Eosinophil extracellular traps: molecular mechanisms and potential roles in chronic rhinosinusitis

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Directed by Professor Chang-Hoon Kim

The Doctoral Dissertation

submitted to the Department of Medicine,

the Graduate School of Yonsei University

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Chi Sang Hwang

June 2020

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June 2020

ACKNOWLEDGEMENTS

I would like to express my deepest appreciation to my advisor Prof. Chang-Hoon Kim for his patience, motivation, enthusiasm, and immense knowledge. His valuable guidance helped me during the conduct of my study. Dong-Joon Park, I am extremely grateful for your advices throughout my study. Those advices have contributed to the improvement of the thesis. I truly thank Prof. Myung Hyun Sohn and Ji-Hwan Ryu for their sustained kind support and insightful comments about my study. Prof. Sang Nam Lee, I sincerely appreciate for always giving me words of encouragement and helpful suggestions.

This thesis work is dedicated to my beloved wife, Hyeri Han, and three sons, Jaeik, Jaeyul, and Jaejoon, whose unyielding love and understanding have inspired me to pursue and complete this study.

Chi Sang Hwang

June, 2020

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ABSTRACT

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Chronic rhinosinusitis (CRS) is a heterogeneous inflammatory airway disease involving non-eosinophilic and eosinophilic phenotypes, which translate to various endotypes. Activated eosinophils and neutrophils are known to generate extracellular traps comprising deoxyribonucleic acid (DNA) and cytotoxic granule proteins. We aimed to investigate the presence of eosinophil extracellular traps (EETs) in human CRS tissues and clarify the association between their quantifiable level and clinical features. Additionally, we investigated whether eosinophils can form EETs in the sinonasal tissue in a murine model of eosinophilic CRS. Finally, we characterized the conditions under which eosinophilic cell lines produce EETs, and the intracellular and

extracellular mechanisms involved were also assessed.

Nasal polyp (NP) or ethmoid tissue slides of 43 subjects undergoing endoscopic sinus surgery for CRS were analyzed. A quantitative analysis of EETs was performed by confocal microscopy using immunofluorescence staining. Regarding correlation study, the presence of NPs, number of infiltrating tissue eosinophils, preoperative Lund–Mackay scores, and other comorbidities were analyzed. Human eosinophilic leukemia cell line was used for artificial stimulation to generate EETs. The role of mitochondrial function was evaluated using JC-1 and Mito-Tracker staining.

EET formation was observed at varying degrees in all CRS groups and was associated with the number of tissue eosinophils ($r = 0.83$, $p < 0.001$), regardless of the presence of NPs. Patients with more EETs demonstrated higher Lund–Mackay scores ($r = 0.51$, $p = 0.009$), blood eosinophil count ($r = 0.80$, $p < 0.001$), and lower olfactory function ($r = -0.65$, $p < 0.001$) than patients with less EETs. EETs are also formed in human eosinophilic leukemia eosinophilic leukemia cell line (EoL-1) cells. Reactive oxygen species play a regulatory role in EET formation by eosinophils *in vitro*. Additionally, more activated EoL-1 cells contribute to higher EET production, which is associated with decreased mitochondrial function.

Eosinophilic CRS indicates the presence of EETs. EET formation could have a role in clinical decision making and prediction of treatment outcomes of CRS, regardless of NP status. Activated eosinophils are observed in DNA traps *in vitro*, which are associated with mitochondrial dysfunction and oxidative damage.

Keywords: chronic rhinosinusitis, eosinophils, extracellular DNA traps, nasal polyps, mitochondria, reactive oxygen species

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I. INTRODUCTION

Chronic rhinosinusitis (CRS) is a common and challenging inflammatory disease that involves non-eosinophilic and eosinophilic phenotypes.[1-3] It is known that the latter are typically marked by a greater disease burden with respect to risk for comorbidities and the likelihood of recurrence after surgical intervention than the former.[4,5] Similarly, it is probable that nasal polyps (NPs) are more frequently observed in patients with tissue eosinophilia compared to patients without tissue eosinophilia.[6] However, there are no standard methods performed to evaluate tissue eosinophilia considering the diversity in geographic conditions and uneven distribution within a tissue.[3,7] Eosinophilic NPs also comprise a variety of inflammatory cells, such as neutrophils and macrophages, that may play a role in the tissue remodeling process in NPs.[8] Therefore, limitations to the subtyping of CRS or NPs based only on inflammatory cells are observed.

A previous study revealed that neutrophils are able to form extracellular

structures comprising deoxyribonucleic acid (DNA) and granule proteins, the so-called neutrophil extracellular traps (NETs).[9] These novel structures are formed by activated neutrophils and can kill invading pathogens before they reach the host cells, a phenomenon that was, at the time of the study, recognized as the third antimicrobial function of neutrophils following phagocytosis and secretion of soluble antimicrobials. Other recent evidences have indicated that activated eosinophils show similar extracellular structures, named eosinophil extracellular traps (EETs), which are able to bind and kill bacteria extracellularly.[10,11] Such extracellular nets are known to form in a reactive oxygen species (ROS)-dependent manner after priming with complement factor 5a, eotaxin, interferon-γ, interleukin (IL)-5, lipopolysaccharides, *Staphylococcus aureus*, or thymic stromal lymphopoietin (TSLP).[10,12,13] Although the mechanism continues to be disputed, their generation has such a distinct pathway from suicidal cell death that such cytolysis could be renamed extracellular trap cell death, the so-called ETosis.[14,15]

In the past, extracellular DNA trap was studied in infectious or autoimmune disease models; however, recently, it was also identified in noninfectious models, such as allergic diseases in relation to EETs.[11,16,17] For the first time, researchers in Belgium and Japan described the presence of EETs in IL-5^{+} NP and eosinophilic sinus secretion, respectively, from human eosinophilic CRS (ECRS) samples.[18,19] These data suggested that EETs can play either a beneficial or harmful role in the field of ECRS, as their increased presence is observed at the site of epithelial barrier defects. EET formation was directly induced on exposure to *S. aureus*, which in turn was able to entrap the bacteria and facilitate a role in the host's defense against microbes.[12] However, compared to NETs, EETs contain smaller amounts of protease that contribute to the stability and high viscosity of eosinophilic mucin, subsequently impairing their clearance by inflammatory cells and antibiotics.[20] As a consequence, EETs might cause

long-lasting adhesive luminal surfaces as postmortem functions, resulting in further compromise of the barrier dysfunction, along with more bacterial aggregation and growth of biofilms and the potential for reinfection and chronicity of disease.

Although EETs in inflamed foci might be involved in the pathogenesis of ECRS, there is a lack of research to date regarding non-ECRS and the aim of clinical correlation. Therefore, the present study aimed to investigate the presence and distribution of EETs in eosinophilic and non-eosinophilic human CRS tissues and to clarify the associations between their quantifiable level and clinical features in patients with CRS. Notably, NETs and EETs were observed in this study because CRS involves various pathomechanisms showing high heterogeneity even in patients with ECRS with a dominant T_H2 profile. Additionally, we investigated whether eosinophils can form EETs in nasal lavage fluids and sinonasal tissues collected from a murine model of ECRS. Finally, we characterized the conditions under which eosinophilic cell lines produce EETs, and the intracellular and extracellular mechanisms involved were also assessed.

II. MATERIALS AND METHODS

1. Subjects

The diagnosis of CRS was based on patient history, nasal endoscopy, and computed tomography (CT) according to a 2012 European Position Paper on Rhinosinusitis and Nasal Polyps.[21] The inclusion criteria in this study were as follows: (1) subjects aged over 19 years; (2) subjects who were refractory to medical treatment, thus requiring bilateral endoscopic sinus surgery; and (3) subjects with available data from a minimum 6-month follow-up period. None of the subjects had taken any form of local or systemic corticosteroid for at least 4 weeks prior to the operation.

