의학석사 학위논문

사람 Th17 세포 분화 및 탈분화에 대한 사람 지방 줄기 세포 유래 엑소좀의 조절 효과

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2019 년 8 월

서울대학교 대학원

의과학과 의과학전공

최 소 원

A thesis of the Master’s degree

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The regulatory effect of human adipose stem cell–derived exosome on human Th17 cell differentiation and subversion

by

So Won Choi

A thesis submitted to the Department of Biomedical Sciences in partial fulfillment of the requirements for the Degree of Master of Science in Medicine at Seoul National University College of Medicine

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Professor _____Chairman

Professor _____Vice chairman

Professor _____

ABSTRACT

Mesenchymal stem cells (MSCs) are multipotent adult stem cells that can be obtained from bone-marrow, fat tissue, umbilical cord, etc. MSCs can differentiate into some kinds of cells and promote tissue regeneration, especially with immune control. Exosome, one of the paracrine factors, is reported to be crucial in the immune-modulatory function of MSCs. For this reason, while a lot of studies have been actively conducted on immune cell suppression by MSC-derived exosomes (MSC-exosomes), relatively few studies have been made on the effect of human MSC-exosomes on human immune cells, especially including Th17 cell. Therefore, in this study, I tried to figure out the effect of human ADSC-exosomes on human Th17 cell differentiation and subversion. Exosomes were isolated from the culture supernatant of the human ADSCs (hADSCs) through differential ultracentrifugation. Isolated exosomes were treated into human $CD4^+$ T cells in the beginning or middle of in vitro differentiation into Th17 cells. As a result, the hADSC-exosomes did not regulate the differentiation of human Th17 cells at the level of mRNA and protein. On the other hand, under the Th17 differentiation conditions, the proliferation of $CD4^+$ T cells was suppressed by the exosomes. Also, when the exosomes were treated to in vitro differentiated Th17 cells, exosome did not significantly reduce the mRNA and protein level of RORγt, a master regulator of Th17 cells, and IL-17a, a typical cytokine of Th17 cell. On the contrary, in the subsequent cytokine secretion assay, the exosomes decreased the secretion of IL-17a from Th17 cells by 6.5 times. This result verified that hADSC-exosomes have the potentiality to control the IL-17a secretion from Th17 cells.

Keywords: Adipose tissue-derived Stem Cells, Exosome, Immunomodulation, Th17

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LIST OF ABBREVIATIONS

BM-MSC: Bone-marrow-derived mesenchymal stem cell

ADSC: Adipose tissue-derived mesenchymal stem cell

ADSC-exosome: ADSC-derived exosome

Th17: T helper 17 cell

IL-17a: Interleukin –17 a

RORγt: RAR-related orphan receptor gamma T

Treg: Regulatory T cell

FACS: Fluorescence-Activated Cell Sorting

CBA: Cytometry bead assay

TGF-β: Transforming growth factor-beta

CD: Cluster of differentiation

FBS: Fetal bovine serum

PBMC: Peripheral blood mononuclear cell

CFSE: Carboxyfluorescein succinimidyl ester

qRT-PCR: Quantitative real time-polymerase chain reaction

PMA: Phorbol-12-myristate-13-acetate

INTRODUCTION

Mesenchymal stromal/stem cell (MSC) is a stromal cell which has multi-potentiality and various functionality. MSC can be acquired from different sources; bone-marrow, adipose tissue, umbilical cord blood and dental pulp, etc. To be classified as MSC, the minimal criteria proposed by the International Society for Cellular Therapy position statement must be met. 1) MSC must be plastic-adherent when maintained in standard culture conditions. 2) MSC must express CD90, CD73 and CD105, and lack expression of CD14, CD34, CD45 or CD11b, CD79α or CD19 and HLA-DR surface molecules. 3) MSC must differentiate to adipocytes (fat cells which give rise to adipose tissue), osteoblasts (bone cells), and chondroblasts (cartilage cells) in vitro. This consensus provided standards to define and characterize MSC.

MSCs are widely used as a cell therapy because they can move to tissue damage sites, and to promote angiogenesis as well as multi-differentiate. It has been known that these abilities are largely due to effects made by cell-to-cell contact, but also by a paracrine manner.

Not only some immunomodulatory substances including prostaglandin E2 (PGE2), indole amine 2,3-oxygenase (IDO), and inducible nitric oxide, exosomes are well known for having a critical role in MSCs' immunomodulation via paracrine manner.

An Exosome is one of the extracellular vesicles which general eukaryotic cells secrete. Exosomes are 50 nm – 100 nm in size and are secreted by the way vesicles release out of the cells as multi-vesicular endosomes are formed and fused with the membrane. Because they contain protein, miRNA, RNA, and DNA, which are information from source cells, they can mimic the function of primitive cells and change the characters and functions of recipient cells.

For this reason, exosome has been considered to alternate a cell therapy and investigated in preclinical studies. In preclinical setting using human samples, human embryonic stem cell(ESC) line-derived exosomes activate an M2 macrophage-like phenotype (1), and human BM-MSC-derived exosomes downregulate Th1 responses and reduce the percentage of Th17 cells from patients with type 1 diabetes (2). The immune-modulatory effects of bone-marrow MSCs (BM-MSCs) and their extracellular vesicles have been well discovered and studied a lot. As well as BM-MSC-derived exosomes, exosomes from adipose-derived stem cells (ADSCs) have also been tested on T lymphocyte proliferation and activity (3). In this bibliography, exosomes from ADSCs shows an anti-proliferative effect on both $CD4^+$ and $CD8^+$ cells (3). Among these MSC-exosome-mediated immune cell modulation, the immune-suppressive functions against Th17 cell which is much related to autoimmune diseases remain unclear.

In our laboratory, it was confirmed that murine bone marrow-MSC derived exosome suppress Th17 cells specifically. The exosome downregulated the level of RORγt by depleting the stability of RORγt identified as the master regulator required for Th17 lineage. Then the exosome inhibited the differentiation of naïve $CD4^+$ T cells to Th17 cells and also induced the subversion of Th17 cells by impairing RORγt. This showed that a specific substance within exosome could regulate RORγt at the protein level. However, these results were confirmed by the *in vitro* and *in vivo* model to determine the effect of murine BM-MSC-exosome on murine Th17 cell not on human Th17 cell.

According to some reports, mechanisms of MSC-mediated immunosuppression vary among different species. As Ren G et al. showed, human and murine MSCs show different aspects of immunomodulatory substances which they secrete after stimulated by inflammatory cytokines (4).

For example, human MSCs mediate immunosuppression using IDO mainly whereas murine MSCs use iNOS under the same culture condition.

MSC characteristics are also dependent on the tissue source from which they were harvested as well as on species-specific factors. MSC can be isolated from many tissues including bone marrow (BM), adipose tissue (AT), placenta (PL), umbilical cord (UC, Wharton's jelly) or umbilical cord blood (UCB), respectively. Most experiments in mice, rats, and monkeys were performed using BM-MSC. In human, BM-MSC is used most frequently, but AT, PL, and especially UCB also serve as sources for MSC isolation (5). Also, differences in the immunosuppressive capacity of MSCs come from different tissues. BM-MSCs of early passage are likely the optimal source of immunomodulation (6). However, in human, sources for BM-MSCs are limited. On the other hand, adipose tissue-derived stem cells (ADSCs) are easier to be acquired from adipose tissue. Adipose tissue is an abundant, expandable, and easily obtained tissue that may prove to be an ideal source of autologous stem cells for regenerating tissues.

Therefore, exosome derived ADSC, which is a sort of human MSC, was isolated in this study. Also, it was tried to confirm whether human ADSC-exosome hinders human CD4+ cell from differentiating to Th17 cell and induces in vitro differentiated human Th17 cell to be subverted as in the murine system. Although current studies are trying to develop effective artificial exosomes by engineering MSCs, the human ADSC-exosomes' effect and function were rarely confirmed in human system. So, this study aims to evaluate the human ADSC-exosomes' effect on human system without any biological modification or environmental condition change to human ADSC.

So, I tried to find out if the exosomes derived from ADSCs can modulate Th17 cells as murine BM-MSC-derived exosomes did.

MATERIALS AND METHODS

1. Cell Culture and Characterization of Human Adipose-derived stem cell (hADSCs)

1) Exosome-depleted FBS

To deplete exosomes contained in fetal bovine serum (FBS, Young In Frontier, Korea), the FBS was diluted into 40% in low-glucose (1 mg/L) Dulbecco's Modified Eagle's Medium (DMEM, GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) and ultra-centrifuged at 100,000 x g for 17 hours at 4℃ using 70Ti rotor (Beckman Coulter, Brea, CA, USA). The exosome-depleted 40 % FBS medium was aliquoted and kept in -20℃.

