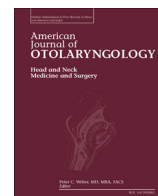




Contents lists available at ScienceDirect

American Journal of Otolaryngology–Head and Neck Medicine and Surgery

journal homepage: www.elsevier.com/locate/amjoto

Intranasal azelastine and mometasone exhibit a synergistic effect on a murine model of allergic rhinitis☆

Do Hyun Kim¹, Boo-Young Kim¹, Ji-Hyeon Shin, Sung Won Kim, Soo Whan Kim^{*}

Department of Otolaryngology–Head and Neck Surgery, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Seoul, Republic of Korea

ARTICLE INFO

Article history:

Received 1 November 2016

Available online xxx

Keywords:

Allergic rhinitis

Corticosteroids

Antihistamines

ABSTRACT

Purpose: The purpose of this study was to compare the anti-allergic effects of the combination of azelastine and mometasone with those of either agent alone in a *Derf*-induced murine model of allergic rhinitis (AR).

Materials and methods: Forty BALB/c mice were divided into five groups: azelastine (A), mometasone (M), a combination of azelastine and mometasone (MA), *Derf*, and control. *Derf* served as the allergen. Allergic symptom scores, eosinophil counts, and serum *Derf*-specific IgE levels were measured. The mucosal levels of mRNAs encoding interferon (IFN)- γ , T-bet, interleukin (IL)-4, GATA-3, Foxp3, IL-17, and ROR- γ t were determined by real-time polymerase chain reaction. The T-bet, GATA-3, Foxp3, and ROR- γ t results were confirmed by Western blotting.

Results: Nose-rubbing motions; the levels of mRNAs encoding IL-4, GATA-3, and ROR- γ t; and tissue eosinophil count were reduced in the MA compared with those in the *Derf* group (all *P* values <0.05). The levels of mRNAs encoding GATA3 and IL-4 mRNA [synthesized by T helper (Th)2 cells] were reduced and that of mRNA encoding Foxp3 was increased in the MA compared with those in the *Derf* and A groups. Western blotting confirmed these findings.

Conclusion: We found that the combination of intranasal azelastine and mometasone synergistically suppressed Th17 responses and (reciprocally) elevated Treg responses. Therefore, this combination not only ameliorated allergic inflammation by suppressing Th2 responses, but also usefully modified the Treg/Th17 balance.

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1. Introduction

Allergic rhinitis (AR) affects >500 million people worldwide [1,2]. It is the fifth most common chronic disease [3] and the most common chronic disease among children [4] in the United States. The direct cost of AR has been estimated to be approximately ~\$7 billion annually in the United States [2], and indirect costs may bring that figure to ~\$9.7 billion [5]. The cost is estimated at €4260 per patient per year in Europe [6]. Comorbid diseases such as asthma and atopy further increase AR-related treatment costs [7,8].

The principal current guideline-based therapies for AR are medical in nature, including corticosteroids and antihistamines (via the intranasal or oral route) or oral leukotriene receptor antagonists [9]. Intranasal corticosteroids are as effective as oral corticosteroids, and the intranasal

route is preferred because there are fewer side effects [1,9]. Intranasal antihistamine therapy has recently become popular, being more efficacious than oral therapy [10]. The onset of action is rapid [11] and the efficacy comparable to that of oral corticosteroids in terms of symptom control [11–13].

However, AR treatment is challenging; many patients have moderate-to-severe disease and do not respond adequately to treatment [14]. Up to 74.4% of all patients use multiple treatments in attempts to achieve symptom relief [8,14–17], although the evidence that such approaches are effective is limited [2]. Physicians tend to prescribe combination treatments featuring oral antihistamines and intranasal corticosteroids [16,18], although evidence of their efficacy is lacking [19–22].

