Regulatory CD25⁺ T Cells in Human Kidney Transplant Recipients

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Abstract. Recent evidence suggests that a population of professional regulatory cells, which limit immune responsiveness, exist in rodents and healthy human subjects. However, their role in disease states remains unclear. A proportion of renal transplant recipients do not demonstrate in vitro reactivity toward their mismatched donor-derived HLA-DR antigens; it was therefore hypothesized that this may be due to such regulatory cells. A cohort of 23 renal transplant recipients was studied at a single institution. In patients with no history of acute rejection, 6 (40%) of 15 demonstrated regulation toward the mismatched HLA-DR allopeptides by CD25⁺ cells. By contrast, only one (12.5%) in eight of those with a history of acute rejection demonstrated regulation. Interestingly, if the patient assays were stratified according to initial in vitro immune responsiveness toward the mismatched allopeptides, 8 (47.1%) of 17 of patient assays with low allopeptide responsiveness (alloreactive T cell frequencies less than 60/million) demonstrated regulation of indirect pathway alloresponses by CD25⁺ cells, whereas 0 of 8 with higher responses (frequencies greater than 60/million) demonstrated no such regulation ($P < 0.05$ by χ² test). The regulatory cells are present in the circulation as early as 3 mo after transplantation and persist for a number of years, despite conventional immunosuppression. Furthermore, induction treatment with anti-IL-2R mAb did not prevent the development of these regulatory CD25⁺ cells. Data from two patients suggest that these cells may also play a role in preventing epitope shifting, implicated in the ongoing immune activation contributing to chronic rejection, and that loss of regulation in a given patient may precede an episode of rejection.

The mechanisms by which tolerance is established toward self-antigens in healthy individuals or toward foreign alloantigens in transplant recipients remain incompletely understood. Experimental systems have demonstrated that there are a number of non–mutually exclusive mechanisms acting on T lymphocytes that can mediate such a state of immunologic unresponsiveness. These mechanisms include deletion of the pathogenic T cells (during immune development and also in the mature immune system), T cell anergy, and immune regulation of effector T cells by soluble factors, such as cytokines, or by professional regulatory cells (1–3). Understanding the contribution that each of these mechanisms makes in human disease states is of great importance for the development of strategies to achieve clinical tolerance after its breakdown in autoimmune diseases or after transplantation.

Recently, confirming early descriptions in rodent transplant models (4), several investigators have described the existence of a unique population of regulatory T cells, expressing both CD4 and CD25 (the IL-2 receptor α chain), which are hyporesponsive and have potent immune regulatory effects in vitro and in vivo (5–11). These cells can efficiently abrogate autoimmune disease and prevent transplant rejection in a number of different experimental animal models (4,7,12–17); more recently, their counterparts have been described in healthy human subjects (10,11,18–21). Only a proportion of CD4⁺ CD25⁺ cells mediate immune regulation; to date, no unique cell markers have been discovered that reproducibly identify these cells, although altered patterns of gene expression may provide a clue (22). However, it appears that in humans at least, they constitute a small subset of CD4⁺ T cells, which express the highest levels of CD25 (CD4CD25high cells) as well as other markers of previous T cell activation (23). The function of these cells in human disease or in clinical transplantation has not yet been elucidated.

Transplant recipients have benefited from dramatic improvements in short-term graft survival; however, in the long-term, they eventually lose their grafts from chronic rejection, currently the major cause of graft failure (24–27). Chronic rejection is a complex process that is believed to be largely immunologically mediated and related to ongoing graft injury initiated by alloreactive T cells, stimulated by processed alloantigens shed from the graft (termed the indirect pathway of allorecognition) (25). Extensive animal studies indicate that indirect allorecognition is sufficient to affect acute skin allograft rejection (28–30) and, in vascularized grafts, priming to the indirect pathway promotes development of chronic rejec-
tion (31,32). In human studies, indirect allore cognition of donor HLA antigens has been demonstrated in renal, cardiac, and lung allograft recipients with chronic rejection (33–36). Furthermore, some of these studies demonstrated peptide immunodominance and a shift of T cell responses toward different allopeptides over time, a process termed epitope spreading (33,34,37). This process may lead to continuous recruitment and activation of naïve CD4⁺ T cells, which react to novel processed allopeptides and orchestrate the immune effector mechanisms leading to progression of chronic rejection (25).

