Feline adipose tissue-derived mesenchymal stem cells pretreated with IFN-γ enhance immunomodulatory effects through the PGE$_2$ pathway

Seol-Gi Park $^{1,†}$, Ju-Hyun An $^{1,†}$, Qiang Li $^{2}$, Hyung-Kyu Chae $^{1}$, Su-Min Park $^{1}$, Jeong-Hwa Lee $^{1}$, Jin-Ok Ahn $^{3}$, Woo-Jin Song $^{4,†}$, Hwa-Young Youn $^{1,†}$

$^{1}$Laboratory of Veterinary Internal Medicine, Department of Veterinary Clinical Science, College of Veterinary Medicine, Seoul National University, Seoul 08826, Korea
$^{2}$Department of Veterinary Medicine, College of Agriculture, Yanbian University, Yanji, Jilin 133000, China
$^{3}$Department of Veterinary Internal Medicine, College of Veterinary Medicine, Kangwon National University, Chuncheon 24341, Korea
$^{4}$Department of Veterinary Internal Medicine, College of Veterinary Medicine and Research Institute of Veterinary Science, Jeju National University, Jeju 63243, Korea

ABSTRACT

**Background:** Preconditioning with inflammatory stimuli is used to improve the secretion of anti-inflammatory agents in stem cells from variant species such as mouse, human, and dog. However, there are only few studies on feline stem cells.

**Objectives:** This study aimed to evaluate the immune regulatory capacity of feline adipose tissue-derived (fAT) mesenchymal stem cells (MSCs) pretreated with interferon-gamma (IFN-γ).

**Methods:** To assess the interaction of lymphocytes and macrophages with IFN-γ-pretreated fAT-MSCs, mouse splenocytes and RAW 264.7 cells were cultured with the conditioned media from IFN-γ-pretreated MSCs.

**Results:** Pretreatment with IFN-γ increased the gene expression levels of cyclooxygenase-2, indoleamine 2,3-dioxigenase, hepatocyte growth factor, and transforming growth factor-beta 1 in the MSCs. The conditioned media from IFN-γ-pretreated MSCs increased the expression levels of M2 macrophage markers and regulatory T-cell markers compared to those in the conditioned media from naive MSCs. Further, prostaglandin E$_2$ (PGE$_2$) inhibitor NS-398 attenuated the immunoregulatory potential of MSCs, suggesting that the increased PGE$_2$ levels induced by IFN-γ stimulation is a crucial factor in the immune regulatory capacity of MSCs pretreated with IFN-γ.

**Conclusions:** IFN-γ pretreatment improves the immune regulatory profile of fAT-MSCs mainly via the secretion of PGE$_2$, which induces macrophage polarization and increases regulatory T-cell numbers.

**Keywords:** Cats; mesenchymal stem cell; macrophage; interferon-gamma; prostaglandin E$_2$

INTRODUCTION

Feline mesenchymal stem cells (MSCs) have anti-inflammatory effects and immunomodulatory functions that make them attractive as novel cell-based therapeutics.
ORCID IDs
Seol-Gi Park https://orcid.org/0000-0002-0404-4911
Ju-Hyun An https://orcid.org/0000-0002-3756-9482
Qiang Li https://orcid.org/0000-0003-2722-1324
Hyung-Kyu Chae https://orcid.org/0000-0002-6876-9460
Su-Min Park https://orcid.org/0000-0002-1709-7000
Jeong-Hwa Lee https://orcid.org/0000-0003-1716-8152
Jin-Ok Ahn https://orcid.org/0000-0002-3300-6084
Woo-Jin Song https://orcid.org/0000-0002-9195-551X
Hwa-Young Youn https://orcid.org/0000-0002-0283-1348

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Conflict of Interest
The authors declare no conflicts of interest.

