Investigation for immunomodulatory and anti-inflammatory mechanisms of mesenchymal stem cells on T cells and dendritic cells in CIA mice

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Investigation for immunomodulatory and anti-inflammatory mechanisms of mesenchymal stem cells on T cells and dendritic cells in CIA mice

Directed by Professor Yong-Beom Park

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Abstract

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Mesenchymal stem cells (MSCs) have profound immunomodulatory properties. Using their properties, MSCs-based therapies have been applied in several inflammatory diseases. The immune modulation of MSCs is related to inhibition of proliferation, differentiation, and activation of various immune cells. Dendritic cells (DCs) play pivotal roles in initiating immune response as DCs are important antigen-presenting cells. DCs are able to mature into inflammatory DCs, sustaining a continuous activation of adaptive immune system. T cells are critical effector immune cells in affecting and regulating immune response to collect signals from DCs. T cells can differentiate into one of several subtypes, including T_H1, T_H2, T_H17 or regulatory T cells

(Tregs), toward different functional subsets. As the effects of MSCs on DCs and T cells have not been fully understood, the immunomodulatory mechanisms of MSCs on DCs and T cells were investigated.

Bone marrow-derived $CD11c^+$ mononuclear cells of DBA/1 mice were differentiated cultured with GM-CSF and IL-4, to immature DCs (iDCs) for 6-7 days. $CD4^+$ T cells were isolated from spleen of DBA/1 mice by negative selection using $CD4^+$ T cell isolation kit. The iDCs were maturated using lipopolysaccharides (LPS), and $CD4^+$ T cells were activated under anti-CD3 and anti-CD28 treatment. Human bone marrow-derived MSCs (BM-MSCs) were co-cultured with mouse DCs and $CD4^+$ T cells directly in the different ratios. The effect of BM-MSCs on DC maturation and T cell differentiation was assessed by maturation markers by flow cytometry, and supernatants for induced production of cytokines were also analyzed. Gene expressions were analyzed by quantitative RT-PCR and microarray. In addition, immunohistochemistry was performed in inflammatory tissues in collagen-induced arthritis (CIA) mice.

Human BM-MSCs significantly inhibited the DCs maturation by decreasing CD86 and major histocompatibility complex (MHC)Ⅱ expressions in both 1:1 and 1:10 co-culture ratio. BM-MSCs decreased the levels of interleukin (IL)-12p70, tumor necrosis factor (TNF)-α, and IL-6 effectively, and increased the levels of IL-10 and transforming growth factor (TGF)-β. Also, the relative gene expressions of indoleamine 2, 3-

dioxygenase (IDO) and heme oxygenase (HO)-1 were increased in 1:1 co-culture ratio at 6 hr. As for T cells, the expression of $CD4^+CD25^+FoxP3^+$ Tregs were highly induced in co-culture condition with BM-MSCs. Human BM-MSCs significantly induced the $CD4^+CD25^+FoxP3^+$ Tregs by increasing programmed death-1 (PD-1) and neuropilin-1 (Nrp-1) expressions, and increased the levels of IL-10 and TGF-β. However, BM-MSCs did not induce the $FoxP3^+$ Tregs in the splenic $CD4^+$ T cells from PD-$1^{-/-}$ mice. Moreover, immunohistochemical analysis of inflamed tissues in BM-MSC treated CIA mice showed significant immunopositive staining for PD-1 on $CD4^+$ T cells.

The results demonstrate that human BM-MSCs inhibit the maturation of DCs and induce anti-inflammatory signals of DCs in *in vitro*. In addition, these data show that human BM-MSCs highly induce the $CD4^+CD25^+Foxp3^+$ Tregs leading upregulation of PD-1 in both *in vitro* and *in vivo.*

Key words: mesenchymal stem cell, immune modulation, dendritic cell, regulatory T cell

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Ⅰ. INTRODUCTION

Mesenchymal stem cells (MSCs) are multipotent progenitor cells that can differentiate into tissues of mesenchymal lineage, including bone, cartilage, and adipose tissue. Most studies of MSCs have focused on the regenerative properties of the cells; however, MSCs also have unique immunoregulatory properties[1-3]. MSCs have potent immunoregulatory effects that might be mediated through direct cell-to-cell contact or secretion of soluble factors. The relative ease of harvesting MSCs and their stable phenotype in culture make the cells an attractive tool for cellular therapy in alloimmunity, autoimmunity, and inflammation. This phenomenon has led to an increasing number of clinical trials, such as in graft-versus-host disease, Crohn's disease,

and multiple sclerosis[4,5].

Mesenchymal stem cells have intermediate levels of HLA major histocompatibility complex (MHC) class I molecules, but do not have HLA class II antigens, FAS ligand, and the co-stimulatory molecules[3,4]. Several articles indicate that T-cell proliferation induced with specific antigens or polyclonal mitogens and allogeneic cells is inhibited by MSCs[6-8]. However, the induction of immune responses is a complicated interaction between T cells and DC, and it has been shown that MSC have inhibitory effects on DC [9-11]**.** However, the precise immunosuppressive mechanisms employed by MSC are not well understood.

T cells play important roles in affecting and regulating immune response as T cells are critical effector immune cells. T cells can differentiate into one of several subtypes, including T_H1, T_H2, T_H17 or regulatory T cells (Tregs), toward different functional subsets. Recently, human MSCs induced $Foxp3^+$ Tregs population in inflammatory disease of animal models according to previous reports[10-12]. However, the immunomodulatory mechanisms of MSCs on Tregs induction remain unclear.

Dendritic cells (DCs) play pivotal roles in initiating immune response as DCs are important antigen-presenting cells[13]. DCs are able to mature into inflammatory DCs, sustaining a continuous activation of adaptive immune system. Human MSC decreased the maturation of DCs in vitro previously[10,11]. However, the effects and mechanisms of MSCs on DC maturations and function have not been fully understood.

In this study, it was investigated the immunomodulatory property of MSCs on DCs maturation and differentiation and the immunosuppressive mechanisms of MSCs on helper T cells and Tregs in collagen-induced arthritis (CIA) mice in *in vitro* and *in vivo*.

Ⅱ. MATERIALS AND METHODS

1. Animals

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 provided by the Institutional Animal Care and Use Committee of Yonsei University Health System, Seoul, Korea. Approval by our institutional review board was obtained (2013-0014-1 and 2010-0372-1, Yonsei University, College of Medicine, Seoul, Korea).

2. Culture of human bone marrow derived-mesenchymal stem cells (hBM-MSCs)

All frozen stocks of MSCs were provided at passage 1 or 2 by the Cell Therapy Center, Severance Hospital (Yonsei University, College of Medicine, Seoul, Korea) after obtained with donor consent. Cells were plated at a density of 1×10^6 cells per 10-cm cell culture dish (BD Biosciences) with Dulbecco's modified Eagle medium (low glucose) supplemented with 10% fetal bovine serum, 1% glutamine, 1% non-essential amino acids, 0.01% 2-mercaptoethanol, and 1% penicillin-streptomycin (all from Invitrogen). MSCs were cultured at 37°C in an atmosphere containing 5% CO_2, and the medium was changed every 3–4 days. All MSCs were cultured until passage 5-6, harvested, and stored in liquid nitrogen until use.

3. CD11c+ dendritic cells (DCs) and CD4+ T cell isolation and co-culture with hBM-MSCs

Bone marrow-derived $CD11c^+$ mononuclear cells of DBA/1 mice (at 6 wk) were cultured in the presence of GM-CSF and IL-4 to immature DCs (iDCs) for 7 days. The iDCs were maturated using lipopolysaccharides (LPS). Human BM-MSCs and mouse

DCs were co-cultured directly with 1:1 and 1:10 ratio. CD4+ T cells were isolated from spleen of DBA/1 mice (at 6 wk) by negative selection using CD4+ T Cell isolation kit Ⅱ (Miltenyi Biotech, German) and then purified CD4+ T cells were cultured in complete RPMI medium containing 10 % of heat-inactivated FBS, 20 mM HEPES, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 ug/ml streptomycin, 1 mM non-essential amino acids, 20 ug/ml Gentamycin and 500 uM β-mercaptoethanol. CD4+ T cells were cultured alone or added at the ratio 1:5 , 1:20, 1:100 in 12 well plate which is coated $1x10^5$ hBM-MSCs and stimulated with 2 ug/ml anti-mouse CD3e/CD28 (BD pharmigen) for 24, 48, 72 hr, and then cell pellet stored at deep freezer still used.

4. Fluorescence activated cell sorting (FACS)

After 6, 18, 24, 48 and 72hr of culture, DC were obtained and after 72hr of culture, CD4+ T cells were obtained at stimulation for 5 hr with 50 ng/ml phorbolmyristate acetate (PMA) and 750 ng/ml Ionomycin and 1 ug/ml Golgi plug. For cell surface antigen stainings, DC and CD4+ T cell were stained by the appropriate antibodies were added and incubated in 100ul suspension for 30 min at 4 ℃. After washing steps, we performed intracellular staining. For that purpose, CD4+ T cells were fixed and permeabilized by Foxp3 Fixation/Permeabilization working solution for 60 min in the dark at 4 ℃. After washing appropriate antibodies were added for Foxp3 staining and incubated in 100ul suspension for 30 min at 4 ℃. The following antibodies were used for T cell experiments: CD4-FITC (clone RM4-5, Ca: 553047, BD Pharmigen), CD25-PerCP (clone PC61, Ca: 551071, BD Pharmigen), IFN-r-PE (Ca: 554412, BD Pharmigen), IL-4-PerCP (clone 11B11, Ca: 560700, BD Pharmigen), IL-17A-PE (Ca: 559502, BD Pharmigen), PD-1-APC (clone J43, Ref: 17-9985-80, eBioscience) and Nrp-1-PE/Cy7 (clone 3E12, Ca: 145212, Biolegend), FoxP3-PE (eBioscience). The

following antibodies were used for DC experiments: CD11C-APC (clone HL3, Ca: 550261, BD Pharmigen), CD86-PE (clone GL1, Ca: 553692, BD Pharmigen) and MHC Ⅱ-FITC (clone 2G9, Ca: 553623, BD Pharmigen). Cells were analyzed on FACS Verse (BD Pharmigen). Data was analyzed by FlowJo Ver10.

5. Enzyme-linked immunosorbent assays (ELISAs)

DC and T cells were co-culture with MSC at the different ratio or without MSC, supernatants were collected on 24, 48, and 72hr. And the amounts of secreted cytokines including murine IL-12p70, IL-6, TNF-α, IL-10 and TGF-β1 in the supernatants were measured using an ELISA kit (eBioscience, BD Pharmigen) according to manufacturer's instructions.

6. RT-PCR and real-time PCR

RNA was isolated using GeneJET RNA purification kit (#K0731, Molecular Biology), and RNA concentration was determined using a Nano-DropND1000 spectrophotometer (Labtech International, East Sussex, UK). And cDNA was synthesized using Maxime RT PreMix (iNtRON, Ca: 25081) as follows: an initial denaturation step (at 94℃ for 3 min), 35 cycles of PCR (95℃ for 30 s, 50℃ for 30 s, 72℃ for 15 s) then a final step at 72℃ for 10 min, using the SimpliAmp™ Thermal cycler (Applied Biosystems, Weiterstadt, Germany). Real-time PCR was performed on an ABI Systems 7500 Fast machine (Applied Biosystems, Weiterstadt, Germany) by using qPCRBIO syGreen Mix Hi-ROX purchased from PCRBIOSYSTEMS, according to the manufacture's recommendations. Amplification conditions were 95℃ for 30 s, followed by 40 cycles at 95℃ for 5 s and 60℃ for 30 s. Relative transcript expression was normalized against GAPDH transcript, where Δ cycle threshold represents the difference in the cycle

threshold values between the target gene and the housekeeping gene. The resulting cycle threshold (Ct) value was processed based on the comparative Ct method, where *gapdh* was used as an endogenous reference gene to normalize the expression level of other genes. Primer sequences for detection of *ctla-4, foxp3, pd-1* and several genes were previously published and as follows (Table 1).

Table 1. The real time PCR primer sequences

	forward	reverse
T-bet	CAACCAGCACCAGACAGAGA	ACAAACATCCTGTAATGGCTTG
Rorγ-t	CAAATACGGTGGTGTGGAG	ACGGTTGGCATTGATGAG
Gata-3	TGACGGAAGAGGTGGACG	CTGGCTCCCGTGGTGG
Pd-1	TTCAGGTTTACCACAAGCTGG	TGACAATAGGAAACCGGGAA
Nrp-1	GAAGGCAACAACAACTATGA	ATGCTCCCAGTGGCAGAATG
Ctla-4	ACCCCTCACAATCACTGTCC	CACCTGCAGGAAGAACTGGT
Lag-3	TCCGCCTGCGCGTCG	GACCCAATCAGACAGCTTGAGGAC
Tgf-β1	AGCAGTGCCCGAACCCCCAT	GGGGTCAGCAGCCGGTTACC
Il-10	AATAAGAGCAAGGCAGTG	CCAGCAGACTCAATACAC
Ido	GTACATCACCATGGCGTATG	CGAGGAAGAAGCCCTTGTC
Ho-1	CTGCTAGCCTGGTGCAAGATACT	GTCTGGGATGAGCTAGTGCTGAT
Pd-l1	GCTGAAGTCAATGCCCCATA	TCCACGGAAATTCTCTGGTTG
Icos	TGA CCC ACC TCC TTT TCA AG	TTA GGG TCA TGC ACA CTG GA
Gapdh	GTGTTCCTACCCCCAATGTGT	ATTGTCATACCAGGAAATGAGCTT

7. Western blot analysis

Cells were collected at 24, 48 and 72 hr and stored at deep freezer for western blot analysis. Procedures for extraction of cellular proteins, T cells were lysed in RIPA Buffer (Lot#R0214711K) with protease inhibitor cocktail tablet (Roche) for 30 min on ice and then using a sonicator for completed lysis. The protein concentrations were measured using the Pierce™ BCA Protein Assay Kit (Ca: 23225 Thermo Scientific) and equalized protein samples were used for the electrophoresis. The proteins were then transferred to polyvinylidene difluoride (PVDF) membrane. The PVDF membranes were blocked in 5% non-fat milk in Tris buffered solution (TBS) for 1 hr. After washing steps, incubated with anti-mouse antibodies during overnight at 4℃, including PD-1, NRP-1, Foxp3 and β-actin at dilutions (1:2,000). The membrane was washed three time with TBS and incubated with appropriate different secondary antibodies (1:3,000), goat anti- rabbit IgG-HRP (Lot # H0614), donkey anti- goat IgG-HRP (Lot # H2113), goat anti-rat IgG-HRP (Lot # E2313), goat anti-mouse IgG-HRP (Lot # G1213) from Santa Cruz Biotechnology. The immunoreactivity was determined using an ECL Western Blotting Substrate (REF W1001, Promega) and then exposure Las 4000. Then, the relative band density of the target protein to β-actin was quantified with image J.

