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Human PD-L1-overexpressing porcine vascular endothelial cells induce functionally suppressive human CD4$^+$CD25^{hi}Foxp3$^+$ Treg cells

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ABSTRACT

In xenotransplantation models, direct activation of hCD4$^+$ T cells by porcine VECs leads to a robust proliferation of T cells. To investigate the underlying mechanisms, human antiporcine MLEC culture was used to investigate cross-species cell interactions, proliferation of hCD4$^+$ T cells, and induction of human cytokines. We report that xeno-antigen presentation by PIEC expands hCD4$^+$ Foxp3$^+$ Tregs and hCD4$^+$ Foxp3$^-$ Teffs, and this process is dependent on porcine MHC-II antigen expression. Stable transfection of hPD-L1 into PIEC inhibits Teff proliferation, but Treg proliferation is not affected. Surprisingly, IL-10 production by hCD4$^+$ T cells is augmented significantly by PIEC$^{hPD\text{-}L1}$. Notably, hPD-L1-induced Tregs have higher suppressive potency and mediate suppressive function partially through IL-10 and CD73. This study opens the possibility of using hPD-L1-overexpressing porcine VECs as a novel therapeutic to allow tolerance of xenotransplants and also supports the possibility of using hPD-L1 transgenic pigs as xenotransplant donors. *J. Leukoc. Biol.* 90: 77–86; 2011.

Introduction

Xenotransplantation offers the possibility of using animal organs (especially from the pig) as substitutes for human organs [1–3]. A ready supply of animal organs, tissues, and cells could resolve the critical shortage of human organs. The creation of genetically modified pigs has made clinical xenotransplantation a greater possibility by avoiding HAR. Notable genetic modifications include α-1,3-galactosyltransferase knockout pigs, which do not express the primary target for primate anti-pig antibodies, and pigs that express human complement-regulatory protein CD46 (membrane cofactor protein) or CD55 (decay-accelerating factor). Although the introduction of genetically modified pigs has increased resistance to HAR, xenografts are also subject to cell-mediated, chronic rejection [4]. Understanding the mechanisms that suppress the human antiporcine response remains an obstacle to xenotransplantation [5].

Xeno-derived hematopoietic APCs, such as DCs and macrophages, are potent activators of xenoreactive CD4$^+$ T cells and are responsible for acute xenograft rejection. However, hematopoietic APCs may decrease in number, thus increasing the importance of xenoantigen presentation to hCD4$^+$ T cells by donor vascular endothelium, which persists for the life of the graft. Direct recognition of MHC-II and costimulators on activated porcine endothelium causes hCD4$^+$ T cell activation and inflammatory cytokine production [6–10].

The PD-L1, also called B7 homologue 1, is a B7 family protein. It mediates immune tolerance by binding to the PD-1 on activated T cells [11–13]. PD-L1-deficienct mice are more susceptible to autoimmune diseases [14], and we also showed previously that PD-L1-overexpressing keratinocytes promote skin allograft survival by inducing IL-10-producing T cells [15]. Moreover, a PD-L1-Ig fusion protein shows potential for alleviating experimental autoimmune encephalomyelitis by inducing IL-10-producing T cells [12]. The relationship between PD-L1 and Foxp3$^+$ Tregs remains controversial. Some data suggest that PD-L1 negatively regulates Tregs during chronic inflammation [16], and other reports suggest that PD-L1 promotes the development of Tregs [17, 18].

In xenotransplantation models, xenografts undoubtedly mount a more robust inflammatory cell infiltration and acute

Abbreviations: 7-AAD=7-amino-actinomycin D, APCP=α,β-methylene ADP, CD73=ecto-5′-nucleotidase, CIITA=MHC-II transactivator, Foxp3=forkhead box p3, h=human, HAR=hyperacute rejection, mCIITA=mutant MHC-II transactivator, MFI=mean fluorescence intensity, MLEC=mixed lymphocyte endothelial cell, MST=median survival time, PD-1=programmed death-1, PD-L1=programmed death ligand-1, PIEC=porcine iliac artery endothelial cell, PIEC$^{hPD\text{-}L1}$=human programmed death ligand-1-overexpressing porcine iliac artery endothelial cell, PIEC^{mCIITA}=mutant MHC-II transactivator transfected into porcine iliac artery endothelial cell, PIEC^{mock}=porcine iliac artery endothelial cell transfected in parallel with an empty vector, SLA=swine leukocyte antigen, Teff=effector T cell, Treg=T regulatory cell, VEC=vascular endothelial cell

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stress response [2]. Extracellular ATP, as an important mediator of inflammation, triggers and maintains inflammation by activating inflammatory cells. Such ATP levels can be 1000-fold higher during inflammation. In the inflammation site, ATP can be converted into ADP by CD39 and finally catalyzed into adenosine by CD73. Notably, adenosine is an efficient anti-inflammatory mediator, dampening excessive inflammation. Recently, CD73 has emerged as an important molecule involved in the suppressive effects of Tregs [19]. It has been shown that expression of CD73 by murine Tregs enhances their suppressive function through the production of adenosine. The suppressive function of CD73 on human Tregs has not been well characterized yet [20, 21].

In this study, we investigated the interactions between PIEC and hCD4$^+$ T cells. Our results show that activated expression of MHC-II on PIEC promotes hCD4$^+$ Foxp3$^+$ Treg and Foxp3$^-$ Teff proliferation. Furthermore, PIEC$^{hPD\text{-}L1}$ inhibits proliferation of Teff without affecting Foxp3$^+$ Treg proliferation. We also demonstrate that PIEC$^{hPD\text{-}L1}$-induced hCD4$^+$ Foxp3$^+$ Tregs exhibit stronger suppressive activity compared with untreated hCD4$^+$ Foxp3$^+$ Tregs and mediate their suppressive activity partially through IL-10 and CD73.

As the vascular endothelium persists for the life of the xenograft, the ability of PIEC$^{hPD\text{-}L1}$ to inhibit Teffs and induce functionally suppressive Treg proliferation is important for xenograft tolerance induction. Our findings help reveal the possibility of using hPD-L1-overexpressing porcine VECs as a novel therapeutic to allow tolerance of xenotransplants. Our data also support the possibility of using hPD-L1 transgenic pigs as xenotransplantation donors.

MATERIALS AND METHODS

Cells and animals

Fresh blood samples were obtained with consent from normal, healthy donors, according to the ethical guidelines approved by the Human Research Ethics Committee of Shanghai Jiaotong University (Shanghai, China).

Bama and Banna miniature pigs were purchased form Shanghai Institute of Agriculture (Shanghai, China) and maintained in the animal facility of Shanghai Jiaotong University, according to institutional guidelines. All animal experiments were performed with the approval of the Shanghai Jiaotong University Animal Experimentation Committee.

Cell transfection

The pcDNA3-hPD-L1 plasmid was a kind gift from Dr. Lieping Chen (Johns Hopkins University, Baltimore, MD, USA). The PIEC line was a kind gift from Dr. Sheng Yun (University College of London, UK), and cells were transfected with pcDNA3-hPD-L1 using Fugene 6 transfection reagent (Roche, Germany) to create a hPD-L1-positive PIEC line (PIEC$^{hPD\text{-}L1}$). PIEC^{mock} served as a control. The transfected cells were selected in RPMI-1640 medium containing 400 μg/ml G418. Selection was performed for 2 weeks, medium was changed every 3 days, and cells were subsequently cloned by limiting dilution. hPD-L1 expression in PIEC was determined by flow cytometry on a FACSCalibur (Becton Dickinson, San Diego, CA, USA) using CELLQuest software. PIEC^{mCIITA} was determined, as described previously [10, 22].

T cell fractionation

hCD4$^+$ T cells were isolated using negative selection with magnetic beads (Miltenyi Biotec, Auburn, CA, USA) from PBMCs of healthy subjects. For some experiments, CD4$^+$ T cells were further separated into CD25$^+$ and CD25$^-$ fractions by using anti-CD25-coupled magnetic beads. The purity of the isolated cells was >98%, as determined by flow cytometry.

CFSE-based proliferation and stimulation of CD4$^+$ T cells

Enriched hCD4$^+$ T cells were labeled with CFSE using the Vybran CFDA SE Cell Tracer kit (Molecular Probes, Eugene, OR, USA), following the manufacturer's protocol. Briefly, CD4$^+$ T cells were suspended in PBS containing CFSE at 2.5 μM and then incubated for 15 min at 37°C. Cells were centrifuged and resuspended in culture medium.

