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EXPERT OPINION

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The therapeutic potential of regulatory T cells for the treatment of autoimmune disease

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Introduction: Immune tolerance remains the holy grail of therapeutic immunology in the fields of organ and tissue transplant rejection, autoimmune diseases, and allergy and asthma. We have learned that FoxP3$^+$CD4$^+$ regulatory T cells play a vital role in both the induction and maintenance of self-tolerance.
Areas covered: In this opinion piece, we highlight regulatory T cells (Treg) cell biology and novel immune treatments to take advantage of these cells as potent therapeutics. We discuss the potential to utilize Treg and Treg-friendly therapies to replace current general immunosuppressives and induce tolerance as a path towards a drug-free existence without associated toxicities.
Expert opinion: Finally, we opine on the fact that biomedicine sits on the cusp of a new revolution: the use of human cells as versatile therapeutic engines. We highlight the challenges and opportunities associated with the development of a foundational cellular engineering science that provides a systematic framework for safely and predictably regulating cellular behaviors. Although Treg therapy has become a legitimate clinical treatment, development of the therapy will require a better understanding of the underlying Treg biology, manufacturing advances to promote cost effectiveness and combinations with other drugs to alter the pathogenicity/regulatory balance.

Keywords: autoimmunity, cell therapy, organ transplantation, peripheral tolerance, suppressor cells, regulatory T cells cells

Expert Opin. Ther. Targets [Early Online]

1. Introduction

Immune tolerance is a fundamental biologic process to avoid autoimmune responses against self-antigens. Two distinct pathways have been described that prevent autoreactive T cells from escaping immune control and promoting autoimmunity. The first pathway is mediated through thymic negative selection (central tolerance) based on the finding that high-affinity self-reactive thymocytes are deleted during development. This deletional mechanism results in elimination of cells directed against common self-antigens expressed widely in the body. In addition, there is a distinct thymic deletional mechanism to eliminate self-tissue-specific thymocytes via a subset of medullary thymic epithelial cells, termed as mTECs. These specialized cells express a transcription factor, autoimmune regulator (*AIRE*) [1], that controls the ectopic expression of a large number of tissue-specific antigens. Together, these deletional pathways in the thymus are effective in eliminating a large percentage of potentially autoreactive T cells.

However, in spite of the efficiency of thymic negative selection, autoreactive cells routinely escape from the thymus [2] as evidenced by T cell receptor analysis using antigenic peptide-MHC-based tetramers, which has shown direct evidence for autoreactive T cells in the periphery that escaped the thymus [3]. A number of peripheral mechanisms have developed to protect against these autoimmune

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Article highlights.

- Both central and peripheral tolerance are essential for immune regulation; however, one specific suppressor T cell subset, regulatory T cells (Tregs), play a master function to control unwanted immunity.
- Tregs are critical in controlling the homeostasis of immune system both systemically and during localized immune responses.
- Tregs can be functionally instable in certain circumstances, for example, autoimmunity and inflammation, leading to increased incidence of autoimmunity.
- Tregs and Treg-friendly immune therapies have shown promising immune tolerant therapeutic effects.

This box summarizes key points contained in the article.

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reactivities. There are deletional mechanisms such as the Fas/FasL and IL-2-mediated activation-induced cell death pathways that lead to peripheral clonal deletion of autoreactive cells. However, the most robust forms of peripheral tolerance depend on specialized T cells that suppress the induction and progression of autoimmunity. These suppressive T cell subsets function to both directly block T cell activation through inhibition of antigen presentation and indirectly through bystander suppression mediated by the suppressive cytokines to regulate autoreactivity beyond the individual specificity of the suppressor cell itself [4]. Moreover, cytokines such as TGF-β and IL-10 can themselves induce additional suppressor T and B cells, which can shut down local autoantigen responses [4].

In spite of the extensive regulatory pathways both in the thymus and periphery, under certain circumstances, tolerance is compromised leading to > 80 autoimmune diseases [5]. In many cases, this occurs as a consequence of genetic, epigenetic and environmental insults leading to a reduction in the number and function of regulatory subsets [5]. In addition, autoimmune pathogenic cells can become resistant to tolerogenic mechanisms [5]. Thus, newly developed immunosuppressive drug therapies are being tested to promote tolerance through mechanisms enhancing regulatory cells. In fact, as discussed below, specific regulatory T cells (Treg) therapies are being tested to re-establish tolerance and block autoimmunity. In this opinion piece, we summarize the current understanding of the nature of suppressor cells (especially Tregs) their attributes, functional characterization and deficiencies in certain disease settings. Finally, we discuss the potential use of regulatory cells as clinical interventions.

2. Suppressive T cell populations controlling autoimmunity

There are a number of suppressor cell populations, including multiple suppressive T cells, regulatory B cells, and multiple immunosuppressive dendritic and macrophage populations described in both humans and rodents. Although all have been shown to be involved in immune regulation, suppressive T cells remain the major cell subsets controlling immunity. T suppressor cells were first introduced > 40 years ago as $CD8^+$ T cells that could suppress helper T cells and induce tolerance [6], but due to the lack of reproducibility or distinguishing genetic markers, the field underwent a rapid demise in the 1980s. The field of suppressor T cells re-emerged 15 years later based on seminal studies looking at $CD4^+$ T cell subsets that controlled immunity [7]. Many of the hallmarks of T cell-mediated immunosuppression, such as inhibition of effector T cells by other T cells, the role of soluble factors (IL-10, TGF-α), and most importantly the importance of suppressor T cells in the maintenance of immune tolerance has been reborn in the current era. Thus, the field was re-energized and with it a renewed interest in the therapeutic implications of harnessing the power of T cell immunosuppression.

