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Elevated T Regulatory Cells in Long-Term Stable Transplant Tolerance in Rhesus Macaques Induced by Anti-CD3 Immunotoxin and Deoxyspergualin

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Regulatory T cells (Tregs) are implicated in immune tolerance and are variably dependent on IL-10 for in vivo function. Brief peritransplant treatment of multiple nonhuman primates (NHP) with anti-CD3 immunotoxin and deoxyspergualin has induced stable (5–10 years) rejection-free tolerance to MHC-mismatched allografts, which associated with sustained elevations in serum IL-10. In this study, we demonstrate that resting and activated PBMC from long-term tolerant NHP recipients are biased to secrete high levels of IL-10, compared with normal NHP PBMC. Although IL-10-producing CD4+ Tregs (type 1 regulatory cells [Tr1]/IL-10 Tregs) were undetectable (<0.5%) in normal rhesus monkeys, 7.5 ± 1.7% of circulating CD4+ T cells of tolerant rhesus recipients expressed IL-10. In addition to this >15-fold increase in Tr1/IL-10 Tregs, the tolerant monkeys exhibited a nearly 3-fold increase in CD4+CD25+ Tregs, 8.1 ± 3.0% of CD4 T cells vs 2.8 ± 1.4% in normal monkeys (p < 0.02). The frequency of CD4+CD25+IL-10+ cells was elevated 5-fold in tolerant vs normal NHP (1.8 ± 0.95% vs 0.30 ± 0.14%). Rhesus CD4+CD25+ Tregs exhibited a memory phenotype, and expressed high levels of Foxp3 and CD38+CD95 compared with CD4+CD25+ T cells. Also, NHP CD4+CD25+ Tregs proliferated poorly after activation and suppressed proliferation of CD4+CD25− effector T cells, exhibiting regulatory properties similar to rodent and human CD4+CD25+ Tregs. Of note, depletion of CD4+CD25+ Tregs restored indirect pathway antidonor responses in tolerant NHP. Our study demonstrates an unexpected increase of Treg populations in tolerant NHP recipients, suggesting that these adaptations may be involved in maintenance of stable tolerance. The Journal of Immunology, 2005, 175: 8060–8068.
15-deoxyspergualin (DSG) therapy is associated with donor-specific indirect pathway unresponsiveness (31) and sustained elevated levels of serum IL-10 (32–34). We found that when tolerant recipients accepted a second donor kidney allotraft without additional immunosuppressive therapy, this was associated with an upsurge in the already elevated serum IL-10 levels (31), suggesting a role for active immune regulation in the maintenance of stable tolerance in this model. Therefore, we hypothesized that presentation of donor Ags by host APC in a milieu dominated by IL-10 may drive the generation and expansion of donor-specific Tregs that mediate stable tolerance induced by anti-CD3 IT plus DSG. In this study, we examined the frequency, phenotype, and function of Tr1/IL-10 and CD4+CD25+ Tregs in normal and long-term tolerant rhesus macaque recipients. To our knowledge, this is the first report demonstrating that CD4+CD25+ Tregs occur naturally in rhesus monkeys and that they are similar in phenotype and function to murine and human CD4+CD25+ Tregs. Notably, the frequency of Tr1/IL-10 Treg and CD4+CD25+ Treg is significantly increased in long-term tolerant rhesus recipients compared with normal cohorts. Furthermore, depletion of CD4+CD25+ Tregs uncovers the response of tolerant recipients’ T cells to cell-free donor negative accessory cells, the non-CD4 fraction was depleted of CD8+/H11022 95% and microbeads were released from CD4+CD25+ T cells were typically 95% and 85% pure, respectively (data not shown). To generate T cell negative accessory cells, the non-CD4 fraction was depleted of CD8+ cells using NHP CD8 microbeads, as recommended by the manufacturer (Miltenyi Biotec).

