Complement C5a impairs phagosomal maturation in the neutrophil through phosphoproteomic remodelling.

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Abstract

Critical illness is accompanied by the release of large amounts of the anaphylotoxin, C5a. C5a suppresses antimicrobial functions of neutrophils which is associated with adverse outcomes. The signalling pathways that mediate C5a-induced neutrophil dysfunction are incompletely understood. Healthy donor neutrophils exposed to purified C5a demonstrated a prolonged defect (7 hours) in phagocytosis of *Staphylococcus aureus*. Phosphoproteomic profiling of 2712 phosphoproteins identified persistent C5a signalling and selective impairment of phagosomal protein phosphorylation on exposure to *S. aureus*. Notable proteins included early endosomal marker ZFYVE16 and V-ATPase proton channel component ATPV1G1. A novel assay of phagosomal acidification demonstrated C5a-induced impairment of phagosomal acidification which was recapitulated in neutrophils from critically ill patients. Examination of the C5a-impaired protein phosphorylation indicated a role for the phosphatidylinositol 3-kinase VPS34 in phagosomal maturation. Inhibition of VPS34 impaired neutrophil phagosomal acidification and killing of *S. aureus*. This study provides a phosphoproteomic assessment of human neutrophil signalling in response to *S. aureus* and its disruption by C5a, identifying a defect in phagosomal maturation and new mechanisms of immune failure in critical illness.

Key words: Neutrophils, Complement system proteins, Staphylococcus aureus, Critical care, Phosphoproteome

Introduction

Critically ill patients who require exogenous organ support as a result of severe physiologic insult, are at high risk of secondary infections (Vincent *et al*, 2009). Critical illness may arise from a variety of sterile or infectious insults. However, despite its varied aetiology, critical illness is often accompanied by stereotyped immune dysregulation, with features of both hyperinflammation and immune-mediated organ damage, as well as impairment of anti-microbial functions (Meakins *et al*, 1977; Conway Morris *et al*, 2013; Hotchkiss *et al*, 2013a). Critical illness is estimated to cause 58 million adult deaths per year globally, (Adhikari *et al*, 2010) and whilst much of the mortality is attributable to the underlying condition, secondary infections make a significant contribution to the eventual outcome (Adhikari *et al*, 2010; Scicluna *et al*, 2015; van Vught *et al*, 2016; Vincent *et al*, 2006).

Impairment of immune cell function predicts secondary infection, (Hotchkiss *et al*, 2013b; Conway Morris *et al*, 2013; Demaret *et al*, 2015; Landelle *et al*, 2010) and failure of neutrophil phagocytosis and bacterial killing has been demonstrated to be one of the strongest predictors of these infections. A key driver of the functional impairment of neutrophils is the anaphylatoxin C5a (Conway Morris *et al*, 2009, 2011; Huber-Lang *et al*, 2002b). However, there remain no efficacious treatments for critical-illness induced immune dysfunction, in part because the mechanisms that underpin C5a-induced dysfunction are incompletely understood.

A wealth of data have demonstrated the importance of C5a in driving classical inflammatory events in neutrophils, including chemotaxis (Ward & Newman, 1969; Ehrengruber *et al*, 1994), generation of reactive oxygen species (ROS) (Suire *et al*, 2006; Mazaki *et al*, 2006; Huber-Lang *et al*, 2002b), phagocytosis (Mollnes *et al*, 2002; Brekke *et al*, 2007), degranulation (Denk *et al*, 2017a, 2017b), and delayed apoptosis (Lee *et al*, 2008; Perianayagam *et al*, 2002, 2004). In critical illness, dysregulated activation of the complement and coagulation cascades occurs, leading to exposure of neutrophils to high concentrations of C5a (Hotchkiss *et al*, 2013a; Venet & Monneret, 2018; Lord *et al*, 2014; Conway-Morris *et al*, 2018; Ward, 2004). In these circumstances, we and others have shown that C5a reduces neutrophil phagocytosis and ROS production in both rodent models and critically ill patients (Conway Morris *et al*, 2009, 2011; Czermak *et al*, 1999; Huber-Lang *et al*, 2002b). Further, C5a exposure has been shown to be associated with nosocomial infection, organ failure, and increased mortality in critically ill patients (Conway Morris *et al*, 2011, 2009, 2013; Czermak *et al*, 1999; Huber-Lang *et al*, 2001, 2002a).

Whilst several signals mediating aspects of C5a-induced neutrophil dysfunction have been established (Conway Morris *et al*, 2011; Denk *et al*, 2017a; Huber-Lang *et al*, 2002b), a global picture of

signalling in neutrophils encountering common pathogens and how this process is perturbed by C5a does not exist. Such studies are challenging in neutrophils owing to their high degradative enzyme content and short *in-vitro* survival times (Luerman *et al*, 2010).

This study aimed to characterise the neutrophil phosphoprotein response to a common nosocomial pathogen, *Staphylococcus aureus*, and investigate how this is perturbed by prior exposure to C5a. Our differential phosphoprotein analysis implicated C5a in altered phagosomal maturation, findings that we confirmed with functional neutrophil assays in C5a-treated healthy donor cells and those from critically ill patients. The phosphoprotein response to *S. aureus* implicated the involvement of the phosphatidylinositol 3-kinase VPS34, hence we continued examined the effects of this enzyme on phagosomal maturation.

Results

C5a induces a prolonged defect in neutrophil phagocytosis of bacteria

C5a induces a defect in phagocytosis of the clinically relevant bacterial species *S. aureus* (Figure 1A) and *E. coli* (1B). Pulse exposure of neutrophils to C5a revealed a persistent defect in phagocytosis lasting at least seven hours (1C), with short pulses inducing a significant defect. These effects were not explained by the loss of cell viability (1D). A similar prolonged defect was identified in the whole blood assay (1E), representing continuous exposure of neutrophils to C5a (which cannot be washed off in this assay). The ability of C5a to inhibit phagocytosis was dependent on the temporal relationship between C5a and *S. aureus* exposure. Only pre-exposure to C5a induced the defect in phagocytosis, whereas co-exposure or the addition of C5a 30 minutes after *S. aureus* addition failed to induce a defect (1F).

To explore the potential mechanisms whereby pre-exposure to *S. aureus* prevents the inhibitory effect of C5a, we examined whether this could be due to reduced C5aR1 expression. Although we could demonstrate a reduction in C5aR1 following *S. aureus* exposure (Figure S1A), this was modest and similar to the reductions induced by other inflammatory mediators including lipopolysaccharide (LPS) and leukotriene A (LTA), neither of which ameliorated the subsequent suppressive effect of C5a (Figure S1B). Further, C5a and not LPS, LTA, granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumour necrosis factor (TNF) reduced neutrophil phagocytosis (Figure S1C). To confirm the functional relevance of C5a-impaired phagocytosis, we demonstrated that C5a pre-treatment reduced bacterial killing of *S. aureus* (Figure S1D).

S. aureus and C5a induce widespread changes in the neutrophil phosphoproteome

Although key signalling 'nodes' have been identified in neutrophils following C5a exposure (Conway Morris *et al*, 2009, 2011), no map of global signalling networks has been produced. Given the rapidity of the C5a-induced phagocytic impairment demonstrated above, and the known signalling kinetics of G-protein coupled receptors (GPCRs) (Lohse *et al*, 2008), we examined post-translational modification by phosphorylation (i.e. a phosphoproteomic approach).

In total, 4859 proteins and 2712 phosphoproteins were identified in peripheral blood neutrophils obtained from four healthy volunteers. C5a-induced suppression of phagocytosis in these donors was confirmed (Figure S2A), and technical reproducibility was high (Figures S2B-E) with the magnitude of phosphorylation changes within the previously reported range (Papachristou *et al*, 2018). Changes in the human proteome were minimal (2 % of total proteome with *S. aureus* treatment) whereas phosphoprotein expression varied markedly (31.6 % of total phosphoproteome with *S. aureus*

treatment, Table S1). Figure 2 shows the top 2.5% most variable phosphoproteins with protein identification, whereas the top 25 % are shown in Figure S3 to demonstrate wider changes within the phosphoproteome. The phosphoproteomic and proteomic datasets are publicly available in the PRIDE database (data available to reviewers, will be made public on acceptance of manuscript).

C5a exposure induces persistent alteration in phosphoproteins across several pathways.

Figure 3A shows a volcano plot comparing neutrophils treated with C5a versus vehicle control. 119 proteins were significantly differentially phosphorylated at 1 hour, indicating persistent signalling, consistent with the prolonged inhibition of phagocytosis seen in Figure 1. Notably, C5aR1 remained highly phosphorylated (a modification key to its internalisation) (Braun *et al*, 2003) and this change has been used to identify C5a-exposed, dysfunctional neutrophils (Conway Morris *et al*, 2009, 2011, 2013, 2018; Schmidt *et al*, 2015; Unnewehr *et al*, 2013). Pathway enrichment using Metascape (Zhou *et al*, 2019) indicated involvement of pathways including membrane trafficking, regulated exocytosis (degranulation), and phosphatidylinositol-3,4,5-trisphosphate (PIP3) signalling which persist one hour after stimulation with C5a (Figure 3B).

***S. aureus* induces a marked alteration in the phosphoproteome which is significantly impacted by C5a exposure**

Exposure of neutrophils to *S. aureus* induced a marked alteration in the phosphoproteome (Figure 4A); 863 proteins (31% of the phosphoproteome) significantly alter their phosphor-status. Pathway enrichment indicated the involvement of multiple pathways, notably Rho-GTPase signalling, endosomal transport, degranulation, and actin cytoskeleton organisation (Figure 4B, with extended heatmap showing top 100 pathways shown in Figure S4).

