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Adipose-derived stem cells attenuate atopic dermatitis-like skin lesions in NC/Nga mice

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Abstract

There is an unmet need in novel therapeutics for atopic dermatitis (AD). We examined the effects of autologous adipose-derived stem cells (ADSCs) on AD-like skin lesions induced by the application of 2,4-dinitrochlorobenzene (DNCB) in NC/Nga mice. Autologous ADSCs and ADSC-conditioned medium (ADSC-CM) were injected intralesionally three times.

Clinical severity and histopathologic findings were compared in sham naïve control, saline-treated, ADSC-treated, ADSC-CM-treated, and 2.5% cortisone lotion-applied animals. The severity index, skin thickness, mast cell number, as well as expression levels of thymic stromal lymphopoietin, CD45, chemoattractant receptor-homologous molecule, chemokine

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ligand 9, and chemokine ligand 20 were significantly lower in mice treated with ADSC, ADSC-CM, or 2.5% cortisone lotion.

Tissue levels of interferon- γ as well as serum levels of interleukin-33 and immunoglobulin E levels were also decreased in those groups. We conclude that autologous ADSCs improved DNCB-induced AD-like skin lesions in NC/Nga mice by reducing inflammation associated with Th2 immune response and interferon- γ .

Key words: Atopic dermatitis; NC/Nga mouse; adipose-derived stem cells; Th2-mediated inflammation; immunomodulation

Introduction

Atopic dermatitis (AD) is characterized by pruritic eczematous lesions, impaired barrier function, Th2 type immune hyperactivation, and frequent elevation of serum immunoglobulin E.(1-3) In the pathogenesis of acute stage AD, Th2 immune response is predominant. Thymic stromal lymphopoietin (TSLP) leads to production of Th2 cytokine, leading to recruitment of immune cells from lymph nodes to skin via trans-endothelial migration. They are mainly chemoattractant receptor-homologous molecule (CRTH2)-positive Th2 lymphocytes.

However, Th1 inflammation is also important. Especially, interferon (IFN)- γ contributes to the severity and chronicity of AD (4) and chemokines induced by IFN- γ (e.g. chemokine ligand 9 [CXCL9] and chemokine ligand 20 [CCL20]) further recruits T lymphocytes from dermis to epidermis, explaining a second step T lymphocyte chemotaxis.(5) AD treatments include non-specific immunosuppressants and anti-inflammatory drugs. Recently, more selective AD therapeutics such as dupilumab, Janus kinase inhibitors, phosphodiesterase

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inhibitors, and others have emerged.(1,6) Although all treatments have some therapeutic benefit, they have limited utility because of adverse events, safety concerns about long-term use, or high cost. Therefore, there is an unmet need in new therapies for AD that would be better than current treatments. Adipose-derived stem cells (ADSCs) are mesenchymal stem cells (MSCs) with characteristics similar to those of bone marrow-derived stem cells.(7) ADSCs can be obtained in sufficient amounts comparatively easier than adult stem cells from other sources. Furthermore, they are not inferior in quality or differentiation ability to bone marrow-derived stem cells and can maintain stem cell characteristics regardless of age. MSCs isolated from various tissues exhibit different biological effects and are especially noted for their immunomodulatory and anti-inflammatory properties.(8) Therefore, in several animal and clinical studies, there have been attempts to treat AD with MSCs, including ADSCs.(9-11) However, previous studies mostly utilized allogeneic or xenogeneic ADSCs, which may have influenced immunologic activity of ADSCs. Thus, we investigated the effect of autologous ADSCs on AD-like skin lesions in NC/Nga mice.

Methods

Animals and treatment

Male 12-week-old NC/Nga mice were purchased from Samtako Inc. (Kyunggi-do, Republic of Korea) and housed in a specific pathogen-free animal facility. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the SMG-SNU Boramae Medical Center (approval number: 2016-0052). The mice were maintained under specific-pathogen-free conditions at 24 ± 2 °C, $55 \pm 10\%$ relative humidity, and 12 h–12 h light-dark cycle. After three weeks of acclimation, NC/Nga mice were randomly divided into five groups: sham naïve control ($n = 3$) mice, as well as animals treated with saline ($n = 6$),

ADSCs ($n = 6$), ADSC-CM ($n = 6$), or 2.5% cortisone lotion (Jiwoo Pharm, Seoul, Republic of Korea) ($n = 6$).

Except for the sham control group, 2,4-dinitrochlorobenzene (DNCB, Sigma, Cat No. 237329, Seoul, Republic of Korea) was applied on the shaved dorsal skin of every mouse for 7.5 weeks to induce AD-like skin lesions. For the first week, 200 μ L of 1% DNCB dissolved in a mixture of acetone and olive oil (3:1) was applied three times per week (days 1, 3, 5). For the next 6.5 weeks, 200 μ L of 0.2–0.4% DNCB was applied on the same site three times per week (days 8–50). AD-like lesions were detected after 20 days of DNCB treatment. Then, ADSCs and or ADSC-CM were injected three times on days 43, 46, and 49, and the mice were sacrificed on day 53.

Harvesting of autologous inguinal fat pads and preparation of ADSCs and ADSC-CM

All surgical procedures were performed under aseptic conditions. On day 17 after DNCB treatment, mice were anesthetized by intraperitoneal administration of a combination of zoletil (Zoletil 100 at 20 mg/kg; Virbac, Seoul, South Korea) and xylazine (Rompun at 10 mg/kg; Bayer, Seoul, South Korea). After the left inguinal region was shaved and draped, a 1-cm incision was made along the inguinal fold. Then, autologous inguinal fat pad (about 1 g) was harvested (Fig. S1a). The isolated fat tissues were washed three times in phosphate-buffered saline (PBS). After rinsing, the fat tissue was digested using 0.25 mL of 0.3 U/mL collagenase (NB6 GMP grade, SERVA, Heidelberg, Germany, 17458) at 37 °C for 1 h under shaking conditions. Collagenase activity was stopped by the addition of 0.5 mL of α -minimal essential medium (α -MEM). Stromal vascular fraction was isolated from the digested fat tissue by centrifugation for 5 min at 1,500 rpm and filtering through nylon meshes with decreasing pore diameter (from 100 μ m to 40 μ m). The isolated cells were washed twice with PBS and plated in a T75 flask in α -MEM. Mouse ADSCs were cultured in α -MEM

