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Efficacy of Budesonide Nasal Spray on Neutrophilic Chronic Rhinosinusitis with Nasal Polyps: A Combined Clinical and Experimental Study

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Keywords
Neutrophilic chronic rhinosinusitis · Nasal polyps · Budesonide · Treatment · Inflammation

Abstract
Background: Neutrophilic chronic rhinosinusitis with nasal polyps (CRSwNP) occur predominantly in Asian subjects. Appropriate treatments for this endotype have not been elucidated. This study aimed to evaluate the efficacy of budesonide nasal spray on neutrophilic CRSwNP. ***Materials and Methods:*** Fifteen neutrophilic CRSwNP patients were included, and then they received budesonide nasal spray treatment for 3 months. Biopsies of nasal polyps (NPs) were obtained from these subjects. Their clinical indexes were scored using Visual Analog Scale (VAS), Sino-Nasal Outcome Test (SNOT)-22, and Endoscopic Appearances (EAs). Histological analyses were used to assess numbers of neutrophils, goblet cells, and submucosal gland cells in NPs. Percentages of $CD8^+$ T cells and $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs) were evaluated using flow cytometry. Mucin 5AC (MUC5AC), MUC5B, myeloperoxidase (MPO), interferon (IFN)-γ, and interleukin (IL)-1β and their mRNAs were also examined. After that, we cultured NP tissues in vitro and evaluated the above-mentioned inflammatory parameters before and after the administration of budesonide. ***Results:*** Budesonide nasal spray did not improve clinical evaluations including VAS, SNOT-22, and EA scores. Numbers of neutrophils and goblet cells, the score of submucosal gland cells, percentages of $CD8^+$ T cells and Tregs, MUC5AC, MUC5B, MPO, IFN-γ, and IL-1β and their mRNAs were not decreased in NPs after the budesonide treatment. Furthermore, the administration of budesonide into NP cultures also did not reduce their levels in comparison with those before the treatment. ***Conclusion:*** These findings demonstrate that budesonide treatment may not alleviate the inflammatory condition in neutrophilic CRSwNP.

Introduction

Chronic rhinosinusitis (CRS) affects 1–9% of the total population throughout the world [1]. This chronic disease affects about 8% of the whole population in mainland China [2]. CRS is a complex disease consisting of 2 clinical phenotypes: CRS with nasal polyps (CRSwNP) and CRS without nasal polyps. The prevalence of CRSwNP

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was found to be approximately 1–4% in the general population [1]. CRSwNP may often be difficult to treat, cause significant morbidity, and has a high impact on quality of life.

In recent years, there have been new findings into the mechanisms of CRSwNP with diverse pathophysiologies and different types of inflammation. Although the etiology of nasal polyps (NPs) has not been clearly elaborated, most NP tissues are found to be characterized by substantial inflammatory cells. Eosinophilic inflammation is always seen in 65–90% of NP patients in Western countries [3–5]. However, neutrophilia and a remarkable increase of T helper (Th)1/Th17 cells characterize a majority of NP cases in East Asian countries, such as China, Korea, and Japan [6–9].

As for the clinical therapies for CRSwNP, both oral and topical glucocorticoids are recognized as effective treatments [10]. However, the therapeutic response rate to steroids varies from 50 to 80% for this chronic disease [10–12]. Unfortunately, few studies have performed relevant analyses to identify the differences between responders and nonresponders [13, 14]. Some studies relevant to asthma have confirmed the differences with the understanding that the therapeutic benefits of corticosteroids are their capability to induce eosinophil apoptosis [15, 16]. In other words, corticosteroids are typically regarded as the primary medical treatment for eosinophilic CRSwNP. However, neutrophilic NPs reduce the response to oral corticosteroid therapy [14]. As regards topical steroid treatment, such as budesonide nasal spray or other analogues, few studies report their clinical efficacy on neutrophilic CRSwNP patients.

The present study aimed to evaluate the efficacy of budesonide nasal spray on neutrophilic CRSwNP in vivo and in vitro. We assessed the clinical symptoms with Visual Analog Scale (VAS), Sino-Nasal Outcome Test (SNOT)-22, and Endoscopic Appearances (EAs) and evaluated cytological characteristics of this inflammatory condition. Furthermore, mucin 5AC (MUC5AC), MUC5B, myeloperoxidase (MPO), interferon (IFN)-γ, and interleukin (IL)-1β were also examined by using ELISA and real-time RT-PCR.

Materials and Methods

Study Design

Fifteen control subjects and 15 patients with neutrophilic CRSwNP were recruited for participation in this investigation. This was a prospective and pilot study, which was performed in accordance with the Helsinki Declaration. The methods and protocols of the study were approved by the Ethical Committee of Huashan Hospital of Fudan University (no. 2016-054). A written informed consent was obtained from all patients. The study was performed between December 2016 and March 2017 in the Department of Otorhinolaryngology-Head and Neck Surgery, Huashan Hospital of Fudan University.

Study Population

Fifteen outpatients with neutrophilic CRSwNP (NP group, 6 men and 9 women), aged between 28 and 58 years (median age 43 years), were recruited in the present study. The diagnosis of CRSwNP was based on the European Position Paper on Rhinosinusitis and Nasal Polyps 2012 [1]. The biopsy of NPs was undertaken 1 week before the relevant treatment [7]. Neutrophilic CRSwNP was identified as <10 eosinophils/per high power field (HPF) and the presence of a focal or diffuse neutrophil infiltrate [17]. Normal mucosa (normal group) samples were collected from the inferior turbinates of 15 subjects (7 men and 8 women), aged between 24 and 56 years (median age 40 years), who underwent nasal septoplasty-inferior turbinoplasty because of the clinical symptom of nasal obstruction. The atopic status of all subjects was assessed according to skin reactivity to house dust mite and other 12 common airborne allergens on the skin prick test (SPT). All the subjects participating in the study had a negative result in SPT. The reaction to the SPT was considered positive if the wheal area was larger than 7 mm^2 (diameter > 3 mm). Exclusion criteria included asthma, history of allergic fungal sinusitis, cystic fibrosis, aspirin intolerance, immunodeficiency, Churg-Strauss syndrome, coagulation disorder and pregnancy, and current use of topical/oral steroids, antihistamines, or antibiotics.

