

## **Pneumolysin activates neutrophil extracellular trap formation**

Jan Gert Nel,<sup>1</sup> Annette J. Theron,<sup>2,3</sup> Chrisna Durandt,<sup>3</sup> Gregory R. Tintinger,<sup>4</sup> Roger Pool,<sup>1</sup> Timothy J. Mitchell,<sup>5</sup> Charles Feldman,<sup>6</sup> Ronald Anderson.<sup>3</sup>

<sup>1</sup>*Department of Haematology, Faculty of Health Sciences, University of Pretoria and Tshwane Academic Division of the National Health Laboratory Service, Pretoria, South Africa.*

<sup>2</sup>*Department of Immunology, Faculty of Health Sciences, University of Pretoria and Tshwane Academic Division of the National Health Laboratory Service, Pretoria, South Africa.*

<sup>3</sup>*South African Medical Research Council Unit for Stem Cell Research, Institute of Cellular and Molecular Medicine, Department of Immunology, Faculty of Health Sciences, University of Pretoria, Pretoria, South Africa.*

<sup>4</sup>*Department of Internal Medicine, Faculty of Health Sciences, University of Pretoria, Pretoria, South Africa.*

<sup>5</sup>*Institute of Microbiology and Infection, College of Medical and Dental Sciences, University of Birmingham, Birmingham, United Kingdom.*

<sup>6</sup>*Division of Pulmonology, Department of Internal Medicine, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg and Charlotte Maxeke Academic Hospital, Johannesburg, South Africa.*

Short title: Pneumolysin-induces NET formation

Correspondence: Dr J.G. Nel  
Dept. Haematology  
PO Box 2034  
Pretoria 0001  
South Africa  
e-mail: [jan.nel@up.ac.za](mailto:jan.nel@up.ac.za)  
telephone: +27-12-319-2641  
telefax: +27-12-323-0732

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**List of abbreviations**

CAP - community acquired pneumonia

CGD - chronic granulomatous disease

DAPI - 4',6-diaminodino-2-phenylindole

DPI - diphenyleneiodonium chloride

End A - endonuclease A

HBSS - Hanks balanced salt solution

IPD - invasive pneumococcal disease

LECL - luminol-enhanced chemiluminescence

MFS - metered fluorescence units

MPO - myeloperoxidase

NADPH - nicotinamide adenine dinucleotide phosphate

NET - neutrophil extracellular trap

PBS - phosphate-buffered saline

Ply - pneumolysin

PMA - phorbol 12-myristate 13-acetate

ROS - reactive oxygen species

TLR - Toll-like receptor

VDC - Vybrant Dye Cycle

## Summary

The primary objective of the current study was to investigate the potential of the pneumococcal toxin, pneumolysin (Ply), to activate neutrophil extracellular trap (NET) formation *in vitro*.

Isolated human blood neutrophils were exposed to recombinant Ply (2.5-20 ng.ml<sup>-1</sup>) for 30-90 min at 37°C and NET formation measured using the following procedures to detect extracellular DNA: i) flow cytometry using Vybrant Dye Cycle Ruby; ii) spectrofluorimetry using the fluorophore, Sytox® Orange (5 µM); iii) and NanoDrop® technology. These procedures were complemented by fluorescence microscopy using DAPI (nuclear stain) in combination with anti-citrullinated histone monoclonal antibodies to visualise nets.

Exposure of neutrophils to Ply resulted in relatively rapid (detected within 30-60 min), statistically significant ( $p < 0.05$ ) dose- and time-related increases in the release of cellular DNA impregnated with both citrullinated histone and myeloperoxidase.

Microscopy revealed that NETosis appeared to be restricted to a subpopulation of neutrophils, the numbers of NET-forming cells in the control and Ply-treated systems (10 and 20 ng.ml<sup>-1</sup>) were 4.3(4.2), 14.3(9.9) and 16.5(7.5) respectively (n=4,  $p < 0.0001$  for comparison of the control with both Ply-treated systems). Ply-induced NETosis occurred in the setting of retention of cell viability, and apparent lack of involvement of reactive oxygen species and Toll-like receptor 4.

In conclusion, Ply induces vital NETosis in human neutrophils, a process which may either contribute to host defence or worsen disease severity depending on the intensity of the inflammatory response during pneumococcal infection.

## Introduction

Despite advances in antibiotic therapy and access to sophisticated intensive care facilities, severe community-acquired pneumonia (CAP), one of the most common infectious disease, carries an unacceptably high mortality rate of 10-15% in hospitalised patients [1]. Those with developing or impaired immune systems are most vulnerable, specifically the very young, the elderly and those with primary and acquired immunodeficiency disorders [1, 2]. Of concern, the threat of CAP-related mortality and morbidity is likely to increase in parallel with a growing elderly population, particularly in developing countries, in whom immunisation procedures against the major viral (influenza virus) and bacterial (*Streptococcus pneumoniae*, also known as the pneumococcus) pathogens are less effective [2, 3].

In the case of invasive pneumococcal disease (IPD), respiratory failure, shock and multi-organ dysfunction syndrome are leading causes of mortality [4]. The major pneumococcal protein toxin, pneumolysin (Ply), contributes significantly to the pathogenesis of acute lung injury, due largely to pro-inflammatory activities and direct cytotoxic effects on pulmonary epithelium and endothelium, which also promote extrapulmonary dissemination of the pathogen [5-7]. These activities result from the cholesterol binding, pore forming activities of the toxin [5-7]. In addition, Ply by a mechanism independent of pore-forming activity, has been reported to activate the production of pro-inflammatory cytokines, tumour necrosis factor  $\alpha$  and interleukin 6, via activation of the pattern recognition receptor, Toll-like receptor 4 (TLR4) on murine macrophages [8]. Ply has also been implicated in the pathogenesis of acute coronary events in patients with bacterial CAP, which are also recognised as a significant cause of mortality [9-11]. Mechanistically, evidence from

experimental animal models of IPD has demonstrated the involvement of Ply in the formation of microlesions in the myocardium, resulting in myocardial toxicity and dysfunction [12, 13].

Dysregulation of neutrophil extracellular trap (NET) formation is another potential mechanism which may exacerbate lung and cardiac injury in IPD [14-18]. The process of NETosis, which is highly conserved throughout evolution [19], involves the extracellular release of a web of hypercitrullinated chromatin impregnated with predominantly granule-derived antimicrobial proteins. NETs entrap, and, in some cases, neutralise, microbial and viral pathogens [20]. However, if excessive, NETosis poses the potential hazard of cell and organ damage, due not only to the cytotoxic actions of histone constituents [17, 21], but also to pro-thrombotic activity [14, 15, 18].

The pro-NETotic activity of Ply as a potential contributor to pulmonary and cardiac dysfunction during severe pneumococcal infection is, however, largely unexplored and represents the primary focus of the current study.

## **Materials and Methods**

### *Recombinant pneumolysin and delta6Ply*

Recombinant native Ply was expressed in *Escherichia coli* and purified from cell extracts [22]. The pneumolysoid, delta6Ply, with a double amino acid deletion (alanine 146 and arginine 147), resulting in attenuation of pore-forming activity, was generated by site-directed mutagenesis [23]. The purities of both toxins were confirmed by sodium dodecyl-sulphate-polyacrylamide gel electrophoresis. The

stock protein concentrations of native recombinant Ply and delta6Ply were 493 and 768  $\mu\text{g}\cdot\text{ml}^{-1}$  respectively and were essentially free of contaminating endotoxin. The respective haemolytic activities (using human erythrocytes, 1% final) were 22,700 and 0 haemolytic units. $\cdot\text{pmol}^{-1}$ .

Unless indicated, all other chemicals and reagents were purchased from the Sigma Chemical Co, St Louis, MO, USA.

### *Ethics Approval*

The study was approved by the Research Ethics Committee of the Faculty of Health Sciences, University of Pretoria (Approval No. 31/2014).

### *Neutrophils*

Neutrophils were obtained from the blood of a total of 19 healthy, non-smoking volunteers, mean age 41, range 23-66. Exclusion criteria included the use of any medication on the day of venepuncture. Neutrophils were prepared as described previously [24, 25]. The cells, which were routinely of high purity (>90%) and viability (>95%), were suspended in indicator-free Hanks balanced salt solution (HBSS, pH 7.4; Highveld Biological Co., Johannesburg, South Africa) and used immediately in assays of NET formation.

