FOXP3-enriched infiltrates associated with better outcome in renal allografts with inflamed fibrosis

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Abstract

**Background.** FOXP3-expressing regulatory T cells (Tregs) play a crucial role in maintaining allogeneic transplant tolerance in experimental models. In clinical transplantation, there are few data about their role in chronic inflammation. We hypothesized that Tregs might accumulate within the graft since enrichment of Tregs has been frequently described in chronically inflamed tissues.

**Methods.** Sixty-seven biopsies, indicated by a rise in creatinine level, were studied. Thirty-four biopsies showing acute T-cell-mediated rejection and 33 displaying inflamed interstitial T-cell infiltrates associated with acute rejection \[10.6 \pm 5.5 \text{ vs.} 2.6 \%, \text{ respectively, } P = 0.005\]. Those with inflammation within scarred areas, the subset of patients with a low FOXP3/CD3 ratio (below the median value) displayed a lower frequency of B-cell-enriched nodular cell clusters \(20\% \text{ versus} 86\%, P = 0.001\) and had a significantly lower graft survival \(\log\text{-rank}, P = 0.02\). In multivariate analysis, the low FOXP3/CD3 ratio remained an independent indicator of outcome \(P = 0.03\). Consistently, the FOXP3+/IL-17+ cell ratio was higher in nodular than in diffuse infiltrates.

**Conclusions.** Our results suggest that Tregs may dampen the graft injury in chronic (versus acute) inflammation and stress the importance of devising strategies to enhance Tregs efficiency.

**Keywords:** FOXP3; nodular infiltrates; regulatory T cell; inflamed fibrosis

Introduction

Regulatory T cells (Tregs) play a crucial role in the control of innate and adaptive immune responses. The expression of a forkhead-winged helix transcription factor FOXP3 has been tightly associated with the best-characterized CD4+CD25+ Treg lineage, which comprises 5–10% of mature CD4+ T cells \[1\]. In mice, FOXP3 is essential for the development and function of Treg \[1,2\] and is specific to the Treg lineage. In humans, although FOXP3 may be transiently induced \textit{in vitro} in activated T cells, stable expression of FOXP3 strongly correlates with the regulatory lineage \[3,4\]. FOXP3 immunohistochemical staining has therefore become a reliable marker for the study of Tregs within tissues \[5,6\].

In rodent models of allogeneic transplantation, Tregs play a crucial role in both induction and maintenance of tolerance \[7,8\]. Most ‘tolerizing’ regimens involve reduction of alloreactive effector T cells and expansion of donor-specific regulatory T cells, leading to enrichment of Treg in lymphoid organs as well as within the graft \[7,8\]. In clinical transplantation, tolerance induction is still an elusive goal, and the few studies available have mostly addressed the issue of whether Tregs may dampen alloimmune responses thus limiting tissue damages \[5,6,9–12\]. Tregs have been involved in donor-specific unresponsiveness \[9\], and their higher frequency in acute and borderline rejections has been correlated with a better outcome \[5,11,12\]. However, the interstitial T-cell infiltrates associated with acute rejection still contain a limited frequency of Tregs, a finding consistent with the destructive potency and fast progression of acute rejection \[6\].

Fewer data concern chronic rejection, and they rely mostly on the study of circulating T-cell subsets. Peripheral blood Tregs have been found to be decreased in patients experiencing chronic rejection, prompting some authors to propose that a deficit of Tregs might favour the development
of chronic rejection [13]. Alternatively, Tregs might accumulate within the graft since enrichment of Tregs has been frequently described within chronically inflamed tissues [14–16]. In this setting, the immune response would be finely balanced between its aggressive and regulatory components, resulting in slow destruction of the target tissue. We hypothesized that Tregs may be preferentially recruited within grafts exhibiting chronic inflammation. To address this issue, we studied the FOXP3/CD3 ratio within tubulointerstitial renal allograft infiltrates in patients with acute rejection and chronic inflammation within scarred areas and investigated whether the frequency of FOXP3+ cells could predict graft outcome.

