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Regulatory T cells: Central concepts from ontogeny to therapy

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Abstract

The balanced differentiation of naïve CD4⁺ T cells into either pro- or anti-inflammatory fates is a central regulator of immune homeostasis, dysregulation of which can lead to inflammatory disease or cancer. Accordingly, the development of diagnostics and therapeutics to measure and modulate this balance is of great interest. In this Review, we focus on the predominant anti-inflammatory subset, regulatory T cells (T_{reg}S), discussing key concepts including development, function, antigen specificity and lineage stability. In particular, we highlight how these notions are shaping the evolution of therapeutics, especially in the context of the transfusion medicine specialist, and identify several key areas that urgently need to be addressed.

Keywords

Regulatory T cell; Immunotherapy; Cell processing; T cell differentiation; Antigen specificity; Lineage stability

Introduction

The immune system faces the fundamental challenge of protecting the individual from foreign pathogens while maintaining tolerance to self. The innate immune system uses a small number of receptors to recognize conserved pathogen-associated molecular patterns, such as lipopolysaccharide (LPS). In more advanced organisms, this is buttressed by the adaptive immune response, which recognizes a much larger diversity of antigens with both specificity and memory, leveraging the error-prone joining of variable region gene segments (i.e. V(D)J recombination) to generate a vast diversity of T- and B-cell receptors using limited genomic space [1]. Because these receptors are generated in an antigen-agnostic manner, they may recognize either self or foreign antigens. Thus, self-tolerance necessitates mechanisms to remove and/or attenuate self-reactive cells. In the thymus, medullary thymic epithelial cells display self-antigens encoded throughout the genome via the activity of AIRE, facilitating the negative selection of self-reactive T cells (i.e. clonal deletion) [2]. Loss of AIRE cripples this process, leading to the autoimmune syndrome known as

Conflict of interest

None.

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autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) in humans [3,4]. Importantly, AIRE can only promote tolerance to antigens encoded within the genome. At the least, our microbiota and food generate antigens that are extrinsic to our genome, and thus to AIRE, that must also be tolerated. Moreover, self-reactive clones can and do escape central deletion. These scenarios highlight the need for extrathymic mechanisms to promote tolerance, i.e. peripheral tolerance.

The CD4⁺ T helper (Th) cell regulates both B and T cell effector functions and is a key player in maintaining an appropriate balance between inflammation and tolerance. This is in large part achieved by the differentiation of naïve CD4⁺ T cells into either pro-inflammatory subsets (e.g. Th1, Th17) or anti-inflammatory subsets (T_{reg}) (Fig. 1) [5]. This lineage decision is dictated by the local cytokine milieu in which naïve Th cells are activated by their cognate MHC-peptide ligands. Thus, T cell receptors are generated in an antigen-agnostic fashion, but their associated functional program (e.g. inflammation vs tolerance) is educated contextually. Appropriate and balanced differentiation into these fates critically impacts immune homeostasis, with excessive bias in either direction leading to pathologic inflammation or cancer [6].

The anti-inflammatory arm is largely represented by regulatory T cells (T_{reg}s), although the contribution of other subsets, including Tr1 cells, is increasingly being recognized. The central role of T_{reg}s in maintaining immune homeostasis is highlighted by patients with deleterious mutations in *FOXP3*, a hallmark transcription factor required for T_{reg} development. These patients develop immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome, which is characterized by a plethora of autoimmune processes that typically lead to early death without treatment [7,8]. Similar autoimmunity arises from FOXP3 defects in mice, as exemplified by the scurfy mutation [9]. In addition to Mendelian disorders like IPEX, a broader role of T_{reg}s in complex human diseases is suggested by the recurring over-representation of T_{reg}-related genes in genome-wide association studies of inflammatory diseases such as inflammatory bowel disease and type 1 diabetes (T1D). This may reflect a quantitative and/or qualitative defect in the T_{reg} axis.

The dominant contribution of T_{reg}s to the anti-inflammatory component makes this a very attractive subset to study, since enhancing T_{reg}s is likely to be broadly relevant to autoimmunity and inflammation, while inhibiting T_{reg}s might be of general utility in cancer. Consistent with this notion, searching registered studies at clinicaltrials.gov reveals 438 entries related to T_{reg}s, equivalent to all other major subsets combined (Th1, Th2 and Th17 with 174, 172 and 93 entries respectively). Of these, 376 T_{reg}-related study entries successfully enrolled patients and collected data. The most studied entities were cancer, autoimmunity and transplant (170, 64 and 34 studies respectively, Fig. 2A). Uniformly, the majority of studies have been observational, typically examining the relationship between peripheral blood T_{reg}s and disease progression or standard therapeutic intervention (Fig. 2A). Importantly, studies aimed at the therapeutic manipulation of T_{reg}s began only 2 years after the first observational studies were initiated, and have been steadily increasing since (Fig. 2B). These have so far been overwhelmingly phase 1 and/or 2 studies in diverse disease entities, reflecting early and general interest, with relative emphasis on T1D and colitis in

