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Review

Treg-based immunotherapy for antigen-specific immune suppression and stable tolerance induction: a perspective

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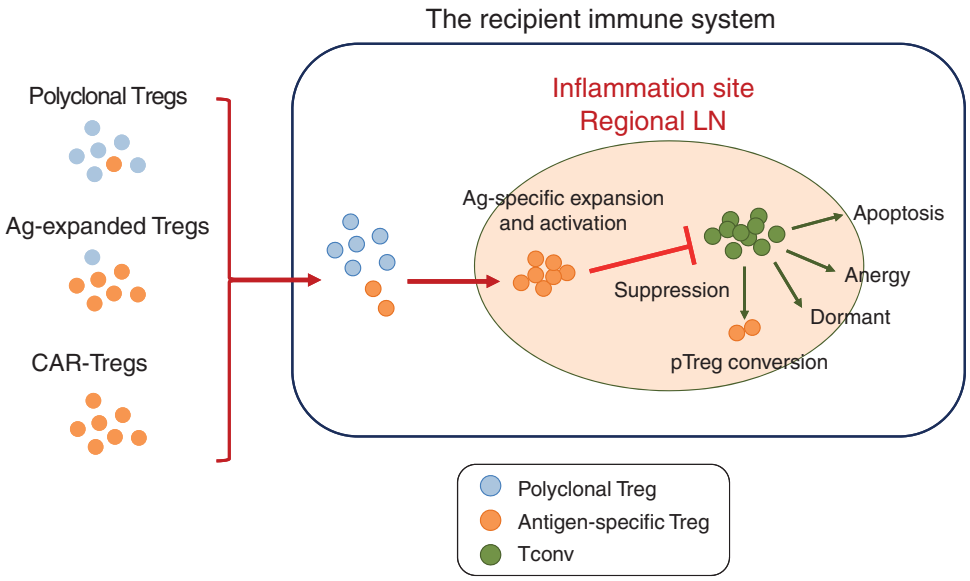
Summary

FoxP3-expressing regulatory T cells (Tregs), whether naturally generated in the immune system or unnaturally induced from conventional T cells (Tconvs) in the laboratory, have much therapeutic value in treating immunological diseases and establishing transplantation tolerance. Natural Tregs (nTregs) can be selectively expanded *in vivo* by administration of low-dose IL-2 or IL-2 muteins for immune suppression. For adoptive Treg cell therapy, nTregs can be expanded *in vitro* by strong antigenic stimulation in the presence of IL-2. Synthetic receptors such as CAR can be expressed in nTregs to equip them with a particular target specificity for suppression. In addition, antigen-specific Tconvs can be converted *in vitro* to functionally stable Treg-like cells by a combination of antigenic stimulation, FoxP3 induction, and establishment of the Treg-type epigenome. This review discusses current and prospective strategies for Treg-based immune suppression and the issues to be resolved for achieving stable antigen-specific immune suppression and tolerance induction in the clinic by targeting Tregs.

Graphical Abstract



Keywords: regulatory T cells, adoptive cell therapy, autoimmune disease, organ transplantation, immune suppression

Abbreviations: ACT: Adoptive cell therapy; APC: Antigen-presenting cell; ATG: Anti-thymocyte globulin; CAR: Chimeric antigen receptor; CDK: Cyclin-dependent kinase; DC: Dendritic cell; EAE: Experimental autoimmune encephalomyelitis; GvHD: Graft-versus-host disease;MBP: Myelin basic protein; MSC: Mesenchymal stem cell; mTOR: Mammalian target of rapamycin; iTreg: In vitro–induced Treg; nTreg: Natural Treg; pTreg: Peripherally derived Treg; Tconv: Conventional T cell; Treg: Regulatory T cell; T1D: Type 1 diabetes

Introduction

Naturally occurring regulatory T cells (nTregs), which specifically and constitutively express the transcription factor FoxP3 in the nucleus and CD25 (IL-2 receptor α-chain) and CTLA-4 on the cell surface, are a unique $CD4^+$ T cell subpopulation specialized for immune suppression [1]. Their indispensable

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role for the maintenance of immunological self-tolerance and homeostasis is best illustrated by spontaneous development of a variety of autoimmune and inflammatory diseases when $CD25^+CD4^+$ T cells are removed from normal rodents at any time of life [2], and by FoxP3 gene mutations that result in congenital Treg cell deficiency in humans and rodents [3–5]. Moreover, adoptive transfer of nTregs is able to achieve long-term inhibition of autoimmune disease development in various animal models [1]. These basic findings have prompted recent attempts to treat chronic autoimmune/inflammatory disorders and establish transplantation tolerance by targeting Tregs.

