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Synthetic Biology in the Engineering of CAR-T and CAR-NK Cell Therapies: Facts and Hopes

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Abstract

The advent of modern synthetic-biology tools has enabled the development of cellular treatments with engineered specificity, leading to a new paradigm in anti-cancer immunotherapy. T cells have been at the forefront of such development, with six chimeric antigen receptor (CAR)-modified T cell products approved by the United States Food and Drug Administration for the treatment of hematological malignancies in the last five years. Natural killer (NK) cells are innate lymphocytes with potent cytotoxic activities, and they have become an increasingly attractive alternative to T cell therapies due to their potential for allogeneic, “off-the-shelf” applications. However, both T cells and NK cells face numerous challenges, including antigen escape, the immunosuppressive tumor microenvironment, and potential for severe toxicity. Many synthetic-biology strategies have been developed to address these obstacles, most commonly in the T-cell context. In this review, we discuss the array of strategies developed to date, their application in the NK-cell context, as well as opportunities and challenges for clinical translation.

Introduction

Surgery, radiation therapy, and chemotherapy have long served as the foundation of cancer therapy, but a number of malignancies have remained resistant to these three pillars of cancer treatment. Over the past decades, immunotherapy has emerged as a fourth pillar in the arsenal against cancer. By harnessing the patient’s own immune system, immunotherapies such as immune checkpoint blockade (1, 2), cancer vaccines (3), and adoptive transfer of immune cells engineered to target tumor antigens, have emerged as promising treatments for malignancies that are refractory to traditional therapies. Among

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Conflict of Interest Statement

Y.Y.C. is an inventor on a patent application for CART19/20 and holds several patent applications in the area of CAR-T cell therapy. Y.Y.C. is a founder of, holds equity in, and receives consulting fees from ImmPACT Bio. She is a member of the scientific advisory board of and holds equity in Catamaran Bio, Notch Therapeutics, Pluto Immunotherapeutics, Prime Medicine, Sonoma Biotherapeutics, and Waypoint Bio. The other authors declare no conflicts of interest.

the different immunotherapy modalities, cell-based immunotherapy has shown particular promise against hematological malignancies, but its application to the treatment of solid tumors remains work in progress (4). Multiple immune cell types, including T cells, natural killer (NK) cells, invariant natural killer T (iNKT) cells, macrophages, and neutrophils have been explored as potential chassis for cell-based immunotherapy, with T cells and NK cells as the most extensively evaluated effector cell types to date.

In contrast to small-molecule drugs, protein biologics, or radiation, cell-based immunotherapies are living drugs with the ability to persist, amplify, and traverse within the patient. These unique characteristics allow cell-based therapies to mount dynamic and complex immune responses against the tumor unattainable by other therapeutic modalities. The ability to specifically program the biological properties of therapeutic cells further expands the capabilities of engineered immune cells as cancer therapeutics. Synthetic biology is a growing discipline that generates biological systems with novel behaviors and functions, by assembling circuitries comprising synthetic biological components and/or naturally occurring biological parts repurposed for new applications. By focusing on the design, construction, and assembly of modular biological components, synthetic biology enables researchers to build biological circuits with programmable input processing and output parameters. In the context of cell therapies, integration of these circuits into immune cells enables development of products equipped with novel therapeutic functions to combat previously ‘undruggable’ or untreatable diseases. Additionally, synthetic biology can accelerate the acquisition of well-controlled biological datasets through the use of clear design rules, thus enabling better understanding of complex biological phenomena and facilitating rational biological engineering principles for translational applications.

Advancements in synthetic biology have been supported by new DNA synthesis and sequencing technologies that enable accurate and high-throughput design, assembly, and testing of biological circuitry (5, 6). Concurrently, gene-editing technologies such as with zinc finger nucleases, transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 have expanded the synthetic-biology toolkit, enabling high-throughput gene screening, targeted gene ablation and transgene insertion, and development of more complicated biological models (7). While the first generation of U.S. Food and Drug Administration (FDA)-approved cell-based cancer immunotherapies largely relies on the expression of a single transgene encoding a tumor-targeting receptor, the incorporation of biological circuits may further expand the safety and efficacy profile of cell therapies to address perpetually intractable diseases.

In this review, we assess the current state of synthetic biology applications in the field of cell-based immunotherapies, focusing specifically on advancements aimed to direct and repurpose T and NK cells against cancer.

Early Development of NK- and T-Cell Immunotherapies

NK and T cells are both lymphocytes with cytotoxic capabilities, including perforin and granzyme-mediated cytotoxicity and pro-inflammatory cytokine release, making them attractive candidates for cell-based anti-tumor therapeutics (8, 9). However, the two effector

cell types also possess differential characteristics that endow each with distinct advantages (Table 1). T cells, the prototypical effectors of adaptive immunity, derive their antigen specificity from genetic recombination of the T-cell receptor, enabling highly specific recognition of foreign peptides presented on host major histocompatibility complex (MHC) molecules to induce clonal T-cell expansion and potent cytotoxicity. Importantly, T cells can establish long-term memory and persistence, thus providing the possibility of long-term surveillance against disease reemergence (10). On the other hand, NK cells are members of the innate immune system that have a relatively short lifespan and more limited proliferative potential, and they do not naturally express receptors with a broad repertoire of antigen specificity (11, 12). Instead, NK cells are regulated by a collection of germline-encoded receptors that can either activate or suppress cytotoxicity upon ligand binding, thus enabling the elimination of virally infected, transformed, or antibody-labeled cells. Importantly, NK cells express many of the signaling molecules downstream of T-cell receptor signaling (13, 14), thus enabling the adaptation of receptors designed for T-cell therapy to the NK cell context.

To date, T-cell therapy development has led the way in oncology applications, culminating in the FDA approval of six autologous chimeric antigen receptor (CAR)-T cell products for B-cell hematological malignancies and hundreds of active clinical trials against various malignancies. However, challenges accompanying autologous cell therapy, such as the risk of manufacturing failure and disease progression by patients awaiting cell manufacturing (15), have motivated research into allogeneic alternatives such as NK cells (Table 2). Unlike T cells, NK cells do not recognize MHC-presented antigens, thus avoiding the potential for graft-versus-host disease that can be triggered by allogeneic adoptive T-cell therapy. This unique property positions NK cell therapies for “off-the-self” use without the need to knock out endogenous receptors. Furthermore, unlike T cells, allogeneic NK cells can be generated from scalable sources such as cord blood, immortalized cell lines, and induced pluripotent stem cells (iPSCs), which can dramatically decrease the cost of adoptive cell therapy (16-18). Lastly, clinical evidence to date suggests NK-cell therapies can achieve anti-tumor efficacy without triggering severe cytokine release syndrome, a serious and commonly observed side effect of T-cell based therapies (17).

