

Immunoglobulin-Like Transcript 3-Fc Suppresses T-Cell Responses to Allogeneic Human Islet Transplants in hu-NOD/SCID Mice

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OBJECTIVE—The aim of our study was to explore the immunomodulatory activity of soluble immunoglobulin (Ig)-like transcript (ILT) 3-Fc in pancreatic islet transplantation and to determine its mechanism of action.

RESEARCH DESIGN AND METHODS—NOD/SCID mice in which diabetes was induced by streptozotocin injection were transplanted with human pancreatic islet cells. Mice in which the transplant restored euglycemia were humanized with allogeneic peripheral blood mononuclear cells and treated with ILT3-Fc or control human IgG or left untreated. The blood glucose level was monitored twice a week, and rejection was diagnosed after two consecutive readings >350 mg/dl. Tolerated and rejected grafts were studied histologically and by immunostaining for human T-cells and insulin production. CD4 and CD8 T-cells from the spleen were studied for suppressor activity, expression of cytokines, and CD40L.

RESULTS—Although human T-cell engraftment was similar in all groups, ILT3-Fc-treated mice tolerated the islets for the entire period of observation (91 days), whereas control mice rejected the graft within 7 weeks ($P < 0.0001$). ILT3-Fc treatment suppressed the expression of cytokines and CD40L and induced the differentiation of human CD8⁺ T suppressor cells that inhibited Th alloreactivity against graft HLA antigens. T-cells allostimulated in vitro in the presence of ILT3-Fc inhibited CD40L-induced upregulation of CD40 in human pancreatic islet cells. Histochemical studies showed dramatic differences between human pancreatic islets from tolerant, ILT3-Fc-treated mice and control recipients rejecting the grafts.

CONCLUSIONS—The data indicated that ILT3-Fc is a potent immunoregulatory agent that suppressed islet allograft rejection in humanized NOD/SCID mice. *Diabetes* 57:1878–1886, 2008

Transplantation of isolated pancreatic islets is a promising approach to curative therapy of type 1 diabetes. However, many immunosuppressive drugs, including corticosteroids, cyclosporin, and tacrolimus, are either diabetogenic or toxic to the islet

cells (1–3). The development of nondiabetogenic regimens that induce immunological tolerance without the hardship of chronic immunosuppressive therapy remains a major goal in islet transplantation. Experimental data suggest that prolonged islet allograft survival can be achieved using biological modifiers, such as monoclonal antibodies and soluble receptor ligands, which block T-cell activation and/or costimulation (4–13). Such attempts include the blockade of the CD40-CD40L costimulatory pathway deemed to be crucial to the activation and differentiation of T effector cells. Because CD40 is expressed by pancreatic islet cells (8), blockade of this pathway may be particularly relevant (6–12).

In previous studies, we have shown that alloantigen-specific human CD8⁺ T suppressor (Ts) cells can be generated both in vitro and in vivo by chronic antigenic stimulation. We generated CD8⁺ Ts cells by multiple in vitro stimulations of human CD3⁺ T-cells with allogeneic, xenogenic, or allopeptide-pulsed autologous antigen-presenting cells (APCs). CD8⁺CD28⁻ Ts cells from such cultures interacted with priming APCs in an antigen-specific, major histocompatibility complex class I-restricted and contact-dependent manner, inducing the upregulation of the Ig-like transcript (ILT) 3 and ILT4 and inhibiting nuclear factor- κ B activation and CD40 signaling in APCs (14–18).

ILT3 and ILT4 have extracellular Ig-like domains responsible for ligand binding at the cell surface and a long cytoplasmic tail containing immunoreceptor tyrosine-based inhibitory motif, which recruits inhibitory phosphatases and transduces a negative signal into the cell (19–21). The crucial role of ILT3 in the induction of T-cell unresponsiveness was documented in experiments showing that ILT3-overexpressing dendritic cells induce anergy in CD4⁺ T helper cells and suppress the differentiation of interferon- γ (IFN- γ)-producing CD8⁺ cytotoxic T-lymphocyte (CTL) (22). Furthermore, membrane-bound and soluble ILT3 (rILT3-Fc fusion protein) elicited the differentiation of CD8⁺ Ts cells in primary 7-day mixed lymphocyte culture (MLC) and in vivo in humanized C.B-17 SCID mice, inducing tolerance to allogeneic human tumor transplants (23). Also, in a rat model of heart transplantation, we demonstrated that tolerance can be induced, maintained, and transferred by CD8⁺ Ts cells (24).

Here, we report that treatment with ILT3-Fc prevented rejection of human pancreatic islets transplanted in NOD/SCID mice, which were reconstituted with human peripheral blood mononuclear cells (PBMCs) (hu-NOD/SCID mice).

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RESEARCH DESIGN AND METHODS

NOD/SCID female mice purchased from Charles River Laboratories were used at 6–10 weeks of age. All protocols involving animal care procedures were approved by the Columbia University Institutional Animal Care and Use Committee. The animals were kept in microisolator cages and were fed autoclaved food and water. Diabetes was induced by intravenous injection of streptozotocin (STZ) (Sigma-Aldrich) at the dose of 180 mg/kg. Blood glucose level was measured twice a week using Ascensia Elite XL Blood Glucose Meter system (Bayer AG). Diabetes was diagnosed after two consecutive glucose measurements >350 mg/dl.

Generation, transplantation, and treatment of humanized NOD/SCID mice. Aliquots of 1,500 islet equivalents human pancreatic islets with >70% purity and >90% viability were transplanted under the kidney capsule of NOD/SCID mice rendered diabetic by STZ injection (25). The viability of islets was determined by fluorescein diacetate and propidium iodine staining. Purity was determined by the percentage of dithizone-positive particles (26,27). Mice that did not restore to euglycemia after transplantation were eliminated from the study on the assumption that the grafted islets were not functional. Seven to 10 days posttransplantation, mice that were restored to euglycemia (glucose level <100 mg/dl) were “humanized” by intraperitoneal injection of 50×10^6 PBMCs, isolated from fresh buffy coats purchased from the New York Blood Center. Concomitant with the humanizing treatment, mice received the first of a series of 10 consecutive intraperitoneal injections of ILT3-Fc or human IgG from Sigma (250 μ g/day) and were assigned to the ILT3-Fc treatment or IgG control group, respectively, as described previously (23). An additional control group of NOD/SCID mice was humanized and transplanted as above but received no treatment. Ten days after humanization, circulating human T-cells were evaluated by flow cytometry using heparinized retro-orbital venous samples. Animals failing to reconstitute (<5% human CD45⁺ PBMCs in the circulation) or developing graft-versus-host disease (hunched back, lethargy, weight loss, and tachypnea) were excluded from analysis by design. To avoid variability between samples, both islets and human PBMCs were administered to mice from the ILT3-Fc and human IgG group in a pairwise fashion. To study human T-cell engraftment and suppressor function, a second cohort of mice administered STZ was humanized, transplanted, and treated with IgG or ILT3-Fc as described above. These mice were killed at the onset or completion of allograft rejection in the control IgG-treated group.

