


Original Article
Regenerative Medicine



Feline adipose tissue-derived mesenchymal stem cells pretreated with IFN- γ enhance immunomodulatory effects through the PGE₂ 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,*}

¹Laboratory of Veterinary Internal Medicine, Department of Veterinary Clinical Science, College of Veterinary Medicine, Seoul National University, Seoul 08826, Korea

²Department of Veterinary Medicine, College of Agriculture, Yanbian University, Yanji, Jilin 133000, China

³Department of Veterinary Internal Medicine, College of Veterinary Medicine, Kangwon National University, Chuncheon 24341, Korea

⁴Department of Veterinary Internal Medicine, College of Veterinary Medicine and Research Institute of Veterinary Science, Jeju National University, Jeju 63243, Korea



Received: Jun 5, 2020

Revised: Dec 12, 2020

Accepted: Jan 7, 2021

*Corresponding authors:

Hwa-Young Youn

Laboratory of Veterinary Internal Medicine,
Department of Veterinary Clinical Science,
College of Veterinary Medicine, Seoul National
University, 1 Gwanak-ro, Gwanak-gu, Seoul
08826, Korea.

E-mail: hyyoun@snu.ac.kr

Woo-Jin Song

Laboratory of Veterinary Internal Medicine
and Research Institute of Veterinary Science,
College of Veterinary Medicine, Jeju National
University, 102 Jejudaehak-ro, Jeju 63243,
Korea.

E-mail: ssong@jejunu.ac.kr

[†]Seol-Gi Park and Ju-Hyun An are co-first
authors and contributed equally.

© 2021 The Korean Society of Veterinary
Science

This is an Open Access article distributed
under the terms of the Creative Commons
Attribution Non-Commercial License (<https://creativecommons.org/licenses/by-nc/4.0>)
which permits unrestricted non-commercial
use, distribution, and reproduction in any
medium, provided the original work is properly
cited.

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-dioxygenase, 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 E₂ (PGE₂) inhibitor NS-398 attenuated the immunoregulatory potential of MSCs, suggesting that the increased PGE₂ 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 PGE₂, which induces macrophage polarization and increases regulatory T-cell numbers.

Keywords: Cats; mesenchymal stem cell; macrophage; interferon-gamma; prostaglandin E₂

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>

Funding

This research was supported by the Program of National Research Foundation of Korea through the Ministry of Education.

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.

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, Parys 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.

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 IFN- γ on the fAT-MSCs, 5×10^5 fAT-MSCs were seeded in 12-well plates in DMEM medium supplemented with 20% FBS and 1% PS. After 24 h, the fAT-MSCs

were treated with 50 ng/mL IFN- 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 IFN- 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 (1×10^6 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 stimulated with 5 $\mu\text{g}/\text{mL}$ concanavalin A (Con A; Sigma-Aldrich) for 24 h to determine the mRNA expression of pro- and anti-inflammatory cytokines. The splenocytes were then collected by centrifugation at 3,000 rpm for 10 min, and they were seeded at a density of 1×10^6 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.

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

Gene	Foward (5'-3')	Reverse (5'-3')	Reference
fGAPDH	ACGATGACATCAAGAAGGTG	CATACCAGGAAATGAGCTTG	[4]
fTGF- β	CCAACAAAATCTATGAGAAAGTCCA	TATTGCTGATTCTGGTACAGCTC	[5]
fHGF	ATTCCATGGGATTATTGCTCTATTT	TTCAAACAAACCATCCATCTACAT	[5]
fCOX2	CGATTCACTCTCATCTGCAATAA	TCAGTTGAACGTTCTTTAGCAGTA	[5]
fIDO	TATTGAATGCAGTAAATGTG AGGA	TGAATTTGTTAAACTCTTCCTTGG	[5]
mGAPDH	AAATGGTGAAGGTCGGTGTG	TGAAGGGGTCGTTGATGG	[4]
mIFN- γ	CACAGTCATTGAAAGCCTAGAAGT	AGTTCCTCCAGATATCCAAGAAGAG	[4]
mIL-6	ACACATGTTCTCTGGGAAATCGT	AAGTGCATCATCGTTGTTTCATACA	[4]
miNOS	GCAGAATGTGACCATCATGG	ACAACCTTGGTGTGAAGGC	[10]
mIL-10	GTGATTTAATAAGCTCCAAGACC	GATCATCATGTATGCTTCTATGCAG	[27]
mIL-1 β	GTCTTCCCGTGGACCTTC	TGTTTCATCTCGGAGCCTGT	[27]
mTNF- α	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG	[27]
mIL-17	GGTCAACCTCAAAGTCTTAACTCC	GAGGGATATCTATCAGGGTCTTCAT	[27]
mArg	CAGAAGAATGGAAGAGTCAG	CAGATATGCAGGGAGTCACC	[27]

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 PBST 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 and were considered statistically significant.

