Article

Locally expressed CTLA4-Ig in a pancreatic beta-cell line suppresses accelerated graft rejection response induced by donor-specific transfusion

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Abstract

Aims/hypothesis. This study examined whether locally expressed CTLA4-Ig can suppress the accelerated islet allograft rejection that is induced by donor-specific transfusion.
Methods. CTLA4-Ig-transfected or parental MIN6 cells were transplanted subcutaneously into the right flank of streptozotocin-induced diabetic C3H/Hej mice with or without donor-specific transfusion. For donor-specific transfusion, spleen cells from C57BL/6 mice were injected i.v. at the time of transplantation. In other experiments, CTLA4-Ig-transfected and parental MIN6 cells were transplanted separately into each flank, together with donor-specific transfusion. Rejection was defined as a blood glucose concentration of more than 300 mg/dl in two consecutive measurements, and graft survival was confirmed by hyperglycaemia after the grafts were removed. The effect of an anti-CTLA4 antibody on the survival of CTLA4-Ig-transfected MIN6 cells was also examined.

Results. In 7 of 12 donor-specific transfusion sensitised mice, CTLA4-Ig-transfected MIN6 cells remained viable 20 days after grafting, whereas all parental MIN6 cells ($n = 10$) were rejected promptly, within 14 days. The prolonged allograft survival was observed even in the absence of detectable levels of serum CTLA4-Ig, while the surviving allografts continued to produce CTLA4-Ig in situ. This protection was abrogated by an anti-CTLA4 antibody, but not by a control antibody. Furthermore, six animals that maintained normoglycaemia after the separate transplantation of parental and CTLA4-Ig-transfected MIN6 cells into each flank all showed abrupt hyperglycaemia after the CTLA4-Ig/MIN6 graft was removed, suggesting that this protection operated locally.
Conclusion/interpretation. A beta-cell line genetically engineered to secrete CTLA4-Ig can protect a graft locally from the alloimmune response induced by donor-specific transfusion. [Diabetologia (2002) 45:831–840]

Keywords: CTLA4-Ig, MIN6, transplantation, donor-specific transfusion, mouse

The insulin-secreting islets comprise only a small fraction of the whole pancreas (~1%); therefore, the

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Abbreviations: APC, Antigen-presenting cell; DST, donor-specific transfusion; mAb, monoclonal antibody; MLR, mixed lymphocyte response; MST, mean survival time; STZ, steptozotocin

transplantation of purified islets rather than the whole pancreas seems to be a reasonable approach to control metabolic dysfunction in patients with Type I (insulin-dependent) diabetes mellitus. Although the survival of vascularised pancreas allografts at one year has significantly improved following the introduction of T cell-specific immunosuppressants such as cyclosporin and FK506 [1], the survival of pancreatic islet grafts under the same immunosuppressive regimen is still under 30% one year after transplantation [2]. However, a current study reports that seven consecutive patients attained sustained insulin independence after pancreatic islet transplantation [3]. All these recipients were

preuremic and given islets alone under steroid-free immunosuppressive regimen. It is still not known whether similar results can be obtained when islets are given together with a kidney allograft into a uremic patient with Type I diabetes because it has been shown that islet grafts are quite vulnerable to alloimmune response [4, 5].

We have previously shown in a rodent islet transplantation model that crude collagenase-digested islets obtained after separation in a Ficoll density gradient are highly immunogenic, and that the removal of contaminants such as lymph nodes and vascular and ductal tissues from the islets by handpicking markedly reduces the islet graft immunogenicity [5]. When transplanted to allogeneic recipient mice, some of the purified islet allografts survived indefinitely without immunosuppression [5]. On the other hand, when the purified islets and contaminants were transplanted together into the same renal subcapsular space or separately into the contralateral renal subcapsular spaces, the purified islet allografts were acutely rejected. These results suggest that purified islets are less immunogenic than crude preparations, but are vulnerable once an immune response is generated. Thus, blood transfusion [4] or simultaneous kidney allotransplantation, which has been carried out in most of previous islet recipients [1], might cause a systemic anti-donor response that would lead to the destruction of islet allografts.

We and others have shown that the site-specific delivery of immunosuppressive molecules could provide an alternative strategy to avoid the hazardous side effects of conventional immunosuppressants [6, 7, 8, 9]. Various immunosuppressive molecules have been tested in a variety of transplantation models and need to be assessed for their effectiveness on graft prolongation. In one of the strategies examined, the blockade of co-stimulatory signals by immunosuppressive proteins has been shown to prolong allograft survival in various transplantation settings [10, 11]. In T cell-mediated immunity, the full activation of naïve T cells requires co-stimulatory signals generated by the ligation of CD28 on T cells with their receptor, B7-1/B7-2, on antigen-presenting cells (APCs) [12]. CTLA4, a homologue of CD28, when expressed on activated T cells, can bind B7-1/B7-2 with a higher affinity than CD28, and compete efficiently for B7-1/B7-2, thereby blocking the co-stimulatory signals for T cells [13]. A chimeric fusion protein, CTLA4-Ig, which is composed of the extracellular region of CTLA4 and the Fc portion of IgG1, similarly blocks the CD28-mediated co-stimulatory pathways, and down-regulates the immune response in various experimental transplantation models. When administered systemically [14, 15, 16, 17] or expressed in engrafted tissues by gene transfer [8, 9, 18], CTLA4-Ig prolongs graft survival, and occasionally induces graft-specific unresponsiveness. However, it has not yet been established whether the prolongation of graft survival is obtained by a local or systemic immunoregulatory mechanism.

In this study, we used the insulinoma cell line MIN6, which retains the physiological characteristics of pancreatic beta cells and secretes insulin in a glucose-dependent manner [19], to examine the effects of local CTLA4-Ig expression on beta-cell survival in diabetic recipient mice. To induce a systemic anti-donor response, we performed a donor-specific transfusion (DST) of spleen cells at the time of transplantation, which causes a dose-dependent accelerated rejection of purified islets [5].

Here we showed that MIN6 cells that were genetically engineered to produce CTLA4-Ig protected engrafted MIN6 cells locally, but did not protect MIN6 cells at a remote site, when an anti-donor response was generated by DST.