ILT3-Fc protein. ILT3-Fc protein expressed and purified as previously described (22) was analyzed by gel electrophoresis and mass spectrometry (MS). Matrix-assisted laser desorption ionization and liquid chromatography–MS/MS analysis of tryptic digests showed no contaminants.

HLA typing. HLA genotypes of human PBMCs and pancreatic islets were determined by PCR with sequence-specific primers using commercially available kits from One Lambda (Los Angeles, CA).

Histology and immunochemistry. Twenty serial paraffin sections of kidney were cut at 4- μ m thickness. Levels 1, 10, and 20 were stained for light microscopic evaluation (hematoxylin-eosin). The remaining sections were used for immunostains including insulin, CD4 (Biogenics, San Ramon, CA), CD3 and CD8 (Dako, Carpinteria, CA), and CD40 (Abcam, Cambridge, MA). Islet quantity and islet inflammatory infiltration (insulinitis) were graded semiquantitatively in blinded fashion by a renal pathologist on a scale of 0 to 3+. The degree of islet inflammation by CD8⁺ T-cells was graded according to the number of CD8 per $\times 40$ high-power field: 0 (none), 1+ (1–10), 2+ (11–25), and 3+ (>25). The results were averaged over at least five high-power fields per slide.

Ts cell assays. Human CD4 and CD8 T-cells were isolated from spleens of humanized NOD/SCID mice using the CD4 or CD8 isolation kits (StemCell Technologies). Sorted CD4 or CD8 T-cells were added at increasing numbers (1–8 $\times 10^4$ /well) to a fixed number (10^4 /well) of unprimed autologous CD3⁺CD25⁻ T-cells and stimulated for 6 days in MLC with irradiated, allogeneic PBMCs sharing HLA-A, -B, and -DR antigens with the islet transplant. [³H]thymidine incorporation was measured (23).

Tissue culture for CD40L induction of CD40 upregulation in islet cells. Responding T-cells were allostimulated with irradiated PBMCs matching the HLA classes I and II of selected islet cultures in the presence of 50 μ g/ml ILT3-Fc. After 7 days, CD8⁺ T-cells were isolated and tested. Nonprimed CD8⁺ T-cells from the same responder served as controls. Pancreatic islets selected as targets were co-incubated overnight with 1) CD40L⁺ D1.1 cells (18), 2) D1.1 cells plus allopecific CD8⁺ Ts cells, or 3) D1.1 cells plus unprimed CD8⁺ T-cells. Islets cultured alone were used to measure the constitutive level of CD40 expression, and islets cultured in tumor necrosis factor- α (10^6 units/l), IFN- γ (10^6 units/l), and interleukin (IL)-1 β (5×10^4 units/l) were used as a positive control for CD40 induction (8). After 18 h, cells were washed, and the T-cells were depleted by incubation with anti-CD3 and anti-CD8 antibodies (Becton Dickinson, San Jose, CA) followed by anti-mouse magnetic beads

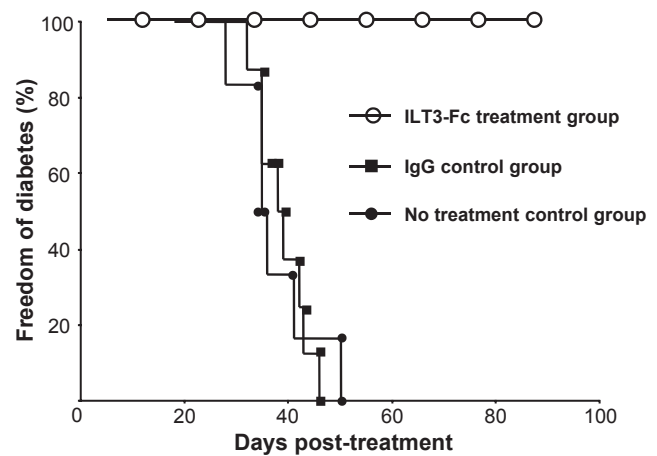


FIG. 1. ILT3-Fc treatment prevents islet allograft rejection in hu-NOD/SCID mice. Diabetic NOD/SCID mice were transplanted under the kidney capsule with human islets. Mice in which euglycemia was restored were injected intraperitoneally with 50×10^6 allogeneic human PBMCs and treated for 10 days with 250 μ g/day of ILT3-Fc (treatment group $n = 8$, \circ), human IgG (control group $n = 8$, \blacksquare), or left untreated (control group $n = 6$, \bullet). Glycemia levels >350 mg/dl were considered indicative of rejection-induced diabetes.

(Invitrogen, Carlsbad, CA). Remaining cells were used for PCR and flow-cytometry studies.

Real-time PCR. Total RNA was isolated with the RNeasy-4PCR kit (Stratagene, La Jolla, CA). Complementary cDNA was synthesized using the 1st Strand cDNA Synthesis Kit for RT-PCR (Roche Diagnostics, Basel, Switzerland). Real-time PCR was performed using proprietary Taqman gene expression primer probes (Applied Biosystems, Foster City, CA). Data were collected and analyzed with the 7300 SDS 1.3.1 software (Applied Biosystems). The relative amount of gene expression was calculated by the formula: $2^{-\Delta Ct}$, where $\Delta Ct = [Ct(\text{gene}) - Ct(\text{glyceraldehyde-3-phosphate dehydrogenase})]$ and Ct is the “crossing threshold” value returned by the PCR instrument for every gene amplification.

