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Neutrophil extracellular traps in nasal secretions of patients with stable and exacerbated chronic rhinosinusitis and their contribution to induce chemokine secretion and strengthen the epithelial barrier

A short running title: neutrophil extracellular traps in chronic rhinosinusitis.

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Summary

Background: Neutrophil extracellular traps (NETs) participate in innate immunity by trapping microorganisms. Their pathophysiological implications have not been defined in chronic rhinosinusitis (CRS).

Objective: We investigated the presence of NETs in nasal secretion of patients with stable or exacerbated CRS and evaluated whether NETs participate in the secretion of chemokines in sinonasal epithelial cells, the epithelial permeability, and transendothelial leukocyte migration, and elucidate whether NETs are released by macrolides and dexamethasone.

Methods: The presence of NETs in nasal secretion and the release of NETs from neutrophils stimulated with macrolides or dexamethasone were evaluated by dsDNA Assay kit and fluorescence microscope. The chemokine secretion, epithelial permeability, and transendothelial leukocyte migration were measured in cultured cells incubated with NETs, the supernatant of unstimulated neutrophils (unstim), NETs inhibitor (DPI), or H3Cit, where the expression of junctional complex proteins and ICAM-1 were evaluated by real time PCR, western blots, and confocal microscope.

Results: The amount of NETs and NETs-forming neutrophils in nasal secretion increased in exacerbated CRS. Epithelial cells treated with NETs or H3Cit secreted chemokines and showed decreased permeability associated with up-regulated junctional complex proteins. Increased transendothelial leukocyte migration associated with up-regulated ICAM-1 was

noted in endothelial cells treated with NETs or H3Cit. These findings were not found in cells treated with unstim, or DPI. NETs were released by macrolides, but not by dexamethasone.

Conclusions and Clinical Relevance: NETs formation increased in exacerbated CRS, inducing chemokine secretion, strengthening the epithelial barrier, and promoting the neutrophils infiltration. Therefore, the release of NETs in CRS might be beneficial or detrimental to CRS patients.

Keywords: neutrophil extracellular traps, chemokines, epithelial permeability, transendothelial leukocyte migration, macrolides, dexamethasone.

1 | INTRODUCTION

Chronic rhinosinusitis (CRS) is a chronic persistent inflammation of the sinonasal mucosa, accompanied by accumulation of inflammatory cells. Although the etiology of CRS still remains unclear, accumulating evidence suggests that dysregulation of innate and adaptive immunity contributes to persistent inflammation of the sinonasal mucosa.¹ Loss of immune barrier, including increased epithelial permeability, is a common feature of CRS. Increased epithelial permeability may allow mucosal pathogens to cross the epithelial barrier, initiating recruitment of adaptive immune cells by the activation of endothelial adhesion molecules and chemokine production.²

Based on endoscopic findings, it is generally accepted that CRS is divided into two types, CRS without nasal polyps (CRSsNP), and CRS with nasal polyps (CRSwNP).^{3,4} Most of the sinonasal mucosa derived from CRSsNP display excessive infiltration of neutrophils and are associated with high Th1 cytokine expression. In western population, the CRSwNP mucosa is associated with predominant infiltration of eosinophil and expression of Th2 cytokines, whereas a considerable portion of CRSwNP in Asian subjects is neutrophil-dominant.^{3,4}

Studies have shown upregulation of IL-8, the main chemokine fostering neutrophils recruitment in both CRSwNP and CRSsNP.⁵⁻⁷ Infiltration of neutrophils, the major cells responsible for inflammation, found in the sinonasal mucosa of patients with CRS suggests that neutrophils may act as the primary pathologic agent in CRS.^{3,7} Nonetheless, the role of the neutrophils in CRS remains unclear.

Neutrophils act as primary effector cells in the innate and acquired immune system. They are equipped for phagocytosis and intracellular lysis of microorganisms, and eventually kill pathogens. Alongside phagocytosis, neutrophils can release DNA and antibacterial proteins such as histones and granule enzymes, all of which form an extracellular fibrous structures called neutrophil extracellular traps (NETs). NETs are considered to play a role in innate immunity by trapping various microorganisms.⁹⁻¹¹ Although NETs function as an effective antimicrobial factor, they themselves can be pathogenic when they are produced in excess, acting as a double-edged sword by contributing to host tissue damage.¹² NETs are directly cytotoxic to pulmonary epithelium and endothelial cells, and accumulation of excessive NETs in alveoli and airway is associated with chronic airway diseases such as asthma and COPD. This suggests that NETs participate in the pathogenesis of chronic pulmonary inflammation.¹³⁻¹⁵ In this respect, NETs may be involved in the pathogenesis of CRS. However, no investigation has yet been made into CRS pathogenesis.

Therapy targeting the pathways of mucosal inflammation in CRS has resulted in elucidation of the use of glucocorticoids and macrolides for treatment. Treatment with glucocorticoids and macrolide antibiotics is recommended for patients with CRSwNP or CRSsNP.³ However, neutrophils are generally considered to be less responsive to glucocorticoids, since pathways involved in neutrophil activation, including adherence, are not effectively inhibited by glucocorticoids.² Furthermore, macrolide is effective in

decreasing neutrophil infiltration and the concentration of neutrophil chemoattractant IL-8 in the nasal secretions of patients with CRS.¹⁶ However, the effect of macrolide and glucocorticoid on the formation of NETs in CRS is not well documented.

Therefore, the purpose of the present study is

- 1) To investigate the presence of NETs in the nasal secretions of patients with CRSwNP or CRSsNP, and acute exacerbated CRSwNP or CRSsNP.
- 2) To evaluate whether NETs participate in the secretion of chemokines in sinonasal epithelial cells, and regulate the epithelial permeability and transendothelial leukocyte migration.
- 3) To elucidate whether macrolide antibiotics (clarithromycin; 14-membered, azithromycin; 15-membered, and josamycin; 16-membered) and dexamethasone have an effect on the formation of NETs in sinonasal mucosa.

