Macrophage Clearance of Neutrophil Extracellular Traps Is a Silent Process

Consol Farrera and Bengt Fadeel

J Immunol published online 31 July 2013
http://www.jimmunol.org/content/early/2013/07/30/jimmunol.1300436

Supplementary Material
http://www.jimmunol.org/content/suppl/2013/07/31/jimmunol.1300436v1.DC1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Macrophage Clearance of Neutrophil Extracellular Traps Is a Silent Process

Consol Farrera and Bengt Fadeel

Neutrophil extracellular traps (NETs) facilitate the extracellular killing of pathogens. However, in recent years, excessive NET formation has been implicated in several pathological conditions. Indeed, NETs that are not removed from tissues or from the circulation might serve to trigger autoimmune responses. We observed that physiological amounts of DNase I do not suffice to completely degrade NETs in vitro, suggesting that additional mechanisms are required for the removal of these extracellular structures. We show in this article that human monocyte-derived macrophages are able to engulf NETs in a cytochalasin D-dependent manner, indicating that this is an active, endocytic process. Furthermore, preprocessing of NETs by DNase I facilitated their clearance by macrophages. In addition, both recombinant C1q and endogenous C1q derived from human serum were found to opsonize NETs, and this facilitated NET clearance. Upon internalization, NETs were apparently degraded in lysosomes, as treatment with chloroquine led to accumulation of extranuclear DNA in human monocyte-derived macrophages. Finally, uptake of NETs alone did not induce proinflammatory cytokine secretion, whereas LPS-induced production of IL-1β, IL-6, and TNF-α was promoted by the uptake of NETs. In summary, we show that macrophages are capable of clearance of NETs and that this occurs in an immunologically silent manner. The Journal of Immunology, 2013, 191: 000–000.

Neutrophils act as sentinels of the innate immune system, ready to be rapidly recruited to the site of infection. These cells are equipped with various strategies to combat a broad range of pathogens. Phagocytosis of pathogens and intracellular destruction has traditionally been thought of as the main weapon of neutrophils, but a novel strategy was described a few years ago, that is, the formation of so-called neutrophil extracellular traps (NETs) (1, 2). NETs are formed on exposure to pathogens including bacteria, fungi, and parasites, and consist of a backbone of nuclear chromatin decorated with granule proteins such as neutrophil elastase (NE) and myeloperoxidase (MPO). Recent studies show that neutrophils stay alive and retain the ability to combat bacteria after expulsion of their DNA (3). NETs are believed to be an effective defense system against infection, as the entrapment of pathogens in NETs may aid in the local confinement of the infection, whereas the granule-derived enzymes facilitate in the extracellular killing of the “netted” pathogens (4).

Several recent reports have implicated the excessive formation of NETs in sepsis, atherosclerosis, or autoimmune diseases such as systemic lupus erythematosus (SLE) or psoriasis (5), suggesting that NET formation can be related to exacerbated immune responses and tissue damage. In fact, Abs against dsDNA or histones are a hallmark of some autoimmune diseases. Defective macrophage clearance of apoptotic cell debris has been suggested to be a source of immunogens (6), but excessive production of NETs and/or inefficient dismantling of these structures may potentially serve as yet another source of immunogens (7). In the case of apoptotic cells, several key steps in the clearance process have been identified, many of which appear to be conserved through evolution, starting with the exposition of “eat-me” signals on the surface of apoptotic cells leading to the specific recognition by the phagocyte, followed by engulfment of the apoptotic prey and its processing in the lysosomal compartment of the engulfing cell, and finally, the modulation of proinflammatory and anti-inflammatory cytokine secretion by macrophages to preclude or resolve inflammation (8). In contrast, NET removal is thought to be accomplished mainly by extracellular DNase I degradation (7). DNase I has been shown to be of vital importance for the clearance of necrotic cell-derived chromatin; deficiencies in this enzyme have been linked to SLE (9). Recent studies have shown that NETs derived from SLE patients contain antibacterial peptides and that SLE NETs activate dendritic cells to produce high levels of IFN-α (10). SLE patients were also found to develop autoantibodies to both the DNA and antimicrobial peptide component of NETs (11). This would support the notion that an active clearance of NETs might be required in addition to the extracellular degradation of these structures to prevent inadvertent immunological responses.