Fluorescence-activated cell sorting analysis. Flow-cytometry studies were performed on a FACSCalibur instrument using six-parameter acquisition (BD Biosciences) as previously described (23).

Statistical analysis. Graft survival curves were computed using the Kaplan-Meier method. Differences between groups were compared by the log-rank test. Student’s *t* test was used to analyze differences in cytokine and cell surface marker expression. The BMDP statistical software package was used for all analyses.

RESULTS

Immunomodulatory effect of ILT3-Fc. NOD/SCID mice with STZ-induced diabetes were transplanted with human islets under the kidney capsule. When they became euglycemic, they were injected intraperitoneally with freshly isolated PBMCs from healthy blood donors and assigned to control groups (receiving human IgG or no treatment) or to the treatment group, which received a daily intraperitoneal injection of ILT3-Fc over a period of 10 days starting the day of PBMC injection, as previously described (23).

Mice from the group not treated ($n = 6$) or treated with IgG ($n = 8$) rejected the graft within 3 to 7 weeks as demonstrated by soaring glucose levels and by histology studies. In contrast, none of the ILT3-Fc-treated hu-NOD/SCID mice ($n = 8$) became diabetic over 91 days of observation (Fig. 1), indicating that ILT3-Fc inhibited rejection of islet allografts in hu-NOD/SCID recipients ($P = 0.0001$).

Human T-cell engraftment in Hu-NOD/SCID mice. Human CD45⁺ PBMC engraftment in NOD/SCID mice recipients of allogeneic islet transplants was analyzed by flow cytometry. Ten days after PBMC injection, the fre-

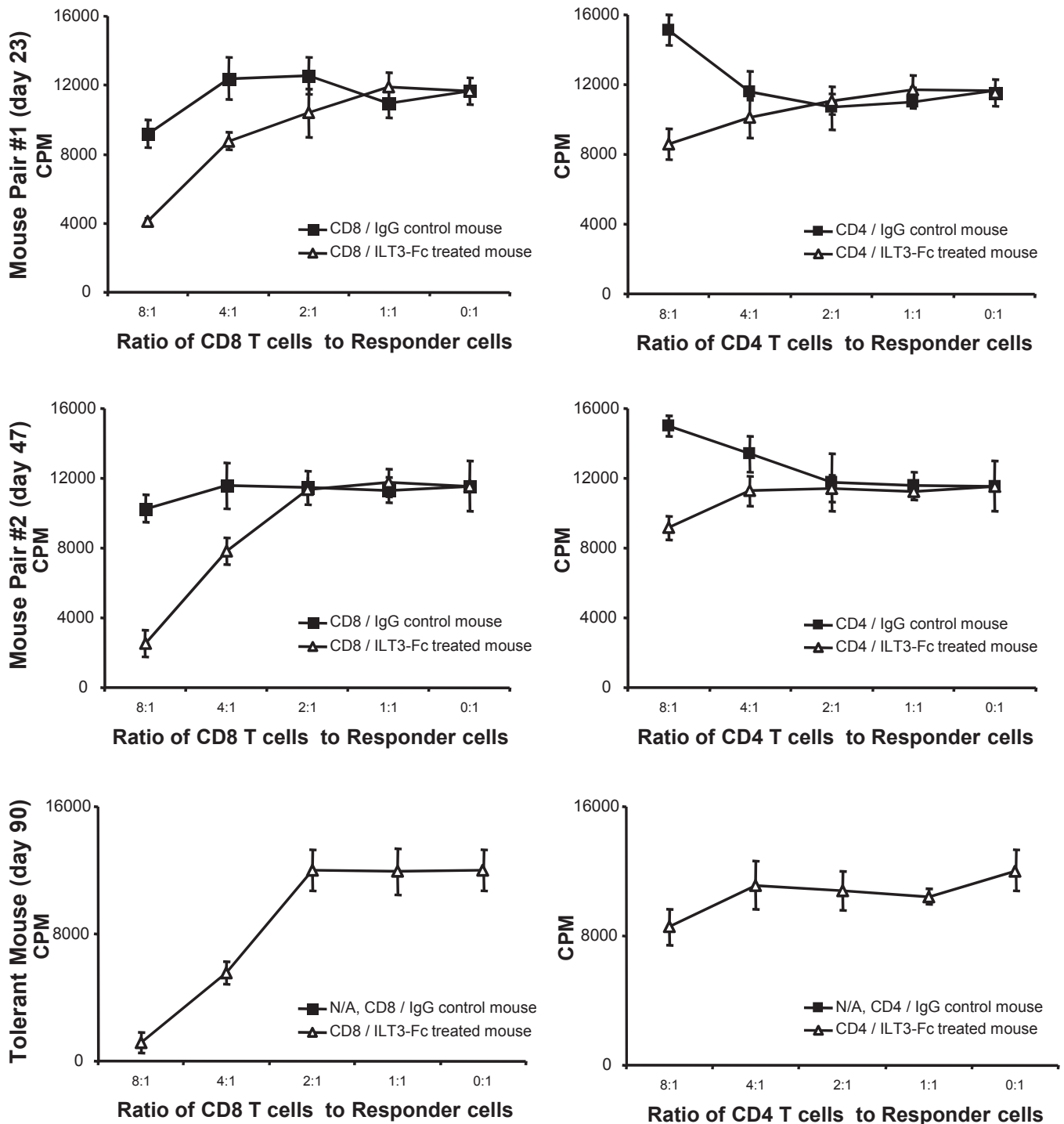


FIG. 2. Ts cell assays in ILT3-Fc-treated (Δ) and human IgG-treated (\blacksquare) hu-NOD/SCID mice recipients of allogeneic islet cells. Mice were killed on days 23, 47, and 91. Human CD4 and CD8 T-cells were isolated from the spleen of recipient mice and added to MLC containing naïve, autologous CD3⁺ T-cells and irradiated allogeneic PBMC HLA matched to the islet donor. Tritiated thymidine [³H], incorporation was measured after 6 days.

quency of human CD3⁺ T-cells in the peripheral blood of hu-NOD/SCID islet allograft recipients was $11 \pm 3\%$ in ILT3-Fc-treated mice ($n = 8$), $10 \pm 2\%$ in untreated mice ($n = 6$), and $10 \pm 4\%$ in mice treated with human IgG ($n = 8$).

