Reversibility with Interleukin-2 Suggests that T Cell Anergy Contributes to Donor-Specific Hyporesponsiveness in Renal Transplant Patients

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Abstract. Data from various rodent models have implicated a role for anergic T cells in the maintenance of self and transplantation tolerance. The relevance of donor-specific T cell anergy to clinical transplantation, however, has not been demonstrated. Previous studies have reported that recipients of solid organ transplant often have reduced frequencies of CD4+ T cells with anti-donor direct pathway allospecificity after transplantation. The underlying mechanism(s) of this donor-specific hyporesponsiveness is unclear but likely to contribute to the diminished immunosuppressive requirement of transplant patients with time after transplantation. This study shows that ex vivo treatment of CD4+ T cells from renal transplant recipients with IL-2 could specifically increase the anti-donor frequency in all the patients with evidence of donor-specific hyporesponsiveness. It also shows that the IL-2–induced recovery of anti-donor frequency is unlikely to result from nonspecific stimulation or selective clonal expansion of activated, allospecific CD4+ T cells. Taken together, the data suggest that T cell anergy plays an important role in the direct pathway hyporesponsiveness that evolves in many human renal transplant recipients.

Heightened interest in clinical transplant tolerance stems from the acquisition of insights into the mechanisms and the advent of novel biologic reagents that may promote such a tolerant state. For these reasons, it is necessary to understand the evolution of anti-donor responses using current approaches to immunosuppression. We and others (1–3) have previously described that a substantial proportion of patients with kidney or other solid organ transplants have markedly reduced numbers of T cells with direct allospecificity, particularly in long-term live-related transplant recipients. Although markedly decreased frequencies of direct pathway T cells does not equate to tolerance, it is tempting to speculate that mechanisms that contribute to peripheral T cell tolerance may be operative in diminishing the anti-donor T cell repertoire. It is also important to note that direct pathway hyporesponsiveness can often be observed in patients with chronic transplant rejection. In these patients, it appears that the indirect pathway of anti-donor alloimmunity provides an immunologic drive to this indolent process.

Two attractive, yet not mutually exclusive, candidate mechanisms of direct pathway hyporesponsiveness are deletion and anergy. In vitro studies using renal epithelial cells as antigen-presenting cells can result in T cell anergy (4), and peripheral deletion has been shown to be an important mechanism in maintaining tolerance to foreign antigens (5,6). In this study, we investigated whether these mechanisms are operative in the maintenance of donor-specific hyporesponsiveness. Because of the lack of known specific marker of anergic cells, we have relied on the functional properties of anergic T cells to distinguish these possibilities. One of the characteristics of anergic T cells is that their reactivity can be restored by cell division driven by added interleukin-2 (IL-2) (7). Thus, if the reduction of anti-donor frequency is a consequence of T cell anergy, ex vivo treatment of T cell with IL-2 may result in restoration of the anti-donor frequency. On the other hand, if deletion is the underlying mechanism of the donor-specific hyporesponsiveness, then the anti-donor frequency will be unchanged with IL-2 treatment.

Materials and Methods

Patients

All patients in this study were adult recipients of a kidney transplant at the Hammersmith Hospital at least 6 mo before the study. All patients included in this study had functional transplants at the time of study. For recipients of cadaveric donor organs, only those with stored donor cells (donor spleen/lymph node cells or peripheral blood mononuclear cells [PBMC]) were studied. Relevant clinical details are given in Table 1. Third party controls were chosen, to the extent possible, so that the number of mismatches for human leukocyte antigen (HLA)-DR was the same as that between the donor and recipient. If the donor and recipient shared a DR allele, a third party
Table 1. Clinical details

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<th>Age (yr)</th>
<th>Cr</th>
<th>BP</th>
<th>Proteinuria</th>
<th>Previous rejection</th>
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<th>Immunosuppressants</th>
<th>No. of mo Since Tx</th>
<th>Proven Chronic Rejection</th>
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Donor-specific hyporesponsiveness

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No hyporesponsiveness (anti-donor freq < anti-third-party freq)

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Cr, serum creatinine (μmol/ml); Tx, transplantation; P, prednisolone; C, cyclosporin A; A, azathiapine; M, mycophenolate; HD, hemodialysis; LRD, living-related transplant; APKD, adult polycystic kidney disease; GN, glomerulonephritis; FSGS, focal segmental glomerular sclerosis; HUS, hemolytic uremic syndrome. Only 1 patient is black (P3). Patients P3, P4, and P8 received ALG induction therapy. Patients P3 and P4 had delayed graft function.
stimulator was chosen, when possible, that expressed the shared DR allotype but expressed a different DR allomantigen. This study was approved by the ethics committee of Hammersmith Hospital.

