

Culture medium from TNF- α -stimulated mesenchymal stem cells attenuates allergic conjunctivitis through multiple antiallergic mechanisms

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Background: The immunomodulatory and anti-inflammatory functions of mesenchymal stem cells (MSCs) have been demonstrated in several autoimmune/inflammatory diseases, but their contribution to allergic conjunctivitis and underlying antiallergic mechanisms remain elusive.

Objective: We sought to explore the clinical application of MSCs to experimental allergic conjunctivitis (EAC) and its underlying antiallergic mechanisms.

Methods: Culture medium from TNF- α -stimulated, bone marrow-derived MSCs (MSC-CMT) was administered topically to mice with EAC, and the related allergic symptoms and biological changes were evaluated. Murine spleen-derived B cells, bone marrow-derived mast cells (MCs), and lung vascular endothelial cells were cultured *in vitro* to investigate the antiallergic MSC-CMT mechanisms.

Results: Topical instillation of MSC-CMT significantly attenuated the clinical symptoms of short ragweed pollen-induced EAC, with a significant decrease in inflammatory cell frequency, nuclear factor κ B p65 expression, and TNF- α and IL-4 production. *In vitro* MSC-CMT significantly inhibited the activation of MCs and B-cell IgE release and reduced histamine-induced vascular hyperpermeability. During EAC, MSC-CMT treatment also decreased IgE production, histamine release, enrichment and activation of MCs, and conjunctival vascular hyperpermeability. The MSC-CMT-mediated inhibition of B cells, MCs, and histamine and its antiallergic effects during EAC were abrogated when MSCs were pretreated with COX2 small interfering RNA.

Conclusions: Our findings provide compelling evidence that MSC-CMT inhibits EAC through COX2-dependent multiple antiallergic mechanisms and support the use of MSC-CMT as a novel strategy for treating allergic conjunctivitis. (J Allergy Clin Immunol 2015;■■■:■■■-■■■.)

Key words: Allergy, mesenchymal stem cells, stem cells, B cells, mast cells, histamine, allergic conjunctivitis

Allergies are increasingly prevalent and have become a major world health problem. Among allergic diseases, allergic conjunctivitis (AC), which includes seasonal and perennial AC, vernal keratoconjunctivitis, and atopic keratoconjunctivitis, is the most prevalent form of mucosal allergy and currently affects more than 20% of the US population.^{1,2} AC is characterized by classical symptoms that include itching, tearing, eyelid swelling, and chemosis and redness of the eyes, and AC symptoms significantly affect a patient's health and quality of life. Topical antihistamines, mast cell (MC) stabilizers, or dual-acting antihistamine/MC stabilizers remain the mainstay of AC therapy. Although corticosteroids are potent antiallergic agents in reducing ocular allergic symptoms, especially in patients with severe and chronic AC, they are associated with various ocular adverse effects, such as secondary infection, increased intraocular pressure, and cataract formation.^{3,4} Additionally, with prolonged use, topical antihistamines might be irritating to the eye, and many antihistamines are known to have anticholinergic effects that can cause ocular drying through muscarinic receptor inhibition.³⁻⁶ Therefore investigating safer and more effective treatments is necessary.

Mesenchymal stem cells (MSCs; also referred to as mesenchymal stromal cells) possess biological functions ranging from tissue repair/regeneration to immunomodulatory actions.⁷⁻⁹ Furthermore, different culture conditions or additional treatment might regulate or enhance the biological functions of MSCs. Because of their multifunctional properties, numerous animal and clinical trials are examining the use of MSCs to treat a wide range of diseases. The antiallergic properties of MSCs have recently received increasing attention, and several studies have reported that MSCs suppress allergic responses in experimental mouse models of asthma,^{10,11} allergic rhinitis,¹² and contact hypersensitivity.¹³ However, the mechanisms by which MSCs mediate antiallergic responses remain largely elusive.

The roles of MSCs in patients with AC have not yet been explored. In previous studies MSCs have been administered for the treatment of ocular surface disorders through various routes, including intravenous injection, application with a special hollow plastic tube, transplantation with an amniotic membrane, and subconjunctival injection.¹⁴⁻¹⁸ However, in a clinical setting these administration routes are not acceptable for treating patients with AC; therefore the development of a clinically feasible MSC administration method for the treatment of AC is desirable.

Although the exact mechanism of MSC immunomodulatory action remains largely unknown, a large number of studies have demonstrated that a variety of soluble factors, such as IL-10,

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Abbreviations used

AC:	Allergic conjunctivitis
ACED:	Autologous serum eye drop
BMSC:	Bone marrow–derived mesenchymal stem cell
EAC:	Experimental allergic conjunctivitis
HRP:	Horseradish peroxidase
IDO:	Indoleamine-2,3-dioxygenase
L-NAME:	N-nitro-L-arginine methyl ester
LVEC:	Lung vascular endothelial cell
MAPK:	Mitogen-activated protein kinase
MC:	Mast cell
MSC:	Mesenchymal stem cell
MSC-CM:	Culture medium from bone marrow–derived mesenchymal stem cells
MSC-CMT:	Culture medium from TNF- α –stimulated, bone marrow–derived mesenchymal stem cells
1-MT:	1-Methyl-tryptophan
NF- κ B:	Nuclear factor κ B
NO:	Nitric oxide
OLF:	Ophthalmic lavage fluid
PGE ₂ :	Prostaglandin E ₂
PMACI:	Phorbol 12-myristate 13-acetate plus calcium ionophore
siRNA:	Small interfering RNA
SRW:	Short ragweed
STAT:	Signal transducer and activator of transcription

TGF- β , prostaglandin E₂ (PGE₂), nitric oxide (NO), TNF-stimulated protein 6, and indoleamine-2,3-dioxygenase (IDO), are involved in the MSC-mediated immunosuppression of various immune responses.⁷⁻⁹ These studies suggest that MSC culture medium, which contains these soluble secreted factors, might be an effective AC treatment. However, recent data suggest that resting MSCs have low immunomodulatory activity, whereas MSCs stimulated by inflammatory cytokines, such as TNF- α , secrete large quantities of soluble mediators and develop their full immunosuppressive potential.⁷⁻⁹ Therefore MSC conditional medium stimulated by inflammatory cytokines might be an effective treatment for AC.

In this study we investigated the therapeutic effects of culture medium from TNF- α –stimulated, bone marrow–derived mesenchymal stem cells (MSC-CMT) on experimental allergic conjunctivitis (EAC) and the underlying cellular and molecular mechanisms of this potential therapy.

METHODS**Animals**

BALB/c mice were supplied by and maintained in the Guangzhou Animal Testing Center and used between 4 and 6 weeks of age. The studies were approved by the Institutional Animal Care and Use Committee of the Zhongshan Ophthalmic Center at Sun Yat-sen University. All procedures involving animal eye studies were conducted in accordance with the Association for Research in Vision and Ophthalmology “Statement for the use of animals in ophthalmic and vision research.” The animals were maintained in specific pathogen-free conditions on a 12-hour light-dark cycle with controlled temperature (23°C $\pm$ 2°C) and humidity (55% $\pm$ 10%).

Isolation and culture of bone marrow–derived mesenchymal stem cells and MSC-CMT preparation

BMSCs were isolated from BALB/c mice, as previously described.¹⁹ Briefly, bone marrow cells were flushed from the bone cavities of femurs

and tibias with α -Minimum Essential Medium (α -MEM; Invitrogen, Grand Island, NY) containing 2% heat-inactivated FBS. Single-suspension murine bone marrow–derived nucleated cells were seeded at a density of 15×10^6 cells in 100-mm culture dishes (Corning Costar, Corning, NY) and maintained at 37°C in a 5% CO₂ atmosphere. Nonadherent cells were removed after 48 hours, and adherent cells were maintained for 16 days in α -MEM supplemented with 20% FBS, 2 mmol/L L-glutamine, 55 μ mol/L 2-mercaptoethanol, 100 U/mL penicillin, and 100 μ g/mL streptomycin. Colonies formed by adherent cells were passaged once for additional experimental use. Flow cytometric analyses demonstrated that bone marrow–derived mesenchymal stem cells (BMSCs) expressed high levels of CD29, CD44, and CD90 but did not express the hematopoietic markers CD34, CD45, or CD11b (eBioscience, San Diego, Calif; data not shown).

BMSCs were cultured in 100-mm dishes until subconfluence to obtain culture medium from bone marrow–derived mesenchymal stem cells (MSC-CM) and TNF- α –stimulated BMSCs (MSC-CMT). The cells were then washed twice with HBSS to remove serum and incubated in 10 mL of α -MEM in the presence or absence of carrier-free recombinant mouse TNF- α (10 ng/mL) for 48 hours. The supernatants were collected and centrifuged to remove cell debris. TNF- α was depleted from MSC-CMT by using anti-TNF- α antibody immobilized on protein G–agarose beads, according to the manufacturer’s instructions. In brief, 0.2 μ g of anti-TNF- α antibody in PBS (30 μ L) was mixed with a protein G–agarose bead suspension (50% slurry) for 1 hour at 4°C with intermittent shaking. After centrifugation, beads bound with the anti-TNF- α antibody were washed 3 times. MSC-CMT was incubated with anti-TNF- α antibody immobilized on protein G–agarose beads for 1 hour at 4°C. The protein G–agarose beads were removed by using centrifugation. TNF- α , IL-10, IL-4, IL-13, and IFN- γ levels were measured (see Fig E1 in this article’s Online Repository at www.jacionline.org). The resultant supernatants were collected and immediately used for experiments.

