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REVIEW

Engineering enhanced chimeric antigen receptor-T cell therapy for solid tumors

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The early clinical success and subsequent US Food and Drug Administration approval of chimeric antigen receptor (CAR)-T cell therapy for leukemia and lymphoma affirm that engineered T cells can be a powerful treatment for hematologic malignancies. Yet this success has not been replicated in solid tumors. Numerous challenges emerged from clinical experience and well-controlled preclinical animal models must be met to enable safe and efficacious CAR-T cell therapy in solid tumors. Here, we review recent advances in bioengineering strategies developed to enhance CAR-T cell therapy in solid tumors, focusing on targeted single-gene perturbation, genetic circuits design, cytokine engineering, and interactive biomaterials. These bioengineering approaches present a unique set of tools that synergize with CAR-T cells to overcome obstacles in solid tumors and achieve robust and long-lasting therapeutic efficacy.

Key words: CAR-T, genetic circuits, synthetic biology, engineered cytokines, biomaterials, vaccine

INTRODUCTION

Chimeric antigen receptor (CAR)-T cell therapy has become a promising therapeutic modality against cancer over the last decade. The early clinical success of CAR-T cell therapy in hematologic malignancies led to a strong commercial investment in establishing adoptive cell therapy as a viable clinical therapy and the first licensure of CAR-T therapies by the US Food and Drug Administration (FDA) in 2017.[1,2] So far, six CAR-T products have been approved by the FDA for the treatment of relapsed and refractory B-cell acute lymphoblastic leukemia,[2,3] non-Hodgkin lymphoma,[2,4] and multiple myeloma,[5] and >1300 registered CAR-T clinical trials (clinicaltrials.gov) are currently ongoing globally.

Despite success in hematologic malignancies, CAR-T cell therapy has been largely disappointing in treating solid tumors.[2,6] Strides have been taken to elucidate the underlying mechanisms of CAR-T failure in solid tumors, and many obstacles have been identified using preclinical models and in early-phase clinical trials (Table 1). In contrast to hematologic malignancies, solid tumors have a number of features that pose inherent challenges for CAR-T cell therapy. For example, in B-cell acute lymphoblastic leukemia, the CD19 antigen is restricted to B-cell lineage, including leukemic and healthy B cells, and B-cell aplasia caused by CD19-CAR-T therapy is clinically manageable.[7] However, most solid tumors do not have such a lineage-restricted surface antigen for CAR to target without causing severe off-tumor toxicity. In rare cases, cancer mutation-derived surface antigen can be highly tumor specific, such as epidermal growth factor receptor variant III (EGFRvIII), yet its expression has extensive intratumoral and interpatient heterogeneity.[8] Loss or downregulation of this tumor-specific antigen expression inevitably leads to tumor escape.[8] In addition, anti-CD19 CAR-T cells experience rapid expansion given their co-existence with $CD19^+$ leukemic cells or healthy B cells in circulation and in lymphoid compartments upon infusion. The abundant antigen stimulation at the early stage of CAR-T infusion has proven critical for robust engraftment and the long-term persistence of CAR-T cells.[9] However, in solid tumors, the initial antigen exposure to CAR-T cells is limited, severely compromising CAR-T expansion, engraftment, and local CAR-T numbers. The dysregulated metabolism in solid tumors and continuous stromal remodeling during tumor

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growth also created an immunosuppressive tumor microenvironment (TME) limiting T-cell infiltration and promoting T-cell dysfunction.[10] Recent evidence also indicated that CAR-T cell-mediated cytotoxicity has a distinct requirement for cell adhesion molecules, which are essential for solid tumor but negligible for leukemia treatment.[11] Besides these solid tumor-specific hurdles, CAR-T cells are also subjected to similar restrictions as they experience in hematologic malignancies, such as chronic stimulation-induced exhaustion and treatment-associated systemic (i.e. cytokine release syndrome) and neuronal toxicity.[2,12]

The detailed investigation of intrinsic and extrinsic determinants of CAR-T cell activity *in vivo*, together with the implementation of smart engineering strategies to steer T-cell responses favorably, is advancing CAR-T cell therapy for solid tumors at a rapid pace. Earlier work on CAR-T cell engineering focused on identifying suitable targets on tumor cells, tuning CAR sensitivity and refining signaling domains. Interestingly, improving the CAR design itself through modulating costimulatory domains barely exceeded the performance of the established second-generation CARs with CD28 or 41BB costimulatory domain,[13-15] indicating dire needs for alternative approaches to achieve combinatorial benefit and synergy with CAR-T cell therapy.

This review will focus on the most recent engineering solutions for enhancing CAR-T cell therapy to address the challenges presented by solid tumors, including targeted single-gene perturbation, rationally designed genetic circuits based on synthetic biology principles, engineered cytokines, and nongenetically encodable biomaterials.

ENGINEERING CAR-T CELLS VIA TARGETED SINGLE-GENE PERTURBATION

The standard CAR-T cell manufacture involves the activation of T cells followed by the introduction of a constitutively expressing CAR via a lentiviral vector, enabling redirection of nontumor reactive T cells against tumor cells expressing a desired surface antigen. As a result, without additional genetic modifications, CAR-T cells are subjected to various T-cell intrinsic limitations. The success of checkpoint blockade therapy in solid tumors has inspired numerous efforts in understanding and reversing T-cell exhaustion (Table 1), and various evidence-based or unbiased genetic screens have been carried out to systemically elucidate genetic limiting factors in T cells to improve the overall fitness of CAR-T cells for enhanced solid tumor therapy.

