Prognostic significance of graft Foxp3 expression in renal transplant recipients: a critical review and attempt to reconcile discrepancies

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ABSTRACT

A large body of evidence has been accumulated from experimental models in the past decade to support the critical role of Foxp3-expressing regulatory T cells (Tregs) in the suppression of alloimmune responses. This has prompted transplant clinicians to investigate whether Foxp3 analysis might be used as an immunodiagnostic tool for better assessment of the significance of graft infiltrate and to predict its impact on graft outcome. However, conflicting results have emerged from these studies and may have generated more confusion than clarification. Foxp3 expression has been antagonistically correlated with either good or poor prognosis. We discuss here how methodological issues and specific clinical settings may have accounted for the discrepancies between the results of these studies. Depending on many factors, including the techniques used, the method of sampling normalization, the extent of intra-graft inflammation, the immunosuppressive regimen and the depletion or repletion of T lymphocyte compartment, the significance of Foxp3 expression may vary. We propose here the conditions to be fulfilled in order to use Foxp3 analysis as a relevant biomarker for graft outcome assessment. Far from challenging the key role of Tregs in dampening alloimmune responses, this review highlights the need for technical harmonization and standards.

INTRODUCTION

The concept of T-cell-mediated immunoregulation arose in the early 1970s following the description of lymphocyte populations and their capability to suppress antigen-specific immune responses [1]. Since then, several studies have validated the crucial importance of this mechanism in the control of T-cell homeostasis and T-cell responses to self-antigens (and thereby the prevention of autoimmune diseases) [2].

In both rodents and humans, there is wide consensus that T-cell immunoregulatory activity is enriched in the CD4+CD25+Foxp3+ T-cell (Treg) population, and this subset will therefore form the focus of the present review. However, it should not be forgotten that regulatory activity has also been reported for CD8+ [3] and other CD4+CD8− [3] T-cell subsets.

There are two categories of Tregs, which differ in their origin, phenotype, plasticity, mode of action and epigenetic
modifications at the Foxp3 locus (Table 1) [4]. Naturally occurring Tregs (nTregs) develop from T-cell precursors with some degree of self-reactivity during the normal process of T-cell maturation in the thymus and survive in the periphery poised for immunoregulation [5]. The second subset of induced Treg (iTreg) develops as a consequence of peripheral activation of classical naive CD4+CD25− T-cell populations under particular conditions [6], notably in the presence of TGFβ (Table 1). It has been proposed that these 2 subsets work in synchronization: nTreg cells are initially recruited and iTreg cells are then induced to further suppress the immune response and to achieve a fine homeostatic balance [7]. Interestingly, Maganto-García et al. [8] recently showed that Foxp3+ iTregs, but not nTregs, interact efficiently with endothelial selectins and transmigrate through activated endothelial monolayers in vitro. In addition, Foxp3+ iTregs adhered to inflamed endothelium in vivo and their secretion products blocked acute inflammation in vivo. These data support the concept that Foxp3+ iTregs are active in inflammatory tissues and also help to regulate inflammation independently of their influence on effector T cells by direct suppression of endothelial activation and leucocyte recruitment [8]. Importantly, this study highlighted the differential capacity of nTregs and iTregs to regulate inflammation. We might envisage that iTregs might be more important in control of alloresponse in organ transplantation than nTregs, though very few studies have investigated this issue [9]. The generation of mice selectively lacking iTregs [10–12] has recently demonstrated the critical role of iTregs in maternal tolerance towards paternally inherited fetal alloantigens [11]. These genetically manipulated mice will undoubtedly constitute a very powerful tool for investigating their role in rodent models of solid organ transplantation. Information about the respective roles of nTregs and iTregs in the regulation of alloimmune responses in humans is even scarcer, though growing evidence suggests that Tregs mainly emerge at the periphery from the memory T-cell pool in healthy adult donors [13] as well as in kidney transplant recipients [14].

The molecular mechanisms that are responsible for the regulation of immune responses by Tregs are not fully understood and are likely to be different according to the context (type of Tregs, type of effector targeted, location etc) (Figure 1). This raises the question of the real site of active suppression and highlights the migration potential of Tregs. Several studies have shown that Treg cells sequentially migrate from inflamed tissues to lymph nodes to suppress alloimmune responses [15, 16]. Zhang et al. showed that Treg cells are first activated in the allograft, and subsequently migrate to the draining lymph nodes where they inhibit dendritic cell migration and suppress the alloantigen-specific immune response. These findings emphasize that Treg infiltration in tissue is a dynamic process that must be kept in mind when interpreting Foxp3 in graft-infiltrating T cells. In this respect, studying the regulatory response in secondary lymphoid organs, as commonly done in experimental models, might be informative, but could not be performed in human settings.

