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Survival of porcine fibroblasts enhanced by human FasL and dexamethasone-treated human dendritic cells *in vitro*

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ABSTRACT

Cell-mediated and acute vascular rejections remain to be one of the primary hurdles to achieve successful xenotransplantation. Fas ligand is known to be an important molecule for the formation of 'immune-privileged' condition and dendritic cells treated with dexamethasone (Dex-DCs) acting like tolerogenic DCs (tDCs) which are known to protect transplanted cells and organs from unwanted immune responses. The present study investigated the possibility that porcine fibroblasts expressing human Fas ligand (PhF) together with human Dex-DCs could induce prolonged survival of porcine fibroblasts *in vitro*. PhF was collected from an ear of human Fas ligand transgenic porcine and cell-line was established by MGEM Inc. PhF labeled with CFSE co-cultured with human peripheral blood mononuclear cells (hPBMCs) were examined with respect to induction of tolerance and cell death when co-cultured with Dex-DCs for 3 days. PhF induced the apoptosis in hPBMCs, especially $CD4^+$ T cells. Dex-DCs showed significant ($P < 0.05$) reduction on the expression of CD80, CD86 and MHC class I/II, and the secretion of IL-12p70, TNF-α and IL-10, but increase of latency-associated peptide (LAP). Survival of PhF was significantly higher than that of WT and it was increased in the presence of Dex-DCs when compared to the other DCs (i.e.,DCs, LPS-treated DCs and LPS/Dex-treated DCs) *in vitro*. Survival of PhF did not change by co-culture with Dex-DCs due to apoptotic cell death of Dex-DCs. Dex-DCs reduced the death of porcine fibroblasts and, at the same time, PhF induced the apoptosis from hPBMCs, but it was not synergistic.

1. Introduction

For xenotransplantation research, Galα1–3Galβ1–4GlcNAc (referred to GalT) knockout pigs were produced in 2002 [1,2] since GalT causes major natural antibodies in human serum, leading to hyperacute rejection (HPR) and acute humoral xenograft rejection (AHXR) [3]. Although baboons were transplanted with heart from a human complement regulatory protein (CD46)-transgenic pigs with B-cell-depleting antibody and an alpha1,3Gal polymer to remove alpha1,3Gal-specific antibodies, rejection was occurred by T cells within 7 days after the operation [4].

FasL, a type II membrane protein, is expressed on activated T lymphocytes [5,6], NK cells [7], and especially at 'immune-privileged' sites including testis, thyroid, and eyes [8]. Some non-hematopoietic cells also express FasL and thereby kill infiltrating Fas-expressed T and NK cells to establish an immune-privileged niche [8–10]. Abnormally increased FasL-mediated killing of Fas^+ bystander cells has been suggested in immune-pathological states, such as hepatitis induced by extensive T cell activation [11]. It has been suggested that transplantation of FasL-expressing islet cells or tissues can induce apoptosis [12]. Nevertheless, only a few reports have addressed the role of transgenic human FasL (hFasL) expression on cell types other than islets in the protection of allo- or xenografts [13–16]. Another study with a transient overexpression of porcine FasL in porcine endothelial cells showed inhibition of human leukocyte-mediated cytotoxicity [6].

Dendritic cells (DCs) are the key player in determining the induction of immunity and tolerance, and essentially required for antigen-specific immune responses. The concept of tolerogenic DCs (tDCs) comes from the observation that human DCs treated with soluble CD83 showing immature phenotype (low expression of MHC class II, CD40, CD80 and interleukin (IL)-12) prolonged more than 100 days of survival time in kidney allograft *in vivo* [17]. Since then, a variety of strategies and pharmacological agents have been exploited to generate tDCs including

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differential use of cytokines (e.g., IL-10 and transforming growth factor (TGF)-β) [18], genetic interference with nuclear factor-kappa B (NF-κB) or expression of co-stimulatory molecules [19], and exposure to immunosuppressive agents including dexamethasone (Dex), corticosteroids, vitamin D3, and rapamycin [20]. It has been suggested that tDCs can induce differentiation and expansion of regulatory T cells (Treg) and suppress effector T cell activities [21].

Dex-DCs have been studied for the potential use in transplantation. Dex-DCs from mice bone marrow-derived DC decreased the secretion of pro-inflammatory cytokines, IL-12 and TNF-α, and prevented the proliferation of $CD4^+$ T cells [22]. Dex-DCs decreased to produce IL-12 and reduced the proliferation of $CD4^+CD25^-$ T cells in rat and human *in vitro* [23].

In the present study, we hypothesized that porcine fibroblasts expressing human Fas ligand (PhF) induce the apoptosis in active human lymphocytes expressing Fas, and together with co-treatment of Dex-DCs would suppress the action of human lymphocytes *in vitro*.

2. Materials and methods

2.1. Cells

Porcine fibroblasts from hFasL transgenic as PhF and normal pigs as wild-type (WT) were kindly provided by MGEN Inc. [24,25]. The cells including PhF and WT were cultured in a complete DMEM purchased from GenDEPOT (Berker, TX, USA) supplemented with 10% fetal bovine serum (FBS) purchased from GIBCO (Grand Island, NY, USA) and 1% antibiotics (GenDEPOT) in a humidified incubator with 5% CO_2 at 39 °C. We used the porcine fibroblasts with passages between 5 and 15. To generate monocyte-derived DCs, peripheral blood mononuclear cells (PBMCs) from heparinized human adult blood (IRB no. 1107/001-005 at Seoul National University) were obtained by using density gradient centrifugation (410 ×*g* for 25 min) with Ficoll-Paque Plus purchased from Amersham Bioscience (Buckinghamshire, UK). $CD14^+$ monocytes were isolated from the hPBMCs using anti-human CD14 antibody together with a magnetic bead-based kit IMag™ purchased from BD Biosciences (San Jose, CA, USA). $CD14^+$ monocytes were suspended in a complete RPMI media and cultured with human recombinant IL-4 (500 U/ml) purchased from CreaGene (Seongnam, Korea) and GM-CSF (800 U/ml) purchased from R&D Systems (Minneapolis, MN, USA) for 6 days with changes of media every 3 days [26].

