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T regulatory Cell–mediated Immunotherapy for Solid Organ Transplantation: A Clinical Perspective

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T regulatory cells (Tregs) play a vital role in suppressing heightened immune response, and thereby promote a state of immunological tolerance. Tregs modulate both innate and adaptive immunity, which makes them potential candidates for cell-based immunotherapy in the suppression of uncontrolled activation of graft-specific inflammatory cells and toxic mediators. Graft-specific inflammatory cells (T effector cells) and other inflammatory mediators (immunoglobulins, active complement mediators) are mainly responsible for graft vascular deterioration followed by acute/chronic rejection. Treg-mediated immunotherapy is under investigation to induce allospecific tolerance in various ongoing clinical trials in organ transplant recipients. Treg immunotherapy shows promising results; however, key issues regarding Treg immunotherapy remain unresolved, including the mechanism of action and specific Treg cell phenotypes responsible for a state of tolerance. This review highlights the involvement of various subsets of Tregs during immune suppression, the novelty of Treg functions, effects on angiogenesis, emerging technologies for effective Treg expansion, and plasticity and safety associated with clinical applications. Altogether, this information will assist in designing single/combined Treg-mediated therapies for successful clinical trials in solid organ transplantations.
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INTRODUCTION

A typical immune response requires a firm balance between activation and attenuation, dependent upon the balance of T effector and regulatory T cell function, in turn dependent on molecular signaling. Alterations in the cell transcriptional phase are critical to the onset of immune self-tolerance (1). Likewise, immunotherapies for organ transplantation face challenges in achieving enough immunosuppression to prevent organ rejection while limiting autoreactivity, without impairing the host's ability to guard against opportunistic infections and malignancies. The immune system defends the host from a broad range of pathogens and foreign tissue antigens while preventing unwarranted and exaggerated immune reactions that would be deleterious to the host tissue (2–4). During an immune response, T and B cells modulate an effective response against foreign tissue antigens, characterized by broad antigen recognition, high specificity, strong effector response and long-term immunologic memory (5,6). An effective immune response balances unresponsiveness to self-antigens (immunological self-tolerance) and the magnitude of adaptive immune responses to non–self-antigens, thereby preventing host tissue destruction (7–9) (Figure 1A). The model of immunotolerance explains how inadequate immune responses against tumor and microbial antigens in chronic infections can be augmented, or how aberrant immune responses to allograft can be regulated. Immunotolerance has been shown to modulate various populations of regulatory cells, which include T regulatory cells ($CD4^+$ $CD25^+FOXP3^+$ Tregs) (5,10), B regulatory cells ($CD19^+CD24^+CD38^+$ Bregs) (11,12), natural killer T cells ($CD16^+CD56^+$ NK T cells) (13) and, finally, dendritic cell–specific intercellular adhesion molecule-3–grabbing non-integrin cells (DC-$SIGN^+$ macrophages) (14).

Treg Subsets

Tregs, produced from naïve $CD4^+$ T cells in the thymus as functionally mature $CD4^+$ T cell subsets, play a vital role in providing immunological tolerance to self-antigens (15,16). The regulatory cells neutralize killer T cells during inflammation (17) and suppress heightened immune responses destructive to host tissue in organ transplant recipients (18–20).

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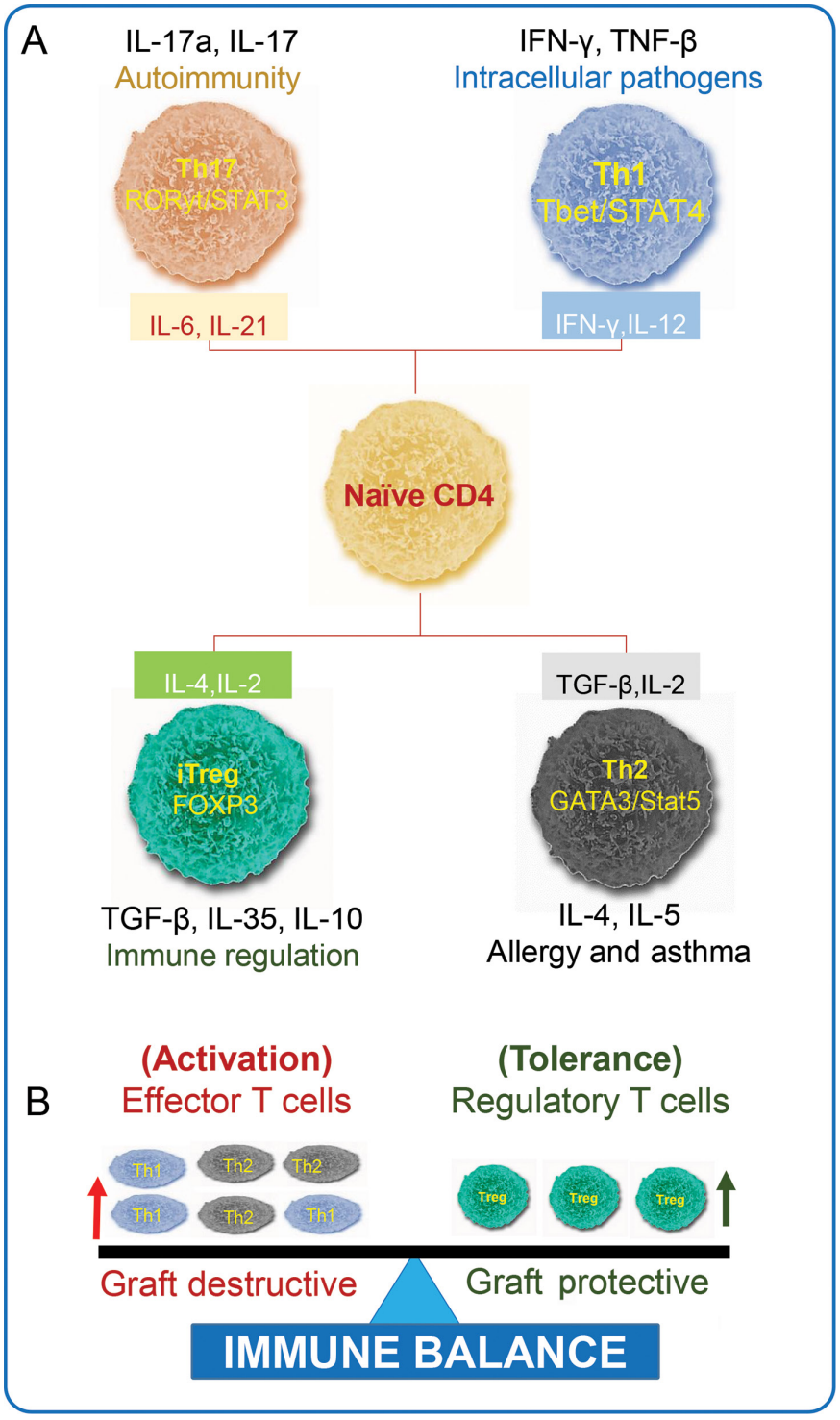

