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SPECIAL FEATURE REVIEW

Switching on the green light for chimeric antigen receptor T-cell therapy

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Abstract

Adoptive cellular therapy involving genetic modification of T cells with chimeric antigen receptor (CAR) transgene offers a promising strategy to broaden the efficacy of this approach for the effective treatment of cancer. Although remarkable antitumor responses have been observed following CAR T-cell therapy in a subset of B-cell malignancies, this has yet to be extended in the context of solid cancers. A number of promising strategies involving reprogramming the tumor microenvironment, increasing the specificity and safety of gene-modified T cells and harnessing the endogenous immune response have been tested in preclinical models that may have a significant impact in patients with solid cancers. This review will discuss these exciting new developments and the challenges that must be overcome to deliver a more sustained and potent therapeutic response.

Keywords: CAR, adoptive cell therapy, solid tumors, T cells

INTRODUCTION

Evidence highlighting the importance of harnessing the immune system for cancer control has become increasingly apparent and is in part attributed to the efficacy of checkpoint blockade therapy such as α-PD-1 and α-CTLA-4 antibodies.[1] More recently, the success of CD19-targeted chimeric antigen receptor (CAR) T-cell therapy in some haematological malignancies, including B-ALL and non-Hodgkin's lymphoma, has further accentuated the potent antitumor potential of T cells.[2–4] However, the impressive clinical success seen with CAR T cells in B-cell malignancy patients has yet to be translated beyond $CD19^+$ malignancies. Both preclinical studies and multicenter clinical trials have been conducted and are still ongoing to test the efficacy of CAR T-cell therapy targeting different antigens in solid tumors. Results thus far have indicated that CAR T-cell therapy for epithelial cancers has not matched the remarkable clinical responses observed in patients with B-cell malignancies.[5] Although not completely understood, the discrepancy between CAR T-cell effectiveness in $CD19^+$ haematological and solid cancers may be due to several factors that include the immunosuppressive tumor environment in solid tumors, the lack of CAR T-cell trafficking and penetration into the solid mass, tumor antigen heterogeneity, as well as the lack of full complement of activation signals required for optimal functional responses by CAR T cells. In addition, the use of CAR T cells in solid tumors faces additional challenges in terms of safety and,

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similarly as with haematological malignancies, the high costs associated with generating a personalised CAR T-cell product. To overcome these challenges, a substantial amount of work has focused on investigating novel strategies to augment CAR T-cell therapeutic efficacy and applicability, including combining CAR T cells with immunomodulatory antibodies, manipulation of the tumor microenvironment (TME), induction of immune responses against antigen-negative tumors, targeting of intracellular tumor antigens, safety and development of 'universal' CAR T-cell strategies. Each of these key aspects will be discussed in this review.

TARGETING TUMOR MICROENVIRONMENT (TME) IN CAR T-CELL THERAPY

The efficacy of CAR T-cell therapy is influenced by both the environment to which CAR T cells are exposed and also the intrinsic functional parameters of CAR T cells which determine whether they can generate effective antitumor responses. The solid tumor landscape presents multiple barriers that can ultimately neutralise CAR T-cell activity. In order to successfully eliminate the tumor cells, CAR T cells must successfully traffic to the tumor site. This could be challenging in the case where there is a mismatch between tumor-derived chemokines and chemokine receptors on T cells. Subsequently, if these CAR T cells do get to the tumor site, the next challenge is to successfully infiltrate the stromal elements for CAR T cells to induce antitumor cytotoxic effects. Therefore, strategies to degrade extracellular matrix in an attempt to improve tumor infiltration by T cells have been explored, for example by engineering CAR T cells to express the heparanase enzyme.[6] Finally, even after successful trafficking and infiltration to the tumor, CAR T cells must then overcome multiple obstacles created by the tumor and/or the host cells in the TME, including the presence of immunosuppressive soluble factors, cytokines and immune cells.[7] Attempts to overcome these obstacles have led to the development of various strategies involving manipulation of the TME.

Using immunomodulatory antibodies to enhance CAR T-cell antitumor responses

Checkpoint inhibitors such as α-CTLA-4 and α-PD-1 antibodies in cancer therapy work by blocking the inhibitory mechanisms in T cells, consequently leading to further T-cell activation and tumor killing. Remarkable results from clinical trials using α-CTLA-4 and α-PD-1 antibodies led to their approval by the Food and Drug Administration (FDA) in 2011 and 2014, respectively.[8–10] Given the clinical success in enhancing T-cell function and antitumor activity, administration of checkpoint inhibitors makes an ideal partner for tumor-targeted engineered CAR T cells. Preclinical studies have tested the combination of CAR T-cell therapy and α-PD-1 mAb against a number of cancers, either through systemic administration of α-PD-1 mAb or genetic modification of CAR T cells to express α-PD-1 single-chain variable fragment (scFv). Combination therapy using α-PD-1 demonstrated superior antitumor efficacy in an *in vivo* model compared to conventional CAR T cells that correlated with enhanced effector function of the CAR T cells such as granzyme B and IFNγ upon PD-1 blockade.[11] More recently, a study involving the combination of CAR T cells, α-PD-1 mAb and additionally an A2AR antagonist that blocks the adenosine immunosuppressive pathway reported an even greater antitumor response in a preclinical model.[12] The clinical translation of CAR T-cell and α-PD-1 mAb is now underway with multiple clinical trials currently recruiting patients.[13] In addition to checkpoint inhibitors, agonistic monoclonal antibodies that activate T-cell costimulatory receptors have also advanced in their development, including, for example, α-4-1BB and α-OX40 mAbs.[14,15] Inclusion of 4-1BB and/or OX40 domains directly in the CAR construct as costimulatory signals has been investigated and demonstrated potent ability to support CAR T-cell activation. Notably, these costimulatory domains significantly impact on T-cell cytokine secretion and proliferation function.[16] Both 4-1BB- and/or OX40-containing CAR T cells have been tested in various preclinical studies; however, comparisons between the two domains remain inconclusive in terms of overall antitumor effect observed given variability in the models used from different groups.[16,17] In the context of costimulation using exogenous antibodies, a recent preclinical study tested the combination of Her2-specific CAR T cells with α-4-1BB therapy against Her2-expressing solid tumors. The combination treatment resulted in significantly enhanced tumor regression compared to CAR T-cell therapy alone or control T cells in combination with α-4-1BB mAb.[18] This study

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highlights the potential of using an agonistic antibody to improve CAR T-cell efficacy in solid tumors, and therefore, testing of other agonistic antibodies in this context is warranted.

