Title

Streptococcus pyogenes M1T1 variants activate caspase-1 and induce an inflammatory neutrophil phenotype

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Summary

Invasive infections due to Group A *Streptococcus* (GAS) advance rapidly causing tissue degradation and unregulated inflammation. Neutrophils are the primary immune cells that respond to GAS. M1T1 GAS isolate 5448 and animal passaged variant 5448AP, containing a *covS* mutation, were assessed for their influence on neutrophils. 5448AP proliferated during neutrophil co-incubation and induced lower neutrophil reactive oxygen species production when compared with 5448. Infection with both strains invoked neutrophil death, however apoptosis was reduced in response to 5448AP. Both strains induced neutrophil caspase-1 activation *in vitro*,

and elicited IL-1β and TNF-α release from neutrophils. GAS infection altered the cell-surface expression of CD16 and CD31 on neutrophils. *In vivo* mouse studies confirmed activation of caspase-1 in neutrophils, while 5448AP infection increased circulating IL-1β. GAS infections that promote an inflammatory neutrophil phenotype may contribute to increased inflammation yet ineffective bacterial eradication, contributing to the severity of invasive GAS infections.

Keywords

Polymorphonuclear leukocyte, PMN, apoptosis, pyroptosis, caspase-1, caspase-3, caspase-4, IL-1β, TNF-α, IL-8, IL-18, cell-death, *covS*, 5448, 5448AP, LEGENDplex, FLICA, inflammation, inflammasome

Introduction

Invasive infections due to the obligate human pathogen *Streptococcus pyogenes* (Group A *Streptococcus*; GAS) are characterised by unregulated inflammation and high mortality (Davies et al., 1996, Lamagni et al., 2008, Nelson et al., 2016, O'Grady et al., 2007, O'Loughlin et al., 2007, Svensson et al., 2000). The highly virulent GAS M1T1 clone 5448 is well studied (Aziz and Kotb, 2008, Tart et al., 2007, Walker et al., 2007). The propensity of M1T1 GAS to cause invasive infections is due, in part, to the increased frequency of spontaneous mutations in the two-component control of virulence regulatory system (covRS) (Walker et al., 2007), resulting in differential expression of multiple virulence factors, many of which are implicated in immune defence (Kilsgård et al., 2016, Sumby et al., 2005, Sumby et al., 2006, Walker et al., 2007). Compelling evidence describes the resistance of *covS* mutant GAS (mutation to the sensor protein of covRS) to killing by human neutrophils (polymorphonuclear leukocytes, PMNs) (Ato et al., 2008, Maamary et al., 2010, Walker et al., 2007). 5448AP (animal passaged) is one M1T1 *covS* mutant strain that shows resistance to killing by human neutrophils, has increased bacterial dissemination and results in decreased survival in mouse models of infection (Fiebig et al., 2015, Maamary et al., 2010, Walker et al., 2007). Due to these characteristics, 5448AP can be used as a

49 model strain to explore *covS* induced hypervirulence and the host response to invasive GAS
50 infection.

51 Neutrophils are the primary innate immune cell-type that defend against bacterial pathogens,
52 with their presence contributing to the overarching inflammatory response (Kobayashi et al.,
53 2018). Proteins of neutrophil origin are highly abundant at sites of invasive GAS infection
54 (Edwards et al., 2018). The neutrophil life cycle is tightly regulated to avoid the release of
55 cytotoxic contents that can damage surrounding tissue and contribute further to inflammation
56 (Epstein and Weiss, 1989, Faurschou and Borregaard, 2003). Under normal conditions cytotoxic
57 reactive oxygen species (ROS) produced by neutrophils kill phagocytosed bacteria (Nguyen et al.,
58 2017). An increased production of ROS signals for the induction of the anti-inflammatory cell-
59 death pathway apoptosis (Lundqvist-Gustafsson and Bengtsson, 1999). During apoptosis,
60 neutrophils are carefully decommissioned, ensuring no unregulated release of material, then
61 cleared by a secondary phagocyte, macrophages, through a process known as efferocytosis
62 (Bratton and Henson, 2011). The cleavage of caspase-3 to the p17 form denotes the execution of
63 apoptosis (Nicholson et al., 1995). Increased neutrophil apoptosis is described during GAS
64 infection (Kobayashi et al., 2003). Pro-inflammatory neutrophil death has also been reported
65 during GAS infection (Tsatsaronis et al., 2015). One form of pro-inflammatory death is pyroptosis,
66 involving the formation of an inflammasome and subsequent caspase-1 or caspase-4 activation,
67 culminating in cell-lysis (Man and Kanneganti, 2016, Sollberger et al., 2012). Deregulation of (or
68 excessive) pro-inflammatory neutrophil-death may therefore contribute further to inflammatory
69 pathology, as demonstrated during infection with other pathogens, including *Pseudomonas*
70 *aeruginosa* (Ryu et al., 2017) and *Staphylococcus aureus* (Ghimire et al., 2018, Greenlee-Wacker
71 et al., 2017, Ryu et al., 2017). Inflammation is also heavily influenced by cell cytokine production,
72 where increased release can invoke neutrophil dysfunction, affecting neutrophil survival,
73 antibacterial function or even migration (Brown et al., 2006, Kipnis, 2013, Kovach and Standiford,
74 2012, Norrby-Teglund et al., 2000). Previous work outlines how GAS take advantage of
75 inadequate or exacerbated immune responses and focus upon the bacterial mechanisms involved

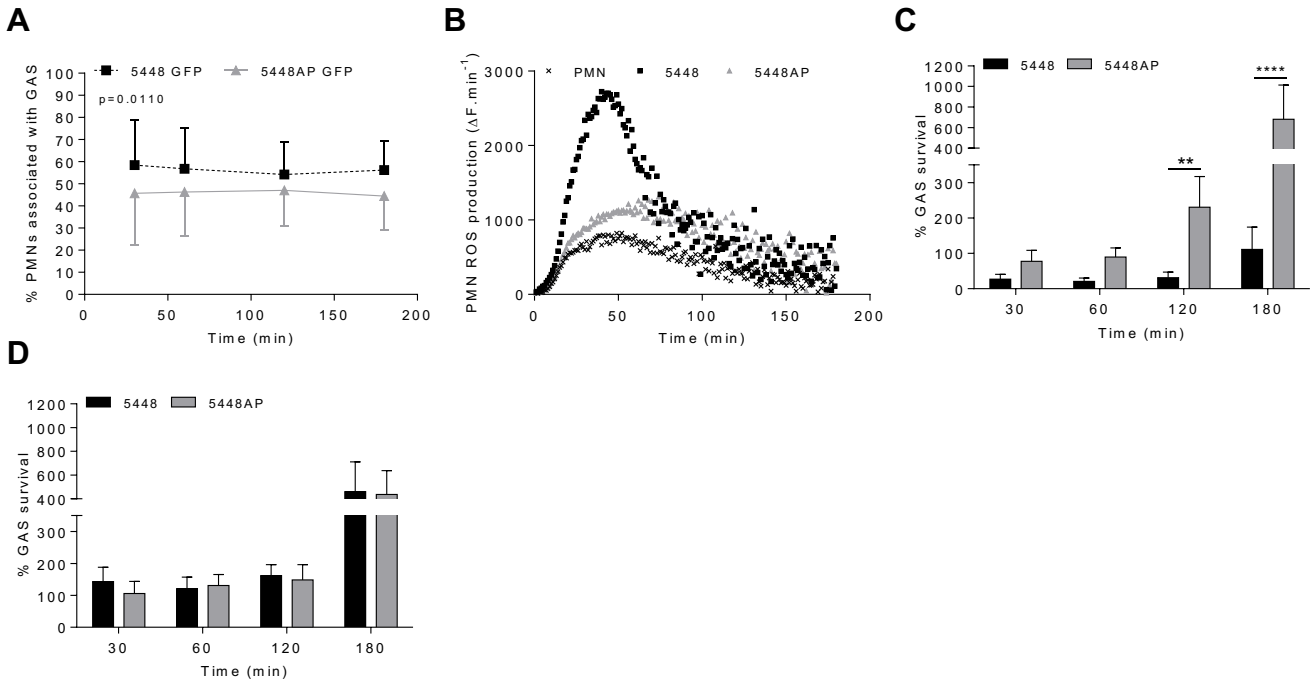
76 (Cole et al., 2011). Although models to investigate leukocyte inflammasome activation have been
77 proposed (Tran et al., 2019), a limited number of studies have investigated the neutrophil
78 response to GAS specifically (Kobayashi et al., 2003). Here, using an *in vitro* model, we temporally
79 describe the effect two M1T1 GAS strains have upon human neutrophil death, signalling and
80 inflammatory profile. We further characterise this interaction in murine neutrophils, using an *in*
81 *vivo* model of intradermal invasive GAS infection. Additionally, we identify differences in the
82 neutrophil response to GAS due to *covS* mutation. We hypothesise that changes to neutrophil
83 function by GAS can promote invasive GAS infection by exacerbating the inflammatory response,
84 decreasing antibacterial capacity and inducing inflammatory neutrophil death.

