Brief Definitive Report

Long-Term Acceptance of Major Histocompatibility Complex Mismatched Cardiac Allografts Induced by CTLA4Ig Plus Donor-specific Transfusion

By Hua Lin,* Steven F. Bolling,* Peter S. Linsley,‖ Ru-Qi Wei,* David Gordon,‡ Craig B. Thompson,¶ and Laurence A. Turka§

*From the Departments of *Surgery, ‡Pathology, and §Medicine, University of Michigan, Ann Arbor, Michigan 48109; ‖Bristol-Myers Pharmaceutical Research Institute, Seattle, Washington 98121; and the ¶Department of Medicine and the Howard Hughes Medical Institute, University of Chicago, Chicago, Illinois 60637*

Summary

Allograft rejection is a T cell–dependent process. Productive T cell activation by antigen requires antigen engagement of the T cell receptor as well as costimulatory signals delivered through other T cell surface molecules such as CD28. Engagement of CD28 by its natural ligand B7 can be blocked using a soluble recombinant fusion protein, CTLA4Ig. Administration of CTLA4Ig blocks antigen-specific immune responses in vitro and in vivo, and we have shown that treatment of rats with a 7-d course of CTLA4Ig at the time of transplantation leads to prolonged survival of cardiac allografts (median 30 d), although most grafts are eventually rejected. Here, we have explored additional strategies employing CTLA4Ig in order to achieve long-term allograft survival. Our data indicate that donor-specific transfusion (DST) plus CTLA4Ig can provide effective antigen-specific immunosuppression. When DST is administered at the time of transplantation followed by a single dose of CTLA4Ig 2 d later, all animals had long-term graft survival (>60 d). These animals had delayed responses to donor-type skin transplants, compared with normal rejection responses to third-party skin transplants. Furthermore, donor-matched second cardiac allografts were well tolerated with minimal histologic evidence of rejection. These data indicate that peritransplant use of DST followed by subsequent treatment with CTLA4Ig can induce prolonged, often indefinite, cardiac allograft acceptance. These results may be clinically applicable for cadaveric organ and tissue transplantation in humans.

Induction of a T cell immune response is required for allograft rejection (1). Antigen-specific T cell activation is initiated through the TCR (2). However, recent evidence has shown that T cells require two signals for activation (3): signal 1, which is provided by stimulation through the TCR, and signal 2 (costimulation), which can be provided by ligation of one or more T cell surface receptors. Engagement of the TCR receptor alone (i.e., signal 1 without signal 2) has been reported to induce a long-lasting state of T cell anergy, rendering cells unresponsive to subsequent antigenic stimulation (3).

The best characterized costimulatory pathway is transduced through the CD28 surface molecule. CD28 is a receptor for B7, a molecule that can be expressed by many types of APCs (4). Stimulation of CD28 by its ligand B7 provides sufficient costimulatory signals to TCR-activated T cells for lymphokine production and cell proliferation (5). CTLA-4 is a gene closely related to CD28 which also serves as a B7-ligand, although the physiologic role of this interaction is not yet defined (6). Recently, a soluble recombinant protein termed CTLA4Ig was produced, which contains the extracellular domain of human CTLA-4 fused to a human Ig Cγ chain (6). CTLA4Ig displays an ~20-fold higher affinity for B7 than does CD28 (6), therefore acting as a competitive inhibitor of CD28 engagement. Although constructed using the human CTLA-4 domain, CTLA4Ig binds efficiently to murine and rat B7. CTLA4Ig inhibits B7-dependent immune responses in vitro (6). In vivo, CTLA4Ig blocks T cell–dependent B cell antibody production, and prevents the rejection of xenogeneic islet and allogeneic cardiac allografts (7–9). In the studies of cardiac allograft rejection we found that animals treated with daily injections of CTLA4Ig for 7 d, initiated at the time of transplantation, had greatly prolonged graft survival (median 30 d, versus 7 d for control animals), although most animals eventually rejected the graft (7). Pretransplant thymectomy did not further extend survival in CTLA4Ig-treated

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animals, indicating that T cells present in the animals at the time of transplantation eventually recovered the capacity to induce rejection.

In this report we have explored other strategies using CTLA4Ig in order to achieve consistent long-term allograft survival. We find that donor-specific cell transfusion (DST) at the time of transplantation, followed by a single dose of CTLA4Ig 2 d later, is sufficient to lead to prolonged, often indefinite, cardiac allograft survival. Since no treatment is required before transplantation, these results may be clinically applicable for cadaveric organ and tissue transplantation in humans.