To determine whether the outcome of the pancreatic islet transplant was influenced by the number of engrafted human T-cells, we analyzed their frequency in spleens of mice killed at the time of rejection or at the termination of the study (day 91). In untreated or IgG-treated animals, the

frequency ranged from 24 to 59% (mean 41 ± 16) and from 25 to 62% (mean 45 ± 16), respectively. In euglycemic animals killed on day 91 ($n = 8$), the frequency ranged from 28 to 71% (mean 50 ± 22). The difference between the groups was not significant. Less than 1% human T-cells was found in the bone marrow of ILT3-Fc-treated or control animals. No engraftment of CD19⁺ B-cells, CD56⁺ natural killer cells, or CD14⁺ monocytes were found in the spleens and bone marrow of these animals. These data indicate that the outcome of the graft was determined not

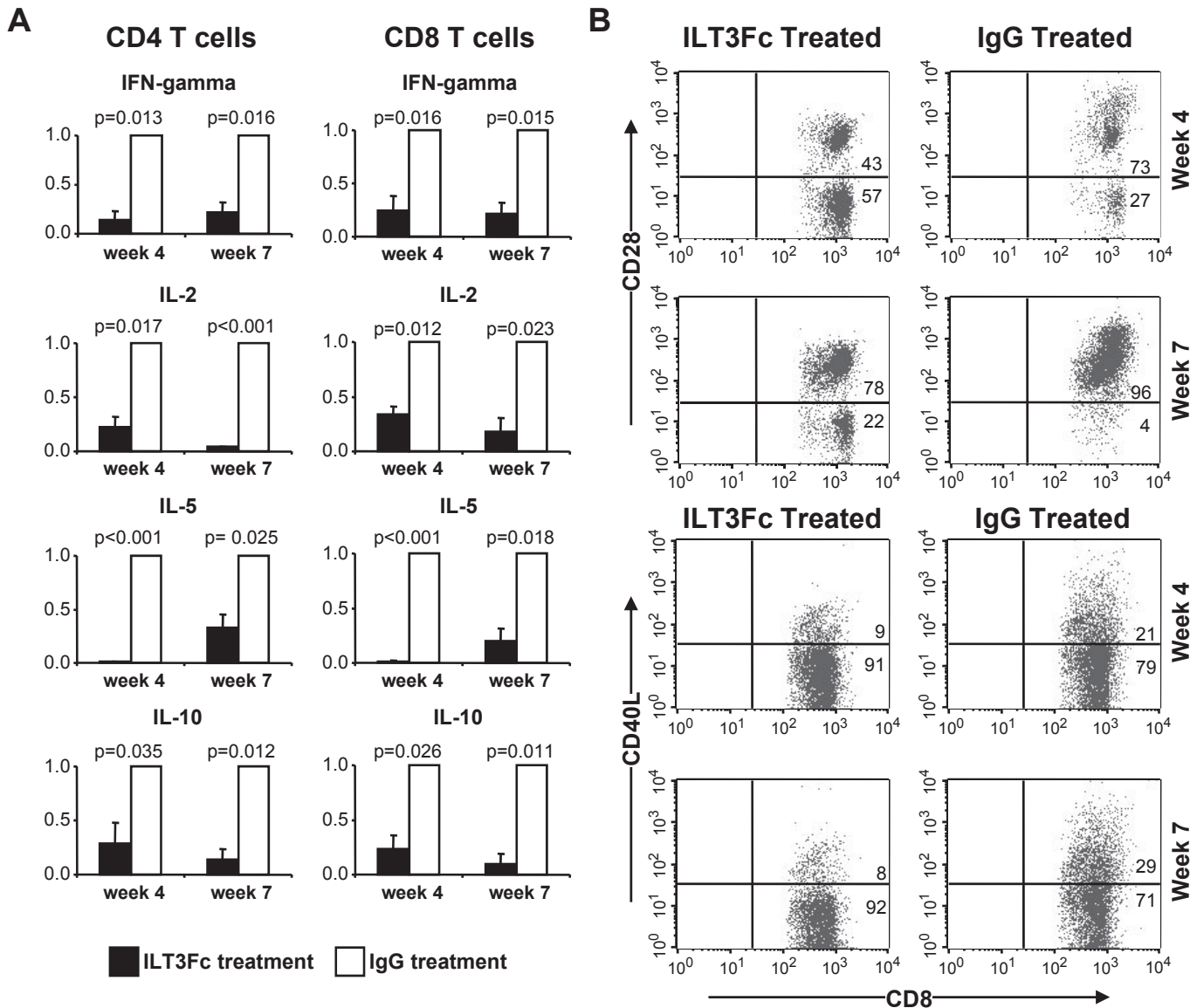


FIG. 3. A: Real-time PCR studies of cytokine transcription by human CD4⁺ and CD8⁺ T-cells from the spleens of ILT3-Fc-treated (■) and human IgG-treated (□) hu-NOD/SCID recipients of islet allografts killed during weeks 4 ($n = 3$) and 7 ($n = 3$) posttransplantation. For clarity, the data generated from each pair of mice were normalized to unity in the control group; mean and SE are shown. **B:** Flow-cytometry analysis of CD28 and CD40L expression on human CD8 T-cells colonizing the spleens of ILT3-Fc- and IgG-treated pairs of animals killed during weeks 4 and 7 posttransplantation. One pair representative of three is shown for each time point.

by the number but, rather, by the functional state of chimeric T-cells in animals treated versus those not treated with ILT3-Fc.

Generation of regulatory T-cells in ILT3-Fc-treated animals. In previous studies, we demonstrated that hu-SCID mice that have been rendered tolerant to allogeneic tumors by treatment with ILT3-Fc develop CD8⁺ Ts cells (23). To determine whether regulatory human T-cells also differentiate in hu-NOD/SCID mice, we tested in parallel the suppressive activity of CD8 and CD4 T-cells magnetically sorted from the spleens of mice that received the pancreatic islets and PBMCs from the same allogeneic donors.

