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IFATS Collection: Immunomodulatory Effects of Adipose Tissue-Derived Stem Cells in an Allergic Rhinitis Mouse Model

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ABSTRACT

Adipose tissue-derived stem cells (ASCs) exhibit immunosuppressive effects in allogeneic transplantation. However, there is no report that evaluates the in vivo immune-modulating effect of ASCs in an experimental allergic rhinitis (AR) model. We investigated whether ASCs migrate to the nasal mucosa in an AR mouse model and evaluated the immune-modulating effect of ASCs in the AR mouse model. Cultured ASCs (2×10^6) were injected i.v. before the first allergen challenge in the AR mouse model. Migration of ASCs to the nasal mucosa was evaluated by immunofluorescence. The immunomodulatory effects of ASCs were evaluated by nasal symptoms, histology, serum ovalbumin (OVA)-specific antibody, and the cytokine profile of the spleen. ASCs migrated to the nasal mucosa in the AR mouse model. ASCs significantly reduced allergic symptoms and inhibited eosinophilic inflammation in the nasal mucosa. ASCs significantly decreased the serum allergen-specific IgE level and the IgG_1/IgG_{2a} ratio and significantly increased the IgG_{2a} level in the AR mouse model. ASCs inhibited interleukin (IL)-4 and IL-5 production from OVA-incubated splenocytes, but enhanced interferon-γ production. In conclusion, ASCs can migrate to the nasal mucosa in the AR mouse model and inhibit eosinophilic inflammation partly via shifting to a T-helper 1 (Th1) from a Th2 immune response to allergens. STEM CELLS *2009;27:259–265*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Mesenchymal stem cells (MSCs) can be isolated from the bone marrow (BM-MSCs) and differentiate into several mesenchymal lineages, such as bone [1–3], cartilage [1, 2], adipose tissue [1, 2], and muscle [1, 4], both in vitro and in vivo. MSCs may be useful in the repair or regeneration of damaged tissues, such as in osteogenesis imperfecta, myocardial infarction, injured lung, and stroke [5–8]. In addition, MSCs possess immunomodulatory properties with therapeutic potential to prevent graft-versus-host disease in allogeneic hematopoietic cell transplantation [9]. Indeed, MSCs can inhibit natural killer cell function [10, 11], modulate dendritic cell (DC) maturation [12], and suppress the allogeneic T-cell response [10] by altering the cytokine secretion profile of DCs and T cells induced by an allogeneic immune reaction.

Adipose tissue contains stromal cells with phenotypic characteristics resembling those of BM-MSCs. Adipose tissue-derived stem cells (ASCs), similar to BM-MSCs, have a multilineage differentiation capacity [2, 13, 14] and also exhibit similar in vitro immunosuppressive properties [15–17].

Allergic rhinitis (AR) is a type I allergic disease caused by an IgE-mediated humoral immune response. AR is characterized by an influx of eosinophils and T-helper 2 (Th2) cells producing interleukin (IL)-4, IL-5, and IL-13 [18]. We hypothesized that ASCs would be capable of immunomodulatory effects on the allergic inflammation of the airways. The purpose of this study was to evaluate whether ASCs can migrate to the nasal mucosa and to evaluate the effects of ASC migration on the development of AR phenotypes, such as inflammation of the nasal mucosa as well as systemic immune responses, including serum antigen-specific IgE, IgG, and spleen cytokines in an AR mouse model.

MATERIALS AND METHODS

Animals

Eight-week-old female BALB/c mice were purchased from Hyochang Science (Daegu, Korea, http://www.dhbiolink.com) and bred in a specific pathogen-free animal facility. The animal study

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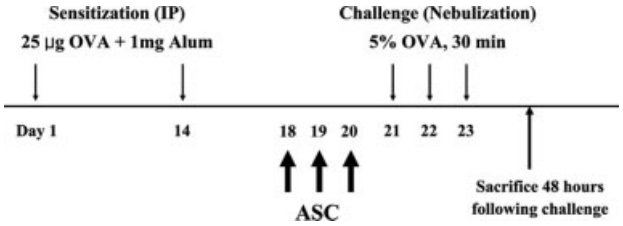

Figure 1. The experimental protocol. The mice were sensitized on days 1 and 14 by i.p. injection of ovalbumin (OVA). Seven days later, the mice were challenged with 5% OVA using the ultrasonic nebulizer for 30 minutes. 2×10^6 purified adipose tissue-derived stem cells (ASCs) were injected via the tail vein on days 18, 19, and 20.

protocol was approved by the Institutional Animal Care and Use Committee of the Pusan National University School of Medicine.

