Achieving Donor-Specific Hyporesponsiveness Is Associated with FOXP3+ Regulatory T Cell Recruitment in Human Renal Allograft Infiltrates

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Achieving Donor-Specific Hyporesponsiveness Is Associated with FOXP3+ Regulatory T Cell Recruitment in Human Renal Allograft Infiltrates

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Exploring new immunosuppressive strategies inducing donor-specific hyporesponsiveness is an important challenge in transplantation. For this purpose, a careful immune monitoring and graft histology assessment is mandatory. Here, we report the results of a pilot study conducted in twenty renal transplant recipients, analyzing the immunomodulatory effects of a protocol based on induction therapy with rabbit anti-thymocyte globulin low doses, sirolimus, and mofetil mycophenolate. Evolution of donor-specific cellular and humoral alloimmune response, peripheral blood lymphocyte subsets and apoptosis was evaluated. Six-month protocol biopsies were performed to assess histological lesions and presence of FOXP3+ regulatory T cells (Tregs) in interstitial infiltrates. After transplantation, there was an early and transient apoptotic effect, mainly within the CD8+ T cells, combined with a sustained enhancement of CD4+ CD25+ T lymphocytes in peripheral blood. The incidence of acute rejection was 35%, all steroid sensitive. Importantly, only pretransplant donor-specific cellular alloreactivity could discriminate patients at risk to develop acute rejection. Two thirds of the patients became donor-specific hyporesponders at 6 and 24 mo, and the achievement of this immunologic state was not abrogated by prior acute rejection episodes. Remarkably, donor-specific hyporesponders had the better renal function and less chronic renal damage. Donor-specific hyporesponsiveness was inhibited by depleting CD4+ CD25+ T cells, which showed donor-Ag specificity. FOXP3+ CD4+ CD25+ Tregs both in peripheral blood and in renal infiltrates were higher in donor-specific hyporesponders than in nonhyporesponders, suggesting that the recruitment of Tregs in the allograft plays an important role for renal acceptance. In conclusion, reaching donor-specific hyporesponsiveness is feasible after renal transplantation and associated with Treg recruitment in the graft.


Increasing long-term allograft survival is a main challenge in organ transplantation. Transplant tolerance is expected to be a way, to both reduce rejection and minimize immunosuppressive side effects, therefore improving graft outcome. A better understanding of basic tolerogenic mechanisms (clonal exhaustion, deletion, and immune ignorance) as well as the capital role that T cells play during transplant rejection has resulted in different strategies trying to induce tolerance (1–6); namely, T cell costimulation blockade, mixed chimerism induction, T cell depletion, and tolerance mediated by regulatory T cells (Tregs). It is to note that some of these approaches have been already successfully proven in murine and in nonhuman primate models (7–12). In the clinical setting, tolerance or "prope" tolerance has been defined as evidence of donor-specific un/hyporesponsiveness with recovered third party response in functional in vitro assays, lack of circulating donor-specific alloantibodies and absence of destructive lymphocyte infiltration in allograft biopsies in patients without or with minimal amount of immunosuppression. In fact, in clinical transplantation, the main therapeutic approach for reaching a tolerogenic state has been based on two sequential steps: first, recipient T cell depletion and then, the use of minimal posttransplant immunosuppression (13). Actually, such strategy has been performed in kidney (14–16) and liver (17) transplantation using tacrolimus (TAC) or sirolimus (SRL) as maintenance immunosuppressive drugs. Nonetheless, calcineurin inhibitors (CNI) have controversial effects on transplant tolerance mechanisms (18–20). Conversely, mammalian target of rapamycin (mTOR) inhibitors and rabbit anti-thymocyte globulin (rATG) have been shown to have relevant immunomodulatory effects such as a proapoptotic T cell effect (21, 22) and the capacity to selectively expand the CD4+ CD25+ FOXP3+ Treg population both in vivo and in vitro (23–26).

Monitoring the alloimmune response is a critical step to carry out protolerogenic immunosuppressive protocols. Certainly, the donor-specific immune evaluation is feasible nowadays; on one hand, donor-specific alloantibodies can be precisely monitored by flow cytometric, cytotoxicity, or ELISA crossmatching techniques, and on the other, high frequency of donor-specific alloreactive T cells can be evaluated by the ELISPOT assay. Indeed, these procedures have been shown to be useful for predicting graft outcome (27–32).

