Regulatory T Cells in Kidney Allograft Infiltrates Correlate With Initial Inflammation and Graft Function

Cécile Taflin, Dominique Nochy, Gary Hill, Thierry Frouget, Nathalie Rioux, Jérôme Vérine, Patrick Bruneval, and Denis Glotz

Background. The relevance of borderline change (BL) and subclinical cellular rejection (SCR) observed in renal transplantation remains to be determined. Several studies have shown in BL and SCR the presence of a Th1 immune response, qualitatively similar to but quantitatively reduced in comparison with infiltrates typical of acute cellular rejection (ACR).

Methods. To elucidate the role of regulatory T cells (Tregs) in the local control of the allogeneic response, we studied their presence by immunohistochemistry in 24 biopsies with graft dysfunction (12 ACR and 12 BL) and in 16 protocol biopsies at 1 year (eight SCR and eight subclinical BL).

Results. The proportion of Tregs in CD4+ T infiltrates was higher in BL and SCR when compared with ACR. Moreover, their presence was correlated with the intensity of interstitial inflammation (r = –0.35, P = 0.027, n = 40) and with graft function at the time of the biopsy (r = –0.37, P = 0.018, n = 40).

Conclusion. These data suggest Treg recruitment at the acute phase of the allogeneic response, where they could act to diminish the interstitial inflammation and its associated lesions.

Keywords: Regulatory T cells, FoxP3, Biopsy, Suppression, Kidney allograft infiltrates.

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found between intragraft FoxP3 mRNA levels and favorable outcome in 28 cases of ACR (14). The role of Tregs has also been studied in borderline change (BL), where it was observed that the FoxP3/Granzyme B ratio was higher compared to that seen in ACR (15). Strong expression of intragraft FoxP3 mRNA was associated with stability of histologic lesions and favorable evolution at 1 month (16). A favorable role for Tregs on the course of subclinical cellular rejection (SCR) was also found (17), and the presence of FoxP3 protein in the graft was correlated with increased graft function at 2 years after transplantation.

The contradictory data on the role of Tregs in the favorable versus unfavorable course of graft function result in part from the lack of specificity of the FoxP3 transcription factor for the Tregs population. In human, transitory low-level FoxP3 expression is seen in the course of effector T-cell activation (18). Immunosuppressive memory Tregs are distinguished by a high level of FoxP3 expression (18, 19). It seems likely that the use of too sensitive techniques to quantify mRNA FoxP3 by reverse-transcriptase polymerase chain reaction detects not only Tregs but also activated T effector cells. Therefore, we investigated the role of Tregs in the local control of the allogenic response, with a previously described validated immunohistochemical technique detecting Tregs in renal grafts (20). Their presence was compared in BL and ACR with or without graft dysfunction. Finally, the associations between the presence of Tregs in the renal transplant, inflammatory response intensity, and graft function at the time of the biopsy were studied.

**PATIENTS AND METHODS**

**Patients and Renal Biopsies**

Our retrospective study includes 40 patients receiving first renal transplants, having had a kidney biopsy between 1994 and 2007. Patients were followed up at the Nephrology Department of the Hôpital Broussais, Paris from 1994 to 2000 and then at the Nephrology Department of the Hôpital St. Louis, Paris. Two patient groups were distinguished:

1. Twenty-four patients underwent a renal biopsy because of renal insufficiency. Twelve of them (50%) had an ACR, and 12 (50%) had BL, according to the Banff diagnostic criteria (21).
2. Sixteen patients with stable renal function had protocol biopsies at 12 months. Eight of these patients (50%) had a SCR, and eight (50%) had subclinical BL (SCBL).

Thirty-five patients had an induction treatment (thymoglobulin in 19 patients and daclizumab in 16 patients). Immunosuppression consisted of a calcineurin inhibitor, azathioprine or mycophenolate mofetil, and additional prednisone for 29 patients (Table 1). To treat the acute episode, patients with ACR, SCR, or BL received methylprednisolone boluses for 3 consecutive days.

Graft function was assessed by serum creatinine (SCr) levels at the time of biopsy.

**Detection of CD4⁺FoxP3⁺ T Cells in Biopsy Material**

CD4⁺FoxP3⁺ cells were detected in biopsies fixed in alcohol-formol-acetic acid embedded in paraffin as previously described (20).