2) Human ADSC Culture

Human adipose-derived stem cells (hADSCs; Catholic University, Department of Medical Life Science, IRB approval no. 17-2018-0030) were cultured in low-glucose DMEM (GE Healthcare Bio-Sciences) supplemented with heat-inactivated and exosome depleted 10% FBS (Young In Frontier) and Antibiotic-Antimycotic (Anti-Anti, Gibco, Waltham, MA, USA). 5 x 10^5 cells/ml of hADSCs were plated per culture dish (150mm diameter) and split every 4-5 days on which cell density reaches 80-90%. The culture supernatant was collected and centrifuged at 300 x g for 10 minutes to remove the cell debris and cells. The supernatant was aliquot and kept at -80℃.

3) Human ADSC Characterization by Flow Cytometry

Human ADSCs were detached with trypsin and washed with Flow Cytometric Assay Cell Staining (FACS) buffer. Then, the cells were incubated with anti-human FcR binding

blocker (eBioscience, Thermofisher Scientific) for 15 minutes and stained with allophycocyanin (APC)/cy7-anti-CD13 (Biolegend, San Diego, CA), Phycoerythrin (PE)-anti-CD44 (Biolegend), PE/cy7-anti-CD73 (eBioscience), Peridinin-Chlorophyll-protein/cy5.5-anti-CD90 (eBioscience), APC-anti-CD105 (eBioscience) for 30 minutes at 4℃ in dark. Cells were analyzed using a Fortessa X-20 (BD Bioscience, San Jose, CA, USA). For fluorescence compensation, one color control was prepared using OneComp eBeads™ Compensation Beads (Thermofisher Scientific). Isotype control antibodies; mouse IgG1, κ – APC/cy7 (Biolegend), mouse IgG1, κ – PE, PerCP/cy5.5, APC, mouse IgG2a, κ - PE/cy7, mouse IgG2b, κ – FITC, rat IgG2a, κ – PE (eBioscience).

2. Exosome Isolation, Characterization, and Quantification

1) Exosome Isolation from the hADSCs culture supernatant by differential centrifugation

To remove the dead cell, the hADSCs culture supernatant was centrifugated at 2,000 x g, 4℃ for 20 minutes and pellets removed. Then, left cell debris was removed by centrifugation supernatant at 10,000 x g, 4℃ for 30 minutes using A50S-8 rotor (high-speed centrifuge, Supra R22, Hanil Science, Korea). Subsequently, the supernatant was ultracentrifuged at 100,000 x g, 4℃ for 150 minutes using 70Ti rotor (Beckman Coulter). The weight balance was the same for each tube to the second decimal place. The resulting pellets, which include exosomes and contaminating proteins, were resuspended in 15 ml PBS and ultracentrifuged at 100,000 x g, 4℃ for 150 minutes using 45Ti rotor (Beckman Coulter). The pellets were re-suspended in about 100 to 200 μl volume of PBS.

2) Quantification of Exosome using Bicinchoninic Acid Protein Assay.

The resuspended exosomes were diluted into 1 to 10, 50, 100 ratios with PBS and then quantified using bicinchoninic acid (BCA) protein assay (Thermofisher Scientific). BSA serial-diluted from 2mg/mL to 0mg/mL was used for standard graph. The results were read at 562nm using Sunrise absorbance microplate reader (Tecan Life Sciences, Switzerland).

3) Imaging of Exosome by Transmission Electron Microscopy

All steps had conducted by an engineer from the Dental Research Institution at Seoul National University. In negative staining consequences, 10ul of exosome suspension was put on formvar/carbon film-coated 200 mesh copper EM grid and incubated for 5 minutes at room temperature (RT). After absorbing the solution on the grid using 3M paper, 2.5% uranyl acetate (UA) solution was filtered with a tightly spaced EM paper and loaded sufficiently to stain. The excess UA solution on the grid was removed by contacting the grid edge with 3M paper. The grid was washed four times with deionized water in 24-well culture plates and dried at RT. The grid was observed with a Transmission electron microscope (JEM-1200EX II, JEOL, Japan) at a magnification of 100,000 times at 80kv.

3. Polarization into Th17 and Exosome treatment

1) $CD4^+$ T cells isolation

Human fresh heparinized blood (40 mL) was obtained from 5 healthy controls (IRB approval no.17-2018-0030). Peripheral blood mononuclear cells (PBMCs) were purified from heparinized blood using a Ficoll gradient (Ficoll-Paque™ PLUS, GE Healthcare) by centrifugation at 900 x g for 30 minutes at RT. After that, $CD4^+$ T cells were sorted negatively

by Magnetic Associated Cell Sorting (MACS, Miltenyi Biotec, Germany) and suspended in RPMI 1640 media (HyClone, GE Health care) supplemented with 10 % FBS (Young In Frontier) and 1 % Anti-Anti (Gibco).

2) CFSE labeling for cell proliferation assessment

Isolated $CD4^+$ T cells were washed with PBS and resuspended in 900 μL of PBS. Carboxyfluorescein-succinimidyl-ester (CFSE) was diluted to 10 μM in PBS. The 100 μL of 10 μM of diluted CFSE was added to CD4+ cells suspension and incubated for 5 minutes at room temperature (RT). Then, the staining was stopped by adding 9 mM of cold T cell-complete media and washing the cells twice with the complete media.

3) $CD4^+$ T cells Polarization into Th17 cells

The $CD4^+$ T cells were seeded at 2.5 x 10^5 cells/well in 48-well-bottom plates (Corning, Durham, NY) and cultured with Dynabeads Human T-Activator CD3/CD28 (Thermofisher Scientific) at a bead-to-cell ratio of 0.1:1 and cytokines that include 5ng/mL of rhIL-1β and 20ng/mL of rhIL-6 (R&D Systems, Minneapolis, MN) and 1ng/mL of rhTGF-β (Peprotech, Rocky Hill, NJ). Cells were used after 5-7 days of culture in a 37℃, 5% CO_2 incubator.

4) Exosome treatment

In order to confirm the effect of exosomes to Th17 differentiation and cytokine secretion, exosomes from 50 μg/mL were treated into the culture supernatant on day 0. The cells were analyzed on day 5. Whereas the effect of exosomes to fully differentiated Th17 cell was explored by treating exosome from 112.5 μg/mL into the culture supernatant on day 5 and cells were analyzed on day 7.

4. Surface Marker, Intracellular Cytokine and Transcription Factor Staining

For intracellular cytokine staining, the differentiated cells were re-stimulated with 50 ng/mL of PMA (Sigma-Aldrich, St.Louis, MO) and 1ug/mL of ionomycin (Sigma-Aldrich) for 5 hours at 37℃ in the presence of Brefeldin A (eBioscience) for the final 4 hours of culture. (All antibodies used for flow cytometric analysis were anti-human Abs. For assessment of surface marker, cells were stained with anti-CD4 antibody (PE/cy7-conjugated, eBioscience) with anti-human FcR binding blocker for 30 minutes at 4℃ in the dark. For intracellular cytokines and transcription factor staining, cells were fixed and permed simultaneously by Foxp3 Transcription Factor Staining Buffer Set (eBioscience) for 30 minutes at RT in the dark. Subsequently, cells were incubated with 2μl of 10% mouse serum to block unspecific binding of mouse IgG antibody and stained with anti-IL-17a antibody (PE-conjugated, eBioscience), and anti-RORγt antibody (APC-conjugated, eBioscience) for 30 minutes at 4℃ in the dark. Samples were analyzed in BD CantoⅡ using FACS DIVA software (BD Bioscience), and results were evaluated using FlowJo software (BD Bioscience).

5. Quantitative real-time PCR

After polarization with or without exosomes, CD4 cells were restimulated with pma (50 ng/mL) and ionomycin (1 μg/mL) for 4 hours at the same concentration as before. In the different sets of experiments, RNA was collected from the CD4 cells using the Trizol® reagent (Invitrogen), according to the manufacturer's protocol. The purity and concentration of RNA were measured by NanoDrop™ 2000/2000c Spectrophotometers (Thermo Fisher Scientific). The RNA was directly used for first-strand cDNA synthesis or kept at -80℃. cDNA was produced from 600 ng of total RNA using the SuperScript™ Ⅲ First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen). Quantitative Real-time PCR (qRT-PCR)

analyses were performed in triplicate and each group included 20μl reaction mixture containing 10ng of cDNA template, the sequence-specific oligonucleotide primers (purchased from Cosmogenetech, Seoul, Korea and listed in Table 1) and the Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The qRT-PCR was performed using the CFX Connect™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The run protocol is equal as follows. 1: 50℃ for 10 minutes. 2: 95℃ for 5 minutes. 3: 95℃ for 10 seconds. 4: 60℃ for 30 seconds and plate read. 5: Go to 3, 39 more cycles. 6: 95℃ for 10seconds. 7: Melt Curve from 65℃ to 95℃; increment 0.5℃ every 5 seconds. 8: plate read.