**Cells and Culture Conditions**

**Culture Medium.** In all in vitro assays involving human cells, RPMI-1640 medium supplemented with l-glutamine (2 mmol/L; Life Technologies BRL, Paisley, UK), penicillin/streptomycin (100 IU/ml and 100 µg/ml, respectively; Life Technologies BRL), amphotericin (500 ng/ml; Life Technologies BRL), and gentamicin (2 µg/ml; Sigma, Poole, UK), referred to as supplemented RPMI, with 10% human AB serum (Harlan Sera-Lab, Loughborough, UK) was used. All cells were incubated at 37°C with 5% CO2 and 95% air.

**CTLL-2 Cells.** This murine cell line responds to murine IL-2 and IL-4 but only to human IL-2. Cells were maintained in culture in supplemented RPMI medium with 10% fetal calf serum (FCS; BioWhittaker, Wokingham, UK) and 10 U/ml recombinant human (rh)-IL-2 (Boehringer Mannheim, Mannheim, Germany). They were subcultured every 2 to 3 d. Cells were reset in medium without IL-2 overnight before use in assays.

**Responder Cells.** PBMC were isolated from peripheral blood samples from patients by density gradient centrifugation over Lymphoprep (Nycomed, Birmingham, UK). For isolation of CD4⁺T cells, PBMC were incubated in medium supplemented with 2% FCS at 37°C on tissue culture dishes for 45 min to remove adherent cells. Nonadherent cells were collected and washed. Non-CD4⁺ cells were depleted by incubation with a cocktail of monoclonal antibodies (anti-CD8 [OKT8], anti-CD19 [BU12], anti-HLA-DR [L243], collected from supernatants of hybridomas, and anti-CD56 [B159; PharMingen, San Diego, CA]) followed by magnetic bead (Goat anti-mouse pan-IgG beads; Dynal, Wirral, UK) separation according to manufacturer’s protocol. The purity of the cell population was measured by flow cytometry and was always above 90%. The responder cells were then separated into two fractions, one being used immediately in limiting dilution analysis (LDA). The other fraction was incubated with 30 U/ml rh-IL-2 for 3 d following by resting in culture medium without exogenous IL-2 for 24 h before being used in LDA.

**Stimulator Cells.** Spleen cells or lymph node cells were provided by the tissue-typing laboratory, Hammersmith Hospital. Cells were isolated from small portions of spleen or lymph node recovered at the time of organ retrieval. The spleen was injected with ice-cold RPMI medium, and the cells were washed and separated by density centrifugation. For live-related donors and third-party controls, peripheral blood samples were obtained, and PBMC were isolated as above. Cells were then aliquoted and cryopreserved until required. All stimulator cells were gamma-irradiated (30 Gy) before use. In the case of autologous mixed lymphocyte reaction culture, irradiated CD3-depleted PBMC were used as stimulator cells. PBMC were incubated with supernatant of anti-CD3 mAb (OKT3), followed by magnetic bead (Dynal) separation.

**Limiting Dilution Assays**

Seven serial dilutions of responder CD4⁺ cells in 24 replicates were co-cultured with 5 × 10⁴ irradiated stimulator cells per well in round-bottomed 96-well plates. For the estimation of anti-tetanus frequencies, 2 × 10⁴ irradiated autologous CD3-depleted PBMC (pulsed with tetanus toxoid [Evans vaccines, UK], at a dilution of 1:1000) were used as stimulator cells. The exact number of responder cells added per well depended on the number of responder cells obtained from each patient, with the top dilutions ranging from 7500 to 80,000 cells per well. After 4 d, 100 µl of supernatant from each well was collected and transferred to another round-bottomed 96-well plate, and IL-2 production was estimated by bioassay using the murine CTLL-2 cells. Essentially, 5000 CTLL-2 cells were added to the supernatant and cultured for 48 h, and thymidine incorporation was added in the last 12 h of the culture. The thymidine incorporation was assessed by liquid scintillation spectrometry. The sensitivity of each assay was determined separately by measuring the thymidine incorporation by the CTLL-2 cells at a titration of rh-IL-2 concentrations. Wells were scored positive if the counts were above three SD of the average count of the control (wells containing only irradiated stimulator cells without responder cells) or the lowest detection limit of the assay (calculated from the sensitivity curve), whichever was higher.

