

Received Date : 16-Aug-2016

Revised Date : 02-Nov-2016

Accepted Date : 20-Nov-2016

Article type : Original Article-Clinical Mechanisms in Allergic Disease

Neutrophil autophagy and extracellular DNA traps contribute to airway inflammation in severe asthma

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Running title: Neutrophil autophagy and NETs in severe asthma

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/cea.12859

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ABSTRACT

Background: Autophagy and neutrophil extracellular DNA traps (NETs) are implicated in asthma; however, their roles in asthma pathogenesis have not been elucidated.

Objectives: We compared autophagy and NET production levels from peripheral blood neutrophils (PBNs) of patients with severe asthma (SA) and non-severe asthma (NSA). Additionally, we investigated the inflammatory effects of NETs on human airway epithelial cells (AECs) and peripheral blood eosinophils (PBEs).

Methods: PBNs from patients with SA (n=30) and NSA (n=38) were treated with interleukin (IL)-8 (100 ng/ml). Autophagy (light chain 3-II expression) and NET production levels were evaluated by western blot, immunofluorescence microscopy, and PicoGreen assay. The effects of NETs on AECs were assessed by investigating cell death, cell detachment, expression of occludin and claudin-1, and IL-8 production; the effects of NETs on PBEs were examined by investigating the activation and release of eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN).

Results: Untreated and IL-8-treated PBNs from the SA group produced higher autophagy and NET levels compared with those from the NSA group ($P < 0.01$). IL-8 increased autophagy and NET levels in PBNs from the SA group but not from the NSA group. NET levels were correlated with autophagy levels in PBNs ($P < 0.001$). IL-8-induced NET production levels negatively were correlated with FEV1/FVC ($r = -0.700$, $P = 0.016$). NETs induced cell death, detachment, degradation of occludin and claudin-1, and IL-8 production from AECs. Higher levels of NET-induced ECP and EDN were released from PBEs in SA compared with NSA groups.

Conclusions & Clinical Relevance: Neutrophil autophagy and NETs could enhance asthma severity by damaging airway epithelium and triggering inflammatory responses of AECs and PBEs. Modulating neutrophil autophagy and NET production may be a new target therapy for SA.

Keywords: asthma; autophagy; eosinophil; neutrophil

INTRODUCTION

Asthma, a common chronic airway disease that affects more than 300 million people, is responsible for 1 in every 250 deaths worldwide [1, 2]. Airway eosinophilic inflammation can cause mild-to-moderate asthma; however, neutrophils are often predominant in the sputum of patients with severe asthma having corticosteroid resistance [3, 4]. Neutrophils in the circulation that are primed by various activating inflammatory mediators exert their biological functions when infiltrating asthmatic airways [5-7]. Interleukin (IL)-8 is a key cytokine found in neutrophilic severe asthma (SA) [5, 8]. In response to allergens, airway epithelial cells could produce IL-8, which then recruits neutrophils to the airway and activates

them [9]. Numerous studies have investigated the contribution of neutrophils to asthma pathogenesis; however, the pathobiological consequence of neutrophil activation in airway inflammation has not been fully elucidated.

Autophagy is a cellular process sequestering and eliminating damaged organelles or proteins to maintain cell survival during starvation and cellular stress [10]. Autophagy is initiated by the formation of an autophagophore, which elongates and sequesters target organelles to form an autophagosome. The autophagosome fuses with the lysosome to degrade the sequestered components using lysosomal enzymes [11]. Autophagy-related gene polymorphisms have been implicated in asthma and neutrophilic airway inflammation [12]. Recently, high levels of autophagy production were reported in the sputum granulocytes, peripheral blood cells, and eosinophils of patients with SA as compared with those with non-severe asthma (NSA)[13]. Additionally, autophagy could regulate neutrophil survival and the formation of neutrophil extracellular DNA traps (NETs) [14].

NET production results from the extracellular release of decondensed chromatin from neutrophils activated by various stimuli including cytokines (e.g. IL-8 or TNF- α), phorbol 12-myristate 13-acetate (PMA), or microbial proteins [15]. NET production was found to be associated with airway obstruction in COPD patients [16]. Recently, several studies have detected increased NETs and NET components in the airways of patients with asthma, suggesting an involvement of NET in asthma pathogenesis [17, 18]. However, the pathobiological function of NET in asthma has not been fully investigated.

We hypothesised that elevated neutrophil autophagy and NET productions in patients with asthma could enhance airway inflammation, leading to a development of SA. Therefore, we evaluated autophagy and NET production levels in the peripheral blood neutrophils (PBNs) of patients with SA compared with those with NSA. Furthermore, we investigated the effects

of NET on inflammatory responses of airway epithelial cells (AECs) and peripheral blood eosinophils (PBEs). The findings in this study provide a potentially novel target for SA treatment with a focus on neutrophil autophagy and NET production.

MATERIALS AND METHODS

Study subjects

We recruited 68 patients with asthma and classified them into SA (n=30) and NSA (n =38) groups at the Allergy and Asthma Centre, Ajou University Hospital, Suwon, South Korea. The patients were separated randomly into two independent cohorts. The first cohort, consisting of 22 SA and 24 NSA subjects, was used to investigate autophagy levels in isolated PBNs using immunoblots. As only a limited number of PBNs could be obtained from each study subject, a second cohort, consisting of 8 SA and 14 NSA subjects, was used to evaluate neutrophil autophagy and NET production levels using confocal microscopy and PicoGreen assay. Asthma was diagnosed by recurrent episodes of wheezing, dyspnea, cough, and evidence of airway hyperresponsiveness to methacholine or variable airway obstruction improved by a short-acting β_2 agonist [19]. All of the subjects had been maintained on inhaled corticosteroid/long-acting β_2 agonist to control their asthmatic symptoms according to the Global Initiative for Asthma guidelines. SA was identified according to the International Guidelines of the European Respiratory Society/American Thoracic Society [20]. Subjects with comorbidities or those having used systemic corticosteroids and other immunosuppressants or biologics were excluded from the study.

Atopy was identified as described previously [12]. Sputum neutrophil and eosinophil counts were evaluated as described in a previous study [12]. Neutrophilic asthma was classified in patients having asthma with sputum neutrophil counts $\geq 65\%$; eosinophil asthma was classified in patients with sputum eosinophil counts $\geq 3\%$.

All study subjects provided written informed consent at the time of recruitment. The study was approved by the Institutional Review Board of Ajou University Hospital (AJIRB-GEN-GEN-09-140).

Human airway epithelial cell (AEC) culture

The human lung carcinoma cell line (A549) and primary small airway epithelial cells (SAECs) were purchased from American Type Culture Collection (Manassas, VA, USA) and cultured as described previously [13].

Antibodies and reagents

The antibodies and reagents are detailed in an online supplementary document of this article.

Isolation of PBNs and PBEs from asthmatic patients

At the time of diagnosis, venous blood (30 ml) samples from patients with SA and NSA were collected into BD Vacutainer[®] tubes containing acid citrate dextrose solution (BD Biosciences, Franklin Lakes, NJ, USA), stored at room temperature (RT), and processed within 2 h after collection.