PIEC, PIEC^{mCIITA}, PIEC$^{hPD\text{-}L1}$, or PIEC^{mock} (1×10^5) was cocultured with 1×10^6 CFSE-labeled or unlabeled CD4$^+$, or CD4$^+$CD25$^-$ T cells. Cells were cultured in 1 ml RPMI-1640 medium supplemented with 10% FBS (Hyclone, Logan, UT, USA), 100 U/ml IL-2 (BD PharMingen, San Diego, CA, USA), 100 U/ml penicillin, and 80 μg/ml streptomycin. Cells were cultured for an additional 6 days. In some cultures, 20 μg/ml anti-SLA-DR/DQ antibody (2E9/13; Serotec, UK) was added. Proliferation was measured by flow cytometric measurement of CFSE.

Treg preparation and functional assessment

PIEC$^{hPD\text{-}L1}$- or PIEC^{mock}-stimulated hCD4$^+$ T cells were labeled with anti-CD4 and anti-CD25 antibodies, and the CD25^{hi} subset was defined as the highest intensity of CD25 expression. CD4$^+$CD25^{hi} T cells were sorted from cocultures using FACSAria (Becton Dickinson) as suppressor cells, and CD4$^+$CD25$^-$ T cells were sorted from fresh PBMCs as responder cells. Fresh CD4$^+$CD25$^-$ T cells were activated with anti-CD3 and anti-CD28 antibodies (Miltenyi Biotec) in the absence or presence of 5′-AMP (5 μmol/L) and/or the selective CD73 inhibitor APCP (100 μmol/L). Cells were stimulated for 72 h, and then small volumes of supernatant were collected for cytokine assays, and ^{3}H-thymidine (1 μCi/well) was added to each well. Cells were harvested 18 h later, and proliferation was analyzed with a β-Trilux scintillation counter.

Cytokine production

Cell culture supernatants were collected at the indicated times and stored at –20°C. Concentrations of IFN-γ, TNF-α, IL-2, IL-4, IL-10, IL-17, and TGF-β in supernatants were determined by ELISA (eBioscience, San Diego, CA, USA).

Determination of cell-surface phenotypes

Cell staining was performed at 4°C unless stated otherwise. PIEC or CD4$^+$ T cells were stained with FITC, PE, APC, or Pcp-conjugated mAb against PD-L1, CD4, and CD25 (eBioscience) and SLA-DQ or SLA-DR (Serotec). Cells were analyzed by flow cytometry.

Intracellular staining for Foxp3

Intracellular Foxp3 staining was performed with the Alexa Fluor 647 anti-human Foxp3 staining kit (eBioscience), according to the manufacturer's instructions.

Apoptosis assay

Apoptosis was determined using annexin-V and 7-AAD staining. Briefly, 10^6 cells were washed in PBS and resuspended in 100 μl Hepes buffer with 2 μl annexin-V-fluorescein solution and 2 μl 7-AAD solution. Alternatively, the cells were stained with annexin-V-fluorescein, 7-AAD, and anti-CD4-PE and anti-CD25-allophycocyanin. After incubating for 15 min at room temperature, the cells were evaluated by flow cytometry. Cells that stained positive for annexin-V but excluded 7-AAD were interpreted as undergoing early apoptosis.

Quantitative real-time PCR

To quantitatively determine levels of pig Foxp3 and IFN-γ mRNA, real-time PCR was performed using the LightCycler RNA Master SYBR Green I kit

(Roche) with the following primers [23, 24]: FOXP3 sense TTCCCAGACTTTCTTTCACAACAT, antisense GCTGCTTCTCTGGAGCCTCCAG; IFN-γ sense TGGTAGCTCTGGGAAACTGAATG, antisense GGCTTTGCGCTGGATCTG; GAPDH sense GTGGAGTCCACTGGTGTCTTCACG, antisense AACTCCCTCTAACAGTATGAAGAG.

RNA was extracted from the freshly prepared PBMCs (4×10^6). Samples were run in triplicate, and the relative expression of Foxp3 or IFN-γ was determined by normalizing to GAPDH expression.

Pig skin transplantation

Skins from inbred Bama minipigs (Shanghai Institute of Agriculture) were transplanted to inbred Banna minipigs (Shanghai Institute of Agriculture). Transplantation was performed as described previously [25]. Briefly, under general anesthesia, four full-thickness skin graft beds (3×3 cm each) were prepared surgically from the lateral dorsal wall of each recipient Banna minipig. Full-thickness skin grafts were harvested from the dorsal wall of donor Bama minipigs and sutured in place using a 6-0 nylon line. $PIEC^{hPD\text{-}L1}$ or $PIEC^{mock}$ (2×10^9) was injected into recipient pigs via the portal vein on the same day. The grafts were observed daily for signs of rejection. It was scored as rejected when the transplanted skin was pale or dark brown and dull, whereas a ruddy and shiny skin graft was scored as surviving.

Statistical analysis

Results are expressed as mean ± SD. Intergroup comparisons were made using one-way ANOVA. A P value <0.05 was considered statistically significant.

RESULTS

Coculture of hCD4$^+$ T cells with PIEC causes proliferation of Tregs and Teffs

To evaluate whether PIEC induces proliferation of hCD4$^+$ T cells, we monitored intracellular dilution of CFSE. MLEC was performed using CFSE-labeled hCD4$^+$T cells as responders and nonirradiated PIEC as APCs. T cell proliferation was measured by flow cytometry at Day 6. As expected, hCD4$^+$T cells rapidly proliferated in response to PIEC. Notably, we noticed a significant increase in the proliferation of Foxp3$^+$ Tregs and Foxp3$^-$ Teffs after 6 days of coculture (**Fig. 1A**). After 6 days without PIEC, control hCD4$^+$ T cell counts were 1.92 ± 0.18% Foxp3$^+$ Tregs (mean±SD of triplicate wells), whereas those cocultured with PIEC had 14.3 ± 1.5% Foxp3$^+$ Tregs. The proliferation rate of Tregs (87.1±4.3%; mean±SD of triplicate wells) was similar to that of Teffs 88±5.2%; $P>0.05$) in response to PIEC, implying that activated PIEC causes robust and equal proliferation of Foxp3$^+$ Tregs and Foxp3$^-$ Teffs. For further verification of this phenomenon, purified hCD4$^+$ T cells from five individuals were used as responders and primed with PIEC in vitro, where all exhibited a similar response as that described above (Fig. 1B), supporting the conclusion that PIEC stimulates equal proliferation of Tregs and Teffs ($P>0.05$). These results indicate that PIEC is an excellent in vitro model in which to study human antiporcine cellular responses.

PIEC-stimulated proliferation of Tregs and Teffs depends on MHC-II stimulation

To develop potential therapeutics that enable xenograft tolerance, it is essential to investigate the molecular mechanisms of PIEC stimulation of hCD4$^+$ T cell growth. PIEC in our cultures expresses MHC-II after activation. After 5 days of coculturing PIEC and hCD4$^+$T cells, SLA-DR expression in PIEC increased 39%; the MFI increased from 3 to 122 ± 26. SLA-DQ expression in PIEC also increased 29%, as the MFI increased from 2 to 99 ± 18 (**Fig. 2A**, left).

