Contributions of Direct and Indirect Alloresponses to Chronic Rejection of Kidney Allografts in Nonhuman Primates


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The relative contribution of direct and indirect allorecognition pathways to chronic rejection of allogeneic organ transplants in primates remains unclear. In this study, we evaluated T and B cell alloresponses in cynomolgus monkeys that had received combined kidney/bone marrow allografts and myeloablative immunosuppressive treatments. We measured donor-specific direct and indirect T cell responses and alloantibody production in monkeys \( n = 5 \) that did not reject their transplant acutely but developed chronic humoral rejection (CHR) and in tolerant recipients \( n = 4 \) that never displayed signs of CHR. All CHR recipients exhibited high levels of anti-donor Abs and mounted potent direct T cell alloresponses in vitro. Such direct alloreactivity could be detected for more than 1 y after transplantation. In contrast, only two of five monkeys with CHR had a detectable indirect alloresponse. No indirect alloresponse by T cells and no alloantibody responses were found in any of the tolerant monkeys. Only one of four tolerant monkeys displayed a direct T cell alloresponse. These observations indicate that direct T cell alloresponses can be sustained for prolonged periods posttransplantation and result in alloantibody production and chronic rejection of kidney transplants, even in the absence of detectable indirect alloreactivity. The Journal of Immunology, 2011, 187: 4589–4597.
various tolerance-inducing immunosuppressive regimens. Lack of alloantibodies and T cell alloresponses were regularly associated with transplant tolerance. Alternatively, sustained T cell alloactivity mediated via both direct and indirect pathways or even the direct pathway alone was always detected along with the production of anti-donor Abs in monkeys undergoing chronic allograft rejection.

Materials and Methods

Animals, conditioning, and transplantations

Eighteen cynomolgus monkeys weighing 3–5 kg were used in this study (Charles River Primates, Wilmington, MA). Details of recipient–donor pair selection were previously reported (34). All the nine recipients were conditioned using our “standard regimen” consisting of total body irradiation (days −6 and −5 (1.5 Gy) followed by thymic irradiation at day −3 and −2 (7 Gy) and three injections of ATG (days −2, −1, and 0) pre-donor cell infusion. In addition to the standard conditioning, the recipients were treated as follows: M1601 received donor splenocytes (200 × 10⁶ cells/kg) as well as two injections of anti-CD40L mAbs (5c8; 20 mg/kg); M1501 was splenectomized at the time of transplantation and received two injections of anti-CD40L mAbs (20 mg/kg); M1900 and M200 were treated with two injections of anti-CD40L mAbs (20 mg/kg); M2800 was treated with anti-CD8 (8 times; 1 mg/kg) and anti-CD40L (6 times; 20 mg/kg) mAbs, the kidney transplant was removed at day 12 because of thrombosis, and a second kidney allograft from the same donor was placed at day 77 and followed for rejection thereafter; M4102 and M2702 were conditioned at day −1 (instead of day −6) and were treated with anti-CD8 (6 times; 1 mg/kg) and anti-CD40L mAbs (6 times; 20 mg/kg); M6601 and M1902 were first transplanted with an allogeneic kidney whose rejection was prevented with FK506, MMF, and prednisone; and prednisone, and they were then conditioned (day 112 and day 126, respectively), injected with donor bone marrow cells (300 × 10⁶ cells/kg), and treated with anti-CD40L mAbs (6 times; 20 mg/kg).

Blood samples

Heparinized blood was obtained from all recipient monkeys prior to transplant and at monthly intervals after postoperative day 100 when they no longer showed therapeutic levels of circulating cyclosporine. Peripheral blood and/or spleen cells from the donors were collected, processed, and frozen in 10% DMSO and stored in liquid nitrogen.

