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Zinc Enhances Neutrophil Extracellular Trap Formation of Porcine Peripheral Blood Polymorphonuclear Cells through Tumor Necrosis Factor-Alpha from Peripheral Blood Mononuclear Cells

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Abstract : Neutrophil extracellular trap (NET) formation is an immune response for the invasion of microbes. The purpose of this study is to examine the effect of zinc on NET formation of porcine peripheral blood polymorphonuclear cells (PMNs). The NET formation of PMNs was measured by fluorescence microplate reader. The production of tumor necrosis factor (TNF)-α in the culture supernatants from zinc-treated peripheral blood mononuclear cells (PBMCs) was measured by enzyme-linked immunosorbent assay (ELISA). Zinc itself did not have no effect on NET formation. However, the NET formation of PMNs was increased by culture supernatants from PBMCs treated with zinc. Also, the NET formation of PMNs was increased by recombinant porcine (rp) TNF-α. The production of TNF-α in PBMCs culture supernatants was shown to increase upon zinc treatments. These NET formations of PMNs increased by either culture supernatant from PBMCs treated with zinc or rpTNF-α were inhibited by treatment of anti-rpTNF-α polyclonal antibody (pAb). These results suggested that zinc has an immunostimulating effect on the NET formation of PMNs, which is mediated by TNF-α released from zinc-treated PBMCs. Therefore, zinc may play an important role for NET formation in the defense of porcine inflammatory diseases.

Key words **:** neutrophil extracellular trap (NET), zinc, peripheral blood mononuclear cells (PBMCs), tumor necrosis factor-α (TNF-α), porcine.

Introduction

Neutrophils represent the major population of circulating leukocytes and play a central role in the defense against bacterial and fungal infections (15). Once polymorphonuclear cells (PMNs) in the circulating blood arrive at the site of infection, they employ different strategies to kill pathogens (4). Primarily, PMNs engulf microorganisms and ingest them by phagocytosis (12). Second, PMNs release reactive oxygen and nitrogen species that perform antimicrobial activity. Third, PMNs form the neutrophil extracellular trap (NET). Activated neutrophils release their DNA, histones, and granule proteins into extracellular space, which is a net-like substance known as NET when they die (5). Microbes are caught in this substance and killed by the neutrophil proteins and histones contained in the NETs (8). This process of NET formation leads to a form of cell death, NETosis, that has been characterized as being different from either apoptosis or necrosis (7). The NET formation is known to be stimulated by chemicals (e.g., phorbol 12-myristate 13-acetate (PMA)), bacterial products such as lipopolysaccharide (LPS) and specific cytokines such as interleukin (IL)-8, tumor necrosis factor-alpha (TNF-α) (22). After stimulation, the neutrophil chromatin goes through decondensation followed by mixing of euchromatin and heterochromatin (7). This process is mediated by enzymes stored in the azurophilic granules, neutrophil elastase (NE) and myeloperoxidase (MPO), which are relocated to the nucleus by a yet unknown mechanism (31).

Zinc is an essential nutrient that is required in mammals for many physiological functions (29). It was reported that low zinc levels can damage macrophage and neutrophil phagocytosis, oxidative burst generation, and natural killer cell activity, and that granulocyte numbers decrease during zinc deficiency (1). Proper zinc treatment induce specific cytokines releases including IL-8 and TNF-α in supernatant from peripheral blood mononuclear cells (PBMCs) (21). These cytokines encourage neutrophil phagocytosis (20) and chemotaxis (14). TNF-α has been identified as an important regulator of the inflammatory response. In particular, many inflammatory conditions were triggered by increased level of TNF-α (9). Similarly, the NET formation was found in inflammatory site. The DNA and granular proteins released by NETosis cause autoimmune diseases (13).

In previous studies, the effects of zinc on phagocytosis (21) and chemotactic activity (28) in PMNs were investigated. However, the effects of zinc on NET formation of PMNs has not been investigated. The present study was performed to determine the effects of zinc treatment on the NET formation of PMNs. For this purpose, PMNs were treated with zinc and culture supernatant from zinc-stimulated PBMCs. We also examined the level of TNF-α production in the culture supernatant of PBMCs treated with zinc.