C5a exposure prior to *S. aureus* reduced the phosphoprotein response to the bacterium considerably (Figure 4C). However, comparing C5a and control treated cells exposed to *S. aureus*, 19 proteins were identified, suggesting selective pathway modulation (Figure 4D). When mapped to known pathways using Metascape (Zhou *et al*, 2019) and manually annotated from the Uniprot database (The Uniprot Consortium, 2019), a pattern of reduced phosphorylation of phagosomal maturation proteins (Tables
Table) and pathways (Figure 4E) emerged. Notably, early endosomal marker ZFYVE16 and its interactor TOM1 had impaired phosphorylation following C5a exposure, as did V-type ATPase subunit G1 (which is critical for phagosomal acidification). ZFYVE16 requires phosphatidylinositol-3-phosphate (PI3P) for recruitment to the phagosome (Sorkin & Von Zastrow, 2009). Another prominent PI3P-responsive protein noted was Ras-related protein 7a (RAB7A), although this protein

was not differentially phosphorylated between the C5a/*S. aureus* and vehicle control/*S. aureus* conditions. Figure S5 shows individual donor data for these key proteins.

Our dataset suggests that C5a exposure that precedes pathogen encounter prevents effective signalling through the phagosomal maturation pathways, and links intracellular signalling to the prolonged functional impairment noted in this context. The other major cluster of differentially phosphorylated proteins were nuclear and nuclear membrane proteins, many of which are involved in mitosis and nuclear envelope integrity.

C5a induces an impairment in phagosomal acidification, distinct from the impairment in ingestion.

The phosphoproteomic signature of altered phagosomal maturation following C5a exposure, and the involvement of V-ATPase suggested that C5a had effects beyond impaired ingestion of bacteria. To disentangle the effects of phagocytic ingestion and phagolysosomal acidification, *S. aureus* bioparticles co-labelled with the pH-insensitive dye AF488 and pHrodo™ red were used. Neutrophils ingested particles, and then subsequently acidified the phagosome, a process which could be ablated by the addition of the V-ATPase inhibitor bafilomycin (Bowman *et al*, 1988) (Figures 5A and B). C5a pre-treatment increased the proportion of neutrophils that failed to ingest particles (Figure 5C) and increased the population that ingested particles but failed to acidify the phagosome (Figure 5D). Recent reports suggest that C5a induces Na^+/H^+ exchanger-1 (NHE-1)-mediated cytoplasmic alkalinisation (Denk *et al*, 2017a). An NHE-1 inhibitor did not alter the C5a-mediated effect on phagosomal acidification (Figure 5E), suggesting that the pathways mediating these two effects of C5a on neutrophils are distinct. Furthermore, we confirmed previous work (Huber-Lang *et al*, 2002b) showing C5a impaired ROS production (Figure S6), which in combination with the current findings, suggests C5a induces a generalised failure of phagosomal maturation in addition to its effect on phagocytic ingestion.

VPS34 inhibition impairs phagosomal acidification

The differential phosphoprotein analysis (Tables
Table) and phagosomal acidification assays (Figure 5) demonstrated impaired phagosomal maturation after exposure to C5a. As noted, several of the phosphoproteins that were differentially phosphorylated are known interactors with PI3P. The phosphatidylinositol 3-kinase VPS34 is the dominant source of PI3P in mammalian cells (Devereaux *et al*, 2013). Although VPS34 itself was detected, its phosphorylation status was not significantly altered. However, the finding that C5a altered the phosphorylation status of PI3P-responsive proteins led us to explore the role of VPS34 in

153 phagosomal acidification. We used the selective inhibitor, VPS34IN1 (Bago *et al*, 2014) to examine
154 the role of this enzyme in phagosomal acidification, and how this related to the defect induced by
155 C5a. VPS34IN1 did not alter the percentage of neutrophils that underwent phagocytosis (Figure 6A
156 and time-course in E) but did lead to a reduction in the overall number of particles ingested (Figure
157 6B) and a more marked reduction in pHrodo signal (Figure 6C and time course in F), indicating
158 VPS34IN1 impairs phagosomal acidification. VPS34 inhibition also led to an impairment in the
159 killing of *S. aureus* (Figure 6D), similar to that observed with C5a (Figure S1D) without a significant
160 reduction in phagosomal ROS production (Figure S7).
161
162
163 **Neutrophils from critically ill patients exhibit defective phagosomal acidification**

164 To establish the relevance of our findings to the clinical setting, we used our assay of phagosomal
165 acidification to interrogate neutrophils obtained from critically ill patients and healthy volunteers. We
166 assessed neutrophil function in critically ill patients, defining neutrophil dysfunction as phagocytosis
167 of <50% in our previously established zymosan assay (Figure 7A), a threshold associated with a
168 markedly increased risk of nosocomial infection (Pinder *et al*, 2018; Conway Morris *et al*, 2009,
169 2011). Using our phagosomal acidification assay, we then compared patients with dysfunctional
170 neutrophils to critically ill patients with functional neutrophils and healthy controls. Dysfunctional
171 neutrophils exhibited a failure of phagosomal acidification (Figure 7B) that was not seen in patients
172 with functional neutrophils. Furthermore, we observed a correlation between C5aR1 expression
173 (decreased after C5a exposure) and phagocytosis (Figure 7C) and an inverse correlation between
174 C5aR1 expression and phagosomal acidification (Figure 7D), though the latter correlation did not
175 reach statistical significance. The patients with dysfunctional and functional neutrophils could not be
176 readily identified by clinical factors such as severity of illness or precipitating insult (Table S2). These
177 data provide evidence of dysfunctional phagosomal acidification in critically ill patients and imply a
178 role for C5a in driving this dysfunction.

179 Discussion

180 Our data demonstrate that C5a induces both a prolonged defect in phagocytosis of relevant pathogens
181 (*S. aureus* and *E.coli*), and persistent signalling across multiple pathways for some hours after the
182 well characterised initial signalling events such as ionised calcium flux (Blackwood *et al*, 1996) and
183 PIP3 generation (Houslay *et al*, 2016). This finding supports the proposal that persistent C5a-induced
184 signalling may mediate the neutrophil dysfunction observed in critically ill patients (Conway Morris
185 *et al*, 2009, 2011).
186

187 To our knowledge, the data presented here (Figures 3-5) represent the deepest sequencing of the
188 human neutrophil proteome and phosphoproteome (Muschter *et al*, 2015; Tak *et al*, 2017; McLeish *et*
189 *al*, 2013). These data provide a phosphoproteomic assessment of the human neutrophil response to *S.*
190 *aureus* and C5a. Unlike transcriptomic data (Juss *et al*, 2016; Rorvig *et al*, 2013; Kobayashi *et al*,
191 2002), phosphoproteomics provides a direct assessment of mediators that are likely to have functional
192 implications, especially in short-lived cells such as neutrophils (Luerman *et al*, 2010; Fessler *et al*,
193 2002) and early pathogen exposure timepoints, as examined in this study.
194
195 The marked changes observed in phosphoproteins in response to *S. aureus* are perhaps unsurprising,
196 as the response to and clearance of bacteria are primary functions of neutrophils. Many of the
197 pathways identified (Figures 4 and S4) are consistent with established literature on neutrophil
198 responses to *S. aureus*, and indeed other bacteria, including activation of PI3K (Li *et al*, 2016), toll-
199 like receptor signalling (Jann *et al*, 2011) and neutrophil degranulation (McGovern *et al*, 2011).
200
201 The enrichment of PI3K and Rho GTPase signalling on C5a stimulation are in keeping with our
202 previous identification of key roles for these molecules in C5a-mediated functional deficits in
203 neutrophils (Conway Morris *et al*, 2009, 2011; Scott *et al*, 2015). The marked suppression of the
204 phosphorylation response to *S.aureus* induced by C5a pre-treatment is not simply a response to
205 reduced particle ingestion. Fifteen minutes after pathogen contact there were limited differences in the
206 ingestion rates between C5a and control treatments, and these became more marked over time (Figure
207 1). Furthermore, the differential analysis of C5a/*S. aureus* versus vehicle control/*S. aureus* conditions
208 identified defects in specific signalling pathways, most notably those involving endosomal trafficking.
209 This led us to examine the process of phagosomal maturation, and to identification of a C5a-induced
210 failure of phagosomal acidification (Figure 5) with similar findings in critically ill patients (Figure 7).
211 Failure of phagosomal maturation and intracellular killing has been described in primary immune
212 deficiency (Buvelot *et al*, 2017), but has not previously been described as part of the immuno-paresis
213 of critical illness. Impaired phosphorylation in pathways involving nuclear envelope breakdown and
214 nuclear pore disassembly by C5a was unanticipated. The functional relevance of these changes
215 remains unclear, though they may be early processes in the formation of non-lethal DNA-containing
216 neutrophil extracellular traps (NETs) (Pilsczek *et al*, 2010).
217
218 Important signalling proteins involved in the process of phagosomal maturation (such as RAB7A,
219 TOM1 and ZFYVE16) can be recruited to the phagosomal membrane by PI3P produced
220 predominantly by VPS34 (Botelho *et al*, 2000; Levin *et al*, 2016; Sorkin & Von Zastrow, 2009). Both
221 ZFYVE16 and TOM1 phosphorylation were impaired by C5a exposure. We investigated the role of
222 VPS34 as a mediator of neutrophil bactericidal function, and found that selective VPS34 inhibition
223 produced a similar impairment in phagosomal acidification to that observed with C5a (Figure 6). The

