Immunology and Cell Biology (2017) 95, 356–363

www.nature.com/icb

REVIEW

CAR T-cell therapy of solid tumors

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The potential for immunotherapy as a treatment option for cancer is clear from remarkable responses of some leukemia patients to adoptive cell transfer using autologous T cells genetically modified to express chimeric antigen receptors (CARs). However, the vast majority of cancers, in particular the more common solid cancers, such as those of the breast, colon and lung, fail to respond significantly to infusions of CAR T cells. Solid cancers present some formidable barriers to adoptive cell transfer, including suppression of T-cell function and inhibition of T-cell localization. In this review, we discuss the current state of CAR T-cell therapy in solid cancers, the variety of concepts being investigated to overcome these barriers as well as approaches aimed at increasing the specificity and safety of adoptive cell transfer.
Immunology and Cell Biology (2017) **95,** 356–363; doi:10.1038/icb.2016.128

INTRODUCTION

The generation of chimeric antigen receptors (CARs) has revolutionized T cell-based immunotherapy for the treatment of some cancers. Developed from the concept of adoptive immunotherapy using tumor infiltrating lymphocytes (TILs), whose T-cell receptors (TCR) recognize tumor-associated antigens (TAA), the genetic modification of peripherally derived T lymphocytes with CARs has produced outstanding results in the treatment of hematological malignancies.[1,2] TCR-modified cells have shown potential in immunotherapy but they have a restricted target-antigen repertoire, requiring major histocompatibility complex (MHC) presentation of immune processed antigens. On the other hand, CARs recognize a range of antigens in a non-MHC context, broadening the clinical application compared with TCR-modified cells. CARs are composed of the antigen-specific region of a single chain variable fragment (scFv) from an antibody fused to the signaling chains of the TCR complex. More specifically, the basic structure of a CAR is comprised of an extracellular scFv region connected to a hinge region, which allows for flexibility. This can then be further linked to a transmembrane region, and most importantly to intracellular signaling moieties, which condition the function, persistence and overall efficacy of the CAR itself.

The ability to recognize antigens in a MHC independent manner is advantageous in that there are no human leukocyte antigen (HLA) compatibility issues between donors and recipients. Although CARs are able to recognize targets such as glycolipids and cell-surface proteins, one disadvantage is that they are rarely able to recognize intracellular processed TCR antigens that can be targeted by engineered TCRs, such as MAGE and NY-ESO-1.[3] First-generation CARs consisted of a singular intracellular signaling chain of CD3ζ. However, as interaction of an MHC-antigen complex in a normal TCR context results in co-stimulation of the T cell, further improvements in the CAR design have included the addition of a secondary and tertiary intracellular signaling chains. These subsequent generations of CARs, with the addition of one or two co-stimulatory domains (in 2nd and 3rd generation CARs, respectively) have shown enhanced activity, persistence and efficacy. A large degree of both inter- and intra-generational variety exists between 2nd and 3rd generation CARs, with a range of co-stimulatory domains being tested (CD28, 41BB, OX40, CD27, ICOS, DAP10, LAT). To add to the complexity, the attributes of each co-stimulatory domain differ in their ability to confer cytokine secretion, cytotoxicity, proliferation, memory development and even metabolism to the modified-CAR T cell.[4,5]

The success of CAR T cells in treating hematological malignancies is impressive, particularly in infants, achieving up to 90% clinical response rates in acute lymphoblastic leukemia (ALL).[6] This has resulted in a large expansion of clinical trials of CAR directed against multiple hematological antigens such as CD19, CD20 and CD22 (reviewed in Holzinger *et al.*[7]). Nevertheless, the clinical efficacy of CAR T cells in solid tumors has been much less rewarding, with multiple cases of toxic side effects and/or a lack of therapeutic response.[8–11] At present, there are 81 active or planned clinical trials using CAR T cells against hematological cancers and only 51 trials for solid tumors (Table 1).

The clinical efficacy of CAR T cells in hematological malignancies is rarely achieved in solid tumors, and the factors necessary for improving its efficacy are currently being determined. Many differences exist between hematological malignancies and solid

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Received 14 December 2016; revised 18 December 2016; accepted 19 December 2016; accepted article preview online 22 December 2016; advance online publication, 17 January 2017

Table 1 List of clinical trials involving CAR T cells directed against solid cancers