Study Protocol

Budesonide aqueous nasal spray (Rhinocort aqua, AstraZeneca R&D, Lund, Sweden) in a dose of 256 μg per day (1.28 mg/mL, 6 μg/spray, 2 sprays in each nostril at awakening and at bedtime) was administered for 3 months (90 days) to all neutrophilic CRSwNP patients. There were 2 study visits. At the first, patients received the evaluations which comprised medical history; nasal endoscopy; scores of VAS, SNOT-22, and EAs; and experimental analyses of cytological characteristics and inflammatory features. EAs were scored on a 0–2 point basis for the presence of polyps (0 = none; 1 = confined to middle meatus; 2 = beyond middle meatus) and discharge (0 = none; 1 = clear and thin; 2 = thick and purulent). Maximum EA score is 8, including the right and left sides [18]. At the second (i.e., after 90-day medical treatment), biopsy specimens of NPs were obtained again. The abovementioned clinical and experimental parameters were also reevaluated. During the study period, all the patients were instructed not to use any other drugs.

Samples Preparation

Samples from inferior turbinates and NPs were obtained and cut into 4 portions. One was for histological analyses, one was analyzed by flow cytometry, one was for examinations of inflammatory substances, and the last one was cultured for the intervention in vitro.

Immunocytochemistry and Confocal Microscopy

Tissue sections were incubated overnight at room temperature with primary antibodies against eosinophil cation protein (ECP) (MyBioSource, Inc., San Diego, CA, USA) or MPO (MyBioSource, Inc., San Diego, CA, USA). After washing with phosphate-buff-

ered saline (PBS), all the samples were incubated using allophycocyanin or fluorescein isothiocyanate-conjugated secondary antibodies at room temperature in the darkness. Then, nuclei were stained by using 10 mg/mL 4′,6-diamidino-2-phenylindole dihydrochloride for 5 min at room temperature. After that, coverslips were mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA). Images were taken through a Leica TCS SP5 confocal laser scanning microscope equipped with the Leica Confocal Software (LCS) 2.61 (Leica Microsystems, Wetzlar, Germany). The microscopic examination of staining was performed at magnifications of ×200 and ×400 by 2 independent observers who were blinded to the subjects. Numbers of eosinophils and neutrophils were detected microscopically in a blinded manner at a HPF of ×400 magnification. Neutrophilic CRSwNP was defined as <10 eosinophils/HPF and the presence of a focal or diffuse neutrophilic invasion.

Histological Analysis

For the immunohistochemical staining, the primary MPO antibody (MyBioSource, Inc., San Diego, CA, USA) was administered to these sections and then stained by using 3,3′-diaminobenzidine chromogen (DAB; Sigma-Aldrich, St. Louis, MO, USA). The numbers of infiltrating neutrophils were determined microscopically in a blinded manner at a HPF of ×400 magnification by 2 independent observers who were blinded to the study.

For the hematoxylin and eosin (HE) staining, sections were immersed in Harris hematoxylin (Thermo Scientific, Waltham, MA, USA), placed in 0.5% acid alcohol, blued in 1% ammonia, and rinsed a final time in deionized water. Finally, the sections were immersed in an alkaline eosin solution. The numbers of goblet cells were examined microscopically in a blinded manner at a HPF of ×400 magnification.

For the periodic acid-Schiff (PAS) staining, sections were stained by a PAS Kit (Sigma-Aldrich, St. Louis, MO, USA). The test was performed according to the manufacturer's protocols. The microscopic evaluation and the scores of staining degrees were performed at a magnification of ×400. The intensities were scored as follows: 0, no staining; 1+, weak staining; 2+, moderate staining; 3+, strong staining. The percentages of staining areas were classified as follows: 0, 0%; 1, 1–10%; 2, 11–50%; 3, 51–100%. The intensity and percentage scores were multiplied to give a composite score of 1–9.

Flow Cytometry Analysis

Cells were harvested for flow cytometry analysis of $CD8^+$ T cells and $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs). For CD3 and CD8 staining, the cells were incubated with CD3 antibody (Abcam, Cambridge, MA, USA) and then with biotinylated goat anti-human IgG followed by R-phycoerythrin-streptavidin. They were incubated with human serum and then with fluorescein isothiocyanate-conjugated CD8 antibody (Abcam, Cambridge, MA, USA). For Tregs analysis, cells were stained for CD4, CD25, and intranuclear Foxp3 (Foxp3 staining kit; eBioscience, San Diego, CA, USA), respectively. Finally, cells were resuspended in 100 µL flow cytometry medium containing 1% bovine serum albumin and 0.1% sodium azide in PBS and analyzed using a FACSAria flow cytometer (BD Biosciences, San Jose, CA, USA) and FlowJo software (TreeStar Inc., Ashland, OR, USA). The percentages of $CD8^+$ T cells in total $CD3^+$ T cells and Tregs in total $CD4^+$ T cells were determined.

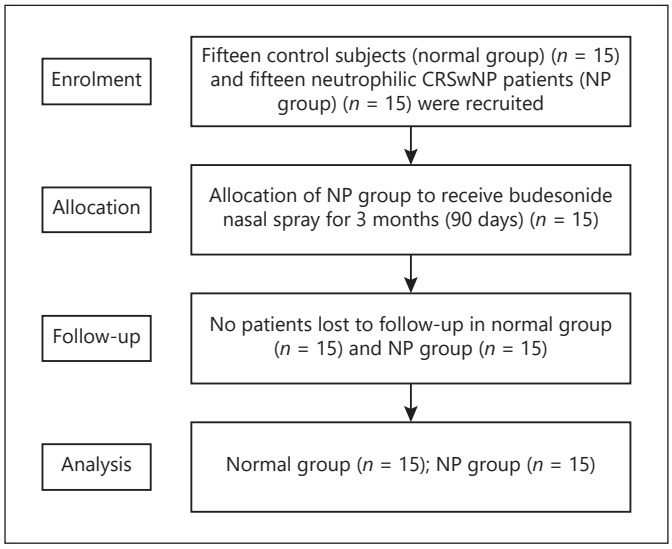

Fig. 1. The study flowchart.

Organ Cultures

Normal mucosa and NP tissues were cultured in vitro in accordance with published procedures [19]. To assess the efficacy of budesonide treatment on NPs, NP tissues were then saturated for 1 h in a culture medium with DMEM, 10% calf serum, and 10 µg/mL gentamicin in the absence or presence of budesonide (1.28 mg/mL) and then placed on a hydrated 1 × 1-cm gelatin sponge with the mucosa facing upward and the submucosa downward. These plates were then placed in a humidified incubator for 24 h. After that, all the cultured tissues were collected and stored at −20°C for further experiments.

ELISA Analysis

Tissue MUC5AC, MUC5B, MPO, IFN-γ, and IL-1β from inferior turbinates and NPs by biopsies or cultures in vitro were evaluated using corresponding ELISA kits purchased from MyBioSource, Inc., San Diego, CA, USA. The ELISAs were performed in accordance with the manufacturer's protocols.