2.2. Expression of hFasL in the porcine fibroblasts

To examine the mRNA expression of hFasL, porcine fibroblasts (WT and PhF) were lysed with 1 ml of Trizol purchased from Invitrogen (Carlsbad, NM, USA). Chloroform was added at 200 μl/ml, the tubes were centrifuged at 12,000 ×*g* for 15 min, and the aqueous phase containing mRNA was transferred into new tubes. The mRNA was precipitated by the addition of 0.5 ml isopropanol followed by centrifugation at 12,000 ×*g* for 10 min. The mRNA was cleaned by washing with 75% ethanol and centrifuged at 7500 ×*g* for 5 min. After removal of ethanol, the mRNA was resuspended in nuclease-free water and then reverse-transcribed into complementary deoxyribonucleic acid (cDNA) with oligo dT purchased from Promega (San Luis Obispo, CA, USA). Amplification of cDNA using PCR was performed in a total volume of 20 μl containing 0.5 unit of Taq polymerase purchased from Bioneer (Deajeon, Korea) and 19 pmol of primers specific for human FasL (forward primer: 5′-GGA ATG GGA AGA CAC CTA TG-3′, reverse primer: 5′-AGA GAG AGC TCA GAT ACG TT-3′), and porcine β-actin (forward primer: 5′-AGA GCG CAA GTA CTC CGT GT-3′, reverse primer: 5′-AAA GCC ATG CCA ATC TCA TC-3′). Amplified PCR products (35 cycles for hFasL and porcine β-actin) were subjected to electrophoresis on 1% agarose gel containing ethidium bromide purchased from Sigma-Aldrich (Saint Louis, MO, USA).

To confirm the protein expression of hFasL, the cells were disrupted by RIPA buffer containing 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS and 50 mM Tris–HCl (pH 7.4), and protease inhibitor cocktail purchased from Roche (Mannheim, Germany) on ice for 30 min. After centrifugation at 28,000 ×*g* for 10 min at 4 °C, the amount of total protein in the supernatant was determined by Pierce™ BCA Protein Assay Kit purchased from Thermo Scientific (Rockford, IL, USA). Samples were boiled at 100 °C for 5 min, separated by 12% SDS–PAGE and transferred onto a polyvinylidene difluoride (PVDF) transfer membrane purchased from Amersham Biosciences (Piscataway, NY, USA) followed by blocking with 10% non-fat milk in TBST (TBS with 0.1% Tween 20) at room temperature for 1 h. The membrane was washed and incubated with rabbit anti-human Fas ligand (1:1,000 dilution) purchased from Cell Signaling Technology (Danvers, MS, USA) and mouse anti-human beta actin (1:20,000 dilution) purchased from Santa Cruz Biotechnology (Delaware, CA, USA) overnight at 4 °C. The membrane was then washed and incubated with a goat anti-rabbit IgG HRP-conjugated or a goat anti-mouse IgG HRP-conjugated antibodies (Santa Cruz) for 1 h at room temperature. The immunoblots were visualized by Amersham™ ECL™ Prime Western blotting detection reagent purchased from GE Healthcare Bio-Sciences AB (Uppsala, Sweden) and analyzed by ChemiDoc™ XRS + system purchased from Bio-Rad (Hercules, CA, USA).

2.3. Preparation of DCs

Immature DCs were treated with Dex (Sigma-Aldrich) at 10^{-6} M in a complete RPMI media containing IL-4 and GM-CSF at day 3 in the presence or absence of LPS, and designated as NDNL (No Dex and No LPS), NDL (No Dex and LPS), DNL (Dex and No LPS) and DL (Dex and LPS), respectively. At day 6, non-adherent and semi-adherent cells were harvested and activated with lipopolysaccharide (LPS, 500 ng/ml) (Sigma-Aldrich) for 24 h [27]. DCs were stained with anti-CD80-FITC, anti-CD86-APC, anti-MHC class I-PE or anti-MHC class II-APC antibodies (all from BD Biosciences) to determine the degree of maturation. All cells were examined using flow cytometry with the Cell Quest software (BD Biosciences) and all flow cytometric data were analyzed with the FlowJo software purchased from Tree Star (San Carlos, CA, USA).

2.4. Analysis of cellular uptake

The uptake capacity was determined in DCs treated with dextan-FITC (Invitrogen) for 1 h followed by extensive washing with phosphate buffered saline (PBS). Final uptake was determined as the geomean fluorescence intensity (MFI) of the cells incubated at 37 °C subtracting the MFI of cells incubated at 4 °C. All cells were examined using flow cytometry with the Cell Quest software (BD Biosciences) and all flow cytometric data were analyzed using the FlowJo software (Tree Star).

2.5. Measurement of cytokine production

Production of TNF-α, IL-10 and IL-12p70 in the culture supernatant of DCs stimulated with Dex (10^{-6} M) and/or LPS (500 ng/ml) for 24 h was determined using ELISA DuoSet kits (R&D Systems). In brief, the cytokine capture antibody was coated on 96-well immuno-plate purchased from Nalgene Nunc International (Rochester, NY, USA) and incubated overnight at 4 °C. The plates were then washed with washing buffer (0.05% Tween 20 in PBS) for three times and blocked with blocking buffer (1% bovine serum albumin in PBS) for 1 h. After washing, the culture supernatants and respective standard proteins were added and incubated for 2 h followed by 2 h incubation with detection antibody conjugated with biotin. The specific binding was examined using streptavidin–HRP (R&D Systems) followed by the addition of the TMB substrate (Sigma-Aldrich). The reaction was stopped with 50 μl of 2 N H_2SO_4. The amount of cytokines was measured at absorbance of 450 nm by using a microplate reader purchased from Molecular Device (Sunnyvale, CA, USA).

