Pneumolysin mediates heterotypic aggregation of neutrophils and platelets *in vitro*

Jan G. Nel*, Chrisna Durandt[+], Annette J. Theron[o], Gregory R. Tintinger[◊], Roger Pool*, Guy A. Richards[˝], Timothy J. Mitchell[▲], Charles Feldman[#], Ronald Anderson[o+]

*Department of Haematology, Faculty of Health Sciences, University of Pretoria, Pretoria, South Africa and Tshwane Academic Division of the National Health Laboratory Service, Pretoria, South Africa

[+]Institute for Cellular and Molecular Medicine, South African Medical Research Council Unit for Stem Cell Research, Department of Immunology, Faculty of Health Sciences, University of Pretoria, Pretoria, South Africa

[o]Department of Immunology, Faculty of Health Sciences, University of Pretoria, Pretoria, South Africa and Tshwane Academic Division of the National Health Laboratory Service, Pretoria, South Africa

[◊]Department of Internal Medicine, Faculty of Health Sciences, University of Pretoria, Pretoria, South Africa

[˝]Department of Critical Care, Charlotte Maxeke Johannesburg Academic Hospital and Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa

[▲]Institute of Microbiology and Infection, College of Medical and Dental Sciences, University of Birmingham, Birmingham, UK

[#]Division of Pulmonology, Department of Internal Medicine, Charlotte Maxeke Johannesburg Academic Hospital and Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa

Correspondence: Dr J.G. Nel, Department of Haematology, PO Box 2034, Pretoria, 0001, South Africa
Telephone: +27-12-319-2641
Telefax: +27-12-323-0732
E-mail: jan.nel@up.ac.za

Highlights

- Pneumolysin (Ply) activates production of PAF and thromboxane A_2 (TxA_2) by neutrophils.
- Ply also promotes formation of pro-thrombotic neutrophil:platelet (NP) aggregates.
- Ply-mediated aggregate formation is independent of PAF and TxA_2.
- P-selectin (CD62P) and protease-activated receptor 1 are involved in Ply-induced NP aggregation.
- Ply-mediated NP aggregate formation may contribute to pulmonary and myocardial injury.

Abstract

Objectives. Platelets orchestrate the inflammatory activities of neutrophils, possibly contributing to pulmonary and myocardial damage during severe pneumococcal infection. This study tested the hypothesis that the pneumococcal toxin, pneumolysin (Ply), activates production of platelet-activating factor (PAF) and thromboxane A_2 (TxA_2) by neutrophils, these bioactive lipids being potential mediators of neutrophil:platelet (NP) networking.

Methods. The effects of recombinant Ply (10–80 $ng.mL^{-1}$) on the production of PAF and TxA_2 by isolated neutrophils were measured using ELISA procedures, and NP aggregation by flow cytometry.

Results. Exposure of neutrophils to Ply induced production of PAF and, to a lesser extent, TxA_2, achieving statistical significance at ≥20 $ng.mL^{-1}$ of the toxin. In the case of NP interactions, Ply promoted heterotypic aggregation which was dependent on upregulation of P-selectin (CD62P) and activation of protease-activated receptor 1

(PAR1), attaining statistical significance at ≥10 ng.mL^{-1} of the toxin, but did not involve either PAF or TxA_2.
Conclusion. Ply induces synthesis of PAF and TxA_2, by human neutrophils, neither of which appears to contribute to the formation of NP heterotypic aggregates *in vitro*, a process which is seemingly dependent on CD62P and PAR1. These pro-inflammatory activities of Ply may contribute to the pathogenesis of pulmonary and myocardial injury during severe pneumococcal infection.

Keywords. Calcium, platelet-activating factor, pneumolysin, P-selectin (CD62P), severe pneumococcal disease.

Pneumolysin (Ply), the cholesterol-binding, pore-forming toxin of *Streptococcus pneumoniae,* is recognised as being the major protein virulence factor of this intransigent respiratory pathogen, the most common bacterial cause of community-acquired pneumonia (CAP) and associated organ damage (1-4). Importantly, Ply has been identified as being a key mediator of both acute lung injury (ALI) (5-8) and myocardial damage (9, 10) in murine models of severe pneumococcal disease. In one such model of ALI, exposure of isolated, perfused lungs to recombinant Ply resulted in the development of pulmonary hypertension and microvascular barrier dysfunction, both of which are key features of this condition in humans (6, 7). The underlying mechanisms appeared to involve increased pulmonary production of the bioactive lipid, platelet-activating factor (PAF), which, in turn, was proposed to activate production of the more potent platelet activator *viz.* the prostanoid, thromboxane A_2 (TxA_2), with resultant vasoconstriction and platelet activation (7). Although the authors speculated that PAF may have originated from Ply-exposed endothelial cells (7), infiltrating neutrophils represent an alternative source of the bioactive lipid. Unlike macrophages, neutrophils express high levels of the PAF-generating enzyme, PAF acetylhydrolase (11). However, to our knowledge a

possible link between Ply, neutrophils, PAF and platelet activation has not been described.

In the context of acute cardiovascular events associated with invasive pneumococcal disease, Ply, via its pore-forming activity, has been reported to inflict injury on myocardium through the formation of cardiac microlesions (9, 10). However, the existence of alternative mechanisms of Ply-mediated cardiotoxicity, possibly related to the pro-inflammatory/pro-thrombotic activities of the toxin are largely unexplored (12-14).

To probe the existence of such mechanisms in the pathogenesis of Ply-mediated ALI and myocardial injury, we have investigated the effects of recombinant Ply on the production of PAF and TxA_2 by isolated, human blood neutrophils *in vitro*. In addition, we have also explored the effects of Ply on the formation of potentially, pro-thrombotic, heterotypic aggregates of neutrophils and platelets (15-19), focusing on the involvement of PAF, TxA_2 and other potent platelet activators, as well as the adhesion molecule, P-selectin (CD62P), in this process.

MATERIALS AND METHODS

Permission to draw blood from healthy, adult human volunteers was granted by the Research Ethics Committee, Faculty of Health Sciences, University of Pretoria.

