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Influence of pharmacological immunomodulatory agents on $CD4^+CD25^{high}FoxP3^+$ T regulatory cells in humans ☆

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ABSTRACT

T regulatory cells (Tregs) play a critical role in the immunologic tolerance to the graft in transplantation. Thus, due to their immunosuppressive capability, *ex vivo* expanded Tregs may be used as a cellular therapy and an attractive novel strategy to control chronic rejection and eliminate need for lifelong pharmacological immunosuppression. Since Treg therapy is still in its infancy, initially Tregs still need to be applied in combination with pharmacological agents to prevent rejection. Fortunately, some of the medications have been shown to enhance the function and number of Tregs. In the clinic, different immunosuppressive regimens are used for individual patients for different types of organ transplantation. In this review, we present the most commonly used pharmacological agents for immunosuppression and discuss how they affect the Treg population. It is extremely difficult to dissect the effect of single agent on Tregs population in clinical settings since usually the combination of several medications is applied at the same time for graft protection. Nevertheless, experimental and clinical data indicate that thymoglobulin as immunosuppressive induction and mTOR inhibitors as immunosuppressive maintenance agents have the most beneficial effect on Treg population in the blood. Among supplemental agents promoting Tregs, anti-TNFα preparations have been in clinical use (in autoimmune diseases) for many years, so they are optimal candidates for testing in transplant settings in combination with Treg based cellular therapy.

1. Introduction

$CD4^+CD25^{high}FoxP3^+$ regulatory T cells (Tregs) act to counterbalance T effector cell activity for immune homeostasis. Function or dysfunction of Tregs plays critical roles in autoimmune disease, cancer, transplantation, allergy, and inflammation. One of the important functions of Tregs is the immunosuppressive regulation of auto-reactive T cells. Naturally occurring Tregs (nTregs), like all other T cells, undergo lineage commitment and maturation in thymus, after which they constitute only 5–10% of $CD4^+$ T cells in the blood [1]. Inducible Tregs (iTregs) are derived from naive $CD4^+$ cells in the periphery.

In the field of organ transplantation, chronic rejection and adverse effects of pharmacological immunosuppression are still a major obstacle to overcome. Thus, due to their immunosuppressive capability, *ex vivo* expanded Tregs may be used as a cellular therapy and are an attractive novel strategy to control chronic rejection and minimize the use of pharmacological agents in maintenance of immunosuppression. Indeed, recently more studies have shown it as a feasible, alternative therapeutic approach. Tregs can physiologically inhibit effector T cells without toxicity and have a lower risk of side effects than immunosuppressive drugs. Furthermore, Tregs may be able to induce long-term immune tolerance [2].

Since Treg therapy is still in its infancy, initially Tregs need to be applied in combination with pharmacological agents to prevent rejection. Fortunately, some of medications have been shown to enhance the function and number of Tregs. In the clinic, different immunosuppressive regimens are used for individual patients for different types of organ transplantation. In this review, we present the most commonly used pharmacological agents for immunosuppression and discuss how they affect the Treg population. For the purposes of this discussion, we have presented these agents in three groups: (1) induction of immunosuppression; (2) anti-inflammatory agents; and (3) maintenance of immunosuppression.

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2. Induction of immunosuppression

2.1. Anti-thymocyte globulin

Anti-thymocyte globulin (ATG) has been the most widely used lymphocyte-depleting preparation in solid organ transplantation for more than 25 years. It targets T cell surface antigens leading to long-lasting T cell depletion. Although it has been registered with the FDA as an agent to treat acute rejection, it is commonly used in the induction of immunosuppression for the prevention of graft rejection [3].

It was shown in animal studies that Tregs were susceptible to cell depletion caused by ATG. However, as compared to conventional T cells, $CD4^+FoxP3^+$ Treg cells were found to be more resistant to mouse ATG treatment, resulting in the Treg/Teff ratio increasing by approximately two-fold in lymph nodes, spleen, and peripheral blood after treatment with ATG [4]. This may be a consequence of higher expression of anti-apoptotic gene Bcl-X_L, which is higher in $FoxP3^+$ cells independent of CD25 expression [5]. This was even more pronounced, when ATG was followed by Sirolimus (SRL) and CTLA4-Ig [6]. Similar increase in the percentage of Tregs was achieved also with TGFβ1 in mice model [7].

