Studies of mitochondrial and nuclear DNA released from food allergen–activated neutrophils. Implications for non–IgE food allergy

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ABSTRACT

Background: *Although adverse food reactions are commonly divided into immunoglobulin E (IgE) mediated food allergy (FA), and non-IgE FA, the current literature is providing support for the role of innate immune responses as an important component of non-IgE FA. Using a commercially available leukocyte activation (LA) assay, a recent quantitative study of total extracellular DNA released in cellular supernatants of human peripheral blood mononuclear cells exposed either to positive or negative tested foods demonstrated that leukocytes exposed to foods with positive LA test results showed higher DNA content than those exposed to foods with negative LA test results. In humans, the origin of DNA might be either the nucleus or the mitochondria. Analysis of emerging data from several laboratories, including our own, suggests that mitochondrial DNA induces inflammatory responses through induction of proinflammatory cytokines.*

Objective: *This pilot study was designed primarily to convey the finding, and relevance of, mitochondrial DNA in the form of neutrophil extracellular traps (NET) as a new pathogenetic mechanism for innate immune-mediated non-IgE FA.*

Methods: *The study population consisted of a total of six subjects, four in a major FA study group and two in a subgroup. Neutrophils were isolated and treated with food antigens that elicited positive and negative LA responses, and the released free DNA was analyzed for the cellular site of origin by using real-time polymerase chain reaction and for leukocyte calprotectin and S100 calcium-binding protein A12 (S100A12) proteins as markers of NETs.*

Results: *We showed that cellular supernatants from neutrophils treated with foods that elicit positive LA responses can contain increased DNA levels of nuclear as well as mitochondrial origin. Supernatants from neutrophils treated with negative tested food (LA) responses did not induce the release of nuclear or mitochondrial DNA.*

Conclusion: *Analysis of our data suggested that the induction of NETs that contain proinflammatory mitochondrial DNA may provide the critical link necessary for a better understanding of the pathogenesis of non–IgE-mediated FA. These discoveries may not only facilitate better diagnostic tests of FA but should also improve clinical management of allergic and other inflammatory diseases.*

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The emerging literature is providing support for the role of innate immune responses in the pathogenesis of non–immunoglobulin E (IgE) food allergy (FA).[1–5] Current diagnostic methods for FA rely on measuring allergen specific IgE antibody, either through skin-prick testing or by *in vitro* serum measurement, followed by formal oral food challenge.[6,7] A large number of commercially available blood tests exist that claim to diagnose FA, although most remain unvalidated.[8] Among the more widely used of these food sensitivity assays purported to measure non-IgE FA are leukocyte activation (LA) tests used in complementary and alternative medicine practices.[9] When using a commercially available LA assay, a recent report by Garcia-Martinez *et al.*[10] quantitated concentrations of total extracellular DNA released in cellular supernatants of human peripheral blood mononuclear cells from healthy volunteers exposed to LA-positive and LA-negative foods. Only foods with positive LA test results demonstrated a higher DNA content when compared with foods with a negative result.

Analysis of emerging data from several laboratories, including our own, suggests that the methylation status of DNA governs the regulation of tolerance or inflammation and that DNA moieties once methylated are immunosuppressive by regulatory T-cell (Treg) activation, whereas, when nonmethylated, they induce inflammatory responses through induction of proinflammatory cytokines.[11–13] In humans, cellular DNA is typically found either inside the nucleus, as nuclear DNA (nDNA), or within the mitochondrion, as

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mitochondrial DNA (mtDNA). Although nDNA is methylated at CpG sites, the CpG methylation in human mtDNA is a rare event at most DNA regions.[14] However, recently, it was demonstrated by next-generation sequencing (NGS)-based bisulfite sequencing that the mitochondrial genome is heavily methylated with predominant non-CpG methylation and that this methylation is associated with multiple DNA methyltransferase enzymes.[15] In summary, mtDNA is a small molecule with methylation patterns discrete from that of nDNA and is present at hundreds of copies per cell.

Mitochondria are functionally versatile organelles. In addition to their conventional role of meeting the cell's energy requirements, mitochondria also actively regulate innate immune responses against infectious and sterile insults. Components of mitochondria, when released or exposed in response to dysfunction or damage, function as damage-associated molecular patterns and can be directly recognized by receptors of the innate immune system and trigger an immune response. The damage-associated molecular patterns that are released by mitochondria include the structural phospholipid cardiolipin, n-formyl peptides, reactive oxygen species, and mtDNA. MtDNA has characteristics consistent with prokaryotic nucleic acid and was shown to activate neutrophils through Toll-like receptor 9/p38 mitogen-activated protein kinase (TLR9/p38 MAPK). This release of mtDNA associated with inflammation is supported by the finding of elevated levels of circulating mtDNA in patients who experienced trauma, rheumatoid arthritis, and femur fracture as well as in animal models of trauma and shock.[16–18]

Neutrophils are recruited early to sites of infection where they kill pathogens (bacteria, fungi, and viruses) by oxidative burst and phagocytosis. However, neutrophils possess another much less recognized means of killing pathogens, the formation of neutrophil extracellular traps (NET).[19] These structures normally function to control microbial infections but, at times, display deleterious inflammatory effects seen in a variety of autoimmune and inflammatory conditions.[20–22] NETs consist of DNA threads attached to a large number of different lytic and antimicrobial enzymes and proteins released from affected cells. Calprotectin and S100 calcium-binding protein A12 (S100A12) are among the dominating proteins.[23] We hypothesize that neutrophils exposed to LA-positive foods promote the release of predominantly mtDNA in the form of NETs, which induce inflammatory cytokines responsible for the clinical expressions of non-IgE FA. Therefore, we studied the cellular sites of origin of the released free DNA found in cellular supernatants of neutrophils treated with foods that elicited positive LA responses.

METHODS

Clinical Subjects

The study was approved by the institutional review board, University of Leipzig, Germany (approval 348-18-ek). After written informed consent was obtained, 20 mL of blood collected in CPDA (citrate phosphate dextrose adenine) blood collection tubes were obtained from each subject. The blood was either collected at the Magdeburg Molecular Detections Laboratory, Magdeburg, Germany or was obtained at the offices of collaborating physicians and sent to the Magdeburg Molecular Detections Laboratory laboratory within 24 hours for isolation of neutrophils. In addition, from each subject, an aliquot of 5 mL of blood was drawn into a serum collection vial for the determination of allergen-specific IgE to eight common food allergens, which consisted of casein, codfish, egg yolk, peanut, soybean, shrimp, wheat, and almond (Thermo Fisher, Uppsala, Sweden).