Early clinical trials evaluating the adoptive transfer of NK cells relied on their innate ability to identify transformed cells for anti-tumor efficacy, leading to modest outcomes (19, 20). However, advances in synthetic biology and the remarkable efficacy of CAR-T cells have provided a template for engineering more targeted and potent NK cell therapies. Early development of synthetic activating receptors in NK cells utilized prototypical CARs developed for T cells and validated the ability to redirect NK-cell cytotoxicity in an antigen-specific manner (21). Similarly, the earliest CAR-NK cell clinical trials utilized prototypical, CD19-targeting CARs (NCT00995137, NCT01974479). While the recent successes with CAR-T cells have reinvigorated the field of cellular therapy, more sophisticated strategies are needed to broaden its therapeutic outlook and synthetic biology is expected to be key in facilitating the engineering of these next-generation therapies.

Synthetic Biology in CAR Engineering

CARs are synthetic receptors that enable immune cells to recognize and initiate antigen-specific cytotoxic responses (22). The adoptive transfer of CAR-T cells has demonstrated remarkable clinical efficacy in treating various B-cell hematological malignancies, becoming the first genetically modified cell therapy to receive FDA approval in 2017 (23). CARs are transmembrane proteins comprising of four major components: an extracellular antigen-binding domain, extracellular spacer domain, transmembrane domain, and intracellular signaling domain (Fig. 1A). This architecture allows for transduction of an extracellular antigen-binding event into an intracellular signaling cascade, resulting in downstream cell activation and subsequent target-cell killing. First-generation CARs, which only utilize CD3ζ as an intracellular signaling domain, failed to elicit potent anti-tumor activity in clinical trials (24, 25). In response, one or two intracellular co-stimulatory domains were incorporated in second- and third-generation CARs, respectively, to enhance cytokine production, proliferation, and *in vivo* persistence (26-28). Although the early development of CAR-NK cells directly adopted CARs developed for T cells, the modular design of CARs has also allowed development of novel CARs containing alternative transmembrane and signaling domains that may be better suited for NK-cell function. For example, “NK-CARs” containing an NKG2D transmembrane domain and a 2B4 co-stimulatory domain, both NK-cell–related proteins, have shown promising anti-tumor function in pre-clinical settings. In comparison to T cells expressing prototypical “T-CARs” comprising CD28 transmembrane and CD28 plus 4-1BB costimulatory domains, iPSC-derived NK cells expressing NK-CARs achieved stronger efficacy and reduced off-tumor in a mouse xenograft model of ovarian cancer (29). These results suggest that tailoring of CAR components for optimal signaling in NK cells may further increase the efficacy of CAR-NK cell therapies for solid tumors.

Regulatable CAR platforms for improved safety

Unlike antibodies and small-molecule drugs, cell-based therapies use living cells that can proliferate, persist, and circulate within the body, thereby mounting dynamic and complex immune responses against target cells. However, the dynamism and potency of cell-based therapies also pose unique safety challenges in the clinic, with the potential for severe toxicities such as cytokine release syndrome and immune-effector cell associated neurotoxicity syndrome (30). A potential strategy to reduce unintended toxicity is to implement regulatory devices that can control either the expression or the function of CAR proteins, and consequently regulate the activity of CAR-expressing cells.

The regulation of gene expression or protein activity using small-molecule drugs has a long history in mammalian synthetic biology (31, 32), and drug-regulatable platforms have been used to enable on-demand cessation of CAR signaling activity without permanently ablating the engineered cell population (Fig. 1B). For example, ON switches can be engineered by splitting the CAR protein into two nonfunctional domains such that CARs are in the inactive OFF state by default, and expression can be turned on through the administration of a dimerizing drug (33-35). Alternatively, OFF switches can be engineered such that CARs fused to degradation domains are in a default ON state, but administration of small-molecule drugs can induce CAR proteasomal degradation to turn expression off (36, 37).

Although drug-regulatable expression systems provide increased flexibility and control, common challenges for these switch designs include: ‘leaky’ (i.e., high baseline) activity in the OFF state, reduced CAR expression and potency compared to constitutive systems, and poor dynamic range between the ON and OFF states. Various engineering strategies are under active evaluation to address these obstacles. A recently reported drug-regulatable platform termed signal neutralization by an inhibitable protease, or SNIP, has been shown to have a tight OFF state, improved dynamic range, and improved potency compared to constitutively active CARs in multiple hematological and solid tumor models (38). Carefully designed clinical trials will be required to evaluate the tunability of drug-regulated CAR designs in human patients, where additional variables such as cell quality, tumor burden, and drug pharmacokinetics could individually and jointly impact the behavior of engineered cells.

Multi-antigen sensing for improved safety and specificity

In addition to toxicities associated with overly active immune responses, off-tumor toxicity presents an additional challenge to cell-based cancer immunotherapy. The lack of targetable tumor-restricted antigens necessitates the targeting of tumor-associated antigens (TAAs) that are not exclusively expressed on tumor cells, thus exposing healthy tissues that express the same antigen to ‘on-target off-tumor’ toxicity. For example, CD19 CAR-T cell and CD19 CAR-NK cell therapies both result in B-cell aplasia in responding patients, as the CD19-targted immune cells simultaneously eliminate CD19-expressing healthy and malignant B cells (17, 39). B-cell aplasia is a clinically manageable condition (40), but analogous on-target, off-tumor toxicities against other antigens such as HER2, mesothelin, and carcinoembryonic antigen have led to early trial terminations and patient fatalities (41, 42).

