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Regulatory T Cells for More Targeted Immunosuppressive Therapies

Hazim Allos, MD, **Basmah S. Al Dulaijan, MD**, **John Choi, MD**, and **Jamil Azzi, MD***

Transplantation Research Center, Renal Division, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA

SUMMARY

Cellular therapy using ex vivo expanded Tregs and immunotherapy using IL-2 to directly stimulate Tregs in vivo is the result of years of conglomerated research into the novel aspects of the immune system. Current trials are largely favorable in efficacy and with paucity of adverse effects shows a great potential compared with traditional therapies. However, further research into efficacy, safety, and, particularly, questions of stability of the Treg population in vivo, is required to fully approach Tregs as bona fide therapies in immune-originated diseases.

Keywords

T-regulatory cells; FOXP3; Immunotherapy

INTRODUCTION

The adaptive immune system thrives on its foundational ability to create an almost innumerable repertoire of randomly assorted T-cell receptors (TCRs), including those toward self-antigens.[1] However, immune homeostasis is a multifaceted system of balanced biological checks that refrain from boisterous autoreactivity and exaggerated activity against pathogens. A lapse in this very delicate, and tightly regulated, homeostatic system in either aspect often results in dire consequences.[2] And, although the structure of immune reactivity has been extensively described in literature, immune regulation has only recently received explicit focus.

Immune tolerance can be achieved via several different pathways, which can be subcategorized into 2 main subsets: cell-intrinsic and cell-extrinsic mechanisms. Avidly self-reactive lymphocytes can accomplish immunotolerance, in the broader sense, by undergoing programmed cell death, genetic rearrangements resulting in a distinctive antigen receptor, peripheral anergy in response to a self-antigen and, finally, increased activation thresholds via increased expression of inhibitory molecules or activation-induced death.[3] These components of the cell-intrinsic pathway for tolerance have been previously properly recognized and already greatly described. The cell-extrinsic mechanisms have been mostly

*Corresponding author. Transplantation Research Center, Brigham and Women's Hospital, 221 Longwood Ave, Boston, MA 02115. jazzi@rics.bwh.harvard.edu.

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unnoticed until recently in 1972, Gershon and colleagues[4] had first provided evidence of a newly discovered, and imperative, player in the immunoregulatory system. The CD4+ T-regulatory cell (Treg) is the first cell subset to be recognized in the cell-extrinsic mechanism of immunotolerance.

DISCOVERY OF T-REGULATORY CELLS

It was first noted that depletion of T cells in adult rats by thymectomies, thereafter treated with low-dose x-ray irradiations, led to the development of autoimmune thyroiditis. When the normal immune cells were repleted, the disease reverted.[5,6] Similarly, Sakaguchi and colleagues[7] showed that thymectomy in neonatal mice at day 3 resulted in autoimmune oophoritis and that this was prevented by a single intraperitoneal injection of spleen cells or thymocytes from nonthymectomized mice. These results lead to the postulation that not only are self-reactive T cells capable of producing autoimmune phenomena present but "suppressor" T cells that counterbalance this inherent autoreactivity are also present. Sakaguchi and colleagues[8] showed that removal of a certain subpopulation of T cells, termed "Lyt+," from mice resulted in extensive autoimmunity. Reconstitution of this cell subset resulted in reversal of auto-immunity. Ultimately, these "Lyt+" cells were found to bear CD4+CD25^{high} (IL-2 receptor α [IL-2Rα] chain) and termed "T-regulatory cells." Furthermore, they were observed to lead to a diminution of self-, nonself, and allergic immune antigen responses.[9,10] However, because CD25 is also highly expressed in activated effector T cells, it is not a specific marker of Tregs. It was not until 2003 that the forkhead box transcription factor FOXP3 was found to be the specific marker of Tregs.[11,12]

The knowledge of FOXP3 stems from a rare monogenic disease; immunodysregulation, polyendocinopathy, enteropathy, X-linked syndrome (IPEX) is an X-linked autoimmune/inflammatory syndrome, caused by genetic defects in the FOXP3 gene. Characteristics of patients with IPEX are very similar to that of scurfy mice that lack Tregs.[13] This led to the vigorous study of FOXP3s' role in the Treg. Initial findings showed that FOXP3 coded for a transcriptional factor that is specifically expressed in naturally arising CD4+ Tregs. In addition, retroviral transfer of FOXP3 gene converts naive T cells toward a Treg-like pathway.[14] These findings led to the conclusion that FOXP3 is the master gene that allows T cells to differentiate into functional Tregs.

