Achieving Permanent Survival of Islet Xenografts by Independent Manipulation of Direct and Indirect T-Cell Responses

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Recent success in pancreatic islet allotransplantation has raised expectations but has equally highlighted the acute shortage of donor tissue. The use of xenogeneic tissue would help to address this shortage; however, strong cellular immunity limits the application of this approach. T-cell responses to xenogeneic tissues involve recognition of intact species-mismatched major histocompatibility complex (MHC) molecules, the direct pathway, and xenogeneic proteins presented as peptides by responder-type MHC molecules, the indirect pathway. In this study, we exploited the species difference to selectively and sequentially inhibit direct and indirect xenoresponses after transplantation of porcine islets into mice. Selective inhibition of the direct response was achieved using porcine CTLA4-Ig, which binds preferentially to pig versus mouse B7 molecules. Selective inhibition of the indirect response was achieved using murine CTLA4-Ig, which binds preferentially to mouse B7 molecules. Administration of porcine CTLA4-Ig alone caused modest prolongation of islet survival. Injection of murine CTLA4-Ig alone had a minimal effect. However, the injection of the porcine fusion protein early and the murine homolog late after grafting led to permanent survival of the porcine islets, in the absence of any other immunosuppression. These results suggest that a similar approach could have clinical utility in porcine islet xenotransplantation. ***Diabetes*** **54:1048–1055, 2005**

Replacement of destroyed insulin-producing tissue by pancreatic islet transplantation can cure the metabolic defects of insulin-dependent diabetes, but this approach is greatly limited by the shortage of available human organs. Because the demand for human islets will very quickly exceed supply, the use of islets from animal donors (xenotransplantation) offers one possible solution to this potentially acute problem. For a variety of reasons, pigs are favored as the best potential donor species. The major obstacle to xenotransplantation of vascularized pig organs is the occurrence of antibody-mediated forms of rejection, referred to as hyperacute and delayed rejection, triggered by the binding of xenoreactive natural antibodies (XNAs) to the endothelium with the activation of the complement cascade (1,2). The majority of XNAs present in humans and Old World primates are directed against the predominant epitope Gal(α1-3)Gal expressed on pig endothelial cells (3). Even when hyperacute and delayed xenograft rejection are prevented, the problems of cell-mediated immunity, which may contribute to acute cellular and chronic rejection, still remain.

The mechanisms leading to xenograft rejection of neovascularized tissue, such as pancreatic islets, in the pig-to-primate combination have been only partially characterized (4,5). Diabetic patients on immunosuppressive treatment and transplanted with porcine fetal islet cell clusters have shown viable β-cells a long time after transplantation (6), which suggests the absence of hyperacute rejection. Other findings in vitro have demonstrated that human and nonhuman primate XNA binding to isolated adult pig islets, freshly isolated or after prolonged culture, is detectable only on intraislet Gal(α1-3)Gal−positive endothelial cells. Moreover, despite complement activation, no cytotoxic effect or alteration in islet endocrine function has been observed after prolonged exposure to intact primate or human serum (7,8). Therefore, there is some evidence that porcine islets may not be hyperacutely rejected by humans; the major obstacle to successful islet xenotransplantation is likely to be the potent T-cell−mediated immune response occurring within days or weeks of grafting (9).

As has already been well demonstrated in response to allogenic tissues, the T-cell xenoresponse involves two distinct pathways: the direct pathway, or donor antigen-presenting cell (APC)-dependent responses, and the indirect pathway, or host APC-dependent response. It has been shown in vitro that both the direct and the indirect pathway of human anti-pig xenorecognition can induce vigorous primary human T-cell responses (9–12). A strong direct response reflects the efficient interaction between porcine costimulatory and adhesion molecules and their human ligands (13). In this context, the ligation of human CD28 by porcine B7 family molecules (CD80 and CD86) is

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APC, antigen-presenting cell; FITC, fluorescein isothiocyanate; MFI, mean fluorescence intensity; MHC, major histocompatibility complex; STZ, streptozotocin; XNA, xenoreactive natural antibody.

an attractive target for immune interaction. The strength of the indirect response reflects the plethora of species-specific proteins that can be presented as peptides. Blockade of CD28-B7 engagement using the B7-binding fusion protein CTLA4-Ig prevents rejection and induces tolerance in some allograft and xenograft models in rodents (14–16). Moreover, the survival of human pancreatic islets has been achieved in mice using CTLA4-Ig (17).

Despite all the problems that xenotransplantation presents, it also provides some unique opportunities, such as developing graft-specific immunosuppression. These strategies exploit the species difference to generate donor-specific blocking antibodies or fusion proteins. With this goal in mind, we recently acquired two sets of promising data. In one study, we generated a porcine CTLA4-Ig (pCTLA4-Ig) fusion protein and compared it with the human CTLA4-Ig (hCTLA4-Ig) in functional assays (18). In response to porcine xenogeneic stimulator cells, hCTLA4-Ig and pCTLA4-Ig were comparably effective at inhibiting human $CD4^+$ T-cell proliferation. In marked contrast, pCTLA4-Ig caused no inhibition of the response of human $CD4^+$ T-cells when costimulation was provided by human B7. For this reason, pCTLA4-Ig may be effective in inhibiting direct, graft-specific T-cell responses when used in xenotransplantation. In a second recent study, we exploited species differences to induce an endogenous, donor-specific, costimulation-blocking antibody response (19). Anti–porcine CD86 antibodies were induced in mice by immunization with peptides containing sequences from pig CD86. The survival of porcine islets was significantly prolonged in the immunized mice.

The aim of the present study was to selectively and sequentially inhibit direct and indirect T-cell responses after porcine islet xenotransplantation.

RESEARCH DESIGN AND METHODS

Male C57BL/6 and BALB/c mice were purchased from Olac Harlan (Biocester, U.K.) and used at age 6–8 weeks. Male major histocompatibility complex (MHC) class II−deficient mice on a C57BL/6 background (MHC $II^{-/-}$) were bred and maintained in the specific pathogen-free Biological Services Unit at the Hammersmith Campus of Imperial College (London, U.K.).

