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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.

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## 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 ($5 \times 10^5$/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 ($5 \times 10^5$/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 ($5 \times 10^5$/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) ($5 \times 10^5$/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 ($5 \times 10^5$/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 ($5 \times 10^5$/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 ($5 \times 10^5$/ml). As controls, THP-M1 or THP-M2 macrophages were incubated without PMA-NETs ($5 \times 10^5$/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 ($5 \times 10^5$/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 ($5 \times 10^5$/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).

### 2.8. Cell death induction in neutrophils

The other type of cell death than NETosis was induced in neutrophils ($5 \times 10^5$/ml) by treatment with 10 μg/ml camptothecin (CPT, Sigma–Aldrich) for 12 h as previously reported [14]. The induction rate of the cell death was evaluated by flow cytometry using the apoptosis detection kit (BD Biosciences). Briefly, the cells were re-suspended in the annexin V-binding buffer and then incubated for 15 min in the dark with FITC-conjugated annexin V and propidium iodide (PI). The cytometer used was Attune™ Acoustic Focusing Cytometer (Applied Biosystems, Tokyo, Japan).

### 2.9. Immunofluorescent staining for citrullinated histone 3

Cells fixed with 4% PFA were exposed to 1:100 diluted rabbit anti-citrullinated histone 3 antibodies (Abcam, Cambridge, MA, USA) for 60 min and were next allowed to react with 1:500 dilution of Alexa Fluor 594-conjugated goat anti-rabbit IgG antibodies (Invitrogen) for 60 min.

### 2.10. Peptidylarginine deiminase 4 (PAD4) knockdown using siRNA

In order to knockdown peptidylarginine deiminase 4 (PAD4) in THP-M1 macrophages, Accell SMART pool siRNAs (GE Healthcare) containing four siRNAs designed for the PAD4 gene (5′-CUGUGAAGCUCUAUUCUGA-3′, 5′-CGAGGAUGAUGAAGUGCUU-3′, 5′-GUGAUGGGUCCAGAUUUUG-3′, 5′-CCUUGACUCAGCUUGACAU-3′) were applied. Briefly, 48 h after plating, THP-M1 macrophages were transfected with 1 μM siRNA and subsequently cultured in Accell siRNA medium (GE Healthcare). The efficiency of the knockdown of the PAD4 protein at 48 h after the transfection was evaluated by immunofluorescent staining with the anti-PAD4 antibody (Abcam). Scrambled siRNAs for PAD4-targeted siRNA were used as a negative control.

### 2.11. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL)

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) was done using the DNA Fragmentation Assay kit (MBL, Nagoya, Japan) according to the manufacturer's instructions.

### 2.12. Caspase inhibition

In order to inhibit apoptosis-mediated caspases, cells were exposed to 100 μM of the pan caspase inhibitor, z-VAD (R&D Systems), for 30 min.

### 2.13. Statistical analysis

Mean ± standard deviation (SD) values were calculated.