85 **Results**

86 **GAS persistence and proliferation occurs during a dampened neutrophil ROS response**

87 Neutrophil phagocytic ability is a predominant and well-established mechanism of immune
88 defence. Both GAS strains 5448 and 5448AP rapidly associated with human neutrophils, with
89 near maximal association observed within 30 min (Figures 1A and S1A-C). Higher percentages of
90 neutrophils were associated with 5448 compared to 5448AP (Figure 1A). 5448 and 5448AP also
91 induced ROS production in neutrophils, with less ROS produced by neutrophils incubated in the
92 presence and absence of 5448AP compared to those incubated with 5448 (Figure 1B). As such,
93 the rate of neutrophil ROS production was significantly increased compared to the neutrophil
94 only control during 5448, but not 5448AP, infection ($p<0.01$, Figure S1D), suggesting dampened
95 antibacterial function in neutrophils in response to 5448AP. This difference in GAS-induced ROS
96 production inversely corresponded to GAS proliferation in the presence of neutrophils, with high
97 numbers of 5448AP observed compared to 5448 over 180 min (Figure 1C). Differential GAS
98 proliferation required viable neutrophils, as 5448 and 5448AP proliferation in the presence of
99 neutrophil lysates was reduced and similar for both strains over 180 min (Figure 1D). Finally,
100 incubation of neutrophils with the phagocytosis inhibitor cytochalasin D significantly increased
101 5448, but not 5448AP, survival ($p<0.001$, Figure S1E), indicating that 5448 killing is

102 predominantly mediated via a phagocytic-related pathway. Collectively, these data indicate that
103 compared to 5448, 5448AP associates less readily with human neutrophils leading to reduced
104 ROS production and increased survival of this strain.



105 **Figure 1. GAS persistence and proliferation occurs during a dampened neutrophil ROS**
106 **response.** (A) Association of fluorescent GAS strains with human neutrophils (PMNs, n=6
107 donors), analysed via flow cytometry (Figure S1A-B). (B) Infection of human neutrophils with
108 GAS invokes ROS production. Representative of triplicate measurements from three separate
109 experiments (n=3 donors), shown as mean change in fluorescence units over time ($\Delta F.min^{-1}$). GAS
110 strains were incubated in the presence of (C) human neutrophils (n=8 donors) or (D) lysed
111 human neutrophils (n=4 donors) over 180 min with surviving bacterial concentration
112 determined as percentage over inoculum. Results are the pooled means±SD (of triplicate
113 measurements for panels C and D). **$p<0.01$ and ****$p<0.0001$.

114 **Neutrophil death is delayed during infection with *covS* GAS**

115 GAS can induce death of human neutrophils (Kobayashi et al., 2003, Tsatsaronis et al., 2015),
116 however the mechanism remains poorly described. Phosphatidylserine (PS) exposure is a
117 hallmark of the induction of multiple death pathways including apoptosis and pyroptosis (Man

118 and Kanneganti, 2016, Wang et al., 2013). Human neutrophils were incubated with 5448, 5448AP
119 or in the absence of GAS then the binding of annexin-V (AV), as a measure of PS exposure, and the
120 binding of Zombie Fixable Viability Kit (Z), as a measure of membrane integrity, were analysed
121 by flow cytometry (Figure 2). Cells were defined as viable (AV^-Z^-), PS exposed (AV^+Z^-) or dead
122 (AV^+Z^+) (Figure 2C). Neutrophils incubated without GAS remained viable over 180 min (Figure
123 2D), with 13% exposing PS (Figure 2E) and 3% dead after this time (Figure 2F). In contrast,
124 incubation of neutrophils with either 5448 or 5448AP resulted in increased PS exposure and cell-
125 death over 180 min, with significantly more cell-death during 5448 infection compared to
126 5448AP infection at 30 and 60 min ($p<0.05$, Figure 2F). Delay in the initial induction of death in
127 response to 5448AP suggests extended neutrophil viability during the early stages of infection
128 (30-60 min).

129 **GAS infection activates caspase-1 in neutrophils but reduced apoptosis occurs in response**
130 **to *covS* GAS**

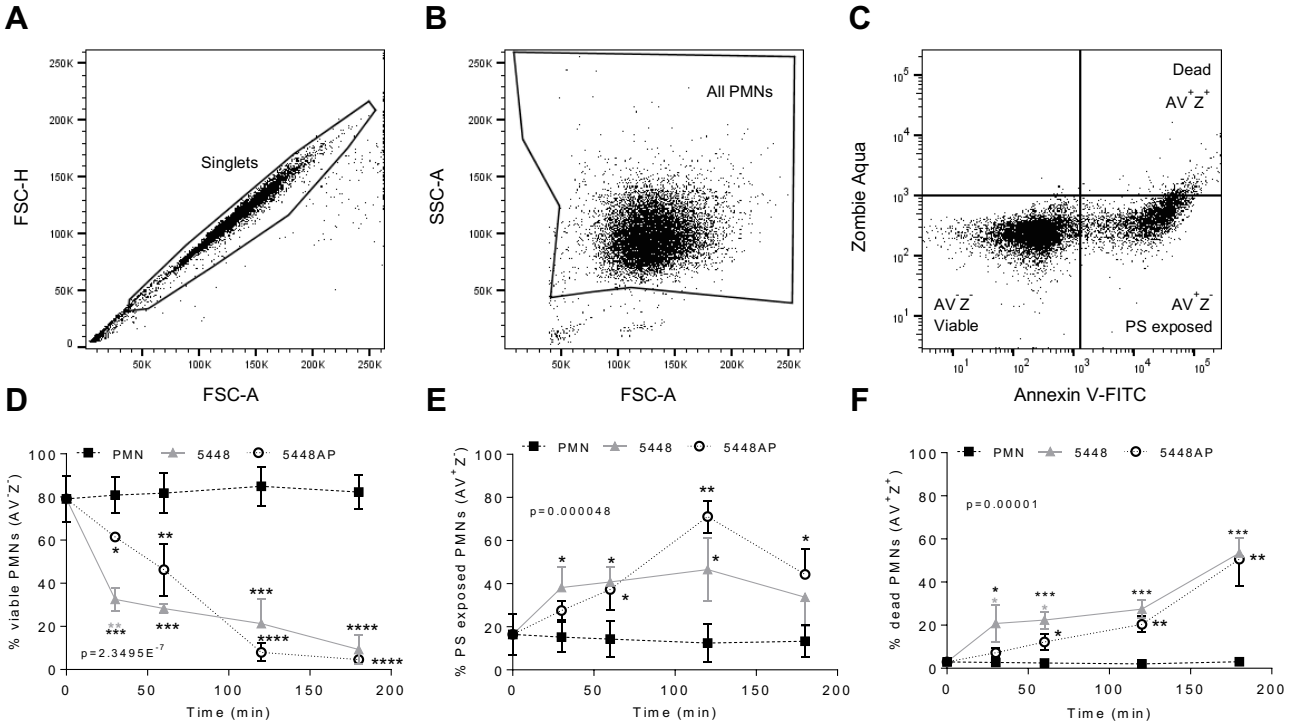
131 The above data (Figure 2) suggests that both GAS strains induce rapid neutrophil death. To
132 explore the mechanism of GAS-induced neutrophil death and whether this differs between GAS
133 strains, human neutrophil lysates were separated by SDS-PAGE and screened for the abundance
134 of active caspase-3, caspase-1 and caspase-4 via immunoblotting and quantified using
135 densitometry (Figures 3A and S2A-D). Cleavage of executioner caspase-3 to p17 is a definitive
136 hallmark of apoptosis induction (Nicholson et al., 1995). Conversely, caspase-1 and caspase-4
137 activity can indicate inflammasome activation, which in turn can cleave downstream effectors of
138 pyroptosis (Man and Kanneganti, 2016, Sollberger et al., 2012). Moreover, a recent report has
139 identified the caspase-1 p46 molecule as the principal active species during inflammasome
140 activation in the cell (Boucher et al., 2018). Neutrophils incubated in the absence of GAS showed
141 limited evidence of caspase-3 cleavage to p17 (Figures 3A and S2A), while levels of caspase-1
142 reduced over 180 min (Figures 3A and S2B-C). Infection of neutrophils with 5448 significantly
143 increased the presence of caspase-3 p17 at 180 min ($p>0.0001$) compared to both 5448AP-

144 infected and uninfected neutrophils (Figures 3A and S2A). In contrast, incubation of neutrophils
145 with either GAS strain caused an upregulation of caspase-1 (Figures 3A and S2B-C). Compared to
146 uninfected neutrophils, 5448 infection significantly increased pro-caspase-1 (50kDa) at 30 min,
147 60 min ($p<0.05$) and 180 min ($p<0.01$), whilst 5448AP infection only significantly increased pro-
148 caspase-1 at 180 min ($p<0.001$, Figures 3A and S2B). Further, 5448 and 5448AP infected
149 neutrophils sustain p46 expression over 180 min, though this difference was not statistically
150 significant (Figure 3A and S2C). Significant differences were not seen in the expression of
151 caspase-1 between 5448 and 5448AP infected neutrophils. Similar to caspase-1, the abundance
152 of full-length caspase-4 in neutrophils incubated in the absence of GAS declined over time
153 (Figures 3A and S2D). In contrast, 5448 infection increased caspase-4 expression at 30 ($p<0.01$),
154 60 ($p<0.001$) and 180 min compared to uninfected neutrophils ($p<0.0001$, Figures 2A and S2D).
155 5448AP infection however, only increased caspase-4 expression at 180 min ($p<0.01$, Figures 3A
156 and S2D).

157 These above data suggest both GAS strains induce pyroptosis in neutrophils, but the response to
158 5448AP is delayed. Therefore, to explore this possibility further, human neutrophils were
159 incubated with 5448, 5448AP or in the absence of GAS and caspase-1 activity assessed using a
160 fluorogenic flow cytometric caspase-1 assay (Figures 3B and S3A-C). 5448 infected neutrophils
161 showed increased active caspase-1 at 60 min and 180 min ($p<0.05$), whilst 5448AP infection
162 increased caspase-1 at 180 min ($p<0.05$) compared to uninfected neutrophils (Figure 3B). No
163 significant differences were observed between strains although caspase-1 activity was slightly
164 reduced during 5448AP infection compared to 5448 (Figure 3B), similar to the immunoblot data
165 for caspase-1 p46 (Figures 3A and S2C). Thus, GAS infection of human neutrophils corresponds

166 to increased caspase-1 activity and caspase-4 expression, consistent with inflammasome

167 activation and pyroptosis.



