

# Adoptive Transfer With In Vitro Expanded Human Regulatory T Cells Protects Against Porcine Islet Xenograft Rejection via Interleukin-10 in Humanized Mice

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T cell-mediated rejection remains a barrier to the clinical application of islet xenotransplantation. Regulatory T cells (Treg) regulate immune responses by suppressing effector T cells. This study aimed to determine the ability of human Treg to prevent islet xenograft rejection and the mechanism(s) involved. Neonatal porcine islet transplanted NOD-SCID IL2 $\gamma$ <sup>-/-</sup> mice received human peripheral blood mononuclear cells (PBMC) with in vitro expanded autologous Treg in the absence or presence of anti-human interleukin-10 (IL-10) monoclonal antibody. In addition, human PBMC-reconstituted recipient mice received recombinant human IL-10 (rhIL-10). Adoptive transfer with expanded autologous Treg prevented islet xenograft rejection in human PBMC-reconstituted mice by inhibiting graft infiltration of effector cells and their function. Neutralization of human IL-10 shortened xenograft survival in mice receiving human PBMC and Treg. In addition, rhIL-10 treatment led to prolonged xenograft survival in human PBMC-reconstituted mice. This study demonstrates the ability of human Treg to prevent T-cell effector function and the importance of IL-10 in this response. In vitro Treg expansion was a simple and effective strategy for generating autologous Treg and highlighted a potential adoptive Treg cell therapy to suppress anti-graft T-cell responses and reduce the requirement for immunosuppression in islet xenotransplantation. *Diabetes* 61:1180–1191, 2012

**P**ancreatic islet transplantation as a treatment for type 1 diabetes received a major impetus with the development of the Edmonton protocol and recent clinical trials demonstrating long-term insulin independence out beyond 5 years after transplantation (1–4), although encouraging this therapy will always be limited by the relatively small number of organ donors available for islet isolation. If islet transplantation is to be made widely available and the current restricted selection criteria

expanded, an alternate and renewable source of  $\beta$ -cells is required. Islet tissue from pigs has been accepted as a potential source of  $\beta$ -cells for transplantation (5,6). The impetus and feasibility of this approach received a significant boost by the demonstration that long-term pig islet xenograft survival could be achieved in primates with chronic immunosuppression (7,8). However, the degree of immunosuppression required was unacceptably high and remains a barrier to clinical application. Thus, for islet xenotransplantation to be successful, the overall burden of immunosuppression must be reduced substantially so that the benefits of improved glycemia control are not outweighed by chronic complications from immunosuppressive therapy. To achieve this, clinically applicable strategies for immunomodulation need to be developed to suppress the T cell-mediated xenimmune response (7–10). CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (Treg) that express FoxP3 transcription factor are critically important for the control of autoimmunity and maintenance of allograft tolerance (11,12). Recent studies have shown that ex vivo expanded human natural Treg can prevent the development of transplant arteriosclerosis and skin allograft rejection in a humanized mouse model (13,14). In addition, human Treg have been shown to be capable of suppressing CD4<sup>+</sup>CD25<sup>-</sup> effector T cell-mediated antipig cellular responses in vitro (15,16). This raises the possibility that Treg may be used therapeutically at the time of xenotransplantation to reduce the requirement of systemic immunosuppression (15,16). However, human natural Treg comprise only 5–10% of peripheral blood CD4<sup>+</sup> T cells (17), and large-scale ex vivo expansion would be required for any future clinical application (18). We have previously demonstrated that ex vivo expanded human natural Treg were superior to their freshly isolated counterparts at suppressing the xenogenic CD4<sup>+</sup> T cell-mediated immune response in vitro, and this suppression by ex vivo expanded human Treg was FoxP3 expression-dependent via an interleukin (IL)-10-involved mechanism (19–21).

In this study, we wished to test the hypothesis that ex vivo expanded human Treg were able to protect islet xenografts from rejection mediated by human effector T cells in NOD-SCID IL2 $\gamma$ <sup>-/-</sup> mice and that IL-10 was an important mediator in this suppression in vivo.

## RESEARCH DESIGN AND METHODS

**Animals.** Newborn pigs from local farms were used for the isolation of neonatal porcine islet cell clusters (NICC). NOD-SCID IL2 $\gamma$ <sup>-/-</sup> mice were housed under specific pathogen-free conditions in the Animal Care Department of Westmead Hospital (Westmead, New South Wales, Australia). Mice between the ages of 6 and 8 weeks at the time of NICC transplantation were used. The study was approved by the Sydney West Area Health Service Human and Animal Research Ethics Committees.

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**Porcine islet isolation and transplantation.** NICC were isolated from the pancreases of 1- to 3-day-old piglets and propagated in culture for 6 days as described previously (22). A total of 5,000 NICC were transplanted into NOD-SCID IL2 $\gamma$ <sup>-/-</sup> mice under the renal capsule of both kidneys.

**Peripheral blood mononuclear cell isolation and expansion of human Treg.** Human peripheral blood mononuclear cells (PBMC) were obtained from healthy donors using density gradient centrifugation over Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden). CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo</sup> cells were isolated from PBMC using a CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>dim/-</sup> Regulatory T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The resulting CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo</sup> cells with >98% purity were expanded as described previously (19). Fresh Treg cells were cultured in 96-well round-bottom plates (5 × 10<sup>4</sup>/well) in RPMI 1640 (GIBCO, Carlsbad, CA), supplemented with 10% human AB serum (Invitrogen, San Diego, CA), 2 mmol/L glutamine, 25 mmol/L HEPES, 50 U/mL penicillin, 50 μg/mL streptomycin, 50 μM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO), and 100 nmol/L rapamycin (Sigma-Aldrich) at 37°C and 5% CO<sub>2</sub>, in the presence of 400 U/mL IL-2 (Chiron, Emeryville, CA) and T cell expander beads (CD3/CD28 Dynabeads; Invitrogen Dynal) at a ratio of four beads per cell for the first week and at a 1:1 ratio thereafter. Cells were split with fresh RPMI 1640 every 3 days. After expansion for 2 to 3 weeks, cells were used in all subsequent experiments. PBMC used for adoptive transfer were depleted of CD25<sup>+</sup> cells (>99% depletion) using a human CD25<sup>+</sup> T cell selection kit (Miltenyi Biotec). CD25<sup>+</sup> cell-depleted human PBMC and porcine PBMC, which were isolated from adult Westran pigs (an inbred syngeneic strain with high genetic homology) (23) were used as responder cells and xenogeneic stimulator cells, respectively, for in vitro proliferation assays.

**In vitro suppression assays.** Treg in vitro suppressive activity was assessed by measuring inhibition of proliferation of autologous PBMC stimulated with xenoantigen. CD25<sup>+</sup> cell-depleted PBMC were labeled with 5 μmol/L 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen). A total of 5 × 10<sup>4</sup> CFSE-labeled PBMC were incubated with 10<sup>5</sup> irradiated (3000 rad) xenogeneic porcine PBMC and serial dilutions of in vitro expanded autologous Treg in round-bottom 96-well plates with RPMI 1640 medium (Invitrogen) containing 10% human AB serum for 5 days. After coculture, cells were stained with allophycocyanin (APC)-conjugated anti-CD25 monoclonal antibody (mAb) prior to measurement of CFSE dilution by fluorescence-activated cell sorting (FACS). Proliferation of xenoreactive PBMC (CD25<sup>-</sup> and CFSE<sup>+</sup> cells) was evaluated based on the percent proliferating xenoreactive PBMC cultured in the absence of Treg compared with the percent proliferating xenoreactive PBMC cultured in the presence of Treg. The percent proliferating xenoreactive PBMC in the absence of Treg was taken as 100% of proliferation and 0% of suppression.

**Adoptive transfer of human cells.** Human PBMC were obtained from healthy volunteers. A total of 2 × 10<sup>6</sup> ex vivo expanded human Treg were injected intravenously into NOD-SCID IL2 $\gamma$ <sup>-/-</sup> mice 3 days after NICC transplantation. Seven days after Treg transfer, recipient mice were treated intravenously with 10<sup>7</sup> CD25<sup>+</sup> cell-depleted autologous PBMC. Peripheral blood, serum, spleen, and NICC grafts were collected from recipient mice at predetermined time points after human PBMC rechallenge to analyze human leukocyte engraftment and NICC graft survival. Graft rejection was defined as no visible intact graft observed by histological examination (24).

