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Review article

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Harnessing the biology of regulatory T cells to treat disease

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Abstract

Regulatory T (T_{reg}) cells are a suppressive subset of $CD4^+$ T cells that maintain immune homeostasis and restrain inflammation. Three decades after their discovery, the promise of strategies to harness T_{reg} cells for therapy has never been stronger. Multiple clinical trials seeking to enhance endogenous T_{reg} cells or deliver them as a cell-based therapy have been performed and hint at signs of success, as well as to important limitations and unanswered questions. Strategies to deplete T_{reg} cells in cancer are also in active clinical testing. Furthermore, multi-dimensional methods to interrogate the biology of T_{reg} cells are leading to a refined understanding of T_{reg} cell biology and new approaches to harness tissue-specific functions for therapy. A new generation of T_{reg} cell clinical trials is now being fuelled by advances in nanomedicine and synthetic biology, seeking more precise ways to tailor T_{reg} cell function. This Review will discuss recent advances in our understanding of human T_{reg} cell biology, with a focus on mechanisms of action and strategies to assess outcomes of T_{reg} cell-targeted therapies. It highlights results from recent clinical trials aiming to enhance or inhibit T_{reg} cell activity in a variety of diseases, including allergy, transplantation, autoimmunity and cancer, and discusses ongoing strategies to refine these approaches.

Sections

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Introduction

Regulatory T (T_{reg}) cells constitute ~2–5% of the adult peripheral blood $CD4^+$ T cell repertoire[1]. Whereas the majority of immune system cells function to promote inflammation to fight pathogens and cancers, T_{reg} cells keep immunity in check to maintain homeostasis and prevent pathology. Dysregulation of T_{reg} cell function is linked with many diseases and the failure of these cells to control antigen-specific effector T cells can lead to autoimmune diseases, allergies and various other hyperinflammatory diseases. When a patient receives a transplant, T_{reg} cells prevent alloreactive effector T cells from attacking the foreign tissue. In contrast, tumours enhance the T_{reg} cell response so that anticancer immunity is suppressed. Therapies focused on T_{reg} cells thus aim to either enhance their function (in the case of autoimmunity, allergy, transplantation and other hyperinflammatory diseases) or hinder it (in patients with cancer). Establishing an appropriate balance between T_{reg} cells and effector T cells is thus critical to human health and underlies the broad clinical potential for T_{reg} cell-based therapies.

T_{reg} cells are characterized by constitutively high expression of FOXP3 and CD25 (the IL-2 receptor α-chain; IL-2Rα). As their lineage-defining transcription factor, FOXP3 is essential for T_{reg} cell development. The absence of functional FOXP3 from birth results in deficient T_{reg} cell function and a disease characterized by widespread autoimmunity and allergy, known as immune dysregulation, polyendocrinopathy, enteropathy and X-linked (IPEX) syndrome[2,3]. FOXP3 has both transcriptional activation and repression functions; for example, it can simultaneously promote *CD25* and suppress *IL2* transcription, resulting in the expression of CD25 (ref. 4) and reliance on environmental IL-2 (refs. 5–8), which are both characteristic of T_{reg} cells.

Like all other T cells, T_{reg} cells express a T cell receptor (TCR), which allows them to detect antigens presented by major histocompatibility complex II (MHC II) on antigen-presenting cells (APCs; commonly dendritic cells or macrophages). Upon recognition of their cognate antigen–MHC on an APC, T_{reg} cell immune modulation can be broadly categorized into three main phases (Fig. 1). First, interactions with APCs reduce their capacity to activate effector T cells. Second, activation-stimulated release of cytokines and metabolites by T_{reg} cells diminishes the pro-inflammatory activity of surrounding immune cells. Third, this re-shaped environment favours the expansion and de novo development of new T_{reg} cell populations, thereby promoting tolerance. T_{reg} cell-focused therapies attempt to target one or more of these modes of action to either enhance cell function (in the case of autoimmunity, allergy or transplantation) or to suppress it (in the case of cancer). An additional therapeutic goal is to change the abundance of T_{reg} cells to re-establish their healthy balance with effector T cells.

In this Review, we first provide a brief overview of the development and function of T_{reg} cells, then outline the challenges associated with their use as a biomarker of immune function and discuss therapeutic approaches to boost endogenous T_{reg} cells. We next discuss T_{reg} cellular therapy as an exciting prospect for the treatment of autoimmunity and to induce transplant tolerance, and describe how the detrimental effects of T_{reg} cells can be targeted to fight cancer. We highlight new, more precise T_{reg} cell-targeting approaches that are beginning clinical testing. Although we focus on $FOXP3^+$ T_{reg} cells, we acknowledge that other types of suppressive T cells, such as IL-10-producing type 1 T_{reg} cells[9] and $CD8^+$ T_{reg} cells[10], also have important roles in immune homeostasis. We review knowledge derived from the study of human T_{reg} cells but refer to selected studies in model systems using mice or non-human primates.

The biology of human T_{reg} cells

T_{reg} cell development in the thymus and periphery

T_{reg} cells arise via two major developmental pathways: selection in the thymus to generate thymic T_{reg} (tT_{reg}) cells (sometimes also referred to as natural T_{reg} cells) or differentiation in the periphery from conventional T cells to generate peripheral T_{reg} (pT_{reg}) cells. In the thymus, T_{reg} cell differentiation is driven by the pattern and strength of TCR and cytokine signalling[11,12], with a general consensus that T_{reg} cells arise from cells bearing 'intermediate' affinity TCRs with hydrophobic antigen–MHC-binding regions that bind self-antigens[13]. These cells with intermediate affinity TCRs bifurcate into two populations: cells receiving continuous antigen signalling develop into autoreactive, IL-2-producing effector T cells, whereas less continuous TCR signalling, for example, interrupted by TGFβ-mediated inhibition, results in cells expressing FOXP3 (ref. 14). The development of these $FOXP3^+$ T_{reg} cells is then locked in by IL-2 produced by their surrounding autoreactive counterparts[14,15]. Studies in mice suggest that most T_{reg} cells bear TCRs that recognize common tissue antigens, rather than highly restricted tissue-specific antigens, resulting in tissue-agnostic migration patterns of the cells[16]. Although FOXP3 is necessary for tT_{reg} development, ongoing expression in fully committed T_{reg} cells might not be essential because knockout of *FOXP3* does not significantly disrupt their transcriptome or methylome[17]. Accordingly, transcription factors other than FOXP3 (for example, TCF1 and SATB1) also have major roles in tT_{reg} cell development, and collectively multiple proteins contribute to lineage-specific epigenetic organization[18–20].

The development of pT_{reg} cells has been best characterized in intestinal tissues, where cells differentiate to induce tolerance to dietary antigens and commensal microbiota. Recently, three studies reported the role of a new type of intestinal APC[21–23], termed $ROR\gamma t^+$ APCs, which share features of both dendritic and epithelial cells. These APCs express integrins αvβ8 and αvβ3, which convert latent TGFβ to its active form. TGFβ then converts conventional T cells into $FOXP3^+$ T_{reg} cells, which proceed to restrain gut-resident T cells. In humans, $ROR\gamma t^+$ APCs are enriched in mesenteric lymph nodes[23] and might be dysfunctional in inflammatory bowel disease[21]. Interestingly, although FOXP3 expression in pT_{reg} cells is essential for suppression of a transcriptional programme associated with T_H17 cells, commitment to the pT_{reg} lineage is reported to be FOXP3 independent[24]. Nevertheless, consistent evidence that people with genetic mutations in *FOXP3* suffer from colitis[25] indicates that this transcription factor has a non-redundant role in maintaining intestinal tolerance.

Whether or not tT_{reg} and pT_{reg} cells are distinguishable in humans has been debated for many years. In mice, surface expression of neuropilin 1 (NRP1) is often used to define tT_{reg} cells as it is not expressed by pT_{reg} cells[26] but, in humans, this receptor does not effectively identify T_{reg} cellular origin[27]. Alternatively, expression of the transcription factor HELIOS (encoded by the *IKZF2* gene) has been proposed as a characteristic of tT_{reg} cells and, in humans, blood-derived T cells co-expressing high levels of FOXP3 and HELIOS are enriched with stable, highly suppressive T_{reg} cells[28].

T_{reg} lineage stability

The stability of the T_{reg} cell lineage and how the biology of thymic versus peripherally derived cells differs are key questions in this field. Understanding T_{reg} cell stability is fundamental for addressing the risk of their conversion into pathogenic effector T cells as well as the longevity of therapeutic effects. The best marker of T_{reg} cell stability is the level of CpG DNA methylation at the T_{reg} cell-specific demethylated region (TSDR), a non-coding DNA sequence element located in

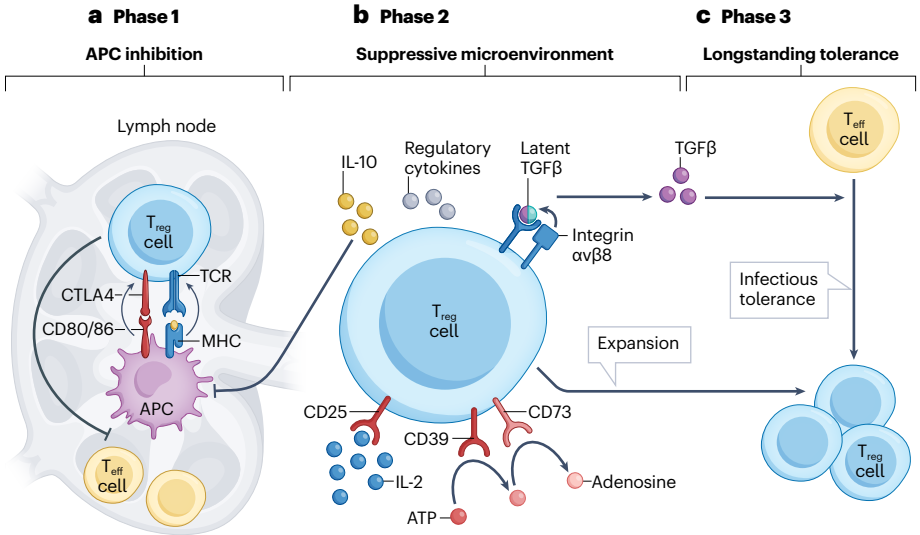

Fig. 1 | Three phases of T_{reg} cell-mediated immune suppression. a, In phase 1, regulatory T (T_{reg}) cells suppress the ability of antigen-presenting cells (APCs) to present antigen to and co-stimulate effector T (T_{eff}) cells. The T cell receptor (TCR) of a T_{reg} cell forms a tight immune synapse with the antigen–major histocompatibility complex (MHC) complex displayed on the APC, physically blocking effector T cell access to the same antigen. Additionally, T_{reg} cell CTLA4 preferentially binds to CD80 and CD86 on the APC, blocking effector T cell access to co-stimulatory signalling through the lower affinity receptor, CD28. Upon retreat from the APC, the T_{reg} cell removes co-stimulatory proteins and antigen–MHC complexes, thereby further abrogating effector T cell activation. These T_{reg} cell–APC interactions are thought to occur predominantly in secondary lymphoid organs (such as lymph nodes) but might also occur in tissues. **b**, In phase 2, T_{reg} cells release a variety of regulatory cytokines into their microenvironment to suppress inflammation. Most notable are IL-10 and TGFβ, which suppress APCs and promote FOXP3 expression, respectively. TGFβ is cleaved from its latent form into its active form by various mechanisms, notably integrin αvβ8 on T_{reg} cells. T_{reg} cells express high levels of CD25, which has a higher affinity for IL-2 than the dimeric IL-2 receptor expressed by conventional T cells. Therefore, T_{reg} cells behave as an 'IL-2 sink', restricting the amount available for conventional T cells. T_{reg} cells also express the ectonucleotidases CD39 and CD73, which work together to convert ATP to adenosine, which has anti-inflammatory properties. **c**, In phase 3, T_{reg} cells mediate 'infectious tolerance' by inducing the expansion of existing T_{reg} cells and/or by converting effector T cells into T_{reg} cells, establishing a long-lasting tolerogenic balance.

the first intron of *FOXP3* (ref. 29). This locus is stably demethylated in T_{reg} cells but not in activated conventional T cells[29]. Of note, since *FOXP3* is on the X chromosome, in females one allele is methylated due to X-inactivation, making it important to account for sex during the analysis. In the context of inflammation, some studies in mice show stable FOXP3 expression and lineage stability[30], whereas others report loss of FOXP3 and gain of an effector T cell phenotype[31,32]. These contrasting results likely arise from differences in the lineage-tracing systems used and because FOXP3 is transiently expressed in activated effector T cells that could be present in putative T_{reg} cell populations[33].

