Regulatory cells and cell signatures in clinical transplantation tolerance
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Experimental models of transplantation provide strong support for the role of regulatory cells in tolerance. However, limited studies of humans who display sustained tolerance following transplantation have not definitively demonstrated a role for regulatory cells in this process. Rather than excluding or minimizing the contribution of regulatory cells to the development of transplantation tolerance, we suggest the possibility that multiple lineages of cells exert regulatory effects that contribute to the development of tolerance, that these regulatory effects are not constant but vary over time, and that the role of regulatory cells varies based on the organ transplanted. More detailed studies will be necessary to elucidate the role of regulatory cells in clinical transplantation and tolerance.

Introduction
Numerous advances in transplantation including the introduction of new immunosuppressive agents have translated into significant improvements in the short-term outcome of nearly all types of organ transplants. However, improvements in the short-term success of renal and extra-renal transplantation have had a minimal impact on the long-term success as the rate of late graft loss is essentially unchanged [1,2]. While there have been no studies directly comparing the outcome of tolerant patients to those who continue to receive immunosuppression, the intuitive advantages associated with the avoidance of chronic immunosuppression continue to generate enthusiasm for implementing approaches to induce tolerance to transplanted organ allografts. Much of our current understanding of the mechanisms promoting tolerance, the barriers to tolerance, and the effect of various immunosuppressive agents on tolerance has been derived from experimental studies in rodents [3]. The mechanisms shown to contribute to transplantation tolerance include ignorance, anergy, exhaustion, deletion, and regulation [4]. This review focuses on regulatory cells in clinical transplantation and tolerance.

Classification of cells with regulatory properties
In 1990 Hall et al. reported that the transfer of cells from rats accepting heart allografts following a short course of cyclosporine to naïve rats conferred donor-specific tolerance [5]. Over the subsequent two decades tremendous strides have been made in our understanding of the immunobiology of regulatory cells and their roles in autoimmunity, infectious diseases, cancers, and transplantation [6–7]. Hematopoietic cells of several different lineages possess regulatory properties that may contribute to tolerance to self-antigens or alloantigens. Many, but not all of these regulatory cells express CD4. The broadest distinction between cells with regulatory properties is based on their expression of the nuclear transcription factor Forkhead box protein 3 (Foxp3), which has been shown to be a key regulator of Treg development in both mice and humans [8–10]. Foxp3+ Tregs are predominantly also CD4+ although CD8+Foxp3+ cells with suppressive properties have been described. CD4+Foxp3+ Tregs can be divided into natural Tregs (nTregs) that develop in the thymus and are selected by self antigens or induced Tregs (iTregs) derived from effector T cells in the periphery that are believed to have been selected by foreign antigens. nTregs have more potent suppressive effects and tend to be more stable than iTregs perhaps due to epigenetic modifications in the Foxp3 gene [11].

Several populations of CD4+Foxp3+ T cells also exhibit powerful suppressive or regulatory properties including firstly, T regulatory type I (Tr1) cells that produce large amounts of the suppressive cytokine IL-10 [12], secondly, T helper 3 (Th3) cells that produce the suppressive cytokine TGFβ [13], and thirdly, T regulatory type 35 (Tr35) cells that produce IL-35, which is related to the IL-12 superfamily and acts to suppress immune responses by a variety of mechanisms [14]. In addition to CD4+ T cells, CD8+CD28– [15], CD3+ double negative (CD3+CD4−CD8−) cells [16], and NKT cells [17] have all been reported to exert regulatory effects on alloimmune responses. T cells expressing the γδ TCR have been reported to possess regulatory properties in
certain autoimmune diseases [18] and following liver transplantation [19].

Until recently the role of B cells in immunity and transplantation was thought to be confined to mediating injurious responses through antibody production, antigen presentation, or cytokine production [20]. However, like T cells, B cells are increasingly recognized as having a dual role in immunity serving both as immune effector and regulatory cells (Breg — reviewed Mauri Nat Rev Rheum 2011). Unlike Tregs, there are no validated molecular or phenotypic markers to define Bregs. Consequently they are currently defined functionally based on their production of IL-10.

### Augmenting regulatory function to promote tolerance

As the fate of transplanted organs is in part determined by the balance between effector and regulatory activities, one approach to promoting tolerance would be to enhance regulatory functions following transplantation. The direct transfer of regulatory cells into transplant recipients would accomplish this goal but is limited by the low frequency of Tregs (estimated to be 1–5% of the peripheral T cell pool). From a practical standpoint obtaining enough Tregs requires that they be isolated and expanded in vitro [21]. The first challenge was to identify markers specific for Treg. The demonstration that a major subset of Treg in mice and humans expressed Foxp3 did much to advance the study of Treg [8–10]. However, Foxp3 is of limited utility for isolating Tregs as it is expressed intracellularly and requires permeabilization of the cell for its detection. Furthermore in humans, unlike mice, Foxp3 may be transiently expressed by recently activated T effector (Teff) cells [22]. These limitations have been somewhat overcome by the demonstration that CD127, the alpha chain of the IL-7 receptor, when used in conjunction with IL-25 expression effectively distinguishes Foxp3+ Teff from Foxp3+ Treg with functional Treg confined to the CD25+CD127– population [23,24].

