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## Xenorecognition and Costimulation of Porcine Endothelium-derived Extracellular Vesicles in Initiating Human Porcine-specific T-cell Immune Responses

Shu Li<sup>1</sup>, Imran J. Anwar<sup>1</sup>, Aidan J. Canning<sup>3</sup>, Tuan Vo-Dinh<sup>3</sup>, Allan D. Kirk<sup>1,2</sup>, Xu He<sup>1,\*</sup>

<sup>1</sup>Departments of Surgery, Duke University School of Medicine, Durham, NC, USA

<sup>2</sup>Immunology, Duke University School of Medicine, Durham, NC, USA

<sup>3</sup>Biomedical Engineering, Duke University School of Medicine, Durham, NC, USA

### Abstract

Porcine vascular endothelial cells (PECs) form a mechanistic centerpiece of xenograft rejection. Here, we determined that resting PECs release swine leukocyte antigen class I (SLA-I) but not SLA-DR expressing extracellular vesicles (EVs) and investigated whether these EVs proficiently initiate xeno-reactive T cell responses via direct xenorecognition and costimulation. Human T cells acquired SLA-I<sup>+</sup> EVs with or without direct contact to PECs, and these EVs colocalized with T cell receptors (TCRs). Although IFN- $\gamma$ -activated PECs released SLA-DR<sup>+</sup> EVs, the binding of SLA-DR<sup>+</sup> EVs to T cells was sparse. Human T cells demonstrated low levels of proliferation without direct contact to PECs, but marked T cell proliferation was induced following exposure to EVs. EV induced proliferation proceeded independent of monocytes/macrophages, suggesting that EVs delivered both a TCR signal and costimulation. Costimulation blockade targeting B7, CD40L, or CD11a significantly reduced T cell proliferation to PEC-derived EVs. These findings indicate that endothelial-derived EVs can directly initiate T cell-mediated immune responses, and suggest that inhibiting release of SLA-I EVs from organ xenografts has the potential to modify xenograft rejection. We propose a secondary-direct pathway for T cell activation via xenoantigen recognition/costimulation from endothelial-derived EVs.

\*To whom correspondence should be addressed: He Xu, MD, Department of Surgery, Duke University School of Medicine, Edwin Jones Building Room 368, Durham, NC 27710 Phone: (919)684-4371, he.xu@duke.edu, allan.kirk@duke.edu.

Authorship:

Shu Li participated in experimental design, conduct of experiments, data analysis, and writing of the paper.

Imran Anwar participated in data analysis and writing of the paper.

Aidan Canning participated in conducting experiments, data analysis, and writing of the paper.

Tuan Vo-Dinh provided consultation to this study.

Allan D. Kirk participated in experimental design and writing of the paper.

He Xu participated in experimental design, conduct of experiments, data analysis, and writing of the paper.

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## Keywords

Xenotransplantation; porcine endothelial cells; extracellular vesicles; swine leukocyte antigen; xeno-recognition; xeno-costimulation

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## Introduction

Clinical allotransplantation is the best treatment for patients with end-stage organ failure. However, the chronic shortage of human allogeneic organ donors has impelled the development of xenotransplantation<sup>(1)</sup>. Pigs have been proposed as a potential source of organs due to their unlimited supply, variable size, similar physiology, and practicality of breeding in specific pathogen-free environment<sup>(2)</sup>. There has been renewed interest in xenotransplantation, driven by newly developed genetically engineered pigs, and prolonged survival of their organs in both kidney and heart xenograft nonhuman primate models<sup>(3–6)</sup>. The first pig-to-human cardiac xenotransplantation was performed recently<sup>(7)</sup> with limited but promising function and survival. Still, several aspects of xenotransplantation remain to be elucidated prior to widespread clinical practice, including ethical/social<sup>(8)</sup>, infectious disease<sup>(9)</sup>, histocompatibility<sup>(10)</sup>, and immune response<sup>(2, 4–7, 11–15)</sup> concerns.

Organ xenograft vascular endothelial cells are the first barrier between xenografts and host immunity and thus form a mechanistic centerpiece in initiating humoral and cellular xenograft rejection (XR)<sup>(2, 4, 11–15)</sup>. PECs are targeted during hyperacute and delayed-XR<sup>(2, 4, 11, 16–17)</sup>, and vascular infiltration of monocytes, macrophages, natural killer (NK) cells, and T cells feature prominently in cellular XR<sup>(12–15, 18)</sup>. Indeed, T cells and NK cells recognize and react with PECs *in vitro*<sup>(19–20)</sup>. It is also known that PECs express SLA class I and II antigens<sup>(21–24)</sup>, and PEC activation can be induced by platelet-derived CD154<sup>(25–26)</sup>. Furthermore, previous studies suggest that PECs constitutively express CD86, and the expression of CD80 can be induced on activated PECs<sup>(27)</sup>. As such, PECs can act as antigen presenting cells (APCs) and are recognized by human CD4<sup>+</sup> and CD8<sup>+</sup> cells resulting in xeno-reactive T cell proliferation<sup>(20, 28–31)</sup>, and T memory (T<sub>m</sub>) cell generation<sup>(28–29)</sup>. In particular, PECs may induce higher immune responses than allogeneic umbilical vein endothelial cells<sup>(31)</sup>. However, in non-sensitized individuals, xenoreactive T cells are predominantly naïve and thus require costimulation<sup>(29, 32–33)</sup>.

Allorecognition is known to occur via both the direct and indirect pathways<sup>(34)</sup>. Additionally, studies have demonstrated that donor-specific T-cell responses can be elicited by host APCs following acquisition of intact donor alloantigens<sup>(35–38)</sup>, a distinct alloantigen presentation pathway termed ‘semi-direct’ pathway allorecognition<sup>(35)</sup>. Importantly, emerging evidence has uncovered a significant role for extracellular microvesicles (EVs), released from donor dendritic cells (DCs), in semi-direct allorecognition and allospecific immune responses<sup>(39–41)</sup>. These immune cell-derived EVs express MHC and costimulatory molecules and are involved in antigen recognition/presentation and costimulation<sup>(42)</sup>. Furthermore, human endothelial cells, as allogeneic APCs<sup>(43)</sup>, can release EVs particularly during vascular injury, inflammation, or thrombosis, and these released EVs can represent up to 15% of circulating EVs<sup>(44–45)</sup>. However, it remains unknown

whether the endothelium-derived EVs play a role in direct xenoantigen presentation and costimulation.

Given that the endothelium is a primary target in xenotransplantation, we hypothesized that PECs could release SLA-expressing EVs, and that direct recognition of SLA<sup>+</sup> EVs by human T cells would induce xeno-reactive T cell activation and proliferation. Herein, we have characterized PEC-derived EVs, evaluated their capability of inducing T-cell proliferation, defined the maturation state of EV-responsive, xeno-reactive T cells, and established their susceptibility to costimulation and adhesion molecule blockade<sup>(5-7, 46-47)</sup>. These findings support a functional role of PEC-derived EVs in direct initiation of xeno-reactive T cell-mediated immune responses.

## Methods

### Monoclonal antibodies (mAb) and reagents

The fluorochrome-labeled monoclonal antibodies (mAbs) specific for human monocyte surface and intracellular molecules were used for flow cytometry analysis. The mAbs specific for T cell surface molecules anti-CD14-BV510 (Clone-M $\phi$ P9), anti-CD3-Alexa-fluor700 (Clone-UCHT1), anti-CD4-PECy7 (Clone-SK3), anti-CD4-V450 (Clone-RPA-T4), anti-CD8-APC (Clone-RPA-T8), anti-TNF- $\alpha$ -PE (Clone-MAb11), anti-IL-6-FITC (Clone-AS12), anti-IL-12-V450 (Clone-C11.5), anti-CD80-FITC (Clone-L307.4), anti-CD81-FITC (Clone-JS-81), Cytotfix/Cytoperm and GolgiPlug were obtained from BD Biosciences (San Jose, CA). Anti-CD8-APC-Fluor-780 (Clone-RPA-T8) was purchased from eBioscience (San Diego, CA). Anti-CD40-PercpCy5.5 was purchased from BioLegend (San Diego, CA). Anti-pig SLA-class-I-FITC (Clone-JM1E3) and anti-human CD9-FITC (Clone-MM2/57) were obtained from Bio-Rad (Hercules, CA). Anti-SLA class-II-DR-FITC (clone 2E9/13) was obtained from LifeSpan BioSciences (Seattle, WA). Fetal bovine serum (FBS), Dulbecco's modified eagle medium (DMEM), and RPMI-1640 medium were purchased from Thermo-Fisher Scientific (Waltham, MA). CD14-microbeads, CD4 cell purification kits, and pan-T-cell purification kits were obtained from Miltenyibiotic (Cambridge, MA). Recombinant human tumor necrosis factor alpha (hTNF- $\alpha$ ) and interferon gamma (hIFN- $\gamma$ ), and porcine IFN- $\gamma$  (pIFN- $\gamma$ ) were purchased from R&D System (Minneapolis, MN).

### Cells

PECs were isolated from aortas collected from outbred pigs (n=3). Cells were cultured in DMEM containing 10% FBS without endothelial cell growth factor followed by subculture as previously described<sup>(25)</sup>. Cultured PECs were verified by surface staining for SLA-I and SLA-DR expression following stimulation by hTNF- $\alpha$ , hIFN- $\gamma$ , and pIFN- $\gamma$ . PECs at passage-4 were diluted with FBS containing 10% DMSO at  $5 \times 10^5$  cells/mL followed by step-down freezing in a  $-80^\circ\text{C}$  freezer, and stored at  $-180^\circ\text{C}$ . All studies were performed using PECs at passage 5–6. All experiments were performed at least three times to obtain reproducible results.

Fresh human blood was collected from normal healthy individuals enrolled under an institutional review board–approved protocol (Pro00062495) following informed consent.

Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation in a vacutainer tube containing Ficoll-gel according to manufacturer's protocol (BD Biosciences). PBMCs were collected and washed before immunoassays.

Human CD14<sup>+</sup> monocytes were isolated from PBMCs using a positive selection kit (Miltenyi Biotec, Cambridge, MA), and the purity of cells was above 94%. Pan-T cells were purified from CD14<sup>+</sup> cell-depleted PBMCs using negative isolation kit (Miltenyi Biotec), and the purity of p-T cells was above 95%. The CD4<sup>+</sup> cells were purified from PBMCs using negative isolation kit (Miltenyi Biotec).

### **Intracellular cytokine staining (ICCS) of porcine endothelial cell-stimulated CD14<sup>+</sup> cells**

To investigate the xenospecific monocyte immune-responses, a 12-hour PBMC-PEC coculture was established in the presence of GolgiPlug. Briefly, PECs were cultured in 24-well plates until confluence.  $5 \times 10^5$  PBMCs were incubated with PECs for four hours followed by adding 1  $\mu$ l/mL GolgiPlug. PBMCs were harvested after 12-hour incubation and stained with mAbs specific for CD14, CD3, CD4, and CD8. Cells were incubated with Cytotfix/Cytoperm followed by ICCS to detect TNF- $\alpha$ , IL-12, and IL-6 producers. Cells were analyzed using fluorescence activated cell sorting (FACS), and data analysis was performed using FlowJo10.0.

