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Combination Immunotherapy with CAR T Cells and Checkpoint Blockade for the Treatment of Solid Tumors

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Summary

Checkpoint blockade (CPB) therapy can elicit durable clinical responses by reactivating an exhausted immune response. However, response rates remain limited, likely secondary to a lack of a tumor-reactive immune infiltrate. Chimeric antigen receptor (CAR) T cells may provide the necessary tumor-targeting immune infiltrate and a highly specific antitumor immune response. This can be further amplified by the addition of CPB agents, which serve to counteract the immune inhibitory environment undermining optimal CAR T-cell efficacy. Herein, we review preclinical and clinical combination therapy with CAR T cells and CPB agents, with a focus on solid tumor malignancies.

Keywords

CAR T cell; immunotherapy; chimeric antigen receptor; PD-1; checkpoint blockade

Introduction

Tumor escape from immune elimination is a hallmark of cancer (Dunn et al., 2002). Escape mechanisms include downregulation of major histocompatibility complex (MHC) receptors, low antigen expression and expression of checkpoint ligands (such as programmed death-ligand 1 [PD-L1]). All together, these elements compose a microenvironment that resists infiltration, persistence, and function of immune cells (Sharma et al., 2017).

Therapeutic targeting with checkpoint blockade (CPB) is the most successful immunotherapeutic intervention to date for cancer (Ribas and Wolchok, 2018). The

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checkpoint signaling that evolved as a means of limiting overactive immune responses and preventing autoimmunity is co-opted by tumor cells to block T-cell immunity, an interaction that CPB targets to reinvigorate antitumor immunity. By reactivating exhausted T cells, CPB has achieved responses in previously untreatable cancers, including long-lasting tumor regressions. However, the enthusiasm generated by the advent of this therapy has been dampened by the recognition that objective response rates to CPB have been limited to <40% for even the most immunogenic malignancies (Zou et al., 2016). Our current understanding of treatment non-responders is that their tumor environment lacks the necessary substrate upon which CPB acts: a preexisting antitumor immune response that can be reactivated by CPB therapy (Huang et al., 2017). Owing to a number of factors, such as decreased antigenicity, MHC downregulation, and resistance to IFN-γ signaling, these "cold" tumor microenvironments do not contain effector T cells to unleash on the tumor. A "hot" tumor microenvironment with T cells that recognize the tumor but have become exhausted may be a prerequisite for response to CPB (Taube et al., 2012). Efforts to combine CPB with another immunotherapy agent that creates an immunogenic tumor microenvironment or directly provides a T-cell infiltrate may therefore provide an ideal combination therapy to increase response rates. This could be accomplished through immunogenic activation (in the form of a cancer vaccine or oncolytic virus, which can release tumor antigens and attract immune effectors) or the direct addition of an antigen-recognizing cellular infiltrate (in the form of adoptive cell therapy). Our focus in this review will be on the adoptive cell therapy approach—in particular, the use of CAR T-cell therapy, which targets T cells directly to tumor cells.

CAR T-cell therapy can achieve remarkably effective—and durable—responses (June et al., 2018). The CAR provides high-avidity binding to cell-surface antigens independent from the MHC receptor, triggering robust T-cell activation and antitumor response (Sadelain et al., 2013). The addition of costimulatory domains to CAR T cells, which enhanced their persistence, catapulted CAR T-cell therapy forward in the clinic. Early trials demonstrated that the CD19targeting CAR could achieve long-lasting remissions in patients with chemotherapy-refractory hematologic malignancies (Park et al., 2018; Schuster et al., 2017). However, this dramatic success has yet to be translated to solid tumors. Solid tumors pose several obstacles, including an immunosuppressive tumor microenvironment that exhausts CAR T cells, making combination therapy with CPB particularly relevant for these malignancies (Joyce and Fearon, 2015). Combining CAR T-cell therapy and CPB may produce a synergistic effect: CAR T cells can provide an infiltrate for immunogenically silent tumors, and CPB can reverse CAR T-cell inhibition and restore functional persistence. In preclinical studies, combination therapy has increased efficacy over each agent alone, supporting the extension to clinical studies in patients (Cherkassky et al., 2016; Hu et al., 2018; John et al., 2013; Strome et al., 2003).

CPB

Checkpoints—Immune system checkpoints play a critical role in balancing T-cell activation and inhibition, as well as preventing autoimmunity. Examples include programmed cell death protein 1 (PD-1; CD279), PD-L1 (CD274; B7-H1), cytotoxic T-lymphocyte–associated protein 4 (CTLA-4; CD154), T-cell immunoglobulin and mucin-

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domain containing-3 (TIM3), and lymphocyte-activation gene 3 (LAG3; CD223). Downregulation or knockout of CTLA-4 causes rampant lymphoproliferation and death from autoimmunity (Tivol et al., 1995), and in cancer models, CTLA-4 signal blockade leads to tumor regression (Leach et al., 1996). In infection models, repeated signaling through the PD-1 receptor causes T-cell inhibition and chronic infection (Barber et al., 2006), whereas in cancer models, upregulation of PD-L1 leads to tumor persistence and PD-L1 blockade results in tumor regression (Curiel et al., 2003; Iwai et al., 2002).

Cancer cells limit antitumor responses by expressing immune checkpoints. The tumor microenvironment is replete with inhibitory PD-L1 signaling, as PD-L1 can be expressed on both tumor cells and host cells, including fibroblasts (Nazareth et al., 2007), dendritic cells, macrophages, and B and T cells. PD-L1 expression can reflect an adaptive cancer response to immune infiltration or can occur independently of immune cell activation (Pardoll, 2012). In the context of adaptive immune resistance, PD-L1 and PD-L2 overexpression are induced by T cell–secreted immunostimulatory cytokines IFN-γ and TNF-α (Spranger et al., 2013; Tumeh et al., 2014). Cancer cells can also express PD-L1 innately in response to disruption of the regulatory 3′ region of the PD-L1 gene (Kataoka et al., 2016) or activation of oncogenic pathways such as PI3K/Akt, EGFR, STAT3, MYC, and cyclin-dependent kinase 5 (Casey et al., 2016; Marzec et al., 2008; Parsa et al., 2007).