autoimmunity as well as melanoma, hematologic malignancies and ovarian cancer in neoplastic diseases (Figs. 2C and 2D). Systemic drugs have been used both to deplete T_{reg} s, such as the anti-CD25 agent denileukin diftitox (Ontak) and metronomic cyclophosphamide, as well as to enhance T_{reg} s, most notably low-dose IL-2. Perhaps of greatest interest to the transfusion medicine practitioner is the extracorporeal manipulation of T_{reg} s, including T_{reg} depletion from donor lymphocyte infusions to increase anti-cancer effects, and T_{reg} enhancement ex vivo, especially to promote transplant tolerance and minimize graft-versus-host disease. Ex vivo T_{reg} differentiation and expansion has particular promise as an emerging tool in the cellular therapy portfolio. In this context, we review concepts of fundamental importance, including T_{reg} identity, mechanism of action, stability and explore how qualitative and quantitative enhancement may be achieved to therapeutic utility. Notably, these concepts are broadly applicable to all endeavors exploring differentiation of Th and other subsets.

Defining and identifying T_{reg} s

As previously discussed, genetic studies in both humans and mice clearly point to Foxp3 as a key transcription factor required for T_{reg} differentiation. Multiple lines of evidence support Foxp3 as a robust marker of T_{reg} s in mice [6]. In humans, Foxp3 is also expressed by activated T cells, and may be less specific a marker of T_{reg} s than in mice [10]. Another key limitation to the use of Foxp3 as a marker is that it is an intracellular protein, detection of which (in humans) requires fixation and intracellular permeabilization. Thus, detection of Foxp3 is not compatible with recovery of live cells in patients and compromises the usability of the labeled cells for in other assays, such as RNAseq studies [11].

To date, no single surface marker has been found that specifically defines T_{reg} s. T_{reg} s were classically identified as $CD4^+CD25^{hi}$ cells, and many studies continue to use this method. However, activated T cells also express CD25, which may impact the specificity of this marker, especially in the setting of ongoing inflammation. At the least, $CD4^+CD127^{low}CD25^{hi}$ should be used to define T_{reg} s. Additional markers including CTLA4 and GITR may also be useful. Taken together, continuing work to identify markers, especially surface proteins, of T_{reg} s in humans will be useful, and should be carefully validated with the recognition that there is likely to be heterogeneity within T_{reg} s. Studies identifying CD15s and TIGIT as markers of suppressive human T_{reg} s represent important efforts in this regard [12,13].

T_{reg} development and function: interventional points

Our understanding of T_{reg} ontogeny is largely informed by murine studies. Many T_{reg} s develop in the thymus (thymic T_{reg} s, tT_{reg} s) and subsequently circulate in the periphery. These tT_{reg} s likely develop in the context of relatively strong TCR-signaling in response to self-peptides presented in an AIRE-dependent manner [14]. T_{reg} s that develop in the periphery (pT_{reg} s) are likely essential for peripheral tolerance and seem to have demonstrable roles in the gut and at the fetal-maternal interface, consistent with the notion that local commensals and paternal-specific proteins represent extra-genomic antigens that pT_{reg} s regulate tolerance of [15,16]. Whether tT_{reg} s and pT_{reg} s are functionally or

mechanistically distinct remains unclear and will require tools to distinguish between these subsets. Expression of the surface marker neuropilin-1 helps distinguish tT_{reg} s from pT_{reg} s in mice, but appears less T_{reg} -specific in humans [17–19]. Mice lacking the CNS1 regulatory region in the *Foxp3* gene can generate tT_{reg} s but not pT_{reg} s, and might reveal additional ways to discriminate between these populations [20].

Induced T_{reg} s (iT_{reg} s) are generated in vitro by stimulating naïve $CD4^+$ T cells in the presence of TGF β [21]. The ability to generate iT_{reg} s in large numbers ex vivo makes them of significant interest as cellular therapeutics; moreover, both iT_{reg} s and pT_{reg} s are thought to arise from the same precursor so that mechanistic dissection of iT_{reg} differentiation may more broadly inform our understanding of pT_{reg} and tT_{reg} differentiation [22]. Transfer of iT_{reg} s attenuates disease in multiple murine models, and human iT_{reg} s exert anti-inflammatory effects in mice with humanized immune systems, although studies suggesting that iT_{reg} s may lack the full genetic, epigenetic and functional signature of tT_{reg} s raise cautionary notes [22–25]. Accordingly, many groups have focused on expanding tT_{reg} s. However, data suggesting that iT_{reg} s play a non-redundant role in tolerance and studies showing that molecules like vitamin C can significantly enhance iT_{reg} function point to the value of continued investigation into the therapeutic potential of iT_{reg} s [26,27].