A NONHOMOGENOUS POPULATION

The CD4+CD25 + CD127lowFOXP3+ Treg population constitutes approximately 5% to 15% of peripheral T-cell population and under close inspection can be divided into 22 distinct population based on differential surface marker expression.[15] However, broadly speaking, Tregs can be divided in 2 main types, denoted by their origin: thymus-derived Tregs and peripherally derived Tregs, also called natural Tregs (nTregs) and induced Tregs (iTregs), respectively.[16]

nTregs compromise most of the Treg population and respond chiefly to self-reactive T cells that have failed to undergo negative selection in the thymus. Naïve nTregs are characterized by CD44low, CD62Lhi, CXCR4low, and CCR7, allowing them to secondary lymphoid

tissue. Local stimulation in the homed lymphoid organ creates a phenotypic switch to express a different array of chemokine receptors, with downregulation of CD62L and CCR7, allowing to traffic to the tissue of interest depending on which chemokine receptor had been expressed.[17–20] This switch marks the creation of a mature effector Treg, characterized by CCR7lowCD62LlowCD44hiKLRG1 +, found in circulation and in tissues harboring immunologic activity.[21–23]

Peripherally, CD4+CD25– T-conventional cells (Tconvs) also have the capacity to become Tregs by induction; these are termed iTregs. There are several differences between nTregs and iTregs. First and foremost, iTregs have a broader TCR repertoire and the cells' differentiation requires their stimulation.[24] Varying conditions present themselves for iTreg differentiation and mainly include bacterial and viral infections, tumors, or in mucosal tissue in the context of oral tolerance.[25–27] However, expression of FOXP3 in these cells is highly unstable and these cells can revert back to Tconvs or even pathogenic Th memory cells once signals and cues, such as IL-2 and TGF-β, abate.[28]

It is worth mentioning that a distinct subset of Tregs was more recently found, the tissue-resident Tregs. Such Tregs have been identified in skin tissue, visceral adipose tissue, and muscle tissue.[29] Each of the tissues mentioned bears a specific Treg with different proportions, phenotypic marker expressions, cytokine secretions, and, therefore, function.[29] However, more research needs to be conducted on the nature of these Tregs and their impact on local tissue immune homeostasis and potential pathogenicity.

ESSENTIAL SIGNALS FOR TREG DEVELOPMENT AND MAINTENANCE

Maturation of T cells, in general, occurs first and foremost in the thymus. There, they undergo processes known as positive and negative selection. Positive selection occurs when a T cell's TCR has the ability to recognize major histocompatibility molecules, thereby allowing it to proceed in its maturation process. Negative selection then eliminates T cells via a process called clonal deletion if they bind, with high affinity, to self-protein antigens and are deemed autoreactive.[30] Physiologically, not all T cells with self-reactive high-affinity TCRs seem to be negatively selected. Indeed, it seems that the alternative pathway for autoreactive T cells is to shunt development into CD4+CD25 + Tregs.[31] However, because self-reactive TCRs are present on both negatively selected T cells and potential Tregs, there should be a secondary signal to induce the Treg pathway.

