

Mechanisms of allergic diseases

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Therapeutic approaches to allergy and autoimmunity based on FoxP3⁺ regulatory T-cell activation and expansion

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Activity Objectives

1. To review how natural and induced human FoxP3⁺ regulatory T (Treg) cells can be invoked in the prevention and treatment of immunologic diseases.

2. To discuss some of the current limitations to therapeutic interventions using Treg cells and propose additional areas of research that could help overcome these problems.

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Forkhead box protein 3–positive regulatory T (Treg) cells are indispensable for the maintenance of self-tolerance and immune homeostasis. They can also be exploited for the treatment of immunologic diseases, including autoimmune diseases and allergy, by way of activating and expanding antigen-specific Treg cells *in vivo*. Cell therapy with *in vitro* activated and expanded Treg cells can be another therapeutic modality. The feasibility of such Treg cell–based therapeutic strategies is

discussed based on recent advances in our understanding of the molecular and cellular basis of Treg cell development and function. (J Allergy Clin Immunol 2009;123:749-55.)

Key words: *FoxP3, regulatory T cell, activation*

A subset of CD4⁺ T cells called natural suppressor/regulatory T (Treg) cells, the majority of which are produced by the thymus as a functionally distinct T-cell subpopulation and characterized as constitutively expressing the transcription factor forkhead box protein 3 (FoxP3), is essential for the maintenance of self-tolerance and immune homeostasis.¹ Illustrating this, FoxP3-mutant or FoxP3-deficient mice spontaneously experience fatal systemic inflammatory/autoimmune syndrome and hyperproduction of IgE.²⁻⁶ Similarly, in human subjects mutations of the FoxP3 gene are responsible for immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX),^{7,8} which is characterized by a wide spectrum of autoimmune diseases, especially type 1 diabetes (T1D), severe allergy (including food allergy), and inflammatory bowel disease. With this unequivocal evidence in human subjects and mice that anomaly of FoxP3⁺ natural Treg cells can cause autoimmune disease, allergy, and inflammatory bowel disease, a key issue is how Treg cells can be exploited for the treatment and prevention of these immunologic diseases.

In addition to thymus-derived FoxP3⁺ natural Treg cell, conventional T cells can also acquire Treg cell phenotype and function in the periphery.⁹ Such induced or adaptive Treg cells, which

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Terms in boldface and italics are defined in the glossary on page 750.

Abbreviations used

APC: Antigen-presenting cell
 CTLA-4: Cytotoxic T lymphocyte-associated antigen 4
 FoxP3: Forkhead box protein 3
 SIT: Specific immunotherapy
 TCR: T-cell receptor
 T1D: Type 1 diabetes
 Treg: Regulatory T

include FoxP3⁺ Treg cells induced in the periphery from naive T cells, IL-10-secreting *T_H1* cells, and *TGF-β*-secreting *T_H3* cells, have been shown, at least in mice, to be capable of preventing autoimmunity.⁹⁻¹¹ It remains uncertain, however, to what extent they contribute to physiologic maintenance of self-tolerance and immune homeostasis. Yet induced Treg cells have the potential for therapeutic use because they allow the preparation of antigen-specific Treg cells from naive T cells.

In this review we focus on human FoxP3⁺ Treg cells, natural or induced, and discuss how they can be exploited for the prevention and treatment of immunologic diseases, in particular autoimmune disease and allergy.

BASIC IMMUNOLOGIC PROPERTIES OF NATURAL Treg CELLS AND THE MOLECULAR BASIS OF Treg CELL-MEDIATED SUPPRESSION

FoxP3⁺CD25⁺CD4⁺ natural Treg cells are characterized by several immunologic properties pertinent to their crucial role in the maintenance of self-tolerance and immune homeostasis. Most, if not all, of them are produced in the thymus as a functionally distinct and mature T-cell subpopulation with suppressive function before antigen encounter.^{12,13} Therefore thymus-derived FoxP3⁺ natural Treg cells are distinct from other adaptive/induced Treg cells that convert from naive T cells after they encounter specific antigen in the periphery. However, some Treg cells that are phenotypically and functionally similar to thymus-produced natural Treg cells might be able to differentiate in the periphery from naive T cells under certain conditions.^{9,14} FoxP3⁺ natural Treg cells bear a T-cell receptor (TCR) repertoire that is as broad and diverse as that of naive T cells yet is more self-reactive than the latter; that is, the TCRs of natural Treg cells bear higher affinity than other T cells for the class II MHC/self-peptide ligands, positively selecting them in the thymus.¹⁵⁻¹⁷ They also express a higher level of accessory molecules, including adhesion molecules, such as lymphocyte function associated 1 (*LFA-1*) and intercellular adhesion molecule 1 (ICAM-1), indicating that

