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Original article

Postprandial induction of neutrophil extracellular trap formation in the blood

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Abstract

***Aims*:** Neutrophils play a key role in infection control via two main mechanisms: phagocytosis and neutrophil extracellular trap (NET) release. Despite their role in infection, NETs can also promote cancer growth and metastasis, inflammatory diseases, and vascular thrombosis. However, the relationship between NET formation and the diet, which are critical factors in the progression of metabolic syndrome, remains unclear. In this study, we examined whether blood NET formation is increased after food intake and sought to identify the dietary components that trigger NET formation.

***Methods*:** Using flow cytometry, we analysed NET formation in the blood of mice fed with four different nutritional diets: normal moderate-fat diet (MF), high-fat diet (HF), high-fat and cholesterol diet (HFHC), and dietary fiber Bacto Agar (BA).

***Results*:** The highest NET formation was found in HF-fed mice, while no increase was found in BA-fed mice. Moreover, we observed peaks in NET formation ~2 h and 6–24 h after meal consumption. NET formation was abolished after intestinal bacteria were depleted by antibiotic treatment, even when the mice were fed the HF meal. Furthermore, we observed NET formation in human peripheral blood 6 h after meal consumption.

***Conclusions*:** These findings suggest that NET formation in peripheral blood may be induced after intake of a high-fat diet, and that this phenomenon is closely associated with the gut microbiota. Further investigations are warranted to unveil the involvement of food components, the gut microbiota, and blood NET formation in the pathogenesis of lifestyle-related diseases.

***KEY WORDS*:** neutrophil extracellular traps, food intake, gut microbiota

Introduction

Neutrophils are a type of granulocyte and account for 45–75% of the total leukocytes in human peripheral blood. They have a strong phagocytic capacity and protect the body against bacterial or fungal infections [1]. Neutrophils migrate to the infection site during an initial immune response and phagocytose pathogens. Then, the phagosomes are fused with lysosomes, forming mature phagolysosomes with full degradative and microbicidal capacity, and the pathogens are killed. Other responses include the formation of reactive oxygen species (ROS) and hydrogen peroxide, which are produced by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and myeloperoxidase (MPO), respectively [2]. Moreover, neutrophils release DNA extracellularly in response to stimuli to capture pathogens; the structures are called "neutrophil extracellular traps (NETs)" [3], and this unique and aggressive mechanism of cell death is known as "NETosis," which is neither apoptosis nor necrosis [4]. NETs contain histones, non-histone proteins, lysozyme, MPO, and neutrophil esterase, all of which contribute to host defense [5]. During the formation of NETs, the collapse of the nuclear membrane occurs after citrullination of histone modification,

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which weakens the association between histones and DNA and makes the chromatin filamentous through the action of peptidylarginine deiminase (PAD), a deiminization enzyme of histone arginine residues, in the presence of Ca^{2+} [6]. NET formation is induced by phorbol myristate acetate (PMA), lipopolysaccharide (LPS), and interleukin (IL)-8 [7].

NETs are also related to the development of various diseases, including cancer growth and metastasis [8], autoimmune diseases [9], and thrombosis [10]. Nevertheless, reduced NET formation is linked to compromised conditions and chronic granulomatosis [10]. To date, it remains unclear whether NET formation can be induced under physiological and healthy conditions in response to daily food intake, drinking, defecation, physical exercise, and mental stress.

In this study, we investigated whether NET formation in the blood is increased after food consumption, which is closely associated with the development of metabolic syndrome. If NET formation is indeed induced by meal intake, the dietary contents that trigger NET formation will need to be identified. Here, we propose a novel concept for the prevention and treatment of inflammatory responses in metabolic syndrome, which consists in controlling the gut microbiota and diet.