Blood was layered onto a LymphoprepTM solution (Axis-Shield, Oslo, Norway), followed by centrifugation at 879 *g*, at 20°C for 25 min without braking. The layer containing red blood cells (RBCs) and granulocytes was sedimented for 30 min in Hank's balance salt solution (HBSS) buffer containing 2 mM ethylenediaminetetraacetic acid (EDTA) and 2% dextran at RT. The neutrophil-rich layer was collected and washed once with HBSS buffer containing 2 mM EDTA. Contaminating RBCs were eliminated by hypotonic lysis. PBNs were maintained in RPMI-1640 medium supplemented with 2% heat-inactivated foetal bovine serum (FBS), penicillin (100 IU/ml), and streptomycin (50 µg/ml). PBEs were isolated from the neutrophil-rich layer using the Eosinophil Isolation Kit and MACS Columns (Miltenyi Biotec Inc., Auburn, CA, USA) according to the manufacturer's protocols. Isolated PBEs were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FBS. Cell viability (>98%) was assessed by trypan blue staining. Cell purity (>95%) was assessed by H&E staining and flow cytometry based on CD68 and CD11b expression (for neutrophils) or Siglec-8 and eosinophil cationic protein (ECP) expression (for eosinophils). After isolation, PBNs and PBEs were placed on ice in the culture medium for 30 min prior to further experiments.

Investigation of autophagy and NET production levels from PBNs

Untreated or IL-8-treated (100 ng/ml) PBNs were incubated for 16 h (for western blot analysis) or 3 h (for confocal microscopy analysis and PicoGreen assay). E64D (10 µM) and pepstatin A (15 µM) (E+P), or hydroxychloroquine (HCQ) were used to block the degradation of autophagolysosomes when indicated.

Evaluation of autophagy and protein expression by western blot

During autophagy, microtubule-associated light chain 3 (LC3)-I is converted into LC3-II and bound to autophagosome membranes. In our experiments, LC3-I was undetected in several cases; consequently, autophagy levels were evaluated by the relative expression of LC3-II to beta-actin. The expressions of LC3-II, tight junction (TJ) proteins including occludin and claudin-1, and ECP were analysed by western blot as described previously [13].

Confocal microscopy analysis

PBNs (2×10^5) were seeded on L-lysine coated slides (Polysciences, Warrington, PA, USA) and treated (or untreated) with the indicated reagents for 3 h. Autophagy was detected using an anti-LC3 antibody as described previously.[13] The cut-off value to determine autophagy-positive cells was calculated by the mean number of LC3 puncta in IL-8-treated PBNs isolated from three patients with NSA (LC3 puncta were counted in 30 PBNs for each sample) [21]. Autophagy-positive cells were defined as neutrophils that expressed ≥ 15 LC3 puncta. Concomitantly, cells were stained with anti-neutrophil elastase (NE) antibody and DAPI to detect NET formation. NET-positive cells were identified by cells that produced extracellular, decondensed DNA in fibres or in a web-like shape colocalised with NE.

Neutrophil migration assay

The rate of neutrophil migration was evaluated using a transwell system (Neuro Probe, Gaithersburg, MD, USA) according to the manufacturer's instructions. Isolated PBNs from healthy donors were suspended in non-phenol red RPMI medium containing 10% FBS. Cells were stained with 2 μ M of calcein-acetomethylester for 30 min at 37°C, followed by one

wash with HBSS. Cells (1×10^6) were pretreated with LY294002, HCQ, clarithromycin (CAM) or dexamethasone (Dex) for 15 min and seeded onto the upper filter plate (pore size of 3.0 μ m). RPMI medium containing 10 ng/ml IL-8 was added to the lower chamber and the transwell plate was incubated at 37°C for 2 h. Medium containing PBNs (100 μ l) from each lower chamber was then transferred to a black-wall plate (Thermo Fisher Scientific, Roskilde, Denmark), and the signal was read at 480 nm of excitation and 520 nm of emission wavelengths under a fluorescent microplate reader (Synergy HT; BioTek Instrument, Inc., Winooski, VT, USA).

Evaluation of NET production by PicoGreen assay

NET production levels were evaluated by measuring the extracellular DNA (ecDNA) concentration in neutrophil culture supernatants. Micrococcal nuclease (MNase, 1 U/ml) was added to the culture plates and incubated for 20 min at 37°C to digest NETs after treatment with IL-8 (100 ng/ml) for 3 h. The reaction was stopped by adding 10 mM EDTA. Cells and cell debris were eliminated by centrifugation at 300 *g* for 5 min, and supernatants containing cell-free DNA were then collected and quantified using Quant-iT™ PicoGreen® dsDNA kits (Invitrogen, Paisley, UK) according to the manufacturer's instructions.

NET induction and isolation for target cell treatment

NETs were induced by PMA and isolated as described previously with minimal modifications [22]. PBNs were isolated from healthy donors and seeded onto six-well plates (9×10^6 cells/well) and treated with 100 nM of phorbol myristate acetate (PMA) for 3 h. Supernatants were discarded carefully by suction and each well was washed twice with RPMI

to eliminate PMA and NET-unassociated substances. To confirm that isolated NETs did not contain residual PMA that could interfere with NETs on target cells, the supernatants from the third wash (wSup) were collected and used to treat target cells as controls. RPMI (1 ml) containing MNase (1 U/ml) was then added to each well to digest NETs at 37°C for 20 min. Supernatants containing NETs were collected after eliminating cell or cell debris by centrifugation. Isolated NETs were quantified by measuring ecDNA concentration using the PicoGreen assay and stored at -70°C for further experiments within 7 days.

Evaluating the effects of NET on A549 cells, SAECS, and PBEs

A549 cells or SAECS were seeded onto 12-well plates or L-lysine coated cover slips to attain 80% confluence. PBEs were isolated from SA (n=3) and NSA (n=3) subjects and seeded onto 12-well plates (5×10^5 cells/well) or L-lysine-coated cover slips. Cells were treated with isolated NETs or wSup at various concentrations for 24 h. When indicated, NETs with or without anti-NE antibody (200 ng/ml) and anti-myeloperoxidase (MPO) antibody (200 ng/ml) were preincubated at 37°C for 1 h prior to cell treatment. Cells were pretreated with Dex, CAM, and a pan-caspase inhibitor (Z-VAD-FMK) for 1 h when indicated. After treatment, cells were lysed in RIPA buffer, and cell lysates were analysed by western blot. Supernatants were collected and stored at -70°C for further analysis. Cell morphology changes were investigated by H&E staining and observed under converted microscopy.

Evaluation of cell death and cell detachment

After treatment with NETs, the cell numbers were assessed using a cell counting kit-8 (CCK-8, Dojindo Molecular Technologies, Kumamoto, Japan) according to the manufacturer's protocol. To evaluate cell detachment, culture plates were washed once with PBS to eliminate detached cells before adding the CCK-8 solution. The % cell detachment = [(cell number in unwashed plates – cell number in washed plate) / cell number in unwashed plates] × 100%.