8. T cell proliferation assay

CD4+ T cells were prepared and cultured in 12-well plates (5×10^5 cells/well) in presence of hBM-MSC (1×10^5 cells/well) for 72 hr. T cells were stained with 5 mM carboxyfluorescein diacetate succinimidyl ester (CFSE; eBioscience). Anti-CD3/CD28 antibodies (5 ug/ml; BD Biosciences, San Jose, CA, USA) were suspended in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) as stimulator.

Proliferation was measured by the reduction in the CFSE concentration using flow cytometry (FACS Verse, BD).

9. Inhibitor study

CD4+ T cells were isolated from spleen of PD-1 knockout mice provided from Dr. Sang-Jun Ha (Yonsei University) to determine the involvement of PD-1 to induce Foxp3+ Tregs in this study. CD4+ T cells were cultured alone or added at the ratio 1:5 in 12 well plate which is coated $1x10^5$ hBM-MSCs and stimulated with 2 ug/ml anti-mouse CD3e/CD28 (BD pharmigen) for 72 hr, and then the expression of Foxp3+ Tregs was analyzed by FACS (FacsVerse, BD Pharmigen).

10. DC function assays

5 x 10^5 DCs were co-cultured with MSCs in 12-well flat-bottomed plates for 6 hr. Then, CFSE labeled-CD4+ T cells were added at 1:10 ratio (DC: T) in MSC-DC co-cultured plates for 72hr. Proliferation of CD4+ T cells and Tregs expression were measured by flow cytometric analysis.

11. Microarray

750 ng of labeled cRNA samples were hybridized to each Mouse WG6 expression v.2 bead array for 16-18 hr at 58°C, according to the manufacturer's instructions (Illumina, Inc., San Diego, USA). Detection of array signal was carried out using Amersham fluorolink streptavidin-Cy3 (GE Healthcare Bio-Sciences, Little Chalfont, UK) following the bead array manual. Arrays were scanned with an Illumina bead array Reader confocal scanner according to the manufacturer's instructions.

The quality of hybridization and overall chip performance were monitored by visual

inspection of both internal quality control checks and the raw scanned data. Raw data were extracted using the software provided by the manufacturer (Illumina GenomeStudio v2011.1 (Gene Expression Module v1.9.0)). Array probes transformed by logarithm and normalized by quantile method.

Statistical significance of the expression data was determined using LPE test and fold change in which the null hypothesis was that no difference exists among groups. False discovery rate (FDR) was controlled by adjusting p value using Benjamini-Hochberg algorithm. For a DEG set, Hierarchical cluster analysis was performed using complete linkage and Euclidean distance as a measure of similarity. Gene-Enrichment and Functional Annotation analysis for significant probe list was performed using DAVID (http://david.abcc.ncifcrf.gov/home.jsp). All data analysis and visualization of differentially expressed genes was conducted using R 3.0.2 (www.r-project.org).

12. Induction of CIA

Male DBA/1J mice (8 wk old; Central Lab Animal, Inc., Seoul, Korea) were injected intradermally at the base of 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 wk later the mice were given intradermal booster injections of 100 μg CII in incomplete Freund's adjuvant (1:1, v/v; Chondrex). Mice were monitored twice weekly for signs of arthritis based on paw swelling and arthritis scores[14]. Clinical arthritis was scored on a scale of 0–4. Each paw was graded and the grades were summed to yield the arthritis score for each animal (maximum possible score, 16). Arthritis scoring was performed by two independent observers. Mice were randomly distributed to each treatment group (n=5) at 1 wk after 2nd booster injection of CII when arthritis scores were between 4 and 6.

13. Treatment protocol for CIA

Treatment was begun after the onset of disease, when arthritis had become well established, approximately 3 wk after the primary immunization, and clinical assessment was continued for following 4 wk. CIA mice were injected intraperitoneally daily with 100 μl phosphate-buffered saline (PBS control, n=5; Invitrogen, Carlsbad, CA, USA) and 100 μl PBS containing 2.5×10^6 BM-MSCs twice over a 3-day interval (total 5×10^6 cells, n=5 per each group). To compare therapeutic efficacy, treatment-control mice (n=5) were injected intraperitoneally with 35 mg/kg methotrexate (MTX) twice weekly for 4 wk. To assess time-dependent changes in therapeutic responses, twenty mice were enrolled, and five mice were sacrificed according to the experimental schedule every week. Finally, all mice were sacrificed 28 days after final MSC injection. The serum, lymph nodes, spleen, and limbs of all animals were collected for analysis.

14. Histological and immunohistochemical assessments of CIA

At day 28 from final MSCs injection, mice were anesthetized with a standard dose of Zoletil (Virbac, Carros, France) and Rompun (Bayer, Barmen, Germany) and euthanized for analysis. Formalin (10%, Merck & Co. Inc, NY, USA)-fixed limbs were decalcified in formic acid (5 %, Sigma) for 2-3 wk and embedded in paraffin. Serial 4-μm sections were cut and stained with specific antibodies directed against murine tumor necrosis factor-α (TNF-α; 1:500; Hycult Biotechnology, Uden, Netherlands), interleukin (IL)-1β (1: 500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), IL-6 (1: 250, Santa Cruz Biotechnology), IL-10 (1:1,000; Abcam, Cambridge, UK) and programmed cell death (PD)-1 (1:500, Biolegend) followed by horseradish peroxidase-conjugated secondary antibodies and staining with diaminobenzidine (DAKO, Glostrup,

Denmark). Antigen retrieval process was done by microwave oven heating and enzyme digestion using protease (Sigma-Aldrich) before the application of the primary antibodies. Expression of markers in the synovial tissues of paw and knee joints was scored semiquantitatively on a four-point scale (0-3) independently by two blinded observers, and the average scores were calculated[15].

15. Confocal microscopy

Nonspecific binding was blocked with 1% normal goat serum in PBS with Tween-20 (Sigma-Aldrich) for 30 min. The sections were incubated with goat anti-mouse CD3 (1:500; Abcam) and rat anti-mouse PD-1 (1:500; Biolegend) at 4 ℃ for overnight, washed 3 times with PBS, incubated with Alexa Fluor 488-labeled donkey anti-goat IgG and Alexa Fluor 568-labeled donkey anti-rat IgG (each 1:200; Invitrogen) at room temperature for 1 h, and washed 3 times with PBS. All sections were mounted in a mounting medium (Vector Laboratories) and examined under a laser scanning confocal microscope (LSM 700; Carl Zeiss). Two pathologists independently assigned semiquantitative scores for the intensity and distribution of immunofluorescence staining for IgG and C3, on a scale ranging 0 - 3 for staining intensity, in which 0 = no staining, 1 = weak staining, 2 = moderate staining, and 3 = strong staining. The average score from the 2 pathologists was calculated.

16. Tregs suppression assay

To measure the suppressive capacity of Tregs generated in the presence of MSCs, $CD4^+CD25^+$ Tregs and $CD4^+$ T cells were isolated from spleens and stained with 5 mM carboxyfluorescein diacetate succinimidyl ester (CFSE; eBioscience). Anti-CD3/CD28 antibodies (5 ug/ml; BD Biosciences, San Jose, CA, USA) were suspended in RPMI

1640 (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) as stimulator. $CD4^+$ T cells were co-cultured with $CD4^+CD25^+$ Tregs at a ratio of 10:1 (1×10^5 $CD4^+$: 1×10^4 $CD4^+CD25^+$). Suppression of T-cell proliferation was measured by the reduction in the CFSE concentration using flow cytometry (FACS caliber, BD).

17. Statistics

All values represent the mean ± standard deviation (SD) or standard error of mean (SEM) for various experimental groups. Statistical analysis was performed using an unpaired Student t test for comparision between two groups or by a one- or two-way analysis of variance (ANOVA), mixed linear tests (GraphPad Prism 5) followed by a Bonferroni correction for multiple comparisions. P values of $P<0.05$ (*), $P<0.01$ (**) or $P<0.001$ (***) were considered statistically significant.

Ⅲ. RESULTS

Part 1. Immune modulation of hBM-MSCs on T cells

1. hBM-MSCs reduced expression of IL-1β, IL-6, and TNF-α and increased expression of IL-10 in inflamed joints of CIA mice

Immunohistochemical analysis of paw and knee joint tissue in untreated CIA mice showed significant immunopositive staining for IL-1β, IL-6, and TNF-α that was localized primarily in synovium. IL-1β, IL-6, and TNF-α staining were decreased in MSC-treated mice compared to untreated mice. IL-10 expression was significantly induced in hBM-MSC-treated mice (Fig. 1A-B).

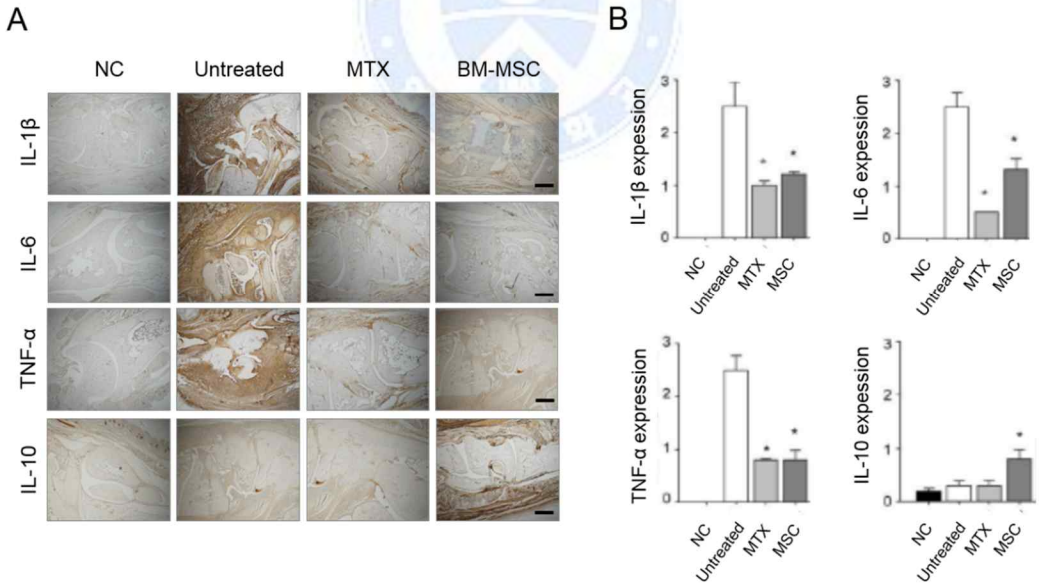


Figure 1. Immunohistochemical analysis of inflammatory cytokines in the joints of CIA mice. IL-1β, IL-6, and TNF-α were highly expressed in the joints of untreated CIA mice.

IL-10 expression was significantly induced in MSC-treated mice. (A) Inflamed tissue were collected from untreated mice, mice treated with hBM-MSCs. (B) Immonopositivity was scored semi-quantitatively on a scale ranging 0 – 3 for staining intensity. All values are the mean ± SD. All scale bars = 200 um. *, $p < 0.05$ versus untreated CIA mice (n=5 per each group).

2. hBM-MSCs induced Tregs expression in mice with CIA

To investigate Tregs expression, the proportion of Tregs in lymph node and spleen of CIA mice at different time points after administration of hBM-MSCs. The induction of Tregs was significantly increased in lymph node (A) and spleen (B) of mice treated with 5×10^6 BM-MSCs (Fig 2). In addition, the induction of Tregs was significantly increased 2 wk after treatment (A-C), when the clinical arthritis scores began to improve and changes were seen in levels of the anti-inflammatory cytokines IL-10 and TGF-β.

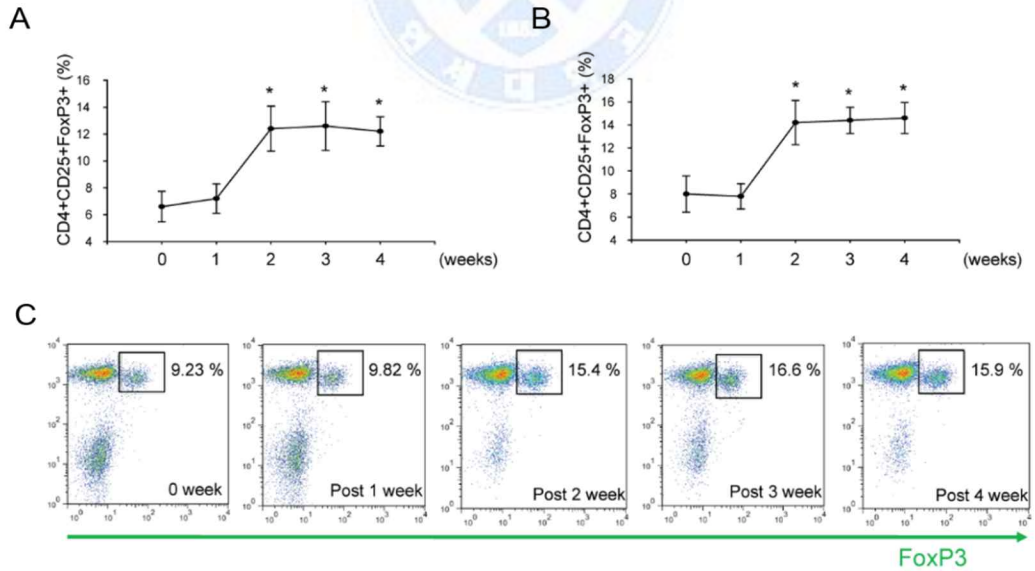


Figure 2. Induction of Tregs treated by hBM-MSCs in mice with CIA. Expression of Tregs was significantly increased in lymph nodes (A) spleen (B) of CIA mice 2 wk after

treatment with 5 x 10^6 hBM-MSCs, when the clinical scores ad levels of anti-inflammatory cytokine IL-10 began to improve (published data). Results of glow cytometric analysis are shown in (C). Values are the mean ± SD. *, $p < 0.05$ versus untreated CIA mice (n=5 per each group).

3. Tregs suppressive activity by hBM-MSCs treatments

To identify the activity of Tregs induced by BM-MSCs treatment, the functionality of Tregs was assessed using an in vitro Treg suppression assay. Tregs showed potent suppressive activity, through inhibition of T-cell proliferation in the hBM-MSCs-treated group compared to untreated CIA mice (Fig. 3).