Previous studies have combined the use of both immune checkpoint inhibitors and agonistic antibodies in preclinical cancer models for increasing the endogenous antitumor immune response (Figure 1). Some of these studies reported increased antitumor effects following the combination of α-PD-1 and α-4-1BB antibodies in a number of murine cancer models,[19–21] and α-PD-1 and α-OX40 antibodies in an ID8 murine ovarian cancer model.[22] However, more recently other studies have reported opposing effects. Two different studies reported that the concurrent addition of α-PD-1 mAb markedly reduced the therapeutic response of α-OX40 mAb.[23,24] Interestingly, however, a study by Messenheimer *et al.* found that when α-OX40 and α-PD-1 antibodies were administered sequentially by treating MMTV-PyMT tumor-bearing mice with α-OX40 mAb before α-PD-1 mAb, the sequential combination therapy resulted in augmented antitumor efficacy. However, this improved effect was not observed when α-PD-1 mAb was administered before α-OX40 mAb, highlighting the importance of timing and sequence of such treatments.[23] Further, another study that tested the combination of α-4-1BB and α-PD-1 antibodies

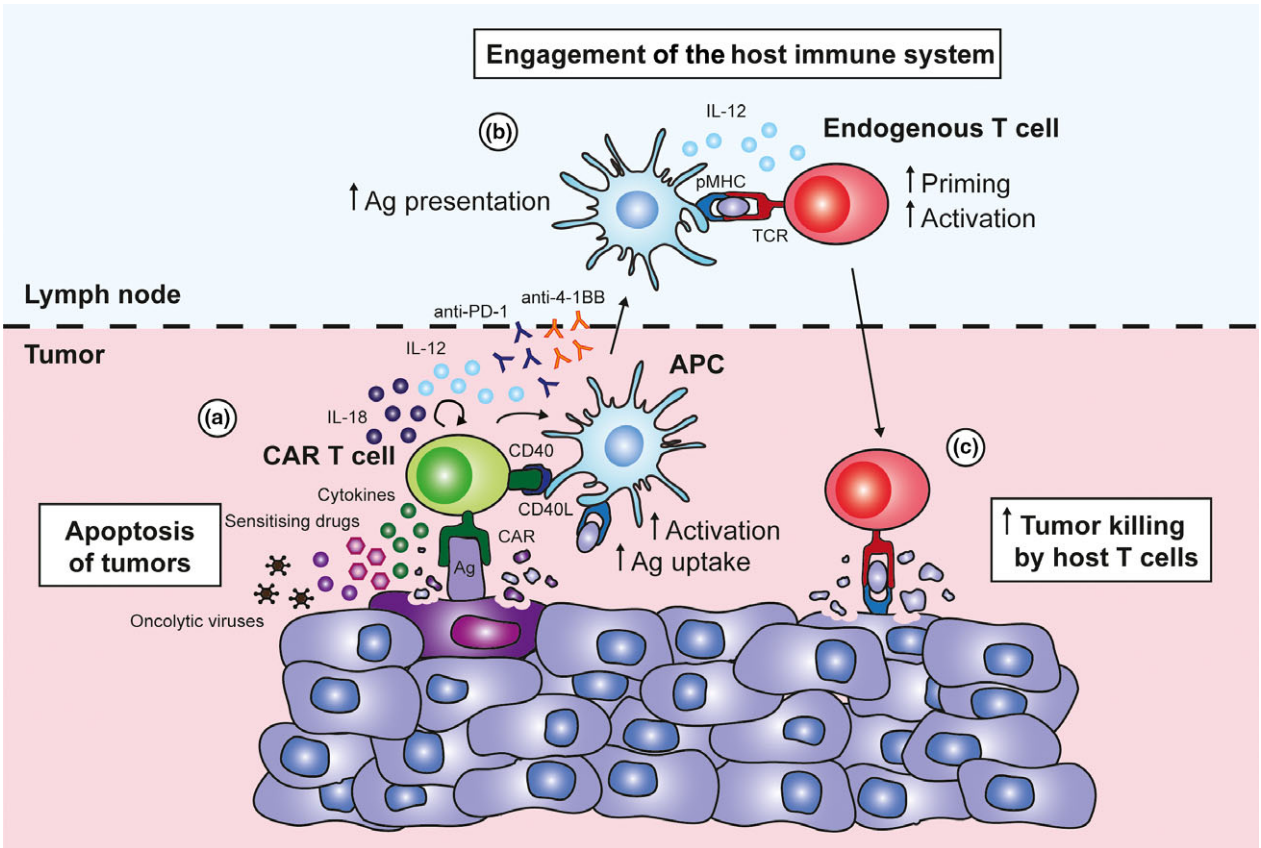

Figure 1. Enhancing tumor killing by engaging host immunity during chimeric antigen receptor (CAR) T-cell therapy. **(a)** CAR T cells lyse tumor cells in an antigen-specific manner and secrete pro-inflammatory cytokines IFNγ and TNFα at the tumor site. Apoptosis-sensitising drugs can be used in combination with CAR T-cell therapy to enhance tumor lysis through TNF-mediated bystander killing. Oncolytic viruses can directly lyse tumor cells, engage host responses and act as carriers of transgenes that facilitate CAR T-cell antitumor activity. Immune-stimulatory antibodies such as anti-4-1BB or anti-PD-1 can be used to increase tumor killing by CAR T cells whilst improving responses by host immune cells. CAR T cells modified to secrete IL-12 or IL-18 not only increase their effector function but also modulate various endogenous immune cell types such as Tregs and myeloid cells at the tumor site. **(b)** CAR T cells can be engineered to express CD40 ligand, which interacts with CD40 on antigen-presenting cells such as DCs to enhance their maturation, antigen uptake and presentation to endogenous T cells. **(c)** This may result in enhanced tumor killing by endogenous T cells, thereby improving the overall efficacy of CAR T-cell therapy.

