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BRIEF COMMUNICATION

CD39-mediated effect of human bone marrow-derived mesenchymal stem cells on the human Th17 cell function

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Abstract This study investigated the immune-modulatory effects of human bone marrow-derived mesenchymal stem cells (hBMSCs) on human Th17 cell function through the CD39-mediated adenosine-producing pathway. The suppressive effects of hBMSCs were evaluated by assessing their effects on the proliferation of Th17 cells and the secretion of interferon (IFN)-γ and interleukin (IL)-17A by Th17 cells with or without anti-CD39 treatment. Changes in CD39 and CD73 expression on the T cells with or without co-culture of hBMSCs were evaluated by flow cytometry. hBMSCs effectively suppressed the proliferation of Th17 cells and the secretion of both IL-17A and IFN-γ from Th17 cells using by both flow cytometry and ELISA, while anti-CD39 treatment significantly reduced the inhibitory effects of hBMSCs on the proliferation and secretion of the Th17 cells. The hBMSCs induced increased expression of the CD39 and CD73 on T cells correlated with the suppressive function of hBMSCs, which was accompanied by increased adenosine production. Our data suggests that hBMSCs can effectively suppress immune responses of the Th17 cells via the CD39-CD73-mediated adenosine-producing pathway.

Keywords Bone marrow-derived mesenchymal stem cells · Th17 cells · CD39 · CD73 · Interleukin-17 · Interferon-γ · Adenosine

Introduction

Bone marrow-derived mesenchymal stem cells (BMSCs) have immuno-modulatory properties that involve the suppression of both innate and adaptive immunity [1–4]. In human

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BMSCs, immunosuppression via direct cell-to-cell contact or in the paracrine pathway is at least partly mediated by the expression of indole amine 2,3-dioxygenase (IDO), transforming growth factor-beta (TGF-β), hepatocyte growth factor (HGF), prostaglandin E2 (PGE2), and others [5, 6]. However, the mechanism underlying immunomodulation by BMSCs in cell-to cell contact has not yet been fully determined.

Current study indicated that CD39 might control cellular immune responses [7]. In line with this investigation, recent studies provided evidences that CD39 mediated the immune suppression of T cells by MSCs in a cell-to-cell contact manner [8, 9]. Adenosine is an anti-inflammatory product of enzymatic hydrolysis of extracellular ATP by CD39 (ecto-ATPase) and CD73 (ecto-5′-AMP nucleotidase). The immunosuppressive activity of adenosine is well-known in T regulatory cells and in the tumor microenvironment [10, 11]. Similar to the effect of T regulatory cells on effector T cell function by CD39 and CD73-mediated suppression, MSCs might exert inhibitory effect on effector T cell function through up-regulation of CD39 or CD73 expression.

Recently, Th17 cells have been suggested to play a critical role in many human diseases including eye disease [12, 13]. Therefore, we aimed to investigate whether Th17 cell function may be modulated by BMSCs. We hypothesized that immune response of Th17 cells may be inhibited by BMSCs, mediated by CD39-dependent promotion of adenosine signaling, using in vitro tests. We showed here that human BMSCs up-regulated CD39 expression on activated T cells and that BMSCs inhibited Th17 cell function through CD39-mediated adenosine production.

Materials and methods

The study was approved by the Institutional Review Board of the Seoul National University Hospital (IRB approval number: H-1007-094-324) and performed in accordance with the guidelines of the Declaration of Helsinki. Human blood was collected from healthy volunteer donors with their written informed consent.

CD4+ T cell isolation and Th17 differentiation

CD4+ human peripheral blood T cells were isolated from freshly collected, heparinized peripheral blood by using CD4 MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's specifications. Th17 cells were differentiated by stimulating 5×10^4 CD4+ T cells on a plate coated with 1 μg/mL of anti-CD3 and anti-CD28 monoclonal antibodies (eBioscience, San Diego, CA, USA; 1 μg/ml) and subsequently cultured with 25 ng/mL IL-1β (R&D systems, Minneapolis, MN, USA) and 25 ng/mL IL-23 (R&D systems) in RPMI 1640 medium (Welgen, Seoul, South Korea) containing 10 % fetal bovine serum (FBS) (Atlanta Biological, Lawrenceville, GA, USA), 100 U/mL penicillin/streptomycin (Gibco, Grand Island, NY, USA), and 10 mM HEPES(Gibco) in a 48-well plate (NUNC, Roskilde, Denmark).

Culturing of BMSCs

A fetal BMSCs line [14] was kindly provided by Professor Kim Seung Up (Medical Research Institute, Chung-Ang University College of Medicine, Seoul, South Korea). The cells were used after six to seven passages. BMSCs were maintained with alpha minimum essential medium (Gibco) containing 10 % FBS and 100 U/mL penicillin/streptomycin (Gibco) at 37 °C in a 5 % CO2 incubator for at least 2 days at 80 % confluency. Subsequently, BMSCs were harvested and were seeded at a density of 5×10^3 cells/well in a 48-well plate (NUNC) for the co-culture with T cells.

