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Leading Edge
Review

The Principles of Engineering Immune Cells to Treat Cancer

Wendell A. Lim[1,*] and Carl H. June[2,*]
[1]Howard Hughes Medical Institute, Department of Cellular and Molecular Pharmacology, UCSF Center for Systems and Synthetic Biology, University of California, San Francisco, San Francisco, CA 94158, USA
[2]Center for Cellular Immunotherapies, the Department of Pathology and Laboratory Medicine at the Perelman School of Medicine, and the Parker Institute for Cancer Immunotherapy, University of Pennsylvania, Philadelphia, PA 19104, USA
*Correspondence: wendell.lim@ucsf.edu (W.A.L.), cjune@exchange.upenn.edu (C.H.J.)
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Chimeric antigen receptor (CAR) T cells have proven that engineered immune cells can serve as a powerful new class of cancer therapeutics. Clinical experience has helped to define the major challenges that must be met to make engineered T cells a reliable, safe, and effective platform that can be deployed against a broad range of tumors. The emergence of synthetic biology approaches for cellular engineering is providing us with a broadly expanded set of tools for programming immune cells. We discuss how these tools could be used to design the next generation of smart T cell precision therapeutics.

Introduction

The emergence of engineered T cells as a form of cancer therapy marks the beginning of a new era in medicine, providing a transformative way to combat complex diseases such as cancer. Within the past few years, clinical trials using T cells engineered to recognize B cell cancers have shown high rates of response (70%–90%) and durability of response that are unprecedented in acute (Brentjens et al., 2013; Maude et al., 2014; Turtle et al., 2016) and chronic leukemia (Kalos et al., 2011). In 2017, we expect to see the first approved engineered T cell therapies coming to market. While poised to revolutionize cancer therapy, the optimism about T cell cancer therapies remains tempered by concerns about safety and off-target toxicity, as well as the development of resistance. Meanwhile, the field also awaits a clear demonstration of clinical efficacy in solid tumors. The developments in this field over the coming years—in the areas of safety, reliability, and efficacy against solid tumors—will ultimately determine how disruptive this new modality can be in the broader battle against cancer.

Living Therapies Can Uniquely Perform Complex Sensing and Response Functions

Engineered T cells are part of a much broader explosion in immuno-oncology, but what perhaps makes these therapies most revolutionary is the concept of using a living cell as the therapeutic platform. Living cells are radically different from inanimate platforms, such as small molecules or antibodies, in that cells are capable of intelligent sensing and response behaviors. At the same time, these cellular devices are more challenging to manipulate, manufacture, and control. In theory, combining a living platform that is capable of complex sensing-response behaviors with the ability to genetically reprogram these behaviors is what generates the disruptive therapeutic potential of this approach.

Engineered T Cells Represent a Convergence of Diverse Areas of Medicine and Science

The engineered T cell therapies of today and the future represent the convergence of diverse areas of medicine and basic science (Figure 1). This new approach combines concepts from three long-standing therapeutic strategies. Engineered antibodies have become a standard platform for recognizing and targeting disease but are largely used to block target protein activity or to target a toxic payload. Vaccination, which uses various methods to awaken the native immune system, has long illustrated the therapeutic power of unleashing complex immune responses. Finally, transplantation has established the paradigm of using a living therapeutic platform (cells or organs), though usually for replacing a defective system rather than for deploying novel, user-targeted functions.

Today, we can now integrate these different therapeutic strategies into a single, more powerful cell therapy platform. The emerging field of synthetic biology is providing us with the components and technology to systematically engineer customized cell regulatory circuits that can generate the sophisticated sense and response behaviors that may be required to effectively combat a complex disease such as cancer (Bashor et al., 2008; Fischbach et al., 2013) (Figure 1).

Envisioning the Next Generation of T Cell Therapies

In this review, we summarize the field of engineered therapeutic T cells and where it is headed. We have focused on forecasting how the tools of synthetic biology could be used to design the best therapeutic cell programs for treating cancer. The fundamental issues that we focusing on in this review—what kinds of sensing and response programs can be encoded into a therapeutic T cell—are very general and therefore largely agnostic to type of cell used, the source of cell, and how it was manufactured.

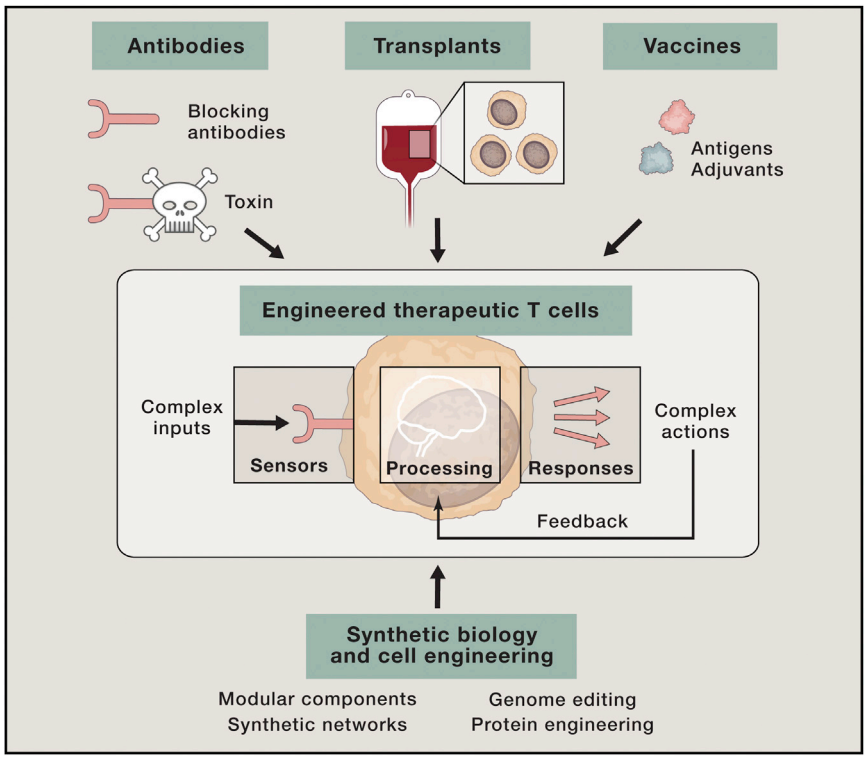

Figure 1. Engineered Therapeutic T Cells Provide a Transformative New Platform for Interfacing with Complex Diseases such as Cancer

Therapeutic T cells combine elements of more traditional therapeutic approaches to yield an integrated smart sense-and-response agent. The emerging field of synthetic biology is providing tools and approaches to program therapeutic cells in diverse ways.

Experience with T Cell Therapy: Lessons Learned

The feasibility and safety of therapeutically infusing non-genetically modified T cells has been validated over the last 20 years. Infusion of ex-vivo-expanded T cells was first used in melanoma patients (Rosenberg et al., 1988) and, shortly thereafter, in patients with disseminated CMV infection (Riddell et al., 1992) and HIV/AIDs (Levine et al., 2002).

Tumor-Infiltrating Lymphocytes

Infusions with tumor-infiltrating lymphocytes (TILs)—T cells isolated from tumors—have been used for several decades in patients with melanoma (Rosenberg et al., 1988). The recent demonstration that TILs can target tumor neo-antigens provides a rationale to target tumors with high mutational loads such as melanoma and lung cancer (Robbins et al., 2013). Several issues, however, have limited the widespread usage of TILs. First, manufacturing of TILs is logistically complex. Second, TILs are dependent on the endogenous T cell receptor (TCR) for cancer recognition. The TCR is an unusual receptor in that the antigen receptor recognizes peptide and self MHC molecules (Figure 2). An advantage of the TCR is that a vast array of distinct peptides can be recognized (Zhang et al., 2016), including tumor-specific mutations (Schumacher and Schreiber, 2015). Thus, the intracellular proteome of cancer cells may represent the largest set of currently untapped targets for new cancer therapies. From a therapeutic perspective, however, there are two principal limitations of the TCR as a recognition modality for T cells given as a cancer therapeutic: (1) the requirement for MHC matching to each patient, as it is currently estimated that humans have > 10,000 HLA alleles (http://hla.alleles.org/nomenclature/index.html) and (2) the affinity of the TCR for cancer targets is typically in the low micromolar range, which can limit the activation and cytotoxicity of tumor specific T cells, as opposed to efficient recognition and discrimination of viral peptides that elicit TCRs with nanomolar affinity (Aleksic et al., 2012).

CD19 CAR T Cells: A Success Story

Chimeric antigen receptors (CARs) are synthetic molecules that allow more flexible user-specified retargeting of T cells (Figure 2). CARs overcome some limitations of the TCR, such as the need for MHC expression, MHC identity, and costimulation. Kuwana and Eshaar first demonstrated that these types of synthetic receptor molecules enabled MHC-independent target recognition by T cells (Gross et al., 1989; Kuwana et al., 1987). One limitation of current CAR T cell strategies is that they require extracellular surface targets on the tumor cells.