168 **Figure 2. GAS induced neutrophil death is delayed during infection with *covS* mutant.**

169 Human neutrophils (PMNs) were stained with Annexin V-FITC and Zombie Aqua Fixable Viability

170 Kit via flow cytometry and initially gated upon (A) 'Singlets' (FSC-A vs. FSC-H, 10,000 events

171 collected). 'All PMNs' (live and dead) were selected using (B) a gate excluding GAS. Cells were

172 then compared for fluorescence of (C) annexin V-FITC/phosphatidylserine (PS) binding against

173 Zombie live/dead viability dye. Purified PMNs were sampled over 180 min to measure (D) 'Viable'

174 (AV^-Z^-), (E) 'PS exposed' (AV^+Z^-), and (F) 'Dead' (AV^+Z^+), neutrophils during GAS infection (n=3

175 donors). Results are pooled means±SD . *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001, with

176 black asterisks denoting significance from control and grey asterisks between 5448 and 5448AP.

177 **Proinflammatory cytokines IL-1β and TNF-α are released by neutrophils in response to**

178 **GAS infection**

179 The release of the inflammatory cytokine IL-1β occurs due to caspase-1 activation and is

180 associated with the induction of pyroptosis (Broz et al., 2010). Uncoordinated release of various

181 inflammatory cytokines can exacerbate infection (Tecchio et al., 2014). During GAS infection,
182 disease severity is negatively correlated to cytokine concentration (Norrby-Teglund et al., 2000).
183 Therefore, human neutrophils were incubated with 5448, 5448AP or in the absence of GAS and
184 supernatants assessed for cytokines using a flow cytometric bead assay. Neutrophils incubated
185 in the absence of GAS failed to release IL-1β (Figure 3C) and TNF-α (Figure 3D) despite releasing
186 increasing amounts of IL-8 over 360 min (Figure 3E). Incubation with GAS induced IL-1β release
187 from neutrophils at 180 min and 360 min, with significantly greater release during 5448AP
188 infection compared to 5448 at 180 and 360 min ($p<0.05$, $p<0.0001$, Figure 3C). Incubation with
189 GAS also induced significant release of TNF-α from neutrophils at 180 min ($p<0.001$) and 360 min
190 ($p<0.0001$), with significantly greater release during 5448 infection compared to 5448AP at 360
191 min ($p<0.0001$, Figure 3D). 5448 induced significant release of IL-8 compared to uninfected from
192 neutrophils at 60 min and 180 min ($p<0.05$), with significantly greater release than 5448AP at 60
193 min ($p<0.05$, Figure 3E). Neutrophils released minimal amounts of IL-18 over 360 min, and
194 infection with both 5448 or 5448AP did not change this response (Figure 3F), suggesting that
195 neutrophils have no major role in IL-18 release over 360 min *in vitro*. These data indicate that
196 both 5448 and 5448AP induce IL-1β release from human neutrophils, consistent with the
197 hypothesis that both GAS strains induce pyroptosis in these cells. Moreover, the results indicate
198 differential cytokine release from human neutrophils infected with GAS, with 5448AP inducing
199 greater IL-1β release and 5448 inducing greater TNF-α and IL-8 release.

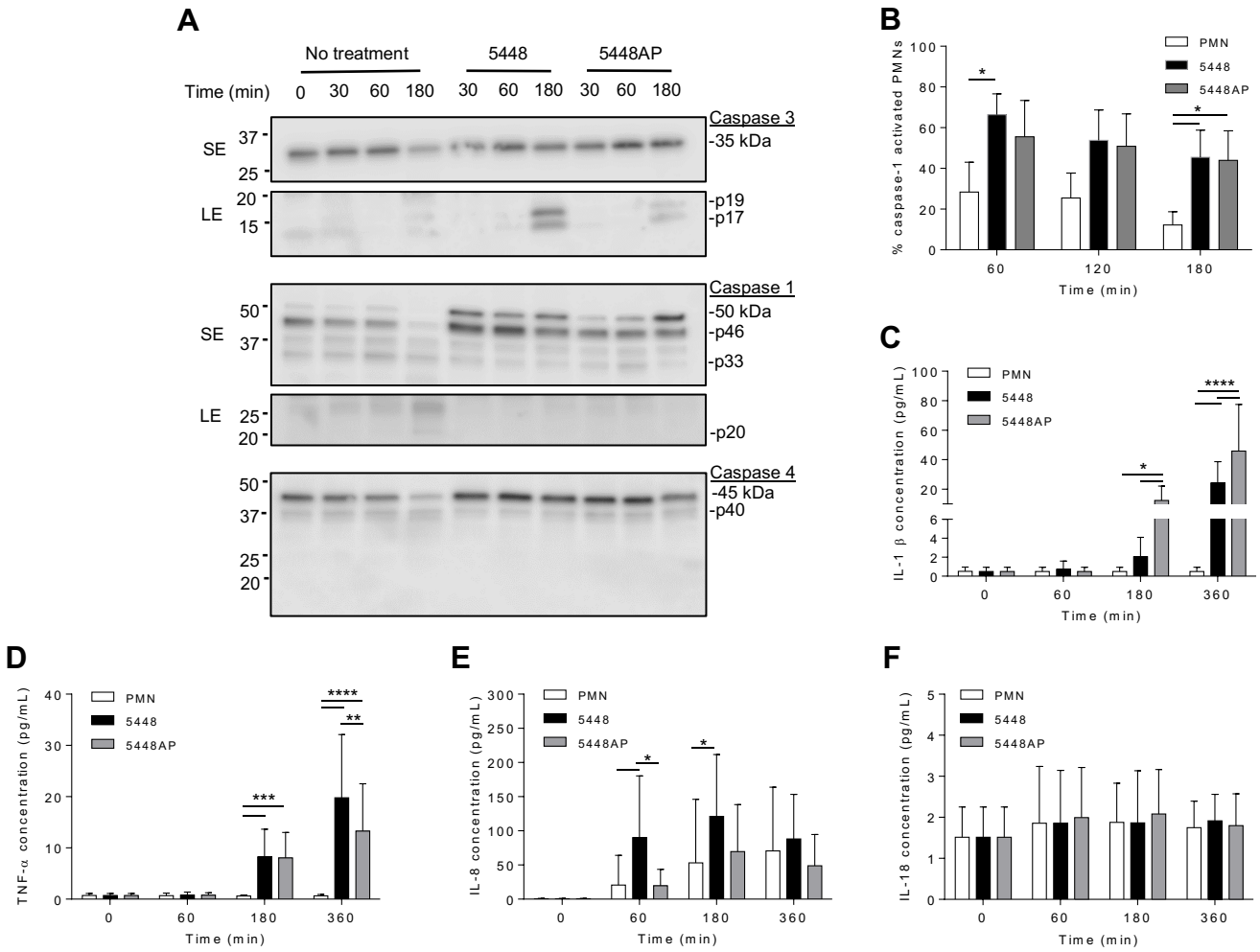


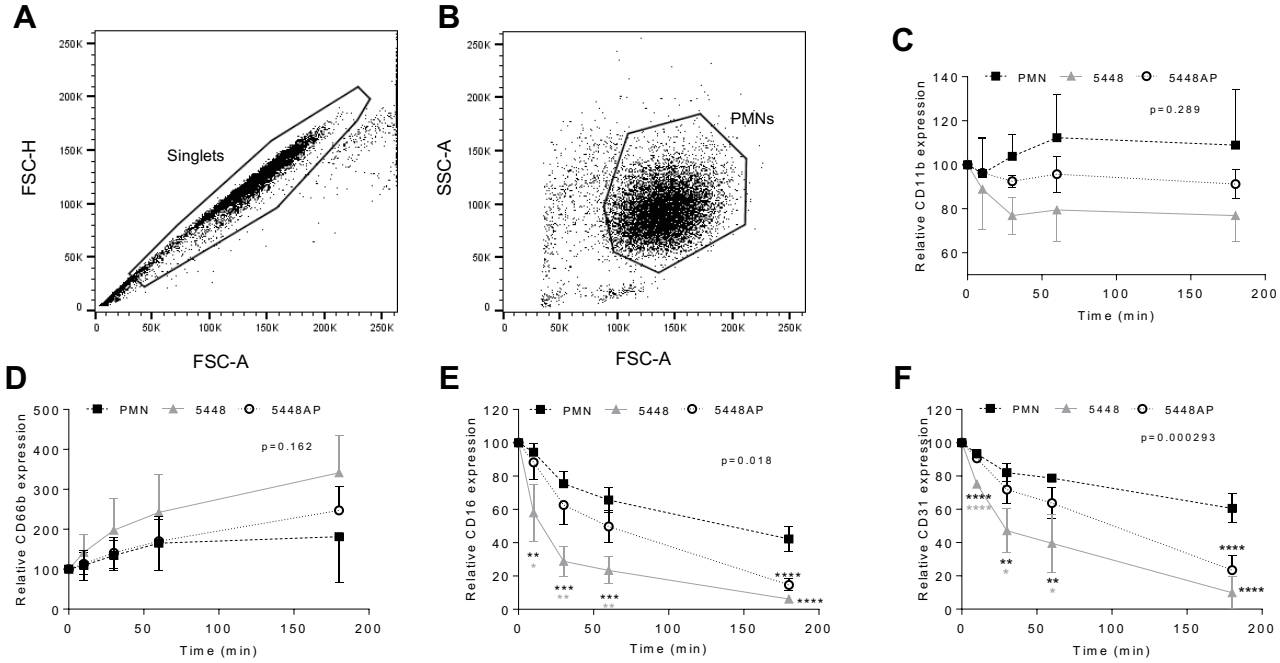
Figure 3. GAS infection activates caspase-1 in human neutrophils and induces proinflammatory cytokine IL-1β and TNF-α release. (A) Human neutrophil (PMN) lysates were prepared at 30, 60 and 180 min during GAS infection and compared to uninfected neutrophils (0, 30, 60, 180 min) via immunoblotting of caspase-3, caspase-1 and caspase-4. Images shown are from a single donor and are representative of triplicate experiments using different donors. Immunoblot bands were quantified (ImageJ) and normalised over total protein (Figure S2). SE=short exposure, LE=long exposure. (B) Caspase-1 activation in human neutrophils due to GAS infection was confirmed via flow cytometry using FLICA (FAM-YVAD-FMK) (n=3 donors). Flow cytometry gating strategy used singlets/PMNs (figure S3A-B) and FAM-FLICA fluorescence (Figure S3C). The release of cytokines from neutrophils during GAS infection was measured using the LEGENDplex™ human inflammation cytometric bead assay over 360 min. Neutrophils differentially released (C) IL-1β, (D) TNF-α, (E) IL-8 and (F) IL-18 in response to GAS