AR Mouse Model

BALB/c mice were sensitized using ovalbumin (OVA) (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com) and aluminum hydroxide (Alum) (Pierce Chemical Co., Rockford, IL, http://www.piercenet.com). Briefly, mice were sensitized on days 1 and 14 by i.p. injection of 25 μg OVA emulsified in 1 mg of Alum in a total volume of 200 μl, as previously described [18, 19]. On days 21, 22, and 23 after the initial sensitization, the mice were challenged with an aerosol of 5% OVA in phosphate-buffered saline (PBS) in a plexiglass chamber through an ultrasonic nebulizer (NE-U12; Omron, Kyoto, Japan, http://www.omron.com) for 30 minutes (Fig. 1).

Isolation and Culture of ASCs

Adipose tissue was obtained from the abdominal fat of BALB/c mice. Because the amount of adipose tissue obtained from one mouse is too small, adipose tissue was collected from 10 BALB/c mice. To isolate ASCs, adipose tissue was washed extensively with an equal volume of PBS and digested with 0.075% collagenase type I (Sigma-Aldrich) at 37°C for 30 minutes. Enzyme activity was neutralized with α-modified Eagle's medium (α-MEM) containing 10% fetal bovine serum (FBS) and the sample was centrifuged at 1,200 $\times$g for 10 minutes to obtain a pellet. The pellet was filtered through a 100-μm nylon mesh to remove cellular debris and incubated overnight at 37°C in 5% CO_2 in control medium (α-MEM, 10% FBS, 100 u/ml penicillin, 100 μg/ml streptomycin). Following incubation, the plate was washed extensively with PBS to remove residual nonadherent RBCs. The resulting cell population was maintained at 37°C in 5% CO_2 in control medium. One week later, when the monolayer of adherent cells reached confluence, cells were trypsinized (0.05% trypsin-EDTA; Sigma-Aldrich), resuspended in α-MEM containing 10% FBS, and subcultured at a concentration of 2,000 cells/cm^3. For experiments, we used the third or fourth passage of ASCs.

Immunophenotypic Analysis

Flow cytometric analysis was used to characterize the phenotypes of the ASCs. At least 50,000 cells (in 100 μl PBS, 0.5% bovine serum albumin [BSA], 2 mmol/l EDTA) were incubated with fluorescein isothiocyanate-labeled monoclonal antibodies against mouse CD105, CD90, CD45, and CD11b (BD Biosciences Clontech, Palo Alto, CA, http://www.bdbiosciences.com) or with the respective isotype control. After washing, the labeled cells were analyzed by flow cytometry using fluorescence-activated cell sorting (FACS)Calibur flow cytometer and Cell Quest Pro software (BD Biosciences, San Diego).

Multilineage Differentiation of ASCs

ASCs were analyzed for their capacity to differentiate toward the adipogenic and osteogenic lineages.

Adipogenic differentiation was induced by culturing ASCs for 2 weeks in adipogenic medium (1 μM dexamethasone, 100 μg/ml 3-isobutyl-1 methylxanthine, 5 μg/ml insulin, 60 μM indomethacin, and 10% FBS in α-MEM) and assessed using an oil red O stain as an indicator of intracellular lipid accumulation. Prior to staining, the cells were fixed for 15 minutes at room temperature in 70% ethanol. The cells were incubated in 2% oil red O reagent for 1 hour at room temperature. Excess stain was removed by washing with 70% ethanol, followed by several changes of distilled water.

Osteogenic differentiation was induced by culturing ASCs for 2 weeks in osteogenic medium (0.1 mM dexamethasone, 10 μM β-glycerophosphate, 50 μg/ml ascorbic acid, and 10% FBS in α-MEM) and examined for extracellular matrix calcification by alizarin red S staining. For alizarin red S staining, the cells were fixed with 70% ethanol and washed with distilled water. The cells were incubated in 2% alizarin red solution for 15 minutes at room temperature. The cells were washed several times with distilled water.

ASC Allograft in the AR Mouse Model

ASCs were washed with PBS and incubated with Cell Tracker CM-Dil (Molecular Probes Inc., Eugene, OR, http://probes.invitrogen.com), 2 μM, in a 37°C incubator for 5 minutes, and then for an additional 15 minutes at 4°C. The cells were washed with PBS and suspended in PBS at a concentration of 2×10^7 cells/ml. To examine the effect of ASCs, 0.1 ml of purified stem cells was injected with a 26-gauge needle via the mouse tail vein on days 18, 19, and 20 (Fig. 1).

Mice were divided into four groups, with five mice in each group: (a) control group mice were sensitized, treated, and challenged with PBS; (b) ASC group mice were sensitized and challenged with PBS, but pretreated with ASCs; (c) OVA group mice were sensitized with OVA plus Alum, pretreated with PBS, and then challenged with OVA; (d) OVA+ASC group mice were sensitized with OVA plus Alum, pretreated with ASCs, and then challenged with OVA. These experiments were performed more than four times according to the same protocol.

