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

XI JIN¹, MIN HU², LINA GONG¹, HUIFANG LI³, YAN WANG³, MING JI⁴ and HONG LI¹

¹Institute of Urology, Department of Urology, West China Hospital, Sichuan University, Chengdu, Sichuan 610041, P.R. China; ²Centre for Transplant and Renal Research, Westmead Millennium Institute, University of Sydney, Westmead, NSW 2145, Australia; ³Cellular Biology Laboratory, Core Facility of West China Hospital, Sichuan University, Chengdu, Sichuan 610041; ⁴Department of Physiology, School of Basic Medical Science, Central South University, Changsha, Hunan 410083, P.R. China

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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)γ− mice. Human cluster of differentiation (CD)4⁺CD25⁺CD127⁻ 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) Vβ 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‑2γr− 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 Vβ4, Vβ10, Vβ18 and Vβ20 in Xeno‑Treg showed restricted 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 potently prevent islet xenograft rejection in humanized NOD‑SCID IL2rγ− 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
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 xenogenetic stimulation are more potent than polyclonally expanded Tregs in protecting against islet xenograft rejection in NOD-SCID interleukin (IL)-2 receptor (IL2r)γ− 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 IL2rγ− 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 IL2rγ− 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− T cells were isolated from PBMCs using the CD4+CD25+CD127dim− Regulatory T Cell Isolation Kit II (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), according to the manufacturer’s protocol. The purity of CD4+CD25+CD127− T cells was ≥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 xenogenotypic stimulation. To obtain large numbers of functional human Tregs with xenogenotypic specificity (Xeno-Treg) from CD4+CD25+CD127− T cells, cells were cultured 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− 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.), 100 U/ml penicillin (Gibco; Thermo Fisher Scientific, Inc.), 100 µg/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 µ/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, 5×104 Tregs were cultured with 2×105 irradiated (30 Gy) porcine PBMCs (xenogeneic PBMC:Treg, 4:1), in the presence of 5×104 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. no. 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 from BioLegend, Inc.) for 30 min at room temperature. Flow cytometric data were acquired using an LSR II flow cytometer (BD Biosciences).

IL-10 analyses. Total RNA was extracted from Tregs using the RNasy 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 Vβ families.

| Gene name | Oligonucleotide sequence |
|-----------|--------------------------|
| BV2       | GAAATCTCAGAAGTCTGAAATAT   |
|           | TCG                      |
| BV3       | CCTAAATCTCCAGAAAGGCTCT   |
| BV4       | CCTGAGTCCCCAACAGC        |
| BV5       | ACCGTATCAAGGACAGGACAG    |
| BV6       | CTCTCTGTGGAGGACCTCC      |
| BV7-2     | GTTTTTAATTTACTCAAAGGCAACA|
| BV7-3     | CAAGGACCCGGTGCCG         |
| BV7-6     | ACTTACTTCAATTGAAAGCACC   |
|           | ACA                      |
| BV7-7     | GAGTCATGCAAACCTTTATTTGT  |
| BV7-8     | AGGGGCCGAGTTTCTGACTTAT   |
| BV7-9     | CTCACACTGAATACACTTGGC    |
| BV9       | AACAGTCTCCGTGACTTCTC     |
| BV10      | TTCTCTATGTGGCCCTTTGTCT   |
| BV11      | GGGTCGAAGGAGTACTCCACT    |
| BV12      | GGTGACAGAGATGGGACAGAAG   |
| BV13      | CATCTGATCAAAGAAAAGGAGGA  |
| BV14      | AGAGTCTAACAGGATGATCGCGG  |
|           | TAT                      |
| BV15      | AGAGTCTAACAGGATGATCGCGG  |
|           | TAT                      |
| BV16      | AAACAGTGATGCCAAGGAAAAGA  |
| BV18      | CAGCCCCATGAAAGGACAGT     |
| BV19      | GGCAAGGGTGGATGATGTG       |
| BV20      | AACATGGCGCTGACCTT        |
| BV23      | TGTCACCCCGGAAAAGGACACATC|
| BV24      | CATGCTCTCTGACAGGCCAC     |
| BV25      | CTCAACATGCGGCTAGTTT      |
| BV27      | CCAAGACCCCGATACTCATCAC   |
| BV28      | GGCTACGCTGATCATTTCCTCA   |
| BV29      | GAGCATCCAGTCTCAAGTCTGATAG|
| BV30      | CTGGATGCCCACAAATCTCT     |
| BC for BV | CTTTCTGTAGGGCTCAACA      |
| BC for BV probe | 6-FAM CACCGAGGTCG MGB   |
|            | NFQ                      |
| BC forward | TCCAGTTCTACGGCTCTC       |
| BC reverse | AGGAATGGTGCGGACAGGAC     |
| BC probe  | 6-FAMACGAGTGCCAGCAAGATA  |
|            | GGCCCA NFQ                |
| GAPDH forward | TGCAACCACAACACTGGTTCG   |
| GAPDH reverse | GGAAGGGCTATGCGAGTGA   |
| GAPDH probe | VIC CCTGGCAAAGGTCATCCAT  |
|            | GACAACTT 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'-GCCTAACATGCTTCTG AGATC-3' and antisense 5'-GGGTTCAGTGACCCCTTC TC-3'. Human GAPDH primer (Sense 5'-TGCAACCACAAAC TGTTTACG-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 Vβ 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 Vβ families, this required 36 PCR amplification cycles, 29 TCR Vβ primers and a Fam-labeled Cβ reverse primer (18). All the TCR Vβ 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 µl) from this reaction was mixed with 12 µl Hi-Di™ Formamide and 0.2 µl 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 µm 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 µM CFSE (Molecular Probes; Thermo Fisher Scientific, Inc.). Responder cells (human PBMCs) were labeled with 0.5 µM CFSE (Molecular Probes; Thermo Fisher Scientific, Inc.) prior to stimulation. CFSE-labeled responder cells (1x10^5) from autologous Treg donors were stimulated with 2x10^5 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).
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 1x10^7 CD25^+ cell-depleted PBMCs with or without 1x10^6 autologous ex vivo expanded human Xeno-Treg or Poly-Treg were injected intravenously into NOD-SCID IL2Rγc−/− 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 µm) 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 ± 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 Vβ repertoire following xenoantigen stimulation. Spectratyping was used to analyze the TCR Vβ families at the CDR3 level and screen for clonal expansion of specific T cells (22-24). All 29 TCR Vβ families were detected in Xeno-Treg or Poly-Treg, and in control CD4+ T cells. However, there were altered TCR Vβ repertoires in both Xeno-Treg and Poly-Treg compared with in CD4+ T cells. Increased expression of TCR Vβ4, Vβ7-9, Vβ20, Vβ28 (>5% in repertoire) and TCR Vβ10 and Vβ18 were observed in Xeno-Treg and Poly-Treg compared with in CD4+ T cells (Fig. 3). In addition, expression levels of TCR Vβ4, Vβ10, Vβ18 and Vβ20 were markedly increased in Xeno-Treg compared with in Poly-Treg (Fig. 3).