The specific nature of Treg activity at the site of inflammation remains unclear. Whether infiltrating Tregs regulate autoimmunity or alloimmunity or both is an important question that needs to be addressed. In a context of inflammation (chronic or acute rejection), infiltrating Tregs might be triggered not only by alloantigens but also by many autoantigens (shared between donor and recipient) in order to prevent autoimmunity, while in a steady state it is possible that the majority of Tregs are active in secondary lymphoid organs to suppress antigen-specific responses.

**Treg contribution to tolerance of allogenic grafts in animal models**

Since the seminal work of Medawar [17], tolerance, i.e., antigen-specific unresponsiveness that is sustained in the absence of chronic immunosuppression, represents the Holy Grail in solid-organ transplantation. Of all of the mechanisms involved in tolerance of allogenic grafts (which include deletion, energy, ignorance and clonal exhaustion), active T-cell-mediated immunoregulation was long ago identified as being crucial [18]. Experimental transplantation models have been critical in the emergence of the concept of T-regulatory cells. In fact, from the initial report by Gershon et al. [1] on the existence of cells with an inhibitory activity to the current time, animal models have supplied many arguments about their existence and involvement in tolerance and transplantation in various degrees with particular differences between species. Most data came originally from rodents, in which Tregs convey dominant tolerance to cardiac allografts from tolerated to naïve animals [19]. In addition, significant CD4/Foxp3 Treg infiltrates have been observed in accepted donor skin allografts suggesting their important role in maintaining tolerance and promoting graft acceptance in the long term [20]. Many other arguments in cardiac and skin transplantation could be cited here (see for review [21, 22]).

However, there are limitations with rodent models because of differences in the control of Foxp3 expression between species: the first difference to be noted between species is at phenotype level, which in mice is CD4+CD25+Foxp3+ for 5–10% of all peripheral CD4 T cells and CD4+CD25HighC-D127LowCTLA-4+Foxp3+ for 3–5% and 1–2% in non-human primates (NHP) and humans, respectively. Foxp3 expression remains restricted to Tregs in mice [23], whereas in NHP and humans, it may also be transiently inducible in non-regulatory human cells upon activation [24]. The second difference is that at least two Foxp3 isoforms are expressed in human T cells. They differ functionally in their capacity to both bind to ROR-γT and inhibit Th17 cell polarization and to allow expansion of Tregs in the presence of rapamycin [25]. Only one Foxp3 isoform has been detected in mouse Tregs. Preclinical large animal models are therefore of major importance in translation of these experimental data into human clinical studies. Large animal models are much rarer and usually involve both renal and cardiac transplantation in NPH. Torreala et al. [26] used an anti-CD3 immunotoxin associated with costimulation blockade using an anti-CD40L mAb in the presence of cyclosporine A or mycophenolate mofetil, with or without donor-specific transfusion. Long-term graft survival until 6 months after immunosuppression withdrawal was achieved for all the animals but later, some rejected...
whereas others displayed very long-term acceptance with an increase in infiltrating regulatory CD4+ TGFβ+ in their kidney allografts. Poirier et al. [27] reported on a strategy of specific CD28 blockade with an anti-CD28 antagonist (sc28AT) with the goal of leaving the CTLA-4/CD80/86 inhibitory pathway intact to modulate the immune response towards tolerance. In combination with tacrolimus, long-term kidney graft survival was achieved even after drug withdrawal and was associated with up-regulation of Tregs in the blood as well as in the graft and donor-specific unresponsiveness.

**Foxp3 as biomarker in human renal transplantation**

Over the past decade, several studies conducted in renal transplant recipients have investigated whether the expression of Foxp3, as surrogate marker for Tregs, correlates with immunological events and/or predict post-transplant outcome (summarized in Table 2). This research interest stemmed from abundant experimental evidence that Tregs could control alloimmune responses in rodent transplantation models. Various clinical settings have been studied including acute cellular rejection (ACR), borderline changes (BL), subclinical inflammation, chronic allograft dysfunction and stable renal transplant recipients, in whom identification of a specific signature for ‘operational tolerance’ would allow safe gradual weaning off immunosuppression. The latter issue has been extensively reviewed in recent years and will not be addressed in the study [28]. Because of space limitations, this review will not cover the significance of increase in Tregs within tumours and the blood of transplanted patients with cancer either [29]. However, recent studies showing a greater frequency of blood and skin Foxp3+ Tregs in renal transplant recipients with cutaneous carcinoma [30, 31] have opened up new and fascinating fields of investigation.