The complement system is yet another effector mechanism of the innate immune system. Circulating complement components can sense “danger” signals, opsonize pathogens or dying cells, and induce the recruitment and activation of the immune system (12). Depending on the sensing mechanism, the complement system can trigger phagocytosis without inflammation, as in the case of apoptotic cell clearance, or its activation may lead to an inflammatory response. C1q is a component of the complement system that is involved in the recognition and clearance of apoptotic cells and cell debris, and defects in C1q have been linked to the development of SLE (13, 14). C1q is able to bind DNA and histones, both of which are present in NETs (1). Hakkim et al. (7) reported that DNase I is required for disassembly of NETs, and that a subset of SLE patients degraded NETs poorly. More recent studies suggested that NETs are potent complement activators and that
Materials and Methods

Isolation and culture of primary human neutrophils and macrophages

Peripheral blood neutrophils were isolated from buffy coats from healthy adult blood donors (Karolinska University Hospital, Stockholm, Sweden) by density gradient centrifugation using Lymphoprep (Nycomed Pharma, Oslo, Norway) as described previously (16). Neutrophils were further separated from erythrocytes by gradient sedimentation in a 5% dextran solution, and residual erythrocytes were removed by hypotonic lysis. Purified neutrophils were cultured in phenol red free RPMI 1640 medium (Sigma), supplemented with 2 mM l-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. Human monocyte-derived macrophages (HMDMs) were prepared as previously described (17). After density gradient centrifugation, the white blood fraction was separated and further incubated with CD14+ MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany). After magnetic bead separation, purified CD14+ monocytes were plated in 24-well plates at a density of 10⁶ cells/ml and differentiated into macrophages by culture in RPMI 1640 (Sigma) medium in the presence of 50 ng/ml recombinant human M-CSF (R&D Systems, Minneapolis, MN) and supplemented with 10% heat-inactivated FBS, 2 mM l-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin for 3 d.

NET induction and isolation

To induce the production of NETs, we incubated freshly isolated neutrophils from healthy blood donors with 25 nM PMA (Sigma) for 2 h at 37°C in RPMI 1640 medium. Cell culture supernatants were removed and fresh media was added to isolate NETs. Then cells and NETs were removed through extensive pipetting. The mixture was centrifuged at 1500 rpm for 5 min to separate neutrophils and NETs containing supernatants were retrieved. For quantification of NET DNA, Hoechst 33342 solution at a concentration of 5 µg/ml (Sigma) was added to the supernatant and fluorescent intensity (excitation wavelength 360 nm, emission wavelength 465 nm) was measured using a Tecan plate reader (Tecan Group, Männedorf, Switzerland).

Immunofluorescence

Neutrophils were seeded in 24-well plates on poly-L-lysine–coated cover slips. After 1 h of attachment, 25 nM PMA (Sigma) was added and neutrophils were cultured for 2 h at 37°C and allowed to produce NETs. After incubation, slides were fixed with 4% paraformaldehyde solution for 30 min at room temperature (RT). Blocking was performed with a 2% BSA PBS solution for 30 min at RT. Staining with primary Abs or the corresponding negative control Abs was performed for 1 h at RT at 1:300 in 2% BSA PBS solution. Secondary staining with FITC-labeled anti-rabbit Abs (Sigma) or goat anti-mouse Alexa 594–labeled Abs (Invitrogen) was performed for 1 h at RT at 1:500 in 2% BSA-PBS solution. After incubation with NETs, DNase I treatment (5 µg/ml) was added. For immunostaining, HMDMs were seeded in 24-well plates, allowed to attach for at least 3 h, and were washed with phenol red free RPMI 1640 media. Purified NETs were then added to the macrophages and incubated for the indicated times. Incubation was performed in the presence of 5 µg/ml DNase I (Sigma), 10 µg/ml cytochalasin D (Sigma), 100 µg/ml C1q protein (Abcam, Cambridge, U.K.), or a mixture of endocytosis inhibitors containing nystatin (25 µg/ml), genistein (200 µM), and brefeldin A (10 µg/ml). After incubation, supernatants containing nonengulfed NETs were recovered through repetitive pipetting and added to a new plate. The slide was then centrifuged for 6 min at 1600 rpm and supernatant was recovered. Micrococcal Nuclease ( Worthington Biochemical, Lakewood, NJ) was added to NETs at a final concentration of 500 mU/ml followed by incubation for 10 min at 37°C. After NE release, N-(methoxyoxycinnaryl)-Ala-Ala-Pro-Val 4-nitroanilide (Sigma) was added at a concentration of 200 µM in assay buffer (0.1 M HEPEs, 0.5 M NaCl, 0.1% Na₂SO₄, 1% BSA, pH 7.5). After incubation for 3 h at RT, absorbance was measured at 405 nm in a Tecan plate reader.