6. Cytokine secretion assay and Cytometric bead array (CBA)

The cells which were differentiated to Th17 for 5 days and co-cultured with hADSC-exo next 2 days. After that, at day 7, the cells were harvested, washed and redistributed 7.5 X 10^4 cells per well (96 well-U-bottom plate). Then, the cells were re-stimulated with Dynabeads Human T-Activator CD3/CD28 (Thermofisher Scientific) at a bead-to-cell ratio of 1:1 for 2 days more. At day 9, the culture supernatants were collected and analyzed by using Human IL-17A Flex Set cytometric bead array (CBA) (BD Bioscience). The supernatants only from Th17 group and Th17-exosome treated group was diluted in half with assay diluent. Standards and samples were prepared as described in manufacture's instruction manual and then analyzed by BD CantoⅡ flow cytometry using FACS DIVA software (BD Bioscience).

RESULTS

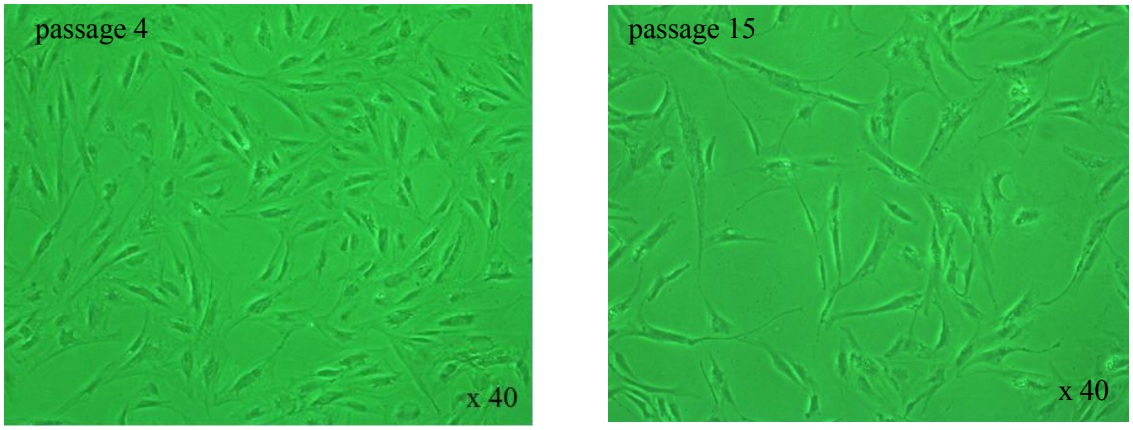
1. Characterization of human adipose-derived stem cells (hADSCs)

Human ADSCs (passage 2 to 4) derived from adipose tissue were provided from the department of plastic and reconstructive surgery in the college of medicine of the Catholic University. The cells were grown sticking on a plastic culture dish and their morphology was flat and spread out like fibroblast (Fig. 1A).

As they grow up, their size was larger and their spindle shape was sharper. To avoid contact inhibition between cells, when the cell density reached 70-80%, the cells were subcultured and the average doubling time was 5 to 7 days in the case within passage 10. Beyond passage 11, the cells hardly expanded and doubling time was over 10 days (Fig. 1B).

For characterization of mesenchymal stem cell, the cells in passage 2 to 4 were cultured for 1-2 weeks and analyzed for the surface marker. Although there are controversial in MSC-specific surface antigen, several antigens including CD73 and CD105/endoglin, were used for MSC characterization. Plus, additional positive markers which can also be found on other cell types include CD13, CD44, and CD90 (8). These positive cell surface markers, together with the absence of hematopoietic, lymphoid and myeloid markers, are now routinely used in MSC characterization (9). Human ADSCs cultured for 1-2 weeks were detached with trypsin and washed with PBS, and then incubated with human Fc-Receptor binding inhibitor to prevent unspecific antibody binding before surface antigen staining. The cells were positive for CD13, CD44, CD73, CD90 and CD105 (Fig. 1C) on the surface but negative for hematopoietic lineage markers such as CD11b, CD45, CD80, CD86, and HLA-DR (Fig. 1D).

A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x 40
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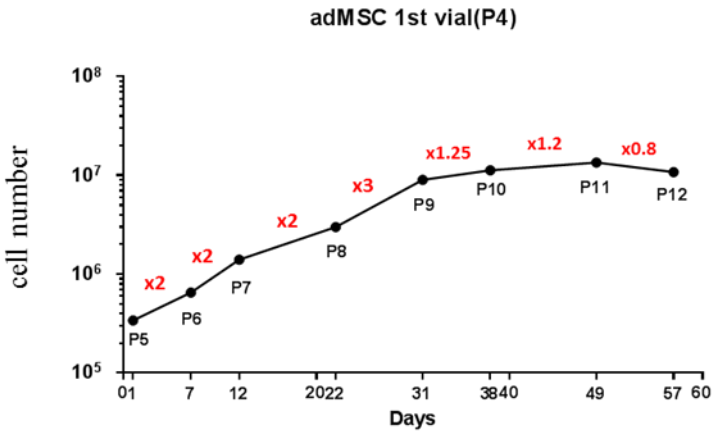
B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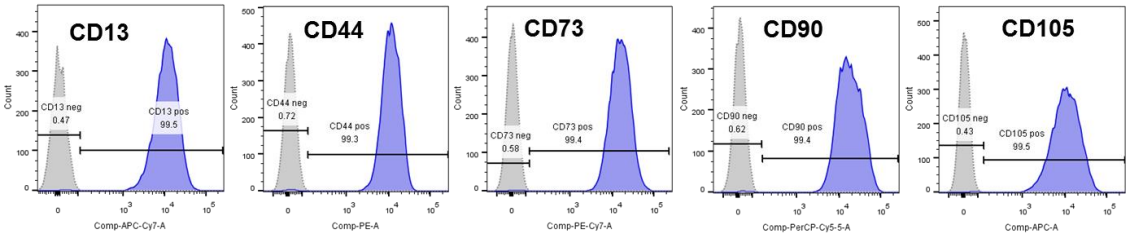

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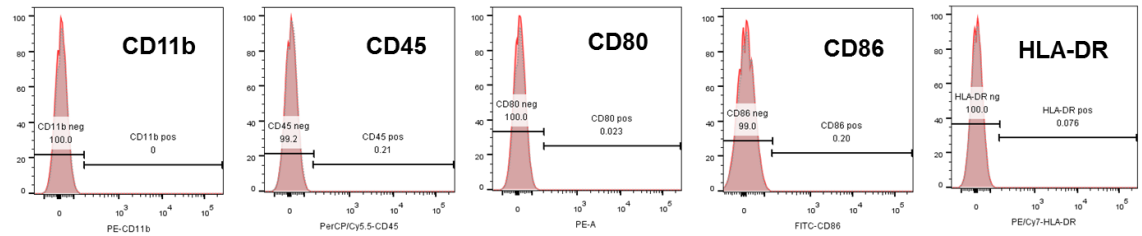


Figure 1. Characterization of human ADSCs

A fibroblast-like and plastic-adherent growing form of human ADSCs in passage 4 (left) and passage 15 (right) (A). When filled up to 80 %, the cells were subcultured and counted. Based on the cell count, doubling time for human ADSCs between passage 5 to passage 12 were decided (B). Cell surface marker expression in human ADSCs (C; positive, D; negative). Grey part of the graph represents each isotype control and all samples were treated with human Fc Receptor binding inhibitor for 15 minutes.

