Probing Neutrophil NETosis

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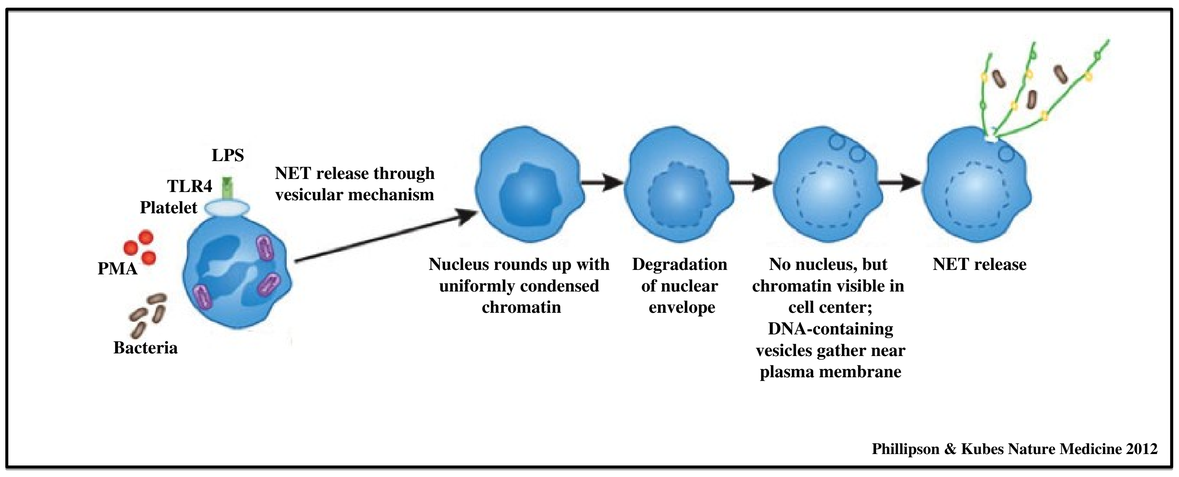
Introduction

•Neutrophil Extracellular Traps (NETs) are produced following the oxidative burst of neutrophils in response to pathogens. (Brinkmann et al. 2007[1])

•NETs are essentially extruded nuclear material, and the process of their extrusion is termed NETosis – an event akin to a cell death mechanism. (Galluzzi et al., 2012[2])

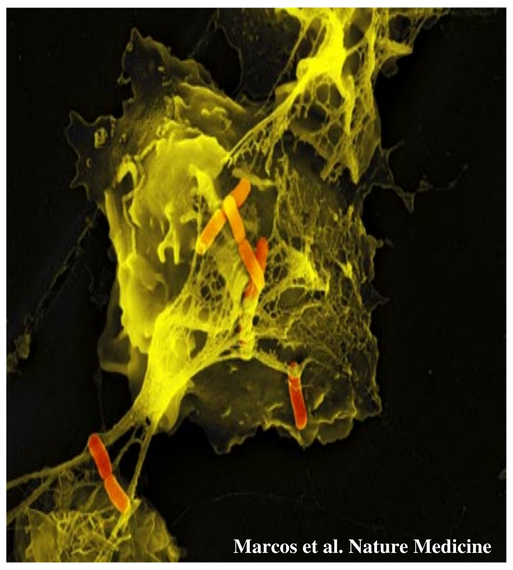
•We have investigated the mechanism using pharmacologic inhibitors of apoptosis and necroptosis, and through imaging using activity-based probes against neutrophil proteases.

•This implicates the neutrophil proteases as mediators of this highly selective cell death process.