Overall spectratyping of PCR products revealed a restricted TCR Vβ repertoire in Xeno-Treg and Poly-Treg compared with in CD4+ T cells, which possessed a diverse TCR Vβ repertoire. Nearly all the TCR Vβ families exhibited a Gaussian distribution, with the exception of TCR Vβ3, Vβ7-7, Vβ19 and Vβ23 (Fig. 4). Spectratypes of TCR Vβ4, Vβ20 and Vβ28 (>5% of the TCR Vβ repertoire and increased expression) possessed restriction and expanded clone at size 205, 196 and 274, respectively (Figs. 5 and 6). Spectratypes of TCR Vβ7-9 (>5% of the TCR Vβ 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 Vβ10 (<5% of the TCR Vβ 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 possessed more potent and xenoantigen-specific suppression, with expression levels 7-fold higher compared with Poly-Treg (Xeno-Treg: 86±16.4  pg/ml; Poly-Treg: 86±16.4  pg/ml; P<0.01, Xeno-Treg vs. Poly-Treg).

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 ± 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.
Figure 3. Gene expression of Treg TCR Vβ 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 Vβ repertoire gene spectratyping of CD4+ T cells. TCR Vβ families (A) TCR Vβ2-Vβ12 and (B) TCR Vβ13-Vβ30 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.
~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 1x10^5 CD25⁺ cell-depleted PBMCs with or without 1x10⁶ autologous ex vivo expanded Xeno-Treg or Poly-Treg were injected intravenously into NOD-SCID IL2γc⁻/⁻ 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±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±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 5. TCR Vβ repertoire gene spectratyping of Poly-Treg. TCR Vβ families (A) TCR Vβ2-Vβ12 and (B) TCR Vβ13-Vβ30 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.](image-url)
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 xenantigen stimulation was developed to selectively expand Xeno-Treg. Following stimulation, spectratyping was conducted to analyze the TCR Vβ 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 Vβ 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).
The present study identified an increase in the expression of TCR V\(^{\beta}4\), TCR V\(^{\beta}10\), TCR V\(^{\beta}18\) and TCR V\(^{\beta}20\) families for Xeno-Treg compared with Poly-Treg. In addition, spectratypes of TCR V\(^{\beta}4\), V\(^{\beta}10\), V\(^{\beta}18\) and V\(^{\beta}20\) 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\(^{\beta}\) 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 infiltration of effector cells and their function via IL-10 (10). In the present study, NOD-SCID IL2r\(^{y}\) mouse 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 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 ± 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.

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 ± 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.

Figure 8. Xeno-Treg suppress rejection of islet xenografts in humanized mice. (A) Percentage of graft survival in mice administered 1x10\(^7\) CD25\(^{+}\) cell-depleted human PBMCs with or without 1x10\(^6\) 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 \(^{y}\) mouse 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 ± 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.
JIN et al.: XENOANTIGEN-STIMULATED HUMAN Tregs PREVENT ISLET XENOGRAFT REJECTION

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 IL2rγc−/− 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 Vβ 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.
The authors declare that they have no competing interests.

Competing interests

All donors provided informed consent.

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