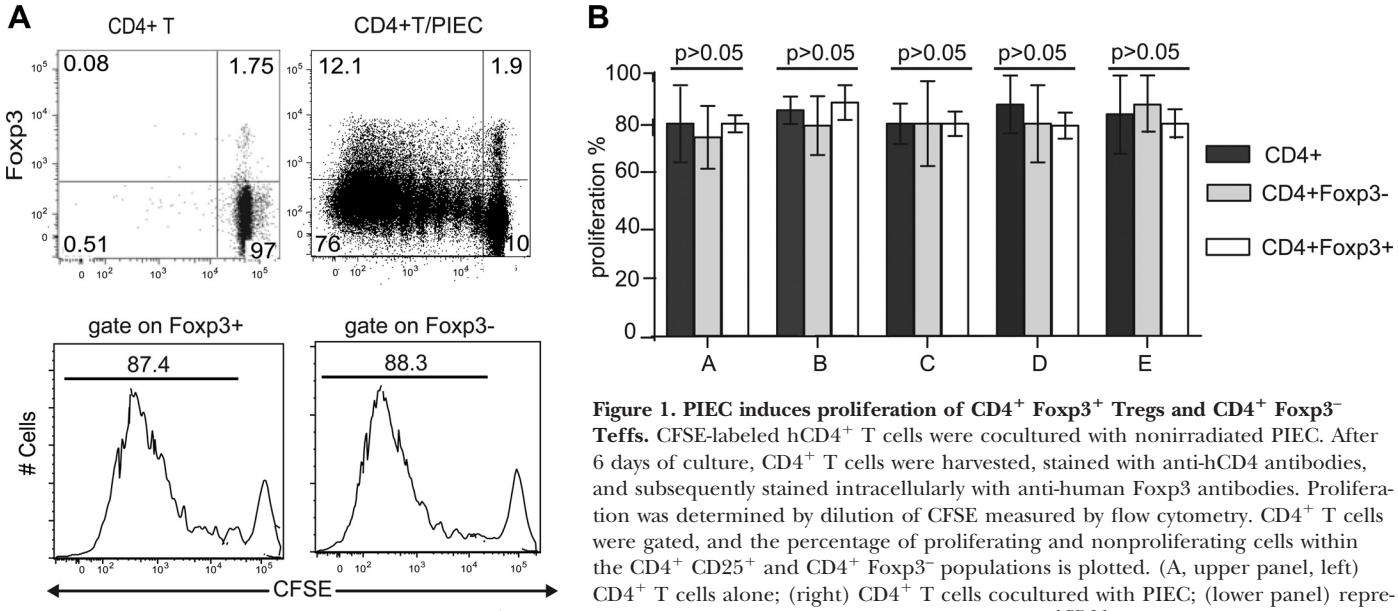

Figure 1. PIEC induces proliferation of CD4$^+$ Foxp3$^+$ Tregs and CD4$^+$ Foxp3$^-$ Teffs. CFSE-labeled hCD4$^+$ T cells were cocultured with nonirradiated PIEC. After 6 days of culture, CD4$^+$ T cells were harvested, stained with anti-hCD4 antibodies, and subsequently stained intracellularly with anti-human Foxp3 antibodies. Proliferation was determined by dilution of CFSE measured by flow cytometry. CD4$^+$ T cells were gated, and the percentage of proliferating and nonproliferating cells within the CD4$^+$ CD25$^+$ and CD4$^+$ Foxp3$^-$ populations is plotted. (A, upper panel, left) CD4$^+$ T cells alone; (right) CD4$^+$ T cells cocultured with PIEC; (lower panel) representative histograms showing proliferation of Foxp3$^+$ or Foxp3$^-$ cells after CD4 cells were stimulated with $PIEC^{hPD\text{-}L1}$. Data represent three independent experiments. (B) Letters A–E represent different individuals used as sources of responders. Proliferation rate is expressed as the percentage of proliferating cells in all CD4$^+$ T cell subsets (total CD4, Foxp3$^+$, Foxp3$^-$). Values are expressed as the mean ± SD from triplicate experiments. $P > 0.05$ between different CD4 subsets.

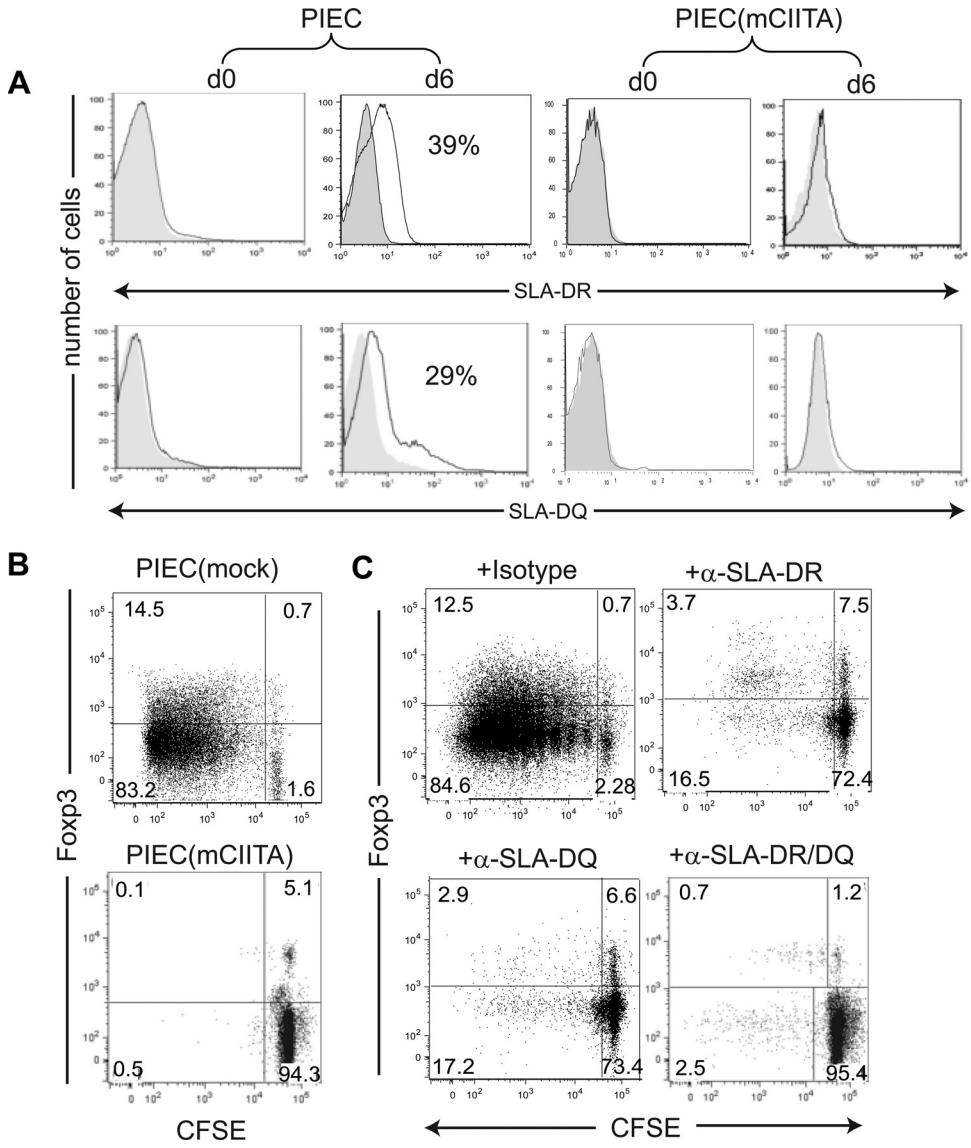

Figure 2. PIEC-induced proliferation of $CD4^+$ $Foxp3^+$ Tregs and $CD4^+$ $Foxp3^-$ Teffs depends on MHC-II expression. (A) Dominant-negative mCIITA suppresses MHC-II expression on PIEC. $hCD4^+$ T cells were cocultured with PIEC, $PIEC^{mCIITA}$, or $PIEC^{mock}$. At the indicated times, activated PIEC, $PIEC^{mCIITA}$, or $PIEC^{mock}$ was harvested and stained with anti-SLA-DR or anti-SLA-DQ-PE antibodies and analyzed by flow cytometry. (Left) Expression pattern of SLA-DR/DQ on activated PIEC from MLEC; (right) expression pattern of SLA-DR/DQ on activated $PIEC^{mCIITA}$ from MLEC. MHC-II expression on Days 0 and 6 are plotted. The expression pattern of MHC-II on $PIEC^{mock}$ is similar to that of PIEC (not shown). (B) CFSE-labeled $hCD4^+$ T cells were cocultured with PIEC in the presence of antiporcine MHC-II antibodies or with $PIEC^{mCIITA}$. After 6 days of coculture, cell surfaces were stained with anti-CD4 and stained intracellularly with anti-Foxp3, and proliferation was assessed by CFSE dilution using flow cytometry. Proliferation within the $CD4^+$ $Foxp3^+$ and $CD4^+$ $Foxp3^-$ subpopulations is plotted. $PIEC^{mCIITA}$ or $PIEC^{mock}$ was used as stimulators in MLEC. (C) PIEC/$CD4^+$ T cell coculture was performed in the presence of anti-SLA (α-SLA)-DR, anti-SLA-DQ, or isotype antibodies. Results are representative of three independent experiments.

