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Minireview

CD4$^+$CD25$^+$ Regulatory T Cells in Transplantation: Progress, Challenges and Prospects

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The involvement of CD4$^+$CD25$^+$ regulatory T cells (Treg) in general immune homeostasis and protection from autoimmune syndromes is now well established. Similarly, there has been increasing evidence for Treg involvement in allograft rejection and current immunotherapies. However, despite significant advances in understanding the development, function, and therapeutic efficacy of Treg in certain well-defined rodent models, the relevance of Treg to clinical transplantation remains unclear. In this review, we summarize our current understanding of the role of Treg in immunity and organ transplantation in experimental and clinical settings. In addition, we review advances in using Treg as a form of immune therapy. The goal is to highlight the complexities and opportunities in the field and to provide evidence to support the use of antigen-specific Tregs in the context of transplantation to facilitate a robust and selective state of immune tolerance.

Key words: Dendritic cell, tolerance induction, transplantation, immunology, T regulatery cells

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Introduction

Evidence for CD4$^+$ T-cell-mediated immunoregulation, especially in transplantation, has been around for more than two decades. In 1985, Hall's group reported the induction of suppressor activity in CD4$^+$ T cells in rats that received a heart transplant and short course of cyclosporine therapy (1). In 1989, a group led by Morris and Wood reported the induction of CD4$^+$ suppressor T-cell activity after donor-specific transfusion (DST) in rats (2). Waldmann's group showed that allospecific tolerance in mice induced by combined anti-CD4 and anti-CD8 antibody treatment was dependent on CD4$^+$ T cells (3) and Wood's group showed that prolongation of allograft survival by DST combined with anti-CD4 antibody in mice was dependent on CD4$^+$ suppressor T cells (4). However, despite reproducible demonstrations of suppressor CD4$^+$ T cells in a variety of rodent models, their very existence remained a matter of debate, partly due to some irreproducible and odd functional properties, but largely due to the lack of reliable distinguishing molecular markers. In 1995, Sakaguchi's group showed in an adoptive transfer model of autoimmunity that suppressor activity resided exclusively in the CD4$^+$CD25$^+$ T cell subset (5). Unlike earlier CD8$^+$ T suppressor cell studies, these so-called 'regulatory T cells' (Treg) were thymically-derived from conventional T cells and expressed typical TCR$\alpha\beta$ receptors. The critical importance of CD4$^+$CD25$^+$ Treg is indisputable, particularly given the recent demonstration that adult mice succumb to massive autoimmune attack after targeted Foxp3$^+$ cell depletion (6). Finally, the involvement of Treg in most models of allospecific tolerance is now well-chronicled (7–9).

Natural CD4$^+$CD25$^+$ Treg

CD4$^+$CD25$^+$ T cells comprise 5–10% of mature T cells (10). Other surface markers such as CD62L, CTLA-4, CD134, CD122, CD103, CD45RB, have been used to define Treg and/or enrich for Treg activity in CD4$^+$CD25$^+$ T cells. However, these markers are also expressed on other T-cell subsets making them unreliable for precisely identifying this cell lineage, especially in humans. In 2003, the forkhead transcription factor, Foxp3, was identified as the 'master regulator' of Treg development and like other transcription factors [Tbet (for Th1 cells), GATA-3 (for Th2 cells) and RORγt (for Th17 cells)] provided a critical lineage-specific transcription factor for this T-cell subset. Foxp3 expression is restricted predominantly to CD4$^+$ CD25$^+$ TCR$\alpha\beta$ cells, although a sizable proportion of Foxp3$^+$ cells do not express any of the defined cell surface markers including CD25 and some are CD8$^+$ (11). Mice and humans deficient in Foxp3 suffer from massive T-cell hyperproliferation, leading to multiorgan autoimmunity and premature death. In addition to the critical role of Foxp3 in Treg development, Rudensky and colleagues recently reported that Foxp3$^+$ Treg play an ongoing critical role through adult life, as deletion of Foxp3$^+$ T cells in adult animals resulted in hyperproliferation, expansion of endogenous dendritic cells and death of mice within 2 weeks (6). Thus, it appears that Foxp3 is the master transcription factor controlling Treg development and function in mice. Unfortunately, the data is less clear in humans as recent studies have shown that

