



The Netosis Formation of HL-60 Cell Differentiated to Neutrophil-Like Cells by LPS



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Ehsan Moghanloo ^a  | Elham Ghorbani ^b | Mohammad Sadegh Beikverdi ^c | Parisa Badameh ^d | Soheila Rezaei ^e | Ahmad Piroozmand ^f | Shahram Teimourian ^j | Minoo Shahidi ^h | Ahmad Khorshidi ^{a,*} 

^a Department of Microbiology and Immunology, School of Medicine, Kashan University of Medical Sciences, Kashan, Iran.

^b Department of Genetics Campus2, Guilan University of Medical Science, Rasht, Iran.

^c Department of Biology Islamic Azad University Central Tehran Branch, Tehran, Iran.

^d Department of Microbiology, College of Basic Science and Biology, Varamin Pishva Branch, Islamic Azad University, Varamin, Iran.

^e Department of Molecular Genetic, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran.

^f Autoimmune Diseases Research Center, School of Medicine, Kashan University of Medical Sciences, Kashan, Iran.

^j Department of Medical Genetics, Iran University of Medical Sciences, Tehran, Iran.

^h Department of Hematology, School of Allied Medicine, Iran University of Medical Sciences, Tehran, Iran.

*Corresponding author: Ahmad Khorshidi

Department of Microbiology and Immunology, School of Medicine, Kashan University of Medical Sciences, Kashan, Iran, 4719115875. Tell: +98-9366883257.

E-mail address: akhorshidi342@gmail.com

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ABSTRACT

Background: Neutrophils are the most abundant white blood cells in humans. Recently, a novel strategy called the formation neutrophil extracellular traps (NETs) was described. NETs is a new strategy for pathogen response. This study focused on whether LPS induced NETs release *in vitro* in the HL60 cell line.

Methods: In this study, the HL60 cell line was used for culture and DMSO for induction and differentiation. Flow cytometry was used to evaluate CD11b in the differentiated cells, and the NBT assay was used to evaluate the functionality of the differentiated HL-60 cells. Neutrophil-like cells were incubated with LPS (200 ng/ml) for 45 min, followed by incubation for 25 min with 100 ng/ml Hoechst 33342. Trypan blue as vital staining was used for viability. The statistical significance of the difference between the control and treated groups was evaluated using a one-way ANOVA.

Results: Our results showed that 75% NETs was produced by HL-60 differentiated neutrophil cells exposed to 200 ng/ml LPS in 45 min.

Conclusion: Consequently, the LPS-induced infection and lethality may occur through various mechanisms. Thus, understanding the molecular mechanisms regulating NET formation in LPS-induced neutrophil-like cells would support the development of new therapeutic methods.

1. Introduction

Neutrophils as the most abundant white blood cells in the human circulation are one of the first-line innate immune defense cells and play a critical role in the host innate immune response against invading pathogens [1].

During inflammation or infection, neutrophils are recruited to the site of injury and provide protection by eliminating potential threats via phagocytosis or release of bactericidal substances [2].

However, improper activation of neutrophils leads to the pathological processes of inflammatory diseases and thrombotic complications; therefore, identifying the key molecular mechanisms involved in neutrophil activation would be helpful. Infectious bacterial diarrhea is the most common and acute type of diarrhea, the two forms of which are watery diarrhea and bloody diarrhea, also known as dysentery [3]. Recently, a novel strategy was described, that is, the formation of so-called neutrophil extracellular traps (NETs). NETs consisting of chromatin and proteins are produced to trap and kill pathogens [4, 5]. NETs prevent the spread of pathogens by trapping. They also enhance viscosity at the site of infection. This barrier along with granules existing on NETs can kill and clear pathogens by destroying the pathogens' cell wall. Prevention of pathogen spreading has a negative effect on their virulence. *In vitro*, NETs are released from neutrophils activated by phorbolmyristate acetate, interleukin-8 (IL8), lipopolysaccharide (LPS), and various pathogens [5]. LPS persuades the release of critical proinflammatory cytokines that are essential to activate potent immune responses [6]. NET formation can be also induced by gram-positive bacteria such as *Staphylococcus aureus*, *Mycobacterium tuberculosis*, and *Streptococcus pyogenes* [7]. Recently, research on NETs has focused not only on exploring their physiological role, but also on their pathophysiological relevance in various diseases including atherosclerosis [8], thrombogenesis [9], cancer metastasis [10] and autoimmune disease [11]. Therefore, this study focused on whether LPS induced NETs release *in vitro* in the HL60 cell line.

