A THESIS FOR THE DEGREE OF MASTER

Pretreatment with IFN-γ enhances the immunomodulatory effect of feline adipose tissue-derived mesenchymal stem cells through the COX-2/PGE_2 increase

인터페론 감마 전처리 고양이 중간엽 줄기세포에 있어서
COX-2/PGE_2 증가에 의한 면역조절 향상

2020년 2월

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수의과대학 수의내과학 전공

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이 논문을 수의학 석사 학위논문으로 제출함

2020년 2월

서울대학교 대학원

수의학과 임상수의학 전공

박 설 기

박설기의 석사 학위논문을 인준함

2020년 2월

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Pretreatment with IFN-γ enhances the immunomodulatory effect of feline adipose tissue-derived mesenchymal stem cells through the COX-2/PGE_2 increase

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Abstract

Preconditioning with inflammatory stimuli is used to improve the secretion of anti-inflammatory agents in stem cells. However, there are only a few studies on feline stem cells. In this study, the immune regulatory capacity of feline adipose tissue-derived mesenchymal stem cells (fAT-MSCs) pretreated with interferon-gamma (IFN-γ) was evaluated. Pretreatment with IFN- γ increased the expression of cyclooxygenase-2 (COX-2), indoleamine 2,3-dioxygenase (IDO), hepatocyte growth factor (HGF), and transforming growth factor-beta 1(TGF-β1) in the fAT-MSCs. To assess the interaction of lymphocytes and macrophages with IFN-γ-pretreated fAT-MSCs, mouse splenocytes, and RAW 264.7 cells were co-cultured with conditioned media. IFN- γ-pretreated

fAT-MSCs increased the expression of M2 markers and regulatory T-cell (Tregs) markers compared to non-preconditioned fAT-MSCs. Further, PGE_2 inhibitor NS-398 attenuated the immunoregulatory potential of fAT-MSCs, suggesting that the increased PGE_2 induced by IFN- γ stimulation is a crucial factor in the immune regulatory capacity of IFN-γ-preconditioning fAT-MSCs. We demonstrate that IFN- γ pretreatment improves the anti-inflammatory status of fAT-MSC by inducing macrophage polarization and increasing Tregs by activating PGE_2 secretion.

Key words: Feline stem cells, Macrophages, Splenocytes, Interferon-gamma, Prostaglandin E2

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1. Introduction

Mesenchymal Stem Cells (MSCs) have anti-inflammatory effects and immunomodulatory functions and could be developed as novel cell-based therapeutics for immune-mediated and inflammatory diseases [1-3]. Several studies have focused on the enhancement of immunoregulatory functions of MSCs, including activation by hypoxia and inflammation. However, the mechanisms underlying the therapeutic effect of enhanced stem cells remain unclear. and further studies are needed to develop these stem cells as anti-inflammatory and immunotherapeutic agents.

One of the strategies[4] to improve the immunoregulatory capacity of MSCs is to pre-treat them with interferon-gamma (IFN-γ). IFN-γ is a pro-inflammatory cytokine secreted by NK cells and T cells which acts on macrophages and lymphocytes [5]. Previous studies suggested that MSCs stimulated with IFN-γ have upregulated immunosuppression functions and show changes in the expression of immunomodulatory factors [6]. Recently Maciej [7] reported that feline MSCs stimulated with IFN-γ showed significantly increased secretion of immunomodulatory factors such as indoleamine 2,3-dioxygenase 1 (IDO1), programmed death-ligand 1 (PD-L1), interleukin-6 (IL-6), cyclooxygenase-2 (COX_2), and hepatocyte growth factor **(**HGF). However, the key factors involved in immunomodulation among these factors have not been identified. Further studies are needed to develop feline stem cells as therapeutic agents for inflammatory and immune-mediated diseases. Therefore, we examined whether MSC derived immune response factors were increased in feline MSCs pretreated with IFN-γ. We also examined whether these factors affect the immune responses and improve the immune regulatory ability of MSCs.

Our results show that the anti-inflammatory activity of stem cells is closely related to M2 polarization in macrophages and the increase in regulatory T cells (Tregs). We demonstrated that IFN-γ-pretreated feline adipose tissue-derived MSCs (fAT-MSCs) could secrete anti-inflammatory agents more efficiently and reprogram and induce macrophage polarization to an anti-inflammatory status as well as enhance the Treg population in mouse splenocytes.

2. Materials and Methods

2.1 Isolation and characterization of fAT-MSCs

Feline adipose tissues were obtained using a protocol approved by the Institutional Animal Care and Use Committee of Seoul National University (SNU; protocol no. SNU-190411-10). fAT-MSCs were cultured in DMEM supplemented with 20% FBS and 1% PS and incubated at 37 °C in a 5% CO_2 humidified atmosphere. The medium was changed every two days until the cells reached a confluence of 70-80%. Previous studies used MSCs in the third or fourth passage, suggesting that early passage cells are more effective [8]. 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 –CD105 (antibody clone SN6, MCA1557F; AbD Serotec), CD90 (antibody clone 5E10, 555596; BD Bioscience) [9], CD44 (antibody clone IM7, 103021, Biolegend) [10], CD34 (antibody clone 1H6, 559369, BD science) [11], and CD45 (antibody clone YKIX716.13, Ebioscience) [12]. For CD105, CD90, and CD44, an indirect immuno fluorescence detection was used with goat anti-rat IgG-PE and goat anti-mouse IgG-fluorescein isothiocyanate (FITC) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Data were analyzed using FlowJo 7.6.5 software (Tree Star, Inc., Ashland, OR, USA). Special differentiation kits (Stem Pro Osteogenesis Diff erentiation, Stem Pro Adipogenesis Differentiation, and Stem Pro Chondrogenesis Differentiation kits; all from Gibco/Life Technologies, Carlsbad, CA, USA) were used for evaluating cellular differentiation following the manufacturer's instructions.

