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CD27-Expressing Xenoantigen-Expanded Human Regulatory T Cells Are Efficient in Suppressing Xenogeneic Immune Response

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Abstract

Clinically, xenotransplantation often leads to T-cell-mediated graft rejection. Immunosuppressive agents including polyclonal regulatory T cells (poly-Tregs) promote global immunosuppression, resulting in serious infections and malignancies in patients. Xenoantigen-expanded Tregs (xeno-Tregs) have become a promising immune therapy strategy to protect xenografts with fewer side effects. In this study, we aimed to identify an efficient and stable subset of xeno-Tregs. We enriched $CD27^+$ xeno-Tregs using cell sorting and evaluated their suppressive functions and stability *in vitro* via mixed lymphocyte reaction (MLR), real-time polymerase chain reaction, inflammatory induction assay, and Western blotting. A STAT5 inhibitor was used to investigate the relationship between the function and stability of $CD27^+$ xeno-Tregs and the JAK3–STAT5 signaling pathway. A humanized xenotransplanted mouse model was used to evaluate the function of $CD27^+$ xeno-Tregs *in vivo*. Our results show that $CD27^+$ xeno-Tregs express higher levels of Foxp3, cytotoxic T-lymphocyte antigen-4 (CTLA4), and Helios and lower levels of interleukin-17 (IL-17) than their $CD27^-$ counterparts. In addition, $CD27^+$ xeno-Tregs showed enhanced suppressive function in xeno-MLR at ratios of 1:4 and 1:16 of Tregs:responder cells. Under inflammatory conditions, a lower percentage of $CD27^+$ xeno-Tregs secretes IL-17 and interferon-γ (IFN-γ). $CD27^+$ xeno-Tregs demonstrated an upregulated JAK3–STAT5 pathway compared with that of $CD27^-$ xeno-Tregs and showed decreased Foxp3, Helios, and CTLA4 expression after addition of STAT5 inhibitor. Mice that received porcine skin grafts showed a normal tissue phenotype and less leukocyte infiltration after reconstitution with $CD27^+$ xeno-Tregs. Taken together, these data indicate that $CD27^+$ xeno-Tregs may suppress immune responses in a xenoantigen-specific manner, which might be related to the activation of the JAK3–STAT5 signaling pathway.

Keywords

antigen-specific Treg, CD27, xenotransplantation, immunosuppression, inflammation, regulatory T cells

Introduction

Transplantation has been adopted as an effective medical procedure for patients who experience organ or tissue failure. The demand for transplantation is on the rise, leading to a worldwide shortage of human organs. Xenotransplantation involves the transplantation of cells, tissues, or organs from animal donors and has been investigated as an alternative to overcome the crisis. However, xenogeneic organs and tissues are more likely to be targeted by the human immune system, leading to life-threatening complications such as T-cell-mediated rejection (TCMR)[1]. TCMR remains one of the major impediments to the clinical application of xenotransplantation, mandating the lifelong use of immunosuppressants. However, the suppression of immune responses in a non-antigen-specific manner may lead to opportunistic infections and malignancies in patients after transplantation[2]. Therefore, advanced immunomodulation strategies need to be developed to prevent nonspecific immunosuppression while protecting xeno-organs.

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Polyclonally expanded $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs) are key regulators of immunological tolerance. Tregs help maintain immune homeostasis by suppressing cell-mediated immunity including xenogeneic responses[3–5]. Previous studies have revealed that Tregs express many distinct mediators such as Foxp3, IL10, cytotoxic T-lymphocyte antigen-4 (CTLA4), CD39, and CD73 that downregulate immune responses[6]. Among these, the transcription factor Foxp3 is the most well-known and specific biomarker for Tregs, and its stable expression is mediated by demethylation of the Treg-specific demethylated region (TSDR) of the Foxp3 gene locus[7]. Furthermore, the expression of other functional markers, such as Helios and CD44, correlated with the higher potency of Tregs in suppressing the proliferation of effector T cells (Teffs), subsequently tuning down host immunity[8,9].

However, to prevent possible infection or tumorigenesis due to global immunosuppression, Tregs must express antigen-specific suppressive functions. Previously animal studies have reported that engineered alloantigen-specific Tregs could efficiently protect allografts when administered at a low dosage[10,11]. Moreover, Tregs induced by xenoantigens could turn down xenogeneic responses more efficiently than can polyclonal Tregs[12], suggesting that xeno-induced Tregs may have xenoantigen-specific activity. Moreover, specific studies have shown that porcine-specific Tregs promote the survival of porcine islet grafts in diabetic mice[13,14]. Therefore, a subset of Tregs could evolve to render a specific suppressive effect on xenogeneic responses when xenoantigens are introduced. However, further studies are needed to decipher the mechanisms underlying the selection of xeno-specific Tregs.

We speculate that CD27, a cytokine receptor belonging to the tumor necrosis factor (TNF) receptor superfamily, may be involved in the immunosuppressive function of xeno-specific Tregs. CD27 is the main player in the CD27/CD70 costimulatory pathway, facilitating the activation, proliferation, and effector functions of T cells[15]. CD27 is constitutively but differentially expressed during T-cell development[16]. An earlier study associated CD27 expression with the survival of Tregs in mice; CD27 expression lowered the tumor-specific T-cell response and alleviated malignancy[17]. Moreover, tissue-resident memory Tregs in human skin show high levels of CD27 and promote full demethylation at the TSDR, stabilizing the expression of the Treg functional marker Foxp3[18]. Conversely, non-CD27-expressing polyclonal Tregs have been reported to be less suppressive and are considered non-Tregs[19,20]. Therefore, CD27 may be a key player in Treg-mediated immunosuppression, and the regulation of CD27 expression may allow Tregs to perform antigen-specific functions. However, the regulation of CD27 expression in xeno-Tregs remains unclear. We hypothesized that CD27 is differentially regulated in xeno-Tregs and that its expression could be correlated with the potency of Tregs to suppress xenoantigen-induced immune responses. Our study aimed to investigate the suppressive function and stability of CD27-expressing xeno-Tregs and their underlying mechanisms. We hope that the information obtained from this study will aid in the development of new strategies to regulate antigen-specific immunosuppression during xenotransplantation.

