Effect of budesonide transnasal nebulization in patients with eosinophilic chronic rhinosinusitis with nasal polyps

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Background: There is little evidence on the efficacy of glucocorticoid transnasal nebulization therapy in patients with eosinophilic chronic rhinosinusitis with nasal polyps (CRSwNP).

Objective: We sought to evaluate the immunologic and remodeling effects of budesonide transnasal nebulization in patients with eosinophilic CRSwNP.

Methods: Sixty patients with eosinophilic CRSwNP were randomized to receive budesonide or placebo treatment for 14 days by means of transnasal nebulization in a double-blind manner. Endoscopic polyp size scores (maximum = 6 points, Kennedy score) and visual analog scale scores for nasal symptoms were assessed before and after treatment. Similarly, polyp samples were evaluated for inflammatory cytokines, matrix metalloproteinases (MMPs), and tissue inhibitors of metalloproteinases (TIMPs) by using an immunoassay; collagen by using histochemistry; eosinophils by using hematoxylin and eosin stain; and T-cell subsets by using flow cytometry.

Results: Budesonide transnasal nebulization significantly reduced polyp size compared with placebo (mean difference between groups, −0.73 units; 95% CI, −1.15 to −0.32 units; $P = .002$) and improved symptoms. Polyp IL-5 and eotaxin expression decreased significantly, whereas TGF-β and IL-10 expression increased. Expression of IFN-γ and IL-17 was not altered. Budesonide transnasal nebulization consistently reduced eosinophil infiltration and T_H2 cell frequency and increased natural regulatory T-cell and type 1 regulatory T-cell frequencies. Indices of remodeling, including albumin, MMP-2, MMP-7, MMP-8, and MMP-9, were significantly decreased, whereas collagen deposition and TIMP-1, TIMP-2, and TIMP-4 levels were significantly increased. Budesonide transnasal nebulization did not suppress the hypothalamic-pituitary-adrenal axis or cause any serious side effects.

Conclusion: Short-term budesonide transnasal nebulization is an effective and safe treatment option in patients with eosinophilic CRSwNP, achieving clinical improvement by regulating remodeling, cytokine expression, and T-cell subset distribution. (J Allergy Clin Immunol 2015;135:922-9.)

Key words: *Eosinophilic chronic rhinosinusitis, nasal polyps, tissue remodeling, inflammatory cytokine, T-cell subsets, budesonide, transnasal nebulization*

Chronic rhinosinusitis with nasal polyps (CRSwNP) is a major health care problem that affects from 0.5% to 4% of the general population.[1] CRSwNP leads to high management costs and poor quality of life of affected subjects and shows a wide diversity of inflammatory phenotypes. Studies have demonstrated that eosinophil infiltration and T_H2-biased cytokine profiles are key features of white patients with CRSwNP,[2,3] whereas nasal polyps (NPs) from southern Chinese patients have been shown to be characterized by neutrophilic inflammation and a significant increase in T_H1/T_H17 cell pattern.[4]

Given the inflammatory nature of CRSwNP, corticosteroids are typically regarded as the mainstay of medical treatment for this condition.[1] Although topical intranasal steroid sprays have proved to be an effective treatment for chronic rhinosinusitis (CRS) and maintain a minimal risk profile,[5] long-term administration of nasal spray is necessary to obtain a persistent therapeutic effect. Furthermore, despite providing more potent and rapid benefits in controlling inflammation associated with CRS,[6,7] systemic corticosteroids have significant side effects, such as hypothalamic-pituitary-adrenal axis dysfunction. Thus a need has arisen for an alternative topical therapy in patients with CRS.

Budesonide inhalation suspension (Pulmicort Respules; AstraZeneca, London, United Kingdom) is another form of corticosteroid therapy, which has been demonstrated to be efficacious and safe in patients with asthma[8] or chronic obstructive pulmonary disease.[9] However, there is little information on the use of budesonide transnasal nebulization in the treatment of inflammatory nasal disease.

Thus the aim of this study was first to evaluate the clinical efficacy and safety of a short-term course of budesonide inhalation suspension delivered by means of transnasal nebulization in patients with CRSwNP. In addition, the study aimed to investigate

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Abbreviations used
CRS: Chronic rhinosinusitis
CRSwNP: Chronic rhinosinusitis with nasal polyps
Foxp3: Forkhead box protein 3
H&E: Hematoxylin and eosin
MMP: Matrix metalloproteinase
NP: Nasal polyp
nTreg: Natural regulatory T
TIMP: Tissue inhibitor of metalloproteinase
TNSS: Total nasal symptom score
T_R1: Type 1 regulatory T
Treg: Regulatory T

the effect of treatment on several markers of inflammation and nasal tissue remodeling (namely differences in the expression of collagen, albumin, TGF-β, matrix metalloproteinases [MMPs], and tissue inhibitors of metalloproteinases [TIMPs]) in these patients.

METHODS

Patients and study design

Sixty patients with eosinophilic NPs were recruited into this prospective, randomized, double-blind, placebo-controlled study (clinical trial registration details available from http://clinicaltrial.gov/show/NCT02024659) from Beijing TongRen Hospital, Beijing, China, from September 2010 to December 2011. At the screening visit, participants were preselected for eosinophilic NPs based on at least 1 of the following parameters: skin prick test response positivity, increased eosinophil counts in peripheral blood, allergic rhinitis, asthma, or aspirin intolerance. The diagnosis of CRSwNP was based on the standard criteria issued in the European Position Paper on Rhinosinusitis and Nasal Polyps guidelines.[10] Polyp biopsy was undertaken 1 week before treatment. Referring to the method of Cao et al,[11] CRSwNP was defined as eosinophilic when the number of eosinophils exceeded 10% of total infiltrating inflammatory cells in the polyp tissue, as evaluated by using hematoxylin and eosin (H&E) staining.

Eligible patients were randomly assigned 1:1 to receive 1 mg of budesonide or placebo (saline solution) transnasal nebulization twice daily for 14 days with a Pari Sinus Nebulizer and Pari Master Compressor (PARI GmbH, Starnberg, Germany). The study medication was delivered in a double-blind manner, with neither the patient nor the investigator knowing the identity of the medication. Nasal symptoms (including nasal obstruction, nasal discharge, loss of smell, and headache/facial pain) were assessed before initiation (1 week after biopsy) and at the end of the 14-day treatment period by using visual analog scales. The total nasal symptom score (TNSS) was calculated as the sum of scores for 4 individual symptoms at the same time. Similarly, the size of NPs was measured by means of endoscopic examination at baseline and the end of the study with the Kennedy score (full details are available in this article's Online Repository at www.jacionline.org). Polyp size was scored from 0 to 3 for each side, and the bilateral polyp grade was obtained as the sum of the individual units for the left and right nasal cavities (maximum score = 6). The physician's assessment of reduction in NP size and the patient's assessment of improvements in symptoms were used as clinical end points.

Biopsy specimens of NPs were obtained again after the 14-day treatment period for assessment of the effect of treatment on several markers of inflammation and nasal tissue remodeling (namely expression of collagen, albumin, TGF-β, MMPs, and TIMPs) as secondary outcome measures. All patients were also assessed before and after treatment for morning serum cortisol levels, and adverse events were reported to the investigator, who assessed the relationship of the event to the study treatment.

Full technical details of the methods used in this randomized, double-blind study design and efficacy end points are provided in the Methods section in this article's Online Repository.