2 | METHODS

2.1 | Subjects

We examined samples of nasal secretions from five groups of subjects (Table 1); i) acute exacerbation of CRSsNP (AECRSsNP), ii) acute exacerbation of CRSwNP (AECRSwNP, non-eosinophilic type), iii) stable CRSsNP, iv) stable non-eosinophilic CRSwNP, v) normal controls.

CRSsNP and CRSwNP were diagnosed based on the criteria of the European Position Paper.³ Acute exacerbation of chronic rhinosinusitis was usually defined as sudden

aggravation of sinonasal symptoms, such as rhinorrhea, stuffiness, and/or facial pain in patients with underlying CRS.¹⁷ Subjects who underwent septoplasty for nasal stuffiness without either sinus disease or pathological lesions was considered to be a normal healthy control.

Normal sinus mucosa was collected from the ethmoid sinus of patients (n=15, M: F= 13: 2, age:19-45 years) with a blowout fracture during endoscopic reduction and was prepared for normal epithelial and endothelial cells culture.

Approval to conduct this study was obtained from our institutional review board and ethics committee, and all participants gave signed informed consent. Endoscopic physical findings and symptoms were evaluated as described previously.^{18, 19} Exclusion criteria included the use of antihistamine, antibiotics, or steroids for the preceding 3 months. Patients with concomitant allergic rhinitis or asthma were also excluded.

2.2 | Nasal lavage, collection of nasal secretions, and NET quantification in nasal lavage fluids

Nasal secretions were obtained from all participants using nasal lavages as previously described.²⁰ After nasal lavage fluids were centrifuged, the supernatants were stored at -75°C for quantification of extracellular DNA (eDNA). Cell pellet was used to prepare for immunofluorescence.

To determine the abundance of NETs in the cell-free supernatant, eDNA was quantified using the Quant-IT™ Picogreen® dsDNA Assay Kit (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions.

For quantitative analysis of NETs in cell pellet, the cells attached to the glass slide were reacted with mouse anti-human neutrophil elastase (NSE, Santa Cruz Biotechnology, Calif., USA) and then incubated with Alexa Fluor 594-conjugated goat anti-mouse secondary antibody (Abcam, Cambridge, UK). For DNA staining, cells were incubated with Sytox green (Invitrogen, Carlsbad, CA). Five images were randomly selected and were taken from different regions of the slides. The images files were loaded as separate image stacks in Image software for cell count. The quantitative analysis of NETs-forming neutrophils was calculated as the average % of NETs-forming neutrophils per total number of neutrophils. The results were expressed as mean % as described previously.²¹

2.3 | Isolation of blood neutrophils and in vitro induction of NET

Neutrophils were isolated from heparinized whole blood of healthy donors by using Polymorprep (Axis-Shield, Oslo, Norway) according to the manufacturer's instruction. Viability of neutrophils was measured 99% by an MTT assay.

Isolated neutrophils (1×10^6 neutrophils) were divided into 3 groups (For control conditions, the first group is cultured and treated with cultured medium without phobol myristate acetate (PMA); The second group is cultured with PMA (100nM) as described previously.²²; To analyze the ability of NET inhibitor to prevent NET release from stimulated neutrophils as described previously,²³ the third group is treated with diphenyleneiodonium chloride (DPI 10 uM, Sigma-Aldrich) for 30 min and followed by PMA stimulation. After neutrophils in 3 groups were cultured for 3 hours respectively, medium was removed and adherent NETs were washed with prewarmed RPMI1640 (Lonza Walkersville Inc.; Walkersville, MD, USA). Thereafter, 1 ml RPMI1640 per well was added to each well and NETs from each well were liberated with 5U/ml micrococcal nuclease (MNase, Sigma-

Aldrich) for 30 min at 37°C. MNase was then inactivated with 5mM EDTA per well. NET-containing supernatants were collected in a 1.5 ml tubes by centrifugation at 300 x g for 5 min at 4°C and were kept at -70°C until further use for the stimulation of cultured cells.

Extracellular DNA concentration in NETs were quantified by the Picogreen® dsDNA Assay Kit and stored at -70°C for further experiments. PMA stimulation resulted in a 250 ± 30 ng/ul NETs production.

2.4 | The effect of NETs on chemokine secretion by sinus epithelial cells

Normal sinus epithelial cells collected from normal sinus mucosa were cultured at an air-liquid interface, as described previously.²⁴ Cultured epithelial cells were incubated with: (1) NETs recovered upon stimulation of 1×10^6 neutrophils (250 ng/ml); (2) with supernatants of neutrophils incubated with NET inhibitor, DPI (10 μ l); (3) with human recombinant citrullinated histone 3, a key component to determine the presence of NETs, (H3Cit, 1 μ M), (Cayman Chemical, Ann Arbor, MI, USA), (4) with supernatants of unstimulated neutrophils (10 μ l) for 24 h, as described previously.^{23, 25} Thereafter, cultured cells and supernatant were collected to evaluate the expression levels of chemokines with real-time PCR and ELISA.

2.5 | The effect of NETs on the epithelial permeability, transepithelial electrical resistance, and epithelial tight junctional protein expression, the expression of ICAM-1 in the endothelial cells, and transendothelial migration of leukocytes

Cultured epithelial cells were treated with each cytokines (20 ng/ml, Th1 cytokines; TNF- α , IL-17A, Th2 cytokines; IL-4, IL-13) for 24 hours in the presence or absence of NETs. Cells were also stimulated with DPI, unstimulated neutrophils, or with human recombinant H3Cit, according to the experimental methods described in the results section.

Epithelial permeability and transepithelial electrical resistance were also measured as described previously.²⁴ Thereafter, cultured epithelial cells were harvested to evaluate the expression levels of epithelial tight junctional proteins (zonular occludens (ZO)-1, ZO-2, and occludin) by real-time PCR and western blotting.

