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Synthetic Biology: Immunotherapy by Design

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Abstract

Cellular immunotherapy holds great promise for the treatment of human disease. Clinical evidence suggests that T cell immunotherapies have the potential to combat cancers that evade traditional immunotherapy. Despite promising results, adverse effects leading to fatalities have left scientists seeking tighter control over these therapies, which is reflected in the growing body of synthetic biology literature focused on developing tightly controlled, context-independent parts. In addition, researchers are adapting these tools for other uses, such as for the treatment of autoimmune disease, HIV infection, and fungal interactions. We review this body of work and devote special attention to approaches that may lend themselves to the development of an "ideal" therapy: one that is safe, efficient, and easy to manufacture. We conclude with a look toward the future of immunotherapy: how synthetic biology can shift the paradigm from the treatment of disease to a focus on wellness and human health as a whole.

Contents

1. INTRODUCTION

1.1. Cellular Immunotherapy

Immunotherapy, the modulation of the immune system to combat disease, is one of the most exciting forms of therapy today, in part due to recent successes providing substantial survival benefits to many cancer patients. Although several types of immunotherapy exist (e.g., cancer vaccines, monoclonal antibodies, and cytokines), checkpoint blockers have become increasingly prominent over the last several years. Checkpoint blockers inhibit the native signaling pathways of immune cells (usually T cells) that serve as "brakes" for immune responses. For example, antibodies that block the activation of the checkpoint receptor cytotoxic T lymphocyte–associated protein 4 (CTLA-4; ipilimumab) and programmed cell death protein 1 (PD-1; nivolumab, pembrolizumab) have shown enormous clinical and commercial success for many cancers, especially lung and skin cancers (1). Although these checkpoint blockade therapies are promising, increasing evidence suggests that they are less effective against tumors with a low mutation load (2, 3). As such, alternative cancer therapies are still needed.

More recently, cellular immunotherapy has shown promising results in clinical trials. In cellular immunotherapy, tumor-targeting T cells are adoptively transferred into the patient to treat diseases. Several methods for generating tumor-targeting T cells exist. One approach that has shown promise in clinical trials for melanoma involves the isolation and reintroduction of tumor-infiltrating lymphocytes (TILs) (**Figure 1*a***). However, the isolation of TILs is challenging and does not work for many tumors, such as liquid tumors. An alternative approach is to redirect the T cell activity by introducing tumor-targeting receptors through genetic engineering. One approach for identifying appropriate receptors involves the screening and directed evolution of native T cell receptors (TCRs). In this way, T cells engineered to express cancer-testis antigen (NY-ESO-1)-specific TCRs have shown clinical response against myeloma in 80% of patients with

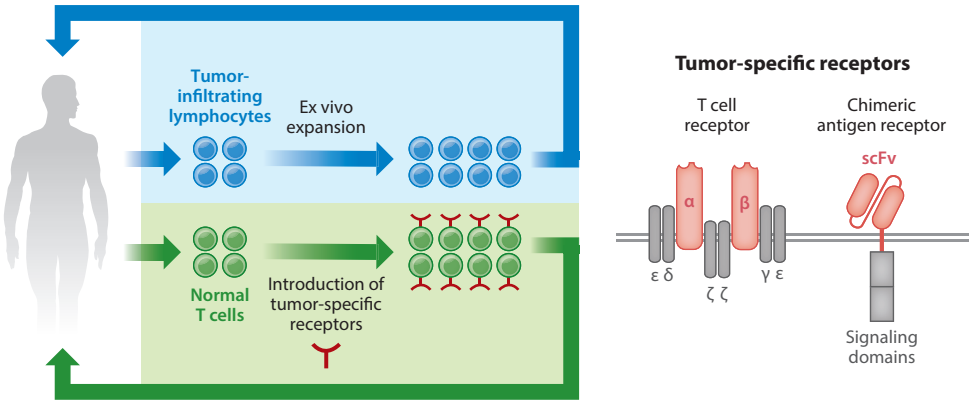


Figure 1

Schematics of cellular immunotherapy and the basic structure of tumor-specific receptors. In cancer cellular immunotherapy, tumor-targeting T cells isolated from the tumor (tumor-infiltrating lymphocytes; blue) or genetically engineered T cells (chimeric antigen receptor T cells; green) that express tumor-specific receptors are adoptively transferred into the patient to clear cancer. Chimeric antigen receptors are composed of cancer-antigen-specific single-chain variable fragment (scFv) and intracellular signaling domains from T cell receptors.

advanced disease (4). Alternatively, a more modular and effective way to create cancer-specific receptors is by fusing the single-chain variable fragment (scFv) of an antibody that targets a cancer antigen to intracellular signaling domains from the TCR and other costimulatory pathways. These fusion receptors are called chimeric antigen receptors (CARs) (5). The scFv on the CAR binds to the tumor antigen and activates the endogenous TCR signaling pathway for the killing of cancerous cells. CAR-modified T cells have yielded a phenomenal response against lymphoid leukemia, even for pediatric patients who failed to respond to or relapsed from other forms of therapy. Independent clinical trials have reported more than 90% complete remission (6, 7), twice the rate observed for standard chemotherapy alone.

In addition to its efficacy against liquid tumors, adoptive T cell immunotherapy is intriguing because it represents the possibility of creating therapeutic agents with unprecedented sophistication. Living cells have the ability to sense many different types of signals, perform complex computations, and produce a wide variety of outputs. Furthermore, immune cells can develop into long-lived memory cells, facilitating the generation of vaccines for diseases. These attributes are very difficult, if not impossible, to engineer in current therapeutic modalities. Given the importance of the immune system in many aspects of human physiology, fully harnessing the potential of cellular immunotherapy could lead to significant progress in treating many complicated diseases. Therefore, the ability to precisely reprogram immune cells is urgently needed. Although there are many options for modulating the immune response, such as through drugs, manipulating the genetics of the immune cells represents one of the most promising and direct routes of achieving precise control of the immune system.

1.2. Synthetic Biology

Synthetic biology is an emerging discipline that focuses on predictable reprogramming of living cells. Broadly, synthetic biology can be considered the forward engineering of biology through the use of tools derived from a variety of disciplines such as genetic engineering, systems biology,

chemistry, biophysics, and computer engineering. Thus, synthetic biology has a strong emphasis on design that is commonplace among engineering disciplines, even though its roots are in molecular cloning and DNA sequencing. The seminal research in synthetic biology includes the engineering of genetic circuits, such as toggle switches (8), oscillators (9, 10), Boolean logic gates (11–13), and feedback controllers (**Figure 2**) (14–16). These circuits have the potential to impart