Evaluation of IL-8, ECP and eosinophil derived neurotoxin (EDN) in cell culture supernatants by enzyme-linked immunosorbent assay (ELISA)

Commercial ELISA kits were used to measure IL-8 production (Endogen, Woburn, MA, USA) and the release of ECP and EDN (Medical & Biological Laboratories, Nagoya Aichi, Japan) in cell culture supernatants according to the manufacturer's protocols.

Statistical analysis

The Mann–Whitney U test was used to compare the data for continuous variables; Pearson's chi-square or Fisher's exact tests were used for categorical variables. To compare autophagy and NET production levels from PBNs between the SA and NSA groups, the generalised linear model was applied to adjust for age as a covariate. Statistical correlations were analysed by Spearman's rank coefficient. All statistical analyses were performed with SPSS software, version 22.0 (SPSS Inc., Chicago, IL, US). *P* values <0.05 were considered to indicate statistical significance. GraphPad Prism 5.0 software (GraphPad Inc., San Diego, CA, USA) was used for graphs, with values presented as the mean ± standard deviation (SD) of at least three independent experiments.

RESULTS

Clinical characteristics of the study subjects

Table S1 shows the clinical characteristics of the subjects recruited to participate in the present study. The mean age of the SA group (49.96 ± 14.8 years) was older than that of the NSA group (42.48 ± 15.7 years, $P = 0.044$). The baseline % FEV1 predicted value of the SA group (77.7 ± 19.1) was significantly lower than that of the NSA group (91.98 ± 13.7 , $P=0.032$). The prevalence of neutrophilic asthma in the SA group (69.2%) tended to be higher than that in the NSA group (37.5%); however, no statistically significant difference was observed ($P = 0.203$).

Autophagy and NET production from PBNs of patients with SA and NSA

In the first cohort, we evaluated autophagy levels by analysing LC3-II expression using western blots after culturing PBNs for 16 h with or without IL-8 treatment (Fig. 1, A&B). E64D + pepstatin A was added to block the degradation of autophagosomes. Both untreated and IL-8-treated PBNs from the SA group exhibited significantly higher relative LC3-II expression levels compared with those from the NSA group ($P=0.027$ and $P=0.022$; respectively). Additionally, IL-8 significantly enhanced LC3-II expression in the SA group ($P=0.041$) but not in the NSA group ($P=0.532$). Moreover, we observed decreased total protein amount from IL-8-treated neutrophils in some cases, which could be due to cell death (NETosis) following NET production

In the second, independent cohort, autophagy levels were further evaluated by a confocal microscopy (Fig. 1, C&D). PBNs were cultured for 3 hr with or without IL-8 treatment. Mean numbers of autophagy positive cells in IL-8 treated PBNs of the SA group were significantly higher than the NSA group ($P=0.012$). Autophagy level in untreated PBNs from

the SA group was higher, but not statistically significant, than those of the NSA group ($P=0.054$). IL-8 remarkably increased autophagy-positive neutrophil numbers in the SA group ($P<0.001$), but weakly increased the neutrophil autophagy level in the NSA group ($P=0.039$).

NET production levels were also evaluated in the second cohort using immunocytochemistry (confocal microscopy) and cell-free ecDNA levels were measured using PicoGreen assays (Fig. 2). IL-8-treated PBNs of the SA group produced a significantly higher level of NET and ecDNA compared with the NSA group as indicated by confocal microscopy (Fig. 2A and B) and PicoGreen assay (Fig. 2C, $P<0.001$ for both). IL-8 significantly increased NET and ecDNA production from PBNs of the SA group ($P<0.001$ in confocal microscopy and $P=0.010$ in the PicoGreen assay) but not from those of the NSA group ($P=0.164$ and $P=0.329$, respectively).

Correlations of NET production levels with autophagy levels and airflow limitation-obstruction

Both naïve untreated and IL-8 treated PBNs showed positive correlations between NETs and autophagy levels ($r = 0.726$ and $r = 0.816$, respectively; $P<0.001$ for both, Fig. 3A and B). Moreover, pretreatment with LY294002 (a PI3 kinase inhibitor) but not HCQ (a protease inhibitor) reduced NET production from the IL-8-treated PBNs of the SA group (data not shown). In addition, we observed a negative correlation between the IL-8-induced NET ecDNA production level with the FEV1/FVC ratio in the study subjects ($r = -0.700$, $P = 0.016$, Fig. 3C), but not with the FEV1 % or FVC % predicted values.

Autophagy inhibitors reduced neutrophil migration

We tested the effect of two autophagy inhibitors (LY294002 and HCQ), as well as CAM and Dex (two compounds used in neutrophilic asthma treatment), on autophagy and IL-8-induced neutrophil migration (Fig. S1). Dex and CAM significantly increased LC3-II expression in PBNs with or without HCQ treatment, which suggested an autophagy inductive effect (Fig. S1 A). In the migration assay, pretreatment with LY294002 and HCQ significantly reduced neutrophil migration towards IL-8 ($P<0.01$ and $P<0.05$, respectively; Fig. S1 B). In contrast, neither CAM nor Dex affected IL-8-induced neutrophil migration.

NETs induced AEC death and detachment

Figure 4 shows the effect of isolated NETs on the survival and detachment of A549 cells (Fig. 4A and B) and SAEs (Fig. 4C and D). NETs at a concentration of 5 $\mu\text{g/ml}$ of cell-free ecDNA induced >30% cell death ($P<0.001$) and >60% cell detachment ($P<0.001$) to AECs. Over 50% of A549 cells treated with 5 $\mu\text{g/ml}$ of NET ecDNA in NETs for 3 h significantly changed their morphology from a triangle or spindle shape to a round shape and detached from the culture plate (Fig. S2). However, wSup at the same volume dilution ratios as NETs did not induce cytotoxicity to A549 cells (Fig. S3 A). Therefore, the NET ecDNA concentration of 5 $\mu\text{g/ml}$ was selected for further experiments.

NETs contain both NE and MPO [22]; therefore, we investigated whether these enzymes contributed to the cytotoxic effect of NETs on AECs. Pretreating NETs with antibodies to NE or MPO (200 ng/ml) significantly recovered cell survival and reduced cell detachment induced by NETs in both A549 cells (Fig. 5, A&B) and SAEs (Fig. 5, D&E), which was not observed in NETs pretreated with isotype control antibody (Fig. S4). Pretreating A549 cells and SAEs with a pan-caspase inhibitor (V-ZAD-FMK) partially recovered cell survival but

did not affect cell detachment induced by NETs (Fig. S3 S5). However, pretreating A549 cells as well as SAECs with Dex further enhanced the toxicity effect of NETs (Fig. 5C and F).

NET-induced degradation of TJ proteins in AECs

TJ proteins such as occludin and claudin-1 are important for maintaining airway epithelial integrity [23, 24]; therefore, we investigated expressions of occludin and claudin-1 in NET-treated SAECs (Fig. 6). NETs dose-dependently down-regulated the expressions of occludin and claudin-1 ($P<0.001$ at 5 $\mu\text{g/ml}$ of cell-free ecDNA concentration). In addition, pretreating NETs with anti-NE or anti-MPO antibody (200 ng/ml) partially, but significantly, recovered the expressions of occludin ($P<0.01$) and claudin-1 ($P<0.001$) in SAECs. A similar observation was found in A549 cells (Fig. S4 S6) However, pretreatment with V-ZAD-FMK could not inhibit NET-induced degradation of occludin and claudin-1 in SAECs (Fig. S5 S7).

