M2 macrophages induced by mesenchymal stem cells provide an anti-inflammatory milieu that induces regulatory T cells in collagen-induced arthritis mice

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M2 macrophages induced by mesenchymal stem cells provide an anti-inflammatory milieu that induces regulatory T cells in collagen-induced arthritis mice

Directed by Professor Yong-Beom Park

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신 용 대 올림

Table of Contents

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

M2 macrophages induced by mesenchymal stem cells provide an anti-inflammatory milieu that induces regulatory T cells in collagen-induced arthritis mice

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(Directed by Professor Yong-Beom Park)

Objective: Mesenchymal stem cells (MSCs) have immunomodulatory properties. Previous studies have shown the therapeutic effects of MSCs in collagen-induced arthritis (CIA) mice and suggested mechanisms underlying the effects of MSC treatment, including the induction of regulatory T cells (Tregs). Many clinical trials for inflammatory diseases are underway; however, the host immune response to MSCs is not fully understood. Herein, the immunomodulatory properties of human MSCs in macrophages in CIA mice and the mechanism by which MSCs affect macrophages were investigated.

Methods: CIA was induced in DBA/1J mice. Human adipose-derived (hAD)-MSCs

were intraperitoneally injected, and the therapeutic efficacy of hAD-MSCs in CIA mice was evaluated. Peritoneal macrophages (PM) and splenic T cells were also analyzed. Macrophages isolated from DBA/1J mice were co-cultured with hAD-MSCs, and macrophage polarization and cytokine expression were analyzed by FACS and ELISA. Protein and mRNA expression levels were examined by western blot and qPCR.

Results: hAD-MSCs ameliorated the severity of disease in CIA mice by inducing M2 macrophages in the peritoneum. The emergence of M2 macrophages preceded that of Tregs in CIA mice. hAD-MSCs enhanced IL-10 and TGF-β1 expression in M2 macrophages. Moreover, hAD-MSCs decreased RAGE and NF-κB expression, but increased STAT6 expression. STAT5 gene expression in macrophages decreased.

Conclusion: The results showed that hAD-MSCs exerted therapeutic effects by inducing M2 macrophages in CIA mice. hAD-MSCs reduced RAGE and NF-κB expression and modulated the STAT family of macrophages. IL-10 and TGF-β1 produced by the induced M2 macrophages might result in an anti-inflammatory milieu that induces Tregs.

Key words: Mesenchymal stem cells, M2 macrophage, Immune modulation, RAGE

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Ⅰ. Introduction

Mesenchymal stem cells (MSCs) are multipotent stem cells that have the ability to differentiate into several cell types, including osteocytes, chondrocytes, adipocytes, and muscle cells. In the last few decades, many studies have shown that MSCs can differentiate into various cell lineages, as well as their potential for clinical application to repair damaged tissues. More recently, MSCs have been found capable of regulating immune responses, including protecting hematopoietic stem cells from inflammation, inhibiting CD11c+ dendritic cell maturation, causing CD4+ and CD8+ T cells to proliferate, and contributing to B cell function. Additionally, MSCs induce regulatory T cells (Tregs) in vitro and in vivo and suppress the differentiation of T

cells into helper subtypes, namely Th1, Th2, and Th17 cells.[1-5]

Macrophages exist in most tissues and play a key role in regulating innate immunity. Monocytes, which respond to chemokines, can differentiate into macrophages in tissues. Macrophages are required for tissue homeostasis and regulate immune responses by triggering injury resolution, inflammation, and phagocytic clearance of apoptotic cells.[6,7] According to their microenvironment, macrophages have distinct functional phenotypes: classically activated (M1) macrophages and alternatively activated (M2) macrophages[8]. M1 macrophages, induced by interferon-gamma (IFN-γ), tumor necrosis factor (TNF), or lipopolysaccharide (LPS), releases pro-inflammatory cytokines, including TNF-α, interleukin-6 (IL-6), and IL-12, express inducible nitric oxide synthase (iNOS), and promote a Th1 response. On the other hand, M2 macrophages activated by IL-4 and IL-13 express mannose receptor (CD206) and high levels of anti-inflammatory cytokines such as IL-10 and TGF-β1. In addition, M2 macrophages have immunomodulatory functions that contribute to tissue repair and wound healing.[9-14] Kim et al. showed experimentally, for the first time, that MSCs induce M2 macrophages from M1 macrophages [8]. They found that macrophages co-cultured with MSCs highly expressed the M2 phenotype marker and produced high levels of IL-10 and low levels of IL-12 and TNF-α. Later, others reported that MSCs utilize Cox2, PGE2, TSG-6, IL-6, IDO, and TGF-β1 for M2 macrophage polarization.[15-18] However, the detailed mechanism underlying macrophage polarization induced by MSCs is still unknown.

Rheumatoid arthritis (RA) is a systemic autoimmune disease that leads to synovial

inflammation and hyperplasia, cartilage and bone destruction, and extraarticular manifestations. Progression of the autoimmune response implies the development of autoreactive Th1 and Th17 cells, which are able to enter joint tissues and release pro-inflammatory cytokines or chemokines that promote the infiltration of macrophages and neutrophils into target tissues and activate these cells.[19] Excessive levels of inflammatory mediators, cytokines, and extracellular matrix-degrading enzymes produced by infiltrating macrophages play a critical role in joint damage.[20]

The current treatment strategy for RA is tightly controlling disease activity with disease-modifying anti-rheumatic drugs (DMARDs) and biologic agents, including TNF-α blockers, anti-IL-6 receptor blockers, and Janus kinase (JAK) inhibitors. However, they have limitations such as incomplete treatment responses and adverse effects.[21] Accordingly, new treatment methods are needed. Clinical research on human MSCs in RA has been conducted, but the mechanism is still not well defined. Therefore, in the previous study, Park et al. determined the therapeutic effects of MSCs derived from three sources: bone marrow (BM), adipose tissue (AD), and cord blood (CB) in collagen-induced arthritis (CIA) mice and found that MSC treatment decreased pro-inflammatory cytokines and increased anti-inflammatory cytokines and Tregs.[22] However, the role of MSCs in the comprehensive host immune response to CIA remains to be explored.

In the present study, the author hypothesized that intraperitoneally injected hAD-MSCs in CIA mice first encounter macrophages, important innate immune effector cells, and affect immunologic responses by functionally modulating macrophages. To

validate this hypothesis, the immunomodulatory properties of hAD-MSCs on macrophages in CIA mice were analyzed, and the mechanism by which hAD-MSCs affect macrophages was examined.

