

CD28-B7 Blockade after Alloantigenic Challenge In Vivo Inhibits Th1 Cytokines but Spares Th2

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Summary

Blocking the CD28-B7 T cell costimulatory pathway with the fusion protein CTLA4Ig inhibits alloimmune responses in vitro and in vivo and induces tolerance to cardiac allografts in mice and rats, but the mechanisms mediating the tolerant state in vivo are unknown. Here, we report the effects and potential mechanisms of CTLA4Ig in the rat renal allograft model. LEW rats were nephrectomized and received renal allografts from major histocompatibility complex-incompatible WF rats. While all untreated and control immunoglobulin (Ig)-treated animals acutely rejected their allografts and died, 86% of rats that received a single injection of CTLA4Ig on day 2 after transplantation had prolonged survival (>60–100 days) with preserved renal function. By contrast, only 29% of animals that received CTLA4Ig on the day of engraftment had prolonged survival. Long-term survivors (>100 days) exhibited donor-specific tolerance, accepting donor-matched WF but acutely rejecting third-party BN cardiac allografts. Immunohistological analysis of grafts sampled at 1 week after transplantation showed that both control and CTLA4Ig-treated animals had mononuclear cell infiltrates, with a higher percentage of CD4⁺ cells in the CTLA4Ig-treated group. However, while this was associated with vasculitis and tubulitis in control grafts, there was no evidence of tissue injury in CTLA4Ig-treated animals. The immune response leading to graft rejection in control animals was characterized by expression of the T helper (Th) type 1 cytokines interleukin (IL)-2 and interferon- γ . In contrast, the persistent CD4⁺ infiltrate without graft rejection in CTLA4Ig-treated animals was associated with increased staining for the Th2-related cytokines IL-4 and IL-10. Furthermore, grafts from CTLA4Ig-treated animals had marked upregulation of intragraft staining for IgG1, but not IgG2a or IgG2b. Administration of rIL-2 to CTLA4Ig-treated animals restored allograft rejection in 50% of animals tested. These results confirm that blockade of the CD28-B7 pathway after alloantigenic challenge induces donor-specific acceptance of vascularized organ allografts, and indicates in this model that CTLA4Ig inhibits Th1 but spares Th2 cytokines in vivo.

Naive T cells require two distinct signals for activation. The first signal is provided by the engagement of the TCR with the MHC molecule plus peptide complex on APC, and the second costimulatory signal is provided by engagement of T cell surface receptors with their ligands on APC. Signaling through the TCR alone without a costimulatory signal leads to a prolonged state of T cell anergy (1). Among the multiple costimulatory pathways identified, interaction of CD28 on T cells with either of two ligands, B7-1 or B7-2, on APC is the most important costimulatory pathway for

the response to alloantigens (2). Ligation of CD28 by B7-1 or B7-2 is blocked by CTLA4Ig, a recombinant fusion protein that contains the extracellular domain of human CTLA-4 (a gene highly homologous to CD28) fused to a human IgG1 heavy chain (2). CTLA4Ig has a 20-fold higher affinity for B7-1 than does CD28, and acts as a competitive inhibitor of CD28 binding to B7-1 or B7-2. Human CTLA4Ig efficiently binds to human, murine, and rat B7 molecules, and inhibits the alloimmune response in vitro (3, 4) and in vivo (3, 5, 6). Previously, we have shown that a single injection

tion of CTLA4Ig, administered 2 d after cardiac transplantation to rats that received donor splenocytes on the day of engraftment, induced donor-specific tolerance of the allografts (7). More recently, CTLA4Ig has been shown to induce tolerance in the mouse cardiac allograft model (8). Despite this evidence that CTLA4Ig can induce tolerance, its mechanisms of action *in vivo* are unknown. We now report the effects and potential mechanisms of CD28-B7 costimulatory blockade by CTLA4Ig in a rat renal allograft model.

Materials and Methods

Animals. Inbred male WF (RT1^u), LEW (RT1^b), and BN (RT1^a) strain rats, 8–12-wk old, were obtained from a commercial source (Harlan Sprague Dawley Inc., Indianapolis, IN).