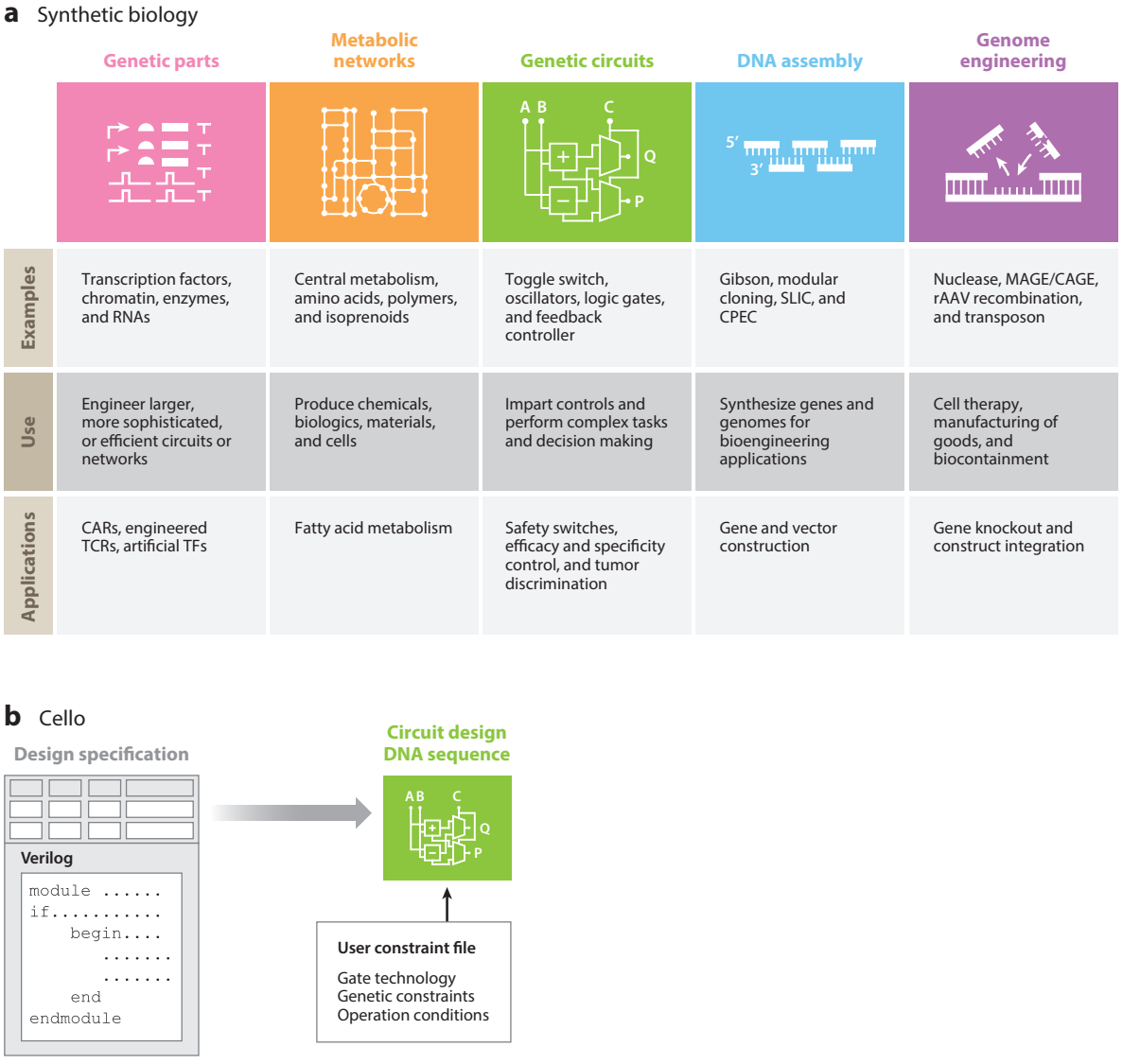


Figure 2

Goals and applications of synthetic biology. (*a*) Synthetic biology places a strong emphasis on developing genetic parts, reprogramming metabolic networks, synthesizing genetic circuits, improving DNA synthesis and assembly techniques, and improving genome engineering tools. (*b*) Automated design of complex genetic circuits with the computer program Cello (Cell Logic). Abbreviations: CAGE, conjugative assembly genome engineering; CARs, chimeric antigen receptors; CPEC, circular polymerase extension cloning; MAGE, multiplex automated genome engineering; rAAV, recombinant adeno-associated virus; SLIC, sequence and ligase independent cloning; TCRs, T cell receptors; TFs, transcription factors.

sophisticated control over various aspects of cellular functions. The reprogramming of metabolic pathways for the production of biofuels (17), pharmaceuticals (18–21), and materials (22, 23) remains a major focus of synthetic biology. Developing and repurposing genetic parts, such as regulatory RNAs (24, 25), recombinases (26), transcription factors (27), and receptors (28, 29), are also major emphases of synthetic biology. These parts can confer distinct functionalities, which can have dramatic effects on the design principles of genetic circuits (11). The demand for high-throughput development of more sophisticated genetic programs has pushed synthetic biologists to develop highly efficient DNA synthesis and assembly techniques, such as Gibson assembly, modular cloning, and open-source automated microfluidic assembly (30–32). Finally, it has become increasingly necessary to modify the endogenous genetic programs of cells to synergize with new synthetic genetic programs to maximize the performance of the engineered cells. As such, significant efforts have been devoted within synthetic biology to develop and improve genome engineering tools, such as zinc-finger (ZF) nucleases, transcription activator–like nucleases, and clustered regularly interspaced short palindromic repeat–associated systems (CRISPR/Cas) (33).

Predictable engineering of living cells has proven to be much more challenging than initially anticipated. Many of the challenges stem from a poor understanding of the substrate or the source materials used in the engineering effort. Furthermore, genetic reprogramming of cells introduces perturbations in biological systems whose consequences are difficult to predict. Although intense effort has been devoted to understanding almost every aspect of biology, especially human biology, the enormous number of components and complexity found in a living cell continue to present major challenges to synthetic biology. As such, various design approaches have been formalized for engineering living organisms. One of the most conservative, yet successful, approaches [elegantly presented by Woodsworth & Holt (34)] involves small, iterative engineering steps introducing well-defined components directly in the cell type of interest. This approach is slow, labor intensive, and not scalable for more complex genetic system designs. Recently, an ambitious and systematic approach was developed for creating large complex genetic circuits with a high success rate and reproducibility, allowing genetic components to be carefully curated and their functionality verified in the cell type of choice. The design of genetic circuits was then automated through a computer program called Cello (Cell Logic) using experimental performance data from each subcomponent as part of the design constraints (35). We envision that, in the future, Cello will incorporate additional genetic components characterized in more cell types to assist in the computer-aided design of genetically engineered cells. Furthermore, with the emergence of cloud-based laboratories for biological experiments, we anticipate that the synthesis and testing of genetic systems will also become automated. Thus, even as synthetic genetic systems are becoming more complex, the engineering of biology is becoming less artisanal and labor intensive, and more predictable and reproducible.

Drug-based immunotherapy, such as checkpoint blockade, is highly successful against many types of cancer. However, checkpoint blockade represents only one tool for modulating the immune system. Through direct reprogramming of immune cells using genetic engineering, many features of the immune system could perhaps be rewired to treat many diseases. Some of the most critical challenges facing cellular immunotherapy are the complexity of living cells, the robust functionality of engineered cells, the efficiency of genetic engineering, and the cost of manufacturing. Many of these challenges fall within the central goals of synthetic biology. Therefore, it is reasonable to assume that synthetic biology will play a pivotal role in the development of cellular immunotherapies.