**Calculation of Precursor Frequencies.** The frequency, confidence interval, and χ² value for each assay were calculated by the maximum likelihood method using GLIM software (NAG Ltd., Oxford, UK). For all data, a probability estimate of the data conforming to “single hit” kinetics was calculated. Hyporesponsiveness was considered significant if the confidence intervals of the anti-donor frequency and the anti–third-party frequency did not overlap and if the measured anti-donor frequency was less than 1 in 10,000. Changes in frequencies after IL-2 treatment were regarded as significant if the 95% confidence limits of the frequencies before and after IL-2 treatment did not overlap.

**Results**

**Donor-Specific Hyporesponsiveness Was Detected in 6 of 13 Patients**

In agreement with previous studies from our laboratory and others, we found that a significant portion of patients had reduced anti-donor, compared with anti–third-party, CD4⁺ T cell frequencies. Thirteen patients were studied, six of which demonstrated donor-specific hyporesponsiveness (Figure 1). In three other patients, the anti-donor CD4⁺ T cell frequencies were significantly lower than anti–third-party frequencies but higher than 1 in 10,000. The maintenance of high anti–third-party frequencies argues against this being the result of nonspecific immunosuppression due to the drug therapy the patients received. Although the anti-donor frequencies pretransplant were not measured in this study, we have previ-
ously reported that reduction in anti-donor CD4⁺ T cell frequency occurred following transplantation (8).

**Treatment of IL-2 Does Not Induce Significant Proliferation of Peripheral Blood CD4⁺ T Cells**

Before investigating the effect of *ex vivo* treatment of CD4⁺ T cells with IL-2 on the anti-donor frequency, we investigated whether IL-2 alone can lead to proliferation of CD4⁺ T cells and/or alter the anti–third-party frequency. To exclude any nonspecific effect of IL-2 in enhancing immune responses of CD4⁺ T cells, we investigated whether incubating purified CD4⁺ T cells with IL-2 would result in significant proliferation and expansion of the cells, which may affect the result of the subsequent limiting dilution analyses. CD4⁺ T cells from peripheral blood of healthy volunteers or renal transplant recipients were purified and were incubated with 30 units of rh-IL-2. Proliferation was measured daily. The cells were then washed thrice and cultured for an additional 3 d without IL-2, and proliferation was again measured daily during this rest period. One healthy volunteer and three patients were studied. In all experiments, the CD4⁺ T cells showed minimal proliferation during the 3-d incubation with IL-2. Upon withdrawal of exogenous IL-2 in the culture, their proliferation returned rapidly to the baseline level after 24 h. Figure 2 shows representative data from a normal subject and a renal transplant recipient.

**Figure 2.** Proliferative responses of CD4⁺ cells to IL-2 in (A) a normal subject and (B) a renal transplant recipient. Purified CD4⁺ cells (1 × 10⁵) were cultured with or without 30 U/ml recombinant human (rh)-IL-2. Proliferation was measured daily. CD4⁺ cells that had been treated with IL-2 were washed twice before proliferation was measured. After 3 d, the cells were washed and cultured for an additional 3 d without exogenous IL-2, and proliferation was measured daily. The responses to phytohemagglutinin (PHA) (2 μg/ml) plus IL-2 (10 U/ml) were used as positive controls and were performed at days 0, 4, and 7.

**Figure 3.** Anti–third-party frequencies before (filled squares) and after (open squares) IL-2 treatment. N1 to N3 are normal subjects, and P00 is a renal transplant recipient.

Treatment with IL-2 Does Not Affect Anti–Third-Party Frequencies as Measured by Limiting Dilution Analysis

Another question concerning the treatment of CD4⁺ T cells with IL-2 was whether it would affect the anti–third-party frequency and make any changes in anti-donor frequency by IL-2 treatment difficult to interpret. Three normal subjects and one renal transplant recipient were studied. In all four cases, the third-party frequencies were remarkably similar before and after IL-2 treatment and their 95% confidence intervals overlapped (Figure 3). Not only did this result exclude nonspecific changes in frequency, but it also indicated that cell viability was maintained during the 4 d of culture before performing the limiting dilution analysis.