A species-specific facet of FOXP3 regulation may account in part for its differential association with T_{reg} s in humans and mice. Although mouse and human FOXP3 are highly homologous, human $CD4^+CD25^+$ T_{reg} s express both *FOXP3* variants containing and missing exon 2 at equivalent levels, unlike mice, which express predominantly full-length *Foxp3* [28]. Importantly, exon 2-containing FOXP3 is required for suppressive activity [28]. In support that this observation applies across species, mice forced to express *Foxp3* lacking both exons 2 & 7 develop multi-organ inflammation comparable to that arising from complete loss of *Foxp3* [29]. $CD4^+CD25^+$ cells in patients with relapsing-remitting multiple sclerosis and T1D have decreased suppressive activity and decreased expression of exon 2-containing *FOXP3*, consistent with decreased T_{reg} numbers and/or activity [30]. Glycolysis helps regulate T_{reg} differentiation; studies showing that the glycolytic enzyme enolase is recruited to the *FOXP3* locus and affects exon 2 splicing suggest novel roles for this enzyme and point to the regulation of *FOXP3* splicing as an interesting area of future study [30].

Several distinct mechanisms have been identified to mediate the suppressive effect of T_{reg} s, including secretion of tolerogenic cytokines (e.g. IL-10, IL-35 and TGF β), local consumption of IL-2 via preferential expression of the high-affinity IL-2 receptor, direct killing of responder T cells (e.g. granzyme B and perforin-1) and modulation of the function of innate immune cells (e.g. dendritic cells and NK cells) [31–35]. IL-10 is a pleiotropic pro-tolerogenic cytokine made by several cell types, but murine models specifically lacking IL-10 in T_{reg} s point to a particular role for T_{reg} -derived IL-10 in regulating mucosal inflammation [32]. Similarly, patients deficient in IL-10 or IL-10-receptor present primarily with early-onset immunosuppression-resistant enterocolitis responsive to hematopoietic stem cell transplant [36]. These and similar data point to the need to consider tissue-specific specializations of T_{reg} function when contemplating their role in diagnosis and therapy.

Most of the assays currently used to assess T_{reg} function largely focus on their suppressive effects on other T cells, and to a lesser extent, innate immune cells. The hallmark in vitro assay of T_{reg} function tests their ability to suppress the proliferation of co-cultured naïve CD4⁺ T cells upon stimulation with anti-CD3 and anti-CD28 antibodies. Beyond these concepts, growing evidence points to T_{reg}s also impacting B cell function. IPEX and DOCK8-deficient patients exemplify defects in T_{reg}s associated with defective B cell tolerance, suggesting a role for T_{reg}s in enforcing peripheral B cell tolerance [37,38]. The particular mechanisms involved remain to be clearly elucidated, and may involve enhancing antigen-specific T follicular helper responses via IL-2 sequestration, signaling to B cells through molecules like CTLA4 or other pathways yet to be described [39,40]. This relationship may explain the overrepresentation of T_{reg}-associated genes in many autoimmune disease genome-wide association studies and impact how we think about mechanisms of autoantibody production.

T_{reg}s may also have physiological functions beyond modulating inflammatory responses. Pioneering studies showing that T_{reg}s in muscle overexpress amphiregulin and promote tissue repair have been buttressed by the finding that T_{reg}-specific ablation of amphiregulin leads to increased tissue damage in an infectious lung injury model independent of T_{reg}-suppressive effects [41,42]. These findings highlight not only potential clinical interest in and utility of T_{reg}s beyond manipulating immune homeostasis, but also the broader importance of understanding compartment- and context-specific specializations of cellular function, that might reveal additional roles for T_{reg}s. It will be important to develop corresponding assays that will not only enable diagnosis of T_{reg} functional defects, but also development of therapeutics to enhance these functions.