212 infection (duplicate measurements, n=6 donors). (B-F) Results are the pooled means±SD.
213 *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

214 **GAS infection may cause neutrophil dysfunction**

215 Neutrophil adhesion and activation are central to the innate immune response, with CD11b (Mac-
216 1) and CD66b (CEACAM8) playing key respective roles (Arnaout, 1990, Power et al., 2001, Skubitz
217 et al., 1996). CD16 (FcγRIII) facilitates opsonisation of pathogens and is down-regulated during
218 apoptosis (Dransfield et al., 1994, Fossati et al., 2002), whilst down-regulation of CD31 (PECAM-
219 1) facilitates clearance of apoptotic neutrophils and the resolution of inflammation (Brown et al.,
220 2002, Kurosaka et al., 2003). Therefore, to further explore the impact of GAS on neutrophil
221 function, human neutrophils were incubated with 5448, 5448AP or in the absence of GAS and the
222 expression of cell-surface CD11b, CD66b, CD16 and CD31 was assessed using flow cytometry
223 (Figure 4A-F). In the absence of GAS, neutrophils displayed minor increases in CD11b expression
224 over time, while incubation with GAS resulted in minor decreases over time, with 5448 inducing
225 a greater loss of CD11b than 5448AP, though these differences were not statistically significant
226 (p=0.289, Figure 4C). Neutrophils incubated in the absence of GAS revealed minor increases in
227 cell-surface CD66b expression over time (Figure 4D). Incubation with GAS further increased
228 expression of CD66b, with 5448 inducing a slightly greater increase than 5448AP, though these
229 differences were not statistically significant (p=0.162, Figure 4D). Incubation of neutrophils in
230 the absence of GAS resulted in a steady decline in CD16 expression over time, a loss significantly
231 increased by co-incubation with either GAS strain 180 min post-infection (p<0.0001, Figure 4E).
232 Notably, incubation of neutrophils with 5448 induced a significantly greater loss of CD16
233 expression than 5448AP incubation at all earlier time points (Figure 4E). Similar to CD16,
234 incubation of neutrophils in the absence of GAS resulted in a steady decline in CD31 expression
235 over time, a loss significantly increased by co-incubation with either GAS strain 180 min post-
236 infection (p<0.0001, Figure 4F). Moreover, incubation of neutrophils with 5448 induced a
237 significantly greater loss of CD31 expression than 5448AP incubation at earlier time points.

238 Collectively, these data indicate that GAS has minimal impact upon human neutrophil CD11b and
239 CD66b, but increases the loss of CD16 and CD31 expression, which may impact opsonisation of
240 GAS and subsequent efferocytosis of neutrophils by macrophages, during infection.

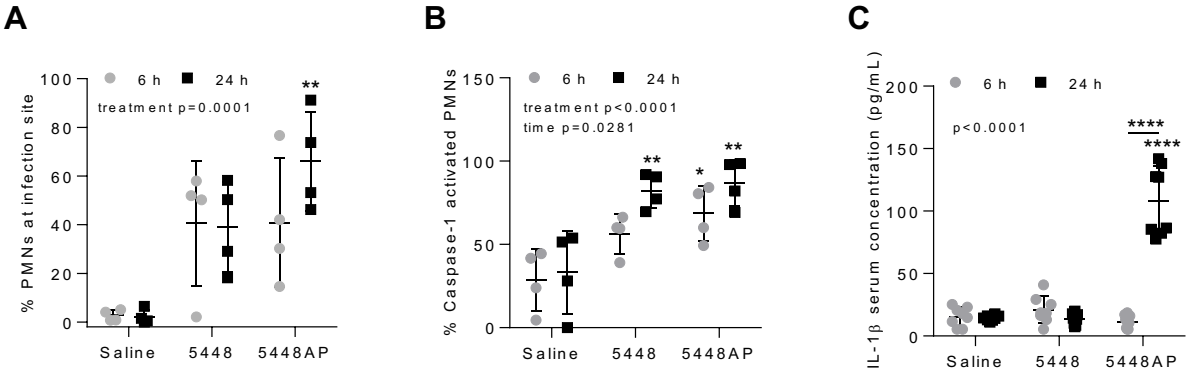


241 **Figure 4. GAS infection changes neutrophil functionality.** Human neutrophils (PMNs) were
242 analysed using flow cytometry, sequentially gated upon (A) 'Singlets' (FSC-A vs. FSC-H, 10,000
243 events collected) then (B) viable 'PMNs' (FSC-A vs. FSC-H). Purified neutrophils infected with GAS
244 were sampled over 180 min for the cell-surface expression of (C) CD11b-FITC (n=4 donors), (D)
245 CD66b-PerCP/Cy5.5 (n=5 donors), (E) CD16-FITC (n=4 donors) and (F) CD31-PE/Cy7 (n=4
246 donors). Results are the pooled means±SD. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001, with
247 black asterisks denoting significance from control and grey asterisks between 5448 and 5448AP.

248 **GAS infection activates caspase-1 in neutrophils *in vivo***

249 The above data indicate that GAS influences human neutrophils *in vitro.* To explore if GAS may
250 alter neutrophils *in vivo*, we assessed the neutrophil response in a mouse model of GAS infection
251 (Ly et al., 2014, Maamary et al., 2010, Tsatsaronis et al., 2014). C57BL/6J mice were injected
252 intradermally with 5448, 5448AP or saline and euthanised at 6 or 24 hours post infection, with
253 neutrophil populations ($CD45^+/CD11b^+/Ly\text{-}6G^+$) and caspase-1 activation characterised by flow

254 cytometry (Figures 5A-B and S4A-F). Infection of mice with either GAS strain increased
255 neutrophil populations at sites of injection, however only 5448AP infection resulted in
256 significantly greater neutrophil numbers than the saline control at 24 h ($p<0.01$, Figure 5A). The
257 percentage of circulating neutrophils in the blood was also significantly increased due to 5448
258 ($p<0.05$) and 5448AP ($p<0.01$) infection at 6 h, however returned to baseline after 24 h (Figure
259 S4G), indicating the importance of neutrophils during the early stages of infection. Murine
260 neutrophils lavaged from sites of saline injection had low amounts of active caspase-1 (Figure
261 5B). Infection with 5448AP, but not 5448, significantly increased caspase-1 activation at 6 h
262 ($p<0.05$), while both strains increased caspase-1 activation at 24 h ($p<0.01$, Figure 5B). Cytokines
263 were assessed in murine serum using a flow cytometric bead assay. IL-1β concentrations
264 remained low for saline control and 5448 infected mice but were significantly increased at 24 h
265 during 5448AP infection ($p<0.0001$, Figure 5C).



266 **Figure 5. GAS infection *in vivo* activates caspase-1 in murine neutrophils.** (A) Percentage of
267 CD45$^+$ cells lavaged from site of injection characterised as neutrophils (PMNs, CD11b$^+$/Ly-6G$^+$)
268 by flow cytometry (Figure S4A-E). (B) Caspase-1 activation in neutrophils at site of GAS infection
269 was confirmed via flow cytometry using FLICA 660 (660-YVAD-FMK, Figure S4F). Each data point
270 represents the results from a single mouse where n=4. (C) The release of IL-1β in mouse serum
271 during GAS infection was measured using the LEGENDplex™ mouse inflammation cytometric
272 bead assay at 6 and 24 h post infection where duplicate measurements are shown and n=4.
273 Results are the mean±SD. **$p<0.01$, ***$p<0.001$ and ****$p<0.0001$ where significance is denoted
274 from control or as indicated by a line.

275 **Discussion**

276 Here we have mapped the human neutrophil response to GAS, addressing cell-death, signalling
277 and the inflammatory profile during the early stages of M1T1 (5448 and 5448AP) infection. For
278 the first time, we describe an inflammatory neutrophil phenotype, as evidenced by caspase-1
279 activation, that may promote inflammation and exacerbate GAS disease. Most notably, changes to
280 the expression of cell-surface proteins CD16 and CD31 may impede the removal of bacteria and
281 clearance of neutrophils at sites of infection, hindering the resolution of inflammation. Further,
282 we elucidate differences between 5448 and 5448AP that aid in understanding the survival and
283 proliferation of GAS harbouring *covS* mutations in the presence of human neutrophils.