2.6. Induction and confirmation of Treg activity

To isolate $CD3^+$ T cells, 1×10^7 hPBMCs in 80 μl of the buffer with PBS (pH 7.2) containing 0.5% BSA and 2 mM EDTA were stained with 20 μl of human anti-CD3 MicroBead purchased from Miltenyi Biotec (Auburn, CA, USA) in dark condition for 20 min at 4 °C and then washed with PBS. $CD3^+$ T cells were separated through LS column (Miltenyi Biotec). To generate Treg, Dex-DCs were co-cultured with $CD3^+$ T cells for 5 days. The cells were stained with anti-CD4-FITC and anti-CD25-APC antibodies. Then, the cells were fixed and permeabilized by fixation/permeabilization solution followed by staining with anti-Foxp3-PE antibody in Perm/Wash™ buffer (all from BD Biosciences) in dark condition for 30 min at 4 °C. To examine regulatory activity, Treg induced by Dex-DCs were co-cultured with CFSE labeled $CD4^+$ T cells that were stimulated with anti-CD2/CD3/CD28 antibody (1:10 for bead-to-cell ratio) (Miltenyi Biotec) for 3 days [28]. The cells were analyzed for the $CFSE^+$ population using flow cytometry (BD Biosciences) and the FlowJo software (Tree Star).

2.7. Determination of apoptosis

To test the apoptosis of porcine fibroblasts, WT and PhF (1×10^5 cells/well) were labeled with carboxy fluorescein succinimidyl ester (CFSE) (Invitrogen) and cultured in 24-well plates for 24 h. Then, the cells were co-cultured with hPBMCs (1×10^5 cells/well) and NDNL, NDL, DL or DNL for 3 days. The cells were stained with AnnexinV-APC and the apoptosis of the porcine fibroblasts was identified as the cells with $CFSE^+$ and AnnexinV-APC^+.

To determine the induction of apoptosis on human $CD4^+$ T cells, WT and PhF were co-cultured with hPBMCs (1×10^5 cells/well) in 24-well plates for 3 days. The apoptosis of human $CD4^+$ T cells was determined by AnnexinV-FITC after staining with human anti-CD4-PerCP and anti-CD95-APC antibodies.

To examine apoptosis of DCs induced by PhF, WT or PhF was labeled with CFSE and co-cultured with DCs in a 24-well plate for 3 days. The apoptosis of the DCs was determined as AnnexinV-APC^+ cells after exclusion of $CFSE^+$ cells (all from BD Biosciences). The cells were examined