Expression of CD39 in BMSCs

MSCs were plated in six-well plates at a density of 3×10^4 cells/well and cultured overnight. mRNA was isolated using the TRIzol (TRIsure, BIOLINE, Tauton, MA, USA) and reversely transcribed using the AccuPower RT PreMix kit (Bioneer, Daejeon, South Korea) according to manufacturer's instructions. cDNA was analyzed for the expression of CD39 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The following primer sets were used for reverse transcription-polymerase chain reaction (RT-PCR) (GenBank Accession number S73813): CD39, 5′-GAC CCA GAA CAA AGC ATT GCC-3′ and 5′-TGT AGT CCT TGC CAT AGA GGC G-3′ and GAPDH, 5′-ACA GTC AGC CGC ATC TTC TT-3′, 5′-TTG ATT TTG GAG GGA TCT CG-3′. BMSCs (5×10^3) were stained with CD39-PE cy7 (eBioscience, CA, USA) and CD73-FITC (eBioscience, CA, USA). Surface expression of CD39 as well as CD73 was measured by flow cytometer with a FACS Vantage Flow Cytometer (BD Pharmingen, San Diego, CA, USA).

In vitro suppression of proliferation assays

To examine the suppressive effect of BMSCs on Th17 cell proliferation, isolated CD4+ T cells were stained with 5 mM carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen, Carlsbad, CA) and were driven to Th17 cells with IL-1β/IL-23 (25 ng/mL). Anti-CD3/CD28 antibody (1 μg/mL; eBioscience) was resuspended in the supplemented RPMI 1640 medium (Welgen) as the stimulator. Th17 cells (5×10^4) were placed in co-culture with BMSCs (5×10^3) for 2 days and were then maintained for additional 3 days after removal of BMSCs. Control Th17 cells without BMSCs were

kept for 5 days in the same conditions. Suppression of Th17 cell proliferation was measured by a reduction in the CFSE concentration by flow cytometer with a FACS Vantage Flow Cytometer (BD Pharmingen). All experiments were performed with six replicates. T cells were isolated from the adhered BMSCs by gentle harvest of the supernatant and were then sorted out by CD4-PE (eBioscience, CA, USA) to exclude BMSCs. To evaluate the changes in CD39 expression on Th17 cells depending on the presence of BMSCs or anti-human CD39 neutralizing antibody, Th17 cells were stained with CD39-PE cy7 (eBioscience, CA, USA) and CD73-PerCP cy5.5 (eBioscience, CA, USA). Then, surface expression of the CD39 as well as CD 73 was then measured.

To determine whether the suppressive effect of BMSCs on Th17 cell function would be blocked by inhibition of CD39 and to compare the effects with the inhibition effect of IDO or nitric oxide synthase 2 (NOS2), cells were treated with anti-human CD39 neutralizing antibody(10 μg/mL, clone A1; AbD Serotec, Langford, UK)[15] or 250 μM 1-methyl-DL-Trp (1-D/L MT; Sigma-Aldrich, St. Louis, MO, USA) or 1 mM 1400w dihydrochloride (Sigma-Aldrich) during the co-culture.

In vitro functional assays of Th17 cell suppression

CD4+ T cells that had been treated with IL-1β and IL-23 for Th17 differentiation were co-cultured with BMSCs for 2 days at a ratio of 10:1 (5×10^4 T cells, 5×10^3, T:BMSCs) under anti-CD3/anti-CD28 treatment. Th17 cells were separated from the adhered BMSCs by gentle shaking of the medium, were collected, and were then maintained in new plates for additional 5 days. Control T cells with treatment of IL-1β/IL-23 and anti-CD3/anti-CD28 were maintained for the same 7 days. After harvest, the T cells were incubated with the CD4-fluorescein isothiocynate (FITC) (eBioscience, CA, USA) and CD45RO-PerCP (eBioscience, CA, USA) for 30 min at 4 °C and cells were washed three times. After the treatment with 50 ng/mL 1-phorbol-12-myristate-13-acetate (PMA, Sigma-A aldrich) and 1 μg/mL ionomycin (Sigma-A aldrich), cells were fixed with a fixation/permeabilization solution for 20 min at 4 °C. After with, the cells were washed with Perm/Wash™ buffer (BD Biosciences. San Jose, CA, USA); they were incubated with IL-17-PE (BD Pharmingen) and IFN-γ-APC (BD Pharmingen) for 60 min at 4 °C. Flow cytometric analysis was performed using a FACS Canto apparatus (Becton Dickinson, San Diego, CA, USA), and the data were analyzed by FlowJo (version 7.6.5, (Treestar, Ashland, OR, USA).

