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Next-generation regulatory T cell therapy

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Abstract

Regulatory T cells (T_{reg} cells) are a small subset of immune cells that are dedicated to curbing excessive immune activation and maintaining immune homeostasis. Accordingly, deficiencies in T_{reg} cell development or function result in uncontrolled immune responses and tissue destruction and can lead to inflammatory disorders such as graft-versus-host disease, transplant rejection and autoimmune diseases. As T_{reg} cells deploy more than a dozen molecular mechanisms to suppress immune responses, they have potential as multifaceted adaptable smart therapeutics for treating inflammatory disorders. Indeed, early-phase clinical trials of T_{reg} cell therapy have shown feasibility, tolerability and potential efficacy in these disease settings. In the meantime, progress in the development of chimeric antigen receptors and in genome editing (including the application of CRISPR–Cas9) over the past two decades has facilitated the genetic optimization of primary T cell therapy for cancer. These technologies are now being used to enhance the specificity and functionality of T_{reg} cells. In this Review, we describe the key advances and prospects in designing and implementing T_{reg} cell-based therapy in autoimmunity and transplantation.

The adaptive immune system has evolved to recognize and destroy a virtually infinite variety of pathogens while remaining unresponsive towards self-tissues; this state is known as immune tolerance. Immune tolerance is maintained by a multilayered, interconnected and redundant array of dominant and recessive mechanisms, ensuring that immune responses are regulated in an effective and timely manner[1,2]. Recessive immune tolerance mechanisms are cell intrinsic and include the deletion of self-reactive immune cells, rendering them non-

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Author contributions

All authors contributed to all aspects of the article. L.M.R.F. and Y.D.M. contributed equally.

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functional (that is, subject to anergy) and increasing the number of inhibitory receptors on immune cells to increase their activation threshold. By contrast, dominant immune tolerance mechanisms are cell extrinsic and are carried out by subsets of specialized immune cells that actively suppress the activation, expansion and function of other immune cells, thereby regulating the intensity and the duration of immune responses.

Anergy

Peripheral mechanism for tolerizing T cells in which they are blocked at the G1 phase of the cell cycle and unable to proliferate.

Autoimmune disorders arise from defects in immune tolerance and affect more than 50 million individuals in the United States alone and more than 4% of the world population. Progress in treating individuals with these diseases has been slow owing to the complex mechanisms underlying the balance between immune reactivity and immune tolerance. Although small-molecule and biologic treatments can alleviate symptoms, they are often non-specific, require long-term administration (and thus long-term exposure to the toxic effects associated with them) and do not account for variability in underlying disease pathogenesis and drug responses. For instance, the mainstay treatment for severe systemic lupus erythematosus, an autoimmune disorder caused by autoreactive B cells, is steroids, which non-specifically suppress inflammation. Living drugs, such as regulatory T cells (T_{reg} cells), may have greater specificity and more complex therapeutic benefits than conventional immunosuppressive drugs (such as steroids and ciclosporin), biologics (such as rituximab and belimumab), antimetabolites (such as azathioprine and methotrexate) and alkylating agents (such as cyclophosphamide), amongst others, and could potentially cure disease by restoring immune tolerance.

T cell-based antigen-specific immune tolerance was first postulated in 1970 (REF.[1]). However, T_{reg} cells were not identified as the main cell type responsible for this phenomenon until the 1990s[2]. T_{reg} cells constitute 5% of circulating $CD4^+$ T cells and can be identified by the lineage marker forkhead box protein P3 (FOXP3). Mutations in the gene encoding FOXP3 (a transcription factor), as well as in genes encoding other molecules that modulate T_{reg} cell function, such as the surface receptors cytotoxic T lymphocyte protein 4 (CTLA-4) and CD25 (also known as IL-2 receptor subunit-α, part of the trimeric high-affinity IL-2 receptor) and the transcription factor signal transducer and activator of transcription 5 (STAT5), lead to the development of severe autoimmune polyendocrine syndromes; the best known example of these syndromes is immunodysregulation polyendocrinopathy enteropathy X-linked syndrome[3]. Moreover, T_{reg} cells can become unstable, losing FOXP3 expression and immunosuppressive function, converting into effector T cells (T_{eff} cells) under extreme inflammatory conditions[4,5]. The inability of T_{reg} cells to produce IL-2 while expressing high levels of CD25 is a cardinal feature of T_{reg} cells. Thus, in the absence of IL-2 produced by other cell subtypes, or signalling by its receptor, there is a decrease in the number and functional activity of the T_{reg} cells, leading to inflammation and autoimmunity[6,7]. Expression of CD127 (also known as IL-7 receptor subunit-α) is inversely correlated with the expression of FOXP3 and the suppressive function of human T_{reg} cells, and is currently used in conjunction with CD25 as a

phenotypic marker in the purification of T_{reg} cells, defined as $CD4^+CD25^+CD127^{low}$ T cells[8,9]. However, the best indicator of a stable T_{reg} cell lineage is demethylation of the T_{reg} cell-specific demethylated region (TSDR), an evolutionarily conserved non-coding regulatory sequence in the *FOXP3* locus; demethylation of the TSDR ensures high, stable levels of FOXP3 (REF.[10]).

Systemic lupus erythematosus

A group of chronic autoimmune disorders defined by inflammation affecting various connective tissues in various organs, including the skin, joints, kidney, lung, nervous system or haematopoietic system.

Autoimmune polyendocrine syndromes

A group of diseases characterized by loss of tolerance and inflammation in endocrine glands, including the thyroid, parathyroid and adrenal glands or the pancreas. They are frequently associated with alopecia, vitiligo, coeliac disease and autoimmune gastritis.

Immunodysregulation polyendocrinopathy enteropathy X-linked syndrome

Specific form of an inherited autoimmune polyendocrine syndrome characterized by a mutation or mutations in the master transcription factor forkhead box protein P3 gene (*FOXP3*), leading to regulatory T cell dysfunction.

Type 1 T helper cell

(T_H1 cell). A type of $CD4^+$ T helper cell expressing TBET as a key transcription factor and defined by its ability to preferentially secrete interferon-γ and induce $CD8^+$T cell and macrophage activation.

Type 2 T helper cell

(T_H2 cell). A type of $CD4^+$ T helper cell expressing GATA3 as a key transcription factor and defined by its ability to preferentially secrete Il-4, IL-5 and IL-13 and promote B cell expansion and antibody class switching.

IL-17-producing T helper cell

(T_H17 cell). A type of $CD4^+$ T helper cell expressing RORγt as a key transcription factor and defined by the production of IL-17, a cytokine important for maintaining mucosal barrier integrity and clearing helminth infections.

The dominance and durability of T_{reg} cell-mediated immune tolerance is underscored by two main features: bystander suppression and infectious tolerance. During bystander

suppression, which was first described by Weiner and colleagues[11] in 1991, T_{reg} cells activated by one antigen suppress immune responses against other antigens. During infectious tolerance, a term coined by Gershon and Kondo[12] in 1971 and expanded by Waldmann and colleagues[13] in 1993, suppressive capacity is transferred from one cell population to another. This process is thought to occur mainly via the production of inhibitory cytokines by T_{reg} cells; these cytokines block dendritic cell (DC) maturation and migration, creating a local tolerogenic environment in which T_{eff} cells undergo apoptosis and naive T cells are converted to induced T_{reg} cells. Molecularly, T_{reg} cells act through pleiotropic mechanisms, depending on their target cells and whether they are in lymphoid organs or in non-lymphoid tissues[14]. Furthermore, studies in mice and humans show that T_{reg} cells become specialized, converting into cells with type 1 T helper cell (T_H1 cell)-like, type 2 T helper cell (T_H2 cell)-like and IL-17-producing T helper cell (T_H17 cell)-like phenotypes, characterized by distinct patterns of chemokine receptor, cytokine and transcription factor expression[15–18]. Genetic deletion experiments in vivo indicate that, by expressing chemokine receptors similar to those on specific T helper cell subsets, specialized T_{reg} cells can more efficiently suppress their targets[19]. In addition to suppressing immune responses, murine T_{reg} cells promote tissue repair following viral infection[19].

Of note, $CD4^+FOXP3^+$ T_{reg} cells are not the only immunosuppressive cells. Other cell types with immunosuppressive functions include $CD8^+$ T_{reg} cells, IL-10-producing type 1 T_{reg} cells (T_R1 cells), transforming growth factor-β (TGFβ)-producing $CD4^+$ T_H3 cells, regulatory γδ T cells, regulatory B cells (B_{reg} cells), myeloid-derived suppressor cells, immunosuppressive plasmocytes, regulatory invariant natural killer (NK) T cells and even subsets of innate lymphoid cells (BOX 1). Yet, to date, $FOXP3^+$ T_{reg} cells are the only known cell lineage arising in the thymus that is exclusively dedicated to inducing and maintaining immune tolerance. Moreover, almost all ongoing clinical trials using cell therapy to induce immune tolerance use $CD4^+FOXP3^+$ T_{reg} cells. Hence, in this Review, we focus on strategies to engineer $CD4^+FOXP3^+$ T_{reg} cells as the next generation of living drugs for treatment of autoimmune and inflammatory diseases. First, we critically assess the current status of T_{reg} cell therapy, including challenges in the isolation and manufacture of T_{reg} cells, finding the best disease indication and the potential crosstalk of T_{reg} cell therapy with other immunosuppressive treatments. Then we discuss the prospects of tailoring T_{reg} cell specificity and function using genome editing and synthetic biology.

Fundamentals of T_{reg} cells as a therapy

T_{reg} cells are an attractive therapeutic candidate for restoring immune tolerance in autoimmune and autoinflammatory diseases, and thus for reducing or replacing immunosuppressive drugs. T_{reg} cells are also being considered as a therapy for inducing tolerance to allogeneic cells and tissues upon the transplantation of haematopoietic stem cells and solid organs. As of July 2019, 51 clinical trials using T_{reg} cells had been registered in ClinicalTrials.gov, of which six have been completed (NCT01634217, NCT00602693, NCT01210664, NCT02166177, NCT02244801 and NCT02129881), five terminated (NCT02428309, NCT00725062, NCT01050764, NCT00376519 and NCT01818479), four suspended (NCT02494492, NCT02991898, NCT02526329 and NCT03773328) and two withdrawn (NCT02118311 and NCT01163201) (FIG. 1). Overall, these studies demonstrate

the feasibility and safety of T_{reg} cell infusion, although their small size, in general, limited the opportunity to assess efficacy. The relatively high rate of prematurely terminated, suspended or withdrawn trials mostly reflects challenges in manufacturing T_{reg} cells and in selecting and recruiting patients, as discussed later in this Review.

T_{reg} cells as living drugs

In traditional pharmacology, a drug is characterized according to its pharmacodynamics, that is, its effect in the body (such as its on-target and off-target interactions), and its pharmacokinetics, that is, the effect of the body on its absorption, distribution and metabolism. Several properties are key to successfully using T_{reg} cells as living drugs, and these can be summed up as the four S's: suppression, survival, stability and specificity (FIG. 2).

T_{reg} cell-meditated immunosuppression—The main goal of T_{reg} cell therapy is to induce or re-establish immune tolerance; this goal requires the suppressive function of T_{reg} cells, which is influenced by factors such as their activation status, their cytokine milieu, the availability of antigen and the affinity of the T cell receptors (TCRs) for the recognized antigens. Although continuous TCR signalling is required for the repressive function of T_{reg} cells[20], and T_{reg} cells are highly sensitive to activation by the recognized antigen[21], the impact of TCR affinity on T_{reg} cell-mediated suppression is unclear. Some studies suggest that T_{reg} cells expressing high-affinity TCRs have a more potent suppressive function than T_{reg} cells expressing low-affinity TCRs[22,23], whereas others have found that T_{reg} cells expressing TCRs with affinities that differ by several orders of magnitude have a similar suppressive function[24].

Mixed lymphocyte reactions

In vitro tests consisting of mixing different subsets of T cells together in the presence of antigen-presenting cells.

There are several mechanisms of T_{reg} cell-mediated suppression, including IL-2 deprivation (wherein T_{reg} cells act as an IL-2 sink, reducing the primary growth and survival factor for T_{eff} cells), the secretion of inhibitory cytokines (such as IL-10 and TGFβ) and the acquisition of costimulatory molecules from antigen-presenting cells (APCs) via high-affinity binding to CTLA-4 (REF.[25]). Currently, the suppressive function of human T_{reg} cells is primarily quantified by measuring the degree to which they inhibit T_{eff} cell proliferation in vitro or prevent graft-versus-host disease (GvHD) in humanized mice. Additional in vitro assays include measuring T_{reg} cell-mediated suppression of mixed lymphocyte reactions, which depends on the interaction of CTLA-4 on T_{reg} cells with DCs[26], and the inhibition of inflammatory cytokine production by T_{eff} cells[27].

Different subsets of T_{reg} cells specialize in suppressing specific T helper cell subsets: T_H1 cell-like T_{reg} cells, which have a similar gene signature to T_H1 cells, are most effective in inhibiting T_H1 cells, T_H2 cell-like T_{reg} cells are most effective in inhibiting T_H2 cells, and so on[14,16]. Furthermore, studies in mice have demonstrated that the expression of chemokine receptors on T_{reg} cell subsets must mimic the expression of chemokine receptors on T helper

cell subsets for specialized T_{reg} cells to preferentially migrate and suppress specific T helper cell-driven immune responses[14]. The approach to maximize the suppressive function of T_{reg} cells will likely need to be tailored for each therapeutic application.

