Chronic Cardiac Rejection in the LEW to F344 Rat Model

Blockade of CD28-B7 Costimulation by CTLA4Ig Modulates T Cell and Macrophage Activation and Attenuates Arteriosclerosis

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Abstract

CTLA4Ig, a fusion protein that blocks CD28-B7 costimulation, was studied in a LEW to F344 rat model of chronic cardiac rejection. In rats treated with a single dose of CTLA4Ig (0.5 mg intraperitoneally) 2 d after transplantation, allografts survived significantly longer (> 70 d in 64%) than in untreated controls or rats treated with control Ig (all rejected within 25 d). Only 25% of grafts from rats treated with a single, high dose of cyclosporine A (25 mg/kg, 2 d after transplantation) survived longer than 70 d. Reverse transcriptase PCR and immunostaining analyses of tissue from 75-d, CTLA4Ig-treated allografts showed reduced expression of the T cell factor IFN-γ and macrophage activation factors monocyte chemoattractant protein-1, inducible nitric oxide synthase, and galactose/*N*-acetylgalactosamine macrophage lectin, as well as TGF-β. Grafts from long-term survivors (> 120 d) treated with CTLA4Ig showed significant reductions in the frequency and severity of arteriosclerosis in comparison with cyclosporine A–treated rats. Thus, T cell activation is a proximal event in the cascade that culminates in the arteriosclerosis of chronic rejection. Strategies for blocking T cell costimulation may help prevent chronic rejection in clinical transplantation. (*J. Clin. Invest.* 1996. 97:833–838). Key words: arteriosclerosis • CD28 • macrophage activation • cardiac transplantation

Introduction

With advances in the control of acute rejection, chronic rejection manifested by arteriosclerosis of donor vessels has become one of the leading obstacles to long-term survival after solid organ transplantation (1, 2). The classic feature of transplant arteriosclerosis is a vasculopathy characterized by accelerated fibrointimal thickening of most graft vessels (3, 4). Although the precise molecular and cellular events that regulate chronic rejection (and acute rejection) are not yet elucidated, a number of alloantigen-dependent and -independent factors have been implicated (2).

The LEW to F344 rat heterotopic abdominal cardiac transplantation model has been used extensively to study chronic allograft rejection and graft arteriosclerosis (5, 6). Chronically rejecting rat allografts develop moderate lymphocyte and macrophage infiltration within the interstitium and perivascular space in association with arteriosclerotic lesions (7, 8). The earliest vascular lesion (days 7–14) involves the adhesion of monocytes/macrophages to the vessel wall (8). Between day 28 and day 75 the intima thickens, primarily because of macrophage accumulation interposed with smooth muscle cells. The last phase involves myointimal thickening with smooth muscle cell accumulation and fewer mononuclear cells.

Because of the early and sustained presence of T cells and macrophages, we have studied T cell and macrophage activation in the LEW to F344 rat model. Intragraft levels of IFN-γ, monocyte chemoattractant protein-1 (MCP-1),[1] inducible nitric oxide synthase (iNOS), galactose/*N*-acetylgalactosamine (Gal/GalNAc) macrophage lectin, and endothelin-1 (9–12) are all increased, and are believed to contribute to development of graft arteriosclerosis.

The interaction of CD28 on T cells with its ligands (B7-1 and B7-2) on antigen-presenting cells is an important costimulatory pathway mediating the response to alloantigens (13, 14). CTLA4Ig is a recombinant fusion protein that contains the extracellular domain of human CTLA4 (a homologue of CD28) fused to a human IgG1 heavy chain (15). CTLA4Ig prevents acute rejection and induces donor-specific tolerance in a number of rodent transplantation models (14–17). However, there have been no studies of the effect that blocking T cell costimulation has on development of chronic rejection. Here we studied the effect of blocking the CD28-B7 costimulatory T cell activation pathway by examining alterations in graft survival, T cell and macrophage activation, and arteriosclerotic lesion development in the LEW to F344 rat model of chronic rejection.