Cell preparation, reagents, and antibodies. The human M1 fibroblast cells (M1.DR1) transfected with porcine CD80 (M1pB7.1) or CD86 (M1pB7.2), CHO cells expressing murine CD80 (CHOmB7.1) or CD86 (CHOmB7.2), and the immortalized porcine endothelial cell line F6 have been previously described (18,20,21). The lipofectamine-based method for generating transfected clones based on the immortalized porcine EC "C29" has been previously described (22), as have the eukaryotic expression vectors containing constructs encoding cDNA for SLA-DRc A and B genes (23). The clone C29-DRc33 used for these studies expresses high levels of SLA-DRc (data not shown) and was maintained in selection medium containing mycophenolic acid (Life Technology, Paisley, U.K.) and G418 (Life Technology).

Murine dendritic cells were generated from bone marrow progenitors by culturing them in RPMI 1640 medium (Life Technology) supplemented with 10% FCS (Globpharm, Guilford, U.K.) in the presence of recombinant granulocyte−macrophage colony-stimulating factor (R&D Systems, Abingdon, U.K.), as previously described (24). Dentric cell maturation was induced by incubation with lipopolysaccharide in the last 24 h of culture on day 8.

pCTLA4-Ig was purified as previously described (18). Murine CTLA4/Fc chimera (mCTLA-Ig) and human IgG1 control antibodies were purchased from R&D Systems and the Binding Site (Birmingham, U.K.), respectively.

Flow cytometric analysis. Cells (5×10^5/well) were incubated with pCTLA4-Ig, mCTLA4-Ig, or human IgG1 control antibodies followed by a secondary fluorescein isothiocyanate (FITC)-conjugated sheep anti-human Ig (Fc specific; The Binding Site). Acquisition and analyses were performed in a Becton Dickinson FACSCalibur running CellQuest software. Data are expressed as mean fluorescence intensity (MFI).

T-cell proliferation assay. Lymphocyte cell suspensions were prepared from lymph nodes harvested from naive C57BL/6 mice in PBS with 0.2% BSA (Sigma, Poole, U.K.). T-cells were purified using mouse $CD4^+$ T-cell enrichment columns (R&D Systems). Responder T-cells (2×10^5/well) were cocultured in 96-well plates with BALB/c splenocytes (4×10^5/well) treated with 30 Gy X-irradiation or the C29DRc33 cell line (2×10^5/well).

Splenocytes were isolated from spleens harvested from naive animals by a density gradient on Lympho Sep (Oxford Biotechnology). After 5 days, cells were pulsed with 1 μCi ^{3}H-thymidine (Amersham, Buckinghamshire, U.K.) for the last 18 h of culture. Proliferation was measured as ^{3}H-thymidine incorporation by liquid scintillation spectroscopy. Results are expressed as mean cpm + SE of triplicate cultures.

Contact sensitivity responses. Male mice were sensitized by the application of 5% oxazolone (Sigma) in absolute ethanol and acetone (4:1; 50 μl) onto the shaved abdomen. The contact sensitivity response was subsequently elicited by applying 0.8% oxazolone in acetone and olive oil (1:4; 10 μl) to the right ear, whereas the left ear was painted with the vehicle alone. At the time of sensitization, mice were injected intraperitoneally with a single dose of 100 μg of pCTLA4-Ig or mCTLA4-Ig, whereas control animals received a human IgG1 antibody. Ear thickness was measured using an electric digital micrometer (Cadar Measurements & Control System, Sheffield, U.K.) and increases in groups of three mice were expressed as means ± SD × mm.

Preparation of T-cells for adoptive transfer. Peripheral lymph nodes and spleens from male C57BL/6 mice were pooled and forced through a 70-μm cell strainer, and erythrocytes were lysed by acetate kinase buffer (0.15 mmol/l NH_4Cl, 1 mmol/l $KHCO_3$, 0.1 mmol/l Na2-EDTA). $CD4^+$ T-cells were negatively selected by incubation with supernatants of the following rat anti-mouse hybridoma culture: anti−MHC class II (M5/114), anti-CD8 (53.6.72), anti-CD45R/B220 (RA3–3A1), and anti-CD16/32 (2.4G2), followed by sheep anti-rat DynaBeads (Dynal, Wirral, U.K.). The purity was determined by flow cytometric analysis. After three washes in PBS, T-cells were injected intravenously (2×10^7/animal) in male MHC class $II^{-/-}$−recipient mice.

Isolation and transplantation of porcine pancreatic islets. Pancreatic islets obtained from female Large White adult pigs (Institute of Animal Health, Compton, U.K.) weighing 150–200 kg were isolated and purified using a semiautomated method, as previously described (7). Briefly, islets were prepared by intraductal injection of collagenase solution (Type V; Sigma) at a concentration of 2 mg/ml, and pancreas digestion was performed at 37°C. Islets were purified over a discontinuous Euro-Ficoll gradient. After being cultured overnight, 800–1,000 hand-picked islets were transplanted under the left kidney capsule of diabetic recipient mice. Mice were rendered diabetic by intraperitoneal injection of streptozotocin (STZ; Sigma) and were considered diabetic when their blood glucose was >20 mmol/l. The function of transplanted islets was assessed by biweekly measurements of blood glucose levels. Islets were considered rejected when blood glucose level was ≥20 mmol/l on two consecutive readings.

In vivo experimental protocols. After islet transplantation, C57BL/6 recipient mice were treated as follows: *1*) intraperitoneal administration of pCTLA4-Ig ($n = 6$), mCTLA4-Ig ($n = 6$), or human IgG1 ($n = 6$) on days 1 and 3 (total dosage 200 μg); or *2*) intraperitoneal administration of pCTLA4-Ig on days 1, 3, and 5 (total dosage 150 μg) followed by mCTLA4-Ig on days 12 and 14 (total dosage 100 μg) ($n = 6$). As controls, recipient mice were treated on days 1, 3, 5, 12, and 14 with a total dosage of 250 μg of pCTLA4-Ig alone ($n = 5$), mCTLA4-Ig alone ($n = 6$), or human IgG1 ($n = 5$).