Ⅱ. Materials and methods

1. Induction and treatment of CIA

7 weeks old male DBA/1J mice (Central Lab. Animal Inc, Korea) were primary immunized through intradermal injection into the tail with 200 μg bovine type II collagen (CII:Chondrex, Redmond, WA, USA) emulsified in Freund's complete adjuvant (1:1, v/v; Chondrex) containing 200 μg Mycobacterium tuberculosis H37Ra (Chondrex). 2 weeks later, the mice were secondly immunized with 100μg CII in incomplete Freund's adjuvant (1:1, v/v; Chondrex).

Treatment was begun after the onset of disease, 5 weeks after the second immunization, and clinical assessment was continued for following approximately 4 weeks. To investigate the therapeutic efficacy of hAD-MSCs and to explore the immune modulatory mechanism of hAD-MSCs, CIA mice were injected intraperitoneally with 100 μl PBS containing 2.5×10^6 hAD-MSCs twice over a 3-day interval (a total of 5×106 cells, n=5). To compare the therapeutic efficacy, 35 mg/kg methotrexate (MTX) was intraperitoneally injected as treatment control mice (n=5) by twice a week for 4 weeks (Figure 1A). Protocols for harvesting hAD-MSCs from patients were approved by the institutional review board (IRB, 4-2010-0236) of Severance Hospital. Informed consent was obtained from all subjects after explanation of the goals and requirements of the study. Mice were monitored twice a week to check the clinical signs of arthritis and to score severity of arthritis using a previously described scoring system indicated by scale from 0 to 4. The arthritis

scores for each paw were summed to yield a total arthritis severity score per mouse. Each paw score was evaluated as follows: score 0= no evidence of erythema and swelling, score 1= erythema and mild swelling confined to the tarsal or ankle joint, score 2= erythema and mild swelling extending from the ankle to the tarsal, score 3= erythema and moderate swelling extending from the ankle to the metatarsal joints, and score 4= erythema and severe swelling encompassing the ankle, foot, and digits or ankyloses of the limb.[23] Clinical scoring was checked by two independent observers. Finally, mice were sacrificed at 1, 2, 3, and 4 weeks after hAD-MSCs injection. The serum, peritoneal macrophages (PM), lymph node, spleen, and limbs of all animals were collected for analysis.

All procedures involving animals were carried out in accordance with the Laboratory Animals Welfare Act, The Guide for the Care and Use of Laboratory Animals, and the Guidelines and policies for Rodent Experiments provide by the institutional Animal Care and Use Committee of Yonsei University Health System, Seoul, Korea. Approval by our institutional review board was obtained (2015-0099, Yonsei University, College of Medicine, Seoul, Korea).

2. Assessment for histological assay of CIA

To evaluate histological score, mice were anesthetized by Zoletil (Virbac, Carros, France) and Rompun (Bayer, Barmen, Germany) and sacrificed for analysis. Formalin (4%, Merck & Co. Inc, NY, USA)-fixed limbs were decalcified in EDTA (30% Merck & Co. Inc) for 4 weeks. Then, tissue sections of 4 um in thickness were

deparaffinized, rehydrated, and washed two times in buffer. To reduce nonspecific background staining due to endogenous peroxidase, the slides were incubated in Hydrogen Peroxide Block for 10 minutes, and washed 4 times in buffer. The primary antibodies hSOX2 (1:200, R&D systems, Minneapolis, MN) were applied and incubated according to the manufacturers' recommended protocols, and the slides were washed 4 times in buffer. The slides were then applied with Primary Antibody Enhancer, incubated for 20 minutes at room temperature and then washed 4 times in buffer. Afterwards, HRP Polymer was applied to the slides and the slides were incubated for 30 minutes at room temperature and washed 4 times in buffer. They were then incubated with hematoxylin for chromogen, washed 4 times in deionized water, and counterstained. To examine morphologic features and determine the histological arthritis score, sections were evaluated histologically and scored for inflammatory cell infiltration, synovial hyperplasia, and bone erosion, according to previously published criteria.[24]

3. Preparation and cultures of peritoneal macrophage

After anesthetizing mice using a mixture of Zoletil and Rompun, 8 ml of ice-cold phosphate-buffered saline (PBS) containing 3% fetal bovine serum (FBS) was injected into the peritoneal cavity, and the peritoneum was gently massaged. After massage, peritoneal fluid was collected using the same syringe, and this fluid was centrifuged at 400g for 5 minutes to collect peritoneal cavity cells. After discarding the supernatant, cells were resuspended with RPMI 1640 medium containing 10%

heat-inactivated FBS, 20 mM HEPES, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1 mM non-essential amino acids, 20 μg/ml gentamycin, and 500 μM 2-mercaptoethanol and were counted using a hemocytometer. Macrophages were adjusted to 1 × 106 cells/well in 6-well plates and incubated at 37℃ for 2 hours. Suspended cells were removed by gently washing three times with warmed PBS.[25] Adherent cells were cultured alone or co-cultured with hAD-MSCs at a ratio of 1:5 for 24 hours and macrophages were characterized by flow cytometric analysis.

4. Flow cytometric analysis

PMs were collected from all experimental animals, and macrophages were cultured for 24 hours. Macrophages were stained with the appropriate antibodies such as CD206-APC (clone: c068c2, Ca: 141708, BioLegend), CD86-PE (clone: GL1, Ca: 553692, BD Pharmingen), CD11b-FITC (clone: M1/70, Ca: 553310, BD Pharmingen), F4/80-PE (clone: T45-2342, Ca: 565410, BD Pharmingen), and F4/80-FITC (clone: BM8, Ca: 123108, BD Pharmingen). CD4+ T cells were obtained from the spleens of all experimental animals by negative selection using a CD4+ T Cell Isolation Kit II (Miltenyi Biotech, German) and then stained with appropriate antibodies, including CD4-FITC (clone RM4-5, Ca: 553047, BD Pharmingen), CD25-PerCP (clone: PC61, Ca: 551071, BD Pharmingen), and FoxP3-PE (clone: NRRF-30, Ca: 12-4771-82, eBioscience). Cells were analyzed using a FACSVerse (BD Pharmingen) and FlowJo Ver10 software (TreeStar, Inc., Ashland, OR, USA).

5. Measurement of cytokines

Serum was isolated from animals in each experimental group, and supernatants were harvested at 24 hours from cultured macrophages that were co-cultured at a 1:5 ratio with hAD-MSCs or without hAD-MSCs and stored at −70°C until analyzed by ELISA. Levels of inflammatory cytokines such as murine IL-1β (Ca: 559603, Lot: 5223506, BD Pharmingen), IL-6 (Ca: 555240, Lot: 2317740, BD Pharmingen), TNF-α (Ca: 558534, Lot: 2324804, BD Pharmingen), and IL-12(p70) (Ca: 555256, Lot: 3119845, BD Pharmingen) in serum and supernatants were measured using a commercially available mouse ELISA kit. Anti-inflammatory cytokines IL-10 (Ca: 88-7105-88, Lot: E17772-105, eBioscience) and TGF-β1 (Ca: 88-8350-88, Lot: E13703-144, eBioscience) were analyzed by ELISA.