T_{reg} cell survival—Unlike small molecules and biologics, living cells can change their identity or undergo apoptosis in response to the levels of oxygen, nutrients and signalling molecules in their new environment. Indeed, T_{reg} cells infused in humans and in non-human primates rapidly (that is, within 2 weeks) decreased in number[28–30]. However, using deuterium to label infused T_{reg} cells, a later study showed that a marked percentage of infused T_{reg} cells are detected in some patients up to 1 year following infusion[31]; these data indicate that better techniques, such as the use of gene-modified cells, are needed to assess, and increase, the survival of human T_{reg} cells in patients. Circulating T_{reg} cells constantly require exogenous IL-2 for their survival, as they do not produce this cytokine themselves[6]. Nevertheless, tissue-resident T_{reg} cells can become IL-2 independent, relying instead on IL-7 and IL-33 for their survival and stability[32,33]. New insights into the metabolic requirements and signalling circuitry of T_{reg} cells may yield strategies to enhance T_{reg} cell survival.

T_{reg} cell stability—FOXP3 expression is central to the T_{reg} cell lineage. Loss of FOXP3 expression and/or ablation of the TSDR in the *FOXP3* locus results in loss of T_{reg} cell identity and systemic autoimmunity[3,34]. In response to proinflammatory or otherwise inhospitable conditions, T_{reg} cells transdifferentiate into T_{eff} cells (also known as 'ex-T_{reg} cells'), including pathogenic T_H1 cells, T_H2 cells and T_H17 cells[4,5,35]. Thus, once administered, antigen-specific T_{reg} cells targeting a specific tissue could convert into pathogenic cells with the same specificity and further exacerbate tissue damage. Strategies to increase T_{reg} cell stability include the ectopic expression of the transcription factors FOXP3, HELIOS and BACH2 (REFS[36–38]), which activate or repress gene transcription, or of a constitutively active form of STAT5 (REF.[6]), as well as the genetic ablation of *PRKCQ* (encoding protein kinase C-θ (PKCθ); PKCθ is found in the immune synapse and controls early T cell activation events critical for T_{reg} cell stability)[39,40], *STUB1* (encoding the E3 ubiquitin-protein ligase carboxy terminus of Hsp70-interacting protein (CHIP)) or *CCAR2* (encoding deleted in breast cancer gene 1 protein (DBC1)); CHIP and DBC1 promote FOXP3 degradation[41,42].

T_{reg} cell specificity—Most clinical trials conducted to date using T_{reg} cell therapy have used ex vivo expanded polyclonal T_{reg} cells. Yet, antigen-specific T_{reg} cells are superior to their polyclonal counterparts in their migration to, and persistence in, the target tissue, and in their execution of a local immunosuppressive response[43,44]. These properties of antigen-specific T_{reg} cells will allow, compared with polyclonal T_{reg} cells, the use of fewer cells, greatly reducing the risk of inducing unwanted, widespread, non-specific immunosuppression. In addition to isolating and expanding endogenous antigen-specific T_{reg} cells, it is also possible to create T_{reg} cells with a desired specificity by TCR gene transfer[23,45–47]. The use of artificial immune receptors, such as chimeric antigen receptors (CARs), to redirect T_{reg} cell specificity towards pathogenic T cells or the affected tissue has expanded this approach[48,49].

Manufacturing T_{reg} cells for therapy

Manufacturing T_{reg} cells involves choosing the source from which T_{reg} cells should be isolated as well as the methods for purifying and expanding T_{reg} cells, product specification and release criteria. Detailed information on protocols for producing T_{reg} cells is often only partially provided in the literature. A 'minimum information about T regulatory cells' document has been generated in a first but critical step towards process reproducibility and standardization[50].

Sources of human T_{reg} cells—Several sources of human T_{reg} cells have been explored. Peripheral blood is the most accessible, and often the only, option for autologous applications. Umbilical cord blood (UCB) has been successfully tested in GvHD using partially human leukocyte antigen (HLA)-matched T_{reg} cells from non-autologous UCB donors[30,51,52]. T_{reg} cells can also be isolated from discarded thymuses removed during paediatric cardiac surgery. Approximately 300×10^6 $CD4^+CD25^+$ T_{reg} cells can be isolated from thymuses from one donor[53], which is equivalent to the number of T_{reg} cells in the entire blood volume of an adult donor. Thus, paediatric thymuses may be an attractive source of non-autologous T_{reg} cells, even though this source is yet to be tested in clinical trials or multiple preclinical models[53].

Methods of purifying T_{reg} cells—If T_{reg} cells are to be expanded in vitro before infusion, the starting T_{reg} cell population must be of high purity. This requirement is because T_{reg} cells proliferate slowly when compared with conventional $CD4^+$ T cells and $CD8^+$ T cells. Minor conventional $CD4^+$ T cell and $CD8^+$ T cell contaminants at the outset of the expansion can allow cells other than T_{reg} cells to overtake the culture, preventing the generation of a pure T_{reg} cell product. The markers initially used to isolate T_{reg} cells were CD4 and CD25. Although expression of these markers is sufficient for isolating T_{reg} cells from UCB, it was not sufficient for isolating T_{reg} cells from adult peripheral blood containing activated antigen-experienced conventional T cells expressing CD25 (REF.[54]). Selection of the T cells on the basis of low CD127 expression greatly increased T_{reg} cell purity and recovery from peripheral blood and lymphoid tissue[8,9]. Additional T_{reg} cell markers are likely to result from efforts aimed at pinpointing the molecular signatures of bona fide T_{reg} cells[55,56].

Two methods have been used to purify T_{reg} cells: magnetic-activated cell sorting (MACS) and fluorescence-activated cell sorting (FACS). Although MACS-based approaches are good manufacturing practice (GMP) compliant and can process billions of cells simultaneously, they are imprecise and often result in T_{reg} cells with insufficient purity, a low rate of T_{reg} cell recovery or both. By contrast, FACS allows the precise identification of T_{reg} cells through the use of multiple markers, ensuring high purity and high recovery; however, the process is slow and limits the number of cells that can be processed to ~109 peripheral blood mononuclear cells (PBMCs) per day, consequently limiting the dose that can be manufactured. In addition, FACS instrumentation, for example, FACSAria (Becton Dickinson), is typically not GMP compliant owing to open air processing and unexchangeable parts that are in contact with cells. Exceptions include the new FX500 fluidics cell sorter (Sony) and the three-laser MACSQuant Tyto cell sorter (Miltenyi Biotec),

closed on-chip sorting systems with exchangeable parts used to enrich and purify the final population. Nonetheless, both MACS and FACS are currently being used to manufacture T_{reg} cells for use in the clinic; the regulatory environment of the country and financial resources of the investigators often dictate which method is chosen.

Methods of expanding T_{reg} cells—Most T_{reg} cell therapies aim to increase, albeit transiently, the number of T_{reg} cells to reset the inflammation-prone state of the recipient. This inhibitory effect is estimated to require an infusion of millions to billions of T_{reg} cells[57] and, thus, requires the expansion of T_{reg} cells before infusion. So far, T_{reg} cells used in clinical trials have been mostly expanded by polyclonal stimulation; that is, anti-CD3 and anti-CD28 beads[30,31,58–60.] To prevent the outgrowth of conventional T cells and to maintain high FOXP3 expression during T_{reg} cell expansion, many centres culture the cells in the presence of rapamycin, an inhibitor of the mechanistic target of rapamycin (mTOR). T_{reg} cells are less sensitive to phosphatidylinositol 3-OH kinase (PI3K) activity (an upstream kinase in the signalling pathway that activates mTOR) than conventional T cells, making them markedly less sensitive to the anti-proliferative effect of rapamycin than T_{eff} cells[61–63]. Including rapamycin in cell cultures is especially important when T_{reg} cells are purified by MACS as it helps to compensate for the considerably reduced initial purity of the T_{reg} cells. Importantly, however, rapamycin also suppresses the expansion of T_{reg} cells, leading to longer culture durations that require repeated stimulation with anti-CD3 and anti-CD28 beads, and sometimes with artificial APCs, to produce sufficient doses of T_{reg} cells. Our laboratories have shown that rapamycin is not needed when T_{reg} cells are purified by FACS with selection for $CD4^+CD25^+CD127^{low}$ T_{reg} cells[58]. Antigen-specific approaches for expanding T_{reg} cell populations are being developed and used. These approaches include the manufacture of clinical grade human alloantigen-reactive T_{reg} cells following expansion in the presence of donor-derived stimulated B cells, which selectively increases the pool of alloantigen-reactive T_{reg} cells that is naturally present in the blood[64,65]. Of note, DCs[66] and K562 cell-based artificial APCs[67] have also been used to expand human T_{reg} cells ex vivo.

K562 cell-based artificial APCs

K562 cells are an immortalized human leukocyte antigen (HLA)-deficient cell line initially isolated from a chronic myelogenous leukaemia. Artificial antigen-presenting cells (APCs) are K562 cells that have been gene edited to express CD80 and/or CD86 and specific HLA alleles to function as APCs.

As T_{reg} cells proliferate poorly in vitro, most protocols for expanding T_{reg} cells rely on the use of strong stimulants to drive cell proliferation and on high concentrations of IL-2 to sustain T_{reg} cell expansion[65]. Yet, considerable variability in the proliferative potential of T_{reg} cells has been observed during ex vivo expansion[68]. This variability may relate to the activation state of the cells, which is reported to be affected by the age of the T_{reg} cell donor, as well as the donor's immunological experiences, inflammatory conditions and current medications. For example, CD45RA-expressing naive T_{reg} cells are in a resting state when isolated, yet readily expand upon in vitro stimulation and maintain their T_{reg} cell lineage after expansion more efficiently than $CD45RA^-$ T_{reg} cells[58,69,70]. Among CD45RA− T_{reg}

cells, recently activated effector T_{reg} cells negative for CC-chemokine receptor type 7 (CCR7) have the least potential for expansion and are the most likely to lose expression of FOXP3 (REF.[71]). Consistent with these observations, UCB-derived T_{reg} cells are mainly naive, bestowing them with a growth advantage over their peripheral blood counterparts[72]. Unexpectedly, T_{reg} cells isolated from infant thymuses, although mostly naive, do not proliferate as effectively as T_{reg} cells from adult peripheral blood[53].

Product specification and release criteria for T_{reg} cells—Most centres conducting T_{reg} cell therapy trials agree that the expanded T_{reg} cell product should remain $CD4^+CD25^+FOXP3^{high}CD127^{low}$, and these markers are widely used to define product identity. Activated conventional human T cells also show a transient increase in FOXP3 expression in response to TCR–CD28 signals, limiting the reliability of this marker alone for the identification of expanded human T_{reg} cells and explaining why lineage-committed T_{reg} cells are identified by demethylation at TSDRs. In activated conventional T cells that transiently upregulate FOXP3, the TSDR remains methylated[73,74]. The ongoing development of validated and quantitative standardized assays for measuring TSDR demethylation in lymphocytes, such as the one performed by Epiontis[75], could soon allow the routine inclusion of this important criterion for T_{reg} cell product release.

Lessons from T_{reg} cell therapy trials

Taming transplant rejection

Thus far, excellent safety profiles have been consistently shown in patients receiving T_{reg} cells[14]. T_{reg} cell therapy was first applied in GvHD, supported by striking efficacy data from preclinical models in the early years of the first decade of this century[76,77]. In 2009, a team in Gdansk, Poland, published the first report of T_{reg} cell therapy in humans. In this study, ex vivo expanded $CD4^+CD25^+CD127^{low}$ T_{reg} cells were infused into one patient with acute (grade IV) GvHD and two patients with chronic GvHD. T_{reg} cells alleviated symptoms, and pharmacological immunosuppression could be reduced in chronic, but not acute, GvHD[59]. Subsequent studies provided encouraging results regarding the use of T_{reg} cells to prevent and treat acute and chronic GvHD[30,51,78–80], although two clinical trials have been withdrawn (NCT01163201, NCT02118311). These studies were halted for reasons other than safety, including logistics, trial design and replacement with a new study.

Alloantigen-reactive T_{reg} cells can attenuate donor-reactive T cells in preclinical models of transplantation, providing robust evidence to justify their evaluation in clinical trials[64,81,82]. As a result, they are being tested in several phase I studies in kidney and liver transplantation[60,83,84] (FIG. 1). Overall, T_{reg} cell infusions have been safe and well tolerated and, although manufacturing the cells in the context of chronic immunosuppressive treatments remains challenging, these studies provide enough safety data to advance T_{reg} cell therapy to phase II trials. Data from these trials, which will include efficacy, will be unveiled in the coming years.