Islets were transplanted into MHC class $II^{-/-}$ diabetic mice that were previously adoptively transferred with $CD4^+$ T-cells from wild-type C57BL/6 mice (2×10^7 cells/animal) and treated with pCTLA4-Ig on days 1 and 3 (total dosage 250 μg; $n = 6$). As controls, recipient mice were treated on days 1 and 3 with mCTLA4-Ig (total dosage 250 μg; $n = 5$), human IgG1 alone ($n = 5$), or in association with adoptive transfer of $CD4^+$ T-cells ($n = 6$).

Statistical analysis. Student's t test was used to assess statistical significance. All reported P values are two-sided. The graft survival was assessed in the experimental groups by a nonparametric analysis. Statistical analysis was performed using a two-sided Mann-Whitney test, and P values were calculated with a Bonferroni correction. The Kaplan-Meier survival analysis was used to assess statistical significance of graft survival ≥100 days. To perform the pair comparisons in the Kaplan-Meier survival analysis, we used the log-rank test. $P \leq 0.05$ was considered statistically significant.

RESULTS

Porcine CTLA4-Ig binds poorly to murine costimulatory molecules. We previously demonstrated that pCTLA4-Ig selectively binds with significantly higher avidity to porcine compared with human CD80 and CD86 costimulatory molecules, due to a leucine for methionine

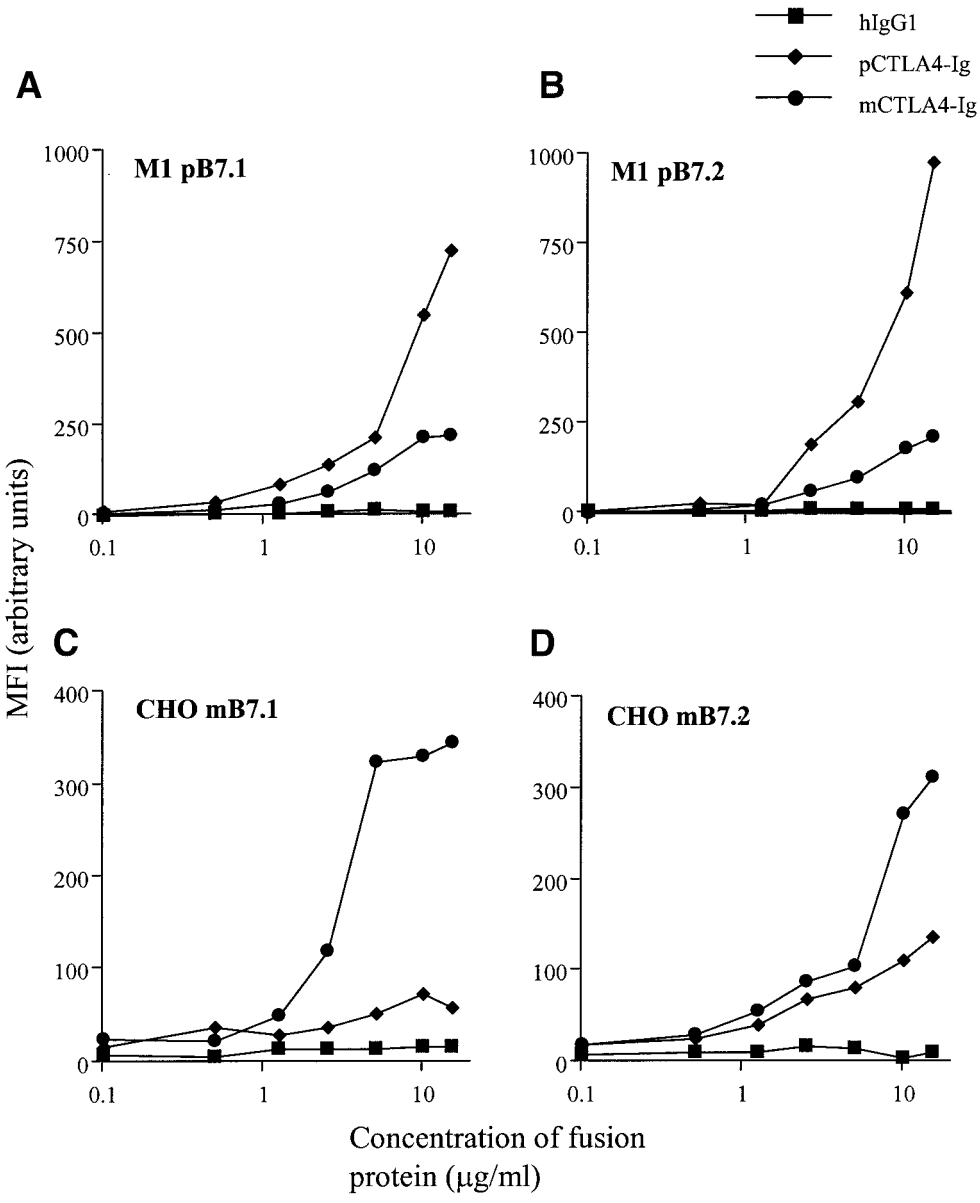

FIG. 1. Comparative binding of pig and mouse CTLA4-Ig to porcine and murine CD80 and CD86. Human M1 fibroblasts transfected with pCD80 (*A*) or pCD86 (*B*) and CHO cells transfected with mCD80 (*C*) or mCD86 (*D*) were stained with a control antibody (■) or porcine (♦) or murine (●) CTLA4-Ig. Antibody binding was revealed by two-layer staining using an FITC-conjugated mouse anti-human IgG monoclonal antibody and assessed by flow cytometry. Results are representative of three separate analyses.

