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T_{reg} cell-based therapies: challenges and perspectives

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Abstract

Cellular therapies using regulatory T (T_{reg}) cells are currently undergoing clinical trials for the treatment of autoimmune diseases, transplant rejection and graft-versus-host disease. In this Review, we discuss the biology of T_{reg} cells and describe new efforts in T_{reg} cell engineering to enhance specificity, stability, functional activity and delivery. Finally, we envision that the success of T_{reg} cell therapy in autoimmunity and transplantation will encourage the clinical use of adoptive T_{reg} cell therapy for non-immune diseases, such as neurological disorders and tissue repair.

The therapeutic potential of a unique FOXP3^{+} immunosuppressive subset of regulatory T (T_{reg}) cells has been demonstrated in various preclinical models of graft-versus-host disease (GVHD)[1–3], solid organ transplantation[4,5], type 1 diabetes mellitus (T1D)[6,7], systemic lupus erythematosus (SLE)[8], inflammatory bowel disease[9,10] and multiple sclerosis (MS)[11]. This has stimulated advances in the clinical development of adoptive cell therapy (ACT) of T_{reg} cells in the clinic. There are now more than 50 active and completed clinical trials testing the safety and efficacy of T_{reg} cell therapy for indications such as kidney or liver transplantation, pemphigus vulgaris, SLE, inflammatory bowel disease, autoimmune hepatitis, allergy and asthma[12]. Although separation and expansion protocols for the T_{reg} cells vary, early clinical trial results report manufacturing success and excellent safety profiles[12–17]. Broadening the therapeutic potential of T_{reg} cell ACT will depend on the use of novel technologies to alter the genome of the cells to enhance functional activity, stability, persistence and antigen specificity.

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Author contributions

The authors contributed equally to all aspects of the article.

Competing interests

J.A.B. is a stock holder and member of the Board of Directors on Rheos Medicines; a stock holder and member of the Board of Directors for Provention Bio; and a stock holder and member of the Scientific Advisory Boards of Vir Therapeutics, Arcus Biotherapeutics, Quentis Therapeutics, Solid Biosciences and Celsius Therapeutics (Founder). J.A.B. owns stock in MacroGenics Inc., Vir Therapeutics, Arcus Biotherapeutics, Quentis Therapeutics, Solid Biosciences and Celsius Therapeutics. C.R. and L.T.V. declare no competing interests. J.A.B is the President and CEO of a newly formed biotech company targeting T_{reg} therapy for the treatment of autoimmune and other immune disorders.

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Numerous preclinical studies have demonstrated that antigen-specific T_{reg} cells are more potent than polyclonal T_{reg} cells in models of T1D[6,7], autoimmune central nervous system disease[18] and transplantation[19–22]. Moreover, antigen-specific T_{reg} cells predominantly localize at the site of antigen presentation, decreasing the risk of generalized immunosuppression. Thus, antigen-specific T_{reg} cells may be both safer and more efficient than unselected polyclonal T_{reg} cells for ACT. Because of their high precursor frequency[23,24], 'antigen-selected' human T_{reg} cells with direct allospecificity, derived from the transplant recipient, can be efficiently expanded in vitro using allogeneic antigen-presenting cells (APCs) from the transplant donor[25]. This is currently being evaluated as a cell-based therapy in several clinical trials[26,27]. However, T_{reg} cells with indirect allospecificity as well as self-antigen-reactive T_{reg} cells have a precursor frequency predicted to be 1,000-fold to 10,000-fold lower than the frequency of direct alloreactive T_{reg} cells and have not been effectively expanded to date[23,28]. Hence, alternative strategies such as genetic engineering are needed to produce T_{reg} cells specific for a particular nominal antigen.

In this Review, we focus on the therapeutic potential of T_{reg} cells to treat and reverse immune-mediated diseases. We provide an overview of adoptive cell therapy with T_{reg} cells and discuss the specificity, function and activity of this small immunosuppressive cell subset, the accumulating evidence that these cells can protect patients from GVHD, autoimmunity and organ transplant rejection, as well as practical aspects of the implementation of T_{reg} cell therapy in the clinic. Finally, we describe current efforts to enhance T_{reg} cell specificity, survival and function as a next-generation therapeutic, using new techniques such as CRISPR-based gene editing and the use of bone marrow, induced pluripotent stem cells and embryonic stem cells to generate T_{reg} cells, in order to advance the use of this essential regulator of immune homeostasis for various immune and non-immune-related diseases.

T_{reg} cell background

The concept of immune regulation by specialized lymphocytes has been a doctrine of immunology for more than four decades. However, the absence of clear molecular markers challenged the dogma and for a long while relegated the notion of suppressor cells to the back-burner of immunology. Then, a series of studies by Sakaguchi et al.[29] revitalized the field by demonstrating that a small subset of cells, identified by the expression of CD4 and CD25, could be used to transfer tolerance in animals that develop autoimmunity as a consequence of neonatal thymectomy. A major break-through came when three groups independently identified the X-linked gene *FOXP3* in mouse and human, as the transcription factor that determines the T_{reg} cell functional programme by inducing a specific gene expression programme and epigenetic signature during T_{reg} cell development[30–32]. The critical role for FOXP3 and, as a consequence, for T_{reg} cells in immune tolerance was clearly established with the discovery that early-onset fatal multi-organ inflammation and autoimmune disorders were observed in patients and mice with a disrupted *FOXP3* gene. In humans, males with deleterious mutations develop immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome and mice with mutant FOXP3 (scurfy mice) develop lethal multi-organ autoimmunity. Both syndromes are a direct consequence of a systemic defect in T_{reg} cells[33–35]. In fact, the first demonstrated utility for

adoptive transfer of T_{reg} cells as a therapy was performed in FOXP3 mutant mice and provides a key rationale for the use of these cells both as a therapy for IPEX patients and in humans with various immune diseases.

T_{reg} cells, which comprise 5–7% of $CD4^+$ T cells, develop both directly in the thymus (tT_{reg} cells) and in the periphery (pT_{reg} cells). pT_{reg} cells develop, especially in the gut, from $CD4^+$ conventional T cells under conditions of high levels of transforming growth factor-β (TGFβ) and retinoic acid in the environment or in response to metabolites produced by microbiota[36,37]. The T cell receptor (TCR) repertoire of the pT_{reg} cell and conventional T cell populations is largely non-overlapping[38–40]. Indeed, tT_{reg} cells mainly recognize self-antigens, whereas the pT_{reg} cell TCR repertoire also includes TCRs specific for 'non-self' infectious antigens or innocuous commensal microbiota-derived antigens, which are important for the maintenance of mucosal tolerance[41]. Unlike in the mouse, where neuropilin 1 (NRP1) has been defined as a marker of pT_{reg} cells[42,43], there is currently no marker able to distinguish tT_{reg} cells from pT_{reg} cells in humans. Thus, T_{reg} cells isolated from peripheral blood are likely a combination of tT_{reg} cells and pT_{reg} cells. Importantly, T_{reg} cells can be found locally in tissues as well as systemically during an inflammatory response. They migrate via afferent lymphatics from the inflamed tissue to the draining lymph node, where they can also function to control antigen presentation. Thus, T_{reg} cells exert their suppressive function both at the tissue site of inflammation and in local secondary lymphoid tissues[44–47].

T_{reg} cells confer immune tolerance via multiple mechanisms[48]. Through the expression of anti-inflammatory soluble mediators, such as IL-10, TGFβ and IL-35, the consumption of IL-2, and the expression of negative regulatory cell surface receptors such as cytotoxic T lymphocyte antigen 4 (CTLA-4), CD39 and CD73 (REF.[49]), they can target T cells directly or indirectly by modulating APCs. For example, CTLA-4 binding to CD80/CD86 on APCs can lead to the induction of indolamine-2,3-dioxygenase (IDO)[50,51]. In addition, T_{reg} binding to APCs can result in stripping of the cell surface molecules (trogocytosis) altering co-stimulation and antigen presentation[52,53]. In fact, some data have suggested that T_{reg} cells, expressing perforin and granzyme B, may directly kill APCs expressing the target antigen[53]. Importantly, many of these suppressive activities of T_{reg} cells can function in an antigen non-specific manner, called dominant bystander suppression, allowing them to suppress effector T cells of diverse specificities[54]. T_{reg} cells can also modulate the tissue microenvironment through the production of some of these same immunosuppressive molecules, promoting the emergence of other immunosuppressive cell populations such as T_{reg} cells with different specificities and T regulatory 1 (Tr1) cells[55–58]. This phenomenon, called 'infectious tolerance', supports the idea that, in a therapeutic setting, adoptively transferred T_{reg} cells may not need to persist indefinitely but for long enough to confer suppressive capacity to other immune cells located in the affected tissue[59]. It is important to highlight that the mechanisms of action of T_{reg} cells and effector T cells differ, with the latter functioning in a largely cell contact-dependent manner directed against antigen-bearing cells (FIG. 1). These differences need to be taken into consideration when designing T_{reg} cell-based therapies.