Real-Time RT-PCR

Real-time RT-PCR was performed to evaluate mRNAs of MUC5AC, MUC5B, MPO, IFN-γ, and IL-1β from inferior turbinates and NPs by biopsies or cultures in vitro. MUC5AC primers were as follows: forward primer 5′-TGATCATCCAGCAGCAGGGCT-3′ and reverse primer 5′-CCGAGCTCA GAGGACATATGGG-3′. MUC5B primers were as follows: forward primer 5′-CTGCGAGACCGAGGTCAACATC-3′ and reverse primer 5′-TGGGCAGCAGGA GCACGGAG-3′. MPO primers were as follows: forward primer 5′-GGTGGGGCTGAGGTACAAAG-3′ and reverse primer 5′-CAGCCCAGCAAGGTCCTAAG-3′. IFN-γ primers were as follows: forward primer 5′-GCAGAGCCAAATTGTCTCCT-3′ and reverse primer 5′-ATGCTCTTCGACCTCGAAAC-3′. IL-1β primers were as follows: forward primer 5′-ACAGATGAAGTGCTCCTTCCA-3′ and reverse primer 5′-GTCGGAGATTCGTAGCTGGAT-3′. GAPDH mRNA prim-

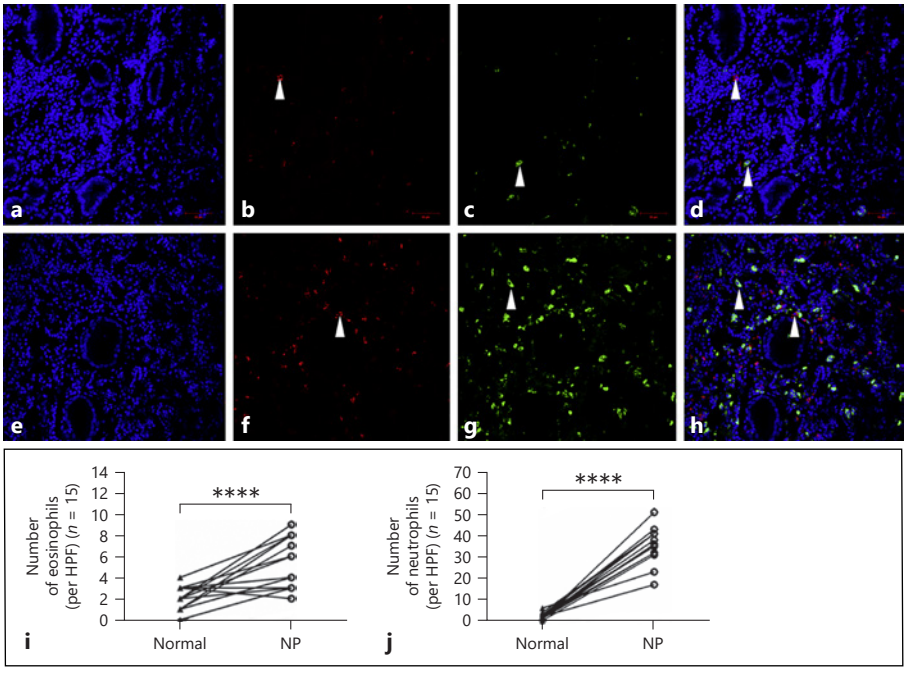

Fig. 2. Confocal immunofluorescence microscopic analysis of infiltrating eosinophils and neutrophils in normal subjects (n = 15) and pretreatment CRSwNP patients (n = 15). **a** ECP and MPO in eosinophils and neutrophils (DAPI) from normal subjects. **b** ECP in eosinophils (allophycocyanin) from normal subjects. **c** MPO in neutrophils (fluorescein isothiocyanate) from normal subjects. **d** ECP and MPO in eosinophils and neutrophils (merged) from normal subjects. **e** ECP and MPO in eosinophils and neutrophils (DAPI) from CRSwNP patients. **f** ECP in eosinophils (allophycocyanin) from CRSwNP patients. **g** MPO in neutrophils (fluorescein isothiocyanate) from CRSwNP patients. **h** ECP and MPO in eosinophils and neutrophils (merged) from CRSwNP patients. **i** Number of eosinophils. **j** Number of neutrophils. Normal, normal mucosa. NPs, nasal polyps; CRSwNP, chronic rhinosinusitis with nasal polyps; ECP, eosinophil cation protein; MPO, myeloperoxidase; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; HPF, high power field; SEM, standard error of the mean. Arrowheads indicate positive staining cells. Scale bars: 50 μm. Original magnification: ×200. The values shown are expressed as mean ± SEM. ****$p < 0.0001$.

ers were as follows: forward primer 5′-CATGTTCCAATATGATTCCACC-3′ and reverse primer 5′-CCTGGAAGATGGTGATGG-3′. Evaluations of data were performed using the threshold cycle (ΔCT) method.

Statistical Analysis

Data obtained were analyzed with the commercially available statistical software Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA). The Kruskal-Wallis test was used for comparisons between normal and NP groups. If the initial Kruskal-Wallis test was significant, a nonparametric Mann-Whitney test was then applied. $p < 0.05$ was considered statistically significant.

Results

Patient Characteristics

Fifteen neutrophilic CRSwNP patients completed the present study. No subjects were excluded (Fig. 1). Patient characteristics are mentioned in detail in the section Study Population.

Adverse Effects

Budesonide nasal sprays were well tolerated by the patients in this study. Adverse effects included epistaxis in 1 patient, dry nose in 3 patients, and nasal irritation in 2 patients. The overall adverse effect incidence was 40%. No serious adverse events were reported during the study period.

Infiltrating Neutrophils in CRSwNP

The results showed that numbers of eosinophils and neutrophils in pretreatment CRSwNP (Fig. 2a–d, i, j) were found to be increased microscopically compared with those in normal mucosa (Fig. 2e–h, i, j). However, the numbers of infiltrating neutrophils were higher than eosinophils. According to a published standard, neutrophilic CRSwNP was identified as <10 eosinophils/HPF, in the meantime with the presence of a focal or diffuse neutrophil infiltration [17]. Obviously, the data indicated the characteristic of the neutrophilic endotype in CRSwNP patients in the current study [16].