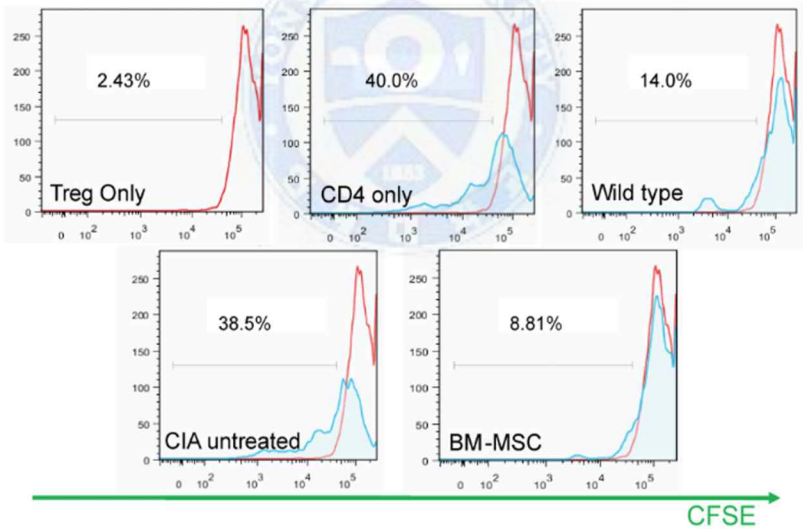


Figure 3. Treg suppressive activity. Treg suppression assay showed that Treg suppressive activity in hBM-MSC-treated mice compared to untreated CIA mice (n=5 per each group). Red line of square box represents Treg only group, and blue line represents the proliferation of CD4+ T cells. CFSE, carboxyfluorescein diacetate succinimidyl ester.

4. hBM-MSCs increased the proliferation of $CD4^+$T cells in co-culture condition

To investigate the effect of hBM-MSCs for regulation of $CD4^+$ T cell population, proliferation assay was done using CFSE staining. CD4+ T cells were co-cultured with hBM-MSCs in 1:5 (MSC: T) ratio for 72 hr. hBM-MSCs increased T cell proliferation compared to $CD4^+$ T cell only (Fig. 4).

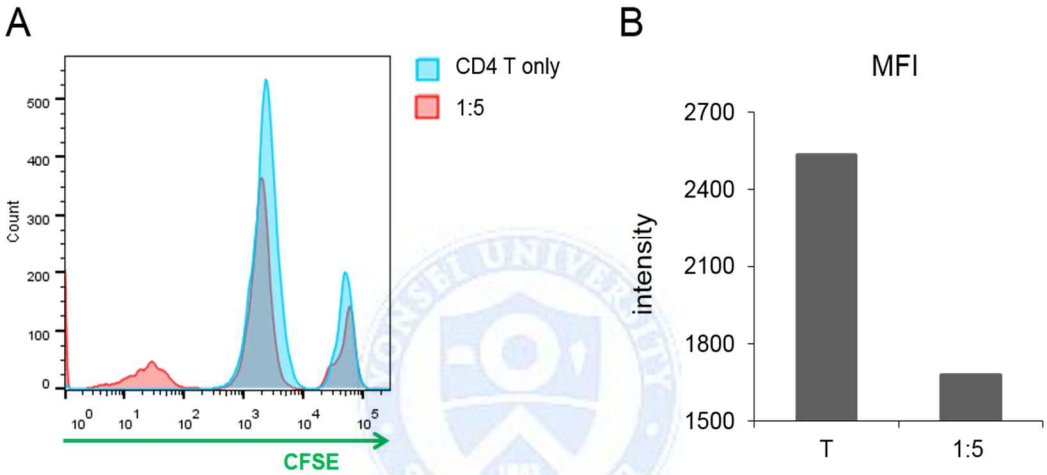


Figure 4. Proliferation activity of $CD4^+$ T cell co-cultured with hBM-MSCs. hBM-MSCs increased the proliferation of $CD4^+$ T cells. CFSE labeled-CD4+ T cells were analyzed by FACS (n=3). CFSE, carboxyfluorescein diacetate succinimidyl ester; MFI, median fluorescence intensity; 1:5, co-culture ratio of MSC:T.

5. hBM-MSCs decreased Th1, Th17, and Th2 responses

To examine whether MSCs has an effect on the inhibition of Th1, Th1, and Th2 differentiation, $CD4^+$ T cells were co-cultured with BM-MSCs in 1:5 (MSC: T) ratio for 72 hr. hBM-MSCs decreased the proportion of $CD4^+$ IFN-r^+ T cells for Th1, $CD4^+$ IL-$17A^+$ T cells for Th17, and $CD4^+$ IL-4^+ T cells for Th2 (Fig. 5A-C). Also, the gene expressions of T-bet, RORr-T, and Gata-3, which are transcription factors for Th1, Th17,

and Th2 respectively were decreased in co-culture with hBM-MSCs (Fig. 5D-F).

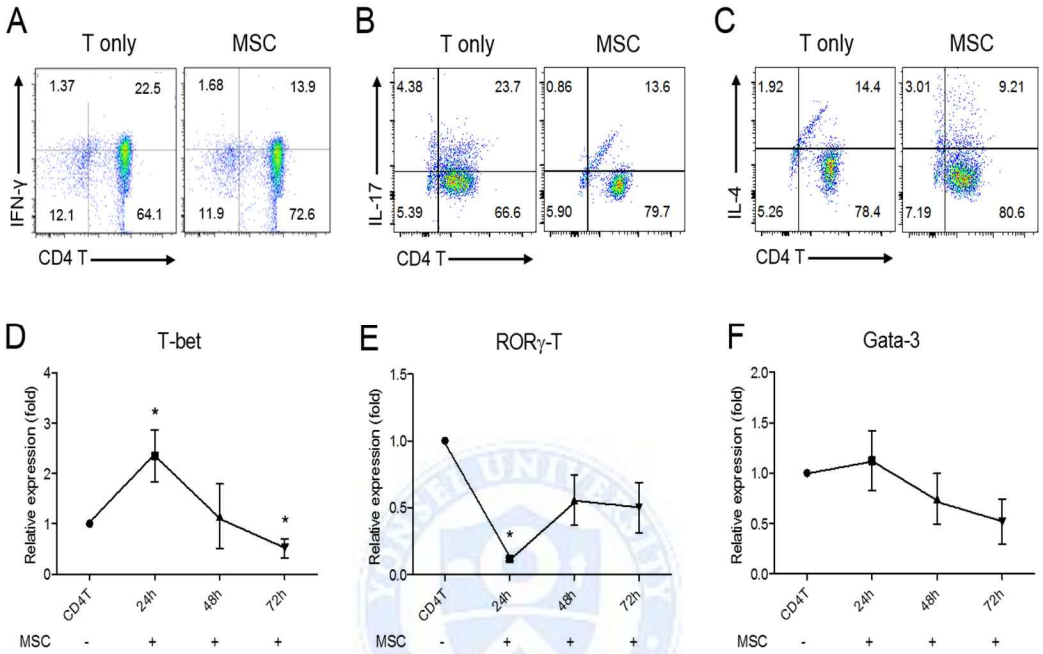


Figure 5. Alteration of T cells population by BM-MSCs. hBM-MSCs decreased the expressions of T-bet (Th1), RORγ-T (Th17), and Gata-3 (Th2) of T cell population. (A-C) $CD4^+$ T cells were isolated from mice and stimulated with anti-CD3 and anti-CD28 for 72 hr. The cells were restimulated with PMA and Ionomycin for 5 h in the presence of GolgiPlug, and then analyzed by intracellular flow cytometry staining to determine the distribution of $CD4^+$ T cells positive for IFN-γ, IL-17A, and IL-4. (D-F) Relative Gene expressions of T cell subset. Values are the mean ± SD. *, $p < 0.05$ versus CD4+ T cells only (n=4).

6. hBM-MSCs increased the expression of $CD4^+CD25^+FoxP3^+$ Tregs in *in vitro*

To examine whether hBM-MSCs has an effect on the propotion of Tregs differentiation, $CD4^+$ T cells were also co-cultured with hBM-MSCs at different ratios for 72 hr. hBM-

MSCs increased the proportion of $CD4^+CD25^+FoxP3^+$ Tregs in a dose dependent manner of co-culture ratios (Fig. 6).

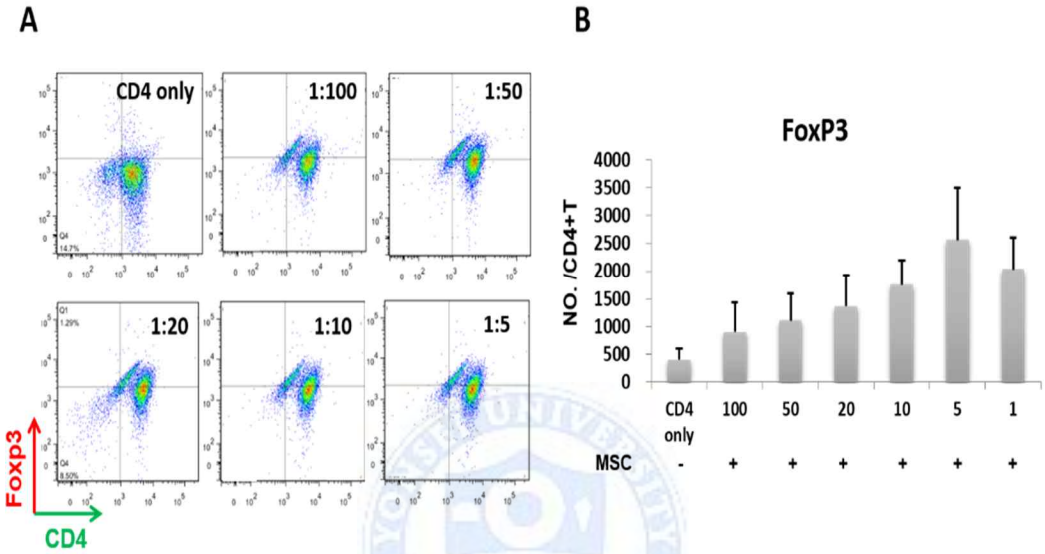


Figure 6. Increase of $CD4^+CD25^+FoxP3^+$ Tregs co-cultured with hBM-MSC. MSC increased the expression of $FoxP3^+$ Tregs in a dose-dependent manner. (A-C) $CD4^+$ T cells were isolated from mice and stimulated with anti-CD3 and anti-CD28 for 72 hr. The cells were restimulated with PMA and Ionomycin for 5 h in the presence of GolgiPlug, and then analyzed by intracellular flow cytometry staining to determine the distribution of $CD4^+$ T cells positive for FoxP3. Values are the mean ± SD. *, $p < 0.05$ versus $CD4^+$ T cells only (n=5).

7. hBM-MSCs increased levels of TGF-β1 and IL-10 in co-cultured supernatant

To elucidate the mechanism of $Foxp3^+$ Tregs induced by MSCs, the two major anti-inflammatory cytokines were measured from co-cultured supernatant with BM-MSC after 24, 48, and 72hr. The anti-inflammatory cytokines TGF-β1 and IL-10 were significantly increased in all co-culture groups at all time points compared to CD4+T

cell only (Fig. 7A-B), and gradually increased in a time dependent manner. Also, the level of TGF-β1 was almost 2 times that of IL-10 at 24hr co-culturing with BM-MSC on $CD4^+$ T cells.

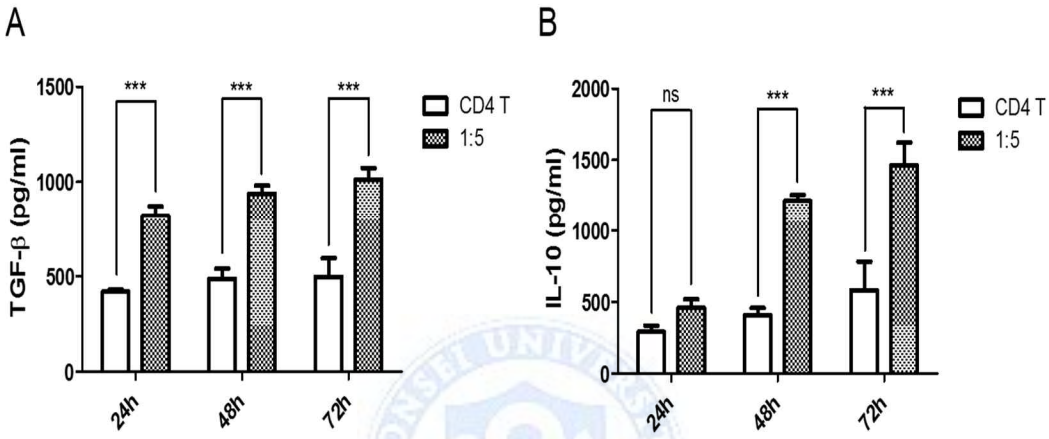


Figure 7. Time-dependent changes in levels of anti-inflammatory cytokines. Expressions of TGF-β1 and IL-10 were significantly increased in all co-culture groups with hBM-MSCs. (A) TGF-β1 and (B) IL-10 levels were assessed in co-cultured supernatant with MSCs on $CD4^+$ T cells. Values are the mean ± SD. ***, $p < 0.001$ versus $CD4^+$ T cells only (n=5), ns means not significant.

8. hBM-MSCs induced PD-1 gene expression of $CD4^+$ T cells

To investigate the mechanisms that induce $Foxp3^+$ Tregs, real time qPCR was performed for several immunoreceptors of $CD4^+$ T cells. Gene expression of programmed cell death 1 (PD-1) was increased in a time dependent manner in 1:5 (MSC: CD4 T) ratio (Fig. 8A). In addition, BM-MSC significantly increased gene expression of Neuropilin-1 (Nrp-1) at 24hr (Fig. 8B), but decreased gene expressions of CD28/cytotoxic T lymphocyte antigen 4 (CTLA-4) and lymphocyte activation gene-3

(LAG-3) in a time dependent manner though were not significant (Fig. 8C-D). Gene expressions of TGF-β1 and IL-10 were also increased as well as protein levels compared to CD4+ T cells only without hBM-MSCs (Fig. 8E-F).