Additionally, *in vitro* studies with thymoglobulin also confirmed that observation: Tregs are more resistant to depletion than T effector cells. After four day *in vitro* incubation, Tregs/Teff ratio increased due to 4-fold increase in the percentage of $CD4^+CD25^{high}FoxP3^+$Tregs [8]. In similar experiment, the same ATG effect was observed only after prolonged exposure of the cells to the agent. While a 6-hour incubation with rabbit ATG did not affect the number and function of Tregs [9], the longer exposure (24 h) induced more Tregs with the suppressive function and up-regulated FoxP3 expression [10,11]. Interestingly, there is evidence that *in vitro* administered ATG can convert $CD4^+CD25^-$ T cells into $CD4^+CD25^+FoxP3^+$ T cells [12].

In the clinic, the recovery of Tregs after ATG related depletion was found more vigorous than T effector cells. By day 90 after renal transplant, the Treg number in ATG-treated patients reached 47% of pre-transplant levels increasing relative Treg/Teff ratio [13]. Kidney recipients who received rabbit ATG (rATG) followed by belatacept/SRL were characterized by having higher number of $FoxP3^+$ Tregs in the blood and enhanced suppressive Treg function as compared to treatment with other immunosuppressive agents one year after the transplant [14]. Interestingly, transplant recipients treated with this ATG protocol possessed higher percentage of $CD4^+CD25^+FoxP3^+$ Treg cells than healthy controls, 10.0% *versus* 6.9%, respectively [15].

Similar results could be found looking at new described Treg markers. ATG may deplete all the T cells but preferentially spare $CD4^+FoxP3^+Helios^+$ cells, leading to a higher Treg/Teff ratio during immune recovery [16,17].

Altogether, in the majority of experimental and clinical studies, ATG depleted all T cells, however increased Treg/Teff ratio due to preferential sparing of Treg cells.

2.2. Anti-CD52 antibody (alemtuzumab)

The anti-CD52 monoclonal antibody alemtuzumab, which is known as Campath-1H, was approved in 2001 for the treatment of hematologic malignancies and has been used in many transplant studies since then [18]. Alemtuzumab targets CD52, a protein expressed on the surface of mature lymphocytes depleting T cells, B cells, monocytes and dendritic cells, therefore it is used as an alternative to ATG for induction therapy in solid organ transplantations. However, alemtuzumab causes more profound and longer lasting blood cell depletion than ATG [19] and the effect is immediate after application. *In vitro*, all T cells, including $CD4^+CD25^+$ cells, were completely depleted only 4 h after Campath-1H exposure [20].

Therefore, in patients, alemtuzumab also induces a profound and unselective depletion of $CD4^+$ T cells, including Tregs [21]. We found in our previous study involving Campath-1H, that after an initial blood count drop, Treg recovery was much slower than other T cell populations [22]. On one hand, it could have been related to the relative resistance of Tregs to homeostatic proliferation, as Tregs do not express receptors for homeostatic cytokines [23]. Low levels of Tregs after profound cell depletion related to alemtuzumab might also be responsible for vigorous homeostatic proliferation of non-regulatory T cells, which is the process normally limited by Tregs and encouraged by Treg deficiency [24].

On the other hand, in this particular study, besides Campath-1H, one of the calcineurin inhibitors (CNI) was also used for the maintenance immunosuppression; therefore, slow recovery of Tregs might have been related directly to the effect of this agent or both [22] (see Calcineurin inhibitors (CNI) section below). The latter might be more likely since the results of other studies suggest that alemtuzumab spares Tregs similarly to ATG. *In vitro* stimulation of PBMCs and exposure to alemtuzumab led to an increased ratio of Treg/Teff due to the relative resistance of $CD4^+CD25^+FoxP3^+$ cells to depletion [25]. This effect was confirmed in patients. The frequency of $CD4^+CD25^+FoxP3^+$ Treg cells increased for up to 90 days in hematopoietic stem cell transplantation patients as well as in IPEX syndrome patients after alemtuzumab treatment [26–28]. In renal transplantation in adults, alemtuzumab was able to increase the percentage of $CD4^+CD25^+FoxP3^+$ Tregs to 20% of all $CD4^+$ T cells 3 months post-transplant [29]. This is of special importance, as the recovery of total $CD4^+$ T cells after depletion is usually delayed and the total number of these cells may be decreased for years after administration of alemtuzumab [25,30]. In pediatric kidney transplants, alemtuzumab also selectively spared $CD4^+CD25^+FoxP3^+$ regulatory T cells. In this steroid-free, CNI-withdrawal protocol the ratio of Treg/Teff increased significantly from baseline to 3 months post-transplant and returned to baseline between 6 and 12 months [31]. As was the case with ATG, the increase in the percentage of Tregs after alemtuzumab can be further enhanced, when sirolimus is included in the immunosuppressive regimen [21,22].