Tumor-targeting specificity can be improved by biological circuitry that computes AND- or AND-NOT–gated Boolean logic, which requires the engagement of multiple TAAs in a specific combination before triggering target-cell killing (Fig. 1C). An AND-gate CAR requires that two or more antigens are present to trigger CAR signaling. One way to achieve this is to distribute the CD3ζ activation domain and co-stimulatory domain (e.g., CD28 or 4-1BB) of a typical second-generation CAR into two different receptors, one first-generation CAR containing only the CD3ζ chain, and a chimeric costimulatory receptor (CCR) containing only the co-stimulatory domain (43). Both receptors must be bound to their respective ligands in order to trigger full-intensity T-cell responses against the target cell. Another approach to achieving AND-gate logic requires sequential detection of multiple antigens, such as with the synthetic Notch (synNotch) or synthetic intramembrane proteolysis receptor (SNIPR) systems (44-46). These designs require two gene-expression cassettes. The first cassette constitutively expresses a synthetic receptor containing a transcription factor that is cleaved and released when the receptor binds its ligand (antigen A). The released transcription factor translocates to the nucleus and drives the inducible expression of a conventional CAR, which subsequently targets antigen B. Such AND-gated designs have been shown to prevent killing of off-tumor targets that express only one antigen but not the other. However, AND-gate designs remain capable of off-tumor killing if healthy tissue expressing the CAR-targeted antigen is colocalized with the tumor cells, as shown in

the case of a synNotch-controlled ROR1 CAR-T cell therapy that triggered severe toxicity in the Raji lymphoma model due to simultaneous destruction of ROR1-expressing lymphoma and healthy tissue in the bone marrow (47).

As an alternative strategy, AND-NOT–gate CARs have also been shown to increase target specificity. AND-NOT gates require both the presence of antigen A and the absence of antigen B in order to trigger cell activation (48, 49). This is accomplished by co-expressing a conventional, activating CAR (aCAR) targeting antigen A with an inhibitory CAR (iCAR) targeting antigen B. iCAR signaling triggered by antigen B overrides aCAR signaling, thus inhibiting cell activation when antigen B is present. By using an aCAR that targets a TAA and an iCAR that targets HLA-A2, this AND-NOT–gate strategy has been applied to target tumor cells that have downregulated MHC expression, thus simultaneously increasing tumor specificity while addressing a potential immune escape mechanism (49, 50).

Despite significant potential advantages, AND- and AND-NOT–gated CAR designs also face important caveats. First, each of the circuits described above require the expression of multiple transgenes, and the increased genetic payload size can significantly reduce the efficiency of transgene integration (51). Second, early synthetic-biology attempts at making computation circuits, exemplified by the synNotch system, often utilized non-human components such as viral transcription factors, and the potential immunogenicity of such designs presents a significant barrier to clinical translation. Finally, by increasing the number of antigens that must be present or absent in a specific combination, the system increases the probability for antigen escape, as the tumor now only needs to alter one of multiple antigens' expression pattern to avoid detection.

Multi-antigen sensing to prevent tumor escape

Although tumor specificity—i.e., ability to specifically recognize tumor cells and not healthy tissue—is a critical consideration in therapy design due to its safety implications, the flip side of the coin—i.e., inability to recognize all tumor cells by targeting a single antigen—also presents a key challenge in cell-based therapy. Antigen escape can arise from either natural tumor heterogeneity or antigen downregulation in response to selective pressure imposed by therapy. For example, a substantial fraction of patients with B-cell malignancies relapse after CD19 CAR-T cell therapy, and up to 94% of relapsing patients have CD19-negative tumors (52-54). Antigen escape poses an even greater challenge in solid tumors such as glioblastoma multiforme (GBM) due to intrinsic heterogeneity in antigen expression (55). Consequently, antigen loss has been observed as a major mechanism of CAR-T cell treatment resistance in patients with GBM (56, 57). Several methodologies have been proposed to address antigen escape, including incorporating tandem bispecific CARs or multiple CARs targeting different antigens into a single cell (OR-gate Boolean logic), or simultaneously or sequentially administering multiple cell products that target different antigens. In most head-to-head comparisons, tandem bispecific CARs have exhibited greater anti-tumor efficacies compared to co-expressing two CARs in a cell or administering a pooled T-cell population (58-60). In OR-gate Boolean logic, therapeutic cells are engineered to recognize two or more antigens, and the presence of any recognizable antigen would trigger cell-mediated toxicity, thus requiring the tumor to lose all recognizable antigens

in order to successfully evade detection. For example, tandem scFv bispecific CARs have demonstrated reduced tumor relapse and superior anti-tumor activity against B-cell malignancies susceptible to CD19 antigen loss (61, 62). Early success with CD19/CD20- and CD19/CD22-targeting bispecific CARs have led to the initiation of numerous ongoing clinical trials (NCT04007029, NCT04700319, NCT03241940). Multi-antigen targeting has also been extended to pre-clinical and clinical studies in other malignancies, including multiple myeloma (e.g., NCT04162353) (60) and GBM (58). It should be noted that, depending on the choice of target antigens, OR-gated CAR designs have the potential to exacerbate off-tumor toxicity, as a wider range of healthy tissue may become recognizable to engineered cells that target multiple antigens.

To date, designs that enable regulated or logic-gated CAR activities have largely been demonstrated in the T-cell setting. However, such designs may become increasingly important in CAR-NK cell engineering as this treatment modality expands beyond B-cell malignancies and into indications with greater toxicity potential or more heterogeneous antigen-expression profiles (63). Despite the largely safe clinical profile of NK-cell therapies reported to date (17, 64), biological designs that enable regulated CAR activity and logic-gated signal computation remain useful resources as CAR-NK cells advance to a broader array of disease indications.

