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T-Regulatory Cells: The Recently Recognized Players of Immunomodulation

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Abstract

Recently a subpopulation of T cells known as regulatory T cells (Tregs) have been identified to play important role of immunomodulation in autoimmune diseases, cancer and also in transplantation immunobiology. Tregs have been identified as biomarkers for graft function status. We have generated Tregs in vitro and also used them in kidney transplantation. We have found their efficacy in safe minimization of immunosuppression in kidney transplantation. Tregs are likely to form an integral part of cell therapy which will completely change the field of clinical transplantation. However there is a wide chasm between basic cell biologists and immunobiologists (who understand the significance of Tregs) and clinicians (who do not appreciate the bedside role of Tregs). This review article discusses the genesis of Tregs, their identification markers and their diagnostic and therapeutic role in transplantation immunobiology.

Introduction

Regulatory T cells (Tregs), formerly known as suppressor T cells, are a subpopulation of T lymphocytes which modulate the immune system by blocking all other types of T-cells. They maintain tolerance to self-antigens, help in inducing tolerance to foreign antigens and prevent malignancy. They have been qualified by expression of CD4, CD25 and *Foxp3* [1]. Takahashi et al described a rodent model wherein they observed that there were special types of T cells with surface markers CD4+ CD25+, which played important role in preventing autoimmune diseases. They observed that absence of these cells led to autoimmune diseases and re-introduction of these cells led to correction of autoimmune disease [2]. Tregs are involved in shutting down immune responses after they have successfully eliminated invading organisms, and also play active role in preventing autoimmunity. The presence of Foxp3 differentiates these Tregs from other suppressor T-cells like Tr1, Th3, CD8+CD28-, and Qa-1 restricted T cells that are generated *in vitro*.

Development and functions

In the process of hematogenesis, T-cells develop from lymphoblasts in the bone marrow. In the beginning they have no marker on their surface and are called double negative (DN) CD4-CD8- T cells. In their process of thymic maturation the T cell receptor genes form a unique, functional molecule in the thymic cortex for a minimal level of interaction with self-major histocompatibility complex (MHC). If they receive these signals, they proliferate and express both CD4 and CD8, becoming double-positive (DP) cells. The selection of Tregs occurs on radio-resistant haematopoietically-derived MHC class II-expressing cells in the medulla or Hassal's corpuscles in the thymus. At the DP stage, they are selected by their interaction with the cells within the thymus and begin the transcription of Foxp3 to become Tregs although they may not begin to express *Foxp3* until the single-positive stage, at which point they are functional Tregs. Tregs have diverse cell receptors compared to other cells. The process of Treg selection is determined by the affinity of interaction with the self-peptide MHC complex. Selection to become a Treg depends upon the intensity of signals received by these cells. A T-cell that receives very strong signals will undergo apoptotic death; a cell that receives a weak signal will survive and be selected to become an effector cell; and a T-cell that receives an intermediate signal will become a Treg. Foxp3+ Treg generation in the thymus is delayed by several days compared to T effector cells (Teff) and does not reach adult levels in either the thymus or periphery until around three weeks post-partum. Tregs require CD28 costimulation, and B7.2 expression is largely restricted to the medulla, the development of which seems to parallel the development of Foxp3+ cells.

Developmentally there are mainly two kinds of Tregs; the induced T-regulatory cells (iTregs) (CD4+ CD25+ Foxp3+) and naturally occurring Tregs (nTregs); iTregs are suppressive cells involved in tolerance and autoimmunity. The iTregs develop from mature CD4+ T-cells outside the thymus, whereas nTregs mature within the thymus. Though iTregs and nTregs share a similar function, little has been known about their exact role in immunotherapy. In an experiment on newborn mice with Foxp3 deficiency manifesting with inflammatory bowel disease, D. Haribhai et al showed that when nTregs alone were administered, the mice continued to have transmural lymphocytic infiltrates, however when they were administered iTregs, the infiltrates reduced significantly in gut, liver and lung also. Thus they deciphered that both iTregs and nTregs are required to induce tolerance [3]. Kim et al had shown that nTregs are essential for maintenance of tolerance [4]. For further studying the effect of depletion of iTregs on tolerance, Haribhai et al created a model of C57BL / 6 transgenic mice that expressed the diphtheria toxin receptor (DTR) under the

