9 *Off-the-Shelf* Chimeric Antigen Receptor Immune Cells from Human Pluripotent Stem Cells

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9.1 The History of CAR-T Therapy

The invention of CAR-T cells and adoptive cell therapy (ACT) is a recent breakthrough. The use of patients' immune cells to treat cancers dated back to 1902 when Blumenthal and E. von Leyden tried to treat their cancer patients with suspension derived from autologous tumor tissue culture. Some beneficial effects can be noted in individuals but without significant disease remission [1]. ACT mainly involves the isolation of the patient's tumor-specific immune cells, especially T cells, genetic modification, the proliferation of these cells in vitro, and infusion back to the patient circulation following a lymphoid-depleting conditioning regimen, such as fludarabine and cyclophosphamide, for cancer treatment [2]. Three forms of adoptive T cell transfer have been developed for cancer immunotherapy, including tumor-infiltrating lymphocytes (TILs), T cell receptor (TCR) T cells, and chimeric antigen receptor (CAR) T cells [3]. There are many approaches to modify immune cells in the laboratory while CAR-T is successfully used in clinical trials. The first use of genetically engineered T cells following the aforementioned ACT canonical workflow for cancer treatment was reported in 1989 [4]. In the mid-1990s, the term CAR-T was first described but the results from the preclinical and clinical study were not satisfactory [5]. Nevertheless, as more and more modifications and improvements were applied to CAR-T design, the promising therapeutic effect of CAR-T therapy has been demonstrated and the huge success of CAR-T therapy emerged. FDA approved the first CAR-T therapy called tisagenlecleucel in August 2017 for children with relapsed B cell acute lymphoblastic leukemia treatment [1].

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CARs as synthetic receptors are generally composed of a specific domain from a monoclonal antibody that can detect corresponding tumor antigen, a T cell activation domain usually derived from the CDζ chain, and a linker domain that bridges the two domains. CAR-T cells can direct tumor cells automatically under the guidance of the antigen detection domain, then the T cell activation domain elicits downstream signals to activate T cells to perform antitumor response [6]. Eshhar's group showed that these CAR-T therapy-related synthetic receptors endow T cells with MHC-independent target recognition compared with engineered TCRs therapy [4, 7]. Eshhar developed the first-generation CAR-T cells targeting 2, 4, 6-trinitrophenyl (TNP)-bearing cells. They removed TCR variable regions and replaced them with antibody variable regions based on a similar structure. These CAR-T cells were composed of VH and VL chains derived from TNP antibody, TCR constant domain, and transmembrane segment. Nevertheless, the results from the initial clinical trial using the first-generation CAR-T cells did not display satisfactory antitumor effects [4]. The first-generation CAR is most likely to fail to fully engage genetically modified T cells because activation is initiated by antigen-dependent signals through the chimeric CD3ζ chain, independent of costimulation through accessory molecules [8]. To enhance the efficacy of CAR-T cells, many modifications were performed, leading to the generation of the second-generation CAR-T cells [9]. Second-generation CARs are improved by the addition of costimulatory domains, such as CD28, OX40, or 4-1BB (also known as CD137), linked with CD3ζ. Although the first-generation CARs displayed disappointing anti-cancer efficacy in clinical trials, the second-generation CARs targeting CD19 with costimulatory domains emerged as a great success in 2011 [10, 11]. CD19 has become a nearly ideal target in CAR-T therapy for B cell malignancies. More and more clinical trials of CAR-T targeting BCMA and CD22 have been carried out and showed significant anti-cancer effects in multiple myeloma and acute lymphoblastic leukemia, respectively [3]. In 2017, FDA has already two autologous second-generation CAR-T cells products due to the promising therapeutic effect in patients with hematologic malignancies, tisagenlecleucel (Kymriah, Novartis) and axicabtagene (Yescarta, Kite Pharma) targeting CD19, for the treatment of relapsed or refractory B cell acute lymphoblastic leukemia (ALL) and relapsed or refractory diffuse large B cell lymphoma and primary mediastinal large B cell lymphoma [12–14]. Third-generation CARs convey two costimulatory domains together to further enhance the antitumor activity [15, 16]. Nowadays, the fourth-generation CARs as the newest version have emerged with additional functional domains, which can precisely control CAR-T cell activity or further effectively enhance CAR-T potency [17]. Diaconu et al. reported that the inclusion of inducible pro-apoptotic protein caspase-9 (iC9) safety switch into the vector encoding the CAR can terminate the effect of CAR-T cells in a humanized mouse model by using chemical inducer of dimerization, which can efficiently eliminate 85%~90% CARs once cytokine release syndrome (CRS) or severe toxicities occur [18]. Phase I trial of fourth-generation anti-CD19 CAR-T cells with iCasp9 suicide switch (4SCAR19) has been carried out [19]. TRUCK T cells refer to CAR-T cells with a transgenic "payload" and belong to another type of fourth-generation CAR. These