**IL-10 and anti-IL-10 Ab treatment.** To determine the role of IL-10 in human Treg-mediated suppression, Treg-transferred recipient mice received 100 μg i.p. rat anti-human IL-10 mAb or rat IgG isotype Ab (BioLegend, San Diego, CA) at days 0, 3, 7, and 10 after human PBMC rechallenge. In separate experiments, recipient mice were treated with 1 μg i.p. recombinant human IL-10 (rhIL-10) (R&D Systems, Minneapolis, MN) per animal on the day of adoptive transfer. Human leukocyte engraftment and NICC graft survival were analyzed as described above.

**Flow cytometry.** Flow cytometric analysis of human antigens in human Treg and leukocytes was undertaken as described previously (21). Fluorochrome-coupled antibodies specific for human antigens CD45, CD4, CD8, CD127, CD62L, cytotoxic T-cell antigen-4 (CTLA-4) (BD Biosciences, San Jose, CA), CD25, glucocorticoid-induced tumor necrosis factor receptor-related protein (GITR), and FoxP3 (eBioscience, San Diego, CA) were used for flow cytometric analysis of human Treg phenotype, human leukocyte engraftment, and graft-infiltrating human leukocytes, respectively. Flow cytometric data were acquired using an LSRII flow cytometer (BD Biosciences) and analyzed using FACSDiva software (BD Biosciences).

**Real-time PCR.** Real-time PCR was performed as described previously (21). PCR primers specific for human GAPDH, FoxP3, IL-2, IL-4, IL-10, IL-17, interferon- $\gamma$  (IFN- $\gamma$ ), transforming growth factor- $\beta$  (TGF- $\beta$ ), GITR, CTLA-4, CD62L, and granzyme A were summarized in Supplementary Table 1. Expression of genes examined was normalized to GAPDH expression in each sample. All experiments were run in triplicate.

**Histology and immunohistochemistry.** Histology and immunohistochemistry of cryostat sections (6–8 μm) were undertaken as described previously (25). Porcine endocrine cells were detected using antiporcine insulin (Dako

Laboratories, Mississauga, Ontario, Canada), guinea pig antiporcine glucagon (Linco Research, St. Charles, MO), goat anti-human somatostatin (Santa Cruz Biotechnology, Santa Cruz, CA), and rabbit anti-human chromogranin (Dako Laboratories) antibodies and the Universal ABC Kit (Vector Laboratories, Burlingame, CA). Graft-infiltrating human leukocytes were stained using mouse anti-human CD4, CD8, CD45 (eBioscience), CD19, and CD68 (Dako Laboratories) antibodies, followed by incubation with horseradish peroxidase-conjugated secondary rabbit anti-mouse Ab (Invitrogen). Sections were visualized with diaminobenzidine (Dako Laboratories). Double immunofluorescence staining of human CD4 and FoxP3 was undertaken with rabbit anti-human CD4 polyclonal Ab (Abcam) and mouse anti-human FoxP3 mAb (Dako Laboratories), followed by secondary incubation with Alexa 562-conjugated goat anti-rabbit and Alexa 488-conjugated goat anti-mouse Abs (Invitrogen). The sections were then stained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich). The sections were viewed under an Olympus FV1000 (Olympus).

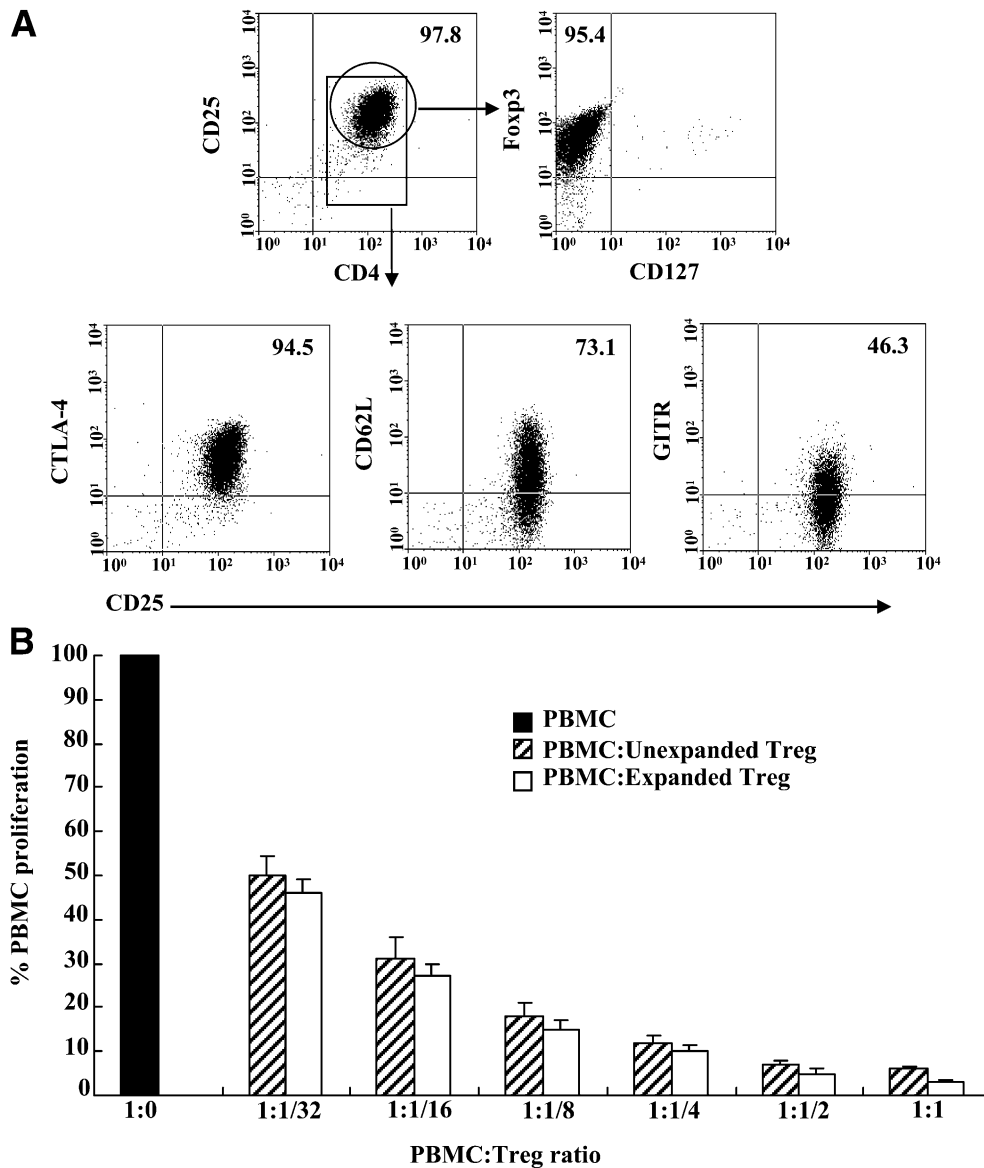
**Cytokine bead array.** Cytokine bead array was performed on serum samples harvested from NICC recipient mice using the human Th1/Th2/Th17 cytokine bead array system (BD Biosciences) following the manufacturer's protocols. A total of 50 μL of sera was incubated at room temperature for 3 h with 50 μL of mixed anti-cytokine mAb-coated and APC-conjugated capture beads of each cytokine and 50 μL of the phycoerythrin-conjugated anti-cytokine detector antibodies. Data acquisition was performed on a FACSCanto flow cytometer (BD Biosciences) and then was analyzed using the BD Cytometric Bead Array software (BD Biosciences). The cytokines detected were IL-2, IL-4, IL-6, IL-10, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), IFN- $\gamma$ , and IL-17. Limits of detection of each cytokine varied between 2.4 and 4.5 pg/mL except for IL-17, which was 18.9 pg/mL.

**Statistical analysis.** Comparisons involving two groups were evaluated using the Student *t* test, and those involving multiple groups were evaluated using ANOVA with the Tukey multiple comparison test (JMP, version 4.02 software; SAC) and presented as mean ± SD. *P* < 0.05 was considered as statistically significant.

## RESULTS

**Ex vivo expanded human Treg retain regulatory characteristics in vitro.** To investigate the potential of human Treg to suppress xenoinnate responses in vivo, CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo</sup> Treg were isolated and underwent in vitro expansion. After 3 weeks in culture, CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo</sup> Treg were expanded up to 1,100-fold (range, 560–1,100-fold expansion) in cell number while retaining their Treg phenotype, with high-level expression of CD25, FoxP3, CTLA-4, GITR, and CD62L and a very low or undetectable expression of CD127 (Fig. 1A). In vitro expanded human Treg demonstrated potent suppression of xenostimulated autologous PBMC. In the presence of Treg, proliferation of xenoreactive PBMC was inhibited significantly in a dose-dependent manner (Fig. 1B), and the suppression of proliferating PBMC by expanded Treg appeared more potent, though not significantly so, than that shown by unexpanded Treg (Fig. 1B). These data showed that ex vivo expanded human CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo</sup> Treg retained both functional and phenotypic characteristics of natural Treg.