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 IFN- γ upregulated the secretion of immunomodulation factors COX2, IDO, TGF- β , and HGF in fAT-MSCs (Fig. 1E).

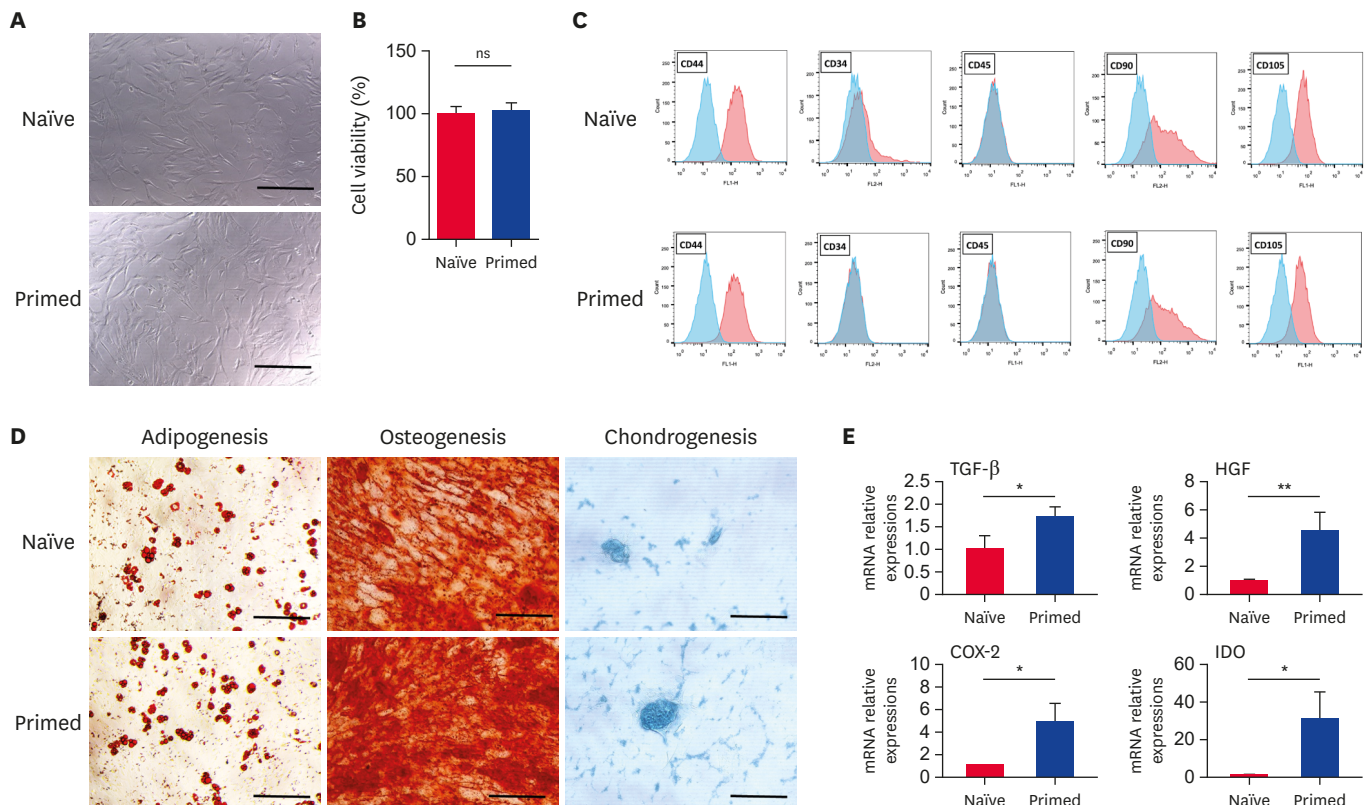


Fig. 1. Characterization of mesenchymal stem cells isolated from feline adipose tissue. (A) Morphology of IFN- γ treated and untreated fAT-MSCs (bars = 20 μ m). (B) Cell viability of IFN- γ treated and un-treated fAT-MSC. (C) Immunophenotypic analysis by flow cytometry. (D) Adipogenesis: intracellular 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 μ m). (E) Increased gene expression of, HGF, COX2, IDO (scale bars = 200 μ m). Results are presented as the mean \pm 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.