Materials and Methods

Animals

Male C57BL/6J mice (6–18-week-old; Charles River, Yokohama, Japan) were used and kept individually (one mouse per cage) in usual stainless cages. The mice were fed a normal moderate-fat diet (MF) (Oriental Yeast Co., Tokyo, Japan) and provided water to drink ad libitum before the experiments. The experimental diets used in the study were as follows: MF (Oriental Yeast Co.; 359 kcal/100 g; protein 23.1%, fat 5.1%), high-fat diet (HF, D12492, Research Diets, New Brunswick, NJ; 524 kcal/100 g; protein 20%, fat 60%), dietary fiber Bacto Agar (BA, BD Sciences, Franklin Lakes, NJ; 0 kcal/100 g, protein 0%, fat 0%), high-fat and high-cholesterol diet (HFHC, D09100301, Research Diets; 449 kcal/100 g; protein 20%, fat 40%, and 1.3% cholesterol). For gut microbiota disruption, the mice were treated with ampicillin (1.0 g/L, Nacalai Tesque, Kyoto, Japan) and neomycin sulfate (1.0 g/L, Nacalai Tesque) for 2 weeks. Peripheral blood was collected from the tail vein of mice and mixed with 5 mM ethylenediaminetetraacetic acid (EDTA). All animal experiments were approved by the Committee on Animal Experimentation of Kanazawa University and performed in accordance with the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Human samples

Blood samples were collected from healthy volunteers (24–47 years old, two males and two females) using a vacuum blood collection tube containing EDTA (Terumo venoject II, Terumo, Tokyo). Blood was collected 12 h after fasting; then, the volunteers were given a meal (one rice ball and one fried chicken). The study complied with the ethical principles set forth in the Helsinki Declaration and Japan's Personal Information Protection Act, and was conducted in accordance with the Ethical Guidelines for Medical Research Involving Human Subjects (Public Notice of Ministry of Education, Culture, Sports, Science and Technology/Ministry of Health, Labor and Welfare). The experimental plan was approved by the ethics committee of Kanazawa University. Informed consent was obtained from subjects prior to the tests, and the subjects sufficiently understood the content of the testing plan and voluntarily expressed their willingness to participate in the test.

Flow cytometry

To prevent non-specific signals, purified rat anti-mouse CD16/CD32 (Mouse BD Fc Block) (1:100, BD Biosciences) was added to 10 µL of EDTA-treated blood, and the mixture was allowed to settle at room temperature (RT) for 15 min in the dark. For the analysis of mouse samples, PE anti-mouse Ly6G/Ly6c (Gr-1) antibody (1:100, BioLegend, San Diego, CA), anti-histone H3 (citrulline R2+R8+R17) antibody (1:50, Abcam), and FITC (fluorescein isothiocyanate) goat anti-rabbit IgG (1:100, BD Biosciences) were added and incubated at RT for 20 min. The samples were then treated with BD FACS Lysing Solution (BD Biosciences), washed with phosphate buffered saline (PBS)/2% fetal bovine serum (FBS), and analyzed using a flow cytometer (BD FACS Aria Fusion), as described previously [11]. For human samples, CD45 monoclonal antibody (HI30) APC-eFluor780 (1:100, eBioscience, San Diego, CA), anti-histone H3 (citrulline R2+R8+R17) antibody (1:50, Abcam, Cambridge, UK), and FITC goat anti-rabbit IgG (1:100, BD Biosciences) were used. The obtained data were re-analyzed using FlowJo v 9.9.5 software (FlowJo, Ashland, OR) [11]. Unstained, single stains, and fluorescence minus one (FMO) controls were used to set compensation and gates.

Giemsa Staining

The smear was covered with absolute methyl alcohol for 5 min (fixation). The alcohol was drained off, and the slide was covered with freshly diluted Giemsa stain solution (Wako Pure Chemical Industries, Osaka, Japan) for 20 min. After washing with water and drying, the slides were enclosed with Entellan® new and observed under an optical microscope.