224 finding that a similar defect could be induced by inhibiting VPS34, the dominant source of PI3P in
225 neutrophils (Devereaux *et al*, 2013), adds further validation to the pathway signature identified in the
226 phosphoproteomic profile.
227
228 Ellson and colleagues (Ellson *et al*, 2001) demonstrated that PI3P plays an important role in targeting
229 neutrophil oxidase components to phagosomal membranes and its importance in phagosomal
230 maturation has also been identified in *Dictyostelium discoideum* (Buckley *et al*, 2019), murine
231 macrophages, and macrophage-like cell lines (Naufer *et al*, 2018). However, the role of VPS34 in
232 human neutrophils has previously been inferred indirectly (Anderson *et al*, 2008), owing to prior lack
233 of selective inhibitors and the difficulties of genetically manipulating human neutrophils. Anderson
234 and colleagues (Anderson *et al*, 2008) demonstrated a role for VPS34 in NADPH oxidase-mediated
235 reactive oxygen species generation in neutrophils. We found a non-significant reduction in ROS
236 production (Figure S7) that was much less marked than the effect on phagosomal acidification. The
237 reasons for these divergent findings are uncertain, though may include differences in ROS
238 measurement assays, our use of a selective VPS34 inhibitor, and differences between primary human
239 neutrophils and cell lines. The mechanism by which VPS34 inhibition impairs killing of *S. aureus*
240 requires further investigation, as phagosomal acidification is not thought to be critical to this process
241 (Lacoma *et al*, 2017) and it is likely that the enzyme inhibition leads to further defects in phagosomal
242 maturation. It is intriguing to note that whilst VPS34 inhibition does not reduce the percentage of cells
243 that undergo phagocytosis (Figure 6A), consistent with previous work (Anderson *et al*, 2008), it does
244 reduce the number of particles ingested (Figure 6B). This suggests a hitherto undescribed relationship
245 between phagosomal maturation and the capacity of cells to ingest particles.
246
247 Our data also demonstrate that the timing of C5a exposure (before, alongside, or after pathogen
248 encounter) has an important effect on neutrophil function. Only pre-exposure to C5a impaired
249 subsequent neutrophil phagocytosis (Figure 2). Reduced C5aR1 availability for ligation by C5a is
250 unlikely to explain this observation, as C5aR1 downregulation is induced by multiple agents that do
251 not have the same effect on phagocytosis (Figure S1). Given the marked phosphoproteomic response
252 to *S. aureus* and its distinction from the response to C5a (Figures 3 and 4), a potential explanation is
253 that signalling induced by *S. aureus* simply overwhelms C5a-induced phosphorylation changes unless
254 they were established prior to *S. aureus* exposure, though this hypothesis requires further
255 experimental validation.
256
257 This study was conducted entirely in primary human neutrophils, using C5a, an established, clinically
258 relevant modulator of neutrophil function that has been linked to a range of adverse outcomes in
259 critically ill patients. The use of clinically relevant pathogens, and the development of a whole-blood
260 bacteraemia model, increases the relevance of our study to the *in-vivo* situation. Impaired ingestion of

261 zymosan by patient neutrophils has been associated with adverse outcomes including development of
262 subsequent nosocomial infection (Conway Morris *et al*, 2011). The finding that patients with such
263 impairment also manifest impaired phagosomal acidification that correlates with markers of C5a
264 exposure (Figure 7) suggests that the identified mechanisms may be clinically relevant.
265
266 Several potential limitations should be highlighted. The phosphoproteomic response to *S. aureus* was
267 evoked with heat-killed bacterial particles, conjugated with fluorescent dyes, and these may not fully
268 reflect the response to live bacteria, although they do allow parallel functional assessment and
269 standardisation of the stimulus between donors and across research sites. Although whole blood is a
270 more physiologically relevant than cell-culture media, it remains an abstraction from the situation *in-*
271 *vivo*, as it must be anticoagulated and does not involve normal flow or interaction with a vascular
272 endothelium. Furthermore, the model may not reflect the function of neutrophils that have migrated
273 into tissues, where most bacterial infections occur. Technical limitations currently prevent efficient
274 phosphoproteomic assessment of cells from whole blood, and therefore isolated cells with the inherent
275 *in-vitro* artefacts must be used.
276
277 In conclusion, we have demonstrated the role of C5a in mediating neutrophil dysfunction in the
278 clinically relevant setting of *S. aureus* and *E. coli* bacteraemia, and demonstrated that the effects of
279 C5a can persist for many hours. We also describe the neutrophil phosphoproteomic response *to S.*
280 *aureus*, and to prolonged exposure to C5a. This approach identified a defective phagosomal
281 maturation signature induced by C5a, likely involving modulation of Class III PI3K-dependent
282 pathways. Further, we have shown the functional manifestation of this phosphorylation signature in a
283 model of bacteraemia. Finally, the clinical relevance of this failure of phagosomal acidification was
284 observed in critically ill patients. A deeper understanding of the biology of neutrophil dysfunction in
285 critical illness is key to developing effective treatments for a phenomenon associated with multiple
286 adverse clinical outcomes.

Materials and methods

Donors

Ethical permission for obtaining peripheral venous blood from healthy volunteers was provided by the Cambridge Local Research Ethics Committee (REC reference 06/Q0108/281) and all donors provided written, informed consent. Critically ill patient blood samples were obtained under an approval granted by the North East-Newcastle & North Tyneside 2 Research Ethics Committee (REC reference: 18/NE/0036). Inclusion and exclusion criteria are detailed in the supplemental methods. Assent was provided by a personal or nominated consultee.

Further details of methods and reagents described below are available in the supplementary materials.

Neutrophil isolation

Neutrophils were isolated from citrated peripheral venous blood by using a modification of the discontinuous plasma-Percoll density gradient centrifugation technique initially described by Böyum in 1968.(Boyum, 1968)

Phagocytosis of pHrodo™ S. aureus and E. coli Bioparticles by purified neutrophils

Purified human neutrophils, suspended in Iscoves Modified Dulbecco's Medium (IMDM) with 1 % autologous serum at a concentration of 5 x 10^6/mL, were incubated in microcentrifuge tubes with purified human C5a or vehicle control. pHrodo-conjugated *S. aureus* or *E. coli* bioparticles were opsonised, in 50 % autologous serum for 30 min prior to be being added to the suspended cells. Analysis was by flow cytometry (Attune NxT, Thermofisher)

No-wash, no-lyse whole blood assay of neutrophil phagocytosis and ROS production

Blood, collected into argatroban 150 µg/mL, was treated with inhibitors or priming agents as indicated in the respective figure legends, before being exposed to *S. aureus* pHrodo™/dihydrorhodamine (DHR) or *E.coli* pHrodo™. Aliquots were stained on ice with anti-CD16 antibody, diluted and analysed by flow cytometry (Attune NxT).

In variations on this assay, *S. aureus* particles labelled with the pH-insensitive dye AlexaFluor (AF)488 or dual labelled with AF488 and pHrodo red were used. pHrodo red conjugation of AF488 *S. aureus* was performed in-house using the pHrodo particle labelling kit (Thermofisher). Fluorescence of extracellular particles was quenched with trypan blue (0.1mg/mlL).

Patient samples were analysed in a different laboratory that did not have access to an Attune Nxt flow cytometer, to fit with established workflows in this laboratory red cells were lysed using Pharmlyse (BD Bioscience, Wokingham, UK) followed by washing twice using a Facswash Assistant (BD Bioscience) prior to undertaking flow cytometry (Fortessa, BD Bioscience).

Bacterial killing assay – whole blood

Methicillin-sensitive *S. aureus* (MSSA) bacteria (strain ASASM6, kind gift from Prof Gordon Dougan, University of Cambridge) were grown to early log-phase. Blood was collected into argatroban and incubated with bacteria for 1 hour. Human cells were lysed by addition of pH 11 distilled water for 3 minutes before plating of serial dilutions on Colombia blood agar.

Preparation of whole human neutrophil lysates for phosphoproteomics

Neutrophils were isolated from whole blood as detailed above, and resuspended in RPMI 1640 media containing 10 mM HEPES with 1 % autologous serum (AS) at a concentration of $1x10^7$ cells/mL.

Proteomic and phosphoproteomic studies

Triplicates of $1x10^7$ neutrophils were treated with vehicle control or C5a (100 nM, 60 minutes) at 37 °C before addition of pHrodo™ *S. aureus* (15 µg/mL). Phagocytosis was allowed to occur for 15 minutes. Aliquots were withdrawn from each triplicate and pooled at the indicated timepoints. Cells were centrifuged at 400 g for 5 min at 4 °C, supernatants aspirated, and cell pellets snap frozen in liquid nitrogen. Cells were lysed by the addition of 0.5 % sodium dodecyl sulphate (SDS)/0.1 M triethylammonium bicarbonate (TEAB) buffer and sonication, before undergoing centrifugation, trypsin digestion, tandem mass tag labelling, fractionation, phosphopeptide enrichment, and liquid chromatography and tandem mass spectrometry (LC-MS/MS) analysis. The experimental schematic can be seen in Supplemental figure S8.

