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Synthesizing a Smarter CAR T cell: Advanced Engineering of T-cell Immunotherapies

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Abstract

The immune system includes an array of specialized cells that keep us healthy by responding to pathogenic cues. Investigations into the mechanisms behind immune cell behavior have led to the development of powerful immunotherapies, including chimeric-antigen receptor (CAR) T cells. While CAR T cells have demonstrated efficacy in treating blood cancers, issues regarding their safety and potency have hindered the use of immunotherapies in a wider spectrum of diseases. Efforts to integrate developments in synthetic biology into immunotherapy have led to several advancements with the potential to expand the range of treatable diseases, fine-tune the desired immune response, and improve therapeutic cell potency. Here, we examine current synthetic biology advances that aim to improve on existing technologies and discuss the promise of the next generation of engineered immune cell therapies.

Keywords

Cellular immunotherapy; Engineered T cells; CAR T-cells; Immunotherapy

Introduction: Potential for immune cells as therapies

Cell-based therapy has advanced far from its early beginnings, with the pace of discovery accelerating over the past decades (Figure 1). Now, cell-based therapy carries great potential

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Conflict of Interest Statement

K.T.R. is a cofounder of Arsenal Biosciences and Dispatch Therapeutics. He is a consultant, SAB member, and stockholder. K.T.Ra. was a founding scientist/consultant and stockholder in Cell Design Labs, now a Gilead Company. K.T.R. holds stock in Gilead. K.T.R. is on the SAB of Alaunos Therapeutics and a compensated advisor to Venrock. D.P. is an employee of Dispatch Therapeutics. I.Z. declares no competing interests.

to advance our ability to treat cancer and other diseases. Patient-derived immune cells have several intrinsic features that make them the most promising platform for the development of engineered living therapeutics. Immune cells constantly survey nearly every organ in the body, responding to a wide variety of potential insults to protect us from disease. The innate immune system rapidly reacts to pathogenic motifs, such as endotoxins or viral RNA, while the adaptive immune system recruits a diverse response against a wider range of antigens and generates immunological memory. A normal immune response is also self-regulating, with inflammation followed by subsequent healing and repair. Practically, immune cells, and in particular T cells, can be quickly isolated from a patient's whole blood and expanded for therapeutic use with minimal risk of graft rejection. These qualities make patient-derived immune cells an ideal candidate for therapeutic engineering.

Current Challenges in the cell-based immunotherapeutic treatment of solid cancers

Chimeric-antigen receptor (CAR) T cells have received FDA approval for the treatment of several hematological cancers. Unfortunately, this success has not been replicated in solid organ cancers. This difficulty can be attributed to a lack of truly solid tumor–specific antigens, leading to "on-target, off-tumor" toxicity, as well as an immunosuppressive tumor microenvironment that promotes T-cell exhaustion and anergy (Figure 2).

On-target, Off-tumor Toxicity

On-target, off-tumor toxicity, where CAR T cells react to cancer antigens expressed on healthy tissues, has been observed since the first published CAR T-cell trials, where CAIX-redirected T cells targeting renal cell carcinomas also attacked the biliary duct, resulting in unacceptable liver toxicity (1). FDA-approved anti-CD19 CAR T-cell therapies for B-cell cancers also produce significant B-cell depletion (2), as well as indirect neutropenia (3) and transient neurotoxicity (4). While the resulting B-cell depletion can be clinically managed by IgG replacement therapy (5), few solid organs are similarly dispensable, necessitating a stringent method for discriminating solid tumors from healthy tissues. Case reports of organ failure and patient mortality via unintended CAR T-cell infiltration also illustrate the need for caution in the choice of target (6).

Tumor microenvironment

To blunt immune rejection, tumors generate highly immunosuppressive microenvironments. Local tumor overproduction of VEGF induces growth of malformed blood vessels that suppresses lymphocyte adhesion, promotes tissue hypoxia, and restricts oxidative metabolism within infiltrating immune cells. Tumors also recruit myeloid-derived suppressor cells (MDSCs), a mixed population of immature and progenitor myeloid cells that produce immunosuppressive factors such as arginase 1, inducible nitric oxide synthase (iNOS), reactive oxygen species (ROS), and peroxynitrite (7). Tumor-associated macrophages (TAMs) typically display an immunosuppressive M2 phenotype, promoting tumor survival, invasion, and metastasis (8). Tumor-expressed chemokines such as CCL5 recruit circulating regulatory T cells (Tregs), while immunosuppressive factors such as TGF-β and IL-10 induce naïve T cells to differentiate into Tregs *in situ* (9). In turn, Tregs

suppress infiltrating cytotoxic T lymphocytes via expression of the immune checkpoints CTLA-4 and LAG-3 and secretion of inhibitory factors such as TGF-β, IL-10, and IL-35. Tumors also directly produce immunosuppressive cytokines as well as exosomes displaying death ligands, such as PD-L1 (10). Finally, tumor cells can evade cellular immunity through upregulation of immune checkpoints (11) and the antiphagocytic marker CD47 (12), while suppression of peptide–MHC presentation (13) can inhibit T-cell recognition of mutated peptides as well as any potential abscopal effects of the initial CAR T-cell therapy. Combined, these factors reduce the potential efficacy of cellular immunotherapies (14).

Exhaustion

Tumor-induced T-cell exhaustion is commonly observed during chronic T-cell stimulation and is characterized by reduced T-cell functionality. Conventional CAR T cells can also display elevated levels of T-cell activation and exhaustion markers (CD25, Lag3, PD-1, CD39, etc.) at baseline (15). This may be attributed to high receptor expression, the choice of costimulatory domain, tonic signaling from extracellular scFv domain clustering (15,16), and lack of transcriptional and post-transcriptional mechanisms to modulate CAR expression analogously to the endogenous T-cell receptor (TCR) (17). High quantitative CAR expression also induces additional mechanisms of T-cell dysfunction, such as Fas-dependent T-cell death and inhibition of long-term central memory T cell (T_{CM}) formation (18). However, clinical trials have suggested that some autonomous signaling may improve outcomes in 4–1BB CAR T cells by preventing T-cell anergy (19). Once in the tumor, long-term exposure to high levels of antigen are associated with CAR T-cell exhaustion (20). Optimally, CAR T cells would be regulated to deliver a powerful inflammatory response specifically at tumor sites while remaining quiescent during manufacture and trafficking.

Systemic toxicity (Cytokine Storm/ICANS)

Currently available CAR T-cell regimens for hematologic malignancies carry “black box” warnings for Cytokine Release Syndrome (CRS), a serious systemic inflammatory response that can lead to TNFα-induced vasodilation, hypoxia, and multi-organ failure. Another frequent side effect of anti-CD19 CAR T-cell therapy is immune effector cell–associated neurotoxicity syndrome (ICANS), which can range in severity from temporary loss of coordination and cognitive ability to seizures, coma, and death (21). Without changes to current CAR T-cell regimens, CRS and ICANS are likely to remain significant risks in the solid tumor setting.