Oral antigen-induced tolerance, mediated by a $CD4^+$ Th3 cells, was among the earliest T suppressor cells identified [8]. This subset mediates suppression by producing high levels of the immunosuppressive cytokine, TGF-β, in response to antigen presented in the gut [8]. These basic studies led to several pre-clinical models, which showed that administering self-antigens orally to mice with autoimmune disease led to the induction of Th3 cells, attenuated autoimmune reactions and tolerance induction [9]. Unfortunately, to date similar clinical efforts in humans have not shown efficacy [9]. A second T suppressor cell subset, Tr1 cells, identified originally in transplantation models produces a high level of IL-10 after T cell receptor (TCR) stimulation leading to robust immune suppression based on contact-independent pathways [10]. Tr1 cells express a unique constellation of activation markers, chemokine receptors, and most recently, two identifying checkpoint regulating molecules, LAG-3 and CD49b [10]. Current efforts are underway by Maria Grazia Roncarolo *et al.* to translate these finding to clinical applications in bone marrow and kidney transplantation [10]. Lastly, a unique subset of suppressive T cells has been identified in mice, iT_R35, which does not require any of the hallmarks of other suppressor T cells including forkhead box 3 (FoxP3), IL-10 or TGF-β to suppress but rather uses a novel cytokine, IL-35, to shut down effector T cell function directly through a Neuropilin-1/Semaphorin A pathway, and data by Vignali *et al.* suggest it may be translatable in human [11,12].

All the suppressive T cell subsets described above are likely to be involved in immune homeostasis and tolerance with potential therapeutic application; however, these cell subtypes are not critical to fundamental immune tolerance as their disruption does not lead to massive autoimmunity. In contrast, one suppressive T cell subset, Treg, has been shown to be essential regulators of immune homeostasis. Removal of the thymus in 1 – 2 day old mice led to the development of multi-organ autoimmunity shortly after weaning [6,13]. Further studies by Sakaguchi and others showed that these

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CD4$^+$ T cells expressed high levels of CD25 [7]. Most importantly, this population expressed a unique transcription factor, FoxP3, which distinguished them from other suppressive T cell subsets [8,10,11,14]. Further studies by Rudensky and their colleagues linked observations that scurfy mice and human IPEX patients, harboring mutations in the FoxP3 gene, led to the multi-organ autoimmunity [15]. Mice and humans that have mutations in the *FoxP3* gene develop multi-organ autoimmunity resulting in the death of the mice within weeks and lethality in many humans within 1 year of age in the absence of a bone marrow transplant to reconstitute the Treg population [16]. As a consequence, Tregs have become the quintessential Treg subset controlling the most fundamental aspects of suppressor cell-regulated immune tolerance. FoxP3 plays an essential role in the peripheral maintenance of Treg stability [17]. Impaired or decreased expression of FoxP3 leads to impaired suppressive function [15,18]. When expression of FoxP3, or its associated chromatin structure, are compromised, a series of downstream consequences, including epigenetic changes, altered gene expression and effector molecule production lead to a profound disruption of Treg function and subsequent autoimmune manifestations [17]. Thus, for the rest of this opinion piece, we will focus on individual subset of CD4$^+$ FoxP3$^+$-expressing Tregs, their biology and therapeutic potential.

3. Treg development and characterization

Tregs can develop in both the thymus and periphery [19]. In the thymus, Tregs utilize their high-affinity TCR to escape thymocyte deletion, resulting in expression of FoxP3, which drives cells to the Treg lineage. These thymic-derived Tregs (tTregs) leave the thymus and become fully developed in the periphery over the first few weeks. tTregs populate both lymphoid and non-lymphoid tissues (such as fat and lung tissue) providing general immune homeostasis. However, FoxP3 can be turned on in peripheral T cells (pTreg), especially in the gut [20], upon exposure to antigens and adjuvants, perhaps derived from the microbiota. In tissues, pTreg manage local inflammatory responses. In the mouse, a series of markers can be used to isolate Tregs and study their function. In humans, the study of Tregs has been limited by the absence of a constellation of markers that led to highly purified Treg population. However, human Tregs express low levels of CD127 (IL-7R) [21], so, when combined with CD4 and CD25, > 95% of the resulting population are bone fide Tregs based on high FoxP3 expression, suppressive activity [21] and epigenetic modifications including demethylation of the Treg-specific demethylation region (TSDR) [22], typically only found in stable Tregs [23]. Together, these markers identify a population of cells that constitute ~ 5 – 10% of peripheral CD4$^+$ T cells in both human and mice [24].

Several markers have been highlighted in the study of distinguishing tTregs and pTregs. Most tTregs express Helios, whereas pTregs are largely Helios negative [19]. But Helios$^+$ Tregs do not exclusively belong to tTreg and vice versa [19]. Although Helios is not the ideal marker to distinguish the two subsets, it is so far widely applied. Mouse tTreg can also be distinguished by the cell surface expression of neuropilin-1 as neuropilin-1$^+$ population overlaps with the Helios$^+$ subset [25,26]. Interestingly, using FoxP3 reporter mice, these cell-based markers and other genetic manipulations (disruption of specific regulatory regions of the FoxP3 gene (termed CNS1 and CNS2)), it is clear that tTregs and pTregs are derived from different cell lineages with pTregs largely developing from conventional T cells in the gut and during inflammatory responses in several mucosal tissues. Finally, there exist memory populations of Tregs that respond more aggressively on re-exposure to self-antigens and are most effective in controlling autoimmunity [27].

Importantly, tTregs and pTregs have largely non-overlapping TCR repertoires [19,28]. This is most likely due to the different sites of development (thymus vs periphery) and the precursor cells (immature thymocytes vs naive T cells). Thus, the nature of the recognized antigens during Treg differentiation may be distinct. In fact, recent studies of autoreactive T effectors cells in the autoimmune setting suggest that the nature of the self-antigenic peptides produced at the site of autoimmune inflammation may be quite different than in the thymus and the peptides may themselves be modified by enzymatic reactions such as citrullination, transglutamination, and so on [29]. These results are consistent with the hypothesis that Tregs at different sites may play very distinct roles ranging from general immune homeostasis to more localized immune regulation at sites of inflammation.