PBMC isolation

PBMCs were isolated from whole heparinized blood using a Percoll density gradient method. Briefly, whole blood was first centrifuged at 2000 rpm for 10 min to obtain an interphase layer of enriched PBMCs just below the upper plasma layer. The interphase layer was harvested and diluted 15-fold in 1× PBS and layered onto a 60% Percoll gradient (Amersham, Little Chalfont, U.K.) at a blood to Percoll ratio of 2:1. After a 30-min spin at 2000 rpm, the PBMC-rich buffy layer was harvested, and contaminating RBCs were removed by water shock treatment. PBMCs were washed and viable cells counted based on eosin-dye exclusion. PBMCs were either frozen at −80˚C and stored in liquid nitrogen or used immediately for assays.

Cell sonication

The method for cell sonication was adapted for the monkey system from established protocols previously reported for murine studies (35). Briefly, 6 million to 12 million PBMCs (adjusted to 3 × 10⁶ cells per milliliter) in 50-ml culture tubes were sonicated over ice for 2–3 min (Microson Cell Disruptor; Misonix) until whole cells were no longer visible under the microscope. PBMCs were washed and viable cells counted based on eosin-dye exclusion. PBMCs were either frozen at −80˚C and stored in liquid nitrogen or used immediately for assays.

Table I. Monkeys used in this study

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Donor</th>
<th>Treatment</th>
<th>Rejection</th>
<th>CHR Detection, Day</th>
<th>Creatinine Elevation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1501</td>
<td>301</td>
<td>STD, SPLX, anti-CD40L</td>
<td>CHR</td>
<td>386</td>
<td>Day 487</td>
</tr>
<tr>
<td>1900</td>
<td>3099</td>
<td>STD, anti-CD40L</td>
<td>CHR</td>
<td>369</td>
<td>None</td>
</tr>
<tr>
<td>1601</td>
<td>2501</td>
<td>Donor splenocytes, anti-CD40L</td>
<td>CHR</td>
<td>231</td>
<td>Day 274</td>
</tr>
<tr>
<td>6601</td>
<td>5001</td>
<td>Post-Tx BMT, anti-CD40L, anti-CD8</td>
<td>CHR</td>
<td>122</td>
<td>Day 369</td>
</tr>
<tr>
<td>4102</td>
<td>4202</td>
<td>STD (day −1), anti-CD40L, anti-CD8</td>
<td>CHR</td>
<td>196</td>
<td>Day 280</td>
</tr>
<tr>
<td>200</td>
<td>1799</td>
<td>STD, anti-CD40L</td>
<td>TOL</td>
<td>—</td>
<td>None</td>
</tr>
<tr>
<td>2800</td>
<td>3800</td>
<td>STD, anti-CD40L, anti-CD8</td>
<td>TOL</td>
<td>—</td>
<td>None</td>
</tr>
<tr>
<td>1902</td>
<td>2102</td>
<td>Post-Tx BMT, anti-CD40L, anti-CD8</td>
<td>TOL</td>
<td>—</td>
<td>None</td>
</tr>
<tr>
<td>2702</td>
<td>1502</td>
<td>STD (day −1), anti-CD40L, anti-CD8</td>
<td>TOL</td>
<td>—</td>
<td>None</td>
</tr>
</tbody>
</table>

Nine recipient–donor pairs were studied. Five recipients of allogeneic kidney transplants developed chronic rejection and displayed an elevation of their serum creatinine levels as indicated in the table. Four recipients never exhibited signs of chronic rejection and had normal creatinine levels throughout the study and were referred to as tolerant. The third column shows the protocols used to treat each recipient.

BMT, bone marrow transplantation; Post-Tx, posttransplantation; SPLX, splenectomy; STD, standard regimen (see Materials and Methods); TOL, tolerant.
microscope. The contents were further centrifuged at 1700 rpm for another 10 min and supernatants harvested. The volumes of supernatants were further adjusted to deliver the desired cell equivalent amounts of PBMC sonicate to be used in the assay.