The study was approved by the medical ethics committee of Beijing TongRen Hospital, and written informed consent was obtained from each patient before participation in the study.

Histologic examination

Human NP tissue was assessed by means of H&E staining to determine eosinophil infiltration. Samples from patients in both groups were evaluated in a blind fashion with respect to the pretreatment and posttreatment sequences. Full technical details are provided in the Methods section in this article's Online Repository.

Immunoassay

Levels of inflammatory cytokines (including IL-5, IFN-γ, IL-17, IL-10, TGF-β, and eotaxin), MMPs (MMP-2, MMP-7, MMP-8, and MMP-9), TIMPs (TIMP-1, TIMP-2, TIMP-3, and TIMP-4), and albumin in tissue homogenates were evaluated by means of immunoassay. Full technical details are provided in the Methods section in this article's Online Repository.

Picrosirius red staining for collagen

The collagen content in the extracellular matrix was assessed by means of picrosirius red staining. Full technical details are provided in the Methods section in this article's Online Repository.

Flow cytometric analysis

T_H1, T_H2, T_H17, type 1 regulatory T (T_R1) cells (the most important subset of inducible regulatory T [Treg] cells, which secrete IL-10 and TGF-β), and natural regulatory T (nTreg) cells, which are thymus derived and characterized by their $CD4^+CD25^+$ forkhead box protein 3 (Foxp3)–positive phenotype, in polyp homogenates were evaluated by means of cytometric analysis. Full technical details are provided in the Methods section in this article's Online Repository. Representative gating figures are also shown in Fig E1 in this article's Online Repository at www.jacionline.org.

Safety assessment

Safety was assessed primarily based on changes in plasma cortisol levels by the end of treatment. Venous blood samples were obtained at 8 AM on the first and last days of treatment and analyzed for plasma cortisol by means of RIA. The secondary safety variable was the incidence and severity of adverse events.

Statistical analysis

The sample size was estimated based on power statistics by using reduction in NP size as the primary outcome measure. On the basis of our preliminary findings of a pilot study, we estimated that by using a parallel-group design, 18 subjects per group would be required to detect a 0.8-unit difference in polyp size reduction between randomized treatments at 2 weeks (combined SD, 0.7) with a power equal to 90% and a 2-tailed α value of .05. Considering a loss of 10% of patients at follow-up for the clinical portion of the study and a dropout rate of 50% for biopsies, we recruited 30 participants in each study group. Statistical analysis was performed with SPSS version 19.0 software (IBM, Armonk, NY). Data were expressed as means and SEMs, unless otherwise specified. Continuous variable differences were analyzed by using the Student *t* test or Mann-Whitney *U* test for unpaired comparisons. A *P* value of .05 or less was considered statistically significant.

RESULTS

Patients' clinical characteristics

Overall, 93 patients were screened for eligibility, of whom 60 met the inclusion criteria and were randomized in equal numbers to receive budesonide or placebo nebulization therapy

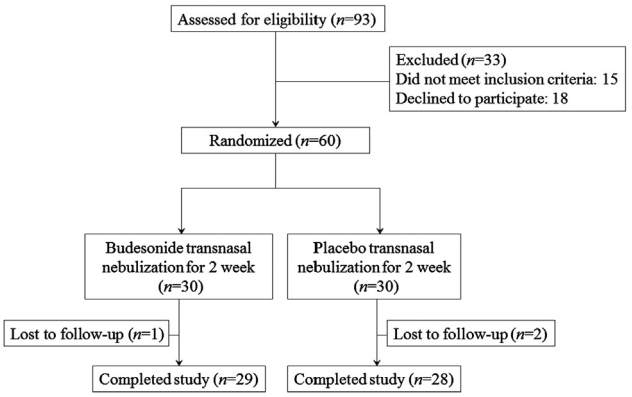

FIG 1. Study patients' flow diagram.

for 2 weeks. One patient in the budesonide group and 2 patients in the placebo group dropped out because of nonadherence (Fig 1). The 2 groups were well matched in demographic characteristics, symptom severity (TNSS), polyp size, and concurrent illness at baseline. Relevant data are presented in Table E1 in this article's Online Repository at www.jacionline.org.

Clinical responses as primary outcomes

By the end of the 14-day treatment period, assessment of the NP size demonstrated that it was significantly decreased in budesonide-treated patients compared with that seen in placebo-treated patients (mean difference between groups, −0.73 units; 95% CI, −1.15 to −0.32 units; $P = .002$; Fig 2, *A*). Patients receiving budesonide transnasal nebulization also showed significant improvements in the 4 major symptoms compared with patients in the placebo-treated group ($P < .001$; Fig 2, *B-E*). Similarly, assessment of TNSSs also indicated that scores were significantly improved at the end of treatment in the budesonide-treated patients compared with the placebo-treated patients ($P < .001$; Fig 2, *F*).

Inflammatory cytokines, mediators, and cellular infiltration as secondary outcomes

Data were not available for all subjects because of the relatively small sizes of individual samples obtained. Thus all polyp tissues were evaluated by means of H&E staining, and then samples from patients in each treatment group were randomly divided into approximately 3 equal groups. The 3 groups of samples were then assessed for inflammatory cytokines, remodeling markers, or T-cell subsets. For the inflammatory cytokines measured by using the Cytometric Bead Array (BD Biosciences, San Jose, Calif), which uses particles with discrete fluorescence intensities to measure the concentration of specific analytes, there were 10 samples each in the active and placebo groups, and for TGF-β levels measured by means of ELISA, there were 14 samples in the active group and 12 samples in the placebo group. Moreover, the concentration of IL-17 in samples from 4 subjects (2 each from the budesonide- and placebo-treated groups) was less than the limit of detection, and thus the value of the lower limit of detection was assigned to these samples. Budesonide transnasal nebulization significantly reduced eotaxin (Fig 3, *A*) and IL-5 (Fig 3, *B*) levels and significantly increased IL-10 (Fig 3, *C*) and TGF-β (Fig 3, *D*) levels in NPs compared with control values. In contrast, IL-17 (Fig 3, *E*) and IFN-γ (Fig 3, *F*) levels were not significantly altered by budesonide transnasal nebulization. Similarly, placebo treatment did not alter the level of any of these cytokines compared with baseline values.

In line with the observed cytokine patterns, eosinophil numbers in NPs were significantly reduced after nebulized budesonide treatment (Fig 4, *A*, and see Fig E2 in this article's Online Repository at www.jacionline.org) but not altered after placebo treatment. Similarly, the frequency of T_H2 cells in NPs was significantly decreased by budesonide treatment (Fig 4, *B*), whereas the frequencies of T_H1 (Fig 4, *C*) and T_H17 (Fig 4, *D*) cells were not altered. Interestingly, budesonide transnasal nebulization also significantly increased T_R1 (Fig 4, *E*) and nTreg (Fig 4, *F*) cell numbers. Placebo treatment did not significantly alter the frequency of either of these cell types. Moreover, there was a significant correlation between the change in TGF-β levels and the change in nTreg cell numbers from baseline in the nebulized budesonide–treated group ($r^2 = 0.56$, $P = .02$, see Fig E3 in this article's Online Repository at www.jacionline.org).