Herein, we report the results of a pilot study performed in twenty renal transplant recipients with the aim of achieving donor-specific hyporesponsiveness using a CNI and steroid-free regimen based on low doses of rATG induction, SRL, and mofetil mycophenolate (MMF). We postulate that a transient T cell depletion by rATG,

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3 Abbreviations used in this paper: Treg, regulatory T cell; SRL, sirolimus; CNI, calcineurin inhibitor; MMF, mofetil mycophenolate; PRA, panel reactive Abs; BPAR, biopsy proven acute rejection; TAC, tacrolimus; rATG, rabbit anti-thymocyte globulin.

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followed by a subsequent regulatory T cell subset reconstitution under SRL, might be a way to promote a “prope” tolerogenic state after renal transplantation.

**Materials and Methods**

**Patients**

This was an investigator-promoted unincentric pilot study to evaluate a clinical strategy to induce donor-specific hyporesponsiveness in renal transplantation. It was done after the approval of the Ethic Committee at the Hospital Universitari de Bellvitge and of the Agencia Española del Medicamento, Ministerio de Sanidad y Consumo. Twenty adult primary renal allograft recipients were included between October 2003 and September 2005. All 20 patients received a deceased HLA-mismatched kidney from donors younger than 60 years. All of them had negative complement-dependent cytotoxic crossmatch at the time of transplantation, panel reactive Abs (PRA) lower than 20% and gave written informed consent. Exclusion criteria were: prior transplant, age > 60 years, fasting triglycerides > 4.6 mmol/L (>400 mg/dL); fasting total cholesterol > 7.8 mmol/L (>300 mg/dL) despite treatment with lipid-lowering therapy, white blood cell count < 2500/mm$^3$, platelet count < 100,000/mm$^3$ or hemoglobin <60 g/L. At the time of inclusion in the study, serologically positive for hepatitis B, C, or HIV, and cold ischemia time > 24 h.

**Immunosuppression**

Patients were given rATG (Thymoglobulin; Genzyme) induction, 1.5 mg/kg on day 0, and 1 mg/kg on days 1 and 2 after transplantation; methylprednisolone 1 mg/kg on day 0 and none thereafter; MMF (Cellcept; Hoffman-LaRoche) 2 g on day 0 followed by 500 mg b.i.d thereafter, with a concentration-controlled monitoring to keep mycophenolic acid C$_{2}$ levels between 2 and 5 $\mu$g/ml (EMIT mycophenolic acid assay; Dade-Behring) and SRL (Rapamune; Wyeth) 15 mg/day on days 0, 1, and 2, then 5 mg/day to reach C$_{2}$ levels 8–12 mg/ml for the first 6 mo after transplantation, followed by 6–10 mg/ml thereafter (HPLC via mass spectroscopy detection). All patients received pneumocystis carinii prophylaxis with daily trimethoprim/sulfam. CMV infection prophylaxis with oral valgancyclovir was done for 3 mo when donor/recipient serology was IgG$^+$/IgG$^-$. HLA typing, PRA- and donor-specific alloantibodies determination

Serologic donor and recipient HLA class I (A, B) and II (DR), as well as PRA determinations were identified by standard methods described previously (33–35). Donor-specific alloantibodies in peripheral blood was evaluated at one year after transplantation by flow cytometric technique using donor PBMCs.

Donor and recipient cells

Donor splenocytes were frozen in liquid nitrogen and subsequently used as stimulator cells. Recipient peripheral heparinized blood samples were obtained pretransplantation and at months 1, 3, 6, and 24 after transplantation. PBMCs were isolated by standard Ficoll density gradient centrifugation. These cells were frozen in liquid nitrogen. Unfractionated responder PBMCs were used for IFN-$\gamma$, and CD4$^+$ T cells for IL-10 ELISPOT assay (CD4$^+$ T cell isolation kit II; Miltenyi Biotec) to avoid the IL-10 production when donor/recipient serology was IgG$^+$/IgG$^-$. **ELISPOT assay**