GLOSSARY

CD25: CD25 is the α chain of the IL-2 receptor and is highly expressed on FoxP3⁺ Treg cells. CD25 associated with the β-chain (CD122) and common γ-chain (CD132) forms the heterotrimeric high-affinity IL-2 receptor. Binding of IL-2 to this receptor induces signaling through the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway, involving the tyrosine kinases JAK1 and JAK3 and STAT5.

CD80/CD86: T-cell activation requires at least 2 signals: (1) through the TCR interacting with MHC molecules loaded with antigenic peptide and (2) a second signal, such as that through CD28-CD80/CD86 or CD40-CD40 ligand interaction. T cells that do not receive a second signal become anergic. Abatacept (CTLA-4-Ig) blocks costimulation by interfering with CD80/CD86-CD28 interactions and by preventing APC maturation. It is approved for the treatment of rheumatoid arthritis.

CTLA-4: CTLA-4 limits activation through the costimulatory molecules CD28-CD80/CD86. Also known as CD152, CTLA-4 is expressed on activated T cells, is a member of the immunoglobulin superfamily, and uses an immunoreceptor tyrosine-based inhibitory motif for signaling that limits IL-2 production. CTLA-4 is constitutively expressed on murine FoxP3⁺ Treg cells.

CYCLOSPORIN: Cyclosporin decreases IL-2 production by binding to cyclophilin and inhibiting calcineurin-induced dephosphorylation of nuclear factor of activated T cells.

IL-6: IL-6 is a major inflammatory cytokine that promotes the formation of *T_H17* effector cells in the presence of *TGF-β* and inhibits the suppressive functions of natural CD4⁺CD25⁺ Treg cells.

IL-10: IL-10 has multiple immune-dampening effects, including suppression of IFN-γ, TNF-α, IL-2, and GM-CSF production from *T_H1* cells; inhibition of CD80/CD86, intercellular adhesion molecule 1, and MHC II expression; and partial inhibition of the induction of activities initiated by IFN-γ, IL-2, TNF-α, and IL-4. IL-10 also promotes the development of B1 cells. IL-10 belongs to class 2 α-helical cytokines that also include IL-19, IL-20, IL-22, IL-24, IL-26, IL-28, IL-29, and interferons.

IL-17: Secretion of IL-17 is one of the defining criteria for *T_H17* cells, which are involved in autoimmunity, especially inflammatory bowel disease, experimental autoimmune encephalomyelitis, and experimental

arthritis. The transcription factor retinoic acid orphan receptor gamma t (RORγt) is essential for the differentiation of *T_H17* cells, whereas signaling through STAT3 maintains the *T_H17* phenotype. *T_H17* cells mainly produce IL-17A, IL-17F, IL-21, IL-22, and CCL20, causing increased neutrophil recruitment and possible fibrosis.

IL-35: A recently identified heterodimeric interleukin constituted by the combination of the IL-12α chain and IL-27β chain (encoded by the EBV-induced gene 3). Production of IL-35 has been proposed as one mechanism for Treg cell-mediated suppression.

LFA-1: Lymphocyte function associated (LFA) 1 allows adhesion and trafficking of leukocytes into inflamed tissues through binding with intercellular adhesion molecule 1 on activated vascular endothelium.

RAPAMYCIN: Rapamycin, also known as sirolimus, blocks lymphocyte proliferation by inhibiting IL-2 signaling. Rapamycin binds to the mammalian target of rapamycin (mTOR; also known as FRAP, RAFT1, or RAPT1) and inhibits its kinase activity.