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supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 5 ng/mL basic fibroblast growth factor, and 100 U/mL penicillin/streptomycin. The flasks were maintained in a cell culture incubator at 37 °C in an atmosphere of 95% air and 5% carbon dioxide. When cultured cells reached over 90% confluence (in about 1 week), they were harvested using trypsin/EDTA solution and frozen in liquid N₂ (P0). Three days before the injection, mouse ADSCs were thawed and cultured at a density of 3×10^5 cells/T75 flask (P1). The medium was changed in 1 day after thawing and maintained for 2 more days. ADSCs adhered to plastic and showed fibroblast-like morphology. ADSCs for cell injection were harvested after a total of 3 days in culture (Fig. S1b, passage 1, 70% confluence). To analyze cultured mouse ADSCs phenotype, flow cytometry analysis was performed on ADSCs (3×10^5). For the detection of ADSC surface makers, cells were treated with FITC-conjugated antibodies against CD45, CD29, CD105, CD73, CD34, CD44, and CD90 (BD Biosciences, San Jose, CA, USA) for 2 min at 4 °C in the dark. After washing with the buffer twice, cells were re-suspended with the buffer and analyzed with FACSCalibur using CellQuest software (BD Biosciences, San Jose, CA, USA). To obtain ADSC-conditioned culture medium, ADSCs (P0) were thawed and seeded at a density of 3×10^5 cells per T75 flask and washed after 1 day with culture medium. Then, 5 mL of the medium was added every 2–3 days for 10. In total, 20–25 mL of ADSC-CM was collected after 10 days and centrifuged at 4,000 rpm for 10 min at room temperature. Cell supernatant was acquired and stored at 4 °C until injections.

Injection ADSCs and ADSC-CM

Autologous ADSCs were detached, suspended in PBS (1×10^5 cells/mL), and 200 µL of the resulting suspension was injected subcutaneously at the top, bottom, right, left, and center of a 3-cm circle of the most protruding DNCB-treated dorsal skin area. ADSC-CM was injected

(5 × 200 µL) using the same protocol. ADSCs and ADSC-CM were injected three times at a 3-day interval (days 43, 46, and 49 after the start of the DNCB treatment). Saline was used as negative control and 2.5% cortisone lotion was used as positive control. Saline injections were done in the same way as injections of ADSCs or ADSC-CM. About 1 mL of 2.5% cortisone lotion was applied with a cotton swab on the AD-like DNCB-induced dorsal skin lesion circle (3 cm in diameter) three times, using the same schedule as described above.

Measurement of skin lesion severity index and skin thickness

The severity of lesions on the dorsal skin was assessed using modified criteria described previously(12). For each of the six signs (erythema/hemorrhage, oozing/crust, swelling/edema, erosion/excoriation, lichenification, and dryness), scores were given (0: none; 1: mild; 2: moderate; 3: severe) by two independent investigators who were blinded to the treatment protocol. A total score was defined as the sum of obtained scores (modified SCORAD index, scale 0–18). Dorsal skin thickness was repeatedly measured during the experiment using a caliper (Mitutoyo®, Kawasaki, Japan).

Harvesting of serum and tissue lysate

Blood was collected by venous sampling from the tail on the first injection day and by cardiac puncture after anesthesia on the sacrifice day. Collected whole blood was left to clot at 25 °C for 30 min and centrifuged (3,000 rpm, 4 °C, 10 min). Serum was collected and stored frozen at –80 °C until use. On the sacrifice day, 60 mg of the dorsal skin was excised, stored in liquid N₂ and homogenized with 300 µL of 0.05% Tween 20-PBS (pH 7.4) by a bullet blender Storm (JCBIO, Seoul, Republic of Korea) at 12,000 rpm for 7 min. Tissue lysates were collected and then stored frozen at –80 °C until use.

Measurement of cytokines and IgE using Luminex and ELISA

For serum IgE measurements, blood was collected on the first injection day before the injection and on the sacrifice day. For interleukin (IL)-4, IL-33, IL-13, and interferon (IFN)- γ , blood and tissue lysate sampling was done only on the sacrifice day. Cytokine levels were measured by the multiplexed magnetic Luminex screening assay (IL-4, IL-33, IL-13, IFN- γ , cat. No. LXSAMSM, R&D Systems, Minneapolis, MN, USA), and IgE levels were determined by a mouse IgE ELISA kit (Abcam, cat. No. 157718) according to the manufacturers' protocols (Supplementary information).

Histopathology

After euthanasia, the dorsal skin was excised, fixed in 4% paraformaldehyde, and embedded in paraffin. Then, 4- μ m-thick sections were cut and stained with hematoxylin and eosin (H&E) and toluidine blue (TB). Epidermal and dermal thickness (H&E, $\times 40$) was analyzed by image analysis software (cellSens Standard, Seoul, Republic of Korea) by averaging three random measurements. TB staining was used to detect mast cells. After deparaffinization, the sections were soaked for 2–3 min in a 1% TB solution (pH 2.2; Sigma, Munich, Germany). The staining solution was then drained off and sections were dehydrated in 96–100% ethanol for 20–30 s, dipped in xylene, and cover slipped carefully. Then, the number of mast cells was counted (TB, $\times 100$) and calculated per 1 mm² of the skin using Leica Qwin image software v.3.2.0 (Leica Imaging Systems Ltd, Cambridge, UK).

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Immunohistochemistry and immunofluorescence experiments

For immunohistochemistry experiments, tissue sections were blocked with PBS containing 0.15% Tween-20, 2% bovine serum albumin, and 5% normal donkey serum for 30 min at room temperature. Sections were then incubated with the following primary antibodies: rabbit polyclonal to CD45 (1:5,000; Abcam, cat. No. ab10558), rabbit polyclonal to TSLP (1:100; MyBiosource, cat. No. MBS9410785), rabbit polyclonal to CRTH2 (1:50; MyBiosource, cat. No. MBS150173), CXCL9 (1:100; Abcam, cat No. ab202961), and rabbit polyclonal to CCL20 (1:100; Abcam, cat No. ab9829) in blocking solution overnight at 4 °C. After washing three times in PBS, the sections were incubated with anti-rabbit horseradish peroxidase-conjugated (1:1,000; DAKO, cat. No. K4003) or FITC-conjugated (1:1,000; Jackson, cat. No. 111-095-003) secondary antibodies for 1.5 h at room temperature. Control sections were incubated with the secondary antibody alone. Slides were visualized on a compound Olympus BX51 microscope using cell Sens standard software (Olympus, Seoul, Republic of Korea) and on a Leica fluorescence DMI4000 B microscope using Leica Application Suite software (v3.8; Leica, Seoul, Republic of Korea). Total pixel intensity was measured using Leica Q win image software (v.3.2.0; Leica Imaging Systems, Ltd, Seoul, Republic of Korea), and the data were expressed as optical densities.