Enforced constitutive expression of key genes that are naturally silenced during CAR-T cell dysfunction represents a promising and straightforward approach to improving CAR-T function. Lynn et al.[16] found that activator protein 1 (AP-1)/basic leucine zipper (bZIP) and bZIP/interferon regulatory factors (IRF) transcription factor (TF) binding motifs were highly enriched in exhausted CAR-T cells as a result of tonic signaling and showed that restoring the classic AP-1 heterodimer c-Fos/c-Jun by overexpressing c-Jun could restore cytokine expression, increase proliferation, and overall functionality of CAR-T cells in solid tumors. Interestingly, overexpressing another AP-1 and activating transcription factor (ATF) TF superfamily member, *BATF*, was also shown to counter exhaustion in tumor-infiltrating CAR-T cells,[17] highlighting the critical role of AP-1 signaling in T-cell exhaustion regulation. Similar enhancement of CAR-T function, reduction of exhaustion, and overall efficacy in solid tumors was also found with overexpression of master TFs regulating T-cell metabolism, such as an engineered *PGC-1α*,[18] or key cytokines and chemokines supporting T-cell fitness and migration, such as interleukin (IL)-12,[19] IL-7, and chemokine (C—C motif) ligand (*CCL19*).[20] Overexpressing alternative genes, such as *LBTR*, identified from a recent unbiased gain-of-function screen for synthetic drivers of T-cell proliferation will likely also contribute to the anti-solid tumor efficacy of CAR-T cells.[21]

Analogous to overexpressing a fitness-promoting gene, genetic ablation of a negative regulator of T-cell responses could achieve a similar outcome. For example, disrupting two T-cell repressors previously identified from a clustered regularly interspaced short palindromic repeats (CRISPR) screen, Regnase-1[22] and Roquin-1,[23] significantly enhanced CAR-T cytotoxicity against pancreatic tumors.[24] Genetic deletion of a T-cell receptor (TCR) signaling negative regulator, *PTNP2*, increased both CAR-T homing and activation in an HER-2^{+} mammary tumor model.[25] Knocking out an epigenetic regulator, PR domain zinc finger protein 1 (*PRDM1*) or DNA (cytosine-5)-methyltransferase 3A (DNMT3A), promoted memory CAR-T expansion, long-term persistence, and improved therapeutic efficacy in multiple tumor models.[26,27] Similarly, eliminating nuclear receptor TF NR4As, the downstream targets of programmed cell death protein 1 (PD-1) and T cell immunoglobulin and mucin-domain containing-3 (Tim-3) signaling, increased CAR-T cell response and antitumor activities.[28] The most intriguing example of increased CAR-T efficacy by inhibiting a negative T-cell regulator comes from a clinical observation that an accidental genetic disruption of *Ten-Eleven Translocation-2* (*TET2*) promoted clonal CD19 CAR-T expansion, memory formation, and increased antileukemia potency,[29] suggesting the potential application of *TET2*-knockout CAR-T for solid tumor therapy. However, the recent report cautioned the risk of uncontrolled proliferation and genomic instability caused by permanent *TET2* disruption.[30] Additional attempts of shutting down negative regulators of CAR-T cell activities include permanent deletion of master inhibitory and exhaustion genes, such as *PD-1* and *TOX*, yet these attempts only lead to transient boosting of CAR-T functionality and rapid loss of *PD-1*- or Tox-deficient cells.[31,32] This paradoxical observation suggests the critical role of the master negative or exhaustion regulators in maintaining T-cell homeostasis potentially by slowing down T-cell differentiation and counteracting overactivation of CAR-T cell-induced cell death.

Table 1. Engineering solutions and the challenges CAR-T therapies encounter in solid tumors

Challenges	Cancer type	Engineering solutions
Tumor heterogeneity	Solid tumors	CAR-T-boosting vaccine;[113,114] BBIR;[50] anti-FITC CAR;[51] RevCAR,[52] BsAb,[53] SAR,[54] IMPACT,[55] Fc-binding CAR;[56] UniCAR,[57] anti-PNE CAR,[58] Co-LOCKR;[59] convertible CAR;[60] SUPRA;[61] SpyCatcher CAR[62]
Antigen escape	Solid tumors and hematological malignancies	BBIR;[50] anti-FITC CAR;[51] RevCAR,[52] BsAb,[53] SAR,[54] IMPACT,[55] Fc-binding CAR;[56] UniCAR,[57] anti-PNE CAR,[58] Co-LOCKR;[59] convertible CAR;[60] SUPRA;[61] SpyCatcher CAR[62]
T-cell persistence	Solid tumors and hematological malignancies	CAR-T-boosting vaccine;[113,114] polymer-nanoparticle hydrogels;[116] implantable macroporous alginate scaffolds;[117] biocompatible nitinol thin films[118]
T-cell exhaustion	Solid tumors and hematological malignancies	TRAC-CAR;[44] IL-10-Fc;[87] knocking out PTNP2,[25] PRDM1,[26] DNMT3A,[27] NR4A,[28] or TET2;[30] overexpressing BATF,[17] c-Jun,[16] LBTR,[21] or PGC-1α[18]
T-cell infiltration	Solid tumors	CAR-T-boosting vaccine;[113,114] IL-7/CCL19 CAR;[20] ICEp;[119] SynNotch-IL-2[41]
Inhibitory TME	Solid tumors	Orthogonal IL-2/IL-2R system;[93] Neo-2/15;[92] cell-tethered IL-12;[106] polymer—nanoparticle hydrogels;[116] implantable macroporous alginate scaffolds;[117] biocompatible nitinol thin films;[118] ICEp[119]
On-target off-tumor toxicity	Solid tumors	Split CARs,[34] iCARs,[35] tanCARs,[36] and dualCARs;[37] synNotch-CAR;[38] SNIPRs;[42] hypoxia-responsive CAR;[47,48] masked CAR;[49] iCasp9-CAR;[64] Tet-On/OFF-CAR;[65] synZiFTR;[66] On-VIPER CAR;[67] SNIP CAR;[68] lenalidomide ON/OFF-switch;[69] DD CAR;[70,71] SWIFF CAR;[70] rapamycin ON-switch;[72] TetCAR;[73] STOP CAR;[74] DARIC,[75] A1120 ON-switch CAR;[76] Blue-Light CAR;[77] ultrasound CAR[78,79]

CAR, chimeric antigen receptor; IL, interleukin.

CONTROLLED REGULATION OF CAR-T CELLS VIA SYNTHETIC BIOLOGY

Synthetic biology-based genetic circuits offer a tunable approach, which overcomes the shortcomings of irreversible genetic alteration of a single gene and enables more sophisticated T-cell regulation *in vivo*. T cells have been equipped with genetic circuits to control cell responses[33] (Table 1), each falling into one of two categories: autonomous programming or external control (Figure 1). Autonomously programmed T cells contain genetic circuits that sense endogenous environmental cues within the organism. Contrastingly, externally controlled T cells are engineered to sense user-administered non-native cues. It is important to note that the same responses can be achieved with either circuit class. While genetic circuits can be designed to modulate various T-cell functions, here we focus exclusively on the regulation of CARs.