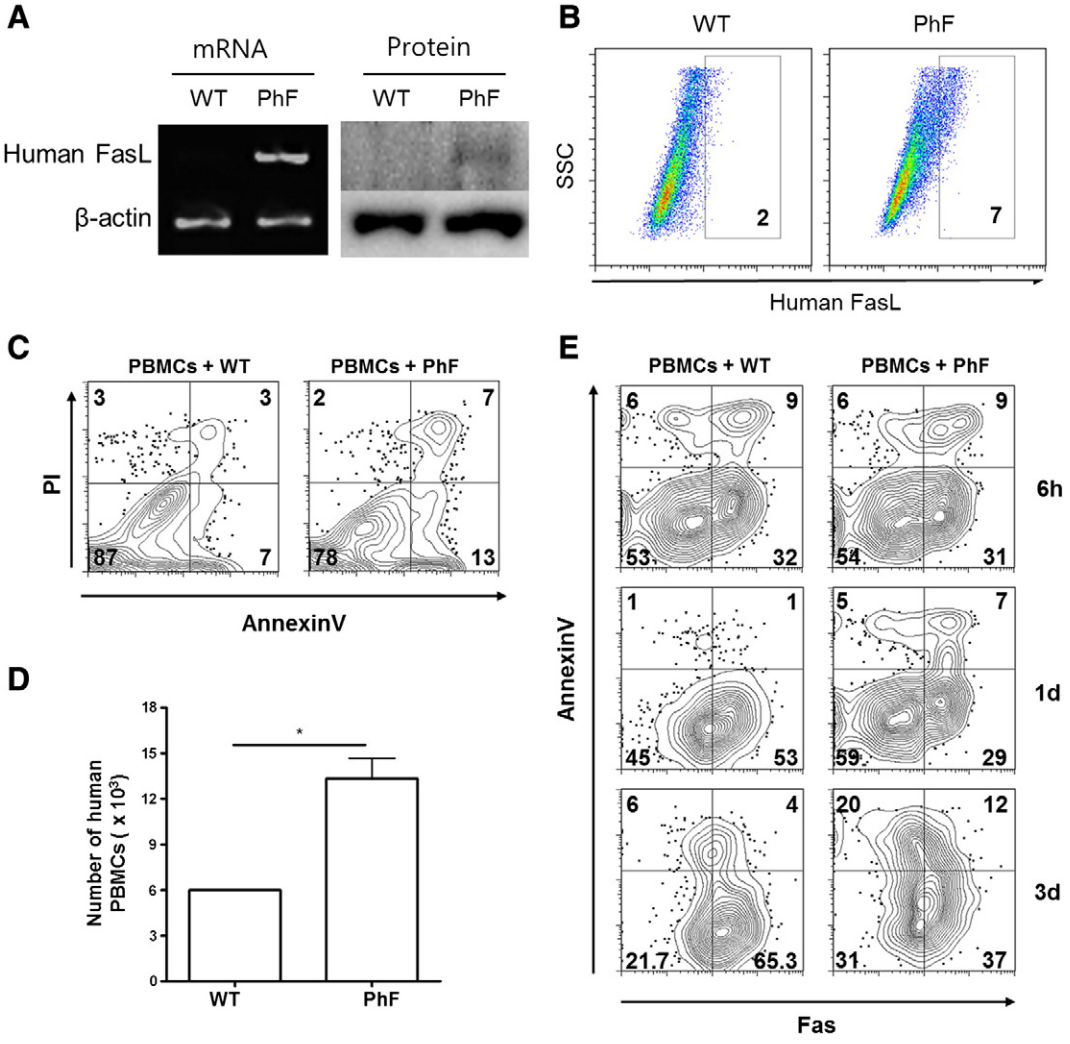

Fig. 1. Porcine fibroblasts expressing human Fas ligand (PhF) induce apoptosis in human PBMCs. Human Fas ligand expression is measured by (A and B) reverse transcription PCR for mRNA and Western blotting for protein level, and (B) flow cytometry for cell level. (C and D) PhF or control porcine fibroblasts were co-cultured with hPBMCs for 1 day. Apoptosis of human PBMCs induced by PhF was measured after (C) AnnexinV-FITC and PI staining in the dark and analyzed by using flow cytometry, and (D) a direct counting after trypan blue staining. * indicates significant difference at $P < 0.05$ (E) To examine the apoptosis of human $CD4^+$ T cells, the cells were stained with AnnexinV-FITC, CD4-PerCP and Fas-APC in dark condition for 20 min at 4 °C. The cells were gated on $CD4^+$ and analyzed for apoptosis by using flow cytometry. In B, C and E, the number in each box indicates the percentage of cells. Data are representative of three independent experiments with similar results.

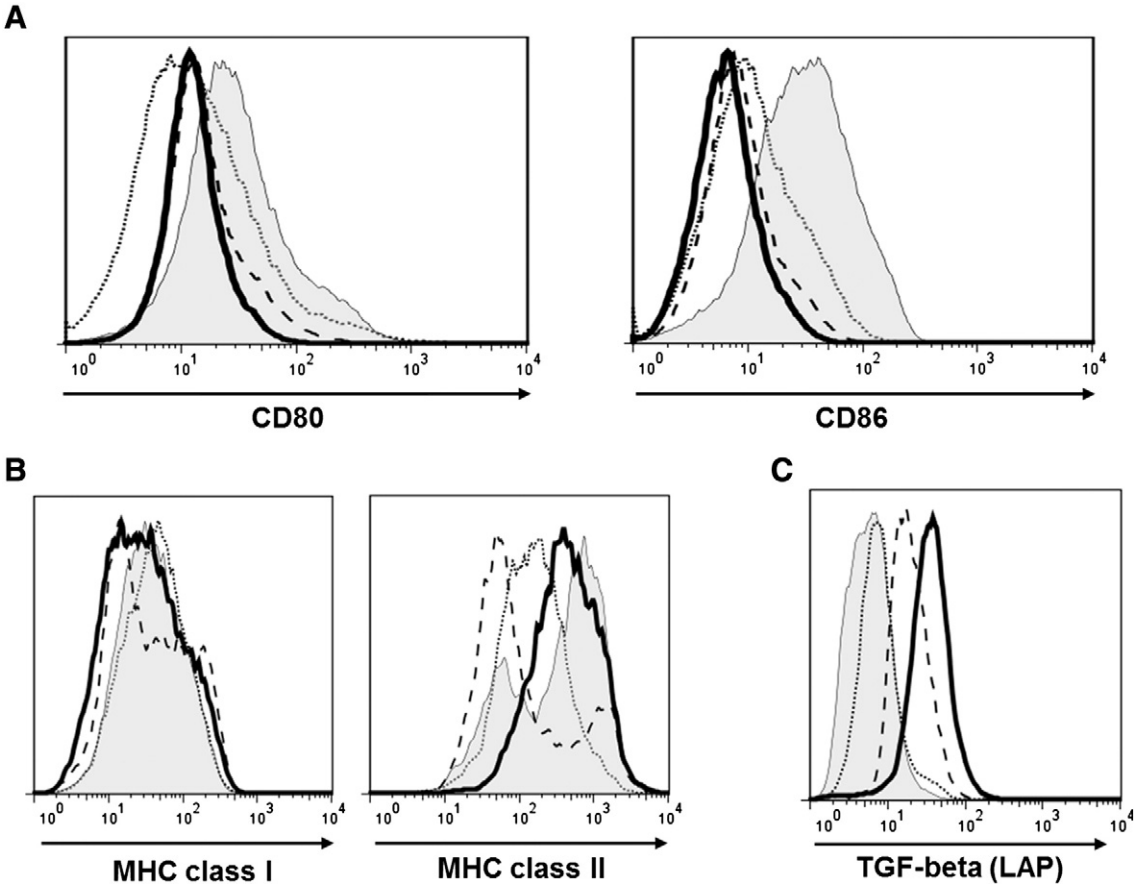

Fig. 2. Dexamethasone (Dex) modulated DCs with tolerance phenotypes. The intensity changes of (A) CD80 and CD86, (B) MHC class I and MHC class II, and (D) LAP were measured in human DCs treated with 10^{-6} M of Dex for 3 days and stimulated with 500 ng/ml of LPS for 24 h. In all cases, *dashed line*: NDNL (no Dex and no LPS treated); *filled area*: NDL (no Dex and LPS treated); *bold line*: DNL (Dex and no LPS treated); *dotted line*: DL (Dex and LPS treated). The cells were stained in dark condition for 20 min at 4 °C and the expression was analyzed by flow cytometry. Data are representative of three independent experiments with similar results.