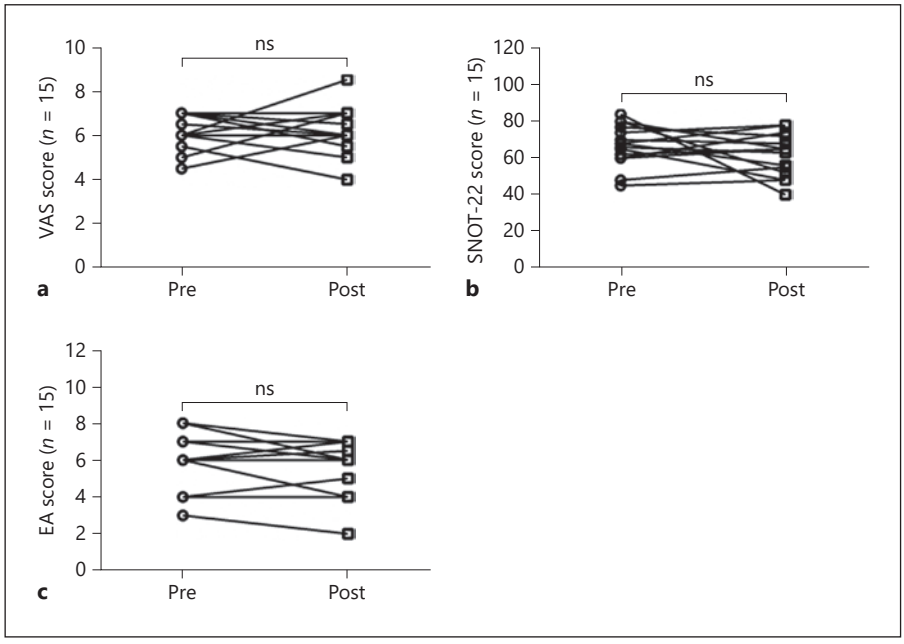

Fig. 3. Comparisons of clinical scores. **a** VAS score. **b** SNOT-22 score. **c** EA score. VAS, Visual Analog Scale; SNOT-22, Sino-Nasal Outcome Test 22; EAs, endoscopic appearances; Pre, pretreatment; Post, post-treatment; SEM, standard error of the mean; ns, not significant. The values shown are expressed as mean ± SEM.

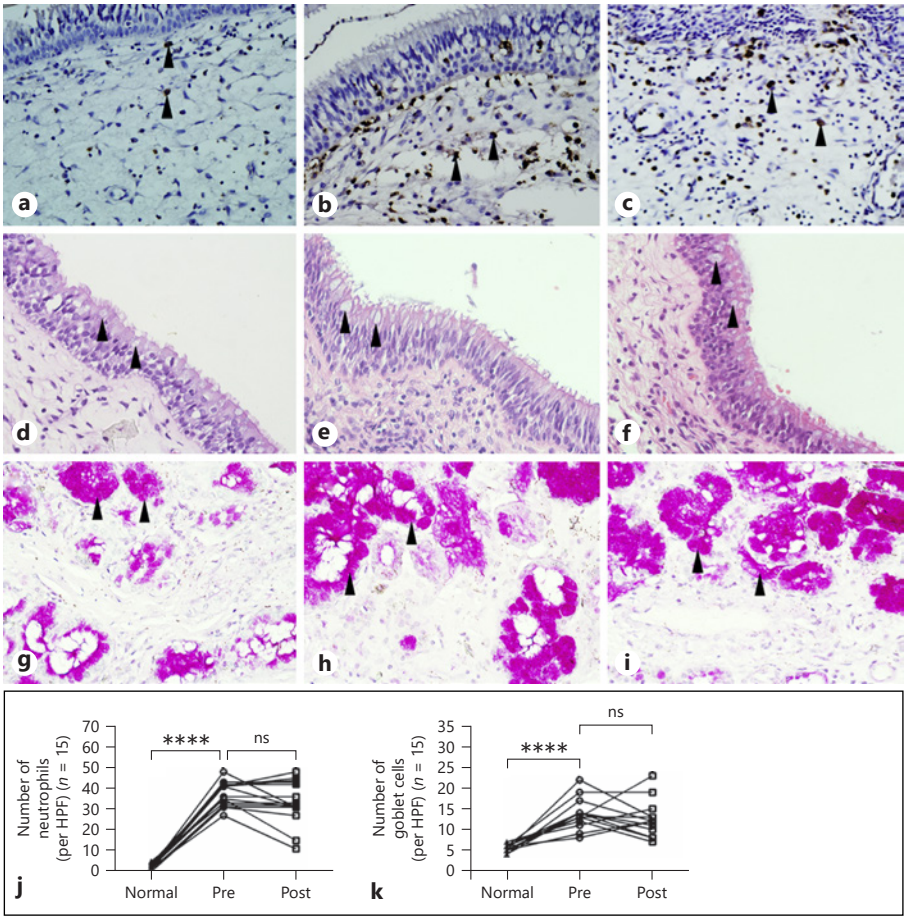

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Fig. 4. Comparisons of histological analyses. **a** Immunohistochemical analysis of normal mucosa. **b** Immunohistochemical analysis of NPs of pretreatment. **c** Immunohistochemical analysis of NPs of post-treatment. **d** HE staining of normal mucosa. **e** HE staining of NPs of pretreatment. **f** HE staining of NPs of posttreatment. **g** PAS staining of normal mucosa. **h** PAS staining of NPs of pretreatment. **i** PAS staining of NPs of posttreatment. **j** Comparisons of number of neutrophils. **k** Comparisons of number of goblet cells. Normal, normal mucosa. NPs, nasal polyps; HE, hematoxylin and eosin; PAS, periodic acid-Schiff; Pre, pretreatment. Post, posttreatment; SEM, standard error of the mean; ns, not significant. Arrowheads indicate neutrophils or goblet cells or submucosal gland cells. Original magnification: ×400. The values shown are expressed as mean ± SEM. ****$p < 0.0001$.

