Video Article

Neutrophil Extracellular Traps: How to Generate and Visualize Them

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URL: http://www.jove.com/video/1724
DOI: doi:10.3791/1724

Keywords: JoVE Immunology, Issue 36, Neutrophil, Granulocyte, Neutrophil Extracellular Trap, NET, isolation, immunolabeling, electron microscopy

Date Published: 2/24/2010

Citation: Brinkmann, V., Laube, B., Abu Abed, U., Goosmann, C., Zychlinsky, A. Neutrophil Extracellular Traps: How to Generate and Visualize Them. *J. Vis. Exp.* (36), e1724, doi:10.3791/1724 (2010).

Abstract

Neutrophil granulocytes are the most abundant group of leukocytes in the peripheral blood. As professional phagocytes, they engulf bacteria and kill them intracellularly when their antimicrobial granules fuse with the phagosome. We found that neutrophils have an additional way of killing microorganisms: upon activation, they release granule proteins and chromatin that together form extracellular fibers that bind pathogens. These novel structures, or Neutrophil Extracellular Traps (NETs), degrade virulence factors and kill bacteria[1], fungi[2] and parasites[3]. The structural backbone of NETs is DNA, and they are quickly degraded in the presence of DNases. Thus, bacteria expressing DNases are more virulent[4]. Using correlative microscopy combining TEM, SEM, immunofluorescence and live cell imaging techniques, we could show that upon stimulation, the nuclei of neutrophils lose their shape and the eu- and heterochromatin homogenize. Later, the nuclear envelope and the granule membranes disintegrate allowing the mixing of NET components. Finally, the NETs are released as the cell membrane breaks. This cell death program (NETosis) is distinct from apoptosis and necrosis and depends on the generation of Reactive Oxygen Species by NADPH oxidase[5].

Neutrophil extracellular traps are abundant at sites of acute inflammation. NETs appear to be a form of innate immune response that bind microorganisms, prevent them from spreading, and ensure a high local concentration of antimicrobial agents to degrade virulence factors and kill pathogens thus allowing neutrophils to fulfill their antimicrobial function even beyond their life span. There is increasing evidence, however, that NETs are also involved in diseases that range from auto-immune syndromes to infertility[6].

We describe methods to isolate Neutrophil Granulocytes from peripheral human blood[7] and stimulate them to form NETs. Also we include protocols to visualize the NETs in light and electron microscopy.

Video Link

The video component of this article can be found at http://www.jove.com/video/1724/

Protocol

1. PMN Isolation from human blood

Use about 24 ml human blood with EDTA or Heparin (10 U/ml) as anticoagulant.

1. Add 6 ml Histopaque 1119 to a 15 ml Falcon tube and carefully layer 5 to 7 ml whole blood on top.
2. Centrifuge for 20 minutes at 800 x g without braking.
3. Aspirate and discard yellowish and clear top layer and transfer lower reddish phase containing granulocytes into fresh Falcon tubes.
4. Wash cells by filling up Falcon tubes with PBS and centrifuge for 10 minutes at 300 x g.
5. In the meantime prepare a 100 % Percoll solution by mixing 18 ml Percoll with 2 ml 10x PBS. With 1x PBS prepare 4 ml solutions of 85 %, 80 %, 75 %, 70 % and 65 % Percoll.
6. Prepare 2 Falcon tubes with a Percoll gradient by layering 2 ml of every percentage on top of each other in decreasing order.
7. After centrifugation remove the supernatant, combine pellets and resuspend sedimented cells in 4 ml of PBS.
8. Carefully layer 2 ml of the resuspension onto each of the gradients.
9. Centrifuge for 20 minutes at 800 x g without braking.
10. After centrifugation remove top layer and most of the 65%-layer with PBMCs and collect white remaining interphases until 85%-layer into new Falcon tubes.
11. Wash cells by filling up the Falcon tubes with PBS and centrifuge for 10 minutes at 300 x g.
12. Remove supernatant and resuspend sedimented cells (usually >95% are PMN) in 2ml of PBS.
13. Count cells using a hemocytometer.

2. Activating PMNs

1. Prepare a 24-well cell culture plate by putting a sterile 13 mm round glass cover slip (# 1,5) into each well

2. Seed 2 x 10^5 cells in 500µl RPMI (containing 2 % human serum albumin) per well and incubate for 1h in CO_2 incubator at 37°C.
3. Meanwhile prepare a 600 nM PMA solution in RPMI and add to cells 100µl per well. Incubate from 15 min up to 4 h in CO_2 incubator at 37°C.
4. Fix cells in 4% (end concentration) paraformaldehyde dissolved in PBS.

PMA serves as a positive control and is until now the most potent agent to induce NET formation. Alternatively, other stimuli or co-cultivation with pathogens can be used for NET induction.

The respective status of NET formation can be checked while the time course is proceeding, if additional parallel samples are prepared. If a non-permeant DNA dye like Sytox Green (Invitrogen) is added to the non-fixed cells, only extracellular DNA will be detected. Since formation of new NETs is somehow impaired in the presence of Sytox, for each time point one parallel sample has to be used.

3. NET detection by immunolabeling

NETs are very fragile even after fixation and have to be manipulated with great care, otherwise the majority will get lost during the preparation.