NET induced IL-8 production from AECs

The effect of NETs on the pro-inflammatory function of AECs was evaluated by measuring IL-8 production levels from NET- treated A549 cells and SAECs (Fig. 7). First, we found that NETs at the treatment concentrations contained a negligible amount of IL-8. However, NETs with 2.5 $\mu\text{g/ml}$ of ecDNA stimulated both A549 cells and SAECs to produce a significantly high level of IL-8 ($P < 0.01$ for both cell types). As NETs containing 5 $\mu\text{g/ml}$ ecDNA significantly decreased cell viability (Fig. 4), the IL-8 production level induced by NETs with 5 $\mu\text{g/ml}$ ecDNA was slightly lower than that induced by NETs with 2.5 $\mu\text{g/ml}$ ecDNA, although the level was significantly higher than that of untreated cells ($P<0.01$ for

both cell types). However, wSup treatment did not induce IL-8 production from A549 cells (Fig. S3 B).

NET-induced PBE activation and degranulation

NETs at 1 µg/ml of cell-free ecDNA caused PBE spreading and the emission of pseudopods (Fig. S6 S8), which are the morphological markers for eosinophil activation [25]. NETs at a higher ecDNA concentration (5 µg/ml) induced primary lysis of PBEs, as evidenced by the release of free eosinophil granules (FEGs). However, wSup induced no morphological changes in PBEs (Fig. S8 E). We also evaluated the effect of NETs on apoptosis and primary lysis of PBEs by measuring the expression of annexin V and propidium iodide (PI) using flow cytometry (Fig. S7 S9). NETs at 1 µg/ml increased annexin V (+)/propidium iodide (PI) (-) cell number ($P<0.001$) and slightly reduced annexin V (+)/PI(+) cell number ($P<0.05$), indicating increased apoptosis or the early phase of primary cell lysis. NETs at 3 µg/ml increased both annexin V (+)/PI (-) and annexin V (+)/PI (+) cell number ($P<0.001$ for both), indicating increased eosinophil primary lysis.

We then evaluated the degranulation effect of NETs on PBEs isolated from patients with SA (n=3) and NSA (n=3) randomly selected from the second cohort (Fig. 8A). EDN secretion levels from PBEs of patients with SA were significantly higher than those from NSA patients ($P<0.001$). Moreover, NETs dose-dependently increased the release of EDN from patients with SA ($P<0.01$) and NSA ($P<0.001$). Additionally, NETs also dose-dependently induced ECP secretion from PBEs of three healthy donors (Fig. 8B). Western blot analysis (Fig. S8 S10) demonstrated an increased ECP level in culture supernatants and a decreased intracellular ECP expression, indicating enhanced eosinophil degranulation rather than increased ECP synthesis.

DISCUSSION

The role of neutrophil in airway inflammation of SA was implicated but not fully understood [3, 4]. A few studies reported autophagy and NET findings in asthma [13, 17]; however, no reports have characterised the roles of autophagy and NETs in neutrophil-mediated airway inflammation in asthma. This is the first study to demonstrate higher levels of autophagy and NET production from PBNs of patients with SA compared with those with NSA. Moreover, inhibiting autophagy could reduce the migration of PBNs towards IL-8. NETs can trigger inflammatory responses by damaging AECs, inducing IL-8 production from AECs and activating eosinophils to release their granules. The findings implicate the important roles of autophagy and NETs in the functions of neutrophils that contribute to the maintenance of airway inflammation in the pathogenesis of asthma, especially in SA.

Autophagy, a well-known cellular process important for cellular function and survival [11], induces mucus secretion and mediate IL-13-induced eotaxin-3 peptide secretion from AECs [26]. Our previous study demonstrated increased levels of autophagy in sputum granulocytes and PBEs of SA patients, suggesting that autophagy could enhance eosinophil activation in SA [13]. Consistently, the present study found increased levels of autophagy in PBNs from patients with SA, suggesting a role of neutrophil autophagy in the pathogenesis of SA. In SA, inflammatory cytokines (such as IL-8, TNF- α , IL-1 β) are up-regulated and associated with neutrophil activation [6, 8, 27]. Previous studies reported that TNF- α and IL-1 β could induce autophagy in human neutrophils [28, 29]. The present study revealed that IL-8 induced autophagy in the PBNs of patients with SA, but it did so less significantly in those with NSA. The findings suggest that PBNs in SA could be primed by pro-inflammatory and inflammatory cytokines to induce a high level of autophagy that could maintain or enhance neutrophil activation.

We investigated the role of autophagy in neutrophil function by evaluating the migration rate of PBNs treated with the two autophagy inhibitors [LY294002, a PI3K inhibitor, and HCQ, a weak base that can inhibit the final step of autophagy (autolysosome degradation)] [13], compared with that of PBNs treated with CAM and Dex. Both autophagy inhibitors significantly reduced the migration of PBNs towards IL-8, whereas CAM and Dex did not. Furthermore, the finding of that Dex and CAM induced autophagy in neutrophils was consistent with the previous reports [30, 31]. We suggested previously that autophagy-related (*ATG*) 5 and *ATG*7 polymorphisms could contribute to neutrophil activation and function [12]. Consistently, deleting *Atg5* and *Atg7* in mice was found to reduce neutrophil degranulation and neutrophil-mediated inflammation [32]. The findings implicate a crucial role of autophagy in the activation and function of neutrophils. Moreover, we suggest that autophagy inhibition could be a novel therapy to treat patients with corticosteroid-resistant asthma.

Autophagy is crucial for NET production [14, 29]; therefore, we evaluated NET production from patients with SA and NSA in the second cohort. We observed greater NET production levels from the IL-8 treated PBNs of patients with SA compared with NSA. Elevated NET production was previously observed in the airways of patients with asthma as well as those with COPD patients [18, 33]. Moreover, we found a significantly negative correlation between IL-8-induced ecDNA production levels and the FEV1/FVC ratio, but this was not the case for the FEV1% or FVC% predicted values. The FEV1/FVC ratio reflects the degree of airflow obstruction in asthma and COPD, whereas the FEV1 % or FVC % predicted value alone may not be sufficient to reflect the degree of airway obstruction [34, 35]. Our finding is supported by other studies that revealed a positive correlation between sputum NET amounts and COPD severity [16], indicating that NETs could be associated with the severity of airway inflammation and obstruction. Additionally, NET was found to cause airway

obstruction by increasing cell-rich plugs in the small airways [36]. Those findings implicate that increased NET formation in patients with asthma may be associated with airway obstruction and asthma severity.

