Comparative Strategies to Induce Long-Term Graft Acceptance in Fully Allogeneic Renal Versus Cardiac Allograft Models by CD28-B7 T Cell Costimulatory Blockade: Role of Thymus and Spleen

MEIKE SCHAUB,* THOMAS H. W. STADLBAUER,* ANIL CHANDRAKER,* JOHN P. VELLA,* LAURENCE A. TURKA,† and MOHAMED H. SAYEGH*
*Laboratory of Immunogenetics and Transplantation, Brigham and Women's Hospital and *Harvard Medical School, Boston, Massachusetts; and †Department of Medicine and Institute of Human Gene Therapy, University of Pennsylvania, Philadelphia, Pennsylvania.

Abstract. Blocking CD28-B7 T cell costimulatory activation by the fusion protein CTLA4Ig prevents rejection and induces long-term graft acceptance in various experimental transplant models. There are reported differences in the efficacy of CTLA4Ig in renal and cardiac rodent allograft models, but it is not clear whether these are due to the strain or species differences investigated in the different studies reported. This study investigates the effect of blocking CD28-B7 T cell costimulation with murine CTLA4Ig in rat models of acute renal and cardiac allograft rejection models, using the same complete major histocompatibility complex-incompatible strain combination. A single injection of murine CTLA4Ig 2 d after engraftment was able to induce long-term graft acceptance (>100 d) in 54% of Lewis rat recipients of Wistar-Furth kidneys. Transferring this protocol into the acute Wistar-Furth to Lewis heart allograft model resulted in a mean graft survival time of 24.7 ± 16.9 d, and all grafts were ultimately rejected. Only concomitant injection of donor cells (4 × 10^7 splenocytes) plus a single injection of CTLA4Ig on the day of transplant could induce long-term graft acceptance in 50% of animals. In both the cardiac and renal transplant models, the thymus and spleen were required for induction of tolerance. The maintenance phase of tolerance, however, did not require an intact thymus but did require the presence of a spleen. These data have important clinical applicability because human studies with T cell costimulatory blockade are being planned. (J Am Soc Nephrol 9: 891–898, 1998)
role in maintenance of tolerance in some models (23,24). There are no reports on the role of the thymus and/or spleen in animals rendered tolerant by T cell costimulatory blockade. In this study, we report on the requirement of the thymus and spleen for induction and maintenance of graft acceptance by CTLA4Ig in vivo.

Materials and Methods

Animals

Inbred male Lewis (LEW) (RT1^a), Wistar-Furth (WF) (RT1^b), and brown Norway (RT1^s) strain rats were purchased from Harlan Sprague Dawley (Indianapolis, IN). Transplant procedures were performed when animals reached a body weight of approximately 200 g. LEW rats served as recipients of renal or cardiac WF allografts, LEW isografts, or third-party BN grafts. Animals were housed according to National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Renal Allograft Model

LEW rats underwent bilateral nephrectomy and received WF renal allograft, as described previously (17). In this model, animals typically reject their graft within 2 wk. Because animals depend on the graft for survival, rejection is defined as death of the animals. Deaths occurring within 48 h posttransplantation were excluded from analysis, assuming surgical technical failure.

Cardiac Allograft Model

Hearts were transplanted to the infrarenal great vessels by standard microvascular techniques, as described previously (25). In contrast to the renal model, in this heterotopic cardiac allograft model, the recipient does not rely on graft function for survival. Graft function was monitored by daily palpation of the transplanted heart; rejection is defined as the day of complete cessation of myocardial contraction, and confirmed by laparotomy.

Thymectomy/Splenectomy

Under anesthesia, a partial sternotomy was performed, and both thymic lobes were removed. Spleen was removed after arterial and venous supply had been ligated.

Experimental Design

Animals that had undergone renal or cardiac transplantation were injected intravenously with CTLA4Ig or control Ig (0.5 mg, with or without donor cells) on the day of transplantation (day 0), 2 d later (day 2), or repeatedly from days 2 to 14 posttransplantation. As indicated, some animals underwent thymectomy or splenectomy 7 to 14 d before engraftment or 14 d posttransplantation.