A second approach to promoting tolerance would be to select immunosuppressive agents that maintain the function of regulatory cells while suppressing effector cells. This rationale explains the frequent avoidance of calcineurin inhibitors (CNI) that may interfere with the development of Tregs and tolerance [25] and the inclusion of sirolimus that supports the development and maintenance of Tregs [26,27] in the design of tolerogenic immunosuppressive regimens. Biologic agents may also have dramatically differing effects on Tregs [28]. Available literature suggest that polyclonal rabbit anti-thymocyte globulin (rATG), alemtuzumab (an anti-CD52 mAb), natalizumab (an anti-VLA-4 mAb), or efalizumab (a anti-LFA-1 mAb) all result in increased frequencies of human Treg in vivo while anti-IL2 receptor antibodies and alefacept (an anti-CD2 mAb) decrease the frequency of Tregs in humans. The effect of belatacept (CTLA4Ig) on Tregs in vivo is uncertain although recently it has been reported that belatacept, when used in combination with rATG and sirolimus, favorably alters the ratio of Treg to Teff [29].

### Regulatory cells in transplantation and tolerance

It is appealing to postulate that an increase in Tregs is mechanistically associated with tolerance and that monitoring the frequency of Tregs may identify tolerant recipients. Surprisingly however, numerous reports suggest that Tregs may be associated with acute rejection. In heart transplant recipients Foxp3 expression was increased in biopsies from recipients with higher grades of rejection relative to those without rejection or with lower grades of rejection [33]. Together these findings suggest that regulatory T cells infiltrate rejecting allografts in an attempt to suppress inflammation. Thus, the detection of increased numbers of Tregs may not always mark individuals predisposed toward tolerance. Consistent with a role for Foxp3+ Treg in limiting alloimmune injury and possibly promoting tolerance, several groups have reported that Foxp3 gene expression and/or the frequency of Foxp3+ Treg was lower in kidney and lung transplant recipients developing chronic injury relative to those who maintained stable function [34–36].

Given the extensive evidence that Tregs play a significant role in experimental models of tolerance together with clinical evidence supporting a role for Tregs in controlling acute and chronic rejection it is surprising that only limited data support a major role for Tregs in clinical transplantation. Studies of children undergoing liver transplantation at the University of Kyoto describe an increase in the frequency of Tregs in the blood and allografts of tolerant patients relative to patients that had failed weaning or healthy volunteers [37–39]. Initial studies of adult liver transplant recipients lead by Dr. Sanchez-Fueyo have revealed over-expression of genes related to NK cells and T cells expressing the γδ TCR in tolerant recipients as well as an increase in the number of potential regulatory T cells in the peripheral blood as defined by expression of CD4 and CD25 [19]. However, more extensive studies but that group failed to confirm the increased frequency of Tregs [40] or γδ T cells [41]. Factors such as differences in recipient age (children versus adults) or donor type (deceased versus living)
may contribute to the discrepant results obtained by in these studies.

A role for Tregs has also recently been described in tolerant patients who underwent combined kidney and bone marrow transplantation from haploidentical donors following nonmyeloablative conditioning [42]. Early during recovery from the induced lymphopenia, the peripheral T cell repertoire of all patients was characterized by T cells with a memory phenotype. A subset of patients displayed an increased frequency of CD4+CD25+CD127−Foxp3+ Treg at early time points that disappeared as reconstitution of the lymphocyte repertoire progressed. The increased frequency of Tregs together with evidence supporting a deletional mechanism of tolerance in this scenario suggest that tolerance may arise from multiple mechanisms working in concert. This concept has important implications for the development of assays to detect tolerance.

Several studies support a role for regulatory mechanisms contributing to operational tolerance following kidney transplantation. Early evidence came from the observation that a cellular component of blood from tolerant patients suppressed the recipient anti-donor response in the trans-vivo DTH assay via the production of TGFβ and/or IL-10 [43]. These data were interpreted as supporting the widely held paradigm that regulatory T cells play a significant, if not dominant, role in transplantation tolerance. A subsequent study of the molecular markers associated with tolerance following kidney transplantation identified 49 genes that distinguished tolerant patients from those with chronic rejection [44]. These genes showed a bias toward T cell-specific expression and regulation during T cell suppression and costimulation as well as cell cycle regulation. Although TGFβ was not differentially expressed at the mRNA or protein level in tolerant patients compared to those with chronic rejection, TGFβ regulates the function of 27% of the 49 identified genes.

