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Key Words. Human gingival • Stromal cells • Immunomodulation • Contact hypersensitivity • Mast cells

ABSTRACT

The immunomodulatory and anti-inflammatory functions of mesenchymal stromal cells (MSCs) have been demonstrated in several autoimmune/inflammatory disease models, but their contribution to the mitigation of contact hypersensitivity (CHS) remains unclear. Here, we report a new immunological approach using human gingiva-derived MSCs (GMSCs) to desensitize and suppress CHS and the underlying mechanisms. Our results showed that systemic infusion of GMSCs before the sensitization and challenge phase dramatically suppress CHS, manifested as a decreased infiltration of dendritic cells (DCs), $CD8^+$ T cells, T_H-17 and mast cells (MCs), a suppression of a variety of inflammatory cytokines, and a reciprocal increased infiltration of regulatory T cells and expression of IL-10 at the regional lymph nodes and the allergic contact areas. The GMSC-mediated immunosuppressive effects and mitigation of CHS were significantly abrogated on pretreatment with indomethacin, an inhibitor of cyclooxygenases. Under coculture condition of direct cell-cell contact or via transwell system, GMSCs were capable of direct suppression of differentiation of DCs and phorbol 12-myristate 13-acetate-stimulated activation of MCs, whereas the inhibitory effects were attenuated by indomethacin. Mechanistically, GMSC-induced blockage of de novo synthesis of proinflammatory cytokines by MCs is mediated partly by the tumor necrosis factor-alpha/prostaglandin E_2 (PGE_2) feedback axis. These results demonstrate that GMSCs are capable of desensitizing allergic contact dermatitis via PGE_2-dependent mechanisms. STEM CELLS *2011;29:1849–1860*

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

INTRODUCTION

Mesenchymal stromal cells (MSCs), previously referred to as mesenchymal stem cells, possess multifunctional properties from tissue repair/regeneration to immunomodulatory functions [1–3]. An increasing body of evidence indicates that MSCs can home and engraft at the injured site and promote tissue repair through a combined downregulation of proinflammatory cytokines and increased production of growth factors, antioxidants, and soluble factors with anti-inflammatory functions [4, 5]. Studies have shown that MSCs exert their immunosuppressive functions via inhibiting the proliferation and activation of different subtypes of effector T cells, natural killer (NK) cells, dendritic cells (DCs), and macrophages or via promoting the differentiation of regulatory T cells (Tregs) [6–10]. These unique properties render MSC a potential novel immunotherapeutic tool for a variety of autoimmune and inflammation-related diseases [3, 4]. Recent studies have reported that MSCs can also suppress allergic responses and chronic inflammation in experimental mouse models of ragweed- and ovalbumin-induced asthma [11, 12] and allergic rhinitis [13]. However, the underlying mechanism of MSC-mediated attenuation of allergic response still remains elusive.

The hapten-induced murine contact hypersensitivity (CHS) is an experimental model for human allergic contact dermatitis (ACD), one of the prevalent skin diseases worldwide with significant economic burden [14, 15]. Comparable to the pathophysiology of human ACD, the murine CHS model comprises three phases: the sensitization phase (also termed as the afferent or induction phase), the elicitation or challenge phase, and the resolution/regulation phase [14]. The sensitization phase is initiated immediately after the first exposure of skin to haptens, during which dermal DCs uptake and process antigens and then migrate to regional draining lymph nodes (dLNs), where they stimulate the differentiation and activation of allergen-specific T lymphocytes, including $CD8^+$ cytotoxic and $CD4^+$ T helper cells. In the elicitation phase, allergen-specific effector $CD8^+$ and $CD4^+$ T cells and various types of innate immune cells, particularly mast cells

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(MCs), are activated and produce a plethora of inflammatory cytokines and mediators that contribute to the appearance of eczematous lesions. The resolution/regulation is characterized by the recruitment and activation of $CD4^+$ Tregs and other potential regulatory immune cells, which contribute to the resolution of inflammatory processes [14]. Currently, topical application of corticosteroid is the first-line palliative measure for ACD with short-term outcome, while allergen identification to improve contact avoidance is still challenging. Therefore, it would be promising to develop a more effective desensitizing tool based on specific cellular targets engaged by multiple types of innate and adaptive immune cells in the complex but distinctively phased pathophysiological processes of ACD. The unique immunomodulatory functions of MSCs on various types of immune cells may render them as a novel approach to desensitize allergic diseases.

We have recently isolated a unique subpopulation of MSCs from human gingival tissues, designated as GMSCs [16], with similar immunomodulatory properties to human bone marrow-derived MSCs (BMSCs), including the inhibition of T-cell proliferation and activation, enhancement of Treg generation and polarization of M2 macrophages [10, 16]. In the present study, we further explored the immunomodulatory functions of GMSCs in an established mouse model of delayed-type allergic responses or CHS and the potential cellular and molecular mechanisms.

Materials and Methods

Animals

BALB/c mice (male, 8-10 weeks old) were obtained from Jackson Laboratories (Bar Harbor, ME, http://www.jax.org) and group-housed at the Animal Facility of University of Southern California (USC). All animal care and experiments were performed under institutional protocols approved by the Institutional Animal Care and Use Committee (IACUC) at USC.

Antibodies and Reagents

Lipopolysaccharides (LPS), phorbol 12-myristate 13-acetate (PMA), A23187, indomethacin, oxazolone, and brefeldin A were from Sigma (St. Louis, MO, http://www.sigmaaldrich.com). Antibodies include anti-CD117-APC, anti-FcεRIα-FITC, anti-interleukin (IL)-6-PE, anti-IL-4-PE, anti-tumor necrosis factor (TNF)-α-PE anti-CD4-PerCP-Cy5.5, anti-CD25-PE, and anti-FoxP3-FITC (eBioscience, San Diego, CA, http://www.ebioscience.com).

Cell Culture

Human gingival samples were collected from healthy subjects following routine dental procedures at USC School of Dentistry under the approved institutional review board. GMSCs were isolated and cultured as previously described [16].

$CD14^+$ cells were negatively selected from human peripheral blood mononuclear cells (PBMCs) (AllCells LLC, Emeryville, CA, http://www.allcells.com) using the MACS CD14 MicroBeads (Monocyte Isolation Kit, Miltenyi Biotec, Auburn, CA, http://www.miltenyibiotec.com) according to the manufacturer's instructions. Purity of isolated monocytes was assessed by flow cytometry. Cells were then cultured in six-well plates in RPMI 1,640 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% L-glutamine (Invitrogen, Carlsbad, CA, http://www.invitrogen.com), and recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF; 20 ng/ml) and IL-4 (20 ng/ml; PeproTech, Rocky Hill, NJ, http://www.peprotech.com).

The human MC line (HMC-1) was kindly provided by Dr. J.H. Butterfield (Mayo Clinic, Rochester, MN) [17]. Cells were maintained in Iscove's modified Dulbecco's medium supplemented with 10% FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin.

Based on previous studies, the inhibitory effects of MSCs on DCs were observed in the range of 1:10-1:1 MSC/monocyte ratios with the maximum inhibitory effect reported at 1:1 ratio [8, 18, 19]. We therefore selected the 1:1 cell ratio of monocyte/DCs or MCs in all the coculture experiments under either direct cell-cell contact or transwell (TW) conditions for the mechanistic studies. Neutralizing Abs specific for human IL-10, transforming growth factor (TGF)-β1, TNF-α, or an isotype-matched monoclonal antibody (10 μg/ml; R&D Systems, Minneapolis, MN, www.RnDSystems.com), or indoamine-2,3-dioxygenase (IDO) inhibitor (1-methyl-L-tryptophan [1-MT]; Sigma-Aldrich) were used for certain experimental purposes. Since endogenous PGE_2 produced by host cells may play an important role in CHS development [20, 21], then under certain conditions we chose to pretreat GMSCs with 5 μM indomethacin (Sigma-Aldrich) in vitro to completely block the exogenous source of PGE_2 release and then injected into mice, wherein the inhibitory effects can be maintained for at least 1 week [22].