2. Materials and Methods

2.1. Cell Culture and Induction

HL-60 cells, obtained from the Cell Bank of the Pasteur Institute of Iran (Tehran, Iran), were grown in a 5% CO₂ incubator in the RPMI 1640 medium (Sigma, Taufkirchen, Germany) supplemented with 2 mmol/l l-glutamine containing 10% (v/v) heat-inactivated fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in 5% CO₂. Exponentially growing cells were induced to differentiate with 1.25% (v/v) DMSO (sigma-Aldrich, Germany) after 6, 7, and 8 days of incubation.

2.2. Flow Cytometry

To assay the differentiation of the HL-60 cells, cellular surface differential antigen CD11b was detected, as previously described by Teimourian and Moghanloo (2015) [12]. Briefly, for flow cytometry, the HL-60 cells (10⁶ cells/ml treated with 1.25% DMSO) were washed in freshly made phosphate-buffered saline (PBS) and incubated with the PE-conjugated CD11b (ITGAM integrin alpha M) antibody (Pharmingen Co., Tehran, Iran) or PE-conjugated IgG1 isotype control antibody at 4°C for 30 min. RPMI 1640 was used as a blank control. Flow cytometry was performed on a FACScan flow cytometer instrument (Becton Dickinson, Mountain View, CA, USA) using the Cell Quest software. The percentage of the CD11b-positive cells and the extent of expression of CD11b on their surface were determined from 1 × 10⁶ cells for each group.

2.3. The NBT Assay

The colorimetric nitroblue tetrazolium assay (NBT, Sigma) was used to assay the functionality of the differentiated HL-60 cells. Cells (5 × 10⁵/well) adhered to cover glasses were treated with 100 µl of the 1 mg/ml NBT solution containing 200 ng/ml LPS and incubated at 37°C in 5% CO₂ for 30 min [13]. Afterwards, the cells were centrifuged for 5 min and then subjected to Wright's staining. Followed by NBT absorption by the cells, the intracellular dye was reduced to insoluble blue formazan crystals by superoxide generated by the NADPH oxidase enzyme [14]. The percentage of cells containing blue formazan particles (NBT-positive cells) was determined by evaluating 200 randomly selected cells under a light microscope (Olympus CX-40, Olympus, Japan).

2.4. Visualization of Neutrophil Extracellular Traps (NETs)

To confirm the presence of the NETs, they were visualized with the DNA Hoechst 33342TM stain, as previously described [12]. Briefly, the cells suspended in the RPMI 1640 medium supplemented with 2% heat inactivated bovine serum were seeded on 12 mm 0.001% polylysine-coated coverslips (2 × 10⁴/well in 24-well plates) and incubated with LPS (200 ng/ml) for 45 min, followed by incubation for 25 min with 100 ng/ml Hoechst 33342 (sigma-Aldrich, Germany) after 6, 7, and 8 days of incubation. Release of the NETs was quantified *in vitro* with Hoechst 33342 staining alone of cells exposed to the control condition. Laser excitation of each well was performed at 350 nm (Hoechst 33342TM – emission 461 nm) prior to microscopy. Fluorescent microscopy was performed using an Olympus IX70 inverted microscope (Olympus, NSW, and Australia) with appropriate filter for Hoechst 33342TM (blue). The differentiated HL-60 cells without stimulation as well as the differentiated HL-60 cells stimulated with 200 ng/ml LPS in the same condition were used as negative and positive controls, respectively.

2.5. Trypan Blue Staining

Viability was studied by the Trypan Blue dye exclusion assay. The PBS-suspended cells were supplemented with 0.4% (w/v) trypan blue solution (Sigma; Taufkirchen, Germany) and incubated for 5 min. Two hundred cells were counted manually for each cell sample, and the proportion of dead (stained) cells in each population was determined.

2.6. Statistical Analysis

Every assay was performed independently with duplicate samples and repeated three times. Statistical analysis was performed using the Mann-Whitney U-test. The statistical significance of the difference between the control and treated groups was evaluated using a one-way ANOVA. The criterion for statistical significance was taken as $P < 0.05$.

3. Results and Discussion

Monitoring CD11b expressed by the DMSO-induced HL-60 showed an increased levels over time (Figure 1). The maximum impact on cell differentiation to neutrophil cells was on the eighth day of incubation with at least 79.2%. Here, also, differentiation showed a stable level after 8 days.