After the four-color compensation, lymphocytes were gated in the forward scatter (FSC)/side scatter (SSC) gate and effector memory cells were then gated by $CD45RO^{hi}$ expression, and finally, the cells were gated by CD4/IFN-γ or CD4/IL-17. To evaluate whether the suppressive effect of BMSCs on the immune response of Th17 cells would be blocked by inhibition of CD39 and to compare the effect with the inhibition effect of IDO or NOS2, cells were treated with 10 μg/mL anti-human CD39 (clone A1; AbD Serotec) as a neutralizing antibody, 250 μM 1-D/L MT or 1 mM/mL 1400w dihydrochloride during the co-culture. All experiments were performed in six replicates with anti-CD39 treatments and in three replicates with 1-D/L MT or 1400w dihydrochloride.

IL-17 and IFN-γ enzyme-linked immunosorbent assay

Levels of IL-17 and IFN-γ were determined in supernatants from the 2-day co-culture of both Th17 cells and BMSCs with or without anti-CD39 treatment using a commercial DuoSet enzyme-linked immunosorbent assay (ELISA) kits following the manufacturers' instructions (R&D Systems). After removal of BMSCs, Th17 cells were maintained with or without treatment for additional 5 days and supernatants were then collected. The concentrations used for the standard curve ranged from 1,000 pg/mL to 15.625 pg/mL, using either human recombinant IL-17 or IFN-γ (R&D Systems). All experiments were performed using six replicates.

Adenosine production in mass spectrometry

Liquid chromatography was performed on an Accela 1250 UPLC™ system (ThermoFisher Scientific, USA) equipped with a quaternary pump, a column oven, and an autosampler. The LC was coupled to a TSQ quantum Access Max, which is a triple quadrupole mass spectrometer (ThermoFisher Scientific) with a heated-electrospray ionization (HESI) interface. All instruments were controlled by Thermo Xcaliver 2.2 SP1.48 version. Analysis was separated using Thermo Fisher Hypersil Gold C18 column (2.1×100 mm, 1.8 μm, ThermoFisher Scientific), and column oven temperature was maintained at 30 °C. The solvent system consisted of 65 % of HPLC-grade water with 0.1 % formic acid and 35 % of acetonitrile with 0.1 % formic acid. The flow rate was set to 400 μL/min, and an injection volume of 10 μL was used. The total retention time was 1.05~1.2 min, and the analysis time was 35 min. All experiments were performed using six replicates.

Changes in CD73 expression by DNA microarray

Microarray hybridization was performed according to the manufacturer's instructions after total RNA extraction from Th17 cells co-cultured with BMSCs and from control Th17 cells induced by IL-1β and IL-23. Gene expression was analyzed with the GeneChip® Human Genome U133 plus 2.0 Array (Affymetrix, Santa Clara, CA, USA), which is composed of over 45,000 probe sets representing 38,500 human genes. Fold changes in CD73 expression were compared between the two groups.