### *Exposure of neutrophils to Ply*

Following pre-incubation for 10 min at 37°C, Ply (5-20 $\text{ng}\cdot\text{ml}^{-1}$ ) or an equal volume of HBSS (40  $\mu\text{l}$ , control system) was added to the neutrophils (4  $\times 10^6$  cells in 4 ml HBSS) and the tubes incubated for 30, 60 and 90 min. After incubation, 1 ml of cell suspension was removed from each tube and allocated for flow cytometric detection

of extracellular citrullinated histone- and myeloperoxidase (MPO)-associated extracellular DNA as described below. The tubes containing the residual volume of 3ml were centrifuged to pellet the cells after which the supernatant fluids were removed and the cell pellets resuspended in 1ml of HBSS and cell viability measured as described below. The supernatants from each tube were then aliquoted into 2.8 ml and 100 µl volumes for detection of extracellular DNA using spectrofluorimetry and NanoDrop® technology as described below.

#### *Flow cytometric detection of NETs*

Neutrophil suspensions were aliquoted into three flow cytometry analysis tubes (Beckman Coulter, Miami, USA) and stained with 5 µl of mouse anti-human CD16-APC monoclonal antibody (Beckman Coulter), 1 µl of Vybrant DyeCycle (VDC) Ruby (final concentration 0.5 µM, Life Technologies, Johannesburg, South Africa) and one of the following in each tube: 5 µl mouse anti-human myeloperoxidase (MPO)-FITC monoclonal antibody (Beckman Coulter), 4 µl of anti-histone H4 (citrulline 3) rabbit polyclonal antibody (Merck Millipore, Bellerica, USA), or 4 µl goat anti-rabbit IgG (H+L)-Alexa Fluor® 488 secondary antibody (Life Technologies). The tubes were gently mixed and incubated in the dark for 10 minutes at room temperature followed by the addition of 4 µl goat anti-rabbit IgG (H+L)-Alexa Fluor® 488 secondary antibody to the tube treated with the purified anti-histone H4 (citrulline 3) rabbit polyclonal antibody. After an additional 10 min period of incubation, flow cytometry analyses were performed using a Gallios flow cytometer (Beckman Coulter). Extracellular DNA was identified as CD16<sup>negative</sup>/VDC Ruby<sup>positive</sup>/MPO<sup>positive</sup> or citrullinated Histone H4<sup>positive</sup> events.

### *Spectrofluorimetric detection of NETs*

In the case of the spectrofluorimetric procedure, the cell-free supernatants (2.8 ml) were mixed with 3 $\mu$ l of the DNA-binding fluorophore, SYTOX® Orange (5 $\mu$ M final, Life Technologies) [26], transferred to cuvettes, and placed in the cuvette holder of a Hitachi 650 10S fluorescence spectrophotometer (Hitachi Ltd, Tokyo, Japan) with the excitation and emission wavelengths set at 530nm and 590nm respectively followed by measurement of fluorescence intensity. The results are expressed as metered fluorescence units (MFUs) following subtraction of the values of time-matched background control systems which were held at room temperature (25°C).

### *NanoDrop® procedure*

Extracellular DNA concentrations were determined in the cell free supernatants using a Nano-Drop ND-1000 spectrophotometer (Thermo Scientific, Johannesburg, South Africa) according to a previously published protocol [27] and results recorded as nanograms (ng). $\mu$ l<sup>-1</sup>.

For this and the spectrofluorimetric procedure a maximum incubation time of 60 min was used, as this gave optimum discrimination between the control and Ply-treated systems.

A series of additional experiments were performed using the spectrofluorimetric and NanoDrop® procedures. These were: i) comparison of the pro-NETotic effects of native Ply with those of delta6Ply (10 and 20 ng.ml<sup>-1</sup>) [23, 28]; ii) measurement of the requirement for extracellular Ca<sup>2+</sup> in the pro-NETotic actions of Ply by suspending the cells in Ca<sup>2+</sup>-free medium; iii) measurement of the requirement for cellular generation of reactive oxygen species (ROS) in Ply-activated NETosis, firstly



by pre-treating the cells with the NADPH oxidase inhibitor, diphenyleneiodonium chloride (DPI, 10 $\mu$ M final), 5 min prior to the addition of the toxin, and secondly, by using cells from a 44 year old male with the autosomal recessive form of chronic granulomatous disease (CGD, deficiency of p47<sup>phox</sup>, GT deletion in exon 2) [29]; iv) a comparison of the pro-NETotic actions of Ply (20ng.ml<sup>-1</sup>) with those of the potent activator of NADPH oxidase, phorbol 12-myristate 13-acetate (6.25 ng.ml<sup>-1</sup>, final) ;and vi) a comparison of the effects of native Ply and the pneumolysoid, delta6Ply, on NETosis.

#### *Microscopic detection of NETs*

Microscopy was performed to confirm that the extracellular DNA demonstrated by other techniques was the product of NETosis. Neutrophil suspension (1.25 x10<sup>5</sup> cells in 250  $\mu$ l) was allowed to adhere to glass cover slips for 30min. Ply (10 and 20ng.ml<sup>-1</sup> final) or an equal volume of HBSS was added to the adherent cells and the coverslips incubated for 90 min at 37°C 5%CO<sub>2</sub>. The cells were then fixed with 4% paraformaldehyde for 10 min, washed 3 times in phosphate-buffered saline (PBS) and blocked with HBSS containing 5% goat serum and 5% bovine serum albumin for 30 min at 37°C. Neutrophils were incubated overnight with polyclonal rabbit anti-histone H4 (citrulline 3, Merck Millipore) and washed three times in PBS. Following addition of Alexa Fluor 488-labeled goat anti-rabbit secondary antibody (Life Technologies), DNA present in the specimen was stained with the DNA-binding dye, 4',6-diamidino-2-phenylindole (DAPI ,Life Technologies), for 2 minutes and analysed for NET formation by fluorescence microscopy using a Zeiss Axio Vert. A1 fluorescence microscope and Axio Vision Software (Zeiss, Johannesburg, South Africa). Representative photomicrographs were taken, and used for the generation of

counts by a single non-blinded observer. Results are expressed as percentage of NET forming cells.

#### *Assessment of pore-forming activity*

The pore-forming activities of Ply (10 and 20 ng.ml<sup>-1</sup>) were determined spectrofluorimetrically according to the magnitude of influx of Ca<sup>2+</sup> into neutrophils using fura-2 acetoxymethylester (fura-2 AM) as the Ca<sup>2+</sup>-sensitive, fluorescent indicator. Briefly, neutrophils (1 x 10<sup>7</sup>.ml<sup>-1</sup> in PBS) were incubated with fura-2 AM (2 μM, final) for 25 min at 37°C, washed and resuspended in HBSS. The fura-2-loaded cells (1 x 10<sup>6</sup>) were then pre-incubated for 10 min at 37°C and transferred to cuvettes which were maintained at 37°C in the Hitachi 650 10S fluorescence spectrophotometer with the excitation and emission wavelengths set at 340 nm and 500 nm respectively. After a stable baseline was obtained (±1 min), Ply, or delta6Ply were added to the cells and alterations in fluorescence intensity monitored over a 5-10 min time course.

#### *Measurement of reactive oxygen species (ROS)*

A luminol (5-amino-2,3-dihydro-1,4-phthalazinedione)-enhanced chemiluminescence (LECL) procedure, which is a composite measure of phagocyte-derived ROS, was used to investigate the generation of ROS by Ply (20 ng.ml<sup>-1</sup>) in the absence and presence of DPI (10 μM). Briefly, the neutrophils (1 x 10<sup>6</sup>.ml<sup>-1</sup>) in HBSS containing 0.1 mM luminol were pre-incubated for 10 min at 37°C, without and with DPI, followed by addition of Ply and measurement of LECL over a 20 min time course using a thermoregulated chemiluminometer (LKB Wallac 1251 Chemiluminometer, Turku, Finland) and results expressed as the peak LECL responses in mV.sec<sup>-1</sup>. In

addition, a lucigenin (bis-N-methylacridium nitrate, 0.2mM, final)-enhanced chemiluminescence method was used to demonstrate the generation of superoxide by PMA ( $6.25\text{ng}\cdot\text{ml}^{-1}$ )-activated neutrophils from the CGD subject.

#### *Cell viability*

This was measured at each time interval following addition of Ply or PMA to neutrophils using a flow cytometric propidium iodide-based dye exclusion assay. The cells ( $4 \times 10^6\cdot\text{ml}^{-1}$ ) were incubated for 5 min with propidium iodide ( $50 \mu\text{g}\cdot\text{ml}^{-1}$ , DNA Prep-Stain, Beckman Coulter) and cell viability assessed flow cytometrically. Results are expressed as percentage viable cells.

#### *Expression and statistical analyses of results*

The results of each series of experiments are expressed as the mean values and standard deviations (SDs), with the exception of the spectrofluorimetric measurements of  $\text{Ca}^{2+}$  influx for which the traces are shown. In all cases, the 'n' values represent the number of different donors used in each series of experiments. The exceptions are studies using CGD cells, in which several replicates were performed on a single donor. For flow cytometric data, statistical analyses were performed using GraphPad Prism 6 (version 6.07; GraphPad Software, San Diego, USA). A 2-way ANOVA with Sidak's multiple comparisons test was used to determine statistically significant differences between the untreated control groups and the Ply-treated groups (various concentrations) over the different time points. For bivariate hypothesis testing, t-tests were used. A p-value of  $<0.05$  was taken as statistically significant.

