Received: 25 May 2023 | Accepted: 3 July 2023

DOI: 10.1111/imr.13252

Immunological Reviews WILEY

INVITED REVIEW

Coengineering specificity, safety, and function into T cells for cancer immunotherapy

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Funding information
Fondation ISREC; Ludwig Cancer Research; Prostate Cancer Foundation; Schweizerischer Nationalfonds zur Förderung der Wissenschaftlichen Forschung, Grant/Award Number: 310030_204326

Summary

Adoptive T-cell transfer (ACT) therapies, including of tumor infiltrating lymphocytes (TILs) and T cells gene-modified to express either a T cell receptor (TCR) or a chimeric antigen receptor (CAR), have demonstrated clinical efficacy for a proportion of patients and cancer-types. The field of ACT has been driven forward by the clinical success of CD19-CAR therapy against various advanced B-cell malignancies, including curative responses for some leukemia patients. However, relapse remains problematic, in particular for lymphoma. Moreover, for a variety of reasons, relative limited efficacy has been demonstrated for ACT of non-hematological solid tumors. Indeed, in addition to pre-infusion challenges including lymphocyte collection and manufacturing, ACT failure can be attributed to several biological processes post-transfer including, (i) inefficient tumor trafficking, infiltration, expansion and retention, (ii) chronic antigen exposure coupled with insufficient costimulation resulting in T-cell exhaustion, (iii) a range of barriers in the tumor microenvironment (TME) mediated by both tumor cells and suppressive immune infiltrate, (iv) tumor antigen heterogeneity and loss, or down-regulation of antigen presentation machinery, (v) gain of tumor intrinsic mechanisms of resistance such as to apoptosis, and (vi) various forms of toxicity and other adverse events in patients. Affinity-optimized TCRs can improve T-cell function and innovative CAR designs as well as gene-modification strategies can be used to coengineer specificity, safety, and function into T cells. Coengineering strategies can be designed not only to directly support the transferred T cells, but also to block suppressive barriers in the TME and harness endogenous innate and adaptive immunity. Here, we review a selection of the remarkable T-cell coengineering strategies, including of tools, receptors, and gene-cargo, that have been developed in recent years to augment tumor control by ACT, more and more of which are advancing to the clinic.

KEYWORDS
cancer, cell activation, chimeric antigen receptor (CAR), cytotoxic, gene-engineering, immunotherapies, T cell receptor (TCR), T cells, tumor immunity

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This article is part of a series of reviews covering Synthetic Immunology; cells, antibodies, sol. Receptors appearing in Volume 320 of *Immunological Reviews*.

1 | INTRODUCTION

$CD8^+$ T cells play a critical role in controlling solid cancers but to do so they must successfully reach and penetrate the tumor, expand and persist within the hostile and suppressive TME. They must also specifically recognize tumor cells and serially kill them while sparing healthy tissues. As we and others have demonstrated, T cells are reliant upon cues to navigate to tumors[1] but there is oftentimes a mismatch between the chemokines locally produced and chemokine receptors (R) expressed by T cells.[2] In addition, T-cell trafficking involves migration along an aberrant tumor vasculature that can upregulate a range of inhibitory ligands/receptors including FasL (CD95L), program cell-death protein ligand 1 (PD-L1), PD-L2, endothelin B receptor (ET_BR), and molecules including adenosine, prostaglandin E2 (PGE_2), and interleukin (IL)-6 that are suppressive to cytolytic T cells.[3] Moreover, dysregulated expression of adhesion molecules along tumor vessels can impede attachment, rolling and transendothelial migration of T cells into the tumor,[4] and cancer-associated fibroblasts (CAFs) can generate and remodel a dense extracellular matrix (ECM)[5] which excludes T cells (Figure 1). Indeed, during extravasation, T cells must actively degrade the sub-endothelial membrane and ECM, including heparan sulphate proteoglycans.[6]

Tumors evolve over time, including in response to infiltrating $CD8^+$ T cells,[7] and establish protumoral and immunosuppressive microenvironments to support their growth and high-jack the endogenous immune system.[8] For T cells that have successfully penetrated the tumor, chronic antigen exposure coupled with insufficient costimulation such as provided by dendritic cells (DCs)[9,10] can quickly render them exhausted and dysfunctional.[11,12] This is compounded by a broad range of inhibitory receptors and molecules that can be expressed by both tumor cells and suppressive immune infiltrate including T regulatory cells (Tregs), M2 tumor-associated macrophages (TAMs), and myeloid derived suppressor cells (MDSCs).[13,14] Tumor cells can also upregulate intrinsic mechanisms of resistance such as to extrinsic apoptosis[15,16] along with inhibitory receptors like PD-L1,[17] poliovirus receptor (PVR/CD155),[18] and V-domain Ig suppressor of T cell activation (VISTA).[19] Moreover, they can generate a range of suppressive molecules including adenosine,[20] PGE_2,[21,22] vascular endothelial growth factor (VEGF),[23,24] and transforming growth factor beta (TGFβ).[25,26] Notably, PGE_2 and adenosine are also released in large quantities by TAMs (some of the most abundant immune cells in tumors) under hypoxic conditions which inhibit T cells by activating G-protein coupled receptors and protein kinase

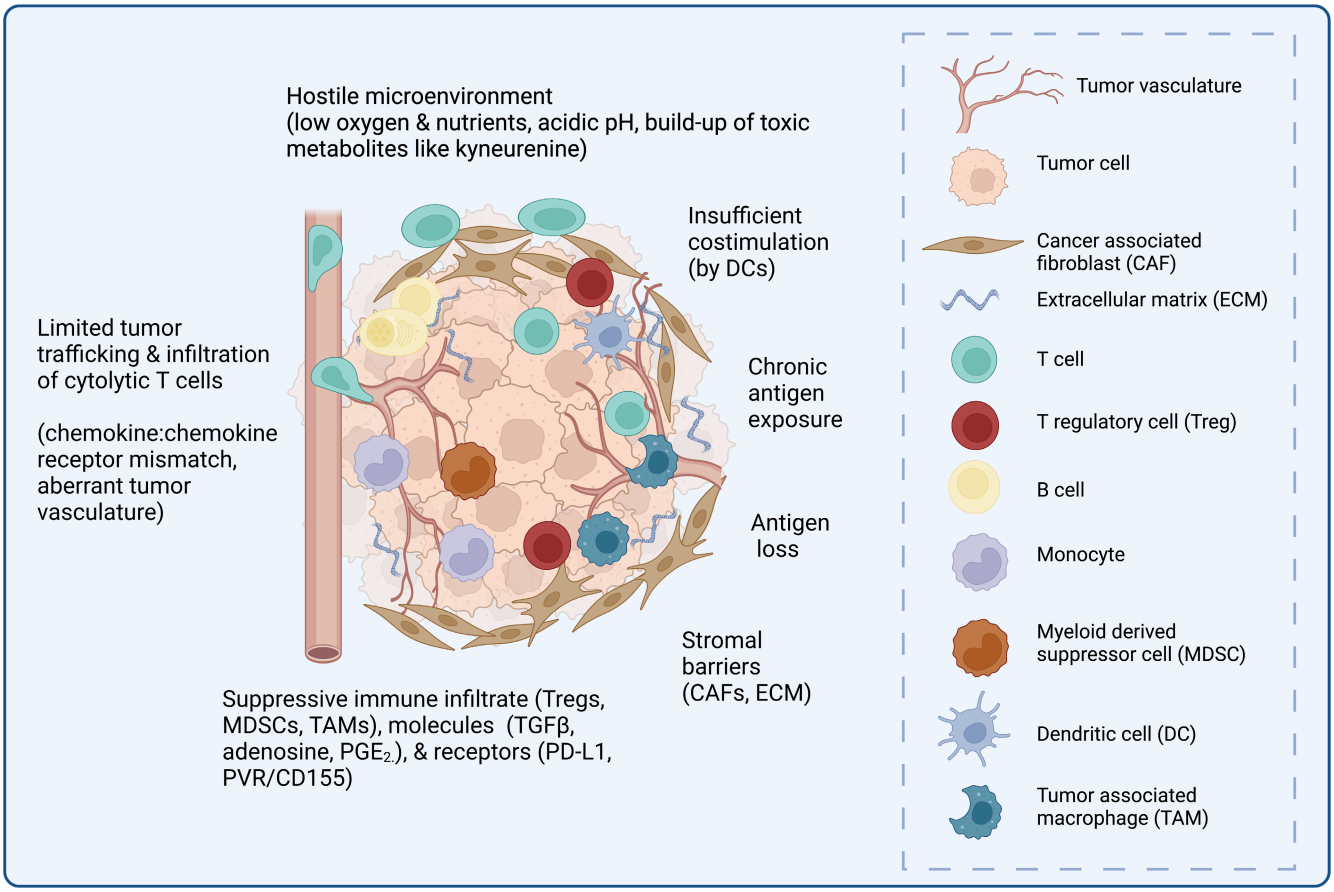

FIGURE 1 Overview of barriers to solid tumor control by T lymphocytes. T cells face challenges in trafficking to, and migrating into, solid tumors. Within the tumor bed T cells typically encounter a hostile environment including low oxygen levels, an acidic pH, and competition for limited nutrients and energy sources. Moreover, T cells can face chronic antigen exposure or antigen loss, insufficient costimulation, and a range of immunosuppressive receptors and molecules generated by both tumor cells and inhibitory immune infiltrate.

A (PKA).[27] Tumor cells also heavily compete for energy supplies and nutrients like glucose and amino acids (e.g., tryptophan, lysine, and arginine) also required by T-cells thus causing them to enter into a stress response.[28] Further, in large part due to vigorous tumor-cell metabolism, the environment is typically acidic which is highly suppressive to T cells.[29,30] Finally, because of its aberrant vasculature, the TME is also usually low in oxygen (i.e., hypoxic)[31] and there can be an important build-up of toxic metabolites such as kynurenine,[32] all of which can be detrimental to T-cell function and survival[33] (Figure 1).

Here, we review innovative approaches that have been taken to coengineer specificity, safety, and function into T cells for cancer immunotherapy. We begin with a brief background on the different viral and non-viral tools available for T-cell engineering,[34] including an optimized dual inverted lentiviral vector that we recently developed to allow efficient and independent coexpression of a TCR or CAR and inducibly expressed gene-cargo.[35] We then discuss the clinical use and efficacy of TCR- versus CAR-T cells, as well as receptor designs to improve TCR and CAR specificity, function, and safety. Finally, we provide an overview of gene-cargo as well as gene-knockouts evaluated in T cells to support their function, harness endogenous immunity or/and overcome barriers in the TME (Figure 1) in order to augment tumor control upon ACT.

2 | TOOLS FOR COENGINEERING T CELLS

Technological advances in cellular engineering are reshaping the clinical landscape. As we have previously reviewed,[34] stable or transient alterations can efficiently be made to T cells, as well as to other cell-types including mesenchymal stem cells (MSCs),[36,37] hematopoietic stem cells,[38] B cells,[39] gamma-delta T cells,[40] natural killer (NK) cells,[41] CAFs,[42] and macrophages,[43] to modify their functional properties and ultimately augment tumor control (or other desired biological outcomes[44]) upon reinfusion. Cellular processes can be disrupted by silencing, correcting, or overexpressing targets in the genome, or by RNA interference of transcribed genes.[45] To evaluate the safety of a new product, messenger (m)RNA electroporation which allows transient alterations to cellular function can be used.[46] For persistent modifications a variety of tools have been developed for genome-editing that have been used in the clinic.[47] Examples include transcription activator like effector nucleases,[48] zinc finger nucleases,[49] clustered regularly interspaced short palindromic repeats (CRISPR)[50,51] and viral vectors such as adenovirus, adeno-associated virus,[52] and γ-retrovirus and lentivirus.[34,53]

In early clinical trials, CRISPR-Cas9 engineered T cells have demonstrated safety both in the context of gene knockout (e.g., *TRAC*, *TRBC*, and *PDCD1*; PD-1)[54] and gene knockin (e.g., neoTCR into the *TRAC* locus).[55] Important advances are rapidly being made for improving the efficiency of CRISPR-based engineering[56–61] poised to revolutionize immunotherapy through cellular reprogramming of T cells.[56] Indeed, CRISPR screens (both loss- and gain-of-function experiments) have enabled important discoveries including the identification of key regulators of T-cell activities such as proliferation in response to stimulation,[62] gene networks controlling IL-2 and IFN-γ production,[63] genes that can be targeted to improve T-cell trafficking to tumors[64] or alleviate exhaustion,[65–67] and new tumor-specific receptors.[68] Importantly, advanced gene-editing tools such as CRISPR are paving the way towards off-the-shelf allogeneic T-cell products that can overcome graft-versus-host disease (GVHD) and host allorejection, and which should substantially decrease the costs of cellular therapies, enable treatment of heavily pretreated patients (i.e., the patients may be lymphopenic and not have sufficient T cells), and allow rapid delivery of a more uniform T-cell product.[69] Notably, off-the-shelf anti-CD7 CAR T cells, CRISPR base-edited by cytidine deamination to target CD52, CD7, and the TCR β-chain, showed potent activity in a recent phase 1 study for relapsed childhood T-cell leukemia.[70]

Lentiviral and γ-retroviral vectors have been widely and safely used for well over a decade in the clinic for generating CAR T cells[34] and important work is ongoing to further optimize manufacturing processes.[71] Indeed, due to their high efficiency and relative ease of use, it is likely that lentiviral and γ-retroviral vectors will be a mainstay for preclinical studies, as well as in the clinic for the foreseeable future, probably also in combination with other emerging technologies like CRISPR. We have put important efforts into optimizing retrovirus and lentivirus transduction protocols for the gene-modification of primary human T cells over the years,[72] and more recently into CRISPR-Cas9 and base-editing. We have also established robust methods for the retroviral transduction and expansion of murine T cells to evaluate coengineering strategies in the context of a fully competent immune system (i.e., in C57BL/6 mice).[73] Indeed, endogenous immune infiltrate can hinder ACT but by rational coengineering or/and combinatorial treatments[73–76] it can be reprogrammed to support tumor control. Syngeneic tumor models thus represent a very important tool for developing effective next-generation T-cell therapies. The details of our methodology can be found in Lanitis et al.[73] Key steps in our protocol include the concentration of retrovirus, anti-CD3/CD28 bead-based activation of the T cells, and robust post-transduction expansion in IL-7/IL-15 rather than IL-2 to favor a central memory phenotype (T_{CM}) and T cells more fit for ACT.

Given our strong interest in the development of coengineering strategies to safely improve TCR- and CAR-based T-cell therapies, we sought to develop a lentiviral vector enabling efficient constitutive expression of a receptor and independent activation-inducible expression of gene-cargo in primary human T cells.[35] Such an all-in-one vector can not only help to keep virus production costs down, but also ensures that all transduced T cells with the receptor also carry the gene-cargo, and vice versa. If both the TCR or CAR and the gene-cargo are constitutively produced they can be expressed from a single promoter and separated on the transfer vector by a picornavirus 2A peptide sequence (P2A)[77] or by an internal ribosome entry site (IRES).[78] However, to limit systemic toxicity, restricting expression of gene-cargo to the TME, such as by placing it under a T-cell activation dependent promoter like Nuclear factor of activated

T cells (NFAT) response elements fused to the IL-2 minimal promoter (6xNFAT),[79,80] may be preferable.

