Journal of Immunological Methods 423 (2015) 104–110

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

A novel image-based quantitative method for the characterization of NETosis

Wenpu Zhao [a,1], Darin K. Fogg [b,1], Mariana J. Kaplan [a,*]

[a] Systemic Autoimmunity Branch, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, MD 20892, United States
[b] EMD Millipore Corporation, Billerica, MA 01821, United States

ARTICLE INFO

Article history:
Received 15 December 2014
Received in revised form 28 April 2015
Accepted 30 April 2015
Available online 20 May 2015

Keywords:
Neutrophil extracellular traps
Flow cytometry
Microscopy
Cell death

ABSTRACT

NETosis is a newly recognized mechanism of programmed neutrophil death. It is characterized by a stepwise progression of chromatin decondensation, membrane rupture, and release of bactericidal DNA-based structures called neutrophil extracellular traps (NETs). Conventional 'suicidal' NETosis has been described in pathogenic models of systemic autoimmune disorders. Recent in vivo studies suggest that a process of 'vital' NETosis also exists, in which chromatin is condensed and membrane integrity is preserved. Techniques to assess 'suicidal' or 'vital' NET formation in a specific, quantitative, rapid and semiautomated way have been lacking, hindering the characterization of this process. Here we have developed a new method to simultaneously assess both 'suicidal' and 'vital' NETosis, using high-speed multi-spectral imaging coupled to morphometric image analysis, to quantify spontaneous NET formation observed ex-vivo or stimulus-induced NET formation triggered in vitro. The use of imaging flow cytometry allows automated, quantitative and rapid analysis of subcellular morphology and texture, and introduces the potential for further investigation using NETosis as a biomarker in pre-clinical and clinical studies.

Published by Elsevier B.V.

1. Introduction

The year 2014 marked the 10th anniversary of the initial description of neutrophil extracellular traps (NETs), a meshwork of chromatin fibers decorated with antimicrobial proteins ejected into the extracellular space to kill or immobilize microbes (Brinkmann et al., 2004). Since then, significant interest has emerged with regard to the role of NET formation as a key mechanism in host defense against microbes. In addition, the putative role of NETs in the induction of autoimmune responses, thrombosis, endothelial cell death and tissue damage is the focus of investigation by many research groups (Knight et al., 2014a, 2014b).

Since its original description, it has become apparent that NET formation is a heterogeneous process. The initial description of NET formation, designated as NETosis, was described as a 'suicidal' process distinct from apoptosis and necrosis (Fuchs et al., 2007). A hallmark of early stage 'suicidal' NETosis is the nuclear translocation of the azurophilic granule proteins neutrophil elastase (NE) and myeloperoxidase (MPO), followed by histone degradation leading to chromatin decondensation (Papayannopoulos et al., 2010; Metzler et al., 2014). As such, the measurement of decondensed nuclei has been used as one of the hallmarks to quantify neutrophils that are undergoing NET formation. Later events in 'suicidal NETosis' include the development of cell lysis and cell membrane rupture, indicating that NETs emerge from dying neutrophils. The classical stimulus that induced 'suicidal' NETosis is phorbol myristate acetate (PMA) after a 4-hour incubation period (Fuchs et al., 2007), a process that depends on production of cellular oxidants.

In addition to this cell death process, an additional mechanism of NET formation was recently described in which the extrusion of chromatin can also occur through an oxidant-independent mechanism termed 'vital' NETosis, whereby NETs are released, leaving behind functional anuclear cells (Yipp et al., 2012). Initial descriptions of 'vital' NETosis in vivo showed the cell nucleus changing from polymorphonuclear to spherical, with NETs then emerging in a localized area of the neutrophil surface through vesicular release. In vivo studies revealed that condensed DNA passed through the cytoplasm without lysing membranes (Pilsczek et al., 2010). Indeed, anuclear neutrophils are a common finding in human abscesses due to bacterial infection (Yipp et al., 2012). As such, 'vital NETosis' is described as 'NETing during patrolling', as these neutrophils undergoing NET formation simultaneously crawl in vivo, as quantified using intravital microscopy (Yipp et al., 2012; Pilsczek et al., 2010; Kolaczkowska and Kubes, 2013). This phenomenon is difficult to quantify in vitro, as the 2-D nature of slides or coverslips likely impair the identification of these "crawling" NETing neutrophils.