Pneumolysin

Recombinant Ply and the pneumolysoid, delta6Ply, attenuated in respect of pore-forming activity, were prepared as described previously (20, 21). The possible influence of contaminating endotoxin was excluded in both Ply preparations using the Endosafe®-PTS™ system (Charles River Laboratories, Wilmington, MA, USA) which is based on the *Limulus* amoebocyte lysate kinetic chromogenic method. Both active Ply and delta6Ply contained <1 endotoxin unit (EU)/µg of protein after purification (1 EU is the lower limit of detection).

Chemicals

PSB 0739, WEB 2086, S 18886 and SCH 79797 antagonists of the platelet purinergic receptor, P2Y12, the PAF receptor, the TxA_2 TP prostanoid receptor, and the protease-activated receptor 1 (PAR1, thrombin activated) respectively were purchased from TOCRIS Bioscience, Bristol, UK. The oral thrombin inhibitor, dabigatran, was provided to one of us (GAR) by Boehringer-Ingelheim Pharma GmbH, Germany. All of these were dissolved to stock solutions of 10 mM in dimethylsulphoxide (DMSO) and used at final concentrations of 10 μM (final DMSO concentrations of 0.1%) in the assays described below. Appropriate DMSO control systems were included in all of the assays in which these receptor antagonists were used.

Mouse anti-human C62P blocking antibody (P-selectin, non-fluorochrome-labelled), was purchased from Biolegend, London, UK. Unless stated, all other chemicals and reagents were purchased from the Sigma Chemical Co., St. Louis, MO, USA.

Preparation of neutrophils

Neutrophils were prepared from heparinised venous blood (5 units preservative-free heparin.mL^{-1}) as described previously (22). Briefly, neutrophil/erythrocyte pellets obtained following centrifugation of whole blood on Histopaque-1077 (Sigma Diagnostics) were resuspended and sedimented in 3% gelatin to remove most of the erythrocytes. Residual erythrocytes were then removed by differential lysis (brief treatment with 0.83% ammonium chloride) and the resultant neutrophil populations of high purity and viability (>90% and >95% respectively) suspended to a concentration of $1x10^7$ cells.mL^{-1} in Hanks' balanced salt solution (HBSS, indicator-free, pH7.4).

PAF and TxA_2

Neutrophils ($2x10^6$) suspended in HBSS were prewarmed for 10 min at 37°C followed by addition of one of the following: i) HBSS (negative control); ii)

recombinant Ply at final concentrations of 10, 20, 40 and 80 nanograms (ng).mL^{-1}; iii) the pneumolysoid, delta6Ply, which is attenuated with respect to pore-forming activity, at a fixed, final concentration of 80 ng.mL^{-1}; or iv) the calcium ionophore, A23187 at 2 μM (final) as a positive control system. The final volume in each test tube was 2 mL. After a further 5 min period of incubation at 37°C, the tubes were transferred to an ice-bath to stop the reactions. Following removal of the cells by centrifugation, the concentrations of PAF and TxA_2 in the cell-free supernatants were measured using commercial sandwich ELISA procedures (Cusabio® Life Science, Wuhan, P.R. China and Abnova GmbH, Heidelberg, Germany respectively) and the results expressed as ng.mL^{-1} and picograms (pg).mL^{-1} respectively. Cell viability was measured using a propidium iodide-based flow cytometric procedure.

Neutrophil:platelet (NP) aggregate formation

In order to minimise spontaneous activation of platelets, NP-enriched buffy coat suspensions, enumerated for both cell types by standard haematological procedures, were used for these studies in keeping with earlier reports which used whole blood (23-25). These cell suspensions were prepared from the heparinised blood of healthy, adult humans by sedimentation at 37°C and diluted 1:50 in HBSS to give a final volume of 1 ml. Following 5 min of preincubation at 37°C, recombinant Ply (10–80 ng.mL^{-1}), delta6Ply (80 ng.mL^{-1}), or adenosine 5'-diphosphate (ADP, 100 μM final, agonist of platelet P2Y12 receptors as a positive control) were added to the cell suspensions which were incubated for a further 5 min at 37°C. Following incubation, the cell suspensions were stained with 5 μl of each of the following murine, anti-human, fluorochrome-labelled monoclonal antibodies to detect neutrophils, platelets and total leukocytes: CD16-allophyocyanin (Biolegend, San Diego, CA, USA), CD42a-phycoerythrin (Becton Dickenson, San Jose, CA, USA) and CD45-Krome Orange (Beckman Coulter, Marseille, France) and incubated for 15 min at room temperature in the dark. This was followed by analysis of the various cell suspensions at a slow flow rate using a Gallios flow cytometer (Beckman Coulter, Miami, USA). NP interactions were determined according to the CD16$^+$/CD42a$^+$ co-expression profiles of CD45$^+$ leukocytes and the results expressed as the relative median fluorescence intensities of CD42a expression of these NP aggregates . Platelet aggregates were excluded prior to the

aforementioned analysis as indicated in Figure 1 which depicts this gating strategy. Note that residual erythrocytes [confirmed by staining with an anti-CD 235a (glycophorin) monoclonal antibody, Becton Dickenson] in the cell suspensions were not lysed prior to flow cytometric analysis to minimise non-specific activation of platelets.