Immunomodulatory effects of IFN- γ primed fAT-MSCs

To determine the immunomodulatory capacity of IFN- γ 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- α), IL-1 β , 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- γ -treated fAT-MSC-conditioned media TNF- α , IL-1 β , 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- γ -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- γ -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- γ -treated fAT-MSC-conditioned media compared to those cultured in fAT-MSC-conditioned media and NS-398-treated IFN- γ -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 β , IFN- γ , and IL-17 in the splenocytes. However, Con A-stimulated splenocytes cultured in IFN- γ -treated fAT-MSC-conditioned media showed decreased expression of IL-1 β , IFN- γ , 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- γ treated fAT-MSC-conditioned media compared to Con A-stimulated splenocytes cultured in IFN- γ -un-treated fAT-MSC-conditioned media (**Fig. 2C**).

PGE₂ concentration levels in various fAT-MSC-conditioned media

PGE₂ ELISA Kit was used to measure PGE₂ levels in the conditioned medium obtained from fAT-MSC treated or untreated with IFN- γ , and NS-398 stimulated fAT-MSC treated with IFN- γ . First, we confirmed that NS-398 (5 μ M) treated fAT-MSCs show normal cell viability (**Supplementary Fig. 1**). PGE₂ was significantly increased in IFN- γ -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- γ pretreatment (**Fig. 3**).

Macrophage polarization and T-cell regulation are associated with PGE₂

To determine the role of PGE₂ 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- γ 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- γ treated fAT-MSC-conditioned media. Pre-treating the fAT-MSCs treated IFN- γ with PGE₂ 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**).