For our study, we began by evaluating classic dual sense[81] and bidirectional vectors[82,83] but observed that they were limited by interference of gene-expression and promoter leakiness in transduced cells, respectively (Figure 2A,B). In an effort to circumvent these issues, we built a dual inverted transfer vector (Figure 2C). For the dual inverted vector we observed high transduction efficiency, and higher coexpression levels of gene-cargo in activated cells than for the other vectors,[35] but the design was associated with low lentivirus titers. We postulated that the low titers were due to Dicer- or Dicer- isoform-mediated cleavage of the dsRNA that is generated as a result of convergent transcription during lentivirus production in HEK293T cells caused by the fact that both the 5′LTR and the inverted hPGK promoter are active (Figure 2D).[84]

We came up with two approaches to overcome low viral titers. For the first, we coexpressed an RNA interference suppressor protein, nodamura virus protein B2 (NovB2; previously demonstrated to inhibit isoforms of Dicer),[85] on the envelope vector and observed an increase in titers. For the second, we sought to address the issue that convergent transcription may restrict the levels of ssRNA viral genome available for packaging. We thus replaced the rous sarcoma virus (RSV)-based truncated 5′LTR promoter with the complete cytomegalovirus (CMV) promoter as it comprises 4 NF-κB binding motifs and included tumor necrosis factor alpha (TNFα) in the culture supernatant which acts as a potent transactivator.[86] We found that TNFα increased viral titers, and that it could be used synergistically with NovB2. Notably, the use of TNFα in culture media may be applicable to augmenting the production of other viruses or recombinant proteins etc. using vectors comprising promoters with NF-κB motifs. We have tested our dual inverted lentiviral vector in the context of both CARs and TCRs and various gene-cargo, both in vitro and in vivo.[35] We next plan to test our dual inverted lentiviral vector in the context of modified HEK293T packaging cells

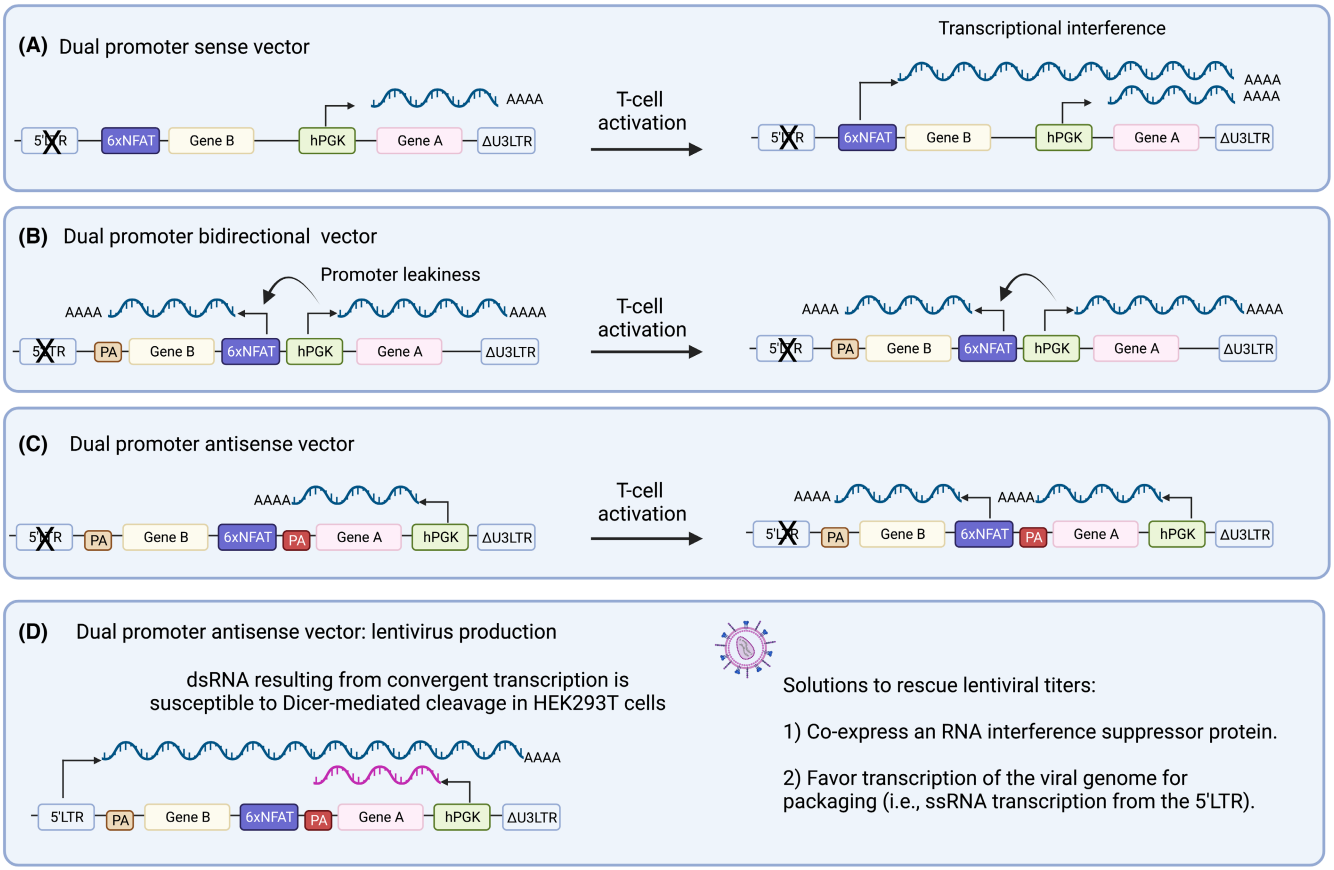

FIGURE 2 Comparison of lentiviral vector designs enabling constitutive expression of a CAR or TCR and activation inducible expression of gene-cargo. (A) For the dual forward sense promoter both 6xNFAT and hPGK are oriented in a forward, sense direction, with hPGK by necessity downstream of the inducible promoter. Upon activation of T cells transduced with the vector both Gene A and Gene B are expressed but there is transcriptional interference. (B) For the bidirectional vector the close proximity of the strong enhancer elements of the constitutive promoter drives transcription from the inducible promoter in non-activated cells. (C) The dual promoter antisense vector is not associated with promoter leakiness or transcriptional interference. (D) Left: the dual promoter antisense vector yields low lentivirus titers due to dsRNA resulting from convergent transcription and consequent Dicer-mediated cleavage in HEK293T cells. Right: solutions to overcome low viral titer are to coexpress an RNA interference suppressor protein (NovB2), and to favor ssRNA transcription by replacing the RSV-based truncated 5′LTR with the complete CMV promoter which harbors four 4 NF-κB binding motifs and including TNFα in the culture supernatant.

developed by Han et al. coined CHEDAR cells (CRISPRed HEK293T to Disrupt Antiviral Response) in which the genes *OAS1*, *LDLR*, and *PKR* are knocked out (factors that impede lentiviral titers) and transcription elongation factors, SPT4 and SPT5 are overexpressed, altogether leading to 11-fold increases in lentiviral titers.[87]

3 | TCRS VERSUS CARS FOR ACT

The two major receptor-types used for ACT of cancer in the clinic are TCRs and second generation (2G) CARs. Briefly, TCRs are a natural heterodimeric transmembrane receptor, with each chain made up of a variable region that mediates target binding, as well as a constant region. The variable region on each chain includes three complementarity-determining regions separated by framework regions. TCR cell-surface expression and function is dependent upon association with the CD3-complex comprising zeta (ζ), gamma (γ), epsilon (ε), and delta (δ) chains (Figure 3A). TCRs recognize intracellularly processed antigenic peptides (p) presented at the tumor-cell (or antigen presenting cell; APC) surface by the human leukocyte antigen complex (HLA; Class I for $CD8^+$ T cells and Class II for $CD4^+$ T cells) which are numerous and highly variable, and productive TCR-HLA-p engagement will trigger an intracellular signaling cascade via the CD3-complex (Figure 3A).[88]

TCRs are also associated with a coreceptor, CD8 in cytolytic T cells and CD4 in helper T cells (T_h cells). The coreceptors engage HLA and can stabilize the TCR:HLA-p interaction as well as enhance lymphocyte-specific protein tyrosine kinase (Lck) delivery to the TCR/CD3 complex, amongst other functions.[89] Some TCRs are dependent upon coreceptor engagement, which itself is a low-affinity interaction, to trigger full T-cell activation upon HLA-p binding, while others are not and this is not necessarily dependent on TCR affinity.[90] It has been demonstrated that transgenic expression of CD8 can rescue/augment TCR-T cell function[91] but increasing the binding affinity of coreceptors (CD8 or CD4) for HLA is not advisable as this can result in non-specific T-cell activation.[92] In collaboration with Prof. Immanuel Luescher, we previously demonstrated that the CD8 coreceptor can exist in both a *cis* and *trans* configuration and that this plays a role in regulating murine T-cell responses.[93] We attempted to enforce a *trans* configuration into the human CD8 coreceptor but we did not observe an increase in T-cell activity levels as compared to T cells transduced with wild-type CD8 coreceptor. However, CD8 coreceptor coengineering could rescue function of a CD8-dependent TCR upon transduction in $CD4^+$ T cells (Scholten et al., unpublished data).

One approach to TCR-T cell therapy is to enrich and expand the autologous T lymphocytes directly from biopsies (i.e., TIL therapy) for reinfusion along with a high-dose IL-2 into patients having received a

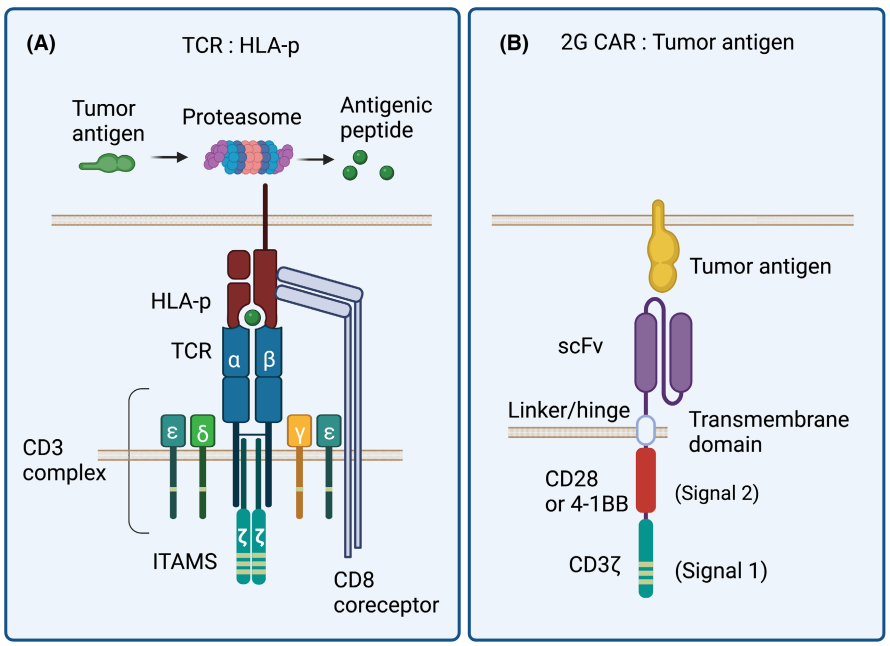

FIGURE 3 Schematic of a TCR versus a 2G CAR. (A) TCRs comprise an α- and β-chain, each made up of a variable region which engages antigenic peptide displayed by human leukocyte antigen receptors (HLA-p), and a constant region. TCRs associate with the CD3 complex proteins including epsilon (ε), gamma (γ), delta (δ) and zeta (ζ) chains. Immunoreceptor tyrosine- based activation motifs (ITAMs) found on the ζ-chain initiate intracellular signaling upon productive TCR-HLA-p engagement. TCRs expressed by $CD8^+$ T cells engage Class I HLA-p. The CD8 coreceptor co-engages HLA. (B) Second generation (2G) CARs are made up of a tumor antigen binding domain, typically a scFv, fused to a linker/hinge, a transmembrane domain, followed by the endodomain which comprises CD3ζ to allow signal 1 of T-cell activation upon target engagement, as well as 1 or more costimulatory endodomains such as from CD28 or 4-1BB that facilitate signal 2. First generation (1G) CARs do not include a costimulatory endodomain while 2G and 3G CARs comprise 1 or more costimulatory endodomains, respectively. CARs engage cell surface expressed target antigen in an HLA-independent manner.

non-myeloablative lymphodepletion pre-conditioning regimen.[94,95] The second approach is to engineer peripheral T cells with a specific TCR, or pool of TCRs.[96] TIL therapy will not be specifically covered in this review but has demonstrated efficacy against melanoma,[97–100] epithelial cancer,[101] cervical cancer,[102] metastatic breast cancer,[103] ovarian cancer,[104] and metastatic lung cancer[105] and may also benefit by enforced gene-overexpression[106–108] or gene-knockdowns.[109]

CARs, on the other hand, are synthetic receptors comprising an extracellular ligand-binding domain, usually an antibody-derived single-chain variable fragment (scFv), fused to intracellular costimulatory and activation domains, typically derived from the cytoplasmic region of CD28[110,111] or/and 4-1BB,[112,113] and CD3ζ, respectively (Figure 3B).[114] Because CARs recognize tumor antigen in a non-HLA-restricted manner, in principle they can be designed to target any antigen provided that it is cell-surface expressed, including proteins, carbohydrates, gangliosides, and even the oncogenic immunopeptidome (i.e., targeting oncogenic peptides within the HLA complex).[115,116] Target engagement triggers CAR dimerization and consequently T-cell activation.[117]

Notably, as comprehensively reviewed by others,[118–121] each CAR component including the scFv used (affinity, epitope proximity, sequence),[122,123] hinge or spacer region (length, flexibility, sequence),[124,125] transmembrane domain (TMD; typically a hydrophobic alpha helix),[126] choice of costimulatory endodomain,[127–130] and the CD3ζ immunoreceptor tyrosine-based activation motifs (ITAM) sequence used,[131,132] can impact CAR T-cell phenotype, fitness and function. Of note, the costimulatory endodomain influences several biological properties of the engineered T cells including, as demonstrated by Prof. Carl June and colleagues, persistence, memory formation, potency, and metabolism.[133] Overall, the CD28 endodomain is associated with greater and faster changes in protein phosphorylation as compared to 4-1BB,[134] and CD28-based CARs are superior against tumors expressing lower levels of TA than 4-1BB-based ones.[132] Seli et al.,[135] also recently demonstrated that chronic activation of CD28-based CARs drives classical T-cell exhaustion programs while 4-1BB-based ones enter into a novel state and that activation of the transcription factor FOXO3 is responsible for impaired function. In addition, it has been shown that the modular structure, or combination of particular hinge, TMD and intracellular signaling modalities,[136] as well as proximity of costimulatory endodomains to the cell membrane,[137] can impact function of CAR T cells through engagement with endogenous receptors and intracellular signaling molecules. Moreover, sequence modification of the CAR endodomain to better engage CD3ε and LAT can enhance T-cell activation in the context of low tumor antigen (TA) density,[138] and the JAK–STAT signaling domain of the IL-2 Receptor beta chain (IL-2Rβ) has been encoded in the CAR endodomain to endow cytokine signaling and improve anti-tumor responses.[139]

4 | TCR T-CELL THERAPY

A variety of TCRs have been tested by ACT, clinically and/or preclinically, including ones targeting tumor associated antigens (TAA) like the cancer testis antigen NY-ESO-1,[140] melanoma antigen recognized by T cells 1 (MART1) and glycoprotein 100 (gp100),[141] MAGE-A3,[142] MAGE-A4,[143] carbonic anhydrase IX (CAIX),[144] and carcinoembryonic antigen (CEA).[145] In addition, TCRs have been evaluated against human virus-derived targets such as human papilloma virus (HPV)-16 E7,[146,147] neoantigens[148,149] (i.e., peptides generated by non-synonymous mutations in tumor cells that are presented by HLA and recognized by anti-tumor T cells[150–152]), mutant KRAS,[153] public neoantigens,[154] and monomorphic MHC class I related protein (MR1) in an HLA-independent manner.[68]