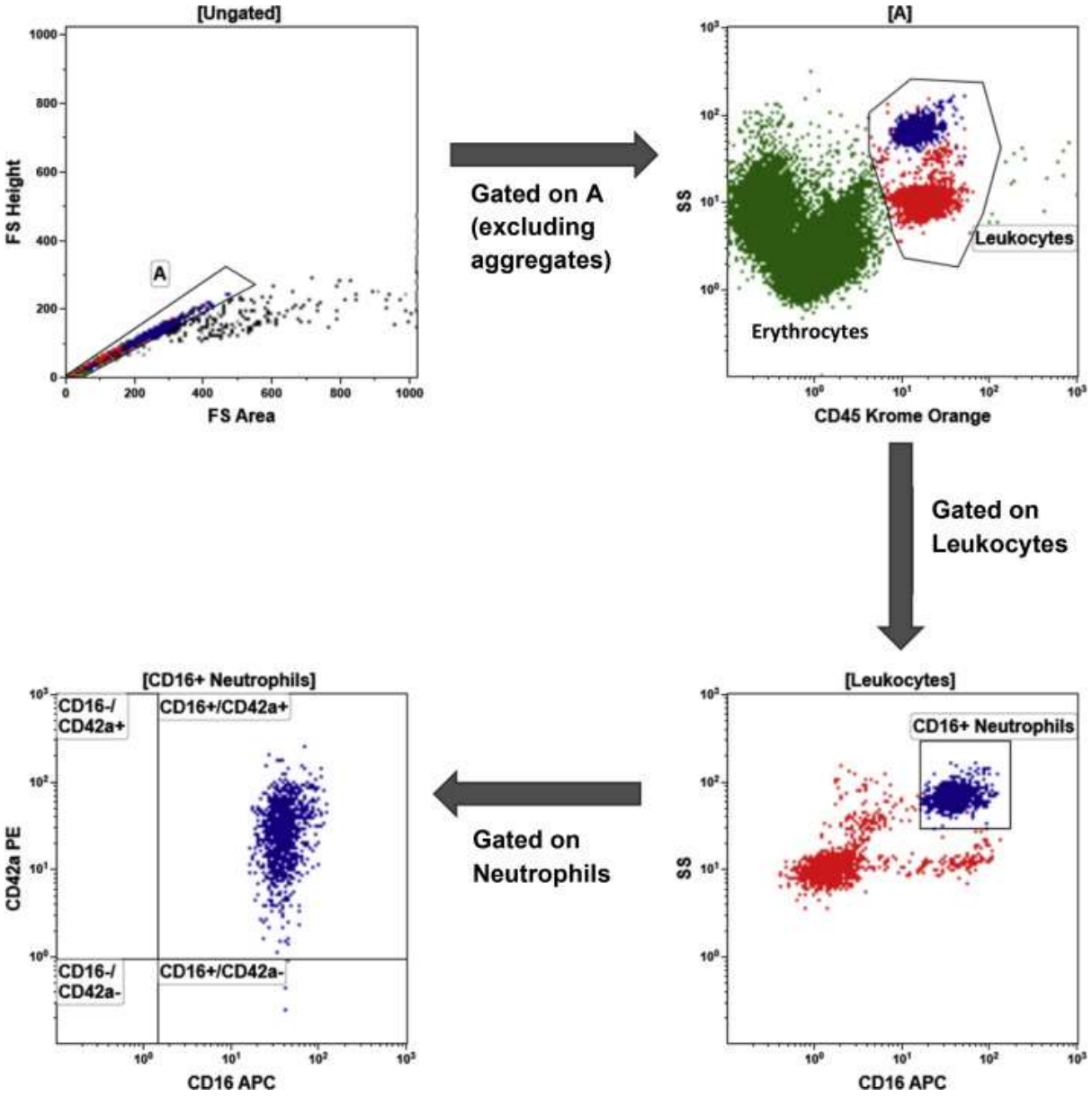

Figure 1. Illustration of the gating strategy followed to identify platelet:neutrophil aggregates. Large aggregates were excluded using a Forward Scatter Area vs Forward Scatter Height plot. This was followed by the identification of $CD45^+$ leukocytes, after which $CD16^+$ neutrophils were identified. The expression intensity of CD42a (marker for platelets) was then measured on the $CD45^+/CD16^+/CD42a^+$ cells. An erythrocyte lysis step was not included in order to minimise non-specific activation of platelets.

In a limited series of experiments (2 in the series) undertaken to ensure the veracity of the various antagonists of platelet P2Y12 and PAF receptors and PAR1 (PSB 0739, WEB 2086, and SCH 79797 respectively), these agents were added to platelet-rich plasma which was incubated for 5 min at 37ºC prior to the addition of the respective receptor agonists, ADP (100 µM), PAF (400 nM), or thrombin (from human plasma, 1.25 NIH units, final). After a further period of incubation for 5 min at 37ºC platelet activation was measured flow cytometrically as described previously according to upregulated expression of the adhesion molecule, CD62P (P-selectin) (14, 25).

The following series of experiments explored the effects of the various platelet receptor (P2Y12, PAF, PAR1, TxA_2) antagonists (all at 10 µM), as well as those of indomethacin (5 µM) and a mouse anti-human CD62 P blocking monoclonal antibody (5 µL per mL of cell suspension), all added prior to preincubation, on NP aggregation activated by Ply (40 $ng.mL^{-1}$, final) measured as described above. The following were also investigated: i) the requirement for extracellular Ca^{2+} in the pro-aggregation activity of Ply; ii) the specificity of the PAR1 receptor antagonist, SCH 79797, which was assessed by measuring the effects of this agent on NP aggregation induced by purified thrombin (1.25 NIH units), as well as on spontaneous aggregation and that activated by ADP (100 µM); and iii) the effects of dabigatran (10 µM) on NP aggregation induced by either thrombin or Ply (40 $ng.mL^{-1}$, final)

Expression and statistical analysis of results

The results of each series of experiments are expressed as median values with interquartile ranges with numbers of different donors and experiments indicated in the text or figure legends. Statistical analyses were performed using GraphPad Prism5 (GraphPad Software, San Diego, USA) using a one-way ANOVA with a Bonferroni correction for multiple comparisons.