Flow Cytometry

Cell surface markers and intracellular cytokine were analyzed using a FACS Calibur (BD Biosciences, San Jose, CA, http://www.bdbiosciences.com) following standard protocols.

Treatment of CHS by GMSCs

The murine CHS model was induced as described previously [23]. Briefly, 20 μl of a 1% oxazolone solution in acetone/sesame seed oil (4:1) was applied to the right ear. Seven days later, the sensitized right ears were challenged with 20 μl of 1% oxazolone. An identical amount of acetone/sesame seed oil (4:1) was administered to the left ear as control. Based on the disease course of CHS, different treatment regimens were performed, whereby GMSCs prelabeled with CM-DiI (2.0×10^6 per mice) were intravenously injected into mice either 1 day before sensitization (treatment regimen I), or 1 day before initiation or challenge (treatment regimen II), or 1 hour after challenge (treatment regimen III) (Fig. 1A). Control group received phosphate-buffered saline only or normal skin-derived fibroblasts ($n = 4$). Ear thickness was measured in a blind way at indicated time points. Mice were sacrificed on day 2 after challenge and ear samples were harvested for further analysis.

Immunohistochemical and Western Blot Analysis

H&E and toluidine blue staining was performed on paraffin-embedded sections for histological and MC examination. Immunofluorescence studies were performed using specific antibody for mice FcεRIα. For semiquantification, positive signals in at least five random high-power fields were visualized and counted. Western blot analysis was performed as previously described [16], with antibodies specific for mice TNF-α, IL-6, IL-4, interferon-gamma (IFN-γ), IL-10 (Biolegend, San Diego, CA, http://www.biolegend.com), or human cyclooxygenase (COX)-2, nuclear factor-kappaB (NF-κB) p65 (Millipore, Billerica, MA) or β-actin (Sigma).

Enzyme-Linked Immunosorbent Assay

The concentration of cytokines in ear lysates and the supernatants of cultured cells were detected using enzyme-linked immunosorbent assay (ELISA) kits (eBioscience), which was normalized according to the protein concentration and cell number, respectively. The production of PGE_2 in supernatants was determined using an EIA kit (Cayman Chemical).

Statistical Analysis

All data are expressed as mean ± SEM from at least three independent experiments, and statistical analyses were performed by means of appropriate one-way analysis of variance or the Student's t test using SPSS software (SPSS 16.0, Inc, Chicago, IL). Differences were considered statistically significant when a p value is less than .05.

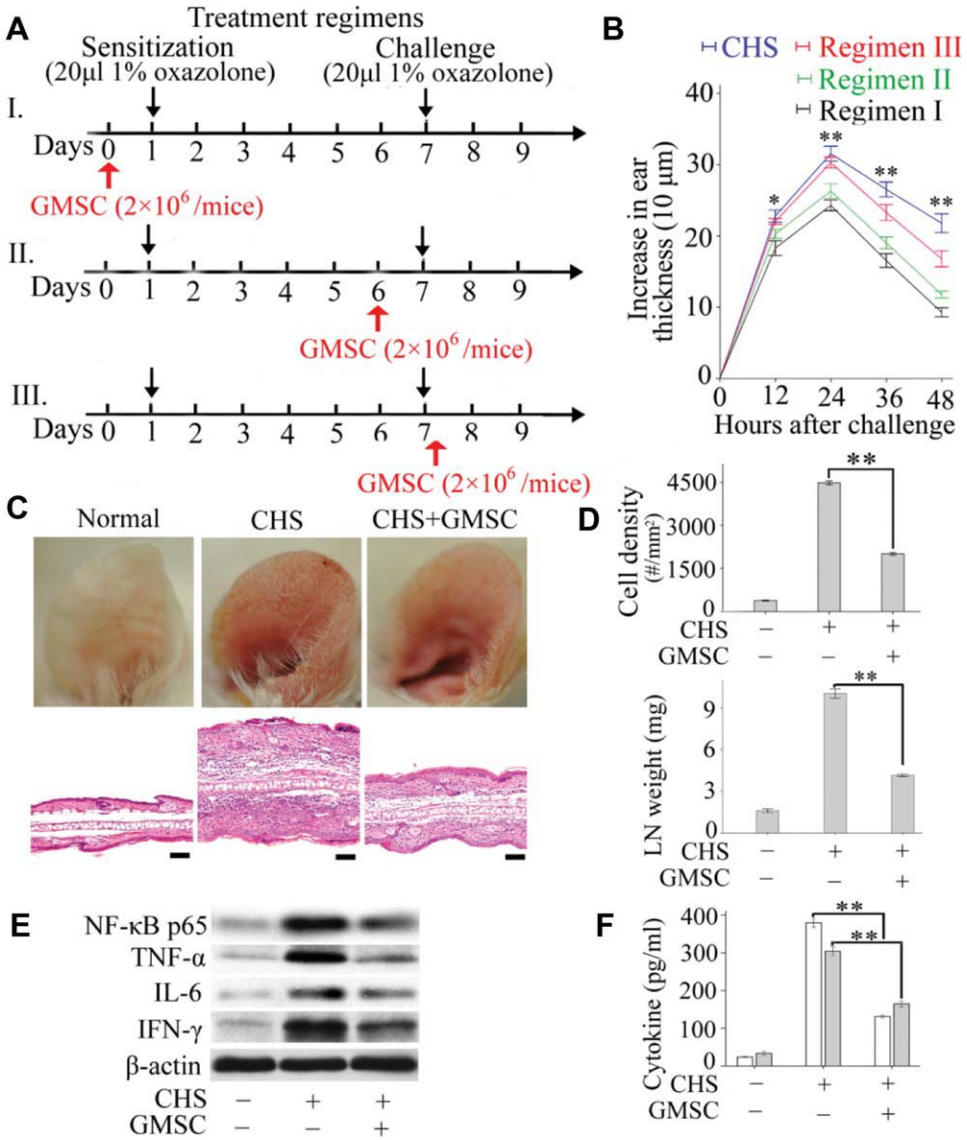

Figure 1. GMSC-based therapy attenuates CHS. **(A):** Experimental protocols showing different treatment regimens using GMSCs. GMSCs (2×10^6 per mice) were systemically injected into mice via tail vein either 1 day before sensitization with 1% oxazolone (regimen I), 1 day before challenge (regimen II), or 1 hour after challenge with 1% oxazolone (regimen III). Forty-eight hours after challenge, ear and local dLN samples were collected for further analysis. **(B):** Ear thickness ($n = 4$) was measured at the indicated times after challenge in different experimental groups. **(C):** Representative photos of the ear and images of H&E staining of ear samples from mice following treatment regiment II with GMSCs. Scale bars, 200 μm. **(D):** Quantification of cellular components in CHS ears and dLNs. **(E, F):** GMSCs inhibited the production of inflammatory cytokines in local ears as determined by Western blot and enzyme-linked immunosorbent assay. The results represent three independent experiments (mean ± SEM). *$p < .05$; **$p < .01$ by one-way analysis of variance (B) and Student's t test (D, F). Abbreviations: CHS, contact hypersensitivity; GMSC, gingiva-derived mesenchymal stromal cell; IFN-γ, interferon-gamma; IL-6, interleukin-6; LN, lymph node; NF-κB, nuclear factor-kappaB; and TNF-α, tumor necrosis factor-alpha.