The study population consisted of a total of six subjects, four in a major FA study group (subjects 1–4) and two in a subgroup (subjects 5–6) (Table 1). In the major FA study group, subjects 1 and 2 had no history of adverse food-related reactions and served as methodologic food negative controls; subjects 3 and 4 both had histories of adverse food-related reactions that consisted of irritable bowel syndrome (IBS) after ingestion of putatively offending foods. Subjects 5 and 6 in the subgroup, were healthy subjects with no history of adverse food-related reactions and served as additional methodologic food negative controls. All allergen-specific IgE test results in these subjects were negative. In addition, for the stimulation of neutrophils with phorbol 12-myristate 13-acetate (PMA) and the neutrophil stimulation experiments with *Candida* control antigens (15 minutes of incubation times), three healthy volunteers, ranging in age from 23 to 60 years, were studied. In later experiments performed for DNA release with a prolonged incubation time of 60 minutes, neutrophils from the four subjects in the major FA cohort were studied.

Measurement of LA

The measurement of LA was performed by using the impedance method of cell counting and sizing.[24] Briefly, peripheral blood leukocytes suspended in an electrolyte buffer are extracted, one cell at a time, by vacuum aspiration and are then passed through a "sensing zone" aperture in which there exists an electromagnetic field. As the cell passes through the sensing zone, it impedes the electrical current in direct proportion to its volume. In this manner, thousands of cells can be rapidly counted and sized in a matter of seconds, which results in a cell distribution based on size represented as a histogram. In the LA assay, some blood samples that do not contain a test reagent

Table 1 **Clinical characteristics of the food allergy cohort study population**

Subject No.	Gender/Age, y	Symptoms	IgE Allergen-Specific Results
1	F/46	No symptoms associated with food ingestion	All negative
2	F/51	No symptoms associated with food ingestion	All negative
3	F/32	More profound symptoms of irritable bowel syndrome after ingestion of foods	All negative
4	F/59	More profound symptoms of irritable bowel syndrome after ingestion of foods	All negative
5	F/60	No symptoms associated with food ingestion	All negative
6	F/54	No symptoms associated with food ingestion	All negative

IgE = Immunoglobulin E.

were included, which served as a methodologic baseline control. Histograms generated by samples that contained the test reagent were compared with those that did not contain the test reagent but which were otherwise treated identically. Tests that induce a change in size and/or number generate a histogram that is distinguishable from that generated by the control specimen. A resident algorithm measurement is used to quantitate the difference. The relative degrees of positivity (*e.g.*, 1+, 2+, 3+, 4+) were determined by statistical analysis. This classification is based on relative degrees of variances of both numbers of cells and alterations in cell volumes that result from the interaction of granulocytes with a food extract. A 1+ response is defined as the least and 4+ as the greatest degree of variances of these defined parameters.

The impedance method, also known as the "Coulter method," is the international standard for cell counting and sizing. Shown in Fig. 1 is a diagrammatic representation of a negative and a positive food reaction as determined by the impedance method of cell measurement used in the LA assay in the present study. Two cellular distributions were generated: (1) a lymphocyte peak (shown on the left), and, more importantly, (2) a predominantly neutrophil peak (shown on the right). Reactivity to foods is observed mainly in the neutrophil fraction where the red line represents baseline control untreated blood samples not exposed to the food antigen. An identically treated sample exposed to the test food antigen creates its own histogram (shown here shaded in blue). The test histogram is then compared with the control histogram. The displacement of the blue histogram to the right and its diminished amplitude shown in Fig. 1*A*, are due to both a decrease in cell number and an increase in cell size. These changes indicate a positive LA response to the respective food antigen. A negative LA response is illustrated in Fig. 1*B*, which shows a response of a subject's granulocytes exposed to a nonstimulating food extract in which the food untreated peak is superimposed on the neutrophil peak.

Isolation of Nonactivated Neutrophils from Whole Blood

Isolation of granulocytes (i.e., neutrophils, basophils, eosinophils) is usually performed by density gradient centrifugation. Although this type of cell isolation methodology poses no problem with freshly drawn blood samples, storage of blood often influences the recovery of neutrophils from density gradients. Moreover, isolation of neutrophils from stored blood by density gradient centrifugation leads to some activation of neutrophil function. Therefore, a neutrophil isolation method for this project was chosen based on negative selection of neutrophils from whole blood. Neutrophils were isolated by using the EasySep Human Neutrophil Isolation Kit (Stemcell Technologies, Grenoble, France), with the fully automated cell separator RoboSep-S (Stemcell Technologies) by negative selection according to the manufacturer's recommendations.

Stimulation of Neutrophils with Food Extracts

A 100 μL suspension of purified neutrophils (PMN) at a concentration of 1×10^6 was added to each well of a 96-well plate LA test cassette that contains food extract antigens and was incubated for 15 minutes at 35°C, 5% CO_2. After incubation, 900 μL RPMI medium was added to each well and, after centrifugation, the supernatants were separated from the cells and analyzed for calprotectin and S100A12 as well as for mtDNA and nDNA release. The mtDNA and nDNA contents were also measured in the cell pellets. The following additional controls were included: (1) 100% control (K100) for determination of the total enzyme content; (2) 100% control (K100) for determination of mtDNA and nDNA, including the relationship of the mtDNA/nDNA ratios.