Flow cytometry

Rhesus whole blood, single cell suspensions of PBMC, or T cell subsets isolated from PBMC by magnetic bead sorting were stained for surface Ags and intracellular IL-10, according to standard methods. Stained samples were acquired on a Coulter Cyanomics FC 500 flow cytometer (Beckman Coulter), and analyses of events in the lymphocyte gate were performed with WinList 3D 5.0 (Verity Software House) or Cytomics RXP software (Beckman Coulter).

Quantitative RT-PCR

Total RNA or mRNA was extracted from PBMC or magnetically sorted T cell subsets using RNaseasy mini kit (Qiagen) or μMACS mRNA (Miltenyi Biotec) isolation kits, respectively, according to conditions stipulated by the manufacturer. First strand cDNA was generated using random hexamers in the Superscript first strand synthesis system, as per the manufacturer’s protocol (Invitrogen Life Technologies). mRNA or cDNA, gene-specific primers, fluorogenic probes, and Universal MasterMix (Applied Biosystems) were combined in a final volume of 20 μl. Rhesus pryrurate dehydrogenase (PDH) forward primer, 5’-GGTTGCAAGAGGCCGCTT-3’; rhesus PDH reverse primer, 5’-GCTTCTTCCATCTACGATTTT-3’; rhesus PDH probe, FAM-5’-CAGCAACTGTCACCTGGCCAGCAG-3’ TAMRA (38). We cloned and sequenced rhesus macaque Foxp3 (G. Balgansuren, et al., manuscript in preparation) and submitted the sequence of a 400-base-long fragment to GenBank® for design and synthesis of a 20X primer (forward and reverse) and FAM-5’-CATTGCCCTGGGACATCCAGTGC-3’ probe, FAM-5’-CATTGCCCTGGGACATCCAGTGC-3’ sequence. The primers and FAM labeled forward primer, 5’-CCTTGTGACAAGCTGAGTACG-3’ were synthesis of a 20X primer (forward and reverse) and FAM-5’-CATTGCCCTGGGACATCCAGTGC-3’ probe, FAM-5’-CATTGCCCTGGGACATCCAGTGC-3’ and sequences were amplified from the diluted T cell subset) were incubated with mature dendritic cells (DC) were harvested and pulsed with cell-free autologous or donor Ags. DC were then cultured for an additional 48 h with a cytokine (i.e., depleted of CD4+CD25+ T cells) or both subsets (3 × 10^6 to 1 × 10^6/well) were stimulated with plate-bound anti-CD3 (FN18, 10 μg/ml) and soluble anti-CD28 (CD28.2, 10 μg/ml). After 5 days, [H]thymidine (1 μCi/well) was added, and cells were harvested 16 h later. Scintillation counting was done to measure proliferation, as described previously (37).

Indirect MLR was done essentially as described previously (31), except that total CD4+ and CD4+CD25+ T cells were used as responders. Briefly, monocytes were isolated from PBMC by plastic adherence and cultured in recombinant human GM-CSF and IL-4 for 7 days. After 7 days, immature dendritic cells (DC) were harvested and pulsed with cell-free autologous or donor Ags. DC were then cultured for an additional 48 h with a cytokine mixture to induce maturation. Freshly isolated CD4+ or CD4+CD25+ T cells (i.e., depleted of CD4+CD25+ subset) were incubated with mature DC at a ratio of 5:1. Proliferation was assessed, as described above, after 5 days’ culture.

Statistical analyses

Comparisons were done using unpaired t tests (GraphPad). Statistical significance was assigned when p value was <0.05.