In this review, we first highlight some of the current applications of cellular immunotherapy. We then summarize the challenges facing cellular immunotherapy and how synthetic biology

can be used to address these challenges. Lastly, we speculate about how the marriage of cellular immunotherapy and synthetic biology could lead to novel applications and discuss the implications of these developments for human health.

2. APPLICATIONS OF CELLULAR IMMUNOTHERAPY

2.1. Cancer Cellular Immunotherapy: Paving the Way

Early efforts to treat cancer with cellular immunotherapy largely involved the purification, expansion, and infusion of TILs. Beginning with a clinical trial in 1988, TILs were extracted from patients and expanded in vitro prior to adoptive transfer, and were found to mediate the regression of metastatic melanoma (36). More recently, immune cells have been genetically modified to incorporate synthetic biology concepts to improve the safety and efficacy of cellular immunotherapy, as well as to expand the number of diseases that can be treated.

Cancer cellular immunotherapy has served as a unique test bed for synthetic biology components in that there is intense interest in rapidly translating advances made in the field, especially CARs. Early clinical trials used CARs comprising scFvs fused to CD3ζ, the native TCR signaling domain. These clinical trials were not very effective; for example, efforts to target ovarian cancer through the folate receptor led to no reduction in tumor burden (37). These failures to reduce tumor burden were in part due to an inability of the engineered T cells to proliferate and persist in the circulation for substantial periods of time. A 2006 study (38) showed that the addition of a CD28 costimulatory domain to CD3ζ led to an increase in T cell proliferation and antitumor effect of these engineered cells. Thereafter, costimulatory domains were introduced to subsequent generations of CARs to enhance the immune response and allow for persistence of the engineered cells. These second-generation CARs were further improved by including multiple costimulatory domains alongside CD3ζ, creating third-generation CARs that exhibited even greater potency against tumor cells in vivo (39). These synthetic receptors have proven effective in a clinical setting in multiple trials, exemplifying the promise that genetic engineering holds for cellular immunotherapies (6, 40, 41). Although there are still challenges that limit the efficacy and adoption of cellular immunotherapy (e.g., safety, application to other cancers and diseases), the success of CAR T cell therapy and the massive interest it has garnered from research entities and governmental organizations illustrate the transformative role that cellular immunotherapy can play in human health.

While the topic of cancer cellular immunotherapy is both fascinating and important, it is not the exclusive focus of this review. We direct the reader to two excellent reviews of this topic for further reading (42, 43).

2.2. Alternative Applications of Cellular Immunotherapy

2.2.1. HIV infection. The first efforts to target HIV-infected cells using adoptive T cell immunotherapy arose from two key observations. First, HIV-specific $CD8^+$ cytotoxic T lymphocytes (CTLs) are present in the peripheral blood mononuclear cells (PBMCs) of seronegative or asymptomatic individuals; second, the loss of these HIV-specific CTLs is associated with disease progression. Therefore, it seemed reasonable that engineered HIV-specific CTLs could be used to combat, and potentially even cure, HIV infection. These early efforts used viral transduction of $CD8^+$ cells to achieve high expression levels of major histocompatibility complex (MHC)-unrestricted chimeric TCRs, which were fusions of the intracellular TCR signaling domain CD3ζ

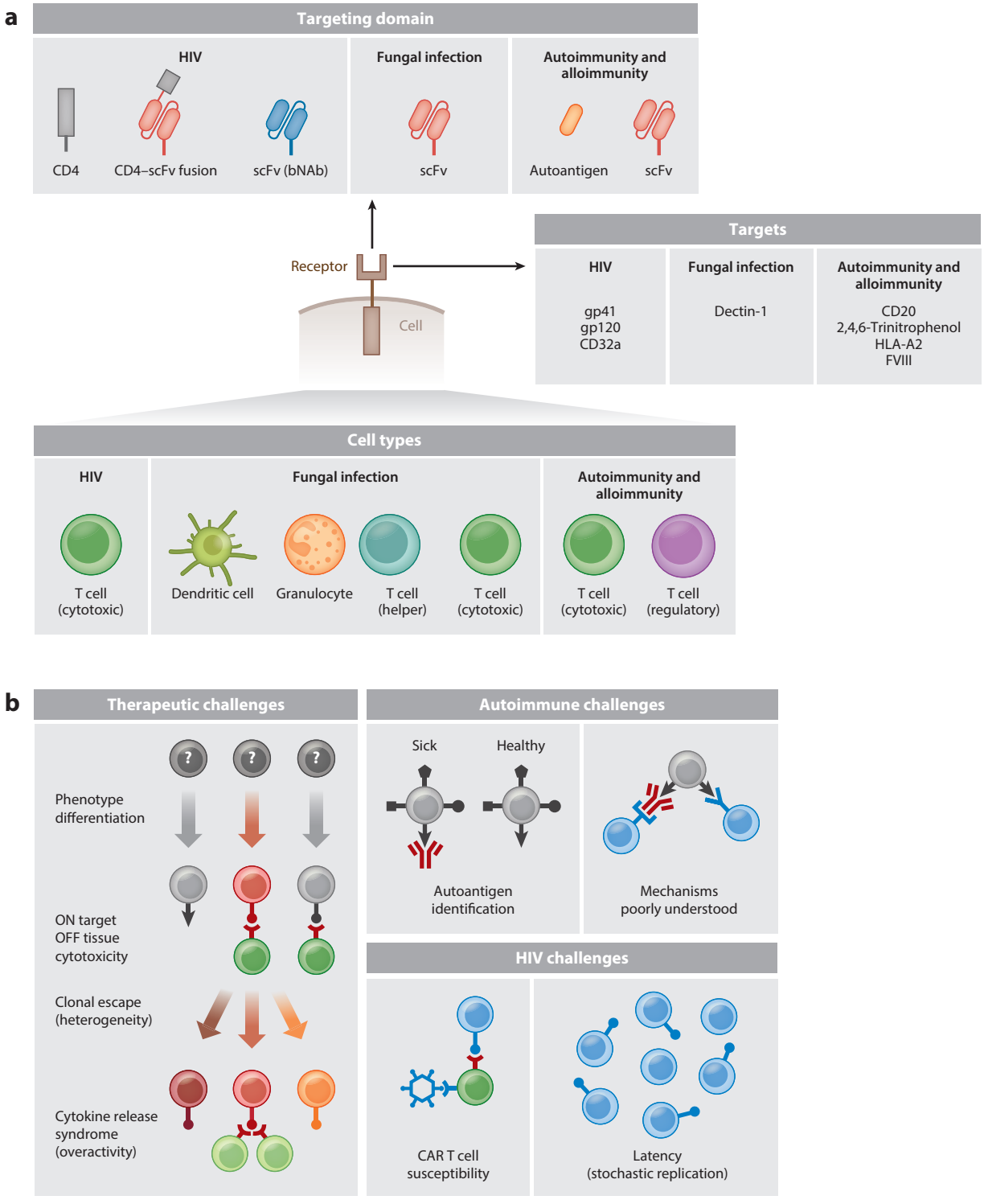
and either the extracellular portion of CD4 or an HIV-specific scFv (**Figure 3*a***) (44). In a clinical study, these cells survived for prolonged durations in vivo, although they did not seem to affect long-term differences in viral reservoirs (45).