Similarly to ATG, alemtuzumab selects for Treg cells at the expense of conventional $CD4^+$ T cells and allows for the preservation of their immunosuppressive function both *in vitro* and *in vivo* [32]. Although alemtuzumab antibody decreases total numbers of all T cells, the reduction in Tregs is far less, resulting in the proportion of Tregs of total $CD4^+$ cells increasing or at least being maintained after the treatment. However, Alemtuzumab seems to promote Tregs to a lower extent, when compared with ATG [12].

2.3. Anti-CD3 antibody

Anti-CD3 antibody binds to the CD3 receptor on the surface of T cells, and is commonly used in clinical depletion strategies [33]. The use of anti-CD3 antibody is considered as an alternative to ATG and alemtuzumab, and is employed less often, usually as a last resort for resistant acute rejection of the graft or as induction therapy [34]. Since, the effect is limited to T cells, this compound is used less often than ATG and Campath-1H in transplantation, but at the same time, it could be used to treat T cell-mediated autoimmunity, like type 1 diabetes (T1D). Similar to ATG and alemtuzumab, anti-CD3 not only depletes but also changes the proportion and activity of the remaining T cells. An experimental autoimmune uveitis mouse model demonstrated that giving anti-CD3 antibodies resulted in an increase in the percentage of regulatory T cells and enhanced the activity of antigen-specific regulatory T cells at day 14th and 30th, whereas the number of autoreactive T cells was selectively reduced [35]. In collagen-induced arthritis, anti-CD3 mAb therapy increased the percentage of $CD4^+CD25^+FoxP3^+$ cells resulting in reduced disease activity [36]. Transient systemic rise in the percentage, but not absolute number of $CD4^+FoxP3^+$ Tregs, was observed particularly in the subset of Helios positive Tregs; this was due to a selective depletion of $CD4^+FoxP3^-$

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conventional T cells *in vivo*, an effect that was also noted in other studies [37]. In addition, anti-CD3 mAbs increased $CD4^+CD25^+FoxP3^+$ levels in diabetes and the effect could be enhanced by the addition of nasal proinsulin [38]. These tolerogenic properties encouraged diabetologists to conduct the PROTÉGÉ Study and administer an anti-CD3 preparation of teplizumab to treat recent onset of type 1 diabetes in humans. Unfortunately, the study was discontinued in phase III since the primary efficacy end-point was not met after the first year [39]. Anti-CD3 mAb has also been used to treat cGVHD-induced lupus nephritis; again, the recovery was associated with an up-regulation of FoxP3 mRNA expression and a down-regulation of effector T cell-related genes in the kidney [40].

2.4. Anti-CD25 (IL-2r) antibody

The interleukin-2 receptor α chain (IL-2Rα, CD25) plays a major role in shaping the dynamics of T cell populations following immune activation [41]. Basiliximab (Simulect, Novartis, USA) and daclizumab are the two antibody-based drugs that directly target the alpha-chain of IL-2R, and have been successfully applied in the clinic to reduce the incidence of acute graft rejection.