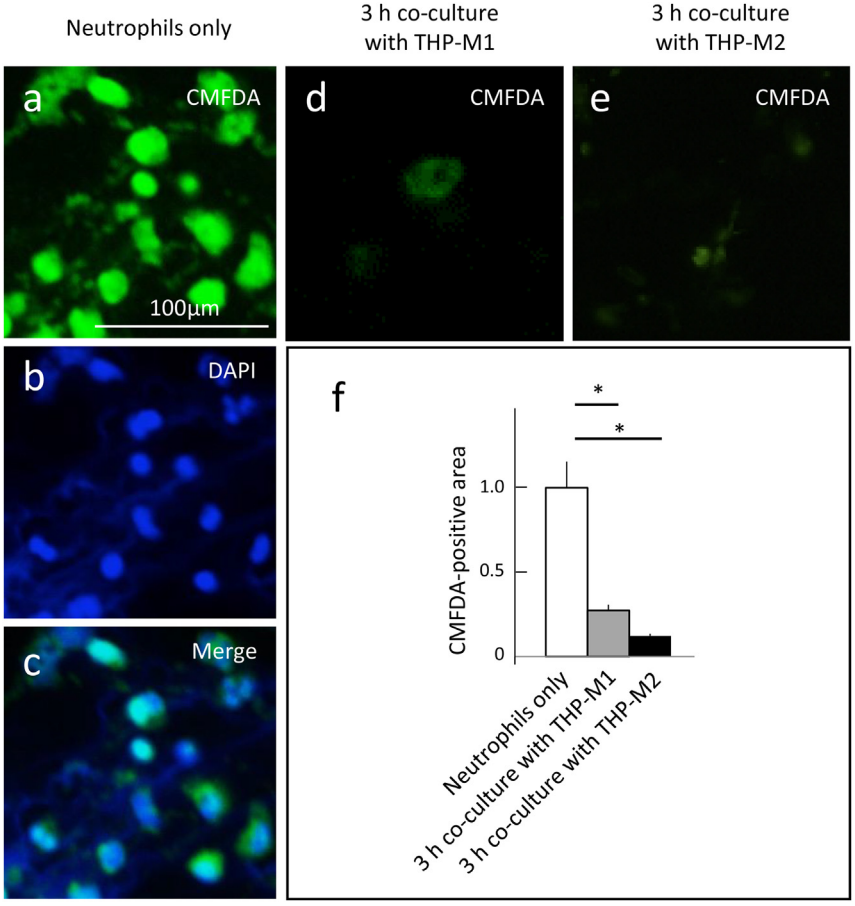



**Fig. 1.** Degradation of NETs by THP-M1/THP-M2 macrophages. **(a–e)** Peripheral blood neutrophils extracted from healthy donors were seeded in BD Falcon™ Culture Slides ($5 \times 10^5$/ml) and then given intracellular labeling with 5 μM CellTracker™ Green CMFDA for 30 min. Thereafter, the neutrophils were exposed to 50 nM PMA for 3 h for NET induction. After removal of culture supernatants containing non-adherent neutrophils without NET formation, THP-M1 or THP-M2 macrophages were added ($5 \times 10^5$/ml). After 3 h of incubation, samples were mounted with solution containing DAPI. Experiments were repeated with neutrophils from three different donors, and similar results were reproduced. Representative photographs for CMFDA (green) and DAPI (blue) are shown. **(f)** Altered NET amounts were substituted by the alteration of the CMFDA-positive areas in 4 random fields of view using Image J software. The mean value of the control (Neutrophils only) was set as 1. *$p < 0.05$, $n = 3$.

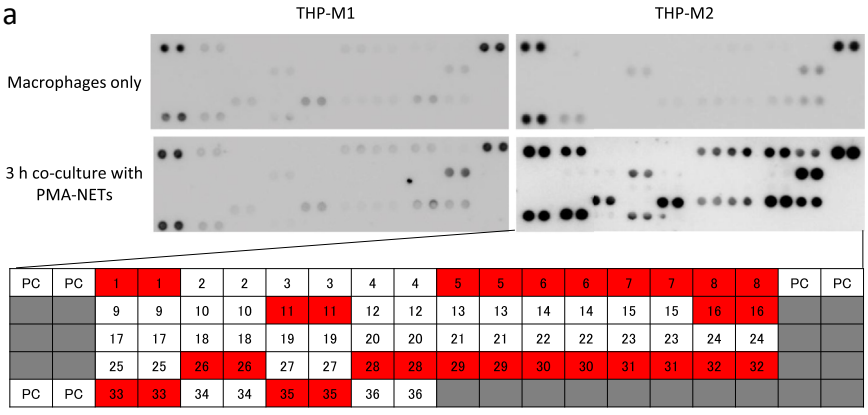
Unpaired Student *t*-tests were used for statistical evaluation with GraphPad Prism 5.0 software. P-values less than 0.05 were regarded as statistically significant.

## 3. Results

### *3.1. Early phase events: NET digestion by macrophages, secretion of pro-inflammatory cytokines/chemokines, and increase in extracellular DNA*

In order to determine the interaction of macrophages and neutrophils that undergo NETosis, co-culture experiments were conducted. First, peripheral blood neutrophils extracted from healthy donors were given intracellular labeling with green fluorescent CMFDA, and then PMA-NETs were induced. After the removal of culture supernatants containing non-adherent neutrophils without NET formation, M1 and M2 macrophages that differentiated from THP-1 cells, THP-M1 and THP-M2 macrophages, respectively, were added. In this study, assays were carried out at 3 h after the incubation to elucidate an early phase interaction of macrophages and NETs. As a result, the CMFDA-positive area was significantly decreased by incubation with both THP-M1 and THP-M2 macrophages (Fig. 1a–f). These findings indicated that NETs could be digested by macrophages regardless of phenotype.

Next, we focused on the secretion of inflammation-related molecules in the supernatants. A variety of pro-inflammatory cytokines/chemokines, including TNF-α, IFN-γ, IL-8, MIF, CCL1, CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES, CXCL1, CXCL10, and CXCL12, were detected in the supernatants of THP-M2 macrophages that interacted with PMA-NETs (Fig. 2a). Since culture supernatants of PMA-NETs were removed before the addition of macrophages and macrophages did not produce significant amounts of cytokines/chemokines when cultured alone, it is considered that THP-M2 macrophages produced the pro-inflammatory cytokines/chemokines after the interaction with NETs. On the contrary, the secretion of inflammation-related molecules was limited when THP-M1 macrophages were incubated with NETs. On the other hand, the amount of extracellular DNA was