IFN-$\gamma$ and IL-10 ELISPOT assays were performed as described previously in detail (36, 37). Briefly, 3 × 10$^5$ responder and irradiated (40 Gy) stimulator cells were placed in triplicate wells. Responder cells were also stimulated with 3 × 10$^5$ irradiated HLA mismatch (A, B, DR) splenocytes as third party. For each patient the same third party was used in all experiments. Responder and donor PBMC were also tested with medium alone and PHA (Sigma-Aldrich) in duplicate wells. The resulting spots were counted using a computer-assisted ELISPOT reader (AID Elispot Reader HR). Results were given as frequencies of IFN-$\gamma$ and IL-10 producing donor-reactive cells. Mean number of IFN-$\gamma$ and IL-10 spots per 3 × 10$^5$ responder cells were calculated by subtracting both the responder and the stimulator control wells from wells containing both the recipient and donor cells. We considered > 25 spots/3 × 10$^5$ responder cells as threshold to define a positive test (38).

**Analysis of spontaneous apoptosis and lymphocyte subsets in peripheral blood**

T cell apoptosis was detected 5, 10, 30 and 90 days after transplantation by the method of Vermes (39), using annexin-V to bind the phosphatidylserine translocated to cell surface during early apoptosis. Lymphocyte subset immunophenotype (CD3$^+$, CD4$^+$, CD8$^+$, CD19$^+$, and CD56$^+$) analysis was done by flow cytometry as described elsewhere (40, 41) using a FACS-calibur flow cytometer (Becton Dickinson) before and at days 5, 10, 30, 90, and 24 mo after transplantation.

<table>
<thead>
<tr>
<th>CD4$^+$ T cell subset analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cells were isolated by cell sorting (Moito, Dako) and purity was &gt;98%. Then, they were used for functional assays.</td>
</tr>
</tbody>
</table>

**Renal histology**

All renal histologies were analyzed following the Banff’03 score (42). In all cases, C4d (Biomedica), FOXP3 (the 86D/D6 mouse mAb, from the mAbs Unit, Biotechnology Program, CNIO) and CD3 (Master Diagnostics) immune-staining in paraffin-embedded tissues were performed as previously described (43). The proportion of FOXP3$^+$ T cells was assessed by FACS analysis (intracellular staining of human FOXP3, clone PCH101; eBioscience). Subsequently, CD4$^+$ CD25$^{high}$ T cells were isolated by cell sorting (Medit, Dako) and purity was >98%. Then, they were used for functional assays.

**Recipient and donors demographic characteristics**

<table>
<thead>
<tr>
<th>Recipients (N = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
</tr>
<tr>
<td>Gender (M/F)</td>
</tr>
<tr>
<td>Race</td>
</tr>
<tr>
<td>Cause of ESRD</td>
</tr>
<tr>
<td>Diabetes (type 2)</td>
</tr>
<tr>
<td>Unknown</td>
</tr>
<tr>
<td>Intestinal nephropathy</td>
</tr>
<tr>
<td>Pretransplant dialysis (yes/no)</td>
</tr>
<tr>
<td>HLA mismatches A, B, DR</td>
</tr>
<tr>
<td>Pre-transplant PRA (%)</td>
</tr>
<tr>
<td>CMV serologic status</td>
</tr>
<tr>
<td>D+/R+</td>
</tr>
<tr>
<td>D−/R+</td>
</tr>
<tr>
<td>D−/R−</td>
</tr>
<tr>
<td>Donors (N = 16)</td>
</tr>
<tr>
<td>Age (years)</td>
</tr>
<tr>
<td>Gender (M/F)</td>
</tr>
<tr>
<td>Cold ischemia time (hours)</td>
</tr>
<tr>
<td>Race</td>
</tr>
</tbody>
</table>