Materials and Methods

Animals. The experiments were conducted using inbred male Lewis (LEW, $RT1^l$), Brown Norway (BN, $RT1^n$) and ACI ($RT1^a$) rats weighing 200–300 g (Harlan Sprague Dawley, Inc., Indianapolis, IN). LEW rats served as heterotopic cardiac allograft recipients. BN and ACI rats were used as cardiac and skin allograft donors, and as a source of splenocytes for DST.

Splenocyte Transfusion. Spleens were harvested and a single cell suspension prepared as previously described (7). Red cells were lysed with water, and the remaining population of mononuclear cells was washed two times in RPMI-1640. Cell viability was consistently >95% as determined by trypan blue exclusion. Varying numbers of splenocytes were injected intravenously in a volume of 0.5–1.0 ml into anesthetized rats at the indicated time points.

Cardiac Transplantation. The rats were anesthetized and mechanically ventilated, after which donor hearts were transplanted into a cervical location in the recipient animals (10). Allograft survival was assessed by daily palpation. The day of rejection was defined as the day of cessation of palpable heartbeat, and verified by autopsy and selective pathological examination. Loss of graft function within 48 h of transplant was considered a technical failure (<5% on the average), and these animals were omitted from further analysis.

Skin Grafting. Animals with long-term (>60 d) cardiac allograft survival received donor-specific and third-party skin grafts placed simultaneously on alternate sides of the flanks. Donor skin was raised as full-thickness grafts from the center abdominal wall, trimmed of fat, cut into standard sizes (2 cm circular skin), and sutured into position as described (11). The grafts were inspected daily, and rejection was said to have occurred when more than 50% of the graft surface became raised, necrotic, or covered by eschar.

Mixed Lymphocyte Reaction (MLR). Lymphocytes were isolated from cervical and axillary nodes by gentle passage of tissue through a nylon mesh. Cells were cultured in RPMI-1640 medium supplemented with 5 mM Hepes, penicillin (10^5 U/liter), streptomycin (100 μg/liter), 50 μM 2-ME, and 10% FCS (GIBCO BRL, Gaithersburg, MD). Next, 3×10^5 each of responder cells and irradiated (3,000 rad; ^{137}Cs source) stimulator cells were cocultured for 4 d in 96-well flat-bottomed microtiter plates as described (7). Proliferation, measured as DNA synthesis, was determined by adding 1 μCi of [^{3}H]thymidine (ICN Radiochemicals, Irvine, CA) to each well for the last 6 h of culture. All assays were performed in quadruplicate.

Results and Discussion

In our initial studies, we found that a 7-d course of CTLA4Ig administered to cardiac allograft recipients starting at the time of transplantation significantly prolonged allograft survival but did not prevent eventual rejection (7). As prior thymectomy failed to induce permanent engraftment in treated animals, T cells present during the course of CTLA4Ig administration were responsible for rejection. We hypothesized that the kinetics of T cell trafficking might not permit all alloreactive cells to encounter donor antigens in the graft or regional lymphoid tissue during the 7-d period of drug administration, and that T cells that escaped an encounter with antigen in the context of B7-blockade could eventually reject the graft. Therefore, we first extended the period of CTLA4Ig treatment. However, animals receiving daily CTLA4Ig for 21 d rejected their grafts at the same time points as those treated for only 7 d (data not shown).

We next considered the possibility that immunosuppression would be most effective if the alloantigen were administered systemically in conjunction with CTLA4Ig, so as to expose all T cells throughout the body to antigen during the drug treatment period. For example, treatment of mice with CTLA4Ig blocks the immune response to systemically administered antigens such as sheep RBCs or KLH (8). Therefore, LEW animals received 10^8 BN lymphocytes intravenously (DST) plus a single dose of CTLA4Ig (0.5 mg) 14 d before placement of a BN cardiac allograft. As shown in Table 1, animals treated with this protocol had significant prolongation of cardiac allograft survival, with median graft survival of 17 d. This effect required both DST and CTLA4Ig, as animals treated with either alone rejected their grafts by 8 d. Furthermore, immunosuppression by DST plus CTLA4Ig was antigen specific. Animals receiving transfusions from third-party ACI animals had a median graft survival of only 9 days. Conversely, when ACI animals were used as heart donors, DST from ACI animals synergized with CTLA4Ig to prolong graft survival ($\geq$20 d), whereas transfusions from BN animals had no effect (rejection by day 7). These data also indicate that CTLA4Ig is not merely acting by depleting the graft of $B7^+$ APCs. First, strain-specific cell transfusions are required for the immunosuppressive effect. Second, our prior analysis of CTLA4Ig pharmacokinetics indicated a serum half-life of 2.8 d (7), and therefore by the time of transplantation circulating CTLA4Ig levels would have fallen far below a therapeutic level.