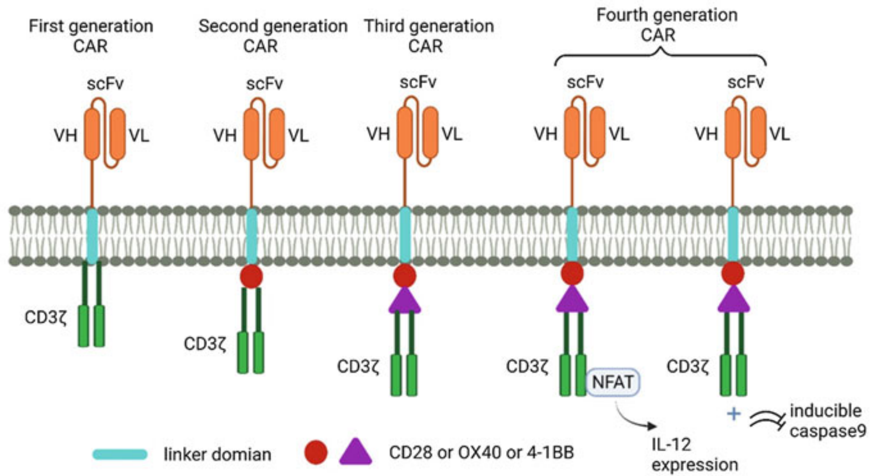

Fig. 9.1 Overview of CAR-T development history

TRUCK T cells can shape the tumor microenvironment by the inducible release of transgenic immune modifiers, such as IL-12, to eliminate antigen-negative cancer cells in the targeted lesion [20]. A dual CAR system has been developed that the first synthetic Notch receptor detected one antigen resulting in the second inducible CAR expression to recognize the other antigen [21]. The SUPERCAR system composed of a zipCAR and zipscFv is another novel CAR system. A zipCAR has a leucine zipper in place of antigen detection domain as the extracellular portion of the CAR. A zipscFv has antigen detection scFv fused to a cognate leucine zipper which can bind with leucine zipper located on the zipCAR. This design endows CAR-T with target antigen flexibility and fine tuneability [22]. After the approval of CAR-T therapy in 2017, increasing numbers of clinical trials have been registered and authorized to develop new products of CAR-T cell therapy. The effect of CAR-T conveyed with a single antigen seems restricted, caused by the limited capacity to discriminate tumor cells from healthy tissue. Researchers have started to study and evaluate the effect of combined sensing approaches by targeting two or more antigens (Fig. 9.1).

9.2 The Achievements and Existing Problems About CAR-T Therapy

CAR-T cells therapy has greatly revolutionized the landscape of hematologic malignancies treatment, especially for acute lymphoblastic leukemia (ALL) and diffuse large B cell lymphoma (DLBCL). In relapsed or refractory cancer patients who have no response to conventional therapy, complete responses (CRs) by CAR-T therapy are approximately 40~60% to aggressive lymphoma and 60 ~80% to ALL [23–25]. However, there are a significant proportion of patients who

do not respond to this treatment regimen. The most important step for CAR-T therapy is to choose the unique antigen based on tumor characteristics. The unique antigen should only be expressed on tumor cells and not on other issues. Although the CD19 CAR-T product has been approved by FDA, it can target not only B malignant cells but also normal B cells. There continues to be a great need for further investigation into proper unique antigen discovery [26]. Severe toxicity, most notably CRS and neurotoxicity, is another hurdle for CAR-T therapy. The frequency of severe CRS and neurotoxicity generally range from 10 to 50%. Lisocabtagene maraleucel as the third product currently being explored in a clinical study for DLBCL treatment shows the exceptionally low frequency of severe adverse events with the same antitumor effect as axicabtagene. In this trial, only one patient showed Grade 3 CRS while the percentage of Grades 3 and 4 neurotoxicity was also low as 12% [25]. The syndrome of CRS includes fever, hemodynamic instability, hypoxia, and end-organ dysfunction, which is similar to systemic inflammatory response syndrome. FDA has approved IL-6 receptor blocker tocilizumab as an option for CRS treatment after CAR-T therapy. Delirium, aphasia, cerebral edema, and seizures are the syndrome of neurotoxicity. Levetiracetam as a type of anticonvulsants can be used for seizure prophylaxis and severe symptoms should be treated with corticosteroids.

CAR-T therapy has shown a promising therapeutic effect in hematologic malignancies while less successful in solid tumors [27]. The reasons why CAR-T therapy shows disappointing outcomes in solid tumors include the following factors. First, it is difficult for CAR-T cells to penetrate solid tumors owing to the massive physical barriers surrounding tumor tissues [28]. Second, the solid tumor forms an immune-suppressive microenvironment to hamper CAR-T antitumor activity by secreting inhibitory cytokines and recruiting immune-suppressive cells [29]. Lastly, tumor-specific antigens are highly heterogenous in a solid tumor, which is hostile to monoclonal antibody-guided therapy [30]. How to improve the antitumor effect of CAR-T cells therapy in solid tumor treatment is an urgent problem that needs to be resolved.