Materials and Methods

Isolation of Peripheral Blood Mononuclear Cells and Expansion of Human Tregs

The collection and use of human blood in this study were approved by the Human Research and Ethics Committee of the Third Xiangya Hospital of Central South University in Hunan, China (No: 2019-S240). Whole blood samples were collected from the healthy adult volunteers after obtaining their consent. Human peripheral blood mononuclear cells (PBMCs) were isolated using gradient centrifugation. $CD4^+CD25^+CD127^{dim/-}$ Tregs were enriched from human PBMCs using the $CD4^+CD25^+CD127^{dim/-}$ Regulatory T Cell Isolation Kit II (Miltenyi Biotec, CA, Germany) and cultured in the presence of h-IL-2 (400 IU/ml; R&D Systems, Minneapolis, MN, USA) and rapamycin (100 nM; Sigma-Aldrich, San Luis, MO USA). To prepare polyclonal Tregs (Poly-Tregs), the cells were stimulated with anti-CD3/CD28 Dynabeads (Invitrogen, USA) only at a 1:1 ratio. To prepare xenoantigen-expanded Tregs (Xeno-Tregs), the Dynabeads were combined with irradiated porcine PBMCs (30 Gy irradiated, from adult Westran pigs) and were used to stimulate the Tregs at a ratio of 4:1 (4×10^5 porcine PBMCs: 1×10^5 Tregs). Irradiated porcine PBMCs were supplied every 7 days. After 21 days, all the cells were harvested for subsequent experiments.

Flow Cytometry and Cell Sorting

To determine Treg phenotypes, flow cytometric analysis was performed by staining the samples with different combinations of the following: APC-H7 mouse anti-human CD4 (#560158; BD Biosciences, San Diego, CA, USA), APC mouse anti-human CD27 (#558664), PerCP-Cy™5.5 mouse anti-human CD25 (#560503), PE mouse anti-human CD152 (CTLA4) (#555853), anti-HLA-DR(L243) FITC (#347363), PE mouse anti-human CD62L (#555544), PE mouse anti-human CD69 (#560968), FITC mouse anti-human CD45RO (#561887), PE mouse anti-human CD278 (ICOS) (#557802), PE mouse anti-human CD197 (CCR7) (#560765), mouse anti-human CD127 (#557938), 4′,6-diamidino-2-phenylindole (DAPI) solution (#564907), PE Foxp3 monoclonal antibody (#PCH101) (#12-4776-42; eBioscience, USA), Helios monoclonal antibody (22F6), PE (#12-9883-42), IL-17A monoclonal antibody-FITC (#11-7179-42), and glucocorticoid-induced TNFR-related (GITR)-PE (R&D Systems, Minneapolis, MN, USA). Intracellular cytokine staining was performed using the Cytofix/Cytoperm™ fixation and permeabilization solution (BD Biosciences, San Diego, CA, USA), and intracellular

Foxp3 staining was performed using the Foxp3 fixation and permeabilization buffer set (eBioscience, USA). Data were acquired using a BD LSRFortessa or Beckman FC500 and analyzed using FlowJo software.

Cell sorting was performed using an FACSAria III (BD Biosciences, San Diego, CA, USA). Xn-Tregs were stained with CD4-APC-H7, CD25-PerCP-Cy5.5, CD27-APC, and DAPI by following the gating strategy (Fig. S3A). To ensure that cell sorting was efficient and accurate, a purity check was performed. Sorted cells were incubated overnight at 37°C in Treg culture medium without interleukin (IL)-2 for subsequent experiments.

Mixed Lymphocyte Reaction Suppressive Assay

A mixed lymphocyte reaction (MLR) suppressive assay was used to assess the ability of Tregs to suppress the proliferation of polyclonally (poly-), allogen (allo-), or xeno-stimulated responder cells[21]. To prepare polyclonally stimulated responder cells (Poly MLR), 1×10^5 carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled (Invitrogen, USA) autologous PBMCs were incubated with anti-CD3/CD28 Dynabeads at a 1:3 ratio. To prepare allo- or xeno-stimulated responder cells (Allo-MLR or Xeno-MLR), CFSE-labeled PBMCs were cocultured with 3×10^5 irradiated (30 Gy) allogeneic or xenogeneic PBMCs, respectively. Tregs were titrated into each MLR-responder cell cultures at four different ratios (1:1, 1:4, 1:16, and 1:64). After inoculation, Poly MLRs were cultured for 3 days[22], and allo- and xeno-MLRs were cultured for 5 days[23]. The proliferation of the responder cells (CFSE-positive cells) in the presence of Tregs was evaluated relative to that of the control. The percentage of proliferating responder cells cultured in the absence of Tregs was considered 100% proliferation and 0% suppression.

Real-Time Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted using an RNeasy Mini Kit (Qiagen, USA). Complementary DNA (cDNA) was synthesized using the Superscript III First-Strand Synthesis System (Life Technologies, Thermo Fisher Scientific, Lithuania). PCR primers specific for human actin, Foxp3, IL-10, TGF-β (transforming growth factor-β), CTLA4, CD39, CD73, CD44, IFN-γ (interferon-γ), IL-17a, and Helios were designed using Primer5 according to the genome sequences obtained from the National Center for Biotechnology Information (NCBI) database (Supplementary Table 1). Primers were synthesized by Sangon Biotech Co. Ltd. (Shanghai, China). The Delta Delta Ct method was used to calculate gene expression, where $2^{-\Delta\Delta Ct}$ represented the relative expression of each target gene.

Western Blot Analysis

The quantity of $CD27^{+/-}$ Tregs was measured using a BCA Protein Assay Kit (23227; Thermo Fisher Scientific, Lithuania). The antibodies used in this study were mouse anti-human Foxp3 (1:1,000, #ab20034; Abcam, UK), rabbit anti-Helios (1:1,000, #42427T; CST, USA), rabbit anti-STAT5 (1:1,000, #ab126832; Abcam, UK), rabbit anti-phosphorylated-STAT5 (p-STAT5, 1:1,000, #ab32364; Abcam, UK), rabbit anti-JAK3 (1:1000, #8827T; CST, USA), and rabbit anti-phosphorylated-JAK3 (1:1000, #8827S; CST, USA). Mouse anti-beta-actin (1:5,000, #66009-1-Ig; Proteintech, Rosemount, IL, USA) was used as an internal control. The blots were visualized using enhanced chemiluminescence (ECL; BioRad, California, USA). Band intensity was analyzed using Quantity One software.

In Vitro *Inflammatory Induction Assay*

To induce the production of inflammatory cytokines, sorted $CD27^{+/-}$ Tregs were cultured in a medium containing 10 IU/ml IL-2, 10 ng/ml IL-1β, 5 ng/ml IL-6, 25 ng/ml IL-21, 25 ng/ml IL-23, and 5 ng/ml TGF-β (R&D Systems, Minneapolis, MN, USA) for 7 days. Cells cultured in medium containing only 10 IU/ml IL-2 were used as the non-induction control[24]. Sorted Tregs were cultured in the upper chamber of a transwell system (pore size:0.4 μm) with or without anti-CD3/CD28 Dynabead-stimulated PBMCs in the lower chamber. Seven days after stimulation, the Tregs were collected and further incubated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, San Luis, MO USA), 1 μg/ml ionomycin (Sigma-Aldrich, San Luis, MO USA), and 1 μl/ml GolgiStop protein transport inhibitor (BD Biosciences, San Diego, CA, USA) for an additional 5 h prior to cytometric analysis of a percentage of IL-17a-secreting cells.