Materials and methods

Patients

The study population consists of 67 clinically indicated renal allograft biopsies performed in 67 kidney transplant recipients in our institution from February 2004 to July 2007. The patients were enrolled after informed consent. Adequate CD4-negative biopsies (containing at least 10 glomeruli and two arterial sections) were selected and divided into two groups, chronic and acute cellular-mediated inflammation, according to the following criteria:

1. T-cell-mediated chronic inflammation: a slow but significant (≥20%) increase in serum creatinine levels over the last months (9 ± 7 months) associated with inflamed fibrosis (inflammation within scarred areas) and no acute rejection according to Banff scores. Transplant glomerulopathy and chronic allograft arteriopathy (chronic intimal fibrosis and mononuclear cell infiltration within fibrosis) were not required for inclusion, but noted when observed.

2. T-cell-mediated acute rejection: a significant increase in serum creatinine (≥20%) and histological criteria according to Banff 05 classification (i score ≥ 2, t score ≥ 2).

BK viral load and urine culture were negative for all patients at the time of graft biopsy, ruling out BK virus and urinary infections, respectively.

Immunohistochemistry

Immunohistochemistry was performed on AFA-fixed, paraffin–embedded tissue. Two serial sections were stained with a specific monoclonal antibody for FOXP3 and CD3. For biopsies with chronic rejection, an additional serial section was stained with an anti-CD20 antibody. Tissue sections were deparaffinized, rehydrated and pretreated. To block endogenous peroxidase, the sections were incubated 30 min in a mixture of H2O2 and 1% methanol. After biotin blockade, an anti-CD3 antibody (polyclonal rabbit anti-human CD3 A0452/DAKOcytomation, Denmark A/S) diluted at 1:50 or the anti-FOXP3 antibody (mouse monoclonal anti-human FOXP3 ab 20034/Abcam, England) diluted at 1:40 or the anti-CD20 antibody (monoclonal mouse anti-human CD20, DAKOcytomation, Denmark) diluted at 1:200 were incubated from 1 h (anti-CD3, anti-CD20) to overnight (anti-FOXP3) at 4°C. After washing, the sections were incubated with the secondary antibody, followed by streptavidin peroxidase (Dako REAL TM Detection System, peroxidase/DAB+, rabbit/mouse, K5001, Denmark A/S) and developed with 3,3′-diaminobenzidine.

IL-17 staining was performed on formalin-fixed removed renal grafts. The sections were incubated for 1 h with anti-IL-17 (monoclonal goat anti-human IL-17, R&D systems, Lille, France) diluted at 1:50. An anti-goat HRP (polyclonal rabbit anti-goat HRP- DakoA cytation, Denmark) diluted at 1:100 was used as a secondary antibody. For FOXP3/IL-17 double staining, the samples were consecutively incubated with anti-FOXP3 and anti-IL-17 antibodies. The anti-FOXP3 antibody was amplified with the biotin–streptavidine system and coloured with either DAB or Fast Blue (Vector Blue Alkaline Phosphatase Substrate Kit III SK-5300). IL-17 positive cells were then marked in DAB colour or by using Fast Blue and a phosphatase alkaline-linked secondary antibody (alkaline phosphatase rabbit anti-goat, Vector laboratories 26628-22-8). Anti-CD8 (monoclonal mouse anti-human CD8, DAKOcytomation, Denmark) was used and revealed using the phosphatase alkaline-linked antibody (Kit envision double stain system, DAKOcytomation, Denmark).

Quantification of infiltrating cells

All biopsies with acute and chronic rejection were evaluated quantitatively in a blinded fashion for the number of FOXP3+ and CD3+ cells in the interstitium. An inflammatory infiltrate was eligible for quantification, if at least 50 CD3+ cells were counted in a ×200 magnified field. The FOXP3+/CD3+ cell ratio was calculated for each infiltrate by counting FOXP3+ and CD3+ cells on two contiguous serial sections. The morphological pattern of inflammatory aggregates was assessed as well, and classified either as type 1 nodular infiltrate (highly compartmentalized CD20+ B-cell cluster) or type 2 nodular infiltrate (lymphoid cell cluster without separated T- and B-cell areas) or diffuse infiltrates (scattered T and B cells). To evaluate FOXP3+/T-cell frequency according to the shape and composition of infiltrates, the FOXP3+/CD3 ratio was calculated separately for each infiltrate. For FOXP3/CD8 double stain, the frequency of FOXP3+ cells was assessed on two separate infiltrates in two biopsies.

