

Adoptive transfer of xenoantigen-stimulated T cell receptor V β -restricted human regulatory T cells prevents porcine islet xenograft rejection in humanized mice

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Abstract. Polyclonal expansion of human regulatory T cells (Tregs) prevents xenogeneic rejection by suppressing effector T cell responses *in vitro* and *in vivo*. However, a major limitation to using polyclonally expanded Tregs is that they may cause pan-immunosuppressive effects. The present study was conducted to compare the ability of *ex vivo* expanded human xenoantigen-stimulated Tregs (Xeno-Treg) and polyclonal Tregs (Poly-Treg) to protect islet xenografts from rejection in NOD-SCID interleukin (IL)-2 receptor (IL2r) $\gamma^{-/-}$ mice. Human cluster of differentiation (CD)4⁺CD25⁺CD127^{lo} Tregs, expanded either by stimulating with porcine peripheral blood mononuclear cells (PBMCs) or anti-CD3/CD28 beads, were characterized by immune cell phenotyping, T cell receptor (TCR) V β CDR3 spectratyping and performing suppressive activity assays *in vitro*. The efficiency of adoptively transferred *ex vivo* human Tregs was evaluated *in vivo* using neonatal porcine islet cell clusters (NICC) transplanted into NOD-SCID IL-2r $\gamma^{-/-}$ mice, which received human PBMCs with or without Xeno-Treg or Poly-Treg. Xeno-Treg, which expressed increased levels of human leukocyte antigen-DR and secreted higher levels of IL-10, demonstrated enhanced suppressive capacity in a pig-human mixed lymphocyte reaction. Spectratypes of

TCR V β 4, V β 10, V β 18 and V β 20 in Xeno-Treg showed restriction and expanded clones at sizes of 205, 441, 332 and 196 respectively, compared to those of Poly-Treg. Reconstitution of mice with human PBMCs and Poly-Treg resulted in NICC xenograft rejection at 63 days. Adoptive transfer with human PBMCs and Xeno-Treg prolonged islet xenograft survival beyond 84 days, with grafts containing intact insulin-secreting cells surrounded by a small number of human CD45⁺ cells. This study demonstrated that adoptive transfer of *ex vivo* expanded human Xeno-Treg may potentially prevent islet xenograft rejection in humanized NOD-SCID IL2r $\gamma^{-/-}$ mice compared with Poly-Treg. These findings suggested that adoptive Treg therapy may be used for immunomodulation in islet xenotransplantation by minimizing systemic immunosuppression.

Introduction

Pancreatic islet transplantation is a potential treatment option for type 1 diabetes mellitus; however, the shortage of human pancreas donors continues to restrict clinical transplantation. The pig represents an alternative source of unlimited organs and tissue, making xenotransplantation a potential strategy for use in humans. However, xenogeneic rejection mediated by T cell responses remains a major limitation to its clinical application (1,2). Long-term survival of xenogeneic islets in large animal models has been achieved with immunosuppression (3,4); however, the high dose of immunosuppressive agents required, accompanied by their side effects (5,6), limits clinical application. Regulatory T cells (Tregs), are critically important for maintaining tolerance and controlling autoimmunity (7-9), therefore they may represent an alternative and novel strategy for achieving transplant tolerance. Previous studies have indicated that adoptive transfer with *ex vivo* polyclonally expanded human Tregs prevents islet xenograft rejection by suppressing effector T cell responses (10), and *in vitro* polyclonally expanded human Tregs maintain their suppressive function in CD4⁺CD25⁺ effector T cells in a xenogeneic-stimulated mixed lymphocyte reaction (11). These findings indicate a possible strategy for overcoming cellular

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xenoresponses *in vitro* and *in vivo*. However, a major limitation to using polyclonally expanded Tregs is that they can cause pan-immunosuppressive effects, leading to opportunistic infections and tumor growth, due to their non-specific suppressive functions. Studies in human and animal models have demonstrated that small numbers of alloantigen-specific Tregs exhibit high efficiency to prevent allograft rejection with fewer side effects (12-14). Therefore, antigen-specific Tregs may hold immense promise for human immunotherapy.

The present study investigated whether *ex vivo* expanded human Tregs receiving xenoantigen stimulation are more potent than polyclonally expanded Tregs in protecting against islet xenograft rejection in NOD-SCID interleukin (IL)-2 receptor (IL2r) $\gamma^{-/-}$ mice.

Materials and methods

Animals. A total of 3 newborn pigs (1 to 3 days old) supplied by Chongqing Enservier Biological Technology Co., Ltd. (Chongqing, China) were used to isolate neonatal porcine islet cell clusters (NICC). A total of 2 adult landrace pigs (male, 18 months old, Chongqing Enservier Biological Technology Co., Ltd.) were used to isolate porcine peripheral blood mononuclear cell as xenoantigen, and were housed in separate cages at 20-26°C, 12-h light/dark cycle with fresh air, and fed pig chow twice a day with free access to water. NOD-SCID IL2r $\gamma^{-/-}$ mice (age, 6-8 weeks, weight, 25-30 g) were obtained from Chengdu Dashuo experimental animals Co. Ltd. (Chengdu, Sichuan, China) and housed under specific pathogen-free conditions (20-26°C, relative humidity, 40-70%, free access to sterile feeds and sterile water and 12-h light/dark cycle) in the approved Experimental Animal Center at Sichuan University (Chengdu, China). The mice were used for porcine islet transplantation. The procedures described in this study were conducted according to the guidelines set by the Institute of Laboratory Animals Resources Guide for the Care and Use of Laboratory Animals (Institutional Animal Care and Use Committee Guidebook) (15).

Porcine islet isolation and transplantation. NICC were isolated from the pancreas of 1-3 day old piglets and cultured for 6 days, as previously described (16). The NICC were pooled and 5,000 clusters (10) were transplanted under the renal capsule of both kidneys of NOD-SCID IL2r $\gamma^{-/-}$ mice.

Peripheral blood mononuclear cell (PBMC) isolation and human Treg isolation. Human PBMCs were isolated from the blood of 4 healthy donors (age, 28-58; gender, 2 male and 2 female) by density gradient centrifugation using Lymphoprep™ (STEMCELL Technologies China Co., Ltd, Shanghai, China). CD4⁺CD25⁺CD127^{lo} T cells were isolated from PBMCs using the CD4⁺CD25⁺CD127^{dim/-} Regulatory T Cell Isolation kit II (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), according to the manufacturer's protocol. The purity of CD4⁺CD25⁺CD127^{lo} T cells was $\geq 98\%$. Porcine PBMCs were isolated from heparinized whole blood of adult landrace pigs by density gradient centrifugation using Lymphoprep™ (STEMCELL Technologies China Co., Ltd.) and used as xenogeneic stimulator cells. Human and animal studies were approved by the Sichuan University Medical

Ethics Committee and Animal Research Ethics Communities. Written informed consent was obtained from all donors.

***In vitro* expansion of human Tregs with xenoantigen stimulation.** To obtain large numbers of functional human Tregs with xenoantigen specificity (Xeno-Treg) from CD4⁺CD25⁺CD127^{lo} T cells, cells were harvested after 7 days of polyclonal stimulation and further expanded for two subsequent cycles (7 days per cycle) by stimulating with irradiated xenogeneic PBMCs. Polyclonal Tregs (Poly-Treg) were solely expanded using CD3/CD28 beads.