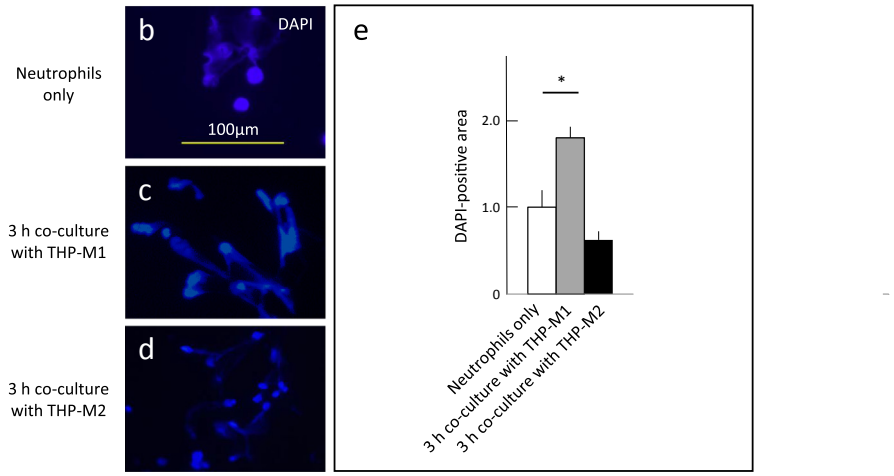




**Fig. 2.** Response of THP-M1/M2 macrophages that interacted with NETs. **(a)** Secreted protein profile in the supernatants of co-culture (THP-M1/M2 macrophages and PMA-NETs) after 3 h was determined by Proteome Profiler Array Human Cytokine Array Panel A. Supernatants of three independent experiments were mixed then subjected for the assay. Red highlights indicate the molecules detected in the supernatants of THP-M2 macrophages that interacted with PMA-NETs. **(b–e)** Increase in distributed DNA after co-culture of THP-M1 macrophages and PMA-NETs. **(b–d)** Experiments were repeated with neutrophils from three different donors, and similar results were reproduced. Representative photographs for DAPI (blue) of the same specimens in Fig. 1. **(e)** Altered DNA distribution was substituted by the alteration of the DAPI-positive areas in 4 random fields of view using Image J software. The mean value of the control (Neutrophils only) was set as 1. $^{*}p < 0.05$, $n = 3$.

noted to increase significantly at 3 h after the incubation of THP-M1 macrophage with PMA-NETs. This property was particular in THP-M1 compared to THP-M2 macrophages (Fig. 2b–e), though NETs were digested by both M1 and M2 macrophages. The collective findings indicated the phenotype-dependent response of macrophages in the interaction with NETs.

Similar phenomenon was observed when M1 and M2 macrophages derived from human peripheral blood monocytes (HMD-M1 and HMD-M2 macrophages) were employed instead of THP-M1 and THP-M2 macrophages, respectively (Fig. S1). Thus, these properties seemed to be general characteristics of M1 and M2 macrophages rather than specific for the THP-1 cell line. These issues could be the theoretical grounds that we used THP-1 cells as the source of human M1 and M2 macrophages in this study.

In the series of experiments, we should pay attention to the possibility that the residual PMA in the co-culture medium affected the responses of macrophages. In order to rule out the possibility, we conducted another set of co-culture experiments employing ANCA-NETs and macrophages. Since macrophages demonstrated similar responses when made to interact with PMA-NETs and ANCA-NETs (Fig. S2), the cells' responses when allowed to interact with neutrophils that undergo NETosis could be induced regardless of the kinds of NETs and the presence of residual PMA, if any, in the co-culture medium.