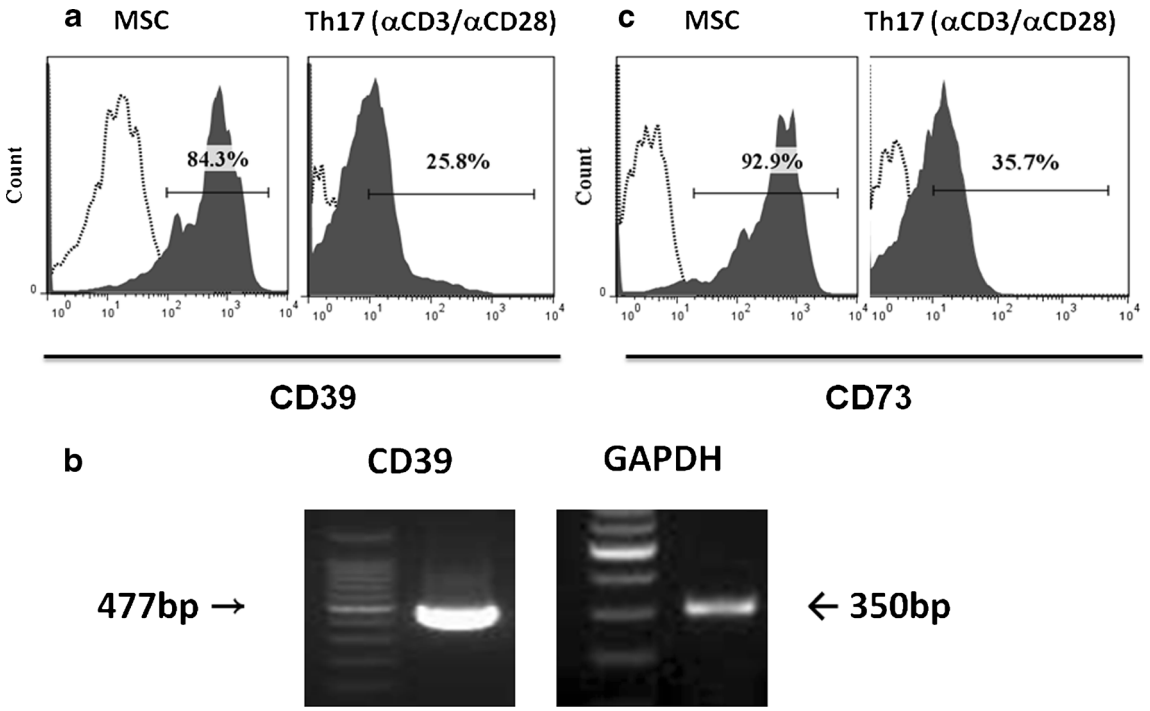

Fig. 1 Flow cytometric histogram (**a**) showed that CD39 as well as CD73 were highly expressed on the human MSCs which corresponded with RT-PCR data showing high expression of CD39 (**b**), while stimulated Th17 cells showed low levels of CD 39 and CD73 expression (**c**)

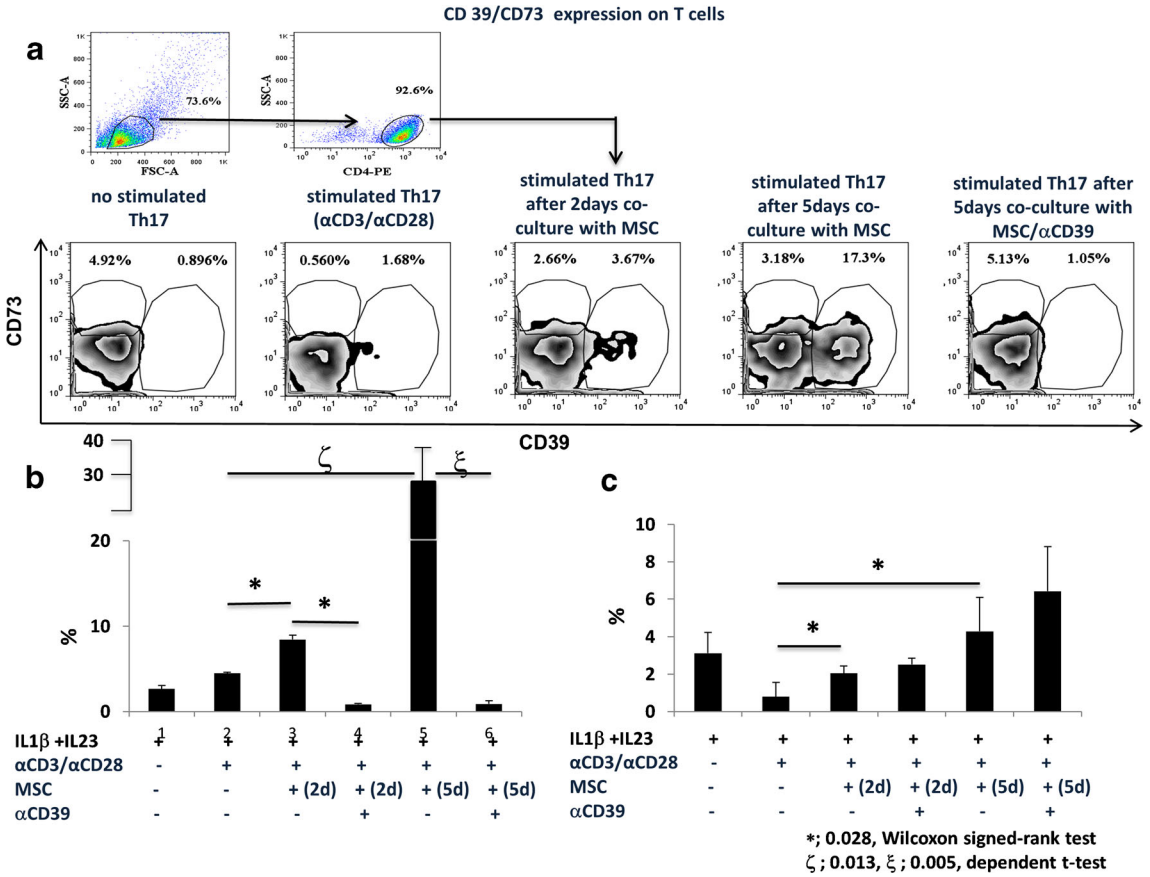

Fig. 2 Expression levels of CD39 on Th17 cells were highly driven by co-culture with BMSCs in a contact time-dependent manner, and these expression levels were significantly reduced by an anti-CD39 neutralizing antibody (**a**, **b**). Low levels of CD73 expression on stimulated Th17 cells switched to high levels of CD73 expression on stimulated Th17 cells with the addition of BMSCs (**a**, **c**). To exclude the contamination of the BMSCs in analysis, T cells were isolated from the BMSCs with CD4 gating after co-culture ($n=6$)

