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*J Immunol* 2004; 172:5753-5764; 
doi: 10.4049/jimmunol.172.9.5753
http://www.jimmunol.org/content/172/9/5753

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*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
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 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 trans-vivo 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) response to an unrelated third-party Ag such as tetanus toxoid (TT) is suppressed when the tolerogen or metastable allograft tolerance. Allograft acceptors display the characteristic feature of linked suppression (2), in which the delayed-type hypersensitivity (DTH) 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 (6–8), rats (9), and humans (10, 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 after transplantation 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 to determine whether their appearance in the periphery or in 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 (Allis, TX), and LABS (Yamasee, 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 DR loci, and bilateral immunosuppression with or without donor-specific transfusion (17). All monkeys in the study had been off all immunosuppressive conventional or experimental medication for >6 mos. 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 human 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 obtained from R&D Systems (Minneapolis, MN). In addition, neutralizing polyclonal Ab to human TGF-β1 (BioSource International, Camarillo, CA). For immunohistochemistry analysis, we obtained from R&D Systems (Minneapolis, MN). In addition, neutralizing polyclonal Ab to human TGF-β1 (BioSource International, Camarillo, CA).

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>YMF (17)</td>
<td>DST+IS</td>
<td>+</td>
<td>&gt;8000</td>
<td>&gt;7635</td>
<td>0.9 1.2</td>
<td>Normal</td>
<td>NT</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 1.2</td>
<td>Susp CR1</td>
<td>Yes</td>
</tr>
<tr>
<td>AV607</td>
<td>IS</td>
<td>+</td>
<td>&gt;1464</td>
<td>&gt;1405</td>
<td>0.7 1.5</td>
<td>Normal</td>
<td>NT</td>
</tr>
<tr>
<td>9HD032</td>
<td>IS</td>
<td>+</td>
<td>&gt;1282</td>
<td>&gt;1223</td>
<td>0.9 2.0</td>
<td>Susp CR1</td>
<td>Yes</td>
</tr>
<tr>
<td>9HD0349 (19)</td>
<td>IS</td>
<td>+</td>
<td>&gt;1282</td>
<td>&gt;1223</td>
<td>0.6 0.7</td>
<td>Normal</td>
<td>Yes</td>
</tr>
<tr>
<td>AW13 (16)</td>
<td>TCD+IS</td>
<td>–</td>
<td>1260</td>
<td>993</td>
<td>1.0 0.9</td>
<td>Rejected</td>
<td>NT</td>
</tr>
<tr>
<td>r142 (18)</td>
<td>DST+TCD+IS</td>
<td>+</td>
<td>&gt;113</td>
<td>996</td>
<td>1.2 0.9</td>
<td>Rejected</td>
<td>CR1</td>
</tr>
<tr>
<td>9HD468 (19)</td>
<td>CoSB+IS</td>
<td>–</td>
<td>1113</td>
<td>883</td>
<td>0.8 0.9</td>
<td>Rejected</td>
<td>CR1</td>
</tr>
<tr>
<td>9HD285 (19)</td>
<td>CoSB+IS</td>
<td>–</td>
<td>623</td>
<td>393</td>
<td>0.8 1.1</td>
<td>Rejected</td>
<td>CR3</td>
</tr>
<tr>
<td>AV02 (16)</td>
<td>TCD+IS</td>
<td>–</td>
<td>745</td>
<td>741</td>
<td>0.8 1.1</td>
<td>Rejected</td>
<td>CR2</td>
</tr>
<tr>
<td>9HD306 (16)</td>
<td>TCD+IS</td>
<td>–</td>
<td>712</td>
<td>709</td>
<td>1.7 1.7</td>
<td>Rejected</td>
<td>CR1</td>
</tr>
<tr>
<td>9HD270 (16)</td>
<td>TCD+IS</td>
<td>–</td>
<td>582</td>
<td>520</td>
<td>1.2 1.2</td>
<td>Rejected</td>
<td>CR3</td>
</tr>
<tr>
<td>9AV8 (19)</td>
<td>CoSB+IS</td>
<td>–</td>
<td>479</td>
<td>249</td>
<td>1.6 1.6</td>
<td>Rejected</td>
<td>CR1</td>
</tr>
<tr>
<td>9HD140</td>
<td>TCD+IS</td>
<td>–</td>
<td>350</td>
<td>288</td>
<td></td>
<td>Rejected</td>
<td>CR2/CR3</td>
</tr>
</tbody>
</table>

* DST, Donor-specific transfusion; TCD, T cell depletion; IS, conventional immunosuppression; CoSB, costimulation blockade.
† 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 Borg 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 isothiocyanate-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 diaminoazobenzidine chromogen (Dako-tomation 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; Novocarsta 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 VectorNovaRed 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 isofoms

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% sodium, 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 electrotherophoretically 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-β, -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 significance 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 co-injected 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 rejectors, 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 co-injected 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
acceptors suggested that circulating, TGF-β1-producing, T regulatory cells may be protecting the allograft from rejection. To determine whether such T regulatory cells were also present in the renal transplant, routine histopathologic monitoring was supplemented with immunohistochemical staining to detect the infiltration of the donor kidney with TGF-β1+ regulatory cells. Fig. 2A shows the clinical course of monkey 98D349 with stable renal allograft function. This monkey received a last dose of immunosuppressive drug on day 60 and maintained low (≤1.0 mg/dl) serum creatinine levels throughout the observation period despite intermittent, low grade, acute rejection biopsy scores. The PBMC showed consistent dAg-linked DTH suppression of response to recall Ag TT, when tested between days 300 and 500 post-transplant (Fig. 2A); the weak DTH responses to dAg during this time period were readily restored to the level of the recall Ag response by neutralization of TGF-β (data not shown).

