Title

Impact of Immune-Modulatory Drugs on Regulatory T Cell.

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Impact of immune-modulatory drugs on Tregs

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Abbreviations:

ADCC, antigen dependent cellular cytotoxicity

APC, antigen presenting cell

ATG, anti-thymocyte globulin

CNI, calcineurin inhibitor

CsA, cyclosporine

GILZ, glucocorticoid-induced leucine zipper

GVHD, graft-versus-host disease

IMPDH, inosine monophosphate dehydrogenase

JAK, Janus-associated kinase

LFA, lymphocyte function-associated antigen

mAb, monoclonal antibody

MLR, mixed lymphocyte reaction

MMF, mycophenolate mofetil

MS, multiple sclerosis

NFAT, nuclear factor of activated T cells

mTOR, mammalian Target of Rapamycin

mTORC, mTOR complex

PI3K, phosphatidylinositol 3-kinase

RA, rheumatoid arthritis

Siglec-10, sialic acid-binding immunoglobulin-like lectins-10

TAC, tacrolimus

Tconv, conventional T cell

TCR, T cell receptor

Teff, effector T cell

TGF-β, transforming growth factor- β

Treg, regulatory T cells

TSDR, Treg-specific demethylated region

Abstract

Immunosuppression strategies that selectively inhibit effector T cells while preserving and even enhancing CD4$^+$FOXP3$^+$ regulatory T cells (Tregs) permit immune self regulation and may allow minimization of immunosuppression and associated toxicities. Many immunosuppressive drugs were developed before the identity and function of Tregs were appreciated. A good understanding of the interactions between Tregs and immunosuppressive agents will be valuable to the effective design of more tolerable immunosuppression regimens. This review will discuss pre-clinical and clinical evidence regarding the influence of current and emerging immunosuppressive drugs on Treg homeostasis, stability, and function as a guideline for the selection and development of Treg-friendly immunosuppressive regimens.

Introduction

The identification of immunosuppressive medications, particularly calcineurin inhibitors (CNI), has allowed the field of transplantation to develop. Contemporaneously, we have learned that the immune system can be re-educated to accommodate changing self and foreign tissues through disarming effector T cells and generation of regulatory T cells (Tregs). There are many types of Tregs including CD4^{+} and CD8^{+} cells expressing the transcription factor FOXP3 and the IL-10-producing Tr1 cells. In this review, we focus on the CD4^{+}FOXP3^{+} subset. These Tregs constitutively express CD25, the α chain of the IL-2 receptor that confers high sensitivity to IL-2. Tregs are essential for immune homeostasis and tolerance to self and foreign antigens including allografts[1,2]. Because of substantial toxicity of immunosuppression medications, there has been increasing interest in promoting transplant immune tolerance so that immunosuppression can be minimized or withdrawn. Many immunosuppressants were designed to broadly mitigate T cell function, including that of Tregs. This review focuses on the impact of immunosuppressive drugs on Tregs with the goal of identifying Treg-supportive immunosuppressive regimens and providing guidelines for rationalized design of therapeutics for promoting immune self regulation in transplantation.

Development, homeostasis, and function of Tregs

Tregs can develop from maturing CD4^{+}CD8^{-} thymocytes and from mature CD4^{+} T cells after they exit the thymus. While Treg development in the thymus (tTregs) and in the periphery (pTregs) both depend on signaling through T cell receptors (TCR), there are differences in the role of TCR signaling intensity on these subsets of Tregs. In the

thymus, strong TCR signaling with CD28 costimulation, just below the threshold for negative selection, promote tTreg lineage commitment[3]. In the periphery, persistent weak TCR stimulation along with IL-2, transforming growth factor-β (TGF-β) or retinoic acid is conducive to pTreg development [4], a process abrogated by strong costimulation. pTregs express FOXP3 and cell surface markers similar to that of tTregs. While tTregs also express transcription factor HELIOS and cell surface protein neuropilin 1, pTregs generally do not, although some exceptions have been reported[5-9]. In addition, DNA in tTregs is demethylated in the Treg-specific demethylated region (TSDR) in the FOXP3 enhancer, whereas TSDR of pTregs is only partially demethylated[7]. The incompletely demethylated TSDR leaves pTregs more prone to lose FOXP3 expression and function. Overall, tTregs are a stable lineage of cells with specificity toward thymically expressed self antigens; whereas pTregs are a more dynamic population recruited to ensure tolerance to new antigens encountered in the periphery. Both populations are essential to immune tolerance[10].

Tregs require IL-2 to maintain their lineage stability, and because Tregs do not make IL-2, they are dependent on IL-2 from other T cells and dendritic cells. Tregs are highly sensitive to IL-2, due to their constitutively high expression of CD25 and amplified intracellular signal transduction downstream of the IL-2 receptor[11]. Tregs can thus be considered the "first responders" to IL-2, competing with conventional T cells (Tconvs) for IL-2 as a mechanism to prevent unwanted immune responses. Defects in the IL-2 receptor, IL-2 signaling, or limited IL-2 availability leads to Treg destabilization. On the other hand, very high levels of IL-2, either provided therapeutically or because of potent immune activation, override Treg suppression and allow immune responses to proceed.

Thus, IL-2 signaling is essential to tolerance mediated by Tregs and the level of IL-2 is a critical determinant of immune activation versus tolerance.