**ELISPOT assays of direct and indirect pathway alloresponses**

Direct and indirect T cell alloresponses were measured as previously described in mice and monkeys (33–37). Briefly, ELISPOT plates were precoated with 5 μg/ml capture Abs against IFN-γ (Mabtech, Mariemont, OH) and stored overnight at 4°C. The plates were blocked for 1 h with PBS containing 1% BSA fraction V (A1933; Sigma) followed by three washes in PBS. Responding PBMCs (3 x 10^5) were added to each well of a 96-well ELISPOT plate in 100 μl RPMI 1640 supplemented with 10% pooled naive monkey serum and L-glutamine, penicillin/streptomycin, and HEPES buffer. The responding cells were cocultured with an equal number of irradiated stimulating cells (for direct allostimulation), or the cell equivalent number of sonicated cells (for indirect allostimulation), or nonstimulated (in medium alone) (Supplemental Fig. 1), or with PHA at 1 μg/ml (Sigma). After a 48-h incubation at 37°C, the plates were washed and biotinylated detection Abs (Mabtech) were added, and the plates were maintained at 4°C for an additional overnight incubation. After four washes with PBS/Tween, streptavidin HRP conjugate in PBS/BSA (no. PO397; Dako, Glostrup, Denmark) was added for at least 2 h at room temperature, followed by an additional six washes. Development was done with aminoethylcarbazole (10 mg/ml in N,N-dimethylformamide) freshly prepared in 0.1 M sodium acetate buffer (pH 5) mixed with 30% H₂O₂. The resulting spots were counted with a computer-assisted ELISPOT image analyzer (Cellular Technology, Cleveland, OH).

**Statistical methods**

Standard deviation of the ratio was estimated by the Δ method.

**Results**

**Chronic rejection versus tolerance in recipients of an allogeneic kidney transplant**

All recipient monkeys exhibited low levels (<5%) and short-term mixed lymphocyte chimerism (<2 mo) (data not shown). None of the nine monkeys selected in this study underwent acute rejection. Kidney transplant biopsies were performed at different time points after transplantation and analyzed for the presence of chronic humoral rejection (CHR). Five recipients developed CHR detected from day 122 to day 386 posttransplantation (CHR monkeys: M1501, M1900, M1601, M6601, M4102), whereas the remaining four recipients never developed any pathological signs of chronic rejection and were subsequently referred to as tolerant (TOL monkeys: M200, M2800, M1902, M2702) (Fig. 1, Table I). Additionally, the creatinine levels were serially monitored after transplantation in each of the recipients. As shown in Fig. 2A, four of the five recipients developing CHR on allograft biopsy displayed elevated creatinine blood levels (>2), which became detectable between 1 and 9 mo after the histopathological diagnosis of CHR (Table I). A slight but not significant increase of creatinine level was detected at day 400 in the fifth recipient M1900 (Fig. 2A). In contrast, none of the tolerant monkeys exhibited any increase of their creatinine levels (Fig. 2B).

**Direct and indirect pathway T cell alloresponses in tolerant versus CHR recipients**

Next, we evaluated the alloresponses by T cells collected from each of the tolerant and CHR monkeys. Direct and indirect T cell alloresponses were tested as previously described (35, 37, 36). Serial measurements of the pre- and posttransplant anti-donor responses recorded in CHR and tolerant monkeys are shown in Figs. 3 and 4 and summarized in Table II. In each panel (Figs. 3, 4), the x axis represents the mean Δ IFN-γ spots per million T cells (Δ spm), corresponding with the mean spm ± SD obtained with T cells stimulated with donor cells/sonicates minus the mean spm obtained with T cells stimulated with medium. In each panel (Figs. 3, 4), the y axis represents the mean stimulation index (SI) ± SD corresponding with the mean spm obtained with T cells stimulated with donor cells/sonicates divided by the mean spm obtained with T cells stimulated with medium. The pretransplant values are represented by open symbols, and the posttransplant values are displayed as closed symbols. This type of scattered representation provides an accurate picture of the response, which takes into account both the actual spm and the SI. Each value corresponds with one time point measured posttransplantation (as indicated in Figs. 3 and 4). We considered as positive all the values that were higher than the mean pretransplant values by more than three times SI and provided a mean Δ spm more than three times the SD obtained with T cells stimulated with medium alone.