**Table I. Recipient and donors demographic characteristics**

**Table II. Biopsy proven acute rejection and borderline changes**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Banff-scored AR</th>
<th>Days after RT</th>
<th>Immunosuppression after AR</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>II A</td>
<td>8</td>
<td>SRL + MMF + PDN</td>
</tr>
<tr>
<td>5</td>
<td>IA</td>
<td>9</td>
<td>TAC + MMF + PDN</td>
</tr>
<tr>
<td>6</td>
<td>B-L</td>
<td>7</td>
<td>SRL + MMF + PDN</td>
</tr>
<tr>
<td>9</td>
<td>B-L</td>
<td>8</td>
<td>SRL + MMF + PDN</td>
</tr>
<tr>
<td>11</td>
<td>IB</td>
<td>11</td>
<td>SRL + MMF + PDN</td>
</tr>
<tr>
<td>13</td>
<td>B-L</td>
<td>15</td>
<td>TAC + MMF + PDN</td>
</tr>
<tr>
<td>15</td>
<td>IA</td>
<td>15</td>
<td>TAC + MMF + PDN</td>
</tr>
<tr>
<td>16</td>
<td>IA</td>
<td>9</td>
<td>SRL + MMF + PDN</td>
</tr>
<tr>
<td>17</td>
<td>II B</td>
<td>10</td>
<td>SRL + MMF + PDN</td>
</tr>
<tr>
<td>20</td>
<td>IB</td>
<td>75</td>
<td>TAC + SRL + PDN</td>
</tr>
</tbody>
</table>

AR, Acute rejection; RT, renal transplantation; B-L, borderline changes; SRL, sirolimus; MMF, mofetil mycophenolate; PDN, prednisone.
CD8, and CD3 (Master Diagnostica, Vision Biosystem Novocastra), as previously described (43). All the histological analysis was done in a blinded fashion.

Statistics

All data are presented as mean ± SD. Groups were compared using the \( \chi^2 \) test for categorical variables, the one-way ANOVA or \( t \) test for normally distributed data and the nonparametric Kruskal-Wallis or Mann-Whitney \( U \) test for nonnormally distributed variables. The statistical significance level was defined as \( p < 0.05 \).

Results

The mean follow-up is 34 months (21–47). Patient baseline demographic characteristics are shown in Table I. The actuarial patient and graft survival are 95 and 85%, respectively. One patient died with a functioning graft 42 mo after transplantation because of a bacterial infection. No significant differences were found regarding both serum creatinine (A) and proteinuria (B). Data are means ± SD. Statistical significance was evaluated using independent samples \( t \) test.

FIGURE 1. Renal function among rejectors and nonrejector patients.

FIGURE 2. Evolution of different lymphocyte subsets after transplantation. There was an important depletion of all lymphocyte subsets during the first 2 mo followed by a progressive recovery afterward. At 24 mo, total lymphocyte count, CD3\(^+\) and CD4\(^+\) T cells remained significantly lower than at baseline (\( p < 0.05 \)). Conversely, CD8\(^-\), CD19\(^+\), and CD56\(^+\) cell count recovered. Data are means ± SD. Statistical significance was evaluated using paired-samples \( t \) test. WBC, peripheral white blood cells.

FIGURE 3. T cell subset apoptosis. A, Representative apoptosis FACS plot of CD4\(^+\)HLA-DR\(^+\) and CD8\(^+\)HLA-DR\(^+\) T cells from a patient at day 5 posttransplantation. In x-axis are annexin-positive cells and y-axis are HLA-DR positive cells. In this particular patient, the CD4 subset has the following results: 26% are annexin-positive (apoptotic cells) and 4% co-express annexin V and HLA-DR (apoptotic and activated cells). Conversely, among the CD8 subset, 17% are annexin-positive (apoptotic cells) and 38% co-express annexin V and HLA-DR (apoptotic and activated cells). B, After induction therapy there was a transient T cell apoptosis which was more profound among CD8\(^+\) T cells than in CD4\(^+\) T cells (46 ± 18 vs 24 ± 10%, * \( p = 0.007 \)), and especially among the CD8\(^+\) expressing HLA-DR\(^+\) lymphocytes (31 ± 15 vs 10 ± 12%, * \( p = 0.03 \)). Data are means ± SD. Statistical significance was evaluated using paired-samples \( t \) test.
pneumonia. Two other patients lost their graft due to chronic allograft nephropathy at 15 and 21 mo after transplantation.

Rejection

Acute rejection was suspected in 12 of 20 patients. In all cases a renal biopsy was performed. Incidence of biopsy proven acute rejection (BPAR) was 35% (7 of 20), borderline changes were observed in 3 of 20 (15%) and acute tubular necrosis without any evidence of acute rejection was diagnosed in 2 of 20 (10%), as illustrated in Table II. C4d staining was negative in all renal tissue samples. All but one acute rejection occurred during the second week after transplantation and were successfully treated with three daily pulses of methylprednisolone (500 mg). In 4 of 7, SRL was switched to tacrolimus (TAC) mainly due to acute vascular damage. In the patient who experienced acute rejection 75 days after transplantation, maintenance immunosuppression was switched to TAC, SRL, and steroids. No correlation with HLA-mismatches between rejectors and nonrejectors was found (data not shown).

