

T-cell activation by the CD28 ligand B7 is required for cardiac allograft rejection *in vivo*

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ABSTRACT Organ graft rejection is a T-cell-dependent process. The activation of alloreactive T cells requires stimulation of the T-cell receptor/CD3 complex by foreign major histocompatibility complex (MHC)-encoded gene products. However, accumulating evidence suggests that, in addition to T-cell receptor occupancy, other costimulatory signals are required to induce T-cell activation. Previously, the CD28 receptor expressed on T cells has been shown to serve as a surface component of a signal transduction pathway that can provide costimulation. *In vitro*, interaction of CD28 with its natural ligand B7 expressed on the surface of activated B cells or macrophages can act as a costimulus to induce proliferation and lymphokine production in antigen receptor-activated T cells. We now report evidence that stimulation of T cells by the CD28 ligand B7 is a required costimulatory event for the rejection of a MHC-incompatible cardiac allograft *in vivo*. These results demonstrate that the B7/CD28 activation pathway plays an important role in regulating *in vivo* T-cell responses.

During an immune response, the initial activation of a quiescent T cell is mediated through the antigen-specific T-cell receptor (TCR) (1). TCR activation occurs either through engagement of an antigenic peptide present in the antigen-binding groove of a self-encoded major histocompatibility complex (MHC) molecule or by engagement of a foreign MHC molecule (1). However, several studies have demonstrated that signals transduced via the antigen receptor are not by themselves sufficient to lead to complete T-cell activation and proliferation but require a second costimulatory signal (2–8). It has been hypothesized that this second signal(s) might be provided through one or more co-receptors on T cells interacting with their nonpolymorphic ligands on antigen-presenting cells (2, 9). One candidate for this co-receptor activity is CD28, a 44-kDa polypeptide that is expressed on resting T cells as a homodimer. *In vitro* studies have demonstrated that CD28 is the surface receptor of a cyclosporin A-resistant T-cell signal transduction pathway (10). Stimulation of CD28 by its natural ligand B7 provides costimulation to TCR-activated T cells, leading to proliferation and lymphokine gene expression (4, 11–14).

Unlike other T-cell co-receptors, CD28 is not colocalized or comodulated with the TCR, nor is CD28-mediated signal transduction dependent on TCR expression (15–17). CD28 is encoded on human chromosome 2 (18). Immediately adjacent to the CD28 gene is a closely related gene, CTLA-4, which shares 32% amino acid identity with CD28 and has a similar genomic organization (19–21). Although CTLA-4 is not expressed on quiescent lymphoid cells and its physiologic role has yet to be defined, a recombinant fusion protein,

CTLA4Ig, which contains the extracellular domain of human CTLA-4 fused to a human immunoglobulin C γ chain, was recently shown to specifically bind the CD28 ligand B7 with high affinity ($K_d \approx 12$ nM) (22). *In vitro*, CTLA4Ig blocks binding of CD28 to B7, and the only molecule that can be immunoprecipitated from 125 I-labeled cell lines with CTLA4Ig is B7 (22). In addition, CTLA4Ig blocks T-cell-dependent antibody production and inhibits the proliferation of activated T cells restimulated with allogeneic lymphoblastoid cell lines (22). Although CTLA4Ig was constructed to contain the extracellular domain of the human CTLA4Ig gene, CTLA4Ig also binds efficiently to mouse and rat B7. In this report, we have used CTLA4Ig to examine the effect of blocking B7-induced T-cell activation on survival of cardiac allografts transplanted into fully MHC-mismatched rats.

MATERIALS AND METHODS

Monoclonal Antibody (mAb) and Reagents. Production of the fusion protein CTLA4Ig has been described (22). L6 is a chimeric mAb that contains a murine variable region and a human Fc region (22) and was used as an isotype-matched control for CTLA4Ig. The mouse anti-rat mAbs R7.3 (anti-TCR) and OX-8 (anti-CD8) were purchased from Harlan (Indianapolis). mAb BWH-4 (anti-CD4) was a kind gift of C. B. Carpenter (Boston). Isotype-matched control antibodies were purchased from Coulter (Hialeah, FL), and fluorescein isothiocyanate-labeled goat anti-mouse mAbs were from Tago. Concanavalin A was purchased from Sigma and was used at 5 μ g/ml.

