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# Monocyte clearance of apoptotic neutrophils is unhindered in the presence of NETosis, but proteins of NET trigger ETosis in monocytes

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ABSTRACT

Resolution of inflammation needs effective and timely removal of dead cells and other toxic products of neutrophils, monocytes, and macrophages. In this study, we evaluated the role of monocytes in the clearance of neutrophil extracellular trap (NET) and apoptotic neutrophils in the inflammation site. For this, monocytes were observed microscopically after exposing them with NETs and/or apoptotic bodies. A subset of monocytes exposed to NETs ejected extracellular traps and this was shown to be mediated by proteins like elastase and citrullinated histones present in NET supernatant. Monocytes showed a preference for the internalisation of the apoptotic body when both NET and apoptotic bodies were present in the medium. The study provides new insight into the role of monocytes in the clearance of NET and apoptotic neutrophils and this information may open up a way in formulating therapeutic strategies for accelerating resolution of inflammation.

## 1. Introduction

The immune system when challenged by pathogens fights back quickly through inflammation. During inflammation, neutrophils infiltrate to the inflammatory milieu and destroy pathogens by oxidative and non-oxidative mechanisms. Monocytes follow neutrophils in the inflammatory response and together they fine-tune the inflammation and co-operatively eliminate the invading pathogens. Monocytes/macrophages are also involved in the timely removal of dead or dying neutrophils [1] and toxic neutrophil components [2] from the site of inflammation, implying unique co-operation between macrophages and neutrophils.

During extracellular trap (ET) formation, neutrophils eject out their nuclear or mitochondrial DNA as a meshwork to trap pathogens [3]. This entrapment prevents the dissemination of bacterial [4], viral [5], fungal [6] and parasitic [7] pathogens and eventually, they are killed by antimicrobial peptides entangled within the ET. Although NETosis enhances the efficiency of the immune system, delayed and defective neutrophil extracellular trap (NET) generation causes infections in neonates [8] and aged [9]. On the other hand, excessive NETosis and impaired NET clearance accelerate inflammation and tissue damage by prolonging the exposure of noxious antimicrobials to the surrounding tissue [10,11].

NET clearance is thought to be performed by macrophages in an immunologically silent manner [2]. NET clearance if delayed, can result in an increase in both cell-free DNA and antibodies against self-antigens entangled in NET-DNA. Circulating cell-free DNA is observed as a marker of NETosis in pathological conditions like chronic obstructive pulmonary disease [12], inflammatory bowel disease [13], pre-eclampsia [14] and type 2 diabetes [15]. Excessive NET formation and NET-mediated complications are also observed in sepsis [16], gout [17] and cystic fibrosis [18]. In addition to an increase in circulating DNA, an increase in antibody titre against NET-DNA and NET-proteins have also been reported in diseases such as type 1 diabetes [19], systemic lupus erythematosus [11], rheumatoid arthritis [20] and antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis [21]. In these conditions, circulating NET components intensify disease complications by activating neutrophils and other immune mediators such as complements. This leads to endothelial damage and tissue necrosis. Another challenging feature of NET-mediated complications is its ability to modulate coagulation status. NET-DNA acts as a scaffold that expresses coagulation mediators (e.g. tissue factor) [22] and stimulates extrinsic or intrinsic coagulation pathway. It also mediates RBC activation as well as platelet activation and aggregation [23]; thereby increasing

*Abbreviations:* ET, extracellular trap; NET, neutrophil extracellular trap; N(ET), NET/ET; NS, supernatant containing the NET components; SCS, *Staphylococcus aureus* cell-free culture supernatant; ETosis, extracellular trap formation by monocyte; NETosis, extracellular trap formation by neutrophil; PCR, polymerase chain reaction; AGE, agarose gel electrophoresis; PBS, Phosphate buffer saline; ANCA, antineutrophil cytoplasmic antibody

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thrombosis-related vascular complications and organ damage in diseases like sepsis [24] and ANCA associated vasculitis [25]. Hence, a balance between NETosis and NET clearance is very essential to prevent both infections and inflammatory diseases.

Neutrophils undergo constitutive apoptosis by a highly regulated caspase-driven pathway and this is inevitable for the resolution of inflammation [26]. Unlike cells undergoing NETosis, apoptotic neutrophils release mediators that suppress the inflammatory response and prevent the leakage of its toxic cell contents to the external environment [1]. If removal of apoptotic neutrophils by monocytes/macrophages via efferocytosis is delayed or inefficient, they serve as a source of auto-antigens and undergo secondary necrosis [27,28]. Ideally, monocytes and macrophages rapidly dispose of apoptotic bodies and keep the inflammation under control.

Peripheral blood monocytes, unlike macrophages, are less differentiated cells and show a difference in phenotype. Though monocytes are said to be the key effector cells of the immune system, their role in defending pathogens has not been investigated. Some reports on their importance in defending infectious pathogens entering blood circulation as in sepsis and in some other kind of infections are, however available [29]. Monocytes play efficient roles in infections of mucosa and other tissues as well [30]. Their role in the removal of apoptotic neutrophils [31] and their ability to generate ET [32] has been recently reported. Since monocytes being the precursor from which macrophages originate, they may also possess the talent to clear NET-DNA; but, to date, their ability to remove NET or their capability to remove apoptotic neutrophils in the presence of NET has not been investigated. Considering the potential of cell-free NET-DNA in causing thrombosis and other complications, their removal from circulating blood is decisive in the clinical outcome. Since monocytes/macrophages are involved in combating infection and removing dead cells, debris and NETs at inflammatory sites, their behavior in the inflammatory setting determines the quantity of cell-free circulating DNA. Hence, this study looks into the behavior of peripheral blood monocytes during the removal of apoptotic neutrophils and NETs.

## 2. Materials and methods

### 2.1. Isolation of neutrophils and monocytes

All experiments were conducted with the approval of the Institutional Human Ethics Committee (IEC/IRB No: 05/IHEC20082015) and in accordance with the declaration of Helsinki. After obtaining informed consent, blood was collected from healthy donors (n = 20, 21–35 years of age) with no recent history of infections or inflammatory diseases. For each experiment, the cells isolated from the blood of at least three different donors were included. The design of the study is given in Fig. 1.

Neutrophils and peripheral blood mononuclear cells were isolated from the blood of healthy human volunteers by dextran sedimentation followed by Ficoll-hypaque density gradient centrifugation [33]. For monocyte isolation, the method described by Raj et al. [34] was employed incorporating slight modifications. Cells were suspended in PBS and viability was analysed by Trypan blue dye exclusion method. The purity of neutrophils and monocytes were confirmed by Giemsa staining.