2.2 Interferon-gamma stimulation

To assess the effects of INF- γ on the fAT-MSCs, 5×10^5 fAT-MSCs were seeded in 12-well plates in DMEM supplemented with 20% FBS and 1% PS. After 24 h, the fAT-MSC were treated with 50 ng/mL INF-γ (Kingfisher Biotech, MN, USA) for 48 h. The cells were then washed four times with DPBS and were then maintained in DMEM supplemented with 20% FBS 1% PS for three days. After incubation, the cell culture supernatant was centrifuged at 1000 rpm for 3 min to remove any debris. The supernatant was collected and stored at -80 °C until use. D-Plus CCK Cell Viability Assay Kit (DonginLS, Seoul, Korea) was used to determine the cell viability of IFN- γ stimulated fAT-MSCs following the manufacturer's instructions.

2.3 Prostaglandin E2 (PGE 2) inhibitor, NS 398 treatment

Previous studies have shown that PGE_2 secreted by fAT-MSCs contributes to the anti-inflammatory and immunosuppressive effect on LPS pre-conditioned RAW 264.7 cells and Concanavalin A stimulated lymphocytes[13]. To confirm the role of PGE_2 produced fAT-MSCs in the anti-inflammatory response, stem cells were treated with PGE_2 inhibitor NS-398 (5 μM; Enzo Life Sciences, Farmingdale, NY, USA) and then stimulated with INF- γ as described above. The cell culture supernatant was then collected to evaluate PGE_2 secretion.

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

RAW 264.7 cells obtained from the Korean Cell Line Bank were treated for 24 h with lipopolysaccharide (LPS) (200 ng/ml; Sigma-Aldrich, St. Louis, MO, USA) and then washed three times with DPBS. LPS-stimulated RAW 264.7 cells were seeded in 6-well plates (1×10^6 cells/well) and cultured in fAT-MSC-conditioned media obtained from fAT-MSCs treated and un-treated with IFN-γ and NS398-treated fAT-MSCs stimulated with IFN-γ for 48 h.

In addition, splenocytes were isolated from mice, as previously described [14]. All procedures were approved by the SNU Institutional Animal Care and Use Committee (Approval number: SNU-190304-1). Briefly, mouse spleens obtained from four mice (C57BL/6, male, 5 W) were mashed using a 1 ml syringe plunger. The cell suspension was then centrifuged for 3 min at 1000 rpm. The cell pellet was resuspended in RBC Lysis buffer (Sigma-Aldrich) and washed with PBS three times. The splenocytes were then resuspended in RPMI-1640 supplemented with 10% FBS and 1% PS. They were stimulated with 5 μg/mL concanavalin A (ConA; Sigma-Aldrich) for 24 h to determine the mRNA expression of inflammatory cytokines. The splenocytes were then collected by centrifugation at 3000 rpm for 10 min and seeded at a density of 1×10^6 cells/well in 6-well plates either in RPMI-1640 supplemented with 10% FBS and 1% PS, conditioned medium from fAT-MSCs, conditioned media from fAT-MSCs treated and un-treated with IFN-γ and PGE_2 inhibitor NS398 stimulated in fAT-MSCs treated by IFN-γ.

2.5 RNA extraction, cDNA synthesis, and RT-qPCR

Easy-BLUE Total RNA Extraction Kit (Intron Biotechnology, Seongnam, 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, Seoul, 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-RO (Enzo Life Science, Lausen, Switzerland), 7.4 μL PCR grade do, 0.8 μL forward and reverse primers (Bionics, Seoul, Korea) (Primers are listed in Table 3), and 1 μL template cDNA. Cytokine mRNA levels were quantified using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control.

2.6 Enzyme-linked immunosorbent assay (ELISA)

The concentration of PGE_2 in the cell culture supernatant was determined using an ELISA kit (Enzo Life Science) 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 PGE_2 ELISA.

2.7 Flow cytometric analysis

Flow cytometry was performed using a FACS Aria II system (BD Biosciences, Franklin Lakes, NJ, USA) and the data were analyzed using FlowJo software (Tree Star, Ashland, OR, 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; Biolengend) and FITC-conjugated anti-$CD206^+$ antibody (clone MRC1, SC376108, Santa Cruz Biotechnology), and evaluated by flow cytometry. To evaluate PGE_2-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, eBiosciences) and APC-conjugated anti-$CD25^+$ antibody (PC61.5, 17-0251-82, eBiosciences) and evaluated by flow cytometry.

2.8 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 four 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 ℃ for 12 h. The cells were rinsed with DPBS three 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 four times and mounted using VECTASHIELD mounting medium

containing 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). The slides were observed under an EVOS FL microscope (Life Technologies, Darmstadt, Germany), and the stained cells in 20 random fields per group were counted.

2.9 Statistical analyses

All data were analyzed using GraphPad Prism v.6.01 software (GraphPad Software Inc., La Jolla, CA, USA). Student's *t*-tests or one-way analysis of variance (ANOVA) were used to determine statistical significance. *P*-values less than 0.05 and were considered statistically significant.

3. Results

3.1 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).

Flow cytometric analyses showed that only a few of the naïve 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. 1B). When specific differentiation media were used, fAT-MSCs differentiated into osteocytes, adipocytes, and chondrocytes (Fig. 1C). There were no differences in the differentiation potential of fAT-MSCs treated with or without IFN-γ MSCs. To evaluate the effect of preconditioning fAT-MSCs with IFN-γ, 48 h after INF-γ stimulation fAT-MSCs were evaluated by CCK-8 assay. IFN-γ-treated fAT-MSCs showed normal cell viability (Fig. 1D). Additionally, 48h treatment with INF-γ upregulated the secretion of immunomodulation factors COX2, IDO, TGF-β, and HGF in fAT-MSCs (Fig. 1E).