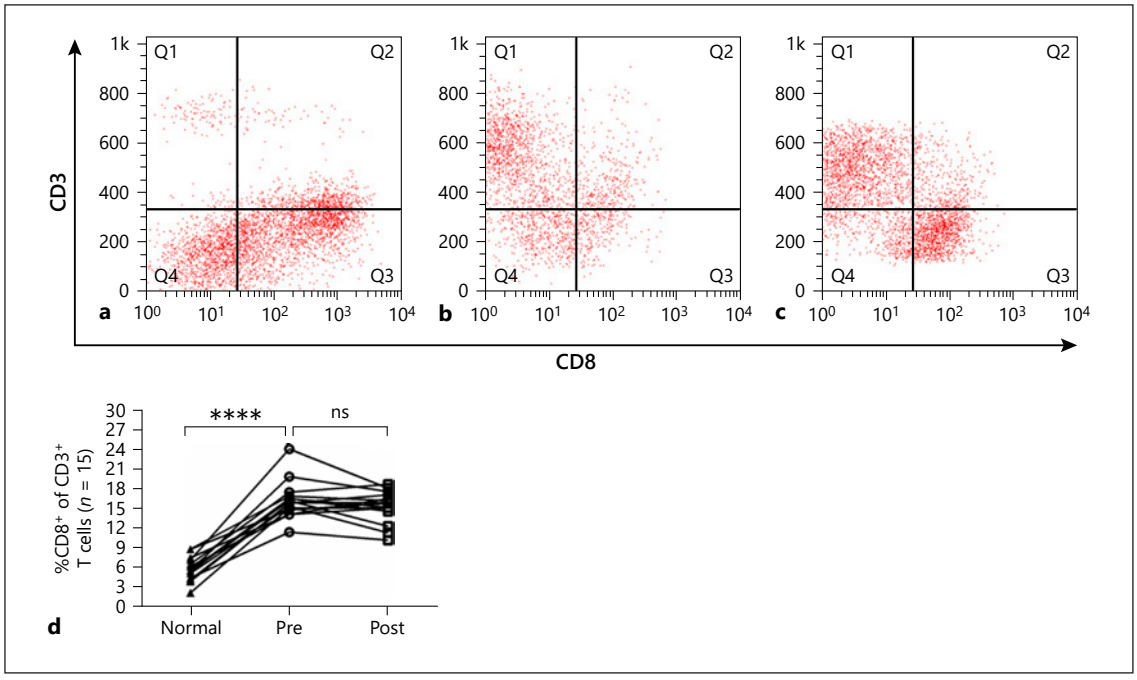

Fig. 5. Comparisons of $CD3^+CD8^+$ T cells. **a** Flow cytometry analysis of $CD8^+$ T cells in total $CD3^+$ T cells in normal mucosa. **b** Flow cytometry analysis of $CD8^+$ T cells in total $CD3^+$ T cells in NPs of pretreatment. **c** Flow cytometry analysis of $CD8^+$ T cells in total $CD3^+$ T cells in NPs of posttreatment. **d** Comparisons of percentage of $CD8^+$ T cells in total $CD3^+$ T cells. Q1 quadrant, $CD3^+CD8^-$ staining. Q2 quadrant, $CD3^+CD8^+$ staining. Q3 quadrant, $CD3^-CD8^+$ staining, Q4 quadrant, $CD3^-CD8^-$ staining. Normal, normal mucosa. NPs, nasal polyps; SEM, standard error of the mean; ns, not significant; Pre, pretreatment; Post, posttreatment. The values shown are expressed as mean ± SEM. ****$p < 0.0001$.

Table 1. Submucosal gland cell staining ($n = 30$)

Staining area	Normal mucosa	Pretreatment	Posttreatment
	Submucosal gland cells	Submucosal gland cells	Submucosal gland cells
Subjects, *n*	15	15	15
Score 1–3	15/15	0/15	0/15
Score 4–9	0/15	15/15	15/15

Clinical Parameters

In order to evaluate the efficacy of budesonide nasal spray on neutrophilic CRSwNP for 3 months, we selected VAS, SNOT-22, and EAs as clinical parameters. We found no statistical differences between pre- and posttreatment in VAS, SNOT-22, and EA scores (Fig. 3a–c).

Histological Analyses

To further estimate the invasion of neutrophils in NPs, we performed the immunohistochemical staining of MPO. As shown in Figures 4a, b, and j, we detected MPO-staining inflammatory cells were increased greatly in neutrophilic NPs compared with those in normal mucosa. We also found these cells were not decreased significantly after budesonide treatment compared with those of pretreatment (Fig. 4c, j). Goblet cells and submucosal glands were reported to play a role in the development of CRS [20] and allergic rhinitis [21, 22]. Therefore, we made experiments to determine these 2 types of cells before and after the treatment with budesonide using HE or PAS staining. The data demonstrated that the numbers of goblet cells were enhanced in NPs compared with those in normal mucosa (Fig. 4d, e , k). However, after the treatment with corticosteroids, there was no significant de-