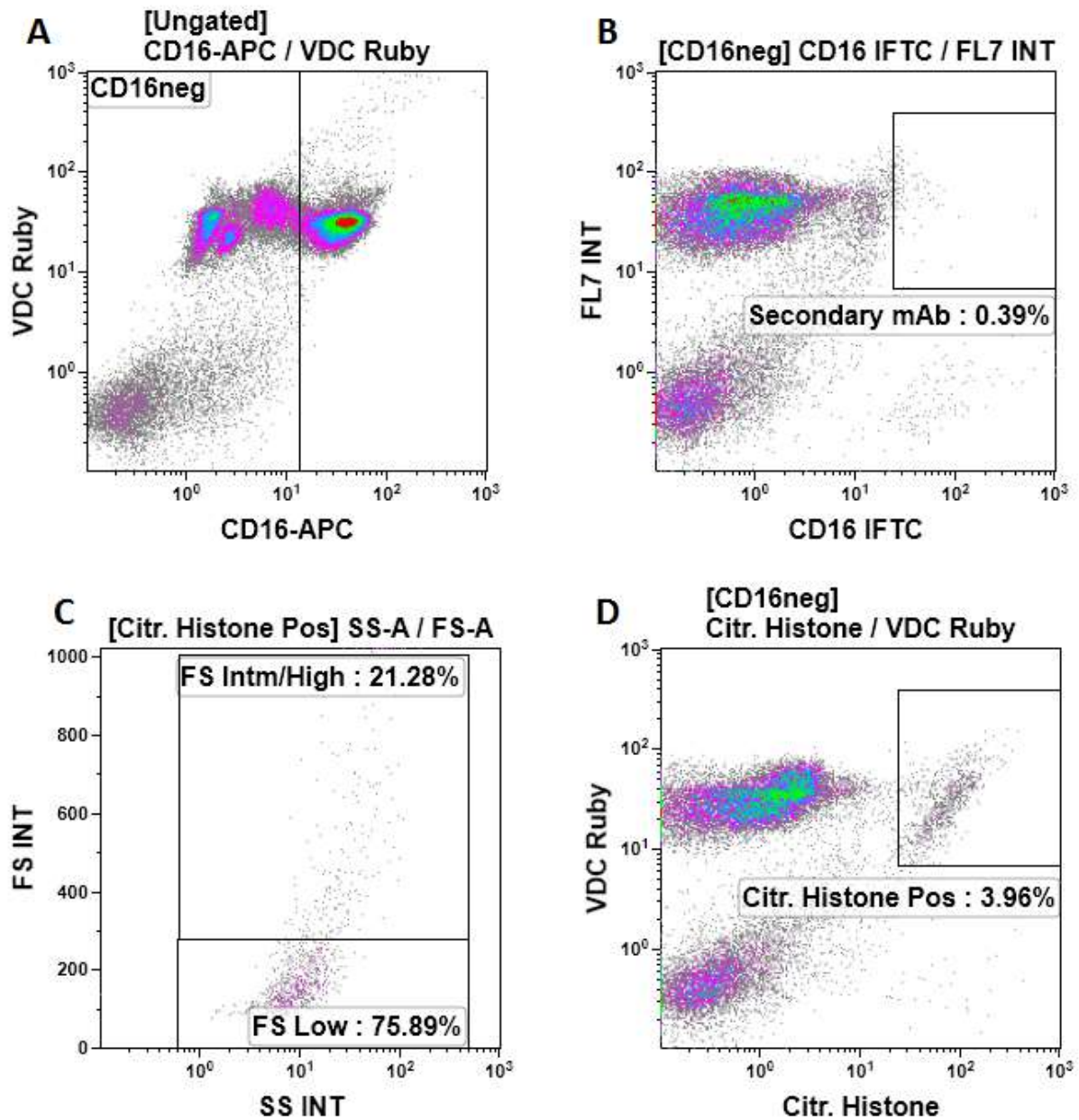
## Results

### *Induction of NETosis by Ply*

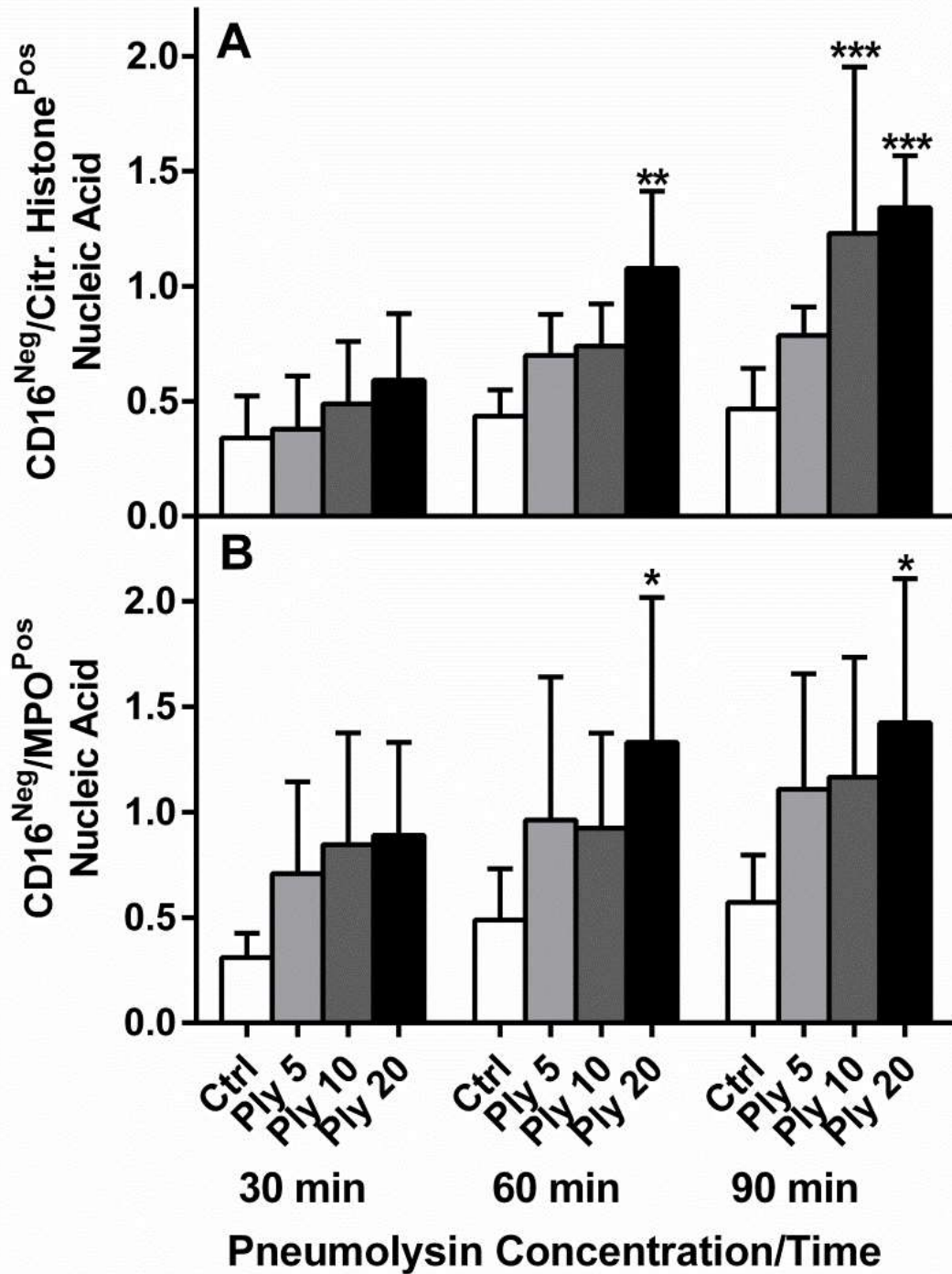
A representative gating strategy used to identify citrullinated histone<sup>pos</sup> / MPO<sup>pos</sup> extracellular DNA fragments following treatment of neutrophils with Ply (20 ng.ml<sup>-1</sup>) for 90 min at 37°C is shown in Figure 1A-D. The various panels show exclusion of intact CD16<sup>pos</sup> neutrophils (A); the non-specific staining control using secondary antibody only (B); indication of extracellular DNA fragment size of CD16<sup>neg</sup>/VDC Ruby<sup>pos</sup>/citrullinated histone<sup>pos</sup> events (C); and identification of CD16<sup>neg</sup>/VDC Ruby<sup>pos</sup> / citrullinated histone<sup>pos</sup> or MPO<sup>pos</sup> extracellular DNA fragments. Probably due to shear pressure applied to the fragments during flow cytometric analysis, the majority of these extracellular DNA fragments, equivalent to 79.59% (7.43), displayed low forward scatter properties (Figure 1D). Only 20.41% (7.43) of the fragments were comparable in size to intact cells (intermediate/high forward scatter).