**Acute cellular rejection.** The recruitment of Foxp3-expressing T cells (presumed to contain mostly true Tregs) into the graft has been well documented as part of the allogenic inflammatory response [32]. This finding has prompted authors to suggest a role for human Tregs in dampening immune-mediated graft injury. However, studies investigating the clinical and prognostic significance of Foxp3+ infiltrate in renal allografts with ACR have yielded conflicting results (Figure 2).

In 2005, Muthukumar et al. [33] reported a much greater number of Foxp3 and CD3 transcripts in the urine of patients with ACR compared with those with chronic allograft nephropathy and stable renal function. Strikingly, they showed that low levels of Foxp3 mRNA in the urinary cells of patients with ACR predicted a poor reversal rate upon steroids and a greater risk of graft failure within 6 months [33]. Further studies based on Foxp3 analysis from graft biopsy cores, using either immunohistochemistry (IHC) or RT-PCR, confirmed higher Foxp3

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**Table 1. Common and distinct features of natural and adaptive regulatory T cells**

<table>
<thead>
<tr>
<th>Natural Tregs</th>
<th>Adaptive Tregs</th>
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<tbody>
<tr>
<td><strong>Ontogeny</strong></td>
<td>Generated in the thymus</td>
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<tr>
<td>Following reception of a TCR signal of intermediate strength coordinated with CD28 co-stimulation</td>
<td>Following reception of a TCR signal coordinated with TGFβ signal</td>
</tr>
<tr>
<td>Foxp3 induction involves NF-κB signalling pathway (binding to CNS3 in the Foxp3 locus)</td>
<td>Foxp3 induction involves TGFβ/Smad3 signalling pathway (binding to CNS1 at the Foxp3 locus)</td>
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<tr>
<td><strong>Phenotype</strong></td>
<td>Foxp3, CD25, GITR, CTLA-4,</td>
</tr>
<tr>
<td><strong>Specific markers</strong></td>
<td>Helios, Nrp1</td>
</tr>
<tr>
<td><strong>Cytokine requirement</strong></td>
<td>IL-2 (proliferation, survival, stability)</td>
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<tr>
<td><strong>Epigenetics</strong></td>
<td>Complete demethylation of TSDR (CNS2) in the Foxp3 locus</td>
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<tr>
<td><strong>Specificity</strong></td>
<td>Enriched in self-reactive T cells. The repertoires of nTregs and Tconv are distinct</td>
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<tr>
<td><strong>Mechanism of action</strong></td>
<td>Cell contact-dependent</td>
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</table>

CNS, conserved non-coding DNA sequence; CTLA-4, cytotoxic T-lymphocyte antigen 4; Dap1, death-associated protein-like 1; GALT, gut-associated lymphoid organ; GITR, glucocorticoid-induced TNFR family-related gene; Igfbp4, insulin-like growth factor-binding protein 4; Ndfip1, Nedd4 family interacting protein-1; NFκB, nuclear factor-kappaB; Nrp-1, neuropilin-1; SLO, secondary lymphoid organ; TCR, T-cell receptor; TGFβ, transforming growth factor-beta.
expression in the grafts exhibiting cellular ACR, compared with stable grafts or those displaying antibody-mediated rejection (AMR) [34, 35]. This finding reflects the close correlation between Foxp3+ and CD3+ infiltrates [36] and CD4+ infiltrates [34]. Nonetheless, these studies failed to demonstrate any potential beneficial effects of Foxp3-enriched infiltrate on graft outcome [34, 37] or even correlated the level of in situ Foxp3 expression with harmful inflammation (tubulitis) [35, 36, 38], higher scarring scores [36] and lower graft survival [35, 38] (Table 2).

However, the impact of Treg-enriched graft infiltrate has also been studied in the context of lower graft inflammation, including BL [39–41] and subclinical acute rejections [39, 42, 43]. Interestingly, the latter studies supported a protective effect of infiltrating Foxp3+ T cells, which inversely correlated with interstitial inflammation and graft function [39]. The Foxp3/granzyme B and Foxp3/CD4 ratios were higher in BL changes than in ACR [39, 41]. This was also true in subclinical settings [39]. In BL changes, strong expression of intra-graft Foxp3 mRNA was associated with the stability of histological lesions and favourable evolution at 1 month after biopsy [40]. These data are in keeping with the recent finding that Foxp3+ cells have a favourable influence on the course of subclinical cellular rejection (SCR), in which they are found in greater proportions than in acute rejection, with hastened decline in graft function [39, 42]. A low Foxp3/CD3 ratio in 6-month protocol biopsies displaying SCR has been correlated with a poor graft function at 2, 3 and 5 years following transplantation [42, 43]. Altogether, these data suggest that Foxp3 analysis may be of value to discriminate between harmful and harmless infiltrating T cells in a context of limited graft inflammation. As discussed later, this restriction is closely related to the technical issue of whether the assay used for Foxp3 analysis could separate Treg-specific and activation-related Foxp3 expression in the context of a major graft inflammation.