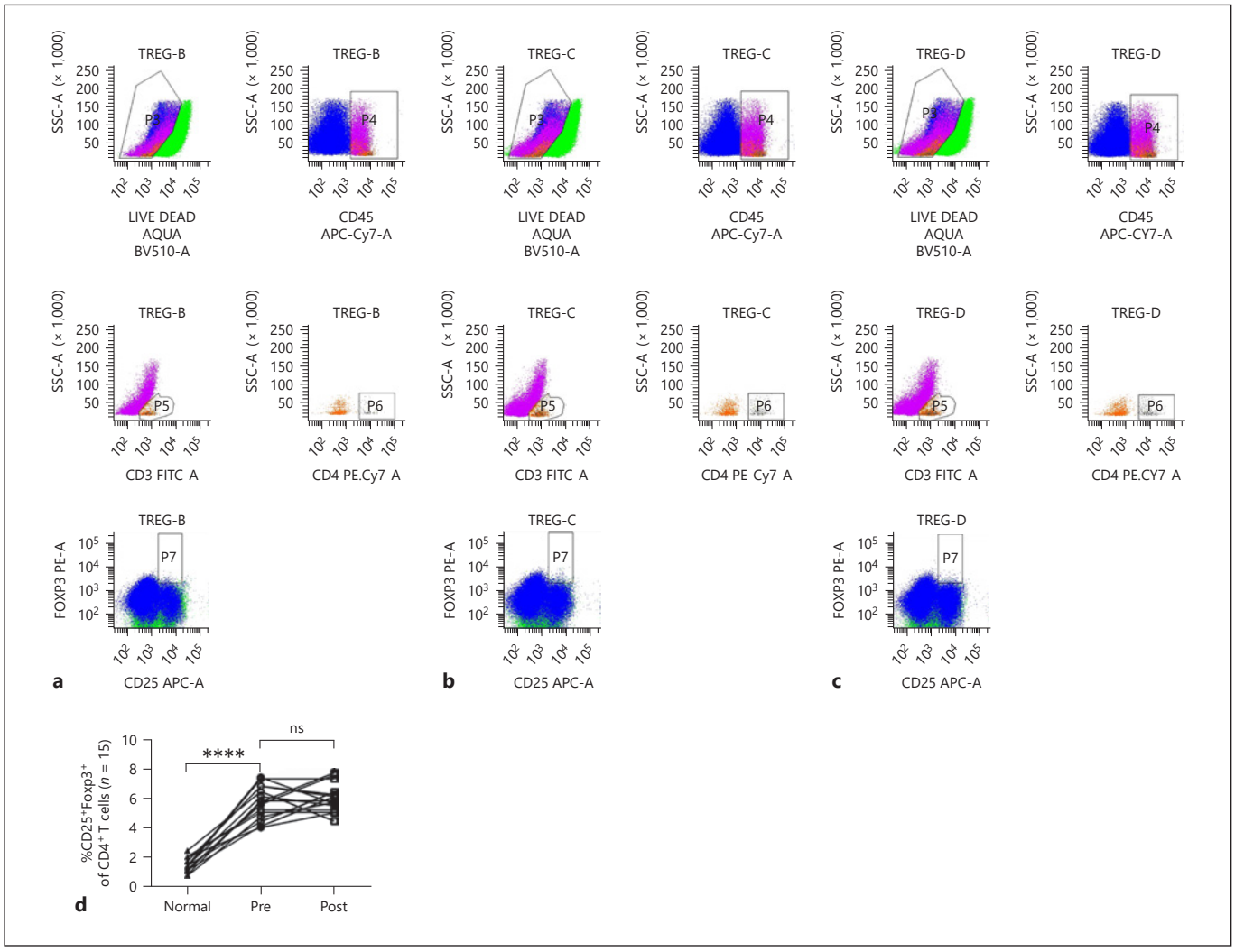

Fig. 6. Comparisons of $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs). **a** Flow cytometry analysis of Tregs in total $CD4^+$ T cells in normal mucosa. **b** Flow cytometry analysis of Tregs in total $CD4^+$ T cells in NPs of pretreatment. **c** Flow cytometry analysis of Tregs in total $CD4^+$ T cells in NPs of posttreatment. **d** Comparisons of percentage of Tregs in total $CD4^+$ T cells. Normal, normal mucosa. NPs, nasal polyps; SEM, standard error of the mean; ns, not significant; Pre, pretreatment; Post, posttreatment. The values shown are expressed as mean ± SEM. ****$p < 0.0001$.

crease in the numbers of these cells (Fig. 4f, k). As for submucosal gland cells, the results showed that composite scores of 1–3 were all included in the normal group, and scores of 4–9 were in the NP group (Fig. 4g, h; Table 1). Furthermore, there were no statistical differences between pre- and posttreatment in the staining of submucosal glands (Fig. 4i; Table 1).

T-Cell Subpopulations in NPs

Asian patients are reported to be more likely to have a type-1 inflammatory profile in NPs compared with patients from Western countries [23]. Tregs can influence the Th1/Th2 balance and decrease the activity of Th1 cells [24]. Consequently, we investigated expressions of T-cell subpopulations including $CD8^+$ T cells and Tregs in NPs with flow cytometry to further assess the effect of the treatment on neutrophilic CRSwNP patients. We found $CD8^+$ T cells (Fig. 5a–d) and Tregs (Fig. 6a–d) were all increased significantly in NP tissues in comparison with those in normal mucosa (Fig. 5a–d). Budesonide nasal spray could not impact the percentages of $CD8^+$ T cells in total $CD3^+$ T cells (Fig. 5a–d) and Tregs in total $CD4^+$ T cells (Fig. 6a–d).

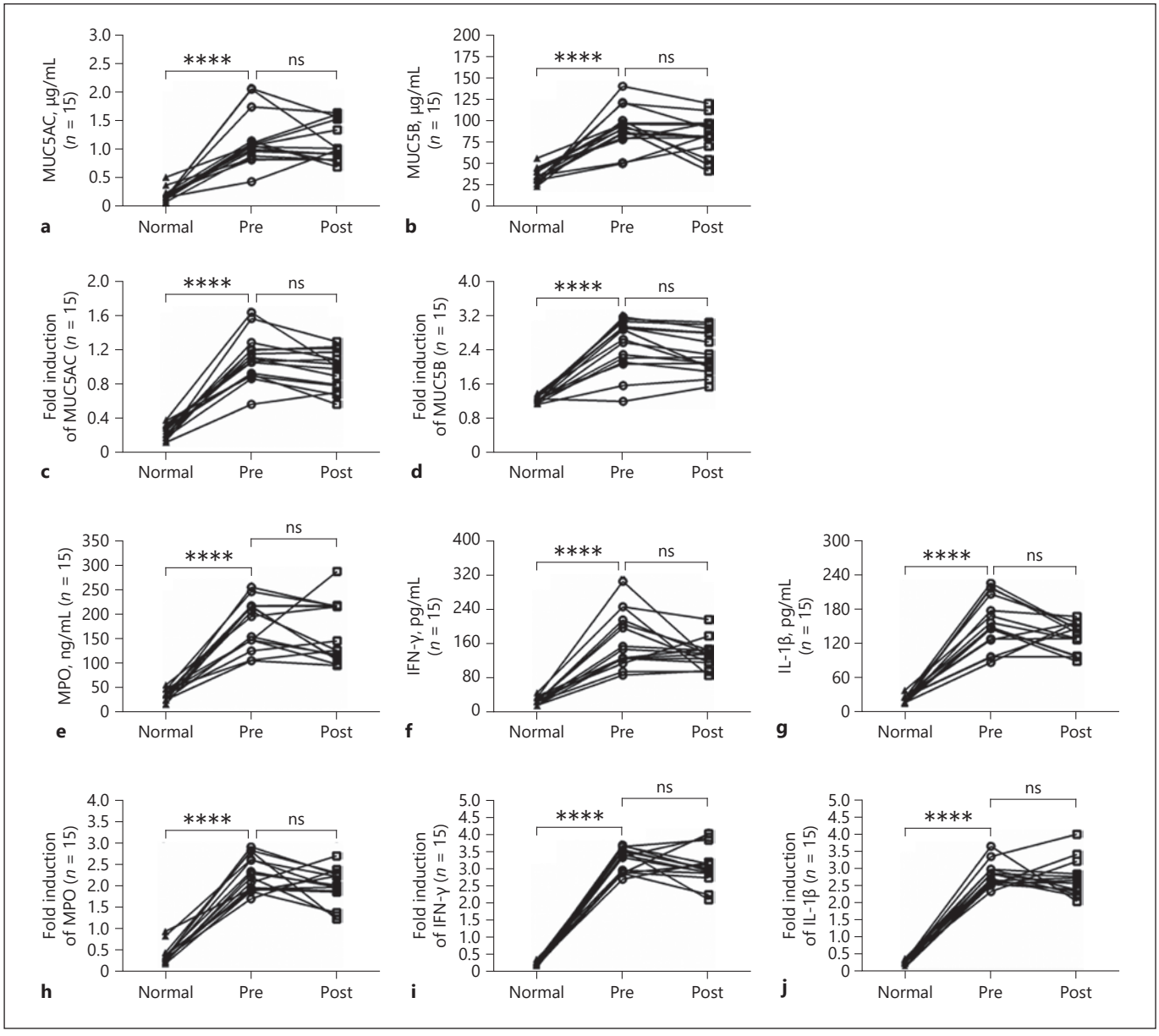

Fig. 7. Comparisons of inflammatory substances. **a** MUC5AC. **b** MUC5B. **c** MUC5AC mRNA. **d** MUC5B mRNA. **e** MPO. **f** IFN-γ. **g** IL-1β. **h** MPO mRNA. **i** IFN-γ mRNA. **j** IL-1β mRNA. Normal, normal mucosa. MUC, mucin; MPO, myeloperoxidase; IFN, interferon; IL, interleukin; Pre, pretreatment; Post, posttreatment; SEM, standard error of the mean; ns, not significant. The values shown are expressed as mean ± SEM. ****$p < 0.0001$.

