The Single-Cell Level Perspective of the Tumor Microenvironment and Its Remodeling by CAR-T Cells

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10.1 Introduction of the Tumor Microenvironment (TME)

Chimeric antigen receptor T (CAR-T) cell therapies show promising efficacy in leukemia and lymphoma [1]. However, CAR-T therapy does not demonstrate efficacy in solid tumors due to the complex milieu in solid cancers, i.e., the tumor microenvironment (TME), which hampers the tumoricidal activity of CAR-T cell [2, 3]. TME is a complicated niche consisting of tumor cells, myeloid-derived suppressor cells (MDSCs) [4, 5], tumor-associated macrophages (TAMs) [6, 7], exhausted T cells [8], immunosuppressive non-cellular components such as cytokines and extracellular matrix (Fig. 10.1) [9–11].

TME contributes to cancer progression and relapse [2, 12]. The presence of tumor-associated MDSCs such as TAMs, neutrophils, and dendritic cells is strongly associated with the failure of cancer immunotherapy. MDSCs play a pivotal role in the invasion and migration of cancer cells. For example, MDSCs interact with cancer stem cells to mediate the immunosuppressive repertoire to CAR-T therapy [4, 13, 14].

Preclinical experiments showed that CAR-T cells became dysfunctional after trafficking into solid tumors [15]. CAR-T cells in TME increased expression of immune-suppressive molecules such as diacylglycerol kinase and Src homology region 2 domain-containing phosphatase-1(SHP-1), programmed cell death protein 1 (PD-1), T cell immunoglobulin, and mucin-domain containing 3 (TIM-3), Lymphocyte-activation gene 3 (LAG-3), and natural killer cell receptor 2B4.

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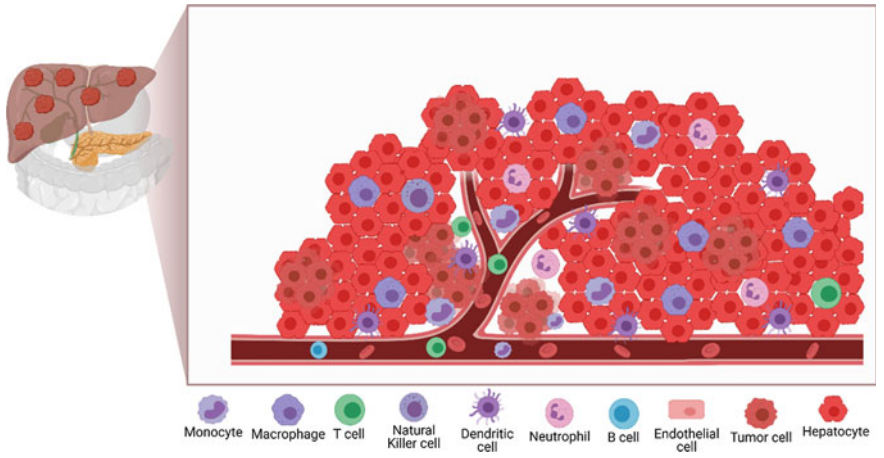

Fig. 10.1 Liver tumor microenvironment. In TME, tumor cells release cytokines that recruit myeloid-derived suppressive cells including monocytes, macrophages, dendritic cells, and neutrophils. T cells are exhausted and lose their antitumor function in TME

Moreover, the dysfunctional T cells could be restored when they were isolated from TME [16], which indicates that TME plays a crucial role in CAR-T immunotherapy.

Here we describe factors and cytokines in the immune-suppressive TME. TGF-β signaling represses Type 2 helper T (Th2) cells and fosters tumor growth by angiogenesis [17]. TGF-β dominant cancers enrich anti-inflammatory macrophage signatures, consistent with an immunosuppressive TME [18]. TGF-β exhausts cytotoxic T (Tc) cells by inducing the expression of PD-1 and TIM-3, differentiates $CD4^+$ T cells to regulatory T cells (Tregs), and inhibits the expression of granzyme and perforin in NK cells [19]. IL-4 fosters tumor progression through upregulating anti-apoptotic genes such as Bcl-xl and cFLIP in tumor cells [20]. IL-4 activates PI3K/Akt pathway for tumor survival and metastasis [21]. A recent study reported that the increased expression level of Notch ligand (DLL4) and receptor (NOTCH2) were responsible for immune suppression of human fetal liver and hepatocellular carcinoma [22]. In line with these results, Notch pathway activation induces IL-4 secretion and polarizes macrophages to immunosuppressive TAMs [23].