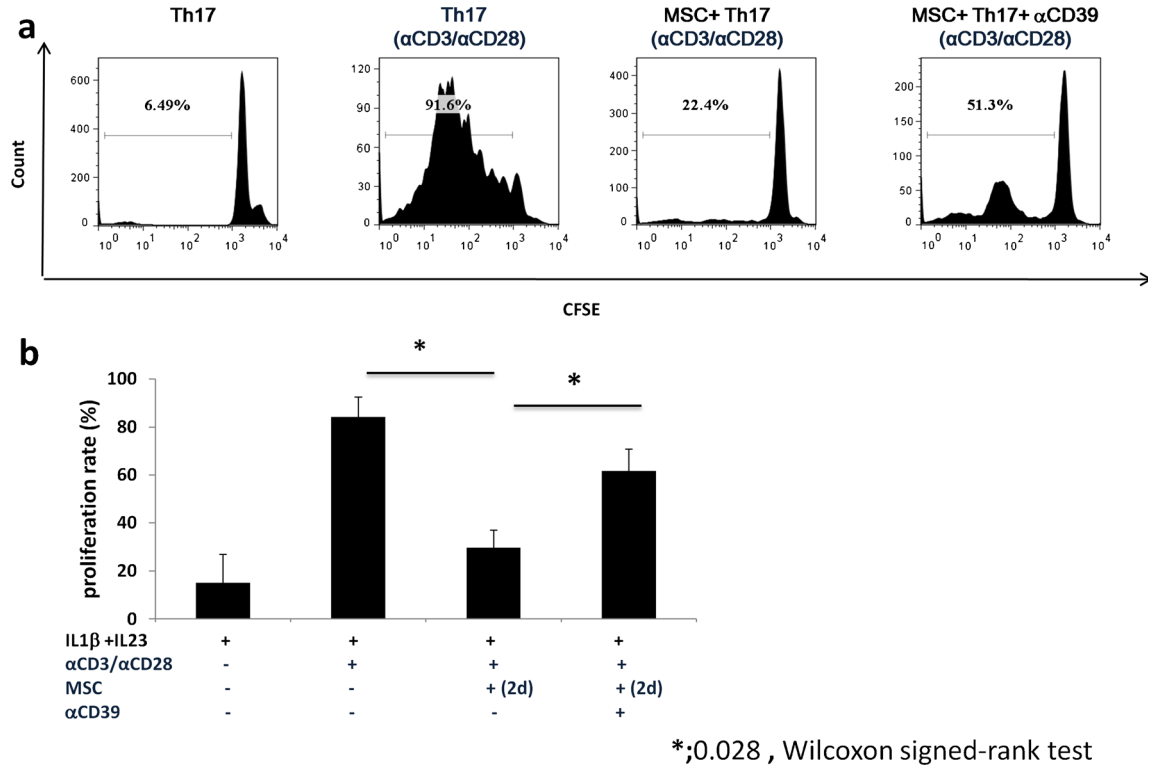

Fig. 3 CFSE assays revealed that hBMSCs effectively suppressed on human Th17 cell proliferation induced by IL-1β/IL-23, which was significantly inhibited by anti-CD39 neutralizing antibody treatment (αCD39) ($n=6$)

Statistical analyses

SPSS 17.0 software (Chicago, IL, USA) was used. Changes in proliferating cells, the expression of CD39/CD73, and IL-17/IFN-γ secreting cells with different treatments were compared using the nonparametric Wilcoxon signed-rank test or dependent t test. IL-17 and IFN-γ concentrations and adenosine concentrations were also compared using the nonparametric Wilcoxon signed-rank test. A p value of <0.05 was considered statistically significant.

Results

CD39 is highly expressed on the BMSCs

We found abundant expression of CD39 on BMSCs (Fig. 1a, b) along with high expression of CD73, which might have proximity-based immune-modulatory effects on neighboring cell function.

CD39 and CD73 expression up-regulation on T cells during co-culture with BMSCs

Next, we assessed changes in CD39 and CD73 expression on activated Th17 cells induced by IL-1ß/IL-23 and stimulated by anti-CD3/CD28 when cultured with BMSCs (Fig. 2). Flow cytometer revealed that expression levels of CD39 on Th17 cells were highly driven on the Th17 cells by the co-culture with BMSCs in a contact time-dependent manner (2 days co-culture, $p=0.028$, Wilcoxon signed-rank test; 5 days co-culture, $p=0.013$, dependent t test), and these expression levels were significantly reduced by an anti-CD39 neutralizing antibody(2 days co-culture, $p=0.028$, Wilcoxon signed-rank test; 5 days co-culture, $p=0.005$, dependent t test). Low levels of CD73 expression on stimulated Th17 cells switched to high levels of CD73 expression on stimulated Th17 cells with the addition of BMSCs (2 days co-culture, $p=0.028$, Wilcoxon signed-rank test; 5 days co-culture, $p=0.028$, Wilcoxon signed-rank test), which were not diminished by the anti-CD39 neutralizing antibody.