Tissue remodeling as a secondary outcome

Assessment of collagen deposition in the extracellular matrix of NP tissues demonstrated that the total collagen amount increased after a 14-day treatment with budesonide transnasal nebulization (Fig 5, *B*, and Fig 6, *A*) but was not altered by placebo treatment. Whereas collagen type 1 was the most abundant collagen type both before and after treatment, collagen type 3 (thinner fibers indicated by green luminescence) was almost absent at baseline (Fig 5, *C*) and upregulated as new fibers were produced after budesonide transnasal nebulization (Fig 5, *D*). In contrast, albumin levels in NPs were significantly reduced by budesonide transnasal nebulization but were unaltered after placebo treatment (Fig 6, *B*). Similarly, expression of MMP-2 (Fig 6, *C*), MMP-7 (Fig 6, *D*), MMP-8 (Fig 6, *E*), and MMP-9 (Fig 6, *F*) was decreased after budesonide treatment compared with that after placebo treatment. Assessment of TIMPs also demonstrated that TIMP-1 (Fig 6, *G*), TIMP-2 (Fig 6, *H*), and TIMP-4 (Fig 6, *I*) concentrations were significantly increased in the budesonide-treated group and not affected by placebo treatment. In contrast, the expression of TIMP-3 in the 2 treatment groups showed no difference (data not shown).

Safety

Plasma cortisol levels varied in the normal range in all participants at baseline. Morning plasma cortisol levels showed no significant difference between the initial treatment and the end of treatment in both groups (18.07 ± 1.98 vs 17.41 ± 2.20 μg/dL in the budesonide transnasal nebulization group and 17.33 ± 1.83 vs 17.02 ± 1.80 μg/dL in the placebo group, respectively). No symptom of adrenal suppression was noted in any of the participants, and no serious adverse events were reported during the study, apart from nasal dryness in 5 patients in the budesonide transnasal nebulization group.

DISCUSSION

Anti-inflammatory agents, such as corticosteroids, are often recommended as the drugs of choice by current CRS guidelines

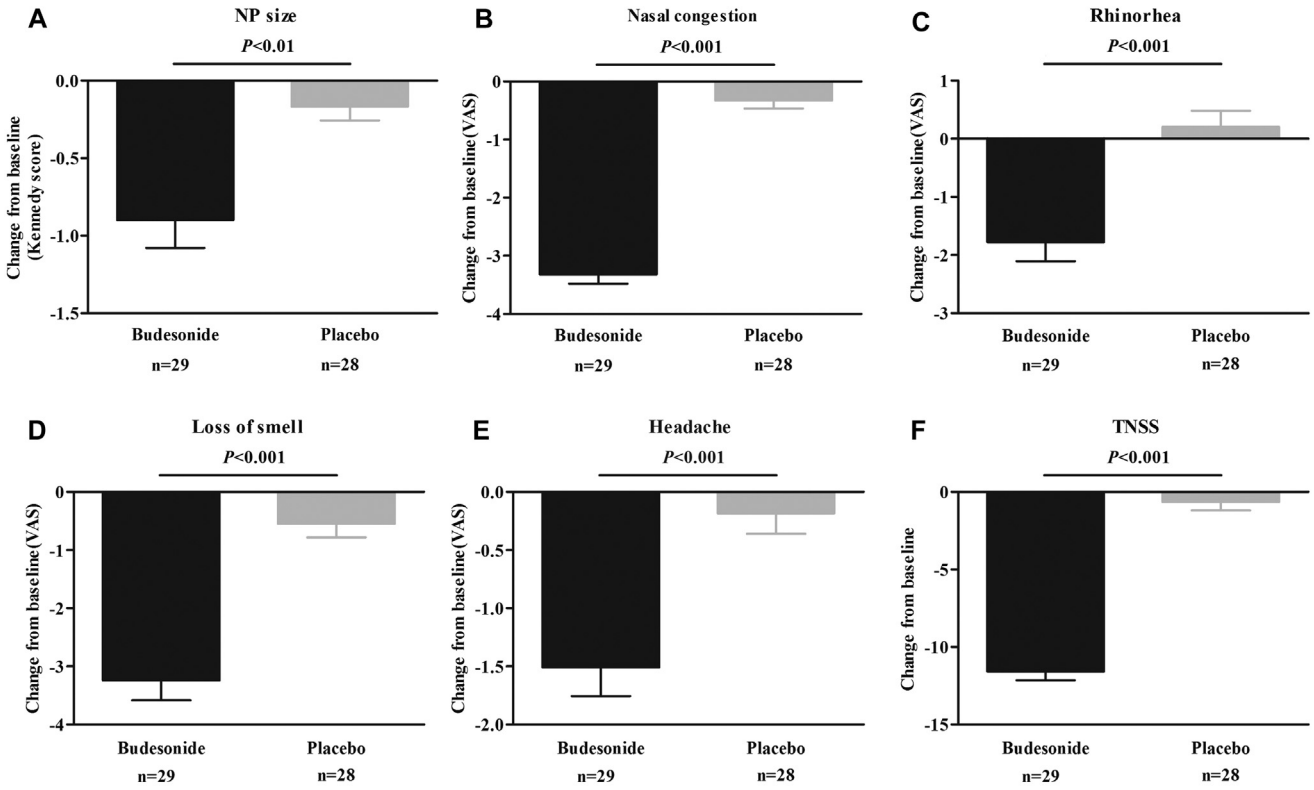

FIG 2. Clinical improvements in the budesonide transnasal nebulization–treated group compared with the placebo transnasal nebulization–treated group. Reductions in NP size and symptom improvements were used as primary end points. **A**, NP size. **B**, Nasal congestion. **C**, Rhinorhea. **D**, Loss of smell. **E**, Headache. **F**, TNSS. *VAS*, Visual analog scale.