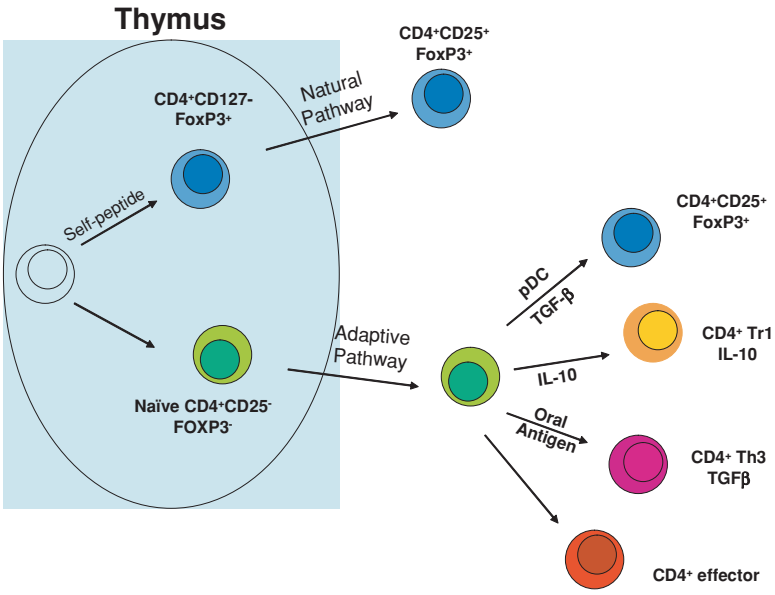

Figure 1: Developmental pathways of natural and adaptive T regulatory cells. pDC = Plasmacytoid dendritic cells.

most T cells turn on Foxp3 during early stages of T-cell activation. This is especially important as multiple human transplant studies are now using the marker to define Tregs in everything from kidney biopsies to urine PCR profiling to *in vitro* assays. Thus, although there is no doubt that this transcription factor is linked to Treg biology, care must be taken with any simple Foxp3 analysis.

Another important practical point to remember is that Foxp3 is a transcription factor localized to the nucleus, and therefore cannot be used as a surface marker to separate Tregs. Thus, there has been a continued search for additional cell surface markers that can distinguish this subset. Recently, we have observed that IL-7 receptor (CD127) expression inversely correlates with Foxp3 expression and Treg activity (12). $CD4^+CD25^+CD127^{lo}$ cells include 3-fold more $Foxp3^+$ T cells than the classic $CD4^+CD25^{hi}$ subset and demonstrat equivalent regulatory activity when compared with conventional $CD4^+CD25^{hi}$ Treg. Most importantly, another potential advantage is that CD127 can distinguish Treg from recently activated $Foxp3^+$ T effectors and memory cells (Bluestone, unpublished observations). In fact, the separation of human T cells based solely on CD4 and CD127 ($CD4^+CD127^{lo}$) are about 50% $Foxp3^+$ and can suppress effector T cell (Teff) responses quite efficiently. This additional marker is a promising tool for human Treg identification and the development of robust Treg isolation protocols essential for eventual clinical development.

Adaptive versus natural Treg

$CD4^+Foxp3^+$ Treg have been shown to develop in the thymus with a repertoire skewed to autoreactive specificities. It has been suggested that these natural Tregs develop as a consequence of high-affinity MHC-self peptide interactions (13) (Figure 1). However, these observations were made in TCR transgenic mice with a limited T-cell receptor repertoire where major thymic deletional events are occurring simultaneously. Thus, it remains possible that Treg develop as a consequence of low-affinity self recognition of cells that have averted negative selection. In fact, increasing evidence during conventional immunity support a model in which low affinity antigenic stimuli can convert $CD4^+CD25^-Foxp3^-$ T cells into $Foxp3^+$ Treg in the periphery. For instance, *in vitro* stimulation of $CD4^+CD25^-Foxp3^-$ T effector cells through the TCR in the presence of TGF-β results in Foxp3 upregulation, generating $CD4^+CD25^+Foxp3^+$ T cells, so-called adaptive Treg, with regulatory properties (14–16) in one case using the Foxp3-GFP knock-in reporter mouse (17). Intriguingly, Bromberg's group has shown that under 'tolerizing' conditions of donor-specific transfusion plus CD40L blockade, host plasmacytoid dendritic cells acquire alloantigen in the graft, migrate to peripheral lymph nodes and induce $CD4^+CD25^+Foxp3^+$ Treg (18). The isolated plasmacytoid dendritic cells (DC) from tolerant mice were shown to convert $CD4^+CD25^-Foxp3^-$ T cells into $CD4^+CD25^+Foxp3^+$ Treg *in vitro*. The notion that $FoxP3^+$ Tregs can develop during immunity, especially when confronted with a regulatory cytokine milieu including TGFβ and IL-10 help to explain and unite many seemingly disparate studies published in the transplant field over the last decade. We suggest that Treg biology is not as complicated as it seems. Treg, like other T-cell subsets, develop during immune responses as a consequence of antigenic exposure linked to antigenic quality, quantity and the environmental milieu. Natural Treg, on the other hand, are fundamentally geared toward controlling overall immune homeostasis and underlying autoreactivity but are less involved in the inflammatory responses created during organ

