Macrophage Clearance of Neutrophil Extracellular Traps (NETs)

J. Paige Gronevelt†, Michael Pisano†, Julie M. Rumble†

†Cayman Chemical Immunology Screening Services, 1180 East Ellsworth Road, Ann Arbor, Michigan, 48108, United States

KEY FINDING

We have developed a sensitive, versatile assay to monitor NET clearance by phagocytic cells *in vitro*.

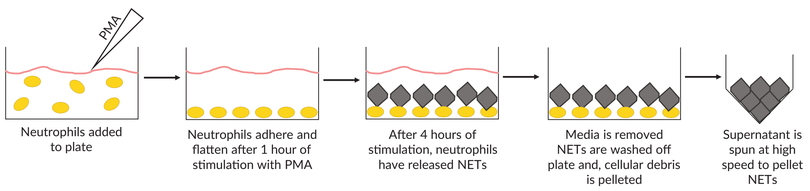
ABSTRACT

Neutrophil extracellular traps (NETs) are formed from dying neutrophils expelling their contents to capture pathogens and limit the spread of infection. While they are beneficial during infection, overproduction of NETs or the inability to degrade them can be pathogenic. Different inflammatory disorders and autoimmune diseases have been shown to be associated with an increase in NET production. Therefore, it is important to understand the method in which NETs are removed or broken down.

Cayman Chemical has developed an imaging-based assay to sensitively assay NET uptake by phagocytic cells. Imaging was found to be an ideal method to monitor NET clearance *in vitro*, in part due to the ability to monitor the uptake of NETs kinetically. Both primary human monocyte-derived macrophages (HMDMs) and differentiated THP-1 cells, which are frequently used as phagocytic cell models, were shown to take up NETs. Putative modulators of NET uptake were tested in the assay.

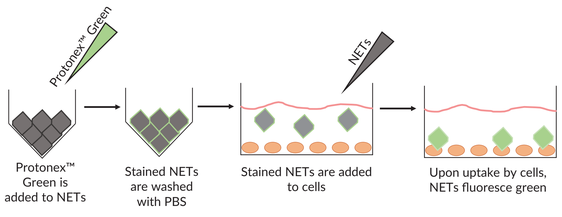
We aimed to develop a method in which the process of NET uptake can be monitored and quantified. Modulators of NET uptake can be tested in this assay, allowing for further studies toward treating autoimmune disorders that are driven by aberrant NETs.

NET FORMATION



NETs are generated by isolating neutrophils from whole blood. Isolated neutrophils are then stimulated with PMA (phorbol 12-myristate 13-acetate). After about an hour of stimulation, neutrophils will have adhered to the plate and begin to flatten. By the time 4 hours of stimulation have passed, neutrophils have released NETs. Media containing PMA is removed and NETs are washed from the plate. This mixture is put through a low-speed spin in order to remove cellular debris. Supernatant is then subjected to a high-speed spin to pellet NETs.

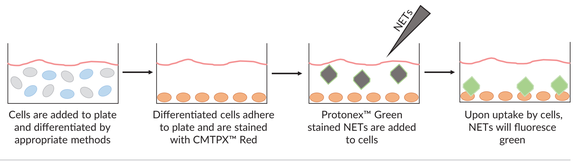
NET STAINING



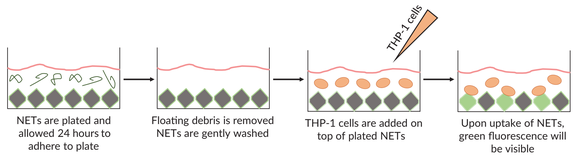
NETs are stained with Protonex™ Green. This is a pH-dependent dye that is activated at acidic, or low, pH. Therefore, when stained NETs remain outside of a cell, they will be neutral, and no fluorescence will be detected. Upon entry into a low pH compartment of a cell, such as lysosomes, endosomes, or phagosomes, stained NETs will emit green fluorescence. This allows for the visualization of stained NET uptake over time.