Next-generation CAR designs should be graded for levels of T-cell activation, proliferation, cytotoxicity, anergy, and exhaustion. Synthetic biology strategies to control the amplitude, duration, and location of CAR T-cell activity may enable cell-intrinsic control of systemic toxicities, thus reducing the risks, cost, and labor of CAR T-cell administration and management.

Engineering Solutions

Optimizing CAR components

All CAR structural components, including the ligand-binding domain (LBD), hinge domain, transmembrane domain (TMD), and costimulatory domains, impact receptor functionality. Interestingly, LBD affinity does not correlate with CAR functionality, as certain CARs with lower affinity LBDs have demonstrated enhanced T-cell proliferation with reduced toxicity (22). The CAR hinge and TMD have been shown to impact receptor expression, cytotoxicity, oligomerization, sensitivity to ligand density, and susceptibility to activation-induced cell death (AICD) (23–27), with CARs including the CD8 and CD28 hinge domain and TMD having produced the most optimal performance to date.

The many contributions of the CAR costimulatory domains have been extensively described. Novel costimulatory domains that promise enhanced T-cell proliferation and reduced exhaustion include both endogenous signaling proteins and synthetic domains with known costimulatory motifs, such as the consensus PI3K binding site YxxM (28–30). Pooled screening approaches have identified additional candidate costimulatory domains that enhance T-cell proliferation and cytotoxicity, such as BAFF-R (29). In addition, mutations to both the CD3ζ and costimulatory signaling domains can improve CAR T-cell cytotoxicity and reduce exhaustion (31,32). Incorporation of the CD28 costimulatory domain (27), the CD3ε chain (33), and the GRB2 adaptor domain, results in improved targeting of antigen^{low} tumors and reduced antigen escape (33). The spatial configuration of costimulatory domains has also been shown to influence CAR efficacy, as second-generation CAR T cells co-expressing additional costimulatory molecules on separate protein chains outperformed their respective third-generation receptor counterparts (34).

Next-generation CAR designs

First-generation CARs signaled solely through the CD3ζ intracellular domain, while second- and third-generation CARs included an additional one or two costimulatory domains, respectively. The “Armored” or “fourth-generation” CAR platform conventionally refers to multicomponent systems bearing both a CAR and a secondary secreted payload. T cells Redirected for Universal Cell Killing (TRUCKs) are an archetypal example, producing the proinflammatory cytokine IL-12 via an NFAT promoter triggered by CAR activity (Figure 2A) (35). A “fifth-generation” CAR, which includes binding sites for STAT3 and CD247, contains additional signaling components whose coordination may amplify natural TCR signaling (36). However, classical CARs remain fundamentally simple receptor designs, with multiple domains connected on a single protein chain.

Newer multichain receptor architectures that better mimic endogenous protein complexes enable more ITAM signaling domains than would be functionally possible on a single chain (Supplemental Table S1). The TCR Fusion Construct (TRuC) design fuses an scFv to an extracellular component of the αβTCR. TRuC T cells have demonstrated improved cytotoxicity as compared to second-generation CAR T cells at equivalent T-cell doses, although with less cytokine secretion (37). Another approach, “AbTCR”, avoids a potential mispairing of an αβ TCR-based design by fusing a Fab to the intracellular signaling

domains of the γδ TCR (38). Like TRuCs, AbTCRs also yielded improved cytotoxicity with decreased cytokine secretion, along with a more stem-like T-cell phenotype. Another design, "Synthetic T-cell receptor and Antigen Receptor (STAR)", employs separate V_H and V_L domains on engineered murine TCRα and TCRβ chains to prevent mispairing with endogenous TCR domains (39). This approach generated more cytokine than conventional CARs, perhaps due to improved overall receptor expression. A fourth strategy, "T-cell Antigen Coupler (TAC)", uses a membrane-tethered tandem scFv pair where the membrane-proximal unit binds to a TCR component and the distal unit binds the target antigen (40). This approach also yielded lower cytokine signaling. Interestingly, when paired with a HER2 binding domain capable of cross-reactivity with endogenous mouse HER2, TAC demonstrated antitumor efficacy comparable to that of a second-generation CAR but with reduced on-target/off-tumor toxicity. TCR-based chimeric receptors can also be inserted directly into the TRAC locus through CRISPR genome editing (41,42). This HLA-Independent T-cell (HIT) receptor displays enhanced sensitivity to low levels of the antigen target and comparable cytokine production to the analogous second-generation CAR. In general, TCR-based chimeric receptors uniformly offer improved cytotoxicity in both *in vitro* and *in vivo* models, particularly against targets with low antigen density.

Determining an optimal CAR architecture will require completeness and consistency in the recorded data. A centralized curated parts repository, consisting of standardized sequences, could be annotated with consistent quantitative measures including cytotoxicity, proliferation, and exhaustion metrics. Optimization of costimulatory domain selection using high throughput library screening represents the first steps in the creation of such a parts catalog suitable for broad comparison of receptor components (28,29,43).

Synthetic signaling platforms in immune cells

Engineering cells with programmable and tunable behaviors will provide the foundation for the next generation of cellular immunotherapies. One of the first AND-gated logic circuits to control CAR T-cell function was the co-expression of a first-generation CAR with a chimeric costimulatory receptor (CCR) combining an scFv against a secondary target (PSMA) with the CD28 costimulatory domain (44). CAR T cells equipped with this circuit effectively targeted tumors expressing both antigens but also demonstrated "off-target" activity against tumors expressing only the antigen targeted by the first-generation CAR. This behavior was accentuated by imbalances in ligand expression between the CAR and CCR targets. A similar strategy can be used to construct NOT gates, whereby an inhibitory chimeric-antigen receptor (iCAR), consisting of an scFv targeting an off-tumor antigen coupled with an inhibitory CTLA-4 or PD-1 intracellular domain, blocked off-tumor CAR activation (45). An alternate approach uses tandem CARs with two low affinity LBDs that depend on simultaneous binding to adjacent epitopes to efficiently transduce signal (46). Recently, "LINK CARs" that utilize elements of the intracellular T cell–signaling cascade have also been described as functional AND-gates (47). Despite the promise of these strategies, full control of engineered immune cells will require a system with easily customizable ligand targeting and programmable cellular function.

Synthetic signaling receptors must transmit an arbitrary signal across the cell membrane and convert it into a programmable cellular behavior. An early major milestone from the Leonard group was a synthetic heterodimer receptor, with a “protease” chain consisting of a membrane-bound tobacco etch virus protease (TEV) and a “target” chain equipped with a TEV-cleavable transcription factor (TF) (48). This receptor class, named Modular Extracellular Receptor Architecture (MESA), is activated when the assembly of the heterodimer around a target ligand induces proximity-mediated TEV cleavage, releasing the TF to traffic to the nucleus. While initial MESA designs were triggered only by synthetic dimerizing agents, subsequent iterations were directed against tumor-relevant targets such as VEGF (49). Although this receptor demonstrates responsiveness to soluble ligands, performance characteristics such as ligand-independent leakage remain challenges.