Phosphorylated STAT5 Inhibition

Tregs cultured for 21 days were collected, and 5, 10, or 20 μmol/l of STAT5 inhibitor (STAT5-IN-2) was added to it along with 20 IU/ml of IL-2 and incubated for 24 h. Inhibition was detected using fluorescence-activated cell sorting (FACS) with BB515 mouse anti-human CD27 (#564642) and PE mouse anti-Stat5 (pY694) (#612567) antibodies. The cells were permeabilized using Perm Buffer III and fixed with Fix Buffer I from BD Phosflow. The live cell percentage was calculated excluding PI-positive cells.

Skin Transplantation and Adoptive Cell Transfer

This animal study was approved by the Ethics Committee of the Third Xiangya Hospital of Central South University.

To prepare for skin transplantation, porcine skin grafts (200 μm thick, black) were obtained from the backs of 3- to 7-day-old piglets [designated pathogen-free (DPF); of either sex; obtained from Hunan Xeno Life Science] using an electric dermatome (Zimmer, Utrecht, The Netherlands). The piglets were anesthetized and sacrificed before surgery. The skin grafts were tailored to small squares (2×2 cm) and kept in ice-cold phosphate-buffered saline (PBS). All grafts were transplanted within 12 h.

NOD/ShiLtJGpt-Prkdcem26Cd52Il2rgem26Cd22/Gpt (NCG) mice (T001475; Gempharmatech, Jiangsu, China)

were housed under specific pathogen-free conditions at the Biological Services Facility of the Cell Transplantation and Gene Therapy Institute. Surgery was performed in 6- to 8-week-old male mice. Animals were subcutaneously injected with 0.05 mg/kg buprenorphine 0.5 h prior to surgery. Lidocaine was administered during and after surgery. During surgery, the animals were kept under complete anesthesia. The skin (2 × 2 cm) was removed from the lower dorsal region of the mice using sterile scissors. Porcine skin of the same size was patched using a medical adhesive (Compont, Beijing, China). The mice were kept warm at 37°C until they regained complete consciousness. The wound was covered with a circumferential abdominal bandage with ibuprofen for at least 7 days after surgery[25].

Thirty days after skin transplantation, the mice were divided into three experimental groups (PBMC, $CD27^+$ Treg, and $CD27^-$ Treg) and one negative control group. The mice in each group were intravenously injected with 1×10^7 human PBMCs with PBS (PBMC, $n = 3$), 1×10^7 human PBMCs with 3×10^6 autologous $CD27^+$ xeno-Tregs ($CD27^+$ Treg, $n = 5$), or 1×10^7 human PBMCs with 3×10^6 autologous $CD27^-$ xeno-Tregs ($CD27^-$ xeno-Treg, $n = 5$), respectively[26,27]. In addition, two mice in the negative control group were injected with 200 µl of saline. Skin grafts were collected from recipient mice at predetermined time points after adoptive cell transfer to assess infiltration of human leukocytes. Human immune cells in the mouse spleen were analyzed using FACS. The levels of human inflammatory cytokines in the mouse serum were detected using a CBA kit (BD Biosciences, San Diego, CA, USA).

Histology

The skin grafts were fixed in formalin, embedded in paraffin, and cut into 4-µm-thick sections using a microtome. The samples were prepared for both hematoxylin and eosin (H&E) staining and immunostaining. To prepare for immunofluorescence staining, the slides were incubated in $CD4^+$, $CD8^+$, and DAPI solutions. All the samples were viewed using a Nikon Eclipse E100 microscope.

Statistical Analysis

The results are presented as mean ± SD of at least three independent experiments from three individuals. The data were analyzed using SPSS version 22.0. Values of $P < 0.05$ were considered as statistically significant.

Results

Xenoantigen-Stimulated Tregs Showed Stronger Suppressive Activity and Higher CD27 Expression in Comparison With That of Polyclonal Tregs

After 21 days, Tregs stimulated by xenoantigen (xeno-Tregs) showed normal expression of surface markers $CD4^+CD25^+$ $CD127^{-/dim}$ and functional markers Foxp3 (99.05% ± 1.77%), CTLA4 (99.15% ± 1.7%), and GITR (98.55% ± 1.77%) as the wild-type polyclonal Tregs (poly-Tregs) *in vitro* (Fig. S1A–D). However, the expansion of xeno-Tregs (134.30 ± 37.85-fold) was much higher than that of poly-Tregs (51.52 ± 14.51-fold) (Fig. S2A, B).

Next, we evaluated the ability of xeno-Tregs to suppress the proliferation of responder cells compared with that of poly-Tregs. MLR showed no significant difference in their suppressive ability when Tregs were mixed with responder cells at ratios of 1:1 and 1:64. However, when xeno-Tregs and responder cells were mixed at ratios of 1:4 and 1:16, xeno-Tregs showed better suppressive activity than that of poly-Tregs ($P < 0.0001$) (Fig. S2C).

To explore whether the elevated xeno-Tregs-induced suppression of responder cell proliferation could be associated with a specific biomarker, FACS analysis was performed on xeno- and poly-Tregs to compare the expression of CD62L (96.36% ± 2.72% and 98.1% ± 0.99%), HLA-DR (87.6% ± 4.43% and 89.86% ± 4.33%), CD39 (77.58% ± 5.05% and 76.7% ± 6.38%), CD45RO (98.7% ± 0.79% and 99.09% ± 0.81%), ICOS (65.78% ± 6.50% and 65.72% ± 5.72%), CCR7 (99.56% ± 0.67% and 99.38% ± 1.15%), and CD27 (42.55% ± 5.30% and 22.05% ± 6.4%, respectively; $P < 0.01$). Among the markers screened, the expression of CD27 was significantly higher in xeno-Tregs than in poly-Tregs (Fig. S1E, F and Fig. 1A). Next, the gate strategy was used to sort xeno-Tregs into $CD27^+$ and $CD27^-$ subpopulations after 21 days of expansion (Fig. S3A). Porcine and non-Tregs cells were removed after sorting. The purity of $CD27^+$ (97.59% ± 1.88%) and $CD27^-$ (93.95% ± 5.60%) xeno-Tregs after sorting was >90% (Fig. S3B). After a day of resting, the viability of the sorted $CD27^+$ and $CD27^-$ xeno-Tregs was 92.17% ± 2.2% and 90.28% ± 4.68%, respectively; the yield of both types of Tregs was 92.17% ± 2.2% and 90.28% ± 4.68%, respectively (Fig. S3C, D). Notably, CD27 expression was observed in approximately half the xeno-Treg population, suggesting that xenoantigen stimulation might be required for the long-term stable expression of $CD27^+$. Taken together, the *in vitro* xeno-MLR results suggested that xeno-antigen-specific Tregs had stronger suppressive activity, which could be attributed to elevated CD27 expression.