Results

Treatment with GMSCs Can Suppress CHS

According to the specific disease course of CHS, we first investigated the efficacy of GMSC treatment intervention at different stages of CHS (Fig. 1A). To this purpose, GMSCs (2×10^6 per mice) were systemically injected into mice either at 1 day before sensitization with 1% oxazolone (treatment regimen I), 1 day before challenge (treatment regimen II), or 1 hour after challenge (treatment regimen III) with 1% oxazolone, and the extent of swelling/inflammation in terms of external ear thickness was carefully measured at the intervals of 12 hours. As shown in Figure 1B, even though GMSC treatment at different phases of CHS yielded variable therapeutic effects, systemic infusion of GMSCs following each of the three treatment regimens consistently led to statistically significant attenuation of CHS appearance when compared with untreated CHS mice from 24 to 48 hours after the first challenge (I or II vs. CHS control, $p < .01$; III vs. CHS control, $p < .05$). Of note, early intervention before antigen sensitization (treatment regimen I) exhibited the best efficacy, followed by treatment regimens II and III, respectively, characterized by a decrease in ear thickness and inflammation that appeared as early as 12 hours (I or II vs. III, $p < .05$), and more apparently in the next 24-48 hours after the first challenge (I or II vs. III, $p < .01$) (Fig. 1B, 1C). Even though the

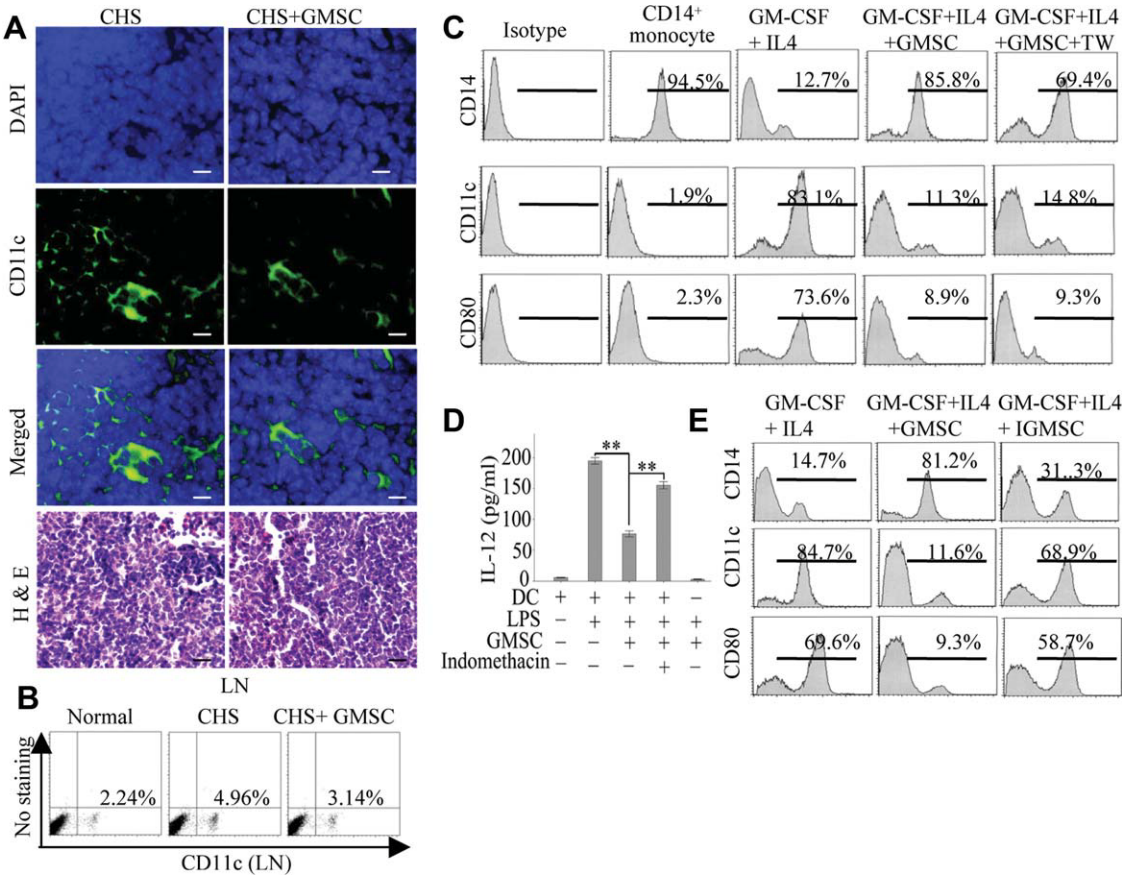

Figure 2. Attenuation of CHS by GMSCs involves PGE_2-dependent inhibition of DCs. **(A):** The infiltration of $CD11c^+$ DCs in the regional draining lymph nodes (dLNs) was determined by immunofluorescence staining. **(B):** The percentage of $CD11c^+$ DCs in dLNs was determined by flow cytometry. **(C):** GMSCs inhibited the differentiation and maturation of DCs. $CD14^+$ monocytes were cultured alone or cocultured with GMSCs (1:1) in direct cell-cell contact or in TWs in the DC-induction medium containing GM-CSF (20 ng/ml) and IL-4 (20 ng/ml). Seven days later, the number of $CD11c^+$ and $CD80^+$ DCs was determined by flow cytometry. **(D):** GMSCs dramatically reduced IL-12 secretion by DCs in response to LPS stimulation. **(E):** GMSCs pretreated with 5 μM indomethacin (IGMSCs) were cocultured with $CD14^+$ monocytes in TWs under the same condition as described in C, and then the number of $CD11c^+$ and $CD80^+$ DCs was determined by flow cytometry. The results represent three independent experiments (mean ± SEM). $^*p < .05$; $^{**}p < .01$ by Student's t test. Abbreviations: CHS, contact hypersensitivity; DAPI; DC, dendritic cell; GM-CSF, granulocyte-macrophage colony-stimulating factor; GMSC, gingiva-derived mesenchymal stromal cell; IGMSC, indomethacin-pretreated mesenchymal stromal cell; IL-4 and -12, interleukin-4 and -12; LN, lymph node; LPS, lipopolysaccharides; and TW, transwell.

therapeutic effect of treatment regimen I appeared more pronounced than that of treatment regimen II, the difference was not statistically significant (I vs. II, $p > .05$). In addition, treatment regimen II in combination with III appeared more effective in suppressing CHS than treatment regimen II alone; however, the difference is not statistically significant ($p > .05$) (Supporting Information Fig. S1A). Similar therapeutic effects were observed after treatment with bone marrow-derived MSCs 1 day before challenge (treatment regimen II), but treatment with skin fibroblasts showed no obvious therapeutic effects on CHS (Supporting Information Fig. S1B). Taken together, these findings suggest that systemic infusion of GMSCs before sensitization and challenge yielded optimal treatment effect on CHS. Therefore, we chose treatment regimen II in most of the following studies unless specifically indicated.

Further analysis of ear specimens and regional dLNs harvested 48 hours after challenge showed a markedly reduction in infiltration of inflammatory cells and gross LN weight in GMSC-treated mice as compared to untreated CHS controls (Fig. 1D). We next investigated the in vivo effects of GMSCs on the production of local inflammatory cytokines at the CHS sites. Results from both Western blot and ELISA showed a significant increase in the expression of NF-κB p65 and proinflammatory cytokines including TNF-α, IL-6, and IFN-γ in tissue lysates of CHS ears when compared with those of normal controls, whereas treatment with GMSCs significantly decreased the local expression of these inflammation-related genes (Fig. 1E, 1F). These findings suggest that GMSCs are capable to harness delayed-type hypersensitivity reactions by suppressing a variety of inflammatory cytokines and gene products.

GMSCs Attenuate CHS via PGE_2-Dependent Inhibition of DCs

Previous studies have demonstrated that DCs play a critical role in the initiation of CHS [24–27], while bone marrow- and adipose-derived MSCs are capable to inhibit their immune functions [8, 18, 19]. Here, we postulated that attenuation of CHS by GMSCs might involve the inhibition of DC functions. To test our hypothesis, GMSCs (2×10^6 per mice) were systemically injected into mice 1 day before sensitization and sacrificed 2 days after challenge. Immunostaining showed that GMSC treatment robustly reduced the number of $CD11c^+$ DCs at both the regional dLNs (Fig. 2A) and the antigen challenged ears (Supporting Information Fig. S1C). The reduction in the number of $CD11c^+$ DCs in LNs caused by GMSC treatment was further confirmed by flow cytometric analysis (Fig. 2B).