**Adoptive transfer of human PBMC results in porcine islet xenograft rejection.** In order to study the mechanisms of human T cell-mediated islet xenograft rejection in vivo, NOD-SCID IL2 $\gamma$ <sup>-/-</sup> mice (26) were transplanted with NICC xenografts and, 3 days after transplantation, reconstituted with 10<sup>7</sup> CD25<sup>+</sup> cell-depleted human PBMC (Fig. 2A). Human PBMC engraftment was confirmed by flow cytometry, with 22.1 ± 4.4% and 38.5 ± 5.5% of cells in the peripheral blood and spleen, respectively, being human CD45<sup>+</sup> by day 28 after adoptive transfer. The majority (>97%) of these cells were CD4<sup>+</sup> and CD8<sup>+</sup> cells (Fig. 2B). Whereas NICC grafts survived for at least 100 days in nonreconstituted recipients, mice reconstituted with human PBMC rejected their xenografts completely within 28 days (Fig. 2C). In human PBMC-reconstituted mice, immunohistochemical analysis revealed no visible insulin positive-staining cells in the rejecting xenografts compared

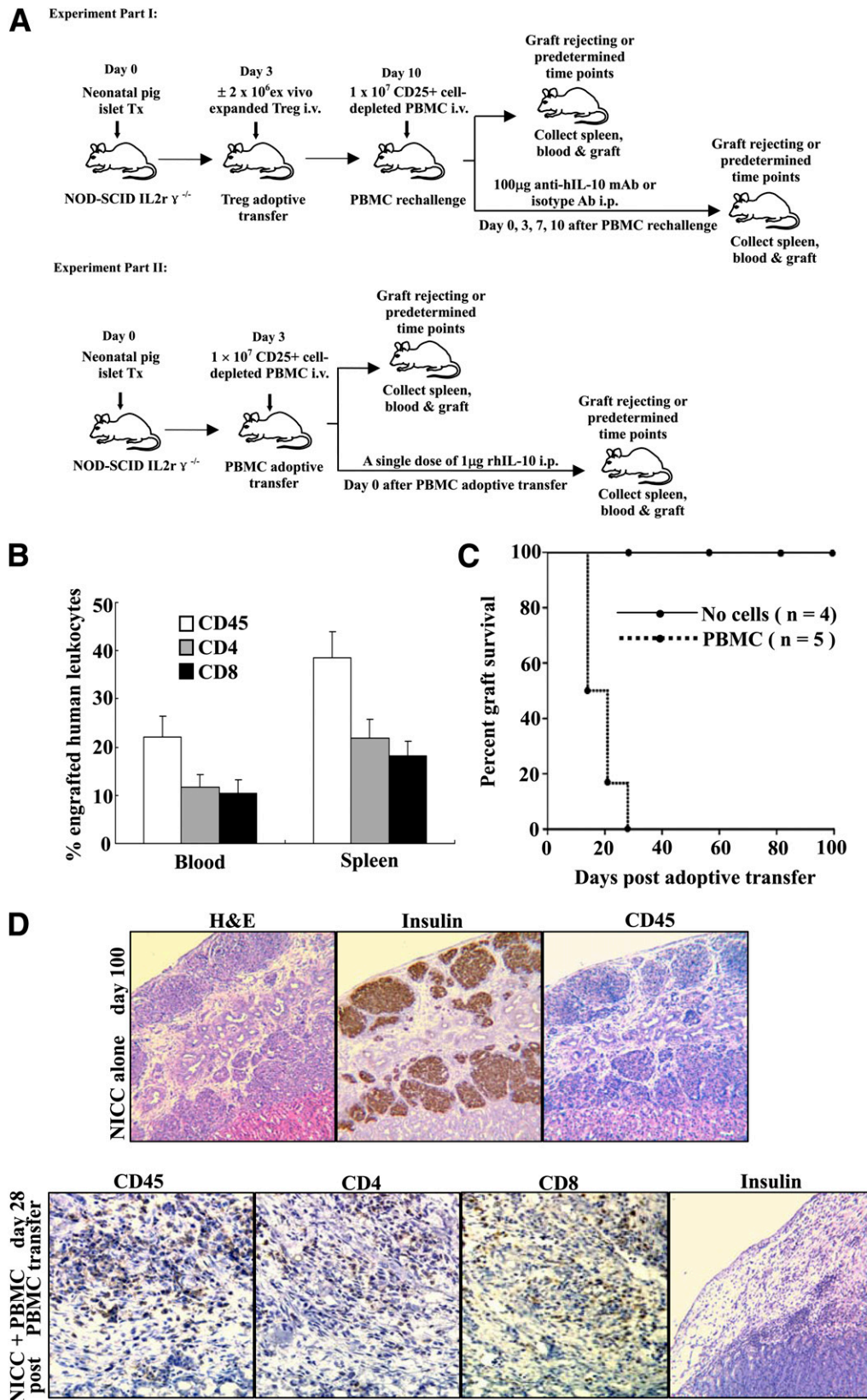


**FIG. 1.** Ex vivo expanded human CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo</sup> cells retain Treg-suppressive characteristics. **A:** Representative FACS analysis of Treg phenotype. Gates were set on CD4<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup> cells, respectively. Foxp3 and other marker expression was shown as the percentage of CD4<sup>+</sup>CD25<sup>+</sup> cells coexpressing individual Treg markers examined. Data represent one of four independent experiments with Treg from four individual donors. **B:** In vitro suppression assay of unexpanded and expanded human Treg. CFSE-labeled CD25<sup>+</sup> cell-depleted human PBMC were stimulated with irradiated xenogeneic pig PBMC in the presence or absence of serial dilutions of unexpanded and expanded autologous Treg for 5 days prior to measurement of xenoreactive PBMC proliferation by CFSE dilution. Data are presented as mean  $\pm$  SD of three independent experiments.

with intact and insulin positive-staining NICC grafts detected in nonreconstituted recipients (Fig. 2D). A large number of graft-infiltrating human CD45<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> cells was detected in human PBMC-reconstituted recipients at the graft-rejecting time point (day 28 after reconstitution) (Fig. 2D). However, no human CD19 and CD68 positive-staining cells were found in the rejecting NICC grafts (data not shown), indicating a predominant T cell-mediated graft rejection. There were very few, if any, mouse CD45<sup>+</sup> cells, confirming that rejection was due to the human T cells (data not shown).

**Ex vivo expanded human Treg prevent rejection of porcine islet xenografts.** Next, we tested the in vivo suppressive activity of ex vivo expanded CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo</sup> human Treg. NOD-SCID IL2 $\gamma$ <sup>-/-</sup> mice were transplanted with NICC xenografts and 3 days later injected with  $2 \times 10^6$

ex vivo expanded human Treg. Seven days after Treg injection, NICC recipients were rechallenged with  $10^7$  CD25<sup>+</sup> cell-depleted autologous PBMC. Human Treg were reconstituted prior to human PBMC transfer in order to achieve enhanced Treg suppression through in vivo antigen-specific expansion during this 7-day period as seen in an allograft setting (27). This hypothesis was supported by preliminary experiments, showing that NICC grafts survived <70 days with 20% incidence of graft-versus-host disease in recipients cotransferred with human PBMC and Treg (data not shown). In all mice reconstituted with human Treg (ratio of 1:5 of Treg/PBMC), pig NICC xenografts survived beyond 100 days after PBMC rechallenge without graft-versus-host disease (Fig. 3A). Human T cell engraftment was confirmed by flow cytometry 100 days after transfer with  $10.2 \pm 2.1\%$  and  $14.7 \pm 3.3\%$  of peripheral blood and



**FIG. 2.** Adoptive transfer of human CD25<sup>+</sup> cell-depleted PBMC leads to porcine islet xenograft rejection in vivo. **A:** A schematic representation of the in vivo-humanized mouse model. **B:** Flow cytometric analysis of percent human leukocytes in the peripheral blood and spleen of mice at day 28 (time of graft rejection) post-human PBMC transfer. Data are shown as mean  $\pm$  SD of three independent experiments ( $n = 4$  mice in each experiment). **C:** Percent graft survival of NICC-recipient mice receiving  $1 \times 10^7$  CD25<sup>+</sup> cell-depleted human or no cells. Graft survival in human PBMC-transferred mice was monitored by histological examination at days 14, 21, and 28 after PBMC transfer, and data for each of time points are pooled from five mice. **D:** Representative immunohistochemical examination of graft samples from mice receiving no cells (NICC alone day 100 posttransplantation) or human PBMC (NICC + PBMC day 28 post-PBMC transfer). Original magnification  $\times 100$  of stains of graft samples from mice receiving no cells for hematoxylin and eosin (H&E), human CD45, and porcine insulin and of graft samples from mice receiving human PBMCs

spleen, respectively, being human CD45<sup>+</sup>, confirming that graft survival was the result of Treg-mediated suppression and not failure of engraftment (Fig. 3A and B).