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in a murine spontaneous B-cell lymphoma model found that simultaneous use of α-PD-1 mAb diminished the antitumor activity of α-4-1BB mAb alone. The mechanism for this effect was thought to be due to a dramatic reduction in the function of effector $CD8^+$ T cells in the presence of α-PD-1 mAb, potentially through induced apoptosis.[25] The reason for the discrepancies observed between different studies involving checkpoint inhibitor and immune agonist combination is not completely understood; however, the dose and timing of the immune-modulating antibody administration may be of high importance. Together, all these observations reveal that whilst supporting T-cell activation using combination of immune agonists and checkpoint inhibitors could generate synergistic antitumor effects, caution is necessary in choosing the optimal timing and sequence in order to achieve maximal therapeutic efficacy particularly in the context of adoptive transfer of gene-modified T cells.

Localised expression of immune-stimulatory molecules by CAR T cells in the TME

The area of synthetic biology is vastly developing and has provided us with the technology to perform customised engineering of cellular pathways, enabling various modifications in cell response behaviours required for more effective T cell-based therapies.[26–29] Strategies to modulate the local TME have led to the generation of 'armored' CAR T cells that provide localised expression of pro-inflammatory cytokines or costimulatory ligands to improve CAR T-cell function within tumors. CAR T cells engineered to secrete IL-12 have resulted in enhanced *in vivo* efficacy in several preclinical models including $CD19^+$ B-cell lymphoma and MUC16-expressing ovarian cancer. In these studies, CAR T cell-secreted IL-12 augmented their cytotoxic function and alleviated regulatory T cell (Treg)-mediated suppression.[30–32] Using a similar approach, CAR T cells secreting IL-18 demonstrated improved antitumor activity, increased proliferation and persistence in an *in vivo* model.[33,34] Other systems involving cytokine-mediated enhancement of CAR T cells include the genetic modification of these cells to express a form of membrane-bound chimeric IL-15, which gave rise to a population of CAR T cells that possessed a T memory stem cell phenotype and a better memory potential even in the absence of antigen stimulation.[35]

Chimeric antigen receptor T cells have also been modified to express immune-stimulatory molecules to influence their interaction with other cell types within the local TME. Constitutive expression of CD40 ligand by CAR T cells not only resulted in their enhanced killing and pro-inflammatory cytokine production but also led to increased maturation and IL-12 secretion by dendritic cells (DCs) (Figure 1). Furthermore, CD40 ligand directly engaged CD40-expressing tumor cells to alter their immunogenicity through the upregulation of surface receptors including MHC molecules and Fas ligand.[36] In other studies, CAR T cells co-expressing 4-1BB ligand and CD80 provided auto-costimulation and induced an additional trans-costimulatory effect on bystander T cells, overcoming the lack of immune-stimulatory signals within the TME that resulted in the eradication of large tumors in preclinical models.[37] A recent study by Rafiq *et al.*[38] demonstrated that CAR T cells secreting α-PD-1 scFv provided localised delivery of the immune checkpoint inhibitor and blocked T-cell PD-1 and tumor PD-L1 binding, enhancing the efficacy of these T cells in both syngeneic and xenograft tumor models. Although not in the context of CAR T-cell therapy, an alternate attempt to overcome immunosuppressive signals involved adoptively transferred T cells overexpressing a dominant-negative TGF-β receptor type II in the treatment of EBV^+ Hodgkin's lymphoma. These cells were found to be immune to the immunosuppressive effects of TGF-β *in vitro*, expanded significantly *in vivo* and resulted in a complete response in 3 of 7 patients.[39] Overall, these studies suggest that therapeutic responses against solid tumors can potentially be augmented by engineering CAR T cells to express additional mediators that boost their local effector function and alter their interaction with surrounding cells in the TME.

Targeting the chemokine milieu to enhance CAR T-cell therapy

Given that trafficking and penetration of CAR T cells into tumor mass are major obstacles that have to be overcome in solid cancers, manipulation of chemokine signalling is another approach that is currently under intense investigation. A recent study by Adachi *et al.* utilised the cytokine modulation approach in combination with chemokine modulation for CAR T cells, combining the overexpression of IL-7 cytokine and CCL19

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chemokine in CD20-targeted CAR T cells. In two different murine CD20-overexpressing tumor models, P815 mastocytoma and 3LL Lewis lung carcinoma, CCL19 and IL-7-secreting CAR T cells were proven superior in promoting tumor clearance in pre-established solid tumors compared to conventional CAR T cells. This superior performance by CCL19 and IL-7-secreting CAR T cells was associated with a marked increase in CAR T-cell and DC infiltration into tumor tissues.[40]