Tregs can modulate the stimulatory capacity of antigen presenting cells (APCs) by removing CD80 and CD86 from their surface through CTLA-4-mediated transcytosis[12]. The resulting reduction of co-simulation increases the threshold for Tconv activation. During an active immune response, TCR and cytokine stimulations induce Treg trafficking to inflammatory sites where they use a broader array of suppressive mechanisms to dampen inflammation and limit collateral tissue damage[13]. Activated Tregs can also induce new pTregs with distinct alloantigen specificity leading to an "infectious" spread of tolerance[14].

Immunosuppressive medications inhibit many of these critical Treg pathways described above. This off-target inhibition of Tregs may impede tolerance while preventing effector T cells from attacking allografts. However, research in Treg signaling in recent years has revealed some distinct intracellular signaling pathways in Tregs versus Tconvs. Knowing these distinctions will guide the use of immunosuppressive drugs to promote Tregs.

Immunosuppression for transplantation

Solid organ transplant recipients typically receive a combination immunosuppressive regimen given at the time of transplantation (induction therapy) and during the maintenance phase[15]. Induction agents may be broadly classified as depleting or non-depleting depending on whether they act by killing or inhibiting immune cells. Depleting induction agents include anti-thymocyte globulin (Thymoglobulin, Genzyme; Atgam,

Pfizer), monoclonal antibodies (mAb) against CD3 (Muromonab, Janssen-Cilag), and anti-CD52 mAb (alemtuzumab; Campath, Genzyme). Non-depleting agents include methylprednisolone and anti-CD25 mAb basiliximab (Simulect, Novartis) and daclizumab (Zenapax, Hoffmann-La Roche). The use of induction immunosuppression strongly suppresses immune response soon after transplant when inflammation associated with surgery and ischemia make the graft most vulnerable to immune attacks[15].

Maintenance therapies vary by type of organ, institutional preference, and organ recipient demographics. A multimodal approach is commonly employed to prevent rejection by blocking immune responses through several pathways. Commonly used immunosuppressive drugs include CNIs, mammalian Target of Rapamycin (mTOR) inhibitors, corticosteroids[16], mycophenolate preparations, CTLA4-Ig, and anti-CD20 mAb. A host of newer agents targeting other cell surface markers and intracellular signaling pathways are at various stages of preclinical and clinical development (Figure 1)[17-19]. Below, we will provide an agent-by-agent review of their effects on Treg maintenance, induction, and function in preclinical models and in clinical settings.

Impact of approved immunosuppressive drugs on Tregs

Anti-thymocyte globulin

Rabbit anti-thymocyte globulin (rATG, Thymoglobulin) is used as an induction therapy in patients with high immunologic risk, to permit delayed introduction of CNI, or to treat steroid-refractory acute cell-mediated rejection[20]. rATG is a polyclonal preparation from rabbits immunized with human thymocytes and has broad specificity against multiple

antigens expressed by thymocytes. It depletes CD4^{+} and CD8^{+} T cells for many months, with CD8^{+} T cells recovering more rapidly and completely than CD4^{+} T cells[21]. Mechanisms of ATG mediated immunosuppression include apoptosis and induction of T-cell anergy at low doses, antibody-dependent cellular cytotoxicity (ADCC) at moderate doses, and complement-mediated lymphocyte lysis at high doses[22-25]. At clinical doses of 1 - 2 mg/kg/day, ADCC is likely to be the primary mechanism while lymphocyte lysis occurs at supra-therapeutic dosage (up to 3.5 mg/kg/day). ATG induction of apoptosis depends on IL-2, which would be limited by concomitant use of medications that inhibit IL-2 or its receptor[22-24]. ATG may further act to inhibit endothelial adhesion and to deplete lymphocyte reservoirs in peripheral lymph nodes[23,26,27].

Treatment of human peripheral blood lymphocytes with low-dose ATG *in vitro* induces expression of CD25 and FOXP3 in CD4^{+}CD25^{-} cells, although whether these *in vitro* stimulated cells acquire immunosuppressive properties seems context dependent[28-31]. ATG induction therapy *in vivo* reduces the absolute number of Tregs, but less than that for Tconvs, favorably altering the Treg/Tconv ratio[29,32,33]. Furthermore, Tregs recover faster during immune reconstitution following ATG treatment, contributing to the sustained elevation of Treg/Tconv ratio[34]. However, high-dose ATG impairs thymic generation of Tconv and Tregs cells in allogeneic hematopoietic stem cell transplantation[34,35]. ATG therapy may also modulate antigen-specific immune responses by inducing memory-like Tregs, as well as other protective T cells such as Th2 and IL-10-producing Tr1 cells[36]. Thus, immunological impact of ATG is not only

limited to T cell depletion, but also in relative preservation of Tregs especially at lower doses.

Anti-CD25 mAb

Anti-CD25 mAb basiliximab and daclizumab are widely used induction agent after solid organ transplantation. Basiliximab is a chimeric mouse-human IgG1 antibody and daclizumab is a humanized IgG1 antibody, both of which block the IL-2-binding site of CD25[37-40]. Anti-CD25 mAb were originally developed to suppress immune response by targeting recently activated effector T cells that express CD25. Anti-CD25 mAb can also inhibit activation of CD25^{-} T cells by blocking CD25^{+} dendritic cells from trans-presentation of IL-2 (a process of donating CD25 complexed IL-2)[41]. Other CD25^{+} pro-inflammatory cell types, such as lymphoid tissue-inducer cells and innate lymphocytes[42], are reduced following daclizumab treatment during multiple sclerosis (MS) flares[43,44]. These immunosuppressive mechanisms may contribute to the positive clinical outcomes of anti-CD25 mAb in MS[37,45,46].