After cultured epithelial cells were stimulated, as mentioned above, the cultured cells were fixed in 3% paraformaldehyde and were incubated with antibodies against ZO-1, ZO-2, and occludin (Invitrogen). Thereafter, the expression and distribution pattern of tight junctional proteins were observed by Confocal laser microscopy.

Sinus endothelial cells were collected from normal sinus mucosa, as described previously,²⁴ and were differentiated in Transwell[®] system.

Migration assay were performed in Transwell plates with 3 μ m pore size filters, as described previously.²⁶⁻²⁸ In brief, endothelial cells were treated with TNF- α , IFN- γ , or IL-17A at a concentration of 20 ng/ml for 24 h. Isolated leukocytes (2×10^6 cells/mL) were then added to the upper compartment. The lower wells below the filter were filled with 20 nM N-formyl-methionyl-leucyl-phenylalanine (fMLP; Sigma-Aldrich, St Louis, MO, USA). After incubation for 4 h, migrated leukocytes were harvested and counted using trypan blue in a hemocytometer. The results of the migration assay are expressed as the ratio of migrated cells in the cells treated with the supernatant of unstimulated neutrophils (unstim), DPI, cytokines, NETs, or H3Cit, in cells co-incubated with cytokines and DPI, NET, or H3Cit, relative to the value for the untreated control cells.

At the end of the stimulation period, cultured endothelial cells were used to evaluate the expression levels of ICAM-1 by real-time PCR and western blotting, using anti-ICAM-1 antibody (R & D systems, Minneapolis, MN, USA).

2.6 | Real time PCR and western blot analysis

Total RNAs were obtained from cultured cells with Qiazol lysis reagent (QIAZEN Inc, CA, USA). Real-time PCR reactions were conducted using SYBR Premix EX TaqTM (Takara bio, Shiga, Japan). The mean threshold cycle (Ct) values were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the relative mRNA levels of target genes were analyzed with the $2^{-\Delta\Delta C_t}$.

For Western blot, proteins were isolated from cultured cells using RIFA buffer and were separated on SDS-PAGE and transferred onto PVDF membranes (BioRad, Bedford, MA, USA). Thereafter, the membranes were incubated with antibodies (ZO-1, ZO-2, occludin, ICAM-1, all antibodies were diluted at 1: 100). The bands were detected with enhanced chemiluminescence.

2.7 | Experiments to determine the effect of clarithromycin, azithromycin, josamycin, and dexamethasone on the NETs release.

The effect of macrolides and dexamethasone on NETs release was performed as described previously.²⁸ In brief, isolated neutrophils (9×10^6 cells/well) were seeded onto six-well plates for quantification of NETs or 8-well Lab Tek Chamber Slides (ThermoFisher Scientific) for NETs visualization and were incubated for 3 h at 37 °C with macrolides or dexamethasone. Thereafter, media was centrifuged to pellet the neutrophils. The quantification of eDNA was measured using the Quant-ITTM Picogreen® dsDNA Assay Kit (Invitrogen).

For NETs visualization, chamber slides were fixed with 4 % PFA. The cells attached to the glass slide were reacted with human anti-mouse neutrophil elastase (NSE, Santa Cruz Biotechnology, Calif., USA) and then incubated with Alexa Fluor 594-conjugated goat anti-mouse secondary antibody (Abcam, Cambridge, UK). For DNA staining, cells were

incubated with Sytox green (Invitrogen, Carlsbad, CA). Five images were randomly selected and were taken from different regions of the slides. The images files were loaded as separate image stacks in Image software for cell count. The quantitative analysis of NETs-forming neutrophils was calculated as the average % of NETs-forming neutrophils per total number of neutrophils. The results were expressed as mean % as described previously.²¹

2.8 | Determination of chemokine secretion by ELISA

Chemokine levels in supernatants were determined by an ELISA (R&D Systems), according to a commercial protocol. The chemokines analyzed are TARC, MCP-1, MIP-1 α , CXCL2, CXCL3, GRO- α , IL-8, GCP-2, ENA-78, eotaxin.

2.9 | Statistical analyses

All statistical analyses were calculated using SPSS for Windows (version 16.0.0; SPSS, Chicago, IL, USA). The age differences, SNOT-20, and endoscopic score among the five groups, as well as all data obtained by real-time PCR, ELISA, and Western blot analysis were evaluated by The Kolmogorov-Smirnov test to inspect the normality and homogeneity of variance. For normally distributed data, ANOVA with a Bonferroni post-hoc test to adjust for multiple comparisons was applied. For non-parametric data, a Kruskal-Wallis test with a Dunn's post-hoc test to adjust for multiple comparison was employed.

3 | RESULTS

The demographic and clinical characteristics of patients enrolled in the study are detailed in Table1. There were no significant differences between the five groups in terms of patients age and gender distribution. The average SNOT-20 and endoscopic scores are also summarized in Table 1.

3.1 | The presence of NETs and NETs-forming neutrophils in nasal secretion

Quantitative and morphological evaluation of NETs in nasal lavage fluids clearly show that the levels of eDNA and the number of NETs-forming neutrophils in nasal secretion increased in nasal secretion of patients with CRS, irrespective of nasal polyp presence, compared with the healthy control (Fig 1, A, B, and C). Interestingly, the level of eDNA and the number of NETs-forming neutrophils were significantly higher in AECRS with or without nasal polyps, compared with stable CRS with or without nasal polyps, respectively (Fig 1, A, B, and C). However, there were no statistical significance in the amount of eDNA and the number of NETs-forming neutrophils between stable or exacerbated CRSwNP and CRSsNP (Fig 1, A, B, and C).