**Chronic allograft dysfunction.** Information about chronic rejection is sparser and mostly comes from studies of circulating blood T-cell subsets. Renal transplant patients with chronic rejection have been found to have lower numbers of peripheral CD4+CD25high T cells than patients with stable graft function [44, 45]. Patients with chronic rejection have been reported to have a decreased frequency of the CD25highCD4+ T-cell subset and a reduced expression of Foxp3 mRNA by CD4+ cells compared with stable or operationally tolerant renal allograft recipients [44, 45]. The question of whether CD4+CD25high Foxp3+ cells in patients undergoing chronic rejection have normal suppressive capacity appears more controversial. In order to address this issue, Braudeau et al. [46] performed a suppression assay using autologous and polyclonal stimulation with anti-CD3. They found that blood CD4+CD25high in patients with chronic rejection elicited suppression similar to that of controls.
and concluded that they were fully functional [46]. On the other hand, Akl et al. [45] assessed the suppressive capacity of blood CD4+CD25^{high} through a donor-specific T-cell assay. By magnetically removing the CD4+CD25^{high} subset from responder T cells, they provided evidence of active donor-specific regulation in stable renal transplant recipients, unlike in patients with chronic rejection. These authors propose that a quantitative, but not qualitative, deficit in Tregs might favour the development of chronic rejection [44, 46]. However, an alternative, but not mutually exclusive, hypothesis might

Table 2. Studies assessing the diagnostic and predictive value of intra-graft Foxp3 expression in renal transplant recipients

<table>
<thead>
<tr>
<th>References</th>
<th>Methods</th>
<th>Patients</th>
<th>Main findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muthukumar [33]</td>
<td>Measurement of urinary Foxp3 mRNA, normalized to HG 18S-rRNA</td>
<td>Prospective study of 83 RTR</td>
<td>Foxp3 expression was higher in the 36 subjects with acute rejection. The levels of Foxp3 mRNA inversely correlated with serum creatinine. Foxp3 expression was significantly higher in the subgroup of steroid-sensitive acute rejection</td>
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<td>Veronese [35]</td>
<td>Number of Foxp3+/mm² calculated using the area per field</td>
<td>Retrospective study of 73 graft biopsies in 73 RTR</td>
<td>A higher density of Foxp3+ cells correlated with worse graft survival</td>
</tr>
<tr>
<td>Yapici [36]</td>
<td>Foxp3+cells/mm² calculated using the area per field measurement of intra-graft Foxp3 mRNA, normalized to HG HPRT1, PPAR and β-actin</td>
<td>Retrospective study of 54 graft biopsies in 54 RTR</td>
<td>Intra-graft Foxp3 expression correlated with tubulitis and interstitial fibrosis. No correlation was found between Foxp3 and response to therapy</td>
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<tr>
<td>Bunnag [38]</td>
<td>Measurement of intra-graft Foxp3 mRNA, normalized to HG HPRT1 Foxp3+ cells/mm² calculated using the area per field</td>
<td>Retrospective study of 83 graft biopsies in 77 RTR</td>
<td>Intra-graft Foxp3 expression correlated with interstitial inflammation, tubulitis, interstitial fibrosis, tubular atrophy, C4d positivity and longer time post-transplant</td>
</tr>
<tr>
<td>Aquino-Dias [55]</td>
<td>Measurement of intra-graft Foxp3 mRNA, normalized to HG β-actin</td>
<td>Prospective study of 48 graft biopsies in 35 RTR</td>
<td>Intra-graft Foxp3 expression correlated with acute rejection in RTR with DGF</td>
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<tr>
<td>Kollins [34]</td>
<td>Number of Foxp3+ cells per μm² CD4+ infiltrate area, independent of density of infiltrate</td>
<td>Prospective study of 55 graft biopsies in 55 RTR</td>
<td>The number of Foxp3+ T cells did not correlate with a milder form of rejection (BL), or with outcome at 1 and 2 years after rejection</td>
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<tr>
<td>Blatsford [37]</td>
<td>Number of Foxp3+ cells per μm² CD3+ infiltrate area, independent of density of infiltrate</td>
<td>Retrospective study of 32 graft biopsies in 23 RTR</td>
<td>The number of Foxp3+ T cells did not correlate with acute rejection episodes, when compared with protocol biopsies</td>
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<tr>
<td>Taflin [39]</td>
<td>Foxp3+/CD4+ cells ratio was scored</td>
<td>Retrospective study of 24 biopsies for cause and 16 protocol biopsies in 40 RTR</td>
<td>The proportion of Foxp3+ cells in CD4+ T cells was higher in BL and SCR when compared with acute rejection. In addition, Foxp3+/CD4+ ratio negatively correlated with serum creatinine and the intensity of interstitial infiltrate at the time of biopsy</td>
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<tr>
<td>Bestard [42]</td>
<td>Foxp3+/CD3+ cells ratio was scored</td>
<td>Retrospective study of 37 protocol graft biopsies with SCR in 37 RTR</td>
<td>Foxp3+/CD3+ positively correlated with graft function at 2 years after transplantation. In addition, patients with no chronic histologic damage had a significantly higher percentage of Foxp3/CD3+ T cells</td>
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Table 2. Continued