Finally, a detailed biological and biochemical understanding of these immunoregulatory T cells, combined with recent advances in T_{reg} cell subset identification (BOX 1), the ability to engineer antigen specificity through TCR and chimeric antigen receptor (CAR) transduction, genome editing to generate more potent and more stable T_{reg} cell populations, and strategies to produce T_{reg} cells de novo from stem cell populations, provides an unparalleled opportunity for ACT. Ultimately, these advances will enable the development of allogeneic T_{reg} cell products for more general use in various therapeutic settings.

Engineering antigen-specific T_{reg} cells

There are currently several approaches to generate and/or expand antigen-specific T_{reg} cells in vitro (FIG. 2). As mentioned, endogenous antigen-specific T_{reg} cells are more potent than polyclonal T_{reg} cells but their expansion is challenging owing to low precursor frequencies. Another approach to generate antigen-specific T_{reg} cells is to redirect polyclonal T_{reg} cells by introducing synthetic receptors. These receptors can take the form of CARs, enabling direct antigen recognition, or engineered TCRs, which target antigens in the context of an antigen–MHC–peptide complex. A third approach is to convert antigen-specific effector T cells into T_{reg} cells through overexpression of FOXP3.

T_{reg} cells with engineered TCRs.

Animal studies have shown that engineered tissue antigen-specific T_{reg} cells are significantly more efficient than polyclonal T_{reg} cells in preclinical models of T1D[6,7], colitis[60], rheumatoid arthritis (RA)[61,62], MS[18] and transplantation[63]. Importantly, TCR-transduced T_{reg} cells accumulate in tissue targeted during autoimmunity in an antigen-driven manner, where they exert both antigen-specific repression and non-specific bystander suppression following activation[18,61,62]. Given that, in many cases, T_{reg} cell populations with a single antigen-specific TCR can suppress polyspecific pathogenic T cell populations, autoantigen-reactive engineered T_{reg} cells may be as effective as polyclonal T_{reg} cells to shut down pathogenic T cells independent of their specificity. However, in some settings, oligoclonal or pauciclonal T_{reg} cells may be more efficacious. In this regard, it was shown that expanded T_{reg} cells with direct allogeneic specificity were more efficient in promoting heart graft tolerance when also transduced with a TCR with specificity for donor antigens presented by host APCs (indirect alloantigen specificity)[63]. Thus, antigen-specific expansion and genetic engineering with recombinant TCRs may be combined to generate dual-specific T_{reg} cells that have higher efficiency in promoting graft tolerance.

These results encouraged the development of TCR transgenic human T_{reg} cells specific for antigens that are abundantly present in affected tissues. For example, human T_{reg} cells with a transgenic TCR specific for the anti-haemophilic factor VIII (FVIII) efficiently suppressed proliferation and cytokine production of FVIII-specific effector T cells and reduced FVIII antibody production in splenocytes of FVIII-immunized HLA-DR1 transgenic haemophilic mice in vitro[64]. Moreover, T_{reg} cells with a transgenic TCR specific for myelin basic protein (MBP) were able to suppress both MBP-specific T cells and T cells with other specificities in a preclinical mouse model of MS[65]. The ability of human T_{reg} cells transduced with TCRs to achieve bystander suppression was also observed in other preclinical studies using TCRs

with diverse antigen specificities[66–68]. Interestingly, the specificity of human T_{reg} cells was also efficiently redirected by class I-restricted transgenic TCRs, which may optimize the effectiveness of the T_{reg} cells in the autoimmune tissue[66,69]. It should be noted that the majority of current efforts have relied on antigen-specific TCRs isolated from effector T cells and transduced into T_{reg} cells. It is possible that the intrinsic affinity and specificity of TCRs isolated from T_{reg} cells can be distinct from effector T cells, affecting migration into specific niches and functional activity. Thus, an approach that utilizes TCRs isolated from T_{reg} cells to redirect antigen specificities in T_{reg} cells may more closely 'replicate' the intrinsic functionality of antigen-specific T_{reg} cells.

Chimeric antigen receptors.

CARs are artificial receptors comprising an antigen binding site of a monoclonal antibody (mAb) in their extracellular domain and T cell stimulatory and co-stimulatory intracellular domains (FIG. 3). CAR-engineered effector T cells (CAR T cells) have demonstrated remarkable efficacy in patients with blood cancers[70–73]. The major advantage of CARs is their ability to recognize whole proteins expressed in target tissues, rather than being restricted to antigens presented in the context of MHC class I or II. Thus, CARs can also provide a unique opportunity to target T_{reg} cells to the site of tissue destruction or to transplanted tissues.

The first preclinical studies with the current generation of CAR-expressing T_{reg} cells were conducted a decade ago in mouse models of colitis and in xenotransplanted mice[74,75], and the first human T_{reg} cells transduced with this generation of CARs were developed around the same time[76]. However, interest for ACT has accelerated after it was shown that T_{reg} cells engineered with a CAR targeting HLA-A2, an antigen commonly mis-matched in transplantation, can induce alloantigen-specific suppression in a humanized mouse transplantation model[77]. Specifically, HLA-A2-specific human CAR T_{reg} cells prevented xenogeneic GVHD caused by HLA-A2-expressing cells in mouse models. In addition, using similar HLA-A2-specific CAR T_{reg} cells, several studies have subsequently shown that these T_{reg} cells can suppress HLA-$A2^{+}$ skin allograft rejection[78–80] and selectively target HLA-A2-bearing tissue with no effect on an HLA-$A2^{-}$ allograft in a side-by-side transplant model[78]. In the haemophilia A setting, human T_{reg} cells expressing an FVIII-specific CAR suppressed FVIII-targeted antibody responses in a xenogeneic mouse model[81]. In addition, in this model, FVIII-specific CAR T_{reg} cells were able to suppress the proliferation of FVIII-specific effector T cells and effector T cells with a distinct antigen specificity, demonstrating their ability to exert bystander suppression.

The unique property of T_{reg} cells to exert bystander suppression enables rational design to target T_{reg} cells to the inflamed tissue, without necessarily targeting cell surface antigens. For example, a CAR was developed that is specific for citrullinated vimentin (CV), a post-translationally modified protein abundantly and almost exclusively present in the extracellular matrix of inflamed joints of patients with RA[82]. Importantly, CV-specific CAR T_{reg} cells expanded when cultured with synovial fluid from the joints of patients with RA, suggesting that CV present in the extracellular matrix is sufficient to trigger CAR-mediated cell activation[82]. Of note, the approach of redirecting T_{reg} cells to the extracellular matrix

instead of targeting antigen-expressing cells may prove beneficial in certain inflammatory settings[83] as direct targeting of the antigen-expressing cells may be detrimental given the reported cytolytic activity of T_{reg} cells in some cases[53].

Comparison of recombinant TCRs and CARs.

Although the introduction of recombinant TCRs or CARs can confer antigen specificity in T_{reg} cells, these engineered receptors possess different mechanistic and functional properties that could represent advantages or disadvantages depending on the context (FIG. 3). For instance, although low antigen expression levels are sufficient to induce TCR-mediated stimulation, some studies of CAR effector T cells suggest that the density of the antigen recognized by the CAR must be high on the target cell to trigger activation[84–86]. In this regard, it is important to restate that although T_{reg} cells can have suppressive function in an antigen non-specific manner, CAR-mediated activation of cells is antigen specific, such that the level of local antigen expression determines whether the cell becomes activated. Thus, TCRs may be more appropriate to target antigens expressed at low levels whereas CARs may be more useful for abundantly expressed antigens on target tissue, with low expression on normal tissues.

Even though there is preclinical evidence that supports the development of both T_{reg} cells engineered with recombinant TCRs and T_{reg} cells engineered with CARs, several key observations may give an indication of their potential clinical success. For example, T_{reg} cells are designed to detect antigen presented in the context of MHC class II and, as such, express class II-binding CD4 molecules. If these cells are engineered to bind to antigen in the context of MHC class I, it is uncertain whether the CD8 molecule will need to be co-expressed on the cells to efficiently engage MHC class I. However, it was shown in in vivo mouse models that T_{reg} cells transduced with MHC class I-restricted TCRs were able to bypass the need for MHC binding by the CD8 co-receptor even with low-affinity TCRs[69]. It remains to be determined whether TCRs can be created routinely with sufficient affinity, and against relevant auto-antigens, to be co-receptor independent as shown in preclinical studies[67,69].