Food-Specific Stimulation

An aliquot of 1×10^6 neutrophils was added to a reaction vial and incubated with different concentrations of soluble pre-prepared food extracts in a total

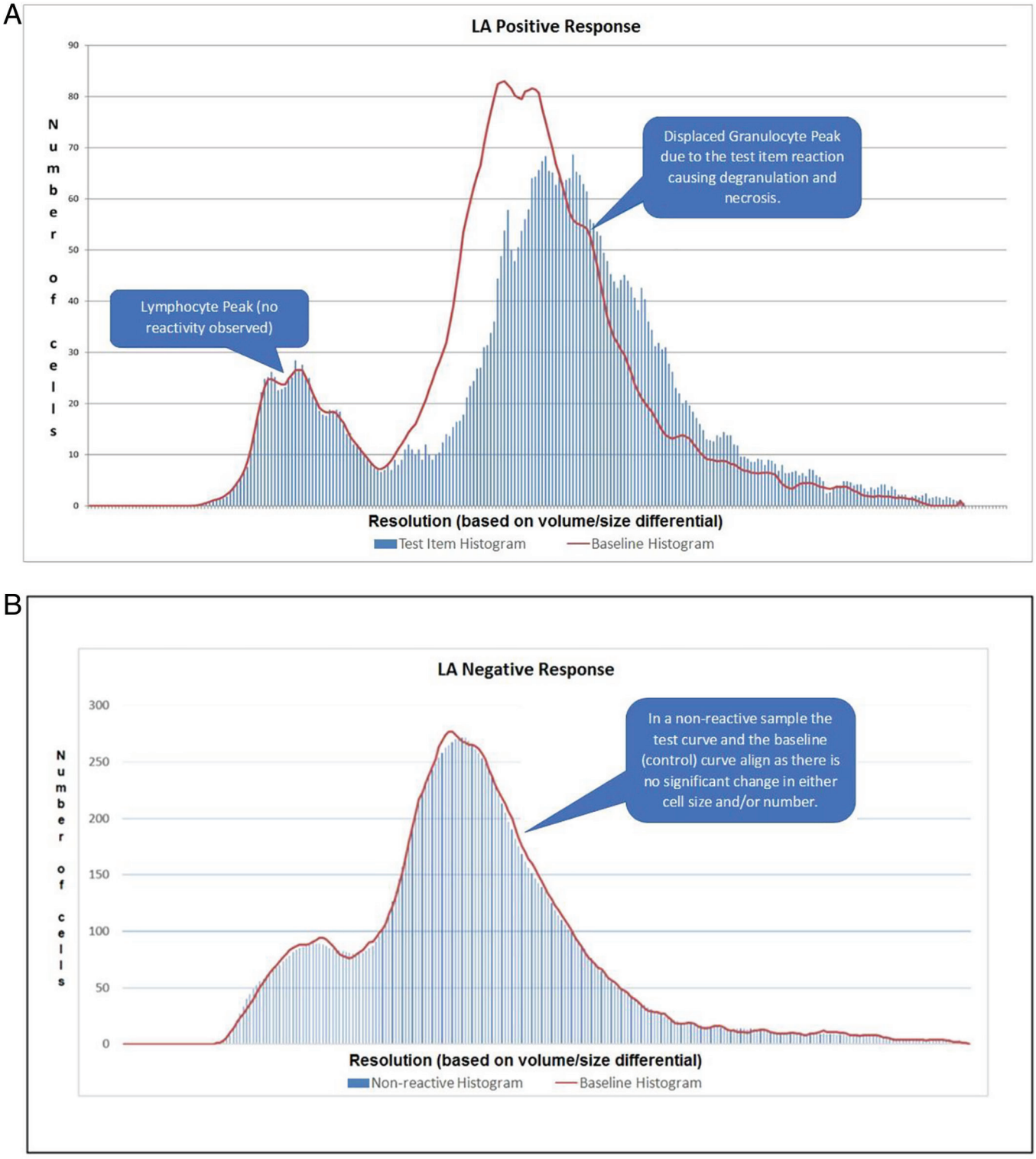

Figure 1. *Diagrammatic representation of positive and negative food reactions as determined by the impedance method of cell measurement used in the LA test. The mean cell size is reflected on the* x*-axis, whereas the cell number is shown on the* y*-axis. Both lymphocyte and granulocyte peaks are shown. Reactivity to foods is observed mainly in the granulocyte fraction. The red line represents the baseline control in the absence of a food extract; the blue graph represents a response of granulocytes exposed to a food extract to which the subject is reactive or nonreactive. (A) LA positive response shows both a decrease in cell number and an increase in cell size, illustrated by a displacement of the granulocyte peak to the right of the food untreated peak. (B) LA-negative response, showing a subject's granulocytes exposed to a nonstimulating food extract in which the food untreated peak is superimposed on the granulocyte peak. LA = Leukocyte activation.*

volume of 1 mL at 37°C at the indicated concentrations and time intervals. Thereafter, stimulation assays were conducted as described above.

Neutrophil Stimulation with PMA

An aliquot of 1×10^6 neutrophils was added to each well of a 96-well plate cassette and was incubated with PMA in a total volume of 1 mL at 37°C at the indicated concentrations and time intervals as described in the Results section. After stimulation, the supernatants and cell pellets were analyzed and were used for testing of various analytes.

Enzyme-Linked Immunosorbent Assay for Measurement of Specific Concentrations of Calprotectin and S100A12

The enzyme-linked immunosorbent assay methodology was used to determine the absolute concentrations of calprotectin and S100A12 in culture supernatants by using the manufacturer's instructions of the enzyme-linked immunosorbent assay kit for calprotectin and for S100A12 (Immundiagnostik, Bensheim, Germany).

Methods for DNA Isolation of Cell-Free DNA

An essential requirement for the research conducted in this study is consistency of the methodology to be used for cell culture and isolation of cell-free DNA (cfDNA) from supernatants of stimulated neutrophils because cfDNA might be composed of a heterogeneous mixture of molecular moieties that range from low-molecular-weight (<1000 bp), primarily uniform fragments (185–200 bp) up to high-molecular-weight DNA constituents (>50,000 bp). Because there might be only small amounts of low-molecular-weight DNA in the cell culture supernatants, an initial set of experiments was performed to determine the efficacy of different DNA extraction systems to isolate low-molecular-weight DNA moieties as well. To determine the optimal method of DNA isolation, five different commercial kits were tested for isolation of cfDNA by using a 100-bp ladder purchased from Promega Corporation (Madison, Wisconsin), ranging from 100 bp up to 3000 bp; and, a 1-kb ladder, ranging from 250 to 10,000 bp in size. Based on the results of these preliminary studies, the QIAamp MinElute ccfDNA Kit from Qiagen (55204; Hilden, Germany) was chosen for the isolation of cfDNA from all neutrophil supernatants generated during this study.[25] The quantification of double- and single-strand cfDNA through fluorimetric assay was performed by using the Qubit 2.0 Fluorometer (LifeTechnologies, Carlsbad, CA).[26]

Determination of mtDNA and nDNA in Culture Supernatants

The origin of the extracellular DNA was determined by amplifying four mitochondrial and three nuclear genes by using the Rotor-Gene SYBR Green polymerase chain reaction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.[26,27] The primer list is provided in full detail in Supplemental Table ST1. All primer pairs showed nearly identical polymerase chain reaction efficiency, ranging from 0.89 ubiquinone oxidoreductase subunit A1 (NADH) to 1.1 ras homolog family member H (rhoh). For the determination of the ratio of mtDNA/nDNA, the ß-actin gene was used to estimate nDNA levels and the ATP synthase-8 mtDNA gene was used to estimate mtDNA levels.