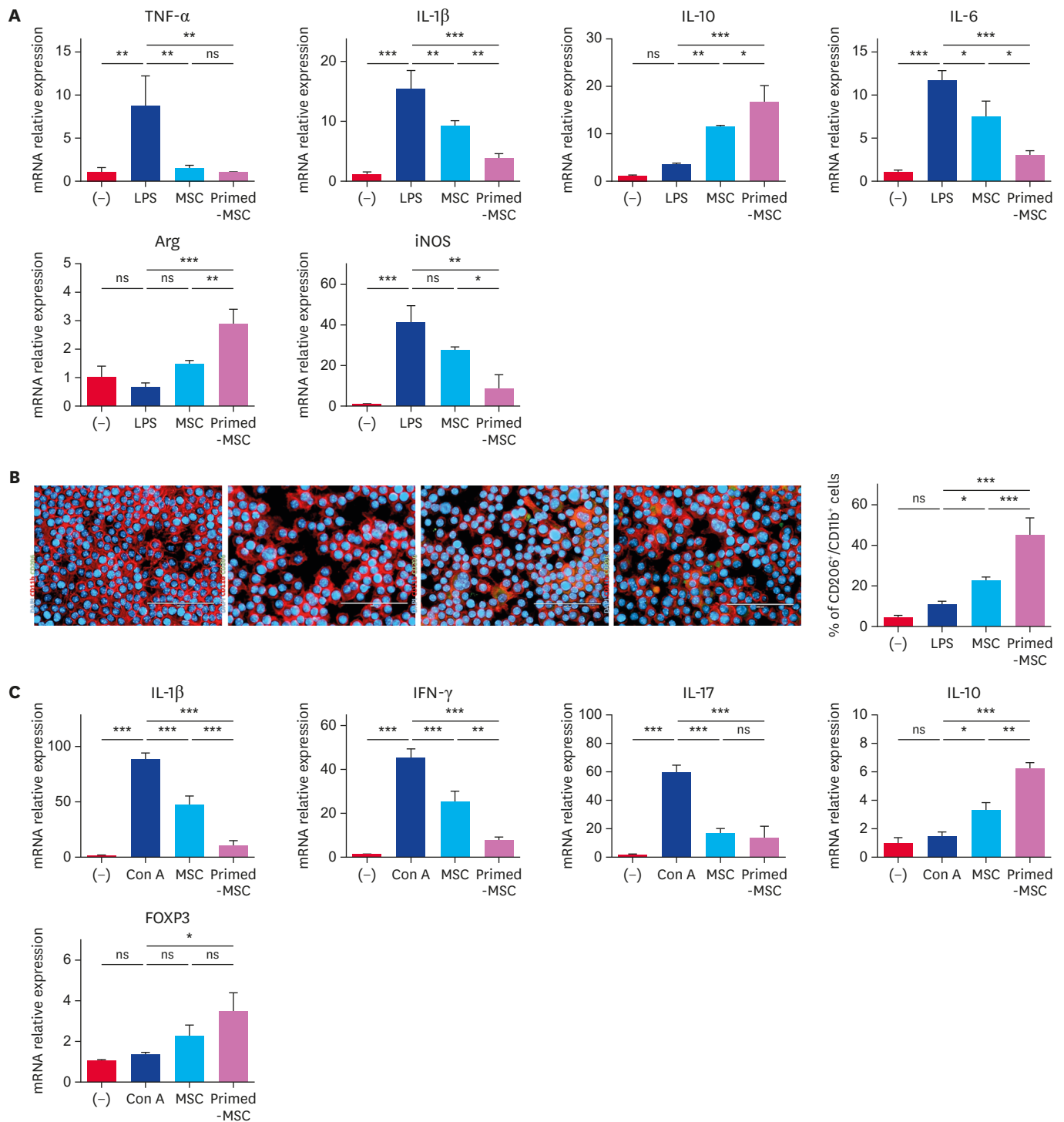


Fig. 2. Immunomodulatory effects of IFN- γ treated fAT-MSCs. (A) Effects of IFN- γ treated fAT-MSCs on the mRNA expression of anti- and pro-inflammatory cytokines by RAW 264.7 cells. (B) M2 macrophage expression of LPS stimulated RAW 264.7 cells cultured with the conditioned media of IFN- γ treated and 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 \pm 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.

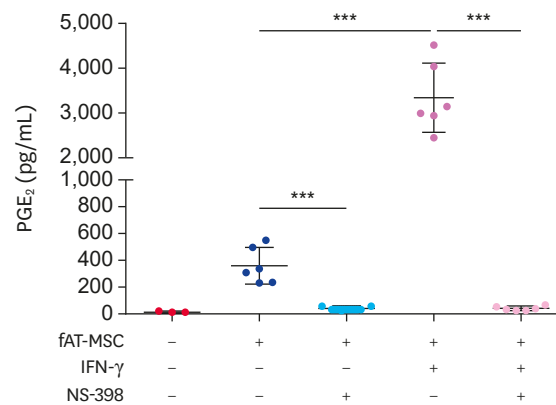


Fig. 3. Levels of PGE₂ in the conditioned media of IFN- γ primed, non-primed fAT-MSCs and the effect of PGE₂ inhibitor NS-398 on fAT-MSCs. Increased levels of PGE₂ production were observed in IFN- γ treated fAT-MSC compared to those in the un-treated fAT-MSC and fAT-MSC treated with IFN- γ and NS-398. Results are presented as the mean \pm standard deviation of 3 independent experiments. PGE₂, prostaglandin E₂; IFN- γ , Interferon-gamma; fAT-MSC, feline adipose tissue-derived mesenchymal stem cell. *** p < 0.001 by one-way analysis of variance analysis.

PGE₂ secreted by IFN- γ treated fAT-MSCs is critical for their immune function

To determine the role of PGE₂, treated IFN- γ treated fAT-MSCs with PGE₂ inhibitor, NS-398, and then compared the mRNA expression of inflammatory cytokines secreted by RAW 264.7 cells cultured in IFN- γ treated fAT-MSC-conditioned media or NS-398-stimulated IFN- γ -treated fAT-MSC-conditioned media. First, we confirmed that 5 μ M of NS-398 effectively inhibits PGE₂ secretion in fAT-MSCs without affecting cell viability (data not shown). TNF- α , IL-1 β , and IL-6 expression levels increased with LPS, which was reversed by culturing in IFN- γ treated fAT-MSC-conditioned media. IL-10 levels were decreased after LPS stimulation, which was reversed by culturing in IFN- γ treated fAT-MSC-conditioned media. However, pre-treating the fAT-MSCs with NS-398 attenuated the effect of IFN- γ treated fAT-MSC-conditioned media (**Fig. 5**).

To confirm the role of PGE₂ in T-cell regulation, we determined the mRNA expression of immune cytokines secreted by splenocytes. IL-1 β , IL-17, and IFN- γ were increased after Con A stimulation, which was reversed by culturing in IFN- γ treated fAT-MSC-conditioned media. Pre-treating the fAT-MSCs with NS-398 attenuated the effect of IFN- γ treated fAT-MSC-conditioned media and increased IL-1 β , IL-17, and IFN- γ levels. IL-10 was decreased after Con A stimulation, which was reversed by culturing in IFN- γ treated fAT-MSC-conditioned media. Pre-treating the fAT-MSCs treated IFN- γ with NS-398 attenuated the effect of IFN- γ 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- γ 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- γ 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- γ stimulation.

