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Metastable Tolerance to Rhesus Monkey Renal Transplants Is Correlated with Allograft TGF-β1⁺CD4⁺ T Regulatory Cell Infiltrates


Approaches that prevent acute rejection of renal transplants in a rhesus monkey model were studied to determine a common mechanism of acceptance. After withdrawal of immunosuppression, all 14 monkeys retained normal allograft function for >6 mo. Of these, nine rejected their renal allograft during the study, and five maintained normal function throughout the study period. The appearance of TGF-β1⁺ interstitial mononuclear cells in the graft coincided with a nonrejection histology, whereas the absence/disappearance of these cells was observed with the onset of rejection. Analysis with a variety of TGF-β1-reactive Abs indicated that the tolerance-associated infiltrates expressed the large latent complex form of TGF-β1. Peripheral leukocytes from rejecting monkeys lacking TGF-β1⁺ allograft infiltrates responded strongly to donor Ags in delayed-type hypersensitivity transvivo assays. In contrast, allograft acceptors with TGF-β1⁺ infiltrates demonstrated a much weaker peripheral delayed-type hypersensitivity response to donor alloantigens (p < 0.01 vs rejectors), which could be restored by Abs that either neutralized active TGF-β1 or blocked its conversion from latent to active form. Anti-IL-10 Abs had no restorative effect. Accepted allografts had CD8⁺ and CD4⁺ interstitial T cell infiltrates, but only the CD4⁺ subset included cells costaining for TGF-β1. Our data support the hypothesis that the recruitment of CD4⁺ T regulatory cells to the allograft interstitium is a final common pathway for metastable renal transplant tolerance in a non-human primate model. The Journal of Immunology, 2004, 172: 5753–5764.

The success of contemporary clinical transplantation has been fueled in large part by the array of pharmacologic agents that are taken lifelong by the recipient to prevent rejection of the allograft. Without chronic administration of such agents, it is expected that cell- and/or Ab-mediated immune rejection of the allograft will occur. When rejection does not happen immediately, however, periods of long term allograft acceptance, or metastable tolerance (1), may ensue.

Studies in rodent allograft models have suggested that active immune regulation plays an important role in maintaining metastable allograft tolerance. Allograft acceptors display the characteristic feature of linked suppression (2), in which the delayed-type hypersensitivity (DTH)³ response to an unrelated third-party Ag such as tetanus toxoid (TT) is suppressed when the tolerogen or specific donor Ag is present (3). This phenomenon was due in part to donor Ag-triggered release of TGF-β and/or IL-10 (4). These features of donor-Ag-linked, TGF-β and/or IL-10-dependent suppression of DTH were reproduced in a small group of human kidney and liver allograft acceptors using a trans-vivo DTH assay (5), suggesting an evolutionarily conserved pathway of peripheral tolerance. The identification of CD4⁺CD25⁺ T regulatory cells in mice (6–9), rats (9), and humans (11) provided a candidate T cell for mediation of allospecific immune regulation. However, the tissue localization, Ag specificity, and mode of action of T regulatory cells are still controversial (12). In particular, there are conflicting data concerning the requirement for TGF-β1 in the regulation mediated by CD4⁺CD25⁺ T cells, with evidence being provided both for (7, 13) and against (14).

We wished to test the hypothesis that T regulatory cells specific for donor alloantigens develop in the periphery in a manner that induces peripheral induction in non-human primates and localize to the graft, and that this tissue localization is essential for maintaining metastable tolerance. We therefore prevented acute rejection graft loss using T cell depletion (15, 16), drug treatment with or without donor-specific transfusion (17, 18), or costimulation blockade (19) in a series of rhesus monkey kidney transplant recipients that were then carefully monitored. Allograft biopsies along with peripheral lymph node and blood samples were obtained over a 1- to 4-year period and analyzed for the presence of TGF-β1-producing T regulatory cells in the graft. The graft was correlated with the absence of rejection. We report in this study that following either mode of induction, renal allograft acceptance was correlated with the presence of both CD4⁺ T and non-T cells expressing latent complex TGF-β1 in the graft interstitium and with a TGF-β1-dependent, regulated DTH response to donor alloantigen in the periphery.
Materials and Methods

Experimental animals

CB-17 SCID mice were purchased from Harlan Sprague Dawley (Indianapolis, IN). Outbred juvenile rhesus monkeys were obtained from University of Wisconsin Regional Primate Center (Madison, WI), Covance (Allentown, TX), and LABS (Yemassee, SC). All animals were housed and treated in accordance with guidelines outlined by University of Wisconsin and National Institutes of Health.

Monkey renal allograft post-transplant monitoring

Monkeys (n = 14; see Table I) underwent heterotopic renal allografts from donors mismatched for one or two MHC class II DRB loci, and bilateral native nephrectomy was performed as previously described (20–22). For immunosuppression, monkeys were treated with T cell deletion using an anti-CD3 immunotoxin in the presence of conventional immunosuppression such as cyclosporine A or mycophenolate mofetil (15, 18), costimulation blockade using an anti-CD40 ligand mAb (19), and conventional immunosuppression with or without donor-specific transfusion (17). All monkeys in the study had been off all immunosuppressive conventional or experimental medication for ≥6 mo. Allograft function was determined by serum creatinine (sCr) level, histological evidence of rejection on biopsy, and clinical condition of the animal. In this study rejection was defined as a rise in sCr of ≥1.5 mg/dl and histological evidence of rejection in the biopsy (23).

Immunization with TT

Monkeys were immunized i.m. with alum-absorbed TT (1.5 Lf/injection) either pretransplant (n = 5) or post-transplant between days 170 and 390 (n = 9). A secondary immunization was performed 2 wk after the primary injection. Evidence of an immune response to TT was determined 2–3 wk later by measurement of anti-TT IgG in serum as previously described (15). Two monkeys (r142 and YM) were not immunized with TT or tested for DTH.