$CD27^+$ Xeno-Tregs Showed Higher Expression of Functional Markers

To further investigate the possible role of CD27 in immunosuppression, we compared the expression of key Treg functional markers in $CD27^+$ and $CD27^-$ xeno-Tregs. Compared with that for $CD27^-$ cells, $CD27^+$ xeno-Tregs showed higher median fluorescence intensity (MFI) for Foxp3 ($CD27^+$: 1,567 ± 122; $CD27^-$: 1,199 ± 85), Helios ($CD27^+$: 1,875±154; $CD27^-$: 1,294 ± 137), CTLA4 ($CD27^+$: 1,898 ± 82; $CD27^-$: 1,221 ± 41), and GITR

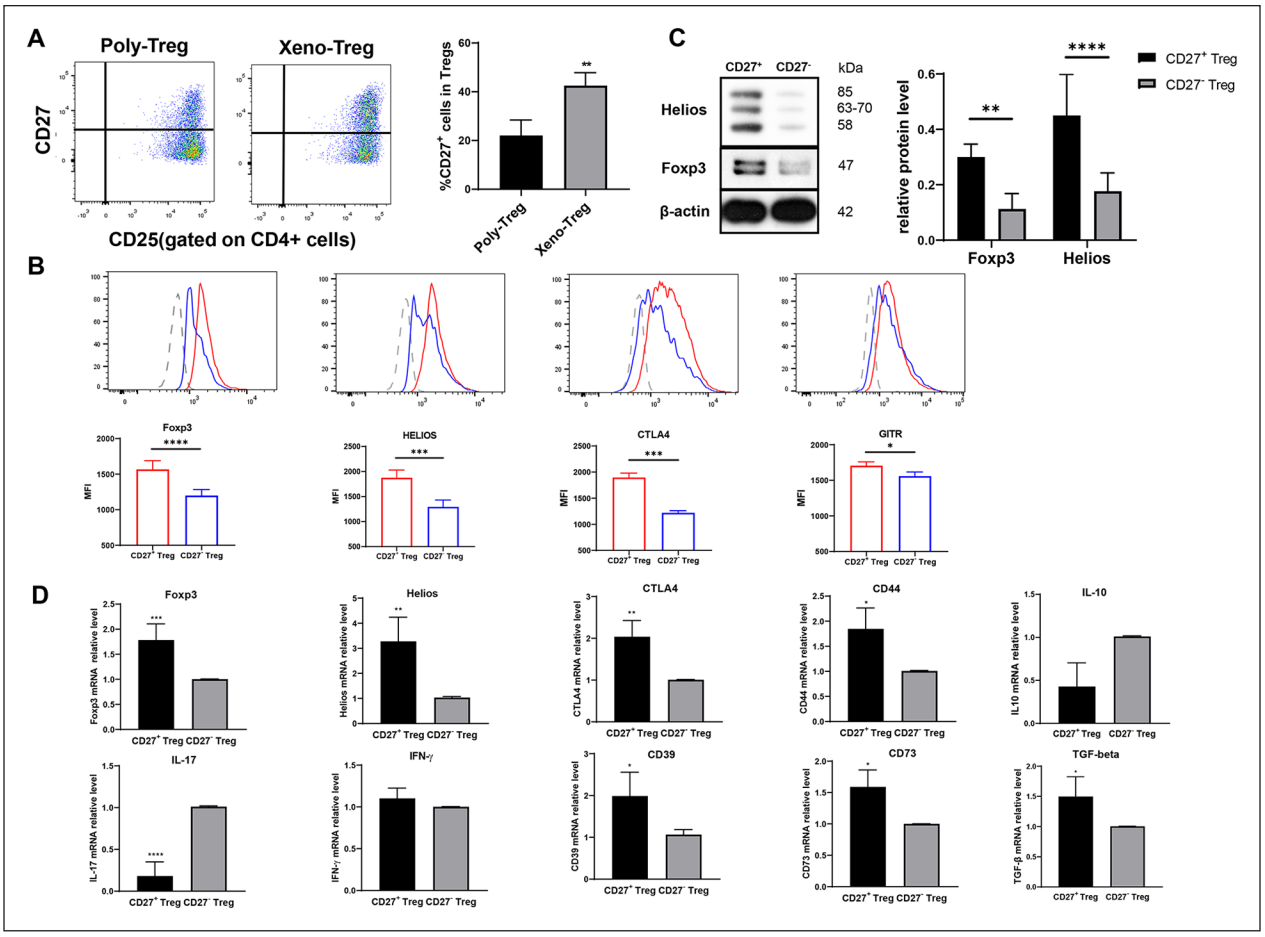

Figure 1. CD27$^+$ xeno-regulatory T cells (Tregs) showed higher expression of functional markers. (A) A fraction of the CD27-expressing poly-Tregs and xeno-Tregs. (B–D) FACS, Western blotting, and RT-PCR results of suppressive markers expressed by CD27$^+$ and CD27$^-$ xeno-Tregs after sorting. Data represent at least three independent experiments from three individuals. FACS: fluorescence-activated cell sorting; RT-PCR: real-time polymerase chain reaction; MFI: median fluorescence intensity; GITR: glucocorticoid-induced TNFR-related; IL-17: interleukin-17; IFN-γ: interferon-γ; TGF-β: transforming growth factor-β. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

(CD27$^+$: 1,706 ± 53; CD27$^-$: 1,560 ± 58) (Fig. 1B). Similarly, Western blotting analysis showed that the protein levels of Foxp3 and Helios were higher in CD27$^+$ xeno-Tregs than in CD27$^-$ xeno-Tregs (Fig. 1C). In addition, CD27$^+$ xeno-Tregs showed significantly increased mRNA expression of Foxp3, CTLA4, Helios, CD39, CD73, CD44, and TGF-β and significantly lower IL-17 mRNA levels (Fig. 1D). In addition, CD27$^+$ xeno-Tregs displayed markedly higher IL-10 mRNA levels than that for CD27$^-$ cells, but this difference was not statistically significant. Similarly, no significant difference was found in IFN-γ mRNA expression between the two Treg subpopulations after expansion. Taken together, our results suggest that CD27$^+$ expression positively correlates with the level of key functional mediators that downregulate immune responses, and CD27$^+$ may be associated with the immunosuppressive activity of Tregs.

CD27$^+$ Xeno-Tregs Have Stronger Xeno-MLR Suppressive Capacity

We performed xeno-MLR to investigate whether CD27 expression was correlated with xeno-Treg-mediated suppression of effector T-cell proliferation. Compared with that observed for CD27$^-$ xeno-Tregs, CD27$^+$ xeno-Tregs demonstrated significantly enhanced potency in suppressing the proliferation of xeno-reactive responder cells when Tregs were mixed with responders at ratios of 1:4 (CD27$^+$: 81.46% ± 5.78%; CD27$^-$: 70.25% ± 11.75%, $P < 0.05$) and 1:16 (CD27$^+$: 47.98% ± 9.42%; CD27$^-$: 25.01% ± 9.60%, $P < 0.0011$) (Fig. 2A). Both CD27$^+$ and CD27$^-$ cells showed greater suppression at a Treg:responder ratio of 1:1 (CD27$^+$: 94.87% ± 1.63%; CD27$^-$: 94.87% ± 1.65%) and poorer suppression at 1:64 (CD27$^+$: 2.16% ± 1.01%; CD27$^-$: 2.38% ± 1.23%) with no significant difference.