For further information on isolation, culture, and activation of B cells; isolation, culture, and activation of bone marrow–derived MCs; isolation and culture of lung vascular endothelial cells (LVECs); *in vitro* and *in vivo* vascular permeability assays; transfection; and EAC treatment with MSC-CMT, see the Methods section in this article’s Online Repository at www.jacionline.org.

The murine EAC model was generated, as previously reported.^{2,20,21} In brief, a mixture of 50 μ g of short ragweed (SRW) pollen (Greer Laboratories, Lenoir, NC) in 5 mg of Imject Alum (Pierce, Rockford, Ill) was applied through footpad injection on the first day. The sensitization procedure was repeated on day 5 to enhance the allergic reaction. The mice were challenged with 1.5 mg of SRW pollen suspended in 10 μ L of PBS in both eyes on days 10 to 14. MSC-CMT (10 μ L) was topically applied once 30 minutes before the SRW pollen challenge and 4 times per day on days 10 to 14 in both eyes. PBS was applied to the control groups in the same manner (n = 6 per group).

For further information on clinical evaluation and immunohistochemistry, Western blot analyses, ELISAs, and real-time PCR, see the Methods section in this article’s Online Repository.

Statistical analyses

The Student *t* test was used to analyze significant differences (SPSS 16.0; SPSS, Chicago, Ill). A *P* value of less than .05 was considered significant.

RESULTS**MSC-CMT treatment suppresses EAC**

SRW pollen–induced EAC is a well-established mouse model of human AC. MSC-CMT (subjected to TNF- α immunodepletion) or MSC-CM was applied to the ocular surfaces of mice with EAC on days 10 to 14 after immunization (day 0) to examine the therapeutic effect of different MSC culture media on AC (Fig 1, A). The effect of MSC-CMT on ocular symptoms in mice was evaluated after topical SRW pollen challenge. Throughout the study, ocular administration of MSC-CMT, but not MSC-CM, significantly alleviated all of the symptoms in the mice with

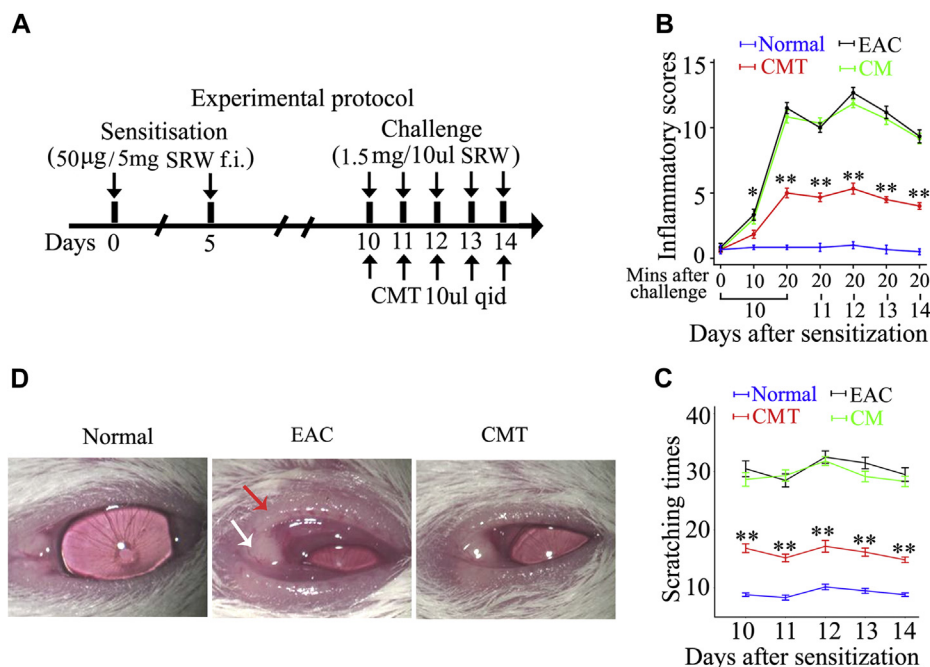


FIG 1. MSC-CMT treatment attenuates EAC. **A**, Experimental protocol. **B** and **C**, Inflammatory scores of EAC (Fig 1, B) and scratch times (Fig 1, C) were evaluated at the indicated time points after challenge in different experimental groups. **D**, Representative images of ocular symptoms in the indicated experimental group 30 minutes after the last challenge. Red arrow, Lid swelling; white arrow, conjunctival edema. $n = 6$ mice for each group. $*P < .05$ and $**P < .01$ between the MSC-CMT and EAC groups. Error bars = means $\pm$ SEMs.

EAC compared with the control group ($P < .01$; Fig 1, B). SRW pollen application also induced a significant increase in the scratching response compared with normal mice, and MSC-CMT administration significantly inhibited the scratch response in mice compared with the control group ($P < .01$; Fig 1, C). Representative images of ocular symptoms demonstrate that MSC-CMT treatment significantly attenuated eyelid swelling, conjunctival edema, and redness (Fig 1, D).

MSC-CMT treatment attenuates inflammation in the mice with EAC

Next, we investigated the effect of MSC-CMT treatment on inflammatory profiles in mice with EAC. Histologic analyses of the conjunctival tissue harvested 14 days after immunization revealed a significantly decreased infiltration of inflammatory cells and eosinophil accumulation in MSC-CMT-treated mice compared with that seen in control animals (Fig 2, A-C). Next, we determined the effects of MSC-CMT on local inflammatory cytokine levels in the conjunctival tissue using real-time PCR and ELISA. The results demonstrated a significant increase in TNF- α , IL-4, IL-5, IL-1 β , and TGF- β expression in the conjunctiva of mice with EAC. MSC-CMT suppressed the upregulation of TNF- α , IL-4, IL-5, and IL-1 β but increased TGF- β expression (Fig 2, D-G, and see Fig E2, A-C, in this article's Online Repository at www.jacionline.org).

Nuclear factor κ B (NF- κ B) is critical for ocular inflammation, including inflammation associated with microbial infections, allergic eye diseases, and dry eye.²² Thus we examined whether MSC-CMT modulates NF- κ B signaling in EAC. Additionally, p38 mitogen-activated protein kinase

(MAPK), caspase-1, and signal transducer and activator of transcription (STAT) 3 and STAT6 expression levels were also measured by means of Western blotting. MSC-CMT treatment reduced phosphorylation of NF- κ B p65, p38 MAPK, and STAT6 and caspase-1 expression but increased STAT3 phosphorylation in the conjunctiva of mice with EAC (Fig 2, H-K, and see Fig E2, D).

MSC-CMT inhibits B-cell IgE release through a COX2-dependent mechanism

B cells play important roles in the pathophysiology of allergic diseases, primarily through their ability to produce IgE antibodies.²³ Thus we investigated whether the antiallergic effects of MSC-CMT might involve inhibition of B-cell function. First, the *in vivo* effects of MSC-CMT on IgE production in mice with EAC were determined. As shown in Fig 3, A, SRW application significantly increased IgE levels of ophthalmic lavage fluid (OLF) in mice with EAC compared with those in normal mice, whereas MSC-CMT treatment significantly reduced IgE levels.

We next performed *in vitro* studies to confirm the inhibitory effect of MSC-CMT on B cells. Purified mouse splenic B cells were cultured with IL-4 and LPS in different medium for 5 days. IgE and IgG₁ levels in culture supernatants were measured by means of ELISA. A significant inhibition of IgE and IgG₁ release by activated B cells was observed in the presence of MSC-CMT but not MSC-CM (Fig 3, B and C). Notably, MSC-CMT did not affect B-cell viability (Fig 3, D).