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Materials and Methods

Chemicals and reagents

Zinc sulfate (Fluka Chemie AG, Buchs, Switzerland) was sterilized by passing through a 0.45-μm filter of membrane (Milipore Co., Bedford, Mass, USA) before being used. Phorbol 12-myristate 13-acetate (PMA), propidium iodide (PI) solution, and RPMI 1640 medium, were purchased commercially from Sigma-Aldrich (St. Louis, MO, USA). Rabbit anti-recombinant mouse (rm) IL-6 pAb (IgG) (Sigma-Aldrich), goat anti-rpTNF-α polyclonal antibody (pAb) (IgG), and recombinant porcine (rp) tumor necrosis factor (TNF)-α (R&D Systems Inc., Minneapolis, MN, USA) were also purchased.

Isolation of porcine peripheral blood mononuclear cells (PBMCs) and polymorphonuclear cells (PMNs)

Clinically healthy crossbred pigs (6-month-old) in slaughterhouse (Donga food Co. Ltd., Cheongju, Chungbuk, Republic of Korea) were donated as blood donors. Peripheral blood in heparinized tube from anterior vena cava, diluted with an equal volume of PBS, and overlaid 1:1 on a Percoll® solution (Sigma-Aldrich; 1.080 gravity). After centrifugation at 400 g for 45 min, the PBMCs in the interface between Percoll® solution and plasma were collected and treated with RBC lysis buffer (iNtRON biotechnology, Seongnam, Gyeonggi, Republic of Korea) for 5 mins. The PMNs were harvested from sedimented erythrocytes after removing the PBMCs layer. To purify the PMNs, one ml of the sedimented erythrocytes was allowed to sediment for 50 min with 1.5% dextran (molecular weight, 200,000; Wako Ltd., Osaka, Japan) in PBS. The floating cells were collected and centrifuged at 900 g for 3 min. The residual erythrocytes were treated by RBC lysis buffer. The resulting PBMCs and PMNs were washed three times with PBS. The viability of PBMCs and PMNs by method of trypan blue dye exclusion, always exceeded 97.5%. All cells were resuspended in medium of RPMI 1640 with 5% heat-inactivated FBS (Gibco Company, Grand Island, NY, USA), 1% 100 U/ml penicillin at 37°C in a 5% CO_2-humidified atmosphere.

PBMCs culture supernatants

The PBMCs seeded at a density of 2×10^6 cells/ml in a twenty-four-multi well plate (Nunc company, Naperville, IL, USA) were cultured with zinc sulfate (100 μM) for 24 h at 37°C in a 5% CO_2-humidified atmosphere. Control cells were treated with the same amount of PBS. After incubation, all culture supernatants were harvested after centrifugation at 900 g for 10 min and stored at −70°C until used.

Quantification of NET formation

NET formation was measured by the enhanced fluorescence emission of propidium iodide (PI) after its interaction with extracellular DNA. The PMNs (8×10^5 cells/ml/well) at each well of a 24-well plate received 800 μl of cells adjusted to 1×10^6 cells/ml were incubated for 4 h with PMA (10 nM) as positive control, zinc sulfate (0-200 μM), culture supernatants from PBMCs and rpTNF-α. The cultured cells were gently harvested, and centrifuged at 900 g for 15 min. Cells were gently washed with warm (37°C) Dulbecco's phosphate-buffered saline containing magnesium and calcium (Gibco Company). Then, cells were mixed with 400 μl of 2.5 μM PI diluted with DPBS. Cells were placed in black 96-well plate (SPL life science, Pocheon, Gyeonggi, Republic of Korea) and incubated for 30 min in the dark. The resulting fluorescence was determined at excitation wavelength (535 nm) and emission wavelength (617 nm) using a SpectraMax M2e microplate reader (Molecular devices, Oreleans Drive Sunnyvale, CA, USA).

Neutralization test

PMNs (8×10^5 cells/ml) were incubated for 4 h either zinc-treated PBMCs culture supernatant (75%) or rpTNF-α (20 ng/ml) in the presence or absence of various concentrations of anti-rpTNF-α (IgG) pAb at 37°C in a 5% CO_2-humidified atmosphere. Rabbit anti-rmIL-6 pAb as a control isotype IgG was used. The effect of this mixed samples on the NET formation of the PMNs was evaluated as described above.

Measurement of TNF-α in culture supernatant from zinc-treated PBMCs

The culture supernatants of PBMCs treated with zinc (100 μM) were collected after 24 h incubation. The amount of TNF-α in the culture supernatants was measured by direct sandwich enzyme-linked immunosorbent assay (ELISA) using Quantikine® porcine TNF-α immunoassay kit (R&D Systems Inc.) by the manufacturer's protocol. All samples containing standard and controls in triplicate were tested. The optical density was determined at 450 nm of wavelength by an automate microplate reader (Elx808, Bio-Tek Instruments Inc., Winooski, Vermont, USA). The TNF-α levels were quantified from standard curves generated with purified porcine TNF-α tested.