Western blot

After incubation with NETs, DNase I treatment (5 µg/ml) for 10 min was performed. Samples were washed twice in PBS; then cells were lysed with RIPA buffer and protein was quantified by BCA assay. Equal amounts of proteins were loaded, separated by SDS-PAGE and transferred to a nitrocellulose membrane, and the membrane was incubated with NE Ab (Santa Cruz Biotechnology) or with β-actin Ab (Sigma). Then, secondary Abs conjugated with HRP (Dako) followed, and bands were detected with ECL (GE Healthcare Europe) on x-ray film (Fujifilm Europe, Düsseldorf, Germany).

Confocal microscopy

Confocal microscopy was used for the visualization and localization of NETs within macrophages. To this end, HMDMs were seeded in 24-well plates containing cover slips and allowed to attach for at least 3 h, and were then incubated in the presence of 10 µM chloroquine (CQ; Sigma) for 30 min to block lysosomal activity. Thereafter, medium was replaced and purified NETs were incubated with HMDMs for 2 h in the presence of 10 µM CQ. Thirty minutes before fixation, 1 µM Lysotracker (Invitrogen) was added, and 10 min before fixation, 5 µg/ml DNase I was added. For detection of Eea-1 and Lamp-1, Abs from Santa Cruz Technology were used and staining was performed as described earlier (see Immunofluorescence section). HMDMs were fixed with 4% formaldehyde, and slides were mounted with DAPI-containing mounting medium and visualized with a ZEISS LSM510META confocal microscope (Carl Zeiss, Oberkothen, Germany).

DNase II gene silencing

SILENCEING OF DNASE II WAS PERFORMED USING THE AMAXA NUCLEOFECTOR (Lonza, Basel, Switzerland) according to the manufacturer’s instructions. In brief, HMDMs were transfected 2 d after their purification with 3 µM of either a Negative Universal Control Stealth RNAi (Invitrogen, San Diego, CA) or DNase II-specific silence RNA (5'-UGGUCCAGAGUAAUCUAGT-3'); Eurofins MWG Operon, Ebersberg, Germany). After transfection, macrophages were cultured for 2 more days in complete cell culture medium containing recombinant human M-CSF. Efficiency of silencing of DNase II was monitored by RT-PCR as described later.
Cytokine production

To analyze the production of cytokines, we used the Luminex system (Bio-Rad) according to the manufacturer’s instructions. In brief, HMDMs were seeded in 96-well plates, allowed to attach for at least 3 h, and washed with phenol red free RPMI 1640 medium, and then purified NETs were added. In some cases, stimulation was performed with 100 ng/ml LPS (Sigma). After 1 h, supernatant was removed and kept at −80°C. Then fresh medium was provided and supernatants were collected again after 24 h and saved at −80°C. Experiments were performed with macrophages from three different blood donors. Detection limits for the cytokines were 1.5 ng/ml for IFN-α, 2.05 pg/ml for IL-1β, 1.66 pg/ml for IL-6, 5.44 pg/ml for TNF-α, and 1.51 pg/ml for IL-10. IFN-α, IFN-β, and TNF-α were also detected by ELISA with detection limits of 7 pg/ml, 2.5 IU/ml, and 13 pg/ml respectively. ELISA kits were from Mabtech (IFN-α and TNF-α) (Stockholm, Sweden) and Invitrogen (IFN-β).