We next investigated the role of MHC-II in the proliferation of $CD4^+$ T cells. CIITA is an essential coactivator for transcription of MHC-II genes. Previous transfection of $PIEC^{mCIITA}$ was able to completely repress IFN-γ-induced porcine MHC-II (SLA-DR/DQ) expression [22, 26, 27]. In this study, we observed that $PIEC^{mCIITA}$ in xenogeneic MLEC was almost suppressed completely (Fig. 2A, right). Additionally, we demonstrated that proliferation of $CD4^+$ $Foxp3^+$ Tregs and $CD4^+$ $Foxp3^-$ Teffs depends on MHC-II when $PIEC^{mCIITA}$ [22] was used as stimulator cells in coculture with $hCD4^+$ T cells. After 5 days in coculture with $hCD4^+$T cells, MHC-II expression was absent in $PIEC^{mCIITA}$. $hCD4^+$T cells ($Foxp3^+$ and $Foxp3^-$ T cells) did not respond to the $PIEC^{mCIITA}$ in MLEC (Fig. 2B). Proliferation of $PIEC^{mCIITA}$-stimulated $CD4^+$ T cells (0.53±0.07%; mean±SD of triplicate wells) was negligible compared with $PIEC^{mock}$-stimulated $CD4^+$ T cells (96.2±2.7%; $P<0.05$).

The importance of $CD4^+$ T cell activation by MHC-II on PIEC was also demonstrated by reduced proliferation of $hCD4^+$ T cells when cocultured with PIEC in the presence of anti-SLA-DR and anti-SLA-DQ antibodies. The addition of anti-SLA-DR and anti-SLA-DQ antibodies prevented proliferation of $CD4^+$ $Foxp3^+$ Tregs and $CD4^+$ $Foxp3^-$ Teffs in cocultures with PIEC (Fig. 2C). Treatment with anti-SLA-DR or anti-SLA-DQ alone reduced proliferation from 96.5 ± 4.5% (isotype-treated; mean±SD of triplicate wells) to 19 ± 2.9% ($P<0.01$) and 18.7 ± 3.2% ($P<0.01$), respectively. However, treatment with anti-SLA-DR and anti-SLA-DQ had the greatest effect (3.5±1.2%; $P<0.01$ vs. isotype). These results demonstrate that $CD4^+$T cells require MHC-II expression by PIEC for proliferation.

$PIEC^{hPD\text{-}L1}$ inhibits the proliferation of Teffs but not Tregs

The suppressive cosignaling molecule PD-L1 may allow protective Treg while minimizing inflammatory Teff responses. As PD-L1 induces the production of IL-10 by $CD4^+$ T cells and increases graft tolerance [13, 28–30], we investigated whether

the immunosuppressive effects of PD-L1 could be achieved by engineering PIEC to overexpress hPD-L1.

In this study, PIEC was transfected with the pcDNA3 vector, with or without the gene encoding the hPD-L1 protein. After four rounds of selection with the antibiotic G418, 10 randomly selected clones were analyzed periodically by flow cytometry to ensure stable expression of hPD-L1. The highest expressing clone ($PIEC^{hPD\text{-}L1}$) was chosen for further analysis. As indicated in **Fig. 3A**, hPD-L1 was expressed nearly uniformly in the selected PIEC clone, as assessed by flow cytometry, and it is noteworthy that the MFI of hPD-L1 expression on the selected clone was also high (MFI=483). This result indicated that almost all PIEC expressed hPD-L1, and the expression level of hPD-L1 in PIEC was also high. The control PIEC (transfected with vector alone, $PIEC^{mock}$) was negative for hPD-L1 expression.

To determine the suppressive capacity of $PIEC^{hPD\text{-}L1}$, $PIEC^{hPD\text{-}L1}$ and $PIEC^{mock}$ were used as stimulators in culture with CFSE-labeled $hCD4^+$ T cells. The proliferative capacity of primed T cells was determined by the CFSE dilution-based proliferation assay described in Materials and Methods. Proliferation of T cells was measured by CFSE dilution using flow cytometry 6 days after stimulation. As expected, $PIEC^{hPD\text{-}L1}$ primed $Foxp3^-$ $CD4^+$ T cells to undergo fewer divisions (87.2±3.9% vs. 94.5±1.9%; mean±SD of triplicate wells; $P<0.05$) based on CFSE dye dilution (Fig. 3B). However, $PIEC^{hPD\text{-}L1}$ enhanced the $Foxp3^+$ Treg proliferation (96.9±3.3% vs. 84.5±1.9%; $P<0.05$). Notably, $PIEC^{hPD\text{-}L1}$-primed hCD4 T cells expressed significantly higher Foxp3 levels (46±3.5%; $P<0.01$) than $PIEC^{mock}$-primed CD4 T cells (13.7±1.6%). Also, the MFI of $PIEC^{hPD\text{-}L1}$-induced Foxp3 (367±34) was much higher than that of $PIEC^{mock}$-primed Foxp3 (114±12; $P<0.05$). To address directly whether the hPD-L1-exposed Tregs are stimulated by the encounter or whether a potent subset selectively survives the encounter, the proliferation histograms of $Foxp3^+$ Tregs from $PIEC^{hPD\text{-}L1}$- or $PIEC^{mock}$-primed $CD4^+$ cells were overlaid and compared in Fig. 3B (right panel). The data clearly show that hPD-L1 significantly stimulates the proliferation of Tregs.

To further investigate the relationship between PD-L1 and apoptosis/necrosis, 7-AAD and annexin-V were measured to determine the percentage of apoptotic cells in the $PIEC^{hPD\text{-}L1}$-primed T cell population. After stimulation with $PIEC^{hPD\text{-}L1}$, the $hCD4^+$ T cells were harvested and stained with annexin-V and 7-AAD. These results demonstrate that PD-L1 enhanced the apoptosis of hCD4+ T cells (Fig. 3C).

Collectively, the results demonstrate that stimulation with $PIEC^{hPD\text{-}L1}$ reduces the proliferative capacity of $CD4^+$ Teffs while increasing their rate of apoptosis. Simultaneously,