Armoring NK and T cells to improve efficacy

To date, much of the synthetic biology efforts in cell-based therapies have focused on the engineering of CAR proteins or circuitries that revolve around CAR proteins. However, numerous biological pathways work in concert to impact the anti-tumor activities of T cells and NK cells, thus providing a wide variety of engineering targets that can potentially enhance efficacy. To date, solid tumors remain intractable challenges for cell-based therapies due to various immunosuppressive and immune-evading mechanisms unique to the tumor microenvironment (TME), such as overexpression of inhibitory ligands and cytokines, hypoxia, a poor nutrient profile, and dysfunctional immune or stromal cells (65). To address this, CAR-expressing immune cells have been “armored” with cytokines, chemokines, receptors, or other transgenic molecules to enhance T-cell cytotoxicity, persistence, or tumor infiltration, or to remodel the TME to a more proinflammatory state that favors anti-tumor activity (66) (Fig. 1D). CAR-T cells have been engineered to co-express cytokines such as interleukin (IL)-12 (67), IL-7 (68), and IL-15 (69), as well as payloads such as bispecific T-cell engagers (70, 71) and bacterial virulence factors (72) to improve targeting of solid tumors with heterogeneous antigen expression. Many armored CAR-T cells are currently being evaluated in the clinic (Table 3), and promising preliminary results have begun to emerge. In a phase-I clinical trial (NCT03198546), six patients with liver, pancreatic, or ovarian cancer were treated with mesothelin- or GPC3-targeting CAR-T cells armored with IL-7 and CCL19. Preliminary report notes one complete response and one partial response among the treated patients, with fever and fatigue as common side effects but “no grade 2–4 adverse events or major complications” reported (73). Although the number of patients treated with armored CAR-T cells to date is relatively small, the potential to achieve response without severe toxicity in patients with solid tumors is highly encouraging.

Likewise, NK cells have been engineered to express armors that exploit their biological characteristics to further improve therapeutic outcomes. In a landmark clinical trial, cord-blood NK cells were engineered to express a second-generation CD19 CAR plus IL-15 to enhance survival, as well as inducible caspase 9 as a safety switch (17). The therapy was efficacious and well tolerated in a large cohort of lymphodepleted patients with relapsed or refractory CD19-postive malignancies, with a 64% complete response rate and CAR-NK cells remaining detectable in peripheral blood for >12 months. This persistence level contrasts with earlier adoptive NK-cell transfer regimens, where NK cell persistence was limited to days or weeks without cytokine supplementation (19, 74). Armoring strategies designed to promote the proliferation and persistence of therapeutic cells may be particularly critical in the allogeneic setting, where the durability of response has been a major concern. Whereas autologous CAR-T cells have been shown to persist in treated patients for a decade (75), the durability of response in patients treated with allogeneic CAR-T and CAR-NK cell is too early to fully assess as clinical data points remain relatively low in number. The fact that NK cells do not establish long-term memory like T cells, and that allogeneic CAR-T cells remain vulnerable to immune rejection despite genetic knockout of endogenous T-cell receptor and/or MHC molecules (76-79), render engineering strategies that can artificially booster cell persistence of particular interest.

In addition to armoring T cells and NK cells with cytokines to drive proliferation, one could also combine therapeutic cells with other forms of immunotherapy to further enhance the immune response. Preclinical studies have suggested that combination of CAR-T cell therapy with anti–PD-1 immune checkpoint blockade could promote the functional persistence of CAR-T cells (80, 81). However, early clinical results reported to date have not yet demonstrated definitive advantage of combining CAR-T cell therapy with checkpoint blockade (82, 83). Pursuing a different modality of combination therapy, an ongoing phase-I clinical trial by BioNTech (NCT04503278) is evaluating the effect of an mRNA vaccine designed to target claudin-6 (CLDN-6) expression to dendritic cells, which can subsequently boost the activation and proliferation of anti–CLDN-6 CAR-T cells. Early results from the trial suggest promising efficacy and safety (84), and the clinical experience generated from such trials will prove highly valuable as the field explores different combination strategies to tackle solid tumors.

Yet another strategy of armoring immune cells is to equip the cells with receptors that can either abrogate immunosuppression or convert suppressive signals in the TME into stimulatory ones for the engineered cells. For example, transforming growth factor-β (TGF-β) is a potent inhibitory cytokine in the TME. To overcome this obstacle, researchers have evaluated strategies to ablate TGF-β signaling, such as CRISPR/Cas9-mediated knock-out of the endogenous TGF-β receptor chain II (NCT04976218). Alternatively, expression of the dominant-negative TGF-β receptor (DNR), which is a truncated TGF-β receptor chain II that lacks the intracellular domain, is also under investigation. The DNR has been co-expressed with tumor-targeting receptors in both T cells (85) and NK cells (86), and preclinical data indicate the DNR can robustly abrogate endogenous TGF-β signaling by both serving as a sink for TGF-β binding and by poisoning the heterodimeric TGF-β receptor complex to abolish signaling. A phase-I clinical trial (NCT03089203) treated patients with prostate cancer using T cells that co-express the DNR with a PSMA-targeting CAR (87). In this

trial, the best response was stable disease, achieved by five of 13 patients. Of note, the only patient to receive the highest dose in the trial (1–3 x 10^8 m^{-2} CAR-T cells) experienced clonal CAR-T cell expansion and a 98% reduction in serum PSA levels, but rapidly developed grade-4 CRS and died due to complications from sepsis and multimodal immunosuppression. The underlying mechanisms that drove the dramatic clonal T-cell expansion and severe toxicity in this individual patient remain unresolved at this time, but this study highlights the need to further explore both the potential and the risks associated with armoring CAR-T cells to overcome immunosuppression.

Taking it one step further, researchers have engineered synthetic receptors that convert TGF-β binding into immunostimulatory signaling. For example, Burga et al. fused the DNR to DNAX-activation protein 12 (DAP12), and demonstrated that NK cells bearing the fusion protein demonstrated improved efficacy and persistence compared to the DNR in mice bearing TGF-β–secreting neuroblastoma xenografts (88). As another example, Chang et al. developed CARs that can respond to soluble antigens and demonstrated the ability to activate T cells in response to TGF-β through the expression of a TGF-β–binding CAR (89). CARs responsive to additional soluble antigens can potentially broaden this signal-conversion strategy to overcome a variety of immunosuppressive cytokines in the TME (90).

Finally, endogenous receptors can also be modified to enhance effector cell function. For example, Zhu et al. demonstrated that the NK-cell activating receptor FcγRIIIa (CD16a), which is responsible for NK-cell–mediated antibody-dependent cellular cytotoxicity (ADCC), can be mutated to prevent proteolytic cleavage by ADAM17 (91). iPSC-derived NK cells expressing the mutated form of CD16a significantly outperformed unmodified peripheral-blood NK cells in controlling Raji xenografts when co-administered with an anti-CD20 antibody (rituximab) in a repeated-dosing study. An NK-cell product (FT596) containing both the “NK-CAR” and optimized CD16a, along with membrane-tethered IL-15, is currently under clinical evaluation. Preliminary results from this trial are encouraging: 20 patients with relapsed and/or refractory B-cell lymphoma were treated with escalating doses of 20–300 million engineered NK cells, with or without co-treatment with rituximab, resulting in >50% objective response rate among 17 efficacy-evaluable patients, including seven complete responses (16). Two patients who achieved a complete response had previously relapsed after CAR-T cell therapy, highlighting the potential for this therapy to serve as an alternative to CAR-T cells. This remarkable outcome underscores the utility of systematically investigating and incorporating synthetic payloads rooted in biological relevance, a methodology that may lead to next-generation cellular therapies equipped to overcome currently intractable malignancies.