NET transfection

Phenol-chloroform extraction was performed to obtain DNA from purified NETs. Phenol-chloroform solution (Invitrogen) was thus added 1:1 to purified NETs. After centrifugation, DNA was precipitated with 100% ethanol, further washed with 75% ethanol, and resuspended in RNase and DNase free water. Quantitation of DNA purified was performed using the NanoDrop (Thermo Scientific, Waltham, MA), and cDNA was obtained by retrotranscription of 1 µg RNA using the Revert Aid-H Minus First Strand cDNA Synthesis Kit (Thermo Scientific). For determination of the amount of DNase II after transfection with control or DNase II–specific small interfering RNA (siRNA), RT-PCR was performed using the Power SYBR Green Reagent Kit (Applied Biosystems, Foster City, CA) was performed. Data are reported as relative mRNA levels normalized to the expression values of the gapdh housekeeping gene. Primers used were as follows: dnaseII forward, 5'-TTTCGCTCTTACAATGACCAAC-3', dnaseII reverse, 5'-GGAAGTTAGGTACACTGTGGACC-3'; gapdh forward, 5'-GGGTCTTTCACTGACGACC-3'; gapdh reverse, 5'-ACCTCCTGTTCGGGGACT-3'. To monitor IFN responses, we used the following primers: IFN-α forward, 5'-GCCTCGCTTTCTTCACT-3'; IFN-α reverse, 5'-CTGTTGGTTCTCAGGAGATCA-3'; IFN-β forward, 5'-ATGACCAAACATGTTCTCCTCC-3'; IFN β reverse, 5'-GGAATCCAAGCAAGTGTTAGCTC-3'. Data are normalized to the expression values of the gapdh gene.

Statistics

Statistical significance was tested with unpaired two-tailed Student t test or one-way ANOVA with Tukey correction using GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego, CA). The level of significance for rejecting the null hypothesis of zero treatment effect was p = 0.05. Data are reported as mean ± SD.

Results

NET production by primary human neutrophils

Neutrophils can die through several mechanisms. First, in the absence of inflammatory stimuli, they have a tendency to undergo apoptosis within 24–48 h (16). Moreover, neutrophils have been described to die during the production of NETs, a process referred to as NETosis (19). Special care should thus be taken so that the study of NETs is not interfered with by DNA from other origins.
unrelated to the release of NETs. We therefore tested a range of concentrations of the NET-inducing stimulus, PMA, as well as a range of time points, and found that incubation with 25 nM PMA for 2 h resulted in the induction of NETs without apoptosis or necrosis of neutrophils. The production of NETs was determined by immunofluorescence staining of the NET components, NE and MPO (Fig. 1A, 1B), and scanning electron microscopy further confirmed the formation of NETs (Fig. 1C). Purified NETs were obtained and quantified using Hoechst 33342 labeling, and NE activity was measured and shown to correlate with the quantification by Hoechst 33342 (Supplemental Fig. 1).

**Partial NET processing by DNase I and human serum**

DNase I is mainly produced in the pancreas and kidneys, and hydrolyzes dsDNA under physiological conditions. Several reports have shown that this enzyme can dismantle NETs, and certain bacteria express DNases to escape from NETs (7, 20–22). Therefore, we tested the ability of this enzyme to degrade NETs. As expected, incubation of NETs with DNase I at a high concentration (5 μg/ml) rendered fully degraded NETs (Fig. 2A). Nonetheless, incubation of NETs with a physiological concentration of DNase I (20 ng/ml) (23) did not lead to degradation of NETs (Fig. 2A), thus demonstrating that under physiological circumstances, the activity of this enzyme might not be sufficient for the complete degradation of NETs. To further test this hypothesis, we incubated NETs for 1 h with 10% serum from normal healthy donors and tested its ability to degrade NETs (Fig. 2B). In addition, we also increased the amount of serum to 20% and the incubation time up to 12 h (Supplemental Fig. 2). Serum from healthy donors (10 or 20%) triggered only partial degradation of NETs after incubation up to 12 h, implying that other mechanisms might be required for the effective dismantling and clearance of NETs.