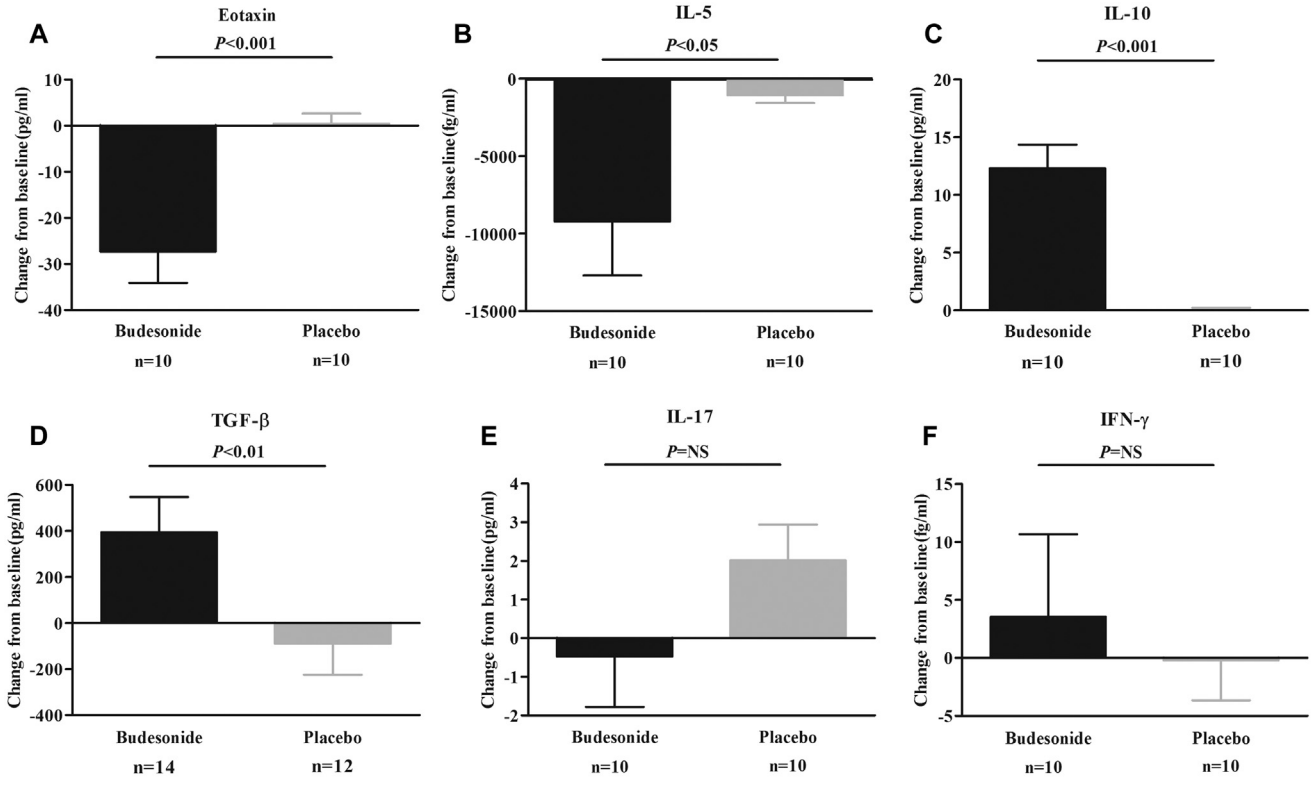

FIG 3. Inflammatory cytokines and mediators in tissue homogenates were determined by using the cytometric bead array (eotaxin, IL-5, IL-10, IL-17, and IFN-γ) and ELISA (TGF-β). **A**, Eotaxin. **B**, IL-5. **C**, IL-10. **D**, TGF-β. **E**, IL-17. **F**, IFN-γ. *NS*, Not significant.

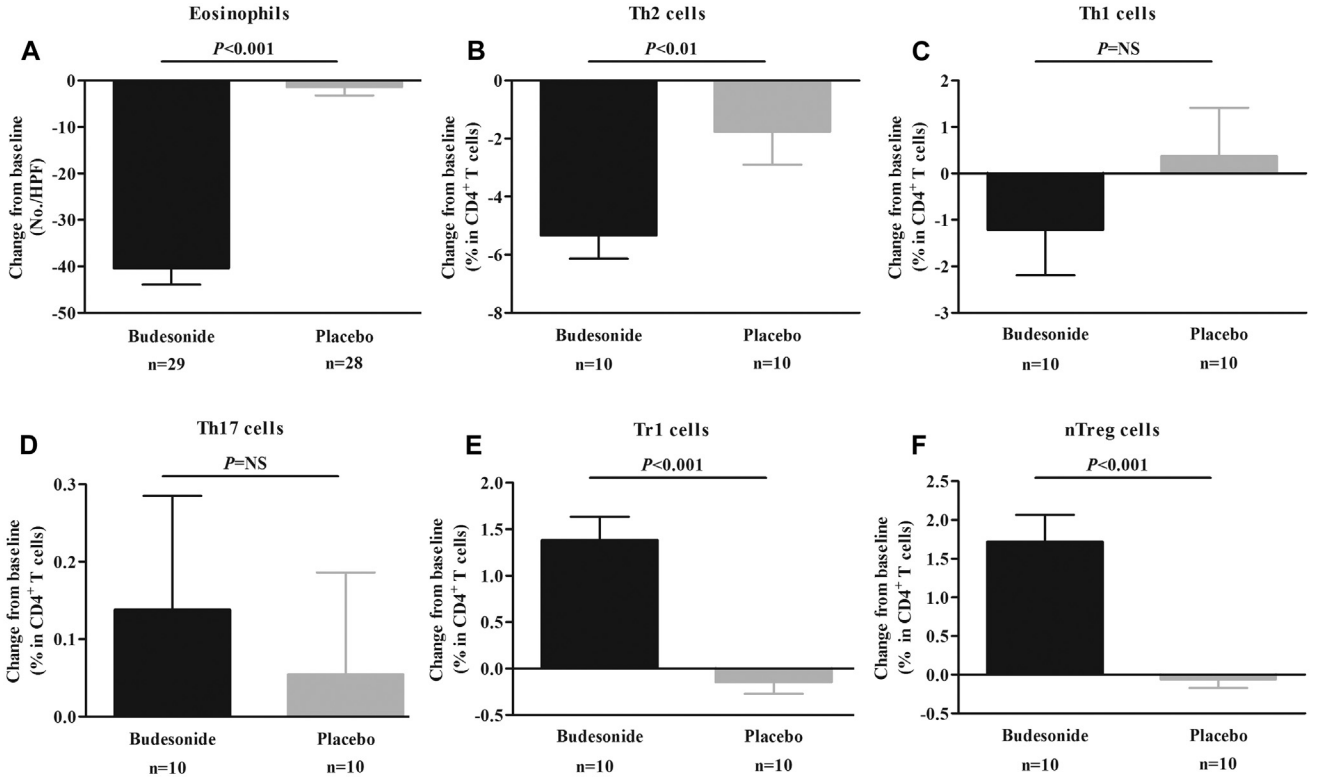

FIG 4. Inflammatory cells in NP tissue were analyzed before and after treatment by using H&E staining (eosinophils) and flow cytometric analysis (T-cell subsets). **A**, Eosinophils. **B**, T_H2 cells. **C**, T_H1 cells. **D**, T_H17 cells. **E**, T_R1 cells. **F**, nTreg cells. *HPF*, High-power field; *NS*, not significant.

because of the inflammatory nature of the disease[1] and are widely used as topical nasal medications administered as nasal drops[12] or delivered as aqueous sprays[5] for sinonasal inflammatory diseases. More recently, a new option for topical corticosteroid treatment in patients with CRS has been the off-label use of budesonide inhalation suspension in nasal lavage or irrigation.[13-16] We have investigated the efficacy and safety, as well as the putative immunologic and tissue-remodeling mechanisms, underlying the effects of short-term budesonide transnasal nebulization treatment in patients with eosinophilic CRSwNP. Overall, our study indicated that budesonide transnasal nebulization led to significant improvements in all the major nasal symptoms and reduced the size of NPs (primary outcome measures) in patients with CRSwNP compared with placebo treatment. Moreover, there were significant improvements in a variety of markers of inflammation and tissue remodeling (secondary outcome measures) in patients treated with budesonide transnasal nebulization compared with placebo-treated patients.

The efficacy and safety of the budesonide inhalation suspension as a means to perform nasal irrigation[13] or lavage[14] in patients with CRS without NPs or CRSwNP has been investigated previously. A pilot study has demonstrated that the addition of budesonide inhalation suspension (0.5 mg twice a day) to nasal saline irrigation produced significant improvements in subjective sinus symptoms and objective evaluation.[13] Other studies have demonstrated that budesonide inhalation suspension neither suppressed the hypothalamic-pituitary-adrenal axis[14,15] nor decreased serum and 24-hour urinary cortisol levels,[16] suggesting that irrigation with budesonide was safe to perform in patients with CRS as an alternative to traditional aerosolized steroid sprays or systemic corticosteroids. Our findings for the clinical efficacy and safety of budesonide transnasal nebulization in patients with CRSwNP are in accordance with the findings of these studies. However, because the dose of budesonide used in the study was twice as high as what has been advocated for maintenance treatment,[17] the possibility of systemic steroid-related side effects if used longer than 14 days cannot be ruled out. Long-term dose-dependent studies with nebulized budesonide will be needed to fully appreciate the benefits of this treatment modality for chronic management of inflammatory nasal disease.