RETINOIC ACID: Nuclear steroid receptors, peroxisome proliferator-activated receptor, and retinoic acid receptors bind retinoic acid. In the intestinal tract, retinoic acid promotes the development of FoxP3⁺ Treg cells.

***T_H3*:** Induced regulatory CD4⁺ T cells that secrete high amounts of *TGF-β*. *T_H3* cells can induce FoxP3 expression in naive cells and render them suppressive. *T_H3* cells can suppress various autoimmune conditions, such as experimental autoimmune encephalomyelitis.

***TGF-β*:** *TGF-β* is produced by a wide variety of cells, including FoxP3⁺ natural Treg cells. However, *TGF-β* is associated with either a proinflammatory or anti-inflammatory state. High production of *TGF-β* by *T_H3* cells resident in the gastrointestinal tract is associated with tolerance to food antigens. Lack of *TGF-β* in mice induces severe autoimmunity. In allergic inflammation, eosinophils can make *TGF-β*, which contributes to airway fibrosis and remodeling.

***T_R1*:** *T_R1* cells are IL-10-producing adaptive Treg cells that can be induced *in vitro* by means of antigenic stimulation in the presence of IL-10. These cells do not express FoxP3.

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natural Treg cells are already phenotypically and functionally antigen primed in the thymus.¹² Some are in a more proliferative state than other T cells in the periphery of normal animals and healthy human subjects, presumably because of their strong recognition of normal self-antigens and commensal microbes.¹⁸ The proliferation and survival of Treg cells is highly dependent on exogenous IL-2 because Treg cells themselves produce little IL-2, and IL-2 deficiency therefore impairs Treg cell survival and produces autoimmunity caused by Treg cell deficiency. Natural Treg cells are also poor producers of proinflammatory cytokines on antigenic stimulation and do no apparent harm to the host, despite their high self-reactivity.^{19,20} These unique immunologic features of FoxP3⁺CD25⁺CD4⁺ natural Treg cells make them well suited for controlling pathogenic self-reactive T cells efficiently and swiftly during episodes when self-antigens are aberrantly or excessively presented in the immune system.

FoxP3⁺ natural Treg cells potently suppress the activation, proliferation, and/or effector functions of other CD4⁺ and CD8⁺ T cells and possibly natural killer cells, natural killer T cells, B cells, and dendritic cells.²¹ They can presumably control such a variety of target cells and at different phases of immune responses by implementing multiple modes of suppression in a multistep way. This includes cell contact-dependent suppression, functional modification or killing of antigen-presenting cells (APCs), and secretion of immunosuppressive cytokines.¹ As the molecular basis of suppression, cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), which is constitutively and highly expressed in FoxP3⁺ Treg cells, plays a crucial role *in vivo* and *in vitro*. Treg cell-specific deficiency of CTLA-4 produces autoimmune diseases similar to those induced by Treg cell depletion and CTLA-4 participates in *in vitro* Treg cell-mediated suppression in part by downmodulating CD80 and CD86 expression on APCs.^{22,23} In addition, constitutive expression of CD25 might contribute to suppression by inducing apoptosis in responder T cells through cytokine deprivation.²⁴ Treg cell production of immunosuppressive cytokines or cell-killing molecules, such as TGF- β , IL-10, granzyme B, and IL-35, might also play a role in suppression.^{21,25}

Natural Treg cells need to be stimulated through the TCR to exert suppression, whereas antigen-activated Treg cells exhibit non-antigen-specific bystander suppression, at least *in vitro*.¹⁹ Assuming the contribution of multiple mechanisms to Treg cell-mediated suppression, it is of note that the mechanisms acting on APCs explain the non-antigen-specific suppression exerted by antigen-specific Treg cells activated by specific antigen. For example, the contact-dependent advantage of Treg cells over naive T cells for recruitment and aggregation around APCs, modification (eg, CD80/CD86 downmodulation) or killing of APCs, or cytokine-dependent alteration of APC function can all hamper the activation of other T cells bearing any antigen specificity when they are recruited to the same APCs.²³ This indicates that preparation of Treg cells for each target antigen is not necessarily required for treatment of autoimmune disease in which multiple self-antigens are targeted (eg, insulin, glutamic acid decarboxylase, and other antigens in T1D).