Tregs bear a high affinity IL-2R consisting of CD25(IL-2Rα), CD122 (IL-2Rβ), and CD132 (IL-2Rγ),[32] and, unsurprisingly, IL-2 is one of the major secondary signals essential for Treg differentiation. This observation stemmed from an experiment using mice that lack CD25, or IL-2 entirely, resulting in significant decrease in FOXP3+ nTreg population.[33] To a lesser degree, other γ-chain (γc) cytokines IL-7 and IL-15 are also deemed important for Treg differentiation and seemed to partially compensate for entire lack of IL-2, resulting in reduced but not absent FOXP3 Treg numbers.[34] Finally, TGF-βs' role in Treg development is somewhat unclear. TGF-βR-deficient mice were associated with the expression of proapoptotic proteins Bax, Bak, and Bim and concomitant low expression of Blc-2, resulting in high rates of apoptosis during negative selection and, furthermore, Bim ablation restores

TGF-b signal deficiency.[35] These results suggested that TGF-β is not necessary to direct differentiation of nTregs but perhaps essential to maintain it.[35] Ultimately, the various interplay of the aforementioned cytokines, and their respective receptors, results in the phosphorylation of transcription factor STAT5, and, finally, induced expression of FOXP3.[36–38]

$CD4+CD25^{-}FOXP3^{-}$ Tconvs are induced, and maintained, toward the Treg pathway by induction via TGF-β.[39,40] As previously mentioned, IL-2 is not absolutely necessary for the development of nTregs and can be replaced by other common γc cytokines, although by similarly conducted studies, it seems to be an irreplaceable signal that is crucial for iTregs.[39,41,42]

FOXP3 AND EPIGENETICS

FOXP3 is an X chromosome–encoded gene and is thought to be the master gene of the Tregs; its expression is induced after TCR stimulation and seems to mainly depend on the intensity of the stimulation.[31] As previously mentioned, IPEX is caused by genetic defects of FOXP3 and experimental scurfy mouse models share the characteristics.[13] Another study showed that because of random inactivation of the X chromosome in women heterozygous for FOXP3, FOXP3–/+ mice produce half the population of functional Tregs, and the other half dysfunctional, which further indicated that FOXP3s' presence is essential for the Treg population.[42] However, various studies have demonstrated that FOXP3 expression in cells is, albeit essential, not sufficient to produce fully functional Tregs. Lin and colleagues[43] demonstrated that mice expressing truncated FOXP3 maintained Treg phenotype but lacked full suppressive abilities. Therefore, there seems to be an independent and distinct factor that compromises a full Treg phenotype and function, in addition to FOXP3.

The FOXP3 locus is a conglomerate of 3 conserved noncoding sequences (CNS1–3) and a promotor region. The CNS are targets of epigenetic modifications causing either reduced or increased sterical hindrance, allowing either, respectively, increased or reduced transcription factor interaction and therefore gene expression.[44] An elucidating study by Ohkura and colleagues[45] demonstrated the role of epigenetics in fully functional Tregs, independent of the presence of FOXP3. According to the study there are 2 independent processes that occur in parallel in the course of Treg development that are necessary for a full-fledged Treg. Continuous TCR stimulation results in TCR-induced CpG hypomethylation of certain DNA regions, in the thymus and the periphery, and is required to obtain genome-wide Treg gene expression pattern. This hypomethylation pattern leads to higher stability and full phenotypic expression and is observed in nTregs and in vivo iTregs but not in vitro iTregs.[45]

SUPPRESSIVE STRATEGIES OF TREGS

Tregs use a variety of methods to impose their suppressive function, most of which are currently controversial and seem to depend on a variety of factors, such as nature of targeted suppression, context of immune response, and anatomic location of suppression. Direct suppression can occur in the absence of antigen-presenting cells (APCs) by direct cell-cell contact causing inhibition of TCR-stimulated IL-2 transcription.[46] Another postulated direct

contact mechanism is via delivery of a highly expressed amount of cytoplasmic cAMP in Tregs to Tconvs by gap junctions.[47] Delivery of granzyme B and perforin, inducing apoptosis, seems to be another observed mechanism.[48,49] Tregs express high-affinity IL-2Rα (CD25) and, concurrent with this fact, it has been observed that Tregs competitively, and preferentially, bind IL-2 limiting the abundance for nonregulatory T cells.[50]