4. Tissue-specific differences in suppressor T cell subsets

Recent studies have focused on Tregs in non-lymphoid tissues, including intestinal mucosa [30], visceral adipose tissue (VAT) [31], muscle [32,33], lung [34] and skin [27]. The frequency of tissue-resident Tregs ranges from 30 to 60% of the total CD4$^+$ T cells and varies according to different tissues [35]. The high Treg frequency is probably caused by Treg migration directly from the thymus in an early developmental wave and from lymphoid tissues. The cells become activated in the tissues, proliferate and differentiate, driven by local self-antigens or cytokines. The tissue-resident Tregs display unique cell surface and functional phenotypes, distinct TCR repertoires indicating an intense Treg proliferation driven by local antigens, and a unique array of transcription factors involved in local cytokine production, metabolism and tissue repair [35].

As stated above, intestinal mucosa is a major non-lymphoid reservoir for tissue-resident Tregs, especially pTregs, which are induced and expanded by the stimulation of commensal microbes [20]. Skin Tregs are preferentially located around hair follicles and display a memory-like phenotype with higher expression of Bcl2 and CD27 [27]. Lung Tregs also

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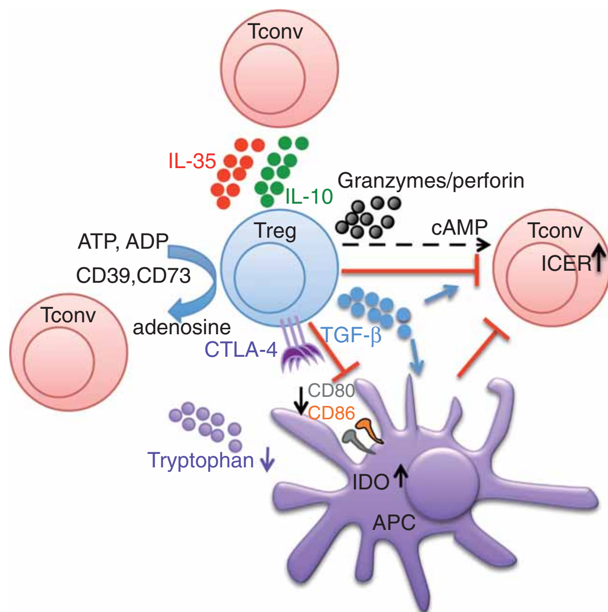

Figure 1. Suppression mechanisms. Tregs suppress Tconv by multiple mechanisms. For direct suppression on Tconv: Treg produce immunosuppressive cytokines (IL-10, IL-35, and TGF-β), induce cell death via Granzyme and perforin, transfer cAMP to Tconv, which increases ICER, and generate immunosuppressive adenosine. For indirect suppression, Tregs downregulate costimulatory molecules and upregulate IDO in APCs via CTLA-4.

APCs: Antigen-presenting cells; ICER: Inducible cAMP early repressor; IDO: Indoleamine 2,3-dioxygenase; Tconv: Conventional T cells; Treg: Regulatory T cells.

show a memory-like phenotype (CCR4^{high}CD62L^{low}CD44-^{high}CD54^{high}CD69^{+}) and they can suppress T cell proliferation but not Th2 cytokine production [34]. In addition, lung-resident macrophages can induce Treg differentiation locally mediated by TGFβ and retinoic acid [36]. Besides these barrier tissues where tolerance is required for immune homeostasis, Tregs also function in metabolic tissue. VAT-resident Tregs express PPAR γ, which has been implicated in the prevention of immune inflammatory disorders and can result in upregulation of genes encoding molecules linked to lipid metabolism. The absence of these cells results in metabolic syndrome due to increased insulin resistance [35]. IL-2-anti-IL2 antibody complex treatment increases IL-10 in VAT and reverses insulin-resistance induced by high-fat diet, indicating a potential broader application of Treg and Treg-friendly therapies in other diseases including type 2 diabetes [35]. Moreover, Tregs are also involved in tissue repair. In fact, it has been recently discovered that muscle Tregs over express amphiregulin [32], and they can control and repair tissue destruction in non-immune diseases such as Muscular Dystrophy. In a mouse muscular dystrophy model (MDX) and in patients affected by muscular dystrophy, there are an increased number of muscle Treg cells at the time of tissue destruction. In the MDX model, elimination of the Tregs exacerbates tissue destruction, whereas treatment with IL-2-anti-IL2 antibody complex leads to reduced disease pathogenesis via promoting Tregs [33].

Finally, it should be noted that the TCR repertoire is significantly skewed under the influence of local tissue antigens, indicating an intense Treg proliferation driven by local antigens [35]. Although tissue-resident Tregs reflect both tTreg and pTregs, the TCR repertoire barely overlaps with conventional T cells (Tconv) or even Tregs from lymphoid tissue [32] indicating that the expansion of Tregs in non-lymphoid tissue might be driven by unique tissue self-antigens, which might be different from the autoantigens recognized by autoreactive Tconv or self-antigens recognized by Tregs in lymphoid tissue.

5. Suppressive mechanisms of Tregs

Tregs have been reported to have multiple suppressive activities that are manifested in different regulatory settings and inflammatory environments (**Figure 1**). Tregs can suppress a variety of immune cells including B cells, NK cells, NKT cells, CD4^{+}, and CD8^{+} T cells, as well as monocytes and DCs leading to a unique role in maintaining immune tolerance in general and controlling unwanted immune responses at various stages of autoimmunity, allergy, and transplant rejection [24]. Cell–cell contact is an important means of Treg-mediated suppression through membrane-bound TGF-β [37] and high levels of expression of cytotoxic T-lymphocyte antigen 4 (CTLA-4). Down-regulation of CD80/CD86 expression on antigen-presenting cells (APCs) by CTLA-4 on Tregs causes an indirect inhibition of Tconv by APCs *in vitro* and *in vivo* [38]. In addition to altering T cell co-stimulation by Tregs, CTLA-4 engagement can lead to an increase in indoleamine 2,3-dioxygenase (IDO) expression in certain human and murine DC subsets [39]. The IDO enzyme catalyzes degradation of tryptophan to kynurenine, inducing the starvation of Tconv.