Donor Cell Preparation

Spleens from naive WF or BN animals were harvested. Cell suspensions were prepared by sieving spleens through a 60-g stainless steel mesh into RPMI 1640 (BioWhittaker, Walkersville, MD). Erythrocytes were lysed by 0.83% Tris-ammonium chloride buffer, pH 7.21. Spleen cells (4 X 10^7) were injected intravenously, and cell viability, as assessed by 0.1% trypan blue exclusion, was consistently >95% (26).

Reagents

The murine fusion protein CTLA4Ig and a corresponding control chimeric recombinant protein L6 were kindly provided by Dr. P. Linsley (Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA). CTLA4Ig or control Ig (0.5 mg) was administered intravenously into the dorsal penile vein. Cyclosporine (CSA; Sandoz Research Institute, East Hanover, NJ) was administered subcutaneously at a dose of 25 mg/kg as induction therapy for 14 d, followed by maintenance therapy of a dose of 5 mg/kg.

Incubation of APC with CTLA4Ig

Donor (WF or BN) spleens were harvested, and cell suspensions were prepared as described above. APC were isolated by plastic adherence as described previously (27). APC (10 x 10^6/ml) were incubated with murine CTLA4Ig at a concentration of 20 μg/ml for 2 h at 37°C, washed twice in RPMI, and 40 x 10^6 cells in 1 ml of RPMI were injected intravenously into LEW recipients on the day of transplantation.

Arteriosclerosis

Beating grafts of different protocols were perfused with phosphate-buffered saline, harvested, serially sectioned into approximately 2- to 3-mm slices, and fixed in 10% formalin. Specimens were embedded in paraffin and stained with elastic van Gieson. By light microscopy, slides were examined by degree of arteriosclerosis/arterial intimal thickening, using a scale from 0 to 5 as described by Adams et al. (28). Only vessels that were cut orthogonally and displayed a clear internal elastic lamina staining were scored.

Statistical Analyses

Differences in survival rates were assessed by log-rank test, and a P value of <0.05 was considered significant. Differences in mean arteriosclerosis scores were analyzed using single-factor ANOVA, and a P value of <0.05 was considered significant.

Results

Comparative Strategies of CD28-B7 T Cell Costimulatory Blockade in Renal and Cardiac Transplant Models

We have previously reported (17) that a single injection of human CTLA1g (0.5 mg) on day 2 posttransplant prevents acute rejection and induces long-term graft survival in the WF into LEW renal transplant model. Interestingly, administration of the same dose of CTLA4Ig on day 0 was not as effective. Recipients were specifically tolerant because they accepted donor (WF) but rejected third-party (BN) heart allografts. In addition, recipients exhibited donor-specific inhibition of cell-mediated and humoral immune responses in vitro and in vivo (29). In this study, we used murine CTLA4Ig. A single injection of 0.5 mg on day 2 posttransplant prevented acute rejection and induced long-term graft acceptance (>100 d) in 54% of recipients, whereas all control unmodified animals and animals treated with a single injection of a murine control Ig acutely rejected their allografts (mean survival time, 10.8 d) (Table 1), confirming results of our original study.