Conclusion

As the synthetic-biology toolkit continues to expand, the generation of increasingly sophisticated biological circuits has become possible in the engineering of cell-based therapies for cancer. Exciting pre-clinical and clinical data for NK and T cell-based therapies are now emerging, demonstrating higher specificity, safety, and durability. Increasing complexity in biological design is frequently coupled with increasing complexity in manufacturing as well as vulnerability to unintended consequences due to system

crosstalk or component failures that compromise overall system function. Therefore, the implementation of multi-layered biological circuits in the cell-based therapy context must be done in a judicious manner to maximize robustness and minimize unnecessary complexity. Nevertheless, the wide array of novel biological functions made accessible by synthetic biology approaches offer potential solutions to many roadblocks currently limiting the broad application cell-based immunotherapies, and provides avenues to extend these therapies to more patients with advanced cancers.

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Abbreviations List:

aCAR	activating CAR
ADCC	antibody-dependent cellular cytotoxicity
CAR	chimeric antigen receptor
CLDN6	claudin-6
CRISPR	clustered regularly interspaced short palindromic repeats
DAP12	DNAX-activation protein 12
DNR	dominant-negative TGF-β receptor
FDA	Food and Drug Administration
GBM	glioblastoma multiforme
iCAR	inhibitory CAR
IL	interleukin
iNKT cells	invariant natural killer T cells
iPSCs	induced pluripotent stem cells
NK cells	natural killer cells
SNIPR	synthetic intramembrane proteolysis receptor
synNotch	synthetic Notch
TAAs	tumor-associated antigens
TALENs	transcription activator-like effector nucleases
TGF-β	transforming growth factor-β

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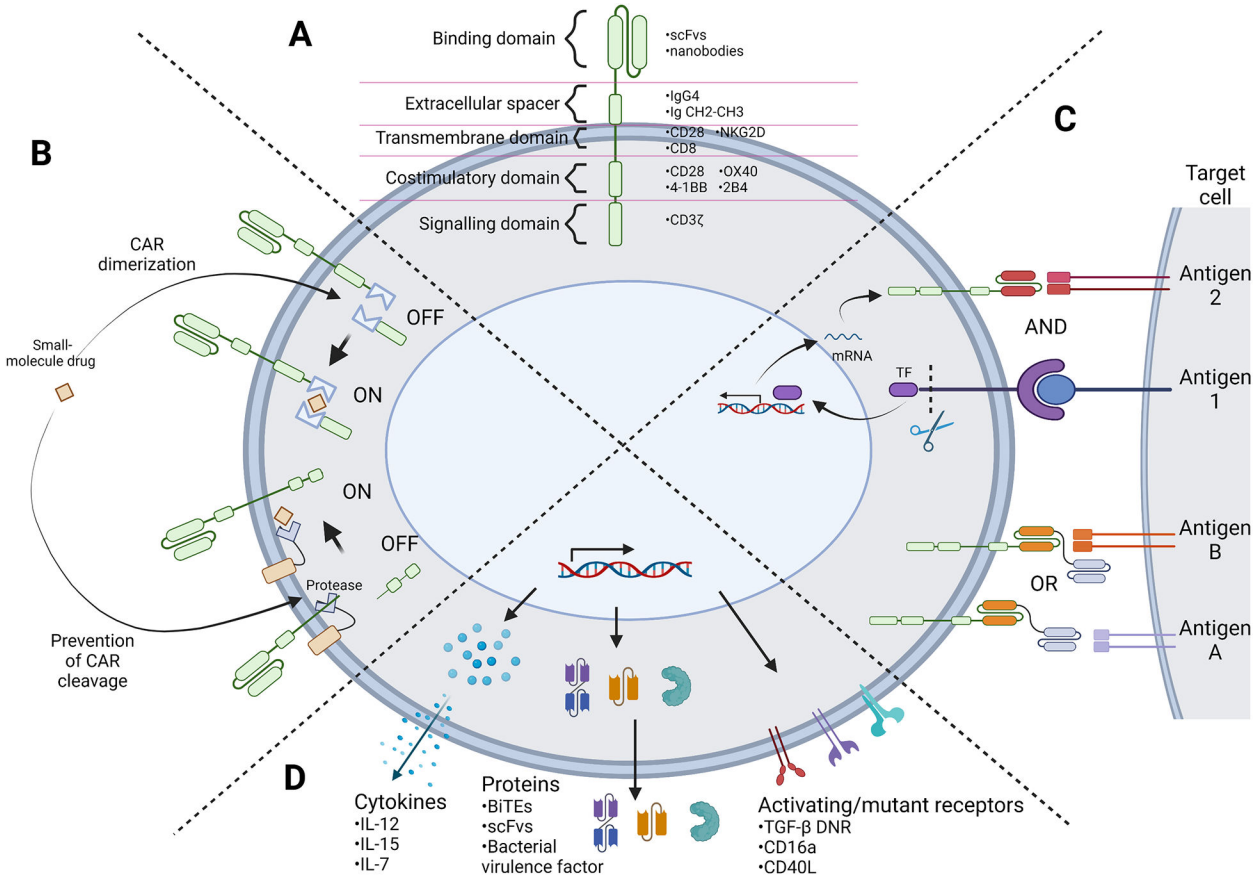

Figure 1.