Methods

LEW–F344 rat model of chronic rejection. Heterotopic abdominal cardiac transplantation was performed and hearts were harvested as de-

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1. *Abbreviations used in this paper:* Gal/GalNAc, galactose/*N*-acetylgalactosamine; iNOS, inducible nitric oxide synthase; MCP-1, monocyte chemoattractant protein-1.

scribed (18). LEW rats (Harlan Sprague Dawley, Indianapolis, IN) were used as donors. F344 rats (Harlan Sprague Dawley) were used as recipients in the allogeneic combination to produce chronic rejection. LEW rats were used in the isogeneic combination to produce control hearts exposed to the same surgical procedure but free of vascular abnormalities. Rejection was defined as the complete cessation of palpable heart beat (19).

Treatment protocols. CTLA4Ig and control human Ig were generously provided by Bristol-Myers Squibb (Seattle, WA). Based on survival data from Sayegh et al. (16) and Lin et al. (17) showing long-term graft survival, one group ($n = 13$) was given CTLA4Ig as a single intraperitoneal injection 2 d after transplantation. The three reference groups included (*a*) untreated recipients ($n = 6$); (*b*) recipients treated with control human Ig given as a single dose on day 2 (0.5 intraperitoneally) ($n = 4$); and (*c*) animals treated with cyclosporine A (25 mg/kg intraperitoneally on day 2) ($n = 9$). For reverse transcriptase PCR analysis, surviving cardiac allografts were harvested after 70–75 d, which coincides with points demonstrated in previous studies to correspond to elevated transcript levels in LEW to F344 cardiac allografts for the various factors analyzed here (11). Serial midventricular sections were frozen in liquid nitrogen for subsequent RNA extraction and immunohistochemical analysis. For morphometric analysis of arteriosclerotic lesion development, we harvested a group of grafts after 100 d, a point at which myointimal thickening had been observed to be maximal in long-term survivors (5). To obtain a significant number of animals with allografts surviving long term, we generated a separate group of F344 recipients ($n = 12$) treated with daily subcutaneous injections of cyclosporine A (5 mg/kg) for 30 d starting on the day of transplantation and harvested for arteriosclerotic lesion analysis after 100 d. Morphometric scores from the long-term surviving grafts ($n = 7$) were compared with those from a group of allografts from recipients treated with CTLA4Ig ($n = 5$) as well as LEW to LEW cardiac isografts ($n = 5$) harvested at 120 d.

Measurement of gene transcript levels. To evaluate the effects of CTLA4Ig treatment on lymphocyte and macrophage activation, we measured gene transcript levels for factors we have previously shown (with the exception of TGF-β) to be upregulated in 75-d cardiac allografts. Relative gene transcript levels were measured by reverse transcriptase PCR assays which are sensitive, require only 2.5 μg of total RNA, and permit replicates to be performed from a single cDNA. Primers and reverse transcriptase PCR conditions (including the logarithmic ranges for cycle number and template dilutions), annealing temperatures, and cycle number for each of the various factors had been optimized already (9–11). The assay for TGF-β was set up similarly, with optimization of the PCR reaction, confirmation of the identity of the product by sequence analysis, and establishment of the logarithmic assay range. The primer sequences used for amplification of TGF-β-1 were 5′ TGA ACC AAG GAG ACG GAA TAC AGG and 3′ TAC TGT GTG TCC AGG CTC CAA ATG generating a single PCR product band of 385 bp at an annealing temperature of 57°C for 24 cycles (20).

To identify relative differences, we derived corrected transcript levels from sets of cDNAs (studied simultaneously) that included negative controls (for which reverse transcriptase had been omitted during cDNA synthesis or for which water had been used instead of cDNA). The 9 cDNAs (experimental set) analyzed were prepared from 75-d cardiac allografts treated in various subgroups with (*a*) a single dose of CTLA4Ig ($n = 3$), (*b*) a single dose of cyclosporine A ($n = 3$), and (*c*) 75-d LEW cardiac isografts ($n = 3$). Each PCR analysis was completed in quadruplicate. The mean value for the corrected levels was obtained by pooling measurements from all animals in a subgroup. Results for each analysis were subjected to multivariate ANOVA without replication. If variance was significant, individual comparisons were made and the level of significance was corrected by the Bonferonni method (11).