Solid tumors can secrete various chemokines which are able to prevent effective T-cell trafficking into the tumor such as CXCL5 and CXCL12.[41,42] Moreover, chemokine receptors expressed on T cells frequently do not match the tumor chemokine signature, leading to limited trafficking to the tumor site.[43] Therefore, matching chemokine receptor(s) expressed on CAR T cells to the tumor chemokine milieu is also an attractive strategy as it may allow for a higher frequency of CAR T cells to traffic to the tumor site. Indeed, a preclinical study by Kershaw *et al.* demonstrated that T cells engineered with CXCR2 were able to migrate towards various tumor cells expressing the cognate chemokine CXCL1.[44] A similar effect has also been observed in other studies utilising CCR2b-bearing CAR T cells in neuroblastoma and mesothelioma xenografts, as well as CCR4-bearing CAR T cells in Hodgkin's lymphoma[45–47] (Figure 2a). One alternative to this strategy may also be to match the tumor chemokine profile to more closely resemble the CAR T-cell chemokine receptor profile.[48–50] For instance, whilst CXCR3 is highly expressed on most activated $CD8^+$ T cells and is critical for their trafficking to the tumor site,[51] intratumoral expression of the CXCR3 ligands CXCL9, CXCL10 and CXCL11 is generally low. However, a recent study utilising a xenograft model of ovarian cancer demonstrated that co-administration of T cells with the EZH2 inhibitor, GSK126, and DNA methyltransferase inhibitor, 5-AZA-dC, led to a significant enhancement in CXCR3-dependent trafficking and antitumor efficacy.[52] This regime was shown to augment T-cell trafficking by inducing tumor cells to express the epigenetically silenced chemokines CXCL9 and CXCL10 (Figure 2b), suggesting that modulating tumor chemokine profile through epigenetic modification may be a viable mechanism to overcome poor trafficking into solid tumors. Alternatively, PD-1 blockade has been shown to

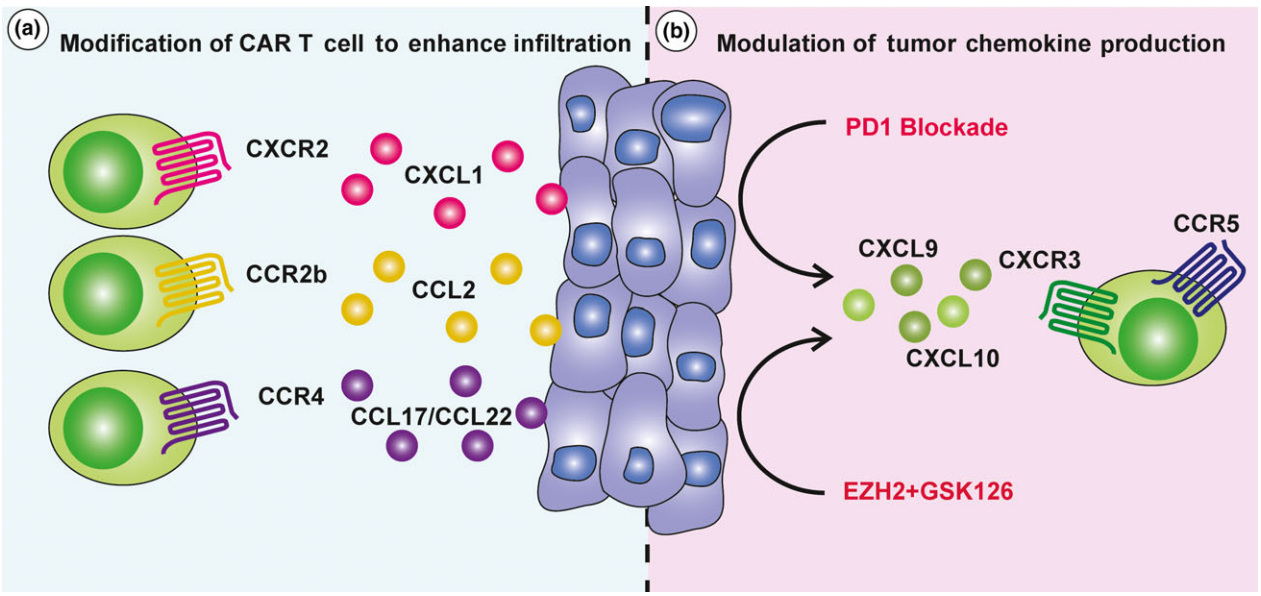

Figure 2. Approaches for enhancing intratumoral chimeric antigen receptor (CAR) T-cell infiltration. Commonly, the chemokine receptors endogenously expressed by CAR T cells do not match the chemokines present in the tumor microenvironment. **(a)** One approach to improve CAR T-cell infiltration therefore is to transduce them with chemokine receptors such as CXCR2, CCR2b or CCR4, better matching the chemokine profile of the tumor microenvironment. **(b)** Alternatively, both PD-1-blockade and epigenetic modifiers, EZH2 and GSK126, have been shown to increase intratumoral expression of chemokines CXCL9 and CXCL10, leading to enhanced CXCR3-dependent trafficking of adoptively transferred T cells.

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significantly enhance adoptive T-cell trafficking to tumors[53] as well as CAR T-cell activation.[11] In the context of adoptive cell therapy (ACT), it was found that enhanced T-cell trafficking was caused by an IFNγ-dependent increase of intratumoral CXCL10.[53] Taken together, based on promising observations from these preclinical studies investigating cytokine and/or chemokine genetic manipulation approach, it is anticipated that more cytokines and chemokines involved in promoting immune cell responses will be explored in the context of CAR T-cell therapy. Importantly, given the chemokine signature may also be influenced by the tumor location, stroma and surrounding cytokine milieu,[54] these factors should be taken into consideration when designing appropriate chemokine receptors for CAR T cells.