Figure 1. Development of Tregs and immune balance. (A) Treg develops from naïve CD4$^+$ T cell population under the influence of IL-4 and IL-2 and characterized by surface expression of CD25 and nuclear expression of FOXP3 compared to other T cell lineages. (B) Immune balance between Tregs (graft-protective cells) and T-effector cells (graft-destructive cells) modulate the effective immune response and immunotolerance to foreign antigens.

Tregs (5–10% CD4$^+$ T cells) are crucial to the regulation of self-tolerance and are capable of inhibiting antigen-specific inflammatory responses (7,21–24) (Figure 1B). Regulatory T cells, originally identified as antigen-specific T suppressor cells, uniquely express surface CD25 and the nuclear *FOXP3* gene (25,26). The *FOXP3* gene is required for immunosuppressive functions and regulation, acting through suppression of cytokines interleukin-2 (IL-2), interferon gamma (IFN-γ) and interleukin-4 (IL-4), and activation of interleukin-10 (IL-10), high-affinity IL-2R, CD25, cytotoxic T-lymphocyte–associated protein–4 (CTLA-4) and glucocorticoid-induced TNFR-related protein family–related genes/proteins (20,21,26–29). The *FOXP3* gene stimulates Treg-associated genes and stabilizes Treg features during antigen-specific activation while inhibiting expression of Th1-, Th2- and Th17-associated genes (26,30).

Distinct subsets of Tregs could play an important immunosuppressive role during rejection (31). Based on surface distribution of various expression proteins and state of origin, Treg subsets include natural Tregs (nTregs), inducible/adaptive Tregs (iTregs), inducible costimulator (ICOS$^+$) Tregs, IL-10–producing type 1 Tregs (Tr1 cells), CD8$^+$ Tregs, IL-17–producing Tregs and CD4$^+$VEGFR1^{HIGH} Tregs (32,33). These subsets share expression of the *FOXP3* gene (except for Tr1 cells) and secretion of inhibitory cytokine IL-10 and/or tumor growth factor beta (TGF-β).

nTREGs. nTregs are characterized by CD4, CD25 and FOXP3 and are involved in inhibiting T cell proliferation, suppressing dendritic cells (DCs) and inhibiting effector Th1, Th2 and Th17 cells. They also suppress mast cells, basophils and eosinophils, interact with resident tissue cells and participate in tissue remodeling thorough the release of IL-10 and TGF-β (26,34).

ICOS$^+$ Tregs. ICOS$^+$ Tregs are generated from nTregs and are characterized by surface expression of CD4, CD25, FOXP3 and ICOS (35). They are involved in suppression of hapten-reactive CD8$^+$ T

cells and release of IL-10, IL-17 and IFN-γ (36,37).

iTregs. iTregs are generated in the periphery and express CD4 FOXP3 as surface markers. They act through IL-10 and TGF-β (38–40).

Tr1 Cells. Tr1 cells, which display CD4 and CD25, are generated from non–Treg cell precursors and draining lymph nodes. They suppress effector Th cell migration and function and suppress mast cells, basophils and eosinophils through the release of IL-10 (41).

CD8$^+$ Tregs. CD8$^+$ Tregs, which display unique CD8, FOXP3, CD25, CD28 and CD122, are generated from CD8 cells. They are involved in blocking activation of naive or effector T cells, suppression of IgG/IgE antibody responses and IL-4 expression, and proliferation of CD4$^+$ T cells through IL-10, TNF-α and IFN-γ release (42,43).

IL-17–producing FOXP3$^+$. IL-17–producing FOXP3$^+$ Tregs, characterized by expression of CD4, FOXP3, chemokine receptor type 6 (CCR6) and RAR-related orphan receptor gamma transcription factor, are differentiated from CD4^{+-} FOXP3$^+$CCR6$^-$ Tregs in peripheral blood and lymphoid tissue. They are mainly involved in inhibiting proliferation of CD4$^+$ effector T cells through IL-17 (44).

CD4$^+$VEGFR1^{high} Treg. Recently, a new CD4$^+$VEGFR1^{high} Treg subset has been reported to suppress proliferation of CD4$^+$CD25$^-$ T cells as efficiently as CD4$^+$CD25^{high} natural Tregs in a contact-independent manner (32).