Furthermore, Tregs can mediate cytolysis and induce apoptosis of effector T cells via secretion of a family of serine proteases, most prominently granzyme-A and B [38]. Tumor necrosis factor-related apoptosis inducing death receptor 5 (TRAIL-DR5) pathways and galectin-1 has also been implicated in Treg-mediated T-cell apoptosis [40,41]. In addition, TGF-β, IL-10 and newly characterized IL-35 are immunosuppressive cytokines produced by Tregs. TGF-β and IL-10 have been extensively studied whereas the role of IL-35 in Treg-mediated suppression has only recently been described. Finally, Tregs mediate suppression via metabolic disruption of target cells. One of the proposed mechanisms is via the consumption of IL-2 [4]. IL-2 is important not only in promoting Treg survival and proliferation but is also required by other T cells. Another metabolic disruptive mechanism involves the expression of CD39 and CD73 ectoenzymes, which hydrolyze extracellular ATP to generate pericelluar adenosine [42,43]. Adenosine can suppress

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T effector cells via adenosine receptor 2A activation and enhance Treg generation by inducing TGF-β secretion and inhibiting IL-6 expression. Tregs also produce high levels of cAMP induce increased in the Tconv upon contact with Tregs, presumably via transfer through gap junctions from murine Tregs to Tconv. It has been suggested that cAMP suppresses Tconv cells by inducing expression of the inducible cAMP early repressor, a repressor of the IL-2 and IL-4 gene loci [44].

6. Treg signaling and stability

As mentioned above, FoxP3 marks Treg lineage commitment and plays a vital role in determining the suppressive function of the cell by maintaining the expression of important molecules involved in suppression [17,18]. However, although FoxP3 expression is critical for Treg suppression, the expression of FoxP3 alone does not insure Treg functional stability [17]. Proper epigenetic modifications are essential for the establishment of positive feedback loop determining stable suppressive function of Tregs [17]. For example, demethylation of FoxP3 CNS2 TSDR is essential to the lineage commitment and function of Tregs by sensing IL-2 and the downstream phosphorylation of STAT5 [45-47]. Epigenetic stability through demethylation is not limited to FoxP3 but also includes several function-associated genes such as *Ctla4, Il2ra, Tnfrsf18 (GITR), Ikzf2 (Helios)* and *Ikzf4 (Eos)*. These demethylated regions are associated with TCR stimulation, contributing to Treg suppressive function [48]. Different from Tregs generated *in vivo*, the *in vitro* converted iTreg show methylated TSDR, and differences have also been observed in H3K27me3 and H3K4me3 [48]. Our lab, and others, have shown that Tregs can become unstable and lose FoxP3 expression in lymphopenic and inflammatory conditions [45,49]. These 'exFoxP3 cells' produce proinflammatory cytokines, for example, INF-γ that can cause tissue destruction [50]. Remethylation of CNS2/TSDR leads to the loss of FoxP3 expression [45]. For these reasons, TSDR analysis has been applied in the quality control of our Treg manufacture in clinical trials [22,24].

Factors facilitating Treg stability include: TCR signaling/Ag exposure, costimulation, IL-2, TGF-β, and so on [23]. All these pathways are critical for the generation and stability of Tregs as well as the conversion of Tconv to pTreg [23,51].

Treg suppression is initiated by antigen stimulation via TCR signaling [23]. Thus, the intensity of Ag-exposure is essential for the Treg suppression function. The optimal TCR signaling for Treg also requires costimulation via CD28 and other costimulatory molecules [52]. Similarly, IL-2 is a critical cytokine for Treg stability, survival and proliferation. IL-2 acts on Treg cells by signaling via STAT5 phosphorylation, which, as described above is often reduced in individuals with autoimmune disease as compared to healthy controls [53-55]. Interestingly, *ex vivo* expansion of Treg with high concentrations of IL-2 can reverse the pSTAT5 defect (Bluestone lab, unpublished). Thus, the factors facilitating Treg stability are promising drug targets to optimize Treg therapies.

7. Treg in autoimmune disease and therapeutic opportunities

As stated above, total loss of Tregs in FoxP3-deficient IPEX patients suggests a vital role for Tregs in the development of this autoimmune disease 1 [5]. However, more minor defects in functional Tregs are associated with multiple autoimmune diseases. In three independent studies, involving a total of 99 individuals with T1D and normal Treg cell numbers, isolated Tregs from patients show defects in IL-2 signaling and a decreased suppressive activity *in vitro*, suggesting that Tregs are functional defective [53-55]. In addition, our lab has shown an increase in FoxP3$^+$INFγ$^+$ Treg frequency in the peripheral blood of T1D patients [56] consistent with an unstable phenotype. Defects in the Treg function have also been identified in other autoimmune diseases including MS, RA, Systemic Lupus Erythematosus, and inflammatory bowel disease (IBD) [5]. These results suggest that Treg and Treg-promoting therapies provide an opportunity for clinical intervention to reestablish immune tolerance in these settings.

The majority of therapeutic approaches to treat autoimmune disease have been focused on using suppressive drugs that suppress immunity systemically. Treatments such as anti-thymocyte globulin modulate autoimmunity, including EAE and spontaneous T1D in the NOD mouse model by both depleting Teff cells and inducing *de novo* pTregs [57-60]. The efficacy of these antibodies has led to clinical trials with Thymoglobulin, (Sanofi, Paris France) a drug approved for use in blocking graft versus host disease and kidney transplant rejection [61]. Monoclonal antibody therapies have shown promising effects as well functioning through their ability to alter the Teff/Treg ratio [5]. One clear example is the anti-CD3-ε humanized mAbs [62]. Anti-CD3 mAb treatment deletes Teff cells, and at the same time spares Tregs and increases TGF-β-dependent pTregs [63,64]. In recently diagnosed T1D patients, the treatment with anti-CD3 reduces disease progression for a long period of time post-therapy and preserves residual endogenous β-cell mass [65]. With this treatment, an increase in the Treg function and IL-10 production has been observed associated with a selective decrease in proinflammatory IFN-γ [63]. Interestingly, in one study we showed that FoxP3 was turned on in CD8$^+$ T cells isolated from anti-CD3-treated patients consistent with a capability of signaling through the TCR leading to upregulation of FoxP3 in the periphery in certain settings [66]. There are other mAbs against cell surface proteins thought to function exclusively by depleting autoreactive cells that are now known to induce Treg. Examples include: anti-CD52 (Campath-1Hs, Wyeth, Madison, NJ, USA) which depletes a variety of lymphocytes but selectively increases reconstitution of Tregs, and an anti-CD45RO/RA mAb that has been shown to

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promote Treg development [5]. All these mAbs have in common the ability to increase the ratio of Treg to Teff cells by either selectively depleting Teff and/or directly promoting tTreg/pTreg development.