For these studies, we used two hu-NOD/SCID mice treated with ILT3-Fc and two IgG-treated controls, which were not included in the computation of actuarial graft and host survival because they were killed by design. These mice were transplanted with pancreatic islets from

a donor expressing HLA-A1, -B8, -DR3/A2, -B44, and -DR7. One pair was killed on day 23 after human PBMC injection, when the glycemia was 240 mg/dl in the IgG-treated mouse, suggesting the onset of rejection, and 72 mg/dl in the ILT3-Fc-treated mouse. The second pair was killed on day 47 with a glycemia of 380 mg/dl in the IgG-treated mouse and 80 mg/dl in the ILT3-Fc-treated mouse.

Human CD8⁺ and CD4⁺ T-cells magnetically sorted from the recipients' spleens were added in increasing numbers to MLC containing unprimed autologous T-cells, used as responders, and irradiated allogeneic (stimulating) PBMCs from an individual who was HLA matched to the islet cell donor.

CD8⁺ T-cells obtained from the ILT3-Fc-treated mice killed on days 23 and 47 suppressed T-cell reactivity in primary MLC by 67 and 78%, respectively, at an 8:1 ratio of regulatory to responding T-cells. At this ratio, CD4⁺ T-cells

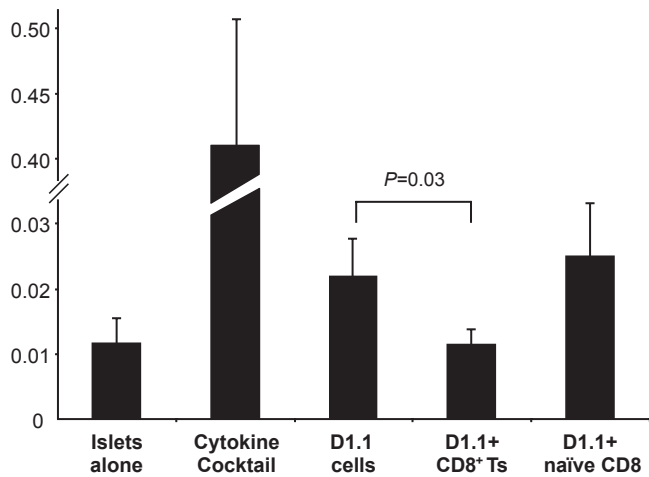


FIG. 4. Real-time PCR analysis of CD40 expression in human pancreatic islet cells. Human pancreatic islet cells were cultured alone or with a mixture of inflammatory cytokines, CD40L-transfected D1.1 cells, D1.1 cells plus allospecific CD8⁺ T cells, or D1.1 cells plus unprimed CD8⁺ T-cells. After 18 h of incubation, T-cells were depleted, and the remaining islets were analyzed by real-time PCR for expression of CD40. The values are expressed as means \pm SD.

from these animals inhibited the MLC response by only 20 and 21%, respectively (Fig. 2).

CD8⁺ and CD4⁺ T-cells from IgG-treated animals killed at the same time had no suppressive activity (Fig. 2). Engraftment of human T-cells into recipients' spleens was similar in the ILT3-Fc- and human IgG-treated mice. These results were reproduced in an additional four pairs of mice killed 4 weeks (days 21 and 26) or 7 weeks (days 43 and 46) after transplantation. The data indicate that ILT3-Fc induced the differentiation of CD8⁺ T cells in hu-NOD/SCID recipients of allogeneic islet transplants. To further determine whether the presence of CD8⁺ T cells is associated with tolerance to the allogeneic islet transplants, we tested CD8⁺ and CD4⁺ T-cells from the spleens of ILT3-Fc-treated euglycemic mice killed on day 91. APCs sharing HLA antigens with the graft were used for stimulating T-cells autologous to the cells tested for suppressive activity. As illustrated in Fig. 2, human CD8⁺ T-cells from mice with long-lasting tolerance ($n = 8$) displayed suppressive activity, inhibiting the MLC response to donor HLA antigens by >70% (mean 85 ± 10). CD4⁺ T-cells from the same animals showed weak inhibitory activity (<20%). The data indicate that ILT3-Fc treatment prevents islet allograft rejection inducing CD8⁺ regulatory T-cells.

Characterization of engrafted human T-cells. To further characterize the phenotype and function of effector and suppressor T-cells from mice rejecting or tolerating human islet allografts, we performed real-time PCR studies on human CD4 and CD8 T-cells sorted from the spleens of six pairs of mice killed during weeks 4 and 7 after humanization. The expression of IFN- γ , IL-2, IL-5, and IL-10 by CD4⁺ and CD8⁺ T-cells was significantly lower in ILT3-Fc- than in IgG-treated recipients (Fig. 3A).

By flow cytometry, the frequency of CD8⁺CD28⁻ T-cells was significantly higher on weeks 4 and 7 ($P = 0.011$ and 0.048), whereas CD8⁺CD40L⁺ was significantly lower ($P = 0.007$ and 0.022) in ILT3-Fc-treated animals compared with paired controls as illustrated in Fig. 3B. This profile of CD8⁺ T-cells from animals treated with ILT3-Fc that have developed T cells is consistent with our previous findings on the CD28-low phenotype of CD8⁺ T cells that act in a cytokine-independent manner (14–16).

Modulation of CD40L-induced upregulation of CD40 in islet cells. Because pancreatic islet cells express a functional CD40 receptor and signaling through this receptor activates nuclear factor- κ B (8), we studied the possibility that allospecific, ILT3-Fc-induced CD8⁺ T cells are able to modulate the expression of CD40 in islet cells. For this purpose, T-cells from healthy blood donors were stimulated for 7 days in MLC with irradiated allogeneic APCs matching the HLA genotype of pancreatic islet cell cultures from three different individuals. ILT3-Fc (50 μ g/ml) was added to the medium at the initiation of the culture. CD8⁺ T-cells primed in the presence of ILT3-Fc differentiated into CD8⁺ T cells, which inhibited the MLC response of autologous CD4⁺ T-cells and induced the upregulation of ILT3 on priming APCs in a pattern consistent with that described previously (22,23). ILT3-Fc-induced CD8⁺ T cells were then added to the corresponding islet cell culture together with CD40L-transfected D1.1 cells at a ratio of 1:1:1. Islets co-incubated with D1.1 alone or with D1.1 cells plus unprimed CD8 T-cells and islets stimulated with the cytokine mixture or left unstimulated were tested in parallel. Real-time PCR analysis showed that the cytokine mixture induced maximal upregulation of CD40 expression. D1.1 cells also induced the transcriptional upregulation of the CD40 costimulatory molecule in pancreatic islets. In three independent experiments, allospecific T cells inhibited to baseline levels D1.1-induced CD40 upregulation (Fig. 4). Unprimed CD8⁺ T-cells had no effect on CD40 triggering by CD40L-expressing D1.1 cells. These results indicate that allospecific CD8⁺ T cells generated in vitro suppress CD40L-induced upregulation of CD40 in human pancreatic islet cells.