CD4⁺CD25⁺CD127^{lo} T cells were expanded in RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% human AB serum (Gibco; Thermo Fisher Scientific, Inc.), 2 mM glutamine (Gibco; Thermo Fisher Scientific, Inc.), 50 μ M 2-mercaptoethanol (2-ME) (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), 100 U/ml penicillin (Gibco; Thermo Fisher Scientific, Inc.), 100 μ g/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.) and 100 nM rapamycin (Sigma-Aldrich; Merck KGaA) at 37°C and 5% CO₂, in the presence of 400 U/ml IL-2 (Novartis Corporation, East Hanover, NJ, USA) and Human T-Activator CD3/CD28 beads (Dynabeads®; Invitrogen; Thermo Fisher Scientific, Inc.) in 96-well U-bottom plates (BD Biosciences, Franklin Lakes, NJ, USA). After 7 days of expansion, Tregs were harvested and used to induce Xeno-Treg. For both cycles of xenoantigen stimulation, 5 $\times 10^4$ Tregs were cultured with 2 $\times 10^5$ irradiated (30 Gy) porcine PBMCs (xenogeneic PBMC:Treg, 4:1), in the presence of 5 $\times 10^4$ Dynabeads®. The cells were split and fresh medium was added every 3 days. After two cycles of expansion, Treg were harvested for all subsequent experiments.

Flow cytometry. Single-cell suspensions were obtained from mouse spleen or peripheral blood at 4-weeks, 9-weeks and 12-weeks following NICC transplantation and were processed using red blood cell lysis buffer (BioLegend, Inc., San Diego, CA, USA), according to the manufacturer's protocol. Human antigen CD45 (368503; BioLegend, Inc.) was used for the flow cytometric analysis of human leukocyte engraftment in the mouse spleen or peripheral blood cell suspension. Human cells were surface stained with fluorescently labeled antibodies specific for the human antigens CD4 (cat. nos. 317407 and 317425), CD25 (cat. nos. 302605 and 302613), CD127 (cat. no. 351323), CD62L (cat. no. 304821), glucocorticoid-induced tumor necrosis factor receptor-related protein (GITR; cat. no. 371223) and HLA-DR (cat. no. 307617) (all from BioLegend, Inc.), in staining buffer at 4°C for 30 min in the dark, followed by fixation and permeabilization (Fix/Perm buffer; BioLegend, Inc.). Intracellular staining was conducted with fluorescently labeled anti-forkhead box P3 (Foxp3) (cat. no. 320105) and -cytotoxic T-lymphocyte antigen-4 (CTLA-4; cat. no. 369603) antibodies (both BioLegend, Inc.) for 30 min at room temperature. Flow cytometric data were acquired using an LSRII flow cytometer (BD Biosciences).

IL-10 analyses. Total RNA was extracted from Tregs using the RNeasy Mini kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's protocol, followed by cDNA synthesis using the SuperScript™ III First-Strand Synthesis system

Table I. Primer sequences of TCR V β families.

Gene name	Oligonucleotide sequence
BV2	GAAATCTCAGAGAAGTCTGAAATAT TCG
BV3	CCTAAATCTCCAGACAAAGCTC ACT
BV4	CCTGAATGCCCCAACAGC
BV5	ACCTGATCAAAACGAGAGGACAG
BV6	CTCTCCTGTGGGCAGGTCC
BV7-2	GTTTTTAATTTACTTCCAAGGCAACA
BV7-3	CAAGGCACGGGTGCG
BV7-6	ACTTACTTCAATTATGAAGCCCA ACA
BV7-7	GAGTCATGCAACCCTTTATTGGTAT
BV7-8	AGGGGCCAGAGTTTCTGACTTAT
BV7-9	CTCAACTAGAAAAATCAAGGCTGCT
BV9	AACAGTTCCCTGACTTGCCTCT
BV10	TTCTTCTATGTGGCCCTTTGTCT
BV11	GGCTCAAAGGAGTAGACTCCACTCT
BV12	GGTGACAGAGATGGGACAAGAAGT
BV13	CATCTGATCAAAGAAAAGAGGGA AAC
BV14	AGAGTCTAAACAGGATGAGTCCGG TAT
BV15	AGAGTCTAAACAGGATGAGTCCGG TAT
BV16	AAACAGGTATGCCCCAAGGAAAGA
BV18	CAGCCCAATGAAAGGACACAGT
BV19	GGGCAAGGGCTGAGATTGAT
BV20	AACCATGCAAGCCTGACCTT
BV23	TGTACCCCCGAAAAGGACATAC
BV24	CAGTGTCTCTCGACAGGCACA
BV25	CTCAAACCATGGGCCATGA
BV27	CCAGAACCCAAGATACCTCATCAC
BV28	GGCTACGGCTGATCTATTTCTCA
BV29	GACGATCCAGTGTCAAGTTCGATAG
BV30	CTGAGGTGCCCCAGAATCTCT
BC for BV	CTGCTTCTGATGGCTCAAACA
BC for BV probe	6-FAM CACCCGAGGTGCT MGB NFQ
BC forward	TCCAGTTCTACGGGCTCTCG
BC reverse	AGGATGGTGGCAGACAGGAC
BC probe	6-FAMACGAGTGGACCCAGGATA GGGCCAA NFQ
GAPDH forward	TGCACCACCAACTGCTTAGC
GAPDH reverse	GGAAGGCCATGCCAGTGA
GAPDH probe	VIC CCTGGCCAAGGTCATCCAT GACAACCTT TAMRA

(Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed on the Bio-Rad CFX Connect Real-Time system (Bio-Rad Laboratories, Inc.,

Hercules, CA, USA) using the Platinum SYBR Green qPCR Supermix-UDG (Thermo Fisher Scientific, Inc.). The reaction was 50°C for 2 min and 95°C for 2 min followed by 40 cycles of 95°C for 15 sec and 65°C for 35 sec. PCR primers specific for human IL-10 were used: Sense 5'-GCCTAACATGCTTCG AGATC-3' and antisense 5'-GGGTTCAGGTACCGCTTC TC-3'. Human GAPDH primer (Sense 5'-TGCACCACCAAC TGCTTAGC-3' and antisense 5'-GGCATGGACTGTGGT CATGAG-3') was used as an internal reference gene and gene expression was normalized to GAPDH expression levels in each PCR reaction (17).

IL-10 in the supernatants collected from Xeno-Treg and Poly-Treg stimulation cultures was measured by ELISA, using the IL-10 Human ELISA kit (cat. no. BMS215-2TEN; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol.

TCR V β CDR3 spectratyping. CDR3 spectratyping was performed as previously described (18). Briefly, PCR amplification of expanded human Tregs with xenoantigen stimulation of polyclonal stimulation was performed until a plateau was reached. For the 29 human TCR V β families, this required 36 PCR amplification cycles, 29 TCR V β primers and a Fam-labeled C β reverse primer (18). All the TCR V β family primers were provided by Professor Stephen I. Alexander (Centre for Kidney Research, The Children's Hospital at Westmead, Sydney, NSW, Australia; Table I). The PCR product (1 μ l) from this reaction was mixed with 12 μ l Hi-Di™ Formamide and 0.2 μ l GeneScan™ 500 TAMRA™ dye Size Standard in a 0.5 ml Genetic Analyzer sample tube (all Thermo Fisher Scientific, Inc.). The sample was denatured by heating at 95°C for 10 min and then rapidly cooled on ice. The sample was then electrophoresed on the ABI Prism® 310 Genetic Analyzer (Thermo Fisher Scientific, Inc.). An electropherogram of the GeneScan-500 Size Standard was generated under denaturing conditions on the ABI Prism® 310 Genetic Analyzer. Fragments were run using the POP-4™ Polymer (Thermo Fisher Scientific, Inc.) at 60°C. When the size of the PCR product was <500 bp, a capillary with dimensions of 47 cm x 50 μ m i.d. (Thermo Fisher Scientific, Inc.) was used. If the signal was too strong, the sample injection time or voltage was decreased; or the sample was further diluted. The data were sized and quantified using ABI Prism 310 Genetic Analyzer with built in software (ABI Prism 310 collection; Thermo Fisher Scientific, Inc.).