**IL-2 Specifically Increased the Direct Anti-Donor Frequencies in all Patients who had Donor-Specific Hyporesponsiveness**

Among the six patients in whom donor-specific hyporesponsiveness was demonstrated, IL-2 significantly increased the frequencies against donor cells in all these patients (Figure 4). In five of these patients, the anti-donor frequency was increased to the extent that the 95% confidence intervals of anti-donor and anti–third-party frequencies overlapped after IL-2 treatment. In the remaining patient, the anti-donor frequency was increased tenfold. The third party frequencies were unaltered. We used Wilcoxon rank test to determine whether the changes in anti-donor and anti–third-party frequencies were statistically significant. The respective P values for changes in anti-donor and anti–third-party frequencies were 0.028 and 0.917, indicating that the change in anti-donor frequencies was statistically significant while the third party frequency was not altered by the treatment with IL-2. Furthermore, while the anti-donor frequencies in this group of patients were significantly lower than the anti-third party frequencies before the IL-2 treatment (P = 0.028), this difference was obliterated after IL-2 treatment (P = 0.753). In contrast, among the patients in whom no donor-specific hyporesponsiveness was demonstrated, treatment with IL-2 *ex vivo* did not increase the
anti-donor frequency \((P = 0.398\); for patient P5, although the anti-donor frequency was marginally increased after IL-2 treatment, the confidence intervals between the anti-third party and anti-donor frequencies were overlapping before and after IL-2 treatment). These observations suggest that the reversal of hyporesponsiveness with IL-2 is not a consequence of nonspecific enhancement of immune reactivity but reflects the reversal of anergy in donor-specific CD4\(^+\) T cells.

Specific Enhancement of Anti-Donor CD4\(^+\) T Cell Frequency Following Treatment with IL-2 Is Unlikely to Be Due to Clonal Expansion of Activated Donor-Specific CD4\(^+\) T Cells

An alternative explanation for the increase in anti-donor frequency after IL-2 treatment was the expansion of small numbers of activated donor-specific T cells. This selective clonal expansion may be difficult to detect. To examine this possibility, we studied the response of a healthy volunteer to tetanus toxoid after a booster dose of tetanus vaccine. We measured the anti-tetanus frequency of CD4\(^+\) T cells before and after the vaccination and after treatment of CD4\(^+\) T cells with IL-2 \textit{ex vivo} post-vaccination. The frequency in the absence of added antigen was used as control. After the booster vaccine, we anticipated an increased number of activated anti-tetanus CD4\(^+\) T cells, creating an ideal situation for the investigation of the responses of activated CD4\(^+\) T cells to the treatment of IL-2. If treatment with IL-2 leads to expansion of activated CD4\(^+\) T cells, then we would expect to observe an increase in anti-tetanus frequency after IL-2 treatment. As
shown in Figure 5, we found that, after vaccination, the anti-tetanus frequency increased more than 20-fold from 1:280,000 to 1:12,500. However, when the CD4+ T cells were treated with IL-2, the anti-tetanus frequency was halved. This implies that the donor-specific increase in frequency with IL-2 treatment observed in renal transplant recipients is unlikely to result from clonal expansion of activated anti-donor CD4+ T cells.

**Discussion**

Ever since the demonstration of anergic T cell clones in vitro (9), there has been widespread interest in their role in the maintenance of self and transplantation tolerance. Several studies using animal models have provided evidence for a role of anergic T cells in the maintenance of transplant tolerance or prolongation of graft survival (10,11), whether T cell anergy play any role in the evolution of anti-donor responses in clinical transplantation has not been examined.

We have previously reported that reduced frequencies of CD4+ T cells with direct anti-donor allo specificity was frequently observed after solid organ transplantation. This reduction of frequencies of anti-donor CD4+ T cells was more pronounced in the CD45RO+ subset of CD4+ T cells. In contrast, the frequencies of anti-third-party T cells were unaltered after transplantation (1,8). One simple explanation for these findings is that anti-donor T cells are sequestered within the graft, resulting in an apparent loss of these cells in peripheral blood. However, Orosz and Bishop (12) used sponge matrix allograft implantation technique and limiting dilution analysis to measure the frequencies of alloantigen-specific T cells at the graft site and in peripheral blood. They found that the frequencies of alloreactive helper T lymphocytes in peripheral blood and at the graft site were similar. Thus, the question of why donor-specific hyporesponsiveness is such a common phenomenon in patients with solid organ transplantation remains unanswered. Although markedly decreased frequencies of direct pathway T cells does not equate to tolerance, it is tempting to speculate that mechanisms that contribute to peripheral T cell tolerance may be operative in diminishing the anti-donor T cell repertoire.

