

1 Article

2 Human neutrophil response to *Pseudomonas* 3 bacteriophages

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12

13 **Abstract:** The immune system offers several mechanisms of response to remove harmful
14 microbes that invade the human body. As a first line of defense, neutrophils can remove
15 pathogens by phagocytosis, inactivate them by the release of reactive oxygen species
16 (ROS) or immobilize them by neutrophil extracellular traps (NETs). Although, recent
17 studies have shown that bacteriophages (phages) make up a large portion of human
18 microbiomes and are currently being explored as human antibacterial therapeutics,
19 neutrophilic responses to phages are still elusive. Here, we show that exposure of isolated
20 human resting neutrophils to high concentration of the *Pseudomonas* phage PAK_P1 led to
21 a 2.8 fold increase in interleukin-8 (IL-8) secretion. Importantly, phage exposure did not
22 further affect resting neutrophil apoptosis or induce necrosis, CD11 expression, oxidative
23 burst, and NETs. Similarly, inflammatory stimuli activated neutrophil effector responses
24 were unaffected by phage exposure. Our work suggest that phages are unlikely to
25 inadvertently cause excessive neutrophil responses that could damage tissues and worsen
26 disease. Because IL-8 functions as a chemoattractant directing immune cells to sites of
27 infection and inflammation, phage-stimulated IL-8 production may boost host immune
28 responses.

29 **Keywords:** innate immunity, microbiota, virome, immunophage, phage therapy, viruses

30

31 1. Introduction

32 As a key component in the innate immune system, neutrophils are generally
33 considered as rapid responders in the first line of defense against pathogen invasion. They
34 are the predominant population among leukocytes and can clear pathogens by a number of
35 mechanisms, including phagocytosis, production of reactive oxygen species (ROS) and
36 other antimicrobial products, and neutrophil extracellular traps (NETs) (1,2). The role of
37 neutrophils has long been considered restricted to the initial phase of defense. However,
38 recent evidence indicates that there is a functional heterogeneity and plasticity among
39 neutrophils that shape both innate and adaptive immune responses by the secretion of
40 cytokines (3,4). To this end, neutrophils are regularly associated with inflammation and
41 disease, while also promoting inflammation resolution and homeostasis.

42 The human microbiota is the aggregate of more than 100 trillion symbiotic
43 microorganisms that live on and within the body, including bacteria, archaea, eukaryotic
44 viruses and bacteriophages (phages) (5,6). It is now acknowledged that the human
45 microbiota affects host physiology to a great extent, including host immunity and
46 homeostasis (5,7). Recently, resident phages – a diverse group of viruses that infect bacteria
47 – community structure and composition have been shown to be altered during
48 inflammatory diseases, such as inflammatory bowel disease (8,9), periodontal disease (10)
49 and diabetes (11). Although phages are not human pathogens, Duerkop and Hooper
50 hypothesize that phages may also trigger antiviral defenses (6) because they can elicit
51 immune responses (12). More recently, phages were proposed to be implicated in the
52 efficacy of fecal microbiota transplantations (13). This suggests phages have an unidentified
53 role in affecting host immunity.

54 Moreover, phages has been proposed as a human antibacterial therapy, namely phage
55 therapy, to address the rise of multidrug resistant infections (14,15). The mechanism by
56 which phages exert their therapeutic action has generally been considered to be via their
57 capacity to lyse bacterial cells. However, studies have shown that phages and the innate
58 immune system work synergistically to eliminate bacterial infections (16,17). Interestingly,
59 neutrophils were deemed necessary for the successful phage therapy in an animal model
60 (17). Neutrophils may be keystone in determining the outcome of phage therapy; however,
61 phage-neutrophil interactions are poorly studied.

62 Here, we describe the interactions between resting human peripheral blood
63 neutrophils and the *Pseudomonas aeruginosa* infecting phage PAK_P1. We also determine
64 the effect of phage exposure while activated neutrophils respond to several biological and
65 chemical inflammatory stimuli.

66 2. Materials and Methods

67 2.1. Bacteria and phage culture

68 *P. aeruginosa* strain PAK was cultured in Luria Broth (LB; BD Biosciences) at 37°C. The
69 pseudomonas phage strain PAK_P1 (17) was propagated on PAK grown in LB medium
70 and purified following cesium chloride density gradient ultracentrifugation (18). After
71 buffer (10mM Tris, 150mM NaCl, pH 7.5) dialysis, phages were passaged 3x through an
72 endotoxin removal spin column (EndoTrap, Hyglos) to obtain residual endotoxin levels
73 <math><1.5 \text{ EU mL}^{-1}</math> (EndoZyme II recombinant factor C, Hyglos). Phage stocks were 0.22 μm filter
74 sterilized and enumerated on PAK. Before experimentation, phage numbers were adjusted
75 in Hank's balanced saline solution (HBSS no calcium, no magnesium; Fisher Scientific).