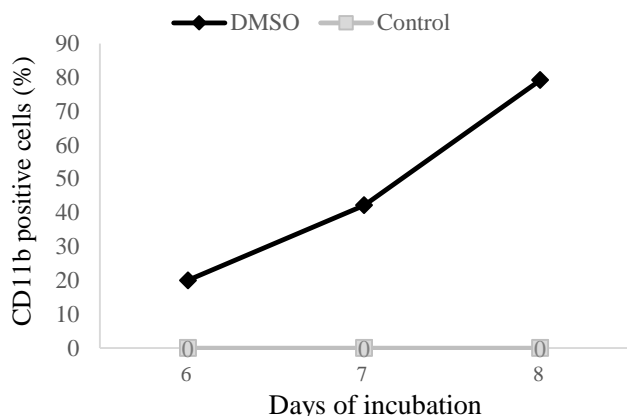


Figure 1: The number of neutrophil cells differentiated from the HL-60 cells by DMSO after 6, 7, or 8 days of incubation; control: untreated HL60

The representative flow cytometric profiles of the CD11b positive HI-60 cells (Figure 2) after 8 days of incubations also showed that differentiation reached almost the maximum level during these days.

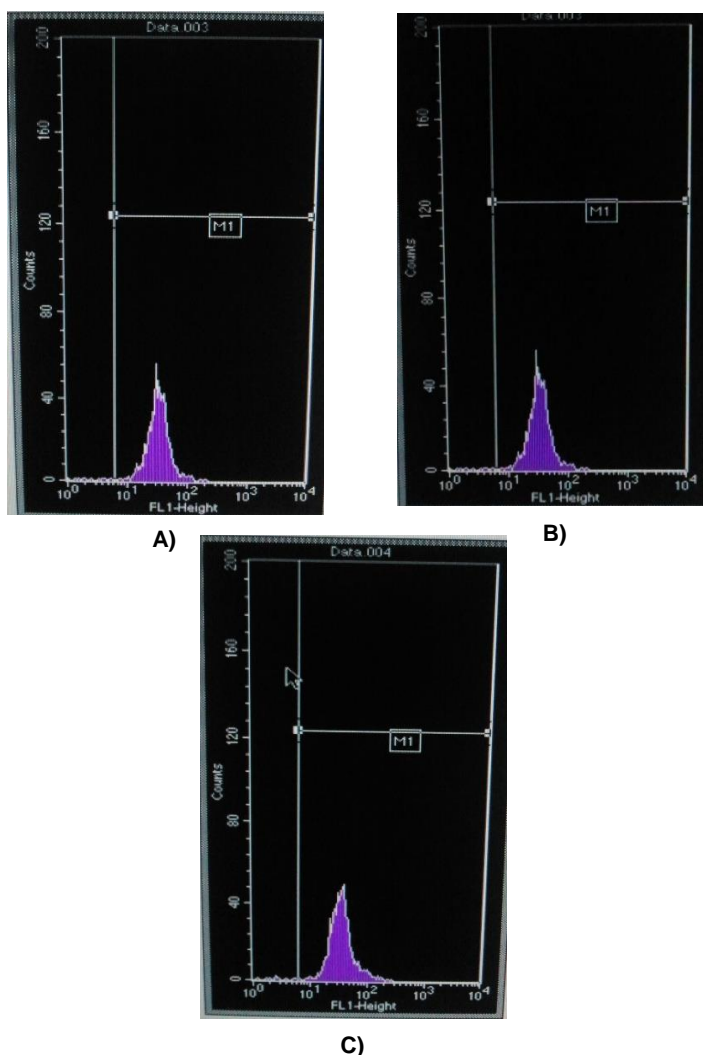


Figure 2: The representative flow cytometric profiles of the CD11b positive HI-60 cells after A) 6, B) 7, and C) 8 days

The nitro blue tetrazolium (NBT) test was performed to study functionality. Here, the ability to reduce NBT after incubation with LPS was determined by microscopically evaluating formazan deposition in the 200 randomly selected cells. An NBT slide to study the functionality of the differentiated HL-60 cells showed nearly similar results as flow cytometry. The NBT slide was dark blue with no NET generation in the absence of DMSO in parallel. As shown, The DMSO treatment dramatically increased the Farmazon formation (Figure 3), meaning that the percentage of the HL-60 cells able to reduce NBT and express CD11b increased during the days of differentiation.