Author Contributions
Conceptualization: Park SG, An JH, Song WJ, Youn HY; Data curation: Li Q, Chae HK, Park SM, Lee JH; Formal analysis: Park SG, An JH, Li Q; Methodology: Park SG, An JH, Ahn JO, Song WJ, Supervision: Song WJ, Youn HY; Writing - original draft: Park SG, An JH; Writing - review & editing: Song WJ, Youn HY.

MATERIALS AND METHODS
Isolation and characterization of feline adipose-tissue derived (fAT)-MSCs
Feline adipose tissues were obtained from 3 healthy cats during ovariohysterectomy at the Seoul National University Veterinary Medical Teaching Hospital, and their owners provided informed consent for research use. All procedures were approved by the Institutional Animal Care and Use Committee of Seoul National University (protocol No. SNU-190411-10), and the protocols were performed in accordance with approved guidelines. fAT-MSCs were cultured in Dulbecco’s Modified Eagle Medium (DMEM; PAN Biotech, Germany) supplemented with 20% fetal bovine serum (FBS; PAN Biotech) and 1% (w/v) penicillin-streptomycin (PS; PAN Biotech) and incubated at 37°C in a 5% CO₂ humidified atmosphere. The medium was changed every 2 days until the cells reached a confluence of 70%-80%. Previous studies used MSCs in the third passage or fourth passage, suggesting that early passage cells are more effective [10]. Therefore, we used 3–4 passages for fAT-MSCs. After adhering to culture plates and achieving a fibroblast-like morphology, the fAT-MSCs were characterized by flow cytometry using antibodies against the following proteins—CD44 (antibody clone IM7, fluorescein isothiocyanate [FITC] rat anti-mouse/human, 103021; Biolegend, USA), CD34 (antibody clone 1H6, phycoerythrin [PE] mouse anti-dog, 559369; BD Biosciences, USA), CD45 (antibody clone YKIX716.13, FITC rat anti-dog; eBioscience, USA), CD90 (antibody clone 5E10, PE mouse anti-human, 555596; BD Biosciences), and CD105 (antibody clone SN6, FITC mouse anti-human, MCA1557F; AbD Serotec, USA). Cell fluorescence was analyzed with FACS Aria II (BD Biosciences). In addition, special differentiation kits (Stem Pro osteogenesis differentiation, Stem Pro adipogenesis differentiation, and Stem Pro chondrogenesis differentiation kits; all from Gibco/Life Technologies, USA) were used for evaluating cellular differentiation following the manufacturer’s instructions.

IFN-γ stimulation
To assess the effects of INF-γ on the fAT-MSCs, 5 × 10⁵ fAT-MSCs were seeded in 12-well plates in DMEM medium supplemented with 20% FBS and 1% PS. After 24 h, the fAT-MSCs

for immune-mediated and inflammatory diseases such as gingivostomatitis, chronic kidney disease, and asthma [1-3]. In addition, we previously showed the mechanisms of anti-inflammatory functions of feline MSCs [4,5]. However, few studies have focused on the enhancement of immunoregulatory effects and their mechanisms of feline MSCs.

One of the strategies to improve the immunoregulatory capacity of MSCs is to pre-treat them with interferon-gamma (IFN-γ) [6]. IFN-γ is a pro-inflammatory cytokine secreted by natural killer cells and T cells which acts on macrophages and lymphocytes [7]. Previous studies suggested that MSCs stimulated with IFN-γ have upregulated immune suppressive functions and show changes in the expression of immunomodulatory factors [8]. Recently, Parsys et al. [9] reported that feline MSCs stimulated with IFN-γ showed significantly increased secretion of immunomodulatory factors such as indoleamine 2,3-dioxygenase (IDO), programmed death-ligand 1, interleukin (IL)-6, cyclooxygenase-2 (COX2), and hepatocyte growth factor (HGF). In this study, however, underlying mechanisms of anti-inflammation have not been identified.