3.2 | Effect of NETs on the chemokine secretion by cultured epithelial cells

Since the sinonasal epithelium is a major source of chemokine secretion in CRS, the functional significance of NETs was assessed by analyzing the chemokines secreted from epithelial cells. After the cultured epithelial cells were treated with the supernatants of unstimulated neutrophils, DPI, NETs, and H3Cit, the expression levels of various chemokines were analyzed in harvested cells and supernatants by real-time PCR and ELISA. The results showed that the levels of MCP-1, MIP-1 α , CXCL2, CXCL3, GRO- α , IL-8, GCP-2, ENA-78, and eotaxin are prominently up-regulated in NETs-treated cells, compared with cells treated with the supernatants of unstimulated neutrophils. (Fig 2, A and B). The expression levels of chemokines upregulated by NETs were also noted in cells stimulated with H3Cit, whereas their levels were not increased in cells treated with DPI (Fig 2, A and B).

3.3 | Effect of NETs on the permeability and transepithelial electrical resistance in cultured epithelial cells, and the expression of intercellular junctional complex proteins

In order to evaluate the effect of NETs on immune barrier, we measured the epithelial permeability and transepithelial electrical resistance using cultured epithelial cells. Cultured cells stimulated with NETs showed a marked decrease in epithelial permeability associated with increased electrical resistance, compared to cells treated with supernatant of unstimulated neutrophils or non-treated control cells (Fig 3, A and B). Decreased epithelial permeability associated with increased electrical resistance was also found in cultured cells treated with H3Cit, but not in cells treated with DPI (Fig 3, A and B).

Furthermore, epithelial permeability was increased in cells stimulated with Th1 (TNF- α or IL-17A) and Th2 cytokine (IL-4 or IL-13), but this increase was significantly blocked in cells co-stimulated with each cytokines and NETs or H3Cit, as shown in Fig 4. In addition, electrical resistance was also decreased in cells treated with Th1 (TNF- α or IL-17A) and Th2 cytokine (IL-4 or IL13), but decreased resistance was recovered in cells co-treated with each cytokine and NET or H3Cit respectively, but not in cells stimulated with DPI, as shown in Fig 4.

To understand the mechanism underlying decreased epithelial permeability, the expression levels of intercellular junctional complex proteins, including ZO-1, ZO-2, and occludin, in epithelial cells were evaluated after treatment with NETs, DPI, or H3Cit, respectively. The data showed that the levels of ZO-1, ZO-2, and occludin are increased in cells treated with NETs or H3Cit, when compared with non-treated control cells.

Additionally, their levels in cells stimulated with DPI or the supernatants of unstimulated neutrophils were not significantly different from non-treated control cells (Fig 3, C-K).

Furthermore, confocal microscopy showed a strong positivity for ZO-1, ZO-2, and occludin

in non-treated control cells and cells stimulated with supernatant of unstimulated neutrophils or DPI, where they appear as contiguous ring around the peripheral margins of the cells, colocalized at sites of cell-cell contact (Fig 3, E, H, K) The distribution pattern of ZO-1, ZO-2, and occludin in cells treated with NETs or H3Cit has an intact appearance which is similar to pattern observed in non-treated control cells or cells stimulated with supernatant of unstimulated neutrophils or DPI (Fig 3, E, H, K).

Exposure to TNF- α , IL-17A, IL-4, or IL-13 induced a noticeable downregulation of ZO-1, ZO-2, and occludin in control cells, which was confirmed by real time PCR and western blots. Interestingly, cells co-stimulated with each cytokine and NET or H3Cit displayed up-regulated levels of ZO-1, ZO-2, and occludin, compared with those in cell treated with cytokines only, or co-stimulated with each cytokine and DPI (Fig 5). Consistent with these quantitative data, confocal microscopic findings showed that exposure of the cells to these cytokines resulted in a loss of ZO-1, ZO-2, and occludin immunoreactivity from the cellular border. However, this effect was blocked by co-stimulating with each cytokine and NETs or H3Cit, showing intact distribution of ZO-1, ZO-2 and occludin (Fig 5, C, F, and I).

3.4 | Effect of NETs on the expression of ICAM-1 in cultured endothelial cells and the transendothelial leukocyte migration

In order to evaluate the effect of NETs on the infiltration of neutrophils into the sinonasal mucosa, the levels of ICAM-1 in endothelial cells were assessed after treatment with NETs, H3Cit, or DPI, respectively. The levels of ICAM-1 were increased in endothelial cells stimulated with NETs or H3Cit, when compared with cells treated by DPI or the supernatants of unstimulated neutrophils (Fig 6, A and B). Furthermore, the expression levels of ICAM-1 increased in endothelial cells treated with TNF- α , IFN- γ , and IL-17A. However, ICAM-1

levels in endothelial cells co-stimulated with these cytokines and NETs or H3Cit, as indicated in Fig 6A and B, were higher than that in endothelial cells treated with the cytokine only. In accordance with these results, the number of leukocytes migration in endothelial cells stimulated with NET or H3Cit was higher than the number in untreated control cells or cells stimulated by the supernatants of unstimulated neutrophils or DPI (Fig 6, C). Furthermore, the number of leukocyte migrated through the endothelial cells co-treated with TNF- α , IFN- γ , and IL-17A and NET or H3Cit respectively was also higher than that in endothelial cells treated with cytokine only or co-treated with each cytokines and DPI (Fig 6, C).

3.5 | Effect of macrolides and dexamethasone on NET release

Macrolides and glucocorticoids are widely used for treating CRS depending on their mechanisms of action including anti-inflammatory and immunomodulatory properties. We studied whether the action of macrolides and glucocorticoids can be mediated through the generation of NETs and investigated the ability of different macrolide subgroups to generate NETs, according to various concentrations. The concentrations of eDNA in clarithromycin-treated neutrophils were increased in dose-dependent pattern. In azithromycin-treated samples, eDNA concentration is highest at the dose of 50 μ M whereas josamycin-treated samples showed highest eDNA concentrations at the dose of 10 μ M. However, although neutrophils were treated with varying doses of dexamethasone, the amount of eDNA in dexamethasone-treated neutrophils was similar in that in non-treated control neutrophils (Fig 7, A, B, and C). These results were observed in fluorescence microscopy (Fig 7, C).