RESULTS

Stimulation of Neutrophils with PMA

According to the publication by Brinkmann *et al.*,[19] stimulation of neutrophils with PMA, a protein kinase C activator, leads to the release of NETs. It is not known, however, whether mtDNA is also simultaneously released and/or is degraded directly by PMA treatment. To resolve this possibility, a separate set of experiments was performed to analyze the effect(s) of PMA on neutrophils with regard to nDNA and mtDNA release as well as for NET specific enzymes, *e.g.*, calprotectin.

Activation of neutrophils with PMA at a concentration of 80 nM for 240 minutes according to the publication by Brinkmann *et al.*[19] led to a huge loss in viability and release of predominantly nDNA (data not shown). Next, because it was important to determine if PMA stimulation was associated with a dose/time-dependent activation of neutrophils and with the release of nDNA and/or mtDNA release from human neutrophils, a set of experiments was performed in which freshly prepared neutrophils were stimulated by using two different PMA concentrations (40 nM and 80nM) for only 40 minutes at 37°C.

Release of Granule Constituents. Calprotectin belongs to a group of enzymes that are released from neutrophils during the formation of NETs and has been suggested as a diagnostic aid in non-IgE FA.[28] In this regard, PMA, as an inducer of NETs, leads to a dose-dependent release of calprotectin. The results of calprotectin release from neutrophils of a healthy subject are provided in **Supplemental Figure SF1.**

Quantitative Distribution of the Release of mtDNA and nDNA. The ratio of mtDNA to nDNA was measured in unstimulated neutrophil suspensions as well as in PMA-stimulated neutrophils in both cell supernatants and cell pellets. In PMA-stimulated neutrophils, the mtDNA/

nDNA ratio in supernatants was similar to the neutrophil mtDNA/nDNA ratio found in pellets. Thus, mtDNA and nDNA were found in the neutrophil supernatants after PMA stimulation mainly as a consequence of dying cells (**Supplemental Figure SF2**).

Results of LA

The results of neutrophil activation are presented at five levels: activation of neutrophils with food extracts (level 1), food control studies with *Candida* cells (level 2), food-specific experiments with study subjects (level 3), enzyme release (calprotectin, S100A12) (level 4), and DNA release (level 5).

Activation of Neutrophils with Food Extracts: Level 1

An impedance method was used as the basis for the LA assay in the present studies, which consisted of food-induced deformability changes of blood leukocytes exposed to various food antigens (see the Methods section).[24] Leukocytes from study group subjects (Table 1) were added to test cassettes with different food extracts. Exposure of leukocytes to *Candida* antigen was used as a positive control in the LA assay. Leukocytes from healthy subjects of the FA cohort (subjects 1 and 2) were simultaneously added to additional test cassettes with different *Candida* antigen concentrations (P0, 0 μg; P1, 5 μg; P2, 3.3 μg; P3, 2.5 μg).

Food Control Studies with *Candida* Cells: Level 2

In this set of experiments performed in subjects from the healthy volunteer control group, neutrophils were incubated with *Candida* for 15 minutes at 37°C according to the standard procedure of the LA assay described in the Methods section. Although the impedance measurement showed a clear positive result, neither the release of calprotectin nor S100A12 could be detected, which indicated that *Candida* cells were being phagocytized without formation of NETs. We, therefore, next analyzed whether mtDNA and/or nDNA was released from neutrophils incubated in the *Candida* test cassettes and determined the relationship of mtDNA/nDNA ratios in control neutrophils (control), in the supernatants of nonstimulated (P0), and in *Candida*-stimulated neutrophils (P1, P2, P3). The neutrophils from subjects 5 and 6 had mtDNA/nDNA ratios of 121.94 and 85.63, respectively. The supernatants of unstimulated neutrophils (P0) of both subjects showed either a slight decrease or no change of the mtDNA/nDNA ratio in comparison with the mtDNA/nDNA ratio of neutrophils of both subjects (control) before incubation without/with candida antigen, which suggested that some neutrophils die during incubation. The supernatants of *Candida*-stimulated neutrophils showed a diminished mtDNA/nDNA relationship compared with unstimulated neutrophils, which indicated the release of predominantly nDNA. However, because the decrease in the mtDNA/nDNA ratio showed an inverse dose-dependence, with the highest nDNA release at the lowest *Candida* antigen concentrations (Fig. 2), this suggested that cell death and NET formation were dose-dependent on the *Candida* antigen concentrations.

In the next set of experiments performed with leukocytes from the subjects in the FA cohort, the incubation times were extended, and neutrophils were incubated with the *Candida* test cassettes for 60 minutes at 37°C. Although the neutrophils from these subjects did not show a significant enzyme release of calprotectin and S100A12, they did release predominantly nDNA into the supernatant fluids after incubation with *Candida* antigen (data not shown). After an incubation time of 1 hour, the amount of DNA in the supernatants was independent of the antigen concentration used as a stimulus. All *Candida* concentrations induced the release of DNA with a fragment size of ~600 bp, which indicated the release of nDNA. The results from one representative healthy subject (subject 2) are provided in Supplemental Table ST2.