Autonomously programmed CAR-T cells

A number of autonomously programmed T-cell circuits have been developed to date, such as split CARs,[34] iCARs,[35] tanCARs,[36] and dualCARs,[37] and one of the advanced designs among them is the synthetic Notch receptor (synNotch)[38] (Figure 1A). Inspired by the endogenous Notch receptor, the synNotch receptor was developed by connecting an extracellular antigen-binding domain (e.g. scFv) to an intracellular orthogonal transcription regulatory domain (ICD) through the Notch core that retains the mechanosensing property to induce ICD cleavage and release upon extracellular target binding.[39] This design enables custom control of CAR expression and activation of T cells via a dual-antigen input 'AND' gate and permits CAR-T cells to sense antigen density with an ultrasensitive threshold.[40] In addition, more complex circuits comprising multiple constitutively expressed synNotch receptors can be used to induce therapeutic payload expression, including cytokines, therapeutic antibodies, cytotoxic agents, or apoptotic proteins. In follow-up work, synNotch exhibited superior performance over TCR—nuclear factor of activated T cells (NFAT) signaling to induce productive IL-2 autocrine signaling in CAR-T cells, promoting T-cell infiltration into T-cell-excluded pancreatic tumors.[41] Synthetic intramembrane proteolysis receptors (SNIPRs) were recently developed to improve the synNotch system by using optimized components to minimize immune rejection, ensure sufficient receptor expression and activity, and reduce circuit size to enable superior clinical efficacy.[42] These circuits have improved target specificity to address on-target, off-tumor toxicity associated with CAR-T cell therapy in solid tumors. Although advanced designs enable higher specificity, there are potential limitations that need to be considered. For example, 'AND' logic gates would require only loss of a single antigen to prevent CAR-T function, which could enable faster tumor escape. While these types of sophisticated circuits are exciting, they will require further optimization for clinical use.

Autonomous programming could also be achieved by directly harnessing endogenous genetic regulatory mechanisms. The CRISPR—CRISPR-associated protein 9 (Cas9) technology can efficiently create DNA double-strand breaks in the genome and facilitate transgene knock-in at a desired locus through homology-directed recombination.[43] One of the first CRISPR-Cas9 applications in T-cell engineering involves inserting a CAR cassette into the endogenous TCR α constant (TRAC) locus, named TRAC-CAR[44] (Figure 1A). Traditionally, the CAR transgene is placed under an exogenous promoter to allow for constitutive expression, which encourages tonic signaling, leading to T-cell exhaustion and limited persistence.[45] By contrast, the TRAC-CAR 'hijacked' the endogenous feedback regulation that a TCR naturally receives upon T-cell activation. The oscillation of CAR surface expression in TRAC-CAR-T cells in response to antigen stimulation prevented tonic signaling and exhaustion, resulting in enhanced CAR-T functionally and long-term persistence.[44] This method allows for predictable and

