

Suppression of xenogeneic innate immune response by a membrane-type human surfactant protein-A

CHIYOSHI TOYAMA¹, AKIRA MAEDA¹, SHUHEI KOGATA¹, RIHO YAMAMOTO¹,
KAZUNORI MASAHATA¹, TAKEHISA UENO¹, MASAFUMI KAMIYAMA¹, YUKO TAZUKE¹,
HIROSHI EGUCHI¹, HIROOMI OKUYAMA¹ and SHUJI MIYAGAWA^{1,2}

¹Department of Pediatric Surgery, Osaka University Graduate School of Medicine, Suita, Osaka 565-0871;

²International Institute for Bio-Resource Research, Meiji University, Kawasaki, Kanagawa 214-8571, Japan

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Abstract. Macrophage-mediated xenogeneic rejection is a major immunological obstacle. We recently reported that membrane-type surfactant protein-D (SP-D) on swine endothelial cells (SECs) suppressed macrophage-mediated rejection. Similar to SP-D, the carbohydrate recognition domain of surfactant protein-A (SP-A) can induce inhibitory signals in effector cells. The present study aimed to examine the suppressive effect of SP-A on macrophage-mediated xenogeneic rejection. Naive SECs and SPA-transfected SECs (SEC/SP-A) were co-cultured with THP-1 cells and cytotoxicity was evaluated. To investigate the effect of SP-A on phagocytosis, human macrophages were co-cultured with SEC or SEC/SP-A, and the extent of phagocytosis and production of reactive oxygen species were assessed via flow cytometry. The mRNA expression levels of inflammatory cytokines in macrophages were determined using reverse transcription-PCR. Additionally, the effects of THP-1-Lucia NF- κ B cells on transcription

factors were evaluated. The cytotoxicity and phagocytosis of SEC/SP-A were significantly decreased compared with those of naive SEC. Furthermore, the co-culture of human macrophages with SEC/SP-A decreased reactive oxygen species production, and the mRNA expression levels of TNF α were decreased in macrophages, whereas those of IL-10 were increased. In addition, NF- κ B transcription was decreased in SEC/SP-A compared with that in SEC. In conclusion, the ectopic expression of human SP-A in porcine cells represents an attractive method for suppressing macrophage-mediated cytotoxicity.

Introduction

Xenotransplantation is one of the most attractive strategies to overcome the shortage of organ donors. α 1,3-galactosyltransferase gene-deficient-knockout (α GalTKO) pigs have been produced to provide sufficient protection against hyper acute rejection, which is mediated by natural antibodies to xenogeneic antigens and complements (1-4). The expression of complement regulatory proteins, such as C1 esterase inhibitors, decay accelerating factors, and membrane cofactor proteins on porcine cells, downregulates complement-mediated cytotoxicity (5-8). However, a limited number of studies have been conducted on cellular xenogeneic rejection (CXR). Histopathological studies have demonstrated that the mechanisms involved in xenogeneic graft rejection are significantly different from those associated with allogeneic graft rejection. In allograft rejections, cytotoxic T lymphocytes are the main infiltrating cells, while xenografts mainly induce the infiltration of neutrophils, NK cells, and macrophages (9-11). CXR with innate immune cells particularly causes severe rejection in xenotransplantation. Macrophages are activated under xenogeneic conditions through both antibody-dependent and -independent mechanisms. Immunocomplexes of porcine cells with xenogeneic antigen bind to the Fc gamma receptor on macrophages and activate macrophages (12,13). Macrophages are also activated by interaction with neutrophils, NK cells, and Th1 cells in an antibody-independent manner (14,15). Furthermore, damage-associated molecular patterns (DAMPs) from dead porcine cells activate macrophages in an antibody-independent manner (16). Martin *et al* demonstrated that the infiltration of neutrophils and macrophages in α GalTKO

Correspondence to: Dr Akira Maeda, Department of Pediatric Surgery, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan
E-mail: zul06720@nifty.com

Abbreviations: CL-P1, collectin placenta 1; CL-SPA, CL-P1 with the carbohydrate recognition domain of SP-A; CRD, carbohydrate recognition domain; CXR, cellular xenogeneic rejection; PBMC, peripheral blood mononuclear cell; PMA, phorbol-12-myristate-13-acetate; ROS, reactive oxygen species; SEC, swine endothelial cell; SEC/SP-A, CL-SPA-transfected SEC; SP-A, surfactant protein-A; SP-D, surfactant protein-D; α GalTKO, α 1,3-galactosyltransferase gene-deficient-knockout; DAMPs, damage-associated molecular patterns; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; SIRP α , signal inhibitory regulatory protein α ; RPMI, Roswell Park Memorial Institute; FACS, fluorescence-activated cell sorter; TLR, toll-like receptor; NET, nuclear extracellular trap

Key words: surfactant protein, macrophages, SP-A, xenotransplantation, phagocytosis

islets was not different from that in wild-type islets in a dual islet transplantation model, suggesting that antibody-independent mechanisms are important in CXR (17). Thus, it is important to develop strategies to suppress the action of innate immune cells, such as macrophages and neutrophils, for successful clinical application of xenotransplantation (18-24).

Surfactant protein-A (SP-A) and SP-D are epithelial cell-derived immune modulators that are C-type collagen-like lectins. SP-A and SP-D can be detected in the mucosal surfaces of various organs, and both proteins play important roles in immune responses (25-29). While ligation of N-terminal collagen domains with the calreticulin/CD91 receptor complex induces pro-inflammatory responses, binding of the carbohydrate recognition domain (CRD) to signal inhibitory regulatory protein α (SIRP α) prevents inflammation (30). We have previously reported the preparation of cDNA for the membrane-type protein, collectin placenta 1 (CL-P1), with the CRD of SP-D (CL-SPD) and its transfection into swine endothelial cells (SECs). The hybrid molecule significantly suppresses macrophage-mediated cytotoxicity in SECs (31). Additionally, *in vitro* studies have indicated that the suppressive function of CL-SPD in macrophage-mediated cytotoxicity is more potent than that of CD47. However, the effect of CRD of SP-A on the xenogeneic condition remains unknown.

This study aimed to examine the suppressive effect of CRD of SP-A on macrophage-mediated xenogeneic innate immune responses. Pro-inflammatory cytokines are key factors in innate immune responses; hence, the activation of pro-inflammatory cytokines and production of reactive oxygen species (ROS) in macrophages were the focus of this study.

Materials and methods

Ethical approval. The present study was approved [approval no. 18395(T1)] by the Ethics Committee of Osaka University (Osaka, Japan). All the participants enrolled in the study provided signed written informed consent. The participants were over 20-year-old healthy volunteers, and their clinical data were blinded.

Cells and generation of human macrophages. A swine endothelial (SEC) line MYP30 was cultured in Dulbecco's modified Eagle's medium (DMEM, Nacalai Tesque, Kyoto, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS). THP-1 cells were cultured in Roswell Park Memorial Institute (RPMI, Nacalai Tesque) 1640 medium containing 10% FBS, and THP-1-Lucia NF- κ B cells were cultured in RPMI-1640 containing 10% FBS and 25 mM HEPES.