Antigen	*Type of cancer*	*Pre-conditioning regimen*	*Additional information*	*Phase*	*ID*	*Cited in (PMID)*
CD133	Liver, brain, breast, AML, ALL	Unknown	Comparing CD3ζ to CD3ζ-CD137	1	NCT02541370	27009301
CD138	Multiple myeloma	Unknown	CD3ζ and CD3ζ-CD137	1 and 2	NCT01886976	26574053
CD171	Neuroblastoma, ganglioneuroblastoma	Chemotherapy	2nd and 3rd generation CARs	1	NCT02311621	26451319
CD70	Renal and other CD70 expressing cancer	C, F	IL-2 at 720 000 IU kg^{-1}	1 and 2	NCT02830724	27803044
CEA	Lung, colorectal, gastric, pancreatic	Unknown	Unknown	1	NCT02349724	27000958 27550819 26574053
CEA	Colorectal adenocarcinoma	Unknown	Minor responses in 2 of 7 patients	1	NCT00004178	23880905
EGFR	Lung, colorectal, ovary, pancreatic	Unknown	CD3ζ-CD137 CAR	1 and 2	NCT01869166	26968708 26574053
EGFR	Advanced glioma	C, F, IL-2	Lentiviral vector, +IL-2	1	NCT02331693	27000958 26574053
EGFRvIII	Glioblastoma	R	Lentiviral vector, CD3ζ-CD137, +TMZ	1	NCT02209376	25696001 25829274
EGFRvIII	Glioblastoma	R	TMZ	1	NCT02664363	
EGFRvIII	Malignant glioma, glioblastoma	C, F	IL-2, CD28-CD137-CD3ζ	1 and 2	NCT01454596	22780919
EPCAM	Liver neoplasms+stomach neoplasms	Lymphodepletion		1 and 2	NCT02725125	
EphA2	Malignant glioma	Unknown		1 and 2	NCT02575261	27009301
FAP	Malignant pleural mesothelioma	Palliative chemotherapy	1×10^6 CAR T cells into pleural effusion	1	NCT01722149	23259649 26574053
GD2	Neuroblastoma	C, F	3rd generation CAR. iCASP9 gene. Autologous NKs	1	NCT02439788	26390167
GD2	Sarcoma, osteosarcoma, neuroblastoma, melanoma	C, AP1903		1	NCT02107963	26425336 26574053
GD2	Neuroblastoma	C, F	4th generation lentivirus	2	NCT02765243	
GD2	Neuroblastoma	C, F, P	iC9-GD2-CD28-OX40	1	NCT01822652	26574053
GD2	Relapsed/refractory Neuroblastoma	C, F	1RG-CART	1	NCT02761915	
GD2	Osteosarcoma	Unknown	iC9-GD2-CAR-VZV-CTls plus vaccine for VZV	1	NCT01953900	26110321 26574053
GD2	Metastatic melanoma	Vemurafenib concurrently	Patients with BRAF V600E+ or V600K+ tumors	1	ACTRN12613000 198729	
GD2	Neuroblastoma	Submyeloablative	Completed. Viral-specific CTLs used.	1	NCT01460901	24333408 25734008
GD2	Neuroblastoma	No lymphodepletion	EBV-specific CTLs	1	NCT00085930	21984804
GPC3	HCC			1 and 2	NCT02723942	27669301
GPC3	HCC	C, F		1	NCT02905188	
GPC3	Lung squamous cell carcinoma	C, F		1	NCT02876978	
GPC3	HCC	C	41BB included	1 and 2	NCT02715362	
GPC3	HCC	Unknown		1	NCT02395250	27000958
HER2	Glioblastoma	Unknown	Up to 1×10^8 CAR T intratumoral	1	NCT02442297	27411023
HER2	Breast cancer	Lymphodepletion	CD28-CD3ζ	1 and 2	NCT02547961	27009301
HER2	Glioblastoma multiforme	Unknown	CMV T cells, CD28-CD3ζ	1	NCT01109095	26574053
HER2	Her+ cancers	Unknown	TGFβ-resistant HER2/EBV-CTLs	1	NCT00889954	25425467 26574053
HER2	Breast, ovarian, lung, pancreatic	Unknown		1 and 2	NCT02713984	
HER2	Breast, gastric, HCC, endometrial, refractory to chemotherapy and Her2 antibody	Unknown		1 and 2	NCT01935843	25050207 26968709
HER2	Advanced sarcoma	C, F	Up to 1×10^8 CAR T cells, repeat infusions	1	NCT00902044	
IL13Ra2	Glioma		CD137-CD3ζ, truncated CD19 marker	1	NCT02208362	
Lewis-Y	AML	F	Completed, two minor responses	1	NCT01716364	23831595
Mesothelin	Pancreatic adenocarcinoma, ovarian cancer, malignant epithelial pleural mesothelioma	C	CD137-CD3ζ CAR	1	NCT02159716	27000958 26574053
Mesothelin	Pancreatic cancer	C	Transcatheter arterial infusion CD137 included in CAR	1	NCT02706782	
Mesothelin	Malignant mesothelioma	Unknown	CD137-CD3ζ CAR	1	NCT02580747	27550819
Mesothelin	Metastatic Her2- breast	C	+ iCASP9	1	NCT02792114	
MG7	Liver metastasis	C	Intratumoral delivery	1 and 2	NCT02862704	

Table 1 (Continued)

Antigen	*Type of cancer*	*Pre-conditioning regimen*	*Additional information*	*Phase*	*ID*	*Cited in (PMID)*
MUC1	HCC, NSCLC, pancreatic carcinoma	Unknown	CAR NK cells	1 and 2	NCT02839954	
MUC1	Glioma, colorectal carcinoma, gastric carcinoma	Unknown		1 and 2	NCT02617134	27550819
MUC1	HCC, NSCLC, pancreatic	Unknown		1 and 2	NCT02587689	27550819
PSCA	Non-resectable pancreatic cancer	AP1903		1	NCT02744287	
PSMA	Prostate cancer	C, F	Completed, two partial responses	1	NCT01929239	27324746
T4	SCCHN	None	Locoregional disease	1	NCT01818323	26738472 24099518
ALPHA FR	Metastatic ovarian	C, F	Completed, no responses, anti-CAR responses	1	NCT00019136	17062687 25505964
CAIX	RCC	None	Completed	1	DDHK97-29	25505964
CEA	Liver metastases	Unknown	Completed, hepatic artery delivery, some CEA decrease	1	NCT01373047	25850950
GD2	Neuroblastoma	Lymphodepletion	Completed. Viral-specific CTLs used	1	NCT01460901	25734008

