

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

Nel, Jan G.; Durandt, Chrisna; Theron, Annette J.; Tintinger, Gregory R.; Pool, Roger; Richards, Guy A.; Mitchell, Timothy J.; Feldman, Charles; Anderson, Ronald

DOI:

[10.1016/j.jinf.2017.02.010](https://doi.org/10.1016/j.jinf.2017.02.010)

License:

Creative Commons: Attribution-NonCommercial-NoDerivs (CC BY-NC-ND)

Document Version

Peer reviewed version

Citation for published version (Harvard):

Nel, JG, Durandt, C, Theron, AJ, Tintinger, GR, Pool, R, Richards, GA, Mitchell, TJ, Feldman, C & Anderson, R 2017, 'Pneumolysin mediates heterotypic aggregation of neutrophils and platelets *in vitro*', *Journal of Infection*, vol. 74, no. 6, pp. 599-608. <https://doi.org/10.1016/j.jinf.2017.02.010>

[Link to publication on Research at Birmingham portal](#)

General rights

Unless a licence is specified above, all rights (including copyright and moral rights) in this document are retained by the authors and/or the copyright holders. The express permission of the copyright holder must be obtained for any use of this material other than for purposes permitted by law.

- Users may freely distribute the URL that is used to identify this publication.
- Users may download and/or print one copy of the publication from the University of Birmingham research portal for the purpose of private study or non-commercial research.
- User may use extracts from the document in line with the concept of 'fair dealing' under the Copyright, Designs and Patents Act 1988 (?)
- Users may not further distribute the material nor use it for the purposes of commercial gain.

Where a licence is displayed above, please note the terms and conditions of the licence govern your use of this document.

When citing, please reference the published version.

Take down policy

While the University of Birmingham exercises care and attention in making items available there are rare occasions when an item has been uploaded in error or has been deemed to be commercially or otherwise sensitive.

If you believe that this is the case for this document, please contact UBIRA@lists.bham.ac.uk providing details and we will remove access to the work immediately and investigate.

Accepted Manuscript

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

Jan G. Nel, Chrisna Durandt, Annette J. Theron, Gregory R. Tintinger, Roger Pool, Guy A. Richards, Timothy J. Mitchell, Charles Feldman, Ronald Anderson



PII: S0163-4453(17)30076-2

DOI: [10.1016/j.jinf.2017.02.010](https://doi.org/10.1016/j.jinf.2017.02.010)

Reference: YJINF 3893

To appear in: *Journal of Infection*

Received Date: 30 November 2016

Revised Date: 17 February 2017

Accepted Date: 24 February 2017

Please cite this article as: Nel JG, Durandt C, Theron AJ, Tintinger GR, Pool R, Richards GA, Mitchell TJ, Feldman C, Anderson R, Pneumolysin mediates heterotypic aggregation of neutrophils and platelets *in vitro*, *Journal of Infection* (2017), doi: 10.1016/j.jinf.2017.02.010.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

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

3
4 Jan G. Nel*, Chrisna Durandt⁺, Annette J. Theron^o, Gregory R. Tintinger[◇], Roger
5 Pool*, Guy A. Richards^ˆ, Timothy J. Mitchell[▲], Charles Feldman[#], Ronald
6 Anderson^{o+}

7
8 *Department of Haematology, Faculty of Health Sciences, University of Pretoria,
9 Pretoria, South Africa and Tshwane Academic Division of the National Health
10 Laboratory Service, Pretoria, South Africa

11
12 ⁺Institute for Cellular and Molecular Medicine, South African Medical Research
13 Council Unit for Stem Cell Research, Department of Immunology, Faculty of Health
14 Sciences, University of Pretoria, Pretoria, South Africa

15
16 ^oDepartment of Immunology, Faculty of Health Sciences, University of Pretoria,
17 Pretoria, South Africa and Tshwane Academic Division of the National Health
18 Laboratory Service, Pretoria, South Africa

19
20 [◇]Department of Internal Medicine, Faculty of Health Sciences, University of Pretoria,
21 Pretoria, South Africa

22
23 ^ˆDepartment of Critical Care, Charlotte Maxeke Johannesburg Academic Hospital
24 and Faculty of Health Sciences, University of the Witwatersrand, Johannesburg,
25 South Africa

26
27 [▲]Institute of Microbiology and Infection, College of Medical and Dental Sciences,
28 University of Birmingham, Birmingham, UK

29
30 [#]Division of Pulmonology, Department of Internal Medicine, Charlotte Maxeke
31 Johannesburg Academic Hospital and Faculty of Health Sciences, University of the
32 Witwatersrand, Johannesburg, South Africa

33

34 Running title Pneumolysin promotes neutrophil:platelet adhesion

35

36

Correspondence:

Dr J.G. Nel

37

Department of Haematology

38

PO Box 2034

39

Pretoria

40

0001

41

South Africa

42

43

Telephone:

+27-12-319-2641

44

Telefax:

+27-12-323-0732

45

E-mail:

jan.nel@up.ac.za

46

Abstract

48

49 Objectives. Platelets orchestrate the inflammatory activities of neutrophils, possibly
50 contributing to pulmonary and myocardial damage during severe pneumococcal
51 infection. This study tested the hypothesis that the pneumococcal toxin, pneumolysin
52 (Ply), activates production of platelet-activating factor (PAF) and thromboxane A₂
53 (TxA₂) by neutrophils, these bioactive lipids being potential mediators of
54 neutrophil:platelet (NP) networking.

55 Methods. The effects of recombinant Ply (10–80 ng.mL⁻¹) on the production of PAF
56 and TxA₂ by isolated neutrophils were measured using ELISA procedures, and NP
57 aggregation by flow cytometry.

58 Results. Exposure of neutrophils to Ply induced production of PAF and, to a lesser
59 extent, TxA₂, achieving statistical significance at ≥ 20 ng.mL⁻¹ of the toxin. In the case
60 of NP interactions, Ply promoted heterotypic aggregation which was dependent on
61 upregulation of P-selectin (CD62P) and activation of protease-activated receptor 1
62 (PAR1), attaining statistical significance at ≥ 10 ng.mL⁻¹ of the toxin, but did not
63 involve either PAF or TxA₂.

64 Conclusion. Ply induces synthesis of PAF and TxA₂ by human neutrophils, neither of
65 which appears to contribute to the formation of NP heterotypic aggregates *in vitro*, a
66 process which is seemingly dependent on CD62P and PAR1. These pro-
67 inflammatory activities of Ply may contribute to the pathogenesis of pulmonary and
68 myocardial injury during severe pneumococcal infection.

69

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

72

73

74

75 Pneumolysin (Ply), the cholesterol-binding, pore-forming toxin of *Streptococcus*
76 *pneumoniae*, is recognised as being the major protein virulence factor of this
77 intransigent respiratory pathogen, the most common bacterial cause of community-
78 acquired pneumonia (CAP) and associated organ damage (1-4). Importantly, Ply has
79 been identified as being a key mediator of both acute lung injury (ALI) (5-8) and
80 myocardial damage (9, 10) in murine models of severe pneumococcal disease. In
81 one such model of ALI, exposure of isolated, perfused lungs to recombinant Ply
82 resulted in the development of pulmonary hypertension and microvascular barrier
83 dysfunction, both of which are key features of this condition in humans (6, 7). The
84 underlying mechanisms appeared to involve increased pulmonary production of the
85 bioactive lipid, platelet-activating factor (PAF), which, in turn, was proposed to
86 activate production of the more potent platelet activator *viz.* the prostanoid,
87 thromboxane A₂ (TxA₂), with resultant vasoconstriction and platelet activation (7).
88 Although the authors speculated that PAF may have originated from Ply-exposed
89 endothelial cells (7), infiltrating neutrophils represent an alternative source of the
90 bioactive lipid. Unlike macrophages, neutrophils express high levels of the PAF-
91 generating enzyme, PAF acetylhydrolase (11). However, to our knowledge a
92 possible link between Ply, neutrophils, PAF and platelet activation has not been
93 described.

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

100

101 To probe the existence of such mechanisms in the pathogenesis of Ply-
102 mediated ALI and myocardial injury, we have investigated the effects of recombinant
103 Ply on the production of PAF and TxA₂ by isolated, human blood neutrophils *in vitro*.
104 In addition, we have also explored the effects of Ply on the formation of potentially,
105 pro-thrombotic, heterotypic aggregates of neutrophils and platelets (15-19), focusing
106 on the involvement of PAF, TxA₂ and other potent platelet activators, as well as the
107 adhesion molecule, P-selectin (CD62P), in this process.