Statistical analyses

GraphPad prism 6 software (GraphPad software, San Diego, CA, USA) was used to perform all statistical analyses. Comparisons in two groups were performed by the student's *t*-test. To test the statistical significance of differences between control and treated groups, one-way analysis of variance (ANOVA) was employed and followed by a Dunnett's *post hoc* test. Results were expressed as means ± standard deviations (SD) and considered to be statistically significant at $p < 0.05$.

Results

Zinc does not increase directly the NET formation of PMNs

To evaluate the direct effect of zinc on NET formation of PMNs, freshly isolated PMNs were incubated with zinc at concentrations ranging from 0 to 200 μM for 4 h. The treatment of PMA (10 nM) as a positive control, significantly ($p < 0.001$) increased the NET formation of PMNs compared to control cells treated without zinc. But the direct treatment of zinc did not show any effect on NET formation as compared with control cells (Fig 1).

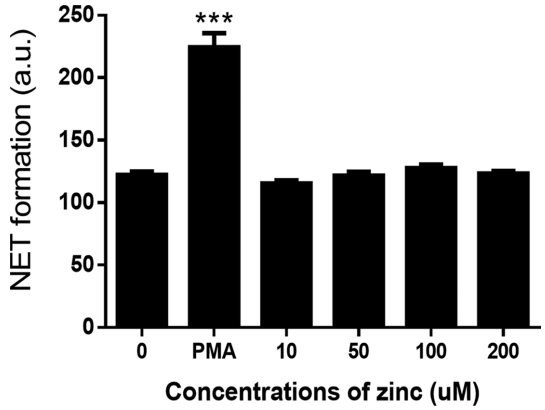

Fig 1. The effect of zinc on NET formation of PMNs. PMNs (8×10^5 cells/well/mL) were treated with 10 nM PMA (positive control) or zinc (0-200 μM) for 4 h. Control cells were incubated with the same amount of PBS. The NET formation was quantified using the membrane impermeable fluorescent DNA dye propidium iodide (PI). The data represent means ± SD (n = 3). One-way ANOVA was used for statistical analysis, followed by a Dunnett's *post hoc* test. Comparison of two groups was made by student's *t*-test. a.u.: arbitrary units. ***$p < 0.001$ *vs*. control (0 μM).

Culture supernatants from PBMCs treated with zinc increase the NET formation of PMNs

We investigated whether the culture supernatant from zinc-treated PBMCs has any effect on the NET formation of PMNs. Freshly isolated PMNs were incubated with culture supernatants (0-100%) from PBMCs for 4 h. The treatment of PMA significantly ($p < 0.001$) increased the NET formation of PMNs compared to control. The NET formation of PMNs were significantly (25 and 50%; $p < 0.05$, 75 and 100%; $p<0.01$) increased in a dose-dependent fashion by culture supernatant from PBMCs treated with zinc as compared with control (0%) (Fig 2). The NET formation of PMNs was climaxed at 75% of culture supernatant from PBMCs treated with zinc. On the other hand, the NET formation of PMNs treated with 100% culture supernatant was slightly reduced as compared with that of 75% culture supernatant but it did not show statistical significance.

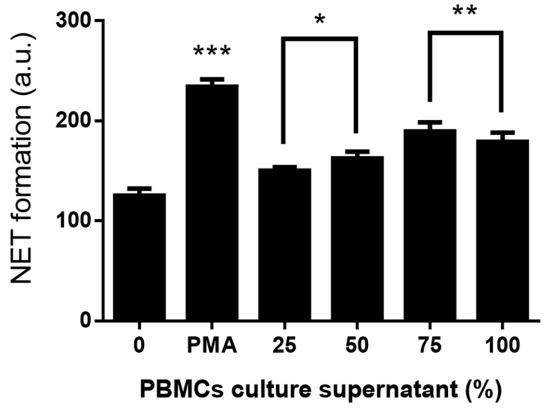

Fig 2. The effect of zinc-treated PBMCs culture supernatant on NET formation of PMNs. PMNs (8×10^5 cells/well/mL) were treated for 4 h with 10 nM PMA (positive control) or culture supernatants (0-100%) from PBMCs (2×10^6 cells/mL) treated with zinc (100 μM) for 24 h. Control cells were treated with the same amount of PBS. The NET formation was quantified using the membrane impermeable fluorescent DNA dye propidium iodide (PI). The data represent means ± SD (n = 3). One-way ANOVA was used for statistical analysis, followed by a Dunnett's *post hoc* test. Comparison of two groups was made by student's *t*-test. a.u.: arbitrary units. *$p < 0.05$ *vs*. control. **$p < 0.01$ *vs*. control. ***$p < 0.001$ *vs*. control (0%).