The effects of addition of Ply (5-20 ng.ml<sup>-1</sup>) on NETosis, over a 90 min time course, using the flow cytometric procedure are shown in Figure 2. Addition of Ply to the cells resulted in a dose-and time-dependent activation of NETosis measured according to the release of citrullinated histone-positive, extracellular DNA from the cells, which achieved statistical significance at a concentration of 20ng.ml<sup>-1</sup> (p<0.05- p<0.01) at 60 min, and at concentrations of 10 and 20ng.ml<sup>-1</sup> Ply after 90 min exposure (p<0.01 and p<0.001 respectively). Similar trends, were observed with respect to detection of MPO-positive, extracellular DNA.

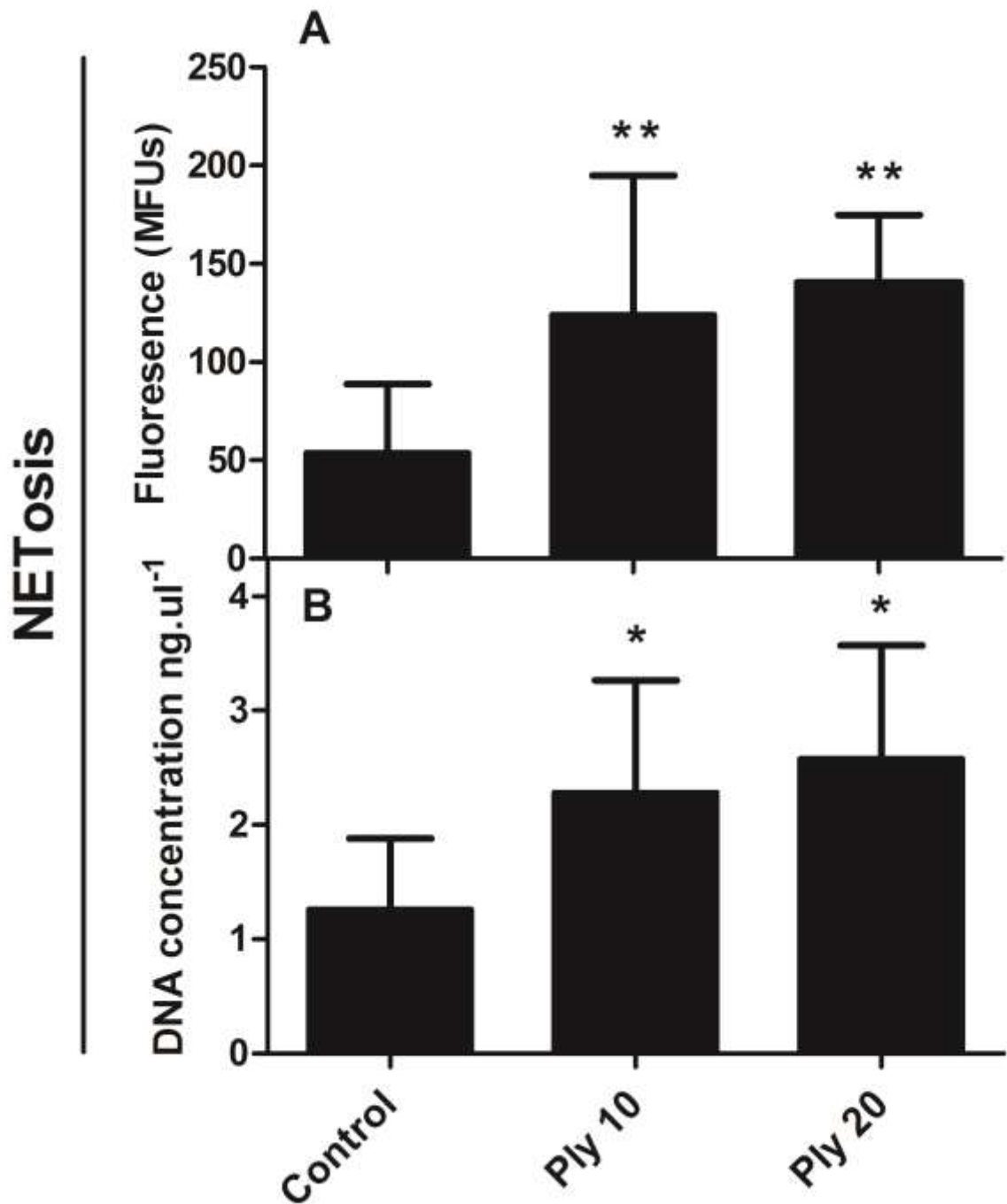
The effects of Ply (10 and 20 ng.ml<sup>-1</sup>) on NETosis, measured using the spectrofluorimetric and NanoDrop® procedures and a fixed incubation time of 60 min, are shown in Figure 3.



**Figure 1:** The flow cytometric gating strategy and staining approach to detect extracellular DNA fragments are shown in panels A-D. Exclusion of intact CD16<sup>pos</sup> neutrophils is shown in A; staining with goat anti-rabbit IgG (H+L)-Alexa Fluor<sup>®</sup> 488 secondary antibody only (non-specific staining control) in B; indication of extracellular DNA fragment size of CD16<sup>neg</sup>/VDC Ruby<sup>pos</sup>/citrullinated (citr). histone<sup>pos</sup> events in C; and identification of CD16<sup>neg</sup>/VDC Ruby<sup>pos</sup>/citrullinated. histone<sup>pos</sup> events in D.



**Figure 2:** Flow cytometric determination of Ply (5, 10 and 20 ng.ml<sup>-1</sup>)-activated NETosis over a 30–90 min time course. The results of 5 separate experiments (n=5) are expressed as the mean percentages of total VDC Ruby<sup>Positive</sup> events (SD), including intact nucleated cells. \*p<0.05; \*\*p<0.01; \*\*\*p<0.001. A 2-way ANOVA with Sidak's multiple comparison test were used to determine significance of the various Ply concentrations at different time points.



**Figure 3:** Measurement of Ply (10 and 20 ng.ml<sup>-1</sup>)-activated NETosis according to measurement of extracellular DNA after 60 min incubation using the spectrofluorimetric (A) and NanoDrop® (B) procedures. The results of 10 separate experiments are expressed as the mean values and SDs. as metered fluorescence units (MFUs) or ng DNA.μl<sup>-1</sup>. \*p<0.01; \*\*p<0.0001. Data shown in both graphs A and B, data was analysed using the t-test.

