Blockade of T-cell costimulation prevents development of experimental chronic renal allograft rejection

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ABSTRACT Blocking CD28-B7 T-cell costimulation by systemic administration of CTLA4Ig, a fusion protein which binds B7 molecules on the surface of antigen-presenting cells, prevents rejection and induces tolerance in experimental acute allograft rejection models. We tested the effect of CTLA4Ig therapy on the process of chronic renal allograft rejection using an established experimental transplantation model. F344 kidneys were transplanted orthotopically into bilaterally nephrectomized LEW recipients. Control recipients, receiving a single injection of CTLA4Ig treated animals, as compared with cyclosporine-alone treated controls. These data confirm that early blockade of the CD28-B7 T-cell costimulatory pathway prevents later development and evolution of chronic renal allograft rejection. Our results indicate that T-cell recognition of alloantigen is a central event in initiating the process of chronic rejection, and that strategies targeted at blocking T-cell costimulation may prove to be a valuable clinical approach to preventing development of the process.

The most common cause of human allograft failure after the first year posttransplant is the incompletely understood clinicopathological entity of chronic rejection (1–3). This process affects all types of solid organ grafts and is characterized morphologically by obliterator vasculopathy, interstitial fibrosis with variable degrees of mononuclear cell infiltration, and in the case of the kidney, glomerulosclerosis. Risk factors for its development include HLA mismatching, acute rejection, early graft ischemia, differences in donor and recipient size, adequacy of immunosuppression, and viral infections. The exact mechanisms responsible for its pathogenesis are unknown, although there is evidence that chronic graft dysfunction is mediated by both alloantigen-dependent as well as alloantigen-independent mechanisms (4). At this moment no specific therapies are available.

T-cell recognition of alloantigens is the key initial event which ultimately results in allograft rejection (5, 6). T cells require two signals for full activation. The first signal is produced by engagement of the T-cell receptor with the foreign antigen presented as a peptide by antigen-presenting cells, thus providing antigen-specificity to the immune response. The second is a “costimulatory” signal mediated, among other interactions, by the T-cell accessory molecule CD28 interacting with the B7 family (B7-1 and B7-2) of molecules on antigen-presenting cells (7–9). In vitro, blockade of costimulatory signals inhibits T-cell activation and induces a state of antigen-specific unresponsiveness. In vivo, agents which block CD28-B7 costimulation, including the fusion protein CTLA4Ig or anti-B7 monoclonal antibodies, have been shown to inhibit the immune response in experimental models of acute transplant rejection (10). In this study we have investigated the role of T-cell costimulatory blockade in an established model of chronic renal allograft rejection.

MATERIALS AND METHODS

Animals. Inbred 200–250 g male LEW rats (RT1™) were used as graft recipients and F344 (RT11™) rats served as donors (Harlan–Sprague–Dawley).

Kidney Transplantation. Kidneys were transplanted (11) orthotopically to the left renal vessels and the left ureter of the host by end-to-end anastomosis using 10-0 prolene. The right kidney was removed 10 days later. Since the animals are dependent on the transplanted kidney function, complete allograft failure is defined as death of the animal.

Reagents. The human fusion protein CTLA4Ig and control chimeric recombinant fusion protein L6 were a generous gift of P. Linsley (Bristol–Myers Squibb, Seattle). Cyclosporine (CsA) was obtained from Sandoz Research Institute (East Hanover, NJ).

