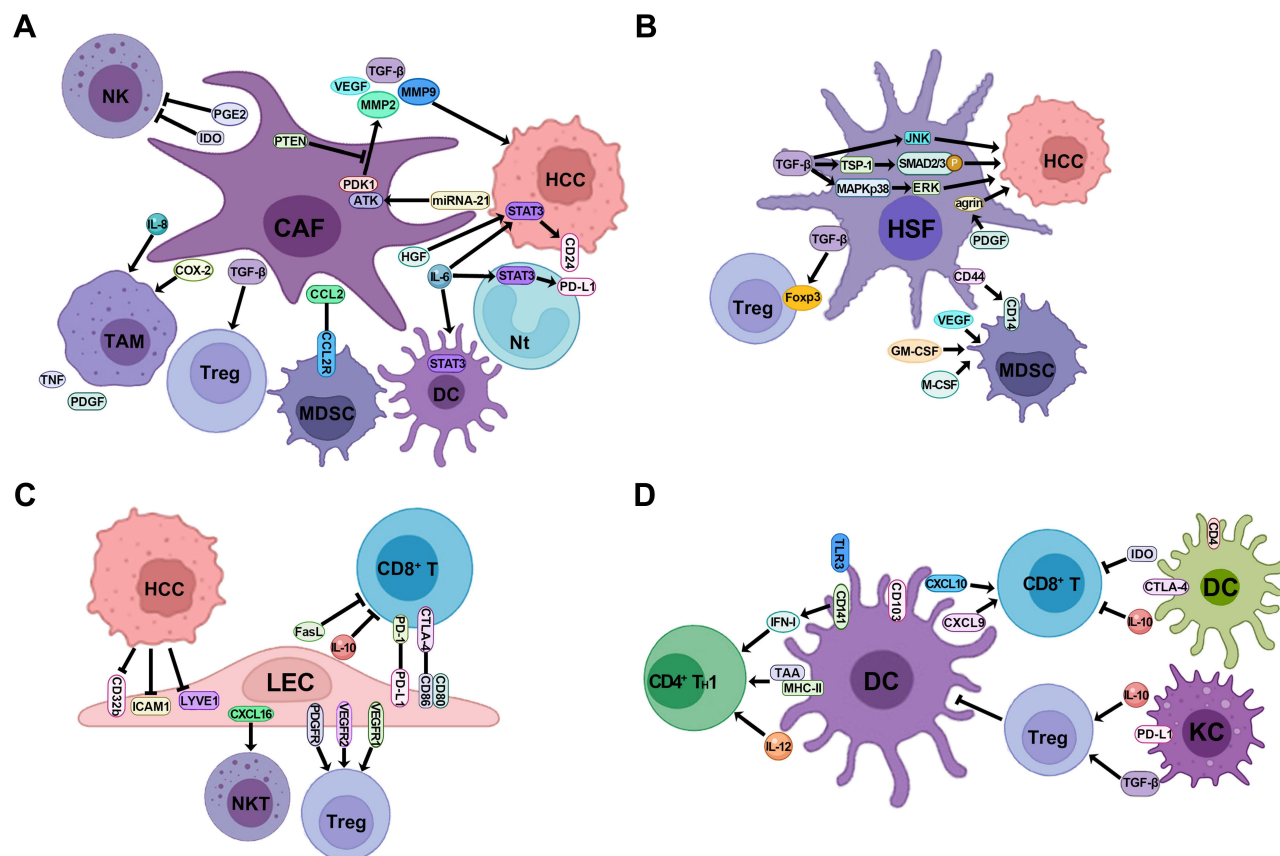
Figure 3 The mechanism of stromal cells in the TME to promote HCC formation. (**A**) CAFs promote the proliferation of HCC cells, recruit a variety of suppressive immune cells including TAM, MDSC, and Treg cells, and also inhibit the activity of NK, DC and Nt cells through the expression of various cytokines and chemokines. (**B**) HSFs promote the proliferation of HCC cells through the multiple TGF-β signaling pathways, and recruit MDSC and Treg cells through the release of cytokines and chemokines. (**C**) LECs conduct antitumor effect by releasing CXCL16 to stimulate the activity of NKTs, and are reprogrammed to inhibit CD8+ T cells when interacting with HCC cells to recruit Treg cells, release immunosuppressive cytokines and express immune checkpoints. (**D**) DCs act as the APCs to stimulate CD8+ T and CD4+ TH1 cells immune response to HCC, while the subtype of CD4+ DCs suppress CD8+ T cells via the expression of CTLA-4 and immunosuppressive cytokines. KCs serve as another type of APCs to stimulate Treg cells through the secretion of IL-10 and TGF-β, which inhibit the immune stimulation of DCs.