Stool DNA extraction and polymerase chain reaction (PCR)

Fresh mouse stool samples were collected in 1.5 ml tubes. The samples were heated at 95°C for 30 min in a 50 mM NaOH solution (300 µL). Then, 1.0 M Tris-HCl (pH 7.0) (100 µL) was added, the tubes were centrifuged at 15,000 rpm for 10 min, and DNA was extracted. To semi-quantify the number of intestinal bacteria, PCR targeting 16S rDNA was performed using 16S rDNA forward (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse (5'-GGTTACCTTGTTACGACTT-3') primers (35 cycles; 98°C for 10 s, 53°C for 20 s, and 72°C for 60 s). The PCR products were separated by agarose gel electrophoresis and detected by ethidium bromide staining.

Statistical analysis

Results are presented as means ± SEM (standard error mean). Comparisons between groups were assessed using Student's *t*-test or one way analysis of variance (ANOVA) combined with Scheffe's multiple comparison test ($p < 0.05$).

Results

Flow cytometric detection of NET formation in mouse blood

We performed flow cytometric analyses to detect NET formation in mouse blood, as described previously [11]. The cluster of granulocytes was gated using forward versus side scatter (FSC vs. SSC), and the single cell population was fractioned for further analysis (***Fig. 1***). Subsequently, Gr-1-positive and citrullinated histone H3 (CitH3)-positive neutrophils were determined to be NET-forming cells (***Fig. 1***). We first examined the appearance rates (%) of NET cells in total Gr-1-positive neutrophils from mice fed a standard chow diet (MF). At fasting, the basal rate was 1.42 ± 0.47% (***Fig. 2-a***). Then, a first peak (6.41 ± 2.02%) was observed 2 h after consumption of the MF meal (***Fig. 2A***). In addition, a second peak and plateau were observed 6–24 h after the meal (14.41 ± 4.63% at 24 h) (***Fig. 2-a***). Next, we examined the effect of different types of meals. The HF meal (60% fat, containing lard) increased the rate of NET formation to 10.55 ± 4.13% and 29.01 ± 5.30% at 2 and 12 h postmeal, respectively (***Fig. 2-b***). The HFHC meal (40% fat, containing cocoa butter and 1.3% cholesterol) induced two peaks of NET formation, 6.75 ± 2.66% and 8.80 ± 4.60% at 2 and 6 h postmeal, respectively (***Fig. 2-c***). The group fed the BA meal (0 kcal/100 g, protein 0%, fat 0%), which was used as a dietary control meal, showed a slight increase in the rate of NET cells 2 h after meal ingestion. However, the increase was not statistically significant and NET formation decreased thereafter (***Fig. 2-d***). The largest increase was observed in the HF group (***Fig. 2***).

The gut microbiota is associated with NET formation in the blood

To examine the association of diet-induced NET formation with the gut microbiota, we treated mice with ampicillin natrium (1.0 g/L) and neomycin sulfate (1.0 g/L) orally for 2 weeks to disturb their gut microbiota. After confirming the effects of the antibiotics by PCR analysis of the 16S rDNA in stools, we selected mice with reduced numbers of gut bacteria and fed them with HF (***Fig. 3-a***). FACS analysis clearly demonstrated that the NET appearance rates in the blood were dramatically reduced in the mice during the observation period, including at the 0 h baseline (***Fig. 3-b***).