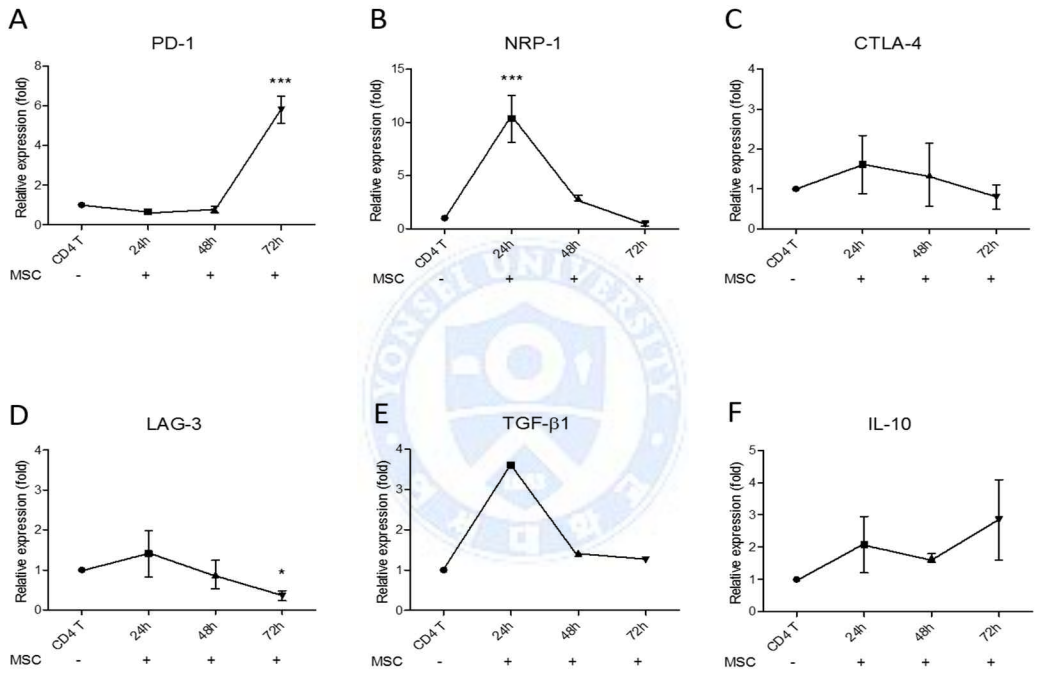


Figure 8. Changes of gene expressions of $CD4^+$ T cells by BM-MSC. BM-MSC significantly increased gene expressions of PD-1, Nrp-1, TGF-β1, and IL-10. (A-D) Relative gene expressions of PD-1, Nrp-1, CTLA-4, and LAG-3. Each experiment was conducted in triplicates. (E-F) Relative gene expressions of TGF-β1 and IL-10. Each experiment was conducted in triplicates. Values are the mean ± SD. *, $p < 0.05$ and ***, $p < 0.001$ versus $CD4^+$ T cells only as control of each time points (n=5).

9. PD-1$^+$ Tregs were highly induced by hBM-MSCs

As well as gene expression, the surface expression of PD-1 of CD4$^+$Foxp3$^+$ Tregs was examined using FACS markers. The expression of CD4$^+$CD25$^+$FoxP3$^+$ PD1$^+$ Tregs were highly induced in co-culture condition compared to CD4$^+$ T cells only. And PD-1 expression was increased in a dose dependent manner in co-culture condition with BM-MSCs (Fig. 9).

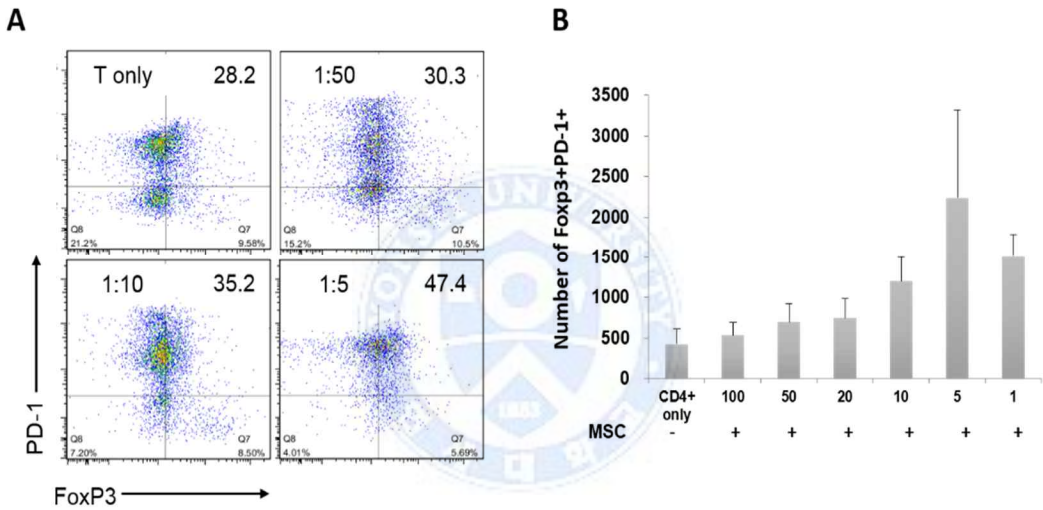


Figure 9. PD-1$^+$ Tregs expression. hBM-MSCs increased CD4$^+$CD25$^+$FoxP3$^+$ PD-1$^+$ Tregs expression in co-culture condition compared to CD4$^+$ T cells only. (A) Frequency of FoxP3$^+$ PD-1$^+$ Tregs and (B) absolute number of FoxP3$^+$ PD-1$^+$ Tregs co-cultured with BM-MSCs for 72 hr. Values are the mean ± SD. *, $p < 0.05$ versus CD4+ T cells only (n=5).

10. hBM-MSCs increased the protein expressions of Foxp3, PD-1 and Nrp-1

To examine the roles of Foxp3, PD-1 and Nrp-1, protein expressions were evaluated by immunoblot. Human BM-MSC increased the protein level of FoxP3 (A), PD-1 (B)

and Nrp-1 (C) of CD4+ T cell in MSC: T (1:5) co-culture condition (Fig. 10).

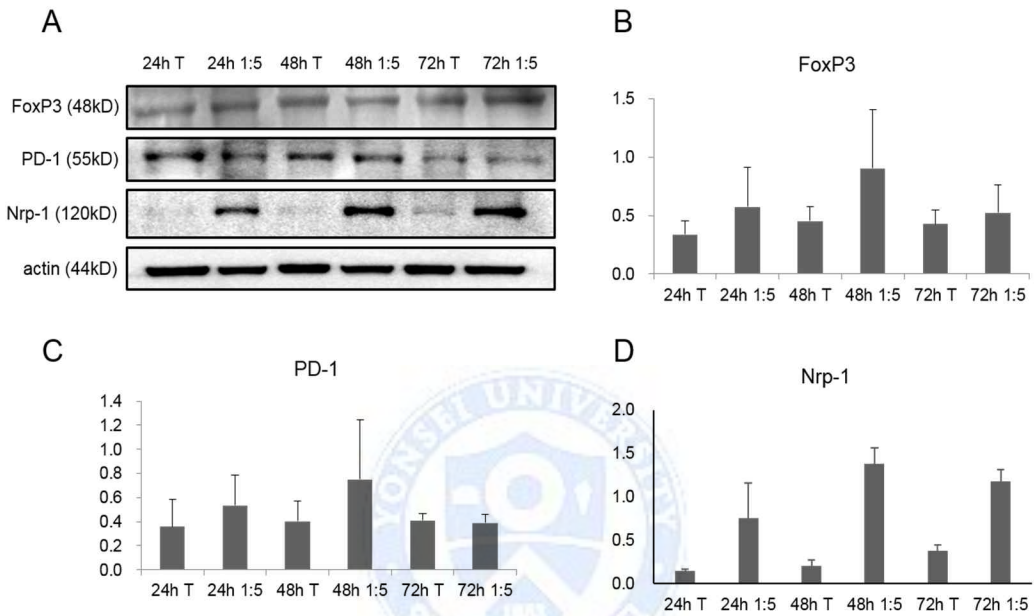


Figure 10. Increased expression of Foxp3, PD-1 and Nrp-1 proteins by hBM-MSCs. (A) Western blot and (B-D) densitometric data of FoxP3, PD-1, and Nrp-1. Values are the mean ± SD. *, $p < 0.05$ versus CD4+ T cells only (n=4).

11. PD-1 is required for Tregs induction by hBM-MSCs

To evaluate the role of PD-1 toward FoxP3+Tregs induction by hBM-MSCs, PD-1 inhibition was conducted by treating PD-1 neutralizing antibody (20 ug/ml, Peprotech) into MSC-T cells co-culturing media for 72 hr. The expression of $FoxP3^{+}$Tregs was not increased when co-cultured with MSC (1:5 ratio) treated with PD-1 neutralizing antibody (Fig. 11). According to data, $FoxP3^{+}$Tregs induction by hBM-MSCs might be dependent on PD-1 expression of $CD4^{+}$ T cells.

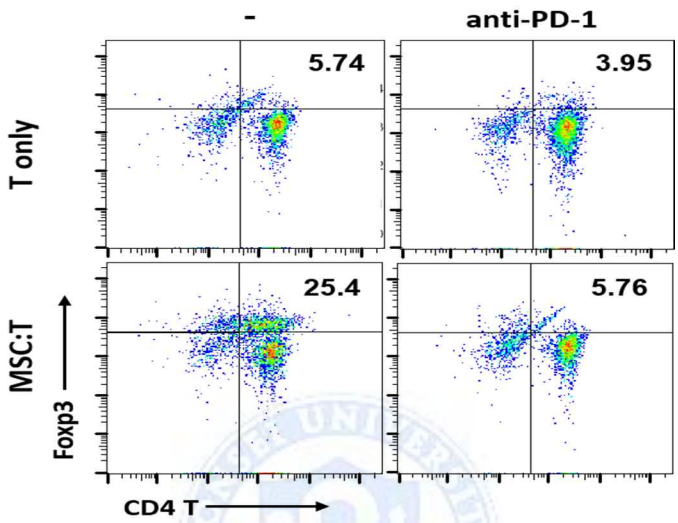


Figure 11. Effect of FoxP3$^+$ Tregs expression using PD-1 neutralizing antibody in vitro. FoxP3$^+$Tregs expression was not increased when co-cultured with BM-MSC treated with PD-1 neutralizing antibody. Splenocytes were isolated from mice and stimulated with anti-CD3 and anti-CD28 for 72 hr. The cells were restimulated with PMA and Ionomycin for 5 hr in the presence of GolgiPlug or GolgiStop, and then analyzed by intracellular flow cytometry staining to determine the distribution of CD4$^+$ T cells positive for Foxp3.

12. PD-1 is critical for Tregs induction by BM-MSCs in PD-1 knockout mice

Next, to confirm the role of PD-1 inducing Foxp3$^+$ Tregs, the CD4$^+$CD25$^+$FoxP3$^+$ Tregs expression was assessed in splenic CD4+ T cells of PD-1$^{-/-}$ mice. According to data, Foxp3$^+$ Tregs were increased in co-culture with BM-MSCs for 72 hr compared to without MSC in wildtype mice. However, Foxp3$^+$ Tregs expression was not increased in co-culture with BM-MSCs and CD4$^+$ T cells isolated from spleen of PD-1$^{-/-}$ mice (Fig. 12).

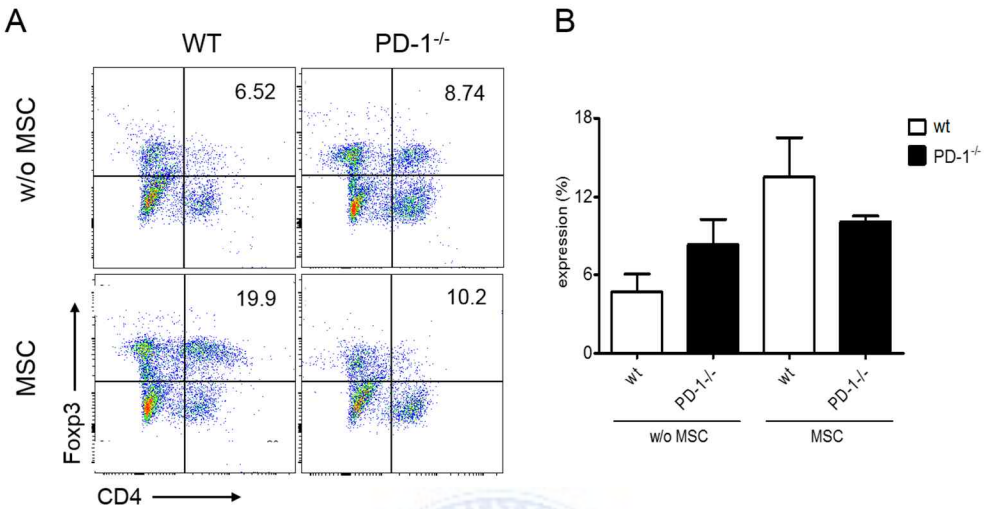


Figure 12. Effect of FoxP3$^+$ Tregs expression in PD-1 knockout mice. (A) Foxp3$^+$ Tregs expression was not increased in co-culture with BM-MSCs and CD4$^+$ T cells isolated from spleen of PD-1$^{-/-}$ mice. (B) Quantitative analysis of CD4$^+$CD25$^+$FoxP3$^+$ Tregs in spleen of WT and PD-1$^{-/-}$ mice. Splenic CD4+ T cells were isolated from PD-1$^{-/-}$ mice and stimulated with anti-CD3 and anti-CD28 for 72 hr with or without hBM-MSCs. The cells were restimulated with PMA and Ionomycin for 5 hr in the presence of GolgiPlug or GolgiStop, and then analyzed by intracellular flow cytometry staining to determine the distribution of CD4$^+$ T cells positive for Foxp3. WT, wild type mice.

13. PD-1 expression was increased in hBM-MSCs-treated CIA mice

Treatment with hBM-MSC attenuated the severity of arthritis significantly during the progression of CIA. In addition, human BM-MSC treated CIA mice showed significant immunopositivity for PD-1 on T cells (Fig. 13).

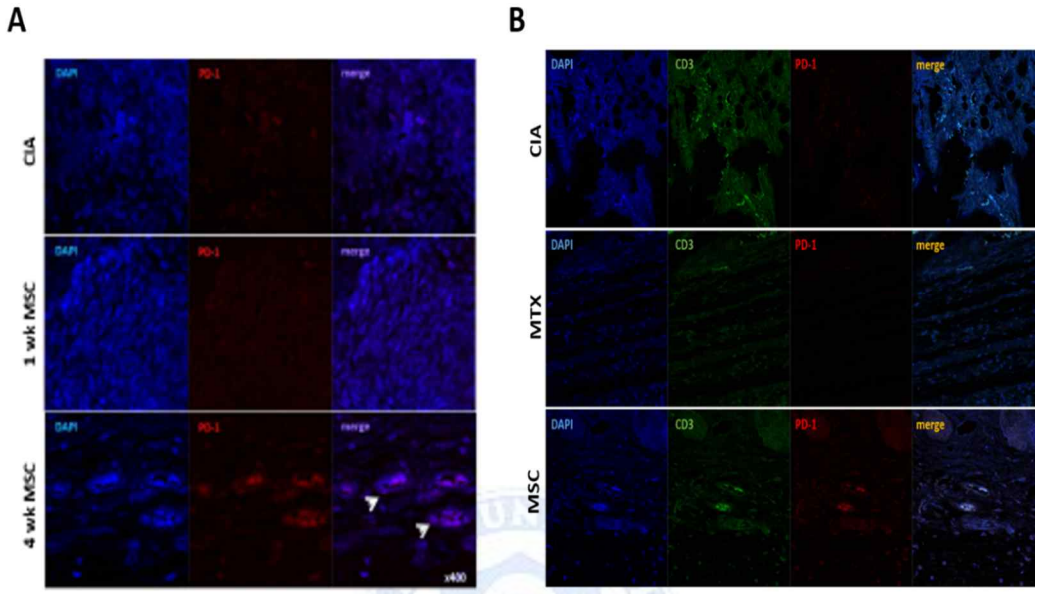


Figure 13. Immunohistochemical analysis of inflamed tissues in CIA mice. hBM-MSC treated CIA mice showed significant immunopositivity for PD-1 on T cells (A) Laser scanning confocal microscopy was used to assess the intensity and distribution of immunofluorescence staining for DAPI (blue) and PD-1 (red) and (B) immunofluorescence staining for CD3 (green) and PD-1 (red) in the inflamed tissue from untreated CIA mice, mice treated with BM-MSCs.