### **Detection of monocyte-derived costimulatory molecule expression**

To evaluate the surface expression of monocyte-derived CD40 and CD80 in response to xenoantigens, a 24-hour PEC-PBMC coculture was established in 24-well plates containing PECs and  $5 \times 10^5$  PBMCs/well with or without transwell-insert. PBMCs were harvested and surface stained with mAbs specific for human CD40 and CD80 followed by FACS analysis.

### **Assays of intact PEC-derived SLA-I and SLA-DR transfer**

To verify SLA-I and SLA-DR expression before studies, the PEC monolayers with or without stimulation by cytokines (TNF- $\alpha$  or IFN- $\gamma$ ) were stained with anti-SLA-I-FITC or anti-SLA-DR-FITC mAb followed by FACS analysis demonstrating the expression of SLA molecules. The porcine splenocytes were used as controls. To evaluate PEC-derived EVs, PECs were cultured in T75-flask until sub-confluence, and treated with or without pIFN- $\gamma$ . Cells were washed, and 10 mL cultured medium that was pre-filtered by 0.20  $\mu$ m filter (Corning, NY) was added to the culture followed by incubation at 37°C. The culture media were collected and underwent centrifugation followed by enrichment with a protein concentrator (Thermo Scientific, Rockford, IL) with 100 kilodalton membrane filter pore size. 200  $\mu$ L enriched PEC-EVs were collected and then stained with FITC-labeled mAb specific for SLA-I or SLA-DR or isotype Ig for 30 minutes on ice. The SLA-I e or SLA-DR-expressing EVs were validated by FACS analysis after wash with filtered PBS buffer. Isolated-EVs were also characterized by FACS analysis after staining with anti-CD9 and anti-CD81 mAb (Fig S1a). Furthermore, a NanoSight-NS500-system (Malvern Panalytical, Malvern, UK) was utilized to characterize the size distribution of isolated-EVs (Fig S1b). The isolate-EVs were evaluated by Transmission Electron Microscopy (Fig S1c). The PECs were stained with live/dead dye, and analyzed by FACS analysis to confirm the lack of cell death (Fig. S1d).

To determine the direct acquisition of PEC-derived SLA-I antigens by human monocytes and T cells (n=6), the resting PECs were pre-labeled with FITC-conjugated anti-SLA-I mAb or isotype Ig control followed by extensive washing to remove unbound mAbs. A PEC-PBMC coculture with or without transwell-inserts were carried out for 12 hours. Cells were collected and stained with mAbs specific for T cell and monocyte surface markers followed by FACS analysis. The SLA-I expression on PECs at the end of cocultures was also verified by FACS analysis.

To determine the acquisition of PEC-derived SLA-expressing EVs by human T cells and monocytes, EVs were isolated from supernatants collected from resting PECs or pIFN- $\gamma$ -stimulated PECs as described above. The EVs were pre-labeled with anti-SLA-I-FITC or anti-SLA-DR-FITC mAbs or isotype Ig and washed to remove unbound mAbs. EVs were incubated with PBMCs (n=6) for four hours. The cells were washed twice and stained for CD3, CD4, CD8, and CD14 followed by FACS analysis.

To evaluate the correlation between PEC-derived EVs and TCR, the purified pan-T cells (n=3) were incubated with SLA-I-FITC labeled EVs for four hours and stained with mAb specific for CD3 and TCR- $\alpha\beta$ . Cells were acquired with ImageStream<sup>®</sup>X Mark-II-Imaging-FACS (Amnis Corp. Seattle, WA) and analyzed using ImageStreamX Mark-II Software IDEA 4.0 to measure colocalization of SLA-I with TCR- $\alpha\beta$  on CD3<sup>+</sup> cells.

### **Xenogeneic T cell proliferation assays**

To investigate and define the xeno-recognition, costimulation, and proliferation of human T cells with or without direct contact to PECs, a VPD450-based T cell proliferation was performed. First, the proliferative responses of PBMCs with or without CD14<sup>+</sup> cells and purified pan-T cells following direct contact with PECs were measured by a mixed-lymphocyte-endothelial cell reaction assay as described previously<sup>(35)</sup>. Cells were collected after 6 days incubation, and surface stained with mAbs followed by FACS analysis. In selective experiments, PBMCs were added into 1  $\mu$ M pore sized transwell inserts and placed in a 12-well plate containing PEC monolayers followed by coculture for 6 days to determine whether T cell proliferation can be induced without direct contact to PECs.

To investigate whether the PEC-derived EVs could induce xeno-specific T cell proliferation, the enriched PEC-EVs were prepared as described above. The 200  $\mu$ L of enriched PEC-EVs (75 cm<sup>2</sup> cultured PECs) was reconstituted with 1.8 mL T-cell culture medium. 200  $\mu$ L PEC-EVs (7.5 cm<sup>2</sup> cultured PECs) were added to 96-well round-bottom plates followed by incubation with  $2 \times 10^5$  VPD-450-labeled PBMCs or pan-T cells for 7 days. In additional experiments, the EVs were pre-treated with RNase at 1  $\mu$ g/mL at 37°C for 30 minutes. Untreated and RNase-treated EVs were used to stimulate VPD-450 labeled PBMCs, and the T cell proliferation was measured by FACS analysis. In selected cultures, 100  $\mu$ g/mL belatacept, 100  $\mu$ g/mL anti-CD154 (5c8), or 20  $\mu$ g/mL anti-human CD11a (TS1/22) mAb were added into the cultures to determine the effects of costimulation blockade in preventing xeno-specific T cell proliferation. The 10 ng/mL tacrolimus, 10 ng/mL rapamycin, and 10  $\mu$ g/mL solu-medrol were added into the cultures to determine the effects of conventional immunosuppressant in inhibiting T cell activation.

To determine the role of CD14<sup>+</sup> monocytes in indirect xeno-presentation and costimulation, purified CD14<sup>+</sup> cells were pulsed with 200  $\mu$ L PEC-EVs for 24 hours and washed to remove unbound EVs. EVs-pulsed CD14<sup>+</sup> or un-pulsed CD14<sup>+</sup> cells were incubated with  $2 \times 10^5$  CD14-depleted-PBMCs pre-labeled with VPD450. The T cell proliferation was measured by FACS analysis after a six-day incubation. Unstimulated and EVs stimulated CD14-depleted PBMCs were used as negative and positive controls.

To determine the role of direct xenorecognition of resting PEC-derived EVs with barely detectable SLA-DR expression in initiating CD4<sup>+</sup> cell activation, CD4<sup>+</sup> cells were purified by negative selection method, and  $1 \times 10^5$  purified CD4<sup>+</sup> cells were stimulated with 200  $\mu$ L PEC-EVs for 24, 48, and 72 hours. Cells were collected followed by FACS analysis to detect surface OX40 and CD25 expression.

### Statistical analysis

A two-sample Student's *t*-test was performed to determine the statistical significance for proliferation and inhibitor effects of regents on both CD4<sup>+</sup> and CD8<sup>+</sup> cells in response to xenogeneic stimulation. A *p* value of less than 0.05 was considered statistically significant.

## Results

### Xeno-recognition and proliferation of human T cells in response to porcine endothelial cells

We first verified that intact PECs could serve as APCs and activate human T cells, and that this could be augmented through PEC activation. PECs upregulated SLA-I following hTNF- $\alpha$  stimulation, and surface SLA-DR expression was induced following stimulation by pIFN- $\gamma$  but not hIFN- $\gamma$  (Fig. S2a). PBMCs (*n*=13) were stimulated with PECs, and both CD4<sup>+</sup> and CD8<sup>+</sup> cells proliferated and acquired a memory phenotype in response to PEC exposure (Fig. S2b and c), a finding consistent with our previous observations<sup>(29)</sup>. These proliferative responses were independent of monocytes, as CD14-depletion or T-cell purification failed to prevent proliferation (Fig. S2d and e).

### Monocytes and T cells acquire SLA-I antigens leading to contact-independent proliferation.

To assess the 'semi-direct' presentation pathway, we established a transwell system to assess the contact-dependence of PEC-specific proliferation. As shown in Fig. 1a, CD14<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> cells became positive for SLA-I following direct-contact with SLA-I mAb-prelabeled PECs. Importantly, CD14<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> cells also acquired SLA-I without direct PEC contact (Fig. 1a). Both CD4<sup>+</sup> and CD8<sup>+</sup> T cells proliferated following direct-contact with PECs, but notably, significant xenospecific T cell proliferation was also induced without direct-contact, though less than that seen with direct-contact (Fig 1b).

### PEC-derived extracellular vesicles carry SLA-I and SLA-DR antigens to T cells and monocytes

Previous studies have found that the EVs, released from B-cell lines or DCs, bear MCH class-II and costimulatory molecules<sup>(48–50)</sup>. We therefore characterized PEC-derived EVs.

PEC-derived EVs, assessed by FACS, ranged in size from 0.2 to 2.0  $\mu\text{M}$  (Fig. 2a), and expressed SLA-I (Fig. 2b). The expression of SLA-DR on EVs, derived from unstimulated PECs, was barely detectable (Fig. 2c), but was induced by pIFN- $\gamma$  but not hIFN- $\gamma$  stimulation (Fig. S2). Thus, EVs derived from SLA-DR expressing PEC expressed SLA-DR (Fig. 2d). It has been recognized the challenge in isolation of EVs particularly obtaining the different types of EVs. The use of ultracentrifugation-based technique remains the standard for the exosome isolation. The method, described in the present study, successfully and rapidly isolated EVs from culture medium, and these PEC-derived EVs were characterized by surface CD9 and CD81 expression (Fig S1a), and the nanoparticle tracking analysis defined the size distribution of isolated-EVs (Fig S1b).

To assess the role of PEC-derived EVs in initiating T-cell activation, PEC-derived EVs were pre-labeled with fluorescent-conjugated mAbs specific for SLA-I and SLA-DR, incubated with human PBMCs and interrogated by FACS analysis. As shown in Fig. 3a, SLA-I expressing EVs, derived from unstimulated-PECs, bound to CD14<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> cells. However, T cells and CD14<sup>+</sup> cells did not become positive for SLA-DR following incubation with SLA-DR-labeled EVs that derived from pIFN- $\gamma$ -activated PECs (Fig. 3b). To evaluate the binding relationship between SLA-I antigens and T-cell receptors alpha-beta (TCR- $\alpha\beta$ ), an imaging FACS was performed following incubation of SLA-I labeled EVs and purified pan-T cells. As shown in Fig. 3c, T cells expressed SLA-I following incubation with SLA-I expressing EVs, and this co-localized with surface TCR- $\alpha\beta$  on CD3<sup>+</sup> cells.

Enriched PEC-derived EVs stimulated robust human T-cell proliferation (Fig. 3d). Proliferation was concentration dependent and decreased following the dilution of EVs. Proliferating T cells acquired a predominantly T<sub>EM</sub> phenotype (Fig. 3e).