We had initially chosen to administer CLTA4Ig plus DST 14 d before transplantation because of the possibility that it might take several days after exposure to this regimen for a state of antigen-specific nonresponsiveness to develop. However, although graft survival was significantly prolonged in animals treated with this protocol, eventually most grafts were rejected. Therefore, we next considered whether a state of tolerance was being induced, but that this state was temporary, and had largely waned by 2 wk. This concept was supported by two sets of data. First, in studies of anergy induction in T cell clones, anergy, although reproducible, was not a permanent state, as the cells could spontaneously regain responsiveness after a finite period of time (12). Second, recent in vivo studies have demonstrated that maintenance of anergy requires the persistence of antigen (13). Thus we reasoned that induction of anergy by DST plus CTLA4Ig might best

Table 1. *Effects of Pretransplant DST plus CTLA4Ig on Cardiac Allograft Survival*

Heart donor	DST donor	DST day	CTLA4Ig day	Graft survival
				d
BN	None			5,7,7,7,7,7,7,7,7
BN	BN	−14		6,6,7,7,8,8
BN	None		−14	6,8,8
BN	BN	−14	−14	14,14,15,16,17,19,46,>100
BN	ACI	−14	−14	7,8,9,9,10,13
ACI	None			5,5,6,7,8
ACI	ACI	−14	−14	23,26,58,>100
ACI	ACI	−14	None	1,2,5,5,6,7
ACI	BN	−14	−14	6,6,7,7,7

Allograft recipients were LEW animals, some of which received DST (10^8 cells) via intravenous injection, CTLA4Ig (0.5 mg) by intraperitoneal injection, or both 14 d before transplantation as indicated. The day of transplantation was defined as day 0.

prolong graft survival if transplantation was performed simultaneously. However, when CTLA4Ig plus DST (4×10^7 cells) were administered on the day of transplantation, graft survival was not improved over the previous protocol, and was no different than in animals treated with a single dose of CTLA4Ig without DST (Table 2).

At present, knowledge of the target of action of CTLA4Ig is limited. Its only known ligand is B7, whose expression is restricted to cells of hematopoietic origin, in particular those which can serve as APCs. In the case of vascularized organ allografts, there are two routes of alloantigen presentation using distinct APCs (14, 15). In the indirect route, donor alloantigens, shed from the surface of donor parenchymal cells, are presented by host APCs in regional LNs. In the direct route, APCs resident within the graft directly stimulate host T cells. Although the relative contribution of these two routes is the subject of debate, the importance of direct sensitization has been demonstrated by the fact that graft survival is significantly prolonged by depleting tissues and organs of resident APCs (for a review see reference 16). In this regard, it is notable that whereas a short course of CTLA4Ig frequently induced indefinite survival of human islet xenografts in mice, anti-human B7 antibody was effective as well, indicating that much of the immune response was initiated against donor-type APCs (9).

One of the most potent types of APC known is the dendritic cell (17). Compared with activated B cells and macrophages, extremely small numbers of dendritic cells are sufficient to stimulate T cell responses. Furthermore, in contrast to B cells and macrophages, dendritic cells constitutively express B7 (18, 19). Larsen et al., (20) have shown that after cardiac allograft placement, donor dendritic cells migrate into host lymphoid organs, and it is possible that this may be responsible for initiating graft rejection. The migration of dendritic cells to LNs was found to peak 2 d after transplantation. Therefore, we next administered DST (4×10^7 cells) on the day of transplantation, followed by a single dose of CTLA4Ig 2 d later (Table 2). Whereas DST alone was not immuno-

Table 2. *Effects of Peri-transplant DST plus CTLA4Ig on Cardiac Allograft Survival*

DST donor	DST day	CTLA4Ig day	Graft survival
			d
–	–	–	5,7,7,7,7,7,7,7,7
BN	0	–	7,7,9,10,11
–	–	0	8,10,18
BN	0	0	8,11,19
–	–	2	8,14,17,25,28
BN	0	2	89,>100,>100,>100,>100
ACI	0	2	14,23,26,28,56

Allograft recipients were LEW animals and heart donors were BN animals. Selected recipient animals received DST (4×10^7 cells) via intravenous injection, CTLA4Ig (0.5 mg) by intraperitoneal injection, or both at the times indicated. The day of transplantation was defined as day 0.