3.2 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. TNF-α, interleukin-1β (IL-1β), interleukin-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-1β, 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-1β, 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-1β, 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).

3.3 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-γ, and NS398 stimulated fAT-MSC treated with IFN-γ. PGE_2 was significantly increased in IFN-γ-treated fAT-MSC-conditioned media treated compared to that in untreated fAT-MSC-conditioned media and NS398-stimulated IFN-γ-treated fAT-MSC-conditioned media (Fig. 3).

3.4 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-γ treated fAT-MSC-conditioned media. 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 PGE_2 inhibitor, NS398 attenuated the effect of fAT-MSC-conditioned media on RAW 264.7 cells significantly increasing $CD11c^+$ and decreasing $CD206^+$ RAW 264.7 cells.

3.5 PGE_2 secreted by IFN-γ treated fAT-MSCs are critical for their immune function.

To determine the role of PGE_2, treated IFN-γ treated fAT-MSCs with PGE_2 inhibitor, NS398, 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 NS398-stimulated IFN-γ-treated fAT-MSC-conditioned media was evaluated. TNF-α, IL-1β, and IL-6 expression levels increased with LPS treatment, 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 NS398 attenuated the effect of IFN-γ treated fAT-MSC-conditioned media (Fig. 5).

To confirm the role of PGE_2 in T-cell regulation, we determined the mRNA expression of immune cytokines secreted by splenocytes. IL-1β, 1L-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 NS398 attenuated the effect of IFN-γ treated fAT-MSC-conditioned media and increased IL-1β, 1L-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 NS398 attenuated the effect of IFN-γ treated fAT-MSC-conditioned media and decreased IL-10 levels (Fig. 5).

4. Discussion

Numerous studies have reported that MSCs pre-conditioned with pro-inflammatory cytokines [10,15-17] have increased immunomodulatory effects. 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[6,18,19]. 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.

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 [20]. Lymphocytes and macrophages are involved in various inflammatory and immune-mediated disease and have been studied in relation to the immune system [9]. Mouse splenocytes have been used for studying various aspects of human, dog, and rat immune systems[11,18,21]. Therefore, we used mouse splenocyte to evaluate T-cell regulation in IFN-γ treated fAT-MSCs.

Anti-inflammatory T-helper (Th)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.

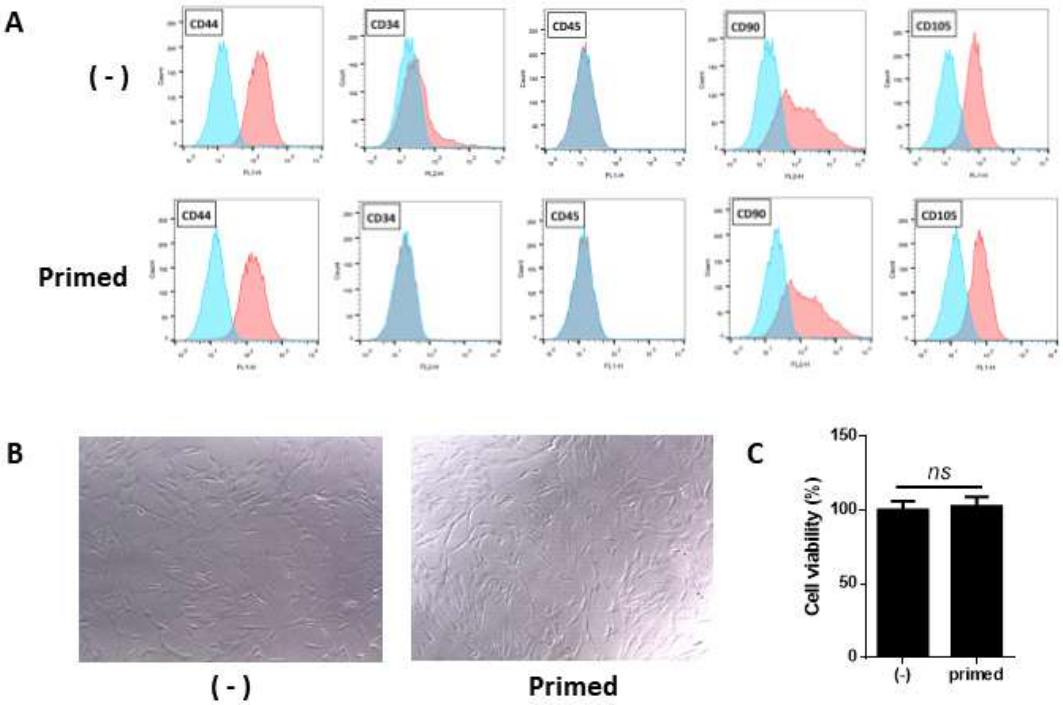
Many soluble factors secreted by MSCs such as TGF-β, HGF, PGE-2, IL-6, IL-10, IL-1, iNOS, IDO, Gal-1, and HLA-G regulate the immune system and modulate the immune cell activity and Relieve the inflammatory environment [8,12-14,22-27]. In particular, previous studies have reported that PGE_2 plays a critical role in the immune responses [28,29] and is associated with macrophage polarization and T-cell regulation [30]. However, no studies have been done to determine whether the increased PGE_2 secretion is a key regulator of the immune regulation mediated by the IFN-γ treated fAT-MSC. Our results confirmed the significant increase of PGE_2 in the IFN-γ treated fAT-MSC-conditioned media. In addition, pretreatment with PGE_2 inhibitor, NS 398, significantly decreased the level of PGE_2 in the IFN-γ treated fAT-MSC-conditioned media. Pretreatment with NS 398 attenuated PGE_2 as so decreased the M2-macrophage polarization, and T-cell regulation induced by the IFN-γ treated fAT-MSC-conditioned media.