Stability of T_{reg}s

The stability of differentiated Th fates is an ongoing investigation, with controversial findings that have clinically important implications. If administered T_{reg}s have significant capacity to subsequently adopt pro-inflammatory fates, therapeutic benefit may be muted or even negative. Lineage-tracing experiments using Foxp3-GFP-Cre-BAC transgenic *Rosa26^{loxP-stop-loxP-YFP}* mice allowed indelible YFP labeling of all cells that expressed *Foxp3* at any point in their ontogeny. In these mice, 10% – 15% of YFP⁺ cells (previously expressed *Foxp3*) did not maintain FOXP3 expression; furthermore these ex-*Foxp3* cells drove pathogenic inflammation in models of T1D and multiple sclerosis [43,44]. A related suite of experiments used a tamoxifen-inducible Foxp3^{GFP-Cre-ERT2} to label *Foxp3*-expressing cells, finding fewer but detectable ex-*Foxp3* cells 14 days or 5 months after tamoxifen treatment (2% – 5%); notably, sorted GFP⁺YFP⁺ T_{reg}s maintained *Foxp3* expression (>99%) in the face of multiple stresses [45]. One possible explanation for these differences arises from the observation that T_{reg} and Th17 cells can arise from a common FOXP3⁺RORγT⁺ precursor, thus some of the exFoxp3 cells observed might reflect normal ontogeny of Th17 cells. Another contributing factor may be differing fidelity of the Foxp3-GFP-Cre-BAC transgene, as opposed to the Foxp3^{GFP-Cre-ERT2} knock-in allele.

Whereas the above studies examine the plasticity of T_{reg}s, this may be more pressing in iT_{reg}s, which data suggest exhibit decreased stability due at least in part to decreased

hypomethylation of key T_{reg}-associated loci (including *Foxp3*, *Tnfrsf18*, *Ctla4* and *Ikzf4*) which occurs in a *Foxp3*-independent fashion [25]. Recent studies identified the ten-eleven-translocation (TET) family of enzymes as key mediators of DNA demethylation; this family comprises 3 members, of which TET2 and TET3 act redundantly to demethylate T_{reg} regulatory regions [27]. Loss of both TET2 and TET3 impairs *Foxp3* expression stability, while enhancement with either vitamin C or hydrogen sulfide increases both *Foxp3* expression stability and T_{reg} function, suggesting immediate applications to improve iT_{reg} therapeutics [27,46].

T_{reg} stability is important, exemplified by spontaneous focal inflammation in mice where this has been impaired by deletion of the *Foxp3* regulatory element CNS2 [47]. However, transition of T_{regs} to other lineages may not necessarily be pathologic; stability appears dependent on full demethylation of CNS2, which is observed in mature but not immature T_{regs} [47–49]. This may allow evolving cytokine contexts a window to drive Th cells to the final desired effector state. In this regard, concomitant inflammation can condition naïve CD4⁺ T cells towards T_{reg} polarization, so that if a subsequent infection took some time to “build up” pro-inflammatory cytokine levels, some degree of lineage flexibility might be physiologically useful [50].

Although these observations were made in murine models, the potential clinical ramifications have evoked concerns regarding human applications. Because of the increased concern of iT_{reg} lineage instability, many early studies have focused on expanded tT_{regs} as a cellular therapeutic. Tracking the fate of the transferred cells is clearly a key issue; methods used range from examining chimerism in the T_{reg} population (using variable number tandem repeat analysis) to labeling transferred T_{regs} with deuterium [51,52]. As Th therapeutics expand beyond T_{regs}, it is useful to recognize that lineage stability is a broadly relevant issue, and transitions of Th2 to Th1 and Th17 to non-inflammatory regulatory fates have been described, amongst others [53–55].

Antigen specificity of T_{regs}

Most methods currently used assess T_{reg} function independent of their MHC-peptide specificity, ranging from the standard in vitro suppression assay (used to assess both mouse and human T_{regs}) to animal models, such as colitis and airway inflammation [56–59]. Given the energetic expenditure to generate a TCR that passes thymic selection, specificity might be expected to impact T_{reg} function. In further evidence that the TCR is not a vestigial feature of T_{regs}, loss of TCR and associated signaling leads to increased prevalence of inflammatory T cells despite persistence of Foxp3+TCR⁻ cells, demonstrating a persistent requirement of the TCR for T_{reg} function [60]. Consistent with this notion, studies of the BDC2.5 transfer model of diabetes revealed potent and consistent suppression of disease with transfer of 2×10⁶ specific T_{regs}, as compared to modest delay of disease using 8×10⁶ non-antigen-specific T_{regs} (no higher doses tested) [61]. Studies in a humanized model of alloimmune skin graft injury reveal that alloantigen-specific human T_{regs} (enriched by surface expression of CD69 and CD71) protect from injury more efficiently and potently than non-alloantigen-specific T_{regs} [62]. Together, these studies suggest that antigen-specific T_{regs} may represent a therapeutic with increased potency as compared to polyclonal T_{regs}.

Clues from human disease

Mendelian diseases pointing to the critical anti-inflammatory role of T_{reg} s include IPEX and Wiskott-Aldrich syndrome, where defects in T_{reg} development and function, respectively, lead to inflammation in multiple compartments including the gut [63]. Genome-wide association (GWA) studies suggest a broader role for T_{reg} s in complex human diseases including inflammatory bowel disease and T1D, with numerous genes known to be important for T_{reg} differentiation and/or function being associated with disease risk (IL-10, IL-10R, IL-2, STAT3, PTPN22, etc) [63]. FOXP3 is not typically implicated in these GWA studies, potentially because significant defects cause more devastating disease; sequencing studies may find rare disease-associated variants with subtler effects, such as a polymorphism in the 3' untranslated region recently associated with both atopic and autoimmune disease in a smaller cohort [64].