CAR T cells targeting CD19 have emerged as the lead paradigm for engineered T cell therapies in cancer and illustrate the synergies of combining synthetic biology with T cell biology. There are several features that have contributed to the success in the clinical studies targeting CD19. CD19 was chosen as the initial target not only for its frequent and high-level expression in B cell malignancies, but also because it is required for normal B cell development in mice and humans (Engel et al., 1995; van Zelm et al., 2006). CD19 is a nearly ideal target, since a loss of normal B cells is tolerable given replacement antibody therapy (IVIG). Patients successfully treated with CD19 CARs have profound B cell aplasia (Porter et al., 2011), and loss of B cell aplasia often heralds relapse (Kochenderfer et al., 2010). These results demonstrate that CAR T cells can have on-target off-tumor effects, a feature that may be mitigated by employing on-off gated CAR T cells, as described below. Preliminary results with other CARs indicates that the CD19 off-tumor cross-reactions will not be a singular example but may be generally observed with other lineage-dependent targets. For example, in ongoing trials (clinicaltrials.gov NCT02546167) with CARs targeting B cell maturation antigen (BCMA, TNFRSF17, or CD269), multiple myeloma patients have shown eradication of non-malignant plasma cells that also express BCMA, in addition to eradication of the myeloma cells (Ali et al., 2016). How tolerable this kind of off-tumor reaction is will depend on the types of non-tumor cells that are targeted.

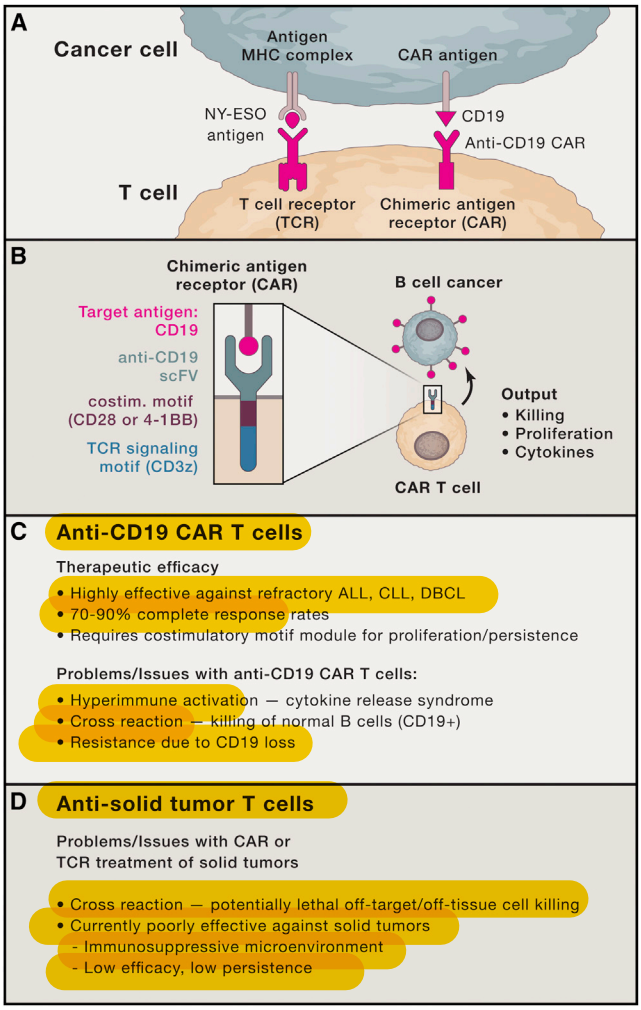

Figure 2. Platforms for Redirecting T Cells to Cancer

(A) T cell receptors (e.g., anti-NY-ESO1) or chimeric antigen receptors (e.g., anti-CD19 CAR).

(B) CAR structure includes an extracellular antigen recognition domain fused to intracellular TCR signaling domains (CD3z) and co-stimulatory domains (e.g., CD28 or 4-1BB).

(C) Current status of CD19 CAR therapies.

(D) Current status of engineered T cells directed toward solid tumors.

CD19 CAR T Cells: The Remaining Issues

Despite their success, several issues remain for CD19 CAR T cells (Figure 2C). In acute lymphoblastic leukemia (ALL), since > 80% of patients enter remission, the issues are to decrease infusional toxicity and increase durability of responses (Maude et al., 2014; Turtle et al., 2016). Infusional toxicities have been limited to cytokine release syndrome (Fitzgerald et al., 2016; Teachey et al., 2016) and a poorly understood neurological syndrome. Cytokine release syndrome occurs more frequently in patients with higher disease burdens at time of infusion and rapidly resolves after cytokine directed therapy that targets IL-6 (Lee et al., 2014).

In contrast, in chronic lymphocytic leukemia (CLL), approximately 50% of patients benefit from CARs, and the remissions have been durable for more than 5 years in the responding patients (Porter et al., 2015); however, further research is required to increase response rates. One approach currently undergoing clinical evaluation is to combine CAR infusions with targeted therapies such as ibrutinib or with checkpoint therapies such as PD1 antagonists. Another approach is to use allogeneic sources of T cells (Brudno et al., 2016), which may overcome cell-intrinsic mechanisms of resistance such as T cell exhaustion or senescence in patients with CLL (Riches et al., 2013).

An evolving issue is understanding and managing the long-term consequences of acquired B cell aplasia in patients with persisting CARs. In patients with congenital B cell aplasia, live viral vaccines are contraindicated, and most patients are managed with intravenous immunoglobulin replacement therapy (Winkelstein et al., 2006). Thus, the consequences of CAR-induced B cell aplasia may be more severe in children who have not fully established memory B cells and long-lived plasma cells before receiving ablative CAR therapy. The use of switchable rather than constitutive CARs should overcome the limitations of B cell aplasia, as discussed below (in Anti-cancer Cell Therapies Must Solve Multi-dimensional Challenges).

In the case of B-cell-directed CARs, two forms of resistance have emerged in patients with ALL. In a subset of patients, the CAR T cells become undetectable, followed by loss of B cell aplasia and leukemia recurrence within the first 3 months after infusion (Maude et al., 2014). Early loss of engineered T cells is often due to induction of host immunity to the transgene. In contrast, in 10%–20% of patients, the patients relapse late after treatment with target loss, manifested by ALL blasts comprised of CD19 loss variants (Yu et al., 2017). In more extreme cases, tumor escape has occurred by lineage switch from lymphoid to myeloid leukemia (Jacoby et al., 2016).

The Solid Tumor Challenge

The largest challenge to the field of immuno-oncology is designing T cell therapies to effectively and safely treat solid tumors such as adenocarcinoma and sarcoma (Figure 2D). Here, we consider the evidence that the immune system can kill the cells comprising solid tumors, the lessons from trials conducted with adoptive T cell transfer, and opportunities for the design of T cells optimized for solid tumor immunotherapy.

Lessons from Previous Immunotherapy of Solid Tumors with Natural T Cells

The powerful antitumor effects of allogeneic (non-self) hematopoietic stem cell (HSC) transplantation were initially thought to be due to the use of super lethal chemotherapy and irradiation with stem cell rescue. Only later, when identical HSC transplantation procedures were performed using monozygotic twin or sibling donors, was it realized that the antitumor effects were due to allogeneic immune responses because the leukemia-free survival of twin transplant recipients was unexpectedly found to be inferior compared to the survival of sibling transplants (Weiden et al., 1979). Definitive evidence for the antitumor effect of allogeneic T cells was finally provided when the adoptive transfer of donor T cells was shown to be sufficient to induce complete remissions in patients with chronic myeloid leukemia (Collins et al., 1997).

The primary toxicity of allogeneic T cell infusions is graft versus host disease (GVHD), which occurs when donor T cells respond

to host antigens. GVHD can be rapidly lethal, with prominent immunopathology occurring in skin, gastrointestinal track, liver, lymph nodes, bone marrow, and lungs, with sparing of kidneys, muscle, and brain (Ferrara et al., 2009). The relative sensitivity and resistance of various tissues to the effects of allogeneic T cell infusions is relevant to strategies attempting to generate antitumor effects using genetically engineered autologous T cells, as discussed below. The mechanisms for the allogeneic effect are complex and relate to the direct effects of foreign T cells on targets in the host, as well as an indirect effect of the allogeneic T cells reawakening host T cells (Symons et al., 2008).

Based on the discovery of the powerful antitumor effects conveyed by the allogeneic immune effects, patients with various solid tumors have been treated with allogeneic HSCs on several clinical trials. The overall results were disappointing after allogeneic HSCs and allogenic T cell infusions (Bregni et al., 2006), although there are a small number of patients with long-term survival (Omazic et al., 2016).

