CELL THERAPY

Harnessing regulatory T cells to establish immune tolerance

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Engineered regulatory T (T_{reg}) cells have emerged as precision therapeutics aimed at inducing immune tolerance while reducing the risks associated with generalized immunosuppression. This Viewpoint highlights the opportunities and challenges for engineered T_{reg} cell therapies in treating autoimmune and other inflammatory diseases.

INTRODUCTION

The immune system must defend the body against an enormous array of foreign agents without inadvertently attacking self-tissues. This rapid, specific, and powerful immune cascade is checked by redundant mechanisms that have evolved to control autoreactivity, known collectively as "self-tolerance." When self-tolerance breaks down, as in autoimmunity, it can unleash an aberrant response against self or otherwise innocuous environmental or food-borne antigens. Left unchecked, the persistence of self-immunoreactivity leads to chronic inflammation and tissue damage.

The ability to preserve immune self-tolerance requires concerted processes during immune cell development (central tolerance) and throughout life (peripheral tolerance). In particular, immune homeostasis is highly dependent on a small population of $CD4^+FoxP3^+$ regulatory T (T_{reg}) cells, because nonsense mutations in the lineage-defining *FOXP3* gene result in defective T_{reg} development and cause a severe and lethal inflammatory disorder termed immune deficiency, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome. The importance of T_{reg} cells in establishing tolerance underscores their potential as a therapeutic for diseases where tolerance breaks down. In this Viewpoint, we provide a roadmap for developing such T_{reg}-based therapies, focusing on the scientific, regulatory, and clinical challenges along the path toward translation.

T_{REG}-MEDIATED TOLERANCE: HISTORY AND MECHANISMS

Although the phenomenon of immune self-control dates to the late 1960s (*1*), T_{reg} cells were not unequivocally identified until 2003, when the Sakaguchi, Rudensky, and Ramsdell laboratories independently demonstrated that mutations in a single gene, *Foxp3*, abrogated T_{reg} development and led to spontaneous development of fatal multi-organ autoimmunity in scurfy mice (*2*–*4*). Importantly, these investigators showed that autoimmune disease was a direct consequence of a T_{reg} deficiency, given that the infusion of FoxP3-replete $CD4^+CD25^+$ T cells was sufficient to restrain autoreactivity (*2*–*4*). It is important to note that CD4 and CD25 [the IL-2 receptor α (IL-2Rα) chain] had previously been used to identify a regulatory population of T cells in mice. Around the same time, nonsense mutations in the human *FOXP3* gene were linked to IPEX syndrome, which is strikingly similar in clinical presentation to scurfy in FoxP3-deficient mice (*5*, *6*). Because of these seminal studies, the field of immune regulation has gained increasing traction, with an ever-expanding constellation of cells capable of mediating many of the same regulatory functions. However, to date, $FoxP3^+$ T_{reg} cells remain the only population that has been genetically proven to be essential to maintain immune tolerance in both rodents and humans.

T_{reg} cells have multiple attributes that underlie their potential as therapeutics. They exhibit diverse inhibitory activities that enable this relatively small cell population to exercise efficient control over entire immune cell networks (*7*). This includes the production of suppressive cytokines, such as interleukin-10 (IL-10) and transforming growth factor–β (TGF-β); consumption of IL-2 that is essential for effector T (T_{eff}) cell development; degradation of proinflammatory adenosine 5′-triphosphate; and inactivation of antigen-presenting cells (APCs) by delivering negative signals such as indoleamine 2, 3-dioxygenase (IDO) and removing key activating molecules, such as CD80, CD86, and major histocompatibility complex (MHC) class II by trogocytosis. These diverse functions lead to both direct and bystander suppression, alter the inflammatory microenvironment, and elicit other immunosuppressive cell types to amplify and prolong the tolerogenic effects (*7*, *8*). In fact, T_{reg}-mediated reprogramming of local environments and resident cell communities can persist long after tolerance is initially established, a unique activity termed "infectious tolerance" (*8*). In their seminal study, Qin and colleagues (*9*) showed that adoptively transferred T_{reg} cells induced tolerance and could subsequently be depleted without affecting the tolerant state. Importantly, infusion of T cells from these long-term tolerant mice induced tolerance in a second animal, suggesting that the tolerogenic properties of T_{reg} cells can be magnified and long lived. T_{reg} cells also have the ability to repair damaged tissues by producing reparative molecules, such as amphiregulin, and proteins that control tissue function, such as peroxisome proliferator–activated receptor γ (PPARγ) (*10*, *11*).