4 | DISCUSSION

The present study shows the presence of NETs and NET-forming neutrophils in nasal secretion of patients with CRSwNP or CRSsNP, exacerbated or stable. The level of eDNA expression and the number of NETs-forming neutrophils in nasal secretion were significantly higher in AECRSsNP or AECRSwNP, compared to stable CRSsNP or CRSwNP. The SNOT-20 and endoscopy score were higher in patients with AECRS than those with stable CRS. Sinonasal epithelial cells stimulated with NETs induced secretion of chemokines, which are involved in the migration of inflammatory cells such as neutrophils, eosinophils, and lymphocytes. Furthermore, sinonasal epithelial cells treated with NETs or H3Cit showed decreased permeability associated with increased electrical resistance. In parallel with these findings, the expression of tight junctional complex proteins such as ZO-1, ZO-2, and occludin were increased in NETs or H3Cit-stimulated epithelial cells. Endothelial cells treated with NETs or H3Cit showed increased transendothelial leukocyte migration which was associated with increased ICAM-1 level. Taken together, the current study indicates that NETs in CRS induces chemokine secretion from superficial epithelial cells, strengthen epithelial barrier function and participates in the further infiltration of neutrophils into the sinonasal mucosa. Therefore, it is uncertain whether reducing the release of NETs in CRS might be beneficial or detrimental to CRS patients. Moreover, we found that macrolides exert a releasing effect of NETs from isolated neutrophils.

Patients with underlying symptoms of CRS commonly experience episodic aggravation of symptoms, which is mainly triggered by viral and bacterial infections on underlying chronic inflammatory mucosa.^{3, 29, 30} Indeed, the bacterial species isolated from patients with AECRS is anaerobic and is similar to those found in CRS.³¹ The incidence of AECRS commonly increases during the winter season compared with other seasons, suggesting that AECRS has a typical pattern of virus seasonality.³² In the present study, NETs levels and the number of

NETs-forming neutrophils were increased in nasal secretion of patients with AECRS, when compared with stable CRS. Increased release of NETs in AECRS may be in the response to the infectious process triggered by viral and bacterial infection, acting as anti-microbial defense. This interpretation is supported by evidence that microorganisms including bacteria and virus induce the release of NETs, and NETs act to kill microbes to prevent microbial dissemination.³³ In this respect, increased NETs levels in CRS or AECRS may be beneficial to fighting infection.

The sinonasal epithelium can produce chemokines after exposure to mucosal pathogens, which induce the recruitment of immune cells, initiating mucosal inflammation.² As a general rule, members of the CXC chemokines are chemotactic for neutrophils, and CC chemokines are chemotactic for monocytes and sub-set of lymphocytes.³⁴ In the present study, CXC chemokines such as GRO- α , ENA-78, GCP-2, IL-8, CXCL2, and CXCL3, and members of CC chemokines, including MCP-1, MIP-1 α , and eotaxin were increased in cells stimulated with NETs or H3Cit. However, these findings were not observed in cells treated with supernatants of non-stimulated neutrophils or DPI. Therefore, our findings indicate that NETs can exert proinflammatory effects on sinonasal epithelial cells, contributing to the further recruitment of inflammatory cells such as neutrophils, lymphocytes, and eosinophils by inducing the chemokines secretion. Similarly, a previous study reported that NETs induces the production of IL-8 and IL-6 in alveolar A549 or bronchial epithelial cells, and suggested that NETs might participate in the amplification of inflammation by stimulating airway epithelial cells to produce inflammatory mediators.²³ On the other hand, Hahn et al reported that cytokine/chemokine-cleaving effects of NETs essentially depend on neutrophil density, and suggest that if reaching a critical density, neutrophils orchestrate the switch to resolution of inflammation by NET formation and the cleavage of inflammatory cytokines and

chemokines.³⁵ Collectively, these results add further weight to considerations of manipulating NETs release for therapeutic purposes in CRS.

The excessive NETs in the lung cause varying degree of damage, resulting in impaired pulmonary function. The severity of these patients' condition is positively correlated with the level of NETs in chronic pulmonary diseases.^{12, 15, 36, 37} Actually, NETs could enhance asthma severity by damaging airway epithelium.³⁷ In COPD, NETs formation correlates with the severity of airflow limitation.³⁸ Moreover, it has been reported that NETs are directly cytotoxic to pulmonary epithelial and endothelial cells.¹³ Thus, existing researches indicates that inhibiting NETs formation is an attractive strategy for preventing the deleterious effects of NETs. Nevertheless, on the basis of present literature, the role of NETs is ambiguous, acting between bactericidal defense and host tissue damage.^{11, 39-41} On the other hand, loss of immune barrier, including increased permeability of sinonasal epithelium, is a common feature of any form of CRS.⁴²⁻⁴⁴ In light of these previous results, we investigated whether NETs may contribute to increased epithelial permeability in CRS. By contrast to the aforementioned studies,⁴²⁻⁴⁴ our data showed that NETs and H3Cit, a key component of NETs, strengthen the epithelial barrier even at the basal level, and also reverse cytokine-induced increases in sinonasal epithelial permeability. Therefore, the present study suggests that NETs may have beneficial effects on the epithelial permeability, possibly contributing to the maintenance of epithelial integrity. This suggestion is confirmed by current data that the expression levels and distribution patterns of ZO-1, ZO-2, and occludin are well-preserved even in cytokine-stimulated cells after treatment with NETs or H3Cit. More comparable studies are required on this subjects.