Trials with Engineered T Cells to Date: A Note for Cautious Optimism?

To date, the most powerful immune effect ever provoked by natural T cells has been the induction of alloimmunity: in a matter of weeks, the infusion of donor T cells can lead to the eradication of leukemia or lethal GVHD. However, as was noted above, not all cancers regress, and some organs are spared from the effects of allogeneic T cells. Infusions of T cells with altered TCRs or with CARs are now showing that these rules can be broken.

The first tests were performed with T cells expressing modified TCRs targeted to antigens that are expressed at high levels on various cancers, but also expressed on normal tissues. A series of trials were conducted in melanoma patients, testing T cells transduced with TCRs targeting MART-1 (melanoma antigen recognized by T cells). One low-affinity TCR that was HLA-A2 restricted was found to be well tolerated but had low antitumor effects (Morgan et al., 2006). A higher-affinity TCR, tested in a second trial, showed very different results: most patients developed severe toxicity, including sloughing of skin, inflamed eyes, and loss of hearing that required treatment with steroids (Johnson et al., 2009). In addition, increased antitumor efficacy was suggested with the higher-affinity TCR.

The avidity of a TCR for its target cell is determined by several contributing factors, including the number of TCRs on the surface, the density of cognate antigen on the target cell, the presence of co-receptors such as CD4 or CD8, as well as the affinity of the TCR for the peptide-MHC complex. Many basic immunology studies have shown that affinity is important for specifying T cell function, so there may be a defined affinity window that optimizes tumor recognition while avoiding the emergence of autoimmunity (Zhong et al., 2013). Studies have shown that, in humans, the affinity of TCRs targeting self-antigens often expressed on tumors cells are, on average, 30-fold lower in affinity of TCRs for foreign pathogens (Aleksic et al., 2012).

A trial is ongoing in the Netherlands, testing a TCR that binds to MART-1 and HLA-A*0201 that is not affinity enhanced. One patient died from cytokine release with a clinical syndrome like that observed with CAR T cells (van den Berg et al., 2015). Whether MART-1 is a valid target for engineered T cells remains an open question. Experiments testing a TCR redirected to the onco-fetal antigen carcinoembryonic antigen CEA were conducted in patients with metastatic colorectal cancer (Parkhurst et al., 2009). After treating three patients, although anti-tumor activity was observed in one patient, all three patients developed severe inflammatory colitis in the colon, resulting in early termination of the trial due to toxicity (Parkhurst et al., 2011).

Given the anti-tumor activity observed in the above results, cancer testis antigens have become one of the most promising classes of targets for cancer immunotherapy (Coulie et al., 2014). The first clinical trial conducted with an affinity-engineered TCR was done with the NY-ESO-1 HLA-A*02-restricted TCR developed by Jakobsen and colleagues (Zhao et al., 2007). Objective clinical responses were observed in synovial cell sarcoma, melanoma, and myeloma in the first two trials (Rapoport et al., 2015; Robbins et al., 2011). Per clinicaltrials.gov, there are currently nine clinical trials using gene-engineered T cells redirected with the affinity engineered NY-ESO-1 TCR to treat a variety of cancers, and none have reported severe toxicity.

Cross-Reactive Toxicity

However, the results have not been as optimistic in patients treated with T cells redirected to the MAGE family of cancer testis antigens. Three trials are ongoing or have been reported with three different TCRs. At the NCI, a TCR was isolated from transgenic mice vaccinated with MAGE-A3 peptides, and patients treated with T cells expressing this TCR developed severe neurotoxicity. At autopsy, extensive necrotizing leukoencephalopathy associated with T cell infiltration was observed (Morgan et al., 2013). The TCR was shown to cross-react with several homologous proteins, including MAGE-A12, which is normally expressed in human brain (Chinnasamy et al., 2011). In a second trial testing an independently derived MAGE-A3 TCR isolated from a patient that was affinity enhanced by phage display, patients died from cardiac toxicity (Linette et al., 2013). The toxicity was due to off-target recognition of an unrelated muscle-specific protein, Titin, leading to a new form of molecular mimicry (Cameron et al., 2013; Raman et al., 2016). A trial testing, T cells transduced recognizing MAGE-A4 and HLA-A24 in patients with esophageal cancer has been safe (Kageyama et al., 2015); however, no significant tumor regressions were observed.

CAR Engineered T Cells Move beyond CD19

The first CAR T cell trials for solid tumors were conducted in patients with ovarian, neuroblastoma, and kidney cancer. CAR T cells targeting folate receptor 1 alpha and CD171 in advanced ovarian cancer and neuroblastoma did not have toxicity or efficacy; however, the short persistence of the infused CAR T cells precludes interpretations on the safety of these targets (Kershaw et al., 2006). In contrast, a study using a more physiologic T cell culture methodology of patients with metastatic renal cell carcinoma testing CAR T cells directed against carbonic anhydrase IX encountered severe liver toxicity, while paradoxically, there were no objective antitumor responses (Lamers et al., 2006, 2013). There were host-directed immune responses against the CAR T cells in these trials (Lamers et al., 2011), indicating that immunogenicity will be a more serious limitation in trials not targeting B cells.

Two groups have reported trials testing CARs targeting the receptor tyrosine-protein kinase erbB-2 (also referred to as

ERBB2, human epidermal growth factor, or HER2). In a trial using a high dose of CAR T cells and a third generation signaling domain comprised of CD28 and 4-1BB, a patient died with severe toxicity consisting of cytokine storm and, potentially, on- and off-tumor target recognition of HER2 (Morgan et al., 2010). In contrast, a trial using a lower-affinity CAR targeting HER2 using a second-generation signaling domain and lower doses of T cells was safely completed with evidence of clinically beneficial antitumor effects (Ahmed et al., 2015).

Looking Forward: Lessons to Target Solid Tumors

There are several lessons from the trials to date targeting solid tumors with engineered T cells. First, specificity of the infused T cells is of paramount importance. For decades, investigators have attempted to induce tumor regression using approaches that target antigens shared on tumors and normal tissue (Pardoll, 1999), hoping that the T cells will somehow ignore the normal tissue expression. It is now retrospectively appreciated that those approaches were largely ineffective and would induce unacceptable toxicity if it were not for the nature of the low-affinity TCRs that were induced and the induction of checkpoint resistance that prevented ongoing effector activity. Thus, engineered T cells for solid tumors that will be endowed with high-affinity receptors and that will be resistant to checkpoint inhibition will require precision targeting and control mechanisms to avoid off-tumor effects while retaining on-target effects.

Second, the results from initial trials suggest that toxicity from T cells employing high-affinity TCRs is difficult to predict. Two types of toxicity may be especially problematic. First, the occurrence of cardiac destruction from an engineered T cell that acquired off-target recognition of a related peptide expressed only in muscle points to a need for improved preclinical testing methods. The large size of the human proteome expressed on the genetically heterogeneous MHC complex in humans makes this a daunting task. In physiology, the thymus screens T cells for endogenous reactivity so that presumably, TCRs that are recovered from other healthy humans can be safely deployed in patients with cancer. Various investigators are now developing screens to test for off-target recognition of engineered TCRs (Hickman et al., 2016; Stone et al., 2015). In contrast, for CARs, unexpected toxicity is less of an issue because robust technology has been developed to screen for antibody reactivity to normal tissues. The other issue with the use of engineered heterodimeric TCRs is that they can potentially pair with the endogenous TCR chains, creating novel specificity for unknown targets that were not previously selected for tolerance in the host (Bendle et al., 2010). This theoretically serious issue has not yet occurred in human trials, but it remains as a serious concern. One approach to obviate this risk is the use of various gene editing technologies to ablate the endogenous TCR (Provasi et al., 2012).

Third, as noted above, in some cases, unexpected toxicities have emerged in the trials to date with engineered T cells. This is not unexpected given the history of drug therapy, where new toxicities are often revealed, not in preclinical testing, but rather, only upon clinical testing in humans. However, to date with engineered T cells, the mechanisms for the toxicities are rapidly uncovered so that improved technologies can be developed. For example, regarding the cardiac toxicity that emerged from a MAGE A3 TCR and the molecular mechanism that was rapidly uncovered (Cameron et al., 2013). It is instructive to compare this to cardiac toxicity from anthracycline therapy, where the mechanisms that cause cumulative dose-dependent anthracycline-cardiotoxicity remain controversial and incompletely understood after more than 40 years of investigation (Gianni et al., 2008). Thus, an important and distinct advantage of engineered T cells is that when toxicities or other problems are uncovered, they can be "debugged"—their engineered sensing and response programs can be systematically and iteratively improved in a rational way.