The poly-pharmaceutical attributes of T_{reg} cells are difficult to emulate or replace with simple drugs. For example, pharmaceuticals and biologics aimed at disrupting inflammatory signaling pathways compensate for just a small fraction of T_{reg}-mediated effects.

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Multicomponent immunosuppressive drug cocktails, such as those used to prevent allogeneic transplant rejection, are needed to counteract the redundant processes involved in immune activation. However, these cocktails systemically and nondiscriminatively stifle immune cell activities, leaving patients susceptible to infections and malignancies, emphasizing the need to devise immune tolerogenic therapies. In fact, tissue-resident T_{reg} cells can adaptively acquire local niche attributes to better control individual immune responses mediated by distinct T cell and innate cell subsets (*12*). Thus, the current challenge to developing T_{reg}-based therapeutics is how to harness the properties of these cells to durably re-establish immune tolerance in acute and chronic inflammatory diseases.

ADOPTIVE T_{REG} CELL THERAPY: A PARADIGM FOR PROMOTING IMMUNE TOLERANCE

The deep molecular and mechanistic understandings of T_{reg} biology have inspired the notion that the repair or replacement of these cells might be a powerful approach to treating autoimmune disorders, organ transplant rejection, and inflammation-associated neurodegenerative diseases. For example, because constitutively high IL-2Rα (CD25) expression is a hallmark of T_{reg} identity (*13*), low-dose IL-2 therapies aim to preferentially confer proliferative and survival advantages to T_{reg} cells. IL-2 and IL-2 muteins have been shown to increase T_{reg} numbers in patients with autoimmune diseases and graft-versus-host disease (GvHD) and have clinical activity (*14*, *15*). However, some recent efficacy studies have been disappointing, because it is increasingly clear that even moderately low doses of IL-2 can trigger unintended signaling in non-T_{reg} cells, particularly activated T_{eff} cells that transiently up-regulated CD25 expression, aggravating inflammatory responses (*16*).

In contrast, adoptive T_{reg} cell therapy has several potential advantages over conventional biologics. Live cells are naturally motile and adaptive, enabling "living therapies" to specifically traffic into affected sites and execute dynamically tuned responses to complex combinations of physiological stimuli. The self-renewal capacity of living cells can also substantially enhance the longevity of cell-based therapies and potentially obviate the need for frequent repeat administrations. In some ways, this parallels the chimeric antigen receptor (CAR) T cell experience in patients with cancer, supporting feasibility as well as the potential for long-term cures. Last, as highlighted above in studies by Qin *et al.* (*9*), T_{reg} therapies can educate and propagate endogenous cells to take on suppressive activities (*17*), thereby orchestrating long-lasting tissue protection even without indefinite survival of the infused T_{reg} cells.

Numerous preclinical studies have provided a proof of concept that T_{reg} therapies not only prevent but also reverse disease activity (*18*–*20*). Initially, adoptive T_{reg} therapy was shown to prevent GvHD in patients, given an allogeneic hematopoietic stem cell transplant. Advances in the isolation and ex vivo expansion of human T_{reg} cells have since enabled dozens of T_{reg} therapy trials for a broad range of autoimmune and transplant indications (*21*). Although most clinical trials to date have not been designed to specifically evaluate therapeutic efficacy, these studies have repeatedly demonstrated the engraftment of infused T_{reg} cells, the safety of T_{reg} infusion without evidence of systemic immunosuppression, and, in some instances, signs of efficacy (*22*, *23*).