Inflammatory Reactions

MUC5AC and MUC5B are major components of respiratory secretion in CRS and play important roles in the pathogenesis of sinus hypersecretion in this chronic disease [25]. Accordingly, we detected the expressions of MUC5AC and MUC5B in NPs. We found the proteins and their mRNAs of these 2 mucins were upregulated in NPs when compared with those in normal nasal mucosa (Fig. 7a–d). Notwithstanding, after the treatment with budesonide, their expressions were not reduced statistically in polyp tissues (Fig. 7a–d). It is well known that neutrophilic CRSwNP is a type-1 inflammatory response [20]. Hence, we made relevant experiments to determine the concentrations of type-1inflammatory mediators in-

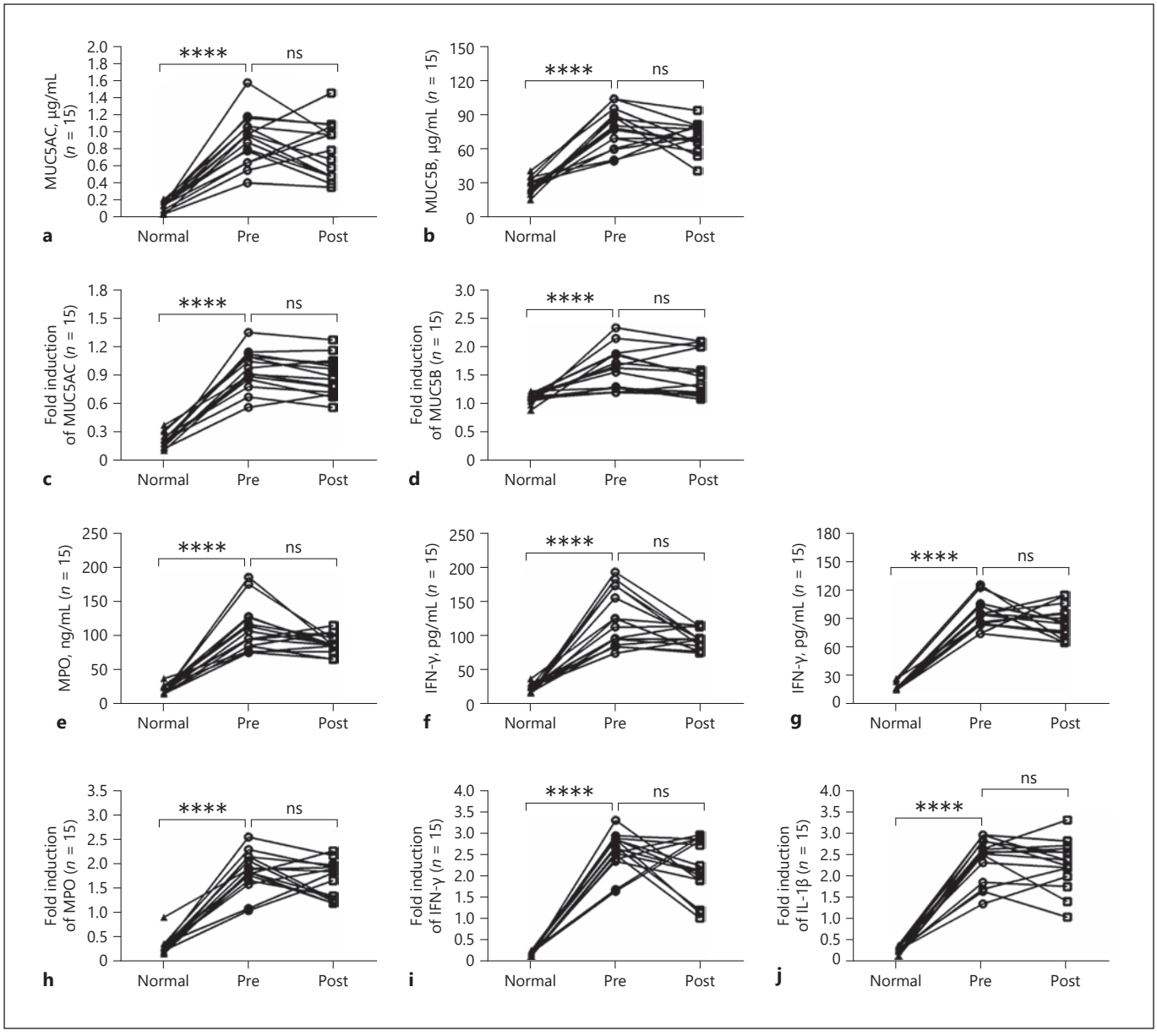

Fig. 8. Comparisons of inflammatory substances in cultures. **a** MUC5AC. **b** MUC5B. **c** MUC5AC mRNA. **d** MUC5B mRNA. **e** MPO. **f** IFN-γ. **g** IL-1β. **h** MPO mRNA. **i** IFN-γ mRNA. **j** IL-1β mRNA. Normal, normal mucosa. MUC, mucin; MPO, myeloperoxidase; IFN, interferon; IL, interleukin; Pre, pretreatment; Post, post-treatment; SEM, standard error of the mean; ns, not significant. The values shown are expressed as mean ± SEM. ****$p < 0.0001$.

cluding MPO, IFN-γ, and IL-1β. The data we obtained clearly showed that MPO, IFN-γ, and IL-1β and their mRNAs were all heightened in neutrophilic NPs in contrast with those in normal tissues (Fig. 7e–j). Additionally, the expressions of the abovementioned substances were not downregulated irrespective of the clinical budesonide intervention (Fig. 7e–j).