Human CD4<sup>+</sup> T-cell proliferative responses stimulated by intact PEC monolayers were significantly lower than CD8<sup>+</sup> cell proliferation (Fig. S2b), confirming and expanding on previously reported findings<sup>(29)</sup>. In contrast, CD4<sup>+</sup> cell proliferation induced by resting-PEC-derived EVs (that barely expressed SLA-DR) was similar to the CD8<sup>+</sup> cells (Fig 3d). It has been suggested that stimulation of the TCR/CD3 complex by MHC antigens is required to induce OX40 expression<sup>(51)</sup>. As shown in Fig. 3f, purified CD4<sup>+</sup> cells upregulated OX40 expression within 24 hours following stimulation with EVs derived from unstimulated-PECs and was consistently maintained for up to 72 hours. Furthermore, CD4<sup>+</sup>OX40<sup>+</sup> cells, induced by resting PEC-derived EV-stimulation, upregulated IL-2 receptor- $\alpha$  (CD25) expression (Fig. 3f). Increasing studies have suggested that EVs contain mRNAs and miRNAs and may play important roles in modulating cell function<sup>(52)</sup>. As shown in Fig. 3g, untreated EVs stimulated T cell proliferation respectively. In contrast, pre-treatment of EVs with RNase did not alter the effects of EVs in inducing xeno-reactive T cell proliferation.

### **Xenogeneic porcine endothelial cells and extracellular vesicles induce CD14<sup>+</sup> monocyte immune responses.**

Given the prominent role of monocytes and macrophages in XR<sup>(12, 53)</sup>, we evaluated the behavior of CD14<sup>+</sup> monocytes during their initial interaction with PECs. Human PBMCs (n=7) were stimulated with PECs followed by intracellular cytokine staining (ICCS) to detect IL-6, IL-12, and TNF- $\alpha$  production in CD14<sup>+</sup> cells (Fig. S3a). PEC-stimulated

CD14<sup>+</sup> monocytes increased their production of IL-6, IL-12, and TNF- $\alpha$  intracellularly (Fig. 4a). In contrast, neither CD4<sup>+</sup> nor CD8<sup>+</sup> cells increased TNF- $\alpha$  production following PEC-stimulation (Fig. S3b), a finding consistent with our prior work<sup>(29)</sup>.

It is well-recognized that costimulation signaling plays a critical role in initiating T cell activation<sup>(54)</sup>, and monocyte-derived costimulatory molecules induce cell-mediated immunoresponses<sup>(55)</sup>. We therefore examined the expression of CD40 and CD80 on CD14<sup>+</sup> cells with or without direct-contact to PECs. As shown in Fig. 4b, CD14<sup>+</sup> cells demonstrated substantial upregulation of CD40 and CD80 surface expression after 24-hour incubation of PBMC with PECs directly, or separated by a transwell insert (Fig. 4c).

Human CD14<sup>+</sup> monocytes acquired PEC SLA antigens during their interaction with PECs or PEC-derived EVs. Purified CD14<sup>+</sup> monocytes were pulsed with PEC-derived EVs for 24 hours and incubated with CD14-depleted T cells after removal of unbound EVs to detect T cell proliferation. As shown in Fig. 5, T cells without CD14<sup>+</sup> monocytes demonstrated proliferative responses to EVs. In contrast, there was a lack of T cell proliferation in T cells incubated with EV-pulsed CD14<sup>+</sup> monocytes, indicating that EV-dressed monocytes could not promote T-cell proliferation. Rather, the T cell response to EVs was direct.

### **PEC-derived EVs provide direct xeno-recognition and costimulation for purified human T cell activation.**

We have previously demonstrated that purified human naïve and memory T cells directly recognize and proliferate in response to cultured PECs<sup>(29)</sup>. To assess the role of PEC-derived EVs in direct xeno-recognition and costimulation, PBMCs and purified pan-T cells were stimulated by PEC-derived EVs, and interrogated by FACS. As shown in Fig. 6a, both T cells in PBMC and purified T cells demonstrated significant proliferation following stimulation by PEC-derived EVs. The proliferating CD4<sup>+</sup> cells upregulated OX40 expression (Fig 6b) and acquired a predominantly T<sub>EM</sub> cells (Fig. 6c). Analysis of proliferating T cells showed significantly higher expression of surface CD2 and CD11a, a feature of costimulation-independent T cells (Fig. 6d).

To assess the costimulation dependence of these interactions, human PBMCs or purified pan-T cells were stimulated by resting PEC-derived EVs in the presence of inhibitors specific for B7, CD154, or CD11a. The conventional immunosuppressants and mTOR inhibitor were also tested to inhibit EVs induced xeno-reactive T cell proliferation. As shown in Fig. 6e, the proliferation of both CD4<sup>+</sup> and CD8<sup>+</sup> cells induced by PEC-derived EVs was significantly inhibited by costimulation blockade, and the inhibitory effects on CD4<sup>+</sup> cells appeared greater than CD8<sup>+</sup> cells, suggesting that this costimulation-resistance phenotype was acquired as a result of stimulation rather than a pre-existing phenotypic trait. The T cell proliferation to EVs was dramatically inhibited by the conventional immunosuppressants as well as mTOR inhibitor (Fig 7f).

## **Discussion**

Direct- and indirect-pathways for antigen-recognition and costimulation are known to be important in allograft transplantation<sup>(34)</sup>. However, migrating donor professional APCs

from transplant organs are found in very low numbers in draining lymphoid tissue, challenging the role of these passenger leukocytes as the sole inducers of allo-specific T-cell activation<sup>(39–40)</sup>. Indeed, donor passenger cells such as DCs can be eliminated rapidly by host NK cells as well as cytotoxic T cells<sup>(56–57)</sup>. Therefore, additional sources of stimulatory antigen and costimulation seem likely following organ transplantation. Endothelial cells of transplanted organs are an initial barrier to host cell- and antibody-mediated immunity, and not only act as APCs, providing antigen and costimulation, but also as targets of rejection<sup>(2, 11, 43, 58–59)</sup>. Although previous studies have demonstrated the *in vitro* acquisition of allogeneic endothelial MHC-I by DCs<sup>(35)</sup> or monocytes<sup>(38)</sup>, the migration of endothelial cells from transplanted organs to recipient lymphoid tissue post-transplantation has not been reported. Recent investigations have demonstrated that the release of endothelial-derived EVs can induce inflammatory responses in numerous conditions<sup>(44–45, 60–62)</sup>. Their importance in promoting alloimmunity in allotransplantation is emerging<sup>(39–40)</sup>, particularly in the setting of vascular injury<sup>(63)</sup>. Given the prominent potential for vascular injury in discordant xenotransplantation, we sought to understand if EVs could promote xenospecific T cell responses.

Our data demonstrate that EVs derived from PECs are capable of directly inducing human xenogeneic T-cell-mediated immune responses *in vitro*. We have showed that EVs, derived from unstimulated or more so from stimulated PECs, can directly bind to human T cells, colocalizing SLA-I with TCR- $\alpha\beta$  molecules on T cell surface and inducing proliferative responses of purified human CD4<sup>+</sup> and CD8<sup>+</sup> cells independent of the presence of host professional APCs. While monocytes and macrophages may acquire EVs, we find that they are not able to stimulate a direct T cell response. These findings are relevant to the transition from initial direct xenorecognition by donor APCs (direct-pathway) to an additional direct pathway, what we are calling a “secondary direct” pathway of xenorecognition, via organ endothelial-derived EVs after the initial phase of xenograft transplantation.

This is consistent with findings in allotransplantation<sup>(39–40)</sup>. However, we are concerned that the liberation of EVs in a discordant xenotransplant setting may perhaps exceed that seen in crossmatch negative allotransplantation, and thus suggest that this may provide a means of ongoing xenospecific T-cell activation in xenograft recipients with ongoing endothelial cell injury evoked by residual natural antibody or complement regulatory protein incompatibilities<sup>(2, 4–7)</sup>. Indeed, in our study, PECs released EVs with or without endothelial activation, suggesting that persistent liberation and acquisition of EV-carried SLA-I antigens by recipient immune cells posttransplantation that may threaten the long-term xenograft survival in humans. The reduction or elimination of SLA antigen expressing EVs may be an approach to mitigate EV-induced xeno-specific cell-mediated immune responses, and recent investigations have shown that surface SLA-I and SLA-DR expression can be reduced by genetic means<sup>(64–65)</sup>. Although activated PECs induced by pIFN- $\gamma$  stimulation released SLA-DR expressing EVs, we were not able to show robust binding of SLA-DR expressing EVs with human immune cells.

Unlike the rapid T<sub>H</sub> cell-mediated allogeneic immune responses that can be attributed to prior alloimmune exposures and heterologous immunity, there appears to be a lack of rapid cytokine-producing T<sub>H</sub> cell responses to PECs in most humans<sup>(29)</sup>. In this study, PEC-

and PEC EV-specific proliferating cells acquired a T<sub>EM</sub> cell surface phenotype, including acquisition of surface CD11a, CD2, and OX40, all critical for memory cell-mediated immune responses<sup>(31, 51)</sup> and associated with costimulation blockade resistance<sup>(32–33)</sup>. As such, we see EV stimulation as a potential way of migrating the xeno-specific T-cell repertoire from an easily controlled naïve population to a more recalcitrant T<sub>M</sub> population. We believe this warrants attention, and underscores the importance of early vascular protection in clinical xenotransplant studies, particularly to the extent that early clinical trials will rely on costimulatory pathway blockade. Fortunately, EV-induced naïve T cell proliferation, particularly of CD4<sup>+</sup> T cells, seems to be controlled by CD154-CD40, CD28-B7, and CD11a-CD54 costimulation blockade. Preventing migration from a naïve to a memory phenotype should also be top of mind in translational and clinical trials, where conventional immunosuppressants (CNIs and mTOR inhibitors) may be required. At this juncture, we do not mean to represent that EV-mediated activation is more or less important than standard direct presentation; only that it exists and is worthy of consideration.