In addition to DNase I, serum may contain inhibitors of this endonuclease. Moreover, proteins such as LL-37 and HMGB1 have been shown to bind to NETs and protect them from degradation (10, 11). However, we could not find any HMGB1 bound to NETs produced under the conditions used in this study (Supplemental Fig. 3), indicating that the presence of this protein in NETs might occur only under certain conditions, for example, during NETosis. We also tested the capacity of serum to prevent NET degradation and found that incubation of NETs with 10% serum did not protect from DNase I when applied at a concentration of 5 μg/ml (Fig. 2C).

**NET clearance by monocyte-derived macrophages**

During inflammation, macrophages are recruited shortly after the initial recruitment of neutrophils to assist in the clearance of pathogens and cellular debris. We hypothesized that macrophages might aid in the clearance of NETs in such a scenario. To test this hypothesis in our in vitro system, we incubated HMDMs with NETs purified from neutrophils in serum-free media. After incubation for 1 h, NETs internalized by macrophages were identified using Abs against NE and macrophages were counterstained with DAPI and phalloidin (Fig. 3A). Confocal microscopy was used to confirm macrophage ingestion of NETs (Supplemental Fig. 4). Notably, an aggregation of actin filaments can be seen at the point of interaction of NETs and the engulfing cell. About 15% of macrophages were found to be engaged in clearance of NETs under these conditions. Moreover, it was common to find several macrophages collaborating in the engulfment of the same NET (Fig. 3A). Macrophage clearance of NETs was further quantified using the measurement of NE activity as an indirect readout, given that NE present in NETs has been shown to be active and to correlate to the amount of NETs (Supplemental Fig. 1) (18). Incubation with HMDMs resulted in a decrease in the amount of NE activity in cell culture supernatants, and this was shown to reach a plateau at 2 h (Fig. 3B).

NETs are typically larger structures as compared with macrophages (or neutrophils), and we hypothesized that the dismantlement of NETs through the actions of DNase I might facilitate their subsequent clearance by macrophages. To test this, we monitored NET clearance in the presence of DNase I. Indeed, the addition of this endonuclease accelerated the clearance of NETs by macrophages (Fig. 3C). Furthermore, to determine whether macrophage clearance of NETs was an active process, cytochalasin D, an agent that blocks the rearrangement of actin filaments, was added to HMDM cultures. Incubation with cytochalasin D prevented NET...
clearance by macrophages, showing that this is an active, endocytic-phagocytic mechanism (Fig. 3C). Preincubation with a mixture of endocytosis inhibitors (nystatin, genistein, and brefeldin A) also suppressed uptake of NETs (Fig. 3D). The inhibitors alone were noncytotoxic (data not shown). To confirm the presence of NET components within macrophages, we performed immunoblot analysis on macrophage cell lysates retrieved on incubation with NETs. As shown in Fig. 3E, NE was detected in macrophages incubated with NETs, but not in macrophages alone. The presence of residual amounts of PMA in the NET suspension could not be excluded; therefore, a possible influence of PMA on macrophage clearance of NETs was assessed. Increasing amounts of PMA (up to 25 nM) were added to macrophages in the NETs clearance assay, showing no influence of PMA on the clearance of NETs by macrophages (data not shown).

**NET opsonization by complement factor C1q**

As reported earlier, HMDMs are able to clear NETs under serum-free conditions, but it remains possible that serum-derived factors could facilitate this process. C1q has been shown to bind DNA and histones, and we hypothesized that C1q could also opsonize NETs and target them for clearance. In fact, a recent publication has shown the ability of recombinant C1q to bind NETs (15). Indeed, as shown in Fig. 4A, recombinant C1q is able to bind to NETs. Furthermore, brief (10-min) incubation of NETs with serum derived from healthy human donors loaded the NETs with C1q (Fig. 4B). C1q is the recognition subunit of the C1 complex, which is the initiator of the classical pathway of the complement. Binding of C1q to its targets can lead either directly to the engulfment of the target or to conformational changes within the C1 complex, leading to the activation of the complement cascade (24). We therefore tested the ability of recombinant C1q to facilitate the clearance of NETs. Using the NE assay, we determined macrophage clearance in the presence or absence of 100 μg/ml recombinant C1q (Fig. 4C). C1q promoted the clearance of NETs, indicating that the binding of C1q to these structures targets them for recognition by HMDMs.