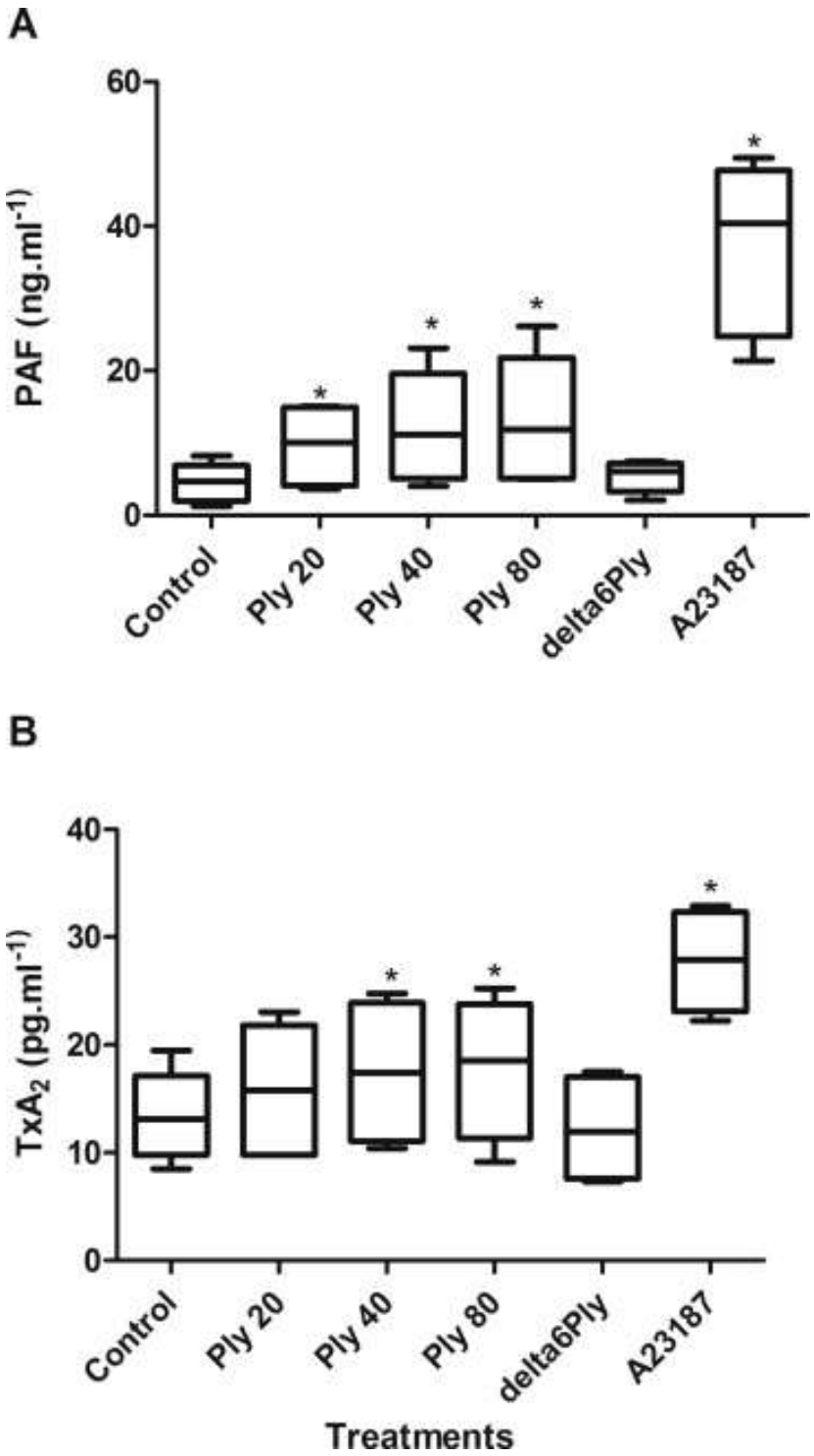

Figure 2. The effects of addition of pneumolysin (Ply) at concentrations of 10–80 ng ml^{-1}, as well as those of delta 6 pneumolysin (delta6Ply, 80 ng ml^{-1}) and the calcium ionophore A23187 (2 μM), to neutrophils on the production of platelet-activating factor (PAF) and thromboxane A_2 (TxA_2) are shown in Figs. 2A and B respectively. The data from 5 different experiments, using cells from 5 different individuals, are expressed as the median values with interquartile ranges. BG = background value for unstimulated cells. *$p < 0.05$–$p<0.002$.

RESULTS

Production of PAF and TxA_2 by Ply-activated neutrophils

These results are shown in figures 2A and 2B for PAF and TxA_2 respectively. As shown in figure 2A, exposure of neutrophils to Ply resulted in dose-related activation of generation of PAF which achieved statistical significance at concentrations ≥20ng.mL^{-1} of the toxin, while the non-physiological positive control, A23187, as expected was extremely potent, and delta6Ply ineffective. The corresponding data for TxA_2 production by neutrophils are shown in figure 2B, which demonstrate similar, albeit lesser, effects.

Neutrophil viability

These results are shown in Figure 3. Exposure of neutrophils to the highest concentrations of Ply (40 and 80 ng.mL^{-1}) or to A23187 caused modest, but nevertheless statistically significant, loss of viability. The median viability values for the control, untreated system and for systems treated with Ply at 40 and 80 ng.mL^{-1} or A23187 were 98.9% (IQR 98.5-99.4%), 93.9% (p<0,005), 91.0% (p<0,001), and 95.4% (p<0.01) respectively (data from 4 experiments using cells from 4 different donors).

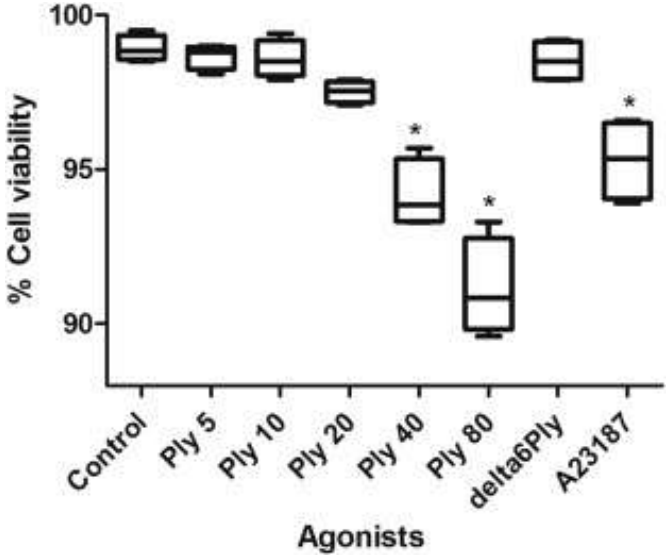

Figure 3. The effects of the addition of pneumolysin (Ply), at concentrations of 5–80 ng ml^{-1}, as well as those of delta 6 pneumolysin (delta6Ply, 80 ng ml^{-1}) and the calcium ionophore A23187 (2 μM, positive control) on neutrophil viability. *p < 0.01–p<0.001.