Many studies have tested medical combinations for the treatment of AR. However, none of intranasal steroid-plus-oral antihistamine, oral antihistamine-plus-leukotriene receptor antagonist, or intranasal steroid-plus-leukotriene receptor antagonist treatment has afforded any pronounced clinical benefit [9]. Although an intranasal steroid-plus-intranasal oxymetazoline regimen afforded some relief, rebound concerns indicate that intranasal oxymetazoline should be limited to a few days [9]. However, combinations of intranasal steroids and antihistamines have been suggested to be more effective than monotherapies when

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^{*} Corresponding author at: Department of Otolaryngology–Head and Neck Surgery, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Banpo-daero 222, Seocho-gu, Seoul 137-701, Republic of Korea.

E-mail address: kshent@catholic.ac.kr (S.W. Kim).

¹ Do Hyun Kim and Boo-Young Kim are equally responsible for this work.

treating AR [23–26]. In addition, the safety and tolerability profiles of the combinations were favorable [27]. These supposedly superior results have not yet been investigated at the immunological level. Thus, in the present study, we compared the effects of a combination of intranasal steroid and antihistamine with those of either agent alone in a murine model of AR.

2. Materials and methods

2.1. Experimental animals

Six-week-old healthy female BALB/c mice (20–30 g) were used. All experiments were performed with the approval of the Institutional Animal Care and Use Committee of the Catholic University of Korea.

2.2. Reagents

Dermatophagoides farinae (Derf) crude body extract (Arthropods of Medical Importance Resource Bank, College of Medicine, Yonsei University, Seoul, South Korea) was dissolved in water prior to use. Mice were assigned to receive azelastine (Bukwang Pharm., Co., Ltd., Seoul, South Korea), mometasone (Hanmi Pharm., Co., Ltd., Seoul, South Korea), or Motesone® (mometasone furoate 50 mcg, Azelastine HCl 140 mcg; Hanmi Pharm., Co., Ltd.).

2.3. The AR model and the treatment protocol

Forty mice were randomized into five groups: control ($n = 8$), Derf (AR, $n = 8$), M (mometasone administered after challenge, $n = 8$), A (azelastine administered after challenge, $n = 8$), and MA (Motesone® administered after challenge, $n = 8$). Allergen sensitization and challenge in the context of the murine AR model are summarized in Fig. 1. Briefly, on days 0, 7, and 14, all mice except those in the control group were immunized via intraperitoneal injection of 100 μ g of Derf and 1 mg of aluminum hydroxide (Thermo Scientific, Waltham, MA, USA). After 1 week, all sensitized mice were intranasally challenged with 20 μ g of Derf daily for 6 consecutive days. Mice in the M group received intranasal mometasone (0.2 μ g) on days 21, 22, 23, 24, 25, and 26. Mice in the A group received intranasal azelastine (0.14 μ g) on days 21, 22, 23, 24, 25, and 26. Mice in the MA group received intranasal Motesone® (0.58 μ g; 0.56 μ g of azelastine and 0.2 μ g of mometasone) on days 21, 22, 23, 24, 25, and 26. The control group received intranasal PBS.

2.4. Allergic symptoms induced after allergen challenge

The numbers of sneezes and nose-rubbing motions during 15-min periods after final allergen challenge were recorded and compared among experimental groups by blinded observers.

2.5. Derf-specific immunoglobulin E (IgE) levels in serum

Serum Derf-specific IgE levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Indoor Biotechnologies, Cardiff, UK).