In conclusion, we have demonstrated the release of EVs from PECs, and shown that these particles lead to T cell activation and maturation without the intervening requirement of professional APCs. Costimulation inhibitors prevent EV-induced T cell proliferation. Our observations lead us to propose a model for secondary direct pathway xenorecognition and costimulation by EVs. We believe that this may occur in the circulation or within secondary lymphoid tissue (Fig. 7). We suggest that the release of PEC-derived EVs is augmented in times of endothelial cell injury, but believe, based on the growing knowledge of EVs released in non-transplant settings, that they likely persist in the circulation for the life of the xenograft. As a continuous source of xenostimulation, this may be a source of indolent induced xenospecific T-cell cytotoxicity and help for induced xenospecific antibody, presenting a barrier to long-term xenograft survival.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

<b>PECs</b>	Porcine vascular endothelial cells
<b>SLA</b>	Swine leukocyte antigen
<b>XR</b>	Xenograft rejection
<b>EVs</b>	Extracellular vesicles
<b>TCRs</b>	T cell receptors
<b>NK</b>	Natural killer

<b>APCs</b>	Antigen presenting cells
<b>T<sub>M</sub></b>	T memory
<b>DCs</b>	Dendritic cells
<b>mAbs</b>	Monoclonal antibodies
<b>PBMCs</b>	Peripheral blood mononuclear cells
<b>TNF-<math>\alpha</math></b>	Tumor necrosis factor alpha
<b>IFN-<math>\gamma</math></b>	Interferon gamma
<b>FACS</b>	fluorescence activated cell sorting
<b>ICCS</b>	Intracellular cytokine staining
<b>VPD450</b>	Violet proliferation dye 450

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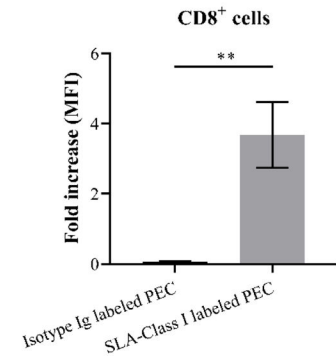
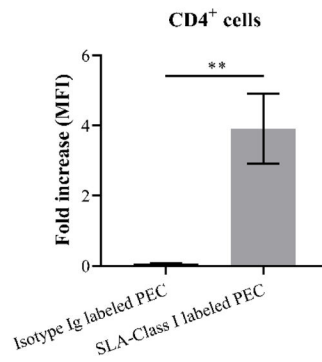
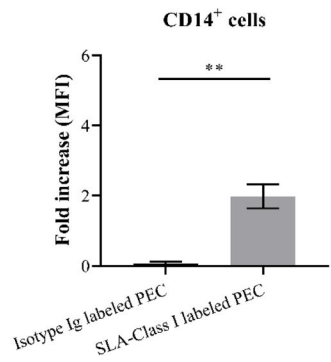
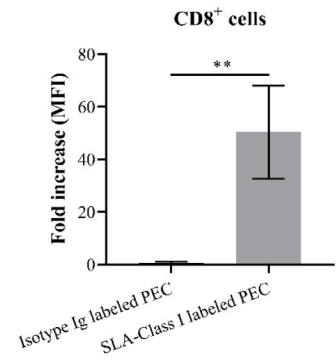
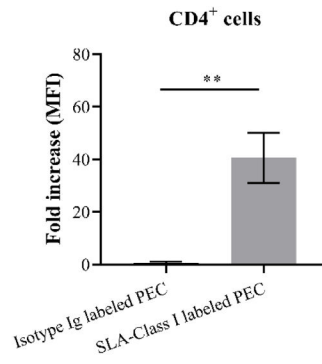
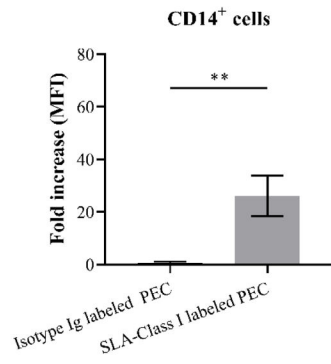
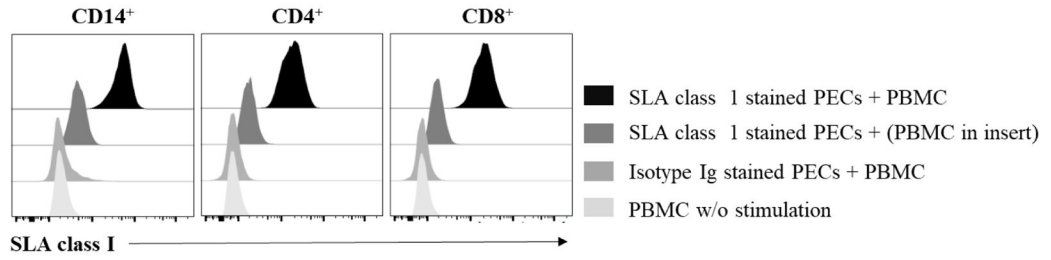
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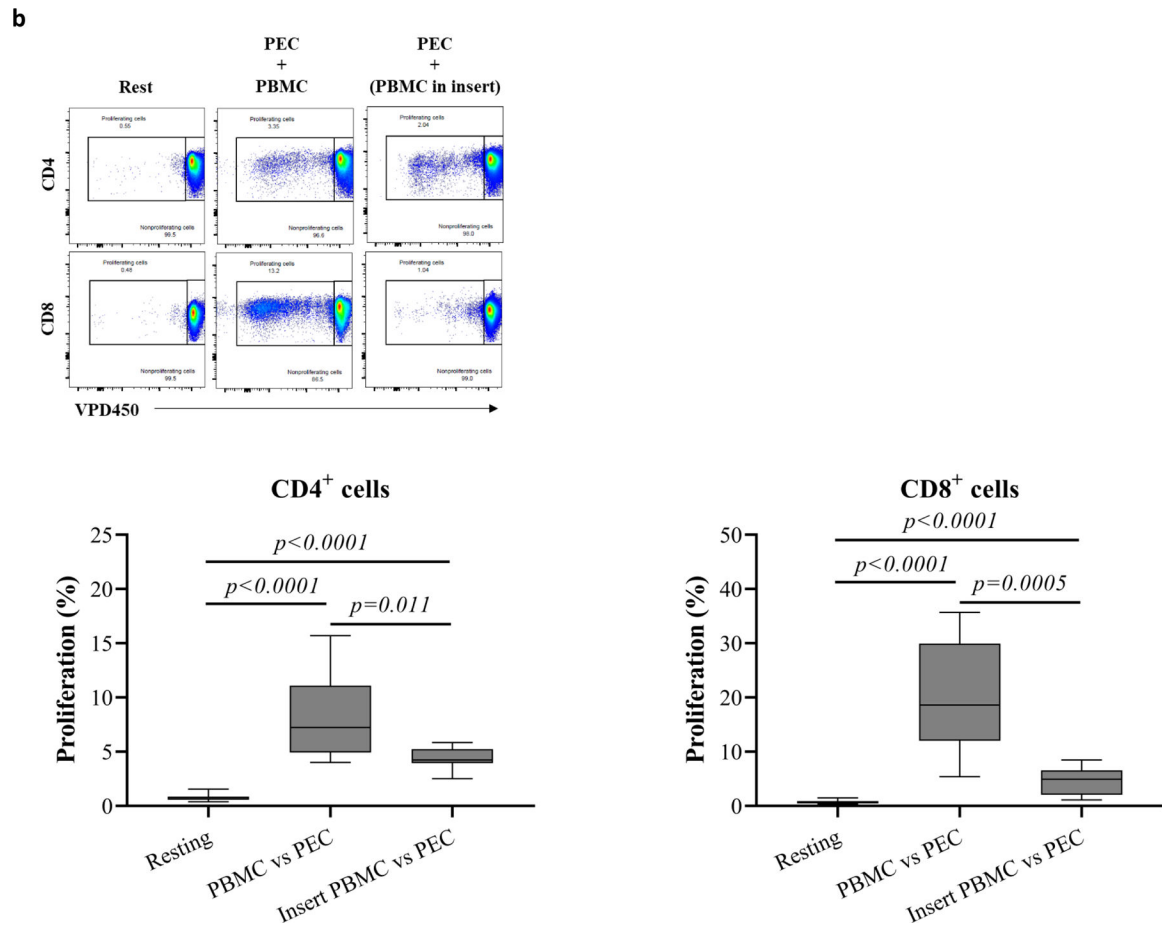
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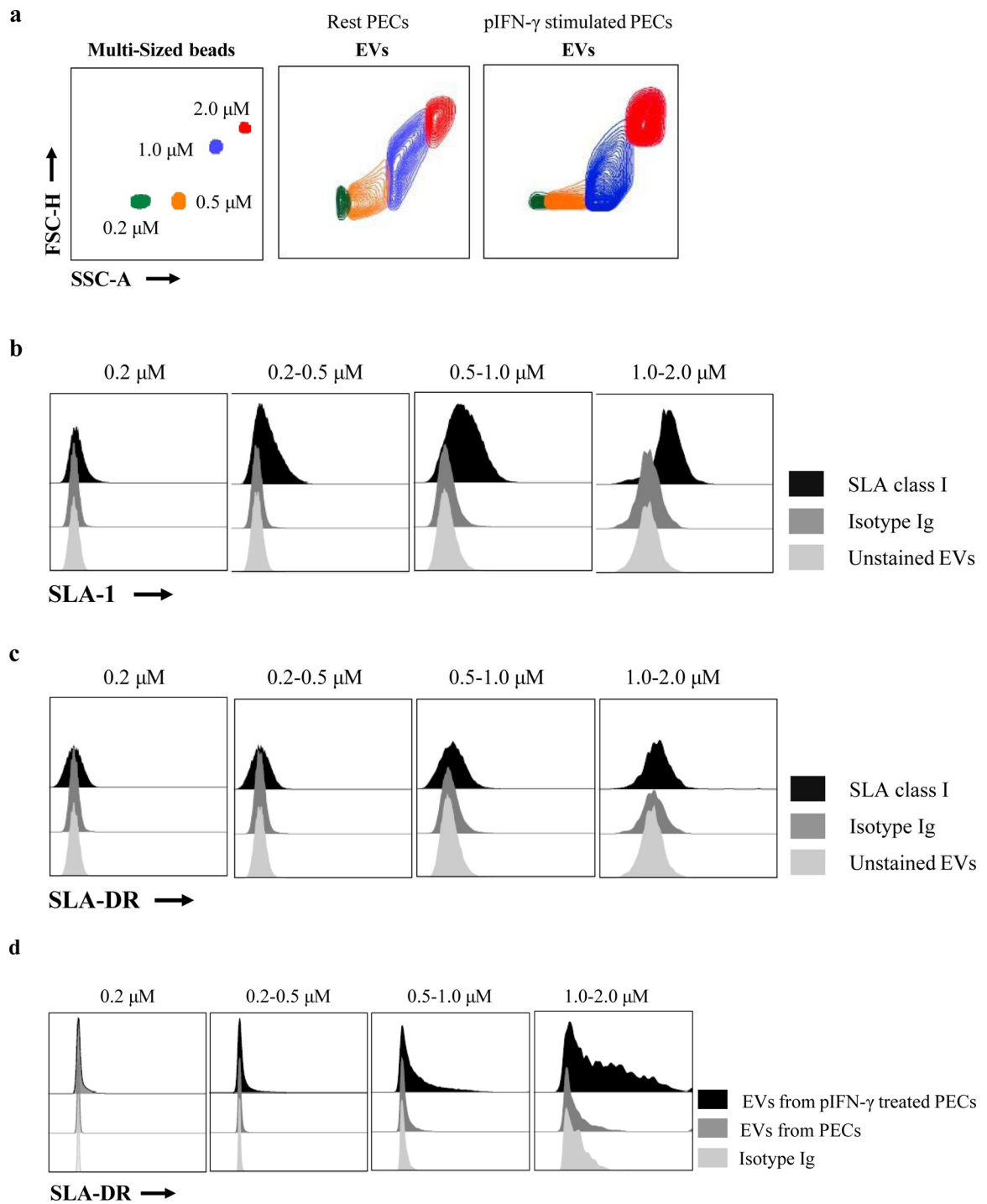
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**Figure 1. Contact-dependent and independent acquisition of SLA-I antigens and xeno-specific T cell proliferation.**

(a) PECs, pre-labeled with SLA-I-FITC mAb, were incubated with human PBMCs with or without transwell insert followed by flow cytometry analysis. A representative experiment was shown on the top panel. The direct interaction between PBMCs (n=6) and PECs demonstrated significant binding of SLA-I to CD14<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> cells when compared to isotype Ig stained PECs (middle panel). The interaction between PECs and PBMCs that were placed in transwell insert demonstrated significant binding of SLA-I to human immune cells when compared to isotype Ig stained PECs (bottom panel) (\*\* $P < 0.01$ ). (b) Contact-dependent and independent T cell proliferation in response to PECs. Confluent PECs were incubated with VPD450-labeled human PBMCs (n=9) with or without transwell insert, and analyzed by flow cytometry at the end of stimulation. A representative experiment was shown on the top panel. Human CD4<sup>+</sup> and CD8<sup>+</sup> cells proliferated following direct contact with PECs respectively. A lower degree but significant proliferation of CD4<sup>+</sup> and CD8<sup>+</sup> cells in inserts was observed without direct contact to PECs.