Clinical Success of CPB—The first CPB therapy to emerge and ultimately receive FDA approval was the CTLA-4–blocking agent ipilimumab, a fully human IgG1 antibody that was first approved for melanoma in 2011. Although the objective response rates were low, excitement over the potential promise of CPB came in the form of durability of response, reflecting activation of a long-lasting antitumor immune attack attributable to T cells (Ribas et al., 2005). The most impressive clinical responses were associated with immune cell infiltration and resulting extensive tumor necrosis (Hodi et al., 2003), establishing the ability of CPB to activate an immune response. Phase III studies confirmed that ipilimumab, alone or in combination with other agents, produced stronger anticancer responses than traditional methods such as chemotherapy (Hodi et al., 2010; Robert et al., 2011).

Pembrolizumab, a humanized anti–PD-1 antibody, has since demonstrated even more impressive results and a more manageable toxicity profile. Pembrolizumab achieved an overall objective response rate of 19.4% in patients with non-small cell lung cancer (NSCLC), 45.2% in patients with PD-L1–positive tumors, and 33% in patients with melanoma (Garon et al., 2015; Ribas et al., 2016). Pembrolizumab has also shown efficacy in patients with refractory head and neck cancer (Bauml et al., 2017) and relapsed classic non-Hodgkin lymphoma (Chen et al., 2017), with complete response rates as high as 22%. Nivolumab, another fully human anti–PD-1 antibody, has also produced encouraging results, with response rates of 18%−28% in patients with NSCLC, renal cell carcinoma, and melanoma (Borghaei et al., 2015; Topalian et al., 2012). Of note, a considerable portion of the patients who respond to PD-1 blockade experience long-lasting tumor regression, which suggests the involvement of memory T cells in an ongoing antitumor response (Lipson et al., 2012). CPB is under continued investigation and is now part of the standard therapy for advanced melanoma, NSCLC, Merkel cell carcinoma, head and neck squamous cell carcinoma, urothelial and kidney cancers, refractory Hodgkin lymphoma, hepatocellular

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carcinoma, gastric cancer, and cancers with high microsatellite instability (MSIH) (Topalian et al., 2012; Zappasodi et al., 2018).

Biomarkers of Response to CPB—Despite the durable responses achieved with CPB, response rates remain under 50%, highlighting the need to identify biomarkers of immune activation that predict response and suggest mechanisms by which we can augment efficacy.

PD-L1.: PD-L1 expression on tumor cells, the first validated predictor of response to CPB, has been investigated in prospective cohorts of patients treated with CPB (Herbst et al., 2014; Tumeh et al., 2014). Higher tumor PD-L1 expression is an established biomarker associated with improved clinical response to CPB (Garon et al., 2015; Robert et al., 2015; Topalian et al., 2012). But in these studies, there were some patients without tumor PD-L1 expression who nevertheless responded, suggesting that PD-L1 expression specifically on tumor cells is neither necessary nor sufficient for response. Instead, immune cells (myeloid-derived suppressor cells [MDSCs], other T cells, antigen-presenting cells) may act as the primary or at least a redundant source of PD-L1, establishing immune resistance (Tang et al., 2018). Additional reasons for why the general correlation between tumor PD-L1 and CPB response is not exact include the following: alternative ligands, such as PD-L2, or other inhibitory pathways may act as immune silencers (Taube et al., 2015); PD-L1 expression may be stimulated *de novo* following influx of T-cell infiltrate such that pretreatment sampling does not reflect expression over the course of tumor regression (Herbst et al., 2014; Powles et al., 2014) and there may be sampling bias (Taube et al., 2012).

Results of randomized clinical trials also suggest the limitations of screening by PD-L1 tumor staining (Wolchok et al., 2013). Overall, PD-L1 staining of a single specimen may misrepresent what is fundamentally being sought: evidence of an existing immune infiltrate that can be reactivated.

Tumor Mutational Burden (TMB) and Neoantigens.: Tumors often and to varying degrees express neoantigens within the context of MHC as a result of mutations that generate altered proteins. These neoantigens can be recognized as non-self and have binding affinity for MHC to allow representation by antigen-presenting cells (Rizvi et al., 2015; Schreiber et al., 2011). As TMB is correlated with quantity of neoantigens, TMB correlates with response to immunotherapy. Patients whose NSCLC tumors had higher levels of nonsynonymous mutations—namely, mutations that result in the production of a different amino acid and, subsequently, a different protein—were more responsive to PD-1 blockade (Rizvi et al., 2015). Other tumors with high TMB demonstrate high response rates to CPB therapy, including desmoplastic melanoma (Eroglu et al., 2018), virally induced Merkel cell and hepatocellular carcinoma (El-Khoueiry et al., 2017; Nghiem et al., 2016), and carcinogen-induced cancers (Garon et al., 2015).

The clearest demonstration of the association between TMB and response to CPB is seen in MSI-H colorectal cancers, with an overall response rate of 53% in MSI-H tumors (Le et al., 2017). These results led to FDA approval of CPB for any MSI-H tumor in 2017, which marked the first approval of CPB based on a biomarker regardless of tissue histologic profile.

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Simply assessing the overall mutational burden misses the nuance of the antigen quality and suggests *the potential for* a response, as opposed to an actual response (Blank et al., 2016). Clonal antigens, for example, which occur early in tumor development (McGranahan et al., 2016), and neoantigens that are cross-reactive with known microbial epitopes can elicit a stronger immune response (Balachandran et al., 2017), compared with antigens without those qualities. In addition, tumors can be heterogenous in terms of mutational load, which means that a biopsy prone to sampling bias may not determine the actual potential to elicit an immune response (Alexandrov et al., 2013).