Effect of Ply on the formation of NP heterotypic aggregates

The median neutrophil and platelet counts of the buffy coats used in these and subsequent experiments were 4.67 x 10^{3}.μL^{-1} and 451 x 10^{-3}.μL^{-1} respectively, and the results are shown in Figure 3. Addition of Ply to mixed NP suspensions resulted in dose-related formation of NP aggregates which achieved statistical significance at concentrations of ≥20 ng.mL^{-1} of the toxin and was greater than that observed with ADP, while delta6Ply was ineffective (Figure 4).

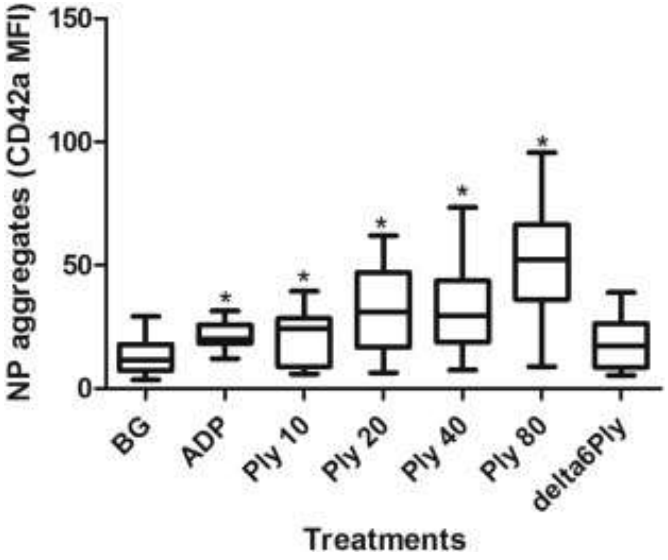

Figure 4. The effects of the addition of ADP (100 μM, positive control) or pneumolysin (Ply, 10–80 ng ml^{-1}) or delta 6 pneumolysin (delta6Ply 80 ng ml^{-1}) on the formation of heterotypic neutrophil:platelet (NP) aggregates. The results of 35 experiments, using cell suspensions from 13 different donors are expressed as the CD42a median fluorescence intensity (MFI) with interquartile ranges. The aggregates assessed were positive for co-expression of CD16, CD42a and CD45. *p < 0.001–p < 0.0001.

Assessment of the veracity of the various platelet receptor antagonists

Prior to assessing their effects on Ply-mediated NP aggregate formation, the efficacy of the various platelet-receptor antagonists (PSB 0739,WEB 2086, SCH 79797, all at 10 μM) was measured in a series of preliminary experiments, using platelet-rich plasma. Following addition of the corresponding, respective receptor agonists ADP (100 μM), PAF (400 nM) or thrombin (1.25 NIH units), platelet activation was measured flow cytometrically according to the level of expression of

the adhesion molecule, CD62P. The results, which are shown in Figure 5, demonstrate the activities of the receptor agonists, with PAF being the least potent, as well as the inhibitory activities of the various receptor antagonists. Importantly, upregulation of expression of CD62P by ADP-, PAF- or thrombin-treated platelets was significantly attenuated by PSB 0739, WEB 2086, and SCH 79797, confirming receptor antagonism.

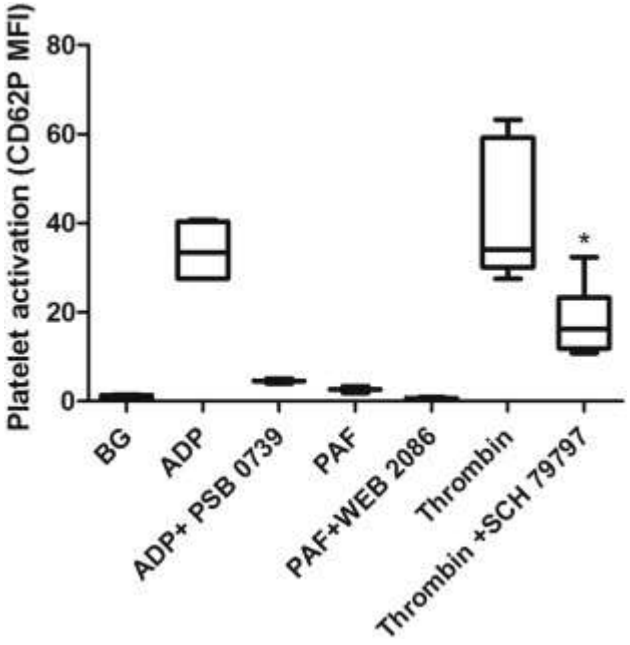

Figure 5. The effects of addition of ADP (100 μM), platelet-activating factor (PAF, 400 nM) or thrombin (1.25 NIH units ml^{-1}) in the absence and presence of their respective receptor antagonists (PSB 0739, WEB 2086, SCH 79797 all at 10 μM) to platelet-rich plasma on expression levels of the adhesion molecule CD62P. The results of 6 experiments, using platelet rich plasma from 2 different donors are expressed as the median $CD16^+/CD42a^+/CD45^+$ fluorescence intensities with interquartile ranges.

Effects of the various platelet receptor antagonists, indomethacin, Ca^{2+}depletion, and an anti-CD62P monoclonal antibody on Ply-mediated NP aggregation

The effects of the various platelet receptor antagonists and indomethacin, as well as those of suspension of the cells in Ca^{2+}-free HBSS, on Ply (40 $ng.mL^{-1}$)-activated formation of NP aggregates are shown in Figure 6A, while those of inclusion of the anti-human CD62P blocking monoclonal antibody are shown in Figure 6B. Exposure of the cells to the PAR1 antagonist, SCH 79797, as well as suspension of the cells in Ca^{2+}-free medium resulted in significant attenuation of Ply-