Materials and methods

Mice. Male 6- to 8-week-old C3H/Hej (H-2^k) and C57BL/6 (H-2^b) mice were purchased from Clea Japan (Osaka, Japan). Mice were reared in a specific pathogen-free environment at the Institute of Experimental Animal Sciences, Osaka University Graduate School of Medicine. C3H/Hej mice in which diabetes was induced with streptozotocin (STZ) (Sigma Chemical, St. Louis, Mo., USA) (300 mg/kg body) were used as recipients. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Osaka University Graduate School of Medicine.

Cell lines. A pancreatic beta-cell line (MIN6) was previously established from an insulinoma that had developed in a transgenic mouse (C57BL/6) expressing the SV40 T-antigen gene under the control of the human insulin-promoter [19]. The MIN6 cells were cultured in DMEM (Sigma Chemical, St. Louis, Mo., USA) supplemented with 25 mmol/l glucose, 15% heat-inactivated fetal calf serum (FCS) (Dainippon Pharmaceuticals, Osaka, Japan), 100 U/ml penicillin, 100 µg/ml streptomycin, 10 mmol/l HEPES, and 50 µmol/l 2-mercaptoethanol (Life Technologies, Carlsbad, Calif., USA). MIN6 cells were used at 15–20 passages. B7.1-transfected P815 cells [20], which were kindly donated by Dr. H. Yagita (Department of Immunology, Juntendo University School of Medicine, Tokyo, Japan) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 10 mmol/l HEPES, 2 mmol/l L-glutamine, 1 mmol/l sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.1 mmol/l non-essential amino acids, and 50 µmol/l 2-mercaptoethanol, as described previously [20].
Plasmid preparation. CTLA4-Ig consists of the extracellular portion of murine CTLA4 and the Fc portion of human IgG1, as previously described [21]. The cDNA fragment encoding CTLA4-Ig was excised from the expression vector CDM8-CTLA4Ig. The ends were blunted and ligated with an EcoRI linker. The cDNA fragment was then inserted into the expression vector pCXN [22], which contains the chicken beta actin promoter and the neomycin phosphotransferase II gene, to generate the expression plasmid pCXN-CTLA4-Ig.

Transfection of COS7 cells with CTLA4-Ig. To obtain CTLA4-Ig protein, COS7 cells were transfected with pCXN-CTLA4-Ig using a DEAE-dextran method [23] (Pharmacia Biotech, Uppsala, Sweden). The CTLA4-Ig protein was purified from se-

rum-free conditioned medium by 50% saturated ammonium sulfate precipitation and protein G-Sepharose affinity chromatography (MAbTrapTMGII; Pharmacia Biotech). Purified CTLA4-Ig were separated by SDS-PAGE and quantitated by densitometry of Coomassie blue-stained gels.

Preparation of spleen cells. The spleen was aseptically removed and mechanically teased in 10 ml of Hank's balanced salt solution containing 2% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. Cell suspensions were filtered through 100-μm nylon mesh, and then treated with $NH_4Cl/Tris$ buffer to remove red blood cells. Thereafter, cells were washed three times and prepared for DST in Hank's balanced salt solution.

Transfection of MIN6 cells with CTLA4-Ig. To generate stable transfectants, pCXN-CTLA4-Ig was transfected into MIN6 cells (passage 15) using Lipofectamin (Life Technologies). As a negative control, the pCXN vector without the CTLA4-Ig insert was transfected into MIN6 cells. Selection was begun 2 days after transfection by adding G418 (Sigma) to the media at 600 μg/ml. Surviving colonies were cloned and screened for secretion of CTLA4-Ig by flow cytometry as described below. Three independent MIN6 transfectants that constitutively produce similar amounts of CTLA4-Ig in vitro were selected. In the preliminary in vivo transplantation experiments (see below), these transfectants gave similar results. Transfectants were cultured for at least 2 months before transplantation. A MIN6 transfectant (CTLA4-Ig/MIN6) was selected and used in this study.

Detection of CTLA4-Ig in vitro by flow cytometry. B7.1-transfected P815 cells were incubated with culture supernatant from CTLA4-Ig-transfected or parental MIN6 cells for 1 h, then washed, and incubated for 30 min with FITC-labelled polyclonal goat anti-human IgG (Cappel, Melvern, Pa., USA) diluted to 1:100. The cells were then washed and analysed on an EPICS-XL flow cytometer (Coulter Electronics, Hialeah, Fla., USA).

Mixed lymphocyte reaction (MLR). Mesenteric lymph node cells from recipient mice (C3H/Hej) suspended in complete RPMI 1640 medium (5×10^5/well) were cultured with the same number of 20-Gy γ-irradiated splenocytes in 96-well flat-bottom microtitre plates. Serial dilutions of CTLA4-Ig obtained from a COS7 transfectant or culture supernatant of MIN6 cells transfected with CTLA4-Ig were then added. After 4 days of culture, the cells were pulsed with [methyl-^{3}H]-thymidine (TdR) (Amersham International, Buckinghamshire, UK) for 6 h (1.0 μCi/well). The cells were then harvested onto glass fibre filters, and ^{3}H-TdR uptake was measured in a liquid scintillation counter (Wallac, Turku, Finland).

Insulin secretion in vitro. The amount of insulin secreted in vitro by CTLA4-Ig-transfected or parental MIN6 cells was determined as previously described [19]. Briefly, cells (5×10^5) were plated onto six-well plates and grown in DMEM growth medium for 2 days. The cells were washed twice in DMEM containing 0.5 mmol/l glucose and 15% FCS and incubated for 1 h. The medium was then replaced with fresh DMEM containing 5% FCS and 0, 5.6, or 25 mmol/l glucose. The culture supernatants were collected after 12 h of incubation, spun to remove cell debris, and stored at –20°C. Samples were later analysed for insulin content using an insulin ELISA kit (Morinaga, Yokohama, Japan).

Transplantation of CTLA4-Ig transfectants. CTLA4-Ig-transfected or parental MIN6 cells (1×10^6) were transplanted subcutaneously into the right flank of STZ-induced diabetic C3H/Hej mice. Spleen cells (1×10^6) from the donor strain (C57BL/6) were injected i.v. at the time of transplantation to accelerate the immune response to the MIN6 cells (DST). Some of the recipients were given i.p. injections of either an anti-CTLA4 monoclonal antibody (mAb) (UC10–4F10) [24], which was kindly given by Dr. J. A. Bluestone (Committee on Immunology, The University of Chicago, Chicago, Ill., USA) or a control hamster IgG mAb (0.2 mg on day 0 and 0.1 mg on days 3, 6, 9, 12, and 15). The function of the graft was monitored by measuring the blood glucose concentration. Rejection was defined as a blood glucose concentration higher than 300 mg/dl in two consecutive measurements.