inflammatory cytokines and growth factors filled in the immunosuppressive TME activate HSCs to transform into myofibroblast-like cells with proliferative, migratory and invasive capability.[108] TGF-β has been evidenced to be one of the key cytokines that drive HSCs to form hepatic fibrosis through the regulation of multiple fibrotic-related signaling pathways.[109] Thrombospondin-1 (TSP1) is one of the signals that mediate the activity of TGF-β signaling, inducing phosphorylation of SMAD2 and 3 to upregulate downstream pro-fibrotic genes.[110] TGF-β can also activate MAPKp38/ ERK and JNK signaling pathways to provoke HSC activation, promoting the fibrosis and tumorigenesis in HCC. PDGF is another essential cytokine involved in HSC activation, and provokes the secretion of agrin, a fibrotic proteoglycan, in the activated HSCs. Agrin promotes HCC cell proliferation, metastasis, and invasion, which was inhibited by blocking the binding of PDGF to its receptor.[111] These studies reveal the role of activated HSCs in inducing fibrosis and HCC.

Activated HSCs also secrete anti-inflammatory mediators to cause the proliferation of suppressive immune cells, thus interfering with effector T cell immunity.[112] HSCs produce and secrete a number of soluble cytokines, such as GM-CSF, M-CSF, and VEGF, which function to induce MDSCs.[113] It was also reported that HSCs could promote MDSC recruitment and proliferation via the transfer of complement factor C3 in mouse model.[114] A study indicated that HSC activation could increase the expression level of CD44, leading to the generation of CD14+HLA-DR−/low MDSCs, while the knockdown of CD44 in HSCs could dampen the proliferation of MDSCs. Moreover, the specificity of CD44 caused by differential splicing determined the function of HSC on the induction of MDSCs, and distinct sets of CD44 splice variants affected the migratory ability of HSCs.[115] Another research indicated that HSCs secreted TGF-β to induce the differentiation of CD4+ T cells to Tregs.[107] Dunham et al demonstrated that the released TGF-β endowed the ability of

antigen presentation to HSCs, which provoked the expression of Foxp3 on T cells for Treg conversion. Blockade of TGF-β secretion could inhibit the direct and indirect effect of HSC-mediated immune tolerance in HCC.[116] Therefore, HSCs are the main producers of extracellular matrix in the liver, which play a key role in HCC progression by stimulating both MDSCs and Treg cells (Figure 3B).

Endothelial Cells

Endothelial cells in the liver are specialized stromal cells that are characterized with the lack of the basement membrane but the presence of open fenestrate. Liver endothelial cells (LECs) play a key role in maintaining liver function through the communication with other cells in the local microenvironment. These cells function as APCs to affect effector T cell, and regulate immune response through the release of cytokines.[117] During HCC progression, LECs lose their fenestrae and form a basement membrane, accompanied with the loss of ICAM1, LYVE1 and CD32b.[118] Meanwhile, LECs participate in angiogenesis, procoagulation and fibrinolytic process during tumorigenesis. These changes are representative of LECs differentiation and are suggestive of their role in promoting HCC development.