Additionally, subjects with a history of mucociliary disorder or with immunocompromised status were also excluded. The present study was approved by the Institutional Review Board (IRB) of Yonsei University College of Medicine (IRB no. 4-2017-0408). Informed consent was obtained from all individual subjects prior to enrolment in the present study. All procedures performed in studies involving human subjects were performed in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Declaration of Helsinki and its later amendments, or with comparable ethical standards.

The classification of CRS was based on the NP and the Japanese Epidemiological Survey of Refractory Eosinophilic Chronic Rhinosinusitis (JESREC) scoring systems.[4] According to the JESREC scoring system, the following four categories of preoperative clinical features were used to assess ECRS: (1) bilateral disease sites were given a score of 3, (2) presence of NP was given a score of 2, (3) a dominant shadow of ethmoid sinus on a CT scan was given a score of 2, and (4) eosinophilia in the peripheral blood was given a score of 4 (> 2% and ≤ 5%), 8 (> 5% and ≤ 10%), or 10 (> 10%). A JESREC score value higher than 11 points was determined to indicate ECRS. Patients in the ECRS group were stratified into three subgroups according to severity (neither A or B = mild, A or B = moderate, A and B = severe) by adding the refractory score as follows: (A) blood eosinophilia (> 5%) and ethmoid-dominant shadow in CT and (B) comorbidity of bronchial asthma, aspirin, or non-steroidal anti-inflammatory drug intolerance.

2. Sample collection

CRS biopsy tissues of 43 patients archived as 4% paraformaldehyde-fixed and paraffin-embedded samples obtained during routine functional endoscopic sinus

surgery at Yonsei University Severance Hospital from January 2017 to December 2017 were analyzed. Ethmoid tissues were obtained from CRS without NPs (n = 22), and NPs were obtained from CRS with NPs (n = 21). Additionally, inferior turbinates from five healthy subjects who underwent septal surgery because of anatomic deviations were collected as control tissues. Finally, 43 nasal tissues samples and five control tissues were classified into four groups and analyzed with clinical parameters. The clinical and demographic data of the patients are summarized in Table 1.

Table 1. Characteristics of the study population. All continuous data are presented as mean ± standard deviation.

Characteristic	Non-ECRSsNP (n = 11)	Non-ECRSwNP (n = 10)	ECRSwNP (n = 11)	ECRSsNP (n = 11)	Control (n = 5)
Mean age, years	51.0 ± 9.1	48.1 ± 14.6	40.6 ± 14.5	45.0 ± 14.1	28.2 ± 9.8
Sex, M/F	8/3	7/3	4/7	5/6	5/0
BMI, kg/m^2	25.1 ± 3.1	25.0 ± 3.2	23.3 ± 3.0	22.0 ± 1.8	23.7 ± 2.6
JESREC†	4.5 ± 2.1	7.4 ± 1.9	17.3 ± 1.3	15.9 ± 2.2	4.0 ± 0
LM score	11.5 ± 6.7	17.4 ± 4.5	19.2 ± 3.1	17.6 ± 5.6	0
KVSS II score	25.7 ± 7.6	13.8 ± 10.3	12.4 ± 8.8	19.2 ± 8.3	31.8 ± 2.6
IgE, KU/L	132.3 ± 93.2	47.0 ± 48.2	234.6 ± 153.9	165.3 ± 130.0	28.1 ± 21.5
Eosinophils in peripheral blood, %	1.9 ± 1.1	2.5 ± 1.4	10.9 ± 3.4	10.5 ± 3.6	2.2 ± 1.2
Allergy, −/+	6/5	6/4	0/11	1/10	4/1
Asthma, −/+	3/0	7/0	7/4	6/5	5/0
Aspirin (NSAIDs) intolerance, n	0	0	0	0	0

†The sum of JESREC score and refractory score

Abbreviations: ECRS, eosinophilic chronic rhinosinusitis; NP, nasal polyp; ECRSsNP, ECRS without NPs; ECRSwNP, ECRS with NPs; BMI, body mass index; JESREC, Japanese Epidemiological Survey of Refractory Eosinophilic Chronic Rhinosinusitis; LM, Lund–Mackay; KVSS II, Korean Version of the Sniffin' Stick test II; NSAIDs, non-steroidal anti-inflammatory drugs

3. Assessment of medical conditions

Regarding the correlation study, preoperative taste and smell tests, preoperative Lund–Mackay CT score,[22] number of tissue eosinophils, comorbidities, and surgical outcomes were assessed. Using the recently developed gustatory function test (YSK taste function test kit, RHICO Medical Co., Seoul, Korea), the sum of the recognition threshold for five tastants, including sweet, butter, salty, sour, and umami, was scored. Subjects with scores lower than 12 were considered to have hypogeusia, according to previously described protocols.[23] The olfactory function test comprised the Korean version of Sniffin' Stick II (KVSS II, Burghart, Wedel, Germany), and the sum of threshold, discrimination, and identification scores was defined as KVSS II score. KVSS II scores 0 to 20 were defined as "anosmia," 20.25 to 27 as "hyposmia," and 27.25 to 48 as "normosmia." Such criteria were determined based on the criteria used in previous studies.[24] Patients were considered to have allergy if the skin prick test was positive for at least one of the batteries of standardized aeroallergens in South Korea. Asthma was defined based on lung function analysis, including methacholine challenge testing.

4. Murine models of nasal polyp

C57BL/6N female mice aged 6 weeks were purchased from Orient Bio (Seongnam, Korea) and maintained under specific pathogen-free conditions. The mice were intranasally administered with multiple airborne allergens three times weekly for 4, 8, 12, and 16 consecutive weeks in the ECRS group. Phosphate-buffered saline (PBS)-treated mice were used as the control group. The multiple allergens included a mixture of 20 μg of house dust mite extract (*Dermatophagoides pteronyssinus*; Greer

Laboratories, Lenoir, NC, USA), 20 μg of *Aspergillus fumigatus* (Greer Laboratories), 20 μg of *Alternaria alternata* (Greer Laboratories), and 1 μg of protease from *S. aureus* (Abnova, Taipei) dissolved in sterile PBS to a total volume of 30 μl, 15 μl of which was instilled in each nostril. The mice were analyzed at 4, 8, 12, and 16 weeks (n = 6 per time point for the control and ECRS groups, respectively, based on the previous studies of sinusitis murine model using 3–8 mice per experimental group)[25,26] (Figure 1).

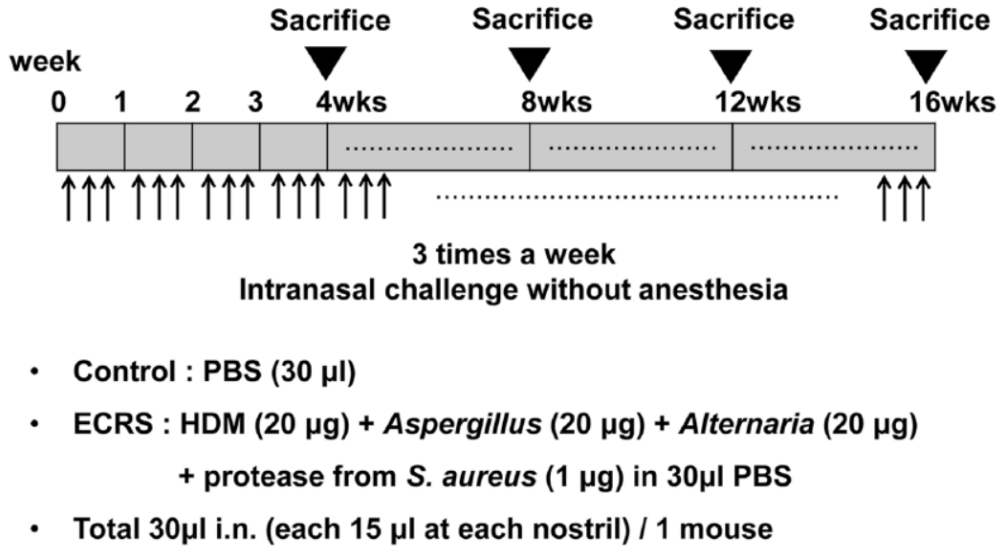

Figure 1. Experimental protocol for the development of eosinophilic chronic rhinosinusitis (ECRS) in mice. Mice were subjected to intranasal challenge by multiple airborne allergens (house dust mite, *Aspergillus fumigatus*, *Alternaria alternata*, and protease from *Staphylococcus aureus*) three times weekly to induce ECRS. PBS was used for the control group. Assessments were performed at 4, 8, 12, and 16 weeks (wks) after the first intranasal challenge. In our previous publications, this model showed typical histological changes of human ECRS such as eosinophil infiltration, goblet cell