Representation of synthetic biology strategies for engineering next-generation T and NK cell therapies. (**A**) Schematic depicting the CAR protein architecture along with commonly employed domains in each modular component. (**B**) Example of CAR regulation strategies. A small-molecule drug can act as a dimerizing agent to assemble a full CAR protein, or stabilize CAR protein expression by inhibiting a protease to prevent CAR protein cleavage. (**C**) AND and OR Boolean logic-gate strategies for multi-antigen sensing. AND-gated strategies require the sensing of multiple tumor antigens to trigger target cell killing. For example, recognition of antigen 1 can prompt the release of a transcription factor (TF) that triggers expression of a CAR specific to antigen 2, thus both antigens 1 and 2 must be present in order to trigger CAR-T cell activation. OR-gated strategies require the sensing of any one antigen among multiple antigens to trigger target cell killing. For example, tandem bispecific OR-gate CARs have two binding domains that recognize two different antigens, such that recognition of either antigen A or B will induce activation of the CAR-expressing cell. (**D**) Armoring strategies in which transgenic payloads are expressed together with the CAR transgene. These payloads can be cytokines, chemokines, receptors, or other proteins that aim to improve the function of the T or NK cell and/or neighboring immune cells, frequently with the goal of reprogramming the surrounding tumor microenvironment. (Figure created with BioRender.)

Table 1.

List of advantages and disadvantages for T and NK cell therapies.

Cell Type	Advantages	Disadvantages
T Cells	• Robust *ex vivo* expansion • Robust cytotoxicity • Proven efficacy in hematological malignancies • Possibility for long-term memory	• Allogeneic implementation remains a challenge
NK Cells	• Innate ability to recognize transformed cells • Can be generated from scalable allogeneic sources • Potential to achieve efficacy without triggering CRS	• Short lifespan • Limited proliferative potential

Table 2.

List of CAR-NK cells in clinical trials.

Clinical Trial Identifier	Trial Name	Antigen Target	Disease Indication	NK Cell Source	Trial Status	Year Trial Started	Armor
NCT03056339	Umbilical & Cord Blood (CB) Derived CAR-Engineered NK Cells for B Lymphoid Malignancies	CD19	Stem Cell Transplant Patients with Relapsed and/or Refractory B-Cell Lymphoma or Leukemia	Cord Blood	Active, not recruiting	2017	iCasp9 and IL-15
NCT03692663	Study of Anti-PSMA CAR NK Cell (TABP EIC) in Metastatic Castration-Resistant Prostate Cancer	PSMA	Metastatic Castration-Resistant Prostate Cancer	Unknown	Recruiting	2018	N/A
NCT04623944	NKX101, Intravenous Allogeneic CAR NK Cells, in Adults With AML or MDS	NKG2D Ligands	Relapsed or Refractory Acute Myeloid Leukemia or Intermediate, High and Very High Risk Relapsed or Refractory MDS	Either Haplo-Matched Related Donor Derived or Unrelated Off-the-Shelf Donor Derived	Recruiting	2020	Membrane-Bound IL-15
NCT04887012	Clinical Study of HLA Haploidentical CAR-NK Cells Targeting CD19 in the Treatment of Refractory/Relapsed B-cell NHL	CD19	B-cell Non-Hodgkin's lymphoma	HLA haploidentical	Recruiting	2021	N/A
NCT05008575	Anti-CD33 CAR NK Cells in the Treatment of Relapsed/ Refractory Acute Myeloid Leukemia	CD33	Acute Myeloid Leukemia	Unknown	Recruiting	2021	N/A
NCT05008536	Anti-BCMA CAR-NK Cell Therapy for the Relapsed or Refractory Multiple Myeloma	BCMA	Multiple Myeloma, Refractory	Umbilical and Cord Blood	Recruiting	2021	N/A
NCT04847466	Immunotherapy Combination: Irradiated PD-L1 CAR-NK Cells Plus Pembrolizumab Plus N-803 for Subjects With Recurrent/Metastatic Gastric or Head and Neck Cancer	PD-L1	Gastroesophageal Junction Cancers Advanced HNSCC	NK-92	Recruiting	2021	N/A
NCT04796675	Cord Blood Derived Anti-CD19 CAR-Engineered NK Cells for B Lymphoid Malignancies	CD19	Relapsed or Refractory Hematological Malignancies	Cord Blood	Recruiting	2021	N/A
NCT05020678	NKX019, Intravenous Allogeneic Chimeric Antigen Receptor Natural Killer Cells (CAR NK), in Adults With B-cell Cancers	CD19	Relapsed or Refractory Non-Hodgkin Lymphoma, Chronic Lymphocytic Leukemia or B cell Acute Lymphoblastic Leukemia	Either Haplo-Matched Related Donor Derived or Unrelated Off-the-Shelf Donor Derived	Recruiting	2021	Membrane-Bound IL-15
NCT05247957	NKG2D CAR-NK Cell Therapy in Patients With Relapsed	NKG2D	Recurrent Refractory Acute Myeloid Leukemia	Umbilical Cord	Recruiting	2022	N/A

Clinical Trial Identifier	Trial Name	Antigen Target	Disease Indication	NK Cell Source	Trial Status	Year Trial Started	Armor
	or Refractory Acute Myeloid Leukemia						
NCT05410717	CLDN6-CAR-NK Cell Therapy for Advanced Solid Tumors	Claudin6	Advanced Solid Tumors	Autologous PBMC	Recruiting	2022	IL7 + CCL19 or scFvs Against PD1/ CTLA4/ Lag3
NCT05213195	NKG2D CAR-NK Cell Therapy in Patients With Refractory Metastatic Colorectal Cancer	NKG2D	Refractory Metastatic Colorectal Cancer	Unknown	Recruiting	2022	N/A
NCT05215015	Study of Anti-CD33/ CLL1 CAR-NK in Acute Myeloid Leukemia	CD33/ CLL1	Acute Myeloid Leukemia	Unknown	Recruiting	2022	N/A
NCT05194709	Study of Anti-5T4 CAR-NK Cell Therapy in Advanced Solid Tumors	5T4	Advanced Solid Tumors	Unknown	Recruiting	2022	N/A
NCT05410041	Anti-CD19 CAR-Engineered NK Cells in the Treatment of Relapsed/Refractory B-cell Malignancies	CD19	Recurrent or Refractory CD19-Positive B-Cell Malignant Tumors	Unknown	Recruiting	2022	N/A
NCT05379647	Natural Killer (NK) Cell Therapy for B-Cell Malignancies	CD19	Relapsed or Refractory B-Cell Acute Lymphoblastic Leukemia and Relapsed or Refractory B-cell Lymphoma	Unknown	Recruiting	2022	N/A
NCT05182073	FT576 in Subjects With Multiple Myeloma	BCMA	Multiple Myeloma	iPSC	Recruiting	2022	Modified CD16, IL-15 Receptor Fusion, Elimination of CD38

Table 3.