THE NEXT GENERATION OF T_{REG} THERAPY: GENETIC ENGINEERING

Despite a number of phase 1 clinical trials for autoimmune diseases testing infusion of polyclonal T_{reg} cells, there have been no successful placebo-controlled phase 2 trials published demonstrating clinical efficacy, perhaps due in part to low target tissue specificity or local persistence (*24*). Preclinical studies have emphasized the enhanced efficacy of directing the T_{reg} cells to tissue-specific antigens, which often exhibit order(s)-of-magnitude greater potency than polyclonal populations. Several strategies have been developed to increase the frequency of tissue reactive T_{reg} cells ex vivo. Donor allo-antigen–reactive T_{reg} cells (e.g., for transplant) can be naturally enriched by stimulation with donor-derived APCs; T_{reg} cells can also be genetically engineered to obtain a desired tissue reactivity through stable integration of constructs encoding antigen-specific T cell receptors (TCRs) or CARs (*25*, *26*). Ectopically expressed antigen-specific TCRs are being deployed in the clinic to increase specific activity in multiple diseases, including type 1 diabetes (T1D) (QuellTX, Abata Therapeutics, PolTREG, AstraZeneca, and GentiBio), multiple sclerosis (MS) (Abata Therapeutics and PolTREG), inflammatory bowel disease (IBD) (Sonoma Biotherapeutics, AstraZeneca, Sangamo Therapeutics, and Tract Therapeutics), and hemophilia (Baudax Bio). TCRs may have advantages in some settings, where sensitivity to a relatively low abundance of peptide-MHC complexes is critical; TCRs also offer the ability to target intracellular proteins, expanding the repertoire of tissue-specific candidate antigens. Engineered TCR-T_{reg} cells can also take advantage of multiple functional activities using naturally evolved interactions between the TCR/CD3 signaling complex and MHC on APCs, especially in the draining lymph nodes, which have been implicated in continuous T_{eff} seeding of target tissues (*27*).

CAR-T_{reg} cells are also in the early stages of development. Originally developed to redirect conventional $CD4^+$ and $CD8^+$ T cells against tumor antigens, CAR designs fuse an extracellular antigen-recognition domain with intracellular T cell signaling domains, a modular architecture that has been adapted to expand the breadth of targetable antigens for T_{reg} therapy. CARs have advantages in enabling MHC-independent targeting, tunable receptor–induced signaling (based on affinity and signaling domain modifications), and the potential ability to recognize antigens present in the extracellular matrix (*28*) or microbial products (*29*). One notable illustration is the development of CAR-T_{reg} cells targeting human leukocyte antigen (HLA)–A2, an MHC class I molecule naturally expressed on nearly all cells from $HLA\text{-}A2^+$ donors. T_{reg} cells expressing an anti–HLA-A2 CAR have been shown in animal models to selectively traffic to $HLA\text{-}A2^+$ grafts and suppress rejection (*30*, *31*). Similar approaches have been proposed for other diseases, including IBD and MS.

CARs that target MHC presenting a specific peptide antigen, or HLA-independent TCRs (HITs), with antibody-based binding domains grafted onto TCR chains are also being developed to redirect T cell specificity. In addition, there are a number of targets for TCRαβ and TCRγδ that are restricted to non-polymorphic MHC molecules, allowing a broad recognition, not unlike CARs. Increasingly sophisticated synthetic antigen receptor platforms also have the potential to specifically redirect T_{reg} activities against targets with even greater precision. For example, a peptide epitope-targeting universal CAR (UniCAR) enables temporal control over antigen selection through administration of epitope-tagged targeting moieties (*32*), whereas synthetic Notch (synNotch) and the related synthetic intramembrane proteolysis receptor (SNIPR) link target antigen recognition to programmed

transcriptional outputs, thereby restricting gene expression within target tissues through Boolean logic (*33*). Importantly, efforts are underway to refine both CAR and TCR design features to drive optimal T_{reg} function, including tissue-homing, intracellular signaling, and cell-cell adhesion, because several of these parameters are likely different between T_{reg} and T_{eff} cells (*34*).