amino acid substitution in the B7-binding motif (L[M]YP PPY) (18). In this study, we first investigated whether the difference in the sequence between pig and mouse CTLA4 would lead to a different pattern of binding to porcine and murine CD80 and CD86. Various cell lines expressing CD80 and CD86 of the two species were stained with pCTLA4-Ig and mCTLA4-Ig. Human transformed fibroblasts (M1.DR1) transfected with pig CD80 or CD86 and CHO cells expressing mouse CD80 or CD86 were incubated with pCTLA4-Ig or mCTLA4-Ig (at 1:2 serial dilutions from 15 μg/ml downwards). As a control, cells were incubated with a human IgG1 control antibody. As shown in Fig. 1*A* and *B*, pCTLA4-Ig bound with high avidity to pig CD80 and CD86, whereas mCTLA4-Ig displayed a poor binding. In contrast pCTLA4-Ig exhibited a weaker binding to mouse CD80 and CD86 than mCTLA4-Ig (Fig. 1*C* and *D*). These findings indicated that pCTLA4-Ig displayed a preferential binding for porcine costimulatory molecules, which might be determined by the methionine-to-leucine substitution at position 97, as was previously speculated for the weak binding to human B7 (18).

The transfectants used for these experiments expressed unphysiologically high levels of B7 molecules. Therefore, primary cells expressing physiological levels of costimulatory molecules were used as a model to analyze the binding of pCTLA4-Ig to porcine and murine APCs. Mature bone marrow–derived dentric cells and porcine endothelial cells constitutively expressing B7 (F6) (23) were stained using the same protocol as for transfectants. (The expression by flow cytometric analysis of B7 on these cells was ~10-fold less than on transfectants; data not shown.) As shown in Fig. 2*A*, pCTLA4-Ig failed to bind to mature murine dendritic cells, whereas it efficiently bound to porcine B7-expressing endothelial cells. Correspondingly, mCTLA4-Ig displayed a weaker avidity for pig B7 molecules than did pCTLA4-Ig (Fig. 2*B*). Taken together, these data illustrate the relative low avidity of pCTLA4-Ig for mouse B7 receptors, as was observed for human B7.

Porcine CTLA4-Ig is inefficient at inhibiting murine T-cell alloresponses. Having demonstrated clear preferential binding of the porcine and murine CTLA4-Ig to species-matched B7 molecules, these reagents were tested

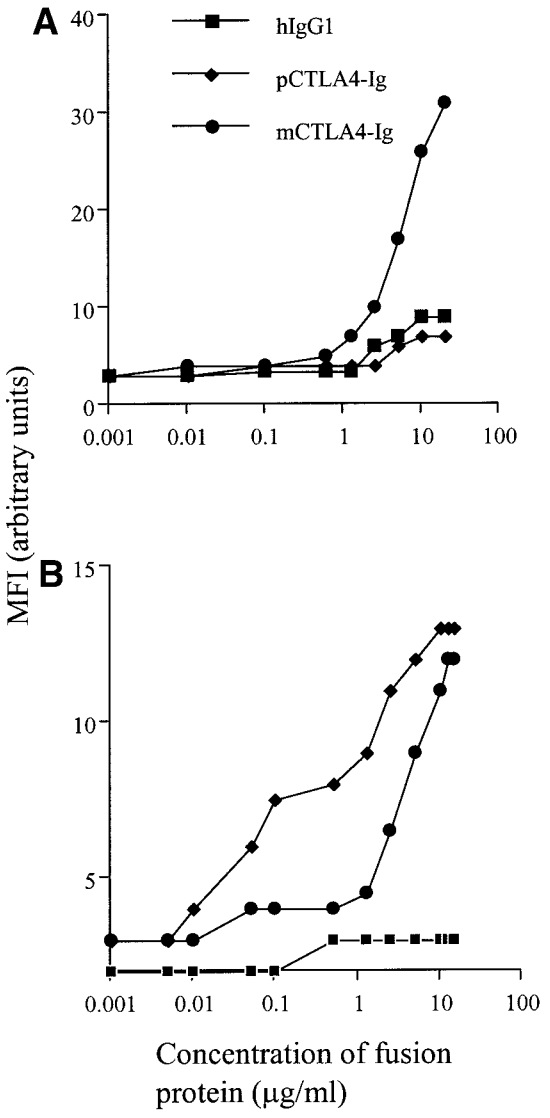

FIG. 2. Flow cytometric analysis of control antibody (■) or porcine (♦) or murine (●) CTLA4-Ig binding to mouse mature dendritic cells (*A*) and the F6 porcine endothelial cell line (*B*). Results are representative of four separate experiments.

