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IgE, but not allergens, induces the release of neutrophil extracellular traps

Immunoglobulina E, ale nie alergeny, indukuje wyrzut zewnątrzkomórkowych sieci neutrofilowych

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ABSTRACT

Introduction: Neutrophils play an important role in innate immunity. To eliminate pathogens they use several mechanisms, such as phagocytosis, degranulation, production of reactive oxygen species (ROS), and release of neutrophil extracellular traps (NETs). It was recently shown that neutrophils can degranulate under the influence of immunoglobulin E (IgE) as they express receptors for IgE. The impact of IgE on the release of NETs has not been investigated thus far.

Aim: To investigate if neutrophils can release NETs following IgE, anti-IgE or allergen stimulation.

Material and methods: Neutrophils were isolated by gradient centrifugation from peripheral blood obtained from sensitized patients and healthy blood donors. Basophil activation was measured using flow cytometry. The presence of NETs following IgE, anti-IgE and allergen stimulation was evaluated quantitatively by fluorometry and qualitatively by fluorescent microscopy.

Results: IgE stimulated NETs release in a concentration-dependent manner, causing the greatest release at 2.86 µg/ml. NETs release under IgE stimulation was significantly stronger in allergic subjects comparing with non-allergic ones, and seemed to be independent of FcεRI expression. IgE-dependent NETs release was significantly decreased following treatment with N-acetylcysteine and 4-aminobenzoic acid. Anti-IgE and allergens did not affect NETs release.

Conclusions: Neutrophils can release NETs in the presence of a high concentration of IgE, which appears to be partially dependent on the allergic status of the patient and the production of ROS. This finding may contribute to better understanding of inflammatory processes accompanied by atopic diseases.

KEY WORDS

allergy, neutrophils, immunoglobulin E, anti-IgE, neutrophil extracellular traps.

STRESZCZENIE

Wprowadzenie: Neutrofile odgrywają kluczową rolę w procesach odporności nieswoistej. Dysponują szeregiem mechanizmów w obronie przeciw patogenom, takich jak fagocytoza, degranulacja, produkcja reaktywnych form tlenu i wyrzut zewnątrzkomórkowych sieci neutrofilowych (NETs). W ostatnich latach wykazano, że neutrofile mogą ulegać degranulacji pod wpływem immunoglobuliny w klasie E (IgE), ponieważ wykazują na swojej powierzchni receptory dla fragmentu Fc tej immunoglobuliny. Jednak do tej pory nie przeprowadzono badań, w których oceniano wpływ IgE na uwalnianie NETs z granulocytów obojętnochłonnych.

Cel pracy: Ocena, czy neutrofile mogą uwalniać NETs pod wpływem IgE, anty-IgE i alergenów.

Materiał i metody: Komórki do badania były izolowane w procedurze wirowania w gradiencie gęstości z krwi pełnej osób uczulonych i zdrowych krwiodawców. Aktywacja bazofilów pod wpływem alergenów była mierzona za pomocą cytometrii przepływowej. Wyrzut NETs pod wpływem IgE, anty-IgE i stymulacji alergenowej był oceniany metodami jakościowymi (mikroskopia fluorescencyjna) i ilościowymi (fluorymetria).

Wyniki: Wykazano, że IgE stymuluje neutrofile do wyrzutu NETs w sposób zależny od stężenia, wywołując najsilniejszy efekt przy stężeniu 2,86 µg/ml. Wyrzut NETs pod wpływem IgE był silniejszy u pacjentów z potwierdzoną alergią niż u osób nieuczulonych i wydawał się niezależny od ekspresji receptora o wysokim powinowactwie do fragmentu Fc IgE (FcεRI). Wyrzut NETs indukowany IgE był hamowany przez zastosowane inhibitory: N-acetylocysteinę i kwas 4-aminobenzoesowy. Wyrzutu NETs nie indukowały alergeny, na które byli uczuleni uczestnicy badania, ani anty-IgE.

Wnioski: Na podstawie przeprowadzonych badań wykazano, że neutrofile mogą uwalniać NETs pod wpływem wysokich stężeń IgE. Proces ten wydaje się zależy od profilu alergicznego pacjenta oraz produkcji reaktywnych form tlenu. Wyniki niniejszych badań mogą przyczynić się do lepszego zrozumienia procesów zapalnych przebiegających u osób z atopią.

SŁOWA KLUCZOWE:

alergia, neutrofile, immunoglobulina E, anty-IgE, zewnątrzkomórkowe sieci neutrofilowe.

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INTRODUCTION

Neutrophils, the most abundant pool of leukocytes during adulthood, are formed in the bone marrow, from where they enter the peripheral blood. They circulate in the bloodstream, where they are present for approximately 19 h [1]; however, neutrophils can leave the vascular bed and enter tissues where they can live for several days. During inflammation, neutrophils activated by various stimuli, are the first cells to migrate to the site of pathogen penetration or tissue damage, where they fight with microbes [2]. To kill pathogens, neutrophils employ several strategies, including phagocytosis, degranulation, production of reactive oxygen species (ROS), and release of neutrophil extracellular traps (NETs). The phenomenon of NETs release was discovered in 2004 by the team of Arturo Zychlinsky; however, the first report about NETs existence was published in 1996, when Takei *et al.* described a type of neutrophil death other than apoptosis and necrosis following stimulation with phorbol acetate myristate (PMA) [3, 4]. Neutrophil extracellular traps are web-like structures composed of chromatin and antimicrobial proteins released outside the cell as a result of intracellular enzymatic cascade and cell membrane rupture. They are involved in defending the body against pathogens by forming a physical barrier that prevents pathogens from further spreading and creating a high local concentration of biocidal proteins. Despite its crucial role in innate immunity, NETs impact on development and exacerbation of autoimmune diseases as they constitute a source of autoantigens, trigger inflammatory response or even contribute to formation of cancer metastases [5].