### 2.2. Induction of neutrophil extracellular trap

Two traditional NET inducing agents; phorbol myristate acetate (PMA, 50 nM) and 6–12 h-old *Staphylococcus aureus* cell-free culture supernatant (SCS, 20 µl) were analysed for the generation of NET. SCS was obtained by centrifugation of *S. aureus* culture at 12,000 rpm for 30 min.

To induce NET from healthy neutrophils, freshly isolated neutrophils ($2 \times 10^6$ cells/ ml) were stimulated with either of the two stimulants for 3 h at 37°C. After inducing NET release, the cells were vortexed for a second to detach the extracellular DNA and centrifuged at 1500 rpm

for 5 min. The supernatant containing the NET components (NS) was transferred to a clean

sterile tube. NS was then quantified for extracellular DNA using Hoechst 33,258 stain [2] and analysed for the extent of protein citrullination by citrullination assay [35]. It was also analysed for myeloperoxidase (MPO) [36] and peptidyl arginine deiminase 4 (PAD4) [37] activities.

### 2.3. Staining of extracellular trap

$2 \times 10^6$ cells/ml were seeded on gelatin-coated cover-slips and after different treatment conditions (described in section 2.5), the slides were viewed under a fluorescent microscope (Olympus BX43 F, Japan) or a light microscope (Labomed, India) to study N(ET) release. For fluorescent microscopy the cover-slips were washed with PBS and fixed with 4% formaldehyde after incubation. The cover-slips were then stained with Sytox green (1:30,000) and observed under a fluorescent microscope at 400x magnification. For light microscopy, the fixed cells were stained with Hoechst 33,258 (1:50,000) for 15 min followed by Giemsa (1:1) for 30 min and observed under 1000x magnification. The additional staining step with Hoechst 33,258 improved the clarity during observation.

### 2.4. Neutrophil apoptosis

Neutrophils ($2 \times 10^5$ cells/ml) were kept at 43°C for half an hour in a water bath and thereafter at 37°C for 2½-3 h [38]. Prolonged maintenance of cells at the above-mentioned conditions may lead to necrosis rather than apoptosis. Therefore, care was taken not to exceed the time limit. Apoptosis was confirmed in these cells by acridine orange/ethidium bromide (100 µg/ml) double-staining [39] and by the banding pattern of the apoptotic DNA in agarose gel electrophoresis (AGE) (see Supplementary Fig. 3).

### 2.5. Microscopy

NET clearance by monocytes ($2 \times 10^6$ cells) was analysed microscopically by examining the following groups: 1) monocytes treated with unstimulated-neutrophil supernatant 2) monocytes treated with NS 3) monocytes treated with proteinase K-treated (10 mg/ml) or preboiled NS 4) monocytes treated with DNase-treated NS.

To study efferocytosis, the above groups were simultaneously treated with apoptotic neutrophils in another set of experiment. Both groups were incubated for 10 or 30 min at 37°C, washed, fixed and stained as already described in subsection 2.3.

In another experiment, for ruling out the possibility of S. aureus components in inducing monocyte ET formation, neutrophils were adhered on a cover-slip before inducing NETosis. After NET release (30 min, 1 h, 2 h or 3 h), the medium was discarded and the cover-slip was washed gently with PBS and placed over adhered monocytes ($2 \times 10^6$ cells/ml) on a slide. After another 30 min, the cover-slip was removed carefully and the monocytes were analysed for ET release by light and fluorescent microscopy.

### 2.6. Quantification of extracellular DNA

To study NET clearance, monocytes were exposed to NS for 30 min, after which,

extracellular DNA was quantified using Hoechst 33,258 (5 µM) as reported earlier [2]. For this study, 4 experimental groups were included: 1) monocytes incubated with NS 2) monocytes incubated with NS in the presence of cytochalasin D (phagocytosis inhibitor, 10 µg/ml) 3) monocytes incubated with proteinase K-treated NS and 4) monocytes

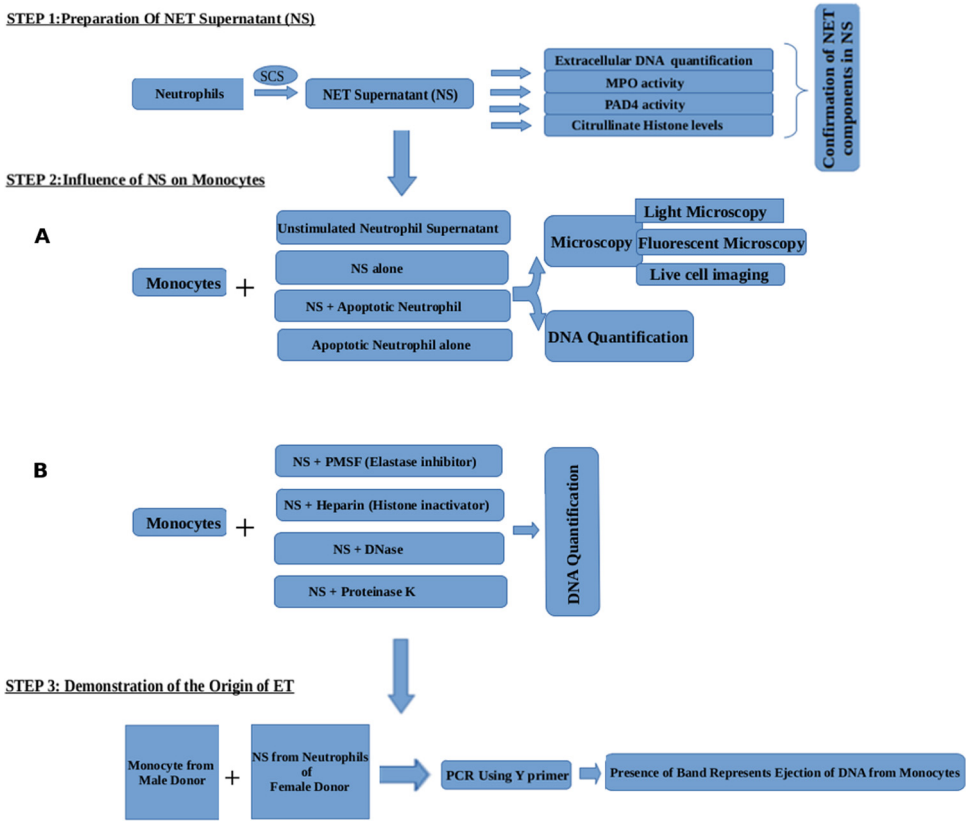


**Fig. 1.** Flowchart showing experimental design. Experiments were conducted in three steps. Step 1: Preparation of NET supernatant (NS), step 2: Influence of NS on monocyte, step 3: Demonstration of the origin of ET.

incubated with SCS (positive control). To study the effect of NET on efferocytosis, NET-DNA quantification was performed with monocytes incubated with apoptotic neutrophil in the presence or absence of

NS. Extracellular DNA in the supernatant after different treatments were quantified using Hoechst 33,258 stain.