284 The temporal expression of neutrophil cell surface markers has not previously been investigated
285 in the context of GAS infection. Our data show that significant reductions to both CD16 and CD31
286 occur in neutrophils due to GAS infection *in vitro*. CD16 functions as a receptor for opsonised
287 bacteria and immune complexes (Fossati et al., 2002), whilst the loss of CD16 has also been
288 associated with the induction of apoptosis (Dransfield et al., 1994). The induction of apoptosis
289 following bacterial phagocytosis is advantageous to the host as it promotes controlled removal of
290 bacteria and the resolution of inflammation (Kobayashi et al., 2018). We hypothesise that the
291 retention of CD16 during infection promotes a reduction in apoptosis and prolonged
292 degranulation at the site of infection. Further, CD31 expression is reduced during GAS infection,
293 facilitating the eventual removal of neutrophils from sites of infection (Kobayashi et al., 2003).
294 Parallel to CD16, CD31 is retained during 5448AP infection of neutrophils to a greater extent than
295 5448 infection. This may result in neutrophils remaining at sites of infection longer, further
296 increasing inflammation. Additionally, we report a trend towards increased cell-surface
297 expression of CD11b and CD66b during GAS infection. Migration of neutrophils to sites of
298 infection, facilitated by CD11b-mediated neutrophil adhesion (Arnaout, 1990), may therefore be
299 affected during GAS infection. Down-regulation of CD11b can prevent accumulation of
300 neutrophils at inflammatory sites (Huston et al., 2009), however retention, such as that seen

301 during 5448AP infection may contribute to prolonged inflammation. Neutrophil activation can be
302 determined through the expression of cell-surface CD66b following stimulation (Skubitz et al.,
303 1996). Reduced cell-surface CD66b expression in response to 5448AP infection may therefore
304 indicate a reduction to neutrophil function and further explain the resistance to neutrophil-
305 mediated killing. Collectively, the neutrophil response is altered and inflammation prolonged at
306 sites of *covS* mutant GAS infection.

307 Disruption of the role neutrophils play during the resolution of GAS infection and induction of
308 inflammatory death pathways has been reported previously (Kobayashi et al., 2003, Tsatsaronis
309 et al., 2015). Proinflammatory activation of the NLRP3 inflammasome in macrophages has been
310 demonstrated for M1 GAS (Valderrama et al., 2017), here we provide the first evidence that GAS
311 activates caspase-1 in human and murine neutrophils, consistent with inflammasome activation
312 and pyroptosis. Caspase-1 activation was consistent during infection with both M1T1 GAS
313 isolates, however the release of inflammatory cytokine IL-1β, a hallmark of clinical invasive
314 infection (LaRock and Nizet, 2015), was significantly increased in response to 5448AP infection
315 *in vitro* and *in vivo*. In murine bone marrow-derived dendritic cells caspase-1 activation is
316 essential for IL-1β release (Schneider et al., 2017). The activation of caspase-1 in human and
317 murine neutrophils does not correlate directly to the amount of IL-1β released, and an alternative
318 mechanism may facilitate IL-1β maturation. Type I interferons are known regulators of IL-1β in
319 GAS infected mice (Castiglia et al., 2016), where increased IL-1β release in murine serum seen in
320 this study may also be attributed to disruption of this homeostatic relationship. Increased IL-1β,
321 being a neutrophil chemoattractant, therefore may further promote inflammation at sites of
322 infection following release (Chen et al., 2007), supporting the hypothesis that *covS* mutant GAS
323 promote greater levels of inflammation during GAS infection.

324 Previous studies have characterised differences between 5448 and 5448AP, demonstrating
325 resistance to neutrophil-mediated killing *in vitro* and increased bacterial dissemination and
326 decreased survival *in vivo* following the acquisition of *covS* mutations (Fiebig et al., 2015,

327 Maamary et al., 2010, Walker et al., 2007). Here, we demonstrate that 5448AP is not only resistant
328 to neutrophil-mediated killing but proliferates in the presence of functional neutrophils. GAS
329 survival has been demonstrated intracellularly in murine neutrophils and human macrophages
330 (Medina et al., 2003a, Medina et al., 2003b, O'Neill et al., 2016) and can even be facilitated by host
331 red blood cells (Wierzbicki et al., 2019). Increased 5448AP proliferation may result from the
332 reduction in neutrophil ROS response during infection. GAS has previously been shown to display
333 resistance to ROS through numerous virulence mechanisms (Henningham et al., 2015). The
334 absence of a hostile environment may further permit bacterial replication. Additionally, the
335 increased production of ROS can induce neutrophil death via apoptosis (Geering and Simon,
336 2011). Reduced neutrophil ROS production during 5448AP infection may therefore explain the
337 reduction seen in caspase-3 activation (apoptosis). Previously it has been demonstrated that GAS
338 M1 strain MGAS5005 modulates neutrophil apoptosis (Kobayashi et al., 2003), a finding which is
339 supported by the current study.

340 Changes to the function of neutrophils during the initial innate immune response have the
341 potential to hinder mechanisms essential for the resolution of infection. Disruption to these
342 processes during GAS infection may induce this neutrophil phenotype we have described, with
343 increased inflammatory properties and potential to exacerbate infection. Caspase-1 activation
344 during GAS infection of neutrophils indicates possible inflammasome activation and death via
345 pyroptosis. Further studies should explore the precise cell death mechanism. A reduction in
346 apoptosis is evident during *covS* GAS infection, as has been reported for non-M1T1 GAS
347 (Tsatsaronis et al., 2015), whilst modulation of apoptosis has previously been implicated as a
348 factor contributing to GAS survival (Kobayashi et al., 2003). The current study aids in
349 understanding the complex host-pathogen interaction identifying host factors that may
350 contribute to severe pathology of invasive GAS infection. Future studies may be able to isolate
351 host-specific targets that could be exploited to control inflammation and tissue damage during
352 invasive GAS infection.

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Author Contributions

Conceptualisation, M.S.S, R.S., J.A.T., J.G.W.; Methodology, M.S.S., R.S., J.G.W., J.G., J.D.M., D.L.; Investigation, J.G.W., D.L., N.J.G., H.K.N.V.; Data and figure curation, J.G.W., D.L., R.S., M.S.S.; Writing-Original draft, J.G.W.; Writing-Review and editing, M.S.S., R.S., J.G.W., D.L., N.J.G., J.D.M., H.K.N.V., J.G., J.A.T.; Funding acquisition, M.S.S and D.L.; Resources, M.S.S., J.D.M.; Supervision, M.S.S., R.S., J.G.

Declaration of Interests

The authors declare no competing interests.

Materials and Methods

Ethics statement

All experiments involving the use of human blood were conducted with informed consent of healthy volunteers, approved and authorised by the University of Wollongong Human Research Ethics Committee (Protocol HE08/250). All experiments involving the use of animals were approved and authorised by the University of Wollongong Animal Ethics Committee (Protocol AE18/10).

Bacterial strains and culture

Escherichia coli MC106 were grown in Luria–Bertani Broth (LB) at 37 °C with constant shaking. M1T1 *S. pyogenes* invasive clinical isolate 5448 (*emm1*) and hypervirulent animal passaged

376 5448AP (containing a non-functional control of virulence regulator mutation) have been
377 described previously (Aziz et al., 2004, Chatellier et al., 2000). GAS strains were routinely cultured
378 at 37°C on horse-blood agar (Thermo Fisher) and enumerated on yeast supplemented (1% w/v)
379 Todd-Hewitt (THY, Benton Dickson) agar. Static overnight cultures were grown at 37°C in THY
380 then sub-inoculated (1:10) into fresh THY and grown to mid-logarithmic phase, antibiotics were
381 added when required (100 μg/ml ampicillin and 200 μg/ml kanamycin). Bacterial pellets were
382 washed twice with phosphate buffered saline (PBS) then resuspended at the specified
383 multiplicity of infection (MOI) and assay specific media conditions.

384 **Construction of GFP expression vector pLZ12Km2-P23R-TA:GFP for GAS**

385 In brief, stable GFP expression by GAS was created by synthesizing the ribosomal binding site
386 (RBS) and *gfp* gene from pDC*erm*-GFP (Ly et al., 2014) into the pUC57 plasmid (GenScript),
387 resulting in pUC57-RBSGFP plasmid. RBS and *gfp* from pUC57-RBSGFP were then sub-cloned
388 using NotI into the toxin–antitoxin (TA) stabilized expression plasmid pLZ12Km2-P23R:TA,
389 kindly provided by Associate Professor Thomas Proft (University of Auckland, New Zealand) (Loh
390 and Proft, 2013), to produce pLZ12Km2-P23R-TA:GFP. The resultant plasmid system utilises the
391 *Streptococcal* ω–ε–ζ TA cassette to achieve segregational plasmid stability under non-selective
392 conditions (Lioy et al., 2010).

393 Specifically, plasmids pLZ12Km2-P23R:TA and pUC57-RBSGFP (engineered with RBS and *gfp*
394 from pDC*erm*-GFP) were first transformed into chemically competent *E. coli* MC106 via
395 electroporation. The plasmids were then retrieved and purified from *E. coli* cultures using a
396 Wizard® Plus SV Minipreps DNA Purification System (Promega). The pLZ12Km2-P23R:TA
397 plasmid was digested with 20 U NotI enzyme and further treated with 5 U shrimp alkaline
398 phosphatase. The pUC57-RBSGFP plasmid was also incubated with 20 U NotI to excise the RBS
399 and *gfp* gene from pUC57-RBSGFP, and these were then ligated into digested pLZ12km2-P23R:TA
400 plasmid. The resulting plasmid, pLZ12Km2-P23R-TA:GFP, was then transformed and cloned in
401 MC106 *E. coli*, and later extracted and purified. pLZ12Km2-P23R-TA:GFP was transformed into

402 GAS using standard GAS electroporation techniques (McLaughlin and Ferretti, 1995).
403 Transformed GAS was confirmed for GFP expression via flow cytometry.