Food-Specific Experiments with FA Study Subjects: Level 3

In this series of studies, a specialized set of experiments was performed that evaluated the release of calprotectin and S100A12 as well as mtDNA and nDNA from food allergen–activated neutrophils from the subjects in the FA study cohort (Table 1). Based on clinical history and the presence or extent of clinical symptomatology, subjects 1 and 2 were designated as nonsymptomatic controls and subjects 3 and 4 were designated as subjects who were food symptomatic. The results of LA studies of the four study subjects are expressed in terms of severity of changes in impedance with specific food items (Table 2). It can be seen that the prevalence of milder reactions in subjects 1 and 2 and more heightened degrees of activation in subjects 3 and 4 correlate with the degree of clinical severity.

Enzyme Release (calprotectin, S100A12): Level 4

In the next set of experiments, calprotectin and S100A12 release was measured in neutrophils of healthy subjects 1 and 2 from the control group, and subjects 3 and 4 from the FA study cohort; samples from each were treated with different concentrations of various specific food allergens for 1 hour at 37°C. The following food extracts were chosen: wheat, peanut, casein, and shrimp. The neutrophils from only the two subjects who were food symptomatic (subjects 3 and 4) reacted with calprotectin release after stimulation with food antigens; S100A12 release was not detected. The data from these two subjects are shown

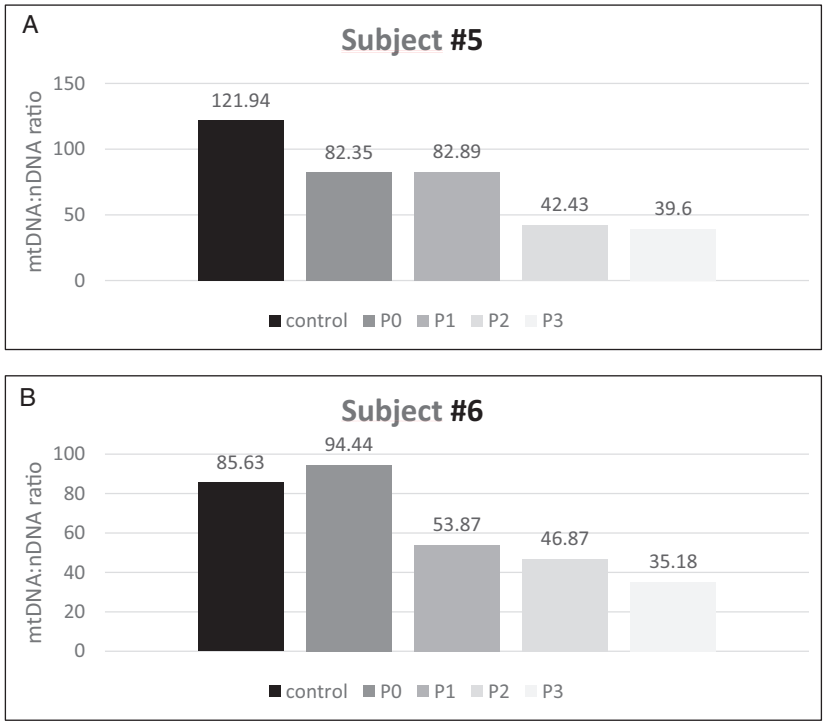

Figure 2. *mtDNA/nDNA in supernatants from neutrophils of (A) subject 5 and (B) subject 6 incubated in the absence (P0) or presence of Candida antigen (P1, P2, P3) in the Candida test cassette. Control: mtDNA/nDNA in neutrophils of patients, respectively. The antigen concentrations (P1, P2, and P3) used were 5 mg, 3.3 mg, 2.5 mg, respectively. mtDNA = Mitochondrial DNA; nDNA = nuclear DNA.*

Table 2 **Relative degrees of leukocyte activation (mild, moderate, severe) with specific food items in study subjects**

Subject No.	Severe (+++)	Moderate (++)	Mild (+)
1	—	Walnut	Almond, clam, halibut, strawberry
2	—	—	Casein*
3	Wheat*	Peanut,* sesame, strawberry, walnut	Baker¨s yeast, cashews, soybean
4	—	Peanut,* wheat*	Almond, baker¨s yeast, cashew, clam, halibut, pecan, shrimp, casein

**The food extracts used in neutrophil stimulation assays.*

in Figs. 3 and 4. The results clearly showed that subject 3 reacted to wheat and peanut, with an enhanced release of calprotectin from neutrophils in a dose-dependent manner. Subject 4 reacted to peanut, with an enhanced release of calprotectin from neutrophils in a dose-dependent manner. Thus, the LA results concurred with the results of calprotectin release.

DNA Release: Level 5

The next set of experiments, conducted in leukocytes from subjects in the FA study cohort, was performed to determine if the released DNA from neutrophils of all four subjects was either nDNA or mtDNA in origin. For this purpose, the mtDNA/nDNA ratios were determined in the supernatants from unstimulated and stimulated neutrophils from each of these subjects. No changes could be detected in the mtDNA/nDNA ratios in the supernatants from stimulated neutrophils of subjects 1 and 2 compared with those seen in unstimulated neutrophils from these subjects. Subject 3, in contrast, showed dose-dependent changes in the mtDNA/nDNA ratio after stimulation of neutrophils with wheat and peanut (Fig. 5). Stimulation of neutrophils with the antigen casein, which induced no positive LA response (control antigen), did not change the mtDNA/nDNA ratio in the supernatants from neutrophils (Fig. 5). Of particular interest was the finding that wheat was the same food allergen that led to a dose-response–related decrease in the *in vitro* mtDNA/nDNA ratio (not seen with the control casein) and was also responsible for the clinically severe reactions seen when wheat was ingested (Table 2), which supported its role both in active release of nDNA from