Interestingly, neutrophils can be activated by exposure of the host to various allergens, hence their accumulation in the site of inflammation, especially in the

respiratory tract, is commonly observed [6]. Patients with asthma have been reported to have an increased number of neutrophils in peripheral blood, which further correlates with disease severity [6]. Neutrophils express high-affinity receptors (FcεRI) for immunoglobulin E (IgE) on their surface and can therefore participate in allergic reactions following activation due to IgE binding [7]. As a result, neutrophils are involved in the process of allergen presentation and release a wide range of substances causing the inflow of other cells involved in the development of inflammation. It has been demonstrated that binding of IgE to the neutrophil FcεRI receptor increases their survival and further emphasizes their activation. It also plays a significant role in the inflammation process that occurs in the course of asthma [7]. NETs have been found in pulmonary alveoli [8]. Furthermore, sputum of asthma patients presents an increased influx of eosinophils accompanied by neutrophil infiltration and extracellular trap structures, the number of which correlates with the symptoms' severity [9]. In addition, neutrophils are capable of producing eosinophil cationic protein (which contributes to airway remodeling in atopic asthma) and interleukin (IL)-8 [9, 10] which is widely known as an activator of NETs release [5].

The role of NETosis in asthma remains unclear. It has been suggested that NETs release may take part in the defense against pathogens or, on the contrary, may participate in respiratory tract damage [11].

AIM

In view of the limited number of studies, we aimed to evaluate the impact of IgE, anti-IgE, and allergens on the release of NETs by neutrophils isolated from allergic patients and healthy individuals.

MATERIAL AND METHODS

REAGENTS

Roswell Park Memorial Institute (RPMI) 1640 medium, phosphate buffered saline (PBS), HEPES, Sytox Green, Sytox Orange, and dihydrorhodamine 123 (DHR) were purchased from Thermo Fisher Scientific (Waltham, USA). Allergenicity Kit, anti-IgE and anti-CD203c-PE antibodies were purchased from Beckmann Coulter (Brea, USA). Anti-MPO FITC-conjugated antibody (ab11729) was purchased from Abcam (Cambridge, UK). Monoclonal antibodies for flow cytometry: anti-CD45-AmCyan A V500, anti-CD13- APC, anti-CD33- PerCP-Cy5-5A, anti-CD14-PeCy7-A, and anti-CD15-V450 were purchased from Becton Dickinson, and anti-FcεRI-FITC from BioLegend. Histopaque 1077, polyvinyl alcohol, calcium ionophore A23187 (CI), diphenyleiodonium (DPI), N-acetylcysteine (NAC), DNAse, paraformaldehyde, Triton X-100, bovine serum albumin (BSA), IgE monoclonal antibody from human myeloma (401152), Tween 20, and 12-myristate 13-acetate (PMA), were purchased from Merck (St. Louis, MO, USA). Allergen solutions were purchased from Allergopharma-Nexter and 4-aminobenzoic acid hydrazide (ABAH) from Cayman Chemical.

ENROLLED SUBJECTS

Three groups of subjects were included in this study. The first one comprised of 27 allergic children, 15 girls and 12 boys, aged 11.07 ±0.53 years, who were patients of the Department of Pediatric Allergology and Pneumonology. Among the enrolled children, 18 were sensitized to birch pollen, 24 to rye pollen, and 19 to grass and grain pollens. The second group comprised of 8 allergic young adults, 5 women and 3 men, aged 22.6 ±1.4 years old, among whom 5 were allergic to birch pollen, 7 were allergic to rye pollen, and 8 were allergic to grass and grain pollens. The third (control) group comprised of 9 young adults, 6 women and 3 men, aged 23.89 ±1.59 years, who denied any history of allergic disease and in whom sensitization against tested allergens was excluded based on a basophil activation test. A sample of peripheral blood (1.8 ml) was collected from each enrolled subject into a tube containing 3.2% sodium citrate for neutrophils isolation and further assessment of NETs release and 1 mL of blood was collected into a tube containing K2-EDTA for the basophil activation test and complete blood count. Exclusion criteria for participation in the study were as follows: active inflammation, autoimmune or any systemic disease, leukopenia, granulocytopenia, and anemia. Written informed consent was obtained from every participant of the study, signed by the participant or caregiver. The study protocol was accepted by the Bioethical Committee of Medical University of Warsaw, no. KB/41/2018.

Where stated, experiments presented in the study were also performed on neutrophils isolated from 9 ml of whole blood of healthy donors. Blood was purchased from a local blood donation center in accordance with Polish legal regulations; each blood donor signed informed written consent, which entitled the blood donation center to sell their blood samples for scientific purposes.