Methods to detect NETosis have been based on classical microscopy analysis requiring that cells are attached to a slide or coverslip. Identification is typically based on the classical appearance of "beads-on-a-

* Corresponding author at: Systemic Autoimmunity Branch, Intramural Research Program, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, 10 Center Drive/ 6D47C, Bethesda, MD 20892, United States. Tel.: +1 301 496 9650; fax: +1 301 402 0765.
E-mail address: mariana.kaplan@nih.gov (M.J. Kaplan).
[1] These authors equally contributed to manuscript and share first authorship.

http://dx.doi.org/10.1016/j.jim.2015.04.027
0022-1759/Published by Elsevier B.V.

string" captured with conventional fluorescence microscopy. While this technique reveals the endpoint of nuclear extrusion and can assess extracellular coexpression of nuclear material with granular proteins through immunolabeling, it does not easily lend itself to objective quantification and introduces possible sampling bias (see Supplemental Fig. 1). Due to the relatively low throughput and subjective nature of conventional microscopy, the results obtained from different laboratories are difficult to compare and the results are not significantly quantitative. Furthermore, the process can be laborious and time-consuming. More automated assays currently used (such as the Sytox green plate assay) lack specificity, as externalization of DNA cannot be equated to NETosis. Also, as mentioned above, the resulting alteration of cellular morphology likely affects nuclear motility causing the failure to easily visualize 'vital' NETosis in vitro. While some on these limitations may be overcome by a recent protocol using dual channel fluorescence staining and image segmentation, there are still concerns regarding discrimination between enlarged nuclei from NETosis and mitotic or apoptotic events (Brinkmann et al., 2012; Knight et al., 2013). Therefore, a high-throughput, objective, and quantitative approach to imaging cells undergoing NETosis is required to further study this novel neutrophil process.

Multispectral Imaging Flow Cytometry (MIFC) combines features of fluorescence microscopy and flow cytometry by providing unbiased acquisition and analysis of large number of images from cells in suspension. CCD camera technology and time-delayed integration (TDI) allows high-speed, high resolution image capture in multiple wavelengths simultaneously (Basiji et al., 2007). MIFC has been widely used in cell studies including nuclear-cytoplasmic translocation (George et al., 2006), quantification of apoptosis based on the changes in nuclear morphology (Henery et al., 2008) and assessment of autophagy (de la Calle et al., 2011). A morphology-based method for measuring NETosis has not yet been reported using this novel technology.

Here we present a novel and rapid image-based method that employs MIFC to study and quantify 'suicidal' and 'vital' NETosis by using transmitted light (Brightfield), side-scatter (SSC) and multiple fluorescence images of cellular components.

2. Results

2.1. 'Suicidal' NETosis is defined and quantified with imaging flow cytometry

We established a streamlined method for high-speed imaging in a fluid stream, followed by semi-automated image analysis to discover features characteristic of neutrophils undergoing 'suicidal' and 'vital' NETosis. We studied neutrophils obtained from peripheral blood from healthy controls, as well as distinct neutrophil subsets isolated from patients with systemic lupus erythematosus (SLE), an autoimmune disease characterized by an enhanced percentage of granulocytes undergoing NET formation. Overall, the morphological features observed in healthy control and lupus neutrophils were similar and representative images from lupus patients are used in the figures.