Statistical analysis

Data were expressed as mean ± SD. For continuous variables, statistical significance was assessed by Student’s t-test or the non-parametric Wilcoxon test, according to their distribution. Binary variables were analysed by either the chi-square or Fisher exact test. Survival analysis was performed on the study biopsy from each patient using Kaplan–Meier analysis with log-rank testing. Logistic regression models were used to test possible associations between outcome and histological or biological lesions. The multivariate model including parameters whose P-value was <0.10 in univariate analysis was based on a backward stepwise elimination procedure.

Results

Higher frequency of infiltrating FOXP3+ T cells in inflamed fibrosis than in acute T-cell rejections

Sixty-seven transplant recipients, whose characteristics are presented in Table 1, were included in the study. Of those, 34 and 33 biopsies were performed on patients exhibiting acute or chronic T-cell infiltrates, respectively. The time from transplant to the graft biopsy was significantly higher in the group of inflamed fibrosis (8.3 ± 7.6 versus 1.7 ± 3.4 years, P < 0.05). Creatinine levels and proteinuria at the biopsy were not different, as well as the number of HLA mismatches. The t and v BANFF scores in grafts versus acute versus inflamed inflammation groups were significantly lower, respectively. 0.8 ± 0.8 versus 2.4 ± 0.7 and 0.2 ± 0.4 versus 2.6 ± 0.7. In contrast, IF/TA and cg (1.2 ± 1.3 versus 0.2 ± 0.6) scores were significantly higher in the chronic inflammation group (P < 0.05). Chronic allograft arteriopathy was observed in 27 out of 33 biopsies with chronic rejection and in 12/34 with acute rejections. A total of 121 infiltrates were scored, 68 and 53 in the acute and chronic inflammation groups, respectively. A mean of 309 (range: 50–700) CD3+ cells were counted per infiltrate. Fifteen, 16 and 2 biopsies in the chronic inflammation group and 5, 24 and 5 in the acute inflammation group had 1, 2 or 3 infiltrates, respectively. The mean FOXP3/CD3 ratio was calculated from all infiltrates in the same biopsy. The ratio of FOXP3+/CD3+ cells within interstitial infiltrates was significantly higher in the chronic than in acute inflammation group (10.6 ± 6.8 versus 5.5 ± 2.6%, P = 0.005) (Figure 1A). By counting the absolute
We sought to determine if enrichment of FOXP3-expressing cells predominate within tertiary lymphoid structures (Figure 1C). The highest frequency of FOXP3-expressing cells was restricted to the group of chronic inflammation biopsies and not to those exhibiting acute rejection (Figure 1E). In acute rejection biopsies, FOXP3+ cells accounted for 7.6 ± 3.5 and 5 ± 2.2% in nodular and diffuse infiltrates, respectively (Figure 1E). We, therefore, hypothesized that FOXP3+-enriched nodular cellular aggregates observed in chronically inflamed grafts might correspond to well-organized tertiary lymphoid structures well described in other chronic inflammation settings [17].

Tertiary lymphoid organs fulfill morphological and functional criteria of germinal centres and therefore display the typical dense B-cell area surrounded by T-cell area. The FOXP3/CD3 ratio was assessed according to the microanatomical organization of infiltrates (Table 2, Figure 1D). Nodules were separated into two groups: those with well-organized concentric B- and T-cell areas (type 1) and those containing no or few B cells and devoid of any T- and B-cell zones (type 2) (Figure 1D). As depicted in Figure 1E, the highest frequency of FOXP3-expressing cells was found in the most organized type 1 nodular aggregate (20.1 ± 6.6%, n = 9), a hallmark of chronic inflammation, as compared with type 2 nodules (11.9 ± 5.9%, n = 20) and diffuse infiltrates (7.3 ± 3.8%, n = 24). Of note, in an attempt to assess with enough statistical power, the correlation between the FOXP3 frequency and the shape of infiltrate we have included all infiltrates as independent events. To eliminate a cluster effect, we conducted a mixed linear regression of the FOXP3/CD3 ratio (with a logit transformation ensuring normal distribution) to account for the hierarchical structure of the data. The fixed effect associated with the shape of the infiltrates remained very significant [estimate (95% CI) = 0.512 (0.270; 0.754) for logit (FOXP3/CD3) or 0.625 (0.567; 0.680) for FOXP3/CD3, P = 3.4×10−5], which confirmed a higher FOXP3/CD3 ratio for nodular aggregates, independent of cluster effect.