transplant rejection. One clear example of this adaptive Treg biology is seen during anti-CD3 mAb therapy. In collaboration with Lucienne Chatenoud and colleagues, we have observed that reversal of diabetes in the nonobese diabetic (NOD) mice, results in part from the induction of a regulatory T-cell subset that expresses low levels of CD25 and Foxp3 (19) and is responsible for tolerance in this model. These adaptive regulatory T-cell subsets have some features in common with natural Tregs but include a cytokine-dependent suppressive mechanism, induction as a consequence of suboptimal signal transduction, and selective tissue expression that mimics other regulatory T-cell subsets. In fact, the adaptive Treg are far more critical to suppression in this model than natural Treg (19). Thus, although natural Treg are an important lineage of cells that prevent massive lymphoproliferation and control immune homeostasis, the ability to induce adaptive Treg (Figure 1) with similar functional activity may have more important implications for regulating ongoing immune responses, especially in autoimmunity and transplantation.

Other adaptive regulatory T-cell subsets have been reported to develop as a consequence of antigen exposure in the periphery (Figure 1). Tr1 cells can be generated from naïve, mature CD4$^+$ T cells by antigenic stimulation in the presence of IL-10, both *in vitro* and *in vivo* (20). Tr1 cells have also been generated *in vitro* using a combination of antigenic stimulation, vitamin D3, and dexamethasone (21). Tr1 cells secrete IL-10 and TGF-β, which have been demonstrated to be critical for their immunosuppressive effect. Th3 cells were initially identified after the induction of oral tolerance as TGF-β secreting T cells in Peyer's patches and mesenteric lymph nodes (22). Their relationship to the other Treg subsets is not yet characterized, though a recent report has shown that oral tolerance induction generates both Foxp3$^+$CD25$^+$ as well as Foxp3$^-$CD25$^-$ T cells with regulatory activity (23). CD8$^+$ T cells with regulatory activity were identified almost three decades ago. After falling into disrepute in the early 1980s, there is now ample evidence in mice and humans that these cells [in some cases Foxp3$^+$, (24)] exist as potential suppressor cells. In one set of studies, these cells, also coined "Ts" cells, appear to induce the inhibitory receptors ILT3 and ILT4 on APC, rendering them "tolerogenic" (25). Finally, other minor subsets of T cells, including NKT cells and CD4$^-$CD8$^-$ T cells (DN Treg) (26) have been shown to have suppressive activity. Although these other regulatory T-cell subsets have been observed in various immune processes, and have demonstrated therapeutic efficacy in some transplant models, the relative importance of these subsets has yet to be defined. Thus, although the Tr1, Th3, CD8$^+$ Ts and other lymphocyte subsets do not generally express Foxp3, and lack characteristic surface markers that allow selective identification and isolation, they are likely to all be part of the family of suppressor cells that are critical in the control of allograft rejection.