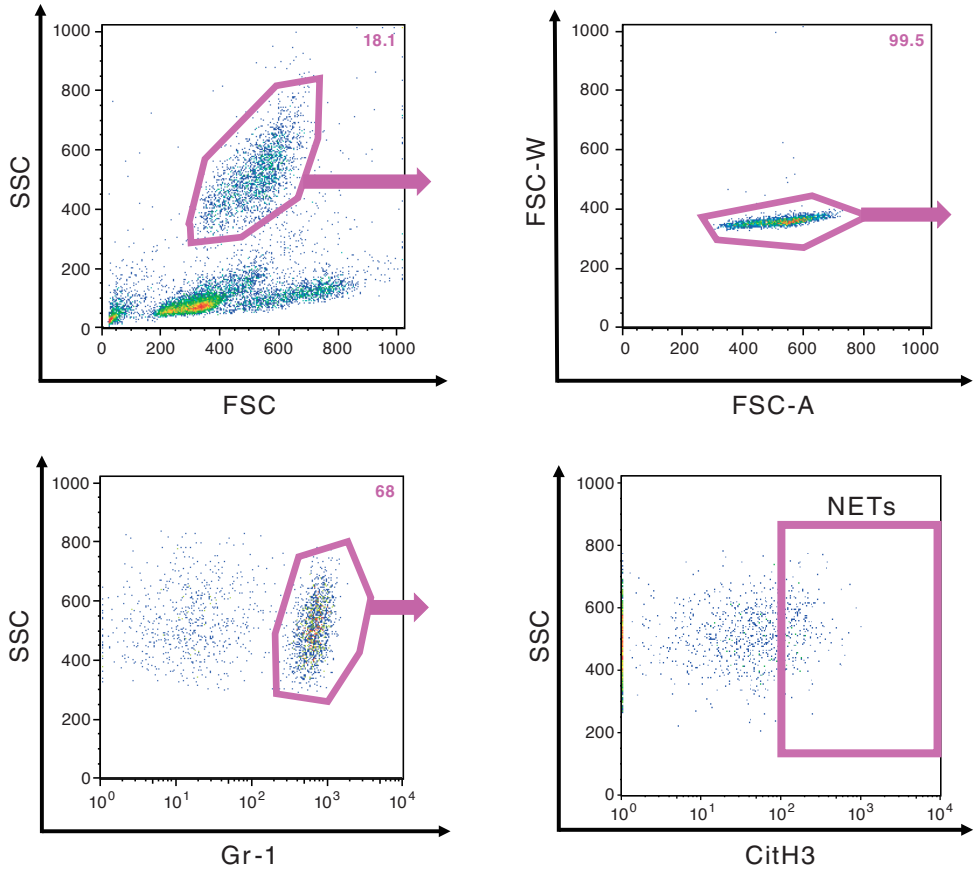

Fig. 1. **Flow cytometry.**

NETs were detected in mouse peripheral blood using PE anti-mouse Ly6G/ Ly6c (Gr-1) antibody, anti-histone H3 (citrulline R2 + R8 + R17) antibody, and FITC goat anti-rabbit IgG by flow cytometry. CitH3, citrullinated histone H3; NETs, neutrophil extracellular traps; FITC, fluorescein isothiocyanate; SSC, side scatter; FSC, forward scatter.