Immunohistochemistry showed that the long-term surviving grafts contained intact endocrine-secreting cells, with positive staining for insulin, glucagon, somatostatin, and chromogranin (Fig. 3C), and were surrounded but not infiltrated by a small number of CD45<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> human leukocytes (Fig. 3C). The number of intra-graft human effector CD4<sup>+</sup>CD25<sup>-</sup> T cells but not CD8<sup>+</sup> T cells was markedly reduced in human Treg transferred and PBMC rechallenged mice when compared with those receiving human PBMC alone (Fig. 3D), indicating a potent suppression of a predominantly CD4<sup>+</sup> T cell-mediated cellular xenograft rejection (24,28,29). To determine the effect of human Treg on effector T cell function, IFN- $\gamma$  levels were measured by cytokine bead array and real-time PCR. In mice reconstituted with Treg, the serum concentration of IFN- $\gamma$  was reduced when compared with that seen in mice with rejecting NICC grafts that were reconstituted with PBMC and no Treg (Fig. 4A and B). Consistent with this, intra-graft IFN- $\gamma$  gene expression was inhibited significantly in Treg-treated mice (Fig. 4C). Furthermore, the suppressed levels of IFN- $\gamma$  gene expression correlated with the presence of FoxP3-expressing CD4<sup>+</sup> cells and increased expression of FoxP3, CTLA-4, CD62L, GITR, TGF- $\beta$ , and granzyme A within the xenografts (Fig. 5A and B).

**IL-10 is required for human Treg to inhibit the human antipig islet response in vivo.** IL-10 production was measured by cytokine bead array and real-time PCR. In NICC-recipient mice reconstituted with Treg, there was a marked upregulation of intra-graft IL-10 mRNA (Fig. 5C) and elevated serum levels of human IL-10 when compared with mice reconstituted with PBMC alone (Fig. 5D and E), suggesting the involvement of IL-10 in suppression of xenograft rejection in vivo by human Treg. However, IL-2, IL-4, IL-6, and IL-17 were not detected in the serum, and elevated IL-4, IL-6, and IL-17 gene expression was not detected within the graft by real-time PCR regardless of reconstitution with human PBMC and/or Treg. Furthermore, human IL-2 mRNA was reduced in recipients receiving both human Treg and PBMC compared with those reconstituted with human PBMC alone (Fig. 4D and E). Taken together, these data suggest that the adoptive transfer of Treg prevented xenograft rejection by suppressing the effector response of CD4<sup>+</sup>CD25<sup>-</sup> T cells via an IL-10-involved mechanism(s).

Next, the importance of IL-10 for human Treg activity in our humanized mouse model was investigated. Seven days after adoptive transfer with  $2 \times 10^6$  ex vivo expanded human CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo</sup> Treg, NICC-recipient NOD-SCID IL2 $\gamma$ <sup>-/-</sup> mice were rechallenged with  $10^7$  CD25<sup>+</sup> cell-depleted PBMC and treated with anti-human IL-10 mAb or IgG isotype control Ab. Even in the presence of Treg, mice treated with anti-IL-10 mAb rejected their xenografts completely 50 days after human PBMC rechallenge (Fig. 6A). The rejecting grafts were heavily infiltrated with human CD45<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> cells with no insulin staining, similar to that seen in CD25<sup>+</sup> T cell-depleted PBMC-reconstituted mice but at a later time point. (Fig. 6B). As expected, human CD45<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> cells were detectable in isotype Ab-treated mice, although they did not cause rejection

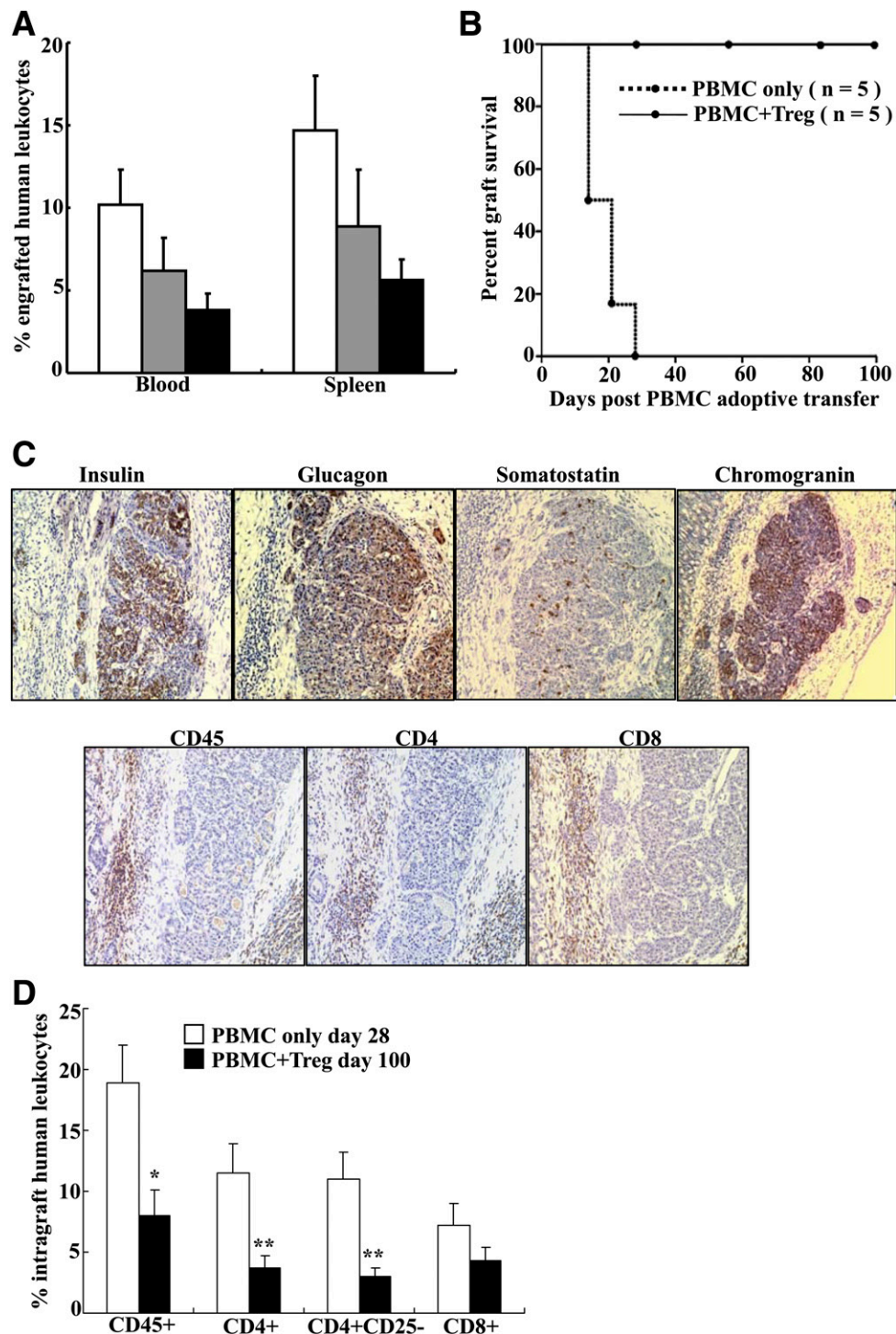
(Fig. 6B). Furthermore, the proportion of graft-infiltrating human CD45<sup>+</sup>, CD4<sup>+</sup>, and CD4<sup>+</sup>CD25<sup>-</sup> but not CD8<sup>+</sup> cells was significantly greater in mice treated with anti-IL-10 mAb when compared with that seen in isotype Ab-treated mice (Fig. 6C). Again, both intra-graft IFN- $\gamma$  mRNA expression (Fig. 6D) and IFN- $\gamma$  production (Fig. 6E and F) were increased substantially in mice receiving anti-IL-10 mAb, whereas intra-graft mRNA expression of FoxP3, CTLA-4, CD62L, GITR, TGF- $\beta$ , and granzyme A was reduced (Fig. 7A). In addition, the levels of both intra-graft IL-10 gene expression and IL-10 secretion in the serum of mice treated with anti-IL-10 mAb were reduced (Fig. 7B–D), confirming the impact of IL-10 neutralization on expression of Treg molecules. To further confirm the importance of IL-10 in the human Treg-mediated prevention of islet xenograft rejection, a single dose of 1  $\mu$ g rhIL-10 per mouse was given to NICC-recipient NOD-SCID IL2 $\gamma$ <sup>-/-</sup> mice that received  $10^7$  CD25<sup>+</sup> cell-depleted human PBMC alone. In the presence of human PBMC engraftment (Fig. 8A), rhIL-10 treatment prolonged NICC xenograft survival from 28 to at least 56 days after human PBMC adoptive transfer (Fig. 8B). The surviving grafts in these mice were surrounded but not infiltrated by human CD45<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> cells (Fig. 8B). The prolonged graft survival correlated with upregulated levels of IL-10 along with reduced IFN- $\gamma$  in their serum when compared with that seen in mice transferred with human PBMC alone (Fig. 8C). Real-time PCR analysis showed reduced IFN- $\gamma$  and upregulated IL-10 gene expression in the NICC grafts from mice receiving human PBMC and rhIL-10 compared with that detected in the rejecting grafts from recipients receiving human PBMC alone (Fig. 8D). Collectively, these data demonstrate that IL-10 was required for human CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo</sup> Treg to suppress xenogeneic responses in vivo.