To investigate whether CD27 expression could be correlated with xenoantigen-specific suppression mediated by xeno-Tregs, we further performed allo-MLR and poly-MLR. In comparison with that of xeno-MLRs, $CD27^+$ and $CD27^-$ xeno-Tregs showed similar suppressive potency in poly-MLR (Fig. 2B) and allo-MLR (Fig. 2C). Taken together, our results suggest that $CD27^+$ xeno-Tregs were able to carry out xenoantigen-specific immunosuppression. Furthermore, CD27 can be used as an effective marker to identify Tregs with higher potency in suppressing the proliferation of xenoantigen-specific Teffs.

$CD27^+$ Xeno-Tregs Are Stable In Vitro *Under Inflammatory Conditions*

Tregs have been previously reported to transdifferentiate into Th17 (IL-17-producing $CD4^+$ T cells) and mediate inflammatory responses after adoptive transfer[28]. After 7 days of culture under Th17 polarized conditions *in vitro*, a small percentage of $CD27^+$ Tregs were found to be IL-$17a^+$ ($0.4\% \pm 0.34\%$) compared with cells cultured without Th17 polarization, whereas $8.06\% \pm 0.11\%$ of $CD27^-$ xeno-Tregs were converted to IL-17a-secreting cells (Fig. 3A). To further mimic the physiological conditions *in vitro*, Tregs were cultured with anti-CD3/CD28-stimulated PBMCs in a Transwell system for 7 days and similar results were obtained; a greater proportion of the $CD27^-$ xeno-Tregs were IL-$17a^+$ ($3.81\% \pm 0.94\%$ vs $0.34 \pm 0.10\%$) than that of the $CD27^+$ xeno-Tregs (Fig. 3B). Moreover, RT-PCR results showed that the mRNA levels of IL-17 and IFN-γ were elevated in $CD27^-$ xeno-Tregs, suggesting inflammation (Fig. 3C). Therefore, $CD27^+$ xeno-Tregs remain functionally stable *in vitro* and are less likely to transdifferentiate into inflammatory Th17 cells.

Higher Functional Marker Expression in $CD27^+$ Xeno-Tregs May Be Related to JAK3–STAT5 Signaling Pathway

Next, we explored the possible mechanisms underlying the stability of $CD27^+$ xeno-Tregs. STAT5 promotes Foxp3 expression[29] while restricting IL-17 production[30]. Protein analysis of $CD27^+$ Tregs showed higher expression levels of phosphorylated JAK3 (pJAK3) and STAT5 (pSTAT5) than that in $CD27^-$ Tregs; however, no significant difference was discerned in total JAK3 and STAT5 levels (Fig. 4A). Xeno-Tregs treated with predetermined concentrations of the pSTAT5 inhibitor STAT5-IN-2 (Fig. S4) showed that the expression of Foxp3, Helios, and CTLA4 was significantly decreased in $CD27^+$ Tregs but not in $CD27^-$ Tregs (Fig. 4B). This suggests that $CD27^+$ xeno-Tregs mediate their immunosuppressive function while bypassing transdifferentiation via JAK3–STAT5 signaling.

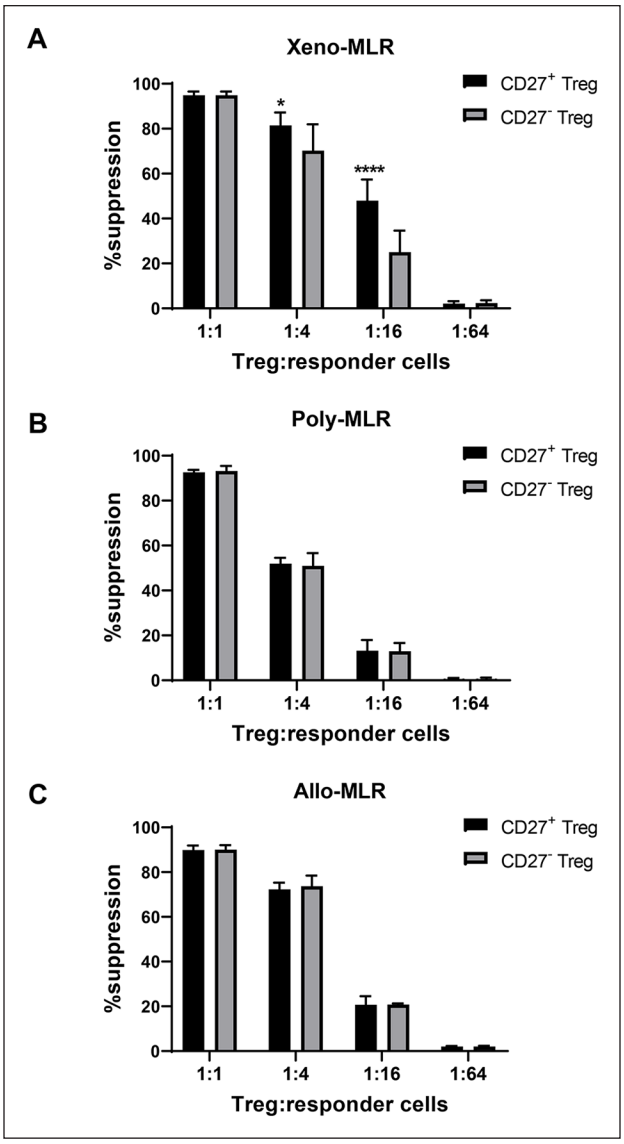

Figure 2. Suppressive capacity of $CD27^+$ and $CD27^-$ xeno-Tregs. Suppression of $CD27^+$ and $CD27^-$ xeno-Tregs in (A). Xeno-MLR, (B) poly-MLR, and (C) allo-MLR. Data represent at least three independent experiments from three individuals. MLR: mixed lymphocyte reaction. $^*P < 0.05$; $^{****}P < 0.0001$.