Human peripheral blood mononuclear cells (PBMCs) were obtained from healthy volunteer donors using differential density gradient centrifugation (lymphocyte isolation solution; Nacalai Tesque). PBMCs were incubated in a 6-well plate at 37°C for 1 h as a plastic adhesion method of isolating human monocytes. To generate macrophages, human monocytes were cultured in the presence of 100 ng/ml rM-CSF (PeproTech) for 6 days.

Plasmid construction. cDNA of human SPA-CRD (GenBank accession no. M68519.1) was replaced with that of membrane-type protein CL-P1 without the cytoplasmic tail but

with a flag-tag between the CRD and integrin domain (Fig. 1A). The cDNA was inserted into the expression vector pCXN2 (chick beta-actin promoter + CMV enhancer + neomycin resistance), which was transfected into SECs (Fig. 1B).

Preparation of SEC transfectants via lipofection. Five micrograms of a plasmid containing SPA-CRD were mixed with 40 μ l Lipofectamine™ LTX with Plus™ Reagent (Thermo Fisher Scientific, Tokyo, Japan) and incubated with SECs at 37°C for 3 h (Fig. 1C). Transfected cells were selected by culturing in DMEM containing 400 μ g/ml G418 (Thermo Fisher Scientific, Inc.). The CL-SPA-transfected SEC (SEC/SP-A) was either bulk type (SEC/SP-A bulk) or a clonal line (SEC/SP-A #11 and #23) obtained via limiting dilution. The SEC/SP-A bulk, #11 or #23 was used for the assay after 2 months of transfection. SEC without lipofection was used as a control because we have confirmed that there is no difference between SEC without lipofection and SEC with lipofection by an empty vector in the cytotoxicity assay.

Semi-quantitative RT-PCR of SP-A. Total RNA was extracted from SEC and SEC/SP-A #11 and #23 with the TRIZOL reagent (Invitrogen). cDNA was synthesized from total RNA using Prime Script™ II 1st strand cDNA synthesis kit (Takara Bio Inc.). The specific PCR products for *Sus scrofa* GAPDH and human SP-A were amplified with KOD FX Neo (Toyobo) using Veriti 96 well Thermal Cycler (Applied Biosystems, Waltham, MA). PCR products were loaded on 1% agarose gel. After electrophoresis, the agarose gel was visualized under ultraviolet light. The sequences of the primers were as follows: *Sus Scrofa* GAPDH F, 5'-ACCACGGTCCATGCCATCACTGC-3'; *Sus Scrofa* GAPDH R, 5'-TCCACCACCCTGTTGCTGTAGCC-3'; Primer1: 5'-GAGAAGGTGTTCAGCAGCAACG-3', Primer2: 5'-GCCACACCAGCCACCACCTTCTG-3'. The sites of Primer1 and 2 in the transfecting plasmids were shown in Fig. 1A.

Cytotoxicity assay. Naive SEC and SEC/SP-A were plated at a concentration of 1.0×10^4 or 1.3×10^4 cells/well in a flat-bottom gelatin-coated 96-well plate at 37°C on Day 0. On Day 1, THP-1 or THP-1-Lucia cells (5.0×10^4 cells/well) were added to each well, and the cells were co-cultured with 200 nM phorbol-12-myristate-13-acetate (PMA). On Day 2, 10 μ l of WST-8 reagent solution was added to each well, and the absorbance was measured at 450 nm using a microplate reader (Thermo Fischer Scientific, Inc.).

Phagocytosis assay. Human PBMCs were plated at a concentration of 3×10^6 cells/well in a flat-bottomed 6-well plate on Day 0 and cultured in the presence of 100 ng/ml rM-CSF for 6 days. SECs or SEC/SP-As (3×10^5) were labeled with calcein-AM (Nacalai Tesque) by incubating with 2 μ l/ml calcein-AM at 37°C for 10 min. Calcein-AM-stained SEC or SEC/SP-A was added to plates, and the cells were co-cultured for 24 h. The cells were then harvested and stained with APC-labeled anti-human CD14 antibody (1:40; cat. no. 982506; BioLegend). The stained cells were analyzed using a fluorescence-activated cell sorter (FACS) FACSVerse™ flow cytometer (BD Bioscience) using the BD FAC Suite software (ver. 10.6). The percentage of phagocytosis was calculated as (CD14⁺ and calcein-AM⁺ cells)/(CD14⁺ cells) x100(%).