Although these cells functioned well in vitro, they were not efficient enough in vivo to warrant widespread adoption of these therapies. One plausible reason for the compromised clinical efficacy is that the choices of antigen-binding domains were not ideal. The use of CD4 as an HIV-specific domain rendered the engineered cells susceptible to infection and provided a pool of infected cells for the virus to spread within. Furthermore, the potency of scFvs derived from antibodies found in the sera of HIV-seropositive individuals was limited by the availability and breadth of coverage of these antibodies. Although these engineered T cells were capable of killing T cells, their use was not capable of curing or drastically curtailing disease and was thus never widely adopted. Despite the limited potential of these engineered T cells, recent studies have shown that they are safe and can be detected in the blood of HIV-seropositive individuals for more than a decade postinfusion (46).

A more recent effort to develop engineered HIV-specific CTLs used a CD4–scFV fusion as the CAR antigen-binding domain, rendering the cells bispecific (**Figure 3*a***). Importantly, these cells were found to not be susceptible to HIV infection. Over the past few years, researchers have again attempted to create engineered CTLs capable of killing HIV-infected cells and have looked to the more recent discovery of broadly neutralizing antibodies (bnAbs) as inspiration for antigen-binding domains with higher potency and breadth (47, 48). Although these cells have greater potency due to their breadth of coverage, they are also efficient at killing cells reactivated from latency using latency-reversing agents (LRAs). LRAs are not completely efficient, but they do allow for the targeting of a previously untouchable population of latently infected cells.

In 2017, researchers cleverly introduced such bnAb-derived CARs into the endogenous CCR5 locus, disrupting the expression of the primary coreceptor for HIV infection (**Figure 3*a***) (49). This approach results in the creation of HIV-specific, HIV-resistant CTLs, thus rendering the clinical applications of such a therapy much more feasible. Finally, the identification of cell surface markers specific to latently infected cells is a goal that, if met, would revolutionize the field of HIV immunotherapy. Researchers are making progress toward this goal; in 2017, researchers in France identified CD32a as a marker of a reservoir of cells containing replication-competent proviruses (50). However, whether CD32a is present on all such reservoirs remains unknown. Additionally, CD32a is a coreceptor found on healthy B cells; therefore, an additional synthetic biology endeavor would be required to target only the latently infected T cell population using logic implemented at the cellular level.

2.2.2. Fungal infection. Beyond HIV, cellular immunotherapy has implications in other types of infection. Despite advances in antifungal therapy, the mortality rates from major fungal infections (e.g., invasive aspergillosis, invasive candidiasis, and cryptococcal meningitis) remain as high as 40% (51, 52). Invasive fungal infections often occur in immune-compromised individuals who have undergone hematopoietic stem cell transplantation and intensive chemotherapy. Current non-cell-based methods to treat fungal infections include the use of cytokines [e.g., interferon (IFN)-γ)] (53–55) and antibody-based therapies (56–58). For cell-based therapies, research has shown that *Aspergillus*-pulsed dendritic cells induce antifungal resistance in vivo (59), and several studies have indicated that transfusion of granulocytes can effectively treat invasive *Aspergillus* infections (**Figure 3*a***) (60–62). Adoptive transfer of fungus-specific T cells has been considered for the treatment of fungal infections, as the T helper 1 cytokines IFN-γ and tumor necrosis factor α have demonstrated efficacy against invasive fungal infection. Clinical trials have shown that the adoptive transfer of fungus-specific T cells is effective against *Aspergillus* (63). Once the efficacy



(*Caption appears on following page*)

Figure 3 (*Figure appears on preceding page*)

Applications and challenges of cellular immunotherapy. (*a*) Summary of cellular immunotherapy as it applies to HIV, fungal infection, and autoimmunity and alloimmunity. These therapies differ in the choice of target antigen, targeting domain, and cell type used for genetic engineering. In some therapies, only endogenous targeting domains are used. (*b*) Challenges associated with current cellular immunotherapies, including challenges unique to HIV and autoimmunity. Abbreviations: bnAb, broadly neutralizing antibody; CAR, chimeric antigen receptor; HLA, human leukocyte antigen; scFv, single-chain variable fragment.

of antifungal cellular immunotherapy was demonstrated in clinical trials, several ways to isolate and expand fungus-specific T cells were reported (64–66). Until recently, CD4 helper T cells were used primarily to combat fungal infection. However, cytotoxic CD8 T cells engineered to express CARs against the fungal pattern-recognition receptor Dectin-1 of the *Aspergillus* cell wall were shown to be effective in a mouse model (67). Moreover, CARs could be developed to target other fungal infections or potentially bacterial infections, including *Mycobacterium tuberculosis*, once distinct target antigens that are specific to each disease are identified (68).

2.2.3. Autoimmunity, alloimmunity, and others. Cellular immunotherapy has been applied within the field of autoimmune disease, in which engineered immune cells are programmed to attack immune cells that aberrantly recognize and initiate an immune response against healthy cells and tissues. In some cases, this syngeneic reactivity can be attributed to B cells, which subsequently differentiate into plasma cells that secrete autoantibodies. These autoantibodies complex with self-antigens and trigger local inflammation, which then activates antigen-presenting cells and recruits effector T helper cells to eliminate the antigen-bearing cells. In other cases, autoreactive T cells are able to escape elimination in the thymus and react against self-antigens. Due to their involvement in sustaining the autoimmune reaction, both T and B cells have been investigated for use in cell therapies.

In order to specifically eliminate autoreactive cells, the immune system must be directed toward markers discriminating autoreactivity. One method of treating B cell–mediated autoimmune disease has involved the depletion of the general B cell population, which was first suggested with the creation of rituximab—an antibody against a B cell antigen named CD20 (**Figure 3*a***). The presence of rituximab causes transient depletion of all B cells, leading to significant improvement of symptoms in patients with rheumatoid arthritis (69). Furthermore, chimeric antibodies have been used to specifically silence double-stranded DNA–specific B cell receptors. These receptors were cross-linked with an inhibitory CD32 receptor through an immune complex, thus rendering the B cells inactive (70). More recently, efforts have been made to mitigate B cell–mediated autoimmune disease by use of cellular immunotherapy to delete autoreactive B cells. Due to the specificity they provide, autoantigens have become a desirable target for cellular autoimmune therapies. Recent research has shown that chimeric autoantibody receptors (CAARs), which are generated by fusing an autoantigen to T cell signaling domains, are capable of selectively killing autoreactive B cells in vivo (71).

Cellular immunotherapy has also been used to address aberrant immune responses in addition to autoimmune disease. Modified regulatory T cells (Tregs) have been engineered to express a 2,4,6-trinitrophenol (TNP)-specific chimeric receptor targeting effector T cells that could be used for the treatment of trinitrobenzenesulfonic acid colitis (**Figure 3*a***). These engineered murine Tregs successfully suppressed effector T cells in vivo (72). In addition to their usefulness in suppressing autoimmunity, Tregs have been used to mitigate alloimmunity. Infusion of ex vivo expanded $CD4^+CD25^+$ Tregs into T cell– and B cell–deficient mice significantly inhibited graft-versus-host disease (GVHD) caused by $CD4^+$ cells originating from a different mouse strain (73).

