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• REVIEW •

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Hurdles of CAR-T cell-based cancer immunotherapy directed against solid tumors

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Recent reports on the impressive efficacy of chimeric antigen receptor (CAR)-modified T cells against hematologic malignancies have inspired oncologists to extend these efforts for the treatment of solid tumors. Clinical trials of CAR-T-based cancer immunotherapy for solid tumors showed that the efficacies are not as remarkable as in the case of hematologic malignancies. There are several challenges that researchers must face when treating solid cancers with CAR-T cells, these include choosing an ideal target, promoting efficient trafficking and infiltration, overcoming the immunosuppressive microenvironment, and avoiding associated toxicity. In this review, we discuss the obstacles imposed by solid tumors on CAR-T cell-based immunotherapy and strategies adopted to improve the therapeutic potential of this approach. Continued investigations are necessary to improve therapeutic outcomes and decrease the adverse effects of CAR-T cell therapy in patients with solid malignancies in the future.

chimeric antigen receptor, T cells, solid tumors

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INTRODUCTION

T cells with engineered/modified chimeric antigen receptors (CARs) have recently emerged as a promising tool for treating tumors. These CAR-T cells have been modified with a recombinant receptor molecule to recognize cell-surface antigens directly and are independent of major histocompatibility complex (MHC) restrictions—a commonly observed mechanism of tumor immune escape (Elkord et al., 2009; Garrido et al., 1997).

The prototype CAR is composed of an extracellular target-binding domain, a hinge, a transmembrane domain, and one or more intracellular signaling domains. Most CARs use an antibody-derived single-chain variable fragment (scFv) for targeting specific tumor-associated antigens (TAA). The hinge is important for CAR expression on the cell surface because it affects flexibility of the scFv and its interaction with the ligand. First-generation CARs were conjugated with an intracellular signaling domain alone, typically the CD3 ζ chain. Some groups used signaling domains derived from the Fcγ receptor (Kershaw et al., 2006). Second- and third-generation CARs harbor one or two costimulatory molecules in their intracellular regions, such as CD27, CD28, CD134 (OX40), CD137 (4-1BB), CD244, or ICOS, which may augment the effects of ζ chain signaling and hence enhance T cell proliferation and persistence (Altvater et al., 2009; Guedan et al., 2014; Hombach et al., 2012; Milone et al., 2009; Song et al., 2011, 2012). Recent-

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ly, fourth-generation CARs, also called TRUCK T cells, were developed involving two separate transgenes, with the CAR gene and a T cell activation responsive promoter linked to a cytokine, such as IL-12 (Chmielewski and Abken, 2015; Chmielewski et al., 2014).

CAR-T cells that specifically recognize CD19 are efficacious in clinical studies aimed at treating CD19-positive hematological malignancies (Brentjens et al., 2011, 2013; Grupp et al., 2013; Kochenderfer et al., 2012; Porter et al., 2011), and the United States Food and Drug Administration granted "breakthrough therapy" designation to anti-CD19 CAR-T cell therapy (Gill and June, 2015). Currently, an increasing number of clinical trials in CAR-T cell therapy are being extended to target solid malignancies (Li et al., 2015). Experience with solid tumors is more limited, and here we review past results on CAR-T cell therapy against solid malignancies by focusing on facts that might aid in improving therapeutic responses and overcome current limitations.

CAR-T CELL THERAPY FOR SOLID MALIGNANCIES

Solid malignancies represent a relatively large proportion of the total cancer burden. Surgery, radiotherapy, and chemotherapy are mainstays of treatment modalities, but the mortality rate remains high for most patients with advanced or metastatic disease. Cancer immunotherapy, evaluated as a scientific breakthrough in 2013, focuses on the development of novel therapies employing the immune system to generate an effective immune response against cancer, such as using monoclonal antibodies against immune-checkpoints and adoptive transfer of CAR-T cells. While CAR-T cell therapy was first attempted against solid tumors (Kershaw et al., 2006; Lamers et al., 2006), the most exciting results from CAR-T cell therapy up to now have been derived from trials of patients with hematological malignancies (Brentjens et al., 2011; Kochenderfer et al., 2012; Porter et al., 2011). In this review, we discuss the possible obstacles and difficulties faced by CAR-T cell therapy when targeting solid tumors.