Anti-cancer Cell Therapies Must Solve Multi-dimensional Challenges

The explosive growth of knowledge in the field of immuno-oncology, including the recent clinical experiences with engineered T cells described above, has led to a much deeper understanding of which functions a T cell must have in order to serve as an effective cancer therapy. An overarching point is that all cancers are complex multifaceted diseases (Hanahan and Weinberg, 2011), and thus, any effective T cell therapy will always need to simultaneously solve multiple functional challenges. Some of these challenges may be more important than others, depending on the type and class of cancer. Here, we review the five major classes of functional challenges that most T cell therapies will need to address (Figures 3A and 3B).

Trafficking

It goes without saying that a T cell therapy must be able to traffic to the site of the tumor cells in order to kill them. While trafficking is not a major issue for blood cancers (such as those targeted by CD19 CAR T cells), this is likely to be a more significant issue for solid tumors. Some tumors are thought to be more fibrotic and more difficult to penetrate physically, while other tumors may also suppress chemokine signaling that helps to mediate T cell infiltration. It is clear that in many patients that do not respond to other forms of immunotherapy (e.g., checkpoint inhibitors), there is often a dearth of T cell infiltration into the tumor. However, it is difficult to say how much migration and trafficking is the problem as opposed to cell proliferation and survival once the T cells enter the tumor. Some work has indicated that introduction of chemokine receptors into CAR T cells can improve their trafficking to tumors that produce the cognate chemokines (Di Stasi et al., 2009; Moon et al., 2011). However, overall, relatively little work has been done to develop more generalized strategies for improving trafficking. It seems likely that our basic understanding of immune cell chemotaxis and migration could be harnessed in novel ways to generate T cell detection and homing circuits.

Tumor Recognition and Bystander Discrimination

The ability to redirect T cell recognition to user-specified antigens represents one of the core advances of CARs. Engineered CARs and TCRs provide a way to retarget T cells, both in their activation and their cytotoxic action. Yet today, the fundamental question is no longer whether it is possible to redirect T cells to new targets (we can), but rather, whether we can identify new disease targets that provide sufficient discrimination. In nearly all T cell therapies brought to trial, there has been evidence for some cross-reaction and killing of bystander non-cancer cells.

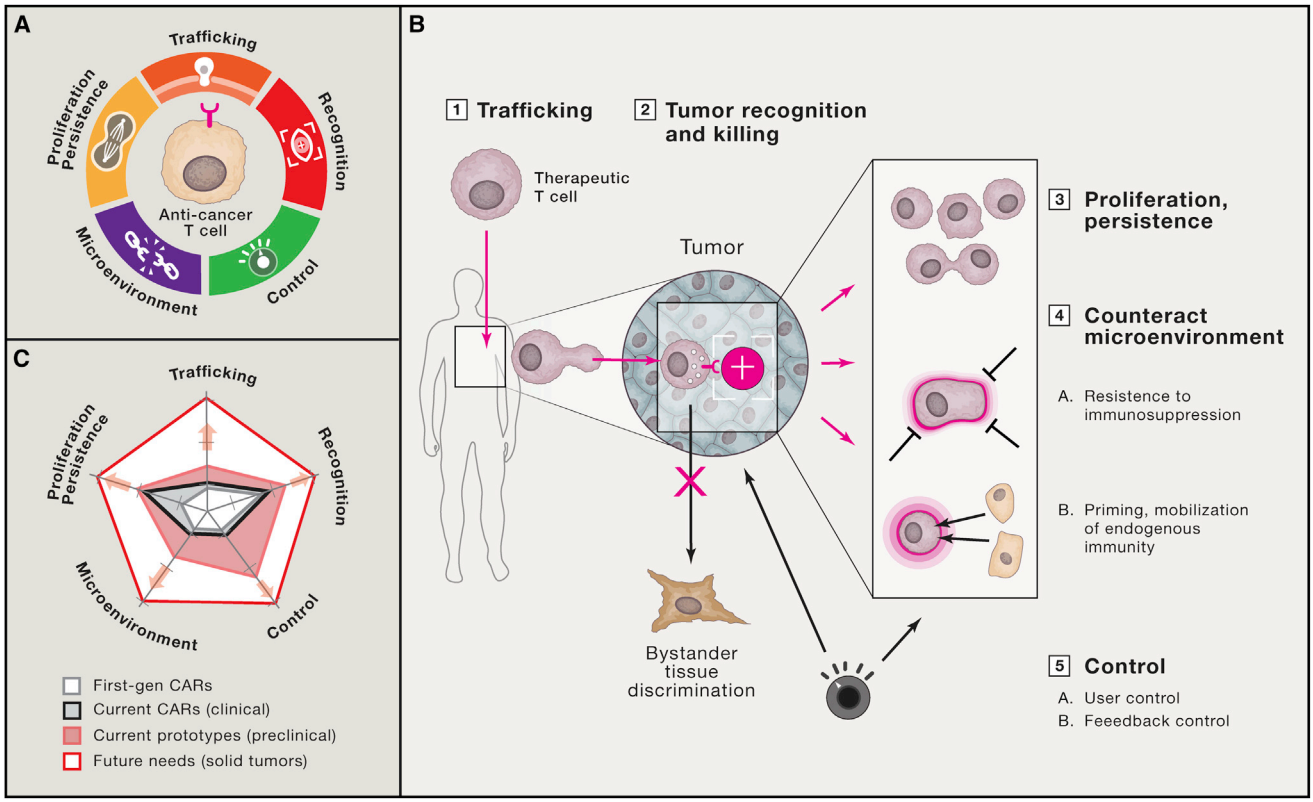

Figure 3. Functional Needs for Optimal Anti-cancer T Cell Therapy

(A) Five major functional challenges for a therapeutic T cell.

(B) Breaking down how, when, and where these five challenges arise as a therapeutic T cell interacts with cancer.

(C) Diagram of different types of therapeutic T cells and how well they address these five challenges. The distance of points from the center indicate how effective a cell is at addressing a particular challenge. The overlaid plots trace early generations of CAR T cells, as well as ongoing and projected next-generation improvements.

In the case of the CD19 CAR T cell, this cross-reaction encompasses killing of normal B cells, which also express CD19, although the resulting B cell aplasia is not life threatening. On the other hand, there have been a handful of trials involving CAR and TCR cells targeted to solid tumors, in which cross-reaction with bystander cells has led to lethal side effects caused by T cell attack of essential tissues. In principle, there are two forms of bystander cross-reaction that are possible. *ON-target OFF-tissue* cross-reaction occurs when a targeted tumor antigen (e.g., HER2) is also expressed on other tissues at a level that can still be recognized by the engineered T cell. In these cases, the antigen is simply not truly tumor specific. *OFF-target* cross-reaction occurs when the engineered receptor simply cross-reacts with an unanticipated stereochemically related antigen that is present on an essential tissue.

Thus, it remains unclear as to whether there exists for any tumor an absolutely tumor-unique "magic bullet" antigen that could be safely targeted by CARs (Klebanoff et al., 2016). There are many constraints that limit identification of an ideal single antigen target: CAR recognition is only restricted to surface antigens; many "tumor-specific" antigens are unique, but are expressed heterogeneously in only a subpopulation of tumor cells (e.g., the EGFR-viii splice variant antigen that is found in glioblastoma); finally, many overexpressed tumor antigens are expressed more widely in a number of normal tissues, albeit at lower levels. Thus, it is imperative that improved methods of recognition be developed. The doubts regarding suitably specific CAR antigens have led some to suggest that engineered TCRs, which have the potential to recognize the broader range of intracellular tumor neoantigens, might provide a better solution for tumor cell targeting (Robbins et al., 2013; Klebanoff et al., 2016). However, as we will discuss below, exciting new strategies are being developed to engineer MHC-independent T cells that recognize multi-antigen signatures or antigen densities, which have the potential to dramatically improve tumor recognition specificity.

Proliferation and Persistence

The history of CAR T cells has empirically shown the absolute clinical importance of T cell proliferation and persistence. An effective proliferative response is the best predictor of clinical efficacy. Without the addition of co-stimulatory domains in the intracellular portion of the receptor, one does not observe T cell proliferation, persistence, or clinical efficacy, either in vitro or in vivo. Simply put, the T cells need to expand to achieve the required effector:target ratio to eliminate the tumor in vivo. In the case of the CD19 CAR T cells, when the T cells

are infused, they rapidly encounter CD19 targets and begin to be activated and to proliferate. Yet what happens in solid tumors? How can we ensure that sufficient proliferation takes place to accumulate enough T cells to eliminate the tumor? Moreover, how can we ensure that cells persist for the weeks or more time that could be required to eliminate a tumor? Improvement of proliferation is, thus, a major focus area of current research.