We began by examining the size and staining intensity and texture of neutrophil nuclei before and 4 h after treatment with PMA. While untreated cells displayed normal multi-lobular nuclei, PMA treatment led to a subset of neutrophils displaying enlarged nuclei with less pronounced lobulation and diffuse staining intensity (Fig. 1a). The average area of nuclei increased from 40 μm^2 for the unstimulated cells to 125 μm^2. This so-called 'nuclear decondensation' was quantified using the Bright Detail Intensity (BDI) feature, identifies areas of peak fluorescence intensity after subtraction of background fluorescence. The feature used measured bright intensity spots with a radius of 7 pixels or fewer, known as BDI R7, within the IDEAS software. Decondensed nuclei are identified by low BDI and high area, while normal nuclei showed high or variable BDI and low area (Fig. 1b). The total Intensity of the Hoechst nuclear stain was similar between normal and decondensed nuclei, however the latter showed slightly lower SSC Intensity (Fig. 1b), perhaps due to diffusion of granular proteins throughout the decondensed nuclei. Similar morphological changes were observed in a small percentage of neutrophils exposed in vitro to LPS for 1 h (Fig. 2). To address whether MPO may be relocating to DNA, as has been described for 'suicidal' NETosis, a feature called Similarity Score was then calculated to quantify the degree of nuclear translocation of MPO, using the Hoechst nuclear stain to identify the nuclear region (Fig. 2b). The median Similarity Score for the unstimulated cells was found to be around −0.12, indicating an anti-correlation between MPO and Hoechst images, and therefore the absence of nuclear localization. In contrast, the median Similarity Score for the gated subpopulation with decondensed nuclei was +1.0, indicating a positive correlation between the MPO and Hoechst images, and therefore increased co-localization of MPO and Hoechst had occurred. As an additional stimulus, we treated neutrophils with the calcium ionophore A23187, an antibiotic that induces NET formation and increases cytoplasmic Ca^{2+} level. As shown in Supplemental Fig. 2, this stimulus primarily induced features of 'suicidal' NETosis.

Overall, this new method defines 'suicidal' NETotic cells with features of large nuclear area and low BDI, as well as colocalization of MPO with the DNA.

2.2. A novel morphology is identified as 'vital NETosis' in vitro

In addition to the expected nuclear decondensation described above, we also observed a striking phenotype in neutrophils treated in vitro with LPS for 1 h. Rather than a normal round cell shape, approximately 50% of the neutrophils were elongated, with large membrane blebs visible at one pole, and cell contents, including nucleus and granules (by SSC) at the other (Fig. 3a). Notably, the chromatin remained condensed and multi-lobular, and displayed nuclear area and BDI similar to unstimulated neutrophils (Figs. 3d and 2c). In order to quantify the observed morphological change, we used the Fisher's Discriminate Ratio (RD) optimization tool within the IDEAS software (so-called 'Feature Finder'). This tool allows comparison of a battery of default and user-defined image analysis algorithms to determine which of those best differentiates two input populations. In our case, we hand-selected images of cells displaying the normal expected neutrophil morphology (Truth Set 1, see Supplemental Fig. 3), and those of cells displaying the elongated morphology including the membrane blebs (Truth Set 2, see Supplemental Fig. 4). The greatest RD value, 1.49, was observed for Aspect Ratio of the Brightfield image, which is calculated as the minor axis/major axis, as illustrated in Fig. 3b and Supplemental Fig. 5. Because the elongated phenotype displayed one cell pole with a high degree of pixel intensity and contrast, and the opposite pole with a very low intensity, low contrast membrane bleb, a feature was calculated to compare the Centroid XY position for the Brightfield Image versus the Intensity-weighted Brightfield Image. This feature, termed 'Delta Centroid XY BF, BF Intensity', will be referred to henceforth simply as Delta Centroid XY (see Fig. 3b and Supplemental Fig. 6). The RD value for Delta Centroid was also high at 1.43, therefore the two features were used in a bivariate plot of Truth Populations 1 and 2 to reveal clear separation of these two populations (Fig. 3b). The same features were then used to plot at least 20,000 cells of untreated and LPS-treated samples, and we observed that while only ~8% of cells from the untreated group fell into the 'elongated' gate, ~50% of cells from the LPS-treated group had this phenotype. Notably, the Nuclear Area and Hoechst BDI values of the elongated cells were similar to those of normal neutrophil nuclei (Fig. 3d), implying that these cells are not undergoing apoptosis.

MPO did not appear to colocalize with DNA in the cells displaying the elongated phenotype and the mean Similarity Score comparing MPO and DNA pixel overlap in elongated cells was −0.5, indicating the absence of translocation of MPO to the nucleus compared to +1.5 in 'suicidal' NETosis (Fig. 4a). Intriguingly, cell-free chromatin was visualized and identified with low SSC and high intensity of Hoechst (Supplemental