Inflammatory Reactions in vitro

For going a step further in comprehension of glucocorticoid treatment on neutrophilic inflammation of NPs, we cultured NP tissues in vitro and tested contents of MUC5AC and MUC5B and inflammatory substances, such as MPO, IFN-γ, and IL-1β. The results displayed that MUC5AC and MUC5B and their mRNAs were ele-

vated in NPs cultured in vitro in contrast with those in normal mucosa (Fig. 8a–d) and were not reduced markedly after the administration of budesonide (Fig. 8a–d). Similarly, type-1 mediators such as MPO, IFN-γ, and IL-1β and their mRNAs were also heightened in neutrophilic polypoid tissues when compared with those in normal tissues (Fig. 8e–j) and showed no significant changes after the application of steroids (Fig. 8e–j).

Discussion

Most earlier studies would like to pay attention to CRSwNP patients of European descent, such as European and US studies. As has been described in these studies, histological analysis would reveal tissue eosinophilia in the majority of polyp specimens. The presence of eosinophilic NPs is even more prominent in patients with concomitant asthma, aspirin sensitivity, or both [26, 27]. CRSwNP in white patients always shows a Th2 polarization with high IL-5 and immunoglobulin E concentrations [1]. More recently, some studies have shown that NP samples from Asian patients living in Asia or second-generation Asians residing in the USA have an increasing type-1 inflammatory environment with enhanced levels of IFN-γ and reduced levels of IL-5 [6, 23]. It remains unclear why Asian NP subjects are more likely to have type-1 inflammatory responses in polyp tissues compared with patients from European countries and the USA. Some scholars inferred that genetic factor might play a role in the regulation of eosinophilia [23].

Some studies have demonstrated that neutrophilic NPs are less frequently related to atopy and asthma [28, 29]. In addition, neutrophilic CRSwNP patients have a lower rate of recurrence [9] and a lower blood eosinophil number [28, 29]. It should be emphasized that macrolide antibiotics are the primary management for neutrophilic NPs; however, corticosteroids are the main therapy for eosinophilic CRSwNP [30]. The purpose of corticosteroid treatment in CRSwNP is to alleviate the local inflammation through the direct decrease of eosinophilic activation [16] and the indirect effect on the secretion of chemotactic cytokines from the nasal mucosa and polyp's epithelial cells [31, 32]. As regards neutrophilic type of NPs, studies have reported that increased neutrophils reduce the response to oral corticosteroid therapy [14]. However, whether topical steroids are efficacious on neutrophilic NPs is yet to be identified. Thus, we made this investigation.

In this study, we used a confocal immunofluorescence microscope to analyze the endotype of CRSwNP. We found <10 eosinophils/HPF and the presence of a focal or diffuse neutrophil infiltrate in all NP specimens from the enrolled patients. As a result, these patients were identified as neutrophilic CRSwNP subjects. We did not select the Lund-Mackay CT score as one of clinical evaluations because these patients did not intend to receive endoscopic sinus surgery and unnecessary exposure to radiation. Scores of VAS, SNOT-22, and EAs are always selected as clinical parameters in many relevant clinical trials. So, we also used these score systems in this clinical research. Based on our data, we found no statistical differences between pre- and posttreatment with budesonide nasal spray in VAS, SNOT-22, and EA scores. The data suggested that budesonide might not improve clinical symptoms of neutrophilic CRSwNP subjects irrespective of the 3-month therapy.

To determine histological changes, in the first place, we performed immunohistochemical staining of MPO to investigate the effect of budesonide treatment on neutrophilic infiltrate in NPs. We found that neutrophil numbers were not decreased significantly after the steroid treatment compared with those of pretreatment. In the second place, we conducted a study on mucosecretory cells in NPs. Mucins have a significant role in airway immunity by capturing infectious viruses and bacteria and expelling them through mucociliary [21]. CRS has a feature of mucus hypersecretion of the upper airway. It has been reported that MUC5AC and MUC5B are major components of respiratory secretions in CRS and may play vital roles in the pathogenesis of sinus hypersecretion in this condition [25]. MUC5AC is in superficial airway goblet cells, and MUC5B is in submucosal gland mucous cells [21]. Thus, we measured histological changes of these 2 types of cells. Surprisingly, we found there was no significant decrease in the numbers of goblet cells after the corticosteroid therapy. Furthermore, the results from the PAS staining scores indicated that the intensity of staining of submucosal gland cells was not reduced significantly in NPs by the treatment. These findings implied that topical budesonide could not lead to significant histological changes in neutrophilic NP tissues.

The differences between eosinophilic and neutrophilic NPs seem to go beyond the numbers of eosinophils and neutrophils. A study has shown that Th cells involved in NP inflammation also characterize the differences between these 2 types of NPs [6]. NPs from white and Asian patients are both characterized by T-cell activation and impaired Treg function. However, effector T cells from

white patients were Th2 biased, whereas the cells from Asian ones demonstrated a Th1/Th17 polarization [6]. On the basis of the above investigations, we examined T-cell subpopulations including $CD8^+$ T cells and Tregs in NPs to make further efforts on the evaluation of the budesonide treatment. The results demonstrated that this local glucocorticoid could not influence the percentages of $CD8^+$ T cells in total $CD3^+$ T cells and Tregs in total $CD4^+$ T cells. We concluded that budesonide could not inhibit the type-1 biased inflammatory responses in neutrophilic CRSwNP.

As for inflammatory reactions, we performed experiments to examine levels of mucins including MUC5AC and MUC5B and inflammatory mediators including MPO, IFN-γ, and IL-1β in vivo and in vitro. We found that the expressions of the abovementioned substances were not decreased after the clinical administration of budesonide. The results suggested that the treatment with steroids could not impact neutrophilic inflammation in NPs in vivo. For exploring further the glucocorticoid intervention on neutrophilic inflammation, we cultured polypoid tissues in vitro and assessed contents of these inflammatory substances. The data reflected no significant changes after the application of budesonide. The findings again suggested that the steroid intervention did not limit the inflammatory condition in neutrophilic NPs in vitro.

This study has some limitations, such as a small number of subjects and a relative short duration. Furthermore, the study is not a multicenter randomized control trial. So, the level of evidence is somewhat low.

Conclusion

In conclusion, budesonide nasal spray did not improve the clinical evaluations including VAS, SNOT-22, and EA scores and could not influence the inflammatory conditions in neutrophilic NPs in vivo and in vitro.

Statement of Ethics

The study was performed according to the Helsinki Declaration and approved by the Ethics Committee of Huashan Hospital of Fudan University (No. 2016-054). A written informed consent was obtained from all patients.

Disclosure Statement

The authors declare that they have no conflicts of interest.

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