Animals and Cells. Inbred adult male rats weighing 250–300 g (Harlan–Sprague–Dawley) were used. Lewis rats (RT1^l) served as responders in mixed lymphocyte cultures and as recipients of heart allografts. Brown Norway rats (RT1ⁿ) served as stimulators in cultures and as heart graft donors. ACI and Long–Evans rats were used as third party control animals. Lymphocytes were isolated from cervical and axillary nodes by gentle passage of tissue through a nylon mesh.

Cell Culture. Cells were cultured in medium consisting of RPMI 1640 medium supplemented with 5 mM Hepes, penicillin at 10⁵ units/liter, streptomycin at 100 μ g/liter, 50 μ M 2-mercaptoethanol, and 10% fetal calf serum (GIBCO). For mixed lymphocyte responses, 3 \times 10⁵ each of responder cells and irradiated (3000 rads; 137 Cs source) (1 rad = 0.01 Gy) stimulator cells were cocultured for 4 days in 96-well flat-bottomed microtiter plates as described (23). Proliferation, measured as DNA synthesis, was determined by adding 1 μ Ci of [3 H]thymidine (1 Ci = 37 GBq) (ICN) to each well for the last 6 hr of culture. All assays were performed in quadruplicate. Unless otherwise indicated, *P* values were determined

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Abbreviations: MHC, major histocompatibility complex; TCR, T-cell receptor; mAb, monoclonal antibody.

by Student's *t* test and values of >0.05 were considered nonsignificant (NS).

Heart Allografting and Thymectomy. Heterotopic cardiac allografts were performed as described (24). Briefly, the donor rats were anesthetized and mechanically ventilated, the right carotid artery was cannulated, and the heart/lung preparation was removed while being retrograde perfused. Next, the recipient rat was anesthetized and the donor heart was anastomosed to the carotid artery and the external jugular vein. After transplantation, heart transplants were assessed daily by palpation for mechanical function. When in doubt, graft function was independently assessed by electrocardiogram. Graft rejection was said to occur on the last day of palpable contractile function. When specified, recipient animals underwent thymectomy 3 days before heart transplantation.

Cell Staining and Flow Cytometry. Cells were stained with primary mouse anti-rat mAbs and counterstained with fluorescein isothiocyanate-conjugated goat anti-mouse antibody. Cells were analyzed on a FACScan (Becton Dickinson).

Cardiac Histology. After the animals were sacrificed, their hearts were excised and fixed in formalin. The fixed tissue was paraffin embedded, and tissue sections were stained with hematoxylin and eosin.

Determination of Serum CTLA4Ig Levels. The serum concentration of CTLA4Ig in treated animals was determined by binding to B7⁺ CHO cells as measured by flow cytometry (12). CTLA4Ig concentrations were quantified by comparing the degree of binding with that of known concentrations of CTLA4Ig. The lower limit of detection by this assay is a serum level of ≈ 50 ng/ml.

RESULTS AND DISCUSSION

The effects of CTLA4Ig on the primary immune response to alloantigen were initially examined in a one-way mixed lymphocyte culture between Lewis rats (RT1^l, responder) and Norway rats (RT1ⁿ, stimulator) (Fig. 1). In this assay, a proliferative response will normally occur in 1–5% of cells as a result of activation through their cell-surface TCR in response to allogeneic MHC (25). CTLA4Ig was able to block proliferation in a dose-dependent fashion with virtually complete inhibition observed at a concentration of 1 μ g/ml. Consistent with these results, alloreactive T-cell responses