In this study, we only confirmed the immunomodulatory effects of PGE_2. Further studies should explore the role of other soluble factors such as IDO, TSG-6, and HGF. However, the immunoregulatory capacity of the IFN-γ treated

fAT-MSC-conditioned media was decreased with PGE_2 inhibition, PGE_2 may be considered to be a major contributor to the immunomodulatory potential of the IFN-γ treated fAT-MSC-conditioned media.

5. Conclusions

In conclusion, IFN-γ pretreatment enhanced the ability of fAT-MSCs to induce M2-macrophage 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-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.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IDO
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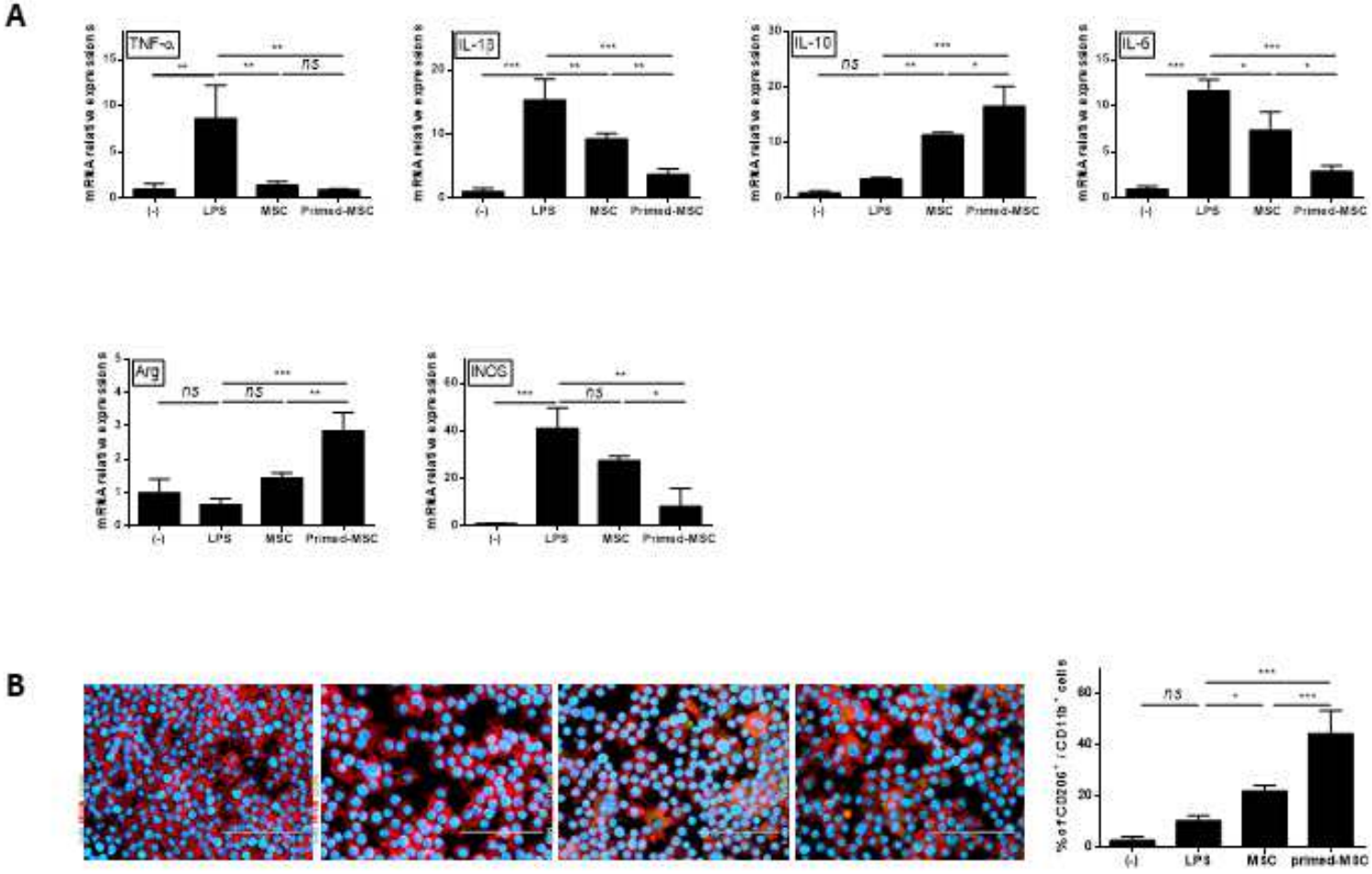
Figure 1. Characterization of mesenchymal stem cells (MSCs) isolated from feline adipose tissue.