Another study demonstrated that NETs could promote further leukocyte recruitment to lesion sites and thus potentiate the regional inflammatory response.⁴⁵ In agreement with these previous studies, our data showed that neutrophils migration increases in endothelial cells treated with NETs or H3Cit. Endothelial cells treated with NETs or H3Cit showed up-regulation of ICAM-1 levels, supporting the notion that NETs induce neutrophil migration. This is in line with a recent study showing that human saphenous vein and umbilical vein endothelial cells treated with NETs exhibit higher concentration of ICAM-1.⁴⁵ Therefore, NETs can mediate inflammatory responses by increasing the neutrophil migration through the increase of the endothelial cells surface expression of ICAM-1 in CRS.

NETs formation is triggered by different physiological and non-physiological compounds.⁴⁶ It is also suggested that antibiotics could be non-physiological compounds that influence NETs release. Bystrzycka et al reported that amoxicillin induces NETs formation, whereas clindamycin has no influence on NETs release.⁴⁷ On the contrary, pretreatment of neutrophils with azithromycin and chloramphenicol caused a significant decrease in NETs release after stimulation with PMA.⁴⁸ In these studies, they did not compare the amount of extracellular DNA of neutrophils treated with antibiotics to that of unstimulated neutrophils. However, we measured the amount of extracellular DNA released from macrolide or corticosteroid-treated neutrophils without PMA stimulation, and found that clarithromycin, azithromycin, and josamycin induces NETs release under the experimental conditions applied in the present study. A similar study has been performed by Knostantinidis et al., who tested the influence of macrolide antibiotics on the NETs release.²⁸ They showed that *in vitro* stimulation of neutrophils with clarithromycin and azithromycin induces the formation of NETs. Increased *ex vivo* NETs formation was demonstrated in neutrophils collected from patients with *H. pylori* positive gastritis 5 days after eradication therapy with clarithromycin.²⁸ Therefore, these findings suggest indirect antimicrobial action of

clarithromycin and azithromycin through the induction of NETs generation. Furthermore, our data demonstrated that josamycin can also induce release of NETs, requiring further comparable studies. On the other hand, NETs are among major mechanisms used by neutrophils to eradicate bacteria.¹¹ In addition, our data showed the effect of NETs on the epithelial permeability and leukocyte migration that is considered as a beneficial tool to eradicate chronic inflammation. Collectively, these data suggest that macrolides treatment may exhibit anti-inflammatory activity by generating NETs, contributing to control chronic rhinosinusitis. On the other hand, considering the evidence that NETs and inflammation are concomitant events, several studies explored the effects of dexamethasone on NETs formation, but showed conflicting results.^{49,}⁵⁰ In an equine model of asthma, dexamethasone decreased PMA-induced NETs formation, suggesting that NETs formation is sensitive to glucocorticoid.⁴⁹ However, another study showed no effects of dexamethasone on the NET release.⁵⁰ Our data also showed that the NETs is not released by application of glucocorticoids. More comparable studies are needed to explore the effect of corticosteroid on the NETs formation.

Although NETs were identified as an alternative mechanism of neutrophils to kill pathogens, the persistent accumulation of NETs can promote injury to the host. In the present study, the amount of NETs in nasal secretion increased in patients with AECRS, compared with stable CRS. In sinonasal mucosa, NETs induces chemokine secretion, tightens the epithelial barrier, and promote further infiltration of inflammatory cells into the mucosa. Furthermore, macrolide showed the action to induce NETs release. More research is required to understand whether the release of NETs may be beneficial or detrimental to CRS patients.

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CONFLICT OF INTEREST

Authors declare no conflicts of interest.

AUTHOR'S CONTRIBUTIONS

Jae Woong Hwang, Sang Hag Lee: contributed to conception, study design, data interpretation and writing of manuscript.

Jae Hyung Kim, Hyun Jung Kim, In Hak Choi, Hye Min Han; conducted data acquisition, analysis and interpretation.

Ki Jung Lee, Tae Hoon Kim; conducted study design and draft manuscript.

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Table 1 Characteristics of study groups

Study group	Age Mean (range)	Sex (M/F)	SNOT-20 score	Endoscopy score
Normal control (n= 31)	37.93±3.02	16/15	17.34 ± 1.55	0
CRSsNP (n= 26)	44.12±3.86	16/10	29.52 ± 3.54	2.55±1.89
CRSwNP (n= 18)	42.66 ± 2.84	11/7	38.83± 6.0	6.31±2.14
CRSsNP (exacerbated) (n= 7)	33.9 ± 11.9	4/3	35.7 ± 2.8	5.8±2.3
CRswNP (exacerbated) (n= 8)	32.1 ± 12.9	5/3	45.5 ± 4.9	7.1±2.2

FIGURE 1 A, The concentration of eDNA in nasal secretion of healthy subjects (cont), patient with CRSsNP, CRSwNP, AECRSsNP, and AECRSwNP. B, The number of NET-forming neutrophils in nasal secretion of healthy subjects (cont), patient with CRSsNP, CRSwNP, AECRSsNP, and AECRSwNP. C, The presence of NETs in nasal secretion of healthy subjects (cont), patient with CRSsNP, CRSwNP, AECRSsNP, and AECRSwNP was evaluated with immunofluorescence. Data are presented as means $\pm$ standard error of the mean (SEM) (* means $P < 0.05$). Red color indicates neutrophils stained with anti-NSE antibody. Green color represents NETs appearing as a web-like extracellular structure (arrow heads) stained with Sytox green which were released from neutrophils. Original magnification x 200.

FIGURE 2 The expression levels of chemokines secreted from cultured epithelial cells stimulated with the supernatants of unstimulated neutrophils (unstim), DPI, NET, and H3Cit was evaluated by real time PCR (A) and ELISA (B). In graph A and B, data from six independent experiments are presented as means $\pm$ SEM and are derived from six individuals. (* indicates $P < 0.05$).

FIGURE 3 Epithelial permeability (A) and transepithelial electrical resistance (B) in cultured epithelial cells stimulated with the supernatants of unstimulated neutrophils (unstim), DPI, NET, and H3Cit.