Prior to transplantation, some recipients exhibited a substantial direct response against their donor, a phenomenon that is associated with the presence of alloreactive memory T cells in these monkeys (36). These cells have been shown to derive from prior cross-reactive expansion/differentiation after exposure to microbes (38–40). The levels of posttransplant direct alloreactivity increased in these monkeys as well as in the other monkeys that subsequently developed CHR (Fig. 3, left panels). Conversely, no posttransplant
direct alloreactivity was detected in three of the four tolerant monkeys. Only M1902 displayed a high and sustained direct alloresponse for up to 700 d posttransplantation (Fig. 3, right panels). In contrast, no indirect alloresponse was detected pre-transplantation in any of the nine monkeys tested (Fig. 4), a result

**FIGURE 3.** Kinetics of direct alloresponses in CHR and tolerant monkeys. The direct alloresponses were measured using recipient PBMCs collected pre-transplantation (day 0) and at different time points posttransplantation in a series of monkeys developing CHR (left panels) and tolerant monkeys (right panels). To measure direct alloreactivity, recipient T cells were stimulated in vitro with donor APCs as described in Materials and Methods. The x axis represents the mean Δ spm (IFN-γ spots per million T cells) ± SD corresponding with the mean numbers of spm obtained with T cells exposed to donor cells ± SD minus the mean numbers of spm ± SD obtained with T cells exposed to medium. The y axis represents the mean SI ± SD corresponding with the mean numbers of spm obtained with T cells exposed to donor cells ± SD divided by the mean numbers of spm ± SD obtained with T cells exposed to medium. Closed symbols correspond with the values measured posttransplantation. Open symbols correspond with the values measured pre-transplantation. For values considered as positive (i.e., higher than pretransplant values and at least three times higher than both the Δ spm and SI), the time point is indicated. TOL, tolerant.

**FIGURE 4.** Kinetics of indirect alloresponses in CHR and tolerant monkeys. The indirect alloresponses were measured using recipient PBMCs harvested pretransplantation (day 0) and at different time points posttransplantation in a series of monkeys developing CHR (left panels) and tolerant monkeys (right panels). To assess the indirect alloresponse, recipient T cells were stimulated with donor-derived sonicates as described in Materials and Methods. The x and y axes represent the mean Δ spm ± SD and mean SI ± SD established as described in the legend of Fig. 3. For values considered as positive (i.e., higher than pretransplant values and at least three times higher than both the Δ spm and SI), the time point is indicated. TOL, tolerant.
consistent with previous observations in mice and swine (35, 41). Posttransplantation, two of the five CHR monkeys, M1900 and M1601, mounted a vigorous indirect alloresponse, first detectable 200–300 d after placement of allogeneic kidneys (Fig. 4, left panels). In contrast, no indirect alloreactivity was detected in the three other CHR recipients (M1501, M6601, and M4102) (Fig. 4, left panel) or in any of the four tolerant monkeys (Fig. 4, right panels).

Alloantibody responses in tolerant versus rejecting monkeys

Next, we investigated the presence of donor-specific alloantibodies in serum samples serially collected from CHR and tolerant recipients. A mean fluorescence intensity (MFI) greater than 55 was considered positive as described elsewhere (42). The results of these experiments are shown in Fig. 5 and summarized in Table II. No alloantibodies were detected pretransplantation. A mean fluorescence intensity (MFI) greater than 55 was considered positive as described elsewhere (42). The results of these experiments are shown in Fig. 5 and summarized in Table II. No alloantibodies were detected pretransplantation. No alloantibodies were detected pretransplantation. No alloantibodies were detected pretransplantation.

