Title: T regulatory cell mediated immunotherapy for solid organ transplantation: A clinical perspective

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Running title: Treg therapy in transplantation.

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ABSTRACT:
T regulatory cells (Tregs) play a vital role in suppressing heightened immune responses, and thereby promote a state of immunological tolerance. Tregs modulate both innate and adaptive immunity, which make them a potential candidate for cell-based immunotherapy to suppress uncontrolled activation of graft specific inflammatory cells and their toxic mediators. These grafts 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 is showing promising results but the key issues regarding Treg immunotherapy are not yet fully resolved including their mechanism of action, and specific Treg cell phenotype responsible for a state of tolerance. This review highlights the involvement of various subsets of Tregs during immune suppression, novelty of Tregs functions, effects on angiogenesis, emerging technologies for effective Treg expansion, 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.
INTRODUCTION:

A typical immune response requires a firm balance between activation and attenuation, and the balance of T effector and Treg functions reliant on various molecular signals, therefore any alterations in the cell transcriptional phase is critical to the onset of state of immune self-tolerance (1). Likewise, most immunotherapies for organ transplantation face the challenges of achieving enough immunosuppression to prevent organ rejection or limit auto reactivity without impairing the host's ability to guard against opportunistic infections, and malignancies. 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 cells, B cells, which are characterized by a broad range in antigen recognition, high specificity, strong effector response, and long-term immunologic memory, modulate an effective immune response against a foreign tissue antigens (5) (6). A major function of an effective immune response is to balance unresponsiveness to self-antigens (immunological self-tolerance) and magnitude of adaptive immune responses to non-self-antigens, and thus prevent host tissue destructions (7-9) (Figure 1A). The state 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. Immune tolerance has been shown to modulated by various population of regulatory cells, which mainly include T regulatory cells CD4⁺CD25⁺FOXP3⁺ Tregs (5, 10), CD19⁺CD24⁺CD38⁺ B regulatory cells (Bregs) (11, 12), CD16⁺CD56⁺ NKT cells (13), Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN⁺) macrophages (14) etc.

TREG-SUBSETS:

Tregs are produced from naïve CD4⁺ T cells in the thymus as a functionally mature CD4⁺ T cell subsets, and play a vital role in providing immunological tolerance to self-antigens (15, 16), in neutralizing killer T cells during inflammation (17) and in suppressing heightened immune responses
destructive to the host tissue in organ transplant recipients (18-20). Tregs consist of 5-10% of CD4+ T cells, are crucial to regulation of self-tolerance, and are also capable of inhibiting antigen-specific inflammatory responses (7, 21-24) (Figure 1 B). Tregs were originally identified as antigen specific T suppressor cells and later recognized as Tregs, which uniquely express surface CD25, and nuclear FOXP3 gene (25, 26). FOXP3 gene is required for the immunosuppressive functions and regulates through the suppression of IL-2, IFN-γ, and IL-4 cytokines, but activates the IL-10, high-affinity IL-2R, CD25, CTLA-4, and the GITR family-related gene/protein (20, 21, 26-29). FOXP3 gene stimulate Treg-associated genes and stabilize Treg features during antigen specific activation while inhibiting the expression of Th1, Th2 and Th17 associated genes (26, 30). There are different subsets of Tregs have been reported, which could play an important immunosuppressive role during rejection (31). Based on the surface distribution of various expression proteins and state of origin, Treg subsets include natural Tregs (nTreg), inducible/adaptive Tregs (iTreg), inducible costimulator ICOS+ Tregs, IL-10-producing type 1 Tregs (Tr1 cells), CD8+ Tregs, IL-17-producing Tregs and CD4+VEGFR1HIGH Tregs (32, 33). These Treg subsets share expression of FOXP3 (except for Tr1 cells), and secretion of inhibitory cytokine IL-10 and/or TGF-β. In addition, nTreg are characterized by CD4, CD25, FOXP3, and mainly involve in inhibiting T cell proliferation, suppression of DCs, inhibition of effector Th1, Th2, and Th17 cells; suppress mast cells, basophils, and eosinophils; interact with resident tissue cells and participate tissue remodeling through the release of IL-10, TGF-β (26, 34). ICOS+ Treg are generated from nTregs and basically characterized by the surface expression of CD4, CD25, FOXP3, ICOS (35), and has been involved in suppression of hapten-reactive CD8+ T cells and mainly release IL-10, IL-17, IFN-γ (36, 37). iTregs are generated in periphery and express CD4 FOXP3 as surface markers and act through IL-10 and TGF-β (38-40). Tr1 cells, which display CD4, CD25 are generated from non-Treg cell precursors and draining lymph nodes suppress effector Th cell migration and functions; suppress mast cells, basophils, and eosinophils through the release of IL-10 (41). Beside these regulatory subsets, CD8+ Tregs, which display unique CD8, FOXP3, CD25, CD28, CD122 are generated from CD8 cells, mainly involve in blocking activation of naive or
effector T cells; suppress IgG/IgE antibody responses, IL-4 expression and the proliferation of CD4+ T cells through IL-10, TNF-α, IFN-γ release (42, 43). Other Treg subsets include IL-17-producing FOXP3+ Treg, which are characterized by expression of CD4, FOXP3, CCR6, RORGTF differentiated from CD4+FOXP3+CCR6- Tregs in peripheral blood and lymphoid tissue mainly involve in Inhibiting the proliferation of CD4+ effector T cells through IL-17 (44). In addition, recently a new CD4+VEGFR1high Treg subset has been reported to suppress the proliferation of CD4+CD25− T cell as efficiently as CD4+CD25high natural Tregs in a contact-independent manner (33).