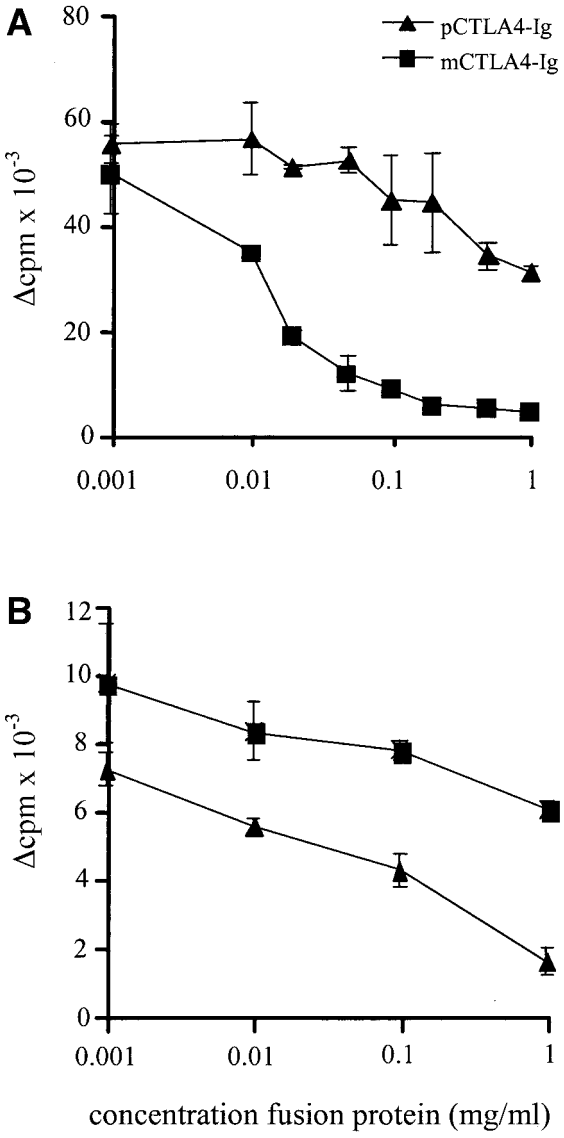

FIG. 3. Inhibition of mouse CD4$^+$ T-cell responses by pCTLA4-Ig (▲) and mCTLA4-Ig (■). T-cells (2×10^5/well) from naive C57BL/6 mice were stimulated with splenocytes (4×10^5/well) purified from BALB/c donors (*A*) or with the porcine cell line C29DRc33 (2×10^5/well) (*B*). The plates were incubated for 5 days. ^{3}H-thymidine was added for the last 18 h of culture. Data are mean cpm ± SE of triplicate cultures. Results are representative of three independent experiments.

in functional assays in which costimulation was provided by porcine or murine CD80 and CD86. Figure 3 shows that the response to porcine stimulators was significantly weaker, as expected for a xenoresponse compared with an alloresponse. Moreover, pCTLA4-Ig was highly ineffective at inhibiting T-cell proliferation in a mouse allogenic mixed lymphocyte reaction (Fig. 3). Compared with mCTLA4-Ig, a concentration approximately two logs higher was required to achieve 50% inhibition of the maximal response (Fig. 3*A*). The same concentration of porcine fusion protein caused almost complete inhibition of mouse T-cell proliferation when costimulation was provided by porcine xenogenic stimulators (Fig. 3*B*). These findings demonstrate that pCTLA4-Ig can regulate T-cell responses in vitro only when costimulation is provided by porcine ligands and minimally affects mouse T-cell activation by allogenic stimulator cells.

To determine whether pCTLA4-Ig was capable of inhibiting mouse T-cell immunity supported by mouse costimulatory molecules in vivo, we used a model of delayed-type hypersensitivity. Mice were sensitized to oxazolone by applying it to the abdomen; at the time of priming, mice were injected with murine or porcine CTLA4-Ig (100 μg/mouse). Animals were challenged on one ear 1 week later, and their ear swelling was measured. As shown in Fig. 4, although mCTLA4-Ig completely prevented delayed-type hypersensitivity sensitization, pCTLA4-Ig had no effect. These data demonstrate that, at the dosages used here, pCTLA4-Ig did not interfere with T-cell responses induced by host APCs.

pCTLA4-Ig prolongs survival of xenogeneic pancreatic islet grafts. It has been previously reported that pancreatic islets are subject to rejection mediated by T-cells with direct and indirect pathway xenospecificity (25). The immunogenicity of pancreatic islets is in fact enhanced by contaminating passenger leukocytes and xenogeneic endothelial cells that are capable of providing

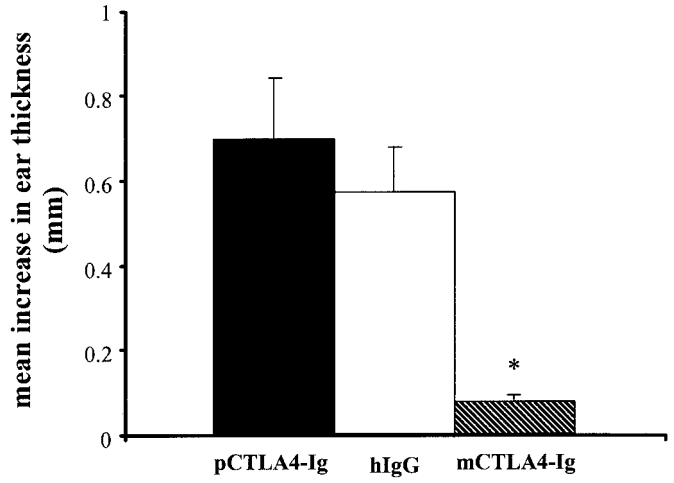

FIG. 4. Effect of porcine CTLA4-Ig on the contact hypersensitivity response. Contact hypersensitivity lesions were elicited with 0.8% oxazolone in acetone and olive oil (1:4) on the right ear of mice sensitized 4 days before with 5% oxazolone or vehicle (ethanol and acetone, 4:1). As a control, the left ear was treated with vehicle alone. After being sensitized, animals were treated with 100 μg of pCTLA4-Ig (■), mCTLA4-Ig (▨), or isotype-matched control monoclonal antibody (□). Data are expressed as the increase in ear thickness and represent means + SD of three independent experiments. *$P < 0.05$ vs. control mice.

costimulation (8,26,27). To analyze whether pCTLA4-Ig is capable of inhibiting costimulatory interactions between mouse and pig cells in vivo, and therefore of interfering with direct presentation of xenoantigens, we used a model of porcine pancreatic islet transplantation. STZ-induced diabetic mice were transplanted with 800–1,000 adult pig islets, placed under the left kidney capsule. Recipients were then treated 1 and 3 days after transplantation with pCTLA4-Ig, mCTLA4-Ig, or human IgG1 control (200 μg/animal). Injection of pCTLA4-Ig led to substantial prolongation of islet graft survival (mean graft survival 36 ± 8.5 days), whereas mCTLA4-Ig had only a minimal effect (mean graft survival 15 ± 1.5 days) (Fig. 5). The prolongation of islet survival with this dosage of pCTLA4-Ig was comparable with that observed in our previous study, in which direct xenospecific T-cell responses were inhibited by means of an endogenous donor-specific costimulation-blocking antibody (19).