INDUCING TUMOR ERADICATION BEYOND CAR T-CELL ANTIGEN RECOGNITION

Treatment with engineered CAR T cells has predominantly focused on targeting one single tumor antigen.[45,55–57] This has been an effective strategy in the case where the target antigen is ubiquitously expressed by tumor cells, which is the case for CD19 in B-cell malignancies. However, even in the most successful case of CD19-CAR T cells targeting B-cell malignancies, a significant fraction of patients relapse not long after T-cell infusion. A proportion of the relapsed patients experience antigen-negative disease recurrence, manifested by the outgrowth of $CD19^-$ tumor cells.[58,59] This phenomenon therefore highlights the important subject of tumor antigen heterogeneity, even when the target antigen is uniformly expressed on all tumor cells. Investigation into the mechanisms leading to this antigen loss phenomenon revealed an alternative splicing mechanism of the CD19 mRNA, resulting in the loss of the cognate epitope on the CD19 protein required for recognition by CD19-CAR T cells.[60] Similar evidence of this splice variant process was reported in the context of melanoma cells resistant to vemurafenib, together indicating splice-based adaptations by tumor cells as an escape mechanism that consequently leads to outgrowth of tumor variants.[61] These findings suggest that CAR T-cell therapy, or any other targeted therapy more generally, may promote the outgrowth of tumor escape variants. Antigen loss renders CAR T cells ineffectual and may have significant ramifications for the wider success of CAR T-cell therapy. Thus, the ability to induce additional antitumor responses recognising alternative tumor antigens, especially in the context of solid tumors where antigen expression is more heterogeneous, will be pivotal for CAR T cells to have a universally efficacious antitumor effect.

Several strategies have been developed in an attempt to overcome the problem of antigen-negative relapse. For example, the generation of dual CAR T cells targeting two different antigens, CD19 and CD22, has been shown to increase antitumor effects compared to CAR T cells targeting either single antigen, where mice were co-inoculated with a mixture of $CD19^-$ $CD22^+$ and $CD19^+$ $CD22^-$ NALM6 leukaemia cells. Importantly, it was demonstrated that having two CARs in the T-cell population was able to offset antigen escape.[62,63] Other dual and even trivalent CAR T cells have also been investigated in preclinical studies, including CAR T cells targeting CD19 and CD123 in a leukaemia model,[64] and CAR T cells targeting Her2, IL13Rα2 and EphA2 in a glioblastoma model,[65] respectively. Despite the promising potential of this approach, however, it may be difficult for most malignancies to find multiple different tumor antigens on one tumor cell that can be targeted by CAR T cells in a safe and effective manner.

Alternatively, an antitumor response against a wider range of targets may be achieved through the recruitment and stimulation of the endogenous immune response, as this involves various effector cells with a broad spectrum of recognition capabilities and antitumor functions. Indeed, engagement of endogenous cellular immunity has been reported to induce antigen-negative tumor cell killing following antigen-specific T-cell therapy.[59] One strategy to potentially promote tumor killing beyond a CAR T-cell antigen-specific response has been through further modification of these cells to secrete pro-inflammatory cytokines (Figure 1). A study by Chmielewski *et al.* elegantly demonstrated the capacity of CAR T cells engineered to secrete the IL-12 cytokine that enabled responses against both antigen-positive and antigen-negative tumor cells. This effect was accompanied by accumulation of macrophages, which were shown to be a critical facilitator for the observed antitumor response.[66] One other potential mechanism to increase the targeting of antigen-negative tumors may also be to combine CAR T-cell treatment with apoptosis-sensitising drugs such as birinapant, leading to

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enhanced TNF-mediated bystander killing of tumor cells[67] (Figure 1). Further, given previous preclinical studies suggesting that the combination of CAR T cells with CD40 ligand or α-4-1BB mAb works in part through the activation of host DCs, approaches that engage the endogenous immune system may be an effective approach to induce antitumor responses beyond CAR T-cell target antigen[18,36] (Figure 1). Interestingly, in the context of PD-1 blockade, PD-1-blocking scFv secreted by CAR T cells in a preclinical model was shown to bind to bystander tumor-specific T cells, resulting in an overall enhanced antitumor effect.[38]

Another approach to enhance the efficacy of CAR T-cell therapy is in combination with oncolytic viruses (OVs). OVs can help to overcome the immunosuppressive tumor microenvironment by providing pathogen-associated molecular patterns, upregulating MHC class I machinery as well as directly lysing tumor cells, releasing danger-associated molecular patterns and tumor-associated antigens (TAAs) that help prime host antigen-presenting cells and engage endogenous T-cell responses.[68–70] Moreover, OVs can be modified to express transgenes that improve the antitumor responses of CAR T cells. By administering gp100-expressing recombinant vaccinia virus vaccination with dual-specific T cells comprising gp100-specific TCR and anti-Her2 CAR, one group reported significant antitumor responses against both antigen-positive and antigen-negative tumors, suggesting the potential of epitope spreading following combination therapy.[71] OVs engineered to express cytokines IL-2 and TNFα, chemokines such as CXCL11, or in some cases a combination of both IL-15 and RANTES/CCL5 have also been shown to increase recruitment of CAR T cells to the tumor and enhancement of antitumor activity in preclinical models.[72–74] Likewise, CAR T cells displayed superior therapeutic efficacy when combined with OVs expressing localised anti-PD-L1 minibody in comparison with systemically delivered anti-PD-L1 antibody.[75] Another study combined antifolate receptor alpha (FRα) CAR T cells with OVs expressing bispecific T-cell engager targeting a second tumor antigen epidermal growth factor receptor (EGFR) and showed that both endogenous and CAR T cells were successfully redirected against $EGFR^{+}$ $FR\alpha^{-}$ tumors, overcoming tumor heterogeneity and prolonging survival of mice.[76]

Taken together, it is well established that tumors can enhance their capacity for immune escape by loss of a targeted antigen or epitope. This makes antigen heterogeneity a major challenge that urgently needs to be overcome in the context of antigen-targeted therapy, more specifically CAR T-cell therapy. Furthermore, in CAR T-cell clinical trials for solid malignancies, eligibility criteria for patients to be enrolled often requires only partial expression of the CAR target antigen on a patient's tumor. For example, enrolment criteria in one of the Lewis Y-specific CAR T-cell clinical trials specified Lewis Y to be expressed on a minimum of 20% of tumor blast cells, meaning that the majority of the tumor cells would not be recognised by the CAR T cells in some patients.[77] Therefore, engagement of endogenous antitumor immunity appears to be a promising approach to increase the likelihood of epitope spreading, which may potentially lead to eradication of antigen-negative tumor cells, and subsequently decreased risk of antigen escape variants emerging.