These findings on the cellular and molecular basis of Treg cell generation, survival, and function, as well as antigen specificity, can be exploited to treat autoimmune disease and allergy through expanding antigen-specific Treg cells by using various strategies discussed below.

THERAPEUTIC STRATEGIES BASED ON *IN VIVO* Treg CELL ACTIVATION AND EXPANSION

In vivo antigen-specific activation and expansion of Treg cells

Because FoxP3⁺ natural Treg cells are present in the immune system as a functionally distinct and mature population with a diverse TCR repertoire, mere clonal expansion of Treg cells through appropriate pathways of antigenic stimulation leads to induction of antigen-specific immunosuppression. For example, Treg cells specific for islet antigen were shown to be more potent in suppressing diabetes in NOD mice than polyclonally activated Treg cells.^{26,27} Also, allergen-specific immunotherapy (SIT), which consists of repeated subcutaneous or sublingual administration of allergen, has been widely used and proved effective for the management of allergies.²⁸ The mechanism of the therapeutic effect of SIT is not completely understood²⁹ but might involve the activation and expansion of natural Treg cells,^{30,31} along with the induction of adaptive Treg cells, such as IL-10-secreting T_R1 cells.^{32,33} Regarding the mechanism of SIT, it is of note that Treg cells can be activated to exert suppression at a much lower concentration of antigen than required for activation of naive T cells.¹⁹ This is presumably due to the fact that natural Treg cells are functionally in an antigen-primed state before antigen encounter, as discussed above, and suggests that there must be a range of antigen concentrations in which a small dose of antigen can activate Treg cells but not naive or resting effector T cells. It is also possible that certain peptides structurally suboptimal to activate effector T cells could still activate natural Treg cells. Therefore specific activation of Treg cells by vaccinating autoimmune patients with low-dose autoantigen peptide or modified antigen peptide, together with additional inhibition of the activation of effector T cells (see below), is conceivable as a possible therapeutic strategy for autoimmune diseases with known target self-antigens, such as insulin for T1D, acetylcholine receptor for myasthenia, thyroglobulin and thyroperoxidase for Hashimoto thyroiditis, thyrotropin receptor for Graves disease, 21-hydroxylase for Addison disease, and hydrogen/potassium adenosine triphosphatase for pernicious anemia and autoimmune gastritis.

Another strategy to bring about antigen-induced Treg cell-mediated tolerance is to administer antigen through the oral or sublingual routes. It has long been known that oral administration of antigen can establish antigen-specific tolerance. Involvement of Treg cells, including natural and induced cells, has been demonstrated.^{32,34,35} Recent studies have also revealed the role of *retinoic acid*, which is secreted by a particular subset of dendritic cells in the gut-associated lymphoid tissue. Retinoic acid induces FoxP3-expressing cells from naive T cells in the presence of TGF- β and represses IL-6-dependent differentiation of naive T cells to IL-17-secreting T_H17 cells.³⁶⁻³⁸ It is clinically known that retinoic acid has a therapeutic effect in human psoriasis.³⁹ Although the mechanism of action is not completely understood, it might act by inducing FoxP3⁺ Treg cells. It remains to be examined whether oral administration of target antigen along with retinoic acid effectively induces antigen-specific tolerance and whether additional manipulation of cytokine production (eg, reduction of IL-6 production) in the gut-associated lymphoid tissue can enhance tolerance induction.

By exploiting the immunologic characteristics that differentiate natural Treg cells from other T cells, the expansion of antigen-specific Treg cells can be achieved by controlling antigen dose,

the route of antigen administration, and cytokine formation while suppressing the activation and expansion of effector T cells.