Renal Transplantation and Graft Function. LEW rats underwent bilateral nephrectomies and received MHC-incompatible WF renal allografts. In this model animals typically suffer acute rejection, become oligoanuric, and die from uremia within 2 wk (9). The survival time of the renal allograft is defined as the time from transplantation to the time of death. Only animals dying from surgical technical failures within the first 24 h after transplantation were excluded from analysis. Renal allograft function was assessed by measuring serial serum creatinine levels at different time intervals after transplantation. Serum was obtained by tail vein puncture, and serum creatinine was measured by the alkaline picrate method.

Antibodies and Other Reagents. The fusion protein CTLA4Ig (Bristol-Myers Squibb Pharmaceutical Research Institute) has been previously described (2). Details of Abs and their use in immunohistology were described previously (10–12). Unless otherwise specified, all murine mAbs and monospecific polyclonal Abs were obtained commercially from Sera-Lab (Accurate Chemicals and Science Corp., Westbury, NY) or made by us (BWH-4 and 1D10). Abs were directed against all rat leucocytes (CD45, OX-1), T cells (TCR- α/β chains, R73), CD4 (BWH-4, made by us) and CD8 (OX-8) subsets, and macrophages (ED1). Activation of mononuclear, endothelial, or parenchymal cells was assessed using Abs to class II MHC antigens (OX-3); p55 chain of the IL-2R (CD25, ART-18, courtesy of Dr. T. Diamantstein, Free University, Berlin, Germany); intercellular adhesion molecule (ICAM-1, CD54, 1A29) (Seikagaku America Inc., Rockville, MD) and the cytokines IL-2 (1D10, made by us and will be made available for research use); IL-4 (BL-4P), IL-6 (LP-716) (Genzyme Corp., Cambridge, MA); IL-10 (AB-417-NA) (R&D Systems, Inc., Minneapolis, MN); IFN- γ (DB-12, courtesy of Dr. P. van der Meide, TNO Primate Center, Rijswijk, Holland); TNF- α (IP-300) (Genzyme Corp.) plus PGE₂ (Sigma Chemical Co., St. Louis, MO). Mouse hybridomas secreting mAbs to rat Ig isotypes were purchased from American Type Culture Collection (Rockville, MD): RG11/39.4 (anti-IgG1, does not react with human IgG1, which is present in CTLA4Ig), RG7/1.30 (anti-IgG2a), and RG7/11.1 (anti-IgG2b). Polyclonal Abs to rat Ig-adsorbed goat anti-mouse immunoglobulin were obtained from Sigma Chemical Co., and rabbit anti-goat Ig, goat peroxidase-antiperoxidase (PAP), swine anti-rabbit Ig, and rabbit PAP were obtained from Dako Corp. (Carpinteria, CA).

Immunohistology. Renal transplants (three/group) were harvested at day 7 or day >100 (allografts), or at day 7 (isografts), sliced into small pieces, quick-frozen in liquid nitrogen, and stored at -80°C in preparation for immunohistologic studies (9). Cryostat sections were fixed in paraformaldehyde-lysine-periodate for staining of leucocytes and activation antigens or fixed in acetone for localization of cytokines, and stained by a three-layer (polyclonal Abs) or four-layer (mAbs) PAP method. Sections were incubated overnight with

primary antibodies at 4°C , followed by incubations at room temperature with bridging antibodies, PAP complexes, and the substrate, diaminobenzidine, dissolved in a 1 M imidazole-0.1 M Tris buffer, pH 7.6. Labeled cells within 20 high-power fields ($\times 400$) per section per rat were assessed with the aid of an ocular grid micrometer. Cytokine and endothelial labeling were judged semiquantitatively, due to the presence of extracellular (cytokines) or continuous (endothelium) labeling, as <1%, <5%, 10–20%, 20–50% or >75% of the cells indicated. Ig deposition was assessed semiquantitatively by end-titer analysis of the primary anti-rat IgG isotype-specific Ab. The specificity of labeling was assessed using isotype-matched mAbs or purified Ig and a control for residual endogenous peroxidase activity in each experiment. In addition, specificity of cytokine staining was confirmed by antibody absorption with corresponding recombinant proteins using IL-4 (204-IL; Genzyme Corp.), IL-10 (417-ML; R&D Systems, Inc.), or IFN- γ (courtesy of Dr. P. van der Meide) (9–12).