These GWA studies may help prioritize genes to study for roles in T_{reg} differentiation and/or function. The relative dearth of T_{reg} -specific therapies is likely at least in part due to an insufficiently complete understanding of disease-relevant T_{reg} pathways. Studies showing significant interindividual variation in $CD4^+$ T cell responses to activation, only ~25% of which is ascribable to simple genetic effects, highlight the challenge and importance of these efforts [65]. Efforts to highlight regulatory elements conserved across species may help focus subsequent studies in conjunction with disease associations [66].

An interesting approach is the use of unbiased high-throughput methods to identify compounds that modulate T_{reg} differentiation and/or function. Traditional genetic methods have been hampered by the resistance of naïve $CD4^+$ T cells to viral transduction, and by the proliferation that accompanies Th differentiation, which limits the utility of siRNA approaches. Small molecule approaches offer the potential dual benefit of not only learning novel biology, but also the potential utility of the identified compound as a therapeutic lead. Such an approach identified DYRK1A as a novel regulator of T_{reg} differentiation, suggesting an explanation for the hypofunctional T_{reg} s seen in Down Syndrome (*DYRK1A* is on chromosome 21) and potential clinical utility of DYRK1A inhibitors [23].

An important consideration is that the majority of current approaches assess quantitative differences in T_{reg} s in the peripheral blood, for technical and logistic reasons. This may not correlate well with observations in local tissues which is more relevant to disease pathobiology [67]. Better tools are needed to assess T_{reg} s in tissues, especially those not typically biopsied. Furthermore, an enhanced suite of tools to assess T_{reg} function is urgently needed. In diseases where T_{reg} s are hypofunctional (e.g. Down syndrome) or normofunctional (e.g. relapsing-remitting multiple sclerosis) it is unclear that currently available approaches of quantitatively increasing T_{reg} s, especially polyclonally, will be effective [68,69]. Additional tools, beyond the in vitro suppression assay, are needed to diagnose qualitative T_{reg} defects and how they might be rescued.

Methods to manipulate T_{reg}s

Taken together, defects in the T_{reg} axis can broadly be classified into quantitative defects versus qualitative defects, layered on top of which are considerations of antigen specificity (a variant of quantitative defect) and stability (features of both quantitative and qualitative defects). Understanding the nature of the defect in a disease-, tissue- and patient-specific manner is necessary to devise the best intervention. Most of the interventions available in the foreseeable future are aimed at enhancing T_{reg} numbers, but other tools may be more important in patients who generate hypofunctional T_{reg}s or who cannot direct appropriate antigen specificities to the T_{reg} lineage.

Systemic T_{reg} therapies

Many existing tools are directed towards well-described core components of the T cell signaling machinery (e.g. CD3 and IL-2 signaling) that can be manipulated in relatively T_{reg}-dominant manners, although more pleiotropic effects should always be considered. Treatment with anti-CD3 increases pT_{reg} induction and induces remission of autoimmune diabetes in mice [70,71]. Encouragingly, treatment of 24 – 80 patients with new-onset T1D with anti-CD3 therapy in placebo-controlled phase II trials demonstrated improved metabolic control and residual insulin production 12 months to 4 years later [72–74]. Phase III trials have been less publicly successful, owing at least in part to the use of unvalidated end-points and drug dosages, although re-analysis of the teplizumab trial using the previously validated end-points showed benefit of therapy at 1 and 2 years post therapy [75–77]. Dosage was changed in part to avoid toxicity, again pointing to the value for continuing to search for more T_{reg}-specific interventions. Results from revised phase III trials and testing in the context of other autoimmune diseases remain anticipated.

Although IL-2 is broadly essential for the maintenance and survival of most T cell subsets, T_{reg}s are particularly dependent on exogenous IL-2 because FOXP3 represses *IL2* expression [78–80]. Expression of the high-affinity IL-2 receptor CD25 facilitates T_{reg} survival and may modulate local levels of IL-2 available for other cells, including NK cells [34,35]. This underlies the use of low-dose IL-2 (<1.5–3×10⁶ IU daily, as compared to high dose 1.8×10⁶ IU/kg daily in cancer) to attenuate the inflammation in graft-versus-host disease and HCV vasculitis [81–83]. These studies observed clinical improvement accompanied by increased circulating T_{reg}s after IL-2 infusion, returning to baseline by 4 weeks after IL-2 discontinuation. Low-dose IL-2 (1.5–3×10⁶ IU x5 days) has also shown early promise in alopecia areata, where the persistent increased T_{reg}s specifically in skin biopsies (2 months after last treatment) point to the value of looking at target tissue, and in systemic lupus erythematosus, where progress in stratifying patients is likely to help select those most likely to benefit from T_{reg} therapies [84–86]. In patients with T1D, IL-2 (4.5×10⁶ IU 3 times/week × 1 month) was used together with rapamycin, resulting in increased circulating T_{reg}s and NK cells but without improvement in islet β-cell function [87]. This was likely due in part to the need to further reduce IL-2 dosing; subsequent trial (phase I/II) of 3 lower IL-2 doses (0 – 3×10⁶ IU/day x5 days) showed safe, dose-dependent T_{reg} increase without negatively impacting β-cells [88].