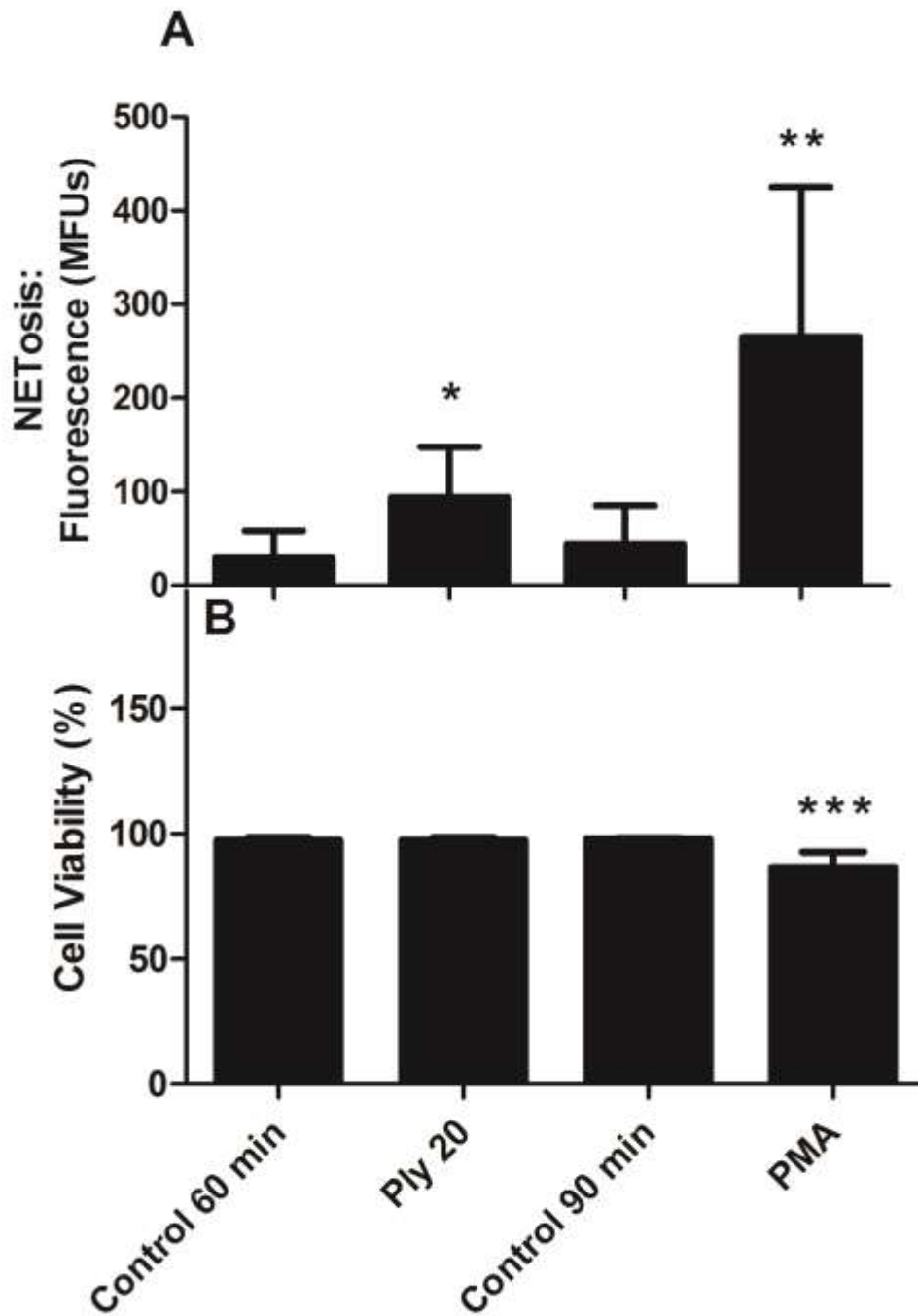
Using both procedures, exposure of neutrophils to Ply at both concentrations resulted in significant increases ( $p < 0.01$ - $p < 0.0001$ ) in release of extracellular DNA. Importantly, no loss of neutrophil viability was observed following treatment of neutrophils with Ply for up to 60 min, the mean percentages viability (SD) being 97.4(0.9), 98.2(0.5), and 97.7(0.7), for the control and Ply (10 and 20  $\text{ng}\cdot\text{ml}^{-1}$ )-treated systems respectively ( $n=6$ ).

A comparison of Ply (20 $\text{ng}\cdot\text{ml}^{-1}$ )- and PMA (6.25  $\text{ng}\cdot\text{ml}^{-1}$ )-activated NET formation using the spectrofluorimetric procedure are shown in Figure 4A with the corresponding cell viability data shown in Figure 4B. Fixed incubation times of 60 min and 90 min were used for Ply and PMA respectively as meaningful PMA-activated NETosis only became detectable at 90 min. PMA, which was a more effective activator of NETosis than Ply, was also associated with loss of viability. A similar time course of PMA-activated NETosis has been described [30].

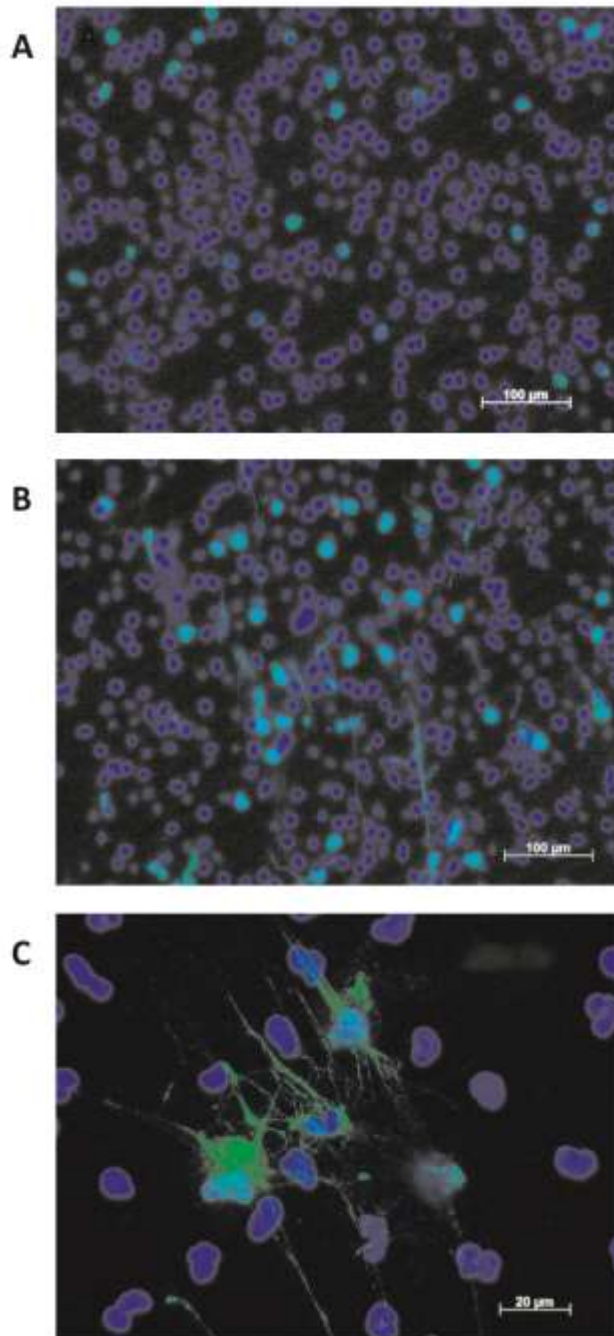
#### *Microscopic detection of NETosis*

According to microscopic analysis, the mean percentages (SD) of NETotic cells in the control system and systems treated with 10 and 20  $\text{ng}\cdot\text{ml}^{-1}$  Ply following 90 min incubation were 4.3(4.2), 14.3(9.9) and 16.5(7.5) respectively ( $n=4$ ,  $p < 0.0001$  for comparison of the control with both Ply-treated systems). A photomicrograph demonstrating Ply (20 $\text{ng}\cdot\text{ml}^{-1}$ )-induced NETs is shown in Figure 5.





**Figure 4:** Comparison of the effects of Ply (20 ng.ml<sup>-1</sup>) and PMA (6.25 ng.ml<sup>-1</sup>) on: i) NETosis using the spectrofluorimetric procedure and exposure times of 60 min and 90 min for systems treated with Ply and PMA respectively (A); and ii) cell viability using the same exposure times (B). The results of 5 experiments (n=5) are presented as the mean values as metered fluorescence units (MFUs) or percentage viability and SD for measurement of NETosis and viability respectively. \*p<0.03; \*\*p<0.02; \*\*\*p<0.007. Data was analysed using the t-test.



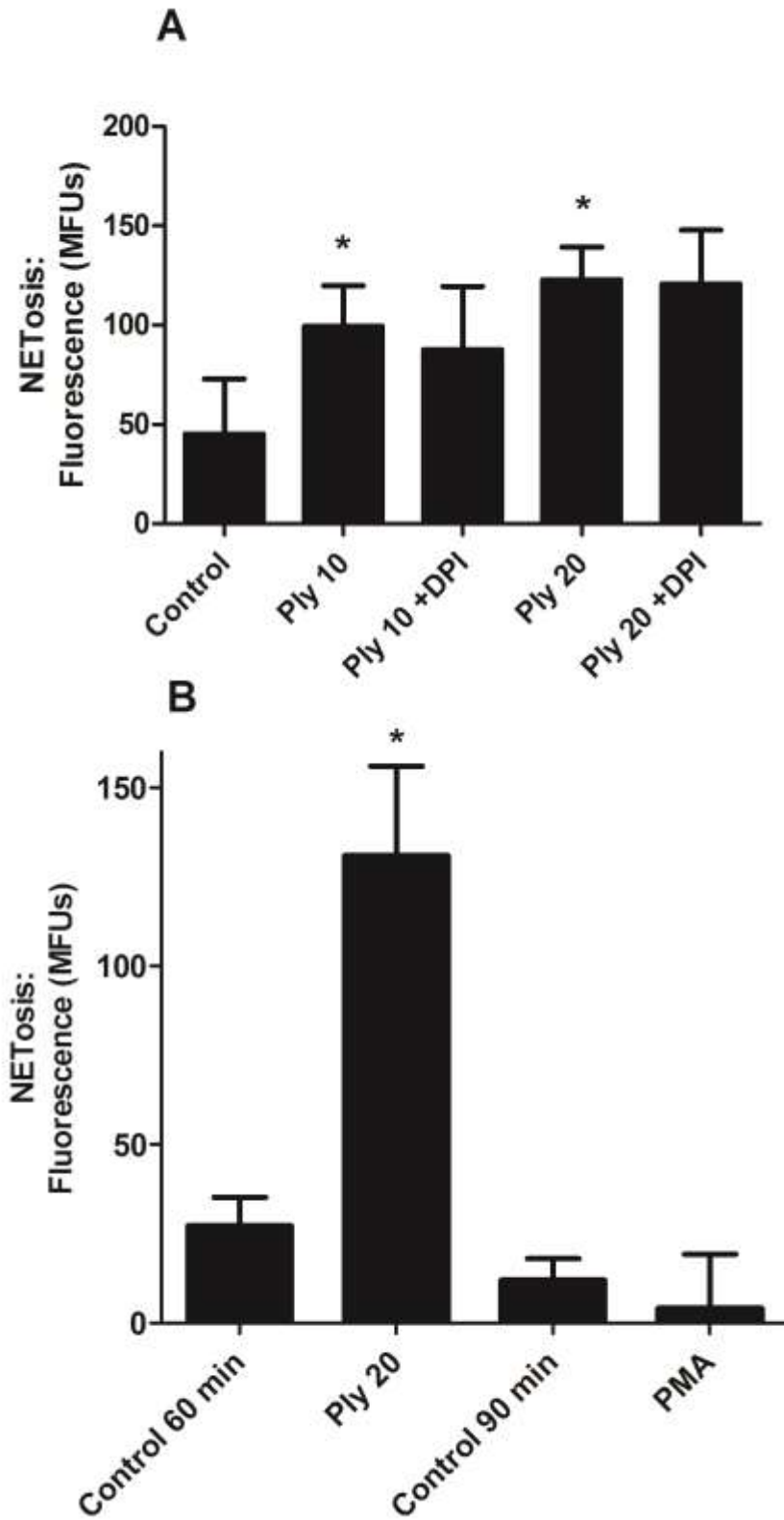
**Figure 5:** Fluorescence microscopic images of neutrophil extracellular trap formation by control neutrophils (A) and cells treated for 90 min with pneumolysin at a concentration of 20 ng.mL<sup>-1</sup> (B) stained with DAPI (nuclear stain) and citrullinated histone antibody viewed at 20x magnification. Image C shows NETotic cells at a higher magnification (40x).