Influence of MHC matching on chronic rejection versus tolerance of kidney allografts

MHC gene disparity between donors and recipients represents an essential element in the acute rejection of allogeneic transplants. However, the influence of MHC gene matching/mismatching on chronic allograft rejection and susceptibility to teratogenesis is not well understood. To address this question, we determined whether the MHC class I and II genes were expressed by each of the recipient–donor monkey pairs. The predicted MHC class I (A and B) and II (DP, DQ, and DR) alleles were expressed by our Mauritian-origin cynomolgus monkeys and were characterized using a microsatellite-based genetic technique (43). The results of the genotyping for each recipient–donor monkey pair are shown in Fig. 6. Each color represents a distinct haplotype.

The degree of haplotype matching between donors and recipients is shown in Table II. For MHC class I, we determined the number of MHC class I alleles shared by the donor and the recipient. The second subcolumn indicates the presence (+) or absence (−) of haplo-matching.

Table II. Summary of the results obtained in CHR and tolerant monkeys

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Donor</th>
<th>Rejection</th>
<th>MHC Class I</th>
<th>MHC Class II</th>
<th>Haplo-matching</th>
<th>Direct Response</th>
<th>Indirect Response</th>
<th>Anti-donor Abs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1501</td>
<td>301</td>
<td>CHR</td>
<td>1</td>
<td>2</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1900</td>
<td>3099</td>
<td>CHR</td>
<td>2</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1601</td>
<td>2501</td>
<td>CHR</td>
<td>0 or 1</td>
<td>2</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6601</td>
<td>5801</td>
<td>CHR</td>
<td>2 or 3</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4102</td>
<td>4202</td>
<td>CHR</td>
<td>2</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2000</td>
<td>1799</td>
<td>TOL</td>
<td>2</td>
<td>2</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>2800</td>
<td>3800</td>
<td>TOL</td>
<td>1</td>
<td>0</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>1902</td>
<td>2102</td>
<td>TOL</td>
<td>2</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2702</td>
<td>1502</td>
<td>TOL</td>
<td>0 or 1</td>
<td>2</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

The T cell responses, Ab responses, and MHC matching results for each of the transplanted monkey pairs displaying CHR or tolerance (TOL) are shown. The first two subcolumns of the MHC matching column indicate the number of MHC class I and II alleles shared by the donor and the recipient. The third subcolumn indicates the presence (+) or absence (−) of haplo-matching. The last three columns show the presence (+) or absence (−) of a direct response, an indirect response, and anti-donor Abs.

Discussion

In this article, we report our investigations of the T and B cell alloresponses in nine cynomolagus renal allograft recipients, which had received donor cells and peri-transplant immunosuppressive treatments. None of these monkeys developed durable mixed chimerism (data not shown). Despite this, they all displayed allograft survival with no signs of chronic rejection for at least 3 mo after withdrawal of immunosuppression. Four recipients never displayed signs of chronic rejection and were considered tolerant. Several monkeys had a very low direct alloresponse pretransplantation, which is indicative of the absence of significant numbers of donor-specific memory T cells, a feature presumably...
associated with lack of acute rejection (36, 37, 45–49). In turn, M1902 (tolerant) displayed a fairly high direct alloresponse pretransplantation (400 spots/million cells). This direct response remained high and even increased around 200 d posttransplantation. Unlike the three tolerant monkeys that received conditioning and DBM in conjunction with the kidney transplant, M1902 had received conditioning and DBM 4 mo after transplantation. It is possible that some memory T cells recognizing alloantigens directly had been generated or expanded during this interval. The fact that this monkey subsequently remained tolerant (i.e., devoid of acute and chronic rejection for years) supports the hypothesis that regulatory mechanisms appear to develop and suppress these allospecific T cells.

All the recipients, which developed CHR, mounted an anti-donor alloantibody response, a result consistent with previous observations in the same model (42). The presence of anti-donor Abs was always detected prior to the onset of CHR, which itself preceded elevation in serum creatinine levels (Table III). Strikingly, all the CHR monkeys displayed a direct T cell alloresponse detectable in some instances for more than 2 y after transplantation. This shows that the ability to mount a direct response can be sustained in recipients and even increase overtime after transplantation. Unlike naive T cells, these memory T cells could be activated by allo-MHC Ags presented by donor professional APCs such as endothelial cells. In addition, presentation of captured allo-MHC molecules by recipient dendritic cells (semidirect pathway) may also contribute to the maintenance of such direct allospecificity long after donor passenger leukocytes have vanished (50–52).