### 2.7. Live cell imaging

For live cell imaging, apoptotic neutrophils ($2 \times 10^5$ cells), NS or NS + apoptotic neutrophils were added just before imaging, to wells containing $2 \times 10^6$ monocytes. A set of monocytes that received no treatment was taken as control. Imaging was performed in an inverted phase contrast microscope (Olympus 1 × 51, Japan) using Q capture pro 7 software (Washington, USA). The images were sequentially captured in time-lapse mode at an interval of 30 s.

### 2.8. Polymerase chain reaction (PCR)

To analyse the origin of extracellular DNA, PCR was performed using Y primer as described by Choi et al. [40] for the following samples: a) ET-DNA of monocytes (female donor) b) ET-DNA of monocytes (male donor) c) ET-DNA of monocytes (from male donor) obtained after treatment with NET-DNA (collected from neutrophils of a female donor) d) NET-DNA of neutrophils (female donor). Primers specific to the Y chromosome were Y1.5 (5′-CTA GAC CGC AGA GGC GCC AT-3′) and Y1.6 (5′-TAG TAC CCA CGC CTG CTC CGG-3′). PCR was performed with an initial denaturation for 2 min at 94°C, followed by 30 cycles of 94°C for 30 s; 60°C for 60 s; 72°C for 60 s and a final extension at 72°C for 10 min.

### 2.9. Inhibition studies

To analyse the contribution of elastase and histones (major components of NET) in triggering monocyte ETosis, inhibition studies were conducted. For that, NS was pre-incubated for 30 min either with PMSF (0.1 mM) for inhibiting elastase or with heparin (100 μg/ml) for inactivating histones. ET formation was then analysed by incubating monocytes with pre-treated NS for 30 min and DNA was quantified using Hoechst 33,258 as described in section 2.6.

### 2.10. Statistical analysis

All experiments were done at least in triplicates and all the data were expressed as Mean ± SEM (Standard error of the mean). Comparison between groups was performed using GraphPad Prism version 5.03 (San Diego, CA) and the minimal value of significance was set at a p-value of $< 0.05$.

## 3. Results

### 3.1. NET supernatant induces ETosis in unstimulated monocytes

When PMA and SCS were used for the generation of NET, the latter triggered more NET generation (see Supplementary Fig. 1). Hence, SCS was used as the NET stimulant throughout the study. Both neutrophils and monocytes released extracellular DNA when stimulated with SCS.

NS isolated from the neutrophils of different donors invariably showed a significant increase

in PAD4 activity ($p < 0.0001$) (Fig. 2 C) and MPO activity ($p < 0.05$) (Fig. 2 B) when compared to the

control i.e., supernatant from unstimulated neutrophil. A significant increase in extracellular DNA ($p < 0.0001$) (Fig. 2A) and citrullinated

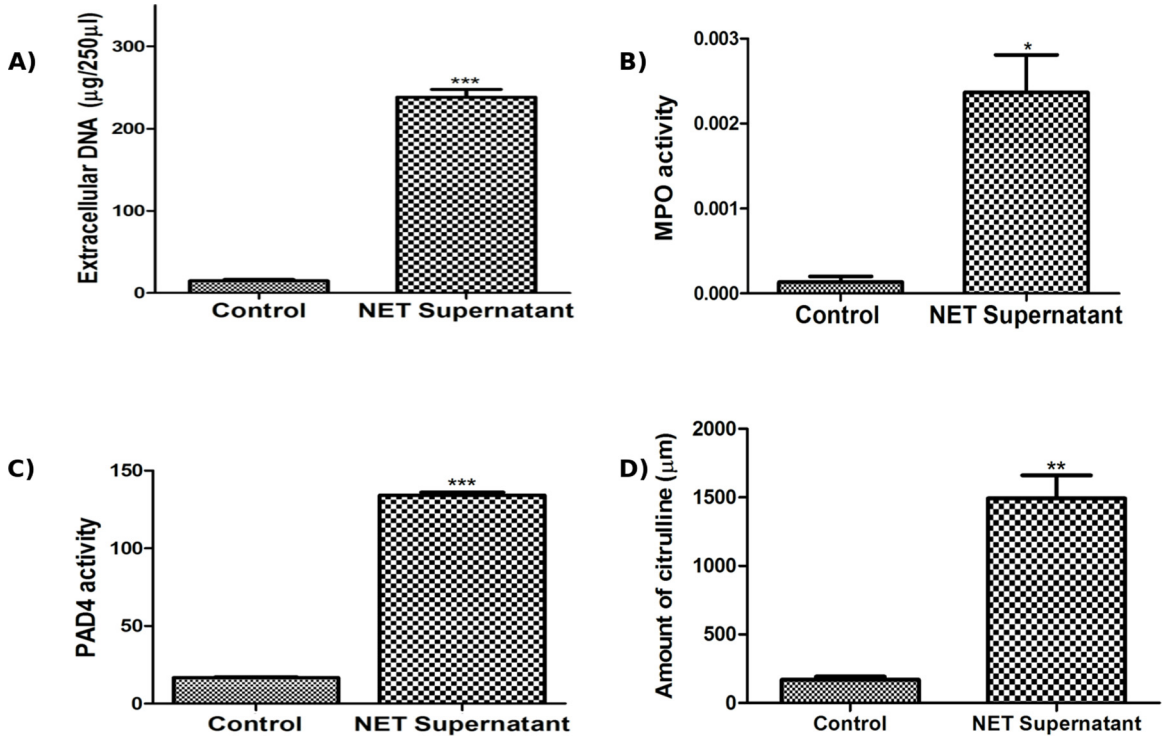


**Fig. 2.** Characterization of supernatant containing NET components (NS). NS was assayed for the presence of extracellular DNA (A), MPO activity (B), PAD4 activity (C) and extent of protein citrullination (D). Supernatant from unstimulated neutrophils served as the control. NS showed a significant amount of NET-DNA. NS also showed an increase in MPO activity and PAD4 activity. The extent of citrullinated proteins were also found to be high in NS.