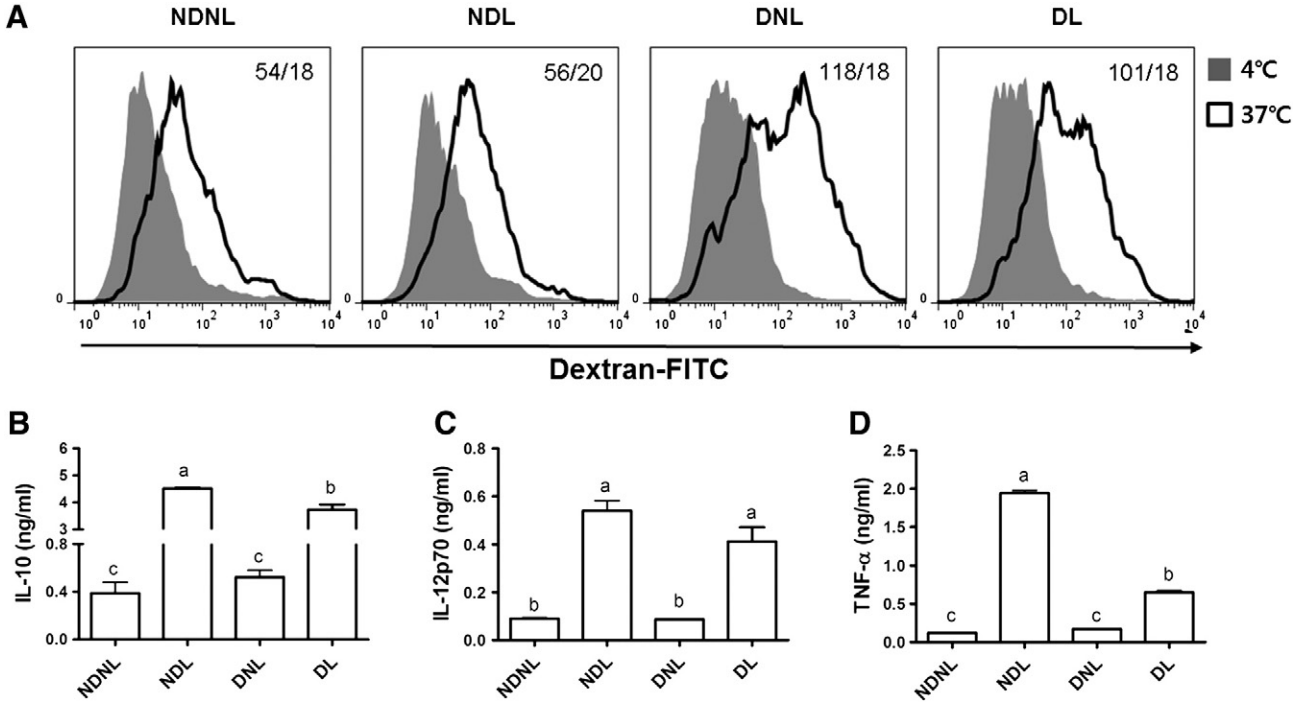

Fig. 3. Dex suppressed the activation of DCs. The intensity changes of (A) Dextran-FITC were measured in DCs treated with LPS or Dex. Dextran-FITC was co-cultured with DCs for 1 h at 37 °C (*bold line*) or 4 °C (*filled area*). The numbers in each panel indicate MFI value at 37 °C/4 °C. The concentration changes of (B) IL-10, (C) IL-12p70 and (D) TNF-α were measured in supernatants from DCs treated with LPS or Dex. A different character indicates significantly different at $P < 0.05$.

using flow cytometry with the Cell Quest software (BD Biosciences) and the FlowJo software (Tree Star).

2.8. Statistical analysis

Statistically significant differences were determined using one-way ANOVA with Tukey's test. Differences were considered significant when P value was less than 0.05.

3. Results

3.1. Porcine fibroblasts expressing human Fas ligand induce the apoptosis in human PBMCs

We confirmed the expression of hFasL in mRNA and protein (Fig. 1A), and at cellular level (Fig. 1B). It has been shown that over-expression of either decoy human Fas antigen or membrane-bound human FasL in porcine endothelial cells significantly alleviates $CD8^+$ T cell-mediated cytotoxicity [29]. To determine whether PhF induces the apoptosis in human cells, PhF was co-cultured with hPBMCs for 24 h, and the death of hPBMCs was measured by flow cytometric analysis and direct counting after trypan blue staining. The death of hPBMCs co-cultured with PhF was significantly ($P < 0.05$) higher than that with WT (Fig. 1C and D). Total of AnnexinV single positive, PI single positive and double positive hPBMCs (i.e., dead cells) in hPBMCs co-cultured with WT was about 13%, while that in PhF was 22% (Fig. 1C). Absolute number of trypan blue stained cells (i.e., dead cells) in hPBMCs with PhF was also significantly ($P < 0.05$) higher (Fig. 1D) than that of WT. The death of $CD4^+$ T cells in hPBMCs co-cultured with PhF was about 32% compared with 10% on WT at day 3 (Fig. 1E). These results showed that PhF contributes, at least partially, to induce the apoptosis in human leukocytes.