We previously demonstrated in a separate cohort of renal transplant recipients that stable patients, with no history of acute rejection, had low frequencies of indirect pathway alloreactive T cells (less than 60/million by ELISPOT) in the peripheral blood (33,38). By contrast, patients with at least one episode of acute graft rejection, who are at high risk for development of chronic rejection (39,40), generally had increased frequencies of indirectly primed alloreactive T cells (greater than 60/million) (38). We therefore investigated whether hyporesponsiveness to donor alloantigens in renal transplant recipients with or without a history of acute rejection and receiving conventional immunosuppressive drugs may be mediated by regulatory CD25⁺ T cells. We studied a new cohort of adult renal transplant recipients and tested the reactivity to donor antigens, in the presence or absence of putative regulatory cells. Furthermore, in so doing we developed an assay that can equally well be applied to investigate the role of such regulatory mechanisms in patients with autoimmune or malignant disease.

**Materials and Methods**

This study was performed under the approval of the institutional review board for human investigation at the Brigham and Women’s Hospital. Twenty-three renal transplant patients, followed at the Brigham and Women’s Hospital, who received a donor kidney with at least one HLA-DR mismatch were studied. Tissue typing of donors and recipients was performed by standard DNA techniques in the Tissue Typing laboratory, Brigham and Women’s Hospital. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll (Amersham Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation. HLA phenotypes were determined by standard serotyping techniques. The frequency of cytokine-producing indirectly primed alloreactive T cells was measured using ELISPOT analysis with peptides corresponding to the mismatched donor HLA-DR molecules, as described previously where the validity and reproducibility of the assay was demonstrated (38). Peptides (20 to 25 mers) corresponding to the hypervariable regions of the β chains of five different HLA-DR molecules (DR0101, DR1501, DR0301, DR0401, DR0701) based on the most frequent HLA frequencies of donors in the New England Organ Bank were synthesized (Quality Controlled Biochemicals, Hopkinton, MA). ELISA-spot plates (Cellular Technology Limited, Cleveland, OH), coated with capture antibodies against IL-10 (PharMingen, San Diego, CA), or IFN-γ (Endogen, Woburn, MA) in phosphate-buffered saline (PBS), and left overnight at 4°C. The plates were blocked with 1% PBS-BSA for 1 h and then washed with PBS. Between 2.5 and 5 × 10⁶ PBMC were added to each well in 100 μl of complete RPMI 1640 medium containing 10% human serum, streptomycin (BioWhittaker, Walkersville, MD) and 50 mM 2-mercaptoethanol (Sigma). Control wells contained responder PBMC plus medium alone. Cells were also tested in triplicate wells (or duplicates if cell number was limited), against mumps antigen (BioWhittaker) and the panel of HLA-DR peptides (each at 10 μg/ml) corresponding to the donor HLA-DR type. After 48 h, the plates were washed, biotinylated detection antibodies were added, and the plates were left for a further overnight incubation at 4°C. After further washing, horseradish peroxidase-conjugate (DAKO, Carpinteria, CA) was added for 2 h at room temperature. Development was with AEC (Sigma). The resulting spots were counted on a computer-assisted ELISAspot Image Analyzer (Cellular Technology Limited). The frequencies were then expressed as the mean cytokine-producing cells per million PBMC plus the SEM.