hyperplasia, epithelial thickening, and polypoid lesions.[27]

5. Cell lines and cell culture

Eosinophilic leukemia cell line (EoL-1) eosinophilic cells were maintained in RPMI 1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) in 5% carbon dioxide at 37°C. EoL-1 cells were induced to differentiate by the addition of butyric acid (Sigma-Aldrich, St. Louis, MO) for 6 days, as described previously.[28] The cell concentration was adjusted to 5×10^5/ml every 3 days. Cell differentiation was assessed by granule formation and nucleus shape after staining with Diff-Quick (Sysmex, Kobe, Japan). For morphological analysis, cultured EoL-1 cells were spun at 500 rpm for 5 min on glass slides (Cytospin 3, Shandon, Pittsburgh, PA, USA). The slides were air-dried, stained with Diff-Quik stain solution, and observed using the CX41 microscope (Olympus, Tokyo, Japan). For cell viability measurement, the conventional Cell Counting Kit-8 assay (Dojindo Laboratories, Kumamoto, Japan) was applied using a culture supernatant following the manufacturer's protocol.

6. Immunofluorescence staining

EETs and NETs were identified as previously described.[12,13,17] EETs and NETs were visualized in 5-μm-thick sections of paraffin-embedded tissue slides by indirect immunofluorescence, followed by counterstaining for DNA. The sections were blocked by 1% bovine serum albumin and 1% normal donkey serum and subsequently incubated with anti-eosinophil cationic protein (ECP) polyclonal antibody (BioLegend, San Diego, CA) and anti-myeloperoxidase (MPO) polyclonal antibody (R&D Systems, Minneapolis, MN), respectively. Alexa Fluor 568 and Alexa Fluor 488 (both Invitrogen,

Carlsbad, CA) were used for secondary incubation, respectively. Negative controls were incubated with a nonimmune serum instead of a primary antibody. DNA was visualized with 4′6-diamino-2-phenylindole (Invitrogen, Carlsbad, CA). After washing with PBS, the slides were mounted in a drop of fluorescent mounting medium (Dako, Glostrup, Denmark). Images were obtained using a confocal laser scanning microscope (LSM 700; Carl Zeiss Microimaging, Jena, Germany) and analyzed using ZEN software (Carl Zeiss, Jena, Germany). EET-positive cells were identified by cells that produced extracellular, decondensed DNA in fibers or in a web-like shape colocalized with ECP.[11] Eosinophils and EETs were counted in 10 high-power fields (HPFs) as previously described.[16,17] Values are expressed as mean ± standard error of the mean (SEM) per HPF. Similar to EET, NET was defined as extracellular DNA released by neutrophils in conjunction with MPO in a fashion similar to the structures previously described in patients with asthma.[17] All assessments of image processing were performed by two independent investigators in a blinded manner.

To detect EET in culture conditions, 2×10^5/100 μl EoL-1 cells were seeded into 48-well plates on 0.01% poly-L-lysine (Sigma-Aldrich, St. Louis, MO)-coated glass sides. Phorbol 12-myristate 13-acetate (PMA; Calbiochem) was used at a final concentration of 100 nM for 3 hours in SYTOX (cell-impermeable DNA-specific dye, Invitrogen, Carlsbad, CA)-containing medium and studied with fluorescence microscopy.

7. Quantification of released double-stranded deoxyribonucleic acid (DNA) in culture supernatants

Released double-stranded DNA (dsDNA) was quantified as previously described.[29] Briefly, EoL-1 cells (4×10^6/mL) were stimulated and treated with a low

concentration of DNase I (2.5 U/mL; Worthington) and proteinase K (0.2 mg/mL; Roche). Reactions were stopped by the addition of 2.5 mM ethylenediaminetetraacetic acid (pH, 8.0). Cells were centrifuged at 500 × g for 5 min. Supernatants were transferred to black, glass-bottom 96-well plates (Greiner Bio-One GmbH). Moreover, the fluorescent activity of PicoGreen dye bound to dsDNA was excited at 502 nm, and the fluorescence emission intensity was measured at 523 nm using a spectrofluorometer (SpectraMax M2, Molecular Devices, Biberach an der Riss, Germany), according to the instructions described in the Quant-iT™ PicoGreen® Assay Kit.

8. Reactive oxygen species (ROS) measurements

ROS was measured by staining with the fluorescent dye dichlorofluorescin diacetate (Sigma-Aldrich, St. Louis, MO). Cells were incubated with Hank's balanced salt solution containing 0.1 mM dichlorofluorescin diacetate. After 20 min, cells were washed two times with Hank's balanced salt solution, and fluorescence was visualized using an FV1000 confocal microscope (Olympus). The effect of an ROS scavenger was evaluated by pretreatment with 100 or 500 μm of N-acetyl cysteine (NAC) (Sigma-Aldrich) to culture media 0.5 h before EoL-1 cell stimulation.

9. Evaluation of mitochondrial dysfunction

Mitochondrial function was evaluated by the mitochondrial membrane potential ($\Delta\psi m$) and mitochondrial morphology, which were examined using the fluorescent probe JC-1 (BD Pharmingen, Franklin Lakes, NJ) and Mito-Tracker probes (Invitrogen, Waltham, MA), respectively. Briefly, cells were seeded in 96-well plates that were treated with PMA at different conditions. Cells after treatment were washed and incubated with 10 μM JC-1 or Mito-Tracker for 15 min in darkness. Subsequently,

cells were washed and imaged under an inverted fluorescence microscope. The green fluorescence intensity after JC-1 staining was quantified by ImagesPlus software. A high 590/530 nm ratio indicates active, polarized mitochondria, and a drop in the ratio indicates a drop in mitochondrial membrane potential. All data and images were obtained from three independent trials and were conducted in accordance with the relevant guidelines as published previously.[30]

10. Statistical analyses

For between-group comparisons, the Mann–Whitney *U* test or Kruskal–Wallis test with a two-tailed test was used. To describe the correlation between variables, linear correlation analysis was used to determine bivariate associations with a Spearman correlation test. Data are expressed as mean ± SEM. All statistical analyses were performed using the Statistical Package for the Social Sciences version 18.0 (International Business Machines Corp., Armonk, NY). A p value < 0.05 was considered statistically significant.