Abbreviations: ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; AP1903, rimiducid; C, cyclophosphamide; CAIX, carboxy-anhydrase-IX; CEA, carcinoembryonic antigen; CMV, cytomegalovirus; CTL, cytotoxic T lymphocyte; EBV, Epstein–Barr virus; EGFR, epidermal growth factor receptor; EPCAM, epithelial cell adhesion molecule; F, fludarabine; FAP, fibroblast activation protein; FR, folate receptor; GPC3, glypican-3; HCC, hepatocellular carcinoma; HER2, human epidermal growth factor receptor 2; iCASP9, inducible caspase-9; MG7, glycosylated protein of CEA; MM, multiple myeloma; MUC1, mucin1; NK, natural killer cell; NSCLC, non-small cell lung cancer; P, pembrolizumab (anti-PD-1); PSCA, prostate stem cell antigen; PSMA, prostate-specific membrane antigen; R, radiation; RCC, renal cell carcinoma; SSCHN, squamous cell cancer of the head and neck; TGFβ, transformation growth factor beta; TMZ, temozolomide; Vemurafenib, BRAF inhibitor; VZV, Varicella zoster virus.
Trial information can be located using trial ID at https://clinicaltrials.gov.

tumors; and while each aspect is being investigated individually, it seems more likely that a combination of modifications will likely be necessary to optimize CAR therapies for the latter indication. Hematological malignancies are often disseminated, and as such are lacking many of the physical immunosuppressive factors that hamper adoptively transferred cells from reaching solid tumors. Furthermore, target antigens that are present on hematological cancers are often homogenous and expressed in a majority if not all of the tumor population. In contrast, target antigens on solid tumors are often heterogenous, differing not just within one tumor but also between both primary and metastatic tumors.

CAR T-cell therapy for solid tumors therefore faces multiple hurdles, starting from the very first step of administration wherein CAR T cells must encounter the correct chemotactic signals to traffic to the tumor in sufficient numbers. Abnormal vasculature impedes efficient infiltration, and physical barriers from both surrounding stroma and infiltrating pro-tumor immune cells prevent adequate penetration. Finally, the multitude of immunosuppressive factors such as checkpoint pathways, cytokines and by-products from an altered metabolism all accumulate into what seems to be an almost impossible challenge for CAR T cells. This review will outline some of the problems associated with CAR T-cell therapies for solid tumors as well as novel CAR T-cell innovations that will help tackle these challenges (Figures 1 and 2).

TRAFFICKING AND PENETRATION: CHEMOTACTIC MANIPULATION AND BREAKING DOWN BARRIERS

The first of many hurdles encountered in the use of adoptively transferred cells is the difficulty in migrating to and adequately penetrating into the tumor to unleash their cytotoxic function. One factor that may contribute to the high level of efficacy observed using CAR T cells against hematological malignancies is that both tumor and effector cells share hematopoietic origins, and thus have a higher propensity to migrate to similar areas such as bone marrow and lymph nodes. In contrast, solid tumors are known to secrete chemokines, such as CXCL12 and CXCL5 which inhibit T-cell migration into the area,[12,13] and often the chemokine receptors present on T cells do not adequately match the chemokine signature of the tumors, resulting in little migration to the tumor site.[14] Profiling the chemokine signature of a tumor and genetically modifying CAR T cells to express the appropriate chemokine receptor(s) may allow a greater proportion of cells to home to the tumor. Indeed, T cells genetically modified to express CXCR2 have been demonstrated to migrate towards a range of tumor cells expressing CXCL1.[15] This effect has also been observed in mesothelioma and neuroblastoma xenografts using CAR T cells bearing a CCR2b receptor,[16,17] and in Hodgkin's lymphoma with CCR4-bearing CAR T cells.[18] In addition, as the surrounding stroma is also capable of secreting different chemokines,[19] the tumor location and the local 'normal' cytokine milieu may also dictate the chemokine repertoire and should be taken into consideration.

As an alternative to changing chemokine receptors on CAR T cells, chemokine secretion from tumors can be modulated to correlate with the chemokine receptors that are naturally present on CAR T cells. The injection of an oncolytic adenovirus expressing RANTES and IL-15 directly into neuroblastoma tumors led to an increase in CAR T-cell infiltration and greater tumor control.[20] Similarly, EGFR-CAR NK-92 cells combined with an oncolytic herpes simplex virus produced promising results in a metastatic pre-clinical model,[21] and work from our laboratory has demonstrated extensive T-cell infiltration and solid tumor eradication with the combination of CAR T cells and oncolytic virus.[22] However, to apply this type of approach to both primary and metastatic lesions, other viral carriers (such as vaccinia virus) or alternative modes of administration, such as cellular delivery vehicles, may need to be used.[23,24] Future work aimed at altering the tumor microenvironment (TME) such that it becomes intrinsically more accessible to T cells is needed.