In addition, putative CD25⁺ regulatory cells were depleted from the PBMC. Cells were incubated with anti-CD25 monoclonal antibody (Clone M-A251, PharMingen) for 1 h at 4°C, washed twice in PBS, and depleted using magnetic bead separation according to manufacturer’s instructions (Dynabeads, Dynal, Lake Success, NY). Depletion was confirmed by flow cytometry using directly conjugated anti-CD4 and anti-CD25 monoclonal antibodies (both PharMingen) using a FACSCalibur (Becton Dickinson, San Jose, CA) and analyzed with Cellquest software (Becton Dickinson). The change in peptide-specific IFN-γ and IL-10 frequencies after depletion of CD25⁺ cells was quantified and compared with pre-depletion values. Statistical analyses were performed using a paired t test or one-way ANOVA test where duplicate wells were analyzed. Comparison of responses between the patient groups was by a two-tailed χ² test.

Regulatory cell phenotype was assessed on the pre-depleted samples, by performing three color flow cytometry and comparing the CD4CD25high cells with the CD4CD25⁻ cells. PBMC were stained with anti-CD4-APC, anti-CD25-FITC (both PharMingen) and control Ig-PE, anti-CD134-PE, anti-CD152-Cychrome (all PharMingen), anti-PD1-PE, anti-PD-L1-PE, anti-ICOS-PE (all eBioscience, San Diego, CA). Cells were analyzed as above. The results are from one or two representative patient samples.

**Results**

The patient characteristics are summarized in Tables 1 and 2. There were eight patients with a history of acute rejection and 15 without. All recipients were receiving steroids. In addition, 18 were receiving calcineurin inhibitors, 22 mycophenolate mofetil, one patient azathioprine, and six induction therapy with the anti-IL-2 receptor monoclonal antibody, Daclizumab. Patients with no history of clinical and/or biopsy-proven rejection had stable renal function as judged by serum creatinine (mean serum creatinine 1.48 ± 0.12 mg/dl), whereas those with a history of rejection had a significantly higher serum creatinine (2.2 ± 0.27; P = 0.016 by Mann Whitney U test). Two patients were repeatedly tested. One retained stable graft function (patient 7), but the second developed acute rejection and lost his graft 1 yr after transplantation (patient 5). These assays were considered separately for statistical purposes. The timing of the assay in relation to the time of transplantation is also shown in Tables 1 and 2.

Depletion of the CD25⁺ cells was successful in all cases and resulted in >90% reduction of CD4CD25high cells (mean depletion, 96.9% ± 1.6) and 30 to 40% reduction of CD4CD25low cells (mean depletion, 39.9% ± 7) (Figure 1). In six of the fifteen stable patients, we found a statistically sig-
significant increase in IFN-γ frequencies to some of the HLA-DR peptides after depletion of CD25<sup>+</sup> cells (Figures 2, 3a, and 3c). In one recipient of a two HLA-DR mismatched graft (patient 7), this increase was only seen with one of the sets of HLA-DR peptides (Figure 3c). More importantly, the effect of depletion was alloantigen-specific because frequencies to the recall antigen mumps either did not change or decreased after depletion of CD25<sup>+</sup> cells in these patients. In two patients, serial assays were performed (patient 5 at 3 and 7 mo, and patient 7 at 3 and 4 yr). In one (patient 5) the regulatory cells were present at 3 mo, the earliest time point we have so far looked at, but were lost by 7 mo (Figure 3, a and b). Shortly afterward, this patient presented with a BANF grade III rejection and lost his allograft. In addition, the second patient (patient 7) demonstrated that new epitopes may be regulated over time, from within the same HLA molecule and from a second HLA molecule, suggesting that these cells may also serve to limit both intramolecular and intermolecular epitope spreading (Figure 3, c and d). Three of the patients (in four assays) demonstrating regulation (patients 7, 13, and 15) had received induction therapy with the anti-IL2-R monoclonal antibody. Of the other stable patients, five had very low frequencies of peptide-reactive T cells and showed no measurable changes in the frequency of IFN-γ producing alloreactive lymphocytes after CD25<sup>+</sup> depletion (not shown), suggesting that CD25<sup>+</sup> regulatory T cells do not play a significant part in maintaining the hyporesponsiveness to the grafts in all stable transplant recipients.