(A) Morphology of IFN-γ treated and untreated feline adipose tissue-derived mesenchymal stem cells (fAT-MSCs). (B) Immunophenotypic analysis by flow cytometry. (C) Adipogenic differentiation; Intracellular lipid vacuoles were stained pink with Oil Red O. Osteogenic differentiation; fAT-MSCs stained positive for calcium deposits with 1% Alizarin red. Chondrogenic differentiation; Proteoglycans were stained with Alcian Blue. Bars = 20 μm.
(D) Cell viability of IFN-γ treated and untreated fAT-MSC. (E) Stimulation of fAT-MSC with 50 ng/mL of IFN-γ results in significant increasing the gene expression of TGF-β, HGF, COX-2, IDO. Results were shown as mean ± standard deviation (*$P < 0.05$, **$P < 0.01$ by one-way ANOVA analysis).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MSC
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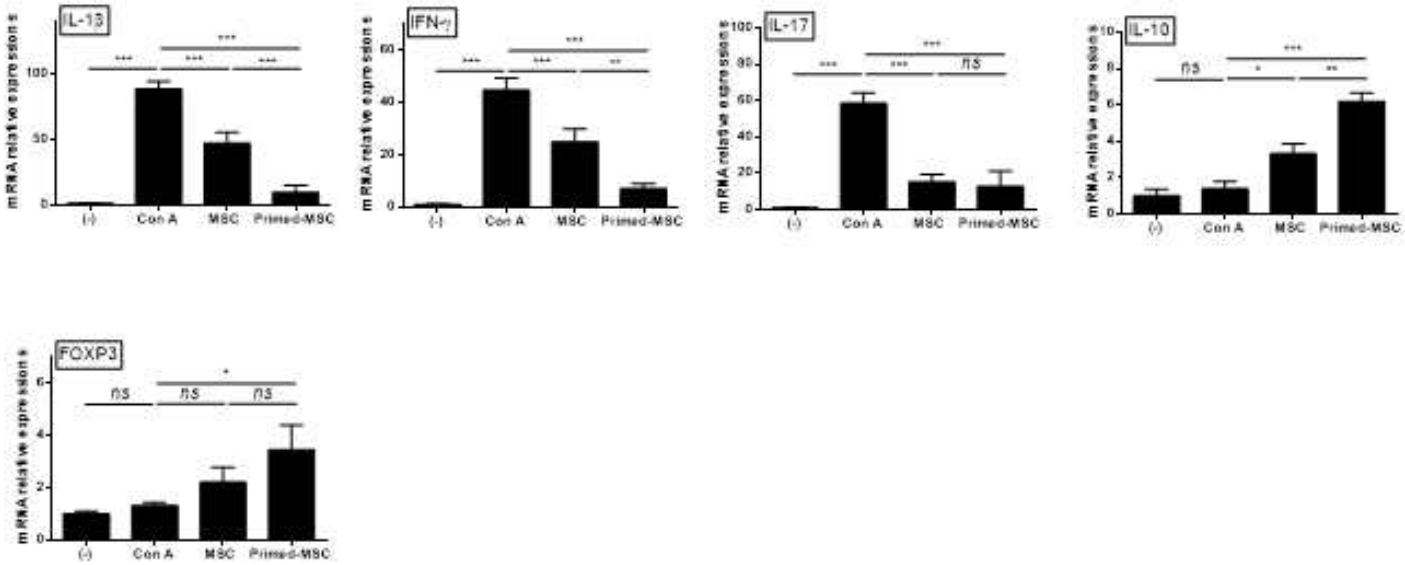
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Figure 2. Immunomodulatory effects of IFN-γ treated fAT-MSCs.

(A) Effects of IFN-γ treated fAT-MSCs on mRNA expression of anti- and pro-inflammatory cytokines secreted by RAW 264.7 cells. Comparing of mRNA expression levels in LPS-stimulated RAW 264.7 cells cultured with conditioned media of IFN-γ treated and untreated fAT-MSCs. (B) M2 macrophage expression of LPS-stimulated RAW 264.7 cells cultured with CM of IFN-γ treated and untreated fAT-MSCs. $CD206^+$ and $CD11b^+$ cells detected in RAW 264.7 cells by immunofluorescence. The number of $CD206^+$ and $CD11b^+$ cells in RAW 264.7 cells. The nuclei of the corresponding cells were visualized with DAPI. observed under an EVOS FL microscope (Life Technologies, Darmstadt, Germany), and the stained cells in 20 random fields per group were counted. (C) T-cell regulation ability of IFN-γ treated fAT-MSCs on mRNA expression of anti- and pro-inflammatory cytokines secreted by splenocytes. Comparing of mRNA expression levels in conA stimulated splenocytes cultured with conditioned media of IFN-γ treated and untreated fAT-MSCs. Results were shown as mean ± standard deviation (*P < 0.05, **P < 0.01, ***P < 0.001 by one-way ANOVA analysis).