Another hurdle that can be faced by CAR T cells before entering the immunosuppressive TME is a physical barrier prohibiting efficient infiltration into the tumor. Immunosuppressive myeloid cells can be attracted into the tumor microenvironment, hindering T-cell infiltration.[25] Tumor fibroblasts and myeloid cells can also contribute to the development of a pro-tumoral fibrotic extracellular matrix,

which may hinder T-cell penetration. Heparanase (HPSE) is an enzyme integral for degradation of heparin sulfate proteoglycans, which constitute a majority of the extracellular matrix. As loss of HPSE has been observed in T cells post *in vitro* culture, overexpression of HPSE in CAR T cells, or alternatively targeting the surrounding non-malignant stroma using CAR T cells directed against the TAA 'fibroblast activation protein' can overcome these physical barriers, enhancing T-cell infiltration into the TME.[26]

In addition, the antigens targeted by CARs need not be limited to just TAAs. Targeting and disrupting the vasculature can restrict blood flow and nutrient supplies to the tumor, impeding its development, whereas at the same time enhancing T-cell infiltration. As demonstrated by Chinnasamy *et al.*,[27] simultaneously targeting tumor antigens and VEGFR-2, expressed on angiogenic endothelial cells and myeloid suppressor cells, resulted in eradication of B16 melanoma in mice together with increased infiltration of T cells into tumors. Furthermore, CARs incorporating ligands for angiogenic vessel-associated molecules such as αvβ3, an integrin commonly expressed on tumor vascular endothelium,[28] demonstrate enhanced migration. The use of echistatin CAR T cells (which target the αvβ3 protein) in combination with nanoparticles increase nanoparticle deposit in the tumor, indicating the possibility of using vasculature-targeted CAR T cells to enhance drug delivery.[29] Similarly, an approach using anti-VEGFR CAR T cells able to secrete IL-12 resulted in increased accumulation of CAR T cells and tumor regression in multiple pre-clinical models.[30] However, as tumor regression in this system was dependent on the presence of both the cytokine and CAR, vasculature-targeting alone may not be sufficient to drive an efficient anti-tumor response. Prostate-specific membrane antigen (PSMA) is found on malignant prostate cells and the endothelium of some tumor vasculature but not on normal vasculature, making it an ideal target for immunotherapy. Administration of PSMA-CAR T cells into a murine ovarian tumor model resulted in tumor regression, but did not achieve complete responses due to the heterogeneous expression of PSMA on the tumor vasculature.[31] This study indicates that CAR T cells can induce anti-tumor responses without targeting a tumor-restricted antigen and suggests a combination of vasculature-targeting and tumor-restricted antigen targeting CAR T cells may be required to achieve complete eradication. This approach may enhance the efficacy of CAR T cells against solid tumors with heterogeneous or low levels of antigen expression.

COMBATING IMMUNOSUPPRESSION

The tumor microenvironment is comprised of multiple cellular and molecular components that reduce efficient anti-tumor immune function. This active orchestration of immunosuppression can severely inhibit the effector functions of CAR T cells. Notably though, this effect is tightly dependent on the tumor microenvironment, as removal of CAR TILs from the tumor restores their anti-tumor functions.[32] These data strongly suggest that appropriate inhibition of immunosuppression or altering the immunosuppressive TME may