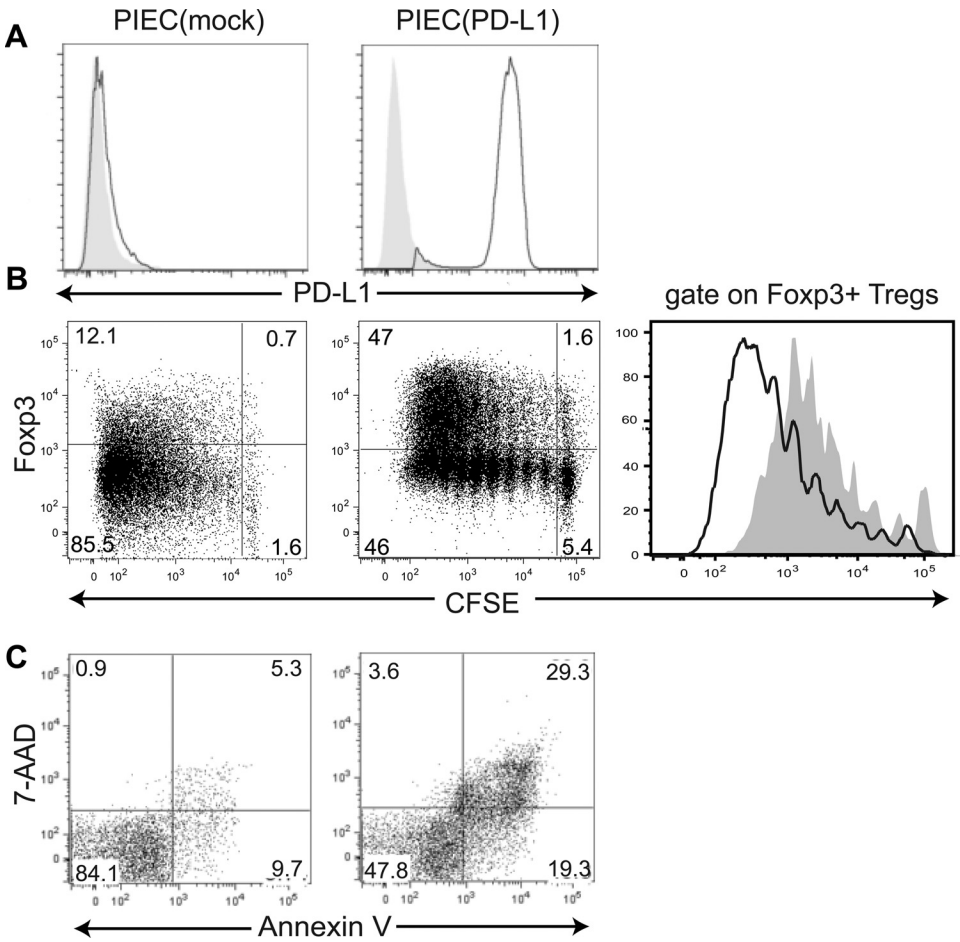

Figure 3. $PIEC^{hPD\text{-}L1}$ inhibits the proliferation of $CD4^+$ $Foxp3^-$ Teffs but not $Foxp3^+$ Tregs. (A) Phenotypic characterization of hPD-L1 transfectants. PIEC were transfected with the pcDNA3-hPD-L1 ($PIEC^{hPD\text{-}L1}$) plasmid or vector lacking the gene ($PIEC^{mock}$). After selection of stable transfectants with G418, $PIEC^{hPD\text{-}L1}$ and $PIEC^{mock}$ were stained with anti-hPD-L1-PE or control antibody and analyzed by flow cytometry. Shaded areas represent control antibody, and black lines represent anti-hPD-L1. (B) CFSE-labeled $CD4^+$ T cells were cocultured with $PIEC^{hPD\text{-}L1}$ or $PIEC^{mock}$. Proliferation was measured as described. Overlay of histograms (right panel) comparing the proliferation of $Foxp3^+$ Tregs in $PIEC^{hPD\text{-}L1}$ (black line)- or $PIEC^{mock}$ (shaded area)-primed $CD4^+$ T cells. (C) $CD4^+$ T cells were cocultured with $PIEC^{hPD\text{-}L1}$ or $PIEC^{mock}$, and the cells were then stained with annexin-V-FITC, 7-AAD, and anti-CD4-PE and evaluated by flow cytometry. Cells that stained for annexin-V but excluded 7-AAD were interpreted as undergoing early apoptosis. Cells staining for annexin-V-FITC and 7-AAD were undergoing late apoptosis or necrosis. One of three independent experiments is shown.

PIEC$^{hPD\text{-}L1}$ permits proliferation of CD4$^+$Foxp3$^+$ Tregs and therefore, could have implications for therapeutic strategies regarding immunosuppression and graft tolerance.

PIEC$^{hPD\text{-}L1}$ increases the number of Tregs by inducing the proliferation of CD4$^+$ Foxp3$^+$ Tregs but not through the conversion of CD4$^+$ Foxp3$^-$ Teffs into Foxp3$^+$ Tregs

To determine whether the increased number of Tregs in the MLEC was a result of expansion of the existing Foxp3$^+$ Tregs, a conversion of Foxp3$^-$ Teffs to Foxp3$^+$ Tregs, or a combination of both, MLEC was performed with purified hCD4$^+$ CD25$^-$ T cells in the absence of exogenous cytokines. In this experiment, CD4$^+$ CD25$^+$ and CD4$^+$ CD25$^-$T cells were fractionated from hCD4$^+$ T cells, and CD4$^+$ and CD4$^+$ CD25$^-$ T cells were stimulated with PIEC^{mock} or PIEC$^{hPD\text{-}L1}$ for 6 days. The expression of the transcription factor Foxp3, a specific marker for Tregs, was determined by intracellular staining flow cytometry with anti-human Foxp3 antibodies. As expected, there was a high expression of Foxp3 in fresh CD4$^+$ CD25$^+$ T cells. In contrast, the fresh CD4$^+$ CD25$^-$ T cell population was negative for Foxp3 expression (**Fig. 4, A** and **B**). To identify whether the increase in the CD4$^+$ Foxp3$^+$ Tregs was a result of the conversion of Foxp3$^-$ Teffs to Foxp3$^+$ Tregs or the proliferation of existing CD4$^+$ Foxp3$^+$ Tregs, we cultured purified CD4$^+$ CD25$^-$ T cells with PIEC, PIEC^{mock}, or PIEC$^{hPD\text{-}L1}$. After 6 days of coculture, ~30% of the CD4$^+$ T cells expressed CD25 at an intermediate level (CD25$^+$; data not shown), but expression of Foxp3 was undetectable, indicating proliferation of existing Foxp3$^+$ Tregs rather than conversion from CD4$^+$CD25$^-$ Teffs (Fig. 4, A and B).

PIEC$^{hPD\text{-}L1}$ enhances IL-10 but inhibits inflammatory Th1 and Th17 cytokine production by hCD4$^+$ T cells

PD-L1 has been shown previously to up-regulate IL-10 production by CD4$^+$ T cells [12, 15, 31, 32]. In the present study, highly enriched hCD4$^+$ T cells were cultured with PIEC$^{hPD\text{-}L1}$ or PIEC^{mock} in the absence of exogenous cytokines. After 6 days of coculture, supernatants were collected, and cytokines were analyzed by ELISA (**Fig. 5**). As expected, high levels of the human Th1 cytokines (IFN-γ, 3100 pg/ml; TNF-α, 300 pg/ml) and Th17 cytokine (IL-17, 160 pg/ml) were detected only in cultures containing PIEC^{mock}. Cocultures containing PIEC$^{hPD\text{-}L1}$ preferentially promoted IL-10 production (IL-10, 780 pg/ml) by hCD4$^+$ T cells. Increased IL-10 generally corresponded with decreased IFN-γ, IL-17, and TNF-α production. Also noteworthy is that the levels of IL-4 and TGF-β were very low or undetectable, and negligible amounts of TGF-β suggest that expansion of Foxp3$^+$ Tregs by PIEC$^{hPD\text{-}L1}$ is not through the classical TGF-β signaling pathway. Therefore, using PIEC$^{hPD\text{-}L1}$ as inducers engages hPD-L1 receptors on CD4$^+$ T cells, which inhibits Teff proliferation and enhances the production of IL-10 leading to apoptosis.

PIEC$^{hPD\text{-}L1}$-induced CD4$^+$ Foxp3$^+$ Tregs exhibit enhanced suppressive activity

Unlike murine Tregs, TGF-β-induced hCD4$^+$ Foxp3$^+$ T cells have been reported to be not suppressive [33–36]. As described above, TGF-β was undetectable in the culture. Thus, PIEC$^{hPD\text{-}L1}$-induced Tregs are not induced through the classical TGF-β pathway. We next asked whether PIEC$^{hPD\text{-}L1}$-induced CD4$^+$CD25^{hi}Foxp3$^+$ Tregs exhibit suppressive activity in vitro. CD4$^+$ CD25^{hi} (>85% Foxp3$^+$; data not shown) cells were sorted from cocultures of hCD4 cells with PIEC$^{hPD\text{-}L1}$ or