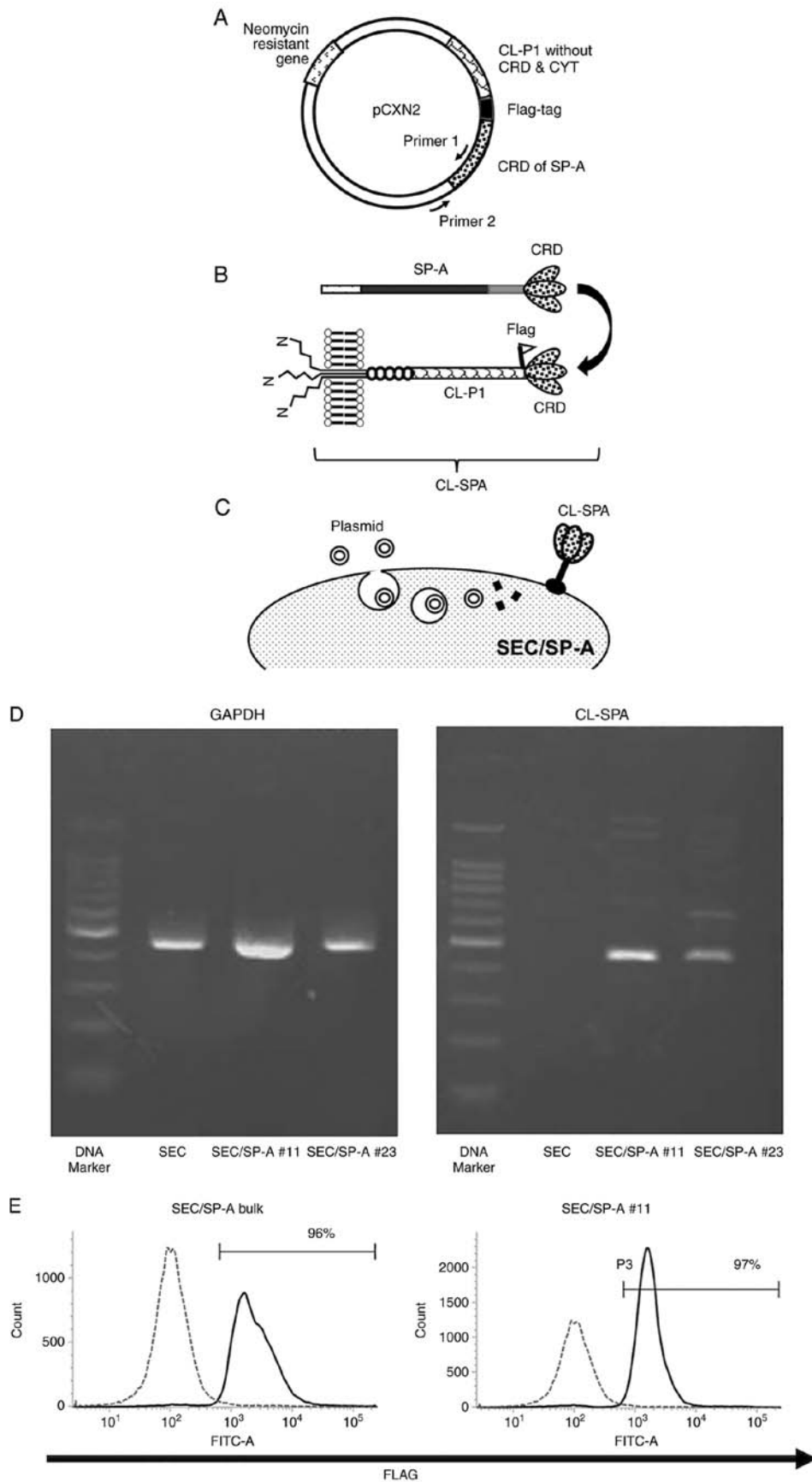


Figure 1. The design of CL-SPA and creation of SEC/SP-A. (A and B) cDNA for human SPA-CRD was replaced with that of membrane-type protein CL-P1 without the cytoplasmic tail but with a flag-tag between the CRD and integrin domain. (C) CL-SPA was transfected into SECs and (D) RNA expression of CL-SPA in cells was confirmed by semi-quantitative RT-qPCR. The RNAs of CL-SPA were expressed in the cell lines (SEC/SP-A #11 and SEC/SP-A #23). (E) The expression of FLAG (SP-A marker) on SEC/SP-A was measured via flow cytometry. SEC/SP-A bulk and SEC/SP-A #11 expressed 96 and 97% of FLAG, respectively. SEC, swine endothelial cell; SP-A, surfactant protein-A; CL-SPA, collectin placenta 1 with carbohydrate recognition domain of surfactant protein-A; SEC/SP-A, CL-SPA-transfected SEC.

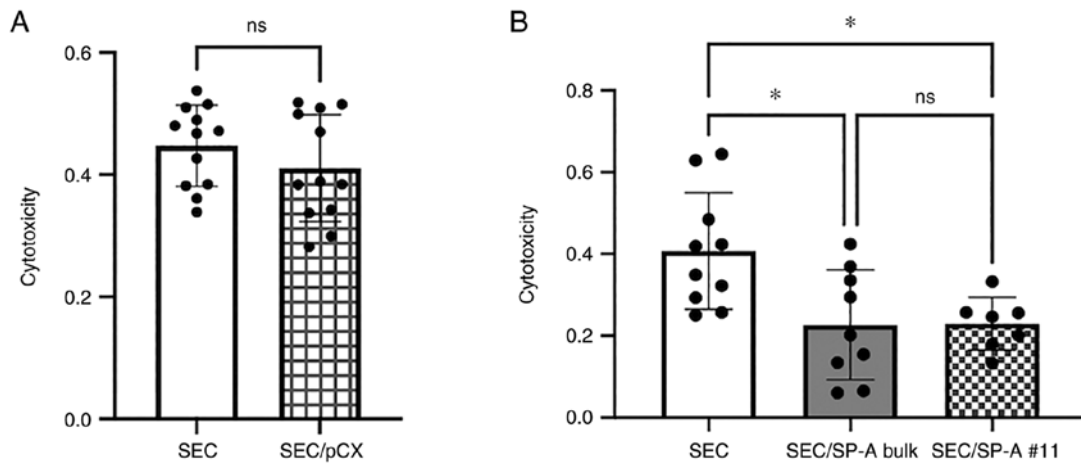


Figure 2. THP-1-induced cytotoxicity with SEC and SEC/SP-A. After co-culturing SEC or SEC/SP-A with THP-1 cells, cytotoxicity was evaluated using WST-8 assay. (A) First, we confirmed that SEC without lipofection and SEC with lipofection by the empty vector (SEC/pCX) had no difference in the cytotoxicity assay. (B) Using SEC as a control, CL-SPA on SECs significantly suppressed THP-1-induced cytotoxicity. Bars represent mean \pm SD. n=7-10, *P<0.05. SEC, swine endothelial cell; SP-A, surfactant protein-A; CL-SPA, collectin placenta 1 with carbohydrate recognition domain of SP-A; SEC/SP-A, CL-SPA-transfected SEC.