6. Western blotting

Macrophages were extracted from CIA mice or harvested from macrophages co-cultured 1:5 with hAD-MSCs at 24 hours. Cells were stored at −70°C until western blot analysis. For the western blot, the following antibodies were used: iNOS (1:100, Ca: ab15323, Abcam), Arg-1 (1:2,000; Ca: 610709, BD Pharmingen), receptor for advanced glycation end products (RAGE) (1:500, Ca: ab30381, Abcam), NF-κB (1:2,000; Ca: #8242, Cell Signaling), and β-actin (1:3,000; Ca: sc-47778, Santa Cruz). Secondary antibodies used were goat anti- rabbit IgG-HRP (1:2,000; Lot # H0614, Ca: sc-2004, Santa Cruz), donkey anti-goat IgG-HRP (1:2,000; Lot # H2113, Ca: sc-2020, Santa Cruz), goat anti-rat IgG-HRP (1:2,000; Lot # E2313, Ca: sc-2006, Santa

Cruz), and goat anti-mouse IgG-HRP (1:2,000; Lot # G1213, Ca: sc-2005, Santa Cruz). Immunoreactivity was determined by an ECL western blotting substrate (Ca: W1001, Promega) and then scanned by Las 4000. The band densities of the target protein relative to those of β-actin were quantified by ImageJ.

7. Real-time PCR

For the real-time PCR analysis, total RNA was extracted from macrophages using a GeneJET RNA Purification Kit (Ca: #K0731, Thermo Scientific), and cDNA was synthesized by Maxime RT PreMix (Ca: 25081, iNtRON) according to the manufacturer's recommendations. Real-time PCR was performed using an AB Applied Biosystems platform with qPCR BIO Screen Mix Hi-ROX (PCR Biosystems), and mRNA expression levels were determined using an ABI Systems 7500 Fast Real-Time PCR System (Applied Biosystems, Weiterstadt, Germany). Then, transcript levels were normalized to those of GAPDH. The primer probe sequences used in the real-time PCR are presented in Table 1.

Table 1. The real time PCR primer sequences

Gene	Forward primer	Reverse primer
Mouse iNOS	CCCTTCAATGGTTGGTACATGG	ACATTGATCTCCGTGACAGCC
Mouse Arg-1	GAACACGGCAGTGGCTTTAAC	TGCTTAGCTCTGTCTGCTTTGC
Mouse RAGE	ACGCAGAAGGACATCAAACC	TCTCCAAGAGGACGACTGG
Mouse CD11b	GGATCATAGGCGCCCACTT	TCCTTACCCCCACTCAGAGACT
Mouse CD86	TTGTGTGTGTTCTGGAAACGGAG	AACTTAGAGGCTGTGTTGCTGGG
Mouse CD206	TCTTTGCCTTTCCCAGTCTCC	TGACACCCAGCGGAATTTC
Mouse STAT1	CAGGAATCTCTCCTTCTTCCTG	TTCAGACCTCTCTTGGTGACTG
Mouse STAT3	GAAACAACCAGTCTGTGACCAG	CACGTACTCCATTGCTGACAAG
Mouse STAT5	ATGGACTCACACCCACAAGGA	CACTGCTACAAGGCTACACAAAACC
Mouse STAT6	GCATTGTTCAGACTTCCTTATGCTT	TGTTGGCTAATACAGCCTGTTCAT
Mouse IL-10	AATAAGAGCAAGGCAGTG	CCAGCAGACTCAATACAC
Mouse TGF-β1	AGCAGTGCCCGAACCCCCAT	GGGGTCAGCAGCCGGTTACC
Human IL-10	CCAAGCCTTGTCTGAGATGA	TGAGGGTCTTCAGGTTCTCC
Human TGF-β1	GAGGTGACCTGGCCACCATT	TCCGCAAGGACCTCGGCTGG

8. Statistical analysis

All values represent the mean ± standard deviation (SD). The statistical significance of differences between two groups were compared using an unpaired Student's t-test, and differences between arthritis scores were analyzed by a two-way ANOVA, followed by post-hoc Bonferroni multiple comparison testing. Data was analyzed using GraphPad Prism 5 software (GraphPad, San Diego, CA, USA), with the level of significance set at $p < 0.05$ (*), $p < 0.01$ (**), or $p < 0.001$ (***).

Ⅲ. Result

1. hAD-MSCs ameliorated the severity of disease in an experimental arthritis model

CIA was induced in DBA/1J mice by immunization with bovine type II collagen and Freund's complete adjuvant. The incidence of arthritis was 95% 9 weeks after the second immunization (Figure 1B). Clinical scores of untreated CIA mice increased beginning 1 week after the second immunization. The severity of arthritis in hAD-MSC-treated mice was gradually attenuated beginning 3 weeks after hAD-MSC injection (Figure 1C). Each foot image was taken at 4 weeks after treatment, and stained histologic slides are shown (Figure 1D). Histological evaluation showed that cell infiltration, synovial hyperplasia, and bone erosion were significantly reduced in mice in the hAD-MSC-treated group compared to those in the untreated group (Figure 1E). Serum cytokine levels in all experimental mice were investigated. Levels of inflammatory cytokines, such as IL-12(p70), IL-6, and TNF-α, decreased significantly in MTX-treated and hAD-MSC-treated mice compared to those in untreated CIA mice. In contrast, levels of anti-inflammatory cytokines IL-10 and TGF-β1 increased in hAD-MSC-treated mice relative to those in MTX-treated mice and untreated CIA mice (Figure 1F).