Initially, when allograft biopsies showed moderate focal tubulitis that was left untreated on day 313 (Fig. 2B, top left), the absence of TGF-β1 (TB21mAb) graft interstitial leukocytes was noted (Fig. 2B, bottom left). Beginning with the biopsy on day 389 (acute rejection IA) and continuing to day 430 (suspicious), the appearance of TGF-β1+ graft-infiltrating leukocytes coincided with gradual resolution of tubulitis and absence of rejection (Fig. 2B, top right). In these and subsequent biopsies, TGF-β1+ cells were found scattered in the interstitium (Fig. 2B, bottom right), adjacent to peritubular basement membranes and showed abundant cytoplasmic and surface staining (Fig. 2B, bottom right, inset). This monkey is currently healthy, with a serum creatinine level of 1.6 mg/dl and evidence of mild to mild histologic chronic rejection (Table I).

Fig. 3A shows the clinical course of monkey 97D285 that received a last dose of immunosuppressive drug on day 230 post-transplant. In contrast to 98D349, monkey 97D285 had excellent renal function and a TGF-β1+ (TB21mAb) graft infiltrate on day 370, but lost this phenotype on subsequent biopsies during a transition from low grade acute rejection (day 480) to combined acute and chronic rejection (day 517). A parallel loss of regulated anti-donor DTH phenotype was seen in PBMC samples obtained over the period between days 370 and 623 post-transplant as the sCr rose from 0.8 to 2.3 mg/dl. Interestingly, in this transition period (days 370–517) some TGF-β1+ leukocytes remained in the graft on day 480, but became rare by day 517, coinciding with clinical/histological acute rejection and loss of regulated anti-donor DTH. 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-β1+ mononuclear 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.

To determine the identity of the TGF-β1+ mononuclear cells seen in stable allografts, we used a double-immunolabeling technique. Depicted in Fig. 4 are day 370 and day 517 biopsy samples from monkey 97D285. On day 370, a time of excellent allograft function and dAg-linked regulation of DTH, the biopsy contained CD4+ interstitial T cells (Fig. 4, top left, green) that coexpressed TGF-β1 (Fig. 4, middle left, red). The superimposition of the two images yielded a pronounced yellow fluorescence (Fig. 4, overlay,
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-β1-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 TGF-β1, which specifically binds to extracellular matrix (28). To define what forms of TGF-β 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. 5, 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, dAg-linked DTH unresponsiveness in long term allograft acceptors. PBMC from three long term allograft acceptors were mixed with dAg 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 dAg 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 naïve controls. The specimens were double labeled by immunoperoxidase for the latent form of TGF-β1 (TB21 Ab) and markers for CD4+ and CD8+ lymphocytes and CD68+ macrophages. The slides were scored, obtaining the ratio of positive cells per tubule in six microscopic high power fields. As depicted in Fig. 6, very low numbers of TB21+ mononuclear cells were observed in both naïve tissue (p < 0.001) and biopsy/necropsy tissue with acute rejection (p < 0.001) compared with biopsies with normal or suspicious histopathology (Fig. 6A). In contrast, biopsies scored as normal or borderline (suspicous for rejection) had a group higher numbers of TB21 cells per tubule. Of the three categories of CD markers evaluated by dual IP (CD4, CD8, and CD68), only CD4+ 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+/TB21+ cells per tubule was significantly greater in biopsies scored as normal/suspicious than in sections...
with acute rejection (Fig. 6B; p < 0.05). Approximately half the graft-infiltrating latent TGF-β1+ cells (TB21+ cells) in the normal/ suspicious 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-β1 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-β1-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 T 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-β1 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-β1 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-β1 in vivo (38) and with the recent finding that CD4+CD25+ as well as CD4+CD25+ T 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

FIGURE 6. Normal allograft histology coincides with CD4+ graft-infiltrating cells coexpressing latent TGF-β1. Twenty-nine kidney allograft specimens from 14 monkeys were single- or double-labeled with TB21 Ab (anti-latent TGF-β1) and CD4, CD8, or CD68 markers. The ratios of positive interstitial cells/tubules are shown for TB21 single-staining cells (A), double-labeled TB21/CD4 cells (B), single CD4+ lymphocytes (C), single CD8+ lymphocytes (D), and single CD68+ macrophages (E). The biopsy categories were nontransplanted kidney (NTx), normal/suspicious transplant biopsy (N/S), acute rejection biopsy (ARB), and acute rejection necropsy (ARN). Each data point represents the averaged counts from an individual monkey’s biopsies that had same biopsy score. The p values shown are for the comparison of N/S vs NTx and N/S vs ARB/ARN samples.
out, the CD3T/latent TGF-β1 T double staining showed similar number of CD4T/latent TGF-β1 T cells, suggesting a non-T cell phenotype for the other 50% of graft-infiltrating TGF-β1 T 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-β1 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-β1 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-β1 (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-β cells in their graft (Fig. 7). Monkeys with low, but detectable, levels of peritubular TGF-β 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/)

**FIGURE 7.** A low anti-donor DTH response is correlated with TGF-β-positive MNC in kidney biopsies. Immunohistochemical staining for TGF-β1 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-β1) 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.

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present study that lost their grafts due to early acute rejection (<60 days) (J. Torrealla, J. H. Fechner, S. J. Kecht, and W. Burlingham, unpublished observations).

The remarkable similarity in TGF-β1+ interstitial 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-β1 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 thymobosin. Histologic and functional characterization of intragraft regulatory cells, including both CD4+ T regulatory 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