We next performed a series of in vitro studies to confirm the inhibitory effect of GMSCs on DCs. To this end, PBMC-derived $CD14^+$ monocytes were cocultured with GMSCs (1:1) under the condition of direct cell-cell contact or in a TW system in the DC-induction medium, whereas $CD14^+$ monocytes cultured alone under the same condition were served as controls. After 7 days, DC differentiation was assessed by flow cytometric analysis for expression of CD11c and the costimulatory molecule, CD80, both are cell surface molecules expressed by matured DCs. As shown in Figure 2C, GMSCs cocultured with $CD14^+$ monocytes under both cell-cell contact and TW conditions significantly inhibited the differentiation and maturation of DCs, characterized by a reciprocal increase in the expression of CD14 and a decrease in the expression of CD11c and CD80, when compared with $CD14^+$ monocytes-derived DCs cultured alone (Fig. 2C). Functionally, we found coculture with GMSCs dramatically reduced IL-12 secretion by DCs in response to LPS stimulation (Fig. 2D), which was further confirmed by flow cytometry after intracellular staining (Supporting Information Fig. S1D). These results suggest that soluble factors contribute to GMSC-mediated inhibitory effects on the differentiation and activation of DCs.

To further define specific secretory factors involved in GMSC-mediated inhibition of DCs, monoclonal neutralizing antibodies specific for TGF-β1 and IL-10 (10 μg/ml), or specific inhibitor of IDO (1-MT, 500 μM), or specific inhibitor of COX-1/2 (indomethacin, 5 μM), were applied to the coculture system. We found that neutralizing TGF-β1 or IL-10 and pretreatment with 1-MT showed no obvious effects on GMSC-mediated inhibitory effects on DC differentiation (Supporting Information Fig. S1E, S1F) and LPS-stimulated IL-12 secretion by DCs (Supporting Information Fig. S1G). On the contrary, high levels of COX-2 expression and PGE_2 production were detected in GMSCs cocultured with $CD14^+$ monocytes under DC differentiation conditions (Supporting Information Fig. S1H, S1I), whereas pretreatment of GMSCs with 5 μM indomethacin significantly but not completely reversed their inhibitory effect on DC differentiation (Fig. 2E) and LPS-stimulated IL-12 secretion by DCs (Fig. 2D; Supporting Information Fig. S1D, S1G); while this concentration of indomethacin was sufficient to completely abolish the increased PGE_2 production in GMSCs stimulated by cocultured $CD14^+$ monocytes without obvious cytotoxicity (Supporting Information Fig. S1H-S1J). These results suggest that GMSC-derived PGE_2 plays a crucial role in GMSC-mediated inhibitory effect on DCs.

Next, we asked whether PGE_2 is essential for GMSCs-mediated desensitizing of CHS and the associated decrease in the number of DCs at the regional LNs. We first demonstrated that most GMSCs homed to the inflammatory sites still constitutively expressed COX-2 (Supporting Information Fig. S2A). Next, we pretreated GMSCs (2×10^6 per mice) with indomethacin and systemically injected them into mice 1 day before sensitization (treatment regimen I) or 1 day before challenge (treatment regimen II). Our results indicated that GMSCs pretreated with indomethacin lost their inhibitory capacity on DC infiltration in LNs collected following treatment regimen I (Supporting Information Fig. S2B); and more importantly, indomethacin treated GMSCs lost their capacity to attenuate CHS under both treatment regimens when compared with untreated GMSC (Supporting Information Fig. S2C, S2D). In addition, subcutaneous administration of 16,16-dimethyl PGE_2 (dmPGE_2; 5-20 μg/kg) led to a dose-dependent suppression of CHS appearance, but the treatment response was not as significant as that mediated by GMSC infusion (Supporting Information Fig. S2E). These compelling findings support the notion that PGE_2 is indispensable to GMSC-mediated inhibition of DC functions both in vitro and in vivo, which might contribute, at least in part, to the underlying mechanism of GMSC-induced immunosuppression in CHS.

Attenuation of CHS by GMSCs is Associated with Their Modulatory Effects on Multiple Effector T Cells

Besides DCs, multiple subtypes of T lymphocytes, including $CD8^+$ cytotoxic T cells [28, 29] and $CD4^+$ T helper cells, especially T_H-17 [30–32] and Tregs [33, 34], partake different roles in the elicitation and resolution of CHS [14]. Here, we postulate that GMSC-mediated immunomodulatory effects on multiple types of T cells also contribute to their desensitizing therapeutic effects on CHS. As expected, systemic injection of GMSCs into mice 1 day before challenge (treatment regimen II) significantly reduced the infiltration of $CD8^+$ T cells at the regional dLNs and challenged CHS ears as demonstrated by immunostaining (Supporting Information Fig. S3A-S3C) and flow cytometric analysis (Fig. 3A). Specifically, we observed a reduction in the number of infiltrated T_H-17 cells in the LNs and a parallel decreased expression of IL-17 in CHS ears after treatment with GMSCs, when compared with CHS controls (Fig. 3B, 3C). Conversely, when compared with CHS controls, GMSC treatment significantly increased not only the expression of FoxP3 (the specific transcriptional factor expressed by Tregs) and the anti-inflammatory cytokine IL-10 (a signature cytokine of Tregs) at the challenged ears but also the number of infiltrated Tregs at regional LNs as determined by Western blot, ELISA, and flow cytometry, respectively (Fig. 3C, 3D; Supporting Information Fig. S3D). Since our results have shown that PGE_2 is essential for GMSC-mediated attenuation of CHS and the associated inhibition of DC functions (Supporting Information Fig. S2), we ask whether PGE_2 plays a similar role in GMSC-mediated inhibition of effector T cells. As expected, GMSC-mediated inhibition of $CD8^+$ and T_H-17 cell infiltration at the LNs was partially reversed when GMSCs were pretreated with indomethacin before systemic injection into mice (Supporting Information Fig. S3C, S3E).

Attenuation of CHS by GMSCs is Associated with Their Inhibitory Effects on MCs

In addition to DCs and multiple subtypes of effector T cells, recent studies have implicated the critical role of MCs in delayed-type allergic reactions [35–43]. Thus, we further investigated whether GMSCs had any effects on MC functions and their potential role in GMSC-mediated attenuation of CHS. We first explored the interactions of homed GMSCs with host MCs at the inflammatory/challenged ears following systemic infusion of GMSCs prelabeled with CM-DiI 1 day before challenge. Our results showed a more robust homing of labeled GMSCs to the challenged ear when compared with the opposite normal ear (Fig. 4A); the homed GMSCs were in close proximity with FcεRIα positive MCs as shown by laser confocal microscopy (Fig. 4B). We next investigated the in vivo effects of GMSCs on the degranulation functions of MCs at the challenged ears. Toluidine blue staining showed that the number of MCs and the percentage of degranulated MCs dramatically decreased in GMSC-treated mice ears when compared with the untreated group (Fig. 4C, 4D), whereas such inhibitory effects on MCs were significantly attenuated when GMSCs were pretreated with indomethacin before systemic injection into mice (Fig. 4D). Altogether, these findings suggest that PGE_2 provides a functional link between GMSCs and MCs and potentially contributes to GMSC-mediated reversal of delayed-type hypersensitivity (DTH) in CHS model.