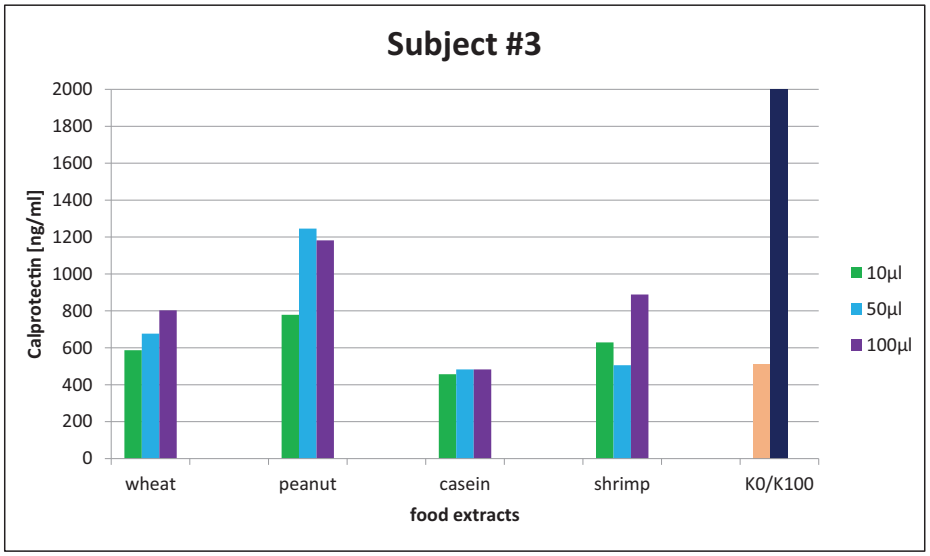

Figure 3. *Calprotectin release from isolated neutrophils (subject 3) incubated in the absence (K0) or in the presence of distinct food extracts at different concentrations (10 µL, 50 µL, and 100 µL) for 1 hour at 37°C. The antigen concentrations of the stock solutions for wheat, peanut, casein, and shrimp were 957.8 µg/mL, 5882 µg/mL, 1000 µg/mL, and 4450 µg/mL. K0 = negative control; K100 = total neutrophil calprotectin content in neutrophils.*

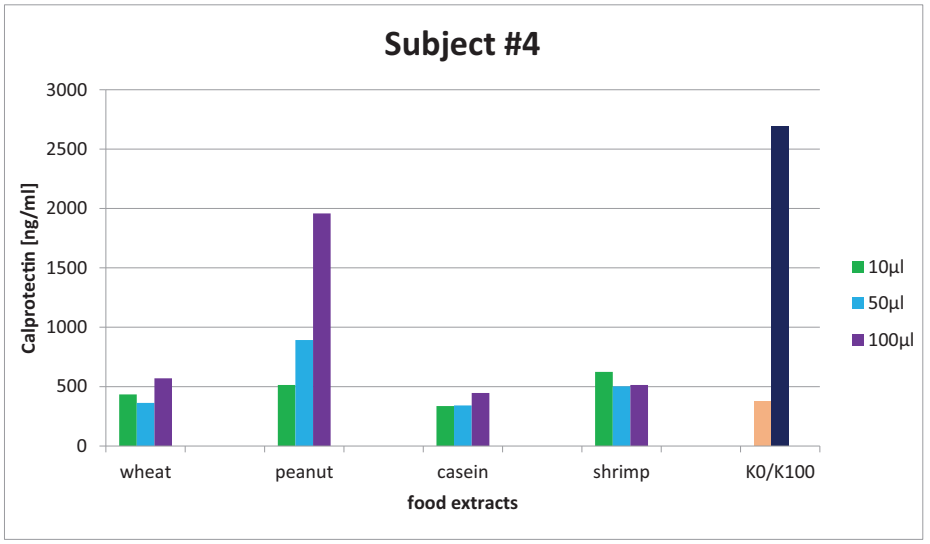

Figure 4. *Calprotectin release from isolated neutrophils (subject 4) incubated in the absence (K0) or in the presence of distinct food extracts at different concentrations (10 µL, 50 µL, and 100 µL) for 1 hour at 37°C. The antigen concentrations of the stock solutions for wheat, peanut, casein, and shrimp were 957.8 µg/mL, 5882 µg/mL, 1000 µg/mL, and 4450 µg/mL. K0 = negative control; K100 = total neutrophil calprotectin content in neutrophils.*

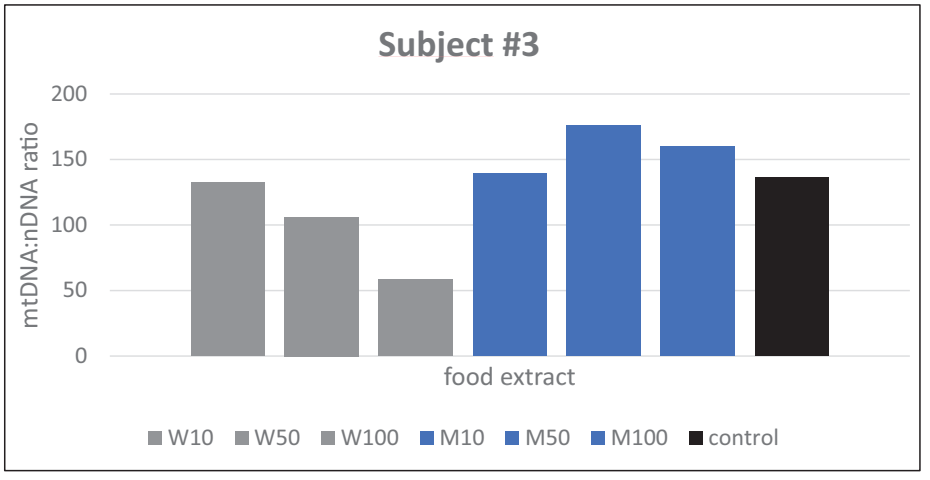

Figure 5. *The mtDNA/nDNA ratio in supernatants from neutrophils of subject 3 incubated in the absence (control) or presence of wheat (W) antigen and the control antigen M (casein) at different concentrations (10 µL, 50 µL, and 100 µL): the total antigen concentrations for wheat were 9.58 µg, 47.89 µg, and 95.78 µg; for casein were 10 µg, 50 µg, and 100 µg. mtDNA = Mitochondrial DNA; nDNA = nuclear DNA.*