1. Carefully remove glass cover slips with attached cells from 24-well culture plate by lifting it up at the edge with a curved needle and seize it with a fine forceps. Put the cover slip upside down on a drop of PBS. This can be done on a Parafilm sheet covering a test tube stand. Wash like this 3 times for 5 min.
2. Incubate cover slips in the same manner in a drop of 0.5 % Triton X-100 for 1 min at RT to permeabilize cells. Wash 3 times in PBS for 1 min.
3. Prepare a humid chamber with Parafilm and a wet tissue. Lay the cover slips upside down on a drop of blocking buffer (5% donkey serum) and incubate for 30 min at 37°C.
4. Dilute primary antibodies, e.g. ms anti Histon and rb anti Neutrophil Elastase, in blocking buffer.
5. Transfer cover slips in the humid chamber directly from blocking buffer onto a drop of primary antibody and incubate for 1 h at 37°C.
6. Wash 3 times for 5 min with PBS.
7. Dilute secondary antibodies, e.g. dk anti ms Cy2 and dk anti rb Cy3, in blocking buffer.
8. Transfer cover slips into the humid chamber onto a drop of secondary antibody and incubate for 1 h at 37°C.
9. Wash 3 times for 5 min with PBS. Depending on your fluorescence microscopy options, stain DNA for 5 min either with Hoechst 33342 (1 µg/ml) which will need UV excitation or with Draq5 for far red excitation and wash twice with dist. water.
10. Set a 20µl drop of Mowiol onto a glass slide and mount cover slips with cells upside down. The cells have to be between the cover slip and the slide. The drop of Mowiol will form a thin layer of homogenous thickness when the cover slip is positioned on the drop. Normally, there is no need to press the sample. If you want to use non-immersion lenses, the specimen is ready for inspection. For microscopic analysis with immersion lenses, let the specimen dry for about 1 hour until the Mowiol has solidified at the edge of the sample.

4. Preparing NETs for Scanning Electron Microscopy (SEM)

1. Post fix cells on the glass cover slips in 24-well plate with 2,5 % glutaraldehyde.
2. Remove glass cover slips containing cells from 24-well culture plate and put upside down on a drop of water. Wash like this 3 times for 5 min.
3. Transfer cover slips back into 24-well cell culture plate containing 0,5% OsO4 and incubate for 30 min.
4. Remove glass cover slips containing cells from 24-well culture plate and put upside down on a drop of water. Wash like this 3 times for 5 min.
5. Transfer cover slips back into 24-well cell culture plate containing 1% tannic acid and incubate for 30 min.
6. Repeat steps 4.2 to 4.4
7. Dehydrate through the following regimen (5min each):
    - 30 % ethanol
    - 50 % ethanol
    - 70 % ethanol
    - 80 % ethanol
    - 90 % ethanol
    - 100% ethanol
    - 100% ethanol
    - 100% ethanol
8. Transfer cover slips into critical point dryer and dry samples.
9. Coat surface of specimen with 5nm platin/carbon layer using thin layer evaporator

5. Representative Results:

The isolation method usually yields unstimulated viable neutrophils with a purity greater than 95%. When fixed at different time points after stimulation, the immunostaining protocol shows the sequence of morphological changes during NETosis cell flattening, loss of nuclear lobules, loss of granule and nucleus integrity which leads to an increasing overlap of nuclear (i.e. histone) and granular (i.e. Neutrophil Elastase) staining. This protocol can serve as a starting point to analyze the specific interactions of pathogens with neutrophils. This interaction can be dissected in greater detail using the preparation protocol for Scanning Electron Microscopy.

NET Fluorescence

Sample of stimulated neutrophils stained for NET components (blue = DNA, red = histone, green = Neutrophil Elastase). The images show, besides the NET localization, the nuclear localization of DNA and histones and the granular pattern for Neutrophil Elastase.

SEM PMA stimulation

Scanning electron micrograph showing non stimulated and PMA-stimulated neutrophils. After stimulation, the neutrophils flatten out and produce NETs.

SEM NETs and Shigella

Higher resolution SEM image of Shigella bacteria trapped in NETs.

Discussion

The provided protocol will allow the isolation of non stimulated neutrophils at considerable purity, the induction of NET formation and the analysis of morphological changes during NETosis. When handling the specimens with care, i.e. avoiding harsh washing conditions which will result in the loss of most of the loosely attached NETs, the amount of NET formation under different stimulation conditions (duration, stimulus) can be compared. In this respect, the provided protocols can serve as a starting point to establish methods to analyze more sophisticated scenarios: neutrophil/pathogen interactions, sequence of stimuli, interplay with other immune cells.

References

1. Brinkmann, V., *et al.* Neutrophil extracellular traps kill bacteria. *Science* **303**, 1532-1535,(2004).
2. Urban, C. F., Reichard, U., Brinkmann, V. & Zychlinsky, A. Neutrophil extracellular traps capture and kill Candida albicans yeast and hyphal forms. *Cell Microbiol* **8**, 668-676 (2006).
3. Guimaraes-Costa, A. B., Nascimento, M.T.C., Froment, G.S., Soares, R.P.P., Morgado, F.N., Conceicao-Silva, F., and Saraiva. E.M. Leishmania amazonensis promastigotes induce and are killed by neutrophil extracellular traps. *PNAS* **106**:6748-6753, (2009).
4. Buchanan, J.T., Simpson, A.J., Aziz, R.K., Liu, G.Y., Kristian, S.A., Kotb, M., Feramisco, J., and Nizet, V. DNase expression allows the pathogen group A Streptococcus to escape killing in neutrophil extracellular traps. *Curr Biol*. **16**:396-400 (2006).
5. Fuchs, T. A., *et al.* Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol* **176**, 231-241 (2007).
6. Brinkmann, V., & Zychlinsky, A. Beneficial suicide: why neutrophils die to make NETs. *Nat Rev Microbiol* **5**, 577-582 (2007).
7. Aga, E., Katschinski, D.M., van Zandbergen, G., Laufs, H., Hansen, B., Muller, K., Solbach, W., and Laskay, T. Inhibition of the spontaneous apoptosis of neutrophil granulocytes by the intracellular parasite Leishmania major. *J Immunol*. **169**:898-905 (2002).