3.2. DCs treated with dexamethasone become tolerogenic DCs in vitro

One of the major obstacles in organ transplantation is that grafted organs are often attacked by the host immune system, which could be overcome by tolerogenic DCs to retain tolerance after the transplantation [19]. To investigate whether Dex induces tolerance in DCs *in vitro*, monocyte-derived DCs were generated and cultured in the absence or presence of LPS since tolerogenic DCs are resistant to mitogen-induced maturation [30]. DNL (e.g., Dex-DCs with No LPS) showed low expression levels of CD80/86 (Fig. 2A). MHC class I expression on DNL was no different compared with those in other DCs and MHC class II expression was higher than NDNL and DL (Fig. 2B). It has been suggested that latency-associated peptide (LAP), an important immune-regulatory molecule, is increased in $CD4^+CD25^+Foxp3^+$ T cells [31]. Our results showed that the highest level of LAP was expressed on DNL (Fig. 2C). The iDCs have higher antigen uptake ability than mature DCs (mDCs) [32] and our results also indicated that it was the highest in DNL (Fig. 3A). NDL (No Dex-DCs treated with LPS) induced increase of IL-12p70, TNF-α and IL-10 while DL (Dex-DCs treated with LPS) produced less cytokines (Fig. 3B). Taken together, our results pointed out that DNL has tolerogenic characterization.

It has been demonstrated that the most considerable feature of tDCs is the generation and expansion of regulatory T cells (mostly $CD4^+CD25^+Foxp3^+$), which contributes to the maintenance of the tolerance [19]. $CD3^+CD4^+CD25^+Foxp3^+$ T cells (Treg) with DNL were increased by about 8% compared with NDNL, 27% (NDL) and 12% (DL) *in vitro* (Fig. 4A). Consequently, these results demonstrated that Treg induced by Dex-DCs suppressed the proliferation of $CD4^+$ T cells by 21% compared to those induced by NDL *in vitro* (Fig. 4B).

3.3. PhF and Dex-DCs modulate the survival of porcine fibroblasts

Use of tDCs can be a good strategy for successful (xeno)transplantation [19,33], and we showed that DNL exhibited tolerogenic characteristics. Our subsequent aim, therefore, was to test whether DNL could modulate the survival of the porcine fibroblasts *in vitro*. To examine the ability of DCs affecting the mortality of porcine fibroblasts, PhF was co-cultured with DNL and allogeneic hPBMCs. Apoptosis of hPBMCs co-cultured with WT and DNL was higher (about 9%) than that of PhF and DNL until day 3 *in vitro* (Fig. 5A). Interestingly, DNL induced higher apoptosis in PhF than DL, for instance, about 8% with DNL versus 6% with DL at day 3, while the opposite results occurred in WT throughout the whole experimental period. PhF may affect DNL rather than DL. Therefore, PhF was co-cultured with DNL. The apoptosis of DNL was increased when co-cultured solely with PhF at day 3 (Fig. 5B). Taken together, the protective ability of PhF was better than that of WT, and DNL treatment up-regulated survival rate (i.e., down-regulation of $AnnexinV^+$ cells) of WT *in vitro*.