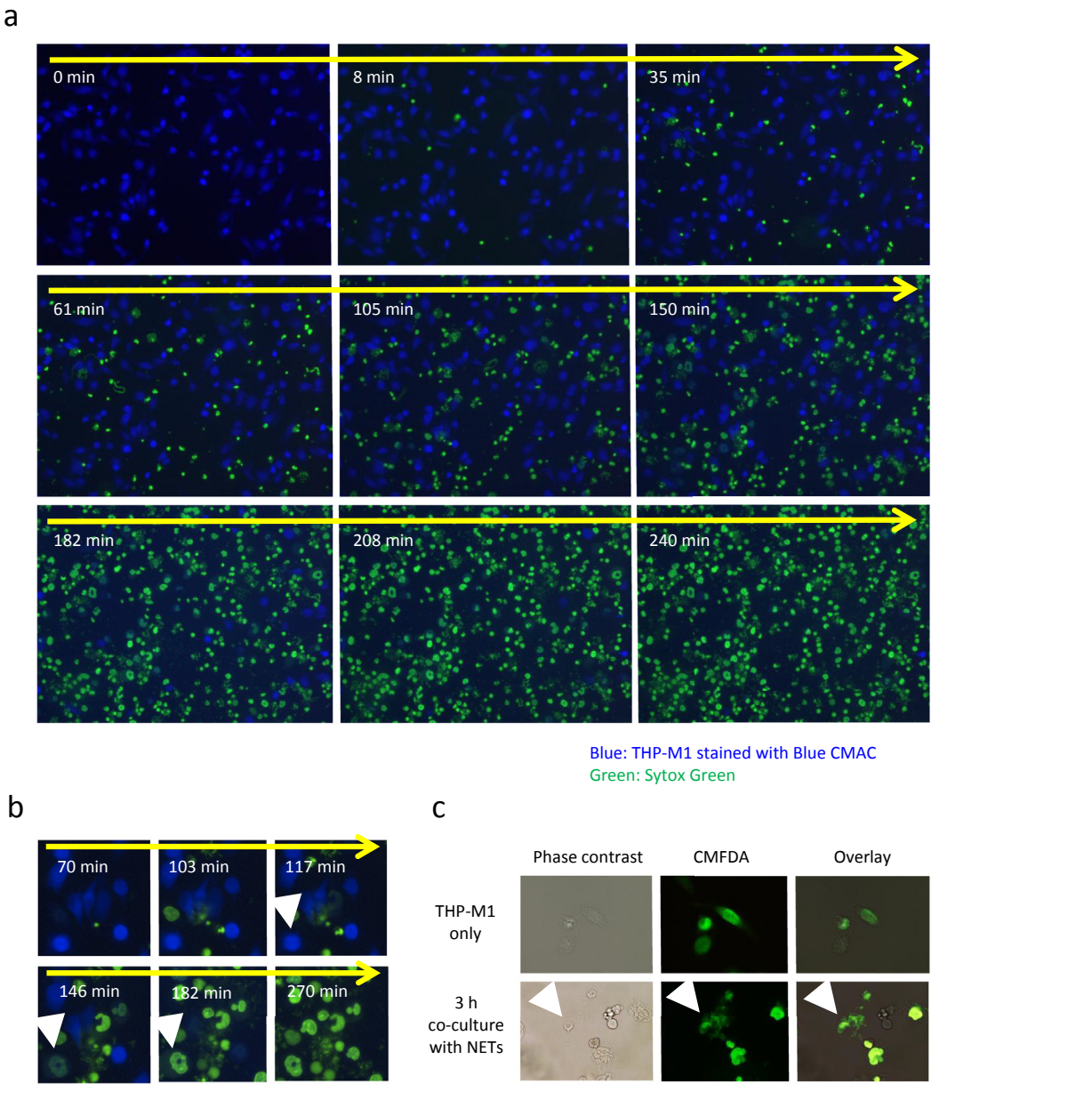



**Fig. 3.** Additional extracellular DNA derived from THP-M1 macrophages after interaction with PMA-NETs. **(a)** PMA-NETs were induced in neutrophils ($5 \times 10^5$/ml). Adherent neutrophils forming NETs were collected using a cell scraper and then centrifuged at 400 G for 5 min. After the supernatants were removed, the pellets were re-suspended in RPMI 1640 medium containing 1 μM Sytox Green and co-cultured with THP-M1 macrophages ($5 \times 10^5$/ml). Prior to the co-culture, macrophages were stained with 10 μM CellTracker™ Blue CMAC. The live cell image was recorded under the BioRevo BZ-9000 microscope for 240 min (original magnification: × 200). Representative scenes in the live cell image (Movie S1) are shown. **(b)** High power fields of view. Arrowheads indicate the chronological color change of the THP-M1 macrophage. **(c)** Phase contrast view at 3 h after the co-culture. Arrowheads indicate a rupture of plasma membrane of the THP-M1 macrophage.

### 3.2. Origin of extracellular DNA

Next, we determined where the additional extracellular DNA came from. THP-M1 macrophages were stained with blue fluorescent CMAC, a reagent that could stain the live cell cytoplasm, and then were incubated with a pellet of unstained PMA-NETs in the medium containing Sytox Green, which could stain extracellular DNA. After the incubation was set-up, cells were observed under the live cell image monitor for 240 min (Movie S1). Approximately at 150 min later, THP-M1 macrophages began to lose the intracellular blue staining, and then the color changed to green. Finally, at 240 min later, green spots that represented extracellular DNA covered the entire field (Fig. 3a). The magnified images showed that the cytoplasm of macrophages (Fig. 3b, arrowhead) was temporally stained in pale green and thereafter in dense green along the nucleus. Phase contrast images showed a rupture of the plasma membrane of the macrophages (Fig. 3c, arrowhead). In order to rule out the effects of residual PMA in the mixed culture, THP-M1 macrophages were exposed directly to 1 nM PMA. However, 3 h exposure to PMA did not induce the DNA release from THP-M1 macrophages (data not shown). The collective findings suggested that decondensated DNA was released from M1 macrophages after interaction with NETs, though the DNA was not drastically released like NETs.

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.jaut.2015.08.018.

### 3.3. Mechanism of increase in extracellular DNA

In order to determine if the M1 macrophage-derived DNA release could be dependent on the interaction with NETosis, we conducted the co-culture experiment of THP-M1 macrophages and neutrophils that undergo other types of cell death. Since PI-positive cells including annexin V-positive cells were increased, late-stage apoptosis seemed to be induced by the CPT treatment (Fig. 4a and b). Contrary to the incubation with NETosis, DNA release from THP-M1 macrophages was limited when THP-M1 macrophages were incubated with the CPT-treated neutrophils (Movie S2, Fig. 4c). Therefore, the M1 macrophage-derived DNA release occurred specifically when these cells interacted with NETs.

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.jaut.2015.08.018.

The DNA release of M1 macrophages after interaction with NETs morphologically showed the decondensation of chromatin fibers (Fig. 3b). Since the decondensation of chromatin fibers was associated with the citrullination of histones induced by PAD4 during NET formation [15], we examined the contribution of PAD4 to the DNA release of M1 macrophages after interaction with NETs. Firstly, citrullinated histone 3 was detected in the additional extracellular DNA derived from THP-M1 macrophages after interaction with PMA-NETs (Fig. 5a). Although THP-1 cells did not express PAD4, the expression of PAD4 accompanied the M1 phenotype induction (our unpublished data). Secondly, when THP-M1 macrophages knocked down for PAD4 by siRNA (Fig. 5b) were incubated with PMA-NETs, the DNA release from the macrophages was markedly inhibited (Movie S3, Fig. 5c). On the contrary, DNA was released from the control siRNA-transfected THP-M1 macrophages after interaction with PMA-NETs (Fig. S3), as well as when non-treated THP-M1 macrophages were co-cultured with PMA-NETs. These findings suggested that the DNA release from M1 macrophages after interaction with NETs occurred in a PAD4-dependent manner.

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.jaut.2015.08.018.