Experimental Groups. Five experimental groups of F344 into LEW renal allograft recipients were examined and compared. Group 1 (n = 4) received no treatment. Group 2 (n = 14) received a single injection of CTLA4Ig (0.5 mg, i.p.) on day 2 posttransplant, a dose based on our recent studies in an acute renal allograft model showing optimal effect if CTLA4Ig administration is delayed until 2 days after transplantation (12). Group 3 (n = 6) received L6 control Ig (0.5 mg, i.p.) also on day 2 posttransplant. Group 4 (n = 19) received a short term low dose regimen of CsA (5 mg/kg/day × 10 days, s.c.), as described (13, 14). Group 5 (n = 13) received the low-dose CsA protocol and day 2 CTLA4Ig therapy in combination. Two additional control groups (n = 4 per group) of LEW into LEW renal isograft recipients were studied, of which one group was unmodified and the other received the low CsA protocol. Grafts (n = 2–4) were harvested from randomly selected animals of the different groups at 8, 16, or 24 weeks posttransplant, for morphological and reverse transcriptase–PCR (RT–PCR) studies.

Function. Urine (24 h) was collected every 2–4 weeks from the time of transplantation. Protein excretion was determined by measuring precipitation following interaction with 3% sulfo salicylic acid. Turbidity was assessed by absorbance at a

Abbreviations: CsA, cyclosporine; RT–PCR, reverse transcriptase–PCR; IL, interleukin; IFN-γ, interferon γ; TNF-α, tumor necrosis factor α; TGF-β, transforming growth factor β; MCP-1, monocyte chemoattractant protein 1.

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wavelength of 595 nm using a Beckman Du-64 spectrophotometer, as described (11).

**Morphology.** Kidney grafts from each recipient group (n = 3–4 per group per time point) were fixed in 10% buffered formalin and paraffin embedded. Sections were stained with hematoxylin/eosin and examined by light microscopy for cellular infiltrates, glomerulosclerosis and vascular intimal hyperplasia (11, 14).

**Immunohistology.** Sections of the kidney grafts were snap-frozen in liquid nitrogen, cut (4 μm), fixed in acetone for 10 min, air-dried and stained with mouse anti-rat monoclonal antibodies (Bio Products for Sciences, Indianapolis). The sections were then stained with rabbit anti-mouse IgG by the alkaline phosphatase, anti-alkaline phosphate methods, and counterstained with hematoxylin. Cell populations and cell surface markers were assessed using antibodies to CD4 (W3/25), CD8 (OX-8), and macrophages (ED-1). Results are expressed as mean ± SD of positive cells/field of view; more than 20 field of view/section/specimen were evaluated at ×400 (11).

**PCR Analysis.** Frozen kidney RNA samples were prepared using a guanidinium isothiocyanate/phenol-chloroform isolation method (ULTRA-spec.; Biotec, Houston). Total RNA was used for first strand cDNA synthesis employing oligo-dT$_{12-18}$ and RT according to supplier-recommended conditions (GIBCO/BRL). Nonlooping, nonoverlapping, oligonucleotide primer pairs from separate exons were prepared for each gene studied [interleukin 2 (IL-2), interferon γ (IFN-γ), IL-2 receptor, IL-6, IL-12, tumor necrosis factor α (TNF-α), monocyte chemoattractant protein 1 (MCP-1), transforming growth factor β (TGF-β)] by CLONTECH or by Genosys (The Woodlands, TX). The competitive PCR for quantification of mRNA was performed as described. Amplification was begun with incubation at 94°C for 2 min and was optimized for each cytokine. PCR products (5 ml) were run on an ethidium bromide-stained 1.5% agarose gel and gene-specific bands were visualized with UV light. We performed control experiments for each primer pair by substituting water for cDNA and omitting RT during the cDNA synthesis. The quantities of MIMIC or competitor fragments (CLONTECH) and target cDNA are compared using a PC SCANJET with analysis by Adobe PHOTOSHOP software (Adobe Systems, Mountain View, CA). Absolute amounts of cytokine PCR products as determined by competitive PCR were corrected by dividing by amounts of β-actin PCR product. Each sample was repeated twice and values are expressed as mean ± standard deviation (11, 15).