In vitro Treg suppression assay. The suppressive capacity of Tregs was assessed by measuring inhibition of proliferation in mixed leukocyte reaction (MLR) assays. Proliferation was evaluated using the CellTrace™ Carboxyfluorescein succinimidyl ester (CFSE) Cell Proliferation kit (Invitrogen; Thermo Fisher Scientific, Inc.). Responder cells (human PBMCs) were labeled with 0.5 μ M CFSE (Molecular Probes; Thermo Fisher Scientific, Inc.) prior to stimulation. CFSE-labeled responder cells (1x10⁵) from autologous Treg donors were stimulated with 2x10⁵ irradiated xenogeneic stimulator PBMCs or purified anti-human CD3 antibody (BD Biosciences). Tregs were titrated into the cultures at different ratios. After 7 days of culture, the proliferation of responder cells was analyzed by flow cytometry (LSR II; BD Biosciences).

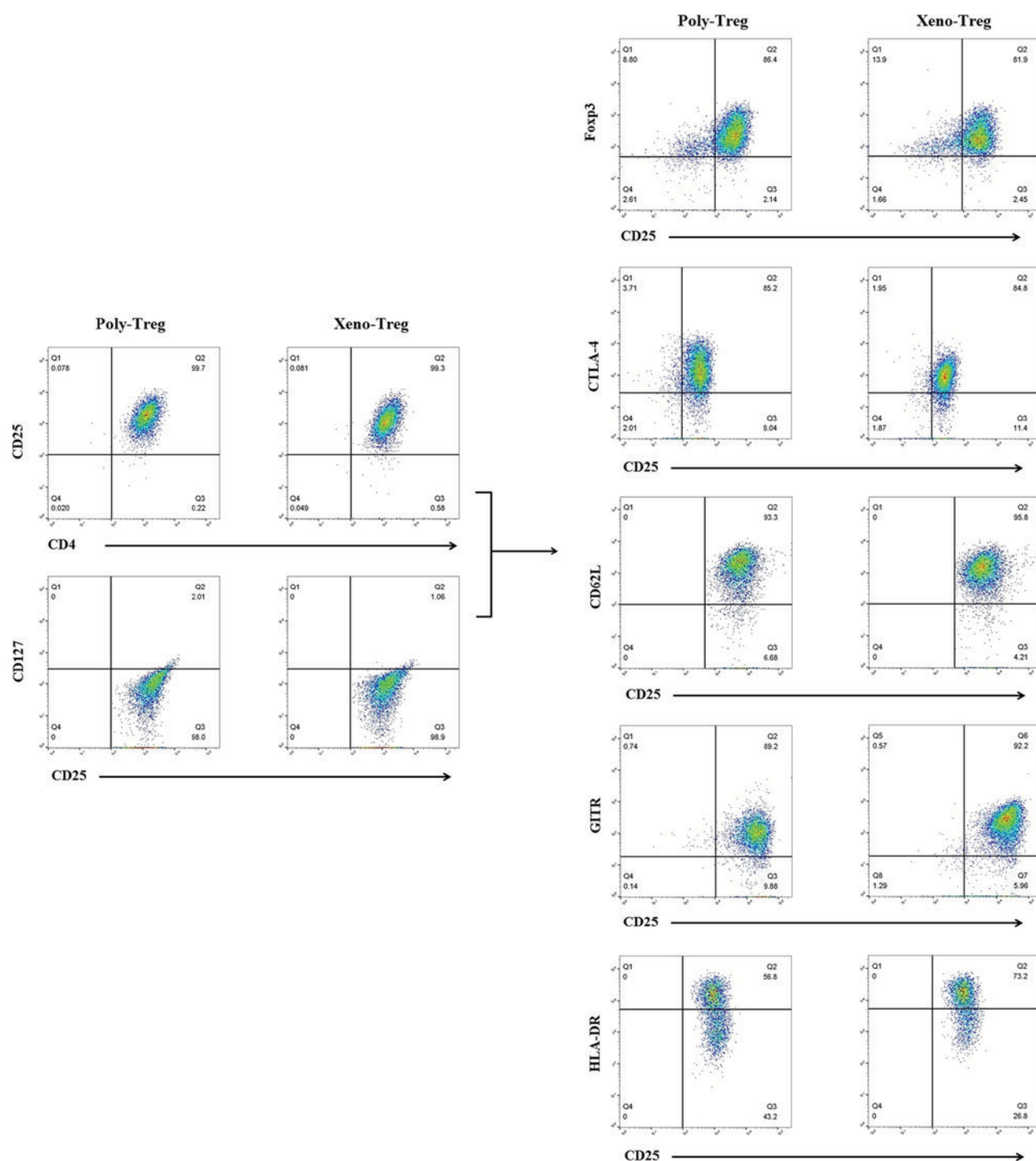


Figure 1. Phenotypic characterization of expanded Tregs. Gates were set on CD4⁺CD25⁺ cells. Cell surface expression of CD25, CD127, CD62L, GITR and HLA-DR, and intracellular expression of Foxp3 and CTLA-4 in Xeno-Treg or Poly-Treg are presented as the percentage of CD4⁺CD25⁺ cells co-expressing individual Treg markers tested. Data are representative of three independent experiments. CD, cluster of differentiation; CTLA-4, cytotoxic T-lymphocyte antigen-4; FoxP3, forkhead box P3; GITR, glucocorticoid-induced tumor necrosis factor receptor-related protein; HLA-DR, human leukocyte antigen-DR; Poly-Treg, polyclonal Treg; Tregs, regulatory T cells; Xeno-Treg, Treg with xenoantigen specificity.

Adoptive transfer of human cells. Adoptive transfer of human cells was performed as previously described (10). Human PBMCs were isolated from the blood of healthy donors. A total of 1×10^7 CD25⁺ cell-depleted PBMCs with or without 1×10^6 autologous *ex vivo* expanded human Xeno-Treg or Poly-Treg were injected intravenously into NOD-SCID IL2 γ ^{-/-} mice 3 days after NICC transplantation. Peripheral blood, spleen

and NICC grafts were collected from recipient mice at predetermined time points to analyze human leukocyte engraftment and NICC graft survival. Graft rejection was defined as no visible intact graft observed by histological examination (19).

Histology and immunohistochemistry. Histology and immunohistochemistry of cryostat section (6–8 μ m) were undertaken

as described previously (10). Porcine endocrine cells were detected using anti-porcine insulin antibody (IR00261-2 insulin; 1:100; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) and the VECTASTAIN[®] ABC kit (Vector Laboratories, Inc., Burlingame, CA, USA). Graft-infiltrating human leukocytes were stained using anti-human CD45 antibody (14-9457-82; 1:200; eBioscience; Thermo Fisher Scientific, Inc.), followed by incubation with horseradish peroxidase-conjugated secondary rabbit anti-mouse antibody (31451; 1:200, Thermo Fisher Scientific, Inc.), then analyze using a Zeiss microscope (AX10; Zeiss AG, Oberkochen, Germany).

Statistical analysis. Results comparisons involving two groups were analyzed using Student's t-test (two-tailed) and those involving multiple groups were analyzed using one-way analysis of variance with the Tukey multiple comparison test by SPSS version 22 (IBM Corp., Armonk, NY, USA). Graft survival was evaluated using Kaplan-Meier analysis. The data were presented as the means \pm standard deviation. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Human Tregs expanded ex vivo with xenoantigen stimulation retain Treg phenotype and secrete IL-10. The phenotype of Xeno-Treg was examined by flow cytometry. After two cycles of xenoantigen stimulation, Xeno-Treg retained the classic Treg phenotype, as did Poly-Treg, which is characterized by high levels of CD25, Foxp3, CTLA-4, CD62L and GITR expression, and very low or undetectable CD127 expression (Fig. 1). However, compared with Poly-Treg, Xeno-Treg expressed more HLA-DR, which has been described as an effector marker of Treg (20,21) (Fig. 1).