$CD27^+$ Xeno-Tregs Could Suppress Immune Responses and Prevent Rejection-Induced Tissue Injury In Vivo

Thirty days after xeno-skin graft transplantation, NCG mice were adoptively transfused with human PBMCs with PBS, $CD27^+$, or $CD27^-$ xeno-Tregs (PBMC, $CD27^+$ Tregs, and $CD27^-$ xeno-Tregs) (Fig. 5A). Porcine skin grafts were rejected following the injection of human PBMCs (Fig. 5B). $CD27^+$ Tregs showed stronger suppression of porcine graft rejection, but the difference was not statistically significant

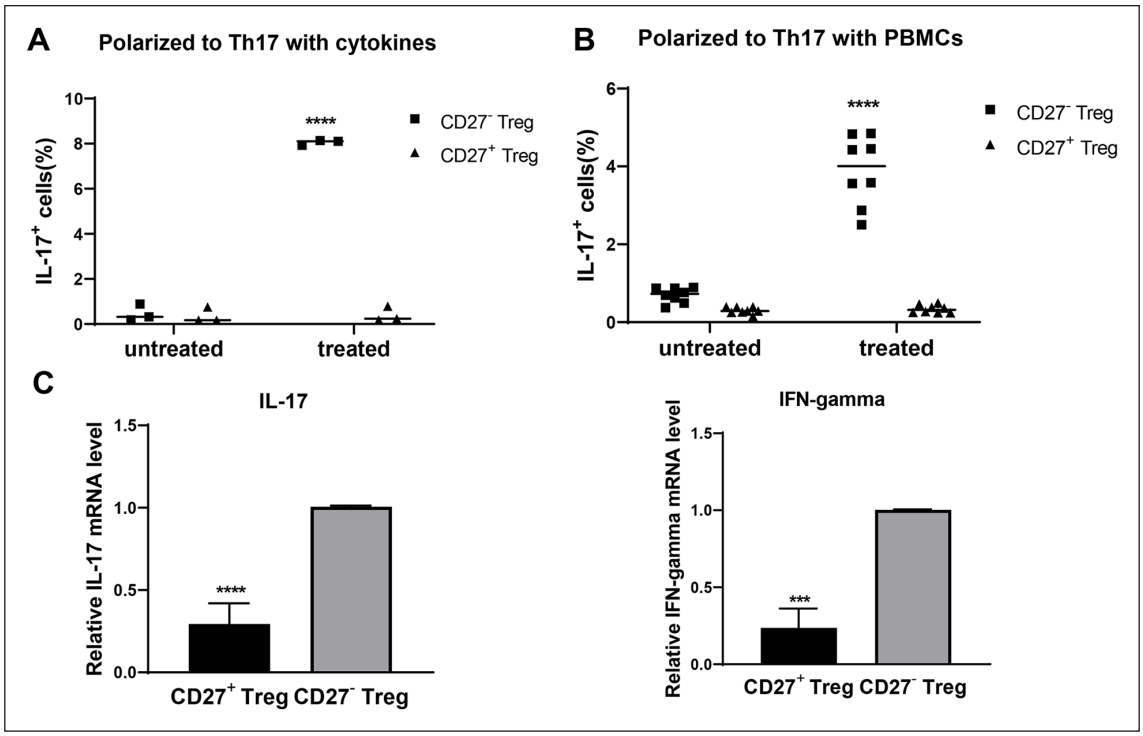

Figure 3. Stability of CD27$^+$ and CD27$^-$ xeno-Tregs under inflammatory condition. (A) Cells treated with proinflammatory cytokines (polarized Th17). (B) Cells treated with anti-CD3/CD28 Dynabeads stimulated with human PBMCs in a Transwell system with cytokine exchange only. (C) mRNA expression of CD27$^+$ and CD27$^-$ xeno-Tregs after induction by PBMCs. PBMCs: peripheral blood mononuclear cells; IL-17: interleukin-17; IFN-gamma: interferon gamma. ***$P < 0.001$; ****$P < 0.0001$.

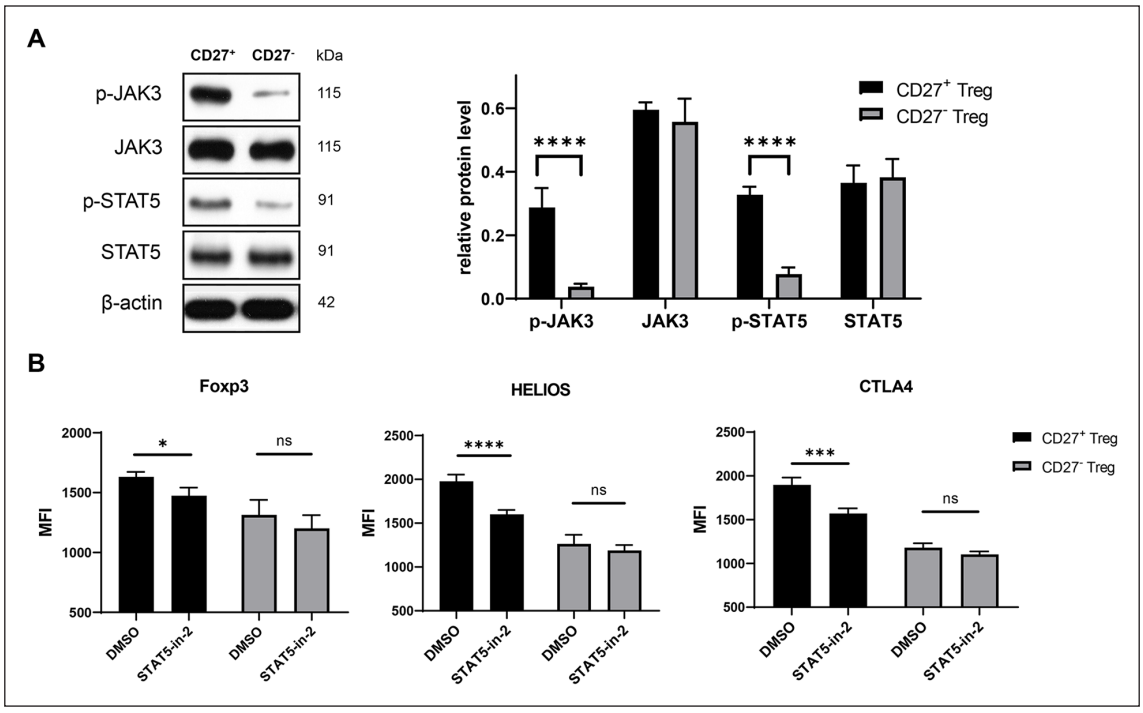

Figure 4. CD27$^+$ xeno-Tregs maintained stable *in vitro* expression of the functional markers related to JAK3–STAT5 pathway. (A) Protein expression of JAK3–STAT5 signaling in CD27$^+$ and CD27$^-$ xeno-Tregs. (B) Functional marker expression in CD27$^+$ and CD27$^-$ xeno-Tregs following pSTAT5 inhibition. MFI: median fluorescence intensity; ns: not significant; DMSO: dimethyl sulfoxide. *$P < 0.05$; ***$P < 0.001$; ****$P < 0.0001$.