We next examined the mechanisms involved in MSC-CMT-mediated inhibition of IgE production by B cells. In these

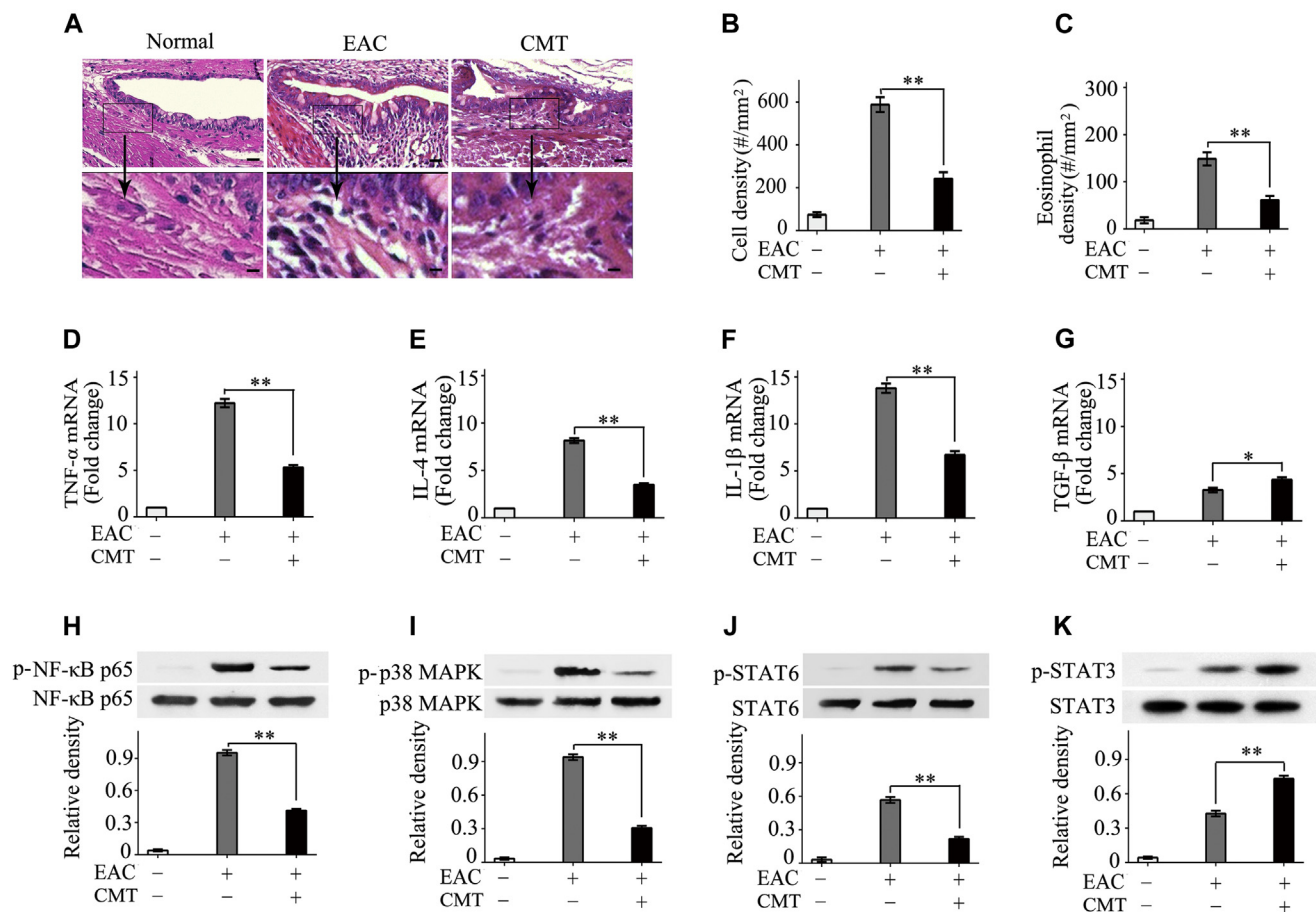


FIG 2. Treatment with MSC-CMT attenuates inflammation and alters the cytokine profile in mice with EAC. **A**, Representative images of hematoxylin and eosin staining of conjunctival samples from mice in the indicated experimental groups. Scale bars represent 200 μm (upper panels) and 600 μm (lower panels). **B** and **C**, Quantification of cellular components (Fig 2, B) and eosinophils (Fig 2, C) in the conjunctivas of mice in the indicated experimental groups. **D-G**, TNF- α , IL-4, IL-1 β , and TGF- β mRNA expression in conjunctival tissue was determined by using real-time PCR. **H-K**, Phosphorylated NF- κB p65, phosphorylated p38 MAPK, phosphorylated STAT6, and phosphorylated STAT3 levels in conjunctival tissue was determined by means of Western blotting. * $P < .05$ and ** $P < .01$ between the MSC-CMT and EAC groups. Error bars = means $\pm$ SEMs.

experiments neutralizing mAbs specific for TGF- β 1 or IL-10 and specific inhibitors of IDO (1-methyl-tryptophan [1-MT]), NO (N-nitro-L-arginine methyl ester [L-NAME]), or COX2 (NS-398) were used. TGF- β 1 and IL-10 neutralizing antibodies and BMMSC pretreatment with 1-MT or L-NAME did not affect MSC-CMT-mediated inhibitory effects. In contrast, BMMSC pretreatment with NS-398 significantly but incompletely reversed the inhibitory effect on IgE production (Fig 3, E, and see Fig E3, A, in this article's Online Repository at www.jacionline.org). These results suggest that COX2/PGE₂ signaling in BMMSCs plays an important role in MSC-CMT-mediated B-cell inhibition.

To further investigate the role of COX2/PGE₂ in MSC-CMT-mediated B-cell inhibition, we first exposed BMMSCs to different concentrations of exogenous TNF- α for 24 hours and then analyzed COX2 expression and PGE₂ production using Western blot analysis and ELISA, respectively. TNF- α treatment led to dose-dependent increases in COX2 expression and PGE₂ production by BMMSCs (Fig 3, F and G). To further confirm the role of

COX2/PGE₂ in MSC-CMT-mediated B-cell suppression by BMMSCs, we used small interfering RNA (siRNA) to knock down COX2 expression in BMMSCs, and the inhibitory capacity of MSC-CMT on B-cell IgE production was significantly decreased in BMMSCs treated with COX2 siRNA but not control siRNA (Fig 3, H, and see Fig E3, B).

EAC attenuation by MSC-CMT is associated with MC inhibition

We next examined whether the antiallergic effects of MSC-CMT involve inhibition of MC function. We first explored the *in vivo* effects of MSC-CMTs on MC functions in challenged conjunctiva. Toluidine blue staining revealed that the numbers of MCs and percentages of degranulated MCs decreased dramatically in the conjunctiva of MSC-CMT-treated mice compared with those seen in control mice (Fig 4, A-C). Additionally, MSC-CMT treatment significantly decreased histamine production in mice with EAC compared with that seen in control mice (Fig 4, D).

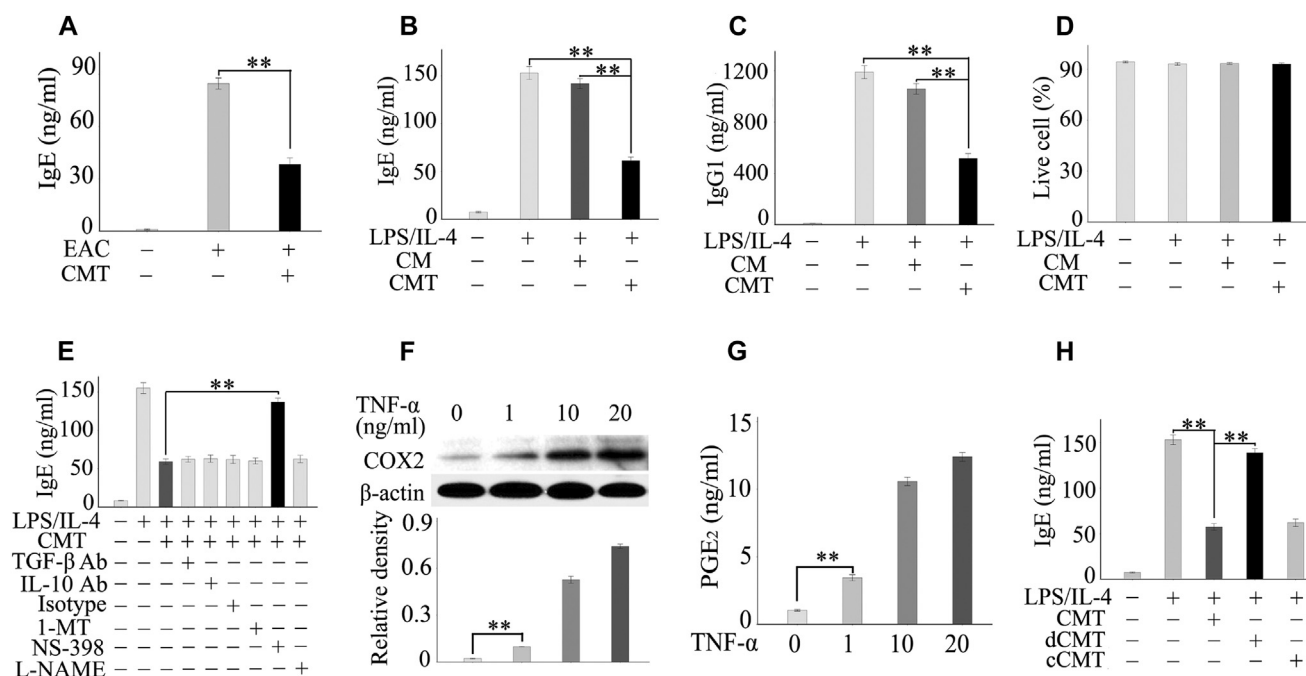


FIG 3. MSC-CMT inhibits IgE release by B cells through a COX2-dependent mechanism. **A**, OLF was collected after the last challenge, and IgE levels were determined by using ELISA. BALB/c splenic B cells were cultured with different media for 24 hours. **B** and **C**, After stimulation with LPS/IL-4 for 5 days, IgE (**B**) and IgG₁ (**C**) levels in supernatants were determined by using ELISA. **D**, Cell viability was assayed by using trypan blue exclusion. **E**, MSC-CMT was added with specific neutralizing antibodies for TGF-β1 or IL-10 (10 μg/mL, isotype used as controls), or MSC-CMT was collected after pretreating BMMSCs with a specific IDO inhibitor (1-MT, 500 μmol/L), a specific NO inhibitor (L-NAME, 1 mmol/L), or a specific COX2 inhibitor (NS-398, 1 μmol/L). **F** and **G**, Exogenous TNF-α-induced dose-dependent increases in COX2 expression and PGE₂ production in BMMSCs. **H**, MSC-CMT was collected after pretreating BMMSCs with COX2 siRNA (dCMT) or control siRNA (cCMT). ***P* < .01. Error bars = means ± SEMs.