Antibodies

Neutralizing polyclonal rabbit anti-human TGF-β Ab, neutralizing polyclonal goat anti-human IL-10 Ab, and paired normal IgG controls were all obtained from R&D Systems (Minneapolis, MN). In addition, neutralizing rat anti-human IL-10 mAb and normal rat IgG were purchased from BD PharMingen (San Diego, CA), as this Ab is also known to cross-react with rhesus IL-10 (24). For neutralization studies, 25 µg of each Ab was used per injection site. The following mouse mAbs were also used in DTH experiments: TSP Ab-4, specific for human thrombospondin-1 (Neomarkers, Fremont, CA); and TB21, specific for human TGF-β1 (BioSource International, Camarillo, CA). For immunohistochemistry analysis, we used a 1/75 dilution of TB21, a 1/8 dilution of the anti-TGF-β1/β2/β3 mouse monoclonal ID11 (R&D Systems), or a 1/15 dilution of a biotinylated goat Ab to the latency-associated peptide (LAP) of human TGF-β1 (R&D Systems). Secondary Abs were either goat anti-mouse IgG-HRP or goat anti-rabbit IgG-HRP.

Trans-vivo DTH assay

The trans-vivo DTH assay was described previously (5, 26). Briefly, 7–10 × 10^8 PBMC, LNC, or SPL were coincubated with Ags and/or Abs into the footpads of naive SCID mice in a total volume of 20–35 µl. When the number of cells isolated from a given blood sample was inadequate, cells from the same animal at two different time points <3 wk apart, but with the same state of graft function, were pooled and used for a single experiment. Ag-induced swelling was measured after 24 h using a dial thickness gauge. TGF-β1 was used as a positive control recall Ag and to test for linked suppression in the presence of donor Ags. DTH reactivity was measured as the change in thickness, using units of 10^(-4) inches over the pre-injection reading. Typical background swelling for the injection of responder cells, Ab, or Ag alone was 10–25 × 10^(-4) inches; by subtracting this value from a test value, a corrected value, or net DTH swelling in response to Ag or Ag/Ab mixture, was obtained.

H&E histopathology and TGF-β1 immunohistochemistry

Core renal biopsies containing at least one vessel and one glomerulus were considered adequate for evaluation. We followed the definitions of Banff-97 criteria (23) to score acute (tubulitis, intimal arteritis, interstitial inflammation, and glomerulitis) and chronic (tubular atrophy, interstitial fibrosis, and chronic glomerulopathy) allograft rejection changes in H&E-stained, paraffin-embedded sections. Paraffin-embodied or frozen sections of biopsy sections were immunolabeled using mAbs against TGF-β1 or with a polyclonal Ab to human LAP. For paraffin sections only, the heat-induced epitope retrieval method (27) using a pH 8.0 EDTA buffer (Borg; Biocare Medical, Walnut Creek, CA) was used. The positive immunostaining of interstitial cell-associated TGF-β1 was scored in a blinded fashion. The ratio of TB21^+ TGF-β1^- cells per renal tubule was obtained as an average.

Table I. Survival of renal allograft recipient monkeys without continuous immunosuppression

<table>
<thead>
<tr>
<th>Monkey (Ref no.)</th>
<th>Treatment</th>
<th>DR Matching</th>
<th>GST (days)</th>
<th>Days IS</th>
<th>sCr (mg/dl) at</th>
<th>Recent or Final Banff Score</th>
<th>Donor Skin Acceptance</th>
</tr>
</thead>
<tbody>
<tr>
<td>YM (17)</td>
<td>DST+IS</td>
<td>+</td>
<td>&gt;8000</td>
<td>&gt;7635</td>
<td>0.9</td>
<td>1.2</td>
<td>Normal</td>
</tr>
<tr>
<td>EEF (15,16)</td>
<td>TCD+IS</td>
<td>–</td>
<td>&gt;2240</td>
<td>&gt;2238</td>
<td>0.9</td>
<td>1.2</td>
<td>Susp</td>
</tr>
<tr>
<td>AV67</td>
<td>IS</td>
<td>+</td>
<td>&gt;1464</td>
<td>&gt;1405</td>
<td>0.7</td>
<td>1.5</td>
<td>Normal</td>
</tr>
<tr>
<td>98D032</td>
<td>IS</td>
<td>+</td>
<td>&gt;1282</td>
<td>&gt;1223</td>
<td>0.9</td>
<td>0.9</td>
<td>Susp/CR1</td>
</tr>
<tr>
<td>98D349 (19)</td>
<td>IS</td>
<td>+</td>
<td>&gt;1282</td>
<td>&gt;1223</td>
<td>0.6</td>
<td>0.7</td>
<td>Normal</td>
</tr>
<tr>
<td>AW13 (16)</td>
<td>TCD+IS</td>
<td>+</td>
<td>1260</td>
<td>993</td>
<td>1.0</td>
<td>2.0</td>
<td>Susp</td>
</tr>
<tr>
<td>r142 (18)</td>
<td>DST+TCD+IS</td>
<td>+</td>
<td>113</td>
<td>993</td>
<td>0.8</td>
<td>0.9</td>
<td>Susp</td>
</tr>
<tr>
<td>97D468 (19)</td>
<td>CoSB+IS</td>
<td>–</td>
<td>623</td>
<td>393</td>
<td>0.8</td>
<td>1.1</td>
<td>Rej/1</td>
</tr>
<tr>
<td>97D285 (19)</td>
<td>CoSB+IS</td>
<td>–</td>
<td>623</td>
<td>393</td>
<td>0.8</td>
<td>1.1</td>
<td>Rej/1</td>
</tr>
<tr>
<td>AV02 (16)</td>
<td>TCD+IS</td>
<td>–</td>
<td>745</td>
<td>741</td>
<td>0.8</td>
<td>Rej</td>
<td>Rej/2</td>
</tr>
<tr>
<td>96D306 (16)</td>
<td>TCD+IS</td>
<td>–</td>
<td>712</td>
<td>709</td>
<td>1.7</td>
<td>Rej</td>
<td>Rej/2</td>
</tr>
<tr>
<td>97D270</td>
<td>TCD+IS</td>
<td>–</td>
<td>582</td>
<td>520</td>
<td>1.2</td>
<td>Rej</td>
<td>Rej/2</td>
</tr>
<tr>
<td>AV98 (19)</td>
<td>CoSB+IS</td>
<td>–</td>
<td>479</td>
<td>249</td>
<td>1.6</td>
<td>Rej</td>
<td>Rej/2</td>
</tr>
<tr>
<td>97D140</td>
<td>TCD+IS</td>
<td>–</td>
<td>350</td>
<td>288</td>
<td>1.6</td>
<td>Rej</td>
<td>Rej/2</td>
</tr>
</tbody>
</table>