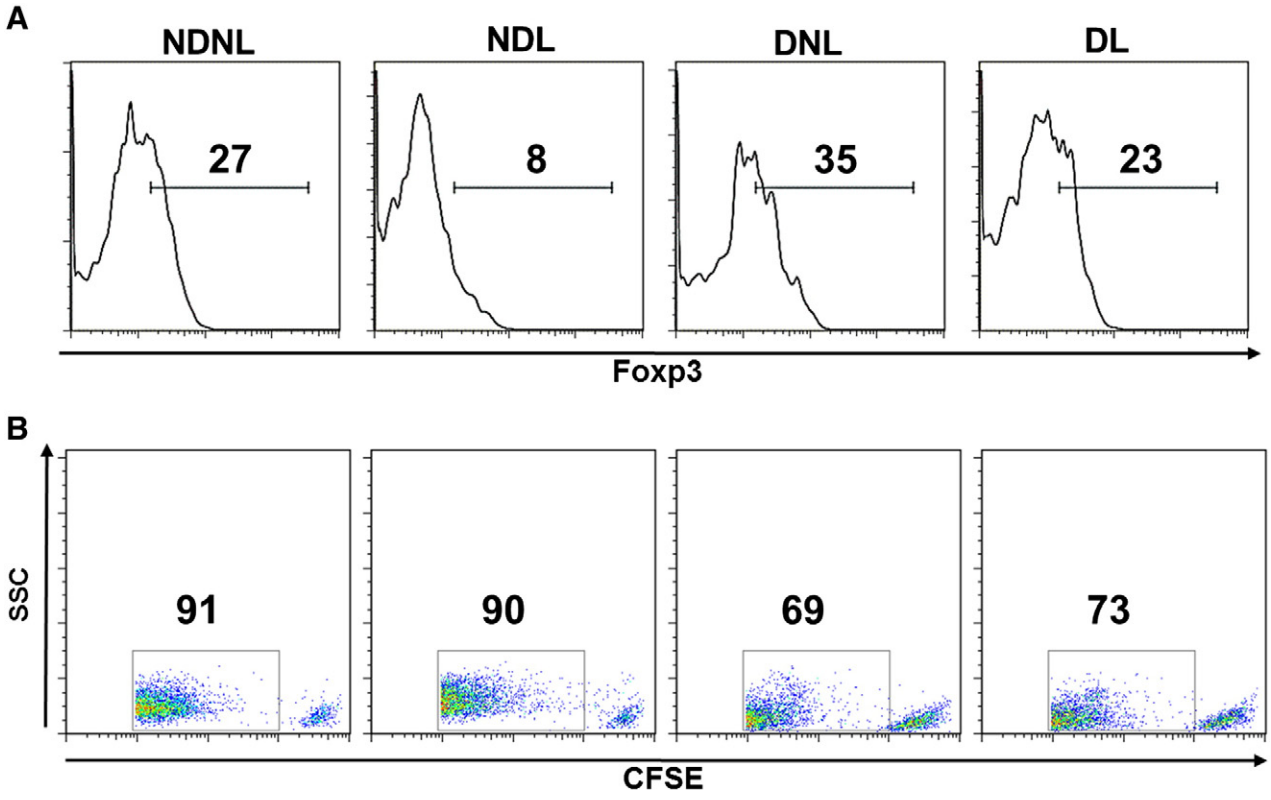

Fig. 4. DNL (Dex-DCs) induced $CD3^+CD4^+CD25^+Foxp3^+$ T cells *in vitro*. The induction of (A) $CD3^+CD4^+CD25^+Foxp3^+$ T cells when $CD3^+$ T cells were co-cultured with DCs. hPBMCs were stained with anti-human CD3-bead and then $CD3^+$ T cells were isolated by bead separation through a column. These cells were co-cultured with DCs for 5 days and then stained with CD4-FITC, CD25-APC and Foxp3-PE in dark condition. The number of each panel indicates the percentage of Foxp3 expression in $CD3^+CD4^+CD25^+$ cells. (B) Suppressive property of Treg induced by Dex-DCs tested that CD4 + T cells sorted from the mixture of $CD3^+$ cells with DCs were co-cultured with CFSE labeled $CD4^+$ T cells and CD2/CD3/CD28 activation antibodies for 3 days. Proliferation of $CD4^+$ T cells was analyzed by $CFSE^+$ population. The number of each panel indicates the percentage of proliferation. Data are representative of three independent experiments with similar results.

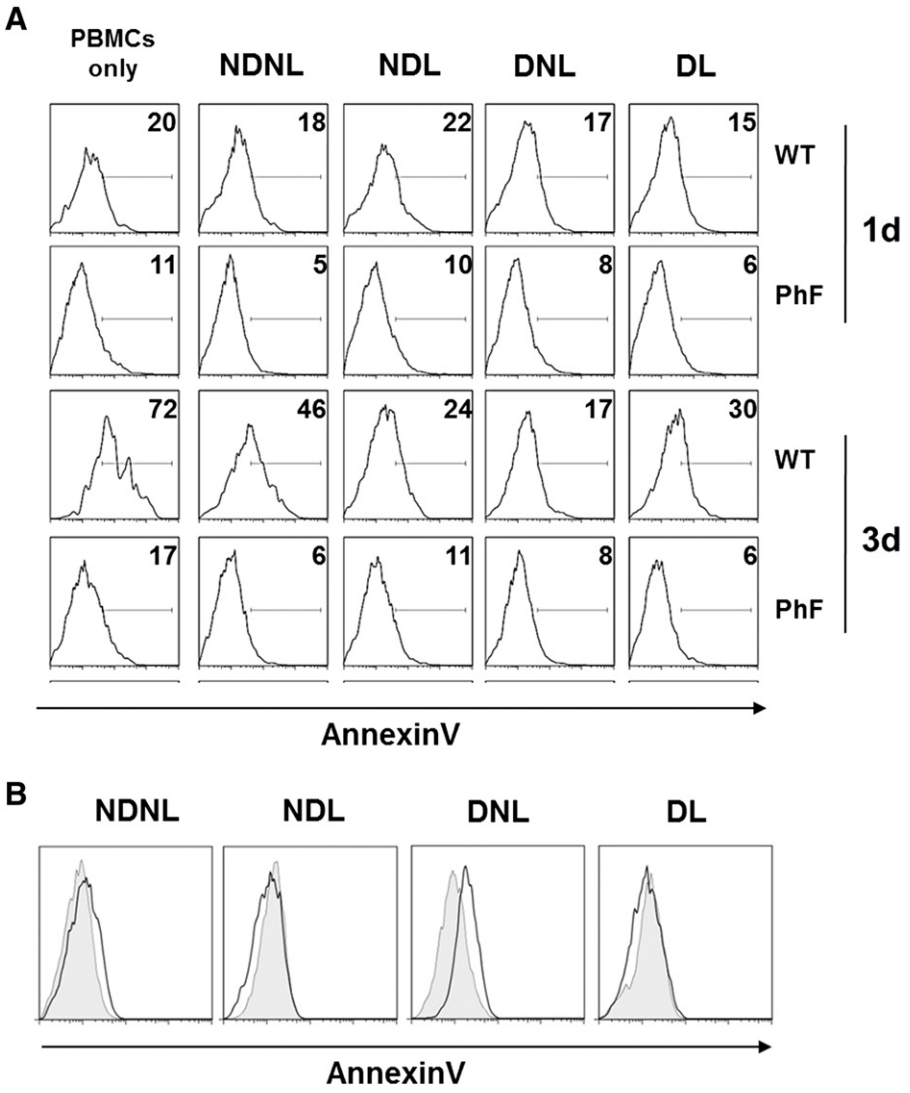

Fig. 5. PhF or DNL (Dex-DCs) enhanced the survival of porcine fibroblasts *in vitro*. (A) hPBMCs were co-cultured with DCs for 3 days and the changes of the apoptosis in porcine fibroblasts were examined. Porcine fibroblasts were labeled with CFSE before co-culture with hPBMCs together with DCs or Dex-DCs, and stained with AnnexinV-APC in dark condition. The cells were gated on CFSE positive population. The number in each panel indicates apoptosis. (B) The apoptosis of DCs was co-cultured with CFSE labeled porcine fibroblasts for 3 days. The cells were analyzed by AnnexinV-APC gating on CFSE negative population. The number of each panel indicates the percentage of proliferation. Data are representative of three independent experiments with similar results.

4. Discussion

We started this study from a concept that an organ from human FasL expressing porcine as a donor could evade partially cell-mediated immune responses in human as a recipient and tDCs suppressed immune responses of recipients who received an organ from human FasL expressing porcine. Subsequently, an organ from human FasL expressing porcine cells might survive longer with Dex-DCs *in vitro*.