Notably, tumor-specific TCRs, although typically much weaker in affinity and expressed at lower density than CARs,[155] can be triggered to induce full T-cell activation in response to fewer than 100 peptide-HLA complexes.[156] In contrast, CAR-T cell activation requires more than 1000 targets per APC.[157,158] Given such sensitivity, it comes as no surprise that ACT of TCR-T cells targeting TAA have led to toxicity in patients. For example, MART1- and gp100-specific TCR-T cell transfer was associated with damage to the skin, eyes, and ears,[141] anti-CEA TCR-T cells led to severe transient inflammatory colitis,[145] anti-CAIX TCR-T cells caused liver toxicity.[144] In addition, affinity-enhanced MAGE-A3 targeted TCR-T cells caused severe toxicity and two fatalities as a result of cross-reactivity with MAGE-A12 expression in the brain in one trial[142] and lethal cardiac toxicity in a second trial due to cross-reactivity with a peptide derived from the protein titin.[159,160] In contrast, for epithelial cancers, HPV-associated antigen E6 or E7 targeted TCRs (i.e., targeting tumor specific antigen; TSA) have demonstrated clinical efficacy, with better responses for higher functional avidity TCR-T cells.[146,147] Notably, a first-in-human clinical trial (NCT02876510) recently demonstrated safety and feasibility of treating patients with autologous personalized TCR-T cells targeting multiple HLA-p cancer targets.[161]

ACT of autologous T cells transduced to express an affinity-enhanced HLA-A2 restricted NY-ESO-1 specific (A2/NY) TCR has shown promise in the treatment of metastatic synovial sarcoma (an immune desert), with a 50% overall response rate. Interestingly, following comprehensive immune monitoring, Interestingly, Frankiw et al.[162] recently observed for the first time that at the time of disease progression in an HLA-A2$^+$ patient with an NY$^+$ undifferentiated pleomorphic sarcoma treated by A2/NY TCR-T cells (along with NY-peptide pulsed DC vaccination and PD-1 blockade) there was extensive methylation of the promoter region of NY and its tumor expression was completely lost. Antigen loss or downregulation is of course a well-known and frequent problem for CAR therapy of liquid tumors[163,164] and also occurs for solid tumors.[165] Another interesting recent paper is from Dr. Eleftheriadou and colleagues who examined the biomarker correlates of response in synovial sarcoma patients treated by high affinity NY-TCR-T cells (NCT01343043). They showed that responders have higher IL-15 levels pre-infusion and a higher number of transduced effector memory (CD45RA$^-$CCR7$^-$) CD8$^+$ T cells.[166]

We have worked extensively with an A2/NY TCR (BC1) originally isolated from an immunodominant T-cell clone of a long-surviving cancer patient in Lausanne, Switzerland[167,168] (in collaboration with Profs. Pedro Romero, Nathalie Rufer, and Daniel Speiser). NY-ESO-1

is expressed by a broad range of cancers including synovial sarcoma (as mentioned above), melanoma, and epithelial ovarian cancer[169,170] but in healthy adult tissues it is restricted to male germ cells.[171] A2/NY TCRs have demonstrated safety, persistence, and anti-tumor activity in the clinic (e.g., against melanoma and sarcoma), including TCRs that have been affinity-enhanced.[140,172–175] The BC1 TCR is near identical in sequence to the well-studied 1G4 TCR, and crystal structures of 1G4 and 1G4 variants, in complex with A2/NY have been solved.[176,177] Previously, by structure-based computational design (using the crystal coordinates for 1G4:A2/NY, 2BNR in the Protein Databank) and free-energy calculations[178] we (with Profs. Olivier Michielin and Vincent Zoete) developed a panel of increasing affinity A2/NY TCR comprising one to four amino acid replacements in the variable regions of the β- and α-chains[179,180] (Figure 4A).

Interestingly, we observed increased effector function for affinity-enhanced TCR-T cells but beyond the upper limit of natural TCR affinity (~5-1 μM) activity levels decreased (Figure 4A, *right*). This attenuation in activity is presumably in part due to impaired serial TCR triggering[181] as we could rescue function for high affinity TCR-T cells by pulsing HLA-A2$^+$ target cells with increased amounts of NY peptide. We found that the activity levels of T cells engineered to express a supraphysiologic affinity TCR (TCR wtc51m 0.015 μM), comprising four amino acid replacements identified by Dunn et al.[182] via phage display screening, were the most highly abrogated (Figure 4A, *right*). We have since demonstrated significantly improved tumor control (against A2$^+$/NY$^+$ melanoma Me275 and A375) by T cells expressing A2/NY TCRs in the upper limit of natural affinity (e.g., TCRs DMβ and A97L)[181] as compared to wild-type, and

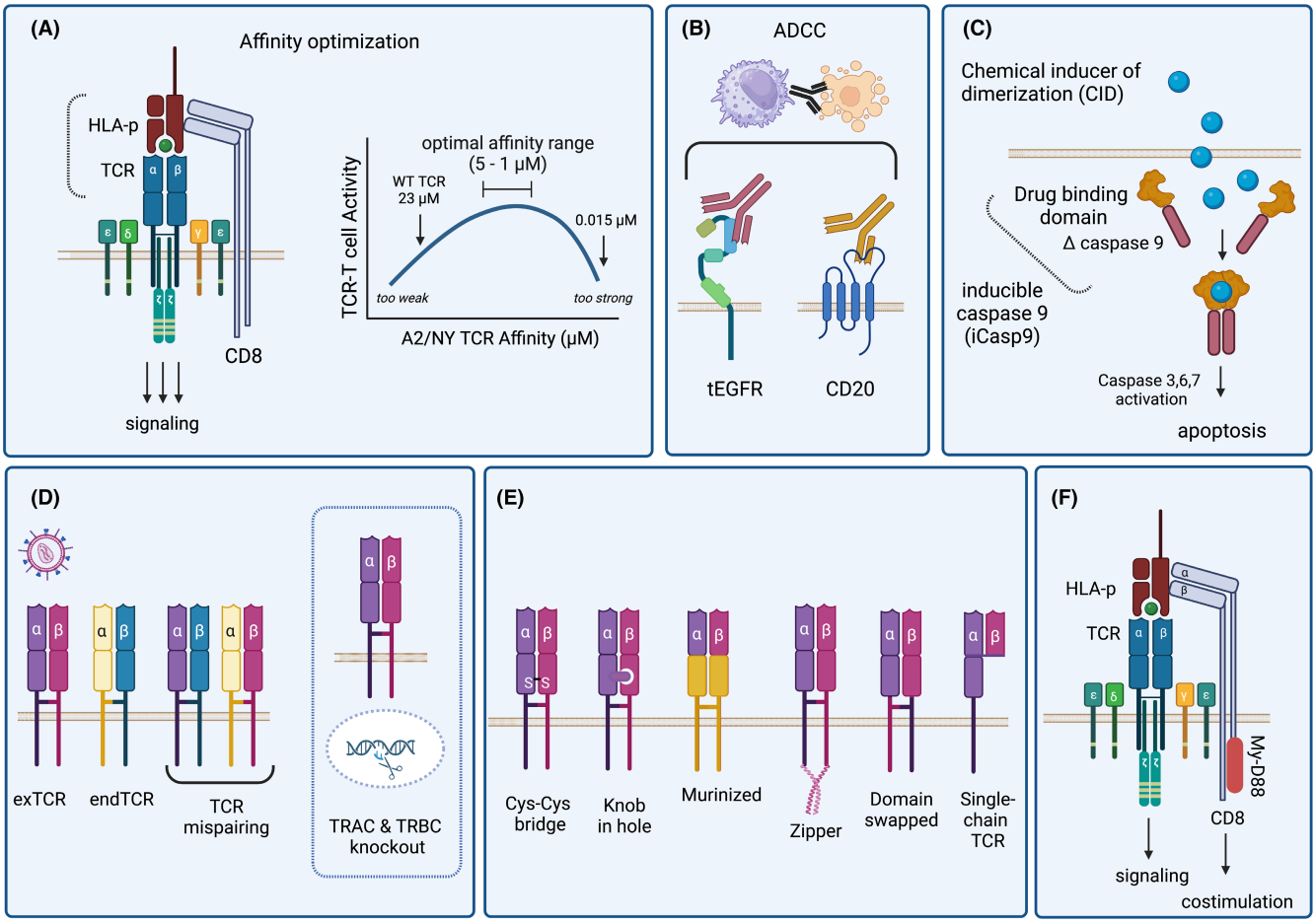

FIGURE 4 Strategies to improve the function and safety of TCR-T cells. (A) TCRs can be affinity optimized (left) to enhance the function of engineered T cells but beyond an affinity threshold activity levels of TCR-T cells are abrogated (right). (B) T cells can be coengineered to express a suicide switch such as truncated (t)EGFR[185,186] or CD20[187] which can be targeted for ADCC via monoclonal antibody administration. (C) The inducible (i)Casp9 system triggers apoptosis upon application of a small molecule that dimerizes the fusion proteins.[188,189] (D) Introduction of an exogenous (ex) TCR into a T cell can lead to mispairing with the endogenous (end)TCR chains but this can be circumvented by knockout of the T cell receptor alpha chain (TRAC) and beta chain (TRBC) loci.[59,195] (E) Other approaches to overcome chain mispairing (or/and improve association with the CD3 complex) include the introduction of a non-native disulfide bridge,[197] inversion of a "knob in hole" in the constant region to sterically hinder association with the endogenous chains,[198] TCR murinization or introduction of full murine constant regions,[199–201] the use of a zipper,[202–204] domain swapping,[205] or by developing a single chain TCR.[206] (F) Costimulation can be introduced into TCR-T cells by fusing a costimulatory endodomain to the CD8 coreceptor α-chain. Enforced expression of the CD8 coreceptor can also enable a CD8-restricted TCRs to function in CD4$^+$ T cells.[210]

no benefit upon ACT of T cells bearing the supraphysiologic affinity TCR wtc51m (Stefanidis et al., in revision, Semilietof et al., in preparation). Notably, one of our dual β-chain amino acid replacement TCR designs (DMβ; along with endogenous TCR knockdown) has been translated to the clinic by others and mediated tumor response.[175]

Taken together, it is evident that binding-enhanced TCRs can augment T-cell function but beyond an affinity-threshold activity levels are abrogated and specificity is lost (Figure 4A). Presumably, the optimal affinity range will vary from TCR to TCR. Of note, in collaboration with Prof. Pedro Romero and his group, we have shown that the overexpression of the microRNA (miR)-155, which is critical for T-cell expansion and survival,[183] can increase T-cell tumor control in the context of weak affinity TCR:HLA-p interactions.[184] Clearly, it is critical that TCRs selected and developed for T-cell engineering are carefully evaluated for function and cross-reactivities, especially against vital organs,[159,160] and it is further advisable that they are coengineered with some sort of suicide- or safety-switch. Examples of suicide-switches include coexpression of truncated (t)EGFR[185,186] or CD20[187] such that in the event of a severe adverse response the corresponding monoclonal antibody (Ab) can be administered to trigger Ab-dependent cell-mediated cytotoxicity (ADCC) and thereby eliminate the transferred T cells (Figure 4B). Another approach is the inducible caspase 9 (iCasp9) system which comprises a fusion of Casp9 with modified human FK-binding protein that dimerizes upon small molecule administration and triggers T-cell apoptosis[188,189] (Figure 4C). Notably, both tEGFR and iCasp9 are currently being employed in clinical studies (Table 1). A major disadvantage of a suicide-switch is that using it will terminate a very expensive therapy in the patient, probably in quite an advanced stage of disease. Hence, approaches that can be used to reversibly tune up or tune down activity levels, such as with a CRASH-IT switch (Chemically Regulated – SH2-delivered Inhibitory Tail),[190] a fusion protein coengineered into T cells comprising the PD-1 tail that inhibits TCR- or CAR T-cell activation in the absence of drug, may be a more favorable option.

As more and more TCRs enter the clinic, it is prudent that strategies are implemented to prevent exogenous (ex)TCR chain mispairing with the endogenous (end)TCR α- and β-chains which may potentially generate autoreactive receptors in a patient.[191–194] This can be achieved, for example, by exTCR integration into the TRAC locus and concomitant TRBC knockout,[59,195] or dual TRAC and TRBC knockout and virus-mediated exTCR expression (Figure 4D). Alternatively, shRNA or miR-based approaches can be used to knock-down the endogenous (end)TCR chain(s).[35] An additional advantage of eliminating the endTCR is that the exTCR does not need to compete for assembly with the CD3 complex in the endoplasmic reticulum[196] which can be limiting to cell-surface expression levels of the exTCR.[191] Other strategies previously proposed to prevent mispairing (or favor CD3 complex association and thereby increase cell-surface expression/stability of the exTCR), include the introduction of a non-native disulfide bridge,[197] inversion of a "knob-into-hole" at the interface of the α- and β-chain constant regions,[198] the use of murine constant regions[199,200] or murinized TCRs,[201] jun-fos zippers,[202–204] domain swapping,[205] a single-chain TCR format,[206] and framework region engineering[207] (Figure 4E).

As previously mentioned, some TCRs are coreceptor dependent, meaning that a TCR originating from a $CD8^+$ T cell may not be functional if engineered into in $CD4^+$ T cells and vice versa. However, it may be desirable for ACT to have a mix of both $CD8^+$ and $CD4^+$ TCR-T cells.[166] One solution is to coengineer with both the TCR and its corresponding coreceptor. Moreover, costimulation can be built directly into the CD8 coreceptor endodomain to improve responses against TA and augment tumor control by TCR-T cells[208] (Figure 4F). As an alternative solution for integrating costimulation, TCR-T cells can be coengineered with a chimeric costimulatory receptor[209] (CCR; i.e., a CAR that does not include the CD3ζ endodomain).

5 | CAR T-CELL THERAPY

As mentioned, anti-CD19-CAR T-cell therapy of certain B-cell malignancies represents the most successful form of ACT to date,[211,212] with some curative responses confirmed.[213] Remarkably, complete remission rates of greater than 80% have been reported for relapsed or refractory (R/R) B-cell acute lymphoblastic leukemia (B-ALL) in multiple independent treatment facilities.[214,215] However, patient relapse is problematic as exemplified by the fact that only one third of mature lymphoma patients have long-term responses.[213] While non-hematological solid tumors remain a challenge (Figure 1) for CAR therapy, encouragingly several trials, as recently summarized,[119] have demonstrated varying levels of clinical efficacy including for CARs targeting human epidermal growth factor receptor to (HER2, for treating sarcomas),[216] disialoganglioside GD2 (for treating neuroblastoma and diffuse midline glioma),[217,218] IL-13Ra2 (for glioblastoma),[165] EGFR (for biliary tract cancers),[219] mesothelin (for malignant pleural disease),[220] Claudin-18.2 (for gastric and pancreatic cancer),[221] and prostate-specific membrane antigen (PSMA, for treating metastatic castration-resistant prostate cancer).[222] Excitingly, an overall response of 63% for anti-GD2-CAR T-cell treatment of R/R-neuroblastoma was reported this year.[223] In addition, anti-GD2 CAR natural killer T (NKT) cells demonstrated objective responses and safety in patients with R/R neuroblastoma.[224]

The choice of target TA is a major determinant a priori of both the efficacy and safety of CAR therapy. Ideally the target is tumor-specific (i.e., a TSA) and not a TAA, the latter of which can also be present, albeit at low levels on healthy tissues. Moreover, it is also favorable that the TA is homogeneously and stably expressed to circumvent tumor escape.[225–227] Like for TCRs, most antigens targeted by CARs are TAA rather than TSA. We recently built CAR T cells against N-glycoslylated ganglioside monosialic 3 (NGcGM3)[228] with previously described scFv derived from the 14F7 monoclonal antibody (mAb) developed at the Center of Molecular Immunology (Havana, Cuba).[229,230] NGcGM3 is present on the surface of a range of cancers including ovarian, breast, melanoma, and lymphoma as a result of metabolic incorporation from dietary sources and it is associated with tumor growth and immune suppression.[231] In humans NGcGM3 represents a TSA because, unlike for most mammals, humans lack the enzyme cytidine monophospho-N-acetylneuraminic acid

TABLE 1 Clinical trials involving coengineering strategies to improve CAR T-cell safety.