The double immunofluorescence CD4⁺FoxP3⁺ was analyzed using a Zeiss LSM 510 microscope with confocal laser. All images were taken using a 60× objective. CD4⁺FoxP3⁺ cells were counted using a quantitative score. Mean percentages of CD4⁺FoxP3⁺ cells were established by counting five fields at 60×. The repartition and proportion of the CD4⁺FoxP3⁺ cells were similar between the five fields analyzed in each biopsy.

**Histopathology**

Graft biopsies were stained routinely for light microscopic study. The cellular infiltration was analyzed on immunoperoxidase

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**TABLE 1.** Baseline demographic data and clinical characteristics of patients

<table>
<thead>
<tr>
<th></th>
<th>ACR (n=12)</th>
<th>SCR (n=8)</th>
<th>BLSC (n=8)</th>
<th>BL (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Recipient age</strong></td>
<td>33.53 ± 10.53</td>
<td>41.38 ± 10.94</td>
<td>41.63 ± 13.65</td>
<td>40.33 ± 10.55</td>
</tr>
<tr>
<td>(year ± SD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Recipient gender</strong></td>
<td>10/2</td>
<td>5/3</td>
<td>5/3</td>
<td>9/3</td>
</tr>
<tr>
<td>(M/F)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Recipient ethnicity</strong></td>
<td>10/2</td>
<td>4/4</td>
<td>3/5</td>
<td>7/5</td>
</tr>
<tr>
<td>(E/A)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Type of donor</strong></td>
<td>10/2</td>
<td>6/2</td>
<td>6/2</td>
<td>11/1</td>
</tr>
<tr>
<td>(DD/LD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>HLA mismatch</strong></td>
<td>2 (1–4)/1 (0–2)</td>
<td>2 (0–4)/1 (0–2)</td>
<td>2 (2–4)/2 (0–2)</td>
<td>2 (2–4)/1 (0–2)</td>
</tr>
<tr>
<td>(class I/class II⁺)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>No. transplant</strong></td>
<td>12/0</td>
<td>8/0</td>
<td>8/0</td>
<td>12/0</td>
</tr>
<tr>
<td>(1/2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><strong>HLA antibodies</strong></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>at the time of biopsy (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><strong>Immunosuppression regimen (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcineurin inhibitor</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Induction therapy</td>
<td>100</td>
<td>75</td>
<td>100</td>
<td>75</td>
</tr>
<tr>
<td>Treatment of the acute episode (%)</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Time to biopsy (wk)</td>
<td>56.67 ± 100.8</td>
<td>52 ± 0</td>
<td>52 ± 0</td>
<td>25.5 ± 42.38</td>
</tr>
<tr>
<td>Serum creatinine (μmol/L; mean ± SD)</td>
<td>126.2 ± 20.36</td>
<td>116 ± 31.18</td>
<td>114 ± 22.9</td>
<td>122.3 ± 13.9</td>
</tr>
<tr>
<td>Before the biopsy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>At the time of biopsy</td>
<td>273.3 ± 102</td>
<td>118.8 ± 33.1</td>
<td>125.6 ± 24</td>
<td>244.9 ± 149</td>
</tr>
<tr>
<td>a Mode (range).</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

M, male; F, female; E, European; A, African; DD, deceased donor; LD, living donor; ACR, acute cellular rejection; SCR, subclinical cellular rejection; BL, borderline change; BLSC, subclinical borderline change; HLA, human leukocyte antigen.
Infiltrates of both groups. These observations show a qualitative difference in the infiltrates posttransplant. Their presence compared with the BL group (1.92% of CD4+ cells, n=12, vs. 0.61% of CD4+ cells, P=0.001), whereas the CD4+FoxP3+ effector cell population was comparable between the two groups (69.82±16 cells/field, n=8, vs. 66.75±13.4 cells/field, n=12, P=0.69; Table 2). Similarly, the SCBL group had a higher percentage of Tregs compared with the BL group (8.98±2.03% of CD4+ cells, n=8, vs. 4.11%±2.49%, n=12, P=0.002) but with no difference in the CD4+FoxP3− cell number (29.84±5.1 cells/field, n=8, vs. 31.95±20.1 cells/field, n=12, P=0.98).

CD3, CD8, and CD20 were also analyzed to compare the nature of the effector response in inflammatory infiltrates with (ACR and BL) or without (SCR and SCBL) graft dysfunction. No difference in the proportion of CD3+CD8−, CD3−CD8+ cell population between the two groups was observed (Table 2). These data suggest that the effects of ACR and BL on renal function depend not only on the effector response but also on the presence of Tregs permitting regulation of the effector response.