Therefore, the aim of our study was to evaluate enhancement of the immunomodulatory effects of feline MSCs pre-treated with IFN-γ. In addition, we assessed the mechanisms by which the immunoregulation is induced.
were treated with 50 ng/mL INF-γ (feline recombinant protein; Kingfisher Biotech, USA) for 48 h. The cells were then washed 4 times with Dulbecco’s phosphate-buffered saline (DPBS) and were then maintained in DMEM supplemented with 20% FBS 1% PS for 3 days. After incubation, the supernatant was collected and centrifuged at 1,000 rpm for 3 min to remove any debris, and stored at −80°C until use. D-Plus CCK Cell Viability Assay Kit (DonginLS, Korea) was used to determine the cell viability of IFN-γ stimulated fAT-MSCs following the manufacturer’s instructions.

**Prostaglandin E2 (PGE₂) inhibitor, NS-398 treatment**

To assess the role of PGE₂ produced by fAT-MSCs in the anti-inflammatory response, fAT-MSCs were treated with PGE₂ inhibitor NS-398 (5 μM; Enzo Life Sciences, USA) for 24 h and then stimulated with INF-γ (50 ng/mL for 48 h). After that, the supernatant was collected and stored as described above.

**Immune cells were cultured with conditioned media obtained from fAT-MSCs**

RAW 264.7 cells obtained from Korean Cell Line Bank were treated for 24 h with lipopolysaccharide (LPS) (200 ng/mL; Sigma-Aldrich, USA) and then washed 3 times with DPBS. LPS-stimulated RAW 264.7 cells were seeded in 6-well plates (1 × 10⁶ cells/well) and cultured in conditioned media described above.

In addition, splenocytes were isolated from mice, as previously described [11]. All procedures were approved by the Seoul National University Institutional Animal Care and Use Committee (approval number: SNU-190304-1). Briefly, mouse spleens obtained from 4 mice (C57BL/6, male, 5 W) were mixed and mashed using a 1 mL syringe plunger. The cell suspension was then centrifuged for 3 min at 1,000 rpm. The cell pellet was resuspended in RBC Lysis buffer (Sigma-Aldrich) and washed with phosphate-buffered saline (PBS) 3 times, and the splenocytes were then resuspended in RPMI-1640 supplemented with 10% FBS and 1% PS. The splenocytes were then centrifuged at 3,000 rpm for 10 min, and they were seeded at a density of 1 × 10⁶ cells/well in 6-well plates in conditioned media described above.

**RNA extraction, cDNA synthesis, and RT-qPCR**

Easy-BLUE Total RNA Extraction Kit (Intron Biotechnology, Korea) was used to extract total RNA from fAT-MSCs, RAW 264.7, and spleen cells following the manufacturer’s instructions. The extracted RNA was transformed into cDNA using LaboPass M-MuLV reverse transcriptase (Cosmo Genetech, Korea) according to the supplier’s instructions. Cytokine mRNA levels were measured using RT-qPCR. The reaction mixture consisted of 10 μL AMPIGENE RT-qPCR Green Mix Hi-ROX with SYBR green dye (Enzo Life Sciences, Switzerland), 400 nM each of forward and reverse primers (Bionics, Korea) (primers are listed in Table 1), and 1 μL template cDNA. Cytokine mRNA levels were quantified using glyceraldehyde 3-phosphate dehydrogenase as a house-keeping control.

**Enzyme-linked immunosorbent assay (ELISA)**

The concentration of PGE₂ in the cell culture supernatant was determined using an ELISA kit (Enzo Life Sciences) following the manufacturer’s instructions. Culture supernatants from fAT-MSCs treated and un-treated with IFN-γ and NS-398 stimulated fAT-MSCs treated and un-treated with IFN-γ were collected after 48 h of stimulation for PGE₂ ELISA.