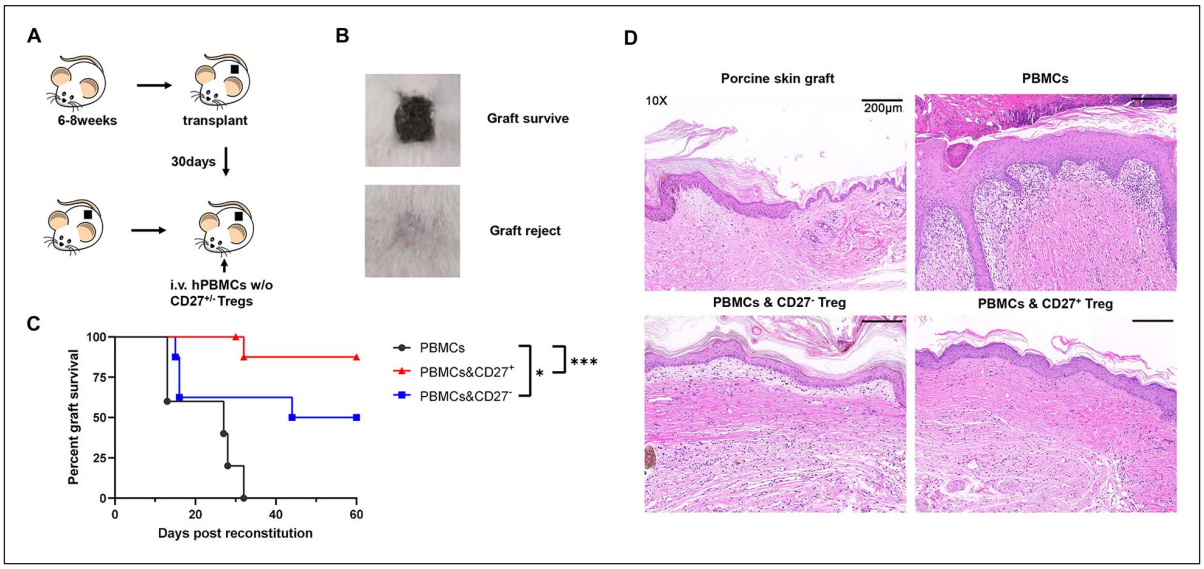

Figure 5. CD27^{+} xeno-Tregs protect xenograft in humanized mouse model. (A) The schematics for the animal experiment. (B) Appearance of skin grafts and (C) survival of the skin graft. (D) H&E staining showing the junctions of porcine skin graft and mouse skin and mononuclear cell infiltration after adoptive cell transfer. H&E: hematoxylin and eosin; hPBMCs: human peripheral blood mononuclear cells; PBMCs: peripheral blood mononuclear cells. *$P < 0.05$; ***$P < 0.001$.

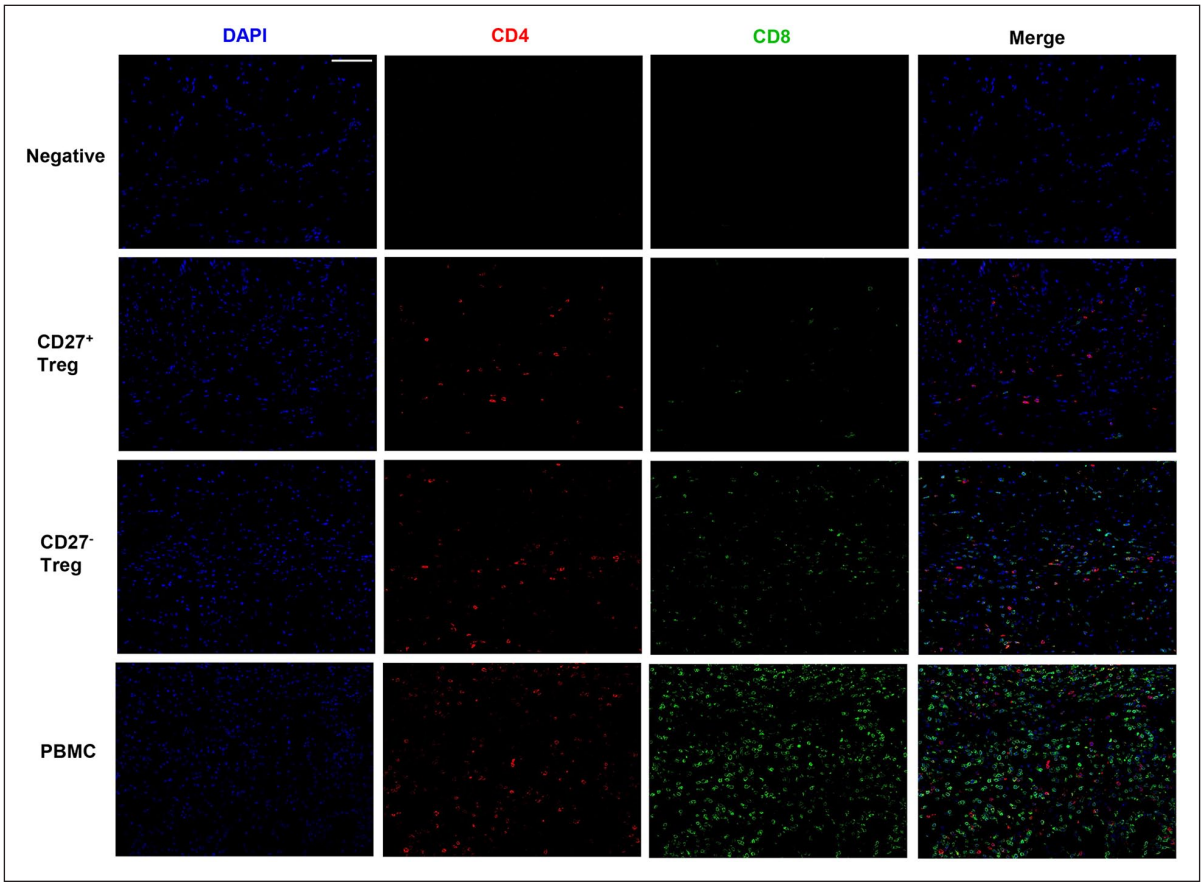

Figure 6. Immunofluorescence staining of skin xenografts. Magnification: 20×, scale bar: 100 μm. Red: human CD4^{+} T cells; green: human CD8^{+} T cells; blue: DAPI. PBMC: peripheral blood mononuclear cell; DAPI: 4′,6-diamidino-2-phenylindole.

($P = 0.07$). Both groups injected with Tregs significantly inhibited graft rejection compared with the PBMC-only group (Fig. 5C). H&E staining of the skin graft showed that the skin grafts in mice injected with PBMCs only depicted an irregularly thickened epidermis and dermal infiltration with more mononuclear cells, suggesting nonlethal graft rejection (Fig. 5D). In contrast, all mice injected with Tregs retained a normal epidermal thickness. Notably, $CD27^+$ Treg mice exhibited less mononuclear cell infiltration than did the $CD27^-$ Treg mice, suggesting that $CD27^+$ Tregs suppressed immune responses and helped bypass rejection-induced tissue injury.