In comparison, in the heterotopic WF into LEW cardiac allograft model, a single injection of 0.5 mg of murine CTLA4Ig on day 2 prolonged graft survival but did not lead to
long-term graft acceptance; all animals rejected their grafts within 54 d (Table 1). Administration of CTLA4Ig (0.5 mg) on the day of transplant was also only marginally effective; all animals rejected their allografts within 33 d (Table 1). Control unmodified animals and animals treated with a similar protocol of control Ig rejected their allografts within 11 d (Table 1). Therefore, using the same donor-recipient combination, we can demonstrate that a similar regimen of CTLA4Ig was significantly less effective in the cardiac compared with the kidney allograft model. It has been hypothesized that because the kidney contains a larger number of donor-derived passenger APC than the heart, it may be easier to “tolerize” to renal rather than cardiac allografts (17). In fact, in the BN into LEW vascularized cardiac allograft model, Lin et al. (14) reported previously that administration of donor cells on the day of transplant followed by a single injection of CTLA4Ig 2 d later induced long-term graft acceptance in the majority of recipients. In our model, administration of 4 × 10⁷ donor (WF) splenocytes on the day of transplant followed by a single injection of CTLA4Ig (0.5 mg) 2 d later did not result in significant prolongation of graft survival when compared with day 2 CTLA4Ig alone (Table 1). However, when both donor splenocytes and CTLA4Ig were administered on the day of transplant, 50% of recipients had long-term graft acceptance (>100 d) (Table 1). Administration of donor cells alone did not prolong graft survival; all grafts were rejected within 9 d (Table 1). In addition, donor specificity was confirmed by administration of third-party (BN) splenocytes (4 × 10⁷) in combination with CTLA4Ig (0.5 mg) given on the day of transplant; allograft survival in these recipients was not different from that in animals treated with CTLA4Ig alone (Table 1).

Because in the cardiac transplant model a single injection of CTLA4Ig on day 2 posttransplant did prolong allograft survival but could not induce permanent graft acceptance, we studied the effect of repeated administration of CTLA4Ig. Recipients received 0.5 mg of CTLA4Ig every other day from day 2 to 14 for a total of seven doses. In contrast to the day 2 single-injection protocol, this regimen resulted in 30% long-term survivors (>100 days). To demonstrate true donor-specific tolerance, we transplanted second grafts into two long-term survivors (>100 d). A third-party (BN) graft was rejected on day 8 postengraftment, whereas a donor-strain (WF) heart was permanently accepted (>100 d). Long-term graft acceptance in this tolerizing regimen could be improved to 67% of recipients when 4 × 10⁷ donor-splenocytes were infused on the day of transplantation following the 2-wk course of CTLA4Ig (Table 1).

In recent studies in an islet cell transplant model, Steurer et al. (30) showed that ex vivo preincubation of donor islet cells with CTLA4Ig prevents their rejection when transplanted into allogeneic recipients in the absence of systemic administration of CTLA4Ig. Similar observations were also reported in the autoimmune encephalomyelitis model, in which ex vivo preincubation of APC with CTLA4Ig and the encephalitogenic