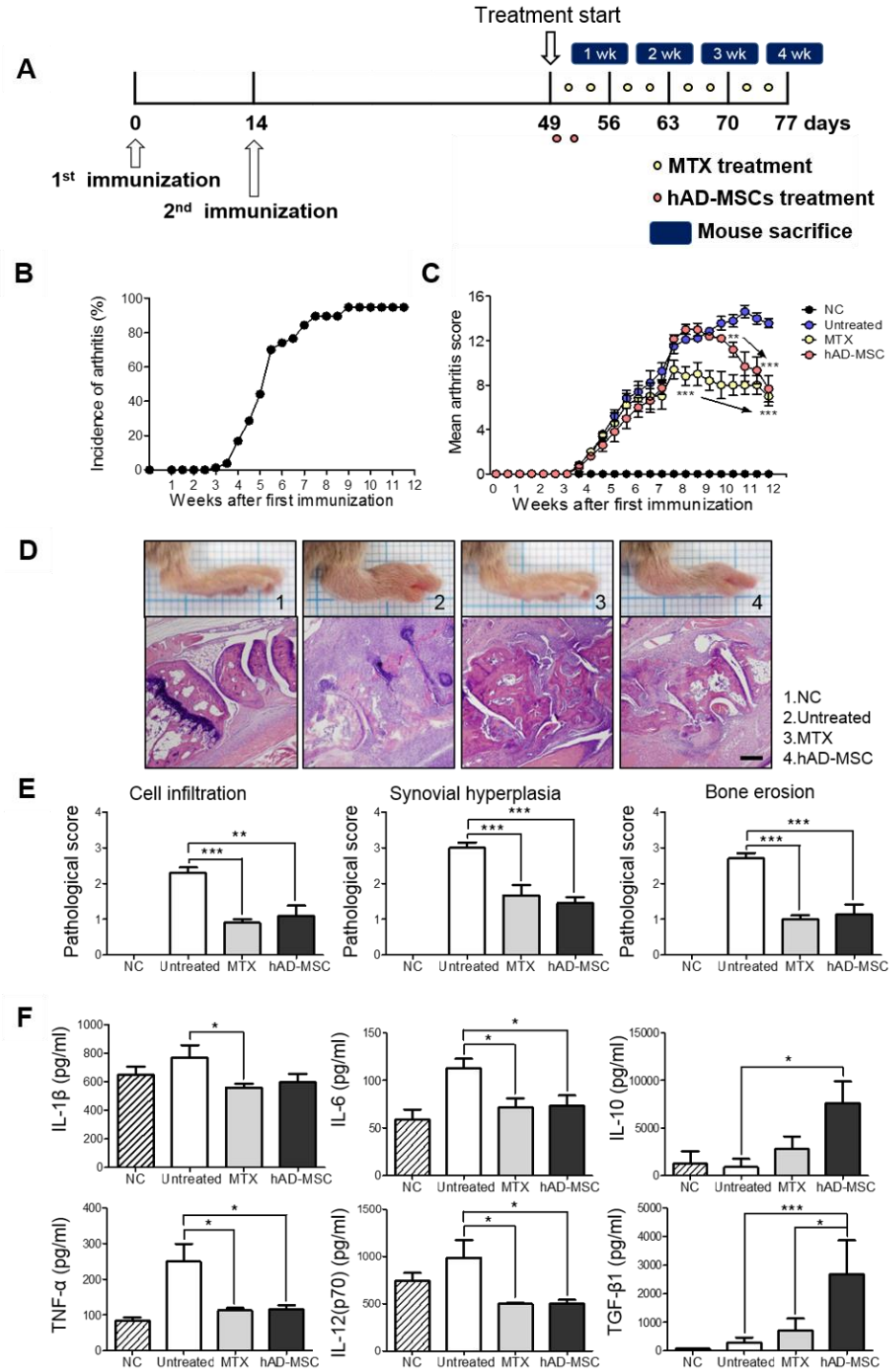

Figure 1. Therapeutic effects of hAD-MSCs in CIA mice. (A) Scheme for inducing CIA mice and the treatment schedule. CIA mice were intraperitoneally injected with 2.5×10^6 hAD-MSCs twice over a 3-day interval and 35 mg/kg MTX twice weekly for 4 weeks for treatment. (B) CIA-induced DBA1/J mice showed an arthritis incidence of 95% (n = 80). (C) Three weeks from treatment, clinical arthritis scores were significantly reduced in hAD-MSC-treated mice compared to those in untreated CIA mice. (D and E) Histological analysis showed severe inflammation of the hind legs of untreated CIA mice. However, mice in the hAD-MSC-treated group showed reduced inflammatory cell infiltration, synovial hyperplasia, and bone erosion compared to those in the untreated group (n = 5 per group). (F) Pro-inflammatory and anti-inflammatory cytokines in serum were measured by ELISA. IL-1β, IL-6, TNF-α, and IL-12(p70) were reduced in hAD-MSC-treated mice and MTX-treated mice. IL-10 and TGF-β1 were significantly increased in hAD-MSC-treated mice relative to those in MTX-treated mice and untreated CIA mice (n = 5 per group). NC, normal control; untreated, untreated CIA mice; MTX, MTX-treated CIA mice; hAD-MSC, hAD-MSC-treated CIA mice. Scale bars: 200 μm. *, $p < 0.05$, **, $p < 0.01$, or ***, $p < 0.001$ versus untreated CIA mice. Values represent the means ± SD.

2. hAD-MSCs induced M2 macrophages in the peritonea of CIA mice

Next, the relative expression levels of selected inflammation-associated proteins in PMs were analyzed at 4 weeks after treatment. iNOS expression in M1 macrophages decreased slightly in the hAD-MSC-treated group. In contrast, Arg-1 expression in

M2 macrophages was highly increased in the hAD-MSC-treated group. Moreover, expression of RAGE and NF-κB in PMs was reduced in hAD-MSC-treated CIA mice (Figure 2A and 2B). It was confirmed that M2 macrophages were induced in the peritoneal cavities of CIA mice.

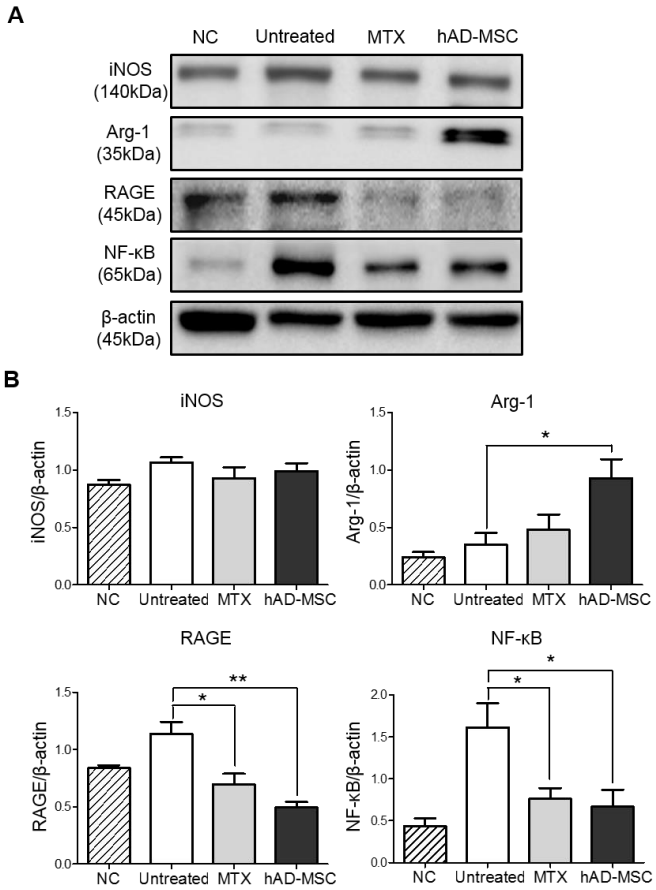

Figure 2. Induction of M2 macrophages following administration of hAD-MSCs in CIA mice. Levels of proteins, including iNOS, Arg-1, RAGE, and NF-κB, in PMs were determined in CIA mice. (A) Western blot bands. (B) Quantification of each protein normalized to β-actin. Arg-1 was significantly increased and RAGE and NF-