108

109 MATERIALS AND METHODS

110

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

113

114 Pneumolysin

115

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

123

124 Chemicals

125

126 PSB 0739, WEB 2086, S 18886 and SCH 79797 antagonists of the platelet
127 purinergic receptor, P2Y₁₂, the PAF receptor, the TxA₂ TP prostanoid receptor, and
128 the protease-activated receptor 1 (PAR1, thrombin activated) respectively were
129 purchased from TOCRIS Bioscience, Bristol, UK. The oral thrombin inhibitor,
130 dabigatran, was provided to one of us (GAR) by Boehringer-Ingelheim Pharma
131 GmbH, Germany. All of these were dissolved to stock solutions of 10 mM in
132 dimethylsulphoxide (DMSO) and used at final concentrations of 10 μM (final DMSO
133 concentrations of 0.1%) in the assays described below. Appropriate DMSO control
134 systems were included in all of the assays in which these receptor antagonists were
135 used.

136

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

141

142

143 **Preparation of neutrophils**

144

145 Neutrophils were prepared from heparinised venous blood (5 units
146 preservative-free heparin.mL⁻¹) as described previously (22). Briefly,
147 neutrophil/erythrocyte pellets obtained following centrifugation of whole blood on
148 Histopaque-1077 (Sigma Diagnostics) were resuspended and sedimented in 3%
149 gelatin to remove most of the erythrocytes. Residual erythrocytes were then
150 removed by differential lysis (brief treatment with 0.83% ammonium chloride) and the
151 resultant neutrophil populations of high purity and viability (>90% and >95%
152 respectively) suspended to a concentration of 1x10⁷ cells.mL⁻¹ in Hanks' balanced
153 salt solution (HBSS, indicator-free, pH7.4).

154

155 **PAF and TxA₂**

156

157 Neutrophils (2x10⁶) suspended in HBSS were prewarmed for 10 min at 37°C
158 followed by addition of one of the following: i) HBSS (negative control); ii)
159 recombinant Ply at final concentrations of 10, 20, 40 and 80 nanograms (ng).mL⁻¹; iii)
160 the pneumolysoid, delta6Ply, which is attenuated with respect to pore-forming
161 activity, at a fixed, final concentration of 80 ng.mL⁻¹; or iv) the calcium ionophore,
162 A23187 at 2 μM (final) as a positive control system. The final volume in each test
163 tube was 2 mL. After a further 5 min period of incubation at 37°C, the tubes were
164 transferred to an ice-bath to stop the reactions. Following removal of the cells by
165 centrifugation, the concentrations of PAF and TxA₂ in the cell-free supernatants were
166 measured using commercial sandwich ELISA procedures (Cusabio[®] Life Science,
167 Wuhan, P.R. China and Abnova GmbH, Heidelberg, Germany respectively) and the
168 results expressed as ng.mL⁻¹ and picograms (pg).mL⁻¹ respectively. Cell viability was
169 measured using a propidium iodide-based flow cytometric procedure.

170

171 **Neutrophil:platelet (NP) aggregate formation**

172

173 In order to minimise spontaneous activation of platelets, NP-enriched buffy
174 coat suspensions, enumerated for both cell types by standard haematological
175 procedures, were used for these studies in keeping with earlier reports which used
176 whole blood (23-25). These cell suspensions were prepared from the heparinised

177 blood of healthy, adult humans by sedimentation at 37°C and diluted 1:50 in HBSS
178 to give a final volume of 1 ml. Following 5 min of preincubation at 37°C, recombinant
179 Ply (10–80 ng.mL⁻¹), delta6Ply (80 ng.mL⁻¹), or adenosine 5'-diphosphate (ADP, 100
180 µM final, agonist of platelet P2Y12 receptors as a positive control) were added to the
181 cell suspensions which were incubated for a further 5 min at 37°C. Following
182 incubation, the cell suspensions were stained with 5 µl of each of the following
183 murine, anti-human, fluorochrome-labelled monoclonal antibodies to detect
184 neutrophils, platelets and total leukocytes: CD16-allophycocyanin (Biolegend, San
185 Diego, CA, USA), CD42a-phycoerythrin (Becton Dickenson, San Jose, CA, USA)
186 and CD45-Krome Orange (Beckman Coulter, Marseille, France) and incubated for
187 15 min at room temperature in the dark. This was followed by analysis of the various
188 cell suspensions at a slow flow rate using a Gallios flow cytometer (Beckman
189 Coulter, Miami, USA). NP interactions were determined according to the
190 CD16⁺/CD42a⁺ co-expression profiles of CD45⁺ leukocytes and the results
191 expressed as the relative median fluorescence intensities of CD42a expression of
192 these NP aggregates . Platelet aggregates were excluded prior to the
193 aforementioned analysis as indicated in Figure 1 which depicts this gating strategy.
194 Note that residual erythrocytes [confirmed by staining with an anti-CD 235a
195 (glycophorin) monoclonal antibody, Becton Dickenson] in the cell suspensions were
196 not lysed prior to flow cytometric analysis to minimise non-specific activation of
197 platelets.

198

199 In a limited series of experiments (2 in the series) undertaken to ensure the
200 veracity of the various antagonists of platelet P2Y12 and PAF receptors and PAR1
201 (PSB 0739, WEB 2086, and SCH 79797 respectively), these agents were added to
202 platelet-rich plasma which was incubated for 5 min at 37°C prior to the addition of the
203 respective receptor agonists, ADP (100 µM), PAF (400 nM), or thrombin (from
204 human plasma, 1.25 NIH units, final). After a further period of incubation for 5 min at
205 37°C platelet activation was measured flow cytometrically as described previously
206 according to upregulated expression of the adhesion molecule, CD62P (P-selectin)
207 (14, 25).

208

209 The following series of experiments explored the effects of the various platelet
210 receptor (P2Y12, PAF, PAR1, TxA₂) antagonists (all at 10 µM), as well as those of

211 indomethacin (5 μM) and a mouse anti-human CD62 P blocking monoclonal
212 antibody (5 μL per mL of cell suspension), all added prior to preincubation, on NP
213 aggregation activated by Ply (40 $\text{ng}\cdot\text{mL}^{-1}$, final) measured as described above. The
214 following were also investigated: i) the requirement for extracellular Ca^{2+} in the pro-
215 aggregation activity of Ply; ii) the specificity of the PAR1 receptor antagonist, SCH
216 79797, which was assessed by measuring the effects of this agent on NP
217 aggregation induced by purified thrombin (1.25 NIH units), as well as on
218 spontaneous aggregation and that activated by ADP (100 μM); and iii) the effects of
219 dabigatran (10 μM) on NP aggregation induced by either thrombin or Ply (40 $\text{ng}\cdot\text{mL}^{-1}$, final)
220

221

222

223 **Expression and statistical analysis of results**

224

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

230

231

232 **RESULTS**

233

234 **Production of PAF and TxA_2 by Ply-activated neutrophils**

235

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

243

244

245 **Neutrophil viability**

246

247 These results are shown in Figure 3. Exposure of neutrophils to the highest
248 concentrations of Ply (40 and 80 ng.mL⁻¹) or to A23187 caused modest, but
249 nevertheless statistically significant, loss of viability. The median viability values for
250 the control, untreated system and for systems treated with Ply at 40 and 80 ng.mL⁻¹
251 or A23187 were 98.9% (IQR 98.5-99.4%), 93.9% (p<0,005), 91.0% (p<0,001), and
252 95.4% (p<0.01) respectively (data from 4 experiments using cells from 4 different
253 donors).

254

255

256 **Effect of Ply on the formation of NP heterotypic aggregates**

257

258 The median neutrophil and platelet counts of the buffy coats used in these
259 and subsequent experiments were 4.67 x 10³.μL⁻¹ and 451 x 10³.μL⁻¹ respectively,
260 and the results are shown in Figure 3. Addition of Ply to mixed NP suspensions
261 resulted in dose-related formation of NP aggregates which achieved statistical
262 significance at concentrations of ≥20 ng.mL⁻¹ of the toxin and was greater than that
263 observed with ADP, while delta6Ply was ineffective (Figure 4).