ASSAY METHODS

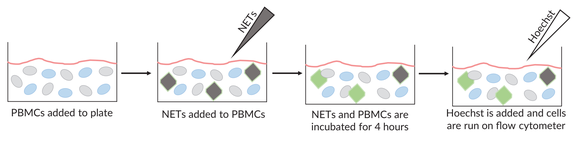
Method 1: Plating cells then adding NETs (Imaging)



Method 2: Plating NETs then adding cells (Imaging)



Method 3: Flow cytometry



IMAGING ASSAY

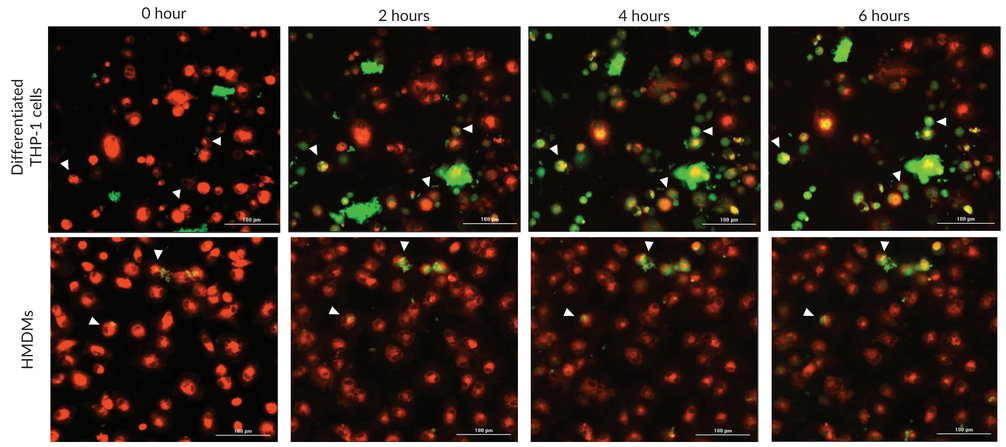


FIGURE 1 – NETs are cleared by macrophages.
Differentiated THP-1 cells and HMDMs were stained with CytoTrace™ Red CMTPX. 0.5 ng/µl Protonex™ Green stained NETs were added. Kinetic imaging was performed over 6 hours using a BioTek Cytation 5 Multi-Mode Imaging Plate Reader, with 465 LED/GFP filter cube and 590 LED/Texas Red filter cube. Upon uptake by macrophage-like cells, NETs will fluoresce green. White arrows point to cells that, over time, are taking up NETs.

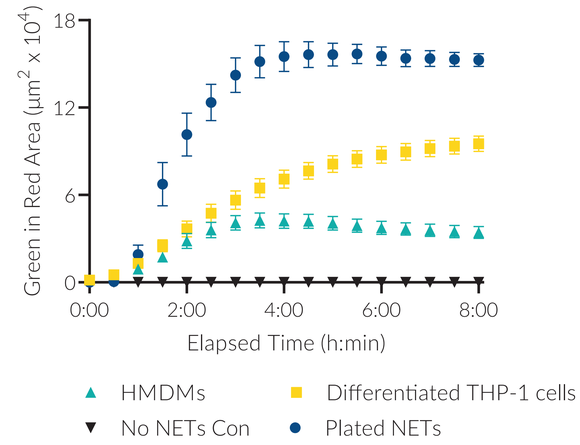


FIGURE 2 – Quantitative analysis of NET clearance by imaging assay.
Total green in red area (µm², representing uptake of green stained NETs into red stained cells) over an elapsed time of 8 hours using 0.5 ng/µl Protonex™ Green stained NETs with plated cells, including both HMDMs (light blue triangles; Mean ± SEM, N=15) and differentiated THP-1 cells (yellow squares; Mean ± SEM, N=9), as well as plated NETs with THP-1 cells parachuted in (dark blue circles; Mean ± SEM, N=9).