Part 2. Immune modulation of hBM-MSCs on DC

14. Inhibition of CD86 and MHCⅡ expressions on DC by hBM-MSCs

To examine the effects of hBM-MSCs on DC maturation, the expressions of CD86 and MHCⅡ were assessed by FACS analysis. The surface expressions of CD86 and MHC Ⅱ highly induced under LPS (100 ng/ml) treatment. But, co-culture in the presence of hBM-MSCs resulted in decreased expression of CD86 (Fig. 14A-B) and MHCⅡ (Fig. 15A-B) in both 1:1 and 1:10 co-culture ratio in different time windows. Activation of immature DCs by LPS in the presence of MSCs did not result in upregulation of costimulatory molecules.

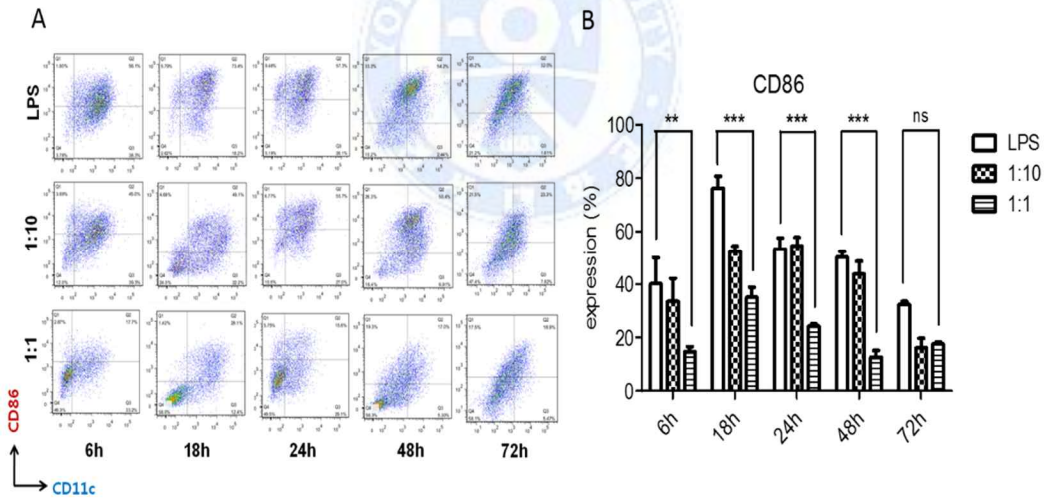


Figure 14. Inhibition of CD86 expression on DC co-cultured with hBM-MSCs. BM-MSCs decreased CD86 expression in both 1:1 and 1:10 co-culture ratio. (A) Flow cytometry plots showing percentages of $CD11c^+$ and $CD86^+$ DC in murine DC co-cultured with hBM-MSC. (B) Quantification of CD86 expression. Values are the mean ± SD. **, $p < 0.01$ and ***, $p < 0.001$ versus LPS treated DC (n=5). ns, not significant.

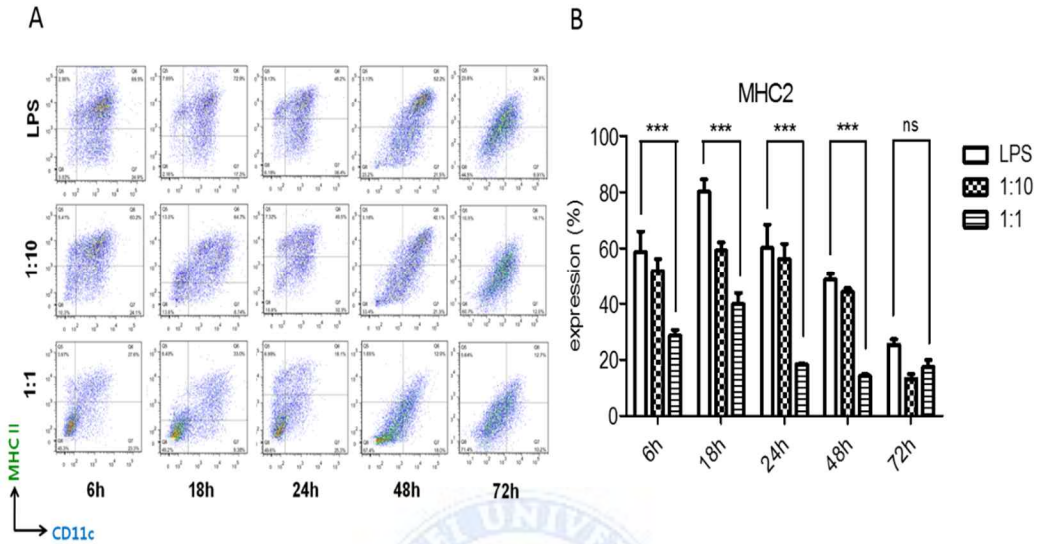


Figure 15. Inhibition of MHC Ⅱ expressions on DC co-cultured with hBM-MSCs. BM-MSCs decreased of MHC Ⅱ expressions in both 1:1 and 1:10 co-culture ratio. (A) Flow cytometry plots showing percentages of $CD11c^{+}$ and MHC $Ⅱ^{+}$ DC in murine DC co-cultured with hBM-MSC. (B) Quantification of MHC Ⅱ expression. Values are the mean ± SD. ***, $p < 0.001$ versus LPS treated DC (n=5). ns, not significant.

15. Pro- and anti-inflammatory cytokine changes of co-culture supernatant

Because the secretion of IL-12p70 is critical for the maturation and function of DCs, it was investigated whether BM-MSCs could interfere with IL-12 production by DCs including other cytokines. Human BM-MSCs decreased the levels of IL-12p70, TNF-α, and IL-6 effectively in both 1:1 and 1:10 co-culture ratio. In contrast, hBM-MSCs increased the levels of IL-10 and TGF-β in 1:1 and 1:10 co-culture ratio with DC.

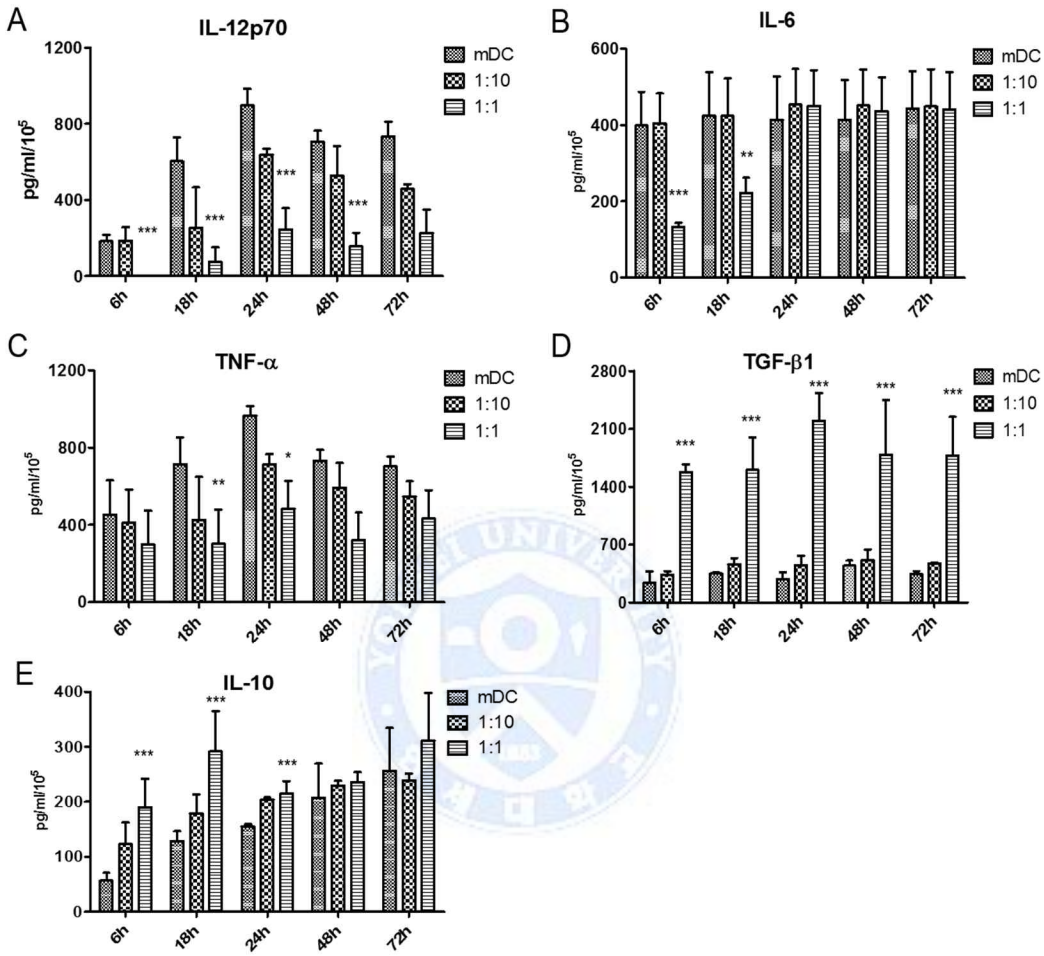


Figure 16. Cytokine expressions of DC co-cultured with hBM-MSCs. BM-MSCs decreased the levels of IL-12p70, TNF-α, and IL-6 effectively, but increased the levels of IL-10 and TGF-β in both 1:1 and 1:10 co-culture ratio. The supernatant from mature DC with (1:10 and 1:1) or without (LPS) MSC co-culture were used to quantify the cytokine levels in this experiments. (A-C) Expression of pro-inflammatory cytokines including IL-12p70, IL-4, and TNF-α. (B) Expression of anti-inflammatory cytokines of TGF-β1 and IL-10. Values are the mean ± SD. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus LPS treated DC (n=5).

16. Gene expressions of anti-inflammatory DC (antiDC) induced by hBM-MSCs

To explore whether the inhibition of BM-MSCs on DC maturation requires the intracellular signal for anti-inflammatory DC, qPCR was used to find important regulators or transcription factors which regulates DC status in various co-culture time windows. The relative gene expressions of TGF-β1, IL-10, indoleamine 2, 3-dioxygenase (IDO) and heme oxygenase-1 (HO-1) were highly increased in 1:1 co-culture ratio at 6 hr. Also, the relative gene expressions of PD-1 ligand 1 (PD-L1) and inducible T cell costimulator (ICOS) were significantly increased in 1:1 co-culture ratio.

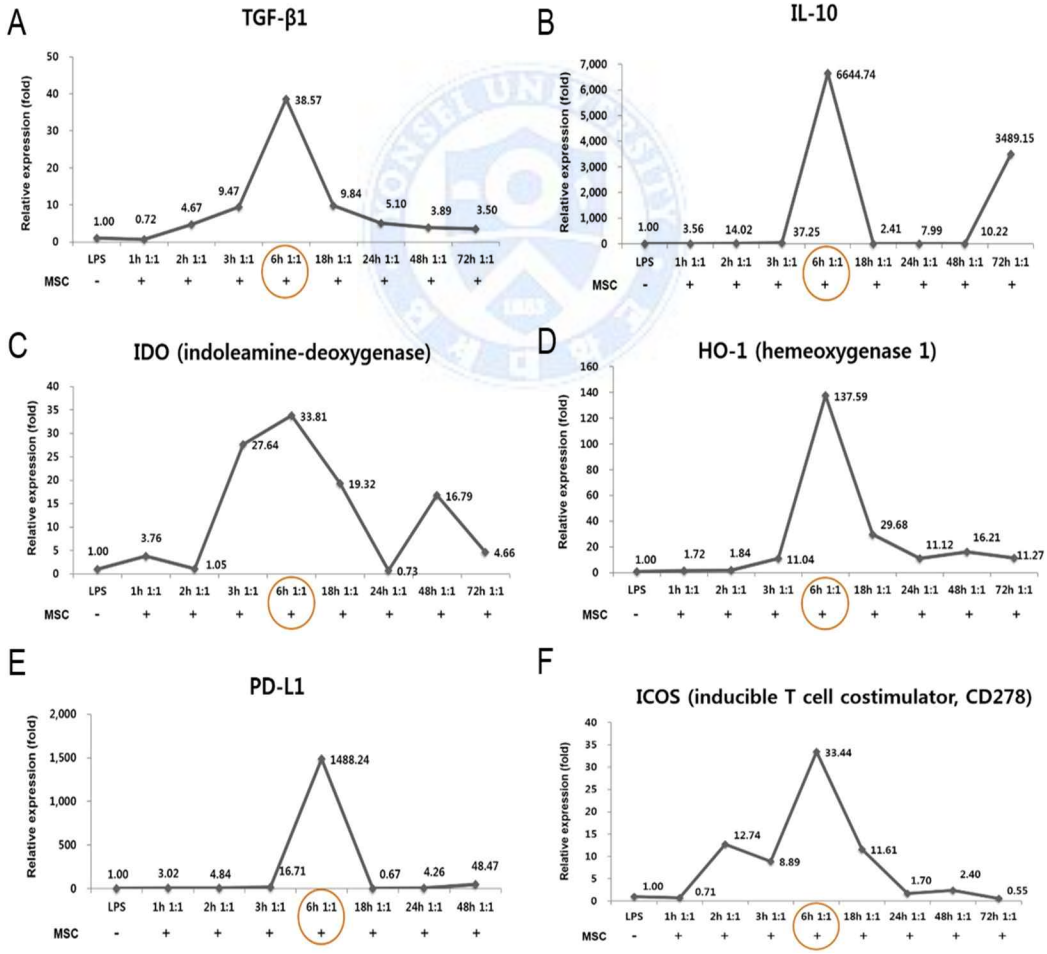


Figure 17. Gene expressions of DC co-cultured with hBM-MSC. The relative gene expressions of TGF-β1, IL-10, IDO, HO-1, PD-L1, and ICOS were highly increased in 1:1 co-culture ratio at 6 hr. DC were isolated from 1:1 co-culture with hBM-MSCs, and then the cells were assessed by real time qPCR to determine the anti-inflammation and tolerance of DC. Each experiment was conducted in triplicates. Values are the mean ± SD. *, $p < 0.05$ and ***, $p < 0.001$ versus mature DC as control of each time points (n=5).