Tumor-Infiltrating Lymphocytes (TILs).: Perhaps the most predictive biomarker is the target and end effector of CPB therapy: TILs. TILs are an indicator of a "hot" or immune-inflamed tumor and can indicate whether an immune response is present and directed at the tumor. TIL grade is associated with disease-specific survival in melanoma (Azimi et al., 2012), colorectal cancer (Galon et al., 2006), ovarian cancer (Zhang et al., 2003), and lung adenocarcinoma (Suzuki et al., 2013). Therefore, the presence of an infiltrate is itself a biomarker; furthermore, the characteristics or quality of the infiltrate can also predict response (Melero et al., 2014). The presence of $CD8^+$ T cells has been associated with improved response to chemotherapy and, more recently, CPB (Danilova et al., 2016). A high density of $CD8^+$ T cells on the leading tumor edge has been associated with improved response to immunotherapy (Gajewski et al., 2010; Tumeh et al., 2014).

Need for an Immune Infiltrate—Even the most immunogenic malignancies, such as melanomas, which generally exhibit high levels of both neoantigens and TILs, fail to respond to CPB in significant numbers. One explanation is that these immune features are not prominent in the tumor microenvironment (Obeid et al., 2016). These features need an antigen-sensitive immune infiltrate that can be reactivated. Adoptive cell therapy can serve this need, and its ability to establish an infiltrate has been shown to be feasible.

CAR T Cells

In CAR T-cell therapy, genetically engineered autologous T cells expressing a CAR are administered to the patient. CARs bind antigen in an MHC-independent manner and, therefore, are not susceptible to MHC downregulation, a mechanism often used by malignant cells to evade eradication. The "chimeric" namesake refers to the fusion of two separate protein domains: one that provides the T cell with the antigen recognition to bind to tumor antigen and one that provides signaling for T-cell activation (a CD3ζ intracellular signaling domain with or without costimulatory signaling) (Sadelain et al., 2003). CAR expression is most often achieved using retroviral- or lentiviral-mediated gene transfer. As the T cell is capable of high-fidelity transgene expression and is a potent antitumor effector, it is an ideal host cell for the CAR. The T cell's rapidly dividing nature facilitates viral integration, its transcriptional machinery promotes high-level transgene expression from viral promoters, and its ability to establish memory serves to reinforce long-lasting transgene expression.

Improved Persistence with Costimulation of CAR T Cells—First-generation CARs designed with only the CD3ζ intracellular signaling domain were prone to cell death and

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anergy. Second- and third-generation CARs, which were designed with one or multiple costimulatory domains (e.g., 4–1BB, CD28, ICOS, and OX40) in addition to the CD3ζ domain (Sadelain et al., 2013), have demonstrated superior antitumor efficacy, which has been associated with enhanced T-cell accumulation, cytokine secretion, cytotoxicity, and long-term persistence (Adusumilli et al., 2014).

Clinical Success of CAR T Cells in Liquid Tumors—CD19 CAR T-cell therapy has achieved a durable clinical benefit in a subset of patients with chronic lymphocytic leukemia (Brentjens et al., 2011; Kochenderfer et al., 2012; Schuster et al., 2015) and non-Hodgkin lymphoma (Geyer and Brentjens, 2016; Kochenderfer et al., 2012; Kochenderfer et al., 2010; Savoldo et al., 2011), with the most dramatic results—complete response rates of 80%—achieved in patients with acute lymphoblastic leukemia (Davila et al., 2014; Gardner et al., 2017; Lee et al., 2015; Maude et al., 2014). The FDA recently approved CD19 CAR T-cell therapy for the treatment of relapsed or refractory pediatric and young-adult B-cell acute lymphoblastic leukemia (Kymriah [tisagencleucel]; Novartis) and relapsed or refractory adult large B-cell lymphoma (Yescarta [axicabtagene ciloleucel]; Kite Pharma).

Limited Success of CAR T Cells in Solid Tumors—Although hematologic malignancies have a high burden of disease, they are within the same peripheral blood compartment as intravenously administered T cells and do not feature a dense tumor microenvironment that is difficult to infiltrate. In contrast, solid tumors express antigens heterogeneously, are sequestered in organ tissue, and feature an immunosuppressive tumor microenvironment in which T cells function suboptimally (Moon et al., 2014; Morello et al., 2016). In addition, CAR T cells have to overcome several hurdles to infiltrate the tumor and remain activated (Figure 1), as discussed below.

Trafficking of CAR T Cells to the Tumor—The recognition that the efficacy of CPB relies on the reactivation of an existing antitumor T-cell response underscores the importance of achieving high numbers of tumor-infiltrating T cells. With this goal as a priority, our research group employed regional administration of CAR T cells to augment T-cell infiltration into the tumor (Figure 2A), which promoted early intratumoral accumulation and enhanced antitumoral efficacy in a clinically relevant model of mesothelioma (Adusumilli et al., 2014). Furthermore, we demonstrated that regional administration resulted in systemic circulation of CAR T cells that retain their functional activity, establishing a long-term systemic presence capable of eradicating metastatic tumor sites. In our phase I clinical trial in patients with pleural mesothelioma and breast and lung primaries metastatic to the pleura (NCT02414269), we have demonstrated that intrapleural administration results in circulating CAR T cells detectable in systemic circulation up to several months following a single infusion. This ability of locally administered T cells to circulate and persist in the periphery may open new avenues of treatment for other metastatic cancers with accessible tumor sites, which can translate into sustained responses in more inaccessible tumors. Examples include intraperitoneal administration of MUC16-targeted CAR T cells in an animal model of ovarian peritoneal carcinomatosis (Koneru et al., 2015), hepatic vascular infusion of CEA-targeting CARs in a model of CEA-expressing colorectal liver metastases (Burga et al., 2015), and intracranial administration targeting IL13α2-expressing glioblastoma (Brown et

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al., 2012). Local administration of CAR T cells is being tested in clinical trials for the treatment of other solid tumors (mesothelioma [NCT02414269], glioma [NCT02208362], liver cancer and metastases [NCT03130712], and head and neck cancers [NCT01818323]).