**Figure 2. Expression of SLA-I and SLA-DR on extracellular vesicles, derived from PECs.**

The confluent PECs were washed with PBS followed by incubation with pre-filtered culture medium. The PEC-derived EVs were enriched from supernatants, and stained with anti-SLA-I-FITC or anti-SLA-DR-FITC. EVs were measured by flow cytometry analysis. **(a)** The sizes of EVs, derived from unstimulated or pIFN- $\gamma$  activated PECs, were measured based on the control reference sized beads. **(b)** The expression of SLA-I on unstimulated

PEC-derived EVs with the sizes ranged from 0.2 to 2.0  $\mu\text{M}$ . **(c)** The barely detectable of SLA-DR expression on unstimulated PEC-derived EVs with the sizes ranged from 0.2 to 2.0  $\mu\text{M}$ . **(d)** Flow cytometry analysis of EVs, derived from PECs treated with pIFN- $\gamma$ , demonstrated SLA-DR expression largely on EVs with the sizes ranged from 0.5–2.0  $\mu\text{M}$ .

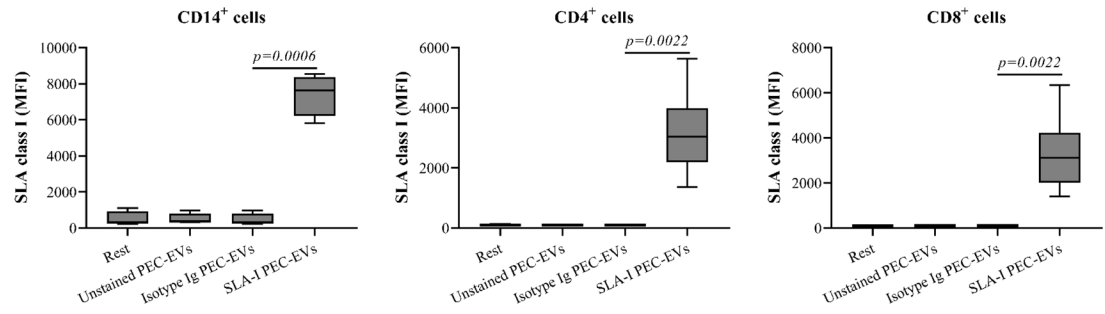
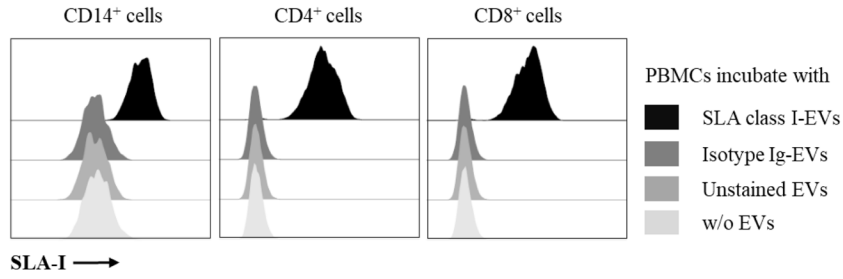
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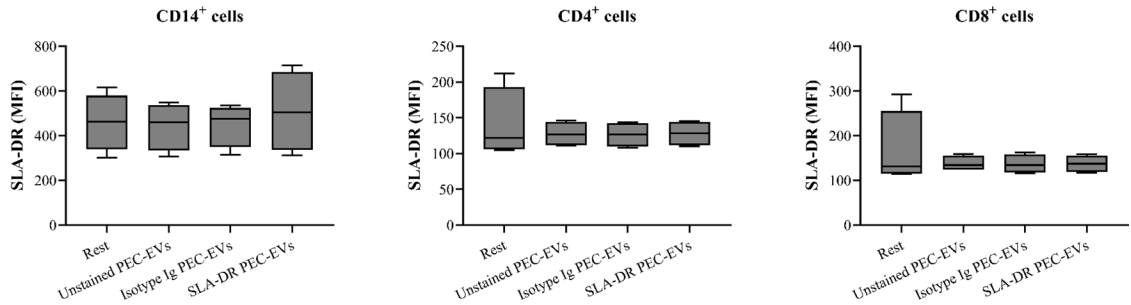
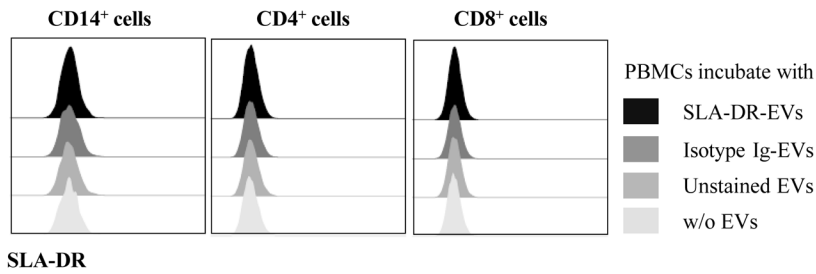
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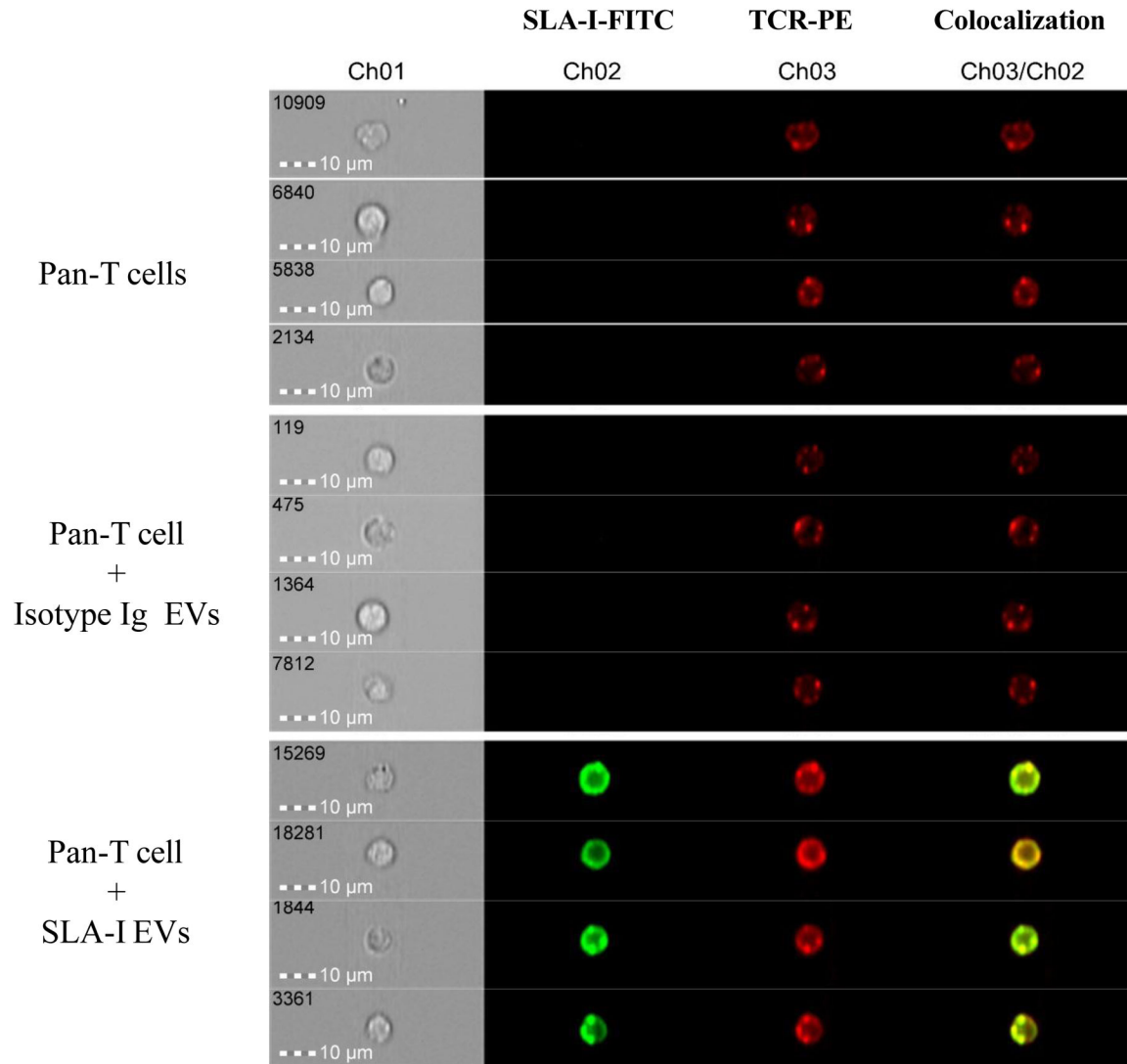
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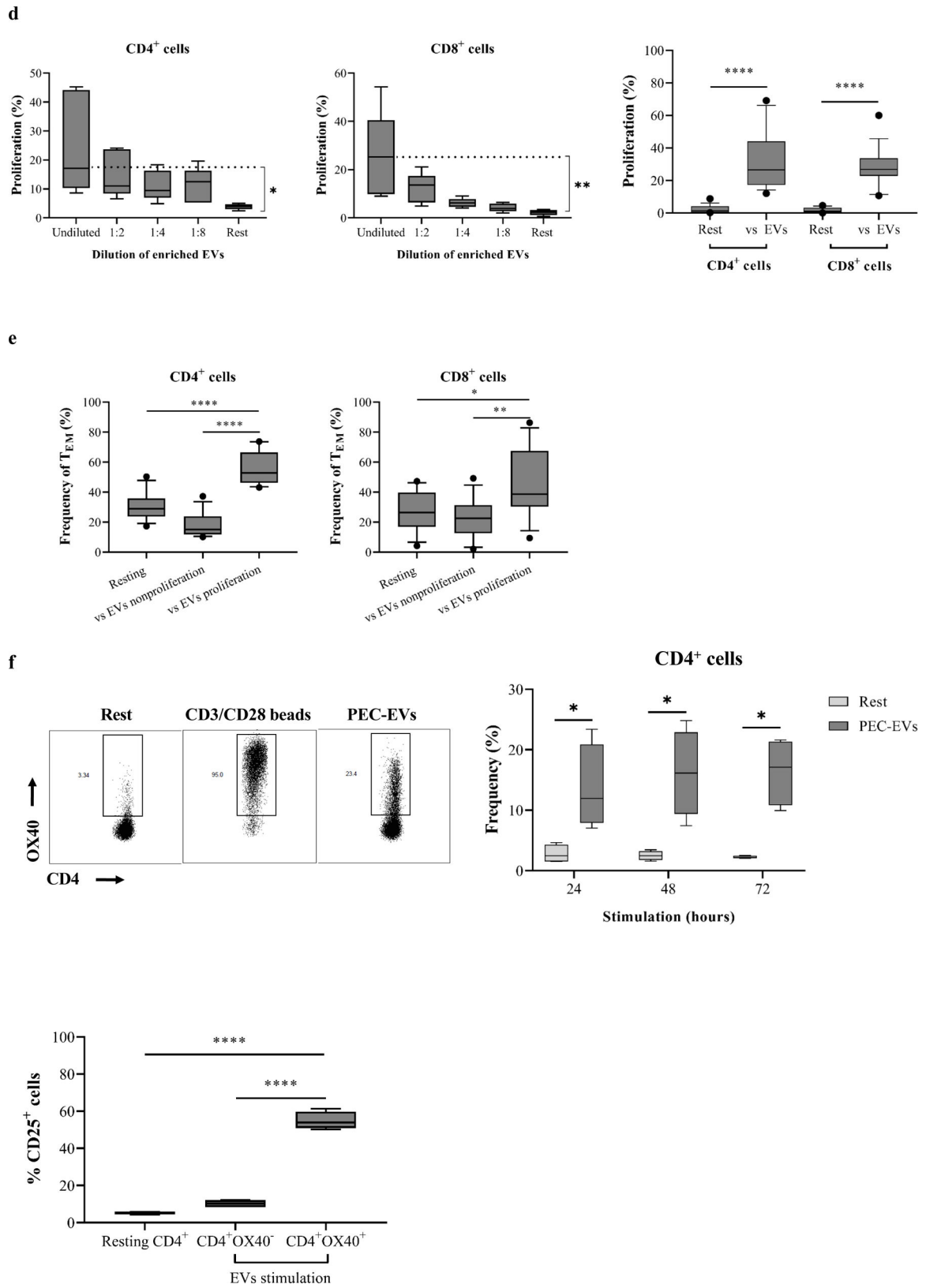