SELECTING TUMOR ANTIGENS

A critical hurdle in fighting solid malignancies with CAR-T cell therapy is the availability of adequate target antigen. Ideally, the targeted antigen would be exclusively expressed on the surface of the malignant cells. However, most of the selected antigens are not restricted to tumor cells and are expressed by normal host tissues. Early trials of anti-ErbB2 (Her2/neu) CAR-T cells against solid tumors yielded several adverse events soon after adoptive transfer (Morgan et al., 2010).

The characteristics of solid tumor antigens are summarized in Table 1. Mutated antigens in solid tumors may be ideal targets for CAR-T cells—for example, mutated epidermal growth factor receptor (EGFRvIII) (Sampson et al., 2010), tumor-specific glycosylation patterns of MUC-1 (Maher and Wilkie, 2009), and erythropoietin-producing hepatocellular A2 receptor (EphA2) epitopes (Coffman et al., 2003)—since they are strictly expressed on tumor cells. However, such target antigens are rare and some mutated molecules are not expressed on the cell surface. Currently, most of the antigens recognized by CAR-T cells are also expressed by healthy cells, including tissue/lineage-specific antigens, developmental antigens normally expressed during fetal development, and antigens that are overexpressed on tumor cells compared to non-malignant host cells. Screening and identification of more biomarkers for solid tumors is a critical step in extending the promise of this immunotherapeutic approach to solid malignancies. The heterogeneity of solid tumors increases the complexity of antigen selection. Unlike hematologic malignancies, solid tumors are composed of cancer cells and stromal cells. Even the cancer cells do not uniformly express the selected antigen with/without mutations.

Tumor-associated stroma, occupying up to 90% of the tumor mass (Dvorak, 1986), has garnered increasing attention for its role in supporting tumor cell growth, invasion, and angiogenesis (Bhowmick et al., 2004; Orimo et al., 2005; Santos et al., 2009; Zhang et al., 2011b). The stroma is composed of heterogeneous cell types including tumor fibroblasts, connective tissue cells, vascular endothelial cells, and immune subtypes such as lymphocytes, granulocytes, and macrophages. Kakarla et al. and Wang et al. have consistently reported that inhibiting tumor growth by targeting tumor stroma with CAR-T cells directed to fibroblast activation protein (FAP) can be safe and effective (Kakarla et al., 2013; Wang et al., 2014). Targeting tumor vasculature provides another means for therapy against multiple solid

Table 1 Solid tumor antigens for CAR-T cell based cancer immunotherapy

Solid tumor antigens	Examples
Overexpressed antigens	CEA, ErbB2 (HER2), FRα, GD2, Mesothelin, VEGFR2, CSPG4, EpCAM, PSMA, EGFRvIII, MUC-1, MUC-16, EphA2, etc
Mutated antigens	Mutated EGFRvIII, glycosylation patterns of MUC-1, EphA2 epitopes
Tissue/Lineage-specific antigens	Prostate specific cancer antigen (PSCA)
Developmental antigens	MAGE family members, NY-ESO-1
Tumor-associated stroma	Fibroblast activation protein (FAP)

tumor types. T cells transduced with VEGFR-2 CAR showed durable tumor regression in preclinical models (Chinnasamy et al., 2010).

Consequently, target antigen selection is a key challenge for CAR-T cell therapy. There may be several possible ways to find a more promising, safer target. CAR-T cells can be genetically modified to recognize two or more tumor-associated antigens, which can enhance discrimination between abnormal and healthy tissue. One example is split-signal CARs, which can limit full T cell activation to tumors expressing multiple antigens (Kloss et al., 2013; Wilkie et al., 2012). Other strategies include tandem CARs (TanCARs), which contain ectodomains with two scFvs (Grada et al., 2013), also limiting the risk of immune escape. Another alternative approach is co-expression of inhibitory CARs (iCARs) directed against molecules in healthy organs together with their activating counterparts. Inhibitory signaling may be provided by checkpoint molecules such as CTLA-4 and PD-1. Still, there is a growing exigency to find novel antigen targets as become more potent.