## DISCUSSION

These data demonstrate that ex vivo expanded human Treg have the capacity to inhibit the T cell-mediated rejection of porcine islet xenografts in vivo. Human PBMC-reconstituted mice demonstrated a predominant T cell engraftment with very few CD19<sup>+</sup> or CD68<sup>+</sup> cells. As a result, the rejection was predominantly T cell-mediated, as has been demonstrated in other islet xenotransplantation models (7–10). Reconstitution of mice with naive Treg prior to PBMC reconstitution was able to suppress this response. After adoptive transfer of Treg, recipient mice had high serum levels of human IL-10 and high levels of IL-10 gene expression within the accepted graft. The suppressive capacity of Treg could be blocked by administering anti-IL-10 mAb at the time of adoptive transfer, suggesting that Treg suppression of xenograft rejection is dependent upon an IL-10-mediated mechanism. Supporting this was the observation that rhIL-10 could in part substitute for the transfer of Treg. Animals receiving rhIL-10 at the time of transplant showed prolonged graft survival, reduced production of the proinflammatory cytokine IFN- $\gamma$ , and increased human IL-10 gene expression within the graft of treated animals. These findings are consistent with in vitro data demonstrating the importance of IL-10 in Treg-mediated suppression of xenogeneic proliferative responses (21). Inhibiting IL-10 production by Treg prevented

for porcine insulin, respectively (*top*). Original magnification  $\times 200$  of stains of graft samples from mice receiving human PBMC for human CD45, CD4, and CD8, respectively (*bottom*). i.p., intraperitoneal injection; i.v., intravenous injection; Tx, transplantation. (A high-quality digital representation of this figure is available in the online issue.)

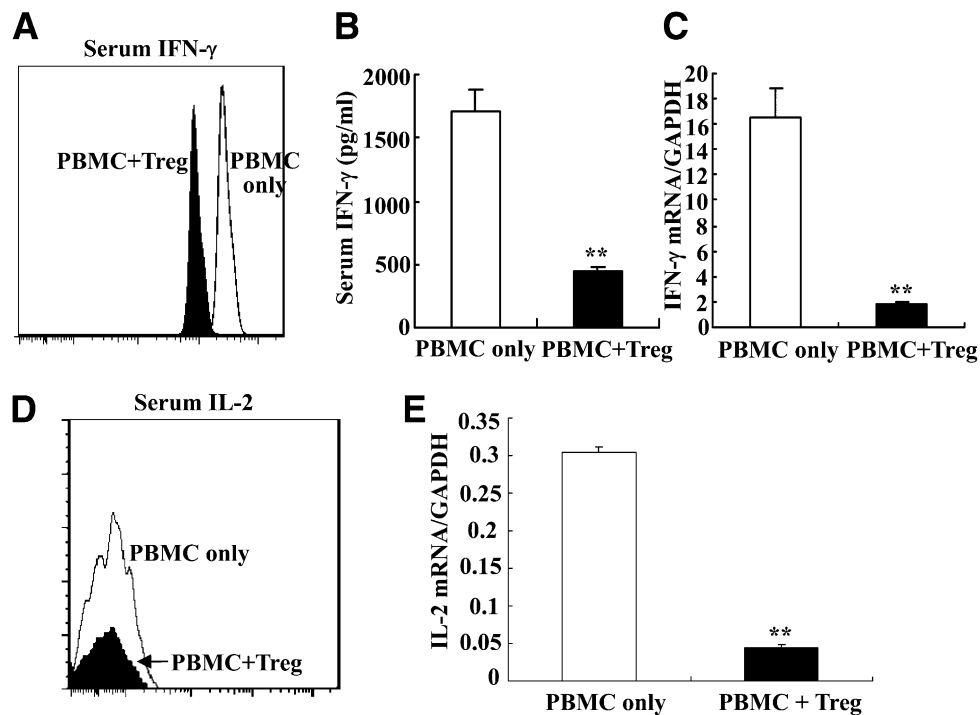




**FIG. 3.** Human Treg suppress the rejection of islet xenografts in humanized mice. **A:** Flow cytometric measurement of percent human leukocytes in the peripheral blood and spleen of mice receiving human Treg and PBMC 100 days after PBMC rechallenge. Data are shown as mean  $\pm$  SD of three independent experiments ( $n = 4$  mice in each experiment). **B:** Percent graft survival in mice receiving  $1 \times 10^7$  CD25<sup>+</sup> cell-depleted human PBMC (PBMC only) alone or with  $2 \times 10^6$  autologous Treg (PBMC+Treg). Graft survival was monitored by histological examination at days 14, 21, and 28 or days 28, 56, 84, and 100, after human PBMC transfer for mice receiving PBMC alone or both PBMC and Treg, respectively ( $n = 5$  mice for each time point). **C:** Representative immunohistochemical staining of graft samples from the same mice as in **B** for human CD45, CD4, and CD8 and porcine insulin, glucagon, somatostatin, and chromogranin. Original magnification  $\times 200$ . **D:** Flow cytometric analysis of percent intragraft human leukocytes in mice receiving PBMC alone or with autologous Treg at day 28 (PBMC only day 28) or 100 (PBMC+Treg day 100), respectively, after PBMC transfer. Data are represented as mean  $\pm$  SD of nine graft samples from three independent experiments. \* $P < 0.05$ ; \*\* $P < 0.01$  compared with PBMC only. (A high-quality digital representation of this figure is available in the online issue.)

their ability to suppress CD4<sup>+</sup>CD25<sup>-</sup> effector T cells in a xenogeneic mixed lymphocyte reaction, whereas the addition of rhIL-10 suppressed xenogeneic proliferative responses (21). Treg have been widely investigated for their

suppressive capabilities in vitro and in vivo (30–32) and for their therapeutic potential in the regulation of autoimmunity, allergy, and immune-mediated transplant rejection [reviewed previously (33)]. Recently, ex vivo expanded



**FIG. 4.** Long-term graft survival correlates with impaired effector function. *A*: Representative cytokine bead array for assessment of human IFN- $\gamma$  in serum samples from NICC-recipient mice receiving human PBMC alone (PBMC only) ( $n = 3$ ) at day 28 or PBMC and Treg (PBMC+Treg) ( $n = 3$ ) at day 100 after PBMC transfer, respectively, is shown. Open histogram represents PBMC only, and filled histogram represents PBMC+Treg. *B*: Serum IFN- $\gamma$  level as assessed in *A* is expressed as mean  $\pm$  SD of three independent mouse samples. *C*: Measurement of intra-graft mRNA expression of IFN- $\gamma$  by real-time PCR in NICC grafts from the same recipient mice as in *A*. Data are shown as mean  $\pm$  SD of three independent graft samples. *D*: Representative cytokine bead array assay for human IL-2 in serum samples from the same NICC recipients as in *A*. Open histogram represents PBMC only, and filled histogram represents PBMC + Treg. *E*: Real-time PCR measurement of intra-graft mRNA expression of human IL-2 within NICC grafts from the same recipients as in *A*. Data are shown as mean  $\pm$  SD of three independent graft samples. \*\* $P < 0.01$  compared with PBMC only.