Treg-Mediated Immunosuppression

Treg activation is antigen-specific, which suggests that their immunosuppressive properties are a function of antigen exposure during an inflammatory response. *In vitro* experiments have shown that the suppressive property of Tregs requires T cell receptor (TCR)–mediated activation (45,46). In addition, antigen specificity modulates Treg proliferation and expansion in lymph nodes (47). Immunotolerance is linked with donor-specific Treg proliferation and expansion, possibly through the reconstitution of donor-antigen specific Tregs. This may prolong allograft survival and induce immunotolerance, as reported in different preclinical and clinical conditions of transplantation (48–52). Treg modulates antigen presentation of antigen-presenting cells (APCs) to conventional T-cells, which in turn become either anergic or regulatory cells (53–55). Therefore, empowering Treg at the expense of CD4$^+$ T can induce a state of immune privilege that could facilitate long-term graft survival.

The mechanism of Treg suppressive functions remains contentious. The major differences between *in vitro* and *in vivo* outcomes, specifically with the IL-10 and TGF-β inhibitory cytokines, have fueled the dispute (56). Under antigen-stimulated *in vitro* conditions, Tregs suppress the proliferation and cytokine production of effector T cells irrespective of antigen specificity (56). Tregs also play specialized regulatory functions during inflammatory conditions and are key regulators in switching off the immune response after the onset of the inflammatory phase (18). Under normal circumstances nTregs prevent autoimmune diseases while iTregs actively modulate transplantation tolerance (9,57). *In vivo* and *in vitro* Tregs are characterized by an anergic state with suppressive functions and are able to inhibit multiple stages of target cell activities (7,16). nTregs have the potential to suppress proliferation and differentiation of naïve T (CD4$^+$ and CD8$^+$) cells into effector T cells, and also suppress effector activities, differentiation and functions of NK cells, NK T cells, B cells, macrophages, osteoclasts and DCs (5,20,58). As reported in preclinical and clinical studies, immune modulation through Treg regulation is a decisive factor in allografts due to the insufficient number of Tregs, which favor allograft injury and rejection in organ transplantations (59–62). Various modes of Treg-mediated suppression have been proposed demonstrating that Tregs adopt various mechanisms for immunosuppression, including cell-contact–dependent secretion of immunosuppressive cytokines and local consumption of growth factors (8,58). Cell-cell interactions of Tregs and dendritic cells trigger the release of IFN-γ, a key inducer of indole amine 2, 3 dioxygenase, which catalyzes the conversion of tryptophan to kynurenine (63). In turn, release of kynurenine triggers generation of T regulatory cells (64,65).

During the process of immune inflammation, Tregs release a myriad of molecular mediators, which include several cytokines (TGF-β1, IL-35, IL-10), the cytotoxic molecule perforin and granzymes, to mitigate immunosuppressive T cells and control inflammation (45,66) (Figure 2). TGF-β1 is one of the key mediators, performing both offensive (67) and defensive (68,69) functions of Tregs during immunosuppression. Tregs utilize TGF-β1 to suppress T cell activation and differentiation to dampen inflammatory response (70). In addition, TGF-β1 released from Tregs has the potential not only to convert naive T cells into iTregs and Th17 to assist in their fight against local inflammatory condition, and defend Tregs against apoptosis and destabilization during inflammatory phase (68,69), but also to affect the activity of cytotoxic T cells and APCs (71) (Figure 2). During a regulatory response, TGF-β1 plays a key role in inflammation, T cell lineage, antibody production, immunosuppression and maintenance of tolerance (70). TGF-β1 is also critical to the development and differentiation of FOXP3$^+$ Tregs (72–74). In addition, TGF-β1 is essential for the generation of IL-17–producing Th17 cells (70), and recent findings indicate that TGF-β1 is involved in the generation of IL-9–producing Th9 cells (75). These observations highlight the role of TGF-β1 in T cell proliferation and differentiation (75). Furthermore, TGF-β1 suppress cytokine released by activated CD4$^+$ T cells without restricting differentiation and apoptosis, while IL-10 assists activated T cells to TGF- β1 response through the expression of TGF receptors (71). Various Treg surface markers have been proposed for this direct interaction. These include glucocorticoid-induced TNFR-related