TREG-MEDIATED IMMUNOSUPPRESSION:

Activation of Tregs is antigen-specific, which suggests that immunosuppressive properties are dependent on antigen exposure during inflammatory response and in-vitro experiments have shown that suppressive property of Tregs requires TCR mediated activation (45, 46). In addition, antigen specificity also modulate 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, which may prolong allograft survival and induce immunotolerance as reported in different preclinical and clinical conditions of transplantation (48-52). Treg modulates antigen presentation of 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 can facilitate long-term graft survival.

The mechanism of Treg suppressive functions remain scanty and contentious, and the major differences between in-vitro and in-vivo outcomes, specifically with the IL-10 and TGF-β inhibitory cytokines have fueled the argument (56) as 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 therefore a key regulator to switch off the immune response after the onset of inflammatory phase(18). Under normal circumstances natural Treg (nTreg) prevent autoimmune diseases, while
peripherally induced Treg (iTreg) actively modulate transplantation tolerance (9, 57). *In-vivo* and *in-vitro* Tregs are characterized by an anergic state with suppressive functions, and able to inhibit multiple stages of target cell activities (7, 16). nTregs have potential to suppress the proliferation and differentiation of naïve T (CD4+ and CD8+) cells in to effector T cells, and also suppress effector activities, differentiation, functions of NK cells, NK T cells, B cells, macrophages, osteoclasts, and DCs (5, 20, 58). As reported in pre-clinical and clinical studies that immune modulation through Tregs regulation is a decisive factor in allograft because of the insufficient number of Treg favors allograft injury and rejection in organ transplantations (59-62). There are different modes of Treg mediated suppression have been proposed; which demonstrate that Tregs adopt various mechanism to immunosuppression, mainly includes cell-contact-dependent, secretion of immunosuppressive cytokines, and local consumptions of growth factors (8, 58). Cell –cell interactions of Treg and dendritic cell trigger the release of IFN-γ, a key inducer of indole amine 2, 3 dioxygenase (IDO) which catalyzes the conversion of tryptophan in to kynurenine (63), and the release of kynurenine triggers the generation of T regulatory cells (64, 65).

During the process of immune inflammation, Tregs release myriads of molecular mediators, which include cytokines TGF-β1, IL-35, IL-10, and cytotoxic molecules perforin, and granzymes to mitigate immuno suppressive T cells and control inflammation (45, 66) (Figure 2). Of which, TGF-β1 is a key mediator, which performs both offensive (67) and defensive (68, 69) functions of Tregs cells 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 not only has potential to convert naïve T cells into iTregs and Th17 to assist in their fight against local inflammatory condition, defend Tregs against apoptosis and destabilization during inflammatory phase (68, 69) but also 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, immune suppression, and tolerance (72, 73), and also critical for the development and differentiation of FOXP3+ Tregs (74-76). In addition, TGF-β1 is essential for the generation of IL-17 producing Th17 cells (23, 26, 77), and recent finding
indicates that TGF-β1 is also involved in the generation of IL-9 producing Th9 cells (78). These observations highlighted the role of TGF-β1 on T cell proliferation and differentiation (78). Furthermore, TGF-β1 suppress cytokine released by activated CD4⁺ T cells without restricting differentiation and apoptosis, whilst IL-10 assist activated T cells to TGF-β1 response through the expression of TGF receptor (71). There are various Treg surface markers have been proposed for this direct interaction, which includes GITR, CTLA-4, membrane bound TGF-β, LAG-3 and the cytolytic molecules Fas and granzymes B (79) but constitutive expression of CD25 by Tregs give them an initial competitive advantage for the consumption of IL-2 over naïve T cells, which express CD25 only after TCR stimulation (8, 57). As reported earlier, nTregs predominantly produce immunosuppressive IL-35, a new member of the IL-12 family, which confers suppressive activity of Treg (80).