In this study, we have demonstrated that donor-specific hyporesponsiveness can be specifically reversed by ex vivo treatment of recipient CD4+ T cells with IL-2 in all patients, consistent with the hypothesis that anergy contributed to the decrease in anti-donor frequencies. The third-party frequencies were unaffected. The consistency of the anti-third-party frequencies not only demonstrated the reproducibility of the assays but also indicated that the increase in anti-donor frequency after IL-2 is unlikely to be due to nonspecific stimulatory effect of IL-2. In this study, the cell number after IL-2 treatment of the CD4+ cells was usually reduced rather than increased. In addition, the poor proliferative response to IL-2 of the CD4+ T cells argues against such an explanation. Moreover, the ability of IL-2 to increase the anti-donor frequency was restricted only to patients who exhibited donor-specific hyporesponsiveness. Nevertheless, it remains possible that IL-2 treatment had expanded the numbers of direct allo specific T cells rather than reversed anergy. However, our observation that IL-2 treatment in vitro led to a decrease in anti-tetanus frequency in a volunteer who had a recent booster vaccine argues against such an explanation. This reduction in frequency may be a result of activation induced cell death in response to IL-2 (13,14).

It is interesting that in the three patients in whom the anti-donor CD4+ T cell frequencies were above 1 in 10,000 but nevertheless significantly reduced to anti-third party frequencies, IL-2 treatment failed to increase the anti-donor frequencies. One explanation is that other mechanism(s) is/are operative; for example, deletion and/or regulation. We have recently shown that a naturally occurring subpopulation of CD4+ T cells, characterized by the constitutive expression of CD25, has the ability to regulate allo responses (15). Interestingly, unlike anergic T cells, CD4+CD25+ cells do not proliferate in response to exogenous IL-2, and they remain hyporesponsive and retain their regulatory function after exogenous IL-2 is withdrawn. Second, it has been suggested that there are different levels of T cell unresponsiveness that are associated with different degrees of reversibility (16). Finally, it has been reported that cyclosporin may prevent the induction of anergy (17,18). However, the effect of cyclosporin on anergy induction is far from clear; many studies have reported the induction of anergy in the presence of cyclosporin (19–23). The reason for the discrepancies between different studies is unclear but may be related to the method of anergy induction and the dose of cyclosporin used. In this study, we did not find any correlation between the use of cyclosporin and the reversibility of donor-specific hyporesponsiveness with IL-2.

Another interesting question is the lifespan of anergic cells. In vitro data suggest that anergic T cell clones can be maintained for weeks (7). Our data suggest that either anergic cells have a long lifespan, or, alternatively, a dynamic process of encounter of recipient T cells with donor MHC molecules is in operation to maintain a cohort of anergic direct pathway T cells.

The finding that IL-2–driven cell division can reverse hyporesponsiveness in direct pathway T cells may have relevance to the link between systemic infections such as CMV or local infections of the urinary tract and acute rejection episodes. Conceivably, infection of the urinary tract can result in production of IL-2 locally or in the draining lymph nodes, leading to reversal of the anergic state of the allospecific T cells and consequent acute rejection. Theoretically, anergy reversal of allospecific T cells could also contribute to the process of chronic rejection; however, there was no correlation between reversible hyporesponsiveness and chronic rejection in our study. The observation that chronic rejection occurs despite hyporesponsiveness in the direct pathway T cells indicates that an alternative mechanism, such as the indirect pathway of the anti-donor T cell response, may play a key role in orchestrating the chronic rejection process. Thus, it is important to devise strategies to induce tolerance to both the direct and indirect allospecific pathway if clinical tolerance is to be achieved.

In conclusion, our findings are consistent with the existence of anergic direct pathway T cells in a proportion of renal transplant patients and suggest that anergy may be an important
contributory mechanism to the maintenance of donor-specific hyporesponsiveness in clinical transplantation.

Acknowledgments

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References