### 3.4. Late phase event: clearance of extracellular DNA

The amount of extracellular DNA derived from M1 macrophages reached a peak at 3–4 h and began to diminish thereafter. Majority

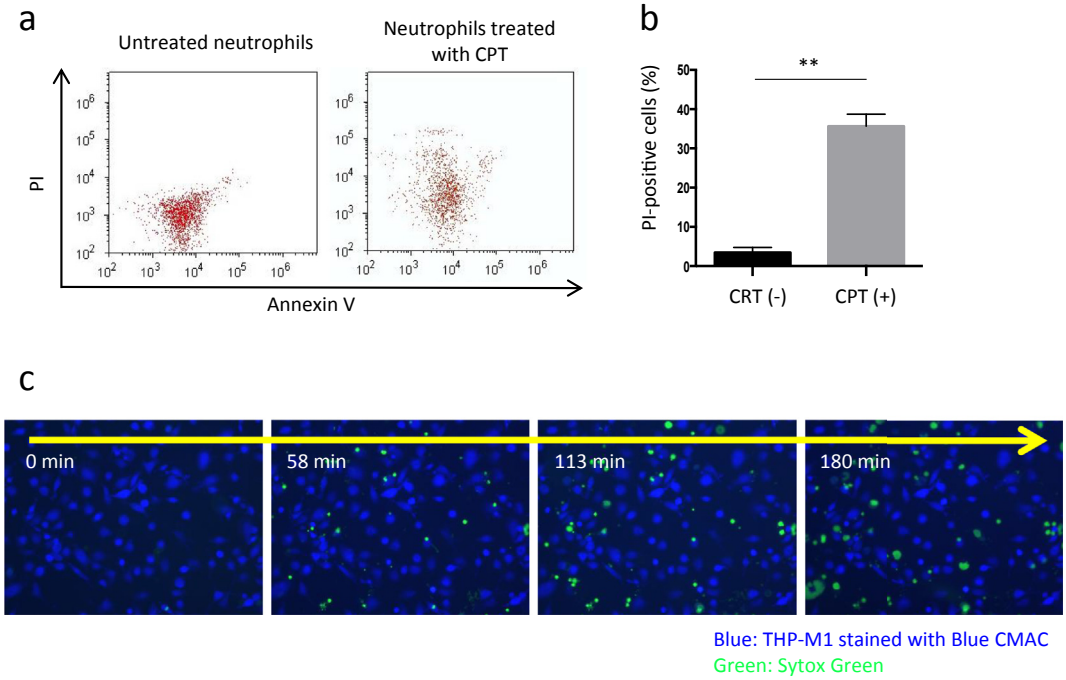



**Fig. 4.** No induction of extracellular DNA release from THP-M1 macrophages that interacted with CPT-treated neutrophils. **(a)** Cell death was induced in neutrophils ($5 \times 10^5$/ml) by treatment with 10 μg/ml CPT. The induction of cell death was evaluated by flow cytometry using the apoptosis detection kit. Experiments were repeated with neutrophils from three different donors, and similar results were reproduced. Representative histograms are shown. **(b)** The percentage of PI-positive cells was compared between the neutrophils that were treated with or without CPT. $^{**}p < 0.01$, $n = 3$. **(c)** Representative scenes in the live cell image of co-culture of THP-M1 macrophages and the CPT-treated neutrophils (Movie S2) are shown.