We found strong positive correlations between autophagy and NET production levels in PBNs, consistent with previous studies demonstrating a concomitant production of autophagy and NET [14, 29]. Furthermore, the inhibition of autophagy could attenuate NET production, suggesting a crucial role of autophagy in NET production. However, we did not observe different NET production levels from untreated PBNs between the two study groups despite a higher autophagy level in the naïve untreated PBNs of the SA group versus the NSA group. The findings suggest that: 1) autophagy is induced in the early stages of neutrophil activation, which is followed by NET production in response to further stimulation (e.g. by IL-8); and 2) autophagy is crucial for NET production, but the induction of autophagy in neutrophils does not always lead to the generation of NETs. We found that IL-8 strongly induced neutrophil autophagy and NET production in the SA group, but it did so less effectively in the NSA group. Our findings are inconsistent but not contradictory with previous studies showing that IL-8 induced NET production from healthy subjects [15, 37]. We speculate that the response of PBNs to IL-8 is dependent on the host environment in which the cells have been generated and developed. Further studies are required to reveal the factors that affect autophagy production levels in PBNs of patients with SA, and the mechanism by which IL-8 induces neutrophil autophagy differently in various extracellular environments.

In the airways of patients with SA, activated neutrophils can adhere to airway epithelium and exert an inflammatory function [38, 39]. Damaged airway epithelium could result in airway obstruction and remodelling [40, 41]. We demonstrated that NETs could impair the integration of airway epithelium by causing AEC death and detachment, leading to increased transepithelial permeability. Consistently, a previous study reported NET-induced apoptosis

of A549 cells [22]. The survival of AECs requires adhesion to basement membranes; consequently, cell detachment is followed by cell death [42]. In the present study, NETs down-regulated the expression of epithelial TJ proteins, occludin and claudin-1, which could be a mechanism of NET-induced AEC detachment followed by cell death. Furthermore, we found that NETs stimulated AECs to produce IL-8 that could generate a positive feedback loop to recruit more neutrophils to the airway and activate them, thereby aggravating the inflammatory responses [38].

MPO and NE are two major enzymes present in NETs, which could damage the airway epithelial integrity [43, 44]. Previously, Saffarzadeh *et al.* [22] demonstrated that MPO inhibitor, but not NE inhibitor, reduced the cytotoxic effect of NETs on A549 cells. However, the present study found that two specific antibodies against MPO and NE significantly reduced NET-induced AEC death, cell detachment and TJ protein degradations. The pan-caspase inhibitor Z-VAD-FMK partially recovered cell survival, but did not affect cell detachment and TJ protein degradations induced by NETs. The findings suggested that NETs induced AEC death by activating caspase pathways and degrading TJ proteins. Moreover, TJ proteins were directly degraded by NET components such as MPO and NE rather than by caspase activity. Other components of NETs, such as histone [22], could also contribute to airway epithelium impairment as anti-MPO and anti-NE antibodies did not completely inhibit NET-induced AEC damage. Interestingly, pretreating AECs with Dex further enhanced the cytotoxicity effect of NETs, whereas Dex alone did not exert toxicity effect on AECs. This finding could explain the corticosteroid resistance found in asthma cases with a neutrophilic phenotype, in which NET production levels are high. Consequently, therapeutic intervention using NET production inhibitors should be considered asthmatic patients having predominantly local and/or systemic neutrophilia.

Eosinophil activation, degranulation, and primary lysis (with release of FEGs) are implicated in severe asthma [25, 45]. Moreover, the numbers of eosinophils and neutrophils are positively correlated in the sputum of severe asthmatics, suggesting that neutrophil activation could induce an eosinophilic airway inflammation in SA [46]. In the present study, we found that NETs induced eosinophil activation at low concentrations and caused primary eosinophil lysis to release FEGs at higher concentrations. Moreover, NETs dose-dependently induced EDN and ECP secretions from PBEs, which are markers for eosinophil degranulation. These findings suggest that NET-producing neutrophils in asthmatic airways could induce eosinophil activation and degranulation, which could maintain and/or enhance airway inflammation in SA.

Our present study had limitations related to evaluating the exact level (concentration) of the NETs produced *in vivo* in asthmatic airways that could help determine the concentrations of NETs that have effects on AECs and PBEs. Standardised methods are required of quantifying *in vivo* NET production levels, which could be used as a biomarker to predict SA phenotype and applied to *in vitro* diagnostic investigations. Additionally, the factors that enhance neutrophil autophagy and NET production levels in PBNs of patients with SA should be further investigated.

In conclusion, neutrophils from SA patients produce high levels of autophagy and NET, which could contribute to the development of SA by enhancing neutrophil/eosinophil activation, damaging airway epithelium and triggering the inflammatory responses of AECs and eosinophils. Modulating neutrophil autophagy and NETs may be new targets for SA treatment.

Authors' Contribution

L.D. Pham designed and performed the experiments, analysed data, and wrote the manuscript; G.Y. Ban collected patient samples and joined in analysing the data; S.H. Kim joined in designing the experiments and interpreting data; Y.M. Ye joined in collecting patient samples and interpreting data; Y.J. Chwae and H.S. Park supervised the process and revised and approved the manuscript for submission.

Funding

This study was supported by a grant from the Korean Health Technology R&D Project, Ministry of Health & Welfare, Republic of Korea (HI16C0992).

Conflicts of interests

None declared.

Ethics approval

This study was approved by the Institutional Review Board of Ajou University Hospital (AJIRB-GEN-GEN-09-140).

REFERENCES

1. Virchow JC. Diagnostic challenges of adult asthma. Curr Opin Pulm Med. 2016;22:38-45.

2. Chen Y, Wong GW, Li J. Environmental exposure and genetic predisposition as risk factors for asthma in China. *Allergy Asthma Immunol Res.* 2016;8:92-100.
3. Pelaia G, Vatrella A, Busceti MT, Gallelli L, Calabrese C, Terracciano R, et al. Cellular mechanisms underlying eosinophilic and neutrophilic airway inflammation in asthma. *Mediators Inflamm.* 2015;2015:879783.
4. Jo EJ, Kim MY, Lee SE, Lee SY, Kim MH, Song WJ, et al. Eosinophilic airway inflammation and airway hyperresponsiveness according to aeroallergen sensitization pattern in patients with lower airway symptoms. *Allergy Asthma Immunol Res.* 2014;6:39-46.
5. Bruijnzeel PL, Uddin M, Koenderman L. Targeting neutrophilic inflammation in severe neutrophilic asthma: can we target the disease-relevant neutrophil phenotype? *J Leukoc Biol.* 2015;98:549-56.
6. Mann BS, Chung KF. Blood neutrophil activation markers in severe asthma: lack of inhibition by prednisolone therapy. *Respir Res.* 2006;7:59.
7. Kim SR, Lee YC. Endoplasmic reticulum stress and the related signaling networks in severe asthma. *Allergy Asthma Immunol Res.* 2015;7:106-17.
8. Silvestri M, Bontempelli M, Giacomelli M, Malerba M, Rossi GA, Di Stefano A, et al. High serum levels of tumour necrosis factor-alpha and interleukin-8 in severe asthma: markers of systemic inflammation? *Clin Exp Allergy.* 2006;36:1373-81.
9. Wang Y, Bai C, Li K, Adler KB, Wang X. Role of airway epithelial cells in development of asthma and allergic rhinitis. *Respir Med.* 2008;102:949-55.
10. Mizushima N, Levine B. Autophagy in mammalian development and differentiation. *Nat Cell Biol.* 2010;12:823-30.