Nevertheless, using a similar NP grading score as used in the present study, budesonide nasal spray[18] has been shown to produce similar reductions in polyp size after 4 weeks of treatment, whereas mometasone furoate nasal spray[5] achieved similar improvements after 2 months of treatment. Collectively, these results suggest that application of budesonide transnasal nebulization might confer a faster onset of action, although this would need to be confirmed in further studies comparing doses similar to those used in nasal sprays.

In the present study we have also explored the immunologic mechanisms underlying the beneficial clinical effects of budesonide transnasal nebulization in patients with eosinophilic CRSwNP. The attenuated production of eotaxin in our study provided evidence of less eosinophil accumulation in NPs. Furthermore, the anti-inflammatory potential of budesonide inhalation suspension in this study was strengthened by the observation that there was a significant reduction in T_H2 cell numbers in the local tissue. This finding is in accordance with the findings of Van Zele et al,[19] which demonstrated that oral

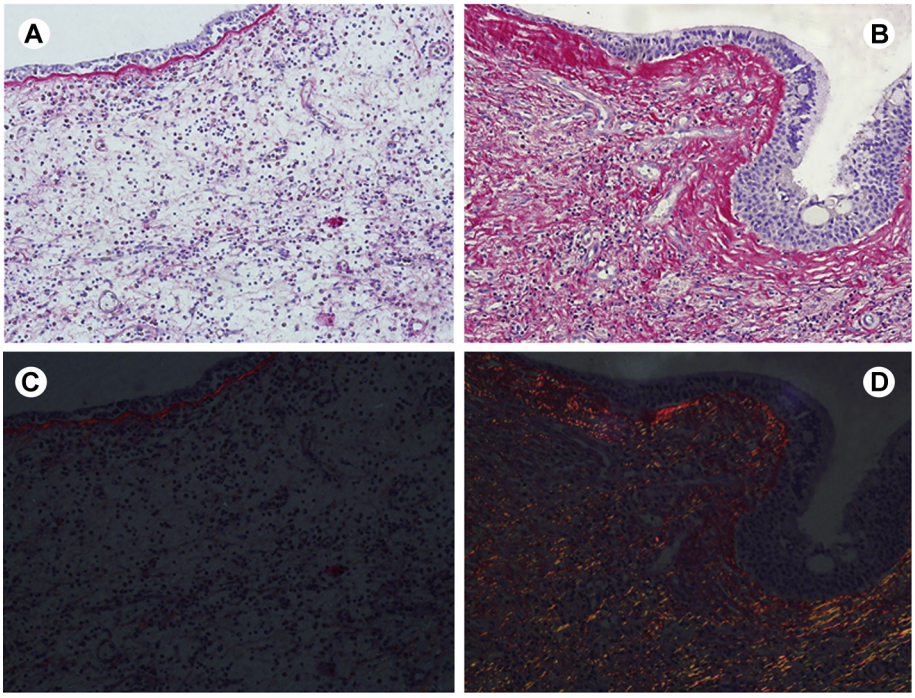

FIG 5. Picrosirius red–stained sections of polyp tissues from the same patient before and after budesonide transnasal nebulization were assessed for total collagen by using bright-field microscopy (**A** and **B**) and for collagen fibers by using polarized light microscopy (**C** and **D**) at ×200 magnification.

methylprednisolone administrated to patients with NPs significantly reduced eosinophil cationic protein, IL-5, and IgE levels in nasal secretions in these patients. However, our study has demonstrated that nebulized budesonide therapy was not equally effective in inhibiting T_H1/T_H17-biased inflammation as T_H2-polarized inflammation. Our findings are in accordance with previous studies that suggested T_H1/T_H17 cell infiltration correlates with reduced sensitivity to corticosteroid therapy.[20]

The aforementioned data suggest a redirection of the cytokine balance *in vivo*, which results in reversal of exaggerated T_H2 cytokine expression after corticosteroid therapy. We speculated that Treg cells might play a critical role in this rebalance and therefore assessed nTreg and T_R1 cells in NP tissue. Our result of an increase in nTreg cell numbers after budesonide transnasal nebulization was also consistent with findings of other authors for intranasal mometasone.[21] The finding of a significant correlation between the change in TGF-β levels and the change in Treg cell numbers in the present study suggests that TGF-β upregulation in NP tissue promoted the induction of Treg cells.[22] Additionally, our finding for significant upregulation of T_R1 cells and associated suppressor cytokine IL-10 and TGF-β levels in NPs from budesonide-treated patients support the notion that T_R1 cells are likely to play an anti-inflammatory role or roles in the pathogenesis of CRSwNP.

TGF-β might be thought of as a double-edged sword, not only inhibiting T-cell activation[23] but also initiating structural remodeling.[22] Studies by Mastruzzo et al[23] have demonstrated that an increase in TGF-β^+ cell numbers in NPs after corticosteroid treatment was accompanied by significant decreases in IL-4^+ and IL-5^+ cell numbers, as well as significant inhibition of ongoing inflammatory responses. Additionally, other studies have suggested that TGF-β was likely to play a critical role in airway remodeling through induction of different profibrotic processes and attraction of fibroblasts.[22] Our findings for excessive collagen production and thickening of collagen fibers, coinciding with a significantly increased concentration of TGF-β in the patients treated with budesonide transnasal nebulization, are in accordance with these studies and suggest that increased TGF-β expression and collagen deposition might reflect an enhancement of tissue repair process. On the other hand, it is possible that the increased collagen deposition noted in this study might reflect a decrease in tissue edema to some extent. Whether chronic use of budesonide is associated with fibrosis in NPs remains unclear[24] and needs to be explored further.

The balance between MMPs and TIMPs is important for homeostasis of collagen synthesis and breakdown, and lower expression of MMPs and higher amount of TIMPs were likely to be regulated by increased TGF-β expression.[25] Several studies have shown that after corticosteroid treatment in patients with asthma and nasal polyposis, there was a significant decrease of MMP-2 and MMP-9 levels, respectively, combined with an increase in TIMP-1 levels.[26,27] Moreover, one study suggested that tissue MMP-2/TIMP-1 and MMP-9/TIMP-1 ratios correlated with the severity of NPs.[26] The findings from the present study are in accordance with these previous studies. It is possible that budesonide treatment downregulated the expression of MMPs at their main cell source level, including fibroblasts, eosinophils, and mast cells,[28] although this needs to be confirmed in future studies.

Our study showed that albumin content in NPs was also significantly reduced in the budesonide-treated group compared with the placebo-treated group. Thus is consistent with the findings of Bachert et al,[3] who demonstrated that oral glucocorticoids significantly reduced albumin levels compared with no treatment with glucocorticoids, which might lead to shrinkage of NPs, an effect observed in our study.