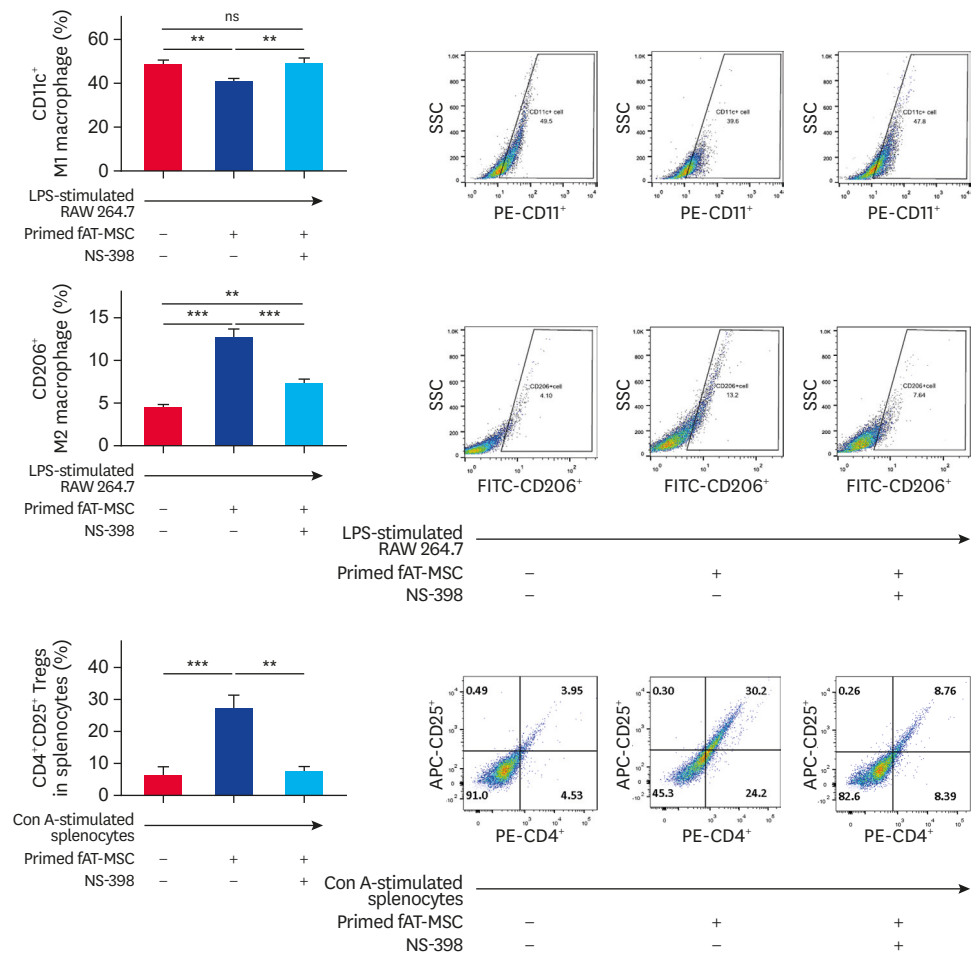


Fig. 4. Macrophage polarization and T cell regulation associated with PGE₂. CD11c⁺ were significantly increased in NS-398 stimulated and IFN- γ treated fAT-MSCs. However, CD206⁺ were decreased in RAW 264.7 cells stimulated by LPS and were increased in RAW 264.7 cells stimulated by LPS and incubated in the conditioned media of IFN- γ treated MSC. Moreover, CD206⁺ significantly decreased in NS-398 and IFN- γ treated fAT-MSCs. Results are presented as the mean \pm standard deviation of 3 independent experiments. PGE₂, prostaglandin E₂; IFN- γ , Interferon-gamma; fAT-MSC, feline adipose tissue-derived mesenchymal stem cell; LPS, lipopolysaccharide; MSC, mesenchymal stem cell; ns, not significant; PE, phycoerythrin; FITC, fluorescein isothiocyanate; SSC, sideward scatter; APC, allophycocyanin; Con A, concanavalin A. ** $p < 0.01$, *** $p < 0.001$ by one-way analysis of variance analysis.

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.