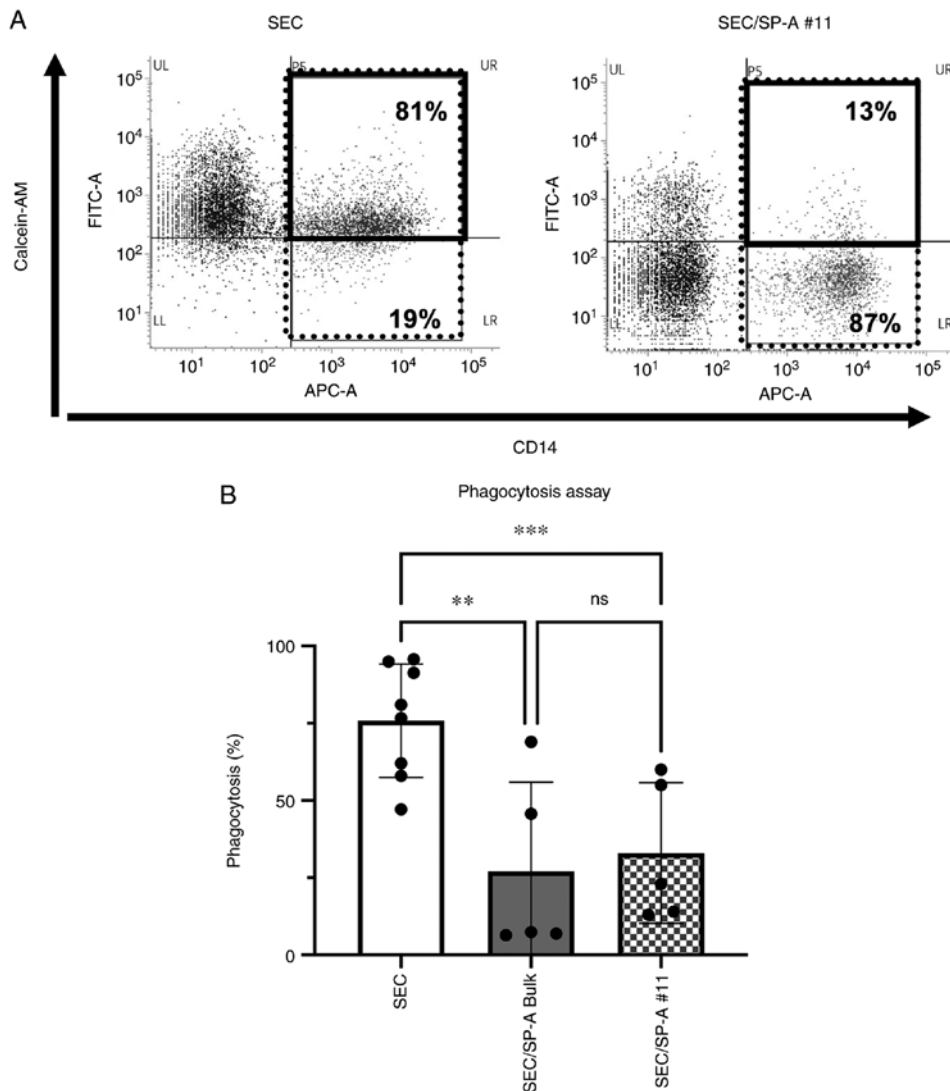


Figure 3. Suppression of macrophage-mediated phagocytosis with CL-SPA. Human macrophages were generated by culturing monocytes with M-CSF (100 ng/ml), and subsequently, the cells were co-cultured with SEC or SEC/SP-A for 24 h. (A) Phagocytosis of SEC or SEC/SP-A #11 was assessed via flow cytometry. Data are representative of five different experiments. These histograms showed the percentage of calcein-AM-positive cells within CD14-positive cells. The percentage of phagocytosis was calculated as (CD14⁺ and calcein-AM⁺ cells, thick-bordered box)/(CD14⁺ cells, thin box) x100(%). (B) CL-SPA induced a significant suppression of phagocytosis. Bars represent SD. n=5-8, **P \leq 0.01, ***P \leq 0.001. SEC, swine endothelial cell; SP-A, surfactant protein-A; CL-SPA, collectin placenta 1 with carbohydrate recognition domain of SP-A; SEC/SP-A, CL-SPA-transfected SEC.

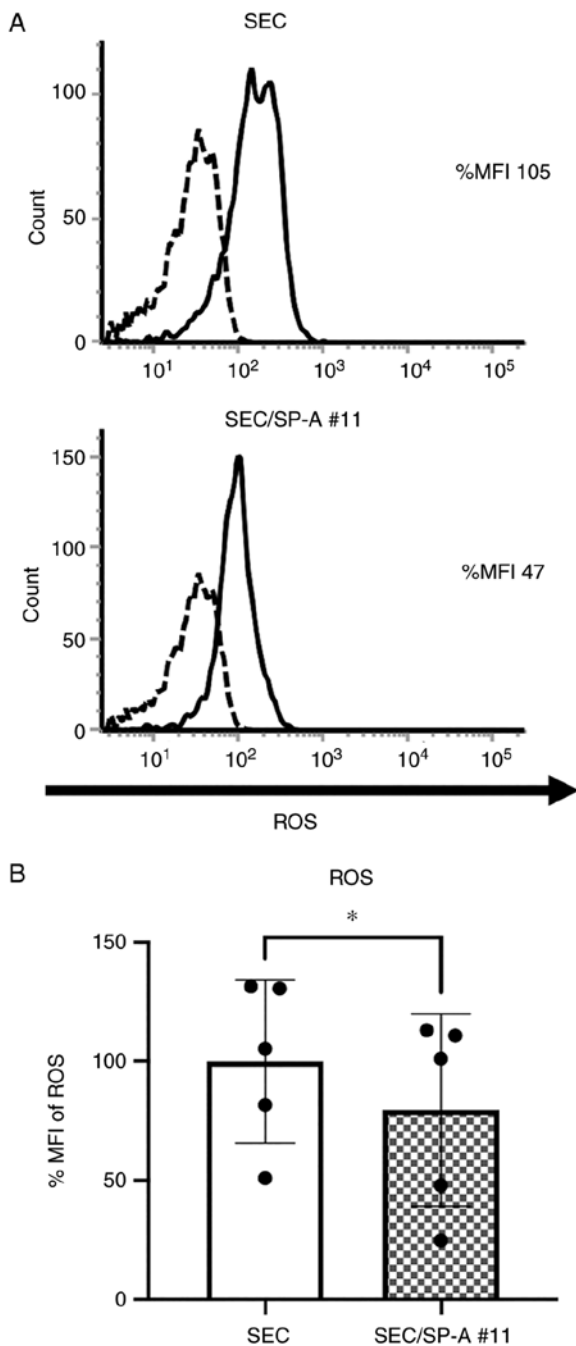


Figure 4. Production of ROS in macrophages under xenogeneic conditions. Human macrophages were co-cultured with SEC or SEC/SP-A #11 for 24 h. The MFI percentages were calculated as (MFI of SEC/SP-A #11)/(median MFI of SEC) \times 100(%). (A) Representative data are shown and (B) production of ROS in macrophages under xenogeneic conditions was significantly suppressed with CL-SPA. Bars represent mean \pm SD. n=5, *P<0.05. SEC, swine endothelial cell; SP-A, surfactant protein-A; CL-SPA, collectin placenta 1 with carbohydrate recognition domain of SP-A; SEC/SP-A, CL-SPA-transfected SEC; ROS, reactive oxygen species; MFI, mean fluorescence intensity.

Detection of ROS in macrophages. ROS levels were quantified after staining with CellROX™ Green Reagent (Life Technologies). CellROX™ reagent was added to cells at a final concentration of 5 μ M, and this was followed by incubation in the dark at 37°C for 30 min. The stained cells were then resuspended in phosphate-buffered saline, and fluorescence images were evaluated using a FACSVerser™ flow cytometer (BD Biosciences).

Luciferase assay and PMA-induced activation of THP-1-Lucia NF- κ B cells. To evaluate the PMA-induced activation of THP-1-Lucia NF- κ B cells, PMA was added to the cell culture at concentrations ranging between 20 nM and 2 μ M. After 24 h, a 10 μ l aliquot of supernatant from each well was placed into a 96-well white plate. Following this, 50 μ l of QUANTI-Luc™ assay solution, the substrate for luciferase reaction, was added to each well, and light signals were measured using a luminometer (Centro XS3 LB960, Berthold Technologies). The obtained values are expressed as relative light units.