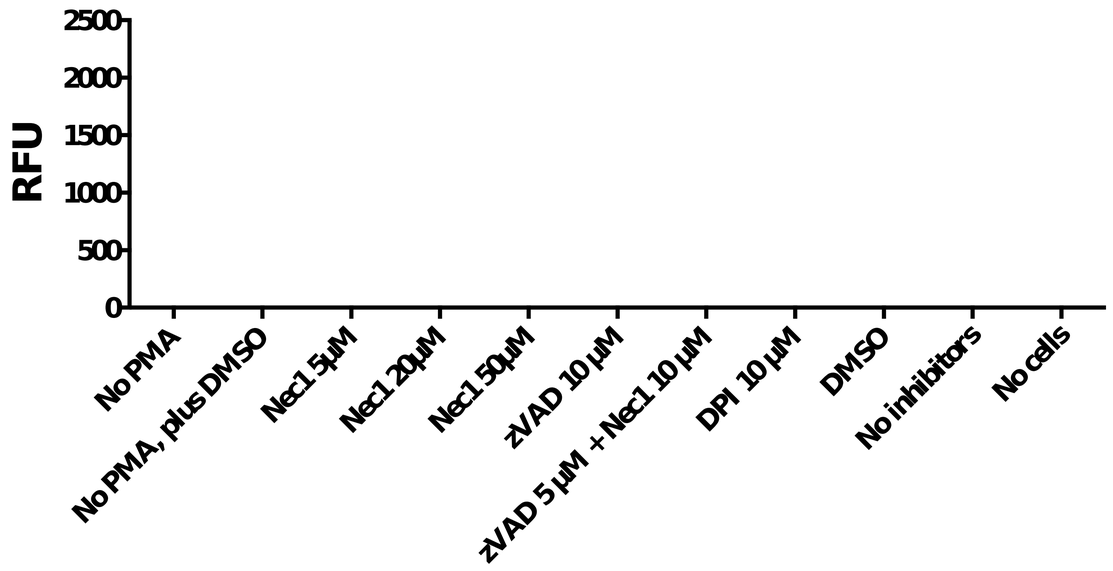
Methods and Results

1) NETs (neutrophil extracellular traps)



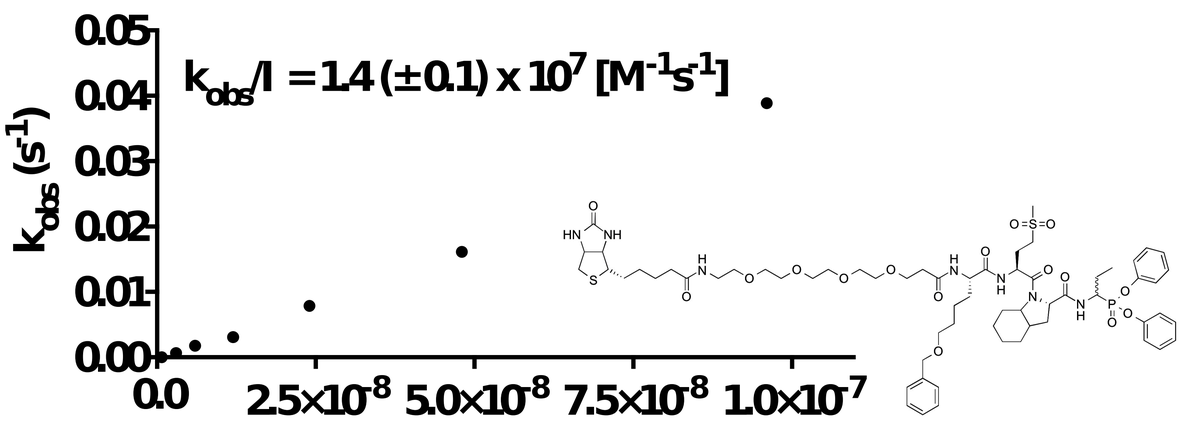
Human immune cells known as neutrophils release granule proteins and DNA into the extracellular space to trap bacteria or viruses during an infection. NETs disarm pathogens with antimicrobial proteins such as neutrophil elastase. In addition to their antimicrobial properties, NETs may serve as a physical barrier that prevents further spread of pathogens. NETs (yellow) entrapping Mycobacterium tuberculosis (red).