76 2.2. Neutrophil isolation and stimulation

77 Neutrophils were separated from human peripheral blood collected from healthy
78 donors on EDTA by negative magnetic sorting (MACSxpress, Miltenyi Biotec) according to
79 manufacturer's instructions. Neutrophils were washed and resuspended at $3 \times 10^6 \text{ mL}^{-1}$ in
80 HBSS, and purity >98% and viability >99% was confirmed by flow cytometry. Equal
81 volumes of phage solution or buffer control and neutrophils were mixed to give a final
82 neutrophil concentration of $1.5 \times 10^6 \text{ mL}^{-1}$. Inflammatory responses were triggered with
83 either 25 nM phorbol myristate acetate (PMA), 5 μM calcium ionophore (A23187), 20 μM
84 platelet activating factor (PAF), 100 $\mu\text{g/mL}$ Zymosan A, 5 $\mu\text{g mL}^{-1}$ Staphylococcus aureus

85 peptidoglycan (PGN), or 100 μ g mL⁻¹ ovalbumin/anti-ovalbumin immune complex at a 1:5
86 ratio in PBS. All stimuli were from Sigma-Aldrich.

87 2.3. Quantification of cell surface CD11b expression

88 Neutrophil CD11b expression was measured after 15 min saturation in 4% BSA PBS
89 and membrane staining with PE-conjugated anti-CD11b (Becton-Dickinson) for 30 min at
90 4°C in darkness. Mean fluorescence intensity (MFI) of washed cells was measured on an
91 Attune Nxt cytometer (ThermoFisher).

92 2.4. Phagocytosis quantification

93 Neutrophil phagocytosis was measured after 90 min incubation in darkness with
94 increasing concentrations of pHrodo Red Zymosan BioParticles (ThermoFisher) conjugates
95 at 5-50 μ g mL⁻¹, as per supplier's instructions. Phagocytosis was monitored by an increase
96 in particle fluorescence in acidic compartments using flow cytometry.

97 2.5. Oxidative burst response

98 ROS production was measured by chemiluminescence as previously described (19).
99 Briefly, cells were incubated with phage with or without noxious stimuli for 40 min at 37°C
100 in the presence of 100 mM luminol in a 96-well microplate (Corning). A Tristar™ LB941
101 microplate reader (Berthold Technologies) measured luminescence every 1 min and area
102 under the curve (AUC) was calculated for each sample tested in triplicate.

103 2.6. Neutrophil Extracellular Traps release

104 Extracellular DNA release was measured by Sytox Green fluorescence, as described
105 previously (19). Briefly, the neutrophils were incubated with phage with or without
106 inflammatory stimuli in presence of 5 μ M of Sytox Green (Fisher Scientific) in a 96-well
107 microplate (Corning) and samples were tested in triplicate. A microplate reader measured
108 DNA release over 3 h and background fluorescence was subtracted from values.

109 2.7. Interleukin-8 (IL-8) production

110 Neutrophils were cultured for 18 h at 37°C in the presence of phages, alone or with
111 PAF or PGN to stimulate the neutrophils. IL-8 levels were measured in cell-free culture
112 supernatant by ELISA (hIL-8 Quantikine kit, Bio-technie), according to manufacturer's
113 instructions, and measured on a Multiskan EX spectrophotometer (Thermo Labsystems).

114 2.8. Apoptosis and necrosis

115 Neutrophils were incubated with phages for up to 18 h at 37°C. Cell apoptosis and
116 necrosis were measured by annexin V/ 7-AAD (Annexin V Apoptosis Detection Kit I, BD
117 Pharmingen), according to the manufacturer's instructions. Cell viability was also
118 evaluated using Trypan blue (Sigma-Aldrich) staining.

119 2.9. Statistical analyses

120 Experimental groups were analyzed by Mann and Whitney unpaired U-test and
121 Kruskal-Wallis test. Results are shown as mean \pm SEM. A p-value < 0.05 was considered
122 statistically significant.

123 2.10. Ethics statements

124 Fresh whole blood samples were collected from healthy blood donors who gave their
125 oral consent, in agreement with the French regulations and provided by Etablissement
126 Français du Sang.