III. RESULTS

1. All groups had eosinophils that were found to form eosinophil extracellular traps in tissues

EET-releasing eosinophils were observed in both the ECRS and non-ECRS groups. The confocal microscopy images clearly showed the presence of extracellular DNA nets in association with the granular protein ECP in the non-ECRS group (Figure 2A). In the ECRS group, EET formation was more prominent than NET formation (Figure 2B). The numbers of EET-releasing eosinophils were higher in the apical part

of the subepithelial region compared with those in the basal part or deep in the stroma. For the ECRS group, in the basal parts of the epithelium or deep in the stroma, eosinophils were found to be mostly intact, and EET formation was significantly rare (Figure 2C), although in the subepithelial regions with single-cell layers and missing epithelial cells, eosinophils were often observed in clusters and were often released as massive and clustered EETs (Figure 2D). Subepithelial regions without denuded epithelium were located where eosinophils were found to be intact, and EET formation was significantly rare (Figure 2E). However, none of the CRS groups containing neutrophils formed NETs in this study. Even in the non-ECRS tissues, where a high number of neutrophils exceeding the number of eosinophils were observed, NETs were not found in the subepithelial region or the stroma (Figure 2F and 2G).

Regarding the quantifiable level of ECP–DNA complex in each individual CRS subtype, Figure 3 shows a mean value of EET-releasing eosinophils per HPF. EETs were expressed as a mean value of eosinophils generating EETs relative to the total amount of those present in 10 HPFs. No EET-releasing eosinophils were observed in the healthy control group.

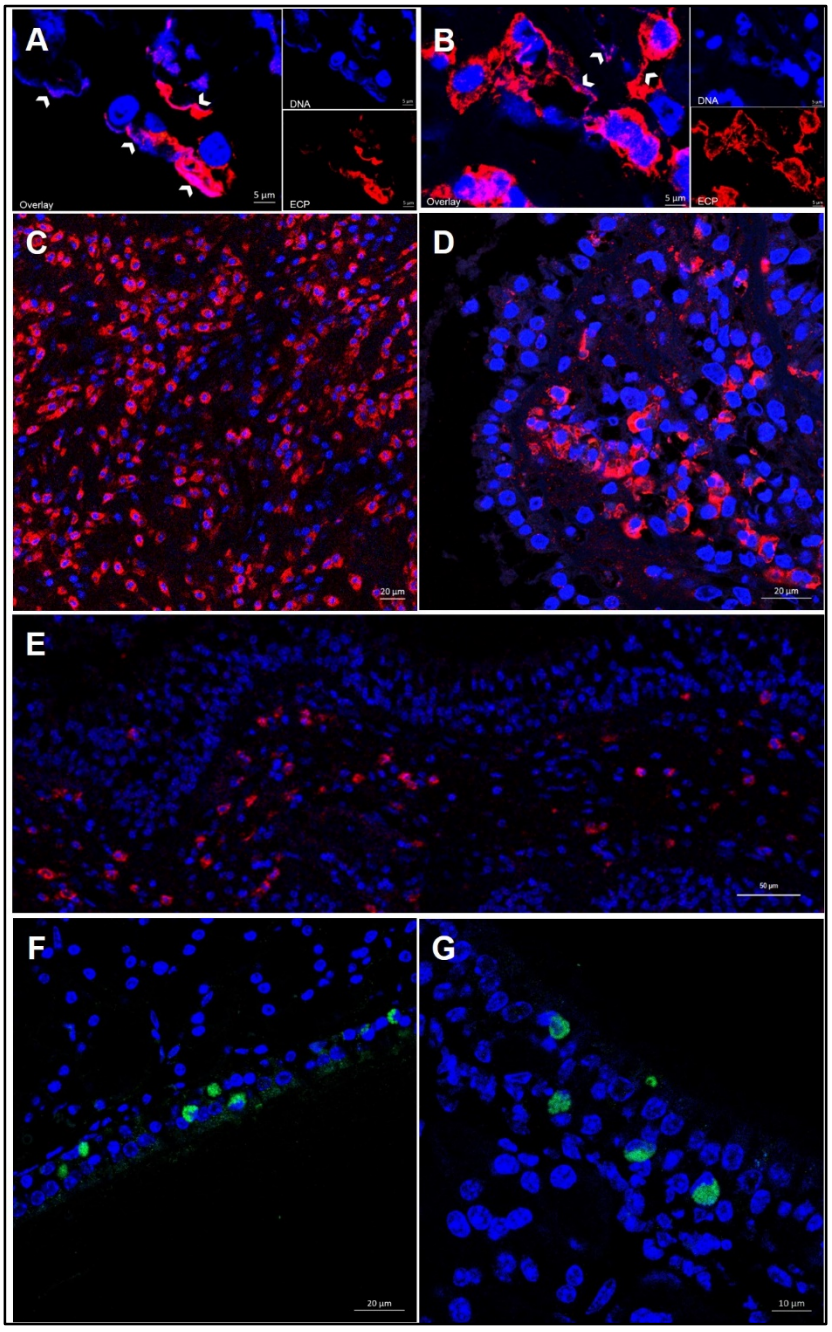

Figure 2. Representative image of endoscopic sinus surgery specimens from subjects with chronic rhinosinusitis (CRS) showing eosinophil extracellular traps (EETs). (A) The extracellular deoxyribonucleic acid was colocalized with eosinophil cationic protein (indicated by the arrowheads) in the non-ECRS without the nasal polyps (NPs) group. *Scale bar* = 5 μm. (B) In the case of ECRS with NPs, EET formation was more prominent (indicated by the arrowheads). *Scale bar* = 5 μm. (C) For the ECRS group, eosinophils located deep in the stroma were found to have mostly intact granules, and EET formation was significantly rare. (D) EETs were mainly observed in the subepithelial regions, with clusters of eosinophils underneath the epithelial barrier defect. *Scale bar* = 20 μm. (E) Subepithelial regions without denuded epithelium were located where eosinophils were found to be intact, and EET formation was significantly rare. *Scale bar* = 50 μm. However, none of the groups were found to form NETs, even with the increased presence and alignment of neutrophils in the non-ECRS with NPs (F) or in the non-ECRS without NPs (G) groups. *Scale bar* = 20 μm.

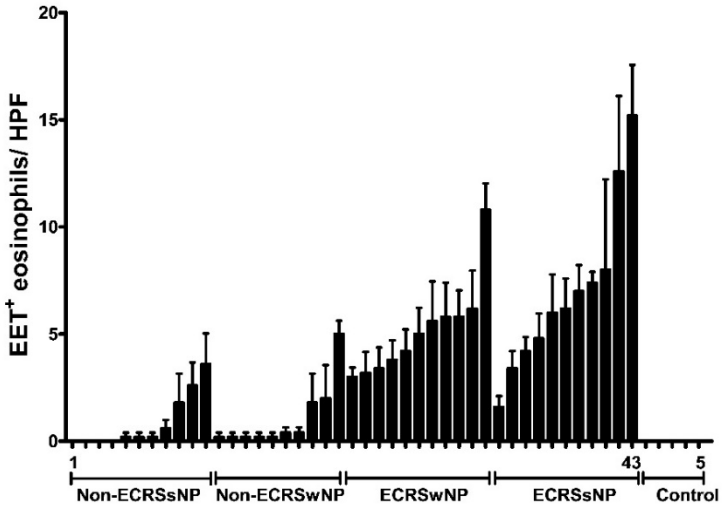

Figure 3. Column bar graph illustrating that eosinophil extracellular trap (EET)-releasing eosinophils could be found in all chronic rhinosinusitis (CRS) subtypes (subjects 1–43) but not in healthy control tissues. The proportion of eosinophils releasing EETs was significantly higher in the eosinophilic CRS (ECRS) group than that in the non-ECRS group. However, the presence of nasal polyps (NPs) exhibited no significant differences between the CRS groups, which indicates that patients with ECRS may be more easily activated to produce EETs regardless of NP status.