Last, allogeneic, off-the-shelf T_{reg} therapies are being developed to treat acute diseases, including acute respiratory distress syndrome (ARDS), stroke, and myocardial infarctions. Because of the sudden onset of these pathologies, there is insufficient lead time to generate tailored products derived from patient cells, necessitating an off-the-shelf solution. In addition, these conditions are acute and self limited, and thus elimination of allogeneic cells is not of the same concern as with chronic autoimmune diseases. In one recent report, T_{reg} cells expanded from umbilical cord blood (UCB) were administered in successive doses under compassionate use to two patients with coronavirus disease 2019 (COVID-19), each suffering from critical and worsening ARDS (*35*). Preliminary results suggested clinical effects.

Technologies for induced pluripotent stem cell (iPSC)–derived T_{reg} differentiation are also under development aiming to enable off-the-shelf treatment of both acute and chronic indications. These iPSC lines can be genetically engineered to reduce immunogenicity and modified for manufacturing T_{reg} cells tailored to different disease indications and patient characteristics. The allogeneic iPSC approaches would potentially reduce cost-of-goods; correct genetically encoded T_{reg} deficiencies in certain disease settings; and leverage the innate inflammation-homing, anti-inflammatory, and wound-healing properties of T_{reg} cells.

CLINICAL TRANSLATION: MISCONCEPTIONS, CHALLENGES, AND OPPORTUNITIES

Clinical manufacturing is an essential pillar of adoptive T_{reg} therapy that can be undermined by defects or scarcity in source material. Although early clinical trials have largely demonstrated the feasibility of expanding patient-derived T_{reg} cells beyond minimum clinical dosages with high $FoxP3^+$ purity, there have also been documented cases of inadequate T_{reg} expansion, resulting in failure to initiate product infusion (*36*). Similarly, long-standing chronic inflammation may alter the phenotype of $FoxP3^+$ T_{reg} cells in patients with autoimmune disease, complicating T_{reg} selection and increasing the risk of conventional $CD4^+$ T cell contamination during initial isolation. To address this challenge, some groups have improved the technologies for isolating and expanding natural T_{reg} populations, whereas others have explored alternative starting cell populations that are more abundant or well characterized. Naïve conventional $CD4^+$ T cells can be induced to adopt various T_{reg}-like states, either through in vitro differentiation into iT_{reg} cells or through genetic modification, including ectopic *FOXP3* expression in conventional T cells (*37*). Data suggest that these modified $CD4^+$ T cells can replicate the essential elements of the T_{reg} epigenome to ensure phenotypic and functional stability as well as potency. Importantly, the T_{reg} product generated in the Bacchetta and Roncarolo laboratories, which also expresses a surface marker gene and therefore can be tracked in vivo (*38*), is in a phase 1 trial in patients with *FOXP3* mutations (NCT05241444), and results from this trial may contribute to addressing some of the T_{reg} cell therapy's challenges.