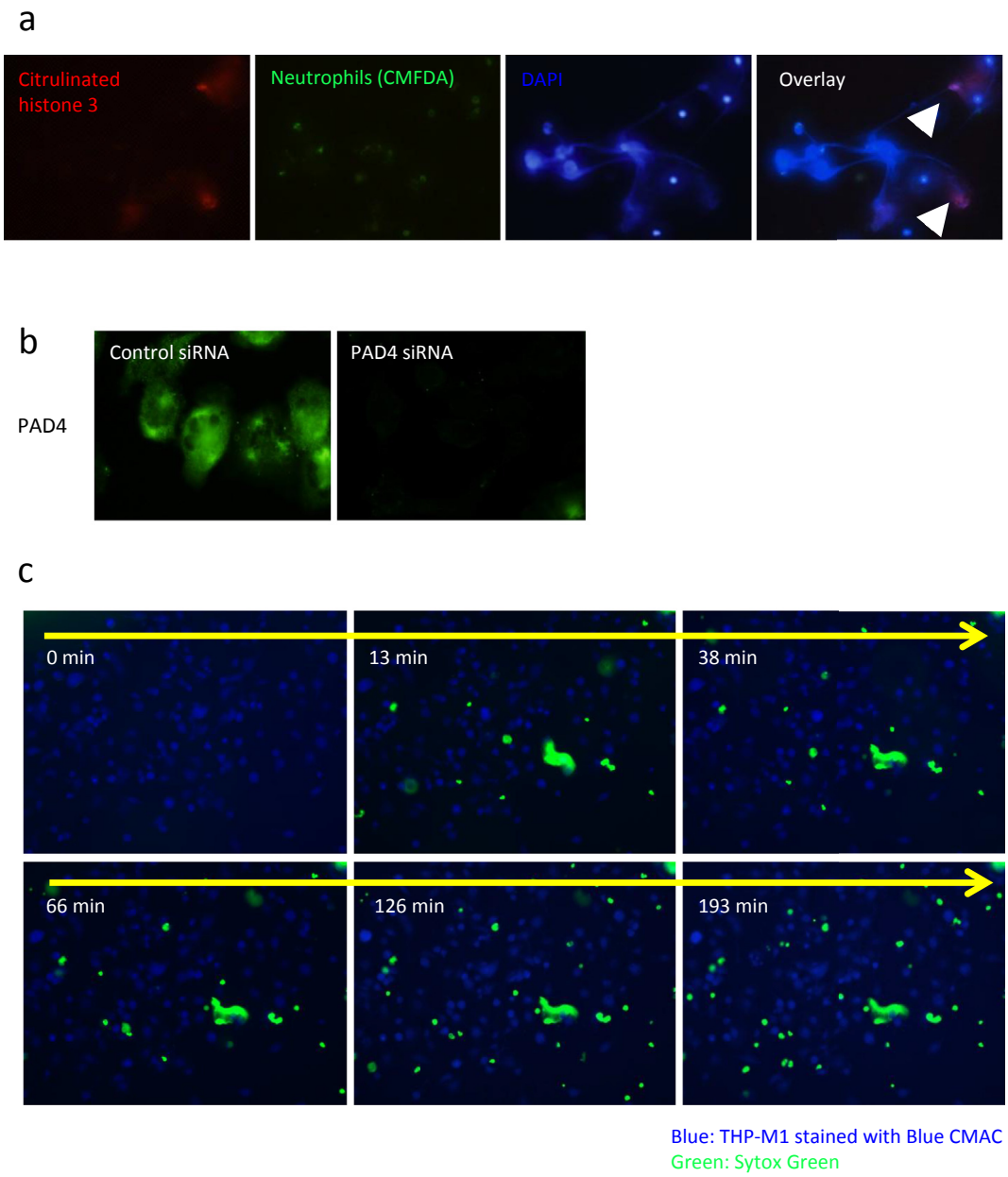



**Fig. 5.** PAD4-dependency of extracellular DNA release from THP-M1 macrophages that interacted with NETs. **(a)** Peripheral blood neutrophils extracted from healthy donors were seeded in BD Falcon™ Culture Slides ($5 \times 10^5$/ml) and then given intracellular labeling with 5 μM CellTracker™ Green CMFDA for 30 min. Thereafter, the neutrophils were exposed to 50 nM PMA for 3 h for NET induction. After removal of culture supernatants containing non-adherent neutrophils without NET formation, THP-M1 macrophages were added ($5 \times 10^5$/ml). After 3 h of incubation, immunofluorescent staining for citrullinated histone 3 was done followed by mounting with DAPI. Experiments were repeated with neutrophils from three different donors, and similar results were reproduced. Representative photographs for citrullinated histone 3 (red), CMFDA (green), and DAPI (blue) are shown. Arrowheads indicate citrullinated histone 3-positive cells other than CMFDA-positive neutrophils, namely macrophages. **(b)** Efficiency of PAD4 knockdown in THP-M1 macrophages. Forty-eight hours after the transfection of PAD4 siRNA or control siRNA, immunofluorescent staining for PAD4 was done. **(c)** Representative scenes of the live cell image of co-culture of the PAD4-knockdown THP-M1 macrophages and PMA-NETs (Movie S3) are shown.

of extracellular DNA disappeared within 24 h (Fig. 6a and b). In order to determine the type of cell death of M1 macrophages after interaction with NETs, we performed TUNEL staining. The positive TUNEL staining for THP-M1 macrophages after interaction with PMA-NETs suggested the apoptotic feature of the cell death (Fig. 7a). Since caspase-activated DNase (CAD) could function during apoptosis, we hypothesized that CAD would be involved in the clearance of extracellular DNA. When the pan-caspase inhibitor, z-VAD, was added to the co-culture of THP-M1 macrophages and PMA-NETs, the diminution of extracellular DNA was significantly inhibited (Fig. 7b and c). These findings suggested that the activation of CAD in M1 macrophages could be involved at least in part of the clearance mechanism.

## 4. Discussion

Ten years have passed since the discovery of NETs in 2004 [1]. During this decade, many studies have revealed the biological roles of NETs. Although NETs are essential for the innate immunity, especially for the elimination of microbes, the extracellular release