**Evidence for lysosomal degradation of NETs**

To provide further evidence of macrophage clearance of NETs, we sought to visualize NET DNA in macrophages. To this end, DNA was stained using DAPI. However, we could not detect any accumulation of extranuclear DNA in macrophages after coincubation with NETs for 1 h (Fig. 5B), suggesting that once NETs are

\*\*\*
ingested, these structures are efficiently processed within the engulfing cell. Therefore, we prestimulated macrophages with CQ to block lysosomal activity (25) before the addition of purified NETs. DNase I (5 μg/ml) was also added during the final 10 min of coculture of HMDMs and NETs to eliminate any extracellular DNA. After this treatment, an accumulation of extranuclear DNA dots was seen in macrophages (Fig. 5C, 5D). In an attempt to define the localization of these extranuclear dots, cells were stained with the early endosomal marker Eea-1, but we found no evidence of extranuclear dots being localized in the early endosomal compartment (data not shown). Staining with the late endosomal marker Lamp-1 showed a redistribution of this protein in macrophages after ingestion of NETs with the appearance of vesicular structures (Fig. 5A, 5B). After treatment with CQ, some of the extranuclear DNA dots were localized within Lamp-1+ vesicles (Fig. 5C). Furthermore, using Lysotracker to identify lysosomes, we noted that the majority of accumulated DNA dots colocalized with lysosomes (Fig. 5D). The latter results suggest that lysosomal enzymes are responsible for the intracellular degradation of NETs in macrophages. DNase II is a key enzyme in lysosomes that is responsible for digestion of DNA derived from apoptotic cells and erythroid precursors (26, 27). To test its involvement in the clearance of NET DNA, we silenced DNase II expression in HMDMs using specific siRNA (Fig. 5E) and then asked whether this would lead to an accumulation of extranuclear (nondegraded) DNA in macrophages after ingestion of NETs. However, we were unable to visualize an increase in undigested DNA under these conditions (data not shown). Kawane et al. (28) reported that DNase II–deficient macrophages that cannot degrade DNA from apoptotic cells produce TNF-α. However, the production of proinflammatory TNF-α in response to NET internalization was not increased in macrophages that lack expression of DNase II (Fig. 5F).

No proinflammatory cytokine production upon NET clearance

To further decipher macrophage responses after NET clearance, we analyzed a panel of cytokines in cell culture supernatants of macrophages after ingestion of NETs. We could only detect a very minor induction of IFN-α at 24 h postingestion, but not after 1 h (Fig. 6A). No induction of the proinflammatory cytokines, IL-1β, IL-6, and TNF-α, was detected (Fig. 6B–D), and no induction of IL-10 (data not shown). These results indicate that NET clearance by macrophages does not lead to an inflammatory response. Nonetheless, under physiological conditions, the production of NETs is likely to be triggered by bacteria or other pathogens, and we thus attempted to mimic this situation by stimulating macrophages with bacterial LPS (100 ng/ml) + NETs. As expected, the induction of the proinflammatory cytokines IL-1β, IL-6, and TNF-α was noted for LPS-stimulated macrophages (Fig. 6B–D). Moreover, a marked potentiation of the LPS-triggered cytokine response was seen when macrophages were cocultured with NETs for 1 h. As shown in Fig. 5F, silencing of DNase II in macrophages reduced the production of TNF-α in response to bacterial LPS + NETs, but did not affect responses to NETs alone.