List of armored CAR-T cells in clinical trials.

Clinical Trial Identifier	Trial Name	Antigen Target	Disease Indication	T Cell Source	Trial Status	Year Trial Started	Armor
NCT01822652	3rd Generation GD-2 Chimeric Antigen Receptor and iCaspase Suicide Safety Switch, Neuroblastoma, GRAIN (GRAIN)	GD2	Relapsed or Refractory Neuroblastoma	Autologous	Active, not recruiting	2013	iCaspase9
NCT01953900	iC9-GD2-CAR-VZV-CTLs/Refractory or Metastatic GD2-positive Sarcoma and Neuroblastoma (VEGAS)	GD2	Advanced Osteosarcoma and Neuroblastoma	Autologous Varicella Zoster Virus (VZV) Specific T Cells	Active, not recruiting	2013	iCaspase9
NCT02498912	Cyclophosphamide Followed by Intravenous and Intraperitoneal Infusion of Autologous T Cells Genetically Engineered to Secrete IL-12 and to Target the MUC16ecto Antigen in Patients With Recurrent MUC16ecto+ Solid Tumors	MUC16	MUC16-Positive Solid Tumors	Autologous	Active, not recruiting	2015	fIL-12
NCT03356782	Safety and Efficacy Evaluation of 4th Generation Safety-engineered CAR T Cells Targeting Sarcomas	CD133, GD2, Muc1, CD117 or Others	Relapsed or Late-Staged Sarcoma	Autologous	Recruiting	2017	anti-PD-1 Ig, anti-PD-L1 Ig, iCaspase9
NCT03198546	GPC3-CAR-T Cells for Immunotherapy of Cancer With GPC3 Expression	GPC3 and/or Soluble TGFβ	GPC3-Positive Hepatocellular Carcinoma	Autologous	Recruiting	2017	IL7 + CCL19 or scFvs Against PD1/ CTLA4/ TIGIT
NCT03016377	Administration of Autologous CAR-T CD19 Antigen With Inducible Safety Switch in Patients With Relapsed/Refractory ALL	CD19	Relapsed or Refractory Acute Lymphoblastic Leukemia	Autologous	Recruiting	2017	iCaspase9
NCT03373071	Anti-CD19 CAR T Cells in Pediatric Patients Affected by Relapsed/ Refractory CD19+ ALL and NHL	CD19	Pediatric Relapsed or Refractory B-Acute Lymphoblastic Leukemia or Non-Hodgkin's Lymphoma with Measurable Bone Marrow Involvement	Autologous	Active, not recruiting	2017	Suicide switch
NCT03089203	CART-PSMA-TGFβRDN Cells for Castrate-Resistant Prostate Cancer	PSMA	Metastatic Castrate Resistant Prostate Cancer	Autologous	Recruiting	2017	TGFβ DNR
NCT03721068	Study of CAR T-Cells Targeting the GD2 With IL-15+iCaspase9	GD2	Relapsed or Refractory	Autologous	Recruiting	2018	IL-15 + iCaspase9

Clinical Trial Identifier	Trial Name	Antigen Target	Disease Indication	T Cell Source	Trial Status	Year Trial Started	Armor
	for Relapsed/Refractory Neuroblastoma or Relapsed/Refractory Osteosarcoma		Neuroblastoma or Osteosarcoma				
NCT03602157	Study of CAR-T Cells Expressing CD30 and CCR4 for r/r CD30+ HL and CTCL	CD30	Relapsed or Refractory CD30+ Hodgkin's Lymphoma and cutaneous T-cell lymphoma	Autologous	Recruiting	2018	CCR4
NCT03778346	Integrin β7, BCMA, CS1, CD38 and CD138 as the Single or Compound Targets for the Fourth Generation of CAR-T Cells	Multiple	Relapsed or Refractory Multiple Myeloma	Autologous	Recruiting	2018	IL-7, CCL19
NCT03635632	C7R-GD2.CART Cells for Patients With Relapsed or Refractory Neuroblastoma and Other GD2 Positive Cancers (GAIL-N)	GD2	Relapsed or Refractory Neuroblastoma and other GD2-positive Solid Cancers	Autologous	Recruiting	2018	IL-7 Receptor
NCT03696784	Anti-CD19 CAR-T Cells With Inducible Caspase 9 Safety Switch for B-cell Lymphoma	CD19	Relapsed or Refractory B-Cell Lymphoma	Autologous	Recruiting	2018	iCaspase9
NCT03741127	Long-Term Follow-Up Study for Subjects Treated With P-BCMA-101	BCMA	Multiple Myeloma	Autologous	Active, not recruiting	2018	iCaspase9
NCT03932565	Interventional Therapy Sequential With the Fourth-generation CAR-T Targeting Nectin4/FAP for Malignant Solid Tumors	Nectin4/FAP	Nectin4-Positive Advanced Malignant Solid Tumors	Autologous	Recruiting	2019	IL-7, CCL19, or / and IL-12
NCT04186520	CAR-20/19-T Cells in Patients With Relapsed Refractory B Cell Malignancies	CD19/CD20	Relapsed, refractory B-Cell Non-Hodgkin Lymphoma or Chronic Lymphocytic Leukemia	Autologous	Recruiting	2019	IL-7 + IL-15
NCT03814447	The Fourth Generation CART-cell Therapy for Refractory-Relapsed Ovarian Cancer	MSLN	Refractory or Relapsed Ovarian Cancer	Autologous	Recruiting	2019	Unknown
NCT04016129	CAR-T Immunotherapy Targeting CD19- ALL	CD22/CD123/ CD38/CD10/ CD20/TSLPR	Patients Who Have Relapsed after CD19 CAR-T Therapy or Have CD19-Negative B cell Malignancies	Autologous	Recruiting	2019	Unknown
NCT04099797	C7R-GD2.CAR T Cells for Patients With GD2-expressing Brain Tumors (GAIL-B)	GD2	GD2-Positive or H3K27M-Mutant Diffuse Intrinsic Pontine Glioma, High Grade Glioma, Embryonal Tumors, or	Autologous	Recruiting	2019	IL-7 Receptor