Similar concerns have also been raised about the stability of human T_{reg} cells, particularly in the context of T_{reg} cell therapy products[34] (discussed below). Patients with IPEX have autoreactive, TSDR-demethylated effector T cells that resemble destabilized T_{reg} cells[35]. These destabilized T_{reg} cells do not emerge in the presence of wild-type FOXP3$^+$ T_{reg} cells, showing that FOXP3 is important for T_{reg} cell stability and that functional T_{reg} cells dominantly assert tolerance in a heterogeneous population of unstable progenitors[35]. A consideration is that it is difficult to attribute changes in phenotype or function to true lineage instability versus effects driven by heterogeneous cell populations that include conventional T cells. Nevertheless, numerous studies in humans found that T_{reg} cells resist lineage instability and maintain suppressive function in the presence of inflammatory cytokines, including IL-6, TNF or IL-12 (refs. 36,37). In addition, multiple preclinical models of adoptive T_{reg} cell therapy have not revealed significant loss of tolerogenic properties[38–40].

HELIOS expression is also associated with lineage stability, potentially via its ability to suppress IL-2, IFNγ and TNF production, as demonstrated in patients with biallelic or dominant negative *IKZF2* mutations[41,42]. Indeed, ex vivo culture of human FOXP3$^+$ HELIOS$^+$ T_{reg} cells in pro-inflammatory cytokines does not destabilize their phenotype and rather enhances their proliferation[36,37]. However, knockout of *IKZF2* in human T_{reg} cells does not change cell phenotype or function, suggesting that HELIOS might be a T_{reg} stability marker but its ongoing expression is not required for fully functional cells[28].

Overall, the conflicting reports regarding T_{reg} cell stability are likely attributed to different T_{reg} cell enrichment and tracking protocols used between studies as well as to differences between mice and humans. To date, there are no reports of a T_{reg} cell product losing its suppressive capacity in patients, suggesting that current clinical T_{reg} cell isolation protocols derive functionally stable products.

Mechanisms of action

Multiple mechanisms are associated with the suppression and/or promotion of tissue repair by T_{reg} cells (Box 1). Gene knockout studies in mice support mechanistic roles for IL-2 consumption[43] and the expression of CTLA4 (ref. 44), IL-10 (ref. 45) and/or TGFβ[46]. Below, we summarize some of the best-characterized mechanisms of action grouped into three phases (Fig. 1). More comprehensive summaries of additional mechanisms are found in recent reviews[47,48]. Precisely which mechanisms control immune homeostasis in different tissues and disease contexts, especially in humans, remains undefined.

Box 1 | T_{reg} adoptive cell therapy in non-traditional diseases

Regulatory T (T_{reg}) cells control immune tolerance but also generally suppress inflammation and contribute to tissue regeneration through cytokines such as amphiregulin, a low-affinity epidermal growth factor receptor ligand[105,262]. Leveraging the ability of T_{reg} cells to reduce inflammation while promoting tissue repair offers a new approach to managing a range of inflammatory diseases.

Human studies

- During the COVID-19 pandemic, polyclonal allogeneic T_{reg} cell therapy was shown to be safe and potentially effective at reducing mortality in patients with acute respiratory distress syndrome[143].
- In a mouse model of Alzheimer disease, β-amyloid-specific T_{reg} cells accumulated in the brain, reducing β-amyloid depositions and microglial-driven inflammation, while enhancing cognitive function[263]. This T_{reg} cell therapy is now in a phase I clinical trial (NCT05016427).
- T_{reg} cell frequency in the blood inversely correlated with amyotrophic lateral sclerosis (ALS) progression in humans[264] and now multiple clinical trials are under way for the treatment of ALS, including the REGALS trial (NCT05695521; Cellenkos) using cryopreserved, allogenic cord blood-derived T_{reg} cells with neurotropic homing markers.

Mouse studies

- After myocardial infarction in mice, T_{reg} cells reduce inflammation and release factors that promote cardiomyocyte proliferation, thereby reducing pathological burden and promoting repair[265]. Similarly, T_{reg} cells promote neovascularization following ischaemic injury in models of obesity and type 2 diabetes mellitus[266].
- T_{reg} cells reside in the mouse brain, where they reduce neuroinflammation and promote tissue repair in models of ischaemic stroke, traumatic brain injury and multiple sclerosis, reducing disease severity and accelerating healing[267,268]. Similarly, co-transplanting T_{reg} cells with midbrain dopamine neurons promotes neuronal survival and function in a preclinical model of Parkinson disease[269].
- Attempts to expand endogenous T_{reg} cells in mice have been able to delay the onset of hindlimb paralysis in a preclinical model of ALS[264].

Phase 1: APC modulation. T_{reg} cells inhibit activation of effector T cells by physically blocking their access to cognate antigens and depleting co-stimulatory proteins from APCs, which primarily reside in secondary lymphoid organs, including lymph nodes (Fig. 1a). Live microscopy reveals that T_{reg} cells form tight bonds with APCs that are presenting their cognate antigen, thereby blocking effector T cells with the same antigen specificity from accessing the APC and preventing T cell activation[49]. Furthermore, when T_{reg} cells pull away from APCs they remove the antigen–MHC complex from the APC surface, further preventing effector T cell activation[49]. Beyond these effects on antigen presentation, T_{reg} cells limit the ability of APCs to provide co-stimulatory signals (via CD80 and CD86) to effector T cells by expressing high levels of the coinhibitory receptor CTLA4, which outcompetes the effector T cell co-stimulatory receptor CD28 for binding to CD80 and CD86 and depletes them from the APC surface[50,51]. As a result, T_{reg} cells from people with genetic mutations in *CTLA4* have decreased suppressive capacity, contributing to their autoimmune phenotypes[52]. Moreover, combined blockade of CD28 and CTLA4 with the CTLA4–Ig fusion protein inhibits the therapeutic effect of T_{reg} cells in a humanized mouse model of skin transplantation[53].

Phase 2: microenvironment regulation. T_{reg} cells produce multiple anti-inflammatory cytokines and metabolites, most notably TGFβ and IL-10 (Fig. 1b). TGFβ is produced by many T cells but often remains in its latent form, tethered to the cell membrane. However, T_{reg} cells express αvβ8 integrin, which can release TGFβ from its inhibitory complex into its active form[54]. Activated autocrine TGFβ then maintains and promotes FOXP3 expression[46,55]. T_{reg} cells also produce IL-10, which suppresses production of innate, pro-inflammatory cytokines (such as TNF, IL-6 and IL-1β) and APC function[45,56]. Furthermore, T_{reg} cells produce IL-35 (Ebi3–p35 heterodimer), which diminishes inflammation and promotes T_{reg} cell function[57]. An additional anti-inflammatory mechanism involves expression of the ectonucleotidases CD39 and CD73, which work together to convert extracellular ATP into immunosuppressive adenosine[58,59], contributing to T_{reg} cell suppression.

Not only do T_{reg} cells produce anti-inflammatory mediators, they also behave as an 'IL-2 sink'. In T_{reg} cells, the IL-2Rα subunit (CD25) complexed with IL-2Rβ and IL-2Rγ subunits form a trimeric receptor with 100-fold higher affinity for IL-2 than the dimeric IL-2Rβ–IL-2Rγ form that is typically expressed by effector T cells[60]. Therefore, T_{reg} cells deplete IL-2 from their environment and deprive conventional T cells of this essential cytokine. Experiments in mice with a T_{reg} cell-specific deletion of CD25 revealed that T_{reg} cell-mediated IL-2 deprivation was primarily required for suppression of $CD8^+$ T cells[43], whereas the effect on $CD4^+$ T cells was nuanced. Specifically, only $CD4^+$ T cells with weak TCR signalling and low IL-2 production are suppressed via this mechanism[61]. A therapeutic consideration is that combining T_{reg} cell adoptive transfer with low-dose IL-2 therapy might be deleterious[62] because provision of the exogenous cytokine could override the IL-2 sink effect.

Phase 3: infectious tolerance. 'Infectious tolerance' refers to the establishment of independent, long-lasting tolerance in response to a treatment[63] (Fig. 1c). The concept is that enhanced T_{reg} cell function leads to a permanent change in the balance of T_{reg} versus effector T cells, favouring tolerance induction. An additional angle is that enhancing T_{reg} cells with one antigen specificity can induce infectious tolerance to a distinct antigen via a process known as 'linked suppression', which is mediated by local APC modulation. The notion of infectious tolerance is crucial for T_{reg} cell therapy because it raises the possibility that a transient treatment could lead to long-lasting tolerogenic effects. Although the mechanisms by which tT_{reg} and/or pT_{reg} cells mediate infectious tolerance remain to be determined, the process is likely TGFβ dependent[64,65] and enhanced in the absence of CD28 signalling[66]. In humans, it is difficult to study infectious tolerance in vivo, and therefore evidence is from studies of autoimmunity and transplantation in mice[67–69]. With more sophisticated cell tracing and gene-editing tools emerging, more research to fully understand this process is warranted.