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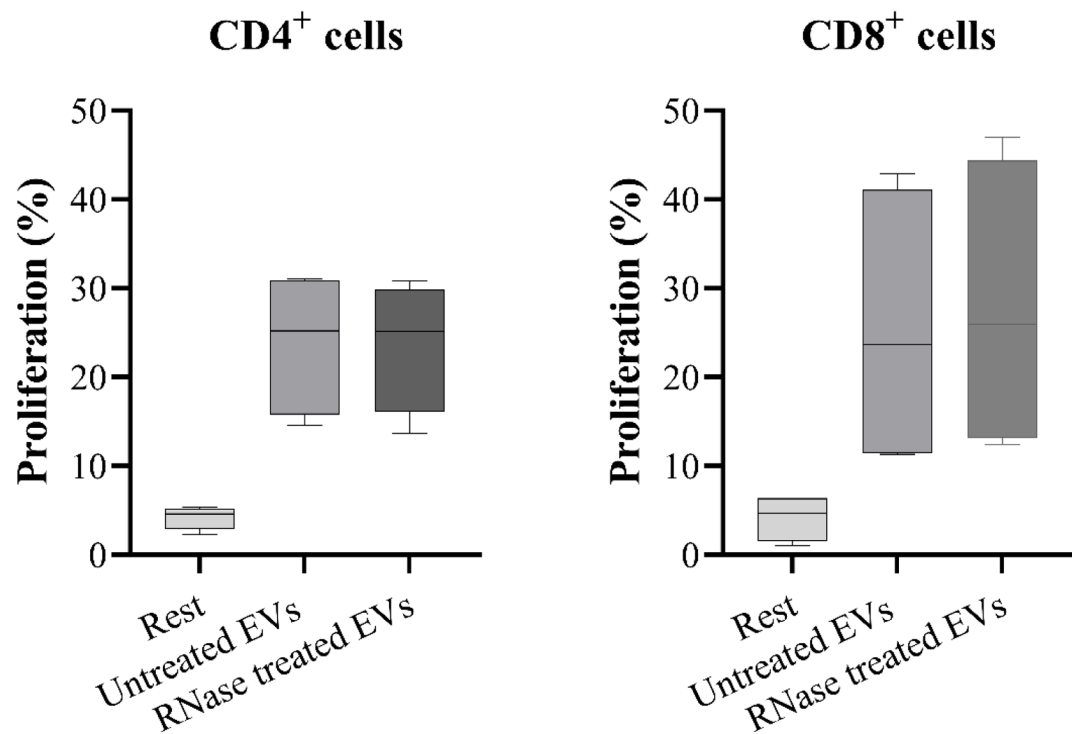


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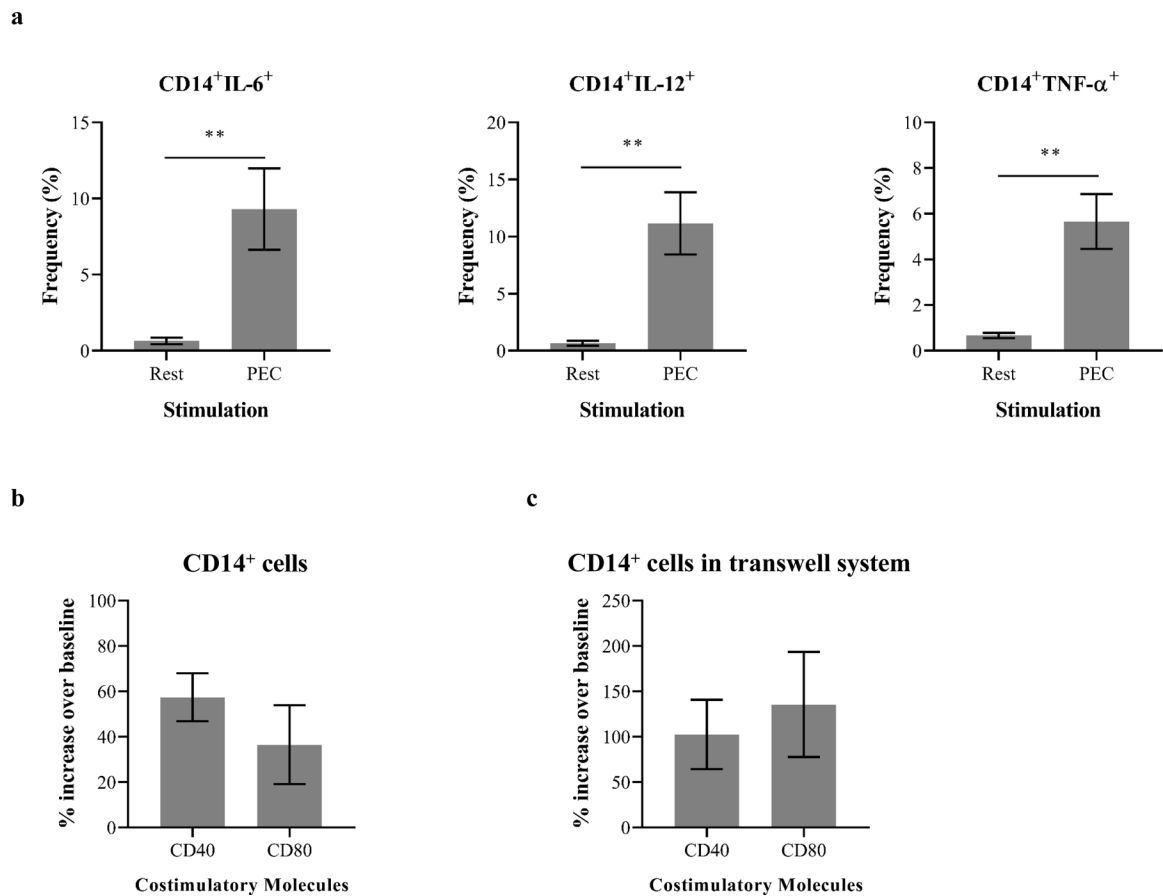
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**Figure 3. PEC-derived extracellular vesicles bound to human T cells and induced T cell proliferation.**