17. Identification of a unique DC signature co-culture with hBM-MSC

To identify a unique DC signature at 6 hr co-culture with hBM-MSC, gene profiling of DC explored BM-MSCs for 6 hr was performed and quantitative mass spectrometry analysis was conducted. Gene array identified almost 10,000 genes that were enriched in DC co-cultured with hBM-MSCs (Fig. 18A-B). 228 genes were significantly increased and 108 genes were significantly decreased in mature DC (mDC) compared to immature DC (iDC). 291 genes were significantly increased and 17 genes were decreased in DC co-cultured with hBM-MSCs (antiDC) compared to mDC.

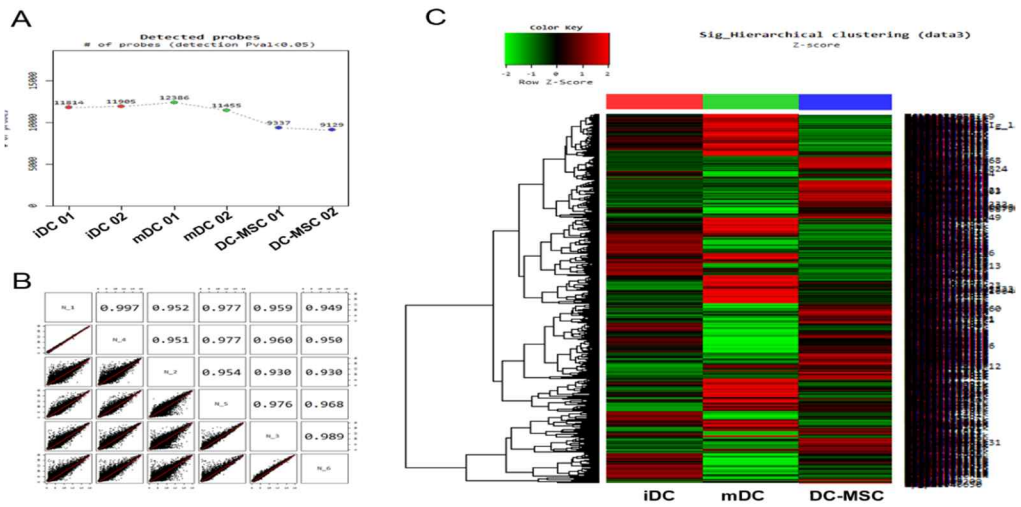


Figure 18. Identification of a DC signature co-cultured with hBM-MSCs by gene expression and quantitative mass spectrometry. (A) Affymetrix genearray expression profile of murine DC identified almost 10,000 genes (> 1.5 fold, $p < 0.05$, Student's t test, two tailed). (B) Scatter plots of gene expression of antiDC. The number means the significant correlation between all samples. (C) Heat map of total gene expression of antiDC based in hierarchical clustering. Each lane represents the average expression value of two biological duplicates per group.

18. Functional gene selection of DC experienced with hBM-MSC

In biological process, many immune response-regulating signal transduction were identified including T cell activation and antigen receptor-mediated signaling and so on (Fig.19 A). In cellular component, receptor complex was identified as well T cell receptor. In molecular function, nucleotide binding was mostly identified (Fig. 19C).

19. Decreased gene profiles in antiDC versus mDC in comparision to iDC control

Next, the gene expression was compared between mDC and antiDC for 6 hr. First, up-regulated genes of mDC were selected compared to iDC under LPS (100 ng/ml) treatment, then down-regulated genes of antiDC were chosen compared to mDC genearray data. Finally, 102 genes identified that were uniquely expressed in antiDC including *IL-1α*, *cxcl10*, *zbtb32* and *lcn2* (Fig. 20, 21). *IL-1α* also known as hematopoietin-1 is responsible for the production of inflammation, maturation of dendritic cells. *Cxcl10* is a CXC chemokine known to favor the recruitment and activation of Th1-polarized cells. BM-MSC reduced the levels of *IL-1α* and *cxcl10* in mDC of co-culture condition for 6 hr.

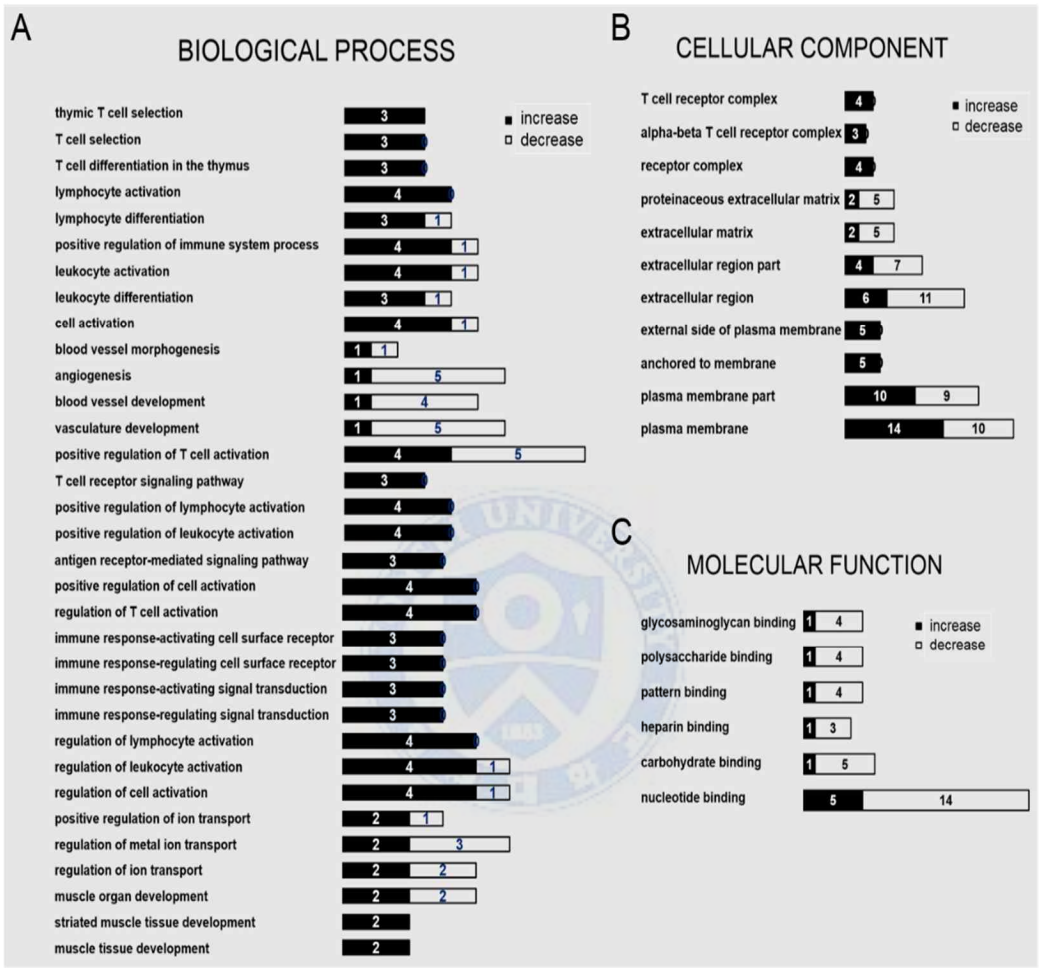


Figure 19. Functional categorization of significantly differential expressed genes of antiDC experienced hBM-MSCs. (A) Biological process, (B) cellular component, and (C) molecular function of antiDC.

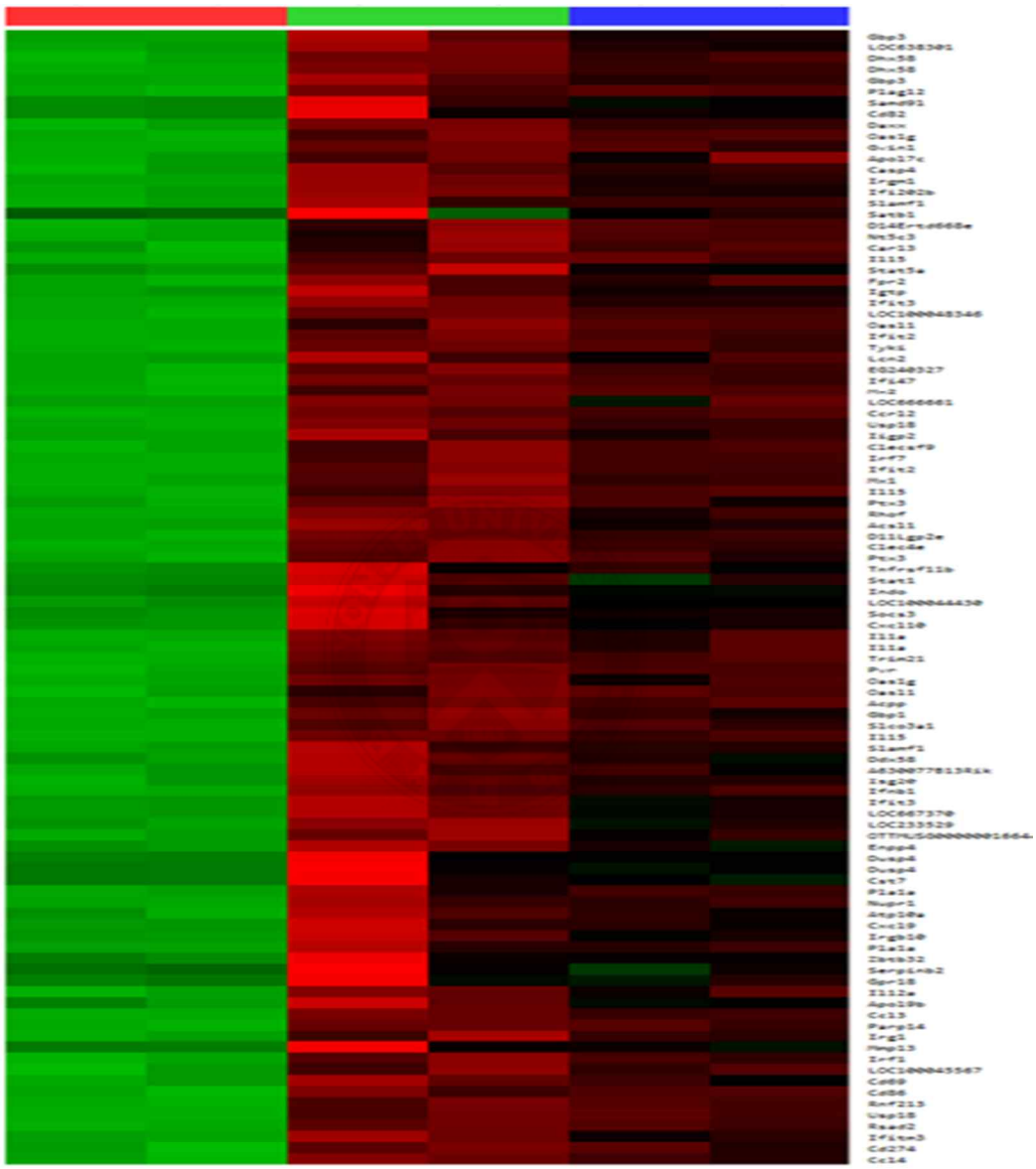
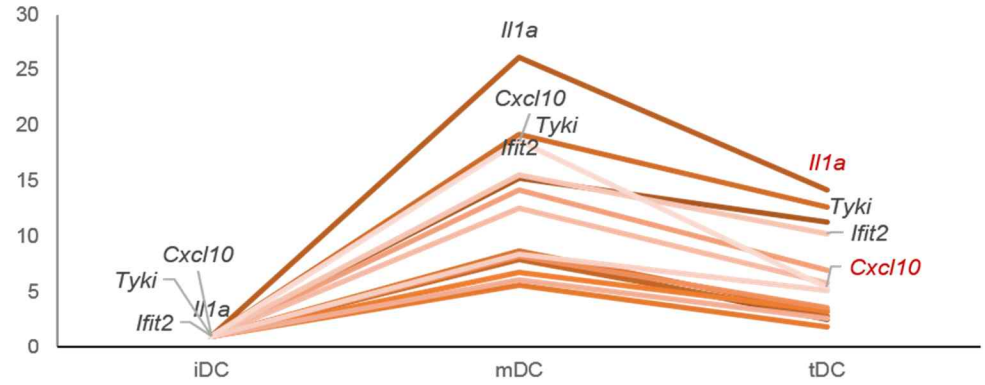


Figure 20. Hierarchical clustering of dependent and nondependent base changes in gene expression; 102 genes whose expression was significantly up-regulated by hBM-MSCs. Heat map of total gene expression of antiDC based in hierarchical clustering. Each lane represents the average expression value of two biological duplicates per group.



Gene	Denfintion	mDC vs iDC	p-value	tDC vs iDC	p-value
Indo	indoleamine-pyrrole 2,3 dioxygenase (Indo), mRNA.	8.2	0.0	2.6	0.0
Rsad2	radical S-adenosyl methionine domain containing 2 (Rsad2), mRNA.	15.3	0.0	11.3	0.0
Il1a		26.2	0.0	14.2	0.0
LOC100044430	PREDICTED: similar to Interferon activated gene 205 (LOC100044430), mRNA.	8.0	0.0	2.8	0.0
Tyki		19.2	0.0	12.7	0.0
LOC667370	PREDICTED: similar to interferon-induced protein with tetratricopeptide repeats 3 (LOC667370), mRNA.	8.7	0.0	3.2	0.0
Serpinb2	serine (or cysteine) peptidase inhibitor, clade B, member 2 (Serpinb2), mRNA.	5.6	0.0	1.9	0.0
LOC638301	PREDICTED: similar to interferon activated gene 204 (LOC638301), mRNA.	6.8	0.0	3.3	0.0
Igtp	interferon gamma induced GTPase (Igtp), mRNA.	8.2	0.0	3.6	0.0
Lcn2	lipocalin 2 (Lcn2), mRNA.	14.2	0.0	7.0	0.0
Zbtb32	zinc finger and BTB domain containing 32 (Zbtb32), mRNA.	6.1	0.0	2.7	0.0
Ifit3	Mus musculus interferon-induced protein with tetratricopeptide repeats 3 (Ifit3), mRNA.	8.1	0.0	3.0	0.0
Ifit2	Mus musculus interferon-induced protein with tetratricopeptide repeats 2 (Ifit2), mRNA.	15.6	0.0	10.3	0.0
Usp18	Mus musculus ubiquitin specific peptidase 18 (Usp18), mRNA.	8.4	0.0	5.1	0.0
Cxcl10	Mus musculus chemokine (C-X-C motif) ligand 10 (Cxcl10), mRNA.	18.6	0.0	5.5	0.0

Figure 21.Relative gene expressions between three groups, and top 15 gene list of down-regulated genes of DC-MSC. Kinetics of the expression of genes encoding transcriptional regulators down-regulated 1.5-fold or more in antiDC relative to their expression in mDC and iDC control.