Safety mechanism incorporated into CAR T cells	Active or recruiting trials
iCasp9 suicide switch	NCT04429438, NCT04249947, NCT03373071, NCT04196413, NCT03373097, NCT03696784, NCT03016377, NCT02414269, NCT01822652, NCT04016129, NCT04432649, NCT05432882, NCT05436496, NCT05436509, NCT05438368, NCT05437328, NCT05437341, NCT05437315, NCT01953900
Truncated EGFR suicide switch	NCT05625594, NCT02028455, NCT02311621, NCT03103971, NCT03710421, NCT02159495, NCT01683279, NCT01815749, NCT02051257, NCT02146924, NCT04109482, NCT02153580,
Truncated CD19 suicide switch	NCT03244306, NCT04483778, NCT03070327, NCT03618381, NCT04185038, NCT03500991, NCT03330691
Truncated Her2 suicide switch	NCT04661384, NCT04003649, NCT04119024, NCT04214392, NCT04510051, NCT02208362, NCT03389230
RQR8 suicide switch	NCT03618381, NCT03330691
Coexpression of an inhibitory CAR	NCT05211557, NCT03590574
Dimerizing ON switch	NCT02442297
	NCT05105152

Note: Data collected from clincaltrials.gov, accessed until 27.04.23. Filtered for active and/or recruiting trials. Search terms used: "CAR T cell" or "TCR". Trials with published results: *NCT03373097*,[223] *NCT03016377*,[241] *NCT02414269*,[220] *NCT01822652*,[242] *NCT02028455*,[243] *NCT02153580*,[244] *NCT02208362*,[165] *NCT04185038*,[245] *NCT03500991*.[246]

hydroxylase (CMAH) and thus cannot convert N-acetylneuraminic acid (NAc) GM3 (NAcGM3) to NGcGM3. An important open question is if dietary modifications can improve tumor control by such CAR T cells.

We have also evaluated CAR T cells targeting PSMA[232] and vasculature endothelial growth factor receptor 2 (VEGFR-2) to mediate vascular disruption. Indeed, the tumor vasculature represents an appealing CAR target for a variety of reasons including that TAA expressed by endothelial cells lining the vessels are more readily accessible to circulating T cells, and the TAAs are typically more stably and homogeneously expressed than ones on tumor cells.[163,233] However, while the collapse of blood vessels may deprive tumors of nutrients, elevated hypoxia may make the tumor cells even more aggressive, create a favorable environment for TAMs,[234] limit T-cell infiltration and confer resistance to immunotherapy.[235] In line with work done by others,[236] we observed limited B16 melanoma tumor control in C57BL/6 mice by anti-VEGFR-2-CAR T cells as a monotherapy. We ultimately determined, however, that soluble VEGF-A physically blocked engagement of the CAR with their shared target VEGFR-2, and we could rescue function of the CAR-T cells in the presence of anti-VEGF-A antibody.[74]

For CAR therapy, some of the most common side effects observed in patients are cytokine release syndrome (CRS), off-target effects, the immune effector cell-associated neurotoxicity syndrome (ICANs), anaphylaxis, tumor lysis syndromes, and infections.[237–240] Most cases are generally managed with supportive care, steroids, and immunosuppressive drugs, but they can be associated with substantial morbidity, with some patients requiring intensive care. CAR T cells further coengineered to secrete potent immunomodulatory molecules like IL-12 may generate even stronger adverse side effects.[106] For this reason, the use of a suicide-switch (Table 1, Figure 4B,C) and/or safety-enhanced CAR designs, such as logic gated or remote control CARs as described below, will be critical in safely advancing effective next-generation CAR therapies to the clinic.

6 | LOGIC GATED CARS FOR ENHANCED SAFETY AND FUNCTION

CARs were first invented by Eshhar and colleagues in the late 1980s[247] with the goal of enabling T cells to recognize and respond to target antigen in a non-HLA-restricted manner. CARs have evolved over the years, importantly to include built-in costimulation as described above (i.e., 2G & 3G CARs, Figure 3B), enabling superior persistence and tumor control.[248] In recent years, a wide range of innovative logic gated[134] CAR designs have been described that allow for enhanced safety or/and T-cell function. For example, in the split-CAR design (Figure 5A) two receptors targeting a pair of TAs are coexpressed, with one CAR including the CD3ζ endodomain and the other costimulation (i.e., the second is a CCR).[249] Thus, only when both CARs engage their respective TA can the T cells be fully activated (i.e., an AND-gate). Whereas, if T cells are engineered to express two fully functional CARs (dual CARs)[250] (Figure 5B) or a tandem CAR comprising two scFv targeting different TA[251,252] (Figure 5C), the engagement of either TA can trigger T-cell activation (i.e., an OR-GATE).[253] The innovative SynNotch system (an IF-THEN circuit) developed by Roybal et al., comprises a synthetic Notch receptor against one antigen which upon engagement drives the transcription of a conventional CAR against a second antigen[254,255] (Figure 5D). The intent of the above AND- and IF-THEN designs is to favor CAR T-cell activation directly in the TME and minimize toxicity in patients. Whereas, the OR-gate CARs help guard against TA

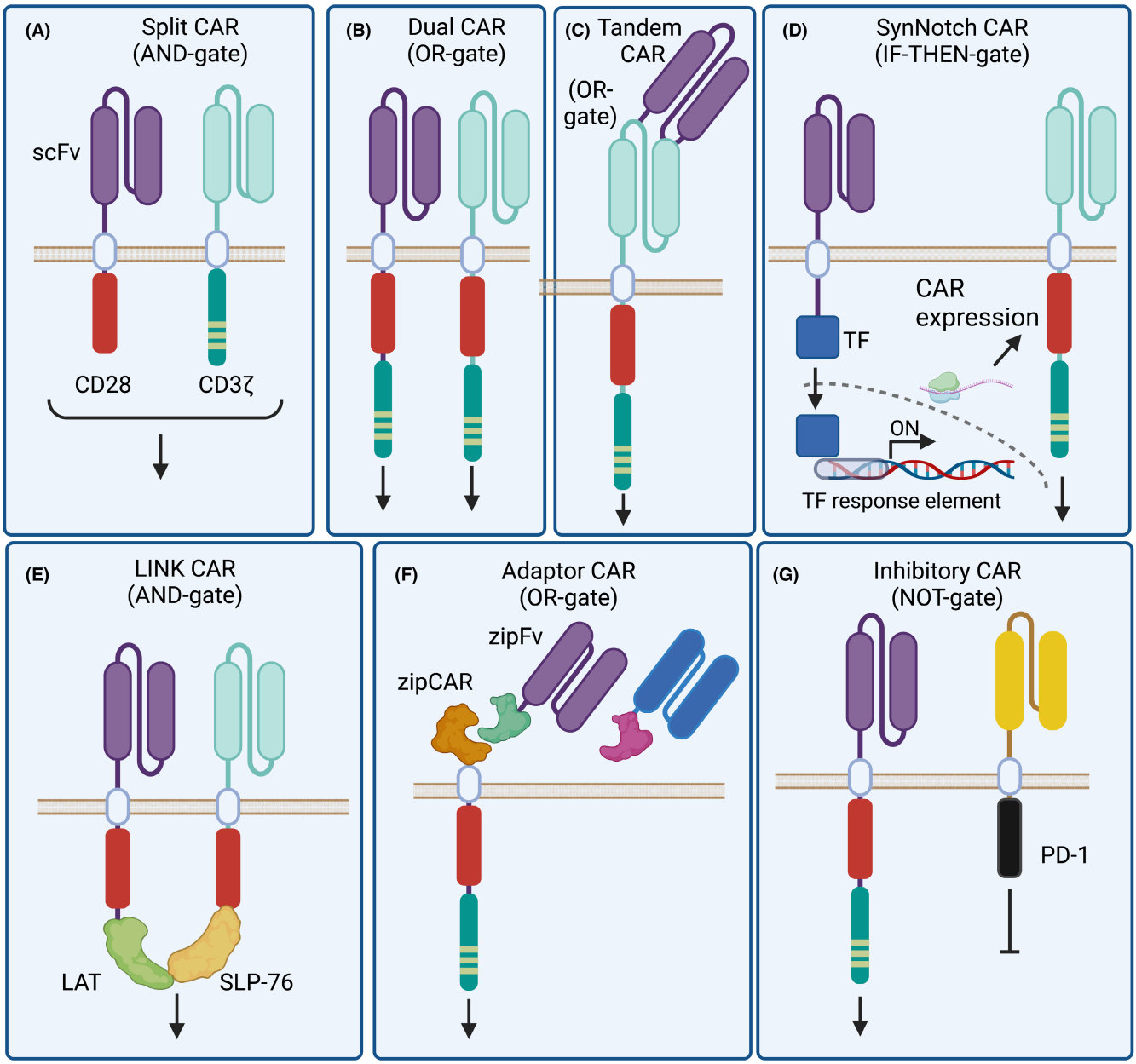

FIGURE 5 Examples of logic gated CARs. (A) For Split-CARs both receptors must be coengaged with their respective target antigens to trigger full T-cell activation.[249] (B) For Dual CARs, T-cell activation can occur if either CAR is engaged with target antigen.[253] (C) Tandem CARs comprise two scFvs in the same receptor targeting two different antigens and the engagement of just one will suffice for T-cell activation.[251,252] (D) For the SynNotch CAR, antigen binding triggers the transcription and cell-surface expression of a conventional CAR against a second antigen.[254,255] (E) LINK CAR signaling is driven by the association of LAT and SLP-76 upon coengagement of antigens by the two CARs.[257] (F) For adaptor-based CARs, the T cells do not directly bind tumor cells. Instead, the CAR specifically engages a tagged adaptor molecule which binds the tumor antigen. SUPRA CARs, for example, comprise a zipper in their ectodomain and require administration of tumor-targeting scFv fused to a zipper (adaptor protein).[266] (G) For the inhibitory (I)CAR[268] a scFv targeting an antigen found on healthy tissue cells is fused to an inhibitory endodomain such as from PD-1 to block T-cell signaling should the 2G CAR bind to its target but off-tumor.

loss, including via trogocytosis, a process in which target antigen is actively transferred from the tumor cell to the CAR T cell.[256]

More recently, Tousley et al., of the Prof. Robbie Majzner group developed the very creative and effective LINK platform (logic gated intracellular network; an AND-gate) comprising a dual CAR system but with one chain linked to LAT and the other to SLP76. Upon TA coengagement these signaling molecules colocalize and trigger T-cell activation starting downstream from ZAP-70[257] (Figure 5E). Additional logic gate approaches include so-called universal CARs in which the T cells themselves do not engage TA but instead rely on an adaptor molecule (i.e., OR-gates).[258] Examples of universal CARs include UniCARs,[259,260] RevCARs,[261] convertible CARs,[262]

PNE (peptide neoepitope specific) CARs,[263] SpyCatcher-based CARs,[264] and AdCARs,[265] amongst others. More recent examples include the SUPRA (split, universal, and programmable) CAR comprising zip-CAR T cells (there is a leucine zipper in the extracellular domain) and a zipFv (a tumor-targeting scFv adaptor with a zip that binds the zip-CAR T cells)[266] (Figure 5F), and the Co-LOCKR platform (the colocalization-dependent protein switches) requiring binding to a defined combination of TA to enable activation (i.e., an AND-gate).[267] Finally, a safety approach from the group of Prof. Michel Sadelain for preventing off-tumor toxicity is to coexpress an inhibitory (i)CAR comprising a scFv that recognizes an antigen found on healthy tissues fused to an inhibitory endodomain (i.e., a NOT-gate) such as from PD-1 or CTLA-4[268] (Figure 5G). In this way, if the 2G CAR binds to its target antigen but on a healthy tissue the iCAR will abrogate, or at least dampen, T-cell activation.

An obvious disadvantage of the combinatorial AND-gate sensing strategy is that loss of either antigen will lead to tumor escape. Whereas, as previously mentioned, dual and tandem CARs can help to guard against TA loss, and are being utilized in numerous ongoing clinical trials for hematological malignancies (e.g., CD19/CD20 and CD19/CD22, clinicaltrials.gov). In contrast, logic gates involving protein adaptors offer high versatility as multiple different TA can be targeted, but there is the added cost of generating recombinant proteins, challenges with respect to pharmacokinetics and biodistribution, and the risk of immunogenicity, in particular upon repeated administration.

7 | REMOTE CONTROL CARS FOR ENHANCED SAFETY AND FUNCTION

Remote control CAR designs, in which a small molecule is administered to specifically either turn on or tune down engineered T cells, provide an interesting alternative to logic gates as they do not depend on combinatorial antigen sensing. Instead, remote control CARs would rely on careful patient monitoring and the clinician's expertise to administer appropriate levels of the regulating small molecule (dosage, timing, pausing). Indeed, tumors are highly variable in their properties within and across indications, and at baseline there can be important patient to patient variation in the quality of the T cells available for engineering, making it difficult to correlate dosing with the pharmacokinetic properties and activity levels of CAR T cells. However, it has been observed that in patients with high tumor burden there is a greater initial expansion and risk for developing severe CRS, and continuous antigen exposure will drive the CAR T cells to exhaustion and defective memory formation.[269–271] In theory, with a remote control CAR design, the clinician could fine-tune CAR T-cell activity levels immediately following transfer, and periodic resting could help to alleviate exhaustion[272] as well as optimize memory induction and expansion.[273]

For the ON-CAR (Figure 6A), first conceived by Prof. Wendell Lim and colleagues[274] and since adapted by others,[275] the scFv and the CD3ζ endodomain are separated on two independent transmembrane chains (i.e., a receptor chain and a signaling chain) that require priming by a small molecule for them to heterodimerize and only then can the ON-CAR T cells become activated in the presence of TA.[131,132] Specifically, Wu et al.[274] utilized the FK506 binding protein (FKBP) domain and the T2089L mutant of the FKBP-rapamycin binding domain (FRB*) that heterodimerize in the presence of a rapamycin analog (AP21967). Taking inspiration from their work, we developed a so-called STOP-CAR (Figure 6B). For the STOP-CAR, we similarly dissociated TA binding from signal 1 of T-cell activation (i.e., the CD3ζ endodomain) on two separate chains. However, in the native state, the STOP-CAR receptor and signaling chains associate via a computationally designed, chemically disruptable heterodimer (CDH) developed by our collaborators Prof. Bruno Correia and Dr. Pablo Gainza.[72] Hence, in the presence of TA, STOP-CAR T cells are fully functional, but upon coadministration of a disruptive small molecule (A1155463)[276] the two chains disassociate and the T cells cannot be activated. We demonstrated that the effect was reversible as withdrawal of the small molecule allowed reactivation of the STOP-CAR T cells, both in vitro and in vivo. Together, we have since developed STOP-CARs comprising lower affinity interfaces in the CDH and that are responsive to clinically approved small molecules, and an ON-CAR (manuscripts in preparation).