2. Formation of eosinophil extracellular traps associated with the number of tissue eosinophils regardless of the presence of nasal polyps

All surgical samples selected in this study were stained with hematoxylin and eosin. Eosinophils were observed rarely in the tissue of the control subjects. Although there were substantial differences between subjects, overall, the ECRS group was infiltrated with a significantly higher number of tissue eosinophils than the non-ECRS group (53.6 ± 8.0 vs. 2.5 ± 0.6, respectively, $p < 0.0001$ [Figure 4A–D]). Moreover, the ECRS group had significantly higher number of EET-releasing eosinophils than the non-ECRS group (5.1 ± 0.5 vs. 0.6 ± 0.2, respectively, $p < 0.0001$ [Figure 5A]). However, EET formation was not associated with the presence of polyps (3.6 ± 1.1 vs. 2.5 ± 0.6, $p = 0.402$ [Figure 5B]), although EETs were associated with the total number of tissue eosinophils ($r = 0.829$, $p < 0.0001$, Figure 5C). Considering all subjects, the mean fraction of EET-releasing eosinophils was 13.5% ± 8.8% (range, 0%–35%) of the total amount of eosinophils infiltrating the nasal mucosa epithelium.

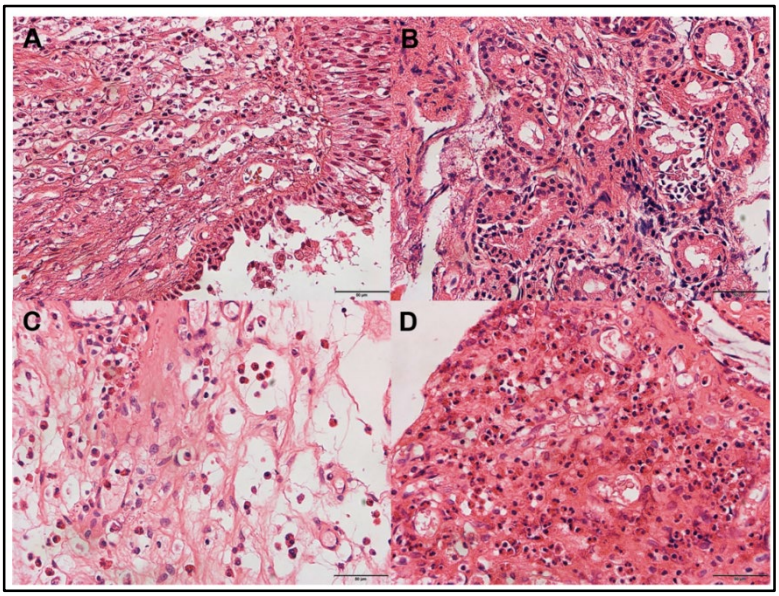

Figure 4. A photomicrograph showing that eosinophil distribution is uneven in surgical samples according to chronic rhinosinusitis (CRS) phenotype. Different from the (A) non-eosinophilic CRS (ECRS) without nasal polyps (NPs) and (B) non-ECRS with NPs groups, (C) the ECRS with NPs and (D) ECRS without NPs groups were infiltrated with a higher number of eosinophils, although their numbers varied among individual subjects (hematoxylin and eosin staining, *scale bar* = 50 μm).

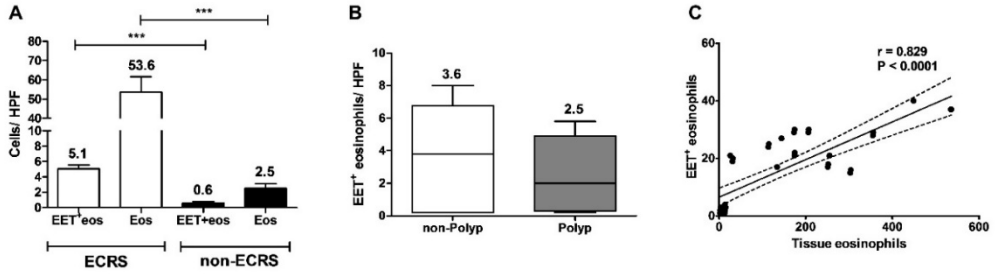

Figure 5. Quantitative analysis of infiltrating eosinophils and eosinophil extracellular traps (EETs) according to chronic rhinosinusitis (CRS) subtype. (A) The number of EET-releasing eosinophils and the total number of tissue eosinophils were significantly higher in the eosinophilic CRS (ECRS) group than those in the non-ECRS group. (B) However, the presence of nasal polyps was not associated with EET formation. (C) Linear regression analysis between the numbers of EET-forming and total infiltrating eosinophils showed a statistically significant positive association. The lines indicate the mean with 95% confidence interval.

3. Eosinophil extracellular traps appear to reflect activated eosinophils with a greater disease burden

EET-releasing eosinophils were associated with some clinical features in patients with CRS. We observed a higher number of infiltrating EET-releasing eosinophils in CRS specimens from subjects with a significantly high preoperative total Lund–Mackay CT score and a high proportion of peripheral blood eosinophils ($r = 0.510$, $p = 0.009$ and $r = 0.795$, $p < 0.0001$ [Figure 6A and 6B], respectively). In particular, a significant negative association was observed between the level of olfactory function and the EET-releasing eosinophils ($r = -0.653$, $p < 0.0001$ [Figure 6C]). The number of tissue-infiltrating eosinophils and EET-releasing eosinophils tended to be higher in patients with bronchial asthma than in those with no bronchial asthma. However, the difference was not statistically significant (total number of tissue eosinophils: 48.8 ± 9.9 vs. 24.7 ± 8.1, $p = 0.186$, EET-releasing eosinophils: 4.5 ± 0.5 vs. 2.9 ± 0.7, $p = 0.257$ [Figure 6D]). None of the other clinical variables including the taste test results and postoperative endoscopic findings were significantly different with regard to the number of EET-releasing eosinophils in this study.

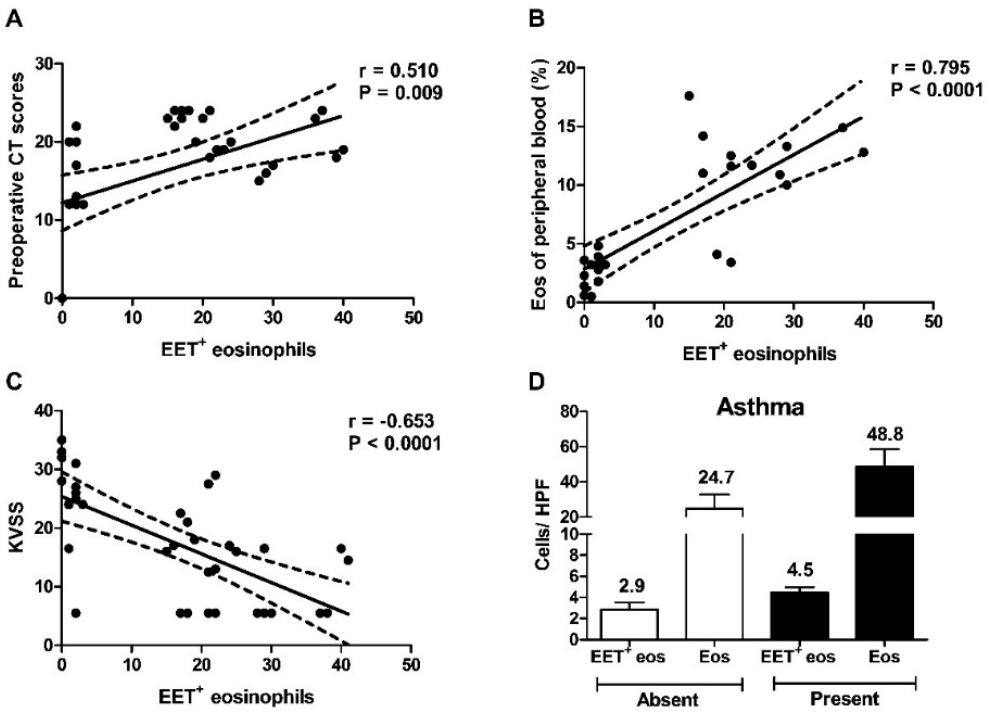

Figure 6. The extent of eosinophil extracellular trap formation is associated with (A) preoperative computed tomography score, (B) decreased olfactory function, and (C) proportion of peripheral blood eosinophils, (D) but not with the presence of asthma. The lines indicate the mean with 95% confidence interval.