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Flow cytometric analysis of mouse immune cells

Flow cytometry was performed using a FACS Aria II system (BD Biosciences) and the data were analyzed using FlowJo software (Tree Star, USA). To determine M2 macrophage polarization, conditioned medium from fAT-MSCs, fAT-MSCs treated with IFN-γ, or NS-398 stimulated fAT-MSCs treated with IFN-γ were used to stimulate RAW 264.7 treated with or without LPS. The RAW 264.7 cells were harvested after stimulation and suspended in DPBS. The cells were stained with PE-conjugated anti-CD11c+ antibody (clone N418, 117307; Biolegend) and FITC-conjugated anti-CD206+ antibody (clone MRC1, SC376108; Santa Cruz Biotechnology, USA), and evaluated by flow cytometry.

To evaluate PGE₂-mediated induction of T-cell regulation by MSCs, conditioned medium from fAT-MSCs, fAT-MSCs treated with IFN-γ, or NS-398-treated fAT-MSCs stimulated with IFN-γ were used to stimulate splenocytes treated with or without Con A. The splenocytes were harvested after stimulation and suspended in DPBS. The cells were then stained with PE-conjugated anti-CD4+ antibody (clone RM4-5, 12-0042-82, eBioscience) and APC-conjugated anti-CD25+ antibody (PC61.5, 17-0251 -82, eBioscience) and evaluated by flow cytometry.

Immunofluorescence analyses

RAW 264.7 cells cultured on coverslips were fixed using 4% paraformaldehyde (in PBS, pH 7.2) at room temperature for 15 min and then washed with DPBS 4 times. The cells were then incubated in a blocking buffer containing 1% bovine serum albumin in PBS for 30 min. The cells were then incubated with antibodies against CD206+ and CD11b+ at 4 h for 12 h. The cells were rinsed with DPBS 3 times and were incubated with corresponding fluorescein-conjugated secondary antibodies (1:200; Santa Cruz Biotechnology) or Texas red-conjugated secondary antibodies (1:200; Santa Cruz Biotechnology) for 1 h at room temperature in the dark. The coverslips were then washed 4 times and mounted using VECTASHIELD mounting medium containing 4’,6-diamidino-2-phenylindole (Vector Laboratories, USA). The slides were observed under an EVOS FL microscope (Life Technologies, Germany), and the stained cells in 20 random fields per group were counted.

Statistical analyses

All data were analyzed using GraphPad Prism v.6.01 software (GraphPad Software Inc., USA). Student’s t-tests or one-way analysis of variance were used to determine statistical significance. The p values less than 0.05 were considered statistically significant.

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### Table 1. Sequences of polymerase chain reaction primers used in this study

| Gene    | Forward (5’-3’)              | Reverse (5’-3’)              | Reference |
|---------|------------------------------|------------------------------|-----------|
| fGAPDH  | ACGATGACATCAAGAAGGTG          | CATACCCAGAAAATGAGCTTG         | [4]       |
| fTGF-β  | CCAAAAAATCTAAGAAAGGTTCCA     | TATTCGTATTATCTGTTACACCTGTC   | [5]       |
| fNGF    | ATCCAGGATGTAATTGCTCTATTT     | TTTCAACACTACATCCACTCTACAT    | [5]       |
| fCOX2   | CGAATGTCCTCTTCCATCGAAT       | TGACTGACTCATGTTGCTCATACAT    | [4]       |
| fIDO    | TATTGAATGCAGCCAAAGGTG         | TTGAATTTGTTTAAACTCTTCCTTGG   | [5]       |
| mGAPDH  | AAAGGGTGTCGCTGTG             | TGAAGGGTGTCGCTGTG            | [4]       |
| mIFN-γ  | CACAGTCATGAAAGGGCTCAAAGAT    | GCATCTCCACATCACCCAGACC       | [4]       |
| mIL-6   | ACACATTGCTCTGGGAAACTG        | AAGTGCATACCATGTTGTTCCATACA   | [4]       |
| mNOS    | GCAGATGGTACCATGATG          | ACAAACCTGGTGGTTGAAAGGC       | [10]      |
| mIL-10  | GTATTGTTAAAGCCTCCAGACC       | GATCATCATGATGCTTCCATGAGC     | [27]      |
| mIL-1β  | GTCTTCGCCGTCGCTTCCCTC       | GTTTCTTCGGAAGCCCTGT          | [27]      |
| mTNF-α  | CCCTGAGTGCTGCTTCCCAAGACC    | GCTAGACGTGGGCTACAG           | [27]      |
| mIL-17  | GGTCACCTCAAGTCTTCTATACCCTC  | GAGGGATATCTATCAGGTTCTTCAT    | [27]      |
| mArg    | CAGAAGAATGGAAAGGCATTG        | CAGATATGCAAGGAGTCACC         | [27]      |
RESULTS