More recently, OFF-switch CARs comprising degrons that can be rapidly and reversibly targeted for degradation upon administration of lenalidomide[277–279] (Figure 6C) have been developed. Briefly, thalidomide and its analogs lenalidomide and pomalidomide act as molecular glue by bringing together $CRL4^{CRBN}$ E3 ubiquitin ligase and degron-tagged (motifs derived from C2H2 zinc finger domains) proteins[280,281] which are subsequently ubiquitinylated and degraded by the proteasome. Notably, Profs. Benjamin Ebert and Marcela Maus[278] also developed a split ON-switch CAR comprising a ZFP91-1KZF3 hybrid zinc finger (mutated to protect from ubiquitinylation) in the endodomain of the receptor chain, and CRBNΔ3 in the endodomain of the signaling chain which dimerize upon administration of lenalidomide.

Additional, examples of remote control CARs are destabilizing domain (DD)-controlled and protease-based. Briefly, the group of Prof. Crystal MacKall incorporated a FK506 binding protein 12 (FKBP) DD into a CAR[282] (Figure 6D) that induces rapid degradation in the absence of a stabilizing drug which acts in a dose- and time-dependent manner.[282,283] Importantly, they also demonstrated in their study that transient rest of CAR T cells restores functionality via epigenetic remodeling.[272] In addition, the MacKall group developed a protease-based CAR termed SNIP (signal neutralization by an inhibitable protease), comprising a standard CAR including a protease cleavage sequence, and a second transmembrane chain including NS3 protease in the cytoplasmic region. In the absence of small molecule (grazoprevir) the CAR will be cleaved by the protease and become non-functional (Figure 6E). The group of Prof. Wilson Wong also developed protease-based CARs referred to as the VIPER (Versatile ProtEase Regulatable) system, in both an ON- and OFF-switch format[284] (Figure 6F,G). Other protease-based systems include SMASh CARs (small molecule-assisted shutoff), also known

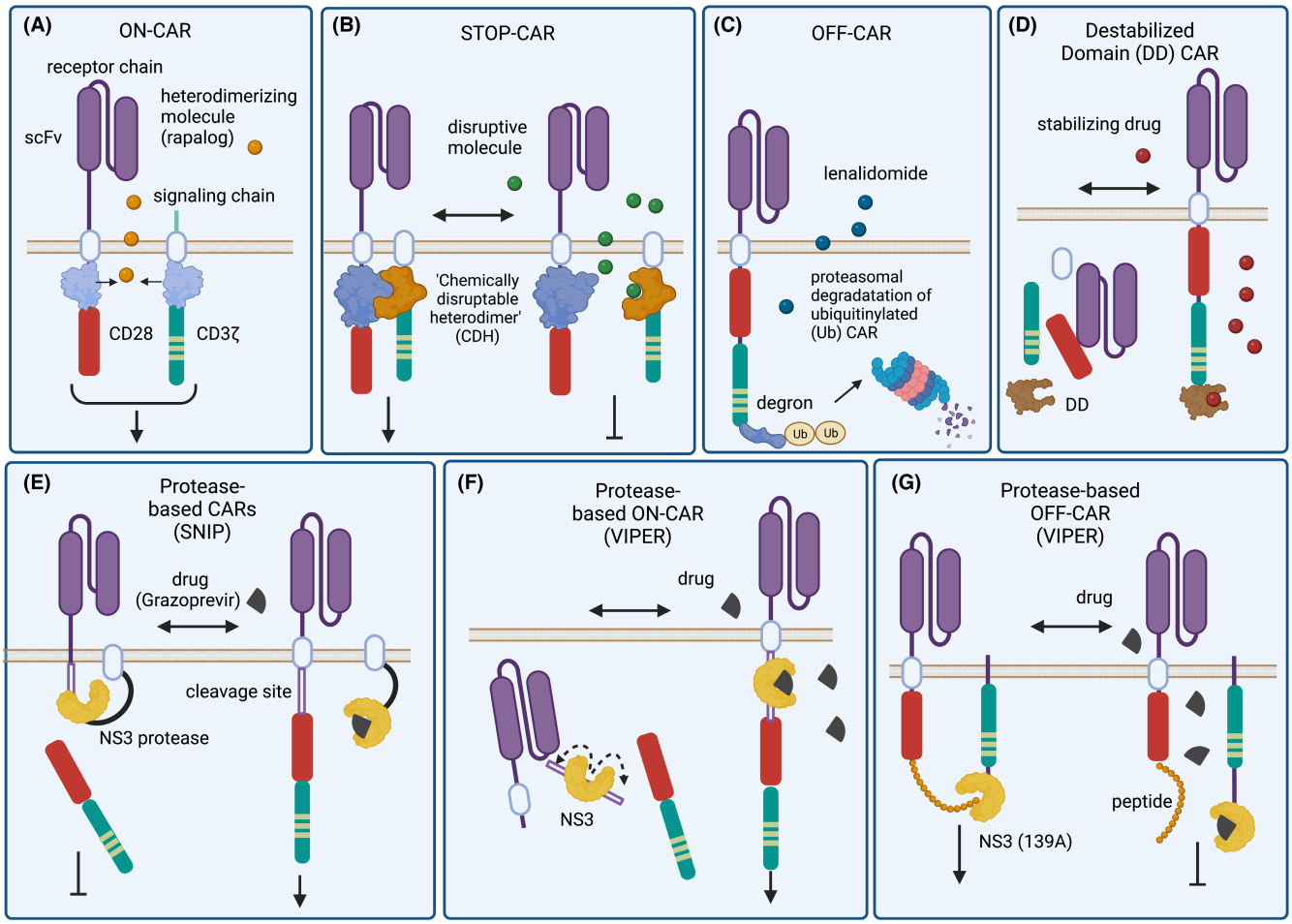

FIGURE 6 Examples of remote control CARs. (A) For the ON-CAR, the tumor antigen binding moiety and the CD3ζ endodomain are separated on the receptor and signaling chains, respectively, The two chains must be brought together by a heterodimerizing small molecule (rapamycin analog AP21967) before the T cells can be activated.[274] (B) The STOP-CAR is a heterodimeric receptor that dissociates tumor antigen binding on the receptor chain, from CD3ζ (signal 1 of T-cell activation) on the signaling chain. In the native state the two chains associate via a computationally designed chemically disruptable heterodimer (CDH) and the STOP-CAR can be activated in the presence of target tumor cells. Upon application of a disruptive small molecule (A1155463)[276] the two chains dissociate and the STOP-CAR T cells cannot be activated.[72] (C) For the degron-based OFF-CAR, the administration of lenalidomide leads to ubiquitinylation and consequently proteasomal degradation of the receptor.[278] (D) The destabilized domain (DD) CAR comprises FK506 binding protein 12 (FKBP) DD at its C-terminus[282] which induces rapid degradation in the absence of a stabilizing drug.[272] (E) The SNIP CAR (signal neutralization by an inhibitable protease), comprising a standard CAR including a protease cleavage sequence, and a second transmembrane chain comprising NS3 (non-structural protein 3) protease in the cytoplasmic region. (NS3 is derived from the hepatitis C virus which cleaves the viral polyprotein at junction sites.) Approved drugs including grazoprevir and danoprevir block the proteolytic activity of NS3.[300] In the absence of small molecule the CAR will be cleaved by the protease and non-functional. (F) The VIPER (Versatile ProtEase Regulatable) CARs comprise the NS3 protein flanked by cleavage sites. Hence in the presence of the drug (grazoprevir) the CAR is intact and functional. (G) The heterodimeric OFF-CAR VIPER format comprises a scFv receptor chain including an NS3-binding peptide and a signaling chain including the DAP10 ectodomain, catalytically dead NS3 (139A) and the CD3ζ signaling endodomain.[284]

as SWIFF-CARs (switch-off CARs). For this design, both a protease target site and a protease are encoded in the CAR construct along with a degron. When the CAR is in an ON-state the target site is cleaved, the degron is removed, and the CAR is cell-surface expressed. Upon administration of a protease inhibitor, however, the CAR-protease-degron complex will be degraded putting the cells in an OFF-state.[285] As a final example of remote control designs, Park et al.,[286] developed CARs using camelid antibody (V_{HH}) in which TA recognition can be directly reversibly blocked by administration of a disruptive small molecule that also binds the V_{HH}.

Ideally the components of remote control CARs are not immunogenic in nature, the small molecules used have sufficient half-lives, are well-tolerated by patients, and reversibly block function of only the engineered T cells. Overall, the system should allow simple, fast, reliable, and flexible control. While the tyrosine kinase inhibitor dasatinib has been proposed as an OFF-switch for CAR T cells (via Lck inhibition),[287] dasatinib will suppress the activity of all T cells and as such may not be suitable for longer term administration due to risk of infection and other side effects.[272,288] ON- and OFF-/STOP-CARs each have distinct advantages, disadvantages, and conditions for

preferential usage. For example, an ON-CAR may be the better choice when on-target but off-tumor toxicity is a potential issue. However, continued activity of the ON-CAR T cells requires long-term drug administration. Whereas, for previously tested CAR tumor targets that are considered safe, OFF-switch or STOP-CAR designs can enable reversible suppression of T-cell activity in the event of a CRS, as well as transient rest to alleviate exhaustion. Ideally, there is also spatiotemporal confinement and activation of the CAR T cells to circumvent on-target but off-tumor toxicity.[289] Synthetic biology and biophysical methods are being developed to address this, including the synNotch CAR as described above, as well as inducible CAR gene expression triggered by small molecules,[290–292] mechanosensory input,[293] light,[294] temperature,[295,296] ultrasound,[297] and hypoxia.[298,299]

8 | COENGINEERING APPROACHES TO TACKLE INHIBITORY LIGANDS AND MOLECULES

Numerous gene-cargo, including combinations,[75,301] have been developed to directly improve T-cell function or/and harness endogenous immunity such as through cellular repolarization[302] and epitope/antigen spreading.[303] Moreover, as mentioned, CRISPR-Cas9 screens have revealed T-cell intrinsic checkpoints that can be overcome by gene knockouts that rewire biological circuitry.[62] Excitingly, more and more coengineering strategies, several of which will be mentioned in this review, are being evaluated in the clinic (Table 2). In general, the function of T cells upon ACT can be directly augmented either by blocking inhibitory signals, like the PD-1/PD-L1 immune checkpoint axis[271,304] and TGFβ,[25,26] or by providing costimulation to the T cells (or/and endogenous immunity) such as by enforced secretion of cytokines.[305] Moreover, innovative synthetic biosensing switch receptors[306] can convert inhibitory into costimulatory signaling in T cells.

Here, we begin by presenting examples of design strategies for targeting both the PD-1/PD-L1 axis and TGFβ. Engagement of PD-1 on T cells with PD-L1 or PD-L2 (on immune or tumor cells) transduces signals that inhibit T-cell proliferation, cytokine production and cytolytic function, and monoclonal antibody (mAb)-mediated immune checkpoint blockade (ICB) of this axis has been a game-changer in cancer immunotherapy.[304] Several coengineering strategies have been taken to rewire this axis. For example, the PD-1 ectodomain has been fused to the CD28 or/and 4-1BB endodomain to convert inhibitory into costimulatory signaling (Figure 7A).[307–310] As previously mentioned, it is also possible to generate an iCAR by fusing the scFv targeting a healthy tissue antigen to the endodomain of PD-1. In this way, if the TCR or CAR coengineered in the same T cell engages its target antigen but on a healthy tissue, the iCAR can suppress on-target but off-tumor activity (Figure 7B).[268] Alternatively, it is possible to block this inhibitory axis by coengineering the tumor-redirected T cells to express either anti-PD-1 scFv or Ab[311,312] (Figure 7C, *left*) or anti-PD-L1 Ab.[313] High affinity PD-1 ectodomain variants have also been developed that can be used as decoys for engineering T cells.[314] These secreted scFvs, Abs and decoys can serve

TABLE 2 Clinical trials involving coengineering strategies to improve TCR- or CAR T-cell function and fitness.

Improving T cell persistence/ TME remodeling	Active or recruiting trials
Coexpression of cytokines	
IL-7/CCL19	NCT04381741, NCT05659628, NCT03198546
IL-15	NCT03294954,
IL-18	NCT04684563
IL-12	NCT04509726
Coexpression of a constitutively active cytokine receptor:	
IL-7R	NCT04099797, NCT03635632
Coexpression of a dominant negative receptor (DNR)	
TGFβR:DNR	NCT03089203, NCT05155189, NCT02650986, NCT04526509
PD-1:DNR	NCT04577326
Coexpression of a switch signaling receptor	
PD-1:CD28	NCT04850560, NCT05451849
IL-4R:/IL-2Rβ	NCT01818323
CD4 TCR cells coexpressing CD8α	NCT04526509
Coexpression of a secreted BiTE	NCT05660369
α EGFR	
Coexpression of a costimulatory ligand	NCT05693844
CD40L	
Coexpression of an NK inhibitory CAR	NCT05066022
Receptor/molecule knockout	
TGFβR	NCT04976218
PD-1	NCT04213469, NCT04768608, NCT03198546, NCT05732948
HPK1	NCT04037566, NCT03198546
CBL-B	NCT05169489
TCR	NCT03250325
Coexpression of a secreted immune checkpoint inhibitor	
αPD-1	NCT05373147, NCT04139057
αPD-L1	NCT04556669
Epi-R manufacturing to improve stemness/fitness	NCT04526509
Improving T cell homing	Active or recruiting trials
Coexpression of a chemokine receptor	
CXCR5	NCT05060796
CCR4	NCT03602157
Improving T cell metabolism	Active or recruiting trials
Coexpression of metabolic enzymes	
GOT2	NCT05120271
Multi-strategy combinations to improve ACT	Active or recruiting trials

TABLE 2 (Continued)

Improving T cell persistence/ TME remodeling	Active or recruiting trials
Coexpression of IL-15 & undisclosed suicide switch	NCT03907527
Coexpression of IL-15 & iCasp9	NCT05103631, NCT04377932, NCT03721068
Coexpression of IL-12 & EGFRt	NCT02498912
Coexpression of 4-1BBL & EGFRt	NCT03085173
Coexpression of αPD-1, αPD-L1 & iCasp9	NCT03356782
Coexpression of IL-15, HER1t switch & additional PD-1 downregulation	NCT05694364
Coexpression of IL-17/ CCL19 and/or αPD-L1/ αCTLA-4/ αTIGIT in PD-1/HPK1 knockdown T cells	NCT03198546
Coexpression of TGFβDNR & PD-1:CD28 switch receptor	NCT05489991

Note: Data collected from clincaltrials.gov, accessed until 27.04.23. Filtered for active and/or recruiting trials. Search terms used: "CAR T cell" or "TCR". Trials with published results: *NCT04213469*,[318] *NCT03085173*.[319]

not only prevent inhibition of the engineered T cells but also other endogenous, bystander immune cells in the TME (Figure 7C, *middle*). Finally, one can knockout[315] or knock-down[316] PD-1 in the T cells, or generate a dominant negative receptor (DNR) of PD-1 to abrogate negative signaling[316,317] (Figure 7C, *right*).