Overcoming the Suppressive Microenvironment

Particularly for solid tumors, it is clear that only focusing on targeting a T cell population to the tumor is unlikely to be sufficient for clinical efficacy. Many solid tumors have an immunosuppressive microenvironment. In cases where tumors downregulate antigen presentation, it is possible that engineered T cells targeted to tumor-specific antigens would be effective. However, in cases where the tumors produce an immunosuppressive environment that directly downregulates T cells (Joyce and Fearon, 2015), it is likely that additional functionality would be required to allow engineered T cells to persist, proliferate, and effectively execute their cytotoxic program. Thus, a major question is whether there are ways to engineer T cells that are resistant to the suppressive microenvironment. In addition, there is also the opportunity to engineer T cells that more proactively remodel the microenvironment to prime the endogenous immune system, enhancing its ability to recognize and respond to the tumor (i.e., for the engineered T cell to act, in part, like a vaccine).

Control Mechanisms

So far, most efforts at T cell engineering have been focused on redirecting their targeting to tumor antigens and enhancing the strength of their response and proliferation. However, in almost all clinical trials, there have been serious adverse effects—some tolerable, and others lethal—indicative of the incredible power of activated T cells. Moreover, as we begin to engineer T cells with the enhanced proliferation and activity that is likely to be required for solid tumors, the potential for adverse side effects will increase. Thus, although less effort has been put into learning how to control the amplitude and timing of T cell activity, such regulatory capabilities are now appreciated as increasingly important. After all, much of the natural regulatory machinery that has evolved in T cells is used to keep T cell activity in check.

Control is a major issue because T cells are autonomous—once they are transferred to the patient, there is little that can be done to control them. Thus, researchers are developing a growing number of *user-control* regulatory strategies that will potentially allow a physician to modulate the survival of T cells, as well as the timing, strength, and location of their activity. Looking even further ahead, it may ultimately be possible to engineer *feedback control systems* into therapeutic T cells, which allow them to autonomously monitor when adverse outcomes reach a critical stage.

Different Cancers, Different T Cell Needs

Any effective T cell therapy will need to address the above five functional areas shown in Figure 3C, where the distance along each radial line represents how potent a T cell therapy is at addressing each of the functional areas. However, since every cancer is different, it is possible that particular facets will be more critical for each target disease (Teng et al., 2015). Identifying the most critical challenges that must be overcome for each cancer type will be important—the size of genetic payloads that can be inserted into T cells is currently limited. A major constraint will be determining how to most efficiently use the payload so that the T cells are equipped with the most essential capabilities.

Early CAR T cells focused unidimensionally on targeting tumor antigens and little else; hence, they were ineffective (Figure 3C). However, the current generation of CD19 CAR T cells, by incorporating co-stimulatory motifs into their receptors, improved cell proliferation and persistence, leading to clinical efficacy. Current preclinical prototypes of engineered T cells are now expanding capabilities in many of these functional dimensions, but we anticipate that the most challenging solid tumors will require significantly enhanced function in all of the dimensions. Below, we discuss some of the areas in which significant new capabilities have been developed, and we discuss how they may help shape the next generation of therapies.

Improving Recognition: New Receptors and Recognition Programs

Exciting new developments in engineering more sophisticated recognition receptors and recognition circuits may lead to dramatic improvements in our ability to design therapeutic T cells that can effectively recognize target tumor cells yet discriminate against normal cells (Figure 4A). As mentioned above, advances in engineering TCRs or CARs that recognize peptide MHC complexes are providing powerful new ways to potentially recognize a broader range of intracellular tumor neoantigens, although this recognition will be restricted to specific MHCs. Here, we focus on advances in engineering CAR-based circuits that improve recognition specificity by integrating information about multiple antigens: combinatorial antigen recognition. Bioinformatic analysis suggests that even recognizing relatively simple combinations of two or three antigens would dramatically improve the capability for discriminating most tumor cells from normal tissues (Lim and Troyanskaya, personal communication).

AND-Gate Circuits: Recognition of Multi-antigen Combinations

Several strategies have emerged to engineer CAR T cells that require a specific combination of two or more antigens for activation. One general strategy has been to express two separate CARs—one for each target antigen—but where one receptor bears the CD3zeta signaling chain of the TCR and the other bears the co-stimulatory motif (Kloss et al., 2013; Lanitis et al., 2013; Wilkie et al., 2012). The rationale here is that full activation, including proliferation, will only take place when both receptors are engaged at the immune synapse, not unlike normal TCR + co-stimulatory signaling. While such dual CAR AND-gates can indeed show much stronger activation by cells expressing both target antigens, there is often significant activation with a single antigen alone, and tuning of antigen expression and affinity is often required to observe strong discrimination between dual- and single-antigen target cells.

A newer strategy for AND-gate recognition involves a two-receptor circuit in which activation of one receptor induces the expression of a second receptor (a CAR or TCR) that mediates cell killing. Only when both antigens are present does the priming and T cell activation occur in a sustained manner. This kind of priming function can be executed by using the recently developed synthetic Notch receptor (Morsut et al., 2016; Roybal

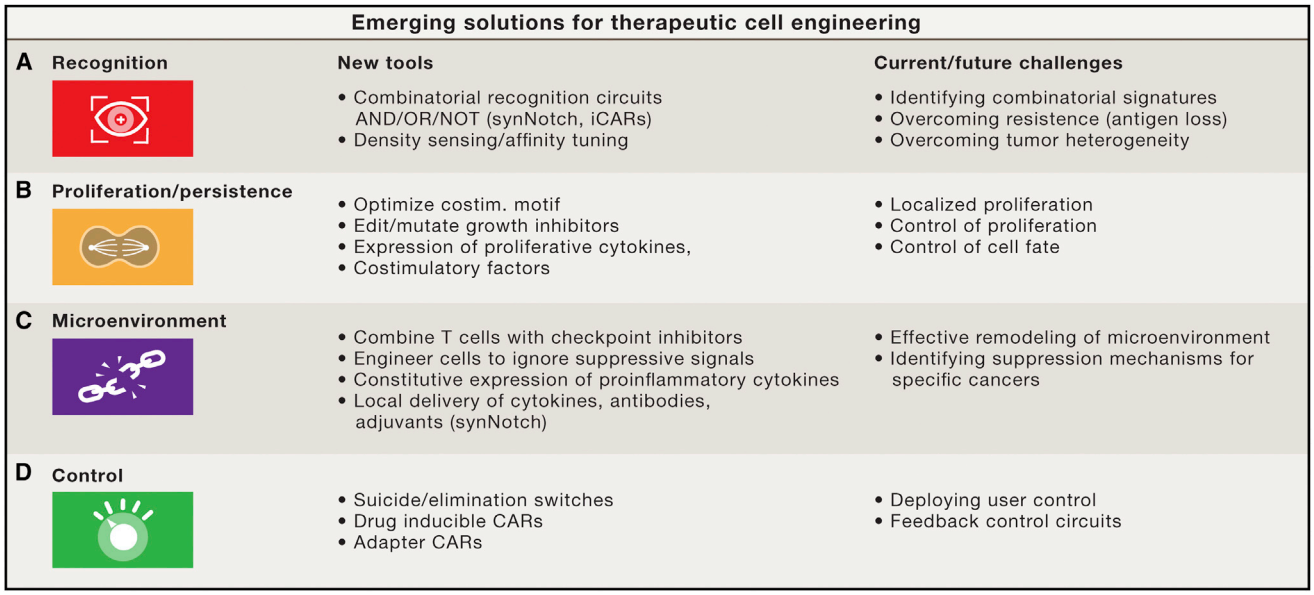

Figure 4. Emerging New Engineering Solutions for Addressing the Functional Challenges of Anti-cancer T Cells
(A–D) New molecular tools for improving therapeutic T cell tumor recognition (A), proliferation and persistence (B), remodeling of the tumor microenvironment (C), and control (D).

et al., 2016a). This powerful new class of chimeric receptors recognizes a target surface antigen, but upon engagement, the class of receptors activates transcription. synNotch receptors contain the regulatory transmembrane region of Notch but bear a novel extracellular recognition domain and a novel intracellular transcriptional activator domain. Like Notch, when the synNotch receptor engages its ligand on an opposing cell (juxtracrine signaling), this induces intramembrane cleavage, releasing the intracellular transcriptional domain, allowing it to enter the nucleus to activate transcription of target genes. Prototypes of this type of synNotch → CAR priming-killing dual-antigen recognition circuit have shown robust discrimination between dual- and single-antigen tumors, clearing a dual-antigen tumor while leaving the single-antigen bystander tumor in the same animal virtually untouched. Similar preclinical results have been observed in synNotch circuits that drive expression of other targeted cytotoxic proteins, such as bispecific antibodies rather than CARs (Roybal et al., 2016b). Identification of combination antigens that can be targeted by such AND gate circuits provides a promising avenue for far more precise recognition that will be required for solid tumors.