Table 1. Allograft survival

<table>
<thead>
<tr>
<th>Category</th>
<th>Survival (days)</th>
<th>Median</th>
</tr>
</thead>
<tbody>
<tr>
<td>Renal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTLA4Ig day 2</td>
<td>14, 17, 18, 20, 30, 30, &gt;100 (n = 7)</td>
<td>54% &gt;100 days&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CTLA4Ig day 0</td>
<td>5, 10, 20, 55, &gt;100 (n = 3)</td>
<td>43% &gt;100 days&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>control Ig</td>
<td>4, 5, 6, 7, 32</td>
<td>6</td>
</tr>
<tr>
<td>control unmodified</td>
<td>5, 13, 13, 14</td>
<td>13</td>
</tr>
<tr>
<td>Cardiac</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTLA4Ig alone day 0</td>
<td>13, 13, 13, 14, 14, 33</td>
<td>13.5&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>CTLA4Ig alone day 2</td>
<td>10, 16, 16, 16, 36, 54</td>
<td>16&lt;sup&gt;b,d&lt;/sup&gt;</td>
</tr>
<tr>
<td>donor cells + CTLA4Ig day 0</td>
<td>12, 13, 16, 22, 36, 58, &gt;100 (n = 6)</td>
<td>50% &gt;100 days&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>donor cells + CTLA4Ig day 2</td>
<td>8, 11, 17, 18, 38, 41</td>
<td>17.5&lt;sup&gt;d,e&lt;/sup&gt;</td>
</tr>
<tr>
<td>BN cells + CTLA4Ig day 0</td>
<td>14, 18, 32, 35</td>
<td>25&lt;sup&gt;c.e&lt;/sup&gt;</td>
</tr>
<tr>
<td>irradiated donor cells + CTLA4Ig day 0</td>
<td>13, 13, 16, 24</td>
<td>14.5&lt;sup&gt;d,e&lt;/sup&gt;</td>
</tr>
<tr>
<td>donor APC incubated with CTLA4Ig day 0</td>
<td>7, 7, 7, 7, 10, 13</td>
<td>7&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>donor cells alone day 0</td>
<td>6, 8, 8, 9</td>
<td>8&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>CTLA4Ig days 2 to 14</td>
<td>65, 68, 70, 72, 89, &gt;100 (n = 2)</td>
<td>30% &gt;100 days&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>donor cells + CTLA4Ig days 2 to 14</td>
<td>9, 10, &gt;100 (n = 4)</td>
<td>67% &gt;100 days&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>control Ig days 2 to 14</td>
<td>6, 8, 9, 10</td>
<td>8.5</td>
</tr>
<tr>
<td>control unmodified</td>
<td>6, 6, 7, 10, 11, 11</td>
<td>8.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> BN, brown Norway rat; APC, antigen-presenting cell.
<sup>b</sup> P < 0.001 compared with organ-specific control groups.
<sup>c</sup> P < 0.01.
<sup>d</sup> P < 0.05.
<sup>e</sup> P < 0.01.
<sup>f</sup> P < 0.001 compared with the protocol CTLA4Ig + donor cells day 0 in the cardiac model (assessed by log-rank test).
<sup>g</sup> P < 0.05.
peptide prevented induction of disease (27). The hypothesis is that exposure of T cells to APC in the absence of costimulatory molecules renders these T cells “unresponsive” in vivo. The efficacy of such an approach has not been reported in vascularized allografts. We therefore incubated $4 \times 10^7$ WF splenic APC with CTLA4Ig (20 μg/ml) and administered these cells intravenously on day 0 without any additional systemic injection of CTLA4Ig. This ex vivo treatment could not abrogate acute vascularized allograft rejection; animals rejected their grafts within 13 d (Table 1).

Therefore, compared with the kidney transplant model in which a single injection of CTLA4Ig appears to be sufficient to prevent acute rejection and induce long-term graft acceptance in >50% of recipients, donor cells followed by repeated administration of CTLA4Ig provide the best strategy in the cardiac allograft model. These results have important clinical applications, because human trials with CTLA4Ig are being planned.

Because chronic graft rejection, manifested by development of graft arteriosclerosis, is a major determinant of graft function and is currently the most important limiting factor in achieving long-term graft acceptance (31,32), we investigated the degree of arterial occlusion in heart allografts of long-term survivors (>100 d) treated with donor cells plus CTLA4Ig. These grafts displayed a very mild graft arteriosclerosis (mean score, 0.83 ± 0.1) that was not significantly different from isograft controls (mean score, 0.37 ± 0.2). As a reference, a separate group of LEW recipients was treated with a clinically relevant protocol of CsA (5 mg/d for 14 d followed by 1 mg/d for 100 d). These animals developed moderately severe graft arteriosclerosis even when harvested earlier (mean vessel score, 2.4 ± 0.4, n = 7; mean harvest time, 111 ± 2 d) (Figures 1 and 2) with a significant difference ($P < 0.05$) compared with CTLA4Ig-treated animals. These findings in CsA-treated animals were not due to direct toxic effects of CsA, because isograft controls treated with a similar protocol of CsA did not develop graft arteriosclerosis (Figure 2). These data suggest that long-term graft acceptance induced by T cell costimulatory blockade is associated with attenuation of development of chronic rejection in fully allogeneic recipients.