Determination of the serum level of CTLA4-Ig in recipients. Serum concentrations of CTLA4-Ig in mice were assayed by ELISA. Serum samples were collected periodically from the tail vein. Goat anti-human IgG (10 μg/ml in PBS; preabsorbed with mouse IgG) (American Qualex, San Clemente, Calif, USA) was coated onto 96-well microplates (50 μl/well) (Sumilon MS-8596F; Sumitomo Bakelite, Tokyo, Japan) at 4°C overnight. The wells were then blocked with 3% bovine serum albumin in PBS for 2 h. A test serum sample or standard CTLA4-Ig was added to each well and incubated for 30 min. After washing, biotinylated hamster anti-mouse CTLA4 mAb (1.0 μg/ml; 1B8; Southern Biotechnology Associates, Birmingham, Al., USA) was added to each well and incubated for 30 min. After washing, peroxidase-conjugated streptavidin (1 μg/ml; Zymed, San Francisco, Calif., USA) was added. For detection, *o*-phenylenediamine (0.4 mg/ml; 50 μl/well) was added, and the chromogenic reaction was allowed to proceed for 10 min at room temperature. The reaction was stopped with 25 μl 8 N H_2SO_4, and the plates were then read at 490 nm in a microtitre plate reader (InterMed, Tokyo, Japan).

Immunohisitochemistry. For immunohistochemical analysis, MIN6 or CTLA4-Ig/MIN6 cells (1×10^7) were transplanted into STZ-induced diabetic C3H/Hej mice with or without DST. DST similarly induced acute rejection of MIN6 grafts under this condition. Grafts were harvested 8 days after transplantation. For detection of insulin in the graft, the paraffin sections were stained using the Histofine SAB-PO insulin kit (Nichirei, Tokyo, Japan). For detection of T cells, paraffin sections were pretreated with microwave-heating for 15 min and incubated with rabbit anti-CD3 antibody (1:100; DAKO, Copenhagen, Denmark) at 37°C for 60 min [25]. The sections were washed in PBS, and incubated with 4 μg/ml peroxidase conjugated anti-rabbit IgG (Nichirei) for 30 min at room temperature. Antibody binding was visualised using a Histofine SAB-PO kit (Nichirei) with diaminobenzidine as a substrate. The slides were counterstained with hematoxylin. For detection of MHC class I (H-2K^b), cryostat sections fixed in acetone were incubated with the biotin-conjugated anti-mouse H-2K^b mAb (1 μg/ml; AF6–88.5; Pharmingen, San Diego, Calif., USA) at 4°C overnight. After washing with PBS, the sections were incubated with peroxidase-conjugated streptavidin and diaminobenzidine as a substrate. Control tissue sections were incubated in the same way, with the first step omitted.