*Assessment of the involvement of Toll-like receptor 4 (TLR4), ROS and extracellular  $Ca^{2+}$  in the pro-NETotic actions of Ply*

Exposure of neutrophils to delta6Ply, which like Ply activates TLR4 [8], failed to induce NETosis, the MFU values (SD) being 15.3(4.1), 96(52.3) and 10.1(10.48), for the control system and the systems treated with 20 ng.ml<sup>-1</sup> Ply or delta6Ply respectively (n=3). Similar results were obtained with the NanoDrop® procedure (not shown). These results apparently exclude involvement of TLR4 in the pro-NETotic actions of Ply.

The effects of the NADPH oxidase inhibitor, DPI (10 µM), on Ply-activated NETosis, as well as the effects of the pore-forming toxin on CGD cells, are shown in Figure 6. Treatment of neutrophils with DPI had modest, statistically insignificant effects on Ply-activated NETosis, while exposure of CGD neutrophils to the toxin, but not to PMA, resulted in significant NETosis. These results appear to exclude meaningful involvement of NADPH oxidase-derived ROS in the pro-NETotic actions of Ply, while confirming their role in PMA-activated NETosis.

Exposure of neutrophils suspended in Ca<sup>2+</sup>-free medium to Ply (10 ng.ml<sup>-1</sup>) for 60 min resulted in significant cytotoxicity accompanied by a massive increase in extracellular DNA (SD) with values of 0.41 (0.25) and 10.35 (1.74) ng DNA.µl<sup>-1</sup> for the control and Ply-treated systems respectively using the NanoDrop® procedure, with corresponding mean percentages (SD) viability of 97.5 (0.5) and 11.7 (11.6) n=3.



**Figure 6:** Measurement of the involvement of reactive oxygen species in Ply ( $20 \text{ ng.ml}^{-1}$ )-mediated NETosis using: i) neutrophils from healthy individuals ( $n=4$ ) treated with the NADPH oxidase inhibitor diphenyleneiodonium chloride (DPI,  $10 \mu\text{M}$ ) shown in A; and ii) neutrophils from a single patient with

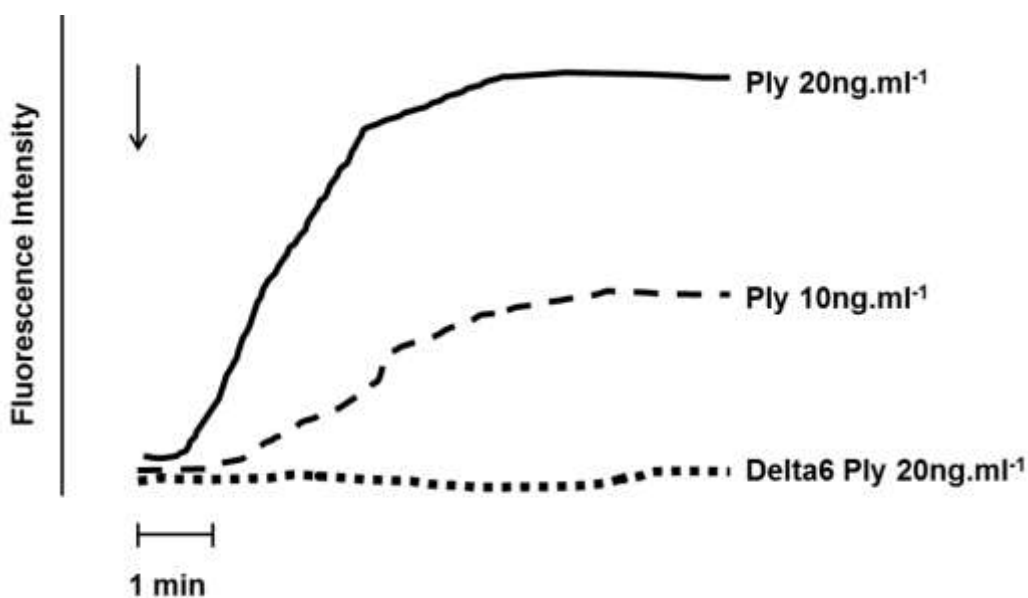
chronic granulomatous disease (CGD) exposed to Ply ( $20\text{ng.ml}^{-1}$ ) or PMA ( $6.25\text{ ng.ml}^{-1}$ ) for 60 and 90 min respectively (4 replicates in each experimental system) as shown in B.

\* $p < 0.03$  for comparison of the Ply-treated systems at both  $10$  and  $20\text{ ng.ml}^{-1}$  with the untreated control system. Inclusion of DPI had no statistically significant effects on NETosis-activated by Ply at either  $10$  or  $20\text{ ng.ml}^{-1}$  (A).

\* $p < 0.03$  for comparison of the responses of Ply-exposed CGD cells with the corresponding control system, while no significant differences were evident between the control and PMA-activated systems (B). Data from both A and B was analysed using the t-test.

### *Assessment of the pore-forming activities of Ply and delta6Ply*

Pore-forming activity was assessed spectrofluorimetrically using fura-2/AM as the  $\text{Ca}^{2+}$ -sensitive, fluorescent probe and these results, which are typical traces from a single representative experiment, are shown in Figure 7. Following a short lag, treatment of neutrophils with native Ply, but not delta6Ply, caused significant influx of  $\text{Ca}^{2+}$ .



**Figure 7:** Fura-2/AM fluorescence traces from a single representative experiment showing the effect of addition (denoted by the arrow, ↓) of either Ply ( $10$  and  $20\text{ ng.ml}^{-1}$ ) or delta6Ply ( $20\text{ ng.ml}^{-1}$ ) on the influx of  $\text{Ca}^{2+}$  into neutrophils recorded as increased fluorescence intensity as a measure of pore-forming activity.

### *Measurement of ROS*

Exposure of neutrophils to Ply resulted in a modest increase in the generation of ROS which was completely abolished by treatment of the cells with DPI ( $p=0.03$ ). The mean peak values (SD) for the control Ply-free system and those treated with  $20\text{ng}\cdot\text{ml}^{-1}$  Ply without and with DPI, were  $1140(286)$ ,  $1427(376)$  and  $36(36)$   $\text{mV}\cdot\text{sec}^{-1}$  respectively ( $n=3$ ). In the case of superoxide generation by the CGD cells, the peak values for unstimulated and PMA ( $6.25\text{ ng}\cdot\text{ml}^{-1}$ )-activated neutrophils from a healthy control subject were  $877(212)$ ,  $5100(246)$   $\text{mV}\cdot\text{sec}^{-1}$  respectively, while the corresponding values from the CGD subject were  $87(43)$  and  $72(35)$   $\text{mV}\cdot\text{sec}^{-1}$ .

### **Discussion**

This study, using, a range of mutually confirmatory procedures, has demonstrated that exposure of human neutrophils to Ply at pathologically relevant concentrations [31] results in activation of NETosis. If such interactions occur in the clinical setting, this may explain, at least in part, the mechanisms underlying the cardiopulmonary complications of severe pneumococcal disease which are associated with a high mortality despite seemingly appropriate antimicrobial therapy [9, 32].