Autoimmune and autoinflammatory disease

There is much interest in using T_{reg} cells to treat autoimmune diseases, particularly patients with type 1 diabetes (T1D). Strong preclinical data were obtained in the non-obese diabetic (NOD) mouse model[43]. A single infusion of ex vivo expanded islet-specific T_{reg} cells prevented autoimmunity and restored sustained self-tolerance in mice with recent diabetes onset. The natural pool of T_{reg} cells in NOD mice is numerically normal, preferentially expands in the pancreatic lymph nodes of prediabetic mice and migrates to inflamed islets. Yet, T_{reg} cells fail to control islet destruction owing to a survival disadvantage in chronically inflamed islets[85]. Moreover, NOD T_{eff} cells are more resistant to T_{reg} cell-mediated suppression than their B6 mouse counterparts[86]. The first clinical trial testing polyclonal T_{reg} cell therapy in T1D showed T_{reg} cell administration to be safe and well tolerated, and the children into whom T_{reg} cells had been infused had markedly higher C-peptide levels and lower insulin requirements than children in the untreated group[28]. In a second phase I clinical trial conducted in the United States, ex vivo expanded polyclonal T_{reg} cells were infused into 14 patients with T1D in doses ranging from 5×10^6 to 2.6×10^9 cells. Infusions were well tolerated, and C-peptide levels in most patients remained stable for 1 year, although efficacy could not be conclusively shown. Notably, cell pharmacokinetic analysis of T_{reg} cells labelled with deuterium during ex vivo expansion showed that a small but visible percentage of the infused T_{reg} cells persisted in peripheral blood for at least 1 year without evidence of deuterium-positive T_{eff} cells; this finding demonstrates that the T_{reg} cells remained phenotypically stable after infusion[31]. Nonetheless, a phase II clinical trial (NCT02691247) performed by Caladrius Biosciences, in which 113 newly diagnosed (that is, less than 100 days since T1D diagnosis) adolescents with T1D into whom autologous ex vivo polyclonally expanded T_{reg} cells had been infused failed to show that T_{reg} cell infusion led to preservation of C-peptide production 1 year after the start of treatment (see Related links). The trial is ongoing but these negative results are in agreement with the finding in preclinical models that antigen-specific, and not polyclonal, T_{reg} cells are required to reverse T1D as the frequency of islet-specific T_{reg} cells in blood is extremely low. One strategy is to artificially redirect peripheral blood T_{reg} cells to specific antigens (see later).

C-peptide

Short polypeptide connecting the A chain of proinsulin to the B chain. After packaging in vesicles in pancreatic beta cells, C-peptide is removed from proinsulin, leaving the A chain and the B chain linked by a disulfide bridge. Blood C-peptide levels are used to monitor endogenous insulin expression in patients with diabetes.

Amyotrophic lateral sclerosis

Neurodegenerative disorder characterized by the progressive loss of motor neurons, causing muscle weakness, atrophy and eventually death.

Pemphigus vulgaris

Group of skin disorders characterized by the formation of blisters induced by autoantibodies targeting intercellular adhesion molecules on keratinocytes.

Guillain–Barré syndrome

Immune-mediated polyneuropathy, usually started after an infection, sharing cross-reactive epitopes with peripheral nerves (myelin or axonal membrane).

Alzheimer's disease

Neurogenerative disorder leading to progressive dementia associated with the accumulation of amyloid-β plaques in nervous cells of the central nervous system.

Marginal zone

High-transit area constituted by B cells, macrophages, dendritic cells and granulocytes, interposed between lymphoid tissues and the circulation, serving as a sentinel for blood-borne antigens.

Two smaller studies assessing the use of T_{reg} cells in the treatment of autoimmune and autoinflammatory diseases have also been reported. The first study focused on one patient with systemic lupus erythematosus and active skin disease who received 1 × 108 autologous T_{reg} cells expanded by polyclonal stimulation[87]. The T_{reg} cells migrated into the affected areas of the skin and markedly attenuated the activity of the interferon-γ (IFNγ) pathway while enhancing the activity of the IL-17 pathway[87]. Of note, IL-17 may be involved in barrier homeostasis. Thus, a shift from IFNγ to IL-17 may lead to reduced skin inflammation and tissue repair. In the second study, three patients with amyotrophic lateral sclerosis, who received eight consecutive infusions of T_{reg} cells while undergoing IL-2 therapy, showed reduced disease progression[88]. In short, the field is steadily moving forward, with several phase I clinical trials aiming to test T_{reg} cell therapy in autoimmune hepatitis (NCT02704338), pemphigus vulgaris (NCT03239470), inflammatory bowel disease (NCT03185000), Guillain–Barré syndrome (NCT03773328) and Alzheimer disease (NCT03865017).

Co-medication and Treg cell therapy

IL-2 therapy and T_{reg} cells—In the 1990s, and counterintuitively at the time, mice deficient in the crucial T cell growth factor IL-2 displayed uncontrolled T cell activation and succumbed to widespread autoimmunity[89]. Later experiments revealed that IL-2 is required for the development, homeostasis and suppressive function of T_{reg} cells[90], explaining this observation and suggesting that IL-2 could be used to expand T_{reg} cells in vivo in patients with autoimmune disease[6]. Although other immune cells — such as conventional T cells, NK cells and some subsets of innate lymphoid cells — express the IL-2 receptor, as T_{reg}

cells constitutively express CD25 (a component of the high-affinity IL-2 receptor), low-dose IL-2 should preferentially affect T_{reg} cells[91]. Thus far, the best evidence that this phenomenon occurs in humans comes from an uncontrolled phase I/IIa clinical trial in patients with hepatitis C-mediated vasculitis that is resistant to treatment with IFNα and the antiviral ribavirin[92]. Low-dose IL-2 treatment had efficacy in eight out of ten patients, as judged by an increase in T_{reg} cell frequency and a concomitant decrease in the proportion of marginal zone B cells[92]. A concurrent study revealed that daily low-dose IL-2 treatment increased T_{reg} cell numbers and alleviated chronic GvHD symptoms in 12 of the 23 patients treated[93]. Finally, an uncontrolled trial using a low dose of IL-2 to treat patients with systemic lupus erythematosus also showed efficacy, with the expansion of T_{reg} cells, T follicular helper cells and T_H17 cells, but not of T_H1 cells or T_H2 cells, along with decreased disease activity[94].

Currently, nine trials are actively testing the efficacy of low-dose IL-2 treatment for several autoimmune diseases. The TRANSREG study goes even further, comparing, in a multicentre, uncontrolled, open-label study, 14 autoimmune diseases (NCT01988506). In T1D, low-dose IL-2 therapy is safe and augments T_{reg} cell numbers; however, the numbers of NK cells and eosinophils were also increased, and efficacy is yet to be demonstrated[95–97]. Hence, IL-2 therapy could be combined with T_{reg} cell therapy to increase efficacy, and three clinical trials are currently testing this hypothesis in the treatment of T1D (NCT02772679), steroid refractory chronic GvHD (NCT01937468) and amyotrophic lateral sclerosis (NCT03241784). Future approaches may include the use of IL-2–anti-IL-2 complexes that selectively expand T_{reg} cells[98]. Alternatively, engineering an orthogonal IL-2 molecule specific to an orthogonal IL-2 receptor is feasible and would allow the selective expansion and survival of the infused engineered cells[99].

Crosstalk with immunosuppressive regimens—Patients with autoimmune disease are often treated with several immunosuppressive drugs, including corticosteroids (such as prednisone), antimetabolites (such as methotrexate, azathioprine, mycophenolate mofetil and leflunomide), alkylating agents (such as cyclophosphamide), blocking monoclonal antibodies that target cytokines or their receptors (such as anti-tumour necrosis factor (TNF), anti-IL-1β, anti-IL-4 receptor subunit-α (which simultaneously inhibits the IL-4 and IL-13 pathways), anti-IL-5, anti-IL-6 and anti-IL-17), abatacept (which blocks CD80 and CD86 in APCs), and B cell-depleting antibodies (such as rituximab and belimumab). In the setting of transplantation, the interactions of different immunosuppressive drugs (for example, ciclosporin, rapamycin, anti-CD25 and thymoglobulin) with T_{reg} cells need also to be evaluated, as reviewed elsewhere[100]. Immunosuppressive drugs may impact the number, expansion and function of T_{reg} cells. If fewer T_{reg} cells can be isolated and expanded from patients with autoimmune disease and transplant recipients compared with healthy individuals, the minimum number of T_{reg} cells required for product release, a typical hurdle in trials for T_{reg} cell therapy in transplantation, may not be achieved[101]. Furthermore, the dosage of these treatments can vary widely during disease treatment, likely with variable effects on the T_{reg} cell compartment. Encouraging preliminary results obtained from patients who underwent allogeneic kidney transplantation suggest that dialysis, immunosuppression and acute rejection episodes affect T_{reg} cell maturation, resulting in a reduced percentage of

mature T_{reg} cells in these patients, but do not preclude ex vivo T_{reg} cell expansion and the generation of a final T_{reg} cell product with therapeutic properties[102]. Future research will shed light on this potential limitation as we gather data on the unwarranted interactions between immunosuppressive drug regimens and infused T_{reg} cells.

Engineering T_{reg} cell specificity

T_{reg} cells could potentially be manipulated to restore immune tolerance in the treatment of autoimmunity. T_{reg} cells must migrate to appropriate sites and respond to their cognate antigen to effectively suppress immune responses. Studies in transgenic NOD mice genetically engineered to express the BDC2.5 diabetogenic TCR revealed that relatively small numbers of antigen-specific T_{reg} cells, but not of polyclonal T_{reg} cells, are sufficient to prevent and even reverse T1D[43]. Strikingly, in a B6 mouse model where insulitis and autoimmune diabetes were induced by expression of the proinflammatory cytokine TNF in islets, as few as 2,000 T_{reg} cells isolated from the pancreatic draining lymph node could prevent diabetes[103]. These seminal studies established the importance of using antigen-specific T_{reg} cells for therapy. Yet, only minute numbers of antigen-specific T_{reg} cells relevant for a given condition are found in peripheral blood. Moreover, most antigen-specific T_{reg} cells reside in tissues, making them difficult to isolate and their cognate antigens laborious to identify. These obstacles have fuelled interest in devising strategies to artificially direct T_{reg} cells to a desired target (FIG. 3).

HLA restriction

The presentation of a peptide by a specific human leukocyte antigen (HLA) to a T cell's receptor.

Currently, engineered TCRs and CARs are the main receptors employed to impart T_{reg} cell specificity. TCRs have lower affinity than CARs yet can recognize just one molecule per target cell[104], whereas cells must have at least 100 target molecules for CARs to recognize them[105]. However, CAR targets tend to be moderately to highly expressed surface proteins. Thus, the difference in affinity between TCRs and CARs is compensated for by different expression levels of the ligands on target tissues. CARs are not subject to HLA restriction or dependent on a co-receptor, but whether the high affinity and downstream signalling of CARs are ideal for T_{reg} cell function is unclear. The relative advantages and disadvantages of each receptor continue to be explored (TABLE 1).

Targeting TCRs to engineer T_{reg} cells

The right TCR for a T_{reg} cell—Perhaps the most straight-forward way to redirect T_{reg} cell specificity is to engineer a TCR recognizing a peptide of interest in the target tissue (FIG. 4). The TCR is a heterodimer comprising an α-chain and a β-chain, each of which is composed of a variable region (VDJ) and a constant region. Each T cell expresses a unique TCR, owing to stochastic V(D)J recombination, which can generate up to 1×10^{61} different TCR sequences in humans[106]; detailed amino acid sequences of each individual region of many TCRs can be found in the IMGT database. Although most T cells with strong reactivity against self-peptide–major histocompatibility complex (MHC) class II complexes

are clonally depleted in the thymus (an example of recessive immune tolerance), a subset of these cells acquire FOXP3 expression when the CNS2 enhancer in the first intron of the FOXP3 gene is demethylated[107,108]. Thus, the TCR repertoire of thymically derived T_{reg} cells is distinct from that of T_{eff} cells, which have been selected for weak peptide-bound MHC–TCR interactions. Autoimmune regulator (AIRE), a transcriptional regulator driving tissue-specific antigen expression in the thymus, promotes T_{reg} cell development by skewing autoreactive T cells towards a T_{reg} cell phenotype[109,110]. Still, some autoreactive T_{eff} cells escape clonal deletion and they may share a common pool of self-reactive TCRs with T_{reg} cells[111]. Distinct T_{reg} cell TCRs can be specific to non-overlapping peptides derived from the self-antigen that is targeted by autoantibodies, suggesting that T_{reg} cells recognize only a few proteins deemed most susceptible to autoimmune recognition[112].

As peptides from any protein (including intracellular proteins) can potentially be bound by MHC and recognized by the TCR, TCRs can be isolated or designed to recognize an almost infinite number of targets. Another advantage of the TCR is that the peptides it recognizes can be derived from proteins that are post-translationally modified. Post-translational modification by oxidation[113], deamidation[114], citrullination[115] or phosphorylation[116] generates unique epitopes that typically circumvent central T cell deletion[117,118]. Hybrid peptides (that is, products of the fusion of the amino terminus of one peptide to the carboxy terminus of another peptide by a peptide bond) have also been uncovered as important self epitopes[119,120].

Importantly, how TCRs can be engineered to impart T_{reg} cell specificity may be different depending on whether T_{reg} cells function primarily in lymphoid organs to restore immune tolerance or in tissues to suppress autoinflammation or induce tissue repair. In the mouse, continuous, steady-state TCR stimulation in lymphoid organs mediates suppression[20,121], whereas in tissues that lack TCR stimulation and have low levels of IL-2, $CD44^{hi}CD62L^{low}CCR7^{low}$ T_{reg} cells depend mainly on costimulation through inducible T cell costimulator (ICOS) for their maintenance[71,122]. Additional signals that are important for the maintenance of tissue-resident murine T_{reg} cells have been identified; T_{reg} cells in adipose tissue rely on IL-33 (REF.[33]), whereas memory T_{reg} cells in the skin require IL-7 for survival[32].

