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• REVIEW •

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Treatment of solid tumors with chimeric antigen receptor-engineered T cells: current status and future prospects

Shengmeng Di & Zonghai Li*

State Key Laboratory of Oncogenes & Related Genes, Shanghai Cancer Institute, Renji Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200032, China

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Chimeric antigen receptors (CARs) are artificial recombinant receptors that generally combine the antigen-recognition domain of a monoclonal antibody with T cell activation domains. Recent years have seen great success in clinical trials employing CD19-specific CAR-T cell therapy for B cell leukemia. Nevertheless, solid tumors remain a major challenge for CAR-T cell therapy. This review summarizes the preclinical and clinical studies on the treatment of solid tumors with CAR-T cells. The major hurdles for the success of CAR-T and the novel strategies to address these hurdles have also been described and discussed.

solid tumor, adoptive cell therapy, T cell, chimeric antigen receptor

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INTRODUCTION

The concept of a chimeric antigen receptor (CAR) was first described by Kuwana and colleagues in 1987 (Diamond et al., 2011). Since its introduction, remarkable improvements have been made to increase the specificity, persistence, and activity of CAR-T cells.

CARs are broadly categorized into three generations. First-generation CARs mediate modified T cell activation via the CD3 zeta chain (CD3ζ) signaling domain, leading to pro-inflamma- tory cytokine secretion, T cell proliferation, tumor cell lysis *in vitro*, and the eradication of transplanted tumors in *in vivo* mouse models. Clinical trials, however, have only shown modest efficacy of first-generation CARs. Incomplete T cell activation and lack of costimulation are thought to be the primary causes of the weak response in patients; tumors frequently do not provide ligands for costimulatory molecules on T cells, and the signal through the CD3ζ chain alone is insufficient for priming resting T cells. Next, second-generation CARs contain costimulatory signaling domains derived from T cell costimulatory, with receptors such as CD28 being the most common choice. CD28-mediated CAR signaling is preferred in inducing IL-2 secretion and promoting T cell amplification (Chmielewski et al., 2011). Besides CD28, other costimulatory molecules, such as tumor necrosis factor receptor superfamily member 9 (TNFRSF9, 4-1BB), tumor necrosis factor receptor superfamily, member 4 (TNFRSF9, OX40), inducible T-cell CO Stimulator (ICOS), and CD27, may be included in CAR construction to take advantage of their associated functions in regulating T cell proliferation, survival, and antitumor response. In particular, 4-1BB costimulation induces IL-2 and IFN-γ production and enhances $CD8^+$ T-cell responses. Moreover, 4-1BB signaling seems to be essential for the persistence of the memory $CD8^+$ T cells (Campana et al., 2014). By some researchers, 4-1BB costimulation is considered more effective than CD28 costimulation, as the former reduces exhaustion induced by

*Corresponding author (email: zonghaili@shsmu.edu.cn)

persistent CAR signaling (Annenkov et al., 2002). Nevertheless, an advantage of CD28 signaling is the potential to circumvent CTLA-4 inhibition (Dubinski et al., 2015). Finally, third-generation CARs are constructed from two costimulatory molecular signaling regions. However, more evidence is needed to demonstrate the superiority of third-generation CAR-T cells over second-generation CAR-T cells.

Recent results from clinical trials indicated that CAR-T therapy is remarkably successful in treating hematological malignancies (Davila et al., 2014; Kalos et al., 2011; Lee et al., 2014; Shannon et al., 2014; Maude et al., 2014). However, the benefits of CAR-T cell therapy on solid tumors were far less clear. In this review, we will discuss the current progress in CAR-T cell therapy, including improvements to target antigen specificity, results from preclinical and clinical studies, as well as novel strategies to increase the method's safety and efficacy against solid tumors.