Detection of in situ production of CTLA4-Ig. Frozen sections were fixed with acetone and were incubated with 5 μg/ml goat anti-human IgG (American Qualex) for 2 h. The sections were washed three times in PBS, and incubated with peroxidase-labelled rabbit anti-goat IgG (American Qualex) for 30 min. Antibody binding was visualised using metal-enhanced diaminobenzidine (Pierce, Rockford, Ill., USA). The slides were counterstained with Giemsa solution.
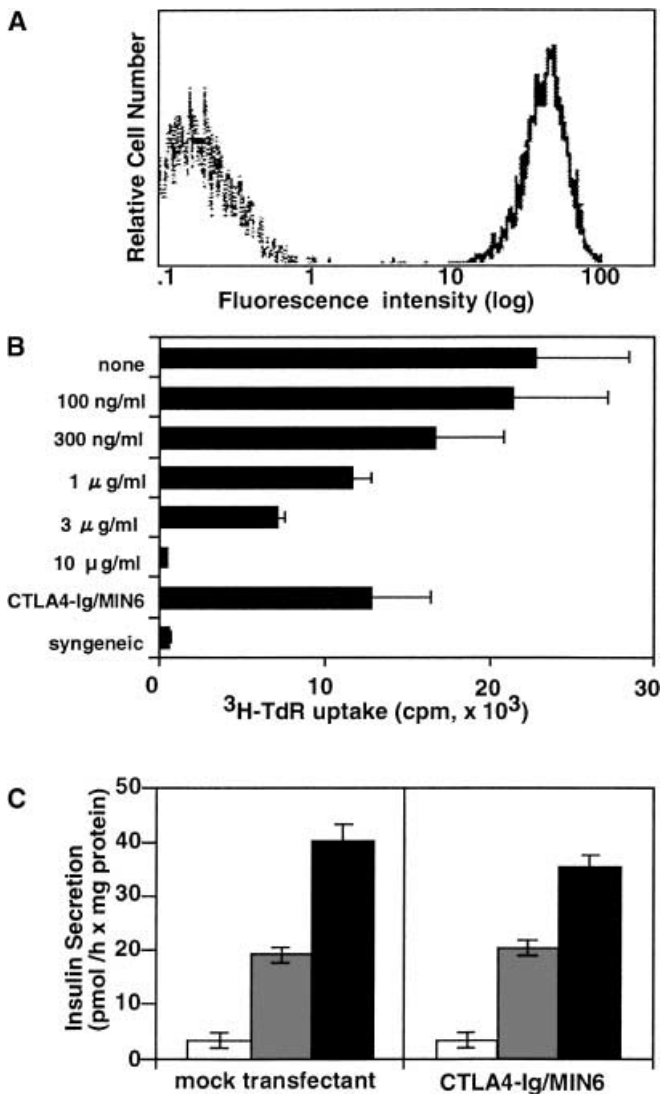
Fig. 1A–C. Production of functional CTLA4-Ig and regulated insulin secretion in CTLA4-Ig/MIN6 cells. **A** Recombinant CTLA4-Ig bound to B7-expressing cells (*dotted line*: supernatant from MIN6; *solid line*: supernatant from CTLA4-Ig/MIN6). **B** Recombinant CTLA4-Ig inhibited the allogeneic MLR in a dose-dependent manner. Lymphocytes from recipient mice (C3H/Hej) were incubated with irradiated stimulator splenocytes and supernatant from CTLA4-Ig/MIN6 (final dilution: 25%) or serial dilutions of purified CTLA4-Ig for 4 days in triplicate wells. After a 6-h pulse of 1.0 μCi of [³H]-TdR, lymphocyte proliferation was measured as ³H uptake (cpm). Culture supernatant of CTLA4-Ig/MIN6 cells containing 2.2 μg/ml CTLA4-Ig was used in this experiment. Similar results were obtained in two independent experiments. **C** Insulin secretion by CTLA4-Ig/MIN6 cells. Mock transfected or CTLA4-Ig/MIN6 cells were preincubated in medium containing 0.5 mmol/l glucose for 1 h and further incubated in medium containing 5% FCS and *open bars* 0, *shaded bars* 5.6, or *solid bars* 25 mmol/l glucose for 12 h. The culture supernatants were collected and assayed for insulin content. Results obtained from one of the two independent experiments were shown
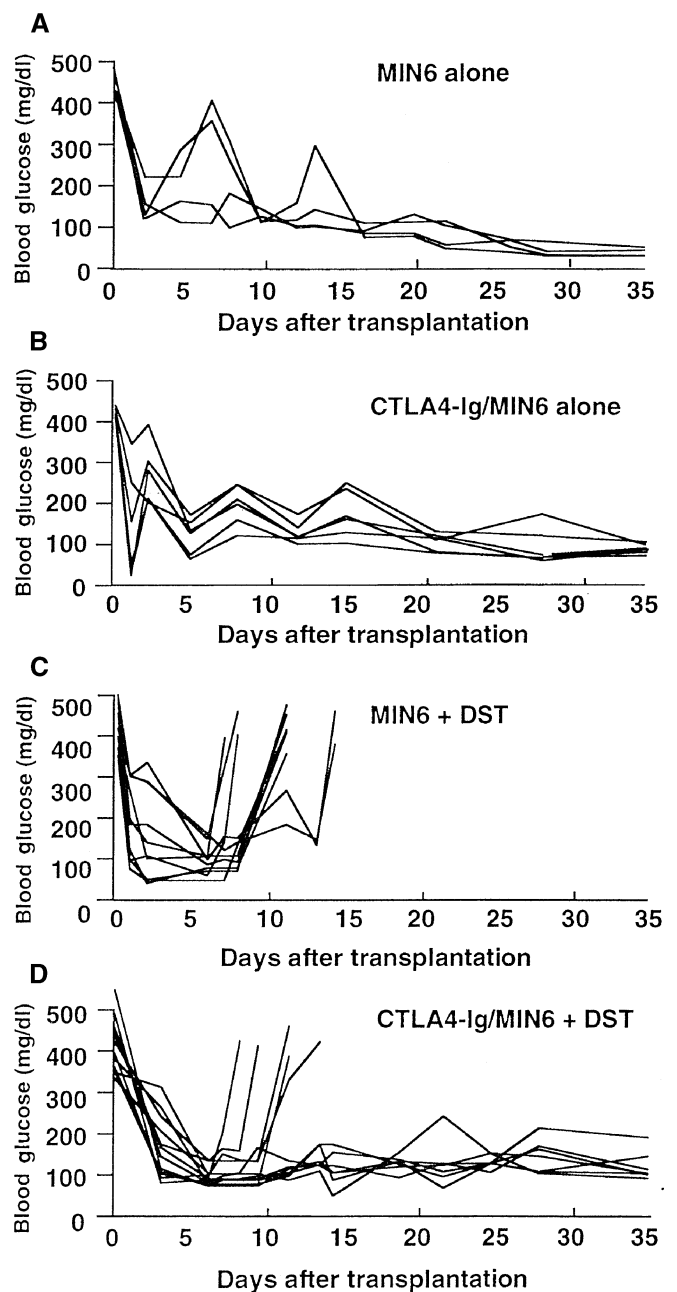
Fig. 2A–D. Blood glucose concentrations of animals given MIN6 or CTLA4-Ig/MIN6 with or without simultaneous DST. MIN6 cells (**A**: $n = 4$) or CTLA4-Ig/MIN6 cells (**B**: $n = 6$) were transplanted (1×10^6 cells) into the lateral flank of STZ-induced diabetic C3H/Hej mice without DST. MIN6 cells (**C**: $n=10$) or CTLA4-Ig/MIN6 cells (**D**: $n=12$) were transplanted similarly but with DST (1×10^6 splenocytes). MIN6 cells and CTLA4-Ig/MIN6 cells survived in non-sensitised diabetic C3H/Hej mice and restored euglycaemia (**A**, **B**)