Synthetic Notch-like receptors

In 2016, Morsut and Roybal reported the development of the synthetic Notch receptor (synNotch), a high-performance single-chain receptor that couples arbitrary ligand sensing to downstream transcriptional activity. Unlike the fully transgene-encoded cleavage apparatus of MESA, the Notch-derived regulatory apparatus of synNotch activates through regulated intramembrane proteolysis (RIP), a process involving sequential cleavages mediated by ADAM and ɣ-secretase, followed by release of a membrane-bound intracellular TF (50). By replacing the endogenous Notch ligand binding and transcriptional domains with orthogonal components, it was possible to retarget the engineered modular receptor to other membrane-bound ligands, such as CD19 and membrane bound eGFP, and induce several potential immunotherapeutic genes, including CARs and cytokines (50). Subsequent studies demonstrated the potential versatility of synNotch receptor circuits for targeting several tumor models (Supplemental Table S2).

SynNotch receptor circuits reduce the risk of on-target, off-tumor CAR toxicity by restricting CAR activity to sites that express both the synNotch and CAR ligands in spatial proximity (51). Additionally, the lack of constitutive CAR expression and concomitant tonic signaling can improve the fitness of the therapeutic product, with a higher fraction of T cells differentiated into a T_{cm} phenotype and reduced expression of exhaustion markers (52). Coupling low affinity synNotch receptors with high affinity CARs can generate T cells capable of ligand density discrimination with an ultrasensitive threshold, potentially mitigating on-target/off-tumor toxicity without sacrificing therapeutic potency (53). Therapeutic payload candidates include CARs, natural cytokines (54), and designed cytokine analogues (55).

Further synNotch engineering efforts aim to improve receptor dynamic range and deliverability while reducing immunogenicity. Reintroduction of the endogenous Notch RAM7 domain was shown to mitigate ligand-independent activation at the cost of reduced overall activation (56). More recently, we have developed an optimized Notch-like receptor platform called SyNthetic Intramembrane Proteolysis Receptors (SNIPRs) (Figure 3). These receptors are comprised of small, modular, and highly tunable extracellular, transmembrane, and juxtamembrane components whose output level can be readily matched to the target application and demonstrate better sensitivity to lower ligand levels than do prior designs

(57). Elimination of the regulatory domain also decreases receptor size by nearly 1 kb, rendering the receptor more amenable for gene therapy applications in which viral vector payload capacity is limited.

Humanized components

Engineered receptors, by their nature, often contain components that are immunogenic as they are not normally expressed in human cells (58). The original synNotch receptor is composed entirely of non-human domains: the murine Notch regulatory core, the yeast Gal4 DNA binding domain, and the HSV1-derived VP64 transactivation domain, which has been specifically noted to engender a high probability of immune rejection (59). To mitigate this risk, human analogues to these components can be incorporated, though this strategy often requires performance re-optimization. An alternative strategy to evade host immunity entails the application of “universal donor” MHC knockout (KO) technologies to reduce the risk of immunogenicity (60).

Building on previously published efforts in yeast (61), parallel efforts by Donahue (62) and Israni (63) produced toolkits of orthogonal, customizable, and clinically viable zinc-finger based TFs with reduced immunogenicity potential. Our optimized SNIPR platform also utilizes domains sourced from human proteins, such as CD8α and the hNotch1 transmembrane domains, thus reducing immunogenicity risk (57). Furthermore, we have also shown that unlike synNotch, SNIPRs are compatible with humanized and human-like TFs, including zinc finger–based programmable TFs (Figure 3) (57).

Shifting Gears: Additional Synthetic Biology Strategies

In addition to CARs, MESA, synNotch, and SNIPRs, other protein engineering strategies have attempted to enhance T-cell therapies in solid cancers (Figure 4). Dominant negative receptors (DNRs) with mutated signaling domains sequester immunosuppressive ligands by binding them without inducing downstream signaling. TGF-β DNR-equipped anti-PSMA CAR T cells have been shown to outperform conventional CAR T cells in pancreatic cancer models expressing high levels of TGF-β (64). Cell-intrinsic checkpoint inhibition via PD-1 DNRs has also been shown to overcome PD-1–mediated immunosuppression in preclinical studies (65), and phase I trials of anti-CD19 CAR T cells co-expressing PD-1–targeted DNRs demonstrate safety and efficacy (66). Switch receptors (SRs) flip the effects of ordinarily immunosuppressive factors by coupling their binding with an immunostimulatory signaling domain. A 4–1BB switch receptor targeting TGF-β enhanced T-cell proliferation and tumor clearance in a TGF-β–overexpressing A375 melanoma model (67). Co-expression of IL-4/IL-7 and TGF-β/4–1BB SRs may overcome complex immunosuppressive tumor microenvironments (68). The “iTurbo” platform uses a small molecule to initiate signaling from a chimeric receptor to provide drug-inducible JAK/STAT signaling, which was shown to improve T-cell persistence (69). A novel strategy from the Garcia laboratory utilizes macromolecular noncovalent crosslinkers to manipulate common kinase signaling networks by localizing phosphatases to target kinases. For example, recruiting CD45 phosphatase to PD-1 could reduce immunosuppressive signaling (70). A subsequent report from the same laboratory incorporated the previously established orthogonal IL-2 (orthoIL-2) cytokine/receptor pair, which can induce the IL-2 cascade independently of endogenous IL-2,

into the SR archetype (71). Fusing the orthoIL-2 receptor extracellular domain to the signaling domain of IL-9 resulted in a SR that induced a more stem-like T-cell phenotype and improved antitumor efficacy in response to both injected and adenovirally-expressed orthoIL-2.

Endogenous T-cell activation can also be exogenously retargeted through a plethora of switchable receptor strategies. The most generalizable approach employs bispecific T-cell engagers (BiTEs), a class of tandem scFvs, to noncovalently crosslink endogenous patient T-cell TCRs with target antigens (72). Blinatumomab, a CD19-targeted BiTE, gained FDA approval in 2014 for refractory acute lymphoblastic leukemia (ALL), but pharmacological constraints may limit the efficacy of BiTE monotherapy in solid tumors (73). BiTEs may also help to supplement CAR T-cell therapy, as an EGFR-targeted BiTE allowed EGFRviii-targeted CAR T cells to fully clear a heterogenous glioblastoma tumor in mouse model (74).

A more constrained strategy employs adapter-based “switchable” or “universal” CARs, in which T cells can be transduced with a constant extracellular moiety to which a soluble targeting protein can subsequently bind. Examples of this approach include Avidin CARs that bind biotinylated adaptors (75), peptide-tagged “αE CARs” (76), “sCARs” bearing scFvs that bind FITC-labeled targeting modules (77), and leucine zipper-bridged “UniCARs” (78). Switchable synNotch platforms have also been described (79). These modalities offer targeting flexibility and reduced risk of CRS and ICANS through real-time dosage control. However, the variable biodistribution of adaptor molecules and the periodic need for adaptor reinfusions render their potential benefit unclear.