NOT-Gate Circuits: Negative Discrimination against Normal Cell Antigens

It may also be possible to achieve more precise tumor recognition by minimizing the ability of T cells to mistarget cross-reactive cells. One promising strategy to achieve this is through engineering the T cells to override activation when they encounter a cell that has a positive targeting antigen but also a negative antigen that is present on bystander cells, but not the cancer cells. An elegant strategy for constructing this kind of NOT-gate circuit involves combining a CAR for one antigen (the killing antigen) with an inhibitory CAR (iCAR) for a second antigen, the overriding antigen (Fedorov et al., 2013). iCARs have an extracellular antigen recognition domain, but their intracellular domains, instead of containing activating TCR derived domains, have the signaling domains from immune inhibitory receptors such as PD-1 and CTLA-4. When a T cell expressing both the CAR and the iCAR encounters a cell expressing only the CAR antigen, the T cell kills the target, but when both the CAR and iCAR antigens are encountered at the same immune synapse, the iCAR negative regulatory signaling will override or dampen the CAR signaling, resulting in poor T cell activation. Such NOT gates could, in principle, achieve specific recognition through negative discrimination of non-cancer signals.

OR-Gate Circuits and Dual-Antigen Targeting CARs

A number of researchers have now also constructed CARs that contain two independent antigen recognition domains (Grada et al., 2013; Zah et al., 2016). Interestingly, these dual-headed CARs appear to serve as OR-gates, in that they can be activated by two different antigen ligands. This kind of OR-gate functionality might be particularly useful for preventing the development of resistance through loss of the target antigen. For example, CARs that target two B cell specific antigens CD19 or CD22 (or CD19 or CD20) appear to be less sensitive to resistance via loss of CD19. The detailed geometry for optimal target recognition is still poorly understood, and the configuration of the receptor and location of the recognized antigen epitope may determine how effective dual-targeting CARs can be.

Affinity-Tuned Receptor: Improving Sensing of Antigen Densities

Another approach for improving recognition specificity is to enhance CAR T cell discrimination of cancer from normal cells based on antigen density. Common oncogenic proteins such as the surface antigen HER2 are highly overexpressed in many

cancers, but HER2 is expressed at lower levels in a number of normal epithelial tissues. Most CARs have been built based on high-affinity therapeutic antibodies, like the HER2 antibody trastuzumab (Herceptin). Not unexpectedly, such high-affinity CARs can be relatively poor at density discrimination, as they are triggered by normal cells expressing lower levels of the HER2 antigen. Improved discrimination based on HER2 expression levels can be achieved, however, by affinity tuning the receptor—CARs built using anti-HER2 scFv's, with affinities reduced by several orders of magnitude, show significantly improved ability to discriminate between high HER2 or EGFR cancer cells and low HER2 or EGFR normal cells in mouse models (Caruso et al., 2015; Liu et al., 2015). The idea that the highest affinity recognition domains may not make the most ideal receptors seems obvious in hindsight, but to harness this, we will need to screen for antibodies that have lower affinities but still maintain high epitope specificity, a goal that is challenging given current methods for antibody screening.

Driving Proliferation and Persistence

The proliferation and persistence of CAR T cells in vivo correlates with durable remission of leukemia (Porter et al., 2015). Thus, different ways to stimulate proliferation present an important opportunity to improve the function of adoptively transferred T cells.

Incorporation of Costimulatory Domains

CAR T cells expressing a zeta chain in the absence of a costimulatory domain have been shown to persist for more than a decade without adverse effects after adoptive transfer in patients with acquired immunodeficiency (Mitsuyasu et al., 2000; Scholler et al., 2012), providing a proof of concept that engineered T cells can engraft and persist for years with safety in humans. However, in cancer patients, CAR T cells expressing the same intracellular signaling domain in the absence of costimulation had only brief persistence (Kershaw et al., 2006), likely due in part to the toxic nature of the tumor microenvironment (Rooney et al., 2015).

To date, the recent successes with CAR T cells in the clinic have been with CARs that incorporate CD28 or 4-1BB costimulatory domains. In preclinical studies, these CARs induced more IL-2 secretion, increased T cell proliferation, and mediated greater tumor rejection. In patients with refractory ALL, both CAR designs have similar rates of remission; however, some differences in the clinical responses are emerging. The time until the onset of fever and the rapidity of remission induction appear to be earlier with the CD28 CARs. In contrast, the persistence of T cells expressing 4-1BB CARs is greater than in patients treated with CARs expressing CD28 signaling domains. Thus, it is possible that CD28-based CARs may be better for remission induction or as a "bridge to transplant," while 4-1BB based CARs may be more useful as a definitive therapy or to serve for immunosurveillance to prevent tumor recurrence, where the CARs persist beyond the initial tumor elimination. However, with the advent of switchable CAR technologies, it is possible that a single T cell infusion could accomplish both features, i.e., rapid tumor elimination and long-term persistence with immunosurveillance.

In addition to CD28 and 4-1BB, many other costimulatory domains, including CD27, OX40, and ICOS, have been incorporated into CARs to further enhance the costimulatory properties (Guedan et al., 2014; Hombach et al., 2013; Song et al., 2012). ICOS costimulation drives human T cells to a Th17 cell phenotype (Paulos et al., 2010), and in certain models, Th17 cells are preferable for adoptive transfer (Muranski et al., 2008). Signaling modules derived from the innate immune system such as MyD88 and CD40 have also been used in CAR domains (Narayanan et al., 2011). Inducible CARs with split signaling domains have been developed with a MyD88 and CD40 costimulatory switch that provides user-controlled induction of CAR T cell proliferation and the potential for regulated long-term CAR T cell engraftment (Spencer et al., 2016).

Considerations on Human T Cell Replicative Lifespan

There are fundamental differences in the regulation of immunosenescence and replicative capacity in T cells of mice and humans that have important implications for adoptive cell transfer (June, 2007). p53 isoforms regulate T cell senescence in human, but not mouse, T cells (Mondal et al., 2013). Human T cells have finite clonal lifespans in vitro, and human naive CD4+ T cells have telomeres that are, on average, 1.4 kb longer than those of human memory T cells (Weng et al., 1995). Subsets of human T cells with longer telomeres may be preferable for adoptive therapy (Fritsch et al., 2005; Pulko et al., 2016), as studies have shown that telomere length correlates with persistence and antitumor efficacy in melanoma patients after TIL therapy (Zhou et al., 2005). Gene transfer can enhance telomere length in T cells (Rufer et al., 2001), which may be an attractive strategy for adoptive transfer, particularly in elderly patients with a limited telomere reserve. In addition, CD28 costimulation can enhance telomere lengths in T cells (Barrett et al., 2014), and restoration of CD28 expression has been proposed as a strategy to regenerate senescent T cells (Topp et al., 2003). IL-15 activates telomerase activity in human memory CD8+ T cells (Li et al., 2005) and, when expressed in T cells, promotes a stem cell memory phenotype (Hurton et al., 2016).

Modulation of T Cell Exhaustion

Under conditions of chronic antigen exposure and inflammation, T cells become functionally incapable of performing effector activities, a condition now termed T cell exhaustion (Wherry, 2011). T cell exhaustion and senescence are often used interchangeably; however, they are now considered as distinct states of differentiation (Akbar and Henson, 2011; Crespo et al., 2013). Increasing evidence indicates that tumor-specific T cells in many patients are in various states of exhaustion (Lee et al., 1999; Schietinger and Greenberg, 2014). Studies indicate that T cells acquire characteristics of exhaustion early in tumorigenesis and that, in advanced states, the exhaustion is irreversible (Schietinger et al., 2016). A hallmark of the exhausted state is that the T cells eventually lose the capacity to proliferate (Im et al., 2016). Interruption of PD-1 ligand binding early in the process can restore T cell function and tumor targeting, although rescue may be incomplete or transient (Schietinger et al., 2012). In mice, the adoptive transfer of PD-1-deficient T cells leads to enhanced function and resistance to exhaustion (Odorizzi et al., 2015).

Recent studies suggest that exhausted T cells represent a distinct lineage of lymphocytes that is different at the transcriptional and epigenetic levels from T_{CM} and T_{EM} subsets of

memory T cells (Peng et al., 2015; Roychoudhuri et al., 2016). The transcriptional status of exhausted and tumor-tolerant T cells differs from healthy T_{CM} and T_{EM} cells; however, the gene signature reports differ, likely reflecting tumor-specific effects (Schietinger et al., 2016; Singer et al., 2016). The transcription factor Bach2 may have a central role in the differentiation process toward exhaustion (Richer et al., 2016; Roychoudhuri et al., 2013). Exhausted human and mouse T cells have sustained expression of PD-1 that is regulated by an exhaustion-specific enhancer that contains motifs from RAR, T-bet, and Sox3 (Sen et al., 2016), and the stability of the epigenetic changes leads to resistance to PD-1 antagonists (Pauken et al., 2016). These results suggest that genetic and epigenetic modulation of T cells has the potential to delay or prevent the induction of exhaustion by tumors, presenting a large opportunity for the field of cellular engineering.