Table 3. *Skin Graft Survival in Rats with Long-Term Functioning Cardiac Allografts*

Animal	Day of skin grafting	Skin graft survival	
		BN skin	Third-party (ACI) skin
1	64	19	10
2	64	19	10
3	66	22	9
4	72	19	
5	83	19	

Skin graft recipients were LEW animals that had received BN cardiac allografts and immunosuppression with DST plus CTLA4Ig. Skin grafts from donor-strain animals (BN) and from third-party animals were performed simultaneously on the indicated day after cardiac transplantation.

suppressive, and CTLA4Ig by itself had a modest effect on graft survival, the combination reproducibly induced long-term engraftment. Similar to pretransplantation DST plus CTLA4Ig (Table 1), the immunosuppressive effect of this protocol was also antigen specific, as LEW rats that received transfusions from ACI animals rejected BN cardiac allografts at a median of 26 d.

Additional transplants were performed on these animals with stable allografts. Skin transplants from donor-type BN animals or from third-party ACI animals were simultaneously placed on opposite flanks (Table 3). The ACI grafts were all rejected by 10 d, the normal time for rejection of ACI skin grafts in LEW animals. In contrast, the BN grafts survival for a median of 19 d. This demonstrates that these animals remained immunocompetent, yet had donor-specific hyporesponsiveness. In four of five rats, rejection of the BN skin grafts did not induce rejection of the BN hearts, although it is notable that the single animal that rejected its primary