11. Mizushima N. Autophagy: process and function. *Genes Dev.* 2007;21:2861-73.
12. Pham DL, Kim SH, Losol P, Yang EM, Shin YS, Ye YM, et al. Association of autophagy related gene polymorphisms with neutrophilic airway inflammation in adult asthma. *Korean J Intern Med.* 2016;31:375-85.
13. Ban GY, Pham DL, Trinh HK, Lee SI, Suh DH, Yang EM, et al. Autophagy mechanisms in sputum and peripheral blood cells of patients with severe asthma: a new therapeutic target. *Clin Exp Allergy.* 2016;46:48-59.
14. Remijsen Q, Vanden Berghe T, Wirawan E, Asselbergh B, Parthoens E, De Rycke R, et al. Neutrophil extracellular trap cell death requires both autophagy and superoxide generation. *Cell Res.* 2011;21:290-304.
15. Brinkmann V, Zychlinsky A. Neutrophil extracellular traps: is immunity the second function of chromatin? *J Cell Biol.* 2012;198:773-83.
16. Grabcanovic-Musija F, Obermayer A, Stoiber W, Krautgartner WD, Steinbacher P, Winterberg N, et al. Neutrophil extracellular trap (NET) formation characterises stable and exacerbated COPD and correlates with airflow limitation. *Respir Res.* 2015;16:59.
17. Dworski R, Simon HU, Hoskins A, Yousefi S. Eosinophil and neutrophil extracellular DNA traps in human allergic asthmatic airways. *J Allergy Clin Immunol.* 2011;127:1260-6.
18. Wright TK, Gibson PG, Simpson JL, McDonald VM, Wood LG, Baines KJ. Neutrophil extracellular traps are associated with inflammation in chronic airway disease. *Respirology.* 2016;21:467-75.

19. Hwang EK, Jin HJ, Nam YH, Shin YS, Ye YM, Nahm DH, et al. The predictors of poorly controlled asthma in elderly. *Allergy Asthma Immunol Res.* 2012;4:270-6.
20. Chung KF, Wenzel SE, Brozek JL, Bush A, Castro M, Sterk PJ, et al. International ERS/ATS guidelines on definition, evaluation and treatment of severe asthma. *Eur Respir J.* 2014;43:343-73.
21. Klionsky DJ, Abeliovich H, Agostinis P, Agrawal DK, Aliev G, Askew DS, et al. Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. *Autophagy.* 2008;4:151-75.
22. Saffarzadeh M, Juenemann C, Queisser MA, Lochnit G, Barreto G, Galuska SP, et al. Neutrophil extracellular traps directly induce epithelial and endothelial cell death: a predominant role of histones. *PLoS One.* 2012;7:e32366.
23. Park do Y, Kim S, Kim CH, Yoon JH, Kim HJ. Alternative method for primary nasal epithelial cell culture using intranasal brushing and feasibility for the study of epithelial functions in allergic rhinitis. *Allergy Asthma Immunol Res.* 2016;8:69-78.
24. Kojima T, Go M, Takano K, Kurose M, Ohkuni T, Koizumi J, et al. Regulation of tight junctions in upper airway epithelium. *Biomed Res Int.* 2013;2013:947072.
25. Muniz-Junqueira MI, Barbosa-Marques SM, Junqueira LF, Jr. Morphological changes in eosinophils are reliable markers of the severity of an acute asthma exacerbation in children. *Allergy.* 2013;68:911-20.
26. Zeki AA, Yeganeh B, Kenyon NJ, Post M, Ghavami S. Autophagy in airway diseases: a new frontier in human asthma? *Allergy.* 2015.

27. Barnes PJ. The cytokine network in asthma and chronic obstructive pulmonary disease. *J Clin Invest.* 2008;118:3546-56.
28. Pliyev BK, Menshikov M. Differential effects of the autophagy inhibitors 3-methyladenine and chloroquine on spontaneous and TNF-alpha-induced neutrophil apoptosis. *Apoptosis.* 2012;17:1050-65.
29. Mitroulis I, Kambas K, Chrysanthopoulou A, Skendros P, Apostolidou E, Kourtzelis I, et al. Neutrophil extracellular trap formation is associated with IL-1beta and autophagy-related signaling in gout. *PLoS One.* 2011;6:e29318.
30. Troncoso R, Paredes F, Parra V, Gatica D, Vasquez-Trincado C, Quiroga C, et al. Dexamethasone-induced autophagy mediates muscle atrophy through mitochondrial clearance. *Cell Cycle.* 2014;13:2281-95.
31. Konstantinidis T, Kambas K, Mitsios A, Panopoulou M, Tsironidou V, Dellaporta E, et al. Immunomodulatory role of clarithromycin in acinetobacter baumannii infection via formation of neutrophil extracellular traps. *Antimicrob Agents Chemother.* 2015;60:1040-8.
32. Bhattacharya A, Wei Q, Shin JN, Abdel Fattah E, Bonilla DL, Xiang Q, et al. Autophagy is required for neutrophil-mediated inflammation. *Cell Rep.* 2015;12:1731-9.
33. Pedersen F, Marwitz S, Holz O, Kirsten A, Bahmer T, Waschki B, et al. Neutrophil extracellular trap formation and extracellular DNA in sputum of stable COPD patients. *Respir Med.* 2015;109:1360-2.
34. Pellegrino R, Viegi G, Brusasco V, Crapo RO, Burgos F, Casaburi R, et al. Interpretative strategies for lung function tests. *Eur Respir J.* 2005;26:948-68.

35. Vestbo J, Hurd S, Agustí A. Global strategy for the diagnosis, management and prevention of COPD, Global Initiative for Chronic Obstructive Lung Disease (GOLD), 2014. 2015.
36. Cortjens B, de Boer OJ, de Jong R, Antonis AF, Sabogal Pineros YS, Lutter R, et al. Neutrophil extracellular traps cause airway obstruction during respiratory syncytial virus disease. *J Pathol.* 2016;238:401-11.
37. Schorn C, Janko C, Latzko M, Chaurio R, Schett G, Herrmann M. Monosodium urate crystals induce extracellular DNA traps in neutrophils, eosinophils, and basophils but not in mononuclear cells. *Front Immunol.* 2012;3:277.
38. Ciepiela O, Ostafin M, Demkow U. Neutrophils in asthma--a review. *Respir Physiol Neurobiol.* 2015;209:13-6.
39. McDonald RJ, St George JA, Pan LC, Hyde DM. Neutrophil adherence to airway epithelium is reduced by antibodies to the leukocyte CD11/CD18 complex. *Inflammation.* 1993;17:145-51.
40. Davies DE. The role of the epithelium in airway remodeling in asthma. *Proc Am Thorac Soc.* 2009;6:678-82.
41. Bergeron C, Tulic MK, Hamid Q. Airway remodelling in asthma: from benchside to clinical practice. *Can Respir J.* 2010;17:e85-93.
42. White SR. Apoptosis and the airway epithelium. *J Allergy (Cairo).* 2011;2011:948406.
43. Sugamata R, Dobashi H, Nagao T, Yamamoto K, Nakajima N, Sato Y, et al. Contribution of neutrophil-derived myeloperoxidase in the early phase of fulminant acute

respiratory distress syndrome induced by influenza virus infection. Microbiol Immunol. 2012;56:171-82.