Clinical Trial Identifier	Trial Name	Antigen Target	Disease Indication	T Cell Source	Trial Status	Year Trial Started	Armor
			Ependymal Tumors				
NCT04162119	Safety and Efficiency Study of BCMA-PD1-CART Cells in Relapsed/Refractory Multiple Myeloma	BCMA	Relapsed or Refractory Multiple Myeloma	Autologous	Recruiting	2019	Mutant PD-1Fc Fusion Protein
NCT04489862	αPD1-MSLN-CAR T Cells for the Treatment of MSLN-positive Advanced Solid Tumors	MSLN	MSLN-Positive Solid Tumors	Autologous	Recruiting	2020	Secreted anti-PD-1 Nanobody
NCT04684563	huCART19-IL18 in NHL/CLL Patients	CD19	Non-Hodgkin Lymphoma and Chronic Lymphocytic Leukemia	Autologous	Recruiting	2020	IL-18
NCT04429438	Multi-CAR-T Cells Targeting B Cell Lymphomas	CD19 + Others	Primary Mediastinal B Cell Lymphoma and B Cell Lymphoma Involving the Central Nervous System	Autologous	Recruiting	2020	iCaspase9
NCT04430530	4SCAR-T Therapy Post CD19-targeted Immunotherapy	CD22/CD123/ CD38/CD10/ CD20	CD19 Negative B-cell Malignancies	Autologous	Recruiting	2020	iCaspase9
NCT04381741	CD19 CAR-T Expressing IL7 and CCL19 Combined With PD1 mAb for Relapsed or Refractory Diffuse Large B Cell Lymphoma (CICPD)	CD19	Diffuse Large B-cell Lymphoma	Autologous	Recruiting	2020	IL-7, CCL19
NCT04430595	Multi-4SCAR-T Therapy Targeting Breast Cancer	Her2, GD2, or CD44v6	Breast Cancer	Autologous	Recruiting	2020	iCaspase9
NCT04577326	Mesothelin-targeted CAR T-cell Therapy in Patients With Mesothelioma	MSLN	MSLN-Positive Malignant Pleural Mesothelioma	Autologous	Active, not recruiting	2020	PD-1 DNR
NCT04650451	Safety and Activity Study of HER2-Targeted Dual Switch CAR-T Cells (BPX-603) in Subjects With HER2-Positive Solid Tumors	HER2	Previously Treated, Locally Advanced or Metastatic HER2-Amplified/ Overexpressed Solid Tumors	Autologous	Recruiting	2020	iCaspase9
NCT04249947	P-PSMA-101 CAR-T Cells in the Treatment of Subjects With Metastatic Castration-Resistant Prostate Cancer (mCRPC) and Advanced Salivary Gland Cancers (SGC)	PSMA	Metastatic Castration-Resistant Prostate Cancer and Advanced Salivary Gland Cancers	Autologous	Recruiting	2020	iCaspase9
NCT04227275	A Study of CART-PSMA-TGFβRDN in Patients With Metastatic Castration Resistant Prostate Cancer	PSMA	Metastatic Castrate Resistant Prostate Cancer	Autologous	Active, not recruiting	2020	TGFβ DNR

Clinical Trial Identifier	Trial Name	Antigen Target	Disease Indication	T Cell Source	Trial Status	Year Trial Started	Armor
NCT04377932	Interleukin-15 Armored Glypican 3-specific Chimeric Antigen Receptor Expressed in T Cells for Pediatric Solid Tumors	GPC3	Relapsed or Refractory GPC3-Positive Solid Tumors	Autologous	Recruiting	2021	IL-15
NCT04706936	Novel BCMA-targeted CAR-T Cell Therapy for Multiple Myeloma	BCMA	Relapsed or Refractory Multiple Myeloma	Autologous	Recruiting	2021	Unknown (4th gen)
NCT04842812	Engineered TILs/CAR-TILs to Treat Advanced Solid Tumors	HER2, MSLN, PSCA, MUC1, Lewis-Y, GPC3, AXL, EGFR, Claudin18.2/6, ROR1, GD1, or B7-H3	Advanced Solid Tumors	Tumor Infiltrating Lymphocytes	Recruiting	2021	scFvs that Target PD1 and CTLA4
NCT04850560	Sequential Low-dose Decitabine With PD-1/ CD28 CD19 CAR-T in Relapsed or Refractory B-cell Lymphoma	CD19	CD19-Positive Relapsed or Refractory B-Cell Lymphoma	Autologous	Recruiting	2021	PD-1/ CD28 Switch Receptor
NCT04960579	P-BCMA-ALLO1 Allogeneic CAR-T Cells in the Treatment of Subjects With Multiple Myeloma (MM)	BCMA	Relapsed or Refractory Multiple Myeloma	Allogeneic T cells	Recruiting	2021	iCaspase9
NCT05166070	An Exploratory Clinical Study of RD133 in Subjects With Relapsed or Refractory MSLN-Positive Solid Tumors	MSLN	Relapsed or Refractory MSLN-Positive Solid Tumors	Autologous	Recruiting	2021	TGFβ DNR
NCT05141253	Safety and Efficacy of RD133 in Subjects With Relapsed or Refractory MSLN-Positive Solid Tumors	MSLN	Relapsed or Refractory MSLN-Positive Solid Tumors	Autologous	Recruiting	2021	TGFβ DNR
NCT05373147	αPD1-MSLN-CAR T Cells for the Treatment of MSLN-positive Advanced Solid Tumors	MSLN	Advanced Solid Tumors with >10% MSLN	Autologous	Recruiting	2022	Secreted anti-PD-1 Nanobody
NCT05239143	P-MUC1C-ALLO1 Allogeneic CAR-T Cells in the Treatment of Subjects With Advanced or Metastatic Solid Tumors	MUC1-C	Advanced or Metastatic Solid Tumors	Allogeneic T cells	Recruiting	2022	iCaspase9
NCT05487495	Donor-Derived CD5 CAR T (CT125B) Cells for Relapsed or Refractory T- Cell Acute Lymphoblastic Leukemia/Lymphoma	CD5	Relapsed or Refractory T-Cell Acute Lymphoblastic Leukemia/ Lymphoma	Autologous	Recruiting	2022	Thymidine Kinase (HSV-TK) Suicide Switch