Tregs constitutively express cytotoxic T lymphocyte antigen 4 (CTLA-4), and there is evidence that Tregs use a CTLA-4–dependent manner to downregulate CD80/86 on APCs. This results in a limitation in antigen presentation and, therefore, activation of naïve T cells via CD28.[51] Perhaps most intuitively, Tregs secrete IL-10, which seems to be essential to keep immune responses at homeostasis in environmental interfaces such as the colon and lungs. IL-35 has been similarly implicated in tolerance in the gut. And, finally, TGF-β also seems to suppress Th1 responses.[52]

T-REGULATORY ADOPTIVE CELL TRANSFER THERAPY

The earliest therapies that implemented knowledge of Tregs' immunosuppressive abilities used transfer of Tregs into the peripheral blood of patients with varying conditions.

The first of these novel approaches was conducted by Trzonkowski and colleagues,[53] where ex vivo expanded Tregs were transferred into 2 cases of either acute or chronic graft-versus-host disease (GVHD). Despite triple suppressive therapy, insulin injections, bronchodilators, and multiple failed attempts of reduced dosing, the patient afflicted with chronic GVHD improved following transferred Treg therapy. Complete removal of one suppressive agent, insulin injections and bronchodilators, and reduction of prednisone was achieved as a direct consequence of Treg therapy. Moreover, 6 months posttransfer, percentages of Tregs remained high in peripheral blood (2.5% before transfer to 5%). In the case of acute GVHD, unfortunately, although modest improvements were initially seen, the lack of available Tregs halted further treatment.[53] Similarly, using umbilical cord blood as a source of Tregs, Brunstein and colleagues isolated,[54] expanded, and injected patients with acute GVHD at certain intervals. Results indicated that Treg infusion not only prevented GVHD manifestations but also inferred no increased risk of opportunistic infection or other obvious adverse effects. In another study involving 28 human leukocyte antigen (HLA)-haploidentical stem cell transplantation engrafted patients, 26 of 28 patients remained acute GVHD free, all remained chronic GVHD free, and CMV infection rates were significantly lower than with traditional therapies.[55]

Adoptive Treg transfer has also been used in autoimmune diseases, for example, in the setting of recently diagnosed diabetes mellitus type 1. Similarly, the results showed significantly decreased hemoglobin A1C levels; requirements of exogenous insulin, with 2 patients completely not requiring insulin; and increase in circulatory Tregs. And, moreover, no significant increased risk to infections or any adverse effects were observed.[56] A follow-up study also reported increased β-cell islet survival with repeated Treg administration over a course of 1 year.[57] However, a common limitation to all these studies is the questionable stability and eventual wane of the Treg populations in peripheral blood, and, therefore also

their effects. Accordingly, more exploration of Treg stability and optimum conditions for persistence in peripheral blood is required to apply Tregs in immunotherapy more adequately.

This article elucidates, at least partially, a mechanism that explains this inherent Treg instability. On Treg activation, granzymes A and B are highly expressed and tend to leak out of compartmentalized granules, ultimately leading to cell intrinsic apoptosis. Blood samples drawn from renal transplant recipients undergoing rejection demonstrate an increase in chemokine receptors that home Tregs to the tissue-harboring inflammatory activity, receptors that are shared with inflammatory Th1 and Th17 cells, which mediate rejection. Similarly, increased granzyme B expression is seen in these Tregs as well.[58] Possibly, this may be a homeostatic phenomenon because the ability of antigen-specific T-cell clones to later be reactivated to the same antigen depends on an inflammatory milieu that Tregs directly counteract.[58]

T-REGULATORY CELL IL-2 IMMUNOTHERAPY

More recent trials for Treg immunotherapy stems from knowledge that IL-2 is imperative to the cell lines' integrity and maintenance in the periphery. $CD4^+CD25^+$ Tregs express all 3 components of the IL-2 receptor: CD25 (IL-2Rα), CD122 (IL-2Rβ), and CD132 (IL-2Rγ).[32] Complexes consisting of CD122 and CD132 create a low-affinity IL-2R; however, the trimeric complex present on Tregs constitutes a high-affinity IL-2R.[32] IL-2 has the ability to both expand and amplify the Treg or conventional T cells depending on concentration. Tregs and Tconvs are contradictory in function and have been shown to react differently under varying IL-2 concentrations.[59] Indeed, in vivo, low-dose IL-2 was shown to preferentially stimulate Tregs, likely because of their high-affinity trimeric IL-2R. In contrast, high-dose IL-2 stimulated Tconvs and Tregs, allowing for dual utilization of IL-2 for either immunosuppressive or immunopro- moting functions, respectively.[59]