Tregs are also involved in growth factors consumption, cytokine deprivation, and thus favors target cells apoptosis. This phenomenon of Treg-mediated immunosuppression mainly involve competitive consumption of IL-2, however, under in-vitro conditions, Tregs can immunosuppress IL-2R deficient T cells in presence of exogenous IL-2, which favors target T cells to proliferate in the presence of Tregs although endogenous target T cells production of IL-2 remains suppressed (81-83). Tregs have been characterized to display 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 (84). In addition, Tregs actively produce extracellular adenosine (ADP and AMP), and promote suppression of T effector cells through A2AR (adenosine receptor) signaling (85, 86). CTLA-4 is a costimulation receptor and constitutively play a crucial role in the development of T cell anergy, and some studies reported that engagement of CTLA-4 with its B7 on the antigen presenting cells (APC) leads to the activation of the Treg (87), which further facilitate the release of several inhibitory cytokines such as IL-10 and TGF-β(88). Furthermore, proliferation of Tregs may be induced, leading to a positive feedback mechanism that ultimately results in the
downregulation of T effector cell response in an antigen specific manner (89). Tregs can either inhibit effector activity of conventional T cells or down-regulate APC function of target cells (58). Additionally, Treg mediated immunosuppression may also operate through various mechanism, which not only involve cellular components but also involve unique proteins known as Sirtuin (90). These cellular and molecular signals require close spatial proximity between Tregs and effector T cells. Sirtuin 1 (Srt1) has an anti-inflammatory property, and its therapeutic targeting may be a valuable factor in organ transplantation (90-93). Activated Treg cells show downregulated Sirt1 and this may be a key process to stabilize FOXP3 expression and Treg phenotype (92), which may have clinical benefits in autoimmunity and transplantation (90, 91, 94). The role of sertuins in immune system has been scanty, 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 (94, 95). These investigations explained that the loss of Srt1 led to upregulation of pro-inflammatory cytokines and is also required for deacetylating RelA/p65 in Tregs which is required for increased immunosuppressive capacity of Tregs (95). Therefore, Srt1 targeting can achieve important therapeutic options for T cell-dependent immune responses in experimental models of transplantations, which enable allograft survival through enhanced Treg function in the fl-Sirt1/ FOXP3+ cre model (94-96). Notably, effector T cell function was practically unaffected by Srt1 deletion. Furthermore, transfection of Srt1 and FOXP3+ into HEK 293 cancer cells, prevent its proteasomal degradation through the deacetylation of FOXP3+ (91, 92). Studies in Sirtuin1 knockout mice reported that native Tregs express high FOXP3+ and inhibiting or deleting Srt 1 can favor formation of acetylated FOXP3+, which is protected from proteasomal degradation (97).