Statistical analysis

To compare SCORAD indices, skin thickness values, and IgE levels, we performed the analysis of covariance (ANCOVA), adjusting for baseline values, to see the difference between groups at each time point. In cases when the interactions were significant, additional one-way analysis of variance (ANOVA) followed by the *post hoc* Bonferroni test was performed at each time point.

When we determined epidermal and dermal thickness values, mast cell infiltration, TSLP, CD45⁺ cells, CRTH2⁺ cells, CXCL9, CCL20, IL-33, IL-4, IL-13, and IFN- γ expression, we performed ANOVA followed by the *post hoc* Bonferroni test. All analyses were conducted using SAS software (v.9.2; SAS Institute Inc., Cary, NC, USA) with a significance level of $\alpha = 0.05$ ($P < 0.05$).

Results

Cell culture

ADSCs cultured for 3 days were used for intralesional injection. Their flow cytometric analyses showed that all cells constitutively expressed CD29, CD105, CD73, CD44, and CD90, which are markers of progenitor cells and ADSCs, respectively, but were negative for hematopoietic cell markers CD45 and CD34 (Fig. S1c). ADSC-CM was collected after culturing ADSCs for 9 days.

Skin lesion severity index and skin thickness

AD-like lesions were successfully induced after DNCB application. Skin lesions were ameliorated in mice treated with ADSCs, ADSC-CM, or cortisone, compared to the state of the lesions in saline-injected group (Fig. 1a).

AD severity index (Fig. 1b) was significantly lower in groups treated with ADSCs, ADSC-CM, or cortisone, compared to that in saline-treated group on day 49 ($P < 0.001$, $P = 0.024$, $P < 0.001$, respectively) and on day 53 ($P < 0.001$ for all respective comparisons). Interestingly,

the severity index was lower in ADSC-CM-treated group than in the groups that received ADSCs or cortisone on day 53 ($P = 0.005$ in both cases).

Skin thickness reflecting disease severity was measured by a caliper (Fig. 1c). On day 46, only cortisone group mice demonstrated thinner skin than saline-treated mice ($P = 0.035$). However, on days 49 and 53, all three treated groups (ADSC, ADSC-CM, and cortisone) had thinner skin than mice in saline-treated group ($P \leq 0.001$ for all respective comparisons). Furthermore, on day 53, whereas there was no significant difference in skin thickness between cortisone- and ADSC-CM-treated groups ($P = 0.175$), cortisone decreased skin thickness more strongly than ADSC treatment ($P = 0.006$).

Histopathology

Epidermal thickness (Fig. 2a, b) was decreased by a similar extent by the injections of ADSCs ($41.40 \pm 8.54 \mu\text{m}$, $P < 0.001$) and ADSC-CM ($34.80 \pm 7.33 \mu\text{m}$, $P < 0.001$), as well as by cortisone application ($45.88 \pm 4.49 \mu\text{m}$, $P < 0.001$) compared to the value observed in saline-treated mice ($123.13 \pm 19.44 \mu\text{m}$). Likewise, dermal thickness values were comparably lower in mice that were treated with ADSCs ($416.22 \pm 8.54 \mu\text{m}$, $P < 0.001$), ADSC-CM ($451.83 \pm 110.20 \mu\text{m}$, $P = 0.001$), or cortisone ($307.20 \pm 73.25 \mu\text{m}$, $P < 0.001$) than in saline-treated animals ($671.68 \pm 65.73 \mu\text{m}$).

As shown in Fig. 2d, mast cell infiltration was significantly ameliorated in animals injected with ADSCs (40.20 ± 9.44 cells, $P < 0.001$), ADSC-CM (47.33 ± 13.13 cells, $P < 0.001$), or treated with cortisone (51.00 ± 21.46 cells, $P < 0.001$) compared with saline group (134.00 ± 7.42 cells).

Immunohistochemistry and immunofluorescence analyses of major AD effectors

TSLP expression level (Fig. 3a, b) in saline group was higher (11.7 ± 1.69) than in mice that were administered ADSCs (5.07 ± 0.76 , $P < 0.001$), ADSC-CM (4.52 ± 1.33 , $P < 0.001$), or received cortisone treatment (6.72 ± 1.66 , $P < 0.001$).

CD45 expression (Fig. 3c) was elevated in DNCB-induced AD-like region in saline-treated group (1.68 ± 0.29), but it was reduced by the injections of ADSCs (0.38 ± 0.18 , $P < 0.001$), ADSC-CM (0.19 ± 0.17 , $P < 0.001$), or by the applications of cortisone (0.37 ± 0.15 , $P < 0.001$).

In a similar fashion, CRTH2 expression levels (Fig. 3d) were lower in animals that received injections of ADSCs (3.79 ± 2.08 , $P < 0.001$), ADSC-CM (1.58 ± 1.84 , $P < 0.001$), or applications of cortisone (4.10 ± 3.32 , $P < 0.001$) compared to CRTH2 expression in saline-injected mice (19.19 ± 5.54). CXCL9 expression levels (Fig. 3e) were lower in injection of ADSCs (4.49 ± 1.25 , $P < 0.01$), ADSC-CM (8.78 ± 0.51 , $P < 0.01$), or application of cortisone (15.83 ± 3.94 , $P < 0.05$) compared to saline-injected mice (41.2 ± 4.15). CCL20 expression (Fig. 3f) was higher in saline-treated group (61.18 ± 1.97), but it was reduced by the injections of ADSCs (23.99 ± 2.46 , $P < 0.001$), ADSC-CM (25.98 ± 3.19 , $P < 0.001$), or by the applications of cortisone (28.87 ± 3.1 , $P < 0.001$).

Serum IgE level (Fig. S2)

Whereas IgE level was increased by a continuous DNCB treatment in saline injection group, IgE levels were slightly reduced in mice that were treated with ADSCs, ADSC-CM, or

cortisone. IgE levels in ADSC-treated and cortisone applied groups were significantly lower than in saline-injected animals.

Suppression of pro-inflammatory cytokines

Tissue levels of IFN- γ (Fig. 4a) were decreased by the treatments with ADSCs (66.20 ± 14.51 pg/mL, $P < 0.001$) or ADSC-CM (36.27 ± 3.26 pg/mL, $P < 0.001$) compared to that in saline group (Fig. 4a–c) (117.14 ± 45.84 pg/mL). Similarly, serum levels of IL-33 (Fig. 4b) were lower in ADSC group (32.20 ± 3.04 pg/mL; $P < 0.001$) and ADSC-CM group (42.83 ± 6.07 pg/mL, $P < 0.001$) than in saline-treated animals (64.06 ± 8.58 pg/mL).