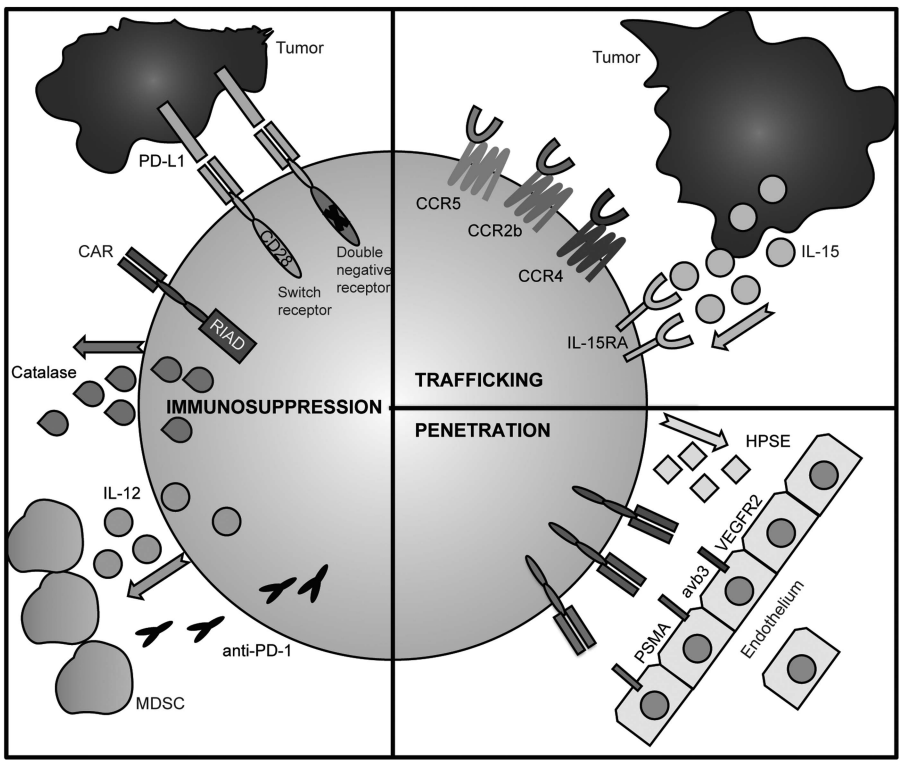

Figure 1 Additional features of CAR T cells that combat the obstacles presented by solid cancers. *Trafficking;* improvements to trafficking of CAR T cells to the tumor can be enhanced through expression of chemokine receptors CCR5, CCR2b, CCR4 or through induction of IL-15 from the tumor itself. *Penetration;* expression of heparanase (HPSE) or CARs targeting vasculature antigens such as vascular endothelial growth factor receptor (VEGFR-2), integrin alpha V beta 3 (αvβ3) or prostate-specific membrane antigen (PSMA) can also aid CAR T-cell infiltration into the tumor. *Immunosuppression;* CAR T cells modified to secrete mAbs such as anti-PD-L1 or T cell redirected for universal cytokine killing (TRUCKs) secreting IL-12 have shown to improve CAR T-cell function in an immunosuppressive environment. CAR T cells secreting catalase can also repolarise the pro-tumoral metabolic environment. Lastly, novel CAR designs involving downstream inhibitory molecules such as the RIAD-CAR, switch receptors or double negative receptors (DNRs) can protect CAR T cells from immunosuppression in the tumor microenvironment. A full color version of this figure is available online at the *Immunology and Cell Biology* website.

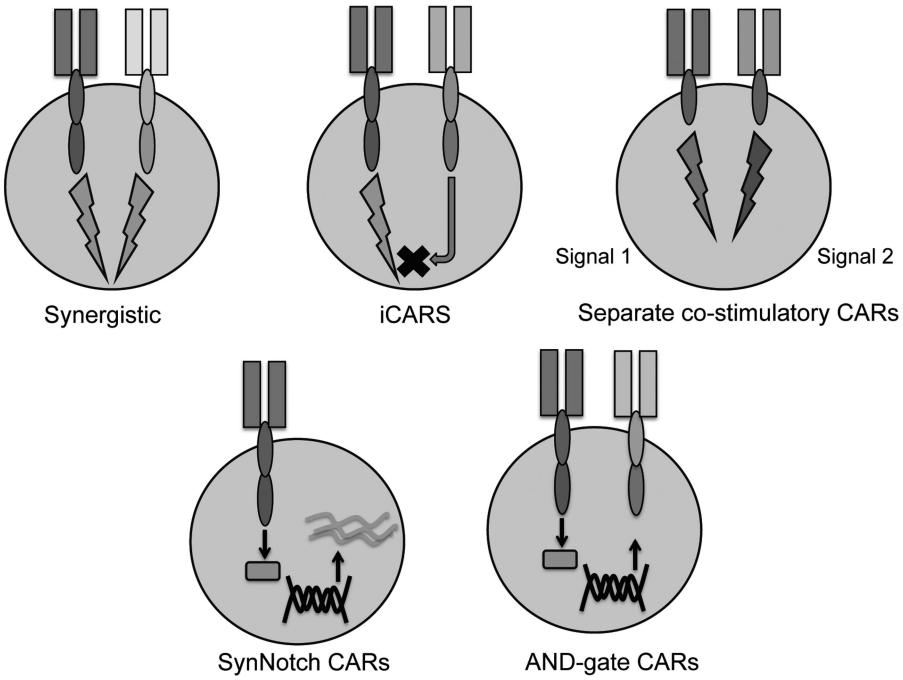

Figure 2 Novel CAR designs to reduce off-tumor effects. *Synergistic;* two distinct CARs with different antigens. Stimulation of one CAR results in suboptimal activity thus the presence of two tumor antigens is required for a full anti-tumor effect. *iCARs;* inhibitory CARs targeted towards a non-tumor antigen results in downstream inhibition of the tumor-targeted CAR, therefore normal tissue expressing basal levels of tumor antigen are protected. *Co-stimulatory CARs;* two separate CARs targeting different tumor antigens, CAR 1 has an intracellular signaling domain for activation, whereas CAR 2 contains co-stimulatory domains. Only in the presence of two tumor antigens will both activation and co-stimulation occur, resulting in full effector functions. *SynNotch CARs;* CAR recognition of the tumor antigen results in downstream cleavage of the intracellular transcriptional domain. This allows for transcription of the target genes (such as chemokines/inhibitors) only after tumor antigen recognition. *AND-gate CARs;* using the SynNotch CAR technology, the extracellular CAR is specific for tumor-localized antigen (based on the tumor location and thus normal antigen profile), which on antigen recognition results in the transcription and expression of a CAR directed against the tumor antigen. A full color version of this figure is available online at the *Immunology and Cell Biology* website.

rescue hypofunctional CAR T cells and open new therapeutic avenues for improving CAR T-cell function.