In inflammatory conditions, such as in murine autoimmune diabetes, T_{reg} cells specific to islet-derived antigens clonally expand in inflamed islets but not in the spleen or lymph nodes[123]. T_{reg} cells with high-affinity or low-affinity TCRs can be found in islets, and evidence supports the hypothesis that these T_{reg} cell subsets have complementary roles in immune homeostasis[22]. High-affinity T_{reg} cells expressed high levels of suppressive molecules (namely IL-10, LAG3 and TIGIT), whereas low-affinity T_{reg} cells expressed amphiregulin, a key factor in tissue repair[22]. Furthermore, T_{reg} cell subsets characterized by distinct cytokine profiles and transcription factor dependencies have been shown to feature TCR signalling of differing strengths[124].

TCR immunosequencing as a route to engineering TCRs—Progress in engineering TCRs will also depend on advances in TCR immunosequencing, at the single-cell level, and thus in predicting which peptides they recognize. High-throughput

immunosequencing of the TCRα and TCRβ chains of $CD4^+$ T cells, $CD8^+$ T cells and T_{reg} cells, in pancreatic tissues and blood, have uncovered a population of pancreas antigen-specific T cells selectively in the pancreatic islet tissue[125]. The local accumulation of these T cells suggests that they are reactive to islet antigens, making their TCRs potential candidates for redirecting T_{reg} cells to peptides selectively expressed in the pancreas. However, knowledge of the sequence of both TCRα and TCRβ and of how they are paired to form heterodimers in a single T cell is needed to reconstitute TCR specificity. The cost of single-cell paired TCR sequencing technology is rapidly decreasing, permitting the routine use of this approach for characterizing TCR repertoires on various T cell subsets, including T_{eff} cells and T_{reg} cells. Additionally, a high-throughput method to pair TCRα and TCRβ genes without the need for single-cell technologies has been reported[126]. These new technologies will help rigorously define the TCR repertoire of T_{reg} cells and, eventually, of autoimmune T cells, which can be used to engineer T cells for therapeutic purposes (FIG. 4). However, predicting the epitope specificity of TCRs will be key, and several algorithms can now cluster TCR sequences on the basis of conserved motifs and complementarity-determining regions[127,128].

As had been previously shown with conventional T cells[129], introducing a TCR from another antigen-specific T cell to T_{reg} cells can effectively redirect them towards a known antigen specificity. In one example, T_{reg} cells were transduced with TCRs that conferred them with specificity for a transplant antigen. Specifically, a TCR specific for a peptide derived from an MHC class I molecule (H-$2K^d$ from the BALB/c strain) presented by an MHC class II molecule (H-$2A^b$ from the B6 strain) was transduced into B6 T_{reg} cells, leading to peptide–MHC specific cells that induced the long-term survival of fully MHC-mismatched (MHC class I and MHC class II) BALB/c heart grafts in immunocompetent B6 mice[130]. In another study, T_{reg} cells from chicken ovalbumin (OVA) antigen-specific, OT-II, transgenic mice, suppressed the in vitro proliferation of T_{eff} cells on stimulation with DCs presenting the relevant OVA peptide. Moreover, in an in vivo model of rheumatoid arthritis, redirected T_{reg} cells from the same OT-II mice homed to the inflamed joint (following an intra-articular injection of OVA in the knee), reduced the number of T_H17 cells in the draining lymph node and decreased inflammation and bone damage[131].

Complementarity-determining regions

Parts of the variable chain of the T cell receptor or of an antibody that determine specificity to their cognate antigen.

To date, several TCRs have been successfully used to redirect the specificity of human T_{reg} cells while preserving their in vitro suppressive function. Target antigens include insulin, glutamic acid decarboxylase and factor VIII[23,45,46]. T_{reg} cells transduced with a high-affinity TCR have superior suppressive function compared with T_{reg} cells transduced with a low-affinity TCR specific for the same peptide[23]. Strikingly, T_{reg} cells transduced with a high-avidity HLA-A2-restricted tyrosinase-specific TCR from $CD8^+$ T cells recognized an MHC class I-presented antigen while maintaining their capacity to suppress T_{eff} cell responses against that same antigen[47]. This result suggests that TCRs for translational applications

using T_{reg} cells can be obtained from cells other than T_{reg} cells or conventional $CD4^+$ T cells.

Chimeric TCRs—TCRs are complex to engineer, in part owing to their heterodimeric nature; introduced subunits can recombine with the endogenous TCRα or TCRβ chain. This complexity raises the risk of generating TCRs with unknown specificities and off-target effects. Several strategies have been devised to address this problem. One approach is to insert extra cysteine residues in the constant region of engineered TCRα and TCRβ chains to form a disulfide bridge between the two to promote preferential pairing of the transduced TCR[132,133]. Another strategy is to replace the human constant region in TCR chains with a mouse constant region, as interspecies pairing between a murine TCR chain and a human TCR chain has never been observed. This approach also facilitates signal transduction, as the mouse constant region has higher affinity for the TCR–CD3 complex than its human counterpart. Building on this approach to avoid xenogeneic immunogenicity, two independent groups systematically identified the minimum set of residues in the murine TCR constant chains required to 'murinize' the human TCR constant chain. The resulting transgenic TCR had enhanced stability and avidity[134,135]. A final strategy is to use vectors with strong promoters to express the engineered TCR[136].

Currently, the most promising approach to prevent mixed TCR dimer formation is to use genome editing to engineer the endogenous TCR locus. One or both endogenous TCR chains could be knocked out before a new antigen receptor is introduced[137]. A more elegant method, however, would be to replace the endogenous TCR with the new TCR by knocking it into the TCRα constant region locus (*TRAC*), ablating expression of the native TCR and endowing the new TCR with the same genomic location and transcriptional regulation as the endogenous gene. Indeed, this was accomplished with reasonable efficiency in primary human T cells by CRISPR–Cas9-mediated editing coupled with a double-stranded DNA template coding for a tumour antigen (NY-ESO)-specific TCR for homology-directed repair[138].

An additional challenge inherent in engineering TCRs is that they require costimulation to fully activate downstream signalling. To overcome this issue, two groups have generated chimeric TCRs. One group linked a melanoma antigen-specific TCR to the transmembrane and intracellular portions of CD28 followed by CD3ε, observing enhanced expression of TCR at the cell surface, the formation of immune synapses, IL-2 secretion, survival of the engineered T cell and tumour clearance[139]. Another group fused a soluble TCR to a CAR transmembrane and signalling domain, demonstrating that this TCR–CAR was functional in primary T cells and in an NK cell line[140].

Engineered CAR Treg cells

Overview of early CAR T_{reg} cell milestones—Next-generation T_{reg} cell therapy will undoubtedly benefit from the field of immuno-oncology, in particular CAR T cell therapy. Since 2017, when the FDA approved CD19 CAR T cell therapy for paediatric acute lymphoblastic leukaemia and non-Hodgkin lymphoma, there have been 241 CAR T cell therapy clinical trials worldwide (91 in the United States; 14 in Europe, 128 in China, 3 in

Canada, 2 in Japan; 2 in Australia and New Zealand, and 1 in Israel) as listed in ClinicalTrials.gov as of January 2018 (REF.[141]). The first clinical trial using CAR T cells targeted the GP120 region of the HIV envelope glycoprotein and was completed 2 years before CD19 CAR T cells were reported to eradicate tumours in animal models[142]. The GP120 CAR consisted of the CD4 co-receptor (which binds HIV GP120) fused to CD3ζ[143,144]. However, although HIV-specific cytotoxicity was shown in vitro, GP120 CAR T cell therapy failed to reduce viral load in patients with HIV infection[145].

As mentioned earlier, robust T cell activation requires the interaction of the TCR with its cognate antigen and the binding of a costimulatory receptor (such as CD28) to its ligand (such as CD80 or CD86) on the surface of an APC. First-generation CARs contained solely a TCR CD3ζ endodomain, which, despite being able to induce T cell activation, could not drive robust T cell expansion[146]. Including the CD28 signalling domain in second-generation CARs allowed sustained CAR T cell expansion on repeated exposure to antigen, and these CARs displayed superior cytolytic function[142]. Incorporating an additional costimulatory domain (whether it be 4–1BB (also known as CD137), OX40 or another costimulatory domain) into a CD28–CD3ζ CAR creates a third-generation CAR (containing two costimulatory domains, as opposed to only one in second-generation CARs), further fine-tuning CAR signalling; addition of the intracellular domain of 4–1BB minimizes CAR-induced exhaustion[147,148], and including the intracellular domain of OX40 (a costimulatory molecule belonging to the TNF receptor family) decreases CAR-induced IL-10 secretion, limiting the inhibition of antitumour activity[149].

Engineering murine CAR T_{reg} cells—The first described efforts to engineer T_{reg} cell specificity involved creating a transgenic mouse line expressing an artificial chimeric receptor comprising an extracellular peptide-bound MHC complex linked to an intracellular TCR ζ-chain signalling domain[49]. The peptide antigen chosen was $MBP_{89–101}$, an autoantigen derived from myelin basic protein (MBP) that induces experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis. This receptor design allowed $CD4^+CD25^+$ T_{reg} cells to directly recognize and be activated by the TCR of pathogenic self-reactive T cells[49]. Upon activation by $MBP_{89–101}$-reactive T cells, these engineered T_{reg} cells secreted high levels of anti-inflammatory IL-10 and TGFβ and low levels of proinflammatory IFNγ, consistent with a regulatory function. Moreover, the level of cytokines produced in response to anti-CD3ε or $MBP_{89–101}$-reactive T cell-mediated activation were similar, indicating that the chimeric receptor mimics physiological TCR signalling[49]. Strikingly, adoptive transfer of engineered T_{reg} cells not only prevented $MBP_{89–101}$-induced EAE but also treated it when performed 11 days or even 31 days (after epitope spreading) following disease induction in an example of bystander suppression[49]. Subsequent work revealed that these T_{reg} cells expressing chimeric receptors functioned primarily by inducing MBP-specific T cells to produce IL-10. The adoptive transfer of non-transgenic MBP-specific T cells from mice treated with engineered T_{reg} cells could prevent $MBP_{89–101}$-induced EAE in naive mice, indicating that pathogenic T cells differentiated into antigen-specific T_{reg} cells in an example of infectious tolerance[150].

A different chimeric receptor design emerged a few years later in 2009, aimed at engineering T_{reg} cells to prevent colitis[151]. Intrarectal administration of 2,4,6-trinitrobenzenesulfonic

acid (TNBS) is a common strategy to induce colitis in mice. Ethanol disrupts the intestinal barrier, allowing TNBS to interact with proteins and render them immunogenic, generating the hapten 2,4,6-trinitrophenyl (TNP). The study authors created a tripartite chimeric receptor in which a recognition unit (an antibody single-chain variable fragment (scFv) specific for the hapten TNP) was fused to the transmembrane and cytoplasmic domains of CD28 and to the signalling domain of Fc receptor-γ. Unlike the aforementioned work, this approach allowed T_{reg} cells to directly recognize a disease-specific tissue antigen. TNBS-induced colitis resulted in only 10% mortality in transgenic mice expressing the receptor in T cells, compared with 50% mortality in control mice. Moreover, T_{reg} cells expressing the chimeric receptor were activated specifically by TNPylated cells and suppressed T_{eff} cell proliferation in vitro. When adoptively transferred into wild-type mice 16 h after colitis induction, transgenic T_{reg} cells migrated to the site of colonic damage and prevented colitis. Even though transgenic T_{reg} cells could suppress colitis induced only by TNBS, indicative of their antigen specificity, they could also ameliorate colitis induced by other agents when trace amounts of TNBS were present, which is a hallmark of bystander suppression[152]. Of note, mice treated with transgenic T_{reg} cells also displayed increased survival after a second round of TNBS-induced colitis (25% mortality compared with 66% mortality in controls). Whether this phenomenon was due to persistence of the modified cells or infectious tolerance was not explored[151].

These initial chimeric receptor designs gave way to the next generation CARs first used in T_{eff} cells for cancer therapy, with two groups generating CD28–CD3ζ CAR T_{reg} cells specific for carcinoembryonic antigen (CEA)[153,154]. CEA expression is upregulated in benign colon inflammation (ulcerative colitis), as well as in colon cancer in humans[155], and the first report focused on induced colitis[153]. Similarly to the TNP-specific modified T_{reg} cells developed by the same group[151,152], CEA CAR T_{reg} cells localized to the colon, suppressed the production of inflammatory cytokines and prevented T cell-mediated colitis in mice when coadministered with CEA CAR $CD4^+$ T_{eff} cells[153]. As CEA is expressed in the lungs as well as the intestine, the second group tested the function of CEA CAR T_{reg} cells in asthma[154]. Using a transgenic mouse model in which the human CEA gene promoter drives CEA expression in the pulmonary and gastrointestinal epithelium, the study authors induced allergic airway inflammation via immunization with OVA. CEA CAR T_{reg} cells potently secreted IL-10 and suppressed T_{eff} cell proliferation in vitro, and localized to the lung on intravenous administration into CEA transgenic mice. Moreover, administration of CEA CAR T_{reg} cells 1 week after initial sensitization, followed by four consecutive days of challenge with OVA, almost completely prevented airway hyper-reactivity and reduced mucus production, eosinophil lung infiltration and the production of T_H2 cell-type cytokines[154].