4. Multiple airborne allergen-induced airway eosinophilic inflammation

Multiple airborne allergens significantly induced higher eosinophil numbers in the sinonasal tissue in the experimental group than in the control group (Figure 7A and B, respectively). Histological analysis of multiple airborne allergen-exposed mice revealed the more typical pathologic features of ECRS compared to control mice.

However, EET formation was not observed in any groups, even with the increased presence and alignment of eosinophils in the ECRS groups (Figure 7C and D, respectively).

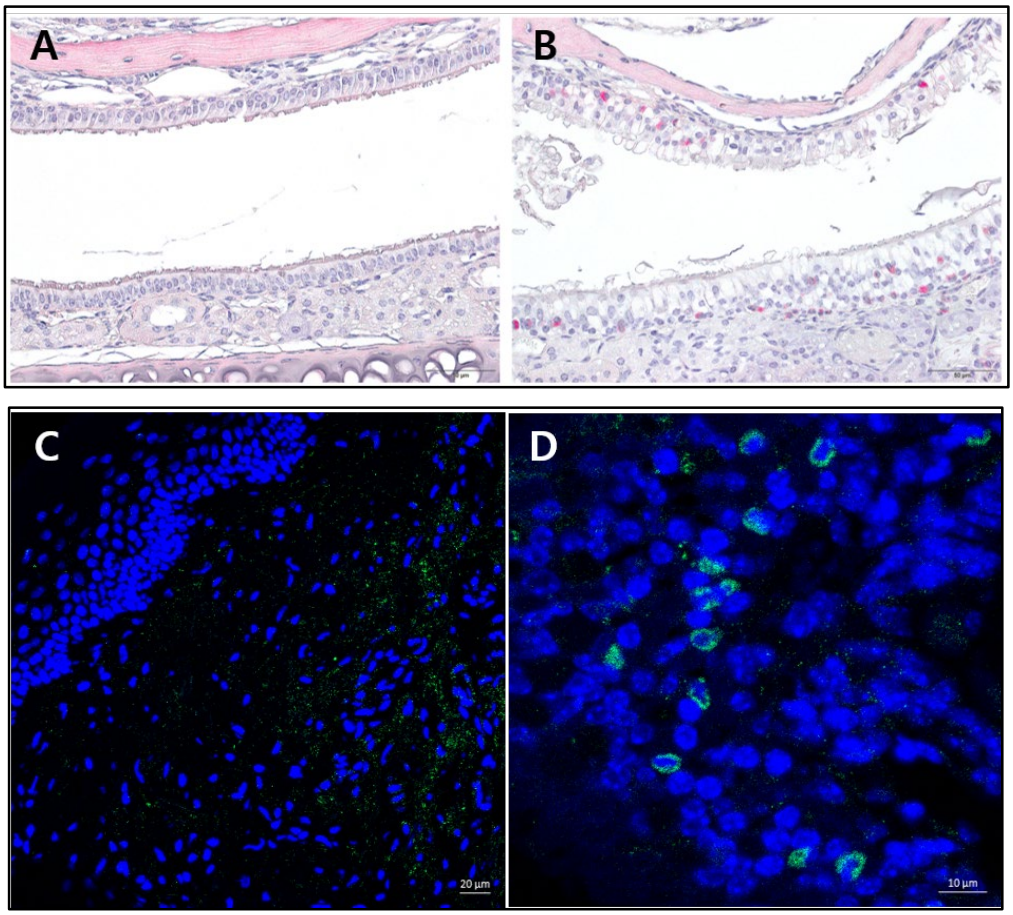

Figure 7. Formalin-fixed sinonasal sections from eosinophilic chronic rhinosinusitis (ECRS) mouse model at 16 weeks were stained with sinus red (A and B) and anti-MBP (1: 250), followed by anti-rabbit FITC (1: 100) (C and D; original magnification: × 400). Deoxyribonucleic acid was visualized with 4′6-diamino-2-phenylindole.

Epithelial disruptions were characterized mainly by the presence of eosinophils. Numbers of eosinophils infiltrating the sinonasal mucosa were significantly higher in the experimental groups (B and D) than those in the control group (A and C). However, none of the groups were found to form eosinophil extracellular traps, even with the increased presence and alignment of eosinophils in the ECRS groups.

5. Phorbol 12-myristate 13-acetate (PMA)-stimulated eosinophil extracellular trap formation

To investigate whether PMA triggers EET formation, EoL-1 eosinophilic cell lines were stimulated with PMA at different concentrations. The greatest proportion of DNA-releasing eosinophils was observed on stimulation with 100 nM PMA after 3 hours. Without PMA stimulation, no DNA release was detected (Figure 8 A–C).

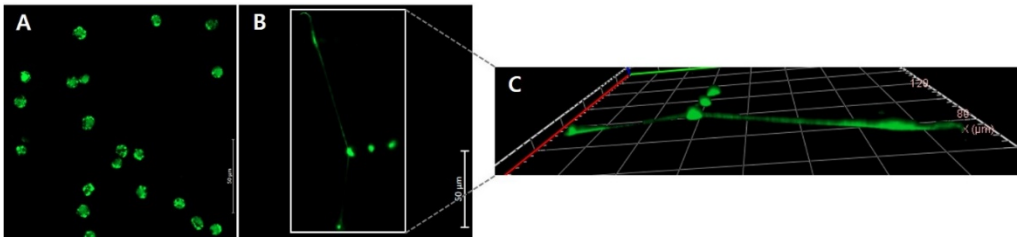

Figure 8. Release of deoxyribonucleic acid (DNA) traps from eosinophils with phorbol 12-myristate 13-acetate (PMA). Compared to the control group (A), (B and C) PMA (100 nM/ml for 3 hours) stimulated eosinophils to release DNA traps (DNA stained with SYTOX green).

6. DNA release depends on ROS production

As there is evidence that ROS are involved in extracellular DNA release[11], we

investigated whether DNA release following PMA treatment in EoL-1 cells also shows ROS dependence. Inhibition of ROS production with NAC prior to PMA stimulation significantly blocked DNA release (Figure 9A). The quantitation of ROS production demonstrated that ROS levels measured on PMA stimulation is positively associated with the level of released double-stranded DNA concentration ($r^2 = 0.615$, $p < 0.0001$ [Figure 9B]).