Serum IL-4 level (Fig. 4c) in mice injected with ADSC-CM (30.03 ± 8.39 pg/mL, $P < 0.001$) was significantly decreased compared to that in saline group (51.97 ± 2.24 pg/mL). Serum IL-13 level (Fig. 4d) was decreased in ADSC-injected group (0.27 ± 0.03 pg/mL, $P < 0.05$) compared to that in saline group (3.68 ± 1.40 pg/mL).

Discussion

Many studies of MSCs have been published since the first report about these cells in 1970.(13) Initially, the focus was on the self-renewal capacity of stem cells, and the possibilities of damaged tissue replacement by MSCs were intensively investigated. In 2002, the discovery that bone-marrow-derived MSCs suppressed T cell proliferation *in vitro* and prolonged skin graft survival *in vivo*(14) expanded the research focus and prompted examination of immunosuppressive properties of MSCs.(8) Subsequent studies revealed that MSCs suppress innate and adaptive immune systems.(15,16) Because of their

immunomodulatory functions, MSCs are considered for the treatment of patients with diseases that involve inflammatory manifestations, such as graft-versus-host disease,(17) multiple sclerosis,(18) inflammatory arthritis,(19) and systemic lupus erythematosus.(20) Because IFN- γ is required for MSC-mediated immunosuppression,(21-23) initially, the diseases with prominent Th1 immune response were mostly selected for treatment with MSCs,(11) whereas the effects of MSCs on diseases with Th2 cell role, such as AD, have been examined less frequently.(11,24)

Here, we investigated whether subcutaneous injections of ADSCs or ADSC-CM could improve DNCB-induced AD-like skin lesions in NC/Nga mice. The latter is the most extensively studied animal model of AD that closely recapitulates some human AD manifestations. Although AD-like lesions can develop spontaneously, we additionally applied DNCB for better reproducibility.(25) Previous studies of MSCs and their conditioned medium in human AD or mouse AD models utilized variable regimens, including different schedules of topical application, subcutaneous, or intravenous injections. However, the distribution of stem cells *in vivo* after intravenous systemic administration has not been established. Some studies have reported free distribution and homing effects of ADSCs to the damaged tissues, but others have shown that stem cells become entrapped in the lung and exhibit low viability at the systemic level.(13,26) Kim *et al.* also commented that subcutaneous local application of human MSCs exerted a stronger therapeutic effect in a mouse AD model compared with that afforded by intravenous MSC administration.(24) Topical application of MSCs might also be a reasonable option considering that AD is a skin disease. However, it was highly doubtful whether materials can be efficiently delivered to the damaged tissue because DNCB application induced the formation of a very thick crust in NC/Nga mice. Therefore, we selected the subcutaneous route of injection and demonstrated that ADSCs successfully alleviated AD-like lesions.

AD pathogenesis has not been fully elucidated yet and is currently attributed to complicated interactions of the environment, host genetic susceptibility, altered skin barrier function, and immune system factors.(27) Increased TSLP expression is considered to be a pivotal immunological pathogenic factor in AD.(28) Skin injury, allergens, or unknown signals likely stimulate keratinocytes to produce TSLP, which acts directly on T cells, natural killer T cells, eosinophils, basophils, and mast cells, potentiating the production of Th2 cytokines, such as IL-4 and IL-13.(28-31) In the presence of IL-4, TSLP-activated dermal dendritic cells migrate, prime naïve T-helper cells in lymph nodes, and induce expansion of skin homing CCR2⁺ Th2 cells.(28,32) Th2 cytokines activate B cells, resulting in IgE production. Then, IgE-mediated mast cells induce the release of various mediators, including histamine and cytokines,(33,34) thereby contributing to AD symptoms. Additionally, IL-33 promotes Th2 cytokine response and, therefore, has important roles in Th2 biased disease.(35,36) Likewise, Th2 response is predominant in the acute phase of AD. However, Th1 response is also important in AD. In particular, IFN- γ contributes to the severity and chronicity of AD.(4,37) Previous studies revealed that Th2 type cytokine IL-4 was always observed together with Th1 type cytokine IFN- γ within identical AD-involved areas of the same patients, suggesting the role of both subsets in pathogenesis of AD.(4,38) However, *in situ* expression of IFN- γ only, but not that of IL-4, correlated with the clinical severity of AD.(4,39) Furthermore, *in situ* expression of IFN- γ was reduced to levels of uninvolved skin after successful treatment of AD patients (regardless of therapy used), whereas it was not significantly decreased in patients unresponsive to treatment, indicating its usefulness as a treatment response marker. IFN- γ strongly upregulates chemokines such as CXCL9 and CCL20 in keratinocytes and these chemokines attract specific T cells with expression of chemokine receptor 3, leading to apoptosis of keratinocytes.(5) Then these keratinocytes release chemokines that recruit more T cells from dermis to epidermis, which further potentiates inflammation and keratinocyte

apoptosis. In this way, T cell and keratinocyte interaction makes a vicious cycle and maintains inflammation.

In the present study, injections of ADSCs or ADSC-CM decreased expression levels of TSLP, CD45, CRTH2, CXCL9, and CCL20, as well as reduced mast cell infiltration. Also, these treatment decreased tissue levels of IFN- γ as well as serum levels of interleukin-33 and immunoglobulin E levels. Taken together, we think that subcutaneous injections of ADSCs and ADSC-CM were effective in reducing TSLP, which, in turn, inhibited Th2 potentiation and inflammatory cell recruitment, and led to subsequent amelioration of AD-like lesions in NC/Nga mice. Furthermore, given that ADSC and ADSC-CM treatments decreased IFN- γ and chemokines induced by IFN- γ , they may not only decrease severity of AD but also prevent AD chronicity.

Interestingly, subcutaneous injections of ADSC-CM tended to exhibit similar efficacy compared to that afforded by ADSC injection or topical steroid therapy in our study. Similarly, recent experiments have revealed that the benefits of MSC therapy could be due to the secretome, i.e., bioactive factors produced by MSCs and secreted into the extracellular space.(8) Indeed, the secretome or the medium conditioned by the presence of adipose-derived stem cells (ADSC-CM) significantly ameliorated AD lesions in dogs and mice.(2)

However, we cannot completely exclude the possibility that the culture medium itself worked and additional experiments with preconditioned medium control group are necessary. Additionally, further research is needed to determine the components of ADSC-CM that improved AD-like lesions in our study. Furthermore, regulatory requirements for manufacturing, quality control, and standardization of MSCs or their media with regard to MSC origin, isolation methods, and culture conditions are necessary to establish the safety and efficacy profile of these products.(40)