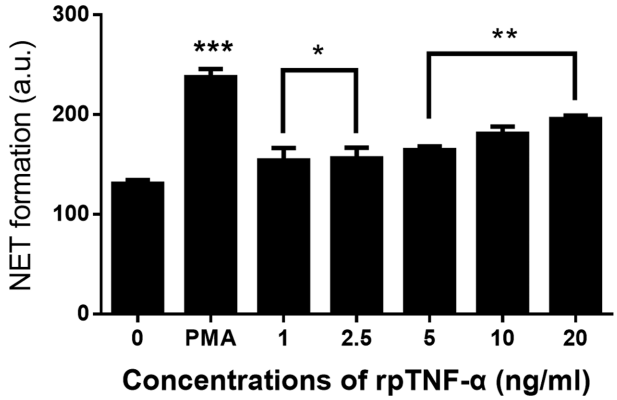

Fig 3. The effect of rpTNF-α on NET formation of PMNs. PMNs (8×10^5 cells/well/mL) were treated with 10 nM PMA (positive control) or rpTNF-α (0-20 ng/ml) for 4 h. Control cells were treated with the same amount of PBS. The NET formation was quantified using the membrane impermeable fluorescent DNA dye propidium iodide (PI). The data represent means ± SD (n = 3). One-way ANOVA was used for statistical analysis, followed by a Dunnett's *post hoc* test. Comparison of two groups was made by student's *t*-test. a.u.: arbitrary units. *$p < 0.05$ *vs*. control. **$p < 0.01$ *vs*. control. ***$p < 0.001$ *vs*. control (0 ng/ml).

TNF-α also increases the NET formation of PMNs

The NET formation of PMNs in response to TNF-α was also investigated. The NET formation of PMNs by rpTNF-α significantly (1 and 2.5 ng/ml; $p < 0.05$, 5, 10 and 20 ng/ml; $p < 0.01$) increased in a dose-dependent fashion as compared with control (Fig 3).

Anti-rpTNF-α pAb decreases the NET formation of PMNs

To evaluate whether the enhanced NET formation of PMNs by the culture supernatant from zinc-treated PBMCs may be due to TNF-α, the neutralization test using the anti-rpTNF-α pAb was performed. The NET formation of PMNs in response to culture supernatant from PBMCs treated with zinc was significantly (10, 25 and 50 μg/ml; $p < 0.05$, 100 μg/ml; $p < 0.001$) inhibited in a dose-dependent fashion by the addition of anti-rpTNF-α pAb as compared with culture supernatant (75%) from PBMCs treated with zinc (Fig 4). Similarly, the increased NET formation of PMNs by rpTNF-α at 20 ng/ml was also significantly (0.1 and 1 μg/ml; $p < 0.01$, 10 μg/ml; $p < 0.001$) reduced by the addition of anti-rpTNF-α pAb in a dose-dependent fashion (Fig 5). However, in the test of nonspecific inhibition for immunoglobulin isotype, IgG, any NET formation of PMNs to either culture supernatant (75%) from PBMCs treated with zinc or rpTNF-α (20 ng/ml) was not inhibited by high concentration (100 μg/ml)