Our results suggest the increase in the survival of porcine fibroblasts as they escape human immune cell attack through the induction of hFasL on porcine fibroblasts or together with the use of Dex-DCs, but not by two factors (e.g., hFasL and Dex-DCs). It could be that PhF induced the apoptosis of Dex-DCs. Dex exhibits an inhibition of NF-κB activity to synthesize anti-apoptotic molecules including cellular inhibitor of the apoptosis protein 2 (cIAP2) [34] and FLICE inhibitor protein (FLIP) [35]. These anti-apoptotic molecules further inhibit caspase-8 activation [36,37], which may cause non-canonic inflammasome [38] resulting to more susceptibility to apoptotic signal. We postulated that apoptosis of Dex-DCs may have occurred when they were co-cultured with PhF, due to down-regulation of anti-apoptotic molecules induced by Dex.

There was a similar attempt in a xenotransplantation study. Matter-Reissmann et al. transfected hFasL into porcine cell lines; however they failed to protect hFasL-transfected cells from attack by human NK cells [39]. Our results showed that the porcine fibroblasts expressing hFasL (PhF) induced apoptosis in human $CD4^+$ T cells and therefore porcine fibroblasts were protected when co-cultured with hPBMCs. It is because the hFasL we transfected did not contain metalloprotease cleavage site [24] and sFasL was not detected in concentrated culture supernatant (data not shown), whereas there was cleavage site and therefore soluble FasL was secreted in the aforementioned study [39] suggesting that soluble FasL inhibited apoptosis mediated by membrane-bound FasL [40].

Our results showed that PhF induced apoptosis in human $CD4^+$ T cells. A number of studies have shown similar observations by using bovine epithelial cells transiently transfected with hFasL [13], and porcine epithelial cells expressing hFasL [39] which were able to induce apoptosis in Jurkat cells. It has been shown that human $CD4^+$ T cells are a major population among PBMCs when treated with porcine cell lysates [14]. Furthermore, activation of $CD4^+$ T cells led to up-regulation of Fas expression [41] suggesting the reason PhF induced apoptosis of human $CD4^+$ T cells the most.

It is known that absence of $CD4^+$ T cells impairs the cytotoxic property of $CD8^+$ T cells, because CD40 signaling by $CD4^+$ T cells is crucial to activate $CD8^+$ T cells [42]. Therefore, the induction of apoptosis in $CD4^+$ T cells can help induce tolerance. Indeed, the expression of Fas on human $CD4^+$ T cells was remarkably increased at day 3 when co-cultured with PhF in the present study. The reason the induction of apoptosis by membrane-bound FasL occurred at an early time point (within 6 h) in the present study, as contrast to the previous report [43], is unclear at the moment and requires further investigation.

Dex is a well-known anti-inflammatory agent that induces glucocorticoid-induced leucine zipper (GILZ) in thymocytes [44]. GILZ of human DCs is responsible for the down-regulation of CD80/83/86, suppressess the proliferation of $CD4^+$ T cells [45] and induces Treg [46]. In mouse model, Dex-DCs induces increase of $Foxp3^+$ cells in lymph node [22] and Dex treatment increased numbers of both Treg and immature DCs [47].We showed that Foxp3 expression was up-regulated in $CD3^+CD4^+CD25^+$ T cells when co-cultured with Dex-DCs *in vitro*.

Whether latency-associated peptide (LAP) is a unique feature of Dex-DCs has been uncertain. In general, the expression of LAP is referred to as the expression of TGF-β because LAP bound to TGF-β that is anchored to glycoprotein A repetitions predominant (GARP) on the surface of Treg [48,49]. It has been suggested that the signaling to produce TGF-β is mediated by CD69 [50] or TGF-β itself [51]. Since glucocorticoid up-regulated TGF-β1 in human T lymphocytes [52] and in gastric gland [53], it is possible that Dex-DCs induced Treg by producing TGF-β that was confirmed from the supernatant of Dex-DCs (data not shown). Notably, Dex-DCs expressed higher MHC class II than DNDL in our results. MHC class II, known to up-regulate Dex-DCs [30], is an important molecule for supporting Treg proliferation [54]. It is possible that MHC class II on Dex-DCs helps to induce Treg.

Low GM-CSF-treated DCs show maturation resistance to LPS or TNF-α and prolong mouse cardiac allograft survival [55] suggesting that maturation-resistance DCs are one of the important characteristics for tDCs in the transplantation practice, because organ graft leads to the induction of inflammation against non-self-organ in the recipient. In this regard, Dex plays a role on maturation-resistance to human DCs when stimulated with LPS or CD40 ligand [30]. Our results in the present study also suggested that the expression of surface markers and cytokines produced in Dex-DCs involved resistance against maturation when treated with LPS.

Although Dex-DCs are known to be a good candidate for immune suppression in cell therapy and transplantation, there are potential hurdles to apply this in clinical trials. In rodent allogeneic graft model, donor derived Dex-DCs were not successful for pancreatic islet transplantation due to induction of Dex-DCs' specific antibody and active NK cells [56] even though they induced hyporesponsiveness on T cells [27]. It has been demonstrated that Dex-DCs derived from F1 (donor × recipient) sustained 100% survival for 100 days with CTLA4Ig treatment [57] suggesting that Dex-DCs derived from both donor and recipient should be considered in xenotransplantation field.

In conclusion, PhF induced the apoptosis in human immune cells, especially in $CD4^+$ T cells, and Dex-DCs increased the tolerance *in vitro*. Our study is the first, to the best of our knowledge, to suggest that immune tolerance induced by using hFasL-expressed porcine fibroblasts and Dex-DCs would be a good solution for the xenotransplantation.

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