Involvement of non-T cells in Treg function

An ongoing question in the Treg field is whether Treg act directly upon Teff or whether they require an intermediary cell type *in vivo*. *In vitro*, it is clear that activated Treg can suppress Teff proliferation and cytokine production in the absence of any other cell types via direct cell-cell contact (27). However, Treg have also been shown to modulate the phenotype of dendritic cells *in vitro*, downregulating or preventing the expression of MHC class II, costimulatory molecules and IL-12. Ligation of CD40 or activation of Toll-like receptors (TLR) on DC appears to abrogate the effect of Treg (28), highlighting again the important relationship between innate immune responses and Treg function (Figure 2). Two recent *in vivo* imaging studies have demonstrated that Treg interact specifically with antigen bearing DC *in vivo* and prevent stable contacts between Teff and DC, a requirement for efficient priming of Teff (29,30). Notably, there was no evidence of specific interactions between Teff and Treg *in vivo*, supporting the notion that Treg act predominantly via DC *in vivo*, rather than through direct T-T interactions. As alluded to above, mature DC are resistant to the effect of Treg, and promote Teff priming. However, mature DC have been shown to cause proliferation of Treg *in vitro* (31), and are involved in stimulating Treg expansion in inflamed tissues, although Teff continue to outnumber and outperform Treg as a consequence of an increase in absolute numbers and a Treg resistant phenotype (32,33). These findings suggest that depletion or inactivation of Teff along with therapies that facilitate Treg expansion will be key components to the induction of transplantation tolerance. Thus, a model can be envisioned in which Treg interact with specific alloantigen-presenting cells, most likely donor and host DC, resulting in Treg activation. The antigen-specific activated Treg then interact with other DC, directly suppressing DC function and eliciting regulatory cytokines, therby mediating so-called 'bystander suppression'.

A recent study has suggested an important role for mast cells in the function of Treg. Although classically viewed as effectors in allergic immune responses, mast cells are increasingly recognized as important regulators of innate and adaptive immunity (34). Recently, Lu et al. have demonstrated increased numbers of mast cells and Treg in tolerated skin grafts when compared with rejecting skin grafts in a DST anti-CD154 model (35). Genetically mutant mice with diminished numbers of mast cells could not be made tolerant; however, reconstitution of mast cells was able to partially restore the ability to induce tolerance. The authors demonstrated that both natural and adaptive Treg secrete IL-9, a potent chemoattractant for mast cells, and that administration of anti-IL-9 antibody could prevent the prolongation of skin graft survival by Treg in an adoptive transfer system. It will be important to establish whether the requirement for mast cells in this model holds true for other types of transplants, particularly those tissues that do not normally have a large population of mast cells.

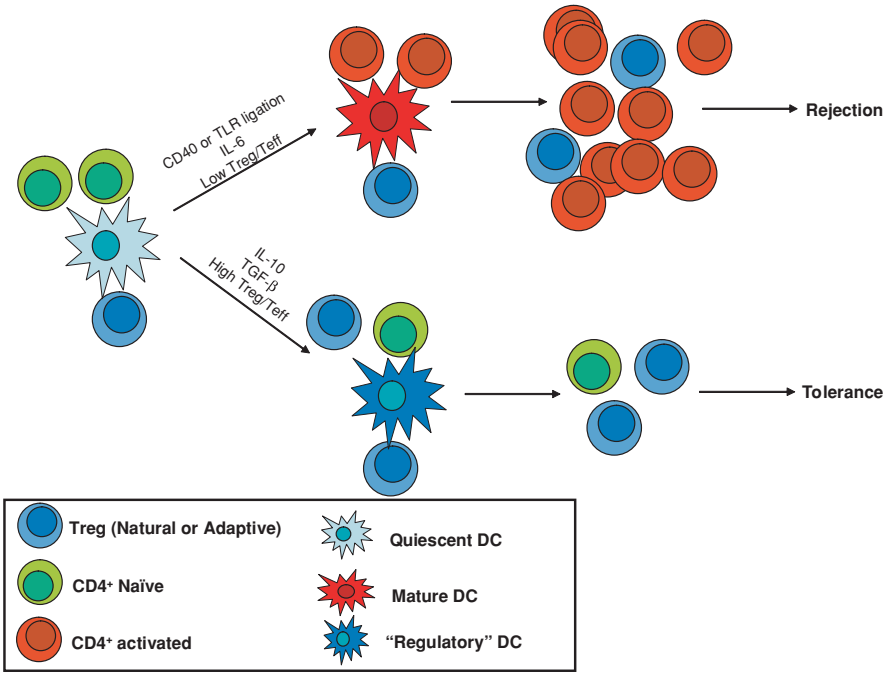

Figure 2: A Treg-centric model of rejection versus tolerance.