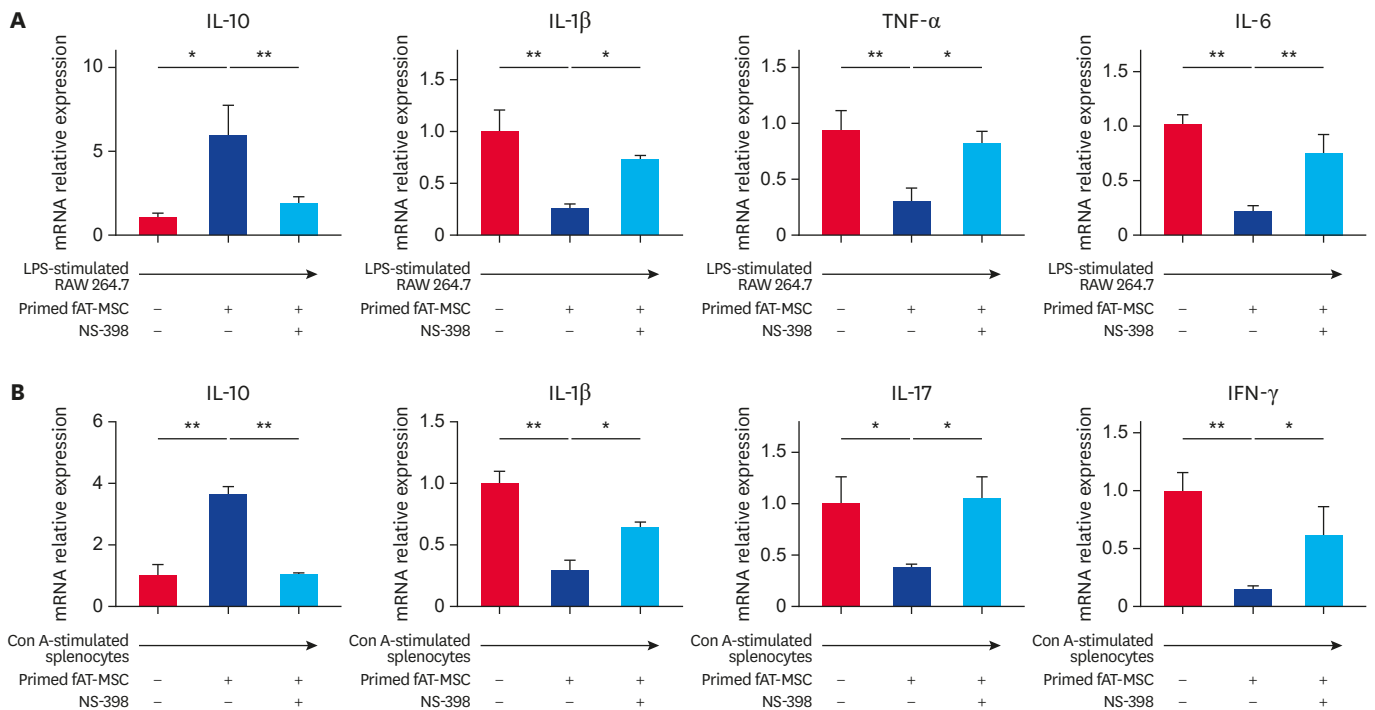


Fig. 5. Effects of immune responses for PGE₂ secreted by IFN- γ treated fAT-MSCs. PGE₂ secreted from IFN- γ treated fAT-MSCs affects the degree of inflammatory cytokine mRNA levels in RAW 264.7 cells (A) and splenocytes (B). Results are presented as the mean \pm standard deviation of 3 independent experiments. 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.

Anti-inflammatory T-helper 2 cytokines suppresses the immune responses and are critical regulators of the immune system. The expression levels 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.

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 PGE₂ in this study. Although we showed here that PGE₂ might be considered to be a major contributor to the immunomodulatory potential of the IFN- γ 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- γ 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 PGE₂ is a major factor contributing to the immunomodulatory function of IFN- γ -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.

ACKNOWLEDGMENTS

We would like to acknowledge the Research Institute for Veterinary Science and the BK21PLUS Program for Creative Veterinary Science, Seoul National University and by the Research Institute of Veterinary Science, Jeju National University. This study was also supported by the Program of National Research Foundation of Korea through the Ministry of Education.

SUPPLEMENTARY MATERIAL

Supplementary Fig. 1

NS-398 (5 μ M) treated fAT-MSCs with or without IFN- γ stimulation show normal cell viability.