IL-10 expression in Tregs was assessed by RT-qPCR. Xenoantigen stimulation led to an upregulation of IL-10 expression, with expression levels 7-fold higher compared with Poly-Treg (Fig. 2A; $P < 0.01$). In addition, IL-10 secretion was measured in the cell culture supernatants of Tregs receiving xenoantigen or polyclonal stimulation. Consistent with mRNA expression, IL-10 secretion by Xeno-Treg was enhanced compared with Poly-Treg (Xeno-Treg: 175 ± 18.9 pg/ml vs. Poly-Treg: 86 ± 16.4 pg/ml; Fig. 2B; $P < 0.01$). These results demonstrated that Xeno-Treg may retain a Treg phenotype, but secrete higher levels of IL-10 compared with Poly-Treg, which may result in greater suppressive potency.

Tregs exhibit restricted TCR V β repertoire following xenoantigen stimulation. Spectratyping was used to analyze the TCR V β families at the CDR3 level and screen for clonal expansion of specific T cells (22-24). All 29 TCR V β families were detected in Xeno-Treg or Poly-Treg, and in control CD4⁺ T cells. However, there were altered TCR V β repertoires in both Xeno-Treg and Poly-Treg compared with in CD4⁺ T cells. Increased expression of TCR V β 4, V β 7-9, V β 20, V β 28 (>5% in repertoire) and TCR V β 10 and V β 18 were observed in Xeno-Treg and Poly-Treg compared with in CD4⁺ T cells (Fig. 3). In addition, expression levels of TCR V β 4, V β 10, V β 18 and V β 20 were markedly increased in Xeno-Treg compared with in Poly-Treg (Fig. 3).

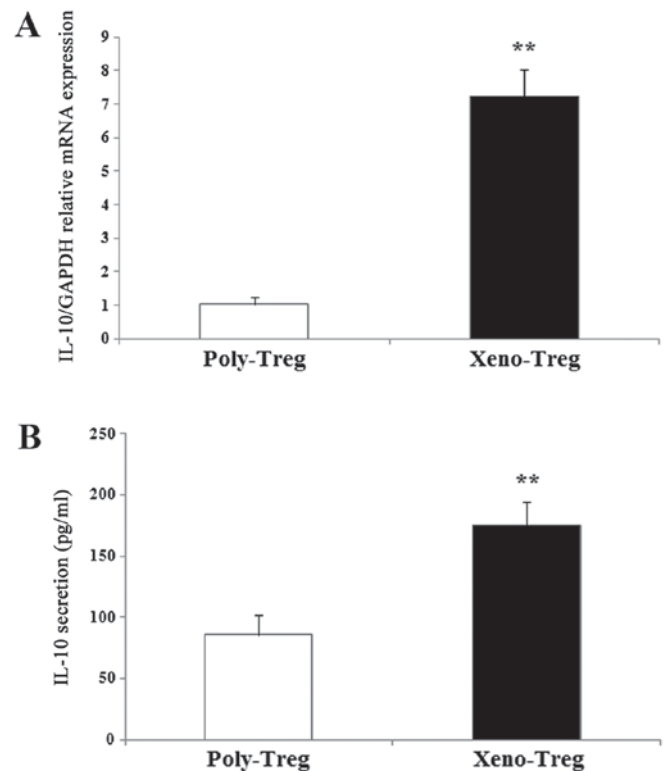


Figure 2. IL-10 gene expression and secretion by expanded Tregs. (A) Treg IL-10 gene expression, as determined by reverse transcription-quantitative polymerase chain reaction. (B) IL-10 concentration in Treg culture medium after 3 weeks expansion with polyclonal or xenoantigen stimulation. Data are presented as the means \pm standard deviation of three independent experiments with Tregs from three individual donors. ** $P < 0.01$, Xeno-Treg vs. Poly-Treg. IL-10, interleukin 10; Poly-Treg, Polyclonal Treg; Tregs, regulatory T cells; Xeno-Treg, Treg with xenoantigen specificity.

Overall spectratyping of PCR products revealed a restricted TCR V β repertoire in Xeno-Treg and Poly-Treg compared with in CD4⁺ T cells, which possessed a diverse TCR V β repertoire. Nearly all the TCR V β families exhibited a Gaussian distribution, with the exception of TCR V β 3, V β 7-7, V β 19 and V β 23 (Fig. 4). Spectratypes of TCR V β 4, V β 20 and V β 28 (>5% of the TCR V β repertoire and increased expression) in Xeno-Treg and Poly-Treg demonstrated restriction and expanded clone at size 205, 196 and 274, respectively (Figs. 5 and 6). Spectratypes of TCR V β 7-9 (>5% of the TCR V β repertoire and increased expression) exhibited restriction and expanded clone at size 234 in Poly-Treg and at size 237 in Xeno-Treg. In addition, spectratypes of TCR V β 10 (<5% of the TCR V β repertoire and increased expression) possessed restriction and expanded clone at size 432 in Poly-Treg and at size 432 and 441 in Xeno-Treg.

Human Tregs expanded ex vivo with xenoantigen stimulation exhibit enhanced suppressive capacity. To determine whether Xeno-Treg possessed more potent and xenoantigen-specific suppressive capacity against xenoimmune responses compared with Poly-Treg, their suppressive function was assessed in an MLR assay using CFSE-labeled PBMCs as responder cells. In a xenoantigen-driven MLR (Xeno MLR) assay, Xeno-Treg exhibited an enhanced xenoantigen-specific suppressive capacity compared with Poly-Treg, as evidenced by the

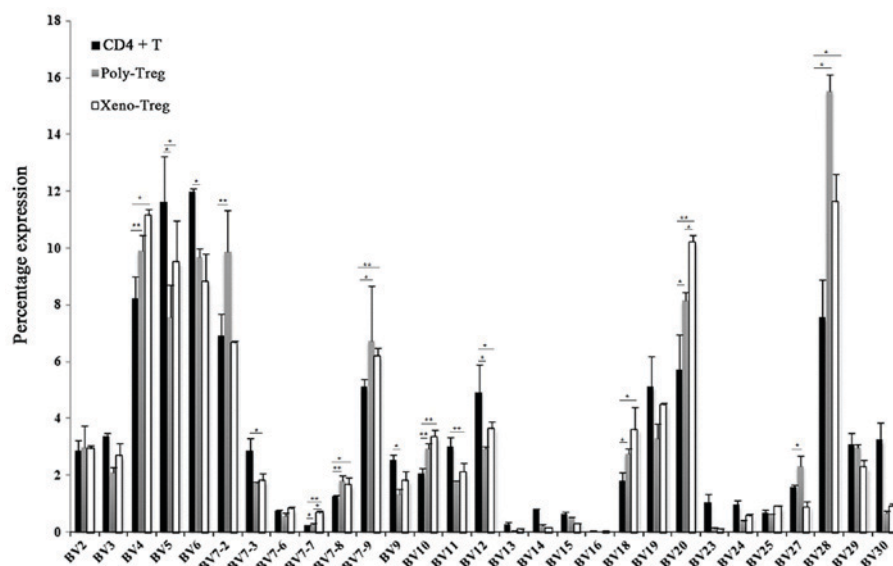


Figure 3. Gene expression of Treg TCR V β repertoires. Reverse transcription-quantitative polymerase chain reaction was conducted to detect cDNA from Tregs expanded with polyclonal or xenoantigen stimulation for 3 weeks, using a single BC primer and 29 BV primers, compared with CD4⁺ T cells. CD, cluster of differentiation; Poly-Treg, Polyclonal Treg; TCR, T cell receptor; Tregs, regulatory T cells; Xeno-Treg, Treg with xenoantigen specificity. * $P < 0.01$, ** $P < 0.05$.