Along with coengineering strategies built around inhibitory receptors and ligands, it is also possible to guard against suppressive soluble factors in the TME, and further build switch-signaling receptors to augment T-cell function in their presence. TGFβ, for example, is one of the most dominant suppressive factors in the TME as it drives macrophage polarization to an immunosuppressive M2 phenotype,[320] pushes T-cell differentiation into Tregs,[321] supresses T-cell expansion, effector function, and migration through the upregulation of PD-1 and TIM-3,[322] and can promote tumor growth.[26,323] The group of Prof. Yvonne Chen demonstrated the construction of CARs reactive to TGFβ, and that response to soluble ligands in general is dependent on ligand-mediated CAR dimerization[117] (Figure 8A). Notably, the TGFβ-responsive CAR T cells protected nearby cells from the inhibitory effects of the molecule, probably via its sequestration.[324] Others have coengineered CAR T cells to secrete TGFβRII traps (Figure 8B)[325] or the trap fused anti-PD-1 scFv (Figure 8C),[326] knocked out TGFβRII by CRISPR (Figure 8D),[327] or expressed a TGFbRII-DNR (Figure 8E).[325,328,329]

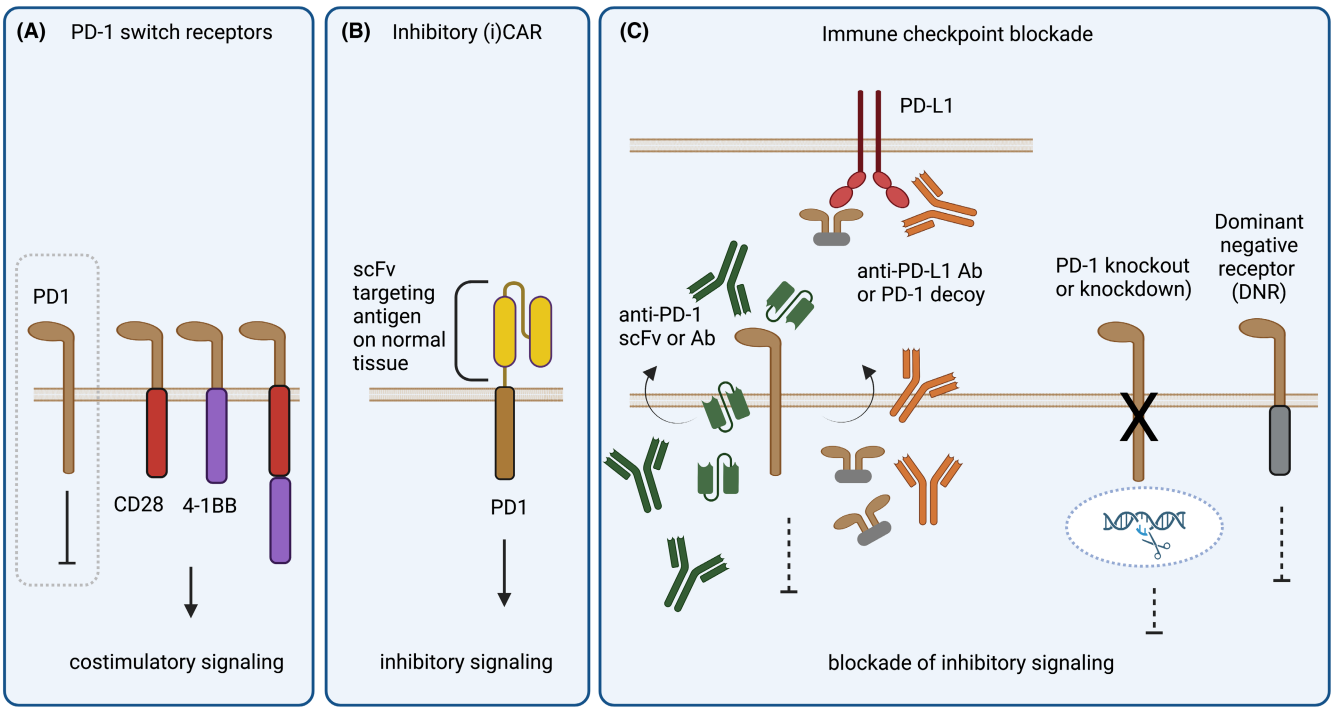

FIGURE 7 T-cell coengineering approaches for targeting the PD-1/PD-L1 axis. (A) Fusion of the PD-1 ectodomain to endodomains such as from CD28 or 4-1BB can convert inhibitory- to costimulatory signaling.[309] (B) Fusion of a scFv targeting an antigen found on healthy tissue to the endodomain of PD-1 can be used to generate an inhibitory (i)CAR that can serve as a safety mechanism to prevent on-target but off-tumor toxicity of a coexpressed conventional CAR.[268] (C) Left: In order to block suppressive PD-1 signaling, T cells can be coengineered to secrete anti-PD-1 scFv or Ab,[311,312] or anti-PD-L1 Ab[313] or PD-1 decoys.[314] Middle: alternatively, PD-1 can be knocked out[315] or knocked down[316] in the T cells. Right: or a dominant negative receptor (DNR) of PD-1 can be engineered into the T cells.[316,317]

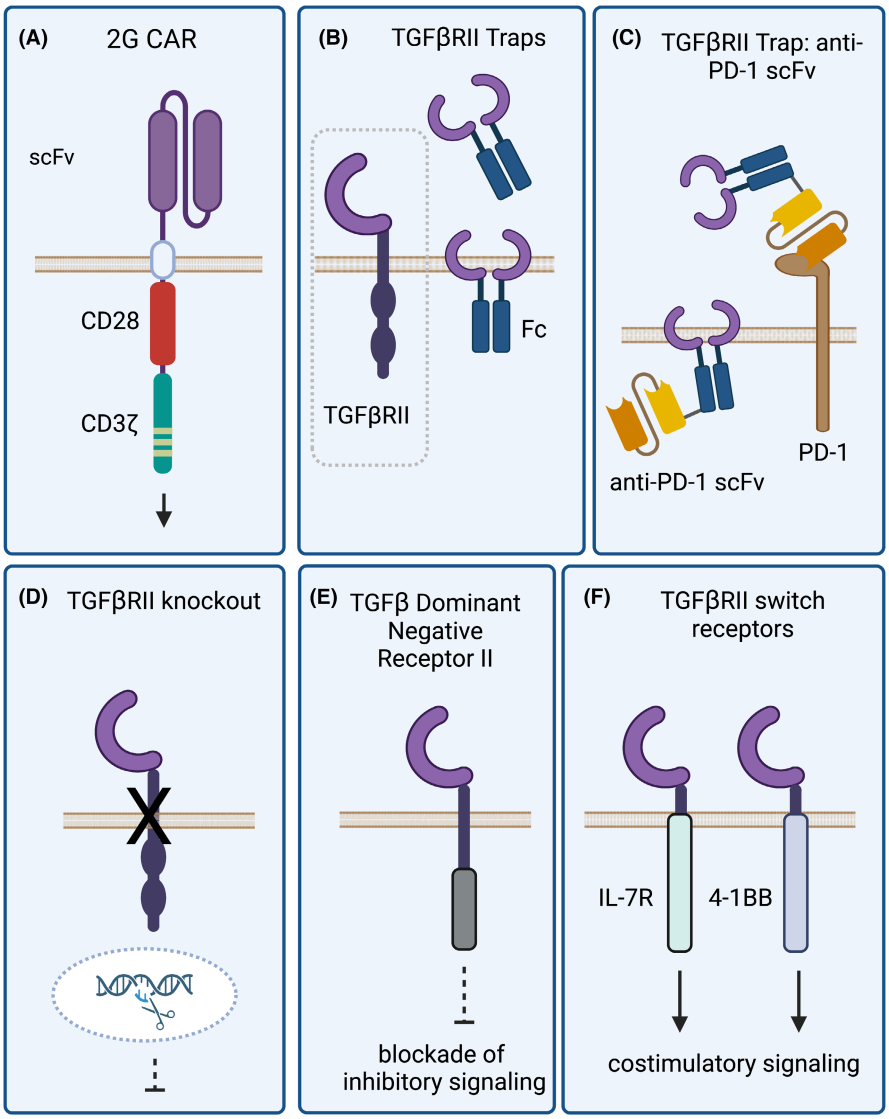

FIGURE 8 T-cell coengineering approaches for overcoming TGFβ-mediated suppression. (A) Anti-TGFβ 2G CARs can respond to soluble TGFβ.[117] (B) Traps comprising the TGFβRII binding domain fused to Fc tails can be used to sequester TGFβ in tumors.[325] (C) Traps made up of the TGFβRII binding domain fused to anti-PD-1 scFv[326] can be used to sequester TGFβ. (D) TGFβRII can by knocked out by CRISPR-Cas9 to rescue T-cell function[327] in the presence of TGFβ. (E) TGFβ DNRII can sequester TGFβ and abrogate inhibitory signaling.[325,328,329] (F) TGFβRII switch receptors comprising the transmembrane and endodomains of IL7Rα[330] or 4-1BB[67] can be used to covert an inhibitory signal into one that provides costimulation to the T cells.

Finally, switch receptors have been generated in which the TGFβRII ectodomain is fused to the transmembrane and endodomain regions of IL7Rα (Figure 8F, *left*),[330] and a CRISPR-Cas9 screening evaluating transgene knockins into the TRAC locus identified a TGFβRII:4-1BB switch receptor (Figure 8F, *right*) as a lead candidate to improve T-cell fitness and ability to control solid tumors.[67] Unfortunately, a recent clinical trial in which anti-PSMA CAR T cells were coengineered with a TGFβDNRII resulted in lethal toxicity in two patients as a result of ICANs,[222,328,331] thus underscoring the potency of next-generation CAR T cells and the importance of robust safety/control mechanisms (e.g., logic gate or remote control CARs, tEGFR, iCasp9, etc.) as more such therapies are translated to the clinic.

9 | T-CELL COENGINEERING STRATEGIES TO AUGMENT TUMOR CONTROL

As previously discussed, T-cell control of solid tumors is restricted post-transfer by a variety of biological processes (Figure 1). Next, as summarized in Figure 9, we will discuss coengineering strategies to specifically: (i) increase tumor homing and infiltration, (ii) enhance T-cell expansion and persistence, and, overcome (iii) suppressive signals, (vi) metabolic barriers, and (v) states of anergy and exhaustion, in the TME.

9.1 | Strategies to improve T-cell trafficking into tumors

T-cell homing via the tumor vasculature is a multi-step cascade broadly comprising (i), tethering and rolling via adhesive interactions of T cells with the endothelial cell surface lining the blood vessels, (ii) chemokine-chemokine-R mediated signaling which activates and enables firm T-cell adherence and cell arrest, and (iii), transendothelial migration of the T cells into the tumor.[332,333] However, several barriers hinder optimal T-cell trafficking into tumor tissue including a mismatch between the chemokines secreted by tumors and the chemokine-R expressed by T cells, the aberrant nature of the

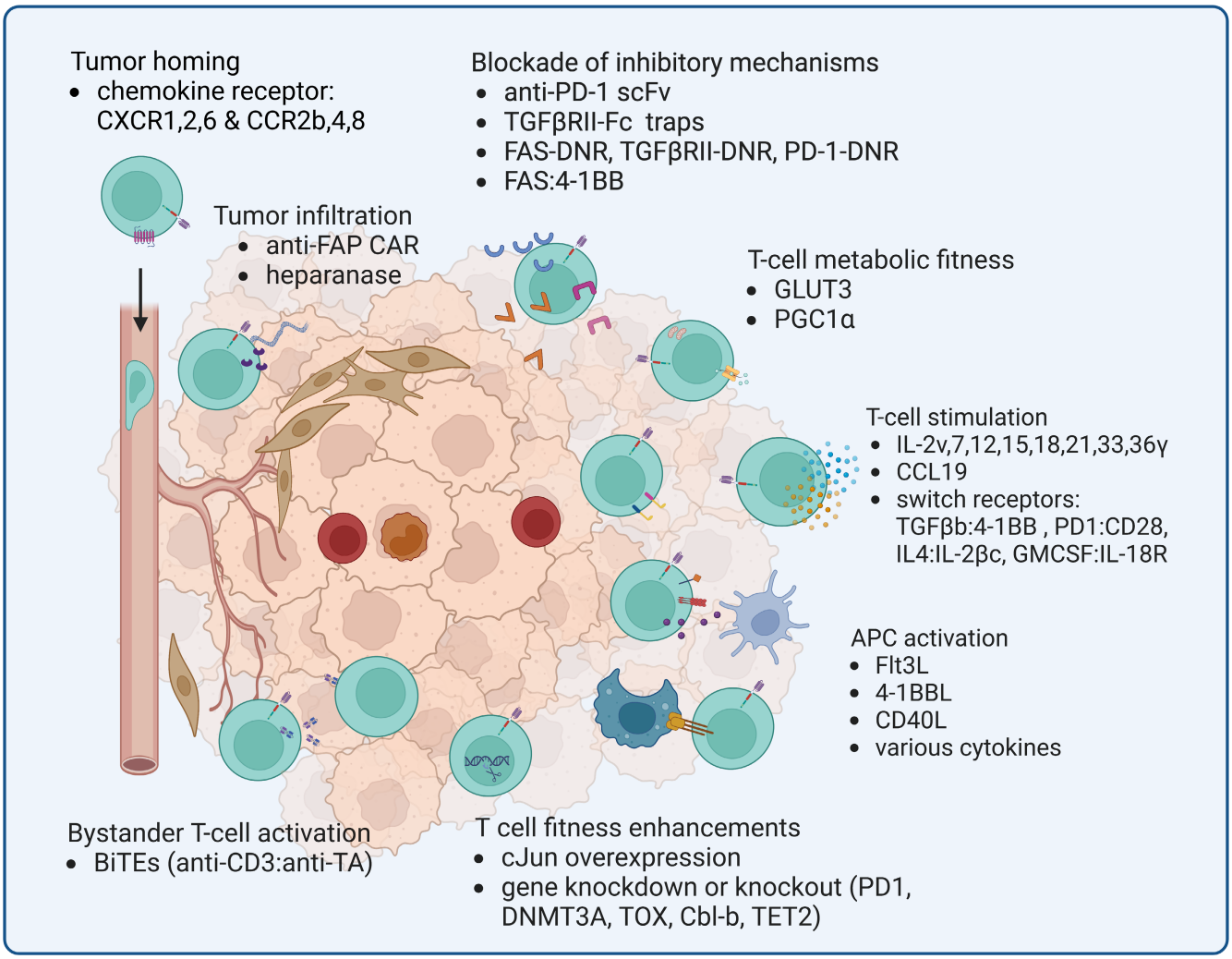

FIGURE 9 Examples of T-cell coengineering strategies to augment tumor control. Gene-modification strategies have been developed to improve T-cell trafficking and penetration into tumors, to block inhibitory mechanisms, to improve the metabolic fitness of T cells, to provide costimulation to T cells either directly or indirectly via the activation of APCs, to improve resilience to anergy and exhaustion, and to activate bystander T cells. Limited examples are shown. Oftentimes immunomodulatory factors secreted in the TME favorably reprogram multiple different immune cell types (e.g., T cells and APCs) but this complexity is not depicted. Moreover, T-cell therapies can comprise combined gene-modifications that can act synergistically but this is not shown.

tumor vasculature, and physical and cell-associated barriers of the stroma including the ECM and CAFs, respectively. Notably, there are a broad spectrum of CAFs that are highly suppressive in nature and can remodel the ECM into a dense matrix[334] (Figure 1).