127 **3. Results**

128 3.1. Neutrophilic response to phages

129 We first established the characteristic neutrophil activities towards purified phage
130 PAK_P1 compared to the HBSS diluent control. Phage PAK_P1 was found to not have a
131 significant effect on neutrophil apoptosis after either short (3 h) or long (18 h)
132 co-incubations (Fig. 1a & b). In addition, phages did not induce necrosis, even at the high
133 cell:phage ratio of 1:10,000 (Fig. 1c and Suppl. Fig. 1). Phages also did not significantly
134 induce neutrophils to upregulate CD11b cell surface expression (Fig. 1d), which regulates
135 leukocyte adhesion and migration. Fig. 1e shows that phages did not induce ROS
136 production, which is a crucial reaction that occurs in neutrophils to degrade internalized
137 particles and microbes. Neutrophils have also been shown to kill pathogens outside of the
138 cell, rather than engulfing them. This occurs by neutrophils releasing web-like structures of
139 chromatin and granules, called NETs (1). Phage PAK_P1 alone did not trigger NETs (Fig.
140 1f). Together, this suggests human neutrophils were unresponsive to phage PAK_P1.
141 However, we found that neutrophils secreted 2.8 fold higher amounts of IL-8 after 18 h of
142 co-incubation with phage PAK_P1 when at the 1:10,000 cell:phage ratio compared to the
143 control (Fig. 1g). Because IL-8 is involved in neutrophil activation and chemoattraction of
144 other immune cells, human neutrophils may sense phage virions as foreign invaders.

145 3.2. Neutrophilic responses to inflammatory stimuli are not influenced by phages

146 We then tested if phage exposure caused an overly lively neutrophil response towards
147 inflammatory chemical (PMA and A23187), microbial (PGN and Zymosan), soluble
148 immune complexes, and PAF stimuli. Phage PAK_P1 exposure did not affect the percent of
149 pHRodo positive cells at either a low ($5\mu\text{g mL}^{-1}$) or high ($50\mu\text{g mL}^{-1}$) Zymosan coated
150 particle dose (Fig. 2a), which suggests phagocytosis was not perturbed. Furthermore,
151 phage exposure did not alter ROS production in response to Zymosan or PMA (Fig. 2b).
152 Indeed, the combination of phages and an inflammatory stimulus could induce a more
153 exuberant response like NETs (1). However, co-incubation did not change the amount of
154 extracellular chromatin released from neutrophils in response to both strong (PMA and
155 A23187) and weak (PAF and IC) stimuli (Fig. 2c and 2d, respectively). Interestingly,
156 activated neutrophil IL-8 production in response to PAF and PGN did not increase with
157 phage exposure (Fig. 2e), despite phages alone causing an increase in IL-8 production in
158 resting neutrophils (Fig. 1g). Nevertheless, our results suggest that phage PAK_P1
159 exposure neither enhances nor dampens activated neutrophil responses.

161 **4. Discussion**

162 In this study, we show that a high concentration of purified *Pseudomonas* phage
163 PAK_P1 can trigger low IL-8 production in freshly isolated human blood neutrophils.
164 Nonetheless, phage exposure did not further affect resting neutrophil apoptosis or induce
165 necrosis, CD11b expression, oxidative burst, and NETs. Similarly, activation-induced

166 neutrophil effector responses were unaffected by phage exposure. Our findings agree with
167 those from Borysowski *et al.* who similarly found that exposure to the *Staphylococcus aureus*
168 phage A3/r did not increase granule marker expression in neutrophils (20). However,
169 another study found that exposure to the *E. coli* phage T4, elicited a weak neutrophil
170 oxidative burst (21). Furthermore, Miedzybrodzki *et al.* showed that co-exposure of the
171 phage T4 with either *E. coli* or LPS derived from *E. coli*, had the beneficial effect of
172 dampening neutrophil ROS production (22). Neutrophil antiviral defense remains
173 controversial because it mediates both detrimental and beneficial effects to the host (23).