Dosing and preconditioning

Early phase 1 clinical trials using nonengineered polyclonal or antigen-specific T_{reg} cells to treat an assortment of autoimmune, inflammatory, GvHD, and transplant contexts have been conducted. These studies all reported high tolerability without maximal tolerated dose reached, with the highest infused dose in the billions. Both preclinical and early clinical studies have shown that T_{reg} cells reach the site of inflammation in response to various chemokine and adhesion molecules (*39*). Thus, unlike CAR-T_{eff} therapies, there is no evidence to date that preconditioning to make "space," with drugs such as cyclophosphamide and fludarabine used in CAR T protocols, is required for T_{reg} cell product access to inflamed tissues (*18*, *40*), although it will be critical to demonstrate that the cells will persist once they get to the target tissue. Some studies have also demonstrated persistence of T_{reg} products for at least a year in blood and have been shown to increase T_{reg} numbers in transplanted kidneys, correlating with clinical benefit for up to 6 years (*40*). Encouragingly, biomarker analyses have suggested therapeutic impacts in several of these trials, mostly in the reduction of effector cytokine production in peripheral blood or biopsy samples (*25*, *41*).

Lineage stability and potential adverse events

One liability of CAR-T_{eff} cells to treat cancer has been the serious adverse events due to cytokine release syndrome (CRS). Although the T_{reg} cytokine secretion profile is anti-inflammatory under normal conditions, the development of T_{reg}-based therapeutics with engineered antigen specificities has raised concerns over whether T_{reg} cells may become functionally unstable within inflamed sites and begin to produce pro-inflammatory cytokines. T_{reg} instability has been observed in murine models in which chronic inflammatory exposure leads to silencing of *Foxp3* expression and epigenetic reprogramming in a small subset of T_{reg} cells, resulting in acquisition of proinflammatory functions and the capacity to exacerbate tissue damage (*42*–*44*). T_{reg} instability has also been observed in nonhuman primate and human T_{reg} cells in vitro and in vivo (*45*). It is important to note that much of the T_{reg} instability observed in animal models may be a consequence of FoxP3 dysregulation in peripherally derived T_{reg} (pT_{reg}) cells, which arise from naïve $CD4^+$ T cells exposed to T_{reg}-polarizing signals in peripheral tissues and therefore lack the full T_{reg} epigenetic imprinting received during thymic development (*46*).

Last, several groups are introducing ectopic *FOXP3* into conventional $CD4^+$ T cells to lock in a T_{reg} phenotype (*47*–*49*). In this context, it will be important to show that the ectopic *FOXP3*-expressing $CD4^+$ T cells replicate hallmarks of T_{reg} identity, functional stability, and potency (*49*, *50*). In some instances, enhanced expression of *FOXP3* in T_{reg} cells appears to generate a stable phenotype (*48*–*50*). However, *FOXP3* expression may not be the only feature of phenotypic or functional T_{reg} stability, because other deficiencies, such as reduced IL-2R expression, can play a role (*51*).

For safety, many products include the expression of suicide genes and targetable, sometimes regulatable, surface proteins and chimeric receptors that allow the management of rogue "ex-T_{reg} cells" should they develop, as well as management of the small number of T_{eff} cells that may be present in the final product. Thus, continued characterization of T_{reg} cell phenotypic stability and purity at the target tissues or within highly inflammatory settings is warranted as engineered T_{reg} products are introduced into the clinic. Importantly, ongoing work to identify cell surface markers and cell culture

additives (e.g., rapamycin) could facilitate isolation and expansion of T_{reg} subsets having greater stability and purity.

Persistence and therapeutic durability

Durability of the T_{reg} product is another potential opportunity for enhancement. T_{reg} survival depends on periodic TCR and costimulatory signaling, as well as the presence of growth and survival factors. The most prominent factor for both T_{reg} survival and maximal FoxP3 expression is IL-2. In multiple preclinical disease settings, the paucity of IL-2 at the site of inflammation has been linked to reduced T_{reg} numbers and, in some cases, loss of function because of reduced FoxP3 expression. T_{reg} cells do not make IL-2 and instead rely on constitutive expression of the high-affinity IL-2Rα, CD25, to compete for extrinsic IL-2 secreted by conventional T cells. In some settings, injection of IL-2 systemically has been shown to increase T_{reg} numbers and FoxP3 expression in the inflamed tissues, although as mentioned above, there remains a risk of T_{eff} cell activation. Thus, strategies to promote T_{reg} persistence and FoxP3 expression have centered on delivering IL-2 signaling intrinsically, in constitutive or regulatable fashion, within the T_{reg} therapy. This includes multiple approaches, ranging from the use of synthetic IL-2/IL-2 receptor pairs, tethered IL-2, and switch receptors to enhance T_{reg} survival and stabilize expression of FoxP3 in settings where there is not sufficient IL-2 or other growth factors, such as IL-33 (*52*). Clinical trials will soon be underway to determine the need for these modifications and their effects on T_{reg} durability and stability.