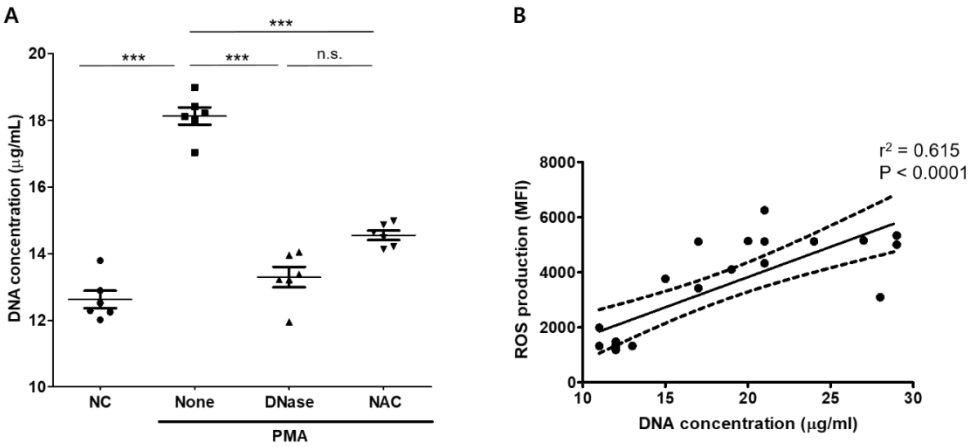

Figure 9. Quantification of released double-stranded deoxyribonucleic acid (dsDNA) in supernatants of activated eosinophils using a PicoGreen fluorescent dye. Data are presented as mean ± standard error of the mean of at least 3 independent experiments. (A) DNA traps were abolished after deoxyribonuclease treatment and also blocked with pretreatment of the reactive oxygen species (ROS) scavenger N-acetyl cysteine. (B) The extent of eosinophil extracellular trap formation is significantly associated with the ROS levels. The lines indicate the mean with 95% confidence interval.

7. Mitochondria's role in PMA-induced DNA release of eosinophilic leukemia cell line cells

To elucidate the role of mitochondria in PMA-induced DNA traps of EoL-1 cells, the Δψm and mitochondrial morphology were detected using JC-1 and Mito-Tracker probes, respectively. As shown in Figure 10, PMA treatment induced significant loss of Δψm, as reflected by the fluorescent shift from red to green (Figure 10A–C). Moreover, the mitochondrial movement decreased as the PMA concentration increased (Figure 10D–F).

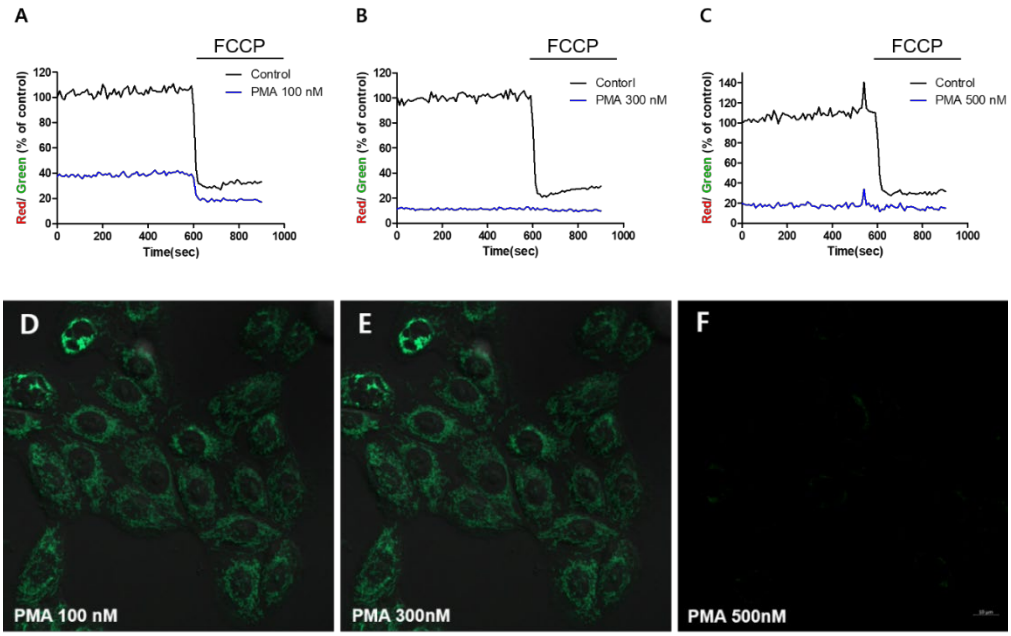

Figure 10. The mitochondrial membrane potential (Δψm) and mitochondrial morphology were detected using JC-1 and Mito-Tracker probes, respectively. (A–C) Phorbol 12-myristate 13-acetate (PMA) treatment induced significant loss of Δψm, as reflected by the fluorescent shift from red to green. Treating cells with FCCP (carbonyl cyanide-4-[trifluoromethoxy] phenylhydrazone, an ionophore uncoupler of oxidative phosphorylation) rapidly depolarizes the mitochondrial membrane potential and eliminates the JC-1 staining. (D–F) The mitochondrial movement decreased as the

PMA concentration increased. Visualization of intracellular mitochondrial movement using confocal microscopy of eosinophilic leukemia cell line cells pre-treated with PMA and Mito-Tracker Green (*Scale bar* = 20 μm).

IV. DISCUSSION

The application of precision medicine in the field of CRS can be more effective after establishing the endotype. This is because CRS involves a diverse spectrum of inflammatory diseases showing high heterogeneity, which causes different therapeutic responses. Although eosinophil activation in inflamed foci is involved in the pathogenesis of ECRS, the factors that perpetuate the inflammation are not completely understood.

The present study demonstrates that some eosinophils in the inflamed nasal mucosa in both ECRS and non-ECRS patients form EETs, which comprise released filamentous chromatin structures in conjunction with eosinophil granule proteins in the extracellular matrix. In the group analysis, the ECRS group had a significantly higher presence of EET-releasing eosinophils than did the non-ECRS group. Unsurprisingly, EET formation was more often observed when the tissue eosinophils appeared more heavily extended. However, the presence of NPs was not a key component for the quantitative difference in EETs. This was a significant finding since previous publications examined only the tissues or secretions from patients with ECRS with NPs.[12,18,19,31,32] In contrast to CRS subjects, none of the control biopsy specimens expressed EETs. EETs were observed mainly in association with disruption of the mucosal interface, which may indirectly confirm the findings of previous studies,[12,16] which reported that extracellular polymers are released as a net, similar to a spider web, and trap invaded bacteria, thus participating in the host's defense against microbes.

Taken together, these findings, despite the lack of evidence of biological investigations, suggest that EETs might play a role in barrier function of the nasal mucosa via inflammation response in the pathogenesis of CRS, but are not directly associated with the tissue remodeling process in NPs.

Possible trigger for EET formation in our non-ECRS group remains unclear. Given the subclassification of CRS by only using NP status and several preoperative clinical features in this study, it was difficult to simplify and dichotomize heterogeneous inflammation patterns when comparing the ECRS and non-ECRS groups. One recent study depicted that even CRS without NP comprises extremely diverse cytokine patterns, with 23%, 36%, and 15% of them showing T_H1-, T_H2-, and T_H17-type inflammation, respectively, which indicate that the overall frequency of type 2 inflammation is higher than that of type 1 inflammation.[33] In our study, the ECRS group evidently had higher number of EET^+ eosinophils and EET^- eosinophils than the non-ECRS group. Moreover, the ratio of EET^+/total eosinophils was lower (5.1/53.6 vs. 0.6/2.5) in the ECRS group than that in the non-ECRS group, although there was no statistically significant difference between the two groups. Considered as one of the limitations of this study, we assume that the more activated status in eosinophils could be an essential part of EET formation rather than simply tissue eosinophil counts. This effect was observed in both the ECRS and non-ECRS groups, with the majority of EETs being focused primarily at the site of epithelial damage. For the ECRS group, several eosinophils deposited in the basal parts of the epithelium or deep in the stroma were found to have mostly intact granule, and EET formation more significantly diminished in the basal parts of the epithelium or deep in the stroma compared to the subepithelial region. Epithelial cytokines, such as TSLP, IL-25, and IL-33, can stimulate innate immune cells, such as eosinophils.[13,34,35] Additionally, TSLP, IL-25, and IL-33 are known to skew Th2 immune response, resulting in eosinophil infiltration

and activation.[36,37] Thus, we hypothesized that in both the ECRS and non-ECRS groups, eosinophils are activated, at least partially, as a consequence of epithelial barrier defects and the subsequent innate immune responses, resulting in EET formation.