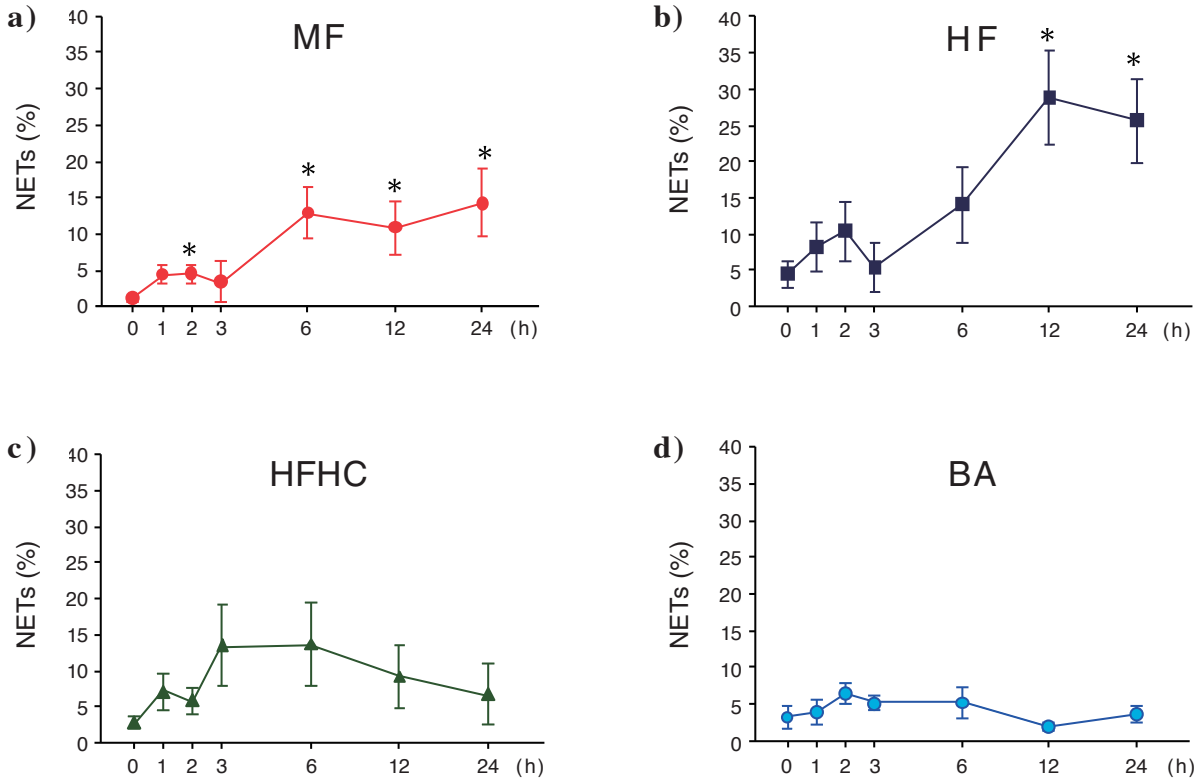

Fig. 2. **The formation of NETs in the blood from mice fed different chow diets.**

The appearance rates (%) of NET cells in total Gr-1-positive neutrophils from mice fed a standard chow diet (MF, n = 14). **(a)** High fat diet (HF, n = 14). **(b)** High fat and high cholesterol diet (HFHC, n = 13). **(c)** Dietary fiber Bacto Agar (BA, n = 9). We observed the NET formation from 0 (fasting) to 24 hours after feeding. Data represent mean ± SEM. *$p < 0.05$ vs basal level (0 hour). NETs, neutrophil extracellular traps; SEM, standard error mean.

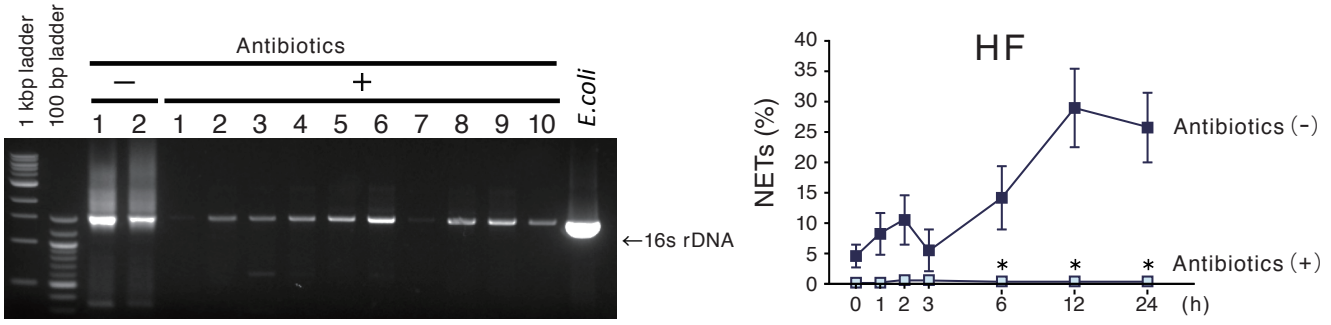

Fig. 3. **The gut microbiota and NET formation in the mouse blood.**

(a) PCR analyses for 16S rDNA. Mice were treated with or without antibiotics (1.0 g/L ampicillin sodium and 1.0 g/L neomycin sulfate in the drinking water) for 2 weeks. Without antibiotics, Antibiotic (–); with antibiotics, Antibiotic (+). **(b)** The appearance rates (%) of NET cells in total Gr-1-positive neutrophils from mice fed a high fat diet (HF) with or without antibiotics in the drinking water. Antibiotic (+), n = 10; Antibiotic (–), n = 14. Data represent mean ± SEM. *$p < 0.05$ between Antibiotic (+) and Antibiotic (–). NET, neutrophil extracellular trap; PCR, polymerase chain reaction; SEM, standard error mean.