a DST, Donor-specific transfusion; TCD, T cell deletion; IS, conventional immunosuppression; CoSB, costimulation blockade.

b Donor-recipient pairs were matched for one DRβ1 allele (+) or were completely DR mismatched (-).
of six high power fields (×400; microscope DMLS; Leica, Deerfield, IL) per biopsy or necropsy tissue.

Double immunolabeling

Double immunoperoxidase or fluorescent immunolabeling was performed following the manufacturer’s directions (Jackson ImmunoResearch Laboratories, West Grove, PA). Briefly, paraffin-embedded tissue was deparaffinized and rehydrated, and Ag retrieval was performed with Biocare’s Borq in a Decloaking Chamber (Biocare Medical). Sections were incubated with primary Ab (TB21, Ab at 1/75 dilution) for 1 h at room temperature, and signal was detected with either a secondary tetramethylrhodamine iso-thiocyanate-labeled Ab (Alexa 546; 1/400; Molecular Probes, Eugene, OR) or secondary HRP-labeled goat anti-mouse IgG (Mach 2 polymer; Biocare Medical, Walnut Creek, CA) and diaminobenzidine chromogen (Dakocytomation California, Carpinteria, CA). To saturate any open Ag binding sites on the first secondary Ab, sections were then incubated with normal mouse serum (1/400; Jackson ImmunoResearch Laboratories) for 30 min at room temperature. Fab goat anti-mouse IgG (Jackson ImmunoResearch) were then added to block any unbound primary Ab. Second primary Abs, anti-CD68 (1/400; DAKO, Carpinteria, CA), anti-CD4 (1/20; Novocastra Laboratories, Newcastle, U.K.); distributed by Vector Laboratories, Burlingame, CA), and anti-CD8 (1/25; Biocare Medical) were applied, sections were incubated for 1 h at room temperature, and signal was detected with a secondary FITC-conjugated Ab (Alexa 488; 1/400; Molecular Probes, Eugene, OR) or secondary Abs goat anti-mouse IgG-HRP Mach 2 polymer (Biocare Medical) and Vector NovaRed chromogen (Vector Laboratories). Sections were visualized with a fluorescent microscope (BX51, Olympus, Melville, NY), and images were analyzed with image software (Diagnostic Instruments, Sterling Heights, MI). Double-immunoperoxidase-labeled sections were averaged as the ratio of single- and double-labeled cells per tubule in six high power fields (×400; Leica microscope, DMLS).

Western blot analysis of TGF-β isozforms

Three kinds of TGF-β were used: human platelet-derived TGF-β1 (Oncogene Research Products, San Diego, CA), recombinant active human TGF-β1, and recombinant latent human TGF-β1 (both from R&D Systems). Each was mixed with nonreduced 2× sample buffer (0.008% bromphenol blue, 20% glycerol, 3% SDS, and 125 mM Tris (pH 6.8), without 2-ME), incubated at 37°C for 30 min, and applied to 8–16% Tris-glycine gradient gel (Invitrogen, Carlsbad, CA). After 2-h electrophoresis, the gels were electrophoretically transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) for 75 min. The blots then were incubated with mouse anti-human TGF-β clone TB21; mouse anti-TGF-β1, -2, or -3 Ab (clone 1D11); biotinylated goat anti-human LAP (TGF-β1) Ab; or mouse anti-human latent TGF-β-binding protein 1 (LTBP-1) Ab (the last three from R&D Systems). Then the blots were incubated with proper secondary Ab (for biotinylated Ab ExtrAvidin peroxidase-conjugated; all others, goat anti-mouse IgG peroxidase-conjugated; both from Sigma-Aldrich, St. Louis, MO), Substrate (SuperSinal West Femto Maximum Sensitive; Pierce, Rockford, IL) was added before exposing blots to the x-ray film.

Statistical analysis

For analysis of DTH-linked suppression data, groups with rejecting (creatinine ≥1.5 mg/dl and histological evidence of rejection) vs stable functioning allografts were compared using Wilcoxon’s rank-sum test. Wilcoxon’s signed-rank test was used to determine whether there was a positive response of PBL to donor Ag (dAg) in the presence of anti-IL-10 or anti-TGF-β compared with isotype controls. ANOVA comparing immunohistochemical staining of acute rejection (biopsy and nephrectomy combined samples), nontransplanted kidney, and normal/suspicious transplanted kidney samples was performed, and pairwise comparisons were made using the Fisher protected least significant difference test. Some monkeys, whose status changed during the course of this study, contributed values to different sample groups. For this analysis, we assumed that these data points were independent. In tests comparing biopsy TGF-β1 with DTH suppression status, a simple ANOVA was used, and a pairwise comparison was performed by a Tukey’s multiple comparison test. For this analysis, two animals each produced observations at different time points, which fell into different comparison groups. These single repeated measure samples were treated as two independent samples. All analyses were performed using SAS statistical software (SAS Institute, Cary, NC).