Interference of immune surveillance

Apart from manufacturing feasibility, functional stability, and therapeutic durability, there have also been concerns with the possibility that T_{reg}-based therapeutics could become too potent and broadly immunosuppressive, leading to infection or cancer. However, T_{reg} therapies are unlike conventional immunosuppressants that act indiscriminately throughout the body. T_{reg}-based therapeutics can be engineered to target defined antigens, limiting off-tissue drug activities. Furthermore, T_{reg} cells naturally exist in equilibrium with other immune cells, expanding and contracting as necessary to allow immunity against infectious agents while repressing overactive, runaway immune responses (*53*). In addition, the risk of deleterious loss of immune surveillance at the targeted tissue is mitigated by dual requirements of CD3ζ stimulation and IL-2 cytokine support, both of which are extinguished when the immune activation is controlled, thus making this theoretical risk inherently time-limited. In fact, the concern about loss of global immune surveillance induced by antigen-specific or polyclonal T_{reg} cells has been robustly addressed in multiple clinical and preclinical experiments without evidence to date of any untoward effects. A common post–kidney transplantation complication, BK polyoma virus–associated nephropathy, was not elevated in patients receiving T_{reg} therapy (*54*). To the contrary, reduced rate of viral infection in kidney transplant recipients who received T_{reg} therapy has been reported, likely due to reduced need for conventional immunosuppressive drugs (*54*).

Importantly, multiple clinical trials spanning an assortment of autoimmune, inflammatory, GvHD, and transplant contexts have