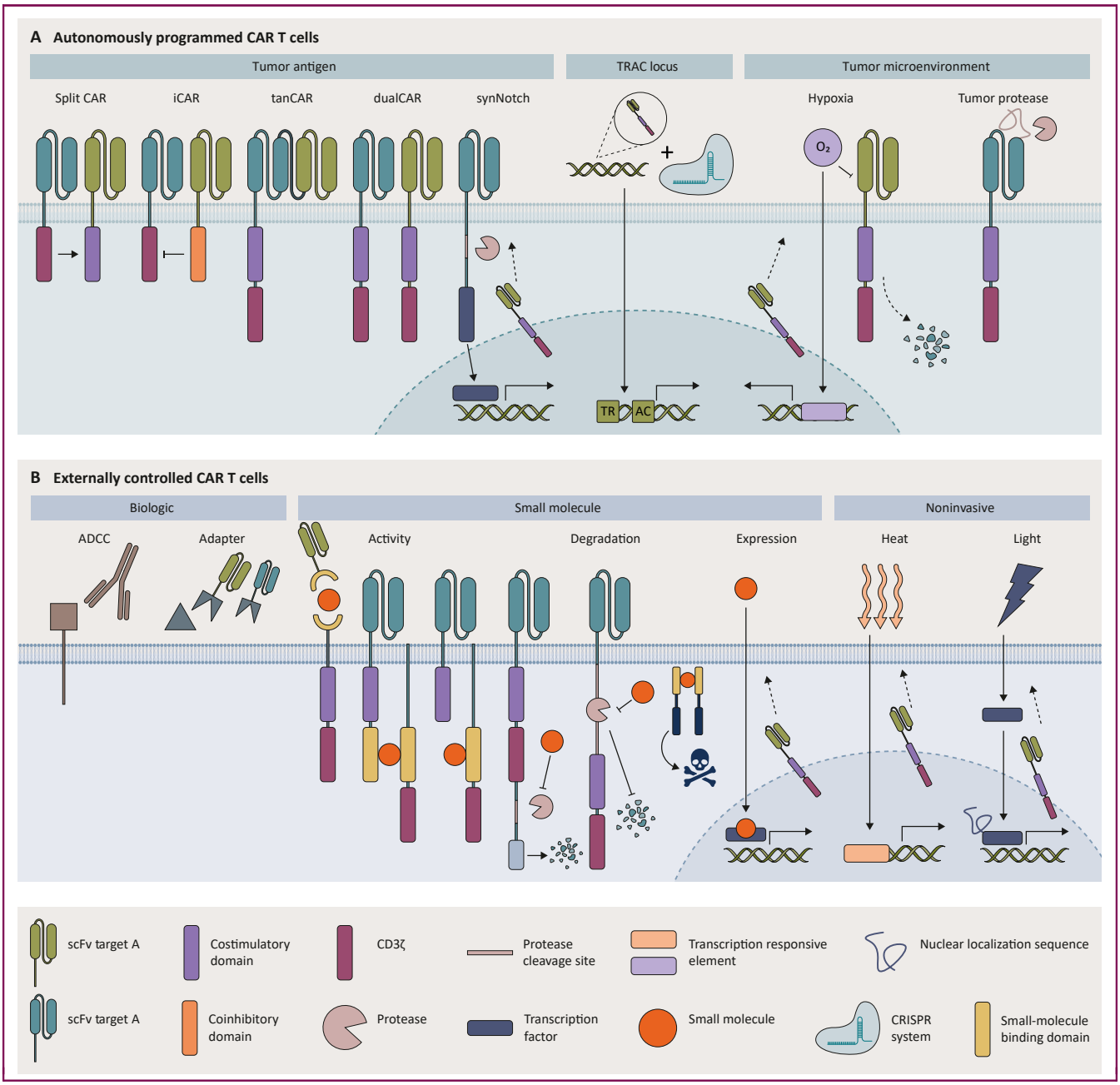

Figure 1. Overview of controlled regulation of chimeric antigen receptor (CAR)-T cells via synthetic biology. (A) Autonomously programmed CAR-T cells are engineered to respond via tumor antigen-dependent Boolean logic gates, TCR alpha (TRAC) locus regulation, or tumor microenvironment factors. (B) Externally controlled CAR-T cells require an administered biologic, small molecule, or noninvasive agent to initiate or silence the CAR signaling. Created with BioRender.com. ADCC, antibody-dependent cellular cytotoxicity; CRISPR, clustered regularly interspaced short palindromic repeats.

uniform CAR expression while prohibiting endogenous TCR expression, which also minimizes the risk of graft-versus-host-disease and increases the potential for allogeneic CAR-T generation.

Beyond tumor antigen recognition, T cells have been developed to sense and respond to TME factors (Figure 1A). One such factor is hypoxia, which is caused by rapid oxygen consumption by tumors coupled with poor nutrient delivery by abnormal vasculature.[46] CARs have been placed under hypoxic control at the transcriptional and translational levels through incorporating hypoxia-responsive elements upstream of the CAR promoter and an oxygen-dependent degradation domain to the CAR itself, respectively.[47,48] Another approach, known as 'masked CAR-T', involves tethering a blocking peptide to the CAR via a proteolytic linker that can be cleaved by proteases overexpressed in the TME, resulting in antigen binding and subsequent CAR-T cell activation.[49] The spatial control of CAR-T activity via TME-responsive designs increases the therapy's safety profile by decreasing on-target, off-tumor toxicities, further expanding the target antigen selection pool. While TME circuits are highly promising, their activation timing and kinetics largely depend on the strength, prevalence, and persistence of these environmental cues in solid tumors. For

example, the degree of hypoxia may vary throughout the tumor, and therefore the therapeutic delivery will not be uniform throughout the tumor. Besides, the types of proteases could vary between tumor types or even between patients, which could result in distinct treatment outcomes. Further advances in TME circuits engineering and *in vivo* validation in various tumor models would help determine the robustness of this approach.

Externally controlled CAR-T cells

External mechanisms that control T-cell activity include using biologic, small molecule, or noninvasive cues. Most biologic-based CAR-T control mechanisms rely on genetically encodable adaptors (Figure 1B), with the exception of biotin and fluorescein isothiocyanate (FITC)-based strategies (BBIR[50] and anti-FITC CAR[51]). Adaptors consist of a tumor-targeting domain fused to a T-cell engaging domain, such as an scFv or antibody (RevCAR,[52] BsAb,[53] SAR,[54] IMPACT,[55] Fc-binding CAR[56]), peptide (UniCAR,[57] anti-PNE CAR,[58] Co-LOCKR[59]), orthogonal ligand (convertible CAR),[60] leucine zipper (SUPRA),[61] or reaction substrate (SpyCatcher).[62] With adaptor-based redirecting technology, T cells only need to be engineered once with a generic CAR and can be given universally to patients. A unique advantage of this platform is that it permits target switchability through the administration of a new adaptor targeting a different tumor antigen in case of the loss of the original antigen on tumor cells. Furthermore, switchable CAR-T cells allow for reversible control as well as titratable response by varying the dosage intervals, affinity, and concentration of adaptors. SUPRA CAR has recently been shown to take advantage of these characteristics to assemble elaborate circuits for more stringent control.[61]

Despite these sophisticated control mechanisms in activating T cells, a major concern in engineered T-cell therapy is the potential toxicity caused by aberrant growth and activation of adoptively transferred T cells. Earlier work leveraged antibodies targeting unique surface proteins on adoptively transferred T cells, known as elimination markers, to deplete them via antibody-dependent cellular cytotoxicity.[63] Small-molecule strategies activating suicide pathways, such as iCasp9, were also used as a 'safety switch' to shut down inappropriately activated CAR-T cells.[64] However, these strategies resulted in irreversible T-cell depletion. Newer circuits have been engineered to use small molecules to reversibly induce or inhibit CAR expression, degradation, or activity through the inhibition of proteases or the activation of the ubiquitin—proteasome pathway (Figure 1B). For example, in the Tet-On system, the small molecule binds to a reverse Tet transactivator protein, which then binds to a tetracycline response element located upstream the promoter to drive CAR expression.[65] The synZiFTR platform is a more recently developed approach that uses small molecules to control zinc finger transcription regulator and downstream CAR expression.[66] By contrast, on-VIPER CAR, which includes protease and cleavage sites in the CAR structure itself,[67] is continuously cleaved and silenced until a small-molecule protease inhibitor is administered to prevent cleavage and stabilize the protein. SNIP CAR is featured by a dual-chain system such that the CAR only contains cleavage sites, and the protease is a separate membrane-bound entity.[68] This configuration reduced leaky activity in the off state likely due to enhance protease proximity. The lenalidomide OFF-switch leverages the ubiquitin—proteasome pathway to control the CAR protein. In this system, the small molecule recruits a ubiquitin ligase to trigger CAR ubiquitin-mediated degradation.[69] DD CAR functions opposite of the lenalidomide OFF-switch CAR such that the small molecule in this system binds to a ubiquitin-target degradation moiety, termed degron, to prevent degradation.[70,71] SWIFF CAR utilizes proteases and the ubiquitin—proteosome pathway simultaneously. Upon administration of a small-molecule protease inhibitor, degron cleavage is blocked, which leads to ubiquitin-mediated degradation of the CAR.[70] Lastly, small molecules can encourage or competitively disrupt interactions of immune signaling domains and therefore modulate CAR activity. The lenalidomide ON-switch,[69] rapamycin ON-switch,[72] TetCAR,[73] and STOP CARs[74] induce or disrupt dimerization of costimulatory and T-cell-activation domains to trigger or prevent downstream CAR signaling. Small molecules coupled with protein-based adaptors modulate CAR-T cell activity through a multilayer regulation (DARIC,[75] A1120 ON-switch CAR[76]). Unlike autonomously programmed T cells, tumor penetration, half-life, toxicity, and the immunogenicity of small molecules or biologic components of the externally controlled circuits can impact the therapeutic benefit of these designs.