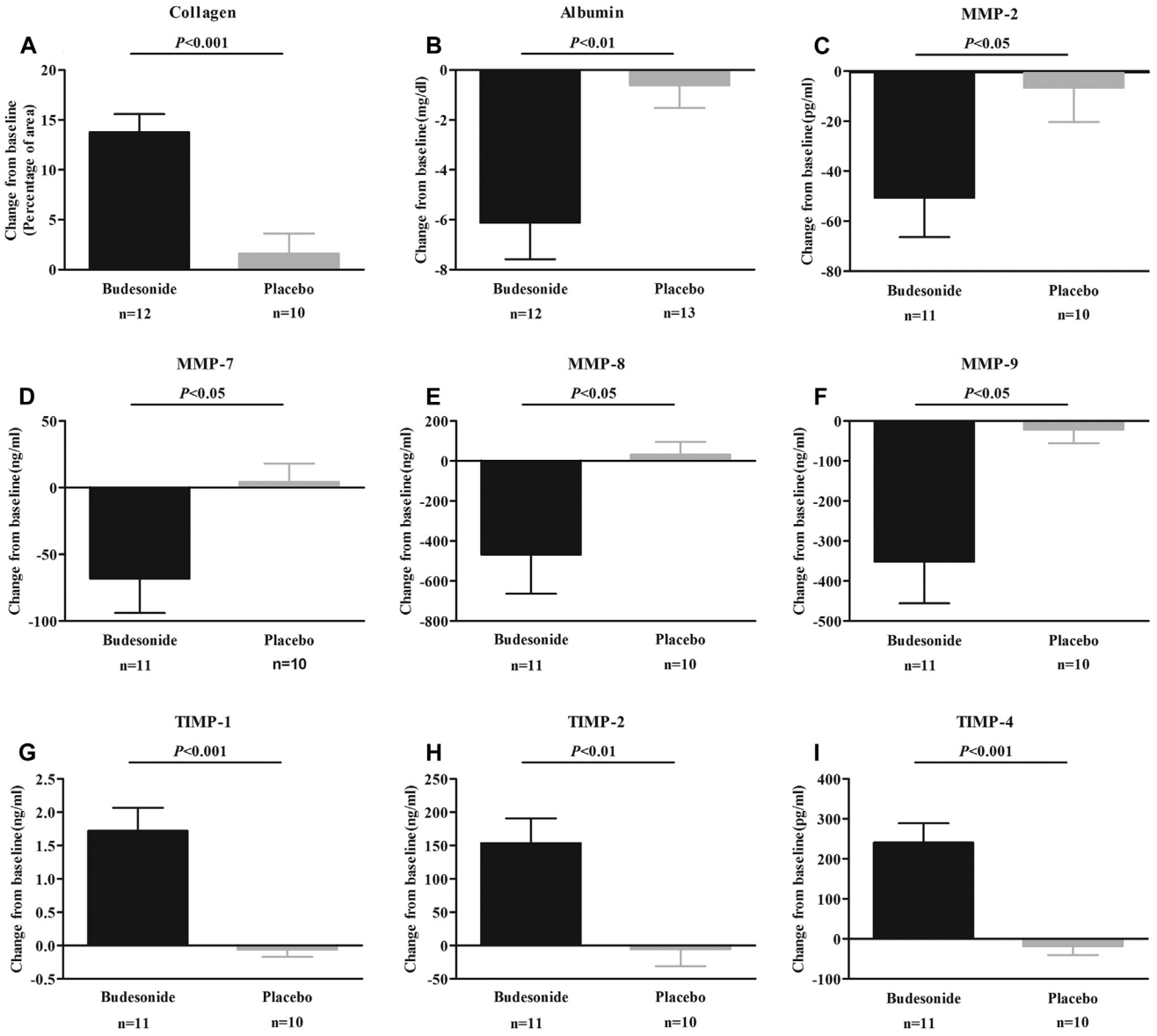

FIG 6. Remodeling pattern shifts of NP tissues were evaluated in the budesonide transnasal nebulization– and placebo transnasal nebulization–treated groups. **A**, Collagen. **B**, Albumin. **C**, MMP-2. **D**, MMP-7. **E**, MMP-8. **F**, MMP-9. **G**, TIMP-1. **H**, TIMP-2. **I**, TIMP-4.

This study is somewhat limited in the absence of adjustments of significance levels for multiple comparisons. Although the probabilities of type I error were low in primary outcomes, false-positive results can occur in some secondary outcomes, including IL-5, MMP-2, MMP-7, MMP-8, and MMP-9, as a result of the lack of adjustment of the significance level. Thus the findings for these secondary outcomes need to be interpreted with caution and confirmed in further studies with larger patient cohorts.

Consistent with a previous report,[29] the current study also demonstrated budesonide transnasal nebulization therapy to be safe and well tolerated, as evidenced by a lack of suppressive effects on adrenal function (ie, normal posttreatment serum cortisol levels) and the absence of serious side effects.

In conclusion, the results of this study indicate that short-term budesonide inhalation suspension through a pulsating atomization device (ie, budesonide transnasal nebulization) is an effective and safe treatment in patients with eosinophilic CRSwNP, as evidenced by significant improvements in symptom scores and inflammatory indices, reductions in polyp size, and the absence of hypothalamic-pituitary-adrenal axis suppression or any serious side effects.

Clinical implications: Short-term budesonide transnasal nebulization significantly improves nasal symptoms, inflammation, and tissue remodeling in the absence of any serious side effects and thus offers an alternative treatment option for patients with eosinophilic CRSwNP.

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METHODS

Patients and study design

Sixty patients aged 19 to 76 years with eosinophilic NPs were recruited into this prospective, randomized, double-blind, placebo-controlled study (registration details are available at http://clinicaltrial.gov/show/NCT02024659) from the Otolaryngology Clinic at Beijing TongRen Hospital, Beijing, China, from September 2010 to December 2011. All participants were outpatients at the otolaryngology clinic and received diagnoses of CRSwNP or symptoms of rhinosinusitis (eg, nasal obstruction, nasal discharge, loss of smell, and headache/facial pain) for at least 12 weeks[E1] from ear, nose, and throat specialists, which were confirmed by using computed tomographic scanning for abnormalities in all sinuses. The presence of bilateral NPs was also confirmed by using nasal endoscopy.

Patients who had undergone surgical treatment for NPs were not included in the study, as were patients who had taken any steroids or antibiotics for at least 4 weeks before sample collection. Patients with cystic fibrosis, any serious or unstable concurrent disease, or psychological disorder and pregnant women were also excluded from the study.

At the screening visit, participants were preselected for eosinophilic NPs based on at least 1 of the following parameters: skin prick test response positivity, increased eosinophil counts in peripheral blood, allergic rhinitis, asthma, or aspirin intolerance. The diagnosis of CRSwNP was based on the standard criteria issued in the European Position Paper on Rhinosinusitis and Nasal Polyps guidelines.[E1] Polyp biopsy was undertaken 1 week before treatment. Referring to the method of Cao et al,[E2] CRSwNP was defined as eosinophilic when the number of eosinophils exceeded 10% of total infiltrating inflammatory cells in the polyp tissue, as evaluated by using H&E staining.