In addition to mAbs directed at cell surface antigen, recent studies have focused on the use of proinflammatory cytokine-specific mAb treatment to promote Tregs. Tissue damage in autoimmunity is caused by cytokines such as IFN-γ and TNF-α. TNF-α antagonist mAb is approved for the treatment of RA and Crohn's disease via enhancing the Treg function [67,68]. Treatment with anti-TNF-α mAb in RA patients reduces inflammation, inducing an increase in the Treg number, and promoting Treg differentiation *in vivo* in a TGF-β-dependent manner [69]. These results provide an example of proinflammatory antagonist drugs that are able to alter the balance of Teff cells versus Tregs.

Pro-tolerogenic cytokines such as IL-10 and TGF-β are critical for Treg functions [4]. TGF-β promotes FoxP3 expression, Treg development and stability, so it has a therapeutic potential [23,51], but it can also cause several side effects including fibrosis [70]. The promising results from IL-10 therapy in several inflammatory diseases tested in experimental models led to its clinical application (Ilodecakin; Schering-Plough Corp., Kenilworth, NJ, USA). IL-10 is well tolerated without serious side effects and it has been shown to induce Tr-1 *in vitro* [5,71]. As described above, Tregs express high levels of CD25 and IL-2 is a critical growth and survival factor. There has been a significant interest in using IL-2 as a therapy *in vivo* to promote Tregs. It is well known that IL-2 is a T cell growth factor and activates CTLs [72]. In fact, recombinant human IL-2 (Proleukin) is an FDA-approved drug and high dose has been approved in treating renal cell carcinomas [73]. The high expression of CD25 on Tregs suggested to Boyman and Sprent [72] that a lower dose might preferentially result in the expansion of Tregs over effector T cells resulting in an efficacious treatment for autoimmunity. In fact, in studies we, and our colleagues, performed in the NOD mouse model of spontaneous T1D, low dose of IL-2 was efficacious in both preventing and reversing T1D [74,75]. Importantly, we observed that high dose of IL-2 enhanced immune responses and exacerbated autoimmune destruction of islets supporting the notion that the *in vivo* effect of IL-2 can vary depending on the dosing regimen, the endogenous level of IL-2, and the levels of activated Teff cells, NK cells, and Tregs in the host [74]. Other studies have been performed by several groups in humans supporting the use of low-dose IL-2 for the treatment of autoimmunity [76]. Thus, combination therapies including IL-2 might provide optimal treatments to ensure the desired outcome. For example, in an autoimmune setting, combining IL-2 with Teff-depleting treatments may prevent potential disease exacerbation. In this regard, rapamycin is a particularly attractive drug [73]. The drug binds to the FK506-binding protein, which in turn complexes with the mammalian target of rapamycin to block cell-cycle progression and cytokine signal transduction [77]. Some studies have shown that this combination is quite effective in reversing diabetes in NOD mice [78]. Another study involving a combination of a mutant IL-15Fc plus IL-2Fc and rapamycin has been shown to induce long-term islet allograft acceptance in the NOD islet allograft model, by eliminating Teffs and promoting Treg development [79]. Studies to engineer or modify IL-2 to improve its therapeutic potential in promoting Tregs are underway including IL-2 muteins that lack an ability to bind the IL-2R complex, anti-IL-2 mAbs that can be complexed with IL-2 to prolong the half-life and alter the conformation of the growth factor to selectively increase Tregs, as shown in the mouse setting [80]. Finally, a number of groups are attempting to develop antigen-specific Treg vaccines. For instance, GAD65 and insulin have been studied as Treg vaccine candidates by altering the route of peptide injection and/or adjuvant [81]. These are just a few examples of multiple approaches that are being developed.

8. Treg cell therapy

Much of the work summarized above has focused on drugs that promote Treg development and expansion at the expense of effector cells. The goal is to promote immune tolerance and reduce autoimmunity or other unwanted immune responses such as GvHD and organ transplant rejection. With that notion in mind, we and other groups set out several years ago to determine if we could use Tregs directly by isolating and expanding *ex vivo* a pure population of cells that would block T effector cell differentiation and function upon re-administration. The advantages of Treg therapy for autoimmune diseases are significant. The cells, especially antigen-specific Tregs, would be expected to block local autoimmunity without requiring systemic immune suppression or massive deletion of immune cells non-specifically as occurs with current immunosuppressive therapies. In addition, the potential for bystander suppression and infectious tolerance might be expected to lead to a robust tolerogenic therapy in the absence of long-term survival of the transferred cells. The Tregs would be expected to modify APCs to become immunosuppressive, convert Tconv into pTreg by providing TGF-β, and recruit other regulatory cell subsets to the site of inflammation.