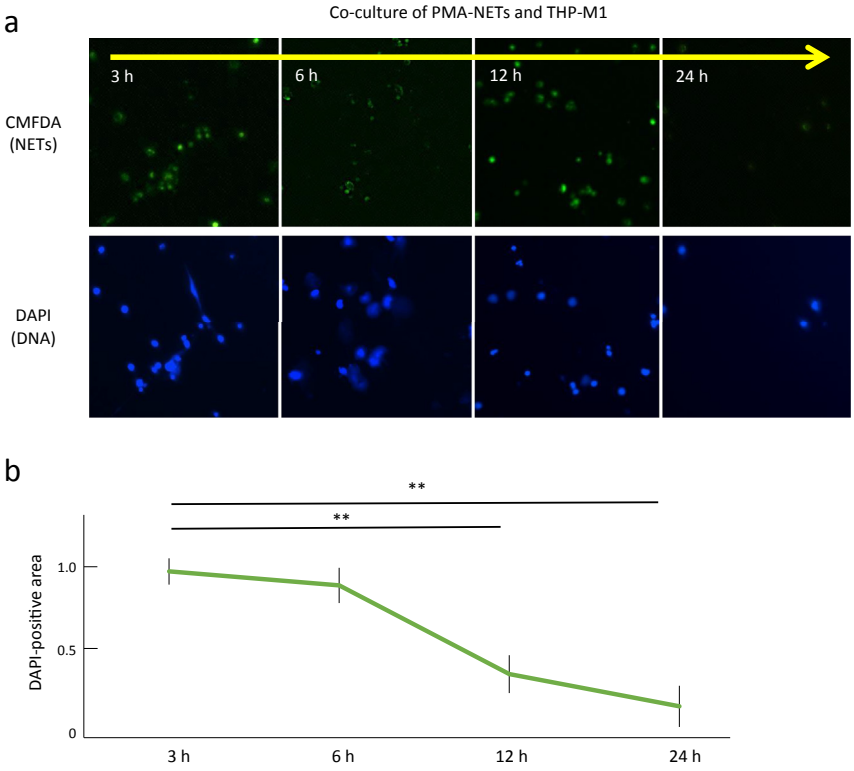



**Fig. 6.** Clearance of extracellular DNA derived from THP-M1 macrophages. **(a)** The co-culture of THP-M1 macrophages and PMA-NETs was followed up at 3, 6, 12, and 24 h later. Experiments were repeated with neutrophils from three different donors, and similar results were reproduced. Representative photographs for CMFDA (green) and DAPI (blue) are shown. **(b)** Chronological decrease in DAPI-positive area determined by Image J software. $^{**}p < 0.01$, $n = 3$.

of DNA and intra-cytoplasmic/nuclear proteins can, on the other hand, result in some adversities to the hosts. Therefore, NETosis is adequately regulated *in vivo*. Recently, it has been shown that macrophages via phagocytosis could remove neutrophils that underwent NETosis, as well as other cells that underwent apoptosis or necrosis [6]. Although Farrera et al. demonstrated a silent removal of PMA-NETs by macrophages, the residual PMA in the co-culture could affect the response of macrophages. Therefore, it remains elusive what response would exactly occur in macrophages after interaction with NETosis.

According to the evidence that patients with chronic granulomatous disease (genetic deficiency of nicotinamide adenine dinucleotide phosphate oxidase, an enzyme which is essential for NET generation) suffer from severe and sometimes life-threatening infections [16], NETs are surely indispensable for microbial elimination from the hosts. Concerning this issue, we suggest two diverse mechanisms that reinforce the role of NETs with the contribution of macrophages. The first mechanism is an induction of inflammation. Several pro-inflammatory cytokines/chemokines are induced when macrophages (particularly M2 phenotype) interact with NETs. A local inflammation induced by pro-inflammatory cytokines/chemokines is regarded as one of the host defense mechanism against microorganisms. The second mechanism is an increase in extracellular DNA. M1 macrophages can increase the amount of extracellular DNA after interaction with NETosis, which is derived from themselves. Since extracellular DNA is the critical antimicrobial component of NETs [17], the additional extracellular DNA can augment the potential of NET-related immunity.

On the other hand, excessive or persistent NETs have been shown to be involved in the pathogenesis of diverse disorders, including autoimmune diseases [4,5] and impaired wound healing [18]. Although serum DNase I is shown as an important regulator of NETs [4], it is unclear whether this enzyme can function in the tissues, as well as in the blood. Concerning this issue, our study demonstrates an interesting clearance mechanism of extracellular DNA mediated by M1 macrophages, which can work in the tissues. Initially, M1 macrophages that interact with NETs induce an increase in extracellular DNA in the surrounding tissues at first and thereafter digest the extracellular DNA by their own CAD. This transient increase and subsequent clearance mechanism of extracellular DNA seems very reasonable in terms of the double-edged sword-like property of NETs. Interestingly, this action of M1 macrophages can be initiated specifically by NETosis.

The evidence presented in this study suggests a novel phenotype- and time-dependent regulatory mechanism of NETosis by macrophages (see Graphical abstract). In this regard, it is important to note that both M1 and M2 macrophages are present in the milieu under physiological condition. According to the following evidence, M1 and M2 macrophages which interact with neutrophils that undergo NETosis can be considered to cooperate via both non cell–cell and cell to cell interactions under physiological condition. Firstly, macrophage chemotactic factors, such as MIF, CCL2/MCP-1, CCL3/MIP-1$\alpha$, and CCL4/MIP-1$\beta$, were produced from THP-M2 cells that interacted with netting neutrophils. Since these soluble factors have been shown to recruit circulating monocytes or to trap macrophages within tissues [19], M1 macrophages could infiltrate into the site where M2 macrophages interacted with neutrophils that undergo NETosis under physiological condition. Secondly, the increase in cell death induced by THP-M1 cells that interacted with netting neutrophils is recognized by M2 macrophages. In this interaction, damage-associated molecular patterns, such as adenosine triphosphate, released from dying cells can activate M2