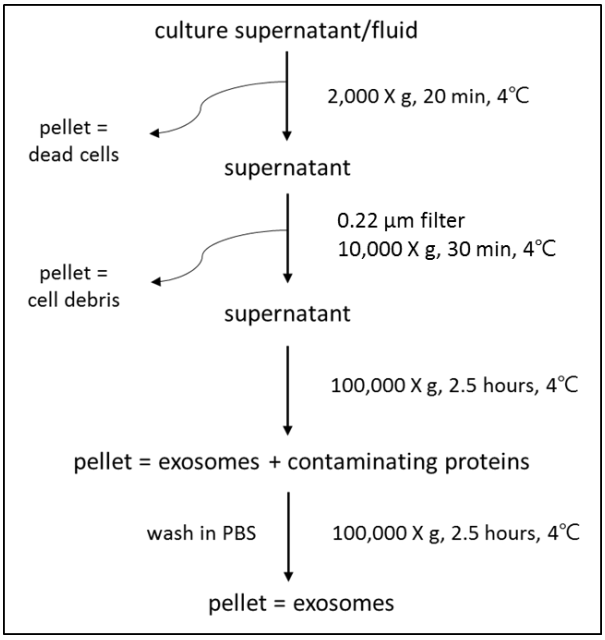
Isolation and observation of exosomes from human ADSCs

Human ADSCs were cultured in low-glucose DMEM containing 10 % exosome-depleted FBS and subcultured every 7-8 days. To rule out the effect from FBS-exosome, 40 % FBS in low-glucose DMEM was centrifuged at 100,000 x g for 15 hours, and then the only supernatant was collected and used for culture media. The culture supernatant was collected every subculture and kept at –80℃ and only supernatant of cells between passage 3 and 11 was used for exosome isolation.

Exosomes were isolated from the supernatant via differential centrifugation (Fig. 2A). The exosome isolation process is precisely delineated in material & methods. Purified Exosomes from about 600 mL- supernatant were re-suspended in 200 μl of sterile PBS, and immediately quantified using BCA assay or stored at –80℃. On average, about 55 μg of exosome was acquired from 600 mL-supernatant.

To determine the existence and size of exosomes, they were negatively stained with uranyl acetate (UA) solution on the formvar/carbon film-coated mesh copper grid. Negative staining method is convenient in the regard that an ultrathin section is unnecessary, so it takes a short time compared to block preparation method. After negatively stained, the Exosomes were captured using a transmission electron microscopy (TEM) at 80-kilovoltage. It was verified that lots of exosomes existed and their sizes were in 50-100 nm (Fig. 2B).

A



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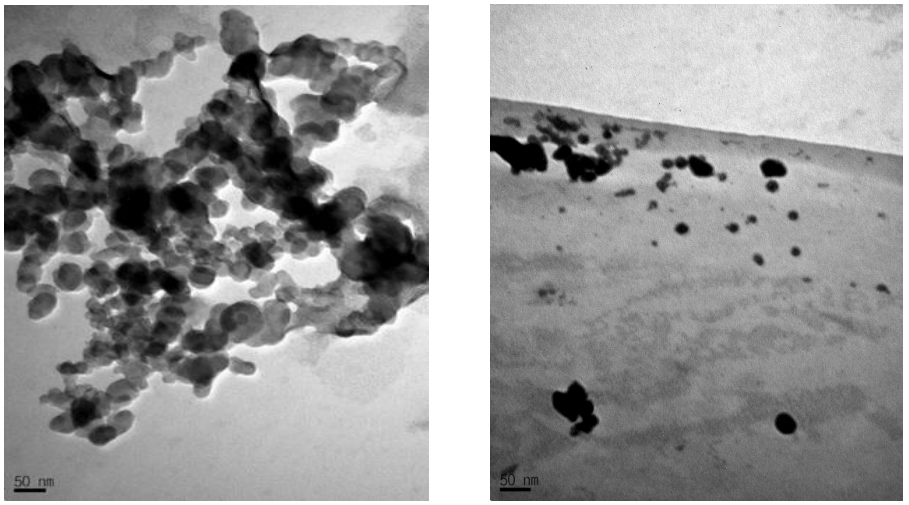


Figure 2. Isolation and observation of exosomes from human ADSCs

Exosomes were purified from the culture supernatant of hADSC from passage 3 to passage 7 through differential centrifugation (A) and then kept or analyzed by BCA assay for quantification. Exosomes' morphology was observed by transmission electron microscopy at 80-kilo voltage (Scale bar = 50nm) after negative staining (B).

Set-up for Th17 polarization condition to verify exosomes' effect over human Th17 cells.

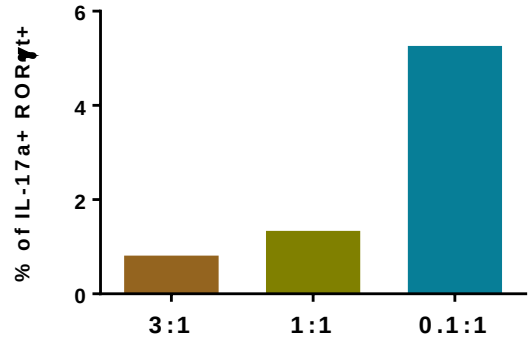
In the murine model, in vitro Th17 polarization condition is well established and consented internationally. However, in human, in vitro Th17 differentiation condition is little different from that in mice and not well built up relatively. So, the proper Th17 polarization condition was decided for testing hADSC-exosomes' effect. Plus, to rule out the impact of non-$CD4^+$cells, including $CD8^+$ T cell, B cell, monocyte and dendritic cell, $CD4^+$ cells were isolated from human PBMCs by magnetic-activated cell sorting (CD4 negative).

As a strength of TCR stimulation is associated with Th17 differentiation (10), human T cell expansion bead concentration was determined first. The anti-CD3/CD28 T cell activator beads to cells ratio was conducted in three ways (3:1, 1:1, 0.1:1) and cells were incubated for 6 days with cytokines which include IL-1β (5 ng/mL), IL-6 (25 ng/mL), IL-23 (25 ng/mL), and TGF-β (10 ng/mL). On day 6, the frequency of each cell positive for IL-17a, and RORγt was analyzed by flow cytometry. Among three ratios, the IL-17a and RORγt positive cell population increased most at a ratio of 0.1:1 on day 6 (Fig. 3A). This result correlated with previously reported data (10).

Following to the TCR stimulation strength optimization, two conditions including different cytokine combination were tested. First condition includes IL-1β (5 ng/mL), IL-6 (25 ng/mL), TGF-β (10 ng/mL), and IL-23 (25 ng/mL), while second condition includes IL-1β (5 ng/mL), IL-6 (20 ng/mL), and TGF-β (1 ng/mL). Although $CD4^+$IL-$17a^+$$ROR\gamma t^+$ cells were generated little more with the second condition when the cell population was confirmed on day 4 and 6, the difference was not great (Fig.3B). But, IL-23 affects the stability of RORγt and makes Th17

keeping their character strongly. Since this study aims to figure out that ADSC-exosome can regulate the RORγt at the protein level, the condition including IL-23 was excepted.

A



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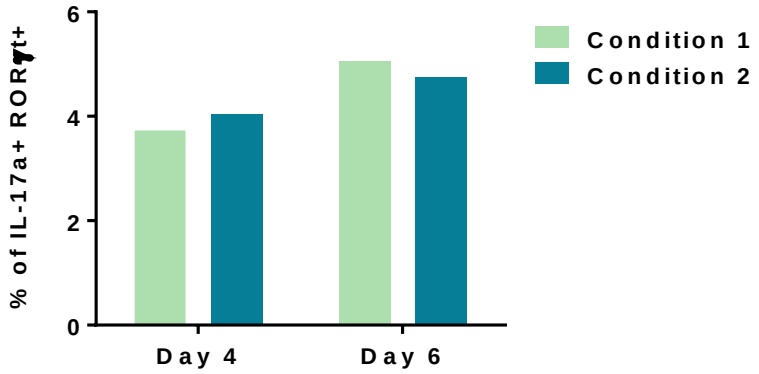


Condition	Cytokine Combination
Condition 1	IL-1β (5 ng/ml), IL-6 (25 ng/ml), TGF-β (10 ng/ml), IL-23 (25 ng/ml)
Condition 2	IL-1β (5 ng/mL), IL-6 (20 ng/mL), TGF-β (1 ng/ mL)

Figure 3. FACS analysis of $CD4^+$ T cells under several Th17 differentiation setting.

Th17 cell population among $CD4^+$ T cell after 5 day-incubation under Th17 polarization with different strength of TCR (anti-CD3/CD28) stimulation (A) or cytokine combinations (B). These data are drawn from a single experiment.