**Role of the Thymus and Spleen**

The thymus plays the major role in induction of self tolerance, although in the past few years there has been a renewed interest in studying the role of the thymus in acquired tolerance (21). In addition, the exact mechanisms mediating graft acceptance induced by CD28-B7 blockade in vivo remain unclear, although anergy/deletion (33) and regulatory cells (17) have been suggested. In that regard, we investigated the role of the thymus and spleen in the induction of graft acceptance by CTLA4Ig in the renal and cardiac transplant models. LEW recipients of WF cardiac allografts underwent thymectomy or splenectomy 7 d before engraftment. Both thymectomy and splenectomy completely abrogated the tolerizing effect of concomitant infusion of CTLA4Ig and donor splenocytes; all grafts were rejected (thymectomy day 7: mean survival time, 17.5 ± 1.3 d, n = 4; splenectomy day 7: mean survival time, 13.5 ± 3.8 d; n = 4) (Table 2). Similar results were observed in the kidney allograft model. Induction of long-term graft acceptance by a single shot of CTLA4Ig 2 d after transplantation was abrogated when animals had undergone thymectomy 7 d before transplantation; all grafts were acutely rejected within 13 d (n = 4). Splenectomy before engraftment had a similar effect: Only one animal went on long-term, and all other grafts were acutely rejected within 15 d (n = 4) (Table 2). In contrast to this “induction phase” of permanent graft acceptance, thymectomy during the so-called “maintenance phase,” i.e., 14 d after engraftment, did not influence long-term graft survival: Three of four animals went on long-term, and only one animal rejected its graft 8 d after thymectomy. However, when splenectomy was performed 14 d after engraftment, four of five animals rejected their allograft within 15 d after splenectomy, and only one animal accepted its graft permanently. These data indicate that the induction phase of tolerance requires an intact thymus and spleen, whereas the maintenance phase requires only the spleen.

**Discussion**

Blocking T cell costimulatory activation pathways is an effective strategy to inhibit cell-mediated immune responses in autoimmune diseases and transplantation. In particular, it has been reported in several rodent models that blockade of CD28-B7 by CTLA4Ig prevents graft rejection and can lead to permanent graft acceptance and even tolerance in some models (13–18). Despite these achievements, studies in different animal models and strain combinations revealed varying requirements of CTLA4Ig for induction of long-term graft acceptance, regarding organ transplant model, timing of administration, and requirement for concomitant administration of donor antigen. Our study is the first to investigate and to compare the different requirements for induction of long-term allograft acceptance in the acute kidney and heart allograft model by using...
CD28-B7 Blockade in Vascularized Transplantation

Isograft

Figure 2. Representative sections of coronary arteries from isograft controls, allografts treated with a single dose of CTLA4Ig+ donor cells on the day of engraftment, and from allografts treated with CsA (from top to bottom, respectively, elastic van Gieson; magnification, ×100).

Allograft, CTLA4Ig + Cells

Table 2. Effect of thymectomy or splenectomy on allograft survival in CTLA4Ig-treated animals

<table>
<thead>
<tr>
<th>Category</th>
<th>Survival (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymectomy day ≤7</td>
<td></td>
</tr>
<tr>
<td>kidney</td>
<td>6, 8, 8, 12, 13</td>
</tr>
<tr>
<td>heart</td>
<td>16, 17, 18, 19</td>
</tr>
<tr>
<td>Thymectomy day +14</td>
<td></td>
</tr>
<tr>
<td>kidney</td>
<td>22, &gt;100 (n = 3)</td>
</tr>
<tr>
<td>Splenectomy day ≤7</td>
<td></td>
</tr>
<tr>
<td>kidney</td>
<td>7, 9, 15, 136</td>
</tr>
<tr>
<td>heart</td>
<td>8, 14, 16, 16</td>
</tr>
<tr>
<td>Splenectomy day +14</td>
<td></td>
</tr>
<tr>
<td>kidney</td>
<td>18, 21, 26, 29, &gt;100 (n = 1)</td>
</tr>
</tbody>
</table>