Results

Metastable tolerance mediated via TGF-β1 is induced by a variety of regimens

Table I shows the 14 monkeys included in our analysis of long term renal allograft acceptance. The group included 12 monkeys transplanted at University of Wisconsin (Madison, WI) and two (r142 and YM) transplanted at Biomedical Primate Research Center (Rijswijk, The Netherlands). Recipients underwent bilateral nephrectomy of their native kidneys at the time of transplant and were discontinued from immunosuppressive drugs after an initial induction regimen. Both depleting (anti-CD3 immunotoxin) and nondepleting (drug alone or anti-CD154/CD40 ligand Ab) induction regimens were used (see references in Table I for details). In two cases (r142 and YM), donor-specific transfusions were combined with T cell depletion or short term cyclosporine treatment (18). With the exception of monkey YM (graft survival since 1982), none of the regimens has achieved lifelong tolerance, but in eight of 14 monkeys, transplanted kidneys survived with excellent function for >1000 days. The latter group included all (n = 5) monkeys receiving a kidney from either an MHC haplotype-matched donor or a donor matched for one MHC class II DRB1 Ag (Table I). Monkeys that were challenged with a donor skin graft after day 180 accepted them (Table I) while rejecting third-party skin (data not shown); however, challenge with donor skin before day 180 has been previously shown to precipitate renal and skin allograft loss (18). These results are consistent with a metastable form of allotolerance, subject to change over time, with the most common outcome being eventual graft loss, or, in rare instances, transition to a more stable form of long-term tolerance.

To determine whether metastable renal allograft tolerance in monkeys given diverse induction treatments is associated with a common immune regulation mechanism, a trans-vivo DTH assay was used to evaluate peripheral immune reactivity (Fig. 1A). A strong positive swelling response (>30 × 10−4 inches over PBS background) was seen when PBMC were coincubated with the recall Ag TT in both animals with stable function and those with rejection (p = NS). However, when PBMC or LNC from monkeys with stable graft function were compared with those from allograft rejection, a significantly (p < 0.01) lower swelling response to donor Ags was observed. Furthermore, the strong responses to TT by responder cells from stable recipients were markedly inhibited when TT was coincubated with dAg (p < 0.05, acceptors vs rejectors; Fig. 1A). A similar pattern of weak anti-donor response as well as linked suppression of anti-TT response was observed in allograft acceptors using sonicates of donor kidney fibroblasts as a source of dAg (data not shown).

To determine whether cytokine neutralization could restore a donor-reactive DTH response in monkeys with stable allografts, Abs to TGF-β or IL-10 were included in footpad injections of PBMC and dAg. As shown in Fig. 1B, addition of a neutralizing rabbit anti-TGF-β Ab to dAg challenge significantly increased the DTH response compared with the response to dAg in the presence of control IgG and compared with the response elicited by the anti-TGF-β Ab alone (both p < 0.001; Fig. 1B). In contrast, coinjection of either of two anti-IL-10 Abs known to react to rhesus monkey IL-10 did not uncover a significant anti-donor DTH response.

Localization of CD4+*, TGF-β1* T cells to the graft during metastable tolerance

The presence of donor Ag-linked suppression and a TGF-β-regulated DTH response to donor alloantigens in PBMC of allograft
The clinical course of monkey 98D349 with stable renal allografts is shown. Responses to TT, dAg, and dAg plus TT using a Wilcoxon rank-sum test are shown for a single monkey. The data demonstrate that such T regulatory cells were also present in the renal transplants.

FIGURE 1. Regulation of anti-donor trans-vivo DTH responses of rhesus monkeys with stable renal allografts. A, Analysis of dAg-linked DTH suppression using leukocytes (LC) as sources of dAg and responder PBMC from rejecting (□, △, and ○; n = 5) or stable functioning (■, ▲, and ●; n = 7) monkeys; each point represents the mean of one to three determinations for a single monkey. The p values for differences between animals with stable graft function and those with biopsy-proven rejection in responses to TT, dAg, and dAg plus TT using a Wilcoxon rank-sum test are shown. ns, not significant. B, Effect of rabbit anti-TGF-β (RbαTGF-β), goat anti-IL-10 (GtαIL-10), and rat anti-IL-10 (RtαIL-10) neutralizing Ab on donor-reactive DTH using PBMC from monkeys with stable renal allografts. Only the significant p values for differences between the response in the presence vs the absence of dAg or isotype vs test Ab are shown for each anti-cytokine or control IgG.

The invasive interstitial MNC were uniformly TGF-β-negative (Fig. 3A, open arrow heads, right middle panel). A high power view of some tubules undergoing rejection (Fig. 3B, solid arrow heads, lower right) revealed TGF-β1 at the apical/luminal surface of the tubules, but no interstitial mononuclear cells were positive for TGF-β1.