The application of cellular immunotherapy in treating GVHD has also been exemplified with the use of a human leukocyte antigen (HLA)-A2 CAR T cell. In this study, an HLA-A2-specific CAR was expressed in Tregs and shown to prevent HLA-A2-mediated GVHD in vivo without tissue damage, highlighting the potential use of cellular immunotherapy in dealing with allogeneic transplant rejection (74). An alternative approach to the use of CAR T cells involves masking the transplanted cells themselves. In vivo studies have been performed to force expression of a minimally polymorphic HLA-E on pluripotent stem cells and their derivatives to eradicate expression of HLA-A, -B, or -C. These cells escaped recognition by $CD8^+$ T cells and avoided natural killer–mediated lysis (75).

Apart from autoimmunity and alloimmunity, Tregs have been engineered to provide specificity in other forms of immunosuppression. Previous research has involved the modification of Tregs to suppress FVIII-specific T and B cell responses through a recombinant TCR. These cells have been used to treat patients with hemophilia A, with the added benefit of avoiding global immunosuppression (76). Furthermore, FVIII-specific CAR Tregs have been generated that would also bypass the MHC restriction of TCRs. This could impart a universality to cellular therapy, as patients with differing HLAs could be treated with the same cells (77).

As more autoantigens, alloantigens, and other aberrant inflammatory antigens are being discovered, additional avenues are opening for cellular therapies involving cellular immunotherapy. An example is the identification of tetraspanin-7, an autoantigen recently implicated in type 1 diabetes (78). A cell therapy for type 1 diabetes could be developed by substituting the scFv domain of a CAR with an autoantibody targeting tetraspanin-7. Similarly, specific target antigens for certain allergies could be incorporated into CAR Tregs to suppress the immune response to them. However, note that diseases are not associated with single antigens, and furthermore not all antigen-expressing cells are culpable, thus necessitating synthetic genetic circuits to sense and logically respond to multiple antigens.

3. CURRENT LIMITATIONS OF CELLULAR IMMUNOTHERAPY

3.1. Safety

Although immunotherapy has shown promising results in various applications, several challenges still need to be addressed. In particular, safety is a primary area of concern. Aberrant activation caused by overstimulation or hypersensitivity can be quite severe, leading to hypoxia and neurologic disorders, and can even be fatal (**Figure 3*b***). In cellular therapies targeting large populations of cells with high rates of proliferation, such as cancer, overactivity of the engineered immune cells becomes a concern. This overactivation leads to high levels of cytokine production, which in turn promotes a systemic hyperinflammatory state known as cytokine release syndrome (CRS). The toxicity of overactive T cells remains a prevailing complication in cancer immunotherapy clinical trials (79). In a 2015 phase I trial, patients with acute and chronic lymphocytic leukemia and B cell lymphomas were treated with anti-CD19 CARs. In this trial, CRS occurred in 16 of 21 patients, with 3 patients experiencing grade 4 CRS (80). In severe cases, CRS can cause systemic organ failure and fatality.

Knowing what to target is another challenge inherent to immunotherapy. One of the bottlenecks facing autoimmune cellular therapies involves knowing the exact mechanisms and antigens underlying autoreactivity (**Figure 3*b***). Once the autoantigens and mechanisms are known, CARs or other receptors could feasibly be synthesized to target them. However, in the case of cancer immunotherapy on-target, off-tumor effects occur when the engineered cells recognize cells expressing low levels of the target antigen. In a phase I/II clinical trial held in 2013, two metastatic

cancer patients treated using anti-MAGE-A3 (melanoma-associated antigen 3) TCR-engineered T cells experienced severe neurotoxicity and subsequently died. It was later found that one of the targeted antigens, MAGE-A12, is also expressed in the brain (81), demonstrating that choosing the right target antigen is vital to the success of cellular immunotherapies. Often, extensive screening is needed to determine how specific the antigen is to the target cell type and whether it is expressed on healthy tissue.

Unlike cancer and autoimmune disease cellular immunotherapy, antigen specificity may not be a major concern for antifungal cellular therapy. Much of the research on antifungal adoptive immunotherapy has focused on purifying and expanding fungus-specific T cells from the blood, where they exist in low numbers (52, 82). The antigens used to stimulate these cells are derived from fungal lysate or fungus-specific peptides that resemble pathogen-associated molecular patterns. It is unlikely that cells recognizing these antigens will cause significant off-target toxicity because these antigens are not normally present in human cells. Rather, more emphasis may need to be placed on controlling overstimulation of antifungal T cells.

Safety concerns regarding the use of engineered T cells to treat HIV infection are distinct from concerns for the treatment of other diseases. Although a decade-long study has demonstrated the persistence of T cells engineered to express a CD4 CAR, the cells were unable to clear HIV cells sufficiently to reduce viral burden (46). This is partly because the engineered T cells still expressed CD4 and CCR5, receptors necessary for HIV infection of T cells, so the engineered cells were themselves susceptible to infection (**Figure 3*b***). Therefore, it is highly desirable to engineer cells that are both capable of recognizing HIV-infected cells and immune to infection. One method of rendering the cells resistant to infection involves fusing to the CAR an antibody fragment highly specific for the CD4 epitope recognized by gp120 (83). An alternative method involves the genomic disruption of the receptors, as illustrated by the simultaneous knockdown and introduction of a CAR into the endogenous CCR5 locus through targeted nuclease disruption and homology-directed recombination (49).

3.2. Efficacy

Historically, efforts to treat cancer using genetically modified T cells have varied wildly in their success, owing largely to the vast heterogeneity of environments in which different cancers exist. While T cells bearing anti-CD19 CARs have led to a complete response rate of up to 90% (7), efforts to treat solid malignancies have been much less successful. Solid tumors are more difficult to target via adoptive immunotherapy for many reasons. For example, vasculature in solid tumors is often deformed, making it difficult for T cells to penetrate the tumor. This issue is exacerbated by the downregulation of adhesion molecules such as intercellular adhesion molecule 1 and vascular cell adhesion molecule 1 on endothelial cells lining the vasculature (84). Even if T cells are able to enter the tumor, they face an array of issues compromising their efficacy.

The same deformed vasculature that hampers tumor penetration is also unable to adequately perfuse the surrounding tissue with oxygen, creating a hypoxic tumor microenvironment (TME). The hypoxic conditions lead to upregulation of hypoxia-inducible factor 1α, which in turn promotes glycolysis as the main source of T cell energy by promoting lactate dehydrogenase A activity and inhibiting oxidative phosphorylation (85). However, inadequate perfusion of the tumor, coupled with high consumption and growth rates of tumor cells, creates a TME devoid of essential nutrients such as glucose and amino acid precursors to cytokines. Thus, tumor-infiltrating cytotoxic T cells become anergic, as they are unable to undergo glycolysis and become metabolically exhausted. Furthermore, hypoxia-induced accumulation of extracellular adenosine triggers immune suppression through the A2 adenosine receptor (A2AR). Genetic deletion of A2AR

improves the inhibition of growth and destruction of metastases in in vivo tumor models, suggesting that the hypoxic TME causes A2AR-mediated suppression of cytotoxic T cells (86, 87). Furthermore, the TME can cause metabolic exhaustion of effector T cells through loss of mitochondrial function, presumably because of a progressive loss of peroxisome proliferator-activated receptor γ coactivator 1α (88).