Histology. Comparison of islet-transplanted kidneys 23 days after human PBMC administration showed that the quantity of islets was greater in the ILT3-Fc-treated (3+) than in the human IgG-treated (2+) animals (Fig. 5A–D). There was insulinitis by CD8⁺ T-cells in the human IgG- but not ILT3-treated mouse (2+ vs. 0.5+) (Fig. 5D and C). Immunostaining for CD40 showed diffuse membrane staining in IgG-treated mice and rare focal membrane staining in islets from the ILT3-Fc-treated pair (Fig. 5F and E).

Pairwise comparison on day 47 showed that islet quantity was greater in treated (mean 3+) than control (mean 1+) mice (Fig. 6A and B). Insulinitis by CD8⁺ T-cells was markedly reduced in treated (mean 0.5+) vs. control (mean 2.5+) mice (Fig. 6C and D). By light microscopy, islets with insulinitis from control animals exhibited scattered apoptotic bodies. By immunostains in both groups, the lymphocytes infiltrating and surrounding the islets were CD8⁺ human T-cells.

Pairwise comparison of insulin immunostains on day 47 showed markedly reduced expression of insulin in islet β -cells from the IgG-treated mouse, indicating impaired function (Fig. 7B and D), compared with strong and diffuse expression in the ILT3-Fc-treated animal (Fig. 7A and C). At 3 months, the tolerated islets displayed strong and diffuse staining for insulin, indicating that they were functionally active and well tolerated (Fig. 7E). In ILT3-Fc-treated mice killed on day 91, there was a large quantity of islets (3+) and no insulinitis (0) (Fig. 7F), consistent with the notion that the graft was well tolerated. Taken together, these findings indicate that ILT3-Fc treatment inhibits the onset and progression of islet allograft rejection.

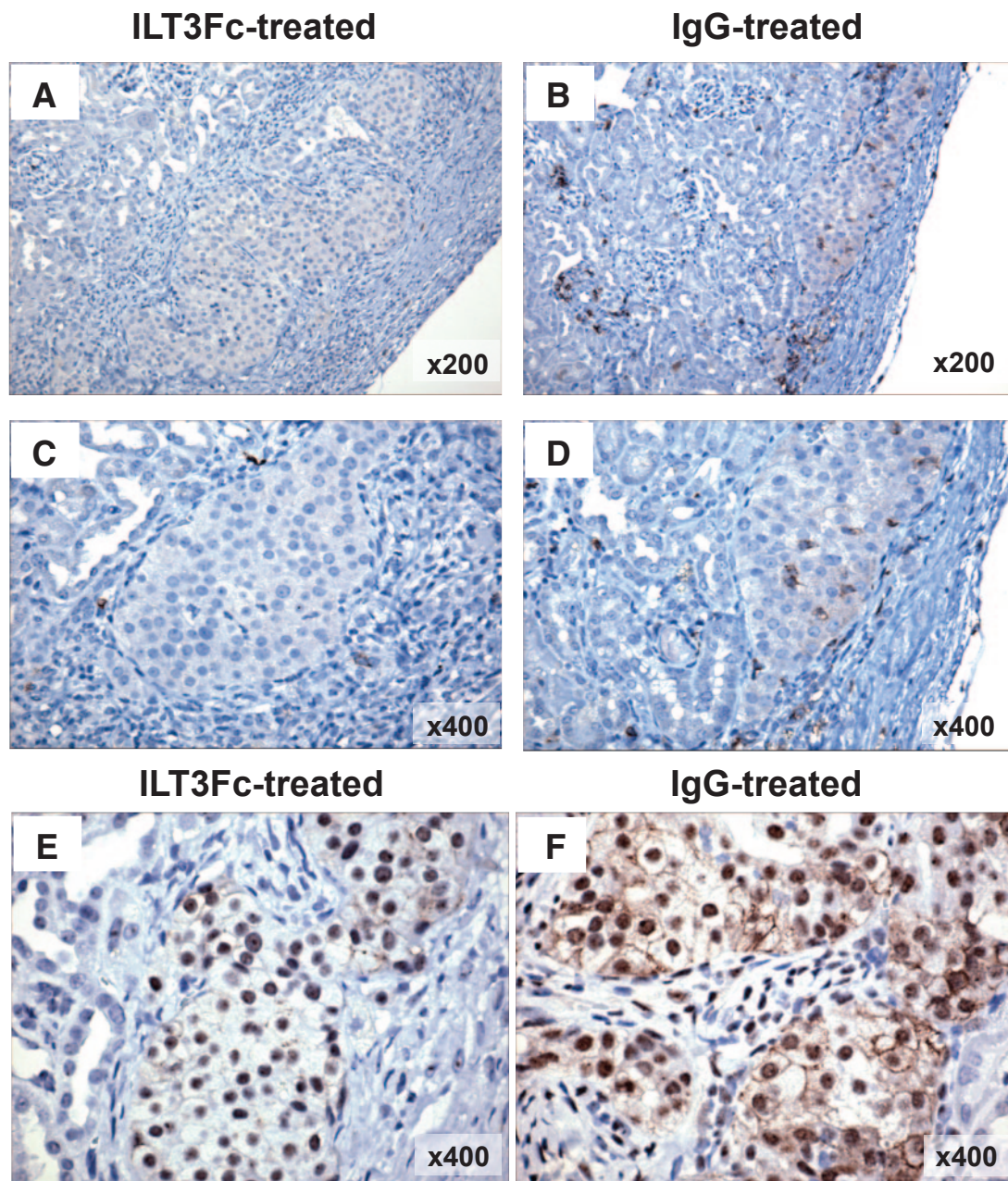


FIG. 5. Immunostaining of CD8⁺ T-cells (A–D) and CD40 (E and F) in islet-engrafted kidneys from ILT3-Fc-treated (A, C, and E) and IgG-treated (B, D, and F) NOD/SCID mice 23 days after humanization.