The monkey was sacrificed on postoperative day 623 with histologic evidence of severe chronic rejection. During the deterioration of renal function in monkey 97D285, the immunohistopathology pattern of the biopsy changed dramatically. As shown in Fig. 3B (left panels), leukocytes were found in the interstitium during the period of excellent renal function and DTH bystander regulation on day 370 (H&E stain, top left). Immuno-staining with mAb TB21 revealed numerous TGF-β-mono-nuclear cells (MNC) scattered throughout the interstitium at this time point. At higher power, TGF-β1+ MNC expressed this epitope both in the cytoplasm and at the cell surface (arrowheads, lower left panel, Fig. 3B). In contrast, at the time of allograft rejection (day 517; Fig. 3B, right panels), there were numerous foci of interstitial and intratubular leukocytes, with some tubular destruction (top panel; H&E stain). The invasive interstitial MNC were uniformly TGF-β1-negative (Fig. 3B, open arrow heads, right middle panel). A high power view of some tubules undergoing rejection (Fig. 3B, solid arrow heads, lower right) revealed TGF-β1 at the apical/luminal surface of the tubules, but no interstitial mononuclear cells were positive for TGF-β1.
white arrow) that appeared to localize either at or just beneath the cell surface. In contrast, the day 517 kidney biopsy taken at the time of mixed acute and chronic rejection shows both interstitial (white arrow, right panels) and intratubular (black arrow) CD4 \(^+\) T cells that were negative for TGF-\(\beta\) expression. Similar studies were performed on different sections of the same biopsies using immunoperoxidase labeling (bottom panels) showing colocalization of CD4 (brown) and TGF-\(\beta\) (purple) at the graft acceptance time point (day 370), whereas CD4 single-positive cells (brown) predominated at the rejection time point (day 517).
FIGURE 3. Clinical findings, trans-vivo DTH responses, and allograft histopathology of a recipient monkey with late allograft rejection. A, Serum creatinine (solid line), biopsy scores (text), and trans-vivo DTH responses (bar graphs) of a representative monkey with late allograft rejection and renal dysfunction (97D285). The TGF-β status of graft-infiltrating leukocytes (GIL) is indicated as low (shaded circles) or positive (●). Abbreviations for biopsy scores are: ar, acute rejection; cr, chronic rejection; sus, suspicious. The time when the last dose of immunosuppression was given is indicated by an arrow. The time when the animal was euthanized is indicated by a skull and crossed bones. B, Photomicrographs of renal allograft biopsies from monkey 97D285 at 370 days (left panels) and 517 days (right panels) post-transplant are shown. The day 370 kidney biopsy scored as suspicious revealed intact renal tubules (RT) and a mild interstitial MNC infiltrate (H&E stain; ×400). In the same sample, immunolabeling with anti-human TGF-β1 mAb (TB21) reveals scattered TGF-β-positive interstitial MNC (×400; filled arrowheads). Two mononuclear leukocytes in the renal interstitium (Ri) with surface and cytoplasmic TGF-β1 staining are shown (×1000; arrowheads). In contrast, the day 517 biopsy scored as acute rejection (left panels) had a significant MNC infiltrates with foci of moderate tubulitis (H&E stain; ×400; arrowhead) with little or no staining of interstitial MNC with the TB21 mAb (×400; open arrowheads). The TGF-β signal localized at the apical surface of the tubular epithelial cells and within tubular lumens in acute rejection (×1000; arrowheads).
The absence of staining for active TGF-β in the interstitial cells along with the positive staining for LAP suggested that interstitial cells in accepted renal allografts might produce the large latent complex form of TGFβ1, which specifically binds to extracellular matrix (28). To define what forms of TGFβ were recognized by the different Abs used for immunohistochemistry analysis, we performed a Western blot using commercially available TGF-β preparations separated on SDS-PAGE. As shown in Fig. 5B, the mAb 1D11 binds strongly to the 25-kDa homodimer of active TGF-β1 found in both the TGF-β1 purified from human platelets and in recombinant active human TGF-β1 derived from transfected CHO cells (Fig. 5B, lanes 1 and 2). In contrast, the mAb TB21 did not bind to active TGF-β1, but instead recognized a 200-kDa doublet (lane 4) or a single band (lane 6) found only in platelet-derived and full-length latent recombinant TGF-β1, but not in recombinant active TGF-β1. The 200-kDa band could also be recognized by Abs to LAP and LTBP, suggesting that TB21 binds to the large latent complex (LLC) form of TGF-β1 as previously suggested by immunoprecipitation analysis (29). Taken together, the results shown in Fig. 5 suggest that the dominant form of TGF-β1 expressed by the graft-infiltrating cells during allograft acceptance is the LLC form of TGF-β1.

**Role of latent-to-active TGF-β conversion in DTH regulation during allograft acceptance**

The identification of interstitial leukocytes positive for LLC-TGF-β1 (TB21”) or LAP along with the generally negative staining of the same cells with Ab to active TGF-β (1D11) caused us to re-examine the mechanism of TGF-β1-dependent, ιΔg-linked DTH unresponsiveness in long term allograft acceptors. PBMC from three long term allograft acceptors were mixed with ιΔg and neutralizing rabbit anti-TGF-β IgG, mAb TB21, or an mAb to thrombospondin-1. As a control, goat anti-IL-10 Abs were also tested. As shown in Fig. 5C, both TB21 and anti-thrombospondin-1 Abs partially uncovered a DTH response to ιΔg in allograft acceptor monkeys; as negative controls, neither mouse IgG isotype control (data not shown) nor anti-IL-10 Abs had any uncovering effect. The partial uncovering of anti-donor DTH by TB21 and anti-thrombospondin suggests a key role for latent-to-active TGF-β conversion by a thrombospondin-dependent pathway in the DTH regulation mechanism.