Naive SECs or SEC/SP-As were plated in a 96-well plate as in the cytotoxicity assay on Day 0. Following this, THP-1-Lucia NF- κ B cells (5.0×10^4) were added to each well on Day 1. After co-culturing, a 10 μ l aliquot of supernatant in each well was measured using 50 μ l of the QUANTI-Luc™ assay solution and a luminometer.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from cells using TRIzol® reagent (Invitrogen) and used for RT-qPCR experiments. mRNA was amplified using a One-Step SYBR® Prime Script® RT-PCR kit (Takara Bio Inc.), and it was quantified using a Light Cycler 96 (Roche). A total of 20 μ l of PCR mixture was prepared containing 50 ng RNA, 2 U TaKaRa Ex Taq HS, 0.4 μ l PrimerScript RT enzyme Mix II, and the corresponding paired primers at a concentration of 0.2 μ mol/l of each primer. The thermal cycling conditions were as follows: 42°C for 5 min, followed by 95°C for 10 s and then 40 cycles at 95°C for 5 s and 60°C for 20 s. The amount of mRNA was normalized to that of GAPDH mRNA and analyzed (32). The primer sequences for each gene were the same as previously reported (33): iNOS-Fwd, ATTCTGCTGCTTGCTGAGGT; iNOS-Rev, TTCAAGACCAAATTCACCAC; IL-1 β -Fwd, ACAGATGAAGTGCTCCTTCCA; IL-1 β -Rev, GTCGGAGAT TCGTAGCTGGAT; TNF- α -Fwd, CCCAGGGACCTCTCT CTAATC; TNF- α -Rev, ATGGGCTACAGGCTTGTCAC; ARG-1-Fwd, GTTTCTCAAGCAGACCAGCC; ARG-1-Rev, GCTCAAGTGCAGCAAAGAGA; IL-10-Fwd, GGCGCTGTC ATCGATTTCTT; IL-10-Rev, GGCTTTGTAGATGCCTTT CTCTTG; GAPDH-Fwd, TAAAAGCAGCCCTGGTGAC, GAPDH-Rev, CTCTGCTCCTCCTGTTTCGAC.

Statistical analysis. GraphPad Prism9 software [GraphPad Prism version 9.0.0 for Mac; GraphPad Software, Inc.] was used to perform statistical tests and to generate graphs. Comparisons between two groups were performed using a two-tailed Welch's t-test or unpaired Student's t-test. Comparisons between multiple groups were evaluated using a two-way ANOVA multiple comparisons test and Tukey's test. Statistical significance was set at P<0.05. Data in the figures are presented as mean \pm standard deviation. P-values are shown as; *P<0.05; **P \leq 0.01; and ***P \leq 0.001.

Results

CL-SPA expression on SEC/SP-A. To investigate the effect of CL-SPA on macrophage-mediated xenogeneic rejection, a plasmid containing cDNA for CL-SPA was transfected into SECs. The RNA expression of CL-SPA in SEC/SP-A was confirmed by semi-quantitative RT-PCR (Fig. 1D). The expression of a flag on SEC/SP-A itself was measured using

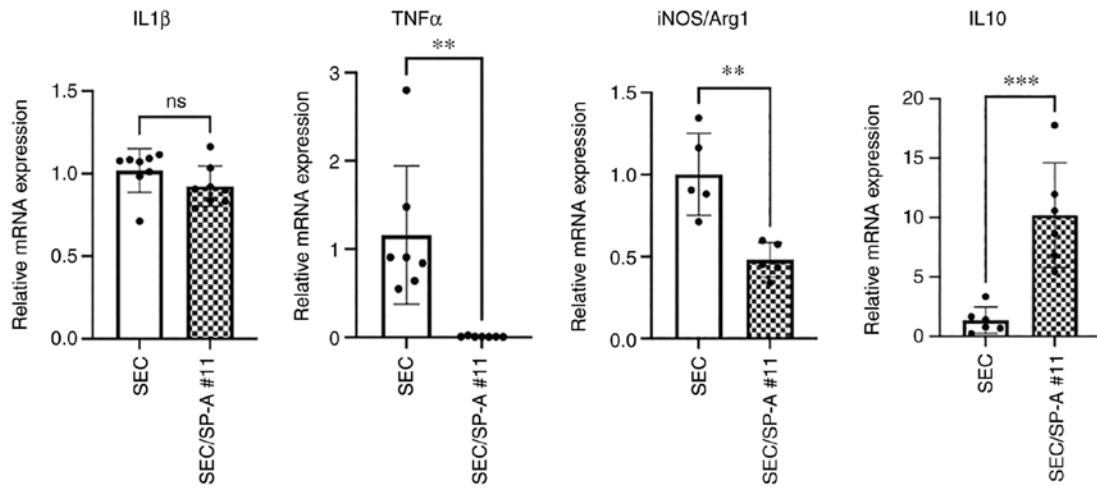


Figure 5. mRNA expression of pro-inflammatory and anti-inflammatory cytokines in macrophages. Macrophages were co-cultured with SEC or SEC/SP-A for 24 h, and they were analyzed via RT-qPCR. A significant suppression of TNF- α expression and upregulation of IL-10 expression were observed in macrophages cultured with SEC/SP-A as compared to those in macrophages cultured with SEC. The balance between iNOS and Arg-1 was significantly suppressed with CL-SPA. Bars represent SD. n=5-8, **P \leq 0.01, ***P \leq 0.001. SEC, swine endothelial cell; SP-A, surfactant protein-A; CL-SPA, collectin placenta 1 with carbohydrate recognition domain of SP-A; SEC/SP-A, CL-SPA-transfected SEC.

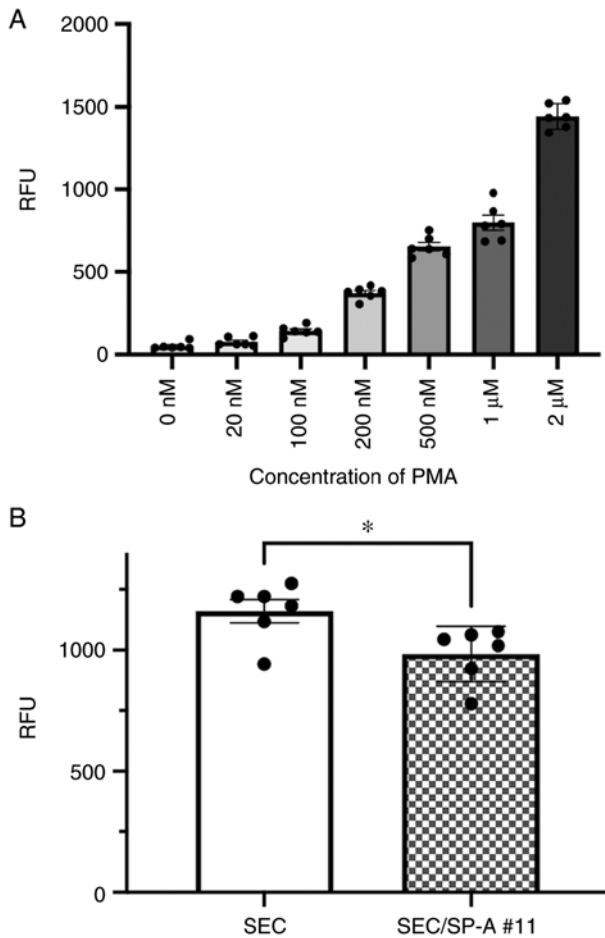


Figure 6. Activation of THP-1-Lucia NF- κ B cell with PMA and suppression with CL-SPA. (A) THP-1-Lucia NF- κ B cells were incubated with PMA at concentrations ranging between 20 nM and 2 μ M for 24 h. RLU were obtained in a dose-dependent manner. (B) THP-1-Lucia NF- κ B cells and SEC or SEC/SP-A were co-cultured with 200 nM PMA and incubated for 24 h. CL-SPA on SEC suppressed PMA-induced NF- κ B activation as compared to SECs. Bars represent SD. n=6, *P<0.05. PMA, phorbol-12-myristate-13-acetate; SEC, swine endothelial cell; SP-A, surfactant protein-A; CL-SPA, collectin placenta 1 with carbohydrate recognition domain of SP-A; SEC/SP-A, CL-SPA-transfected SEC.