BMSCs suppressed Th17 cells proliferation via up-regulation of CD39 expression

CFSE assays demonstrated that BMSCs effectively suppressed Th17 cells proliferation by 64.7 % ($p=0.028$, Wilcoxon signed-rank test; Fig. 3a, b). The suppression effect was significantly abrogated by anti-CD39 treatment ($p=0.028$, Wilcoxon signed-rank test). The inhibitory effect of anti-CD39 treatment on the BMSC-mediated suppression of T cell proliferation appeared to be higher than the blocking effect of

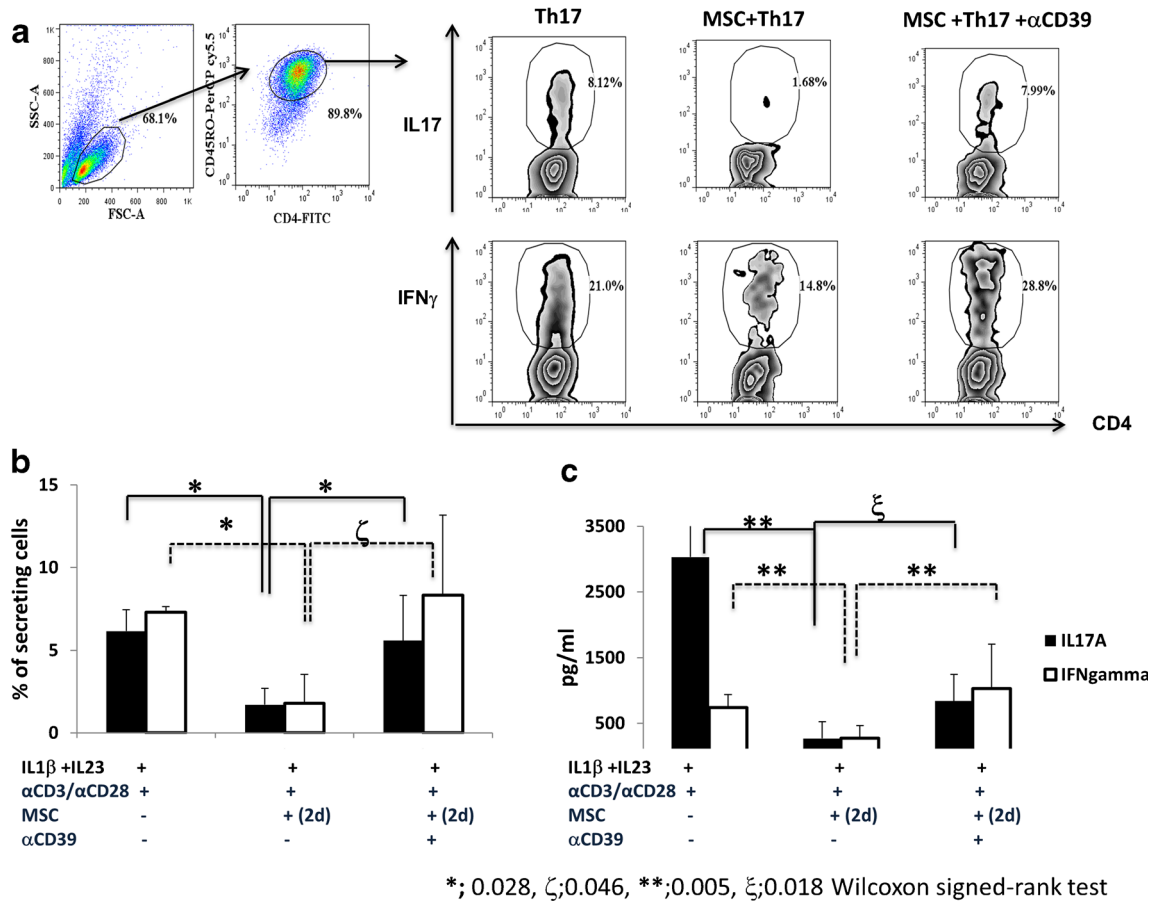

Fig. 4 The suppressive effect of BMSCs on the secretion of both IL-17A and IFN-γ by Th17 cells was significant, which was markedly reduced by anti-CD39 treatment (**a**~**b**) ($n=6$). Reduced secretions of IL-17A and IFN-γ were also shown in MSCs co-cultured supernatants by ELISA, which were significantly reversed by anti-CD39 treatment (**c**) ($n=6$). M indicates BMSCs, T indicates Th17 cells, and αCD39 presents anti-CD39 treatment

either 1-D/L MT or 1400w dihydrochloride (Supplementary Fig. S1).