Off-the-shelf CAR-T cells will solve the issue of donor availability. Patient-derived autologous T cells have been the source of CAR-T. Autologous T cells have long persistence after adoptive transfer because they can evade host allogeneic immune response. However, autologous CAR-T cells therapy requires a bespoke manufacturing process for every patient after leukapheresis and display certain disadvantages. It takes approximately 3 weeks to produce enough CAR-T cells for autologous CAR-T cells therapy and the cost of CAR-T cells therapy is inevitably expensive [6]. Moreover, T cell quality is variable for cancer patients and is susceptible to be impaired by chemotherapeutic agents. Dysfunctional T cells isolated from the immunosuppressive tumor microenvironment in certain cancer patients lead to CAR-T cell therapy failure [31]. The application of 'off-the-shelf' allogeneic CAR-T cells has many potential advantages compared with autologous T cells if the inherent barriers caused by MHC mismatch can be resolved. Allogeneic CAR-T cells are usually derived from healthy donors who have a robust immune function, which can overcome immune defects of

autologous T cells from cancer patients. Moreover, harnessing allogeneic CAR-T cells makes it possible to perform more rapid and less expensive treatment, which also simplifies the manufacturing process and standardizes CAR-T products [32]. In addition, parts of allogeneic CAR-T cells can be stored by cryopreservation when they have been manufactured; thus cancer patients can be simultaneously treated with the combination of CAR-T cells targeted different antigens. Peripheral blood mononuclear cells (PBMCs) from healthy donors are the main source of allogeneic CAR-T cells. In very rare cases, umbilical cord blood (UCB) can also be the source of allogeneic CAR-T cells. Indeed, T cells from UCB have a unique antigen-naïve condition associated with decreased incidence and severity of graft-versus-host disease (GVHD) [33]. Nowadays, more and more studies focus on self-renewable pluripotent stem cells such as induced pluripotent stem cells (iPSCs) or embryonic stem cells (ESCs) as the new source of allogeneic CAR-T cells [34]. These pluripotent stem cells can proliferate indefinitely and theoretically produce all other cells in the human body. Harnessing pluripotent stem cells to produce therapeutic cells has been of keen interest to regenerative medicine [35–37]. Application of iPSCs as the source can generate more homogeneous CAR-T cells because they are produced from one clonal engineered pluripotent cell line. Antibody-mediated graft rejection usually causes organ transplantation failure and the presence of donor-specific anti-HLA antibodies (DSAs) appears to impede the successful engraftment of donor cells [38]. For allogeneic CAR-T cells transfer, the levels of DSAs as the major barrier need to be assessed carefully [39]. The allogeneic approach leads to two major issues that need to be addressed promptly. First, it may cause life-threatening GVHD. GVHD is the main reason for morbidity in allogeneic CAR-T transplantation and αβ-T cells play the central role in the pathogenesis of both acute and chronic GVHD [40–43]. In GVHD, T cells express TNF family molecules and secret intracellular granule contents to damage target organs [44, 45]. HLA mismatches between donor and recipient elicit immune recognition, potentially causing graft rejection and GVHD. HLA-restricted TCR repertoire can recognize subtle structural differences of allogeneic HLA molecules, leading to T cell alloreactivity. The generation of allogeneic CAR-T cells by deletion of endogenous TCR is expected to reduce the chance of GVHD [46]. Second, these allogeneic CAR-T cells have a high chance to be eliminated by the host immune system, hampering the antitumor effect [34]. The antitumor effect of allogeneic CAR-T cells is determined by the initial expansion, length of persistence, and host immune rejection. According to the first-in-human report with CAR19-T cells manufactured using *piggyBac* transposon system, *piggyBac* CAR19-T cells induced CAR-T cell lymphoma in two of ten patients, while the same phenomenon has not been found with CAR19-T cells produced by viral vector [47]. This incidence indicates the needs of either lentiviral vectors for primary T cells, or safe-harbor loci (such as AAVS1 and human ROSA26) in pluripotent stem cell-derived T cells.

The reasons leading to CAR-T therapy failure include immune-suppressive tumor microenvironment, tumor antigen escape, CAR-T cell exhaustion, and persistence reduction. Individual conventional CAR-T cells can only recognize one