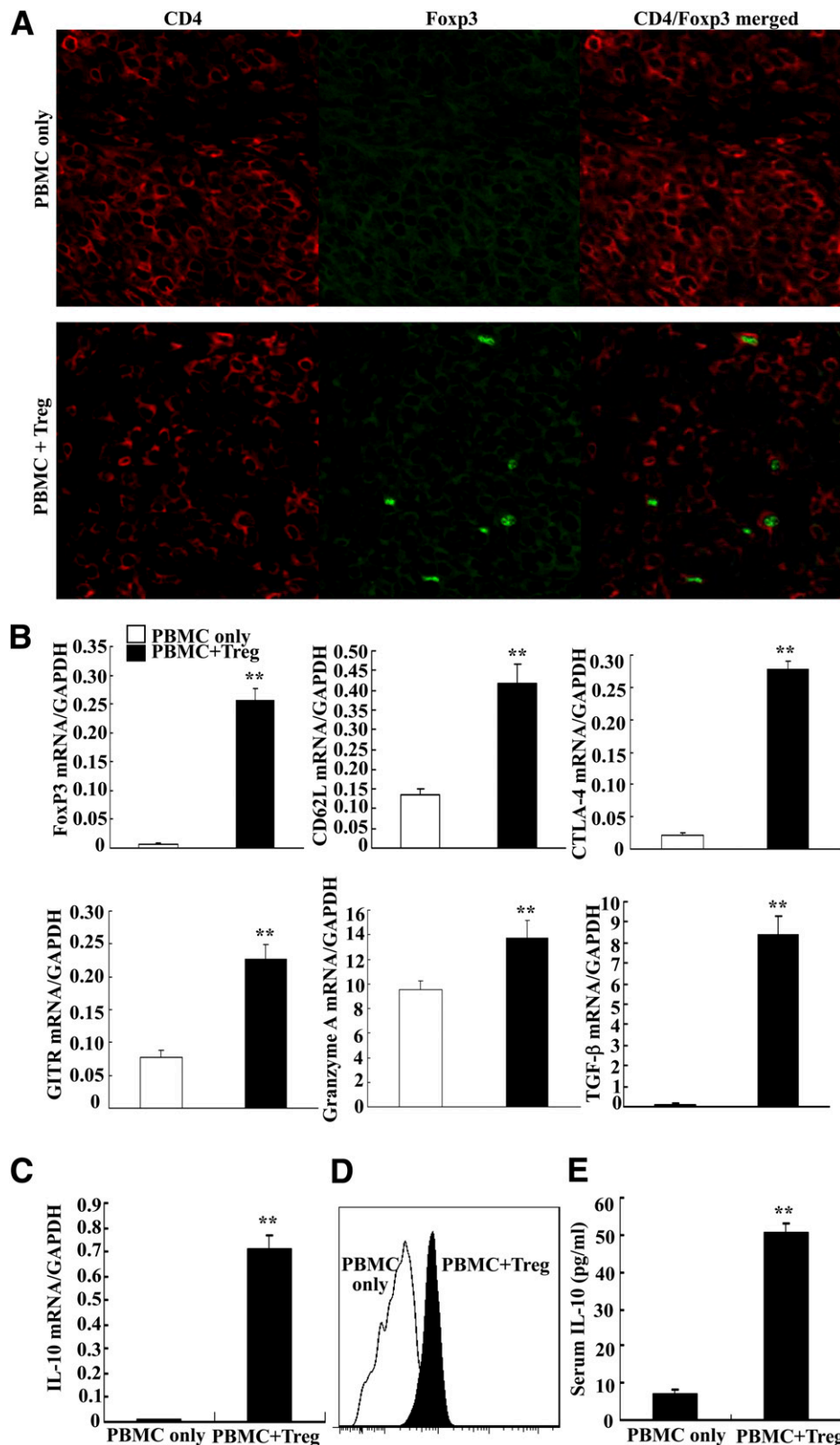
human CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup>CD127<sup>lo</sup> Treg have been shown to inhibit transplant arteriosclerosis and prevent skin allograft rejection in humanized mice (13,14). These studies emphasized the importance of selecting CD127<sup>lo</sup> and CD62L<sup>high</sup> Treg cells in order to optimize their suppressive ability (13). Consistent with these reports, we further demonstrated in this study that, after adoptive transfer, ex vivo-expanded human CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup>CD127<sup>lo</sup> Treg can home to and prevent porcine islet xenograft rejection by impairing effector function and graft T-cell infiltration. Furthermore, IL-10 was identified as an important mediator of this suppressive response.

Although the precise molecular mechanisms of suppression by human Treg remain to be determined, several mechanisms have been proposed. These include cell contact-dependent mechanisms such as CD80–CD86 interactions with CTLA-4 on the Treg and effector-cell lysis and apoptosis via a granzyme- or perforin-dependent pathway. In addition, there are cytokine-mediated suppression mechanisms involving production of immune suppressive cytokines TGF- $\beta$ , IL-10, and IL-35 by Treg [reviewed previously (33)]. Although cell–cell contact pathways are essential for Treg function in vitro, in vivo studies support a role for secreted cytokines such as TGF- $\beta$  and IL-10 (34,35). For example, IL-10–deficient Treg were unable to suppress inflammatory bowel disease in a mouse model (34). Blockade of IL-10R and neutralization of TGF- $\beta$  abolished Treg-mediated inhibition of inflammatory bowel disease (36). In contrast, IL-10–deficient Treg suppressed autoimmune gastritis produced by Treg depletion (37). Collectively, this suggests that IL-10–involved mechanisms may contribute differently to Treg-mediated suppression in different clinical

settings. IL-10–producing Treg have been identified in humans, particularly human cancers and children with severe combined immune deficiency (38–42).

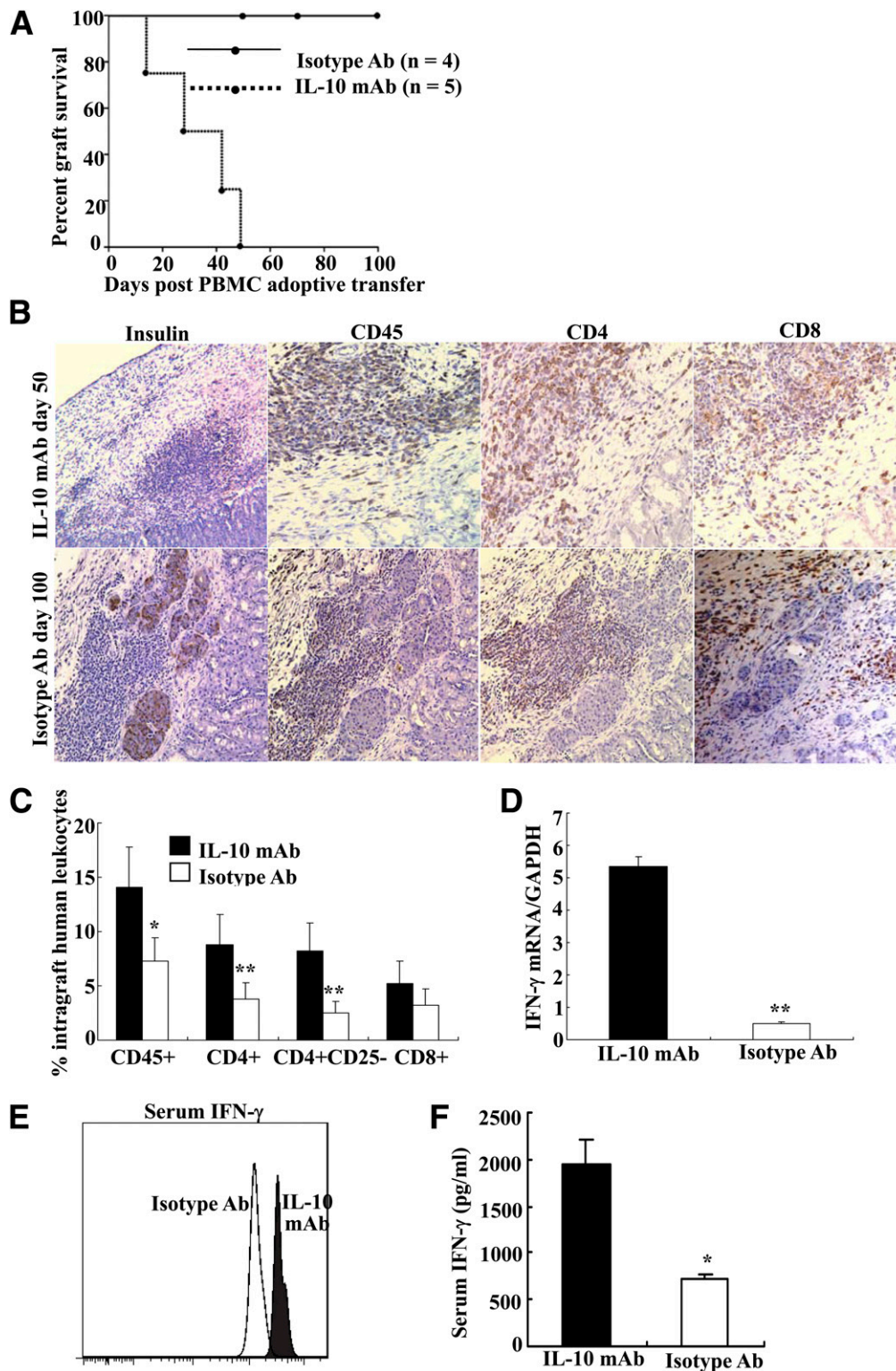
IL-10 is a well-characterized anti-inflammatory cytokine and plays a central role in controlling inflammation, suppressing T-cell responses, and maintaining immunological tolerance after transplantation (43). In addition, IL-10 is involved in the induction of adaptive T regulatory type 1 (Tr1) cells from naive T cells while in the presence of antigen (43–45). Tr1 cells can be induced in vivo by exogenous IL-10 (45), which may explain the observation in this study that administration of rhIL-10 led to increased IL-10 gene expression within the graft and increased serum concentrations of IL-10 in islet transplant recipients given rhIL-10 without Treg at the time of transplant. Tr1 cells express several cell-surface markers shared with natural Treg, including GITR and CTLA-4, but do not constitutively express FoxP3 (45–48). They produce large quantities of IL-10 and TGF- $\beta$  and are capable of regulating adaptive immune responses both in mice and humans [reviewed previously (44)]. Hence, it is possible that the suppression of xenogeneic responses by human Treg in the current study may be due to natural Treg-inducing Tr1 cells from CD4<sup>+</sup>CD25<sup>–</sup> T cells via secretion of IL-10. In fact, preliminary FACS phenotyping of T cells from the spleens of IL-10–treated mice show that there were more CD4<sup>+</sup>CD25<sup>–</sup> T cells secreting IL-10 and expressing FoxP3 without an increase in FoxP3<sup>+</sup> Treg, which may be consistent with the generation of Tr1-like T cells (data not shown).