Treatment with basiliximab in transplant patients can lead to a transient reduction of both Tregs[47-49] and down-modulation of CD25 expression on Tregs without deleting the cells or impairing their functions[50,51,52]. A recent study finds that donor-reactive Tregs is minimally impacted in lung transplant patients after basiliximab induction[53]. Tregs normally express low levels of the IL-7 receptor α chain CD127[54,55]. In MS patients, CD25^{lo} Tregs are able to increase CD127 expression and IL-7 responsiveness following daclizumab treatment, explaining Treg rescue in the absence of IL-2 signaling[56]. Moreover, daclizumab increase CD56^{hi} NK cells by increasing the bioavailability of IL-2.

These CD56^{hi} NK cells suppress immune responses by killing autologous activated T cells or allogeneic antigen presenting cells[40,43,56-59]. Overall, anti-CD25 induction inhibits effector T cells and its effect on Tregs appears to be transient and of uncertain clinical significance. Anti-CD25 mAb may also promote Treg-independent tolerogenic mechanisms that may offset anti-CD25 mAb impairment of Tregs.

Anti-CD52 mAb

Alemtuzumab (CAMPATH-1H, Genzyme) targets CD52 that is highly expressed on T cells and B cells[60,61]. Although alemtuzumab gained FDA approval for the treatment of leukemia, it has found off-label efficacy as an induction agent in transplantation due to its profound depletion of T and B cells[62-65]. The mechanisms of action and effects of alemtuzumab are quite similar to those reported for ATG. ADCC and complement activation via anti-CD52 are the most likely mechanisms for alemtuzumab-mediated killing of CD52^{+} cells, with cell death proportional to surface expression of CD52[60,66-69]. Alemtuzumab preferentially depletes activated Tconvs over Tregs, resulting in a transient elevation of the Treg to Tconv ratio[70-73]. Alemtuzumab can induce conversion of Tconv into Tregs[73-75], increase anti-inflammatory cytokines IL-4, IL-10 and TGF-β, and suppress pro-inflammatory cytokines IFN-γ and IL-17[76].

CD52 is shed from the cell surface through a phospholipase C-dependent mechanism[77]. Soluble CD52 (sCD52) prevents T cell activation via binding to the inhibitory molecule sialic acid-binding immunoglobulin-like lectins-10 (Siglec-10) on activated T cells[77,78]. Addition of *s*CD52 to activated Tconvs *in vitro* inhibits T cell proliferation through a Siglec-10 dependent mechanism[78-80]. sCD52 also prevents leukocyte adhesion to

vascular endothelium by blocking Siglec-10 interaction with vascular adhesion protein-1, further modulating the local immune response[81]. These findings suggest immune regulatory functions for sCD52 protein, which may also be blocked by alemtuzumab.

CNI

CNIs, tacrolimus (FK506) and cyclosporine (Cyclosporin A, CsA), are the most commonly used drugs for maintenance immunosuppression following solid organ transplantation[82]. They act by inhibiting the intracellular phosphatase calcineurin, which dephosphorylates cytosolic nuclear factor of activated T cells (NFAT) to allow for its nuclear translocation and transcriptional activation of cytokine genes such as IL-2[83,84]. IL-2 is a regulator of proliferation, survival, and maturation for all T cell subtypes, including Tregs[85]. While the function of IL-2 on Tconvs can be substituted by other cytokines, IL-2 is indispensable for Treg development, homeostasis, and function[86].

CNIs impair Tregs by directly inhibiting Treg activation, inhibiting the generation of pTregs, and indirectly by limiting IL-2 production by Tconvs. CNIs inhibit Treg proliferation in a dose-dependent fashion *in vitro*[87]. The FOXP3 promoter and enhancers contain multiple NFAT binding sites. NFAT binding to the CNS1 enhancer is important to pTreg induction[88] and its binding to CNS2 enhancer is critical for Treg stability[89-91]. Decreased FOXP3 mRNA expression in Tregs exposed to cyclosporine correlates with reduced suppressor activity[92]. Treatment with tacrolimus also increases FOXP3⁻ TSDR-demethylated "ex-Tregs", suggesting that CNIs may transform Tregs into Tconvs[91,93].

The effects of CNIs on Tregs are dose and duration dependent. High-dose, but not low-

dose, CNI exposure alters gene expression in Tregs[87]. A portion of NFAT constitutively resides in the nucleus of most Tregs, making Tregs resistant to short-term low-dose action of CNIs[94-97]. CNIs also indirectly affect Tregs by inhibiting IL-2 expression from Tconvs. The decrease of FOXP3 expression in Tregs by cyclosporine is restored by addition of IL-2[92,98]. Combining IL-2 with CsA resulted in an increase of Tregs by permitting IL-2-induced Treg expansion and function while preventing antigen-specific Tconv proliferation *in vivo*[98]. These studies suggest that a combination of low-dose CNIs and low-dose IL-2 may achieve the desired effect of selectively inhibiting Tconvs while sparing Tregs. A recent report in non-human primates demonstrates that IL-2 therapy broke kidney allograft tolerance induced by mixed bone marrow chimerism despite an increase in Tregs[99]. Breaking tolerance in this study was IL-2 dose dependent and required a lower dose in primates with lymphoid aggregates in the allografts than in those with pristine grafts. In comparison, following autologous stem-cell transplantation, low-dose IL-2 therapy was found to be effective in promoting Tregs and ameliorating GvHD[100-102]. The disparities in these findings may be rooted in the dose of IL-2 used and the immunological status of the recipients. None of these studies evaluated the concurrent use of CNI with low-dose IL-2 as an approach to reduce impairment of Tregs by CNI.