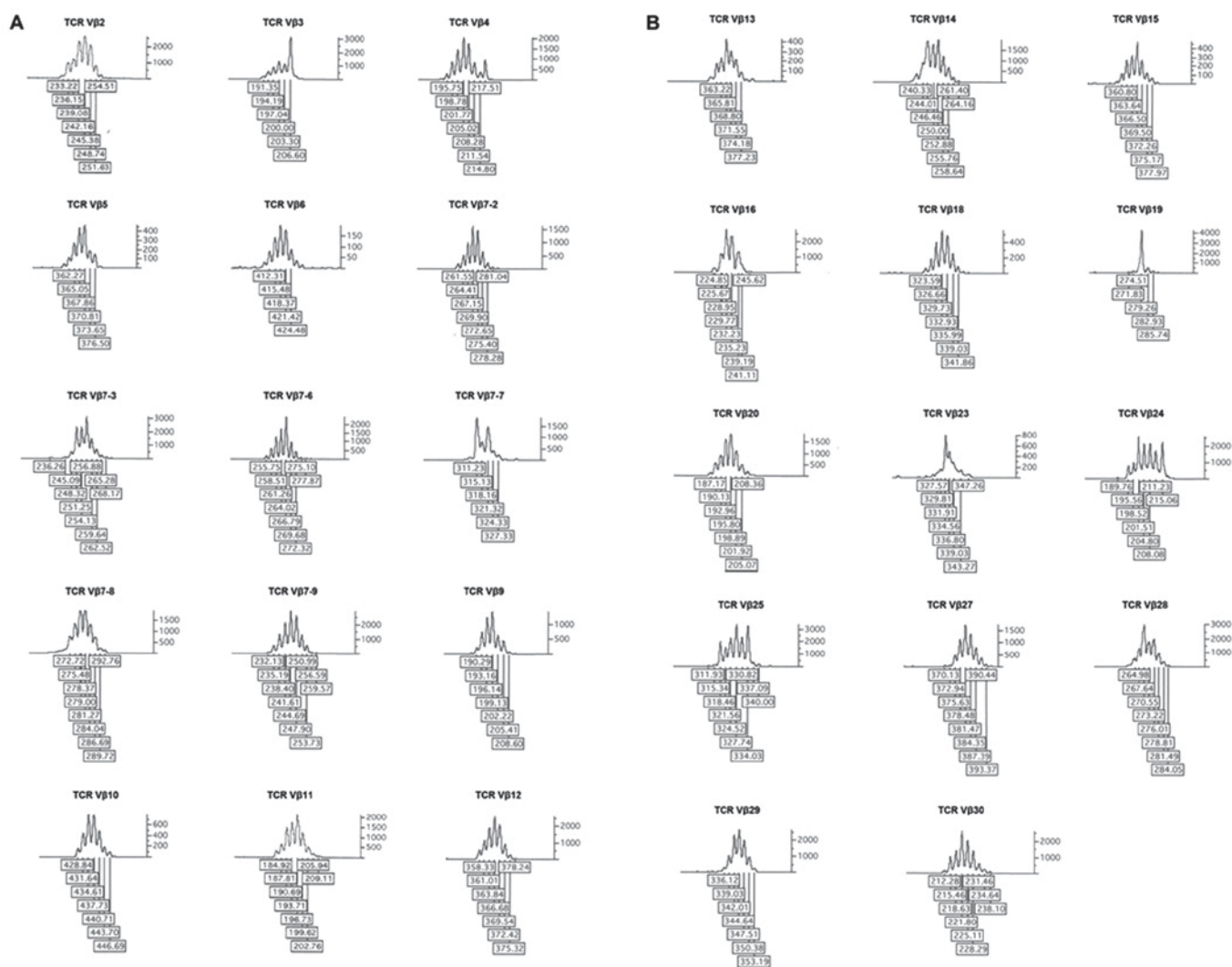


Figure 4. TCR V β repertoire gene spectratyping of CD4⁺ T cells. TCR V β families (A) TCR V β 2-V β 12 and (B) TCR V β 13-V β 30 at CDR3 level were selected to screen for clonal expansion of CD4⁺ T cells. X-axis represents the size of polymerase chain reaction products and Y-axis represents fluorescence density. CD, cluster of differentiation; TCR, T cell receptor.

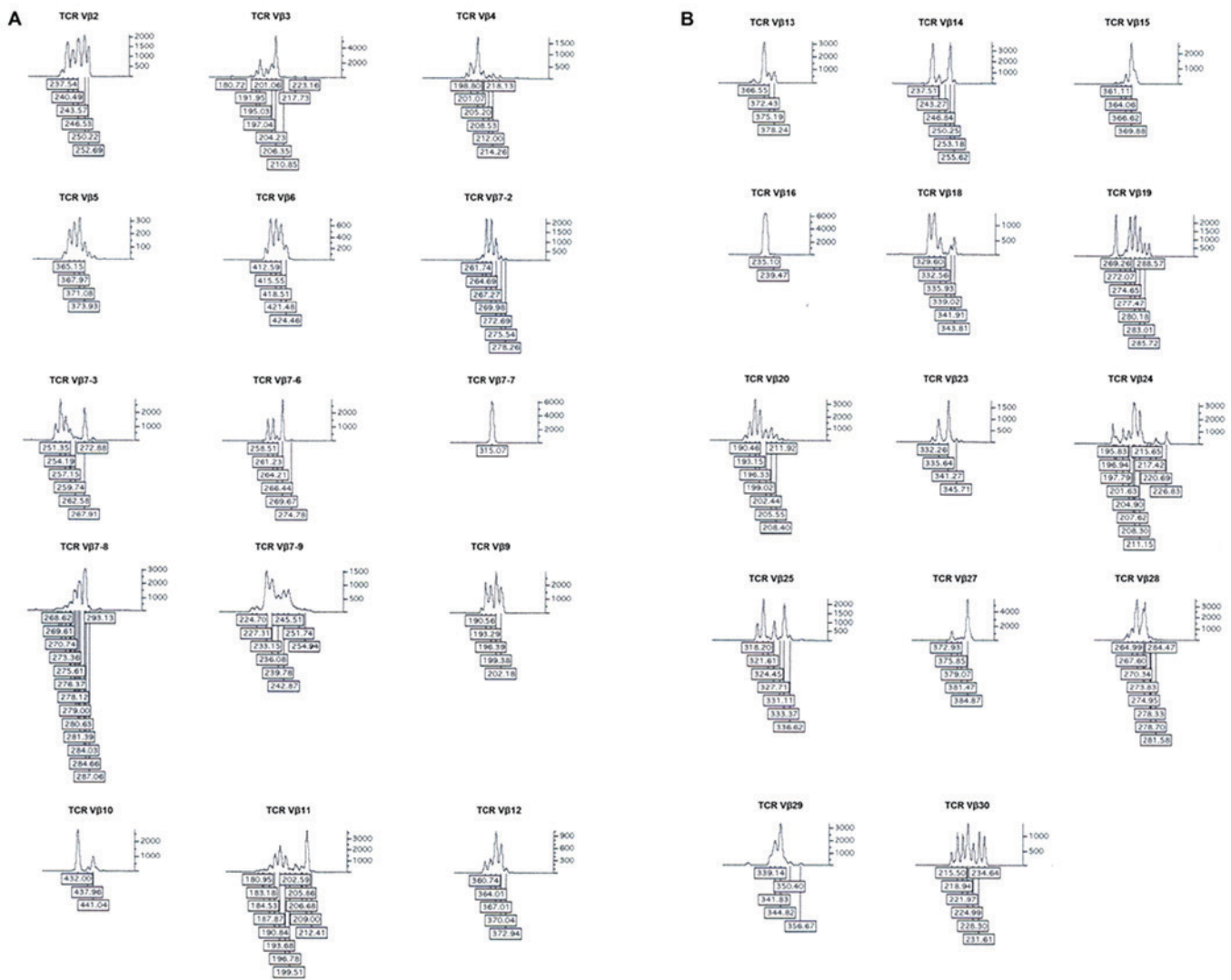


Figure 5. TCR V β repertoire gene spectratyping of Poly-Treg. TCR V β families (A) TCR V β 2-V β 12 and (B) TCR V β 13-V β 30 at CDR3 level were selected to screen for clonal expansion of Poly-Treg. X-axis represents the size of polymerase chain reaction products and Y-axis represents the fluorescence density. CD, cluster of differentiation; Poly-Treg, Polyclonal Treg; TCR, T cell receptor; Tregs, regulatory T cells.