Engineering human CAR T_{reg} cells—These encouraging results in mouse disease models moved the field to work in human cells. The first study using a CAR to redirect human T_{reg} cells also involved CEA. However, instead of preventing or alleviating disease in the intestine or the lung, human CEA CAR T_{reg} cell-mediated suppression in vivo was demonstrated via partial protection of a CEA-expressing tumour from CEA CAR T cell-mediated death in immunodeficient mice[156]. One immediate caveat of this setting was that

both CAR T_{reg} cells and CAR T cells, which were administered at a 1:1 ratio, targeted the same antigen[156]. Thus, the observed partial protection could have been due to competition between these cell types for antigen rather than due to true suppression. Shortly afterwards, a different group generated human CD19 CAR T_{reg} cells that suppressed the proliferation and cytotoxic activity of CD19 CAR T_{eff} cells in vitro and migrated to CD19-expressing B cell-derived tumours in mice, preventing CD19 CAR T_{eff} cell-mediated tumour killing in vivo at a ratio as low as one CAR T_{reg} cell per 16 CAR T_{eff} cells[157].

The most immediate application of CAR T_{reg} cells is poised to be in GvHD and organ transplant rejection. Unlike in most autoimmune disorders, there are very clear targets in transplantation in the form of HLA molecules. Moreover, CARs remove the need to activate recipient T_{reg} cells with donor-derived APCs[158]. In 2016, the first HLA-A2 CAR T_{reg} cells were reported; as HLA-A2 is present in 50% of the population, creating tolerance for this allele is advantageous. The study authors demonstrated that HLA-A2 CAR T_{reg} cells suppress T_{eff} cell proliferation and prevent HLA-$A2^+$ PBMC-mediated GvHD in immunodeficient NSG mice. HLA-A2 CAR T_{reg} cells did not kill HLA-$A2^+$ cells in short-term in vitro assays but completely prevented HLA-$A2^+$ PBMC engraftment, leaving open the possibility that they might kill HLA-$A2^+$ cells in vivo[48]. Two subsequent studies also generated HLA-A2 CAR T_{reg} cells[159,160] and introduced new models to test HLA-A2 CAR T_{reg} cells in vivo. One group demonstrated that HLA-A2 CAR T_{reg} cells suppressed a mixed lymphocyte reaction between HLA-$A2^+$ and HLA-$A2^-$ PBMCs in vivo, reducing ear swelling and preserving HLA-$A2^+$ cells in mice. Of note, this result was accomplished at an HLA-A2 CAR T_{reg} cell to HLA-$A2^+$ PBMC ratio of 1:10, indicating that CAR-mediated suppression of GvHD is similar in potency to TCR-mediated suppression of GvHD[161]. Moreover, HLA-A2 CAR T_{reg} cells prevented the rejection of HLA-$A2^+$ skin grafts by HLA-2^- PBMCs[159]. The other group also used HLA-$A2^+$ skin graft rejection as a model, and found that HLA-A2 CAR T_{reg} cells reduced alloimmune damage, as assessed by a reduction in the number of keratinocytes in the graft and increased blood vessel integrity[160]. Recently, a panel of fully humanized HLA-A2 CARs was generated and tested in T_{reg} cells. Several humanized HLA-A2 CAR T_{reg} cells rapidly migrated to, and persisted in, HLA-$A2^+$ skin grafts, delaying HLA-A2 skin graft rejection[162].

Introducing B cell-targeting antibody receptor T_{reg} cells—New applications for CAR T_{reg} cells are still emerging. B cell-targeting antibody receptor (BAR) T_{reg} cells have been described recently. Human factor VIII (FVIII) injections are used to treat patients with haemophilia A. Over time, anti-FVIII neutralizing antibodies develop, causing morbidity and death[163]. A BAR containing an immunodominant domain of FVIII, either A2 or C2, as the extracellular domain, was designed to target FVIII-specific B cells; the intracellular signalling domain remained as CD28–CD3ζ as in a previous FVIII CAR design by the same group[164]. Strikingly, in vitro, human BAR T_{reg} cells displaying either the A2 domain or the C2 domain suppressed antibody production by splenocytes isolated from mice that had been immunized with recombinant FVIII. Furthermore, intravenous administration of human FVIII BAR T_{reg} cells into mice the day before immunization with recombinant FVIII prevented anti-FVIII antibody formation in vivo, whereas infusion of the cells into mice already producing anti-FVIII antibodies decreased antibody titres. BAR T_{reg} cells could thus

suppress antibody production even in a xenogeneic setting, both in vitro and in vivo. Whether these cells prevented T follicular helper cells from helping B cells, directly targeted FVIII-specific memory B cells or interacted with APCs to indirectly affect B cells was unclear. To address these questions, T cells and B cells were isolated from mice immunized with FVIII and treated with either BAR T_{reg} cells or control OVA BAR T_{reg} cells. B cells isolated from mice treated with control T_{reg} cells produced anti-FVIII antibodies regardless of the source of the T cells, whereas B cells from mice treated with FVIII BAR T_{reg} cells did not. BAR T_{reg} cells are thus likely to act directly on B cells, either by suppressing them or by killing them[165].

Alternative targets for CAR T_{reg} cells—CAR T_{reg} cell targets need not be present on the cell membrane. Our laboratories generated a CAR specific for citrullinated vimentin, a modified protein unique to the inflamed joints of more than 50% of patients with rheumatoid arthritis[166]. Citrullinated vimentin CAR T_{reg} cells recognized and were activated by citrullinated vimentin present in synovial fluid from patients with rheumatoid arthritis; this approach may provide a strategy to restore homeostasis at the site of inflammation[167]. Currently, citrullinated autoantigens are thought to be present in the extracellular milieu primarily as a result of neutrophil extracellular trap (NET)-associated cell death (NETosis). A unique form of programmed cell death, NETosis involves intracellular content extrusion, particularly of genomic DNA-rich NETs of neutrophils infiltrated in synovial tissue[168].

Adapting CAR signalling for T_{reg} cells—Early experiments in the field found that incorporating CD28 signalling into CD19 CAR T cells, by switching from CD3ζ alone to a bipartite CD28–CD3ζ intracellular domain, made these CAR T cells resistant to T_{reg} cell-mediated suppression, resulting in higher proliferation of CAR T cells and increased CAR T cell-mediated killing of B cell-derived leukaemia cells[169]. Yet, subsequent experiments reported that CEA CAR T cells containing a CD28–CD3ζ intracellular domain were more susceptible to T_{reg} cells in a solid tumour compared with CAR T cells containing CD3ζ only. Deleting the binding site for the tyrosine-protein kinase LCK on the CD28 moiety of the CEA CAR ablated IL-2 secretion while supporting CAR T cell proliferation and preventing suppression by tumour-infiltrating T_{reg} cells[170]. The seemingly contradictory results of the two studies might reflect how CAR T cells interact with liquid tumours that express CD80 and CD86 (CD19 CAR for B cell-derived leukaemia) and with solid tumours that do not (CEA CAR for non-blood cancer).

Efforts have been made to find the ideal CAR signalling architecture for CAR T cells by comparing how different costimulatory domains induce CAR T cell proliferation and longevity, as well as their ability to secrete specific cytokines and to kill tumour cells (reviewed elsewhere[171]). However, this is not the case for CAR T_{reg} cells. After the first chimeric receptor design for T_{reg} cells, in which CD3ζ alone was used as a signalling moiety[49], subsequent CAR T_{reg} cell studies in mice and humans used the prototypical CD28–CD3ζ CAR, with the exception of the use of CD3ζ alone in CD19 CAR T_{reg} cells[157]. Yet, different CAR signalling modalities are now being tested in human T_{reg} cells. One group constructed a CAR recognizing dextran and tested the function of different signalling domains in it using 4–1BB expression as a marker of T_{reg} cell activation[172].

Curiously, 4–1BB–CD3ζ CARs and OX40–CD3ζ CARs induced CAR T_{reg} cell activation, whereas CD28–CD3ζ CARs and CARs containing other signalling architectures did not[172]. This observation is at odds with results obtained with a 'universal' CAR (in which the scFv binds to a targeting module, which in turn recognizes a target antigen) featuring CD3ζ, CD28–CD3ζ or 4–1BB–CD3ζ as a signalling domain[173]. All three CAR T_{reg} cell populations suppressed T_{eff} cell proliferation with the same efficiency and were not cytotoxic in vitro. However, CD28–CD3ζ CAR T_{reg} cells uniquely secreted detectable IFNγ and TNF and were activated to a greater extent than 4–1BB–CD3ζ CAR T_{reg} cells, as assessed by expression of the CD69 early T cell activation marker. 4–1BB–CD3ζ CAR T_{reg} cells efficiently protected a tumour from CAR T cell-mediated killing in a humanized mouse model[173]. In contrast, a study by a different group found that 4–1BB–CD3ζ CAR T_{reg} cells are poor suppressors of T_{eff} cells in vitro and fail to protect skin xenografts in vivo, whereas CD3ζ CAR T_{reg} cells and CD28–CD3ζ CAR T_{reg} cells performed well in both assays. All three CARs activated T_{reg} cells to the same extent, as assessed by their upregulation of CD69 expression[174]. The discrepancies observed in these preliminary studies may be due to the use of different experimental models and also to the different activation markers used; the levels of 4–1BB, the activation marker used in the first study[172], may be more sensitive to activation downstream of TNF receptor family members than the levels of CD69, which was the activation marker used in the second and third studies[173,174]. Current work in our laboratories focuses on systematically studying the impact of different signalling domains on CAR T_{reg} cell and CAR T_{eff} cell survival, stability and function[175]. From a clinical standpoint, it would be valuable to develop a CAR that maximizes the properties of T_{reg} cells and is simultaneously a poor inducer of cytotoxic and proinflammatory responses even if it is inserted in a T_{eff} cell; such responses could contaminate T_{reg} cell preparations and thus jeopardize the efficacy and safety of CAR T_{reg} cell therapy.

Finally, the choice of scFv binding domain may also impact CAR signalling as varying the affinity of scFv modulates CAR T cell function[176]. Work comparing a CD19 CAR with a GD2 CAR (GD2 is a disialoganglioside expressed in neuroblastomas) found that the latter is substantially more prone to aggregation, tonic signalling and subsequent T cell exhaustion than the former[147]. Thus, tailored signalling architectures may be required to accommodate differences in the affinity of, and the propensity for self-aggregation amongst, scFv chains. Indeed, 4–1BB costimulation was superior to CD28 costimulation in diminishing GD2 CAR tonic signalling-induced exhaustion[147]. Curiously, in the studies discussed above, CEA CD28–CD3ζ CAR T_{reg} cells were used to suppress CEA CD28–CD3ζ CAR T cells[156], whereas CD19 CD3ζ CAR T_{reg} cells were used to suppress CD19 CD28–CD3ζ CAR T_{eff} cells in a different study[157]. The rationale behind using different CAR signalling domains for the same scFv in T_{reg} cells and T_{eff} cells in the second article was not given. Intriguingly, T_{reg} cells expressing only anti-HLA-A2 scFv migrated to and protected an HLA-$A2^{+}$ skin graft in NSG mice to the same extent as CD28–CD3ζ HLA-A2 CAR T_{reg} cells, possibly indicating the occurrence of bystander suppression via polyclonal endogenous TCR signalling[160]. Whether CAR-mediated signalling is always required for T_{reg} cell function, whether there is an ideal signalling architecture for CAR T_{reg} cells and whether such a signalling architecture is dependent on a specific scFv require further investigation.

Tonic signalling

Low level of signalling independent of activating antigen in resting T cells.

Next-generation T_{reg} cell engineering

Synthetic biology

Developing T_{reg} cells as living drugs for autoimmune diseases need not be limited to use of TCRs and CARs. Synthetic immunology has produced a number of artificial receptors and systems that warrant testing in T_{reg} cells. These systems include a T cell antigen coupler that recruits the endogenous TCR complex to a non-MHC target via a linked scFv[177], CARs optimized to bind and be activated by soluble ligands[178], and a split, universal and programmable (SUPRA) CAR system that fine-tunes the strength of T cell activation[179]. CAR variants may be indispensable in some of these systems. For instance, the SUPRA CAR system encompasses a receptor that recognizes multiple targets and is activated only if all of the targets are present. This feature is especially important when one is targeting tissue-specific antigens and solid tumours, as discovering a single antigen that is uniquely expressed in that tissue is highly unlikely. Another hurdle to optimal CAR function is tonic signalling and concomitant exhaustion. Synthetic Notch is a gene circuit in which binding to one target elicits translocation of an engineered Notch transcription factor into the nucleus, which in turn activates the transcription of a CAR receptor that recognizes a different antigen[180]. This system thus allows for greater specificity, by requiring two distinct antigens to elicit T cell activation, and prevents tonic signalling and undesired binding to CAR by only expressing CAR if the first antigen is recognized.