Analysis of ASC Migration

ASCs were labeled with Cell Tracker CM-Dil and injected via the tail vein after OVA sensitization. Mice were sacrificed 48 hours after the final OVA challenge by means of anesthetic overdose with ketamine hydrochloride (1 mg/kg) and xylazine (0.5 mg/kg). Nasal mucosa was removed and immediately frozen in optimum cutting temperature medium. Sections (7-μm thick) were cut at -20°C and washed, and then examined by fluorescence microscopy.

Measurement of Nasal Symptoms

Nasal symptoms were evaluated by counting the number of sneezing and nasal rubbing occurrences for 15 minutes following an OVA challenge in a blind manner by three observers.

Histologic Analysis of Nasal Mucosa

Nasal mucosa was removed, perfused with 1.0 ml of 10% neutral-buffered formalin, immersed in the same fixative for 24 hours at room temperature, and then embedded in Technovit 7100 resin (Heraeus Kulzer, Wehrheim, Germany, http://www.kulzer-technik.de). Sections were stained with hematoxylin and eosin and assessed through the use of light microscopy with a high-power field. Eosinophils were counted by two individuals who were unaware of the treatment. After five randomly selected tissue sections per mouse were counted, the absolute numbers of eosinophils were counted as the mean $\pm$ standard error of the mean (SEM).

Measurement of Serum OVA-Specific IgG and IgE

At 48 hours after the last OVA challenge, blood samples were obtained from mice via cardiac puncture. OVA-specific IgE, IgG_1, and IgG_{2a} were determined by enzyme-linked immunosorbent assay (ELISA). Ninety-six-well microtiter plates (Nunc Maxisorb; Nunc, Roskilde, Denmark, http://www.nuncbrand.com) were coated with 100 μl/well of OVA (10 mg/ml) in PBS (pH 9.8) and incubated at 4°C overnight. Wells were then blocked with blocking buffer (4% BSA in PBS) at room temperature for 1 hour. Serially diluted serum samples (100 μl/well) were added to each well, followed by incubation for 2 hours at room temperature. After washing with 0.1%