~55 and 45% suppression of responder cell proliferation at low responder cell: Treg ratios of 1:1/8 and 1:1/16, respectively (Fig. 7A). Even at a higher responder cell: Treg ratio of 1:1/32, Xeno-Treg still demonstrated >35% suppression of responder cell proliferation, which was 2.5-fold higher compared with Poly-Treg (Fig. 7A). These data revealed that Xeno-Treg possess enhanced and xenoantigen-specific suppressive capacity. However, both Xeno- and Poly-Treg demonstrated similar ability to suppress responder cell proliferation in a polyclonally-stimulated MLR (Poly MLR) assay (Fig. 7B), thus suggesting that xenoantigen stimulation did not alter the capacity to suppress polyclonally-stimulated responses.

Human Treg expanded ex vivo with xenoantigen stimulation prevent rejection of porcine islet xenografts. To determine the *in vivo* suppressive capacity of *ex vivo* expanded Xeno-Treg, a total of 1×10^7 CD25⁺ cell-depleted PBMCs with or without 1×10^6 autologous *ex vivo* expanded Xeno-Treg or Poly-Treg were injected intravenously into NOD-SCID IL2r $\gamma^{-/-}$ mice 3 days after NICC transplantation. Nonreconstituted mice were used as a control. Mice that were reconstituted with human

PBMCs, rejected their xenografts completely within 28 days of transplantation, whereas NICC grafts survived for ≥ 84 days in nonreconstituted recipients (Fig. 8A). In mice reconstituted with Xeno-Treg and PBMCs (Xeno-Treg:PBMC ratio of 1:10), 75% of NICC xenografts survived beyond 84 days (Fig. 8A). In contrast, in mice reconstituted with Poly-Treg and PBMCs (Poly-Treg:PBMC ratio of 1:10), 75% of NICC xenografts survived ≥ 48 days, 25% of NICC xenografts survived until day 56, and all xenografts were rejected by day 63 (Fig. 8A). These results suggested that Xeno-Treg may prolong NICC xenograft survival.

Human leukocyte engraftment was confirmed by flow cytometry. Following human Poly-Treg and PBMC adoptive transfer, the spleen and peripheral blood was engrafted with 27.3 ± 6.3 and $14.7 \pm 4.2\%$ of human CD45⁺ cells respectively, by day 63 (Fig. 8B). However, following human Xeno-Treg and PBMC adoptive transfer, the spleen and peripheral blood was engrafted with 15.2 ± 3.8 and $10.5 \pm 3.2\%$ of human CD45⁺ cells respectively, by day 84 (Fig. 8B). Decreased engraftment of human CD45⁺ cells in mice reconstituted with Xeno-Treg and PBMCs may indicate that graft survival is as a result of

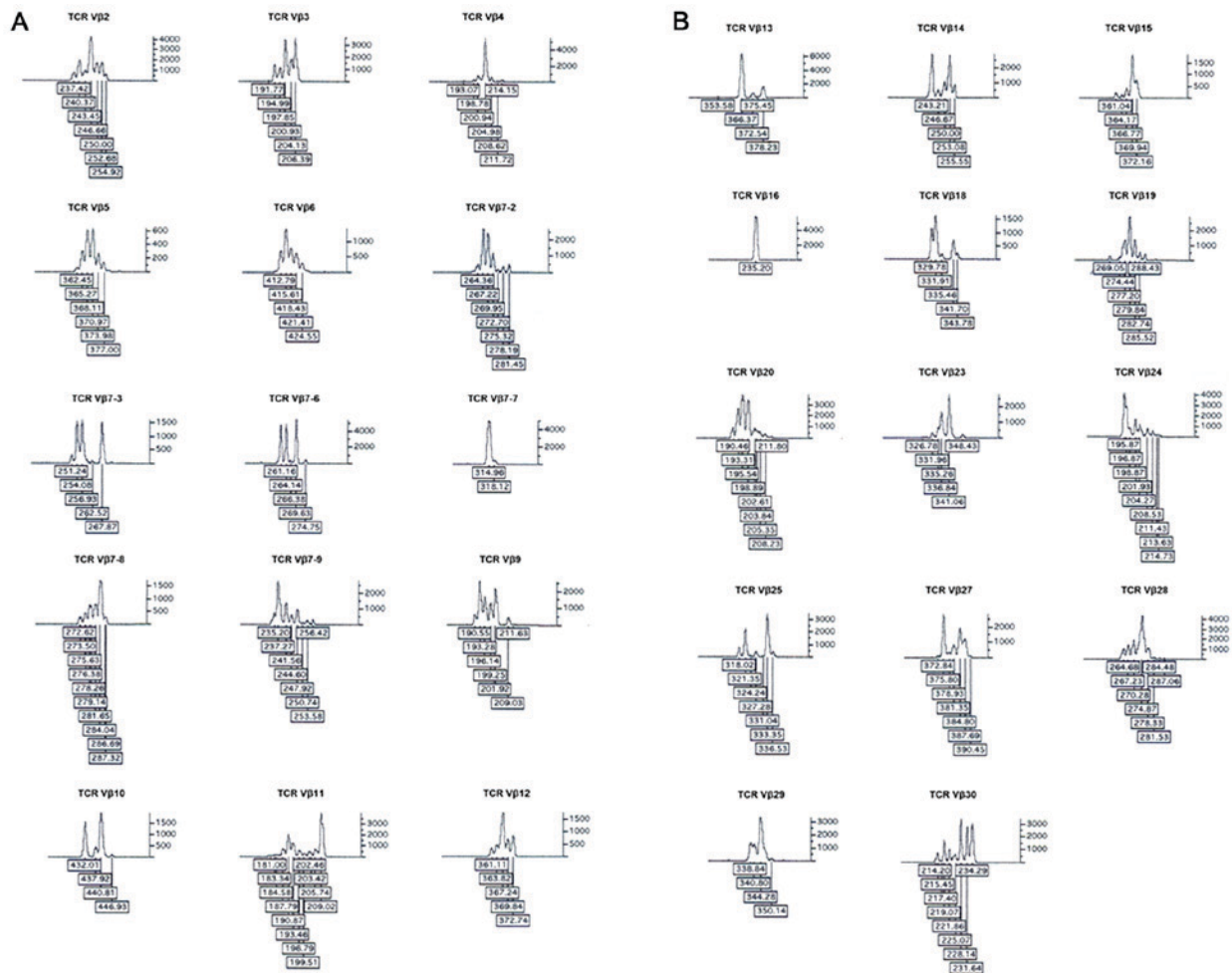


Figure 6. TCR V β repertoire gene spectratyping of Xeno-Treg. TCR V β families (A) TCR V β 2-V β 12 and (B) TCR V β 13-V β 30 at CDR3 level were selected to screen for clonal expansion of Xeno-Treg. X-axis represents the size of polymerase chain reaction products and Y-axis represents the fluorescence density. CD, cluster of differentiation; TCR, T cell receptor; Tregs, regulatory T cells; Xeno-Treg, Treg with xenoantigen specificity.

xenoantigen-specific Treg-mediated suppression and not due to engraftment failure.