Importantly, TCRs are MHC restricted, which requires matching of the patient MHC genotype, whereas antigen recognition through the CAR is MHC independent and could be applicable to a larger number of patients. However, CAR T_{reg} cell activity would potentially be more limited to the site of inflammation, although one might consider using an antibody for the creation of the CAR that is directed at an autoantigenic peptide–MHC class II complex, which would be expressed on APCs in both the inflamed site and, potentially, the draining lymph node. Finally, although no cytotoxicity was observed in the initial preclinical CAR T_{reg} cell studies, it was recently reported that CAR T_{reg} cells can have cytotoxic activity in vitro[77,83]. Direct recognition of an antigen on target cells by CARs could also lead to T_{reg} cell-mediated targeted killing via the perforin/granzyme B pathway as previously shown[87,88]. Thus, the killing potential of CAR T_{reg} cells needs to be further investigated and may make the direct recognition of molecules on target tissues problematic.

Ectopic overexpression of FOXP3 in antigen-specific CD4+ T cells.

An alternative strategy to antigen receptor engineering is the enforced expression of FOXP3 to convert antigen-specific conventional $CD4^+$ T cells into a pool of antigen-specific T_{reg} cell-like cells. It has been shown that lentiviral-based transduction of antigen-specific conventional T cells with *FOXP3* leads to high levels of FOXP3 expression that do not fluctuate with the state of T cell activation. This results in suppressive cells that are as potent as bona fide T_{reg} cells both in vitro[89,90] and in vivo[31,91–93]. Importantly, FOXP3 overexpression suppresses effector cytokine production in conventional $CD4^+$ T cells. This approach has been applied to convert $CD4^+$ T cells from patients with IPEX syndrome, which is caused by FOXP3 deficiency, into T_{reg} cells, and in preclinical models of autoimmunity[30–32,91–93]. However, the induction of $FOXP3^+$ T_{reg} cell-like cells poses the potential risk of unstable or intermediate cell types that may still possess effector function owing to epigenetic imprinting in the effector T cells. Moreover, there is little understanding of the differences in TCR signalling and affinity between T_{reg} cells and effector T cells and how these affect function in T_{reg} cells as compared with conventional T cells. Indeed, there is experimental evidence suggesting that numerous functional epigenetic differences found in T_{reg} cells are present in T_{reg} cells before FOXP3 expression but may not be replicated when FOXP3 is overexpressed in conventional T cells[94–96]. Moreover, the consequences of the expression of certain cell surface molecules that regulate anergy and exhaustion pathways in effector T cells versus bona fide T_{reg} cells remain unclear. For example, CTLA-4 and programmed cell death 1 (PD-1) are constitutively expressed on functional T_{reg} cells and are essential for their suppressive activity, whereas these checkpoint molecules play a distinct role as inhibitory molecules in effector T cells. Thus, the fidelity of such converted cell types will need to be rigorously evaluated across various inflammatory settings using advanced ACT platforms[97,98].

Stability, durability and trafficking

Most T_{reg} cell engineering approaches have been limited to the generation of defined antigen specificities. However, with the increasing capabilities of CRISPR and other gene editing approaches, it is easy to envision multiple editing events in a single cell and, as a consequence, additional alterations that can modulate T_{reg} cell function, stability, persistence and trafficking, or add payloads that may deliver soluble molecules to the inflamed site to complement T_{reg} cell suppressive activity.

T_{reg} cell plasticity.

T_{reg} cells have remarkable phenotypic plasticity and can acquire different transcriptional programmes in response to a changing environment, notably through cytokine signalling[99]. The basis of this phenotypic plasticity lies in their ability to express different master regulatory transcription factors. T_{reg} cells can adopt these alternative transcriptional lineage programmes, which may generate functionally distinct subsets that can localize to specific sites of inflammation and acquire suppressive and tissue repair programmes that effectively control ongoing tissue-specific immune responses[99]. For example, the expression of alternate lineage transcription factors, such as T-bet, GATA3 and RORγt, activates transcriptional programmes that drive the differential expression of chemokines, chemokine

receptors and adhesion molecules to suppress local inflammation mediated by functionally 'matched' effector cells. Several mouse studies have suggested that a small subset of T_{reg} cells can lose FOXP3 expression (termed exFOXP3 cells), especially in the presence of inflammatory cytokines and IL-2 deficiency, leading to the acquisition of an effector T cell phenotype and potential pathogenicity of the exFOXP3 population[100–103]. Importantly, pT_{reg} cells were reported to be less stable than tT_{reg} cells under lymphopenic conditions, suggesting that tT_{reg} cells may represent a better population for ACT[104].

The key role of FOXP3 in T_{reg} cell stability is exemplified by certain *FOXP3* gene mutations in IPEX patients. Although a majority result in loss of FOXP3 protein and, thus, the inability to generate the T_{reg} cell lineage, some mutations result in a population of T_{reg} cells expressing an altered FOXP3 protein that causes the rewiring of the T_{reg} cell transcriptional programme and the transdifferentiation of the T_{reg} cells into other effector T cells. In one example, a patient with a FOXP3 mutation in the putative domain swap interface of the protein dimer presented with an autoimmune syndrome that resulted from an unrestrained T helper type 2 (T_H2) cell immune response by the mutant T_{reg} cells owing to a specific derepression of the T_H2 transcriptional programme, leading to the generation of T_H2-like T_{reg} cells producing type 2 cytokines[105]. Indeed, a valid concern for antigen-specific T_{reg} cell therapy is potential FOXP3 destabilization and possible pathogenic conversion of T_{reg} cells into antigen-specific effector T cells that can exacerbate tissue destruction. In this case, small molecules targeting epigenetic modifiers, such as DNA methyltransferase, histone deacetylase, histone demethylase or methyltransferase (for example, the methyltransferase EZH2)[106], could potentially be used in combination with ACT to help stabilize T_{reg} cells in vivo[107,108]. However, this idea still needs to be validated in vivo and in the clinic.

Thus, FOXP3 overexpression or modulation of epigenetic pathways that regulate FOXP3 expression may be viable strategies to stabilize T_{reg} cells. Studies aiming at understanding the molecular mechanisms behind T_{reg} cell alternative fate programmes are needed to gain better insight into how to control and maintain T_{reg} cell stability, especially in inflammatory settings.

Selective gene knockouts to promote T_{reg} cell stability.

Pro-inflammatory molecules have been reported to destabilize T_{reg} cells[109,110] and could therefore serve as targets for T_{reg} cell modifications. One example is IL-6, a pleiotropic cytokine that is a central regulator of the balance between T_{reg} cells and T_H17 cells.

Together with TGFβ, IL-6 induces the development of T_H17 cells from naive T cells. By contrast, IL-6 inhibits TGFβ-induced T_{reg} cell differentiation[111]. IL-6 triggers signal transducer and activator of transcription 3 (STAT3) signalling, which induces the expression of the DNA methyltransferase DNMT1, resulting in methylation of the *FOXP3* locus and downregulation of its expression[112,113]. Functionally, IL-6 signalling has been shown to impede immune suppression by T_{reg} cells in psoriasis[114], and anti-IL-6R therapy prevented the development of T_H17 cells and arthritis in a collagen-induced arthritis model[115] and suppressed pathology in experimental autoimmune encephalomyelitis[116]. In humans, IL-6R-targeted antibodies represent a therapeutic strategy for inflammatory and autoimmune diseases such as RA[117], Crohn's disease[118] and SLE[119]. Thus, due to the key role of IL-6 in

the T_H17/T_{reg} cell paradigm, knocking out IL-6R or STAT3 in T_{reg} cells may represent a viable strategy to improve the stability of T_{reg} cells and make them resistant to potential changes induced by IL-6 signalling.

However, IL-6R knockout may be counterproductive in certain disorders, as it was reported that particular T_{reg} cell subsets actually require IL-6 signalling for development. For instance, a recent study demonstrated that in addition to promoting T_H17 responses in a mouse model for crescentic glomerulonephritis, signalling through IL-6Rα was also essential for the generation of a protective RORγt$^+$ T_{reg} cell population with enhanced immunosuppressive properties[120]. Therefore, strategies to stabilize T_{reg} cells via IL-6/IL-6R modulation for ACT must be context specific.

Promoting persistence via IL-2.