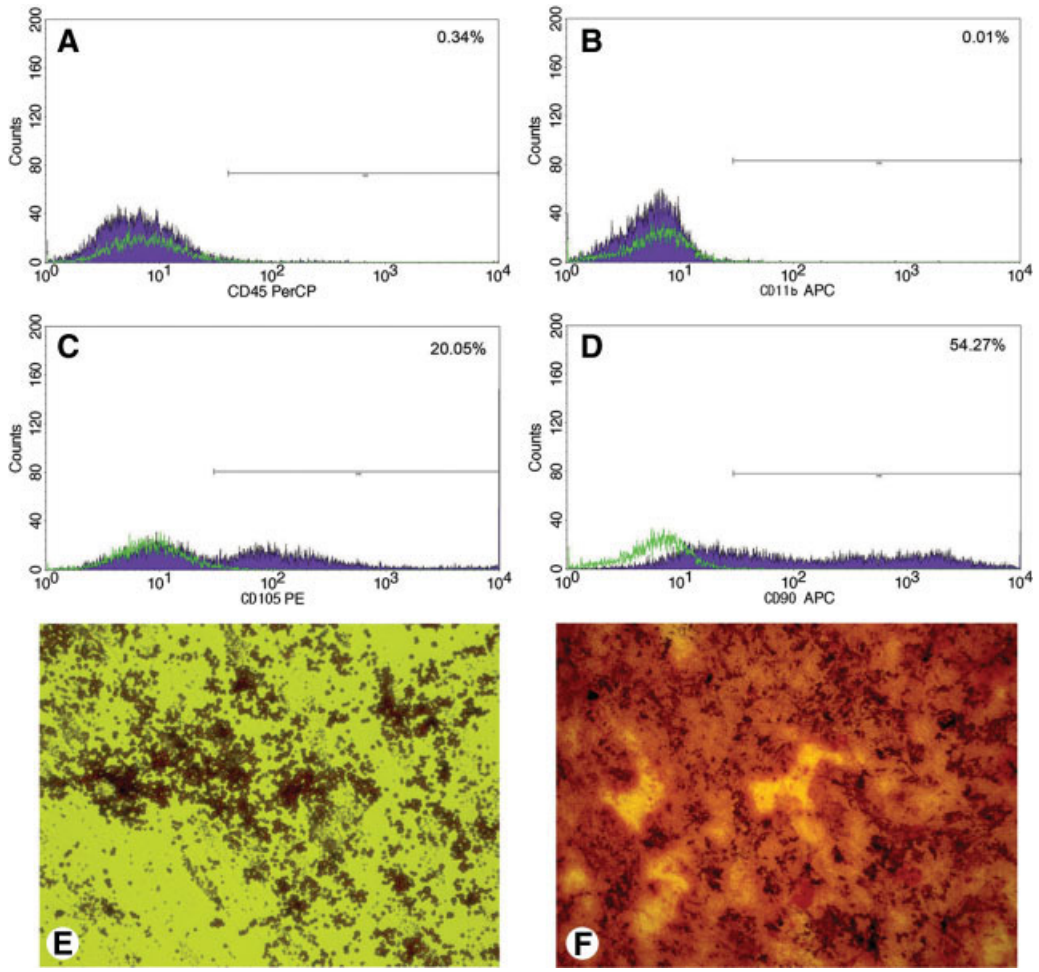

Figure 2. Analysis of the immunophenotype and differentiation capacity of adipose tissue-derived stem cells (ASCs). ASCs were negative for CD45 **(A)** and CD11b **(B)** and positive for CD105 **(C)** and CD90 **(D)**. Adipogenesis was detected by the formation of multiple, intracellular lipid-filled droplets stained with oil red O **(E)**. Osteogenesis was demonstrated by several red regions, indicative of calcified extracellular matrix **(F)** (magnification, 200×).

Tween 20 in PBS (PBS-T), horseradish peroxidase-conjugated anti-mouse IgE (Pharmingen, Hamburg, Germany, http://www.bdbiosciences.com//pharmingen) (1:4,000), IgG_1 (1:5,000), or IgG_{2a} (1:5,000) was added to each well and incubated at room temperature for 1 hour. After washing with PBS-T, all wells were developed with 100 μl tetramethyl benzidine (Sigma-Aaldrich). Absorbance (450 nm) was measured with an ELISA plate reader (Molecular Devices Corporation, Sunnyvale, CA, http://www.moleculardevices.com).

Expression of Cytokines in the Spleen

The spleen was removed 48 hours after the last OVA challenge, placed in a tissue culture petri dish containing 3 ml of culture medium, and pressed with the plunger of a 5-ml syringe. After splenocytes were treated with NH_4Cl-$KHCO_3$ to deplete erythrocytes, single-cell suspensions of splenocytes (2×10^5 cells/well in 96-well culture plates) were cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine, 1 mM sodium pyruvate, and antibiotics with OVA (100 ng/ml). After 48 hours of culture, IL-4, IL-5, IL-10, and interferon (IFN)-γ levels in the supernatants were determined by sandwich ELISA (R&D Systems Inc., Minneapolis, http://www.rndsystems.com) as per the manufacturer's guideline.

Statistical Analysis

Data are presented as mean ± SEM. Statistical significance was assessed by an unpaired *t*-test using the SPSS software package version 12.0 (SPSS Inc., Chicago, IL, http://www.spss.com). A p-value $< .05$ was considered significant.

RESULTS

Immunophenotypic and Differentiation Characterization of ASCs

ASC cultures were analyzed for expression of cell-surface markers. ASC cultures were negative for CD45 (Fig. 2A) and CD11b (Fig. 2B) and expressed the surface proteins CD105 and CD90. The percentage of $CD105^+$ cells in ASCs was 20.1% (Fig. 2C) and the percentage of $CD90^+$ cells was 54% (Fig. 2D).

To examine the multilineage capacity of ASCs, cells were differentiated toward the adipogenic and osteogenic lineages using lineage-specific induction factors. ASCs cultured in adipogenic media were reproducibly induced toward the adipogenic lineage as early as 2 weeks postinduction. A significant fraction of the cells contained multiple, intracellular lipid-filled droplets that stained with oil red O (Fig. 2E). Calcification appeared as red regions within the cell monolayer. Consistent with osteogenesis, several red regions with alizarin red S staining, indicative of calcified extracellular

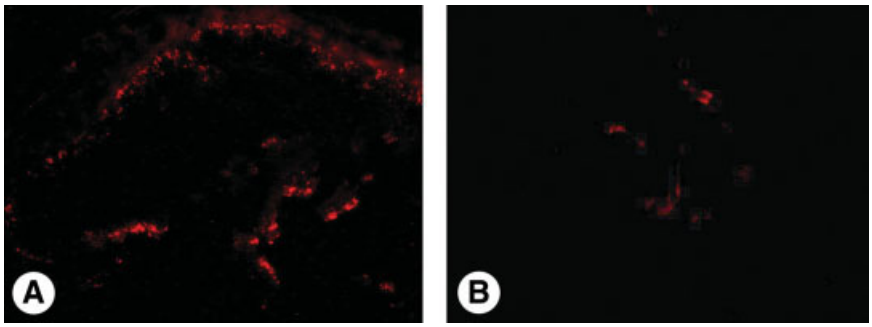

Figure 3. Immunofluorescence of migrated adipose tissue-derived stem cells (ASCs) in the nasal mucosa. ASCs labeled with Cell Tracker CM-Dil (red) migrate into the nasal mucosa of the mice. Migration was greater in the ovalbumin-challenged mice **(A)** than in the phosphate-buffered saline-challenged mice **(B)** (magnification, 200×). Results are representative of four independent experiments.