Immunohistochemistry of NICC grafts from nonreconstituted recipients revealed intact insulin-positive cells with no CD45⁺ cells infiltration (Fig. 9A-C). Numerous graft-infiltrating human CD45⁺ cells were detected in the rejected xenografts from PBMC-reconstituted mice; however, no insulin-positive cells were visible (Fig. 9D-F). Long-term surviving grafts from Xeno-Treg- and PBMC-reconstituted mice contained intact insulin-secreting cells surrounded by a small number of human CD45⁺ cells (Fig. 9G-I). On day 63, immunohistochemistry of NICC grafts from Poly-Treg- and PBMC-reconstituted mice revealed small, damaged and insulin-positive cells with numerous graft-infiltrating human CD45⁺ cells (Fig. 9J-L). These results suggested that adoptive transfer of *ex vivo* expanded Xeno-Treg may possess a greater capacity to reduce xenograft damage and prevent rejection of porcine islet xenografts compared with Poly-Treg.

Discussion

The use of efficient antigen-specific Tregs may reduce the number of Tregs required for therapy and lower the risk of systemic immunosuppression (25,26). Numerous

studies have investigated strategies for large-scale expansion of alloantigen-specific human Tregs (27-29), and *ex vivo* alloantigen-specific Tregs were shown to possess enhanced suppressive capacity in allogeneic responses *in vitro* and *in vivo* (30,31). In the present study, a strategy using one cycle of polyclonal stimulation followed by two subsequent cycles of xenoantigen stimulation was developed to selectively expand Xeno-Treg. Following stimulation, spectratyping was conducted to analyze the TCR V β families of Tregs at the CDR3 level and screen for clonal expansion of specific T cells. CDR3 spectratyping is a well-described method for measuring oligoclonality within T cell populations (32). Normal PBMC samples of a single V β family display a Gaussian distribution of 6-11 peaks, each separated by three nucleotides. Each peak corresponds to a TCR transcript with a given CDR3 length that may contain numerous sequences (32). The number of TCR transcripts with a specific CDR3 length is proportional to the area under each peak. An increase in the height and area of a size peak typically indicates oligoclonal or monoclonal expansion in the polyclonal T cell background. Oligoclonal T cells give fewer peaks in a restricted distribution. Single clones give a single peak. This method has been used previously to screen PCR products in an efficient manner for possible T cell clonal expansion following MLR, renal biopsies and urine at the time of rejection (22,33).

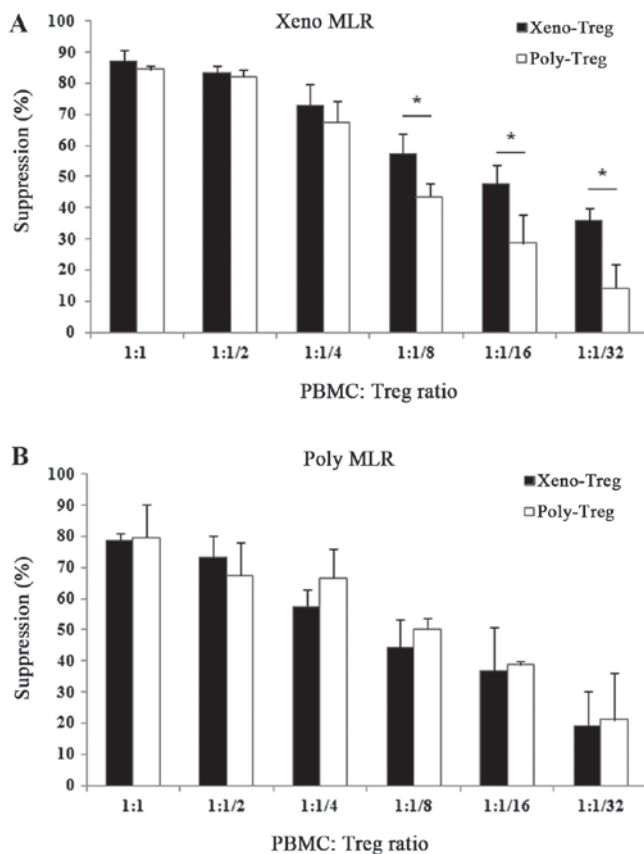


Figure 7. Treg suppressive capacity assessed using MLR assays. Xeno-Treg and Poly-Treg were assessed for suppressive capacity using (A) Xeno MLR or (B) Poly MLR assays. Data are presented as the means \pm standard deviation of three independent experiments. * $P < 0.05$, Xeno-Treg vs. Poly-Treg. MLR, mixed leukocyte reaction; Poly MLR, polyclonally-stimulated MLR; Poly-Treg, polyclonal Treg; Tregs, regulatory T cells; Xeno MLR, xenoantigen-driven MLR; Xeno-Treg, Treg with xenoantigen specificity.

The present study identified an increase in the expression of TCR V β 4, TCR V β 10, TCR V β 18 and TCR V β 20 families for Xeno-Treg compared with Poly-Treg. In addition, spectratypes of TCR V β 4, V β 10, V β 18 and V β 20 in Xeno-Treg demonstrated restriction and expanded clone at size 205, 441, 332 and 196, respectively, which indicated that Treg acquired xenoantigen specificity following xenoantigen stimulation by identifying the specific expanded clones in TCR V β families. Furthermore, Xeno-Treg were acquired and showed enhanced suppressive capacity in the xenoimmune response detected in a MLR.

The mechanisms underlying human xenoantigen-specific Treg suppressive functions *in vivo* remain largely unknown. Previous studies have revealed that IL-10 serves a critical role in Treg-mediated suppression of xenogeneic responses *in vivo* and *in vitro* (10,34,35). In the present study, Xeno-Treg upregulated the expression of IL-10 and produced more IL-10, compared with Poly-Treg, in the culture medium. Therefore, it may be hypothesized that the suppressive functions of Xeno-Treg in the Xeno MLR assay are mediated by IL-10. Furthermore, the results demonstrated that Xeno-Treg expressed increased levels of HLA-DR, thus suggesting that IL-10 secretion of Xeno-Treg was associated with the upregulated effector marker. Yi *et al* demonstrated that adoptive transfer with expanded autologous Tregs prevents islet xenograft rejection in human PBMC-reconstituted mice, by inhibiting graft