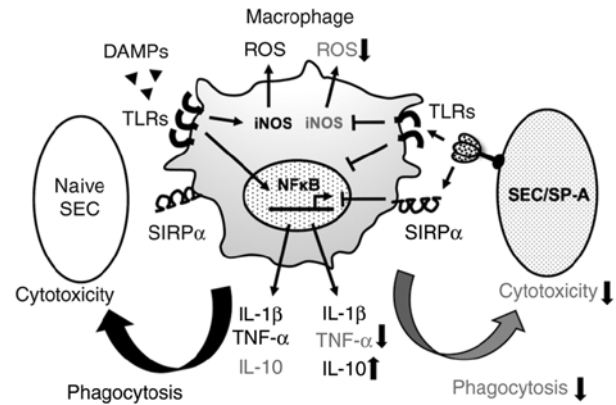


Figure 7. The suppressive effects of THP-1 and macrophages with CL-SPA on SECs. SEC-derived DAMPs induce inflammatory cytokines and ROS production in macrophages via TLRs. CL-SPA suppressed the cytotoxicity and phagocytosis of macrophages, including the reduction in mRNA expression of TNF α and ROS production. As a result, macrophage-mediated xenogeneic rejection was suppressed by CL-SPA on SECs. DAMPs, damage-associated molecular patterns; SEC, swine endothelial cell; SP-A, surfactant protein-A; CL-SPA, collectin placenta 1 with carbohydrate recognition domain of SP-A; SEC/SP-A, CL-SPA-transfected SEC.

flow cytometry because CL-SPA is a membranous protein (Fig. 1E). We used SEC/SP-A bulk cells and a clonal line that expressed more than 95% of CL-SPA.

Suppression of macrophage-mediated cytotoxicity via CL-SPA. We confirmed that SEC without lipofection and SEC with lipofection by empty vector (SEC/pCX) had no difference in the THP-1-induced cytotoxicity assay (SEC: 0.447 vs. SEC/pCX: 0.410, P=0.371, Fig. 2A). SECs without lipofection were used as a control.

To evaluate the cytotoxicity of macrophages against SEC cells, THP-1 cells were used as effector cells (Fig. 2B). After co-culturing SEC or SEC/SP-A with THP-1 cells, we evaluated the cytotoxicity of effector cells using a WST-8 assay. CL-SPA significantly suppressed THP-1-induced cytotoxicity (SEC:

0.407 vs. SEC/SP-A bulk: 0.2265 or SEC/SP-A #11: 0.2293, $P=0.0131$, $P=0.0252$, respectively).

Suppression of macrophage-mediated phagocytosis via CL-SPA. To evaluate macrophage-mediated phagocytosis against SECs, macrophages were generated by culturing monocytes with 100 ng/ml rM-CSF, and then co-cultured with SEC or SEC/SP-A. Significant phagocytosis was induced in SECs (75.9%), and CL-SPA significantly suppressed phagocytosis by macrophages (SEC/SP-A bulk: 27.1%, SEC/SP-A #11:33.0%, $P=0.0085$, $P=0.0010$, respectively, Fig. 3).

Suppression of innate immune responses via CL-SPA. To evaluate the suppressive effect of CL-SPA on innate immune responses, macrophages were cultured with SEC or SEC/SP-A. The production of ROS in macrophages under xenogeneic conditions was significantly suppressed by CL-SPA (SEC: 99.9 vs. SEC-SP-A #11:79.5, $P=0.0138$, Fig. 4). The expression of pro- and anti-inflammatory cytokines in macrophages was analyzed using RT-qPCR. Significant suppression of TNF- α expression (SEC: 1.160 vs. SEC/SP-A #11:0.009, $P=0.0022$) and upregulation of IL-10 expression (SEC: 1.357 vs. SEC/SP-A #11:10.20, $P=0.0007$) were observed in macrophages cultured with SEC/SP-A as compared to that in those cultured with SECs (Fig. 5). The balance between iNOS and Arg-1 was significantly suppressed via CL-SPA (SEC: 1.002 vs. SEC/SP-A #11:0.4792, $P=0.0027$), indicating that CL-SPA on porcine cells inhibited the differentiation of peripheral blood monocytes into inflammatory M1 macrophages. Additionally, NF- κ B activation in macrophages was evaluated with a luciferase assay using THP-1 Luc NF- κ B cells. CL-SPA significantly suppressed PMA-induced NF- κ B activation as compared to THP-1 cells co-cultured with SEC (SEC: 1160 vs. SEC/SP-A #11:983.8, $P=0.0258$, Fig. 6).

Discussion

The study findings indicated that SP-A induced a significant suppression of macrophage-mediated phagocytosis. This is the first report evaluating the suppressive function of SPA-CRD.

Xenotransplantation using a porcine with transgenic modifications has been reported recently (34). The genetically engineered porcine harbored ten genetic modifications, including the targeted insertion of two human complement regulatory genes (hDAF, hCD46), two human anti-coagulant genes (hTBM, hEPCR), and two immunomodulatory genes (hCD47, hHO1) as well as deletion (knockout) of 3 pig carbohydrate antigens and the pig growth hormone receptor gene. Our study suggested that the control of macrophage-mediated xenogeneic reaction by SPA-CRD may potentially be a new approach to genetic modification of xenografts.