ANTIGEN CHOICE IN SOLID TUMORS

Tumor antigens targeted by CAR-modified T cells could be cell surface proteins and glycolipids, carbohydrates, and sometimes the major histocompatibility complex 1 (MHC-I). An ideal tumor antigen for CAR-T therapy should target only cancer cells, but few truly tumor-specific antigens exist. Most antigen candidates for CAR-T cells are expressed in both tumor cells and some normal cells. Consequently, the selection of tumor antigens requires balancing antitumor efficacy with the risk of "on-target, off-tumor" toxicity in normal tissue. Selecting an appropriate target is a critical step to ensure CAR-T function, as evidenced by the extremely positive outcomes in CAR-T treatments of hematological malignancies, attributed in part to the choice of CD19, an ideal target widely expressed by acute lymphoblastic leukemia cells. While also found in normal cells, CD19 expression is restricted to B cells and their progenitors, allowing the use of gamma-globulin to protect the patients from infection. To date, more than 20 antigens have been used as the targets of CAR-T cells for solid tumors (Table 1). In addition, neoantigens are emerging as possible biomarkers in cancer immunotherapy. Formed by peptides that are entirely absent from the normal human genome, their presence provides an incentive for developing neoantigen-specific CAR-T cells (Schumacher and Schreiber, 2015).

Generally, CAR-T cells recognize and bind to targeted antigens via the single chain antibody expressed on the target cell surface. Thus, antigen intensity appears to be a factor that influences CAR-T cell function. Typically, CAR-T cells target highly expressed antigens while ignoring those that are lowly expressed (Usanarat Anurathapan et al., 2013). This property, however, is a double-edged sword. An

Table 1 Antigens targeted by CAR-T for the treatment of solid tumors

Antigen	Full name	Cancer
AC133	–	Glioblastoma (Liao et al., 2015)
B7H6	–	Ovarian melanoma (Wu et al., 2015c)
CAIX	Carbonic anhydrase IX	Metastatic renal cell carcinoma (Lamers et al., 2013)
CEA	Carcinoembryonic antigen	pancreatic tumors (Chmielewski et al., 2012)
CSPG4	Chondroitin sulfate proteoglycan-4	Melanoma, HNSCC, breast carcinoma (Geldres et al., 2013)
EGFR	Type III variant of the epidermal growth factor receptor	Glioblastoma (Miao et al., 2014)
EphA2	Erythropoietin-producing hepatocellular A2	Lung cancer (Kakarla et al., 2013), glioblastoma (Chow et al., 2013)
FAP	Fibroblast activation protein	Cancer-associated stromal cells (Yarden and Sliwkowski, 2001)
Alpha FR	Alpha folate receptor	Ovarian cancer (Song et al., 2011)
GD2	Disialoganglioside 2	Neuroblastoma (Craddock et al., 2010; Pule et al., 2008)
GPC3	Glypican-3	Hepatocellular carcinoma (Gao et al., 2014)
Her2	Human epidermal growth factor receptor 2	Ovarian cancer (Lanitis et al., 2012a), medulloblastoma (Ahmed et al., 2007)
IL13Ra2	Interleukin-13Ra2	Glioblastoma (Krebs et al., 2014)
L1-CAM (CD171)	Adhesion molecule L1-CAM	Neuroblastoma, medulloblastoma, ovarian adenocarcinoma (Hong et al., 2014)
LMP-1	Latent membrane protein 1	nasopharyngeal carcinoma (Tang et al., 2014)
Mesothelin	–	Malignant pleural mesotheliomas (Beatty et al., 2014)
MUC-1	–	Breast carcinoma (Wilkie et al., 2008)
NKG2D	Natural killer group 2 member D	Ovarian cancer (Spear et al., 2013)
PSMA	Prostate specific membrane antigen	Prostate cancer (Stephan et al., 2007)
PSCA	Prostate stem cell antigen	Pancreatic cancer (Abate-Daga et al., 2014)
TRAIL-receptor 1	TNF-related apoptosis-inducing ligand (TRAIL) receptor 1	Colon cancer, ovarian cancer (Kobayashi et al., 2014)
VEGFR-1	Vascular endothelial growth factor receptor-1	Neovascularization (Wang et al., 2013)
EGFR	Epidermal growth factor receptor	Epithelial carcinoma, non-small cell lung cancer (Zhou et al., 2013)

advantage is the avoidance of on-target, off-tumor toxicity, as normal tissues often express low levels of tumor-associated antigens. However, a clear disadvantage is the failure of lowly expressed of antigens to fully activate CAR-T cells, which then cannot efficiently eliminate tumor cells with low densities of target antigens.