FLOW CYTOMETRY ASSAY

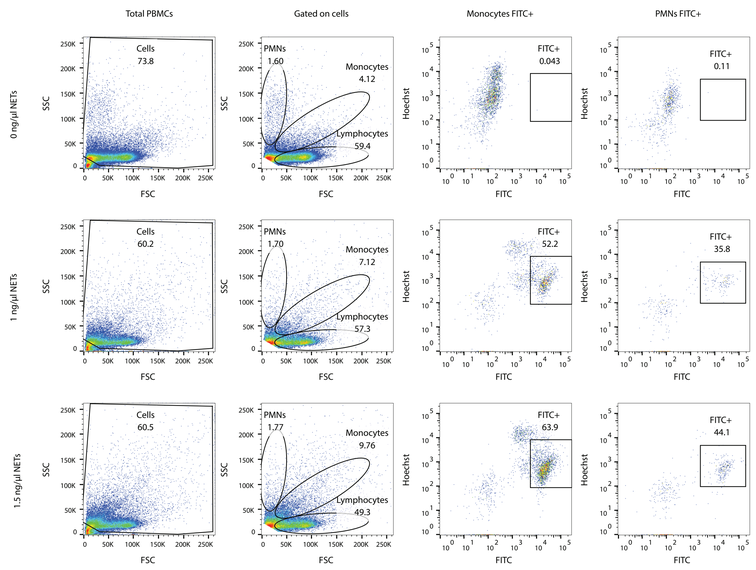


FIGURE 3 – Clearance of NETs by phagocytic cells.
Total peripheral blood mononuclear cells (PBMCs) were isolated and then incubated with 0, 1, or 1.5 ng/µl Protonex™ Green-stained NETs. After 4 hours of incubation at 37°C, cells were stained with 5 µM Hoechst and run on a Miltenyi MACSQuant flow cytometer using V1 and B1 channels. Total PBMCs were gated on first to capture all cell populations. Next, each cell population was determined by FSC and SSC. Within the PMN and monocyte cell populations, FITC positive and Hoechst positive was determined, indicating cells that phagocytosed NETs. Data were analyzed using FlowJo (TreeStar Inc.).

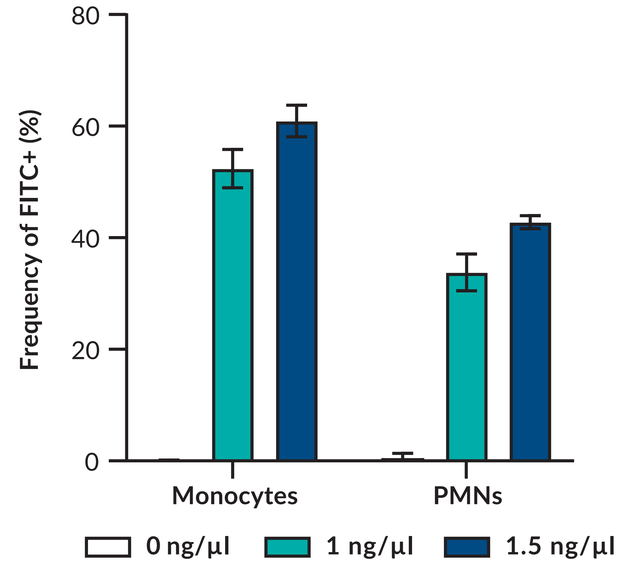


FIGURE 4 – Quantitative analysis of NET clearance by flow cytometry assay.
Frequency of FITC positive live cells within different leukocyte cell populations using 0, 1, or 1.5 ng/µl Protonex™ Green-stained NETs after a 4-hour incubation.

VERSATILE ASSAY

Cell type	Pro
Macrophage-like cell line	Easy to use
Primary differentiated macrophages	More physiological, representative of tissue NET clearance
Whole PBMC	Representative of blood NET clearance

Assay type	Pro
Plated cells, soluble NETs – Imaging assay	Kinetic uptake readout, adhered cells
Plated NETs, parachuted cells – Imaging assay	Kinetic uptake readout, suspended cells
Flow cytometry	Complex cell populations (such as PBMCs)

REFERENCES

1. Farrera, C. and Fadeel, B. Macrophage clearance of neutrophil extracellular traps is a silent process. *J. Immunol.* **191(5)**, 2647–2656 (2013). DOI: 10.4049/jimmunol.1300436
2. Najmeh, S., Cools-Lartigue, J., Giannias, B., *et al.* Simplified human neutrophil extracellular traps (NETs) isolation and handling. *J. Vis. Exp.* **98**, 52687 (2015). DOI: 10.3791/52687

Download this poster & learn more about our NETosis Studies & Analysis Services