**High FOXP3+ cell frequency in T-cell infiltrates within scarring predicts better graft outcome**

None of the 33 patients with biopsy-proven inflamed fibrosis had received any therapeutic intervention following the biopsy. The follow-up was 29.9 ± 13.5 and 23.1 ± 15.8 months for the high- and low-FOXP3/CD3 cell ratio groups, respectively. We performed a Kaplan–Meier survival analysis, considering as events either an allograft loss or a persistent (>3 months) doubling in plasma creatinine levels. As shown in Figure 2, patients with higher frequency of Tregs had a significantly lower risk for event than those with lower FOXP3/CD3 ratio. The 3-year graft survival was 82 versus 40% in patients with higher FOXP3 frequency in

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**Table 1.** Characteristics of renal transplant recipients in acute rejection and inflamed fibrosis groups.

<table>
<thead>
<tr>
<th></th>
<th>Acute rejection (34)</th>
<th>Inflamed fibrosis (33)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epidemiological data</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recipient age (yrs)</td>
<td>44.2 ± 14.0</td>
<td>37.2 ± 13.7</td>
<td>0.04</td>
</tr>
<tr>
<td>Recipient gender (M/F)</td>
<td>24/11</td>
<td>22/11</td>
<td>ns</td>
</tr>
<tr>
<td>Deceased/living donor</td>
<td>28/7</td>
<td>28/5</td>
<td>ns</td>
</tr>
<tr>
<td>HLA-mismatch</td>
<td>3.1 ± 1.4</td>
<td>3.1 ± 1.2</td>
<td>ns</td>
</tr>
<tr>
<td>DR HLA-mismatch</td>
<td>1.2 ± 0.7</td>
<td>1.2 ± 0.8</td>
<td>ns</td>
</tr>
<tr>
<td>Immunosuppressive drugs</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Steroids</td>
<td>31</td>
<td>30</td>
<td>ns</td>
</tr>
<tr>
<td>Calcineurin inhibitors (CsA/FK)</td>
<td>12/19</td>
<td>14/13</td>
<td>ns</td>
</tr>
<tr>
<td>Sirolimus</td>
<td>2</td>
<td>7</td>
<td>ns</td>
</tr>
<tr>
<td>At the time of the graft biopsy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transplant to biopsy interval (yrs)</td>
<td>1.7 ± 3.4</td>
<td>8.3 ± 7.6</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Creatinine levels (µmol/L)</td>
<td>226.6 ± 140.8</td>
<td>187.0 ± 63.8</td>
<td>ns</td>
</tr>
<tr>
<td>Proteinuria (g/day)</td>
<td>0.71 ± 1.55</td>
<td>0.85 ± 1.25</td>
<td>ns</td>
</tr>
<tr>
<td>BANFF scores</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Banff grades of acute rejection (BL1A/1B2A/2B3)</td>
<td>0/10/13/9/0/2</td>
<td>6/0/0/0/0/0</td>
<td>ns</td>
</tr>
</tbody>
</table>

BL = borderline; CsA = cyclosporine A; F = female; FK = FK506 (tacrolimus); M = male; ns = non-significant; yrs = years.

number of infiltrating cells, we found that the decreased percentage of FOXP3+ cells in acute rejection rather resulted from a marked influx (or expansion) of FOXP3− cells into the graft than from a poor recruitment of FOXP3+ cells (Figure 1B). Since it has been reported that FOXP3 may be expressed upon the activation of human CD4+ and CD8+ T cells, we sought to determine if the in situ expression of FOXP3 was restricted to presumed regulatory CD4+ T cells or was broadly detected in activated CD4 and CD8+ T cells. In FOXP3/CD3 double stains, only few FOXP3+ cells were found that expressed CD8 (3.9 ± 2.8%), suggesting that in situ FOXP3 expression is mostly restricted to the CD4+ compartment (Figure 1C).