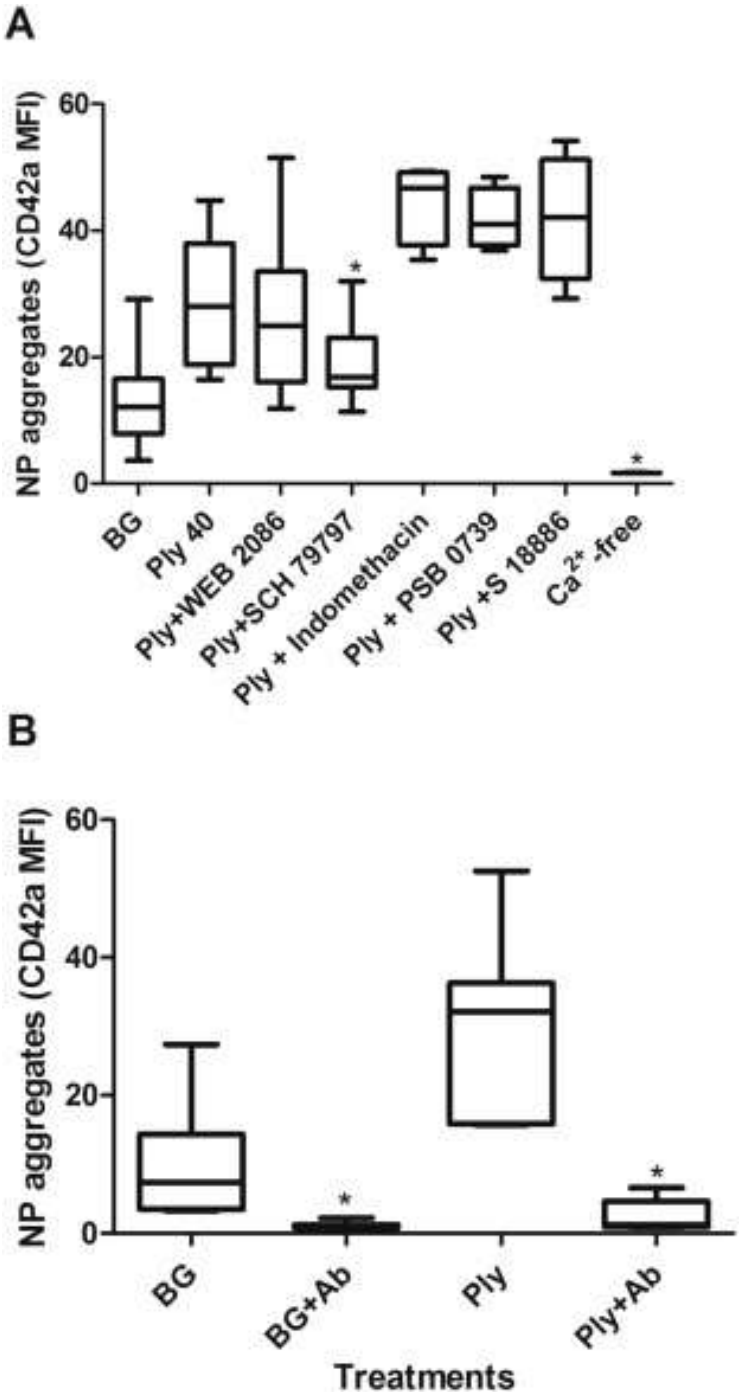

Figure 6. The results in the upper figure (6A) show the effects of pneumolysin (Ply 40 ng ml^{-1}) only or in the presence of WEB 20186, SCH 79797, indomethacin, PSB 0739 or S 18886, all at 10 μM), as well as the effect of calcium depletion from the cell suspending medium, on the formation of neutrophil; platelet (NP) heterotypic aggregates. The results of 18 experiments using cells from 7 donors are expressed as the median $CD16^+/CD42a^+/CD45^+$ fluorescence intensities with interquartile ranges. The results in the lower figure (6B) show the effects of addition of an anti-CD62P monoclonal antibody to buffy coat suspensions on the spontaneous (BG) and pneumolysin (Ply 40 ng ml^{-1})-activated formation of neutrophil:platelet heterotypic aggregates. *$p < 0.006$ For comparison with the corresponding Ply-treated, drug-free control system. *$p < 0.0004$ For comparison of the control and corresponding anti-CD62P-treated systems.

activated formation of NP aggregates, while the other receptor antagonists and indomethacin were ineffective (Figure 6A). Inclusion of the anti-CD62P antibody caused almost complete attenuation of Ply-mediated NP aggregate formation (Figure 6B). Depletion of Ca^{2+}, as well as inclusion of the anti-CD62P antibody also caused significant reductions in basal NP aggregation, underscoring the involvement of both Ca^{2+} and CD62P in basal aggregation. These observations demonstrate significant involvement of CD62P, as well as PAR1, but not the P2Y12, PAF or TxA_2 receptors in Ply-mediated NP aggregate formation.

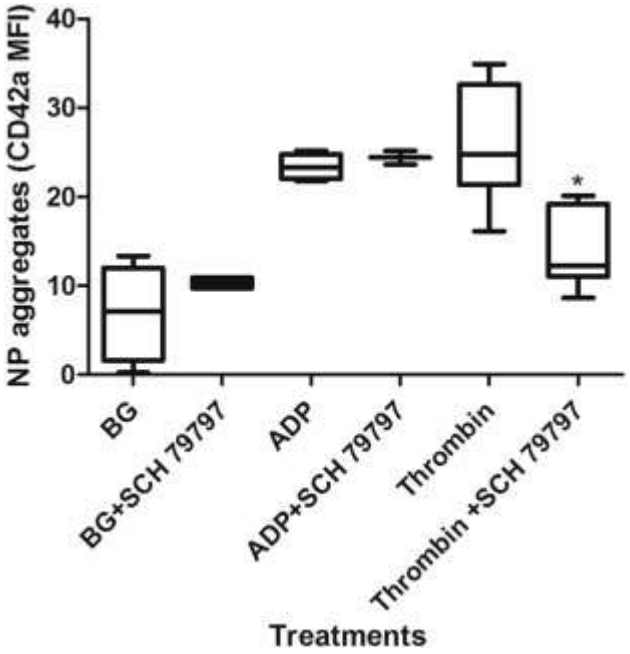

Figure 7. Measurement of the effects of addition of the PAR1 receptor antagonist, SCH 79797 (10 μM), to buffy coat suspensions on the spontaneous (background) and ADP (100 μM)- or thrombin (1.25 NIH units ml^{-1})-activated formation of neutrophil platelet (NP) heterotypic aggregates. The results of 4 experiments using cells from 4 donors are expressed as the CD42a median fluorescence intensity (MFI) with interquartile ranges. The aggregates assessed were positive for co-expression of CD16, CD42a and CD45. *p < 0.001 For comparison of the thrombin-activated systems without and with SCH 79797