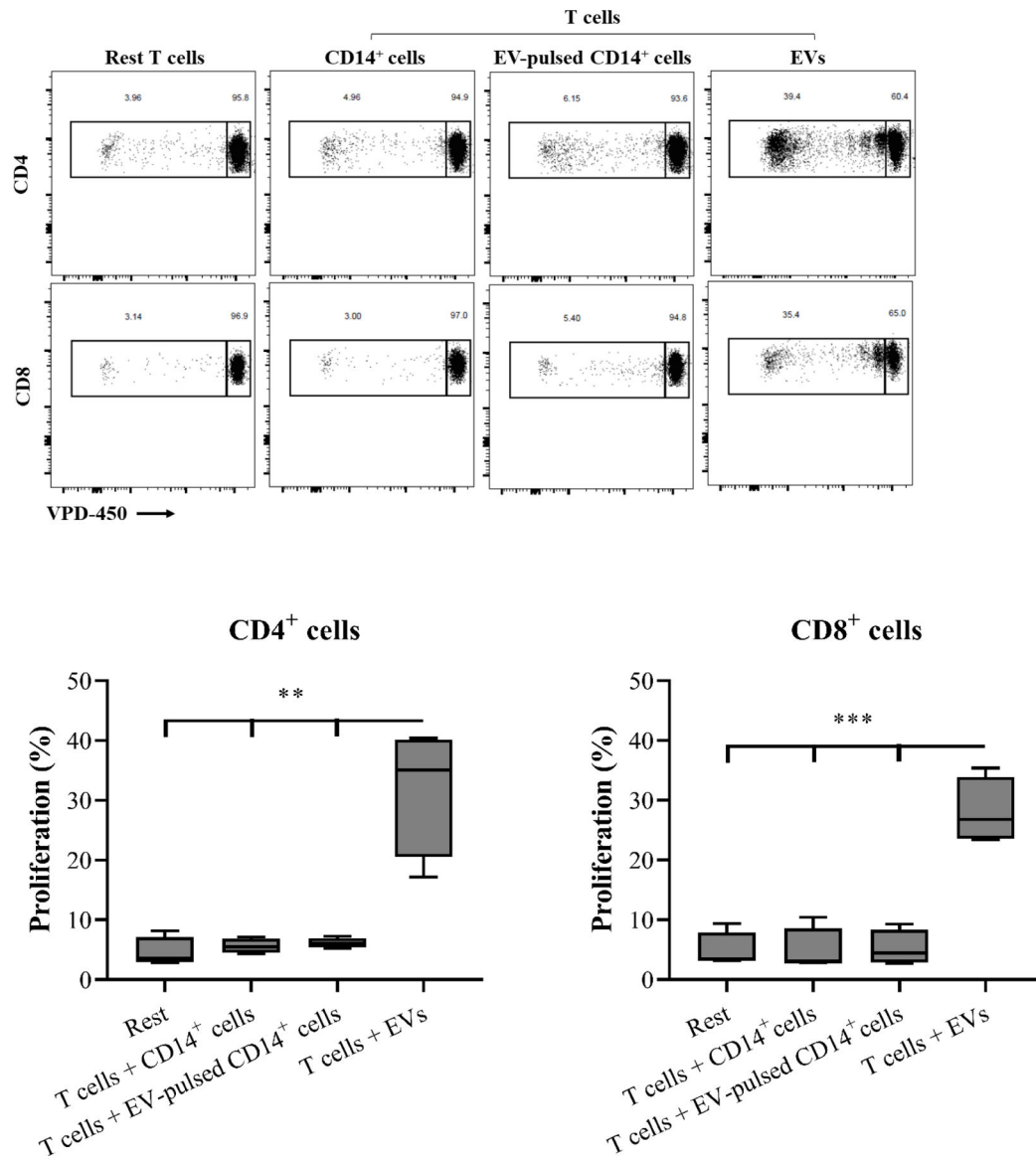
(a) Assessment of the binding of SLA-I<sup>+</sup> EVs to human immune cells. The EVs, enriched from resting PEC culture medium, were pre-labeled with anti-SLA-I-FITC mAb followed by incubation with human PBMCs. The binding of SLA-I expressing EVs to human immune cells were measured by flow cytometry analysis (n=6). A representative experiment is shown on the top panel. The SLA-I expressing EVs demonstrated significant binding to CD14<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> cells when compared to isotype Ig stained EVs (bottom). Rest PBMC and PBMC, incubated with unstained EVs were also used as controls. (b) Evaluation of binding of SLA-DR expressing EVs to human immune cells. SLA-DR expressing EVs, derived pIFN- $\gamma$ -stimulated PECs, were incubated with human PBMCs (n=4) followed by flow cytometry analysis. A representative experiment is shown on top panel. There was a lack of significant binding of SLA-DR-expressing EVs to monocytes and T cells. (c) Imaging flow cytometry analysis for colocalization of SLA-I and TCR- $\alpha\beta$  on CD3<sup>+</sup> cells. Purified pan-T cells were incubated with SLA-I expressing EVs, derived from resting PECs. Cells were stained with anti-CD3 and TCR- $\alpha\beta$  mAb, and then measured by ImageStream<sup>®</sup>X Mark-II-Imaging-cytometry. The colocalization of SLA-I with TCR- $\alpha\beta$  was detected. Pan-T cells, incubated with isotype Ig stained EVs and resting cells were used as negative controls. (d) Resting PEC-derived EVs induced xeno-specific T cell proliferation. The PEC-derived EVs were enriched from supernatants (1 mL/7.5 cm<sup>2</sup>).  $2 \times 10^5$  VDP-450-labeled human PBMCs (n=5) were stimulated by undiluted EVs (7.5 cm<sup>2</sup>) and diluted EVs, and the proliferation was determined by the dilution of VPD450 on T cells. The significant proliferation of CD4<sup>+</sup> and CD8<sup>+</sup> cells was observed in EVs stimulated PBMCs. (e) The xenospecific

proliferating CD4<sup>+</sup> and CD8<sup>+</sup> cells, induced by PEC-derived EVs, were phenotypically CD45RA<sup>-</sup>CCR7<sup>-</sup> effector memory cells based on CD45RA and CCR7 classification. **(f)** The activation of purified CD4<sup>+</sup> cells in response to resting PEC-derived EVs that lack SLA-DR expression. CD3/CD28 bead-stimulated cells were used as controls. Purified CD4<sup>+</sup> cells (n=4) demonstrated upregulation of OX40 expression for over 72 hours, and the predominant OX40 expressing cells expressed CD25. **(g)** VPD-450 labeled human PBMCs were stimulated with untreated or RNase-treated EVs followed by flow cytometry analysis to detect T cell proliferation. (\* $P<0.05$ , \*\* $P<0.01$ , \*\*\*\* $P<0.0001$ ).

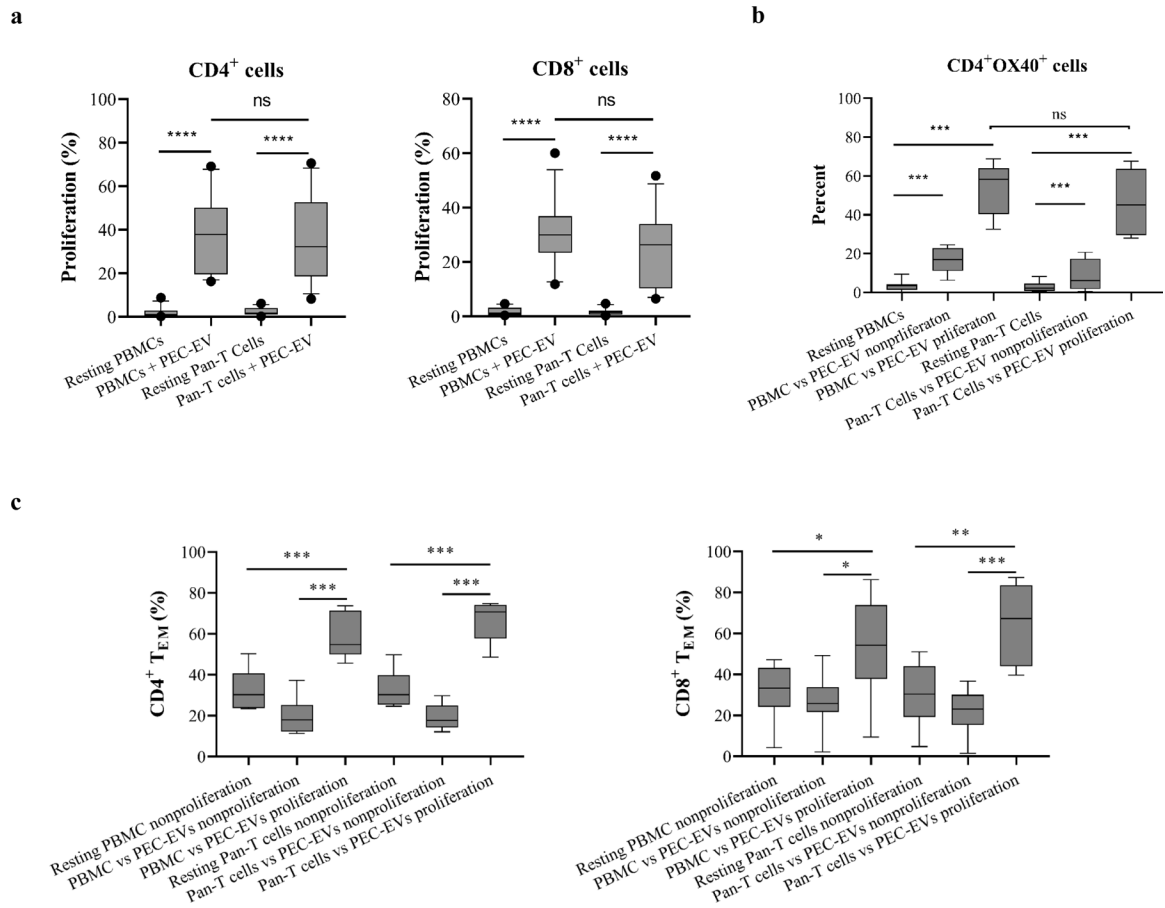


**Figure 4. Porcine endothelial cells activate human CD14<sup>+</sup> monocytes.**

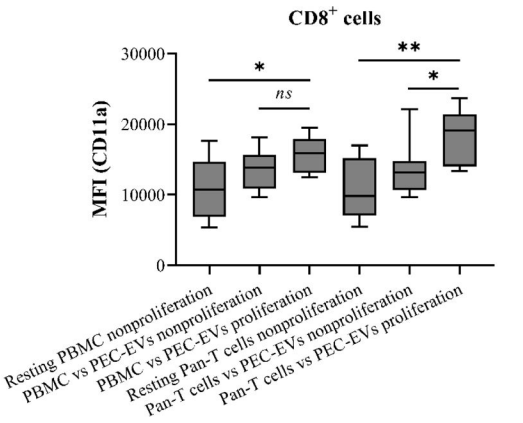
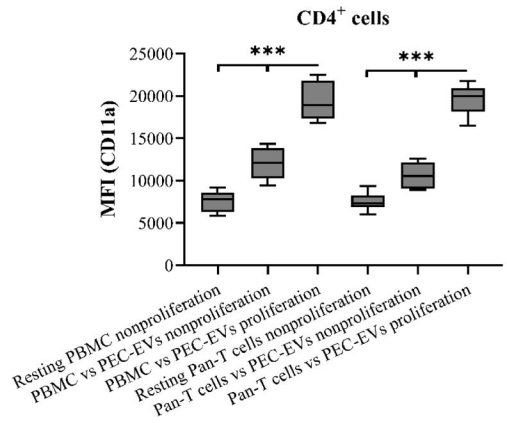
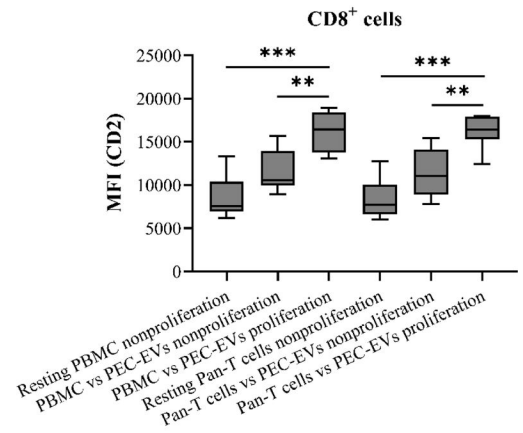
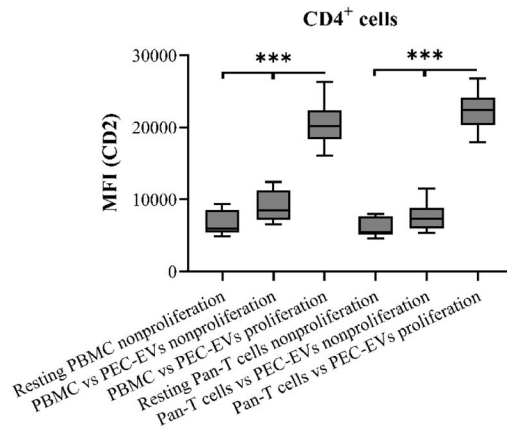
(a) Human PBMCs were stimulated by PEC monolayer in the presence of protein transport inhibitor followed by ICCS to detect IL-6, IL12, and TNF- $\alpha$ . The activation of CD14<sup>+</sup> monocytes was identified based on expression of intracellular cytokines (\*\* $P < 0.01$ ). (b) Human CD14<sup>+</sup> cells ( $n=4$ ) following direct stimulation by PECs upregulated surface CD40 and CD80 expression as measured by MFI (mean fluorescence intensity). (c) Upregulation of surface CD40 and CD80 expression on CD14<sup>+</sup> cells after indirect (transwell insert) interaction with PECs.