404 **Isolation of human neutrophils**

405 Venous human blood was drawn into 10 mL lithium heparin-coated Vacutainer (Benton Dickson)
406 tubes and layered over equal volumes of Polymorphprep (Axis Shield) and centrifuged as per
407 manufacturer's instructions. The resulting layer of neutrophils was isolated and erythrocytes
408 hypotonically lysed. Isotonic concentration was restored with Hank's Balanced Salt solution
409 (without Ca^{2+} or Mg^{2+}, Corning Inc.). Prior to experimentation neutrophils were resuspended at
410 specified concentrations in complete medium, Roswell Park Memorial Institute (RPMI)-1640
411 medium (Life Technologies) containing 2% heat-inactivated autologous plasma and 2 mM L-
412 glutamine (Life Technologies), unless otherwise stated. Neutrophil viability was assessed via
413 Trypan Blue (Sigma-Aldrich) staining and neutrophil purity was assessed using a Benton Dickson
414 LSR Fortessa X-20 flow cytometer via distinct forward and side scatter profiles or CD66b-
415 peridinin chlorophyll protein (PerCP)/Cy5.5 (clone G10F5, BioLegend) expression. Neutrophils
416 were maintained at room temperature throughout processing.

417 ***In vitro* infection of neutrophils with GAS**

418 Purified human neutrophils were seeded in either 96-well plates at MOI (GAS:neutrophil) 1:10
419 (survival and proliferation) or 10:1 (ROS production and cytokine release), 24-well plates at 10:1
420 (phagocytosis, Annexin-V/Viability staining, CD expression and FAM-FLICA caspase-1 activation)
421 or 6-well plates at 10:1 (immunoblotting) and incubated at 37°C in 5% CO_2.

422 **GAS survival and proliferation**

423 Human neutrophils were co-cultured with GAS or lysed via three freeze-thaw cycles prior to
424 incubation. To block phagocytosis neutrophils were preincubated with 10 µm cytochalasin D
425 (Cayman Chemicals) in complete medium at 37°C for 30 min prior to infection. At indicated time

426 points neutrophils were hypotonically lysed and surviving bacteria serially diluted before plating
427 on THY agar.

428 **GAS phagocytosis**

429 Human neutrophils were co-cultured with GAS expressing GFP for the times indicated in
430 complete medium. Cells were removed and washed twice with 10% (v/v) heat-inactivated foetal
431 bovine serum (FBS, Bovogen Biologicals) diluted in PBS, followed by data acquisition via flow
432 cytometry.

433 **ROS production**

434 ROS production was assessed as previously described (Kobayashi et al., 2003). Briefly, prior to
435 infection, human neutrophils were incubated in complete medium containing 25 μM
436 dichlorofluorescein (DCF, Molecular Probes) for 45 min at room temperature (RT). ROS
437 production was measured fluorometrically ($_{ex}$485 nm $_{em}$520 nm) using a POLARstar Omega plate
438 reader (BMG Labtech).

439 **Flow cytometry**

440 For human neutrophil in vitro assays, flow cytometry data was acquired using a BD LSR Fortessa
441 X-20 with excitation lasers; violet (405 nm), blue (488 nm), yellow/green (561 nm) and red (640
442 nm). Bandpass filters for Zombie Aqua Fixable Viability Kit (525/50), fluorescein isothiocynate
443 (FITC)/GFP/FAM FLICA (525/50), PerCP-Cy5.5 (695/40) R-phycoerythrin (PE, 586/15),
444 propidium iodide (PI, 610/20), PE-Cy7 (780/60) and allophycocyanin (APC, 670/30) were used.
445 For murine infection studies, flow cytometry data was acquired using an Invitrogen Attune NxT
446 with excitation lasers; violet (405 nm), blue (488 nm), yellow (561 nm) and red (638 nm).
447 Bandpass filters VL1 (440/50), VL2 (512/25), BL1 (530/30), YL1 (585/16), YL3 (695/40) and
448 RL2 (720/30) were used. Data was analysed using FlowJo software V10.6.1 (TreeStar Inc.).

449 **Annexin-V/viability staining**

450 To assess cell viability human neutrophils at indicated times were washed with PBS and
451 incubated with Zombie Aqua Fixable Viability Kit (BioLegend) for 15 min at RT. Cells were
452 washed again once with PBS, then 10% (v/v) FBS in PBS and stained with Annexin-V-FTIC
453 (BioLegend) for 15 min at RT. Cells were analysed immediately via flow cytometry.

454 **Immunoblotting and antibodies**

455 Following co-culture in the presence or absence of GAS, human neutrophil lysates were prepared
456 in RIPA buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris, 1.0% (v/v) Triton X-100, 0.1% (w/v) SDS,
457 0.5% (w/v) sodium deoxycholate, 2x cOmplete protease inhibitor cocktail (Roche), 1 mM
458 phenylmethylsulphonyl fluoride, 5 mM sodium pyrophosphate, 5 mM sodium molybdate and 5
459 mM β-glycerophosphate) following co-culture in the presence or absence of GAS and incubated
460 for 30 min on ice with intermittent vortexing. Soluble fractions were separated via centrifugation
461 at 4°C, snap frozen in liquid N_2 and stored at -80°C. Protein concentrations were determined by
462 comparison to bovine serum albumin standards using the DC Protein Assay (Bio-Rad) and
463 absorbance (A_{750nm}) measured using a POLARstar Omega plate reader. Neutrophil lysates (20 µg)
464 were separated on 4-20% TGX Stain-Free protein gels (Bio-Rad) as per the manufacturer's
465 running conditions then activated for 5 min under UV to determine total protein (Bio-Rad
466 ChemiDoc XR, Image Lab Software). Proteins were transferred to PVDF membranes (Bio-Rad),
467 blocked, then incubated overnight at 4°C with caspase-1 polyclonal (1:1000, #2225), caspase-3
468 polyclonal (1:1000, #9662), caspase-4 polyclonal (1:1000, #4450) or caspase-8 monoclonal
469 (1:1000, #4790) antibodies (Cell Signalling Technology). PVDF membranes were washed thrice
470 between incubations for 5 min with tris-buffered saline containing 0.1% (v/v) Tween 20. PVDF
471 membranes were incubated with horseradish peroxidase-conjugated goatαrabbit IgG (1:5000,
472 Invitrogen) for 1 h at RT and detected using Clarity or Clarity Max Western ECL Blotting Substrate
473 (Bio-Rad) and imaged (Amersham AI600). Bands were quantified using ImageJ software
474 (National Institutes of Health) as area under the peak and normalised over UV determined total
475 protein.

476 **FAM-FLICA caspase-1 activation**

477 Caspase-1 activation was measured using FAM-FLICA Caspase-1 assay kit (ImmunoChemistry
478 Technologies) as per the manufacturer's instructions. In brief, following co-culture of human
479 neutrophils with GAS, cells were removed and incubated in serum free medium (RPMI-1640)
480 containing 1 x FAM-YVAD-FMK for 60 min at 37°C. Cells were then analysed for caspase-1
481 activation via flow cytometry.

482 **Cytometric cytokine bead assay**

483 Human neutrophil supernatants were at indicated times during GAS infection, snap frozen with
484 liquid N_2 and stored at -80°C for a period no longer than 5 days. Neutrophils were assessed for
485 the release of cytokines IL-1β, IL-8, IL-18 and TNF-α using the LEGENDplex™ human
486 inflammation panel bead-based immunoassay (BioLegend) and flow cytometry as per
487 manufacturer's instructions. In brief, samples and standards were incubated with beads and
488 detection antibodies for 120 min at room temperature, with shaking (600 rpm) protected from
489 light. PE-conjugated streptavidin was then added and incubated for a further 30 min at room
490 temperature, with shaking (600 rpm) protected from light. Beads were washed twice with
491 LEGENDplex™ wash buffer and data collected via flow cytometry. Beads were initially gated upon
492 FSC-A and SSC-A (A and B populations). Cytokines have signature APC fluorescence with
493 quantitative expression determined using PE fluorescence in comparison to cytokine standards.
494 Data was analysed using the LEGENDplex™ software V8.0 (VigeneTech Inc.).

495 **Cluster of differentiation expression**

496 Human neutrophil cell surface CD expression was assessed during GAS infection. Neutrophils at
497 indicated times were washed with PBS then routinely incubated with Zombie Aqua Fixable
498 Viability Kit for 15 min at RT. Cells were washed once again in PBS, followed by 10% (v/v) FBS in
499 PBS. Neutrophils were stained with fluorochrome-conjugated antibodies CD11b-FITC (Clone
500 ICRF44), CD31-PE/Cy7 (Clone WM59), or CD66b-PerCP/Cy5.5 (Clone G10F5) (BioLegend) and

501 CD16-FITC (Clone 3G8, Benton Dickson) for 15 min at RT. Neutrophils were washed with 10%
502 (v/v) FBS in PBS then analysed via flow cytometry.