264

265 **Assessment of the veracity of the various platelet receptor antagonists**

266

267 Prior to assessing their effects on Ply-mediated NP aggregate formation, the
268 efficacy of the various platelet-receptor antagonists (PSB 0739, WEB 2086, SCH
269 79797, all at 10 μM) was measured in a series of preliminary experiments, using
270 platelet-rich plasma. Following addition of the corresponding, respective receptor
271 agonists ADP (100 μM), PAF (400 nM) or thrombin (1.25 NIH units), platelet
272 activation was measured flow cytometrically according to the level of expression of
273 the adhesion molecule, CD62P. The results, which are shown in Figure 5,
274 demonstrate the activities of the receptor agonists, with PAF being the least potent,
275 as well as the inhibitory activities of the various receptor antagonists. Importantly,
276 upregulation of expression of CD62P by ADP-, PAF- or thrombin-treated platelets

277 was significantly attenuated by PSB 0739, WEB 2086, and SCH 79797, confirming
278 receptor antagonism.

279

280 **Effects of the various platelet receptor antagonists, indomethacin,**
281 **Ca²⁺ depletion, and an anti-CD62P monoclonal antibody on Ply-mediated NP**
282 **aggregation**

283

284 The effects of the various platelet receptor antagonists and indomethacin, as
285 well as those of suspension of the cells in Ca²⁺-free HBSS, on Ply (40 ng.mL⁻¹)-
286 activated formation of NP aggregates are shown in Figure 6A, while those of
287 inclusion of the anti-human CD62P blocking monoclonal antibody are shown in
288 Figure 6B. Exposure of the cells to the PAR1 antagonist, SCH 79797, as well as
289 suspension of the cells in Ca²⁺-free medium resulted in significant attenuation of Ply-
290 activated formation of NP aggregates, while the other receptor antagonists and
291 indomethacin were ineffective (Figure 6A). Inclusion of the anti-CD62P antibody
292 caused almost complete attenuation of Ply-mediated NP aggregate formation (Figure
293 6B). Depletion of Ca²⁺, as well as inclusion of the anti-CD62P antibody also caused
294 significant reductions in basal NP aggregation, underscoring the involvement of both
295 Ca²⁺ and CD62P in basal aggregation. These observations demonstrate significant
296 involvement of CD62P, as well as PAR1, but not the P2Y₁₂, PAF or TxA₂ receptors
297 in Ply-mediated NP aggregate formation.

298

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

301

302 To probe the receptor-targeted veracity of SCH 79797 (10μM) in the context of
303 NP aggregate formation, the effects of this agent on spontaneous, ADP- or thrombin-
304 activated NP aggregation were investigated and these results are shown in Figure 7.
305 Addition of SCH 79797 to the mixed neutrophil and platelet suspensions during pre -
306 incubation resulted in statistically significant formation of thrombin-activated
307 heterotypic aggregates, but had no effect on either spontaneous or ADP-activated
308 formation of NP aggregates. These findings confirm the selectivity of SCH 79797 for
309 PAR1 and the probable involvement of this receptor in Ply-mediated NP aggregate
310 formation, possibly via thrombin activation.

311 Effect of dabigatran on Ply-mediated NP aggregation

312

313 The thrombin inhibitor, dabigatran, was used to explore the possible
314 involvement of thrombin in Ply-mediated activation of PAR1 and these results are
315 shown in Figure 8. Dabigatran was found to attenuate thrombin-, but not Ply-
316 mediated formation of NP aggregates, apparently excluding the involvement of
317 thrombin derived from either the plasma or cellular elements of the buffy coat
318 preparations in Ply-activated NP aggregation.

319

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

321

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

333

334

335

336

337

338

339

340

341

342

343

344

345 **DISCUSSION**

346

347 The results of the current study demonstrate that exposure of neutrophils to
348 Ply, at concentrations representative of those measured in the cerebrospinal fluid of
349 patients with pneumococcal meningitis (26), caused dose-related generation of
350 production of PAF, reaching levels which were about 3-fold higher than those of the
351 untreated control system at the highest concentrations tested (40 and 80 ng.mL⁻¹).
352 The pneumolysoid, delta6Ply, was ineffective, while the calcium ionophore, A23187,
353 included as a positive control system, was more potent than Ply. Similar, but less
354 impressive trends were observed in the case of TxA₂, possibly indicative of intense
355 competition for arachidonic acid by the range of prostanoid/eicosanoid/PAF-
356 generating enzymes present in activated neutrophils. Although not shown, similar
357 effects were observed with TxB₂, excluding conversion of TxA₂ to TxB₂ as a possible
358 cause of the lesser effect of Ply on production of TxA₂ by neutrophils relative to PAF.
359 Although exposure of neutrophils to the toxin at concentrations of 40 and 80 ng.mL⁻¹
360 resulted in loss of viability, these effects were modest and unlikely to have
361 contributed to the observed activation of production of PAF and TxA₂. In this context,
362 it is noteworthy that some types of mammalian cell can withstand the cytotoxic
363 actions of Ply due to the existence of a mechanism which promotes microvesicle
364 shedding of toxin pores (27). Ply-mediated pore formation in the plasma membrane
365 of inflammatory cells does, however, result in an influx of extracellular Ca²⁺ which
366 either activates or sensitises the cells for increased pro-inflammatory activity (12-14).

367

368 A possible association between the production of PAF and TxA₂ by Ply-
369 treated neutrophils and activation of neighbouring platelets was explored by
370 investigating the effects of the toxin on the heterotypic aggregation of these cells in
371 the absence and presence of a PAF or TP receptor antagonist, as well as
372 antagonists of other types of receptor which mediate platelet activation, these being
373 P2Y₁₂ and PAR1. Exposure of mixed NP suspensions to Ply resulted in significant
374 dose-related heterotypic aggregation of these cells which was maximal at 40-80
375 ng.mL⁻¹ of the toxin, exceeding that observed with ADP, and dependent on the
376 presence of extracellular Ca²⁺, while delta6Ply was ineffective. With respect to the
377 effects of the various receptor antagonists, only SCH 79797, somewhat surprisingly,
378 was found to attenuate Ply-mediated NP heterotypic aggregation, while blockade of

379 the PAF, P2Y₁₂, and TP receptors, as well as inhibition of cyclooxygenases with
380 indomethacin, were all ineffective. The selectivity of SCH 79797 for the PAR1 was
381 confirmed by the absence of effects of this agent on either spontaneous or ADP-
382 activated NP aggregation. In addition, SCH 79797 did not interfere with the pore-
383 forming activity of Ply, excluding non-specific inactivation of the toxin as a possible
384 mechanism of interference with NP aggregation. In this context it is noteworthy that
385 antagonism of PAR1 has recently been reported to decrease the levels of
386 pulmonary, pro-inflammatory cytokines/chemokines and to attenuate alveolar leak in
387 a murine model of experimental pneumococcal pneumonia (28).

388

389 The apparent involvement of triggering of PAR1 on platelets in NP heterotypic
390 aggregation was an unexpected finding, which is most likely a secondary, albeit
391 important, amplification mechanism resulting from interaction of the receptor with
392 putative activators derived from Ply-activated platelets and/or neutrophils, reinforcing
393 and sustaining NP adhesion. Possible contenders include prothrombin released
394 from platelet α -granules (29), which may be converted to thrombin by the action of
395 pro-thrombinase expressed on neighbouring monocytes (30). Activation of PAR4
396 which is also expressed on platelets and activated by thrombin, albeit at a slower
397 rate than PAR1, may also contribute to NP aggregation (31). However, the lack of an
398 effect of the thrombin inhibitor, dabigatran, on Ply-mediated NP aggregation appears
399 to exclude any meaningful involvement of thrombin activation of PARs. An
400 alternative, albeit unexplored mechanism, implicates the serine proteinases, elastase
401 and proteinase 3, as well as the matrix metalloproteinases 8 and 9 expressed by Ply-
402 exposed adherent neutrophils (13, 32) all of which are known activators of PAR 1
403 (33, 34), while cathepsin G has been reported to activate PAR 4 (35). Addressing
404 this issue is, however, beyond the scope of the current study given the spectrum of
405 neutrophil-derived proteinases and their probable interactions, compounded by the
406 requisite large number of enzyme inhibitors.