**Normal allograft histology coincides with CD4+ T lymphocytes coexpressing the latent form of TGF-β1**

Twenty-nine kidney allograft specimens (24 core biopsies and five necropsy tissues) were obtained from 12 experimental animals and two naive controls. The specimens were double labeled by immunoperoxidase for TGF-β1 (purple) and CD4 (brown) and CD markers evaluated by dual IP (CD4, CD8, and CD68), only CD4” T lymphocytes coexpressed the latent form of TGF-β1; neither CD8” lymphocytes nor CD68” macrophages did, consistent with their low frequency in nonrejection tissue samples (Fig. 6, D and E). When the subgroups were compared, the mean number of double-labeled CD4”/T lymphocytes per tubule was significantly greater in biopsies scored as normal/suspicious than in sections

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**FIGURE 4.** Metastable renal allograft tolerance is associated with CD4”-TGF-β” cell infiltrates. Depicted are the monkey 97D285 day 370 (left panels) and day 517 (right panels) kidney biopsies (×1000) at the time of allograft acceptance and rejection, respectively. The upper panels depict the same field at each time point immunolabeled with anti-CD4 Ab and anti-TGF-β mAb TB21, and analyzed for colocalization of both markers (overlay). Peritubular CD4” T cells are indicated by the white arrows, whereas a single intratubular (tubulitis) CD4” T cell is indicated by a solid arrow on day 517. The two bottom panels show a different section of the same biopsies, stained by immunoperoxidase for TGF-β1 (purple) and CD4 (brown) RT, renal tubular lumen. Inset, Higher power view of cell indicated by solid arrow.

**Mononuclear infiltrates during allograft acceptance express the large latent complex form of TGF-β1**

To define the form of TGF-β expressed by interstitial infiltrates of allograft acceptors, we compared the immunostaining and Western blotting patterns of mAb TB21 (the Ab used for immunolabeling of Figs. 2B and 3B), mAb 1D11 (an Ab specific for active forms of TGF-β1, -2, and -3) and Ab to human LAP (TGF-β-LAP). Fig. 5A shows the immunostaining patterns of anti-LAP (upper left), 1D11 (lower two panels) and TB21 (upper right) in accepted monkey kidney allografts. As shown in Fig. 5A, left top panel, strong positive staining for LAP was observed in paraffin sections of a portion of the graft interstitial infiltrating cells of monkey 97D285 on day 370. In contrast (Fig. 5A, left bottom panel), the majority of interstitial cells in the same biopsy were 1D11 negative, with rare exceptions (open arrow), whereas tubules were occasionally positive (filled arrowhead). A similar pattern was seen in frozen sections of a graft biopsy from monkey r142 obtained on day 948 post-transplant (Fig. 5A, right panels). This monkey’s status was stable, with normal sCr and a low histopathologic score at this time point. A characteristic distribution pattern of TB21” cells scattered throughout the interstitium was seen (top right, filled arrowheads). In contrast, the vast majority of interstitial MNC in the same biopsy were negative for active TGF-β, as indicated by the low frequency of cells staining with mAb 1D11 (Fig. 5A, right bottom panel, filled arrowhead). As in monkey 97D285, active TGF-β was expressed by some of the renal tubules of r142 (a normal finding).
FIGURE 5.  A. Renal allograft acceptance is associated with TGF-β1-LAP-positive interstitial cells that are largely negative for the active form of TGF-β1. The monkey 97D285 day 370 biopsy was immunolabeled with TGF-β1-LAP Ab (left top panel; ×1000) and with anti-active TGF-β Ab. 1D11 (left bottom panel; ×400). A TGF-β1-LAP positive (closed arrowhead) and an adjacent TGF-β1-negative (open arrowheads) MNC in the renal interstitium are shown. The same biopsy immunolabeled for the active form of TGF-β (1D11 mAb) demonstrated active TGF-β-positive renal tubular cells (closed arrowhead), whereas most interstitial cells were negative (left bottom panel, open arrowhead). The monkey r142 day 948 TB21-immunolabeled frozen biopsy section (right top panel; ×200) revealed scattered TGF-β1+ mononuclear cells in the renal interstitium (closed arrows). The same biopsy immunolabeled for the active form of TGF-β (1D11 mAb; right bottom panel; ×200) shows only an occasional positive cell and staining of some tubular epithelial cells (closed arrow). B, TB21 Ab binds the latent form of TGF-β1 in Western blots. Human platelet TGF-β1 (a), recombinant human TGF-β1 (b), and recombinant human latent TGF-β1 (c) were analyzed in nonreducing SDS-PAGE (8–16%), with 100 ng/lane (lanes 4–6) and 20 ng/lane (lanes 1–3, 7, and 8) of the indicated Abs. The blots were probed with two mouse anti-TGF-β Abs, TB21 and 1D11; biotinylated anti-human LAP (TGF-β1) Ab; and anti-human LTBP-1 Ab. C, Trans-vivo DTH responses (mean ± SD) of long term allograft acceptors (n = 3) using PBMC mixed with dAg and neutralizing rabbit anti-TGF-β IgG, mAb TB21, mAb to thrombospondin-1, or goat anti-IL-10 Abs. The observations are paired with an appropriate isotype control. The shaded area indicates the mean response to dAg alone.

with acute rejection (Fig. 6B; p < 0.05). Approximately half of the graft-infiltrating latent TGF-β1+ cells (TB21+) in the normal/suspicous group of biopsies of monkeys with normal allograft function were CD4+ T lymphocytes. When labeled with anti-CD3, the number of T lymphocytes coexpressing latent TGF-β1 was similar to the CD4+/TB21+ count (data not shown), suggesting that the second half of TB21+ cells corresponds to non-T cells. Overall, the numbers of CD4+, CD8+ lymphocytes, and CD68+ macrophages were significantly greater in tissues scored with acute allograft rejection (Fig. 6, C–E). Remarkably, the highest density of graft-infiltrating latent TGF-β1+ cells (1.25 cells/tubule) was seen in a monkey >22 years post-transplantation (YM) without evidence of chronic allograft nephropathy.