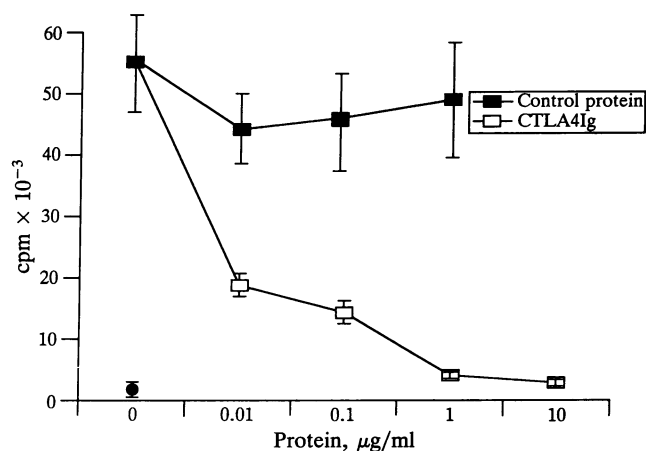


FIG. 1. Effect of CTLA4Ig on a one-way mixed lymphocyte culture. Lymphocytes from a Lewis rat strain were cocultured in microtiter wells with equal numbers of irradiated lymphocytes from a Norway rat strain in the presence of graded concentrations of CTLA4Ig or isotype-matched control mAb L6. ●, Spontaneous proliferation is the incorporation of thymidine by Lewis rat cells in the absence of Norway rat stimulators. Results are expressed as cpm of [³H]thymidine incorporation \pm SD.

have been reported to be inhibited by nonstimulatory Fab' fragments of an anti-CD28 mAb (9). Together, these data suggest that to mount a proliferative response *in vitro*, alloreactive T cells must be stimulated not only through MHC engagement of the TCR but also through costimulation of the CD28 receptor by B7 engagement.

CTLA4Ig was next used in a rat model of organ transplantation to ascertain its ability to block alloantigen responses *in vivo*. Recipient animals received a heterotopic cardiac allograft, which was anastomosed to vessels in the neck. Animals were treated with daily injections of CTLA4Ig or isotype-matched control mAb L6 for 7 days. This period of drug administration was selected because untreated Lewis rats reject heterotopic Norway rat allografts in 6.8 ± 0.3 days ($n = 10$). Therefore, as an initial test animals were treated until they were past the point at which rejection occurs in untreated animals. The allografts in CTLA4Ig-treated animals remained functional after completion of drug administration, whereas untreated animals, or animals treated with the L6 control antibody, uniformly rejected their grafts by day 8 ($P < 0.0001$) (Table 1). CTLA4Ig-treated rats manifested no observable acute or chronic side effects from administration of the protein. No gross anatomic abnormalities were observed in CTLA4Ig-treated animals at autopsy. Pharmacokinetic studies initiated 24 hr after the administration of 0.5 mg of CTLA4Ig revealed a CTLA4Ig serum half-life of 2.8 days.

An untreated animal and a CTLA4Ig-treated animal were sacrificed on day 4 for histological examination of the allografts (Fig. 2). The donor heart removed from the untreated animal showed histological findings of severe acute cellular rejection, including a prominent interstitial mononuclear cell infiltrate with edema formation, myocyte destruction, and infiltration of arteriolar walls. In contrast, the transplanted heart from the CTLA4Ig-treated animal revealed only a mild lymphoid infiltrate. Frank myocyte necrosis and evidence of arteriolar involvement were absent. The native heart from each animal showed no histological abnormalities.

