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The responses of macrophages in interaction with neutrophils that undergo NETosis

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ABSTRACT

Neutrophil extracellular traps (NETs) are net-like chromatin fibers decorated with antimicrobial proteins, which are released from dying neutrophils. The death of neutrophils with NET formation is called NETosis. Although NETs play important roles in the innate immunity, especially in the elimination of microbes, the extracellular release of DNA and intra-cytoplasmic/nuclear proteins can, on the other hand, result in diverse adversities to the hosts. Therefore, NETosis is adequately regulated *in vivo*. Currently, two mechanisms, namely DNase I-dependent digestion and phagocytosis by macrophages, have been shown as such regulatory mechanisms. In this study, we focused on the interaction of macrophages and neutrophils that underwent NETosis. Results demonstrated that macrophages displayed a phenotype-dependent response after degradation of NETs. Several hours after the interaction, M2 macrophages induced a pro-inflammatory response, while M1 macrophages underwent cell death with nuclear decondensation. This nuclear decondensation of M1 macrophages occurred in a peptidylarginine deiminase 4-dependent manner and resulted in a local release of extracellular DNA. Thereafter, M1 macrophages degraded DNA derived from themselves in a caspase-activated DNase-dependent manner resulting in the clearance of extracellular DNA within 24 h. This transient increase and subsequent clearance mechanism of extracellular DNA seems very reasonable in terms of the double-edged sword-like property of NETs. The collective findings demonstrate a novel phenotype- and time-dependent regulation of NETosis by macrophages.

1. Introduction

Neutrophil extracellular traps (NETs) are net-like chromatin fibers decorated with antimicrobial proteins, such as myeloperoxidase (MPO), which are released from dying neutrophils [1]. Microorganism-triggered neutrophils die in due course with the formation of NETs. Consequently, neutrophils can trap and kill microorganisms by NETs even after they died [2]. The death of neutrophils with NET formation is called NETosis.

Although NETs play critical roles in the innate immune system, a persistent or excessive formation of NETs can induce diverse adversities in the hosts [3]. Therefore, NETosis is strictly regulated *in vivo*. One of the most important regulators of NETs is serum DNase I [4]. NET DNA can be digested by normal serum *in vitro*, whereas some SLE patients with low activity of serum DNase I cannot eliminate NETs adequately. This condition can be involved in the pathogenesis of SLE, at least in part, via induction of anti-DNA antibodies. Furthermore, the anti-thyroid drug, propylthiouracil-induced DNase I-resistant NETs can lead to the production of anti-MPO antibodies (MPO anti-neutrophil cytoplasmic antibody: MPO-ANCA) and subsequent development of ANCA-associated vasculitis (AAV) [5]. These findings clearly indicate that dysfunction of DNase I can cause the following: 1) persistent NETs, 2) production of autoantibodies against NET components, and 3) subsequent development of autoimmune diseases, such as SLE and AAV.

Recently, it has been suggested that NETs could be removed by macrophages via phagocytosis [6]. Macrophages are divided into phenotypically distinct two populations, namely M1 macrophages and M2 macrophages. In general, M2 rather than M1 macrophages

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are considered to be preferentially implicated in the clearance of dead cells [7]. Moreover, macrophages can induce an anti-inflammatory response through phagocytosis of apoptotic cells [8–10], whereas they can induce a pro-inflammatory response after phagocytosis of necrotic cells [11]. However, the phenotype-dependent roles of macrophages in the interaction with neutrophils that undergo NETosis have not been determined.

In this study, we investigated the interaction of macrophages and neutrophils that underwent NETosis. Results demonstrated that both M1 and M2 macrophages could digest NETs, but they displayed different responses. In an earlier period (3–4 h after the interaction with NETs), the secretion of pro-inflammatory cytokines/chemokines was detected in the supernatants of M2 macrophages, whereas M1 macrophages induced a transient increase in extracellular DNA, which was derived from themselves. Interestingly, the extracellular DNA was degraded completely by M1 macrophages in a later period (within 24 h). This study demonstrated a novel phenotype- and time-dependent regulatory mechanism of NETosis by macrophages.

2. Materials and methods

2.1. Neutrophil isolation and NET induction

Human neutrophils were obtained from peripheral blood of healthy volunteers by density centrifugation using Polymorphprep (Axis-Shield, Dundee, Scotland). The obtained cells were re-suspended in RPMI 1640 medium supplemented with 5% FBS, 10 mM Hepes, 100 U/ml penicillin, and 100 mg/ml streptomycin (5×10^5/ml). NETs were induced by the following two diverse methods. PMA-NETs: The neutrophils were exposed to 50 nM phorbol myristate acetate (PMA) (Sigma–Aldrich, St. Louis, CA) for 3 h. ANCA-NETs: The neutrophils were primed by treatment with 5 ng/ml TNF-α (Sigma–Aldrich) for 15 min and then exposed to 250 μg/ml IgG eluted from the serum of patients with MPO-AAV for 3 h.