In addition to the biologic and small molecule-based regulation, noninvasive control methods have been explored (Figure 1B). For example, an optogenetic approach was developed to induce CAR expression via blue-light stimulation; however, this approach is limited by its minimal tissue penetration depth.[77] Thermal circuits, responsive to heat generated by focused ultrasound, have been constructed by placing a CAR under the control of a heat shock protein promoter or a minimal promoter that contains heat shock enhancer elements.[78,79] While this design allows for precise delivery of ultrasound, more solid tumor models will need to be tested to determine how tumor location and tumor type impact the efficacy of this treatment. The introduction of these noninvasive circuits exemplifies the vast range of genetic approaches that have been investigated to date for therapeutic T-cell engineering. As the synthetic biology field continues to advance, engineers will need to balance the therapeutic output and circuit complexity in terms of its size, number of parts, and source.

ENGINEERING CYTOKINES TO SUPPORT CAR-T CELL THERAPY

T-cell activation involves first recognizing an antigen through a TCR or CAR (signal 1), then receiving costimulation (signal 2), usually CD28, together with cytokine support (signal 3).[80,81] While these signals are present

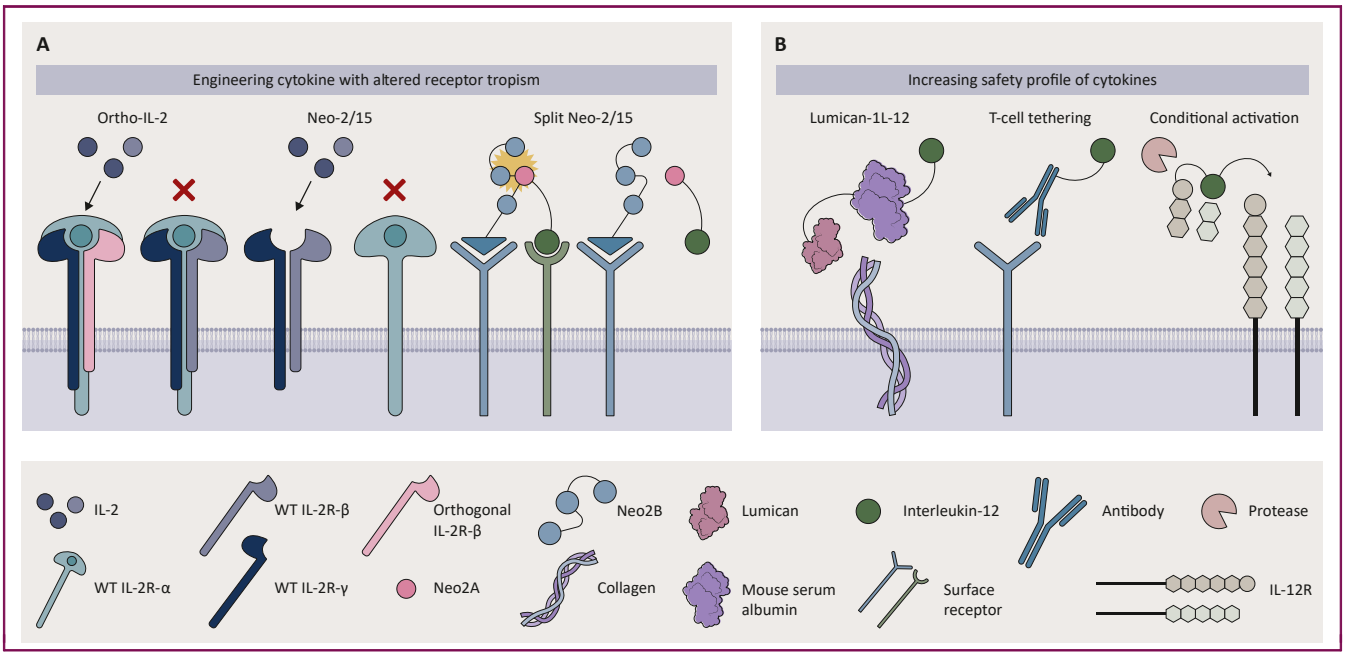

Figure 2. Cytokine engineering to enhance chimeric antigen receptor (CAR)-T cells. (A) Examples of altering receptor tropism through engineering include orthogonal receptor/ligand systems such as Ortho-IL-2/IL-2R, *de novo* designed interleukin mimics such as Neo-2/15, and conditionally activated cytokines such as split Neo-2/15. (B) Examples of efforts to increase the safety profile of proinflammatory cytokines include tethering IL-12 to the tumor environment via lumican or Von Willebrand factor, tethering IL-12 to the T cell via antibodies, or conditionally activating an IL-12R-masked IL-12 via a protease-cleavable linker. Created with BioRender.com. IL, interleukin; WT, wild type.