Initially, therapeutic approaches involved high-dose IL-2 administrations. Ahmadzadeh and colleagues[60] showed that high-dose IL-2 administration to patients with cancer resulted in a nearly 6-fold increase in circulatory Tregs, with substantial increase in FOXP3 expression. However, with further expanse of IL-2 immunotherapy trials, high-dose IL-2 administration was repeatedly associated with a multitude of adverse effects.

One of the earliest studies that clearly showed the safety profile and potential for efficacy of low-dose IL-2 therapy was conducted in Hepatitis C-induced vasculitis patients. Low-dose IL-2 administration to 10 patients resulted in clinical improvement in 80% of patients, increase in total percentage of Tregs, and the attenuation of overall inflammatory markers. Moreover, it was found to be clinically safe with adverse effects more commonly seen at the higher IL-2 doses.[61]

Koreth and colleagues[62] also demonstrated that low-dose IL-2 therapy preferentially expanded the Treg lines rather than conventional CD4+ T cells in patients with GVHD. Continued low-dose IL-2 administration was shown to be safe; it created sustained Treg expansion, with peak values at 4 weeks, and led to an overall reduction of GHVD

manifestations. In another study that involved GVHD in allogeneic hematopoietic stem cell transplantation (HSCT) by Matsouka and colleagues,[63] the mechanisms of IL-2 therapy on Treg homeostasis was revealed, at least partially. The research group demonstrated that chronic GVHD is characterized by constitutive phosphorylation of Stat5 in Tconv with association of elevated levels of IL-7 and IL-15 and functional deficiency of IL-2. Low-dose IL-2 administration, over an 8-week period, resulted in discriminatory increase of Stat5 phosphorylation in Treg and a decrease in Tconv. Furthermore, IL-2 has been shown to induce a multitude of reactions from Tregs including increased thymic export, proliferation, and enhanced resistance to apoptosis.[63]

One of the major concerns of any suppressive immunotherapy resulting in an abated immune response is the potential for opportunistic infections. Kennedy-Nasser and colleagues, in a prospective cohort study, demonstrated the use of low-dose IL-2 therapy in allogeneic HSCT to treat GVHD. Patients were given low-dose IL-2 within a time frame of less than 30 days post-HSCT and continued for 6 to 12 weeks. No patient developed grade II-IV GVHD or grade ¾ toxicities. More importantly, only 15% of the patients developed viral infections, compared with 63% of patients without IL-2 therapy, following standard protocol.[64] From this study, it seems that patients given low-dose IL-2 therapy seem to retain the ability to recognize and respond to viral antigens, unlike patients given standard post-HSCT protocol therapy. Two other studies in patients with GVHD further revealed the potential of low-dose IL-2 for efficacy and safety.[65,66]

IL-2 therapy has also been briefly studied in autoimmune diseases. Castela and colleagues[67] showed the potential of lowdose IL-2 therapy in patients with alopecia areata, with 4 out of 5 patients showing signs of clinical improvement. An objective decrease in CD8+ T cells infiltration in scalp biopsies, increase in circulatory Tregs, and no major signs of toxicity were also observed. In the more systemically affected autoimmune diseases, He and colleagues[68] showed that the utilization of recombinant human IL-2 in systemic lupus erythematosus, over the course of 2 weeks, resulted in significant decrease in disease manifestations, increase in Treg circulatory cells, and a drop in T follicular helper and TH17 cell percentages.