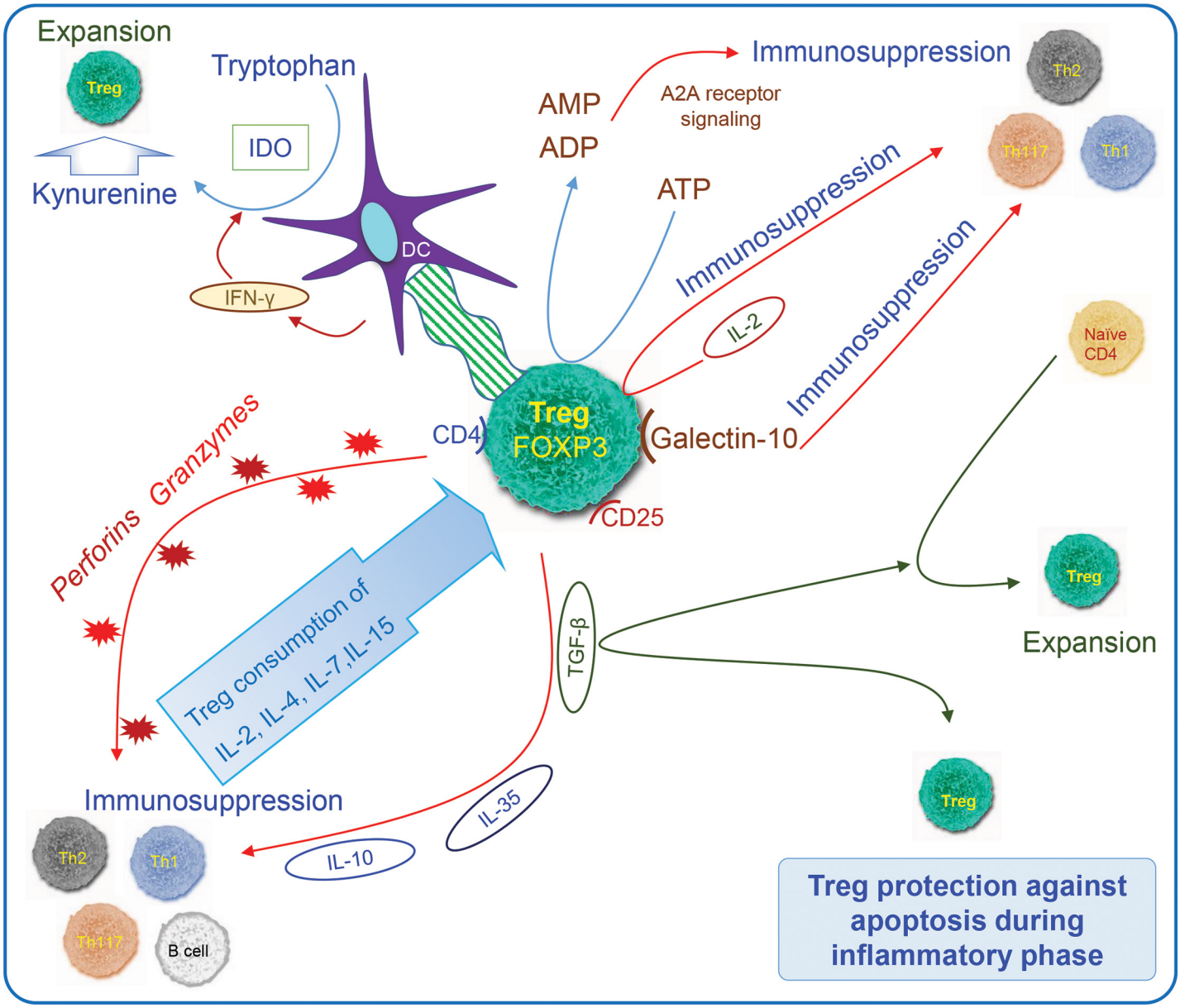

Figure 2. Array of Treg-mediated immunosuppression. This demonstrates different mediators of Treg-mediated immunosuppression, which mainly includes IL-10, TGF-β and IL-35, consumption of IL-2, IL-4, IL-7 and IL-35, or release of perforins/granzymes. TGF-β also plays both offensive (immunosuppression) and defensive (Treg protection) roles as it suppress T-effector functions but protects Tregs from the surrounding inflammatory environment.

protein, CTLA-4, membrane-bound TGF-β, LAG-3 and the cytolytic molecules Fas and granzyme B (76). However, constitutive expression of CD25 by Tregs gives them an initial competitive advantage for the consumption of IL-2 over naïve T cells, which express CD25 only after TCR stimulation (58). As reported earlier, nTregs predominantly produce immunosuppressive IL-35, a new member of the IL-12 family, which confers a suppressive activity of Tregs (77).

Tregs are also involved in growth factor consumption and cytokine deprivation, and thus favor target cell apoptosis. This phenomenon of Treg-mediated immunosuppression involves competitive consumption of IL-2. However, under *in vitro* conditions, Tregs can immunosuppress IL-2R–deficient T cells in the presence of exogenous IL-2. This favors target T cell proliferation in the presence of Tregs while endogenous target T cell production of IL-2 remains suppressed (58). Tregs

have been characterized as displaying a wide range of immunosuppressive mediators, including TGF-β, CTLA-4, IL-10 and galectin-1, although it is still uncertain which mode of immunosuppression is the main mediator of immunoregulatory properties (78). In addition, Tregs actively produce extracellular adenosine (ADP and AMP) and promote suppression of T effector cells through adenosine receptor signaling (79,80). CTLA-4 is a costimulation receptor and plays a crucial role in the development of T cell anergy. Some studies report that engagement of CTLA-4 with B7 on the APC leads to activation of the Treg (81), which further facilitates the release of several inhibitory cytokines such as IL-10 and TGF-β (82). Furthermore, proliferation of Tregs can be induced, leading to a positive feedback mechanism that ultimately results in the downregulation of T effector cell response in an antigen-specific manner (83). Tregs can either inhibit effector activity of conventional T cells or downregulate APC function of target cells (58). Additionally, Treg-mediated immunosuppression can also operate through various mechanisms that involve not only cellular components but also unique proteins known as sirtuins (84). These cellular and molecular signals require close spatial proximity between Tregs and effector T cells. Sirtuin 1 (Srt1) has antiinflammatory properties, and its therapeutic targeting may be a valuable factor in organ transplantation (84–87). Activated Treg cells show downregulated Sirt1, and this may be a key process in stabilizing FOXP3 expression and Treg phenotypes (86), which may have clinical benefits in autoimmunity and transplantation (84,85,88). Information regarding the role of sertuins in the immune system has been sparse. However, enhanced Treg immunosuppressive activity and attenuated immune responses as a result of Srt1 deletion in $CD4^+$ T and Treg cells have been reported, and both *Srt1* deletion and *Srt1* inhibition cause prolonged allograft survival (88,89). These investigations explain that the loss of *Srt1* led to upregulation of proinflammatory cytokines and was required for deacetylating RelA/p65 in Tregs, which is required for the increased immunosuppressive capacity of Tregs (89). Therefore, *Srt1* targeting can offer important therapeutic options for T cell–dependent immune responses in experimental models of transplantation, which enable allograft survival through enhanced Treg function in the fl-Sirt1/$FOXP3^+$ cre model (88–90). Notably, effector T cell function was practically unaffected by *Srt1* deletion. Furthermore, transfection of Srt1 and $FOXP3^+$ into HEK 293 cancer cells prevents its proteasomal degradation through the deacetylation of $FOXP3^+$ (85,86). Studies in sirtuin1 knockout mice reported that native Tregs express high $FOXP3^+$, and inhibition or deletion of *Srt1* can favor formation of acetylated $FOXP3^+$, which is protected from proteasomal degradation (91).