Improved exogenous control of CAR T-cell function through small-molecules may help to mitigate both systemic and on-target/off-tumor toxicity. “Drug-On” systems that activate CAR activity may improve the CAR T-cell fitness analogously to SynNotch-mediated inducible expression by reducing exhaustive TCR signaling prior to administration, while “Drug-Off” systems may be used to prevent or reduce systemic toxicities. However, these systems increase therapeutic complexity and require drug bioavailability for full therapeutic efficacy. The potential for a “Drug-On” system was demonstrated in 2015 when the CD3ζ domain was separated from the main CAR scaffold via the chemically-inducible heterodimer FKBP/FRB* and reconstituted under small molecule control (80). A similar approach placed rapamycin-inducible heterodimerization domains on the CAR extracellular domain (81). However, rapamycin-based control systems are limited by the drug’s broadly immunosuppressive activity as well as its limited biodistribution and half-life (82). Subsequent designs utilized more bioavailable drugs to modulate the association (83) or dissociation (84) of the CAR transmembrane and intracellular domains. Another “Drug-On” CAR design relies on the interaction between hRBP4 and an orally bioavailable drug (85). The “CRASH-IT” approach introduces an inhibitory PD-1 intracellular domain that can be removed by a Asunaprevir-regulated degron (86). A flexible design, “AvidCAR” uses drug-induced dimerization of low affinity CARs to toggle CAR activity; however, the requirements for low-affinity, non-scFv binders limit its versatility (46).

Degrons can be used for “Drug-Off” systems where degron activation degrades the CAR itself (63,87). Another example of a “Drug-Off” approach modulates assembly of a

CAR extracellular chain and intracellular CD3ζ domain through a modified Bcl-XL/BIM heterodimer that is dissociable via small molecule (88). This “STOP-CAR” performed similarly relative to a conventional CAR but did not display full drug-induced inhibition.

Many previous strategies do not demonstrate clean switch-like behavior between active and inactive T-cell states, use drugs that interact with multiple intracellular targets, and sacrifice important parameters such as receptor expression, cytotoxicity, and cytokine production for the goal of drug inducibility. The modularity of these designs is also hampered by dependence on the choice of costimulatory domains (46,84). Recently, the Mackall group developed an NS3 protease–regulated “SNIP CAR” system triggered by grazoprevir, which demonstrates an impressive dynamic range and improved potency relative to conventional CAR controls, albeit with a potentially immunogenic viral component (89). The SNIP CAR uses dimeric tandem chains to keep the protease and cleavage site in proximity while also keeping CAR signaling domains near the plasma membrane.

In addition to regulating CAR activity at the protein level, small molecules can also control CAR expression at the transcriptional level. The third-generation Tet-On system allows for induction of CAR expression via doxycycline administration. However, this platform demonstrates substantial basal cytotoxicity and makes use of the xenogeneic rtTA transactivator (90). A tamoxifen-regulated zinc-finger platform has been used to drive the CAR transgene with impressive dynamic range, though this construct also employed a xenogenic transactivator, VP64 (91). The Khalil lab has developed synthetic zinc finger transcription regulators (“SynZiFTR”s), a set of zinc finger TFs with human-derived transactivation domains regulated by viral NS3 protease, plant abscisic acid pathway dimerizers, and the tamoxifen-responsive human ERT2 module (63). The SynZiFTR platform offers impressive performance and multiple orthogonal channels with which to regulate CARs and other payloads, and the ERT2-regulated form offers a transgene expression system comprised of human-derived components.

Drug-inducible CAR platforms offer an option for titratable control of CAR function with a wide dynamic range, but their application is complicated by issues of drug bioavailability, distribution, half-life, and expense. Additionally, the availability of drugs that reversibly modulate T-cell activity, such as dasatinib, may render more complex engineering approaches moot (92).

As alternatives to using small molecules to control CAR function, methods focused on the use of light and sound have also been devised. Optogenetically-controlled CAR T cells can enable physician-guided spatiotemporal control of therapeutic activity (93). However, the difficulty of light to penetrate deep tissue currently limits these modalities to surface-localized tumors, although additional progress may allow for more clinically-compatible photoreceptors (94). Additionally, the need to constantly apply light to activate split CARs mediated by reversible photo-dimerizing domains may limit clinical practicality. More tissue-penetrant forms of energy, such as Focused Ultrasound (FUS), can induce a highly localized mild hyperthermia at a tumor site, specifically activating T cells that express CARs or cytokines under heat shock promoter control (95,96). Image-guided immunotherapeutics may facilitate targeting of tumors lacking an unique antigen signature,

but careful consideration must be given to the design strategy of transiently active versus state-switching mechanisms, as the former incurs clinical expense due to needing long or multiple therapeutic sessions while the latter may be prone to toxicity caused by activated immune cells escaping the region of optical or sonic stimulation.

The advent of robust computational protein design has facilitated a novel strategy to engineer T cells responding to complex antigen signatures. Lajoie et al. demonstrated that a co-localized protein switch system, named Co-LOCKR, could produce AND, OR, and NOT logic gating by undergoing conformational changes and exposing target residues in response to a series of designed inputs (97). By using a CAR directed against the conditionally exposed peptide, the Co-LOCKR could act as a soluble multi-input T-cell engager. Future implementations of LOCKR may allow for transcriptional control, but its fully synthetic nature may raise immunogenicity concerns, and the dual-chain architecture may increase stoichiometry-associated unintended activation.

Visiting New Destinations: Novel Targets for CARs and Co-receptors

Enhancing cancer treatment may require targeting antigens in the immunosuppressive tumor stroma, such as FAP (98) or FRβ (99). Tumor-associated soluble factors are also emerging as viable targets: anti–TGF-β CAR-T-cells were found to activate through TGF-β–mediated dimerization (100) and could augment the function of neighboring cytotoxic T cells (101).

Efforts are underway to apply features of CAR T cells, such as the production of pro-inflammatory cytokines, toward improving tumor-infiltrating lymphocyte (TIL) therapy (102). Additionally, scFvs targeting MHC–peptide complexes can be employed to generate “TCR-like” CARs (103). However, these strategies are hindered by tumor cells downregulating MHC to escape T cell–mediated rejection along with the significant polymorphism among human MHC genes (104).

While oncogenic mutations often help cancers avoid immune rejection, they may also render tumor cells vulnerable to other strategies. For example, oncolytic viruses that selectively replicate in cancer cells by leveraging defects in transcriptional regulation and innate immune signaling have been under investigation for decades. Arming these viruses with inducers of adaptive immunity, such as inflammatory cytokines, may enhance the antitumor response (105). Studies have also engineered subcellular exosomes (106) and lytic granules (107) with custom therapeutic payloads. The latter strategy was subsequently explored in a method, named COVERT, in which lytic granules carrying a Granzyme B–SUMO protein regulated by a proteolytically-cleavable linker selectively induced apoptosis in cancer cells that upregulated the protease SENP1 (108). Leveraging synthetic biology strategies at both the transcriptional and protein levels will allow greater control of cellular immunotherapies.