Rewiring cytokine signalling

Not surprisingly, cytokines play a key part in the outcome of an immune response. T_{reg} cells constitutively express CD25, the high-affinity chain of the IL-2 receptor, effectively depriving T_{eff} cells of IL-2. With this fact in mind, T_{reg} cells have been used to deprive T_{eff} cells of additional cytokines by expressing chimeric high-affinity cytokine receptors in engineered T_{reg} cells. Use of receptors in which the extracellular domain of one cytokine receptor is fused to the intracellular domain of a different receptor prevents the corresponding proinflammatory cytokine from having biological efficacy. This strategy successfully converted IL-4 signalling, which limits T cell persistence and effector function in the tumour microenvironment, into IL-7 signalling, augmenting antitumour activity[181]. Converting proinflammatory cytokine signalling into IL-2 or IL-10 signalling in engineered T_{reg} cells could increase the suppression of inflammation[182]. Indeed, designer cells that could sense TNF and IL-22, which in turn elicited secretion of IL-4 and IL-10, resolved local inflammation in a mouse model of psoriasis[183]. Ultimately, T_{reg} cells could incorporate entire synthetic gene circuits that secrete anti-inflammatory cytokines in response to proinflammatory cytokines, effectively remodelling the cytokine milieu.

Genome editing

Genome editing, which uses engineered DNA endo-nucleases programmed to recognize and bind to a specific site in the genome, allows genomes to be precisely modified and holds promise in next-generation cell therapy. The past two decades have witnessed the development of several generations of engineered nucleases that offer great opportunities for gene therapy, including zinc-finger nucleases and transcription activator-like effector nucleases, although these technologies are laborious to implement. The field changed dramatically with the advent of CRISPR as a tool to edit the genome of human cells[184]. The CRISPR–Cas9 system includes the Cas9 nuclease, which induces double-stranded DNA breaks at specific locations in the genome; Cas9 is directed to target locations by a single guide RNA, which can be designed to interact with any 20-base-pair (bp) DNA sequence upstream of an NGG (where N is any nucleobase and G is guanine) protospacer-associated motif through Watson–Crick base pairing. These properties make CRISPR–Cas9 the cheapest, most efficient and most scalable genome editing technology to date.

Since the first demonstration that genes in primary human T cells could be edited with use of CRISPR–Cas9 (REF.[185]), tremendous progress has been made in our capacity to perform CRISPR-mediated gene modifications in human T cells; the achievements include biallelic gene knockout with efficiencies greater than 80%[186], for example, of the *TRAC* locus, and the knock-in of multiple genes at precise genomic locations in primary human T cells[138]. This progress has been achieved in part by improved delivery methods; electroporation of Cas9 protein complexed with guide RNA (ribonucleoprotein (RNP)) is the delivery method of choice for T cells.

Several preclinical studies using CRISPR–Cas9 to disrupt genes in human T cells have been published. These include knocking out the gene encoding C-C chemokine receptor type 5 (CCR5) in $CD4^+$ T cells, which generated T cells that were resistant to HIV infection[185]; knocking out the gene encoding CD7 in CD7 CAR T cells, which prevented fratricide, as T cells themselves express CD7 (REF.[187]); knocking out the gene encoding programmed cell death protein 1 (PD1) in CD19 CAR T cells, which improved tumour clearance in a humanized mouse model[188]; and a step towards the generation of ‘universal’ exhaustion-resistant CAR T cells by ablation of the genes encoding β_2-microglobulin, TCR and PD1 (REF.[189]). Clinical trials using CRISPR technology in the United States started recently. The first CRISPR clinical trial in the United States, launched by CRISPR Therapeutics and Vertex, is treating patients with the blood disorder β-thalassaemia (NCT03655678) (see Related links). The first T cell cancer immunotherapy trial is being conducted by the University of Pennsylvania in partnership with Tmunity and the Parker Institute for Cancer Immunotherapy (NCT03399448). These studies have proceeded despite recent reports that CRISPR–Cas9 editing can provoke large genomic deletions and rearrangements away from the target site[190]. Efforts towards engineering high-fidelity versions of Cas9 and alternative CRISPR–Cas systems are under way. Of note, CRISPR-mediated editing is not limited to altering genomic sequence; new CRISPR–Cas systems and its variants have been engineered for targeted DNA methylation, gene activation and direct RNA editing[191–193].

Improving delivery

Currently, the manufacture of CAR T cells uses retroviral and lentiviral transduction to deliver and integrate genetic material into T cells (FIG. 5). Lentiviruses, which are members of the retrovirus family, have a diploid RNA genome. Safe use of such viruses is achieved by splitting the three main coding regions of the genome — *gag* (encoding structural proteins), *pol* (encoding reverse transcriptases and integrases) and *env* (encoding virus envelopes) — into separate packaging plasmids that are combined only during the transfection process for viral production. This strategy allows the gene of interest, flanked by long terminal repeat sequences, to be integrated into the genome of T cells while prohibiting viral replication[194]. Despite progress in the use of lentiviruses to genetically modify T cells, this approach features several drawbacks, including the random integration of the gene of interest, a different number of gene copies per cell and the use of a constitutive and strong promoter to drive gene expression, resulting in non-physiological gene regulation. Indeed, CAR expression levels in cells transduced by lentiviruses differ between patients and are often supraphysiological, prompting CAR T cell exhaustion[195]. Inserting the CAR into the *TRAC* locus by means of CRISPR–Cas9 normalized CAR expression between patients, averted tonic CAR signalling and allowed the internalization and re-expression of the CAR on the cell surface upon CD19 recognition, mimicking the surface expression dynamics of physiological TCR[195].

In the study that inserted the CAR into the *TRAC* locus[195], recombinant AAV (rAAV) was used to deliver the CAR DNA homology repair template into the T cells (FIG. 5). AAVs are single-stranded linear DNA viruses that can be safely produced, similarly to lentiviruses, by splitting the main coding regions — *rep* (encoding replication proteins), *cap* (encoding capsid proteins) and *aap* (encoding assembly proteins) — into separate packaging plasmids during transfection for virus production. In contrast to lentiviruses, however, after transduction, the inverted terminal repeat-flanked transgene of the rAAV persists inside the cell as an episome rather than being integrated into the genome[196] (FIG. 5). rAAV is therefore lost over time in replicating cells, making it ideal to deliver a homology repair template to introduce specific mutations or knock in a gene cassette of interest.

There are nine serotypes of rAAV (rAAV1–rAAV9), each of which has a different tropism. Currently, rAAV6 is the most efficient serotype for T cell transduction[138,197]. Several groups are working on bioengineering the rAAV capsid via multiplexed sequential directed evolution screens using capsid libraries with the goal of making rAAVs with novel cell specificities[198,199]. Thus, in the future, specific rAAVs could be made to selectively target distinct T cell (or even T_{reg} cell) subtypes, vastly improving the precision and safety of gene editing for T cell therapy.

A non-viral approach to knock in DNA sequences more than 1 kilobase (kb) long at a specific genomic site in human T cells has also been reported[138] (FIG. 5). Contrary to expectation, double-stranded DNA templates were not toxic when co-electroporated with CRISPR RNPs. This method corrected a pathogenic CD25 mutation in cells from patients with a monogenic autoimmune disease, inserted *GFP* downstream of several genes and inserted a transgenic TCR into the *TRAC* locus of T_{eff} cells, demonstrating its potential[138]. This approach holds great promise, as manufacturing and testing clinical grade lentivirus or

rAAV is time-consuming, expensive and plagued by safety issues. However, this approach is limited by the fact that editing efficiency decreases as the size of DNA templates increases, limiting the electroporated construct to an ~1.5-kb insert, which includes two homology arms of 300 bp long. CAR and TCR constructs are ~1.5 kb long and may therefore be too large to be efficiently integrated into DNA by this method; a *GFP* tag, by contrast, is only 750 bp long. Single-stranded rAAV6 templates have a packaging capacity of 4.7 kb, leaving up to 3.6 kb available for the gene construct after exclusion of the homology arms and inverted terminal repeat regions[200]. In conclusion, the methods used for gene editing differ depending on cell type, the insert size and the editing efficiency required. Furthermore, viral approaches can be combined with CRISPR RNP complexes to disrupt genes and/or to insert new cassettes, such as CAR or TCR transgenes.

Designer T_{reg} cells

CRISPR–Cas9 genome editing could potentially knock in antigen receptors in precise genomic locations while simultaneously editing multiple genes that regulate T_{reg} cell function (FIG. 2). As discussed earlier, one hurdle facing T_{reg} cell therapy is ensuring the survival of the cells following infusion. Deleting the gene encoding JUN amino-terminal kinase 1 (JNK1) in murine T_{reg} cells was shown to make them resistant to apoptosis, and JNK1-deficient T_{reg} cells secreted higher levels of IL-10 and TGFβ and protected transplanted islets from rejection 100 days longer than their wild type counterparts[201]. Additionally, T_{reg} cells can become unstable and transdifferentiate into pathogenic T_H17 cells in inflammatory milieus[4]. PKCθ, the most abundant PKC isoform in T cells, is activated downstream of TCR and CD28, resulting in the induction of nuclear factor of activated T cells and nuclear factor-κB. Deletion of *PRKCQ*, which codes for PKCθ, or pharmacological inhibition of PKCθ reduced the propensity of T_{reg} cells to differentiate to T_H17 cells while preserving the suppressive function of T_{reg} cells in mice and humans[39,40]. A complementary strategy could involve stabilizing high FOXP3 levels. T_{reg} cells can have diminished levels, or even loss, of FOXP3 expression as a result of increased methylation of the TSDR locus over prolonged periods of in vitro culture[202], or because of the CHIP-dependent ubiquitylation of FOXP3 in response to proinflammatory cytokines[41]. Indeed, genetically ablating *STUB1* (the gene encoding CHIP) in murine T_{reg} cells prevented FOXP3 degradation, whereas *STUB1* overexpression abrogated T_{reg} cell function in vitro and in vivo[41]. DBC1 also promotes FOXP3 destabilization by associating with FOXP3 to trigger its degradation in response to TNF by activating caspase 8. Accordingly, T_{reg} cells in DBC1-deficient mice were present in larger numbers, and were more potent suppressors of EAE and colitis upon adoptive transfer, than T_{reg} cells from wild-type mice[42]. Several other proteins interact with FOXP3 (REF.[203]), and the list continues to grow. Future mechanistic studies are likely to yield additional targets for modulating FOXP3 stability. Finally, an alternative approach would be to enforce the expression of factors shown to preserve T_{reg} cell identity. One example is BACH2, a transcriptional repressor that reduces the differentiation of naive T cells into T_H1, T_H2 and T_H17 cells[38]. Another example is STAT5-CA, a constitutively active form of signal transducer and activator of transcription 5, a key molecule that is activated downstream of IL-2 signalling and is essential for the suppressive function of T_{reg} cells[204].

As well as optimizing the suppressive properties of T_{reg} cells, genome editing could be used to tailor T_{reg} cells to different needs. In particular, there is growing evidence that T_{reg} cells have an important role in tissue repair by producing amphiregulin and keratinocyte growth factor, amongst other molecules[205,206]. Including these and other factors in engineered T_{reg} cells can further increase their therapeutic value. Finally, the recent generation of hypoimmunogenic human pluripotent stem cells via genome editing[207,208], coupled with ongoing efforts to differentiate stem cells into T_{reg} cells[209], could revolutionize engineered T_{reg} cell therapy.

Conclusion

Improper immune reactivity and inflammation underlie many currently incurable diseases. Using synthetic bio logy to fine-tune and augment the properties of immune cells will accelerate the implementation of curative cell-based therapies for autoimmunity and, ultimately, help achieve one of the most elusive goals in immunology: immune tolerance.

Currently, T_{reg} cell manufacturing is still not ideal, largely due to the lack of tailored instruments and reagents. A process that combines MACS with FACS would allow the bulk processing and precision isolation of highly pure T_{reg} cells as a starting material for manufacturing. The low proliferation rates of T_{reg} cells in vitro are in sharp contrast to their highly proliferative state in vivo[210–212], suggesting that poor proliferation is not an intrinsic characteristic of human T_{reg} cells but likely a result of the suboptimal conditions currently used to expand T_{reg} cells. Culture media, growth factors and stimulants that are suited for T_{reg} cell biology and that can accommodate donor variability are yet to be developed. Moreover, current T_{reg} cell manufacturing processes are costly and labour-intensive. Maximizing automation will not only decrease costs but will also improve reproducibility and lead to standardization.

It is the right time to design therapies using T_{reg} cells. Recent decades have witnessed dramatic progress in our knowledge of basic T_{reg} cell biology and autoimmune diseases, and also in our capacity to build artificial immune receptors and edit the genome of primary immune cells. However, many questions remain in the field of T_{reg} cell biology and T_{reg} cell therapy. For example, it will be important to identify markers that can distinguish lineage-committed thymic T_{reg} cells from peripheral T_{reg} cells and to determine whether certain T_{reg} cell subsets are more suited to engineering for T_{reg} cell therapy than others. Indeed, it is currently unclear whether the plasticity of T_{reg} cells reflects the initial heterogeneity of a T_{reg} cell population and whether only a subset of T_{reg} cells (or all T_{reg} cells) can become T_{eff} cells. The origin of T_{reg} cells that control local inflammation (that is, whether they are tissue resident or circulating) also needs to be determined. Moving into the clinic, a better understanding of how T_{reg} cells maintain tissue integrity during homeostasis and in autoimmunity and organ transplantation, whether (and how) T_{reg} cells change their identity in autoimmunity and whether T_{reg} cells from patients with autoimmune disease are intrinsically defective, and thus unsuitable for therapeutic use, will also be critical.