**Statistical Analysis.** For urinary protein excretion one way ANOVA was performed at each time point. The group effects were all significant at the 0.05 level for P values. To examine individual differences further, comparison between every pair of groups was performed. Since more than one comparison must be made at each time point, a Bonferroni adjustment was used for this pairwise multiple comparisons. The unpaired Student’s t test was used for data from immunohistology and PCR.

**RESULTS**

**CD28-B7 Blockade Prolongs Renal Allograft Survival.** First we analyzed the effects of CD28-B7 blockade by CTLA4Ig on renal allograft survival (Fig. 1). Most (90%) of control unmodified recipients (group 1) and animals treated with L6 control human Ig (group 3) died by 8 weeks posttransplant, with only one animal surviving more than 100 days (mean survival, 33 ± 31 days). Seventy percent of animals treated with the low dose CsA protocol (group 4) survived >8 weeks (Fig. 1). CTLA4Ig therapy alone (group 2) or in addition to the transient low-dose CsA protocol (group 5) prevented early graft loss and improved long-term survival with 90% of animals surviving >8 weeks, similar to the LEW into LEW isograft recipients. These data confirm our previous results in an acute rejection model which indicated that CD28-B7 T-cell

![Fig. 1. Graft survival. The figure shows graft survival of the different experimental groups as a function of time posttransplant. Since the native kidneys of all animals have been removed, graft failure is defined as death of the animal. Survival data for LEW into LEW isograft controls treated with or without CsA (n = 4 per group) were combined.](image-url)
costimulatory blockade by a similar protocol of CTLA4Ig prevented acute rejection and induced tolerance to renal allografts (12). Treatment with the conventional immunosuppressive agent CsA prevents early loss and prolongs survival of renal allografts in the F344 to LEW model; such grafts, however, develop functional and morphological changes of chronic rejection (11, 13, 14). The question to be answered is whether blocking CD28-B7 T-cell costimulation by CTLA4Ig prevents development of chronic renal allograft rejection.

Functional Studies. Although several assays of sequential renal function have been studied in the established F344 into LEW model of chronic allograft rejection, urinary protein excretion is the most convenient and accurate (13). Fig. 2 shows the 24-h protein excretion data from surviving animals. Group 4 control animals treated with low-dose CsA develop progressive proteinuria starting around 8–12 weeks posttransplantation. Animals treated with CTLA4Ig alone (group 2) or in addition to CsA (group 5) never developed proteinuria throughout the follow-up period \( P < 0.005 \) versus CsA alone treated animals at 12, 16, and 24 weeks. In fact, protein excretion in these two groups was not significantly different from isograft controls. To our knowledge, this is the first demonstration indicating that early short-term blockade of T-cell costimulation prevents development of late functional deterioration associated with chronic renal allograft rejection.

Graft Morphology. By 16 weeks, allografts in the group receiving short term CsA alone (group 4) had developed the characteristic features of chronic rejection, including mononuclear cell infiltration in perivascular and periglomerular areas, early transplant arteriosclerosis, and glomerulosclerosis. Some 10–20% of cortical arteries contained mild intimal proliferation (<20% of vessel luminal cross-section), and all arteries had ballooning of endothelial cells, medial vacuolization, and perivascular edema. More than 50% of glomeruli showed focal and segmental proliferation, as well as focal and segmental glomerulosclerosis. Sections of allografts from rats receiving either CTLA4Ig alone (group 2), or CTLA4Ig plus CsA (group 5), showed normal arteries, minor glomerular hypercellularity and only low intensity interstitial mononuclear cell infiltrates. By 24 weeks, the differences between the animals given CsA alone and either group receiving CTLA4Ig had become markedly accentuated (Fig. 3). Essentially all the arteries in allografts from rats given CsA alone showed 10–30% luminal occlusion by neointimal expansion, and in many vessels >50% encroachment was seen (Fig. 3f). All glomeruli showed focal and segmental proliferation and sclerosis, and most were markedly hypertrophied (Fig. 3g). In contrast, allografts from animals which received CTLA4Ig with or without CsA demonstrated only minor, nonspecific vascular changes (focal ballooning of endothelial cells, focal medial vacuolization); no intimal proliferation was observed. Glomeruli in both groups were of normal size, showing only minor hypercellularity and patent capillary loops (Fig. 3 h and i). Minor, nonspecific tubular changes were seen in each of the three groups, and interstitial fibrosis was not apparent by 24 weeks.