Since some tumors (such as retroperitoneal tumors including sarcoma) may not be as amenable to regional administration and because local administration may still not be sufficient to penetrate the dense solid tumor stromal network or effectively perfuse the distorted and immature blood vessels that constitute many solid tumors, other strategies may be necessary to further augment T-cell infiltration into primary and metastatic tumor sites. Promoting a cellular infiltrate can be achieved by employing traditional cancer treatments such as radiotherapy and chemotherapy to induce a local inflammatory state that increases cellular presence within the tumor bed (Ariyan et al., 2018; DeSelm et al., 2018). Achieving the correct chemokine receptor and ligand pairing to effectively target homing to tumor sites is another strategy to achieve infiltration, either by the use of virus to deliver chemokine ligand expression to tumor (Moon et al., 2018) or, conversely, by expressing a chemokine receptor on CAR T cells that is complementary to the most abundant chemokine ligand within the tumor environment (Moon et al., 2011). Other groups have employed strategies targeting the stromal matrix by administering stromal degradation enzymes such as heparanase (Caruana et al., 2015) to enhance infiltration or employing FAP (fibroblast activating protein)–directed CARs to directly target the dense stromal matrix that serves as a barrier to infiltration in many solid tumors (Schuberth et al., 2013; Wang et al., 2014). Furthermore, using T-cell receptors with the highest affinity for target antigen can achieve infiltration and efficacy in tumors known for their high-density stromal matrix (pancreatic ductal adenocarcinoma) (Stromnes et al., 2015), and anti-angiogenic therapy targeting VEGF, D276, or endothelin B receptor may normalize tumor vasculature and promote T cell delivery (Daenen et al., 2009; Yang et al., 2018). Furthermore, we must recognize that metastatic sites are often different from the primary tumor with respect to ease of infiltration, sensitivity to therapy, and degree of immunosuppression. Attention to efficacy at both primary tumor and metastases is therefore needed to optimize long-term oncologic outcomes.

Limited Functional Persistence of CAR T Cells in the Tumor Microenvironment—Even upon achieving an adequate T-cell infiltrate, we and many others have demonstrated the relevance of an additional profound hurdle to effective therapy—overcoming the immunosuppressive tumor environment. An understanding of this profoundly T-cell–inhibiting environment justifies the rational use of CAR T-cell and CPB combination therapy. Immunologically, large tumor burdens are similar to chronic viral infections in the large antigenic burden stressing T-cell function, requiring persistent function upon repeated antigen stimulation in an immunosuppressive environment to achieve successful elimination. Chronic viral infection models were the first to characterize endogenous T-cell exhaustion resulting from repeated antigen stimulation, which is characterized by loss of cytokine secretion and lytic function with simultaneous expression of inhibitory receptors and exhaustion-related transcription factors. Chronic antigen stimulation by tumor can similarly lead to CAR T-cell exhaustion, which we have characterized as an environment replete with CAR T cells that are nonetheless hypofunctional (Cherkassky et al., 2016). Therefore, T-cell

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persistence alone may not reflect the more relevant need of functional persistence for solid tumor regression and costimulation alone still does not prevent long-term exhaustion resulting from the expression of inhibitory ligands.

Evidence for Checkpoint-Mediated CAR T-Cell Inhibition—Overexpression of PD-L1 and PD-L2 on tumor cells directly inhibits CAR T-cell effector functions, especially after chronic antigen stimulation (Cherkassky et al., 2016). This effect is seen regardless of the CAR costimulation domain. Both 4–1BB and CD28 CAR T cells appear to be sensitive to PD-L1–mediated inhibition, even though 4–1BB CAR T cells express lower levels of PD-1, compared with CD28 (Cherkassky et al., 2016) and other markers of exhaustion, such as TIM-3 and LAG-3 (Long et al., 2015), following chronic antigen stimulation.

Combination Therapy

T cells naturally undergo activation-induced upregulation of coinhibitory pathways, which can limit antitumor immune responses when the T cells bind their complementary ligands. Tumor cells upregulate the expression of coinhibitory ligands, such as PD-L1, following exposure to T cell–secreted Th1 cytokines. Expression of these ligands can then limit the antitumor response in favor of tumor survival. Strategies that combine adoptive T-cell therapy with CPB hold promise to achieve functional persistence of CAR T cells (Figure 1).

Methods of Combination: Cell Extrinsic vs. Cell Intrinsic

Cell-Extrinsic Strategies (Figure 2B).: In a breast cancer model, the use of HER2-targeted CAR T cells upregulated PD-1 *in vitro* after incubation with target cells. PD-1 blockade enhanced *in vitro* T-cell proliferation, IFN-γ production, granzyme B expression, and *in vivo* activity of CAR T cells (John et al., 2013). A study of an anti-GD2 CAR showed that the use of pembrolizumab improved T-cell function and survival following repeated antigen stimulation against PD-L1–positive tumor cells (Gargett et al., 2016). Our group demonstrated that PD-1 CPB antibodies can rescue the effector function of exhausted CAR T cells (Cherkassky et al., 2016). However, tumor relapse was observed following cessation of treatment, which suggests that the efficacy of the PD-1 antibody relies on repeated antibody administration.

PD-L1 CPB was also effective in enhancing CAR T-cell therapy by blocking PD-L1 expression on MDSCs. The combined use of CAR T cells and PD-L1 antibody with MDSC-depleting antibodies (anti-Gr1 or anti–GM-CSF) further enhanced the efficacy of the therapy (Burga et al., 2015) and highlights the immunosuppressive role that PD-L1–positive MDSCs can play in CAR T-cell therapy. To avoid the possible toxicities associated with systemic administration of CPB, Tanoue et al. combined anti-HER2 CAR T-cell therapy with an oncolytic adenovirus expressing a PD-L1–blocking mini-antibody that allows for local production of PDL1 antibodies within the tumor microenvironment (Tanoue et al., 2017). In a subcutaneous mouse model of prostate cancer, this PD-L1 oncolytic strategy was more efficient at reinvigorating CAR T cells than systemic injection of PD-L1 antibodies.

Two phase I clinical trials have investigated the efficacy of combining CAR T cells and PD-1/PD-L1 CPB (Chong et al., 2017; Heczey et al., 2017). In the study by Chong et al., 1

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patient with refractory diffuse large B-cell lymphoma received cyclophosphamide lymphodepleting chemotherapy followed by autologous CD19 CAR T cells. Following treatment, the tumor continued to progress and strongly expressed PD-L1. Beginning on day 26 after T-cell infusion, pembrolizumab was administered every 3 weeks (2 mg/kg), after which the percentage of T cells expressing PD-1/EOMES decreased, expansion of CART19 cells and TCRβ T-cell clones was observed, and regression of multiple lesions occurred by day 45 (Chong et al., 2017).