Results

A Single Injection of CTLA4Ig on Day 2 After Transplantation Induces Donor-specific Acceptance of Renal Allografts. LEW recipients of MHC-incompatible WF renal allografts received a single i.p. injection of CTLA4Ig (0.5 mg) either on the day of or on day 2 after transplantation. This protocol was selected based on our recent data in the rat cardiac allograft model (7). Control animals were untreated or received a single injection of control human Ig (0.5 mg i.p.). While all control untreated animals, and animals treated with a single injection of control Ig on day 2 after transplantation, died on day 8.9 ± 1.2 ($n = 7$) and day 6.2 ± 1.7 ($n = 6$), respectively, six out of seven animals that received a single injection of CTLA4Ig on day 2 after transplantation survived >60–100 d (Fig. 1). By contrast, only two out of seven animals that received a single injection of CTLA4Ig on the day of engraftment survived >100 days; the remainder of the animals

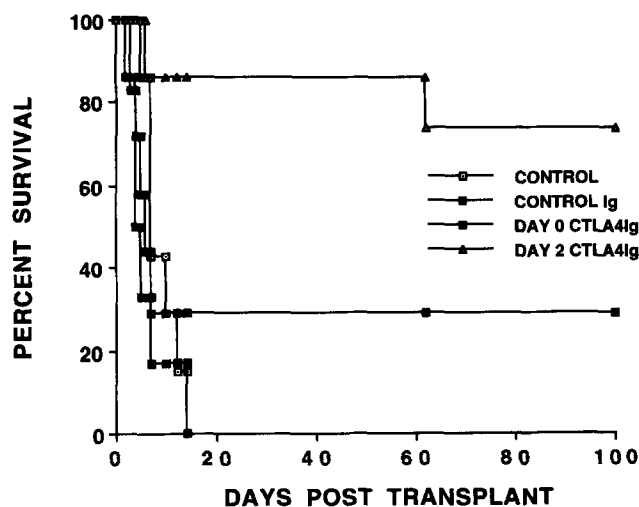


Figure 1. Survival of LEW recipients of WF renal allografts. The control group were unmodified ($n = 7$). The control Ig group received 0.5 mg i.p. of control human Ig ($n = 6$) on day 2 after transplantation. The day 0 CTLA4Ig and day 2 CTLA4Ig groups received 0.5 mg i.p. of CTLA4Ig on the day of engraftment ($n = 7$) or 2 d after transplantation ($n = 7$), respectively.

died within 7 d (Fig. 1). We then compared the effects of a single injection of a relatively high dose of the conventional immunosuppressive agent cyclosporine to that of CTLA4Ig. LEW rats received a single injection of 25 mg/kg cyclosporine i.m. on day 2 after transplantation. Four out of five animals died within 13 d; the remaining animal survived >60 d.

In our model, the renal allograft is life sustaining, and death of the animal occurs only with almost complete loss of graft function. Thus, the parameter of animal survival would not detect mild or even moderate degrees of acute rejection. This was of particular concern since our previous work in the cardiac model indicated that the grafts were sustaining at least some degree of immunologically mediated damage (7). Therefore, we assessed renal allograft function more quantitatively by measurement of serial serum creatinine levels. While untreated

control animals had an elevated serum creatinine level of 4.99 ± 1.35 mg/dl ($n = 4$), compared with isograft controls (serum creatinine = 0.69 ± 0.12 mg/dl, $n = 3$) on day 7 after transplantation, day 2 CTLA4Ig-treated animals had normal allograft function with a serum creatinine level of 0.75 ± 0.07 mg/d ($n = 6$). In addition, day 2 CTLA4Ig-treated rats continued to have preserved renal allograft function with serum creatinine of 0.72 ± 0.13 mg/dl at 1 mo and 1.05 ± 0.04 mg/dl at 3 mo. These levels were not significantly different from serum creatinines of age-matched isograft controls (0.76 ± 0.13 and 1.08 ± 0.18 mg/dl, respectively).

To test whether CTLA4Ig-treated allograft recipients surviving for >100 d were "tolerant," we rechallenged six animals with heterotopic WF or third-party BN cardiac allografts.