Characterization of fAT-MSCs

Cells isolated from feline adipose tissue were characterized by immunophenotyping and multilineage differentiation. Three days after seeding, the cultured cells exhibited a fibroblast-like morphology. There was no difference in the morphology of fAT-MSCs treated with or without IFN-γ (Fig. 1A). In addition, IFN-γ-treated fAT-MSCs showed normal cell viability (Fig. 1B).

Flow cytometric analyses showed that only a few of the naïve or IFN-γ-primed fAT-MSCs expressed the known hematopoietic markers CD34 and CD44, but more than 95% expressed the known MSC markers CD90, CD44, CD9, and CD105 (Fig. 1C). When specific differentiation media were used, fAT-MSCs differentiated into osteocytes, adipocytes, and chondrocytes. The abilities of osteogenic and chondrogenic differentiation were enhanced in IFN-γ-treated fAT-MSCs compared with naïve fAT-MSCs (Fig. 1D). Additionally, 48 h treatment with INF-γ upregulated the secretion of immunomodulation factors COX2, IDO, TGF-β, and HGF in fAT-MSCs (Fig. 1E).

[Fig. 1. Characterization of mesenchymal stem cells isolated from feline adipose tissue. (A) Morphology of IFN-γ treated and untreated fAT-MSCs (bars = 20 μm). (B) Cell viability of IFN-γ treated and untreated fAT-MSC. (C) Immunophenotypic analysis by flow cytometry. (D) Adipogenesis: intracellular lipid vacuoles were stained pink using Oil Red O. Osteogenesis: fAT-MSCs were stained positive for calcium deposits using 1% Alizarin red. Chondrogenesis: proteoglycans were stained using Alcian Blue (bars = 20 μm). (E) Increased gene expression of, HGF, COX2, IDO (scale bars = 200 μm). Results are presented as the mean ± standard deviation of 3 independent experiments.

IFN-γ, Interferon-gamma; fAT-MSC, feline adipose tissue-derived mesenchymal stem cell; HGF, hepatocyte growth factor; COX2, cyclooxygenase-2; IDO, indoleamine 2,3-dioxygenase; ns, not significant; TGF-β, transforming growth factor-β.

*p < 0.05, **p < 0.01 by one-way analysis of variance analysis.]

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Immuno\-modulatory effects of IFN-$\gamma$ primed fAT-MSCs

To determine the immunomodulatory capacity of IFN-$\gamma$ treated fAT-MSCs, we quantified the mRNA expressions of anti- and pro-inflammatory cytokines secreted by RAW 264.7 cells. Tumor necrosis factor-alpha (TNF-$\alpha$), IL-1$\beta$, IL-6, and inducible nitric oxide synthase (iNOS) were upregulated in RAW 264.7 cells stimulated with LPS compared to that in unstimulated RAW 264.7 cells. When RAW 264.7 cells stimulated by LPS were cultured with fAT-MSC- and IFN-$\gamma$-treated fAT-MSC-conditioned media TNF-$\alpha$, IL-1$\beta$, IL-6, and iNOS expression levels were reduced. We also measured the mRNA expressions of M2 markers, IL-10, and arginase. The levels of these M2 markers were significantly increased in LPS-stimulated RAW 264.7 cultured in IFN-$\gamma$-treated fAT-MSC-conditioned media compared to that LPS-stimulated RAW 264.7 cultured in untreated fAT-MSC-conditioned media (Fig. 2A).