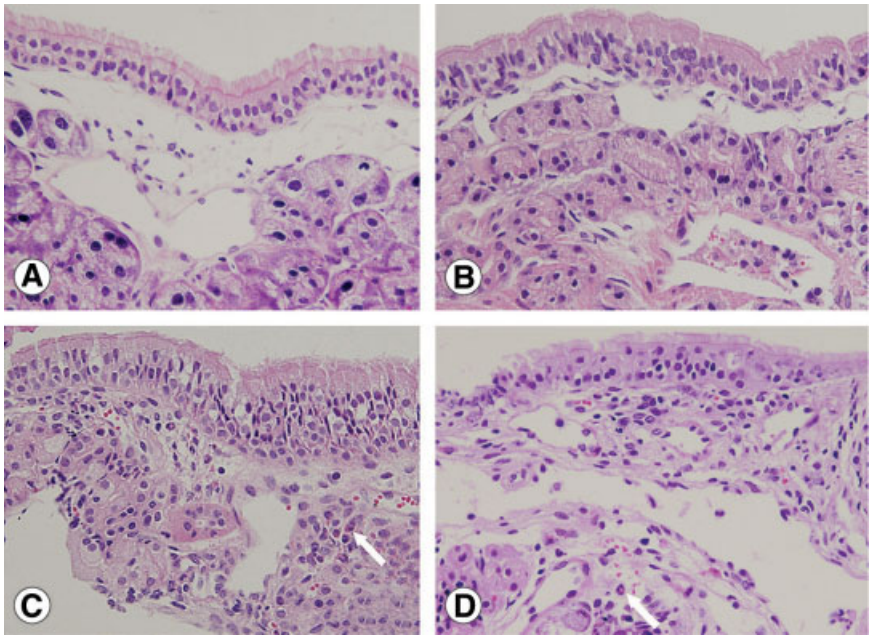

Figure 4. Histology of nasal mucosa. The control **(A)** and adipose tissue-derived stem cell (ASC) animals **(B)** had no inflammatory changes. The ovalbumin (OVA) group **(C)** showed that the nasal mucosa was infiltrated with eosinophils (arrow). Eosinophilic infiltration was significantly lower in the OVA+ASC group **(D)**. Sections were stained with hematoxylin and eosin (magnification, 400×). Results are representative of four independent experiments.

matrix, were observed in ASCs treated for 2 weeks in osteogenic medium (Fig. 2F).

Detection of ASCs in Nasal Mucosa

To determine whether injected ASCs migrated into the nasal mucosa in the AR mouse model, five nasal mucosa sections for each animal were examined with fluorescence microscopy. Purified stem cells (2×10^6) were injected i.v. before the first allergen challenge in the AR mouse model. Immunofluorescence microscopic examination showed that red CM-Dil-positive ASCs were detected in both the epithelial and subepithelial layer of nasal mucosa, especially in the OVA+ASC group, in comparison with the ASC group (Fig. 3).

Number of Nasal Allergic Symptoms

To know whether injected ASCs affect changes in nasal symptoms, clinical parameters were measured. The sum of sneezing and nasal rubbing occurrences was increased significantly by daily OVA challenge. The number of nasal symptoms was significantly higher in the OVA group than in the control group ($p < .001$). Interestingly, ASC pretreatment significantly decreased the number of nasal symptoms in the OVA group ($p = .009$) (data not shown).

Histological Analysis of Nasal Mucosa

To evaluate histological changes in the nasal mucosa after ASC injection, the infiltrating inflammatory cells were counted. There was no inflammatory cell infiltration into the nasal mucosa in the control or ASC group. However, histological examination showed that inflammatory cells, such as eosinophils and mononuclear cells, infiltrated into the nasal mucosa in the OVA group at 48 hours after the last OVA challenge. Eosinophil infiltration was significantly lower in the OVA+ASC group than in the OVA group ($p = .009$) (Figs. 4, 5).