Interestingly, if we considered the patients according to their initial immune responsiveness toward the HLA-DR allelopeptides (38), those considered hyporesponsive (less than 60 alloreactive cells/million) demonstrated regulation in 8 (47.1%) of 17 assays, whereas none of those classified as responsive (0 of 8) demonstrated regulation ($P = 0.018$ by two-tailed $\chi^2$ test).

**Table 1.** Characteristics of stable patients and timing of assay in relation to date of transplantation

<table>
<thead>
<tr>
<th>Patient&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Age (yr)</th>
<th>Gender</th>
<th>Immunosuppression&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Graft</th>
<th>HLA-DR Mismatch</th>
<th>Serum Cr (mg/dl)</th>
<th>Time of Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>56</td>
<td>M</td>
<td>P, TAC, MMF</td>
<td>CAD</td>
<td>3</td>
<td>0.9</td>
<td>8 mo</td>
</tr>
<tr>
<td>2</td>
<td>52</td>
<td>M</td>
<td>P, TAC, MMF</td>
<td>CAD</td>
<td>1.3</td>
<td>1.0</td>
<td>7 mo</td>
</tr>
<tr>
<td>3</td>
<td>38</td>
<td>M</td>
<td>P, CSA, MMF</td>
<td>LRD</td>
<td>15</td>
<td>1.7</td>
<td>6 yr</td>
</tr>
<tr>
<td>4</td>
<td>69</td>
<td>M</td>
<td>P, CSA, MMF</td>
<td>LRD</td>
<td>7</td>
<td>1.5</td>
<td>6 yr</td>
</tr>
<tr>
<td>5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>26</td>
<td>M</td>
<td>P, TAC, MMF</td>
<td>CAD</td>
<td>3</td>
<td>1.3</td>
<td>3 mo/7 mo</td>
</tr>
<tr>
<td>6</td>
<td>73</td>
<td>F</td>
<td>P, CSA, MMF</td>
<td>CAD</td>
<td>7</td>
<td>1.1</td>
<td>2 yr</td>
</tr>
<tr>
<td>7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>64</td>
<td>M</td>
<td>P, MMF, DAC</td>
<td>CAD</td>
<td>3.7</td>
<td>2.2</td>
<td>3 yr/4 yr</td>
</tr>
<tr>
<td>8</td>
<td>34</td>
<td>M</td>
<td>P, TAC, MMF</td>
<td>LURD</td>
<td>7</td>
<td>1.5</td>
<td>12 mo</td>
</tr>
<tr>
<td>9</td>
<td>46</td>
<td>F</td>
<td>P, CSA, MMF</td>
<td>LURD</td>
<td>1.15</td>
<td>1.5</td>
<td>2 yr</td>
</tr>
<tr>
<td>10</td>
<td>58</td>
<td>F</td>
<td>P, CSA, MMF</td>
<td>CAD</td>
<td>4</td>
<td>2.0</td>
<td>4 yr</td>
</tr>
<tr>
<td>11</td>
<td>53</td>
<td>M</td>
<td>P, CSA, MMF</td>
<td>LRD</td>
<td>15</td>
<td>1.0</td>
<td>12 mo</td>
</tr>
<tr>
<td>12</td>
<td>52</td>
<td>M</td>
<td>P, CSA, MMF</td>
<td>CAD</td>
<td>3.7</td>
<td>2.5</td>
<td>9 yr</td>
</tr>
<tr>
<td>13</td>
<td>57</td>
<td>F</td>
<td>P, MMF, DAC</td>
<td>LRD</td>
<td>3</td>
<td>1.3</td>
<td>4 yr</td>
</tr>
<tr>
<td>14</td>
<td>57</td>
<td>M</td>
<td>P, MMF, DAC</td>
<td>LURD</td>
<td>4</td>
<td>1.3</td>
<td>4 yr</td>
</tr>
<tr>
<td>15</td>
<td>56</td>
<td>F</td>
<td>P, MMF, DAC</td>
<td>CAD</td>
<td>7</td>
<td>1.2</td>
<td>4 yr</td>
</tr>
</tbody>
</table>