Initial studies on the potential of Treg therapies have been conducted in a series of animal models including GvHD, transplantation, MS, RA, T1D, and IBD with good success [5,24,82]. Based on these preclinical studies, we, and others, set out to manufacture Tregs as clinical therapeutics. To isolate high-purity Treg cells, we have taken advantage of three markers, CD4, CD25, and CD127 to isolate by flow cytometric cell sorting a pure population of $CD4^{+}CD25^{+}CD127^{lo/-}$ cells that are generally > 95% $FoxP3^{+}$ [21]. On average, we can isolate ~ 1 million Treg cells from 100cc of peripheral blood [83]. Next, we use a standard protocol to expand the human Tregs using two rounds of stimulation by anti-CD3 and anti-CD28-coated beads combined with high-dose IL-2. After

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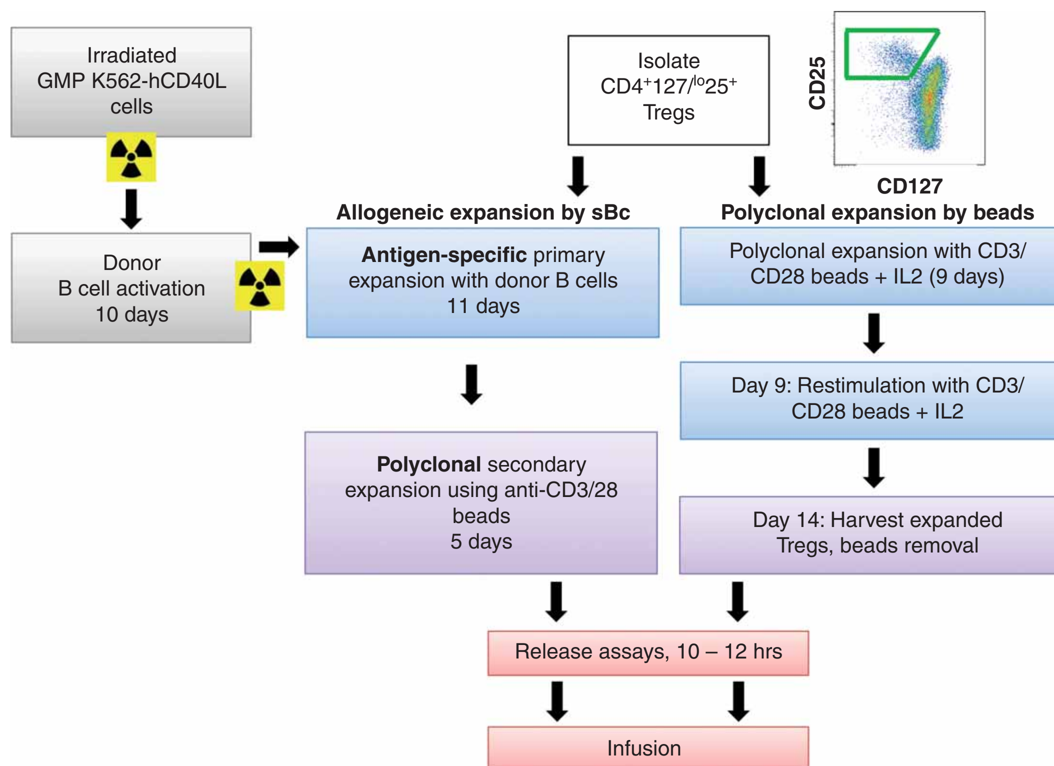

Figure 2. *Ex vivo* manufacture Tregs. Autologous Tregs are isolated from peripheral blood. Le7, allogeneic expansion of Tregs: Tregs are s5mulated by donor ac5vated B cells (sBc), then s5mulated by an5-CD3/CD28 beads; Right, Polyclonal expansion of Tregs: s5mulated polyclonally with an5-CD3/CD28 beads and IL-2 for 9 days, and challenged with the s5mula5on beads again on day 9. The cell product is harvested on day 14. Quality controls are performed before infusion into pa5ent.

GMP: Good manufacturing practices; Treg: Regulatory T cells.

14 – 16 days in culture, up to 3 billion expanded human Tregs can be generated with, on average, > 90% FoxP3+ cells [83]. The Tregs are both highly stable and functional based on TSDR demethylation, low levels of IFN-γ or other effector cytokines producing cells, and typical Treg cell surface phenotype (high levels of CTLA-4 and Helios [22] and another Treg marker, GITR and highly suppressive in both *in vitro* and *in vivo* T suppressor assays (**Figures 2** and **3A**) [83].

These efforts and those of other have led to a number of clinical trials to test the safety and efficacy of Tregs in patients with a number of immunological diseases. **Table 1** provides a list of reported/ongoing clinical trials using Treg cell therapies [84-89]. We just completed a dose escalation, Phase I trial of expanded polyclonal Tregs for the treatment of T1D. To date, there have been no significant safety concerns, a significant subset of the *ex vivo* expanded Tregs survive long-term *in vivo* (at least 12 months) and no untoward loss of pancreatic islet cell function has been observed [24]. These results are similar to those produced by a group in Poland that have examined lower doses of Tregs in a T1D population using a similar expansion protocol [84,89]. However, unlike our studies in T1D, another group examining expanded cord blood Tregs in the GvHD setting suggested limited persistence of the infused Tregs perhaps due the HLA-mismatch or limited IL-2 *in vivo* in this immune-deficient setting [85]. Importantly, none of the studies to date have reported an increase in the risk of infection or cancer. In this regard, a recent study demonstrated that the efficiency of influenza vaccine immunization was similar between untreated control subjects and those infused with expanded Tregs [90]. This indicates that the immune system retains a robust ability to protect the host from foreign pathogens even in the face of the polyclonal Treg infusion.

9. Next steps – antigen-specific Treg therapy

In order to reach the full potential of therapeutic Tregs, it is likely that antigen-specific Treg cell population will be required. The use of antigen-specific Tregs will reduce the risk of off-target immunosuppression that might increase the risk of cancer and infection. In addition, the antigen-specific Treg should exhibit an increased specific activity in the target tissue. In fact, initial studies in mouse models have suggested that antigen-specific Treg therapy can increase efficacy by 20 – 100-fold [91,92]. As the antigen-specific Tregs are more efficient in controlling unwanted immune responses,