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</thead>
<tbody>
<tr>
<td>Bestard [43]</td>
<td>Foxp3+/CD3+ cells ratio was scored Quantitative assessment of both methylated and demethylated TSDR in Foxp3 gene</td>
<td>Retrospective study of 37 protocol biopsies with SCR and 68 control biopsies in 105 RTR</td>
<td>Higher Foxp3+/CD3+ ratio was observed in patients with SCR compared with patients with BPAR when assessed by TSDR. Numbers of intra-graft Foxp3+ cells positively correlated with Foxp3 demethylation at TSDR in patients with SCR, unlike in patients with BPAR. The presence of Foxp3+ in biopsies of patients with SCR correlated with better 5-year graft function</td>
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<td>Mansour [40]</td>
<td>Measurement of intra-graft Foxp3 mRNA, normalized to HG GAPDH</td>
<td>Retrospective study of 46 graft biopsies with BL in 46 RTR</td>
<td>Increased Foxp3 expression was found in patients with stable renal function compared with patients who progressed to BPAR</td>
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<tr>
<td>Grimbert [41]</td>
<td>Foxp3/GZB mRNA ratio was scored</td>
<td>Retrospective study of 36 graft biopsies in 36 RTR</td>
<td>Median Foxp3/GZB ratio was significantly lower in biopsies from patients BPAR compared with BL changes</td>
</tr>
<tr>
<td>Zuber [47]</td>
<td>Foxp3+/CD3+ cells ratio was scored</td>
<td>Retrospective study of 67 graft biopsies in 67 RTR</td>
<td>Foxp3+ cells were enriched in chronic compared with acute T-cell-mediated rejection. In patients with infamed fibrosis, low Foxp3/CD3 ratio was associated with lower graft survival</td>
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BL, borderline acute rejection; DGF, delayed graft function; GZB, granzyme B; HG, house-keeping gene; RTR, renal transplant recipients; SCR, subclinical T-cell-mediated acute rejection; Tregs, regulatory T cells; TSDR, Treg-specific demethylated region.

postulate that the reduced number of blood Tregs in patients with chronic rejection might originate from their enhanced homing and selective recruitment into chronically inflamed grafts [38, 47], as demonstrated in other chronic inflammatory settings [48, 49]. This hypothesis may explain the absence of donor-specific blood Tregs accumulating within the graft in renal transplant recipients undergoing chronic rejection [45, 46].

Following these lines, slow-evolving T-cell-mediated chronic rejection has been associated with a significantly higher recruitment of Foxp3+ T cells into the graft than ACR [47]. Of note, the highest frequency of Foxp3+ cells was found in the most organized nodular aggregate, where T cells surrounded a central B-cell area, recognized as a hallmark of chronic inflammation [47, 50]. This fits well with the observation that intra-graft Foxp3 expression correlates with time after transplantation [38, 51]. Interestingly, three independent studies reported a higher frequency of infiltrating Foxp3+ cells in chronically rejected grafts, characterized by scar tissue inflammation, with a better graft outcome [47, 52, 53].