**FOXP3-expressing cells predominate within tertiary lymphoid structures**

We sought to determine if enrichment of FOXP3-expressing cells in chronic inflammation biopsies correlated with any particular clinical or pathological feature. Biopsies from patients with chronic inflammation were split into two groups according to the mean FOXP3/CD3 ratio calculated from the whole biopsy, taking the median value (7.4%) as the threshold for high (n = 17) or low (n = 16) (Table 2). The two groups were remarkably similar for epidemiological data, HLA mismatches, immunosuppressive regimen, creatinine levels and proteinuria as well as for conventional anatomopathological features. The only difference was the occurrence of nodular cell clusters, which accounted for 86 and 20% of infiltrates in biopsies with high and low frequency of FOXP3+ cells, respectively (Table 2, Figure 1D, P = 0001). Banff scores were not significantly different between the two groups (Table 2).

The significant association between nodular infiltrates and FOXP3+ cells was restricted to the group of chronic inflammation biopsies and not to those exhibiting acute rejection (Figure 1E). In acute rejection biopsies, FOXP3+ cells accounted for 7.6 ± 3.5 and 5 ± 2.2% in nodular and diffuse infiltrates, respectively (Figure 1E). We, therefore, hypothesized that FOXP3+-enriched nodular cellular aggregates observed in chronically inflamed grafts might correspond to well-organized tertiary lymphoid structures well described in other chronic inflammation settings [17].
Fig. 1. Frequency of FOXP3+ cells within grafts during acute and chronic cellular rejections. AR = acute rejection, IF = inflamed fibrosis, dif. = diffuse, nod. 1 = type 1 nodular infiltrate (well-organized aggregate with distinguishable B- and T-cell zones), nod. 2 = type 2 nodular infiltrate (cluster-like structure without B- and T-cell segregation). (A) Comparison of the FOXP3/CD3 cell ratio between acute rejection and inflamed fibrosis groups. (B) Enumeration of FOXP3+ and FOXP3−CD3+ cells. The absolute number of Tregs and non-regulatory T cells were counted in 3 high power fields. (C) Representative double stain for FOXP3 (brown) and CD8 (blue) (200 × magnification). Double positive CD8+ FOXP3+ cells are barely detected (arrow head). Insert shows 400 × magnification of two cell clusters. (D) Representative CD20, CD3 and FOXP3 staining on contiguous serial sections in biopsies displaying inflamed fibrosis with type 1 and 2 nodular infiltrates. (E) Comparison of the FOXP3/CD3 cell ratio according to the microanatomical organization of lymphoid infiltrates. *P = 0.005, **P < 0.001.

Comparison to the other group (log-rank, P = 0.02). In an attempt to study clinical and histological data associated with graft loss or doubling creatinine level, univariate and multivariate analyses were performed. The calculated odds ratios (OR) and corresponding 95% confidence interval (CI) are indicated in Table 3. In univariate analysis, the low FOXP3/CD3 ratio [OR (CI) = 6.00 (1.22–29.41), P = 0.03] and to a less extent proteinuria at the time of the biopsy [OR (CI) = 1.97 (0.94–4.10), P = 0.07] were associated with bad outcome. Other relevant variables, such as creatinine level at the time of the biopsy, BANFF scores and presence of type 1 and 2 nodular infiltrates, were not risk factors for declining renal function. To further address the prognostic value of B-cell infiltrates, we performed a multivariate analysis (Table 3) with any factor likely to be associated with outcome according to the univariate analysis (P ≤ 0.20). After a backward stepwise elimination procedure discarding factors not significantly associated with the outcome (P > 0.10), the final model kept the low FOXP3/CD3 ratio [OR (CI) = 7.47 (1.22–46.08), P = 0.03] and proteinuria at the time of the biopsy [OR (CI) = 2.29 (0.94–5.63), P = 0.07] as best predictors of bad outcome. Adding the presence of B-cell-enriched type 1 nodular aggregate to this model provided evidence that this factor was not associated with the outcome (P = 0.98).