<sup>a</sup> Patients in bold indicate those demonstrating regulation.  
<sup>b</sup> Patients had two assays performed.  
<sup>c</sup> P, prednisolone; CSA, cyclosporin; TAC, tacrolimus; MMF, mycophenylate mofetil; DAC, daclizumab; A, azathioprine.

**Table 2.** Characteristics of patients with a history of rejection and timing of assay in relation to date of transplantation

<table>
<thead>
<tr>
<th>Patient&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Age (yr)</th>
<th>Gender</th>
<th>Immunosuppression</th>
<th>Graft</th>
<th>HLA-DR Mismatch</th>
<th>Serum Creatinine (mg/dl)</th>
<th>Time of Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>25</td>
<td>F</td>
<td>P, MMF</td>
<td>LRD</td>
<td>7</td>
<td>3.4</td>
<td>3 yr</td>
</tr>
<tr>
<td>17</td>
<td>34</td>
<td>F</td>
<td>P, CSA, MMF</td>
<td>LRD</td>
<td>7</td>
<td>2.6</td>
<td>6 yr</td>
</tr>
<tr>
<td>18</td>
<td>47</td>
<td>M</td>
<td>P, TAC, MMF</td>
<td>LURD</td>
<td>1.4</td>
<td>2.0</td>
<td>1 yr</td>
</tr>
<tr>
<td>19</td>
<td>39</td>
<td>M</td>
<td>P, CSA, MMF</td>
<td>LRD</td>
<td>7</td>
<td>2.6</td>
<td>3 yr</td>
</tr>
<tr>
<td>20</td>
<td>55</td>
<td>F</td>
<td>P, CSA, MMF, DAC</td>
<td>LRD</td>
<td>1</td>
<td>2.6</td>
<td>4 yr</td>
</tr>
<tr>
<td>21</td>
<td>53</td>
<td>F</td>
<td>P, TAC, MMF, DAC</td>
<td>LRD</td>
<td>7</td>
<td>1</td>
<td>4 yr</td>
</tr>
<tr>
<td>22</td>
<td>53</td>
<td>F</td>
<td>P, TAC, MMF</td>
<td>LURD</td>
<td>15</td>
<td>2.2</td>
<td>3 yr</td>
</tr>
<tr>
<td>23</td>
<td>41</td>
<td>M</td>
<td>P, CSA, A</td>
<td>CAD</td>
<td>1</td>
<td>1.3</td>
<td>11 yr</td>
</tr>
</tbody>
</table>

<sup>a</sup> Patients in bold indicate those demonstrating regulation.
The mechanism of suppressive action of CD4+CD25+ cells may be through secretion of suppressive cytokines such as IL-10 (14,41,42), although other reports suggest activity is independent of soluble cytokines such as IL-10 or TGF-β (23). In one of two patients tested in whom there was an increase in the frequency of IFN-γ producing alloreactive T cells after CD25+ depletion, there was a decrease in frequency of IL-10 producing alloreactive T cells (not shown). Thus, it appeared, at least in that one patient, that the regulatory CD25+ cells were significant producers of the Th2 cytokine IL-10.

The phenotype of these regulatory cells was examined by flow cytometry concentrating on signals that may serve to maintain the survival and expansion of this population. We compared CD4+CD25high and CD4+CD25low cells for their expression of costimulatory molecules. Interestingly, in unstimulated cells, there were almost twice as many CD134+ cells in the CD4+CD25high compared with the CD4+CD25low population, whereas ICOS was present on a smaller percentage of the CD4+CD25high cells, and PD-1, PD-L1, and CTLA4 expression were not markedly different in the two populations (Figure 4).