Inflammation within solid tumors leads to recruitment of a moiety of leukocytes, many of which are immunosuppressive in nature and lead to the proliferation, survival, and migration of tumor cells (89). Increasing evidence has shown that tumor-associated macrophages (TAMs) are implicated in the immunosuppression of cytotoxic T cells in addition to their canonical role as tumor-targeting cells. A transcriptome analysis of TAMs shows downregulation of activating cytokines and increased expression of interleukin (IL)-10, which induces programmed death ligand 1 (PD-L1) expression in monocytes and thus inhibits T cell activation. Furthermore, TAMs secrete inhibitory molecules such as transforming growth factor β (TGF-β) (90). Inhibition of macrophage tumor infiltration in response to recruitment by factors secreted by epithelial cells is associated with improved survival of tumor-bearing mice (91). Additionally, redirecting these macrophages to an antitumor phenotype using histidine-rich glycoprotein to redirect macrophage polarization leads to a decrease in tumor growth and metastasis, improving the efficacy of traditional chemotherapy. Foxp3$^+$ Tregs can also reside within many solid tumors, leading to an immunosuppressive state that can inhibit the efficacy of tumor-infiltrating lymphocytes (92). The presence of these Tregs is directly associated with breast cancer disease progression and can be used to identify high-risk patients (93).

3.3. Manufacturing

Genetically engineered autologous cell therapies, such as CAR T cell therapy, are arguably some of the most complex therapeutic agents to ever reach the market. The manufacture of these therapies involves many complicated steps, including cell harvesting, purification, modification, expansion, and characterization. First, blood is harvested and the immune cells are separated from the rest of the plasma. Second, purified immune cells are activated, typically through the use of microbeads conjugated to activating antibodies. Third, genetic modifications are made to introduce antigen-targeting receptors or modify the expression of endogenous genes. These modifications are achieved through various methods, such as the introduction of retroviruses, messenger RNAs (mRNAs), and proteins. Further cell expansion and cultivation steps are then required to generate a sufficient number of cells to be therapeutically viable.

Currently, clinical-grade cell production involves both open-loop and manual cell processing (94). However, standardization and characterization of this labor-intensive process remain major challenges. To further increase the scale of production and improve the quality control, closed-loop automation of the manufacturing process will be required. In addition to the complexity of the manufacturing process, the quality and consistency of the reagents used in the process represent major areas of concern. For example, autologous T cells collected from individual patients are highly variable at the point of origin, which can create variability in the final manufactured cells. Moreover, biologics such as antibodies, cytokines, and media used to activate and expand the T cells have not been rigorously standardized. At each point in the manufacturing process and for each reagent used, variability could compromise the quality of an already complicated manufacturing process and the therapeutic benefit of an already complex treatment.

CAR T cell therapy, the first cellular therapy using genetically engineered cells, is expected to be one of the most expensive therapies, with an estimated cost of $500,000 to $1,000,000 per patient when benchmarked against the cost of hematopoietic stem cell transplantation. This cost

represents a marketing challenge for pharmaceutical companies. If the therapy is priced too high, its sales could be too low to justify continued production. UniQure's gene therapy Glybera, which costs around $1,200,000 per patient, was ultimately pulled from the market due to poor sales. Furthermore, it remains uncertain when, if ever, insurance companies will start covering cellular immunotherapy. Therefore, how patients will be able to afford such an expensive therapy is a major concern for the scientists developing the therapy.

4. SYNTHETIC BIOLOGY TOOLS FOR CELLULAR IMMUNOTHERAPY

In the context of immune cell engineering, synthetic biology tools encompass the use of conventional transcriptional tools developed for genetic regulation in mammalian cells, as well as protein engineering and receptor design. While the concepts behind engineering tools for microbial organisms and mammalian cells are the same, the implementations are necessarily different due to differences in the underlying biological processes (e.g., localization of transcription factors, transcriptional and posttranscriptional regulation, and promoter architecture). Therefore, mammalian synthetic biology focuses on developing tools and novel gene circuits to control and reprogram different functions of mammalian cells (95). These tools include devices for controlling RNA and protein expression, synthetic transcription factors capable of carrying out user-defined gene expression programs, tools for editing the genome, and tools that enable rewiring of signaling pathways.

Many RNA devices have been developed to tune gene expression and to perform logic based on RNA interference or microRNAs (96, 97). Other types of RNA control devices include RNA aptamers and ribozymes used to regulate the stability of mRNA transcripts. Additionally, synthetic biologists have created tools to control protein activity and turnover that could be harnessed for cellular immunotherapy. Degron domains, which affect the regulation of protein degradation, have been used to control the degradation kinetics, and thus levels, of protein in the cell (98, 99). Alternatively, ligand-inducible domains can be used to control the degradation or dimerization of proteins of interest in a tunable manner (100–102). Recently, Lim and colleagues (101) demonstrated the use of heterodimerization domains to develop CARs that can be controlled by small molecules, effectively creating an ON switch. Furthermore, light-inducible dimerization domains could be useful in cellular therapy to enable spatiotemporal control of cell activity (103–108).

On the transcription level, there are many tools available for regulation, such as DNA-binding domains developed by fusing natural transcription factors (e.g., TetR and GAL4) to transcriptional activator or repressor domains (109, 110). Synthetic transcription factors could also be designed to regulate endogenous transcription using ZFs (27, 111, 112), transcription activator–like effectors (113–115), or CRISPR/Cas (116–118). Additionally, these systems enable highly specific and efficient genome editing at defined genomic loci (119, 120). These tools are already being used to enhance cellular immunotherapies. For example, a recent study demonstrated that CD19 CARs precisely integrated at the endogenous T cell receptor α–constant (TRAC) locus using CRISPR/Cas9 resulted in uniform expression of the CAR and increased T cell potency (121). Furthermore, evidence suggests that simultaneous genomic disruption and integration of an HIV-specific CAR into the CCR5 locus help engineered T cells resist HIV infection (49).

Another main goal of synthetic biology is to rewire cell-sensing pathways to create novel input–output relationships. To achieve this goal, investigators have developed synthetic receptors that can couple specific inputs (e.g., small molecules or surface antigens) with user-defined outputs (e.g., transcription programs or cell signaling) (**Figure 4*a***). One of the first classes of synthetic receptors is activated solely by synthetic ligands (RASSLs). RASSLs are genetically engineered G protein–coupled receptors (GPCRs) that can respond to synthetic ligands (122)