***In vivo* polyclonal activation and expansion of Treg cells**

Non-antigen-specific polyclonal activation of FoxP3⁺ natural Treg cells *in vivo* might also be used to control autoimmune disease and allergy. Nonmitogenic anti-CD3 mAb has been reported to be a potent inducer of anergy and tolerance and able to treat T1D in NOD mice.^{40,41} Notably, treatment with modified anti-CD3 mAb (hOKT3γ1[Ala-Ala] and ChAglyCD3) ameliorated the disease process in human patients with T1D.⁴² It significantly, although not completely, reduced the need for insulin required to control the disease.⁴³ The modified anti-CD3 mAbs were also able to reverse experimental autoimmune encephalomyelitis in mice, presumably by activating Treg cells.⁴⁴ It remains to be determined to what extent natural or induced Treg cells are responsible for the therapeutic effects of anti-CD3 mAb. Recent studies have indicated that tolerance induction by anti-CD3 mAb was independent of natural FoxP3⁺ Treg cells⁴⁵ but rather related to the activation of induced FoxP3⁺ Treg cells,⁴⁶ FoxP3⁺CD8⁺CD25⁺ Treg cells, or both.⁴⁷ Another study showed that combining anti-CD3 and intranasal administration of insulin peptide in diabetes-prone mice could expand FoxP3⁺CD4⁺ Treg cells.⁴⁸ This indicates that anti-CD3 treatment might be useful as an adjuvant to low-dose antigen vaccination to activate antigen-specific Treg cells, whether natural or induced, and re-establish antigen-specific tolerance in autoimmune disease and presumably in allergy.

***In vivo* expansion of Treg cells through cytokines, small molecules, or mAbs**

In addition to the antigen- or TCR-based approach to Treg cell expansion discussed above, control of activation, proliferation, and death of Treg cells, effector T cells, or both by using cytokines, drugs, or biologic methods (eg, mAbs) is conducive to establishing Treg cell-mediated dominant self-tolerance and immune homeostasis. This would occur either by expanding Treg cells, reducing effector T cells, or both.

Administration of IL-2, a key cytokine for the development and, in particular, the maintenance of natural Treg cells might facilitate the expansion of antigen-stimulated natural Treg cells in the circulation or at the site of inflammation. IL-2 is pleiotropic in function; in addition to maintaining natural Treg cells, it induces activation-induced cell death in activated T cells, maintains CD8⁺ cytotoxic T cells (especially memory cells), and inhibits T_H17 cell differentiation.¹ Therefore *in vivo* treatment with IL-2 or small molecules modifying signals from IL-2 receptor in T cells might have different outcomes, either immunosuppressive or immune enhancing, depending on the type and phase of immune responses to be controlled. IL-2 might better be used in combination with other strategies rather than for monotherapy for *in vivo* Treg cell expansion.

In addition to Treg cell-targeted expansion, selective enrichment of Treg cells can be achieved by reducing effector T cells while preserving Treg cells. **Rapamycin**, a macrocyclic lactone commonly used in organ transplantation to prevent acute rejection,⁴⁹ is useful in this regard. The enrichment of Treg cells by rapamycin treatment can be attributed to their relative resistance to

apoptosis/necrosis because of different modes of activation in the phosphoinositide 3-kinase/Akt/mammalian target of the rapamycin (mTOR)-signaling pathway in Treg cells and effector T cells, although the drug could also downregulate the expression of FoxP3 in TGF-β-dependent induction of Treg cells from naive T cells.⁵⁰⁻⁵³ Because rapamycin appears to have a low rate of infectious or malignant adverse effects,⁴⁹ it is a good adjuvant for antigen-based Treg cell expansion and resulting antigen-specific Treg cell-mediated immunosuppression.

Similarly, *in vivo* and *in vitro* proliferation of antigen-stimulated Treg cells appears to be more resistant than naive T cells to costimulation blockade (eg, by non-cell-depleting anti-CD4 or blocking anti-CD40 ligand mAb). This leads to a relative increase in the number of antigen-specific Treg cells after antigenic stimulation in the presence of these antibodies.⁵⁴

Thus rapamycin, anti-CD4mAb, and anti-CD154 mAb similarly inhibit *in vitro* expansion of effector T cells, but not Treg cells, although through different molecular mechanisms. This differential effect on the expansion of antigen-reactive Treg cells and effector T cells, with resulting enrichment of the former, can be exploited to design new agents and regimens for treating autoimmune disease and allergy. In particular, some biologic agents already in clinical use as immunosuppressants, such as anti-CD25 mAbs, CTLA-4-Ig, and **cyclosporine A**, might not only target effector T cells but also modify Treg cell function. They might be used to establish dominancy of antigen-specific Treg cells over effector T cells by controlling the dose, timing of administration, and combination with other reagents.