To optimize T_{reg} cell engineering, we must ascertain whether there is a minimal set of signalling pathways that, when incorporated into an artificial immune receptor such as a

CAR, guarantees the survival, stability and suppressive function of engineered antigen-specific T_{reg} cells. Advancing the field of engineered CAR T_{reg} cell therapy also requires a better understanding of whether the inflamed tissue, the pathogenic T cells causing the immune response or the APCs activating the pathogenic T cells are the best target for engineered T_{reg} cells, and whether the target molecule on these cells can be a soluble antigen instead of a surface molecule.

In the next decade, as some of these outstanding questions are addressed, we can expect to see continued improvements in the manufacture of T_{reg} cells and an increased alliance with synthetic biology and genome editing to tailor T_{reg} cell therapies to a growing number of conditions.

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Competing interests

J.A.B. and Q.T. acknowledge support from the Juvenile Diabetes Research Foundation, Caladrius Biosciences, Becton, Dickinson and Company (BD), Juno Therapeutics and Pfizer. L.M.R.F. has patents and pending patents on genome editing of T cells, haematopoietic stem cells and pluripotent stem cells. J.A.B. and Q.T. have patents and pending patents on polyclonal and antigen-specific regulatory T cells and con- sult for Third Rock Ventures and Juno Therapeutics in this area. J.A.B. serves on the board of Pfizer. Y.D.M. has no competing interests.

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Box 1 |

The increasing diversity of immunosuppressive cell types

A successful immune response entails the eradication of the pathogen and the timed contraction of activated immune cell populations to avoid excessive tissue damage. The best understood subsets of immunoregulatory cells that, in addition to $CD4^+FoXP3^+$ regulatory T cells (T_{reg} cells), play a role in suppressing immune responses and maintaining immune homeostasis are briefly summarized here.

Type 1 T_{reg} cells

Inducible type 1 T_{reg} cells (T_R1 cells) are $CD4^+$ $FoXP3^-$ T cells that secrete the immunosuppressive molecule IL-10. Some T_R1 cells also express granzyme B and can kill myeloid cells[213]. T_R1 cells are enriched by the simultaneous expression of CD49b and lymphocyte activation gene 3 protein (LAG3)[214]. In vitro, T_R1 cell expansion protocols use vitamin D_3, dexamethasone[215] or IL-10-producing antigen-presenting cells to generate antigen-specific T_R1 cells[216]. In 2012, in the first open-label uncontrolled, multicentre, single-infusion dose-escalation phase I/IIa clinical trial using T_R1 cells, ovalbumin-specific T_R1 cells were injected into 20 patients with refractory Crohn's disease; the injection was well tolerated and showed dose-related efficacy[217]. A multicentre phase II trial using T_R1 cells was completed in 2016 (NCT02327221; results not yet available). T_R1 cell-based cell therapy has also been successful in haematological diseases, preventing graft-versus-host disease and improving immune reconstitution[218].

$CD8^+$ T_{reg} cells

In 1970 one group established that T cells could suppress antibody responses[1], and hypothesized that suppressor T cells existed[219]. T cells expressing CD8α (Ly-2 at the time) and CD8β (Ly-3), but not CD4 (Ly-1), were later found to have suppressive functions[220]. $CD8^+$ T_{reg} cells were thus the first suppressor cells to be identified. $CD8^+$ T_{reg} cells share markers with activated conventional $CD8^+$ T cells, making it difficult to isolate them or dissect their function. Nevertheless, human $CD8^+FoXP3^+CD45RC^{low}$ T_{reg} cells are potent suppressive cells in graft-versus-host disease and solid organ transplantation[221], and $CD8^+FoXP3^+CD25^+TNFR2^+$ T_{reg} cells are suppressive in patients with type 1 diabetes treated with teplizumab, a humanized anti-CD3 antibody[222]. FoXP3 expression may allow the identification of bona fide human $CD8^+$ T_{reg} cells. In mice, the transcription factor HELIOS is required for the stability of both $CD4^+$ and $CD8\alpha\alpha^+$ T_{reg} cells in a proinflammatory milieu[223]. $CD8\alpha\alpha^+$ T_{reg} cells reside in the intestine, recognize the nonclassical major histocompatibility complex molecule Qa1 (HLA-E in humans) and protect against $CD4^+$ T cell-mediated colitis[224,225]. $CD8^+$ T_{reg} cells may primarily suppress activated T cells by killing them directly or by secreting inhibitory cytokines.

Type 3 T helper cells

Transforming growth factor-β (TGFβ)-producing type 3 T helper cells (T_H3) were identified when SJL mice, which are susceptible to experimental autoimmune encephalomyelitis (EAE), were fed myelin basic protein (MBP), and MBP-specific $CD4^+$

T cell clones were isolated from mesenteric lymph nodes[226]. These clones secreted low levels of interferon-γ (a hallmark of T_H1 cells), IL-4 (a hallmark of T_H2 cells) and IL-10 and high levels of TGFβ. Adoptive transfer of these T cell clones suppressed EAE in mice immunized with MBP in a TGFβ-dependent manner. The study authors named these mucosal regulatory cells T_H3 cells[226]. T_H3 cell-derived TGFβ can prevent and reverse autoimmune encephalomyelitis by inducing T_{reg} cell differentiation[227]. T_H3 cells may have an important role in controlling autoimmunity and allergy in humans.

Regulatory B cells

Regulatory B cells (B_{reg} cells) secrete anti-inflammatory cytokines, mainly IL-10, and suppress the proliferation of lymphocytes, including effector T cells[228]. B cell-mediated tolerance was originally hypothesized in the 1970s[229], but it was not until 1996, when the genetic ablation of B cells was shown to decrease the frequency of spontaneous recovery from EAE, that a regulatory role for B_{reg} cells was proposed[230]. B cells in mice that recovered from EAE were found to secrete IL-10 in response to self-antigen, and the genetic ablation of IL-10 in B cells prevented spontaneous recovery from EAE[231]. Inflammation seems to trigger the induction of B_{reg} cells through the production of IL-10 by suppressive cells at the inflamed site[232]. Accordingly, individuals with chronic inflammation and autoimmunity display deficiencies in the number and function of suppressive cells circulating and at the inflamed site[233]. Importantly, B_{reg} cells skew T cell differentiation towards T_{reg} cells[234,235] and promote T_{reg} cell expansion[236,237], suggesting that they act by promoting T_{reg} cell activity.

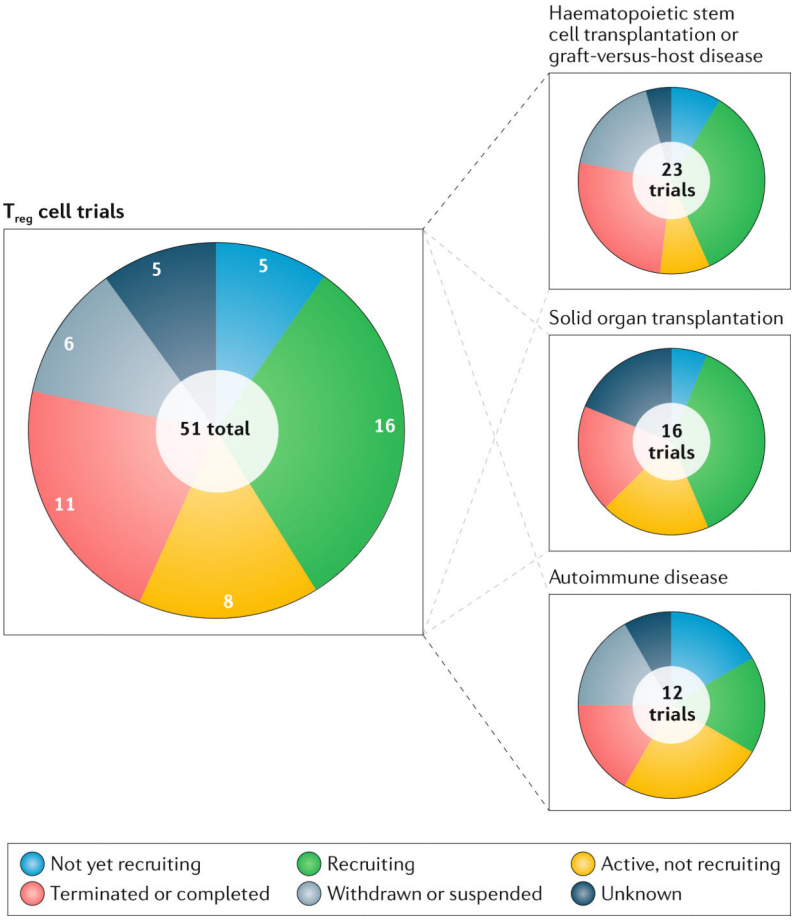

Fig. 1 |. Registered clinical trials using regulatory T cells.

The number and status of registered clinical trials based on regulatory T cell (T_{reg} cell) infusion were searched for in ClinicalTrials.gov and are summarized here (the numbers represent the data available in July 2019). The large circle shows the overall number of registered trials, divided into coloured segments that represent the proportion of these clinical trials with their status defined as not yet recruiting (blue), recruiting (green), active not recruiting (yellow), terminated or completed (pink), withdrawn or suspended (light grey) and unknown (dark grey). Unknown status corresponds to studies, the last known status of which was recruiting, not yet recruiting, or active, not recruiting, but that have passed their completion date but have not had their status verified within the past 2 years (http://clinicaltrials.gov). Small circles are categorized by indication: hematopoietic stem cell transplantation or graft-versus-host disease, solid organ transplantation and autoimmune disease.

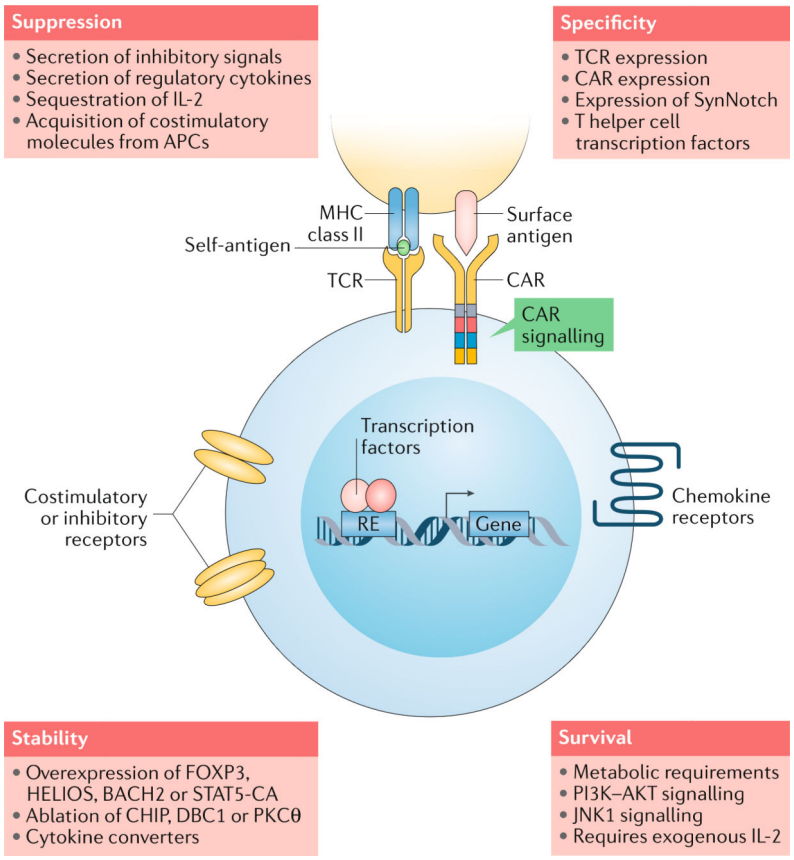

Fig. 2 |. Regulatory T cells as living drugs.

Four key properties are needed to successfully use regulatory T cells (T_{reg} cells) as living drugs, summed up as the four S's: suppression, specificity, stability and survival. In terms of suppression, T_{reg} cells act through multiple suppression mechanisms (including IL-2 deprivation from the milieu, secretion of inhibitory cytokines and interactions with antigen-presenting cells (APCs)), which could be tailored to specific conditions or diseases by, for example, forcing the expression of specific transcription factors. Regarding specificity, it is possible to create T_{reg} cells with a desired specificity using T cell receptor (TCR) gene transfer or artificial immune receptors, such as chimeric antigen receptors (CARs). Specificity can be made conditional by using synthetic Notch (SynNotch) receptors. Overexpressing transcription factors characteristic of T helper cell subsets can also enhance the specificity of T_{reg} cells. With respect to stability, forkhead box protein P3 (FOXP3) expression is central to the lineage of T_{reg} cells. Strategies to increase T_{reg} cell stability include the ectopic expression of the transcription factors FOXP3, HELIOS and BACH2 or of a constitutively active form of signal transducer and activator of transcription 5 (STAT5-CA), as well as the ablation of carboxy terminus of Hsp70-interacting protein (CHIP), deleted in breast cancer gene 1 protein (DBC1) or protein kinase C-θ (PKCθ) to prevent the degradation of FOXP3. Finally, the survival of T_{reg} cells depends on exogenous IL-2, metabolic requirements and tonic signalling mediated by the TCR and costimulatory molecules. Targeting T_{reg} cell metabolic requirements or manipulating the phosphatidylinositol 3-OH kinase (PI3K)–AKT or JUN amino-terminal kinase 1 (JNK1)

signalling pathways may increase T_{reg} cell survival after infusion. MHC, major histocompatibility complex; RE, regulatory element.