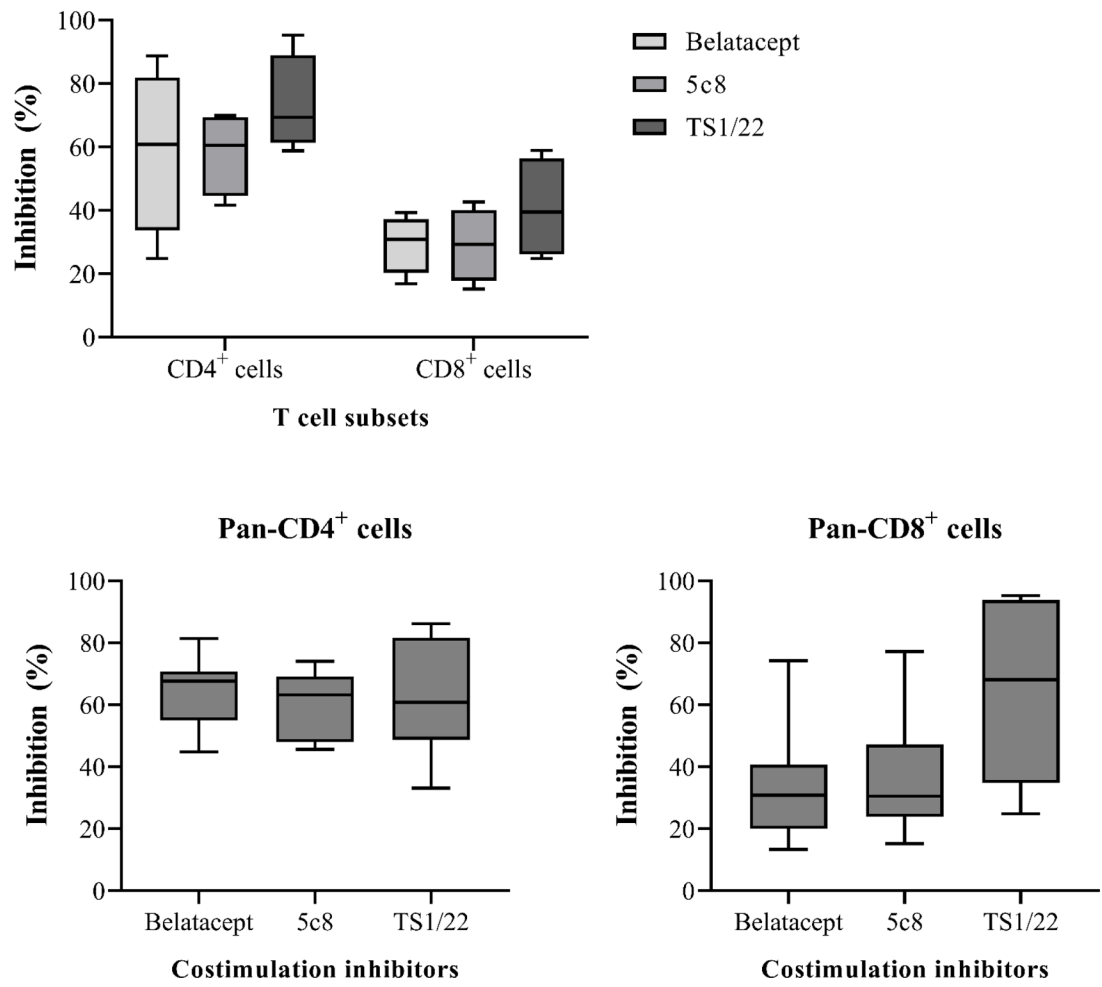
**Figure 5. Extracellular vesicle-pulsed CD14<sup>+</sup> monocytes did not induce T cell proliferation.** To assess the role of CD14<sup>+</sup> monocytes in providing xeno-presentation for T cells, the purified CD14<sup>+</sup> monocytes were pre-pulsed with resting PEC-derived EVs for 24 hours, and then incubated with VPD450-labeled T cells. The proliferation was measured by VPD450 dilution in T cells (n=4). CD14-depleted PBMCs, stimulated by EVs were used as positive controls. CD14-depleted PBMCs, incubated with un-pulsed CD14<sup>+</sup> cells, were used as negative controls (\*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

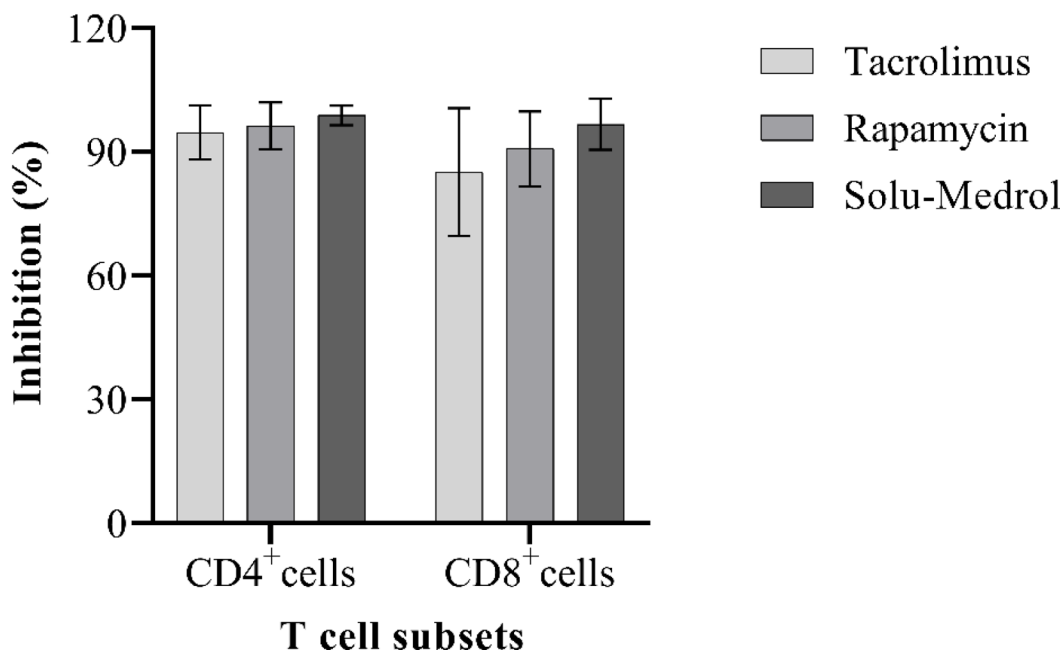


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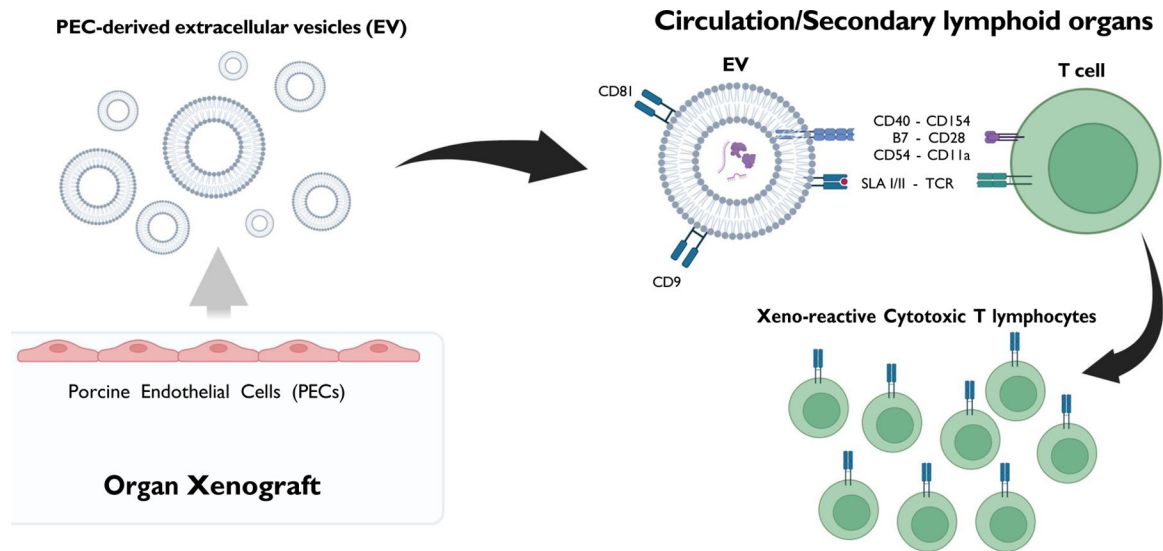
e



**f**

**Figure 6. Direct xeno-recognition/presentation and costimulation by resting PEC-derived EVs to human T cells.**

(a) Pan-T cells showed similar proliferative responses to PEC-derived EVs when compared to PBMCs. (b) The proliferating CD4<sup>+</sup> cells induced by EVs demonstrated upregulation of surface OX40 expression when compared to nonproliferating and resting cells. (c) The proliferating CD4<sup>+</sup> and CD8<sup>+</sup> cells were largely effector memory cells based on surface CD45RA and CCR7 expression. (d) The proliferating CD4<sup>+</sup> and CD8<sup>+</sup> cells demonstrated higher surface expression of CD2 and CD11a when compared to nonproliferating and resting cells. (e) The proliferation of human PBMCs and T cells in responses to resting PEC-derived EVs were partially inhibited by costimulation inhibitors specific for B7-CD28, CD40-CD154, and CD54/CD11a pathways. (f) The proliferation of T cells induced by PEC-derived EVs were dramatically inhibited by tacrolimus (10 ng/mL), rapamycin (10 ng/mL), and solu-medrol (10 µg/mL) as determined by flow cytometry analysis. (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ )



**Figure 7. A model for the role of porcine endothelial cell-derived extracellular vesicles in xenograft rejection.**

Following reperfusion of organ xenografts, the xenograft endothelial cells release extracellular vesicles into circulation. The extracellular vesicles traffic to lymph nodes and directly present intact SLA antigens via a direct-pathway, and provide costimulatory signals through intact costimulatory molecules to generate a cytotoxic T cell response leading to xenograft rejection.