BASOPHIL ACTIVATION TEST

All tests were carried out within 2 h of blood collection, in accordance with the recommended time between blood collection and basophil processing. CD203c-induced expression was evaluated using the Allergenic-

ity Kit (Beckman Coulter, Brea, CA, USA) according to the manufacturer's instructions and as previously described [12]. Allergens (Allergopharma-Nexter, Germany) – birch pollens and rye pollens, 47.62 standardized biological units (SBU)/ml and grass/grain pollens, 95.24 SBU/ml in phosphate-buffered saline – were used for the *in vitro* challenge. EDTA-anticoagulated peripheral blood aliquots (100 µl) stained with 20 µl of mixture of monoclonal antibodies (CRTH2–FITC, CD203c –PE, CD3-PC7) and Activation Solution (100 µl) were stimulated (37°C) for 15 min with 20 µl of optimal dilution of allergens; antibody directed against the high affinity IgE receptor (FcεRI) was used as a positive control and PBS as a negative control. After incubation, the reaction was stopped with Stop Solution. Erythrocytes were lysed with Lysing Solution for 10 min at room temperature (RT). Suspension was centrifuged (5 min, 300 x g) after lysing, washed with PBS, once more centrifuged and resuspended in 500 µl Fixative Solution. During acquisition, basophils were selected as CD203c positive/CRTH2 high/CD3-negative population using FL1/FL2 and SS/FL5 dot plots. The negative control threshold for positivity was set at less than 5% of activated cells. An LSR Fortessa (Becton Dickinson, Franklin Lakes, NJ, USA) flow cytometer was used to measure basophil activation following *in vitro* allergen challenge.

FCεRI SURFACE EXPRESSION EVALUATION

Full blood from several subjects was stained with monoclonal antibodies against CD45, CD13, CD14, CD15, CD33, CD203c and FcεRI, incubated for 20 minutes at RT, then lysed with Erythrocyte-Lysing Reagent without Fixative, EasyLyse™ (Agilent, Santa Clara, CA, USA) and washed according to the cell lyser manufacturer's instruction. Multicolor staining with monoclonal antibodies was performed to distinguish between different peripheral blood leukocytes (neutrophils, basophils, eosinophils, monocytes and lymphocytes) populations with or without FcεRI expression. Cell populations were identified by CD45/side scatter analysis and specific antigen presence – CD13, CD15, CD33 for neutrophils, CD14 for monocytes and CD203c for basophils. An LSR Fortessa (Becton Dickinson, Franklin Lakes, NJ, USA) flow cytometer was used to measure FcεRI expression on neutrophils, basophils and eosinophils.

NEUTROPHILS ISOLATION

Rich-platelet plasma was discarded and blood was diluted with saline and layered onto Histopaque 1077 and centrifuged. Subsequently, the layer containing red blood cells and granulocytes was mixed with 1% polyvinyl alcohol solution and after 20-minute sedimentation neutrophil-enriched supernatant was collected. Residual red blood cells were lysed by hypotonic lysis for 40 s. Neutrophils were washed twice and suspended in protein-free RPMI-1640 without phenol red supplemented with 10 mM HEPES. The purity of neutrophil isolation was estimated for 98% (basing on flow cytometric analysis using CD45, CD15 and CD33 staining).

NETS QUANTIFICATION

Cells were seeded into 24-well plates (5×10^4 cells/well; 12.5×10^4 cells/ml) and allowed to settle for 30 min in 5% CO_2 atmosphere at 37°C. Subsequently, cells were stimulated with PMA (100 nM), IgE antibody (0.29, 0.57, 1.43 and 2.86 µg/ml), anti-IgE antibody (0.0014 mg/ml), birch pollens and rye pollens (47.62 SBU/ml) and grass/grain pollens (95.24 SBU/ml) and incubated for 3 h. To assess whether IgE induces NETs release in a ROS-dependent mechanism, cells were incubated with inhibitors diphenyleiodonium (DPI, 20 µM), N-acetylcysteine (NAC, 5mM), and 4-aminobenzoic acid (ABAH, 100 µM) for 30 min prior to cells stimulation. Unstimulated cells (Un) were used as controls. After incubation, extracellular DNA release was measured fluorometrically using a FLUOstar Omega plate reader (BMG Labtech, Ortenberg, Germany), as described previously [13].

NETS VISUALIZATION

Cells were allowed to settle for 30 min in Lab-Tek chamber cover slides (2.5×10^4 cells/chamber; 6.25×10^4 cells/ml) and then NETs formation was stimulated with PMA (100 nM), IgE (2.86 µg/ml), anti-IgE (0.0014 mg/ml), and allergens (47.62 SBU/ml of rye pollens or 95.24 SBU/ml of grass/grain pollens). After a 3-hour stimulation, samples were fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.1% Triton X-100 and blocked with 1% bovine serum albumin. Slides were incubated overnight with 1 : 500 anti-MPO antibody at 4°C and then DNA was counterstained with 1 µM Sytox Orange. A Leica DMi8 fluorescent microscope equipped with a 40× and a 10×magnification objective lens was used to visualize NETs.

OXIDATIVE BURST MEASUREMENT

Kinetic measurements were performed using dihydrorhodamine 123 (DHR 123) – non-fluorescent dye, which once oxidized within the cell to cationic rhodamine emits green fluorescence. Cells were seeded into the wells of black 96-well plates (10^5 cells/well; 5×10^5 cells/ml), loaded with 4 µg/ml DHR 123 for 30 min at 37°C in darkness