Statistical analysis of wet laboratory data

Data are presented as individual data points with summary statistics (median and interquartile range (IQR) or mean and standard deviation (SD) according to whether data are normally distributed. Parametric or non-parametric statistical tests were applied as appropriate after data were tested for normality using the D'Agostino-Pearson test. Tests used for comparisons are indicated in figure legends. Two-tailed P values were computed, $P < 0.05$ was considered statistically significant. Non-significant differences have not been indicated in figures for clarity. Statistical analyses were undertaken using GraphPad Prism v8.0 (GraphPad Software; San Diego; California).

Statistical analysis of phosphoproteomics data

Spectral .raw files from data dependent acquisition were processed with the SequestHT search engine on Thermo Scientific Proteome Discoverer™ 2.1 software. Data were searched against both human and *S. aureus* UniProt reviewed databases at a 1 % spectrum level false discovery rate (FDR) criteria using Percolator (University of Washington). MS1 mass tolerance was constrained to 20 ppm, and the fragment ion mass tolerance was set to 0.5 Da. TMT tags on lysine residues and peptide N termini (+229.163 Da) and methylthio (+45.988 Da) of cysteine residues (+45.021 Da) were set as static modifications, while oxidation of methionine residues (+15.995 Da) and deamidation (+0.984 Da) of asparagine and glutamine residues were set as variable modifications. For TMT-based reporter ion quantitation, we extracted the signal-to-noise (S:N) ratio for each TMT channel. Parsimony principle was applied for protein grouping.

Peptide and phosphopeptide intensities were normalised across conditions using median scaling and then summed to generate protein and phosphoprotein intensities. Proteins and phosphoproteins were independently identified and quantified in all samples from all four donors; species not meeting these criteria were excluded from subsequent analysis. Log base 2 fold change (Log2FC) was calculated between conditions of interest, compared across n = 4 donors and tested for statistical significance by limma-based linear models with Bonferroni's correction for multiple testing. Hierarchical clustering using Euclidean distance was undertaken on the entire dataset. Heatmaps and volcano plots were generated as shown in Results. Statistical analyses were performed in RStudio (RStudio Team, 2016) using the qPLEXanalyzer (Papachristou *et al*, 2018) package, and plots were produced using the ggplot2 (Wickham, 2016) package.

Data sharing statement

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol Y *et al*, 2019) partner repository with the dataset identifier PXD017092 and will be made public on acceptance after peer-review

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Authorship contributions (CRediT):

AJTW: Conceptualisation, formal analysis, investigation, methodology, validation, visualisation, writing – original draft, review and editing.
AV: Investigation, methodology, writing - review and editing.
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CZ: Investigation, writing - review and editing.
CGT: Investigation, methodology, writing - review and editing.
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CSDS: Project administration, resources, supervision, writing - review and editing.
AJS: Project administration, resources, supervision, writing - review and editing.
DKM: Project administration, resources, supervision, writing - review and editing.
CS: Methodology, validation, project administration, resources, supervision, writing - review and editing.
ERC: Conceptualisation, formal analysis, funding acquisition, methodology, project administration, resources, supervision, writing - review and editing.
KO: Project administration, resources, supervision, writing - review and editing.
ACM: Conceptualisation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, supervision, validation, writing – original draft, review and editing.

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Figure legends

Figure 1: C5a induces a prolonged defect in neutrophil phagocytosis of bacteria

A and **B**: Isolated neutrophils were pre-treated with 100 nM C5a or vehicle control for 60 min before incubation with *S. aureus* (A) or *E. coli* (B) bioparticles. Data are presented as the median phagocytic index for each condition for n=7 (A) or 6 (B) independent donors, **P* = 0.016 (A) and 0.031 (B) by Wilcoxon's matched-pairs signed rank test.
C: Neutrophils were pulsed with 100 nM C5a or PBS control for the indicated periods of time, followed by 2 washes. *S. aureus* bioparticles were then added and cells were incubated for the indicated time points. Data are presented as the mean and SD of the phagocytic index of C5a-treated cells relative to their paired vehicle control for n=5 independent experiments. $P < 0.0001$ for time and $P = 0.0186$ for treatment by two-way ANOVA. ****P* = 0.0001 *****P* < 0.0001 by Dunnett's multiple comparison test.
D: Data are presented as the mean and SD of the percentage of DRAQ7 positive, dead cells for n=5 independent experiments. $P = 0.378$ for time *and* $P = 0.349$ for treatment by two-way ANOVA.
E: Anticoagulated whole blood was pre-treated with 300 nM C5a or control for the indicated duration before phagocytosis was measured as previously indicated. Data are presented as the mean and SD of the cumulative phagocytic index for 4 independent experiments. $P < 0.0001$ by two-way ANOVA, *****P* < 0.0001, ****P* < 0.001 by Sidak's multiple comparisons test.
F: *S. aureus* particles were incubated with isolated PMNs in the presence of 100 nM C5a or PBS added at the indicated time points, with time 0 representing the time of addition of *S. aureus* bioparticles. Experiments proceeded for the indicated time points and phagocytic index quantified. Data are presented as the mean and SD of the phagocytic index of C5a-treated cells relative to their paired vehicle control for n=5 independent experiments. $P < 0.0001$ for time and $P = 0.0186$ for treatment by two-way ANOVA. *****P* < 0.0001 by Dunnett's multiple comparisons test.

Figure 2: *S. aureus* and C5a induce widespread changes in the neutrophil phosphoproteome

Heatmap of phosphoprotein intensity relative to baseline ($\log_2$ fold change) across the four experimental conditions shows phosphoproteins with variance across conditions in the top 97.5th centile with dendrograms clustered by Euclidean distance. Increased phosphoprotein expression is indicated in red, decreased in blue. Only phosphoproteins detected in all four donor samples were included.

Figure 3: C5a exposure induces persistent alteration in phosphoproteins across several pathways

A: Proteins with adjusted *P*-values < 0.05 are shown in blue and the 20 proteins with the highest absolute $\log_2$ fold change are labelled. *P*-values were computed by limma-based linear models with Bonferroni's correction for multiple testing. **B:** Metascape(Zhou *et al*, 2019) enrichment heatmap showing functional clusters of phosphoproteins affected by C5a treatment.

Figure 4: *S. aureus* induces a marked alteration in the phosphoproteome that is significantly impacted by C5a exposure

A, B, D: Proteins with adjusted *P*-values < 0.05 are shown in blue and the 20 proteins with the highest absolute $\log_2$ fold change are labelled. *P*-values were computed by limma-based linear models with Bonferroni's correction for multiple testing. **C:** Metascape(Zhou *et al*, 2019) enrichment heatmap showing functional clusters of phosphoproteins affected by *S. aureus* exposure. **E:**

Metascape(Zhou *et al*, 2019) bar graph showing top non-redundant functional clusters of phosphoproteins enriched in the vehicle control/*S. aureus* condition versus C5a/*S. aureus* condition.

Figure 5: C5a induces an impairment in phagosomal acidification, distinct from the impairment in ingestion

A: Exemplar flow cytometry plots of whole blood pre-treated with vehicle control or bafilomycin A (60 min; 100nM) prior to exposure to 5 µg/mL co-labelled AF488/pHrodo red *S. aureus* for 120 min. Both phagocytosis (x-axis) and phagosomal pH (y-axis) can be measured simultaneously in the same population of cells. pHrodo™ fluorescence increases with decreasing pH, indicating phagosomal maturity as shown.
B: Conditions as in A. Data are shown as individual data points with mean for n=7 individual donors. $P = 0.016$ by Wilcoxon's test.
C: Whole blood was pre-treated with vehicle control or C5a (300 nM; 60 minutes) prior to exposure to phagocytosis probe for 180 min. Phagocytosis without maturation (i.e. AF488 signal) is shown. Data are shown as mean and SD of n = 5 individual donors. ****$P < 0.0001$ by repeated-measures two-way ANOVA with Bonferroni's multiple comparisons test.
D: Conditions as in C. The percentage of *S. aureus* particle positive (AF488+) cells with low pH (mature) and high pH (immature) phagosomes is shown for control and C5a-treated conditions. Data are shown as mean and SD of n = 5 individual donors. ***$P < 0.001$ by repeated-measures two-way ANOVA with Bonferroni's multiple comparisons test.
E: Whole blood was pre-treated with C5a, NHE-1 inhibitor (5µM), or both, then exposed to maturation probe for 60 min. The percentage of AF488+ cells with high pH (immature) phagolysosomes is shown. Data are shown as individual data points with median from n = 7 individual donors. $P = 0.0080$ by Friedman's test, *$P < 0.05$ for Dunn's test of multiple comparisons, ns = non-significant.

Figure 6: VPS34 inhibition impairs phagosomal acidification

Whole blood was pre-treated with vehicle control or VPS34IN1 (1 µM; 60 min) prior to addition of 5 µg/mL maturation probe (A-D), or live *S. aureus* (E), for 120 minutes prior to analysis.
A: Percentage of neutrophils that have phagocytosed bioparticles. $P = 0.31$ by Wilcoxon's test. n = 6 individual donors.
B: MFI of ingested particles, indicating relative quantity of phagocytosis. $P = 0.03$. by Wilcoxon's test. n=6 individual donors.
C: pHrodo™ Median Fluorescent Intensity (MFI), indicating phagosomal acidification. $P = 0.03$. by Wilcoxon's test. n=6 individual donors.
D: After phagocytosis of live bacteria, human cells were lysed in alkaline dH_2O and surviving bacteria were incubated overnight on blood agar. Bacterial survival was quantified by counting colonies. $P = 0.03$ by paired t-test, n=5 individual donors.
E-F: Whole blood was processed as above with quantification of phagocytosis (E) and acidification (F) at the indicated time points. There was a reduction in phagosomal acidification as shown but no change in percentage of cells that underwent phagocytosis. Data are shown as mean and SD of n=5 individual donors. **$P = 0.0058$ for drug treatment by repeated measures two-way ANOVA with Bonferroni's multiple comparisons test.