20. Increased gene profiles in antiDC versus mDC in comparision to iDC control

Then, the other unique gene expression was compared between mDC and antiDC for 6 hr. First, down-regulated genes of mDC were selected compared to iDC under LPS (100 ng/ml) treatment, then up-regulated genes of antiDC were chosen compared to mDC genearray data. As a results, 20 genes identified that were uniquely expressed in antiDC including *ccdc80* and *Rn18s* (Fig. 22, 23). *Ccdc 80* (coiled-coil domain containing 80) is a protein-coding gene suggesting CCDC80 protein is a peroxiredoxin as ubiquitous family of antioxidant enzymes that control cytokine-induced peroxide levels and thereby mediate signal transduction in mammalian cells according to bioinformatics analysis. BM-MSC reduced *ccdc80* level in mDC of co-culture condition for 6 hr.

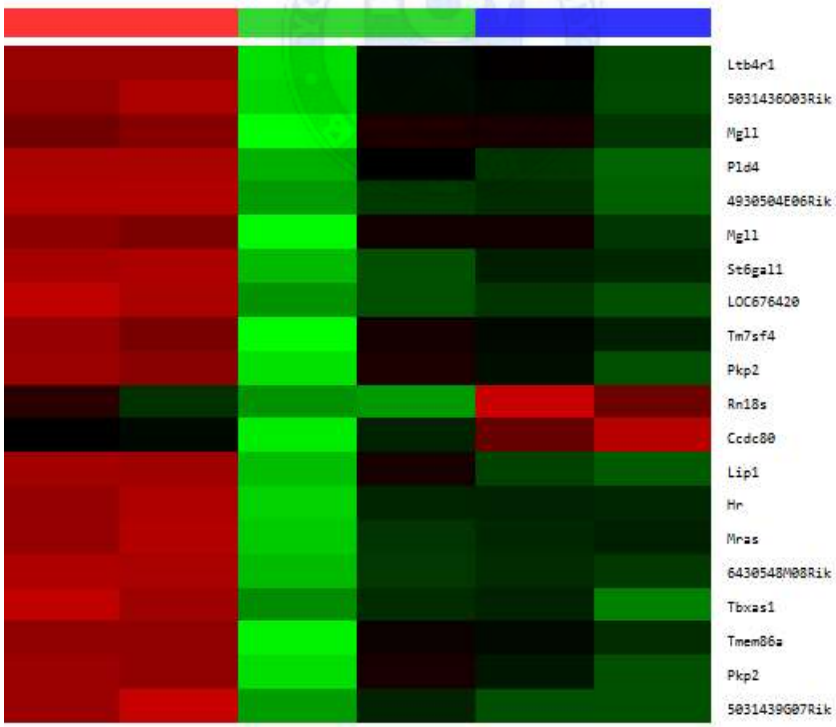
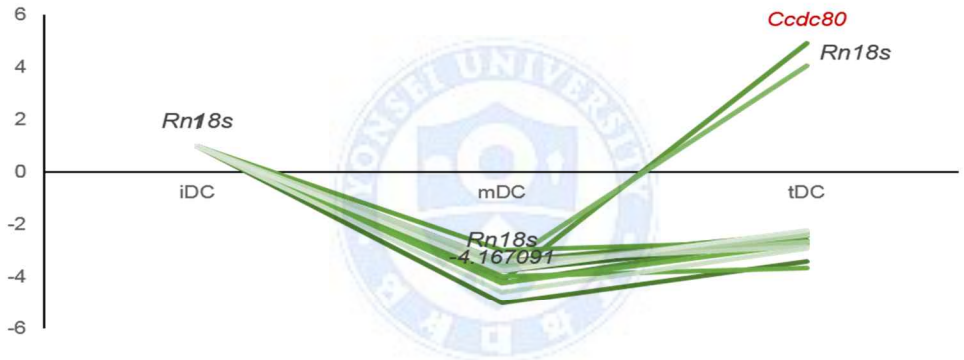


Figure 22. Hierarchical clustering of dependent and nondependent base changes in gene expressions; 20 genes whose expression was significantly increased by hBM-MSC. Heat map of total gene expression of antiDC based in hierarchical clustering. Results were log-transformed, normalized (to the mean expression of zero across samples) and centered, and populations and genes were clustered by pairwise centroid linkage with the Pearson correlation. Each lane represents the average expression value of two biological duplicates per group.



Gene	Denfintion	mDC vs iDC	p-value	tDC vs i DC	p-value
5031436O03Rik		-3.77	0.01	-2.63	0.00
6430548M08Rik		-5.03	0.00	-3.40	0.00
Ltb4r1	leukotriene B4 receptor 1 (Ltb4r1), mRNA.	-3.40	0.02	-2.34	0.00
Tm7sf4	transmembrane 7 superfamily member 4 (Tm7sf4), mRNA.	-4.22	0.00	-2.44	0.00
Ccdc80	coiled-coil domain containing 80 (Ccdc80), mRNA.	-4.17	0.01	4.91	0.00
4930504E06 Rik	RIKEN cDNA 4930504E06 gene (4930504E06Rik), mRNA.	-2.98	0.05	-2.61	0.00
5031439G07Rik		-3.97	0.01	-3.66	0.00
Mras	muscle and microspikes RAS (Mras), mRNA.	-4.25	0.01	-2.72	0.00
Rn18s	18S RNA (Rn18s), non-coding RNA.	-3.54	0.02	4.08	0.00
Tmem86a	transmembrane protein 86A (Tmem86a), mRNA.	-3.60	0.03	-2.34	0.00
Pkp2	plakophilin 2 (Pkp2), mRNA.	-3.46	0.02	-2.66	0.00
St6gal1	beta galactoside alpha 2,6 sialyltransferase 1 (St6gal1), mRNA.	-3.65	0.01	-2.41	0.00
LOC676420	PREDICTED: similar to ceramide kinases (LOC676420), misc RNA.	-3.41	0.02	-2.83	0.00
Hr	hairless (Hr), mRNA.	-4.59	0.00	-2.90	0.00
Mgll	monoglyceride lipase (Mgll), mRNA.	-3.36	0.02	-2.04	0.00

Figure 23.Relative gene expression and top 15 gene list of up-regulated genes of DC-MSC. Kinetics of the expression of genes encoding transcriptional regulators up-regulated 1.5-fold or more in antiDC relative to their expression in mDC and iDC control.

21. Gene profiles in inflammatory responses of antiDC in comparision to iDC and mDC

To clarify the degree to which regulate the inflammatory responses of DC by hBM-MSCs, profiling was analyzed using DAVID program that categorizes inflammatory responses. Almost 180 gene were identified in innate immune response, also T cell tolerance induction was involved in inflammatory response (Fig. 24)

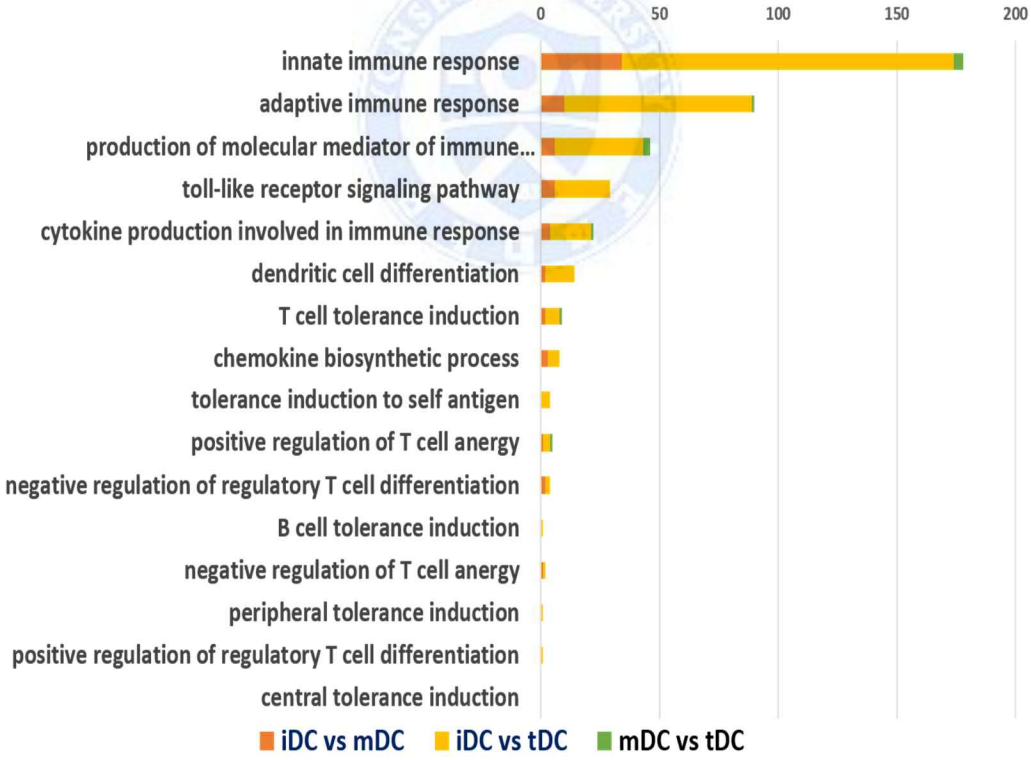


Figure 24. Biological function of antiDC in the category of inflammatory responses.

22. Selected gene expression of antiDC co-cultured with hBM-MSCs

To further characterize antiDC distinguishing from other DC phenotypes, mostly up-regulated genes of both mDC and antiDC were selected compared to iDC. Also, down-regulated genes of both mDC and antiDC were selected compared to iDC. Then, RT-PCR was performed to quantify selected gene expressions. The expressions of *Lamb2*, wars involving regulation of cell cycle and cell proliferation decreased in DC co-cultured with hBM-MSCs. In addtion, the expressions of *ccr7*, *cxcl4*, irf1 involving inflammatory response also decreased in DC co-cultured with hBM-MSCs for 6 hr.

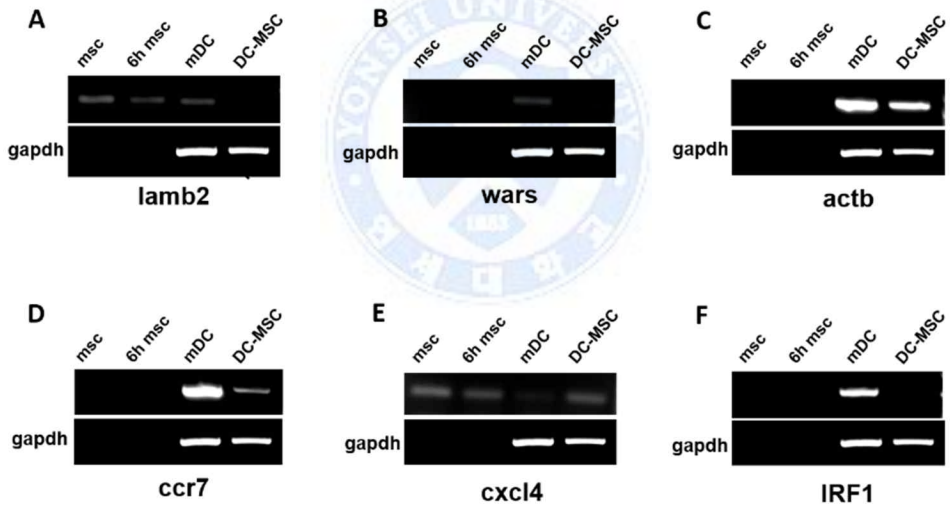


Figure 25. RT-PCR of antiDC co-cultured with hBM-MSC.RT-PCR analysis of *lamb2*, *wars*, *actb*, *ccr7*, *cxcl4*, and *irf1* expression was decreased in antiDC experienced BM-MSC for 6 hr. msc, healthy BM-MSC at passage 5 ;6h msc, hBM-MSC alone for 6 hr.

23. Function study of antiDC

To confirm the function of DC co-cultured with hBM-MSCs, DC function study was finally performed in triple co-culture system. DC cultured with hBM-MSCs for 6hr at first, and $CD4^+$ T cells were added into the co-culturing DC with MSCs for another 72 hr. BM-MSC increased T cell proliferation compared to immature and mature DC, also the expression of $FoxP3^+$ Tregs was increased in DC co-cultured with hBM-MSCs (Fig. 26).

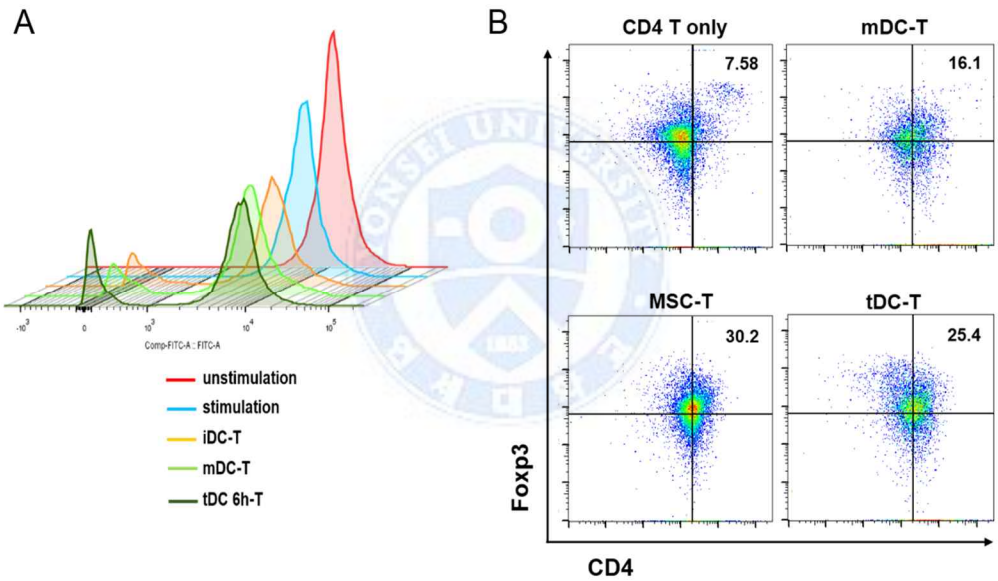


Figure 26. Function study of antiDC. antiDC proliferated the T cell population, and also increased $Foxp3^+$ T cells. (A) Proliferation assay of antiDC assessed by CFSE stained $CD4^+$ T cells. (B) $Foxp3^+$ Tregs expression of antiDC by BM-MSCs. CFSE, carboxyfluorescein diacetate succinimidyl ester.