To determine the duration of graft survival after CTLA4Ig treatment, animals treated for 7 days with daily injections of CTLA4Ig were observed without additional therapy until cessation of graft function. Graft survival was 18–40 days in animals treated with 0.05 mg of CTLA4Ig per day. This failure to induce permanent engraftment did not appear to be due to inadequate dosing of CTLA4Ig, as animals treated with a 10-fold higher dose (0.5 mg/day) showed a similar graft survival curve (Fig. 3), with one animal maintaining long-term graft function (>50 days). Furthermore, serum CTLA4Ig levels during treatment in this group were in excess of 10 μ g/ml, a concentration that is maximally suppressive *in vitro* (Fig. 1). Serum measurements of CTLA4Ig fell below detectable levels by day 24. Histological examination of the

Table 1. Heterotopic cardiac allografts were performed with Lewis rats (RT1^l) as recipients and Norway rats (RT1ⁿ) as heart donors

	Graft survival at day 8	Significance
Untreated	0/10	} $P < 0.0001$
CTLA4Ig	18/18	
Control protein	0/5	} $P < 0.0001$

Animals were untreated or received 7 daily injections of either CTLA4Ig or L6, an isotype-matched control mAb. Grafts were monitored by daily palpation for mechanical function, and graft rejection was taken to have occurred on the last day of palpable contractile function. CTLA4Ig was administered by intraperitoneal injection at doses of 0.015 mg/day (five animals), 0.05 mg/day (five animals), and 0.5 mg/day (eight animals). L6 was given at 0.5 mg/day. *P* values were calculated by χ^2 analysis.

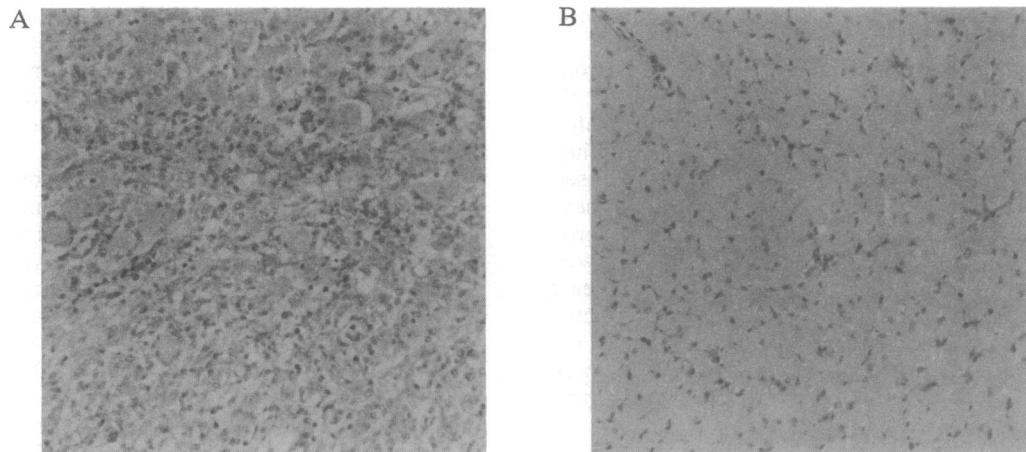


FIG. 2. Histopathology of cardiac allografts. Cardiac allografts were removed from an untreated animal (A) and a CTLA4Ig-treated animal (0.5 mg/day) (B) 4 days after transplantation. Allografts were fixed in formalin, and tissue sections were stained with hematoxylin and eosin. ($\times 200$.)

allografts from CTLA4Ig-treated animals whose grafts ceased functioning displayed typical signs of acute cellular rejection of the same degree of severity as seen in control animals that had rejected their hearts after 7 days. The animal with continued graft function was sacrificed on day 57, and the allograft from this animal failed to reveal any histological abnormalities. The thymus and spleen from the day 57 animal with continued graft function were similar in size and cell number to those of control rats, and flow cytometric analyses of thymus, lymph nodes, and spleen revealed percentages of CD4⁺ and CD8⁺ T cells similar to those found in normal controls. When the proliferative response of this animal's lymphocytes was measured in comparison to a nontolerant animal, a significant decrease in the response to Norway rat cells was observed, 1700 ± 1380 (cpm; mean \pm SD) versus 8798 ± 2057 ($P < 0.01$). Responses to the third party ACI rat were equivalent— 2283 ± 529 versus 2800 ± 1982 (NS). Both animals responded well to concanavalin A— $18,606 \pm 4893$ versus $22,579 \pm 2461$ (NS).