Human ADSC-exosomes have no significant effect on Th17 differentiation

Based on optimized Th17 differentiation conditions, $CD4^+$ T cells were first isolated and differentiated into Th17 cells under the predetermined differentiation condition. A total of 2.5 x 10^5 cells per well (48-well) were seeded and stimulated with predetermined Th17 cell polarization condition as described "Material and Method" section and co-cultured either alone or with the hADSC-exosomes at the final concentration of 50 μg/mL. Undifferentiated $CD4^+$ T cells were determined as a negative control, and Th17-differentiated $CD4^+$ T cells without exosomes were used for positive control. At day 5, $CD4^+$ cells were collected and re-activated with pma (50 ng/mL) and ionomycin (1 μg/mL) for 5 hours and brefeldin A (BFA) for last 4 hours. And then, CD4 surface antigen was stained first and the cells were permeabilized and fixed. Then, the cells were stained with anti-IL-17a and anti-RORγt fluorescence-conjugated antibody for flow cytometry analysis.

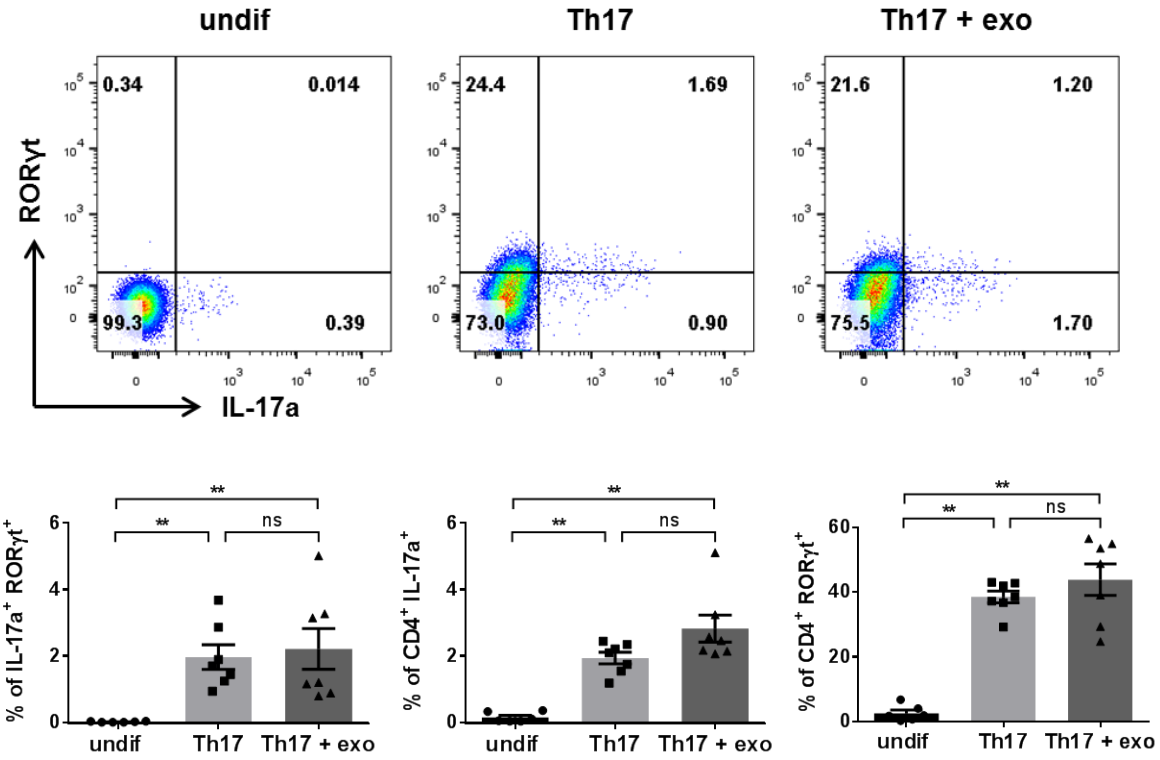
Dot plots of all groups are shown and a numerical value of each target is described as a graph (Fig. 4A). Undifferentiated group and non-exosome treated group were determined as negative control and positive control respectively. The population of IL-$17a^+$ RORγt^+ cell was gated based on the population in the negative control. As a result, the percentage of IL-$17a^+$ RORγt^+ cell among $CD4^+$ T cell showed no significant difference between positive control and exosome-treated group.

It has been reported that exosomes contain various bioactive molecules which modulate immune cells at transcription level as well as protein level. For this reason, the hADSC-exosomes' effect on mRNA level of IL-17a and RORγt in $CD4^+$ T cells stimulated with Th17 polarization condition was also evaluated via qRT-PCR. According to some studies about pma/ionomycin stimulation and mRNA level, the stimulation augments only very low-level transcripts but not change the

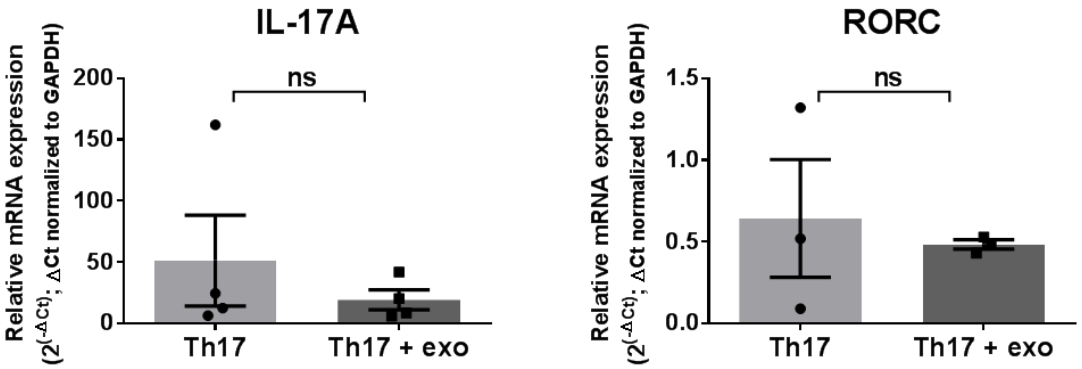
transcript expression pattern inversely. So, $CD4^+$ T cells were also collected and re-activated with pma (50 ng/mL) and ionomycin (1 μg/mL) (BFA wasn't added) for 5 hours. And then, RNA was purified by using Trizol as described in "Materials and Methods" section. The synthesized cDNA (complementary DNA) of 30 ng/μl was used for qRT-PCR. The primers' information is provided in Table 1. All target mRNA levels were normalized by GAPDH mRNA level and $2^{(-\Delta Ct)}$ value is represented in a graphical scheme (Fig. 4B). The graph shows that relative expression levels of IL-17A and RORC mRNA are not significantly different between exosome-treated group and positive control.

In summary, hADSC-exosomes fail to downregulate IL-17a and RORγt at mRNA and protein level significantly. So, hADSC-exosomes do not affect human Th17 differentiation.

A


undif
Th17
Th17 + exo
RORγt
IL-17a
0.34
0.014
99.3
0.39
24.4
1.69
73.0
0.90
21.6
1.20
75.5
1.70
% of IL-17a+ RORγt+
% of CD4+ IL-17a+
% of CD4+ RORγt+
**
ns
undif
Th17
Th17 + exo

B


IL-17A
RORC
Relative mRNA expression
(2(-ΔCt); ΔCt normalized to GAPDH)
ns
Th17
Th17 + exo

‘

Figure 4. Human ADSC-exosomes show no effect on Th17 differentiation.

$CD4^+$ cells were stimulated with Th17 differentiation condition in the absence or presence of hADSC-exosomes for 5 days and analyzed for IL-17a and RORγt expression level using flow cytometry. The percentages of $CD4^+$ cells positive for $IL\text{-}17a^+$ and $ROR\gamma t^+$ intracellular staining are indicated with representative flow cytometry data (A). The values of $2^{(-\Delta Ct)}$ are represented. The value of ΔCt was calculated by subtracting Ct value of GAPDH from that of the target gene (IL-17A and RORC) (B). Data represent two independent experiments in triplicate, mean ± SEM (n=2). Mann-Whiney test (*p< 0.05, **p< 0.01, ***p< 0.001).

Target Name	Forward primer	Reverse primer
hGAPDH	5’- AAG CCT GCC GGT GAC TAA C -3’	5’- GCA TCA CCC GGA GGA GAA AT -3’
hIL-17A	5’- CCC GGA CTG TGA TGG TCA AC -3’	5’- GCA CTT TGC CTC CCA GAT CA -3’
hRORC	5’- GGC AAA TAC GGT GGC ATG G -3’	5’- AAG GCA CTT AGG GAG TGG GAG A -3’

Table 1.