IV. DISCUSSION

In the present study, hBM-MSC significantly increased $CD4^+FoxP3^+PD\text{-}1^+$Tregs expression in MSC-CD4 T co-culture condition in *in vitro*. In addition, gene expression of PD-1 was increased in a time dependent manner in 1:5 (MSC: CD4 T) ratio. BM-MSC significantly increased Nrp-1 gene expression at 24hr, and decreased CTLA-4 and Lag-3 gene expressions in a time dependent manner. BM-MSC significantly increased protein expressions of FoxP3, Nrp-1, and PD-1 in MSC-CD4 T co-culture condition, and also increased the immunopositivity of PD-1 on T cells in inflamed tissue of CIA mice treated with hBM-MSCs.

Tregs induction when co-cultured with hBM-MSCs was consistent with our previous findings that induced $CD4^+Foxp3^+$ Tregs in CIA mice *in vivo*[12]. Tregs play a crucial role in controlling autoimmunity by inducing tolerance, and they prevent the development of chronic inflammatory and autoimmune diseases[16]. FoxP3 is a master transcription factor specific to Treg cells and supports their differentiation and functions[16]. *In vitro* and *in vivo* studies with human, baboon, and murine MSCs demonstrated that MSCs suppress the proliferation of T cells induced by alloantigens or mitogens, and induce $Foxp3^+$ Tregs, which are pivotal to maintaining self-tolerance and preventing autoimmunity[12,18,19]. However, the mechanisms of $Foxp3^+$ Tregs induced by hBM-MSC are derived from naturally occurring thymus-derived Tregs (nTregs) or peripherally induced Tregs (iTregs) that develop from naive T cells under different conditions are not well defined that the origins and signalings.

In addition to CD25 and Foxp3, Treg cells express various surface molecules, such as CD28/cytotoxic T lymphocyte antigen 4 (CTLA-4), lymphocyte activation gene-3 (LAG-3), Neorupilin-1 (Nrp-1), and programmed death-1 (PD-1), on the cell surface or in the

intracellular region. These molecules expressed by Treg cells might have profound effects on the suppressive function of Treg cells[16-21]. Also, CTLA-4, LAG-3, and PD-1, are also highly upregulated on exhausted T cells, and they mediate the inhibition of pathogen-specific T cell function[19,20]. Among the various inhibitory receptors, PD-1 is a hallmark of exhausted T cells in chronic pathogen infection[19,20]. In the present study, BM-MSCs increased PD-1 expression of $CD4^+$ T cells when the $Foxp3^+$ Tregs induced by BM-MSCs in *in vitro*. PD-1 is an immunoreceptor belonging to CTLA-4 family, provides negative costimulation to antigen stimulation, and play a critical role in Foxp3+Tregs induction[21]. In this study, $Foxp3^+$ Tregs did not increased when co-cultured with BM-MSC and $CD4^+$ T cells from PD-1 knockout mice. PD-1 may play an important role in $Foxp3^+$ Tregs induction in vitro.

Nrp-1 is a cell surface molecule that can be efficiently used to separate inducible versus nTregs subsets, and nearly 70% of $Foxp3^+$ T reg cells expressed Nrp-1 in WT NOD or B6 mice[22,23] suggesting marker of nTregs because of high levels on nTregs cells[23]. In the present study, the expression of Nrp-1 was significantly increased at 24 hr earlier than that of PD-1 in $CD4^+$ T cells co-cultured with hBM-MSCs even though in vitro experiment. However, Nrp-1 that is proper Tregs marker of natural or induced Tregs is now controversial in distinct immune settings. Therefore, the roles of PD-1 and Nrp-1 in $Foxp3^+$ Tregs induction need to be further determined.

The polyclonal activation of T cells in Ctla4 knockout mice is most likely a result of the dysfunction of Treg cells, and deletion of the CTLA-4 gene in $FoxP3^+$ Treg cells recapitulated the fatal disease of the Ctla4 knockout mice[24]. Thus, CTLA-4 might be related to Tregs induction recently. Also, LAG-3 was recently reported to negatively regulate the function of $CD4^+$ T cells, $CD8^+$ T cells, and plasmacytoid DCs and to regulate the homeostatic proliferation of $CD8^+$ T cells and the suppressive activity of T

reg cells[20]. However, the effect of LAG-3 in these assays is relatively small, and the actual contribution of LAG-3 to the regulation of immune system has been unclear. In the current study, the expressions of CTLA-4 and LAG-3 were not significantly changed by hBM-MSC, and the roles of these two inhibitory immunoreceptors that induce $Foxp3^+$ Tregs under experiencing MSCs will be elucidated.

It is well known that MSCs exert profound immunosuppressive properties on T cell proliferation. However, their effect on the initiators of the immune response, the DCs are relatively unknown. DCs play an important role in the initiation of primary immune responses and in the induction of tolerance, depending on their stage of activation and maturation. In this present study, hBM-MSCs significantly inhibited the DCs maturation by decreasing CD86 and MHCⅡ expressions in both 1:1 and 1:10 co-culture ratio. Human BM-MSCs also decreased the levels of IL-12p70, TNF-α, and IL-6 effectively in both 1:1 and 1:10 co-culture ratio. And, BM-MSCs increased the levels of IL-10 and TGF-β in 1:1 and 1:10 ratio. The gene expressions of IDO, HO-1, PD-L1 and ICOS which explored BM-MSCs for 6 hr were increased in 1:1 co-culture ratio.

IDO is a catabolitic enzymes that degrades the essential amino acid tryptophan (trp), which can suppress T cell proliferation by producing metabolic products including kynurenine. The potential role of IDO in immune suppression first gained attention because of its involvement in Treg induction[25]. Recently, as well as DC tolerance, the induction of Tregs have shown to increase the IDO expression or activity in vitro and in vivo[24]. HO-1 is a microsomal enzyme with antioxidant, antiapoptotic, and immunoregulatory functions in the degradation of heme to biliverdin, carbon monoxide, and iron[26]. Recently, it is also involved in immune regulation and anti-inflammation through a single reported clinical case of HO-1 deficiency show that a

systemic lack of HO-1 results in profound immune dysregulation, characterized by a number of autoimmune lesions. HO-1 plays a role in the active inhibition of T cell responses by inducing tolerogenic DCs[27]. In the present study, gene expressions both IDO and HO-1 were significantly increased in DC co-cultured with BM-MSCs suggesting that the involvement of IDO and HO-1 on immunomodulatory function of BM-MSCs against DC maturation.

ICOS is critical in the regulation of humoral response, and has also been linked with Treg maintenance in mice and mucosal tolerance[28]. In human ICOS plays a predominant role in pDC/T-cell interaction and participates in naive $CD4^+$ T cell polarization into IL-10-secreting Tr1-like cells. ICOS was also associated with Treg homeostasis in nonobese diabetic (NOD) mice and with increased Treg proliferative capacity and immunosuppressive functions. In the present study, the gene expression of ICOS was significantly increased in DC co-cultured with BM-MSCs suggesting that the involvement of ICOS on anti-inflammatory DC functions after experiencing BM-MSCs.

PD-L1 is found on resting T cells, B cells, macrophages, and dendritic cells, a negative regulatory pathway that results in T-cell exhaustion. PD-1: PD-L signaling also plays an important role in the maintenance of peripheral cell tolerance and the generation of induced regulatory T cells[29]. The PD-1–PD-L1 pathway exerts its effects during the initial phase of activation and expansion of autoreactive T cells by attenuating self-reactive T cell responses during presentation of self-antigen by DCs.

Treg cells also upregulate PD-1 expression on their surfaces during chronic viral infection[29]. PD-1: PD-L1 interaction has a critical role in promoting the conversion of conventional T cells into Treg cells and maintaining them. In this study, the expression of PD-L1 was highly increased in DC co-cultured with BM-MSCs suggesting that the

pivotal role of PD-L1 to induce tolerance and Tregs.

This is first reports that show total gene expressions of DC co-cultured with hBM-MSCs assessed by microarray. Also, a unique genetic signature of DC co-cultured with BM-MSCs was identified that distinguish from classical DC and other DC subsets using various approaches. Many biological functions involved the immune modulation of BM-MSCs on DC, of course, including inflammatory responses. Despite of new features of DC explored MSCs, the genuine function of DC maintained under co-culture condition. Detailed regulations and functions of selected genes will be required to determine the extent to which major factor alter DC differentiation and to define which factors might be a good marker for regulatory DC.

In vitro studies with human, baboon, and murine MSCs demonstrated that MSCs suppress the proliferation of T cells induced by alloantigens or mitogens. Furthermore, MSCs have been reported to induce T cell division arrest, to inhibit the differentiation and maturation of dendritic cells (DCs), and to decrease the production of inflammatory cytokines by various immune cell populations. Several factors and molecules secreted by MSCs have been linked to the immunoregulatory function of these cells[30]. These include TGF-β, NO, IDO, TSG6, prostaglandin E2, IL-1 receptor antagonist, IL-10 and an antagonistic variant of the chemokine CCL2. Also, several studies have examined their direct effects on T cells by determining how MSCs affect proliferation, apoptosis, differentiation and regulatory mechanisms in T cells, including the induction of Treg cells mediated by iNOS or IDO[30]. Therefore, further studies need to be investigating the immune modulatory mechanism of MSCs from a perspective of the direct effects on other immune cells or their secretomes.

In this present study, human BM-MSCs and murine DC or CD4+ T cells were co-cultured to explain the innunosuppressive functions of MSCs in in vitro. According to

previous published data, MSCs are not rejected by the immune system, even after allogenic or xenogenic transplantation[5,6,12], because T-cells do not recognize them. They express few or no class II MHC molecules or costimulation molecules, such as CD80 and CD86[1-3]. Nevertheless, the mechanism study using co-culture system of hBM-MSC and human DC or CD4+ T cells needs to be further investigated.

According to data, human BM-MSCs significantly increased Foxp3+Tregs in co-culture with $CD4^+$ T cells in vitro. The cells decreased pro-inflammatory cytokines and up-regulated anti-inflammatory cytokines and induced PD-1^+ Tregs. These results show that the immune modulatory effect of MSCs was associated with Tregs induction, consistent with the increase in anti-inflammatory cytokine expression. In addition, human BM-MSCs significantly suppressed the maturation of DC, and increased the anti-inflammatory cytokines and tolerogenic genes relating to increase of anti-inflammatory and regulatory DC in peripheral tissues under inflammatory condition. Therefore, these findings provide evidences into the mechanisms of $Foxp3^+$ Treg induction, and new insights into anti-inflammatory and regulatory DC functions.

Ⅴ. CONCLUSION

These data showed that human BM-MSCs inhibit the maturation of DCs and induce anti-inflammatory signals of DCs in *in vitro*. Moreover, our data showed that human BM-MSCs induced the $CD4^{+}Foxp3^{+}$ Tregs in both *in vitro* and *in vivo*.

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Abstract (in Korean)

콜라겐 유도 관절염 마우스 모델에서 T 세포와 수지상세포에 대한 중간엽줄기세포의 면역조절 및 항염증 기전 규명

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문 진 희

중간엽줄기세포는 면역조절능을 가진 줄기세포로, 이를 이용한 세포치료가 많은 염증성 질환 모델에서 연구되고 있다. 중간엽줄기세포의 면역조절능은 면역세포의 증식과 활성화를 저해하는 것과 관련되어 있다. 수지상세포는 선천 면역반응을 매개하는 중요한 면역세포로 항원전달기능을 가지고 있다. 수지상세포는 염증성 수지상세포로 성숙할 수 있고 이는 후천면역반응을 일어나게 한다. T 세포는 Th1, Th2, Th17 또는 조절 T 세포로 분화할 수 있으며, 항원전달세포의 신호를 받아 후천면역반응을 조절하는 중요한 면역세포이다. 본 연구는 중간엽줄기세포가 가진 수지상세포와 T 세포에 대한 면역조절능 및 그 기전에 대해 연구하였다.

DBA/1 마우스의 비장에서 CD11C 양성 수지상세포를 분리하여 GM-CSF와

IL-4 처리하여 7일 간 미성숙 수지상세포로 분화하였다. CD4 양성 T 세포는 CD4 양성 T 세포 분리키트를 이용하였다. 다양한 공배양 비율로 수지상세포와 T 세포 각각 인간 골수 유래 중간엽줄기세포와 공배양하였다. 수지상세포의 성숙 및 T 세포 분화에 대한 중간엽줄기세포의 영향은 flow cytometry로 확인하였고, 공배양 상층액에서 사이토카인의 발현을 확인하였다. 유전자 발현은 qRT-PCR 및 microarray로 확인하였으며, 콜라겐 유도 관절염 마우스 모델에서 조직학적 염색을 시행하였다.

중간엽줄기세포와 공배양한 조건에서 CD4+CD25+FoxP3+조절 T 세포의 발현이 높게 증가하였다. 또한, 중간엽줄기세포는 PD-1, Nrp-1 유전자 발현 역시 증가시켰다. 항염증성 사이토카인인 IL-10, TGF-β의 발현 또한 증가하였으며, 중간엽줄기세포를 처치한 콜라겐 유도 관절염 마우스 모델의 발 조직에서 PD-1의 발현이 증가하였다.

골수 유래 중간엽줄기세포는 1:1 및 1:10 공배양 조건에서 CD86 및 MHCⅡ의 발현을 감소시킴으로써 수지상세포의 성숙을 저해하였다. 또한, 염증성 사이토카인인 IL-12p70, TNF-α, IL-6의 발현을 감소시키고, 항염증성 사이토카인인 IL-10, TGF-β의 발현은 증가시켰다. 또한, 1:1 비율에서 6시간 동안 중간엽줄기세포와 공배양한 수지상세포에서 IDO, HO-1 유전자발현이 증가하였다.

본 연구를 통하여 골수 유래 중간엽줄기세포가 수지상세포의 성숙을 억제할 뿐만 아니라, 염증성 수지상세포를 항염증성 수지상세포로 유도함을 증명하였다. 또한, 골수 유래 중간엽줄기세포는 세포 공배양 및 동물 모델 연구를 통해 CD4+CD25+FoxP3+ 조절 T 세포 발현을 증가시켰다.

핵심되는 말: 중간엽줄기세포, 면역조절, 수지상세포, 조절 T 세포

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