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control of the Foxp3 promoter (*Foxp3*DTR mice). When *Foxp3*DTR mice were injected a single dose of 50 μg/ kg diphtheria toxin, these mice lost 90% of their peripheral nTregs within 24 hours. Further, *in vitro* conversion experiments demonstrated that 94% of Foxp3+ iTreg cells expressed the DTR. Then they bred *Foxp3*DTR mice with CD45.1+ *Foxp3* enhanced green fluorescent protein (EGFP) mice and used their progeny as a source of marked CD4+ Tconv cells capable of expressing both EGFP and the DTR upon appropriate stimulation *in vivo*. They treated newborn CD45.2+ C57BL/6 *Foxp3*ΔEGFP mice with 1×10^6 CD45.2+ EGFP+ nTreg cells mixed with 4×10^6 CD45.1+ EGFP–DTR– Tconv cells. Littermate controls received 1×10^6 CD45.2+ EGFP + nTreg cells mixed with 4×10^6 CD45.1+ EGFP-Tconv cells that lacked the DTR transgene. Both groups of mice gained weight up to 50 days. From 50th day, all mice were injected with 50 μg/kg of DT every other day for 15 days and analyzed 5 days later. Only those mice where the transferred Tconv cells carried the DTR transgene lost weight. Thus they showed that acute depletion of the iTreg cell pool in mouse models resulted in inflammation and weight loss. The exact contribution of nTregs versus iTregs in maintaining tolerance is unknown, but both are important. Epigenetic differences have been observed between nTregs and iTregs, with the former having more stable Foxp3 expression and wider demethylation. Baron et al and Thornton et al have shown a highly specific DNA methylation pattern within the Treg-specific demethylated region (TSDR) of the Foxp3 gene. According to them, this gene gets demethylated in nTregs and it gets methylated in iTregs [5]. Moreover, Helios, a member of the Ikaros transcription factor family, has been identified as a marker for nTregs [6].

The molecular mechanism by which Tregs exert their suppressor/ regulatory activity has not been definitively characterized. *In vitro* experiments have given mixed results regarding the requirement of cell-to-cell contact with the cell being suppressed. The immunosuppressive cytokines TGF-beta and Interleukin 10 (IL-10) have been implicated in Treg function.

Molecular characterization

All the Tcells develop in thymus. Tregs are defined by the expression of fork head family transcription factor Foxp3 which also controls the genetic program of the cell's fate [7]. The large majority of Foxp3-expressing Tregs are found within the MHC class II restricted CD4-expressing (CD4+) population and express high levels of IL-2 receptor alpha chain (CD25). In addition to the Foxp3-expressing CD4+ CD25+, there also appears to be a minor population of MHC class I restricted CD8+ Foxp3-expressing Tregs. Unlike conventional T cells, Tregs do not produce IL-2 and are therefore anergic at baseline.

For identifying Tregs, high expression of CD25 and CD4 surface markers was considered as their hallmark, however it was later found that non-regulatory T-cells also express CD25 on immune activation. As defined by CD4 and CD25 expression, Tregs comprise about 5-10% of the mature CD4+ T cell subpopulation in mice and humans, while about 1-2% of Tregs can be measured in whole blood. The additional measurement of cellular expression of Foxp3 protein allowed a more specific analysis of Tregs (CD4+CD25+Foxp3+ cells). However, Foxp3 is also transiently expressed in activated human effector T cells, thus complicating a correct Treg analysis using CD4, CD25 and Foxp3 as markers in humans. Therefore, some research groups use another marker, the absence or low-level expression of the surface protein CD127 in combination with the presence of CD4 and CD25. Several additional markers have been described, e.g., high levels of CTLA-4 (cytotoxic T-lymphocyte associated molecule-4) and GITR (glucocorticoid-induced TNF receptor) are also expressed on Tregs, however the