**TREGS AND ANGIOGENESIS:**

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 (98). Tregs play a key role in suppressing excessive immune responses during an inflammatory response, and also support a
vascular repair at different levels (99). Loss of microvasculature may be an unappreciated root cause of chronic rejection for all solid organ transplants (2, 100). In clinical conditions, ischemia phase favors the process of neovascularization including vasculogenesis and angiogenesis, characterizes the tissue microvascular repair and remodeling during allograft rejection (100-103). In addition to specific initial activators, neovascularization requires growth factors, chemokines, and proteases that play distinct roles in promoting and refining tissue repair and regeneration. Most of cellular machineries of the immune system play a key role during the process of microvascular repair (104) and the involvement of T lymphocytes are also shown in microvascular and vessel developments, and as reported, T cells deficient nude mice exhibit a distinct reduction in microvascular and vessel growth (105, 106). Furthermore, CD4+ and CD8+ cells have been suggested to play a key role in vascular remodeling as CD4- and CD8-deficient mice display a major reduction in vessel growth (107). In addition, Leukocytes also release a bunch of angiogenic mediators including VEGF and pro-inflammatory cytokines TNF-α and IL-1β, which initiate neovascularization, microvascular establishment and organize the tissue response to ischemic condition (108, 109). A number of cytokines secreted by pathogenic T cells affect the survival and function of Treg cells specifically IL-2 release by peripheral pathogenic T cells after CD28 interaction multiply the Treg cell population (74). Numerous costimulatory signals have been involved in inflammatory T-cell activation and differentiation, of which, the B7/CD28 and the CD40L/CD40 pathways play key roles, which suggested 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 (110). In addition, release of the immunosuppressive cytokines TGF-β and IL-10 by Treg cells suppress dendritic cells, which further inactivate effector T cells and monocytes (8, 71). Expression of IL-10 and TGF-β by Tregs offer a possible mechanism explaining how these cells limit inflammatory injury, and possibly accelerate recovery (1). Furthermore, Tregs also modulates inflammatory responses of innate immune
cells including macrophages, monocytes, DCs, NK cells and complement activation system, which highlighted that immunoregulatory role of Tregs is not limited only to adaptive immune system (111).

Complement pathway is a unique part of innate immunity and their 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 (101, 112). Inhibition/ or genetic deficiency of both C3aR/C5aR on nTreg cells augment their in-vitro and in-vivo suppressive activity and prolongs skin allograft survival (113). In addition, C3aR/C5aR deficiency/ or inhibition further triggers the activation of murine iTreg cells, stabilizes expression of FOXP3 gene, preclude iTreg conversion to IFN-γ/TNF-α producing T effector cells, and thereby limit graft versus host diseases (114, 115). Liu et al. showed an antagonistic relation between CD4+CD25− T cells and CD4+CD25+ Treg cells on the polarization of macrophage phenotypes(116). They highlighted that CD4+CD25+ Treg favors M2 macrophage polarization whereas the M1 macrophages can be induced by CD4+CD25− T effector cells (117). Phenotypically, M2 macrophages play a crucial anti-inflammatory and reparative role by secretion of IL-10, IL-1β, and TGF-β, which regulates both tissue repair and promote angiogenesis through VEGF secretion (118). Tregs affected angiogenesis through both indirect and direct mechanisms and have potential to stimulate angiogenesis indirectly by Th1 cells suppression through the release of TNFα and IFN-γ cytokines, as well as interferon-induced chemokines such as CXCL9, 10 and 11(119). Further, CD4+CD25+ Treg cells release surplus of VEGF at the steady state as well as under hypoxic conditions when compared with CD4+CD25−T cells, while demonstrated capillary formation in vitro through VEGF signaling (120). In addition, supernatants of hypoxic Tregs were able to promote angiogenesis in-vivo in cell-free Matrigel implants (120). As reported in left lung ischemia model, an increase in CD4+CD25+FOXP3+ Tregs cells was observed 3-5 days after the onset of ischemia in C57Bl/6 WT mice (99). Further experiments with adoptive transfer of CD4+CD25+ lymphocytes into FOXP3+ Treg depleted mice showed an almost complete recovery of the angiogenic phenotype (99). Furthermore, recent studies
highlighted a relationship between Tregs and vascular wall function in cardiovascular diseases (121), which showed that an increase in apoptotic Tregs triggers the induction of vascular inflammation and impaired endothelium-dependent relaxation in coronary arterioles in hypertension, whereas reconstitution of Tregs subdue arterial blood pressure and improves coronary arteriolar endothelial functions through the release of IL-10 (121) (Figure 3).