Current clinical trials are exploring CNI-sparing protocols to minimize nephrotoxicity of CNIs[103]. Although the data are promising for restoration of Tregs, some patients need to resume CNIs because of rejection. As a result, the OPTN/SRTR Annual Data Report in 2013 recommend against CNI withdrawal[82]. The finding that Tregs are resistant to

low-dose CNI and combing CNIs with sirolimus restore Tregs[93,104] suggest that reducing CNI dose may not only spare patients from the nephrotoxicity, but also spare Tregs.

Mycophenolate

Mycophenolate Mofetil (MMF) is a mainstay of immunosuppression regimens in transplantation, typically in combination with CNI and prednisone. Following *in vivo* conversion to its active form of mycophenolic acid, MMF inhibits *de novo* purine synthesis by blocking the enzyme inosine monophosphate dehydrogenase (IMPDH)[105]. B cells and T cells are dependent on this pathway for proliferation, because they cannot bypass this requirement using the salvage pathway of purine biosynthesis[106]. Additionally, the type II isoform of IMPDH, found in activated T and B cells, is five times more sensitive to mycophenolic acid than the type I isoform present in all cells, including resting lymphocytes[106,107]. MMF may also act by down-regulating costimulatory ligands on dendritic cells, indirectly impairing T cell activation[108]. Further mechanisms have implicated MMF in induction of T cell apoptosis, inhibition of IL-1 expression, and impairment of nitric oxide production[106].

Treatment of murine Tregs with MMF *in vitro* does not impair the viability or function of Tregs in MLR[109], and treatment of activated human PBMC with MMF does not alter Treg phenotype but inhibits pro-inflammatory Th1 and Th17 response[110]. MMF treatment promotes Treg predominance over Th17 cells by inhibiting T cell Ig mucin-1 expression, a protein that promote differentiation into effector T cells than Tregs[111,112]. However, administration of supra-therapeutic MMF monotherapy to mice receiving Treg cell therapy reduced the efficacy of Tregs[113]. In liver transplant recipients, conversion

from CNI to MMF with a one-time dose of daclizumab showed an increase in the percentage of Tregs from baseline[114]. Analysis of kidney transplant patients on stable immunosuppression regimens identified higher levels of $CD4^+CD25^{high}FOXP3^+$ Tregs in patients receiving MMF versus everolimus[115]. Overall, preclinical and clinical evidence thus far suggest that MMF is compatible with Treg homeostasis and function.

Corticosteroids

A multitude of anti-inflammatory mechanisms have been attributed to corticosteroids. Corticosteroids bind their cytosolic glucocorticoid receptor, translocate to the nucleus, and inhibit NF-κB-mediated transcription, resulting in broad suppression of pro-inflammatory cytokines[116-118]. Glucocorticoids may also bind to specialized receptors on T cells, which uncouple TCR signaling from downstream signal transduction pathways[118,119]. Extracellular steroid binding can impair T cell interaction with APCs, down-regulate leukocyte rolling and adhesion, and disrupt the T cell cytoskeleton to inhibit migration[120,121]. Corticosteroids also alter the balance of T cell subsets to favor a predominance of Th2 cells and Tregs[122,123].

In a murine model of MS, treatment with dexamethasone and IL-2 expands Tregs[124]. Patients treated with glucocorticoids for autoimmune and atopic diseases have likewise demonstrated an increase in Treg percentage amongst T cell subsets[125,126]. In transplantation, treatment with methylprednisolone during kidney rejection episodes alters T cell composition to favor highly-suppressive $DR^{high}CD45RA^-$ Tregs[127]. Steroids are able to induce pTregs by promoting the expression of glucocorticoid-induced leucine zipper (GILZ), which facilitates TGF-β signaling and FOXP3 expression[128]. GILZ

appears to be a strong inducer of FOXP3^{+} expression, but deletion of GILZ does not completely inhibit FOXP3^{+} expression in Tregs, suggesting that glucocorticoids promote Tregs through multiple, redundant mechanisms[129]. Overall, corticosteroids likely benefit Treg prevalence and activity. Additionally, steroids may also create a favorable immune environment for Tregs through modulation of local cytokine expression.

mTOR Inhibitors

Two mTOR inhibitors are currently FDA approved for transplantation, sirolimus (rapamycin, Rapamune) and everolimus (Zortress). Rapamycin, first identified as a potent antifungal isolated from *Streptomyces hygroscopicus* bacteria, was also found to have immunosuppressive capabilities by inhibiting the serine/threonine kinase mTOR downstream of phosphatidylinositol 3-kinase (PI3K) and Akt[130,131]. mTOR acts through two distinct complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Rapamycin is more effective at inhibiting mTORC1, and is able to inhibit mTORC2 only after prolonged exposure[132-134]. The two mTOR complexes mediate distinct cellular activities. Th1 and Th17 differentiation of CD4^{+} T cells are dependent on mTORC1, whereas Th2 differentiation requires mTORC2[135]. However, both mTOR complexes promote glycolytic metabolism in CD8^{+} T cells and drive effector CD8^{+} T cell differentiation at the expense of memory CD8^{+} T cell formation[136]. Thus, mTOR deficiency or rapamycin treatment inhibits CD8^{+} effectors but paradoxically enhances CD8^{+} T cell memory response[137].