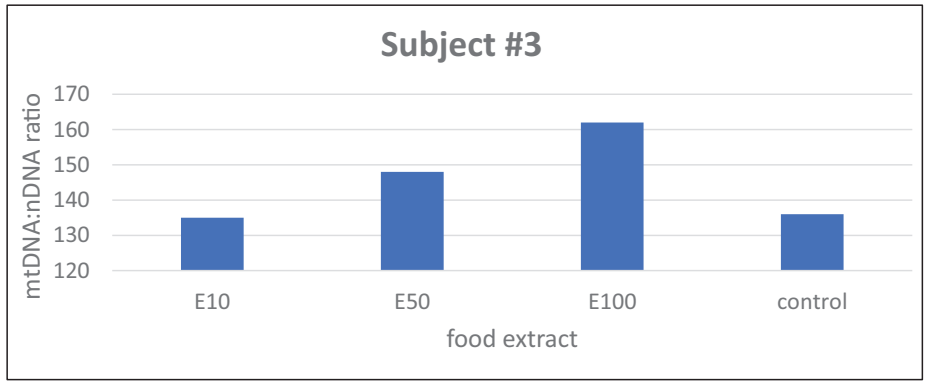

Figure 6. *The mtDNA/nDNA ratio in supernatants from neutrophils of subject 3 were incubated in the absence (control) or the presence of peanut (E) antigen at different concentrations (10 μL, 50 μL, and 100 μL), and the total antigen concentrations were 58.82 μg, 294.10 μg, and 588.2 μg. mtDNA = Mitochondrial DNA; nDNA = nuclear DNA.*

neutrophils as well its putatively clinically role in FA. Indeed, only nDNA (ß-actin) increased in the neutrophil supernatants after stimulation with wheat antigen (data not shown). However, after stimulation, the neutrophils from study subject 3 treated with peanut showed an increased mtDNA/nDNA ratio (Fig. 6). Only mtDNA (*ATP8*) increased in the culture supernatants from neutrophils stimulated with peanut (data not shown).

With regard to subject 4, dose-dependent mtDNA/nDNA changes were observed only after stimulation of neutrophils with peanut (data not shown). In concordance with findings from the study of subject 3, mtDNA, but not nDNA, was released after stimulation of neutrophils with peanut. The release of mtDNA was verified by quantification of two different mitochondrial genes, the *ATP8* (mitochondrially encoded ATP synthase membrane subunit 8'; data not shown) and the ND1 [a gene of the mitochondrial genome coding for the NADH-ubiquinone oxidoreductase chain 1 (ND1) protein] (Fig. 7). Shown in Table 3 is a summary of the differential responses of the release of nDNA versus mtDNA after LA of neutrophils with specific food extracts from each of the four study subjects. It can be seen that a greater degree of calprotectin, nDNA, and mitochondrial release were observed in the more clinically symptomatic study subjects, 3 and 4, which correlated with positive food LA responses.

DISCUSSION

Adverse food reactions are commonly divided into immune-mediated reactions (*i.e.,* IgE-mediated FA (IgE FA), and nonimmune mediated reactions (*i.e.,* non-IgE FA). In contrast to the more commonly seen symptoms of IgE-FA allergic conditions that occur within minutes after allergen challenge that affect many target organ systems, non–IgE-mediated FAs are caused by reactions that do not appear immediately after the ingestion of the offending food and usually relate to responses primarily restricted to the gastrointestinal tract. Emerging literature is providing evidence for the role of innate immune responses in the pathogenesis of non-IgE FA.[1–5] Although the innate immune system is presumed to be the most likely arm of the immune system involved, the precise mechanism of non–IgE FA is not well understood. In the interest of bringing further mechanistic clarification in this area, the present report focuses on studies of the role of neutrophil activation in the pathogenesis of non-IgE FA.

Neutrophils play an important role in innate immune responses. They are rapidly recruited in tissues during infections, where they kill bacteria or at least inhibit their growth. In recent years, it has been recognized that microbial killing by neutrophils not only occurs intracellularly after phagocytosis, accompanied by oxidative intracellular mechanisms, but also can occur in the extracellular space by the formation and release of structures from the neutrophil, referred to as NETs.[19,20] NETs are networks of extracellular fibers, primarily composed of DNA from neutrophils that play an important beneficial role in protective antimicrobial immunity by binding and killing pathogens by using activation of metabolic pathways.[19] NETs can also have a deleterious effect on the host and play an important role in the development of several immunologically mediated diseases, such as the autoimmune disorders, *e.g.,* systemic lupus erythematosus[29]; in preeclampsia, a pregnancy-related inflammatory disorder in which neutrophils are known to be activated[30]; in the pathogenesis of vasculitis[31]; and in the colon mucosa of patients with inflammatory bowel disease, *e.g.,* ulcerative colitis.[32] Calprotectin, the major constituent of NETs has been shown recently to be a biomarker in cow's milk protein allergy in children.[27]

The relationship of NETS to allergic disease has been less well studied and had not been reported in the literature until recently with a seminal publication by Garcia-Martinez *et al.,*[10] who described a novel mechanism of tissue injury associated with non-IgE FA caused by the extracellular release of DNA from innate immune peripheral blood leukocytes activated by food allergens. By using a commercial LA screening assay that identified a list of foods categorized as positive, negative, or intermediate for each of 20 subjects, a

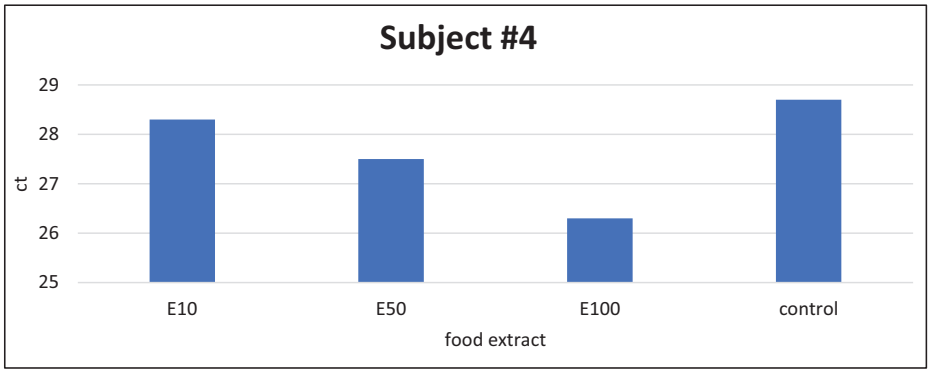

Figure 7. *The mitochondrial* ND1 *[a gene of the mitochondrial genome coding for the NADH-ubiquinone oxidoreductase chain 1 (ND1) protein] gene was analyzed by quantitative real-time polymerase chain reaction in supernatants from neutrophils of subject 4 incubated in the absence (NK) or presence of peanut (E) antigen at different concentrations (10 μL, 50 μL, and 100 μL). The antigen concentrations of the stock solutions for peanut were 5882 μg/mL. Ct = crossing point (a decrease in ct values means an increase in concentration).*