Continuing discovery of targetable antigens on solid tumors will also help to develop better CAR T-cell therapies. Extensive existing data sets, such as the Human Protein Atlas and the Cancer Genome Atlas, can be mined for differential expression between cancerous and healthy tissues (109). Additionally, the emerging field of surfaceomics aims to directly discover surface targets via strategies that enrich for proteins with extracellular domains (110,111). Such methodologies can be coupled with downstream computational

analysis to produce multiplexed “molecular fingerprints” amenable to the logic gating strategies described previously (112). Beyond conventional overexpressed proteins and mutated neoantigens, the relatively unexplored realm of non-protein antigens, including lipids and carbohydrates, may also yield a wealth of additional CAR targets. A leading target within this class is the ganglioside GD2, which has been investigated in the context of neuroblastoma (113,114). Surface proteins displaying aberrant glycosylation patterns, such as the Tn form of Muc1 (115), also present as intriguing targets.

Tune ups under the hood: Beyond receptor engineering

Advances in CAR design can be coupled with further intracellular modifications to generate cell therapies with augmented cytotoxicity, stemness, and exhaustion features. The impact of intracellular modifications was initially demonstrated when a patient with chronic lymphocytic leukemia and infused with anti-CD19 CAR T cells displayed near total monoclonal dominance two months after their second CAR infusion (116). Evaluation of these clones revealed that the CAR vector had integrated into and prematurely terminated *TET2*, a hematopoietic master switch, while the second *TET2* allele natively bore a hypofunctional mutation. The resulting CAR T cells were more resistant to exhaustion, suggesting that manipulation of *TET2* and other endogenous genes could improve CAR T-cell functionality. However, biallelic *TET2* loss was recently shown to promote antigen-independent, BATF3-dependent clonal CAR T-cell proliferation and reduced effector functionality (117), indicating the importance of understanding how genetic perturbations affect global T-cell phenotypes.

The use of CRISPR knockout (KO) libraries has uncovered additional negative regulators of T-cell activity. Screening through a KO panel of metabolic regulatory genes revealed that loss of the nuclease REGNASE-1 improved T-cell proliferation, effector function, and memory formation by increasing BATF and TCF-1 activity (118). Furthermore, co-transduction of CAR and BATF resulted in increased T-cell potency and exhaustion resistance (119). Also, analysis of upregulated genes in hypofunctional T cells revealed that TOX1 and TOX2 serve as markers of exhaustion whose downregulation enhanced T cell–mediated tumor control (120). Similarly, a KO of the family of NR4A TFs also improved tumor control (121), although the redundancy between the three TFs necessitates a difficult triple KO. KO of the DNA methylator DNMT3A in CAR T cells conferred increased proliferation and efficacy, primarily due to an increase in IL-10 expression (122). Combined *in vitro* and *in vivo* CRISPR KO screens have identified chromatin remodeling factors such as Arid1a and cBAF as additional critical regulators of exhaustion (123). Suppression or KO of IDO1, the rate-limiting enzyme in tryptophan metabolism, was also found to boost T-cell metabolism and cytotoxicity (124).

In addition to genetic KO, knock-in of new open-reading frames (ORFs) can also improve T-cell fitness. Using a lentiviral genome-scale ORF library, Legut et al. found that several transgenes, including lymphotoxin-β receptor (LTBR), enhanced T-cell proliferation, cytokine secretion, and exhaustion resistance (125). Study of AP-1 transcriptional complex dysregulation during T-cell exhaustion found that CAR T cells overexpressing c-Jun, a key AP-1 component, were rendered exhaustion-resistant (126). Furthermore, an engineered

enzyme that breaks down kynurenine, an inhibitory product of IDO1 activity, enhanced naïve $CD8^+$ T-cell proliferation and infiltration (127), and CAR T cells engineered to constitutively express catalase protected co-cultured natural killer (NK) cells under H_2O_2 stress while demonstrating enhanced direct effector function, although no results were shown *in vivo* (128).

Along with modification of intracellular T-cell factors, T cells can also be engineered to secrete immunomodulatory factors into the tumor microenvironment. While TRUCKs are currently designed to secrete an induced pro-inflammatory cytokine payload (35), payloads could also be constitutively expressed, trading tumor-localized expression for higher overall cytokine levels (129). Interleukin payloads demonstrated to improve CAR T-cell efficacy include IL-15 (130) and IL-18 (131,132). Engineered cytokines, exemplified by derivatives of IL-2, can maintain cytotoxic lymphocyte stemness (133), selectively activate desired T-cell subsets while avoiding stimulation of Tregs (134), or only activate engineered T cells (135) expressing an orthogonal cognate receptor. Cytokine payloads can also be engineered for improved stability, as demonstrated with IL-7 (136). Spatial delivery of these payloads theoretically allows for localized inflammation at the tumor microenvironment, conferring a safety advantage when compared to systemic routes of administration (137). Genetic elements such as the internal ribosome entry site and the 2A self-cleaving peptide also provide a compact method to deliver synergistic payloads, such as IL-7 and CCL-19 (138) or Super-IL2 and IL-33 (139). Future work should include a systematic comparison between CAR T cells bearing different combinatorial payloads and the development of guidelines for optimizing cytokine cargos with specific disease contexts.

In addition to the secretion of cytokines and chemokines, Durgin et al. found that CAR T-cell secretion of *C. perfringens*–derived neuraminidase to cleave immunosuppressive sialic acid moieties on the surface of tumor cells improved overall CAR T-cell function (140). T-cell secretion of heparanase (141) and hyaluronidase (142) has also been shown to improve immune cell trafficking by locally remodeling tumor extracellular matrix without the toxicity observed during systemic administration (143). Recently, CAR T cells were engineered to transcribe an immunostimulatory RNA molecule, package it into exosomes, and deliver it to surrounding myeloid cells (144). Delivery of these molecular payloads can be triggered using promoters downstream of the TCR pathway such as NFAT, or through orthogonal cell surface receptors such as SNIPR and MESA.

Modifications may also improve allogenic CAR T-cell safety. T cells with a deleted TCRα would have non-functional TCRs and would be unlikely to provoke graft-versus-host disease due to their inability to recognize peptide–MHC complexes (145). However, this potential safety benefit is countered by a decreased persistence relative to conventional CAR T-cell controls (146). Deletion of β2M and CIITA, components of MHC class I and II respectively, can also eliminate patient T cell–mediated rejection. Doing so also results in CAR T-cells that are camouflaged from host T cells (60). However, the resulting MHC hypoexpression would render these cells vulnerable to NK-cell attack, a complication that can be mitigated through overexpression of CD47 or another NK-inhibitory ligand (147). Combinations of these intracellular modifications with logic-gated targeting modalities may result in safer allogenic cell therapeutics, but higher gene delivery efficiencies will be required for clinical

relevance. Progress in stem cell–derived CAR T-cell products (148) may also enable the step-wise delivery of a panel of beneficial transgenes to a pool of renewable precursor cells prior to final differentiation into a therapeutic product.

In addition to direct T-cell engineering, several efforts have improved the efficiency and safety of manufacturing T-cell therapeutics. Currently, CAR T-cell therapies rely on lentiviral- or retroviral-mediated integration, which preferentially integrates DNA at transcriptionally active sites (149). In contrast, advances in CRISPR knock-in allow for targeted insertion of CAR genes at specific locations in the genome, such as the *TRAC* locus (150). The advantages of this method include eliminating potential cross-talk of the endogenous TCR as well as standardizing copy number. However, potential issues include off-target genomic editing events and p53-mediated cell-cycle arrest (151).