The growth factor IL-2 is essential for T_{reg} cell generation, survival, stability and function[121], and in its absence T_{reg} cells undergo apoptotic death, leading to autoimmunity[122]. Notably, IL-2 deficiency contributes to intra-islet T_{reg} cell dysfunction and has been associated with disease progression in the non-obese diabetic (NOD) mouse model of diabetes, suggesting that IL-2 deficiency at the site of autoimmune insults may lead to T_{reg} cell instability[123]. Multiple approaches have therefore been developed to use IL-2 as a therapeutic pathway to enhance T_{reg} cell efficacy, stability and survival in vivo[121,124]. In fact, studies have suggested that T_{reg} cells are ‘wired’ to have a reduced IL-2 receptor (IL-2R) signalling threshold[125,126]. Thus, it has been hypothesized that the administration of low doses of IL-2 would preferentially activate T_{reg} cells and limit the activation of effector T cells that was previously observed in response to treatment with high-dose IL-2 in the clinic[123,127–129]. Consequently, the first successful clinical trials testing the therapeutic efficacy of low-dose IL-2 were performed in patients with hepatitis C virus-induced vasculitis[130] and in patients with GVHD[131,132]. Treatment with low-dose IL-2 has been examined in ulcerative colitis[133], alopecia areata[134], SLE[135,136] and T1D[137] as well.

The effect of low-dose IL-2 on the expansion, function and survival of autologous adoptively transferred T_{reg} cells in patients with T1D is currently being investigated[138]. However, it should be noted that there is evidence for activation of effector cells in some patients even at moderate IL-2 doses. This off-target effect is due to the pleiotropic nature of the cytokine in promoting the expansion of other immune cell types including CD4$^+$ T cells[139–141], CD8$^+$ cytotoxic T cells[142], natural killer (NK) cells[143,144] and other innate lymphoid cell populations[145]. Therefore, several groups have developed IL-2 mutants, PEGylated IL-2 and IL-2-directed mAbs that promote T_{reg} cell expansion in vivo[146–148]. Notably, altered forms of IL-2 have been developed to prolong the circulation half-life of IL-2 and hold the promise of selectively inducing the extended expansion of T_{reg} cells in vivo. Despite encouraging preclinical studies, the success of these approaches in the clinic remains to be determined[149,150].

Several mAbs targeting IL-2 have been described that selectively promote the proliferation of a specific T cell subset by altering the conformation of IL-2 in such a way that they modulate the interaction of IL-2 with its receptor subunits[151,152]. A fully human anti-IL-2 antibody, called F5111.2, was recently developed[128]. By blocking IL-2Rβ binding and

reducing IL-2Rα interaction, F5111.2 induces the preferential expansion of human T_{reg} cells. This antibody–IL-2 complex was effective in mouse models of T1D and experimental autoimmune encephalomyelitis, as well as in GVHD[128]. Of note, to simplify the clinical administration of the mAb–IL-2 complex, a covalently linked version of IL-2 and an IL-2-specific mAb (JES6–1) was developed, which selectively expanded T_{reg} cells and demonstrated superior disease control in a murine colitis model compared with a non-covalently linked complex of IL-2 and JES6–1 (REF.[153]). Current efforts to develop tethered human anti-IL-2 mAbs are underway.

Numerous other strategies for manipulating the IL-2 pathway have been described, and these are further discussed in detail elsewhere[121,154].

Regulating T_{reg} cell function using synthetic receptors.

A number of synthetic receptors have been engineered in order to manipulate T_{reg} cell function. For example, a receptor–ligand orthogonalization approach was used to engineer a mutant IL-2 cytokine and mutant IL-2R that specifically bind to one another but not to their wild-type counterparts[155]. In this interaction, cells are transduced with the mutant IL-2Rβ, replacing the endogenous wild-type gene, which then selectively enables cells to respond to the mutant IL-2 that has little to no activity on wild-type IL-2Rβ, expressed by the endogenous cells. An effector T cell ACT approach with orthogonal IL-2Rβ-$CD4^+$ and orthogonal IL-2Rβ-$CD8^+$ T cells promoted a robust antitumour immune response in a preclinical cancer model[155]. In a complementary approach, it was demonstrated that transduction of a mutant version of STAT5, which is constitutively activated, can enhance T_{reg} cell survival and function in preclinical models[156]. Currently, studies are underway to determine whether mutant human IL-2 and IL-2R orthogonal pairs can be used in T_{reg} cell ACT to enhance the survival and efficacy of the transferred cells.

Another strategy to control T_{reg} cell function and expansion has been the development of dual-antigen activating systems, such as synthetic Notch (SynNotch) receptors that induce custom transcriptional activation in response to a chosen antigen[157]. An initial signal drives the transcription of CAR expression; thus, the antigen specificity is controlled by this gated expression and provides better control and regulation of the T cell.

Although the published studies have been performed on effector T cells in preclinical cancer mouse models, there is no a priori reason why this may not be effective in the T_{reg} cell setting. Similar approaches have been used that employ altered cell surface proteins that form bioactive protein switches[158]. Moreover, these systems could be combined with the orthogonal IL-2/IL-2Rβ approach by placing the expression of the orthogonal IL-2 under the control of an antigen-dependent SynNotch receptor to generate self-sufficient orthogonal IL-2Rβ^+ T_{reg} cells that would produce orthogonal IL-2 in an antigen-dependent positive-feedback loop manner[155].

Enhancing trafficking and other functions to target tissue sites.

Direct administration of T_{reg} cells to the site of inflammation is not feasible for many autoimmune disorders owing to tissue inaccessibility or the presence of multiple affected

sites. This prompts the need to ensure homing of infused T_{reg} cells to the desired sites to limit the risk of off-target activity.

Chemokine receptors, such as CXCR3 and CCR5, have been reported to be important for the recruitment of immune cells in MS[159] and T1D[160,161], whereas CCR6 plays a role in the pathogenesis of psoriasis[162]. Thus, enforced co-expression of a chemokine receptor that recognizes chemokines expressed in the inflammatory environment may enhance T_{reg} cell activity. In analogous studies, chemokine receptor overexpression improved CAR T cell homing to the tumour and enhanced antitumour activity and survival[163–169]. Moreover, co-expression of CXCR4 and a CAR specific for KIT, an antigen expressed by haematopoietic stem cells, in T cells enabled the efficient homing of these cells to the bone marrow and achieved haematopoietic stem cell depletion for allogeneic bone marrow transplantation without irradiation in a mouse model[170].

In patients receiving effector T cell ACT for meta-static melanoma, the therapeutic potential of lymphodepletion followed by the ACT of tumour-infiltrating lymphocytes expressing CXCR2, in combination with high-dose IL-2, is currently being evaluated in a phase I/II clinical trial[171]. Another approach is the manipulation of cytokine receptors and certain transcription factors as described above. Migration properties of human T_{reg} cells can be tailored during in vitro expansion by adding cytokines and/or metabolites to the culture, while maintaining T_{reg} cell stability and function[172]. Thus, combining ectopic chemokine receptor, cytokine receptor or transcription factor expression with antigen specificity via a CAR or TCR might improve T_{reg} cell homing and restrict their activation to target tissue, thereby limiting the risk of off-target activity. An alternative strategy to specifically control the trafficking of adoptively transferred cells to the site of interest would be to design an orthogonal ligand–protein pair engineered to constitutively express the orthogonal chemokine receptor, whereas the expression of the orthogonal chemokine would be triggered by the activation of a CAR or a SynNotch receptor in an antigen-dependent manner. The first engineered T_{reg} cells reaching the site of interest would become activated and secrete the orthogonal chemokine, generating a chemokine gradient that would specifically attract the engineered T_{reg} cells.

In addition to trafficking, several other functional activities can be engineered into T_{reg} cells. This includes incorporating additional payloads, such as selected suppressive factors or tissue repair molecules that may enhance T_{reg} cell efficacy.

Safety and regulatability

Cells used for ACT represent living drugs and may develop unwanted activities. T_{reg} cells may become unstable and a subset could produce effector cytokines that may cause damage, even if the risk would seem less of a concern than that with effector T cell ACT used in cancer immunotherapy. Another concern is the risk of tumorigenesis associated with viral integration near oncogenes. Therefore, it is essential to consider how these cells may be eliminated should they acquire deleterious activities.

Suicide cassettes.