Only two (M1900 and M1601) of the five recipients developing CHR and alloantibodies displayed an indirect alloresponse. It is at first glance surprising that three of five CHR monkeys (M1501, M6601, and M4102) had no detectable indirect alloreactivity, which is traditionally considered the driving force behind alloantibody production and CHR (22–26). This is supported by the view that cognate T–B cell cooperation requires Ag recognition on self-MHC class II molecules. In our study, it is important to note that the three CHR monkeys with direct but no indirect alloreactivity shared MHC class II genes with their donors (Fig. 6). It is possible that sharing MHC class II between donors and recipients may allow cognate interactions between recipient B cells and directly activated T cells recognizing peptides on the shared MHC class II molecules. In this scenario, it is conceivable that direct T cell allorecognition can trigger and/or perpetuate an alloantibody response by host B cells and contribute to the chronic rejection process.

At first glance, our observations of persistence of direct alloactivity and its involvement in the chronic rejection process, in the absence of indirect alloresponses, may appear to be in disagreement with a previous report by Lechler and colleagues (19). It should be emphasized, however, that loss of direct and maintenance of indirect alloresponses were observed in that study in chronically immunosuppressed patients who developed allograft nephropathy. In contrast, in our allograft recipients, all immunosuppressive treatment had been discontinued 28 d after transplantation.

In summary, this study shows that, unlike the traditionally accepted mechanistic basis for long-term allospecificity, the direct pathway can be long-lived, presumably perpetuated by the presence of the allograft. This response could be maintained by recipient memory T cells recognizing allogeneic MHC molecules.

### Table III. Sequence of the events detected in CHR monkeys

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Donor</th>
<th>Rejection</th>
<th>Alloantibodies, Day</th>
<th>CHR, Day</th>
<th>Creatinine Rise, Day</th>
</tr>
</thead>
<tbody>
<tr>
<td>1501</td>
<td>301</td>
<td>CHR</td>
<td>236</td>
<td>386</td>
<td>487</td>
</tr>
<tr>
<td>1900</td>
<td>3099</td>
<td>CHR</td>
<td>168</td>
<td>369</td>
<td>None</td>
</tr>
<tr>
<td>1601</td>
<td>2501</td>
<td>CHR</td>
<td>225</td>
<td>231</td>
<td>274</td>
</tr>
<tr>
<td>6601</td>
<td>5801</td>
<td>CHR</td>
<td>114</td>
<td>122</td>
<td>369</td>
</tr>
<tr>
<td>4102</td>
<td>4202</td>
<td>CHR</td>
<td>114</td>
<td>196</td>
<td>280</td>
</tr>
</tbody>
</table>

Columns 3, 4, and 5 show the earliest time point (day posttransplant) at which alloantibodies, CHR, and creatinine increases were detected, respectively, for each recipient of a kidney allograft having developed chronic rejection.
on donor nonprofessional APCs. Most importantly, in some monkeys, this direct response was associated with the production of donor-specific alloantibodies presumably due to T–B cognate interactions rendered possible by sharing of MHC class II genes between donor and recipients, and it correlated with the presence of chronic allograft rejection of kidney transplants. This suggests that whereas MHC class II gene matching between donor and recipients is likely to reduce the risk of acute rejection and presumably favor the activation/expansion of some regulatory T cells (53, 54), it could increase the risk of chronic rejection.

**FIGURE 6.** MHC gene expression in donors and recipients. The MHC class I (A and B) and class II (DR, DQ, and DP) genes expressed by each donor and recipient were determined using molecular techniques. The MHC genes expressed by each chromosome of each pair of monkeys are shown side by side (left panel, CHR; right panel, tolerant). Each color corresponds with one haplotype. TOL, tolerant.
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Disclosures
The authors have no financial conflicts of interest.

References