Advances in manufacturing may also overcome technical and logistical hurdles in generating sufficient CAR T-cells for treatment. Many cancer patients, especially those pretreated with chemotherapy, have hypofunctional immune cells which poorly expand *ex vivo* (152). Novel viral vectors specifically targeting T cells may enable *in situ* CAR T-cell generation, bypassing a complex and expensive manufacturing process and producing a more universal and economical treatment modality (153,154).

Beyond the Conventional CAR T-cell Model

Conventional cellular immunotherapies entail the use of a single T-cell population modified with a single receptor targeting a unique cancer-associated ligand. Broader antigen-targeting efforts include an OR-gated CAR targeting either CD19 or CD20, which demonstrated reduced antigen escape (155). In addition, multiple engineered T-cell populations, including a CAR library, demonstrated more robust tumor control than a single CAR directed against an overexpressed target, although severe toxicity in an allogenic model suggest only a limited panel of receptors would be clinically feasible (156). In the long term, robust antitumor therapies may encompass entire synthetic immune systems, with engineered macrophages (157), neutrophils (158) NK cells (159), γδ T cells (160), NKT cells (161), and B cells(162).

Perspective

After over a decade of synergistic efforts between synthetic biology and immunotherapy, we have a range of strategies for controlling immune cell function (Figure 5). Advances in CAR design, notably via the high-throughput screening of signaling domains, will generate novel receptors that maximize cytotoxicity and proliferation while minimizing AICD, exhaustion, and anergy. Clinically optimized priming receptors such as SNIPRs improve targeting to sites of disease and are currently in clinical trials for ovarian cancer (NCT05617755). The toolkit of clinically relevant molecular parts to improve cell therapies is advancing rapidly along with the ability to insert larger gene payloads into cells.

To further reduce the risk of on-target, off-tumor toxicity, future proteomic studies must generate multifactor combinatorial “molecular fingerprints” that more accurately distinguish diseased from healthy tissue, including identification of carbohydrates and

lipids. Meanwhile, careful consideration must be paid to the functional consequences of asynchronous (IF → THEN) versus synchronous (AND) sensing. SynNotch and SNIPR employ the former approach, wherein the therapeutic power offered by the flexibility in payload selection must be balanced against potential on-target, off-tumor toxicity in Receptor → CAR configurations when the priming antigen is spatially close to healthy tissues that are positive for the CAR antigen (51). In contrast, the recently described LINK-CAR platform is limited to conventional TCR-pathway signaling but functions robustly as a synchronous AND gated CAR (47). Protein-based logic gates could be extended beyond the TCR pathway to regulate arbitrary transgenes or circuits via synthetic enzymatic signaling cascades to bypass the delay imposed by transcriptional logic circuits (163).

The sheer breadth of strategies for better T-cell immunotherapy currently far exceeds our capacity to efficiently compare these methods. Our field would benefit from a standardized comparison of how they affect parameters such as T-cell function, stemness, and bystander recruitment. Additionally, the potential for combinatorial modification of payloads has been greatly underexplored despite potentially offering synergistic benefits that could propel these therapies over the clinical finish line. Given the large array of available perturbations, it may be necessary to regard each transgene, knockout, and circuit as a module that can be installed into a T cell or another clinically relevant immune cell type, such as an NK cell, to generate a highly specialized final product.

Regardless of the strategy used, the synergy between synthetic biology and immunotherapy is driving the next generation of cell therapies. Future cell therapies will depend on these efforts for improving safety, feasibility, and antitumor efficacy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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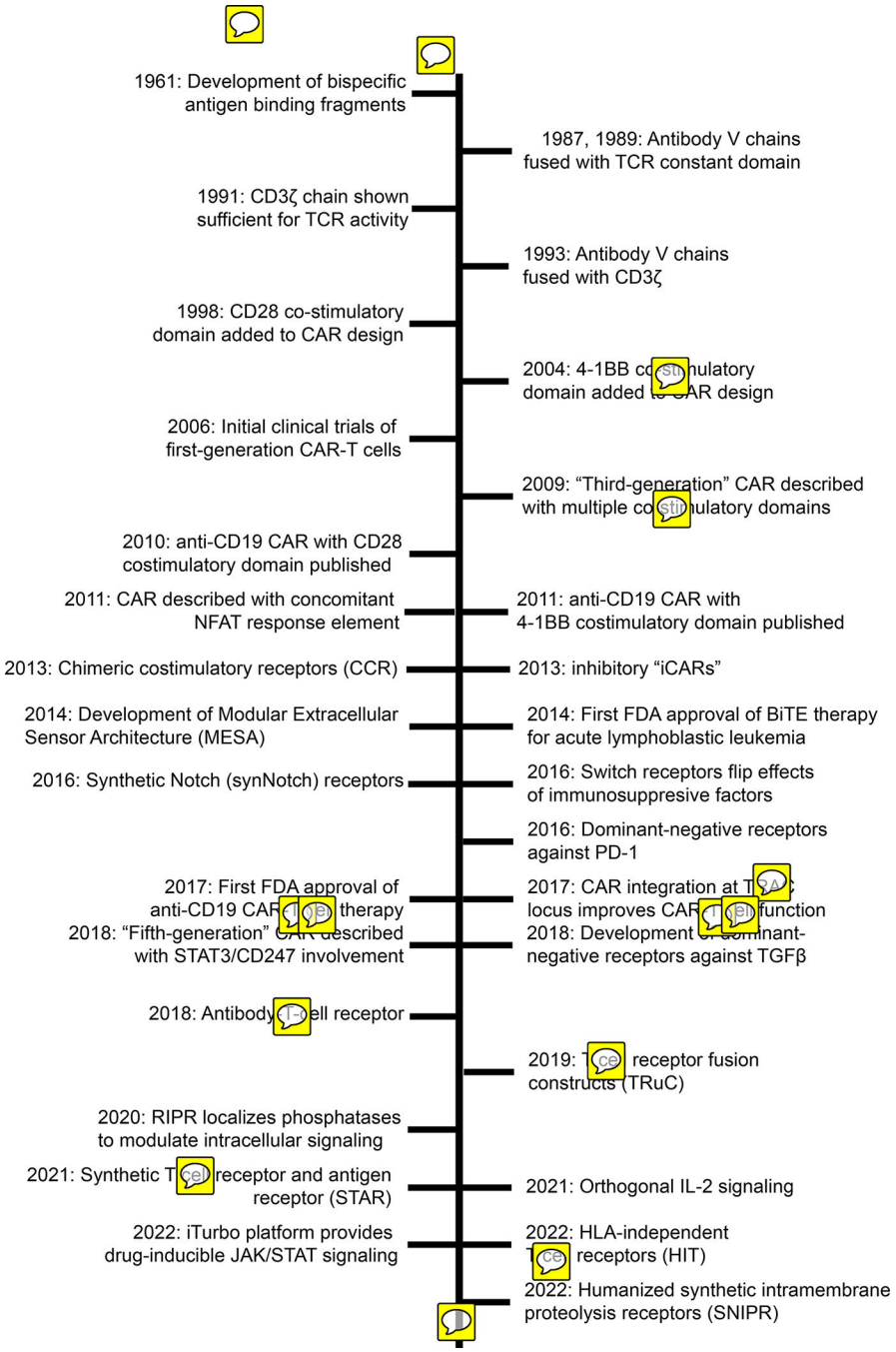

Figure 1. Timeline of selected milestones in cellular immunotherapy.