Several studies have reported that NETs are associated with various pathologies, including autoimmune inflammation, infections with thrombosis in sepsis, and allergic diseases.[9,38] Recently, NETs were identified in bronchial biopsies from asthmatic patients with a high number of infiltrating neutrophils.[17] However, no extracellular neutrophilic DNA with colocalizing MPO was found in this study. Although this finding showed similarities with those of the previous publications,[31] we thought that this result was due to the limited or insufficient number of infiltrating neutrophils in our subjects. Further investigations with more tissue samples characterized by a neutrophil-dominant cell type and cell-specific knockout approaches may be required to determine the relative contribution of NETs to the pathogenesis of CRS.

In the present study, we adapted the JESREC scoring system established by a multicenter study in Japan and classified subjects into the ECRS and non-ECRS groups using preoperative clinical features of disease side, CT scan, presence of NPs, and peripheral blood eosinophils.[4] Although ECRS is more likely to be associated with the presence of tissue eosinophilia, there is no agreed standard system for the definition of tissue eosinophilia worldwide. The most important reason for this disagreement is that the distribution of eosinophils is typically uneven throughout the tissue. If a certain biomarker is to be used for endotyping, the difference in clinical outcome depending on the endotype should be determined. Our study showed that an increased number of EET-releasing eosinophils indicated the presence of a greater disease burden with respect to the blood and eosinophilia and increased CT severity score and decreased olfactory function.

These clinical characteristics of EETs may be derived from their functional attributes. The expanding DNA traps in the inflamed nasal mucosa contribute to the properties of highly viscous eosinophilic mucin and impairments in its clearance, resulting in long-lasting inflammation and formation of a secondary epithelial barrier defect.[15] As a result, the odorant might be interfered with the passage from the nasal cavity into the olfactory nerve of the olfactory epithelium. Decreased olfaction function in association with EET formation could also suggest a possible role for tissue eosinophilia or eosinophil-associated mucous cytokines that induce cell damage at the neuronal level, likely from inflammatory infiltrate in CRS-associated olfaction dysfunction.[39,40]

Evidence that mitochondrial and non-nuclear DNA is released by eosinophils was obtained from live-cell imaging analyses showing that the DNA was derived from extranuclear cell structures and several molecular biological techniques.[11,38] However, its underlying mechanism has not been well elucidated yet. Hence, in the present study, PMA-induced DNA traps and the underlying mechanisms were evaluated in EoL-1 cells and ECRS mouse model, which are both accepted as good models when investigating the EETs *in vitro* and *in vivo*, respectively. The results indicated that activated eosinophilic cells displayed significant EET formation *in vitro*. Moreover, we demonstrated that mitochondrial dysfunction was associated with oxidative damage as an intracellular mechanism of releasing EETs in activated eosinophils. A previous study in Europe reported that low levels of cellular adenosine triphosphate in optic atrophy 1 (OPA1)-deficient neutrophils are insufficient for microtubule network and NET formation, thus resulting in a reduced antibacterial defense capability in an *in vivo* model of *Pseudomonas aeruginosa* lung infection.[41] OPA1 is a mitochondrial inner membrane protein that has an important role in mitochondrial fusion and structural integrity.[42,43] One recent study in the United States showed that oligomerization of

voltage-dependent anion channels (VDACs) under oxidative stress conditions forms mitochondrial pores to release mitochondrial DNA fragments.[44] Additionally, in a mouse model of systemic lupus erythematosus, an inhibitor of VDAC oligomerization, decreased NET formation, type I interferon signaling, and disease severity.[44] Taken together, the findings in our study might provide further evidence regarding the role of mitochondria in the biology of eosinophils, particularly for EET formation, and the impact of a mitochondrial membrane function to the innate immune response. Further studies, including those evaluating the possible role of EETs in CRS, should be conducted to designate new biomarkers that could reflect activated eosinophils with a greater disease burden.

V. CONCLUSION

In summary, we describe the presence and clinical characteristics of EETs representing a novel feature of activated eosinophils infiltrating the nasal mucosa in both ECRS and non-ECRS subjects *in vivo*. Activated eosinophils are observed in DNA traps *in vitro*, which are associated with mitochondrial dysfunction and oxidative damage. The concept of EETs also could have novel implications for potential therapeutic targets, clinical decision-making, and prediction of treatment outcomes.

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ABSTRACT (IN KOREAN)

만성비부비동염에서 호산구 세포외 트랩에 의한 선천면역 및 염증반응 조절에 관한 연구

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황 치 상

세포외 트랩은, 2004년 호중구 세포외 트랩(neutrophil extracellular traps; NETs)으로 처음 알려졌으며 세포외공간에 호중구에서 분출된 DNA와 구상단백으로 구성되어 있는 실 같은 그물망 구조를 일컫는 것으로, 주로 침입한 병원균과 결합하여 제거하는 역할을 하지만 SLE 같은 자가면역질환이나 혈전 형성을 통한 패혈증에서의 병인과 관련된 역할도 밝혀지고 있다. 호중구 외에도 호산구(eosinophil extracellular traps; EETs), 비만세포, 대식세포에서도 세포외 트랩의 존재가 알려져 있으나, 그 역할에 대한 연구는 아직 부족하다. 호산구 세포외 트랩은 호중구 세포외 트랩과 다르게 단

백분해효소가 적고 호산구성과립이 비교적 온전하게 세포외 공간으로 방출되어 안정성이 높고, 점도가 높아진 점액의 분비로 인해 정상적인 점액섬모청소(mucociliary clearance) 능력이 저하되어, 이차적 세균감염을 야기하게 된다고 보고되어 있다.

따라서 우리는 호산구 세포외 트랩이 만성비부비동염의 발생 및 중증도에 영향을 미칠 수 있으며, 알러지 발생과정에 대한 이전 연구로 볼 때, 활성산소를 포함한 세포내 미토콘드리아의 기능이 이 과정에서 중요한 역할을 할 것으로 가정하였다. 이를 위하여 우리는 (1) 질환을 대변하는 비부비동염 수술 환자의 코 점막 시료를 가지고 임상 중증도와 호산구 세포외 트랩 간의 상관 연구를 진행하였고, (2) 호산구성 비부비동염 마우스 모델을 이용하여 동물 모델에서도 호산구 세포외 트랩이 관찰될 수 있고 관련된 기전 연구가 가능할지 살펴보았으며, (3) 마지막으로 인간 호산구성 세포 유래주 (Eosinophilic leukemia cell line, EoL-1)를 성숙된 호산구 세포로 분화시킨 뒤, 이를 이용하여 호산구 세포외 트랩의 발생과 활성산소 및 미토콘드리아 관련 병리 기전을 분석하였다.

비부비동염 환자 수술 시료를 통한 분석에서는 호산구 세포외 트랩이 조직리모델링으로 대변되는 비용종의 발생과는 관계없이, 만성 염증과 관련된 호산구 비부비동염의 질병 중증도 인자(후각 기능 점수, CT 점수, 조직내 침윤된 호산구 세포 수)와 통계학적으로 유의한 연관성이 있다는 것을 알 수 있었다. 또한, 호산구 세포주를 이용한 실험으로 호산구 세포외 트랩이 활성화 산소의 발생량과 미토톤드리아 막 전위 저하의 정도와 유의한 연관성을 가진다는 것을 관찰하였다.

따라서 우리는 호산구 세포외 트랩의 주 기전인 활성화 산소 발생이 미토콘드리아 기능 저하를 통하여 발생시키게 되며, 이는 임상적인 중등도에도 영향을 주는 것으로 결론 지었다.

핵심되는 말: 만성비부비동염, 호산구, 세포외 DNA 트랩, 비용종, 미토콘드리아, 활성화 산소

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