407

408

409 Together with the observation that inclusion of an anti-CD62P monoclonal
410 antibody caused almost complete attenuation of Ply-mediated NP aggregate
411 formation, the aforementioned observations appear to be consistent with a sequence
412 of events whereby exposure of platelets to Ply results in influx of extracellular Ca²⁺,

413 as described previously (14), Ca^{2+} -dependent mobilisation of α -granules,
414 upregulated surface expression of CD62P, and adhesion of neighbouring
415 neutrophils. In this context, interactions between CD62P expressed on platelets and
416 its counter ligand, P-selectin glycoprotein ligand-1 (PSGL-1) expressed on platelets
417 and other cell types, are considered to be the primary mediators of platelet
418 homotypic and heterotypic aggregation (15, 17, 24, 36). Although platelet-derived
419 CD40 ligand has also been reported to mediate this type of interaction, the results of
420 the current study appear to implicate CD62P as being the major player in the pro-
421 adhesive actions of Ply (37, 38). Although speculative, initial CD62P-dependent NP
422 adhesion is then reinforced by neutrophil proteinase-mediated activation of platelet
423 PAR1, resulting in the formation of more stable NP aggregates. Given that
424 endothelial cells also express PAR1 (39), it is likely, albeit unexplored that exposure
425 of endothelium to Ply also results in Ca^{2+} influx and upregulation of endothelial
426 CD62P. This, in turn, may promote the binding of neutrophils and NP aggregates
427 favouring activation of endothelial PAR1 and endothelial dysfunction (39).

428

429

430 In agreement with the findings of the current study, CD62P-dependent
431 formation of NP aggregates following exposure of whole blood to the bacterial pore-
432 forming toxins, streptolysin-O or *Staphylococcus aureus* α -hemolysin, has been
433 described previously (23, 24). In the case of the former, the authors proposed a link
434 between streptolysin-O production, formation of NP aggregates, and vascular
435 occlusions and tissue damage during infection with group A *streptococci* (23). In the
436 case of α -hemolysin, heterotypic aggregate formation was linked to alveolar capillary
437 destruction in haemorrhagic/necrotising pneumonia caused by community-
438 associated, methicillin-resistant *S. aureus* (24). However, unlike the current study,
439 neither of these earlier studies, investigated the pathophysiological mechanisms
440 underpinning toxin-mediated aggregate formation. Very recently, Zhang *et al.* in a
441 study focused primarily on the *S. suis* pore-forming toxin, suilysin, reported that this
442 toxin, as well as Ply, promoted NP aggregation *in vitro* by a Ca^{2+} - and P-selectin-
443 dependent mechanism as described in the current study (40). However, in the study
444 reported by Zhang *et al.* Ply was used at a concentration considerably higher (800
445 ng.mL^{-1} , fixed) than those used in the current study (10-80 ng.mL^{-1}), while these

446 authors did not investigate the involvement of platelet-activating receptors in either
447 suliyisin- or Ply-mediated NP aggregation (40)

448

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

463

464

465 **REFERENCES**

- 466 1. Mitchell TJ, Dalziel CE. The biology of pneumolysin. *Subcell Biochem.* 2014;80:145-60.
467 PubMed PMID: 24798011. Epub 2014/05/07. eng.
- 468 2. File TM, Jr., Marrie TJ. Burden of community-acquired pneumonia in North American adults.
469 *Postgrad Med.* 2010 Mar;122(2):130-41. PubMed PMID: 20203464. Epub 2010/03/06. eng.
- 470 3. Welte T, Torres A, Nathwani D. Clinical and economic burden of community-acquired
471 pneumonia among adults in Europe. *Thorax.* 2012 Jan;67(1):71-9. PubMed PMID: 20729232. Epub
472 2010/08/24. eng.
- 473 4. Musher DM, Rueda AM, Kaka AS, Mapara SM. The association between pneumococcal
474 pneumonia and acute cardiac events. *Clin Infect Dis.* 2007 Jul 15;45(2):158-65. PubMed PMID:
475 17578773. Epub 2007/06/21. eng.
- 476 5. Feldman C, Mitchell TJ, Andrew PW, Boulnois GJ, Read RC, Todd HC, et al. The effect of
477 *Streptococcus pneumoniae* pneumolysin on human respiratory epithelium in vitro. *Microb Pathog.*
478 1990 Oct;9(4):275-84. PubMed PMID: 2097494. Epub 1990/10/01. eng.
- 479 6. Witzentrath M, Gutbier B, Hocke AC, Schmeck B, Hippenstiel S, Berger K, et al. Role of
480 pneumolysin for the development of acute lung injury in pneumococcal pneumonia. *Crit Care Med.*
481 2006 Jul;34(7):1947-54. PubMed PMID: 16715037. Epub 2006/05/23. eng.
- 482 7. Witzentrath M, Gutbier B, Owen JS, Schmeck B, Mitchell TJ, Mayer K, et al. Role of platelet-
483 activating factor in pneumolysin-induced acute lung injury. *Crit Care Med.* 2007 Jul;35(7):1756-62.
484 PubMed PMID: 17522574. Epub 2007/05/25. eng.
- 485 8. Garcia-Suarez Mdel M, Florez N, Astudillo A, Vazquez F, Villaverde R, Fabrizio K, et al. The
486 role of pneumolysin in mediating lung damage in a lethal pneumococcal pneumonia murine model.
487 *Respir Res.* 2007;8:3. PubMed PMID: 17257395. Pubmed Central PMCID: 1790890. Epub
488 2007/01/30. eng.
- 489 9. Brown AO, Mann B, Gao G, Hankins JS, Humann J, Giardina J, et al. *Streptococcus*
490 *pneumoniae* translocates into the myocardium and forms unique microlesions that disrupt cardiac
491 function. *PLoS Pathog.* 2014 Sep;10(9):e1004383. PubMed PMID: 25232870. Pubmed Central
492 PMCID: 4169480.
- 493 10. Alhamdi Y, Neill DR, Abrams ST, Malak HA, Yahya R, Barrett-Jolley R, et al. Circulating
494 Pneumolysin Is a Potent Inducer of Cardiac Injury during Pneumococcal Infection. *PLoS Pathog.* 2015
495 May;11(5):e1004836. PubMed PMID: 25973949. Pubmed Central PMCID: 4431880. Epub
496 2015/05/15. eng.
- 497 11. Howard KM. Differential expression of platelet-activating factor acetylhydrolase in lung
498 macrophages. *Am J Physiol Lung Cell Mol Physiol.* 2009 Dec;297(6):L1141-50. PubMed PMID:
499 19837851. Pubmed Central PMCID: Pmc2793189. Epub 2009/10/20. eng.
- 500 12. Cockeran R, Durandt C, Feldman C, Mitchell TJ, Anderson R. Pneumolysin activates the
501 synthesis and release of interleukin-8 by human neutrophils in vitro. *J Infect Dis.* 2002 Aug
502 15;186(4):562-5. PubMed PMID: 12195386. Epub 2002/08/27. eng.
- 503 13. Cockeran R, Theron AJ, Steel HC, Matlola NM, Mitchell TJ, Feldman C, et al. Proinflammatory
504 interactions of pneumolysin with human neutrophils. *J Infect Dis.* 2001 Feb 15;183(4):604-11.
505 PubMed PMID: 11170986. Epub 2001/02/15. eng.
- 506 14. Nel JG, Durandt C, Mitchell TJ, Feldman C, Anderson R, Tintinger GR. Pneumolysin Mediates
507 Platelet Activation In Vitro. *Lung.* 2016 Aug;194(4):589-93. PubMed PMID: 27192991.
- 508 15. Yokoyama S, Ikeda H, Haramaki N, Yasukawa H, Murohara T, Imaizumi T. Platelet P-selectin
509 plays an important role in arterial thrombogenesis by forming large stable platelet-leukocyte
510 aggregates. *J Am Coll Cardiol.* 2005 4/19;45(8):1280-6.
- 511 16. Ed Rainger G, Chimen M, Harrison MJ, Yates CM, Harrison P, Watson SP, et al. The role of
512 platelets in the recruitment of leukocytes during vascular disease. *Platelets.* 2015;26(6):507-20.
513 PubMed PMID: 26196409. Epub 2015/07/22. eng.
- 514 17. Sreeramkumar V, Adrover JM, Ballesteros I, Cuartero MI, Rossaint J, Bilbao I, et al.
515 Neutrophils scan for activated platelets to initiate inflammation. *Science.* 2014 Dec