*Thymectomy or splenectomy was performed 7 to 14 d before or 14 d after transplantation. In the renal allograft model, CTLA4Ig was given on day 2, in the cardiac model CTLA4Ig and donor cells were concomitantly administered on the day of engraftment.*

Allograft, Cyclosporine

the same MHC class I and class II incompatible rat strain combination. As we have shown previously, a single injection of CTLA4Ig 2 d after engraftment prevents acute renal allograft rejection and results in long-term graft acceptance in >50% of recipients (17). Applying this protocol to the acute cardiac allograft rejection model in the same WF to LEW strain combination, we were not able to induce permanent graft acceptance. Only administration of donor splenocytes in combination with CTLA4Ig given on the day of transplantation resulted in long-term allograft survival in >50% of recipients, indicating that there are different requirements for induction of long-term graft survival in renal and cardiac allograft recipients. Our data are consistent with studies of Lin et al. (14) in a different rat strain combination, in which they showed that additional infusion of donor splenocytes seems to be necessary to induce long-term allograft survival in rat recipients of cardiac allografts treated with CTLA4Ig. However, in that study, the best outcome was obtained when CTLA4Ig administration was delayed to day 2 posttransplant. Therefore, the most appropriate timing of T cell costimulatory blockade may vary with the strain combination, and may be related to temporal patterns of expression of costimulatory molecules in the target organ. Indeed, expression of B7–1 and B7–2 has been shown to follow distinct temporal patterns after activation in vitro and in vivo (34–36). In addition, expression of the negative regulatory molecule CTLA4 occurs after initial T cell activation (37). Although the mechanisms of how donor cells synergize with costimulatory blockade to promote graft acceptance remain unknown (14,16), it is possible that administration of donor alloantigens results in upregulation of B7 molecules, rendering the animal more susceptible to costimulatory blockade. Alternatively, donor antigen administration may result in earlier upregulation of expression of CTLA4, which may be important in induction of tolerance (38).

Premature donor bone marrow-derived cells (dendritic cell progenitors) that express MHC class II molecules but are deficient of B7 costimulatory molecules induced alloantigen-
specific hyporesponsiveness in vitro (39). In contrast to reported studies in a model of islet cell transplantation (30), the approach of ex vivo blockade of B7 molecules by incubation of donor APC with CTLA4Ig could not abrogate acute rejection in our vascularized cardiac allograft model. One possible explanation is that treatment of donor APC with CTLA4Ig will block B7 molecules on donor cells, but not on recipient APC. To the extent that CD28-mediated costimulation is most efficiently provided by the cell presenting the antigen, this would block direct allore cognition (recipient T cell recognizing allo-MHC on donor cells), but not indirect allore cognition (recipient T cell recognizing processed allo-MHC-derived peptides presented on self-MHC molecule) (40). Therefore, in contrast to islet cells (30), blocking direct allore cognition alone may not be sufficient to prevent rejection of vascularized solid organ allografts.

Clinically, grafts that survive more than 1 yr posttransplant develop chronic rejection manifested by progressive graft arteriosclerosis, glomerulosclerosis, and variable degrees of interstitial inflammation and fibrosis. This poorly understood clinicopathologic entity, which is not responsive to conventional immunosuppression, becomes the major determinant of allograft function and long-term outcome (31,32). Studies in experimental chronic rejection have shown that graft interstitial and vessels are infiltrated by macrophages and T lymphocytes (41). In addition, there is significant upregulation of intragraft expression of activation and inflammatory cytokines, chemokines, and growth-factors (19,42,43). It has been suggested that chronic rejection is mediated by both alloantigen-dependent and alloantigen-independent factors (44). We have previously shown that blocking CD28-B7 T cell costimulatory activation prevents development of experimental chronic rejection in both cardiac and renal allograft models of non-fully allogeneic (LEW-F344) strain combinations (19,20). In the current study, long-term surviving cardiac allografts demonstrated minimal degrees of arteriosclerosis with very mild intimal thickening and little luminal compromise, similar to isograft controls. In the same strain combination, a clinically relevant protocol of chronic CsA regimen did not affect development of chronic rejection. These observations indicate that costimulatory blockade by CTLA4Ig in combination with donor-specific cell transfusion is a powerful way not only to induce long-term graft acceptance, but also to minimize the degree of transplant vasculopathy. In addition, our data underscore the key role of T cells in initiating the process of chronic rejection and highlight the importance of developing tolerance strategies rather than immunosuppression as a means to prevent chronic rejection (45).