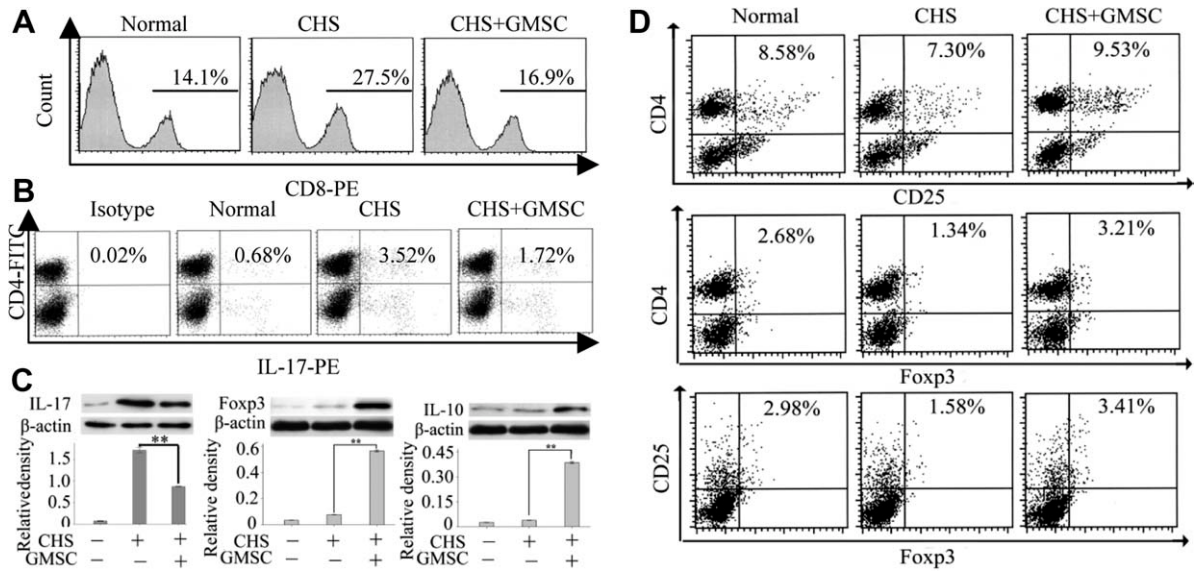

Figure 3. Attenuation of CHS by GMSCs is associated with their modulatory effects on multiple effector T cells. Following treatment regimen II with GMSCs, ear and local draining lymph node (dLN) samples were collected at 48 hours after challenge for further analysis. **(A, B):** The infiltration of $CD8^+$ T cells and T_H-17 in the regional dLNs was determined by flow cytometry. **(C):** The expression of IL-17, Foxp3, and IL-10 in CHS ears was determined by Western blot, wherein the graphs showed their relative density after normalization to the intensity of β-actin bands. **(D):** The infiltration of regulatory T cell in the regional dLNs was determined by flow cytometry. The results represent three independent experiments (mean ± SEM). $^*p < .05$; $^{**}p < .01$ by Student's t test. Abbreviations: CHS, contact hypersensitivity; FITC; Foxp3; GMSC, gingiva-derived mesenchymal stromal cell; IC-17; IL-10, interleukin-10; and PE.

Mechanisms Underlying GMSCs-Mediated Inhibition of MC Functions In Vitro

Next, we performed a series of in vitro studies to explore the interplay between GMSCs and MCs and their potential mechanisms. To this purpose, HMC-1 cells, an established human MC line, were cocultured with GMSCs at different ratios of cell density under direct cell-cell contact for 72 hours, followed by stimulation with PMA and calcium ionophore (PMACI) for another 18 hours. The production of TNF-α in

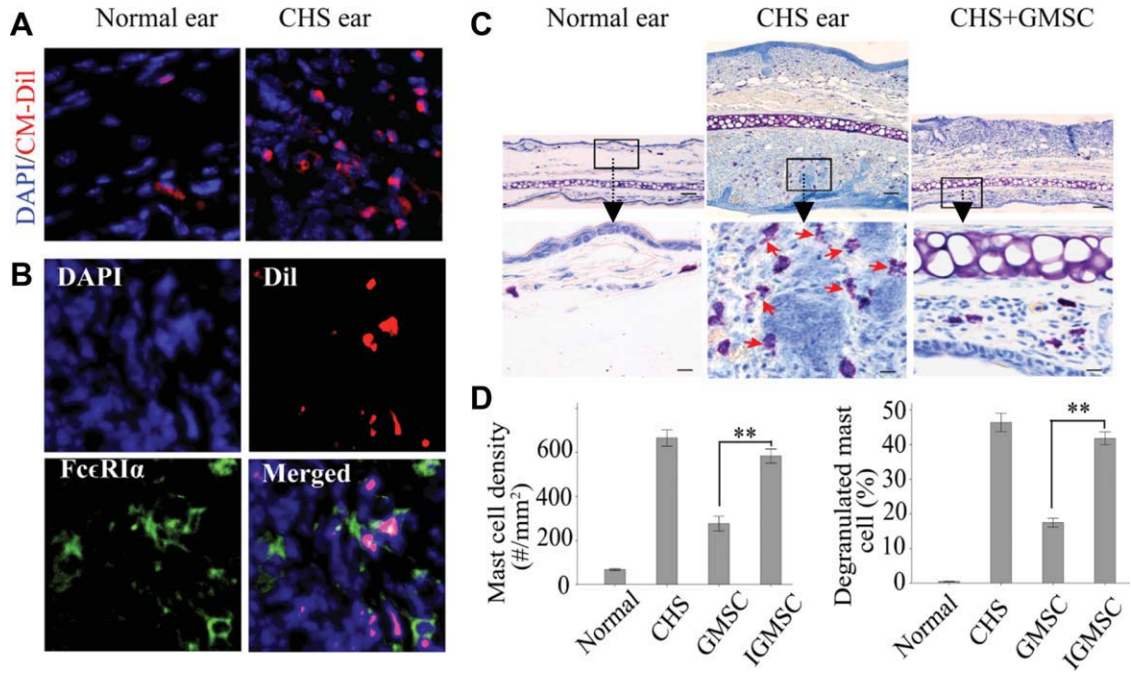

Figure 4. Interplay of homed GMSCs and mast cells (MCs) in CHS model. GMSCs prelabeled with CM-DiI were systemically injected into mice 1 day before challenge (regimen II). Forty-eight hours after challenge, ear samples were collected for further analysis. **(A, B):** GMSCs (red) homed to the inflammatory sites were in proximity with MCs (green) as determined by immunofluorescence staining with a specific antibody for FcεRIα. **(C, D):** GMSCs treatment decreased the number and degranulation of MCs as determined by toluidine blue staining. (red arrow: degranulated MCs). The results represent three independent experiments (mean ± SEM). $^*p < .05$; $^{**}p < .01$ by Student's t test. Abbreviations: CHS, contact hypersensitivity; CM; DAPI; DiI; FcεRIα; GMSC, gingiva-derived mesenchymal stromal cell; and IGMSC, indomethacin-pretreated mesenchymal stromal cell.