Tregs and Angiogenesis

The process of angiogenesis in ischemic tissues is controlled by immune cells, which require Tregs and macrophages, with chemokines playing a key role in new vessel growth (92). Tregs play a key role in suppressing excessive immune response during an inflammatory response, and also support vascular repair at different levels (93). Loss of microvasculature may be an unappreciated root cause of chronic rejection for all solid-organ transplants (2,94). In clinical conditions, the ischemic phase favors the process of neovascularization (including vasculogenesis and angiogenesis) and characterizes the tissue microvascular repair and remodeling during allograft rejection (94–97). In addition to tissue-specific initial activators, neovascularization requires growth factors, chemokines and proteases that play distinct roles in promoting and refining tissue repair and regeneration. Most of the cellular machinery of the immune system plays a key role during the process of microvascular repair (98). The involvement of T lymphocytes is also shown in microvascular and vessel development and, as reported, T cell–deficient nude mice exhibit a distinct reduction in microvascular and vessel growth (99,100). Furthermore, it has been proposed that $CD4^+$ and $CD8^+$ cells play a key role in vascular remodeling, as CD4- and CD8-deficient mice display a major reduction in vessel growth (101). In addition, leukocytes release angiogenic mediators, including vascular endothelial growth factor (VEGF) and proinflammatory cytokines TNF-α and IL-1β, which initiate neovascularization and microvascular establishment and organize the tissue response to ischemic conditions (102,103). A number of cytokines secreted by pathogenic T cells affect the survival and function of Treg cells, specifically IL-2 released by peripheral pathogenic T cells after CD28 interaction multiplies the Treg cell population (72). Numerous costimulatory signals have been involved in inflammatory T cell activation and differentiation, of which the B7/CD28 and CD40L/CD40 pathways play key roles, which suggests that loss of Treg cell–mediated self-tolerance affects both T and B cell–mediated tolerance (24). Of note, CD28 interactions with the ligands B7-1 (CD80) and B7-2 (CD86) are also vital for the development of $CD4^+CD25^+FOXP3^+$ Treg cells (104). In addition, release of immunosuppressive cytokines TGF-β and IL-10 by Treg cells suppresses dendritic cells, which further inactivates effector T cells and monocytes (8,71). Expression of IL-10 and TGF-β by Tregs offers a possible mechanism to explain how these cells limit inflammatory injury and possibly accelerate recovery (1). Furthermore, Tregs also modulate inflammatory responses of innate immune cells, including macrophages, monocytes, DCs, NK cells and the complement activation system, which highlights that the immunoregulatory role of Tregs is not limited to the adaptive immune system (105).

The complement pathway is a unique part of innate immunity, and the receptors C3aR and C5aR are present in a variety of cells, including Tregs, and signaling through both C3aR and C5aR on nTregs cells has been reported to inhibit regulatory functions of Tregs (106). Inhibition or genetic deficiency of both C3aR

and C5aR on nTreg cells augments their *in vitro* and *in vivo* suppressive activity and prolongs skin allograft survival (106). In addition, C3aR/C5aR deficiency or inhibition further triggers the activation of murine iTreg cells, stabilizes expression of the *FOXP3* gene and precludes iTreg conversion to IFN-γ/TNF-α–producing T effector cells, thereby limiting graft-versus-host disease (106). Liu et al. showed an antagonistic relation between $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ Treg cells on the polarization of macrophage phenotypes (107). They highlighted that $CD4^+CD25^+$ Treg favors M2 macrophage polarization, whereas M1 macrophages can be induced by $CD4^+CD25^-$ T effector cells (108). Phenotypically, M2 macrophages play a crucial antiinflammatory and reparative role by secretion of IL-10, IL-1β and TGF-β, which regulate tissue repair and promote angiogenesis through VEGF secretion (109). Tregs affect angiogenesis through both indirect and direct mechanisms and have the potential to stimulate angiogenesis indirectly by Th1 cell suppression through the release of TNFα and IFN-γ cytokines, as well as interferon-induced chemokines such as CXCL9, -10 and -11 (110). Further, $CD4^+CD25^+$ Tregs release a surplus of VEGF at the steady state as well as under hypoxic conditions when compared with $CD4^+CD25^-$T cells, while demonstrating capillary formation *in vitro* through VEGF signaling (111). Also, it was recently reported in a mouse model of orthotopic tracheal transplantation that adoptive transfer of $CD4^+CD25^+FOXP3^+$ Tregs ameliorated functional microvascular blood flow between donor and recipient grafts, which further facilitated allograft recovery from the severe hypoxia phase (112,113). In addition, supernatants of hypoxic Tregs were able to promote angiogenesis *in vivo* in cell-free Matrigel implants (114). As reported in a left lung ischemia model, an increase in $CD4^+CD25^+FOXP3^+$ Tregs cells was observed 3–5 d after the onset of ischemia in C57Bl/6 WT mice (93). Further experiments with adoptive transfer of $CD4^+CD25^+$ lymphocytes into $FOXP3^+$ Treg–depleted mice showed almost complete recovery of the angiogenic phenotype (115). Furthermore, recent studies highlighted a relationship between Tregs and vascular wall function in cardiovascular disease (116), showing that an increase in apoptotic Tregs triggers induction of vascular inflammation and impaired endothelium-dependent relaxation in coronary arterioles in hypertension, whereas reconstitution of Tregs subdues arterial blood pressure and improves coronary arteriolar endothelial function through the release of IL-10 (116) (Figure 3).