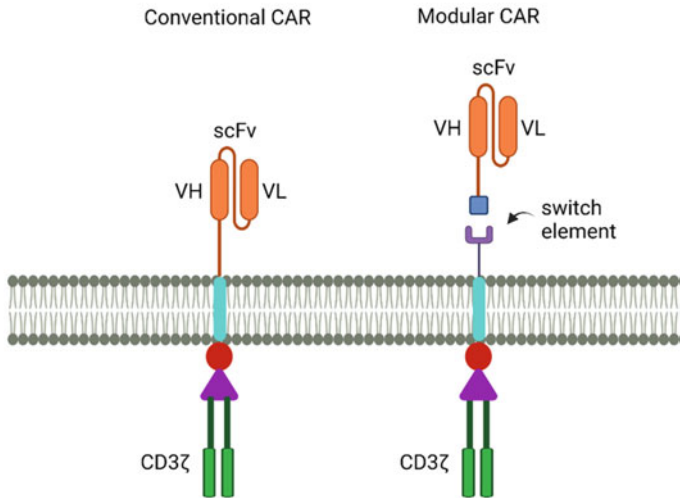

Fig. 9.2 The structure of conventional CAR and modular CAR

specific tumor antigen because of the fixed, single-antigen targeting capacity. Antigen loss of tumor tissue usually leads to therapy failure [48, 49]. The manufacturing of CAR-T cells targeting diverse tumor antigens is a promising approach to address this issue. Compared with the traditional CAR-T system, the modular or universal CAR-T technology utilizes a switch molecule to separate targeting and signaling elements. An adaptor or switch element in modular CAR-T cells replaces the antigen detection domain in conventional CAR-T cells. By choosing specific targets, the strategy would achieve better efficiency in the cold TME. This adaptor can be assembled with any specific tumor antigen and is required to bridge the immunological synapse [50] (Fig. 9.2).

According to the antigen expression of the patient's tumor, the modular CAR-T system can be flexibly adjusted with the corresponding tumor antigen, allowing for tailored therapy. Meanwhile, the modular or universal system can precisely control CAR-T activity by managing the adaptor function. The ability to titrate on adaptors enables halting of the administration of the adaptor, resulting in the blockade of CAR-T function without the effect on other T cells (Table 9.1).

9.3 Generation of CAR-immune cells from PSCs (examples, advances)

In 1998, the human ESCs were established by the James Thomson group for the first time [51]. In 2006, Shinya Yamanaka discovered that mouse somatic cells are capable to be reprogrammed to ESCs-like status by transducing four pivotal transcription factors (Klf4, Oct4, Sox2, and c-Myc), these cells are termed as iPSCs

Table 9.1 CAR-T clinical trial for solid tumors

Solid tumor	Target antigen	Target cell	CAR	Clinical trials
Glioblastoma	IL13Rα2	T cell	IL13Rα2 scFv-4-1-BB-CD3ζ	NCT02208362
Glioblastoma	EGFRvIII	T cell	EGFRvIII scFv-CD8 Hinge&TM-4-1BB-CD3ζ	NCT02209376
Neuroblastoma	L1-CAM	T cell	L1-CAM scFv-CD3ζ	NCT00006480
Neuroblastoma	GD2	T cell	GD2 scFv-CD3ζ	NCT00085930
Carcinomas	CD133	T cell	CD133 scFv-CD8a Hingle&TM-4-1BB-CD3ζ	NCT02541370
Colon cancer	CEA	T cell	CEA scFv-CD8 Hinge-CD28-CD3ζ	NCT01373047
Colon cancer	HER2	T cell	HER2 scFv-CD8 Hinge&TM-CD28-4-1BB-CD3ζ	NCT00924287
Pancreatic cancer	Mesothelin	T cell	Mesothelin scFv-4-1BB-CD3ζ	NCT01897415
Renal cell carcinoma	CAIX	T cell	CAIX scFv-CD16γ TM&Signal domain	Phase I/II
Prostate cancer	PSMA	T cell	PSMA scFv-CD3ζ	Phase I
Seminal vesicle cancer	MUC1	T cell	MUC1 scFv-Fc-IgD Hinge-CD28 TM-4-1BB-CD3ζ	NCT02587689
Ovarian cancer	FRα	T cell	FRα scFv-CD16γ TM&Signal domain	Phase I
DLBCL	CD19	T cell	CD19 scFv-CD8a Hinge&TM-4-1BB-CD3ζ	NCT02445248
Non-Hodgikin lymphoma, CLL	CD19	NK cell	iCasp9-2A-CD19 scFv-CD28-CD3ζ-2A-IL15	NCT03056339

[52]. Soon after, human iPSCs have been successfully established from fully differentiated somatic cells, even from cells in the urine [53–55]. Human immune cells can also be differentiated and generated from human iPSCs for immune cell therapy, especially to treat tumors that are incurable by conventional approaches. CRISPR/Cas9 system as the gene-editing technology can be used to modify genes associated with immune responses during the production of human pluripotent stem cell-derived immune cells. Notably, the primary immune cells are refractory to gene editing and difficult to expand afterward. Compared with primary immune cells, human pluripotent stem cells can easily be edited by transfection, and could be an ideal source for CAR-immune cell generation. Moreover, deleting MHCs will offer a universal source for "off-the-shelf" immunotherapeutic cell differentiation [6]. More studies established hypoimmunogenic universal donor iPSCs to avoid immune rejection after adoptive transfer [56, 57]. Employing the advantage of amenable and expandable features, universal iPSCs were designed by deleting immunogenic MHCs, offering the possibility to generate universal CAR-immune cells for all patients. MHC I plays a core role in mediating immune rejection after allogeneic transplantation. The deletion of the *B2M* gene leads to the loss of