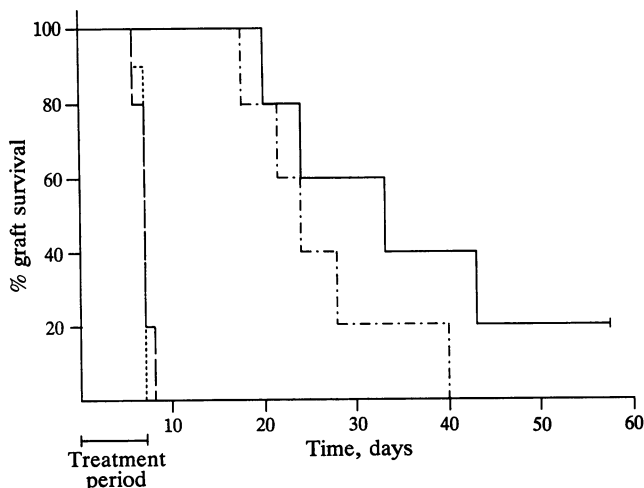


FIG. 3. Effects of CTLA4Ig treatment on cardiac allograft survival. Lewis rat recipients of Norway rat heterotopic cardiac allografts received either CTLA4Ig (0.5 mg/day \times 7 days; —), CTLA4Ig (0.05 mg/day \times 7 days; - - -), isotype-matched control mAb L6 (0.5 mg/day \times 7 days; ---), or no treatment (---). In all cases, treatment was initiated at the time of transplantation. Graft survival was assessed daily and is displayed as the last day of graft function. Animals treated with a dose of 0.015 mg/day \times 7 days have a mean survival of 13.2 ± 2.5 days ($n = 5$).

The fact that 9 of 10 allograft recipients treated with the highest doses of CTLA4Ig (0.05–0.5 mg/day \times 7 days) rejected their grafts within the study period indicated that blockade of the costimulatory molecule B7 for a finite period of time did not induce permanent graft acceptance. One potential explanation was that while CTLA4Ig treatment may have induced a state of nonresponsiveness in circulating T cells by allowing target antigen recognition without B7-dependent costimulation, newly matured T cells emerging from the thymus after cessation of CTLA4Ig treatment could mediate graft rejection as a result of B7-costimulated T-cell alloreactivity. To test this hypothesis, rats were thymectomized 3 days before cardiac transplantation and treated with daily injection of CTLA4Ig (0.5 mg/day \times 7 days) after transplantation. These animals rejected their grafts between days 28 and 33 ($n = 3$), indicating that allograft recipients were not dependent on the influx of new T cells to initiate an alloimmune response. This suggests that CTLA4Ig-induced nonresponsiveness in host T cells may be only temporary. Alternatively, the kinetics of T-cell trafficking might not allow for all alloreactive cells to encounter donor antigens in the graft or in regional lymphoid tissue during the 7-day period of B7 blockade with CTLA4Ig. T cells that escaped this process could subsequently encounter graft antigens in the absence of B7 blockade and effect rejection.

Although CTLA4Ig blocks B7-dependent T-cell costimulation *in vitro*, it was important to verify that prolongation of cardiac allograft survival *in vivo* was not merely due to depletion of antigen-presenting cells from the allograft. Therefore, 1×10^8 Norway rat spleen cells were injected intravenously into Lewis animals that simultaneously received 0.25–0.5 mg of CTLA4Ig. Control animals received spleen cells alone without CTLA4Ig. Fourteen days later, the animals received Norway rat heterotopic cardiac allografts without further immunosuppressive treatment. The 14-day delay between donor cell plus CTLA4Ig treatment and transplantation was chosen since, based on the pharmacokinetics of CTLA4Ig elimination, this is a time point at which serum levels are below the limit of detection. Graft survival in the control animals was 7–9 days ($n = 3$), whereas allografts in the CTLA4Ig-treated animals functioned for at least 15 days ($n = 6$) with two long-term survivors (>90 days). As an additional control, CTLA4Ig was administered alone at day –14 without accompanying donor cell transfusion. This protocol failed to prolong graft survival beyond 7 days.