Table 3 **Relationship of nuclear vs mitochondrial DNA release, specific food extracts, and food LA in the four study subjects**

	Subject No.					
	1	**2**	**3**		**4**	
Allergen						
Peanut	Negative	Negative	Peanut		Peanut	
Wheat	Negative	Negative		Wheat		Wheat
Casein	Negative	Negative				
Metabolite released						
Calprotectin	Negative	Negative	Positive	Positive	Positive	Negative
Nuclear DNA	Negative	Negative	Negative	Positive	Negative	Negative
Mitochondrial	Negative	Negative	Positive	Negative	Positive	Negative
Food LA	Negative	Negative	2+	2+	4+	2+

LA = Leukocyte activation.

series of experiments were conducted that measured the release of DNA in the form of NETs from neutrophils activated by these food allergens. A greater release of DNA in the cellular supernatants of immune cells exposed to positive foods than by negative foods was seen providing a potential new innate immune system biomarker for the measurement of non-IgE FA.[10] The same group also described similar correlations and reported significant reduction in disease-related symptoms as well as reduction in serum levels of neutrophil elastase (a major NET component) in patients with the IBS treated with elimination diets guided by LA tests.[15] These data are consistent with and support the findings of our investigations in the present study.

In humans, there is a dual intracellular location of DNA, one contained in the nucleus (nDNA) and the other within the mitochondrion (mtDNA). DNA from both sites can be released into the extracellular environment in a dysregulated way by cell death or in a regulated way, such as during the release of DNA NETs by neutrophils, a phenomenon referred to as NETosis. Results of previous reports suggest that mtDNA has a much lower level of CpG methylation than the nuclear genome.[14,15] In the present study, we confirmed and extended the findings of Garcia-Martinez *et al.*,[10] and showed that, in the subjects with non-IgE FA who showed positive LA test results to food allergens, neutrophils exposed to positive tested foods are accompanied either by the release of nDNA or by mtDNA in the form of NETs. These differential release responses of either nDNA or mtDNA suggest a novel predictive marker for identification of disease outcomes in individuals at risk of FA caused by non-IgE mechanisms.

In support of these findings are a series of associated research observations made by our group related to the controlling role of DNA methylation on inflammation or tolerance, which interface with those described above.[11–13] Because CpG motifs commonly found within bacteria are known to be predominantly unmethylated and proinflammatory in contrast to CpG moieties in mammalian DNA, which are largely methylated and noninflammatory, and

lack immunogenicity, it indicated that DNA methylation could be a key molecular determinant that controls the regulatory properties of the nDNA or mtDNA structures released from allergen-induced activation of leukocytes in the present study. A report by Notley *et al.*[33] supports this possibility and describes how the methylation status of cellular DNA governs the regulation of tolerance or inflammation. DNAs that are methylated were immunosuppressive by regulatory T-cell activation either directly or through dendritic cells. Nonmethylated DNAs induced inflammatory responses through induction of proinflammatory cytokines exerting their effects *via* Toll-like receptor 9 (TLR9) inflammasome activation. In a study by Patil *et al.*,[15] human mitochondrial genomes have been shown to be extensively methylated predominantly at non-CpG sites The pathogenic relationship of NETs has now extended to new infectious diseases that appear in several new reports that describe their pathophysiologic role in COVID-19 infection.[34–42] The inflammatory-promoting effects of these molecules may not only lead to a better understanding of the pathogenesis but may also provide new modalities for therapy of this devastating modern-day plague.

The impedance measurement used in the assay of LA in the present studies formed the basis for the analysis of these food-induced deformability changes of blood leukocytes exposed to various food antigens.[27] However, because current flow cytometers used to measure fluorescence are expensive and complicated, and require trained personnel, which makes them unsuitable for point of care applications, one of the more promising alternative approaches used in LA assays is impedance-based cytometry, which measures food allergen–induced cellular changes by electrical impedance as deformed cells flow through a sensing aperture. Counters that use the Coulter principle have the advantage of a simple, cost-effective, and label-free electronic detection method that lends itself to point of care applications. The formation of NETs represents one reason for the deformability changes of blood leukocytes.

The molecular requirements for NETosis have only recently begun to be elucidated and can be stimulated with a variety of factors, including bacteria or yeast, monosodium urate crystals associated with gout, platelet-activating factor, bacterial ionophores, or lipopolysaccharides, or can be pharmacologically induced with phorbol ester, *e.g.*, PMA.[19] After their ingestion by phagocytic leukocytes, *Candida* cells are known to be one of the most potent stimulants of NETosis. However, there might be dose- and time-dependent effects that determine the formation of NETs, the release of nDNA and/or mtDNA.

In this preliminary study, we attempted to demonstrate that the analysis of cfDNA found in cellular supernatants of neutrophils treated with foods that elicit positive LA responses may shed new insight into the innate immune mechanisms that underlie non-IgE FA. The association of innate immune cell measurement with cfDNA may hold an important key to understanding the role of innate immunity in non–IgE-mediated food reactivity. Further studies, however, that correlate the results of double-blind, placebo-controlled oral food challenges (DBPCFCs) with the LA and NET assays would be important.

Our study had several limitations. Because it was a pilot study, aimed primarily at conveying the finding and relevance of NET-induced tissue damage as a new pathogenetic mechanism for innate immune mediated non-IgE FA, only a small number of subjects with IBS with limited characterization were included as a prototype of a larger group of better characterized patients who have non-IgE FA. As a single-center study on a limited number of patients with IBS from Germany, further studies, therefore, are needed to reproduce these findings on larger numbers of patients, preferably from different geographic populations and possibly on non-IgE FA disorders other than IBS.

CONCLUSION

Future well-designed and empowered clinical studies that evaluate the pathogenetic role of NETs in larger numbers of better characterized subjects who had non-IgE FA, *e.g.*, patients with food protein–induced enterocolitis syndrome, should be able to confirm and extend our findings and, as such, offer new diagnostic and therapeutic modalities for the management of these complex and clinically challenging patients.

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