The phase I study reported by Heczey et al. enrolled 11 patients with relapsed or refractory neuroblastoma. The patients were treated with CAR T cells alone (n=4), CAR T cells plus cyclophosphamide and fludarabine (Cy/Flu; n=4), or CAR T cells, Cy/Flu, and pembrolizumab n=3). All infusions were well tolerated, and no dose-limiting toxicities were observed. Cy/Flu lymphodepletion significantly increased levels of IL-15 and CAR T-cell expansion, but the addition of pembrolizumab did not further enhance this accumulation or persistence. Although a complete response was observed in 1 patient in the pembrolizumab-treated cohort, no conclusions can be drawn from this small group of patients (Heczey et al., 2017).

Overall, the clinical trials exploring CAR T-cell therapy to date have yielded encouraging results, with several studies ongoing (Table 1). The use of PD-1/PD-L1 described above is FDA approved, which can simplify logistics; however, several factors can limit this approach, including the short half-life of PD-1 antibodies, requiring multiple administrations; variable tumor penetration and pharmacokinetics inherent to antibodies; and the risk of systemic nontargeted therapy and its associated toxicity. T cell–intrinsic strategies centered on genetically modifying CAR T cells to induce sustained resistance to PD-1 signaling can address some of these limitations.

Cell-Intrinsic Strategies (Figure 2C).: A cell-intrinsic model could eliminate the need for separate dosing (Table 2). It would also equip the patient with a self-replicating and constitutive biological treatment that could continuously provide CPB. One cell-intrinsic strategy that has been investigated is the use of a bicistronic lentiviral vector to induce expression of an anticarbonic anhydrase IX CAR and anti–PD-L1 scFv antibodies in primary human T cells. This promoted accumulation of anti–PD-L1 antibodies in the tumor microenvironment and blocked PD-1 signaling. The CAR T cells had decreased expression of inhibitory receptors, such as PD-1, TIM-3, and LAG-3, and enhanced effector activity in an orthotopic model of human renal cell carcinoma (Suarez et al., 2016). Similarly, CART19 cells engineered to locally secrete an anti–PD-1–blocking scFv were more efficacious *in vivo* (Li et al., 2017; Rafiq et al., 2018b).

Our group developed a dominant negative receptor of PD-1 that consists of the extracellular domain of PD-1 without the intracellular signaling domain. When expressed in combination with an anti-mesothelin CAR that harbors CD28 and CD3ζ domains, PD-1 dominant negative receptor competes for PD-L1/PD-L2 with the endogenous PD-1 receptor and significantly enhances CAR T-cell antitumoral activity in mesothelioma and lung cancer mouse models (Cherkassky et al., 2016). In the “switch receptor” approach, the extracellular domain of PD-1 is fused with the transmembrane and intracellular domains of CD28 to

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create an artificial receptor that, upon PD-L1 recognition, provides costimulation. When co-expressed with an anti-mesothelin CAR T cell, PD-1:CD28 chimera enhanced CAR T-cell effector functions and persistence *in vivo*, thus leading to durable efficacy and tumor eradication of established tumors (Liu et al., 2016). More recently, we compared intrinsic and extrinsic strategies and found similar efficacy between the dominant negative receptor and multiple doses of anti–PD-1 antibody; these findings led to the development of our upcoming clinical trial investigating a mesothelin-targeting CAR featuring a PD-1 dominant negative receptor (Dozier et al., 2018).

Genome-editing approaches are also being used to induce resistance to PD-1 signaling, taking advantage of the CRISPR/Cas9 (Rupp et al., 2017) and TALEN (Gautron et al., 2017) systems. PD-1–knockout CAR T cells demonstrate improved cytotoxic activity *in vitro* and tumor clearance *in vivo*. A similar approach combined multiplex genome engineering of three loci (TCR, β−2 microglobulin, and PD-1) and CAR expression to generate universal CAR T cells resistant to PD-1 signaling and with enhanced effector function *in vivo* (Ren et al., 2017). There are some limitations specific to the gene-editing approach: In a mouse model of chronic viral infection, complete absence of PD-1 signaling may counterintuitively produce terminal differentiated exhausted T cells (Odorizzi et al., 2015), and so it may be best for T cells to upregulate some amount of PD-1 that is then blocked by cell-intrinsic or cell-extrinsic CPB. Also, off-target cleavage must be identified and limited (Kleinstiver et al., 2016; Slaymaker et al., 2016; Tsai et al., 2015), and added PD-1 gene editing, in addition to CAR transgene, may produce genotoxicity, with negative effects on T-cell proliferation and effector functions.

CAR T cells have been modified to secrete PD-1–blocking scFvs, which may avoid the need to administer two treatments separately but would achieve similar levels of each *in vivo* (Chong et al., 2017; Rafiq et al., 2018a). The safety and effectiveness of PD-1 cell-intrinsic therapy using CAR T cells that secrete PD-1 antibodies are being investigated for the treatment of EGFR-positive advanced solid malignancies, including lung cancer, gastric cancer, and liver cancer (NCT02862028). Similarly, PD-1–knockout genome-editing strategies to knock out the PD-1 receptor in adoptively transferred T cells are also being investigated (NCT03081715, NCT02867332, NCT02867345, NCT02793856, NCT03044743).

The cell-intrinsic approach is attractive for its simplicity in drug administration: the repeated administrations required for persistence of CPB effect when the antibody/cell-extrinsic strategy is used can be replaced by a single administration of CPB-enabled T cells bolstered by the long-term transgene expression achieved by viral vector integration and reinforced by the long-lasting persistence of T-cell memory. However, when considering the limitations, it is unclear which might provide the most efficacious and least toxic of the two approaches. The cell-intrinsic approach may limit recruitment of the endogenous T-cell response, which may provide the polyclonal antitumor immunity most resistant to antigen escape and optimal for what are often highly heterogeneous tumors. Whereas the cell-intrinsic strategy may be better in limiting autoimmunity to off-target epitopes, in the end, it may be that antibody-mediated CPB should be used in tandem with the cell-intrinsic strategy to both prevent CAR

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T-cell exhaustion and reactivate what is an initially quiescent endogenous immune response but which is then recruited via epitope spreading.

