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Neutrophil Extracellular Trap Formation: A Single Cell Event?

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Abstract

In 2004, neutrophil extracellular traps (NETs) have been described as fundamental immune defense of neutrophils against various microbes. Since that time publications are increasing that characterize the stimuli and cellular mechanisms which can activate the cells to release NETs. However, it is still not entirely clear whether NET formation starts as a single cell event, that spreads by cell to cell communication or if neighboring cells undergo NET formation simultaneously caused by a common trigger. Using immunofluorescence microscopy, we here show that at lower cell density only single cells were detected that released NET structures. However, the higher the cell density, the higher was the x-fold-increase of NET formation in PMA stimulated cells compared to unstimulated cells. This might give a hint that NET formation might start as a single cell event but is able to spread due to cell communication. To fully understand the mechanisms mediating NET formation, future experiments should focus on single cell analysis to characterize the detailed cellular events that mediate formation of NETs in single cells within a population and to differentiate the signaling process that leads to NET formation in contrast to other antimicrobial strategies as phagocytosis or degranulation.

Keywords: Neutrophils; Neutrophil extracellular traps; Cell density; Immunofluorescence microscopy; Phorbol-12-myristate 13-acetate

Commentary

Within the innate immune system, neutrophils are a key player of the first line of defense against pathogens. They exert a variety of intra- and extracellular antimicrobial functions and additionally contribute in processes of tissue remodeling and tissue repair [1]. Besides phagocytosis and degranulation, extracellular trap formation has been described as an additional fundamental antimicrobial activity of neutrophils [2]. Those neutrophil extracellular traps (NETs) are decondensed chromatin structures which contain antimicrobial components like histones and proteases as well as antimicrobial peptides and can enable the immobilization of microbes. NET formation can be activated by pathogens or microbial derived factors like lipopolysaccharide (LPS), proinflammatory cytokines (IL-8) [2] or proinflammatory substances like phorbol-12-myristate 13-acetate (PMA) [3]. PMA is often used as a positive control in in vitro experiments [2-4], since PMA has been shown to efficiently induce NET formation via increasing the oxidative burst in the cell [3-6]. However, the regulatory mechanisms mediating NET formation are still not fully understood. Whether NET formation starts as a single cell event that spreads by cell to cell communication or if several cells undergo NET formation simultaneously, caused by a common trigger, is still not entirely clear.

Here, we want to highlight that only single cells may initiate the formation of NETs in a population and, thus, single cell analysis is needed in the future to understand the detailed mechanisms in NET formation and to differentiate the process of NET formation from other antimicrobial strategies as phagocytosis or degranulation. Therefore, ex vivo NET induction experiments using human primary blood-derived neutrophils were conducted. The neutrophils were isolated from freshly heparinized blood of healthy donors as previously described [7] and seeded on poly-L-lysine-coated (0.01%, Sigma) cover slides in 24-well plates at a concentration of 5×10^4, 1×10^5 or 5×10^5, cells/well (250 µl/well). RPMI 1640 (PAA) was used for cultivation of the cells at 37°C and 5% CO_2. 25 nM PMA (InvivoGen Corp., San Diego, CA, US), as NET inducing agent, was applied as positive control and RPMI 1640 medium alone as negative control. After incubation, the cells were fixed by adding PFA (#15710 Electron Microscopy Science) at a final concentration of 4% for 15 min at room temperature. Subsequently the samples have been immune stained with a 1:5000 dilution of a mouse monoclonal anti-histone antibody H1-DNA complex (Millipore MAB3864, 2.2 mg/ml in 2% BSA in 0.2% Triton X-100/PBS), additionally incubated with an Alexa-Fluor-488-labelled goat-anti-mouse antibody (1:1000; Invitrogen), mounted in ProlongGold® antifade with DAPI (Invitrogen) and analyzed by confocal fluorescence microscopy using a Leica TCS SP5 confocal microscope with a HCX PL APO 40 × 0.75-1.25 oil immersion objective. Different cell densities were used to determine if there is a difference in NET formation detectable depending on the amount of cells seeded.

As already well known from the literature [3,7], the fluorescent micrograph in Figure 1 confirms that only single cells (arrow) in the population release NET structures (green) after 2h of incubation with PMA. Interestingly, experiments comparing different cell densities show that the higher the cell density, the more NETs were detected in PMA stimulated cells compared to non-stimulated cells (Figure 2 and Supplemental Figure 1): The x-fold NET induction after PMA treatment compared to the control significantly increases with higher cell densities (Figure 2).

Based on these data, it may be hypothesized that during NET formation, neutrophils release factors that stimulate neighboring neutrophils to additionally form NETs and thereby increase the overall NET formation at higher cell densities in a population. Those triggering factors might be any proteins that have been found to be associated with NETs e.g. elastase, myeloperoxidase (MPO) or LL-37, since it is also

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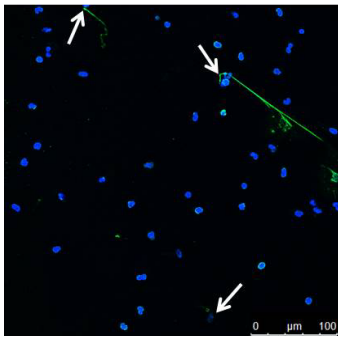

Figure 1: Representative fluorescent micrograph of neutrophils after a 2h incubation with 25 nM PMA at 37°C, 5% CO_2. Formation of NETs (white arrow) was visualized microscopically, using an antibody against H1-DNA complexes (green) in combination with DAPI (blue) to stain the nuclei.

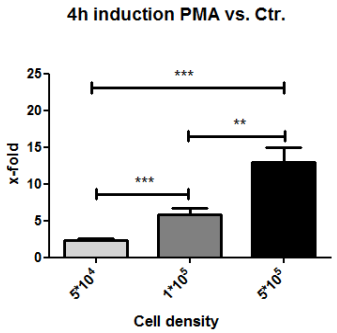

Figure 2: NET release after 4h of incubation at 37°C, 5% CO_2 for different cell densities. The x-fold increase in the amount of PMA treated cells releasing NETs in comparison to unstimulated cells increases with the cell density. NET releasing cells were counted using the Image J software. Data were analyzed using Excel 2010 (Microsoft) and GraphPad Prism 5.0 (GraphPad Software). Due to donor-specific variations in spontaneous NET release, each experiment was performed with neutrophils derived from a minimum of three independent healthy blood donors. For each preparation, a minimum of six randomly selected images were acquired per slide and used for quantification of NET producing cells. Differences were analyzed by using a one-tailed Student's t-test. The significance is indicated as **p<0.005 and ***p<0.001.

known that especially those three factors contribute to NET formation: Whereas MPO and elastase have been shown to mediate degradation of histones associated with nuclear decondensation prior to NET formation [8], the cathelicidin LL-37 seems to be involved in disruption of the nuclear membrane and thereby facilitates formation of NETs [9]. Thus, it might be hypothesized that the closer the proximity of the individual neutrophils, the stronger might be the impact of those factors to subsequently also trigger the release of NETs in neighboring neutrophils. Current work should therefore also focus on the question, if and how activated neutrophils can stimulate neighboring cells to release NETs. But to evaluate and understand the exact mechanisms of NET formation and especially to differentiate the process of NET formation from other antimicrobial strategies in the same neutrophil population as phagocytosis or degranulation, it will be very important to perform single cell analysis in future experiments.

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