Flow cytometric detection of NET formation in human blood

We next investigated the formation of postprandial NETs in human blood. NETs were defined as CD45- and CitH3-positive granulocytes in the peripheral blood (***Fig. 4-a***). After fasting for 12 h, a meal (fried chicken and rice with tuna mayonnaise) was given to four healthy volunteers (24–47 years old, two males and two females). Flow cytometry analyses revealed that the NET appearance rate in the blood was higher 6 h after meal intake (2.475 ± 0.975 %) than at fasting (0.775 ± 0.048 % at 0 h); however, the difference was not statistically significant (***Fig. 4-b***). Next, we conducted morphological observations of NET formation in human peripheral blood. We used blood smear samples collected 6 h after meal ingestion and performed Giemsa staining. As seen in ***Fig. 5-b***, we detected neutrophils with nuclei released outside the cell membrane in the blood.

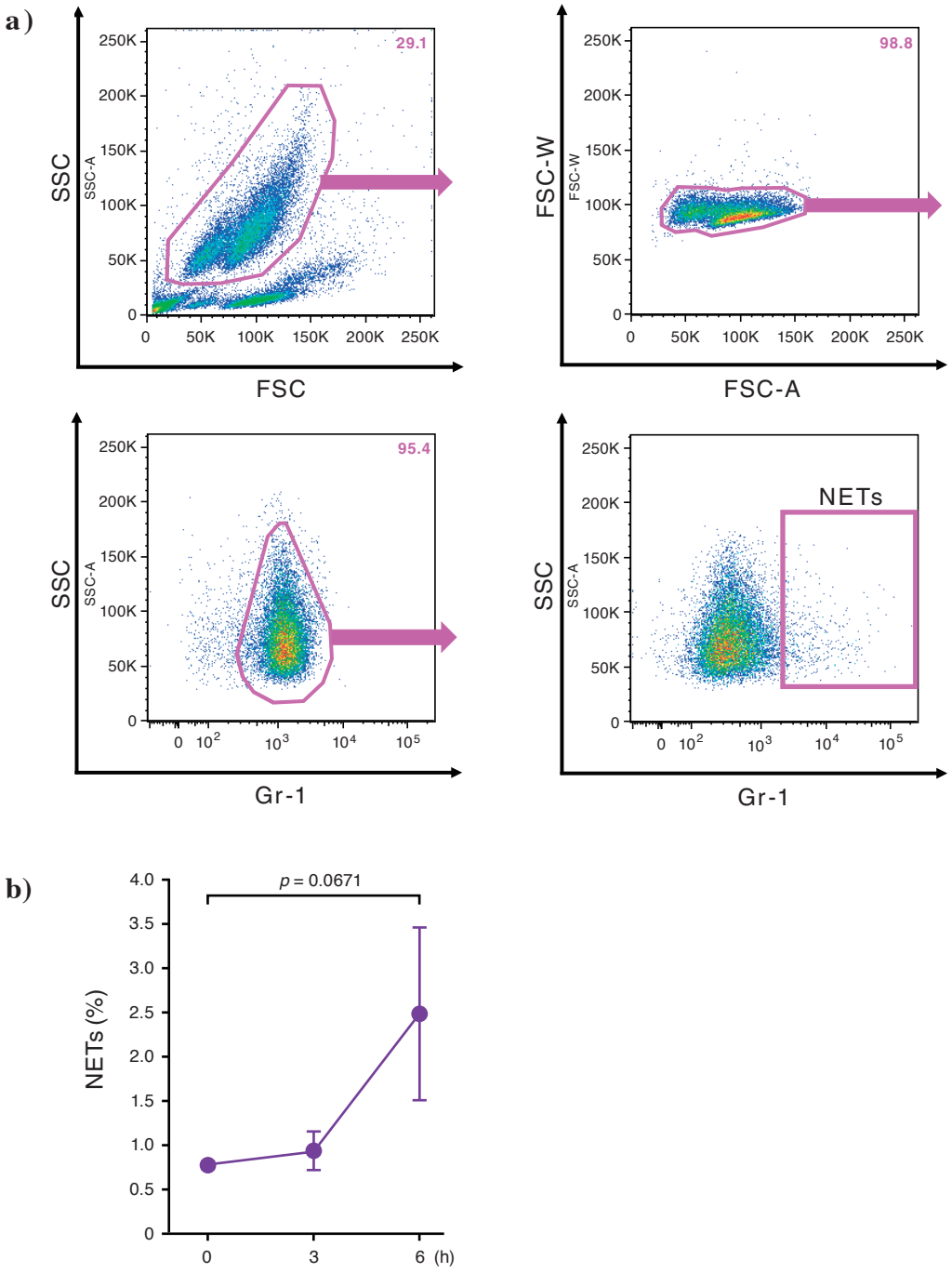

Fig. 4. **The formation of NETs in the human blood.**

(a) Gating strategy. NETs were detected in human peripheral blood using CD45 monoclonal antibody APC-eFluor780, anti-histone H3 (citrulline R2+R8+R17) antibody, and FITC goat anti-rabbit IgG by flow cytometry **(b)** The appearance rates (%) of NET cells in CD45-positive granulocytes from healthy subjects (n = 4). Data represent mean ± SEM. CitH3, citrullinated histone H3; NETs, neutrophil extracellular traps; FITC, fluorescein isothiocyanate; SSC, side scatter; FSC, forward scatter; SEM, standard error mean.

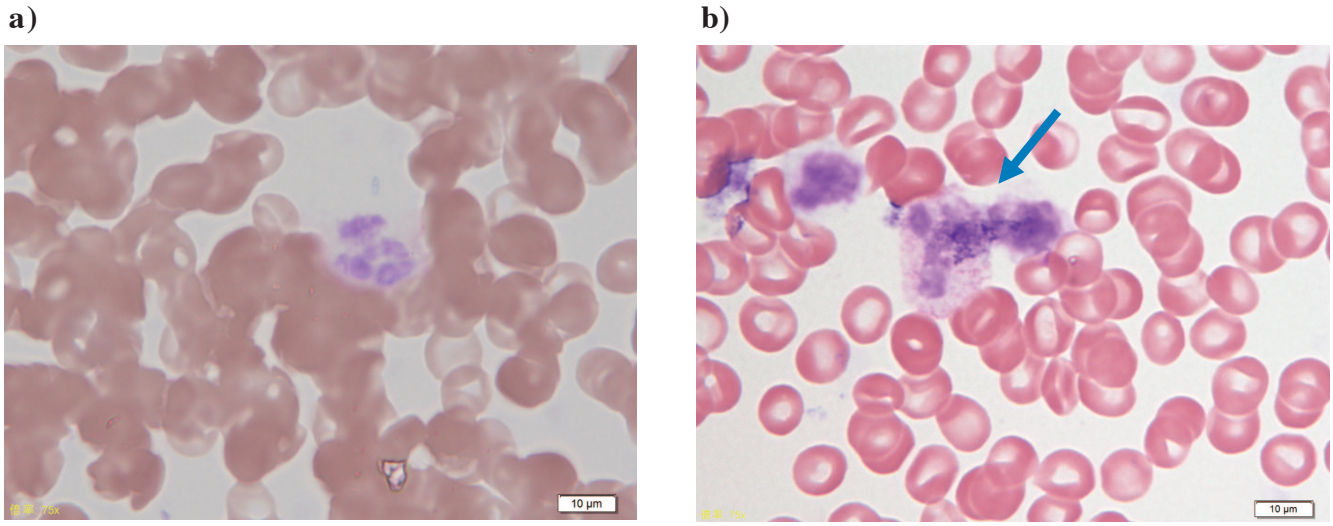

Fig. 5. **Morphological observation of NETs in human blood.**
Giemsa staining. **(a)** Normal neutrophils and **(b)** neutrophils showing neutrophil extracellular traps (NETs).