Current Trials of Combination Therapy

At present, several trials are exploring combination therapy with CPB and CAR T cells. These trials vary in terms of agents used, dosing, and schedules. Models to explore these elements in the preclinical setting have yet to be developed.

Our group investigated the safety of anti–PD-1 CPB (off trial) following treatment with mesothelin CAR T cells (on trial) in patients with malignant pleural mesothelioma (Adusumilli et al., Cancer Res 2019; 79(13 Suppl): abstract CT036). In a subcohort of 11 patients with mesothelioma who received cyclophosphamide preconditioning followed by a single dose of CAR T cells and subsequent anti–PD-1 agent (at least 3 doses) with a minimum of 3 months of follow-up, 8 patients showed response (response rate, 72%), including complete metabolic responses in 2 patients. The observation of a complete metabolic response in tumors of which only 40% expressed the targeted antigen raises the intriguing possibility of combination therapy–induced neoantigen response and epitope spreading (Figure 1)—possibilities that have not yet been investigated.

Future Directions of Combination Therapy

Combination immunotherapy with CAR T cells and CPB is poised to be the next frontier in immunotherapy as it addresses the two elements necessary for a robust immune attack: the presence of immune cells and functional persistence. CAR T cells can provide an infiltrate, and PD-1/PD-L1 blockade can ensure sustained persistence and function. In this section, we recognize that even this combination may not be sufficient to attain the T-cell infiltration and effector function necessary to optimally combat solid tumors. In turn, we describe some additional barriers to effective combination therapy. Also, we address the innate problem that arises whenever a targeted therapy's efficacy is enhanced—that of toxicity to normal tissues expressing the same target as tumor, or on-target off-tumor toxicity.

Enhancing Functional Persistence and Other Obstacles.—Other inhibitory receptors, such as TIM-3 and LAG-3, are upregulated in T cells after chronic antigen stimulation (Schildberg et al., 2016) and PD-1 blockade (Huang et al., 2017; Koyama et al., 2016), which suggests that additional combinations of blocking antibodies may optimize function. In order to offset the immunosuppression imposed by multiple inhibitory signaling pathways (Huang et al., 2017), cell-extrinsic (infusions of multiple checkpoint targeting antibodies) or cell-intrinsic (delivery of multicistronic elements targeting multiple pathways) strategies may be necessary to offset redundant inhibitory signaling. Other newly described inhibitory receptors, such as B7-H3, VISTA, and B7S1, provide alternative pathways to modulate T-cell responses (Ni and Dong, 2017), and recruiting other elements of the immune system—both innate and adaptive—has great potential to induce a more robust antitumor response (Moynihan et al., 2016).

Still, other barriers to effective therapy exist, such as antigen heterogeneity—an obstacle that must be addressed to successfully target solid tumors often characterized by heterogenous

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antigen expression. Overcoming this obstacle begins with the selection of a target antigen that is as homogenously and highly expressed as possible on tumor tissue and can be further countered by infusing CAR T cells with the ability to target multiple antigens (Majzner and Mackall, 2018) or by adding radiation therapy that results in TRAIL-mediated death or even targeting of antigen negative tumour cells (DeSelm et al., 2018).

The requirement for a functionally persistent effector response, the principle underlying combination CPB and T-cell therapy, will guide the next steps in the preclinical and clinical settings. More-accurate and clinically relevant models are needed for investigation of CAR T-cell efficacy. To address the current shortcomings in the currently available models, our lab has investigated CAR T-cell persistence and function in malignant pleural effusions (Tano et al., 2018).

Limiting On-Target Off-Tumor Toxicity.—Most CARs targeting solid tumors are aimed at antigens shared by normal tissues, carrying the risk of on-target off-tumor toxicity (Lamers et al., 2006; Morgan et al., 2010). And as with all functional enhancements to a targeted therapy, combination CPB enhances efficacy but risks increasing toxicity. In addition to the methods already in clinical practice such as use of steroids to reduce the number of CAR T cells, incorporating suicide genes (iCaspase-9, epidermal growth factor receptor mutation) can be used to mediate T-cell elimination upon detection of side effects but may squander T-cell efficacy. An optimal solid-tumor antigen target is one whose expression is restricted to expendable cells or, better yet, tumor cells only, such as the mutated form of EGFR expressed on glioblastoma multiforme tumors (Johnson et al., 2015; Sampson et al., 2014), or neoantigen peptide: MHC complexes, which have yet to be developed but theoretically could selectively target unique tumor mutations. Other carefully selected antigens are those occurring at high densities on tumor but very low levels in normal tissues, an example being our target, mesothelin—chosen for its overexpression in mesothelioma, triple-negative breast cancer, and pancreatic cancer, and with only low levels of expression by the pleural and pericardial lining. With this selection strategy, we have demonstrated anti-tumor responses but have not as of yet experienced any on-target off-tumor toxicities in our ongoing phase I clinical trial. As scientists and clinicians are faced with a limited availability of efficiently targetable antigens, other strategies can be used to improve the specificity and safety of CAR T cells, even while targeting tumor-associated antigens that are shared by normal cells, such as a trans-signaling approach that splits CD3ζ signaling from costimulatory signaling (Kloss et al., 2013; Wilkie et al., 2012), engineering a requirement for dual antigen activation (Roybal et al., 2016), or activating inhibitory signaling upon binding of a normal tissue antigen (Fedorov et al., 2013).

As we take the next steps in combining CAR T-cell therapy and CPB agents, the current clinical trials will provide the best insights into the future of this approach for the treatment of solid tumors and will shine light on strategies to develop new methods of studying combination therapy and providing these groundbreaking treatments to our patients.

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Declarations of Interest

P.S.A. has received research funding from ATARA Biotherapeutics and OSE Immunotherapies and has patents on mesothelin chimeric antigen receptor and PD-1 dominant negative receptor. All other authors have no interests to declare.