Treg-Mediated Immunotherapy in Transplants

Organ transplantation can be a life-saving procedure for patients with end-stage disease of the lung, heart, kidney or liver. Unfortunately, this treatment strategy is limited by chronic rejection of the transplanted organ, which occurs when the patient's immune system continually attacks and impairs the organ and ceases vascular flow required for graft survival (117,118). This process affects nearly all patients in the first 10 years following transplantation, and there is no effective therapy for this condition. Treg cell–based therapy can be achieved by administering Tregs cells to diseased patients (119). Tregs ($CD4^+CD25^+FOXP3^+$) play a crucial role in self-tolerance and graft immunity as well as in controlling infections, and outcomes of preclinical models have recognized them as a vital candidate for cell therapy, e.g., for the treatment of transplant-related complications such as graft-versus-host disease following allogeneic stem cell transplantation (15,120). Currently, different translational approaches have been utilized to induce Tregs though anti–TNF receptor super family member 25 (121), IL-2/mAb complexes (122), anti-CD45RB (123), rapamycin-mediated (124), mitomycin C–incubated myeloid blood cells (MICs), regulatory macrophages (Mregs) (125) and regulatory dendritic cells (DCregs) (126), which show positive therapeutic effects in preclinical studies (127,128). Tregs are mainly isolated and expanded *ex vivo*, but few preclinical studies have successfully expanded Treg populations through *in vivo* treatment with either anti-CD45RB antibody or anti–TNF receptor super family member 25 antibody or IL-2-IL2 complex or recombinant IL-33 (123,129–132) (Figure 3). *TNFRSF25*, also known as DR3, is constitutively and highly expressed by $CD4^+FOXP3^+$ Tregs, which are mostly involved in autoimmunity (121), while CD45 is a protein tyrosine phosphatase receptor type C that regulates T and B cell antigen receptor signaling and lymphocyte activation (133), and anti-CD45RB is a potent tolerogenic molecule that works by boosting the Treg number (123). This immune modulation of Tregs occurs by specifically inducing proliferative expansion of Tregs *in vivo*, and recent data have suggested that this occurs through specific enhancement of interactions between Tregs and APCs via an unknown mechanism but could potentially be a milestone discovery for *in vivo* Treg expansion if replicated in humans (123). In contrast, *ex vivo* Treg cell–based therapy is a clinically approved strategy to harness the immunosuppressive properties of Tregs for therapeutic use (134). In this therapeutic approach, Tregs are isolated from a patient, enriched, expanded *ex vivo* and adoptively transferred to the patient (135) (Figure 3). This cell-based therapeutic approach is beneficial because the expanded Treg cell population can be screened phenotypically and functionally prior to adoptive transfer under controlled conditions (135).

Several immunotherapeutic strategies implicating the use of Tregs have been developed, some of which have been used in clinical trials in organ transplantation (Table 1) (135). Preclinical and clinical studies have demonstrated the therapeutic relevance of Tregs in allograft survival in different transplantation models (52,136–138). Several clinical studies have shown an increase in peripheral $CD4^+CD25^{high}$ T cells in operationally tolerant liver transplant