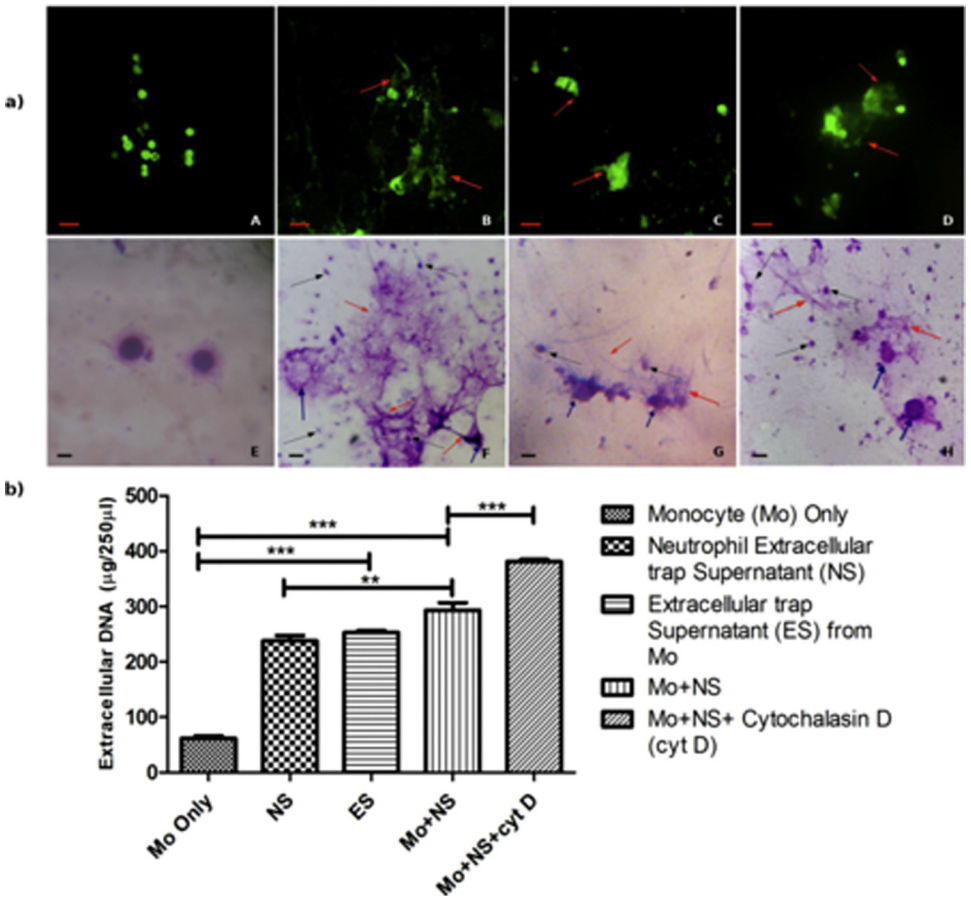


**Fig. 3.** Fluorescent and Light microscopic images of monocyte ETosis after NS treatment (a) For fluorescent microscopy, cells were stained with Sytox green and for light microscopy, cells were stained with Hoescht 33,258 + Giemsa. Fluorescent image (400x) of unstimulated monocyte (A), monocytes releasing extracellular traps (red arrow) after SCS treatment (B), monocytes after 10 min of NS treatment (C), monocytes after 30 min of NS treatment (D). Light microscopic image (1000x) of unstimulated monocytes (E), monocytes (blue arrow) releasing ET (red arrow) and vesicles (black arrow) after SCS treatment (F), monocytes exposed to NS for 10 min (G) and monocytes exposed to NS for 30 min (H). Quantification of extracellular DNA in monocyte cell supernatant subjected to different treatment conditions (b). Monocyte + NS showed a significant increase in DNA compared to NS (**P < 0.01). SCS induced extracellular trap formation in monocytes (ES) (***P < 0.0001). Likewise, in the absence of SCS, NS-treated monocytes (***P < 0.0001) showed significant extracellular DNA release. Treatment of monocytes with NS in the presence of phagocytic inhibitor cytochalasin D resulted in higher levels of extracellular DNA (***P < 0.0001) suggesting phagocytosis of NET-DNA in the absence of the inhibitor. **p < 0.01, ***p < 0.0001. Scale bar 2 µm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