As noted earlier, clearance of NETs by macrophages does not lead to type I IFN production. These observations are in contrast with several previous results showing the capacity of mammalian DNA to stimulate IFN production (25, 29). To further investigate the capacity of NETs to induce type I IFNs, we transfected NETs or purified NET DNA into HMDMs and analyzed type I IFN expression at 24 h using RT-PCR. By this approach, we could observe the induction of expression of both IFN-α and IFN-β after transfection of NETs or NET DNA into macrophages (Fig. 7A), and these results were further confirmed by ELISA (Fig. 7B). However, incubation of macrophages with NETs, leading to their engulfment, did not trigger the induction of these cytokines, nor did cotreatment with CQ, leading to an accumulation of extranuclear DNA in macrophages that are engaged in NET clearance. This experiment demonstrates that the DNA component of NETs can potentially induce a type I IFN response and suggests that macrophages are able to process NETs in an immunologically silent manner when NETs enter via the endocytosis-phagocytosis route.
The mechanisms that drive NET formation have been studied in detail (30–32), but not much attention has been paid to their ultimate fate once these structures have been released into extracellular space. We report for the first time, to our knowledge, that macrophages actively engulf NETs, and that this event is facilitated by the preprocessing of NETs by DNase I. Our studies underscore the importance of using physiological concentrations of DNase I when assessing the capacity of this enzyme to degrade NETs. Hence, high concentrations of DNase I, which are typically used in most studies (7, 18, 33), can completely digest NETs, but physiological concentrations of the enzyme do not. This obser-

FIGURE 5. NETs are processed in lysosomes. HMDMs were incubated with NETs for 2 h, and Lamp-1 staining (red) together with DNA (DAPI, blue) was performed in control macrophages (A) or macrophages incubated with NETs (B). Note the redistribution of Lamp-1 upon ingestion of NETs (B). (C and D) HMDMs were pretreated with or without 10 μM CQ for 30 min to block lysosomal function. Then purified NETs were added to macrophages in the presence of CQ (10 μM). Ten minutes before fixation, DNase I (5 μg/ml) was added to avoid interference from extracellular DNA. (C) Lamp-1 staining (red) was performed and visualized by confocal microscopy together with DNA (DAPI, blue). White arrows mark extranuclear DNA dots contained within Lamp-1+ vesicular structures. (D) Cells were stained to visualize lysosomes (Lysotracker) in red and DNA (DAPI) in blue, and visualized using a confocal microscope. The merged image is shown to facilitate visualization of extranuclear dots indicating the colocalization (white arrows) of DNA and lysosomes. Original magnification ×60. (E) DNaseII mRNA expression in macrophages transfected with specific DNase II siRNA or control siRNA as described in Materials and Methods was determined using RT-PCR. Data are normalized to the expression values of the gapdh gene. One-way ANOVA analysis was performed with Tukey correction and significant differences in reference to the corresponding control (***, p < 0.001). (F) TNF-α secretion after incubation of macrophages with or without a reduction of DNase II expression with 100 ng/ml LPS, purified NETs, or LPS + NETs. Cell culture supernatants were harvested after 24 h, and cytokine levels were determined by ELISA. Results shown are the mean values ± SD of triplicate values from one representative experiment. Unpaired, two-tailed Student t test analysis was performed, and significant differences between the indicated samples and the corresponding control are indicated (***, p < 0.001).
vation is potentially relevant in an in vivo setting as the rapid and complete digestion of NETs might prevent NETs from performing their actual task, that is, to ensnare and kill pathogens, whereas a lower or slower rate of degradation may be required to facilitate NET clearance by macrophages.

The complement factor, C1q, has been shown to act in cooperation with DNase I in the clearance of chromatin from necrotic cells (34). Moreover, C1q is known to act as an opsonin for apoptotic cells (14). Our data are in accordance with these findings as we have been able to show opsonization of NETs with C1q and the facilitation of macrophage clearance of NETs decorated with C1q. Leffler et al. (15) reported that NETs are a potent complement activator and suggested that this may play an important role in SLE insofar as sera from a subset of patients with active SLE had a reduced ability to degrade in vitro–generated NETs and displayed lower levels of complement proteins C4 and C3. In our study, serum from healthy blood donors could only partially degrade NETs, as determined by agarose gel electrophoresis. In previous studies from Leffler et al. (15) and Hakkim et al. (7), degradation was monitored by fluorescence spectrometry of DNA content in cell culture supernatants; the latter assay may not report complete degradation of NETs.