Assessing human T_{reg} cells in clinical studies

Identifying T_{reg} cells

Perhaps one of the biggest challenges to the study of human T_{reg} cells is the difficulty in tracking their numbers and function in health versus disease, or in response to therapy, due to the lack of a single definitive phenotypic marker (Fig. 2). Human T_{reg} cells are commonly identified as $CD25^{high}$, $CD127^{-}$ and $FOXP3^{+}$ (ref. 70); however, these proteins are technically difficult to measure via flow cytometry as they have gradients of expression rather than clear positive and negative populations (Fig. 2a). Moreover, this pattern of expression is also characteristic of activated effector T cells. Thus, multiple phenotypic markers need to be assessed to increase confidence in accurate T_{reg} cell identification. Simple approaches include co-staining for HELIOS and/or CD45RA[70]. CD45RA is particularly useful in separating resting ($CD45RA^{+}FOXP3^{low}$) or activated ($CD45RA^{-}FOXP3^{high}$) T_{reg} cells from conventional T cells expressing low levels of FOXP3 ($CD45RA^{-}FOXP3^{low}$)[71]. More complex approaches, such as ascertaining the absence of IL-2 production[33] and/or using gene signatures, can further help identify T_{reg} cells. For example, nanoString or RNA sequencing has been used to identify and track changes in the transcriptomic signature of T_{reg} cells in people with type 1 diabetes mellitus (T1DM)[72] or heart transplant recipients[73] (Fig. 2b).

Measuring T_{reg} cell antigen specificity

T_{reg} cell therapy is thought to be most effective if it enriches the function of disease-relevant, antigen-specific cells. While TCR sequencing provides information on clonality changes, the use of multimers and/or activation-induced marker (AIM) assays can quantify antigen-specific T_{reg} cells (Fig. 2d). Multimers are complexes of antigen–MHC conjugates that bind to antigen-specific T_{reg} cells so they can be detected by flow cytometry. Multimers have been used to track T_{reg} cells in T1DM, revealing that people with protective MHC haplotypes have an increased frequency of islet-specific T_{reg} cells[74]. However, multimer-based methods are limited by manufacturing difficulties as well as limited knowledge of relevant antigens. Moreover, this technology is highly influenced by cell freezing[75], making its use in human studies challenging.

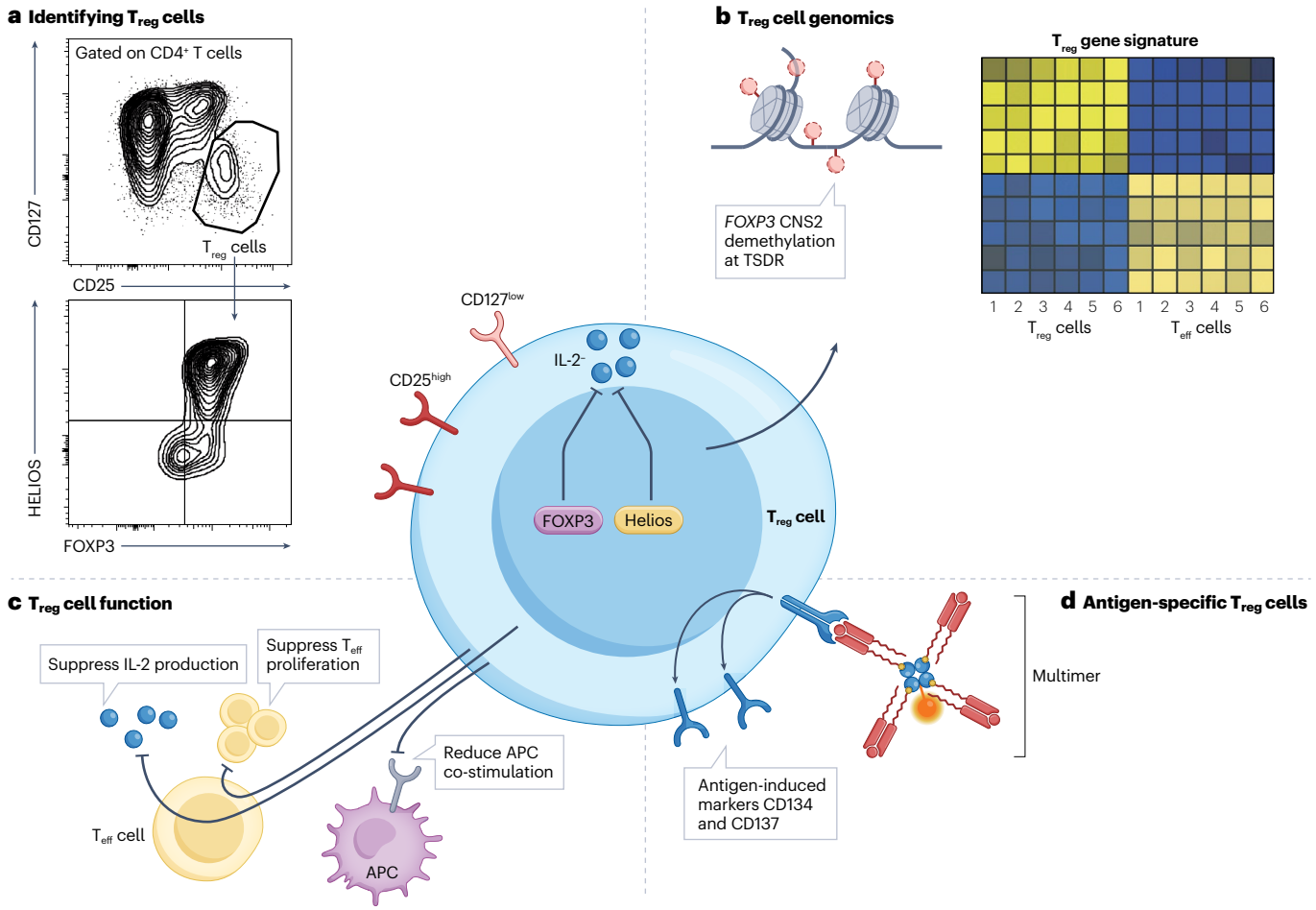

Fig. 2 | Tracking T_{reg} cells in clinical studies. a, Human regulatory T (T_{reg}) cells can be identified based on their surface expression of $CD4^{+}CD25^{high}CD127^{low}$, intracellular expression of FOXP3 and HELIOS, and lack of IL-2 production (inhibited by FOXP3 and HELIOS). **b**, Stable T_{reg} cells have demethylated DNA at the CNS2 region of *FOXP3* (the T_{reg} cell-specific demethylation region (TSDR)) and a characteristic gene signature comprised of a combination of high and low gene expression compared to effector T (T_{eff}) cells. **c**, T_{reg} cell function can be evaluated ex vivo by measuring reduced expression of co-stimulatory molecules on co-cultured antigen-presenting cells (APCs) and/or the ability to reduce T_{eff} cell proliferation and IL-2 production. **d**, Antigen-specific T_{reg} cells can be tracked with fluorescently labelled multimers or antigen-induced marker assays to quantify antigen-stimulated expression of activation proteins, including CD134 and CD137.

Table 1 | Selected clinical studies reporting effects of oral allergen-specific immunotherapy on antigen-specific T_{reg} cells

Clinical trial, refs.	Allergen	Exit allergen tolerance test	Method to detect antigen-specific T_{reg} cells	Impact on antigen-specific T_{reg} cells and other effects
NCT02635776 (ref. 87)	Peanut	67.2% tolerance (vs 4% placebo)	$CD154^-CD137^+CD134^+$ cells	No effect on T_{reg} cells Decreased peanut-specific $CRTH2^+$ effector T cells
No linked NCT[88]	Peanut	87% tolerance (vs 0% placebo)	Proliferation	Increased frequency Increased in vitro suppression Decreased *FOXP3* DNA methylation
NCT01750879 (ref. 257)	Peanut	65% tolerance (vs 0% placebo)	$CD154^+$ and/or $CD137^+$	No effect on T_{reg} cells Decreased peanut-specific effector T cells
NCT00932828 (ref. 258)	Peanut	NA	Proliferation	Transient increased T_{reg} and effector T cell frequency
NCT01290913 (ref. 89)	Peanut+omalizumab (anti-IgE antibody)	92.3% tolerance	Proliferation	Increased unstable/T helper 2-like T_{reg} cells Increased peanut-specific in vitro suppression No change in *FOXP3* DNA methylation
No linked NCT[259]	Caesin (from cow milk)	NA	$CD137^+$ and proliferation	Increased frequency Increased FOXP3 Casein-reactive T_{reg} cell frequency negatively correlated with time to tolerance development

NA, not available; T_{reg}, regulatory T.

As an alternative, many groups are turning towards AIM assays in which whole proteins or mixtures of peptides are added to blood or peripheral blood mononuclear cells, resulting in rapid upregulation of antigen-stimulated surface proteins. Antigen-specific T_{reg} cells can be identified using a variety of markers, including CD25, CD134 (OX40) and CD137 (4-1BB), and are distinguished from conventional T cells based on the absence of CD154 (CD40L) expression[76–79]. With AIM assays growing in popularity[76], we predict that they will increasingly be incorporated into immune monitoring for antigen-specific T_{reg} cells.

Functional assays

T_{reg} cells are functionally defined by their suppressive capability; their study was revolutionized by Thornton and Shevach who developed an in vitro suppression assay, which, in its original form, tested for the ability of T_{reg} cells to prevent proliferation and IL-2 production by stimulated effector $CD4^+$ T cells[80] (Fig. 2c). Although many variations on this assay have since been developed and the assay is considered the 'gold standard' to assess T_{reg} cell function, it is limited by two major caveats. First, the mechanisms of suppression measured in this assay and the assay's physiological relevance are unclear. Experiments testing roles for TGFβ, IL-10, CTLA4 and CD39–CD73 in suppression generally show no, or only partial, effects. Second, activated effector T cells can also exhibit suppressor-like activity via unknown mechanisms[81]. An alternate functional assay that is gaining popularity is to co-culture T_{reg} cells with APCs and assess the subsequent downregulation of co-stimulatory markers on the latter[82] (Fig. 2c). The development of improved in vitro assays where T_{reg} cell mechanisms of action are defined and reflect relevant in vivo effects is urgently needed. Systems such as organoid-based co-cultures[83], live tissue slices[84] and organs on chips[83] could offer new approaches to these old issues.

Boosting endogenous T_{reg} cells

Given that T_{reg} cells are essential for maintaining immune homeostasis and that most hyperinflammatory diseases can, in part, be ascribed to insufficient T_{reg} cell efficacy, there is significant interest in devising ways to increase T_{reg} cell activity. In this section, we focus on strategies to enhance T_{reg} cells in vivo either by harnessing natural mechanisms or by pharmacological approaches.

Harnessing natural mechanisms

Allergen immunotherapy. Allergen immunotherapy (AIT) for foreign antigens, including food and aero-antigens[85,86], seeks to reverse or prevent allergic symptoms mediated by antigen-specific T helper 2 (T_H2) cell immunity and enhance T_{reg} cells by repeated, low-dose allergen exposure to restore tolerance (Table 1). AIT is particularly effective for peanut allergy, as shown in the large PALISADE trial[87], where 67.2% of treated patients, compared to only 4% of placebo-treated patients, could eat peanut protein without symptoms at the end of the trial. Although the effects of AIT are presumed to be at least partly due to the induction of T_{reg} cells, consistent evidence supporting this mechanism in humans is lacking, possibly because few studies tracked changes in antigen-specific T_{reg} cells or considered changes in ratios of T_{reg} cells to allergen-specific TH2 cells (Table 1). For example, the PALISADE trial did not find increased peanut-specific circulating T_{reg} cells, although there was a decrease in peanut-specific T_H2 cells[87], potentially resulting in a beneficial balance between these cells. Indeed, a study of T_{reg} cells in birch allergy found that ratios of antigen-specific T_{reg} to T_H2 cells best correlated with clinical phenotypes[77]. On the other hand, another study of peanut AIT did find an increase in circulating antigen-specific T_{reg} cells using assays that showed decreased TSDR methylation and enhanced in vitro suppression[88]. Administration of peanut AIT in combination with omalizumab, an anti-IgE antibody that reduces allergen-triggered inflammation, also led to enhanced antigen-specific in vitro suppression by T_{reg} cells[89]. Of note, continued efficacy of AIT seems to be reliant on consistent antigen exposure, suggesting that, if T_{reg} cell-mediated tolerance is enhanced, it might not be permanently re-set for life, contrary to the theory of infectious tolerance.