The PI3K-Akt-mTOR signaling axis critically controls Treg development, homeostasis and function[138]. Activation of CD4^{+} Tconvs while blocking PI3K, Akt, or mTOR signaling

leads to Treg induction *in vitro*[139]. In committed Tregs, this signaling axis is suppressed by high expression of PTEN, an intracellular phosphatase that inhibits PI3K-Akt-mTOR signaling. Excessive activation of this axis, as seen in PTEN-deficient Tregs, leads to Treg destabilization due to mTORC2 over activation[140,141]. Tregs alternatively employ the kinase PIM2 for cell growth and activation[142], and as a result, Tregs are able to proliferate in the presence of rapamycin[143-145]. This property of rapamycin has been exploited for manufacturing Tregs for therapeutic applications. Addition of rapamycin in Treg expansion cultures inhibits Tconv outgrowth while preserving Treg identity, yielding purer and more potent cellular products[146]. In kidney transplant recipients, patients on sirolimus maintenance immunosuppression show a four-fold increase in circulating Tregs when compared to patients receiving cyclosporine[147]. Additionally, converting from CNIs to a rapamycin-based regimen produces a sustained increase in peripheral Tregs for months following conversion[93,104]. However, this increased proportion of Tregs failed to correlate to significant improvement in clinical outcomes, with a mild protective benefit in GFR but no significant difference in rejection, graft loss, or development of neoplasm[147-149]. It is worth noting that rapamycin does not promote Treg expansion, and the enrichment of Tregs seen *in vitro* and *in vivo* by rapamycin is due a higher sensitivity of Tconv to rapamycin than Tregs. Treg resistance to mTOR inhibition is not absolute. In fact, addition of rapamycin to *ex vivo* Treg cultures dramatically reduces Treg proliferation, but less so than it inhibits Tconv proliferation[146]. Selective deletion of mTORC1 in mouse Tregs leads to impairment of Treg function and systemic autoimmunity, demonstrating that mTORC1 is required for proper Treg activation[150]. Taken together, targeting mTOR may promote pTreg induction and

selectively suppress Tconvs while sparing Tregs. This Treg favoring effect is dose-dependent and high doses of mTOR inhibitors will also negatively impact Treg function.

CTLA4-Ig

Productive T cell activation requires TCR signaling along with costimulation through CD28 interaction with CD80 or CD86 on APCs[18]. At the same time, CTLA-4, a CD28 homolog expressed on activated T cells, bind to CD80 and CD86 with high affinity to inhibit T cell activation. CTLA4-Igs are genetically engineered soluble fusion proteins that bind to CD80 and CD86, thereby inhibiting CD28 costimulation. Two CTLA4-Igs, belatacept and abatacept are currently available; both consist of the extracellular domain of human CTLA4 linked to a modified Fc of human IgG1, with belatacept containing modifications to increase affinity for CD80 and CD86. Currently, belatacept is FDA approved as an immunosuppressant for kidney transplantation[151].

While CD28 costimulation is critical for Tconv activation, it is also essential for tTreg development and homeostasis[152]. CD28-deficient mice have reduction in Tregs leading to exacerbated autoimmune diseases in autoimmune-prone mice[152-154]. Effector T cell activation requires higher CD80 and CD86 expression than is needed for maintaining Treg homeostasis; thus, partial CD80 and CD86 blockade prevents the emergence of effector T cells while permitting Treg homeostasis in mouse models and in kidney transplant patients[47,155]. Moreover, while complete absence of CD80 and CD86 abrogate pTreg genesis, strong costimulation through CD28 drives effector T cell programming at the expense of pTreg induction. Thus, partial CD80 and CD86 blockade using CTLA4-Ig may favor pTreg development[156]. CTLA4-Ig may also operate

independently of blocking CD28 signaling by inducing dendritic cell expression of indoleamine 2,3-dioxygenase, leading to tryptophan catabolism, T cell apoptosis, and activation of Tregs[157,158]. CTLA4-Ig is shown to promote nitric oxide production by macrophages, contributing to Treg generation while inhibiting pro-inflammatory cytokines[159-161]. Long-term clinical follow-up of kidney transplant patients treated with belatacept demonstrates superior graft function at 5 years without differences in graft or patient survival when compared to CNI-based immunosuppression[162,163]. Studies in liver transplantation are less encouraging[164] and CTLA4-Ig has not been well studied in other solid organ transplants. Overall, CTLA4-Ig is compatible with Treg function at the right dose and can be an effective replacement for CNI at thwarting rejection with minimal renal toxicity.