Stem cells from various sources, e.g., bone marrow, umbilical cord blood, and muscle,(9,11,24,41,42) have been utilized in the past. However, these types of cells often require invasive or complicated extraction processes with low yield. Moreover, such cells are disadvantageous owing to limited cell availability and low efficacy.(43) In this study, we used autologous ADSCs that could be easily acquired in large amounts by only minimally invasive procedures. Although MSCs are considered to be hypoimmunogenic,(44,45) it is still unclear whether these cells differentiate. Differentiation can lead to a change in immunogenicity and elicit chronic rejection.[6, 37] A previous study using intravenously administered autologous, allogeneic, and xenogeneic (human) MSCs for pig myocardial infarction revealed that the injection of autologous cells was most effective in reducing infarct size, whereas allogeneic and xenogeneic cells had weaker effects.(46) Grinnemo *et al.* transplanted human MSCs into the site of myocardial infarction of rats and concluded that xenogeneic MSCs induced transplant rejection.(47) Furthermore, in striking contrast to the general notion, Nauta *et al.* indicated that allogenic MSCs are not intrinsically immunoprivileged and induce a memory T-cell response, resulting in the rejection of allogeneic stem cell graft.(48) Therefore, we used autologous ADSCs harvested from mice in order to minimize the effects of allogeneic or xenogeneic immune responses. Cell-free therapies using MSC secretome preparations may resolve these immune compatibility issues.

Our study had several limitations. First, it was a study in an animal model, therefore it could not completely reflect all human AD manifestations. However, the pathology induced by repetitive DNCB applications in NC/Nga mice has been so far one of the most reproducible AD models that closely recapitulated at least some human AD signs. Second, the components of ADSC-CM were not analyzed, and the effect of the medium itself was not compared to that of ADSC-CM. Third, we did not evaluate pruritus or barrier function in the mice and

focused on immunological changes only. Further studies to elucidate in greater detail the mechanism of stem cell therapy in AD and to evaluate various aspects of AD are required.

In summary, subcutaneous injection of autologous ADSCs improved DNCB-induced AD-like skin lesions in NC/Nga mice by immunomodulatory effects that reduced Th2 response-related inflammation. Our findings can help to develop a new AD treatment strategy and warrant further studies elucidating exact therapeutic mechanisms of ADSCs.

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Competing interests

The authors declare that they have no competing interests.

Authors' contributions

HSP, HYS, MHC, HSH, and JUP performed the experiments and analyzed the data; HSP, YS, SK, HSH, MHC, and JUP designed and supervised the study; SK, YS and JUP provided crucial input for the project; HSP, HYS, and JUP wrote the manuscript. All authors read and approved the final manuscript.

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Figure legends

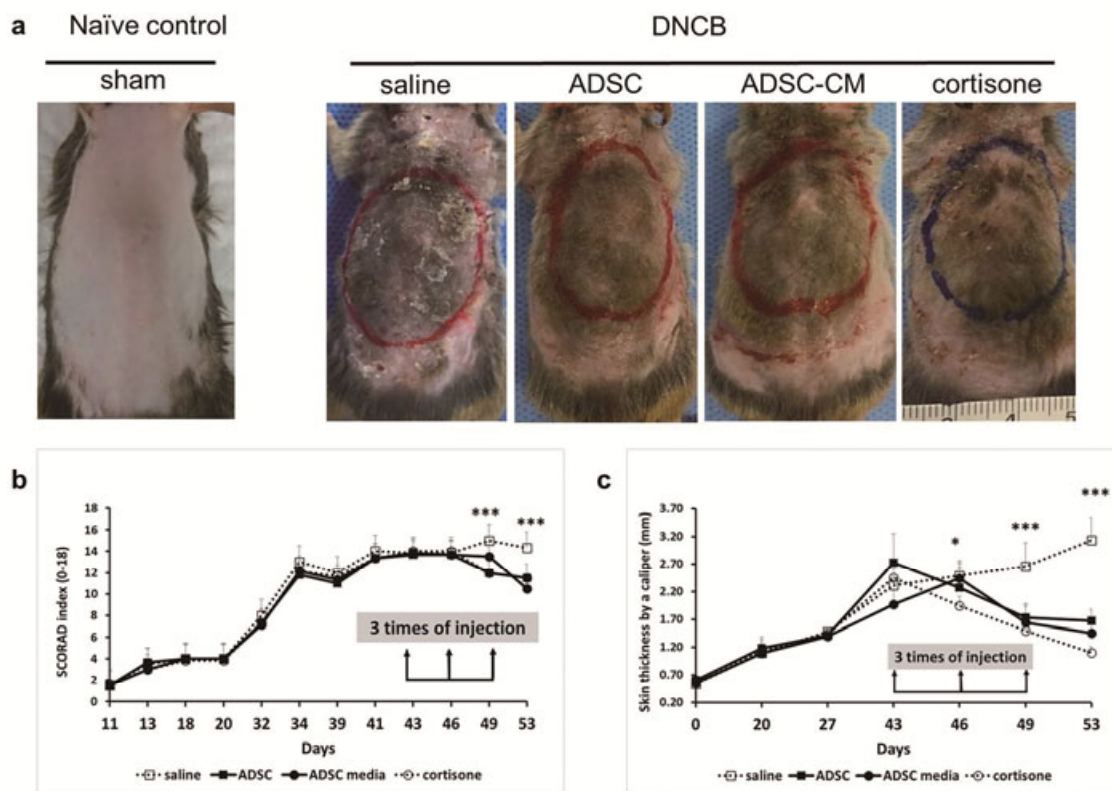
Fig. 1. Clinical effect of adipose-derived stem cells (ADSCs) or ADSC-conditioned medium (ADSC-CM) in atopic dermatitis-like skin lesions of NC/Nga mice. (a) Photographs of the morphological features of NC/Nga mice that were sham-treated (naïve control), received ADSCs, ADSC-CM, or 2.5% cortisone lotion (positive control) at the time of sacrifice. (b) Clinical skin severity scores (SCORAD) of the mice. (c) Skin thickness was calculated with a caliper. Data are presented as the mean $\pm$ standard error of the mean ($n = 6$ in each group except sham, where $n = 3$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