174 Human neutrophils generally cannot sense DNA viruses, largely because of lacking
175 the DNA sensing Toll-like receptor 9 (23). DNA phages are the most abundant viruses in
176 microbiomes and are used as therapeutic agents; therefore, it is unlikely neutrophils sense
177 phages by their DNA content. Despite that, neutrophils have been implicated in the anti
178 DNA-viral immune response (23). For example, herpes simplex virus (HSV) can cause a
179 rapid neutrophil infiltration to the site of infection, and neutrophil depletion results in
180 increased HSV load (24). Epstein-Barr virus (EBV) induces IL-8 production in neutrophils,
181 which was found to be dependent on the interaction between the viral envelope and cell
182 surface (25). A major difference however is that DNA phages lack a viral envelope. Another
183 key difference is that HSV and EBV elicits neutrophilic effector responses (e.g., apoptosis
184 and oxidative burst) in parallel to IL-8 production (24,25). This was not observed during
185 phage PAK_P1 exposure.

186 A caveat of deciphering anti-phage immune response is that phages propagate in
187 bacteria. Importantly, neutrophils are highly bacterial responsive phagocytes, expressing
188 an abundance of bacteria-specific receptors, including the endotoxin (i.e. LPS) sensing
189 Toll-like receptor 4 (1). Thus, trace amounts of LPS in the phage preparation might be
190 responsible for IL-8 secretion in neutrophils (26). However, LPS tends to enhance
191 neutrophil sensitivity to subsequent inflammatory stimuli (27), which was not observed
192 after exposure to the phage PAK_P1 preparation. It has also been shown that phage tail
193 fibers can bind free LPS making it less inflammatory (28), which could thereby scavenge
194 trace endotoxin after the LPS removal procedures used in this study during phage
195 purification.

196 Although neutrophils are the first and predominant immune cell population recruited
197 to an affected site after infection, it remains unclear if this occurs when triggered by phage
198 signals. Importantly, our results suggest that phages are unlikely to stimulate neutrophils
199 to inadvertently release their intracellular toxic contents and cause collateral tissues
200 damage. As a major component of the human microbiota (6), phages do not appear to play
201 a major role in diseases where neutrophils have been implicated (29,30). As antibacterial
202 therapeutic agents, phages have been shown to work in concert with neutrophils to cure
203 bacterial infection (17). But, phages do not appear to enhance neutrophil effector responses
204 during treatment. However, because IL-8 has other biological functions besides a central
205 role in inflammation, such as immune cell chemotaxis, angiogenesis, and hematopoiesis (4),
206 phage-stimulated IL-8 production may boost immune responses.

207 The interaction between human immune cells and phages that either make up part of
208 the human microbiota or are administered as human therapeutics, remains
209 underappreciated. A better understanding of phage-stimulated immune responses could
210 open new treatment options for infectious and inflammatory diseases.

211

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214 prepared the manuscript.

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219 conflict of interest.

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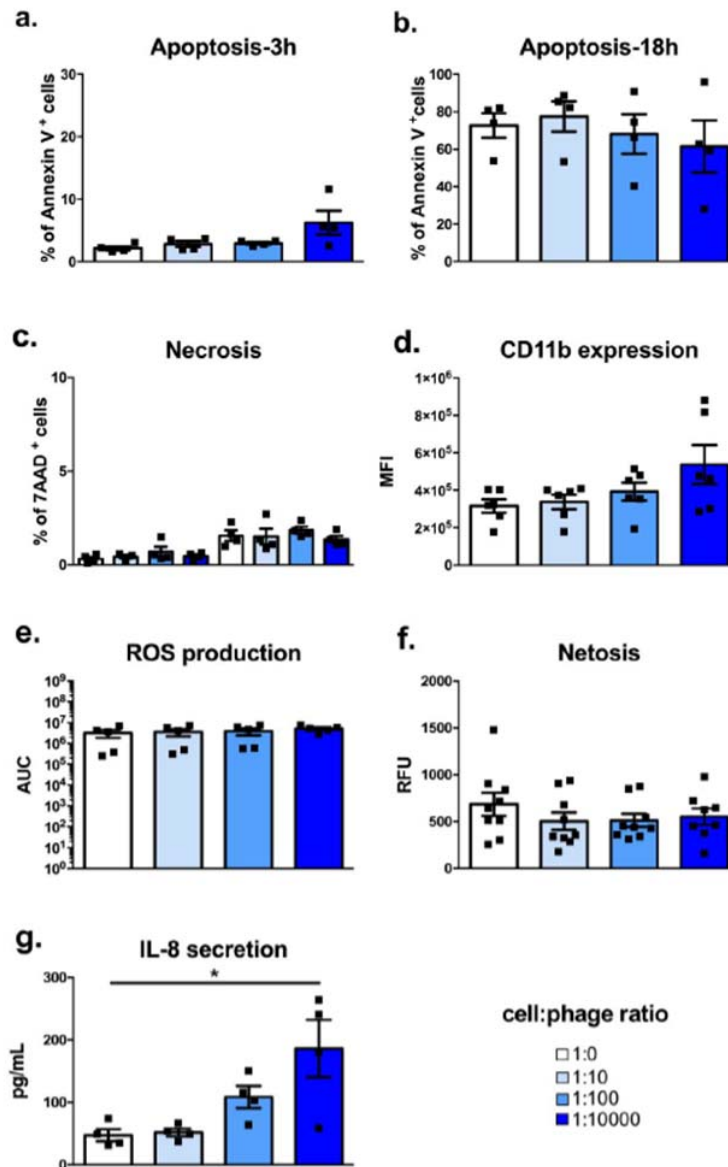
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300 **Figures**