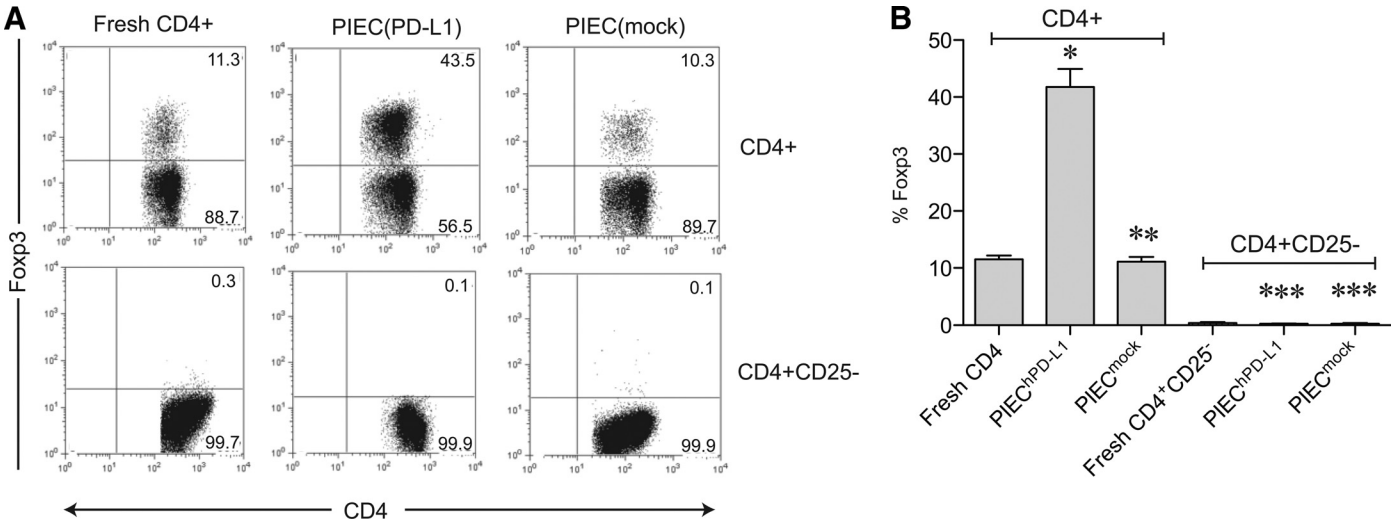

Figure 4. PIEC and PIEC$^{hPD\text{-}L1}$ increase the number of Tregs by inducing proliferation of CD4$^+$ Foxp3$^+$ Tregs rather than conversion of CD4$^+$ Foxp3$^-$ Teffs into Foxp3$^+$ Tregs. Enriched CD4$^+$ and CD4$^+$ CD25$^-$ T cells were cocultured with PIEC, PIEC^{mock}, or PIEC$^{hPD\text{-}L1}$ for 6 days. In vitro-stimulated CD4$^+$ and CD4$^+$ CD25$^-$ T cells were surface-stained with anti-hCD4 and intracellularly stained with anti-human Foxp3 antibodies. (A) Cells were analyzed by flow cytometry, and analysis of Foxp3 expression was performed after gating CD4$^+$ T cells. Data are representative of three independent experiments. (B) Frequency of cells expressing Foxp3 in CD4$^+$ or CD4$^+$ CD25$^-$ T cells that were treated as indicated. Data are the mean ± SD of three indpendent experiments. *$P < 0.05$ versus fresh CD4; **$P > 0.05$ versus fresh CD4; ***$P > 0.05$ versus fresh CD4$^+$ CD25$^-$.

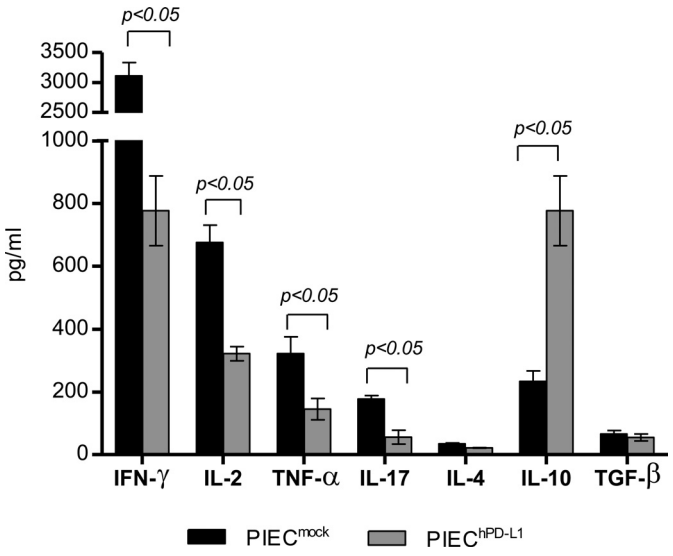

Figure 5. PIEC$^{hPD\text{-}L1}$ enhances IL-10 production but inhibits production of inflammatory cytokines Th1 and Th17 by hCD4$^+$ T cells. Enriched hCD4$^+$ T cells were cocultured with PIEC$^{hPD\text{-}L1}$ or PIEC^{mock} for 6 days, and culture supernatants were collected. Cytokine (IFN-γ, IL-2, TNF-α, IL-17, IL-4, IL-10, TGF-β) concentrations were determined by standard sandwich ELISA. Results are expressed as mean pg ± SD of triplicate cultures.

PIEC^{mock} and tested for their capacity to suppress activation of conventional CD4$^+$ CD25$^-$ T cells. In this experiment, fresh CD4$^+$ CD25$^-$ T cells were stimulated with anti-CD3/anti-CD28 mAb in the presence of unstimulated PIEC$^{hPD\text{-}L1}$- or PIEC^{mock}-induced CD4$^+$ CD25^{hi} T cells. The proliferation in the presence of PIEC$^{hPD\text{-}L1}$-induced Tregs was markedly lower than that in the unstimulated Treg controls ($P<0.05$; **Fig. 6A**). In agreement with previous reports, untreated or PIEC^{mock}-treated CD4$^+$ CD25^{hi} T cells had limited suppressive function. Thus, xenogenic PIEC$^{hPD\text{-}L1}$ markedly augments the suppressive potency of CD4$^+$CD25^{hi}Foxp3$^+$ Tregs.

PIEC$^{hPD\text{-}L1}$-induced CD4$^+$ Foxp3$^+$ Tregs mediate immunosuppression partially through IL-10 and CD73

To investigate the key suppressive factors for PIEC$^{hPD\text{-}L1}$-induced Tregs, the effect of IL-10 in the MLEC was tested using neutralizing mAb. The suppressive effect of TGF-β was not considered, as it was undetectable in the supernatant of the MLEC. In this study, CD4$^+$ CD25$^-$ cells were stimulated with anti-CD3/anti-CD28 mAb in the presence of PIEC$^{hPD\text{-}L1}$-induced CD4$^+$ CD25^{hi} T cells, and neutralizing anti-IL-10 mAb were added to block the suppressive cytokine IL-10. Subsequently, proliferation was assessed by ^{3}H-thymidine incorporation assay. As expected, PIEC$^{hPD\text{-}L1}$-induced Tregs inhibited the proliferation of Teffs by 80% ($P<0.05$; Fig. 6A), and anti-IL-10 mAb partially reversed the proliferation of Teffs by 50% ($P<0.05$; Fig. 6B) and IFN-γ production by 80% ($P<0.05$; Fig. 6D). These data indicate that PIEC$^{hPD\text{-}L1}$-induced Foxp3$^+$ Tregs mediate immunosuppression partially through IL-10.

CD73 has recently been described to be enriched in murine Foxp3$^+$ Tregs and involved in the suppressive function of Tregs [19, 37]. Our results indicate that PIEC$^{hPD\text{-}L1}$-induced hCD4$^+$ Foxp3$^+$ Tregs express more CD73 (56%) compared with unstimulated or PIEC^{mock}-induced cells (13%; Fig. 6C). To verify