**Higher Percentage of Memory Tregs in Infiltrates in the Absence of Graft Dysfunction**

We then studied the influence of Tregs on the clinical expression of the infiltrates posttransplant. Their presence compared with the same immunohistochemical technique in infiltrates with (ACR) or without graft dysfunction (SCR). As shown in Figure 2, more Tregs were present in the SCR group when compared with the ACR group (2.49%, n=8, vs. 1.92% of CD4+ cells, P=0.001). Similarly, the SCBL group had a higher percentage of Tregs compared with the BL group (8.98±2.03% of CD4+ cells, n=8, vs. 4.11%±2.49%, n=12, P=0.002) but with no difference in the CD4+FoxP3− cell number (29.84±5.1 cells/field, n=8, vs. 31.95±20.1 cells/field, n=12, P=0.98).

**The Presence In Situ of Tregs Is Correlated With the Intensity of Interstitial Inflammation and Graft Function at the Time of the Biopsy**

Finally, we addressed the question of whether intragraft Tregs had an impact on the intensity of the inflammatory response and graft function at the time of biopsy. No corre-

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**RESULTS**

**Borderline Change Are Enriched in Memory Tregs as Compared With Acute Cellular Rejection**

The markers CD3, CD8, CD20, and CD68 were compared in 12 BL and 12 ACR. The infiltrates in both were primarily composed of CD3+ cells (Table 2). Of these, more than 70% were CD4+, with CD8+ cells primarily located in tubules and vessels. The remainder of the infiltrates were composed of CD68+ cells, and rare CD20+ nodular infiltrates (<10%) were seen. Eosinophils were absent from the infiltrates of both groups. These observations show a qualitatively similar but quantitatively lower effector response in the BL group compared with the ACR group.

Next, we evaluated the presence of Tregs in these infiltrates. The FoxP3+ cells quantified through confocal microscopy on paraffin sections were analyzed within the CD4+ infiltrates of both groups. We have previously shown that this technique on fixed specimens only detects FoxP3bright cells, thus identifying memory Treg cells, the FoxP3low cells (including naïve Tregs and effector T cells) remaining below the threshold of detection (20). As shown in Figure 1, the Treg proportion was lower in the CD4+ infiltrates in ACR when compared with BL (1.58%±1.92% of CD4+ cells, n=12, vs. 4.11%±2.49%, n=12, P=0.015).

These results show that Tregs are associated with a lower inflammation response, suggesting that they are implicated in the lower effector response in the BL group compared with the ACR group.

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**TABLE 2.** Percentage of the different effector cell populations in the allogenic infiltrate

<table>
<thead>
<tr>
<th></th>
<th>ACR (n=12)</th>
<th>BL (n=12)</th>
<th>SCR (n=8)</th>
<th>BLS (n=8)</th>
<th>P&lt;sup&gt;a&lt;/sup&gt;</th>
<th>P&lt;sup&gt;b&lt;/sup&gt;</th>
<th>P&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total CD3&lt;sup&gt;+&lt;/sup&gt; (%)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>74.17±10.2</td>
<td>72.92±10.7</td>
<td>73.13±9.6</td>
<td>71.88±10.6</td>
<td>0.93</td>
<td>0.87</td>
<td>0.79</td>
</tr>
<tr>
<td>CD3&lt;sup&gt;+&lt;/sup&gt;CD8&lt;sup&gt;−&lt;/sup&gt; (% of CD3&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>71.25±18.3</td>
<td>70.83±8.7</td>
<td>72.5±9.3</td>
<td>73.75±12.7</td>
<td>0.75</td>
<td>0.98</td>
<td>0.87</td>
</tr>
<tr>
<td>CD3&lt;sup&gt;+&lt;/sup&gt;CD8&lt;sup&gt;+&lt;/sup&gt; (% of CD3&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>28.75±8.3</td>
<td>29.17±8.8</td>
<td>27.5±9.2</td>
<td>26.25±13</td>
<td>0.75</td>
<td>0.98</td>
<td>0.87</td>
</tr>
<tr>
<td>CD68&lt;sup&gt;+&lt;/sup&gt; (%)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>18.75±7.42</td>
<td>17.92±6.9</td>
<td>17.5±7.1</td>
<td>19.38±8.2</td>
<td>0.56</td>
<td>0.72</td>
<td>0.72</td>
</tr>
<tr>
<td>CD20&lt;sup&gt;+&lt;/sup&gt; (%)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>7.08±8.64</td>
<td>9.16±8.4</td>
<td>9.37±9.4</td>
<td>8.74±5.82</td>
<td>0.70</td>
<td>0.61</td>
<td>0.95</td>
</tr>
<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt;FoxP3&lt;sup&gt;−&lt;/sup&gt; (n)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>66.75±13.4</td>
<td>31.95±20.1</td>
<td>69.82±16</td>
<td>29.84±5.1</td>
<td>0.001</td>
<td>0.69</td>
<td>0.98</td>
</tr>
</tbody>
</table>