Peripheral TGF-β1-dependent DTH regulation associates with intragraft TGF-β1+ mononuclear cells and lack of histologic rejection

Based on the striking pattern of TGF-β1 immunostaining in the graft, corresponding to TGF-β1-mediated regulation of anti-donor DTH in blood, we wanted to further explore the relationship between latent-TGF-β1+ graft-infiltrating leukocytes and the TGF-β1-dependent DTH regulation detected in the periphery. Immunohistochemical staining for TGF-β1 using mAb TB21 was performed on 12 biopsy and necropsy specimens from a subset of nine animals previously tested in the DTH assay. The results of immunohistochemistry and DTH analyses were compared in instances when PBMC were collected within 1 wk of biopsy or necropsy. As shown in Fig. 7, the specimens were subdivided according to the number of graft-infiltrating TB21+ cells in three groups. Necropsy tissues with low numbers of TGF-β1+ cells per tubule (<0.1; Fig. 7, left panel) from the three animals tested at the time of sacrifice due to acute and chronic rejection revealed high DTH responses to both TT and donor Ag. In contrast, monkeys with stable allograft function (normal creatinine values) and biopsies with normal histology showed greater numbers of graft-infiltrating TB21+ cells (>0.4) and low donor-Ag specific peripheral DTH response, with normal response to TT (Fig. 7, right panel). In between, there was a group of monkeys that, despite histologic diagnosis of acute rejection, had peripheral donor-Ag specific DTH regulation. This group of animals showed intermediate numbers of graft-infiltrating TB21+ cells (Fig. 7, center panel). Monkey 98D349 represents an interesting case. This monkey had normal allograft function throughout the study. However, it gradually converted from an allograft with low TB21+ cell numbers and histologic acute rejection (Fig. 7, left panel) to an allograft with intermediate levels of TB21+ cells (middle panel) and finally to high TB21+ cell numbers and no evidence of histologic rejection (Fig. 7, right panel). This finding suggests that the allograft infiltration of regulatory latent TGF-β1+ cells is a dynamic process and indeed helps to resolve subclinical (histologic only) allograft rejection and stabilize graft function.

Discussion

Acute and chronic renal allograft rejection have previously been associated with TGF-β1-dependent tubulitis (29, 30) and interstitial fibrosis (30, 31), respectively. Production of active TGF-β by renal tubular epithelial cells is markedly increased in the rejecting kidney, inducing CD103 expression on activated graft-infiltrating T cells (32). Indeed, this TGF-β-mediated process appears to be required for development of tubulitis and CTL lysis of renal tubular epithelial cells (33). The benign interstitial infiltrates in renal
transplants of graft recipients without maintenance immunosuppression (34) are therefore puzzling, because they may also be associated with elevated intragraft expression of TGF-β1 (35, 36). Our findings offer a solution to this paradox; namely, that CD4 regulatory cell-derived TGF-β is continuously present in the interstitium of accepted allografts, but in latent complex form, awaiting conversion to active TGF-β at the point of encounter with donor Ag and APC. A similar suggestion was made based on earlier, in vitro studies of contact-dependent inhibition by CD4+CD25+ T regulatory cells in mice (7) and humans (11).

Exactly how the immunosuppressive potential of latent TGF-β is harnessed to inhibit the DTH response of the graft acceptor remains unclear. The rabbit TGF-β-neutralizing polyclonal Ab used for uncovering of latent DTH responses to dAg (Fig. 1B), like mAb 1D11 used in immunostaining, binds primarily to the low molecular mass (25-kDa) active dimer form of TGF-β in Western blot analysis (Q. Xu and W. Burlingham, unpublished observations). This suggests that active TGF-β is indeed the final form required for immunoregulation. In contrast, mAb TB21, which could also restore anti-dAg DTH responses in acceptor monkeys (Fig. 5C), is known to have relatively weak neutralizing activity for active TGF-β in bioassays (37), but binds strongly to 200-kDa LLC-TGF-β1 forms (Fig. 5B). The apparent contradiction between the requirement for TGF-β to be active to perform its immunosuppressive functions and the expression of mainly latent forms of TGF-β1 by CD4+ T regulatory cells (7, 11) can be reconciled if there is a further requirement for processing of latent-to-active TGF-β for the regulated anti-donor DTH response to occur. The partial restoration of anti-donor DTH response in allograft acceptor monkeys by anti-thrombospondin-1 mAb (Fig. 5C) is consistent with the known critical role of thrombospondin-1 in processing latent-to-active TGF-β in vivo (38) and with the recent finding that CD4+CD25+ as well as CD4+CD25− regulatory cells in mice coexpress surface latent TGF-β1 and thrombospondin (13). As latent TGF-β1 is converted to an active form only when the TCR of the T regulatory cell is engaged, a catalytic role of CD36-bound thrombospondin on the APC (for example, macrophage) cell surface in this process is also possible (39).

To date, we have been able to identify only CD4+ T cells (Figs. 4 and 6), not CD8+ T cells or CD68+ macrophages, expressing latent complex TGF-β1 among cells infiltrating accepted monkey renal allografts. CD4+latent TGF-β1+ cells accounted for ~50% of the total number of latent TGF-β1+ cells in graft infiltrates. Although double-negative regulatory cells (40) could not be ruled...
out, the CD3+/latent TGF-\(\beta\)-1+ double staining showed similar number of CD4+/latent TGF-\(\beta\)-1+ cells, suggesting a non-T cell phenotype for the other 50% of graft-infiltrating TGF-\(\beta\)-1+ cells. This non-T cell portion may include regulatory dendritic cells (41, 42), NK cells (43), and possibly CD68-negative, monocyte lineage cells. Interestingly, monkey YM (Table I and Fig. 6) had latent-TGF-\(\beta\)-1, NK cells (43), and possibly CD68-negative, monocyte lineage cells. Interestingly, monkey YM (Table I and Fig. 6) had latent-TGF-\(\beta\)-1 (primarily non-CD4+ T cell-type) graft-infiltrating cells present >21 days post-transplant without any evidence of chronic allograft rejection, indicating that the mere presence of TGF-\(\beta\) in the allograft does not necessarily translate into chronic rejection and allograft loss.