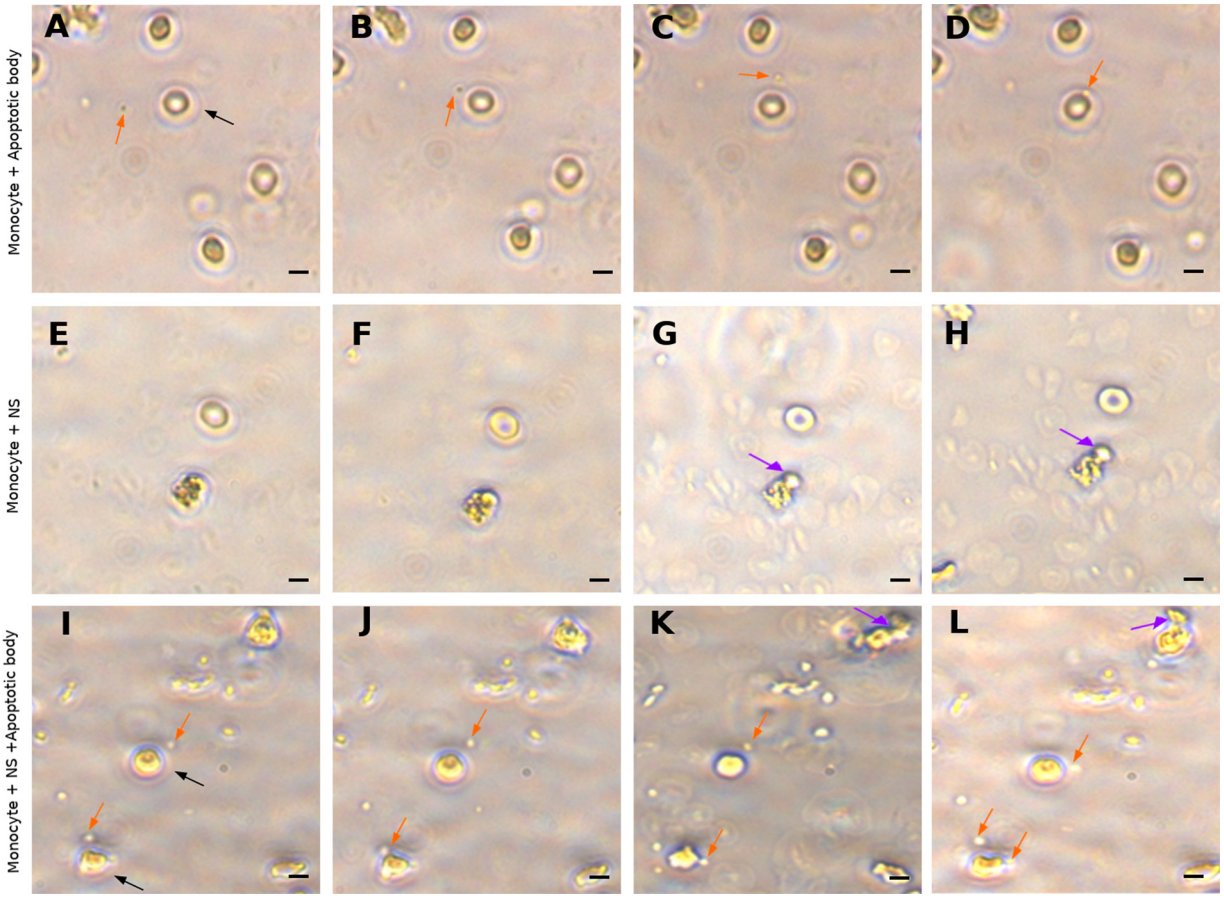


**Fig. 4.** Live cell imaging demonstrating the behavior of monocyte in presence of apoptotic body (A–D), NS (E–H) or apoptotic body + NS (I–L). Arrows indicate monocyte (black arrow), apoptotic body (orange arrow), ET-DNA release (purple arrow). Monocyte establishing contact with the apoptotic body and initializing the uptake of the apoptotic body (B,C; I–K). The apoptotic body inside monocyte (D, L). In presence of NS, monocyte release ET and the ET-DNA released by the monocyte is represented by the purple arrow (G, H). In presence of the NS + Apoptotic body, the monocyte phagocyting apoptotic bodies (orange arrow) and monocyte undergoing ETosis (purple arrow) (I–L) can be seen. Scale bar 2 μm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

proteins ($p < 0.01$) (Fig. 2 D) was also noticed in NS. Together, these results confirmed the presence of NET components in the NS.

Monocytes exposed to NS ejected out ET-DNA within 10 min (Fig. 3a C, G). An extensive ET release, in both classical and vesicular form, was observed after 30 min (Fig. 3a D, H). The morphological changes in these cells were clearer under the light microscope than under the fluorescent microscope. ETing monocytes were devoid of an intact nucleus and they also showed mixed cytoplasmic-nuclear contents (Fig. 3a G, H). Live-cell imaging also provided evidence for the ET release (Fig. 4G, H). In addition, DNA content of corresponding cell supernatant was found to be higher when compared to that of unstimulated monocytes ($p < 0.0001$). To validate phagocytosis of NET-DNA by monocytes, cytochalasin D, a phagocytosis inhibitor, was added to the monocytes before the addition of NS. A significant increase in extracellular DNA ($p < 0.0001$) was observed (Fig. 3b) upon inhibition of phagocytosis.

For confirming extracellular DNA generation from monocytes upon NS treatment, monocytes from male donors were treated with NS collected from neutrophils of female origin. Then, the DNA isolated from the supernatant was amplified using a Y-specific primer. A prominent positive band confirming the expulsion of extracellular DNA from monocytes was observed (Fig. 5).

To rule out the effect of *S. aureus* components in triggering monocyte ETosis, in another set of experiments, neutrophils attached to cover-slip was induced to release NET and the cover-slip was washed repeatedly to remove possible *S. aureus* components. The cover-slip containing attached NET-DNA (confirmed by microscopy) was then placed over monocytes adhered on a

slide. These monocytes also (at all time intervals) expelled out ET-DNA ruling out the effect of *S. aureus* component in triggering ET formation (Supplementary Fig. 2 C, D).

### 3.2. Protein fraction of NET is responsible for NET- induced ETosis in monocytes

To investigate the constituents of NET responsible for triggering ETosis, monocytes were treated for 10 or 30 min with DNase-treated NS, proteinase K-treated NS or pre-boiled NS. Proteinase K-treated NS or pre-boiled NS did not induce ET generation from monocytes, however, these cells showed altered morphology with loss of cell membrane integrity. The shape and density of the nucleus in them were found to be compromised (Fig. 6a A, B). Fluorimetric quantification also showed a significant reduction in extracellular DNA levels ($p < 0.0001$). Furthermore, to assess the participation of elastase and histone in ETosis, NS pre-treated with PMSF or heparin was given to monocytes. In the presence of both PMSF-treated NS ($p < 0.0001$) and heparin-treated NS ($p < 0.01$), the amount of ET released from monocyte was significantly reduced (Fig. 6b).

Interestingly, monocytes exposed to DNase-treated NS released ET efficiently; however, released ET-DNA was found to be broken, possibly due to leftover DNase present in the DNase-treated NS. In these slides, broken DNA strands were found to be scattered (Fig. 6a C) in all the

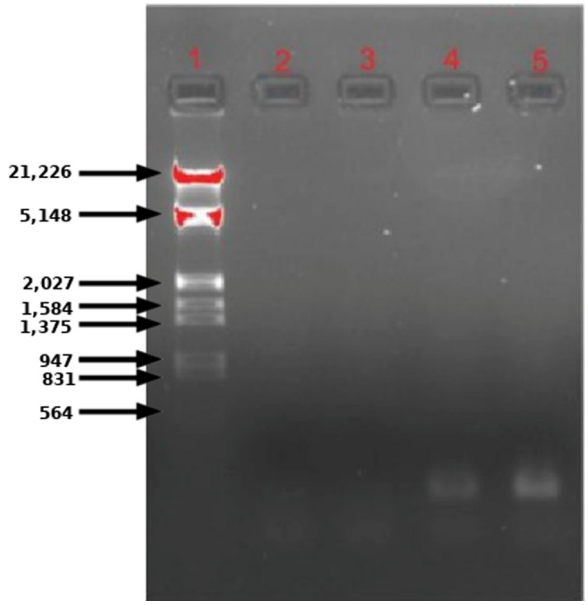


**Fig. 5.** Analysis of DNA on AGE after PCR amplification: DNA isolated from neutrophils and monocytes were loaded on 1.2% agarose gel after PCR amplification using Y primer. Lane 1: Marker DNA (λ DNA EcoRI Hind III Double digest, Genie, Bangalore, India). Lane 2: ETotic DNA released from monocytes (female donor) upon stimulation with SCS. Lane 3: NETotic DNA (female donor) upon stimulation with SCS. Lane 4: ETotic DNA from monocytes (male donor) after stimulation with SCS. Lane 5: ETotic DNA from monocytes (male donor) exposed to NS (female donor). The amplicon size is ˜200 bp.