Figure 7: Neutrophils from critically ill patients exhibit defective phagosomal acidification

A: Zymosan-based assay demonstrating differentially impaired phagocytosis in critically ill patients. Data are shown as individual patients/controls with median values indicated. n = 6 patients with dysfunctional neutrophils and 5 patients with functional neutrophils respectively. ** P=0.004 by Mann-Whitney U-test.
B: Neutrophil phagosomal acidification was assessed in whole blood from critically ill patients using the maturation probe. Patients were classed as dysfunctional using the assay from A. Data are shown as individual patients/controls with mean from n = 6 patients with dysfunctional neutrophils, 5 patients with functional neutrophils and 10 healthy controls respectively. P = 0.04 by one-way ANOVA. **P < 0.01 by Holm-Sidak's test of multiple comparisons.
C, D: C5aR1 expression was assessed by flow cytometry and correlated (Spearman) with phagocytosis (C) and phagosomal acidification (D) for n = 12 patients. NB: One patient's cells did not adhere to tissue culture plastic for the zymosan assay, thus they could not be assigned to dysfunctional or non-dysfunctional groups shown in A and B. C5aR1 expression and maturation probe data was available to allow inclusion in correlation analyses in C and D, hence the difference in numbers between these figures.

Figures

Figure 1

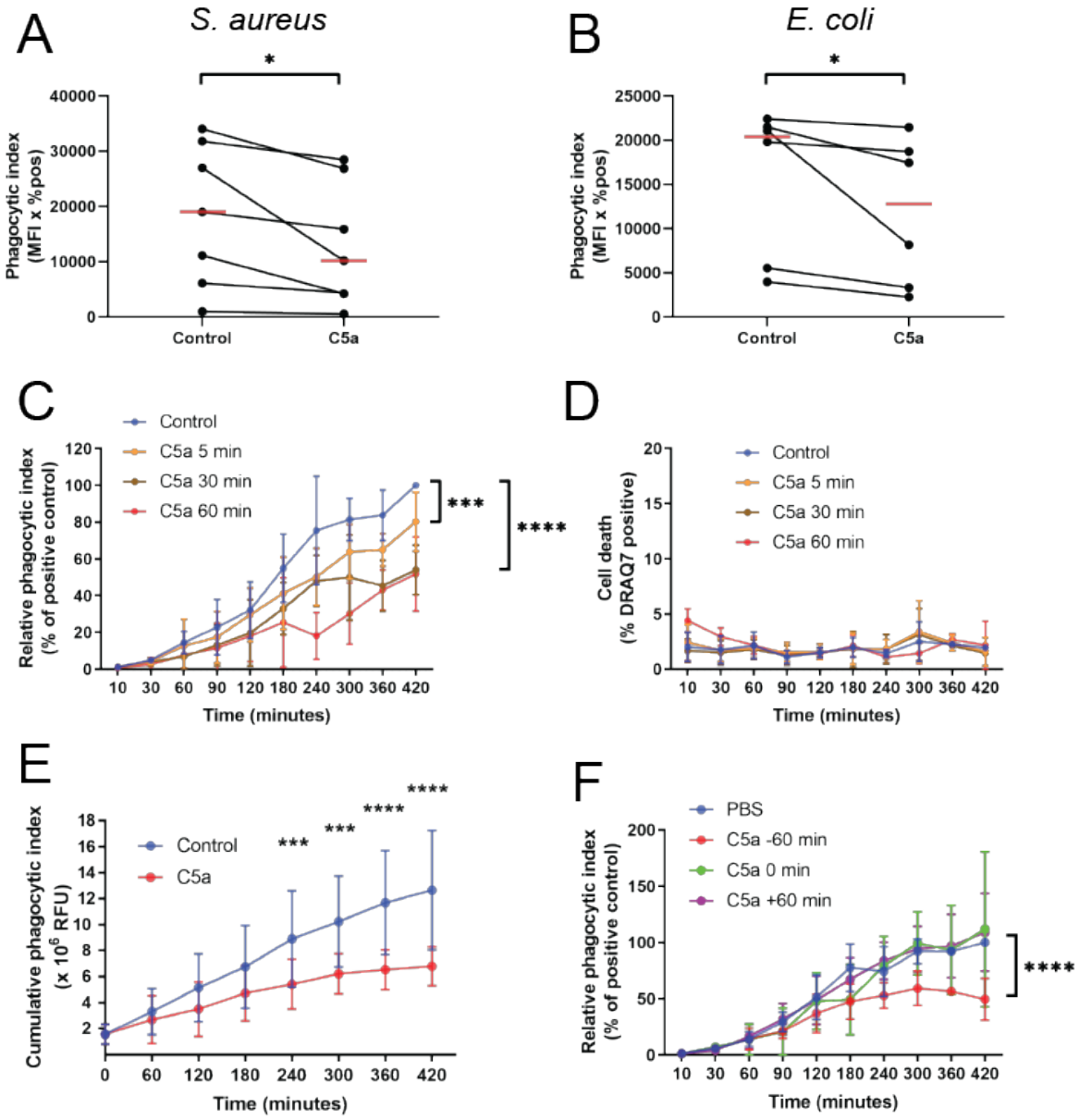


Figure 2

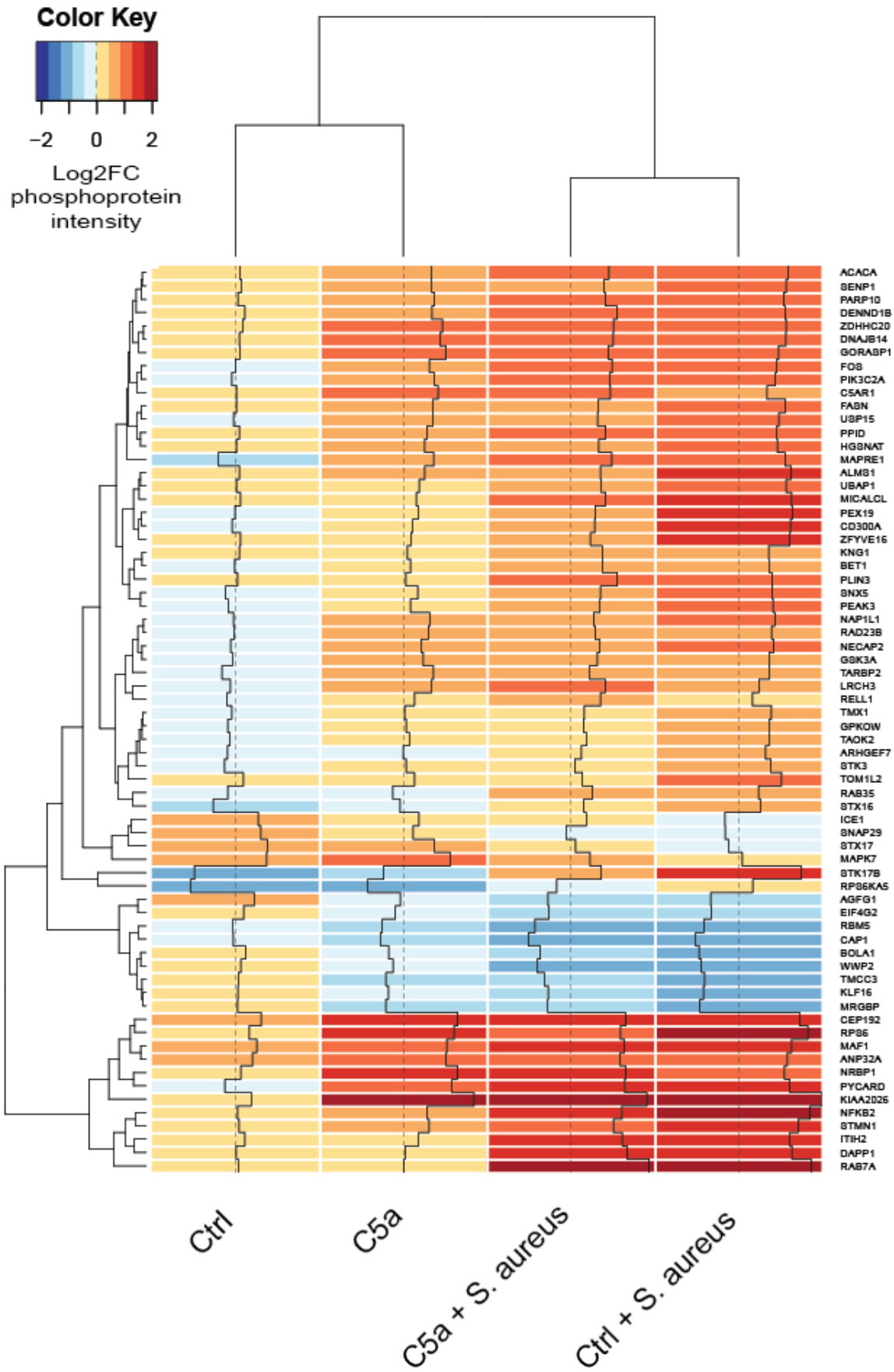

Color Key
−2
0
2
Log2FC phosphoprotein intensity
ACACA
SENP1
PARP10
DENND1B
ZDHHC20
DNAJB14
GORASP1
FOS
PIK3C2A
C5AR1
FASN
USP15
PPID
HGSNAT
MAPRE1
ALMS1
USAP1
MICALCL
PEX19
CD300A
ZFYVE16
KNG1
BET1
PLIN3
SNX5
PEAK3
NAP1L1
RAD23B
NECAP2
GSK3A
TARBP2
LRCH3
RELL1
TMX1
GPKOW
TAOK2
ARHGEF7
STK3
TOM1L2
RAB35
STX16
ICE1
SNAP29
STX17
MAPK7
STK17B
RPS6KA5
AGFG1
EIF4G2
RBM5
CAP1
BOLA1
WWP2
TMCC3
KLF16
MRGBP
CEP192
RPS6
MAF1
ANP32A
NRBP1
PYCARD
KIAA2026
NFKB2
STMN1
ITIH2
DAPP1
RAB7A
Ctrl
C5a
C5a + S. aureus
Ctrl + S. aureus