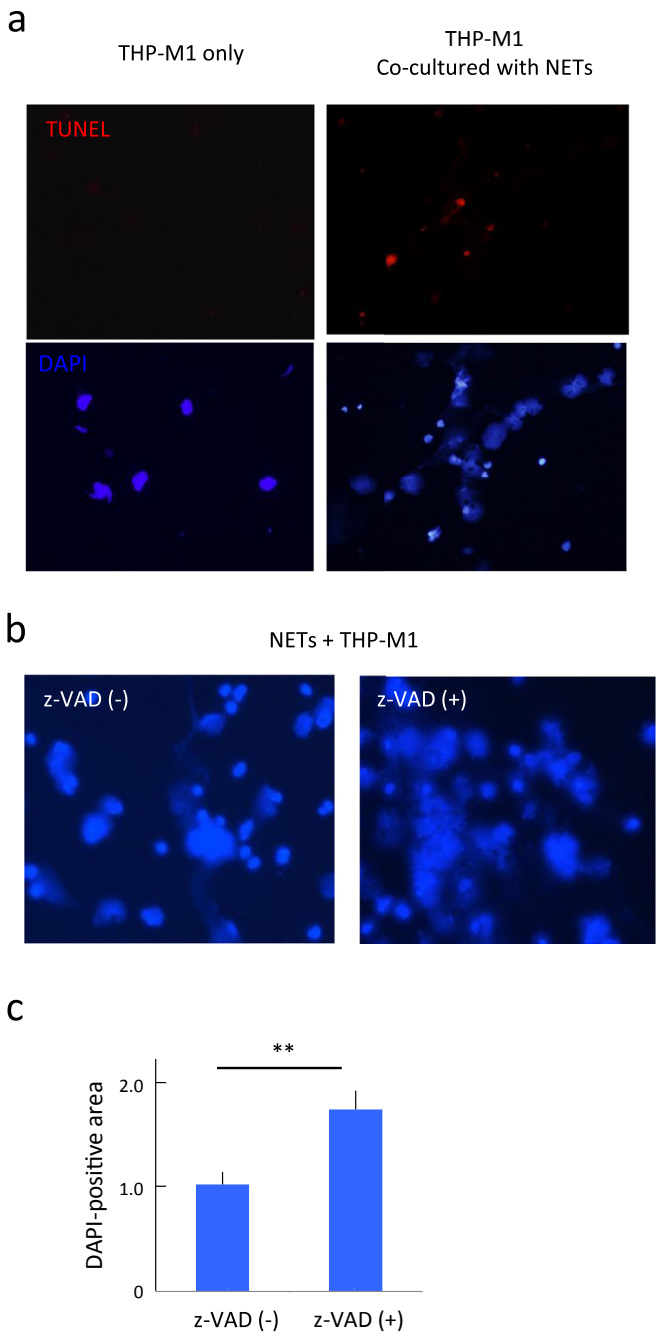



**Fig. 7.** Apoptosis-mediated CAD-dependent clearance of extracellular DNA. **(a)** Three hours after co-culture of THP-M1 macrophages and PMA-NETs, cells were subjected for TUNEL followed by mounting with DAPI. Experiments were repeated with neutrophils from three different donors, and similar results were reproduced. Representative photographs for TUNEL (red) and DAPI (blue) are shown. **(b)** Inhibitory effect of z-VAD on the clearance of extracellular DNA. Experiments were repeated with neutrophils from three different donors, and similar results were reproduced. Representative photographs for DAPI (blue) at 3 h after the co-culture of THP-M1 macrophages and PMA-NETs **(c)** Comparison of residual DAPI-positive area at 3 h after the co-culture of THP-M1 macrophages and PMA-NETs in the presence or absence of z-VAD. $^{**}p < 0.01$, $n = 3$.

macrophages [20–22]. Thereafter, the dead cells that express eat-me signals, such as phosphatidylserin, on the cell surface are trapped by the activated M2 macrophages [23]. Therefore, these responses can be mediated by both non cell–cell interaction and cell to cell contact under physiological condition.

One of the pathogeneses of autoimmune diseases has been considered as a disorder of macrophages that remove apoptotic cells [24–26]. Actually, the mice deficient in milk fat globule-EGF-factor 8, the anchor molecule for apoptotic cells secreted from macrophages, cannot eliminate apoptotic cells by phagocytosis resulting in the development of autoimmune disorders, such as glomerulonephritis [27]. Moreover, deficiency of CAD in macrophages resulted in increased anti-DNA antibody titers in lupus-prone mice [28]. Since macrophages contribute to the removal of not only apoptotic cells but also neutrophils that undergo NETosis, a certain functional disorder of macrophages can likely cause the NET-associated pathogenesis. Further studies are vital to reveal the involvement of dysfunction of macrophages in the pathogenesis of NET-related diseases, including SLE and AVV.

Recent studies have advocated diverse strategies for therapeutic NET regulation. At first, the inhibition of PAD4 was challenged to prevent the uncontrolled NET formation [29]. The critique for this strategy is the possible inhibition of essential NET formation simultaneously. In this regard, however, Martinrod et al. demonstrated that the prevention of NET formation by inhibiting PAD4 did not lead to increased susceptibility to bacterial infections; hence, a therapeutic potential is suggestive [30]. More recently, the role of phosphatase and tensin homolog (PTEN) in NET formation has been revealed [31]. Therefore, PTEN can be suggested as an alternative therapeutic target in NET-related diseases. Although further studies are needed, the present data also suggest the possibility of amendment of macrophage function as another novel therapeutic strategy for NET-related diseases.

## Disclosure statement

The authors declare that they have no conflict of interests.

## Author contributions

D.N. carried out most of the experiments. H.S., Y.K., and A.M. contributed to the preparation of NETs. S.N., U.T., and T.A. analyzed the data. D.N., U.T., and A.I. designed the research and wrote the manuscript.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jaut.2015.08.018.

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