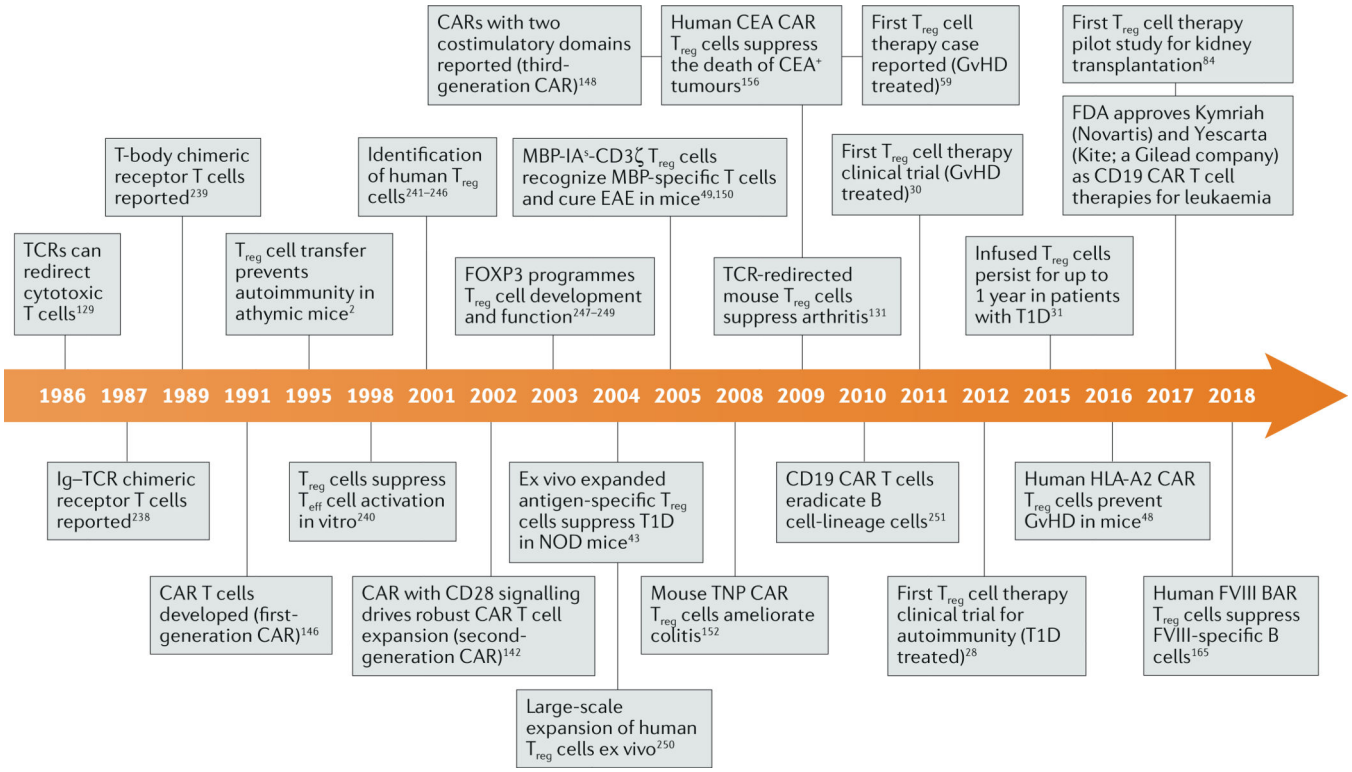

Fig. 3 |. Timeline of events in the development of regulatory T cell therapy.

A timeline of key developments leading to the use of regulatory T cell (T_{reg} cell) therapy in the clinic. BAR, B cell-targeting antibody receptor; CAR, chimeric antigen receptor; CEA, carcinoembryonic antigen; EAE, experimental autoimmune encephalomyelitis; FOXP3, forkhead box protein P3; FVIII, factor VIIII; GvHD, graft-versus-host disease; HLA, human leukocyte antigen; IA, murine major histocompatibility complex class II molecule I-A; Ig, immunoglobulin; MBP, myelin basic protein; NOD, non-obese diabetic; T1D, type 1 diabetes; TCR, T cell receptor; Teff cell, effector T cell; TNP, 2,4,6-trinitrophenyl.

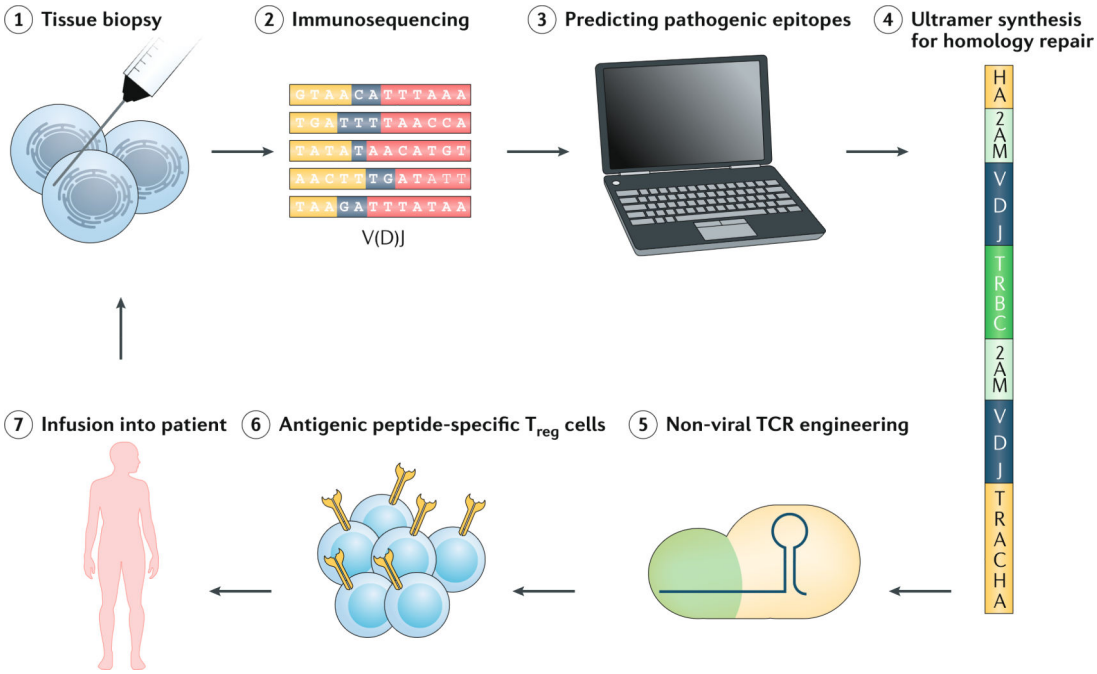

Fig. 4 |. Redirecting regulatory T cells by engineering T cell receptors.

A tissue biopsy sample is taken from a patient with an autoimmune disease (step 1) and subjected to single-cell paired T cell receptor (TCR) sequencing to characterize the TCR repertoire of the regulatory T cells (T_{reg} cells) or, alternatively, of the autoimmune T cells (step 2). On the basis of algorithms identifying conserved motifs and complementarity-determining regions, pathogenic epitopes are predicted (step 3) and selected TCR ultramer templates are synthesized (step 4). Using a non-viral approach, the ultramer and a Cas9 ribonucleoprotein complex targeting the endogenous TCR via homology repair are electroporated into peripheral blood-derived T_{reg} cells (step 5). Edited T_{reg} cells are further expanded in vitro (step 6) and finally reinfused into the patient (step 7).

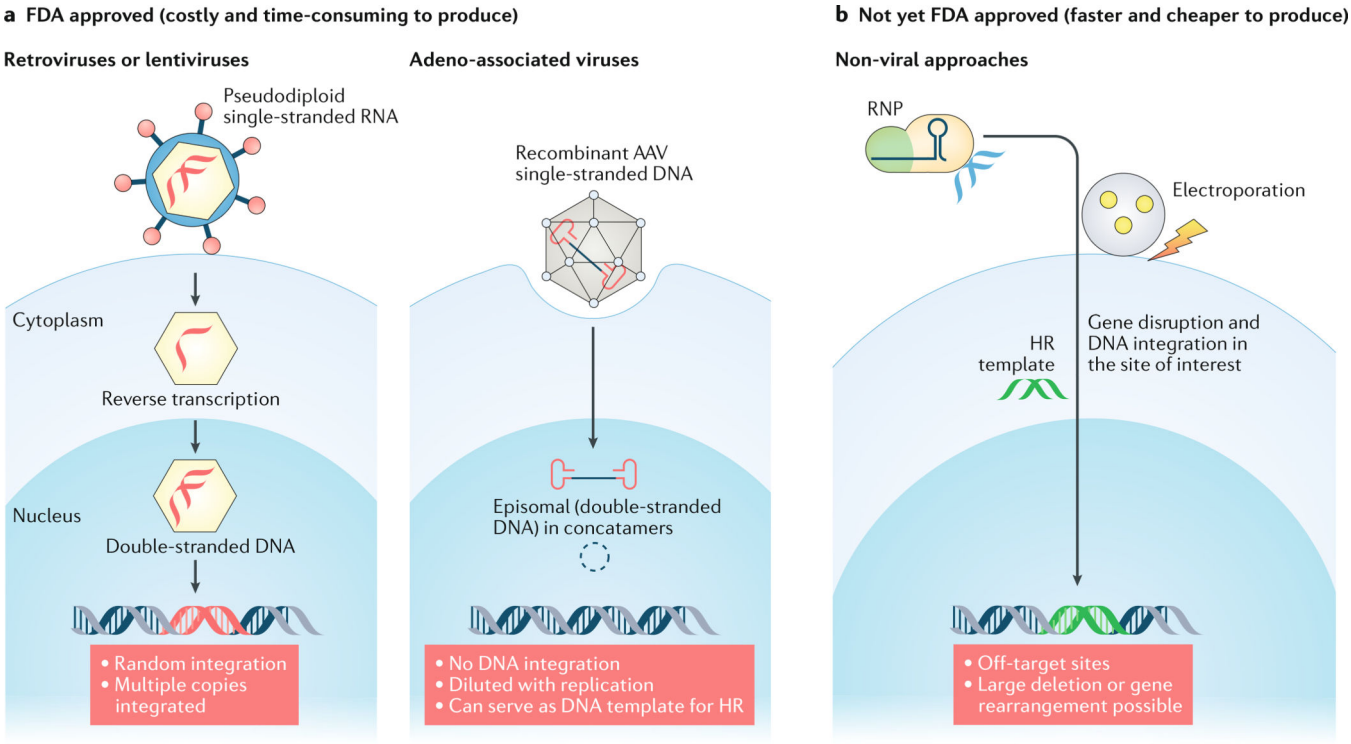

Fig. 5 |. Methods for delivering genetic material to engineer regulatory T cells.

a | The first FDA-approved method for delivering genetic material to engineer regulatory T cells uses retroviruses or lentiviruses, which are pseudodiploid single-stranded RNA viruses. Genomic integration of the gene cassette of interest is random, resulting in non-physiological gene regulation with often multiple copies of the gene per cell. The second FDA-approved method uses recombinant adenoassociated viruses (AAVs); these viruses persist inside the cell as a double-stranded DNA episome that is not integrated into the genome and will therefore be diluted over time in replicating cells. Many groups combine use of recombinant AAVs with use of CRISPR-based technologies, as the episome can serve as a homology repair (HR) template, an approach recently approved by the FDA for clinical trials. **b** | A third method, which is purely non-viral but not FDA approved yet, electroporates the desired cassette as double-stranded DNA or an ultramer (single-stranded DNA) template together with a Cas9–ribonucleoprotein (RNP) complex. Although this method leads to a single copy of the gene being precisely integrated at a desired location in the genome, it also typically results in lower modification efficiencies.

Table 1 |

Advantages and disadvantages of TCRs and CARs in engineering regulatory T cell specificity

Property	TCRs	CARs
Specificity	Peptide–HLA complex	Any surface antigen or multivalent soluble antigen
HLA restriction	Yes	No
Co-receptor required	Yes (CD4 or CD8)	No
Affinity[a]	$K_D = 10^{-6}$–10^{-4} M[252]	$K_D = 10^{-10}$–10^{-6} M[253]
Sensitivity	<10 molecules per target cell[104,254]	100–10,000 molecules per target cell[105,255–257]
Signalling	Via endogenous CD3 complex (comprising six chains)	Via synthetic modular signalling domain
Expression challenges	Endogenous TCR might pair with exogenous TCR chains	Protein aggregation; aggregation of single-chain variable fragments leading to tonic signalling

CAR, chimeric antigen receptor; TCR, T cell receptor.

[a] K_D, equilibrium dissociation constant; the lower the K_D value, the higher the affinity.