Immunocytochemistry. Leukocyte populations and expression levels of various gene products were evaluated in serial cryostat sections by immunoperoxidase labeling (peroxidase-antiperoxidase technique) (10, 16). This analysis included the use of mouse mAbs to rat mononuclear phagocytes (ED-1; Accurate Chemicals, Westbury, NY) and IFN-γ (DB-10; Dr. P. van der Meide, Rijswijk, Holland) and polyclonal rabbit antibodies to iNOS (Transduction Laboratories, Lexington, KY), TGF-β (R and D Systems, Minneapolis, MN), and MCP-1 (Dr. Barrett Rollins, Dana-Farber Cancer Institute, Boston, MA).

Morphometric analysis. Sagittal sections of cardiac allografts stained with Verhoeff's elastin were compared by using the 0–5 grading system described by Adams et al. (5). In brief, a score of 0 indicated a normal vessel, 3 implied 20–50% luminal occlusion, and a score of 5 implied > 80% luminal occlusion. For a more quantitative assessment, the actual percent luminal occlusion for each vessel was determined using NIH-Image (version 1.51) on a Macintosh Centris 650 to trace the internal elastic lamina and the lumen in each vessel from transmitted light images of elastin-stained sections. Percent luminal occlusion was calculated using: (area within the internal elastic lamina − area of the lumen)/(area within the internal elastic lamina) × 100. Mean values for the vessel scores, percentage of diseased vessels, and percent luminal occlusion were then derived by pooling all of the vessels analyzed per graft. A total of 268 vessels were analyzed with an average of 15.7 scored per graft. Mean scores were pooled for all grafts in each group and subjected to multivariate ANOVA as described above.

Results

Cardiac allograft survival. LEW heart allografts (Table I) were rejected by week 4 after transplantation in the three control groups of F344 recipients: untreated, single dose of control Ig on day 2, and single dose of cyclosporine A. Administration of CTLA4Ig 2 d after engraftment resulted in a prolongation of graft survival, with 64% of the allografts surviving longer than 70 d (palpable cardiac impulses were similar to those in isografts). In contrast, in only 22% of the animals that received a relatively high dose of cyclosporine A (25 mg/kg 2 d after transplantation) was graft survival prolonged beyond 70 d. As expected, subcutaneous injection of cyclosporine A (5 mg/kg) daily for 1 mo improved allograft survival, with 58% of the grafts surviving longer than 100 d.

Inflammatory cell activation patterns. To study the effect of CD28-B7 blockade on inflammatory cell activation in the target organ, we measured relative gene transcript levels in 75-d cardiac allografts with reverse transcriptase PCR assays optimized for CD4, IFN-γ, TGF-β, iNOS, MCP-1, and Gal/GalNAc macrophage lectin. For all six factors, transcript levels were significantly higher in cardiac allografts from animals that received cyclosporine A and survived longer than 70 d ($P < 0.001$) than in the age-matched isografts (Fig. 1). As we observed for the previously studied factors, TGF-β transcript levels were higher in allografts (by day 7 and through day 90) than in isografts or host hearts (data not shown). These findings are consistent with reports of increased TGF-β transcript levels in rat aortic allografts and in human endomyocardial biopsy samples (21, 22). In contrast, in the cardiac allografts from recipients treated with CTLA4Ig, transcript levels were significantly lower for most factors studied in comparison with levels in the group receiving cyclosporine A (Figs. 1 and 2). Specifically, CTLA4Ig treatment was associated with reduced gene transcript levels for the cell surface marker CD4. As might be expected, this reduction correlated with a decrease in mononuclear cell infiltration apparent on histologic examination of tissue from the CTLA4Ig group in comparison with that from the cyclosporine A group. There was also a signifi-

Table I. Cardiac Allograft Survival

Treatment	Survival (d)
No treatment	12, 14, 18, 21, 25
Control Ig (0.5 mg intraperitoneally, day 2)	9, 14, 17, 17
CTLA4Ig (0.5 mg intraperitoneally, day 2)	11, 7, 20, 20, > 27, > 27, > 70, > 74, > 76, > 76, > 124, > 125, > 125
Cyclosporine A (25 mg/kg intramuscularly, day 2)	6, 15, 16, 16, 22, 28, 28 > 73, > 73
Cyclosporine A (5 mg/kg subcutaneously for 30 d)	4, 5, 6, 41, 49, 120, 124, 124, 124, > 118, > 124, > 124

cant reduction in gene transcript levels for the T cell activation cytokine IFN-γ, macrophage activation markers (iNOS and Gal/GalNAc macrophage lectin), and TGF-β in the group receiving CTLA4Ig in comparison with the cyclosporine A group ($P < 0.007$). The increase in MCP-1 gene transcript levels was not affected significantly by single-dose CTLA4Ig treatment, suggesting that MCP-1 may not be regulated at the transcriptional level or that it may be regulated by factors other than those in the CD28-B7 cascade.