SP-A has been reported to suppress macrophage phagocytosis by binding to SIRP α in macrophages (30). As expected, SP-A significantly suppressed phagocytosis. In RT-qPCR analysis, SP-A caused suppression of the pro-inflammatory cytokine, TNF- α , and upregulation of the anti-inflammatory cytokine, IL-10, similar to our previous report describing the effect of SP-D on macrophages (31). The graphical abstract is shown in Fig. 7. The CRD of SP-A has been reported to bind to toll-like receptor (TLR)-2,4 and suppress TLR-mediated

inflammation (35-37). Suppression of NF- κ B by SP-A (Fig. 6) might be a result of CL-SPA binding to TLRs. Since our result may involve TLR-mediated inhibitory effects, further investigation of these mechanisms is necessary. Regarding the control of CXR, suppression of pro-inflammatory cytokines from macrophages may also contribute to the suppression of neutrophil-induced rejection. Pro-inflammatory cytokines from macrophages have been reported to contribute to nuclear extracellular trap (NET)osis in neutrophils (38), and ectopic expression of SP-A in SEC is an effective strategy for suppressing the innate immunity associated with xenotransplantation. Neutrophils also contribute to CXR and induce tissue damage under xenogeneic conditions in antibody-dependent and -independent manners (18,39,40). NETs produced by neutrophils cause extensive endothelial cell damage in response to xenogeneic antigen (41-43). However, neutrophil apoptosis contributes to the resolution of inflammation. The phagocytosis of apoptotic cells by macrophages is central to the successful resolution of an inflammatory response, and it is increasingly apparent that the dying neutrophils themselves exert anti-inflammatory effects by modulating surrounding cell responses, particularly the release of inflammatory cytokines from macrophages (44,45). These findings indicate that the ectopic expression of CRD in SP-A can suppress macrophage-mediated cytotoxicity as well as neutrophil-mediated tissue damage. Furthermore, increasing neutrophil apoptosis and decreasing NETosis by NF- κ B suppression would lead to anti-inflammatory conditions and resolution of inflammation.

In conclusion, we reported that the CRD of SP-A suppressed macrophage-mediated cytotoxicity and phagocytosis. Moreover, SP-A suppressed NF- κ B activation and reduced the expression of pro-inflammatory cytokines in macrophages. To further investigate the effects of SP-A on innate immunity in more detail, *in vivo* studies should be performed in the future.

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Availability of data and materials

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

Authors' contributions

CT performed the research and wrote the manuscript. AM and KM participated in study design and contributed materials. SK, RY and KM performed the research. MK, TU, YT, HE and HO participated in data analysis. SM participated in the study design and wrote the manuscript. CT, AM, SK, RY and SM confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experiments were approved by the Osaka University ethics committee [approval no. 18395(T1)]. All the participants enrolled in the study provided signed written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

- Niu D, Wei HJ, Lin L, George H, Wang T, Lee IH, Zhao HY, Wang Y, Kan Y, Shrock E, *et al*: Inactivation of porcine endogenous retrovirus in pigs using CRISPR-Cas9. *Science* 357: 1303-1307, 2017.
- Servick K: Xenotransplant advances may prompt human trials. *Science* 357: 1338, 2017.
- Cooper DKC, Ezzelarab M, Iwase H and Hara H: Perspectives on the optimal genetically engineered pig in 2018 for initial clinical trials of kidney or heart xenotransplantation. *Transplantation* 102: 1974-1982, 2018.
- Cooper DK, Gaston R, Eckhoff D, Ladowski J, Yamamoto T, Wang L, Iwase H, Hara H, Tector M and Tector AJ: Xenotransplantation-The current status and prospects. *Br Med Bull* 125: 5-14, 2018.
- Fukuta D, Miyagawa S, Yamada M, Matsunami K, Kurihara T, Shirasu A, Hattori H, and Shirakura R: Effect of various forms of the C1 esterase inhibitor (C1-INH) and DAF on complement-mediated xenogeneic cell lysis. *Xenotransplantation* 10: 132-141, 2003.
- Miyagawa S, Kubo T, Matsunami K, Kusama T, Beppu K, Nozaki H, Morita T, Ahn C, Kim JY, Fukuta D and Shirakura R: Delta-short consensus repeat 4-decay accelerating factor (DAF: CD55) inhibits complement-mediated cytolysis but not NK cell-mediated cytolysis. *J Immunol* 173: 3945-3952, 2004.
- Miyagawa S, Fukuta D, Kitano E, Kobayashi C, Fumimoto Y, Shirasu A, Hattori H, Shirakura R and Fukuzawa M: Effect of tandem forms of DAF(CD55) on complement-mediated xenogeneic cell lysis. *Xenotransplantation* 13: 433-439, 2006.
- Cardone J, Le Fric G and Kemper C: CD46 in innate and adaptive immunity: An update. *Clin Exp Immunol* 164: 301-311, 2011.
- Cadili A and Kneteman N: The role of macrophages in xenograft rejection. *Transplant Proc* 40: 3289-3293, 2008.
- Wang H and Yang YG: Innate cellular immunity and xenotransplantation. *Curr Opin Organ Transplant* 17: 162-167, 2012.
- Vadori M and Cozzi E: The immunological barriers to xenotransplantation. *Tissue Antigens* 86: 239-253, 2015.
- Candinas D, Belliveau S, Koyamada N, Miyatake T, Hechenleitner P, Mark W, Bach FH and Hancock WW: T cell independence of macrophage and natural killer cell infiltration, cytokine production, and endothelial activation during delayed xenograft rejection. *Transplantation* 62: 1920-1927, 1996.
- Fox A, Mountford J, Braakhuis A and Harrison LC: Innate and adaptive immune responses to nonvascular xenografts: Evidence that macrophages are direct effectors of xenograft rejection. *J Immunol* 166: 2133-2140, 2001.
- Xu XC, Goodman J, Sasaki H, Lowell J and Mohanakumar T: Activation of natural killer cells and macrophages by porcine endothelial cells augments specific T-cell xenoreponse. *Am J Transplant* 2: 314-322, 2002.
- Adams S, van der Laan LJ, Vernon-Wilson E, Renardel de Lavalette C, Döpp EA, Dijkstra CD, Simmons DL and van den Berg TK: Signal-regulatory protein is selectively expressed by myeloid and neuronal cells. *J Immunol* 161: 1853-1859, 1998.
- Itoh T, Hata Y, Nishinakamura H, Kumano K, Takahashi H and Kodama S: Islet-derived damage-associated molecular pattern molecule contributes to immune responses following microencapsulated neonatal porcine islet xenotransplantation in mice. *Xenotransplantation* 23: 393-404, 2016.
- Martin BM, Samy KP, Lowe MC, Thompson PW, Cano J, Farris AB, Song M, Dove CR, Leopardi FV, Strobert EA, *et al*: Dual islet transplantation modeling of the instant blood-mediated inflammatory reaction. *Am J Transplant* 15: 1241-1252, 2015.
- Matsunami K, Miyagawa S, Nakai R, Yamada M and Shirakura R: Modulation of the leader peptide sequence of the HLA-E gene up-regulates its expression and down-regulates natural killer cell-mediated swine endothelial cell lysis. *Transplantation* 73: 1582-1589, 2002.
- Matsunami K, Kusama T, Okura E, Shirakura R, Fukuzawa M and Miyagawa S: Involvement of position-147 for HLA-E expression. *Biochem Biophys Res Commun* 347: 692-697, 2006.
- Maeda A, Kawamura T, Ueno T, Usui N, Eguchi H and Miyagawa S: The suppression of inflammatory macrophage-mediated cytotoxicity and proinflammatory cytokine production by transgenic expression of HLA-E. *Transpl Immunol* 29: 76-81, 2013.
- Maeda A, Kawamura T, Nakahata K, Ueno T, Usui N, Eguchi H and Miyagawa S: Regulation of macrophage-mediated xenocytotoxicity by overexpression of alpha-2,6-sialyltransferase in swine endothelial cells. *Transplant Proc* 46: 1256-1258, 2014.
- Esquivel EL, Maeda A, Eguchi H, Asada M, Sugiyama M, Manabe C, Sakai R, Matsuura R, Nakahata K, Okuyama H and Miyagawa S: Suppression of human macrophage-mediated cytotoxicity by transgenic swine endothelial cell expression of HLA-G. *Transpl Immunol* 32: 109-115, 2015.
- Maeda A, Eguchi H, Nakahata K, Lo PC, Yamanaka K, Kawamura T, Matsuura R, Sakai R, Asada M, Okuyama H and Miyagawa S: Monocytic MDSCs regulate macrophage-mediated xenogenic cytotoxicity. *Transpl Immunol* 33: 140-145, 2015.
- Sakai R, Maeda A, Choi TV, Lo PC, Jiaravuthisan P, Shabri AM, Wang HT, Matsuura R, Kodama T, Eguchi H, *et al*: Human CD200 suppresses macrophage-mediated xenogeneic cytotoxicity and phagocytosis. *Surg Today* 48: 119-126, 2018.
- Bridges JP, Davis HW, Damodarasamy M, Kuroki Y, Howles G, Hui DY and McCormack FX: Pulmonary surfactant proteins A and D are potent endogenous inhibitors of lipid peroxidation and oxidative cellular injury. *J Biol Chem* 275: 38848-38855, 2000.
- LeVine AM and Whitsett JA: Pulmonary collectins and innate host defense of the lung. *Microbes Infect* 3: 161-166, 2001.
- Borron P, McIntosh JC, Korfhagen TR, Whitsett JA, Taylor J and Wright JR: Surfactant-associated protein A inhibits LPS-induced cytokine and nitric oxide production in vivo. *Am J Physiol Lung Cell Mol Physiol* 278: L840-L847, 2000.
- Kremlev SG and Phelps DS: Surfactant protein A stimulation of inflammatory cytokine and immunoglobulin production. *Am J Physiol* 267 (6 Pt 1): L712-L719, 1994.
- Gardai SJ, Xiao YQ, Dickinson M, Nick JA, Voelker DR, Greene KE and Henson PM: By binding SIRPalpha or calreticulin/CD91, lung collectins act as dual function surveillance molecules to suppress or enhance inflammation. *Cell* 115: 13-23, 2003.
- Janssen WJ, McPhillips KA, Dickinson MG, Linderman DJ, Morimoto K, Xiao YQ, Oldham KM, Vandivier RW, Henson PM and Gardai SJ: Surfactant proteins A and D suppress alveolar macrophage phagocytosis via interaction with SIRPalpha. *Am J Respir Crit Care Med* 178: 158-167, 2008.
- Jiaravuthisan P, Maeda A, Takakura C, Wang HT, Sakai R, Shabri AM, Lo PC, Matsuura R, Kodama T, Eguchi H, *et al*: A membrane-type surfactant protein D (SP-D) suppresses macrophage-mediated cytotoxicity in swine endothelial cells. *Transpl Immunol* 47: 44-48, 2018.
- Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
- Noguchi Y, Maeda A, Lo PC, Takakura C, Haneda T, Kodama T, Yoneyama T, Toyama C, Tazuke Y, Okuyama H and Miyagawa S: Human TIGIT on porcine aortic endothelial cells suppresses xenogeneic macrophage-mediated cytotoxicity. *Immunobiology* 224: 605-613, 2019.
- Porrett PM, Orandi BJ, Kumar V, Houpp J, Anderson D, Cozette Killian A, Hauptfeld-Dolejsk V, Martin DE, Macedon S, Budd N, *et al*: First clinical-grade porcine kidney xenotransplant using a human decedent model. *Am J Transplant* 22: 1037-1053, 2022.
- Ohya M, Nishitani C, Sano H, Yamada C, Mitsuzawa H, Shimizu T, Saito T, Smith K, Crouch E and Kuroki Y: Human pulmonary surfactant protein D binds the extracellular domains of Toll-like receptors 2 and 4 through the carbohydrate recognition domain by a mechanism different from its binding to phosphatidylinositol and lipopolysaccharide. *Biochemistry* 45: 8657-8664, 2006.