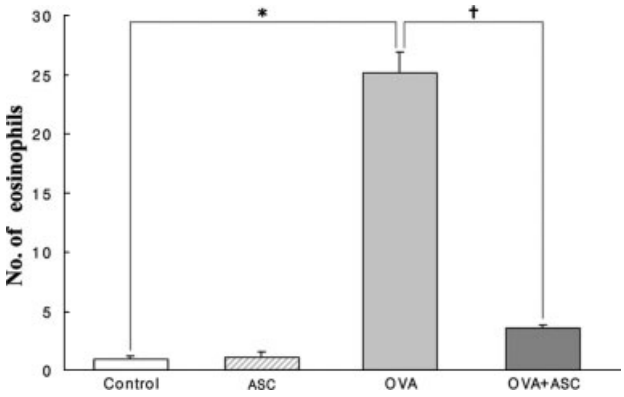

Figure 5. Number of eosinophils in the nasal mucosa. The number of eosinophils was significantly higher in the ovalbumin (OVA) group than in the control group and significantly lower in the OVA plus adipose tissue-derived stem cell (OVA+ASC) group. Data are expressed as the mean ± standard error of the mean ($n = 5$ in each group). Results are representative of four independent experiments. *, $p = .039$; †, $p = .009$.

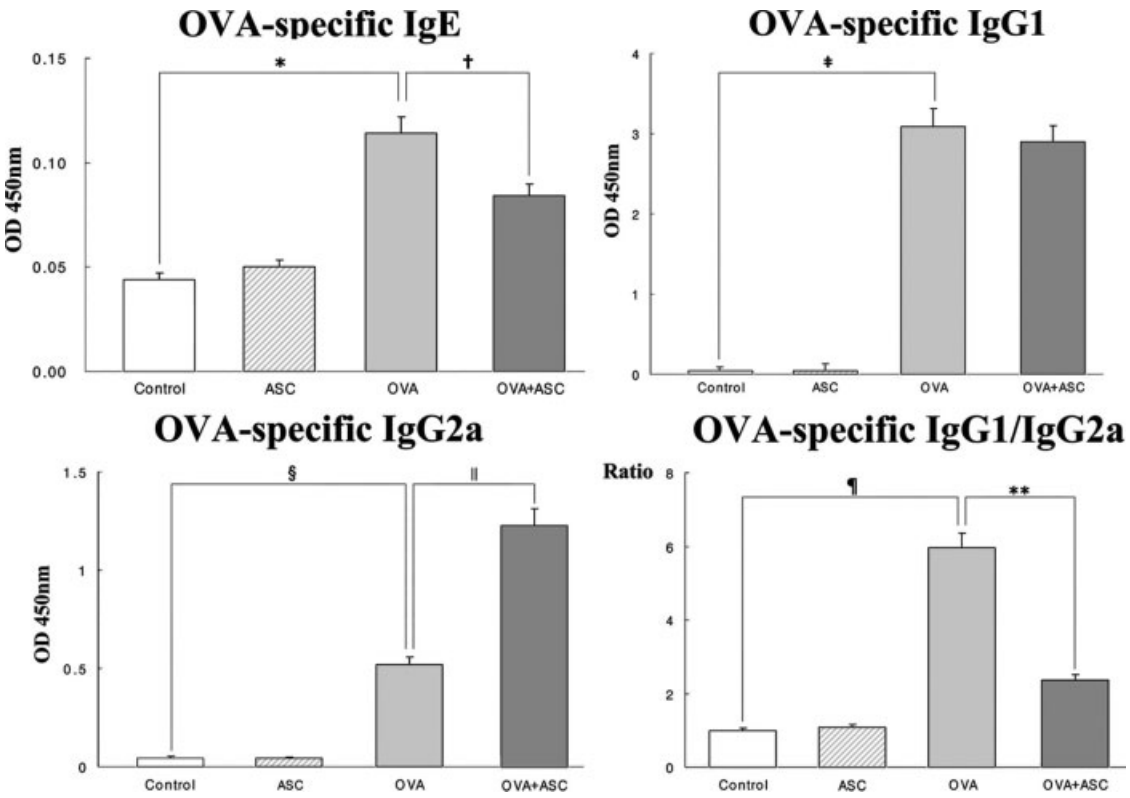

Figure 6. Serum antigen-specific-antibody responses. OVA-specific IgE, IgG_1, and IgG_{2a} levels were higher in the OVA group than in the control group. IgE and the IgG_1/IgG_{2a} ratio were significantly lower in the OVA+ASC group. The IgG_{2a} level was significantly lower in the OVA+ASC group than in the OVA group. Data are expressed as the mean ± SEM ($n = 5$ in each group). Results are representative of six independent experiments. *, $p = .001$; †, $p = .039$; ‡, $p < .001$; §, $p = .039$; ‖, $p = .01$; ¶, $p < .001$; **, $p < .001$. Abbreviations: ASC, adipose tissue-derived stem cell; OD, optical density; OVA, ovalbumin; SEM, standard error of the mean.