MHC I and avoid attacks from CD8$^+$ T cells [57]. The resultant cells could be still attacked by both macrophages and NK cells via innate immune mechanisms that recognize and attack MHC I-null cells. Thus overexpression of immune-tolerant genes avoids attacks from NK cells (via HLA-E single-chain dimers fused to *B2M*) [58] and macrophages (via CD47). Both T cells and NK cells do not express MHC II while macrophages do express MHC II. The expression of MHC II will provoke attacks from CD4$^+$ T cells and potentially challenge the development of the CAR-macrophage approach [59]. The deletion of the *CIITA* gene results in the loss of MHC II and is expected to free CAR-macrophages from the CD4$^+$ T cells [57]. Knocking out the genes encoding TCR α and β subunits prevented the occurrence of GVHD [60] (Fig. 9.3).

T cells play pivotal roles in the adaptive immune system and form the keystone of cellular immunity. They can recognize foreign molecules expressed on the surface of antigen-presenting cells via the interaction between TCR and MHC. CD4$^+$ T helper cells can secret a series of cytokines to regulate other immune cell activity, such as CD8$^+$ T cytotoxic cells, macrophages, and B cells. CD8$^+$ T cytotoxic cells can recognize antigens presented by MHC I or tumor common antigens with the help of their TCRs. TCR α, β subunits together with CD3 γ, δ, ε and ζ subunits constitute the core part of T cell signal transduction [61]. Upon binding to foreign antigens, CD8$^+$ T cytotoxic cells secret perforin, granzymes, and granulysin to trigger the target cell's apoptosis. In addition, activated CD8$^+$ T cytotoxic cells can also induce apoptosis of FAS-expressing cells by FAS ligand expression [62]. The differentiation protocols from human pluripotent stem cells to functional T cells have been invented by several groups. The stromal cell line, such as the mouse bone marrow-derived OP9 cell line, is employed for the differentiation from human pluripotent stem cells to CD34$^+$ hematopoietic cells. Notch signaling

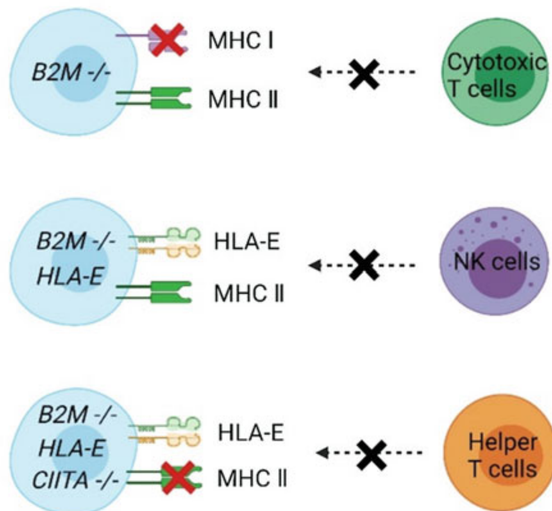

Fig. 9.3 The strategy to generate hypoimmunegenic, universal donor PSCs

determines the further differentiation from $CD34^+$ hematopoietic cells to mature functional T cells. Therefore, the OP9-DLL1 cell line was established by transducing Notch ligand Delta-like ligand 1 into the OP9 cell line. With the help of the OP9-DLL1 cell line, human pluripotent stem cells-derived $CD34^{hi}CD43^{lo}$ cells have the potential to differentiate into $CD4^+$ and $CD8^+$ double-positive TCRαβ T cells. Using OP9-DLL4 in place of OP9-DLL1 cell line for T cells differentiation was reported to be further efficient [63, 64]. However, since TCR rearrangements are random during in vitro differentiation, it is difficult to know their antigen specificity and HLA restriction of these T cells. The advent of CAR technology circumvents this limitation because CARs could redirect T cell specificity in an HLA-independent fashion [65]. The Sadelain group successfully produced CAR-T targeted to CD19 from iPSCs and demonstrated that these iPSCs-derived CAR-T cells potently inhibited tumor progression. The pairwise correlation analysis based on gene expression microarray results suggested that these iPSCs-derived CAR-T cells were more similar to fresh or activated γδ T cells [66].The Crooks group established PSC/ATO (pluripotent stem cells/artificial thymic organoid) system to generate mature functional T cells from human PSCs in vitro system. This 3D organoid system facilitates the differentiation from PSCs to embryonic mesoderm through hematopoietic specification, and then induces T cell lineage commitment to become naïve $CD3^+CD8\alpha\beta^+$ and $CD3^+CD4^+$ conventional T cells. This system can also be used to produce antitumor antigen-specific $CD3^+CD8\alpha\beta^+$ T cells by the introduction of MHC I-restricted in PSCs [67]. The Nakauchi group reported that antigen-specific $CD8^+$ T cells from HIV-1-infected patients showed exhausting phenotypes. However, after reprogramming to pluripotency and redifferentiating into $CD8^+$ T cells, these rejuvenated cells recovered antigen-specific killing capacity and possessed a high proliferative activity [68]. This discovery monumentally provides new insight and ideas for cancer immunotherapy. FT819 as a dual-targeted CAR-T candidate (CD19/CD16) made from a master iPSC cell line is being evaluated in a clinical study [69].