It has been suggested that thymus and spleen play an important role in regulating immune responses (21–24). Intrathy mic injections of donor cells or soluble donor antigen have been shown to induce permanent allograft survival or tolerance (21,22), indicating that the thymus has a central role in the induction of acquired tolerance. More recently, data by Yamada et al. indicate that the thymus is required for induction of peripheral tolerance in a large animal transplant model (46). In our study, rat recipients of WF hearts or kidneys that had undergone thymectomy 7 d before engraftment rejected their allografts in an acute manner. Although the thymus seems to be important during the induction phase of tolerance, its presence in the recipient during maintenance phase, i.e., after the time point of acute rejection, appears not to be mandatory. Seventy- five percent of animals that had undergone thymectomy 14 d after transplantation went on to have long-term survival. The induction phase of permanent graft acceptance is also highly dependent on the spleen; recipients that had been splenecto mized before engraftment almost entirely rejected their grafts acutely. Splenectomy during the maintenance phase, however, resulted in allograft loss within 2 wk after the procedure, indicating that a state of permanent graft acceptance is dependent on the presence of spleen but not thymus. Our data strongly argue against a state of anergy or deletion as the sole mechanism of graft acceptance in our model. Our observations suggest that regulatory cells exist in the spleen, presumably originating initially in the thymus, and that these cells are responsible for maintaining a state of tolerance. The phenotype and regulatory function of these cells are currently under investigation. Whether Th2 cells function as regulatory cells in maintenance of allograft tolerance remain controversial (17,47,48).

In summary, our results suggest that blockade of costimulatory pathway of T cell activation by CTLA4Ig is a potent regimen to induce long-term graft acceptance and even donor-specific tolerance in vascularized organ allografts. Using the same MHC class I and II incompatible strain combination to compare different models of organ engraftment kidney versus heart, our observations indicate that induction of long-term graft acceptance in different organ models requires distinct regimens in blocking costimulatory pathway and address the importance of donor cell transfusion in cardiac transplantation. Our data also highlight the pivotal role of the thymus and spleen in mediating different phases of tolerance to allografts. A greater understanding of these mechanisms may be of important clinical relevance, because clinical trials with costimulatory blockade in transplant recipients are being planned. In particular, the requirement of the thymus for induction of tolerance should be confirmed in large animal studies, because the thymus is known to involute in adult life, and this may limit the efficacy of targeting T cell costimulation as a means to induce tolerance in humans.

**Acknowledgments**

This work was supported in part by National Institutes of Health Research Grants AI 34965-03 and AI 40629-01. Dr. Sayegh is the recipient of the National Kidney Foundation Clinician Scientist Award. Dr. Turka is an established investigator of the American Heart Association, and Dr. Chandraker is the recipient of a research fellowship grant from the Juvenile Diabetes Foundation. Dr. Stadlbauer is supported by a fellowship from Deutsche Forschungsgemeinschaft (Sfa 461/1-1). We thank Cheng Kwok for excellent technical assistance.
References


36. Hancock WW, Sayegh MH, Zheng XG, Peach R, Linsley PS, Turka LA: Costimulatory function and expression of CD40 li-
gand, CD80 and CD86 in vascularized murine cardiac allograft rejection. Proc Natl Acad Sci USA 93: 13967–13972, 1996