We next performed *in vitro* studies to confirm the inhibitory effect of MSC-CMT on MCs. MCs were cultured with different media for 24 hours and then stimulated with phorbol 12-myristate 13-acetate plus calcium ionophore (PMACI) for an additional 12 hours. Our results demonstrated that MSC-CMT, but not MSC-CM, significantly inhibited PMACI-stimulated TNF-α and IL-4 release by MCs (Fig 4, E, and see Fig E4, A, in this article's Online Repository at www.jacionline.org). MSC-CMT did not affect MC viability (see Fig E4, B).

Next, we investigated the mechanisms involved in MSC-CMT-mediated MC inhibition. The MCs were cultured with MSC-CMT for 24 hours and then stimulated with PMACI for another 6 hours. NF-κB activation was subsequently determined by using Western blot analysis. Although PMACI stimulation resulted in increased NF-κB p65 protein production, MSC-CMT significantly inhibited NF-κB p65 expression in MCs (Fig 4, F). Most agents, including PMACI, activate NF-κB through IκB-α phosphorylation, degradation, or both. IκB-α degradation exposes a nuclear localization signal and leads to NF-κB activation.²⁴ Thus we investigated whether MSC-CMT modulates NF-κB activity in MCs by inhibiting IκB-α degradation. As expected, MSC-CMT significantly inhibited IκB-α degradation in MCs (Fig 4, G). Because COX2/PGE₂ signaling was essential for the MSC-CMT-mediated inhibition of B-cell function, we examined whether COX2 plays a similar role in MSC-CMT-mediated MC inhibition. As shown in Fig 4, H, and Fig E4, C, MSC-CMT-mediated MC inhibition was partially reversed when BMMSCs were pretreated with COX2 siRNA.

Antihistamine effects of MSC-CMT

We next determined whether MSC-CMT has an inhibitory effect on histamine function. We initially tested whether MSC-CMT effectively reduced vascular hyperpermeability in mice with EAC. The Evans blue permeability assay demonstrated that MSC-CMT treatment significantly reduced conjunctival vascular hyperpermeability in mice with EAC compared with that seen in untreated control mice (Fig 5, A and B). We next performed a series of *in vitro* studies to confirm the inhibitory effect of MSC-CMT on histamine function. LVECs purified from mouse liver and lung tissue were cultured with or without MSC-CMT in a Transwell system (BD Biosciences, San Jose, Calif) for 24 hours. Cells from each culture condition were stimulated with histamine for 30 minutes and subsequently evaluated in the permeability assay. LVECs cultured with MSC-CMT exhibited significantly decreased vascular hyperpermeability to streptavidin-horse-radish peroxidase (HRP) than LVECs stimulated with histamine alone (Fig 5, C).

We next examined the mechanisms regulating these MSC-CMT-mediated inhibitory effects. VE-cadherin-VE-cadherin homophilic interactions and VE-cadherin-β-catenin binding are crucial to maintaining normal vascular integrity and permeability. Histamine increases vascular permeability by inducing the tyrosine phosphorylation of VE-cadherin and disrupting VE-cadherin-VE-cadherin homophilic interactions.^{25,26} Thus we analyzed total VE-cadherin and phosphorylated VE-cadherin expression in LVECs under different conditions. LVECs

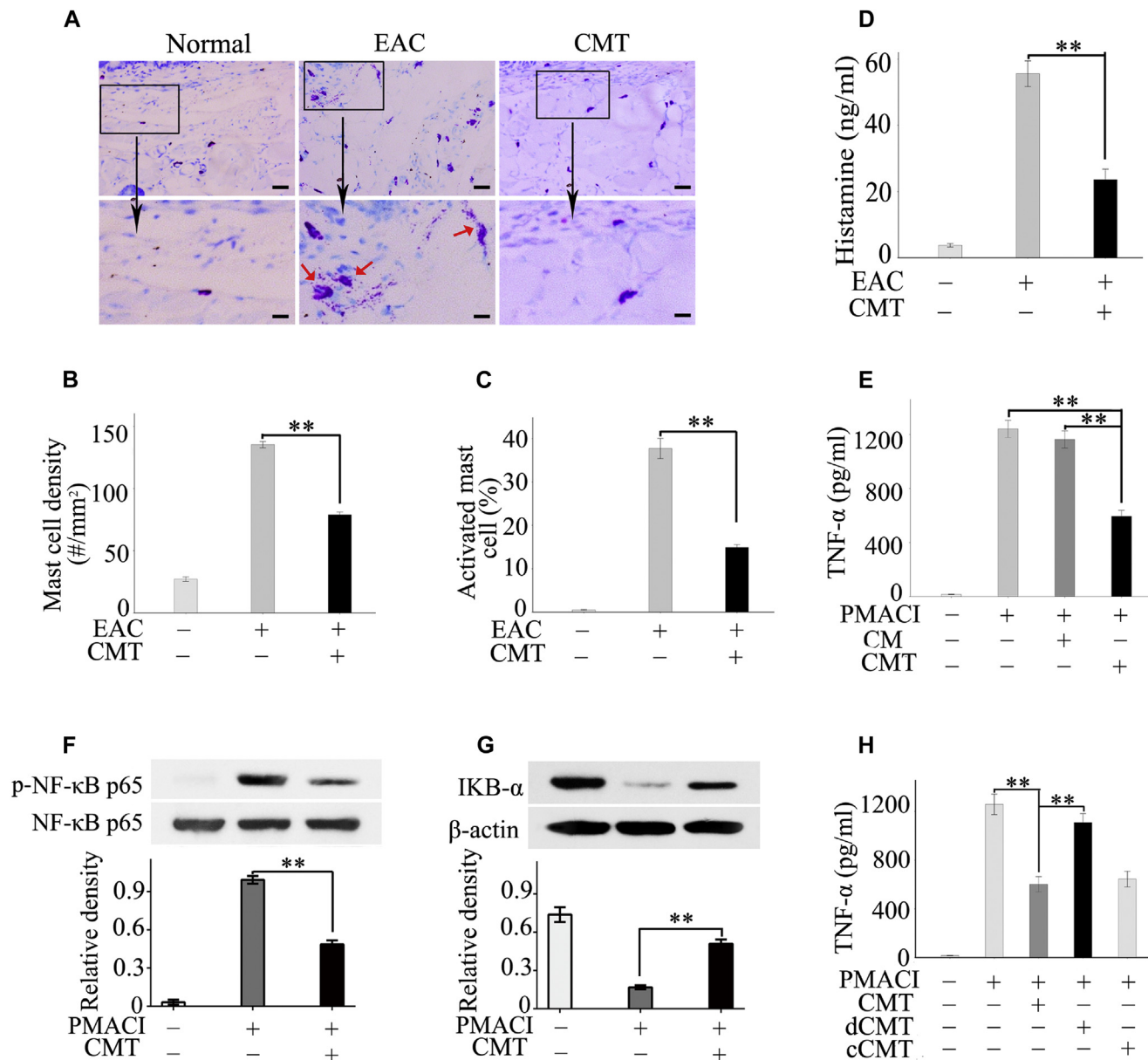


FIG 4. Attenuation of EAC by MSC-CMT is associated with its inhibitory effects on MCs. **A-C**, MCs were identified by using toluidine blue staining, and activated MCs were identified based on their irregular shape (red arrows) in conjunctivas of normal mice, mice with EAC, and MSC-CMT-treated mice with EAC, as indicated. Scale bars represent 200 μm (upper panels) and 600 μm (lower panels). **D**, Histamine levels in OLF were determined by using EIA (n = 6). **E** and **H**, MCs were cultured with MSC-CMT or MSC-CM for 24 hours and then stimulated with PMACI for an additional 12 hours. TNF-α levels in supernatants were determined by means of ELISA. **F** and **G**, IκB-α and NF-κB p65 expression levels in BMMSCs were determined by means of Western blotting. **H**, MSC-CMT was collected after pretreating BMMSCs with COX2 siRNA (dCMT) or control siRNA (cCMT). **P < .01. Error bars = means ± SEMs.

cultured with MSC-CMT exhibited significantly increased total VE-cadherin expression but reduced the phosphorylation of VE-cadherin compared with that seen in control mice (Fig 5, D and E). COX2 is essential for MSC-CMT-mediated inhibition of B-cell function; therefore we investigated whether COX2 plays a similar role in the MSC-CMT-mediated inhibition of histamine. MSC-CMT-mediated vascular hyperpermeability inhibition was partially reversed when BMMSCs were pretreated with COX2 siRNA (Fig 5, F).