Discussion

Our results demonstrate, we believe for the first time, that in 40% of stable renal transplant recipients there is evidence of an antigen-specific regulatory CD25+ cell population suppressing responsiveness toward alloantigens, as judged by the upregulation in frequencies of IFN-γ producing indirect pathway alloreactive peripheral blood lymphocytes. It is possible that the regulatory cell population in some patients may predominately contained within the allograft and thus less amenable to detection in peripheral blood samples, as has been recently demonstrated in a murine model (43). The regulatory cells appear early in the time course after transplantation and may persist for months to years. Preliminary data also suggest that they appear to play a role in limiting the phenomenon of epitope spreading, because depletion at different time points uncovered a shifted set of peptides to which the alloreactive T cells responded. In one patient, after CD25 depletion, reactivity was shown to new peptides within the same HLA molecule and to a second mismatched molecule to which there had been no previous response. However, this needs to be verified in a prospective analysis of a larger group of patients, currently underway. If confirmed, this finding is in keeping with previous reports of epitope spreading in renal (33) and cardiac transplant recipients (34) and is of significant interest because epitope spreading has been implicated as a mechanism for the continual recruitment of naïve alloreactive T cells, which may contribute toward the smoldering process of chronic rejection (25). In one patient, there was evidence of a loss of regulatory activity at 7 mo posttransplantation, which was followed by an acute rejection episode and graft loss with endstage renal failure within 1 yr of transplantation. Whether these two events are causally linked remains to be confirmed in our prospective study, but the data are provocative: if confirmed, they may provide a highly clinically relevant immunologic assay for predicting graft outcome.

Our data suggest that there are other important issues to be considered, such as why only 40% of the stable patients demonstrated regulation by these cells, and what accounts for the low alloreactive T cell frequencies in the others? It is interesting to note that low alloreactive T cell frequencies may occur in patients with or without a history of acute rejection. Our prior study demonstrated that a cut-off alloreactive T cell frequency of <60/million statistically separated those patients without rejection and with stable graft function from those with history of rejection. However, subclinical rejection and ongoing immune reactivity may occur in “stable” patients. Hence, if we stratified our patients according to the degree of immunologic responsiveness toward their mismatched HLA-DR allopeptides (38), then regulation is seen in 8 (47.1%) of 17 low responders compared with 0 of 8 high responders. These data suggest that CD25-mediated regulation is limited to patients in whom there is relative hyporesponsiveness toward their alloantigens. What of the other patients in whom there is relative hyporesponsiveness but no evidence of CD25-mediated regulation? Likely mechanisms acting to limit the alloresponses in these include anergy (possibly secondary to immunosuppres-
sion) and clonal deletion, which have been demonstrated to be important mechanisms for donor hyporesponsiveness in the direct allorecognition pathway (44). In light of recent data demonstrating the presence of regulatory cell populations within the graft, such cells may be sparse in the peripheral circulation, making sampling errors one further reason for a lack of effect in the remaining patients (43). Therefore, further studies are required to investigate the mechanisms of hyporesponsiveness in patients without evidence of regulation.

Our study provides novel data on the potential functions of regulatory cells on indirect pathway alloresponses in transplant patients. Interestingly, these cells do not appear to contribute to direct pathway hyporesponsiveness, demonstrated in patients after transplantation (45,46), where T cell anergy and death are the main regulatory mechanisms (R. Lechler, personal communication). We believe that our observations will stimulate further research into this area in transplant recipients and in patients with other immunologic diseases and malignancies.