Renal function

Delayed graft function (defined as the need for dialysis in the week following transplantation because of a rising serum creatinine, after ruling out other causes of graft dysfunction) was observed in 1 of 20 patients. Regarding renal function, no statistically significant differences between rejectors and nonrejectors were found (Fig. 1).

Immunosuppression

Trough levels of SRL and mycophenolic acid at day 5 were not different between rejectors and nonrejectors (5.4 ± 1.2 and 2.1 ± 0.8 vs 5.0 ± 0.7 ng/ml and 2.2 ± 1.4 mg/L, respectively, p = ns). Fourteen (70%) recipients were CNI-free and seven (35%) remained steroid-free at last time of follow-up. Therefore, 7 of 20 patients (35%) remained on protocol (steroid and CNI-free). Two patients are on SRL monotherapy.

Adverse events

Anemia requiring erythroid-stimulating agents was a common complication. Actually, one third required erythroid-stimulating agents during the first year after transplantation. Persistent leukopenia was present in two patients, so MMF was withdrawn with subsequent recovery of leukocyte counts. Four patients received oral valganciclovir CMV prophylaxis during 3 mo. Only one patient developed CMV infection (her CMV serology was positive and received a CMV positive organ) and was treated with 21 days gancyclovir. In one patient a testicular seminoma was diagnosed 27 mo after transplantation and is currently in good health under a...
SRL-based therapy. Neither other malignancies nor posttransplant lymphoproliferative disease were observed in any patient during the follow-up. One patient under TAC, MMF, and prednisone died 42 mo after transplantation because of a bacterial pneumonia. No major surgical problems were observed; two patients (10%) had superficial wound complications that resolved with topical care and one developed a nonobstructive lymphocele, which was resolved with a percutaneous drainage. There were no cases of post-transplant diabetes mellitus. Hypertension and hyperlipidemia requiring treatment was observed in >50% of the patients.

Peripheral lymphocyte subsets and apoptosis monitoring

The T, NK, and B cell subset evolution after transplantation is illustrated in Fig. 2. After induction therapy, we found a transient T cell depletion associated with a 5-day posttransplant peak of apoptosis (Fig. 3), which was mainly restricted within the CD8\(^+\) cells expressing HLA class II molecules (CD8\(^+\)HLA DR\(^+\)).

Regulatory T cell subset

An early increase of the CD4\(^+\)CD25\(^{high}\) T cells was observed among the CD4\(^+\) lymphocytes and sustained until month 24 after transplantation (Fig. 4\(A\)). There were no differences in CD3\(^+\), CD4\(^+\), and CD8\(^+\) cell counts and percentage of CD4\(^+\)CD25\(^{high}\) between rejectors and nonrejectors (data not shown). Also, no differences were observed in the percentage of CD4\(^+\)CD25\(^{high}\) between donor-specific hyporesponder and nonhyporesponder patients (data not shown). However, at 24 mo, there was a significantly higher expression of the FOXP3 among CD4\(^+\)CD25\(^{high}\)T cells in donor-specific hyporesponders than in non-donor-specific hyporesponder patients (Fig. 4, \(B\) and \(C\)). Patients on SRL had significantly higher expression of FOXP3/CD4\(^+\)CD25\(^{high}\)T cells (Fig. 4\(D\)).

Cellular allorreactivity monitoring (ELISPOT)

ELISPOT IFN-\(\gamma\) assay (Th1 alloresponse). Pretransplant ELISPOT assay could be performed in 14 of 20 patients. High frequency of donor-specific alloreactive IFN-\(\gamma\) producing cells was observed pretransplantation in 6 of 14 patients while 8 of 14
were nonresponders. Interestingly, 5 of 6 donor alloreactive patients developed BPAR after transplantation whereas 1 of 8 non-donor alloreactive experienced BPAR ($p = 0.02$) (Fig. 5A). The 24-mo cellular allograft immune response evolution is illustrated in Fig. 6. At 6 mo, 9 of 14 became donor-specific hyporesponders; all 9 remained donor-specific hyporesponders at 24 mo and were on SRL (four without steroids and five on steroids). We tested whether 6- and 24-mo cellular allograft reactivity was associated with renal function. As illustrated in Fig. 7, serum creatinine was significantly lower in donor-specific hyporesponder patients than in nondonor-specific hyporesponders at 6 mo and tended to be lower at 24 mo. Induction of donor-specific hyporesponsiveness was not completely abrogated in CMV negative recipients receiving a CMV positive donor organ because 1 of 4 CMV−/CMV+ patients achieved this immunologic state.