Eligible patients with eosinophilic NPs were randomly assigned 1:1 to receive 1 mg/2 mL budesonide transnasal nebulization twice daily or placebo transnasal nebulization (saline solution) for 14 days, according to a computer-generated random allocation sequence using block randomization with a block size of 4. An independent, off-site clinical trials pharmacist masked the containers of budesonide inhalation suspension (Pulmicort Respules, AstraZeneca) and placebo. The medications were distributed in sequentially numbered sealed opaque envelopes at the research unit by a laboratory technician and administered to the patients by a nurse, both of whom were not involved with the study protocol, in a double-blind manner with neither the patient nor any investigator connected with the study knowing the identity of the medication. A Pari Sinus Nebulizer and Pari Master Compressor (PARI GmbH) was used. Nebulization of the medication was generally well tolerated and completed over a period of 5 to 10 minutes for each dose. Nasal symptoms (including nasal obstruction, nasal discharge, loss of smell, and headache/facial pain) were assessed before initiation (1 week after biopsy) and at the end of the 14-day treatment period by using visual analog scales. TNSSs were calculated as the sum of 4 individual symptoms at the same time. Similarly, the size of NPs was measured by means of endoscopic examination. Biopsy specimens of NPs were obtained again at the end of the 14-day treatment period. In view of the relatively small sizes of individual samples, all samples were evaluated by using H&E staining, and then samples from patients in each treatment group were randomly divided into approximately 3 equal groups. The 3 groups of samples were then assessed for inflammatory cytokines, remodeling parameters, or T-cell subsets. All patients were also assessed for pretreatment and posttreatment morning serum cortisol levels to evaluate adrenal function, and adverse events were reported to the investigator, who assessed the relationship of the event to the study treatment.

Clinical evaluation

As the primary outcome, the physician's assessment of reduction in NP size was used. The investigator assessed the size of bilateral polyps by using rhinoscopic examination at baseline and the end of the study with the Kennedy score. Polyp size was scored as 0 (no polyps), 1 (polyps in the middle meatus not reaching below the inferior border of the middle turbinate), 2 (polyps reaching below the inferior border of the middle turbinate but not below the inferior border of the inferior turbinate), or 3 (large polyps reaching below the inferior turbinate). Bilateral polyp grade was obtained as the sum of individual grades for the left and right nasal cavities, with a possible total score of 6.

The patient's assessment of symptom improvements was used as the coprimary end point. Four major symptoms, including nasal obstruction, discharge, loss of smell, and headache/facial pain, were assessed by the patient using an ordinal scale visual analog score of 0 to 10, where 0 indicated the absence of any symptoms and 10 signified the presence of the most severe nasal obstruction, nasal discharge, alteration in sense of smell, and headache/facial pain. An overall assessment of symptom severity was shown as the TNSS, which was the sum of 4 individual symptoms.

Histologic examination

Paraffin sections were stained with H&E and observed by means of bright-field light microscopy at ×400 magnification. Numbers of infiltrating eosinophils were counted and reported as the mean of counts for at least 10 randomized fields by 2 independent observers who were blind to the clinical diagnosis and characteristics of the patient. In case of disagreement (ie, difference >10% between the 2 counts), the specimen was observed further by using a multihead microscope, and consensus was reached on the count accordingly.

Immunoassay

Samples to be assessed by means of immunoassay were weighed, and a total of 1.0 mL of PBS supplemented with 0.05% Tween 20 (Sigma-Aldrich, St Louis, Mo) and 1% protease inhibitor cocktail (Sigma-Aldrich) was added for every 100 mg of tissue. All samples were homogenized with a standard bench-top homogenizer (Polytron PT 2100, Kinematica, Switzerland) at 1000 rpm for 5 minutes, and the homogenates were centrifuged at 1500*g* for 10 minutes at 4°C. The supernatants were collected from each sample and stored at −80°C until further analysis for a variety of inflammatory cytokines, MMPs, TIMPs, and albumin.

Expression of MMPs (MMP-2, MMP-7, MMP-8, and MMP-9) and TIMP isoforms (TIMP-1, TIMP-2, TIMP-3, and TIMP-4) was assessed by using Fluorokine MAP Multiplex Kits (R&D Systems, Minneapolis, Minn) and analyzed on a Luminex 100 analyzer (Luminex 100 System; Luminex, Austin, Tex). IL-5, IFN-γ, IL-17, IL-10, and eotaxin concentrations were measured by using the BD Cytometric Bead Array Human Enhanced Sensitivity Flex Set System (BD Biosciences), which uses particles with discrete fluorescence intensities to obtain the concentration of analytes. Albumin and TGF-β levels in tissue homogenates were determined by using commercially available ELISA kits (R&D Systems).

Picrosirius red staining for collagen

The collagen content in the extracellular matrix was assessed by using picrosirius red staining, as reported by Li et al.[E3] Briefly, paraffin sections were deparaffinized, hydrated, and stained with picrosirius red for 60 minutes. The stained sections were washed in 2 changes of acidified water and dehydrated in 3 changes of 100% ethanol before analysis by means of microscopy with an Olympus microscope (CX-40) equipped with filters to provide circularly polarized illumination. The tissue images were viewed under both bright-field and polarized light conditions at ×200 magnification and recorded with a digital camera (Olympus BX-41).

Collagen content was calculated in the digital images taken during bright-field microscopy. The percentage of positively stained area was determined from 10 randomly selected fields per sample to analyze total collagen content, and collagen type 3 fibers present in the tissue were determined by using polarized light microscopy.

Flow cytometric analysis

Fresh polyp tissues were washed and cut into small fragments before teasing apart to allow dispersion of the nasal cells into RPMI 1640 media (Life Technologies, Rockville, Md). The dispersed samples were passed through a 40-μm pore size mesh to obtain a single-cell suspension, and after

rinsing with fresh RPMI 1640 medium, cells were adjusted to a concentration of 2×10^6 cells/mL.

T-cell subsets in polyp tissues were phenotyped by means of flow cytometry (FACSAria Flow Cytometer, BD Biosciences), according to the manufacturer's instructions. We gated on lymphocytes first based on forward scatter and side scatter and then gated on $CD4^+$ cells with anti-CD4–allophycocyanin H7 (12 μg/mL), which could be stained only on live cells, excluding dead cells/doublets. Each antibody was matched with a respective isotype IgG_1 as a control, and the gating threshold was set accordingly. Cells were labeled with specific mAbs in different combinations as follows: anti-CD25–PerCP CY7 (12 μg/mL), anti–IL-4–Alexa Flour 488 (0.125 μg/5 μL), anti–IL-10–phycoerythrin (0.03 μg/20 μL), anti–IL-17A Alexa Flour 647 (0.25 μg/20 μL), anti–IFN-γ–PerCP-CY5.5 (0.06 μg/5 μL), and Foxp3–Horizon V450 (50 μg/mL; all from BD PharMingen, San Jose, Calif). T-cell subsets were selected for detailed phenotypic analysis as follows: (1) T_H1 cells were IFN-γ^+IL-4^-CD4^+ T cells; (2) T_H2 cells were IFN-γ^-IL-4^+CD4^+ T cells; (3) T_H17 cells were IL-$17A^+CD4^+$ T cells; (4) T_R1 cells were IL-10^+IL-4^-CD4^+ T cells; and (5) nTreg cells were $CD4^+CD25^+Foxp3^+$ T cells. Representative gating figures are shown in Fig E1.