functional significance of this expression remains to be defined. There is a great interest in identifying cell surface markers that are uniquely and specifically expressed on all Foxp3-expressing Tregs. However, to date no such molecule has been identified. In addition to the search for novel protein markers, a different method to analyze and monitor Tregs more accurately has been described in the literature. This method is based on DNA methylation analysis. Only in Tregs, but not in any other cell type, including activated Teff cells, a certain region within the foxp3 gene (TSDR, T_{reg}-specific-demethylated region) is found demethylated, which allows to monitor Tregs through a PCR reaction or other DNA-based analysis methods [8]. Recent evidence suggests that mast cells may be important mediators of Treg-dependent peripheral tolerance [9].

Tregitopes

Tregitopes were discovered in 2008 when De Groot et al were searching for potential effector T-cell epitopes in monoclonal antibodies [10,11]. By using special mapping tools they found out that there were putative T-cell epitope sequences highly conserved across Fc and Fab domains of IgG antibodies and that these sequences were found to suppress immune responses when co-cultured with T cells. The expanded cells thereof, demonstrated characteristics of Tregs. Thus Tregitopes are established as linear sequences of amino acids within monoclonal antibodies and immunoglobulins, and are crucial for the activation of nTregs [12]. Tregitopes are peptides having the following four characteristics: (i) their sequences are highly conserved in similar autologous proteins, (ii) they almost all exhibit "EpiBars" or a pattern (measured by EpiMatrix) that suggests promiscuous MHC binding [12], (iii) T cells responding to these Tregitopes exhibit a T regulatory phenotype (CD4+CD25+FoxP3+) and secrete IL-10, TGF-β and MCP-1 [10], and (iv) coincubation of Tregitopes with immunogenic peptides inhibits T cell proliferation *in vitro* and suppresses the secretion of effector cytokines and chemokines in response to the immunogenic peptides. The hypothesized potential applications of Tregitopes are transplant tolerance, autoimmunity and allergies [13-15].

Tregs and disease

In mouse model studies, it has been observed that although Tregs are essential to induce tolerance and prevent autoimmune diseases, they are down-regulated during an infectious episode to facilitate elimination of the infection like tuberculosis, Leishmania and malaria [16,17]. Increased number of Tregs has been associated with poor prognosis in breast, colorectal and ovarian cancers [18].

Tregs and Autoimmunity

Takahashi et al showed that elimination of CD25 (IL-2 alpha receptor chain) expressing T cells which constitute about 5-10% of CD4+ cells and <1% of CD8+ cells in normal naive mice produced different autoimmune diseases similar to those in human beings like autoimmune gastritis, insulin-dependent diabetes mellitus and thyroiditis [2]. They prepared spleen and lymph node cell suspensions from 8 week old BALB/c null + and COG transgeneic mice. They isolated two groups of cells, CD4+ CD25+ and CD4+ CD25- T cells from these suspensions. They found that CD4+ CD25+ T cells exhibited no proliferative response to *in vitro* stimulation in contrast to CD4+ CD25- T cells which exhibited 2-3 times proliferation as compared to un-separated CD4+ cells. Further they also elicited that CD 4+ CD25+ T cells exert suppressive effect on CD4+ CD25- T cells via antigen presenting cell by direct cell-to-cell contact leading to reduced IL-2 formation. They found that breakage/ anergy of suppression of CD4+ CD25+ T cells evoked activation of self-reactive