PRECLINICAL STUDIES

CAR-T cells targeting several tumor-associated antigens have been advanced into clinical trials. Here, we describe several preclinical studies on these target-redirected CAR-T cells.

Her-2-specific CAR-T cells

Human epidermal growth factor receptor 2 (Her-2, also called neu or ErbB2) is a member of the epidermal growth factor receptor (EGFR) family. It is one of the most commonly studied tumor-associated antigens for cancer immunotherapy, as it is widely expressed on medulloblastoma, osteosarcoma, glioblastoma, and breast cancer cells. Initial studies using N29, a first-generation CAR derived from Her2-specific scFv, demonstrated specificity and lysis activity dependent on Her2 presence (Stancovski et al., 1993). Another such CAR (FRP5) resulted in sustained regression of established medulloblastomas in an orthotopic, xenogeneic mouse model (Ahmed et al., 2007). Subsequent data confirmed the functionality of second-generation (CD28) CARs expressing canine T cells on Her-2^+ osteosarcoma (Mata et al., 2014). Further study revealed that CD28-modified second-generation CAR-T cells significantly inhibited tumor growth in mice bearing osteosarcoma or breast tumor xenografts (Sun et al., 2014). Taken together, these results highlight the clinical potential of Her-2 targeted CAR-T therapy.

EGFRvIII-specific CAR-T cells

The epidermal growth factor receptor variant III (EGFRvIII) is an oncogenic variant of EGFR, absent in normal tissue but exclusively expressed in tumors from several cancer types, including glioblastoma (GBM), breast cancer, and non-small cell lung cancer (NSCLC). Most EGFRvIII-redirected CAR-T studies were assessed in glioblastoma models. Early preclinical studies demonstrated the potential antitumor effect of first-generation CARs targeting EGFRvIII (González-Navajas et al., 2012; Jacoby et al., 2015). Subsequent second- and third-generation CAR-T cells validated their antitumor activity on EGFRvIII$^+$ glioblastoma cells. Furthermore, intracerebral injection of third-generation CAR-T cells successfully inhibited tumor growth (Choi et al., 2014). Intriguingly, after systemic delivery, EGFRvIII-targeted third-generation CAR-T cells could localize to intracranial tumors, suppress tumor growth, and enhance the survival of mice carrying established GBM xenografts (Miao et al., 2014).

Mesothelin-specific CAR-T cells

Mesothelin is a tumor-associated antigen that is overexpressed in many cancer types (e.g., malignant pleural mesotheliomas (MPM), pancreatic cancers, and ovarian cancers) and lowly expressed on normal peritoneal, pleural, as well as pericardial mesothelial surfaces (Beatty et al., 2014). Thus, immunotherapy that targets mesothelin has yielded promising results. For example, a recent study (Carpenito et al., 2009) engineered T cells with high affinity for mesothelin (CD28-41BBz) and transferred them intratumorally or intravenously into immunodeficient mice, engrafted with tumors pre-established by serial passage of primary pleural effusion cells from mesothelioma patients. As a result, the engineered T cells reduced the tumor burden and in some cases completely eradicated the tumors at low effector-to-target ratios (Carpenito et al., 2009). Similarly, intratumoral injection of RNA CAR electroporated T cells mediated regression of large, vascularized flank-mesothelioma tumors in mice (Zhao et al., 2010), although these T cells are less potent in the same model than lentivirus-transduced T cells, which cure most mice (Carpenito et al., 2009). Furthermore, adoptive transfer of mesothelin-targeted first-generation CAR-T cells mediated regression of ovarian cancer in a xenogeneic model (Lanitis et al., 2012b). These findings support the potent clinical application of mesothelin-specific CAR-T cells for MPM, ovarian cancer, and other solid tumors.

GD2-specific CAR-T cells

Ganglioside GD2 is a tumor-associated surface antigen found in a broad spectrum of human cancers and stem cells, including pediatric embryonal tumors and adult cancers. Because its distribution in normal tissue is restricted, GD2 is safe for immunotherapy targeting (Krug et al., 2015). Third-generation CAR-T cells, based on GD2-specific antibody sc14.G2a, secreted significant levels of cytokines upon antigen recognition and exhibited anti-melanoma activity both *in vitro* and in xenograft models (Yvon et al., 2009). Additionally, first-generation CAR-T cells co-expressing chemokine receptor CCR2b was constructed and improved homing, as well as greater anti-tumor activity, to CCL2-secreting GD2$^+$ neuroblastoma *in vivo* was observed (Craddock et al., 2010).