2.6. Real-time polymerase chain reaction (PCR)

Nasal mucosae were removed and real-time PCR was used to quantify the levels of mRNAs encoding interferon (IFN)- γ , T-bet, interleukin (IL)-4, GATA-3, Foxp3, IL-17, and ROR- γ t. Total RNA was extracted from nasal mucosa using TRIzol reagent (Invitrogen, Waltham, MA, USA); the first strands were reverse-transcribed using random primers (Takara, Otsu, Japan). The oligonucleotide primer sequences were as follows: IFN- γ forward, 5'-AGAGCCAGATTATCTCTTCTACCTCAG-3' and IFN- γ reverse, 5'-CCTTTTTCGCCTTGCTGTG-3'; T-bet forward, 5'-GCCAGGGAACCGCTTATA-3' and T-bet reverse, 5'-CCTGTGTGTTGGTGAGCTTTA-3'; IL-4 forward, 5'-TCAACCCAGCTAGTTGTC-3' and IL-4 reverse, 5'-AAATATGCGAAGCACCTTGG-3'; GATA-3 forward, 5'-CTGGATGGCGGCAAAGC-3' and GATA-3 reverse, 5'-GTGGGCGGGAAGGTGAA-3'; Foxp3 forward, 5'-GAAAGCGGATACCAATGA-3' and Foxp3 reverse, 5'-CTGTGAGGACTACCGAGCC-3'; ROR- γ t forward, 5'-ACCTCCACTGCCAGCTGTGTGCTGTC-3' and ROR- γ t reverse, 5'-TCATTTCTGCACTTCTGCATGTAGACTGTCCC-3'; IL-17 forward, 5'-TTTAACCTCCCTTGGCGCAAAA-3' and IL-17 reverse, 5'-CTTCCCTCCGATTGACAC-3'; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward, 5'-GCACAGTCAAGGCCGAGAAT-3' and GAPDH reverse, 5'-GCCTTCTCCATGGTGGTGAA-3'. The levels of mRNAs encoding IFN- γ , T-bet, IL-4, GATA-3, Foxp3, IL-17, ROR- γ t, and GAPDH were determined by real-time PCR using the CFX96 Real Time PCR platform (Bio-Rad, Hercules, CA, USA) and an iQ SYBR Green Supermix kit (Bio-Rad). All results were normalized to GAPDH expression and are shown as fold increases over control expression levels.

2.7. Western blotting

Western blots were used to quantify the relative expression levels of T-bet, GATA-3, Foxp3, and ROR- γ t in nasal mucosae from the control, Derf, M, A, and MA groups. We calculated grayscale ratios between the expression levels of target genes and GAPDH; these indicate the relative expression levels of target genes. The antibodies used were the anti-IFN- γ antibody sc-59,992, the anti-T-bet antibody sc-21,003, the anti-IL-4 antibody sc-1260, and the anti-GATA-3 antibody sc-9009 (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.8. Statistical analysis

All data are expressed as means $\pm$ standard deviations (SDs). Among-group differences were analyzed using the Kruskal-Wallis test.

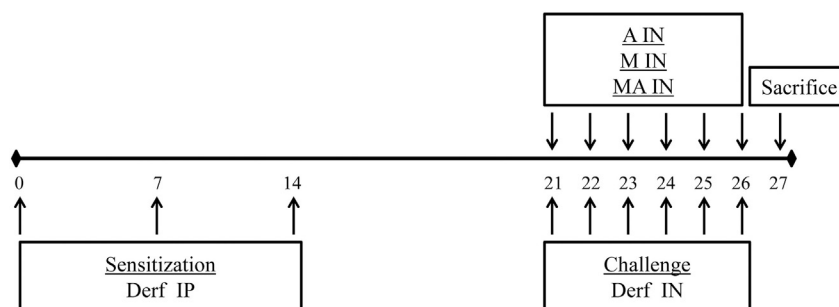


Fig. 1. Schematic representation of our experimental allergic rhinitis model and treatment protocol. A, azelastine; M, mometasone; MA, Motesone® (mometasone furoate 50 mcg/dose, azelastine HCl 140 mcg/dose); Derf, *Dermatophagoides farinae*; IP, intraperitoneal administration; IN, intranasal administration. The time units are days.