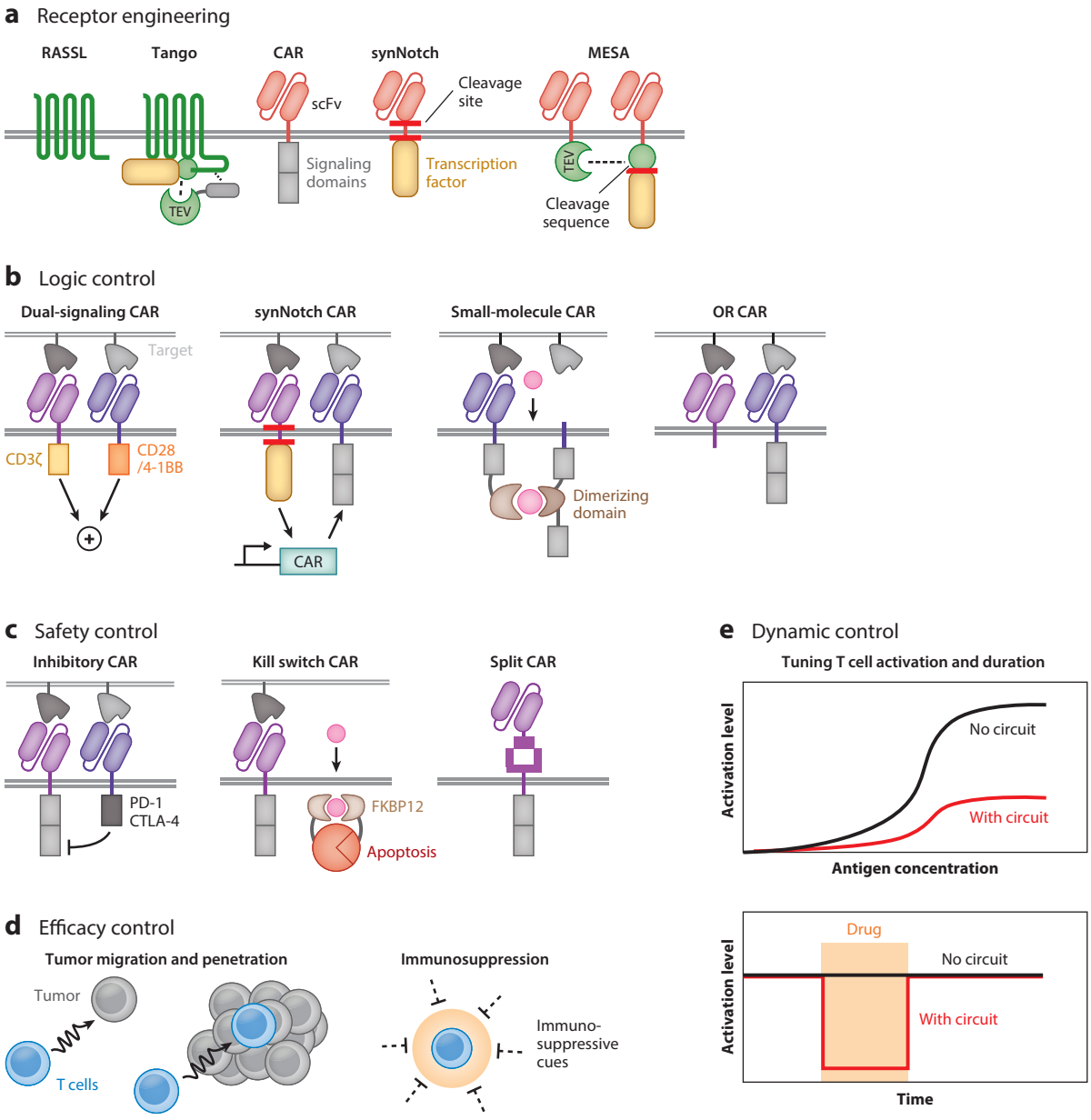


Figure 4

Applications of synthetic biology in cancer cellular immunotherapy. (*a*) Receptor engineering. Different synthetic receptor designs developed to reprogram cells with user-defined input–output relationships. (*b*) Logic control. Different chimeric antigen receptor (CAR) designs that can perform logic to enhance tumor specificity or to tune T cell activity. The tumor antigen is shown in dark gray. (*c*) Safety control. Different CAR designs that can mitigate CAR/T cell–related toxicities. (*d*) Efficacy control. Development of novel gene circuits or CARs to increase effectiveness of CAR T cell therapy by engineering T cell mobility or mitigating immunosuppressive cues in the cancer microenvironment. (*e*) Dynamic control. Engineering cells with gene circuits that can tune T cell activation and its duration. Abbreviations: CTLA-4, cytotoxic T lymphocyte–associated protein 4; MESA, modular extracellular sensor architecture; PD-1, programmed cell death protein 1; RASSL, receptor activated solely by synthetic ligands; scFv, single-chain variable fragment; synNotch, synthetic Notch; TEV, tobacco etch virus protease.

with native GPCR signaling. Additionally, Tango receptors have been developed to enable transcriptional output from three different classes of receptors (GPCRs, receptor tyrosine kinases, and steroid hormone receptors) (123). Tango receptors tether synthetic transcription factors to the membrane by using linkers containing a protease cleavage sequence. Upon ligand binding, the receptor recruits a signaling protein fused to the appropriate protease, which subsequently cleaves the transcription factor from the receptor and allows it to carry out a defined transcription program. Moreover, the modular extracellular sensor architecture (MESA) has been used to sense soluble ligands through ligand-induced dimerization of a transcription factor–bearing receptor chain and a protease-bearing receptor chain. The MESA system has recently been used to rewire human T cells to sense vascular endothelial growth factor and to produce IL-2 or other programmable transcriptional outputs using the dCas9 system (28). This particular input–output function demonstrates how synthetic receptors can be used to direct the immune system to respond against normally immunosuppressive cues. Furthermore, synthetic receptors built upon a minimal proteolytic core of the Notch receptor (synNotch) enable the programming of both inputs and outputs useful for cell-based therapies (29, 124). Different tools, gene circuits and synthetic receptors developed can be used to tackle some of the most important challenges that cellular immunotherapy is currently facing, namely safety, efficacy, and manufacturing.

4.1. Tools for Improving Safety

One of the main limitations of cellular immunotherapy, particularly evident in the use of CAR T cells to treat solid tumors, is the lack of a single, sufficient tumor-specific antigen, which compromises both the safety and efficacy of the therapy. A dual CAR system that can perform combinatorial antigen detection has been developed to increase the specificity of the engineered T cells (**Figure 4*b***). In this design, one CAR contains the CD3ζ signaling domain and the other CAR contains the CD28/4–1BB costimulatory domains, such that the T cell responds only to tumor cells that express both antigens. This novel strategy could help to avoid ON target/OFF tissue toxicities observed in CAR T cell therapy. Another example of combinatorial antigen detection uses the synNotch receptor to build combinatorial antigen-sensing circuits (125). Binding of the synNotch receptor to the tumor antigen releases an intracellular transcription factor, which then induces the expression of a CAR specific for a second tumor antigen. Furthermore, dimerizing domains that are responsive to small molecules were used to precisely regulate the timing and strength of the T cell response (101).

Tumor antigen escape is another challenge that CAR T cell therapy will need to overcome (126). In order to mitigate the effect of antigen escape on therapy, bispecific receptors that can be triggered by CD19 and Her2 or CD19 and CD20 have been developed (127). Thus, even if tumor cells are able to escape detection of one antigen through loss of expression, the cells will still be recognized via the second antigen. Furthermore, many CARs used in clinical trials have a fixed CAR design that is impossible to alter without reengineering the T cells (128). Several split CAR designs have been introduced to enable increased control over T cell activation during the course of treatment (**Figure 4*c***) (129–132). In these split designs, adaptor antibodies mediate binding between the antigen and the CAR. These split designs promote temporal control of T cell activity and enhance the flexibility of the therapy by allowing T cells to target multiple antigens without reengineering the receptors.