CEA-specific CAR-T cells

CEA is a 180-kD tumor-associated glycoprotein. It is a well-known tumor-inducer that is overexpressed in many epithelial cancers, most notably in colorectal adenocarcinoma and pancreatic tumors. A recent study reported rejection of carcinoembryonic antigen (CEA)-positive pancreatic tumors in CEA-transgenic mice that expressed CEA as self-antigen in healthy gastrointestinal cells (Chmielewski et al.,

2012). Adoptive therapy with CEA-targeted first-generation CAR-T cells eliminated CEA^+ tumors in a primary response. Moreover, upon rechallenge, cured mice produced an efficient, long-term recall response towards CEA^+ tumor cells. The antitumor response of CD28-costimulatory CAR-T cells was also validated in an animal model for CEA^+ colorectal cancer (Emtage et al., 2008).

EphA2-specific CAR-T cells

EphA2 has emerged as an attractive target for GBM immunotherapy because it is overexpressed in glioma and promotes malignancy. Studies found that EphA2 signaling plays an important role in glioma cell proliferation (Liu et al., 2006), migration, and invasion (Miao et al., 2009). EphA2-specific CAR-T cells (CD28z) could recognize EphA2-positive glioma cells and were potent against human glioma-initiating cells, preventing neurosphere formation and destroying intact neurospheres in coculture assays. Additionally, adoptive transfer of EphA2-specific CAR-T cells resulted in the regression of glioma xenografts in a mouse model (Chow et al., 2013). When combined with FAP (fibroblast activation protein)-specific T cells (CD28z), these EphA2 specific CAR-T cells significantly enhanced overall antitumor activity and conferred a survival advantage over either treatment alone (Kakarla et al., 2013).

NK cell activating receptor-based CAR-T cells

It is well known that NK cells employ activating receptors, such as NKG2D, NKp30, and DNAX accessory molecule-1 (DNAM-1), to recognize stress-induced ligands expressed on various tumor types. Therefore, CARs composed of the ligand-binding region of these receptors or the scFv against these ligands were developed and the resulted CAR-T cells have been evaluated in preclinical animal models, with encouraging antitumor results (Wu et al., 2015b, 2015c; Zhang et al., 2012). However, sometimes lethal toxicity was observed (VanSeggelen et al., 2015).

Beside these commonly used targets, many other antigens are emerging as potential CAR-T targets in solid tumors. For example, we demonstrated that GPC3 is a suitable antigen for the development of CAR-T cells to treat hepatocellular carcinoma (Gao et al., 2014) and lung squamous non-small cell lung cancer (Li K, et al., 2015). Other research has shown that interleukin-13Ra2 specific CAR-T cells may be used to eliminate glioblastoma (Krebs et al., 2014). In general, existing preclinical data strongly indicate that CAR-T cells are extremely robust antitumor reagents.

CLINICAL TRIALS

To date, about 40 clinical trials using CAR-T to treat solid tumors have been performed (Table 2). These clinical trials include first-, second-, and third-generation CAR-T cells.

First-generation CAR-T cells

As early as 2006, Kershaw et al. conducted a phase I clinical trial using CAR-T cells to target the ovarian cancer-associated antigen, α-folate receptor (FR) (Kershaw et al., 2006). However, tumor burden was not reduced in any patient after infusion with first-generation CAR-T. In the same year, Lamers et al. reported another clinical trial that treated metastatic clear cell renal cell carcinoma (RCC) using carboxy-anhydrase-IX (CAIX)-targeted CAR-T cells (Lamers et al., 2006). An unexpected liver toxicity was observed, caused by the CAR-T cells attacking the $CAIX^+$ bile duct epithelial cells. This study demonstrated that CAR-T cells redirected to targets shared by normal and cancer cells would cause on-target off-tumor toxicity. Thus, to reduce such risks, additional safety mechanisms must be incorporated into these CARs to reduce the risk. However, another clinical trial using first-generation CAR-T cells targeting L1-cell adhesion molecule (L1-CAM) also showed no significant toxicities and toxicities and limited efficacy when treating six children with recurrent/refractory neuroblastoma (Park et al., 2007). Besides on-target off-tumor side effects, infused CAR-T cells may be immunogenic because of the associated transgene and the retroviral vector-encoded epitopes (Lamers et al., 2011). Thus. it is essential to attenuate the immunogenicity of both transgene and vector.