Primers list used for qRT-PCR against human IL-17a and RORC

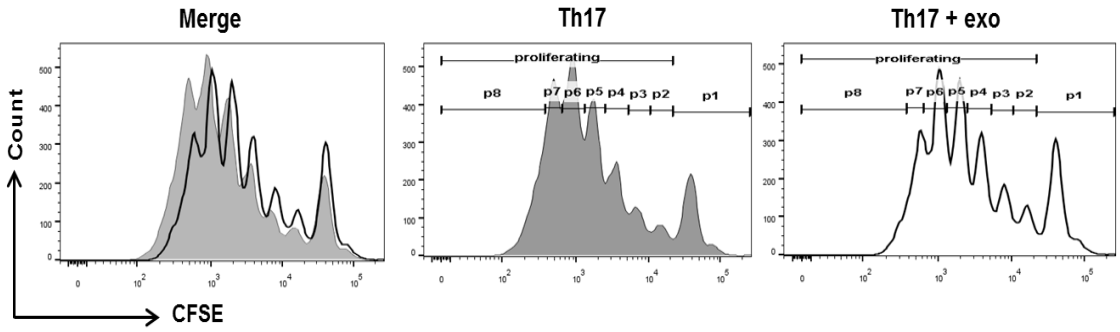
hADSC-derived exosomes attenuate the proliferative ability of CD4+ T cells under Th17 polarization condition.

To figure out whether the reduction of IL-17a producing subset by hADSC-exosomes is down to either diminished proliferative ability or viability, I aimed to verify their effect over the proliferation of CD4+ T cells under modified Th17 differentiation condition. For that, a total of 8 x 10^4 CFSE-labeled $CD4^+$ T cells were stimulated under Th17 polarization condition as referred in "Materials and Methods' section and co-cultured with hADSC-exosomes of 50μg/mL concentration. The proliferation ability was determined by CFSE dilution assay.

Undifferentiated $CD4^+$ T cells were set as a negative control, and Th17-differentiated $CD4^+$ T cells without exosomes were assigned for positive control. In positive control, the CFSE dilution peaks were detected up to a maximum 8 times. As shown in Figure 4A, when human $CD4^+$ T cells were cultured in the presence of hADSC-exosomes under Th17 polarization condition, the proliferation rate was diminished. If non-diluted CFSE peak is regarded as 1st passage and most diluted CFSE peak is determined as 8^{th} passage, in total cells, the percentage of cells belonging to a passage of less than 5 was higher in a group of hADSC-exosomes treated cells than a group of exosomes-untreated cells. Meanwhile, the percentage of cells belonging to the passage of more than 6 was much lower in a group of hADSC-exosomes-treated cells than a group of exosomes-untreated cells. A representative histogram (Fig. 5A) and a graphical presentation showing the percentage of cells belonging to each passage is provided (Fig. 5B). The percentage of total proliferating cells were significantly lower in exosome-treated group than control group. Also, the cell percentage of exosome-treated group was significantly higher in passage 2 and lower in passage 8 than control group.

Through this result, we can guess that hADSC-exosomes restrain the proliferation of $CD4^+$ T cells under Th17 polarization condition via hindering the first trigger of TCR stimulation. These results would indicate that the hADSC-exosomes abrogate proliferation of $CD4^+$ cells being stimulated with Th17 cell polarization condition.

A



B

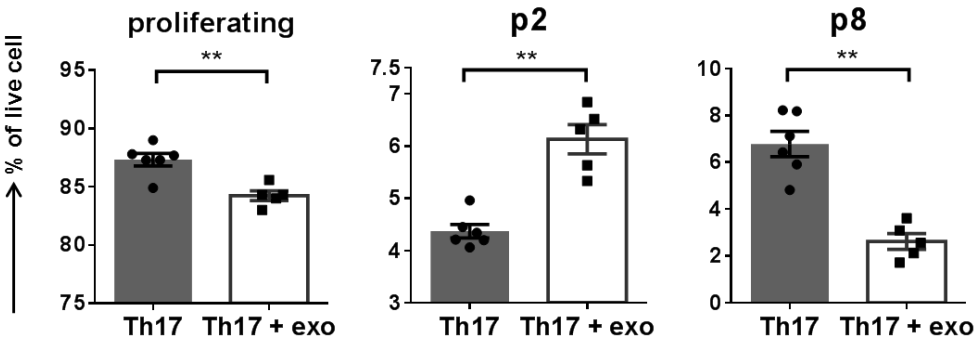


Figure 5. The proliferative ability of CD4+ T cells under Th17 cell polarization condition by hADSC-exosomes.

For assessment of proliferative ability of CFSE-labeled $CD4^+$ T cells were cultured either alone or co-cultured with hADSC-exosomes under Th17 polarization condition (anti-CD3/anti-CD28 bead to cell ratio = 1 : 10, IL-1β (5ng/mL), IL-6 (20ng/mL), and TGF-β (1ng/mL)). At day 5, $CD4^+$ T cells were collected and analyzed by CFSE-dilution peak. A detailed representation of $CD4^+$ T cells showing a representative histogram (A), as well as the percentage of the proliferating cell and the cells belong to some division passages (indicated as p2 and p8) is described (B). Data represent two independent experiments in triplicate, mean ± SEM (n=2). Mann-Whiney test (*p< 0.05, **p< 0.01, ***p< 0.001).

The hADSC-exosomes induce subversion of Th17 cells by preventing Th17 cells from secreting IL-17a.

For therapeutic applications of human MSC-exosomes to Th17 cell-mediated diseases, it is required that the exosomes attenuate fully-differentiated Th17 cell's function and character as well as differentiation of naïve T cell to Th17 cell. In order to investigate exosomes' ability to control pre-differentiated Th17 cells, the exosomes were added at the final concentration of 112.5 μg/mL on day 5 post-differentiation and the effect was analyzed on day 7. The final concentration was determined with an account of increased cell counts. Negative and positive control was determined as in Figure 4. As a result, the population of IL-17a$^+$ RORγt$^+$ cell among CD4$^+$ cell decreased by an average value of 0.6 % in exosomes-treated (day 5) group (Fig. 6A). Plus, the percentage of CD4$^+$ IL-17a$^+$ cell or CD4$^+$ RORγt$^+$ cell (single positive) also showed similar aspect with that of double-positive cell (Fig. 6A).

The mRNA relative expression was also evaluated likewise method in Figure 4. The mRNA relative expression in all targets on day 7 increased compared to that of day 5 (Ratio of mRNA relative expression level on day 7 to day 5 in positive control: IL-17A; 5.78 : 1, RORC; 4.3 : 1) indicating that CD4$^+$ cells differentiated sufficiently to Th17 cells under referred condition.

The mRNA relative expression level of IL-17A in exosomes-treated (day 5) group was 2.72 times less than those of positive control, respectively. Additionally, the results correlate with the mRNA level of a transcription factor. The mRNA relative expression level of RORC in exosomes-treated (day 5) group was lower than those of positive control (Fig. 6B).

Briefly, the hADSC-exosomes decrease the production of IL-17a in pre-differentiated Th17 cells and down-regulate the mRNA level of IL-17A and RORC in pre-differentiated Th17 cells even the exosomes are treated after $CD4^+$ cells polarized to Th17 cells for 5 days.