10.2 Tumor-Associated Macrophages in TME

TAMs play a key role in TME via tumor growth, immunosuppression, invasion, and metastasis (Fig. 10.2) [6, 24]. In the following, we are going to introduce how TAMs regulate TME.

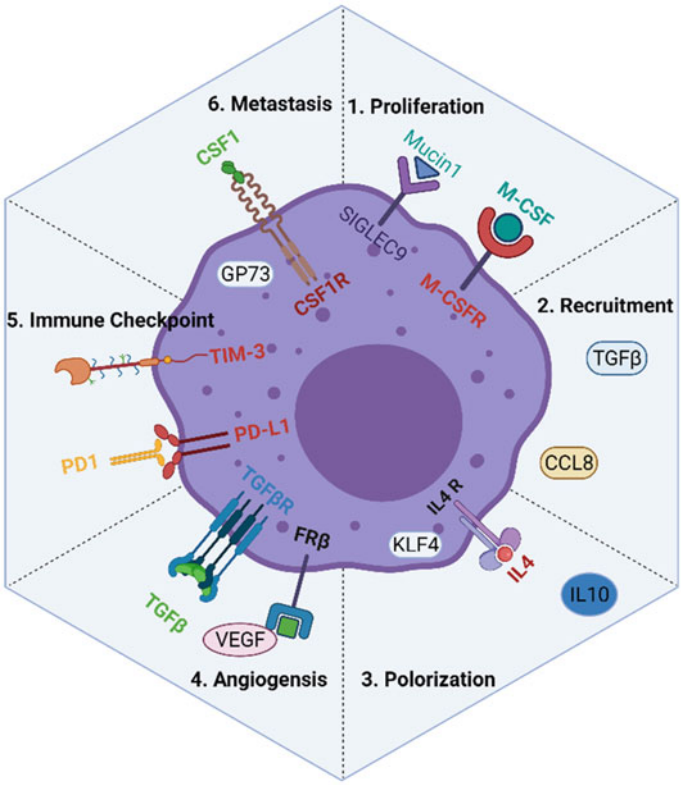

Fig. 10.2 Properties of tumor-associated macrophages. The repertoire of tumor-associated macrophages facilitates tumor progression in the TME. (1) Mucin1 induces the proliferation of TAMs and expression of anti-inflammatory markers such as M-CSFR, CD206 leading to tumor progression. (2) TAMs secrete the TGFβ and CCL8 to facilitate the recruitment of monocytes leading to the accumulation of TAMs in TME. (3) TAMs polarize surrounding macrophages into anti-inflammatory phenotype by IL4, IL10, or IL13. KLF4 is involved in the induction of the anti-inflammatory phenotype. (4) $FR\beta^{+}$ macrophages release VEGF to promote angiogenesis of tumor. And TGFβ reprogrammes macrophages into TAMs leading to angiogenesis progression. (5) TAMs express the immune checkpoints such as PD-L1 and TIM3 to exhaust cytotoxic T cells. (6) $CSF1R^{+}$ TAMs enhance the invasion of myeloid cells, leading to the metastasis of tumor cells

Macrophages can be polarized to pro-inflammatory macrophages (M1 phenotype) induced by lipopolysaccharide of microbes or interferon γ [25]. On the other hand, macrophages become alternatively anti-inflammatory macrophages (M2 phenotype) induced by IL-4, IL-13, or TGF-β [26]. The pro-inflammatory macrophages have antitumor activity, whereas the anti-inflammatory macrophages have tumor-promoting properties.

TAMs secrete TGFβ and IL-10 to promote tumor cell growth and angiogenesis through the PI3K pathway [27]. TAMs produce CCL8 to promote the recruitment of monocytes, resulting in more macrophages becoming immunosuppressive

TAMs [28]. Hedgehog signaling facilitates the communication of TAMs and tumor cells leading to polarizing the macrophage toward anti-inflammatory phenotype. The study suggested that KLF4 and NF-kB mediate the anti-inflammatory macrophages polarization [29].