The majority of nTregs are produced by the thymus as a functionally distinct and mature population (thymus-derived Tregs [tTregs]), forming a functionally stable cell lineage from the thymus to the periphery. Immunosuppressive Tregs can also be physiologically generated from Tconvs under certain conditions in the periphery (peripherally derived Tregs [pTregs]), especially in the intestinal mucosa, by stimulation with commensal microbes or diet proteins in the presence of tissue TGF-β, retinoic acid, and other FoxP3-inducing substances (e.g. short-chain fatty acids). It is difficult at present to distinguish between tTregs and pTregs by reliable molecular markers; hence both are still termed as nTregs. $FoxP3^+$Tregs can also be induced from Tconvs *in vitro*, for example, by antigenic stimulation in the presence of TGF-β and IL-2 (induced Tregs [iTregs]) [6, 7]. Since $FoxP3^+$ nTregs and iTregs possess antigen-specificities, they can be used as a 'living drug' with target specificity to circumvent clinical problems such as increased vulnerability to opportunistic infections associated with current antigen-non-specific immunosuppressive drugs that mainly deal with disease-mediating Tconvs.

In addition to $FoxP3^+$ Tregs, $FoxP3^-$ T cells with an immunosuppressive activity can be induced from antigen-specific Tconvs. For example, $CD4^+$ T cells antigen-stimulated *in vitro* in the presence of high-dose IL-10 are able to differentiate into IL-10/TGF-β-producing $CD4^+$ T cells called Tr1 cells [8]. IL-10-producing B cells can also exert immune suppression [9]. It remains to be determined what degree of functional specialization and stability such $FoxP3^-$ suppressive T and B cells would possess in long-term immune suppression and whether they could be functionally synergistic with $FoxP3^+$ Tregs, which are the main focus of this review.

In this article, we discuss how $FoxP3^+$ Tregs including nTregs and iTregs can be utilized to treat immunological diseases and avert rejection of organ transplants for stable establishment of antigen-specific immune tolerance, which is the Holy Grail of clinical immune suppression.

Key immunological features of Tregs as a target of immune suppression

Tregs, in particular nTregs, possess unique immunological features, as summarized below, that distinguish them from Tconvs, hence enabling differential control of nTregs and Tconvs toward dominance of the former over the latter for immune suppression. The features can be exploited *in vivo* and *in vitro* for polyclonal or antigen-specific expansion of nTregs and for *de novo* generation of pTregs and iTregs from antigen-specific Tconvs mediating physiological or pathological immune responses.

Development, TCR repertoire, and functional state of nTregs

Developing tTregs positively selected by self-peptide/MHC ligands differentiate into a functionally distinct and mature T-cell subpopulation that is partially 'antigen-primed' state (e.g. high expression of T-cell accessory molecules) within the thymus, contrasting with thymic production of Tconvs as an antigen-non-primed naive T cell population.

tTregs possess a TCR repertoire that is as broad as that of Tconvs but skewed, to some extent, toward recognizing thymic self-peptide/MHC ligands more strongly than Tconvs selected by the same ligands [10–12]. Given that pTregs are induced in the periphery by non-self-antigens, nTregs (tTregs and pTregs) could recognize *in toto* a broad spectrum of self- and non-self antigens.

In the periphery, nTregs can adaptively change their clonal composition of the TCR repertoire to a substantial degree in response to antigen exposure. For example, mice having recovered from experimental autoimmune encephalomyelitis (EAE) induced by myelin basic protein (MBP) immunization become resistant to reinduction of EAE apparently via an expansion of MBP-specific nTregs [13]. Similar 'memory'-like Treg expansion has been implicated in antimicrobial host defense and maternal-fetal tolerance in rodents [14]. In humans, TCR repertoire comparison between naive and effector Tregs in the peripheral blood revealed clonal differentiation and expansion [15]. A sizable fraction of effector type nTregs are indeed actively proliferating presumably by recognizing self-antigens and possibly non-self-antigens derived from commensal microbes [15, 16].

Thus, the broad TCR repertoire and the semi-antigen-primed state, together with high IL-2 sensitivity (discussed below), enable nTregs to be readily activated upon antigen stimulation in the periphery to exert dominant suppression over Tconvs with various antigen specificities.

Treg expansion and survival

As cardinal features of Tregs, they do not produce IL-2 because of FoxP3-dependent gene repression especially upon TCR stimulation, while they constitutively express the high affinity IL-2 receptor composed of α, β, and γ chains from the stage of their production in the thymus, maintaining the expression in the periphery [17, 18]. They are therefore highly dependent on exogenous IL-2 for their expansion and survival, as illustrated by the finding that IL-2 neutralization selectively reduces actively proliferating, presumably self-reactive Tregs in the thymus and the periphery, thereby eliciting autoimmune diseases similar to those inflicted by Treg depletion [17]. In addition, IL-2 absorption by Tregs from the surroundings via the high affinity IL-2 receptor ('cytokine sink'), together with no IL-2 production by activated Tregs, hence scarcely providing IL-2 to responder Tconvs, is fundamental to Treg-mediated suppression, at least *in vitro*, as illustrated by abrogation of *in vitro* suppression by addition of IL-2 [19, 20] (see also below). Tregs are also highly IL-2 sensitive in their activation as indicated by their intrinsic activation of STAT5-dependent signaling pathways (e.g. higher STAT5 phosphorylation than Tconvs in physiological states) [21, 22]. They are therefore able to quickly sense IL-2 produced by antigen-reactive Tconvs in an early stage of an immune response and co-localize with them to suppress their further activation in a dominant manner [23, 24].