Several suicide programmes that have been developed for CAR effector T cell therapy to prevent or block potential adverse events could also be used for CAR or recombinant TCR T_{reg} cell ACT. Although the risk of cytokine release syndrome reported in some CAR T cell clinical trials appears to be very small with T_{reg} cells as they do not produce pro-inflammatory cytokines, suicide cassettes may be needed in the event that an inflammatory microenvironment induces dysfunction or unstable pathogenic conversion of adoptively transferred T_{reg} cells. Current clinical-grade suicide gene strategies for CAR effector T cell therapy include programming the cells to express a truncated version of epidermal growth factor receptor (EGFRt) that is co-expressed with the CAR on the cell surface; these cells can be eliminated both early and late after transfer through the administration of the EGFRt-directed mAb cetuximab[173,174]. Another suicide gene is RQR8, a 136-amino-acid epitope-based marker engineered by combining epitopes from both CD34 and CD20 antigens, which enables the tracking and deletion of cells in the case of adverse events by making them highly susceptible to lysis by the CD20-directed mAb rituximab[175]. Moreover, Bellicum Pharmaceuticals has developed a safety switch technology, CaspaCIDe, which is a construct composed of a CID-binding domain and a signalling domain derived from caspase 9 (iCasp9), an initiating enzyme in the apoptotic pathway. This construct can be engineered into CAR T cells before infusion and, in the event of toxicity, administration of the small molecule rimiducid triggers the dimerization of the CID domain, which leads to the activation of iCasp9, inducing selective apoptosis of the CaspaCIDe-containing cells. Lastly, a recent study reported that the tyrosine kinase inhibitor dasatinib can be used to induce an immediate blockade of CAR T cell function and that this effect was reversible upon dasatinib removal[176]. Notably, dasatinib treatment was able to halt cytokine production, and more studies are needed to determine whether it may be used as an emergency drug to stop cytokine release syndrome. This on/off switch system represents an attractive tool for CAR T_{reg} cell ACT, notably in the case of unintentional bystander suppression, such as during a local viral infection.

Integration sites.

A critical limitation of recombinant TCR and CAR approaches in T_{reg} cell ACT is the current use of recombinant viral vectors to insert the transgene. In terms of manufacturing, the production and testing of viral vectors are time consuming and costly. Moreover, the transgene integrates randomly in the genome, which carries the risk of causing oncogenic genetic changes. The advent of genome editing technologies such as CRISPR–Cas9 has enabled alternative strategies to improve the safety, efficacy and manufacturing of genetically modified antigen-specific T cells. Using a CRISPR–Cas9-mediated genome targeting system in human primary T cells, it was shown that the endogenous TCR locus can be replaced by a recombinant TCR[177]. Moreover, again in the context of cancer immunotherapy, it was shown that targeting a CAR to the endogenous T cell receptor-α constant (TRAC) locus enables physiologically relevant expression and regulation of CAR recycling in T cells, resulting in superior T cell activation and in vivo activity, as well as delaying T cell differentiation and exhaustion[178]. Importantly, targeting the TRAC locus disrupts the expression of the endogenous TCR, thereby reducing the risk of dual antigen specificity as a consequence of mispairing of α- and β-chains between the native and

recombinant TCRs in the context of engineered TCRs. Moreover, CAR transgene insertion in the TRAC locus enables endogenous expression of the transgene via the TRAC promoter, which prevents overactive CAR signalling and delays effector T cell exhaustion[178]. However, how these targeted gene editing strategies may affect T_{reg} cell homeostasis and function remains to be investigated. Nevertheless, the elimination of the endogenous T_{reg} cell TCR may eliminate the potential for off-target immunosuppressive activity as the T_{reg} cell TCRs are presumed to be selected for the recognition of self-antigens.

Towards universal donor T_{reg} cells

Ongoing clinical trials will continue to validate the therapeutic potential of T_{reg} cell-based therapies. However, an important step towards the broad applicability of ACT is an off-the-shelf T cell product. The source of autologous T_{reg} cells is limiting and current strategies require autologous T_{reg} cell expansion under good manufacturing practice-compliant conditions, which, at present, is very costly. As T_{reg} cell-based therapies move forward, there are several approaches to circumvent the need for autologous T_{reg} cell manufacturing. The use of third-party (unrelated to the donor or recipient) T_{reg} cells has been a strategy in the clinical setting of GVHD in which T_{reg} cells derived from cord blood were used as a short-term solution before endogenous T_{reg} cell generation from donor bone marrow cells. Thus, one approach to off-the shelf T_{reg} cell products would be the generation and use of a bank of T_{reg} cells generated to match different MHCs as well as possible. These could be used as a short-term solution sufficient to enable the establishment of a suppressive environment (that is, infectious tolerance)[179,180]. However, this approach may still be prone to host-mediated elimination of the transferred cells, preventing a durable response. Thus, the generation of T_{reg} cells that escape immune recognition by the host will offer unique opportunities in the development of off-the-shelf products. One approach has been the use of CRISPR–Cas9 technology to render cells HLA deficient, and thereby less immunogenic. However, cells that do not express canonical HLA class I or II molecules are subjected to NK cell killing. One solution may be to ectopically express HLA-E or HLA-G, non-canonical HLA genes expressed during maternal–fetal tolerance that are ligands for inhibitory receptors on NK cells, to bypass NK cell-mediated killing[181]. Another option would be to artificially express the ‘do-not-eat-me-signal’ CD47 in adoptively-transferred T cells to prevent phagocytosis by macrophages[182,183].

Targeting multiple genes in primary T lymphocytes poses technical challenges owing to the substantial safety validation required for each cell product; therefore, banking master pluripotent stem cells with engineered properties may be a viable alternative. Human pluripotent stem cells represent a potentially inexhaustible source of clinically useful cells and are amenable to gene editing[184,185]. To date, there have been a few studies demonstrating the feasibility of T cell generation from pluripotent stem cells[186–190]. These reports have largely focused on the development and characterization of T cell progenitors ($CD4^+CD8^+$) or $CD8^+$ effector T cells for cancer immunotherapy. Furthermore, differentiation of T cells from pluripotent cultures is almost exclusively biased towards $CD8\alpha\alpha^+$ T cells, with little $CD8\alpha\beta^+$ T cell development[189,191]. A better understanding of the mechanisms that control the development of distinct T cell subsets will lay the foundations for the differentiation of therapeutically useful T cells from pluripotent stem

cells, including $CD4^+$ T_{reg} cells. Furthermore, human pluripotent stem cells could be engineered to be exhaustion and inflammation resistant. Genetic engineering approaches have been developed to generate universal donor pluripotent stem cell lines that lack β_2-microglobulin, and therefore MHC class I molecules, and express HLA-E to inhibit NK cell-mediated cytotoxicity[181,192,193]. In principle, these universal donor lines could be differentiated to deliver off-the-shelf T cell therapies; however, the major challenge remains understanding the fundamental pathways that distinguish distinct T cell subsets, including T_{reg} cells, and their precursors.

Conclusion

Within the past decade, we have witnessed the transformative therapeutic potential of adoptive T cell therapy for cancer. The continued validation and success of CAR T cell therapy have paved the way for T_{reg} cell-based therapies for autoimmune diseases and solid organ and bone marrow transplantation. Results from clinical trials focused on T_{reg} cell-based therapies for T1D, SLE and inflammatory bowel disease are emerging[12]. The initial success of T_{reg} cell therapy in autoimmune diseases will likely promote the use of T_{reg} cells to treat non-autoimmune disorders. Transgenic mouse models in which T_{reg} cells are specifically ablated have uncovered a non-traditional role for tissue-resident T_{reg} cells in modulating processes that promote tissue repair and wound healing in several settings independent of their suppressive activity[194–197]. T_{reg} cells have also been shown to have neuroprotective effects in mouse models of Alzheimer disease[198,199], and there are ongoing small phase I clinical trials to evaluate the safety and tolerability of ACT with expanded polyclonal T_{reg} cells in patients with amyotrophic lateral sclerosis[200,201]. However, T_{reg} cell therapy alone may be insufficient to induce tolerance and it is unlikely that T_{reg} cells will be used as a single therapeutic (BOX 2).

Finally, we anticipate that the use of polyclonal T_{reg} cell therapies will shift to antigen-specific T_{reg} cells generated with improved non-viral gene editing approaches[177] and efforts towards deriving off-the-shelf products (FIG. 4). CRISPR–Cas9-mediated technology allows the rapid and efficient generation of genetic perturbations to identify novel pathways regulating T cell stability, proliferation and function[202]. Beyond the therapeutic application of T_{reg} cells for autoimmune diseases, T_{reg} cells may be exploited for more generalized inflammatory diseases and in combination therapies. Autoinflammatory adverse events are often associated with cancer immunotherapy (for example, checkpoint inhibitors) and therefore T_{reg} cells may be used concurrently[203]. However, such T_{reg} cells should be engineered to possess target-tissue specificity to prevent or treat the adverse autoimmune event and not dampen the antitumour response. There remain several unanswered questions related to fundamental T_{reg} cell biology, T_{reg} cell engineering and clinical translation (BOX 3). However, this is an exciting era of gene and cell therapy, with numerous new tools and strategies to accelerate T_{reg} cell-based immunotherapies. Finding the proper balance between immunosuppression and immune surveillance will be key to success as we move towards T_{reg} cell-based therapies as an immunotherapeutic modality.