Limitations of AIT include significant risks, side effects and patient compliance; therefore, various modifications of AIT are being explored. For example, a short regimen of allergen peptides modified to be less inflammatory increases the proportion of activated

T_{reg} cells and decreases grass allergy symptoms[90]. Similarly, less immunogenic mannan-coupled 'allergoids' that are taken up by dendritic cells and induce T_{reg} cells[91] are effective in treating patients with dust mite allergy[92].

Microbiome and environment. T_{reg} cells are strongly influenced by the microbiome and also control immune homeostasis to commensal bacteria. Therefore, there is significant interest in developing microbiota or other environmental interventions to enhance T_{reg} cell function. As an example, allergic infants lacking a series of *Clostridiales* species had fewer T_{reg} cells in their blood than healthy controls[93]. When these bacteria species were introduced to allergy-prone mice, there was an increase in protective T_{reg} cells. The reverse approach is also possible, with T_{reg} cell-promoting therapies facilitating beneficial microbiome changes. In mice, faecal microbiota transplant from animals previously treated with low-dose IL-2 to expand T_{reg} cells offered increased protection from colitis and T1DM compared to controls[94]. Similarly, in a small number of patients with autoimmune disease, treatment with low-dose IL-2 promoted a T_{reg} cell-permissive intestinal microbiome[94].

Vitamin D, obtained from the diet or generated in the epidermis upon ultraviolet B light exposure, also influences T_{reg} cells. Daylight influences the number of circulating T_{reg} cells in healthy people, and a study conducted in the northern hemisphere showed that peak T_{reg} cells in July and August correlated with the highest blood vitamin D levels[95]. A systematic review of the impact of oral vitamin D supplementation concluded that it increases the number of circulating T_{reg} cells relative to placebo and enhances their suppressive function[96]. A study of more than 25,000 healthy people showed supplementation with 2,000 IU of vitamin D per day resulted in a 22% drop in the incidence of autoimmune disease compared to placebo after 5 years[97]. Although these studies remain correlative, they suggest that manipulation of micronutrients could be a promising approach to enhance T_{reg} cell function.

Diet and exercise. A so-called Western lifestyle consisting of high-fat, salt and caloric intake might be a causative factor driving rising rates of autoimmunity. High levels of salt inhibit T_{reg} cells, which might be due to effects on mitochondrial respiration[98]. In terms of obesity, adipose tissue in overweight mice and humans is characterized by low-grade inflammation and decreased T_{reg} cell frequency and function relative to lean individuals[99–101]. Lean individuals fed a high-fat diet for 2 weeks had decreased T_{reg} cell frequencies and elevated markers of inflammation in their subcutaneous fat, relative to baseline[100]. In a pilot trial that studied the effects of diet and the microbiome in people with multiple sclerosis, 2 weeks of intermittent fasting led to microbiome changes that were similar to those seen in mice following the same diet and to a small but significant increase in proportions of circulating T_{reg} cells[102].

In mice, exercise promotes T_{reg} cell function by inducing expansion of the muscle T_{reg} cell compartment, thereby limiting the production of IFNγ, an inflammatory cytokine that impairs exercise-induced performance enhancement[103]. Therapeutic boosting of T_{reg} cells in mice improves insulin sensitivity in obesity[99] and muscle regenerative capacity[104,105]. At least some of the beneficial effects of exercise on T_{reg} cells are mediated by IL-6 released from muscle during exercise[104], challenging the concept that IL-6 might destabilize T_{reg} cells and aligning with human T_{reg} cell-based studies showing beneficial effects of inflammatory cytokines[36]. Further investigation into how T_{reg} cells promote muscle recovery might reveal relevant mechanisms that could be harnessed to treat diseases such as sarcopenia, sterile muscle injury and tocilizumab-associated muscle weakness.

Pharmacological approaches

Drawbacks to the non-pharmacological interventions discussed above include poor compliance and highly heterogeneous, non-T_{reg} cell-specific effects. Thus, a significant area of growth is in developing traditional drug-based approaches to boost T_{reg} cells in vivo.

Low-dose IL-2. IL-2 is a critical cytokine that is indispensable for T_{reg} cell function and survival. Although T_{reg} cells are unable to produce their own IL-2, they express the high-affinity trimeric IL-2 receptor (including CD25)[4]; therefore, multiple clinical trials have tested the effect of administering low doses of IL-2 to promote T_{reg} cell expansion[106]. T_{reg} cells with their high-affinity IL-2 receptor should outcompete $CD25^{-}$ effector T cells for IL-2 administered at low doses. Although low-dose IL-2 therapy is well tolerated, evidence for its efficacy is mixed. In trials for chronic graft-versus-host disease (GVHD)[107] and hepatitis C virus-induced vasculitis[108], more than half of patients showed improved symptoms after IL-2 therapy. Similarly, the TRANSREG study, which enroled patients with 11 different autoimmune diseases, reported decreased disease activity[109]. Other studies treating rheumatoid arthritis[110], systemic lupus erythematosus (SLE)[111,112] and T1DM[113] also reported beneficial effects. In transplantation, low-dose IL-2 administered to two face transplant recipients resulted in enhanced T_{reg} cell frequency in the skin graft and higher suppressive capacity[114].

On the other hand, several studies found no benefit or detrimental effects of low-dose IL-2. The LITE trial, which aimed to decrease immunosuppression in liver allograft recipients, was halted due to a potentially increased risk of rejection with no evidence for alloreactive T_{reg} expansion[115]. In the TILT study, autologous polyclonal T_{reg} cells plus low-dose IL-2 were combined to treat new-onset T1DM but showed no metabolic benefit and increased circulation of pro-inflammatory cells[62].

These heterogeneous results might be partly explained by the widely varying dosing regimens between studies, both in terms of timing and amount of cytokine. Given the short half-life and potential negative feedback pathways of the cytokine, small differences in these parameters could have major effects on outcomes[116]. Another consideration is that low-dose IL-2 could also expand other, non-suppressive immune cells leading to deleterious impacts. It consistently causes an increase in circulating $CD56^{bright}$ natural killer cells and eosinophils, although these appear transient and not harmful[117]. However, a study found that low-dose IL-2 increases inflammatory granzyme B^{+} lymphocytes and clonally expanded $CD8^{+}$ T cells[62], potentially fuelling hyperinflammation. Overall, the lack of a true T_{reg}-specific effect and undesirable pharmacodynamics makes low-dose IL-2 a challenging strategy to boost endogenous T_{reg} cells.

Modified IL-2 for T_{reg} selective effects. Given the challenges with natural IL-2, strategies to modify IL-2 to increase its specificity for T_{reg} cells and extend its half-life are being pursued with three general approaches. The first is to mutate the IL-2 protein so it is more selective for CD25, resulting in so-called 'muteins'. Mutant forms of IL-2 are often combined with a second approach of adding a half-life-extending moiety. Efavaleukin alfa (previously AMG 592) is a mutated form of IL-2 with decreased binding to the IL-2Rβ subunit and increased reliance on the IL-2Rα subunit (CD25) of the IL-2R; it is also fused to an immunoglobulin Fc domain to extend its half-life. This drug was in clinical trials for ulcerative colitis, but was terminated due to meeting a prespecified futility rule (NCT04987307). An earlier trial of efavaleukin alfa in SLE (NCT03451422) showed T_{reg} cell expansion with minimal off-target effects on conventional T cells and natural killer cells[118],

Table 2 | Clinical-stage nanomedicines to boost T_{reg} cells

Lead company	Nanoparticle content	Anticipated impact on T_{reg} cells	Disease/conditions	NCT and phase
Moderna	mRNA-6231: mRNA encoding IL-2 mutein	IL-2-mediated T_{reg} cell expansion[260]	Healthy volunteers	NCT04916431 (I)
COUR (Takeda partnership for TAK-101)	TAK-101: gliadin	Nanoparticles release antigens in a tolerogenic manner, inducing T_{reg} cells[261]	Coeliac disease	NCT03486990 (I) and NCT03738475 (IIa)
	CNP-201: peanut protein		Peanut allergy	NCT05250856 (Ib/IIa)
	CNP-104: PDC-E2		Primary biliary cholangitis	NCT05104853 (IIa)
	CNP-106: undisclosed antigen		Myasthenia gravis	NCT06106672 (Ib/IIa)
Selecta Biosciences; now merged with Cartesian Therapeutics	ImmTOR: rapamycin; administered with non-encapsulated pegylated uricase	Creates tolerogenic environment; delivered antigen protected from antibody-mediated degradation[131]	Gout	NCT02959918 (II)
TOPAS Therapeutics	TPM502: major gluten epitopes	Antigen-conjugated nanoparticles targeted to the liver; antigen presentation is tolerogenic and induces T_{reg} cells	Coeliac disease	NCT05660109 (II)
	TPM203: undisclosed antigen		*Pemphigus vulgaris*	EudraCT Number: 2019-001727-12 (I)

T_{reg}, regulatory T.

but clinical development was also halted due to a likelihood of it being ineffective. Similarly, development of other Fc-fusion IL-2 muteins was stopped by Roche and Bristol Myers Squibb due to a lack of efficacy in ulcerative colitis and psoriasis, respectively. Merck, Cugene and Xencor also performed trials with Fc-fusion IL-2 muteins in ulcerative colitis (NCT04924114) or healthy volunteers (NCT05328557, NCT04857866), with results pending.

Exemplifying a second approach to modify IL-2, rezpegaldesleukin (NKTR-358) consists of wild-type IL-2 conjugated to polyethylene glycol to extend its half-life. Compared to unmodified IL-2, it has improved T_{reg} cell selectivity, a longer half-life and consistently expands T_{reg} cells, with only a few people exhibiting $CD56^{bright}$ natural killer cell expansion[119]. A phase II study to treat SLE (NCT04433585) showed some symptom improvement[120] and there is reported success in treating patients with eczema (NCT04081350) or psoriasis (NCT04119557)[120].

A third approach, which is still in preclinical development, is to couple IL-2 with another moiety for increased functionality. For example, an anti-human IL-2 antibody (F5111) was converted into a single-chain antibody and complexed with human IL-2, creating a so-called 'immunocytokine' (licensed by Cartesian Therapeutics). This immunocytokine preferentially activates and expands T_{reg} cells, showing efficacy in mouse models of colitis and immune-checkpoint inhibitor-induced diabetes mellitus[121]. In another approach, IL-2 has been fused to other beneficial cytokines. For example, IL233 is a hybrid cytokine linking IL-2 and IL-33 (licensed by Slate Bio) so that IL-2 can induce IL-33R expression on T_{reg} cells, making them IL-33 responsive. The combined effects of IL-2 and IL-33 expand T_{reg} cells in vivo, protect against autoimmunity and reduce inflammation[122,123].