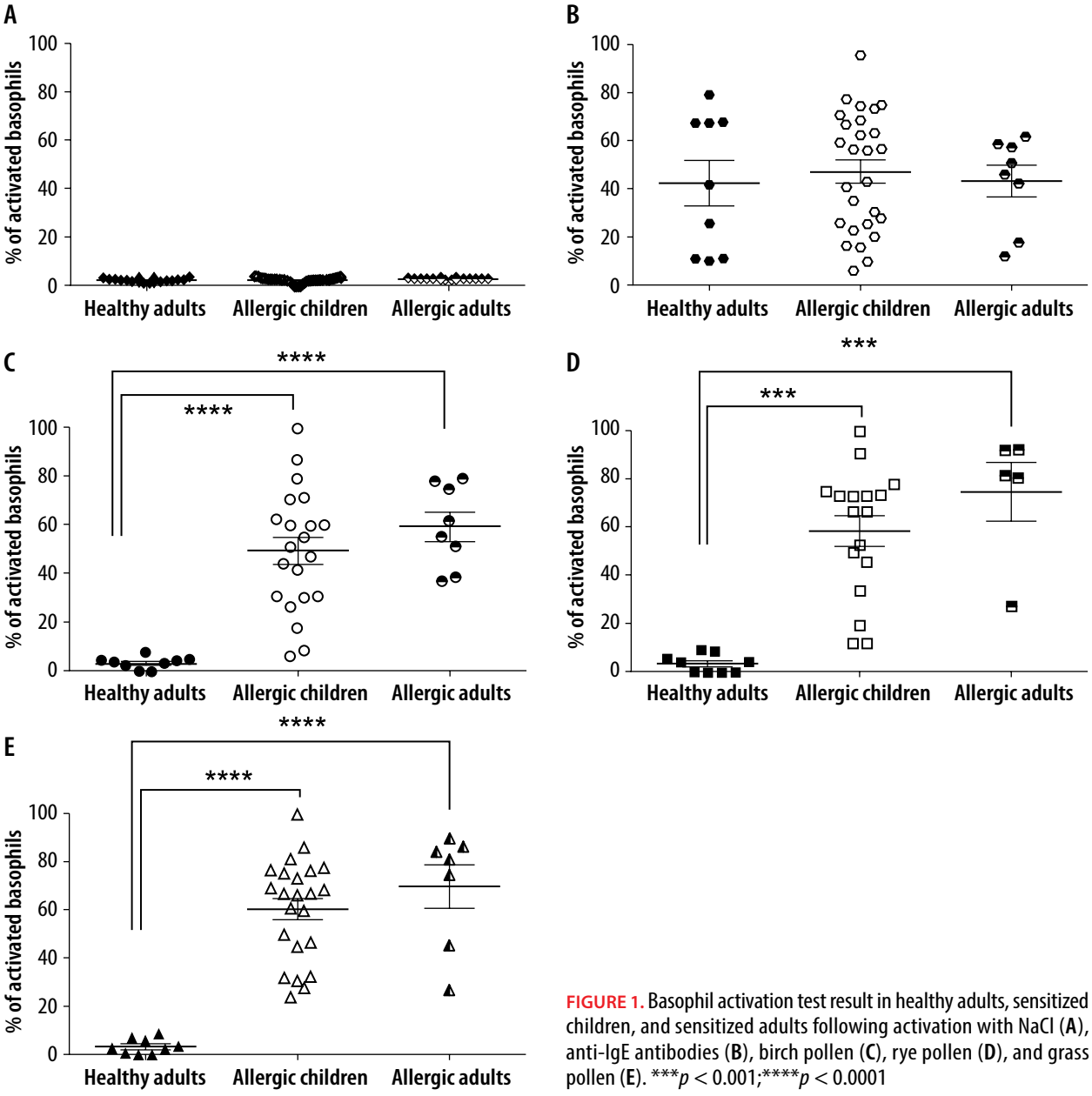

FIGURE 1. Basophil activation test result in healthy adults, sensitized children, and sensitized adults following activation with NaCl (**A**), anti-IgE antibodies (**B**), birch pollen (**C**), rye pollen (**D**), and grass pollen (**E**). ***$p < 0.001$;****$p < 0.0001$

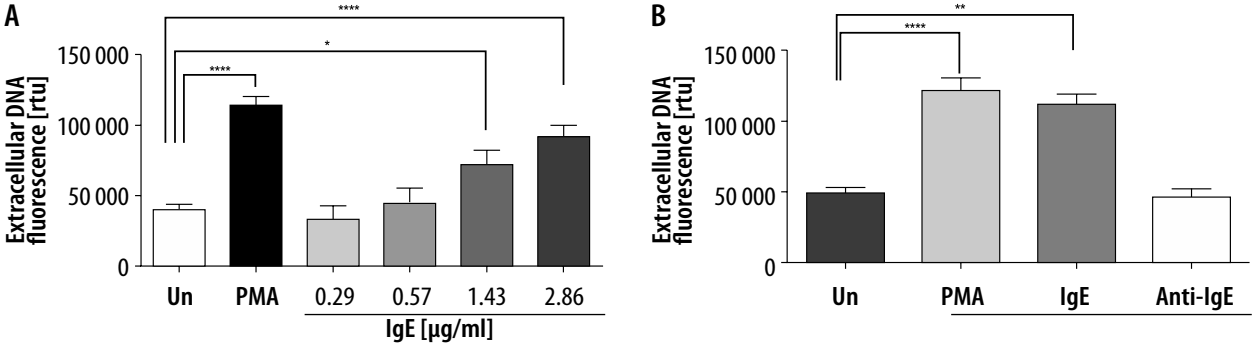

FIGURE 2. IgE stimulates release of neutrophil extracellular traps (NETs) from cells isolated from healthy adult subjects in a concentration-dependent manner (**A**). Similarly, neutrophils from sensitized children significantly released NETs under the influence of IgE in a concentration of 2.86 µg/ml. Anti-IgE did not induce NETs release. PMA served as a positive control (**B**). $n \geq 6$; *$p < 0.05$; ****$p < 0.0001$

and washed. Cells were allowed to settle and stimulated with PMA (100 nM) or IgE (2.86 µg/ml). Fluorescence was monitored every 15 min for 3 h using a FLUOstar Omega plate reader.