microscopic fields. Monocytes treated with commercial calf-thymus-DNA showed no extracellular trap formation (Fig. 6b).

### 3.3. Apoptotic bodies are cleared efficiently even in the presence of NET

When monocytes were incubated with NS + apoptotic body, a group of cells exhibited typical ET-DNA release in 10 min and extensive ETosis within 30 min (˜20% cells) (). Apoptotic bodies entangled in N (ET) like structures (Fig. 7a B) were also observed. Monocytes actively phagocyting apoptotic neutrophils (Fig. 4I–L) and monocytes with phagocytosed apoptotic bodies were frequently observed (˜40% cells) (Fig. 7a A). Monocytes phagocyting NET-DNA and monocytes phagocyting both NET-DNA and apoptotic neutrophils were also present (Fig. 7a C).

The apoptotic bodies were cleared efficiently by monocytes without an increase in extracellular DNA content. Monocytes incubated with apoptotic body + NS (Fig. 7b) also showed a significant decrease ($p < 0.05$) in extracellular DNA level than monocytes treated with NS alone.

## 4. Discussion

Monocytes were recently recognized as one of the key cells in early innate immune response. They were shown to play an important role in eliminating pathogens from blood [41],

body fluids [42] and tissues [30]. Recently, the ability of monocyte to release ET was also established [32]. In an inflammation site, phagocytic cells may stumble upon both apoptotic cells and NETing cells. Hence, timely removal of both NET and apoptotic neutrophils is essential for controlling inflammation. Though monocytes were shown to remove apoptotic bodies, their role in the removal of NET remains unknown. Information about the efferocytic function of monocytes in an inflammation setting overwhelmed with the presence of these toxic inflammatory components gives insight on their contribution to exacerbated inflammation observed in inflammatory diseases.

We induced NET formation in neutrophils using both PMA and SCS. Consistent release of elevated levels of NET-DNA was observed in SCS-treated neutrophils. SCS induced rapid NETosis in neutrophils, possibly with the help of Panton-Valentine leukocidin protein and leukotoxins GH [43,44]. In response to SCS, we observed both conventional fiber-like NET and rapid vesicular NET (see supplementary Fig. 2) just as observed by others [43]. Since NS was prepared using neutrophils collected from different donors, to confirm the presence of NET components in it, the presence of extracellular DNA and citrullinated histone was established. Significant activities of MPO and PAD4 enzymes also suggest the presence of NET components in NS. SCS is a complex mixture of proteins and it may also induce degranulation in neutrophils. As similar molecules are released during NETosis and degranulation [45], and as all cell contents are released eventually during NETois we did not rule out degranulation in them.

For better visualization of cell morphology during the internalization of N(ET)-DNA or apoptotic bodies, we relied on light and fluorescent microscopy. For improving clarity, we used a differential staining technique using both Hoechst 33,258 and Giemsa stain. Combination of Hoechst and Geimsa was earlier reported for staining sister chromatids and had shown to increase the resolution of its banding pattern [46]. By this technique, light microscopic images provided a clearer view of cell morphology and N(ET) release, when compared to fluorescent microscopic images. In these images, DNA strands were observed as deep purple threads. In our experiment to examine the extent of monocyte-mediated removal of NET-DNA, an increased extracellular DNA level was observed in contrary to our expectation. This extra DNA was found to be originated from the monocytes as a result of ET formation, which was initiated within 10 min of NS exposure. In order to rule out the possibility of residual SCS components that might be present in NS in inducing similar effects, we conducted the cover-slip experiment. In this, after SCS treatment on neutrophils, the residual SCS-components were washed off with PBS. These neutrophils when given to monocytes, showed the release of extracellular DNA confirming that NET components could trigger monocyte ETosis.

Though about 20% of monocytes underwent ETosis, some of the monocytes still were seen engaged in phagocytosis and removal of NET-DNA effectively. This was evident from the results of cytochalasin D treatment wherein we observed increased extracellular DNA. Cytochalasin D is an inhibitor of actin polymerization and thereby it inhibits phagocytosis. Since the concentration of cytochalasin D (10 μg/ml) used in this study does not interfere with extracellular trap formation [47], this elevated extracellular DNA content could be solely due to the inhibition of monocyte uptake of NETs. So, this observation together with microscopic observation suggests the involvement of at least a subset of monocytes engaged in phagocytosis and another subset involved in ETosis. Just as observed in the case of monocytes, macrophage subsets involved in the removal of NET-DNA and a subset releasing ET were already reported [48]. But they observed ET release within 3–4 h unlike the more rapid (within 10 min) ET release of monocytes observed by us. The ability of monocyte to release ET is recently known [32], but the involvement of monocytes in NET removal and subsequent ET release from a subset is a new finding. Though histone, a major component of DNA was shown to inhibit monocyte efferocytosis [49], we did not observe any such direct influence of NS on monocytes efferocytosis.