Immunocytochemical analysis. The extent to which increased levels of gene transcripts resulted in corresponding expression of gene products was determined by immunoperoxidase labeling (Fig. 2). The 75-d allografts from recipients

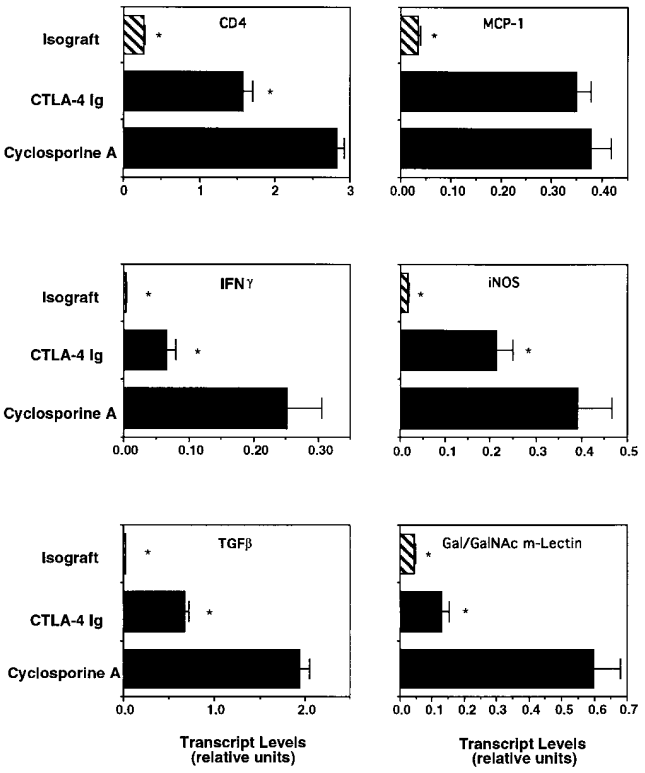


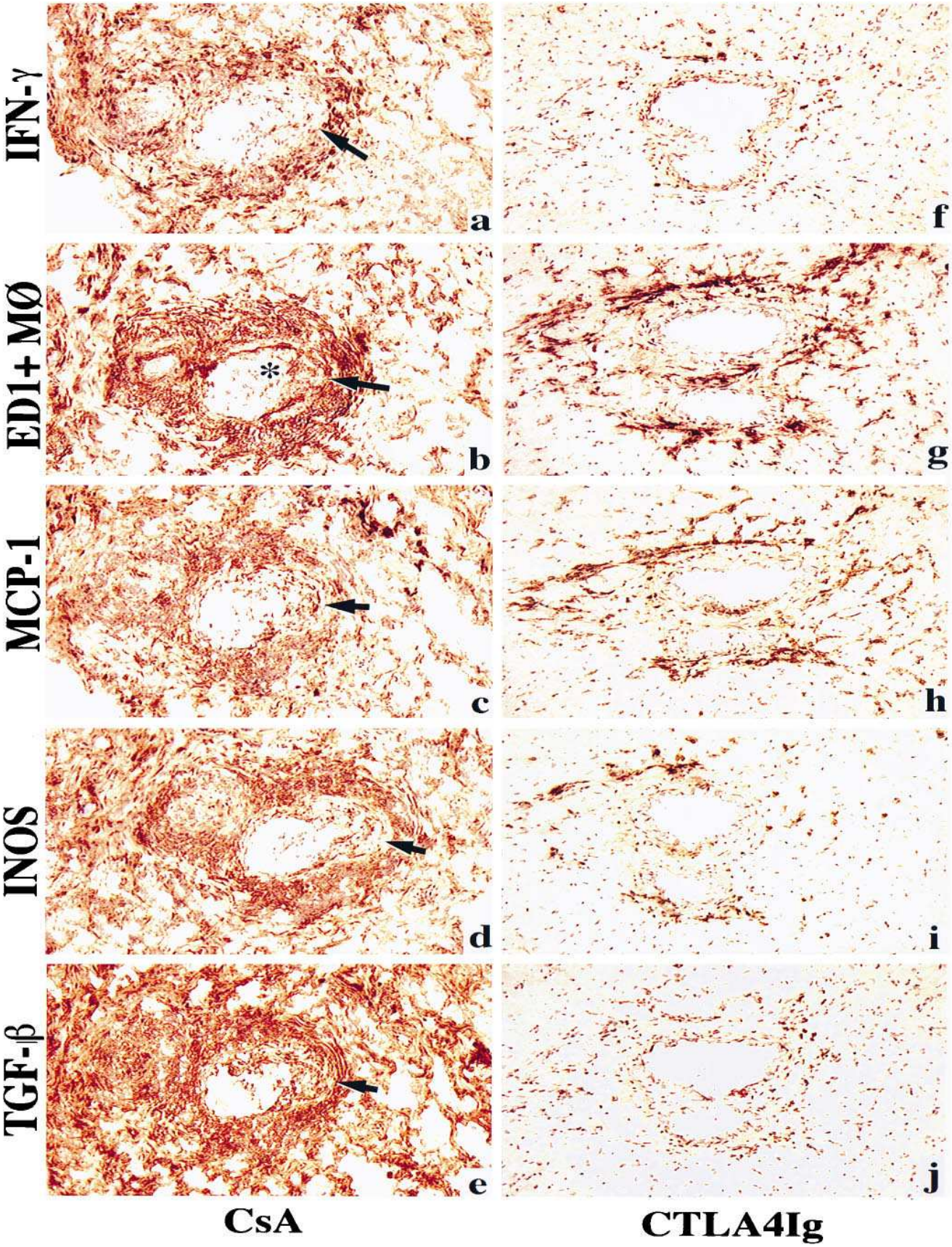
Figure 1. CTLA4Ig suppresses upregulation of inflammatory cell transcripts in LEW to F344 rat cardiac allografts. ^{32}P–reverse transcriptase PCR assays normalized with G3PDH comparing relative levels of the indicated gene transcript in cDNAs derived from 75-d allografts treated with cyclosporine A or CTLA4Ig in contrast with LEW rat isografts. Each bar represents the mean±SEM for each subgroup ($n \geq 3$) and represents four separate PCR analyses per sample. *$P < 0.007$ in comparison with cyclosporine A.

treated with a single dose of cyclosporine A showed marked mononuclear cell infiltration involving many ED-1–positive macrophages (Fig. 2 *b*) associated with patchy fibrosis in the myocardium and perivascular space. Intimal thickening typical of chronic rejection was present in the group of allografts from recipients treated with cyclosporine A (two vessels in *b*). In the smaller vessel (*left*) the lumen was almost completely obliterated, whereas in the larger vessel the neointima had expanded (*asterisk*). Mononuclear cell infiltration was associated with striking staining for IFN-γ, MCP-1, and iNOS (Fig. 2 *a*, *c*, and *d*). Also, for iNOS some smooth muscle cells in some vessel subsets were also positive, as we have observed elsewhere in control allografts (11). Finally, in the cyclosporine A group (Fig. 2 *e*) there was dense staining for TGF-β associated with mononuclear and neointimal cells and matrix. In contrast, in 75-d cardiac allografts from recipients treated with CTLA4Ig there were far fewer cells staining positive and a decrease in the intensity of staining for all five factors (Fig. 2, *f–j*). Of the inflammatory cell factors studied, staining for MCP-1 was still intense, in spite of the clear reduction in level in comparison with the cyclosporine A–treated group. One possible explanation for the discordance between the levels of MCP-1 transcripts (which were not significantly lower) and those of the MCP-1 gene product (which were reduced in the CTLA4Ig-treated allografts) is that small decreases might not be identified in the reverse transcriptase PCR assay. Alternatively, MCP-1 may be regulated at the translational level in these allografts.