T cells from CD25- dormant states. They also found that the antigen concentration required for stimulation of CD4+ CD25+ T cells to exert their suppressive effect was much lower than concentration required for activation of self-reactive T cells. Thus it is clear that even low dose of CD4+ CD25+ T cell is enough to control/prevent autoimmune disease. Sakaguchi et al. reported that *in vivo* and *in vitro* studies showed that there were multiple mechanisms by which Tregs carry out their suppressive action on cells causing autoimmunity [19]. There are various molecules secreted or expressed on the surface of Tregs which directly contribute to their action of self tolerance and immune homeostasis. Each Treg has been assigned a particular suppressive mechanism and several complementary mechanisms which may be CTLA-4 dependent or IL-2 dependent. Thus depending upon the situation a particular cell will be directed to carry out its suppressive activity in addition to other activities and in congruence with neighbouring cells. This symphony maintains the state of self-tolerance.

Tregs and Organ Transplantation

Tregs as biomarkers for graft dysfunction: Kidney transplantation has now become a well-accepted therapy for chronic renal failure. Surgical skills are now well established however there is still no definite consensus to prevent the graft loss due to immune causes. To date there are no non-invasive tests established that can reflect the functioning of the graft in terms of immunological and non-immunological status. Biopsy still remains the gold standard to diagnose the pathology in a functioning graft. However it is not always possible to predict the prognosis by examining a graft biopsy. In addition there is a small risk of complications of performing a graft biopsy ranging from hematuria to arterio-venous fistula and eventual graft loss [20]. Hence the problem of diagnosing graft dysfunction especially using non-invasive techniques remains unsolved.

Various novel biomarkers like urinary neutrophil gelatinase associated lipocalin (NGAL), β-2 microglobulin, N-acetyl-beta-D-glucosaminidase (*NAG*), interleukin-18, kidney injury molecule (KIM-1), vascular endothelial growth factor (VEGF), serine protease inhibitor-9 (PI-9) or cystatin C have been tried to diagnose graft dysfunction, however none of them has withstood the test! Measurement of donor specific/nonspecific HLA antibodies in serum has shown promising results. However in our experience when a renal allograft recipient is subjected to stem cell therapy in these circumstances even if there is high level of DSA, the graft stays protected from immune injury [21]. T-regs with Foxp3 are believed to hold promise of becoming dependable biomarkers.
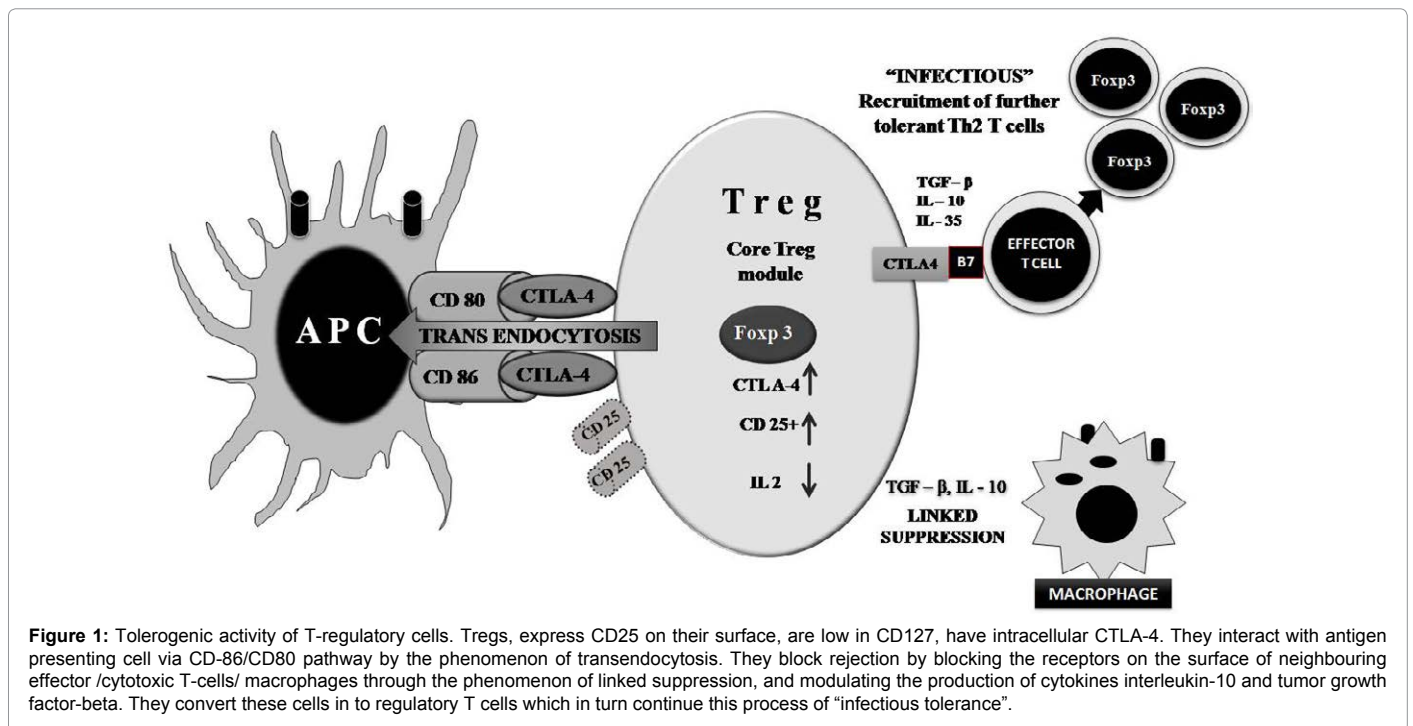
In a study by Amirzargar et al. 57 renal allograft recipients with stable graft function for 6 months posttransplant were selected for a prospective study of cellular changes to identify their immune status [22]. Their peripheral blood CD4+CD25+FoxP3+ and CD8+CD28- Tregs, myeloid dendritic cells type 1 (MDC1) and type 2 (MDC2), and plasmacytoid dendritic cells (PDC) were measured pre-transplant, and at 2 weeks, 1 month, 3 months, and 6 months after transplantation. They observed that mean Tregs decreased 2 weeks after transplantation and then these values increased subsequently within 2 months of transplantation. The frequency of Tregs and MDCs at 3 months could predict the frequency at 6 months posttransplant. Interestingly donor-recipient relationship/HLA match and GFR did not correlate with graft function status. Thus Tregs were found to be more reliable predictors of stable graft function. The authors have deciphered that initial heavy immunosuppression has led to drop in Tregs at 2 weeks posttransplant. Subsequently with stability of the graft function and lower immunosuppression requirement/ dosage of calcineurin