Determination of Serum OVA-Specific IgE, IgG_1, and IgG_{2a} Levels

To determine whether injected ASCs affect serum OVA-specific antibodies in the AR mouse model, the OVA-specific IgE, IgG_1, and IgG_{2a} levels and IgG_1/IgG_{2a} ratio were determined. OVA-specific IgE and IgG_1 levels were higher in the OVA group than in the control group ($p = .001$ and $p < .001$, respectively). OVA-specific IgE levels were significantly lower in the OVA+ASC group ($p = .039$). There were no significant differences in serum OVA-specific IgG_1 levels between the OVA+ASC and OVA groups. OVA-specific IgG_{2a} levels were significantly higher in the OVA+ASC group than in the OVA group ($p = .01$). The IgG_1/IgG_{2a} ratio was also higher in the OVA group than in the control group ($p < .001$). This ratio was significantly lower in the OVA+ASC group than in the OVA group ($p < .001$) (Fig. 6).

Analysis of Cytokines in the Spleen

To determine whether injected ASCs affect cytokine production, the cytokines in the spleen were analyzed. IL-4, IL-5, and IL-10 levels in the supernatants of OVA-incubated splenocytes were significantly higher in the OVA group than in the control group ($p = .01$, $p = .042$, and $p = .009$, respectively). Interestingly, IL-4 and IL-5 levels were lower in the OVA+ASC group than in the OVA group ($p = .03$ and $p = .046$, respectively). In contrast, IFN-γ levels in the supernatants were significantly higher in the OVA+ASC group than in the OVA group ($p = .004$). There was no significant difference in IL-10 levels between the OVA+ASC and OVA groups (Fig. 7).

DISCUSSION

MSCs have been reported to have a strong immune-suppressive effect [9–12, 15]. Previous evidence suggests that ASCs play an immunosuppressive role in terms of the inhibition of mixed lymphocyte reactions and lymphocyte proliferative response to mitogens [15–17]. ASCs and BM-MSCs can both repair damaged tissue and promote immunosuppression [6, 7, 15–17, 20]. Although BM has been the main source for the isolation of multipotent MSCs, adipose tissue is another alternative source that can be obtained in larger quantities [13, 21].

We demonstrated that ASCs administered i.v. migrated in higher numbers into the nasal mucosa in the AR mouse model, compared with control mice. These results suggest that immune responses to allergens may improve the ASC recruitment environment at the allergen entry site.

Allergic airway inflammation leads to mucous gland hyperplasia and eosinophilic infiltration [18]. Here, eosinophilic inflammation of the nasal mucosa was higher in the OVA group than in the control group, and ASCs inhibited this inflammation. These data indicate that ASCs inhibit eosinophilic inflammation.

In the present study, serum OVA-specific IgE, IgG_1, and IgG_{2a} levels, which are B-cell immune responses that are regulated by cytokines from Th cells, were significantly increased by OVA challenge. Interestingly, IgE and the IgG_1/IgG_{2a} ratio, which represent the Th2 immune response, were significantly decreased by pretreatment with ASCs. In contrast, OVA-specific IgG_{2a} levels, which indicate a Th1 immune response, were significantly increased by ASC pretreatment. These data suggest