- 516 5;346(6214):1234-8. PubMed PMID: 25477463. Pubmed Central PMCID: Pmc4280847. Epub
517 2014/12/06. eng.
- 518 18. McMahon TJ. Platelets promote pulmonary pull of polys. *Blood*. 2015 Nov 5;126(19):2174-5.
519 PubMed PMID: 26542252. Pubmed Central PMCID: PMC4635113. Epub 2015/11/07. eng.
- 520 19. Guo Y, Mishra A, Howland E, Zhao C, Shukla D, Weng T, et al. Platelet-derived Wnt
521 antagonist Dickkopf-1 is implicated in ICAM-1/VCAM-1-mediated neutrophilic acute lung
522 inflammation. *Blood*. 2015 Nov 5;126(19):2220-9. PubMed PMID: 26351298. Pubmed Central
523 PMCID: PMC4635118. Epub 2015/09/10. eng.
- 524 20. Mitchell TJ, Walker JA, Saunders FK, Andrew PW, Boulnois GJ. Expression of the pneumolysin
525 gene in *Escherichia coli*: rapid purification and biological properties. *Biochim Biophys Acta*. 1989 Jan
526 23;1007(1):67-72. PubMed PMID: 2642385. Epub 1989/01/23. eng.
- 527 21. Kirkham LA, Kerr AR, Douce GR, Paterson GK, Dilts DA, Liu DF, et al. Construction and
528 immunological characterization of a novel nontoxic protective pneumolysin mutant for use in future
529 pneumococcal vaccines. *Infect Immun*. 2006 Jan;74(1):586-93. PubMed PMID: 16369015. Pubmed
530 Central PMCID: 1346677. Epub 2005/12/22. eng.
- 531 22. Gravett CM, Theron AJ, Steel HC, Tintinger GR, Cockeran R, Feldman C, et al. Interactive
532 inhibitory effects of formoterol and montelukast on activated human neutrophils. *Eur Respir J*. 2010
533 Dec;36(6):1417-24. PubMed PMID: 20413544. Epub 2010/04/24. eng.
- 534 23. Bryant AE, Bayer CR, Chen RY, Guth PH, Wallace RJ, Stevens DL. Vascular dysfunction and
535 ischemic destruction of tissue in *Streptococcus pyogenes* infection: the role of streptolysin O-
536 induced platelet/neutrophil complexes. *J Infect Dis*. 2005 Sep 15;192(6):1014-22. PubMed PMID:
537 16107954. Epub 2005/08/19. eng.
- 538 24. Parimon T, Li Z, Bolz DD, McIndoo ER, Bayer CR, Stevens DL, et al. *Staphylococcus aureus*
539 alpha-hemolysin promotes platelet-neutrophil aggregate formation. *J Infect Dis*. 2013 Sep
540 1;208(5):761-70. PubMed PMID: 23698812. Pubmed Central PMCID: PMC3733505. Epub
541 2013/05/24. eng.
- 542 25. Mauler M, Seyfert J, Haenel D, Seeba H, Guenther J, Stallmann D, et al. Platelet-neutrophil
543 complex formation-a detailed in vitro analysis of murine and human blood samples. *J Leukoc Biol*.
544 2015 Nov 17. PubMed PMID: 26578648. Epub 2015/11/19. Eng.
- 545 26. Wall EC, Gordon SB, Hussain S, Goonetilleke UR, Gritzfeld J, Scarborough M, et al.
546 Persistence of pneumolysin in the cerebrospinal fluid of patients with pneumococcal meningitis is
547 associated with mortality. *Clin Infect Dis*. 2012 Mar 1;54(5):701-5. PubMed PMID: 22238165.
548 Pubmed Central PMCID: Pmc3275762. Epub 2012/01/13. eng.
- 549 27. Wolfmeier H, Schoenauer R, Atanassoff AP, Neill DR, Kadioglu A, Draeger A, et al. Ca²⁺(+)-
550 dependent repair of pneumolysin pores: A new paradigm for host cellular defense against bacterial
551 pore-forming toxins. *Biochim Biophys Acta*. 2015 Sep;1853(9):2045-54. PubMed PMID: 25219550.
552 Epub 2014/09/16. Eng.
- 553 28. Jose RJ, Williams AE, Mercer PF, Sulikowski MG, Brown JS, Chambers RC. Regulation of
554 neutrophilic inflammation by proteinase-activated receptor 1 during bacterial pulmonary infection. *J*
555 *Immunol*. 2015 Jun 15;194(12):6024-34. PubMed PMID: 25948816. Pubmed Central PMCID:
556 PMC4456635. Epub 2015/05/08. Eng.
- 557 29. Blair P, Flaumenhaft R. Platelet alpha-granules: basic biology and clinical correlates. *Blood*
558 *Rev*. 2009 Jul;23(4):177-89. PubMed PMID: 19450911. Pubmed Central PMCID: PMC2720568.
- 559 30. Robinson RA, Worfolk L, Tracy PB. Endotoxin enhances the expression of monocyte
560 prothrombinase activity. *Blood*. 1992 Jan 15;79(2):406-16. PubMed PMID: 1730086. Epub
561 1992/01/15. eng.
- 562 31. Nieman MT. Protease-activated receptors in hemostasis. *Blood*. 2016 Jul 14;128(2):169-77.
563 PubMed PMID: 27127302. Pubmed Central PMCID: PMC4946198.
- 564 32. Cockeran R, Mitchell TJ, Feldman C, Anderson R. Pneumolysin induces release of matrix
565 metalloproteinase-8 and -9 from human neutrophils. *Eur Respir J*. 2009 Nov;34(5):1167-70. PubMed
566 PMID: 19880617. Epub 2009/11/03. eng.

- 567 33. Mihara K, Ramachandran R, Renaux B, Saifeddine M, Hollenberg MD. Neutrophil elastase
568 and proteinase-3 trigger G protein-biased signaling through proteinase-activated receptor-1 (PAR1).
569 J Biol Chem. 2013 Nov 15;288(46):32979-90. PubMed PMID: 24052258. Pubmed Central PMCID:
570 PMC3829148. Epub 2013/09/21. eng.
- 571 34. Lee EJ, Woo MS, Moon PG, Baek MC, Choi IY, Kim WK, et al. Alpha-synuclein activates
572 microglia by inducing the expressions of matrix metalloproteinases and the subsequent activation of
573 protease-activated receptor-1. J Immunol. 2010 Jul 1;185(1):615-23. PubMed PMID: 20511551. Epub
574 2010/06/01. Eng.
- 575 35. Sambrano GR, Huang W, Faruqi T, Mahrus S, Craik C, Coughlin SR. Cathepsin G activates
576 protease-activated receptor-4 in human platelets. J Biol Chem. 2000 Mar 10;275(10):6819-23.
577 PubMed PMID: 10702240. Epub 2000/03/04. eng.
- 578 36. de Bruijne-Admiraal LG, Modderman PW, Von dem Borne AE, Sonnenberg A. P-selectin
579 mediates Ca(2+)-dependent adhesion of activated platelets to many different types of leukocytes:
580 detection by flow cytometry. Blood. 1992 Jul 1;80(1):134-42. PubMed PMID: 1377047. Epub
581 1992/07/01. eng.
- 582 37. Andre P, Nannizzi-Alaimo L, Prasad SK, Phillips DR. Platelet-derived CD40L: the switch-hitting
583 player of cardiovascular disease. Circulation. 2002 Aug 20;106(8):896-9. PubMed PMID: 12186789.
584 Epub 2002/08/21. eng.
- 585 38. Gerdes N, Seijkens T, Lievens D, Kuijpers MJ, Winkels H, Projahn D, et al. Platelet CD40
586 Exacerbates Atherosclerosis by Transcellular Activation of Endothelial Cells and Leukocytes.
587 Arterioscler Thromb Vasc Biol. 2016 Mar;36(3):482-90. PubMed PMID: 26821950. Epub 2016/01/30.
588 eng.
- 589 39. Jose R, Williams A, Sulikowski M, Brealey D, Brown J, Chambers R. Regulation of neutrophilic
590 inflammation in lung injury induced by community-acquired pneumonia. Lancet. 2015 Feb 26;385
591 Suppl 1:S52. PubMed PMID: 26312874. Epub 2015/08/28. eng.
- 592 40. Zhang S, Zheng Y, Chen S, Huang S, Liu K, Lv Q, et al. Suliyisin-induced Platelet-Neutrophil
593 Complexes Formation is Triggered by Pore Formation-dependent Calcium Influx. Sci Rep. 2016 Nov
594 10;6:36787. PubMed PMID: 27830834. Epub 2016/11/11. Eng.
- 595 41. Sharma A, Helft G, Garg A, Agrawal S, Chatterjee S, Lavie CJ, et al. Safety and efficacy of
596 vorapaxar in secondary prevention of atherosclerotic disease: A meta-analysis of randomized control
597 trials. Int J Cardiol. 2017 Jan 15;227:617-24. PubMed PMID: 27810296. Epub 2016/11/05. eng.
- 598 42. Anderson R, Steel HC, Cockeran R, von Gottberg A, de Gouveia L, Klugman KP, et al.
599 Comparison of the effects of macrolides, amoxicillin, ceftriaxone, doxycycline, tobramycin and
600 fluoroquinolones, on the production of pneumolysin by Streptococcus pneumoniae in vitro. J
601 Antimicrob Chemother. 2007 Nov;60(5):1155-8. PubMed PMID: 17848373. Epub 2007/09/13. eng.