STATISTICAL ANALYSIS

All data were analyzed using GraphPad Prism Software 6.0 (GraphPad Software, La Jolla, CA, USA). All data are presented as mean ± standard error of mean. The effect of IgE and anti-IgE on NETs release was analyzed using one-way ANOVA. The mechanisms of IgE-dependent NETs release were analyzed with a Kruskal-Wallis test. Results of the basophil activation tests were analyzed using Student's *t*-test. A *p*-value of < 0.05 was considered significant for all statistical analyses.

RESULTS

BASOPHIL ACTIVATION TEST

Sensitization against all tested allergens was confirmed or excluded in all enrolled subjects based on the basophil activation test. Allergens did not cause activation of basophils in healthy adults but did activate basophils in sensitized children (birch pollen: 58.5 ±26.49%; rye pollen: 60.76 ±20.83%; and grass pollen: 49.69 ±25.09% of activated basophils) and sensitized adults (birch pollen: 74.76 ±27.17%; rye pollen: 70.10 ±24.04%; and grass pollen: 59.74 ±16.97% of activated basophils). Anti-IgE antibodies, which served as a positive control, caused activation of basophils in healthy and allergic children and adults (Figure 1).

EFFECT OF MONOCLONAL IGE AND ANTI-IGE ON NETS RELEASE

Monoclonal IgE stimulated NETs release from neutrophils isolated from healthy subjects in a concentration-dependent manner, significantly inducing release at 1.43 and 2.86 µg/ml ($p < 0.05$ and $p < 0.0001$, respectively) (Figure 2 A). The effect of IgE (2.86 µg/ml) and anti-IgE (0.0014 mg/ml) on NETs release was analyzed in neutrophils isolated from blood obtained from allergic children. The mean fluorescence of extracellular DNA was 45970 ±20439 rfu for negative control, 121274 ±36882 rfu for PMA stimulation, 98622 ±40713 rfu for IgE antimulation and 46482 ±20994 rfu for anti-IgE stimulation (Figure 2 B).

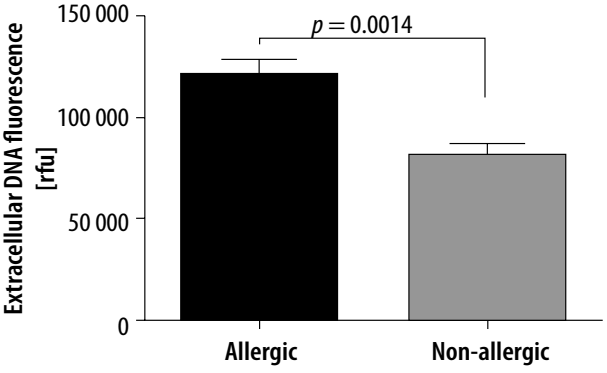

FIGURE 3. The difference between release of NETs from neutrophils isolated from allergic and non-allergic adults was statistically significant, $p = 0.0014$

RELEASE OF NETS IN ALLERGIC AND NON-ALLERGIC ADULT SUBJECTS UNDER THE INFLUENCE OF IGE

In the next step, we analyzed if there is a difference in NETs release under the influence of IgE between allergic and non-allergic adults. Here we show that release of NETs under the influence of IgE differs significantly between allergic and non-allergic adults. In allergic subjects, mean fluorescence of extracellular DNA after IgE stimulation was 120289.6 ±21502.93 rfu, whereas in non-allergic subjects it was 80971.11 ±18149.45 rfu, $p = 0.0014$ ($n = 7$) (Figure 3). Basing on these results we decided to analyze expression of FcεRI on neutrophils isolated from enrolled subjects with and without allergy and surprisingly found that this expression did not differ between both groups (1.7 ±0.68% in the allergic group and 2.4 ±0.99% in the non-allergic group, $p > 0.05$).

ANALYSIS OF MECHANISMS OF IGE-DEPENDENT NETS RELEASE

To analyze the mechanism of NETs release following monoclonal IgE stimulation, we decided to inhibit known pathways of NETs generation and measure IgE induced NETs release. DPI (NOX inhibitor), ABAH (MPO inhibitor) and NAC (ROS scavenger) strongly inhibited NETs release (Figure 4 A). Immunofluorescent microsopy observations were confirmed by quantitative analysis, where all of the tested inhibitors caused a decrease of IgE-mediated NETs release of 45.22, 75.31, and 85.19%, respectively (Figure 4 B). However, the effect was significant only for ABAH and NAC. Moreover, we decided to analyze if monoclonal IgE may induce reactive oxygen species production and found no effect of IgE on the generation of ROS, measured by DHR fluorescence (Figure 4 C).

NETS RELEASE FOLLOWING ALLERGEN STIMULATION

Next, we decided to assess if allergen specific IgE, that may be bound to its receptor on neutrophil surface, may be involved in NETs release. For this purpose, neutrophils from sensitized and non-sensitized subjects were incubat-