Generally, the first-generation CAR-T cells do not survive well *in vivo*. Therefore, Epstein-Bar virus (EBV)-specific CTLs were engineered to express a chimeric antigen receptor directed at the disialoganglioside GD2, a tumor-associated antigen expressed by human neuroblastoma cells (Pule et al., 2008). They found that virus-specific CAR-T cells persisted in higher numbers and for longer periods after infusion to patients than CAR-T cells that lack EBV specificity (Pule et al., 2008). The characteristics associated with virus-specific CAR-T cells were linked to subsequent tumor necrosis or sustained complete remission. Thus, virus-specific CAR-T cells may offer distinct advantages as tumor- directed effector cells.

Second- and third-generation CAR-T cells

Several second- and third-generation CAR-T cells have advanced into clinical trials. As one example, a phase I/II clinical study was conducted in which patients with recurrent/refractory sarcoma received escalating doses of T cells expressing an Her2-specific chimeric antigen receptor, with a CD28-CD3ζ signaling domain (Ahmed et al., 2015). Although the second-generation Her2-CAR-T cells persisted for six weeks in patients without evident toxicity, the clinical outcome was limited. Third-generation CARs specific to Her2 was also tested in clinical trials. However, these CARs caused lethal on-target, off-tumor toxicity in a patient with colon cancer (Morgan et al., 2010). This serious side effect prompted researchers to modify CAR-T cells with a "safety switch" to limit toxicity. Such switches include caspase-9

Table 2 Summary of CAR-T clinical trials in solid tumors[a)]

Number	Antigen	Disease	CAR generation	Phase	Clinicaltrials.gov identifier
1	CAI	Clear cell renal, cell carcinoma	–	–	NCT00602862
2	CD171	Neuroblastoma, ganglioneuroblastoma	Second/Third (41BB/CD28-41BB)	I	NCT02311621
3	CEA	Adenocarcinoma	–	I	NCT00004178
4	CEA	Colorectal carcinoma	Second (CD28)	I	NCT00673322
5	CEA	Breast cancer	Second (CD28)	I	NCT00673829
6	CEA	Solid tumor	–	I	NCT01212887
7	CEA	Liver metastases	Second (CD28)	I	NCT01373047
8	CEA	Adenocarcinomas	Second (CD28)	II	NCT01723306
9	CEA	CEA^+ solid tumors	–	I	NCT02349724
10	EGFRvIII	Glioblastoma	Third (CD28-41BB)	I/II	NCT01454596
11	EGFRvIII	Glioblastoma	–	I	NCT02209376
12	EGFR	EGFR positive advanced solid tumors	Second (41BB)	I/II	NCT01869166
13	EGFR	Advanced glioma	–	I	NCT02331693
14	FAP	Malignant pleural mesothelioma	–	I	NCT01722149
15	FR	Ovarian cancer	–	I	NCT00019136
16	GD2	Neuroblastoma	First	I	NCT00085930
17	GD2	Neuroblastoma	–	I	NCT01460901
18	GD2	Neuroblastoma	Third (CD28-OX40)	I	NCT01822652
19	GD2	Osteosarcoma	Third (CD28-OX40)	I	NCT01953900
20	GD2	Non-neuroblastoma, $GD2^+$ solid tumors	Third (CD28-41BB)	I	NCT02107963
21	GD2	Neuroblastoma	Third (CD28-OX40)	I	NCT02439788
22	GPC3	Hepatocellular carcinoma	–	I	NCT02395250
23	Her2	Lung malignancy	Second (CD28)	I	NCT00889954
24	Her2	Advanced osteosarcoma	Second (CD28)	I	NCT00902044
25	Her2	Metastasized $Her2^+$ cancer	Third (CD28-41BB)	I	NCT00924287
26	Her2	GBM	Second (CD28)	I	NCT01109095
27	Her2	Solid tumors	First and second (4-1BB)	I/II	NCT01935843
28	Her2	Her2 positive malignancy	–	I	NCT00889954
29	IL 13 zetakine	Brain and CNS tumors	–	I	NCT00730613
30	IL13Rα2	Glioma, neoplasm	Second (4-1BB)	I	NCT02208362
31	Mesothelin	Pancreatic cancer	Second (4-1BB)	I	NCT02465983
32	Mesothelin	Pancreatic (ductal) adenocarcinoma, ovarian cancer, mesothelioma	Second (4-1BB)	I	NCT02159716
33	Mesothelin	–	–	I	NCT02388828
34	PSMA	Prostate cancer	First	I	NCT00664196
35	PSMA	Prostate cancer	Second (CD28)	I	NCT01140373
36	ErbB	Head and neck cancer	Second (CD28)	I	NCT01818323