SPECIFIC HURDLES IN THE PROCESS OF TARGETING TUMORS

While CAR-T cells are genetic modified, the last step is infusing them into patients, the key process in fighting tumors. However, this process can be frustrating and complicated. As a fundamental prerequisite for therapeutic efficacy, CAR-T cells need to be transported to the tumor lesion. Once they accumulate in the vicinity, they must efficiently infiltrate the tumor. When migrating into the solid tumor lesion, CAR-T cells face a highly immunosuppressive microenvironment. The solid tumor microenvironment is extremely inhospitable and capable of inducing anergy in CAR-T cells. As shown in Figure 1, CAR-T cells must therefore overcome many obstacles and use countermeasures to fight solid tumors.

T cell trafficking

The presence of tumor-infiltrating lymphocytes (TIL) has been reported to correlate well with positive clinical outcomes in some patients with various solid cancers (Galon et al., 2006; Kim et al., 2013; Kmiecik et al., 2013; Piersma et al., 2007). In fact, improved antitumor responses have been shown to positively correlate with increased cytotoxic T lymphocyte (CTL) infiltration. CTL trafficking is a tightly controlled process, whose homing could be influenced by many factors, such as mismatching of chemokine-chemokine receptor pairs, down-regulation of adhesion molecules, and aberrant vasculature (Slaney et al., 2014). CAR-T cell therapy is a personalized treatment involving genetic modification of autologous CTLs, enabling specific recognition and targeting of tumor-associated antigens expressed by the tumor cells or the tumor stroma. Previous

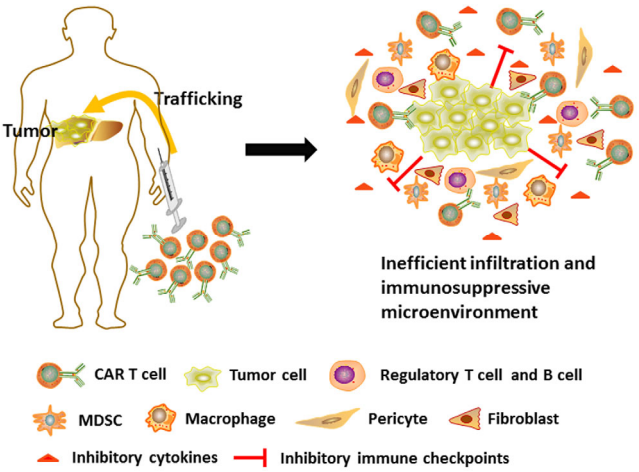

Figure 1 Specific hurdles in the process of targeting solid tumors. The efficacy of CAR-T cells is dependent on their capacity to be transported to the tumor tissue, efficiently infiltrate into the tumor lesion, and resist immunosuppressive signals within the tumor microenvironment.

studies have shown that $CD8^+$ T cells are recruited from the blood to the site of infection by a variety of distinct processes involving attachment/adhesion, rolling/tethering, chemotaxis, and extravasation (Masopust and Schenkel, 2013; Nolz et al., 2011). In addition, the tumor is a hostile microenvironment for T lymphocytes, and T cell trafficking is markedly reduced compared to an infectious disease setting due to both intrinsic and extrinsic factors (Bellone and Calcinotto, 2013). Consequently, new strategies are required to enhance the trafficking of these gene-modified CAR-T cells to the tumor microenvironment.