DISCUSSION

Although many biological mechanisms are similar in rodents and humans, there are several structural and functional differences that render the extrapolation of experimental results to clinical practice quite difficult. Multiple transgenic, knockout, and reconstituted models of autoimmune diseases have been developed over the past 2 decades. The creation of humanized mice, defined as immunodeficient mice engrafted with human hematopoietic stem cells or PBMCs, provided a powerful tool for preclinical testing of new immunomodulatory agents and study of human immune responses (25,28–36). This is particularly true in the case of ILT3, which, like other members of the Ig gene superfamily, has no ortholog in rodents.

In addition to T- and B-cell deficiency, NOD/SCID mice

have functional defects of macrophages and natural killer cells (31–34) and high rates of human lymphocyte engraftment (34), providing a tool for studying human islet allograft rejection and the effect of immunomodulatory agents (25,28,35,36). The rate of T-cell engraftment observed in our study was similar to that described by other authors studying the same strain of mice (34). Using ILT3-Fc treatment, we prevented rejection of pancreatic islet transplants in 100% of hu-NOD/SCID recipients over a 91-day observation period. To our knowledge, this is the highest rate of successful transplantation of allogeneic human islets in a preclinical model in which the efficacy of a biological agent was tested alone without any pharmaceutical immunosuppression. Tolerance to the islets was mediated by CD8⁺ T_s cells as demonstrated by the capacity of human CD8⁺ T-cells, sorted from the spleen of

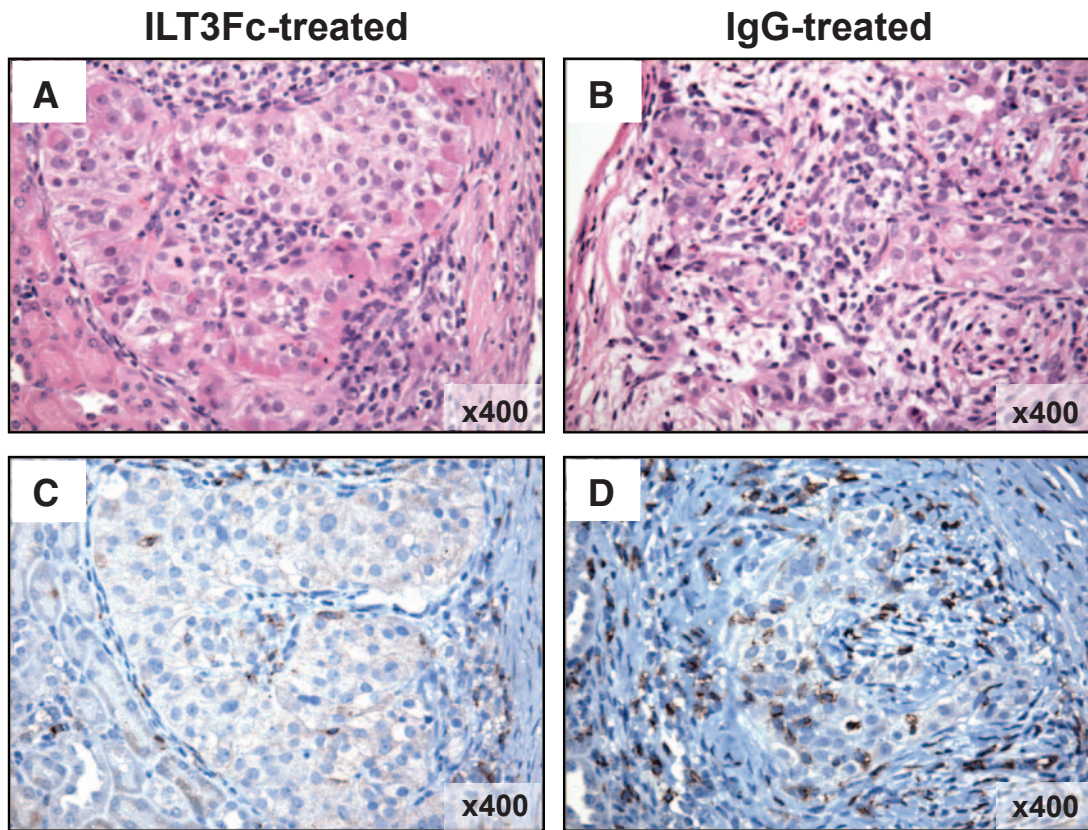


FIG. 6. Hematoxylin-eosin and CD8 immunostaining of islet-engrafted kidneys from ILT3-Fc-treated (A and C) and human IgG-treated (B and D) NOD/SCID mouse 47 days after humanization.

ILT3-Fc-treated hu-NOD/SCID islet recipients, to inhibit the response of autologous T-cells to donor HLA antigens in primary MLC. Most of these CD8⁺ Ts cells displayed a CD28-low phenotype reminiscent of Ts cells generated in vitro by multiple allostimulations (14,22,23). Treatment with ILT3-Fc inhibited the capacity of both CD4 and CD8 T-cells to produce Th1-type (IFN- γ and IL-2) and Th2-type (IL-5 and IL-10) cytokines. Inhibition of Th2-type cytokines is important in islet transplantation because alloantibodies against donor HLA antigens and autoantibodies against the pancreas compromise not only graft survival but also chances for retransplantation (37).

Because only a few CD8⁺ T-cells were found within the islet graft in ILT3-Fc-treated animals, the way in which they mediate tolerance to the graft is not quite clear. It is obvious, however, that direct interaction between human CD8⁺ T-cells and graft HLA class I antigens is required for priming and takes place within the graft. We postulate that in the absence of ILT3-Fc, CD8⁺ T-cells differentiate into effector CTLs, which proliferate within the graft, produce cytokines, induce inflammation, and promote the destruction of the islets. Because they express CD40L, a positively enhancing immunostimulant, they may provide the alarm signal that attracts other T-cells to the site of rejection (14,38). In contrast, primed CD8⁺ T-cells from ILT3-Fc-treated animals differentiate into Ts cells, which do not proliferate within the graft, produce no inflammatory cytokines, have low CD40L expression, and may thus be unable to trigger danger signals from the graft. The absence of CD8⁺ T-cell infiltrates and inflammatory changes of the graft supports this possibility. Because mice were humanized only after the graft was healed, trauma-related danger signals were unlikely to occur.