<sup>a</sup> P value between ACR and BL.

<sup>b</sup> P value between ACR and SCR.

<sup>c</sup> P value between BL and BLS.

<sup>d</sup> Mean percentage of effector cell populations in cellular infiltrate±SD.

<sup>e</sup> Mean cell number±SD/field.

ACR, acute cellular rejection; SCR, subclinical cellular rejection; BL, borderline change; BLS, subclinical borderline change.
Normalization was observed between the percentage of Tregs and the different demographic parameters (age, sex, ethnicity, donor origin, number of human leukocyte antigen mismatches, time to biopsy, and the use or the nature of induction treatment). However, the percentage of Tregs was negatively correlated with SCr at the time of biopsy ($r = -0.37$, $P = 0.018$, $n = 40$; Fig. 3). Similarly, there was a negative correlation between the intensity of the interstitial infiltrate and the percentage of Tregs ($r = -0.35$, $P = 0.027$, $n = 40$; Fig. 3). These data suggest a protective role of Tregs during the allogenic response in controlling interstitial inflammation and its associated lesions.

**FIGURE 1.** Increased percentage of regulatory T cells in borderline change. Comparison of the percentage of CD4$^+$ cells (green membrane staining) with FoxP3$^+$ (red nuclear staining) among CD4$^+$ cells on paraffin sections of acute cellular rejection (ACR) and borderline change (BL). Graph shows results for 12 ACR compared with 12 BL, with $P$ calculated from Mann-Whitney $U$ test. Magnification $\times 600$.

**FIGURE 2.** Augmentation of infiltration by regulatory T cells in subclinical lesions. Confocal analysis of the percentage of CD4$^+$FoxP3$^+$ cells on paraffin sections. Graph shows results of comparison of acute cellular rejection (ACR; 12 patients), subclinical cellular rejection (SCR; eight patients), and of BL (12 patients) compared with subclinical BL (eight patients), with $P$ calculated by Mann-Whitney $U$ test. Magnification $\times 600$. © 2010 Lippincott Williams & Wilkins
associated with the Th1 response, including the transcription factor Tbet, peripherally diminished in the latter. Similarly, Hoffmann et al. have shown, in a transgenic mouse model in which tolerance was induced after skin grafting, that there is a migration of allospecific Tregs into the graft, leading to local suppression of the generation of memory T cells from effector T cells already present in the graft. Memory T cells are characterized by marked proliferation in response to antigens and increased secretion of certain chemokines, such as regulated on activation normal T-cell expressed and secreted (RANTES), interferon-γ, and granzyme B. Interestingly, the nature of the infiltrates into the graft, such as Tregs, that are falsely identified as mediators of rejection. Thus, to address the question of whether Tregs in the graft and graft function at the time of biopsy suggest that they also play a role in the inhibition of parenchymal insult associated with the allogenic inflammatory reaction. For example, apoptosis of tubular epithelial cells via the interaction of Fas and its ligand expressed by activated CD4+ cells is one of the mechanisms described in graft dysfunction in ACR. In view of the transcriptional data observed in SCR, showing diminished expression of Fasl in comparison with ACR, it may be hypothesized that effector T-cell activation is locally suppressed in SCR, thus inhibiting apoptosis of renal tubular cells and permitting maintenance of renal function.

Moreover, the observation in this study of an increase in Tregs in SCR and of a negative correlation between their presence in the graft and graft function at the time of biopsy suggest that they also play a role in the inhibition of parenchymal insult associated with the allogenic inflammatory reaction. For example, apoptosis of tubular epithelial cells via the interaction of Fas and its ligand expressed by activated CD4+ cells is one of the mechanisms described in graft dysfunction in ACR. In view of the transcriptional data observed in SCR, showing diminished expression of Fasl in comparison with ACR, it may be hypothesized that effector T-cell activation is locally suppressed in SCR, thus inhibiting apoptosis of renal tubular cells and permitting maintenance of renal function.