Porcine CTLA4-Ig prevents rejection of porcine islets in CD4$^+$ T-cell–reconstituted major histocompatibility complex class II–deficient mice. To further test the efficacy of pCTLA4-Ig in inhibiting the direct xenoresponse, MHC class II–deficient C57BL/6 mice were reconstituted with CD4$^+$ T-cells from wild-type C57BL/6 mice. In these animals, the lack of MHC class II molecules renders them incapable of indirect presentation of porcine xenoantigens; however, the adoptively transferred CD4$^+$ T-cells should be capable of direct recognition of porcine MHC class II molecules. As shown in Fig. 6, the MHC class II–deficient mice accepted porcine islets indefinitely, indicating that the CD8$^+$ T-cell response was insufficient to cause rejection. However, reconstitution with CD4$^+$ T-cells led to rejection in all animals, with a mean survival time of 36 ± 8 days. Similar survival time was obtained in mice treated with human IgG1 after T-cell adoptive transfer (data not shown). When pCTLA4-Ig (250 μg/animal) was administered on the 1st and 3rd day after transplantation, indefinite graft survival was observed in all recipients, whereas animals treated with mCTLA4-Ig using the same regimen as pCTLA4-Ig rejected islet grafts after 21–37 days (survival time 31 ± 6 days), indicating that porcine B7 costimulation is required for the direct anti-pig xenoresponse. These data demonstrate that the direct mouse anti-pig xenoresponse is sufficient to cause islet graft rejection, and that pCTLA4-Ig is capable of completely inhibiting this response.

Sequential administration of porcine and murine CTLA4-Ig leads to indefinite survival of porcine islets. The data presented thus far demonstrate that pCTLA4-Ig binds preferentially to porcine B7 family molecules and is capable of preventing rejection of porcine islets mediated by direct T-cell xenoresponse. However, administration of pCTLA4-Ig to wild-type mice was insufficient to induce permanent graft survival. We assumed that the delayed rejection of the porcine islets in the

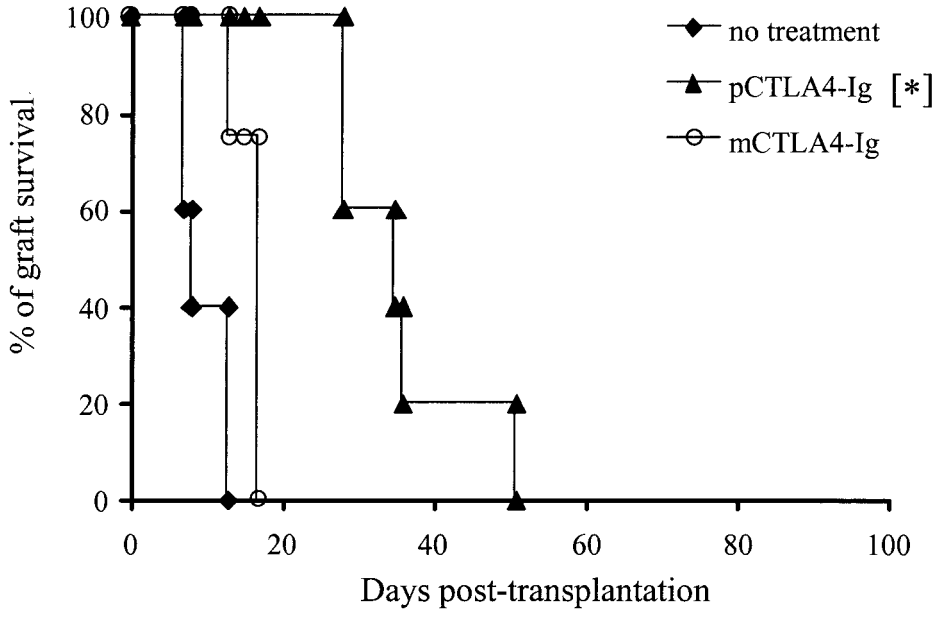

FIG. 5. Treatment with pCTLA4-Ig prolongs survival of transplanted porcine pancreatic islets. Adult porcine islets were transplanted in STZ-induced diabetic C57BL/6 mice (800–1,000 islets/animal). Then, 24 and 72 h later, recipients received a single injection of 100 μg of pCTLA4-Ig ($n = 6$; ▲), mCTLA4-Ig ($n = 6$; ○), or control antibody ($n = 6$; ♦). *$P < 0.008$ vs. control mice.