How to reconcile the discrepant conclusions from human studies

Although experimental data in animal models largely support a critical role of Foxp3+ cells in dampening alloimmune responses, the conclusions drawn from human studies are perceived as discrepant and may be confusing for non-expert readers. However, it is worth remembering how the method used for Foxp3+ analysis and how the clinical settings may dramatically influence the interpretation of the results. In our opinion, most of these conflicting results could be attributed to methodological factors and/or could be explained by the current understanding of Treg biology.

Methodological issues. Two different approaches have been proposed with respect to the quantification of Foxp3+ cells within the graft, regardless of the kind of assay used (RT-PCR or IHC). Simply, Foxp3 expression has been normalized either to the whole graft tissue sampling [32–37, 54, 55] or to the T-lymphocyte compartment infiltrating the graft [39–42, 47] (Figure 1). In the former approach, Foxp3 expression is indicative of the extent of the inflammatory infiltrate rather than of the contribution of the regulatory component to the lymphocyte infiltration. These studies, therefore, usually conclude that the more inflamed the graft, the higher the level of Foxp3 expression [32, 35, 55] (Figure 1). In a provocative way, some authors have even proposed that Foxp3 expression might be paradoxically used as an informative biomarker for acute rejection [55]. In contrast, the second approach takes into consideration the balance between the harmful and harmless arms of alloimmune responses. Most of these studies conclude that high Foxp3 expression positively correlates with better outcome [40–42, 47], lower inflammation grade [39] or donor-specific hyporesponsiveness [53] (Figure 2). The
finding that the frequency of Foxp3+ cells was lower in acute rejection than in slow-evolving chronic rejection, though their absolute number was higher, illustrates how these two different approaches may lead to conflicting conclusions (Figure 3A) [47]. Similarly, the Foxp3/granzyme B mRNA ratio was significantly higher in the biopsies showing BL compared with those exhibiting acute rejection, though the absolute Foxp3 levels were lower [41].

Interestingly, Bunnag et al. [38] found a poor correlation between Foxp3+ cells counted on biopsy sections and Foxp3 mRNA quantification. The authors attributed this mainly to the patchiness of the infiltrate and the limited number of samples with suitable material for immunostaining. However, this poor correlation might also be explained by the low sensitivity of the IHC technique, which preferentially identifies highly Foxp3-expressing cells. Several data have suggested that these Foxp3high cells might be activated Tregs [56, 57], whereas the Foxp3low subpopulation includes conventional activated effector T cells [39, 56–59]. One caveat for the PCR method is, therefore, its inability to assess the fraction of Foxp3high cells. Of note, IHC studies have found that virtually all Foxp3+ infiltrating cells were CD4+ [34, 35, 39, 42, 47]. If we hypothesize that activation-induced Foxp3 expression involves both CD4+ and CD8+ cells [60, 61], this would be another argument to suggest that IHC does not count conventional Foxp3-expressing l activated T cells. Consequently, in a rejection biopsy where presumably many infiltrating cells are activated effector T cells, measuring the fraction of highly Foxp3-expressing cells takes into greater consideration the balance between effector and regulatory arms of alloimmune responses and might be more relevant than simply quantifying Foxp3 by RT-PCR. Finally, the poor correlation between Foxp3+ cells counted on biopsy sections and Foxp3 mRNA quantification from inflamed grafts suggests that Foxp3 analysis using RT-PCR should be interpreted with caution [38].

More recently, assessment of epigenetic changes of the Foxp3 locus has been proposed as the most reliable way to quantify bona fide Tregs in humans [62]. Indeed, an evolutionary conserved region within the Foxp3 locus, upstream of exon-1, has been found to be demethylated in stable human nTregs, unlike in iTregs and activated T cells [63]. Consistent with this, quantitative assessment of the natural Foxp3 Treg-specific demethylated region (TSDR) has been positively correlated with the circulating ‘bona fide’ Treg CD127- CTLA-4 population in renal transplant recipients [64] and with the determination of infiltrating Foxp3+ cells in grafts with subclinical acute rejection [65]. On the other hand, Bestard et al. [43] showed a lack of correlation between IHC and Foxp3 TSDR with respect to intra-graft Foxp3 quantification in biopsy-proven acute rejection (BPAR). This finding suggests that activated T cells may be mistaken for Tregs in highly inflammatory settings even when assessed by IHC, depending on the threshold of the technique used in each centre. Importantly, however, the epigenetic approach is based on the restrictive assumptions that all the circulating or infiltrating Tregs are thymically-derived and that no post-transcriptional/post-translational events affect the correlation between Foxp3 locus opening and Foxp3 expression. In respect with this, Akimova et al. [64] recently showed that these assumptions may be false in solid-organ transplant recipients given immunosuppressive drugs. For instance, iTregs,