Dual enrichment of FOXP3 and IL-17-expressing cells within terminally rejected grafts

To further understand the mechanism through which the FOXP3+ cell enrichment of infiltrates in chronic rejection may influence the graft outcome, we investigated the presence of markers typical of a pro-inflammatory response. We devoted particular attention to the study of IL-17-secreting cells because they share the common developmental pathway with Tregs, they are found in chronic inflammation sites [18] and also appear to be involved in germinal centre formation [19]. For technical reasons, IL-17 staining could not be performed on AFA-fixed graft biopsies and was performed on formalin-fixed transplant nephrectomy specimens removed for terminal chronic rejection (Figures 3A–D). Twenty-six infiltrates (15 diffuse and 11 nodular) were assessed. As expected, IL-17 and FOXP3 antibody stains, respectively, cytoplasm and nucleus (Figure 3A). The IL-17-secreting cells were readily identified in the rejected grafts, in the interstitium as well as in the intratubular compartment (Figure 3B). Recent data suggest that FOXP3-expressing cells can differentiate into IL-17-producing cells in an appropriate pro-inflammatory microenvironment [20]. We thus examined the possibility that some cells could co-express IL-17 and FOXP3, but we did not find any double positive (Figure 3C). In contrast, our
### Table 2. Characteristics of renal transplant recipients according to the frequency of FOXP3-expressing cells within interstitial infiltrates

<table>
<thead>
<tr>
<th>Variable</th>
<th>Category</th>
<th>Odd ratio</th>
<th>CI 95%</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteinuria</td>
<td>Y/N</td>
<td>0.70</td>
<td>0.34–1.44</td>
<td>0.33</td>
</tr>
<tr>
<td>Nod. 1 (Y/N)</td>
<td>Y/N</td>
<td>1.19</td>
<td>0.48–2.93</td>
<td>0.71</td>
</tr>
<tr>
<td>Diffuse</td>
<td>Y/N</td>
<td>1.01</td>
<td>0.99–1.02</td>
<td>0.20</td>
</tr>
<tr>
<td>FOXP3low</td>
<td>Y/N</td>
<td>0.70</td>
<td>0.34–1.44</td>
<td>0.33</td>
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<tr>
<td>Diffuse</td>
<td>Y/N</td>
<td>1.19</td>
<td>0.48–2.93</td>
<td>0.71</td>
</tr>
<tr>
<td>FOXP3low</td>
<td>Y/N</td>
<td>1.01</td>
<td>0.99–1.02</td>
<td>0.20</td>
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Table 3. Variables associated with allograft loss or a persistent (>3 months) doubling in plasma creatinine levels by univariate and multivariate logistic regression analyses in the inflamed fibrosis group

#### Discussion

Recent studies have pointed out that fibrosis associated with inflammation represents an important pejorative prognostic factor. Infiltrates in either area, unscarred [21,22] or scarred [23], are associated with lower allograft survival. Therefore, while inflammatory infiltrates within fibrosis had been neglected for long time, in 2007 it was proposed to include a ‘total inflammation’ (ti) score into Banff classification [24]. The underlying concept is that inflammatory infiltrate, whatever its localization, should not be considered as harmless but rather indicative of an active alloreactive immune response. The significance of the ti score is under evaluation, but here we considered inflamed fibrosis as a marker of chronic active T-cell-mediated rejection. We, therefore, focused our study on biopsies showing inflamed fibrosis and not fulfilling Banff criteria for acute rejection [24].

This study shows that FOXP3-expressing cells are enriched in grafts with chronic active versus acute cellular inflammation. Since the presence of FOXP3+ T cells within infiltrates during borderline and acute rejections correlates with a better graft outcome [5,11,12], it is likely that regulatory T cell enrichment may dampen graft injury in chronic (versus acute) inflammation and may contribute to the slow-evolving process. On the other hand, persistence of overt chronic inflammation, despite significant enrichment in FOXP3-expressing cells, raises the question of why these Tregs are not capable of efficiently suppressing the alloimmune response.
Fig. 3. TH17/FOXP3+ ratio within nodular and diffuse infiltrates in terminally rejected renal grafts. (A) Representative nuclear FOXP3 (arrow) and cytoplasmic IL-17 (arrow head) staining on two contiguous serial sections (400× magnification). (B) Intratubular IL-17 secreting cells. (C) Representative double stain for FOXP3 (blue) and IL-17 (brown) (400× magnification). (D) Representative double stain for FOXP3 (brown) and IL-17 (blue) in nodular and diffuse aggregates (left panel). Comparison of the IL-17/FOXP3 cell ratio according to the microanatomical organization of lymphoid infiltrates (right panel).