36. Henning LN, Azad AK, Parsa KV, Crowther JE, Tridandapani S and Schlesinger LS: Pulmonary surfactant protein A regulates TLR expression and activity in human macrophages. *J Immunol* 180: 7847-7858, 2008.
37. Agrawal V, Smart K, Jilling T and Hirsch E: Surfactant protein (SP)-A suppresses preterm delivery and inflammation via TLR2. *PLoS One* 8: e63990, 2013.
38. Nakazawa D, Shida H, Kusunoki Y, Miyoshi A, Nishio S, Tomaru U, Atsumi T and Ishizu A: The responses of macrophages in interaction with neutrophils that undergo NETosis. *J Autoimmun* 67: 19-28, 2016.
39. al-Mohanna F, Collison K, Parhar R, Kwaasi A, Meyer B, Saleh S, Allen S, al-Sedairy S, Stern D and Yacoub M: Activation of naive xenogeneic but not allogeneic endothelial cells by human naive neutrophils: A potential occult barrier to xenotransplantation. *Am J Pathol* 151: 111-120, 1997.
40. Sachs UJ, Andrei-Selmer CL, Maniar A, Weiss T, Paddock C, Orlova VV, Choi EY, Newman PJ, Preissner KT, Chavakis T and Santoso S: The neutrophil-specific antigen CD177 is a counter-receptor for platelet endothelial cell adhesion molecule-1 (CD31). *J Biol Chem* 282: 23603-23612, 2007.
41. Sayah DM, Mallavia B, Liu F, Ortiz-Muñoz G, Cadrillier A, DerHovanessian A, Ross DJ, Lynch JP III, Saggat R, Ardehali A, *et al*: Neutrophil extracellular traps are pathogenic in primary graft dysfunction after lung transplantation. *Am J Respir Crit Care Med* 191: 455-463, 2015.
42. Huang H, Tohme S, Al-Khafaji AB, Tai S, Loughran P, Chen L, Wang S, Kim J, Billiar T, Wang Y and Tsung A: Damage-associated molecular pattern-activated neutrophil extracellular trap exacerbates sterile inflammatory liver injury. *Hepatology* 62: 600-614, 2015.
43. Liu FC, Chuang YH, Tsai YF and Yu HP: Role of neutrophil extracellular traps following injury. *Shock* 41: 491-498, 2014.
44. Kennedy AD and Deleo FR: Neutrophil apoptosis and the resolution of infection. *Immunol Res* 43: 25-61, 2009.
45. Fox S, Leitch AE, Duffin R, Haslett C and Rossi AG: Neutrophil apoptosis: Relevance to the innate immune response and inflammatory disease. *J Innate Immun* 2: 216-227, 2010.