BMSCs significantly attenuated IL-17A/IFN-γ secretion in Th17 cells via up-regulation of CD39 expression

The suppressive effect of BMSCs on the secretion of both IL-17A and IFN-γ by Th17 cells was significant ($p=0.028$ and $p=0.028$, respectively, Wilcoxon signed-rank test; Fig. 4a, b). The suppression effect was significantly reduced by anti-CD39 neutralizing antibody treatment ($p=0.028$ and $p=0.046$, respectively, Wilcoxon signed-rank test; Fig. 4b). 1-D/L MT, an inhibitor of the IDO pathway, or 1400w dihydrochloride, an inhibitor of NOS2, also diminished the suppression by IFN-γ-treated BMSCs on IL-17A and IFN-γ secretion by Th17 cells. However, the diminished behavior was statistically insignificant and the effect of inhibition was lesser than the blocking effect of anti-CD39 treatment (Supplementary Fig. S2). Supernatants from the 2-day co-cultures with BMSCs also reduced secretion of IL-17A and IFN-γ, based on ELISA results ($p=0.005$ and $p=0.005$, respectively, Wilcoxon signed-rank test; Fig. 4c). The reductions were significantly reversed by anti-CD39 treatment ($p=0.018$ and $p=0.005$, respectively, Wilcoxon signed-rank test; Fig. 4c). Treatment with 1400w dihydrochloride also increased IFN-γ levels in supernatants, albeit in a statistically insignificant manner (Supplementary Fig. S2).

Suppressive effect of BMSCs on the Th17 cell function is involved in adenosine production

Mass spectrometry (Fig. 5a) presented enhanced adenosine production in supernatants from co-culture of Th17 cells and BMSCs ($p=0.028$, Wilcoxon signed-rank test), with a significant reduction in adenosine production after anti-CD39 treatment ($p=0.028$, Wilcoxon signed-rank test). Microarray data (Fig. 5b) showed increased CD73 expression on co-cultured Th17 cells with BMSCs, which corresponded well with flow cytometric data shown in Fig. 2c. These outcomes suggest that BMSC-mediated suppression on Th17 cell function is more involved in CD39-dependent promotion of adenosine signaling (P1 signaling) rather than in CD39-dependent ATP signaling termination (P2 signaling).