Immunofluorescence staining showed that the epidermis of the skin grafts from positive control mice showed concurrent infiltration of both $CD4^+$ and $CD8^+$ T cells, with $CD8^+$ T cells covering most of the skin graft (Fig. 6). The negative control mice showed no traces of human $CD4^+$ or $CD8^+$ T cells. Mice injected with the Treg subpopulation showed concurrent infiltration of T cells, which was less than that of the positive control. Notably, the skin graft from the $CD27^+$ group was mainly infiltrated with $CD4^+$ cells, whereas the skin graft from the $CD27^-$ group was mainly infiltrated with $CD8^+$ cells.

The serum level of IFN-γ significantly decreased in $CD27^+$ Treg-reconstituted mice (1.04 ± 0.4 pg/ml) compared with that in $CD27^-$ Treg mice (32.16 ± 10.41 pg/ml) and PBMC mice (256.7 ± 53.59 pg/ml) (Fig. S5A). The leukocyte infiltration of the mouse spleen was similar to that observed in the skin graft, and the $CD27^+$ Treg group had a higher proportion of $CD4^+Foxp3^+$ cells in human CD45+ cells than in the other groups (Fig. S5B–D).

Discussion

Xenotransplantation is a promising solution to the organ shortage crisis. However, T-cell-mediated immune response remains an obstacle to the clinical application of xenotransplantation[1]. Adoptive immunotherapy is an alternative to immunosuppressants. *In vitro*–generated Tregs are the most promising agents for adoptive immunotherapy as they efficiently reducing the need for immunosuppressants while averting graft-versus-host disease (GvHD)[31,32]. Consistent with a previous study[12], we showed that compared with that observed for polyclonal Tregs, xenoantigen-expanded Tregs showed stronger immunosuppressive effect in their xenogeneic responses. We also reported that CD27 was upregulated in xenoantigen-expanded species, implying its importance as a marker of Treg-mediated xeno-antigen-specific immunosuppressive activity. More specifically, $CD27^+$ xeno-Tregs showed a higher expression of functional markers. Furthermore, $CD27^+$ xeno-Tregs exerted stronger xeno-specific suppression of the proliferation of xeno-MLR responder cells than did the $CD27^-$ xeno-Tregs *in vitro*. Although $CD27^+$ xeno-Tregs maintained their functional integrity *in vitro*, porcine skin–grafted mice exhibited alleviated inflammatory tissue damage caused by xenograft rejection after injection of $CD27^+$ xeno-Tregs. This suggests that $CD27^+$ xeno-Tregs could potently suppress the proliferation of xeno-specific Teffs both *in vitro* and *in vivo*, downregulate potential inflammatory tissue damage due to xenotransplantation, and protect the donor cells, tissues, and organs.

Tregs are natural mediators of immunosuppression[33]. However, enhancing their functional stability is crucial for the application of artificially expanded Tregs in clinical application. In this study, $CD27^+$ species performed better in suppressing the xenogenic response, implying that CD27 signaling is crucial for the regulation of Tregs and the subsequent downstream inflammatory responses. The signaling of CD27 was mediated through the CD27/CD70 costimulatory pathway, which provides an important regulatory mechanism for Treg stability[15–17]. CD27 belongs to the TNF receptor superfamily, accounting for both the generation and maintenance of T-cell immunity[17]. Specifically, the binding between CD27 and its unique ligand CD70 activates a signaling cascade that results in the differentiation and clonal expansion of T cells[17]. Consistent with our results, previous studies have reported that CD27 signaling prevents skin-resident Tregs from converting to proinflammatory Th17 cells during *Candida albicans* infection, thus downregulating the downstream inflammatory response[34]. Furthermore, we reported that $CD27^+$ xeno-Tregs suppressed the proliferation of xeno-stimulated responder cells. Specifically, we showed that CD27 expression lowered the level of $CD8^+$ T-cell infiltration, suggesting the suppression of skin graft rejection. Previous studies have shown that CD27 may have opposing effects on cell survival under different circumstances. For example, CD27 promoted or counteracted apoptosis on interacting with proapoptotic molecules such as SIVA1 or upregulating antiapoptotic factors such as Bcl-xl[35,36]. Therefore, induction by xenoantigens could cause CD27 to interact with proapoptotic molecules to suppress the survival of $CD8^+$ T cells. Taken together, the expression of CD27 on Tregs plays an important role in its functional stability, and xeno-specific stimulation is likely to be responsible for the regulation of effector T-cell proliferation.

Foxp3 expression is also crucial for Treg functional integrity. Tregs that lost Foxp3 expression acquired the ability to produce proinflammatory cytokines[37]. Helios is a signature marker present in thymus-derived Tregs, and $Helios^+$ Tregs express a more highly demethylated TSDR, facilitating the stable expression of Foxp3[9]. Our study reported that $CD27^+$ xeno-Tregs exhibited higher levels of Helios, which subsequently promoted the stable expression of Foxp3 and prevented the transdifferentiation of Tregs into Teffs such as Th17. Therefore, these xeno-Tregs are more likely to retain their functional stability during inflammation *in vitro*. However, further research is needed to determine whether a direct correlation exists between Helios and CD27 expression.

Furthermore, we propose that stable Foxp3 expression could be mediated through modulation of the JAK3–STAT5

signaling pathway. The JAK3–STAT5 signaling pathway affects T-cell differentiation and immune homeostasis by promoting the expression of Foxp3[38] and inhibiting the production of proinflammatory cytokines such as IL-17[30]. In our study, we observed that $CD27^+$ xeno-Tregs displayed high levels of phosphorylated JAK3 and STAT5, suggesting upregulation of this signaling pathway. A previous study associated CD27 expression with IL-2 production in $CD4^+$ Teffs[39]. Subsequently, IL-2-related cytokines facilitate the activation of the JAK3–STAT5 signaling pathway[40]. The $CD27^+$ xeno-Tregs showed a significant decrease in Foxp3, Helios, and CTLA4 after treatment with a pSTAT5 inhibitor, suggesting that the expression of these functional markers by $CD27^+$ Tregs is related to the activation of the JAK3–STAT5 signaling pathway.

In summary, our study showed that $CD27^+$ Tregs stimulated using xenoantigens were functionally stable Treg species that could mediate immunosuppression in a xenoantigen-specific manner. We believe that the information obtained from this study would improve our understanding of xenoantigen-expanded Tregs and open new avenues for the development of xenotransplantation and cell-mediated immunosuppressive therapies.

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Availability of Data and Materials

The datasets used and/or analyzed in the current study are available from the corresponding author upon reasonable request.

Ethical Approval

The study was approved by the Ethics Committee of the Third Xiangya Hospital, Central South University, Hunan Province (China batch number: 2019-S240).

Statement of Human and Animal Rights

All procedures in this study were conducted in accordance with the guidelines of the Ethics Committee of the Third Xiangya Hospital, Central South University, Hunan Province (China batch number: 2019-S240).

Statement of Informed Consent

Verbal informed consent was obtained from the healthy donors for their anonymized information to be published in this article.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplemental Material

Supplemental material for this article is available online.

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