TREGS CELL-BASED IMMUNOTHERAPY IN TRANSPLANTS:

Organ transplantation can be a life-saving procedure for patients with end-stage diseases of lung, heart, kidney and liver. Unfortunately, this treatment strategy is limited by the occurrence of chronic rejection of transplanted organ, which occurs when the patient’s immune system continually attacks and impairs the transplanted organ, and cease vascular flow required for graft survival (122, 123). This process affects nearly 100% of patients in the first ten years of following transplantation, and there is not any effective therapy for this condition. Treg cell-based therapy can be achieved by administration of Tregs cells in to diseased patients (124). Tregs (CD4+CD25+FOXP3+) play an crucial role in self-tolerance and graft immunity as well as in controlling infections, and outcomes of pre-clinical models have recognized them a vital candidate for cell therapy, e.g. for the treatment of transplant-related complications, such as GVHD following allogeneic stem cell transplantation (15, 125). Currently, different translational approaches have been utilized to induce Tregs though anti-TNFR25 (126), IL-2/mAb complexes (127), anti-CD45RB (128), rapamycin mediated (129), mitomycin C-incubated myeloid blood cells (MICs), regulatory macrophages (Mregs)(130), and regulatory dendritic cells (DCregs)(131) which shows positive therapeutic effects in preclinical studies (132, 133). Tregs are mainly isolated and expanded ex-vivo but few pre-clinical studies successfully expanded Treg population through in-vivo treatment either with anti-CD45RB antibody or anti-TNFR25 antibody or (IL-2-IL2 complex) or recombinant IL-33 (128, 134-137) (Figure 3). TNFRSF25; also known as DR3, is constitutively and highly expressed by CD4+FOXP3+ Tregs, which mostly involved in autoimmunity (126) while CD45 is a protein tyrosine phosphatase receptor type C.
(PTPRC) that regulates T- and B-cell antigen receptor signaling and lymphocyte activation (138), and anti-CD45RB is a potent tolerogenic molecule that works by boosting the Treg number (128). This immune modulation of Tregs occurs by specifically inducing proliferative expansion of Tregs \textit{in-vivo}, and latest data has suggested that this occurs through specific enhancement of interactions between Tregs and antigen presenting cells (APCs) with unknown mechanism but could be a potentially a milestone discovery for \textit{in-vivo} TREG expansion if replicated in humans (128). In contrast, \textit{ex-vivo} Treg cell-based therapy is a clinically approved strategy to harness the immunosuppressive properties of Tregs for therapeutic use (139). In this therapeutic approach, Tregs are first isolated from a patient, enriched, expanded \textit{ex-vivo}, and adoptively transfer to patients (140) (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 (140).

Several immunotherapeutic strategies implicating the use of Tregs have been developed, some of which has already been under clinical trials in organ transplantations (Table 1) (140). Preclinical and clinical studies have demonstrated the therapeutic relevance of Tregs in allograft survival in different transplantation models (52, 141-143). Several clinical studies have shown an increase in peripheral CD4^{+}CD25^{high} T cells in operationally tolerant Liver Transplant Recipients (60, 144, 145), Initial observation that CD4^{+}CD25^{high} T cells play a pivotal role in transplantation tolerance has been well demonstrated in mouse models (20). Sakaguchi S et al showed that depletion of CD4^{+}CD25^{high} T from enhanced graft rejection, while dose dependent reconstitution of CD4^{+}CD25^{high} T cells prolonged allograft survival, (59, 146, 147). 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 (148, 149). Moreover, Tregs known to be crucial in the maintenance of peripheral immune tolerance are a critical modulator of post-ischemic neovascularization in hind limb ischemia model (150). In human, 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), allowed a clear variation between Treg activated CD4^{+}CD25^{+} T
cells (151, 152), further this marker could also be used in patients after solid organ transplantation in liver and kidney [127,128] and CD127 based characterized Treg and activated T cell subsets have been reported to differentially distributed in healthy individuals as compared to transplant recipients (153). However, ratio of the activated T cell subsets among CD4^{+}CD25^{high} T cells was augmented in stable liver and kidney transplant recipients as compared to healthy individuals(154). The use of expanded Tregs and iTregs compared to nTregs required more phenotypic evaluation for safety and quality control. CD127 is less useful after strong activation, for example, as it can down-regulate on conventional T cells (155). As it has been discussed earlier that the plasticity and safety of expanded Tregs mainly depends on their state of FOXP3 expression (156). 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 tolerized graft and these cells can have indirect allospecificity for donor antigens (156). In patients transplanted with lungs, 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 (157) and based on these observations, it is widely accepted that Tregs play a pivotal role for the induction of transplantation tolerance (158-160). Therefore, this supported the possibility of using Tregs cells as a biological therapy to maintain tolerance to alloantigens. Preclinical research findings reported that reconstitution of Treg cells has been shown to ameliorate graft-versus host diseases and facilitate engraftment of the bone marrow (161-163). Originally, natural Tregs have to maintain immunological self-tolerance but deficiency or dysfunction of these cells may be leads to onset of autoimmune disease (5). However, it was then realized that a decline in their number or function can also provoke tumor immunity (164), whereas their antigen-specific subset expansion can potentiate transplantation tolerance (5). Furthermore, Treg mediated immunotolerance has been implicated in other pathological conditions including allergy, microbial infections, and foeto-maternal tolerance (125, 165, 166) and in organ transplantation(52). Based on these outcomes, elevating Treg number or their suppressive property may be a key feature
for treating autoimmune diseases 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 (167).