Overall, boosting T_{reg} cells with IL-2, using either the native cytokine or various modified forms, has not resulted in the major success originally hoped for based on data from preclinical models. Apart from the continuing challenge that T_{reg} cells are not the only cells expressing CD25, a fundamental question with this approach is whether transiently increasing T_{reg} cells in an antigen non-specific way is sufficient to induce tolerance. The answer to this question likely depends on the disease context and patient-specific factors. More work is needed to understand how IL-2 therapy might enhance disease-relevant T_{reg} cells and how it could be combined with other approved therapies for synergistic effects.

Nanomedicine-based therapy to boost T_{reg} cells. Thanks to the success of the COVID-19 mRNA vaccines, there has been an explosion of interest in using biodegradable nanomedicines to target T_{reg} cells, with most approaches seeking antigen-specific T_{reg} cell induction or expansion[124] (Table 2). In mice, nanoparticles loaded with autoantigen-encoding mRNA[125] or complexed with autoimmune peptides[126] prevent progression of experimental autoimmune encephalomyelitis and T1DM, respectively, leading to T_{reg} cell expansion. Nanoparticles can also be conjugated with antibodies to target them to certain locations. For example, antigen-complexed nanoparticles targeting scavenger and mannose receptors on liver sinusoidal endothelial cells promote T_{reg} cell expansion and subsequent suppression of airway inflammation in mice[127]. Moreover, nanoparticles can be loaded with T_{reg}-promoting substances such as rapamycin[128], TGFβ[129] or F5111 immunocytokine[130].

This preclinical success has led to multiple companies entering clinical-stage testing of such nanomedicines to treat allergy and autoimmunity (Table 2). Each approach uses nanoparticles with immunomodulatory properties expected to increase T_{reg} cell activity. For example, both Moderna and Cartesian Therapeutics have developed nanoparticles that deliver IL-2 muteins. Furthermore, rapamycin nanoparticles (ImmTOR) that reduced the development of anti-drug antibodies specific for uricase (used to treat gout)[131] were shown to induce significant T_{reg} cell expansion when combined with an IL-2 mutein[132]. TOPAS Therapeutics and COUR (in partnership with Takeda) are developing nanoparticles loaded with tolerogenic autoantigens that induce T_{reg} cells to treat a variety of autoimmune disorders and allergies. Given the broad interest in nanoparticle-based therapies as well as in modulating T_{reg} cell activity, there are likely to be increased numbers of clinical trials in this area in the future.

T_{reg} cellular therapy

The strategies discussed above to expand T_{reg} cells in vivo are relatively low cost and feasible but the effects could vary significantly depending on the immunological history of a patient and the environmental context. An alternate approach to bolster T_{reg} cells is to administer them as a cell-based therapy (Fig. 3). Numerous approaches are being developed for obtaining, expanding and engineering T_{reg} cells for optimal therapeutic application.

Sources of natural T_{reg} cells for therapy

Early clinical trials investigating the safety and feasibility of T_{reg} cell therapy were focused on autologous T_{reg} cells isolated from peripheral blood. T_{reg} cells were enriched from peripheral blood mononuclear cells using magnetic beads to deplete $CD8^+$ T cells and enrich $CD25^+$ cells. However, because of the relatively low purity of T_{reg} cells achieved with this approach, these cells must be expanded in the presence of rapamycin to limit growth of contaminating conventional T cells and yield a potent, immunosuppressive T_{reg} cell product[133]. An alternate approach is to use flow cytometry-based cell sorting to obtain a purer starting population of T_{reg} cells, with sorting typically based on a combination of CD4, CD25 and CD127. Sorting strategies can also further select naive T_{reg} cells based on CD45RA[134,135], CD226 (ref. 136) or GPA33 (ref. 137) to enrich for cells co-expressing FOXP3 and HELIOS.

In an effort to decrease the cost and complexity of T_{reg} cell therapy, several groups are exploring the possibility of manufacturing T_{reg} cells from allogeneic donors, with a focus on two tissues that contain a large proportion of naive T_{reg} cells: umbilical cord blood (UCB) and thymus. Advantages of UCB T_{reg} cells include an increased TCR repertoire diversity and lineage stability relative to blood-derived cells[138,139]. UCB T_{reg} cell therapy also decreases the risk of acute GVHD[140,141]. Furthermore, fucosylation (the addition of a Siayl-Lewis X moiety onto P-selectin) has been used to alter the homing potential of UCB-derived T_{reg} cells, with promising preliminary data and an ongoing phase II clinical study[142]. A key question is the requirement for HLA matching with these allogeneic cells. In the studies by Brunstein et al.[140,141], the administered T_{reg} cells were partially HLA matched (4–6 of 6 HLA antigens), whereas a recent study of UCB T_{reg} cells for COVID-19-associated acute respiratory distress syndrome did not perform intentional HLA matching and did not observe an increase in anti-HLA antibodies compared to the placebo group[143]. UCB-derived polyclonal T_{reg} cells developed by Cellenkos are being used to treat refractory bone marrow failure syndrome (NCT03773393). Preclinical studies are also testing UCB T_{reg} cells in a variety of non-traditional contexts such as traumatic brain injury[144] and lung inflammation[145].

Thymuses are routinely removed from infants during cardiac surgery procedures and contain large numbers (0.3–3 billion) of T_{reg} cells. Thymus-derived T_{reg} cells are more suppressive than adult blood-derived cells[146], leading to the development of protocols for their isolation and expansion that are compatible with good manufacturing practices[147–149]. Recently, the first testing of thymus-derived T_{reg} cells was reported in a single patient, with the delivery of 20×10^6 cells/kg autologous cells 9 days after a heart transplantation. Notably, despite ongoing treatment with calcineurin inhibitors as immunosuppressants, the patient maintained T_{reg} cells at higher levels than before the transplantation throughout the 2-year follow-up period[150], suggesting this approach stably increased the T_{reg} cell pool.

Generating therapeutic T_{reg} cells from conventional T cells

Given the difficulties in isolating pure populations of T_{reg} cells, another approach is to re-programme the more numerous conventional T cells into T_{reg} cells. To increase FOXP3 expression, conventional $CD4^+$ T cells can be cultured in the presence of rapamycin, retinoic acid and/or TGFβ to generate induced T_{reg} (iT_{reg}) cells, which express FOXP3 for a period of time. Therapeutic administration of iT_{reg} cells ($CD4^+CD25^-$ T cells cultured in rapamycin and TGFβ1) was shown to be safe in patients at risk of GVHD[151]. However, there is some doubt about the stability of iT_{reg} cells because their levels of TSDR demethylation never approach those of ex vivo T_{reg} cells and they do not express HELIOS[152,153].

Recently, two groups generated iT_{reg} cells via differentiation of induced pluripotent stem (iPS) cells. $CD4^+$ T cells were first derived from iPS cells, and then $FOXP3^+$, TSDR-demethylated T_{reg} cells were generated by expanding the $CD4^+$ T cells in a cocktail of CDK8 and CDK19 inhibitor, rapamycin, TGFβ, and an agonistic TNFR2 antibody[154] or, as reported in a preprint, by expanding them in TGFβ plus all-trans retinoic acid[155]. Given that iPS cells can theoretically give rise to unlimited numbers of well-characterized cells, these methods will undoubtedly be of significant interest.

T_{reg} cells can also be obtained by overexpressing FOXP3 in conventional T cells. To achieve the desired effect, *FOXP3* must be controlled by a strong, constitutive promoter so that expression does not significantly diminish as cells enter a resting state[7,156]. CRISPR-based methods are also being attempted, whereby a strong promoter is inserted immediately upstream of the endogenous *FOXP3* locus to drive constitutive

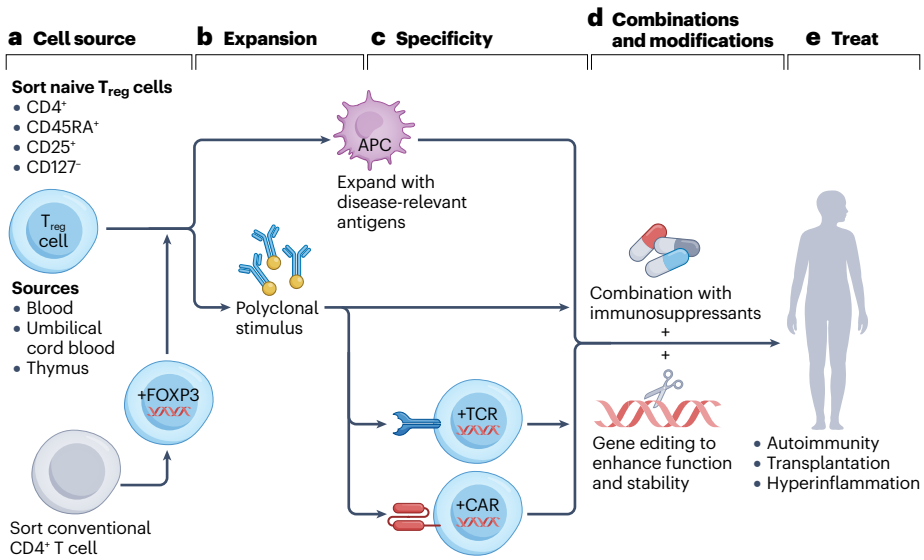

Fig. 3 | T_{reg} cell therapy steps and considerations. **a**, Regulatory T (T_{reg}) cells are commonly sorted from blood, umbilical cord blood or thymus based on their expression of $CD4^+CD25^{high}CD127^-$ and, commonly, $CD45RA^+$. Alternatively, conventional $CD4^+$ T cells can be genetically engineered to express FOXP3. **b**, T_{reg} cells can be expanded ex vivo either using a polyclonal general T cell stimulus or by antigen-presenting cells (APCs) presenting disease-relevant antigens. The latter approach also enriches T_{reg} cells for antigen specificity. **c**, T_{reg} cell antigen specificity can also be modified by genetically engineering cells to express a T cell receptor (TCR) or chimaeric antigen receptor (CAR). **d**, T_{reg} cell therapies are often administered to patients in combination with existing immunosuppressive regimens. In the future, gene editing could be used to incorporate orthogonal receptors and/or remove deleterious proteins. **e**, Bespoke T_{reg} cell products with optimal function, stability and persistence hold promise to treat many diseases.

expression[157]. *FOXP3* manipulation can also promote T_{reg} cell differentiation from haematopoietic stem and progenitor cells (HSPCs). Interestingly, constitutive overexpression of FOXP3 in UCB-derived $CD34^+$ HSPCs promotes stem cell quiescence and impairs T cell differentiation[158], but this can be overcome by delivering *FOXP3* with its regulatory elements to recapitulate physiological, non-constitutive expression[159]. Further, genome engineering to co-express FOXP3 and HELIOS can generate more functional T_{reg} cells than engineering to express FOXP3 alone[160].