Effects of SCH 79797 on spontaneous, ADP- and thrombin-activated NP aggregation

To probe the receptor-targeted veracity of SCH 79797 (10μM) in the context of NP aggregate formation, the effects of this agent on spontaneous, ADP- or thrombin-

activated NP aggregation were investigated and these results are shown in Figure 7. Addition of SCH 79797 to the mixed neutrophil and platelet suspensions during pre - incubation resulted in statistically significant formation of thrombin-activated heterotypic aggregates, but had no effect on either spontaneous or ADP-activated formation of NP aggregates. These findings confirm the selectivity of SCH 79797 for PAR1 and the probable involvement of this receptor in Ply-mediated NP aggregate formation, possibly via thrombin activation.

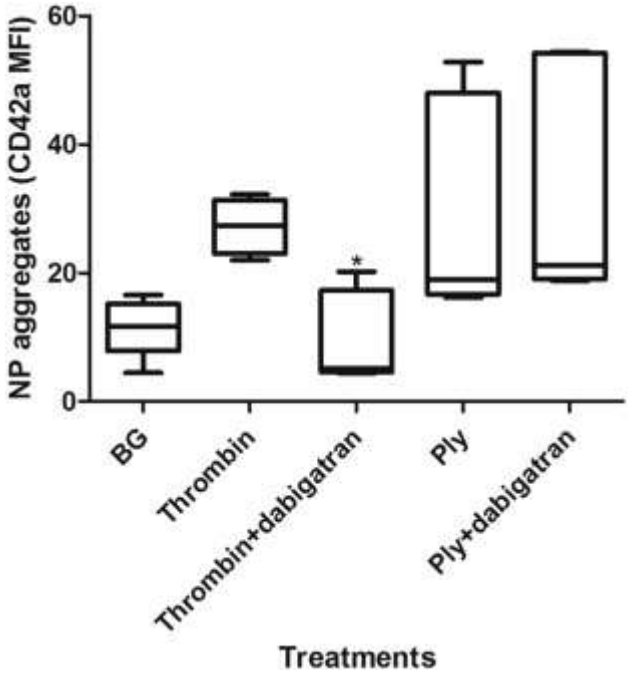

Figure 8. Measurement of the effects of dabigatran (10 μM) added to buffy coat suspensions on the formation of heterotypic neutrophil:platelet (NP) aggregates, activated by either thrombin (1.25 NIH units ml^{-1}) or pneumolysin (Ply 40 ng ml^{-1}). The results of 6 experiments using cells from 2 donors are expressed as the median $CD16^{+}/CD42^{+}/CD45^{+}$ fluorescence intensities with interquartile ranges. *p < 0.02 For comparison of the thrombin-activated systems in the absence or presence of dabigatran.

Effect of dabigatran on Ply-mediated NP aggregation

The thrombin inhibitor, dabigatran, was used to explore the possible involvement of thrombin in Ply-mediated activation of PAR1 and these results are shown in Figure 8. Dabigatran was found to attenuate thrombin-, but not Ply-mediated formation of NP aggregates, apparently excluding the involvement of

thrombin derived from either the plasma or cellular elements of the buffy coat preparations in Ply-activated NP aggregation.

Effect of SCH 79797 on the pore-forming activity of Ply

An erythrocyte haemolysis assay was used to exclude possible interference of SCH 79797 with the pore-forming activity of Ply. Erythrocytes are particularly vulnerable to the lytic action of Ply. Briefly, the toxin (20 ng.mL^{-1}) was pre-incubated with SCH 79797 (10 µM) for 5 min at 37ºC followed by the addition of a 0.5% suspension of human erythrocytes in a final volume of 1 mL HBSS. Following 5 min incubation, the remaining erythrocytes were pelleted by centrifugation and haemoglobin in the supernatant fluids measured spectrophotometrically at a wavelength of 490 nm. The mean percentages haemolysis of Ply-treated erythrocytes in the absence or presence of SCH 79797 were 24% and 25% respectively (NS), clearly indicating lack of interference of the PAR1 antagonist with the pore-forming activity of Ply.

DISCUSSION

The results of the current study demonstrate that exposure of neutrophils to Ply, at concentrations representative of those measured in the cerebrospinal fluid of patients with pneumococcal meningitis (26), caused dose-related generation of production of PAF, reaching levels which were about 3-fold higher than those of the untreated control system at the highest concentrations tested (40 and 80 ng.mL^{-1}). The pneumolysoid, delta6Ply, was ineffective, while the calcium ionophore, A23187, included as a positive control system, was more potent than Ply. Similar, but less impressive trends were observed in the case of TxA_2, possibly indicative of intense competition for arachidonic acid by the range of prostanoid/eicosanoid/PAF-generating enzymes present in activated neutrophils. Although not shown, similar effects were observed with TxB_2, excluding conversion of TxA_2 to TxB_2 as a possible cause of the lesser effect of Ply on production of TxA_2 by neutrophils relative to PAF. Although exposure of neutrophils to the toxin at concentrations of 40 and 80 ng.mL^{-1} resulted in loss of viability, these effects were modest and unlikely to have contributed to the observed activation of production of PAF and TxA_2. In this context,

it is noteworthy that some types of mammalian cell can withstand the cytotoxic actions of Ply due to the existence of a mechanism which promotes microvesicle shedding of toxin pores (27). Ply-mediated pore formation in the plasma membrane of inflammatory cells does, however, result in an influx of extracellular Ca^{2+} which either activates or sensitises the cells for increased pro-inflammatory activity (12-14).