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Direct allospecificity

A process by which donor-derived antigen-presenting cells present allogeneic major histocompatibility complex–peptide complexes.

Indirect allospecificity

A process by which host-derived antigen-presenting cells present self-major histocompatibility complex–allogeneic peptide complexes.

Trogocytosis

A process whereby lymphocytes physically extract surface molecules from antigen-presenting cells.

Infectious tolerance

A phenomenon in which a tolerance-inducing state is transferred from one cell population to another.

Pauciclonal

The presentation of limited clonal variation.

Domain swap interface

The interface of a three-dimensional process by which two identical protein chains exchange part of their structure to form an intertwined dimer or higher-order oligomer.

Box 1 |

Considerations for T_{reg} cell subset selection

Early efforts to purify T_{reg} cells using only the CD4 and CD25 markers resulted in contamination by activated effector T cells. However, the identification of an additional marker, CD127 (also known as IL-7 receptor), allowed to distinguish effector T cells from T_{reg} cells as the $CD4^+CD25^+CD127^{lo/-}$ population is highly enriched for $FOXP3^+$ cells[204]. T_{reg} cell-associated epigenetic changes, including >95% of DNA demethylation of a conserved non-coding region upstream of FOXP3, named the T_{reg} cell-specific demethylated region (TSDR), characterize the most stable T_{reg} cells[205–208]. Subsequently, other markers were identified (for example, CD49d) to purify T_{reg} cells but yields have not been as reliable as T_{reg} cells purified using the CD4, CD25 and CD127 markers[209]. In fact, there is an increasing number of T_{reg} cell subsets that develop both as a consequence of their maturation state and the environment where they develop.

Human T_{reg} cells can be subdivided into naive, effector and memory cell compartments based on the expression of CD45RA, CD45RO, CCR7, HLA-DR, CD27 and L-selectin[210,211]. Several studies have suggested that naive $CD45RA^+$ T_{reg} cells have a greater proliferative capacity when expanded ex vivo, display a more stable phenotype and have a higher suppressive capacity compared with effector or memory T_{reg} cells[212,213]. Moreover, at present, most of the clinical applications of T_{reg} cells for adoptive cell therapy (ACT) have been limited to unseparated cord blood-derived and adult peripheral blood-derived T_{reg} cells. However, the percentage of naive T_{reg} cells decreases with age and the majority of the T_{reg} cells in adult blood are $CD45RA^-$, hampering the ability to expand naive T_{reg} cells from older patients for ACT[211,214]. Therefore, the clinical application of T_{reg} cells to date has been limited to unselected T_{reg} cell subsets as described below. More importantly, recent studies have suggested that mature T_{reg} cells can take on characteristics similar to effector T cell subsets. For instance, in the presence of T helper type 1 (T_H1) cytokines, T_{reg} cells express the transcription factor T-bet, which controls genetic programmes that affect chemokine receptor expression and other T_H1-like attributes, whereas T_H2 or T_H17 immune responses are best controlled by T_{reg} cells expressing GATA3 or RORγ, respectively. In this context, recent studies also revealed the existence of tissue-specific T_{reg} cells in visceral adipose tissue[215], skin[216,217], skeletal muscle[218,219], colonic lamina propria[220], placenta[221,222] and other non-lymphoid tissues in both mouse and human. These appear to be from diverse origins (both the thymus and periphery)[41,218,222,223] and possess a distinct and clonally expanded T cell receptor repertoire, suggesting that they accumulate in tissues in an antigen-dependent manner[215,218,224].

To acquire a tissue-specific phenotype, T_{reg} cells undergo extensive epigenetic reprogramming, leading to the expression of tissue-specific transcription factors and molecular mediators[223,225,226]. The role of tissue-resident T_{reg} cells and their mechanisms of action remain to be fully elucidated. These may inform novel T_{reg} cell-based therapies for general inflammatory disease settings but would only be suitable for ACT if there was an effective way to isolate and expand them or to engineer equivalent cells. Thus, the subsets and mechanisms of action of T_{reg} cells are quite diverse and

future efforts will be devoted to exploiting these different subtypes of T_{reg} cells directed to local environments and broad inflammatory activities rather than direct antigen-targeted responses (FIG. 1).

Box 2 |

T_{reg} cell-compatible therapeutics

As discussed in the main text, combination approaches for regulatory T (T_{reg}) cell adoptive cell transfer include the concomitant use of IL-2 receptor and signal transducer and activator of transcription 5 (STAT5) agonists, but other approaches may include debulking the intrinsic effector T cell responses (which are often associated with resistance to T_{reg} cell-based therapies[227]) through the use of anti-T cell therapies including anti-thymocyte globulin (for example, THYMOGLOBULIN (Sanofi)) or inhibitors of tumour necrosis factor (TNF), a cytokine that triggers pro-inflammatory autocrine loops[228]. However, it should be noted that the physiology of T_{reg} cells and effector T cells can overlap. Paradoxically, on occasion, patients with autoimmune conditions treated with TNF inhibitors develop aggravated disease or a new autoimmune disease, including severe chronic inflammatory arthritis[229–231], and polymorphisms in the *TNFR2* gene have been strongly correlated with several autoimmune diseases, such as rheumatoid arthritis[232,233], systemic lupus erythematosus[234], Crohn's disease and ulcerative colitis[235], perhaps owing to the expression of high levels of TNFR2 (REF.[236]) on T_{reg} cells, which plays a role in T cell receptor-mediated proliferation and suppressive function under inflammatory conditions[237–239]. This is one example that demonstrates the conflicting roles for certain signalling pathways in human T_{reg} cells versus effector T cells. Similar challenges have been observed with IL-2 therapy, mTOR inhibitors and other potential combination treatments where it will be critical to study the effects of concurrent inflammation and effector T cell inhibitors in the clinic. It may be necessary to alter timing and specificity (consider TNFR1 antagonists rather than systemic TNF inhibitors, for instance) when inhibiting pro-inflammatory pathways during T_{reg} cell adoptive cell transfer treatment of organ transplant rejection and autoimmune diseases.

Box 3 |

Unanswered questions important for human T_{reg} cell-based therapy

T_{reg} cell biology

- Which markers can be used to distinguish tissue regulatory T (T_{reg}) cells from peripheral T_{reg} cells?
- Where is the site of action of T_{reg} cells (that is, tissue and/or draining lymph nodes)?
- What are the relevant mechanisms of action of T_{reg} cells in vivo?
- Is infectious tolerance real?
- What are the mechanisms that regulate T_{reg} cell stability?

T_{reg} cell manufacturing and engineering

- What are the optimal T_{reg} cell source, isolation method and culture condition to generate the most suitable T_{reg} cells for therapy?
- What are the best co-stimulatory domains in chimeric antigen receptor (CAR) constructs for T_{reg} cells (for example, optimal activation, absence of tonic signalling and stability)?
- Will deletion of the endogenous T cell receptor (TCR) affect CAR or TCR-engineered T_{reg} cell homeostasis?
- How can functional T_{reg} cells be generated from induced pluripotent stem cells?
- Does CAR-mediated T_{reg} cell suppression differ from TCR-mediated suppression in vitro and in vivo (for example, in terms of mechanisms of action, efficacy)?

T_{reg} cell therapy clinical outcome

- What is (are) the optimal dose(s) and timing for T_{reg} cell administration?
- Will patients need to be preconditioned to create space for infused T_{reg} cells and/or reduce the number of pre-existing memory T cells?
- will T_{reg} cells persist after infusion into patients?
- will T_{reg} cells destabilize and convert into effector T cells under pro-inflammatory conditions in vivo?
- Is there a risk of generalized immunosuppression with polyclonal T_{reg} cells?
- Is cytokine release syndrome a risk after CAR T_{reg} cell infusion?
- Is there a risk of unintentional bystander suppression with antigen-specific T_{reg} cells (for example, to a local viral infection)?
- Can T_{reg} cell-based therapy be used for non-autoimmune disorders?