Our results suggest a beneficial effect of Tregs in the graft, inhibiting acute allogenic inflammation and tissue injury, and raise the question of their diagnostic value for rejection in kidney transplantation. Presently, the diagnosis of rejection in kidney transplantation, but also in other solid organ transplantation such as heart transplantation, is made on histologic evaluation of the grafted tissue. Consequently, the decision to treat depends on the infiltrate and not on the deterioration of graft function. The observation presented here may have an important bearing in the clinical situation, especially on the treatment of subclinical infiltrates. Indeed, it is likely that the systematic treatment of these infiltrates results in disappearance not only of effectors of allogenic inflammation but also in some cases of beneficial mononuclear cells infiltrating the graft, such as Tregs, that are falsely identified as mediators of rejection. Thus, to address the question not only of the diagnostic but also of the prognostic value of Tregs in the allogenic inflammatory infiltrate, a prospective multicenter study is needed, comparing two groups of patients with subclinical infiltrates, treated or not, where the functional and histologic evolution can be correlated with the level of Tregs in the initial biopsy. Definitive data on this question would establish the putative beneficial effect of Tregs infiltration in allogenic inflammation, allowing accurate assessment of individual patients’ immunologic risk to tailor immunosuppressive treatments to these different levels of risk.

DISCUSSION

The Banff classification has permitted the establishment of uniform therapeutic strategies for treatment of ACR and more recently antibody-mediated rejection. However, the nature and significance of borderline and subclinical changes remain uncertain. Recently, microarray analysis has permitted a more precise study of the nature of the inflammatory response associated with these infiltrates. Halloran and coworkers have shown that the expression of transcripts associated with the T-dependent allogenic response was qualitatively the same in ACR and BL, but quantitatively diminished in the latter. Similarly, Hoffmann et al. have shown the expression of transcripts associated with the T-dependent allogenic response was qualitatively the same in SCR and ACR, but with higher expression of Tbet and FasL in ACR. These data suggest an immune response that is qualitatively similar but quantitatively reduced in BL and SCR compared with ACR. Our study revealed an equivalent proportion of CD4+ and CD8+ T cells, B cells, macrophages, and the absence of eosinophils in BL and ACR, with or without graft dysfunction, support this hypothesis. Moreover, we have observed a higher percentage of Tregs in BL, and more generally a negative correlation between the presence of Tregs in the graft and the intensity of interstitial inflammation. These results suggest a local suppression of the inflammatory reaction by allospecific Tregs previously activated in the lymphoid organs and migrating into the graft. Two mechanisms might thus explain the diminution of interstitial inflammation: either suppression of effector T-cell recruitment into the graft or inhibition of their proliferation in situ. Recently, Wood and coworkers have shown, in a transgenic mouse model in which tolerance was induced after skin grafting, that there is a migration of allospecific Tregs into the graft, leading to local suppression of the generation of memory T cells from effector T cells already present in the graft. Memory T cells are characterized by marked proliferation in response to antigens and increased secretion of certain chemokines, such as regulated on activation normal T-cell expressed and secreted and inducible protein-10 (26, 27). These chemokines play a key role in the recruitment of allospecific T cells into the graft (28). It is, therefore, possible that the Tregs suppress memory T cells and thus decrease the intensity of the inflammatory reaction, leading to BL and not to full-blown ACR.

FIGURE 3. Correlation of regulatory T cells (Tregs) in situ with interstitial inflammation and graft function. The percentage of CD4+ Tregs in the graft was correlated with interstitial inflammation at the time of the biopsy as quantified by the Banff score (i0–3) (P values calculated from the Spearman rank correlation test, n = 40) and graft function at the time of biopsy (P values calculated from the Pearson product moment correlation test, n = 40).
In conclusion, we have shown that BL and SCR have equivalent proportions of the different effector cell types compared with ACR, but they are distinguished from the latter by an increased percentage of Tregs in the CD4\(^+\) infiltrate. The presence of Tregs in the graft was correlated with the intensity of the inflammatory reaction and renal function at the time of biopsy. These observations suggest an initial beneficial effect of Tregs in the graft and need to be extended to determine the correlation with graft outcome.

**ACKNOWLEDGMENTS**

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**REFERENCES**