TREG PLASTICITY AND SAFETY:
The FOXP3+ plays an important role in the development and immunosuppressive functions of Treg (8, 86, 168). Treg functions are modulated through transcription as well as post-translation modifications, including lysine residue acetylation, which protects FOXP3+ from degradation and thus promotes to optimum Treg functions (168). Acetylation process of FOXP3+ gene is structured by the histone/protein acetyltransferases and histone/protein deacetylases (95). Interestingly, targeted deletion of Sirtuin1 upregulated the process of acetylation and ultimately the expression of FOXP3+, and augments the immunosuppressive mechanism of Tregs (94). However, deletion/ or inhibition of Sirtuin1 attenuates allograft rejection, and prolongs survival of murine cardiac allografts (91). Based on the origin, there are two different forms of FOXP3 Tregs exist, of which, naturally occurring CD4+CD25+FOXP3+ Tregs (nTregs) originated in the thymus after TCR stimulation through MHC/self-antigen complex interaction, followed by signaling through CD28 and CD25 (169), while Induced Tregs (iTregs) develops from naive CD4+ T cells through TGF-β1 stimulation (169), and more specifically the generation of iTregs has been explained in the GALT, spleen, lymph node, chronically inflamed tissues, and transplanted tissues (170, 171). Functionally, under Th17 or Th1 cell-polarizing conditions, nTreg cells differentiated in to a substantial fraction of IL-17+FOXP3+ or IFNγ+FOXP3+ cells arose without down-regulation of FOXP3 expression (172-174). In contrast, under Th17 cell-polarizing conditions TGF-β-induced Tregs lost their FOXP3 expression and acquired IL-17 expression (173). It is notion that the difference in FOXP3 stability between nTreg cells and iTreg cells is due to epigenetic modifications at the FOXP3 locus (175), which contains a highly conserved CpG-rich region upstream of exon −1 is referred to as the Treg cell-specific demethylated region.
(176-178). 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 (169).

Tregs have been shown to display a unique feature, which depends on their FOXP3 expression and demethylation state of the CNS2/TSDR and Treg cell representative regions. This feature is the indicator of Treg exposure to specific antigen, the strength, and duration of costimulation and T cell receptor signaling. Therefore, genetically 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 is usually affected by the balance between their intrinsic FOXP3 expression, stabilizing, and destabilizing signals which regulate their effective immunosuppressive functions. The association of these key functions is likely to be apparent, and influenced by the developmental, environmental, and, the type of inflammatory microenvironment, and state of tolerance, thereby generating different mediators to initiate Treg stability or instability (179).