This study did not attempt to address the molecular mechanisms by which these regulatory cells exert their effect; rather, it asks whether these cells do exist in transplant recipients and are functional in alloimmune responsiveness. In one of two patients demonstrating regulation, there was evidence for the CD25\(^+\) cells producing significant amounts of the inhibitory cytokine IL-10, although no firm conclusions can be drawn from this because of the small numbers. Numerous publications have attempted to address the mechanisms of action of these regulatory cells, although they remain poorly defined. In general, it appears that the cells require direct cell-cell contact with their targets and that their effect is mediated in certain cases through inhibitory cytokines such as IL-10 (14,41,42), although this is not a universal finding (22). In addition, others have emphasized a central role for the inhibitory costimulatory molecule CTLA4 (18–20). Moreover, recent claims have been made for a number of TNF superfamily members, especially GITR and its receptor playing a central role (47,48).

Our phenotype analysis demonstrated that there appear to be interesting differences in the expression of molecules required...
for cell activation and survival. In particular, more of the CD4^+CD25^{high} cells express CD134 (implicated in survival of memory cells), whereas fewer express ICOS, and there was no difference in expression of CTLA4, PD-L1, or PD-1 (two negative signaling pathways). The functional significance of these findings is currently being addressed, although they may partly explain the persistence and relative hyporesponsiveness of these cells. The suggestion that these cells may act through expression of PD-L1 ligation PD-1 or on activated T cells seems unlikely as equivalent percentages of regulatory CD4^+CD25^{high} cells expressed PD-L1 as CD4^+CD25^{-} cells. This is in keeping with a report demonstrating minimal loss of suppressive ability in the presence of blocking anti-PD-L1 mAb (23).

Further investigation is required to fully understand the molecular basis of regulation in humans with autoimmune diseases and in transplant recipients. It is interesting to note that regulatory cells are functional in stable transplant patients despite treatment with conventional immunosuppression. This is consistent with the observations that the indirect pathway of allorecognition is more resistant to the effects of conventional immunosuppressive agents (45), so it may not be surprising that indirect pathway regulatory cells are similarly unaffected. Furthermore, this resistance makes these cells an even more attractive target for future manipulation and therapy. Moreover, despite induction therapy with an anti-IL-2 receptor antibody, regulatory CD25^{+} cells can still be generated, demonstrated in three (50%) of six patients who received such

Figure 3. Graphs showing the changes in regulation by CD25^{+} cells of allopeptide responses over time in patient 5 at 3 mo (a) and at 7 mo (b), and patient 7 at 3 yr (c) and at 4 yr (d).
induction therapy. Further analysis of patients treated with anti-IL2 antibody induction therapy is required to establish whether such therapy may be not only permissive for the generation of regulatory cells but may actually promote their production.

One of the major challenges in developing new strategies to minimize immunosuppression or induce tolerance in transplant recipients is the lack of reproducible assays to detect a hypo-responsive state. Recently, several reports have been published describing novel assays that are useful in diagnosing rejection noninvasively or possibly detecting incipient rejection (49–51). VanBuskirk et al. (52) also reported on a human-to-mouse trans-vivo delayed-type hypersensitivity response assay that may be useful in the diagnosis and/or monitoring of transplant patients for allograft acceptance. We now report, using a novel assay system, the existence and functional significance of regulatory cells in human transplant recipients. Our assay system is potentially applicable to patients suffering from autoimmune diseases in which the antigenic target is known. If validated in prospective trials, such assays, perhaps coupled with the previously published trans-vivo delayed type hypersensitivity assay (52) may at last provide the rationale to develop protocols for tapering immunosuppression in stable transplant recipients or patients with quiescent autoimmune disease, and they may be useful for monitoring patients enrolled in ongoing and future tolerance trials (3).

Acknowledgments

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References


Figure 4. Flow cytometry analysis of unstimulated CD4+CD25high (left) and CD4+CD25− cells (right), demonstrating altered levels of CD134 and ICOS expression, but similar levels of PD-L1, PD-1, and CTLA4.


29. Dolloul AH, Chmouezis E, Nko F, Fun-Leung W-P: Adoptively transferred CD4+ lymphocytes from CD8–/– mice are sufficient to mediate rejection of MHC class II or Class I disparate skin grafts. *J Immunol* 156: 4114–4119, 1996