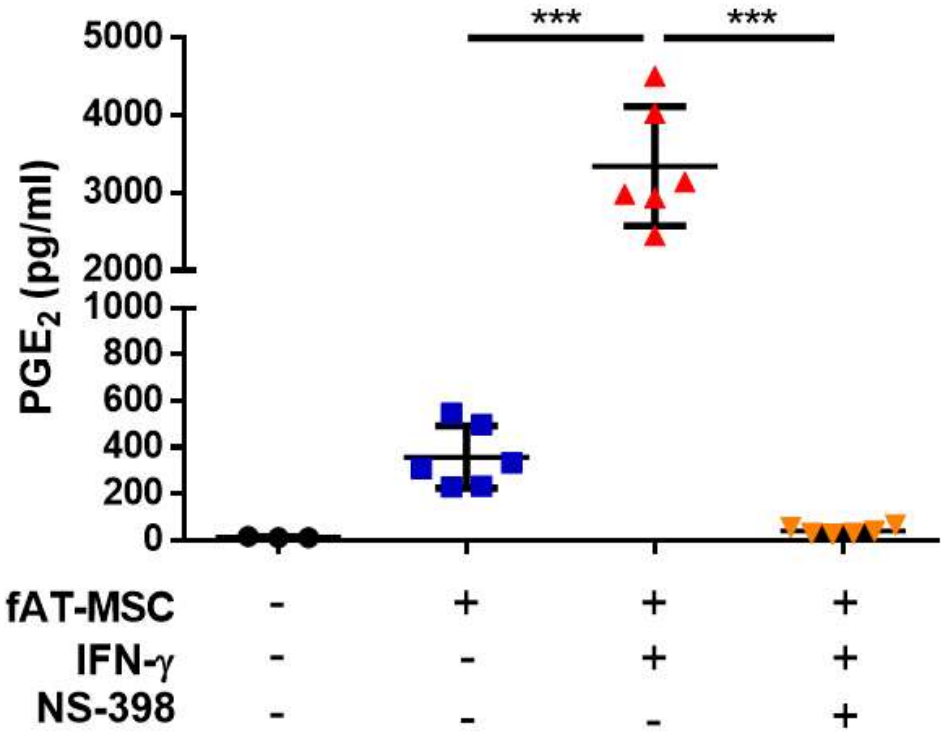
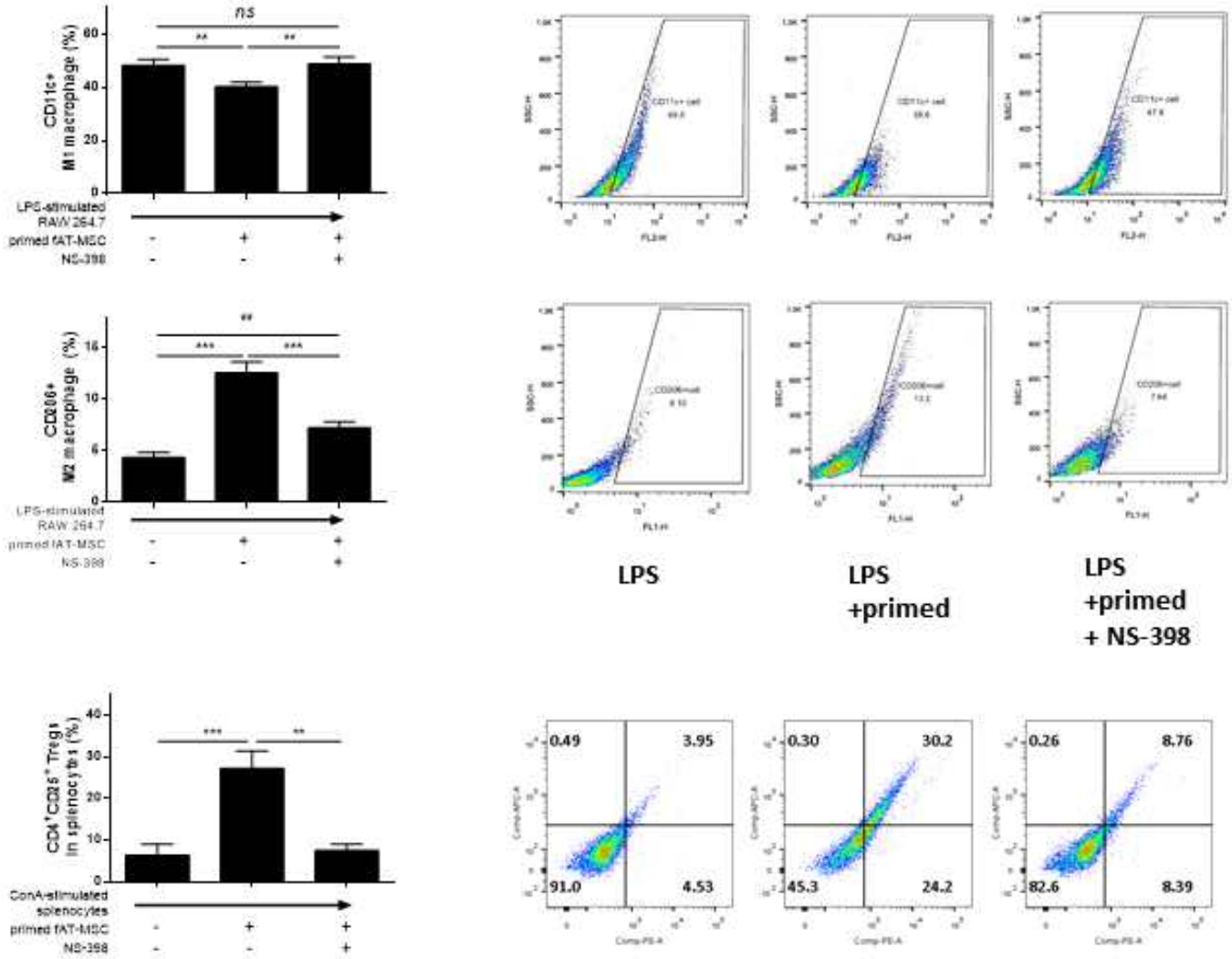


Figure 3. PGE_2 concentration levels in conditioned media of IFN-γ primed, non-primed fAT-MSCs and PGE_2 inhibitor NS398 on IFN-γ primed fAT-MSCs.

PGE_2 concentration found in conditioned media from fAT-MSCs treated or untreated IFN-γ and PGE_2 inhibitor NS398 stimulated fAT-MSCs treated IFN-γ. Increasing levels of PGE_2 production in IFN-γ treated fAT-MSC compared untreated IFN-γ fAT-MSC and stimulated NS398 treated IFN-γ. (**P < 0.01, ***P < 0.001 by one-way ANOVA analysis)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CD11c+ M1 macrophage (%)
60
40
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LPS-stimulated RAW 264.7
primed fAT-MSC - + +
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CD206+ M2 macrophage (%)
16
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LPS-stimulated RAW 264.7
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NS-398 - - +
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CD4+CD25+ Tregs in splenocytes (%)
40
30
20
10
0
ConA-stimulated splenocytes
primed fAT-MSC - + +
NS-398 - - +
0.49
3.95
91.0
4.53
0.30
30.2
45.3
24.2
0.26
8.76
82.6
8.39

Figure 4. Macrophage polarization and T-cell regulation both associated with PGE_2.