Immunosuppressive cytokines, such as TGF-β or IL-10 secreted by both pro-tumoral immune cells and the tumor cells themselves, can orient immune activity away from a robust cytolytic response. To repolarise the tumor microenvironment, 'armored' CAR T cells or 'TRUCKs' (T cells redirected for universal cytokine killing) have been explored in pre-clinical studies. These improved armored-CARs and TRUCKS have been designed to secrete pro-inflammatory cytokines such that they can better function in an immunosuppressive microenvironment. Specifically, co-expression of the single chain IL-12 on CAR T cells resulted in tumor regression through the repolarisation of the tumor microenvironment, even in the absence of exogenous IL-2. This effect was mediated by alterations in the number and function of myeloid cells present in the tumor.[30] In addition to repolarising the TME, constitutive IL-12 signaling enhances T-cell cytotoxicity and cytokine secretion, promoting resistance against Treg immunosuppression.[33] The use of TRUCKs to deliver a range of cytokines, and their potential to change the TME, warrants further exploration (summarized in Chmielewski and Abken[34]).

Checkpoint inhibitory proteins, such as PD-L1, which normally function to regulate the immune response are often upregulated on tumors. On interaction of PD-L1 with its receptor PD-1, which is upregulated on exhausted T cells and TILs, T lymphocytes become hypofunctional. Our group has previously demonstrated an enhanced efficacy of CAR T cells when used together with monoclonal antibodies against checkpoint molecules. Addition of an anti-PD-1 antibody reduced the infiltrating myeloid derived suppressor cell (MDSC) population in the TME and resulted in a more potent CAR T-cell anti-tumor response.[35] Following on from this study, we recently reported that co-blockade of both PD-1 and CD73/adenosine pathways using specific adenosine 2A receptor antagonists could further augment CAR T-cell responses *in vivo*. Another recent study generated CAR T cells capable of secreting anti-PD-L1 antibodies, surpassing the need to co-transfer anti-PD-L1 mAbs.[36] In addition to significantly reducing tumor growth in a humanized renal cell carcinoma mouse model, local secretion of anti-PD-L1 antibodies from CAR T cells increased migration of adoptively transferred human NKs into the tumor. NK cells were shown to exhibit an anti-tumor role through ADCC as well as by providing IFNγ stimulation to $CD8^+$ T cells. Therefore, enhancing the infiltration of non-T cell anti-tumor immune subsets into the TME through local antibody secretion can improve CAR T-cell therapy.

Another approach to combating immunosuppression is the generation of novel CARs incorporating mutant or nullified cell-surface dominant negative receptors (DNRs) that can override the inactivating signals encountered in the TME. DNRs maintain the extracellular region of a membrane receptor but generally harbor a mutation in the intracellular chain, resulting in an absence of downstream signal transduction and subsequent loss of function.[37] As such, DNRs are often able to compete with their endogenous receptors for target ligands, thus prohibiting the full effect of target/receptor binding. The use of DNRs for immunosuppressive factors such as TGF-β has endowed transduced EBV cells with resistance to immunosuppression, as monitored by proliferation and cytokine secretion.[38,39] Similarly, a DNR for PD-1 on CAR T cells

rescued the effect of checkpoint blockade and restored effector functions.[40] As PD-1/PD-L1 blockade is normally achieved through antibody blockade, which due to its broad target range can lead to autoimmune effects, the use of PD-1 'insensitive' DNR T cells may overcome this issue.

Switch receptors offer yet another alternative approach to circumvent immunosuppression. These contain the extracellular portion of an antibody specific for an immunosuppressive molecule, such as PD-1 or CTLA-4, fused to an intracellular activating signaling molecule, such as CD28, which reinforces the effector function of the cell.[41,42] The infiltration and anti-tumor efficacy of CAR T cells were enhanced when bearing a PD-1-CD28 switch receptor as compared with parental CAR T cells. Interestingly, Liu *et al.*[41] also observed a reduction in other checkpoint inhibitors, namely LAG3, TIM-3 and CEACAM1 expression and an increase in IL-2 signaling, perhaps suggesting the gain in function may result from an overall 'younger', less exhausted population. Unlike DNR receptors, however, this effect was dependent on efficient CD28 signaling, as mutated PD-1-CD28 CAR T cells showed similar efficacy to that of CAR T cells, suggesting the addition of another signaling domain can further augment CAR T-cell function. This holds promise to alleviate the effects of immunosuppression in the TME and to further boost the cytotoxic function of CAR T cells.

In addition, endogenous modification to nullify inhibitory signaling pathways in T cells has shown promise in re-initiating T-cell function. A recent study by Newick *et al.*[43] showed that inhibition of Protein Kinase A with Ezrin using a 'regulatory subunit 1 anchoring disruptor' (RIAD-CAR) resulted in an up-regulation of CXCR3 and CD49D integrin (VLA-4) that translated to enhanced RIAD-CAR T-cell trafficking to tumors and better migration to CXCL10 *in vitro*. In addition, RIAD-CAR cells expressed higher levels of both IFNγ and cytotoxicity when exposed to adenosine *in vitro*, and were more resistant to immunosuppressive adenosine in the tumor microenvironment, resulting in an enhanced anti-tumor response as compared with CAR T cells alone. Preventing T-cell inactivation may therefore increase T-cell infiltration and facilitate a more potent and effective response.