Antigen specificity of Treg

An early controversy in the field was whether Treg need to be antigen-specific to function. Stockinger and colleagues suggested that cognate antigen activation might not be essential for Treg activity (36). However, multiple studies have now shown that Treg suppressive function requires TCR activation, including a need for CD28-mediated co-stimulation (37). However, as stated above, although Treg must be activated by an antigen-specific TCR engagement, they can function 'nonspecifically' to inhibit T cells of an irrelevant specificity. Moreover, it should be remembered that Treg appear to preferentially localize to sites of inflammation where the antigen is expressed. Thus, the nonspecific suppressor functions are likely to be limited to localized areas of the graft or draining lymph node and not resulting in pan-suppression. These key concepts underlie the studies that show that antigen-specific Treg are more effective in models of autoimmune diabetes (38), bone marrow transplantation (9), and solid organ transplantation (39) compared to polyclonal Tregs. Thus, it is likely that in most instances, antigen-specific Treg will be required for optimal efficacy. However, the studies support the use of polyclonal Tregs in certain transplant settings due to the high alloreactive precursor frequency.

Prospects for therapeutic use of Treg in transplantation

Despite the abundance of evidence that Treg can be used therapeutically in models of autoimmunity and bone marrow transplantation, it has been difficult to show that Treg can be used to induce allospecific tolerance. Notably, there has been no demonstration of allospecific tolerance induction by infusion of Treg in an otherwise unmanipulated, fully MHC incompatible host. There are many reasons that might explain this negative outcome. First, a significant barrier to Treg-mediated tolerance is the high precursor frequency of alloreactive pathogenic T cells, which is estimated to be as high as 5–10% in fully allogeneic combinations (40). This precursor frequency is at least several orders of magnitude greater than that of nominal antigen-specific T-cell responses. Thus, if tolerance or rejection is in part determined by the balance between Treg and Teff (Treg/Teff ratio, Figure 2), far more Treg may be needed to induce tolerance to alloantigens than autoantigens. This may explain why Treg therapy is highly effective in non-manipulated models of autoimmunity (38), but has been relatively unsuccessful in nonmanipulated major mismatch transplantation models. Interestingly, spontaneous acceptance of cardiac allografts across a single MHC II disparity was shown to be mediated by Treg, consistent with the speculation that, under conditions of diminished precursor frequency, the Treg response can prevail over the Teff response for some graft types (41). The importance of the balance between Treg and Teff may explain why efficacy of therapeutic Treg in transplantation has been limited primarily to co-adoptive transfer models into irradiated or genetically lymphopenic hosts, wherein the number of Teff is dramatically reduced. Thus, it is likely that depletion of alloreactive Teff responses via apoptosis or anergy will be important for the induction of tolerance using Treg. Consistent with this idea, Zheng et al. have shown that combined treatment with rapamycin, agonist IL-2/Fc, and an antagonistic mutant IL-15/Fc could specifically deplete alloreactive T cells while preserving Treg (42). Remarkably, this therapy was shown to induce allospecific tolerance in

a stringent skin transplant model, as well as cardiac and islet allograft models.

A second issue unique to transplantation is the presence of the direct and indirect pathways of antigen presentation. Treg with direct specificity (39) as well as indirect specificity (43) have been shown to prolong allograft survival in murine models. It will be important to determine whether Treg with direct and indirect specificity will have synergistic effects, and whether they will have differential effects on acute and chronic rejection. If the effect of Treg *in vivo* is primarily via modulation of DC, Treg with indirect specificity are not likely to significantly affect donor-derived DC, while Treg with direct specificity may not be able to regulate self-DC presenting donor peptides. Treg with direct specificity are likely to have a much higher precursor frequency than Treg with indirect specificity, similar to the Teff cells. Thus, isolation and expansion of Treg with indirect specificity is likely to be challenging. However, techniques for antigen-specific expansion of Treg have been reported (9,43,44), suggesting that producing sufficient numbers of allospecific Treg for potential therapy will be feasible.