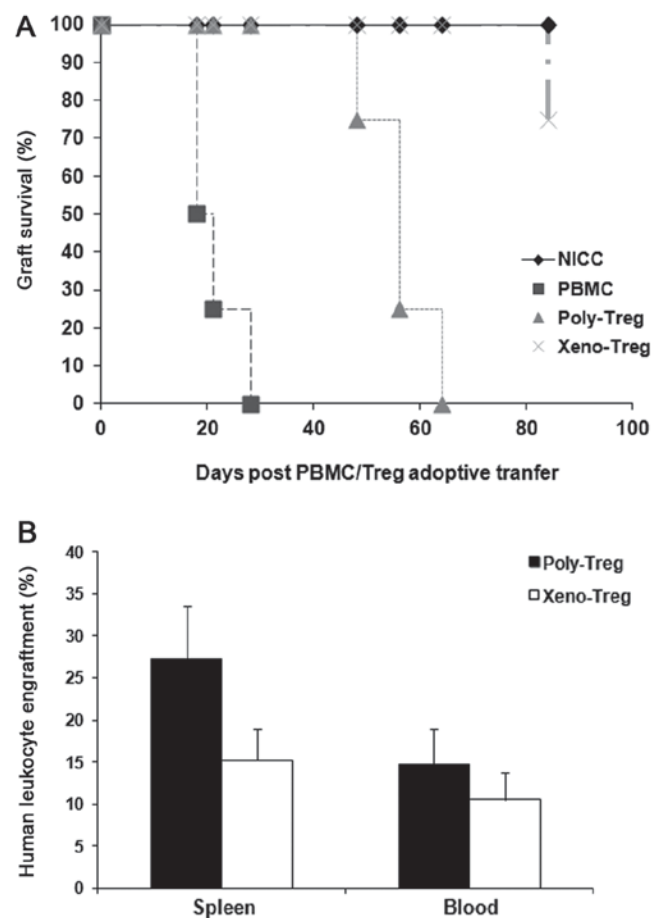


Figure 8. Xeno-Treg suppress rejection of islet xenografts in humanized mice. (A) Percentage of graft survival in mice administered 1×10^7 CD25 $^+$ cell-depleted human PBMCs with or without 1×10^6 Poly-Treg or Xeno-Treg. Graft survival was monitored 18, 21, 28, 48, 56, 63 and 84 days post cell transfer. (B) Flow cytometric analysis of the percentage of human leukocyte engraftment in the spleen and peripheral blood of NOD-SCID interleukin-2 receptor $\gamma^{-/-}$ mice after PBMC plus Treg adoptive transfer. Data were acquired on day 63 for Poly-Treg or day 84 for Xeno-Treg. Data are presented as the means \pm standard deviation of three independent experiments. CD, cluster of differentiation; PBMC, peripheral blood mononuclear cell; Poly-Treg, polyclonal Treg; Tregs, regulatory T cells; Xeno-Treg, Treg with xenoantigen specificity.

infiltration of effector cells and their function via IL-10 (10). In the present study, NOD-SCID IL2 $\gamma^{-/-}$ mice reconstituted with Xeno-Treg and PBMCs at a Xeno-Treg:PBMC ratio of 1:10; 75% of NICC xenografts contained intact insulin-secreting cells, which survived beyond 84 days compared with the Poly-Treg- and PBMC-reconstituted group, in which 75% of NICC xenografts survived to day 48 and by day 63 all xenografts showed small, damaged and insulin positive-staining cells, with a large number of graft-infiltrating human CD45 $^+$ cells. These findings suggested that adoptively transferred *ex vivo* expanded Xeno-Treg may display a greater capacity to reduce xenograft damage and prevent porcine islet xenograft rejection compared with Poly-Treg. However, the mechanisms of human xenoantigen-specific Treg suppressive function *in vivo* remain largely unknown. Further studies are required to explore whether IL-10 has an important role in human xenoantigen-specific Treg suppressive function *in vivo*.

In conclusion, the present study demonstrated that adoptive transfer with *ex vivo* expanded Xeno-Treg exhibited a greater

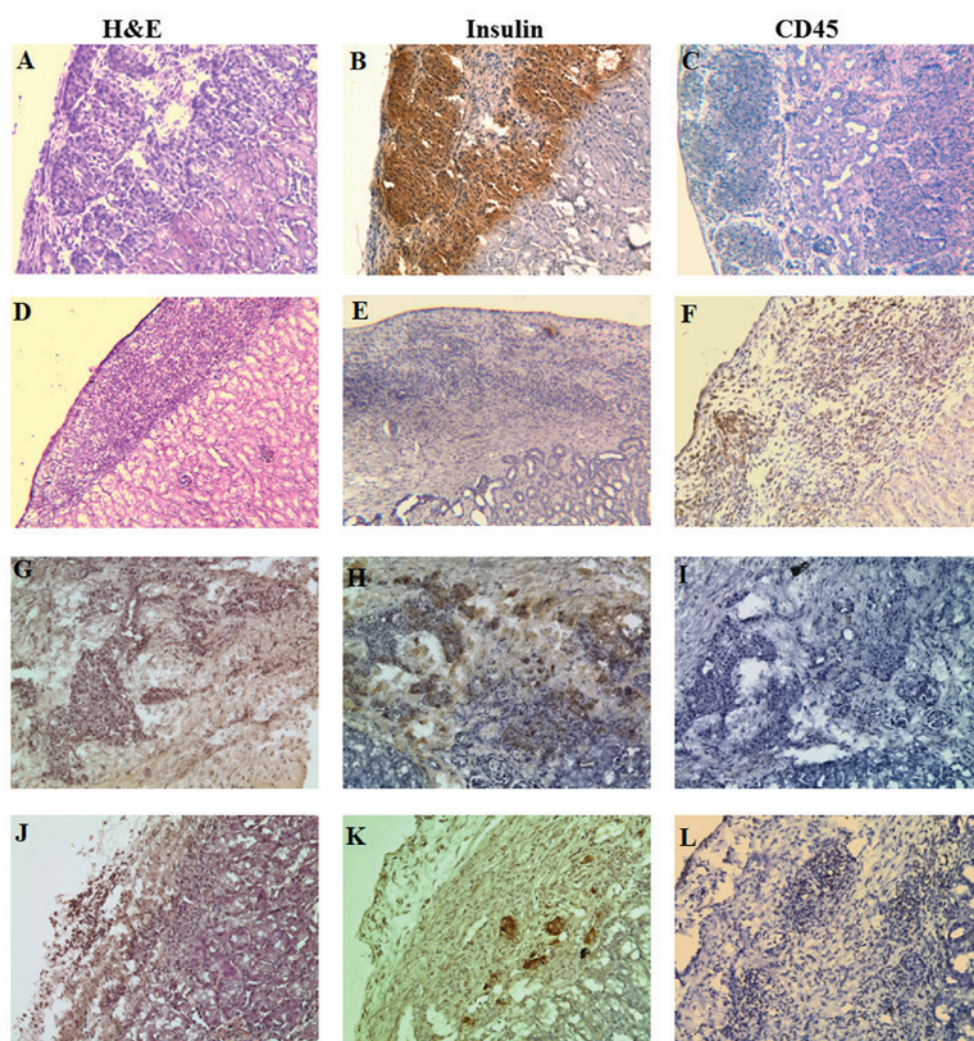


Figure 9. Histology and immunohistochemical analysis of NICC xenografts. Representative hematoxylin and eosin staining images; and immunohistochemical staining images of porcine insulin and human CD45 in NICC xenograft samples from mice receiving (A-C) no human cells (NICC alone 84 days post-transplantation), (D-F) only human PBMCs (NICC + PBMC 28 days post-PBMC transfer), (G-I) human PBMCs and Xeno-Treg (NICC + PBMC + Xeno-Treg 84 days post-cell transfer) or (J-L) human PBMCs and Poly-Treg (NICC + PBMC + Poly-Treg 63 days post-cell transfer). (A, B, E-I, K and L) Magnification, x200; (C, D and J) magnification, x100. CD, cluster of differentiation; NICC, neonatal porcine islet cell clusters; PBMC, peripheral blood mononuclear cell; Poly-Treg, polyclonal Treg; Tregs, regulatory T cells; Xeno-Treg, Treg with xenoantigen specificity.

capacity to prevent islet xenograft rejection in humanized NOD-SCID IL2 $\gamma^{-/-}$ mice compared with Poly-Treg, thus suggesting a novel strategy for adoptive Treg cell therapy for immunomodulation in islet xenotransplantation that may minimize systemic immunosuppression.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

XJ contributed to the design of the present study, data analysis, and helped draft the manuscript. MH conducted the TCR V β CDR3 spectratyping and analyzed the data. LG contributed to the animal work and performed the immunohistochemistry. HFL and YW performed the flow cytometry. MJ participated in the Treg suppression assay and revised the article. HL helped to design the present study and with the data analysis, and participated in reviewing and revising the article.

Ethics approval and consent to participate

All human and animal studies were approved by the Sichuan University Medical Ethics Committee and Animal research Ethics communities. All donors provided informed consent.

Patient consent for publication

Written informed consent was obtained from all donors.

Competing interests

The authors declare that they have no competing interests.

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