a) From https://www.clinicaltrials.gov/.

(Gargett and Brown, 2014), caspase-8 (Khaleghi et al., 2012), Herpes simplex thymidine kinase (Hsv-tk) (Jensen et al., 2010), and CD20 suicide genes (Burnette et al., 2011).

Recently, six patients (with chemotherapy-refractory metastatic pancreatic ductal adenocarcinoma) were treated with autologous T cells, transiently expressing a mesothelin-specific CAR that includes both CD3ζ and 4-1BB costimulatory domains (Beatty et al., 2015). Of the administered CAR-T cell infusions, 98% showed no dose-limiting toxicity. Infusions were well tolerated, without evidence of cytokine release syndrome, pleuropericarditis, or peritonitis. Two of the six patients were categorized as experiencing stable disease under RECIST 1.1 guidelines, and in one patient, liver metastases were no longer detected after therapy. These outcomes suggest that the CARmeso T cells successfully exhibited antitumor activity. Additionally, T cells bearing bispecific CAR were also applied to treat patients with progressive $Her2^+$ GBM (Nabil et al., 2015). Only one out of fifteen patients exhibited partial response and four patients had stable disease after CAR-T infusion.

Overall, the results of clinical trials have demonstrated that CAR-T cells do exhibit antitumor activity in humans. However, clinical responses to CAR-T cell treatment of solid tumors are generally far from satisfactory. Therefore, considerable effort must still be made to increase the safety and efficacy of CAR-T cell therapy.

NOVEL STRATEGIES TO INCREASE THE SAFETY AND EFFICACY OF CAR-T

The difference in the clinical responses between solid tumors and blood cancers, especially leukemia, is very obvi-

ous. There are at least four important reasons for this great difference. The first reason is that CAR-T cells cannot easily penetrate solid tumor tissues to fight the cancer. The second reason is that a solid tumor represents an extremely hostile microenvironment that hampers the infiltration, expansion, survival of the CAR-T cells, because tumor-educated immune cells, mesenchymal cells, and vascular endothelial cells are present along with tumor cells. The third reason is that cancer cells and the target antigens in the solid tumor are generally heterogeneous, which negatively affects CAR-T cell antitumor activities. The fourth reason is that cancer-specific antigens in solid tumor are very limited. All of these problems contribute to on-target off-tumor toxicity, which undeniably limits the therapeutic window of CAR-T cells. Thus, considerable effort is being exerted to develop a variety of strategies aimed at improving the safety and efficacy of CAR-T cell therapy.

Enhanced accumulation of CAR-T cells into the tumor tissues

Several strategies have been designed to increase CAR-T cell migration to the tumor site. One strategy is to engineer T cells with chemokine receptors. For instance, Kershaw et al. showed that deficiencies in T cell chemotaxis towards tumor cells are overcome by transgenic CXCR2 expression, facilitating the recognition of tumor-produced Gro-α (Kershaw et al., 2002). Likewise, results from murine xenograft experiments demonstrated that overexpression of the chemokine receptor CCR4 by CD30-specific CAR-T cells enhances their migration into Hodgkin's lymphoma (Di Stasi et al., 2009). Moreover, CCR2b-modified CAR-T cells redirected to GD2 have improved tumor-specific trafficking and significantly enhanced activity against neuroblastoma xenografts (Craddock et al., 2010).