Macrophages are endowed with several means to ensure effective clearance of nucleic acids from various origins. Our results indicate that NETs are internalized through an endocytic mechanism leading to lysosomal degradation, as shown by blockage of NET clearance by a mixture of endocytosis inhibitors, accumulation of extranuclear DNA on CQ treatment, and colocalization of these extranuclear DNA dots within Lamp-1 vesicles and lysosomes. DNase II resides in the lysosomal compartment and has been shown to digest DNA from apoptotic cells, and Nagata and coworkers have reported that its deficiency leads to activation of the innate immune system (25, 35). Macrophages from DNase II–deficient mice accumulate undigested DNA, resulting in the production of TNF-α and polyarthritis (28). DNase II also forms a barrier against the propagation of horizontally transferred DNA in normal cells (36). In this study, silencing of DNase II did not lead to any discernible accumulation of extranuclear DNA in macrophages nor did this lead to the secretion of proinflammatory TNF-α by macrophages after the ingestion of NETs. Nonetheless, it is noteworthy that TNF-α secretion in response to LPS + NETs was significantly reduced in HMDMs with reduced DNase II expression, suggesting a role for this nuclease in the sensing of NET DNA in cells costimulated through TLRs. Moreover, transfection of NETs or NET DNA inside macrophages leads to the production of type I IFNs, whereas the normal uptake of NETs by macrophages is immunologically silent. Further studies of intracellular DNA-sensing systems, as well as DNA-degrading systems in macrophages that are engaged in the clearance of NETs, are warranted.

In the present model of PMA-triggered NET formation, macrophage internalization of NETs alone did not give rise to a proinflammatory response. PMA is a potent activator of the NADPH
oxidase (17), and NET formation is largely NADPH oxidase-dependent (2, 37), although examples do exist of NADPH oxidase–independent NET induction (38). Of note, the very recent observation that NADPH oxidase (Nox2)–deficient mice display exacerbated lupus-like symptoms implies that NET formation per se does not contribute to SLE (39). Indeed, based on the latter animal study and the current in vitro studies using primary human neutrophils and macrophages, NETosis may be viewed as an inherently noninflammatory form of cell death, in cases of NADPH oxidase–dependent generation of NETs. This is in accordance with recent studies of mice immunized with NETs, which have shown that isolated exposure to NETs prepared using hydrogen peroxide stimulation is insufficient to break immune tolerance (40). How may these observations be reconciled with other recent reports (10, 11) demonstrating that the delivery of NETs to dendritic cells has a proinflammatory effect, resulting in the production of type I IFNs through a TLR-dependent pathway? It is conceivable that the presence of additional microbial and/or cellular factors such as HMGB1 (not present in NETs in our study) is needed. In fact, we noted that the LPS-triggered cytokine response was enhanced when macrophages were cocultured with NETs as compared with LPS stimulation only. Based on these observations, one may speculate that in an inflammatory milieu, NETs loaded with bacteria elicit a stronger proinflammatory response in neighboring monocytes/macrophages than bacteria alone, thus enabling the immune system to mount a more robust and prolonged response against invading pathogens.

In summary, we report that monocyte-derived macrophages are capable of efficient clearance of NETs that have been extruded from neutrophils, and that this process is facilitated by extracellular preprocessing of NETs by DNase I, as well as by the opsonization of NETs with C1q. Following their uptake, NETs are apparently shuttled via phagosomes to lysosomes, and experiments using CQ suggested that degradation occurred in the lysosomal compartment; however, we could not conclusively demonstrate a role for DNase II in the degradation process and we cannot exclude that degradation may be taking place in other compartment(s). Further studies are thus needed to identify the nuclease(s) responsible for NET degradation in macrophages. Importantly, the process of NET clearance is immunologically silent insofar as macrophages do not produce proinflammatory cytokines after ingestion of NETs alone. In this respect, the clearance of NETs resembles the clearance of apoptotic cells, a process that is also “silent” and thus serves to preserve homeostasis in tissues (41). Rapid clearance of NETs by macrophages could act to prevent the induction of autoimmune responses toward NET DNA or other NET components and, consequently, a deficiency in macrophage clearance of NETs may therefore lead to autoimmune disease.

**Acknowledgments**

We thank Dr. Kjell Hultenby (Electron Microscopy Unit, Karolinska University Hospital Huddinge, Stockholm, Sweden) for assistance with scanning electron microscopy. We also thank Ingrid Delin (Institute of Environmental Medicine, Karolinska Institutet) and Dr. Florian Salomons (Department of...
References