An outstanding question is whether overexpression of FOXP3 will fully recapitulate all the functions of natural T_{reg} cells. So far, the functional properties of human, polyclonal conventional T cells overexpressing FOXP3 have only been tested using in vitro suppression assays or in vivo xenogeneic models of GVHD and IPEX[161]. T cells that constitutively express FOXP3 following lentivirus transduction are being investigated to treat IPEX (NCT05241444), and data from this study will be key for understanding the therapeutic potential of FOXP3-expressing conventional T cells as a replacement for T_{reg} cells.

Expanding polyclonal or antigen-specific T_{reg} cells

To acquire a clinically relevant number of cells, autologous T_{reg} cells are usually stimulated and expanded ex vivo. Most clinical trials have used polyclonally expanded cells (Fig. 4), which have been tested in many different diseases (see Bluestone et al. for a recent comprehensive review[162]). Of particular interest is the ONE study, an international, multi-centre study that tested several cell therapies in living donor kidney transplantation with the goal of minimizing the requirement for immunosuppressive drugs. Patients who received T_{reg} cell therapy did not undergo increased levels of rejection despite not being given the complete standard-of-care immunosuppressive therapy and, moreover, they had lower rates of viral infections[163]. As part of the ONE study, Roemhild et al. showed that most (8 out of 11) T_{reg}-treated patients achieved stable immunosuppression with a single drug, whereas a reference group remained on standard dual or triple immunosuppressive drugs[164]. Interestingly, T_{reg} cells in the blood shifted to a less diverse TCR repertoire, suggesting there could be an alloantigen-driven selection process for these cells. The ongoing TWO study (ISRCTN: 11038572) aims to confirm the benefit of polyclonal T_{reg} cell therapy in transplantation by testing whether immunosuppressive drug treatment can be tapered to a low-dose of tacrolimus alone following polyclonal T_{reg} cell infusion in kidney transplant recipients[165]. A subset of patients from this trial showed successful transition to tacrolimus monotherapy without transplant rejection[166].

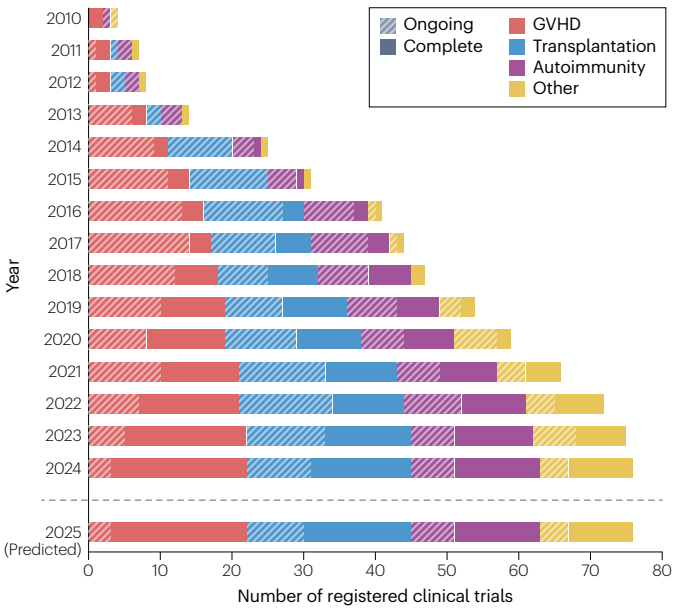

Fig. 4 | Clinical trials of T_{reg} cell therapy. Time course of the number of clinical trials investigating regulatory T (T_{reg}) cell therapy, based on data available from ClinicalTrials.gov and EudraCT. Ongoing (striped bars) refers to the number of clinical trials that were registered as recruiting in each given year. Complete (solid bars) refers to the number of clinical trials that were recorded as complete or terminated in each given year. Trials are separated into disease areas. GVHD, graft-versus-host disease.

Clinical trials exploring the use of polyclonal T_{reg} cells to inhibit GVHD following HSPC transplantation have also yielded promising results, with most treated patients showing clinical improvement and decreased GHVD activity[167–171]. An interesting new approach is the manipulation of the graft itself by infusing a polyclonal T_{reg} cell-enriched product, a strategy being investigated by Orca Bio. In several studies, patients received a $CD34^+$-selected HSPC graft supplemented with T_{reg} cells (sorted from the graft), followed by infusion of defined numbers of donor-derived conventional T cells 2 days later[167,170]. Patients treated with this product had early myeloid engraftment, and low rates of GVHD and relapse compared to standard of care[167,170,171]. A phase III study known as Precision-T (NCT05316701), has completed enrolment with results expected in 2025. These intriguing results suggest that more research is needed to understand how T_{reg} cells can benefit HSPC engraftment. Indeed, the bone marrow is known to be a rich source of T_{reg} cells, yet little is known about their biology and function in this tissue.

In T1DM, there have been mixed reports about the clinical efficacy of autologous polyclonal T_{reg} cells to preserve β-cell function. Some studies showed preservation of insulin production and reduced dependence on exogenous insulin[172,173], particularly when combined with a B cell-depleting antibody (rituximab)[174], whereas others failed to find clinical impacts[62,175,176]. These outcome variations could be due to differences in T_{reg} doses, number of infusions or the timing of treatment with respect to disease onset.

Polyclonal T_{reg} cells have also been sporadically tested in single individuals for the treatment of SLE[177] and ulcerative colitis[178], further confirming the wide applicability and excellent safety profile of this strategy. These studies noted an enrichment of T_{reg} cells in the skin and intestine, respectively, but the low number of patients treated limits the ability to draw meaningful conclusions about therapeutic efficacy. An ongoing phase II clinical trial in amyotrophic lateral sclerosis (NCT05695521) will reveal potential utility beyond traditional immune-mediated diseases (Box 1).

There have been attempts to expand antigen-specific T_{reg} cells using APCs ex vivo. For the majority of autoimmune conditions and inflammatory disorders, the feasibility of this expansion approach is poor as only a small number of disease-relevant T_{reg} cells naturally reside in the periphery. However, in a transplant context, approximately 1–10% of peripheral T_{reg} cells can be activated by donor antigens[179]. Consequently, ex vivo stimulation with graft donor B cells expands autologous T_{reg} cells that predominantly recognize donor-derived antigen–MHC complexes, termed donor-alloantigen reactive T_{reg} ($darT_{reg}$) cells[180]. This approach has been successfully tested in three kidney transplant recipients[181]. However, a separate study in liver transplantation emphasized the difficulties of manufacturing clinically relevant doses using this strategy, with 44% and 33% of recruited

participants yielding insufficient or only partial doses of T_{reg} cells, respectively[182]. Furthermore, despite a promising safety profile from these clinical studies, data from a recent non-human primate heart transplantation model suggested that darT_{reg} cells might not be stable as they lose signature T_{reg} cell markers following infusion[183].

Genetic engineering of T_{reg} cells

The expansion phase of the T_{reg} cell manufacturing protocol provides an opportunity for genetic manipulation and enhancement. Engineering can improve T_{reg} cell function, stability, trafficking and/or persistence in vivo following adoptive transfer. Most research has focused on improving T_{reg} cell potency by conferring antigen specificity with TCRs or chimaeric antigen receptors (CARs). Key considerations for this approach are whether overexpression of a TCR or CAR is more suitable (Table 3) and selection of the target antigen (Box 2).

TCR engineering. Antigen engagement and subsequent TCR signalling by T_{reg} cells drive retention of the cells in the relevant tissue and enhanced suppression; thus, a logical approach to redirect T_{reg} cell specificity is to introduce new TCR α-chain and β-chain genes. This idea has been explored in a variety of contexts, most notably in mouse models of T1DM[184]. Initial studies in human cells focused on modifying blood-derived T_{reg} cells[185], and TCR-engineered blood-derived T_{reg} cells are now being pursued for the treatment of multiple sclerosis by Abata Therapeutics (ABA-101). More recently, TCR delivery has been combined with *FOXP3* editing[157] to generate islet-specific T_{reg} cells from conventional T cells[186]. Mouse TCR-transgenic *Foxp3*-edited T_{reg} cells were shown to home to the pancreas, stably persist, and prevent disease development in an adoptive transfer model of T1DM. This multi-editing approach is now being pursued commercially for the treatment of T1DM by GentiBio (GNTI-122), using conventional T cells engineered to express FOXP3, an islet-specific TCR and a rapamycin-activated, chemically induced IL-2 signalling complex (allowing rapamycin to drive IL-2 signalling in these cells)[187].

CAR engineering. CARs are synthetic fusion proteins that typically bypass the requirement for antigen–MHC interactions through the use of an antibody-derived antigen-targeting moiety attached to TCR signalling domains[188]. Early studies investigating the potential of CARs to confer specificity to human T_{reg} cells were performed in the context of transplantation, where HLA-A2 was targeted as a clinically relevant human MHC molecule that is commonly mismatched. In vivo studies of mouse or human HLA-A2-specific CAR (A2-CAR) T_{reg} cells showed that CAR expression controlled T_{reg} cell homing to transplanted HLA-$A2^+$ skin or islet grafts, in turn delaying transplant rejection[189–191].

Multiple groups also refined CAR design for optimal T_{reg} cell function by testing various intracellular signalling domain configurations. These studies uniformly showed that CARs encoding CD28 co-stimulatory signalling domains but not the CD137 co-stimulatory domain are optimal for T_{reg} cells[82,192,193]. Interestingly, in a skin transplant model, a first-generation CAR with no co-stimulation domain was also effective, revealing that CAR T_{reg} cells respond to endogenous co-stimulation[193]. Overall, the potent effects of A2-CAR T_{reg} cells led to the rapid development of good manufacturing practice-compatible protocols for CAR T_{reg} generation[39], and two clinical trials are testing the safety and efficacy of A2-CAR T_{reg} cells in kidney (NCT04817774) and liver (NCT05234190) transplant recipients.

In the context of GVHD, the B cell antigen CD19 has been pursued as a CAR T_{reg} target[194,195] with the rationale that systemic $CD19^+$ B cells will enable widespread CAR T_{reg} cell stimulation. Remarkably, CD19-specific CAR T_{reg} cells not only reduced GVHD severity but also controlled the growth of $CD19^+$ tumour cells. Importantly, this was achieved in the absence of cytokine release syndrome, a common adverse event associated with CAR T cell therapy but not with CAR T_{reg} cell therapy, given the latter's relative lack of inflammatory cytokine production[196]. CD19-CAR T_{reg} cells might also be suitable for the treatment of autoimmune diseases in which B cells and/or antibodies play pathological roles. Recently, FOXP3-overexpressing, CD19-CAR T_{reg} cells reduced autoantibody generation and delayed lymphopenia in a humanized mouse model of SLE[197].