Figure 3

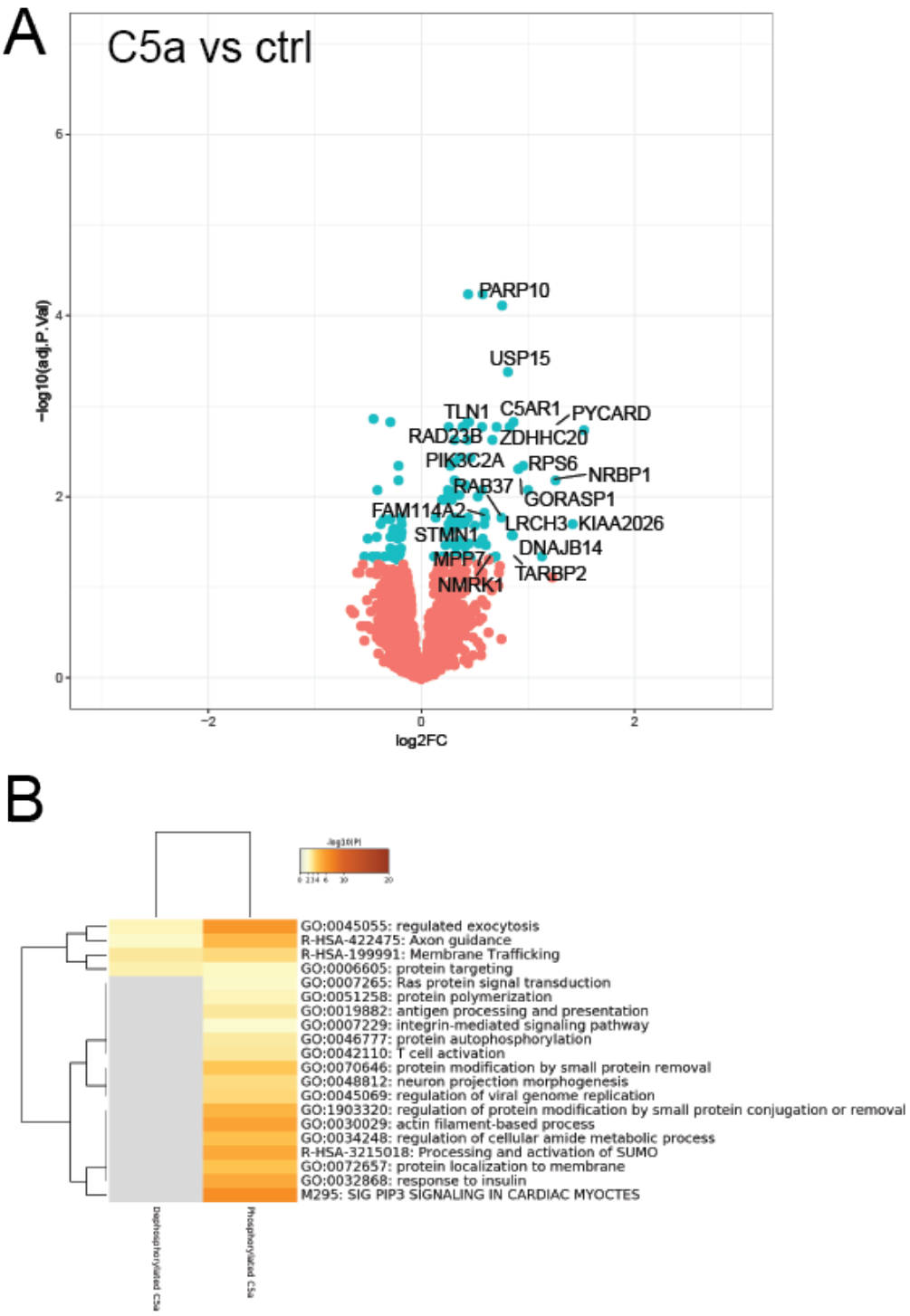

A
C5a vs ctrl
−log10(adj.P.Val)
log2FC
PARP10
USP15
TLN1
C5AR1
PYCARD
RAD23B
ZDHHC20
PIK3C2A
RPS6
NRBP1
RAB37
GORASP1
FAM114A2
LRCH3
KIAA2026
STMN1
DNAJB14
MPP7
TARBP2
NMRK1
B
GO:0045055: regulated exocytosis
R-HSA-422475: Axon guidance
R-HSA-199991: Membrane Trafficking
GO:0006605: protein targeting
GO:0007265: Ras protein signal transduction
GO:0051258: protein polymerization
GO:0019882: antigen processing and presentation
GO:0007229: integrin-mediated signaling pathway
GO:0046777: protein autophosphorylation
GO:0042110: T cell activation
GO:0070646: protein modification by small protein removal
GO:0048812: neuron projection morphogenesis
GO:0045069: regulation of viral genome replication
GO:1903320: regulation of protein modification by small protein conjugation or removal
GO:0030029: actin filament-based process
GO:0034248: regulation of cellular amide metabolic process
R-HSA-3215018: Processing and activation of SUMO
GO:0072657: protein localization to membrane
GO:0032868: response to insulin
M295: SIG PIP3 SIGNALING IN CARDIAC MYOCTES
Dephosphorylated C5a
Phosphorylated C5a