Anti-CD25 antibody can reduce almost all $CD4^+CD25^{high}$ cells and has less effect on $CD4^+CD25^{low}$ cells even at low doses, which has been observed in both *in vitro* and *in vivo* studies [42,43]. In the circulation, a transient loss of both $FoxP3^+CD25^+$ and $FoxP3^-CD25^+$ T cells was found and the total number of FoxP3 + cells was reduced [44,45]. This raises important questions about the use of this therapy in tolerance-promoting therapeutic protocols. Even more, this antibody has been used for Treg depletion in anti-cancer therapies, where a high level of Tregs was responsible for tumor progression [46]. This observation was mainly made for daclizumab, administration of which led to a marked decrease in number and frequency of $CD4^+CD25^+$ cells *in vitro* [47] as well as in metastatic melanoma patients [48–50]. Daclizumab administration depleted Tregs and increased the effector T cell to Treg ratio [41,50–52]. However, researchers also found that the percentage of $CD4^+FoxP3^+$ Tregs and the FoxP3 mRNA expression in PBMCs were not significantly reduced, even though the percentage of $CD4^+CD25^+$ T cells was decreased in the short term after administration of anti-CD25 antibodies [42,43,53]. A similar effect was noted in human transplantation studies, in which induction with basiliximab did not change the proportion of $FoxP3^+$ cells among total $CD4^+$ T cells nor did it change the level of FoxP3 expression. At the same time, the proportion of $CD25^+FoxP3^+$ cells decreased and the proportion of $CD25^-FoxP3^+$ cells increased [54–56]. It might be possible that this is an artifact and in all of those studies, anti-CD25 antibody was simply masking the CD25 receptor by binding to it and $FoxP3^+$ cells were falsely assessed as CD25 negative [57]. Nevertheless, there are reports with confirmed reduction of Treg cells after anti-CD25 treatment. Induction therapy with basiliximab in combination with a CsA for maintenance of immunosuppression reduced the amount of circulating regulatory $CD4^+CD25^{high}FoxP3^+$ T cells in kidney transplant recipients in the long-term follow up [25,58]. On the other hand, graft biopsies showed that basiliximab therapy leads to high expression of FoxP3 locally in the graft after kidney transplantation [59]. There are also reports that the addition of ATG to anti-CD25 induction treatment leads to an increase in systemic levels of $FoxP3^+$ Tregs post-transplant [57].

3. Anti-inflammatory agents

3.1. Anti-TNFα antibodies

Tumor necrosis factor-alpha (TNFα) is a cytokine associated with systemic inflammation and immune response [60]. It has been shown that TNFα can up-regulate CD25 leading to enhanced IL-2 stimulated phosphorylation of STAT5 and subsequent up-regulation of FoxP3 [61]. Adding 10 ng/mL TNFα to a 72-hour *in vitro* culture of mouse $CD4^+$ cells increased (4-fold) the percentage of cells expressing FoxP3 as well as enhanced the suppressive function of Tregs [62]. Another *in vitro* study using human cells revealed that 50 ng/mL TNFα inhibited the number and function of Tregs [63]. This discrepancy may be partly ascribable to differences in the TNFα dosages used and insufficient attention to the possible effects of TNFα on T effectors, and the fact that the cells came from different species.

Another reason might be that TNFα is required only for the development of nTregs but not for iTregs [64]. This could explain the therapeutic efficiency of anti-TNFα in the clinic. Therapy with anti-TNFα antibody increases the number (2–3 fold) of $FoxP3^+$ Tregs (including $CD4^+CD25^+FoxP3^+$ and $CD4^+CD25^-FoxP3^+$) in peripheral blood enhancing their function (tested *in vitro*) in patients with inflammatory bowel disease [65,61]. Treating rheumatoid arthritis patients with anti-TNFα led to a reduction of Teff cells and an enrichment of Tregs, leading to a higher Treg/Teff ratio in the blood even at 24 weeks post-agent application [66,67]. In active chronic uveitis patients, the level of $CD4^+CD25^{high}FoxP3^+$ Treg cells increased in the first 3 months after initiation of the treatment with anti-TNFα preparation, adalimumab [68]. In ankylosing spondylitis anti-TNFα decreased the levels of Th17 cells and related cytokines and increased the Treg/TGFβ axis [69]. Increased expression of type 1 TNFα receptors on Tregs was found in type 1 diabetic children. This was associated with impaired function of Tregs *in vitro* and could be reversed by addition of an anti-TNFα preparation infliximab to the culture [70]. It seems that anti-TNFα antibody may cause a redistribution of Tregs from tissue to blood and directly boost the suppressive function of these cells [61]. Anti-TNFα agents have been tested effectively in inflammatory bowel disease. Two weeks after treatment, the number of circulating Tregs increased twice with a 2–3 fold increase in FoxP3 expression and a 2 fold enhancement in their suppressive function [61]. Interestingly, clinical responders to therapy with anti-TNF agents had durable clinical remission with sustained increases in the number of circulating FoxP3 positive cells [65].