A TGF-\(\beta\)-1-mediated mechanism has also been recently described to restore self-tolerance in nonobese diabetic mice (44). In this model, low numbers of CD4+CD25+ T cells from anti-CD3e-mAb-treated animals protected recipients from diabetes in adoptive transfer studies; this protection was abolished by treatment of the mice with neutralizing anti-TGF-\(\beta\)-1 Ab. In our study, monkeys with subclinical (histologic only) rejection (Fig. 7, middle panel) revealed low, but significant, numbers of latent-TGF-\(\beta\)-1+ graft-infiltrating cells. Preliminary studies of graft-infiltrating cells harvested from monkey kidneys undergoing late acute rejection following a period of metastable rejection indicate that residual latent-TGF-\(\beta\)-1+ cells may delay the acute rejection process by inhibiting the local DTH reaction (E. Jankowska-Gan, J. Torre-alba, and W. Burlington, unpublished observations).

We propose the following model to account for our findings. After the period of early allograft ischemic injury has passed, and the allo-specific host adaptive immune response has been stymied by the induction agent(s), the development of donor-specific CD4+ T regulatory cells begins. T regulatory cell induction is likely to occur first in the peripheral lymphoid tissue by means of the indirect pathway of CD4+ T cell allorecognition that requires host class II+ APC (45) and may be facilitated by MHC class II Ag sharing with the graft-derived, donor APC (46). This idea is consistent with the long term acceptance (>1000 days) of renal transplants in five of five monkeys matched for at least one DR allele with their donor (Table I). Once immunosuppression is withdrawn, T effector cells that have already migrated to the graft may become active and cause tubulitis. CD4+ T regulatory cells that express latent, surface-bound TGF-\(\beta\)-1 then migrate via the peripheral blood to the renal allograft. Once this occurs, the regulatory T cells begin to inhibit by linked suppression the proinflammatory responses of colocalized alloantigen-specific CD8+ and CD4+ T effector cells. Tubulitis ceases, and a stable benign interstitial infiltration is established. A further induction of latent TGF-\(\beta\)-1 complex that is secreted locally by the CD4+ T regulatory cells then coats the surrounding matrix adjacent to the tubules. There it may act as an extracellular sensor to control inflammation and regulate angiogenesis and matrix deposition, without provoking deleterious side effects associated with overexpression of the active form of TGF-\(\beta\)-1 (28).

Recently, Graca et al. (47) were able to transfer CD4+ T regulatory cells from a tolerant to a naive mouse by retransplanting the original tolerated skin graft. The present analysis of monkey renal allograft acceptance strengthens the evidence that alloreactive T regulatory cells are not restricted in distribution to blood, spleen, and lymph node, but migrate into the graft where they play a sentinel role. It is still not clear whether the supply of CD4+ T regulatory cells to the allograft needs to be constantly replenished from the peripheral lymphoid tissues, or, alternatively, if the local CD4+ T regulatory cells may become self-perpetuating, as suggested by Graca et al. (47). However, it is clear that monkeys with high (>0.4 cells/tubule) density of these cells in the interstitium had a significantly lower anti-donor DTH responses than monkeys with undetectable TGF-\(\beta\)-1 cells in their graft (Fig. 7). Monkeys with low, but detectable, levels of peritubular TGF-\(\beta\)-1 cells (0.1–0.4 cells/tubule) included both transitional cases (such as described in Figs. 2 and 3) as well as two monkeys not included in the

![FIGURE 7](http://www.jimmunol.org/)

A low anti-donor DTH response is correlated with TGF-\(\beta\)-positive MNC in kidney biopsies. Immunohistochemical staining for TGF-\(\beta\)-1 was performed using TB21 mAb on 12 renal tissue samples from nine animals. The trans-vivo DTH responses of PBL obtained within 15 days of biopsy against TT (□) and dAg (□) were compared in monkeys with renal biopsies harboring undetectable (Neg), low, or high number of TB21-positive (latent TGF-\(\beta\)-1) interstitial mononuclear cells. *, p < 0.05 for comparison of DTH responses to dAg in TB21 high vs TB21 negative groups. TT responses were not significantly different among the three groups.
present study that lost their grafts due to early acute rejection (<60 days) (J. Torreallaba, J. H. Fechner, S. J. Knechtel, and W. Burlingham, unpublished observations).

The remarkable similarity in TGF-β1 interferilial infiltrates during the long term allograft acceptance phase of both T-depleted and nondepleted monkeys may represent an evolutionarily conserved pathway of peripheral tolerance to Ags like those expressed by a kidney transplant. Cytokine neutralization studies clearly demonstrated that TGF-β1, not IL-10, was the principal mediator of DTH regulation in all the allograft acceptors. A TGF-β bias in regulated anti-donor DTH response may reflect conditions unique to tolerance induced by APCs emerging from TGF-β-rich microenvironments, as previously suggested by studies of mouse kidney transplants (48) and the anterior chamber of the eye (49, 50). Other tolerance mechanisms, including IL-10-producing allospecific T regulatory cells (8), may be required to achieve permanent, as opposed to metastable, tolerance.

In conclusion, we have found that metastable tolerance in the rhesus monkey kidney allograft is associated with a TGF-β-regulated anti-donor DTH response in the periphery and with graft-infiltrating latent TGF-β cells, of which ~50% were CD4+ T cells. These cells may persist in the graft for long periods of time (>21 years post-transplant) without necessarily causing chronic rejection or allograft loss. The Ab blocking studies of peripheral DTH responses strongly support a mechanism by which donor Ag recognition is linked to latent TGF-β1 activation via thrombospondin. Histologic and functional characterization of intrgraft regulatory cells, including both CD4+ T regulatory cells as well as non-T cells, will provide an important step toward a tolerance assay that can be used to monitor the kidney transplant recipient before, during, and after withdrawal of immunosuppressive drugs.

References