To explore whether donor-specific hyporesponsiveness was related to the suppressive activity of Tregs on cytokine production, we made the IFN-γ ELISPOT assay in all patients by depleting the CD4+CD25+high T cell population. As shown in Fig. 8A, donor-specific hyporesponders significantly recovered anti-donor cytokine production after depletion. Furthermore, when these CD4+CD25+high T lymphocytes were transferred to allogenic responder cells (from an HLA A, B, and DR complete-mismatch individual) and cocultured with their specific donor cells, the donor-specific IFN-γ production was significantly abrogated, showing the Ag specificity of these cells (Fig. 8B). Conversely, when donor responders were depleted from these CD4+CD25+high T cells, they showed a significant inhibition of anti-donor cytokine production (Fig. 8A). Moreover, no abrogation of cytokine release was observed when these cells were transferred to an allogeneic mismatch individual (Fig. 8B), suggesting that rather than Tregs, these CD4+CD25+high T cells would mainly be activated/effector T lymphocytes. In fact, the significantly lower expression of the transcription factor FOXP3 within the CD4+CD25+high T cell population in nondonor-specific hyporesponders sustains this observation (Fig. 4C).

**ELISPOT IL-10 assay (Th2/Th3 alloreponse).** We could not find any correlation between pretransplant IL-10 production and BPAR (Fig. 5B). Also, the 6- and 24-mo IL-10 ELISPOT did not correlate with renal function with donor-specific hyporesponsiveness achievement (data not shown).

**Humoral alloreactivity immune monitoring**

All twenty patients had no circulating donor-specific alloantibodies at 1 year after transplantation. Also, no patients had positive C4d immune staining in 6-mo protocol biopsies.

**Table III. Six-month chronic Banff score**

<table>
<thead>
<tr>
<th>Banff Score</th>
<th>N (17)</th>
<th>Previous BPAR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 0 (normal)</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>CAN Ia</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>CAN Ia</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>CAN Iib</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>CAN Iia</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

BPAR, biopsy proven acute rejection; CAN, chronic allograft nephropathy.
Banff IA subclinical rejection. Chronic Banff score is shown in Table III. Also, the association between the alloimmune status and chronic renal damage was assessed. As shown in Table IV, donor-specific hyporesponder patients tended to have a better preserved allograft parenchyma than nondonor hyporesponders.

Moreover, CD3 and FOXP3 staining was blinded evaluated in tubulointerstitial cell infiltrates as markers of T and Treg cells, respectively (Fig. 9, A and B). We observed a significantly higher FOXP3+/CD3+ ratio in donor-specific hyporesponders than in nondonor hyporesponder patients (Fig. 9C). Also, the majority of FOXP3+ cells were CD4+ and CD25+. However, double-labeling could identify some FOXP3+CD8+ cells (Fig. 10D).

Discussion
The aim of this pilot study was to investigate whether an immunosuppressive protocol with protolerogenic properties could induce donor-specific hyporesponsiveness. Herein, we have shown that donor-specific hyporesponsiveness achievement is feasible and may be maintained over time, even in patients who suffered from acute rejection.

Our protocol provided excellent graft and patient survival, favorable graft function and fully acceptable safety profile. There are previous experiences exploring protolerogenic and nonnephrotoxic immunosuppressive strategies based on SRL. In fact, Knechtle et al. (15) using alemtuzumab and SRL found a high incidence of early humoral acute rejection. More recently, Flechner et al. (44) reported the results of an immunosuppressive protocol based on alemtuzumab, SRL and MMF. They described an acute rejection rate of 36%, similar to ours, although some events were Ab-mediated. Thus, the low rATG dose used in our protocol could explain both the slightly high acute rejection rate and the low incidence of over-immunosuppression-related complications.