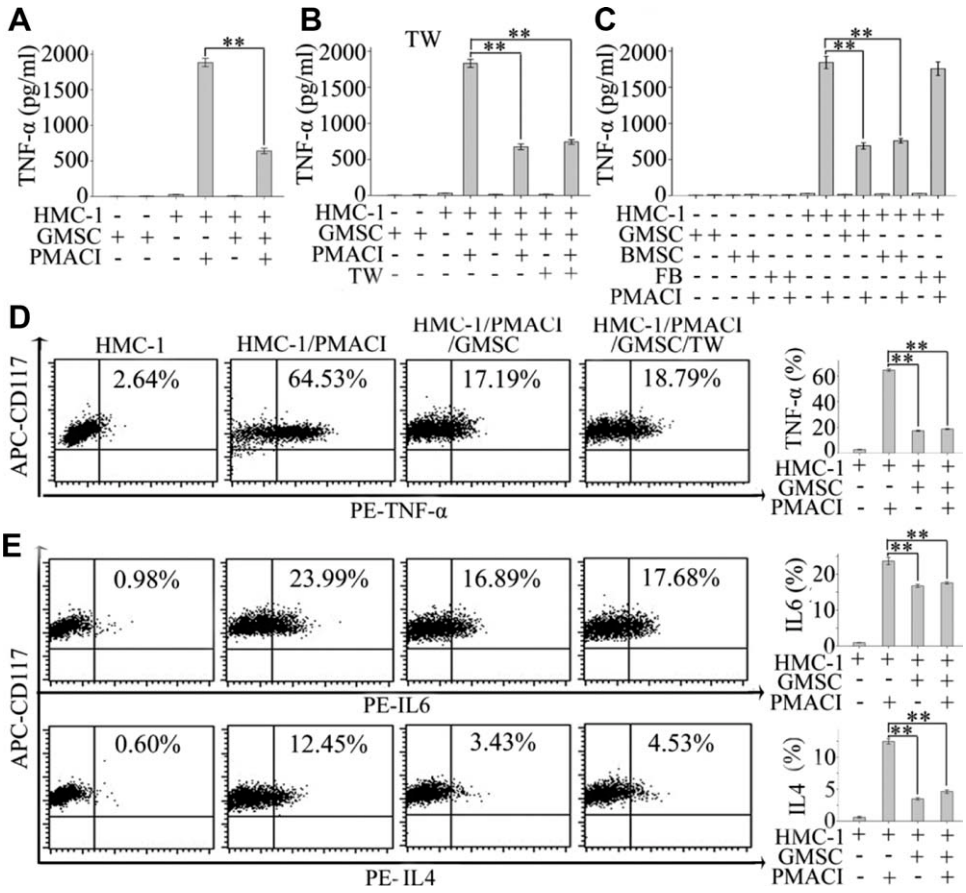

Figure 5. GMSCs suppress inflammatory cytokines release by HMC-1 cells. HMC-1 cells were cocultured with GMSCs (1:1) for 72 hours under direct cell-cell contact or in a TW system. **(A, B):** Following stimulation with PMACI, the secretory TNF-α in the supernatants were determined using enzyme-linked immunosorbent assay (ELISA). **(C):** HMC-1 cells were cocultured with BMSC or skin FBs (1:1) for 72 hours under in TWs. Following stimulation with PMACI, the secretory TNF-α in the supernatants were determined using ELISA. **(D, E):** The PMACI-stimulated expression of intracellular cytokines (TNF-α, IL-4, and IL-6) in HMC-1 after coculture with GMSCs in TWs were determined by flow cytometry. The results represent three independent experiments (mean ± SEM). $*p < .05$; $**p < .01$ by Student's t test. Abbreviations: APC; BMSC, bone marrow-derived mesenchymal stromal cell; FB, fibroblast; GMSC, gingiva-derived mesenchymal stromal cell; HMC-1, human mast cell line; IL-4 and -6, interleukin-4 and -6; PMACI, phorbol 12-myristate 13-acetate and calcium ionophore; TNF-α, tumor necrosis factor-alpha; and TW, transwell.

the supernatants of MCs or GMSCs cultured alone and their cocultures was measured by ELISA. Our results showed that coculture with GMSCs under direct cell-cell contact led to a cell-dose-dependent inhibition of PMACI-stimulated TNF-α release by HMC-1 cells (Supporting Information Fig. S4A), with a maximum inhibition at equal cell density ratio (Supporting Information Fig. S4A; Fig. 5A; $p < .01$). To determine whether inhibition of TNF-α release by GMSCs is dependent on direct cell-cell contact and/or soluble factors, HMC-1 cells and GMSCs were cocultured in the TW system. As shown in Figure 5B, coculture with GMSCs in TWs decreased PMACI-stimulated TNF-α release to a similar extent when compared with direct coculture, implying that soluble factors may play an essential role. However, coculture with an increasing number of GMSCs in TWs had no obvious effect on the proliferation of HMC-1 cells, therefore, ruled out the possibility that GMSC-mediated inhibition of MC activation was due to the inhibition of cell proliferation (Supporting Information Fig. S4B). Similar to above findings, a reproducible reduction of PMACI-stimulated TNF-α production in coculture with bone marrow-derived MSCs was observed ($p < .01$), whereas no obvious changes were detected in cocultures with normal skin fibroblasts ($p > .05$) (Fig. 5C). Substantially, the inhibitory effects of GMSCs on MC activation were further confirmed by flow cytometric analysis, which showed a significant decrease in the percentage of HMC-1 cells expressing TNF-α, IL-6, and IL-4 among total $CD117^+$ (c-kit) HMC-1 cells cocultured with GMSCs under both direct cell-cell contact and TW system following stimulation with PMACI ($p < .01$) (Fig. 5D, 5E). Of note, PMACI-stimulated upregulation of NF-κB p65 was also abolished in HMC-1 cells when cocultured with GMSCs in TW when compared with HMC-1 cells cultured alone (Supporting Information Fig. S4C), which was apparently correlated with GMSC-mediated inhibition of TNF-α secretion by PMACI-activated HMC-1 cells. Taken together, these results suggest that GMSCs can potently inhibit MC activation through secretory soluble factors.

We then aimed to identify the specific secretory soluble factors that potentially contributed to GMSC-mediated inhibition of proinflammatory cytokine synthesis by MCs. To this end, monoclonal neutralizing antibodies specific for TGF-β1 and IL-10 (10 μg/ml), or a specific inhibitor for IDO (1-MT, 500 μM), were added into the coculture. Our results indicated that blocking these factors failed to restore GMSC-mediated inhibition of TNF-α release by HMC-1 cells in response to PMACI stimulation (Fig. 6A). Our results from in vivo

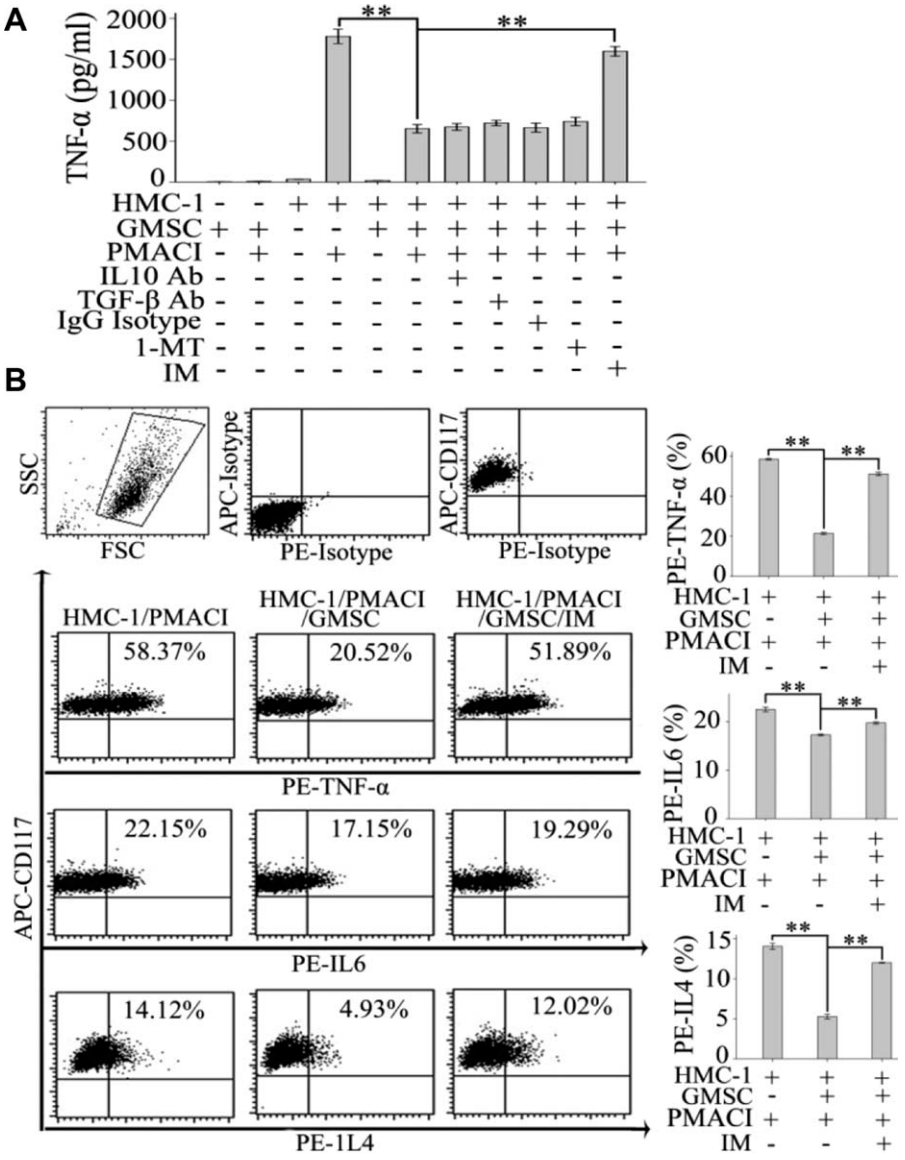

Figure 6. PGE_2 plays a key role in GMSC-mediated suppression of de novo synthesis of inflammatory cytokines in HMC-1 cells. HMC-1 cells were cocultured with GMSCs for 72 hours in transwells in the presence or absence of specific neutralizing antibodies (10 μg/ml) for either TGF-β1 or IL-10, or specific inhibitors for indoamine-2,3-dioxygenase (1-MT) or cyclooxygenase1/2 (IM). **(A):** Following stimulation with PMACI, the secretory TNF-α in the supernatants was determined using enzyme-linked immunosorbent assay. **(B):** PMACI-stimulated expression of intracellular cytokines was determined using flow cytometry. The results represent three independent experiments (mean ± SEM). *$p < .05$; **$p < .01$ by Student's t test. Abbreviations: Ab; APC; FSC; GMSC, gingiva-derived mesenchymal stromal cell; HMC-1, human mast cell line; IL-4, -6, and -10, interleukin-4, -6, and -10; IM, indomethacin; 1-MT, 1-methyl-L-tryptophan; PE; PMACI, phorbol 12-myristate 13-acetate and calcium ionophore; SSC; TGF-β, transforming growth factor-beta; and TNF-α, tumor necrosis factor-alpha.