Simultaneous grafting of CTLA4-Ig transfectants and parental MIN6 cells. CTLA4-Ig-transfected and parental MIN6 cells (1×10^6) were transplanted separately into the right and left flanks of STZ-induced diabetic C3H/Hej mice ($n = 17$). In control mice, only MIN6 cells were transplanted. In both groups, spleen cells (1×10^6) from the donor strain (C57BL/6) were injected i.v. at the time of transplantation. On day 21 after transplantation, survival of the grafted MIN6 cells in both groups
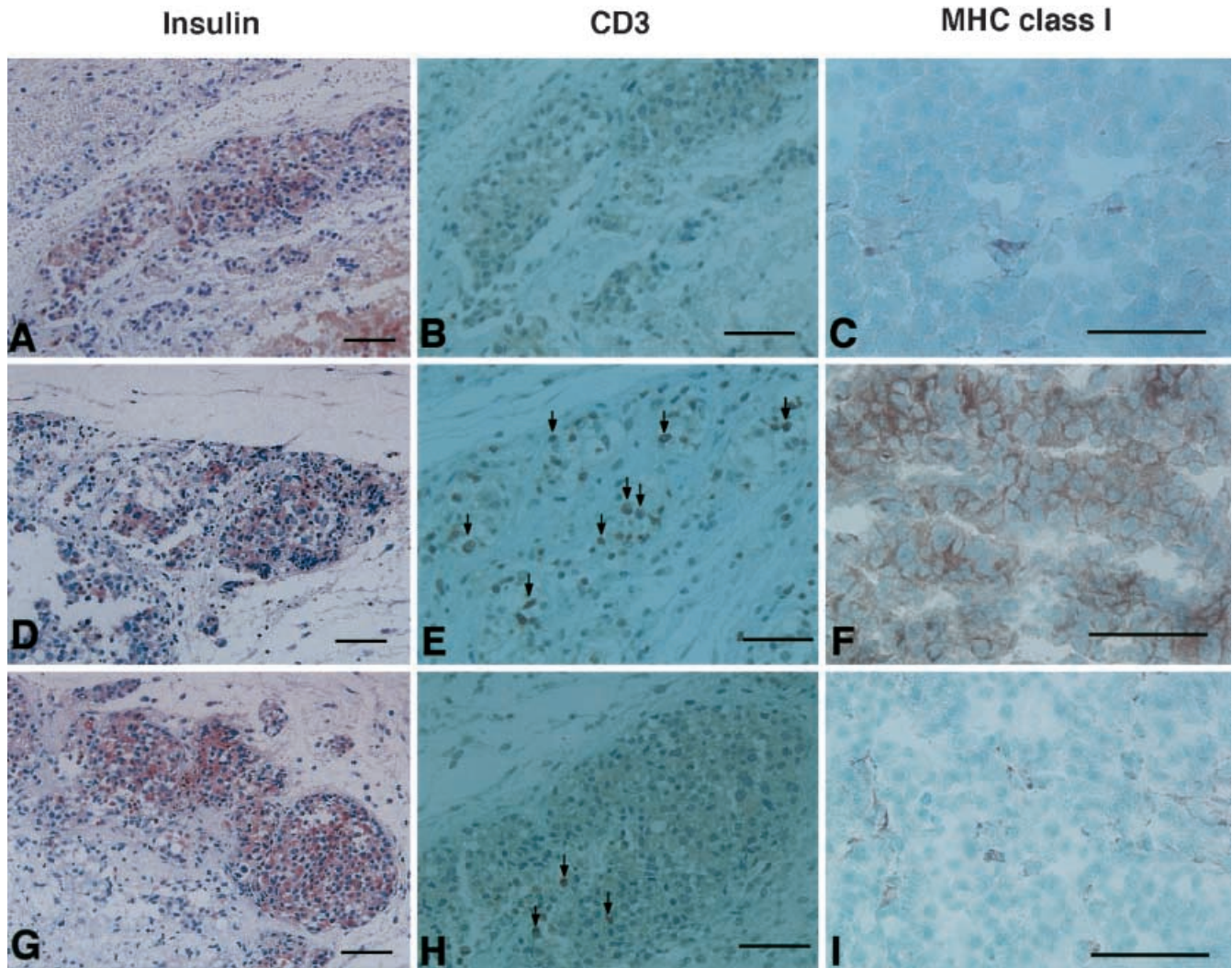
Fig. 3A–I. The histological appearance of subcutaneously transplanted MIN6 or CTLA4-Ig/MIN6 cells with or without DST treatment. MIN6 cells (**A**–**F**) or CTLA4-Ig/MIN6 cells (**G**–**I**) (1×10^7) were transplanted into STZ-induced diabetic C3H/Hej mice with (**D**–**I**) or without DST (**A**–**C**). Grafts were dissected 8 days after transplantation. Paraffin-embedded tissue sections were stained with anti-insulin antibody (**A**, **D** and **G**) or anti-CD3 antibody (**B**, **E** and **H**). Frozen sections were stained with anti-MHC class I (H-2K^b) mAb (**C**, **F** and **I**). *Arrows* indicate CD3$^+$ cells infiltrated into MIN6 or CTLA4-Ig/MIN6 grafts. *Scales bars*, 50 µm

was determined macroscopically. In mice given a simultaneous transplantation of CTLA4-Ig/MIN6 and MIN6 cells, rejection of MIN6 cells was further confirmed by the presence of hyperglycaemia after surgical removal of the visible CTLA4-Ig/MIN6 graft.

Statistical analysis. Data were expressed as the means ± the SD. Graft survival in different experimental groups was compared using the log-rank test. Student's *t* test was used for the comparison of paired and unpaired measurements. A *p* value of less than 0.05 was considered to be statistically significant. All statistical calculations were performed using Statview-J5.0 for Macintosh system 8.6 software.

Results

Transfected MIN6 cells secreted functional CTLA4-Ig. MIN6 cells were transfected with pCXN-CTLA4-Ig to generate stable transformants. After G418 selection, transformants were examined for CTLA4-Ig production. The culture supernatant of a stable transformant cell line (CTLA4-Ig/MIN6) contained CTLA4-Ig that was specifically reactive with B7.1-expressing cells (Fig. 1A). Purified CTLA4-Ig inhibited the MLR with an IC50 of 1 µg/ml. Supernatant from CTLA4-Ig/MIN6 also inhibited the MLR to 58% of control levels when it was diluted to 25% (Fig. 1B). Upon glucose loading, CTLA4-Ig/MIN6 cells showed increased insulin secretion in a dose-dependent manner, similar to original MIN6 or mock cells. Taken together, these results indicate that the CTLA4-Ig/MIN6 cells constitutively produced CTLA4-Ig and were capable of secreting insulin in response to high extracellular glucose (Fig. 1C).

Locally expressed CTLA4-Ig prolonged the survival of MIN6 cells in a model of DST-induced transplantation

Table 1. Effect of anti-CTLA4 mAb on prolongation of graft survival by CTLA4-Ig

Group	Graft and Treatment	n	Graft survival (days)	MST±SD (days)	p[a]
Exp. 1					
1	CTLA4-Ig/MIN6 + DST	4	22, > 40×3	>35.5 ± 9.0	
2	CTLA4-Ig/MIN6 + DST+ control mAb	4	24, > 40×3	>36.0 ± 8.0	NS vs 1
3	CTLA4-Ig/MIN6+DST+anti-CTLA4 mAb	4	12, 17, 17, > 40	21.5 ± 12.6	0.029 vs 1, 2, NS vs 4
4	MIN6+DST	4	12, 22, 22, 24	20.5 ± 5.4	0.041 vs 1, 0.018 vs 2
Exp. 2					
1	MIN6 alone	3	>26×3	>26 ± 0	
2	MIN6 + anti-CTLA4 mAb	5	>26×5	>26 ± 0	
3	MIN6+DST	5	17, 19, 19, 21, 23	19.8 ± 2.3	
4	MIN6+DST+anti-CTLA4 mAb	5	15, 15, 19, 21, 21	18.2 ± 3.0	NS vs 3