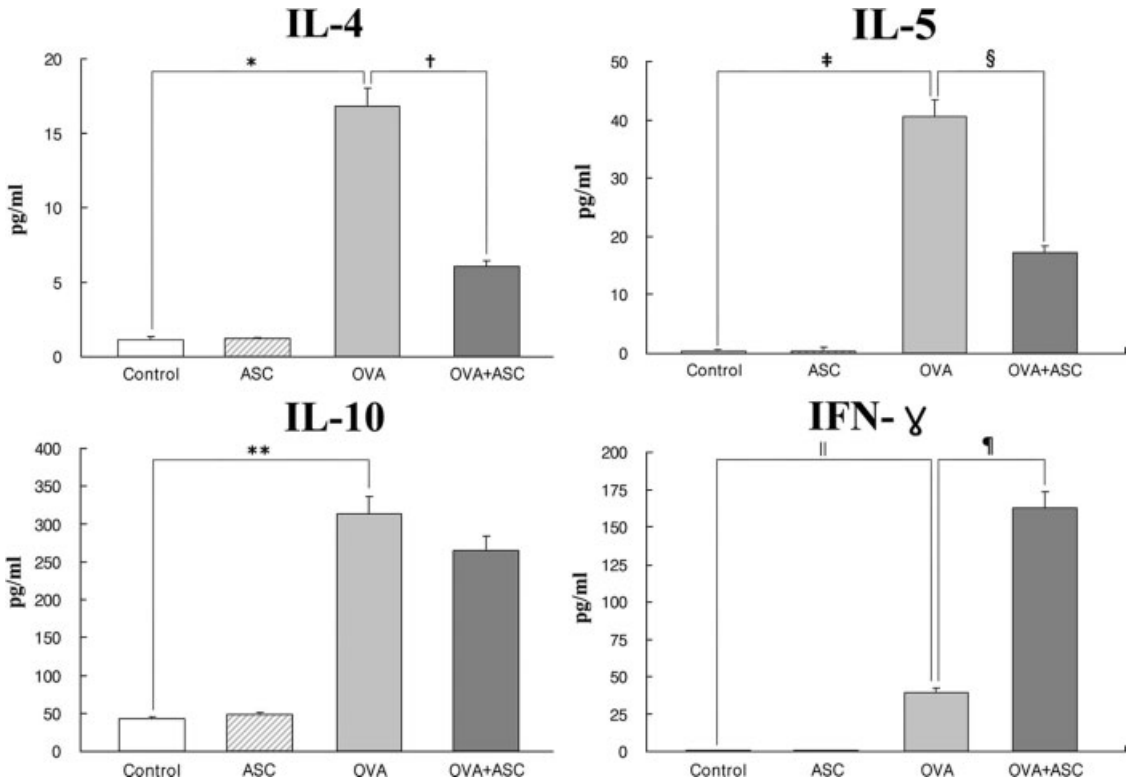

Figure 7. Cytokine responses in supernatants of the spleen. IL-4, IL-5, and IL-10 levels were significantly higher in the OVA group and IL-4 and IL-5 levels were lower in the OVA+ASC group than in the OVA group. IFN-γ levels were significantly higher in the OVA+ASC group than in the OVA group. There was no significant difference in IL-10 levels between the OVA+ASC and OVA groups. Data are expressed the mean ± SEM ($n = 5$ in each group). Results are representative of six independent experiments. *, $p = .01$; †, $p = .03$; ‡, $p = .042$; §, $p = .046$; **, $p = .009$; ‖, $p = .006$; ¶, $p = .004$. Abbreviations: ASC, adipose tissue-derived stem cell; IFN, interferon; IL, interleukin; OVA, ovalbumin; SEM, standard error of the mean.

that ASCs injected i.v. were responsible for the Th2 to Th1 shift in the AR mouse model.

BM-MSCs and ASCs have immunosuppressive effects [15, 22]. Transplanted allogeneic MSCs suppress immune responses by interacting with MSCs and immune cells via inhibiting tumor necrosis factor-α secretion, but enhancing IL-10 secretion, which affects the DC maturation state and function and induces T-cell tolerance rather than T-cell priming [10, 11, 15, 23, 24]. MSCs may also shift Th1 immune responses to Th2 responses in allografts via inhibition of IFN-γ secretion and augmentation of IL-4 secretion [22]. AR is an aberrant Th2 cytokine-mediated immune response, and Th2 cytokines such as IL-4 and IL-5 induce eosinophilic inflammation [18]. In terms of eosinophilic inflammation, Th1 and Th2 cytokines are mutually antagonistic, and selective suppression of Th2 responses may be crucial for protection against allergic inflammation [18, 19]. The present study showed that IL-4 and IL-5 levels were significantly increased in AR mice, indicating that allergic inflammation in the nasal mucosa was generated by Th2 cytokines. Interestingly, ASC pretreatment inhibited IL-4 and IL-5 production from OVA-incubated splenocytes, but enhanced IFN-γ production. Taken together with the inhibition of eosinophilic inflammation by ASCs, these findings indicate that ASCs inhibit the development of allergic inflammation by shifting to a Th1, from a Th2, immune response to allergens. Moreover, taken together with the immune-modulating effects of MSCs in the allogeneic graft model, stem cells behave in an immunosuppressive manner, irrespective of the type of T-cell immune response, although the exact mechanism of a Th2 to Th1 shift in the AR mouse model cannot be fully explained by the present study. Further studies are required to fully characterize the immunomodulatory mechanism of ASCs to skew from a Th2 to a Th1 response by direct contact of ASCs with T lymphocytes or secretion of crucial mediators by ASCs in nasal allergic inflammation.

In conclusion, this study showed that ASCs allotransplanted i.v. can migrate to the nasal mucosa in the AR mouse model. Migrated ASCs have an immunomodulatory effect, which is characterized by inhibition of eosinophilic inflammation in the nasal mucosa partly via downregulation of the Th2 immune response to allergens.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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