Furthermore, during active immune responses, IL-2 produced by activated Tconvs expands Tregs, stabilizes FoxP3 expression, and enhances their suppressive activity to suppress further immune responses in a negative feedback fashion [25].

It is also of note that, in contrast with *in vivo* proliferation of effector-type Tregs in the physiological state, *in vitro* TCR stimulation activates Tregs and evokes suppressive activity, but does not trigger their proliferation [19, 20]. However, proliferation can be elicited in Tregs by strong antigenic stimulation in the presence of high dose IL-2, potent co-stimulation via CD28 or TNFRSF molecules such as GITR and TNFR2 [26–29]. For example, TNFR2 is selectively expressed by nTregs; and anti-TNFR2 agonistic antibody can expand nTregs *in vivo* and *in vitro* in rodents [28]. In addition, Treg proliferation is enhanced by augmentation of TCR signaling by targeting TCR-proximal signaling molecules that are transcriptionally repressed by FoxP3 upon TCR stimulation [30]. Distinctive metabolic features of Tregs, such as a high anabolic state *in vivo*, may also allow differential control of Tregs as shown by mTOR inhibition with rapamycin [31].

Multiple mechanisms of Treg-mediated suppression

Tregs utilize cell-contact-dependent and humoral factor-mediated mechanisms of suppression [1, 32–34] (**Fig. 1**). Among them, CTLA-4-dependent one is highly important because Treg-specific CTLA-4 deficiency produces autoimmune/inflammatory diseases similar to those induced by Treg deficiency [35]. Both Tregs and Tconvs have different manners of controlling CTLA-4 gene expression; i.e. constitutive in the former and activation-induced temporal expression in the latter [36]. Upon antigenic stimulation, Tregs further up-regulate the expression of CTLA-4, which not only competes with CD28 for binding to CD80/CD86 but also down-regulates the expression of CD80/CD86 on antigen-presenting cells (APCs) via trogocytosis and subsequent transendocytosis of CTLA-4-bound CD80/CD86 to Treg cell membrane or cytoplasm, thereby depriving responder Tconvs of CD28 co-stimulatory signal [23, 35, 37, 38]. In addition, CD80 down-regulation dissociates PD-L1 from cis-bound CD80, increasing free-PD-L1 on APCs to suppress PD-1-expressing effector Tconvs as well [38]. Thus, Tregs can determine cell fate of responder Tconvs, depending on the degree of the reduction of co-stimulatory signals on APCs and on the TCR affinity of the responder Tconvs for the antigen; i.e. when APCs presenting a self-peptide/MHC are subjected to Treg-mediated CD80/CD86 down-regulation, self-reactive Tconvs with high affinity TCRs for the self-antigen-peptide/MHC die by apoptosis, those with intermediate affinities are driven to anergy, while those with low TCR affinities remain dormant [39]. Moreover, such Treg-induced CD80/CD86^{low} tolerogenic DCs, which can produce TGF-β, may promote pTreg generation from naive Tconvs [40].

Tregs possess other suppressive mechanisms as well; for example, extracellular adenosine production catalyzed by cell surface CD39/CD73, deprivation of IL-2 from responder T cells (see above), production and activation of immune-suppressive cytokines such as IL-10, TGF-β, and IL-35, and secretion or transduction of intracellular molecules such as granzyme B, cyclic AMP, and IDO [32, 33]. Each mechanism contributes to specific aspects of immune suppression to various degrees, synergistically, and in a tissue-specific manner, although abrogation of each alone does not completely break self-tolerance. For example, IL-10-deficient Tregs only cause colitis but not systemic autoimmune disease, suggesting that Treg-produced IL-10 is required for mucosal homeostasis [41]. In addition, the sensitivity of responder Tconvs to such Treg-mediated suppression may depend on their activation and differentiation status and on the cytokine milieu. Naive Tconvs at the stage of antigen activation by APCs can be more effectively suppressed than activated Tconvs exerting effector functions. Inflammatory cytokines such as TNFα and IL-6 can render responder Tconvs resistant to the suppression via activating protein kinase B/c-akt [42].