Efforts to enhance CAR-T cell trafficking have been made, including efforts to create a match between the chemokine produced by tumors and the chemokine receptors on the effector T cells. Chemokine secretion varies among different tumor types, and successful re-direction of T cell migration depends on matching the chemokine with its appropriate chemokine receptor. Kershaw et al. have demonstrated that engineering the chemokine receptor *CXCR2* (*CXCL1* receptor) into T cells enabled the T cells to efficiently migrate toward a melanoma tumor (Kershaw et al., 2002). Transgenic co-expression of CCR4 improved the homing of CAR-CD30-modified T cells to $CD30^+$ Hodgkin lymphoma that secreted CCL17 (the ligand for CCR4), and thereby improved anti-lymphoma effects (Di Stasi et al., 2009). In addition, previous studies have shown that enhanced CCR2b expression from mesothelin-reactive CAR-T cells and CAR-GD2 T cells led to improved anti-tumor effects against malignant pleural mesothelioma and neuroblastoma (Craddock et al., 2010; Moon et al., 2011). Locoregional disease is the primary cause of disease-related mortality among some malignancies, providing a strong rationale for regional T cell delivery. In contrast to systematic administration, local delivery, such as intra-peritoneal and intra-tumoral injection, may also provide a good meth-

od for CAR-T cell delivery. One example is head and neck squamous cell carcinoma. Intra-tumoral delivery of ErbB-targeted CAR-T cells is currently under Phase 1 clinical trial evaluation (van Schalkwyk et al., 2013). Similarly, ovarian cancer and malignant pleural mesothelioma may both be appropriate candidates for local delivery because of their propensity for localized dissemination within peritoneal and pleural cavities.

T cell infiltration

Once the genetically modified T cells accumulate in the vicinity of the tumor, they must infiltrate into the tumor lesion and efficiently exert an anti-tumor effect. These processes involve a complex sequence of events, including the adhesion of T cells to endothelial cells and chemokine-chemokine receptor interactions modulating their extravasation into antigen-rich tissues (Muller, 2003; Parish, 2006; Yadav et al., 2003). It has been reported that the panel of chemokine produced by solid tumors does not favor T cell infiltration into tumor sites. Thus, better strategies are needed to facilitate T cell infiltration into tumors and enhance the efficacy of CAR-T cell anti-tumor effects.

The extracellular matrix (ECM) is an integral component of the stroma, and the main components of the ECM are heparan sulfate proteoglycans (HSPGs). Therefore, T cells that attack stroma-rich solid tumors must degrade HSPGs in order to access tumor cells and exert anti-tumor effects. Caruana et al. have demonstrated that CAR-T cells engineered to express heparanase (which degrades HSPGs) promoted tumor T cell infiltration and anti-tumor activity (Caruana et al., 2015). Moreover, the endothelin B receptor has been reported to prevent T cell infiltration in ovarian tumors. Kandalaft et al. demonstrated that blocking those receptors improved T cell infiltration into the tumor lesion and enhanced the efficacy of immunotherapy (Kandalaft et al., 2009). Another candidate for enhancing T cell infiltration is VEGF receptor-2, which is overexpressed by tumor-associated endothelial cells (Slaney et al., 2014). Throughout the history of cancer treatment, the long-term therapeutic effect of blocking VEGF receptor-2 has been a major anti-angiogenic pharmacologic intervention and has been fully dependent on $CD8^+$ T cell infiltration in tumors (Manning et al., 2007). T cells transduced with VEGF receptor-2 CAR also showed durable and increased tumor infiltration, correlating with their anti-tumor effect (Chinnasamy et al., 2010).

Immunosuppressive microenvironment

A critical barrier against the use of engineered T cells for the treatment of solid tumors is the tumor microenvironment. It is a key determinant of anti-tumor immunity with the capacity to suppress the infiltration, activation, and effector activity of T cells. The ultimate goal is to be curative in solid tumors, and CAR-T cells must withstand and thrive in the solid tumor microenvironment. Moon et al. have revealed that CAR-T cells were, with varying efficiencies, able to traffic into tumors and proliferate, which slowed tumor growth but did not cause regressions or cures. The CAR TILs underwent rapid loss of cytolytic and cytokine secretion capacity, which is reversible by resting cells *in vitro* within 24 h (Moon et al., 2014). Immune suppressor leukocytes, as well as other obstacles, such as immunosuppressive cytokines and inhibitory immuno-checkpoints, present within the tumor microenvironment can suppress the anti-tumor activity of CAR-T cells.