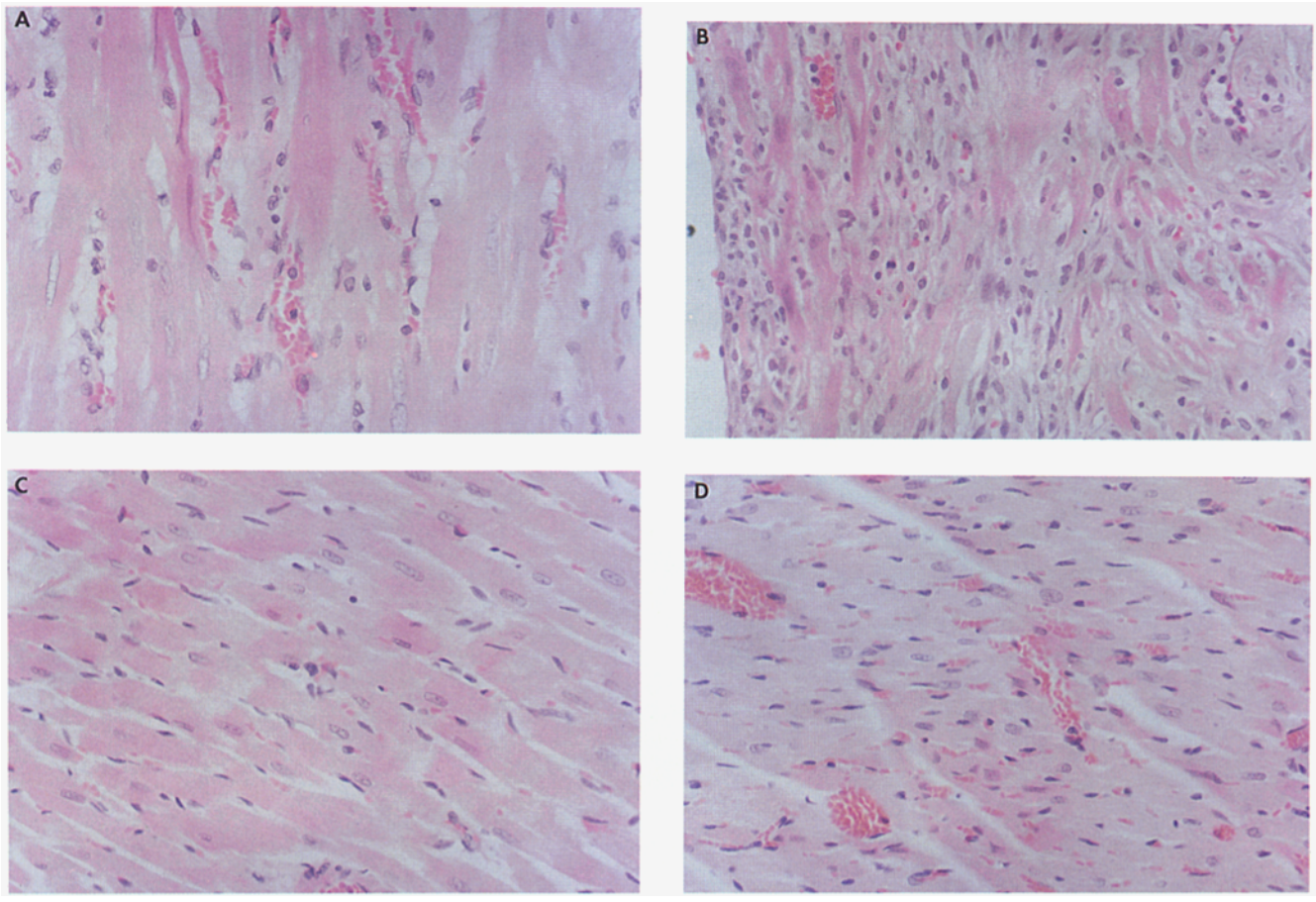

Figure 1. Histopathology of cardiac tissue. Animals were treated with DST on day 0 plus CTLA4Ig on day 2, and hearts were removed for examination at least 60 d after transplantation. First cardiac allografts appeared either histologically normal (*A*) or showed evidence of focal rejection with resulting myocyte loss (*B*). In all cases, second cardiac allografts showed no or minimal histologic evidence of rejection (*C*). The native heart from a LEW animal which received a BN allograft is shown for comparison (*D*). The hearts were fixed in formalin and tissue sections were stained with hematoxylin and eosin. ×100.

cardiac allograft (on day 89) did so 6 d after rejection of a BN skin graft.

Skin grafts are frequently rejected in animals tolerant to vascularized organs, perhaps because of the increased immunogenicity of skin or the existence of skin-specific antigens (21). Second cardiac allografts from BN donors placed into CTLA4Ig plus DST-treated animals, all appeared to function normally until the animals were killed for humane reasons. This occurred on days 17–52 after transplantation of the second heart. In all instances, histologic examination of the second heart revealed only minimal cellular infiltrates consistent with normal histology or focal mild rejection (Fig. 1). Consistent with this minimal response, lymphocytes from engrafted animals have a 50% reduction in their in vitro proliferative response (as measured in an MLR) to donor-type stimulators (data not shown). This data is in agreement with our previous study which found a 50% decrease in MLR responsiveness to donor-type cells in CTLA4Ig-treated animals, with no effect on third-party responses (7). The reduction in proliferation appears to be somewhat modest, given the failure of the animals to reject their cardiac allografts grafts. This suggests either a discrepancy between in vitro and in vivo antidonor reactivity, or split tolerance characterized by CTL activity that is severely depressed in comparison with proliferative capacity.

Together, our data indicate that DST at the time of cardiac transplantation followed by treatment with CTLA4Ig are sufficient to induce long-term cardiac allograft acceptance in rats. These animals remain immunocompetent, but exhibit donor-specific nonresponsiveness. It should be emphasized that although the hearts remain functional as assessed by electrical activity and palpable contractions, some of the grafts have experienced focal rejection with resulting myocyte loss (Fig. 1 *B*). Thus long-term engraftment is not strictly equated with immunologic nonresponsiveness. However, even in animals with significant myocardial damage to their initial allograft, the second graft had minimal histologically abnormalities, suggesting that a previously existing immune response might be relatively quiescent.

A curious finding was that CTLA4Ig was dramatically more effective when given on day 2, than on day 0. Given that the half-life of CTLA4Ig is >60 h, this finding cannot be due to the fact that when administered on day 0, the drug is absent by day 2. Rather, it suggests that the drug is most effective when given after an initial immune response is allowed to begin. The finding that a single dose of CTLA4Ig, without any DST, is more effective when administered at day 2 than at day 0 is consistent with this hypothesis. DST at day 0, which is also required for optimal graft prolongation, may serve to help synchronize and/or maximally stimulate the immune response by distributing alloantigen throughout the lymphoid system. Subsequent treatment with CTLA4Ig could then block costimulatory signals at at time when T cells were maximally sensitive to this maneuver.

Address correspondence to L.A. Turka, University of Michigan Medical Center, 1560 MSRB-II, 1150 West Medical Center Drive, Ann Arbor, MI 48109–0676.

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