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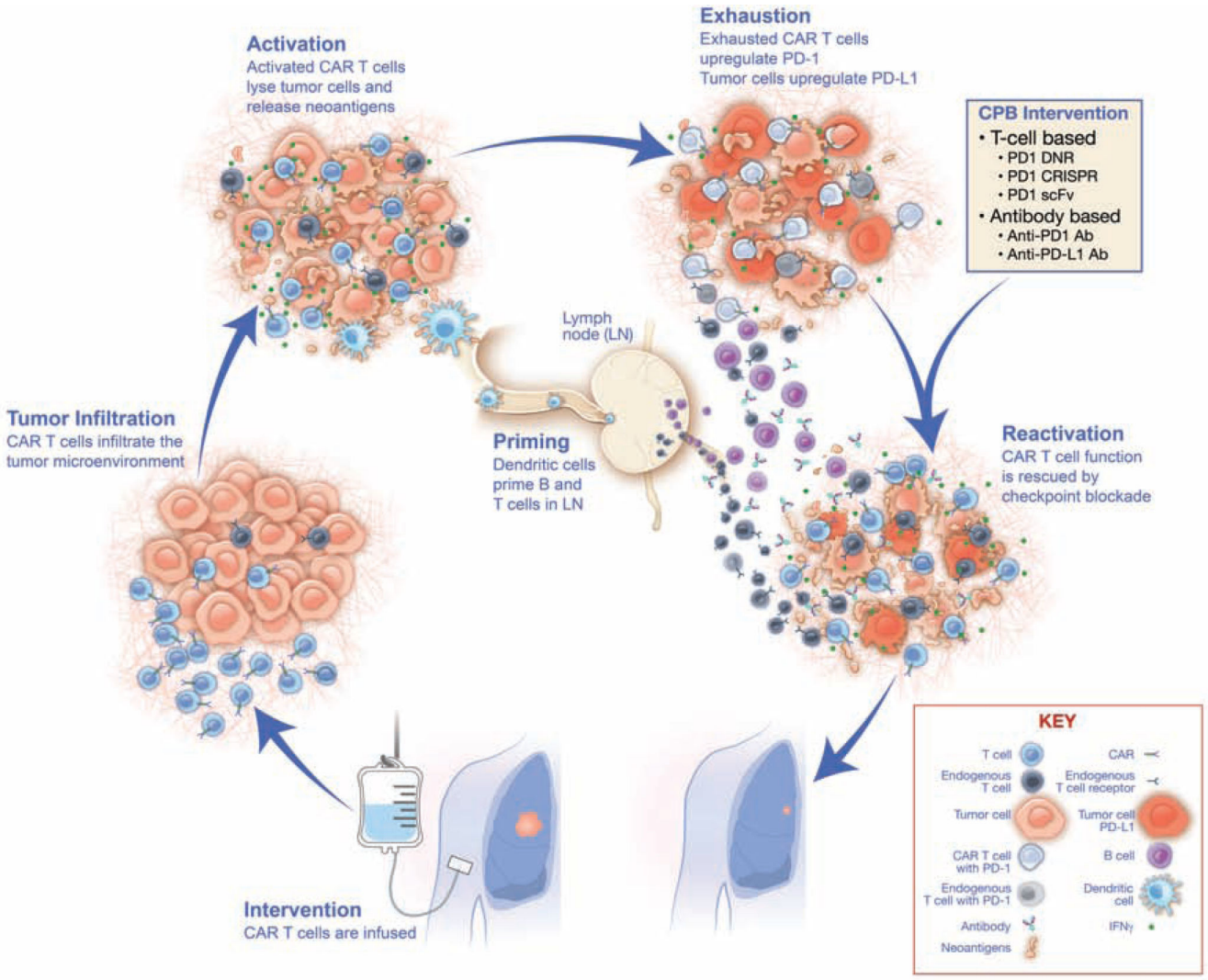

Figure 1. Mechanisms of rescue of CAR T-cell exhaustion with checkpoint blockade

Intervention: Chimeric antigen receptor (CAR) T cells are administered via intravenous infusion. Tumor Infiltration: CAR T cells infiltrate the tumor microenvironment. Activation: Following major histocompatibility complex (MHC)-independent recognition of surface antigen, CAR T cells are activated and begin releasing cytokines (e.g., IFN-γ) and lyse tumor cells. Priming: Released neoantigens from lysed cancer cells are recognized by dendritic cells and carried to the lymph nodes, where endogenous B and T cells are primed, generating additional adaptive responses. Exhaustion: In response to IFN-γ, tumor cells upregulate programmed death-ligand 1 (PD-L1), which interacts with programmed cell death protein 1 (PD-1) on the surface of exhausted CAR T cells. Checkpoint blockade (CPB) Intervention and Reactivation: Upon administration of CPB, CAR T-cell and endogenous T-cell function is rescued to enable killing of tumor cells. Ab, antibody; CRISPR, clustered regularly interspaced short palindromic repeats; DNR, dominant negative receptor; scFv, single chain variable fragment.