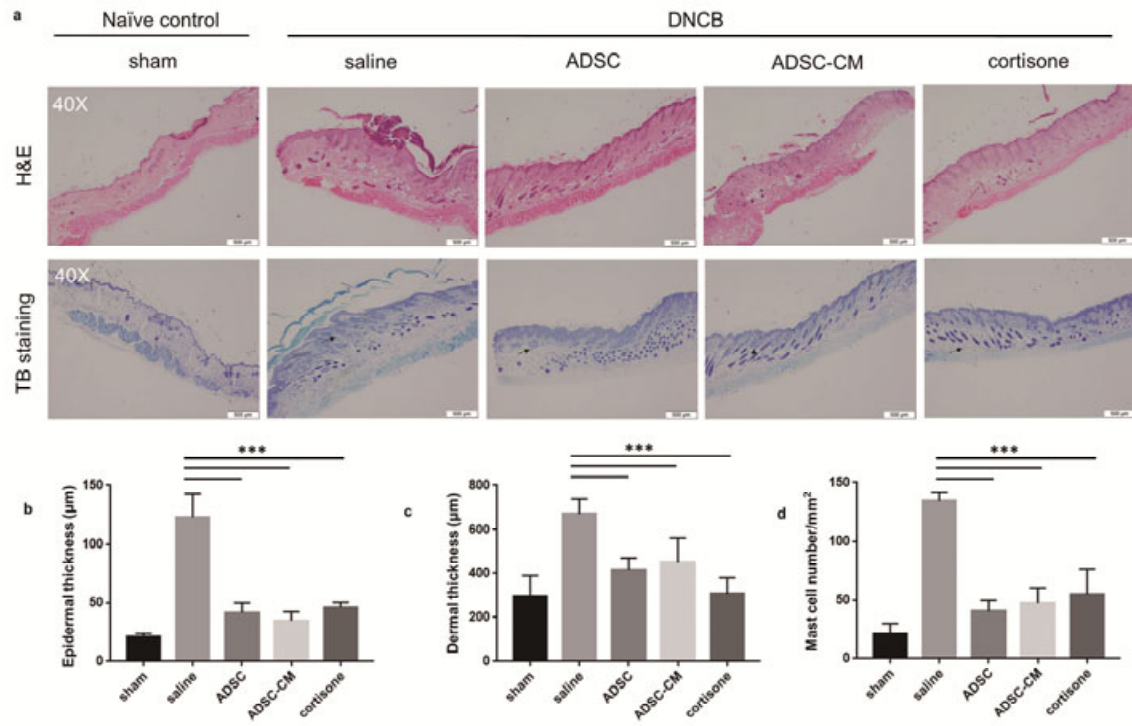
Fig. 2. Effect of adipose-derived stem cells (ADSCs) or ADSC-conditioned medium (ADSC-CM) on skin thickness and mast cell infiltration in atopic dermatitis-like skin lesions. (a) Hematoxylin/eosin (H&E) staining ($\times 40$) provided a histological score of epidermal and dermal thickness; toluidine blue (TB) staining ($\times 100$) reflected mast cell recruitment (arrow). Representative images depicting histological features of the skin collected on day 53 are shown. Epidermal thickness (b), dermal thickness (c), mast cell number (d) in the skin regions with DNCB-induced atopic dermatitis-like manifestations. Data are presented as the mean $\pm$ standard error of the mean ($n = 6$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

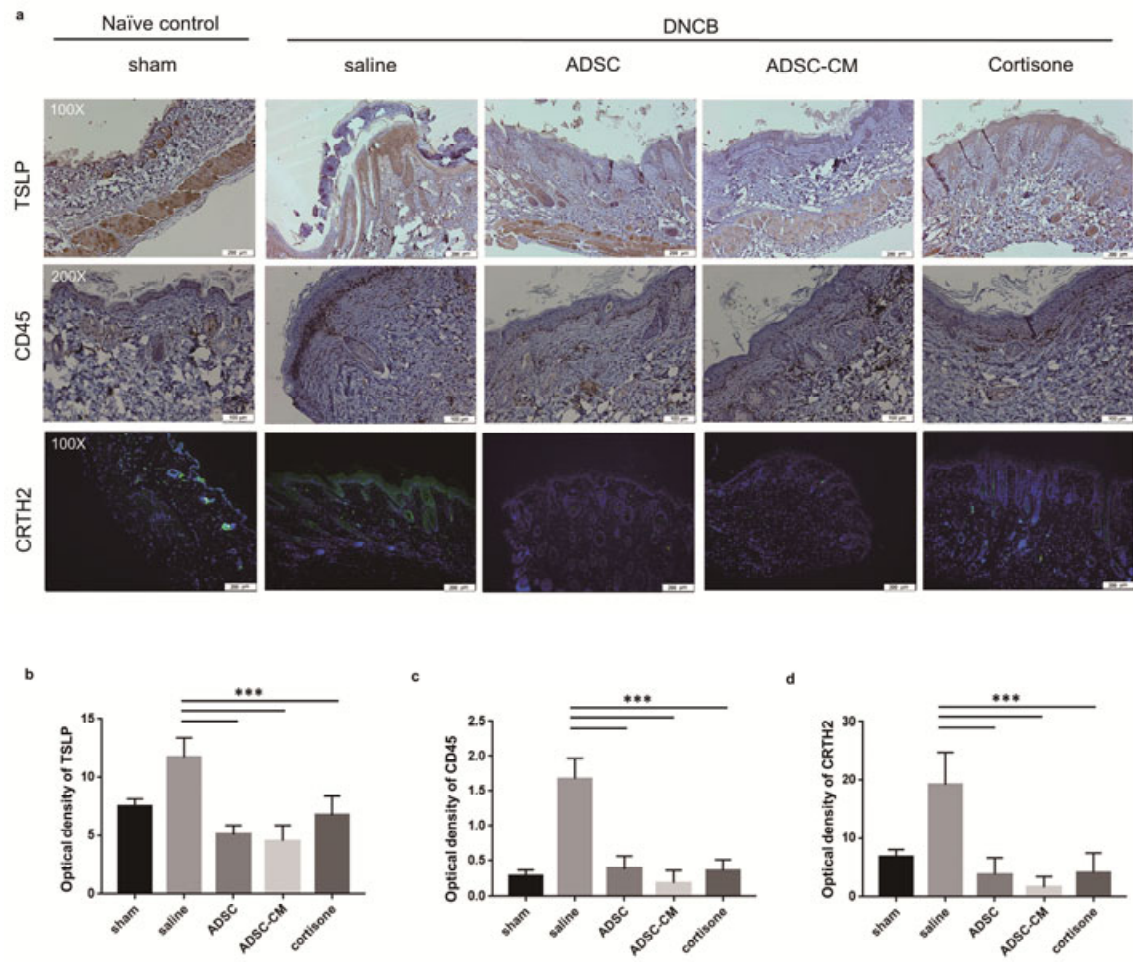
Fig. 3. Expression levels of TSLP, CD45, CRTH2, CXCL9 and CCL20 were determined after injections of adipose-derived stem cells (ADSCs) or ADSC-conditioned medium (ADSC-CM). (a) Representative images of skin lesions. Magnification: TSLP (upper), $\times 100$; CD45⁺ cells (second), $\times 200$; CRTH2 (third), $\times 100$; CXCL9 (fourth), $\times 200$; CCL20 (fifth), $\times 200$. Optical density of TSLP (b), CD45 (c), CRTH2 (d), CXCL9 (e) and CCL20 (f) were