Although this study demonstrates the potential of Treg to suppress T cell-mediated effector function, its findings should be interpreted with caution. Although mice treated

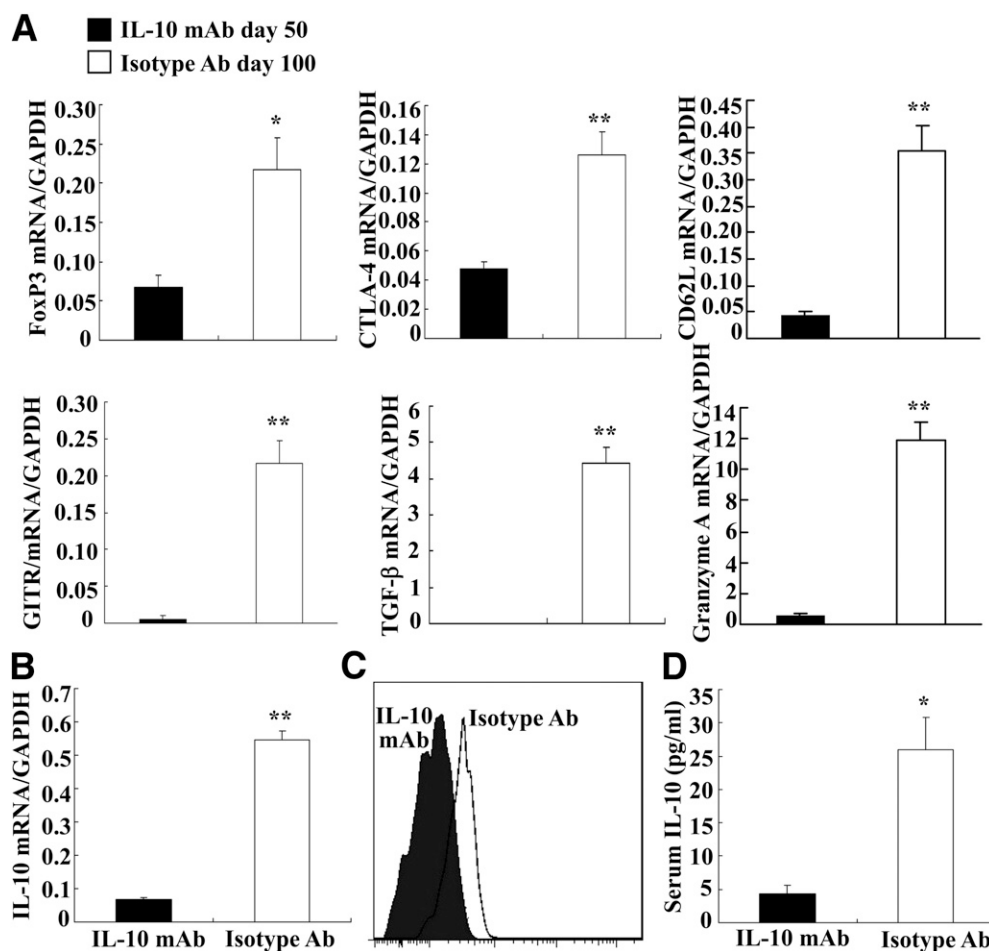


**FIG. 5.** Suppressed graft rejection is associated with intragraft expression of human Treg markers and elevated serum human IL-10. **A:** Representative double immunofluorescence staining for CD4 and FoxP3 of graft samples from NICC-recipient mice receiving human PBMC alone (PBMC only) or with autologous Treg (PBMC + Treg) at days 28 (time of graft rejection) or 100, respectively, after PBMC transfer. Cell-surface CD4-positive staining is in red and intracellular FoxP3-positive staining in green. Original magnification  $\times 200$ . **B:** Real-time PCR analysis of intragraft mRNA expression of Treg markers in the same mice as in **A**. Data are presented as mean  $\pm$  SD of three independent mouse samples. **C:** Real-time PCR analysis of intragraft IL-10 mRNA in the same mice as in **A**. **D:** Representative cytokine bead array assay of human IL-10 levels in serum samples from four independent experiments by using the same mice as in **A** is shown. Open histogram represents PBMC only, and filled histogram represents PBMC+Treg. **E:** Serum IL-10 level as measured in **D** is expressed as mean  $\pm$  SD of four independent mouse samples. \*\* $P < 0.01$  compared with PBMC only. (A high-quality digital representation of this figure is available in the online issue.)





**FIG. 6.** Blockade of human IL-10 impairs the Treg-mediated suppression of xenogeneic effector cells in vivo. **A:** Percent graft survival in recipient mice receiving human PBMC and Treg treated with anti-IL-10 mAb (IL-10 mAb) or isotype control Ab (Isotype Ab). Graft survival was monitored by histological examination at days 14, 21, 42 or 50, 70, and 100, respectively, after human PBMC transfer in mice treated with anti-IL-10 mAb or isotype Ab, respectively ( $n =$  mice for each time point). **B:** Representative immunohistochemical staining of graft samples from anti-IL-10 mAb or isotype Ab-treated mice at day 50 (IL-10 mAb day 50) or 100 (Isotype Ab day 100), respectively, post-PBMC transfer for human CD45, CD4, and CD8 and porcine insulin. Original magnification  $\times 200$ . **C:** Flow cytometric analysis of percent intragraft human leukocytes in the same mice as in **B**. Data are mean  $\pm$  SD of nine graft samples from three independent experiments. **D:** Real-time PCR measurement of intragraft human IFN- $\gamma$  mRNA in the same mice as in **B**. Data are mean  $\pm$  SD of nine graft samples from three independent experiments. **E:** Representative cytokine bead array assay of serum IFN- $\gamma$  from one of three independent experiments is shown. Open histogram represents isotype control Ab treated, and filled histogram represents anti-human IL-10 mAb treated. **F:** Serum IFN- $\gamma$  level as assessed in **E** is presented as mean  $\pm$  SD of three independent samples. \* $P < 0.05$ ; \*\* $P < 0.01$  compared with anti-IL-10 mAb treatment. (A high-quality digital representation of this figure is available in the online issue.)