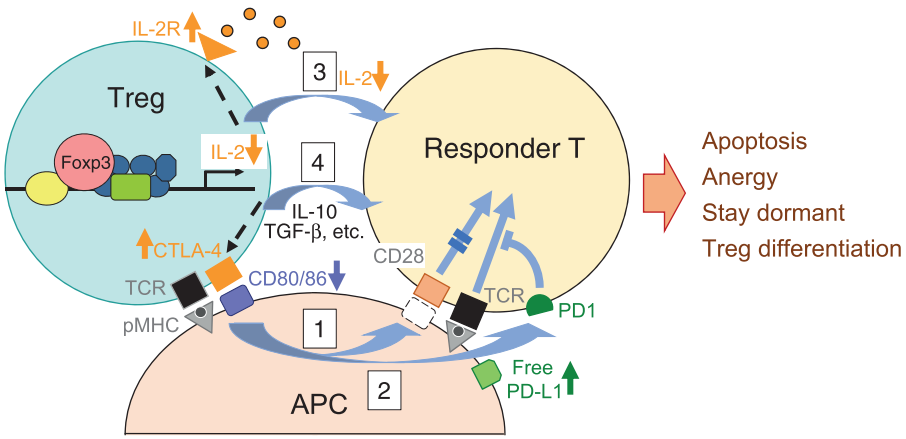

Figure 1. Treg-mediated suppressive mechanisms and cell fates of responder T cells subjected to suppression. Upon activation by antigen recognition, Tregs downregulate the expression of CD80/86 on antigen presenting cells (APCs) via CTLA-4, thereby reducing the availability of co-stimulation molecules for responder T cells [1]. CD80 down-regulation dissociates CD80-bound PD-L1, increasing free PD-L1 that interacts with PD-1-expressing effector T cells [2]. Tregs absorb IL-2 from the surroundings via their expression of the high-affinity IL-2 receptor, depriving IL-2 from responder T cells [3]. In addition, Tregs secrete immunosuppressive cytokines such as IL-10, TGF-β, and IL-35, and also catalyze adenosine production via CD39/CD73. These soluble factors can inhibit activation/maturation of APCs and activation of responder T cells [4]. In the presence of Tregs downregulating CD80/86 expression by APCs, Tconvs with high affinity TCRs for an antigen-peptide/MHC die by apoptosis, those with intermediate affinity TCRs are driven to anergy, and those with low affinity TCRs stay dormant as naive Tconvs. Some of the Tconvs under Treg suppression may be driven to differentiate into Tregs. pMHC, peptide/MHC complex

Treg-mediated modulation of APCs described above, together with Treg production of immunosuppressive cytokines and soluble factors, may exert 'bystander suppression'; i.e. Tregs can suppress not only Tconvs recognizing the same antigen as seen by the Tregs but also other Tconvs recognizing other antigens presented on the same APC or adjacent APCs. That is, given that a Treg needs to be activated by a specific cognate antigen to exert suppression, the afferent phase of Treg suppression (i.e. Treg activation) is antigen-specific whereas the efferent phase (i.e. exertion of suppression) is not antigen-specific. This may form the basis of 'linked suppression' in organ transplantation and enable a Treg with a particular self-antigen specificity to suppress autoimmune Tconvs with other antigen specificities as well in Treg-mediated treatment of autoimmune disease.

Functional stability and adaptability of Tregs

nTregs are functionally stable as illustrated by long-term suppression of autoimmune disease in animal models by adoptive nTreg transfer. The Treg-specific transcription factor network and the Treg-specific epigenetic landscape essentially contribute to Treg-specific stable gene expression, hence their stable maintenance of Treg function and cell lineage continuity. Some nTregs might, however, lose FoxP3 expression and secrete inflammatory cytokines under certain conditions, typically in a lymphopenic environment with IL-2 paucity [43]. In contrast with the high functional stability of nTregs in general, iTregs are functionally unstable mainly because they lack Treg-specific epigenetic changes, in particular Treg-specific DNA hypomethylation, at enhancer regions of *Foxp3* and other Treg signature genes [44, 45]. This has prompted efforts to install nTreg-like epigenome in iTregs for their therapeutic use, as discussed later.

Tregs not only recognize specific antigens but also sense specific inflammatory cues to adaptively control a particular inflammation [46]. For example, Tregs in a type 1 inflammation site express T-bet and CXCR3, those in a type 2 inflammation express GATA-3 and CCR4/CCR8, and those in a type 3 inflammation RORγt and CCR6, as expressed by Th1, Th2, and Th17 cells, respectively. Similarly, T-follicular helper (Tfh) and T-follicular regulatory (Tfr) cells express Bcl-6 and CXCR5. Thus, the expression of chemokine receptors dictated by Th-specific transcription factors, but lack of helper function due to FoxP3-dependent repression of the transcription of Th-specific inflammatory cytokines, may allow Tregs to traffic to the site of a specific type of inflammation and suppress the corresponding effector Tconvs.

In addition, Tregs are not only immunosuppressive but also actively facilitate tissue repair and regeneration, for example, by producing amphiregulin and other growth factors and by promoting tissue stem cell differentiation and proliferation [47].

In vivo and *in vitro* targeting of nTregs for immune suppression

By exploiting the high functionality and stability of nTregs discussed above, they can be simply expanded *in vivo* for immune suppression, used for Treg-based adoptive cell therapy (ACT) after *in vitro* expansion, and also made to express engineered receptors to confer target specificity in ACT (**Table 1**).

Table 1. Immunological features of nTreg, iTreg, and CAR-Treg cells

	nTreg	iTreg	CAR-Treg
Cell source	Thymocytes/Peripheral Tconvs (*in vivo*)	Naïve Tconvs (*in vitro*)	nTregs (*in vitro*)
Treatment	None	TCR stim, IL-2, TGF-β	CAR transduction
Treg-type epigenome	++	+/-	++
Functional stability	++	+/-	++
Antigen Specificity	Self/non-self-Ag	Any Ag	Specified Ag
Expansion (in vitro)	+	++	+
Survival (after in vivo transfer)	++	+	?