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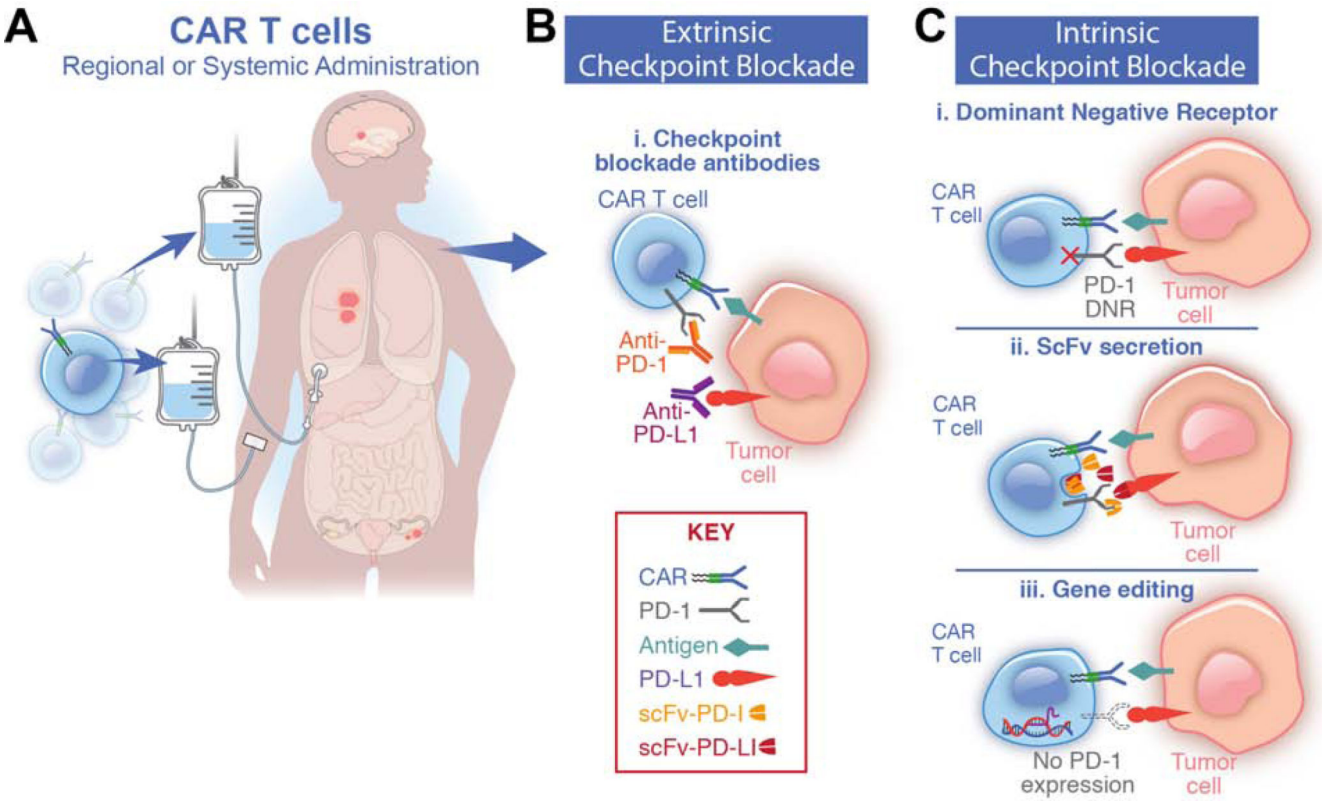

Figure 2. Strategies to combine CAR T cells and checkpoint blockade

(**A**) Chimeric antigen receptor (CAR) T cells are delivered either systemically via intravenous infusion or by regional administration, which enhances primary tumor elimination. Both methods establish systemic immunosurveillance. Combination therapy with programmed death-ligand 1 checkpoint blockade (PD-1 CPB) is provided using either cell-extrinsic or cell-intrinsic strategies.

(**B**) Extrinsic CPB relies on programmed cell death protein 1 (PD-1) receptor/ligand blocking antibodies. A potential advantage of cell-extrinsic CPB is additional enhancement of endogenous antitumor T-cell immunity.

(**C**) Cell-intrinsic PD-1 CPB uses genetic engineering to express nucleic acids or proteins that interfere with PD-1/PD-L1 signaling. A possible advantage to cell-intrinsic CPB is efficacy with a single administration lasting the lifetime of the T cell. (**i**) PD-1 dominant negative receptor (DNR) is constructed lacking the intracellular signaling domain. It acts as a decoy receptor that competes with the native PD-1 receptor, thereby decreasing inhibitory signaling via the native wild-type receptor. (**ii**) CAR T cells can be employed to produce PD-1/PD-L1 blocking single chain variable fragments (scFvs), providing regional antibody blockade of PD-1 receptor/ligand binding. (**iii**) Gene-editing techniques such as CRISPR/Cas9 or TALEN eliminate PD-1 expression by editing the *PDCD1* gene locus.

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Table 1.

Clinical Trials Exploring Combination Therapy with CAR T Cells and Checkpoint Blockade

Trial	Launch	Phase	Center(s)	CPB Agent	CAR Target/Design	Cancer Diagnosis
NCT00586391	2009	I	Baylor	Ipilimumab	CD19/CD19CAR-28-zeta T cells	B-cell lymphoma, chronic lymphocytic leukemia, acute lymphocytic leukemia
NCT01822652	2013	I	Baylor	Pembrolizumab	GD2/iC9-GD2-CD28–OX40 (iC9-GD2) T cells	Neuroblastoma
NCT02650999	2016	I/II	University of Pennsylvania	Pembrolizumab	Anti-CD19 CARs	CD19+ diffuse large B-cell lymphoma, follicular lymphoma, mantle cell lymphoma
NCT02706405	2016	I	Fred Hutchinson	Durvalumab	Autologous anti-CD 19CAR-4-1BB-CD3ζ-EGFRt-expressing $CD4^+/CD8^+$ central memory T-lymphocytes JCAR014	Diffuse large B-cell lymphoma
NCT02926833	2016	I/II	City of Hope, Stanford, Moffitt, Dana Farber, MD Anderson	Atezolizumab	CD19/KTE-C19	Diffuse large B-cell lymphoma
NCT03310619	2017	I/II	City of Hope, Northwestern University, Massachusetts General, University of Nebraska, University of Pennsylvania, MD Anderson	Durvalumab	JCAR017	Lymphoma, non-Hodgkin lymphoma, diffuse large B-cell lymphoma, follicular lymphoma
NCT03726515	2018	I	University of Pennsylvania	Pembrolizumab	CART-EGFRvIII T cells	Glioblastoma

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Table 2.

Comparison of Characteristics of Therapy with PD-1 DNR CAR T Cells vs. Checkpoint Blockade Agents with CAR T cells

Characteristic	PD-1 DNR CAR T cells	CPB Agents with CAR T cells
Mechanism	Cell-intrinsic	Cell-extrinsic
Potential doses	Single	Multiple doses of CPB
Targeted therapy	Yes	No
Potential toxicity	Localized to tumor	Systemic
Limits to tumor penetration	Unlikely	Limits of antibody penetration
Antigen responsiveness	Multiple	Multiple