Table 3 | Comparison of key features of engineered TCRs versus CARs

Feature	TCR	CAR
Affinity	Low	High/variable
Co-stimulation	Exogenous	Incorporated
Sensitivity (number of antigens required on target)	High (low antigen density)	Low (high antigen density)
Specificity	Cross-reactive	High
Endogenous TCR	Prone to mispairing	Independent (typically)
Target antigens	Extracellular and intracellular	Membrane bound and oligomeric (sometimes soluble)
Antigen type	Processed peptides	Range of possible targets
Major histocompatibility complex	Dependent	Independent (typically)
Immunogenicity	Unlikely	Possible

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

A fundamental advantage of using CARs to redirect T cell specificity is the ability to engage target antigens in an MHC-independent manner (Table 3). However, in some cases, ideal antigens might be intracellular or predominantly soluble, making them difficult CAR targets. This limitation was recently overcome by generating so-called 'TCR-like' CARs specific for an insulin peptide presented by MHC class II. In two parallel studies, T_{reg} cells expressing insulin-MHC-specific CARs suppressed pathogenic T cell proliferation in vitro and significantly delayed or prevented T1DM in vivo[198,199].

Depending on the structure and environmental context of an antigen, it is possible to use CARs to redirect T_{reg} cell specificity to non-membrane-bound proteins. For example, CAR T_{reg} cells targeting the blood coagulation factor VIII are efficacious in mouse and humanized mouse models of haemophilia A[200,201]. More recently, CAR T_{reg} cells targeting flagellin, the protein component of bacterial flagella, were explored as a treatment option for inflammatory bowel disease[202]. Flagellin was selected as it is naturally oligomeric and primarily accessible to immune cells during periods of inflammation and gastrointestinal damage. Flagellin-specific CAR expression promoted intestinal trafficking, and CAR T_{reg} cells were significantly more suppressive and could promote intestinal epithelial cell integrity in the presence of their target antigen.

Ensuring stability of antigen receptor-engineered T_{reg} cells

A general concern with T_{reg} cells, particularly those modified to express antigen-specific receptors, is lineage stability. Injection of human CAR

Box 2 | Selecting a CAR T_{reg} cell target antigen

Chimaeric antigen receptor (CAR) regulatory T (T_{reg}) cells incorporate an antibody-derived antigen-targeting moiety, enabling them to engage a range of possible target antigens in a major histocompatibility complex (MHC)-independent manner. Below are some factors that should be considered when selecting a target antigen for CAR T_{reg} cells.

Disease relevance

In oncology, CARs redirect T cells towards antigens that might not be strictly disease-specific, leading to the potential for so-called 'collateral damage'. For T_{reg} cells, whether CAR target antigens need to be disease relevant is unclear. To date, most CAR T_{reg} cell studies selected a disease-relevant antigen, for example, in transplantation targeting allogeneic MHC molecules known to be targets of rejection or in autoimmunity selecting known autoantigens. Because T_{reg} cells are not expected to mediate collateral damage, it might be feasible to redirect specificity in an organ-specific or tissue-specific way but not necessarily in a disease-specific way.

In vivo location of suppression

Another consideration for CAR target selection is knowing where in vivo suppression should occur. In organ transplantation, evidence suggests suppression is needed both in the allograft and in associated lymph nodes. Redirecting CAR T_{reg} cells to allogeneic MHC molecules would be expected to induce suppression in both these locations due to exosome-mediated cross-decoration of donor MHC on host antigen-presenting cells. For autoimmunity, it remains to be defined if CAR T_{reg} cells targeting tissue-specific antigens will also need to have activity in lymph nodes and, if so, how the relevant antigens will need to be displayed.

T_{reg} cells into mice systemically expressing HLA-A2 did not reveal significant loss of FOXP3 (refs. 38,39,190). Similarly, in a non-human primate model of islet transplantation, infusion of autologous Bw6-specific CAR T_{reg} cells into $Bw6^+$ animals did not result in any overt toxicity despite ubiquitous target antigen expression in vivo[40]. Nevertheless, CAR-induced lineage changes are possible as evidenced by the loss of HELIOS (but not FOXP3) expression in mouse and human T_{reg} cells expressing CARs encoding TNFR family co-receptors[82,193].

Strategies to safeguard against instability and promote stable FOXP3 expression include expanding cells in the presence of FOXP3-promoting molecules (such as rapamycin or TGFβ) or genetic manipulation to overexpress *FOXP3*. For example, McGovern et al. developed a method to transduce an enriched population of T_{reg} cells with a vector encoding *FOXP3* and a myelin basic protein-specific TCR under the same promoter, restricting transgenic TCR expression to $FOXP3^+$ cells[203].

Another factor that could influence T_{reg} cell stability is dysfunction induced by overstimulation. In studies of tonic-signalling CARs in T_{reg} cells, chronic stimulation caused an exhausted phenotype and loss of function[204] and, in another study, was associated with loss of FOXP3 and HELIOS expression (although the cells did not acquire an effector phenotype)[205]. This tonic signalling can be mitigated by careful selection of CAR co-stimulatory domains and, possibly, by also using CRISPR technology to insert CARs into the TCRα constant (*TRAC*) locus, resulting in TCR-like transcriptional control and a more physiological level of CAR expression[190].

Combining engineered T_{reg} cells with immunosuppression

A consideration yet to be explored is how to condition patients so they are ideally suited to receive engineered T_{reg} cells. Ideally, T_{reg} cell therapy should be integrated with existing regimens and opportunities identified to combine it with new types of less toxic immunosuppression. As examples, CAR T_{reg} cells can work in synergy with rapamycin[191], and TCR-transgenic T_{reg} cells can be combined with low-dose IL-2 (ref. 206) or anti-CD3 (ref. 207). Ongoing work is also defining the optimal strategy to combine T_{reg} cell therapy with anti-thymocyte globulin[208] or CD28 blockade[53]. Development of combination therapies is a particularly important consideration in transplantation, where patients receive drug-based immunosuppression that could reduce the function of T_{reg} cell therapy[209]. For example, gene editing to knock out FK506-binding protein 12 or CD52 could result in T_{reg} cells that are resistant to tacrolimus[210] or alemtuzumab (an anti-CD52 antibody)[211], respectively. One can also envision bespoke CAR engineering combined with specific types of immunosuppression, for example, to deliver signals blocked by antibodies or recombinant proteins.

Other gene-editing strategies

Building on the success of TCR and CAR engineering, additional gene-editing strategies are being explored to further enhance the efficacy, survival and/or stability of adoptively transferred T_{reg} cells. As previously discussed, IL-2 therapy is an effective strategy to bolster T_{reg} cells. Limitations related to lack of T_{reg} specificity can be overcome by using engineered, so-called 'orthogonal', systems in which synthetic versions of IL-2 and the IL-2 receptor interact with each other but not the endogenous versions[212,213]. Engineered expression of an orthogonal IL-2 receptor in adoptively transferred T_{reg} cells allows selective stimulation in vivo by administration of orthogonal IL-2. The benefits of this approach have been demonstrated in mouse models of GVHD[214] and heart transplantation[215].

A similar approach is the use of chimaeric cytokine receptors, or switch receptors, which detect one extracellular cytokine and deliver the stimulatory signal of another. Although this approach has not yet been reported in T_{reg} cells, a similar concept was developed whereby extracellular detection of pro-inflammatory cytokines triggered CD3ζ and CD28 signalling in T_{reg} cells[216]. T_{reg} cells expressing these so-called artificial immune receptors were significantly more effective at alleviating GVHD in a mouse model.

CAR T_{reg} cell therapy can delay but not prevent tissue rejection[191,217], suggesting the need to further enhance T_{reg} cell-suppressive mechanisms. In an early test of this concept, A2-CAR T_{reg} cells were genetically modified to constitutively express IL-10 (ref. 218), which is normally expressed at relatively low levels in T_{reg} cells compared to IL-10-producing type 1 T_{reg} cells[219]. A similar strategy could be used to introduce other beneficial molecules such as TGFβ[188].

Removal of deleterious genes from therapeutic T_{reg} cell products can also be achieved with gene editing. In oncology, PD1 ablation improves the efficacy of CAR T cells[220,221] and, given the negative impact of PD1 signalling on T_{reg} cells[82], adapting this approach could be beneficial. This strategy could also be used to enhance the function of allogeneic T_{reg} cells to minimize their immunogenicity. For example, in work reported in a preprint, McCallion et al.[222] used a humanized mouse

model of skin transplantation to show that $CD8^+$ T cell-mediated killing of allogeneic T_{reg} cells could be overcome by CRISPR–Cas9-mediated silencing of MHC. Moreover, expression of a non-polymorphic HLA-E–β2-microglobulin fusion protein further protected T_{reg} cells lacking MHC expression from natural killer cell-mediated killing. Other strategies to evade natural killer cells, such as expression of HLA-E[223–225], Siglec ligands[226] or CD47 (ref. 227), are also likely to have applications in T_{reg} cells.

Overall, it is clear that, to fully maximize the potential of T_{reg} cell therapies, gene editing should be employed. Fortunately, the extensive work on gene editing of conventional T cells can be leveraged for T_{reg} cells, albeit for a different purpose.

Depleting T_{reg} cells to treat cancer

Cancer evades immunity by creating an immunosuppressive environment that promotes T_{reg} cells. The prognostic value of T_{reg} cell abundance in tumours varies depending on the type of cancer but, generally, a higher frequency of intratumoural T_{reg} cells is associated with decreased overall survival (reviewed in ref. 228). Therefore, many cancer therapies aim to deplete T_{reg} cells in order to invigorate the anticancer functions of other immune cells.

Targeted T_{reg} cell depletion

As discussed earlier, there have been many attempts to selectively expand T_{reg} cells based on their high expression of CD25. Targeting CD25 has also been used to deplete T_{reg} cells in cancer. Denileukin diftitox (ONTAK) is a human IL-2 protein fused to diphtheria toxin that was tested for many years; however, T_{reg} cell depletion was transient and, in some cases, expansion of antigen-specific effector T cells was halted[229]. E7777 is a new, purer IL-2–diptheria toxin fusion protein under investigation. A recent clinical trial treating patients with relapsed or refractory lymphoma showed an objective response in one-third of participants; however, half of patients experienced a serious adverse event[230]. A different approach tested RG6292, a non-blocking, CD25-depleting antibody that binds CD25 to trigger antibody-dependent cellular cytotoxicity of T_{reg} cells but leaves the substrate-binding domain of CD25 open to receive IL-2 signalling on remaining effector T cells[231]. It exhibited preclinical efficacy without immune-related toxicities and a monotherapy clinical trial in patients with advanced solid tumours was recently concluded (NCT04158583).

Because all therapies targeting CD25 have a risk of off-target effects, several new approaches have been developed to more selectively target T_{reg} cells. For example, AstraZeneca developed an antisense oligonucleotide targeting *FOXP3* (AZD8701) that is in clinical testing (NCT04504669). Preclinical modelling showed effective knockdown of FOXP3 throughout the body, including the tumour, as well as reduced T_{reg} cell-suppressive capacity and a parallel boost in $CD8^+$ T cell antitumour activity[232]. Preclinical models have also demonstrated the efficacy of reducing intratumoural T_{reg} cells by targeting CCR8 (refs. 233,234), a strategy now in clinical testing by Shionogi as a combination therapy with the PD1 inhibitor pembrolizumab (NCT05101070). Interestingly, the controversial relevance of NRP1 in human T_{reg} biology has recently been re-examined, with Chuckran et al.[235] finding its expression is prevalent in tumour-resident T_{reg} cells, supporting the rationale for anti-NRP1 therapy (NCT03565445).