We then performed immunohistological studies to define and compare the nature of the cellular infiltrates in the grafts of the different experimental groups (Table 1). Grafts from control animals (group 4) treated with low dose CsA showed a progressive increase in the number of infiltrating ED1+ macrophages in the periglomerular and perivascular areas after 8 weeks and peaking at 16 weeks posttransplant. Infiltrating T cells of both CD4+ and CD8+ phenotype also peaked at 16 weeks and were localized in the periglomerular and perivascular areas. Grafts from animals treated with CTLA4Ig alone (group 2) or in combination with CsA (group 5), on the other hand, had significantly less infiltration with either macrophages or CD4+ and CD8+ cells at 16 weeks \( P < 0.05 \), a time when cellular infiltrates peak in the control animals treated with CsA alone.

![Fig. 2. Urinary protein excretion. The figure shows 24-h protein excretion of the different experimental groups as a function of time posttransplant (n = 3–11 per group per time point). Protein excretion data for LEW into LEW isograft controls treated with or without CsA (n = 4 per group) were combined.](image-url)
Fig. 3. Representative histologic findings in paraffin sections of rat renal allografts at 24 weeks posttransplant from animals receiving CsA alone (CsA), a single dose of CTLA4Ig (CTLA4Ig), or their combination (CsA/CTLA4Ig). Low power views (a–c) show the extensive mononuclear cell infiltration, arterial luminal occlusion, and glomerular injury associated with therapy with CsA alone, and how these are lacking in the other two groups. Arrowheads indicate vessels and glomeruli shown at high power in the corresponding subsequent panels. In d, in addition to a perivascular focus of mononuclear cell infiltration, extensive intimal expansion encroaching on >50% of the arterial lumen is seen; arrowheads indicate points...
with CsA-treated rejection. The most common cause of chronic rejection is the clinico-pathological entity of chronic rejection (1-3), a process that affects all solid organ allografts, including kidney, heart, liver, and lung. Therefore, development of strategies to prevent and/or interrupt this process should have major implications for the field. It has been difficult to prove that early immune activation in a vascularized organ allograft is responsible for development of the late loss-of-function characteristic of the process of chronic rejection. Failure of a previously functioning renal allograft is now the most common cause of end-stage renal disease beyond the first year posttransplant, accounting for 50–80% of patients who return to dialysis, and 25–30% of those awaiting renal transplantation. Similarly, over 20% of kidney transplants performed in the United States go to patients who have had one or more renal allografts fail. The introduction of CsA therapy has improved short-term renal allograft survival by 10–20% at 1 year posttransplantation; however, the rate of chronic graft loss has not been significantly reduced (3).

Although the process of chronic graft rejection has long been recognized, the mechanisms responsible for the condition remain unclear (1–3). It has been postulated, based on recent experimental studies, that chronic graft dysfunction is mediated by both alloantigen-dependent as well as alloantigen-independent mechanisms (4). The hypothesis, based on serial studies of various models, is that T-cell recognition of alloantigen leads to activation of CD4+ T cells which provide the necessary help for activation of macrophages and alloantibody producing B cells; CD8+ T cells have been shown not to play a critical role in initiation or development of chronic rejection (16). Chemokine receptors, such as RANTES (regulated upon activation, normal T-cell expressed and secreted) and MCP-1, recruit circulating mononuclear cells to the graft. Activated T cells and macrophages in turn secrete the necessary cytokines to activate endothelial cells, which express class II major histocompatibility complex antigens, adhesion molecules, and costimulatory molecules that can present antigen and activate more T cells. Activated graft infiltrating T cells and macrophages, and the endothelial cells themselves, secrete growth factors that cause smooth muscle proliferation leading to vascular intimal occlusion, interstitial fibrosis, and in the case of the kidney, glomerulosclerosis, all typical changes in chronically rejected allografts. Alloantigen-independent mechanisms, such as graft ischemia, contribute by upregulating major histocompatibility complex on the surface of graft cells and inducing upregulation of cytokines and activation markers (17) which amplify alloantigen-dependent mechanisms; the interaction of these several factors lead ultimately to progressive graft failure (4, 18).