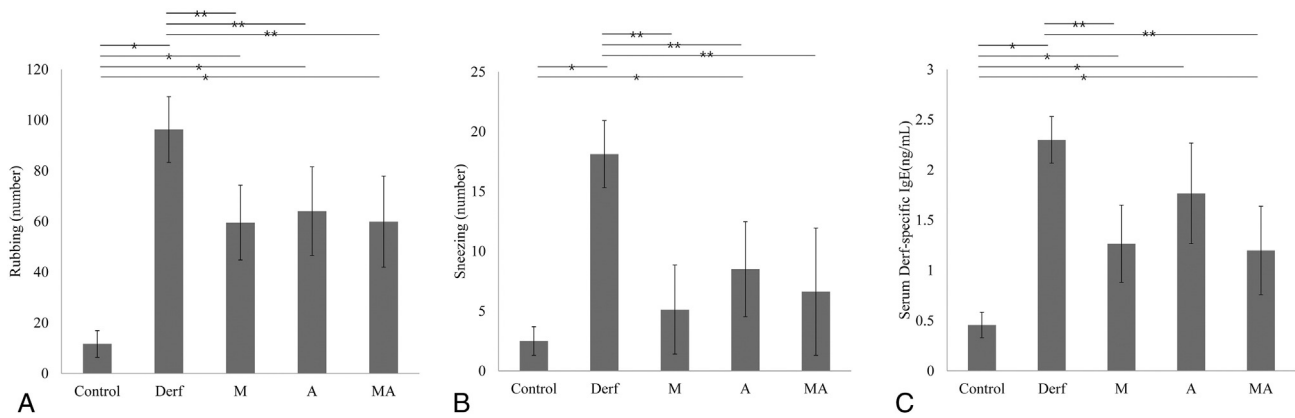


Fig. 2. Nasal symptom scores. Rubbing (A) and sneezing (B). Serum Derf-specific immunoglobulin E levels (C). Error bars: standard deviations. * $P < 0.05$ vs. the control group, ** $P < 0.05$ vs. the Derf group, *** $P < 0.05$ the A vs. the MA group,

If statistical significance was apparent, ranked parameters were compared by one-way analysis of variance followed by Bonferroni's multiple comparison. A P value < 0.05 was considered to reflect statistical significance except when Bonferroni's multiple comparison test was performed, when $P < 0.01$ was considered to reflect significance. All analyses were conducted with the aid of SAS version 9.3 software (SAS Institute, Cary, NC, USA).

3. Results

3.1. Allergic symptoms

The allergic symptoms evaluated were the number of nasal-rubbing motions and sneezes during a 15-min period after final challenge. Rubbing motions numbered 11.63 ± 5.26 in the control group, 96.25 ± 12.98 in the Derf group, 59.50 ± 14.76 in the M group, 64.00 ± 17.51 in the A group, and 59.88 ± 18.01 in the MA group. The numbers of sneezes were 2.50 ± 1.20 in the control group, 18.13 ± 2.80 in the Derf group, 5.13 ± 3.72 in the M group, 8.50 ± 3.96 in the A group, and 6.63 ± 5.32 in the MA group. The number of nose-rubbing motions was significantly lower in the control than in all other groups (all P values < 0.001 ; Fig. 2A) and was significantly higher in the Derf group than in the M, A, and MA groups (vs. M and A, $P = 0.001$; vs. MA, $P < 0.001$). The number of sneezes was significantly lower in the control group than in the Derf, A, MA groups (vs. Derf and A, $P < 0.001$; vs. MA,

$P = 0.028$; Fig. 2B) and significantly higher in the Derf group than in the M, A, and MA groups (vs. M, $P < 0.001$; vs. A and MA, $P = 0.001$).

3.2. Serum Derf-specific IgE levels

The serum Derf-specific IgE level was significantly higher in the Derf group (2.29 ± 0.23 ng/mL) than in the control (0.45 ± 0.13 ng/mL, $P < 0.001$), the M (1.20 ± 0.44 ng/mL, $P < 0.001$) and MA groups (1.19 ± 0.44 ng/mL, $P < 0.001$). The control group had a significantly lower serum Derf-specific IgE level than all other groups (all $P < 0.001$). Also, the MA group showed lower IgE level than A group (1.76 ± 0.50 ng/mL, $P = 0.038$; Fig. 2C).