[a] Determined by the log rank test
CTLA4-Ig/MIN6 or parental MIN6 cells ($1×10^6$) were transplanted into STZ-induced diabetic C3H/Hej (H-2 ^{k}) mice with simultaneous DST ($1×10^6$ splenocytes). Some of the recipients were given i.p. injections of either anti-CTLA4 mAb or control hamster IgG mAb (0.2 mg on day 0 and 0.1 mg on days 3, 6, 9, 12, and 15). Graft survival was monitored by blood glucose concentrations, and rejection was defined as a glucose concentration of >300 mg/dl in two consecutive measurements

rejection. When $1×10^6$ MIN6 cells of C57BL/6 origin or CTLA4-Ig/MIN6 cells were transplanted into the flank of STZ-induced diabetic C3H/Hej recipients, most of the animals showed restored euglycaemia (< 200 mg/dl) within 1 week post-transplantation and maintained it for more than 1 month (Fig. 2A, B). As we previously demonstrated [5], DST of spleen cells accelerated the host immune response, providing a model of pancreatic beta-cell transplantation rejection that is relevant to clinical settings where graft rejection is initiated by contaminating immunogenic non-islet cell populations. Whereas parental MIN6 cells of C57BL/6 origin survived in non-sensitised diabetic C3H/Hej mice and restored euglycaemia, the same cells were acutely rejected when DST was given to the recipient animals simultaneously (MST was 10.7±2.2 days) (Fig. 2C). Histologically, the MIN6 graft showed a minimum level of cellular infiltration by T cells, and was positively stained for insulin (Fig. 3A, B). Following DST, the MIN6 graft showed obvious signs of rejection with infiltration of CD3+ T cells (Fig. 3E). Both CD4+ and CD8+ T cell subsets were almost equally observed in the MIN6 graft (data not shown). Importantly, while the MIN6 graft expresses only marginal levels of MHC class I before DST (Fig. 3C), it came to express MHC class I abundantly after DST (Fig. 3F). The rejecting MIN6 graft was stained only weakly for insulin after DST (Fig. 3D). These results indicate that MIN6 cells, like purified murine islet beta cells, are only weakly immunogenic, but can be rejected by recipient mice by the concomitant sensitisation with immunogenic donor cells.

To determine whether this rejection can be circumvented by local expression of CTLA4-Ig, CTLA4-Ig/MIN6 cells were transplanted into diabetic C3H/Hej mice sensitised with DST at the time of grafting. As shown in Fig. 2D, 7 of 12 CTLA4-Ig/MIN6 grafts survived beyond day 20 after transplantation in allogeneic diabetic C3H/Hej mice given simultaneous DST, whereas none of the grafts of parental MIN6 cells survived beyond this time point in DST-sensitised mice. Histologically, the CTLA4-Ig/MIN6 grafts showed little sign of tissue injury with only slight cellular infiltration even after DST (Fig. 3H), and no marked increase in MHC class I antigen expression (Fig. 3I). The overall survival of the CTLA4-Ig/MIN6 cells (MST was >24.8±12.7 days) was significantly prolonged compared with that of parental MIN6 cells (MST was 10.7±2.2 days). These results indicate that, under our experimental conditions, the forced expression of CTLA4-Ig can successfully suppress the accelerated rejection response induced by DST.

To ascertain whether the prolonged survival was attributable to CTLA4-Ig produced locally by the MIN6 transfectants, the effects of a neutralising anti-CTLA4 mAb were examined. As shown in Table 1, i.p. administration of the anti-CTLA4 mAb, but not an isotype-matched control mAb, induced prompt rejection of the CTLA4-Ig/MIN6 cells. When the subcutaneously inoculated CTLA4-Ig transfected cells were removed from normoglycaemic mice, blood glucose concentrations increased to over 300 mg/dl within 2 days, indicating that the transfected MIN6 cells were responsible for maintaining the normal blood glucose concentrations. In separate experiments, we also tested the effects of anti-CTLA4 mAb on the survival of parental MIN6 cells. While previous studies reported that administration of neutralising anti-CTLA4 mAb enhances T-cell responses by blocking inhibitory functions of CTLA4 [24], we found that the anti-CTLA4 mAb treatment did not affect survival of the parental MIN6 graft in either untreated- or DST-sensitised re-

Table 2. Effect of contralateral transplantation of CTLA4-Ig/MIN6 on MIN6 survival at a remote site. In group 1, MIN6 cells (1×10^6) alone were transplanted into the right flank of STZ-induced diabetic C3H/Hej mice ($n = 5$)

Group	Graft	Duration of normoglycaemia	Number of MIN6 graft surviving on day 21
1	MIN6 alone (right)	11, 17, 19, 21, >21	1/5
2	MIN6 (right)+CTLA4-Ig/MIN6 (left)	11×2, 14×2, 17, 21 >21×4, >21×6[a]	0/6[a]

[a] Normoglycaemic mice bearing a visible CTLA4-Ig/MIN6 graft but no visible MIN6 graft

In Group 2, CTLA4-Ig/MIN6 (1×10^6) and parental MIN6 cells (1×10^6) were transplanted separately into each flank of STZ-induced diabetic C3H/Hej mice ($n = 17$). In both groups, spleen cells (1×10^6) from the donor strain (C57BL/6) were in-

jected i.v. at the time of transplantation. The survival of the parental MIN6 cells was examined on day 21 after transplantation, when only one out of five control animals (Group 1) showed normoglycaemia. At this time point, none of the animals in Group 2 showed a recognisable MIN6 graft

cipient mice in our system (Table 1). Collectively, these results suggest that the prolonged graft survival was dependent on CTLA4-Ig secreted by the transfected cells.

CTLA4-Ig prolonged survival of MIN6 cells via local mechanism. To investigate whether locally expressed CTLA4-Ig is also beneficial for the survival of MIN6 cells at a remote site, we then transplanted MIN6 cells into diabetic C3H/Hej mice with or without the contralateral transplantation of CTLA4-Ig/MIN6 cells. Mice were given DST at the time of grafting, and MIN6 cell survival was examined on day 21 after transplantation. Of mice transplanted with MIN6 cells alone, only one of five animals showed MIN6 cell survival and normal blood glucose concentrations (Table 2). Of mice transplanted with MIN6 and CTLA4-Ig/MIN6 cells simultaneously at separate sites ($n = 17$), 10 were normoglycaemic, while none retained a palpable MIN6 graft. To examine whether the normoglycaemia was due to the survival of the CTLA4-Ig/graft but not the MIN6 graft, six mice with clearly visible CTLA4-Ig/MIN6 grafts were selected and subjected to further analysis. As summarized in Table 2, all six animals showed abrupt hyperglycaemia after removal of the CTLA4-Ig/MIN6 graft, indicating that the CTLA4-Ig/MIN6 graft survived preferentially and that the MIN6 cells had been rejected, even in the presence of the contralateral CTLA4-Ig/MIN6 graft. Together, these results suggest that locally expressed CTLA4-Ig was effective only in situ and did not prolong the survival of MIN6 cells at remote sites.