COX2 signaling in BMMSCs is essential for MSC-CMT-mediated EAC inhibition

Our *in vitro* studies demonstrated that COX2/PGE₂ signaling plays an essential role in the MSC-CMT-mediated inhibition of B cells, MCs, and histamine, and therefore we investigated whether COX2 is also implicated in MSC-CMT-mediated EAC attenuation. COX2 siRNA was used to knock down COX2 expression in BMMSCs, followed by stimulation of the cells with TNF-α. MSC-CMT derived from BMMSCs treated with COX2 siRNA

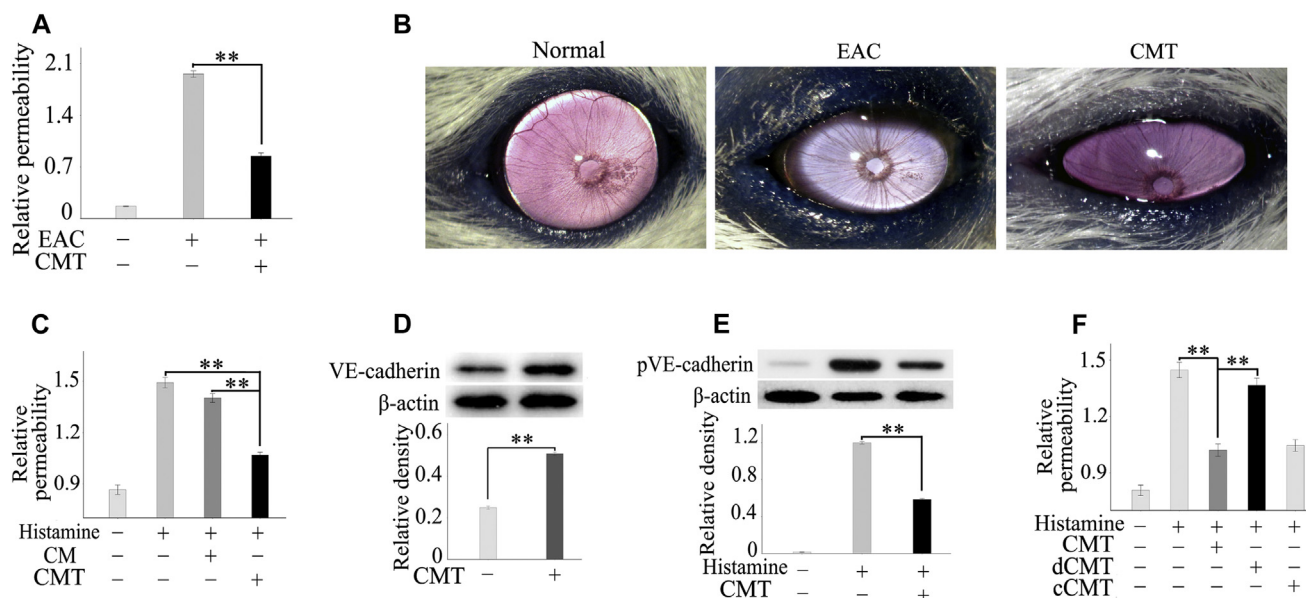


FIG 5. Antihistamine effects of MSC-CMT. **A**, Conjunctival vascular permeability was evaluated by using the Evans blue assay ($n = 6$). **B**, Representative images of eyes after Evans blue injection. **C** and **F**, LVECs were cultured with MSC-CMT for 24 hours. After stimulation with histamine for 30 minutes, the vascular permeability assay was performed. **D** and **E**, Total VE-cadherin (Fig 5, **D**) and phosphorylated VE-cadherin (Fig 5, **E**) expression was determined by using Western blotting. Fig 5, **F**, MSC-CMT was collected after pretreating BMMSCs with COX2 siRNA (*dCMT*) or control siRNA (*cCMT*). $**P < .01$. Error bars = means $\pm$ SEMs.

did not suppress EAC in mice (Fig 6, **A** and **B**). COX2 knockdown in BMMSCs also significantly decreased the inhibitory effects of MSC-CMT on inflammatory cell infiltration, IgE production, MC enrichment and activation, histamine production, and TNF- α and IL-4 production in mice with EAC (Fig 6, **E-G**, and see Fig E5 in this article's Online Repository at www.jacionline.org). Additionally, topical administration of 16,16-dimethyl PGE₂ (20-80 ng/mL in PBS) led to a dose-dependent suppression of EAC appearance, but the treatment response was not as significant as the effect mediated by MSC-CMT treatment (Fig 6, **H** and **I**). As shown in Fig E6 (in this article's Online Repository at www.jacionline.org), our results showed that MSC-CMT reduced the production of TNF- α and IL-1 β by lung epithelial cells stimulated with lipopolysaccharide.

DISCUSSION

MSCs have a striking variety of biological functions; therefore therapeutic applications of MSCs for a wide range of diseases have been studied extensively. AC, a common clinical problem for both ophthalmologists and allergists, is caused by an allergen-induced inflammatory response.^{1,2} Here we demonstrated that MSC-CMT treatment significantly attenuated the clinical symptoms of EAC with a significantly decreased inflammatory cell frequency, decreased TNF- α and IL-4 production, and reduced NF- κ B expression. Furthermore, we determined that MSC-CMT inhibits B-cell, MC, and histamine function. These inhibitory actions might contribute to the therapeutic effects of MSC-CMT on EAC. To our knowledge, these findings provide the first evidence that MSC manipulation is a potential novel strategy for the treatment of AC.

In response to allergen stimulation during type I allergic reactions, allergen-specific T_H2 cells produce cytokines that

initiate B-cell production of allergen-specific IgE, which binds to the high-affinity IgE receptor on MCs. Allergen cross-linking with allergen-specific MC IgE leads to the release of preformed granule-stored allergic mediators, such as histamine. These mediators cause early acute inflammatory responses and activation of the *de novo* synthesis of mediators, such as inflammatory cytokines, which sustain the late inflammatory response phase.²³ Therefore T_H2 cells, B cells, MCs, and histamine play crucial roles in the development of allergic reactions. Several recent studies have reported that the intravenous administration of MSCs during the sensitization phase produces prophylactic anti-allergic effects in patients with allergic rhinitis and asthma.¹⁰⁻¹² The inhibition of antigen-specific T_H2 cell differentiation, which shifts the T_H2/T_H1 balance, has been proposed as the mechanism underlying these effects.

In contrast to studies demonstrating MSC-mediated inhibition of antigen-specific T_H2 cell differentiation during the sensitization phase, the administration of topical MSC-CMT during the effector phase in the present study yielded promising therapeutic effects in SRW pollen-induced EAC. Our results demonstrated that the topical administration of MSC-CMT during the effector phase of EAC reduced IgE production, histamine release, enrichment and activation of MCs, and conjunctival vascular hyperpermeability. These results were further supported by the inhibition of MC activation and B-cell IgE release and reduced histamine-induced vascular hyperpermeability mediated by MSC-CMT *in vitro*. These findings collectively suggest that the inhibition of B-cell, MC, and histamine function contributes to the antiallergic functions of MSC-CMT.

In vitro MC degranulation is always induced in a specific buffer but not in the culture medium.^{27,28} Therefore we were not able to evaluate the *in vitro* role of MSC-CMT in MC degranulation in this study. Instead, we evaluated the roles of MSC-CMT in