One possible explanation is that detected FOXP3+ cells are activated T cells rather than Tregs [3,4]. However, some results argue against this hypothesis. If FOXP3 expression would indicate recently activated T cells, FOXP3+ cells should be expressed by CD4+ as well as CD8+ cells [3,4]. In fact, CD8+ FOXP3+ cells were barely detectable in our biopsies. Furthermore, recent reports suggest that ‘ex-vivo’ FOXP3+-activated T cells express low levels of FOXP3, at levels a log lower than true Tregs [25]. We indeed identified dimly stained T cells in the graft, but we did not count them as positives, considering that they should not correspond to true Tregs.

Another possibility is that FOXP3-expressing cells are true Tregs but that local pro-inflammatory environment might not be favourable to suppression. Although suppression assays using cells isolated from the graft might be helpful to address this issue, we did not perform these experiments due to the difficulty to obtain enough cells from renal biopsies. Nevertheless, failure of Tregs to suppress Teffs within inflamed tissues has been demonstrated in a variety of settings [26,27]. This may not be due to deficient suppressive capacity of Tregs per se, but rather to an acquired resistance of Teffs to suppression often linked to the inflammatory environment [26,27]. This finding highlights that control of an immune response is not only a matter of the Treg/Teff ratio but also of the local microenvironment [27,28]. Since pro-inflammatory cytokines (such as IL-6, IL-1 and TNFα) render Teffs less sensitive to Treg-mediated suppression, outnumbered Tregs may not outperform Teffs in chronically inflamed tissue [27,28].

Strikingly, our study provides also evidence for infiltration of IL-17-producing cells within chronically rejected grafts. IL-17-secreting cells might reduce Treg function, by inducing IL-6 and TNFα [29], while pro-inflammatory cytokines may in turn induce commitment of naive or memory T cells into TH17 lineage [20,30]. In addition, a recent report indicates that IL-17 expression is poorly suppressed, unlike IFNγ, by FOXP3-expressing Tregs [31]. Our study also stresses that the TH17/FOXP3+ ratio may significantly vary according to the type of infiltrate, being higher in diffuse infiltrates. This latter observation is in keeping with recent findings that diffuse infiltrates are more aggressive for the renal structures than nodular aggregates, as evidenced by higher grade of tubulitis and more frequent rise in serum creatinine [32]. This observation raises the question whether specific pro-inflammatory cytokine blockade would enhance efficiency of Tregs and reverse the chronic rejection process. In the field of chronic autoimmune diseases, especially those in which balanced TH17/Treg response has been well documented, innovative treatments neutralizing inflammatory cytokines have demonstrated enhancement of Treg function and reduced TH17 response in parallel with clinical improvement [26,33].

We found a significant enrichment of Tregs within nodular inflammatory aggregates that disclosed morphological characteristics of ectopic germinal centres. These well-organized lymphoid structures (also named tertiary lymphoid tissues) arise in the situation of chronic inflammation and it is presumed that activated infiltrating T and B cells may provide signals required to lymphoid neogenesis [34,35]. A similar co-localization of FOXP3+ cells and
nodular B-cell-enriched infiltrates has also been described in advanced-rejected nonhuman primate kidney transplants [36]. This finding fits well with recent observations that both intra-graft FOXP3 messenger [10] and B-cell associated transcripts [37] correlate with a longer post-transplant course. Altogether, these data suggest that chronic inflammation is associated with a distinct microenvironment, favourable to B-cell and Treg expansion and/or recruitment. Of note, recent evidence indicates that IL-17 can enhance the formation of new germinal centres by favouring B-cell retention [19].