The expression levels and distribution patterns of ZO-1 (C, D, E), ZO-2 (F, G, H), and occludin (I, J, K) in cultured epithelial cells were evaluated with real time PCR (C, F, I), western blot (D, G, J), and confocal microscope (E, H, K) after stimulation with the

supernatants of unstimulated neutrophils (unstim), DPI, NET, and H3Cit. Right lower panel in E, H, and K shows representative bands evaluated with western blot.

Data from six independent experiments are presented as means $\pm$ SEM and are derived from six individuals. * indicates that there are statistically significant differences in epithelial permeability, electrical resistance, and the expression levels of ZO-1, ZO-2, and occludin among control cells, cells treated with unstim, DPI, NETs and H3Cit.

FIGURE 4 Graph A indicates that the epithelial permeability in epithelial cells treated with IL-4, IL-13, TNF- α , and IL-17A or co-treated with cytokines and supernatant of unstimulated neutrophils (unstim) was increased, compared with that of control cell (cont). Down-regulated epithelial permeability was observed in cells co-treated with cytokines and NET or H3Cit (IL-4+NET, IL-13+NET, TNF- α +NET, and IL-17A+NET, or IL-4+H3Cit, IL-13+H3Cit, TNF- α +H3Cit, and IL-17A+H3Cit), compared with that of cells treated with cytokine only or co-treated with cytokines and supernatant of unstimulated neutrophils (unstim).

Graph B shows that transepithelial electrical resistance in epithelial cells treated with IL-4, IL-13, TNF- α , and IL-17A or co-treated with cytokines and supernatant of unstimulated neutrophils (unstim) was decreased, compared with that of control cell (cont). Increased electrical resistance was observed in cells co-treated with cytokines and NET or H3Cit (IL-4+NET, IL-13+NET, TNF- α +NET, and IL-17A+NET, or IL-4+H3Cit, IL-13+H3Cit, TNF- α +H3Cit, and IL-17A+H3Cit), compared with that of cells treated with cytokine only or co-treated with cytokines and supernatant of unstimulated neutrophils (unstim). Data from six independent experiments are presented as means $\pm$ SEM and are derived from six individuals. (** indicates $P < 0.05$).

** indicates that there are statistically significant differences between normal control cells and cells treated with cytokines, co-treated with cytokines and supernatant of unstimulated neutrophils (unstim), or co-treated with cytokines and NET or H3Cit.

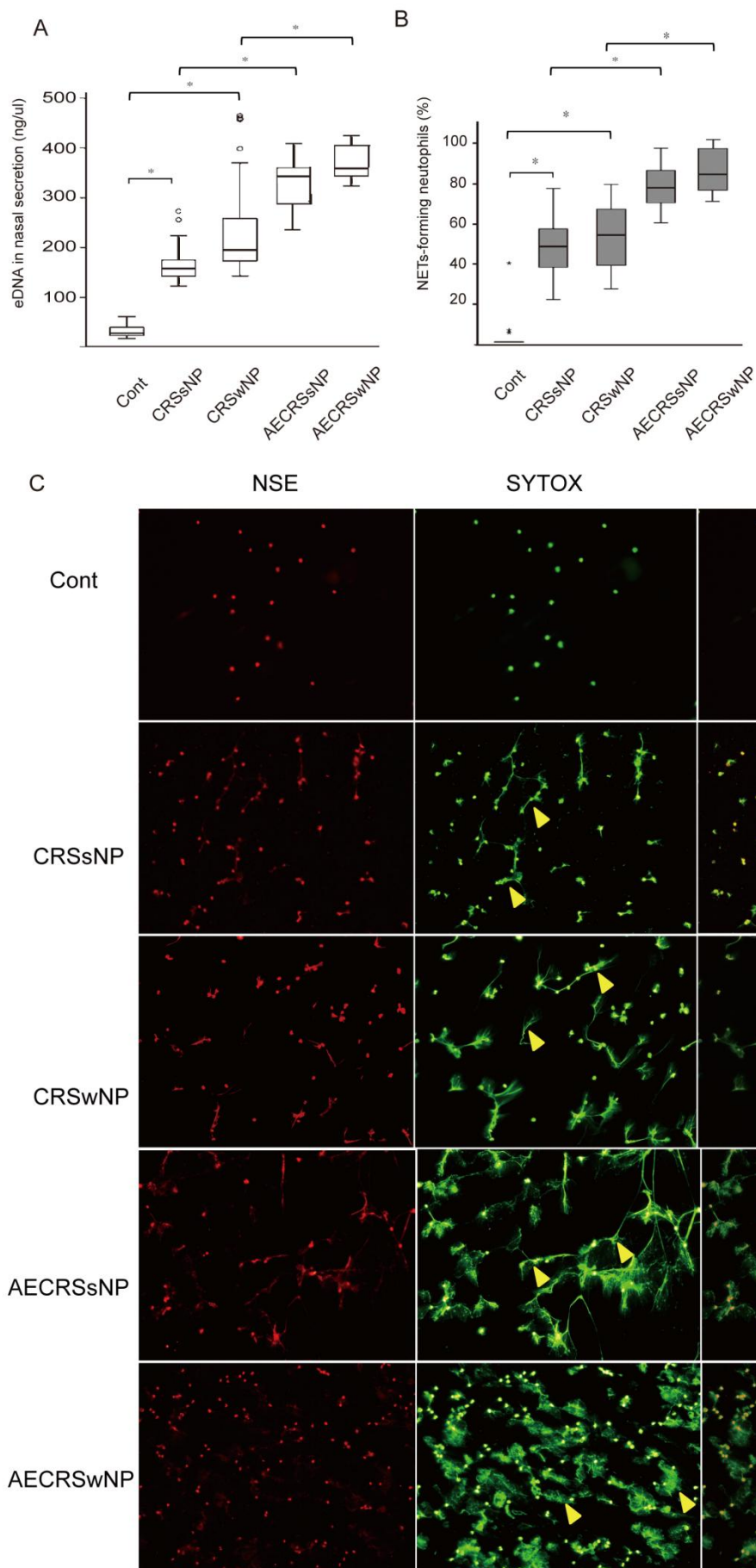
FIGURE 5 The expression levels of ZO-1 (A, B, and C), ZO-2 (D, E, and F), and occludin (G, H, and I) in epithelial cells treated with IL-4, IL-13, TNF- α , and IL-17A or in cells co-treated with these cytokines and supernatant of unstimulated neutrophils (unstim), cytokines and NET, or cytokines and H3Cit evaluated with real time PCR (A, D, G), western blot (B, E, H), and confocal microscope (C, F, I). ** indicates that there are statistically significant differences in the expression levels of ZO-1, ZO-2, and occludin between normal control cells and cells treated with cytokines or cells co-treated with cytokines and unstim. The expression levels of ZO-1 (A, B, and C), ZO-2 (D, E, and F), and occludin (G, H, and I) was increased in cells co-treated with cytokines and NET or H3Cit, compared with that of cells stimulated with only cytokines or cells co-treated with cytokines and unstim. Data from six independent experiments are presented as means $\pm$ SEM and are derived from six individuals. (* indicates $P < 0.05$). The distribution pattern of ZO-1 (C), ZO-2 (F), and occludin (I) observed with confocal microscope in cells stimulated with cytokines, co-treated with cytokines and unstim, co-stimulated with cytokines and NET or H3Cit. (Original magnification x 200).