LECs overexpress PD-L1, as well as the co-stimulatory molecules CD80 and CD86, which bind to PD-1 and CTLA-4 present in T cells, respectively, inhibiting the antitumor activity of effector T cells.[119] The induced immune impotential state of T cell in turn promotes the production of IL-10 in LECs, aggravating the immunosuppressive effect on T cell activity. LECs also express various angiogenesis-associated receptors including VEGFR1, VEGFR2, and PDGFR. The interaction of these receptors with corresponding ligands induces proliferation and migration of endothelial cells, which facilitates the development of immunosuppression via the induction of Treg cells.[120,121] Tumor-associated LECs also express FasL, which contributes to immune evasion by deleting $CD8^+$ T cells without affecting Treg cells, leading to the tumor cell proliferation and invasion.[122]

Blockade of LECs function is a strategy for HCC treatment. Durvalumab, an antibody recognizing PD-L1 in LEC, has been evaluated for the antitumor effect in clinical trial.[123] MiR-3178 expression is downregulated in LECs, while its upregulation could be used as the therapeutic target in HCC treatment.[124] The usage of tyrosine kinase inhibitors in LECs such as cabozantinib or regorafenib also showed improving clinical efficacy in HCC.[123] In addition, simvastatin was found to promote the production of CXCL16 when nano-delivered into LECs, and dampen the fibrotic HCC development via the recruitment of natural killer T (NKT) cells.[125] Therefore, targeting LECs is a potential regimen in the therapy of HCC (Figure 3C).

Dendritic Cells

Dendritic cells (DCs) are the professional APCs that capture, process and present TAAs to T cells, thus initiating an adaptive immune response.[126] In HCC, communication between DCs and T cells is instrumental on stimulating effective antitumor immunity. DCs present the processed TAAs on MHC-II molecules to $CD4^+$ T cells for their proliferation and differentiation into T_H1 upon the synergy of IFN-I and IL-12.[127] In addition, it was reported that the $CD103^+$ DC subtype primarily releases the chemokines CXCL9 and CXCL10 to recruit $CD8^+$ T cells into the TME. Insufficiency of $CD103^+$ DCs impairs T cell migration into the tumor and abated antitumor response.[128] Kupffer cells (KCs) residing in the liver constitute another major APC population. Their antigen presentation can upregulate the expression of PD-L1 and the release of immunosuppressive molecules IL-10 and TGF-β, resulting in the induction of immunosuppressive Tregs and the suppression of T cell immune response.[47]

DCs exert pro-immunogenic function by presenting antigen to lymphocytes for their activation, which are severely inhibited by immunosuppressive specificity of HCC microenvironment filled by Treg cells, lactic acid, VEGF, immunosuppressive cytokines and adenosine.[129] However, a subpopulation of $CD4^+$ DCs has been identified in HCC patients, which specially express high level of CTLA-4, thus being supposed to mediate robust tolerogenic effects via CTLA-4-dependent production of IL-10 and IDO.[61]

Strengthening the cytotoxicity of effector T cells via enhancing the antigen-presenting ability of DCs is one promising approach of HCC immunotherapy. It has been reported that icaritin could promote DC activation via the induction of immunogenic cell death of cancer cells, thus elevating the effect on presenting the captured antigen to $CD8^+$ T cells to stimulate T cell cytotoxicity in HCC.[130] Moreover, the combination with Doxorubicin could further show synergistic effect on activating DCs to prime and stimulate memory T cells response to HCC. In addition, vaccine that is based on DCs has

Table I The Modification of Immune Components in the TME in Clinical Trials

Trial No.	Treatment Method	Targeted Cells in TME	Effect	Reference
NCT02716012	Activating RNA (MTL-CEBPA)	MDSC, M2	Inhibited	[133,134]
NCT02496949	Icaritin	MDSC	Inhibited	[135]
NCT02476123	Mogamulizuma and nivolumab	Treg	Inhibited	[136]
NCT02072486	Sorafenib	Treg	Inhibited	[137]
NCT03755791	Cabozantinib and atezolizumab	MDSC, Treg, TAM	Inhibited	[138]
NCT02699515	Bintrafusp alfa	MDSC, Treg, TAM	Inhibited	[139]
NCT01743469	Tasquinimod	MDSC, TAM	Inhibited	[140]
NCT03163992	Pembrolizumab	CD8$^+$ T	Activated	[141]
NCT03916627	Cemiplimab	CD8$^+$ T	Activated	[142]
NCT03198546	CAR-T	T cell	Activated	[75]
NCT00699816	IL2/anti-CD3 activated NK cells	NK	Activated	[143]
KCT0003973	Autologous NK cells and chemotherapy	NK	Activated	[144]
NCT04297202	Camrelizumab and apatinib	DC	Activated	[145]
NCT01974661	Ilixadencel (immune primer) and sorafenib	NK, CD8$^+$ T, DC	Activated	[146]
NCT00692770	Sorafenib	CD4$^+$ T, NK	Activated	[147]