Because CTLA4-Ig is a secreted protein, its efficacy could be due to systemic immunosuppressive effects, even when it is expressed locally. To examine whether the prolonged survival of the CTLA4-Ig/MIN6 grafts was associated with the presence of CTLA4-Ig in the peripheral blood, the serum concentration of CTLA4-Ig was determined at various time points after CTLA4-Ig/MIN6 transplantation. CTLA4-Ig was readily detectable on day 6 after transplantation, (mean 268 ± 66 ng/ml), but not on days 14 and 21
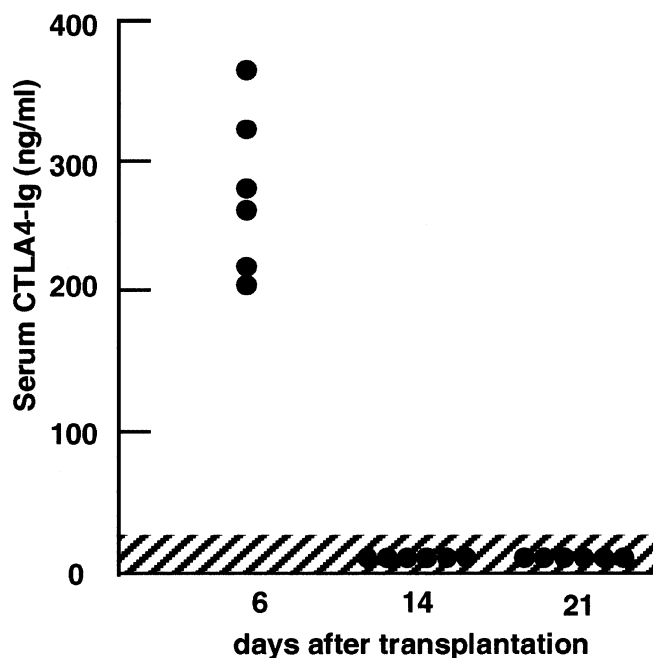
Fig. 4. CTLA4-Ig concentrations in the peripheral blood of mice transplanted with CTLA4-Ig/MIN6 cells. CTLA4-Ig/MIN6 cells (1×10^6) were subcutaneously transplanted into STZ-induced diabetic C3H/Hej mice. Mouse serum samples were collected from CTLA4-Ig/MIN6-transplanted, euglycaemic mice at 6, 14, and 21 days after transplantation. Concentrations of CTLA4-Ig were determined by ELISA. The *shaded zone* indicates the range where CTLA4-Ig concentrations were below the detection level (3 ng/ml)

(Fig. 4), indicating that CTLA4-Ig was present systemically during an early stage after transplantation but disappeared thereafter from the circulation. When in situ CTLA4-Ig production was examined by immunohistochemistry, a strong signal was observed within and around the engrafted CTLA4-Ig/MIN6 cells at 72 days after transplantation (Fig. 5). These results suggest that the prolonged survival of CTLA4-Ig/MIN6 cells was associated with the continued presence of CTLA4-Ig in situ, and that its protective effect was conferred locally.
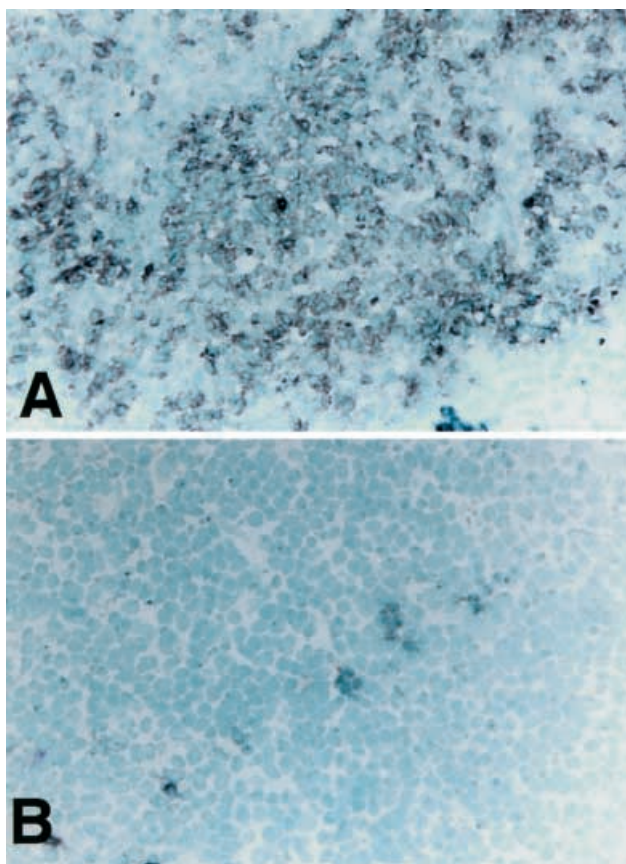
Fig. 5A,B. Long-term production of CTLA4-Ig by a CTLA4-Ig/MIN6 graft in situ. Cryostat sections were made from a CTLA4-Ig/MIN6 graft 72 days after transplantation with simultaneous DST (**A**) and a MIN6 graft 20 days after transplantation without DST (**B**), and incubated with goat anti-human IgG to detect CTLA4-Ig. The sections were then stained with horseradish peroxidase-labelled anti-goat IgG. (original magnifications: ×50)

Discussion

Our study demonstrates that locally expressed CTLA4-Ig in transplanted MIN6 cells significantly prolonged the survival of the beta cells in sensitised recipient mice. Administration of a high dose of CTLA4-Ig was previously reported to suppress immune responses systemically [14, 15,16, 17], and several preceding studies indicate that local delivery of CTLA4-Ig prolongs the survival of islet allografts in mice [8, 9, 18]. Using adenovirus-mediated expression of CTLA4-Ig, investigators [26] recently reported that the production of CTLA4-Ig by islet cells themselves, but not by cells located in remote sites, is effective in prolonging islet allograft survival. Another study [27] showed that ex vivo "coating" of islet allografts with CTLA4-Ig protein before transplantation resulted in a long-term engraftment of the islet allografts. However, little has been known about mechanisms involved in the prolongation of allograft survival in sensitised animals by locally delivered CTLA4-Ig in vivo.