44. Ginzberg HH, Cherapanov V, Dong Q, Cantin A, McCulloch CA, Shannon PT, et al. Neutrophil-mediated epithelial injury during transmigration: role of elastase. Am J Physiol Gastrointest Liver Physiol. 2001;281:G705-17.
45. Persson C. Primary lysis of eosinophils in severe desquamative asthma. Clin Exp Allergy. 2014;44:173-83.
46. Kikuchi S, Nagata M, Kikuchi I, Hagiwara K, Kanazawa M. Association between neutrophilic and eosinophilic inflammation in patients with severe persistent asthma. Int Arch Allergy Immunol. 2005;137 Suppl 1:7-11.

FIGURE LEGENDS

Figure 1. Autophagy levels in PBNs isolated from patients with severe asthma (SA) and non-severe asthma (NSA). (A, B) Autophagy levels evaluated by LC3-II expression using western blot. (C, D) Autophagy levels evaluated using confocal microscopy. PBNs were stained with anti-LC3 antibody and anti-neutrophil elastase (NE) antibody as a neutrophil marker, and nuclear DNA were stained with DAPI. Cells expressing ≥ 15 LC3 puncta were considered autophagy-positive cells. (A, C) Representative data from NSA and SA groups. (B, D) Comparison of autophagy levels between NSA and SA groups. PBNs were untreated (naïve, open circles) or treated with IL-8 (100 ng/ml, closed circles). The horizontal bars in the middle of the dot plots indicate mean value. Scale bar, 50 μm . *P* values were obtained by Mann-Whitney U test. **P* values were adjusted for age as a covariate using a generalised linear model.

Figure 2. Neutrophil extracellular DNA trap (NET) production levels in PBNs isolated from patients with severe asthma (SA) and non-severe asthma (NSA). (A, B) NET levels were evaluated using confocal microscopy. Cells were stained with neutrophil elastase (NE) antibody and DAPI. (C) NET levels were evaluated by measuring extracellular DNA concentrations using PicoGreen assay. (A) Representative data from NSA and SA groups. (B, C) Comparison of NET levels between SA and NSA groups. Cells were untreated (naïve, opened circles) or treated with IL-8 (100 ng/ml, closed circles) for 3 h. The horizontal bars in the middle of the dot plots indicate mean value. Scale bar, 10 μ m. *P* values were obtained by Mann-Whitey U test. **P* values were adjusted for age as a covariate using a generalised linear model.

Figure 3. Correlation between neutrophil autophagy and NETs evaluated using confocal microscopy. PBNs were untreated (A) or treated with IL-8 (100 ng/ml) for 3 h (B). (C) Correlation between NET production levels from IL-8 treated PBNs and FEV1/FVC ratio. Spearman's rank correlation was applied to the tests.

Figure 4. Neutrophil extracellular DNA trap (NET) induces airway epithelial cell death and detachment. NET was produced by PBNs treated with PMA (100 nM) for 3 hr, then collected and quantified by PicoGreen assay. A549 cells (A, B) and primary small airway epithelial cells (C, D) treated with different concentrations of NETs for 24 h. Cell numbers were assessed by CCK8 assay. The assays were performed in triplicate in three independent experiments (n=9). **P*<0.05, ***P*<0.01, ****P*<0.0001, obtained by one-way ANOVA with Bonferroni's post-hoc test; NS, not significant.

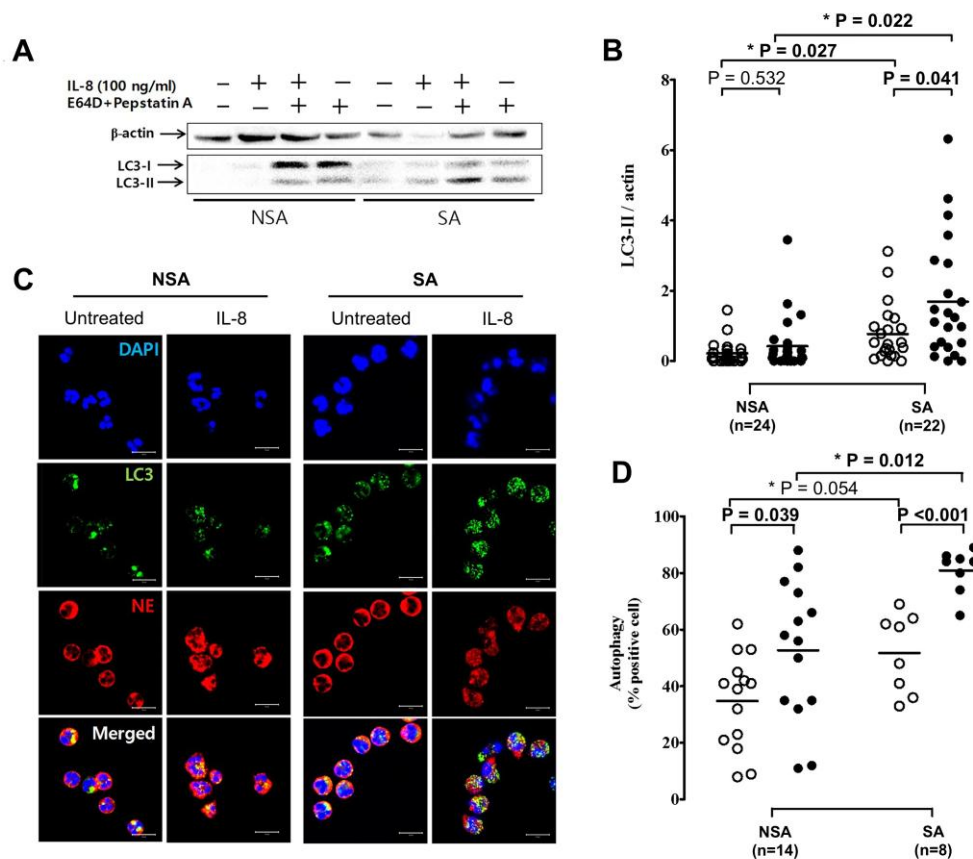
Figure 5. Effects of neutrophil elastase (NE) antibody (Ab), myeloperoxidase (MPO) Ab and dexamethasone (Dex) on NET-induced cytotoxicity. NETs were collected and quantified from PBNs treated with PMA (100 nM) for 3 h. NETs pretreated with MPO antibody or NE antibody were used to treat A549 cells (**A, B**) and primary small airway epithelial cells (SAECs, **D, E**) for 24 h. A549 cells (**C**) or SAECs (**F**) were pretreated with Dex for 1 hr then co-treated with NETs for 24 h. Serum-free medium was use as the control. The assays were performed in triplicate in three independent experiments (n=9). * $P<0.05$, ** $P<0.01$, *** $P<0.0001$, obtained by one-way ANOVA with Bonferroni's post-hoc test; NS, not significant.