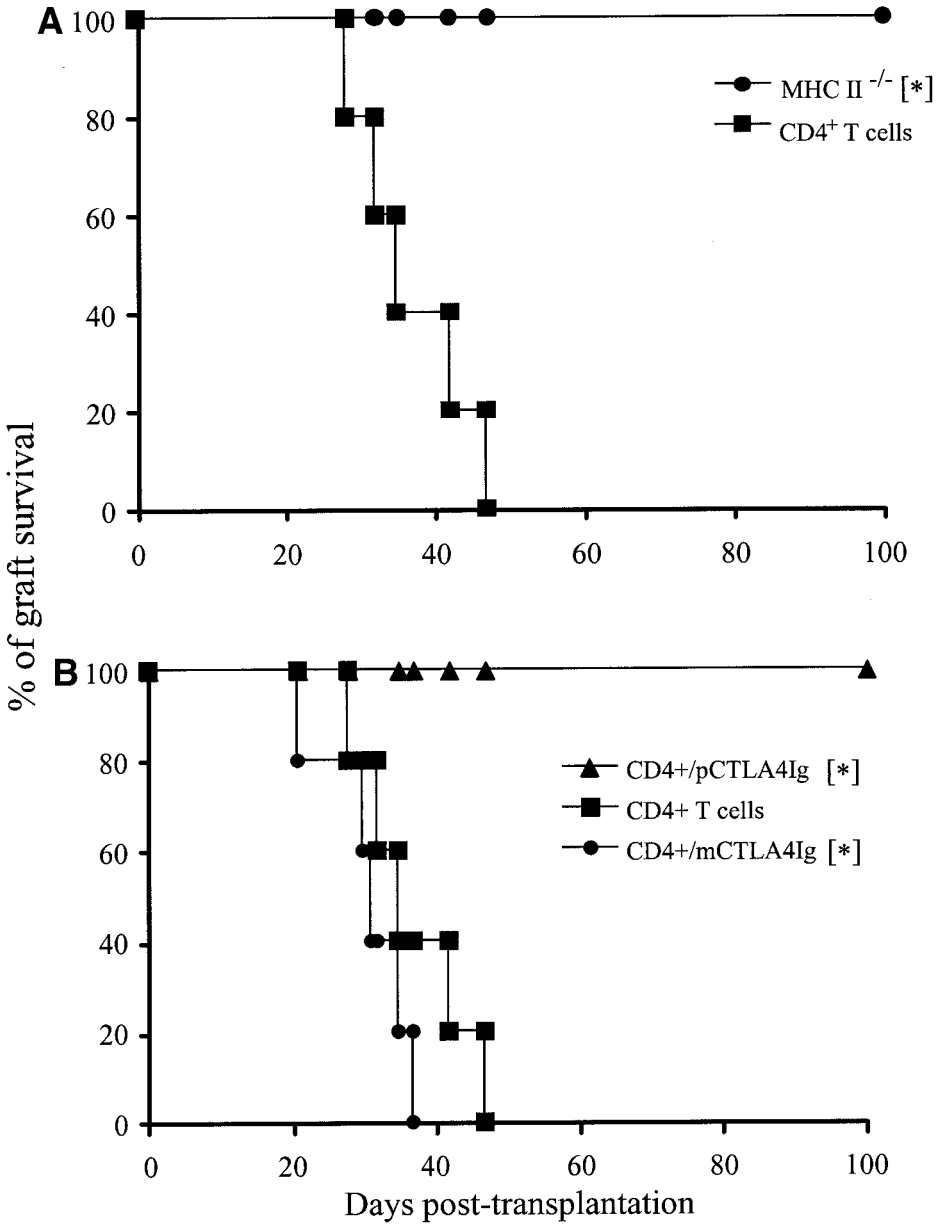

FIG. 6. pCTLA4-Ig induces indefinite survival of porcine pancreatic islets transplanted in adoptively transferred MHC II$^{-/-}$ mice. *A*: Xenograft survival of purified pig islets (800–1,000 islets/animal) transplanted into STZ-induced diabetic MHC II$^{-/-}$ mice (n = 6; ●) or MHC II$^{-/-}$ diabetic mice after adoptive transfer of syngeneic CD4$^+$ T-cells (2 × 10^7 cell/animal; n = 5; ■). *B*: At 24–48 h before porcine pancreatic islet transplantation, MHC II$^{-/-}$ diabetic mice were reconstituted with CD4$^+$ T-cells as described for *A*. After islet transplantation, recipients were treated with pCTLA4-Ig (250 μg total dose; n = 6; ▲) or mCTLA4-Ig (250 μg total dose; n = 5; ●) as described for Fig. 5. *P < 0.007 vs. control mice.

wild-type mice treated with pCTLA4-Ig was due to the indirect response against porcine antigens presented by recipient APCs. To test this assumption, we used a protocol that involved the administration of pCTLA4-Ig early after transplantation, on days 1, 3, and 5 (150 μg/animal), followed by two doses of mCTLA4-Ig on days 10 and 14 (100 μg/animal). The goal was to inhibit the initial direct response, and then to prevent subsequent indirect pathway-mediated rejection. The results are presented in Fig. 7. Mice treated exclusively with pCTLA4-Ig injected on days 1, 3, 5, 10, and 14 all rejected their grafts by day 35. Similarly, mice treated with five doses of mCTLA4-Ig all lost their grafts by day 20. In contrast, the mice treated with pCTLA4-Ig early and mCTLA4-Ig late after grafting all experienced indefinite survival. These results confirm that independent and sequential inhibition of direct and indirect T-cell xenoresponses after porcine islet xenografting is a highly effective strategy.

DISCUSSION

In this study, we showed that pCTLA4-Ig is highly effective at inhibiting the direct mouse anti-pig T-cell response, leaves the host immune system intact to respond to all other antigens, and, in combination with mCTLA4-Ig, leads to permanent survival of porcine islet grafts in normal mice. This work was based on two earlier findings: *1*) that the induction of an endogenous anti–porcine CD86 antibody response in mice leads to significant prolongation of porcine islet graft survival (19), and *2*) that pCTLA4-Ig exhibits clear preferential binding to porcine compared with human B7 family molecules due to a leucine for methionine substitution in the L(M)YPPPY B7-binding motif of CTLA4 (18).

Both fusion proteins were capable of prolonging the survival of porcine islet xenografts after transplantation into C57BL/6 mice, but the impact of each was critically dependent on the time that each was given after trans-