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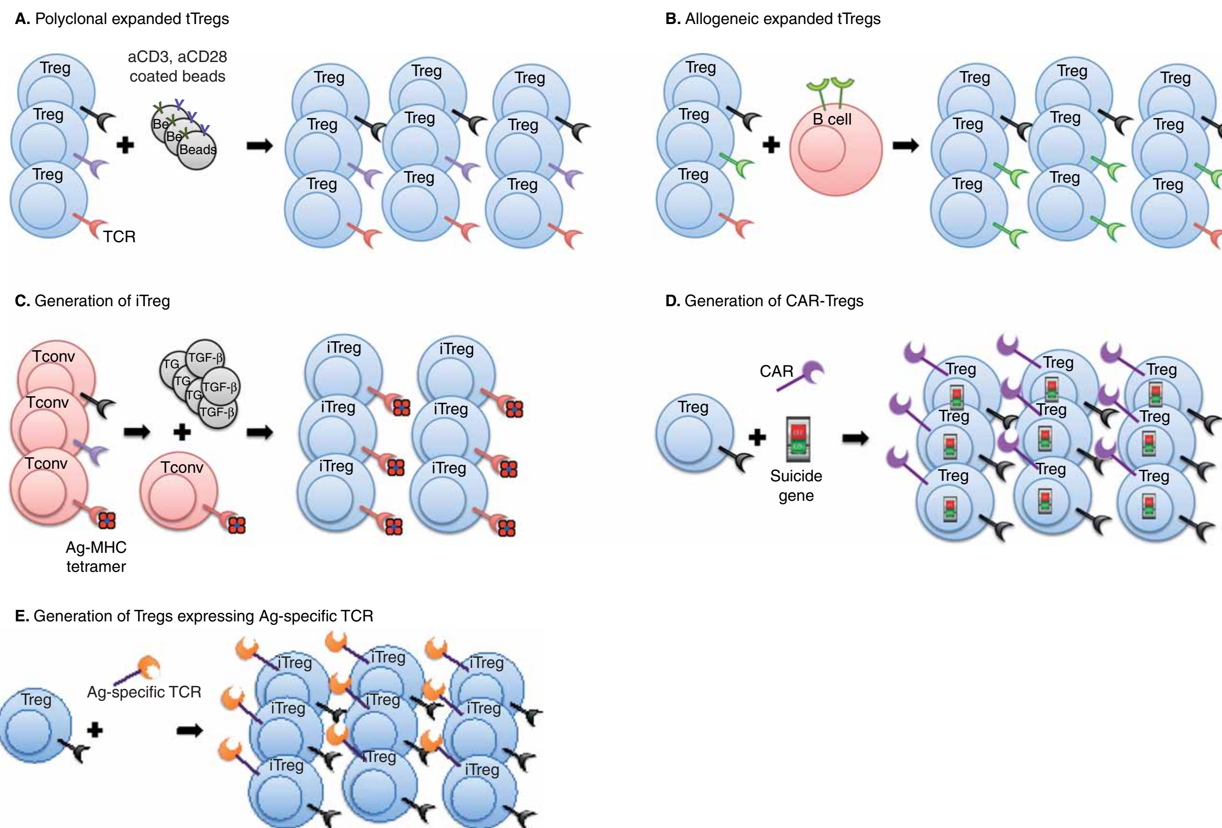

Figure 3. Current and future Tregs as cell therapies. Several approaches have been considered in clinical and preclinical Treg cell therapies. These include: **A**. polyclonal expansion of Tregs with anti-CD3 and anti-CD28 coated beads, **B**. expansion of Tregs using allogeneic B cells, **C**. generation of iTreg from Ag-specific Tconv. **D**. As a future model, CAR-Treg armed with suicide genes will be generated to target tissues for higher suppression efficiency. **E**. Generation of Treg expressing Ag-specific TCR (Brusko TM 2008).

Tconv: Conventional T cells; Treg: Regulatory T cells.

fewer cells may be required, which would reduce the costs of the manufacturing process. There are several approaches being developed to isolate and induce antigen-specific cells. The most reliable approach is the isolation and expansion of antigen-specific Tregs [93]. The isolation of antigen-specific Treg relies on robust antigen-specific Treg expansion and efficient screening assays. In this regard, Tang *et al.* have developed clinical grade manufacture procedures to expand Tregs from potential transplant recipients against the allogeneic HLA of the donor (NCT02188719). Purified Treg are stimulated *ex vivo* against activated allogeneic B cells (**Figure 3B**) followed by additional expansion using anti-CD3 and anti-CD28-coated beads plus IL-2 [22]. Not only is there a significant expansion (300 – 500-fold) in these cultures but the resulting cells are virtually all reactive against the donor antigen, highly suppressive in a donor-specific manner and can selectively block allogeneic skin allograft rejection in a humanized mouse model [22].

Other approaches are also being developed to generate antigen-specific Tregs. Several groups have used dendritic cells and other APCs to generate induced FoxP3$^+$ Tregs [94] and Tr1 cells [95] against a number of autoantigens, which include both the tissue-specific antigens and also ubiquitous stress-induced antigens such as Hsp70 [96]. These cells have been shown to be efficacious in animal models of autoimmune arthritis and other settings such as IBD [96-98]. In this regard, it is interesting to note that Tregs may recognize gut microbiota. Thus, there is an opportunity to expand Tregs against these commensals for therapeutic application in colitis and other IBD indications [99,100]. In another setting, a group has generated ovalbumin-specific Tregs and injected the cells into the intestine which evidence of efficacy, especially when patients were fed ovalbumin [101]. However, it should be noted that the conversion of antigen-specificTconv to iTreg (**Figure 3C**) [102] is not without some risk as there is increasing evidence that there indicating plasticity of these cells in terms of stability and lineage commitment, especially at sites of inflammation [23].

An alternative approach will be to engineer Treg cells to target specific antigens by expressing antigen-specific

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Table 1. Reported Treg cell therapy in humans.