2) Inhibition assay for NET formation



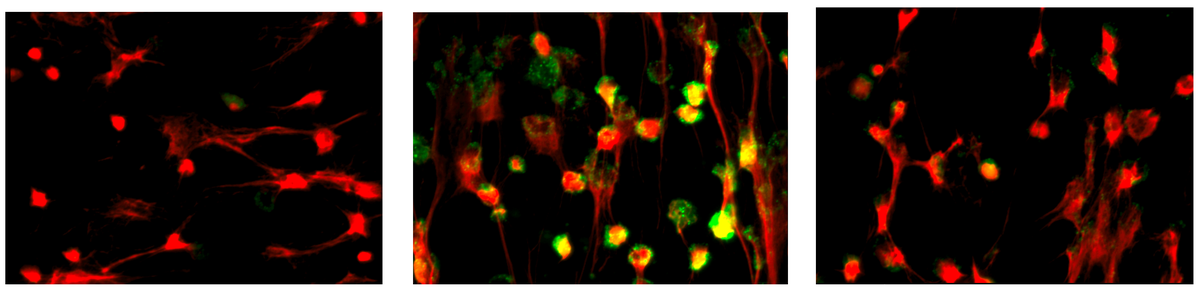
Human neutrophils were stimulated using 20nM Phorbol myristate acetate (PMA). Necrostatin, a necroptosis inhibitor, zVAD-FMK, an apoptosis inhibitor and DPI, a NADPH oxidase inhibitor were added to test for reductions in NET formation. Neutrophils that have extruded their DNA can be stained with SYTOX green. This staining can be measured by fluorescence in the presence or absence of inhibitors.

3) Inhibition rate constant for Neutrophil Elastase and PK101



Using PK101, a potent elastase inhibitor (see poster presented by Paulina Kasperkiewicz), we determined the inhibition rate for elastase by calculating apparent k_{obs}/I (second order rate constant for inhibition) under pseudo first order conditions. We did this by varying inhibitor concentrations at a constant 10 µM optimal substrate concentration. Since we had previously determined the K_m of the optimal substrate for elastase, we were able to calculate the exact k_{obs}/I value for elastase.

4) Staining for DNA (NETs) and Neutrophil Elastase



No probe | 40 nM PK101, 10 min | 40 nM PK101, 10 min pre-treated for 30 min with 1µM Suc-AAPV-CMK

Neutrophils were isolated by dextran sedimentation followed by Ficoll-Paque PLUS centrifugation. To induce NETs, neutrophils were treated with 20nM PMA. Cells were incubated at 37°C for 2.5 hours, then treated at 37°C for 10 min with 40nM PK101. After washing briefly in PBS, cells and NETs were carefully fixed with 4% paraformaldehyde, washed twice with PBS, blocked for 1 hour with 10% (w/v) BSA in PBS, then incubated with 1/1000 dilution of Alexa Fluor® 488 for 1 hour at room temperature in 3% (w/v) PBS. NETs were stained with propidium iodide. PI (red) shows DNA staining and Streptavidin (green) bound to the biotinylated PK101 shows active elastase.

Conclusions

1) NETosis is a cell death mechanism that is blocked by inhibition of NADPH oxidase but only slightly reduced by a combination of apoptosis and necrosis inhibitors. Therefore it appears to be primarily a novel death mechanism. Despite the fact that the neutrophils have extruded their nuclei, it remains unclear whether they are "dead" and this is fruit for future studies.

2) The generation of a highly reactive activity based probe (PK101) that is substantially selective for neutrophil elastase allows us to visualize the distribution of this protease during NET formation.

3) Somewhat surprisingly, elastase and NETs seem to be mutually exclusive. Either the NETs do not contain elastase (in contrast to previous suggestions by Papayannopoulos et al., 2010[3]) or the elastase that is associated with NETs is not active. Development of probes in this series combined with binary imaging techniques should allow us to define the role of neutrophil serine proteases in NET formation.

References

1) Volker Brinkmann & Arturo Zychlinsky. (2007) Beneficial suicide: why neutrophils die to make NETs. Nature Reviews Microbiology 5, 577-582.

2) Galluzzi, L., et al. (2012) Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. Cell Death and Differentiation 19, 107–120.

3) Papayannopoulos, V., et al. (2010) Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. J Cell Biol 191,677-91.

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