3.3. Real-time PCR and Western blot analyses

The nasal mucosal levels of mRNAs encoding T-bet, IFN- γ , GATA-3, IL-4, ROR- γ t, IL-17, and Foxp3 were evaluated by real-time PCR. In terms of the Th1 response, the T-bet mRNA level was significantly higher in the control group than in the Derf, A and MA group (vs. Derf, $P < 0.001$; vs. A, $P = 0.038$; vs. MA, $P = 0.01$). The Derf T-bet mRNA was significant lower than the MA group ($P < 0.038$; Fig. 3A). In addition, the level of IFN- γ mRNA was significantly higher in the control group than in the Derf, M and group (vs. Derf, $P < 0.001$; vs. M, $P = 0.01$; vs. A, $P = 0.01$). Also, the Derf IFN- γ mRNA was significant lower than the M and A group (vs. M, $P = 0.028$; vs. A, $P = 0.01$; Fig. 3B). In

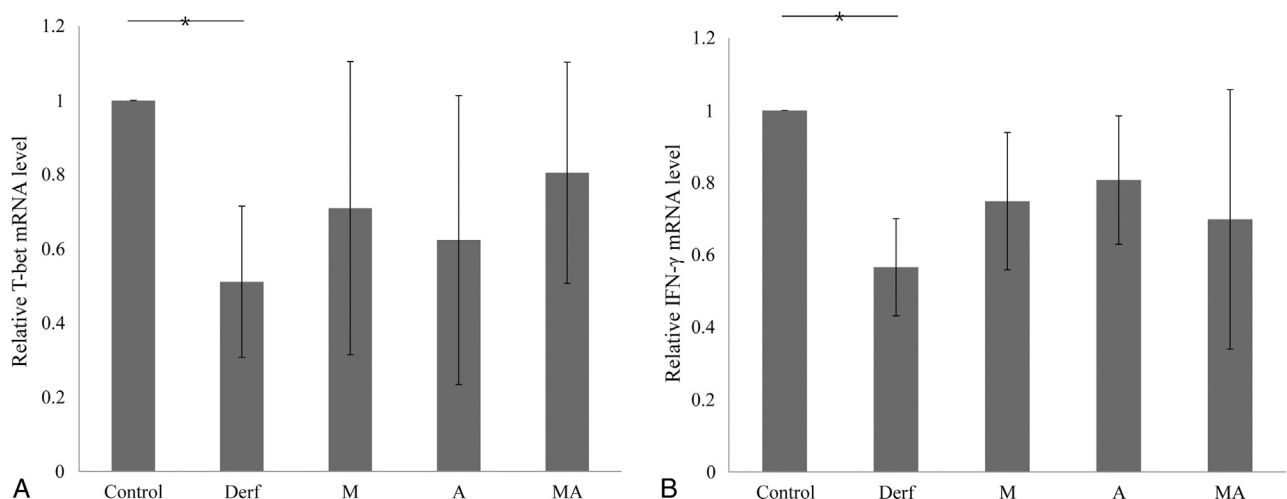


Fig. 3. Quantitative analysis via real-time PCR of cytokine levels in the nasal mucosa of each study group in terms of the Th1 response: (A) T-bet and (B) IFN- γ . The results are normalized to the GAPDH expression levels. Error bars: standard deviations. * $P < 0.05$ vs. the control group, ** $P < 0.05$ vs. the Derf group.