Although chemokine receptor modification improves CAR-T cell migration to tumor sites, it does little to promote cell extravasation, necessary for eventual contact between the effector cells and the target tumor cells. To solve this issue, one study incorporated echistatin into CAR-T cells (Fu et al., 2013). Echistatin binds strongly to αvβ3 integrin, which is highly expressed on the surface of endothelial cells from tumor neovasculature. The echistatin-modified CAR-T was then able to lyse human umbilical vein endothelial cells and tumor cells that express αvβ3 efficiently *in vitro*. Additionally, systemic administration of echistatin-modified CAR-T cells led to extensive bleeding in tumor tissues with no evidence of damage to blood vessels in normal tissues.

Recently, CAR-T cells were found to lose heparanase (HPSE) expression after *in vitro* culture. Heparanase degrades heparin sulfate proteoglycans, the main components of the extracellular matrix. Thus, re-expression of HSPE in CAR-T cells significantly improved their T cell infiltration and antitumor activities (Caruana et al., 2015). Another method of improving CAR-T penetration into tumors is the use of cancer-associated fibroblasts (CAF). Specifically, a recent finding revealed that FAP-specific CAR-T cells, capable of destroying FAP-positive fibroblast cells, could increase the antitumor activity of EphA2-targeted CAR-T cells in lung cancer models (Kakarla et al., 2013).

Armored cytokines to increase immune response

Most immunotherapy trials yield primarily transient tumor regression, likely because we tend to neglect the heterogeneity of tumor lesions at the immunologic level. CAR-T cells themselves are not able to lyse target-negative tumor cells directly. Therefore, it seems necessary to equip CAR-T cells with cytokines such as IL-12, which play a pivotal role in immunoregulatory functions. To mediate tumor lysis, IL-12 can augment the activation of cytotoxic T cells and NK cells, both primary effectors of the adaptive and innate immune responses. A recent study has demonstrated the effectiveness of this method; CEA-specific CAR-T cells, carrying an $NFAT_6$-induced IL-12 transgene, were able to eradicate mixed CEA-positive and CEA-negative tumor cells (Watford et al., 2003).

Optimization of costimulatory signals

Although CD28 and 4-1BB are the most commonly used costimulatory molecules, less conventional options, such as MyD88 and CD40, may ultimately prove to be more suitable for CAR-T cell immunotherapy. In a recent report, MyD88/CD40 was able to promote higher levels of IL2 production in CAR-T cells than CD28 (Lasek et al., 2014). CAR-T cells bearing MYD88/CD40 signals also display enhanced efficacy in $CD19^+$ and $Her2^+$ tumor models, respectively, compared with CAR-T cells bearing the CD28 signal. Costimulation with MYD88 or CD40 resulted in greater T cell proliferation, cytokine production, and antitumor efficacy *in vivo* than control CARs with standard costimulatory molecules. Similarly, CD40L-modified CAR-T (CD28-CD3ζ) exhibited more antitumor activity in a xenotransplant model of $CD19^+$ systemic lymphoma than CD28-CD3ζ cells without CD40L (Curran et al., 2015). Finally, T lymphocytes armed with the uncommonly used CD80 and 4-1BBL provoked potent rejection of large, systemic tumors in a xenotransplant model of $PSMA^+$ prostate cancer (Stephan et al., 2007).

Regional delivery of CAR-T cells

Taking advantage of an orthotopic model that faithfully mimics human pleural malignancy, Adusumilli et al. used M28z CAR to evaluate two routes of mesothelin-targeted T cell administration (Adusumilli et al., 2014). They found that intrapleurally administered CAR-T cells vastly outperformed systemically infused T cells, requiring 30-fold fewer M28z T cells to induce long-term, complete remission. After intrapleural T cell administration, prompt *in vivo* antigen-induced T cell activation allowed robust CAR-T cell

expansion and effector differentiation, resulting in enhanced antitumor efficacy and functional T cell persistence for 200 days. Regional T cell administration also promoted the efficient elimination of extrathoracic tumor sites. The ability of intrapleurally administered T cells to circulate and persist within the periphery opens new avenues of treatment for metastatic cancers with accessible tumor sites, which may serve as "regional charging and distribution centers" for CAR-T cell therapy. Appropriate candidates include cancers that metastasize to the pleural cavity, such as lung and breast cancers, as well as those that metastasize to the peritoneal cavity, such as pancreatic and ovarian cancers. In addition to intrapleural or intraperitoneal administration, Choi et al. implanted EGFRvIII CAR-T cells intracerebrally and successfully treated glioma in mice (Choi et al., 2014).