Proteins associated with NET-DNA are highly toxic, pro-inflammatory and are responsible for severe bystander tissue damage. In line with this, de-proteinated NET-DNA was shown to be less cytotoxic [50] and was shown to abolish cytokine production [51]. Triggering ET release by monocytes as demonstrated here could be another effect of these proteins. Experiments using inhibitors proved that this is at least partly due to neutrophil elastase and citrullinated histones present in the medium. Neutrophil elastase is a granular protein entangled in NET and studies have reported that the translocation of elastase with the aid

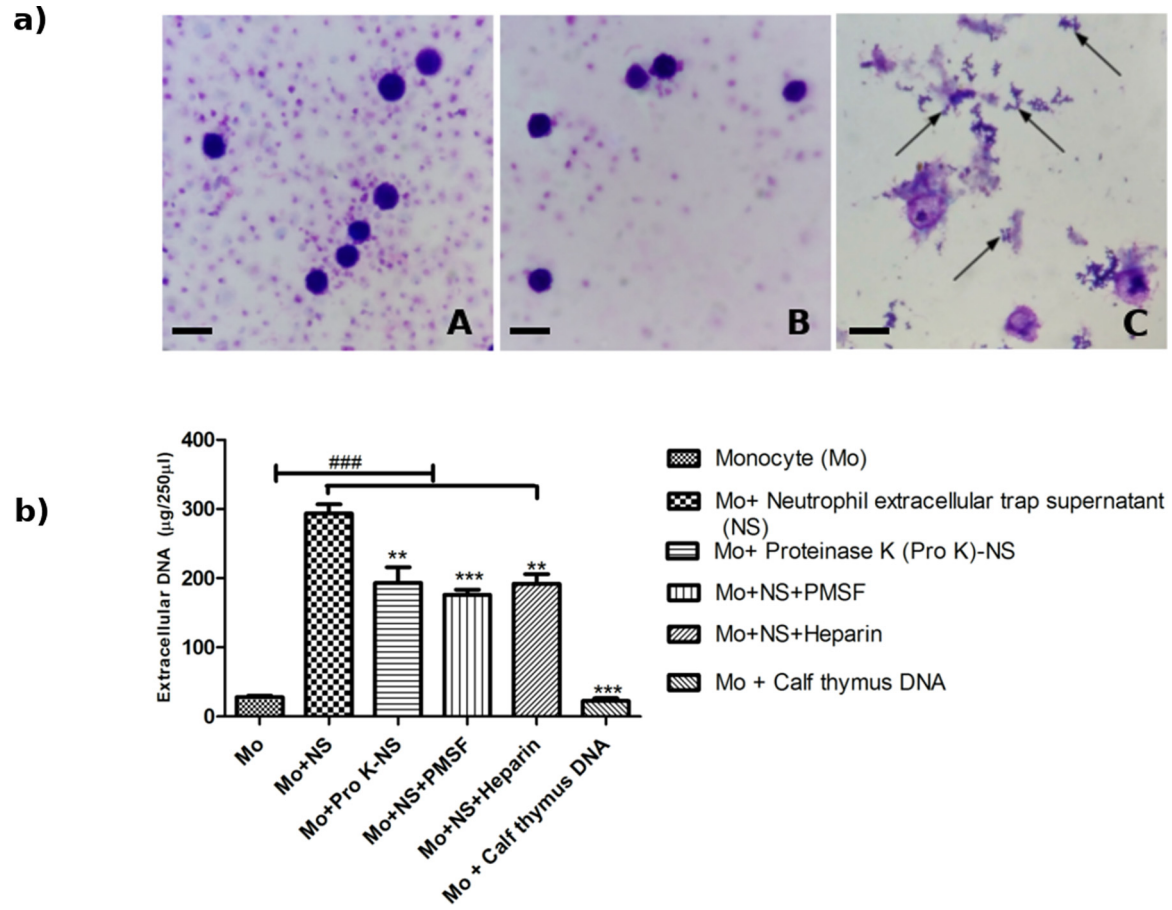


**Fig. 6.** Effect of NET-protein on monocyte ETosis (a). (A) Monocytes treated with proteinase K-treated-NS showing no signs of ET release. (B) Monocytes exposed to boiled NS showing no ET release. (C) Monocytes exposed to DNase-treated-NS showing ET release but released ET-DNA was fragmented (black arrow) (400x). Quantification of extracellular DNA (b). Monocytes in presence of proteinase K-treated-NS, PMSF and heparin showed a significant decrease in extracellular DNA level compared to NS-treated monocyte. Calf thymus DNA treated monocytes showed no DNA release, **p < 0.01, ***p < 0.0001. Scale bar 2 µm.

of MPO is a prerequisite for NET production [52]. In addition to its role in neutrophil NETosis, elastase released from neutrophil influences monocyte differentiation [53], enhances cytokine production [54] and procoagulant protein expression in monocytes/macrophages [55]. Though the effect of citrullinated histone on cell functions was less studied, the ability of histone in cell activation and organ damage has been widely reported [56,57]. Histone also induces NETosis and MPO release in human neutrophils [56]. Altogether, these proteins may initiate the proinflammatory process and may cause tissue damage. Though the effects of NET proteins on monocytes are reported here for the first time, earlier studies has identified some pro-inflammatory effects for NET-DNA on macrophages. Nakazawa et al. (2016) reported a phenotype-dependent increase in extracellular DNA production in macrophages upon exposure to NETing neutrophils. However, these macrophages completely removed extracellular DNA by caspase-activated DNase dependent mechanism [48]. Even so, sustained exposure of NET can result in cell death in macrophages and dendritic cells via mitochondrial damage [58,59]. Extent and nature of NET release vary between different individuals and so does its deleterious effects. The number of blood samples used in the experiment, however, was not adequate in analysing the variations in NET release observed with different donors. As this was beyond the primary objectives of the study, we did not investigate NET release from more individuals.

At the site of inflammation, cells may undergo different modes of cell death such as necrosis [60], apoptosis [61] and NETosis [62]. These modes of cell death occur simultaneously during inflammation and their concurrent presence over a prolonged time will follow autoimmune reactions and hence, these dying cells have to be cleared off at the earliest. These dying cells often communicate with macrophages/monocytes for their proper clearance. Since neutrophils undergo constitutive apoptosis at inflammation site, we used apoptotic neutrophils to assess the ability of monocytes to remove them in the presence of NET. $CD16^+$ monocytes are reported to be responsible for phagocyting apoptotic bodies. They do so in an immunologically silent manner by reducing proinflammatory cytokine production in them [31]. However, in adhered neutrophils, apoptotic bodies induced NETosis and neutrophils phagocyting apoptotic bodies were non-responsive to NET-inducing stimuli [63]. In our study, in the presence of NS, apoptotic bodies were cleared efficiently without an additional increase in the DNA level. Also, we observed a significant decrease in their DNA level when compared to the DNA levels observed in monocytes exposed to NS alone. The recognition of receptors in apoptotic bodies and their internalization along with entangled DNA might have resulted in enhanced NET-DNA clearance. In support of this speculation, we have observed apoptotic bodies entangled in N(ET) threads.