RAW 264.7 Cells were stained with PE-conjugated anti-$CD11c^+$ antibody and FITC-conjugated anti-$CD206^+$ antibody. $CD11c^+$ and $CD206^+$ cells detected in RAW 264.7 cells by flowcytometry. Determining PGE_2 role, stimulated PGE_2 inhibitor NS398 on IFN-γ treated fAT-MSC. $CD11c^+$ were significantly increased in NS 398 stimulated on IFN-γ treated MSCs. However, $CD206^+$ were decreased in RAW 264.7 cells stimulated by LPS and were increased in IFN-γ treated MSC-RAW 264.7 cells stimulated by LPS. Also, $CD206^+$ significantly decreased in NS 398 treated on IFN-γ treated fAT-MSCs. Results were shown as mean ± standard deviation (**P < 0.01, ***P < 0.001 by one-way ANOVA analysis).

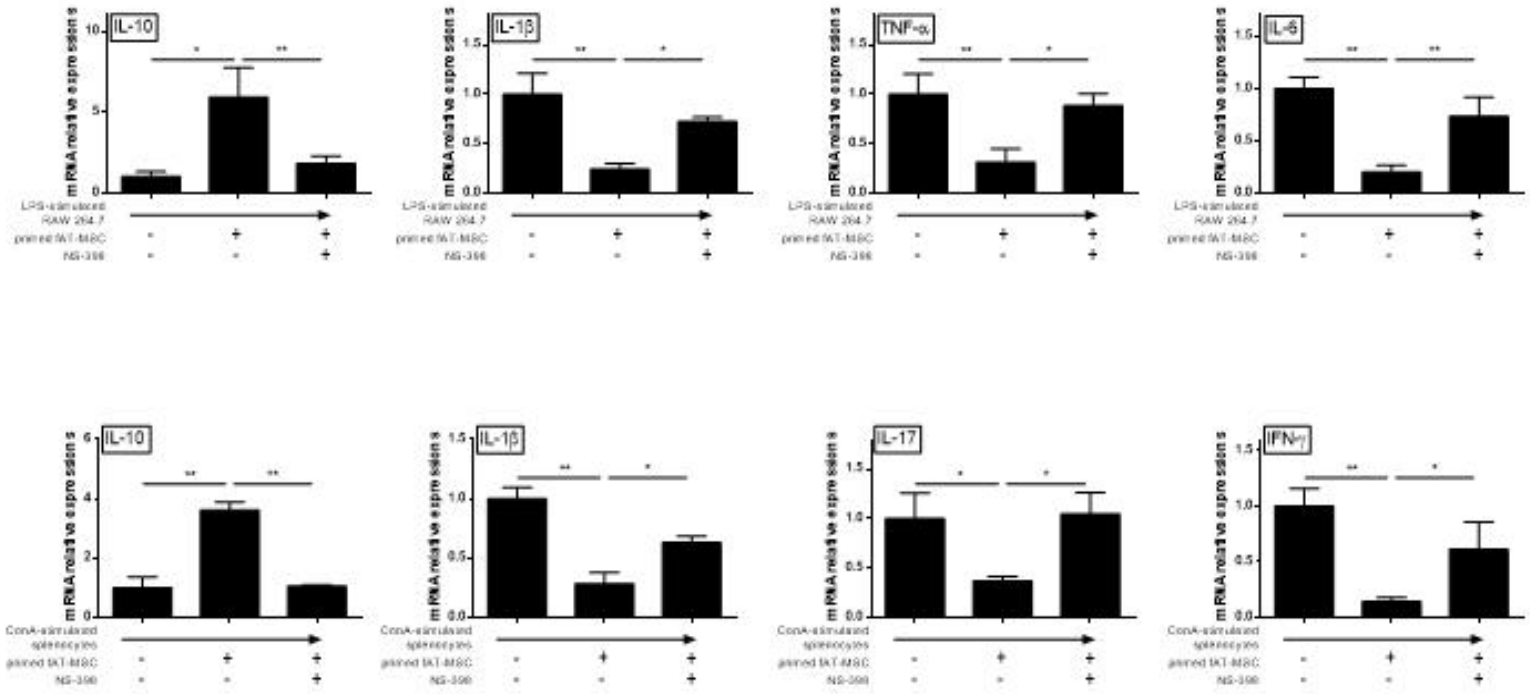
IL-10
IL-1β
TNF-α
IL-6
IL-10
IL-1β
IL-17
IFN-γ
mRNA relative expression
LPS-stimulated RAW 264.7
ConA-stimulated splenocytes
primed BAT-MSC
NS-398

Figure 5. Effects of immune responses for PGE_2 secreted by IFN-γ treated fAT-MSCs.

To confirm the role of PGE2 in immune responses, Compare the mRNA expression of inflammatory cytokines secreted by RAW 264.7 cells and splenocytes seeded on conditioned medium of IFN-γ treated fAT-MSCs and NS398 stimulated on IFN-γ treated fAT-MSCs were determined by qRT-PCR. PGE_2 secreted from IFN-γ treated fAT-MSCs affects the degree of inflammatory cytokine mRNA level in RAW 264.7 cells and splenocytes. A, B Results were shown as mean ± standard deviation (*P < 0.05, **P < 0.01 by one-way ANOVA analysis).

Table 1. Sequences of PCR primers used for cytokine analysis.