Discussion

NETosis is a newly discovered type of aggressive neutrophil cell death that is different from apoptosis and necrosis. NET formation during NETosis allows the release of nuclear substances outside the cells [12, 13]. NETs, which are composed of a network of fibers assembled from chromatin DNA, histones, non-histones, and neutrophil granule proteins, can kill pathogens; however, they can also exhibit toxic actions in the host. For example, activated neutrophils with NET formation enhance autoimmune responses related to a wide range of inflammatory autoimmune diseases by exposing autoantigens in susceptible individuals. Several studies have recently shown that NETs play a pivotal role in the development of sterile inflammation under several chronic inflammatory conditions [14]. Sterile inflammation occurs in the absence of microorganisms and is induced by physical, chemical, metabolic, or antigenic stimuli. When non-infectious stimuli persist and cannot be eliminated, NETs can promote sterile inflammation, becoming a part of the pathophysiology of a variety of diseases, including vascular and immune-related diseases, metabolic disorders, and cancer. Neutrophils can be activated by various stimuli such as bacteria, fungi, viruses, and platelets [14]. In addition to these physiological stimuli, non-physiological small compounds, including PMA and calcium ionophores (CaI), are known to activate neutrophils [14]. However, the types of physiological components, including diet, that can induce NET formation remain unknown.

In this study, we have revealed for the first time that food intake, even with a standard chow diet (MF), can induce NET formation in mouse peripheral blood (***Fig. 2***). The lipid content in the diet, especially lard, significantly enhanced NET formation, as evidenced by the fact that HF-fed mice showed higher NET formation than HFHC-fed mice (***Fig. 2***). In addition, cholesterol did not appear to accelerate blood NET formation (***Fig. 2-c***). Furthermore, we found that diet-induced NET formation consisted of two peaks in blood NET formation, occurring approximately 2 h and 6–24 h after meal consumption (***Fig. 2***). Since the first peak was observed even in calorie-free and lipid-free agar BA (***Fig. 2-d***), we suspect that this may not be caused by the dietary content, but by physical irritation to the intestinal tract or intestinal peristalsis triggered by the entrance of the substance into the intestinal tract. In contrast, the second peak may be induced by the lipid contained in the diet. The experiments with antibiotic treatment suggested that both peaks in blood NET formation may be due to the presence of gut microbiota (***Fig. 4***). Furthermore, we found that dietary intake induces blood NET formation in humans (***Figs. 4*** and ***5***). Vitamin C suppresses inflammation and NET formation, and minerals such as copper and zinc are involved in human NET formation [15]. Moreover, vitamin D supplementation has been shown to inhibit NET formation in patients with systemic lupus erythematosus [16].

In addition to NETosis, which is a lytic pathway of NET formation, nonlytic NET formation has been reported very recently; this nonlytic pathway may occur in the absence of neutrophil death, maintaining the ability of neutrophils to phagocytose and release NETs without plasma membrane disruption [17]. In this study, we used flow cytometry to detect cell surface CitH3-positive granulocytes as NETs; thus, the NET appearance rate (%) obtained in our system included both lytic and nonlytic NETs.

Based on our findings, we propose a novel concept for the prevention and treatment of vascular and immune-related diseases, metabolic disorders, and cancer by focusing on the gut microbiota and diet. Further investigations are required to unveil the molecular and cellular involvement of the gut microbiota and food components such as lipids in the pathogenesis of lifestyle-related diseases through blood NET formation.

Conclusion

Diet-induced NET formation was observed in the peripheral blood of mice and humans. The dietary lipid content and gut microbiota of the host may affect NET formation. This phenomenon may be a biomarker for the progression of lifestyle-related diseases and may open a new avenue for the prevention and treatment of vascular and immune-related diseases, metabolic disorders, and cancer.

Conflict of interest statement

The authors declare no conflict of interest in this study.

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