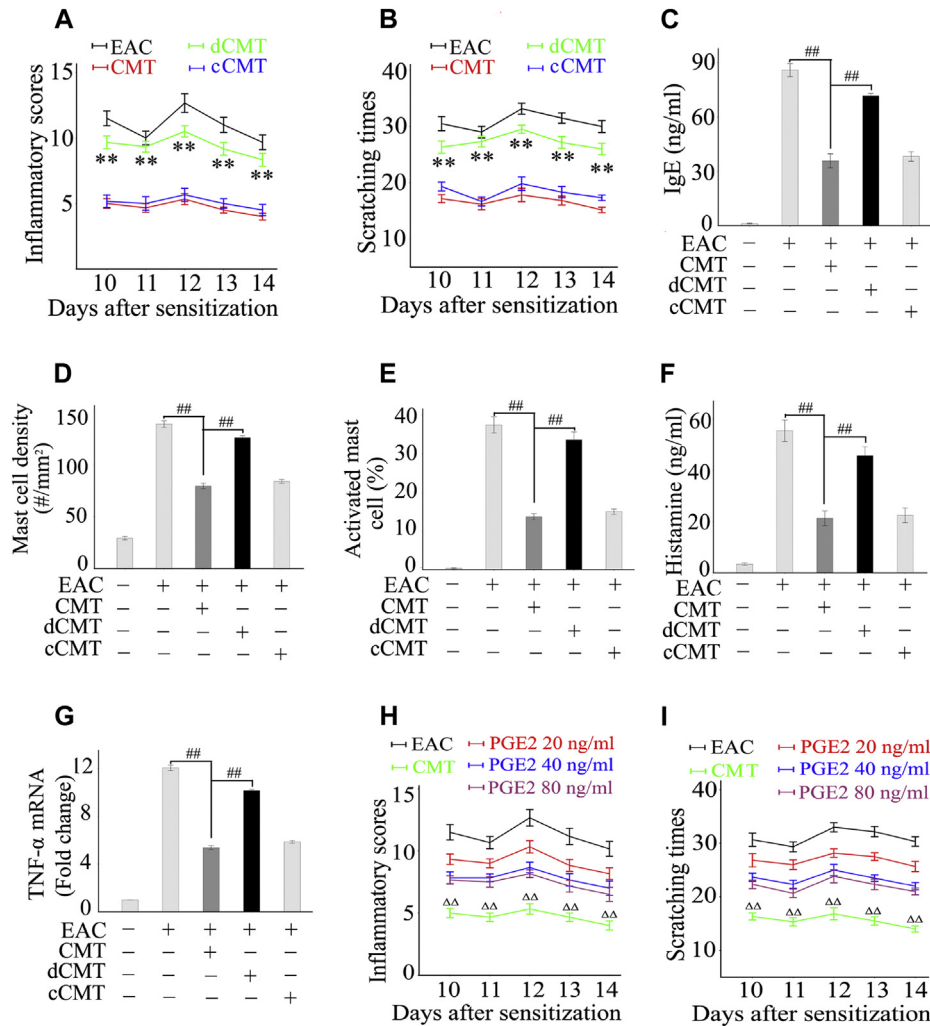


FIG 6. COX2 signaling in BMMSCs is essential for MSC-CMT-mediated inhibition of EAC. EAC was induced as above, and MSC-CMTs collected after pretreating BMMSCs with COX2 siRNA (*dCMT*) or control siRNA (*cCMT*) were administered to mice with EAC. **A** and **B**, Inflammatory scores (Fig 6, A) and scratch times (Fig 6, B) were evaluated at the indicated time points after challenge (n = 6). **C-G**, IgE production (Fig 5, C), MC enrichment and activation (Fig 5, D and E), histamine production (Fig 5, F), and TNF-α expression (Fig 5, G) in EAC were measured as above at the indicated time points after challenge. Topical 16,16-dimethyl PGE₂ (*dmPGE*₂; 20-80 ng/mL) was administered on days 10 to 14 in mice with EAC. **H** and **I**, Inflammatory scores (Fig 5, H) and scratch times (Fig 5, I) were evaluated at indicated time points after challenge (n = 6). **P < .01 between the MSC-CMT and dMSC-CMT groups. ##P < .01 between the indicated groups. ΔΔP < .01 between the MSC-CMT and PGE₂ 80 ng/mL groups. Error bars = means ± SEMs.

PMACI-stimulated MC activation. MSC-CMT significantly inhibited MC inflammatory mediator production by modulating the NF-κB signaling pathway in a COX2-dependent manner. MSC-CMT treatment *in vivo* significantly reduced histamine production and the enrichment and activation of MCs. Together with the aforementioned BMMSC-mediated inhibition of MC degranulation through a COX2-dependent mechanism,²⁹ these data indicate that MSC-CMT inhibits MC function *in vitro* and *in vivo*.

Although the exact mechanism of MSC-mediated immunomodulatory functions remains elusive, many studies have demonstrated that COX2/PGE₂ signaling plays important roles in MSC-mediated immunosuppression in various immune cells, including T lymphocytes, natural killer cells, dendritic cells, MCs, and macrophages.²⁹⁻³³ In the present study we focused on B cells, MCs, and histamine as major players in MSC-

mediated EAC immunosuppression pathways. We demonstrated that the addition of specific neutralizing antibodies for IL-10 and TGF-β1 and a specific inhibitor for IDO or NO did not affect MSC-CMT-mediated inhibition of IgE release by B cells, but pretreatment of MSCs with NS398, a specific inhibitor of COX2/PGE₂, significantly reversed these inhibitory effects on B-cell IgE release. Furthermore, COX2 knockdown in BMMSCs also significantly decreased the inhibitory effects of MSC-CMT on B-cell IgE release, PMACI-stimulated activation of MCs, and histamine-induced vascular hyperpermeability. Additionally, MSCs pretreated with COX2 siRNA before injection into mice lost their suppressive effects on EAC, including the effects on IgE and histamine production and MC infiltration and activation. These results support previous findings demonstrating a dominant role of COX2 signaling

in the MSC-CMT-mediated attenuation of EAC and the underlying immunosuppressive effects on B cells, MCs, and histamine.

PGE₂ exerts its biological functions through 4 subtypes of prostaglandin E receptors, EP1-EP4. The therapeutic effects of PGE₂ or its analogs on allergic inflammation have been previously described³⁴⁻³⁶; however, conflicting results with respect to its efficacy have been obtained, depending on the receptor subtype.³⁷⁻⁴² For example, PGE₂ suppresses allergic inflammation mediated through EP3 or EP2³⁷⁻³⁹ while triggering inflammatory responses through EP4 or EP1.⁴⁰⁻⁴² These discrepancies might limit the clinical application of PGE₂ and its analogs in the treatment of allergic diseases. In the present study we demonstrated that 16,16-dimethyl PGE₂ topical administration partially ameliorated EAC in a dose-dependent manner; however, the suppressive effect was not as significant as the effect observed in animals treated with topical MSC-CMT. This finding might be attributed to the actions of multiple factors, rather than PGE₂ alone, that promote MSC-CMT-mediated inhibition of EAC. Furthermore, Heo et al⁴³ recently reported that topical MSC-CMT enhances cutaneous wound healing by soluble factors.

These findings indicate that MSC-CMT, which possesses both immunosuppressive and trophic activities, suppresses the inflammatory phase of EAC and contributes to the repair of injured tissues, which might provide additional lasting treatment benefits without multiple dosing by using a single pharmacologic drug. The current mainstay of AC therapy includes topical MC stabilizers and antihistamines and has demonstrated variable and limited clinical success,³⁻⁶ possibly because other factors in addition to MCs and histamine, such as T cells, B cells, macrophages, platelets, dendritic cells, and neutrophils, also play important roles in AC. Therefore the simultaneous targeting of multiple inflammatory signaling mediators, cellular components, or both might represent a promising modality for the treatment of this type of allergic disease. Our results demonstrated that in contrast to MC stabilizers and antihistamine drugs, MSC-CMT simultaneously acts on multiple targets, including B cells, MCs, and histamine. Furthermore, MSCs possess multifunctional properties in addition to potent immunosuppressive and anti-inflammatory functions through their interactions with and/or inhibition of different subtypes of T cells, natural killer cells, dendritic cells, neutrophils, and macrophages through release of soluble factors. Taken together, these unique properties suggest that MSC-CMT might function as a multidrug dispensary or “drug store” with multifunctional capacities superior to a single immunosuppressive or antiallergic drug. Future studies should address the detailed method of MSC-CMT preparation and MSC-CMT concentration to optimize MSC-CMT therapeutic effects at both physiologic and pharmacologic levels.

Previous studies have used intravenous infusion, subconjunctival injection, amniotic membranes as a carrier, or a special hollow plastic tube to administer MSCs to the ocular surface.¹⁴⁻¹⁸ However, these methods are not appropriate for clinical AC treatment. By contrast, topical drug instillation is a common and easy clinical administration route for the treatment of ocular surface disorders. Thus we used topical MSC-CMT instillation to exploit the beneficial properties of MSCs in this study. Our results demonstrate that topical MSC-CMT application significantly attenuated EAC. This finding suggests that topical MSC-CMT application is a promising alternative for the clinical application of MSCs to ocular surface disorders.

Recently, autologous serum eye drops (ACEDs) have been shown to possess potential benefits for ocular surface disorders. Several studies reported that the therapeutic benefits of ACEDs in patients with persistent corneal epithelial defects and dry eyes are mediated by their trophic properties that promote wound healing.^{44,45} However, to our knowledge, no articles have reported that ACEDs possess immunomodulatory effects. Thus ACEDs are different from MSC-CMT, which possesses not only trophic properties but also immunomodulatory properties. In addition, the use of amniotic membranes has also been shown to have therapeutic benefits for severe ocular surface disorders, such as ocular chemical burns, corneal ulcers, persistent corneal epithelial defects, and corneal limbal stem cell deficiency,⁴⁶ as well as anti-inflammatory and trophic properties on the ocular surface. Of note, the combination of amniotic membrane treatment and MSCs has shown better therapeutic effects than amniotic membrane treatment alone in patients with ocular chemical burns, skin defects, and articular cartilage defects.^{47,48} Thus for some severe ocular disorders, the combination of amniotic membrane treatment and MSCs or MSC-CMT might be a promising therapy.

In summary, for the first time, our study demonstrates that MSC manipulation can be used to treat AC. Our results show that MSC-CMT inhibits IgE release from B cells and MC activation through COX2-dependent mechanisms. Furthermore, we provide the first evidence showing that MSCs possess antihistamine properties. These findings provide compelling evidence that MSC manipulation inhibits allergic reactions and supports the use of MSCs as part of a novel strategy for treating AC. MSC-CMT obtained from donor BMMSCs and preserved as eye drops might be applied either therapeutically or prophylactically to patients with AC because of its multifunctional capacities. The prophylactic application of MSC-CMT for AC, especially seasonal allergic conjunctivitis and vernal keratoconjunctivitis, might reduce the occurrence rate of AC rather than only alleviating the symptoms.