Gene	Foward(5'-3')	Reverse(5'-3')	References
fGAPDH	ACGATGACATCAAGAAGGTG	CATACCAGGAAATGAGCTTG	(An et al., 2018)
fTGF-β	CCAACAAAATCTATGAGAAAGTCCA	TATTGCTGTATTTCTGGTACAGCTC	(Chae et al., 2017)
fHGF	ATTCCATGGGATTATTGTCCTATTT	TTCAAACTAACCATCCATCCTACAT	(Chae et al., 2017)
fCOX-2	CGATTCAGTCTCTCATCTGCAATAA	TCAGTTGAACGTTCTTTTAGCAGTA	(Chae et al., 2017)
fIDO	TATTGAATGCAGTAAAATGTG AGGA	TGAATTTGTTTAAACTCTTCCTTGG	(Chae et al., 2017)
mGAPDH	AAATGGTGAAGGTCGGTGTG	TGAAGGGGTCGTTGATGG	(Okumachi et al., 2015)
mIFN-γ	CACAGTCATTGAAAGCCTAGAAAGT	AGTTCCTCCAGATATCCAAGAAGAG	(An et al., 2018)
mIL-6	ACACATGTTCTCTGGGAAATCGT	AAGTGCATCATCGTTGTTCATACA	(Kuipers et al., 2004)
miNOS	GCAGAATGTGACCATCATGG	ACAACCTTGGTGTTGAAGGC	(Lee et al., 2018)
mIL-10	GTGATTTTAATAAGCTCCAAGACC	GATCATCATGTATGCTTCTATGCAG	(Song et al., 2017)
mIL-1β	GTCTTTCCCGTGGACCTTC	TGTTCATCTCGGAGCCTGT	(Song et al., 2017)
mTNF-α	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG	(Song et al., 2017)
mIL-17	GGTCAACCTCAAAGTCTTTAACTCC	GAGGGATATCTATCAGGGTCTTCAT	(Song et al., 2017)
mArg	CAGAAGAATGGAAGAGTCAG	CAGATATGCAGGGAGTCACC	(Song et al., 2017)

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국문 초록

인터페론 감마 전처리 고양이 중간엽 줄기세포에 있어서 COX-2/PGE_2 증가에 의한 면역조절 향상

박설기

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중간엽 줄기세포(mesenchymal stem cells) 의 항염증 인자의 분비를 향상시키는 방법으로 염증성인자를 줄기세포에 전 처리하는 방법이 있다. 그러나 고양이의 중간엽 줄기세포에 대한 연구는 부족한 실정이다. 따라서 본 학위 논문은 Interferon-gamma (IFN-γ)로 전처리 된 고양이의 중간엽 줄기세포에서 면역 조절 효과를 평가하고자 하였다.

IFN-γ로 48시간 동안 고양이의 중간엽 줄기세포에 전처리하여 적응용배지(Conditioned media)를 만들었다. 적응용배지를 역전사 중합효소 연쇄반응(qRT-PCR)을 이용하여 평가하였을 시 Cyclooxygenase-2 (COX-2), Indoleamine 2,3-dioxygenase (IDO), Hepatocyte growth

factor (HGF), Transforming growth factor-beta 1(TGF-β1)의 발현이 유의적으로 증가하였다($P < 0.05$). 또한 IFN-γ를 전처리한 고양이의 중간엽 줄기세포와 림프구 및 대식세포의 상호관계를 확인하기 위하여 마우스의 비장세포와 마우스의 면역세포(RAW 264.7)를 Concavalin A 와 LPS에 각각 자극한 뒤, 적응용 배지에서 48시간동안 배양하였다. 역전사중합효소연쇄반응(qRT-PCR), 면역형광염색(immunofluorescence staining), 유세포분석(FACS)을 통하여 IFN-γ 전처리 고양이 중간엽 줄기세포배지와 처리하지 않은 순수한 고양이의 중간엽 줄기세포 배지에서 배양한 마우스의 비장세포와 마우스의 면역세포(RAW 264.7) 비교하였다.

IFN-γ를 전처리한 고양이의 중간엽줄기세포와 처리되지 않은 고양이의 중간엽줄기세포와 비교하였을 때 LPS의 자극을 받아 증가한 Tumor necrosis factor-α(TNF-α), Interleukin 1β(IL-1β), Interleukin 6(IL-6), Inducible nitric oxide synthase(iNOS)의 발현이 IFN-γ 전처리 고양이 중간엽줄기세포와 처리하지 않은 고양이 중간엽줄기세포 적응용 배지에서 배양시에 각각 감소되는 것을 확인할 수 있었다 ($P < 0.01$). 반면 Arginase와 Interleukin 10(IL-10)은 LPS의 자극을 받아 감소되었으나 IFN-γ 전처리 고양이 중간엽줄기세포와 처리하지 않은 고양이 중간엽줄기세포 적응용배지에서 배양시에 발현이 증가되었다 ($P < 0.001$). Enzyme linked immunoassay를 통해 PGE_2를 측정하였을 시 PGE_2는 고양이 중간엽줄기세포에서는 833배 IFN-γ를 전처리한 고양이의 중간엽줄기세포에서는 3833배 증가되는 것을 확인하였으나 NS398을 처리한 고양이 중간엽줄기세포에서는 PGE_2가 증가되지 않았다 ($P < 0.01$). PGE2가 면역조절능력에 영향을 미치는 지 알아보기 위하여 RAW 264.7세포 유세포분석을 실시하였다. IFN-γ 전처리 고양이 중간엽줄기세포 그룹에서는 CD11b+가 감소한 것을 확인하였고, NS398을 처리한 고양이의 중간엽줄기세포 그룹에서는 CD11b+가 증가하였다 ($P < 0.01$). 반면, CD206+는 IFN-γ 전처리 고양이 중간엽줄기세포 그룹에서 증가하였으나 NS398을 처리한 고양이의 중간

엽줄기세포 그룹에서는 CD206+가 감소하였다 ($P < 0.001$). 본 연구는 고양이중간엽줄기세포에 IFN-γ을 전처리하여 면역조절능력이 향상되었음을 확인한 보고로 의의가 있으며 이번 연구는 실제 생체 내 실험 및 임상 적용에 대한 기초가 될 것으로 기대된다.

주요어: 고양이; 대식세포; 비장세포; 중간엽 줄기세포; Interferon-gamma; Prostaglandin E2

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