TAMs express the folate receptor β (FRβ) and mediate immune suppression in TME [30]. $FR\beta^{+}$ macrophages regulate tumor metastasis via secreting vascular endothelial growth factor (VEGF) and facilitate angiogenesis in pancreatic cancer patients [12]. Colony-stimulating factor 1 receptor (CSF-1R)-expressing TAMs are associated with tumor progression and motility [28] due to increased myeloid cell migration and invasion. The anti-CSF-1R antibody treatment inhibited tumor growth and metastasis [31]. Golgi protein 73 (GP73) is a biomarker of invasion and metastasis of hepatocellular carcinoma [32]. GP73 endows the TAMs an anti-inflammatory phenotype. GP73 expression is correlated with the expression of TIM3 and IL18Bpa, immunosuppressive markers in hepatocellular carcinoma (HCC) [33].

Sialic acid-binding Ig-like lectin 9 (SIGLEC9), primarily expressed on monocytes and macrophages, promotes cell growth through its receptor mucin 1 [34]. The study shows that SIGLEC9-mucin 1 signaling converts macrophage to immune-suppressive TAMs by expressing PD-L1, M-CSFR, CD206, and CD163 [35, 36].

10.3 Cellular and Molecular Features that Determine the Response to CAR-T Cells

Herein we describe immune checkpoint molecules that curb CAR-T cells (Fig. 10.3). PD-1 expresses on the surface of the immune cell such as T cells, B

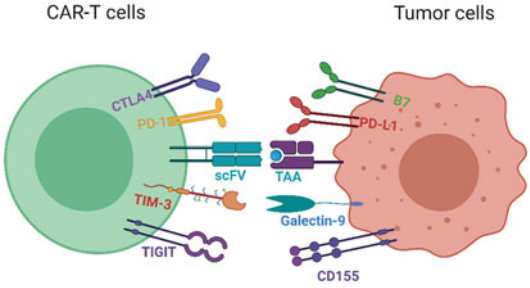

Fig. 10.3 Key immune checkpoints of CAR-T cells engagement with tumor cells. CAR-T cells recognize the tumor cells by tumor antigen-specific scFV. The main four immune checkpoints, CTLA-4, PD-1, TIM-3, and TIGIT, impair the CAR-T cells' antitumor function. CTLA4 binds to costimulation ligand B7(CD80 or CD86) leading to inhibition of T cells. PD-L1 suppresses CAR-T by engaging with PD-1, which results in the apoptosis of CAR-T cells. TIM-3 and TIGIT suppress CAR-T by interaction with galectin-9 and CD155, respectively. CAR-T cells will lose their tumor-killing function through engagement with these molecules

cells, and macrophages. Granulocyte–macrophage colony-stimulating factor (GM-CSF) induces PD-L1 on MDSCs curbing the immune activity of CAR-T cells in liver metastases. The combination of anti-GM-CSF and anti-PD-L1 antibodies restored the efficacy of CAR-T cells [5], which indicates the crucial role of GM-CSF and PD-L1 in CAR-T therapy.

A recent study shows that anti-inflammatory TAMs upregulated immunosuppressive genes such as T cell immunoreceptor with Ig and ITIM domains (TIGIT), CD305, and TIM-3 in HCC. These signals limit the $CD8^+$ T cell infiltration directed to the tumor and are associated with poor clinical prognosis 37]. The low-level expression of PD-1 and CTLA-4 signal in the primary HCC patients correlate with the low efficacy of anti-PD-1 and anti-CTLA-4 immunotherapy in clinical settings [38]. Targeting TIGIT and TIM-3 combined with PD-1 or CTLA-4 may enhance the prognosis of HCC.

TIM-3 is another crucial immune checkpoint molecular [39]. A recent study suggested that TIM-3 induces the exhaustion of $CD8^+$ tumor-infiltrating lymphocytes exhausted in advanced non-small cell lung cancer (NSCLC) patients. The high expression of TIM-3 correlated with the poor efficacy of anti-PD-1 therapy [8]. Clinical study shows that TIM-3 is upregulated on patients' peripheral $CD4^+$ and $CD8^+$ T cells [40]. Combination of anti-TIM-3 and anti-PD-1 therapy increase IFNγ-secreting $CD8^+$ cells and $IFN\gamma^+$ TNFα effector T cells in TME leading to improve survival of glioblastoma [41].

Nuclear receptor subfamily 4A (NR4A) activates the nuclear factor of activated T cell (NFAT) leading to the $CD8^+$ T cell exhaustion. CAR-T cells with NR4A deletion reduced the expression of the PD-1 and TIM-3 and enhanced antitumor efficacy [42].