We have used an established experimental model of chronic renal allograft rejection to show that blocking the CD28-B7 costimulatory pathway of T-cell activation by administering a single injection of CTLA4Ig 2 days after transplantation prevents early rejection and increases renal allograft survival substantially, decreases graft mononuclear cell infiltration and activation, and prevents development of the functional and morphological features of chronic rejection. In particular, targeting this pathway essentially abrogated the pathologic features of chronic renal allograft rejection, including transplant arteriosclerosis, and glomerular hypertrophy and glomerulosclerosis. Blocking CD28-B7 T-cell costimulation by CTLA4Ig has been shown to be effective in preventing acute rejection of vascularized allografts in several experimental models (12, 19, 20). The novelty of our present observations is the finding that early blockade of this pathway prevents the development of late changes of chronic allograft rejection, confirming our recent studies in a chronic cardiac allograft rejection model which showed that CD28-B7 T-cell costimulatory blockade prevents development of graft arteriosclerosis (21). Unlike the cardiac model, the renal model provides the added advantage of being able to follow graft function serially of attenuation or breach in the internal elastic lamina. In contrast, arteries from the other two groups (e and f) show intact internal elastic laminae and an absence of intimal hyperplasia. Characteristic glomerular features from the CsA alone group are seen in g; these include glomerular hypertrophy and hypercellularity, and focal and segmental proliferation and glomerulosclerosis. Note thickening of almost all capillary loops and expanded matrix, as well as adjacent mononuclear cells. Glomeruli from the CTLA4Ig therapy groups (h and i) are largely normal in size and cellularity, and show patent capillary loops. (Hematoxylin/eosin counterstain; a-c, ×30; d-f, ×150; g-i, ×100.)

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*P < 0.05 when compared with CsA (control) group using the Student’s t test. Results are expressed as mean ± SD of positive cells/field of view; more than 20 fields of view/section/specimen.
These recognition of plant recipients findings provide in Grants transplantation, macrophage (TNF-α associated and to show that of regulation of novel ment size that "immunosuppression" T-cell only tolerance process, 21-23).

One of the known consequences of CD28-B7 signaling is the regulation of cytokine production by T cells. In this report, we show that early blockade of CD28-B7 T-cell costimulation disrupts considerably later increases in intragraft gene transcript levels associated with T-cell (IL-2, IFN-γ, and IL-2 receptor) and macrophage (TNF-α and IL-6) activation, chemokattants (MCP-1), and the fibrogenic growth factor TGF-β; all gene products upregulated in allografts undergoing chronic rejection (15, 21-23). TGF-β expression, in particular, has been recently demonstrated to correlate with existence of interstitial fibrosis and chronic dysfunction of human renal allografts (24, 25). Our findings provide clear support for the hypothesis that T-cell recognition of alloantigen and activation are critical early events in the complex cellular and molecular processes leading to development of chronic rejection late after transplantation. These studies should have important implications for clinical transplantation, and clinical trials with CTLA4Ig in kidney transplant recipients are being planned.

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**Fig. 4.** Intragraft expression of T-cell and macrophage products tested by competitive RT-PCR against β-actin and expressed as a function of time posttransplant in control CsA-treated animals (A and C) versus CTLA4Ig-treated animals (B and D) (n = 3 per group per time point).