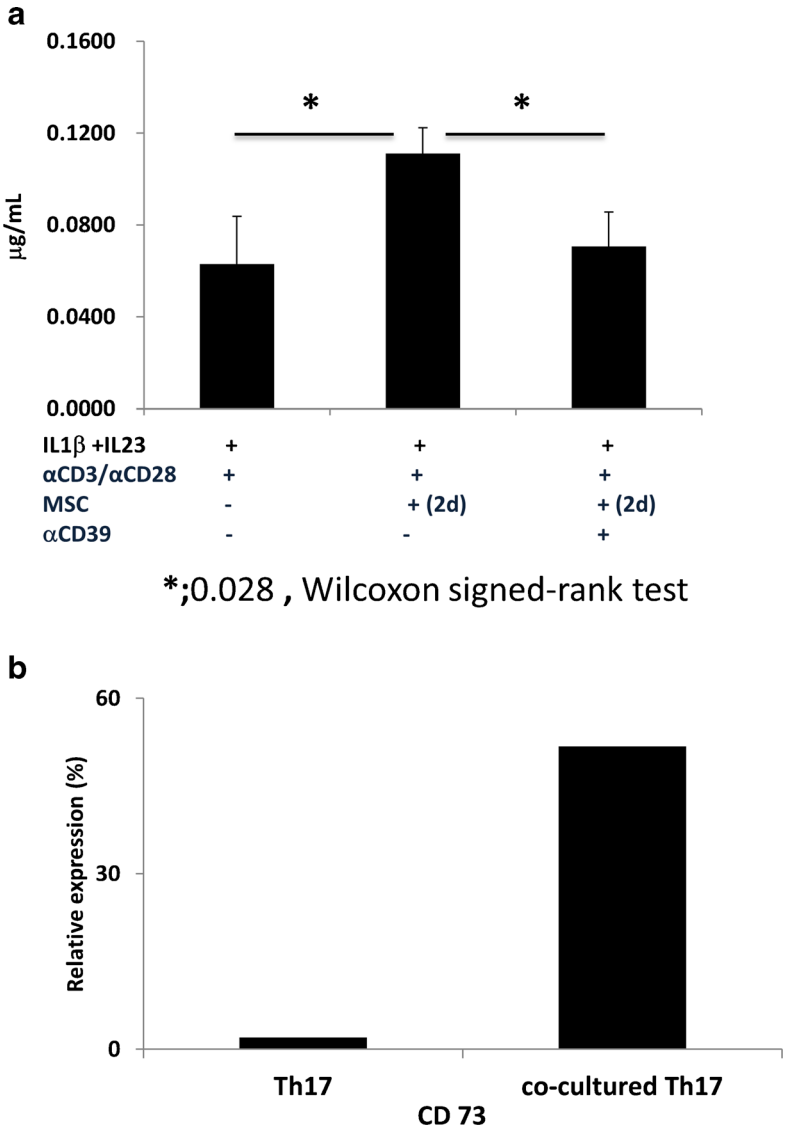

Fig. 5 Mass spectrometry (**a**) presented enhanced adenosine production in supernatants from co-culture of Th17 cells and BMSCs, with a significant reduction in adenosine production after anti-CD39 treatment ($n=6$). Microarray data (**b**) showed increased CD73 expression on co-cultured Th17 cells with BMSCs. It supports that BMSC-mediated suppressive function of human Th17 cells is dependent on CD39–CD73-mediated P1 signaling

Discussion

Th17 cells have been implicated in many human diseases targeted by MSC therapy including multiple sclerosis, autoimmune arthritis, and uveitis [16–18]. Most experimental animal studies have shown that MSCs effectively suppress murine Th1 and Th17 responses, though only a few human studies have corroborated the inhibitory effects of human mesenchymal stem/stromal cells (hMSCs) on human Th1 and Th17 cell responses [19–21]. Conversely, some studies have shown that hMSCs could increase human Th17 responses [18, 22]. This dichotomy prompted the present study, which sought to clarify the responses of human BMSCs on human Th17 cell function to confirm the inhibitory effect of hBMSCs on the adaptive immunity. Our results demonstrate suppressive effects of hBMSCs on IFN-γ and IL-17A secretion by Th17 cells in a CD39–CD73-mediated P1 signaling. Moreover, this reduction effect of hBMSCs via CD39 upregulation was comparable to or greater than the suppressive effect of BMSCs in the IDO- or NOS2-dependent pathways.

In human MSCs, it has been known that immunosuppression is at least partly mediated by the IDO, PGE2, TGF-β, IL-6, IL-10, human leukocyte antigen-G5, leukemia inhibitory factor, heme oxygenase 1, TNF-α stimulated gene/protein 6, nitric oxide, and others [1–4, 23–26]. Recently, some studies have shown that murine MSCs could share the adjacent modulatory function of inflammatory immune responses with Treg cells through the mediation of the CD39/CD73 pathway [8, 9]. CD39 is an extracellular enzyme that catalyzes ATP and ADP to AMP, followed by rapid dephosphorylation of AMP into adenosine by CD73. CD39 as well as CD73 are also present and enzymatically active in both human MSCs and murine MSCs [27], while CD73 that is either co-expressed or supplied by other cells such as lymphocytes, catalyzes the

conversion of AMP to adenosine in order to accomplish immune modulation. Our data support the previous evidence that MSCs modulate immune responses of Th17 lymphocytes mediated by the CD39/CD73 pathway in both human and mice, as mentioned above. In addition, our data reveal that hBMSCs suppressed the immune responses of human Th17 cells by induction of CD39/CD73 on Th17 cells and by increase of adenosine production (P1 signaling).

As expected, the CD39-associated suppression of Th17 cell proliferation based on CFSE study was significant, and the CD39-mediated suppression on the effector function of secretion of IFN-γ and IL-17A in Th17 cells was remarkable. When we evaluated the suppressive effects of BMSCs on the proliferation of naïve T cells, we found that the suppressive effect of BMSCs was lesser than the effect on effector Th17 cells (data unpublished). Taken together, the results indicate that BMSCs seemingly had a greater impact on effector T cell function rather than on naïve T cell function.

Autoimmune diseases including uveitis, scleritis, and Sjogren's syndrome, as well as the damage mediated by chemical burns, are known to be involved in Th17 cells-associated inflammation [12, 13, 28, 29]. In consideration of future human clinical trials, the advantage in the treatment of eye diseases is that hMSCs can be applied topically while other systemic autoimmune or chronic inflammatory diseases require systemic administration with hMSCs. Topical application of hMSCs may be safer than systemic administration, considering that fewer cells are required and that direct contact with the target site is possible without lung entrapment. Therefore, future clinical trials using hMSCs may be more successful in the eye disease than in systemic diseases. To understand how Th17-related eye diseases can be treated with application of hMSCs, our study may be beneficial because it demonstrated proximity-dependent immune-modulatory effects of MSCs on Th17 cell function through CD39-dependent pathway promoting adenosine production.

In conclusion, hMSCs effectively suppressed the effector function of human Th17 cells and this suppression was mediated by the CD39/CD73 pathways in vitro.

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Conflict of interest The authors report no conflicts of interest.

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