In contrast to reports associating Tregs with tolerance following kidney transplantation, three recent studies reported that increases in B cells and B cell-related genes distinguished tolerant from non-tolerant kidney transplant recipients. The major findings of a study of 25 tolerant kidney transplant recipients sponsored by the Immune Tolerance Network (ITN) included firstly, the identification by microarray and real-time PCR of 30 genes, over two-thirds of which are B cell specific, that distinguished tolerant and non-tolerant individuals, secondly, the finding that phenotyping of peripheral blood cells showed an increase in B cells of a primarily naive and transitional phenotype in the tolerant group, thirdly, the demonstration that polyclonally stimulated transitional B cells from tolerant recipients produced more IL-10 in vitro relative to those from non-tolerant transplant reci-pients, and fourthly, expression of CD20 was increased in the urinary sediment cells from tolerant individuals [45**]. Despite the use of different assays and methods the central findings of the study of 11 tolerant kidney transplant recipients conducted by the RISET consortium were very similar and included the demonstration that spontaneously tolerant kidney transplant recipients: firstly, over-expressed B cell-related genes as determined by analysis of microarrays, secondly, displayed expansion of B cells and NK cells together with fewer activated CD4+ T cells in the blood as determined by flow phenotyping, and thirdly, were characterized by donor-specific hyporesponsive CD4+ T cells in vitro [46**]. A third report of 12 tolerant kidney transplant recipients from investigators at the University of Nantes also found that tolerant patients were characterized by an increase in the frequency and absolute number of B cells in the blood that was largely due to an expansion of activated, memory and early memory B cells [47**]. Detailed phenotypic analysis of B cells in tolerant patients demonstrated that they had an inhibitory phenotype as defined by the increased expression of BANK1, CD1, and CD5 as well as a decreased ratio of CD32a/CD32b. A significant difference between these reports associating B cells with tolerance and previous reports linking Tregs to tolerance is the use of transplant recipients with stable function as a comparison group versus patients with chronic rejection. As chronic rejection has been associated with a decrease in Treg frequency [34–36] it may be that the increase in Tregs in tolerant patients actually reflects decreased Tregs in the comparison group of patients with chronic rejection.

Mechanistic considerations linking B cells to suppressed immunity

Based on these three reports it is appealing to attribute a mechanistic role for B cells in the development of tolerance. However, as the reports associating B cells with the tolerant phenotype following kidney transplantation examined patients with well-established, longstanding tolerance, it is not possible to determine whether the ‘B cell signature of tolerance’, proceeded the development of tolerance and thus possibly contributed to its development or whether it arose after tolerance had developed, and could reflect either a tolerant state perse, or a consequence of discontinuation of immunosuppression. These considerations have important implications for the design of tolerance inducing regimens and assays for detecting tolerance.

While the role of B cells in clinical tolerance remains speculative at this time, there is ample evidence that Bregs contribute to homeostasis in autoimmunity, cancer, and infectious diseases [48] and there is emerging experimental data that B cells contribute to transplantation tolerance. Le Texier and colleagues reported that tolerance to heart allografts induced by a deoxyspergualine
analogue in rats was associated with an accumulation of B cells within the allograft [49] Similar to the report from this group describing operationally tolerant kidney transplant recipients [47**] the B cells in these tolerant rats displayed an inhibitory phenotype. Transfer of B cells from tolerant recipients to naïve rats conferred donor-specific tolerance demonstrating that in this model B cells actively contributed to tolerance. Lai and colleagues examining the role of B cells in tolerance induced by treatment of mice with an anti-CD154 mAb and donor-specific transplantation found that depletion of B cells at the time of transplantation abrogated tolerance to heart allografts [50]. Chen and colleagues examined the effect of this regimen on mice reconstituted with bone marrow such that IL-10 production was defective in either the T or B cell compartment. They demonstrated an independent role for IL-10 producing B cells in the maintenance of tolerance [51].

Conclusions

Despite two decades of experimental data demonstrating a role for regulatory T cells in transplantation, definitive evidence linking Tregs and tolerance in human transplant recipients is lacking. Emerging clinical data appear consistent with a mechanism by which Tregs expand in response to rejection in an attempt to limit immune injury. Reduced numbers of Tregs result in failure to adequately control the alloimmune response and the consequent development of chronic rejection. Whether the failure to discover significant differences in Tregs in tolerant patients compared to non-tolerant patients is due to limitations in our ability to sample the correct tissue at the appropriate time, the existence of as yet undefined populations of regulatory cells, or simply reflects their limited role in clinical tolerance remains uncertain. Ongoing coordinated efforts by several groups to identify larger numbers of tolerant transplant recipients and study them in detail should shed light on the mechanisms responsible for transplantation tolerance and the contributions of regulatory cells to this process.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest


A concise summary of the current literature discussing the effects of biologic immunosuppressive agents on regulatory T cells.


An original report describing the molecular and phenotypic characterization of peripheral blood cells in tolerant renal transplant recipients. This report describes a B cell ‘signature’ associated with tolerance following renal transplantation.


An original report describing molecular, phenotypic, and functional characteristics of tolerant renal transplant recipients. The findings reported suggest that differences in B cells distinguish tolerant from non-tolerant renal transplant recipients.


An original report including a detailed phenotypic characterization of B cells in tolerant renal transplant recipients demonstrating that in addition to being numerically increased, B cells in tolerant recipients display an inhibitory phenotype.