602

603

604

605

606

607

608

609

610

611 **Figure legends:**

612 **Figure 1.**

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

619

620 **Figure 2.**

621 The effects of addition of pneumolysin (Ply) at concentrations of 10-80 ng.ml⁻¹, as
622 well as those of delta 6 pneumolysin (delta6Ply, 80ng.ml⁻¹) and the calcium
623 ionophore A23187 (2 μM), to neutrophils on the production of platelet-activating
624 factor (PAF) and thromboxane A₂ (TxA₂) are shown in figures 2A and 2B
625 respectively. The data from 5 different experiments, using cells from 5 different
626 individuals, are expressed as the median values with interquartile ranges.
627 BG=background value for unstimulated cells.

628 *p<0.05-p<0.002

629 **Figure 3.**

630 The effects of the addition of pneumolysin (Ply), at concentrations of 5-80 ng.ml⁻¹, as
631 well as those of delta 6 pneumolysin (delta6Ply, 80ng.ml⁻¹) and the calcium
632 ionophore A23187 (2 μM, positive control) on neutrophil viability.

633 *p<0.01-p<0.001

634 **Figure 4.**

635 The effects of the addition of ADP (100 μM, positive control) or pneumolysin (Ply, 10-
636 80 ng.ml⁻¹) or delta 6 pneumolysin (delta6Ply 80 ng.ml⁻¹) on the formation of
637 heterotypic neutrophil:platelet (NP) aggregates. The results of 35 experiments, using
638 cell suspensions from 13 different donors are expressed as the CD42a median
639 fluorescence intensity (MFI) with interquartile ranges. The aggregates assessed
640 were positive for co-expression of CD16, CD42a and CD45.

641 *p<0.001-p<0.0001

642

643

644

645 Figure 5.

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

652

653 Figure 6.

654 The results in the upper figure (5A) show the effects of pneumolysin (Ply 40 ng. ml^{-1})
655 only or in the presence of WEB 20186, SCH 79797, indomethacin, PSB 0739 or S
656 18886, all at 10 μM), as well as the effect of calcium depletion from the cell
657 suspending medium, on the formation of neutrophil:platelet (NP) heterotypic
658 aggregates. The results of 18 experiments using cells from 7 donors are expressed
659 as the median CD16⁺/CD42a⁺/CD45⁺ fluorescence intensities with interquartile
660 ranges.

661 The results in the lower figure (5B) show the effects of addition of an anti-CD62P
662 monoclonal antibody to buffy coat suspensions on the spontaneous (BG) and
663 pneumolysin (Ply 40 ng. ml^{-1})-activated formation of neutrophil:platelet heterotypic
664 aggregates.

665 * $p < 0.006$ For comparison with the corresponding Ply-treated, drug-free control
666 system.

667 * $p < 0.0004$ For comparison of the control and corresponding anti-CD62P-treated
668 systems.

669

670 Figure 7.

671 Measurement of the effects of addition of the PAR1 receptor antagonist, SCH 79797
672 (10 μM), to buffy coat suspensions on the spontaneous (background) and ADP
673 (100 μM)- or thrombin (1.25 NIH units. ml^{-1})-activated formation of neutrophil platelet
674 (NP) heterotypic aggregates. The results of 4 experiments using cells from 4 donors
675 are expressed as the CD42a median fluorescence intensity (MFI) with interquartile
676 ranges. The aggregates assessed were positive for co-expression of CD16, CD42a
677 and CD45.

678 * $p < 0.001$ For comparison of the thrombin-activated systems without and with SCH
679 79797

680

681 **Figure 8.**

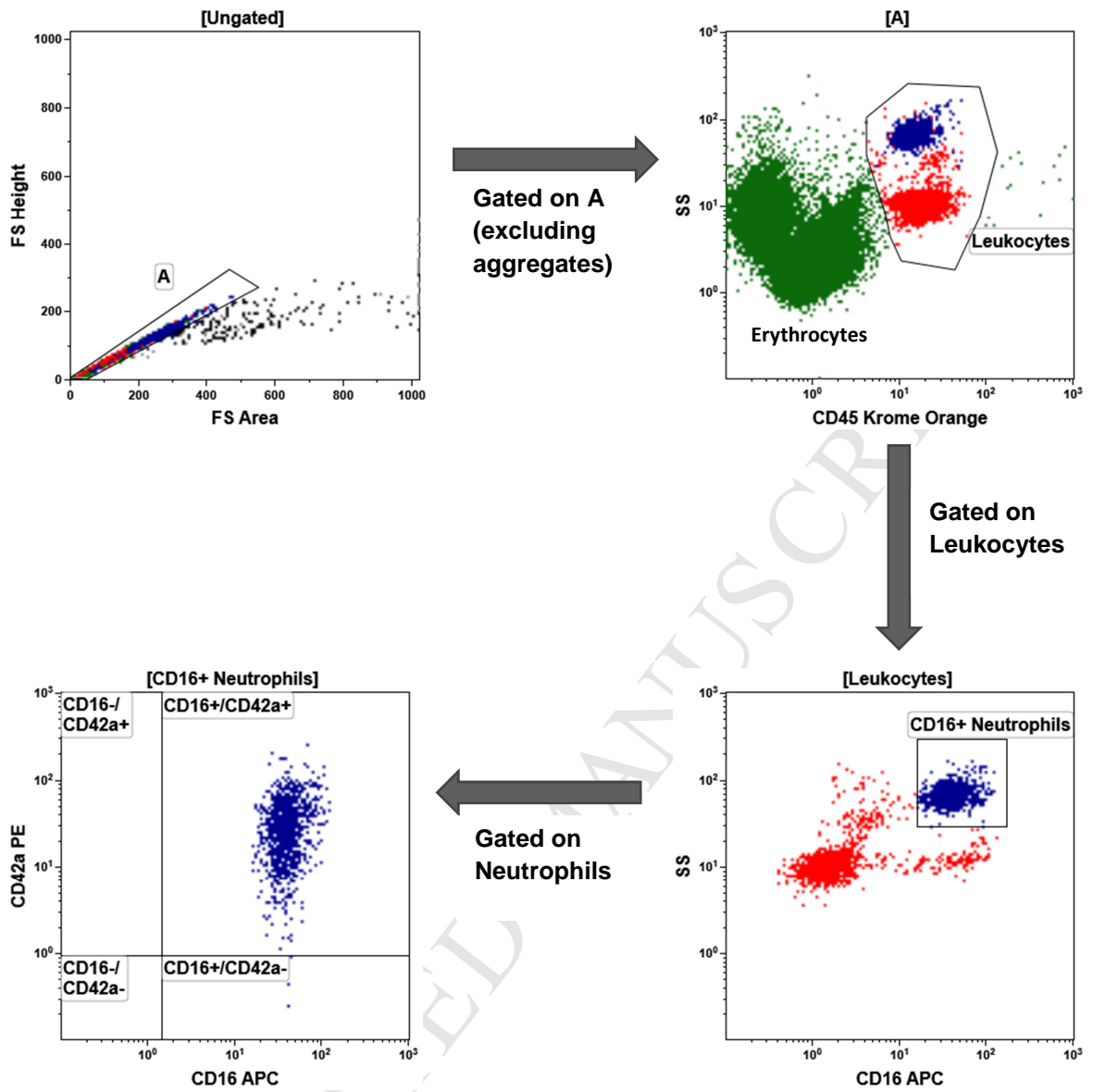
682 Measurement of the effects of dabigatran (10 μ M) added to buffy coat suspensions
683 on the formation of heterotypic neutrophil:platelet (NP) aggregates, activated by
684 either thrombin (1.25 NIH units.ml⁻¹) or pneumolysin (Ply 40 ng.ml⁻¹). The results of 6
685 experiments using cells from 2 donors are expressed as the median
686 CD16⁺/CD42⁺/CD45⁺ fluorescence intensities with interquartile ranges.