Symbols (++, +, +/-, -) indicate the levels of the establishment or efficacy of each parameter.

In vivo nTreg expansion, enrichment, and functional enhancement

Because Tregs constitutively express the high affinity IL-2 receptor, and do not produce IL-2 themselves, IL-2 administration at a low dose is able to selectively expand Tregs while avoiding activation/expansion of Tconvs and side effects of IL-2 itself [48, 49]. Low-dose IL-2 also induces anti-apoptotic Bcl2 expression in Tregs and enhances their suppressive function [50]. To achieve IL-2-dependent selective expansion of nTregs, efforts have also been made to modify the IL-2 molecule to bear a stronger affinity for IL-2R α-chain [51], generate an anti-IL-2 mAb that changes the IL-2 conformation in binding to the IL-2R [52], retain IL-2 longer in the circulation [53], and prepare IL-2/anti-IL-2 mAb complexes to modify IL-2 binding to the IL-2R [54, 55]. In addition, inhibition of Tconv activation/expansion, for example, by co-stimulation blockade with CTLA-4Ig, may allow more selective Treg expansion by modified or unmodified IL-2 [56]. A combination of low-dose IL-2 and rapamycin, which prevents T and B cell activation by inhibiting the mTOR pathway, can also be synergistic in nTreg expansion [57].

Besides various means to expand nTregs, nTregs can be relatively enriched *in vivo* by reducing effector Tconvs, for example, by cell-depleting anti-CD3 mAb, anti-CD45RC mAb, and thymoglobulin (rabbit anti-thymocyte globulin) [58–61].

Thus, Treg dominance over Tconvs for immune suppression can be achieved by targeting Tregs, Tconvs, or both. Considering that Treg suppression is more effective at the initial step of naive Tconv activation by APCs rather than inhibiting ongoing effector functions of activated Tconvs, IL-2-dependent Treg expansion could be more effective when combined with specific reduction of activated Tconvs by some immunosuppressants currently in clinical use. This also needs to be taken into account in Treg ACT discussed below.

In vitro polyclonal or antigen-specific expansion of nTregs for Treg ACT

For nTreg ACT, nTregs purified from patient's blood, or third-party cord blood, can be polyclonally expanded *in vitro* by anti-CD3/CD28 stimulation, or enriched for allo-antigen-specific Tregs by stimulation with allogeneic

stimulator cells, in the presence of high-dose IL-2 [62]. Phase 1 trials of such autologous nTreg cell therapy have already demonstrated that this approach is safe in patients with T1D and graft-versus-host disease (GvHD) after allogeneic hematopoietic stem cells transplantation, and has not accompanied serious non-specific immune suppression [63–66]. The current key issues for developing better nTreg ACT concern the antigen specificity, functional stability, and survivability of Tregs especially after *in vivo* transfer. It has been shown with various models of organ transplantation that allo-antigen-specific Tregs enriched by *in vitro* allo-antigen stimulation are superior to polyclonal Tregs prepared by anti-CD3/CD28 stimulation in preventing graft rejection [67, 68]. In contrast, when nTregs prepared from the HSC donor is used for prevention of GvHD, transferred Tregs would easily and selectively expand via recognizing the ubiquitously expressed recipient histocompatibility antigens, without need for expanding alloantigen-specific Tregs prior to cell transfer.

Upon *in vitro* TCR stimulation of human nTregs, naive type ($CD45RA^{hi}CD25^{lo}FoxP3^{lo}$) nTregs immediately differentiate into effector type ($CD45RA^{lo}CD25^{hi}FoxP3^{hi}$) Tregs and proliferate [15]. The latter are Bcl-2^{lo} and prone to die by apoptosis upon TCR stimulation [69, 70]. A sizable fraction of such *in vitro* activated/expanded Tregs may therefore die soon after *in vivo* transfer mainly because of their translocation from an IL-2^{high} *in vitro* condition to the IL-2^{low} *in vivo* environment [71]. This might explain why transfer of a large number of nTregs generally does not incur general immunosuppression immediately after cell transfer. Notwithstanding the high apoptosis-sensitivity of activated effector-type nTregs, transferred Tregs were detected in the circulation one year later in Treg ACT of T1D [66].

As the cell source of Treg ACT, especially for treatment of GvHD, it needs to be determined which Treg preparation, Tregs prepared from the blood of an adult HSC donor or those from cord blood partially HLA-matched with the HSC donor and the recipient, is therapeutically more effective [63, 64]. Cord blood is abundant in easily expandable naïve-type Tregs [15]. It also remains to be determined in Treg ACT whether infused nTregs might somehow suppress *de novo* development of endogenous tTregs and pTregs in a negative feedback manner (thereby hindering possible 'infectious tolerance' discussed later), as effector Tregs can suppress their further generation from naive Tregs *in vitro* in humans and similarly *in vivo* in mice [15, 72, 73].