Reinforcing Lymphocyte Metabolism

T cell subsets cycle through states of metabolic quiescence and activation. Mature naive T cells are quiescent cells that primarily oxidize glucose-derived pyruvate in their mitochondria via oxidative phosphorylation or fatty acid oxidation to generate ATP. Upon TCR activation and costimulation, the naive T cells undergo metabolic remodeling and a switch to aerobic glycolysis to convert to rapidly dividing effector T cells (Fox et al., 2005). In contrast, memory T cells undergo mitochondrial biogenesis and a switch to oxidative metabolism (van der Windt et al., 2012). The changes can be detected by ultrastructural analysis: effector T cells have small distinct mitochondria, an indication of mitochondrial fission, whereas memory T cells have densely packed fused mitochondria (Buck et al., 2016). Mitochondrial membrane potential is a marker of cells with enhanced stemness and function after adoptive transfer (Sukumar et al., 2016).

Tumors can inhibit antitumor immunity by nutrient depletion. In the tumor microenvironment, there is a competition for glucose between tumors and T cells (Chang et al., 2015). In glucose-limiting microenvironments, T cells have insufficient phosphoenolpyruvate (PEP), and T cells with genetically increased PEP production have enhanced effector functions and restricted tumor growth in mice (Ho et al., 2015).

T cells not only rely on glucose but also depend on amino acids for survival and function. Depletion of glutamine in culture medium blocks T cell proliferation and cytokine production (Carr et al., 2010), and it is likely that glutamine competition in the tumor microenvironment also influences their anti-tumor function. In natural T cells, glutamine and glucose import are CD28 dependent (Carr et al., 2010; Frauwirth et al., 2002). Initial studies indicate that T cell engineering enables the installation of desired metabolic phenotypes. CAR T cells with CD28 signaling domains have enhanced aerobic glycolysis, while CAR T cells with 4-1BB signaling domains have enhanced mitochondrial biogenesis and increased fatty acid oxidation (Kawalekar et al., 2016).

L-arginine is considered as a conditionally essential amino acid. Recent studies indicate that L-arginine has an important role in regulating T cell metabolism, as supplementation of the culture medium with L-arginine resulted in decreased protein expression of glycolytic enzymes, while Krebs cycle and serine biosynthetic pathway enzyme expression was increased (Geiger et al., 2016). T cells cultured with increased L-arginine promoted T_{CM} differentiation and increased arginine levels in the culture medium also resulted in enhanced T cell survival and improved antitumor activity. The effects of L-arginine on T cell survival were mediated in part by the nuclear proteins BAZ1B, PSIP1, and TSN, as CRISPR/Cas9-mediated deletion of these genes was sufficient to abrogate the effects of arginine on T cell survival.

A recent study showed that adenosine, which is a byproduct of metabolic activity and is enriched in the tumor microenvironment, suppresses TCR signaling in a dose-dependent manner (Cekic et al., 2013). The level of potassium ions, the main intracellular cation, in the tumor microenvironment is five to ten times higher than those encountered by T cells in the bloodstream. Potassium released from dying tumor cells potently inhibits T cell activation, and enhancing the removal of potassium from T cells by overexpressing the voltage-gated K+-channel protein Kv1.3 (encoded by the KCNA3 gene) or the calcium-activated K+-channel protein KCa3.1 (encoded by the KCNN4 gene) restores their antitumor activity (Eil et al., 2016). Thus, the decreased availability of certain amino acids and the accumulation of metabolic waste products act in concert to alter the microenvironment and adversely influence T cell function.

The epigenetic state of T cells can modulate T cell proliferative capacity, at least in part through cellular metabolites. Gain-of-function mutations in isocitrate dehydrogenase 1 or 2 lead to accumulation of the oncometabolite 2-hydroxygluatarate. Recent studies show that the S enantiomer of 2-hydroxygluatarate can inhibit T cell effector differentiation, an effect mediated by the epigenetic modifiers Utx and Tet2 (Tyrakis et al., 2016). TCR triggering induces a loss of 5hmC in genomic DNA of T cells, and adoptively transferred T cells supplemented with S-2-hydroxygluatarate have enhanced proliferative capacity and antitumor effects. Thus, improving T cell fitness through control of cellular metabolism has emerged as a key goal in the design of adoptive cellular immunotherapies.

Remodeling the Microenvironment

Perhaps the largest unmet need in engineered anti-cancer T cells is the ability of the cells to overcome or remodel the immunosuppressive microenvironment found in many solid tumors (Figure 4C). Even if an engineered T cell can traffic to and precisely recognize tumor cells, if they are efficiently downregulated by a suppressive microenvironment, they will not be able to effectively attack the cancer. An additional confounding issue is that tumors are heterogeneous in nature; there are likely many different ways to create a immunosuppressive microenvironment, and appropriate countermeasures may need to be tumor specific.

Combination Therapy with Checkpoint Inhibitors

An obvious first way to address this problem is by taking advantage of checkpoint inhibitor antibodies, such as anti-PD-1, PD-L1, and CTLA-4 (and others in development), that have been successful in a significant fraction of melanoma and lung cancer patients. It is possible that some of the non-checkpoint responding patients simply lack an endogenous T cell population that can recognize the tumor, even after the checkpoint proteins are inhibited. Thus, combining engineered CAR T cells with checkpoint inhibitors makes a great deal of sense, and initial

trials appear promising, where PD-1 or PD-L1 antagonists are being co-administered with engineered T cells (Chong et al., 2016), based on synergy observed in pre-clinical models (John et al., 2013).

Engineering Cells to Ignore Suppressive Signals

Several efforts have been made to engineer T cells that are inert to potential suppressive signals. For example, to reduce suppression by TGFbeta, one can express a dominant-negative form of the TGFbeta receptor in T cells (Foster et al., 2008). Similarly, chimeric receptors have been engineered that can reduce suppression by the checkpoint protein ligand, PD-L1. In this case, the extracellular domain of the checkpoint receptor PD-1 has been fused to intracellular costimulatory domains, leading to a receptor that will lead to enhanced T cell activity when it engages the normally suppressive PD-L1 signal (Prosser et al., 2012). Finally, several recent efforts have been made to use CRISPR genome editing to remove the PD-1 receptor from T cells, rendering them non-responsive to PD-L1-mediated suppression (Ren et al., 2016; Schumann et al., 2015). These types of non-suppressive T cells appear to function well and appear to show enhanced anti-cancer cell activity. Nonetheless, these suppressive pathways may play an important role in modulating and downregulating T cell function, especially after a potent response has been mounted, and it is unclear whether such strategies may lead to increased challenges with control. There are many checkpoint molecules that are induced on activated T cells that limit their effector functions (Mahoney et al., 2015), and genetic editing tools permit the efficient disruption of these molecules (Ren et al., 2016). However, it is likely that unexpected toxicities will be encountered, as, for example, when ipilimumab was coadministered with an inhibitor of B-raf (Ribas et al., 2013).

Equipping T Cells with the Ability to Remodel the Microenvironment

Another general strategy is to equip the engineered T cells themselves with new capabilities to counteract the suppressive microenvironment. For examples, so-called ''armored'' CAR T cells constitutively express the potent cytokine IL-12 (Kerkar et al., 2010; Pegram et al., 2012). IL-12 is one of the most potent anti-cancer cytokines, which acts through pleiotropic action on both innate and adaptive immune cells and thus can be a powerful agent to remodel a tumor microenvironment. TILs with engineered NFAT-inducible IL-12 have antitumor activity in melanoma and severe toxicity (Zhang et al., 2015). CAR T cells expressing IL-18 may be a safer version of ''armored'' CARs and TILs. In a recent example, constitutive CAR T cells have been designed that secrete a soluble form of HVEM and remodel the tumor microenvironment (Boice et al., 2016).

More recently, new strategies for delivering agents that can remodel the microenvironment have been developed. For example, the synNotch receptor system described earlier can be used to engineer T cells that produce specific secreted payloads in response to recognition of a target antigen (Roybal et al., 2016b). Thus, the T cells can in principle be programmed to serve as local delivery agents, or ''pharmacytes.'' synNotch T cells can be engineered to locally express a range of interesting payloads, including IL-12, other pro-inflammatory cytokines, checkpoint antibodies, and bispecific antibodies. They can also be engineered to produce adjuvants (e.g., flaggelin) that are expected to stimulate the innate immune response, thus potentially augmenting the host-mediated immune response. Given the flexibility of the synNotch system, it may be possible to drive the delivery of many different payloads, customized for the needs of a particular cancer type. Some of these payloads could act in concert with CAR-killing activity or might act independently to remodel the environment and engage the native host-immune system. Significantly, the localized production of secreted factors driven by synNotch cells might avoid the toxicities observed with systemic or constitutive production of potent factors such as IL12. Such strategies, while promising, are still untested in the clinic.