687 *p<0.02 For comparison of the thrombin-activated systems in the absence or
688 presence of dabigatran.

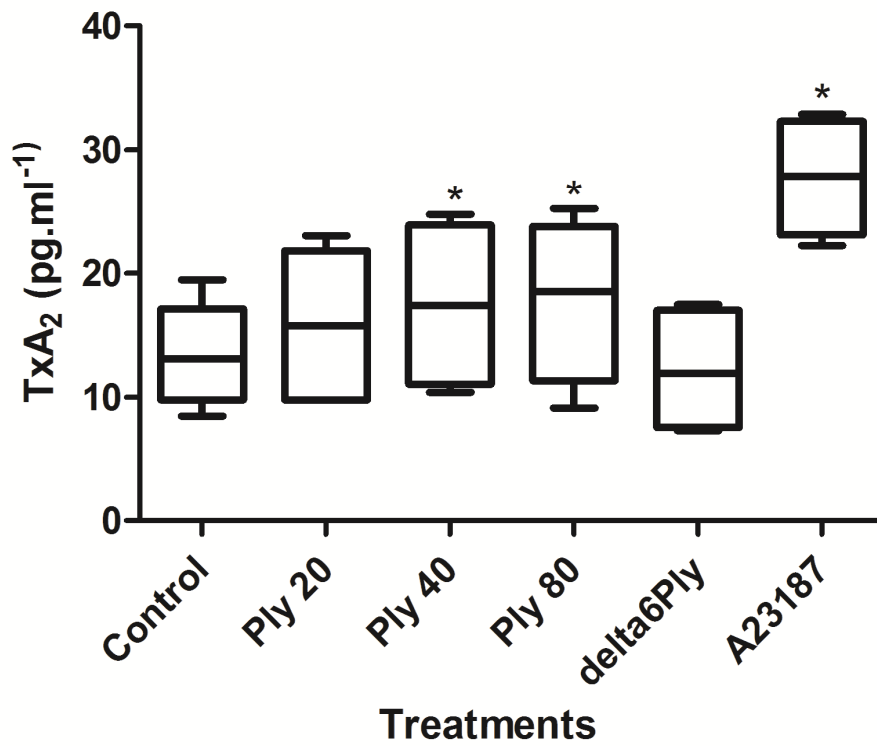
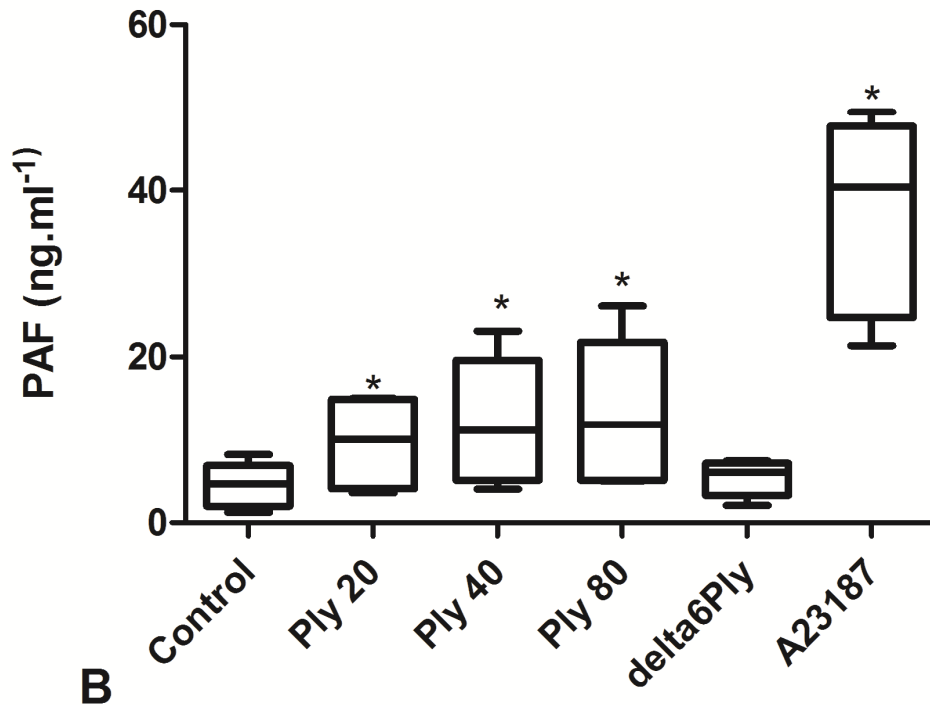
689

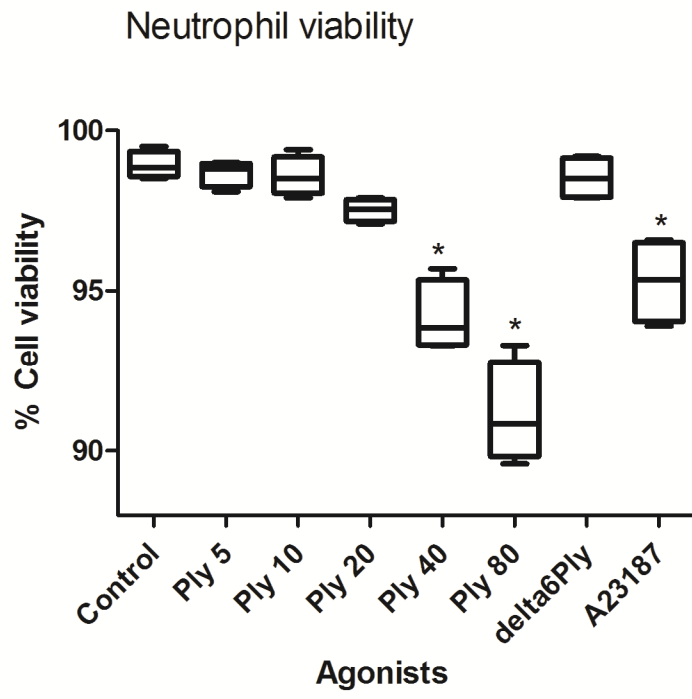
690 **Foot note comments:**

691 None of the authors has any conflict of interest to declare. JGN and CF were funded
692 by research grants awarded by the South African National Research Foundation
693 (NRF).

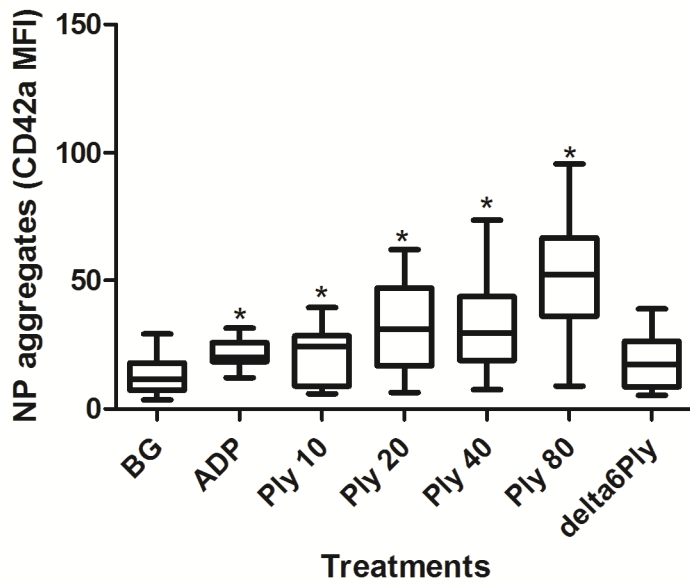


A

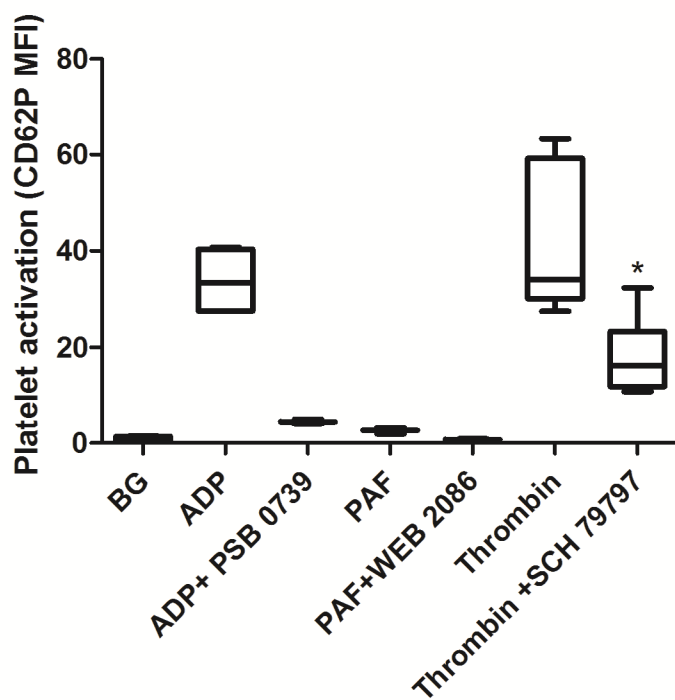




Effects of the addition of Ply, delta6Ply or ADP on neutrophil:platelet aggregation