studies have assumed the potential role of PGE_2 in GMSC-mediated inhibition of MC functions during CHS (Fig. 4). To confirm this, we then pretreated GMSCs with indomethacin for 24 hours and then cocultured with HMC-1 cells in TWs followed by PMACI stimulation. We found that GMSCs pretreated with indomethacin lost their ability to inhibit PMACI-stimulated TNF-α release by HMC-1 cells (Fig. 6A). Flow cytometric analysis further demonstrated that pretreatment of GMSCs with indomethacin reversed their inhibitory effects on PMACI-activated HMC-1 cells in terms of TNF-α, IL-6 or IL-4 expression (Fig. 6B). These findings suggest that PGE_2 plays a critical role in GMSC-mediated inhibition of de novo synthesis of proinflammatory cytokines by MCs.

To further dissect the mechanisms whereby PGE_2 contributes to GMSC-mediated inhibition of MC activation, we first exposed GMSCs to different concentrations of exogenous TNF-α for 24 hours, and the expression of COX-2 and production of PGE_2 were determined by Western blot and ELISA, respectively. Our results showed TNF-α treatment led to a dose-dependent increase in COX-2 expression and PGE_2 production by GMSCs (Fig. 7A, 7B). As expected, indomethacin-pretreated GMSCs failed to produce PGE_2 in response to stimulation with exogenous TNF-α (Supporting Information Fig. S4D). Conversely, treatment with exogenous PGE_2 inhibited PMACI-stimulated TNF-α secretion by HMC-1 cells in a dose-dependent manner (Fig. 7B). In addition, the expression of COX-2/PGE_2 was elevated in cocultured GMSCs and HMC-1 without PMACI stimulation, and such an increase was significantly augmented in the presence of PMACI stimulation (Fig. 7C). Likewise, the enhanced expression of

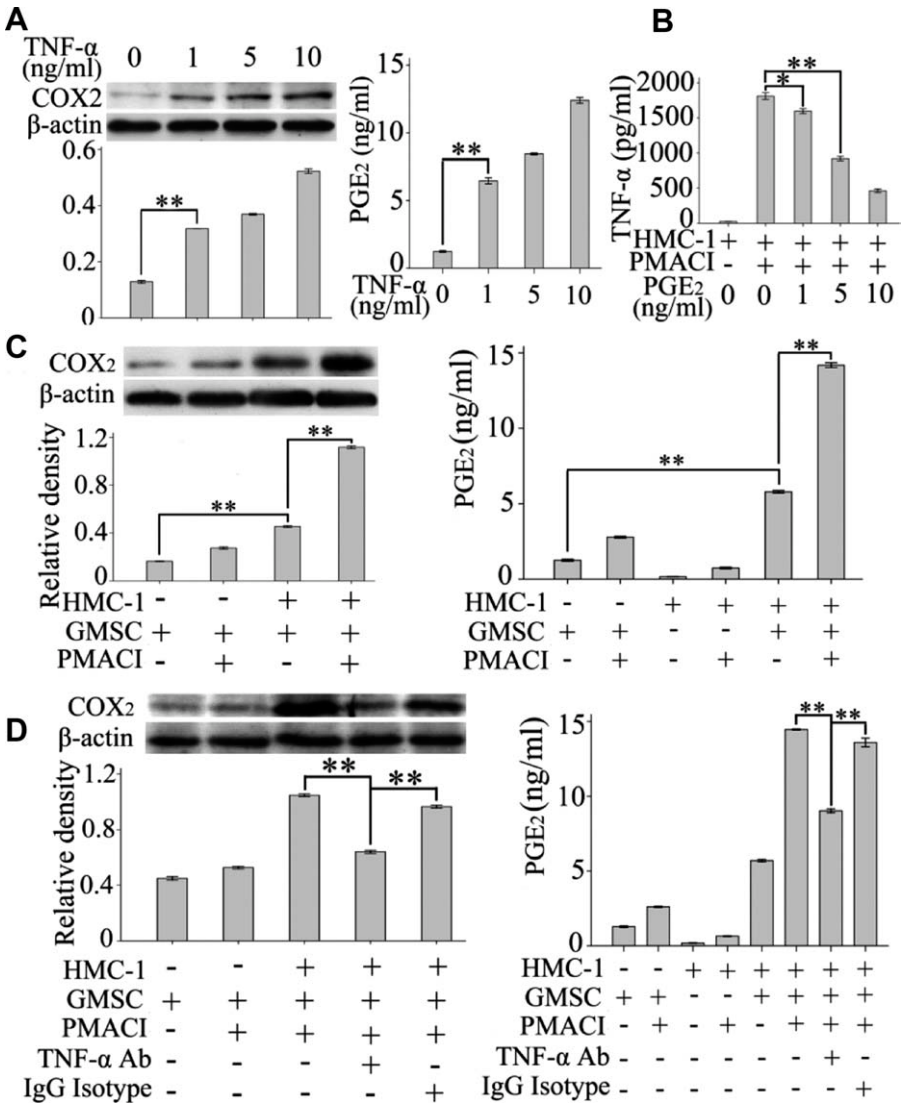

Figure 7. GMSCs interact with HMC-1 cells via TNF-α-PGE_2 feedback loop. **(A, B):** Exogenous TNF-α induces a dose-dependent increase in COX-2 expression and PGE_2 production in GMSCs. (B): Exogenous PGE_2 inhibits PMACI-induced TNF-α expression in a dose-dependent manner. **(C):** Cocultured HMC-1 cells in the presence of PMACI enhanced the COX-2 expression and the PGE_2 production by GMSCs. **(D):** Neutralizing TNF-α decreased COX-2 expression and PGE_2 production by GMSCs cocultured with HMC-1 cells stimulated by PMACI. The results represent three independent experiments (mean ± SEM). *$p < .05$; **$p < .01$ by Student's t test. Abbreviations: Ab; COX2, cyclooxygenase2; GMSC, gingiva-derived mesenchymal stromal cell; HMC-1, human mast cell line; PGE_2; PMACI, phorbol 12-myristate 13-acetate and calcium ionophore; and TNF-α, tumor necrosis factor-alpha.

COX-2/PGE_2 induced by PMACI-activated HMC-1 cells was abolished in the presence of TNF-α neutralizing antibody (Fig. 7D). Taken together, these results suggest that a critical feedback loop conferred by TNF-α/PGE_2 axis might play a key role in the crosstalks between MCs and GMSCs.