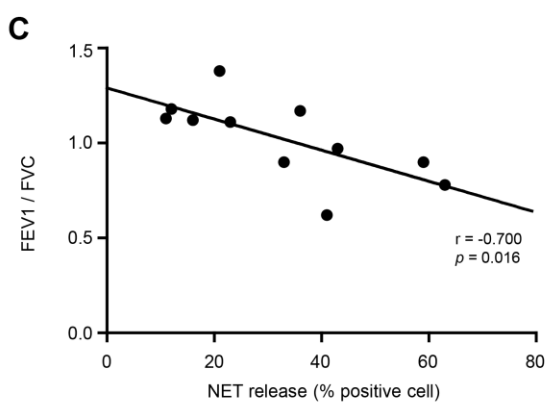
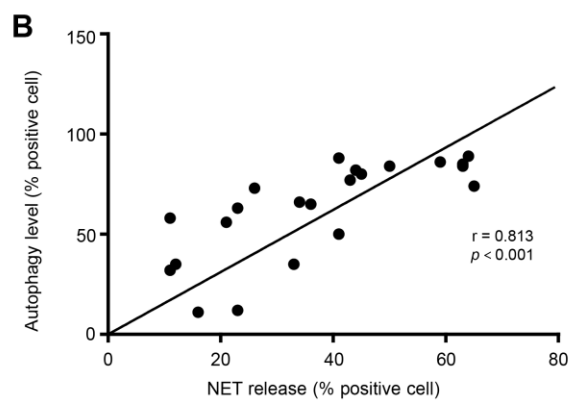
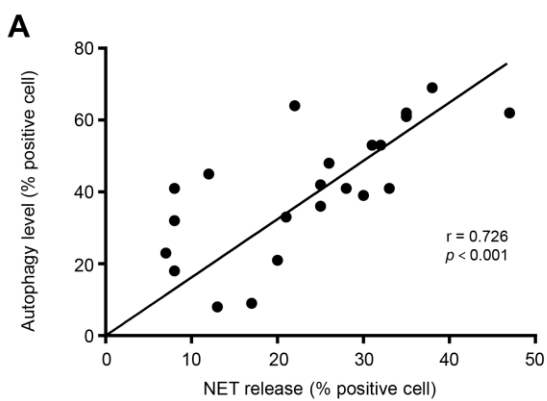
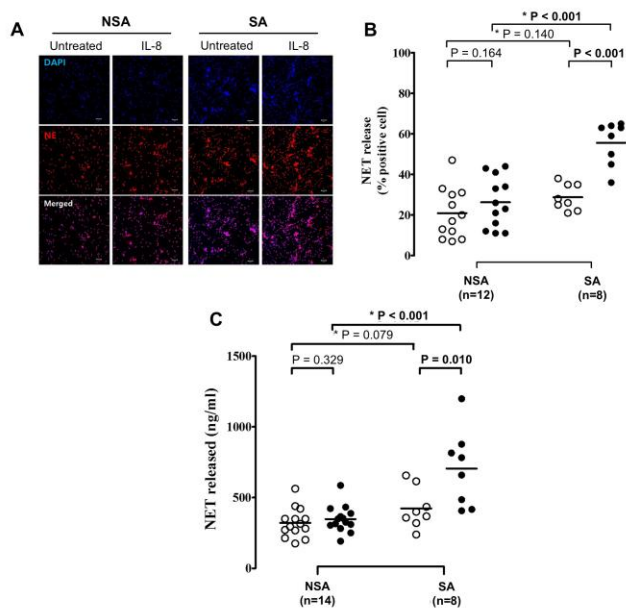
Figure 6. NETs down-regulated tight-junction protein expression. NETs were collected and quantified from PBNs treated with PMA (100 nM) for 3 h. NETs pretreated with MPO antibody or NE antibody were used to treat primary SAECs for 24 h. (**A**) Representative data of at least three independent experiments performed in triplicate (n=9). Intracellular expressions of occludin (**B**) and claudin-1 (**C**) were investigated by western blot. * $P<0.05$, ** $P<0.01$, *** $P<0.0001$, obtained by one-way ANOVA with Bonferroni's post-hoc test.

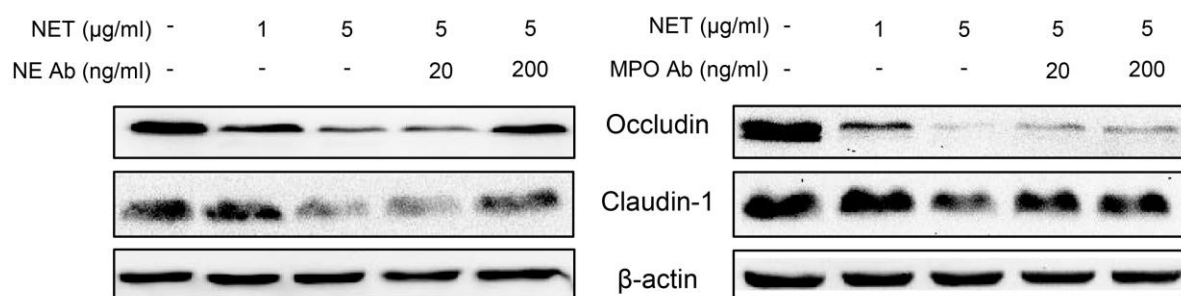
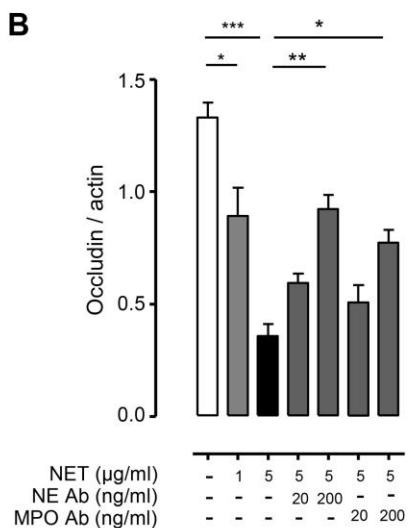
Figure 7. NETs induced IL-8 production from airway epithelial cells (AECs). A549 cells or SAECs were treated with NETs for 24 h. IL-8 levels were measured from culture supernatants and the NET solution. IL-8 production levels from AECs were calculated by subtracting IL-8 levels from the culture supernatants by IL-8 levels in the NET solution at the same concentrations. The assays were performed in triplicate in three independent experiments (n=9). ** $P<0.01$ obtained by ANOVA with Bonferroni's post-hoc test.

Figure 8. The effect of NETs on degranulation of human peripheral blood eosinophils

(PBEs). **(A)** NETs induced eosinophil derived neurotoxin (EDN) release from PBEs of patients with severe asthma (SA) and non-severe asthma (NSA). **(B)** Levels of eosinophil cationic protein (ECP) released from NET-treated PBEs of healthy donors (n=3). The experiments were performed in triplicate for each study subject. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. non-treated cells in the same subject groups, obtained by one-way ANOVA; ## $P < 0.01$, ### $P < 0.001$ when compared between the indicated groups, obtained by Mann-Whitney U test.





A**B****C**