Results

Resting and activated PBMC from long-term tolerant recipients are high IL-10 secretors

We reported previously that long-term, rejection-free tolerance in NHP recipients treated briefly with anti-CD3 IT plus DSG associates strongly with elevated serum levels of IL-10 (33, 34). Tolerant rhesus recipients induced with anti-CD3 IT plus DSG are unable to respond to donor Ags presented by the indirect pathway, but are otherwise fully immunocompetent (31, 32). The sustained
increase in serum IL-10, an immunosuppressive cytokine, in immunocompetent recipients suggests a role for immunoregulation in induction and/or maintenance of anti-CD3 IT plus DSG-induced tolerance. In this study, we determined serum IL-10 levels in tolerant NHP recipients >5 years after transplantation. As shown in Table I, serum IL-10 levels ranged from 71.4 to 872 pg/ml (321.0 pg/ml ± 99.6 SEM) in long-term tolerant recipients (n = 8). In contrast, IL-10 was below the assay detection limit (<15.6 pg/ml) in all normal monkeys (n = 12) examined, p < 0.02 for tolerant vs normal monkeys. Thus, serum IL-10 is significantly elevated in tolerant rhesus recipients >5 years after induction with anti-CD3 IT plus DSG therapy.

Next, we compared the production of IL-10 and proinflammatory cytokines by resting and activated PBMC from normal rhesus macaques vs long-term tolerant recipients. In the resting state, PBMC from tolerant recipients secreted more IL-10 than PBMC from normal monkeys throughout the culture period (p < 0.02, Fig. 1A). A similar trend was observed following TCR-mediated activation of the cells (Fig. 1B), although the difference between normal and tolerant monkeys was not statistically significant (p = 0.05). Tolerant monkeys’ PBMC secreted markedly more IL-10 into the culture supernatant than normal monkeys following polyclonal stimulation with PMA and ionomycin D (Fig. 1C). Thus, resting and activated PBMC from long-term tolerant rhesus recipients secrete more IL-10 than PBMC from rhesus cells from normal cohorts. These findings are consistent with previous reports (33), which suggested that a mean 7.5 ± 1.7% of CD4+CD25+ T cells are enriched in peripheral blood cells from long-term tolerant NHP recipients and show that peripheral blood cells are a source of the high IL-10 production.

Within 1 mo following induction therapy with anti-CD3 IT and DSG, long-term tolerance can be predicted accurately on the basis of elevated serum IL-10 and low to undetectable serum levels of proinflammatory cytokines (34). Consistent with this, polyclonal stimulation of long-term tolerant recipients’ PBMC in vitro elicited high levels of IL-10 and low amounts of the proinflammatory cytokine TNF-α in the culture supernatant (Fig. 2A). In contrast, PBMC from normal monkeys secreted higher amounts of TNF-α than IL-10 after activation with PMA/ionomycin D (Fig. 2B). Therefore, PBMC from tolerant, rejection-free NHP are biased to secrete IL-10 with minimal proinflammatory cytokine production after polyclonal activation. These data suggested involvement of an IL-10-driven immunoregulatory mechanism in maintenance of stable tolerance induced by anti-CD3 IT plus DSG therapy.

Presence of CD4+CD25+ Tr1/IL-10 Tregs in long-term tolerant NHP recipients. To determine the peripheral blood source(s) of IL-10 in long-term tolerant NHP, we stained PBMC for surface Ags and intracellular IL-10 by FACS analysis (Fig. 2C). We found that peripheral blood CD4+CD25+ T cells in long-term tolerant rhesus T cells produce IL-10 (Fig. 3 and data not shown). As expected, we estimate that a mean 7.5 ± 1.7% of CD4+CD25+ T cells from long-term tolerant NHP are Tr1/IL-10 Tregs (i.e., CD4+CD25+ T cells that secrete high levels of IL-10 and low amounts of other cytokines) as compared with <0.5% in peripheral blood of normal macaques (Fig. 3). Thus, Tr1/IL-10 Tregs are expanded in the peripheral blood of drug-free long-term tolerant NHP, years after peritransplant anti-CD3 IT plus DSG therapy.

The frequency of CD4+CD25+ T cells is elevated in long-term tolerant rhesus recipients

Immune regulation mediated by CD4+CD25+ Tregs has emerged as a major mechanism of self and transplantation tolerance (6).