The pace with which advances in cellular immunotherapy are being made has accelerated with the development of synthetically guided modalities for T-cell activation, such as the chimeric-antigen receptor (CAR), as well as additional mechanisms for enhanced control of the activity of the CARs and T-cell engineered to express them.

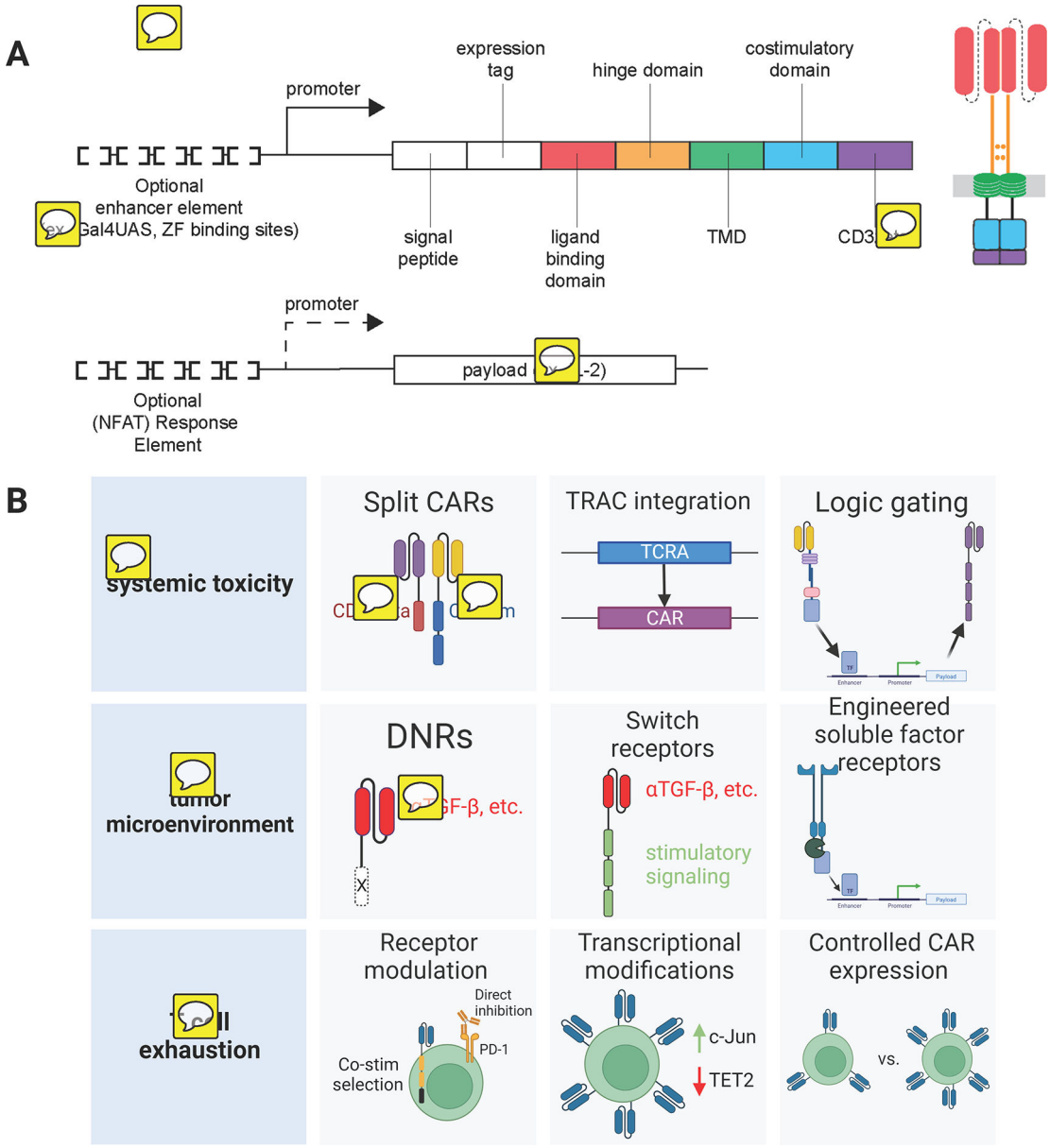

Figure 2. CAR Design, common obstacles, and potential engineering solutions.

A. CARs are conventionally expressed under a constitutive promoter. Integration of synthetic biology concepts has enabled more complex control of expression using inducible enhancer elements under the control of a variety of signaling inputs. The CAR molecule contains ligand-binding domain(s), an extracellular hinge domain, a transmembrane domain, costimulatory domain(s), and an ITAM-containing motif, usually CD3ζ. Fourth-generation CARs include response elements responsive to T-cell activation, such as NFAT-responsive elements, that induce cytokines or other payloads to stimulate an endogenous immune response. B. Systemic CAR toxicity, the immunosuppressive tumor microenvironment, and early T-cell exhaustion all contribute non-optimal CAR performance, especially in solid tumor settings. Here, several solutions are described to address these issues. Systemic toxicity resulting from on-target, off-tumor activity can be mitigated via multi-antigen

targeting and dynamic transcriptional regulation of the CAR. T-cell dysfunction by the TME can be ameliorated by blocking signaling from immunosuppressive components or rewiring their receptors to transduce pro-inflammatory pathways. T-cell exhaustion due to tonic CAR signaling can be reduced by regulating the timing and expression level of the CAR or inducing broad transcriptional changes by perturbing master regulator genes, while exhaustion within the tumor can be blunted by blocking feedback regulatory pathways such as checkpoint inhibition. Created with BioRender.com.