**FIGURE 2:** Main conclusions of the studies correlating Foxp3 expression with graft outcome. This figure illustrates how the predictive value of Foxp3 expression on graft outcome critically depends on the normalization method of Foxp3 expression and on the extent of inflammation within the graft. BPAR, biopsy-proven acute T-cell-mediated rejection; BL, borderline acute rejection; chronic TCMR, chronic T-cell-mediated rejection; SCR, subclinical T-cell-mediated acute rejection.
generated upon mTORi regimen augment the Foxp3/TSDR ratio whereas CNI dampen Foxp3 expression in nTregs despite fully demethylated TSDR (Figure 3B) [64].

Altogether, these data show that activated T cells should not interfere too much with quantification of Foxp3+ regulatory T cells infiltrating the graft when assessed by IHC in a reasonably inflamed graft. Nonetheless, the inconstant ability of RT-PCR and IHC to discriminate between specific Tregs and activation-induced Foxp3 expression remains a problem in grafts exhibiting major inflammation.

**Impact of post-transplant environmental factors on Treg biology.** The understanding of Treg cellular biology has increased exponentially over recent years, driven mostly by in vivo experimental studies in mice. Dynamic back-and-forth exchange between scientists and clinicians has provided important clues to interpreting Foxp3+ analysis in complex clinical settings. In this context, the significance of Foxp3+ analysis in organ transplant recipients treated with immunosuppressive therapies has posed several challenging issues.

First, a significant enrichment inFoxp3+ cells within an inflamed graft does not necessarily imply that alloimmune responses has been skewed towards tolerance, but may instead characterize an inflammatory response evolving to chronicity [38, 47, 48, 52]. This does not necessarily indicate that Tregs are useless for dampening the inflammation [48], but could suggest rather that analysis of Foxp3 alone is not sufficient to assess the strength of the regulatory arm of the immune response. In experimental rodent models, it was recently proposed that the balance of two signals, through STAT3 and STAT5 respectively, integrates the influence of the microenvironment on the fate of T cells, tipping either towards Tregs or towards TH17 lineages [66]. The situation is further complicated by the finding that a pro-inflammatory environment is not favourable to suppression [67]. Failure of natural Tregs to suppress Teffs within inflamed tissues has been well demonstrated in autoimmune settings, emphasizing that control of an immune response is not only a matter of Treg/Teff ratio but also of the local microenvironment (Figure 3A) [68, 69]. In this context, studying the orientation of alloimmune responses in secondary lymphoid organs, as commonly done in

**FIGURE 3:** Interference of post-transplant conditions with Treg biology and function. (A) Though an increase in the absolute number of infiltrating Foxp3+ Treg cells has frequently been observed in acute rejection, the frequency of Tregs is lower compared with other conditions associated with milder inflammation. In addition, effector T cells are much less sensitive to suppression in the presence of pro-inflammatory cytokines, including IL-6, IL-1 and TNFα. (B) TSDR demethylation correlates with bona fide Treg phenotype. However, the Foxp3+ TSDR-demethylated cells ratio (Foxp3/TSDR) may be dramatically influenced by the immunosuppressive regimen. The negative effect of the calcineurin inhibitors (CNI) on the transcriptional and/or post-transcriptional regulation of the Foxp3 gene may account for a lower Foxp3/TSDR ratio. On the other hand, rapamycin-induced iTregs, characterized by methylated TSDR, account for an increase in the total Foxp3+ compartment, contrasting with a low number of cells with demethylated TSDR. (C) Naïve T cells are dramatically depleted by lymphodepleting agents. In contrast, depletion-resistant memory T-cells expand within the first month. The increased frequency of circulating Foxp3+ cells observed in lymphopenic conditions reflects their greater contribution to the memory T-cell compartment. Lymphopenia-induced proliferation, termed “homeostatic proliferation”, renders expanding memory T cells resistant to the suppressive effect of Tregs.
experimental models, would likely be more informative, but this could not be performed in human settings for obvious reasons.