CAR T CELLS TARGETING INTRACELLULAR TUMOR ANTIGENS

Designing a treatment that is effective in facilitating tumor destruction whilst sparing healthy cells is considered the 'holy grail' in cancer immunotherapy. Discovery of tumor antigens that are only expressed by tumors but not by healthy cells remains a key aspect towards improving the specificity and safety of immunotherapies. Tumor antigens can be classified into several categories, namely TAAs such as overexpressed self-antigens (Her2, CD19), tissue-specific antigens (CEA), as well as tumor-specific antigens including mutated antigens (neoantigens) and viral antigens. Most of the tumor antigens targeted by immunotherapies to date are those overexpressed on tumor cells but are also present on healthy cells to a lesser extent. Examples of these include the Her2, CEA, GD2 and Lewis Y antigens that have been used as targets for CAR T-cell therapy.[57,78–81] Given that these antigens are found on healthy cells, their use as immunotherapeutic targets is attributable solely to their preferential expression on tumors. However, caution has to be taken as on-target off-tumor side effects can in certain cases pose a significant limitation on this approach.[82,83]

Careful selection and design of CAR T-cell constructs may potentially alleviate some of the issues associated with differences in antigen expression between healthy and tumor tissues. A

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recent study by Majzner *et al.*[84] demonstrated that CAR T cells targeting the pan-cancer antigen B7-H3 mediated cytolysis of high antigen-expressing tumor cells whilst displaying minimal reactivity towards low antigen-expressing cells. This suggests that antigens that are found on normal tissues may still serve as safe targets, provided that their expression on tumor cells is sufficiently distinguishable by CAR T cells. Nonetheless, extensive pre-evaluation will be required to determine therapeutic efficacy versus safety as antigen density can vary widely across individual patients' normal and tumor tissues.

Another factor is the suitability of tumor antigens as targets for CAR T-cell technology. CAR-engineered T cells are only able to recognise antigens that are expressed on the cell surface. However, a number of tumor antigens are found intracellularly and are therefore considered nontargetable by conventional CAR T cells. One innovative approach to circumvent this problem is the development of CAR T cells using antibody fragments that recognise intracellular tumor antigens based on their surface presentation as peptide epitopes on MHC molecules. These TCR-mimic CAR T cells are designed to specifically engage MHC–peptide complexes found on the surface of target cells. Willemsen *et al.*[85,86] first reported the engineering of CAR T cells using a phage display-derived MAGE-A1/HLA-A1-specific Fab, which induced *in vitro* target lysis and cytokine production against MAGE-A1 expressing HLA-A1$^+$ melanoma cells. These studies provided a conceptual framework for the development of similar CAR T cells targeting tumor antigens NY-ESO-1 and proteinase 3 peptide PR1.[87,88] More recently, two groups have individually described the generation of TCR-mimic CAR T cells targeting intracellular Wilm's tumor 1 antigen in the context of HLA-A2 in preclinical models, with one of these studies demonstrating *in vivo* therapeutic efficacy of these CAR T cells against antigen-expressing leukaemia and ovarian tumors.[89,90] Based on these promising developments, there is the potential to expand the repertoire of tumor antigens targetable by CAR T-cell therapy.

GENERATING UNIVERSAL 'OFF-THE-SHELF' CAR T CELLS

The two recently approved CD19-CAR T-cell products, Kymriah™ and Yescarta™, although highly effective, are very expensive treatments, priced at US $475 000 and US $373 000 for a one-time treatment, respectively.[91] These high costs are partly attributed to the fact that the process from T-cell collection, genetic modification, to CAR T-cell reinfusion is patient specific. To make CAR T-cell therapy more broadly applicable to diverse patient populations, strategies to generate universal off-the-shelf CAR T-cell products that can be safely and effectively delivered to multiple recipients will be a key issue to address.[92] At present, there have been a number of clinical trials evaluating the potential use of allogeneic CAR T cells.[93]

It is anticipated that the use of allogeneic rather than the more personalised autologous CAR T cells will significantly reduce manufacturing costs, as bulk manufacturing of CAR T cells can be achieved in a time-efficient and less labour-intensive manner. Indeed, the feasibility of using 'off-the-shelf' T cells in humans has now been well demonstrated to be both an effective and safe treatment for a number of viral diseases.[94,95] However, to permit such an approach, two inherent challenges associated with allogeneic cell transfer must be overcome. These include graft-versus-host disease (GVHD) and rejection of the infused CAR T cells by the host. Studies have been conducted to address these issues, as exemplified by the development of an approach using Transcription Activator-Like Effector Nucleases (TALEN™) to simultaneously inactivate both the endogenous TCR and CD52 of the adoptively transferred T cells. This approach decreased GVHD risk due to elimination of the endogenous TCR, whilst allowing for persistence of the infused CAR T cells due to depletion of the host T cells upon α-CD52 mAb administration.[96,97] Another report utilised CAR T cells that were additionally transduced with an ER retention signal-containing scFv specific for the CD3ε component of the TCR. This resulted in the surface downregulation of endogenous TCRs on the CAR T cells and a reduced occurrence in GvHD.[98] A recently developed alternative to remove TCR expression on the transferred CAR T cells is by targeting the CAR transgene insertion into the native TCR alpha chain (TRAC) locus, either using TRC1-2 nuclease or CRISPR/Cas9 technology. It was demonstrated that the transduced CD19-specific CAR T cells lacked the endogenous TCR whilst exhibiting robust antitumor responses due to reduced tonic CAR signalling and T-cell exhaustion.[99] In a mouse model of ALL, enhanced tumor rejection was observed following treatment with CD19-CAR T