Impact of alternative immunosuppressive agents on Tregs

Anti-CD20 mAb

Rituximab is an anti-CD20 mAb that depletes B cells, most frequently used in transplantation for management of antibody-mediated rejection and ABO-incompatible kidney transplants[165]. Current data investigating the impact of rituximab on Tregs are mostly derived from autoimmune models, with somewhat conflicting results. B cell depletion with rituximab in a mouse model of arthritis correlated with increases in Treg number and function[166]. Similarly, patients with systemic lupus erythematosus showed an increase in the percentage of $CD4^+CD25^{bright}$ Tregs with rituximab treatment[167,168]. However, another report showed that B cells were critical for maintenance of Tregs in autoimmune disease[169]. Within the transplant population, a study evaluated the

addition of rituximab induction to standard immunosuppression in renal transplantation and revealed no detrimental effect on Tregs[170]. Rituximab may have further use in the transplant population, as it has shown efficacy in both prophylaxis against acute GVHD and treatment of steroid-refractory chronic GVHD in allogenic stem cell transplant patients, which is believed to be a B cell-dependent process[171-173]. More studies within the transplant population are needed to better understand the effects of B cell depletion on Tregs[167,174-176].

LFA-3 Fusion Protein

Alefacept (Amevive, Astella Pharma) is a fusion protein of LFA-3 and human IgG1 to target CD2 that is highly expressed on memory T cells. Alefacept prevents T cell activation by blocking CD2-mediated costimulation and enhances NK cell-mediated lysis of CD2-expressing cells. Alefacept use led to selective loss of CD40RO^{+} memory T cells without affecting native T cells in psoriasis patients[177]. In a more recent phase II trial in patients with type 1 diabetes, alefacept was found to spare Tregs, leading to an increased ratio of Tregs to memory T cells[178]. Alefacept prolonged kidney allograft survival in nonhuman primates when combined with abatacept by targeting CD28^{-} effector T cells[179,180]. However, two follow-up studies in nonhuman primates using an optimized immunosuppressive regimen of belatacept and intramuscular sirolimus found that addition of alefacept conferred no benefit in graft survival and increased risk of opportunistic infections[181,182]. Moreover, dramatic reduction in the frequency of Tregs was observed in monkeys that received additional alefacept. These studies used a higher dose of alefacept than previous studies in addition to the optimized concurrent immunosuppressions. These dosing differences may explain the distinct efficacy

outcomes and discordant findings with respect to Tregs. In human transplant patients, addition of alefacept to the standard immunosuppressive regimen of tacrolimus, MMF, and corticosteroids was well tolerated, but did not reduce acute rejection rates[183]. Unfortunately, this study did not report the impact of supplemental alefacept on Tregs. Since memory T cells pose a significant threat to transplanted grafts, preservation of Tregs while reducing memory T cells is a highly desirable goal for optimizing immunosuppression in transplant patients. Future studies are needed to determine if alefacept could benefit patients with higher immunological risks such as those with autoimmune diseases, HIV infections, or those prone to rejection on a belatacept-based regimen.

JAK3 Inhibitors

Janus associated kinase 3 (JAK3) transduces signals downstream of CD132, which is the common gamma chain shared among many cytokine receptors including receptors for IL-2[184,185]. JAK3 signaling is critical to normal homeostasis and function of T cells, B cells, and NK cells. The importance of JAK3 to normal immune function is demonstrated by the severe combined immunodeficiency in patients with inborn JAK3 mutations[184,186]. Thus, JAK3 inhibitors such as tofacitinib (Xeljanz, Pfizer) can broadly affect many immune cells including various $CD4^+$ T helper subsets, $CD8^+$ T cells, NK cells, as well as Tregs. Although mouse studies of JAK3 inhibitors have shown preservation of Tregs, JAK3 inhibitor use at 30 mg twice daily in transplant patients partially depletes $CD4^+CD25^{bright}$ Tregs[187]. Early clinical trials in kidney transplant patients found that this dose of tofacitinib led to a trend toward higher rejection and increased infections, whereas 15 mg twice daily was comparable to CNI in incidences of

rejection and infections[188]. However, the impact of low-dose tofacitinib on Tregs and other immune cells was not reported. An *in vitro* analysis of tofacitinib inhibition of STAT5 phosphorylation showed that CD25$^{-/dim}$ T cells were nearly twice as susceptible to JAK3 inhibition as CD4$^+$CD25^{bright} T cells, suggesting that lower doses of tofacitinib may preferentially inhibit Tconvs that have lower expression of CD25[189].

Anti-LFA-1 Antibodies

Leukocyte function antigen-1 (LFA-1) is a cell surface adhesion molecule expressed on a variety of leukocytes that controls leukocyte transmigration from blood to peripheral tissues[190]. In T cells, LFA-1 is also essential for the establishment of an immunologic synapse between T cells and APCs. Similar to CD2 described above, LFA-1 expression is higher on memory T cells than on naïve T cells. Anti-LFA-1 antibodies (efalizumab; Raptiva, Genentech) impair the stimulatory interaction of naïve and memory T cells. The addition of efalizumab to full-dose CNI maintenance therapy led to profound immunosuppression and increased incidence of post-transplant lymphoproliferative disorder in kidney transplant recipients[191]. On the other hand, efalizumab allowed reduction of CNI dosing by half without increasing rejection. In islet transplantation, a combination of ATG induction and efalizumab-based maintenance immunosuppression allowed patients to achieve insulin independence on a steroid-free CNI-free regimen[192]. Surprisingly, this regimen also led to a dramatic rise of the percentage of Tregs to 30 to 70% among circulating CD4$^+$ T cells in all study participants[192]. This finding suggests that efalizumab differentially affects Treg versus Tconv homeostasis and/or trafficking. Efalizumab was withdrawn from market because

of incidences of serious infections in psoriasis patients. However, the use of efalizumab in transplantation, particularly as an induction agent, warrants further exploration.