ACT with receptor-engineered Tregs

Another approach to effectively and efficiently generate antigen- or disease-specific Tregs is to engineer nTregs to transgenically express a particular TCR or a CAR, an artificial receptor composed of the antigen-binding region of an antibody and capable of transducing a strong activation signal. Recent studies have demonstrated that nTregs transduced with an alloantigen-reactive TCR-CAR or single-chain Fv-CAR are more efficient than control Tregs in extending skin graft survival and suppressing GvHD in animal models [74–76]. Luciferase-based *in vivo* imaging has indeed shown that CAR-transduced Tregs rapidly accumulated at the targeted tissue, such as a skin graft [76]. Of note is that in CD19-CAR-T therapy of lymphoma, nTregs inadvertently transduced with the CD19-CAR-T construct suppressed $CD8^+$ CAR-T cells to attack tumor cells, unexpectedly providing evidence that CAR-Tregs are feasible for immune suppression in humans [77, 78].

An advantage of CAR-Tregs over nTregs is that the former can be prepared by expressing a single CAR construct in any nTregs and, unlike TCR, target recognition by CAR-Treg is not MHC-restricted. On the other hand, antibody Fab portion of the CAR construct limits target molecule recognition to a single one expressed on the cell surface of target cells. Efforts have been made therefore to guide nTregs efficiently to any inflammation sites, for example, by expressing CAR constructs recognizing cytokines such as TNF-α abundant in inflammation milieu [79].

Advances in the technology would also enable preparing 'Off-the-Shelf' CAR-Treg cell therapeutics by using third-party Tregs or pluripotent stem cell derived Tregs. In addition, combining transgenic expression of an engineered receptor and FoxP3 together would be able to convert antigen-specific Tconvs to Treg-like cells with target specificity and potent suppressive activity.

De novo generation of antigen-specific Tregs *in vivo* and *in vitro*

With physiological pTreg development, in particular in mucosal tissues, attempts have been made to generate antigen-specific pTregs *de novo* and also to prepare iTregs that are as functionally competent and stable as nTregs and can be expanded in an antigen-specific fashion more efficiently than nTregs.

pTreg generation *in vivo*

It has been shown that pTregs can be induced experimentally by antigen presentation on $CD80/CD86^{low}$ tolerogenic dendritic cells (DCs). For example, administration of antigen-conjugated mAb specific for CD205 (DEC205) expressed by immature DCs have the DCs present the antigen to $CD4^+$ Tconvs and drive their differentiation into stable pTregs [80, 81]. The treatment with self-peptide-DEC205 mAb protected autoimmune disease development in animal models [82, 83]. As another approach, a mRNA-based vaccine encoding an encephalitogenic MOG (myelin oligodendrocyte glycoprotein)-peptide and incorporating 1-methylpseudouridine in place of uridine, hence lower innate immune activation via TLR7, was delivered to mice in a non-inflammatory lipoplex carrier; the treatment induced MOG-specific pTregs via induction of tolerogenic DCs and thereby suppressed EAE development in a bystander manner when immunized with MOG or other encephalitogenic peptides [84]. Tolerogenic DCs can also be generated *in vitro* from human peripheral blood mononuclear cells in the presence of GM-CSF, IL-4, and NF-kB inhibitors and pulsed with antigen peptides; intradermal injection of the DCs to patients resulted in an increase in Tregs [85]. In addition, oral, nasal, or intradermal administration of antigen peptides to induce T-cell anergy via tolerogenic DCs is able to generate $FoxP3^+$ pTregs and $FoxP3^-$IL-10-producing cells (Tr1 cells) [86, 87]. It remains to be determined in humans whether peptide drugs such as glatiramer acetate (Copaxone), a mixture of synthetic polypeptides resembling MBP for the control of relapsing multiple sclerosis, involves pTreg generation for its clinical effects [88].

Similar to tolerogenic DCs, mesenchymal stem cells (MSCs), which have been used as a regenerative therapy of various diseases, appear to control inflammation and promote tissue regeneration at least in part by generating Tregs [89–91]. For

example, MSCs can induce pTregs *de novo* apparently via TGF-β or PGE2 secretion [89, 90], and are reportedly able to enhance Treg-specific DNA hypomethylation in a cell-contact dependent manner [91].

Another attempt for generating antigen-specific pTregs *in vivo* is to convert antigen-specific Tconvs, especially memory/effector type T cells mediating an autoimmune disease, into Tregs. A chemical inhibitor of Cyclin-dependent kinases (CDK) 8/19, the regulatory modules of the mediator complex, is able to generate antigen-specific pTregs in mice. The inhibitor can induce FoxP3 only in antigen-stimulated Tconvs but not in non-stimulated ones; pTregs thus induced possess Treg-type epigenome, hence are functionally stable [92] (see below).