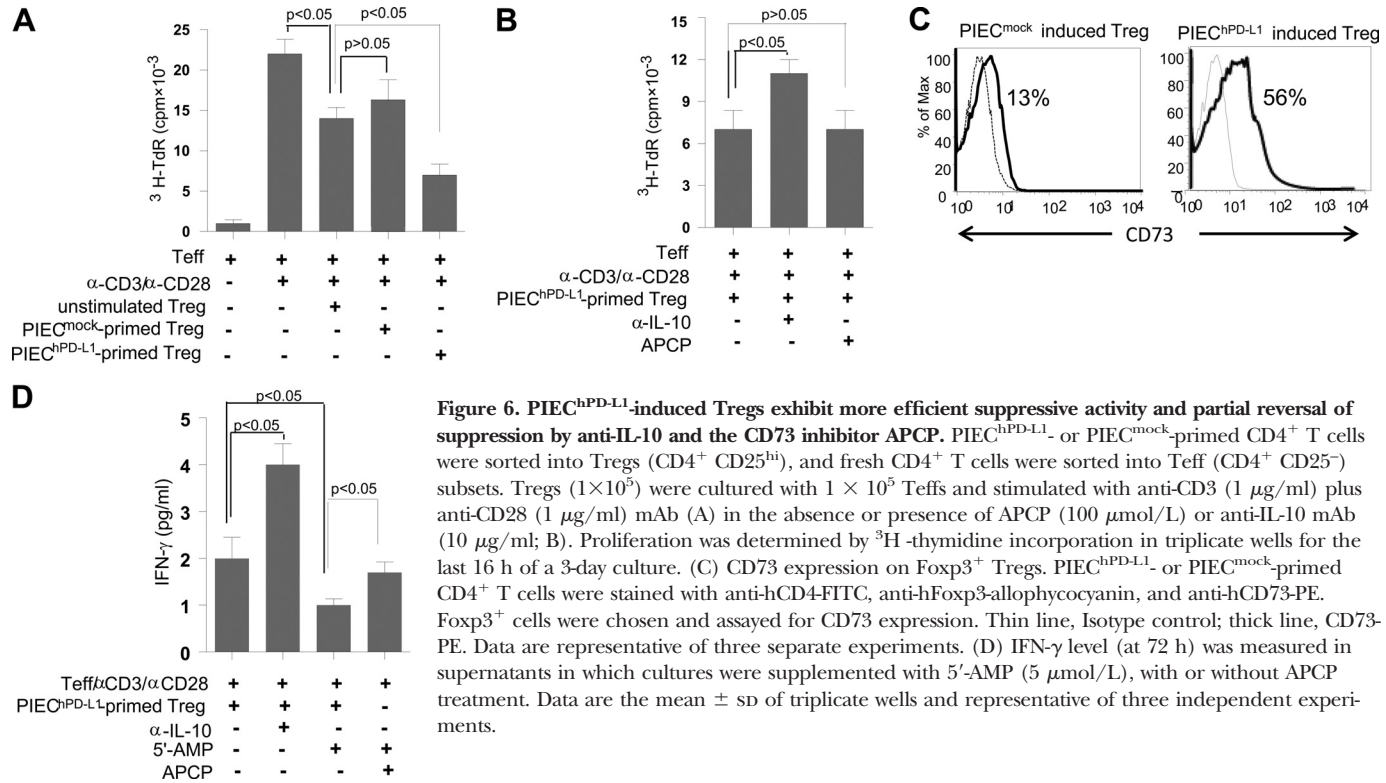

Figure 6. PIEC$^{hPD\text{-}L1}$-induced Tregs exhibit more efficient suppressive activity and partial reversal of suppression by anti-IL-10 and the CD73 inhibitor APCP. PIEC$^{hPD\text{-}L1}$- or PIEC^{mock}-primed CD4$^+$ T cells were sorted into Tregs (CD4$^+$ CD25^{hi}), and fresh CD4$^+$ T cells were sorted into Teff (CD4$^+$ CD25$^-$) subsets. Tregs (1×10^5) were cultured with 1×10^5 Teffs and stimulated with anti-CD3 (1 μg/ml) plus anti-CD28 (1 μg/ml) mAb (A) in the absence or presence of APCP (100 μmol/L) or anti-IL-10 mAb (10 μg/ml; B). Proliferation was determined by ^{3}H -thymidine incorporation in triplicate wells for the last 16 h of a 3-day culture. (C) CD73 expression on Foxp3$^+$ Tregs. PIEC$^{hPD\text{-}L1}$- or PIEC^{mock}-primed CD4$^+$ T cells were stained with anti-hCD4-FITC, anti-hFoxp3-allophycocyanin, and anti-hCD73-PE. Foxp3$^+$ cells were chosen and assayed for CD73 expression. Thin line, Isotype control; thick line, CD73-PE. Data are representative of three separate experiments. (D) IFN-γ level (at 72 h) was measured in supernatants in which cultures were supplemented with 5′-AMP (5 μmol/L), with or without APCP treatment. Data are the mean ± SD of triplicate wells and representative of three independent experiments.

that PIEC$^{hPD\text{-}L1}$-induced Tregs mediated immunosuppressive function through CD73, CD4$^+$ CD25$^-$ T cells were activated with anti-CD3/CD28 mAb in the presence of PIEC$^{hPD\text{-}L1}$-induced CD4$^+$ CD25^{hi} Tregs. Subsequently, the cultures were assessed for proliferation and IFN-γ production in the presence of APCP (a specific inhibitor of CD73 enzymatic activity). Other cultures were tested with the CD73 substrate 5′-AMP. As shown in Fig. 6B, the addition of APCP did not reverse the suppressive activity of Tregs on proliferation (Fig. 6B; $P>0.05$) but was able to reduce suppression of IFN-γ production (Fig. 6D). These functional assays proved that like murine Tregs, human Tregs express CD73 and appear to be able to generate adenosine that suppresses IFN-γ production.

PIEC$^{hPD\text{-}L1}$ induces immunosuppression in vivo

It is well known that some costimulatory molecules can overcome species barriers and mediate productive interactions between different species [38–40]. Cross-species interaction of PD-1 with PD-L1 has been reported before by several studies [41–44]. Then, we asked whether hPD-L1 could cross the species barrier and affect pig immune cells. An in vitro proliferation assay was set up in 96-well culture plates. Irradiated (3500 rad) PBMCs (10^5/well) from inbred Banna minipigs were cocultured with PBMCs (10^5/well) from the inbred Bama minipig in the presence of 2×10^4 PIEC$^{hPD\text{-}L1}$ or PIEC^{mock} as modulatory cells, and proliferation was determined by ^{3}H-thymidine incorporation for the last 16 h of a 7-day culture. We observed an ~50% inhibition of cellular proliferation compared with controls ($P<0.05$; **Fig. 7A**). The results indicate that addition of PIEC$^{hPD\text{-}L1}$ into pig MLRs efficiently inhibited the proliferation of pig PBMCs (Fig. 7A), thus establishing the cross-species effectiveness of hPD-L1 in pig models.

Next, the suppressive effect of PIEC$^{hPD\text{-}L1}$ was examined in vivo using a pig skin transplantation model. As described in Materials and Methods, 2×10^9 PIEC$^{hPD\text{-}L1}$ or PIEC^{mock} was injected into Banna recipient minipigs at the same day when allogeneic Bama minipig skin grafts were transplanted. Daily observation of the transplanted skins revealed that PIEC^{mock} treatment has no effect on skin graft survival with a MST of 9 days ($n=3$ pigs), which was similar to untreated, control recipients ($n=3$; $P>0.05$). However, PIEC$^{hPD\text{-}L1}$ treatment significantly prolonged skin graft survival with a MST of 19 days ($n=3$; $P<0.05$; Fig. 7B). Thus, PIEC $^{hPD\text{-}L1}$ is also effective in vivo.

As described above, PIEC$^{hPD\text{-}L1}$ enhances the expression of the protective Foxp3 and suppresses the proinflammatory IFN-γ in vitro. We further asked whether PIEC$^{hPD\text{-}L1}$ could enhance Foxp3 and inhibit IFN-γ in vivo. Real-time PCR was used to analyze Foxp3 and IFN-γ mRNA expression in PBMCs from skin-transplanted minipigs. Similar to the in vitro data, PIEC$^{hPD\text{-}L1}$-treated recipients have more Foxp3 expression (5.9±1.8; mean±SD; $n=3$ pigs; $P<0.05$) compared with the PIEC^{mock}-treated group (3.5±0.8; $n=3$; Fig. 7C). Furthermore, PIEC$^{hPD\text{-}L1}$-treated recipients have lower proinflammatory IFN-γ (4.8±0.5) than the PIEC^{mock}-treated group (6.6±1.7; $P<0.05$).