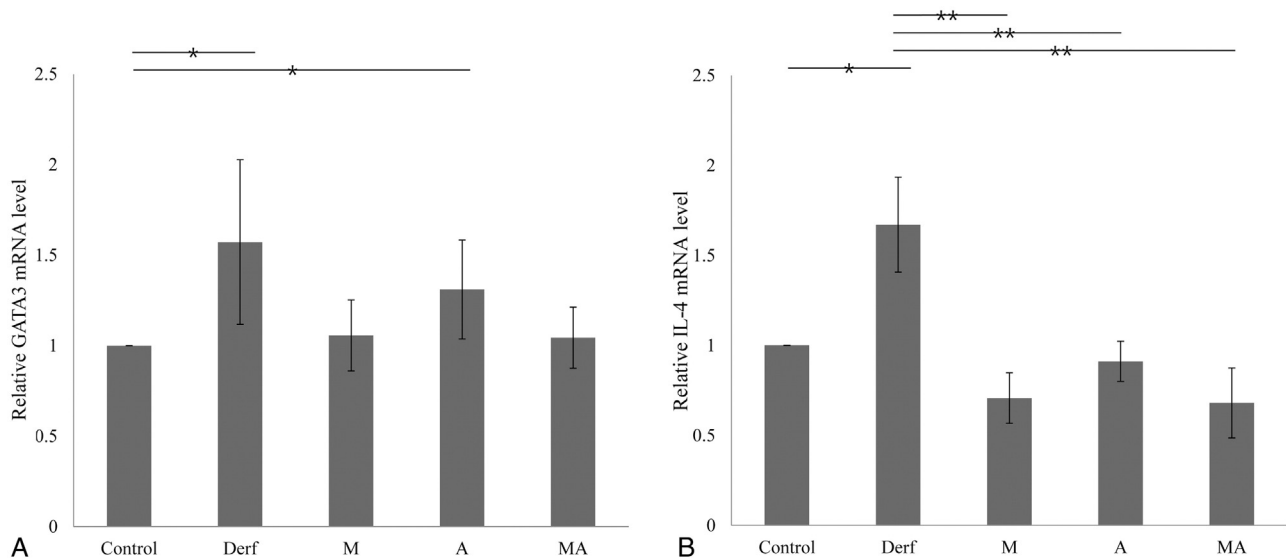


Fig. 4. Quantitative analysis via real-time PCR of cytokine levels in the nasal mucosa of each study group in terms of the Th2 response: (A) GATA-3 and (B) IL-4. The results are normalized to the GAPDH expression levels. Error bars: standard deviations. * $P < 0.05$ vs. the control group, ** $P < 0.05$ vs. the Derf group, *** $P < 0.05$ the M vs. the A group, **** $P < 0.05$ the A vs. the MA group.

terms of the Th2 response, the GATA-3 mRNA level was significantly higher in the Derf, M and A groups than in the control group (vs. Derf, $P < 0.001$; vs. M, $P = 0.01$; vs. A, $P < 0.001$). The M and MA group showed significantly lower the GATA-3 mRNA than Derf group (vs. M, $P = 0.028$; vs. MA, $P < 0.015$). Also, the M and MA group showed lower GATA-3 than A group (vs. M, $P = 0.028$; vs. MA, $P < 0.038$; Fig. 4A). The level of IL-4 mRNA was significantly higher in the Derf group than in all other groups (all $P < 0.001$) and significantly lower in the M and MA group than in the A group (vs. M, $P = 0.007$; vs. MA, $P = 0.028$; Fig. 4A). The M, A, MA group showed lower IL-4 than control group (all $P = 0.01$). In terms of the Th17 response, the ROR- γ t mRNA level was significantly higher in the Derf group than in all other groups (all P values < 0.001). In addition, the level in the control group was significantly higher than that in the M, A and MA group (all $P = 0.01$). The A group showed significantly lower ROR- γ t mRNA level than M group ($P = 0.007$). Moreover, the MA group showed significantly lower ROR- γ t than M and A group (vs. M, $P = 0.001$; vs. MA, $P = 0.028$; Fig.

5A). The IL-17 mRNA level was significantly higher in the Derf group than in M and A group (vs. M, $P = 0.038$; vs. MA, $P = 0.007$), and also significantly higher in the M, A and MA group than in the control group (vs. M, A, $P < 0.001$; vs. MA, $P = 0.01$; Fig. 5B). The Foxp3 mRNA level was significantly higher in the MA group than in the Derf, control and A group (vs. Derf, $P = 0.007$; vs. control, $P = 0.01$; vs. A, $P = 0.021$) and significantly lower in the Derf group than in the control and M group (vs. control, $P = 0.01$; vs. M, $P = 0.038$; Fig. 6).