To investigate this issue, we transplanted CTLA4-Ig/MIN6 cells and MIN6 cells separately into each flank of antigen-sensitised diabetic C3H/Hej mice, and examined the survival of the allografts. The results showed that locally expressed CTLA4-Ig prolonged the survival of the CTLA4-Ig/MIN6 cells, but not of the MIN6 cells at a remote site, suggesting that the protective effect of the CTLA4-Ig was conferred locally. In addition, we found that the prolonged survival of CTLA4-Ig/MIN6 allografts was closely associated with the continued production of CTLA4-Ig in situ, but not with its presence in the systemic circulation. Immunohistochemical analysis confirmed that the surviving CTLA4-Ig/MIN6 allografts continued to produce CTLA4-Ig in situ more than 70 days after transplantation (Fig. 5). In contrast, serum CTLA4-Ig was detectable on day 6 (mean 268 ± 66 ng/ml), but not on days 14 and 21 in the CTLA4-Ig/MIN6-engrafted mice. (The minimum detection level of the assay used was 3 ng/ml.) The level of CTLA4-Ig detected in the peripheral blood was presumably insufficient to suppress the host T-cell response systemically, because our in vitro studies showed that higher concentrations of CTLA4-Ig are required to suppress the anti-alloantigen T-cell responses (Fig. 1B). In support of this notion, mice engrafted with CTLA4-Ig/MIN6 cells showed almost normal T-cell responses to alloantigen in vitro and rejected a donor type skin graft in a first set fashion (data not shown). Taken together, these results suggest that the locally produced CTLA4-Ig by MIN6 cells did not induce systemic antigen-specific unresponsiveness, but rather provided a local microenvironment that facilitated the survival of the engrafted MIN6 cells.

An immunosuppressive effect of the locally produced CTLA4-Ig could be exerted in steps, as follows. First, secreted CTLA4-Ig could help abrogate the initial T-cell responses by interrupting the CD28-B7-dependent co-stimulatory signals. The CTLA4-Ig/MIN6 cells are devoid of B7 antigen expression; therefore, B7+ APCs need to be recruited to the graft site for a CD28-B7-mediated rejection response to take place. Locally produced CTLA4-Ig could suppress the initial T-helper responses by inhibiting the co-stimulatory signals provided by B7+ APCs. Second, locally expressed CTLA4-Ig could help down-regulate the immunogenicity of MIN6 cells. While unstimulated MIN6 cells were poorly immunogenic and could not be recognized by unprimed allogeneic C3H/Hej mice (Fig. 2A), they were rapidly rejected upon simultaneous DST (Fig. 2C). This indicates that MIN6 cells are vulnerable to immunological attack once an accelerated rejection response is generated. While the MIN6 cells barely express MHC class I in unsensitised mice, they express MHC class I abundantly in mice given DST. In sharp contrast, CTLA4-Ig/MIN6 cells did not express readily detectable amounts of MHC class I antigen expression even after DST.

Therefore, by blocking the co-stimulatory signals of B7$^+$ APCs locally, CTLA4-Ig may inhibit production of cytokines, such as TNFα and IFN-γ, that can upregulate MHC expression in MIN6 cells [19].

Although our results suggest that CTLA4-Ig expressed in MIN6 cells prolonged the graft survival via local mechanisms, it is also possible that secreted CTLA4-Ig could affect T-cell functions in the draining lymph nodes, where the immune responses to MIN6 grafts is probably initiated. Because CTLA4-Ig was detected in the circulation during an early stage after transplantation in this study, secreted CTLA4-Ig could affect immune responses in the lymph nodes at this time point. We previously showed that DST induces production of various cytokines and donor microchimerism in lymphoid tissues [28]. Accordingly, it is possible that secreted CTLA4-Ig could modulate DST-induced immune response to alloantigens in lymph nodes during an early stage after transplantation. To gain a better understanding of mechanisms underlying site-specific effects of CTLA4-Ig produced by transfected MIN6 cells, it is necessary in future to examine cytokine production and T-cell responses against allo-antigens in lymphoid tissues including draining lymph nodes at various time points after transplantation.

Although the local expression of CTLA4-Ig in MIN6 cells appeared to be advantageous for their survival, graft rejection was still observed in some recipient mice. There are several possible reasons for the different responses of these mice. For instance, it is now evident that the co-stimulatory signals for T-cells are redundant and that several ligand and receptor pairs including the CD40/CD40 ligand pathway are involved in T-cell activation [29]. Therefore, blockage of the CD28-dependent co-stimulatory signals by CTLA4-Ig alone might not have been sufficient for the complete suppression of T cells. In addition, in the rejected grafts, it is conceivable that the production of CTLA4-Ig was not sufficient to override the accelerated rejection response induced by DST. While we used stably transfected MIN6 cells to produce CTLA4-Ig, the rate at which the CTLA4-Ig is produced in individual grafts in vivo could be affected by their local environment in situ. In addition, the immunogenicity of the human-Ig-based chimeric CTLA4 protein used in this study could have played a role in the outcome. In fact, we found anti-human IgG activity in the serum samples of mice engrafted with CTLA4-Ig/MIN6 cells (data not shown), which could have reduced the efficacy of the CTLA4-Ig. CTLA4-Ig with mouse Fc could be more suitable for inhibiting CD28-dependent co-stimulatory signals in mice.

While we found that locally expressed CTLA4-Ig significantly suppressed the acute rejection response induced by DST, a study [30] reported that the i.p. administration of CTLA4-Ig failed to prolong the allograft survival in mice given DST. Differences in the route of CTLA4-Ig delivery, timing of the DST, or combinations of mouse strains used or both could have contributed to this apparent discrepancy in results.

Under most clinical settings for islet transplantation, which involves simultaneous organ transplantation or islet transplantation from multiple donors sharing MHC antigens, the purified islets might be injured by the systemic anti-donor immune response elicited by the donor antigens. Our results demonstrate that local expression of CTLA4-Ig by the genetically engineered pancreatic beta-cell line MIN6 successfully circumvented the accelerated rejection responses induced by DST, and significantly prolonged the allograft survival in vivo, suggesting that the targeted delivery of CTLA4-Ig could protect the graft without causing systemic immunosuppression. Together with technical advances in targeted gene delivery, precise site-directed immunosuppression could become an important strategy in clinical islet transplantation.

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