FIGURE 6 The expression levels of ICAM-1 evaluated with real time PCR (A) and western blots (B) in cultured endothelial cells treated with the supernatants of unstimulated neutrophils (unstim) and DPI, or in non-stimulated control cells (cont) was compared with that of cells treated with NET, or H3Cit. Furthermore, the expression levels of ICAM-1 of cells treated with cytokines only or co-treated with cytokines and unstim was compared with that of cells co-stimulated with cytokines and NET or H3Cit. The upper panel of B represents

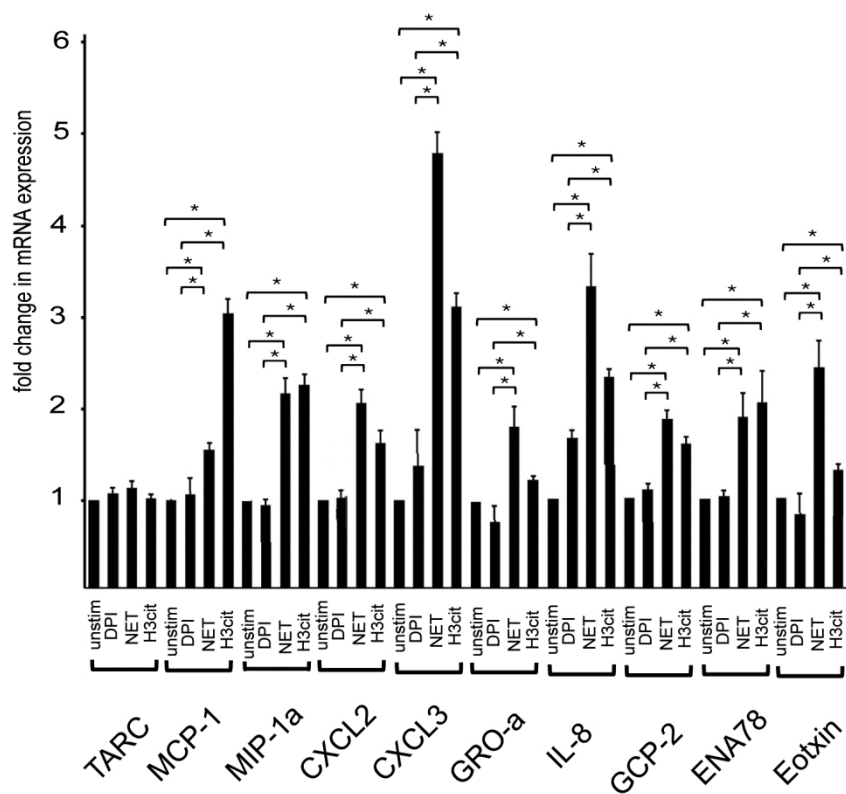
the western bands. C indicates transendothelial leukocytes migration in control cells, cells treated with unstim, DPI, NET, H3Cit, cytokines only, cells co-treated with cytokines and unstim, NET, or H3Cit. Data from six independent experiments are presented as means $\pm$ SEM and are derived from six individuals. (* indicates $P < 0.05$).

FIGURE 7 A, Extracellular DNA concentration evaluated with dsDNA assay kit in neutrophils unstimulated (cont), neutrophils treated with PMA (cont + PMA), neutrophils treated clarithromycin, azithromycin, josamycin, or dexamethasone. Data from six independent experiments are presented as means $\pm$ SEM and are derived from six individuals. (* indicates that there is statistical significant difference in the concentration of extracellular DNA between control and dose of macrolides or glucocorticoid, $P < 0.05$). B, The number of NETs-forming neutrophils evaluated by fluorescence microscopy after isolated neutrophils are incubated with varying doses of macrolides and dexamethasone. Data from six independent experiments are presented as means $\pm$ SEM and are derived from six individuals. (* indicates that there is statistical significant difference in the concentration of extracellular DNA between control and dose of macrolides or glucocorticoid, $P < 0.05$).

C, Visualization of the release of NETs performed by fluorescence microscopy after incubation with clarithromycin (10 μ M), azithromycin (10 μ M), josamycin (10 μ M), and glucocorticoids (0.01 μ M). Red color indicates neutrophils stained with anti-NSE antibody. Green color represents NETs appearing as a web-like extracellular structure (arrow heads) stained with Sytox green which were released from neutrophils stained with Sytox green. Original magnification x 200.



A



B