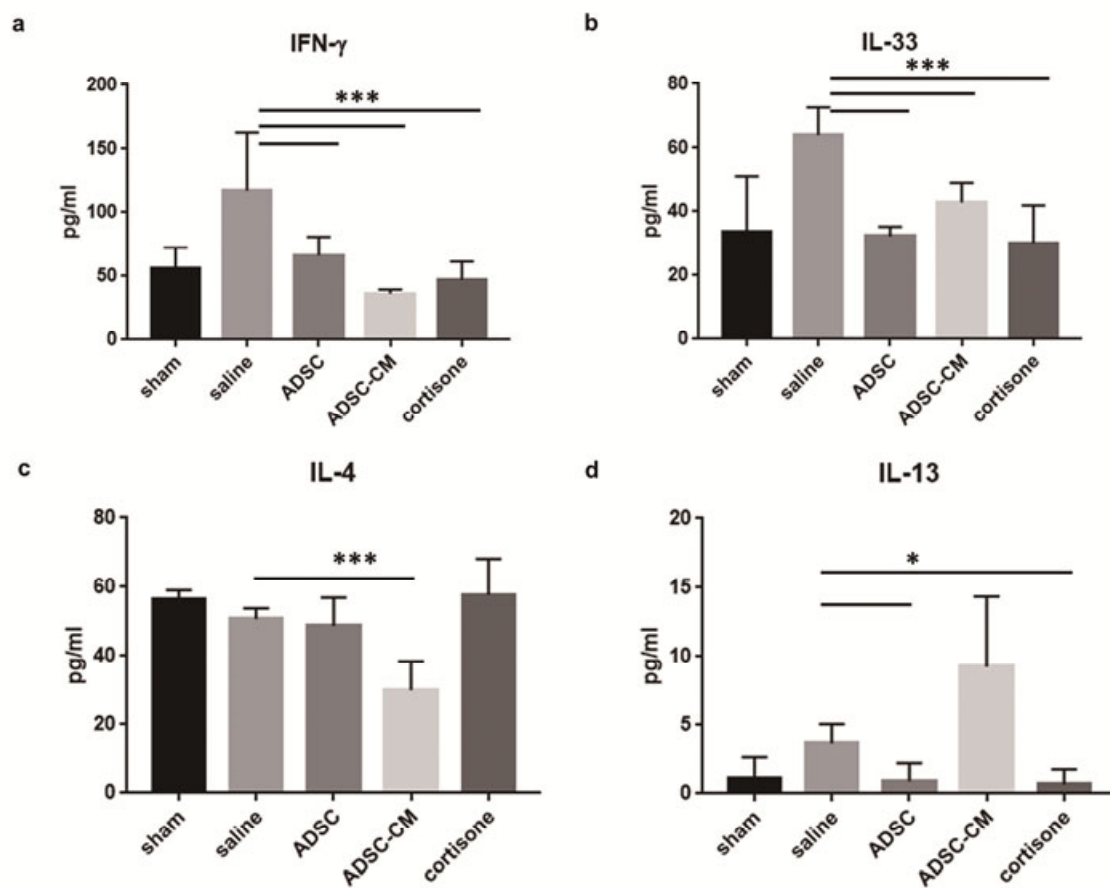
measured. Data are presented as the mean $\pm$ standard error of the mean ($n = 6$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

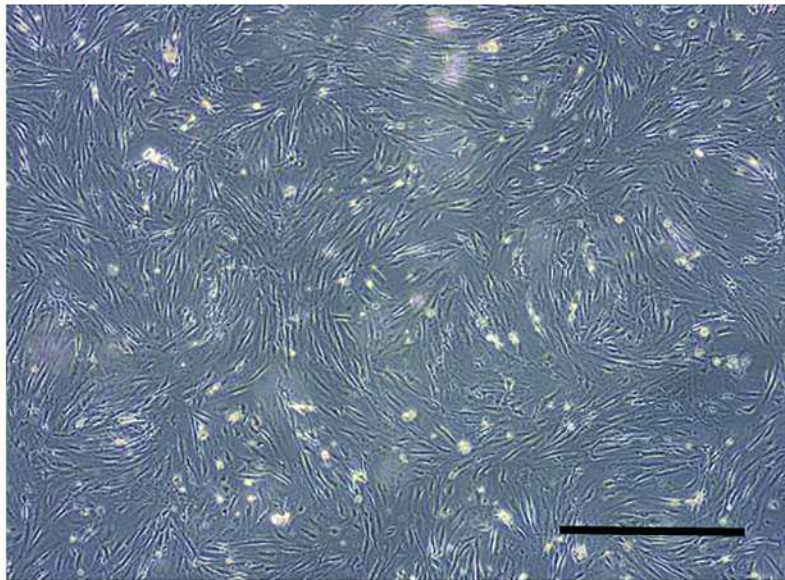
Fig. 4. Effects of adipose-derived stem cells (ADSCs) or ADSC-conditioned medium (ADSC-CM) on the levels of the Th1 and Th2 cytokines. The levels of INF- γ (**a**) in skin tissue and IL-33 (**b**), IL-4 (**c**), and IL-13 (**d**) in the serum. The results are presented as the mean $\pm$ standard error of the mean ($n = 6$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.