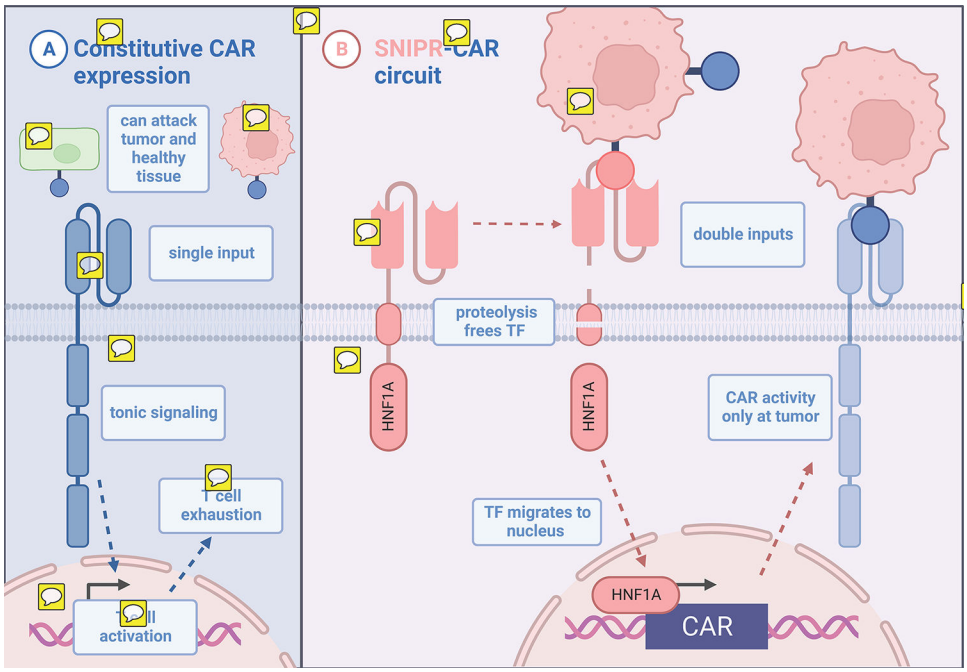

Figure 3. Spatio-temporal control of CAR expression can be achieved with synthetic logic gated receptors.

A. Single input conventional CARs are constitutively expressed and in many cases yield a basal level of tonic CAR signaling, which leads to premature T-cell exhaustion. B. Engineered receptors, such as synthetic intramembrane proteolysis receptors (SNIPRs), have been shown to induce CAR expression specifically at tumor sites, which reduces systemic CAR toxicity, and preserves a more functional and less exhausted T-cell phenotype. Created with BioRender.com.

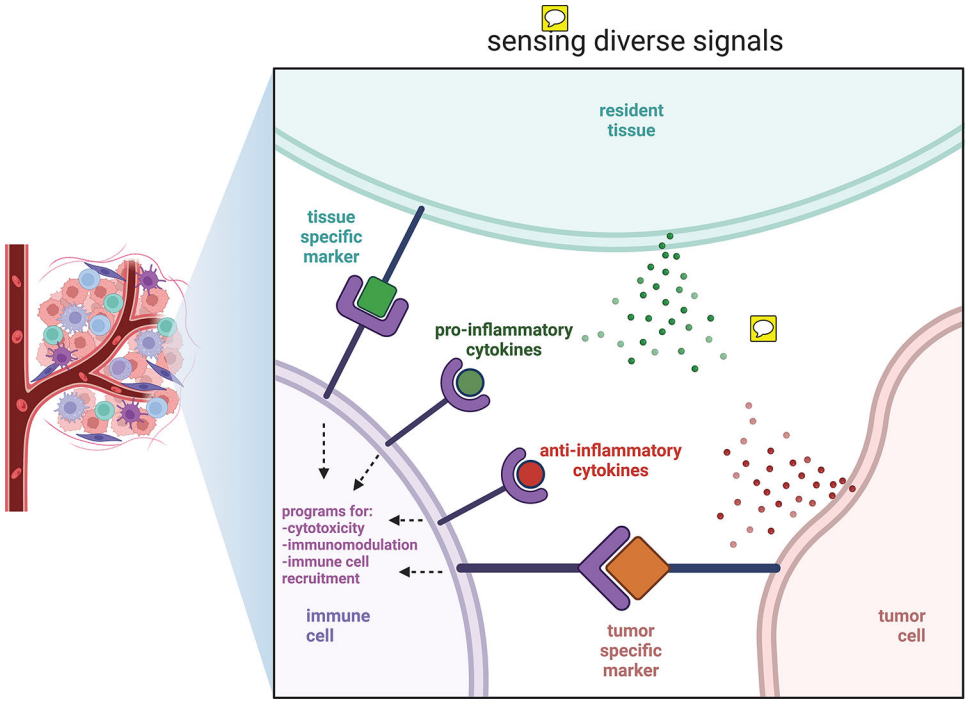

Figure 4. Reprogramming responses to diverse signals.

Using synthetic receptors, responses to normal tissue-specific and tumor-specific molecular markers as well as pro- and anti-inflammatory cytokines can be rewired to enhance engineered immune cell responses to tumors. Examples include CARs specific for tumor-specific antigens, SRs converting the immunosuppressive cytokine TGFβ to a pro-inflammatory signal, and SNIPRs targeting tissue-specific markers to generate a spatially constrained immune response. Created with BioRender.com.

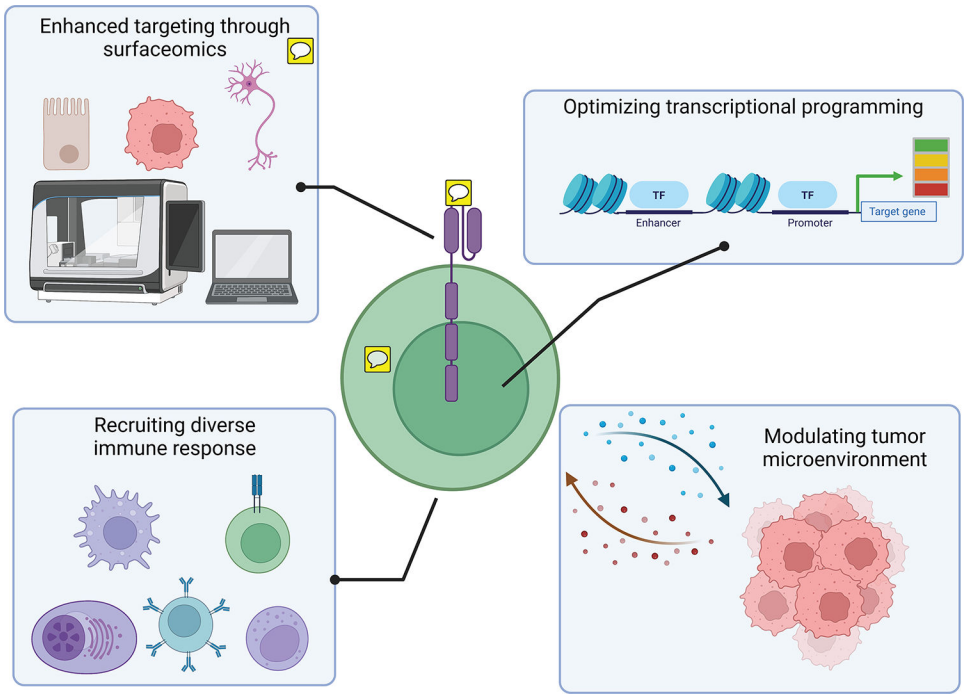

Figure 5. Next-generation cell therapies.

Future breakthroughs in engineered immunotherapeutic cell therapy will include greater tumor specificity as a result of new insights gained through surfaceomics, fully optimized receptor activity as a result of breakthroughs in transcriptional programming, the recruitment and stimulation of a diverse immune response beyond the engineered cell, and the ability to overcome and modulate the immunosuppressive tumor microenvironment. Created with BioRender.com.