Furthermore, conditions that increase the acidity of the TME can negatively impact on intra-tumor T-cell function. This can occur as a result of an increased glycolysis by cancer cells. This condition, known as the 'Warburg effect', refers to the preferential utilization of glucose via glycolysis rather than via oxidative phosphorylation. The former, by increasing lactate production, results in the acidification of the extracellular environment. This altered metabolism observed in cancer cells (reviewed in Vazquez *et al.*[44]) also leads to yet another means of immunosuppression; an increase in oxidative stress and reactive oxygen species.[45] Furthermore, infiltrating pro-tumor myeloid cells such as MDSCs have been known to secrete high levels of reactive oxygen species (ROS), which adds to their repertoire of immunosuppressive abilities. CAR T cells easily succumb to the immunosuppression of oxidative stress, where both proliferation and cytotoxicity are greatly impaired. However, when engineered to secrete catalase (CAT), an anti-oxidant enzyme, into the local environment, CAR-CAT T cells retained their anti-tumor functions.[46] Furthermore, *in vitro* analysis suggested local catalase secretion was sufficient to facilitate a bystander effect, restoring cytotoxic function to NK cells. Modification of the surrounding tumor microenvironment with metabolism based therapies requires further investigation.

CAR T CELLS AND DRUGS; A COMBINATORIAL APPROACH

The concept of using CAR T cells in a combination approach alongside other drugs has opened up new avenues of treatment. Most drug treatments in the clinic are prescribed in the absence of adoptive cell therapy; therefore, although there are many opportunities to combine current treatments with ACT, a rational choice of drugs based on an understanding of drug:immune system interaction is required.

Recent work has suggested that the optimal type of cells for adoptive cell transfer are those which retain their memory/naïve capacities, allowing for a greater boost in proliferation and function *in vivo*.[47] Similarly, using inhibitors of differentiation *in vitro,* such as the BET inhibitor JQ1, preferentially enhanced expansion of central memory and stem cell memory-like T cells. Adoptively transferred *in vitro*-JQ1-treated CAR T cells had higher proliferation, persistence and increased cytokine secretion and significantly increased survival compared with non-treated CAR T cells.[48]

Lenalidomide, a derivative of thalidomide, has demonstrated impressive anti-tumor results in patients with multiple myeloma.[49] Similarly, when used in conjunction with CAR T cells it resulted in increased CAR T-cell infiltration into the tumor site, as well as augmented IFNγ production and cytotoxicity, resulting in complete cures in all treated mice.[50] Conversely, while rapamycin (rapa) and other rapalogs are commonly used as a treatment for dysregulated AKT/mTOR signaling in combination with other therapies for cancer, the effect of rapa on effector T-cell function is counterintuitive; modifying the differentiation of T cells, favouring the expansion of Tregs and prohibiting full effector T-cell functions.[51,52] As such, by expressing a mutant rapa-resistant mTOR (mTorRR) in CAR T cells (CAR.mTorRR), Huye *et al.*[53] were able to demonstrate a sustained ability to secrete IFNγ. Furthermore, as target cells treated with rapa were more susceptible to CAR T-cell killing, the addition of rapa increased the potency of CAR.mTorRR T cells. Therefore, the effect of therapeutic drugs for cancer on CAR T cells must first be established before using them in combination. The potency of a two-pronged approach, targeting both tumors and CAR T cells with the same drug, holds great promise.

CAR T cells can also be used as vectors for drug delivery in targeted therapy. As systemic administration does not always sufficiently localize to the target cells (and may have off-target effects), Boice *et al.*[54] harnessed the antigen-specific nature of CAR T cells to locally secrete soluble herpesvirus entry mediator (HVEM) which, when bound to the B and T lymphocyte attenuator (BTLA) receptor on B cell lymphoma cells inhibited their proliferation. As such, CD19 directed CARs were capable of eliminating their target cells and in addition, the HVEM:BTLA interaction strongly blocked proliferation of target lymphoma cells, which resulted in a greater therapeutic outcome than CD19-CAR T cells alone. Exploiting the antigen-specific localization of CAR T cells and the flexibility provided from our ability to genetically alter the 'contents' or 'passengers' of these cells may lead to new ways of delivering tumor-specific antibodies/drugs into the tumor or TME. Moreover, local delivery via CAR T cells can reduce the large volume of antibodies/drugs needed to appropriately saturate the target compared with systemic delivery and may result in less off-target effects on distant organs or tissues.

Therapeutic use of all-trans retinoic acid (ATRA) for acute promyelocytic leukemia induces the differentiation of immature myeloid blasts, one of the key immunosuppressive players in the TME. Furthermore, targeting the infiltrating MDSC population in the TME can also impact on the efficacy of CAR T therapy as has been observed in pre-clinical models of pediatric sarcoma xenografts.