Importantly, we found that highly pretransplant donor-specific T cell alloreactive patients were at risk for developing acute rejection whereas those non-T cell alloreactive were not. Our finding, in this CNI and steroid-free immunosuppressive protocol is in agreement with other groups (29–32) that showed the deleterious impact of high donor-specific cellular alloresponse pretransplantation on...
graft outcome in CNI-treated patients. Therefore, the assessment of pretransplant donor-specific cellular alloreactivity seems to be an important tool to discriminate those patients at risk for developing acute rejection after transplantation.

Recently, it has been shown that more than just acting as a T cell depletion agent, rATG is able to induce a significant increase of Treg in vitro (25), although it has been suggested that Tregs are quite sensitive to elimination by depleting Abs (45). However, it has also pointed out that the relative resistance of Tregs to apoptosis can promote tolerance through preferential depletion of T-effector cells (46, 47). In contrast, optimal concentrations of rATG for in vitro generation of Tregs appear to occur at significantly lower levels than those achieved in serum after treatment with standard rATG doses in vivo (42, 48). Furthermore, SRL preferentially inhibits growth of T effector cells stimulated in vitro, thus favoring the out-growth of FOXP3+ Tregs (24, 26). In agreement with all these data, in our study we found a transient T cell apoptotic effect mainly restricted among the CD8+ with all these data, in our study we found a transient T cell apoptosis that is not due to a decrease in the number of Tregs but rather to their selective depletion. Furthermore, it is to note that in contrast to other lymphocyte subset populations, which decreased transiently in peripheral blood, the T cell CD4+CD25+high subset significantly increased after transplantation. Thus, the low and short rATG doses given in combination with SRL could explain to some extent these results. It could be argued that higher doses of rATG induction or an initial introduction of a CNI could have minimized acute rejection, as though it seems memory T cells are more susceptible to CNI, especially to TAC (49). However, whether these or other alternative approaches to efficiently prevent acute rejection fit with the induction of a tolerogenic state deserves further investigation.

Here, we show that nearly two-thirds of the studied patients achieved donor-specific hyporesponsiveness at 6 mo and that it was maintained at 24 mo. Interestingly, among donor-specific hyporesponders, two had previously experienced acute rejection and two had had some borderline changes in renal biopsies performed early after transplantation. Regarding their immunosuppression, all donor-specific hyporesponder cases were on SRL, suggesting that the maintenance of SRL seems to facilitate the achievement of this permissive immune state, despite the occurrence of previous acute rejection. However, because patients on TAC had severe acute rejection, it cannot be excluded that these patients were indeed more resistant to become donor-specific hyporesponders.

In this study, we have shown that the achievement of donor-specific hyporesponsiveness under rATG, SRL, and MMF seems to be driven by the presence of naturally occurring Tregs with functional suppressive activity. In fact, we have shown that the donor-specific alloresponsiveness could be recovered after removal of Tregs from donor-specific hyporesponder patients. In addition, these Tregs exhibited donor-Ag specificity, because they were capable of abrogating cytokine production against their paired donor when transferred to an allelogeneic HLA-mismatch responder individual. In accordance to our data, Noris et al. (16) recently showed a significantly higher expansion of FOXP3+ CD4+CD25+high Tregs using alemtuzumab as a T cell depletion agent followed by SRL and MMF compared with alemtuzumab, CNI, and MMF. They demonstrated that donor-specific hyporesponsiveness achievement in the SRL group was due to the expansion and suppressive activity of these Tregs, instead of the induction of an anergic state in the CNI-treated group. Moreover, some experimental data underline the importance of Tregs infiltrating the graft parenchyma to achieve transplant tolerance (50, 51). To our knowledge, we show for the first time, that donor-specific hyporesponder patients have the higher presence of FOXP3+CD4+CD25+ T cells in graft infiltrates. Therefore, our findings strongly support the notion that the Tregs population is playing an active role promoting graft acceptance (52, 53). Besides, rather than just being a static immune condition, the achievement of donor-specific hyporesponsiveness had a beneficial functional translation showed by better graft function and more preserved graft parenchyma.

In summary, we have shown that the achievement of donor-specific hyporesponsiveness is feasible after renal transplantation using rATG low-doses combined with SRL and MMF, and that is associated with enhanced Tregs both circulating in the periphery and directly recruited in the graft.

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Disclosures

The authors have no financial conflict of interest.

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