The pro-NETotic actions of Ply resulted in mobilisation of citrullinated histone- and MPO-associated, nuclear DNA, but in contrast to those of the prototype pro-oxidative activator of NETosis, phorbol ester (PMA) which induces late suicidal NETosis [30, 33], these were detected earlier (within 30-60 min), were independent of activation of NADPH oxidase, and occurred in the setting of retention of cell viability. These findings are consistent with the process of vital NETosis previously reported to be initiated by activators such as the *Staphylococcus aureus* pore-forming toxin,

Panton-Valentine leukocidin (PVL) [33], and the calcium ionophore, ionomycin [34]. In both cases, there are some similarities with those of Ply-activated NETosis [33]. These include: i) relative rapidity of onset; ii) lack of involvement of ROS; iii) mobilisation of nuclear DNA; iv) retention of cell viability; and v) restriction of vital NETosis to <20% of the cell population, consistent with the involvement of a neutrophil sub-population in this process [35]. Previous reports using non-opsonised 6 hour bacterial cultures have suggested that pneumococci are less effective inducers of NETosis than *Staphylococcus aureus* [33]. However, as release of Ply occurs late in the growth of pneumococci due to autolysis, 6 hours of culture is too early for optimal release of Ply. In addition, induction of NETosis occurs most effectively in the presence of opsonised bacteria [36].

With respect to alternative potential mechanisms of Ply-mediated NETosis, the toxin in both its native and toxoid forms, has been reported to interact with and activate TLR4 on macrophages [8], also present on neutrophils, representing a potential mechanism of induction of NETosis. This can be excluded, however, on the basis of the observed inactivity of delta6Ply observed in the current study, underscoring the requirement for the pore-forming activity of the intact native toxin in activation of NETosis. In this context, we have previously reported that Ply augments several early-occurring inflammatory activities of neutrophils by a mechanism dependent on pore-formation and influx of extracellular  $\text{Ca}^{2+}$  [24, 25]. These activities include increased generation of ROS, prostanoids and eicosanoids, as well as release of the primary granule enzyme, elastase [24, 25]. In the current study, however, a link between Ply-mediated NETosis and  $\text{Ca}^{2+}$  influx was difficult to establish. This was due to augmentation of the cytotoxic actions of Ply in  $\text{Ca}^{2+}$ -free medium, a

phenomenon we have observed previously [24], which is seemingly related to the requirement for  $\text{Ca}^{2+}$  to repair Ply-mediated membrane pore formation [37]. The lack of activity of delta6Ply therefore represents the major line of evidence, albeit indirect, supporting a pore-forming/ $\text{Ca}^{2+}$ -dependent mechanism in the pro-NETotic actions of Ply. It is, however, also supported by the similarities to the pro-NETotic actions of ionomycin, which also occur within 30-60 min, in the setting of ~~retention of cell viability and~~ lack of involvement of ROS [34]. In this context, it is noteworthy that neutrophils from a CGD individual were responsive to Ply-induced NETosis in keeping with previous reports of the existence of NADPH-oxidase-independent mechanisms of NETosis operative in CGD neutrophils [16, 38, 39].

Pneumococcal pneumonia is associated with marked neutrophilic infiltration [37] and the presence of NETs in the lungs of mice experimentally-infected with the pneumococcus [40]. Although unproven, the events involved in induction of NETosis in this setting are likely to be related to the pro-NETotic actions of Ply described in the current study, possibly augmented by pneumococcal alpha-enolase, which has also been reported to induce NET formation, albeit via an indirect mechanism[41]. Although NETs are intended to trap and possibly kill microbial pathogens, the pneumococcus via expression of the polysaccharide capsule [42] and the surface endonuclease, EndA [43], is particularly adept at evading and escaping from NETs. Induction and subversion of NETosis therefore represents a potential strategy favouring persistence and possibly invasiveness of the pathogen resulting from alveolar epithelial and endothelial damage mediated by the cytotoxic components of NETs, especially histones [17, 21].



The findings of the current study not only extend the repertoire of potentially harmful pro-inflammatory, pro-infective activities of Ply, but may also provide novel insights into the immunopathogenesis of acute lung injury and acute cardiac events during severe pneumococcal infection. Notwithstanding direct cytotoxic effects [12-18] and pro-inflammatory actions of the toxin [24, 25], indirect activities related to activation of NETosis, including cytotoxic and pro-thrombotic activities of NETs [14, 15, 18] are also likely to contribute to toxin-mediated pulmonary and cardiac injury. These findings underscore the potential of Ply-targeted therapies such as macrolide and macrolide-like antibiotics which are potent inhibitors of the production of Ply [44], as well as cholesterol-rich liposomes which neutralise the toxin [45]. In addition, pharmacological inhibitors of peptidylarginine deiminases, key enzymes in the induction of NETosis, are currently in pre-clinical development [46].

### **Conflict of interest statement:**

None of the authors have any conflicts of interest to declare.

### **References**

1. Steel HC, Cockeran R, Anderson R, Feldman C. Overview of community-acquired pneumonia and the role of inflammatory mechanisms in the immunopathogenesis of severe pneumococcal disease. *Mediators Inflamm* 2013; **2013**:490346.
2. Welte T, Torres A, Nathwani D. Clinical and economic burden of community-acquired pneumonia among adults in Europe. *Thorax* 2012; **67**:71-9.
3. Feldman C, Anderson R. Review: current and new generation pneumococcal vaccines. *J Infect* 2014; **69**:309-25.
4. Aliberti S, Ramirez JA. Cardiac diseases complicating community-acquired pneumonia. *Curr Opin Infect Dis* 2014; **27**:295-301.
5. Feldman C, Munro NC, Jeffery PK, Mitchell TJ, Andrew PW, Boulnois GJ, Guerreiro D, Rohde JA, Todd HC, Cole PJ, et al. Pneumolysin induces the

- salient histologic features of pneumococcal infection in the rat lung in vivo. *Am J Respir Cell Mol Biol* 1991; **5**:416-23.
6. Witzenrath M, Gutbier B, Hocke AC, Schmeck B, Hippenstiel S, Berger K, Mitchell TJ, de los Toyos JR, Rosseau S, Suttorp N, Schutte H. Role of pneumolysin for the development of acute lung injury in pneumococcal pneumonia. *Crit Care Med* 2006; **34**:1947-54.
  7. Garcia-Suarez Mdel M, Florez N, Astudillo A, Vazquez F, Villaverde R, Fabrizio K, Pirofski LA, Mendez FJ. The role of pneumolysin in mediating lung damage in a lethal pneumococcal pneumonia murine model. *Respir Res* 2007; **8**:3.
  8. Malley R, Henneke P, Morse SC, Cieslewicz MJ, Lipsitch M, Thompson CM, Kurt-Jones E, Paton JC, Wessels MR, Golenbock DT. Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection. *Proc Natl Acad Sci U S A* 2003; **100**:1966-71.
  9. Musher DM, Rueda AM, Kaka AS, Mapara SM. The association between pneumococcal pneumonia and acute cardiac events. *Clin Infect Dis* 2007; **45**:158-65.
  10. Corrales-Medina VF, Madjid M, Musher DM. Role of acute infection in triggering acute coronary syndromes. *Lancet Infect Dis* 2010; **10**:83-92.
  11. Corrales-Medina VF, Musher DM, Wells GA, Chirinos JA, Chen L, Fine MJ. Cardiac complications in patients with community-acquired pneumonia: incidence, timing, risk factors, and association with short-term mortality. *Circulation* 2012; **125**:773-81.
  12. Alhamdi Y, Neill DR, Abrams ST, Malak HA, Yahya R, Barrett-Jolley R, Wang G, Kadioglu A, Toh CH. Circulating Pneumolysin Is a Potent Inducer of Cardiac Injury during Pneumococcal Infection. *PLoS Pathog* 2015; **11**:e1004836.
  13. Brown AO, Mann B, Gao G, Hankins JS, Humann J, Giardina J, Faverio P, Restrepo MI, Halade GV, Mortensen EM, Lindsey ML, Hanes M, Happel KI, Nelson S, Bagby GJ, Lorent JA, Cardinal P, Granados R, Esteban A, LeSaux CJ, Tuomanen EI, Orihuela CJ. *Streptococcus pneumoniae* translocates into the myocardium and forms unique microlesions that disrupt cardiac function. *PLoS Pathog* 2014; **10**:e1004383.
  14. Mangold A, Alias S, Scherz T, Hofbauer T, Jakowitsch J, Panzenbock A, Simon D, Laimer D, Bangert C, Kammerlander A, Mascherbauer J, Winter MP, Distelmaier K, Adlbrecht C, Preissner KT, Lang IM. Coronary neutrophil extracellular trap burden and deoxyribonuclease activity in ST-elevation acute coronary syndrome are predictors of ST-segment resolution and infarct size. *Circ Res* 2015; **116**:1182-92.
  15. Borissoff JI, ten Cate H. From neutrophil extracellular traps release to thrombosis: an overshooting host-defense mechanism? *J Thromb Haemost* 2011; **9**:1791-4.
  16. Narayana Moorthy A, Narasaraju T, Rai P, Perumalsamy R, Tan KB, Wang S, Engelward B, Chow VT. In vivo and in vitro studies on the roles of neutrophil extracellular traps during secondary pneumococcal pneumonia after primary pulmonary influenza infection. *Front Immunol* 2013; **4**:56.
  17. Ward PA, Grailer JJ. Acute lung injury and the role of histones. *Transl Respir Med* 2014; **2**:1.
  18. Stakos DA, Kambas K, Konstantinidis T, Mitroulis I, Apostolidou E, Arelaki S, Tsironidou V, Giatromanolaki A, Skendros P, Konstantinides S, Ritis K.