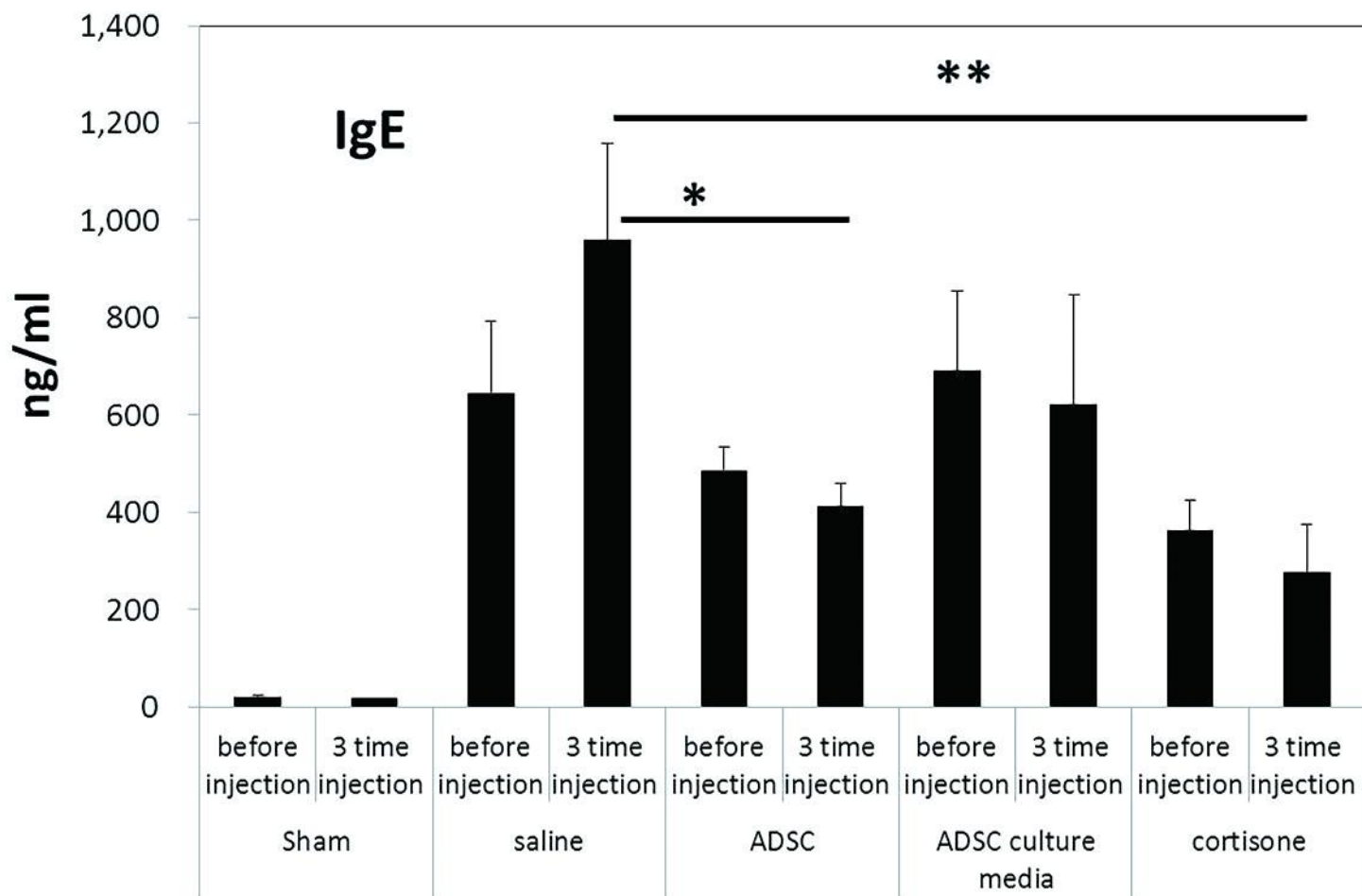






a**b****c**

Marker	Percentage of expression (%)
CD45	1.1 ± 0.07
CD29	94.5 ± 0.06
CD105	88.4 ± 0.1
CD73	78.4 ± 0.2
CD34	1.5 ± 0.04
CD44	98.4 ± 0.5
CD90	95.2 ± 0.08



Supplementary information

Measurement of cytokines and IgE using Luminex and ELISA

In the Luminex assay, the diluted microparticle cocktail was resuspended by inversion or vortexing. Fifty microliters of the microparticle cocktail was added to each well of the microplate followed by the addition of 50 μ L of the standard or sample. The plate was covered with a foil plate sealer and incubated for 2 h at room temperature on a horizontal orbital microplate shaker set at 800 ± 50 rpm. A magnetic device designed to accommodate a microplate was washed by applying the magnet to the bottom of the microplate. After that, the liquid was removed, each well was filled with wash buffer (100 μ L), and the liquid was removed again. The wash procedure was done three times. Fifty microliters of 1:11 diluted biotin antibody cocktail was added to each well. The plate was covered with a foil plate sealer and incubated for 1 h at room temperature on the shaker set at 800 ± 50 rpm. The wash step was then repeated. Thereafter, 50 μ L of diluted streptavidin-phycoerythrin was added to each well. The plate was covered with a foil plate sealer and incubated for 30 min at room temperature on the shaker set at 800 ± 50 rpm. The wash step was repeated. The plate was incubated for 2 min on the shaker set at 800 ± 50 rpm. Within 90 min, the plate was read using a Luminex instrument (Luminex, Austin, TX, USA).

For IgE measurements, 100 μ L of the sample or standard (in duplicate) was added into designated wells. The plate was incubated at room temperature for 30 min, and the samples were aspirated. Then, the plate was washed and aspirated three times. One hundred microliters of streptavidin-horseradish peroxidase was added to each well and incubated at room temperature for 30 min. The wash step was repeated and 100 μ L of 3,3',5,5'-tetramethylbenzidine substrate solution was added into each well. It was incubated in the dark at room temperature for precisely 10 min. Then, 100 μ L of Stop Solution was added to each

well. The absorbance at 450 nm was measured using a plate reader (SpectraMax Plus 384, Molecular Devices, Seoul, Republic of Korea) and calibrated data by standard curve.

Fig. S1. **(a)** Harvesting of the left inguinal fat pad. **(b)** ADSCs (P1) cultured for 3 days before injection. Original magnification: 100 $\times$. Bar: 250 μ m. **(c)** FACS analysis of autologous ADSCs before injection. Cells were positive for the common stem cell markers CD29, CD105, CD73, CD44, and CD90 but negative for the hematopoietic markers CD45 and CD34.

Fig. S2. Effect of adipose-derived stem cells (ADSCs) or ADSC-conditioned medium (ADSC-CM) on total IgE levels in the serum of NC/Nga mice. IgE level was significantly elevated in saline group after DNCB treatment compared with that in sham group without DNCB treatment (on day 43, 644.77 ± 148.85 ng/mL vs. 21.17 ± 4.53 ng/mL, $P < 0.001$). Furthermore, the level of IgE was increased even more by a continuous DNCB treatment in saline injection group (on day 53, 959.19 ± 198.40 ng/mL). On the contrary, IgE levels were slightly reduced in mice that were treated with ADSCs (from 486.06 ± 47.77 ng/mL to 414.12 ± 45.63 ng/mL), ADSC-CM (from 691.62 ± 162.15 ng/mL to 621.34 ± 224.58 ng/mL), or cortisone (from 363.47 ± 162.16 ng/mL to 277.22 ± 97.88 ng/mL). IgE levels in ADSC-treated ($P = 0.010$) and cortisone applied ($P = 0.008$) groups demonstrated were significantly lower than in saline-injected animals, whereas treatment with ADSC-CM did not alter IgE levels significantly ($P = 0.101$). Data are presented as the mean $\pm$ standard error of the mean ($n = 6$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ for comparisons of the values before and after triple treatments.