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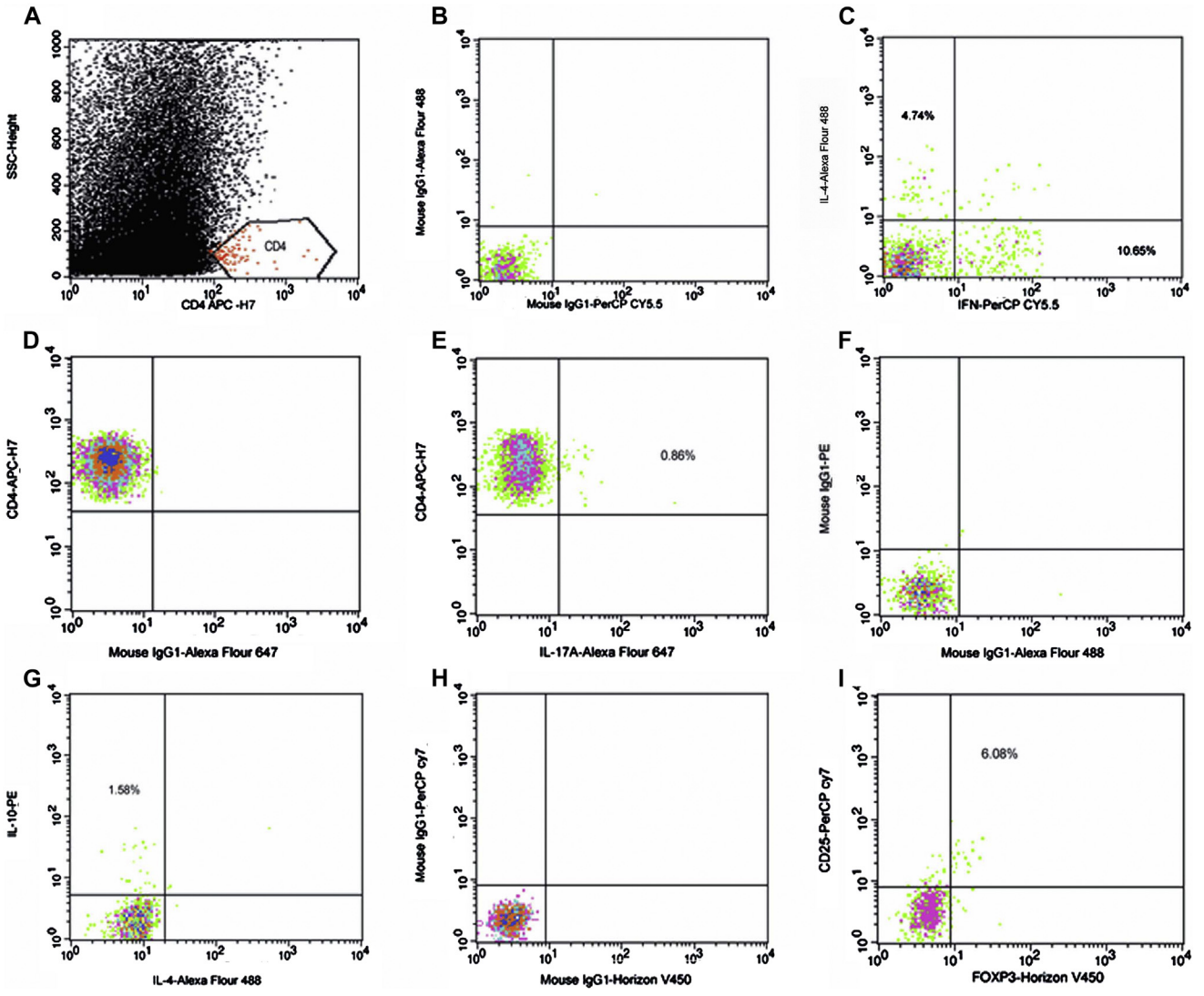

FIG E1. Representative gating figures in flow cytometric analysis of T_H1, T_H2, T_H17, T_R1, and nTreg cells in tissue homogenates. **A,** $CD4^+$ T cells *(scatter dots in the semicircle)* were gated. **B,** Isotype control of T_H1 and T_H2 cells *(left lower quadrant)*. **C,** T_H1 cells ($IFN\text{-}\gamma^+IL\text{-}4^-CD4^+$ T cells, *right lower quadrant*) and T_H2 cells ($IL\text{-}4^+IFN\text{-}\gamma^-CD4^+$ T cells, *left upper quadrant*) were gated in $CD4^+$ T cells. **D,** Isotype control of T_H17 cells *(left upper quadrant)*. **E,** T_H17 cells ($IL\text{-}17^+CD4^+$ T cells, *right upper quadrant*) were gated in $CD4^+$ T cells. **F,** Isotype control of T_R1 cells *(left lower quadrant)*. **G,** T_R1 cells ($IL\text{-}10^+IL\text{-}4^-CD4^+$ T cells, *left upper quadrant*) were gated in $CD4^+$ T cells. **H,** Isotype control of $CD25^+Foxp3^+$ cells *(left lower quadrant)*. **I,** nTreg cells ($CD4^+CD25^+Foxp3^+$ T cells, *right upper quadrant*) were gated in $CD4^+$ T cells. Values in dot plots indicate percentages of different cell subsets in total $CD4^+$ T cells.

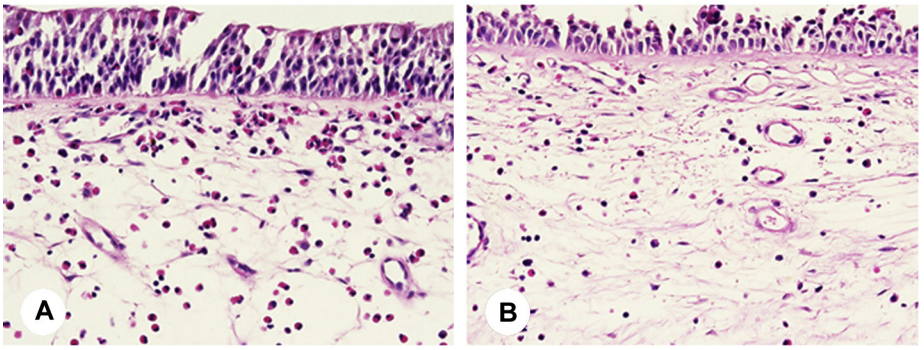

FIG E2. Eosinophil infiltration in polyp tissues (×400 magnification) decreased from before **(A)** to after **(B)** treatment in the budesonide transnasal nebulization group.

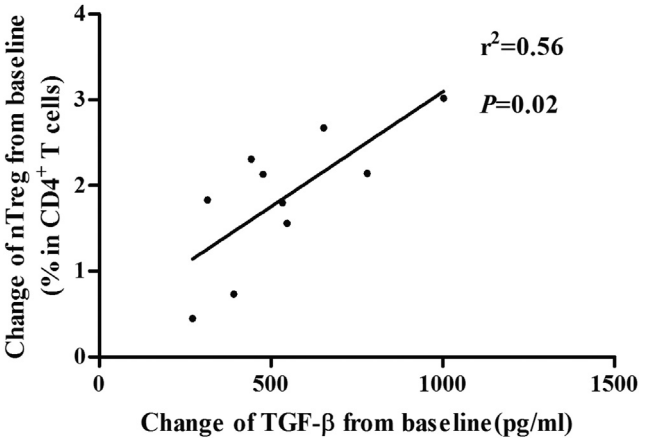

FIG E3. Correlation between the change in TGF-β levels and the change in nTreg cell numbers from baseline in the budesonide transnasal nebulization–treated group.

TABLE E1. Clinical characteristics of patients with NPs at baseline

	Patients with NPs	
Characteristic	**Budesonide group**	**Placebo group**
No.	30	30
Age (y), mean (range)	48.23 (29-68)	44.03 (19-76)
Sex (male/female)	12/18	17/13
Duration (y), mean (range)	10.67 (4-24)	8.57 (2-20)
Asthma in history	11	9
Aspirin intolerance	7	5
Polyp size score, mean (SD)	4.85 (0.69)	4.72 (0.67)
TNSS, mean (SD)	28.52 (2.32)	27.36 (2.15)