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Macrophage Clearance of Neutrophil Extracellular Traps Is a Silent Process

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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.
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 10^6 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 mg/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 500 mU/ml was added to the supernatant and fluorescence (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) 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 500 mU/ml was added to the supernatant and fluorescence (excitation wavelength 360 nm, emission wavelength 465 nm) was measured using a Tecan plate reader (Tecan Group, Männedorf, Switzerland).

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 chloroquine (CQ; Sigma) 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 LSM510 META confocal microscope (Carl Zeiss, Oberkochen, Germany).

DNase II gene silencing

Silencing 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′-UUGUCAACAGAACUUA- GAU(GT)U-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 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′-TTCTCGCTCTTACAATGACACCA-3′, dnaseII reverse, 5′-GGAAAGTATGCACTTGTTGACC-3′; gapdh forward, 5′-GGGTCTTGCAGTCTATGG-3′; gapdh reverse, 5′-ACCTCCCTTCTGGGGACT-3′. To monitor IFN responses, we used the following primers: IFN-α forward, 5′-GCCTCGCCCTTGTGCTACT-3′; IFN-α reverse, 5′-CTGTGCTTCTCAGGAGAATCA-3′; IFN-β forward, 5′-ATGACCAACAAGTTTCCTCC-3′; IFN β reverse, 5′-GGATCTCAAGCAAGTGTAGCTC-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.

Figure 1. NET production by neutrophils. Neutrophils obtained from healthy adult blood donors were incubated in the presence of 25 nM PMA for 2 h at 37°C. After fixation, NETs were detected by the presence of NE (A) in green or MPO (B) in red. DAPI staining was included in the mounting medium for visualization of cell nuclei. The merged images illustrate the characteristic network of the extracellular traps. Original magnification ×40. (C) Scanning electron microscopy images of neutrophils fixed for imaging after treatment with 25 nM PMA for 2 h at 37°C. NET formation is clearly seen. Scale bars, 10 μm (left panel); 2 μm (right panel).
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 μM CQ. β-actin was determined as a loading control.

**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

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**FIGURE 3.** Macrophages engulf NETs. (A) Immunofluorescence image showing HMDMs in the process of engulfing NETs. HMDMs were incubated for 60 min with purified NETs and after fixation, NETs were stained for NE (green) and macrophages were visualized with DAPI (blue) and phalloidin (red). Original magnification ×40. (B) Time course of NET clearance by macrophages as determined using the NE assay. HMDMs were incubated for different time points in the presence of purified NETs. Supernatants were then recovered and NE activity was determined. For each independent experiment, triplicates were performed for each time point. Results shown are the mean values ± SD of three independent experiments. One-way ANOVA analysis was performed with Tukey correction, and significant differences in reference to initial point are indicated (**p < 0.01, ***p < 0.001). (C) NE activity was measured as in (B), but incubation was performed also in the absence or presence of either 5 μg/ml DNase I or 10 μg/ml cytochalasin D. Results shown are the mean values ± SD of three independent experiments. Unpaired, two-tailed Student t test analysis was performed, and significant differences between the indicated points and the corresponding control (**p < 0.01) are indicated. (D) NE activity was measured at different time points in the presence of a mixture of endocytosis inhibitors containing nystatin (25 μg/ml), genistein (200 μM), and brefeldin A (10 μg/ml). Results shown are the mean values of three independent experiments ± SD. One-way ANOVA analysis was performed with Tukey correction, and significant differences in reference to the corresponding value without the mixture are depicted (*p < 0.05). (E) The presence of NE inside macrophages was assessed by Western blot after 2 h of incubation with NETs in the presence of 10 μM CQ. β-actin was determined as a loading control.
of extranuclear dots being localized in the early endosomal compartment (data not shown). Staining with the late endosomal marker Eea-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 not increased in 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.
**Discussion**

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

FIGURE 6. NET clearance by macrophages is noninflammatory. (A) Purified NETs were administered to HMDMs for their ingestion, and macrophage supernatants were recovered after 1 or 24 h and analyzed for the presence of IFN-α. Complete medium (see Materials and Methods) and supernatant from neutrophils nonstimulated with PMA were included as negative controls. Results shown are the mean values ± SD of three independent experiments. One-way ANOVA analysis was performed with Tukey correction, and significant differences in reference to the medium control are indicated (***p < 0.001).

Note the very low levels of IFN-α production. (B–D) HMDMs were treated as in (A) with additional stimulations in the presence of 100 ng/ml LPS as indicated, and supernatants were harvested for the analysis of IL-1β (B), TNF-α (C), and IL-6 (D). Results shown are the mean values ± SD of three independent experiments. One-way ANOVA analysis was performed with Tukey correction, and significant differences in reference to the corresponding LPS control (*p < 0.05, **p < 0.001) are indicated.
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 by DNs in 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 compartments. Further studies are thus needed to identify the nuclease(s) responsible for NET degradation in macrophages. In this respect, the 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.

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 compartments. 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 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.

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Disclosures

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