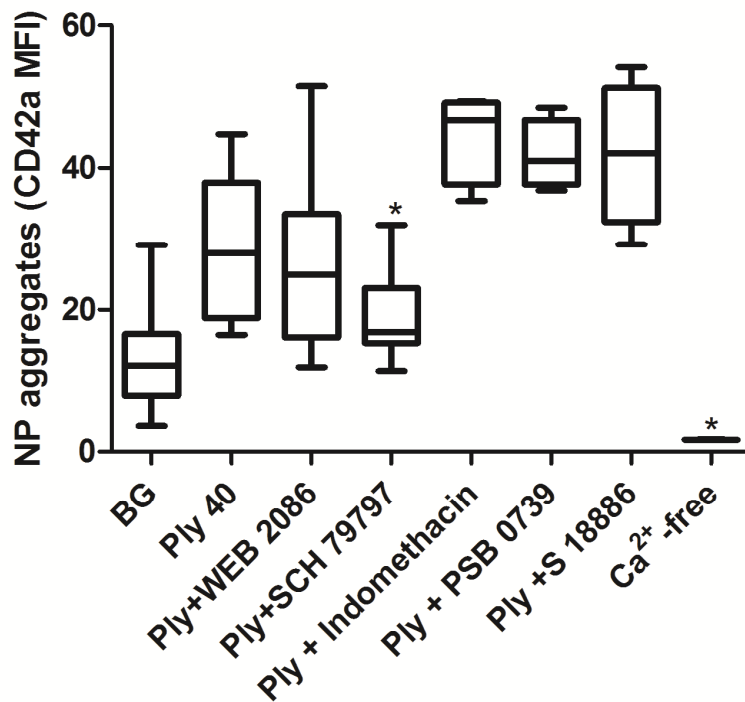


Effects of platelet receptor antagonists on ADP-, PAF- or thrombin-activated upregulation of expression of CD62P

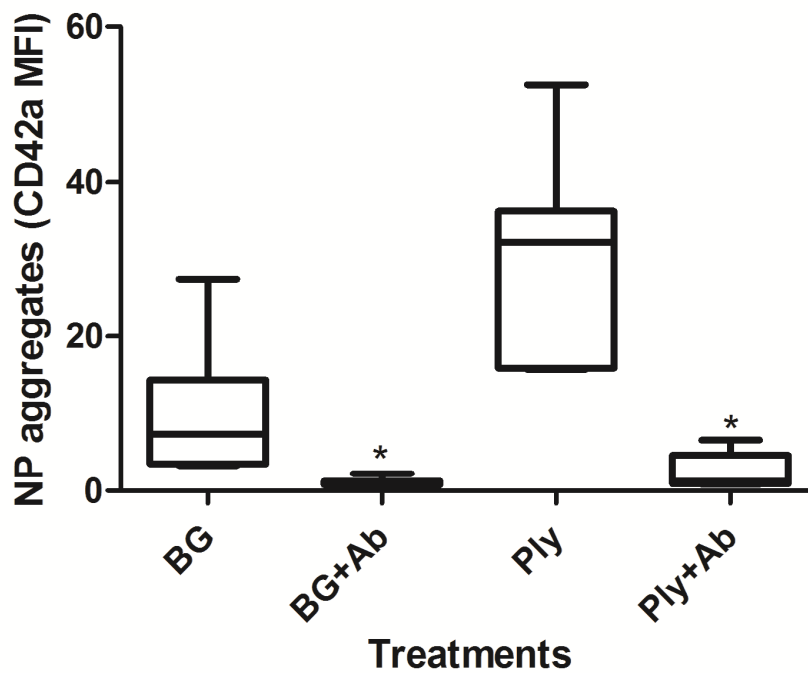


Effects of platelet receptor antagonists, indomethacin or calcium depletion on Ply-induced neutrophil:platelet aggregation

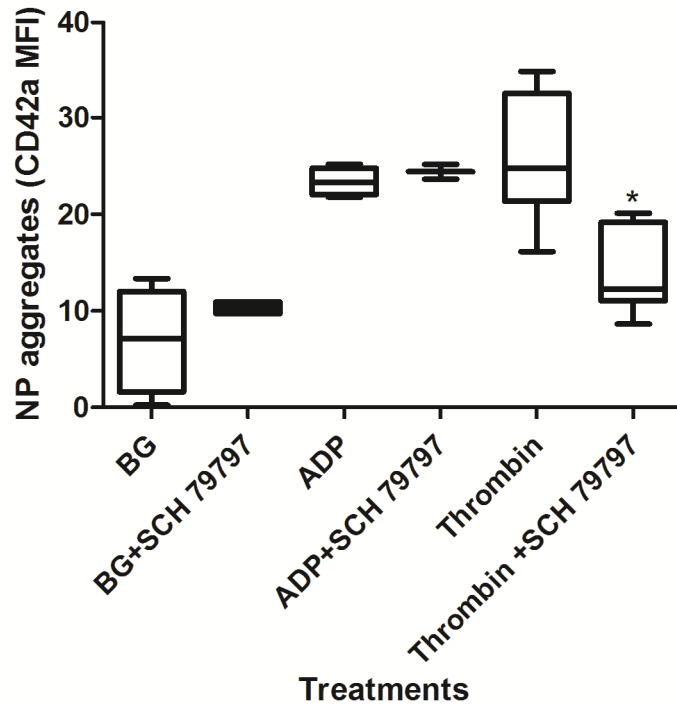
A



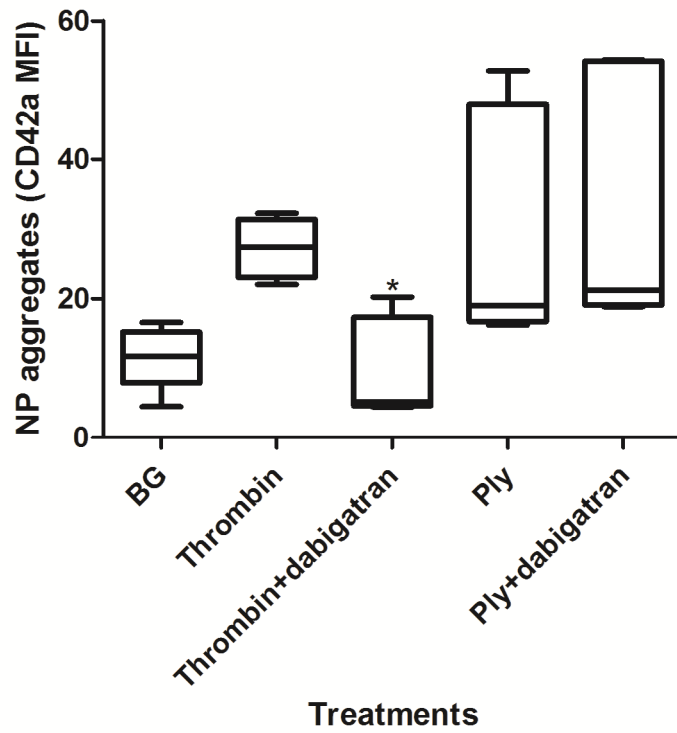
B



Effects of the PAR1 receptor antagonist, SCH 79797 on basal, ADP-, or thrombin-activated neutrophil:platelet aggregation



Effects of dabigatran on thrombin- or Ply-activated neutrophil:platelet aggregation



Highlights

- Pneumolysin (Ply) activates production of PAF and thromboxane A₂ (TxA₂) by neutrophils.
- Ply also promotes formation of pro-thrombotic neutrophil:platelet (NP) aggregates.
- Ply-mediated aggregate formation is independent of PAF and TxA₂.
- 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.