κB were decreased in hAD-MSC-treated CIA mice. NC, normal control; untreated, untreated CIA mice; MTX, MTX-treated CIA mice; hAD-MSC, hAD-MSC-treated CIA mice. *, $p < 0.05$ or **, $p < 0.01$ versus untreated CIA mice. Values represent the means ± SD (n = 4 per group).

3. hAD-MSCs induced M2 macrophages prior to induction of Tregs in CIA mice

The proportions of M1 and M2 macrophages in peritoneal cavities and Treg cells in spleens of CIA mice were evaluated at different time points after the administration of hAD-MSCs. Macrophages were gated on CD11b+ F4/80+ cells and their immunophenotypes characterized by CD86 and CD206 expression. One week after hAD-MSC treatment, the hAD-MSC-treated mice had fewer F4/80+CD86+ macrophages (M1) and significantly more F4/80+CD206+ macrophages (M2) among peritoneal cavity cells than those of the normal control (NC) or untreated control (Figure 3A and 3C). Meanwhile, an increase of CD4+CD25+Foxp3+ Tregs in spleens was observed 3 weeks after hAD-MSC treatment (Figure 3B and 3D). The results imply that the emergence of M2 macrophages preceded that of Tregs in CIA mice treated with hAD-MSCs.

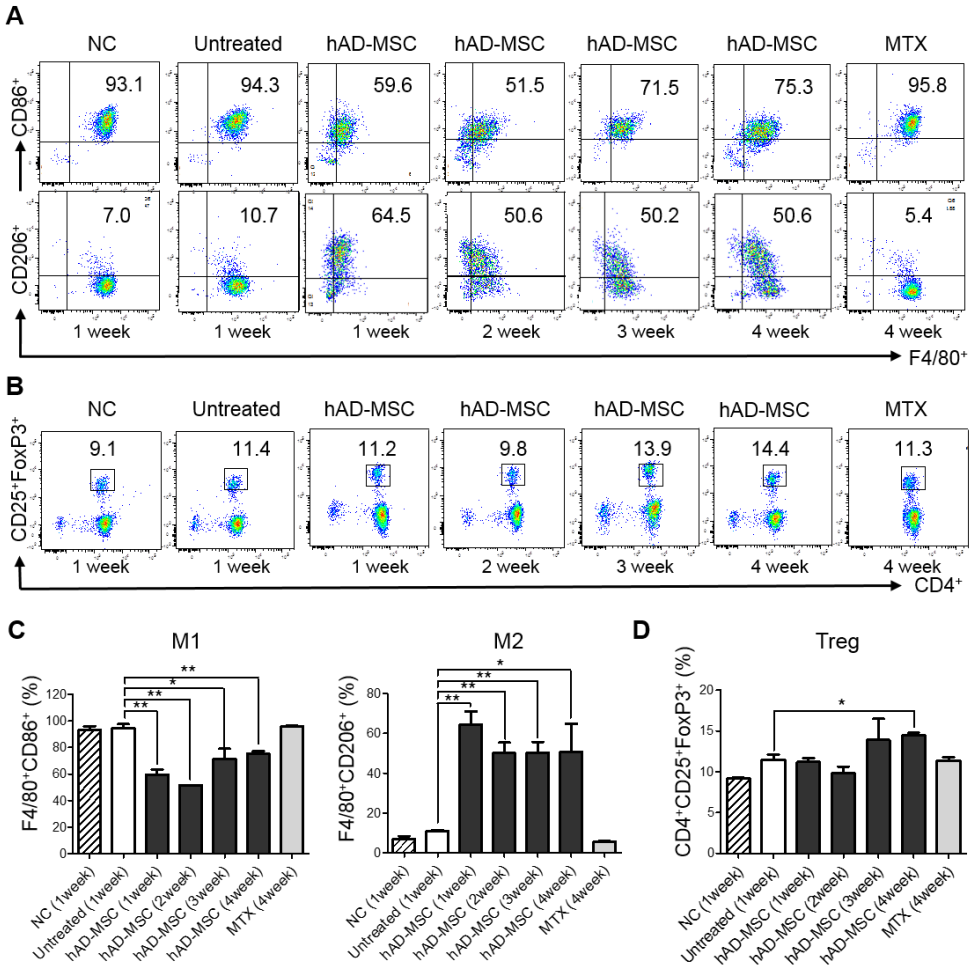

Figure 3. Induction of M2 macrophages prior to Treg induction in CIA mice by hAD-MSCs. The phenotypes of immune cells were evaluated through time after administration of hAD-MSCs. (A) Flow cytometric analysis showed that F4/80+CD86+ expression decreased and F4/80+CD206+ expression increased significantly in cells in the peritoneal cavities of CIA mice 1 week after hAD-MSC treatment compared to those in untreated and MTX-treated mice. (B) An increase in CD4+CD25+FoxP3+ Tregs in spleens was observed 3 weeks after hAD-MSC

treatment. (C and D) Quantitative analysis of F4/80+CD86+, F4/80+CD206+ (C), and CD4+CD25+FoxP3+ expression (D). NC, normal control; untreated, untreated CIA mice; MTX, MTX-treated CIA mice; hAD-MSC, hAD-MSC-treated CIA mice. *, $p < 0.05$ or **, $p < 0.01$ versus untreated CIA mice. Values represent the means ± SD (n = 3 per group).