Additionally, we performed immunofluorescence staining on RAW 264.7 cells to determine whether the population of M2 macrophage increased when cultured in fAT-MSC- and IFN-$\gamma$-treated fAT-MSC-conditioned media. Quantitative analysis of CD206$^+$ and CD11b$^+$ RAW 264.7 cells demonstrated a significant increase in CD206$^+$ cells among the RAW 264.7 cultured in IFN-$\gamma$-treated fAT-MSC-conditioned media compared to those cultured in fAT-MSC-conditioned media and NS-398-treated IFN-$\gamma$-stimulated-fAT-MSC-conditioned media (Fig. 2B).

To determine the ability to induce T-cell regulation, we determined the mRNA expressions of inflammatory cytokines secreted by splenocytes. Con A stimulation increased the expression of IL-1$\beta$, IFN-$\gamma$, and IL-17 in the splenocytes. However, Con A-stimulated splenocytes cultured in IFN-$\gamma$-treated fAT-MSC-conditioned media showed decreased expression of IL-1$\beta$, IFN-$\gamma$, and IL-17 compared to the Con A-stimulated cultured in untreated fAT-MSC-conditioned media. Conversely, IL-10 and FOXP3 expression levels were increased in Con A-stimulated splenocyte cultured in IFN-$\gamma$ treated fAT-MSC-conditioned media compared to Con A-stimulated splenocytes cultured in IFN-$\gamma$-un-treated fAT-MSC-conditioned media (Fig. 2C).

PGE$_2$ concentration levels in various fAT-MSC-conditioned media

PGE$_2$ ELISA Kit was used to measure PGE$_2$ levels in the conditioned medium obtained from fAT-MSC treated or untreated with IFN-$\gamma$, and NS-398 stimulated fAT-MSC treated with IFN-$\gamma$. First, we confirmed that NS-398 (5 $\mu$M) treated fAT-MSCs show normal cell viability (Supplementary Fig. 1). PGE$_2$ was significantly increased in IFN-$\gamma$-treated fAT-MSC-conditioned media treated compared to that in untreated fAT-MSC-conditioned media and NS-398-stimulated fAT-MSC-conditioned media with or without IFN-$\gamma$ pretreatment (Fig. 3).

Macrophage polarization and T-cell regulation are associated with PGE$_2$

To determine the role of PGE$_2$ in the immunomodulatory capacity of fAT-MSCs, we compared pro- and anti-inflammatory cytokines secreted by RAW 264.7 cells and splenocytes. Flow cytometry revealed an increase in CD11c$^+$ RAW 264.7 cells with LPS stimulation, which was decreased by culturing in IFN-$\gamma$ treated fAT-MSC-conditioned media (Fig. 4). However, there was a decrease in CD206$^+$ RAW 264.7 cells with LPS stimulation, which was reversed by culturing in IFN-$\gamma$-treated fAT-MSC-conditioned media. Pre-treating the fAT-MSCs treated IFN-$\gamma$ with PGE$_2$ inhibitor, NS-398 attenuated the effect of fAT-MSC-conditioned media on RAW 264.7 cells significantly increasing CD11c$^+$ and decreasing CD206$^+$ RAW 264.7 cells (Fig. 4).
Fig. 2. Immunomodulatory effects of IFN-γ treated fAT-MSCs. (A) Effects of IFN-γ treated fAT-MSCs on the mRNA expression of anti- and pro-inflammatory cytokines by RAW 264.7 cells. (B) M2 macrophage expression of LPS stimulated RAW 264.7 cells cultured with the conditioned media of IFN-γ treated and untreated fAT-MSCs. (C) The regulatory effects of IFN-γ treated fAT-MSCs on the mRNA expression of anti- and pro-inflammatory cytokines secreted by splenocytes. Results are presented as the mean ± standard deviation of 3 independent experiments.