301

302

Figure 1



303

304 **Figure 1: Resting human neutrophil responses to phage PAK_P1.** Isolated human

305 peripheral neutrophils were co-incubated with increasing amounts of purified phages.

306 **(a&b)** Percent of annexin V positive neutrophils indicative of cell apoptosis after **(a)** 3h and

307 **(b)** 18h co-incubation. **c)** Percent of 7-AAD positive neutrophils indicative of necrosis after

308 co-incubation. **d)** CD11b expression as mean fluorescence intensity (MFI) after 18 h

309 co-incubation. **e)** ROS production shown as luminescence area under curve (AUC) after 40

310 min co-incubation. **f)** Extracellular DNA release shown as relative fluorescence units (RFU)

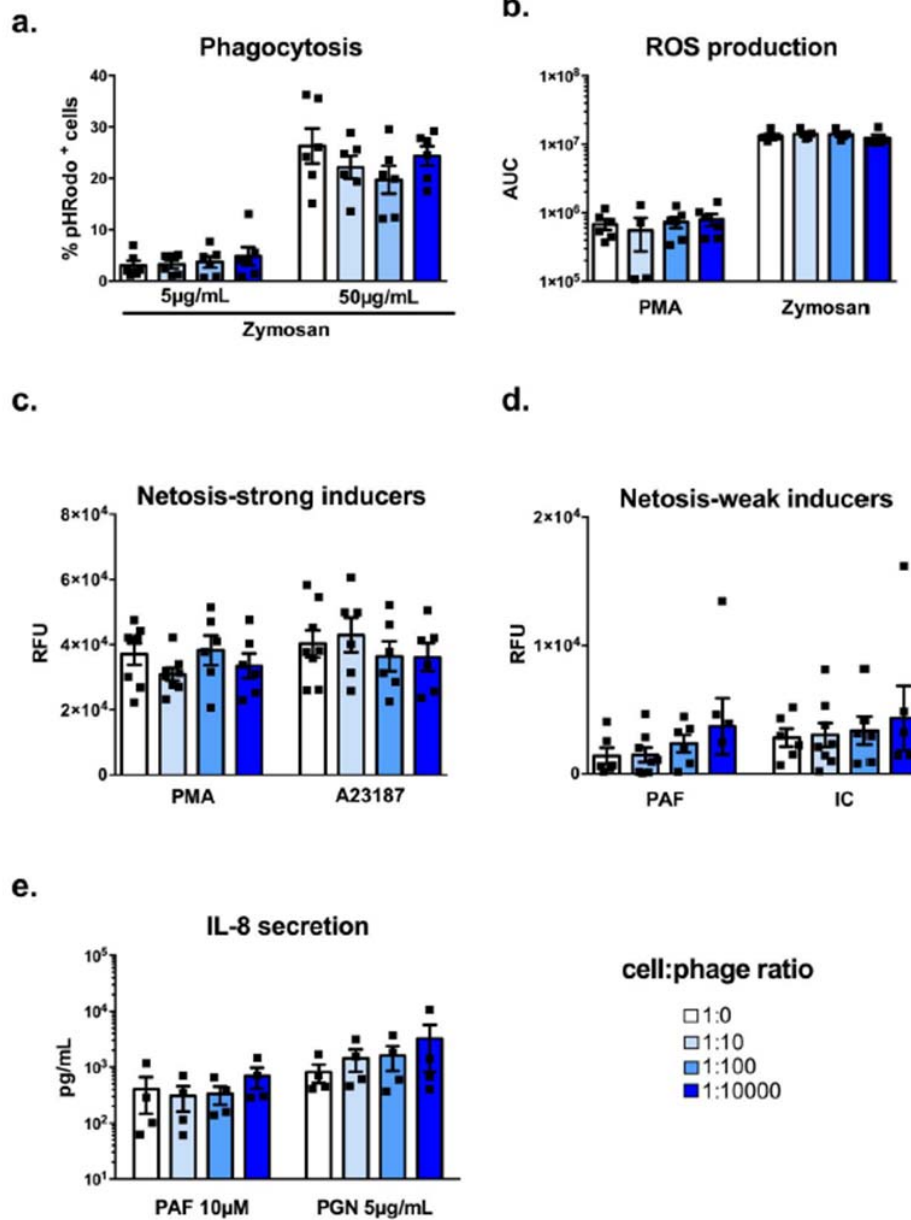
311 after 3 h co-incubation. **g)** Interleukin 8 (IL-8) secretion after 18 h co-incubation. Data