Clinical implications: MSC-CMT might be a novel therapeutic option for treating AC and ocular surface inflammatory diseases.

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METHODS

Reagents and antibodies

LPS, Evans blue dye, histamine, phorbol 12-myristate 13-acetate, calcium ionophore (A23187), NS398, L-NAME, 1-MT, and protein G-agarose beads were purchased from Sigma (St Louis, Mo). Recombinant mouse IL-4, TNF- α , and IL-3 were from PeproTech (Rocky Hill, NJ). α -MEM and RPMI 1640 medium were obtained from Invitrogen (Grand Island, NY). The following antibodies were used: anti-COX2, anti-VE-cadherin, and anti-TNF- α (Abcam, Cambridge, Mass); anti-NF- κ B p65, anti-phosphorylated NF- κ Bp65, anti-I κ B- α , anti-p38 MAPKs, anti-phosphorylated p38 MAPK, anti-STAT3, anti-phosphorylated STAT3, anti-STAT6, anti-phosphorylated STAT6, anti-caspase-1, and anti- β -actin (Cell Signaling Technology, Danvers, Mass) and neutralizing antibodies specific for mouse IL-10 or TGF- β 1 or an isotype-matched mAb (R&D Systems, Minneapolis, Minn).

Isolation, culture, and activation of B cells

BALB/c splenic B cells were purified (purity >95%, as determined by means of flow cytometric analysis of B220 cell-surface expression, data not shown) with a B-cell isolation kit (Miltenyi Biotec, Auburn, Calif), according to the manufacturer's instructions. Purified B cells were cultured in RPMI 1640 containing 10% FBS, L-glutamine, and 55 μ mol/L 2-mercaptoethanol. For B-cell activation, B cells (1×10^6 /mL) were stimulated by the addition of IL-4 (50 ng/mL) and LPS (10 μ g/mL) to culture medium for 5 days. Day 6 supernatants were collected for IgE and IgG₁ analysis.

Isolation, culture, and activation of bone marrow-derived MCs

Bone marrow-derived MCs were derived from BALB/c mice, as previously reported.^{E1,E2} In brief, BALB/c mice (Jackson Laboratory, Bar Harbor, Me) were killed by means of CO₂ asphyxiation and bathed in 70% ethanol. The femurs were then removed with scissors and forceps and placed in 10 mL of 37°C culture medium. After cutting the ends of the femurs, the bone marrow was flushed from the interior of each femur with a syringe and needle into a well containing 5 mL of medium.

Cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, 25 mmol/L HEPES, 1.0 mmol/L sodium pyruvate, nonessential amino acids (BioSource International, Camarillo, Calif), 0.0035% 2-mercaptoethanol, and 30 ng/mL recombinant mouse IL-3 (PeproTech). After 4 weeks, MC purity was evaluated by means of toluidine blue staining, and CD117 (c-Kit) surface staining was evaluated by means of flow cytometry. The purity of the MCs used in this study was greater than 95% (data not shown). The MCs were used after 4 to 6 weeks of culture at 37°C and 5% CO₂. For MC activation, MCs (2×10^5 /mL) were stimulated with phorbol 12-myristate 13-acetate (50 nmol/L) and calcium ionophore (A23187, 1 mg/mL; PMACI) for 16 hours.

Isolation and culture of LVECs

LVECs were isolated from lung tissue of BALB/c mice by using CD45 and CD31 microbeads (Miltenyi Biotec). In brief, blood cells were removed from the lung tissue of BALB/c mice by using PBS perfusion before excision. The lung tissue was minced and digested with collagenase II (Worthington, Freehold, NJ). Cell suspensions were filtered through a 40- μ m cell strainer (Corning Costar). Magnetic labeling and separation were performed according to the manufacturer's instructions. After total lung cell suspensions were depleted of CD45⁺ cells, positive selection for CD31⁺ cells was performed. The enrichment and purity of the endothelial cells were analyzed by using flow cytometry (purity >95%). LVECs were then cultured in EGM-2 Single-Quots (Lonza, Walkersville, Md).

In vitro and in vivo vascular permeability assays

In vitro vascular permeability assays were performed, as described previously.^{E3} In brief, LVECs (2×10^5) were grown in a Transwell plate (BD Biosciences) in 500 μ L of medium until a monolayer formed. LVEC monolayer

permeability was tested 8 hours later by the addition of 7.5 μ L of streptavidin-HRP (1.5 μ g/mL, R&D Systems) to the upper chamber. The monolayers were stimulated with histamine (100 μ mol/L) for 30 minutes before addition of streptavidin-HRP. Medium (50 μ L) in the lower chamber was collected 5 minutes after addition of streptavidin-HRP and assayed for HRP activity by adding 100 μ L of TMB substrate. Color development was detected with a microplate reader at 450 nm.

In vivo vascular permeability assays were performed by administering a 0.1% Evans blue dye solution in PBS (12 mL/kg) through the tail vein after the first challenge. Mice were killed 60 minutes after injection. The eyelid and conjunctival tissue were then removed and collected. Evans blue dye was extracted after incubation for 24 hours. The extract was measured at 620 nm with a microplate reader.

Transfection

COX2 siRNA and fluorescein-conjugated control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif). Three distinct siRNAs with the same specificity were used to recognize and exclude off-target effects. siRNA transfection was performed according to the manufacturer's instructions. In brief, BMMSCs were washed once with 2 mL of siRNA Transfection Medium. The medium was then aspirated. For each transfection, 0.8 mL of siRNA Transfection Medium was added to each tube containing the siRNA Transfection Reagent mixture (Solution A + Solution B). After gentle mixing, the mixture was overlaid onto the washed cells. Cells were then incubated for 5 to 7 hours at 37°C in a CO₂ incubator. Next, 1 mL of normal growth medium containing twice the normal concentration of serum and antibiotics (2 $\times$ normal growth medium) was added without removing the transfection mixture. If toxicity occurred, the transfection mixture was removed and replaced with 1 $\times$ normal growth medium before adding 2 $\times$ normal growth medium. The cells were then incubated for an additional 18 to 24 hours, and the medium was aspirated and replaced with fresh 1 $\times$ normal growth medium. BMMSCs were used 24 to 72 hours after the addition of fresh medium.

EAC treatment with MSC-CMT

The murine EAC model was generated, as previously reported.^{E4,E5} In brief, EAC was induced by using the following protocol: a mixture of 50 μ g of SRW pollen (Greer Laboratories) in 5 mg of Imject Alum (Pierce) was applied through footpad injection on the first day. The sensitization procedure was repeated on day 5 to enhance the allergic reaction. The mice were challenged with 1.5 mg of SRW pollen suspended in 10 μ L of PBS in both eyes on days 10 to 14. MSC-CMT (10 μ L) was topically applied once 30 minutes before the SRW pollen challenge and 4 times per day on days 10 to 14 in both eyes. PBS was applied to the control groups in the same manner ($n = 6$ per group).

A slit lamp was used to examine the eyes throughout the course of the study. After 20 minutes of challenge, blinded researchers recorded and evaluated the clinical reaction according to a published system.^{E4} In addition, allergic symptoms were graded by using a previously published system.^{E4} Conjunctival edema, lid swelling, tearing, and conjunctival redness were graded from 0 to 4 based on the criteria. The clinical score was the sum of the 4 parameters. The scratching times of each animal 15 minutes after the 30-minute challenge were also counted by observers who were blind to the treatment protocol. The scratching response was defined as rapid movements of the hind paws that were precisely directed toward the eye. OLF was collected after the last SRW pollen exposure. PBS (10 μ L) was applied to the eye by using a micropipette without touching the eye. After 2 or 3 forced blinks, OLF was collected. The lavage was repeated 5 times in each eye. The OLF was centrifuged at 400g for 10 minutes, and the supernatant was separated for further analysis. One hour after the last challenge, the mice were killed, and the eyes were removed for further analysis.

Immunohistochemistry

Hematoxylin and eosin staining was performed on paraffin-embedded sections for histologic evaluation. For semiquantification, positive signals in at

least 5 random high-power fields were visualized and quantified with Image Pro-Plus 5.1 (Media Cybernetics cell numbers, Silver Spring, Md).

Western blot analysis and ELISA

Cell lysates or mouse conjunctiva lysates (50–100 µg of total protein) were resolved on polyacrylamide-SDS gels and electroblotted onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, Calif). After blocking with 5% nonfat dry milk/TBS, the membranes were incubated with antibodies against VE-cadherin, COX2, p38 MAPK, STAT3, STAT6, caspase-1, NF-κB p65, or IκB-α, followed by incubation with an HRP-conjugated secondary antibody. The signals were visualized with an enhanced chemiluminescence substrate (Pierce). The blots were then reprobed with β-actin antibody.