Immune suppressor cells

First, solid tumors are usually infiltrated with abundant immune suppressor cells, including M2 tumor-associated macrophages, myeloid-derived suppressor cells (MDSCs), and regulatory T cells (Tregs) and B cells (Bregs), which protect malignant cells from the anti-tumor activity of the immune system (Biswas and Mantovani, 2010; Liyanage et al., 2002; Schmid et al., 2011). Preclinical data supports the hypothesis that the incorporation of co-stimulatory molecules, such as CD28, into CARs may help CAR-modified T cells to overcome the immunosuppressive tumor microenvironment mediated by Treg cells (Koehler et al., 2007; Lee et al., 2011; Loskog et al., 2006). Recently, a study by Burga et al. showed that myeloid-derived suppressor cells expand in response to liver metastases and inhibit the anti-tumor efficacy of anti-CEA CAR-T cells; CAR-T cell efficacy was rescued when mice received CAR-T in combination with MDSC depletion (Burga et al., 2015). Tumor cells have been found to secrete high levels of granulocyte-macrophage colony-stimulating factor (GM-CSF) *in vivo* implicated in MDSC recruitment, and GM-CSF neutralization may be an alternative approach to prevent MDSC expansion (Lesokhin et al., 2012; Schmidt et al., 2013).

Cytokines

Soluble factors, namely cytokines, in the tumor microenvironment are important determinants of immunotherapy for solid tumors. Various immunosuppressive cytokines such as TGF-β and IL-10 are involved in the inhibition of the efficacy of cancer immunotherapy (Rabinovich et al., 2007; Schreiber et al., 2011). TGF-β suppresses $CD8^+$ effector T cells and is capable of modulating the $CD4^+$ helper T cell toward a Treg phenotype. Therapies aimed at inhibiting immunosuppressive cytokines, such as introducing a dominant-negative TGF-β receptor in CAR-T cells, showed improved efficacy (Bollard et al., 2002). In addition, activating cytokines such as IL-2, IL-12, and IL-15 has been shown to mitigate the effect of immunosuppressive factors in the tumor microenvironment and showed remarkable enhancement of CAR-T efficacy. Chmielewski et al. showed that IL-12 secretion by engineered T cells expressing CARs resulted in the destruction of antigen negative cancer cells that may escape from T cell therapy (Chmielewski et al., 2011). Indeed, studies have shown that therapy with CAR-T cells

engineered to express IL-12 could change the tumor microenvironment and enhance anti-tumor function (Kerkar et al., 2010; Zhang et al., 2011a). Moreover, numerous studies have demonstrated that production of cytokines such as IL-2 and IL-15 improved the anti-tumor effects of CAR-T cells (Hoyos et al., 2010; Kershaw et al., 2006; Nishio and Dotti, 2015; Till et al., 2008; Wang et al., 2013).

Inhibitory immuno-checkpoints

It has been reported that a number of inhibitory immune-checkpoint pathways, such as programmed cell death protein 1 (PD1), cytotoxic T lymphocyte antigen 4 (CTLA-4), B7-H family members, or FasL, can shut down the anti-tumor ability of TILs. Interaction between PD1 and its ligand, PDL1, can suppress the activation of CAR-T cells in the tumor microenvironment. Infused CAR-T cells express PD1 and are susceptible to PD1/PDL1 interaction-mediated suppression (Abate-Daga et al., 2013; Kalos et al., 2011). Moon et al. showed that CAR-T cells underwent rapid loss of function in tumors, a process that was associated with up-regulation of intrinsic T cell inhibitory enzymes and expression of surface inhibitory receptors (Moon et al., 2014). The use of checkpoint inhibitors targeting the above pathways, such as anti-PD1 and anti-CTLA-4, has been demonstrated to enhance T cell responses in patients with melanoma, renal cancer, etc. (Hodi et al., 2010; Topalian et al., 2012). At present there are preclinical data showing that blocking PD1 immunosuppression can boost CAR-T cell therapy, likely representing a fruitful area for future study (John et al., 2013a, 2013b). We believe that combining CAR-T cells with other therapies, like blocking antibodies, offers the potential to improve anti-tumor effects, as tumors are heterogeneous and complex.