312 shown as mean +SEM, n= 4-9 per group; *Kruskal-Wallis test p <0.05.

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Figure 2

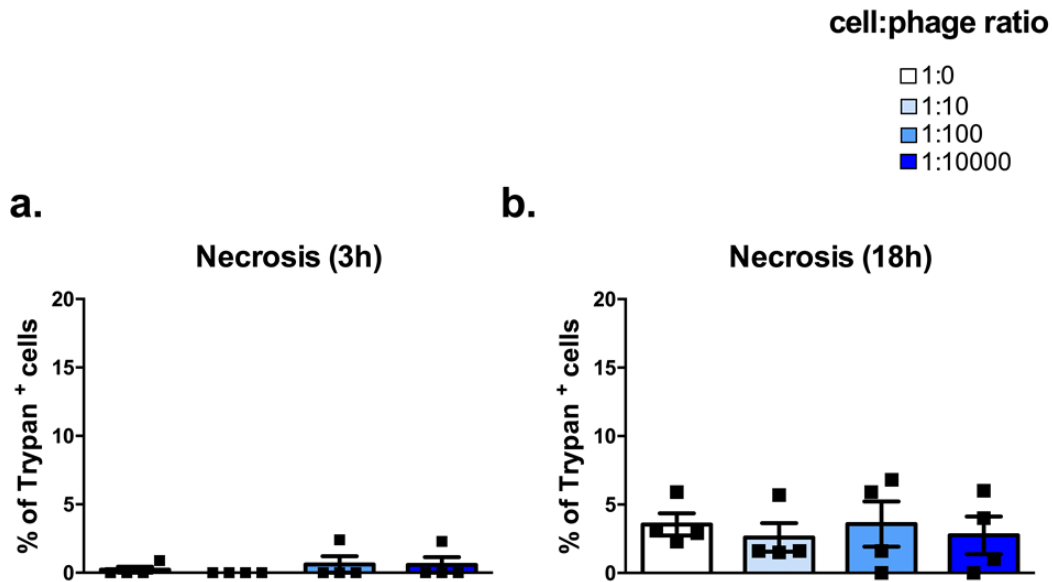


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316 **Figure 2: Activated human neutrophil responses to phage PAK_P1.** Isolated human
 317 peripheral neutrophils were activated with either fungal glycan (Zymosan), phorbol
 318 myristate acetate (PMA), bacterial peptidoglycan (PGN), calcium ionophore (A23187),
 319 soluble immune complexes (IC) or platelet-activation factor (PAF) and co-incubated with
 320 increasing amounts of purified phages. **a)** Percent of pHRodo positive low (5 µg mL⁻¹) or
 321 high (50 µg mL⁻¹) Zymosan stimulated neutrophils after a 90 min co-incubation with
 322 phages. **b)** Mildly (PMA) or strongly (Zymosan) activated neutrophil ROS production
 323 shown as luminescence area under curve (AUC) after 40 min co-incubation with phages.
 324 **(c&d)** Strongly (PMA and A23187) **(c)** or weakly (PAF and IC) **(d)** activated neutrophil

325 extracellular DNA release shown as relative fluorescence units (RFU) after 3 h
326 co-incubation. **e)** Activated neutrophil interleukin 8 (IL-8) secretion after 18 h
327 co-incubation. Data shown as mean +SEM, n= 4-8 per group.
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Supplementary Figure 1



332
333 **Supplementary Figure 1: Resting human neutrophil necrosis after phage PAK_P1**
334 **co-incubation.** Percent of Trypan blue positive human peripheral neutrophils after (a) 3 h
335 and (b) 18 h co-incubation with increasing amounts of purified phages. Data shown as
336 mean +SEM, n= 4 per group.
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