4. hAD-MSCs enhanced IL-10 and TGF-β1 expression from M2 macrophages *in vitro*

To determine the exact cellular source of anti-inflammatory cytokines detected in serum of hAD-MSC-treated CIA mice, PMs were co-cultured with hAD-MSCs in vitro. First, M2-type macrophage polarization was confirmed by co-culture with hAD-MSCs. Macrophages co-cultured with hAD-MSCs or treated with IL-4 showed significantly decreased CD86 expression and increased CD206 expression (Figure 4A and 4B). IL-4 was used to induce M2 macrophages from PMs as a positive control. Cytokine expression levels in supernatants were investigated. hAD-MSCs elevated the levels of anti-inflammatory cytokines IL-10 and TGF-β1 in macrophages compared to those in PM only or IL-4-treated PM (Figure 4C). Based on the results of qPCR using mouse and human primers to search for the source of IL-10 and TGF-β1, mRNA expression levels in mice were only increased in hAD-MSCs and macrophages in co-culture (Figure 4D).

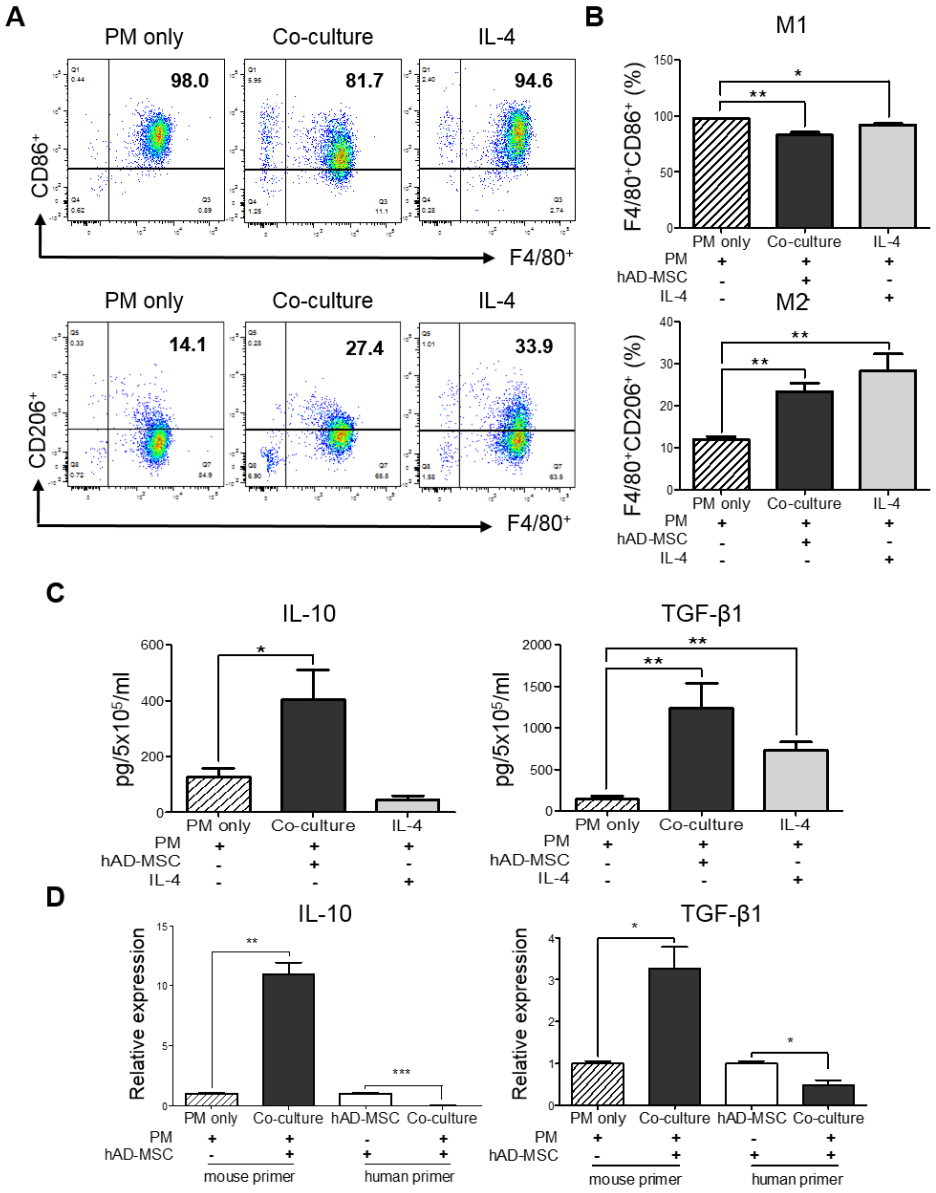

Figure 4. Induction of M2 macrophages that produced anti-inflammatory cytokines in co-culture with hAD-MSCs. PMs were isolated from DBA/1J mouse and co-cultured with hAD-MSCs at a ratio of 1:5 for 24 hours. IL-4 (20 ng/ml) was used to polarize M2 macrophages. (A) Flow cytometric analysis showed that CD86

expression decreased and CD206 expression increased in co-culture conditions (n = 4 per group). (B) Quantitative analysis of F4/80+CD86+ and F4/80+CD206+ expression. (C) Cytokine expression in macrophages was determined from the supernatants of cultured cells. Macrophages co-cultured with hAD-MSCs showed high levels of IL-10 and TGF-β1 compared those in PM only and IL-4-treated PM. (D) qPCR analysis showed that IL-10 and TGF-β1 increased only when mouse primers were used. PMs, peritoneal macrophages; co-culture, macrophages co-cultured with hAD-MSCs at a 1:5 ratio for 24 hours; IL-4, M2 macrophage positive control. *, $p < 0.05$, **, $p < 0.01$ versus PMs only. Values represent means ± SD (n = 4 per group).

5. hAD-MSCs reduced RAGE expression and modulated STAT genes in macrophages in vitro

To understand the molecular mechanism by which hAD-MSCs modulated the M2 macrophage phenotype, macrophages were co-cultured with hAD-MSCs in vitro. Expression levels of a selected set of inflammation-associated proteins were examined by western blot. hAD-MSCs increased Arg-1 and decreased RAGE and NF-κB expression in PMs in vivo (Figure 5A and 5B). In addition, qPCR analysis was performed to evaluate mRNA expression from macrophages. qPCR results showed that expression of Arg-1 increased and that of iNOS and RAGE decreased in a 1:5 co-culture, similar to that observed for protein expression. Furthermore, expression of genes encoding proteins in the STAT family was investigated. hAD-

MSCs significantly increased STAT6 and decreased STAT5 expression in macrophages, inducing M2 macrophage polarization (Figure 5C).