NK cells belong to the innate immune system because of their lack of receptors for antigen specificity and form the first line of defense against tumor cells and virus-infected cells, and they show promising potential in cancer immunotherapy. The activation of NK cells is decided by a balance between activating and inhibitory signals, which does not have a somatically rearranged and antigen-specific TCR [70]. The activating receptors of NK cells include CD94/NKG2C, NKG2D, NKp30, NKp44, and NKp46, which recognize the different ligands expressed on various target cells. The inhibitory receptors of NK cells include polymorphic inhibitory killer cell immunoglobulin-like receptors (KIRs) that bind with MHC class I [71]. The antitumor efficacy of NK cells is limited because NK cells are highly susceptible to the immunosuppressive microenvironment. Upon activation, NK cells localize the site of infection and perform functions by cytokine secretion, the release of cytolytic granules, and death receptor-mediated cytolysis [72]. The cytokines secreted from NK cells include IFNγ, TNFα, GM-CSF, RANTES, and some chemokines, which can regulate the functions of the innate and adaptive immune system [73, 74]. In addition, NK cells can lyse target cells by

secreting perforins and granzymes [75, 76]. They can also express specific ligands to activate death receptors on their target cells [77]. Compared with T cells, NK cells do not depend on HLA matching to perform their function. They can be easily transferred across HLA barriers without causing GVHD. The protocols to differentiate NK cells from hPSCs have been invented. In the early protocols, mouse stromal cells (S17 or M210) were used for hematopoietic differentiation. The differentiated cells were selected and seeded onto EL08-1D2 stromal cells in presence of IL-3, 7, 15, and FLT3L, then $CD45^{+}CD56^{+}$ NK cells were generated [78, 79]. The generated NK cells were able to eradicate human tumor cells by direct cell-mediated killing and secreting antibodies. Considering the use of hPSCs-derived NK cells in clinic for disease treatment, a xeno-free and serum-free protocol needs to be developed. Spin embryoid body method was used for $CD34^{+}CD43^{+}$ hematopoietic progenitor cells generation and the resultant cells were further differentiated using membrane-bound IL-21-expressing artificial antigen-presenting cells [80, 81].The Kaufman group generated CAR-NK from human iPSCs. Human iPSCs were transfected with a plasmid encoding scFv targeted to human mesothelin, 2B4 costimulatory domain and CD3ζ chain. These genetically modified human iPSCs were differentiated to functional CAR-NK cells. Compared with CAR-T cells, CAR-NK cells displayed similar antitumor efficacy, but with less overall toxicity [82]. Nowadays, the design strategy of fourth-generation CAR-T has also been tested in CAR-NK generation [6].

Macrophages belong to the innate immune system with a high infiltration rate and play indispensable roles in inflammation and the protection of our body from outside invaders and tumor cells. The yolk sac, fetal liver, and bone marrow are all the sites for macrophage origination. Yolk sac-derived macrophages not only form microglia in the brain but also populate the fetal liver which produces most of the self-renewing tissue-resident macrophages (TRMs) [83, 84]. After postnatal, macrophages originate from bone marrow myeloid progenitor cells, occurring through differentiation of circulating monocytes in an MCSF- or GMCSF-dependent manner [85]. In general, the life span of bone marrow-derived macrophages is shorter than TRMs [86]. Macrophages are highly plastic cells that perform diverse functions in different organs, including clearance of cell debris, elimination of pathogens, modulation of inflammatory responses, and tissue homeostasis maintenance [87]. Macrophages may undergo M1 or M2 polarization in different tissues encountering different microenvironment stimuli and signals. M1 phenotype which is highly expressed in inflammatory cytokines has strong anti-microbial and tumor activity, while M2 phenotype can promote tumor growth and tissue remodeling [88, 89]. Macrophages can directly recognize outside invaders via pattern recognition receptors (PRRs). PRRs include Toll-like receptors, NOD-like receptors, C-type lectin receptors, and cytoplasmic proteins [90]. After receptors are activated, macrophages provoke intracellular signals to induce actin polymerization and phagocytic cup formation [91]. Then macrophages phagocytose outside invaders or tumor cells and move to lymph nodes to present antigens to T cells, subsequently triggering a series of T cells