**FIG. 7.** Human IL-10 neutralization results in inhibited expression of intragraft Treg function genes and production of suppressive cytokine IL-10. **A:** Measurement of intragraft mRNA expression of Treg marker and function associated molecules by real-time PCR in mice receiving both human PBMC and Treg and treated with anti-IL-10 mAb (IL-10 mAb day 50) or isotype Ab (Isotype Ab day 100) at days 50 or 100 post-PBMC reconstitution, respectively. Data are presented as mean  $\pm$  SD of three individual graft samples from one of three independent experiments. **B:** Intragraft expression of human IL-10 mRNA measured by real-time PCR in the same mice as in **A**. Data are represented as mean  $\pm$  SD of three individual graft samples from one of three independent experiments. **C:** Representative cytokine bead array of measurement of serum IL-10 in the same mice as in **A** is shown. Open histogram represents isotype control Ab treated, and filled histogram represents anti-human IL-10 mAb treated. **D:** IL-10 level in serum samples as assessed in **C** is shown as mean  $\pm$  SD of three independent mouse samples. \* $P < 0.05$ ; \*\* $P < 0.01$ , compared with anti-IL-10 mAb-treated mice.

with Treg maintained long-term graft survival, the islet grafts were surrounded by T cells. Their effector function over time is unknown, and it may result in graft damage. Hence, there may be a requirement for repeated infusions, which would be possible, as the expanded Treg are autologous. In addition, NOD-SCID IL2 $\gamma^{-/-}$  mice are not reconstituted with a full human immune system, lacking human CD19 and CD68 in the rejecting graft. Given the importance of macrophages in experimental islet xenograft rejection, the relative impact of innate immunity and T cell-mediated B-cell responses is unknown (22,49). Despite these caveats, the study supports the clinical feasibility of administering expanded autologous Treg. Furthermore, naive Treg can be expanded 1,000-fold in vitro and still maintain their in vivo function, demonstrating that this strategy is practical. Moreover, it demonstrates the capacity of naturally occurring Treg to modulate effector T responses and the potential for these effects to occur at the site of the graft, which opens up the possibility for targeted rather than systemic therapy. It highlights the importance of administering Treg at the time of antigen challenge, thereby allowing Treg expansion to occur in

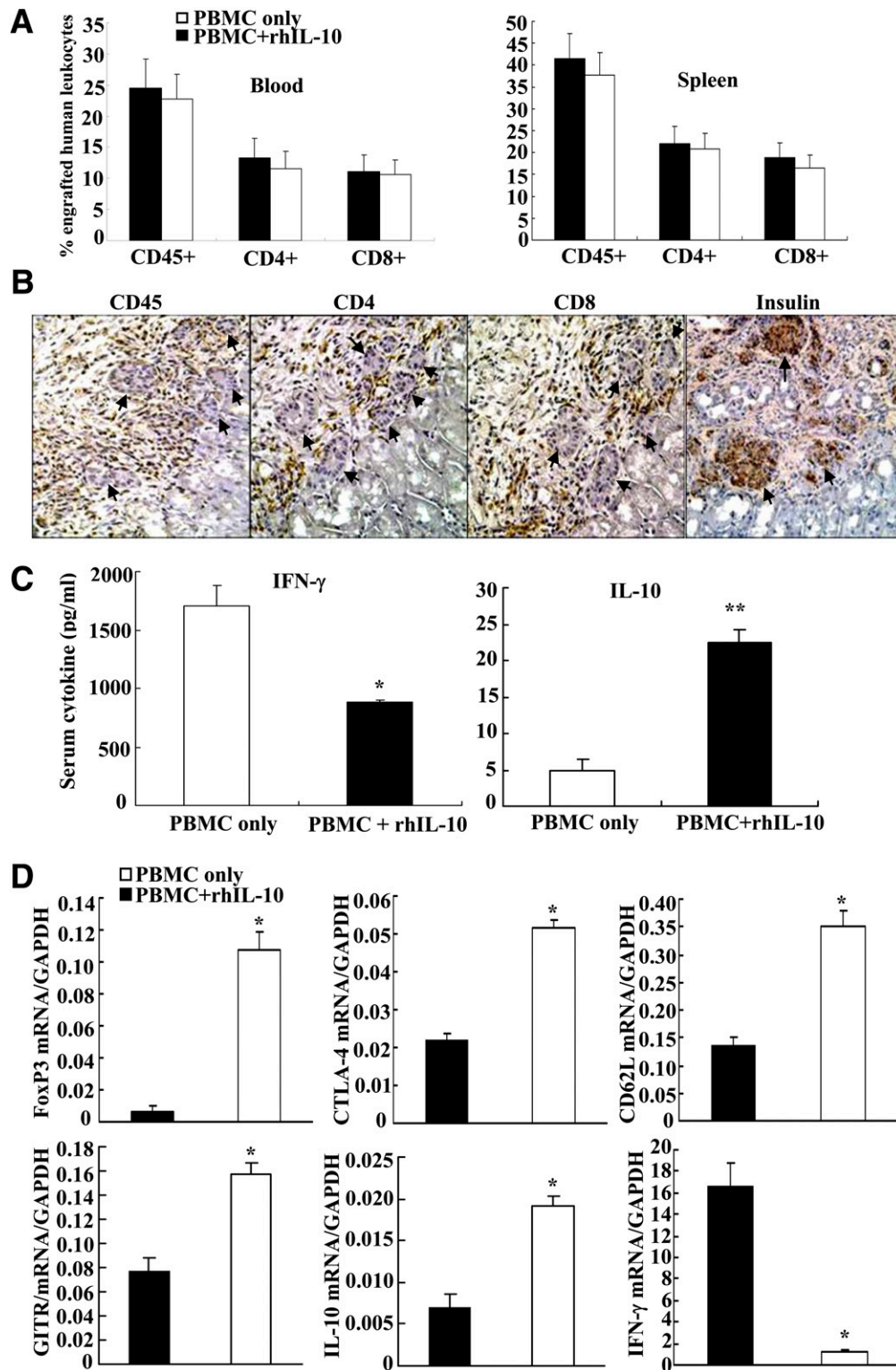
presence of antigen to maximize their response. Importantly, it demonstrates the importance of IL-10 in promoting Treg-suppressive function, possibly through the recruitment and expansion of Tr1 cells in vivo. These findings point to a strategy for using Treg as adjunctive therapy with the potential to reduce the immunosuppressive burden in islet xenotransplantation, thereby increasing the feasibility of clinical trials in the future.

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No potential conflicts of interest relevant to this article were reported.

S.Y. designed experiments, researched data, and wrote the manuscript. M.J. designed and performed experiments, researched data, and edited the manuscript. J.W. and X.M. performed experiments and researched data. P.P. and W.J.H. researched data. P.J.O. designed experiments, contributed to discussion, and reviewed, cowrote, and edited the manuscript. P.J.O. and S.Y. take responsibility for the



**FIG. 8.** rhIL-10 treatment leads to prolonged graft survival in human PBMC-transferred NICC-recipient mice. **A:** Flow cytometric analysis of percent human leukocytes in the peripheral blood and spleen of NICC-recipient mice receiving  $1 \times 10^7$  CD25<sup>+</sup> cell-depleted human PBMC and treated with (PBMC+rhIL-10) or without (PBMC only) rhIL-10 28 days after human PBMC transfer. Data are shown as mean  $\pm$  SD of three independent experiments with total nine mice for each group. **B:** Representative immunohistochemical staining of graft samples from human PBMC-reconstituted and rhIL-10-treated NICC-recipient mice 56 days after PBMC transfer for human CD45, CD4, and CD8 and porcine insulin. Arrows indicate intact NICC grafts. Original magnification  $\times 200$ . **C:** Measurement of IL-10 and IFN- $\gamma$  by cytokine bead array in serum samples from mice receiving human PBMC at graft rejection time (28 days after PBMC transfer) (PBMC only) or PBMC and rhIL-10 at day 56 after PBMC transfer (PBMC + rhIL-10). Data are shown as mean  $\pm$  SD of three individual samples from one of two independent experiments. **D:** Real-time PCR analysis of intragraft mRNA expression of Treg function-associated molecules and effector cytokine IFN- $\gamma$  in NICC grafts from the same mice as in **C**. Data are presented as mean  $\pm$  SD of three individual samples from one of two independent experiments. \* $P < 0.05$ ; \*\* $P < 0.01$  compared with PBMC only. (A high-quality digital representation of this figure is available in the online issue.)

contents of the article. P.J.O. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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