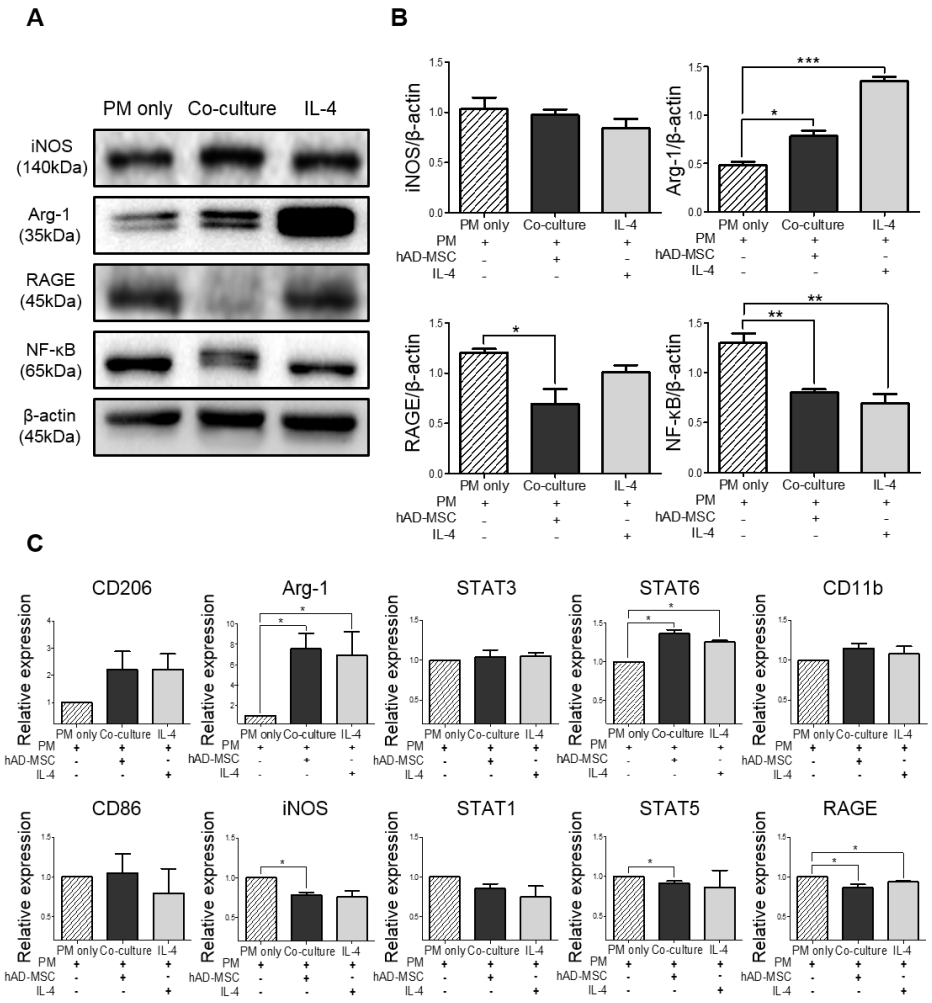

Figure 5. Relative expression levels of selected inflammation-associated proteins and genes in PMs co-cultured with hAD-MSCs. Protein and mRNA expression levels of macrophages co-cultured with hAD-MSCs were investigated. (A) Western blot bands. (B) iNOS was slightly decreased and Arg-1 was increased in a 1:5 co-culture at 24 hours. In addition, hAD-MSCs downregulated RAGE and NF-κB

expression in PMs (n = 4 per group). (C) qPCR analysis showed that hAD-MSCs increased Arg-1 and STAT6 gene expressions and decreased iNOS, RAGE, and STAT5 gene expressions in PMs in co-culture conditions. PMs, peritoneal macrophages; co-culture, macrophages co-cultured with hAD-MSCs at a 1:5 ratio for 24 hours; IL-4, M2 macrophage positive control. *, $p < 0.05$, **, $p < 0.01$, or ***, $p < 0.001$ versus PMs only. Values represent means ± SD (n = 4 per group).

IV. DISCUSSION

MSCs are multipotent stem cells that have the capacity to differentiate into various cell types. Their potent immunomodulatory properties led to their use in treatments for various inflammatory diseases such as Crohn's disease, multiple sclerosis, and RA .[5,26-28] Recently, Park et al. showed the therapeutic effects of MSCs from three different sources in a CIA animal model of arthritis.[22] the optimal cell dosages and injection frequency were determined and the mechanism underlying MSC therapeutic effects mediated by Treg cells in CIA mice was investigated. However, Augello et al. reported that MSCs injected into the peritonea of mice disappeared before Tregs emerged.[29] Therefore, the author hypothesized that hAD-MSCs first contact macrophages, crucial innate immune effector cells, when hAD-MSCs are injected into the peritonea of CIA mice, and that hAD-MSCs modulate macrophage function and the affected macrophages induce Tregs. Thus, the immune modulatory properties of hAD-MSCs on macrophages in the peritonea of CIA mice were analyzed, and the mechanism underlying the effects of hAD-MSCs on macrophages was investigated in the present study.

This study showed that hAD-MSCs had a therapeutic effect on CIA mice. hAD-MSC treatment attenuated the severity of arthritis, including reducing inflammatory cell infiltration, synovial hyperplasia, and bone erosion in joints. Serum levels of inflammatory cytokines such as IL-1β, TNF-α, IL-12(p70), and IL-6 were decreased in hAD-MSC-treated mice and MTX-treated mice compared to those in untreated CIA mice. In contrast, anti-inflammatory cytokines IL-10 and TGF-β1 were

significantly increased in hAD-MSC-treated mice relative to those in MTX-treated mice, but not in untreated CIA mice. IL-1β and TNF-α are key cytokines in chronic joint inflammation because of their role as inflammatory mediators and participation in cartilage destruction. IL-12(p70) can induce the production of TNF-α and IL-1β in a T cell-independent manner, enhancing disease severity in CIA mice. IL-6 is secreted by T cells and macrophages to stimulate immune responses. In addition, IL-10 and TGF-β1 downregulate inflammatory responses by upregulating an inhibitor of IL-1β and TNF-α and either decreasing T cell proliferation or increasing Treg cell numbers, respectively.[30] Therefore, decreased levels of pro-inflammatory cytokines and increased levels of anti-inflammatory cytokines in CIA mice alter the microenvironment, which affects the homeostatic regulation of autoreactive T cell and induces Tregs in vivo.