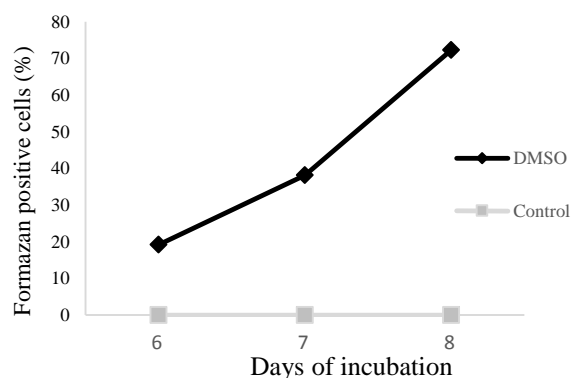


Figure 3: The number of formazan positive cells after 6, 7, or 8 days of incubation; control: untreated HL60

The LPS-induced cells were treated with nuclear indicators Hoechst 33342, which is excited at 380 nm and visualized by fluorescent microscopy (Figure 4).

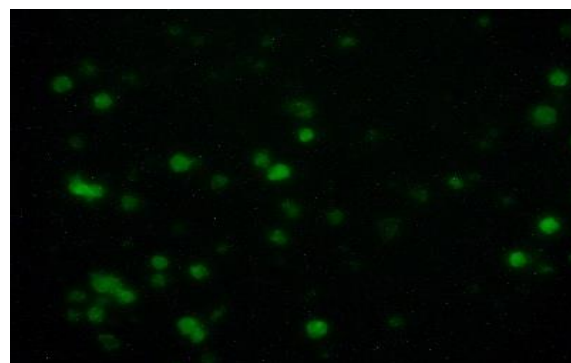


Figure 4: The representative images of neutrophil cells stained with Hoechst33342 and NET release

Our results showed that 75% NETs were produced by the HL-60 differentiated neutrophil cells exposed to 200 ng/ml LPS in 45 minutes (Figure 5). differentiated from the HL-60 cells by DMSO after 7 days of incubation. The percentage of formed NETs is shown on the Y axis. Assay was performed independently with duplicate samples and repeated three times.

To detect viable differentiated HL-60 cells, the trypan blue (TB) method as a common cell viability analysis was used.

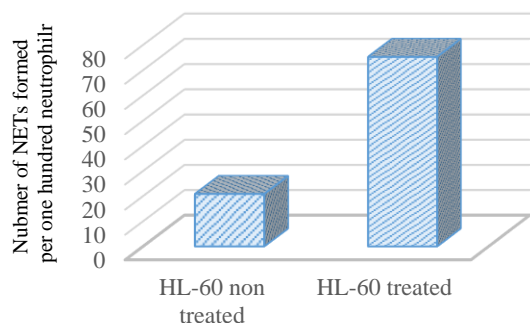


Figure 5: The number of the LPS-stimulated NETs formed per one hundred neutrophil cells

To this end, 104 cells in 24-well plates were incubated for 6, 7, and 8 days. Then, the number of stained cells in each well was determined by 0.4% TB staining. The proportion of viable cells after HL-60 cell differentiation induction by the DMSO is shown subsequent to 6, 7, or 8 days of incubation. Followed by DMSO induction, cell viability was significantly reduced, such that within the seventh day of induction, dead cells were almost 10.24% less than those on the eighth day (Figure 6). Thereupon, the most viable differentiated cells should be found after 7 days of incubation.

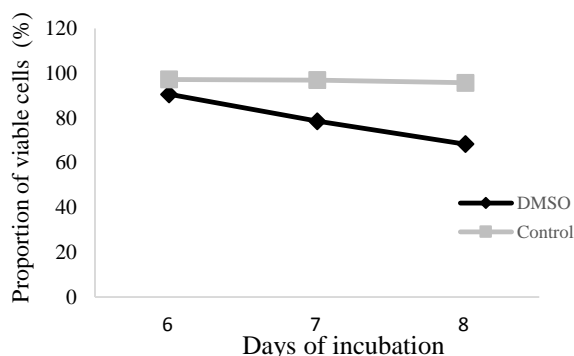


Figure 6: The proportion of viable cells after 6, 7, or 8 days of incubation; control: untreated control