within the initial priming environment in lymphoid organs, signals 2 and 3 are often missing from the TME. Although a built-in costimulation (e.g. CD28 or 41BB) exists within the CAR, it does not appear sufficient for CAR-T cell therapy in solid tumors. Furthermore, the natural regulatory mechanisms within the TME limit the activity of tumor-infiltrating CAR-T cells.[12] For example, regulatory T cells (Tregs) bearing a high-affinity IL-2 receptor, CD25, will consume the surrounding IL-2, a key cytokine for maintaining T-cell survival and proliferation.[82] Tumor cells, tumor-associated macrophages, and stromal cells secrete immunosuppressive cytokines, such as IL-10 and transforming growth factor beta (TGFβ), to inhibit T-cell proliferation and polyfunctionality.[83] Engineering cytokines to specifically act on adoptively transferred T cells or shift the cytokine balance within the TME presents a promising strategy to enhance adoptive T-cell therapy. Although cytokines are essential in maintaining T-cell homeostasis, natural cytokines are pleiotropic and could cause severe dose-limiting toxicity upon systemic administration.[84] Early attempts at cytokine engineering include polyethylene glycol (PEG) or fragment crystallizable (Fc) fusions of major cytokines to improve cytokine half-life.[85,86] For example, IL-10-Fc fusion has recently been shown to improve intratumoral T-cell metabolic fitness with enhanced anti-solid tumor efficacy.[87] However, these methods do not address the issues of systemic toxicity, poor localization to the TME, and undesired pleiotropism. Here, we will focus on IL-2 and IL-12 and discuss cytokine engineering solutions to address these issues and enhance CAR-T cell therapy (Figure 2, Table 1).

Engineering cytokines with altered receptor tropism

The common γ-chain cytokines, including IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, are instrumental in regulating CAR-T and natural T cells.[88] IL-2 and IL-15 from this family have gained substantial engineering focus owing to the traditional interest in their therapeutic potential and the similarities of their signaling mechanisms. However, the use of unmodified IL-2 therapeutically for cancer has been stunted owing to the life-threatening vascular leak syndrome potentially caused by IL-2 binding to IL-2Rα^+ endothelial cells.[89] IL-2 signals through a trimeric receptor containing the subunits α (CD25), β, and γ. The β and γ subunits form an intermediate affinity receptor that is sufficient for signaling and is shared by IL-15. The addition of CD25 (or CD215 in the case of IL-15) induces the formation of the high-affinity receptor.[90,91] As CD25 is transiently expressed on activated lymphocytes but constitutively expressed at high levels on Tregs, IL-2 signaling is naturally biased to promote Treg activation.[90] Although IL-15 can activate T cells without interacting with Tregs, it has lower therapeutic efficacy due to the necessity for trans-signaling, which IL-2 is not limited to.[91,92]

An orthogonal IL-2/IL-2R (ortho-hIL-2) receptor—ligand pair has been developed for both murine and human use through structure-guided directed evolution of the binding interface between wild-type IL-2 and IL-2Rβ[93] (Figure 2A). This system retains CD25 binding yet lacks binding to wild-type IL-2Rβ; therefore it is not expected to induce IL-2R trimer formation in Tregs and subsequence Treg expansion. In a preclinical model of acute lymphoblastic leukemia, the human ortho-hIL-2 system has exhibited the ability to specifically and selectively expand ortho-hIL-2Rβ-expressing

CD19 CAR-T cells while also significantly enhancing the engraftment and antileukemia activity of these CAR-T cells.[94] The ortho-hIL-2 system has the potential to improve the outcome of CAR-T therapy in treating solid tumors by improving CAR-T cell expansion and persistence. However, the ortho-hIL-2 system induced severe TCR-independent toxicity in NSG mice, which was likely caused by the rapid activation of CAR-T cells and the accompanying cytokine release, calling for the need of carefully select ortho-hIL-2 dosing in designing future clinical trials.[94]

Concurrently, a hyper-stable IL-2 and IL-15 mimic (Neo-2/15) was created via *de novo* design to have increased affinities toward the β and γ chains of the IL-2/15 receptors and no binding to CD25 and CD215 (Figure 2A).[92] Neo-2/15 elicited robust downstream signaling through the intermediate IL-2R$\beta\gamma$ complex and exhibited superior therapeutic activity and reduced toxicity compared with wild-type IL-2 in mouse models of melanoma and colon cancer.[92] Given the promises in preclinical models, Neo-2/15 is advancing to a phase I clinical trial (NCT04659629).[95] Neo-2/15 has recently been engineered into a conditionally active system,[96] made possible by the hyperstability of the original molecule. This involves splitting the Neo-2/15 protein into its β- and γ-chain-binding components, each of which is fused to a separate antigen-targeting domain. Split Neo-2/15 only assembles and signals when both components are directed to tumor cells or CAR-T cells to achieve *trans*- or *cis*-activation, respectively (Figure 2A). This split approach offers opportunities to increase tumor-targeting specificity and reduce CAR-T cell therapy-induced systemic toxicities.[96] While these *de novo* engineered cytokines achieved superior specificity and potency, one caveat is that the non-native peptide sequences within these cytokines likely introduce immunogenicity, albeit not observed with Neo2/15,[92] and repeated administration of engineered cytokines likely elicits anticytokine antibodies that may eventually diminish their therapeutic efficacy.[97]

Increasing the safety profile of cytokines

IL-12 is a highly proinflammatory cytokine that has been explored to promote antitumor immunity in immunologically cold tumors.[98] This is mainly through the stimulation of antigen presentation, cytotoxic T-cell activity, and differentiation of helper T cells into IFN-γ-secreting type 1 helper T cell and type 1 cytotoxic T cells. However, IL-12 therapy has shown minimal success owing to dose-limiting toxicity upon systemic delivery.[99] Fourth-generation CARs, termed T cells redirected for antigen-unrestricted cytokine-initiated killing (TRUCK),[100] include IL-12 under an NFAT promoter that is induced upon T-cell activation to stimulate endogenous immune response via the recruitment of primarily cytotoxic T cells and NK cells.[101] However, lack of efficacy and unexpected toxicity in patients led to the termination of the TRUCK clinical trials (NCT01236573 and NCT01457131).[102]