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**Table I. Sustained elevation of IL-10 in the serum of tolerant NHP >5 years posttransplantation**

<table>
<thead>
<tr>
<th>Monkeys</th>
<th>Serum IL-10 (pg/ml)</th>
<th>Mean Serum IL-10 (pg/ml) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (n = 12)</td>
<td>Below detection level (15.6) in all tested</td>
<td>NA</td>
</tr>
<tr>
<td>Tolerant (n = 8)</td>
<td>71.4, 572.5, 274.0, 91.6, 380.0, 228.0, 872.0, 78.2</td>
<td>321.0 ± 99.6</td>
</tr>
</tbody>
</table>

*Serum samples were collected from normal monkeys, and tolerant NHP recipients >5 years after transplantation. IL-10 levels were determined by ELISA. NA, not applicable.*
Recent reports indicate that indirect recognition of donor alloantigens may be involved in the generation of donor-specific Tregs (39, 40). Our previous finding of specific indirect pathway hyporesponsiveness to donor Ags in long-term tolerant NHP recipients led us to hypothesize that Tregs might have a role in induction and/or maintenance of stable tolerance in our model. Because in vitro properties of CD4⁺CD25⁺ Tregs have not been described in NHP, we first characterized this population in normal animals. As shown in Fig. 4A, 2.8% ± 1.4 (range 1.3–5.7%, n = 9) of CD4⁺ T cells in the peripheral blood of normal rhesus monkeys coexpressed CD25. In normal monkey lymph node and spleen, 13.7% ± 2.1 and 18.8% ± 5.6 of CD4⁺ T cells were CD25⁺, respectively (n = 3; data not shown). Thus, like mice and humans, normal monkeys have naturally occurring CD4⁺CD25⁺ Tregs. Also, in normal monkeys, the frequency of these CD4⁺CD25⁺ Tregs is significantly higher in secondary lymphoid organs compared with peripheral blood.

In the case of long-term tolerant NHP recipients, the frequency of CD4⁺CD25⁺ T cells (Fig. 4A) was increased to 8.6% ± 3.1 (range 5.3–15.3%, n = 8, p = 0.002 vs normals). Intracellular IL-10 expression in CD4⁺CD25⁺ T cells from tolerant monkeys was 1.8 ± 0.9%, 5-fold higher than that observed in the normal cohorts (0.4 ± 0.2%, p < 0.001, Fig. 4B). In contrast to the elevated numbers of Tr1/IL-10 Tregs and CD4⁺CD25⁺ Tregs, another regulatory T cell population, Vα24δ1 invariable NKT cells were decreased in the circulation of long-term tolerant monkeys compared with normal controls (0.04% ± 0.04 vs 0.3% ± 0.25, p = 0.0001, Fig. 4C). Thus, Tr1/IL-10 and CD4⁺CD25⁺ Tregs are specifically elevated in the peripheral blood of long-term tolerant NHP recipients.

Rhesus CD4⁺CD25⁺ T cells exhibit a memory phenotype

To further characterize the phenotype of rhesus peripheral blood CD4⁺CD25⁺ T cells, we used four-color flow cytometry. The representative data in Fig. 5, A and B, show that the majority of CD4⁺CD25⁺ T cells in the peripheral blood are negative for CD69, and therefore are not recently activated T cells. In contrast, most of the CD4⁺CD25⁺ T cells coexpressed CD95 (Fig. 5C), indicating a memory phenotype (41). Also, a majority of the
CD4\(^+\)CD25\(^+\) T cells exhibited a CD28\(^+\) phenotype (Fig. 5D), suggesting they are central memory cells. Consistent with this finding, real-time quantitative PCR analysis demonstrated 3-fold higher CCR7 expression in CD4\(^+\)CD25\(^+\) T cells than CD4\(^+\)CD25\(^-\) T cells (data not shown). Therefore, in line with murine and human CD4\(^+\)CD25\(^+\) Tregs (42, 43), rhesus CD4\(^+\)CD25\(^+\) T cells exhibit a heterogeneous phenotype in which central memory cells are prominent.