CELL THERAPY WITH Treg CELLS

***In vitro* activation and expansion of natural Treg cells**

Another possible strategy for separating *in vivo* Treg cell dominance over effector T cells is to expand antigen-specific Treg cells *in vitro* and transfer them back to the host. As previously discussed, an important precaution to take in Treg cell-based therapy is to avoid possible activation of pathogenic effector T cells that might contaminate Treg cell inocula. It should be emphasized that Treg cells are poorly proliferative *in vitro* on TCR stimulation,⁵⁵⁻⁶⁰ and in the presence of IL-2,⁵⁸ the expansion of contaminating effector T cells, if any, might be significant. Moreover, there is recent evidence that not all FoxP3-expressing cells are Treg cells in human subjects.⁶¹⁻⁶³ A sizable fraction of CD45RO⁺FoxP3⁺ cells secrete proinflammatory cytokines and do not have suppressive activity, being apparently an effector population (unpublished data). It is therefore necessary to prepare Treg cells to be as pure as possible before initiating *in vitro* culture to obtain a pure Treg cell population. A combination of CD25 and CD127 expression has been claimed to allow clear separation of FoxP3-expressing CD4⁺ T cells as CD25⁺CD127^{low/-} cells.^{64,65} Yet recent studies have shown that the expansion of CD25⁺CD127^{low/-}FoxP3⁺ cells fails to preserve a pure FoxP3-expressing population and eventually leads to the expansion of a significant proportion of cells that produce either IFN-γ, IL-2, or IL-17.⁶⁶

Efforts by several groups to obtain a functionally pure population of Treg cells have shown that human FoxP3⁺ Treg cells can be separated into 2 subpopulations, naive (ie, CD45RA⁺) and memory (ie, CD45RO⁺) phenotype cells, and Treg cells with a naive phenotype efficiently expand to give rise to a mostly homogeneous population of functionally competent FoxP3⁺ Treg

cells.⁶⁷⁻⁶⁹ Even in conditions using naive Treg cells as a source to expand Treg cells, non-Treg cells secreting proinflammatory cytokines might still contaminate.⁶⁹ Because rapamycin allows selective expansion of Treg cells even if they are not absolutely pure initially,^{50,70,71} selection of CD45RA⁺ naive Treg cells and their *in vitro* expansion in the presence of IL-2 and rapamycin should help to result in pure FoxP3⁺ Treg cells.

It is also of note that the fate of *in vitro* expanded Treg cells after *in vivo* transfer is hardly known in human subjects. In particular, possible *in vivo* conversion of Treg cells into effector T cells that secrete proinflammatory cytokines cannot be excluded. Indeed, it has recently been shown that FoxP3⁺ Treg cells can convert into T_H17 cells *in vitro*.^{72,73} In our hands Treg cells can also be converted easily into IFN- γ -secreting cells when cultured with IL-12 (Miyara and Sakaguchi, unpublished data). Thus cell therapy with *in vitro* expanded Treg cells should be evaluated with careful monitoring of the fate of Treg cells in phase I trials before clinical application for the treatment of allergy or autoimmune disease.

***In vitro* induction of Treg cells from naive T cells**

It has been shown in mice that CD4⁺ T cells induced to express FoxP3 *in vitro* by means of antigenic stimulation in the presence of TGF- β and IL-2 are able to suppress autoimmunity.⁹ Theoretically, a comparable strategy is plausible in human subjects; however, it is now accepted that not all induced FoxP3⁺CD4⁺ T cells are regulatory.⁶¹⁻⁶³ The level of FoxP3 expression is crucial to endowing a suppressive activity to T cells.⁷⁴ Furthermore, human T cells retrovirally transduced with the FoxP3 gene can suppress *in vitro* proliferation of other T cells if and only if they express the highest levels of FoxP3.⁷⁵ For future clinical application of induced Treg cells, it is therefore necessary to determine the culture conditions that effectively lead to the expansion of a homogeneous population of T cells stably expressing very high levels of FoxP3. *In vivo* functional stability of induced FoxP3⁺ Treg cells and their fate after transfer to the host also need to be investigated.