10.4 Single-Cell Sequencing Combined with the Different Approaches Uncovers TME

Bulk RNA sequencing informs the transcriptome of total cells on average, which could have a bias due to the heterogeneity of cells. If some cell populations play a pivotal role in TME but their proportion is low, bulk RNA sequencing could not be informative [43]. Single-cell sequencing could provide a solution to decipher the heterogeneity of cells in TME. The single-cell level perspective of TME provides knowledge about the nature of the tumor property and may lead to innovative cancer therapies [44].

Single-cell transcriptomes identified that Tregs accumulate in brain metastases and resulted in T cell dysfunction by secreting IL-10 and IL-4 to shift TAMs to an immunosuppressive phenotype in TME [45]. A small population of TAMs interacts with $CD40^+CCR7^+LAMP3^+$ dendritic cells and immune stimulation in colorectal cancer patients. The results indicate that targeting these subpopulations can enhance the therapy [46].

Mass cytometry analysis of renal cell carcinoma demonstrated the distribution of PD-1, CTLA-4, and TIM-3 in the TME. This would open up the precision

medicine of cancer immunotherapy to the patients. For example, patients predominantly expressing PD-1/PD-L1 in T cells could choose anti-PD-1 therapy as a preference [47]. Pembrolizumab, a humanized anti-PD-1 drug, shows improved efficacy in PD-L1+ non–small-cell lung cancer patients [48].

Integration of flow cytometry with immunofluorescence imaging on brain tumors demonstrated that T cells with high expression of immune checkpoints such as PD-1, LAG-3, TIM-3, and TIGIT were dysfunctional. Advanced brain metastases accumulated Tregs reflecting the immune-suppressive milieu, while early-stage glioma accumulated immature NK cells reflecting potentially immunologically active state [45, 49].

10.5 Strategies of CAR-T Remodel the TME

CAR-T cell immunotherapy can be improved by applying insights from single-cell RNA sequencing of TME. Blocking highly expressed immune checkpoint molecules such as CTLA-4, PD-1, LAG-3, TIGIT, VISTA in CAR-T cells could rescue them from exhaustion in TME, or rewire surrounding immune cells by converting immunosuppressive signals to stimulant signals. Arming the CAR-T cells with Th1 triggering cytokines such as IL-7, IL-12, IL-15, IL-18, IL-21, or JAK-STAT signal switches the TME to a pro-inflammatory state [50]. This could reprogram surrounding TAMs to pro-inflammatory phenotype, and subsequently remodel the TME to an antitumor niche [51]. Moreover, conveying the T cells with two single-chain variable fragments, i.e., bispecific T cell engagers (BiTEs) could enhance the specificity to target tumor CAR-T cells and could be engineered to secrete BiTEs [52]. In the following, we summarize four approaches to remodel the TME (Fig. 10.4).

First, endowing CAR-T cells with immune checkpoint blockades allows for CAR-T cells to be engineered and secrete anti-PD-1 scFv, which could engage bystander T cells with antitumor activity [53]. They found that PD-1 scFV-secreting CAR-T cells show stronger antitumor efficacy in both Raji-PD-L1 hematologic and SKOV3-PD-L1 solid tumor-bearing mouse models compared to the single CAR-T approach due to the escort of bystander T cells from PD-1 scFV-secreting CAR-T cells.

Second, CAR-T cells can be engineered to secrete antitumor cytokines. IL-12 enhances CAR-T cell responses by sustaining T cell cytotoxicity [54]. Intratumoral delivery of IL-12 in the combination with tumor-targeted CAR-T cell therapy remodeled the TME into a pro-inflammatory state by the production of pro-inflammatory cytokines IFN-γ and TNF, decreasing regulatory T cells and polarization to inflammatory macrophages [55]. CAR-T cells expressing IL-7 and CCL19 showed superior antitumor activity [56]. CAR-T cells coexpressing IL-15 remodeled the TME by activating NK cells and reduced anti-inflammatory macrophages [57]. CAR-T cells expressing the p40 subunit of IL-23 enhanced the tumoricidal function by upregulating the granzyme B and downregulating PD-1