iTregs for ACT

iTregs can be generated *in vitro* by antigenic stimulation of Tconvs in the presence of TGF-β and IL-2 [6, 7]. An advantage of such iTregs over nTregs is the convenience of *in vitro* preparation of a large number of antigen-specific iTregs from $CD4^+$ Tconvs. A disadvantage of iTregs, on the other hand, is their functional instability [93]. In contrast to nTregs, FoxP3 expression in iTregs is unstable mainly because of incomplete epigenetic changes at Treg-specific demethylated regions (Treg-DRs), resulting in their frequent reconversion to effector Tconvs after *in vivo* transfer [44, 45]. It has therefore been a key issue to have iTregs acquire Treg-type epigenetic changes. For example, ascorbate (Vitamin C), a cofactor for Tet (ten eleven translocation) enzyme mediating DNA demethylation, can facilitate *de novo* hypomethylation of *Foxp3* CNS2 in iTregs, enhancing their stability of FoxP3 expression and suppressive activity to prevent mouse GvHD [94, 95]. Ascorbate, however, cannot induce sufficient DNA demethylation in human iTregs [90]. Strong TCR signal activation is a requirement for tTreg development [96], and can induce hypomethylation in iTregs to a certain extent [97]. In addition, iTregs generated by stimulation with allogeneic thymic stromal cells acquire Treg-DR epigenetic changes and efficiently suppress skin allograft rejection [98]. As a possible common mechanism of these findings, a recent study has shown that deprivation of CD28 co-stimulatory signal at an early stage of iTreg generation is able to establish Treg-DR hypomethylation at Treg signature genes including FoxP3 in effector/memory as well as naive Tconvs, facilitating the generation of functionally stable iTregs [99].

Another issue regarding iTreg cell therapy is Treg conversion efficiency. Although TGF-β is widely used to induce FoxP3 in Tconvs, TGF-β-dependent FoxP3 induction is antagonized by proinflammatory cytokines such as IL-4 or IL-6, which, together with TGF-β, promotes IL-9-producing Th9 or IL-17-producing Th17 cells, respectively. Several FoxP3 inducing substances such as rapamycin, retinoic acid and butyrate have been reported; however, all these small molecules require TGF-β for initial FoxP3 induction *in vitro* and the effect is limited under inflammatory conditions [100–102].

By addressing how to induce antigen-specific iTregs in a TGF-β-independent manner even in the presence of proinflammatory cytokines, a recent study has shown that CDK8/19 inhibitors are able to induce FoxP3 in antigen-stimulated effector as well as naïve Tconvs, *in vitro* and *in vivo* (see above), in mouse and human Tconvs, in a proinflammatory cytokine abundant *in vitro* environment, and by a TGF-β independent and STAT5 dependent mechanism [92]. Combinatory use of TGF-β and CDK8/19 inhibitors synergistically up-regulated FoxP3 expression via activating the TGF-β/SMAD pathway and abrogating the CDK8/19-dependent inhibition of STAT5 activation. Since iTregs induced by TGF-β/IL-2 or CDK8/19 inhibition do not possess Treg-DR hypomethylation, a combination with deprivation of CD28 co-stimulation in iTreg induction is likely able to generate functionally stable iTregs for clinical use [92, 99]. Further, genome editing by CRISPR/Cas9-mediated gene deletion could modify the expression of Treg functional molecules advantageous or disadvantageous for iTreg generation and function; for example, deletion of Fas receptor to prevent Treg apoptosis by targeting the gene in Tconvs before Treg induction [103].

It is thus envisaged that antigen-specific iTregs would be generated for clinical use with further improvement in their functionality and stability as well as their therapeutic effectiveness to match and even surpass nTregs.

Perspectives for Treg-based immune suppression and tolerance induction

The equilibrium, not necessarily numbers, between antigen-specific Tregs and Tconvs, with the former stably suppressing the latter, is the hallmark goal of establishing dominant immune tolerance to treat autoimmune disease and avert transplant rejection. For this aim, one ought to ask not only how antigen-specific tolerance can be induced locally at the target tissue or transplanted organ, but also how it can be maintained stably and even strengthened.

Establishment of Treg dominance by antigen-specific or polyclonal Treg immunotherapy

In vivo antigen-specific expansion of nTregs and ACT with antigen-specific nTregs or iTregs appear to be more efficacious than polyclonal expansion of nTregs or iTregs (Fig. 2). It is difficult, however, in autoimmune disease settings to enrich self-antigen-specific Tregs by *in vitro* antigen stimulation presumably because the Tconvs reactive against a particular self-antigen is very small in clone size and self-antigens are generally weak in antigenicity. Besides, target self-antigens are not always known and may be multiple in many autoimmune diseases. Donor cells for *in vitro* allogeneic stimulation are not always available for preparation of allo-specific Tregs for controlling organ rejection by Treg ACT. It thus needs to be determined whether self-antigen-specific or disease-responding Tregs that are presumed to be present in the blood of an autoimmune disease patient can be expanded to some extent by *in vitro* polyclonal stimulation and, for that matter, whether a nTreg isolated from the inflammation site or the draining lymph nodes are better sources for enriching putative self-antigen- or disease-specific Tregs. It also remains to be determined in polyclonal Treg ACT whether such specific Treg clones included in transferred Tregs can be stimulated at inflammation sites and able to expand more readily and become dominant over unstimulated Tregs at the sites, thus attaining stable antigen-specific dominant suppression in the long term. Further, assuming that an ideal way of antigen-specific immune suppression is not only to eliminate antigen-specific effector Tconvs but also to convert them to antigen-specific Tregs, further investigation is required into how activated Tconvs that exist among disease-mediating antigen-specific effector/memory Tconvs can be selectively converted to