**Expression of Foxp3 and CTLA-4 by rhesus CD4\(^+\)CD25\(^+\) T cells**

Foxp3 is expressed by CD4\(^+\)CD25\(^+\) Tregs in mice and humans (15, 16, 44). Thus, we examined the relative expression of Foxp3 mRNA by rhesus CD4\(^+\)CD25\(^+\) and CD4\(^+\)CD25\(^-\) T cells. Our results demonstrate that freshly isolated CD4\(^+\)CD25\(^+\) T cells from long-term tolerant rhesus recipients and normal cohorts expressed high levels of Foxp3 mRNA. In contrast, freshly isolated CD4\(^+\)CD25\(^-\) T, CD8\(^+\), CD14\(^+\), and CD20\(^+\) cells expressed low levels of Foxp3 message that were barely detectable (Fig. 6, A and B). Although the frequency of CD4\(^+\)CD25\(^+\) T cells was significantly elevated in tolerant rhesus recipients compared with normal monkeys, there was no significant difference in the relative Foxp3 mRNA levels within the CD4\(^+\)CD25\(^+\) T cell population from tolerant and normal monkeys (Fig. 6A). Thus, Foxp3, per se, is not up-regulated in tolerant recipients.

CD4\(^+\)CD25\(^+\) Tregs have been demonstrated to express higher levels of intracellular CTLA-4 than CD4\(^+\)CD25\(^-\) T cells (7, 45). Fig. 6C demonstrates that long-term tolerant and normal monkeys’ CD4\(^+\)CD25\(^+\) T cells also express 3- to 6-fold more CTLA-4 mRNA than CD4\(^+\)CD25\(^-\) T cells. Thus, our findings indicate that rhesus CD4\(^+\)CD25\(^+\) T cells, similar to rodent and human CD4\(^+\)CD25\(^+\) Tregs, express higher levels of CTLA-4 mRNA relative to CD4\(^+\)CD25\(^-\) T cells.

**Rhesus CD4\(^+\)CD25\(^+\) T cells exhibit regulatory activity**

Following TCR-mediated stimulation in vitro, rodent and human CD4\(^+\)CD25\(^+\) Tregs are anergic and suppress proliferation of CD4\(^+\)CD25\(^-\) T cells (42). We examined rhesus CD4\(^+\)CD25\(^+\) T cells isolated from normal and tolerant monkeys for evidence of regulatory activity in vitro. In contrast to the strong proliferation of CD4\(^+\)CD25\(^-\) T cells observed after TCR-mediated polyclonal stimulation, expansion of rhesus CD4\(^+\)CD25\(^+\) T cells was reduced, a mean 52% of that of the CD4\(^+\)CD25\(^-\) T cells (range 21–79% in 6 of 6 experiments). The representative experiments in Fig. 7, A and B show the relative suppression. We also demonstrate that coculturing CD4\(^+\)CD25\(^+\) T cells with autologous CD4\(^+\)CD25\(^-\) T cells suppressed proliferation of the latter (range 43–65% suppression in 3 of 6 experiments). Overall, there was no significant difference in the regulatory activity of normal and tolerant monkeys’ CD4\(^+\)CD25\(^+\) T cells. These findings confirm that rhesus macaque CD4\(^+\)CD25\(^+\) cells exhibit regulatory activity in vitro.

**Indirect pathway antidonor responses are restored by depletion of CD4\(^+\)CD25\(^+\) Tregs**

We demonstrated previously that long-term NHP tolerance induced by peritransplant anti-CD3 IT plus DSG therapy is associated with unresponsiveness to donor Ags presented by the indirect pathway. In contrast, T cells from tolerant recipients exhibited robust responses to third party Ags presented by the indirect pathway, as well as to donor Ags presented by the direct pathway (31). In this study, we compared the ability of tolerant recipients’ total CD4\(^+\) or CD4\(^+\)CD25\(^+\) T cells to respond to cell-free donor Ags presented by autologous monocyte-derived DC. The representative data in Fig. 7C demonstrate that the response of total CD4\(^+\) T cells to autologous and donor cell-free Ags is similar. In contrast, depletion of CD4\(^+\)CD25\(^+\) Tregs resulted in significantly greater proliferation of CD4\(^+\)CD25\(^+\) effector cells to cell-free donor Ags relative to autologous Ags (p = 0.005). These results suggest that CD4\(^+\)CD25\(^+\) Tregs may contribute to the suppression of indirect pathway responses to cell-free donor Ags.