downstream responses. Compared with other immune cells, macrophages can penetrate solid tumors easily and interact with almost all cellular components in the tumor microenvironment, which endows them with profound advantages to be developed into CAR-macrophage [88]. The feeder- and xeno-free protocol about the differentiation from hPSCs to functional macrophages has been reported. First, iPSCs were exposed to morphogens and cytokines such as BMP4 and VEGF step-by-step, after specifying the lateral plate mesoderm organoids, the organoids were then exposed to hematopoietic cytokines such as SCF, IL-6, and FLT3 to specify immune cells. The resultant mesoderm organoids will generate $CD34^{+}$ $FLK1^{+}$ endothelial cells (so-called hemogenic endothelium) that will derive the innate immune cells including macrophages [92]. The hPSCs-derived macrophages have the capacity of phagocytosis and polarization, and they can also secret cytokines in response to LPS, indicating the same characteristic and function as macrophages that develop naturally in the body. It has been reported that CAR-macrophages could destroy the extracellular matrix (ECM) of the tumor and facilitate the penetration of T cells into the tumor, thus playing an antitumor role [93]. The Zhang group successfully established CAR-macrophages from human iPSCs. CAR expression endowed iPSCs-derived antigen-dependent macrophages with enhanced phagocytosis of tumor cells and in vivo antitumor activity [36]. The Gill group evaluated the antitumor potential of CAR-macrophages in different animal models and found that they could effectively reduce tumor burden. Moreover, in humanized mouse models, CAR-macrophages were demonstrated to strengthen T cell's antitumor activity and facilitate the formation of a pro-inflammatory environment. For the intracellular domain of CAR-macrophages, the Gill group used CD3ζ chain similar with CAR-T cells [59], while the Tonald Vale group applied the cytosolic domains from Megf10 and FcRγ as the intracellular domain of CAR-macrophages, which showed robust phagocytosis capacity [94] (Table 9.2).

9.4 Potential and Perspectives of CAR-Immune Cells in Cancer Treatment

CAR-T therapy as the earliest CAR-immune cells therapy has achieved great success and become a powerful immunotherapeutic source in hematologic cancer treatment. FDA has already approved four CAR-T-related drugs Kymriah, Yescarta, Tecarta, and Breyanzi from 2017 to 2021 [97, 98]. Lately, CAR-NK therapy has emerged as an alternative therapy option to CAR-T therapy. Compared with CAR-T therapy, allogeneic CAR-NK therapy has reduced risk for GVHD, CRS, and neurotoxicity [99, 100]. That is because activated T cells predominantly produce more cytokines associated with CRS and severe neurotoxicity than activated NK cells [101]. CAR-NK cells may be able to eliminate tumor cells via both CAR-dependent and NK cell receptor-dependent mechanisms. Therefore, CAR-NK cells can form a second line of defense in case tumor cells escape T cells recognition by MHC downregulation. The use of NK cell lines such as NK92 and allogeneic NK cells with CAR engineered functions have been

Table 9.2 The study of CAR-immune cells derived from pluripotent stem cells

Type of CAR	CAR target	CAR structure	Cell line used (in vitro)	Cancer type (in vivo)
CAR-T [66]	CD19	1928z-T-iPSC	EL4 cells ($CD19^{high}$)	Burkitt lymphoma
CAR-NK [82]	mesothelin	SS1-NKG2D-2B4-CD3ζ	K562 cells ($meso^{high}$), A1847 cells ($meso^{high}$)	Ovarian cancer
CAR-macrophage [36]	CD19/meso	scFv-CD8α-CD86-FcγRI	K562 cells ($CD19^{high}$), OVCAR3/ASPC1 cells ($meso^{high}$)	Ovarian cancer
CAR-T (FT819) [69]	CD19 and CD16	not shown	N/A	Clinical study
CAR-NK [95]	GPC3	G2-CD8α-CD28-4/1BB-CD3ζ	SK-Hep-GPC3	Ovarian cancer
CAR-NK [96]	CD19	1928z-NK-autologous HSC	N/A	Clinical study (NCT03579927)
CAR-NK [96]	CD19	1928z-NK-allogeneic UCB	N/A	Clinical study (NCT03056339)
CAR-NK [96]	CD19	not shown	N/A	Clinical study (NCT04245722)

studied only recently [96]. Currently, there are more than 500 CAR-T-related and 19 CAR-NK-related clinical trials being conducted in the world [98]. The majority of CAR-T therapy under clinical evaluation still employs patient-derived autologous T cells, whereas almost all CAR-NK therapy applies to cells from allogeneic donors. The first large-scale clinical trial (NCT03056339) of CAR-NK cells has shown promising and safe results in patients with $CD19^+$ CLL and B cell lymphoma [102]. Although CAR-NK therapy possesses multiple advantages in comparison with CAR-T therapy, CAR-NK therapy still needs to be optimized to improve efficacy. Nowadays, researchers have paid great interest in developing CAR-macrophage for cancer treatment. FDA has already approved one CAR-macrophage clinical trial, which is CT-0508 from CARISMA Therapeutics with anti-HER2 CAR-macrophage in subjects with HER2 overexpressing solid tumors (NCT04660929).