Strikingly, our study found that low FOXP3/CD3 ratio was independently associated with a worse graft survival in the chronic inflammation subgroup. This finding was consistent with previous observations in borderline and subclinical acute rejections [5,11,12]. Although this result should be confirmed in a larger prospective cohort, this suggests that chronically inflamed grafts with a higher Treg/Teff ratio may show a better outcome. In contrast, this suggests that chronically inflamed grafts with a higher Treg/Teff ratio may show a better outcome. In contrast, this suggests that chronically inflamed grafts with a higher Treg/Teff ratio may show a better outcome. In contrast, this suggests that chronically inflamed grafts with a higher Treg/Teff ratio may show a better outcome. In contrast, this suggests that chronically inflamed grafts with a higher Treg/Teff ratio may show a better outcome. In contrast, this suggests that chronically inflamed grafts with a higher Treg/Teff ratio may show a better outcome.

In conclusion, our study shows that chronic inflammation in scarred grafts resembles other chronic inflammatory processes with significant recruitment of FOXP3-expressing regulatory T cells into the target tissue. FOXP3+ cells preferentially segregate within B-cell-enriched nodular lymphoid aggregates, a hallmark of the chronic inflammatory process. The lower FOXP3/CD3+ cell ratio in chronically inflamed transplants is associated with a worse graft outcome. Slow-evolving yet destructive allogeneic responses may result from an imbalance between pro-inflammatory TH17 cells and the FOXP3-expressing Treg, whose efficiency might be hampered by the local microenvironment. These results stress the interest of devising strategies to ‘skew’ immune responses towards their regulatory fate.

Conflict of interest statement. None declared.

References
Exercise capacity and body composition in living-donor renal transplant recipients over time

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Abstract

Background. Renal transplantation (RTx) restitutes the function of the failing organ and induces convalescence of the entire organism. Our study investigates whether this is accompanied by improvements in cardiovascular function and structural changes.

Methods. A total of 25 Caucasian patients (14 male, median age 44.2 ± 9.2 years, BMI 23.7 ± 4.0 kg/m²) were assessed in a prospective trial before, 1, 3 and 12 months after RTx from living donors by clinical examination, cardiopulmonary exercise testing, dual X-ray absorptiometry (DEXA) and analysis of plasma indices.

Results. Creatinine clearance improved from 8.0 ± 3.1 to 60.9 ± 18.1 mL/min at 1 month, but declined at 3 (51.6 ± 16.3 mL/min) and 12 months (53.6 ± 20.8 mL/min, P = 0.04 versus month 1). Body composition shifted from lean towards fat tissue (25.8 ± 12.5–31.2 ± 11.2% body fat content, P = 0.0001). Only baseline lean weight correlated with fat increase over time (r² = 0.28, P = 0.008). Patients with fat content above median (n = 13) had a 3-fold increased hazard ratio of infection (CI 1.04–9.41, P = 0.042) and overall hospitalization (hazard ratio 2.95, CI 1.10–7.93, P = 0.03). PeakVO2 decreased over RTx (23.2 ± 6.0–17.6 ± 5.1 mL/kg/min) and returned to baseline levels not until 1 year later (P < 0.001). After an initial decline, muscle oxidative capacity (peakVO2/lean mass) improved from 33.6 ± 10.1 to 35.0 ± 8.2 mL/kg/min at 12 months after RTx (P < 0.001).

Conclusions. After RTx, body composition shifted continuously towards fat tissue, and baseline lean weight significantly correlated with fat increase over time. Both severe infections and hospitalizations are associated with a higher fat content before RTx. Exercise capacity (peakVO2) worsened after RTx and restitutes during follow-up, with muscle quality (peakVO2/lean) even exceeding baseline levels after 12 months.

Keywords: body composition; exercise; kidney transplantation; metabolism; peakVO2

Introduction

Among all cardiovascular risk factors, renal failure is associated with the highest mortality [1–3]. Renal failure causes a variety of metabolic changes that may damage the cardiovascular system: retention of electrolytes and water, increase in renin and decrease in erythropoietin, kinines and nitric oxide, followed by the release of cytokines and other inflammatory stimuli. Chronic inflammatory activation then induces tissue wasting and leads to a loss in body...