[Click here to view](#)

REFERENCES

1. Arzi B, Mills-Ko E, Verstraete FJ, Kol A, Walker NJ, Badgley MR, et al. Therapeutic efficacy of fresh, autologous mesenchymal stem cells for severe refractory gingivostomatitis in cats. *Stem Cells Transl Med.* 2016;5(1):75-86.
[PUBMED](#) | [CROSSREF](#)
2. Quimby JM, Webb TL, Randall E, Marolf A, Valdes-Martinez A, Dow SW. Assessment of intravenous adipose-derived allogeneic mesenchymal stem cells for the treatment of feline chronic kidney disease: a randomized, placebo-controlled clinical trial in eight cats. *J Feline Med Surg.* 2016;18(2):165-171.
[PUBMED](#) | [CROSSREF](#)
3. Trzil JE, Masseur I, Webb TL, Chang CH, Dodam JR, Cohn LA, et al. Long-term evaluation of mesenchymal stem cell therapy in a feline model of chronic allergic asthma. *Clin Exp Allergy.* 2014;44(12):1546-1557.
[PUBMED](#) | [CROSSREF](#)
4. An JH, Song WJ, Li Q, Kim SM, Yang JI, Ryu MO, et al. Prostaglandin E₂ secreted from feline adipose tissue-derived mesenchymal stem cells alleviate DSS-induced colitis by increasing regulatory T cells in mice. *BMC Vet Res.* 2018;14(1):354.
[PUBMED](#) | [CROSSREF](#)

5. Chae HK, Song WJ, Ahn JO, Li Q, Lee BY, Kweon K, et al. Immunomodulatory effects of soluble factors secreted by feline adipose tissue-derived mesenchymal stem cells. *Vet Immunol Immunopathol.* 2017;191:22-29.
[PUBMED](#) | [CROSSREF](#)
6. Duijvestein M, Wildenberg ME, Welling MM, Hennink S, Molendijk I, van Zuylen VL, et al. Pretreatment with interferon- γ enhances the therapeutic activity of mesenchymal stromal cells in animal models of colitis. *Stem Cells.* 2011;29(10):1549-1558.
[PUBMED](#) | [CROSSREF](#)
7. Krampera M, Cosmi L, Angeli R, Pasini A, Liotta F, Andreini A, et al. Role for interferon- γ in the immunomodulatory activity of human bone marrow mesenchymal stem cells. *Stem Cells.* 2006;24(2):386-398.
[PUBMED](#) | [CROSSREF](#)
8. English K, Barry FP, Field-Corbett CP, Mahon BP. IFN- γ and TNF- α differentially regulate immunomodulation by murine mesenchymal stem cells. *Immunol Lett.* 2007;110(2):91-100.
[PUBMED](#) | [CROSSREF](#)
9. Parys M, Kruger JM, Yuzbasiyan-Gurkan V. Evaluation of immunomodulatory properties of feline mesenchymal stem cells. *Stem Cells Dev.* 2017;26(10):776-785.
[PUBMED](#) | [CROSSREF](#)
10. Lee BY, Li Q, Song WJ, Chae HK, Kweon K, Ahn JO, et al. Altered properties of feline adipose-derived mesenchymal stem cells during continuous *in vitro* cultivation. *J Vet Med Sci.* 2018;80(6):930-938.
[PUBMED](#) | [CROSSREF](#)
11. Kim HW, Song WJ, Li Q, Han SM, Jeon KO, Park SC, et al. Canine adipose tissue-derived mesenchymal stem cells ameliorate severe acute pancreatitis by regulating T cells in rats. *J Vet Sci.* 2016;17(4):539-548.
[PUBMED](#) | [CROSSREF](#)
12. Fan H, Zhao G, Liu L, Liu F, Gong W, Liu X, et al. Pre-treatment with IL-1 β enhances the efficacy of MSC transplantation in DSS-induced colitis. *Cell Mol Immunol.* 2012;9(6):473-481.
[PUBMED](#) | [CROSSREF](#)
13. Lin T, Kohno Y, Huang JF, Romero-Lopez M, Maruyama M, Ueno M, et al. Preconditioned or IL4-secreting mesenchymal stem cells enhanced osteogenesis at different stages. *Tissue Eng Part A.* 2019;25(15-16):1096-1103.
[PUBMED](#) | [CROSSREF](#)
14. Redondo-Castro E, Cunningham C, Miller J, Martuscelli L, Aoulad-Ali S, Rothwell NJ, et al. Interleukin-1 primes human mesenchymal stem cells towards an anti-inflammatory and pro-trophic phenotype *in vitro*. *Stem Cell Res Ther.* 2017;8(1):79.
[PUBMED](#) | [CROSSREF](#)
15. Zimmermann JA, McDevitt TC. Pre-conditioning mesenchymal stromal cell spheroids for immunomodulatory paracrine factor secretion. *Cytotherapy.* 2014;16(3):331-345.
[PUBMED](#) | [CROSSREF](#)
16. Kim DS, Jang IK, Lee MW, Ko YJ, Lee DH, Lee JW, et al. Enhanced immunosuppressive properties of human mesenchymal stem cells primed by interferon- γ . *EBioMedicine.* 2018;28:261-273.
[PUBMED](#) | [CROSSREF](#)
17. Sheng H, Wang Y, Jin Y, Zhang Q, Zhang Y, Wang L, et al. A critical role of IFN γ in priming MSC-mediated suppression of T cell proliferation through up-regulation of B7-H1. *Cell Res.* 2008;18(8):846-857.
[PUBMED](#) | [CROSSREF](#)
18. Cho DI, Kim MR, Jeong HY, Jeong HC, Jeong MH, Yoon SH, et al. Mesenchymal stem cells reciprocally regulate the M1/M2 balance in mouse bone marrow-derived macrophages. *Exp Mol Med.* 2014;46(1):e70.
[PUBMED](#) | [CROSSREF](#)
19. Gao P, Ding Q, Wu Z, Jiang H, Fang Z. Therapeutic potential of human mesenchymal stem cells producing IL-12 in a mouse xenograft model of renal cell carcinoma. *Cancer Lett.* 2010;290(2):157-166.
[PUBMED](#) | [CROSSREF](#)
20. Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood.* 2005;105(4):1815-1822.
[PUBMED](#) | [CROSSREF](#)
21. Di Nicola M, Carlo-Stella C, Magni M, Milanese M, Longoni PD, Matteucci P, et al. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood.* 2002;99(10):3838-3843.
[PUBMED](#) | [CROSSREF](#)
22. Nasef A, Mathieu N, Chapel A, Frick J, François S, Mazurier C, et al. Immunosuppressive effects of mesenchymal stem cells: involvement of HLA-G. *Transplantation.* 2007;84(2):231-237.
[PUBMED](#) | [CROSSREF](#)