3.2. IL-1 inhibitor

Anakinra, a human recombinant IL-1 receptor antagonist, is approved for the treatment of autoimmune and inflammatory conditions such as rheumatoid arthritis [71], Schnitzler's syndrome [72], and other inflammations. After receiving anakinra, the percentages of Th17 cells and Th1 cells were lower and the percentage of Treg cells was higher by 24 weeks [73].

4. Maintenance of immunosuppression

4.1. Calcineurin inhibitors (CNI)

Cyclosporin A and tacrolimus (TAC) are immunosuppressants that act by inhibiting calcineurin, a phosphatase 3 protein, which activates T cells. Calcineurin inhibitors (CNI) are used as immunosuppressive therapy in solid organ transplantation and other immune diseases [74]. In general, CNIs reduce the number of Tregs. For example, decreased number of $CD4^+CD25^{high}FoxP3^+$ Tregs was found in renal and liver graft patients [22,75,76]. However, no direct relationship between FoxP3 expression and CNI has been established. What is more, the use of TAC monotherapy did not influence FoxP3 expression, neither at the mRNA nor the protein level [75]. When dermatitis patients were given TAC, the number of $CD25^+$ cells was reduced, but the number of $FoxP3^+$ cells was not altered [77].

4.1.1. Cyclosporine A

Cyclosporine A (CsA) has been shown to suppress the induction of $CD4^+CD25^+$ Tregs but enhances Treg function in *in vitro* MLR experiments [78]. After giving CsA to healthy mice, the number of Tregs was reduced, and the development and function of $CD4^+CD25^+$ Treg cells were impaired [79]. Both low and high doses of CsA inhibited the proliferation of Tregs, but only a low dose CsA impaired the suppressive

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function [80]. In mice, CsA didn't change FoxP3 expression but caused lower FoxP3/IL17a ratio because of a selective sparing of Th17 cells [81].

4.1.2. Tacrolimus

Tacrolimus (TAC), known also as FK506, is a calcineurin inhibitor first approved for liver transplant in 1994. Like CsA, TAC decreases Treg proliferation *in vitro* [82,83]. In a model of rat liver transplant, tacrolimus also reduced Treg number [84]. In the human setting *in vivo*, an association between low number of Tregs and TAC maintenance was described in kidney transplant recipients [22].

4.2. CTLA4-Ig

Cytotoxic T lymphocyte antigen 4 (CTLA4-Ig) is a CD80/CD86 antagonist which disrupts an important co-stimulatory signal passed from APC to T cells after T cell receptor engagement consisting of an interaction between CD80/86 and CD28 receptors [85]. CD28 signaling in T-regulatory cells was shown to promote FoxP3$^+$ T regulatory cell generation from developing thymocytes [86]. CTLA4-Ig may inhibit the development and expansion of nTreg cells by impairing the CD28 signaling pathway, although in short-term-treated patients the expression of FoxP3$^+$ cells in the tissues is higher compared with CNI-treated patients [87]. Therapy with belatacept, an investigational selective co-stimulation blocker, led to similar patient/graft 1-year survival compared to cyclosporine; however, a higher graft rejection rate was observed in the BENEFIT study. Currently, it is the first biological agent approved for the maintenance of immunosuppression after kidney transplantation [88]. In contrast to CNI-based maintenance immunosuppression, belatacept better preserves kidney graft function (glomerular filtration rate) after first year and may reduce the rate of chronic allograft nephropathy in the long-term [89,90]. A recent study with abatacept – another form of CTLA4-Ig fusion protein – has revealed in type 1 diabetes that the drug is able to preserve pancreatic β-cell function and delay progression of the disease [91].