We next examined the *in vitro* functional responses of lymphocytes from animals treated with donor-specific cell transfusion plus CTLA4Ig. Specifically, we wished to deter-

mine whether these cells exhibited decreased antidonor reactivity and, if so, whether responses to third-party antigens remained intact. For these experiments, animals were sacrificed at day 7, a time at which all animals treated with this protocol exhibited normal graft function and control animals were uniformly undergoing rejection. When the mixed lymphocyte response of the treated animals was measured in comparison to control Lewis rats, a significant decrease in the response to Norway rat stimulators was observed— $44,572 \pm 3584$ (cpm; mean \pm SD; $n = 3$) versus $93,101 \pm 23,585$ ($P < 0.05$). Both sets of animals responded equivalently to the third party stimulator Long-Evans, 9989 ± 2450 versus $10,865 \pm 5610$ (NS). These data suggest that CTLA4Ig-treated animals were specifically hyporesponsive to donor stimulators. However, in most animals this hyporesponsiveness was short lived as four of six animals treated in this manner rejected their transplanted heart within 31 days of transplantation. Even the two long-term survivors were not universally tolerant to Norway rat cells. Both animals rejected Norway rat skin within 3 weeks of skin grafting.

In all of our experiments, the immunosuppressive effect of CTLA4Ig lasted 2–6 weeks beyond the treatment period in most animals. This coupled with the failure of CTLA4Ig to induce permanent engraftments in thymectomized animals suggests that T cells present in the animals during the time of treatment are not rendered permanently tolerant. This finding is consistent with previous *in vitro* studies which have demonstrated that T-cell clones made tolerant by TCR activation in the absence of a costimulating signal can recover antigen-specific responsiveness if treated with interleukin 2 (3, 26, 27). Nevertheless, the fact that deprivation of B7 costimulation at the time of exposure to alloantigen induces at least a temporary state of nonresponsiveness that is specific for donor antigens suggests that this may be a useful immunosuppressive strategy.

Several different approaches have been used to successfully suppress the immune response to allogeneic tissue grafts. These include depleting circulating T cells with toxins or antibodies (28), blocking TCR-mediated signal transduction with drugs such as cyclosporin A (29), and interfering with the process of immune cell adhesion by using mAbs against leukocyte cell-surface molecules (30). The use of B7 blockade with CTLA4Ig to prevent cardiac allograft rejection is likely to be a distinct immunosuppressive strategy for several reasons. First, because B7-dependent costimulation can be delivered independently of TCR stimulation (15–17), it is unlikely that the effects of blocking cell-surface B7 are simply due to interfering with T-cell adhesion. Second, unlike TCR-mediated activation, the CD28 activation pathway is cyclosporin A resistant (10). Third, the immunosuppression observed with CTLA4Ig did not result in significant alterations in circulating T-cell subsets. Finally, CTLA4Ig appears to act by blocking B7-dependent costimulation rather than by merely depleting the graft of B7⁺ antigen-presenting cells.

In conclusion, our data indicate that blockade of B7-dependent T-cell activation with a short course of CTLA4Ig administered at the time of transplantation can significantly prolong cardiac allograft survival. Thus, B7-dependent T-cell activation is required for acute cardiac allograft rejection. Strategies to induce more prolonged graft survival, such as continued deprivation of costimulation, use of CTLA4Ig with other immunosuppressive regimens, and donor-specific transfusion plus CTLA4Ig remain to be investigated.

Note Added in Proof. While this manuscript was in review, two manuscripts reaching similar conclusions were published (31, 32).

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