IFN-γ, interferon-gamma; fAT-MSC, feline adipose tissue-derived mesenchymal stem cell; LPS, lipopolysaccharide; TGF-β, transforming growth factor-β; IL, interleukin; Arg, arginase; iNOS, inducible nitric oxide synthase; MSC, mesenchymal stem cell; ns, not significant; FOXP3, forkhead box protein P3.

*p < 0.05, **p < 0.01, ***p < 0.001 by one-way analysis of variance analysis.
PGE\textsubscript{2} secreted by IFN-\(\gamma\) treated fAT-MSCs is critical for their immune function

To determine the role of PGE\textsubscript{2}, treated IFN-\(\gamma\) treated fAT-MSCs with PGE\textsubscript{2} inhibitor, NS-398, and then compared the mRNA expression of inflammatory cytokines secreted by RAW 264.7 cells cultured in IFN-\(\gamma\) treated fAT-MSC-conditioned media or NS-398-stimulated IFN-\(\gamma\)-treated fAT-MSC-conditioned media. First, we confirmed that 5 \(\mu\)M of NS-398 effectively inhibits PGE\textsubscript{2} secretion in fAT-MSCs without affecting cell viability (data not shown). TNF-\(\alpha\), IL-1\(\beta\), and IL-6 expression levels increased with LPS, which was reversed by culturing in IFN-\(\gamma\) treated fAT-MSC-conditioned media. IL-10 levels were decreased after LPS stimulation, which was reversed by culturing in IFN-\(\gamma\) treated fAT-MSC-conditioned media. However, pre-treating the fAT-MSCs with NS-398 attenuated the effect of IFN-\(\gamma\) treated fAT-MSC-conditioned media (Fig. 5).

To confirm the role of PGE\textsubscript{2} in T-cell regulation, we determined the mRNA expression of immune cytokines secreted by splenocytes. IL-1\(\beta\), IL-17, and IFN-\(\gamma\) were increased after Con A stimulation, which was reversed by culturing in IFN-\(\gamma\) treated fAT-MSC-conditioned media. Pre-treating the fAT-MSCs with NS-398 attenuated the effect of IFN-\(\gamma\) treated fAT-MSC-conditioned media and increased IL-1\(\beta\), IL-17, and IFN-\(\gamma\) levels. IL-10 was decreased after Con A stimulation, which was reversed by culturing in IFN-\(\gamma\) treated fAT-MSC-conditioned media. Pre-treating the fAT-MSCs treated IFN-\(\gamma\) with NS-398 attenuated the effect of IFN-\(\gamma\) treated fAT-MSC-conditioned media and decreased IL-10 levels (Fig. 5).

**DISCUSSION**

Numerous studies have reported that MSCs preconditioned with pro-inflammatory cytokines have increased immunomodulatory effects [12-15]. Despite these studies, studies to enhance the immunomodulatory effects of feline MSCs are lacking. Pretreatment of MSCs with IFN-\(\gamma\) has been reported to have a significant effect on the immunoregulatory effect of the MSCs in humans and mice [8,16,17]. However, the effect of IFN-\(\gamma\) on fAT-MSCs has not been reported. In this study, we determined the mechanism underlying the improvement of immunomodulatory effects of feline MSCs with IFN-\(\gamma\) stimulation.
The concentration of IFN-γ was determined by combining data from previous studies and preliminary experiments. HGF, TGF-β, IDO, and COX2 were significantly higher in fAT-MSC conditioned media pre-stimulated with 50 ng/mL IFN-γ in fAT-MSCs. We also investigated the relationship between IFN-γ preconditioned fAT-MSC and immune cells in order to confirm that the immunoregulatory ability of these cells was effectively increased. RAW 264.7, a murine macrophage cell line, was used to confirm the immunomodulatory effects of IFN-γ treated fAT-MSCs in vitro as macrophages play an important role in the immune system [18]. Lymphocytes and macrophages are involved in various inflammatory and immune-mediated disease and have been studied in relation to the immune system [4]. Mouse splenocytes have been used for studying various aspects of human, dog, and rat immune systems [11,17,19]. Therefore, we used mouse splenocyte to evaluate T-cell regulation in IFN-γ treated fAT-MSCs.
Anti-inflammatory T-helper 2 cytokines suppress the immune responses and are critical regulators of the immune system. The expression levels of anti-inflammatory cytokines arginase and IL-10 in RAW 264.7 cells were significantly increased when they were cultured in IFN-γ treated fAT-MSC-conditioned media. The population of CD206+ RAW 264.7 cells was also increased when cultured in IFN-γ treated fAT-MSC-conditioned media. Culturing in IFN-γ treated fAT-MSC-conditioned media also increased the expression of FOXP3 and IL-10 in splenocytes. In addition, stimulating RAW 264.7 with LPS and splenocytes with Con A confirmed that the activated immunity could be regulated by IFN-γ treated fAT-MSC-conditioned media.