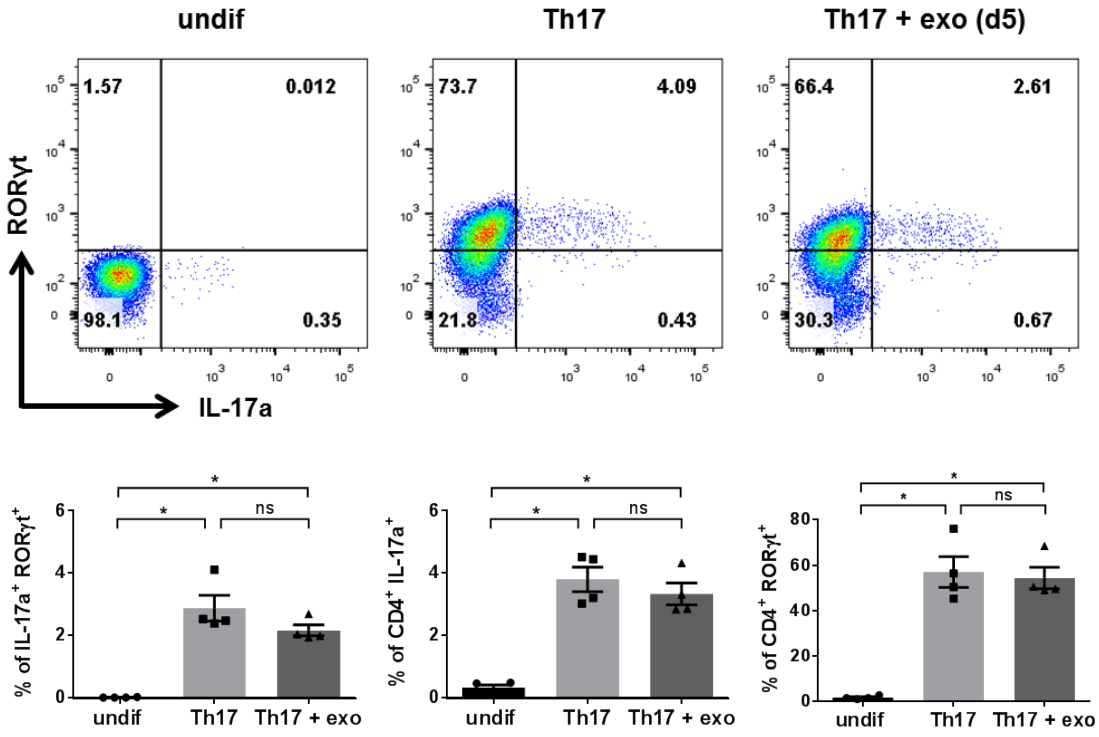
Generally, immune cells' character and function are evaluated with surface marker, specific intracellular cytokine and transcription factor expression, mRNA expression level and cytokine secretion ability, etc. By investigating the amount of secreted cytokine, we can understand more directly what kinds of and how powerful immune responses the immune cells can evoke and how the immune cells affect the local immune system. For assessment of cytokine secretion ability, four-groups (A; undifferentiated group, B; $CD4^+$ cells cultured under Th17 polarization group for 7 days (positive control), C; positive control + exosomes on day 5) were used to stimulation for cytokine secretion. On day 7, all groups were washed and detached from anti-CD3/CD28 beads.

Then, a total of 7.5 x 10^4 cells of each group per well were seeded in triplicate to 96 well-U-bottom TC-treated plate. And then, in order to promote cytokine secretion, the cells were stimulated with anti-CD3/CD28 expander beads at a bead : cell ratio of 1:1 for 2 days. After 2 days, the culture supernatants were collected and estimated by CBA for checking the amount of human IL-17a. As shown in Figure 6C, the exosome treated group (Th17 polarization + exosomes at day 6) secreted 6.5 times lower IL-17a comparing with the B group (Th17 polarization) (Fig. 6C). Cytokine secretion assay showed more dramatic differences between groups w/ or w/o hADSC-exosomes than analysis for intracellular cytokine.

Interestingly, exosome treated group showed markedly low cytokine secretion while that displayed a very little reduction of $CD4^+$ IL-$17a^+$ cells comparing with B group in analysis using flow cytometry. This result indicates the hADSC-exosomes can preclude *in vitro*-differentiated

Th17 cells from secreting IL-17a. In summary, the hADSC-exosomes induce subversion of Th17 cells and dampen the secretion of IL-17a from Th17 cells.

A


undif
Th17
Th17 + exo (d5)
RORγt
IL-17a
1.57
0.012
98.1
0.35
73.7
4.09
21.8
0.43
66.4
2.61
30.3
0.67
% of IL-17a+ RORγt+
% of CD4+ IL-17a+
% of CD4+ RORγt+
undif
Th17
Th17 + exo
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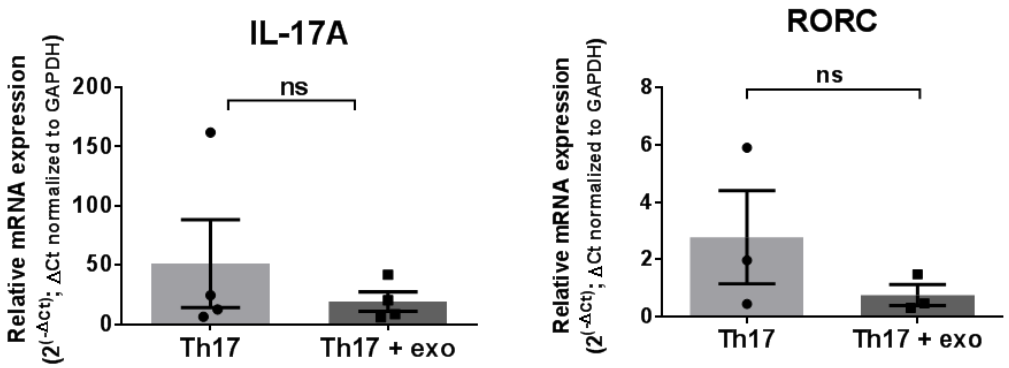
B


IL-17A
RORC
Relative mRNA expression
(2(-ΔCt); ΔCt normalized to GAPDH)
ns
Th17
Th17 + exo

C

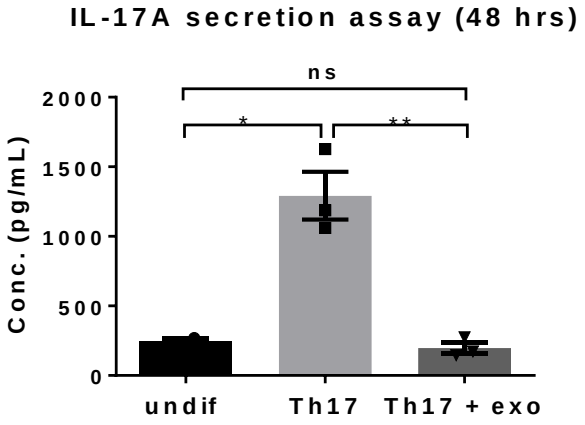


Figure 6. The hADSC-exosomes' effect on the subversion of Th17 cells.

$CD4^+$ cells were cultured under Th17 differentiation condition for 5 days and co-culture with the hADSC-exosomes next 2 days more at the final concentration of 112.5 μg/mL. At day 7, the cells were analyzed by flow cytometry (A) and qRT-PCR (B) as in Figure 4. The supernatant containing cytokines secreted by the cells during re-stimulation were analyzed by CBA (IL-17a flex set). Data represent two independent experiments in single or triplicate, mean ± SEM (n=2). Mann-Whiney test (*p< 0.05, **p< 0.01, ***p< 0.001).

DISCUSSION

My study showed that human ADSC-derived exosome could suppress the proliferation of human $CD4^+$ cells under Th17 differentiation condition and attenuate the ability of *in vitro* differentiated human Th17 cells to secrete IL-17a.

Over the last several decades, multiple studies have reported the potentiality of MSC-exosome to regulate local immune system by modulating immune cells; for example, suppressing the proliferation of $CD3^+$ T cells (3), strengthening Treg function (11), and inhibiting activation of APCs and the development of Th1 and th17 cells (12), etc.

Rebeca (3) showed hASC-exosome has immunomodulatory potential on *in vitro* stimulated T cells. The hASC-exosome inhibit activation of T cell and differentiation to Th1, Th17 cell and reduce the proliferation. After human peripheral blood lymphocytes (PBLs) were stimulated with anti-CD2/CD3/CD28-coated antibody for 6 days in the presence or absence of hASC-exosome, the proliferation of CD4 and CD8 was reduced exosome dose-dependently. Especially, when CFSE-dilution assay was conducted, the percentage of more-divided cells was far low in exosome-treated group. This data is mostly similar to my result in Figure 6. However, on the contrary, Andrea (13) reported that human BM-MSC-EVs have no effect on the proliferation of *in vitro* stimulate T cells from PBMC culture. But, human BM-MSC-EVs induced apoptosis of $CD3^+$ and $CD4^+$ cells.

In addition, Wancheng (14) reported that human BM-MSC-derived exosome inhibits human Th17 cell differentiation dose-dependently. Conversely, in my study, I found that human ADSC-derived exosomes enhance Th17 differentiation from human $CD4^+$ cells. This different results would come from different MSC sources and Th17 cell development conditions. Wancheng group

used only TGF-β and IL-2 for Th17 development while TGF-β, IL-1β, and IL-6 were used in my study. Also, Wancheng group isolated exosomes from the culture supernatant of BM-MSCs under passage 8 strictly, whereas I used ADSC under passage 13. As I mentioned in the introduction section, the ability to modulate immune cells depend on MSCs' source and quality.