The realization that the mTOR inhibitor rapamycin enhances T_{reg} differentiation in vitro, in conjunction with its use in transplant for immunosuppression, has led to a broader consideration of its value as a pro- T_{reg} agent [89,90]. Recent data suggest that rapamycin's growth-inhibitory effects may dominantly contribute to the in vitro results and similarly impact T_{reg} expansion in vitro and in vivo, although there may be ancillary benefits on stronger inhibition of other lineages and reducing production of pro-inflammatory cytokines [23,91,92]. Rapamycin can impair insulin signaling and may have contributed to the transient decrease in β -cell function in the IL-2/rapamycin T1D trial [87,93]. Together, these results exemplify some of the challenges in using broadly acting agents to achieve specific immune goals.

The microbiota has emerged as a potent modulator of immune function, notably with segmented filamentous bacteria (SFB) driving Th17 differentiation and certain clades of *Clostridia* driving T_{reg} differentiation [94–96]. Although a human counterpart of SFB has not yet been identified, human strains of *Clostridia* have been identified with anti-inflammatory activity in murine models of colitis [96,97]. These microbes are thought to promote T_{reg} differentiation through their natural production of short-chain fatty acids such as acetate, propionate and butyrate, raising the notion of using microbes and microbial products as therapeutic agents [97–100].

Adoptive cellular therapy

The emerging field of T_{reg} cellular therapy is perhaps of most obvious pertinence to the transfusion medicine physician, leveraging existing infrastructure to harvest, store, manipulate and deliver blood-based therapeutics. Most of these have been phase 1 studies exploring safety in the context of graft-versus-host prophylaxis. A University of Minnesota study used $T_{reg}s$ ($3 \times 10^6/kg$) expanded from third party cord blood in 23 patients undergoing double-cord blood transplantation and found no T_{reg} -associated toxicities [51]. Although not powered to examine efficacy given co-administration of standard GVHD prophylaxis, a trend towards decreased grade II-IV acute GVHD was observed ($P=0.04$) along with transient increase in peripheral blood T_{reg} frequencies (up to 2 weeks); chimerism analyses were not performed to examine long-term persistence. A University of Perugia study tested freshly isolated donor $T_{reg}s$ ($2 - 4 \times 10^6/kg$) as the sole immunosuppression in 28 haploidentical stem cell transplant recipients, leveraging predicted in vivo expansion of $T_{reg}s$ observed in mouse models [101]. Although no improvement in mortality was seen in this high-risk population, only 2 patients developed GVHD and 1 relapse with seen after median 12 months follow-up. A phase 2 follow-up expanded testing to 43 haploidentical stem cell transplant recipients receiving $2.5 \times 10^6/kg$ donor $T_{reg}s$ as sole immunosuppression finding full engraftment in 95% and acute GVHD grade 2 in 15% with only 2 relapses at median 46 months follow-up [102]. These findings show great promise for the use of $T_{reg}s$ in GVHD prophylaxis – how this can best be applied to treat ongoing GVHD is more challenging and remains to be clarified.

The potential of T_{reg} cellular therapy in T1D is also of active interest. At the Medical University of Gdansk, 10 new-onset patients were treated with autologous expanded $T_{reg}s$ ($10 - 20 \times 10^6/kg$) with no toxicity was noted [103]. Long-term persistence of $T_{reg}s$ was not

examined. Clinical parameters, including clinical remission, insulin need and C-peptide levels, were better in T_{reg} -treated than in untreated patients and stayed improved up to 1 year later [103,104]. A study at University of California San Francisco and Yale, treated 14 recent-onset T1D patients with expanded deuterium-labeled autologous T_{regs} ($5 \times 10^6 - 2.6 \times 10^9$), which was tolerated well [52]. Deuterium labeling allowed tracking of the infused T_{regs} , demonstrating no instability to inflammatory fates (at least in peripheral blood), and persistence of a small proportion of infused T_{regs} in peripheral blood up to a year later. This study was not powered to detect metabolic improvements, but clearly did not exhibit the same precipitous C-peptide decline that accompanied IL-2/rapamycin treatment [52,87].