Although recent multiple leukemia clinical trials with CD19 CAR T cells were successful, limited control over CAR T cell activity could result in severe toxicity, including CRS (79). Because safety concerns are one of the main barriers that keep CAR T cell therapy from extending to cancers other than leukemia, many current CAR designs focus on controlling T cell activation.

One example is the use of coinhibitory domains (e.g., PD-1, CTLA-4) that enable an antigen-specific inhibitory function of the CAR (133). Also, T cells have been engineered to express modified human caspase-9 fused to human FKBP12 to allow dimerization via small molecules. Dimerization induces apoptosis of engineered T cells by activating human caspase-9 (134, 135). Introduction of this kill switch will increase the safety of T cell therapy (**Figure 4*c***).

4.2. Tools for Improving Efficacy

In addition to safety and specificity concerns, the efficacy of cellular immunotherapies is limited by the inability of engineered T cells to migrate through solid tumors, particularly in solid tissues with deformed vasculature (**Figure 4*d***). In order to increase the migration of T cells to tumors, T cells were engineered to express the chemokine receptor CXCR2. These engineered T cells showed enhanced localization to tumors expressing the chemokine CXCL1 in vivo (136). Moreover, this trafficking resulted in significantly improved antitumor efficacy in vivo (137). T cells engineered with photoactivatable chemokine receptor (PA-CXCR4) also showed enhanced directional migration to tumor sites and significantly reduced tumor burden in vivo (138). Another approach to enhancing engineered T cell migration involves restoration of heparanase expression. Ex vivo expanded T cells were found to underexpress heparanase, an enzyme that promotes the degradation of the extracellular matrix. T cells engineered to express heparanase demonstrated improved tumor penetration and antitumor activity (139).

The TME contains many immunosuppressive cues, including inhibitory cytokines (e.g., IL-4 and TGF-β) and cell surface markers (e.g., PD-L1), that inhibit the antitumor activity of engineered T cells. Thus, engineering T cells to overcome these immunosuppressive cues will be critical for effective cancer cellular immunotherapy (**Figure 4*d***). In one study, blockade of inhibitory TGF-β signaling through overexpression of a nonfunctional TGF-β receptor mitigated inhibitory effects (140). Another approach is to convert immunosuppressive cues into immunostimulatory responses. For example, fusion of the inhibitory cytokine receptor IL-4 exodomain to the IL-7 receptor endodomain effectively converted the tumor-derived IL-4 inhibitory pathway to IL-7 immune stimulation (141). T cells have also been engineered to express receptors that comprise the PD-1 exodomain and CD28 endodomain. With this approach, binding of engineered T cells to PD-L1$^+$ tumor cells resulted in increased cytokine secretion and proliferative capacity of the engineered cells (142). Furthermore, tumor-specific T cells have been engineered to conditionally secrete immunostimulatory cytokines (e.g., IL-12) in the TME to promote engineered T cell efficacy (143). However, recent clinical trials indicate a need for better control of IL-12 secretion via novel gene circuit design, as IL-12 showed severe toxicity in a clinical setting (144). Lastly, feedback control has been used to regulate the duration and dynamics of the T cell response (**Figure 4*e***) (14).

4.3. Tools for Improving Manufacturing

Many of the difficulties in manufacturing T cells for immunotherapy arise from the need for autologous cells. Cells need to be extracted from the patient, expanded ex vivo, purified, and rigorously characterized before being infused back into the patient. An appealing alternative to this approach would be the use of cells from an allogeneic T cell bank. Cells could then be produced and characterized at a large scale, reducing both the time and cost associated with manufacturing. However, the use of allogeneic cells poses its own risks, such as the possibility of GVHD and host-mediated rejection. A recent study has addressed this risk by simultaneously introducing a CAR and using transcription activator–like effector nucleases (TALENs) to knock out TRAC

and CD52, rendering the cells resistant to host immunity. These cells were then used to achieve molecular remission in two infants with B cell acute lymphoblastic leukemia (145). An additional challenge during the manufacturing process is the inability to identify transduced cells. This leads to an inability to purify transduced cells and, ultimately, a variable cell product. This problem can be solved through the inclusion of Strep-tag® II sequences into the CAR for rapid identification and purification (146).

5. FUTURE ASPIRATIONS: MOVING BEYOND TREATING DISEASE

5.1. Diagnostics: T Cells as Sentinels for Health

For the most part, the primary focus of medicine to date has been to treat disease. However, it is self-evident that prevention and early detection of disease are highly desirable, both to increase life expectancy and to decrease the financial burden of medical interventions. The economic benefit of a healthy population is enormous, as it can simultaneously lower health care costs and increase national productivity. Therefore, investment in the wellness of the population would reap substantial returns for society. The use of synthetic biomarkers is emerging as an attractive candidate for the early diagnosis of various diseases. For example, nanoparticles functionalized with mass-encoded peptides have been used to detect localized protease expression and convert it to a signal that can then be noninvasively monitored through mass spectrometry of the host's urine (147). Whereas conventional imaging techniques are limited to tumors approximately 1 cm in size, these nanoparticles have been used to detect tumors smaller than 2 mm in size (148). As the body's natural defense system against disease and infection, the immune system is a rational target for engineering diagnosis and early treatment of many diseases. An approach combining the diagnostic sensitivity of synthetic biomarkers with the therapeutic potential of cellular immunotherapy could involve engineering T cells to sense a target antigen and secrete a protease, which will then interact with the synthetic biomarker. This would require synthetic receptors capable of driving protease transcription in genomically integrated reporters. Although several such receptor architectures already exist (28, 29), their ability to achieve clinically relevant differences in gene expression is still being explored. In this way, engineered T cells would serve as sentinels, constantly monitoring and reporting on threats and treatment progression. Furthermore, such an approach would enable detection of more diverse disease phenotypes (i.e., surface antigen expression, combinatorial logic).

5.2. Targeting Senescent Cells: T Cells as Rejuvenants

Cellular senescence, the process by which cells undergo permanent cell cycle arrest, is thought to be an important contributing factor to organismal aging. The loss of proliferative cells leads to a decreased capacity to repair tissues and the production of inflammatory cytokines, causing gradual tissue and organ degeneration. However, selective depletion of senescent cells delayed the onset of tumorigenesis and increased life span in a genetically engineered mouse model (149). Furthermore, depletion of senescent cells through pharmacological inhibition of Bcl-2 and Bcl-xL can rejuvenate senescent stem cells and delay aging in mice (150). These findings suggest that clearance of senescent cells by genetically engineered T cells could potentially slow aging and lead to tissue rejuvenation. The key challenge in this endeavor would be to selectively target senescent cells while leaving normally dividing cells intact. A significant effort within the cancer immunotherapy community has led to the development of synthetic biology tools employing combinatorial logic. These tools may prove useful in the specific targeting of senescent cells for

antiaging therapies. Still, new antigen-targeting domains will undoubtedly be needed in order to discriminate between senescent and nonsenescent cells. We envision that such therapy could be applied to help patients recover from major illnesses.

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Errata

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