503 **Murine intradermal GAS infection model**

504 Intradermal GAS challenge of C57BL/6J mice has been described previously (Ly et al., 2014,
505 Maamary et al., 2010, Tsatsaronis et al., 2014). In brief, equal numbers of 6-8 week old male and
506 female C57BL/6J mice (Australian BioResources) were anesthetised via isoflurane inhalation,
507 followed by two intradermal injections into shaved left and right flanks with either 1 x 10^8 CFU
508 of mid-logarithmic GAS or 100 µL sterile 0.7% saline. Mice were euthanised via slow-fill CO_2
509 asphyxiation at 6 or 24 h post-infection. Blood collected via cardiac puncture was separated into
510 two aliquots for serum and flow cytometric analysis. Serum was collected by clotting for 1 h at RT
511 then placed on ice until centrifugation at 1200 x *g* for 10 min at 4°C, then immediately stored at -
512 80°C until use. Remaining blood samples were mixed with 20 µL 0.5% sodium citrate (w/v) per
513 mL of blood and placed on ice. Equal volume of PBS was added to the blood sample and
514 centrifuged at 350 x *g* for 5 min. For erythrocyte lysis, samples were incubated with 1 mL of
515 ammonium-chloride-potassium lysing buffer (150 mM NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2CO_3, pH
516 7.3) for 5 min with gentle agitation and centrifuged at 350 x *g* for 5 min. This process was repeated
517 and cells were then washed with PBS and resuspended in 500 µL PBS and placed on ice.
518 Additionally, sites of infection were lavaged with 1 mL of sterile 0.7% saline, and fluid collected
519 from both right and left flanks was pooled to increase cell numbers. Pooled samples were kept
520 cold until centrifugation at 350 x G for 5 min, resuspended in 500 uL of PBS and placed on ice.
521 Both blood and lavage fluid samples were analysed via flow cytometry.

522 **Flow cytometric analysis of murine cells**

523 Cell viability was assessed as described above. To assess caspase-1 activation and CD expression,
524 cells were initially washed with 1 mL of PBS, followed by 1 mL of 10% (v/v) FBS in PBS. Cells
525 were then centrifuged and simultaneously stained with 1 x 660-YVAD-FMK (FLICA 660 Caspase-

526 1 assay kit, ImmunoChemistry Technologies) for caspase-1 activation and with fluorochrome-
527 conjugated antibodies CD45-BV421 (clone 30-F11), CD11b-PE/Cy5 (clone M1-70) and Ly-6G-PE
528 (clone 1A8) (Biolegend) in RPMI 1640 media for 30 min at RT . Cells were washed with 10% (v/v)
529 FBS in PBSand analysed via flow cytometry.

530 **Murine serum IL-1β concentration**

531 IL-1β release was measured in murine serum using the LEGENDplex™ mouse inflammation panel
532 bead-based immunoassay (BioLegend) and flow cytometry as described above except shaking
533 was performed at 800 rpm.

534 **Statistical analyses**

535 Graphs were created using Prism 6 (GraphPad Software Inc.) and statistical analysis was
536 performed using Prism 6 and IBM SPSS Statistics 25 (IBM® corporation). Data was analysed
537 using one-way and two-way ANOVA to determine significant differences and adjusted using
538 Tukey HSD corrections or as stated otherwise. To account for the nested nature of the human
539 neutrophil Annexin-V/Zombie and CD data with repeated measures over time in the same donor,
540 a linear mixed model was used to determine significant interaction between treatment and time.
541 Post-hoc tests were performed by multiple one-way ANOVA at each time point where multiple
542 comparisons were adjusted using Tukey HSD corrections. *In vivo* data was analysed by two-way
543 ANOVA where differences are shown between treatment and time, also using Tukey HSD
544 corrections. Values presented for 'p' represent interaction (treatment*time) unless otherwise
545 stated. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

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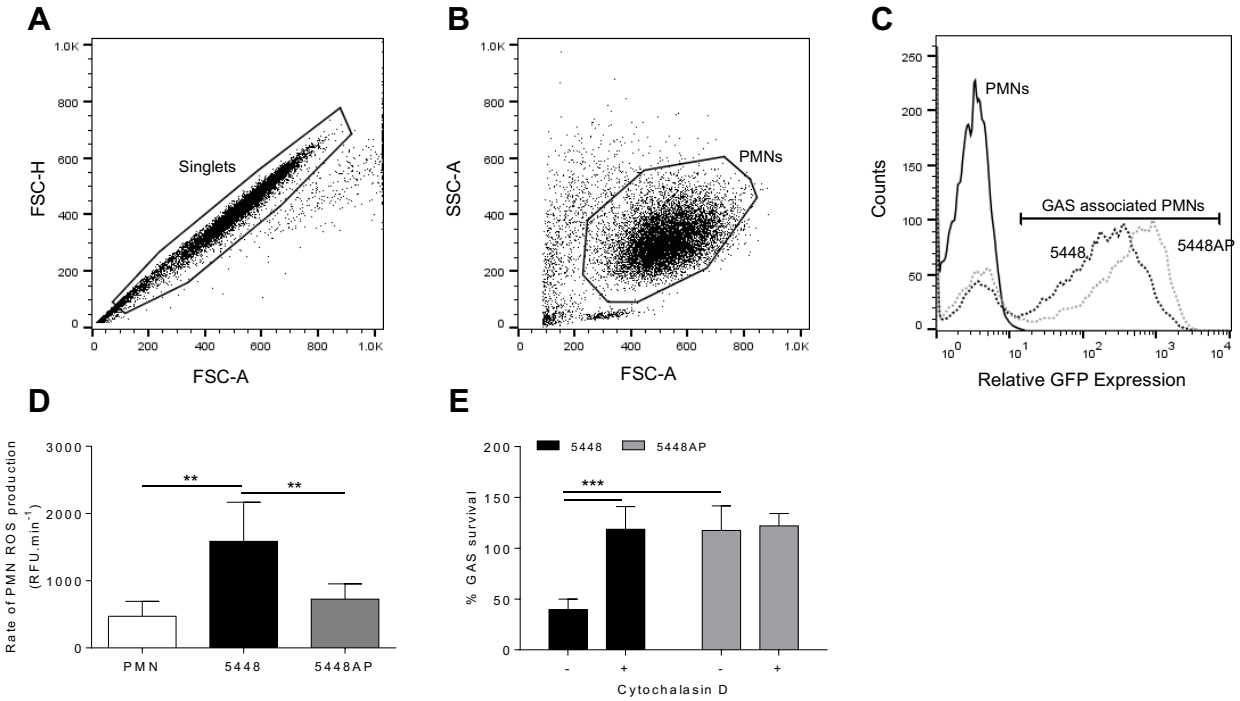
741 **Supplemental Information**



742 **Supplementary figure 1. The association of fluorescent GAS (GFP) to human neutrophils**
743 **determined via flow cytometry.** (A) Primary neutrophil (PMN) 'Singlets' cell selection (FSC-A
744 vs. FSC-H) and (B) viable 'PMNs' discrimination (FSC-A vs. SSC-A). (C) Representative histogram
745 showing relative fluorescence (GFP) of GAS infected neutrophils and gating for the selection of
746 GAS-neutrophil association. (D) Rate of ROS production by neutrophils between 30-60 min (n=6
747 donors). (E) GAS killing by human neutrophils is inhibited at 30 min following pre-incubation
748 with actin polymerising molecule cytochalasin D (n=4 donors, Sidak's multiple comparison).
749 Results are the pooled means±SD (of triplicate measurements for panels D and E). **p<0.01,
750 ***p<0.001.