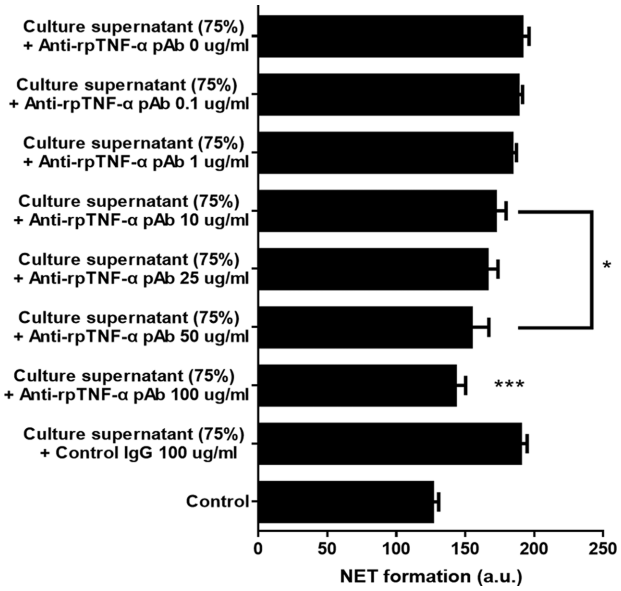

Fig 4. The effect of anti-rpTNF-α pAb with zinc-treated PBMCs culture supernatant on NET formation of PMNs. Anti-rpTNF-α pAb (0, 0.1, 1, 10, 25, 50 and 100 μg/ml) and anti-rmIL-6 pAb (100 μg/ml) were mixed with the zinc-treated PBMCs culture supernatant (75%) for 30 min at room temperature. PMNs (8×10^5 cells/well/mL) were incubated for 4 h with these mixtures. Control cells were treated with the same amount of PBS. The NET formation was quantified using the membrane impermeable fluorescent DNA dye propidium iodide (PI). The data represent means ± SD (n = 3). One-way ANOVA was used for statistical analysis, followed by a Dunnett's *post hoc* test. Comparison of two groups was made by student's *t*-test. a.u.: arbitrary units. $*p < 0.05$, $***p < 0.001$ compared with culture supernatant (75%) alone.

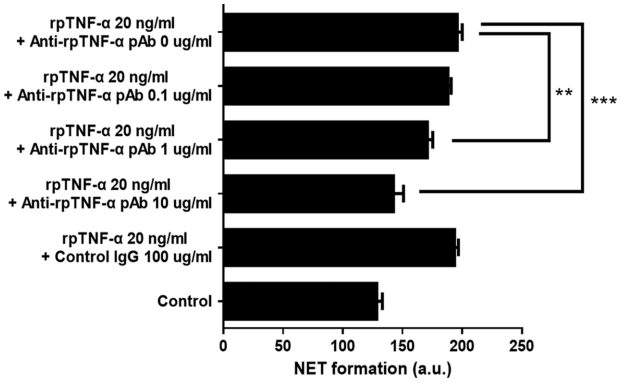

Fig 5. The effect of anti-rpTNF-α pAb with rpTNF-a on NET formation of PMNs. Anti-rpTNF-α pAb (0, 0.1, 1, 10 and 100 μg/ml) and anti-rmIL-6 pAb (100 μg/ml) were mixed with the rpTNF-α (20 ng/ml) for 30 min at room temperature. PMNs (8×10^5 cells/well/mL) were incubated for 4 h with these mixtures. Control cells were treated with the same amount of PBS. The NET formation was quantified using the membrane impermeable fluorescent DNA dye propidium iodide (PI). The data represent means ± SD (n = 3). One-way ANOVA was used for statistical analysis, followed by a Dunnett's *post hoc* test. Comparison of two groups was made by student's *t*-test. a.u.: arbitrary units. $**p < 0.01$, $***p < 0.001$ compared with rpTNF-α (20 ng/ml) alone.

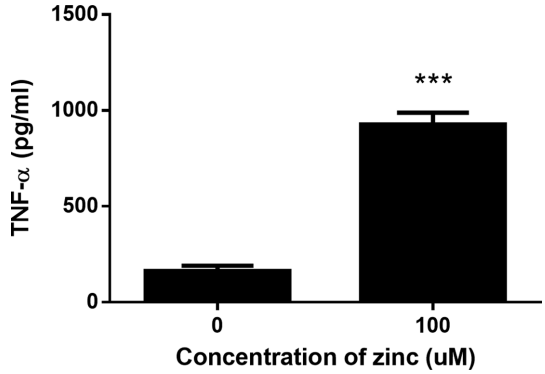

Fig 6. The effect of zinc on TNF-α production of PBMCs. PBMCs (2×10^6 cells/mL) were incubated with zinc sulfate (100 μM) for 24 h. Control cells were treated with the same amount of PBS. The concentration of TNF-α in the culture supernatant from PBMCs was measured by ELISA. The data represent means ± SD (n = 3). Comparison of two groups was made by student's *t*-test. $***p < 0.001$ *vs*. control (0 μM).

of control IgG, anti-rmIL-6 pAb (Fig 4 & Fig 5).