Morphometric analysis. LEW to F344 cardiac allografts older than 100 d showed more advanced and frequent arteriosclerotic lesions after cyclosporine A treatment compared with the group that received CTLA4Ig or isografts. Fig. 3 depicts representative vessels from the isograft group without intimal thickening (*A*), from the cyclosporine A–treated group (*B*) with neointimal thickening obliterating the lumen, and from the CTLA4Ig-treated group (*C*) with markedly reduced thickening. In the cyclosporine A–treated group, the measured percent luminal occlusion, mean intimal thickening score, and number of affected vessels were significantly higher than in vessels from the allografts treated with CTLA4Ig or isografts ($P < 0.0003$)(Table II). Thus, a single dose of CTLA4Ig delivered on day 2 after transplantation affected both late inflammatory cell activation and arteriosclerotic development.

Discussion

Our data demonstrate that blocking the CD28-B7 costimulatory pathway of T cell activation by administering a single in-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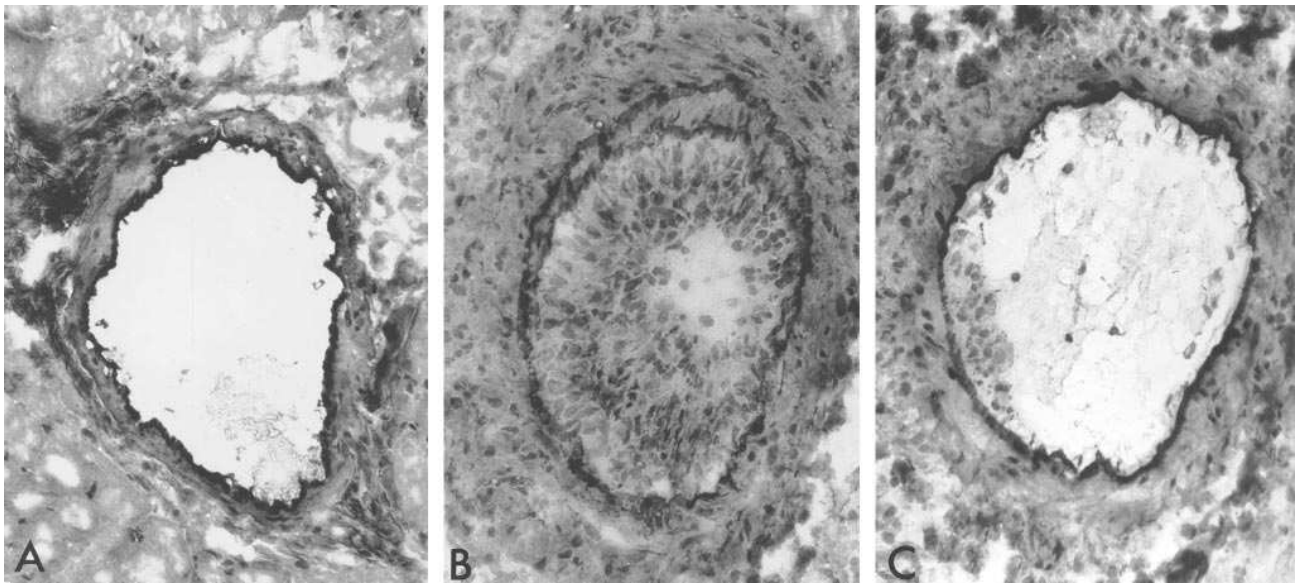

Figure 3. Intimal thickening in representative arteries from LEW heart grafts (≥ 115-d-old). (*A*) No significant thickening is seen in those placed in LEW recipients (isograft controls). (*B*) Obliterative thickening is seen in grafts placed in F344 recipients treated with cyclosporine A. (*C*) Reduced thickening is present in grafts placed in F344 recipients treated with a single dose of CTLA4Ig on day 2 (×400).

jection of CTLA4Ig 2 d after transplantation increased cardiac allograft survival, decreased graft mononuclear cell infiltration, and significantly reduced gene transcript and product levels for key T cell and macrophage activation markers in the LEW to F344 rat model of chronic rejection. Our observations in 75-d allografts indicate that effector cascades associated with both T cell and macrophage activation were disrupted by early administration of CTLA4Ig. Furthermore, our data show that early CD28-B7 blockade attenuates arteriosclerotic development, the sine qua non of chronic rejection, reducing both its severity and the percentage of diseased vessels. Hence, these findings provide clear support for the widely held hypothesis that T cell recognition of alloantigen and activation are critical and proximal events in the complex cellular and molecular processes leading to chronic rejection.