Anti-IL-6R mAb

IL-6 is produced early by a wide variety of immune and non-immune cells in response to acute injury and elicits its cellular actions by binding to IL-6R. IL-6R dimerizes with a transmembrane protein gp130, which is responsible for transmitting intracellular signals. IL-6R can shed from the membrane generating a soluble form of the receptor (sIL-6R) that can complex with IL-6 and activate cells that lack the membrane-bound IL-6R but express gp130. This "trans-signaling" process is known to be important in the transition from acute to chronic phases of inflammation[193]. IL-6 has pleotropic effects including granulopoiesis, B cell growth and maturation, and T cell proliferation and differentiation. Notably, IL-6 occupies a unique position in determining the fate of naïve T cells. In the presence of IL-6, TGF-β drives naive T cells into proinflammatory Th17 cells, whereas in the absence of IL-6, TGF-β induces pTregs[194]. In addition, IL-6 renders Tconvs resistant to Treg suppression and directly destabilizes Treg by inhibiting FOXP3 expression[195-197]. IL-6 has been implicated in frailty, which is an emerging risk factor for poor transplant outcomes[198,199]. Thus, IL-6 critically regulates Treg number and function, and the level of IL-6 in patients may impact to transplant outcomes.

Tocilizumab (Actemra) is a recombinant humanized anti-IL-6R monoclonal IgG1 antibody that prevents IL-6 from binding to membrane-bound and soluble IL-6R. Tocilizumab is currently FDA-approved for the treatment of rheumatoid arthritis and juvenile idiopathic arthritis. Tocilizumab treatment leads to a significant and sustained

increase in the proportion of Tregs in patients with rheumatoid arthritis[200,201]. In transplantation, blocking IL-6 leads to decreased IFN-γ and IL-17 mRNA, reduced alloantigen-stimulated T cell proliferation, increased proportion of Tregs, and prolonged allograft survival in mouse models[202-204]. In humans, IL-6 is associated with acute and chronic rejections and IL-6 levels correlate with the degree of inflammation in the allografts[205-210]. The use of tocilizumab with intravenous immunoglobulin in sensitized kidney transplant patients shows improved donor-specific antibody levels similar to the results obtained in a mouse model[211,212]. Together, anti-IL-6R has multiple anti-inflammatory properties and may favor the function and stability of Tregs. Future studies are needed to determine the effects of tocilizumab on Tregs in transplant patients.

Anti-CD28 Antibodies

CTLA-4Ig blocks CD80- and CD86-mediated costimulation through CD28 but also blocks CTLA-4 engagement with CD80 and CD86 and CD80 with to PDL-1[213,214]. Blockade of T cell checkpoints CTLA-4 and PD-1 is a major breakthrough in cancer treatment, which underscores the importance of these pathways in immune regulation[215]. Anti-CTLA-4 and anti-PD-1 therapies in transplant recipients with metastatic melanoma provide insights on how these pathways contribute to alloimmune responses. So far, 5 published reports described 6 cases of kidney or liver transplant recipients receiving checkpoint blockade. Four patients (two liver and two kidney recipients) received anti-CLTA-4, with all four showing tumor regression without long-term impairment of graft function[216-218]. One kidney transplant recipient showed tumor regression with anti-PD-1 therapy, but lost kidney graft to acute cellular rejection[219].

One kidney transplant recipient received initial anti-CTLA-4 therapy without rejection or tumor regression, but follow-up anti-PD-1 therapy precipitated graft rejection while inducing tumor shrinkage[220]. These early experiences suggest that PD-1, but not CTLA-4, may be crucial in suppressing alloreactive T cells in patients with stable graft function. Nonetheless, blockade of CD28 using anti-CD28 mAbs more specifically targets the immune activation function of this complex network of activators and inhibitors. However, many anti-CD28 mAbs have agonist activities. Although agonist anti-CD28 mAbs in rodents show preferential augmentation of Tregs and therapeutic efficacy in autoimmune diseases, an early human trial revealed life-threatening cytokine storm in healthy volunteers after receiving agonist anti-CD28 mAb[221]. Monovalent anti-CD28 Fab lacking an Fc region abrogates agonist activity and effectively blocks T cell activation[222-226]. When evaluated in animal models of organ transplantation, monovalent anti-CD28 mAbs are able to protect allografts from acute and chronic rejection while increasing the proportion of peripheral CD4$^+$Foxp3$^+$ Tregs[223,225,227]. These favorable pre-clinical findings has led to an ongoing phase I trial in healthy subjects to define the safety and tolerability of this therapy in humans with potential future development in transplantation and autoimmune diseases.

Inhibitors of TNF family ligands and their receptors

TNF family ligands, such as TNFα, CD40L, OX40L, and 41BBL, are primarily proinflammatory by promoting activation of both innate and adaptive immunity. Each member and their respective receptors are all potential targets for immune modulation. We will briefly summarize preclinical and clinical experiences of antagonists targeting

three members of the TNF family that have been most studied in the context of transplantation.