A possible association between the production of PAF and TxA_2 by Ply-treated neutrophils and activation of neighbouring platelets was explored by investigating the effects of the toxin on the heterotypic aggregation of these cells in the absence and presence of a PAF or TP receptor antagonist, as well as antagonists of other types of receptor which mediate platelet activation, these being P2Y12 and PAR1. Exposure of mixed NP suspensions to Ply resulted in significant dose-related heterotypic aggregation of these cells which was maximal at 40-80 $ng.mL^{-1}$ of the toxin, exceeding that observed with ADP, and dependent on the presence of extracellular Ca^{2+}, while delta6Ply was ineffective. With respect to the effects of the various receptor antagonists, only SCH 79797, somewhat surprisingly, was found to attenuate Ply-mediated NP heterotypic aggregation, while blockade of the PAF, P2Y12, and TP receptors, as well as inhibition of cyclooxygenases with indomethacin, were all ineffective. The selectivity of SCH 79797 for the PAR1 was confirmed by the absence of effects of this agent on either spontaneous or ADP-activated NP aggregation. In addition, SCH 79797 did not interfere with the pore-forming activity of Ply, excluding non-specific inactivation of the toxin as a possible mechanism of interference with NP aggregation. In this context it is noteworthy that antagonism of PAR1 has recently been reported to decrease the levels of pulmonary, pro-inflammatory cytokines/chemokines and to attenuate alveolar leak in a murine model of experimental pneumococcal pneumonia (28).

The apparent involvement of triggering of PAR1 on platelets in NP heterotypic aggregation was an unexpected finding, which is most likely a secondary, albeit important, amplification mechanism resulting from interaction of the receptor with putative activators derived from Ply-activated platelets and/or neutrophils, reinforcing and sustaining NP adhesion. Possible contenders include prothrombin released from platelet α-granules (29), which may be converted to thrombin by the action of pro-thrombinase expressed on neighbouring monocytes (30). Activation of PAR4

which is also expressed on platelets and activated by thrombin, albeit at a slower rate than PAR1, may also contribute to NP aggregation (31). However, the lack of an effect of the thrombin inhibitor, dabigatran, on Ply-mediated NP aggregation appears to exclude any meaningful involvement of thrombin activation of PARs. An alternative, albeit unexplored mechanism, implicates the serine proteinases, elastase and proteinase 3, as well as the matrix metalloproteinases 8 and 9 expressed by Ply-exposed adherent neutrophils (13, 32) all of which are known activators of PAR 1 (33, 34), while cathepsin G has been reported to activate PAR 4 (35). Addressing this issue is, however, beyond the scope of the current study given the spectrum of neutrophil-derived proteinases and their probable interactions, compounded by the requisite large number of enzyme inhibitors.

Together with the observation that inclusion of an anti-CD62P monoclonal antibody caused almost complete attenuation of Ply-mediated NP aggregate formation, the aforementioned observations appear to be consistent with a sequence of events whereby exposure of platelets to Ply results in influx of extracellular Ca^{2+}, as described previously (14), Ca^{2+}-dependent mobilisation of α-granules, upregulated surface expression of CD62P, and adhesion of neighbouring neutrophils. In this context, interactions between CD62P expressed on platelets and its counter ligand, P-selectin glycoprotein ligand-1 (PSGL-1) expressed on platelets and other cell types, are considered to be the primary mediators of platelet homotypic and heterotypic aggregation (15, 17, 24, 36). Although platelet-derived CD40 ligand has also been reported to mediate this type of interaction, the results of the current study appear to implicate CD62P as being the major player in the pro-adhesive actions of Ply (37, 38). Although speculative, initial CD62P-dependent NP adhesion is then reinforced by neutrophil proteinase-mediated activation of platelet PAR1, resulting in the formation of more stable NP aggregates. Given that endothelial cells also express PAR1 (39), it is likely, albeit unexplored that exposure of endothelium to Ply also results in Ca^{2+} influx and upregulation of endothelial CD62P. This, in turn, may promote the binding of neutrophils and NP aggregates favouring activation of endothelial PAR1 and endothelial dysfunction (39).

In agreement with the findings of the current study, CD62P-dependent formation of NP aggregates following exposure of whole blood to the bacterial pore-forming toxins, streptolysin-O or *Staphylococcus aureus* α-hemolysin, has been described previously (23, 24). In the case of the former, the authors proposed a link between streptolysin-O production, formation of NP aggregates, and vascular occlusions and tissue damage during infection with group A *streptococci* (23). In the case of α-hemolysin, heterotypic aggregate formation was linked to alveolar capillary destruction in haemorrhagic/necrotising pneumonia caused by community-associated, methicillin-resistant *S. aureus* (24). However, unlike the current study, neither of these earlier studies, investigated the pathophysiological mechanisms underpinning toxin-mediated aggregate formation. Very recently, Zhang *et al.* in a study focused primarily on the *S. suis* pore-forming toxin, suilysin, reported that this toxin, as well as Ply, promoted NP aggregation *in vitro* by a Ca^{2+}- and P-selectin-dependent mechanism as described in the current study (40). However, in the study reported by Zhang *et al.* Ply was used at a concentration considerably higher (800 $ng.mL^{-1}$, fixed) than those used in the current study (10-80 $ng.mL^{-1}$), while these authors did not investigate the involvement of platelet-activating receptors in either suilysin- or Ply-mediated NP aggregation (40)

While the exact clinical significance of the findings of the current study await clarification, they do, however, imply a multifaceted role for Ply in the pathogenesis of lung, heart and other types of organ damage during severe pneumococcal disease. Notwithstanding direct Ply-mediated organ damage (6-10), the effects of the toxin described here are also consistent with a pathogenic role for Ply-mediated formation of large, intravascular NP aggregates with resultant microvascular occlusion. Importantly, activated platelets and NP aggregates may also promote tissue injury by amplifying the inflammatory response. On a cautionary note, however, should these harmful activities of the toxin be evident in the clinical setting, therapeutic targeting may prove difficult given the drawbacks and side effect profile of a commercially available PAR1 antagonist, vorapaxar (41), as well as the current lack of pharmacological agents which directly inhibit Ply. In this context, inhibitors of bacterial protein synthesis, especially macrolide antibiotics, may offer the best therapeutic option (42).

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Foot note comments:

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