LPS was chosen to induce NET formation because it is known to be involved in the pathophysiology of sepsis and other infectious and inflammatory disorders [15]. Moreover, LPS induces histone H3 citrullination and autophagy in mature neutrophils [16]. In a study performed by Liu and his colleagues, LPS showed NETs release against acute lung injury in mice upon direct and indirect contact with neutrophils [17]. Here, to study the LPS role in DMSO-differentiated neutrophil-like cells, viable cells from different populations of differentiated HL-60 cells were stimulated by LPS after 7 days. Reportedly, hypercitrullination of histone H3 plays an important role in chromatin decondensation in an early stage of NETs formation [18]. Therefore, to detect NET formation in vivo, intracellular chromatin decondensation was performed using the Hoechst 33342 stain. The ability to form NETs illustrates the degree of cellular differentiation [15].

The differentiation process of neutrophils could clarify hematopoietic diseases that affect neutrophils and/or myeloid differentiation, such as neutrophil function disorders [19]. The HL-60 cell line is a traditional model appropriate to study the differentiation process. This cell line is a promyelocytic cell line, a subtype of acute myelogenous leukemia, which grows well and differentiates easily into neutrophils by compounds such as dimethyl sulfoxide (DMSO) or all-trans retinoic acid (ATRA) [20]. Based on our previous results, DMSO increases the amount of neutrophil-like cells and proportion of apoptotic cells during 6–8 days of incubation [21]. Therefore, here, DMSO was used to induce the differentiation. The flow cytometry analysis of antibodies specific for CD11b was employed to study the differentiation induced by DMSO since CD11b as a neutrophil adhesion molecule is an appropriate terminal differentiation marker [22]. The maximum impact on cell differentiation to neutrophil cells was on the eighth day of incubation with at least 79.2%, same as the previous report on the effect of RPMI on DMSO-differentiated HL-60 cells [21].

NBT forms formazan derivative in reaction with cellular superoxide ions. Formazan can be monitored spectrophotometrically [23]. The relationship between microbicidal function and dye reduction followed by precipitating the formazan due to NBT reduction indicates that NBT test is a valuable index for functional capacity of neutrophils [24].

TB as a vital stain makes a distinctive blue color in nonvital cells under a microscope since nonvital cells lack functional membrane and absorb dye from their surroundings [25].

Our data revealed an *in vitro* functional mature neutrophil differentiation system and NET formation by LPS. McInturff in consistent with our findings also showed that differentiated HL-60 leukocytes formed NETs in response to LPS stimulation[26]. However, Liu et al. declared that neutrophils failed to form NETs when they directly contacted LPS, suggesting that platelets are necessary for NETosis triggered by LPS [17]. Consistently, Clark et al. reported that LPS induced platelet-neutrophil interactions in vivo only at higher levels and led to neutrophil activation and NETs formation in a platelet-dependent manner [27]. Shimomura et al. also found that neither LPS nor platelets alone caused NETosis in cultured neutrophils obtained from blood samples [28].

It is also found that induced NET formation is stimulated by the expression of hypoxia-inducible factor 1 α (HIF-1 α) protein in a mammalian target of rapamycin (mTOR)-dependent manner, which may be activated by inflammatory agonist such as the platelet-activating factor [26]. The inconsistency why LPS-induced NETs are detected in DMSO-differentiated HL-60 cells but not in blood isolated neutrophils may suggest different pathways to activate mTOR or HIF-1 α regulatory proteins to lead NET formation and clarifying such controversial results need comparing the biochemical pathways involve in stimulating differentiated neutrophil-like HL-60 and natural neutrophil blood cells.

4. Conclusion

Based on our observations, the NETs formation in the HL-60 differentiated cells during LPS-induction suggested the presence of direct interactions between neutrophils and gram-negative bacteria, in addition to indirect LPS-stimulated platelets. Consequently, LPS-induced infection and lethality may occur through various mechanisms, and exploring the molecular mechanisms regulating NET formation in LPS-induced neutrophil-like cells would support the development of new therapeutic methods.

Authors' Contributions

E.M. and A.Kh., designed the manuscript; E.Gh., Performed the statistical analysis; M.S.B., and S.R., wrote the protocol; E.M. and P.B., wrote the manuscript; E.M. and P.B. and M.S.B., Managed the analyses of the study and managed the literature searches. All authors read and approved the final manuscript.

Conflict of Interest

The author report no conflict of interest.

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