been the other way used in cancer immunotherapy including HCC.[131] DC-based vaccines could promote DC maturation and activation, thereby effectively presenting the TAA to CD8$^+$ T cells, which is critical for the induction of anticancer cytotoxic response. To provide sufficient stimulatory signals, most DC-based vaccines are combined with Toll-like receptor ligands or cytokines in the clinical practice. A new subset of DCs that expresses CD141 was identified to show strong ability to present antigens to T cells, and was engineered to express Toll-like receptor 3 (TLR3) and high level of IFN-I, resulting in a robust T$_H$1 response and CD8$^+$ T cell antitumor immunity.[132] Globally, DC-based therapy has proven to be safe and capable of generating immune response to cancer in a substantial proportion of patients (Figure 3D).

Conclusion

It is well summarized that there is a substantial amount of non-parenchymal components consisted of immune and stromal cells accumulated in the liver, which commit multiple functions such as the building of systemic defense. Regardless of whether they conduct immunostimulatory or immunosuppressive effects, they are all primary constructive elements in HCC TME and act as immunological modulators by responding to various cell-surface ligands, producing a wide array of pro- and anti-inflammatory molecules, or directly killing cancer cells to affect the activity of tumor cells. Several clinical trials regarding the modification of immune components in the TME have been ongoing, and some encouraging results have been reported (Table 1). Therefore, therapies that revitalize the surveillance ability in immunostimulatory cells or keep immunosuppressive cells of the innate and adaptive immune systems dormant, and modulate the activity of various types of stroma cell to enhance immune response to tumor in the TME may produce promising effect on treating patients with HCC.

Abbreviations

HCC, hepatocellular carcinoma; RFA, radiofrequency ablation; TAE, transarterial embolization; CTLA-4, cytotoxic T lymphocyte-associated antigen-4; PD-1, programmed cell death protein 1; PD-L1, programmed cell death ligand 1; ICI, immune checkpoint inhibitor; ORR, objective response rate; PFS, progression-free survival; OS, overall survival; TME, tumor microenvironment; CTL, cytotoxic lymphocyte; I-E, infiltrated-excluded; I-I, infiltrated-inflamed; MDSC, myeloid-derived suppressor cell; ROS, reactive oxygen species; NOS2, nitric oxide synthase; NO, nitric oxide; SIRPα, signal regulatory protein alpha; TAM, tumor-associated macrophage; Treg, regulatory T; NK, natural killer; TIM-3, T cell immunoglobulin domain and mucin domain-3; PGE2, prostaglandin; LAG-3, lymphocyte activation gene-3; APC, antigen-presenting cell; TCR, T cell receptor; TAA, tumor-associated antigen; FasL, Fas ligand; CAR, chimeric antigen receptor; T$_H$1, CD4$^+$ T helper 1; T$_H$2, CD4$^+$ T helper 2; DC, dendritic cell; TIL, tumor-infiltrating lymphocyte; DAMP,

damage-associated molecular pattern; PAMP, pathogen-associated molecular pattern; CAF, cancer-associated fibroblast; HSC, hepatic stellate cell; HGF, hepatocyte growth factor; COX-2, cyclooxygenase-2; TSP-1 thrombospondin-1; LEC, liver endothelial cell; NKT, natural killer T; KC, Kupffer cell; TLR3, Toll-like receptor 3.

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Disclosure

The authors report no conflicts of interest in this work.

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