Pre-clinical studies of ATRA therapy has demonstrated that the differentiation of immunosuppressive immature myeloid cells can restore anti-tumor lymphocyte function.[55] Similarly, when combined with CAR T cells targeting the GD2 antigen on osteosarcoma xenografts, both the frequency and function of tumor infiltrating MDSCs were reduced resulting in an overall improved survival compared with mice treated with GD2-CAR T cells alone.[56]

ENHANCING SPECIFICITY AND SAFETY OF CAR T-CELL THERAPY

One major difference between hematological malignancies and solid tumors is the availability and heterogeneity of tumor antigens. One of the reasons CAR T cells against B cell antigens have demonstrated such success is due to the homogenous expression of tumor antigens, CD19 or CD20 on virtually all tumor cells within a given patient (at least before therapy). While non-malignant cells in these patients also express the CD19/CD20 antigen and are thus susceptible to CAR T killing, the on-target/off-tumor effects are manageable and not life threatening, as can be the case with other tumor antigens.[11] As such, novel CARs targets are currently being explored.

Examples of novel targets include the type 1 insulin-like growth factor receptor (IGF1R) and receptor tyrosine kinase-like orphan receptor 1 (ROR1) for sarcoma[57] as well as the L1-cell adhesion molecule (L1-CAM) for ovarian cancer.[58] However, in all cases low-level expression is still present on non-malignant tissue, and thus caution must be used before moving these CARs into clinical trials.

The struggle to identify target antigens expressed uniquely on tumors and absent on all other normal tissue is currently being addressed with alternative CAR designs. While the specificity and avidity of CARs are improving, toxicity due to recognition of low levels of the target antigen on normal tissues is an issue that warrants further attention. As such, multiple groups have taken various approaches to reduce the off-tumor effects based on the presence or absence of two target antigens. Previous work from our lab has focused on the synergistic effects of two individual CARs against two TAAs (folate binding protein and Her-2), with minimal anti-tumor effect in the presence of only one.[59] Although most tumor cells share TAAs with normal tissue, the likelihood of expression of two unique TAAs on normal and tumor tissue is less likely. Dual CAR expressing T cells were reported to secrete almost double the amount of cytokine as compared with CAR T cells expressing a single CAR when co-cultured with dual antigen-expressing tumor targets.[59]

Another alternative to reduce unwanted off-target effects are inhibitory CARs. Inhibitory CARs (iCAR) contain extracellular signaling domains for 'normal' antigens which are bound to cytoplasmic regions of either PD-1 or CTLA-4, thus facilitating downstream inhibition in the presence of the iCAR target antigen.[60] However, as iCAR signaling failed to completely abrogate T-cell function, further modifications such as the inclusion of suicide genes (reviewed in Minagawa *et al.*[61]) may aid to eliminate undesirable toxicity.

Other strategies to reduce autoimmune effects involve separation of intracellular signaling domains 'signal 1' (CD3ζ) and 'signal 2' (co-stimulation) onto different CARs recognizing two alternative target antigens. As clearly demonstrated in first and second generation CARs, the presence of the co-stimulatory domains greatly enhances the overall function of the cell. By separating the two intracellular domains, CAR T cells were only able to function at full capacity in the presence of both target antigens, thus restricting activation in the presence of a single antigen.[62]

Similarly, one of the latest developments in CAR technology also uses a 'one-two' system, relying on the specificity of the CAR to traffic to the tumor target ('one') before unleashing the secondary hit ('two'). SynNotch receptors consist of an extracellular CAR specific for a target antigen, which is then fused to an intracellular cleavable transcriptional domain. Binding to the target antigen resulted in Notch cleavage, and downstream transcriptional activation of Notch-inducible genes.[63] The possibilities for the inducible genes are many, for example secondary CARs to ensure specific targeting of tumor tissue expressing two antigens. Additional possible inducible genes include chemokines, checkpoint blockade antibodies or endogenous factors that may promote the persistence, memory or anti-tumor function of the T cell itself. Subsequent work from the same group further explored this concept, designing the AND-gate T cells. Recognition of a 'tumor-localized antigen' on the primary SynNotch receptor facilitated the expression of the CAR for the tumor antigen, thus both tumor antigen and tumor-localized antigen are required to activate the SynNotch/AND-gate circuit, preventing premature CAR T-cell activation in the presence of single antigen-expressing tumor cells.[64]

CONCLUDING COMMENTS

The potential power of CAR therapy has been validated in hematological malignancies, yet the rate of success in solid tumors is currently low. Novel cutting edge designs for CAR T cells to overcome many of the challenges presented by solid tumors are currently being tested. It is hoped that our growing wealth of knowledge about the tumor microenvironment and the speed of technological advances will promote the development of CAR T cells that are adequately modified to combat the immunosuppressive nature of the tumor microenvironment and deliver a lethal hit to solid tumors.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by grants from the Cancer Council of Victoria, Australia (1066554), The Peter MacCallum Cancer Center Foundation, and the National Health and Medical Research Council (NHMRC) of Australia (1103352). MHK and PKD were supported by Senior Research Fellowships from the NHMRC. CSMY was supported by a PhD Top Up Scholarship from the Cancer Therapeutics Cooperative Research Centre, Australia, and a scholarship from L'Association pour la Recherche sur le Cancer, France.

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