We (16) and others (17, 23, 24) have shown previously that CD28-B7 blockade by CTLA4Ig prevents acute rejection and induces donor-specific tolerance in several experimental models. The data presented here are the first to demonstrate that blocking this costimulatory T cell activation pathway prevents chronic rejection. Of interest are our observations that relatively high doses of cyclosporine A administered in a protocol similar to that for CTLA4Ig were ineffective in prolonging allograft survival and that they had little effect on development of chronic rejection. This is not surprising given that humans receive chronic cyclosporine A therapy and still develop transplant arteriosclerosis. Furthermore, although prolonged daily administration of maintenance doses of cyclosporine A was effective in prolonging allograft survival to a degree similar to that seen with a single injection of CTLA4Ig, cyclosporine A did not prevent development of chronic rejection at this dose. Therefore our data underscore the importance of making detailed examinations of allografts when examining strategies for prolonging graft survival, since survival data alone may not reflect the degree of ongoing chronic rejection. Our observations also emphasize that therapies targeted to induction of tolerance rather than immunosuppression may be essential in the development of novel strategies for preventing chronic rejection (25, 26).

One of the known consequences of CD28-B7 signaling is the regulation of cytokine production by T cells (13, 27). Little is known, however, about the effects of the CD28-B7 costimulatory pathway on macrophage activation. In this report, we show that early blockade of CD28-B7 also disrupts increases in gene transcript levels (Gal/GalNAc macrophage lectin and iNOS) associated with macrophage activation in 75-d cardiac allografts. For those factors whose expression levels were reduced, this might have been caused by a direct inhibition of macrophages through the B7 axis or, more likely, by an indirect effect related to a decrease in levels of T cell cytokines (such as IFN-γ) required for induction of macrophage expression. In LEW to F344 rat cardiac allografts, we have shown previously that upregulation of T cell factors such as IFN-γ and IL-6 occurs in concert with that of macrophage activation factors (MCP-1, iNOS, Gal/GalNAc macrophage lectin, and allograft inflammatory factor-1). Gene transcript levels increase early in allografts (by day 7) and remain persistently elevated (through day 75 at least), and they are low in age-

Figure 2. Immunoperoxidase labeling of serial sections from a representative 75-d LEW to F344 rat cardiac allograft after treatment with a limited course of cyclosporine A (CsA) (*a–e*) or CTLA4Ig (*f–j*). In the cyclosporine A–treated group, consecutive sections show perivascular, adventitial, and occasional intimal and interstitial mononuclear cells and an expanded neointima (to immediate right of *asterisk* in muscular artery in *b* and extending to the *arrow* in *a–e*). The effects of CTLA4Ig therapy include a marked reduction in labeling for IFN-γ (*f*), considerably fewer macrophages (*g*), and greatly reduced labeling for MCP-1, iNOS, and TGF-β (*h–j*). (Cryostat sections, counterstained with hematoxylin and dilute eosin, ×200.)

Table II. Vessel Morphometry in 120-d LEW to F344 Cardiac Allografts

	Percent luminal occlusion	Vessel score	Percent diseased vessels	
	mean±SD	*mean±SD*	*mean±SD*	
Cyclosporine A	32±12	2.0±0.7	94±7	$n = 7$
CTLA4Ig	7±5	0.5±0.3	51±25	$n = 5$
Isografts	5±1	0.4±0.1	40±6	$n = 5$

matched isografts. The sustained expression of these activation factors is in keeping with a chronic alloimmune response, it occurs in concert with development of arteriosclerotic lesions, and it is modulated by other strategies that reduce arteriosclerosis (11).

These studies in the LEW to F344 rat model of chronic cardiac rejection show that CTLA4Ig blockade of the CD28-B7 costimulatory T cell activation pathway modulates the state of chronic inflammatory activation in both T cells and macrophages in allografts. More importantly, our findings demonstrate that CTLA4Ig, in addition to protecting against acute rejection, protects against the arteriosclerotic sequelae of chronic rejection. These observations should have important implications for clinical transplantation, as studies of CTLA4Ig are being planned in humans.

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