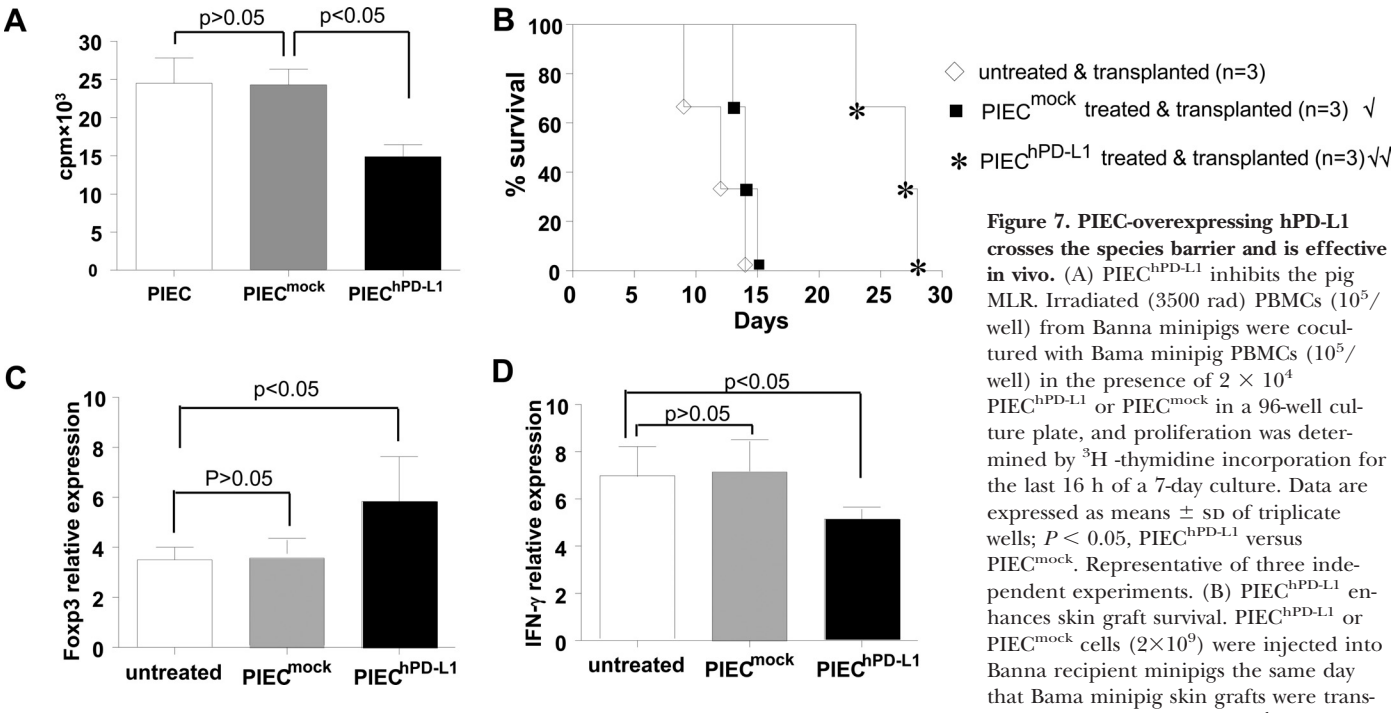

Figure 7. PIEC-overexpressing hPD-L1 crosses the species barrier and is effective in vivo. (A) PIEC$^{hPD\text{-}L1}$ inhibits the pig MLR. Irradiated (3500 rad) PBMCs (10^5/well) from Banna minipigs were cocultured with Bama minipig PBMCs (10^5/well) in the presence of 2×10^4 PIEC$^{hPD\text{-}L1}$ or PIEC^{mock} in a 96-well culture plate, and proliferation was determined by ^{3}H -thymidine incorporation for the last 16 h of a 7-day culture. Data are expressed as means ± SD of triplicate wells; $P < 0.05$, PIEC$^{hPD\text{-}L1}$ versus PIEC^{mock}. Representative of three independent experiments. (B) PIEC$^{hPD\text{-}L1}$ enhances skin graft survival. PIEC$^{hPD\text{-}L1}$ or PIEC^{mock} cells (2×10^9) were injected into Banna recipient minipigs the same day that Bama minipig skin grafts were transplanted. The Kaplan-Meier survival curve was plotted for each transplant group, with three pigs in each group. √, $P > 0.05$, PIEC^{mock} versus untreated; √√, $P < 0.05$, PIEC$^{hPD\text{-}L1}$ versus PIEC^{mock}. (C and D) Real-time PCR analysis of Foxp3 (C) and IFN-γ (D) mRNA expression in PBMCs from untreated or PIEC$^{hPD\text{-}L1}$- or PIEC^{mock}-treated recipient minipigs. Samples were run in triplicate, and the relative expression of Foxp3 and IFN-γ was determined by normalizing to GAPDH expression. Data are expressed as means ± SD ($n=3$ pigs for each group). $P > 0.05$, PIEC^{mock} versus untreated; $P < 0.05$, PIEC$^{hPD\text{-}L1}$ versus PIEC^{mock}.

DISCUSSION

The effectiveness of PD-1/PD-L1 targeting to modulate immune responses to allografts has been shown in various mouse models [28–30]. Generating transgenic pigs that express hPD-L1 might be effective for xenotransplantation. We addressed the question of whether ectopic expression of hPD-L1 in pig APCs alters their capacity to activate human T cells, which could lead to novel strategies to suppress cellular xenogeneic responses through the use of hPD-L1-expressing pigs as donors. We designed a xenogenic coculture system in which PIEC was stably transfected with the hPD-L1 gene and used as stimulators to determine their effects on $hCD4^+$ T cells. We show that the $PIEC^{hPD\text{-}L1}$ significantly inhibits the proliferation of Teffs, and IL-10 production by primed Teffs is increased dramatically. Our findings provide the rationale for using hPD-L1 transgenic pigs as potential xenograft donors. Notably, porcine PD-L1 interacts with hPD-1, which does not however affect our experiments, as PIEC does not express significant levels of pig hPD-L1 (data not shown).

Our in vitro data suggest that transgenic expression of hPD-L1 in pig cells can effectively reduce human Teff responses, which raises questions concerning the outcome of transplanting transgenic porcine organs in humans. If hPD-L1 is strongly expressed on porcine endothelial cells, major effects on the direct route of xenograft sensitization should appear. hPD-L1 on porcine vascular endothelium should directly activate specific T cells (Treg and Teff) and up-regulate PD-1. Among these cells, only inflammatory Teffs should be inhibited by transgenically expressed hPD-L1. As $PIEC^{hPD\text{-}L1}$ has been shown only to induce inhibitory functions if delivered together with TCR signals, it is conceivable that this approach to immune modulation will be graft-specific. Furthermore, we assume that porcine MHC-II-induced $Foxp3^+$ Treg expansion will not be suppressed, potentially allowing sustained control of antixenograft responses. Thus, ectopic expression of hPD-L1 in pig cells might be protective, not only during the sensitization phase of the human anti-pig T cell response but also in the effector phase.

Adenosine is a ubiquitous nucleoside that is usually at low concentrations in the extracellular space. The expression of CD73 on Tregs is important for understanding how metabolism of ATP to adenosine regulates inflammation. The expression of CD73 by murine Tregs and the presence of A2A adenosine receptor on activated Teffs generate immunosuppressive loops, whereby Tregs generate adenosine that inhibits the function of Teffs [19, 37]. The present study demonstrates that $PIEC^{hPD\text{-}L1}$-induced $hCD4^+CD25^{hi}Foxp3^+$Tregs are enriched for the expression of CD73, suggesting that such Tregs contribute to adenosine synthesis, which in turn, mediates anti-inflammatory function. Moreover, the attenuation of the inflammatory response may favor xenotransplant tolerance.

Normally, T cells in transplant rejection are biased predominantly toward the Th1 phenotype, as is evidenced by their ability to produce IFN-γ but little IL-4. We observed that $PIEC^{hPD\text{-}L1}$-induced $CD4^+$ T cells expressing markers associated with Tregs have significantly higher expression of CD39 and CD73. Murine Th cells expressing CD73 act on the 5′-AMP substrate to generate sufficient adenosine to suppress effector Th cell function. The current study confirms the importance of CD73 in regulating immune and inflammatory responses and extends this principle to human Th cells in systemic and mucosal tissues. In summary, the enrichment of CD73 in human Tregs suggests that Tregs contribute to local adenosine accumulation and the control of inflammation. Together, these observations support the conclusion that the production of adenosine and its ability to limit inflammation may contribute to the persistence of the transplant.

It has been reported recently that hPD-L1-overexpressing pig B cells inhibit proinflammatory cytokine production by hCD4 cells and expand Tregs in vitro [45]. However, as a result of ongoing decreasing of donor-derived B lymphocytes in transplantation models, the disadvantages of using genetically modified B lymphocytes as potential therapeutics cannot be ignored, especially as the lifespan of B cells is much shorter compared with other professional APCs. This increases the importance of donor vascular endothelium, which persists for the life of the graft and mediates direct contact with the recipient's peripheral immune system [6–10]. Thus, genetic modification of donor VECs has more advantages than other cells in xenotransplantation. Another contribution of our study is that it demonstrates that PD-L1 augments the expression of CD73 by human Tregs, which could dampen the overzealous immune response via catalyzing the production of adenosine.

Our data suggest a two-level model, whereby $PIEC^{hPD\text{-}L1}$ cells contribute to peripheral tolerance. Inhibition of unwanted, inflammatory T cell responses can be obtained by suppressive PD-1/hPD-L1 signaling, thereby inactivating "dangerous" T cells. However, the capacity of induced Tregs might be essential for the maintenance of long-term tolerance. The potential of the PD-1/hPD-L1 system to control tolerance is of particular interest for its possible applications in xenotransplantation. Organs from hPD-L1 transgenic porcine donors could reduce human antiporcine T cell responses. By inducing Treg proliferation, it might also be possible to control T cells sensitized to the xenograft through the indirect route that is required to achieve long-lasting tolerance.

AUTHORSHIP

Q.D. and L.L. performed the majority of the functional assays. X.Z. and Y.Z. performed partial functional assays and provided advice. Q.D. and K-Y.C. provided overall direction and supervised project planning.

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KEY WORDS:
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