Table 1. Cells, Cytokines, and Other Features of Renal Allografts or Isografts in Control versus CTLA4Ig-treated Rats

	Untreated controls day 7	Single dose of CTLA4Ig on day 2		Isograft controls day 7
		day 7	day >100	
Histology (CD45)	Dense interstitial MNC infiltrate, with severe tubulitis and perivascular aggregates	Marked interstitial MNC infiltrate but no vasculitis or tubulitis	Minor interstitial MNC but moderate perivascular MNC aggregates	Few interstitial MNC
T cells	~20–30% MNC (~50% CD4 ⁺)	~20–30% MNC (>80% CD4 ⁺)	~50% MNC (>80% CD4 ⁺)	Few MNC
Macrophages	>50% MNC	>50% MNC	~50% MNC	Few MNC
IL-2R	5–10% MNC	5–10% MNC	5–10% MNC	Negative
ICAM-1	Dense MNC, all EC, most tubules	Weak MNC, focal EC, tubules	MNC	Few MNC and focal EC
MHC class II	Dense MNC, focal EC, most tubules	Weak MNC plus focal EC	Residual MNC	Interstitial dendritic cells
TNF- α	>50% interstitial MNC, SM	<5% MNC	Negative	Few MNC
IL-2	5–10% MNC	Negative	Negative	Negative
IFN- γ	10–20% MNC	Negative	Negative	Negative
IL-4	<5% MNC	>50% MNC	>50% MNC	Negative
IL-6	10–20% interstitial MNC	>50% MNC	<5% MNC	Few MNC
IL-10	<1% MNC	10–20% MNC	<5% MNC	Negative
PGE ₂	<5% MNC	>50% MNC	>50% MNC	Negative

EC, endothelial cells; MNC, mononuclear cells; SM, smooth muscle cells.

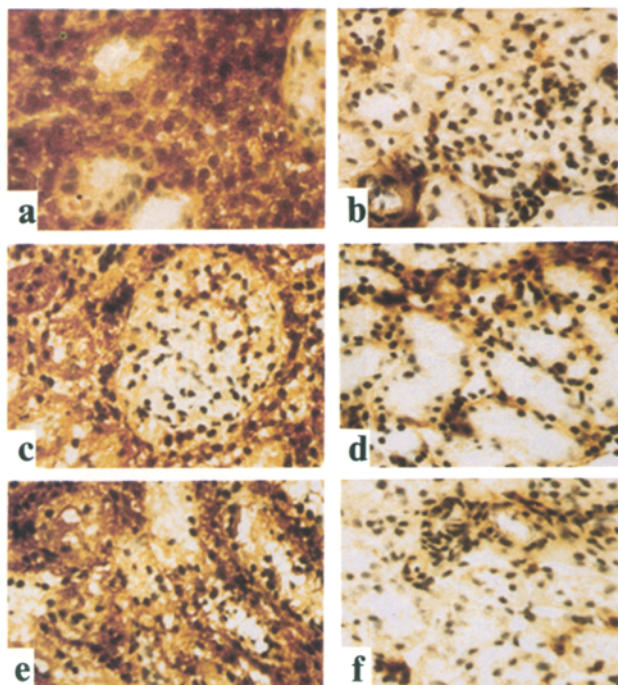


Figure 2. Immunoperoxidase analysis of cellular antigens within day 7 renal allografts of control (*left*) versus CTLA4Ig-treated (*right*) rats. CTLA4Ig treatment reduced graft leukocyte infiltration (CD45, *a* and *b*) and prevented development of tubulitis and glomerulitis. While untreated animals showed upregulation of ICAM-1 (*c* and *d*) on leukocytes, endothelial cells, and tubules, including tubular brush borders, treated animals showed only baseline ICAM-1 expression by interstitial dendritic cells and residual leukocytes. Class II MHC antigens (*e* and *f*) also showed differential expression within allografts of untreated versus treated hosts, in a similar pattern to ICAM-1. Hematoxylin; $\times 630$.

These recipients exhibited donor-specific unresponsiveness, since they accepted WF grafts (two animals died of surgical complications on days 7 and 16 with normal beating WF heart allografts; two other animals survived >60 and >100 d with normal beating WF cardiac allografts, $n = 4$), but they acutely rejected third-party BN grafts (survival 7 and 8 d, $n = 2$).