inhibitors, the peripheral blood levels of Tregs have improved and remained steady.

Bouvy et al. carried out a study on 33 renal transplant patients to study the effect of induction therapy on the homeostasis and function of Tregs [23]. The study was carried out in 2 groups of patients, one group received rabbit anti-thymocyte globulin (r-ATG) in the dose of 2 mg/kgBW on days 1, 2 and 3 posttransplant; and the other group received monoclonal antibody targeting the IL-2R (basiliximab) in the dose of 20 mg on days 0 and 4 post-transplant. All patients were on triple immunosuppression of Tacrolimus, mycophenolate mofetil (MMF) and steroids, tapered after 3 months of transplantation. Peripheral blood of all recipients was collected for analysing CD127$^{low/-}$/CD4+/CD25^{hi} before, and at 1, 3, 6 and 12 months posttransplant. In the rATG arm, there was significant reduction in both Teff and Tregs. These numbers gradually increased after a month, however the pre-transplant levels were not achieved; whereas in the Basiliximab arm, the absolute numbers remained unchanged. For the first 3 months posttransplant, the proportion of CD4 and Tregs was higher in Basiliximab arm vs. rATG arm all along. However from 3 to 6 months, there was a sustained rise in Tregs in rATG arm with drop in CD4+ cells unlike in the Basiliximab arm. While studying the functional capacity of Tregs, there was no difference in IFNγ production capacity however IL-10 production was increased at 1 and 12 months posttransplant after rATG therapy. This increased capacity was noted only at 1 month posttransplant in Basiliximab arm. In addition MLR performed from peripheral blood mononuclear cells of rATG arm patients with 3rd party Teffs exhibited inhibition of proliferation capacity of Teffs. Interestingly 4 out of 5 patients also showed inhibition of donor effector cells but not third party effector cells. In basiliximab arm, Tregs comparably inhibited donor and third party stimulated Teff cells. However due to large variation in suppressive capacity of Tregs, no statistical difference was found between the 2 arms. They also found no correlation between rejection episodes, Tregs and rATG/ basiliximab.