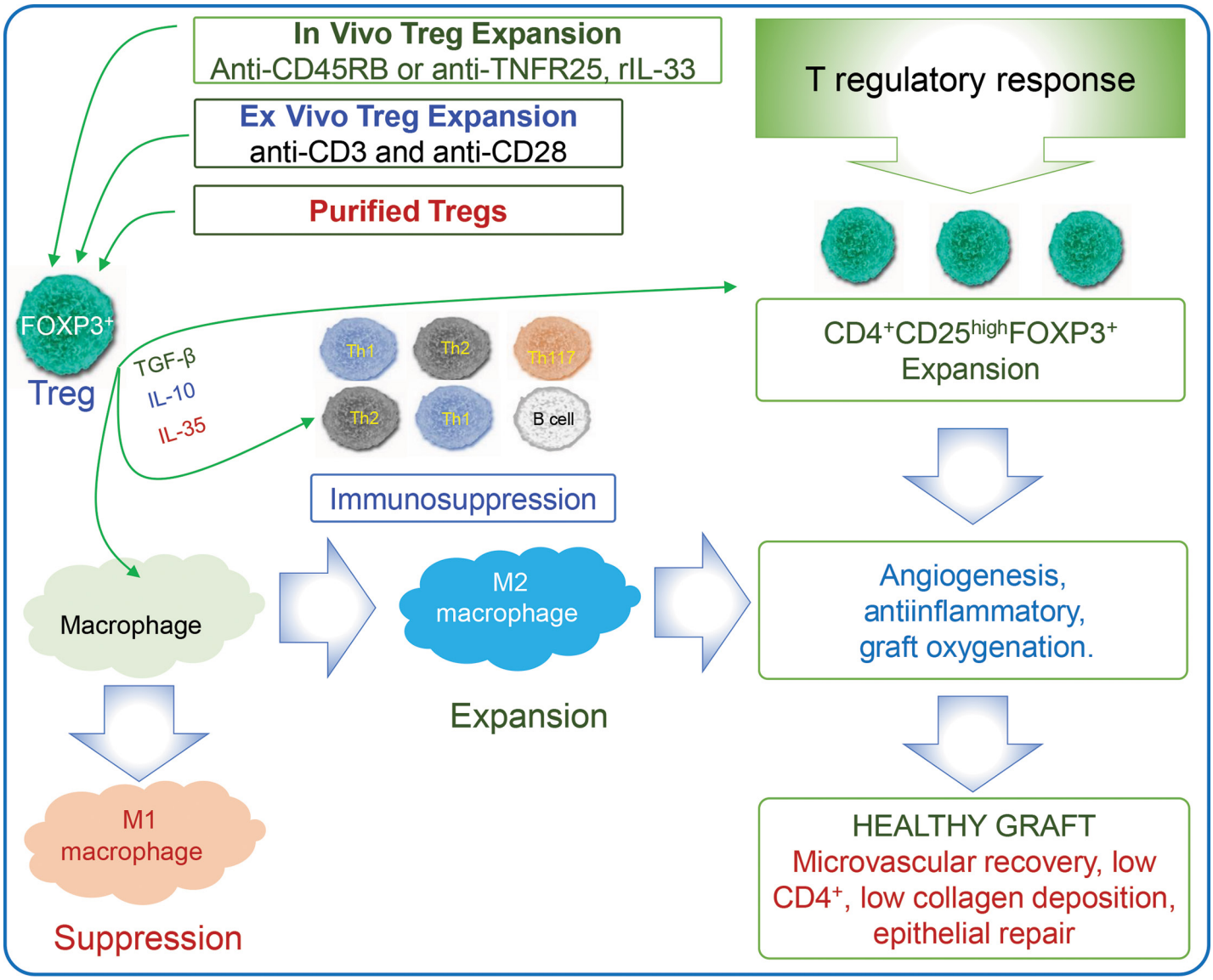

Figure 3. Treg therapy and immunotolerance. This demonstrates *ex vivo* and *in vivo* expansion of freshly isolated Tregs for cell therapy to rescue allograft rejection. Adoptive transfer of Tregs shows downregulation of $CD4^+$ T cells followed by upregulation of Th2 responses, which favor microvascular and tissue repair in rejecting allograft.

recipients (60,139,140), and an Initial observation that $CD4^+CD25^{high}$ T cells play a pivotal role in transplantation tolerance has been well demonstrated in mouse models (20). Sakaguchi et al. showed depletion of $CD4^+CD25^{high}$ T cells from enhanced graft rejection, while dose-dependent reconstitution of $CD4^+CD25^{high}$ T cells prolonged allograft survival (59,141,142). Clinical studies in transplantation have investigated the number and functional properties of regulatory $CD4^+CD25^{high}$ Tregs in relation to immunological quiescence, tolerance and acute or chronic rejection (143,144). Moreover, Tregs known to be crucial in the maintenance of peripheral immune tolerance are a critical modulator of post-ischemic neovascularization in a hind limb ischemia model (145). In humans, there are only few distinctive markers to distinguish $CD4^+CD25^{high}$ T cells from conventional activated T cells. However, the α-chain of the IL-7 receptor (CD127) allows a clear variation between Treg–activated $CD4^+CD25^+$ T cells (146,147); further, this marker could also be used in patients after solid organ transplantation of liver and kidney (127,128), and CD127-based characterized Treg and activated T cell subsets have been reported to be differentially distributed in healthy individuals as compared

Table 1. List of clinical trials of T regulatory cell–based immunotherapy.

Treatment of Children with Kidney Transplants by Injection of CD4+CD25+FoxP3+ T Cells to Prevent Organ Rejection (NCT01446484)
T-Regulatory Cell Infusion Post Umbilical Cord Blood Transplant in Patients with Advanced Hematologic Cancer (NCT00602693)
Infusion of T-Regulatory Cells in Kidney Transplant Recipients (the ONE Study) (NCT02091232)
T-Regulatory Cell Kinetics, Stem Cell Transplantation, REGKINE NCT00578461
Donor-Alloantigen-Reactive Regulatory T Cell (darTregs) in Liver Transplantation (deLTa) (NCT02188719)
Treg Adoptive Therapy for Subclinical Inflammation in Kidney Transplantation (TASK) NCT02088931
Phase 1 Infused Donor T Regulatory Cells in Steroid Dependent/Refractory Chronic GVHD NCT01911039
Donor Regulatory T Cells in Treating Patients with Visceral Acute Graft-versus-Host Disease after Stem Cell Transplant NCT02526329

to transplant recipients (148). However, the ratio of activated T cell subsets among $CD4^+CD25^{high}$ T cells was augmented in stable liver and kidney transplant recipients as compared to healthy individuals (149). The use of expanded Tregs and iTregs compared to nTregs requires more phenotypic evaluation for safety and quality control. CD127 is less useful after strong activation, for example, as it can downregulate on conventional T cells (150). As discussed earlier, the plasticity and safety of expanded Tregs mainly depends on their state of FOXP3 expression (151). The standard therapeutic intervention after transplantation should induce tolerance, and regulatory T cells play a pivotal role in maintaining homeostasis and self-tolerance through the modulation of immune effector functions (7,16). Treg cells can be found inside the tolerated graft, and these cells can have indirect allospecificity for donor antigens (151). In patients transplanted with lung, liver or kidney grafts, a positive correlation between graft survival and the number of circulating Treg cells has been reported in both preclinical and clinical conditions (152), and based on these observations, it is widely accepted that Tregs play a pivotal role in the induction of transplantation tolerance (153–155). Therefore, this supports the possibility of using Tregs as a biological therapy to maintain tolerance to alloantigens. Preclinical research findings report that reconstitution of Tregs has been shown to ameliorate graft-versus-host disease and facilitate engraftment of the bone marrow (156–158). Originally, natural Tregs had to maintain immunological self-tolerance, but deficiency or dysfunction of these cells may lead to the onset of autoimmune disease (5). However, it was later realized that a decline in their number or function can also provoke tumor immunity (159), whereas their antigen-specific subset expansion can potentiate transplantation tolerance (5). Furthermore, Treg-mediated immunotolerance has been implicated in other pathological conditions including allergies, microbial infections and fetomaternal tolerance (120,160,161), and in organ transplantation (52). Based on these outcomes, elevating Treg numbers or their suppressive properties may be key in treating autoimmune disease and preventing allograft rejection. On the other hand, depletion of Treg cells or inhibition of their regulatory function could enhance immunity against tumors and chronic infectious agents (162).