Immunohistological Studies of Renal Allografts. We undertook a detailed immunohistologic evaluation of renal allografts of day 2 CTLA4Ig-treated animals harvested at 1 wk and >100 d after transplantation and compared them with acutely rejecting control grafts from untreated animals (1 wk after transplantation) (Table 1 and Figs. 2 and 3). Acutely rejecting control renal allografts showed a dense and diffuse interstitial mononuclear cell infiltrate with severe tubulitis, glomerulitis, and vasculitis. Infiltrating mononuclear cells, which consisted of macrophages and T cells, showed evidence of immune activation with expression of IL-2R and considerable cytokine expression, including dense labeling for IFN- γ and TNF- α , and to a lesser extent IL-2, but lacked significant labeling for IL-4 or IL-10. Evidence for functional activity of proinflammatory cytokines was indicated by upregulation of intracellular adhesion molecule (ICAM-1) and MHC class II on endothelial cells and renal tubular cells.

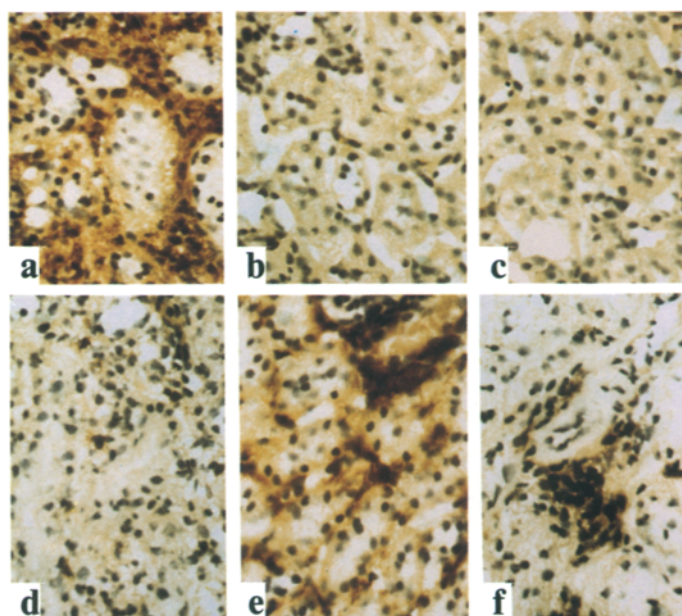


Figure 3. Immunoperoxidase analysis of IFN- γ and IL-4 deposition within renal allografts of control (day 7, *left*) versus CTLA4Ig-treated (day 7, *middle*, and day >100 , *right*) rats. CTLA4Ig treatment essentially prevented expression of IFN- γ (*a-c*) but was associated with markedly increased mononuclear cell staining for IL-4 (*d-f*), including residual perivascular infiltrates in longterm (>100 d, *f*) allografts. Hematoxylin; $\times 630$.

Allografts from CTLA4Ig-treated animals showed persistence of a mononuclear cell infiltrate, although this was less dense than that of untreated controls. Infiltrating mononuclear cells were predominantly CD4 $^{+}$. Grafts lacked evidence of tissue injury as evidenced by absence of tubulitis, glomerulitis, or vasculitis. These grafts also lacked staining for the activation markers MHC class II and ICAM-1, as well as the Th1-derived cytokines IL-2 and IFN- γ . In contrast, in comparison to control grafts, they showed increased staining for the Th2-derived cytokines IL-4 and IL-10 and PGE2. Sections incubated with anticytokine Abs after absorption with corresponding cytokine protein were unstained, as were sections incubated with isotype-matched Ig. Analysis of >100 -d-old allografts showed minimal interstitial cellular infiltrates but persistent perivascular mononuclear cell accumulations. Infiltrates at day >100 lacked staining for IL-2 or IFN- γ , but showed persistent upregulation of the Th2-related cytokine IL-4 and, to a lesser extent, IL-10. Furthermore, both day 7 as well as >100 -d-old grafts from CTLA4Ig-treated animals had marked upregulation of intragraft staining for IgG1, but not IgG2a or IgG2b, compared with rejecting controls (data not shown).

Effects of Recombinant IL-2 on Induction of Tolerance by CTLA4Ig. In the present study, it appeared that animals treated with CTLA4Ig were not immunologically unresponsive to their grafts, in that they mounted a predominantly Th2-type response. Therefore, it was of interest to determine if systemic IL-2 could block tolerance induction (9) in CTLA4Ig-treated animals. To this end, 4 animals received CTLA4Ig on day 2 after transplantation followed by rIL-2 (Collabora-

tive Research Inc., Bedford, MA; 1,000 U/d i.p.) for 5 d starting on day 3 after transplantation. Administration of rIL-2 resulted in partial reversal of the tolerant state; two out of the four animals exhibited delayed renal allograft rejection and died on day 40, while the other two animals survived >100 d. In addition, rIL-2-treated animals had higher serum creatinine levels at 1 wk and 1 mo (1.24 ± 0.31 and 1.62 ± 0.33 mg/dl, respectively, $n = 4$) after transplantation, compared with day 2 CTLA4Ig treated animals alone (0.75 ± 0.07 , and 0.72 ± 0.13 mg/dl, $n = 6$).