CONCLUSIONS:
Recent research investigations highlighted the cellular and molecular basis of Treg developments and functions, and also implicated dysregulation of Tregs in major immunological diseases including allograft rejection (19, 23). On the basis of the information explained above, it should be apparent that Tregs are instrumental in establishing immune tolerance, and are important cellular mediators of cell based therapy for clinical applications(140). Efforts to unravel the complexity of Treg are only just the beginning, and a further understanding of their biology and characterization of targets will undoubtedly enhance future therapeutic opportunities (10, 94, 139). 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 is hampered by inadequate understandings, Treg expansions, cost of GMP Treg manufacturing, safety, and difficulties with stability of the Treg phenotype after adoptive transfer. The present and future therapeutic scope of Treg based therapy
will hopefully minimize drug burden of immunosuppression on solid organ transplant patients. In coming years, Treg cell based therapy will provide novel therapeutic platform in transplantation as well as in other diseases. This will further assist both clinicians, and researchers to test the combinations of current drug based therapies with Tregs, which will further minimize the side effects, and thus morbidity caused by these toxic immunosuppressant (180). The Treg-based immunotherapy assures antigen specific immunosuppression, cell dosage can be tightly controlled, affect long-lasting regulation in-vivo, and can be individualized to each patient with very limited side effects (140). Tregs possess unique defense and offence mechanism, antigen specificity and promising therapeutic potential as reported in both preclinical and clinical studies of solid organ in transplantation but these cells do not have sufficient efficacy as a stand-alone therapy to prevent chronic rejection for solid organ transplantation, and certain factors, which include dose, specificity, and plasticity remain uncertain and challenging areas to uncover. The future of Treg based immunotherapy depends on effective clinical trial designs, technological advancement 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 potential pharmaceutical tool through adoptive cell therapy protocol to rescue patients with inflammatory diseases, chronic inflammation and transplantation. This review highlighted the clinical significance of Treg, and also emphasized the difficulties encountered in transitioning from the bench-to-bedside. Although, Treg therapy is very effective, and does not pose any side effects but various challenges including different methods of Treg expansion, cost of GMP-Treg manufacturing, safety, and difficulties with stability of the Treg phenotypes after adoptive transfer still remain a big issues to completely resolve.
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Abbreviations: APC: Antigen presenting cell; Bregs: B regulatory cells; CNS2: Conserved non-coding region 2; TREG: T regulatory cells; A2AR: Adenosine receptor; CCR6: Chemokine receptor type 6; CTLA-4: cytotoxic T-lymphocyte-associated protein 4; GITR: Glucocorticoid-induced TNFR-related protein; ICOS: Inducible costimulator; IDO: indole amine 2, 3 dioxygenase; TCR: T cell receptor; TSDR: Treg-Specific Demethylated Region; MCP-1: Monocyte chemoattractant protein-1; PTPRC: Protein tyrosine phosphatase, receptor type, C; RORG TF: RAR-related orphan receptor gamma transcription factor; TNFR25: TNF receptor super family member 25.

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FIGURE LEGENDS

FIGURE 1) Development of Tregs and Immune Balance: A) Treg develops from naïve CD4\(^+\) T cells population under the influence of IL-4 and IL-2 and characterized by the 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.

FIGURE 2) Array of Treg-mediated immunosuppression: 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 play both offensive (immunosuppression) and defensive (Treg protection) roles as it suppress Teffector functions but protect Treg from surrounding inflammatory environment.

FIGURE 3) Treg therapy and Immunotolerance: demonstrates an Ex-vivo and In-vivo expansion of freshly isolated Tregs for cell therapy to rescue allograft rejection. Adoptive transfer of Tregs show downregulation of CD4\(^+\) T cells followed by upregulation of Th2 responses, which favor microvascular and tissue repair in rejecting allograft.
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**FIGURE 1**

A. **Naïve CD4**

- **Th1**
  - IFN-γ, TNF-β
  - Intracellular pathogens
  - IL-12, IL-21

- **Th2**
  - IL-4, IL-5
  - Allergy and Asthma
  - IL-2, IL-21

- **Th17**
  - IL-17a, IL-17
  - Autoimmunity
  - IL-6, IL-21

- **iTreg**
  - FOXP3
  - Immune regulation
  - TGF-β, IL-35, IL-10

B. **(Activation) Effector T cells**

- **Th1**
- **Th2**

**Graft destructive**

B. **(Tolerance) Regulatory T cells**

- **iTreg**

**Graft Protective**

**IMMUNE BALANCE**
Treg protection against apoptosis during inflammatory conditions
**FIGURE 3**

**In-Vivo TREG Expansion**
- Anti-CD45RB or anti-TNFR25, rIL-33

**Ex-Vivo TREG Expansion**
- anti-CD3 and anti-CD28

**T regulatory response**
- CD4+CD25highFOXP3+
  - Expansion
  - Angiogenesis, Anti-inflammatory, Graft oxygenation.
  - HEALTHY GRAFT
  - Microvascular recovery, low CD4+, low collagen deposition, epithelial repair

**Immunosuppression**
- Th1
- Th2
- Th17
- B cell

**Expansion**
- M2 macrophage

**Suppression**
- M1 macrophage

**Macrophage**
- TGF-β
- IL-10
- IL-35

**FOXP3+**
- Treg

**Molecular Medicine**
### Table 1: Tregs in Clinical Trials

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