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cells directed to the TRAC locus.[100] Others have reported similar genomic modification strategies combining the basis of TALEN DNA binding with meganucleases (megaTAL) to insert a CAR transgene into the CCR5 locus of primary human T cells.[101] In addition, Cooper and colleagues demonstrated the utility of zinc finger nucleases to specifically disrupt endogenous TCR and HLA genes in T cells, increasing the prospects of generating allogeneic CAR T cells for individuals of disparate HLA.[102,103]

Given rapid advances in gene-editing technology such as CRISPR and other site-specific endonucleases, such genetic modification strategies may be achieved in an efficient and precise manner, allowing for widespread clinical use.[99] Other potential avenues to generate off-the-shelf antigen-specific effector cells have also been investigated, and a promising strategy involved the incorporation of a CAR transgene into NK cells instead of T cells. The use of CAR-expressing NK cells potentially obviates the GVHD issue from allogeneic donors as they do not induce GVHD. Several groups have reported preclinical evaluation of these CAR-expressing NK cells with promising results demonstrated,[104,105] and clinical trials investigating this approach are currently underway.[106]

In addition to improving the widespread applicability of allogeneic CAR T cells, current efforts have also been focused on generating universal CARs to provide greater flexibility for antigen recognition by CAR T cells. These methods generally involve the engineering of a generic receptor on the extracellular portion of the CAR which can then be coupled with a soluble ligand-conjugated, antigen binder of choice. Using the biotin–avidin system, Urbanska *et al.*[107] generated CAR T cells containing extracellular avidin and showed that these cells could elicit antigen-specific effector functions against $EpCAM^+$ ovarian tumors in an *in vivo* model when conjugated with a biotinylated anti-EpCAM antibody. Similarly, another group administered CAR T cells containing an extracellular high-affinity Fc receptor-binding CD16 variant and demonstrated significantly enhanced antitumor efficacy of rituximab or trastuzumab, respectively, against $CD20^+$ or $HER2^+$ expressing tumors in mice.[108] Exploring further on this concept, Cho *et al.* developed split, universal and programmable (SUPRA) CARs in which CAR T cells express an extracellular leucine zipper (zipCAR), which can bind to scFvs containing leucine zippers (zipFvs). Using this approach, zipCARs can simultaneously be endowed with multiple specificities based on the variety of scFvs present, and signal strengths can be adjusted depending on each individual zipper binding affinities. This potentially enables the production of CAR T cells targeting a broad range of antigens without having to further engineer the CARs and may also help to address the issue of tumor escape and toxicity.[109]

SAFETY OF CAR T-CELL THERAPY

In cases where CAR T cells are directed against nontumor-specific TAAs, potential toxicity due to CAR T-cell recognition of low levels of the target antigen on healthy cells remains an important issue to be addressed. Thus, multiple strategies to mitigate the on-target off-tumor effects are currently being investigated. One approach has been to ensure target selectivity by dual CAR T-cell recognition of two different TAAs on the same tumor cell. In this setting, the two CARs are designed to either induce a ζ-chain signal or a CD28 costimulatory signal, allowing for superior T-cell activation upon simultaneous antigen engagement of the two CARs. As a result, this may be a safer approach restricting CAR T-cell full activity to only tumor cells expressing the two antigens at the same time, whereas the potency of signals delivered into the CAR T cells via only one CAR engagement remains below the activation threshold and hence rendered ineffective.[110] A similar approach of dual T-cell recognition involves engineering a T-cell circuit whereby a synthetic Notch receptor for one antigen leads to subsequent expression of a CAR specific for a second antigen. These T cells are only activated when both antigens are present on the tumor cells. Both of these approaches may be suitable for controlling potential on-target off-tumor effects, as dual antigen recognition may allow for more selective tumor elimination, whilst sparing healthy cells that express only a single antigen.

Another strategy employs the principle of an inhibitory CAR (iCAR), a receptor designed to counteract the CAR T-cell activation signal induced by the conventional CAR. This approach involves a signalling combination of two different CARs in the engineered T cell, whereby the iCAR, upon engagement by a specific antigen expressed only on healthy cells, induces a dominant inhibitory signal to limit the T-cell activating signal

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generated by the conventional CAR. In human T cells, PD-1 and CTLA-4 intracellular signalling domains were used in iCARs owing to their ability to reduce TCR signalling, resulting in decreased T-cell cytokine production and lysis upon antigen stimulation on healthy cells.[111] This iCAR T-cell strategy thus provides a self-regulating safety switch that allows for distinction between the tumor and healthy cells, resulting in a more selective elimination of tumor cells. One additional area of investigation has been the generation of 'titratable' CAR T cells that allow exogenous regulation of T-cell function. This has been achieved through the generation of 'On-switch' CAR receptors consisting of separate extracellular and intracellular domains containing an FKBP and FRB domain, respectively, that heterodimerise and signal only in the presence of rapamycin analogues.[112,113] Whilst promising, these preclinical models are limited by their reliance on rapamycin analogues that have unfavorable pharmacokinetic characteristics.[112]