The relative protein expressions of Arg-1 and iNOS from macrophages in peritoneal cavities were examined in CIA mice. Expression of Arg-1 was highly increased and that of iNOS was decreased in PMs of hAD-MSC-treated mice for M2 macrophage induction. Moreover, to examine how hAD-MSCs affected immune cells in CIA mice, subsets of macrophages and Tregs in CIA mice were investigated at different time points after administration of hAD-MSCs. One week after hAD-MSC treatment, the number of M2 macrophages began to increase. At 3 weeks after treatment, clinical scores began to decrease, and the number of Tregs increased. This study showed that injected hAD-MSCs initially transformed macrophages into M2 macrophages, causing an anti-inflammatory milieu to develop before the induction of Tregs. Savage

ND at al. reported that Treg-induced M2 macrophages can suppress T cell proliferation. Their mechanism of suppression is cell-cell-dependent contact, which is mediated by membrane-bound TGF-β1 expressed on Tregs.[31] Therefore, these results indicated that M2 macrophages induced by hAD-MSCs induce Tregs, suppressing T cell proliferation to attenuate the severity of arthritis in CIA mice.

To explore the mechanism by which hAD-MSCs induced M2 macrophages, PMs isolated from DBA/1J mouse were co-cultured with hAD-MSCs at a ratio of 1:5 for 24 hours. CD86 expression on macrophages co-cultured with hAD-MSCs decreased, but CD206 expression increased. In addition, macrophages co-cultured with hAD-MSCs showed significantly elevated levels of anti-inflammatory cytokines such as IL-10 and TGF-β1. To determine the cells that secreted anti-inflammatory cytokines, a qPCR analysis was conducted using mouse and human primers. In this experiment, when mouse primers were used, mRNA expression levels of IL-10 and TGF-β1 were only increased in the co-culture conditions. This study confirmed that the elevated anti-inflammatory cytokines originated not from human MSCs but from mouse PMs. Therefore, the study clearly showed that increased anti-inflammatory cytokines in co-cultures were derived from PMs.

In addition, protein and mRNA expression levels in macrophages co-cultured with hAD-MSCs were examined. Previous reports explained that the mechanisms underlying induction of M2 macrophages by MSCs involve Cox2, PGE2, TSG-6, IL-6, IDO, and TGF-β1, all of which affect M2 macrophages polarization.[15-18] This study showed that hAD-MSCs significantly increased STAT6 and decreased STAT5 gene

expression in macrophages for M2 macrophage polarization. Canonical IRF/STAT signaling is a central pathway in modulating macrophage polarization.[32] STAT5 is a transcription factor that can induce iNOS and IL-12 in macrophages for M1 macrophage activation; in contrast, STAT6 activates M2 macrophages that can transcribe Arg-1 and CD206.[32] However, the exact mechanism by which MSCs regulate the STAT family of macrophages has not been reported. Thus, this study provided evidence for the first time that hAD-MSCs regulate the STAT gene family to induce M2 macrophages.

Furthermore, RAGE expression in macrophages was investigated. RAGE is expressed on cell surfaces and is one of the superfamilies of immunoglobulins that interact with a wide range of ligands, including high-mobility group box-1 (HMGB-1), amyloid β peptide, S100P, S100A12, and advanced glycation end-products (AGEs). Activation of RAGE induces an immediate inflammatory response, leading to chronic inflammation.[33] A previous study showed that MSCs have therapeutic effects in a hyperoxic lung injury model by inhibiting inflammation, and this correlated with the reduced levels of RAGE and NF-κB signaling in lung tissue.[34] However, in their study, RAGE expression was observed in total proteins extracted from homogenized inflamed lung tissue. Thus, it was unclear which cells caused the decreased expression of RAGE. However, the present study showed that RAGE and NF-κB expression levels decreased in PMs of hAD-MSC-treated CIA mice and macrophages co-cultured with hAD-MSCs in vitro. Therefore, the current study proposes that hAD-MSCs are able to downregulate RAGE and NF-κB expression in

macrophages.

Ⅴ. Conclusion

The results showed that hAD-MSCs exert therapeutic effects by inducing M2 macrophages in CIA mice. hAD-MSCs reduced RAGE and NF-κB expression and modulate the STAT family of macrophages. IL-10 and TGF-β1, produced by induced M2 macrophages, might create an anti-inflammatory milieu that induces Treg cells.

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ABSTRACT (IN KOREAN)

콜라겐 유도 관절염 마우스에서 중간엽줄기세포에 의해 유도된 M2 대식세포가 조절 T 세포를 유도하기 위한 항염증성 환경을 제공함

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신 용 대

목적: 중간엽 줄기세포 (mesenchymal stem cell; MSC)는 다양한 세포로 분화할 수 있는 다분화능 세포로 또 하나의 특별한 능력인 면역조절 능력을 갖추고 있다. 이전연구를 통해 콜라겐 유도 관절염(CIA) 마우스에서 MSC의 치료 효과를 확인하였고, 그 기전으로서 조절 T 세포의 증가를 제시하였다. 다양한 염증성 질환에서 MSC의 치료가 많이 시도되고 있으나, MSC의 면역조절에 대해서는 아직 알려진 바가 많지 않다. 이번 연구를 통해, CIA 마우스에서 MSC가 대식세포에 미치는 영향을 조사하고 그 기전을 알아보고자 하였다.

방법: DBA/1J 마우스에 bovine type Ⅱ collagen과 Freund's complete adjuvant를 사용하여 면역반응을 일으켜 collagen induced arthritis

(CIA)를 유도하고, CIA 쥐에 human adipose derived (hAD)-MSC를 복강으로 주사하여 치료 효과를 확인하였다. 또한, in vitro 상에서 DBA/1J 마우스의 복강 막 세포를 분리하여 hAD-MSC와 1:5 비율로 24시간 동안 공배양하고, hAD-MSC가 대식세포의 아류형에 미치는 영향은 유세포분석을 통해 확인하고, 쥐의 혈청과 공배양한 배지에서 사이토카인 발현량은 ELISA를 통해 확인하였다. 그리고 단백질과 mRNA의 발현량은 각각 western blot과 qPCR을 통해 확인하였다.

결과: hAD-MSC는 복강 내의 M2 대식세포를 유도함으로써 CIA 마우스의 질병 도를 개선했다. M2 대식세포의 증가는 Treg의 증가 이전에 일어난다. hAD-MSCs는 M2 대식세포의 IL-10과 TGF-β1의 분비량을 증가시켰다. 게다가 hAD-MSC는 대식세포의 RAGE와 NF-κb의 발현을 감소시키고 STAT gene을 조절함을 알 수 있었다.

결론: 이번 연구를 통해 CIA 마우스에서 hAD-MSC가 M2 대식세포를 유도함을 통해 치료 효과가 나타남을 알 수 있었다. hAD-MSC는 대식세포의 RAGE와 NF-κB 발현을 감소시켰다. 유도된 M2 대식세포는 IL-10과 TGF-β1을 분비함으로써 조절 T세포를 유도하는 항염증성 환경을 만들어 가는 것으로 생각된다.

핵심되는 말: 중간엽 줄기세포, M2 대식세포, 면역조절, RAGE.