Many soluble factors secreted by MSCs such as TGF-β, HGF, PGE-2, IL-6, IL-10, IL-1, iNOS, IDO, Gal-1, and HLA-G regulate the immune system and modulate the immune cell activity and relieve the inflammatory environment [20-29]. In particular, previous studies have reported that PGE2 plays a critical role in the immune responses [5,30] and is associated with macrophage polarization and T-cell regulation [4]. However, no studies have been done to determine whether the increased PGE2 secretion is a key regulator of the immune regulation mediated by the IFN-γ treated fAT-MSC. Our results confirmed the significant increase of PGE2 in the IFN-γ treated fAT-MSC-conditioned media. In addition, pretreatment with PGE2 inhibitor, NS-398, significantly decreased the level of PGE2 in the IFN-γ treated fAT-MSC-conditioned media. Pretreatment with NS-398 attenuated PGE2, as so decreased the M2-macrophage polarization, and T-cell regulation induced by the IFN-γ treated fAT-MSC-conditioned media.

Fig. 5. Effects of immune responses for PGE2 secreted by IFN-γ treated fAT-MSCs. PGE2 secreted from IFN-γ treated fAT-MSCs affects the degree of inflammatory cytokine mRNA levels in RAW 264.7 cells (A) and splenocytes (B). Results are presented as the mean ± standard deviation of 3 independent experiments. PGE2, prostaglandin E2; IFN-γ, Interferon-gamma; fAT-MSC, feline adipose tissue-derived mesenchymal stem cell; IL, interleukin; TNF-α, tumor necrosis factor-alpha; LPS, lipopolysaccharide.

*p < 0.05, **p < 0.01 by one-way analysis of variance analysis.
There were some limitations in this study. First, we used xenogeneic immune cells such as macrophage cell line and splenocytes. Although mouse immune cells such as RAW 264.7 (macrophages) and splenocytes are widely used for studies of immunology, further research using feline immune cells should be needed. Second, we only confirmed the immunomodulatory effects of PGE$_2$ in this study. Although we showed here that PGE$_2$ might be considered to be a major contributor to the immunomodulatory potential of the IFN-$\gamma$ treated fAT-MSC-conditioned media, further studies should explore the role of other soluble factors such as IDO, TSG-6, and HGF.

In conclusion, IFN-$\gamma$ pretreatment enhanced the ability of fAT-MSCs to induce M2macrophage polarization and T-cell regulation. It also regulated the secretion of anti- and pro-inflammatory cytokines in activated immune cells. In addition, we found that PGE$_2$ is a major factor contributing to the immunomodulatory function of IFN-$\gamma$-pretreated fAT-MSCs. Our results provide a theoretical basis for the use of fAT-MSCs as cell-based therapeutics for autoimmune and inflammatory diseases in the future.

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SUPPLEMENTARY MATERIAL

Supplementary Fig. 1

NS-398 (5 μM) treated fAT-MSCs with or without IFN-$\gamma$ stimulation show normal cell viability.

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