Secondly, contrary to what has been intuitively stated, a high frequency of Foxp3+ cells following a T-cell depleting strategy is not synonymous with immune tolerance-prone conditions. Naïve T cells are dramatically depleted in patients treated with alemtuzumab or rabbit antithymocyte globulin (ATG) following renal transplantation [70]. In contrast, depletion-resistant memory T-cells expand within the first month (Figure 3C) [71, 72]. Interestingly, thymic egress of Tregs has been evidenced following lymphocyte-depleting induction [73]. Nonetheless, in our opinion, the increased frequency of circulating Foxp3+ cells observed in these settings reflects their greater contribution to the memory T-cell compartment [56] and possibly T-cell activation [74] rather than selective expansion of Tregs [73]. Furthermore, compelling evidence suggests that lymphopenia-induced proliferation, termed ‘homeostatic proliferation’, hampers effective control of alloimmune responses by Tregs. First of all, memory T cells are much less sensitive to the suppressive effect of Tregs than naïve T cells [72, 75]. In addition, homeostatic proliferation by itself renders the effector T cells resistant to tolerance induction (Figure 3C) [76]. Consistent with this, a therapeutic strategy combining alemtuzumab induction and sirolimus-based maintenance regimen has been associated with a high rate of acute rejection, because of the poor ability of mTOR inhibitors to control memory T cells [72, 77].

Recent data indicate that several commonly used immunosuppressive drugs have adverse effects on the induction and function of Tregs, while other drugs seem to spare these cells or even have a beneficial effect (for extensive review, see [78]). CNI negatively affect Foxp3+ Tregs through different mechanisms, including reduction of the cooperation between NFAT and Foxp3 transcriptional factors [79], blockade of IL-2 secretion [80] and interference with the miR155-mediated enhanced IL-2 signalling pathway in Tregs [81, 82]. This result raises the interesting question of whether weak expression of Foxp3 might be indicative of CNI toxicity. In both animal and human models, calcineurin inhibitors inhibit Treg generation and their ability to suppress [64, 83–86], and therefore limiting the dose and timing the administration of these drugs may be essential for future tolerance induction protocols. In contrast, rapamycin likely promotes Treg survival and function [64, 84, 87] but clear evidence of a beneficial effect of mTOR inhibitor on Treg in human transplant studies is still lacking [88].

**CONCLUSION**

The crucial role of Tregs in tolerance of allografts has been well established by many animal models. However, delineating their role in human transplantation is a hard task. Several authors have tried to address this issue by analysing the expression of Foxp3 in kidney grafts. These studies remain rare and heterogeneous in terms of clinical and methodological settings, and consequently provide conflicting results. In this review, we have attempted to shed light on these discrepant results. The thorough analysis of the literature showed that Foxp3 expression correlates either with good or with poor outcome, depending on whether the graft biopsy exhibits low- or high-grade inflammation, respectively. In addition, we stress the critical need to harmonize methods for Foxp3 analysis, as different methodological strategies may yield opposite conclusions. One of the main problems when studying Foxp3 in inflammatory settings arises from the fact that Foxp3 can be expressed in both recently activated effector T cells and bona fide Tregs. In addition, polarized T-cell lineages appear to be much more plastic than previously thought, resulting in the ability of Tregs to be reprogrammed into effector T cells and vice versa. In our opinion, any attempts to predict graft outcome at the time of acute rejection based solely on the Foxp3 analysis is therefore elusive. However, we still believe that monitoring the regulatory component of alloimmune responses might become a very useful diagnostic and prognostic biomarker for the long-term management of renal transplant recipients. Nonetheless, several conditions are required: first, Foxp3 analysis should not be undertaken concomitantly with an overwhelming inflammatory process; secondly, the Treg/Teff ratio should be interpreted with caution in lymphopenic settings; thirdly, the significance of TSDR demethylation should be rethought in light of the effects of immunosuppressive drugs; fourthly, both the pro- and anti-inflammatory components of alloimmune responses should be monitored; fifthly, a study ideally investigating all aspects of Treg biology, including phenotypic, epigenetic and functional characteristics, would be the most meaningful.

Finally, in clinical renal transplantation, definitive evidence that Tregs contribute to long-term transplant survival in chronically immunosuppressed patients is still lacking. So far, the most convincing evidence for the role of Tregs in experimental models of allotransplantation has been provided through adoptive transfer of tolerance-inducing Tregs from tolerant recipients to naïve animals. Future clinical trials assessing the effects of Treg cell therapy combined with a low immunosuppressive regimen on graft outcome might therefore provide further convincing and definitive arguments about their role in humans.

**CONFLICT OF INTEREST STATEMENT**

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Medullary sponge kidney: state of the art

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Medullary sponge kidney (MSK) is a kidney malformation that generally manifests with nephrocalcinosis and recurrent renal stones; other signs may be renal acidification and concentration defects, and pre-calyceal duct ectasias. MSK is generally considered a sporadic disorder, but an apparently autosomal dominant inheritance has also been observed. As MSK reveals abnormalities in both the lower and the upper

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