Zinc increases TNF-α production from PBMCs

The amount of TNF-α in the culture supernatant (100%) from PBMCs treated with zinc (100 μM) for 24 h was quantified by ELISA. The level of TNF-α in culture supernatant from PBMCs treated with zinc was significantly ($p < 0.001$) higher than culture supernatant from PBMCs without zinc (Fig 6).

Discussion

NET formation is initiated by binding of neutrophil surface receptors to microbes or microbial breakdown products, inflammatory stimuli, or endogenous inducers (16). Binding to receptors causes endoplasmic reticulum (ER) calcium store release that leads to cytoplasmic calcium increases. Increased calcium stimulates protein kinase C (PKC) activity, leading to nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation. These activations cause reactive oxygen species (ROS) and nitric oxide (NO) production and chromatin decondensation (30). A rupture in the plasma membrane allows the release of chromatin traps in extracellular space (5). The NET formation is mostly mediated by ROS production involving NADPH oxidase and myeloperoxidase (MPO) (25,26).

The effect of zinc on NET formation in human has been investigated (12). In the present study, PMA as positive control increased the NET formation of PMNs. PMA induces the NET formation, which differs both biochemically and morphologically from apoptosis and necrosis (27). The NET formation induced by PMA requires for both autophagy and superoxide production, which trigger the intracellular chromatin decondensation following the NET formation (3). To investigate the effect of zinc on the NET formation of PMNs, we directly treated various concentrations (0-200 μM) of zinc to PMNs. Our results showed that zinc itself did not cause any effect on the NET formation of PMNs indicating that

zinc has directly no effect on the NETosis. However, the culture supernatant from zinc-treated PBMCs increased the NET formation of PMNs. This indicates that there are soluble factor(s) in the culture supernatant from zinc-treated PBMCs that can enhance the NET formation of PMNs. The NET formation can be modulated by specific cytokines (17). TNF-α is a influential PMNs priming agent that enhances the phagocytosis of neutrophils (24) and increases the NADPH oxidase dependent superoxide anion production by PMNs (2). Also, TNF-α enhances phagocytosis, degranulation and oxidative burst activity of PMNs, as well as increases migration through endothelium because of up-regulation of endothelial adhesion molecules (10,19,20). Zinc induces specific cytokines including IL-2, IL-8, interferons and TNF-α release in PBMC supernatant (6,11,21). Accordingly, we hypothesized that the soluble factor(s) in the culture supernatant from zinc-treated PBMCs may be associated with TNF-α. We examined the effect of TNF-α on the NET formation of PMNs. Similar to the effect of zinc-treated culture supernatant to the NET formation of PMNs, rpTNF-α could enhance the NET formation of PMNs. This is consistent with a previous report showing that TNF-α increased the NET formation of human PMNs (17). We found that TNF-α level of culture supernatants from PBMCs were enhanced by zinc treatment. Next, we tested whether anti-rpTNF-α pAb neutralize the increment of the NET formation induced by either zinc-treated PBMCs culture supernatant or rpTNF-α. The anti-rpTNF-α pAb neutralized the ability of both culture supernatant from zinc-treated PBMCs and rpTNF-α to increase the NET formation of PMNs. This finding that the culture supernatant from PBMCs treated with zinc can increase the NET formation of PMNs might suggest that the immunostimulating effect of zinc on the NET formation of PMNs is mediated by TNF-α released by zinc-stimulated PBMCs. Thus, zinc stimulates PBMCs to produce TNF-α, which in turn increases the NET formation of PMNs.

In our results, anti-rpTNF-α pAb did not completely neutralize the ability of the culture supernatant from zinc-treated PBMCs to enhance the NET formation of PMNs. Of course, TNF-α increases the NET formation of PMNs. IL-1β and IL-8 are also potent activators that increase the NET formation of PMNs (18). IL-1β induce the NET formation of gout PMNs through MPO release and ROS production (23). Similarly, IL-8 cause the NET formation of PMNs mediated by NADPH oxidase and superoxide production (18). For this reason, it is thought that anti-rpTNF-α pAb in these results could not completely inhibit the ability of the culture supernatant from zinc-treated PBMCs to enhance the NET formation of PMNs.

These data indicate that zinc has immunostimulating effect on the NET formation of PMNs. Several studies also find out the NET formation and its components in autoimmune diseases such as immune-mediated hemolytic anemia, systemic lupus erythematosus (SLE), vessel vasculitis, lupus nephritis, psoriasis, and rheumatoid arthritis (13,31). Therefore, the NET formation may be useful as markers of immune diseases.

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