Malignant and stromal cells, as well as leukocytes within the TME, secrete an array of chemokines (cytokines with chemotactic abilities and involved in regulating migration and trafficking) which attract immune cells expressing the corresponding chemokine-R.[1] However, as previously mentioned, a mismatch between chemokine-R expression by T cells and the chemokines present in the TME often exists. For example, G-protein coupled receptors CXCR3 and CCR5 are frequently expressed by TILs from melanoma, colorectal, and breast cancers[335] but their cognate ligands, CXCL9 and CXCL10, are often absent, requiring stimulation (i.e., by IFNγ, TNFα) for their upregulation.[336] Notably, House et al., demonstrated the upregulation of CXCL9 and CXCL10 was associated with responsiveness to dual PD-1/CTLA-4 blockade, that CXCL9 and CXCL10 were predominantly produced by macrophages, and that both $CD8^+$ T-cell infiltration and therapeutic efficacy were CXCR3 dependent. Moreover, they identified a novel transcriptional signature in macrophages associated with patient response to dual ICB.[337]

The most common strategy to increase homing is to coengineer T cells to express a chemokine-R matching the chemokine secretome of the target tumor[2] (Figure 9). An early proof of principle for this approach was presented by Kershaw and colleagues in 2002.[108] They observed CXCL1 upregulation in a subset of human melanoma samples, transduced T cells with the corresponding chemokine-R, CXCR2 (normally absent from quiescent and activated T cells[338–340]), and demonstrated enhanced chemotaxis towards tumor cells in vitro. In multiple pre-clinical studies since, CXCR2 coengineering of TCR- or CAR T cells has been demonstrated to confer enhanced T-cell infiltration and tumor control in models

of melanoma,[339,341] hepatocellular carcinoma,[340] glioma, ovarian, and pancreatic cancer.[338] CD70 CAR T-cells coengineered to express CXCR2 were able to eradicate later stage orthotopic glioma xenografts, and control subsequent rechallenges.[338] Clinical trials are currently registered for TILs (NCT01740557) and CD70 CAR T cells (NCT05353530) coexpressing CXCR2 (Table 2). The enforced expression of CXCR1,[338] CXCR6,[342] CCR2b,[343,344] CCR4,[345,346] or CCR8[347] have also been reported to improve the homing of CAR/TCR-T cells to a variety of tumor-types, including brain malignancies,[338,343] ovarian cancer,[338] pancreatic cancer,[338,342,345,347] mesothelioma,[344] and lymphoma.[346]

It should be taken into consideration that chemokines are not restricted to tumors and as such enforced expression of a chemokine-R may divert the T cells to undesirable anatomical locations.[348] In addition, the chemokine landscape within a tumor can be quite heterogeneous.[349] Notably, not all chemokines expressed by tumor cells may be favorable targets for engineering. For example, CXCL12 is expressed by a broad range of cancers[350] but the group of Prof. Amanda Lund has recently demonstrated that CXCR4 expression in TILs is associated with T cell egress into tumor associated lymphatic vessels.[351] Mechanistically, her group showed that high-affinity antigen interactions with the $CD8^+$ T cells could downregulate CXCR4 and upregulate ACKR3 (a CXCL12 decoy receptor) thereby reducing CXCL12 sensitivity and favoring T-cell retention. Thus, while CAR T cells have been modified to express CXCR4 in order to enhance their recruitment to CXCL12-rich bone marrow in a patient derived acute myeloid leukemia mouse model,[2] and is being evaluated in the clinic in the context of anti-BCMA CAR T cells against multiple myeloma (NCT04727008), for non-hematological solid tumors, egress could potentially be problematic.

In some instances, loco-regional delivery of CAR T cells is possible, and this can also overcome pulmonary sequestration of intravenously transferred T cells.[352–355] Notably, Hong et al., recently performed an in vivo loss of function screen with a CRISPR-Cas9 pooled library and identified ST3 beta-galactoside alpha-2,3-sialyltransferase 1 (ST3GAL1) as a negative regulator of the cancer-specific migration of CAR T cells. They determined that ST3GAL1 altered lymphocyte function-associated antigen-1 (LFA-1) endocytic recycling and that this could be overcome by enhanced expression of betaII-spectrin, a central LFA-1-associated cytoskeleton molecule. Indeed, CAR T cells overexpressing betaII-spectrin demonstrated improved tumor homing and tumor control.[64] Notably, our collaborator Prof. Christoph Hess recently demonstrated a role for magnesium sensing by LFA-1 and the regulation of T-cell effector function, including of CAR T cells.[356]

As previously described (Figure 1), tumor blood vessels are aberrant in nature (tortuous and leaky with insufficient pericyte coverage) as the neovasculature is usually rapidly formed under conditions of hypoxia. Indeed, low oxygen levels induce hypoxia inducible factor 1α (HIF1α) which subsequently drives the robust upregulation of pro-angiogenic genes such as VEGF-A. As we have previously reviewed,[4] a variety of strategies have been used to target and normalize the tumor vasculature to support T-cell infiltration. For example, TNFα coupled to tumor-vascular-targeting peptide (NGR or RGR peptides) can bind to new angiogenic vessels, causing the upregulation of adhesion molecules such as Intercellular Adhesion Molecule 2 (ICAM-2) and vascular cell adhesion molecule 1 (VCAM-1) on endothelial cells and improved T-cell infiltration.[357] In addition, as further described below, enforced expression of CD40L by T cells is associated with the upregulation of adhesion molecules.[358] Further, as previously mentioned, a variety of CARs targeting the tumor vasculature have been explored.[74,359]

Interestingly, Olivera et al.,[360] recently showed that transferred pools of tumor-specific TCR T cells transiently engineered with mRNA to express either IL-12 or IL-18 in tumor bearing mice conferred changes in the glycosylation profile of surface proteins that enabled adhesiveness to E-selectin (important during tethering and rolling stages of T-cell trafficking). Other changes included enhanced T-cell metabolic fitness and expression of cytokines, as well as elevated miR-155 control on immunosuppressive target genes.[360] Notably, they utilized an IL-18 decoy-resistant variant that is not functionally impaired by IL-18 binding protein (IL-18BP). Indeed, the group of Prof. Aaron Ring recently demonstrated that IL-18BP (a high affinity IL-18 decoy receptor) is frequently upregulated in human and mouse tumors and that it accounts for the poor response in tumor-bearing mice to IL-18. By directed evolution Zhou et al.,[361] developed a decoy-resistant variant of IL-18 (DR-18) and demonstrated anti-tumor efficacy in mice associated with elevated poly-functional effector $CD8^+$ T cells and fewer exhausted TOX^+ $CD8^+$ T cells.

Following successful extravasation into the tumor, T-cell movement and function can be impaired by a dense ECM and suppressive cells in the stroma including MDSCs and CAFs, a particular challenge in cancers such as metastatic pancreatic ductal adenocarcinoma (PDAC) which comprise a desmoplastic stroma.[362] One approach to assist T-cell migration is to engineer them with the ability to degrade components of the ECM. While T cells can naturally produce heparanase, an enzyme with the ability to cleave heparan sulphate proteoglycans deposited in the ECM, expression is lost over time in culture. Caruana et al.,[6] thus enforced expression of heparanase in anti-GD2-CAR T cells for improved motility and tumor control. Others have safely engineered reduced affinity CAR T cells targeting ICAM-1,[363] a molecule that is upregulated in the associated stroma of several carcinomas,[364] and demonstrated rapid tumor elimination. In addition, anti-FAP CARs for targeting CAFs have been shown to supress MDSC recruitment and improve the efficacy of coadministered anti-tumor CAR T cells.[365,366] It should be noted, however, that anti-FAP CAR T cells have been demonstrated to cause cachexia and bone toxicity by targeting FAP^+ stromal cells in the bone marrow in preclinical tumor models.[367] Moreover, there is the risk that CAF depletion can also accelerate tumor growth and metastasis by unleashing tumor cells from a tight nest.[368,369] The use of a remote control ON-CAR design, for example, may provide better control and security when targeting an antigen such as FAP.

9.2 | Strategies to enhance T-cell expansion and persistence in the TME

Following successful trafficking and tumor infiltration, rapid T-cell expansion and persistence are critical as T-cell presence and abundance are highly correlative of clinical efficacy.[211,215,370] A common strategy to support this is enforced expression, either constitutive or inducible, of immunostimulatory cytokines by the TCR- or CAR T cells. Such T cells have been termed "TRUCKS" (T cells redirected for universal cytokine mediated killing) by Prof. Hinrich Abken and colleagues,[371] and "armored" T cells by Prof. Renier Brentjens and associates.[372] We refer to coengineered T cells in general as "GEEPs" (gene-engineered for enhanced performance), as coined by Prof. George Coukos.

Several pre-clinical studies, including our own work, have demonstrated the beneficial impact of enforced IL-15 expression on T cells, and this strategy has been used in the clinic.[73,373–375] We demonstrated not only improved phenotype, fitness and function of IL-15 coengineered CAR T cells, but also favorable reprogramming in the TME including a higher proportion of activated NK cells and fewer M2 macrophages.[73] Others have demonstrated reshaping of the TME and improved function of tumor-redirected T cells coengineered to express IL-7,[376] IL-12,[377–380] IL-18,[381,382] IL-21,[383] and IL-36γ.[384] Notably, enforced expression of IL-7 and IL-15 can also protect against activation induced cell death (AICD) of T cells.[385,386] Interestingly, Profs. Li Tang, Ping-Chih Ho and colleagues recently demonstrated that IL-10-Fc can metabolically reprogram exhausted $CD8^+$ T cells for improved tumor control,[387] and engineering T cells for its local delivery is an appealing strategy. IL-10 engages IL-10Rα with high affinity and IL-10Rβ with lower affinity, thus Gorby et al., engineered an IL-10 variant having higher affinity for the β-chain and subsequently demonstrated that CAR T cells cultured in the presence of it were more effective at killing acute myeloid leukemic cells. Because the level of cytokine or other immunomodulatory molecule delivered to the TME can be critical to efficacy, the development and use of higher affinity cytokine variants for T-cell engineering may help to improve responses, provided that they are not immunogenic.[388]

To improve the safety of TRUCKs a variety of approaches have been taken, including the expression of the cytokines under an inducible promoter such as 6xNFAT as we have done with our dual inverted lentiviral vector (Figure 2),[35,38] transiently expressing them via mRNA electroporation,[360] masking the cytokine such as with a tumor-associated protease substrate,[389] engineering the T cells with orthogonal cytokine and receptor variants so that there is no effect of the molecule on endogenous immune cells,[390] or utilizing rationally designed cytokines such as an IL-2 variant that does not engage CD25 and thus preferentially acts on $CD8^+$ T cells and not Tregs[301,391] (Ortiz-Miranda et al., manuscript under review). Alternatively, Shum et al., for example engineered T cells to express an IL-7R that constitutively activates STAT5 (the downstream signaling molecule of IL-7). This approach provides costimulation to the engineered T cells for improved activation, proliferation, and persistence (and better anti-tumor activity), but not bystander lymphocytes thereby lowering the risk of systemic toxicity. The T cells also had lower levels of the proapoptotic protein Caspase-8 and Fas, both involved in AICD.[392] Similarly, Perna et al., enforced expression of the IL-7Rα chain to restore responsiveness to IL-7 and promote T-cell proliferation.[393] Notably, a primary reason for lymphodepleting regimens prior to T-cell infusions is to eliminate competition by endogenous immune cells for homeostatic cytokines (i.e., IL-2, -7, and -15). Thus, enforced IL7R expression, or of switch receptors that mimic homeostatic signaling, may allow for lower doses of conditioning lymphodepletion agents which are associated with toxicities and risk of infection. This may also be critical for the efficacy of next-generation T-cell products designed to harness endogenous immunity in the TME through the release of immunomodulatory molecules.[301] As a final switch receptor example, the group of Prof. Stephen Gottschalk developed a GM-CSF-IL18 receptor to create an autocrine costimulatory loop (i.e., GM-CSF produced by the T cells binds to the chimeric receptor and induces MYD88 signaling) enabling higher stress resistance and tumor control by coengineered CAR T cells.[394]

It is also possible to devise coengineering strategies to modulate the TME and mediate indirect costimulation/support of the transferred T cells. For example, a study from Profs. Phillip Darcy and Paul Beavis demonstrated that the combination of TCR- or CAR T cells engineered to secrete Fms-like tyrosine kinase 3 ligand (FLT3L), together with immune agonists poly (I:C) and anti-4-1BB, expanded intratumoral conventional type 1 DCs, induced epitope spread, and enabled enhanced tumor control upon ACT.[302] The group of Prof. Renier Brentjens has coengineered CAR T cells to express CD40L[358] (normally only transiently expressed after TCR stimulation), and others have developed a CD40L-CD28 switch receptor.[395] Notably, CD40L coengineering of anti-CD19-CAR T cells was associated with higher expression of HLA, adhesion and costimulatory molecules, and superior tumor control.[358] Furthermore, in a syngeneic tumor model, the Brentjens group went on to show that CD40L engineered T cells can license APCs in lymphatic tissues, and augment the recruitment of DCs and endogenous T cells to both lymphatic tissue and the tumor.[396] Others have coengineered CAR T cells with 4-1BBL and demonstrated reprogramming of the TME, improved T-cell persistence, decreased exhaustion and better tumor control.[397]

Finally, it is possible to engineer T cells with combinations of molecules that can act synergistically. An elegant study comes from the group of Prof. Koji Tamada in which T cells were coengineered to secrete the cytokine IL-7 and the chemokine ligand CCL-19. Their rationale for using this combination is that both molecules are essential for the maintenance of T-cell zones in lymphoid organs. The coengineered T cells exhibited less exhaustion, higher tumor infiltration and superior tumor control in several syngeneic models, and the therapy was dependent upon endogenous immune cell recruitment and activation.[398] Their work has been translated to clinical trials (NCT05659628 and NCT04381741) for targeting relapsed or refractory diffuse B cell lymphoma (Table 2). Finally, we recently demonstrated that ACT of T cells coengineered to secrete an IL-2

variant (that does not engage CD25) and a PD-1 decoy, pooled with T cells having enforced expression of the alarmin IL-33 and a PD-1 decoy, are reprogrammed in vivo to acquire a novel, synthetic effector state that deviates from canonical exhaustion. Importantly, the gene-modified T cells were endowed with superior tumor control, in the absence of host pre-conditioning.[301,399]

9.3 | Strategies to overcome immunosuppressive barriers in the TME

We previously presented a variety of coengineering strategies for targeting PD-1/PD-L1 axis that either convert engagement with PD-L1 into costimulatory signaling, or block inhibitory signaling (Figure 7). Other immune checkpoints have also been targeted by gene-engineering strategies. For example, the group of Prof. Cyrille Cohen fused the ectodomain of TIGIT (T cell immunoreceptor with Ig and ITIM domains; an inhibitory receptor found on T and NK cells that engages PVR/CD155) with the CD28 endodomain and demonstrated improved effector functions and tumor control by tumor redirected T cells.[400]

Interestingly, the group of Prof. Christopher Klebanoff observed that Fas (CD95) is highly expressed on patient-derived T cells used for ACT in the clinic. To circumvent the risk of FasL-mediated AICD in the TME they coengineered TCR- and CAR T cells with a FasDNR and demonstrated enhanced persistence and superior tumor control.[401] More recently, the group of Prof. Philip Greenberg fused the extracellular domain of Fas to the 4-1BB co-stimulatory domain, which they refer to as an immunomodulatory fusion protein (IFP), and demonstrated improved proliferation and persistence of coengineered TCR-T cells in a syngeneic ovarian tumor model, as well as significantly improved survival of the mice.[402]

We previously described engineering approaches to tackle the highly suppressive molecule TGFβ in the TME (Figure 8). Another soluble factor that has been widely exploited in the context of switch receptors is IL-4 which, like TGFβ, also induces M2 polarization,[403] supresses effector T cells,[404] and can exert tumor promoting effects. Notably, the group of Prof. John Maher fused the IL-4Rα ectodomain to the βc receptor subunit (common to IL-2, -7 & -15) and showed that its coexpression could be used to selectively expand CAR T cells in culture in the presence of IL-4.[405] Others have created IL-4 switch receptors comprising the transmembrane and endodomains of IL-7Rα[406–408] or IL-21R.[409] These switch receptors promote the expansion of T cells both in vitro and in vivo, and augment control of tumor xenografts. Interestingly, in a preclinical study the enforced expression of CSF-1R (macrophage colony stimulating factor 1 receptor) in CAR T cells conferred responsiveness to CSF-1 (a monocyte recruiting chemokine abundant in many solid tumors) including enhanced IL-2 driven CAR T cell proliferation and IFNγ production by activated T cells.[410] As CSF-1 recruits myeloid cells such as MDSCs that suppress tumor immunity[411] it would be interesting to determine if sequestration of CSF-1 in the TME by the CSF-1R engineered T cells dampens this effect.