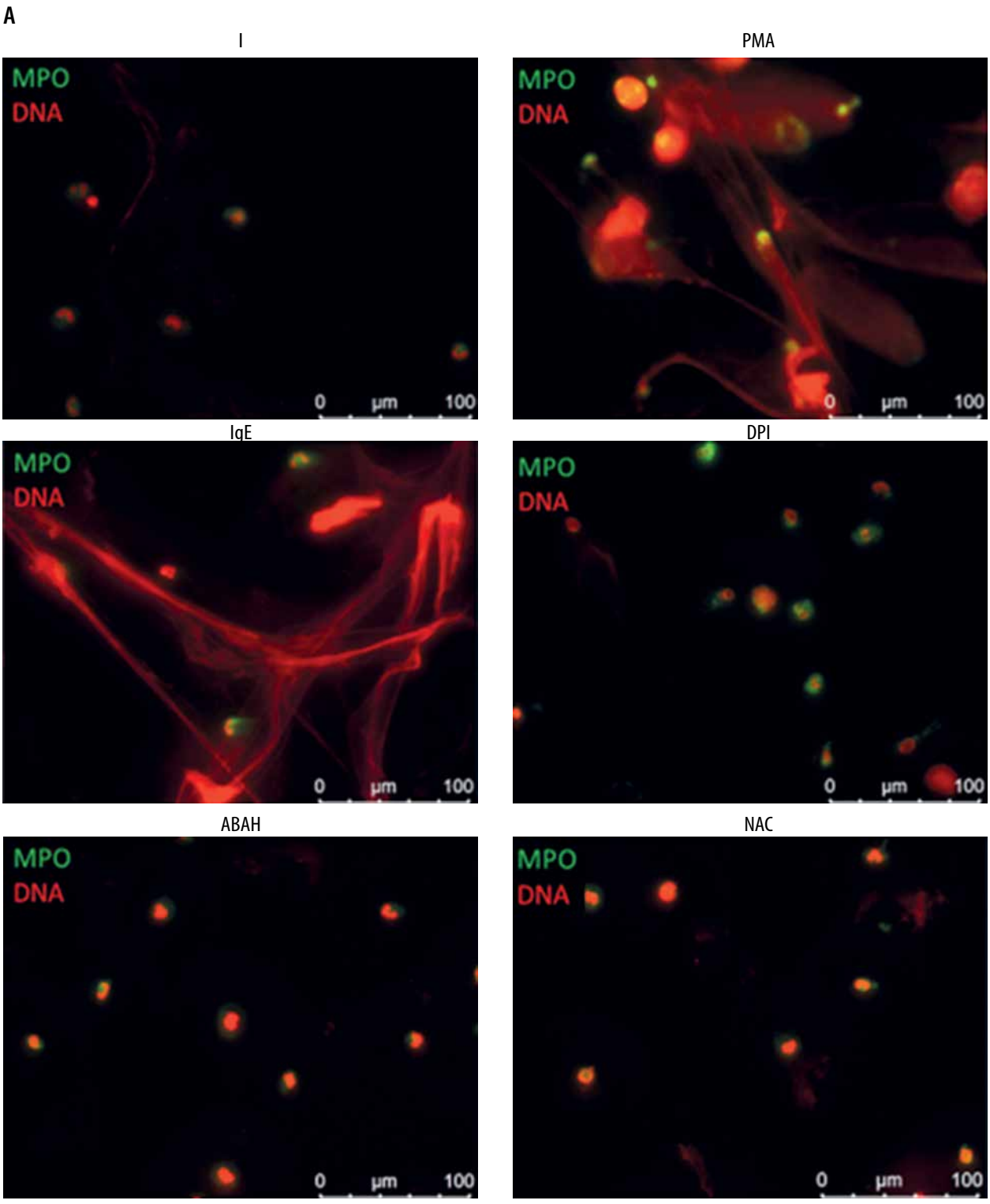

FIGURE 4. Visualization of the release of NETs performed by immunofluorescence microscopy. NETs were induced by 2.86 µm/ml IgE and inhibited by DPI, ABAH and NAC, as observed by microscopy (**A**). $N = 3$; *$p < 0.05$; **$p < 0.01$

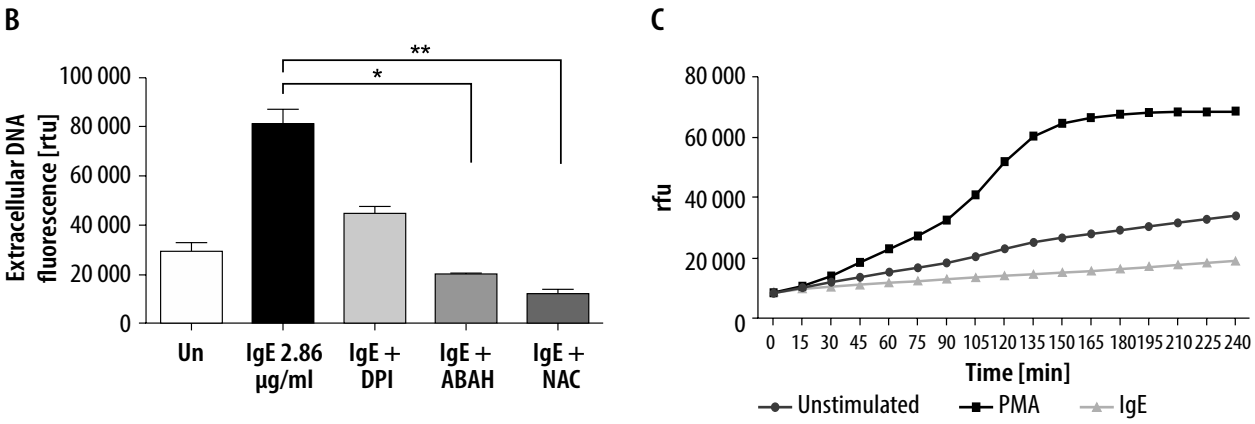

FIGURE 4. Cont. Visualization of the release of NETs performed by immunofluorescence microscopy. NETs were induced by 2.86 µm/ml IgE and inhibited by DPI, ABAH and NAC, as observed by microscopy (**A**) and confirmed by the quantitative method (**B**). IgE did not induce ROS production measured by fluorescence of DHR (**C**). PMA served as a positive control. $N = 3$; $^{*}p < 0.05$; $^{**}p < 0.01$