Discussion

ACD or CHS is one of the most common occupational diseases caused by repeated skin exposure to contact allergens such haptens [14, 15]. CHS belongs to type IV or DTH reactions, in which hapten-specific $CD8^+$ and $CD4^+$ effector or memory T cells secrete INF-γ, IL-17, perforin, granzyme, and other inflammatory mediators, eliciting the efferent limb of the immune responses and amplifying the inflammatory reactions at the re-encounter of the same type of hapten [28–32]. Most recently, several lines of evidence have demonstrated the critical role of MCs in delayed-type allergic reactions, which contribute to CHS development by promoting the recruitment of neutrophils and DCs [38], by enhancing T-cell activation [37] as well as by regulating the magnitude and cytokine microenvironment of CHS response [44]. Meanwhile, emerging evidence also supports the important role of $CD4^+CD25^+FoxP3^+$ T regulatory cells in preventing the development of allergic reactions to haptens/allergens contacting the skin, and in limiting the magnitude of the inflammatory process in already sensitized individuals [33, 34]. These studies have delineated the dynamics and complexity of cellular and molecular inflammatory networks contributory to the pathogenesis of CHS [14]. Therefore, simultaneously targeting multiple inflammatory signaling mediators and/or cellular components and reinducing tolerance to sensitizing haptens represent new modalities for the treatment of this type of allergic disease.

To date, numerous studies have well documented the immunomodulatory and anti-inflammatory properties of BMSCs and various other postnatal tissues [1–5] such as adipose [13, 18] and lung tissues [6], which have been used for the treatment of a variety of immune- and inflammation-related diseases [2–5]. In recent years, several studies have demonstrated that MSCs isolated from human dental tissues, including dental pulp [45, 46], dental follicle [46], apical papilla [47], periodontal ligament [48] and gingiva [16, 49], also exhibit similar in vitro immunosuppressive effects on T-cell functions when compared with BMSCs. Most recently, we and other groups have further reported some overlapping immunomodulatory functions of GMSCs and BMSCs on T cells and macrophages [10, 16, 49]. Herein, we further explored the therapeutic properties of GMSCs in a mouse model of CHS and found that GMSCs, similar to BMSCs, could ameliorate symptoms of CHS in mice (Supporting Information Fig. S1B). The attenuation of CHS induced by GMSC treatment was manifested as a significantly reduced infiltration of DCs, $CD8^+$ T cells and T_H-17 effector cells at the regional draining LNs and local allergic areas, and a markedly increased infiltration of Tregs (Figs. 2, 3). More importantly, we found that treatment with GMSC also dramatically reduced the total number of MCs as well as the percentage of degranulated MCs in the allergic ears (Fig. 4). These findings suggest that GMSCs attenuate CHS through targeting multiple types of innate and adaptive immune cells, which constitute the cellular and molecular inflammatory cascade involved in the pathophysiology of CHS [14]. However, further studies are warranted to dissect the detailed mechanisms by which GMSCs affect the complex interactions among these immune cells during the CHS.

Even though the exact mechanism of MSC-mediated immunomodulatory functions in vivo remains largely unknown, a large number of in vitro studies have demonstrated the involvement of a variety of immunosuppressive factors, such as IL-10, TGF-β1, PGE_2, nitric oxide (NO), and IDO, in MSC-mediated immunosuppression on various types of immune cells, including T lymphocytes, NK cells, DC, and macrophages [1–5, 7]. In the present study, we select to focus on DCs and MCs as major cellular players in GMSC-mediated immunosuppression pathways recognizing that DCs play critical role in the initiation of CHS [24–27] whereas MCs likely dominate CHS progression [35–44]. We showed that addition of specific neutralizing antibodies for IL-10 and TGF-β1 and a specific inhibitor for IDO failed to affect GMSC-mediated inhibition of DC and MC activation (Supporting Information Fig. S1E-S1G; Fig. 6A), but pretreatment of GMSCs with indomethacin, a specific inhibitor for COXs/PGE_2, significantly reversed their inhibitory effects on DC function as well as the PMA-stimulated activation of MCs (Figs. 2, 6; Supporting Information Fig. S1E-S1G). Meanwhile, GMSCs pretreated with indomethacin before injection into mice lost their suppressive effects on CHS and correspondingly the infiltration and activation of DCs and MCs (Figs. 2, 4). These results are in agreement with previous findings that PGE_2 plays a key role in MSC-mediated suppressive effects on DCs [8, 18] and MCs [50]. On the contrary, to our best knowledge, evidence supporting the involvement of IL-10 and TGF-β1 in MSC-mediated suppression of DCs and MCs remains at large; a recent study reported that IDO knockout murine BMSCs could not suppress DC maturation and lacked immunomodulatory functions when compared with wild-type MSCs [51]. Taken together, these findings support the notion that PGE_2, but not IL-10, TGF-β1, and IDO, plays a dominant role in GMSC-mediated attenuation of CHS and the underlying immunosuppressive effects on DCs and MCs.

As well characterized, PGE_2 exerts its biological functions via four subtypes of prostaglandin E receptors, EP1-EP4. The therapeutic effects of PGE_2 or its analogs on skin allergic inflammation have been described [52]; however, its efficacy and controversial results have been cautioned depending on the interaction with the receptor subtypes [21, 53–55]. For instance, PGE_2 suppressed skin allergic inflammation mediated via EP3 [21, 53] while triggering inflammatory response through Th1 cell and Th17 cell expansion via EP4 or EP1 [54, 55]. Such discrepancies may limit the clinical application of PGE_2 and its analogs in the treatment of skin allergic diseases. In the present study, we showed that subcutaneous administration of $dmPGE_2$ could partially ameliorate CHS in a dose-dependent manner; however, the suppressive effect is not as significant as that observed in animals treated with systemic infusion of GMSCs (Supporting Information Fig. S2E). Most importantly, MSCs, capable of both tropic and trophic activities, not only function to suppress the inflammatory and hypersensitivity phase of CHS but also contribute to the regeneration and repair of the injured tissues [56]. It has become increasingly apparent that even transient MSC engraftment may exert favorable effects through their trophic activities [4]. Most recently, we have demonstrated that labeled GMSCs could home to wounded skin sites and be detected even 1 week after systemic infusion [10]; this engraftment may provide additional lasting treatment benefit without the need for multiple dosing using a single pharmacological drug. All together, based on these unique properties, MSCs can function as a site-regulated multidrug dispensaries or "drugstore" [56] with multifunctional capacities, and therefore, are more superior to a single immunosuppressive or anti-inflammatory drug. Further studies should address the therapeutic cell dosage to optimize MSC application at both physiological and pharmacological levels.

In addition, we have evaluated the therapeutic effects of GMSCs on CHS using different treatment regimens and showed that systemic infusion of GMSCs following each of the three treatment regimens consistently attenuated CHS appearance in comparison with untreated CHS mice from 24 to 48 hours after challenge with the antigen, even if early intervention before antigen sensitization (treatment regimen I) and challenge (treatment regimen II) exhibited better efficacy than late intervention (treatment regimen III). This observation is consistent with previous reports in mice models of experimental autoimmune encephalomyelitis [57] and rheumatoid arthritis [58], whereby "prophylactic" administration of MSC, before disease onset, showed either similar or better ameliorative effects on the severity of the disease when compared with "therapeutic" administration of MSCs at early disease course, or at its peak [57, 58]. As reported, ACD is the most common occupational disorder; frequent recurrence and chronic cases have been reported in certain population of ACD patients due to continuous exposure to "unknown" allergens at the occupational settings [14, 15, 59]. Typically, ACD symptoms primed by the first encounter with allergens resolves in 7-21 days; but chronic ACD induced by continual exposure to allergens may persist for weeks to months even after antigen removal [15, 59]. Therefore, both prophylactic and therapeutic administrations of MSCs are necessary and particularly benefit chronic ACD patients in the allergen-exposed occupational settings. Further studies are warranted to explore the practical feasibility for the application of MSC-based therapy for the prevention and treatment of allergic diseases, including allergic rhinitis [13] and asthma [11, 60], knowingly that administration of MSCs during sensitization or before challenge also show significant ameliorative effects in mouse models.

Conclusion

In summary, we have demonstrated that systemic application of GMSCs significantly suppressed both the sensitization and elicitation of CHS through modulating the function of multiple types of innate and adaptive immune cells through the COXs/PGE_2 pathway. These findings further support the notion that GMSCs, a unique population of MSCs with functional similarities to BMSCs, and specifically, their ease of isolation, accessible tissue source, and rapid ex vivo expansion, are a promising cell source for MSC-based therapies of inflammatory and allergic diseases.

Acknowledgments

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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