23. Ortiz LA, Dutreil M, Fattman C, Pandey AC, Torres G, Go K, et al. Interleukin 1 receptor antagonist mediates the antiinflammatory and antifibrotic effect of mesenchymal stem cells during lung injury. *Proc Natl Acad Sci U S A*. 2007;104(26):11002-11007.
[PUBMED](#) | [CROSSREF](#)
24. Pittenger M. Sleuthing the source of regeneration by MSCs. *Cell Stem Cell*. 2009;5(1):8-10.
[PUBMED](#) | [CROSSREF](#)
25. Puissant B, Barreau C, Bourin P, Clavel C, Corre J, Bousquet C, et al. Immunomodulatory effect of human adipose tissue-derived adult stem cells: comparison with bone marrow mesenchymal stem cells. *Br J Haematol*. 2005;129(1):118-129.
[PUBMED](#) | [CROSSREF](#)
26. Sato K, Ozaki K, Oh I, Meguro A, Hatanaka K, Nagai T, et al. Nitric oxide plays a critical role in suppression of T-cell proliferation by mesenchymal stem cells. *Blood*. 2007;109(1):228-234.
[PUBMED](#) | [CROSSREF](#)
27. Song WJ, Li Q, Ryu MO, Ahn JO, Bhang DH, Jung YC, et al. TSG-6 Secreted by human adipose tissue-derived mesenchymal stem cells ameliorates DSS-induced colitis by inducing M2 macrophage polarization in mice. *Sci Rep*. 2017;7(1):5187.
[PUBMED](#) | [CROSSREF](#)
28. Spaggiari GM, Capobianco A, Becchetti S, Mingari MC, Moretta L. Mesenchymal stem cell-natural killer cell interactions: evidence that activated NK cells are capable of killing MSCs, whereas MSCs can inhibit IL-2-induced NK-cell proliferation. *Blood*. 2006;107(4):1484-1490.
[PUBMED](#) | [CROSSREF](#)
29. Yang FY, Chen R, Zhang X, Huang B, Tsang LL, Li X, et al. Preconditioning enhances the therapeutic effects of mesenchymal stem cells on colitis through PGE2-mediated T-cell modulation. *Cell Transplant*. 2018;27(9):1352-1367.
[PUBMED](#) | [CROSSREF](#)
30. Yang HM, Song WJ, Li Q, Kim SY, Kim HJ, Ryu MO, et al. Canine mesenchymal stem cells treated with TNF- α and IFN- γ enhance anti-inflammatory effects through the COX-2/PGE₂ pathway. *Res Vet Sci*. 2018;119:19-26.
[PUBMED](#) | [CROSSREF](#)