Figure 4

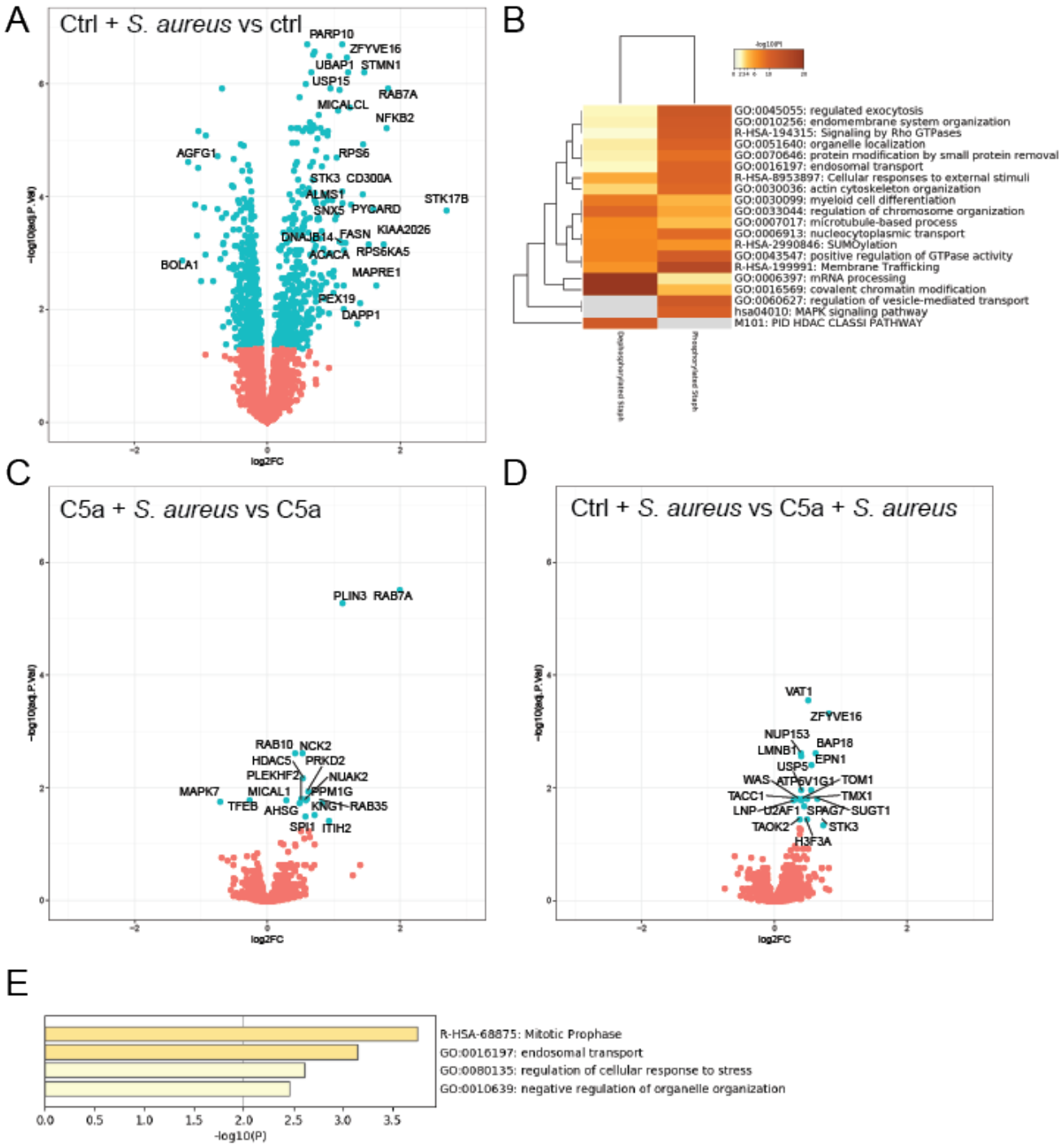

A
Ctrl + S. aureus vs ctrl
PARP10
ZFYVE16
UBAP1
STMN1
USP15
RAB7A
MICALCL
NFKB2
AGFG1
RPS6
STK3
CD300A
ALMS1
STK17B
SNX5
PYCARD
KIAA2026
DNAJB14
FASN
ACACA
RPS6KA5
BOLA1
MAPRE1
PEX19
DAPP1
-log10(adj.P.Val)
log2FC
B
GO:0045055: regulated exocytosis
GO:0010256: endomembrane system organization
R-HSA-194315: Signaling by Rho GTPases
GO:0051640: organelle localization
GO:0070646: protein modification by small protein removal
GO:0016197: endosomal transport
R-HSA-8953897: Cellular responses to external stimuli
GO:0030036: actin cytoskeleton organization
GO:0030099: myeloid cell differentiation
GO:0033044: regulation of chromosome organization
GO:0007017: microtubule-based process
GO:0006913: nucleocytoplasmic transport
R-HSA-2990846: SUMOylation
GO:0043547: positive regulation of GTPase activity
R-HSA-199991: Membrane Trafficking
GO:0006397: mRNA processing
GO:0016569: covalent chromatin modification
GO:0060627: regulation of vesicle-mediated transport
hsa04010: MAPK signaling pathway
M101: PID HDAC CLASSI PATHWAY
C
C5a + S. aureus vs C5a
PLIN3
RAB7A
RAB10
NCK2
HDAC5
PRKD2
PLEKHF2
NUAK2
MAPK7
MICAL1
PPM1G
TFEB
AHSG
KNG1
RAB35
SPI1
ITIH2
D
Ctrl + S. aureus vs C5a + S. aureus
VAT1
ZFYVE16
NUP153
BAP18
LMNB1
EPN1
USP5
WAS
ATP6V1G1
TOM1
TACC1
TMX1
LNP
U2AF1
SPAG7
SUGT1
TAOK2
STK3
H3F3A
E
R-HSA-68875: Mitotic Prophase
GO:0016197: endosomal transport
GO:0080135: regulation of cellular response to stress
GO:0010639: negative regulation of organelle organization
-log10(P)

Figure 5

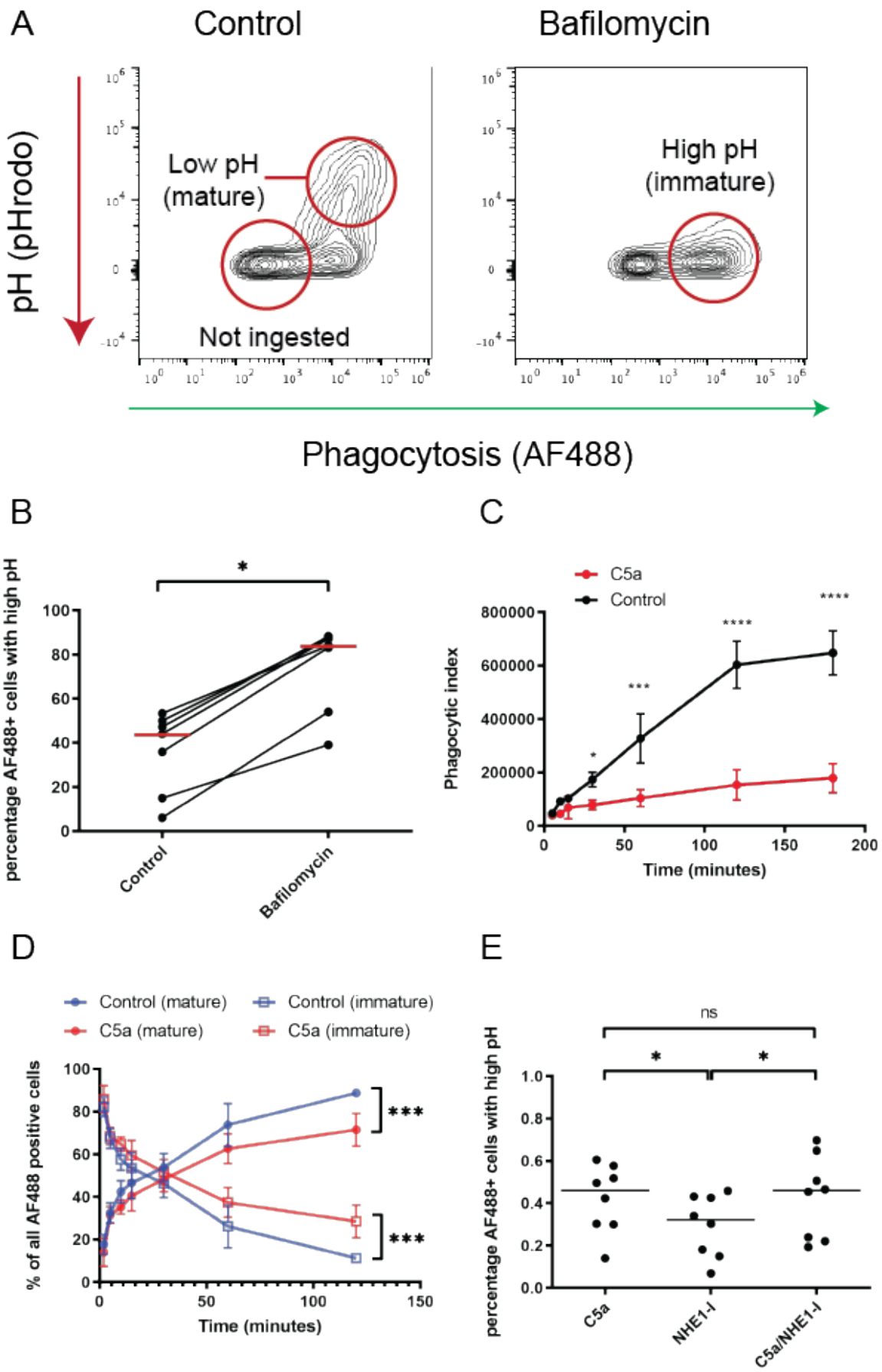

A
Control
Bafilomycin
pH (pHrodo)
Low pH
(mature)
Not ingested
High pH
(immature)
Phagocytosis (AF488)
B
percentage AF488+ cells with high pH
Control
Bafilomycin
C
C5a
Control
Phagocytic index
Time (minutes)
D
Control (mature)
Control (immature)
C5a (mature)
C5a (immature)
% of all AF488 positive cells
Time (minutes)
E
ns
percentage AF488+ cells with high pH
C5a
NHE1-I
C5a/NHE1-I