However, there is a rationale that CTLA4-Ig may have a counterproductive effect on Treg function and tolerance induction, since Tregs rely upon CD28-dependent signals for development and peripheral expansion. It was found in specific CTLA4 knockout mice that the function of Tregs was lost, although the frequency of peripheral Tregs was dramatically elevated [92]. Additionally, administration of CTLA4-Ig significantly decreased the amount of thymus-derived natural Tregs [93]. In a murine skin transplant model, IL-2 mediated Treg expansion was inhibited by CTLA4-Ig because of the starvation of available B7 molecules [94]. Although graft biopsies in belatacept-treated kidney recipients had a significantly greater number of FoxP3$^+$ cells compared with CNI-treated patients [87], it was not confirmed when mRNA levels were measured. Twelve months after renal transplant, FoxP3 mRNA level in graft biopsies was significantly lower in the belatacept group than the CNI group [95]. Of note, other immunosuppressive agents were used simultaneously (anti-metabolite); therefore, observed effects might be a result of combined actions, not purely the result of CTLA4-Ig treatment.

4.3. Mycophenolate mofetil (MMF)

MMF is a reversible inhibitor of inosine monophosphate dehydrogenase, an enzyme, which plays a crucial role in GTP biosynthesis [96]. It is widely used as an immunosuppressant in organ transplantation, autoimmune diseases, and GVHD [97]. MMF specifically reduced the percentage of FoxP3$^+$ Tregs locally in the kidney in an ischemia-reperfusion model [98]. Additionally, the number and percentage of Ag-specific CD4$^+$CD25$^+$FoxP3$^+$ Tregs were also decreased on day 7 in the draining lymph nodes and spleens after treatment with MMF in the ovalbumin-immunized mice [99]. MMF can significantly decrease Treg numbers in a spontaneous hypertensive rat [100].

It is difficult to dissect the pure effect of MMF on Tregs in clinical settings, since this compound is usually supplemental to induction and other maintenance agents. In humans, basiliximab/belatacept/MMF/steroid therapy reduced Treg numbers after renal transplant for up to 1 year [14]. In liver graft recipients, where only CNI is used in addition to MMF, not only the number but also the percentage of Tregs was steadily reduced at 3 years post-transplant [30]. In lung transplant patients treated with protocols containing MMF, the number of CD4$^+$CD25^{high} cells was not significantly reduced [101]. As highlighted above, reduction of Tregs while using immunosuppressive regimens containing MMF might be rather the results of the simultaneous actions of all agents used, and not specifically associated with MMF. For example, in setting of allogenic bone marrow transplantation or symptomatic carotid artery stenosis, MMF did not affect Treg number, function, or FoxP3 expression in both in mice and in clinical settings [102,103]. However, conversion from CNI to MMF due to renal dysfunction was associated with an increase in the percentage of Tregs in liver transplant patients when MMF was used as a single-agent therapy [104].

4.4. Steroids

Glucocorticoids play an important role in the treatment and prevention of organ rejection in transplant patients. Corticosteroids interact with the intracellular receptor, the GC receptor (GR), a ligand-regulated transcription factor that positively or negatively alters the transcription of specific target genes, such as FoxP3 in Tregs [105]. *In vitro*, dexamethasone upregulated mRNA and FoxP3 expression in CD25$^-$ cells and generated CD4$^+$CD25$^+$FoxP3$^+$ Tregs; however, the immunosuppressive function of those cells was not improved [106]. In mouse models, multiple sclerosis or colitis treatment with steroids preserved both the number and function of Tregs [107]. In human studies, steroids were shown to preserve Tregs populations in chronic uveitis patients [68], and even to increase the levels of CD4$^+$CD25$^+$FoxP3$^+$ Tregs in asthmatic children treated with inhaled glucocorticoids, when compared to the controls and to the patients' own Treg levels prior to treatment [108].

4.5. mTOR inhibitor

Sirolimus also known as rapamycin (Rapa) and its analog, everolimus, are mammalian target of rapamycin (mTOR) inhibitors. mTORs display immunosuppressive activity affecting mTOR kinase and arrest the cell cycle in G1 phase, without inhibiting the production of IL-2, a cytokine playing an important role in the generation and activity of Tregs [78]. They preferentially spare Tregs, while simultaneously inhibiting T effector cells [109]. Although in *in vitro* MLRs Rapa slightly decreases the percentage of CD4$^+$CD25^{high} FoxP3$^+$ T cells, it substantially enhances their suppressive function when compared to control or assays treated with CsA [78].