PI/Institution	Indication	Drug	Dose	Status
Trzonkowski 2009/ Medical University of Gdańsk	GvHD treatment (n = 2+)	Polyclonal expanded Tregs	$0.1^{-3} \times 10^6$/kg	Published [84]
Brunstein/U Minnesota	GvHD prophylaxis (n = 23)	Polyclonal expanded Tregs	$0.1^{-6} \times 10^6$/kg	Published [85]
Martelli 2010/Perugia	GvHD prophylaxis (n = 28)	Polyclonal Tregs	$2^{-4} \times 10^6$/kg	Published [86]
Martelli 2014/Perugia	GvHD prophylaxis (n = 43)	Polyclonal Tregs	2.5×10^6/kg	Published [87]
Edinger/U Regensburg	GvHD prophylaxis (n = 9)	Polyclonal Tregs	?	Reported in a review [88]
Negrin/Stanford	GvHD prophylaxis	Polyclonal Tregs	?	Presented at meetings
Trzonkowski 2012/ Medical University of Gdańsk	T1D, children (n = 12)	Polyclonal expanded Tregs	$10^{-30} \times 10^6$/kg 1 or 2 infusions	Published [89]
Bluestone/UCSF	T1D, adult (n = 14)	Polyclonal expanded Tregs	$5 \times 10^6 – 2.6 \times 10^9$	Enrollment and infusion complete, manuscript in preparation
Salomon/INSERM	Uveitis	Polyclonal stimulated Tregs	?	Presented at meetings
Todo/Hokkaido U	Liver Tx (n = 10)	PBMC stimulated with donor PBMC + CoStim blockade	$0.6 – 2.6 \times 10^9$	Presented at meetings

Tregs: Regulatory T cells.

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TCRs [103] or taking advantage of the growing field of Chimeric Antigen Receptors (**Figure 3D**) [104]. To date, most of the CAR studies are focusing on tumor antigen-specific CTLs for the treatment of human cancers. However, several studies in animals suggest that CAR-expressing Tregs can be efficacious in preventing EAE [105] or colitis [106]. CAR Tregs can be engineered to express antigen receptors to target pathogenic cells in an inflammatory site even if key tissue-specific autoantigens have not been identified. Genetic manipulation, combined with engineering to provide better homing, deliver key molecules involved in tissue healing and regeneration, and a regulated on/off function using molecular switches [107], will increase specificity, efficacy and minimize the potential risk.

10. Expert opinion

Two decades ago, the pharmaceutical industry – long dominated by small-molecule drugs – was revolutionized by the discovery that biological processes can be harnessed to make medical products (biologics). Today, biomedicine sits on the cusp of a new revolution: the use of human cells as versatile therapeutic engines. But history suggests that the advent of cellular medicines will require the development of a foundational cellular engineering science that provides a systematic framework for safely and predictably regulating cellular behaviors.

The last decade has seen an explosion of efforts to understand and exploit one cell type, Tregs, for therapeutic use. On the one hand, there is increasing evidence that the inability to reject tumors or mount an anti-viral response is a direct consequence of Treg function. In fact, recent studies suggest that drugs such as Ipilimumab, which targets the CTLA-4 checkpoint regulator, functions in part by eliminating Tregs localized to tumor sites. Moreover, the successful use of many immunosuppressive drugs depends on their ability to promote Tregs at the expense of T effector cells. Finally, Treg therapy has become a legitimate clinical entity capable of selectively blocking unwanted immune and autoimmune responses and in some cases leading to tolerance induction. However, the excitement in the field must be balanced by the many unanswered questions and requirement for further development.

First, will Treg therapy be cost-effective and safe? There is ample evidence that in the inflammatory setting, especially in autoimmune disease with a genetic predisposition, the stability is key to successful application of Treg technology. This is likely to depend on combination therapies that promote Treg stability. Drugs such as rapamycin and IL-2 may meet this need but it will be important to target these drugs to the Treg. Second, what is the correct antigen specificity needed to effect maximal Treg efficacy? Will the antigenic repertoire of tTregs be appropriate given the potential for antigenic modifications at the site of inflammation? Will the bystander suppression and infectious tolerance, mediated by Tregs, be sufficient to induce tolerance? It has become increasingly clear that the essential activities of Tregs differ based on disease indication and site of action. It will be critical to determine which functions are key for any particular diseases – not unlike the challenges of developing cytokine antagonists such as anti-IL-1 versus anti-TNF. Third, Treg subsets, such as other T cell subsets, are controlled by distinct chemokines, adhesion molecules and cytokines depending on site of action. Will we be able to define populations of cells that migrate to the right site and function appropriately to mediate their tolerogenic effects across different tissues? In order to exploit the use of Tregs to promote tissue remodeling and would healing

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in diseases outside those classically considered immune-mediated, will we need to isolate and expand cells from tissue sites or mimic the phenotypic and function attributes of these cells to mimic their tissue-specific activities and antigenic specificities?

So, how will we meet all these challenges? We believe that to fully realize the potential of Tregs, as well as other regulatory cell therapeutics, it will be essential to genetically modify the cells. With the recent advent of gene editing tools such as TALENs [108] and CRISPR [109], the opportunities can now be realized safely and effectively. We can now direct the antigen specificity as well as other key functional properties of the cells. It is feasible to engineer human Treg cells to express antigen-specific TCR and maintain Treg stability [103]. We can take advantage of the growing field of Chimeric Antigen Receptors (**Figure 3D**) [104]. To date, most of the CAR studies are focusing on tumor antigen-specific CTLs for the treatment of human cancers. However, several studies in animals suggest that CAR-expressing Tregs can be efficacious in preventing EAE [105] or colitis [106]. CAR Tregs can be engineered to express antigen receptors to target an inflammatory site even if key tissue-specific autoantigens have not been identified. Genetic manipulations, combined with engineering to provide better homing, deliver key molecules involved in tissue healing and regeneration, and a regulated on/off function using molecular switches [107], will increase specificity, efficacy and minimize the potential risk.

All this can be accomplished in a robust manner, as Tregs are less likely to have off-target effects because they selectively recognize other cell types, migrate to them, and exert their effect in a target-cell-specific manner. The cells can be engineered to be ‘smart’ able to be turned on or off as needed and able to deliver both the wanted tolerogenic activities but also act as Trojan horses to deliver additional payloads to the target sites to enhance suppressive activities and mediate complimentary activities such as tissue repair and regeneration. Thus, in conclusion, we believe that cell therapy, and specifically Tregs have a promising future as therapeutics.

Declaration of interest

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