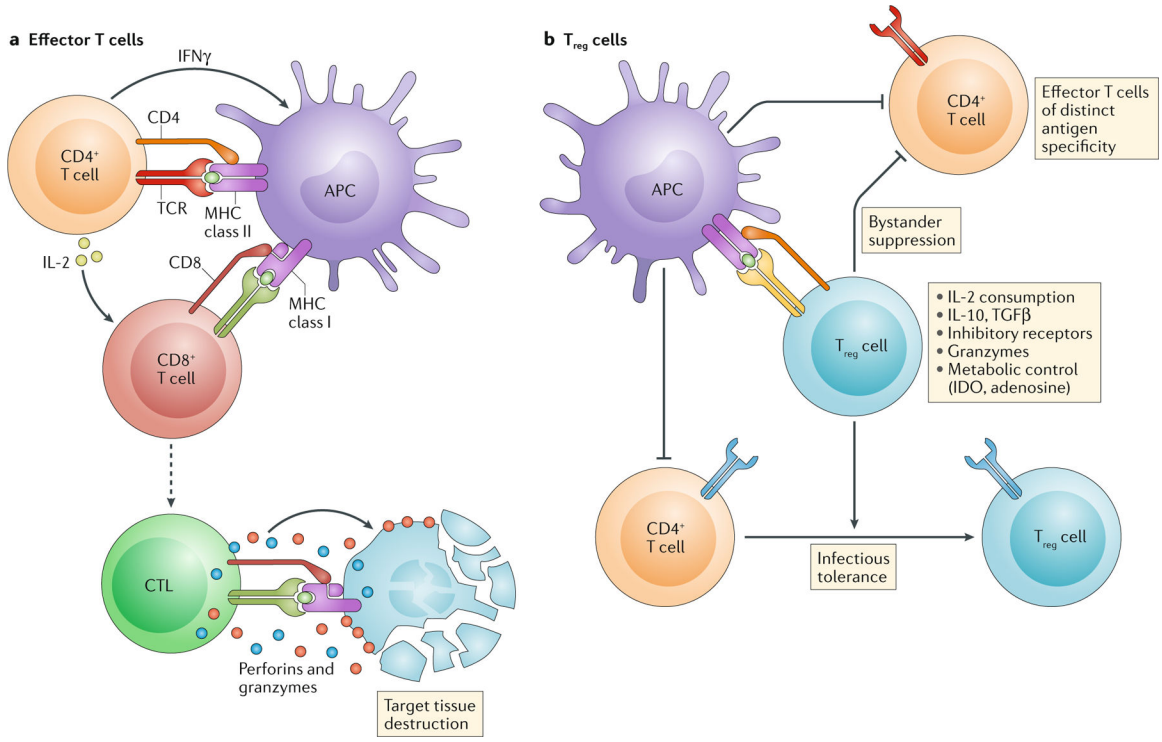


Fig. 1 |. Mechanisms of action of effector T cells versus T_{reg} cells.

a | The recognition of specific antigens presented or cross-presented by antigen-presenting cells (APCs) activates $CD4^+$ and $CD8^+$ T cells, respectively. Following activation, $CD4^+$ T cells produce cytokines that help $CD8^+$ T cells to differentiate into cytotoxic T lymphocytes (CTLs). CTLs then kill target cells by performing their cytotoxic activity in an antigen-specific and cell contact-dependent manner. **b** | Like effector T cells, regulatory T (T_{reg}) cell activation is antigen specific. However, once activated, T_{reg} cells exert bystander suppression, meaning that they have suppressive function regardless of their antigen specificity. T_{reg} cells suppress effector T cells using direct or indirect mechanisms of action. Finally, T_{reg} cells can spread their suppressive properties to neighbouring cells via a phenomenon named infectious tolerance. IDO, indoleamine-2,3-dioxygenase; IFNγ, interferon-γ; MHC, major histocompatibility complex; TCR, T cell receptor; TGFβ, transforming growth factor-β.

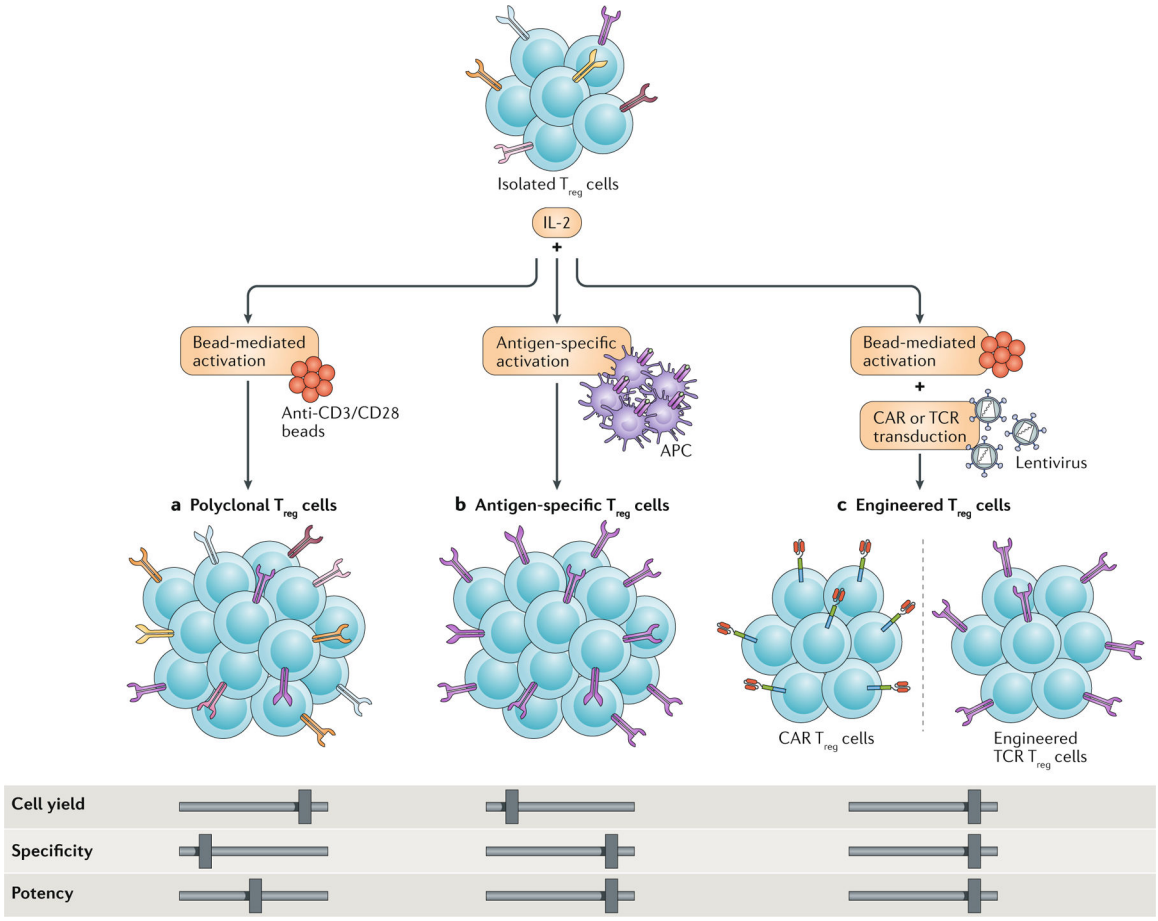


Fig. 2 |. Various T_{reg} cell products for T_{reg} cell-based therapy.

There are currently three main regulatory T (T_{reg}) cell products developed for adoptive cell therapy (ACT). **a** | The most widely used product in clinical trials is expanded polyclonal T_{reg} cells that were isolated from peripheral blood and expanded in vitro using high-dose IL-2 and anti-CD3/CD28 beads to generate a high number of T_{reg} cells for ACT. **b** | Another approach, mainly used in transplantation to prevent graft rejection, is the use of antigen-presenting cells (APCs) from the graft donor to specifically stimulate alloreactive T_{reg} cells from the recipient in vitro. These antigen-specific T_{reg} cells have been proven to be more potent than polyclonal T_{reg} cells. However, the cell yield is relatively low after expansion, and this approach cannot be adapted for the treatment of autoimmune diseases owing to the low precursor frequencies of specific autoantigen-reactive T_{reg} cells. **c** | A third approach is the expansion of polyclonal T_{reg} cells genetically engineered to express a synthetic receptor (chimeric antigen receptor (CAR) or artificial T cell receptor (TCR)) that recognizes a target antigen of interest. With this strategy, a high number of antigen-specific T_{reg} cells can be obtained after expansion.