Combating the suppressive microenvironment

Immunosuppression is a hallmark of cancer; reducing immunosuppressive cells and/or signals can re-invigorate anticancer immunity. Cyclophosphamide, a cytotoxic alkylating agent used to treat malignancies, also selectively depletes T_{reg} cells[236]. Mechanistically, T_{reg} cells have less ATP than effector T cells and thus produce less glutathione, which detoxifies cyclophosphamide, making them more susceptible to this drug. Several clinical trials are testing CD39 and CD73 inhibitors in attempts to increase extracellular, pro-inflammatory ATP levels. One preclinical study showed that antibodies blocking CD39 and CD73 were effective in activating effector T cells and slowing tumour progression; however, there was no apparent impact on T_{reg} cells[58]. Administration of oleclumab, an anti-CD73 antibody, together with an anti-PDL1 antibody (durvalumab) also increased overall response rate and progression-free survival relative to those treated with durvalumab alone[237].

Tryptophan and kynurenine are metabolites that influence the balance of effector cells to T_{reg} cells. Their relative abundance is regulated by the intracellular enzyme indoleamine 2,3-dioxygenase (IDO1), which converts tryptophan into kynurenine. The latter metabolite decreases effector T cell proliferation and survival and promotes pT_{reg} cell differentiation. IDO1 is expressed in APCs and many tumour cells and has become an anticancer drug target. However, although there were high hopes for the IDO1 inhibitor epacadostat, it ultimately failed to have any effect in a phase III clinical trial[238]. Despite this disappointing result, which was possibly related to suboptimal trial design and lack of mechanistic understanding[239], there are multiple new IDO1-targeting candidates in clinical trials (reviewed in ref. 240).

Galunisertib is a TGFβ type I receptor kinase small-molecule inhibitor that can reverse T_{reg} cell suppression in vitro[241]. In preclinical models, it lowered T_{reg} cell abundance and increased lymphoma-related survival[242]. In a phase II trial for patients with rectal cancer, galunisertib in combination with neoadjuvant chemoradiotherapy improved the complete response rate[243]. Interestingly, although the frequency of blood T_{reg} cells increased with treatment, intratumoural T_{reg} density and TGFβ signalling decreased in tumour biopsies over time[243]. Overall, many anticancer therapies are hypothesized to work, at least in part, via suppressing T_{reg} cell function. Continued investigation of the impact of these drugs on T_{reg} cells will provide mechanistic insights that could be leveraged for both pro-T_{reg} and anti-T_{reg} cell therapies.

ICIs and T_{reg} cells

Immune-checkpoint inhibitors (ICIs) are now a mainstay of cancer therapy, working to prevent effector T cell exhaustion and re-invigorate anticancer immunity. For example, inhibitors of the checkpoint proteins PD1 and CTLA4 block effector T cells from receiving inhibitory signals that would otherwise prevent them from killing cancer cells. The effects of these ICIs on T_{reg} cells are just starting to be elucidated. As with effector T cells, PD1 signalling inhibits T_{reg} cells, reducing their proliferation and suppressive effects[244–246]. Thus, an unintended consequence of PD1 blockade can be enhanced T_{reg} cell function. A small fraction of patients treated with a PD1 ICI develop hyper-progressive disease associated with an increased frequency of proliferating T_{reg} cells in the blood[245]. Furthermore, patients who expressed PD1 on over half of their T_{reg} cells had poor survival following ICI treatment[246]. Of note, the ratio of tumour-resident $PD1^+$ $CD8^+$ T cells to $PD1^+$ T_{reg} cells is predictive of clinical response to PD1 ICIs, with a higher ratio predicting favourable outcome[246]. Thus, in the future, identification of tumours with high $PD1^+$ T_{reg} cells prior to treatment might enable personalized approaches to first deplete T_{reg} cells before administering PD1 ICIs.

CTLA4 inhibitors are another major class of ICI in clinical use. As discussed above, T_{reg} cells express constitutively high levels of CTLA4 so it is not surprising that anti-CTLA4 antibodies inhibit them[247,248].

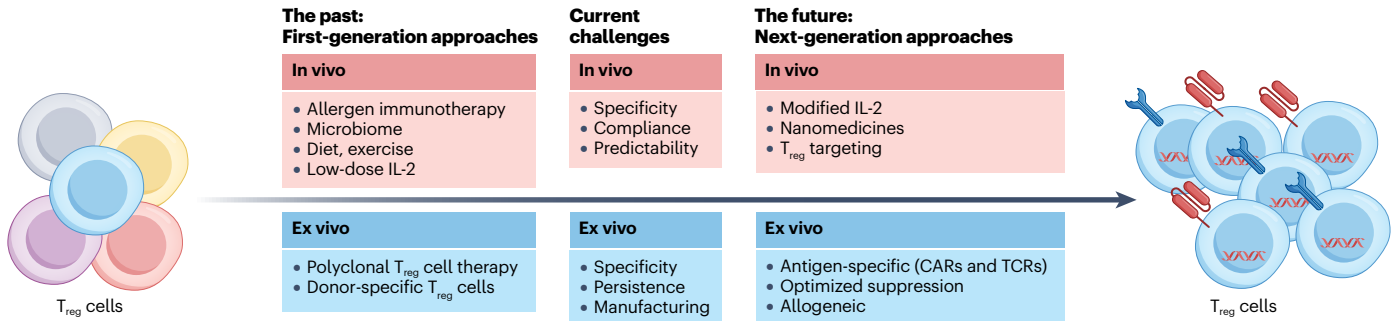

Fig. 5 | Evolution of T_{reg} therapies. Over the last 10 years, regulatory T (T_{reg}) cell-directed therapies have primarily focused on in vivo (red) or polyclonal adoptive cell therapy-based ex vivo approaches (blue) to enhance disease-relevant function. These first-generation approaches are consistently safe but there are significant current challenges such as a lack of antigen specificity and poor control over the ultimate effects. The field is rapidly moving to next-generation approaches focused on more precise and specific T_{reg} cell targeting using protein engineering and nanomedicines for in vivo targeting and gene engineering for optimal adoptive cell therapy approaches. CAR, chimaeric antigen receptor; TCR, T cell receptor.

In mice, the depletion of intratumoural T_{reg} cells by CTLA4 ICIs required the presence of tumour macrophages expressing the IgG Fc receptor FcγRIV[247]. The human homologue of FcγRIV is FcγRIIIA and, in vitro, FcγRIIIA$^+$ monocytes mediate the antibody-dependent cell cytotoxicity of CTLA4 ICI-treated T_{reg} cells[248]. Interestingly, the abundance of these monocytes is higher in tumours of CTLA4 ICI responders than in non-responders, suggesting that T_{reg} cell depletion contributes to clinical benefit. However, results are overall inconsistent, leading to the conclusion that the effects of CTLA4 ICIs on T_{reg} cells vary due to tumour type and time of sampling relative to ICI treatment[248–250].

Many patients treated with ICIs experience immune-related adverse events (IRAEs). These events vary in severity and are essentially due to localized and, in some cases, antigen-specific inflammation[251]. Whether T_{reg} cells are implicated in IRAEs is unclear. In a study of ICI-induced colitis in patients with melanoma, single-cell sequencing analysis of the colon paradoxically found increased frequencies of T_{reg} cells in the patients with colitis compared with ICI-treated patients without colitis[252]. However, these T_{reg} cells were skewed towards an inflammatory and potentially unstable phenotype, perhaps, as the authors suggest, in response to local inflammation[252]. Interestingly, the risk of IRAEs is increased by a history of autoimmune disease or use of a CTLA4 ICI[253], suggesting that T_{reg} cell depletion or destabilization (which would be stronger with a CTLA4 ICI) could tip the balance of tolerance in healthy tissues to become permissive of an inflammatory response. In accordance, IRAE incidence positively correlates with response rate and survival[254], suggesting that a brief loss of tolerance might be required to allow anticancer immunity to function.

Conclusions and future directions

T_{reg} cells are undoubtedly a cornerstone of a healthy immune system; however, tracking their contribution to disease and harnessing their full therapeutic potential has remained somewhat elusive. Major limitations in tracking T_{reg} cells as biomarkers have included the lack of FOXP3 specificity and challenges in quantifying antigen-specific cells. Tools to more accurately identify T_{reg} cells, such as combining FOXP3 and HELIOS staining, measuring TSDR methylation, and gene signatures, can now be routinely applied to better discriminate between T_{reg} and conventional T cells. In terms of specificity, AIM assays and a growing repertoire of multimers provide new ways to follow antigen-specific cells (both in blood and other tissues) so that changes in disease-relevant cells can be more accurately quantified. Early use of such tools in allergy and autoimmunity clearly shows that changes in antigen-specific T_{reg} cells are relevant for human disease[74,77] and malleable in response to therapy (Table 1).

In terms of therapeutic targeting to increase T_{reg} cell activity, the past two decades have focused on in vivo modulation using relatively unspecific tools or non-antigen-specific adoptive cell therapy. These studies have hinted that it is possible to enhance tolerance and reduce reliance on non-specific immunosuppression. The field is now evolving to address challenges and to create more specific and effective approaches (Fig. 5). For adoptive T_{reg} cell therapy, although few phase II trials powered to measure efficacy have yet to be completed, it is a significant advance that polyclonal and darT_{reg} cells can be used to replace or reduce immunosuppressive drugs in the context of organ transplantation[163,181]. Even if T_{reg} cell therapy does not induce long-term tolerance, the ability to reduce toxic immunosuppression would have a major impact on quality of life and morbidity. The potential to expand the application of polyclonal T_{reg} cell therapy to a variety of inflammatory diseases is also a very exciting new direction (Box 1).

However, the most exciting potential lies in the evolution of methods to induce antigen-specific T_{reg} cells either directly in vivo or through adoptive cell transfer. Nanomedicine-based approaches represent a precision approach to tolerance induction; one could envision targeting nanomedicines specifically to T_{reg} cells, for example, through antibody-mediated targeting[255] or mRNA-mediated or microRNA-mediated control of T_{reg} cell-specific expression[256]. These approaches have the potential to be significantly more potent than approaches such as AIT, yet still rely on the patient's own immune system. Delivery of engineered T_{reg} cells by adoptive transfer offers an even more precise approach and we can foresee that, similarly to T cell therapy for cancer, cells that have been progressively more engineered will be tested over the next decade.

Tolerance is undoubtedly a balance and there is a significant opportunity to apply lessons learned from cancer to autoimmunity and transplantation and vice versa. Understanding the cellular mechanisms underlying the side effects of various ICIs has the potential to reveal significant insight into the biology of human T_{reg} cells and hence pathways that can be harnessed for tolerance.

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C.M.W. and M.K.L. conceived the article. All authors contributed to the research, writing and editing of the manuscript.

Competing interests
M.K.L. is a science advisory board member for Anokion, advises for and holds shares in Integrated Nanotherapeutics, and is an inventor on patent applications related to A2-chimaeric antigen receptor regulatory T cells with licensed technology to Sangamo Therapeutics. C.M.W. and D.A.B. declare no competing interests.

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