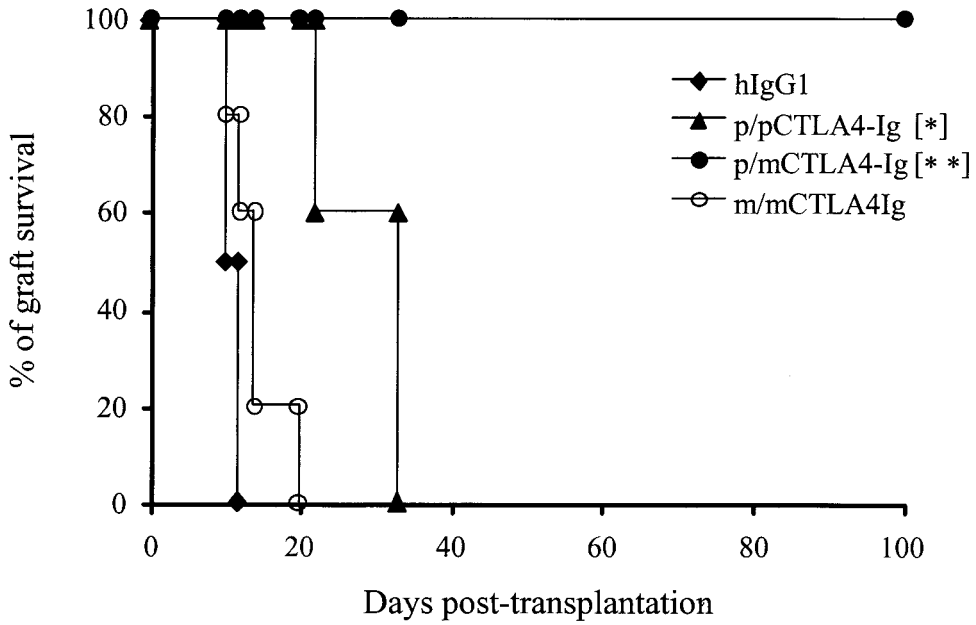

FIG. 7. pCTLA4-Ig, in combination with delayed administration of mCTLA4-Ig, leads to permanent islet graft survival. The survival of porcine islet xenografts was analyzed in C57BL/6 diabetic mice. After islet transplantation, recipients were treated on days 1, 3, and 5 with pCTLA4-Ig (150 μg total dose) in association with delayed administration on days 12 and 14 of mCTLA4-Ig (100 μg total dose; $n = 5$; ●). As controls, recipient mice received a combination of porcine (p) + pCTLA4-Ig ($n = 5$; ▲), murine (m) + mCTLA4-Ig ($n = 6$; ○), or control monoclonal antibody ($n = 5$; ♦) administered at the same time points as described above. *$P < 0.008$ vs. control mice; **$P < 0.01$ vs. control mice.

plantation. The only regimen capable of achieving long-term (>100 days) survival was early administration of pCTLA4-Ig followed by delayed administration of mCTLA4-Ig (Fig. 7). The most intuitive interpretation of these data is that they reflect sequential inhibition of the two pathways, direct and indirect, by which mouse anti-pig T-cell responses are sensitized. The fact that pCTLA4-Ig led to long-term survival in $CD4^+$ T-cell–reconstituted MHC class II knockout mice (Fig. 6), in which islet xenograft rejection could only have been mediated by a donor MHC class II–specific direct xenoresponse, provides strong support for this interpretation. We interpret the observed effect of CTLA4-Ig treatment in terms of steric blockade of B7-mediated costimulation. However, it may be possible that CTLA4-Ig induced expression of indoleamine 2,3 dioxygenase in the recipient dendritic cells, as recently reported in a murine model of islet allograft (28). The timing of the sequential administration of the two fusion proteins was based on what is known of the kinetics of direct pathway T-cell priming, which is likely to be a very rapid event (29,30), and the fact that when rejection is confined to the indirect pathway, it tends to be slower in tempo (31).

The importance of the direct pathway in mouse T-cell xenoresponses is often overlooked. This is because mouse T-cells from a variety of inbred strains fail to mount primary in vitro responses to xenogeneic stimulators due to cross-species molecular incompatibilities that serve to limit the efficiency of direct xenorecognition (32). Even in those studies where a primary response to xenogeneic islets has been documented in vitro, it has often been shown to be dependent on the presence of recipient APCs, implying either a reliance on transcostimulation or an indirect presentation of processed xenoantigens (33). However, as we have previously shown (19) and confirmed again in this study, $CD4^+$ T-cells from C57BL/6 mice are capable of mounting in vitro primary direct anti-pig MHC class II–specific proliferative responses (Fig. 3). Accordingly, after porcine islet xenograft transplantation into C57BL/6 mice, the direct pathway was critically involved in the $CD4^+$ T-cell–mediated rejection response, as evidenced by the impact of pCTLA4-Ig on graft survival. The differential effect of porcine compared with murine CTLA4-Ig in the early posttransplantation period suggests that sensitization via the direct pathway provides the major impetus to graft rejection during this period.

We have previously documented the importance of direct pathway sensitization in this model by showing that pig-specific anti-CD86 antibodies could significantly prolong porcine islet xenograft survival in C57BL/6 mice (19). In a related model, Lenschow et al. (17) documented the importance of the direct mouse anti-human T-cell response using specific anti-human B7 monoclonal antibodies, which delayed graft rejection, in contrast to hCTLA4-Ig, which by binding efficiently to both human and murine B7 family molecules, blocked both direct and indirect pathways of sensitization and led to long-term graft survival.

These results are reminiscent of those seen after experimental allogenic islet transplantation, in which the direct pathway provides the major stimulus to rejection, as illustrated by the prolonged survival noted by many investigators after the depletion of allogenic donor passenger leukocytes (27,34). However, this situation may be unusual after xenogeneic islet transplantation, after which the indirect pathway can provide a potent stimulus to rejection, even in the absence of the direct pathway (35). For example, some have reported that porcine islet xenograft rejection, even in C57BL/6 mice, is mediated entirely by the indirect pathway (36). The difference between our results and these others may be due to the source of the islets, or alternatively the in vitro manipulations that islets were subjected to before being transplanted. For example, neonatal porcine islets or those cultured for periods up to several days after being harvested appear less immunogenic than those used for our studies (36), which were derived from adult pigs and were cultured only overnight before being transplanted.

We have previously hypothesized that the human direct

anti-pig $CD4^+$ T-cell xenoresponse may prove to be a significant and persistent problem after clinical xenotransplantation because of the constitutive expression of MHC class II, CD80, and CD86 molecules by porcine endothelial cells (32). If this hypothesis is correct, there is likely to be less of a decline in the immunogenicity of a vascularized xenograft than occurs with an equivalent human allograft. Human endothelial cells do not express B7 family molecules, and several groups have reported that over time following transplantation, a decline is seen in the frequencies of direct-pathway anti-donor T-cells (37). After a porcine vascularized xenograft, there may be a need for more persistent inhibition of the direct T-cell response. If pharmacological agents are relied upon, this would lead to severe systemic immunosuppression. In contrast, the properties of pCTLA4-Ig, as illustrated here, suggest that this could be administered over a prolonged period, without compromising the recipient's immune competence.

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