PROBLEMS AND PROSPECTS OF Treg CELL-BASED IMMUNOTHERAPY OF IMMUNOLOGIC DISEASE

The ultimate goal of Treg cell-based immunotherapy is to tip the balance between Treg cells and effector T cells toward dominance of the former and to stably sustain the balance, even if effector T cells are not completely eliminated.

Several issues need to be resolved to make Treg cell-based immunotherapy a clinical reality. First, we need to know more precisely the molecular basis of Treg cell activation, proliferation, and survival to differentially control Treg cells and effector T cells mediating immunologic diseases.¹ Even if Treg cells can be expanded *in vitro* by high-dose IL-2,^{58,69} they might die through apoptosis caused by cytokine deprivation when transferred to a host in which IL-2 concentration in the circulation is low. Resting Treg cells with a naive phenotype, which are FoxP3^{low}CD45RA⁺, have a high proliferative capacity and differentiate to FoxP3^{high}CD45RA⁻ activated Treg cells, which exert potent suppression and the majority of which die through apoptosis (Miyara et al, unpublished data). Molecular control of the differentiation and survival of particular Treg cell subsets, rather than whole FoxP3⁺ cells, might be more effective in controlling the magnitude of Treg cell-mediated suppression.

Second, to reliably purify Treg cells for cell therapy, better molecular markers need to be found that can specifically differentiate Treg cells from other T cells, especially activated effector T cells. Recent advances in the methods of purifying and expanding Treg cells *ex vivo* and *in vitro* have enabled a high yield of FoxP3-expressing cells by selecting CD45RA⁺ Treg cells and stimulating them in the presence of rapamycin.⁶⁹ Yet when Treg cells are prepared from patients with autoimmune disease, a minority of activated non-Treg cells contaminating the purified Treg cell population might have the potential to worsen the disease.

Third, the conditions need to be determined by which dominance of Treg cells over effector T cells can be established. It is general consensus that Treg cells can suppress more effectively the activation of naive T cells than the expansion or effector function of already activated effector T cells. This means that in cell therapy with Treg cells, pathogenic effector T cells need to be depleted first as much as possible by the use of biological agents (eg, T cell-depleting mAb) or radiomimetic drugs. Then Treg cells can be transferred to control regeneration of potentially pathogenic precursors and their differentiation to effector T cells. In addition, Treg cells would be effectively used to maintain remission in autoimmune/inflammatory diseases with a relapsing and remitting course (eg, multiple sclerosis, rheumatoid arthritis, and systemic lupus erythematosus). For these therapeutic purposes, it needs to be determined how long the inoculated Treg cells can survive in the host. Ongoing Treg cell therapy for graft-versus-host disease after allogeneic bone marrow transplantation will provide information on Treg cell survival in human subjects.⁶⁹

Fourth, it must be determined which combination of strategies can best establish or re-establish Treg cell-mediated antigen-specific dominant tolerance and immune suppression. In particular, it is necessary to design antigen-based strategies in combination with nonspecific strategies that target Treg cells, responder T cells, or APCs by controlling cytokines, accessory or costimulatory molecules, or signaling molecules.

Finally, caution should be taken for the possibility that *in vivo* expansion of Treg cells might interfere with immune surveillance against cancer.⁷⁶ Treg cell therapy for bolstering self-tolerance might suppress antitumor responses.⁷⁷ Again, it is critical to devise ways to expand antigen-specific Treg cells *in vivo* and *in vitro* to avoid non-antigen-specific general immune suppression.

CONCLUSION

Recent advances in our understanding of the roles of natural or induced Treg cells for the maintenance of self-tolerance and immune homeostasis support the notion that a Treg cell-targeted strategy is a promising modality for the treatment of autoimmune disease and allergy. Judging from our current knowledge on the functional stability of Treg cells, thymus-derived FoxP3⁺ Treg cells might be the best target for such Treg cell-based immunotherapy because of their functional stability and their natural presence in the immune system as a phenotypically definable T-cell subpopulation. Yet it is not our assertion that induced Treg cells are clinically less effective than natural FoxP3⁺ Treg cells, especially in controlling allergy.³³ Further elucidation of the cellular and molecular basis of Treg cell development and function will help in the design of new therapeutic strategies based on controlling natural and induced Treg cells.

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