Whereas the above studies focus on polyclonal T_{regs} , there is likely to be additional benefit to using antigen-specific T_{regs} . Human trials are in progress to assess the efficacy of such antigen-specific T_{regs} in kidney and liver transplantation (the ONE study and NCT02474199). Systemic approaches include peptide-pulsed apoptotic cells and peptide-linked microparticles, which target tolerogenic antigen-presenting cells to induce pT_{regs} in murine models of multiple sclerosis and T1D [105,106]. With regards to cellular therapy-type approaches, ovalbumin-specific T_{reg} clones were generated by incubating PBMCs with ovalbumin and Drosophila S2 cells expressing anti-CD3, CD80, CD58, IL-2 and IL-4 in a phase 1/2a study of 20 patients with refractory Crohn's disease [107]. These T_{regs} were well-tolerated and demonstrated dose-related efficacy. Whether this approach can be effectively generalized using other autoimmune-relevant antigens and/or patient antigen-presenting cells remains to be demonstrated. Antigen-specific T_{regs} could be sorted using HLA/peptide multimers for subsequent expansion, although this is likely to be useful only in diseases with limited pathogenic HLA/peptide combinations [108,109]. One emerging hybrid approach to specificity uses chimeric antigen receptors, which confer specificity using an extracellular antibody domain that recognizes antigen in a non-MHC-specific manner. Murine studies demonstrate antigen-specific disease attenuation in models of colitis and multiple sclerosis [110,111]. Because specificity is conferred by antibodies, this approach is likely most useful to enhance localization of T_{regs} , rather than true TCR-MHC/peptide specificity.

Forced expression of FOXP3 can help generate T_{regs} , either in a polyclonal or antigen-directed manner (if pre-selected for specificity). Early efforts showed retroviral-driven FOXP3 expression in conventional $CD4^+$ T cells confers suppressive activity, albeit not to normal T_{reg} levels [28]. More recent efforts showed lentiviral-driven expression of FOXP3 in $CD4^+$ T cells from IPEX patients drives differentiation into functional and stable T_{regs} [112]. Some concern regarding the general sufficiency of FOXP3 (outside IPEX) is raised by the finding that forced expression of FOXP3 does not necessarily recapitulate the entire T_{reg} genetic signature although efforts to identify ancillary factors like transcription factors that help lock in the T_{reg} signature may propel these efforts [113,114].

One of the more prominent technical barriers to using adoptive T_{reg} cellular therapies is available purification techniques; fluorescence-associated cell sorting (FACS) is routinely used in research and allows for simultaneous positive/negative sorting based on multiple markers but standard sorters are not GMP-compliant [115]. For these reasons, magnetic sorting is typically substituted, but GMP-compliant sorters are eagerly awaited, development

of which may be accelerated by recent philanthropic efforts [116]. Constant evolution of GMP reagents and methods is required to attain therapeutic T_{reg} doses – this is currently achieved by expanding polyclonal tT_{reg} s, although iT_{reg} s can be generated in even larger quantities and may become more attractive, as the ability to enhance their stability and relevance of antigen-specific cellular therapy advance [27]. Regulatory issues also present a burden, especially in a context of a patient-specific therapeutic; studies that demonstrate efficacy of shipped products point to feasible ways to centralize necessary expertise [52,115]. Finally, especially given concerns regarding lineage stability, methods to track transferred T_{reg} s will be of great value. Deuterium labeling is of demonstrable value for peripheral blood analysis, as is VNTR chimerism analysis in allogeneic recipients, but noninvasive techniques like single photon emission computed tomography (SPECT) may eventually allow tracking of Technetium-99-radiolabeled cells in target organs of patients [117].

Further considerations

As previously alluded to, T_{reg} therapies are an aspect of a broader endeavor to modulate immune homeostasis. It is in this context that safety should continue to be evaluated. In this regard, ipilimumab is an antibody against the inhibitory co-receptor CTLA-4 currently used predominantly in melanoma to “break” tolerance to the tumor. Significant risk of immune-related adverse events accompanies ipilimumab use (72% all grades, 24% high grade), especially colitis [118]. In the converse context of pro- T_{reg} therapy, key side effects of concern include infection, decreased graft-versus-leukemia (GVL) effect and impaired immune surveillance leading to neoplastic disease. In non-cancer-related studies, no increased susceptibility to infection has been noted, perhaps best exemplified by the absence of increased viral load in HCV-positive patients treated with low-dose IL-2 [52,83,103,104]. Post-transplant patients are immune-compromised and do develop infections, albeit at similar levels to historical rates where tracked [51]. Settings where T_{reg} therapy may allow decreased immunosuppression may even reduce risk of infection, although this remains to be demonstrated. In the setting of using T_{reg} s to treat GVHD, an attendant concern is inhibition of the GVL effect post-transplant; this was found to be maintained in one study and is worth tracking in subsequent studies [102].