TOXICITY

Unwanted toxicity is a major problem that limits CAR-T cell-based immunotherapy. There are three potentially key routes contributing to the toxicity of CAR-T cells that must be considered (Table 2). The most common is on-target, on-tumor toxicity relating directly to the effects of binding of the CAR to the cognate antigen resident on the target tumor cell, such as cytokine release syndrome (CRS) and tumor lysis syndrome (Bugelski et al., 2009; Howard et al., 2011). CRS is typified by chills, fevers, and hypotension, but can also result in much more severe life-threatening multiple-organ failure (Brentjens et al., 2010; Maude et al., 2014). The severity of CRS seems to correlate with tumor burden, potency, and infusion dose of CAR-T cells. IL-6, IL-10, and IFN-γ cytokines play a major role in CRS. The main strategies used to overcome CRS involve direct targeting of the cytokine action by blocking access to their receptors (e.g. anti-IL6 receptor antibody (tocilizumab)) with or without co-application of non-specific corticosteroids (Grupp et al., 2013). Secondly, on-target, off-tumor toxicity is a major challenge when treating solid cancers. CAR-T cells engage a target antigen that is expressed upon healthy and normal cells; this may result in the destruction of healthy cells expressing the specific target antigen—perhaps even at a level much lower than that of tumor cells—and substantially limit the clinical application. For B-cell malignancies, B-cell depletion after treatment with anti-CD19 CAR-T cells is clinically manageable (Cheadle et al., 2010). In the case of many solid tumors, the CAR target may not be tumor-specific. A case reported by Morgan et al. showed that activation of anti-ErbB2 CAR-T cells against healthy epithelial tissues that included the lung and heart resulted in the patient's death soon after adoptive transfer (Morgan et al., 2010). The third potential mechanism of CAR-T cell toxicity may relate to the response of non-CAR-T cells to the therapy (Cheadle et al., 2014). Integrating vectors based upon retroviral and lentiviral backbones may pose a potential risk of oncogenic events.

To control this, suicide genes—genetically encoded molecules that allow for selective destruction of adoptively transferred cells—are often employed. The most commonly used suicide genes are herpes simplex thymidine kinase (HSV-TK), inducible caspase 9 (iCasp9), and CD20 (Ciceri et al., 2009; Di Stasi et al., 2011; Marin et al., 2012). However, a disadvantage of their use is immunogenicity resulting in unwanted elimination of the modified T cells. In contrast to stable modified cells, transient expression of CARs by RNA transfer may provide temporary redirected T cell activity and limit adverse events in the case of toxicity (Birkholz et al., 2009). Even so, some studies have shown that RNA CAR-T cell activity is limited by ineffective tumor infiltration in solid tumor models (Singh et al., 2014a, 2014b). In addition, dual CAR targeting also provides a means to improve the tumor specificity of CAR-T cells with potential for avoiding antigen escape (Grada et al., 2013). Dual CAR targeting involves the use of one CAR containing a "signal 1" and a second with a "signal 2" activating domain. Both signals are required for full T cell activation.

Table 2 CAR-T-cell therapy-associated toxicities

Categorization of toxicities	Reasons	Examples
On-target, on-tumor toxicity	Binding the CAR to cognate antigen resident on the target tumor cell	Tumor-derived toxicity, e.g. cytokine release syndrome (CRS) and tumor lysis syndrome
On-target, off-tumor toxicity	Destruction of healthy cells expressing the specific target antigen	Organ failure and even death (a case reported by Morgan et al.)
Off-target, off-tumor toxicity	Relating to the response of non-CAR-T cells	Genotoxicity based upon retroviral and lentiviral backbones, e.g. oncogenic events

This strategy may provide increased tumor specificity, thus avoiding on-target, off-tumor toxicity (Kloss et al., 2013).

CONCLUSIONS

In summary, we have outlined how the targeting of CAR-T cells toward solid tumors faces certain obstacles and difficulties that must be overcome for therapeutic success. Currently, efforts aiming to exploit CAR-T cell therapy to treat solid tumors are mainly in the preclinical stage. Strategies to overcome the major challenges must be empirically defined in forthcoming clinical trials with respect to both safety and efficacy in the treatment of solid cancers.

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

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