Fusing IL-12 to collagen-binding proteins such as lumican and the collagen-binding domain of von Willebrand factor has shown promises in anchoring IL-12 to the TME in murine models upon intratumoral injection.[103,104] The collagen-binding module enables slow release of IL-12 without compromising its function (Figure 2B). Anchoring IL-12 to tumors via collagen binding synergized with anti-melanoma CAR-T cell therapy and led to a complete cure of all melanoma-bearing animals with negligible toxicity.[103,104] A detailed follow-up study found that the therapeutic efficacy of collagen-binding cytokines could be impacted by their molecular sizes and affinities toward collagen, revealing the design rules for engineering localized cytokine therapies.[105] IL-12 has also been fused to antibodies targeting the T-cell surface receptors CD45, CD11a, and CD18[106] (Figure 2B). Cell-tethered IL-12 provides autocrine and paracrine support of therapeutic T cells with low toxicity and repolarizes the intratumoral suppressive myeloid cells toward inflammatory phenotypes to sustain antitumor immunity. Analogous to this approach, tethering T cells to the IL-2 or IL-15 superagonist or IL-12 nanogels that were formulated with TME or TCR signaling-responsive chemical linkers enables higher therapeutic payload delivery with controlled release in solid tumors, further improving the therapeutic index of CAR-T cell therapy and reducing immune adverse effects.[107,108] In addition to local delivery of IL-12, a recent work implemented an elegant design to systemically deliver a conditionally active IL-12 that was created by fusing IL-12 to its own receptor (i.e. IL-12R) as a single recombinant protein via a cleavable linker.[109] This way, IL-12 will be silenced in circulation but activated intratumorally upon the release of the masking receptor by tumor-associated matrix metalloproteinases (MMPs; Figure 2B), significantly reducing the toxicities associated with systemic delivery of unmodified IL-12. However, given the enzyme promiscuity and abundance of MMPs in various tissues,[110] unmasking IL-12 by a single MMP likely still elicits unpredictable local toxicity and would need further preclinical evaluations. The next-generation engineered cytokines can potentially benefit from more sophisticated protein logic gate designs to respond to multiple tumor-intrinsic cues and synthetic cues released by CAR-T cells to achieve precise spatial control and greater safety.

ENHANCE CAR-T CELL THERAPY WITH ENGINEERED BIOMATERIALS

Synthetic biology and cytokine engineering are powerful genetic approaches to enhance CAR-T cells intrinsically. Immunomodulatory biomaterials offer a highly complementary approach to not only directly modulate CAR-T cells in a way that genetic approaches cannot but also engage CAR-T cells with the endogenous immune system to overcome therapeutic barriers in solid tumors (Figure 3, Table 1).

Vaccines are excellent examples of how biomaterials can be engineered to specifically modulate CAR-T cells directly *in vivo*.[111] The lymph node (LN) is the natural environment that T cells will home to and receive antigen stimulation from antigen-presenting cells (APCs) together with

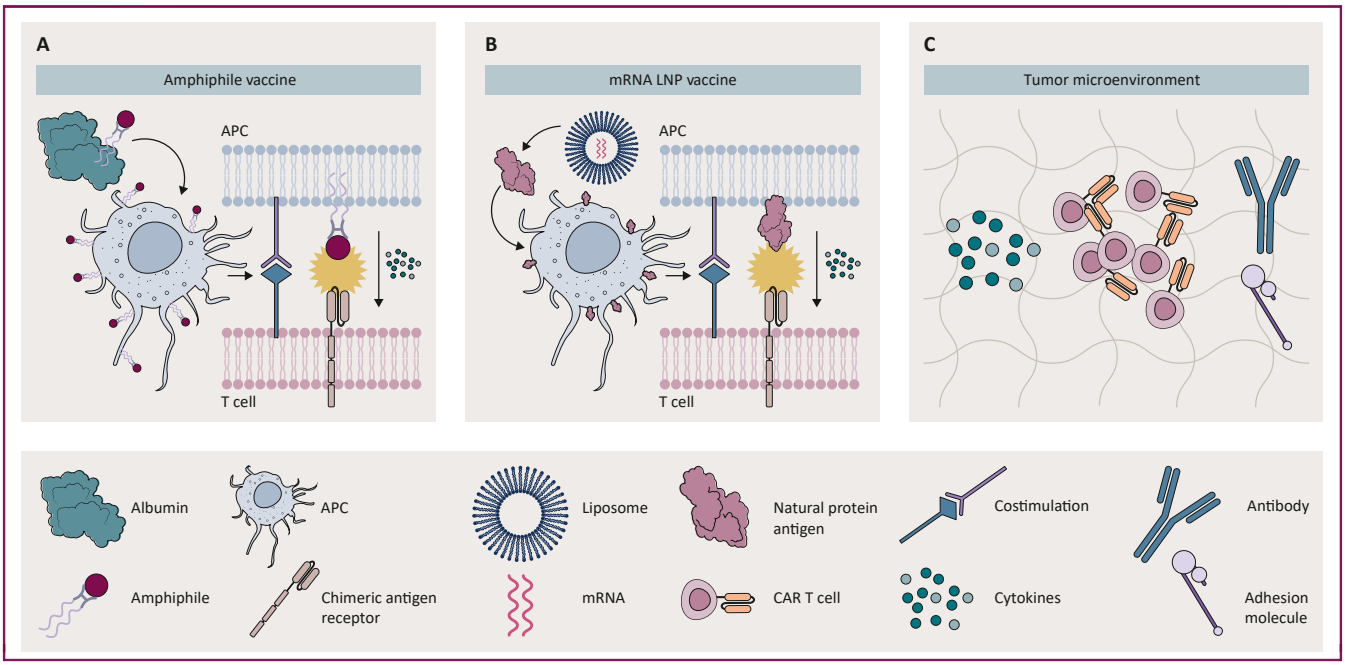

Figure 3. Chimeric antigen receptor (CAR)-T cell modulation with engineered biomaterials. (A) An amphiphile vaccine bearing a CAR ligand hitchhikes albumin for trafficking to the lymph node (LN) where it decorates the surface of LN-resident antigen-presenting cells (APCs) and primes CAR-T cells homing to the LN. (B) A lipid nanoparticle can be used to encapsulate messenger RNA (mRNA) molecules encoding for a CAR-stimulating antigen and efficiently deliver them into the LN, where the mRNA will be transfected into LN-resident APCs and translated into the protein molecules on APC surface for stimulating incoming CAR-T cells. (C) A polymer scaffold can be used to encapsulate CAR-T cells with stimulatory molecules such as cytokines and antibodies to form an inflammatory niche, promoting CAR-T cell expansion and infiltration into tumors. Created with BioRender.com.