With regards to immune surveillance, it bears explicating that patients with cancer or inflammatory disease do not typically exhibit robust perturbations of T_{reg} numbers in peripheral blood. This highlights the importance of considering (T_{reg}) location, specificity and functionality in the context of immune homeostasis, and the value of tools that allow measurement of these parameters. Tumor microenvironments may be particularly supportive of T_{reg} development and the role of T_{reg} s in initiating and/or propagating defects in immune surveillance in individual patients remains to be clearly delineated [119,120]. The risk of neoplastic disease may increase with prolonged pro- T_{reg} regimens, although the chronic inflammation typically being treated is notably itself a risk factor.

Durability of any given pro- T_{reg} regimen is an issue of interest that better tools may help assess. This is typically monitored using the peripheral blood T_{reg} count (which typically returns to baseline within 2–8 weeks after treatment) but this does not inform about location-

or antigen-specific responses [51,81–83,103]. In the setting of adoptively transferred cellular therapy, deuterium labeling is a promising approach that shows detectable persistence of some transferred T_{reg} s in the circulation of many patients up to one year later [52]. Developing quantitative tools will be helpful to monitor both persistence and stability of transferred T_{reg} s, although this method may still be limited to accessible tissues such as blood and perhaps colon and skin. The ability to assess T_{reg} s in other organs such as pancreas is greatly anticipated. These tools may significantly impact regimen design and development of next-generation therapies of increased durability.

Concluding remarks

T_{reg} s are a critical regulator of immune homeostasis, manipulation of which is broadly relevant to both inflammation and cancer. Several studies show great promise of this therapy and progress in key areas will greatly advance our capabilities. There remains significant room and need to identify novel pathways that regulate T_{reg} development and function, especially in the context of human disease. This will help better identify patients who might benefit from T_{reg} therapies, as well as suggest how current T_{reg} therapies might be improved, particularly with autologous therapies. Development of T_{reg} -related interventions must include considerations not only of quantitative and qualitative defects, but also of tissue-localization, specificity and lineage stability. Diagnostically, major challenges include finding improved ways to identify T_{reg} s, especially in tissues, as well as developing better assays to assess T_{reg} function, ideally in a manner that lends itself to current testing platforms.

Current therapeutic modalities focus on increasing T_{reg} s quantitatively either using systemic therapies or adoptive cellular therapies, and show promise in early studies. It is important to keep in mind that these T_{reg} therapies need not and should not be considered in isolation, but rather as a component of a synergistic, coordinated manipulation of the immune system away from the pathologic state. For example, whereas adoptively transferred T_{reg} s may be useful in early-onset type 1 diabetes, more advanced cases may require a different regimen to enable induction of tolerance, followed by a pro- T_{reg} therapy and islet transplant. Adoptive cellular therapies offer unique opportunities to select T cells based on antigen specificity and even modulate their functionality using agents at concentrations that might not be tolerated when administered systemically. Certainly, T_{reg} therapies exemplify the tip of an exciting chapter of therapeutic manipulations of immune homeostasis, in which the transfusion medicine physician plays a key role.

Supplementary Material

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Abbreviations

GWA	Genome-wide association (study)
T1D	Type 1 diabetes
TCR	T cell receptor
Th	T helper
T_{reg}	Regulatory T cell

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Highlights

- Core concepts of T_{reg} differentiation and function.
- Antigen-specificity and lineage stability are important considerations in T_{reg}s.
- Quantitative and qualitative defects in T_{reg}s lead to disease.
- Systemic and cellular therapies can address T_{reg} defects.
- Unique potential of cellular T_{reg} therapies.

Figure 1. Immune homeostasis is maintained by balanced differentiation of naïve $CD4^+$ T cells into pro-inflammatory (e.g. $Th1$, $Th17$) or anti-inflammatory (e.g. T_{reg}) subsets. Excessive skewing leads to either pathologic inflammation or cancer.

Fig. 2A

Fig. 2B

Fig. 2C

Fig. 2D

Figure 2.

Overview of T_{reg} clinical trials documented in clinicaltrials.gov. **2A.** Manual curation of all study entries involving T_{reg} s, with further subdivision according to data accrual, related disease entity, and type of study (observational, Obs, versus therapeutic, Tx). **2B.** Number of observational (Obs) and therapeutic (Tx) study entries initiated in each year. **2C.** Percentage of clinical studies in each phase (1 to 3) according to disease category; total number of studies per category indicated in parentheses. **2D.** Distribution of most-studied diseases/organ systems in each category. CR, colorectal.