In human, the number of Tregs in peripheral blood increased significantly in kidney transplant patients 6 months after switching to monotherapy with rapamycin comparing to monotherapy with TAC or MMF. Moreover, since numbers of antigen-specific Tregs increased in blood as well, the potential for the regulation of donor-specific responses in lymphoid and peripheral tissues was enhanced too [110]. Long-term patients on Rapa had more circulating Tregs at 12 and 24 months after renal transplant in comparison to patients on CsA [111]. Furthermore, patients with liver grafts treated with sirolimus monotherapy had significantly higher percentages of CD4$^+$CD25^{high} FoxP3$^+$ T cells compared with non-sirolimus group, even 3 years after transplantation [30]. After lymphocyte depletion by alemtuzumab or ATG induction, sirolimus but not CsA or TAC, increased the pool of FoxP3-expressing CD4$^+$CD25^{high} cells [16,21]. Moreover, sirolimus was able to increase the Treg number after conversion from TAC in liver and kidney transplant patients [22,112,113]. These data indicate that mTOR

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inhibitors can reverse the reduction of Tregs caused by other immunosuppressive agents. Also, combined use of everolimus and IL-2/IL-2ab complexes makes it feasible to achieve highly effective antigen-driven conversion of naive T cells into Tregs and expand these cells with high purity *in vivo* [114]. Nevertheless, despite a higher number of Tregs in peripheral blood, type 1 diabetes patients treated with IL-2 and sirolimus were characterized by lower pancreatic β-cell function, when compared to untreated controls [115]. However, this effect might be related to the toxicity of sirolimus in the pancreas.

5. Other agents

5.1. Interleukin-2

Interleukin-2 (IL-2) is a cytokine produced by T helper cells that is critical for signal transduction during immune response. It is critical for the function of not only cytotoxic lymphocytes but also Tregs. Tregs express high levels of the IL-2 receptor and IL-2 is essential for peripheral tolerance mediated by them [116]. IL-2 deficient mice had no or decreased numbers of FoxP3$^+$ Treg cells [117]. Treatment with anti-IL-2 antibody or IL-2 immunotoxin systemically also decreases the absolute number of Tregs [118] (also see above discussion of IL-2 receptor antibody). IL-2 therapy results in a higher percentage of CD4$^+$FoxP3$^+$ T cells in mice splenocytes; anti-CD25 can abrogate this effect *in vivo* [119]. In the NOD mice, IL-2 increases FoxP3 expression and the number of Tregs in the periphery, in local lymph nodes, and in the pancreas [118,120–122].

Similar results were found in clinical trials. IL-2 therapy increased the numbers of Tregs in graft-*versus*-host disease and autoimmune vasculitis, but did not affect the number of T effectors; thus, as a result, IL-2 increased the Treg/T effector ratio 4–5 times after therapy [123,124]. Combined with Rapamycin, IL-2 transiently increased both the number and frequency of nTregs in T1D patients. The number of FoxP3$^+$ cells increased from day 0 to day 28 and fell back to baseline on day 56, while the function was preserved [115]. Because IL-2-dependent STAT5 phosphorylation occurs primarily in FoxP3$^+$ regulatory T cells, Tregs receiving IL-2 signals proliferated and developed enhanced suppressive activity and could potentially be used to prevent autoimmunity [125].

6. Conclusion

In conclusion, most of the immunomodulatory agents used in clinical settings influence the function and number of Treg cells. Since Tregs play a vital role in immune adjustment, it is very important to understand the relationship between those agents and Tregs, especially in the clinical application of novel cellular therapy involving adoptive Treg infusion. Knowing the effects of those agents allows us to take advantage and select agents, which enhance Treg action, maintaining at the same time a graft immunoprotective environment. This enables us to plan the number and frequency of Tregs applied in order to achieve clinical effect [126]. In order to establish long-term tolerance, it would (most likely) require use of not one agent but a few drugs in combination in addition to *ex vivo* expanded Tregs. From this perspective, it seems that today ATG as a T cell depletion agent in combination with mTOR inhibitors as maintenance and antiTNFα as supplemental compound should be considered as the candidates with the most potential. Clinical trials utilizing those agents in combination with Tregs are imminent.

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