Discussion

In this study, we show that administration of a single dose of CTLA4Ig induces donor-specific tolerance of MHC-incompatible rat renal allografts in inbred strains. Our results indicate that delaying the administration of the fusion protein after engraftment is an essential component of this immunosuppressive strategy. These results confirm our earlier work (7), where we found that administration of donor splenocytes on the day of transplantation followed by a single dose of CTLA4Ig two d later induced tolerance to vascularized cardiac allografts. A notable difference between those data and the current studies is that administration of donor cells was not necessary for induction of long-term renal allograft survival. This finding may indicate that the kidney allograft itself is supplying the alloantigenic challenge in the form of either passenger APC or shed allo-MHC. The former possibility is consistent with the findings of Austyn et al. (13) in the mouse, which show at least 1-log-fold fewer dendritic cells resident in cardiac compared with renal tissue.

A novel finding of this study was the preservation of intragraft mononuclear cell infiltrates in CTLA4Ig-treated animals in conjunction with inhibition of Th1 and sparing of Th2 (14) cytokines. This, in conjunction with the ability of rIL-2 to partially restore rejection, suggests that induction of tolerance to renal allografts by CTLA4Ig in vivo may be mediated by a state of immune deviation to a predominance of Th2 cell function. Such "deviation" of immune responses to Th2 cell function has been recently reported in several experimental models of immunologic tolerance in vivo (10, 12, 15, 16). In addition, upregulation of PGE2 in grafts of CTLA4Ig-treated animals may help prevent rejection, given

its ability to selectively inhibit Th1-cytokine production (17), and its previously described association with oral tolerance to autoimmune encephalomyelitis (10).

What are potential mechanisms by which CTLA4Ig could lead to a state of immune deviation to a Th2 response? Bousiotis et al. (18) reported that B7⁺ B cells costimulated anti-CD3-activated T cells for IL-2 but not IL-4 production. McArthur and Raulet (19), using T cell clones, also found that CD28 stimulation was not required for IL-4 production. Similarly, Tan et al. (4) showed that CTLA4Ig inhibited accumulation of IL-2 and IFN- γ , but not IL-4, mRNA in the secondary MLR. It has also been reported that IL-2 may be required to prime naive T cells for IL-4 production, and that CTLA4Ig, by blocking IL-2 production, may indirectly inhibit IL-4 production (20). In vivo, a study of the immune response to *H. polygyrus*, a nematode parasite that normally induces a Th2 response, showed that CTLA4Ig blocked IL-4 production in regional lymph nodes in response to infection (21). Taken together with our own data, a model emerges in which delaying the administration of CTLA4Ig may facilitate the subsequent emergence of a Th2 response. The delay period of unhindered T cell activation may allow initial costimulation of IL-2 production by Th1 cells, which enables T cell precursors to produce IL-4. A Th2 response develops and persists in the target organ and protects it from cell-mediated (Th1) rejection.

Our studies do not definitely prove that the tolerant state induced by CTLA4Ig is mediated by Th2 cells. Such a conclusion would require more detailed studies using exogenous administration of other cytokines and anti-cytokine Abs (such as anti-IL-4 or anti-IL-10). Indeed, the inability of rIL-2 to fully restore rejection may reflect the need for other cytokines, such as IFN- γ . Other approaches include cytokine "knock-out" animals, especially IL-4. In addition, the specific role of increased IgG1 deposition in grafts of CTLA4Ig-treated animals, an effect which is most likely mediated by IL-4 (22), needs to be investigated, as antibody-mediated "enhancement" may be important in prolongation of allograft survival.

In summary, our results indicate that delayed administration of CTLA4Ig after alloantigenic challenge induces donor-specific tolerance to renal allografts and inhibits Th1 but spares Th2 cytokines in the target organ. These observations should have important clinical implications for the use of CTLA4Ig in humans.

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