supporting signals to achieve optimal expansion and differentiation. A number of polymer and nanoparticle-based vaccines have been engineered for targeted delivery of antigens to the LN to drive antigen-specific T-cell responses.[112] The CAR-T-boosting amphiphile vaccine (amph-vax) was developed by conjugating an albumin-binding and membrane-inserting lipid polymer to a ligand recognized by a CAR.[113] Constitutive trafficking of albumin to LN effectively concentrates these amph-vax molecules in the draining LN. Upon arrival, the amph-vax molecules directly insert into the cell membrane of LN-resident APCs via their lipid tails, allowing activated APCs to directly present them to CAR-T cells and trigger MHC-independent T-cell activation through the chimeric receptor, analogous to MHC-dependent T-cell activation through the TCR (Figure 3A). This synthetic vaccination triggers marked expansion of CAR-T cells with enhanced polyfunctionality, memory formation, and tumor infiltration, collectively leading to increased solid tumor clearance.[113] Unexpectedly, this CAR-T-specific amph-vax elicited potent priming of host T cells against tumor neoantigens that are not originally targeted by the CAR, that is, antigen spreading, potentially enabling this vaccine to co-engage CAR-T and host T cells to directly target antigenically heterogeneous tumors and achieve durable long-term tumor control.[113,114] Given that the CAR recognizes a genetically encodable surface protein, lipid nanoparticles encapsulating an messenger RNA encoding the cognate protein antigen can also efficiently traffic to the LN and transfect LN-resident APCs (Figure 3B). Upon expression, the cognate protein antigen can be displayed on the APC surface, achieving a similar outcome in boosting CAR-T cells.[115] Notably, a number of factors could impact vaccine boosting of CAR-T cells, including the vaccine dosing, size of vaccine molecules, trafficking, and retention on APC surface, which are beyond the scope of this review. In contrast to messenger RNA lipid nanoparticle vaccines, amphiphile vaccines enable the delivery of nongenetically encodable ligands and small-peptide ligands more efficiently. Presumably, the combination of the switchable CAR-T platform[51] (e.g. anti-FITC CAR) and a compatible amphiphile vaccine[113] (e.g. amph-FITC) could offer an affordable universal solution for *in vivo* CAR-T manufacturing and tumor targeting.

Biomaterials can also shield cellular cargos from harsh environments, serve as a CAR-T manufacturing plant, and enable sustained local release into tumors. For example, polymer-nanoparticle (PNP) hydrogels,[116] implantable macroporous alginate scaffolds,[117] and biocompatible nitinol thin films[118] have been engineered successfully to encapsulate both CAR-T cells and one or multiple supporting molecules, including antibodies, cytokines, and adhesion peptides (Figure 3C). Upon peritumoral implantation, they act as depots with a sustained inflammatory niche to promote CAR-T cell expansion and release into tumors. In addition, immune-supporting molecules can be loaded onto the outer layer of microparticles for specific and potent local stimulation of tumor-infiltrating CAR-T cells upon intratumoral delivery.[119]

CONCLUSIONS AND OUTLOOK

The design of CAR itself and its successful integration of antigen recognition, costimulation, and TCR signal to boost T-cell function and redirect T cells against cancer have embodied how engineering principles can be leveraged to revolutionize cancer immunotherapy. The remarkable clinical success of CAR therapy in hematological malignancies has built a road map for future cellular immunotherapies. However, the early clinical experience and lessons learned from preclinical models have uncovered major obstacles that must be overcome to make engineered CAR-T cell therapy a reliable and efficacious treatment for solid tumors.

Decades of targeted cancer therapy using small-molecule inhibitors has provided valuable insights into how cancer cells continuously evolve and evade a new treatment.[120] The heterogeneity of target gene expression, cancer cell phenotypic plasticity, and redundant cancer cell signaling pathways together make hitting a single target in cancer an undesirable option.[121] Understandably, CAR-T, as a type of targeted immunotherapy directed against a single tumor surface antigen, also led to antigen loss and tumor escape in both hematological malignancies[122] and solid tumors.[8] Yet it is inspiring to see Emily Whitehead, the first individual receiving CD19-directed CAR-T cell therapy, still in remission after 10 years from the initial treatment. Numerous CAR-T trials and other immunotherapy trials converged on an encouraging observation that once a patient with cancer is successfully treated with immunotherapy, sustainable remission could be achieved, and no follow-up treatment is required in contrast to conventional targeted therapy. The reason behind is that once immunotherapy, such as CAR-T, is administered, its effect is pleiotropic rather than solely on cancer cells.[123] The cytokines produced by CAR-T cells will shape the local immune landscape and trigger antigen spreading to prime secondary antitumor immunity to clear residual tumor cells and prevent tumor relapse.

The rise of CAR-T cell therapy coincides with a number of maturing bioengineering fields, including synthetic biology, protein engineering, and biomaterials. The numerous tools and concepts outlined in this review are being actively tested in preclinical tumor models and clinical trials to examine their abilities to meet the needs of treating solid tumors effectively and safely. Individual tumor types present different challenges depending on the anatomic location and clinical stage of the lesions. The route and timing of therapeutics administration need to be tailored for each tumor type to maximize efficacy while reducing immunotherapy-associated toxicity. Multiple engineering approaches will likely need to be combined to create a reliable solution for enhancing CAR-T cell therapy for solid tumors, and this solution should bridge CAR-T more efficiently with endogenous antitumor immunity to overcome tumor antigen heterogeneity and ensure long-term tumor control.

Lastly, although our discussion in this review focused on engineering solutions to enhance CAR-T cell therapy, the principles and approaches presented here could be adapted to enhance other adoptive T-cell therapies in cancer, such as tumor-infiltrating lymphocytes and TCR-transgenic T-cell therapy. As these engineering solutions are being tested in clinical trials and new technologies are to be developed in the coming years, the full potential of adoptive T-cell therapies can be unlocked.

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DISCLOSURE

The authors have declared no conflicts of interest.

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