Human MSCs' optimal culture condition is little different from murine MSCs' and includes a low-glucose DMEM and 10% Fetal Calf Serum (or FBS) (15). High-glucose DMEM has been reported be toxic to human MSCs. In addition, human MSCs expand more slowly than murine MSCs and their doubling time was more than 3-4 days in my cell culture case. However, human MSCs are heterogeneous and depending on the optimization of culture conditions. The multiplication times have been reported to vary widely. In this study, they rarely expanded over passage 9 and then, doubling time exceeded 7 days. As their passage increased, their sizes and shapes became bigger and sharper respectively (Fig. 1a)

Many studies which evaluate the effect of MSCs and MSC-exosomes on Th17 cells used different Th17 polarization condition. The optimal condition for human Th17 differentiation *in vitro* is controversial and has been discussed over several decades. To decide proper differentiation condition for testing exosomes' effects, several *in vitro* environments were tested and evaluated by frequency of IL-17a producing cells (Fig. 3). Generally, in vitro human Th17 differentiation condition includes TCR stimulation against CD3 and CD28 and IL-1β, IL-6, TGF-β, and IL-23 with neutralizing antibody against IFN-γ and IL-4. Each concentration of anti-CD3/CD28 antibody and cytokines varies from experiment to experiment.

First, the strength of T cell activation also dictates the extent of Th17 differentiation significantly. Recently, Harriet A.(10) showed that low-strength stimulation via anti-CD3/CD28 beads or dendritic cells pulsed with super-antigen in the presence of pro-Th17 cytokines IL-1β,

TGF-β, and IL-23 profoundly promoted Th17 responses by enhancing both the relative proportion and a total number of Th17 cells. My data is in consistent with the recent study reported that a low-strength of T cell activation promotes Th17 responses but only in the presence of anti-CD28 (16). In my condition, T cell stimulation was conducted using anti-CD3/CD28 T-cell expander bead at a 1:10 bead : T cell ratio which was more efficient for Th17 polarization than the ratio of 1:3 or 1:1.

In addition, the combinations of cytokines are various and controversial still. According to Mazak et al., a combination of TGF-β and IL-23 with inflammatory cytokines such as IL-6 and IL-1β in serum-free medium is effective to induce human Th17 cell differentiation (17). Nevertheless, Wilson et al. reported that using TGF-β plus IL-6 neither in combination nor with IL-1β plus IL-23 is rather inhibitory to Th17 development. In early studies, TGF-β had been found inhibitory to Th17 differentiation (18). However, recent data utilizing serum-free media and naïve T cells isolated from cord blood have shown that TGF-β is required for the differentiation of human Th17 cells (19), (20), (21). These discrepancies among different studies could also be reflected by the amount of TGF-β and/or presence of serum in culture media. In my study, because FBS was included in T cell media, the first concentration of TGF-β (10 ng/mL) was too high to induce Th17 cell normally and inhibited whole cell proliferation. So, the concentration of TGF-β was lowered to 1 ng/mL (Fig. 3).

Also, the absence of neutralizing antibodies to IFN-γ and IL-4 in my study would induce the differentiation of Th1 and Th2 cells which inhibit Th17 cell development. While the results of Acosta-Rodriguez et al. which used IL-1β, IL-6, and TGF-β for differentiation condition and neutralizing antibodies to IFN-γ and IL-4 accomplished 4.1 % of IL-17a^{+}IFN-γ^{-} cells among CD4^{+}

T cells, my study which used equal cytokine combinations but not neutralizing antibodies accomplished average 3.5 % of total $CD4^+IL\text{-}17a^+$ $ROR\gamma t^+$cells at day 7 (Fig. 6).

In my study, it was assessed whether the human ADSC-derived exosomes could inhibit the polarization of $CD4^+$ T cell into Th17 cell and destabilize the pre-differentiated Th17 cell. Since IL-23 is greatly engaged to keep the stability of RORγt, IL-23 was excluded from the condition for polarizing $CD4^+$ T cell into Th17 cell to observe the influence of exosomes on RORγt level and cytokine production distinctly (Fig.3).

My study showed that when human ADSC-exosomes were treated to in vitro differentiated human Th17 cells and co-cultured with the cells for two days, the exosomes reduced the secretion of IL-17a from in vitro differentiated human Th17 cells by 4 times but didn't downregulate the mRNA and protein level of IL-17a and RORγt significantly. So far, there have not been any papers which reported human Th17 subversion by exosomes; however, there is unpublished data which confirmed that in the mouse system.

Instead, several papers showed that exosomes derived from human and/or mice attenuate the IL-17a secretion from murine splenic mononuclear cells (22) and Th17 cells from cervical lymph nodes in mice with experimental autoimmune uveoretinitis (EAU) (12). Shigemoto-Kuroda (12) showed that human BM-MSC-EVs lower the amount of IL-17a/f secreted from allogenic mixed lymphocyte reaction by about two times. Consistently, I found that human ADSC-exosomes which were treated to in vitro differentiated Th17 cells two days before re-stimulation suppressed the secretion of IL-17a from the Th17 cells (Fig. 6). When comparing my data with Shigemoto's data, BM-MSC-EVs showed the similar suppressive ability to IL-17a secretion compared to ADSC-exosomes though the amount of BM-MSC-EVs treated was 10 times lower than that of ADSC-exosomes. Although it has been reported that their ability to inhibit Th17 depends on

humans or mice, the remarkable inhibitory effect of BM-MSC-EV, as shown in the above data, would mean that BM-MSC is still the best source of exosome with immunosuppression capabilities.

The aim of this study is to verify whether human ADSC-exosomes can modulate human Th17 differentiation and subversion without any genetic modification or environmental optimization. In summary, human ADSC-exosomes failed to modulate human Th17 differentiation but succeeded to suppress the proliferation of $CD4^+$ T cells under Th17 differentiation condition. Also, they succeeded to attenuate the secretion of IL-17a from in vitro differentiated human Th17 cells. These results suggest that hADSC-exosome has the potentiality to be used as a none-cell therapy for curing Th17-mediated diseases.

In order to use ADSCs as an alternative source for EVs or exosomes instead of BM-MSCs, an additional modification for them and their culture environment are needed to make them more anti-inflammatory and immune-suppressive. Plus, the substance which presented the competence to destabilize RORγt would be applied to human MSCs to make human MSC-EVs or exosomes to modulate Th17 cells more specifically.

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국문 초록

중간엽 줄기세포는 다분화능을 가진 성체 줄기 세포로서 골수, 지방조직, 탯줄 등으로부터 얻을 수 있는 것으로 알려져 있다. 중간엽 줄기세포는 다른 세포로 분화할 수 있으며 조직 재생을 촉진하고, 특히 면역 조절 기능이 있는 것으로 알려져 있다. 주변 분비 인자 중 하나인 엑소좀은 특히 중간엽 줄기세포의 면역 조절 기능에서 핵심적이라는 것이 알려졌다. 그래서 중간엽 줄기세포 유래 엑소좀에 의한 면역 세포 억제 등에 대한 연구가 활발히 이루어져왔지만, 사람의 중간엽 줄기세포가 사람의 면역세포에 미치는 영향에 대한 연구는 상대적으로 적게 이루어졌다. 또한 중간엽 줄기세포 유래 엑소좀의 대식세포, 조절 T 세포, Th1 세포에 대한 면역 조절 효과에 대한 연구와 비교하여 Th17 세포에 직접적으로 미치는 영향에 대한 연구는 거의 이루어지지 않았다. 따라서, 본 연구에서는 사람의 지방줄기세포 유래 엑소좀이 사람의 Th17 세포의 분화 및 탈분화에 미치는 영향에 대해서 살펴보았다. 엑소좀은 ultracentrifugation 을 통해 줄기세포의 배양액에서 분리되었다. 분리된 엑소좀은 사람의 $CD4^+$ T 세포가 Th17 세포로 분화되기 시작하는 시점과 *in vitro* 에서 분화가 이루어지고 난 후의 시점에 처리되었다. 결과적으로 사람 지방줄기세포 유래 엑소좀은 사람 Th17 세포의 분화를 mRNA 와 단백질 수준에서 조절하지는 못했다. 그러나 Th17 분화 조건 하에서 $CD4^+$ T 세포의 분열을 억제하였다. 또한 in vitro 에서 분화된 Th17 세포에 사람 지방줄기세포 유래 엑소좀을 처리하였을 때, 엑소좀은 사람 Th17 세포의 주요 전사인자인 RORγt 와 대표적 cytokine 인 IL-17a 의 mRNA 와 protein level 을 통계적으로 유의하게 감소시키지 못했다. 그러나 이후 cytokine secretion assay 를 진행한 결과 exosome 을 처리한 그룹에서 IL-17a 의 분비가 현저하게 감소하였다. 이를 통해 사람 지방줄기세포유래 엑소좀이 Th17 세포의 IL-17a 분비를 조절할 수 있을 것이라는 가능성을 확인하였다.

주요어 : 지방줄기세포, 엑소좀, 면역 조절, Th17

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