- Expression of functional tissue factor by neutrophil extracellular traps in culprit artery of acute myocardial infarction. *Eur Heart J* 2015; **36**:1405-14.
19. Robb CT, Dyrzynda EA, Gray RD, Rossi AG, Smith VJ. Invertebrate extracellular phagocyte traps show that chromatin is an ancient defence weapon. *Nature communications* 2014; **5**:4627.
  20. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, Weinrauch Y, Zychlinsky A. Neutrophil extracellular traps kill bacteria. *Science* 2004; **303**:1532-5.
  21. Saffarzadeh M, Juenemann C, Queisser MA, Lochnit G, Barreto G, Galuska SP, Lohmeyer J, Preissner KT. Neutrophil extracellular traps directly induce epithelial and endothelial cell death: a predominant role of histones. *PLoS One* 2012; **7**:e32366.
  22. Saunders FK, Mitchell TJ, Walker JA, Andrew PW, Boulnois GJ. Pneumolysin, the thiol-activated toxin of *Streptococcus pneumoniae*, does not require a thiol group for in vitro activity. *Infect Immun* 1989; **57**:2547-52.
  23. Kirkham LA, Kerr AR, Douce GR, Paterson GK, Dilts DA, Liu DF, Mitchell TJ. Construction and immunological characterization of a novel nontoxic protective pneumolysin mutant for use in future pneumococcal vaccines. *Infect Immun* 2006; **74**:586-93.
  24. Cockeran R, Theron AJ, Steel HC, Matlola NM, Mitchell TJ, Feldman C, Anderson R. Proinflammatory interactions of pneumolysin with human neutrophils. *J Infect Dis* 2001; **183**:604-11.
  25. Cockeran R, Steel HC, Mitchell TJ, Feldman C, Anderson R. Pneumolysin potentiates production of prostaglandin E(2) and leukotriene B(4) by human neutrophils. *Infect Immun* 2001; **69**:3494-6.
  26. Berkes E, Oehmke F, Tinneberg HR, Preissner KT, Saffarzadeh M. Association of neutrophil extracellular traps with endometriosis-related chronic inflammation. *Eur J Obstet Gynecol Reprod Biol* 2014; **183**:193-200.
  27. Desjardins P, Conklin D. NanoDrop microvolume quantitation of nucleic acids. *Journal of visualized experiments : JoVE* 2010.
  28. Cockeran R, Steel HC, Theron AJ, Mitchell TJ, Feldman C, Anderson R. Characterization of the interactions of the pneumolysoid, Delta6 PLY, with human neutrophils in vitro. *Vaccine* 2011; **29**:8780-2.
  29. Tintinger GR, Theron AJ, Steel HC, Anderson R. Accelerated calcium influx and hyperactivation of neutrophils in chronic granulomatous disease. *Clin Exp Immunol* 2001; **123**:254-63.
  30. Barrientos L, Marin-Esteban V, de Chaisemartin L, Le-Moal VL, Sandre C, Bianchini E, Nicolas V, Pallardy M, Chollet-Martin S. An improved strategy to recover large fragments of functional human neutrophil extracellular traps. *Front Immunol* 2013; **4**:166.
  31. Spreer A, Kerstan H, Bottcher T, Gerber J, Siemer A, Zysk G, Mitchell TJ, Eiffert H, Nau R. Reduced release of pneumolysin by *Streptococcus pneumoniae* in vitro and in vivo after treatment with nonbacteriolytic antibiotics in comparison to ceftriaxone. *Antimicrob Agents Chemother* 2003; **47**:2649-54.
  32. Mongardon N, Max A, Bougle A, Pene F, Lemiale V, Charpentier J, Cariou A, Chiche JD, Bedos JP, Mira JP. Epidemiology and outcome of severe pneumococcal pneumonia admitted to intensive care unit: a multicenter study. *Crit Care* 2012; **16**:R155.

33. Pilszczek FH, Salina D, Poon KK, Fahey C, Yipp BG, Sibley CD, Robbins SM, Green FH, Surette MG, Sugai M, Bowden MG, Hussain M, Zhang K, Kubes P. A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to *Staphylococcus aureus*. *J Immunol* 2010; **185**:7413-25.
34. Parker H, Dragunow M, Hampton MB, Kettle AJ, Winterbourn CC. Requirements for NADPH oxidase and myeloperoxidase in neutrophil extracellular trap formation differ depending on the stimulus. *J Leukoc Biol* 2012; **92**:841-9.
35. Scapini P, Cassatella MA. Social networking of human neutrophils within the immune system. *Blood* 2014; **124**:710-9.
36. Mitchell TJ, Dalziel CE. The biology of pneumolysin. *Subcell Biochem* 2014; **80**:145-60.
37. Wolfmeier H, Schoenauer R, Atanassoff AP, Neill DR, Kadioglu A, Draeger A, Babiychuk EB. Ca<sup>2+</sup>(+)-dependent repair of pneumolysin pores: A new paradigm for host cellular defense against bacterial pore-forming toxins. *Biochim Biophys Acta* 2015; **1853**:2045-54.
38. Rada B, Jendrysik MA, Pang L, Hayes CP, Yoo DG, Park JJ, Moskowitz SM, Malech HL, Leto TL. Pyocyanin-enhanced neutrophil extracellular trap formation requires the NADPH oxidase. *PLoS One* 2013; **8**:e54205.
39. Arai Y, Nishinaka Y, Arai T, Morita M, Mizugishi K, Adachi S, Takaori-Kondo A, Watanabe T, Yamashita K. Uric acid induces NADPH oxidase-independent neutrophil extracellular trap formation. *Biochem Biophys Res Commun* 2014; **443**:556-61.
40. Kadioglu A, Andrew PW. The innate immune response to pneumococcal lung infection: the untold story. *Trends Immunol* 2004; **25**:143-9.
41. Mori Y, Yamaguchi M, Terao Y, Hamada S, Ooshima T, Kawabata S. alpha-Enolase of *Streptococcus pneumoniae* induces formation of neutrophil extracellular traps. *J Biol Chem* 2012; **287**:10472-81.
42. Wartha F, Beiter K, Albiger B, Fernebro J, Zychlinsky A, Normark S, Henriques-Normark B. Capsule and D-alanylated lipoteichoic acids protect *Streptococcus pneumoniae* against neutrophil extracellular traps. *Cell Microbiol* 2007; **9**:1162-71.
43. Beiter K, Wartha F, Albiger B, Normark S, Zychlinsky A, Henriques-Normark B. An endonuclease allows *Streptococcus pneumoniae* to escape from neutrophil extracellular traps. *Curr Biol* 2006; **16**:401-7.
44. Anderson R, Steel HC, Cockeran R, von Gottberg A, de Gouveia L, Klugman KP, Mitchell TJ, Feldman C. Comparison of the effects of macrolides, amoxicillin, ceftriaxone, doxycycline, tobramycin and fluoroquinolones, on the production of pneumolysin by *Streptococcus pneumoniae* in vitro. *J Antimicrob Chemother* 2007; **60**:1155-8.
45. Henry BD, Neill DR, Becker KA, Gore S, Bricio-Moreno L, Ziobro R, Edwards MJ, Muhlemann K, Steinmann J, Kleuser B, Japtok L, Luginbuhl M, Wolfmeier H, Scherag A, Gulbins E, Kadioglu A, Draeger A, Babiychuk EB. Engineered liposomes sequester bacterial exotoxins and protect from severe invasive infections in mice. *Nat Biotechnol* 2015; **33**:81-8.
46. Bozdog M, Dreker T, Henry C, Tosco P, Vallaro M, Fruttero R, Scozzafava A, Carta F, Supuran CT. Novel small molecule protein arginine deiminase 4 (PAD4) inhibitors. *Bioorg Med Chem Lett* 2013; **23**:715-9.