Engineering Tighter Control Systems in Therapeutic T Cells

Control systems that can increase the safety of therapeutic T cell treatments have now become a clinical priority. Many control switches are focused on user-control, whereby through the addition of a small molecule or biologic, a physician can negatively or positively regulate T cell function (Figure 4D).

Suicide or Elimination Switches

The earliest types of control systems developed have been suicide or elimination switches, which can be triggered by physicians to eliminate T cells that are mediating overly toxic effects. One suicide switch, called iCasp9, has been clinically validated in limiting graft versus host disease in patients undergoing hematopoietic stem cell transplants (Di Stasi et al., 2011). iCasp9 has a split form of the apoptotic protein Caspase, which must be homodimerized to become active. In this construct, addition of a small molecule triggers assembly and apoptosis. Another strategy for removing T cells involves expressing a tag that can be used for elimination: expression of the extracellular domain of EGFR allows the T cells to be eliminated by adding the cognate antibody cetuximab (Wang et al., 2011).

Drug-Controlled ON-Switch CARs

A distinct class of control strategies involve engineering T cells that are inactive but then can be switched on by the addition of specific activating agents. For example, a drug inducible version of a CAR can be constructed in which the recognition scFV and the signaling domains (CD3z and costimulatory motifs) are on separate polypeptides, with each containing a partner drug-inducible heterodimerization domain. The split CAR is inactive until the heterodimerizing drug is added, assembling a fully functional receptor. The activity of this split ON-switch CAR can be rapidly titrated and reversed, allowing in principle for a high level of physician remote control (Wu et al., 2015). New constructs optimized for clinical use can be controlled by FDA approved drugs (W.A.L., unpublished data).

Adaptor-Mediated CARs

Adaptor mediated CARs require the addition of an adaptor molecule to target it to the cancer. For example, a CAR with an Fc receptor domain can be targeted to a specific cancer by addition of an antibody that targets a cancer antigen so that the Fc receptor domain will bind the antibody (Kudo et al., 2014). More orthogonal versions of such adaptor CARs have been engineered in which the CAR has a binding domain that recognizes a cognate ligand or peptide (Ma et al., 2016; Rodgers et al., 2016). In this case, targeting antibodies can be converted into highly specific

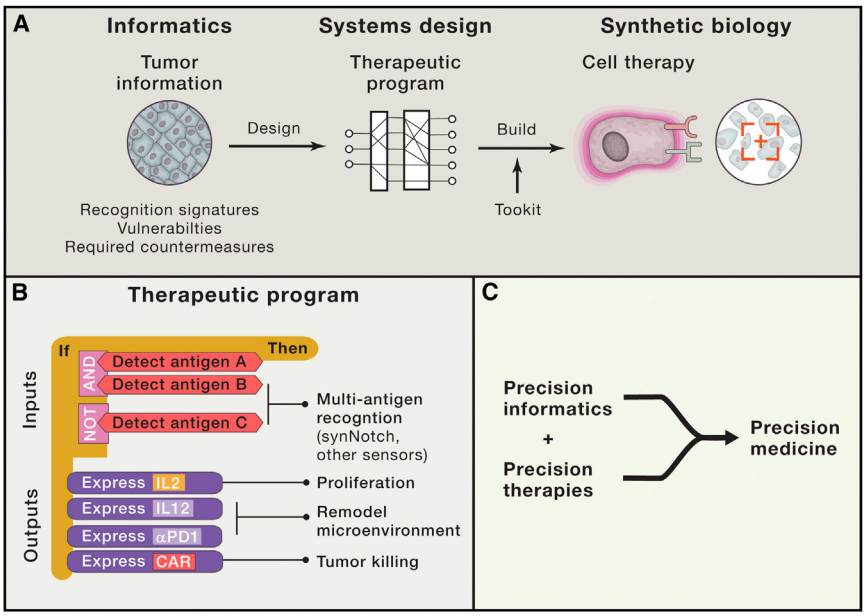

Figure 5. Smart Cell Therapies May Fulfill Promise of Precision Medicine

(A) The design and implementation of therapeutic immune cells will, in principle, combine tumor informatics with systems and synthetic biology to construct cell therapies strategically optimized to recognize discriminating features and to attack tumor vulnerabilities.

(B) The emerging synthetic biology toolkit for cell engineering may allow modular construction of precision therapeutic programs (here modeled by an illustrative program inspired by the Scratch graphical programming language).

(C) Precision informatics combined with custom engineered therapeutic cells has the potential to provide true precision therapies.

adaptor molecules by fusing them to the cognate ligand or peptide. With this strategy, the CAR T cell should be inert until the adaptor antibody is added. An advantage of these adaptor-mediated CARs is their targeting flexibility; they could be retargeted to different targets based on what adaptor antibody is used.

Feedback Control

Most efforts in engineering control over therapeutic T cells have focused on providing ways for the physician to intervene and either eliminate or modulate the T cell function in response to potential side effects. However, ultimately, it may be most powerful to engineer autonomous feedback circuits into T cells that homeostatically control their activity. Full-on maximal effector activity may not be optimal both in eliminating the cancer and certainly not in terms of minimizing adverse effects. For example, activation responses that reach a setpoint or that are pulsatile and dynamically controlled may be optimal for anti-tumor effects. In addition, it would be desirable to engineer feedback control based on signs of toxicity. For example, IL-6 is a major marker of cytokine release syndrome, and it might be possible to engineer synthetic downregulatory feedback circuits that respond autonomously to an excess of IL-6 or other signatures of hyperactivity. Such feedback circuits, which are the subject of much interest in the fields of systems and synthetic biology, have largely been unexplored in the context of therapeutic T cells and represent an area of future growth.

Conclusion: Vision of Immune Cell Therapies in Precision Medicine

The last few years have been an era of exuberance in cancer immunotherapy in general and in the use of engineered immune cells more specifically. At present, the power of T cells as a therapeutic is remarkable, as well as the fact that we can redirect them, but this early clinical experience has revealed the major challenges that must be met to make engineered immune cells a reliable, safe, and effective platform for treating cancer, especially in the realm of solid tumors. Treating cancers is a complex multifactorial problem, in which multiple problems must be simultaneously addressed. Moreover, individual cancer types present different challenges, and thus, the types of engineered behaviors that they need will be different.

Fortunately, the rise of immunotherapy coincides with the maturation of the fields of synthetic biology and genome engineering, and thus, powerful tools and approaches, outlined here, are being developed to address the engineering needs of T-cell-mediated cancer therapy. It is likely that engineered cell therapies will be one of the major testbeds for the application of synthetic biology.

We envision that an array of modular genetically encoded tools will be developed that will allow cell engineers to address the array of functional challenges outlined here. This toolbox will include new molecules (e.g., sensors, switches, etc.) that can be deployed together in different types of circuits to execute sensing-response behaviors that are optimized for the target cancer (Figure 5).

A therapeutic T cell can provide far more multi-faceted actions than a targeted molecular therapeutic. Engineered cells also have the advantage that their response programs can be systematically debugged and improved in an iterative fashion as toxicities and issues arise, potentially providing a more stable risk profile than the development pipeline normally associated with traditional small molecule therapeutics (Fischbach et al., 2013).

Therapeutic immune cells are thus one of the first examples of precision therapeutics (Figure 5A). We have entered an era of remarkable precision bioinformatics, in which we are beginning to access highly detailed information about tumors—which antigens are present, which types of suppressor molecules are present, and how these are distributed in the different cells that make up the tumor ecosystem (Tirosh et al., 2016). We now have the potential to use informatics data to design optimized therapeutic response systems that harness discriminating molecular features and attack the vulnerabilities of a tumor. We can also construct these systems using our emerging synthetic

biology toolkit (Figure 5B). The concept of precision medicine will ultimately be even more fulfilling if we can successfully combine precision informatics with precision cell therapies that make this information more directly actionable (Figure 5C).

Finally, although we have limited our discussion here only on using engineered immune cells to treat cancer, the principles discussed here could also be used to design cell therapies targeted to treat other diseases. Autoimmunity, infection, inflammation, degeneration, and fibrosis are all examples of diseases that, like cancer, could dramatically benefit from a smart cell therapy that can recognize and locally respond to complex tissue pathologies (Fischbach et al., 2013).

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