Concentrations of IgE, IgG₁, TNF-α, and IL-4 in the supernatants or OLF were determined by using ELISA (eBioscience). Concentrations of PGE₂ and histamine in the supernatants or OLF were determined by using ELISA kits (Cayman Chemical, Ann Arbor, Mich).

Real-time PCR

Total RNA was extracted from the tissue lysates with an RNeasy Mini Kit (Qiagen, Valencia, Calif), and cDNA was generated by using an Omniscript RT kit (Qiagen). TNF-α and IL-4 mRNA expression were quantified by using the Absolute SYBR Green ROX mix (Thermo Fisher Scientific, Waltham, Mass). The samples were performed in triplicate, and the relative expression of TNF-α and IL-4 was determined by normalizing the expression of each target gene to β-actin by using the $2^{-\Delta\Delta C_t}$ method.

RESULTS

MSC-CMT reduces inflammatory cytokine release by lung epithelial cells

LA-4, a murine lung epithelial cell line, was used to examine the effects of MSC-CMT on epithelial cells. As shown in Fig E6, our results showed that MSC-CMT reduced the production of TNF-α and IL-1β by lung epithelial cells stimulated with lipopolysaccharide.

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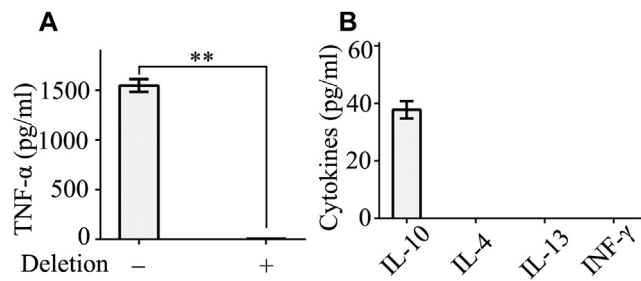


FIG E1. TNF- α , IL-10, IL-4, IL-13 and IFN- γ levels in MSC-CMT. **A**, After TNF- α depletion with antibody, the TNF- α level was detected by using ELISA in MSC-CMT. **B**, IL-10, IL-4, IL-13, and IFN- γ levels were measured by using ELISA in MSC-CMT. ** $P < .01$. Error bars = means $\pm$ SEMs.

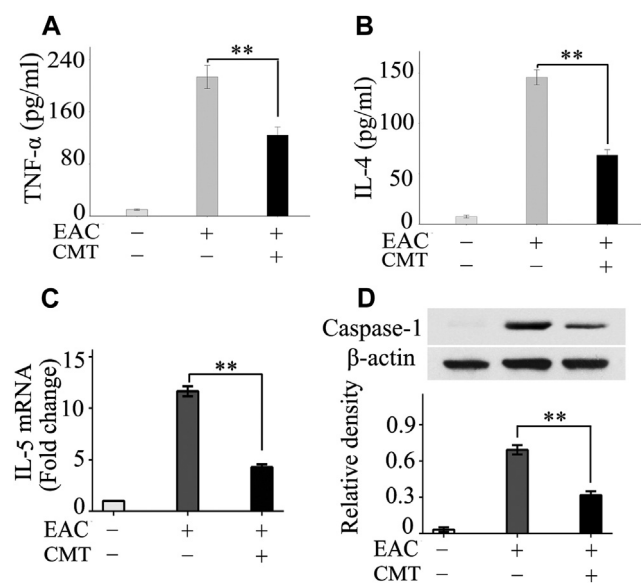


FIG E2. Treatment with MSC-CMT reduces TNF- α , IL-4, and IL-5 production and caspase-1 activation in mice with EAC. **A-C**, TNF- α (Fig E2, A), IL-4 (Fig E2, B), and IL-5 (Fig E2, C) protein expression in conjunctival tissue was determined by means of ELISA. **D**, Caspase-1 expression in conjunctival tissue was determined by means of Western blotting. ** $P < .01$. Error bars = means $\pm$ SEMs.

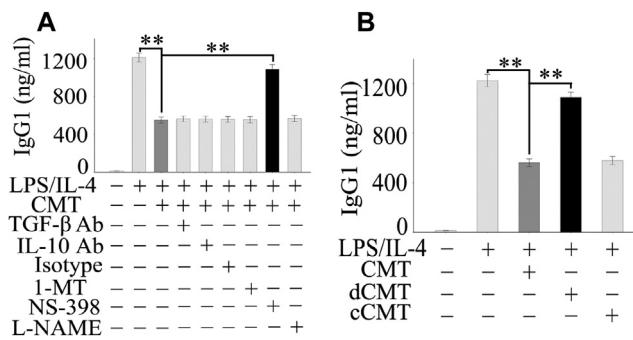


FIG E3. MSC-CMT inhibits IgG₁ release by B cells through a COX2-dependent mechanism. B cells were cultured with different media for 24 hours. After stimulation with LPS/IL-4 for 5 days, IgG₁ levels in supernatants were determined by using ELISA. **A**, MSC-CMT was added with specific neutralizing antibodies for TGF-β1 or IL-10 (10 μg/mL, isotype used as controls), or MSC-CMT was collected after pretreating BMMSCs with a specific IDO inhibitor (1-MT, 500 μmol/L), a specific NO inhibitor (L-NAME, 1 mmol/L), or a specific COX2 inhibitor (NS-398, 1 μmol/L). **B**, MSC-CMT was collected after pretreating BMMSCs with COX2 siRNA (*dCMT*) or control siRNA (*cCMT*). ***P* < .01. Error bars = means ± SEMs.

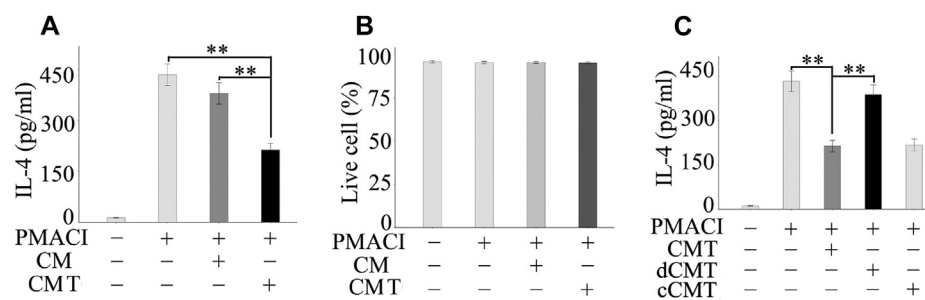


FIG E4. MSC-CMT inhibits IL-4 release by MCs through a COX2-dependent mechanism. **A** and **C**, MCs were cultured with MSC-CMT or MSC-CM for 24 hours and then stimulated with PMACI for an additional 12 hours. IL-4 levels in supernatants were determined by using ELISA. **B**, Cell viability was assayed by using trypan blue exclusion. Fig E4, C, MSC-CMT was collected after pretreating BMMSCs with COX2 siRNA (*dCMT*) or control siRNA (*cCMT*). ** $P < .01$. Error bars = means $\pm$ SEMs.

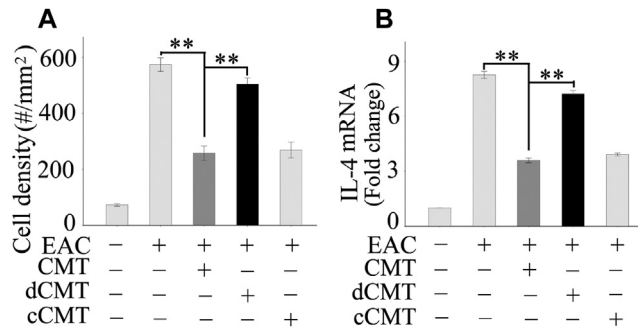


FIG E5. COX2 signaling in BMMSCs is essential for MSC-CMT-mediated inhibition of inflammatory cell infiltration and IL-4 production. MSC-CMTs collected after pretreating BMMSCs with COX2 siRNA (*dCMT*) or control siRNA (*cCMT*) were administered to mice with EAC. Inflammatory cell infiltration (**A**) and IL-4 expression (**B**) in EAC were measured as above at the indicated time points after challenge. ****** $P < .01$. Error bars = means $\pm$ SEMs.

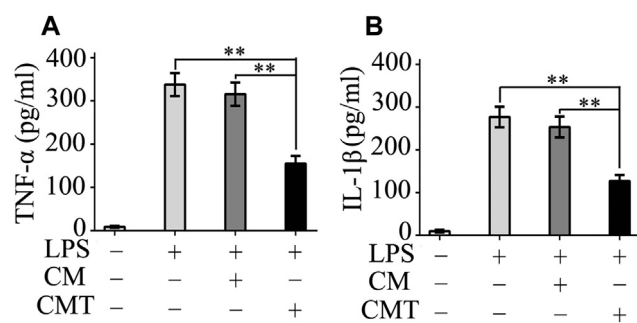


FIG E6. MSC-CMT reduces inflammatory cytokine release by lung epithelial cells. LA-4, a murine lung epithelial cell line, was cultured with MSC-CMT or MSC-CM for 24 hours and then stimulated with LPS for an additional 12 hours. TNF- α (**A**) and IL-1 β (**B**) levels in the supernatants were determined by using ELISA. $**P < .01$. Error bars = means $\pm$ SEMs.