Nguyen et al. carried out a study of pre-transplant Tregs (CD4+ CD25^{hi} Foxp3+) and Teff (CD4+ CD25-) frequencies in 53 deceased donor renal transplant recipients to find out whether Tregs can serve as biomarkers of acute kidney injury (AKI) so that timely and effective management can be instituted in transplant recipients to improve the short term, and more importantly, long-term graft outcome [24]. They divided the patients in 2 groups, AKI group (n=27) of delayed graft function (n=10) and slow graft function (n=17), and group with immediate graft function (IGF) (n=16). They found that there was no difference in frequencies of both cell populations and ratios pre-transplant, however functionality of Tregs was higher in IGF group vs. AKI group. They found that Treg function remained a significant predictor of eGFR only at 14 days after transplantation and not later.

In rodent model studies Jofrre et al. have observed that Tregs offer graft protection through the phenomenon of 'linked suppression' [25]. They interact with antigen presenting cell via CD-86 /CD80 pathway by the phenomenon of transendocytosis (Figure 1). They block rejection by blocking the receptors on the surface of neighbouring effector /cytotoxic T-cells/ macrophages through the phenomenon of linked suppression, and modulating the production of cytokines interleukin-10 and tumor growth factor-beta. They convert these cells in to regulatory T cells which in turn continue this process of "infectious tolerance". Linked suppression requires the presence of new antigen and the transplant antigen to which the host is already tolerant. Thus immune tolerance to transplant antigens mediated by Tregs is highly specific and highly localized. In addition Tregs are highly versatile. They can control immune responses of other cells like NK cells, CD4+ T cells, NKT cells,

Figure 1: Tolerogenic activity of T-regulatory cells. Tregs, express CD25 on their surface, are low in CD127, have intracellular CTLA-4. They interact with antigen presenting cell via CD-86/CD80 pathway by the phenomenon of transendocytosis. They block rejection by blocking the receptors on the surface of neighbouring effector /cytotoxic T-cells/ macrophages through the phenomenon of linked suppression, and modulating the production of cytokines interleukin-10 and tumor growth factor-beta. They convert these cells in to regulatory T cells which in turn continue this process of "infectious tolerance".

B cells and various antigen presenting cells [26]. Thus Tregs are more powerful and therapeutically more potent immunomodulators than standard immunosuppressive agents available in pharmacy today.

Tang and Lee showed that the total population of Tregs in an adult human being is estimated to be 13×10^9 [27,28]. They also established that most of the Tregs reside in lymphoid organs. Further they derived that effective dose of polyclonal Tregs for preventing rejection in humans is estimated to be more than 30×10^9. They found that the number of Tregs circulating in blood is 0.25×10^9.

We have generated Tregs *in vitro* from kidney donor adipose tissue derived mesenchymal stem cells and recipient peripheral blood mononuclear cells and infused them in 30 recipients after transplantation. These patients have finished 11 months and show absence of rejection, have stable graft function with mean serum creatinine of 1.2 mg/dL on immunosuppression of Sirolimus,1 mg/day and Prednisone, 5 mg/day (in press).