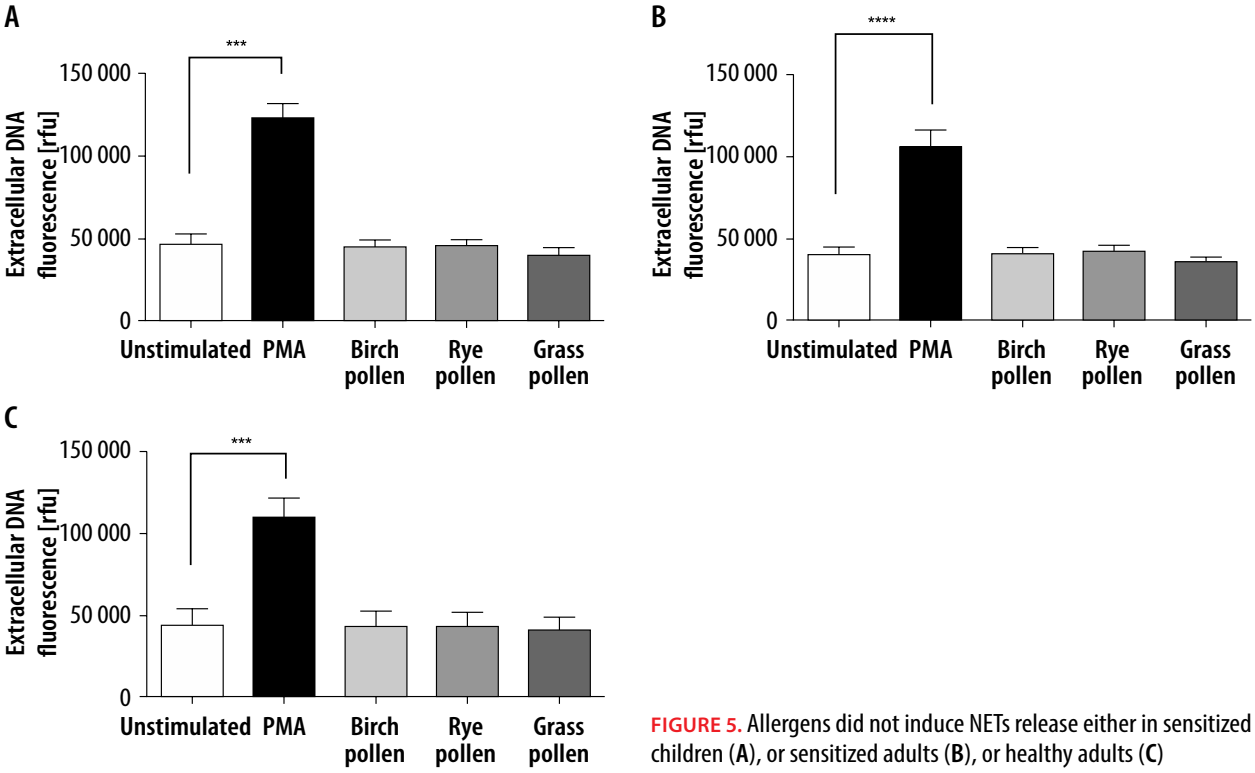

FIGURE 5. Allergens did not induce NETs release either in sensitized children (**A**), or sensitized adults (**B**), or healthy adults (**C**)

ed with allergens matched with the sensitization profile. Allergens did not cause release of NETs from neutrophils isolated from sensitized (Figures 5 A, B) or non-sensitized individuals (Figure 5 C). Qualitative analysis confirmed the results of the quantitative examination.

DISCUSSION

We found that a high concentration of IgE may induce release of NETs in allergic and non-allergic subjects, moreover the effect of IgE is significantly stronger in neutrophils from allergic subjects. Recently, it has been shown that stimulation of FcεRI induced release of lactoferrin from neutrophils isolated from allergic individuals, but the effect of IgE on NETs release has not been reported thus far [14].

In the present study, release of NETs under the influence of monoclonal IgE was concentration-dependent. The highest IgE concentration used in our study (2.86 µg/ml), which potently induced NETs release, corresponds with the total IgE concentration observed in the sera of atopic subjects [15]. Moreover, such a concentration may be also observed in rare cases of IgE plasma cell myeloma. It is worth mentioning that IgE used in our study was from