**Discussion**

Our previous studies demonstrated that brief peritransplant treatment of rhesus macaque recipients with two doses of anti-CD3 IT and a 14-day course of DSG resulted in stable, chronic rejection-free tolerance to MHC-mismatched kidney and islet allografts persisting for >5 years in many animals. Long-term tolerance in this clinically relevant NHP model associates strongly with early and sustained increases in serum IL-10 levels (33, 34). The specificity of tolerance has been demonstrated, with tolerant recipients accepting a second kidney allograft from the original donor without additional immunosuppressive therapy, while rejecting third party grafts in normal first set time. Of note, an upsurge in the elevated serum IL-10 levels occurred following transplantation of a second
graft from the original donor without enhancing an active IL-10-mediated immunoregulatory mechanism (24, 25, 31). These in vivo findings implicate active immunoregulation in the maintenance of long-term rejection-free tolerance in this NHP model.

The data presented in this work extend our previous findings. We have shown that serum IL-10 levels remain elevated in long-term tolerant recipients >5 years posttransplantation. In addition, at the cellular level, resting and activated PBMC from tolerant monkeys are high IL-10 secretors and are biased to secrete higher levels of IL-10 than TNF-α, compared with cells from normal animals. Thus, our in vitro data are in agreement with the in vivo finding that stable, drug-free tolerance associates with high levels of IL-10 than TNF-α, compared with cells from normal animals. Our in vitro data are in agreement with the in vivo finding that stable, drug-free tolerance associates with high levels of IL-10 secretors and is biased to secrete higher levels of IL-10 than TNF-α, compared with cells from normal animals.

The most notable finding in this study is that peripheral blood of long-term tolerant rhesus recipients contains an elevated frequency of Tr1/IL-10 and CD4+CD25+ Tregs relative to normal animals. Examination of our most tolerant islet recipients (up to 12 mo posttransplantation) indicates that the elevation in CD4+CD25+ Treg frequency is observed as early as 2 mo posttransplantation and is sustained thereafter. In contrast, animals that reject their allografts exhibit a short-lived increase in CD4+CD25+ T cells within the first month, followed by rapid decline toward pretransplant levels (data not shown). Thus, the data suggest that adaptations leading to expansion of Tregs may be

**FIGURE 5.** Rhesus CD4+CD25+ T cells display a memory phenotype. PBMC were stained for CD4 and CD25 in combination with CD69 or CD95 or CD28. Gated CD4+CD25+ T cells (A) were analyzed for expression of CD69 (B) or CD28 (C) or CD95 (D). Similar results were obtained when this experiment was performed in >8 tolerant animals and repeated several times in the same animal.
involved in the genesis and maintenance of the durable tolerance induced by anti-CD3 IT plus DSG therapy.