Figure 6

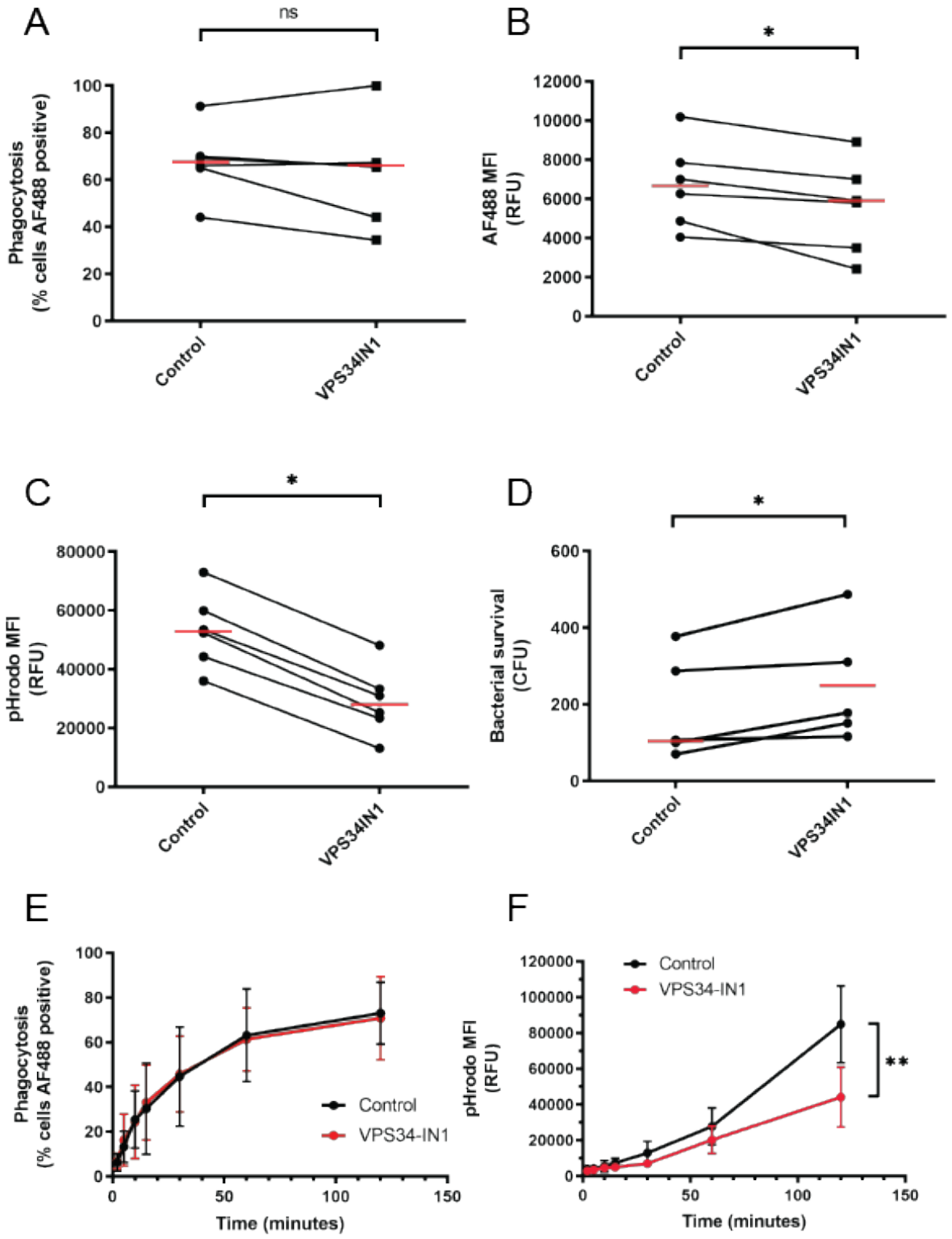
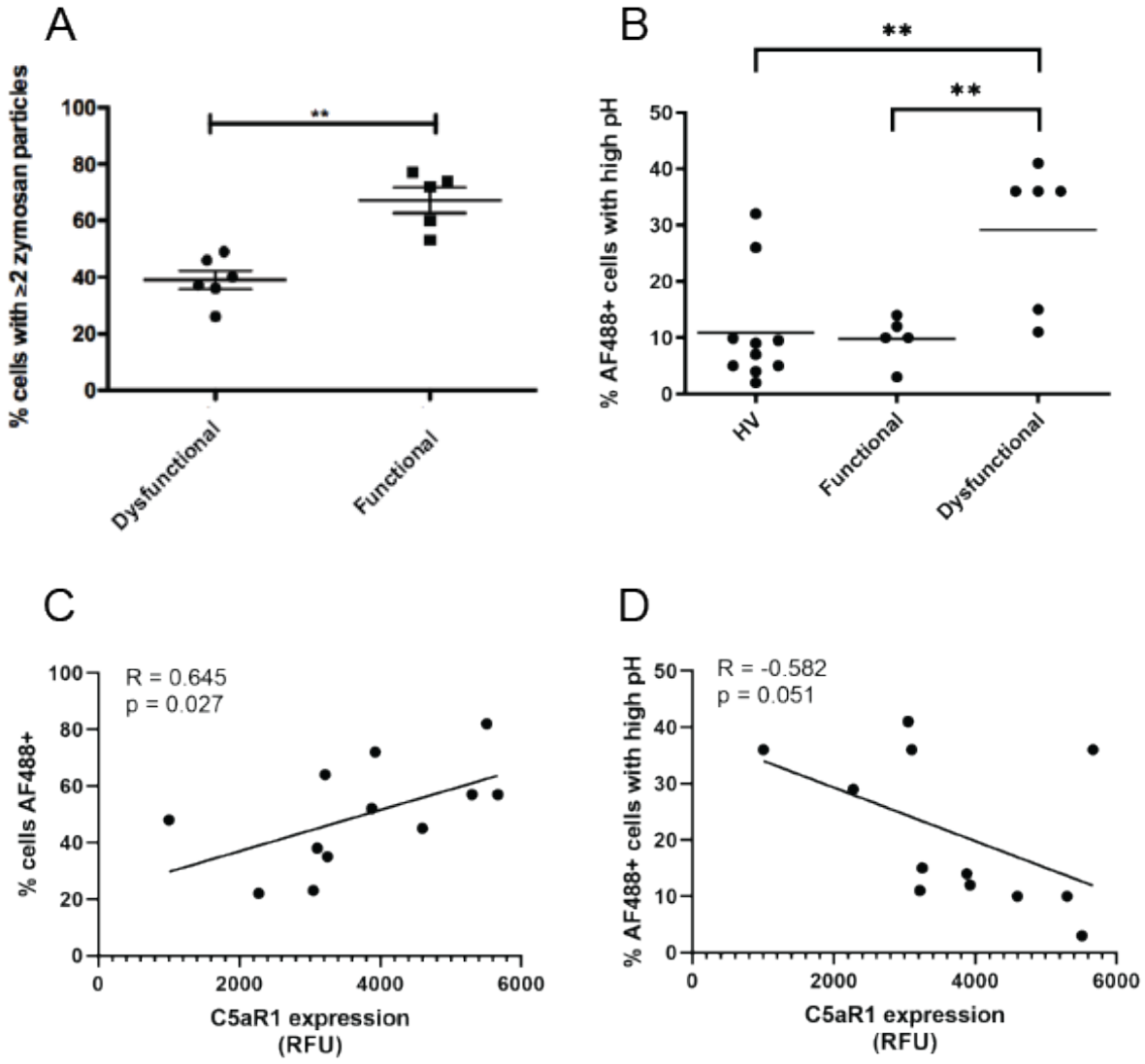

A
ns
Phagocytosis
(% cells AF488 positive)
100
80
60
40
20
0
Control
VPS34IN1
B
*
AF488 MFI
(RFU)
12000
10000
8000
6000
4000
2000
0
Control
VPS34IN1
C
*
pHrodo MFI
(RFU)
80000
60000
40000
20000
0
Control
VPS34IN1
D
*
Bacterial survival
(CFU)
600
400
200
0
Control
VPS34IN1
E
Phagocytosis
(% cells AF488 positive)
100
80
60
40
20
0
0
50
100
150
Time (minutes)
Control
VPS34-IN1
F
pHrodo MFI
(RFU)
120000
100000
80000
60000
40000
20000
0
0
50
100
150
Time (minutes)
Control
VPS34-IN1
**

Figure 7


A
% cells with ≥2 zymosan particles
100
80
60
40
20
0
**
Dysfunctional
Functional
B
**
**
% AF488+ cells with high pH
50
40
30
20
10
0
HV
Functional
Dysfunctional
C
R = 0.645
p = 0.027
% cells AF488+
100
80
60
40
20
0
0
2000
4000
6000
C5aR1 expression
(RFU)
D
R = -0.582
p = 0.051
% AF488+ cells with high pH
50
40
30
20
10
0
0
2000
4000
6000
C5aR1 expression
(RFU)

Tables

Table I: Differentially phosphorylated proteins between C5a and control-treated neutrophils exposed to *S. aureus*.

All 19 phosphoproteins with Bonferroni adjusted p-values < 0.05 for difference in phosphorylation status between the Control plus *S. aureus* vs C5a plus *S. aureus* conditions. Subcellular location and function manually annotated from Uniprot database (The Uniprot Consortium, 2019).

Protein name	Sub-cellular localisation	Functional role
Endosomal/phagosomal		
Zinc finger FYVE domain-containing protein 16 (ZFYVE16)	Early Endosomes	PI(3)P binding FYVE domain, found on early endosomes, targets TOM1 to endosomes
Target of Myb protein 1 (TOM1)	Endosomes/azurophil granule membrane (neutrophil)	Known interactor with ZFYVE16
V-type proton ATPase subunit G 1 (ATP6V1G1)	Endosomes/phagosomes	Proton channel acidifying vacuolar structures
Wiskott-Aldrich protein (WAS)	Cytoplasm, phagosomes	RhoGTPase effector and actin polymerisation
Epsin-1 (EPN1)	Plasma membrane, clathrin coated pits	Regulates receptor-mediated endocytosis, PI(4,5)P binding
Lysosomes		
Ubiquitin carboxyl-terminal hydrolase 5 (USP5)	Lysosome and cytoplasm	Deubiquitination
Synaptic vesicle membrane protein VAT-1 homolog (VAT1)	Lysosomes/azurophil granule (neutrophil)	Neutrophil degranulation
Nuclear		
Nuclear pore complex protein 153 (NUP153)	Nuclear membrane	Phosphorylation occurs during nuclear membrane disassembly
Lamin-B1 (LMNB1)	Nuclear membrane	Phosphorylation occurs during nuclear membrane disassembly
Histone H3.3 (H3F3A)	Nucleus	Histone protein
Protein SGT1 homolog (SUGT1)	Nucleus and kinteochore	Mitosis
Sperm-associated antigen 7 (SPAG7)	Nucleus	Nucleic acid binding
Splicing factor U2AF 35 kDa subunit (U2AF1)	Nucleus	RNA splicing
Chromatin complexes subunit BAP18 (BAP18)	Nucleus	Chromatin organisation
Nuclear/Cytoplasmic proteins		
Serine/threonine-protein kinase 3 (STK3)	Nucleus/cytoplasm	Cycles between nucleus and cytoplasm, influenced by phosphorylation status under control of caspases
Transforming acidic coiled-coil-containing protein 1 (TACC1)	Nucleus, centerosome, microtubules	Microtubule organisation and mitotic spindle formation
Serine/threonine-protein kinase TAO2 (TAOK2)	Nucleus/Cytoskeleton	Regulation of actin cytoskeleton and MAP kinase activity
Endoplasmic reticulum		
Protein lunapark (LNP)	Endoplasmic reticulum	Phosphorylated during mitosis

Thioredoxin-related transmembrane protein 1 (TMX1)	Endoplasmic reticulum	Cell response to oxidative stress