An alternative approach to overcome potential CAR T-cell therapy toxicity is through incorporation of the so-called 'suicide genes' into transferred cells that allow for their targeted depletion. One key approach involves the incorporation of extracellular markers that can then be targeted by antibodies with pre-established clinical use. These markers include codon optimised CD20 (CD20op)[114] and RQR8,[115] both targetable by rituximab; truncated epidermal growth factor (EGRFt),[116] targetable by cetuximab; and HSC-tk, targetable by ganciclovir.[117] Arguably the most specific method, however, involves the integration of an inducible caspase 9 (iCasp9) domain into CAR T cells. The iCasp9 gene consists of an intracellular compartment of the human Casp9 protein, which is a pro-apoptotic molecule, fused to a chemical induction of dimerisation (CID) drug-binding domain. Upon administration of a CID drug, the drug-binding domains of the fused iCasp9 protein are cross-linked, leading to dimerisation of the Casp9 proteins that eventually results in cellular apoptosis induced by the downstream caspase 3 molecule.[118,119] In 2010, Hoyos *et al.*[120] reported the first preclinical study of CAR T cells incorporating the iCasp9 gene. In this study, second-generation CD19-CAR T cells expressing iCasp9 were used *in vivo* and were successfully eliminated within 3 days following administration of a CID drug. In the event of a serious adverse event, this suicide gene strategy may facilitate immediate removal of CAR T cells, alleviating toxicity induced by the CAR T cells. However, it is important to note that there may be other preferred strategies to utilise given that complete removal of CAR T cells may increase the risk of tumor relapse.

FUTURE PERSPECTIVES

Adoptive T-cell therapy holds promising potential for being a standard of care treatment option. This is supported by the recent FDA approval of two CD19-CAR T-cell products for the treatment of patients with B-cell ALL and non-Hodgkin lymphoma who have not responded to, or who have relapsed following at least two other conventional treatments.[121,122] In order for adoptive T-cell therapy to become a first in-line treatment option, however, a number of crucial challenges still need to be addressed.

Overall, clinical responses of patients with B-cell ALL and non-Hodgkin lymphoma to the FDA-approved CD19 CAR T cells have been excellent.[123] However, a significant proportion of patients treated with Kymriah™ have relapsed after several months, and many of the patients treated with Yescarta™ have exhibited only partial responses that eventually waned by 6 months post-treatment.[124] Thus, despite the remarkable success, there are concerns about long-term efficacy of CAR T cells, and it remains unknown how long the responses might last. More clinical data with long-term follow-up will be required to assess the long-term benefit of CAR T cells. Furthermore, in the more complex setting of solid cancers, significant clinical responses are yet to be achieved.[5] Several factors could potentially have an impact on CAR T-cell efficacy, including the variable potencies of the CARs themselves. It has been reported that tonic CAR signalling triggered by the clustering of CAR scFvs independent of antigen is capable of inducing CAR T-cell exhaustion hence limiting antitumor activity. Such activation was observed to varying degrees in multiple CARs studied targeting different antigens, except for the highly efficacious CD19 CAR.[125] Another factor that could influence CAR T-cell effectiveness includes the makeup of the gut microbiome. A recent study demonstrated that the efficacy of adoptive therapy in a mouse model was significantly affected by differences in the composition of gut bacteria.[126] Further, the TME is highly

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immunosuppressive in solid tumors,[127] and hence, combination strategies that can alleviate the immunosuppressive environment will be important to test in future clinical trials.

In addition to the TME, other factors related to the CAR T cells themselves can also have significant impacts on the therapeutic outcome. For example, component variability of the CAR T-cell product such as variable differentiation stages of T cells used for infusion can potentially affect overall CAR T-cell activity, and thus, a more uniform production method may be important in the future. One approach to address this issue may include determining the optimum formulation of T-cell subsets to use for infusion. It is well established that each subset of T cells has a unique function and cytokine profile, which influence their respective antitumor response.[128,129] In the clinic, predefined CD4:CD8 T-cell compositions have been used in clinical trials funded by therapeutic companies including Juno and Celgene.[130,131] Moreover, it is increasingly apparent that the quality and efficacy of T-cell immunity are a result of the diversification of naïve T cells into a number of phenotypically different subsets, including the highly differentiated effector, tissue resident memory, effector memory, central memory and memory stem T cells.[132] Naïve T cells can give rise to long-lived memory stem and central memory T cells that are capable of self-renewal and can provide proliferating populations of more differentiated effector T cells, which are relatively short-lived.[129] This fate framework indicates that CAR genetic modification of less differentiated T-cell subsets may result in achieving a greater and more sustained therapeutic response.[133] Indeed, preclinical studies have reported that receptor engineering of T cells selected from naïve and central memory subsets, or expanding naïve T cells *in vitro* with the addition of factors preventing the differentiation of T cells, can result in cell products possessing superior antitumor effects, proliferation and engraftment following adoptive transfer.[134,135] Further, given that different cytokines commonly used in *in vitro* culture to maintain T-cell survival such as IL-2, IL-7, IL-15 and IL-21 may have different impacts on T-cell differentiation,[136–140] the cytokines used to culture T cells prior to adoptive transfer require further testing and characterisation. These observations together indicate that determining the optimum formulation of T-cell subsets with the most superior antitumor potency for uniform use in adoptive transfer may help improve therapeutic outcome. Furthermore, this strategy may additionally reduce product variability between patients, resulting in a more consistent therapeutic response. Taken together, resolving these concerns and challenges will hopefully allow for more widespread application, as well as acceleration of CAR T-cell therapy to become a standard of care treatment option for various cancer types.

CONCLUDING SUMMARY

The approaches described herein to potentially improve CAR T-cell therapy emphasise the important challenges within both haematological and solid malignancies that need to be overcome. Increasing the specificity and safety of CAR T cells, harnessing the endogenous immune response to extend tumor destruction beyond CAR T-cell recognition and reducing the manufacturing costs will together accelerate the broad application of CAR T-cell therapy in various cancer types. Notably, emerging technologies using nonviral gene transfer such as mRNA electroporation and the *Sleeping Beauty* and *PiggyBac* transposon/transposase systems are currently being explored as inexpensive alternatives for large-scale manufacturing of CAR T cells.[141,142] Insights gained from ongoing research will be important to the growing body of knowledge that provides novel strategies to significantly address some of the existing limitations for the treatment of solid malignancies.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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