Finally, as we have previously reviewed, adenosinergic signaling is highly suppressive to effector T cells (as well as NK cells and DCs).[20] Briefly, ATP levels are typically high in the TME as a result of necrosis, apoptosis, hypoxia, and persistent inflammation, and it can be catabolized by CD39 and CD73 to generate adenosine. Adenosine engages adenosine receptors (AR), predominantly A2AR in T cells. ARs are coupled to G-proteins that directly influence the activity of adenylyl cyclases which convert intracellular ATP into cyclic adenosine monophosphate (cAMP). In T cells, this second messenger, cAMP, then induces the activity of protein kinase A (PKA) which localizes to the immune synapse (by binding to the membrane protein Ezrin) and ultimately inhibits cytokine production and proliferative responses in both $CD4^+$ and $CD8^+$ T cells. Beavis et al.,[412] demonstrated that pharmacologic or genetic targeting (using shRNA) of A2AR in CAR T cells improved efficacy, especially in combination with PD-1 blockade. Taking an alternative approach, Newick et al., demonstrated that enforced intracellular expression of the RIAD (regulatory subunit 1 anchoring disruptor) peptide by T cells, which inhibits the association of PKA with Ezrin, can overcome the inhibitory effects of both adenosine and PGE_2 to T-cell signaling via PKA.[413]

9.4 | Strategies for overcoming metabolic barriers in tumors

T cells require energy and nutrients for survival, expansion and the execution of effector functions in the TME. However, to support proliferation, tumor cells utilize intracellular anabolic pathways to generate de novo macromolecules and robustly acquire building-blocks (lipids, amino acids, nucleotides) and energy (glucose) needed from the circulation, in direct competition with T cells and other immune infiltrate.[414] A variety of gene-engineering strategies have been taken to help T cells overcome metabolic barriers in the TME[415] and improve their fitness. One approach is to better equip the T cells to compete for energy sources and nutrients. For example, in Cribioli et al.,[416] we overexpressed the high-affinity glucose receptor GLUT3 in murine OT1 TCR $CD8^+$ T cells. As summarized in Figure 10, we demonstrated increased glucose uptake in the GLUT3 coengineered T cells as well as of energy storage in the form of glycogen and fatty acids, increased resilience to stress, overall better fitness and superior ability to control B16-OVA melanoma tumors. Moreover, in some mice that were cured by ACT we observed protection from rechallenge.

Tumors, both solid and liquid, also heavily consume the semi-essential amino acid arginine to support their proliferation. T cells are highly sensitive to low arginine levels because of their limited expression of the arginine resynthesis enzymes argininosuccinate synthase (ASS) and ornithine transcarbamylase (OTC). Fultang et al.,[417] thus took the approach of overexpressing ASS and OTC in CAR T cells and demonstrated increased proliferative capacity, as well as enhanced tumor clearance. Recently, Yan et al., revealed high cholesterol (a critical component of most cellular membranes and needed for proliferation) content in tumor cells as well as cells

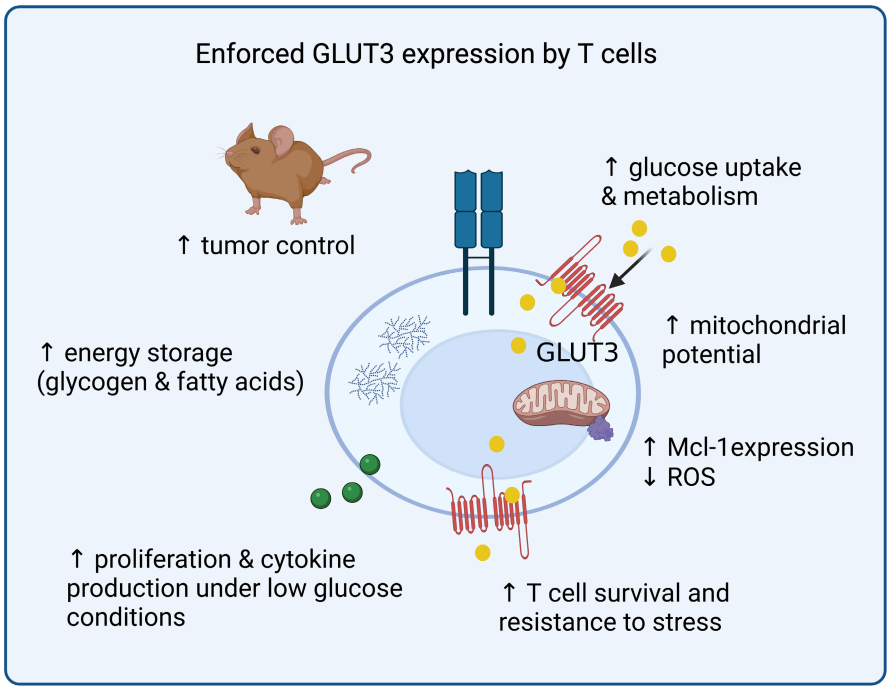

FIGURE 10 Summary of the beneficial impacts of coengineering murine $CD8^+$ T cells to express the high affinity glucose receptor GLUT3. GLUT3 coengineered T cells demonstrated increased glucose uptake and energy storage, higher proliferation and cytokine production under low glucose conditions, increased survival and resilience to stress, increased mitochondrial fitness, reduced ROS levels, higher abundance of the anti-apoptotic protein Mcl-1, and superior in vivo tumor control.[416]

from the myeloid compartment but that TILS had low levels and reduced uptake. They further demonstrated that cholesterol deficiency in the TME contributes to T-cell exhaustion via inhibition of mTORC1 signaling and that knockout of liver X receptor (LXR) in CAR T cells restores their cholesterol levels and augments antitumor function.[418]

As a final example of a metabolic intervention, others have overexpressed the peroxisome proliferator-activated receptor-γ (PPARγ) coactivator-1α (PGC-1α) to improve T-cell fitness. Briefly, PGC-1α is a member of a family of transcription coactivators that plays a critical role in the regulation of cellular energy metabolism, including mitochondrial biogenesis, and it is repressed in TILs.[419] Dumauthioz et al.,[420] demonstrated that PGC-1α overexpression in T cells favors central memory formation and stronger antitumor responses. Notably, however, PGC-1α can be repressed in TILs both at the transcriptional level and via phosphorylation by AKT. To counteract the latter, the group of Prof. Greg Delgoffe recently engineered a variant of PGC-1α that is resistant to post-translational regulation and demonstrated more effector-like programs as well as a long-lived memory state in coengineered human CAR T cells, and superior tumor control.[421]

9.5 | Strategies to overcome states of anergy and exhaustion in the TME

The transcriptional and epigenetic states of T cells regulate their functional properties and thus strongly influence the efficacy of ACT. Indeed, although not discussed here, important research efforts have focused on the generation of less-differentiated CAR T cells enriched with a memory population for ACT to allow superior expansion, persistence and tumor control.[422] Post-transfer, however, transcription factors (TFs), and epigenetic modifiers will continue to process both intrinsic and extrinsic signals into controlled gene expression programs, and in tumors T cells can be driven to enter into undesirable states including of anergy and exhaustion.[423] Coengineering strategies, including the overexpression or knockout of various TFs, or targeting epigenetic modifiers, can be taken to rewire T cells and support the maintenance of stemness properties[424] in the TME.

Anergy, briefly, is an induced hyporesponsive state in T cells resulting from low costimulatory or/and high coinhibitory stimulation and is characterized by incomplete T-cell activation and low IL-2 production.[425] The anergic state in T cells arises from factors that negatively regulate proximal TCR signaling resulting in NFAT homodimer formation due to the absence of activator protein 1 (AP1) transcription factor (AP1 is comprised of Jun homodimers and Jun-Fos heterodimers),[426,427] and the transcription of anergy inducing genes like the E3 ubiquitin ligase GRAIL,[428] Cbl-b (a master regulator of CD28 and CTLA-4 signaling), and ITCH.[429,430] Other anergy associated genes include epigenetic factors like IKAROS (via acetylation)[431] and Sirt1[432] (they are involved in histone modifications that promote anergy), and diacylglycerol kinase alpha (DGKα).[429]

T cell exhaustion, whereas, arises from chronic T-cell activation. Exhausted T cells are characterized by: (i) a progressive loss of effector functions and proliferative capacity, (ii) the upregulation and sustained expression of multiple inhibitory receptors (e.g., PD-1, CLTA-4, TIM-3, LAG-3), (iii) elevated susceptibility to apoptosis, and (iv) a rewiring of the transcriptional and epigenetic state.[11,433] Moreover, (v) exhausted T cells are characterized by suppressed mitochondrial respiration and function as well as decreased glucose uptake and glycolytic flux. Indeed, T-cell exhaustion is a critical barrier to the eradication of tumors by ACT and it is widely associated with poor clinical outcome.[434] The TFs NFAT[435] and TOX[436,437] and

NRF4A[438] have been shown to play a major role in driving T-cell exhaustion upon chronic antigen exposure.

In recent years, a variety of TFs, epigenetic modifiers and other genes have been knocked out, knocked down, or overexpressed, to overcome suppressive T-cell states. For example, the knockout/down of TFs TOX[437,438] NRF4A,[439] FLi1,[440] regulators of DNA methylation including DNM3TA[66] and TET2,[441] members of the BAF chromatin remodeling complex,[65,442] Cbl-b,[443] DGKα,[444] sorting nexin 9 (SNX9),[445] PD-1[446] and combinations of checkpoint inhibitors (PD-1, TIM-3, LAG-3),[447] can overcome exhaustion or/and anergy to rescue T-cell function. In addition, the enforced overexpression of TFs including BATF,[448] cJUN,[449] and Runx3[450] can augment T-cell activity.

Notably, while the targeting of selective inhibitory pathways can confer superior tumor control in preclinical studies, these responses are typically transient and do not necessarily protect against rechallenge due to other suppressive mechanisms at play in the TME.[20] Combinatorial coengineering including gene knockouts of immune checkpoints such as PD-1 and TFs involved in exhaustion like TOX will likely augment clinical responses but come with the risk that removing the brakes could result in uncontrolled proliferation of the T cells and other adverse events. Indeed, Jain et al.,[451] showed that biallelic deletion of TET2 in combination with enforced expression of BATF3 causes antigen-independent CAR T-cell expansion. This underlies the importance of robust preclinical testing and the integration of safety mechanisms in gene-modified cellular therapies.

10 | FUTURE PERSPECTIVES

Next-generation TCR- and CAR T-cell therapies under development against cancer hold tremendous translational potential. Improving clinical outcomes for more patients and cancer-types will require careful choice of target antigen(s), receptor-type(s) and design, personalized gene-cargo to allow better homing, provide costimulation or/and block suppressive mechanisms at play in the TME, and robust safety mechanisms. In addition, as we have previously reviewed, it is possible to combine T-cell therapies with other forms of cancer treatments.[75] Notably, there is evidence of synergy for irradiation with CAR T cells.[452] We recently demonstrated in a preclinical ovarian model that low doses of radiotherapy (LD-RT) which are non-toxic could trigger changes in the TME to reverse immune desertification, reprogram the myeloid compartment, and enable responsiveness to personalized combinatorial immunotherapy.[76] We are keen to explore LD-RT in combination with ACT as others have shown that it can help mitigate antigen escape to CAR therapy in a TRAIL-dependent manner.[453]

It is evident that both adaptive and innate immunity must be harnessed in order to cure solid tumors[454] thus necessitating synergistic combinatorial therapies.[148] Indeed, checkpoints of innate immunity, such as the CD47/Sirpα axis, should be addressed to harness APCs including DCs and macrophages in the TME[455] (Stefanidis et al., manuscript in revision). Recent studies have highlighted a crucial role for certain neutrophil populations in eliminating tumor antigen escape variants and mediating effective cancer therapy.[456,457] NK cells also have the capacity to detect and eliminate tumor cells in a non-HLA-restricted manner and have demonstrated important clinical responses.[458] Notably, in a preclinical study, Wang et al.,[459] recently demonstrated that optimally expanded NK cells can be combined with T-cell transfer to help guard against antigen escape. Hence, one could envision co-transfer of gene-modified NK cells, macrophages,[43] or MSCs,[36,37] with T cells. Or, the NK cells, for example, could be transferred in advance as first responders[460] to help seed a more favorable microenvironment for the T cells. Finally, combining complementary stromal-targeted with immune-targeted treatment modalities, such as coadministration of anti-FAP-CAR T cells, may help facilitate the entry of TA-specific T cells into solid tumors.[365,366,461]

This is a very exciting time in the field of T-cell engineering and of gene-modified cellular therapies in general for cancer and other diseases.[44] Our deeper understanding of tumor immunology and the dynamic nature of the TME, coupled with important breakthroughs in technologies like CRISPR-based genome editing,[462] the development of high through-put screening,[463,464] and implementation of machine learning strategies,[465] as well as advances in protein design[466] and synthetic immunity tools,[289] are enabling the rapid development of function and safety enhanced receptors and coengineering strategies. Not to mention, innovative approaches emerging to expand the capabilities of T cells. For example, Cieniewicz et al.,[467] recently reported a novel chimeric engulfment receptor (CER), comprising the extracellular domain of the phagocytic receptor TIM-4 fused to intracellular signaling domains from TLR2/TIR, CD28, and CD3ζ, and demonstrated enhanced T-cell cytotoxicity and target dependent phagocytic function enabling superior cross-presentation by the CER-T cells. In conclusion, optimized TCRs and CARs along with personalized gene-cargo delivery to address specific barriers at play in a patient's tumor and harness endogenous immunity, coupled with a robust safety strategies, as well as switch-receptors or/and gene knockouts or editing that can improve T-cell resistance to exhaustion and anergy, are certain bring important clinical benefits to patients in the very near future.

ACKNOWLEDGMENTS

We wish to sincerely thank and acknowledge Prof. George Coukos and current and former HI-TIDE T-cell engineering group members for their important contributions to our studies mentioned in the review. We wish to thank members of the Flow Cytometry Platform and the Animal Care Facility of the Epalinges Campus of the UNIL for their excellent support of our research, as well as the Center for Integrative Genomics at the UNIL for all of their sequencing support. We wish to graciously thank Ludwig Cancer Research, the Swiss National Science Foundation to MI (SNSF# 310030_204326), the ISREC Foundation, and the Prostate Cancer Foundation for their generous financial support of our work in the HI-TIDE platform over the years. All figures were made with BioRender.com. Open access funding provided by Universite de Lausanne.

CONFLICT OF INTEREST STATEMENT

Intellectual property filings have been made for our dual inverted lentiviral vector and production protocol as well as various CARs, TCRs, and T-cell coengineering strategies developed in the lab.

DATA AVAILABILITY STATEMENT

Data related to our studies presented in the review are available upon request.

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How to cite this article: Giordano Attianese GMP, Ash S, Irving M. Coengineering specificity, safety, and function into T cells for cancer immunotherapy. *Immunol Rev*. 2023;320:166-198. doi:10.1111/imr.13252