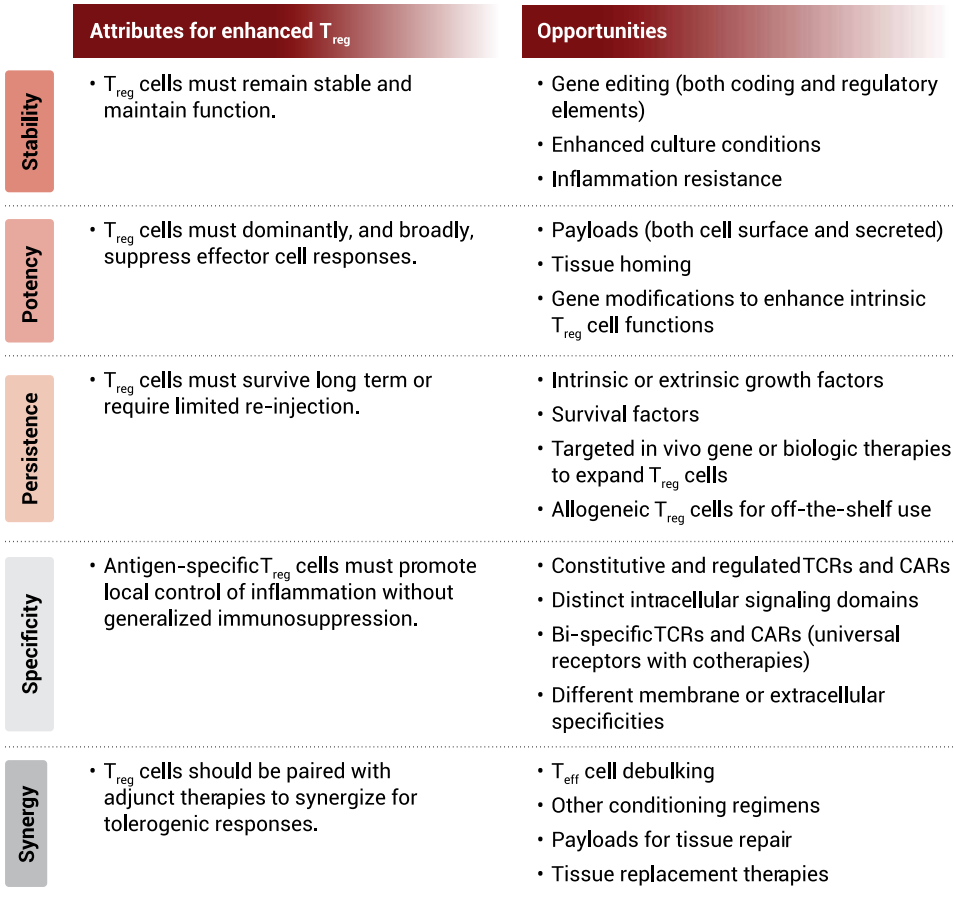

Fig. 1. Multiple T_{reg} attributes should be considered for T_{reg} engineering approaches. Engineering approaches should consider the need for T_{reg} cells to maintain stability and potency, persist long term after infusion, and ideally be antigen specific to avoid general immunosuppression. The impact of combination therapies, including other immunotherapies and tissue regeneration regimens, should also be considered. Each of these attributes offers opportunities to improve on existing T_{reg} cell therapies.

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been conducted with polyclonal T_{reg} cells, with no reported adverse reactions requiring life-saving intervention or increased rates of infection or cancer. Thus, there is ample evidence at present to presume that T_{reg} therapies are likely to be safe and without overt concerns related to either instability or excessive immunosuppression. That said, there will continue to be opportunities to enhance T_{reg} biology to maximize T_{reg} efficacy without increasing the risks involved.

THE NEXT ERA: A PEEK INTO THE FUTURE AND CONCLUDING REMARKS

This is an exciting time for cell therapy in general, as multiple new CAR-T therapies have been approved for hematologic cancers. These advances have provided clinical and scientific validation, as well as financing, to extend cell-based immunotherapies to other cell types and diseases. TCR- and CAR-T_{reg} therapies are among the therapeutics that are now being developed with the goal of transforming the treatment of patients with autoimmune and inflammatory diseases, as well as the treatment of organ transplantation recipients. The opportunities going forward include the continued engineering of T_{reg} cells to enhance specificity, potency, persistence, specificity, and synergy, including regulated receptor expression, incorporation of additional payloads (including factors promoting tissue repair and regeneration), and enhanced functionality (Fig. 1). Moreover, the future will include the use of combination therapies to directly promote T_{reg} function, manipulate the inflamed microenvironment, and provide targeted immune regulation. In this latter case, T_{reg} tolerogenic therapies may enable gene and protein therapies that can repair or replace molecular defects seen in metabolic and other physiologic diseases such as hemophilia, Pompe disease, adrenoleukodystrophy, and others. Cell therapy is currently expensive, but, in many cases, successful induction of tolerance could save millions of dollars spent yearly on chronic immunosuppressive drugs. Importantly, should allogenic, off-the-shelf cell therapies succeed in providing durable, stable tolerance in the absence of retreatment, not only would it markedly reduce the cost of the drugs and increase access but also would enable the use of engineered T_{reg} cells to treat acute diseases. Last, we imagine a time when cell-based therapies will be used early in the course of disease progression, if not before disease onset, should ongoing efforts to define early biomarkers be successful, reestablishing immune balance and preventing subsequent tissue damage and clinical complications. We are still at the "end of the beginning," and it will take partnerships between academia, biotech companies, pharmaceutical companies, regulatory agencies, and the venture capital community to achieve success.

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