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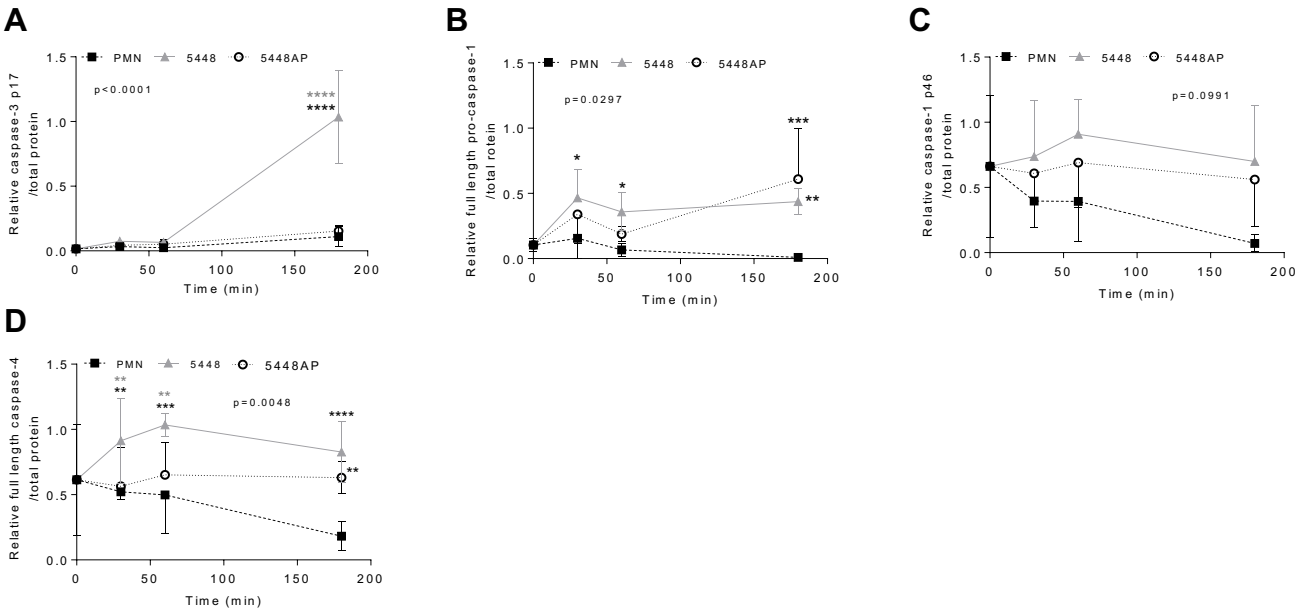
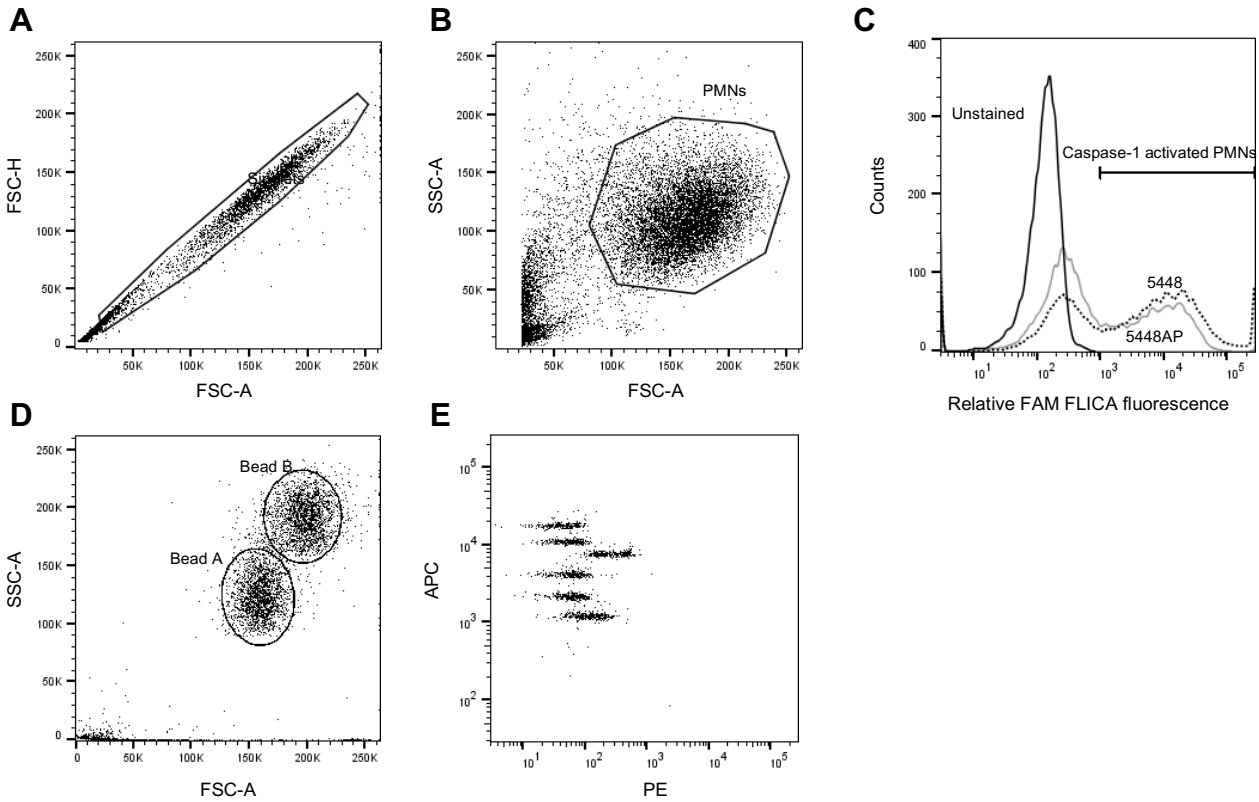
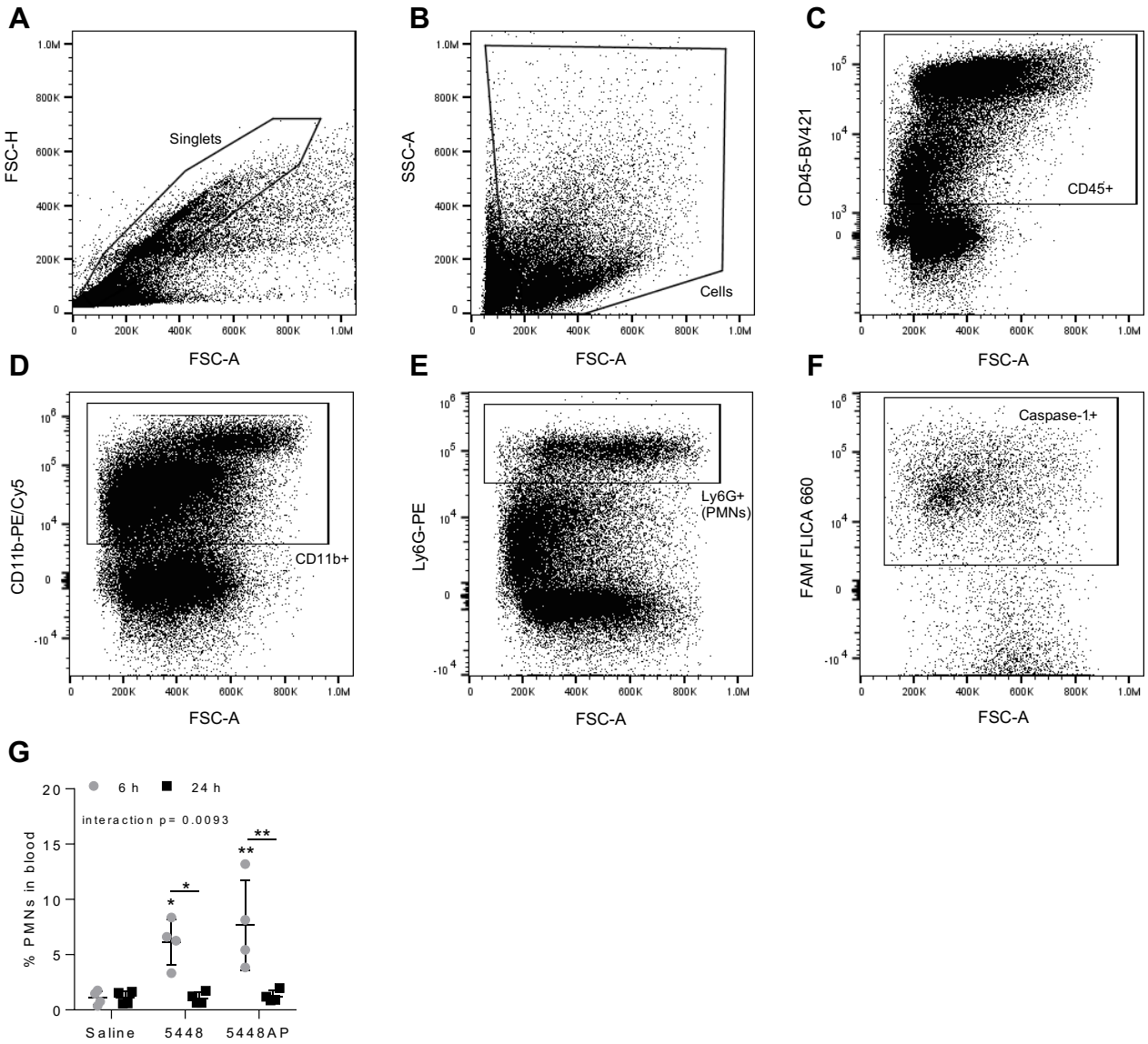


Supplementary figure 2. Quantification of protein bands in human neutrophil lysates identified through immunoblotting. (A) Caspase-3 p17, (B) caspase-1 full-length, (D) caspase-1 p46 and (D) caspase-4 immunoblots were imaged then analysed using ImageJ where area under the curve was normalised over total protein. Results are the means±SD where 3 donors were used. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001, with black asterisks denoting significance to control and grey asterisks between 5448 and 5448AP.



759 **Supplementary figure 3. Human neutrophils were assessed for the activation of caspase-1**
760 **and release of cytokines via and flow cytometry.** (A) Primary neutrophil (PMN) 'Singlet' cell
761 selection (FSC-A vs. FSC-H) and (B) viable 'PMNs' discrimination (FSC-A vs. SSC-A). (C)
762 Representative plot showing unstained and GAS infected neutrophils (Singlets/PMNs) with
763 FLICA (FAM-YVAD-FMK) fluorescence. Gating strategy for LEGENDplex cytometric bead assay
764 where (D) beads A and B were gated (FSC-A vs. SSC-A). Gates for beads A (shown) and B were
765 assessed for (E) PE vs. APC fluorescence where cytokines had unique APC fluorescence and
766 expression was measured via PE fluorescence when compared to a standard curve.

767



768 **Supplementary figure 4. Flow cytometric sequential gating strategy for identification of**
769 **murine neutrophils determining caspase-1 activation.** (A) Primary 'Singlets' cell selection
770 (FSC-A vs. FSC-H) and (B) 'Cells' discrimination (FSC-A vs. SSC-A) of murine blood and lavage
771 fluid. 'Cells' were further gated upon (C) CD45-BV421 expression and (D) CD11b-PE/Cy5
772 expression. Neutrophils (PMNS) were defined as (E) Ly-6G-PE^{+} cells from the CD45^{+}/CD11b^{+}
773 population. Neutrophils were assessed for (F) caspase-1 activation using FLICA 660 (660-YVAD-
774 FMK). (G) Percentage of neutrophils (CD11b^{+}/Ly6G^{+}) of CD45^{+} leukocytes in the blood where
775 each data point represents the result from a single mouse where n=4. Results are the means±SD.
776 *p<0.05 and **p<0.01 where significance is depicted from control or as indicated by a line.