Anti-TNFα mAb infliximab (Remicade, Janssen Biotech) is able to down-regulate pro-inflammatory cytokines in a number of autoimmune diseases, and its use has been shown to increase functional Tregs in RA patients[228-230]. Induction therapy containing anti-TNFα and T cell depleting agents in clinical islet transplantation leads to higher rate of insulin independence when compared to anti-CD25-based induction[231].

CD40 and CD40L have critical functions in regulating T and B cell function, through their co-stimulatory interaction in the activation of naïve T cells and induction of high affinity antibody production. In the non-obese diabetic mouse model, CD40L deficiency impairs effector T cell function without impacting Tregs, and stimulation through CD40 on APC rendered the cells resistant to Treg-mediated suppression[232,233]. These findings suggest that targeting the CD40/CD40L interaction may selectively block effector cell differentiation while preserving function of Tregs. Indeed, CD40L blockade showed remarkable efficacy in promoting allograft tolerance in murine and non-human primate models. Clinical translation of this strategy was halted due to a high incidence of thromboembolism[234]. Since thrombus stabilization by anti-CD40L is dependent on Fc receptor binding and mediated by CD40L binding to beta 3 integrin, not CD40, alternative strategies using anti-CD40L and CD40-targeting antibodies which bypass Fc receptor binding are being evaluated in preclinical and early clinical trials[235-239].

OX40L is constitutively expressed by Tregs and induced on Tconvs after activation. Blocking antibodies to OX40L inhibit memory T cell function and allow for Treg

expansion in murine cardiac allograft models[240,241]. Costimulation through OX40L abrogates conversion of Tconv to Tregs *in vitro* whereas OX40L synergizes with IL-2 to induce proliferation of Tregs that have already developed. OX40 stimulation alone induces moderate Treg proliferation but down-regulates FOXP3 expression and increases expression of PD-1[242-244]. Addition of IL-2 to OX40L induced more robust Treg proliferation while maintaining high FOXP3 expression. In transplant patients on CNI when IL-2 availability may be reduced, inhibition of OX40L may prevent destabilization of formed Tregs and promote pTreg generation.

Vitamin D

Although not normally considered an immunosuppressant, vitamin D has potent immune modulatory activities on many cells types[245]. Conversion of Vitamin D into its biologically active form 1,25-OH vitamin D_3 starts in the skin and completes in the kidney by renal tubule cells. Macrophages are also capable of producing vitamin D_3 from its precursors, providing a local source of vitamin D_3 at site of immune activation. Vitamin D_3 is anti-inflammatory and promotes immune regulation via IL-10 and Treg induction. The immunologic effects of vitamin D deficiency in the kidney transplant population – where end-stage renal disease leads to dysregulation of vitamin D metabolism – can persist well beyond successful transplantation of a renal allograft. Low vitamin D levels are predictive for deterioration in allograft glomerular filtration rate[246,247] and correlate with acute rejection, infection, and mortality in lung transplant patients[248]. Given the potentially significant immunologic impact of vitamin D deficiency and the safety of vitamin D supplementation, normalization of vitamin D levels should be recommended in the transplant population.

Conclusion

Modifying immunosuppression regimens to selectively inhibit effector and memory T cells while permitting Treg development, survival, and function could theoretically allow for minimization of immunosuppressive drugs and favor tolerance induction. Current knowledge of Treg biology reveals numerous distinctions between Tregs and Tconvs that may be targeted therapeutically to favor Tregs (Figure 2). Many of these distinctions, such as IL-2 responsiveness, CD28 and mTOR dependence, CNI sensitivity, and resistance to lymphodepletion are not absolute but quantitative. Thus, selection of Treg-friendly immunosuppressive regimens not only has to consider which drugs to use, but also what dose to apply. Many immunosuppressive drugs currently used in transplant patients are compatible with Tregs at lower doses. It is possible that a combination of multiple immunosuppressants at low-dose would be better able to support Tregs while adequately preventing rejection while minimizing toxicity. When evaluating new immunosuppressive regimens, close monitoring of the numbers, activation status, and function of Tconvs versus Tregs, in addition to clinical outcomes, will help to enrich our knowledge and guide future development of tolerogenic therapies for transplantation. It is important to note that the high proportion of Tregs needed to induce transplant tolerance in pre-clinical models[249] is not likely achievable by titrating doses of immunosuppressive drugs alone. Combining Treg cell therapy, attenuation of effector responses, and Treg-supportive immunosuppression may be needed to induce tolerance.

Figure legend:

Figure 1. Mechanisms of action of immunosuppressive drugs on T cell activation.
Schematic representation of the mechanisms of action by which immunosuppressants control T cell activation. Black font marks molecules expressed by T cells and antigen presenting cells (APC). Red font marks immunosuppressive drugs. Black arrows indicate signaling pathways and red T-bars point to targets of immunosuppressive drugs.

Figure 2. Differential effects of immunosuppressive drugs on the balance between effector T cells and Tregs. Immunosuppressive drugs are represented by colored blocks. The overall length of the block roughly correlates with the drug's immunosuppressive potency. The left-right position of the block indicates the drug's selectivity for effector T cells (Teff) versus Tregs with the center position representing no selectivity and left to the center more suppressive for Teff than for Tregs. The overall height of the balance in the cylinder below correlates with state of immune activity and the tilt of the balance correlates with regulatory activity.

Acknowledgements:

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