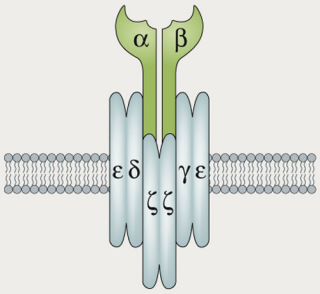
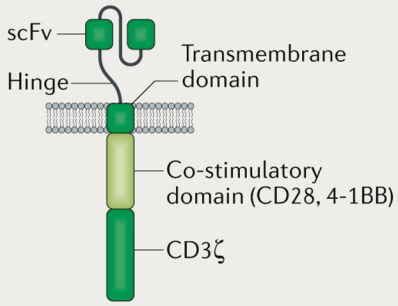
Engineered TCRs	CARs
	
Multi-protein complex	Single protein
Low affinity	High and adjustable affinity
Recognition is MHC-restricted	Recognition is MHC-independent (broader applicability)
Recognize processed extracellular and intracellular antigens bound to MHC molecules	Recognize whole proteins, including MHC–peptide complexes and extracellular antigens present in the ECM
Risk of mispairing with endogenous TCR	CRS reported with CAR T cell ACT but unlikely with CAR T_{reg} cells

Fig. 3 |. Characteristics of recombinant T cell receptors versus chimeric antigen receptors.
T cell receptors (TCRs) are commonly composed of a heterodimer of one α-chain and one β-chain that can bind to a specific peptide–major histocompatibility complex (MHC) and subsequently activate the CD3 complex that is made of four signalling subunits: one dimer of CD3ε and CD3γ chains, one dimer of CD3ε and CD3δ chains, and one homodimer of CD3ζ chains. Recombinant TCRs are generated through the integration of the genes that encode for the α- and β-chains of a TCR specific for an antigen of interest. Second-generation chimeric antigen receptors (CARs) are artificial immunoreceptors composed in their extracellular part of a single-chain variable fragment (scFv) capable of binding a target antigen that is linked to a transmembrane domain via a hinge and in their intracellular part of a co-stimulatory domain (CD28 or 4–1BB) and a CD3ζ domain that together effectively trigger the activation of the CAR-expressing T cell after binding of the antigen. ACT, adoptive cell therapy; CRS, cytokine release syndrome; ECM, extracellular matrix; T_{reg} cell, regulatory T cell.

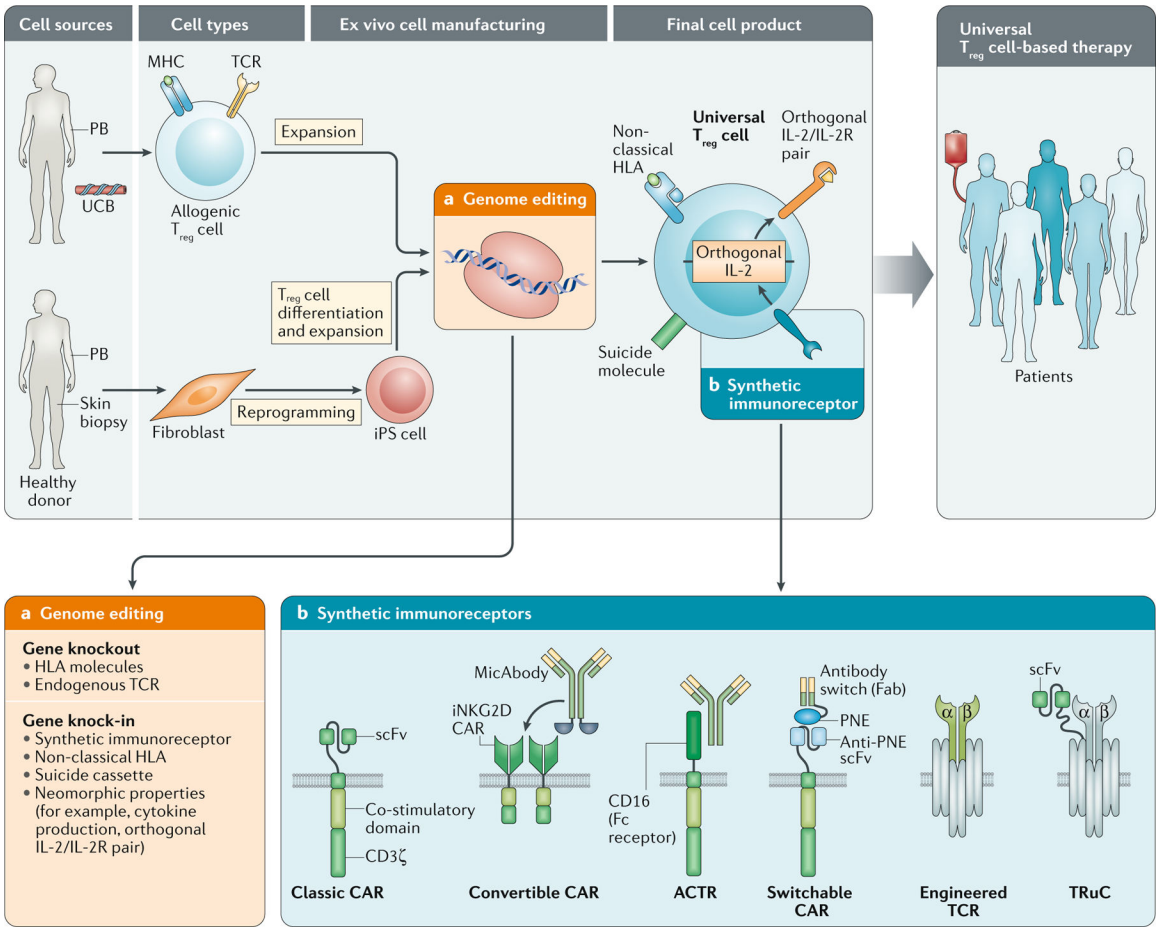


Fig. 4 |. The future of T_{reg} cell-based therapy.

Universal donor regulatory T (T_{reg}) cells for adoptive cell therapy represent the ultimate goal to broaden the use of T_{reg} cells as therapeutics. Recent advances in genome editing and ongoing studies aimed at differentiating various T cell subsets from induced pluripotent stem (iPS) cells have the potential to enable the generation of T_{reg} cells compatible with any major histocompatibility complex (MHC) class and equipped with an adjustable immunoreceptor to confer the appropriate antigen specificity based on patient needs. T_{reg} cells will be isolated from a healthy donor (from peripheral blood (PB) or umbilical cord blood (UCB)) or differentiated from iPS cells, which can be derived from fibroblasts. T_{reg} cells will be expanded in vitro and subjected to genome editing using a non-viral approach, such as CRISPR–Cas9 technology, to equip the cells with a synthetic immunoreceptor and other payloads such as an orthogonal IL-2/IL-2 receptor (IL-2R) pair. **a** | By knocking out the donor HLA molecules and knocking in a non-classical HLA, T_{reg} cells can be made compatible with any HLA profile while being protected from natural killer cell-mediated cytotoxicity. Moreover, based on recent effector T cell studies, we envision that replacing the endogenous T cell receptor (TCR) with a synthetic immunoreceptor will confer the desired antigen specificity to T_{reg} cells to improve their potency while removing the endogenous TCR to prevent mispairing. In addition, the potency of the engineered T_{reg} cells will be optimized by equipping them with neomorphic properties, such as the expression of chemokine receptors to enhance their migration to the target tissue, the production of

immunosuppressive cytokines to improve their suppressive activity as well as the expression of the orthogonal pair of IL-2/IL-2 receptor (IL-2R) to promote their specific survival. Importantly, technologies, such as the Synthetic Notch (SynNotch) system, may be used to control the expression and activity of these neomorphic properties. Finally, in case of an adverse event (for example, cell instability, off-target toxicity), engineered T_{reg} cells will express a suicide cassette to allow efficient and rapid elimination if necessary. **b** | Although most of the effort in the T_{reg} cell field to redirect the cell antigen specificity has been made using classic chimeric antigen receptors (CARs) or TCRs, the ideal engineered immunoreceptor should be universal with an adjustable antigen recognition domain (that is, the antigen recognition domain should be separated from the signalling domain of a conventional CAR, enabling one to pick and even switch the antigen specificity of the cells). To this aim, alternative approaches, such as by Xyphos Biosciences (convertible CAR), Unum Therapeutics (ACTR, antibody-coupled TCR) and CALIBR (switchable CAR), focus on the development of universal binding sites for antigen receptors to redirect tissue targeting. Although these approaches have been developed for effector T cells, they could also be translated to T_{reg} cell engineering to direct the cells to specific sites of inflammation using universal binding platforms. Similar to CARs, engineered TCRs are capable of redirecting the antigen specificity of T_{reg} cells, and novel TCR-based technologies are being evaluated. Notably, TCR2 Therapeutics has designed a T Cell Receptor Fusion Construct (TRuC) platform that consists of the fusion of a single-chain variable fragment (scFv) specific for a surface antigen of interest to the extracellular N termini of one of the TCR subunits, which enables the recognition of the antigen on target cells without the need for HLA presentation and engages the complete TCR machinery. MicAbody, modified MHC-class I-related chain A fused to an antibody; iNKG2D, inert natural killer group 2, member D receptor; PNE, peptide neo-epitope.