Tregs have been demonstrated to prevent allograft rejection and to mediate transplantation tolerance in experimental models (6, 47, 52). Information on the role of NHP Tregs in transplantation is scant, although there is growing information on the role of Tregs in human transplantation. Meloni et al. (24) reported recently that the frequency of peripheral blood CD4$^+$CD25$^+$ Tregs in lung transplant recipients in stable condition was similar to those in normal subjects. In contrast, CD4$^+$CD25$^+$ Treg frequency was significantly lower in lung transplant recipients experiencing chronic rejection compared with normal individuals, suggesting that maintenance of this population in recipients with chronic immunosuppressive therapy is an important variable for successful graft outcome. In our studies, the frequency of CD4$^+$CD25$^+$ and Tr1/IL-10 Tregs was 3- and 15-fold higher in tolerant recipients than normal monkeys, respectively. The difference between our results in NHP and those obtained in the clinical study may be due to the fact that the lung transplant recipients were on immunosuppressive drugs, while tolerant monkeys did not receive immunosuppressive therapy after the initial 2-wk induction period. This viewpoint is bolstered by the report of Li et al. (53), showing an
increased frequency of CD4^+CD25^{high} cells in the peripheral blood of liver transplant patients who maintained graft function after discontinuance of immunosuppressive therapy. Unlike the tolerant human recipients, our tolerant monkeys showed a significant elevation in CD4^+CD25^"Tregs regardless of the intensity of CD25^{expression}. Additionally, the monkeys showed a greater difference in Treg frequency between tolerant recipients and normal cohorts than did those in the human study. The relatively greater increase in frequency in our tolerant monkeys might be related to the longer duration of immunosuppressive drug therapy (1–6 years in humans vs 2 wk in NHP). Also, the rhesus MHC is more complicated than the human MHC, the former having a far greater number of MHC genes per locus (54). The recipient/donor pairs used in our study had multiple MHC mismatches, as defined by rhesus-specific PCR sequence-specific primers for class II DR loci (31, 55), class IA (56), and the class IB locus (our unpublished data). Consequently, the extent of mismatches in our recipient/donor pairs generally exceed those in clinical transplantation and may be part of the reason Treg frequencies are elevated in tolerant rhesus recipients. Relevant to our findings, ~50% of human kidney allograft recipients with indirect pathway donor-specific hyporesponsiveness exhibited increased responses against donor immunogenic peptides after depletion of CD4^+CD25^"T cells (23). Stable tolerance in our preclinical model is associated with donor-specific indirect pathway hyporesponsiveness (31). In this study, we have demonstrated that long-term tolerance also associates with elevated frequencies of Tri1/IL-10 and CD4^+CD25^"Tregs. Furthermore, depletion of CD4^+CD25^"Tregs alleviated suppression of indirect pathway antidonor responses. Frozen normal, pretransplant cells from the long-term tolerant rhesus macaques are no longer available, so we are unable to directly test for suppression of indirect pathway antidonor responses. However, our data suggest that donor-specific CD4 regulatory cells promote allograft survival and suggest the potential of developing donor MHC matched CD4 regulatory cells for immunologic self-tolerance.

Intragraft CD4^+ TGF-β-producing regulatory T cells are restricted to recipients with metastable tolerance following withdrawal of prolonged immunosuppressive therapy (57). Breakdown of tolerance was preceded by disappearance of these from graft biopsies. In contrast, stable, rejection-free tolerance induced in NHP following brief therapy with anti-CD3 IT plus DSG is associated with the absence of TGF-β1 from graft biopsies (32). Thus, the mechanisms underpinning long-term durable NHP tolerance (32, 34, 46) appear to be distinct from NHP metastable tolerance with approaching chronic rejection (57–59).

Acknowledgments

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Disclosures

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Letter of Retraction


We have learned that assessment of kidney allograft function included several animals that possessed an intrinsic kidney and had not undergone bilateral nephrectomies as reported. Consequently, my coauthors and I think that data and conclusions relating to the duration of operational tolerance were overestimated and invalid.

Although, (1) the in vitro cellular and molecular characterization of rhesus regulatory T cells subsets is entirely valid, and (2) the highest frequency of CD4\(^+\)CD25\(^+\) T regulatory cells we reported was in recipient 98R317, who underwent bilateral nephrectomy and is currently functioning 5.4 years on his kidney allograft, the relevance of the findings to stable kidney allograft tolerance remains unproven at this point. Thus, in the interest of scientific accuracy and ethical standards, we retract the article cited above.

We express regret to all in the scientific community whose work on tolerance might have been impacted by this unfortunate error.

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