Inflammatory response accounts for apoptotic, necrotic and NETotic cell death and triggers the release of DNA and DNA-associated proteins into the circulation. Usually, these DNAs are degraded by DNase I and they are cleared by monocytes/macrophages within no time. Elevated inflammation in chronic inflammatory conditions results in panoptic cell death and the level of circulating cell-free DNA is increased with a proportional decrease in its clearance rate. This cell-free DNA can act as danger-associated molecular patterns that can trigger tissue damage. Here, we showed that even in the presence of this cell-free DNA, a set of monocytes can efficiently phagocytose apoptotic bodies or DNA strands, though another set of monocytes can release ETs in response to

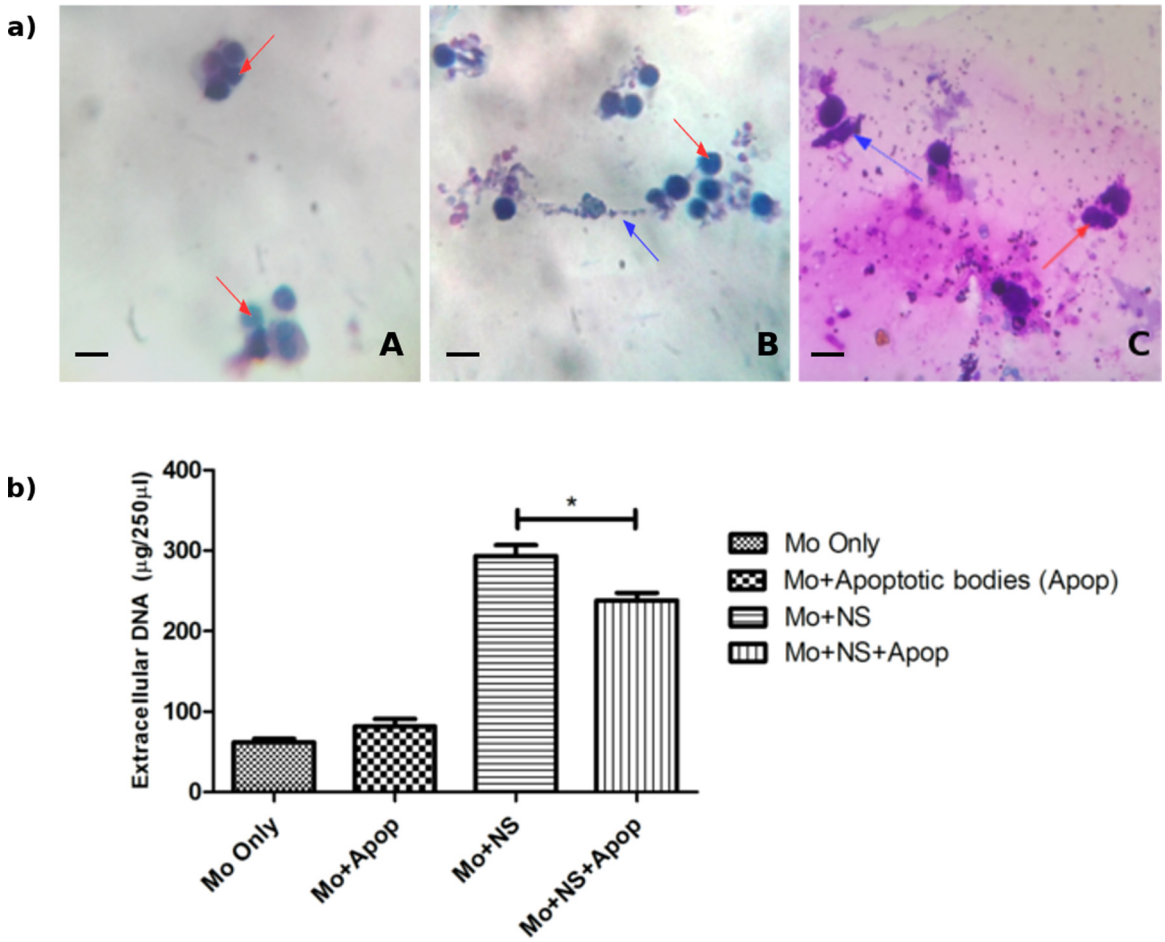


**Fig. 7.** Efferocytosis by NS-treated monocytes under light microscopy (1000x) (a). (A) Monocytes (brown arrow) in presence of NS phagocyting apoptotic neutrophils (red arrow). (B) Apoptotic body entangled in NET-DNA (blue arrow). (C) Monocytes phagocyting NET-DNA (blue arrow) like structures and apoptotic bodies (red arrow). Quantification of extracellular DNA in the supernatant of monocytes treated with NS + apoptotic bodies. (b). Monocytes in presence of Apoptotic bodies and NS showed a significant decrease in DNA compared to NS-treated monocyte (*$p < 0.05$) suggesting an increase in the removal of NET-DNA in presence of apoptotic bodies. Scale bar 2 µm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

the NET-proteins associated with it. So, just like macrophages, there must be different subsets of monocytes that are determined to undergo different modes of cell death. Since protein components of NET are involved in triggering NETosis/ETosis, these proteins can be a therapeutic target for reducing inflammation and treating autoimmune diseases.

## 5. Conclusion

Neutrophil extracellular trap plays a very crucial role in defending pathogens. NET-DNA and NET-related proteins can modulate different cells of innate immunity and thus, can determine the magnitude of inflammatory response. In this study, we report that peripheral blood monocytes undergo ETosis when encountered with NET components, especially the protein part of the NET. However, internalisation of apoptotic neutrophils is not affected in the presence of NET. Contrarily, apoptotic bodies are preferentially taken in by monocytes though it can remove both apoptotic body and NET-DNA. Thus, the study gives insight into the behavior of monocytes in a complex inflammatory milieu overwhelmed with apoptotic bodies, cell-free DNA/proteins and other remnants released from dead cells. The study also demonstrated the involvement of NET and NET components in modulating monocytes.

## Conflict of interest

None of the authors have conflict of interest.

## Authorship

The concept and design of the study were formulated by Y Anie and V H Haritha. Acquisition of data were done by V H Haritha, P Seena and T U Nithin. Result analysis and interpretation was performed by V H Haritha, P Seena, Binchu V Shaji, V N Hazeena and Y Anie. The manuscript was prepared by Binchu V Shaji, V H Haritha, and Y Anie.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imlet.2019.02.001.

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