Western blotting was performed to confirm the expression patterns of T-bet, GATA3, ROR- γ t, and Foxp3. The results were not quantitative; we present general grayscale patterns for each factor (Fig. 7).

4. Discussion

Intranasal corticosteroids are generally considered the most effective drugs in terms of AR treatment. However, time is required to attain peak efficacy [2]. Thus, patients are often dissatisfied and noncompliant;

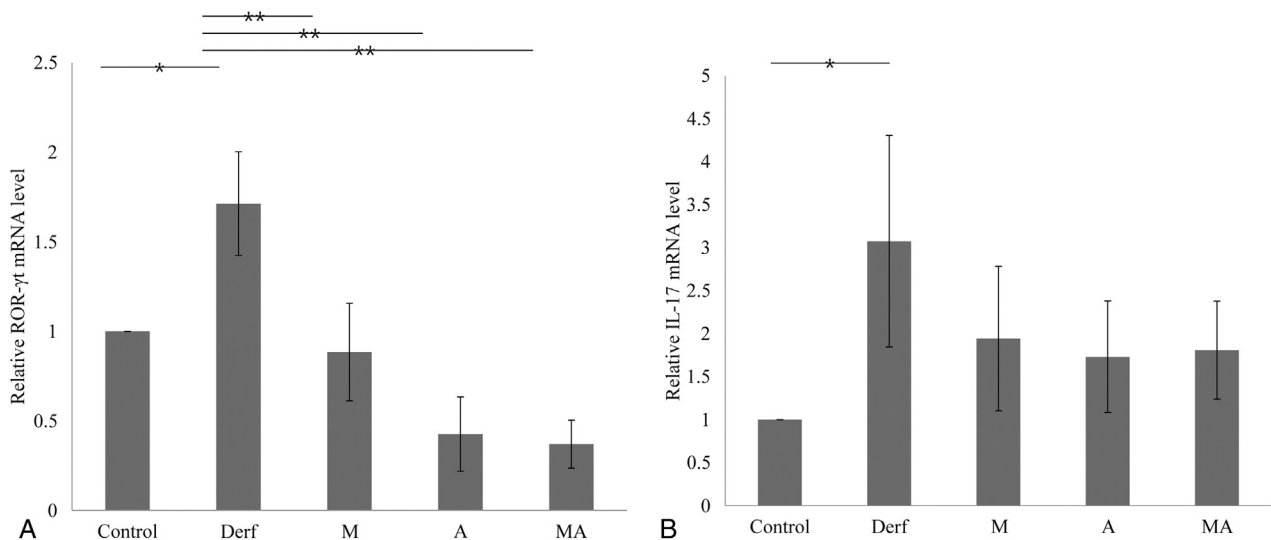


Fig. 5. Quantitative analysis via real-time PCR of cytokine levels in the nasal mucosa of each study group in terms of the Th17 response: (A) ROR- γ t and (B) IL-17. The results are normalized to the GAPDH expression levels. Error bars: standard deviations. * $P < 0.05$ vs. the control group, ** $P < 0.05$ vs. the Derf group, *** $P < 0.05$ the M vs. the A group, **** $P < 0.05$ the A vs. the MA group.

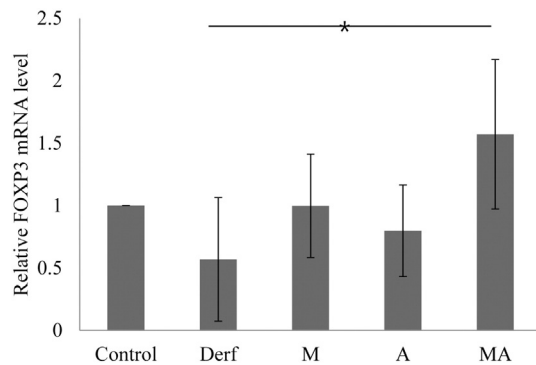


Fig. 6. Quantitative analysis of cytokine levels derived by real-time PCR from the nasal mucosa of each study group in terms of the regulatory T response: Foxp3. The results are normalized to the GAPDH expression levels. Error bars: standard deviations. * $P < 0.05$ vs. the control group, ** $P < 0.05$ vs. the Derf group, *** $P < 0.05$ vs. the A vs. the MA group.