At the very least, this approach may decrease the T cell dose requirement, presenting an advantage when high numbers of CAR-T cells are not attainable (i.e., due to low-yield apheresis, poor *ex vivo* expansion, or low transduction). In the best case, regional delivery may even obviate the need for systematic apheresis.

Increasing the safety index of CARs

The importance of successfully redirecting CAR-T cells to cancer tissues and away from normal tissues cannot be overestimated, because doing so reduces toxicity and increases the therapeutic window. However, as stated previously, cancer-specific antigens like EGFRvIII are very rare. Therefore, numerous strategies have been developed to increase the safety of CAR-T cell therapy. One popular and clinically tested method of controlling CAR-T cells *in vivo* is to include suicide switches in the vectors used to engineer T cells. For instance, the caspase switch efficiently eliminates T cells in patients (Di Stasi et al., 2011; Russo et al., 2012), and the "tumor sensing" approach, which combines two types of antigen recognition with balanced signaling, can potentially reduce on-target off-tumor toxicity (Kloss et al., 2013). Yet another way to circumvent on-target off-tumor toxicity is the recently developed antigen-specific inhibitory chimeric antigen receptors (iCARs) technology (Fedorov et al., 2013). The iCAR has a surface antigen recognition domain combined with a powerful acute inhibitory signaling domain to limit T cell responsiveness despite engagement of an activating receptor. This study demonstrated that CTLA-4- or PD-1-based iCAR could temporarily and selectively limit cytotoxicity induced through the endogenous T cell receptor or an activating chimeric receptor.

Promising methods to increase the therapeutic window include TanCAR, which mediates bispecific activation and targeting of T cells. This novel receptor produces synergistic enhancement of effector functions when encountering two antigens and preserves cytolytic ability upon the loss of one target (Grada et al., 2013). In addition, fine-tuning CAR-T cell affinities may increase their therapeutic index, as low-affinity CAR-T cells appear capable of discriminating tumor cells with high EGFR or Her2 expression from tumors with low expression (Caruso et al., 2015; Liu et al., 2015).

Combination with immune checkpoint inhibitors

The expression and activity of immune checkpoints significantly affect the survival and function of the CAR-T cells. Thus, a potential method of increasing CAR-T cell activity is a combination with immune checkpoint inhibitors. For instance, adding an anti-PDL1 antibody significantly restored the killing activity of CAR-T cells (Moon et al., 2014). Notably, anti-CTLA-4 and anti-PD-1 antibodies have shown clinical promise by derepressing anti-T cell responses in some patients with melanoma, lung, and renal cancer (Carosella et al., 2015). However, researchers have not fully explored the efficacy of joint therapeutic techniques involving both CAR-T cells and immunomodulatory agents such as the anti CTLA-4 or PD-1 checkpoint inhibitors.

FUTURE DIRECTIONS AND CONCLUDING REMARKS

In this review, we summarized the current preclinical and clinical studies on CAR-T therapy against solid tumors. Although the results of clinical studies have thus far yielded limited achievements, we are confident in the potential of solid tumor treatments using CAR-T. We should bear in mind that CAR-T is completely different from other current antitumor therapies, including antibodies and small-molecule drugs. There is tremendous scope for increasing CAR-T cell efficacy and safety, but this cannot be achieved without further clinical trials to validate treatment and tailor them to patients appropriately. Moreover, CAR-T cells are amenable to combinations with other therapy options. For example, low dose irradiation may be used to promote CAR-T infiltration into tumor tissues (Wu et al., 2015a). In conclusion, we believe that with increased effort and attention, CAR-T cells will soon become a potent and efficient treatment method for solid tumors.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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