There is evidence that the constitutive and selective expression of CD40 on the surface of β -cells contributes to autoimmunity and islet allograft rejection by providing costimulatory signals to infiltrating lymphocytes (6,8,10,12,39,40).

It is, therefore, possible that in addition to suppressing islet allograft rejection in diabetic patients, ILT3-Fc may also prevent recurrence of diabetes by inhibiting the CD40-CD40L interaction between pancreatic islet cells and autoaggressive T-cells, primed to diabetogenic islet cell peptides presented by self-APCs. This possibility is strongly supported by our previous studies showing that allospecific CD8⁺ Ts cells inhibit CD40 signaling in APCs and T-cell reactivity to immunogenic peptides (16,41). Because in diabetic patients, selective autoimmune destruction of pancreatic β -cells occurs alone or in combination with rejection of the transplanted islets, the discovery of agents that may block both of these pathological processes would be of paramount importance. Based on our previous findings that CD8⁺CD28⁻ Ts cells are present in the circulation of successfully transplanted heart, liver, and kidney recipients and display the capacity to inhibit CD40L-CD40 interaction, we believe that induction of Ts cells by ILT3-Fc treatment may achieve this goal (rev. in 14).

Taken together, our data demonstrate for the first time that ILT3-Fc is a potent immunoregulatory agent that inhibits human islet allograft rejection. Because sILT3 is a natural product of human APCs, found to be elevated and to induce Ts cells in patients with cancer (23), this biological agent is unlikely to be toxic or have undesirable side effects. Furthermore, because the ligand for ILT3 is expressed only by activated and not by unprimed T-cells

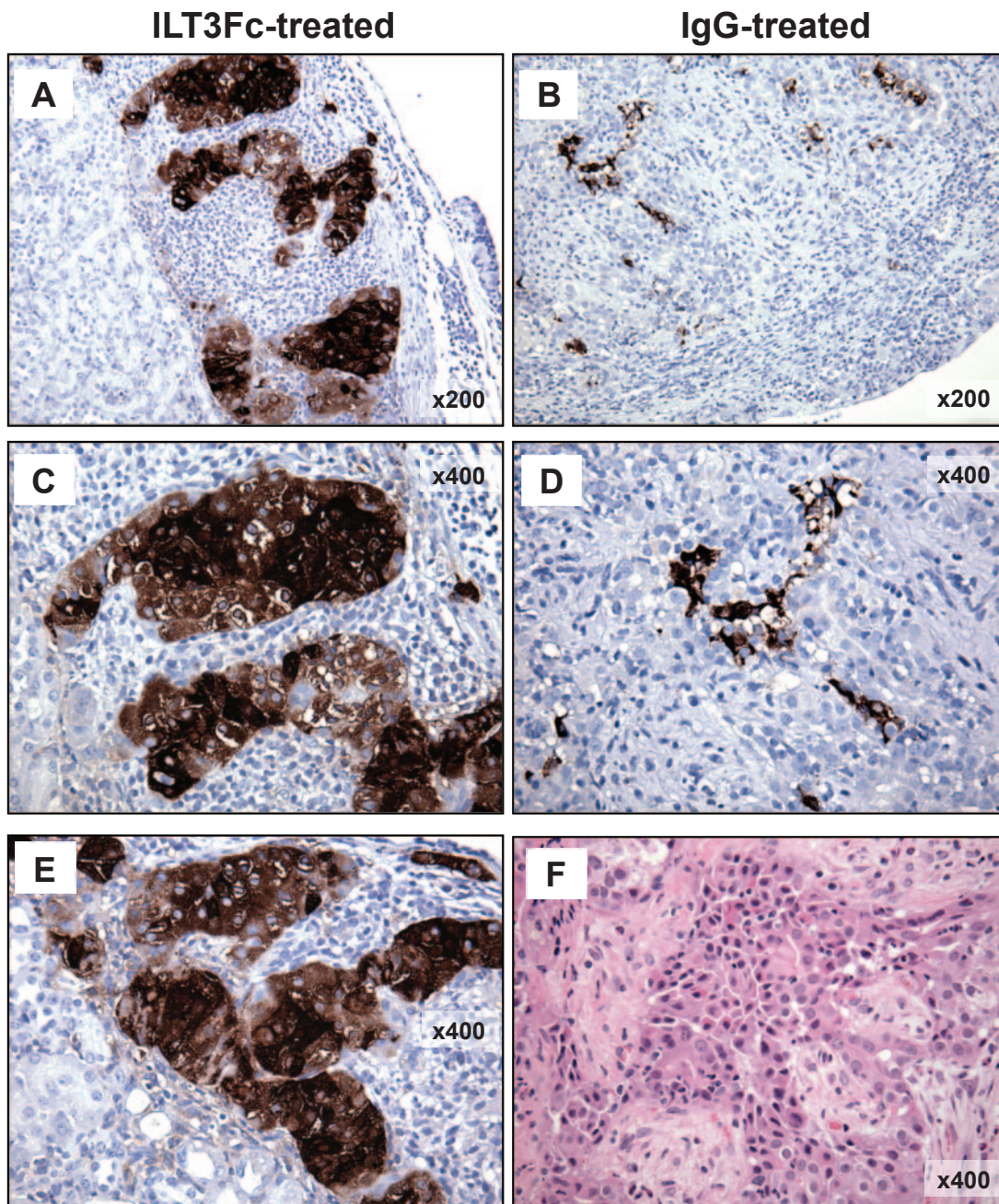


FIG. 7. Immunostaining of insulin expression in islet-engrafted kidneys from ILT3-Fc-treated (A and C) and IgG-treated (B and D) NOD/SCID mice 47 days after humanization. Insulin expression (E) and hematoxylin-eosin staining (F) in tolerated islets on day 91.

(22), this agent is not expected to cripple the immune system by depleting or blocking naïve T-cells. Further research will be necessary to fully assess the potential usefulness of ILT3-Fc for treatment of diabetes and suppression of islet allograft rejection.

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