2.2. Isolation, cultivation, and phenotype induction of human monocyte derived macrophages

To obtain monocytes, peripheral blood mononuclear cells collected from healthy volunteers were separated and further incubated with CD14 MACS beads (Miltenyi Biotec, Tokyo, Japan). After the magnetic separation, purified $CD14^+$ monocytes were cultured in 6-well plates in RPMI 1640 medium with 10% FBS (5×10^5/ml). Thereafter, these monocytes were made to differentiate into the M1 and M2 macrophages (HMD-M1 and HMD-M2 macrophages) by incubation with 10 ng/ml GM-CSF (PeproTech, Rocky Hill, NJ) for 7 days and with 10 ng/ml M-CSF (PeproTech) for 7 days, respectively [12].

2.3. Cultivation and phenotype induction of THP-1 cells

As a source of macrophage cell lines, the human monocytic leukemia cell line, THP-1, was used. The macrophage-like state was induced in THP-1 monocytes by exposing to 100 ng/ml PMA for 48 h (5×10^5/ml in 6-well plates). Thereafter, adherent cells were washed twice with RPMI 1640 medium and incubated for another 24 h to induce a resting state. The resting macrophages were then exposed to 20 ng/ml IFN-γ (PeproTech) and 1 mg/ml LPS (Sigma–Aldrich) for 6 h to differentiate into the M1 phenotype (THP-M1 macrophages) or stimulated by 20 ng/ml IL-4 (Sigma–Aldrich) for 24 h to differentiate into the M2 phenotype (THP-M2 macrophages) as previously described [13].

2.4. Co-culture of macrophages and NETs

In order to examine if macrophages would digest NETs, the following protocol was employed. First, peripheral blood neutrophils extracted from healthy donors were seeded in BD Falcon™ Culture Slides (BD Biosciences, Tokyo, Japan) (5×10^5/ml). These cells were given intracellular labeling with 5 μM CellTracker™ Green 5-chloromethylfluorescein diacetate (CMFDA, Molecular Probes, Tokyo, Japan) for 30 min followed by induction of PMA-NETs or ANCA-NETs. After removal of culture supernatants containing non-adherent neutrophils without NET formation, THP-M1, THP-M2, HMD-M1, and HMD-M2 cells were added consecutively (5×10^5/ml). Prior to the addition, these macrophages had been detached from the wells and the reagents used for the phenotype induction had been washed out thoroughly. After 3 h of incubation, cells were fixed with 4% paraformaldehyde (PFA) for 15 min and then exposed to 1:100 dilution of the mouse anti-CD68 antibody (DAKO, Tokyo, Japan) for 60 min to label macrophages. After washing with PBS, the cells were next allowed to react with 1:500 dilution of Alexa Fluor 594-conjugated goat anti-mouse IgG antibodies (Invitrogen, Tokyo, Japan) for 60 min. Finally, the slides were mounted with the solution containing 4′,6-diamidino-2-phenylindole (DAPI) (Sigma–Aldrich) for DNA staining. The BioRevo BZ-9000 microscope (Keyence, Osaka, Japan) was used for observation of fluorescence.

2.5. Quantification of NETs and DNA

In the co-culture experiments, altered NET amounts and DNA distribution were substituted by the alteration of the CMFDA-positive areas and DAPI-positive areas, respectively, using Image J software.

2.6. Proteome profiler protein array

Peripheral blood neutrophils extracted from healthy donors were seeded in 12-well plates (5×10^5/ml), and then PMA-NETs were induced. After removal of culture supernatants containing non-adherent neutrophils without NET formation, THP-M1 and THP-M2 cells were added subsequently (5×10^5/ml). As controls, THP-M1 or THP-M2 macrophages were incubated without PMA-NETs (5×10^5/ml). Three hours later, the culture supernatants were collected and subjected to detection of secreted proteins using Proteome Profiler Array Human Cytokine Array Panel A (R&D Systems, Minneapolis, MN) according to the manufacturer's instruction. The chemiluminescence was detected using ImageQuant LAS-4000 (GE Healthcare, Tokyo, Japan).

2.7. Live cell imaging

PMA-NETs were induced in neutrophils (5×10^5/ml). Adherent neutrophils forming NETs were collected using a cell scraper and then centrifuged at 400 G for 5 min. After the supernatants were removed, the pellets were re-suspended in RPMI 1640 medium containing 1 μM Sytox Green (Molecular Probes) and co-cultured with THP-M1 (5×10^5/ml). Prior to the co-culture, macrophages were stained with 10 μM CellTracker™ Blue 7-amino-4-chloromethylcoumarin (CMAC, Molecular Probes) for 40 min in PBS with 2% FBS. The co-culture plate was put on the stage of BioRevo BZ-9000 microscope for 240 min (original magnification:×200). Fluorescence overlay videos were recorded using Time Lapse software (Keyence).

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