Issa and Wood report that recipients with operational tolerance in solid organ transplantation show an increased level of Foxp3 cells [29]. However it has been observed paradoxically that in patients undergoing acute rejection in cardiac and kidney transplants there is an increased level of Foxp3 mRNA levels. Similarly the urine of patients with rejecting renal allografts also has shown increased level of Tregs [30-32]. Higher peripheral Foxp3+ cells predict a higher risk of development of skin cancers in renal allograft recipients [33]. The authors believe that increase in Foxp3+cells in acute rejection may be related to its up-regulation on activated effector T cells, or an increase in Tregs in response to high donor-activated effector T cell activity. Conversely, in renal transplant patients undergoing chronic rejection there was a fall in peripheral Foxp3+ cells compared to those with either normal graft function under immunosuppression or those who were operationally tolerant. The increase in Foxp3+ cells in the graft and periphery was most striking in tolerant liver transplant recipients [34-37]. In our experience we have measured Tregs in periphery and

Foxp3 levels in renal allografts of patients with operational tolerance and compared them with patients undergoing rejection. We have observed that patients with operational tolerance show about 3 to3.5% Tregs in their circulation and about 0.3% in their grafts. In event of acute rejection, the levels of Tregs start dropping to ≤ 1% in circulation and Foxp3 in the graft start increasing to ≥ 0.5%, depending upon the severity of injury. Further when chronic rejection sets in, the same patient exhibits high level of Tregs to around 5% to 7 % and plateaus out at that level, along with sustained levels of Foxp3 in the graft to about 1% to 1.5% (unpublished observations). Interestingly we have also seen that in patients who have undergone stem cell therapy, Tregs offer graft protection in spite of high level of DSA. In these patients, even if there is an event of acute rejection, Tregs initially fall in peripheral circulation to migrate to the graft to offer protection and once the graft function stabilises, they move back to their bunkers in lymph nodes and maintain their homeostatic balance of ≈3-4% in peripheral circulation. This phenomenon exhibits the mechanism of action of Tregs. Tregs work through the phenomenon of "linked suppression" and "infectious tolerance".

Regarding the interaction of Tregs with immunosuppressive medications it has been observed that Tregs and T-eff cells preferentially employ different intracellular activation pathways: Tregs utilise IL-2-dependent STAT-5, whereas effector T cells utilise the phosphoinositide 3-kinase/ Akt/mammalian target of rapamycin (mTOR) pathway [38-40]. Immunosuppression with an mTOR inhibitor takes advantage of this distinction. The beneficial effects of Sirolimus on Treg survival and proliferation have been demonstrated *in vitro* and *in vivo* [41-47]. Alemtuzumab (anti-CD52 mAb) may also favour Treg survival, with evidence from one study demonstrating a higher proportional depletion of T eff cells than Tregs [48].

Conclusion

The studies mentioned here have shown that Tregs can be good predictors of stable renal allograft function for the first six months.

However, long term Treg values and correlation with graft biopsy and functional status need to be studied. The percentage of Tregs in recipients with stable graft function also needs to be studied by more workers.

Functionality rather than number of Tregs was found to be more important for immediate graft function. However it has been noted that Foxp3 can be seen with activated T cells also, hence it is important to predefine Tregs as CD127 negative. Secondly the deleterious effect of calcineurin inhibitors (if used) also needs to be considered while establishing the diagnostic role of Tregs. In addition, in a potential set-up of transplantation where antibody activity increases considerably in the immediate posttransplant period in spite of adequate immunosuppression, it is quite possible that some of the cells studied may have been converted in to Teffector cells. When the effect of induction therapy on the homeostasis and function of Tregs is evaluated for transplantation tolerance, it was believed that newly formed iTreg after rATG therapy has donor-specific immunoregulatory properties. Repopulation of Treg after rATG and basiliximab therapy is believed to be the result of homeostatic proliferation and not of thymopoiesis. It is emerging that Tregs are the likely key players of tolerance in transplantation by playing the dual diagnostic and therapeutic role. Both nTregs and iTregs appear to work synchronously to achieve tolerance induction.

Acknowledgement

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