Treg Plasticity and Safety

$FOXP3^+$ plays an important role in the development and immunosuppressive function of Tregs (8,80,163). Treg functions are modulated through transcription as well as post-translation modifications, including lysine residue acetylation, which protects $FOXP3^+$ from degradation and thus promotes optimum Treg function (163). The acetylation process of the *FOXP3*$^+$ gene is structured by the histone/protein acetyltransferases and histone/protein deacetylases (89). Interestingly, targeted deletion of sirtuin1 upregulates the process of acetylation and ultimately the expression of $FOXP3^+$ and augments the immunosuppressive mechanism of Tregs (88). However, deletion or inhibition of sirtuin1 attenuates allograft rejection and prolongs survival of murine cardiac allografts (85). Based on the origin, two different forms of FOXP3 Tregs exist, of which naturally occurring $CD4^+CD25^+FOXP3^+$ Tregs (nTregs) originate in the thymus after TCR stimulation through MHC self-antigen complex interaction, followed by signaling through CD28 and CD25 (164), while induced Tregs (iTregs) develop from naive $CD4^+$ T cells through TGF-β1 stimulation (164), and more specifically, generation of iTregs has been explained in GALT, spleen, lymph node, chronically inflamed and transplanted tissues (165,166). Functionally, under Th17 or Th1 cell-polarizing conditions, nTreg cells differentiated to a substantial fraction of $IL\text{-}17^+FOXP3^+$ or $IFN\gamma^+FOXP3^+$ cells arose without downregulation of *FOXP3* expression (167–169). In contrast, under Th17 cell-polarizing conditions, TGF-β–induced Tregs lost their *FOXP3* expression and acquired IL-17 expression (168). There is a notion that the difference in *FOXP3* stability between nTreg cells and iTreg cells is due to epigenetic modifications at the *FOXP3* locus (170), which contains a highly conserved CpG-rich region upstream of exon –1 and is referred to as the Treg cell–specific demethylated region (171–173). This specific *FOXP3* locus is fully demethylated in nTreg cells but remains methylated in iTreg cells and activated human conventional T cells that transiently express FOXP3 (164).

Tregs have been shown to display a unique feature that depends on their *FOXP3* expression and the demethylation state of the conserved non-coding region 2/ Treg-specific demethylated region and Treg cell representative regions.

This feature is the indicator of Treg exposure to specific antigens, the strength and duration of costimulation, and T cell receptor signaling. Therefore, genetically the Treg population is a mishmash of FOXP3$^+$epigenome$^+$ stable Tregs, potential Tregs (FOXP 3$^-$epigenome$^+$) and transient Tregs (FOXP3$^+$epigenome$^-$). The stability and plasticity of Tregs are usually affected by the balance between their intrinsic *FOXP3* expression, stabilizing and destabilizing signals that regulate their effective immunosuppressive function. The association of these key functions is likely to be apparent and influenced by the developmental, environmental and inflammatory microenvironment and state of tolerance, thereby generating different mediators to initiate Treg stability or instability (174).

CONCLUSIONS

Recent research has highlighted the cellular and molecular basis of Treg development and function, and implicated dysregulation of Tregs in major immunological diseases, including allograft rejection (19,23). Tregs are instrumental in establishing immune tolerance and are important cellular mediators of cell-based therapy for clinical applications (135). Efforts to unravel the complexity of Tregs are only just beginning, and further understanding of their biology and characterization of targets will undoubtedly enhance future therapeutic opportunities (10,88,134). Increasing evidence indicates that Tregs could be used to inhibit pathogenic anti-transplant immunity (in the absence of immune suppression), but mechanisms to accomplish this goal are hampered by inadequate understanding, Treg expansion, cost of GMP Treg manufacturing, safety and difficulties with the stability of the Treg phenotype after adoptive transfer. The present and future therapeutic scope of Treg-based therapy will hopefully minimize the drug burden of immunosuppression on solid organ transplant patients. In coming years, Treg cell–based therapy will provide a novel therapeutic platform in transplantation as well as in other diseases. This will further assist both clinicians and researchers in testing the combinations of current drug-based therapies with Tregs, which will further minimize the side effects and thus morbidity caused by these toxic immunosuppressants (175). Treg-based immunotherapy assures antigen-specific immunosuppression and cell dosage can be tightly controlled and affect long-lasting regulation in vivo, and can be individualized to each patient with very limited side effects (135). Tregs possess unique defensive and offensive mechanisms, antigen specificity and promising therapeutic potential, as reported in both preclinical and clinical studies of solid organ transplantation, but these cells do not have sufficient efficacy as a stand-alone therapy to prevent chronic rejection in solid organ transplantation, and certain factors, including dose, specificity and plasticity, remain uncertain and challenging areas to uncover (176). The future of Treg-based immunotherapy is dependent on effective clinical trials, technological advancements in Treg manufacture/expansion and better mechanistic understanding of Treg biology and transplantation tolerance in humans.

In summary, Treg-based immune control can be implemented as a potential pharmaceutical tool through an adoptive cell therapy protocol to rescue patients with inflammatory diseases, chronic inflammation and transplantation. This review highlights the clinical significance of Tregs and also emphasizes the difficulties encountered in transitioning from bench to bedside. Although Treg therapy is very effective and does not present side effects, various challenges, including different methods of Treg expansion, the cost of GMP-Treg manufacturing, safety, and difficulties with stability of the Treg phenotypes after adoptive transfer mean that its use as a standard therapy remains a challenge.

DISCLOSURE

The author declares that he has no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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