Allogeneic CAR-T therapy has monumental advantages compared with autologous approaches, such as a reduced expense and timesaving production cycle as a result of the implementation of standardized and scaled-up manufacturing processes, in which a host of CAR-T cells can be generated from healthy donors, even the therapeutic CAR-T cells that have already been produced and stored in advance before patients arrive. The applicable targets for allogeneic CAR-T therapy include CD19 and CD22 in ALL and B cell lymphomas, respectively, CD30 in Hodgkin lymphoma and anaplastic large cell lymphoma, BCMA, CS1 and CD38 in multiple myeloma, and CD123, CD33, and CLL1 in AML [103]. Owing to the shorter persistence of allogeneic CAR-T cells, the approaches, such as a systematic strategy of redosing [34], the combination of CAR-T cells targeted different antigens [104] and the combination of CAR-T therapy with immune checkpoint modulators or cancer vaccine [105] can be employed to enhance CAR-T therapy efficacy. To date, the efficacy of CAR-T in solid tumors is much less satisfactory than in hematologic malignancies owing to the sturdy physical barriers, immune-suppressive tumor milieu, and the heterogeneity of inner tumor cells. CAR-T cells coexpressing catalase are able to promote their antioxidative capacity by metabolizing H_2O_2, subsequently more resilient toward the harsh tumor microenvironment caused by abundant reactive oxygen species (ROS), and perform superior over conventional CAR-T cells [106]. Moreover, gene-editing approaches reduce the sensitivity of T cells to negative immune checkpoints. The Moon group generated a new switch receptor construct which introduced truncated extracellular domain of PD-1 and costimulatory domain CD28 into CAR-T cells. They demonstrated that the application of PD-1/CD28 can enhance the antitumor activity of CAR-T cells against solid tumors [107]. The Brentjens group reported CAR-T cells which can secrete PD-1 blocking scFv increased antitumor activity [108]. Targeting chemokine receptors, such as CXCR2 [109] and CCR2B [110], allows CAR-T cells migration to the tumor site. The Dotti group revealed that CAR-T cells expressing heparanase, a heparan sulfate-degrading enzyme, could enhance tumor penetration of T cells, subsequently improving antitumor activity [28]. Constructing CAR-T cells which can secrete cytokines further promote their survival or greater activity. CAR-T cells secreting IL-12 [111], IL-18 [112], and IL-15 [113] have been reported to optimize their antitumor activity by different mechanisms. There is a multitude

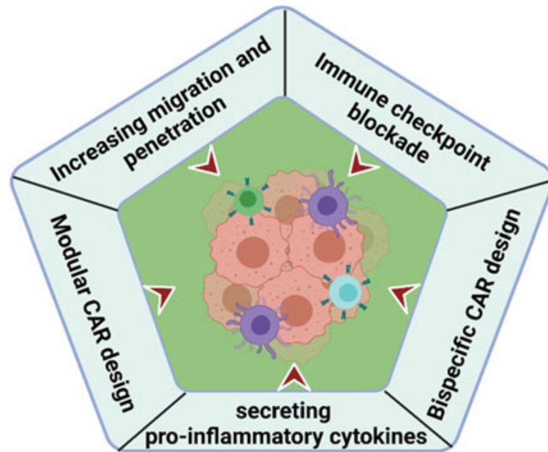

Fig. 9.4 Hallmark of modified CAR-T to target solid tumor

of potential modifications for CAR-T therapy, and the proper modification needs to be selected to implement based on the individual tumor characteristics, which can provide effective ways to eradicate tumors independent of tumor-expressing MHC. More advanced modification techniques, such as modular CAR and dual-targeting approach, are being used in CAR-immune cells design to circumvent therapy resistance and avoid GvHD (Fig. 9.4).

9.5 Future Prospects

CAR-immune cell therapy holds an unprecedented potential to treat cancers that are incurable by conventional treatments. The number of clinical trials involving CAR-immune cell therapy is increasing exponentially, indicating more and more researchers show great enthusiasm for this area [114]. Developing more potent, more cost-effective, and safer CAR-immune cell therapy is the critical goal in the future. Compared with primary immune cells, human pluripotent stem cells-derived immune cells can be easily engineered and have the capacity to proliferate indefinitely, enabling clonal selection and generation of enough clonally-selected therapeutic cells for cancer treatment [115]. The application of gene-editing approaches and fourth-generation CARs can generate CAR-immune cells that are less prone to causing severe CRS [116] and subsequently optimize therapy in terms of safety, cost and potency. However, there is no denying that the generation and application of human pluripotent stem cells-derived CAR-T cells, CAR-NK cells, and CAR-macrophages are still at the early stage. The manufacturing processes from human pluripotent stem cells to functional CAR-immune cells need to be standardized. Moreover, how to improve the efficacy of CAR-immune cells in solid tumors is an inevitable hurdle. Another great challenge in this area is the paucity of preclinical models to carry on the safety and efficacy evaluation of CAR-immune cells before human studies or in response to safety issues that have

been observed in early-phase clinical trials. More basic and translational research need to be dedicated to this area to improve CAR-immune cell therapy and foster new applications beyond oncology in autoimmunity, infectious diseases, and organ transplantation.

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