More interestingly, a recent clinical trial conducted a partial evaluation of the combined effect of adoptive Treg transfer and simultaneous IL-2 injections in patients with chronic GVHD. The 3 patients who received this regime showed increased counts in circulating Tregs and clinical improvements and/or stabilization.[69] Table 1 summarizes various clinical trials involving Tregs. Clearly more research is required to draw conclusions; nonetheless combined therapy may prove to show more favorable results.

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KEY POINTS

- Immune tolerance can be achieved via several different pathways, which can be subcategorized into 2 main subsets: cell-intrinsic and cell-extrinsic mechanisms.
- Avidly self-reactive lymphocytes can accomplish immunotolerance, in the broader sense, by undergoing programmed cell death, genetic rearrangements resulting in a distinctive antigen receptor, peripheral anergy in response to a self-antigen, and, finally, increased activation thresholds via increased expression of inhibitory molecules or activation-induced death.
- These components of the cell-intrinsic pathway for tolerance have been previously properly recognized and already greatly described.
- The CD4+ T-regulatory cell is the first cell subset to be recognized in the cell-extrinsic mechanism of immunotolerance.

Key Points

- History of Tregs
- Treg biology and function
- Ex vivo expanded and transfusion Treg therapy trials
- IL-2 therapy trials

Table 1

Clinical trials with Tregs

Therapy Type	Disease Model	Effects	Source
Adoptive Treg transfer	Chronic GVHD	Reduced chronic GVHD manifestations; dose reduction of various immunosuppressive/symptomatic medications.	Trzonkowski et al,[53] 2009
	Prevention of acute GVHD	Prevention of GVHD manifestations; no increased risk of infection; no obvious adverse effects observed.	Brunstein et al,[54] 2011
	Prevention of acute GVHD	Umbilical cord isolated Tregs; prevention of acute GVHD in 26/28; prevention of chronic GVDH in 28/28; no increased risk of CMV infection.	Di Ianni et al,[55] 2011
	T1DM	Decreased HbA1c level; insulin dose reduction or complete withdrawal in 2 patients; increased Treg circulatory numbers; no increased risk of infections; no observable adverse effects.	Marek-Trzonkowska et al,[56] 2012
	T1DM	Follow-up study reporting increased β-islet cell survival with repeated Treg administration over 1 year.	Marek-Trzonkowska et al,[57] 2014
Low-dose IL-2 therapy	HCV-induced vasculitis	8/10 patients experienced clinical improvement; attenuation of inflammatory markers; increased total Treg percentages; safe with adverse effects observed at high-dose IL-2.	Saadoun et al,[61] 2011
	Chronic GVHD	Safe; sustained Treg expansion with peak values at 4 wk; decreased GVHD manifestations.	Koreth et al,[62] 2011
	Chronic GVHD	Molecular basis revealed discriminatory increase in Stat5 phosphorylation in Tregs and a decrease in Tconv numbers; increased Treg thymic export, proliferation, and resistance to apoptosis.	Matsuoka etal,63 2013
	Chronic GVHD	Decrease in GVHD manifestation development; no serious observable adverse effects; decrease in rate of viral infections compared with standard therapy.	Kennedy-Nasser et al,[64] 2014
	Chronic GVHD	Increased circulating Tregs; clinical improvements in >60% of patients with steroid refractory disease; no increased risk of infection.	Koreth et al,[65] 2016
	Chronic GVHD	Increased circulating Tregs first wk, normalization during second wk despite continued IL-2 administration; mildly decreased GVHD manifestations; safe.	Kim et al,[66] 2016
	Alopecia areata	4/5 patients showed clinical improvements; decreased CD8+ T-cell infiltration of scalp; increased circulatory Tregs; no major adverse effects observed.	Castela et al,[67] 2014
	Systemic lupus erythematosus	Decrease in disease manifestations; increased circulatory Tregs; decrease T follicular helper and TH17 cell percentages.	He et al,[68] 2016
Combined adoptive transfer & low-dose IL-2	Chronic GVHD	Increased circulatory Tregs; observable clinical improvement and/or stabilization.	Theil et al,[69] 2015

Abbreviations: HbA1c, hemoglobin A1C; T1DM, type 1 diabetes mellitus.