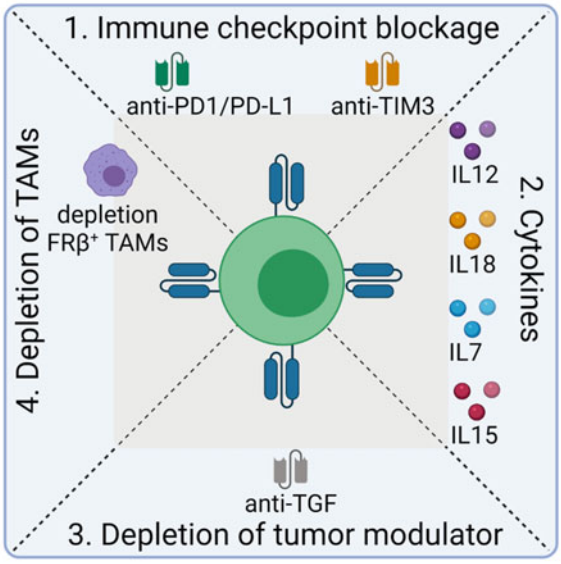

Fig. 10.4 Strategies to remodel TME. The major strategies are blockage or depletion of immunosuppressive factors in the TME by CAR-T cells. (1) CAR-T cells secrete the anti-PD1 antibody, which blocks the PD-1 signal of immune cells, leading to both protecting the CAR-T cell and restoring the bystander T cell. (2) Secreting the immune priming cytokines such as IL12, IL18 can boost the T cell activation and convert the TAMs to a pro-inflammatory state. (3) CAR-T cells block the immune-suppressive cytokines such as TGFβ to improve the enrichment of cytotoxic T cells in the TME. (4) Targeting the immunosuppressive TAMs by CAR-T. Elimination of FRβ$^+$ TAMs increased the infiltration of cytotoxic T cells in the TME

expression [58]. CAR-T cells releasing IL-18 showed superior efficacy of expansion and antitumor by increasing the cytotoxic T cells [59], as well as reversing the exhausted T cell to a tumoricidal Tbet ^{high} FoxO1^{low} T cells [60].

Third, engineering CAR-T cells to antagonize immune-suppressive cytokine. TGF-β, secreted by tumor cells, shapes an immunosuppressive TME, leading to resistance to immunotherapy [61]. Anti-TGF-β therapy reduced the epithelial-to-mesenchymal transition of tumor cells and improved the penetration of T cells into tumors [62]. Selective inactivation of TGF-β1 by SRK-181 antibody facilitated the antitumor activity by enriching the CD8$^+$ T cell and the memory cell in the TME [17, 63]. Co-expression of a dominant-negative TGF-β RII with anti-prostate specific membrane antigen CAR can be resistant to TGF-β dominant TME in PC3-PSMA tumor-bearing mouse model [64]. Anti-TGF-β CAR-T cells protect T cells from immunosuppressive TGF-β into an immunostimulatory phenotype. And what is more, Anti-TGF-β CAR-T cells can reverse the TGF-β from an immunosuppressive molecule toward a stimulator of T cell proliferation in vitro [65].

Table 10.1 Key molecules determine the response of CAR-T cells

Molecular	Cell type enriched	Function	References
Immune checkpoint			
CTLA-4 (CD152)	Activated T cells, Tregs	Binds CD80/CD86 to inhibit the CD28 signal leading to inhibitory function of T cell	[69, 70]
PD-1 (CD279)	T cell (Tregs), B cells, macrophages	Bind to PD-L1 or PD-L2	[70–72]
TIM-3(CD366)	T cells, myeloid cells	Mediate exhaustion of immune cells	[8, 39, 73]
LAG-3(CD223)	T cells, B cells, NK cells	Treg suppressive function	[74, 75]
TIGIT	T cells, NK cells	Inhibit T cell activation	[37, 76]
Cytokines or factors			
TGFβ	Tumor cells, leukocytes, macrophages	Tumor cells, leukocytes, macrophages	[17, 18]
NR4A	T cells, macrophage	Exhaust the $CD8^+$ T cells	[42, 77]

Fourth, targeting TAMs by CAR-T cells. Abolishing $FR\beta^+$ subpopulation of TAMs improved T cell-mediated antitumor immune responses [66].

10.6 Prospective

Precision medicine of cancer immunotherapy will be a major goal of CAR-T technology. In this review, we discussed the molecules and cells which play key roles in the tumor microenvironment and CAR-T therapy. Based on the findings of single-cell sequencing in TME and CAR-T cells, we believe that the identification of novel immune checkpoint molecules and cytokines that hinge the activity of CAR-T cells will offer new targets in cancer immunotherapy. We summarized the four approaches to engineer CAR-T cells to remodel the TME. The insight from the new single-cell technologies will pave the avenue for improving CAR-T immunotherapy to benefit the patients [67]. The spatial multi-omics can define both the transcriptome and proteome of the TME [68]. By defining the TME, one could engineer CAR-T cells to precisely target immune-suppressive molecules in the TME for each patient (Table 10.1).

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