a myeloma cell line. We did not use sera from atopic subjects with a high concentration of IgE, mainly to avoid the effect of different cytokines and protein on NETs release. Recently, Impellizzieri *et al.* showed that sera from allergic donors may inhibit release of NETs from neutrophils isolated from healthy donors, by affecting IL-4 receptor signaling in neutrophils. From this point of view, using sera from allergic subjects to present the effect of IgE on NETs release could be ineffective [16]. In the course of allergic diseases, an effect of IgE was associated with its high affinity interaction with FcεRI. Gounni *et al.* found that the expression of FcεRI on neutrophils was increased after IgE challenge [9]. In our study we found that the expression of FcεRI on resting neutrophils is not dependent on the allergic status of the patient. Moreover, Mora *et al.* showed no correlation between serum IgE concentration and FcεRI expression on neutrophils [15]. On the other hand, IgE-driven activation of neutrophils leads to release of IL-8 from stimulated cells, and IL-8 is a known inducer of NETs release [5]. Monteseirin *et al.* found that allergens induce release of myeloperoxidase (MPO) from neutrophils of allergic subjects *in vitro*, and this process is allergen-specific. Release of MPO from neutrophils isolated from non-sensitized individuals was not observed when cells were incubated with the allergen solution [10]. Release of MPO together with neutrophil elastase from cells of allergic subjects may contribute to the development of asthma and airway remodeling [17, 18]. Our study was focused on NETosis not degranulation, and we did not observe any effect of allergen, which was able to activate basophils in sensitized subjects (confirmed by the basophil activation test based on detection of neutrophil CD203c surface expression), on neutrophil activation leading to NETs release. The study performed by Konig *et al.* showed that neutrophils from sensitized subjects under the influence of food allergens may release both mitochondria-derived (mtNETs) and nuclei-derived NETs. However, it has to be underlined that the source of released DNA was dependent on the sensitizing agent, and mtNETs were more likely to be released in patients sensitized to peanuts than wheat. Moreover, nuclei-derived NETs were not as common as mtNETs in the study group [19]. Subjects selected to the mentioned study were diagnosed with non-IgE food allergy, however peanut allergy in majority of cases is recognized as IgE-dependent, and is the most frequent cause of anaphylaxis due to food exposure [20]. Non-IgE dependent peanut allergy is more rare, but cannot be excluded if there were symptoms of allergy after peanut exposure (i.e. during oral food challenge) and specific IgE is absent [21, 22]. In our study none of the tested allergens induced NETs release from sensitized and non-sensitized children and adults. Alphonse *et al.* found that the expression of FcεRI on neutrophils is season-specific and in allergic subjects is increased only at the time of the highest exposure to sensitizing allergens [23]. In our study we measured surface FcεRI expression on neutrophils and found no difference between allergic and non-allergic subjects, however analysis was made out of the pollen season.

In the present study we found an association between IgE-mediated NETs release and allergy (significantly higher NETs release after IgE stimulation in cells isolated from allergic subjects comparing with healthy individuals). Unfortunately, lack of difference in FcεRI expression between allergic and non-allergic subjects did not allow to state that the mechanism of NETs release might be associated with FcεRI signaling. Moreover, because we observed strong IgE induction of NETs in both study groups, we suggest that this effect of IgE might be dependent on other mechanisms. Referring to the Konig *et al.* study [19] we have to underline that our study protocol was validated for NETs formed of nuclear DNA, since the neutrophil stimulation lasted for 180 min. NETs made of mitochondrial DNA (mtDNA) are released from living cells in a few minutes. Their release is, similarly to nuclei-derived NETs, NADPH-oxidase dependent, although requires also deglutathionylation of actin and microtubules [24].

Consequently, we decided to analyze the mechanism underlying IgE-mediated NETs release by inhibiting known pathways of nuclear NETs induction. DPI, an NADPH-oxidase inhibitor, which has been confirmed to inhibit NETs release [25], inhibited IgE mediated NETs release, what could be observed by microscopy, however the effect was not significant, most likely due to a small study group. This suggests that IgE-activated NETs release may be dependent on ROS production. On the other hand, the experiment with NAC showed that this ROS scavenger and glutathione precursor strongly inhibited NETs release. Radomska-Lesniewska *et al.* showed that NAC inhibited release of IL-8 and matrix metalloproteinase-9 from cells isolated from bronchoalveolar fluid in asthmatic subjects, what stays in line with our neutrophil observations [26]. The observed action of the two inhibitors of ROS suggests that IgE-mediated NETs release requires ROS action, and acts partially on the NOX-dependent pathway. As MPO activation may lead to release of NETs, an ABAH (MPO inhibitor, which diminishes production of HOCl, an important bactericidal compound) was used to assess whether IgE-mediated NETs release requires MPO activation. We found that inhibition of MPO strongly inhibited release of NETs compared with cells treated only with IgE, suggesting that the pathway involving MPO and HOCl may play an important role in IgE-stimulated NETs formation.

Despite the significant inhibition of IgE-mediated NETs release by ROS inhibitors and scavengers, we did not show any influence of IgE on ROS production measured by DHR reduction. Based on our results, we cannot conclude what stage of NETs formation is affected by IgE interaction with the cells.

Vega *et al*. showed that anti-IgE induces NOX-dependent activation of neutrophils isolated from allergic subjects, which leads to prostaglandin and thromboxane A release from cells [27]. Surprisingly, we did not show any influence of anti-IgE antibodies on NETs release in allergic or non-allergic subjects, despite NETosis is known as an NADPH-oxidase dependent process. Mora *et al*. also did not observe peroxide formation following stimulation of neutrophils with anti-IgE [15]. In the present study we also did not observe any effect of sensitizing allergens on NETs release. On the other hand, Vega *et al.* showed that allergens induced activation of neutrophils in allergic patients in an NFAT protein family-dependent pathway.

The results of this study are the first to suggest that a high concentration of IgE may be associated with activation of neutrophils and release of NETs. Previous reports have indicated an impact of NETs on the course of allergic and atopic diseases [11, 28–31]. Here, we add new insight on a possible effect of atopy on innate immunity. However, several limitations of our study have to be pointed out. First of all, we tested neutrophils from patients out of pollen season – this could affect their responsiveness and may not be corresponding with innate immune response during the hay season. Moreover, in our study we used commercially available IgE isolated from multiple myeloma subjects, which may contain some plasma inflammatory contaminants that could affect NETs release. However high dilutions of used reagent should reduce such an effect. Moreover, in further studies, additional methods to assess NETs release might be used, including assessment of mtNETs, to help identify the specific mechanism underlying IgE-dependent NETs release. Despite all limitations we believe that our results are reliable and will contribute to opening the discussion on IgE influence on innate immunity. Future investigations will allow us to shed further light on this process and help understand the mechanism underlying IgE-mediated NETs release.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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