symptoms persist during treatment [28,29]. Therefore, about 70% of all physicians prescribe at least two AR medications in efforts to alleviate symptoms [15]. When it is considered that >500 million subjects have AR, the economic burdens, including the direct costs of physician visits and those of the various medications, and the indirect costs of reductions in productivity, are high [23]. Clinical trials have shown that combinations of intranasal steroids and antihistamines exhibit rapid onsets of action, being more effective than either intranasal steroid or antihistamine monotherapies alone, and are generally well tolerated.

Azelastine is a second-generation H1 receptor antagonist, the biological activities of which include both anti-allergic and anti-inflammatory properties. Topical azelastine inhibits the activation of both mast cells and basophils in vitro [30]. Ciprandi et al. [31] showed that topical azelastine reduced allergen-induced epithelial expression of the intercellular adhesion molecule-1 (ICAM-1) during both the early and the late phases of AR, and reduced late-phase eosinophil and neutrophil recruitment. Combinations of mometasone with newer topical corticosteroids exhibited low systemic bioavailability (0.46%) when administered intranasally. In addition, mometasone did not significantly affect any of the HPA axis, bone metabolism, or basic hematological parameters [32].

We found that MA afforded superior anti-allergic effects in terms of the Th2 cytokine milieu (including GATA3 and IL-4) compared with A. In the Th17 context, when the MA and M groups were compared, the ROR- γ t mRNA level was significantly lower in the former group. The IL-17 level was also somewhat lower in the MA group, but statistical significance was not attained. The MA group exhibited somewhat increased expression of Foxp3-encoding mRNA, showing that CD4⁺CD25⁺Foxp3⁺Treg cells were present in higher numbers than in the M and A groups, but the difference was significant only for the A group. Statistical power may be lacking because of the small sample sizes of all groups ($n = 8$). It has been reported that intranasal

azelastine suppressed IL-17 synthesis by inhibiting ICAM-1 expression [33]. In addition, azelastine has been shown to enhance corticosteroid efficacy by inducing the expression of mitogen-activated protein kinase phosphatase-1 (MKP-1), subsequently suppressing ICAM-1 expression in vitro [33]. We found that this was also relevant in vivo. Naïve T cells may differentiate into either Th17 cells or Treg cells, depending on the immunological environment [34,35]. All of excess Th17 functionality, a defect in Treg function, and reduced numbers of Tregs trigger the development and progression of inflammatory diseases including allergic asthma and AR [36–39]. In such contexts, the combination of azelastine and a corticosteroid may enhance IL-17 suppression and (reciprocally) elevate Treg cell numbers. Therefore, such a combination may afford substantial clinical synergy.

5. Conclusions

We found that the combination of intranasal azelastine and mometasone synergistically suppressed the Th17 response and (reciprocally) elevated the Treg response. Therefore, this combination not only ameliorated allergic inflammation by suppressing the Th2 response, but also enhanced the Treg/Th17 balance.

Financial disclosure

Funded by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science, and Technology (2011-0026915). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflict of interest

None.

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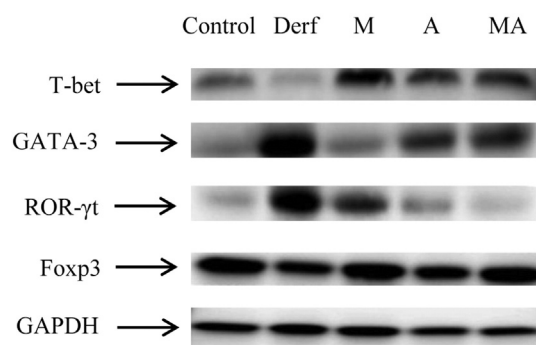


Fig. 7. The cytokines and transcription factors, the levels of which were measured by Western blotting to confirm protein expression.

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