A third issue is that inflammatory signals associated with surgical trauma as well as ischemia–reperfusion injury may alter the inhibitory activity of Treg while augmenting the T effector response (Figure 2). For example, TLR engagement on DC has been shown to prevent suppression by Treg in an IL-6-dependent manner (45). The engagement of TLR and systemic release of IL-6 has been well documented after transplantation (46). IL-6 may also prevent the conversion of $CD4^+Foxp3^-$ cells into $CD4^+Foxp3^+$ Treg (17). Recently, Chen et al. showed that TLR engagement prevents anti-CD154-mediated tolerance in a murine heart transplant model. This result correlated with decreased recruitment of Treg to the graft (47). In other studies, the presence of IL-6 and TGF-β shift T cell differentiation away from Treg toward IL-17-producing, pathogenic Th17 cells (48). Reciprocally, Treg appear to be able to mitigate innate immune responses (49), and therapeutic Treg administration has been shown to decrease innate immune injury and promote engraftment in an islet transplant model (16). These results suggest that minimization of ischemia-reperfusion injury will be a key element of tolerance induction strategies. Further studies will also need to address issues that are important in the clinical setting, but are generally not modeled in rodent studies. For example, the effectiveness of Treg on memory T cells has not been clearly delineated. The effect of Treg on the generation of alloantibodies is also largely unknown.

Finally, application of Treg therapy to the clinical setting will require a better understanding of Treg in clinical transplantation where patients are receiving chronic immunosuppressive therapy. Recent studies have suggested a potential role for Treg in regulating allograft rejection. Suthanthiran's group reported that levels of urinary mRNA for Foxp3 were correlated with the reversal of acute rejection in renal transplant patients receiving conventional immunosuppressive therapy (50). Similarly, we reported last year at the World Transplant Congress that the absolute numbers of Treg were increased in the allograft tissue of patients undergoing acute graft rejection, with higher numbers in those patients that responded effectively to anti-rejection therapy (Belingheri M, Vincenti F, Gross D and Bluestone JA, unpublished observations). The elucidation of drugs that inhibit Teff function while maintaining or even enhancing Treg activity will be critical to Treg therapy. Rapamycin has been shown to promote expansion of human Treg *in vitro* (51), and to preserve Treg numbers in mouse models (52) as well as in renal transplant patients (53). On the other hand, calcineurin inhibitors appear to reduce the numbers of Treg in these patients (53). Mycophenolate mofetil in conjunction with vitamin D3 appears to favor induction of Treg in mouse models, but its effect in humans is not well established. Another commonly used agent, antithymocyte globulin, has recently been shown to preferentially expand Treg *in vitro*, optimally at concentrations generally lower than observed in patients (54). However, a recent short-term study in humans showed that Treg were efficiently depleted by both antithymocyte globulin and aletuzumab (55), which correlates with the lack of convincing data that these agents promote tolerance. Nevertheless, it is possible that these agents will be useful for lymphodepletion before Treg therapy in order to increase the Treg/Teff ratio. The effect of Abatacept (CTLA4Ig) and the related drug, Belatacept, on Treg is not yet clear. There is a concern that Treg may be depleted by B7 blockade, given the demonstration that CD28-B7 interactions are critical to the generation and survival of Treg (56). However, preliminary evidence suggests that combined Belatacept and anti-IL-2 receptor antibody treatment does not chronically deplete Treg after renal transplantation (57), likely because the blockade is nonsaturating.

Conclusions

The emergence of Treg as a central regulator of peripheral tolerance has raised the exciting possibility that Treg can be manipulated for improving outcomes in clinical transplantation. Although data from rodent models cannot be simply extrapolated, several key aspects are likely to be relevant for translation to the clinical arena. First, the importance of antigen specificity for optimal Treg activity *in vivo* is now well established. Second, alloreactive natural Treg exist and adaptive alloreactive Treg can be induced under some conditions. Third, alloreactive Treg can be expanded for therapeutic administration. Fourth, therapeutic administration of Treg alone is not likely to be successful in preventing rejection.

Several aspects of Treg biology that have particular relevance to organ transplantation remain to be fully elucidated. Understanding the effect of Treg on the direct and indirect pathways, on memory cells, and alloantibody

production will afford novel insights into Treg function and may afford new opportunities to harness Treg function to modulate these important players in clinical outcomes. In addition, further studies using fully MHC mismatched rodent models and possibly primate models need to be performed to understand how Treg therapy can be optimized for clinical translation. Questions such as efficacy relative to other immunomodulatory agents, potential synergy/antagonism with conventional agents, and overall immunosuppressive properties of infused Treg remain to be answered. Finally, it will also be essential to define how current immunosuppressive regimens affect Treg in transplant patients and correlate immunologic outcomes to changes in Treg/Teff ratio. Such data may provide an opportunity to design novel immunosuppressive schemes, suggest reliable methods for immunologic monitioring and possibly optimize prospects for tolerance induction with newer Treg-based modalities.

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