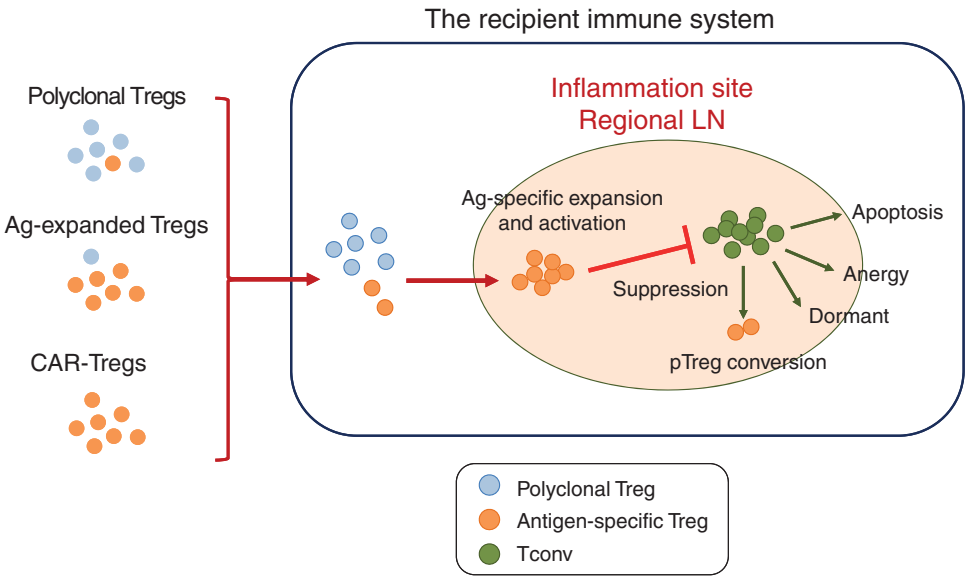

Figure 2. Antigen-specific or polyclonal Treg ACT. In polyclonal or antigen-specific Treg ACTs, whether with nTregs, iTregs, or CAR-Tregs, antigen-specific Tregs expand with antigenic stimulation in the recipient while non-stimulated Tregs in polyclonal Treg ACT may stay dormant or die by apoptosis. Antigen-stimulated Tregs suppress Tconvs and determine their cell fate as shown in Figure 1.

pTregs or iTregs by pharmacological reagents (e.g. CDK8/19 inhibitors) even when target antigens are not known and, after conversion, how they can be maintained to exert stable suppressive function, for example, by installing in them Treg type epigenome.

Local establishment of immune tolerance

Treg-dependent graft tolerance mainly depends on on-site suppression of allo-antigen-reactive effector Tconvs by allo-antigen-specific Tregs recruited to the graft. For example, in mice with Treg-induced long-term stable allo-graft tolerance, some, but not all, rejected the secondary graft from the same mouse strain shortly after transplantation while stably retaining the primary graft [71]. This implies that graft-specific effector T cells can persist in the graft recipient in tolerance and that a stable local balance is established between Tregs and effector Tconvs at the site of the primary graft, but needs to be newly established at the secondary graft site where local tolerance is unstable until reestablishment of the Treg-dominant balance. In addition, transfer of the graft itself that had attained such local Treg dominance was more efficient than transfer of lymph node T cells, both from stably tolerant mice, in adoptive tolerance induction in other mice [104]. With these findings in organ transplantation, it is likely that for Treg-based immunotherapy to succeed in treating organ-specific autoimmune diseases, such as T1D, local Treg dominance over autoimmune Tconvs is essential for re-establishing self-tolerance. Monitoring the local balance between Tregs and effector Tconvs may help in assessing the state of immune self-tolerance.

Stabilization and augmentation of tolerance: 'Infectious tolerance'

Tregs can induce CD80/CD86^{low} tolerogenic DCs by CTLA-4-dependent CD80/CD86 down-modulation and presumably by IL-10/TGF-β-dependent inhibition of DC maturation. Such tolerogenic DCs may in turn drive antigen-specific Tconvs to differentiate into pTregs at the regional lymph nodes of autoimmune inflammation or organ transplantation (**Fig. 1**). This sequence of events, called 'infectious tolerance' that was first demonstrated in organ transplantation [105], may stabilize and augment the process of establishing local immune tolerance and can be extended to self-tolerance as well. It remains to be determined whether ACT with nTregs or iTregs can generate tolerogenic DCs and thereby facilitate local pTreg generation to establish 'infectious tolerance' in the recipient.

Conclusion

The ultimate goal of Treg-based immunotherapy for autoimmune disease is to reestablish stable self-tolerance and, for organ transplantation, to establish graft tolerance as stable as self-tolerance. With better understanding of the development and function of Tregs at the cellular and molecular levels, it is possible that nTregs can be expanded, and functionally stable pTregs as well as iTregs be generated, more efficiently and in an antigen-specific manner for their therapeutic use.

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Conflict of interest

The authors declare no conflict of interest.

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